A molecular mechanism of energy coupling in the oxaloacetate decarboxylase Na+ pump of Klebsiella pneumoniae as inferred from mutational analysis

Author(s):
Jockel, Petra

Publication Date:
1999

Permanent Link:
https://doi.org/10.3929/ethz-a-003824334

Rights / License:
In Copyright - Non-Commercial Use Permitted
A molecular mechanism of energy coupling in the oxaloacetate decarboxylase Na\textsuperscript{+} pump of Klebsiella pneumoniae as inferred from mutational analyses

A dissertation submitted to the

SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZÜRICH

for the degree of

DOCTOR OF NATURAL SCIENCES

presented by

PETRA JOCKEL

Dipl. Biol., Justus-Liebig-University of Giessen (D)

born February 20\textsuperscript{th}, 1970

Federal Republic of Germany

accepted on the recommendation of

Prof. Dr. P. Dimroth, examiner

Prof. Dr. L. Thöny-Meyer, Co-examiner

Zürich 1999
TABLE OF CONTENTS

1 Principles of bacterial energy conservation ......................................................... 5
  1.1 Chemical energy conservation: substrate-level phosphorylation ..................... 5
  1.2 Electrochemical energy conservation: electrontransport coupled phosphorylation .... 5
  1.2.1 Establishing ΔpH or ΔpNa⁺ by aerobic or anaerobic respiration .................... 7
  1.2.2 Establishing ΔpH⁺ or ΔpNa⁺ by combining secondary transport systems with a soluble decarboxylase ................................................................. 9
  1.2.3 Establishing ΔpNa⁺ by primary Na⁺ pumping decarboxylases ....................... 10
  1.3 Oxaloacetate decarboxylase from Klebsiella pneumoniae and its role in citrate fermentation ........................................................... 15
    1.3.1 The oxaloacetate decarboxylase Na⁺ pump of Klebsiella pneumoniae ............ 16
    1.3.2 The membrane-bound β subunit of the oxaloacetate decarboxylase Na⁺ pump of Klebsiella pneumoniae .................................................. 18
  1.4 Topological studies of integral membrane proteins ........................................... 20
    1.4.1 Helix packing in polytopic membrane proteins: the lactose permease of Escherichia coli ................................................................. 22
  1.5 Aims of this work ...................................................................................... 27
  1.6 References ............................................................................................ 29

CHAPTER 2 ............................................................................................................ 40
  2 Membrane Topology of the β-Subunit of the Oxaloacetate Decarboxylase Na⁺ Pump from Klebsiella pneumoniae ......................................................... 41
    2.1 Abstract .............................................................................................. 41
    2.2 Introduction ...................................................................................... 42
    2.3 Experimental Procedures .................................................................... 44
    2.4 Results ............................................................................................ 50
<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>A molecular Mechanism for Energy Coupling in the Oxaloacetate Decarboxylase Na⁺ pump</td>
<td>73</td>
</tr>
<tr>
<td>3.1</td>
<td>Abstract</td>
<td>74</td>
</tr>
<tr>
<td>3.2</td>
<td>Introduction</td>
<td>75</td>
</tr>
<tr>
<td>3.3</td>
<td>Results</td>
<td>78</td>
</tr>
<tr>
<td>3.4</td>
<td>Discussion</td>
<td>88</td>
</tr>
<tr>
<td>3.5</td>
<td>Materials and methods</td>
<td>93</td>
</tr>
<tr>
<td>3.6</td>
<td>References</td>
<td>99</td>
</tr>
<tr>
<td>4</td>
<td>Essential Role of Tyrosine 229 of the Oxaloacetate Decarboxylase β Subunit in the Energy Coupling Mechanism of the Na⁺ Pump</td>
<td>102</td>
</tr>
<tr>
<td>4.1</td>
<td>Summary</td>
<td>103</td>
</tr>
<tr>
<td>4.2</td>
<td>Introduction</td>
<td>104</td>
</tr>
<tr>
<td>4.3</td>
<td>Results</td>
<td>107</td>
</tr>
<tr>
<td>4.4</td>
<td>Discussion</td>
<td>114</td>
</tr>
<tr>
<td>4.5</td>
<td>Experimental procedures</td>
<td>118</td>
</tr>
<tr>
<td>4.6</td>
<td>References</td>
<td>124</td>
</tr>
<tr>
<td>5</td>
<td>Mechanisms of ion translocation through bacterial membrane proteins</td>
<td>127</td>
</tr>
<tr>
<td>5.1</td>
<td>Mechanism of ion translocation through F₀F₁-ATPases</td>
<td>127</td>
</tr>
<tr>
<td>5.2</td>
<td>The K⁺ channel of <em>Streptomyces lividans</em></td>
<td>129</td>
</tr>
<tr>
<td>5.3</td>
<td>The melibiose permease of <em>Escherichia coli</em></td>
<td>131</td>
</tr>
<tr>
<td>5.4</td>
<td>A molecular mechanism for energy coupling in a membrane transport protein, the lactose permease of <em>Escherichia coli</em></td>
<td>132</td>
</tr>
<tr>
<td>5.5</td>
<td>Mechanism of Na⁺ translocation by oxaloacetate decarboxylase of <em>Klebsiella pneumoniae</em></td>
<td>135</td>
</tr>
<tr>
<td>5.6</td>
<td>Conclusions and outlook</td>
<td>139</td>
</tr>
<tr>
<td>5.7</td>
<td>References</td>
<td>140</td>
</tr>
</tbody>
</table>
CHAPTER 6 .................................................................................................................. 144

6 Appendix ................................................................................................................. 144
6.1 Appendix to Chapter 2 .......................................................................................... 144
6.2 Appendix to Chapter 3 and 4 .............................................................................. 150
6.2.1 Effect of Na+ on tryptic hydrolysis of the oxaloacetate decarboxylase β-subunit... 150
6.2.2 Determination of oxaloacetate decarboxylase activity at various Na+ concentrations
and pH values ............................................................................................................. 154
6.3 References ............................................................................................................ 157

Curriculum Vitae ........................................................................................................ 159

LIST OF PUBLICATIONS .......................................................................................... 160
ZUSAMMENFASSUNG

Die Natrium-pumpende Oxalacetat Decarboxylase aus *Klebsiella pneumoniae* besteht aus drei verschiedenen Untereinheiten, welche in der cytoplasmatischen Membran einen stabilen Komplex bilden. Die periphere, Biotin-haltige α Untereinheit ist an die membrangebundene β Untereinheit durch die γ Untereinheit gebunden, welche den Komplex zusammenhält. Die Gene der einzelnen Untereinheiten des Enzyms wurden sequenziert und in *Escherichia coli* Expressions-Plasmide kloniert. Die β Untereinheit ist für die Natrium-Bindung und -Translokation verantwortlich, was bereits in früheren Mutagenese-Studien und durch Schutz vor proteolytischem Verdau in Gegenwart von Natriumionen gezeigt werden konnte.

Zusammenfassung

Das neue Modell platziert den N-Terminus im Cytoplasma und den C-Terminus im Periplasma.


Weiterhin konnte ein 3-dimensional Modell für den Protonentransfer durch die β Untereinheit aufgestellt werden. Diesem liegen Aminosäureaustausche zu Grunde, welche die kinetischen Eigenschaften der Oxalacetat Decarboxylase verändern.
The Na⁺ pumping oxaloacetate decarboxylase of Klebsiella pneumoniae consists of three different subunits, which form a stable membrane-bound complex. The peripheral biotin-containing α subunit is attached to the membrane-bound β subunit via the γ subunit, which stabilizes the complex. The genes of the subunits of the enzyme have been sequenced and cloned into Escherichia coli expression plasmids. The β subunit is responsible for Na⁺ binding and translocation, which was shown in earlier studies by mutagenesis and protection from proteolytic digestion.

In this work the topology and the function of the membrane-bound β subunit has been investigated. The hydrophobic β subunit was previously thought to span the membrane nine times in a zigzag fashion, with the N-terminus placed into the periplasm and the C-terminus placed into the cytoplasm. In order to probe this topology model three different approaches were carried out: 1) comparative hydropathy plots and sequence alignments including several members of the Na⁺ pumping decarboxylase family, 2) gene fusion analysis with the β subunit and the reporter enzymes alkaline phosphatase and β-galactosidase, 3) probing the sidedness of native and introduced cysteine residues with the fluorescent, non-permeable probe 4-acetamido-4'-maleimidylstilbene-2,2'-disulfonic acid. Construction of FLAG-epitope insertion mutants failed, because the resulting enzymes showed no oxaloacetate decarboxylase activity and were therefore not suitable to investigate the topology. The data obtained from these approaches indicated additional membrane-located segments. In contrast to the previous model, two further membrane-spanning helices in the N- and C-terminal part, and an extended hydrophobic segment (about 50 amino acid residues, combining two putative transmembrane segments) in the middle of the protein were identified. The latter does not traverse the membrane, but forms two hairpin structures and seems to be flexible. In this new model the N-terminus is placed into the cytoplasm and the C-terminus into the periplasm.

Using this topology model as a starting point, site-directed mutagenesis studies were performed with the membrane-bound β subunit. In previous studies, the highly conserved residue Asp 203 was found to be essential for Na⁺ binding and translocation.
In a first round, several polar and conserved residues and glycine residues of transmembrane helix VIII, a highly conserved region, were exchanged. Amino acid residues N373, G377, S382 and R389 were identified to have functional significance. Amino acid residue S382 was identified to be directly involved in Na\(^+\) binding and translocation of Na\(^+\) and H\(^+\) across the membrane. Amino acid residues N373 and D203 form together the second Na\(^+\) binding site. By characterisation of these mutants, an improved new model for the coupling mechanism between ion translocation and oxaloacetate decarboxylation could be suggested. The most important differences with respect to a previous model are: 1) two Na\(^+\) binding sites exist, one at the Asp 203, as suggested in the old model, and one at the deprotonated Ser 382, 2) two Na\(^+\) ions are bound at the same time to the β subunit, which is also in accordance with a cooperative effect, found in kinetic studies. In a second round of mutagenesis studies of this work, additional 20 polar and/or conserved amino acid residues and glycine residues have been replaced. A tyrosine residue, Y229, which is essential for proton transfer through the membrane has been identified. Additionally, an amino acid residue was found, which is located in a region of the β subunit that is affected by a conformational change due to binding of Na\(^+\) ions. Further, a three dimensional model for the proton transfer pathway through the β subunit has been proposed. The model is based on amino acid replacements, which affect the kinetic properties of the oxaloacetate decarboxylase.
CHAPTER 1

Introduction

1 Principles of bacterial energy conservation

The energy requirement for most of the endergonic biochemical processes in bacterial cells is covered by ATP hydrolysis. Under physiological conditions, the free energy change for this reaction is -60 to -80 kJ/mol. This energy is used for solute uptake, biosynthesis or flagellar motion. To maintain this energy source and capacity, a continuous complement of consumed ATP is necessary. For this purpose, a great variety of pathways has evolved throughout the biosphere. They can be classified into only two general groups.

1.1 Chemical energy conservation: substrate-level phosphorylation

During catabolism of energy-rich substrates, phosphorylated intermediates with high phosphate group transfer potential emerge. The phosphate group of these intermediates is transferred to ADP to yield ATP. A classical example for this so-called substrate-level phosphorylation is the oxidation of glycerinaldehyd-3-phosphate. Here, glycerinaldehyd-3-phosphate dehydrogenase catalyzes the oxidation of glycerinaldehyd-3-phosphate to 1,3-diphosphoglycerate. In the subsequent reaction, a phosphate group is transferred to ADP. This step is catalyzed by the phosphoglycerate-kinase.

1.2 Electrochemical energy conservation: electrontransport coupled phosphorylation

In cases, where energy sources with an energy yield that is too low for direct synthesis by substrate-level phosphorylation, shall be exploited for ATP regeneration, another principle is applied. Here, ATP is synthesized by a membrane-bound ATP synthase at the expense of the energy potential of an electrochemical ion gradient across the membrane. The universal F$_1$F$_0$-ATPase is capable of synthesizing one ATP per three to four protons translocated from the outer to the inner side of the membrane along the electrochemical H$^+$ gradient. If an energy source bears enough energy to translocate one H$^+$ electrogenically out of the cell, it can serve as source for ATP synthesis. The
membrane-bound ATP synthase is universally present in all organisms with only little variation. There are also ATP synthases known, which exploit an electrochemical Na⁺ gradient. Here, an electrochemical Na⁺ ion gradient is needed. The first example was found in Propionigenium modestum, which possesses an unique type of F₁F₀ ATPase, that uses Na⁺ as the physiological coupling ion (Hilpert et al., 1984; Laubinger and Dimroth, 1987, 1988). Like the proton translocating F-type ATPases this enzyme is composed of a cytoplasmic F₁-part, the catalytic center for chemical synthesis of ATP, and a membrane-bound F₀-part. The ion specificity, meaning a binding site for the cation, is determined by the F₀-part alone. This was indicated by a functional hybrid ATPase consisting of the cytoplasmic F₁-part of the H⁺-translocating ATP synthase from Escherichia coli and the membrane-bound F₀-part of the Na⁺-dependent ATPase from P. modestum, which showed the same specificity as the P. modestum enzyme (Laubinger et al., 1990; Kaim et al., 1997, 1998). The position of the cation binding site and the translocation pathway were identified recently (Kluge and Dimroth, 1993; Kaim et al., 1997, 1998). Na⁺ translocating F₁F₀ ATPases were isolated from Acetobacterium woodii (Rcidlinger and Müller, 1994) and from Ilyobacter tartaricus (Neumann et al., 1998), and a Na⁺ dependent V-type ATPase is known from Enterococcus hirae (for a review see Kakinuma, 1998). V-type ATPases belong to the family of ion-motive ATPases, which is divided into two categories: one which forms phosphorylated intermediates (P-ATPase), per example Na⁺, K⁺-ATPase or Ca²⁺-ATPase, and the other which does not. This second category is divided into two types: F₁F₀-ATPase, which is described above, and vacuolar ATPase (V-ATPase). V-ATPase is known as the proton pump of acidic organelles, such as the vacoules of plants or endosomes of animal cells. Archaeabacteria contain a V-ATPase, which is believed to mediate ATP synthesis (Kakinuma, 1998).

The global structure of the ATP synthase (see Figure 1) is based on the X-ray structure of most of the F₁-part (Abrahams et al., 1994), crosslinking experiments, and electron spectroscopic images (Birkenhäuser et al., 1995; Decker-Hebestreit and Altendorf, 1996). The F₁-part consists of the αβεγδε-subunits, which form the cytoplasmic domain. It is attached to the membrane-bound F₀-part, which consists of the abɔc₁₃-subunits.

In contrast to the universally present pathway for ATP synthesis upon exploiting an electrochemical membrane potential by F₁F₀ ATP synthase, a number of processes have been recognized for the generation of the electrochemical ion gradient.
Introduction

Figure 1: Composition of F$_{1}$F$_{0}$ ATPases. The F$_{1}$-part is composed of five different subunits $\alpha_{3}\beta_{3}\gamma_{8}\delta\epsilon$ and the F$_{0}$-part consists of $ab_{3}c_{10}$. The $\gamma$-subunit is located in the center of the hexagon of $\alpha$- and $\beta$-subunits and extends into the multimeric ring of $c$-subunits in the F$_{0}$-part. Upon translocation of protons (or sodium ions) through F$_{0}$, the $\gamma$-subunit rotates and mediates the movement into the F$_{1}$-part. Here, the ATP synthesis is catalyzed by the rotation.

1.2.1 Establishing $\Delta$$\mu$H$^{+}$ or $\Delta$$\mu$Na$^{+}$ by aerobic or anaerobic respiration

Electrochemical ion gradients are established by electrogenic ion movements across the membrane, driven by either light, respiratory or chemical energy. The most common coupling ions of bacteria are protons, but in some cases, Na$^{+}$ ions play a central role in energy transducing reactions (Dimroth, 1987). Phototrophic bacteria have a photosynthetic apparatus, which enables them to maintain an electrochemical proton gradient ($\Delta$$\mu$H$^{+}$) by conversion of light energy. In aerobic bacteria an electron transport chain exists, by which the free energy of substrate oxidation with O$_{2}$ as terminal electron acceptor is used for H$^{+}$ pumping out of the cells. Also, many anaerobic bacteria generate $\Delta$$\mu$H$^{+}$ by electron transport reactions with electron acceptors other than oxygen (e.g., nitrate, fumarate, sulfate, carbonate).
Sodium bioenergetics is an important link between exergonic and endergonic reactions in the membrane for many bacteria. They create and maintain, like other living cells, a Na\(^+\) concentration in their cytoplasm, that is below that of the environment (Padan and Schuldiner, 1993). Na\(^+\) gradients exist over the bacterial cytoplasmic membrane without regard of extreme differences in salinity in the habitats, where the different species live. Na\(^+\) translocation systems, responsible for the Na\(^+\) extrusion from the cytoplasm to the outside of the cell, operate in the membrane at the expense of an energy source. Usually, Na\(^+\) extrusion is driven by secondary active transport via the Na\(^+\)/H\(^+\) antiporter (Schuldiner and Padan, 1993). Escherichia coli, for example, contains three antiporter genes nhaA, nhaB and chaA, which have been thoroughly studied (Karpel et al., 1988; Pinner et al., 1992; Ivey et al., 1993). However, in some cases, \(\Delta\mu\text{Na}^+\) is not maintained by this secondary mechanism at the expense of \(\Delta\mu\text{H}^+\). Certain bacteria have developed primary Na\(^+\) extruding systems. For example, in some marine Vibrios that live at alkaline pH, respiratory enzymes exist, which were demonstrated to function as primary sodium pumps (Tokuda, 1993). The best investigated example in this field is the Na\(^+\)-pumping NADH-ubiquinone oxidoreductase (NDH I) from V. alginolyticus (Unemoto and Hayashi, 1989). Another type of primary sodium pump was found in some methanogenic and acetogenic archaea, the N5-methyl-tetrahydromethanopterin:coenzyme M methyl-transferase. This enzyme catalyzes the transfer of a methyl group from a N5-methyltetrahydrofolate analogue to the thiol group of 2-mercapto-ethylsulfonate (coenzyme M), and concomitant Na\(^+\)-translocation (Müller and Gottschalk, 1993). Well described examples for this interesting system are found in Methanosarcina mazei Göl and Methanobacterium thermoautotrophicum (Lienhard et al., 1996; Lienhard and Gottschalk, 1998; Weiss et al., 1994; Harms and Thauer, 1997).

A further example for a bacterial primary sodium pump was first described by Dimroth (1982) for the opportunistic pathogenic bacterium Klebsiella pneumoniae. This organism contains a membrane-bound oxaloacetate decarboxylase, which is able to convert the free energy from the decarboxylation of oxaloacetate to pyruvate into \(\Delta\mu\text{Na}^+\).

Due to the central role of this enzyme system for the presented work, decarboxylation based energy conservation by bacteria is described in the following chapter in more detail.
1.2.2 Establishing $\Delta \mu H^+$ or $\Delta \mu Na^+$ by combining secondary transport systems with a soluble decarboxylase

The free energy change of a decarboxylation reaction is small (17 - 26 kJ/mol). This is equivalent to only one third of the energy required for ATP-synthesis from ADP and $P_i$ to ATP under physiological conditions (+60 - +80 kJ/mol). Therefore, decarboxylation energy cannot be conserved by substrate-level phosphorylation (Thauer et al., 1977). But it may be converted into an electrochemical potential, which then can serve as energy source for ATP synthesis, which has been termed decarboxylation phosphorylation (Dimroth and Schink, 1998).

Bacteria have evolved two substantially different systems to meet this target. Next to the above mentioned primary sodium-pumping decarboxylases, a combined system of a soluble, non ion-translocating decarboxylase and an electrogenic substrate/product antiporter is used to establish $\Delta \mu H^+$. This kind of machinery was first investigated at the example of Oxalobacter formigenes, which is able to grow by decarboxylation of oxalate to formate (Allison et al., 1985; Figure 2). Here, oxalate is first chemically activated, and thus prepared for decarboxylation by CoA-transfer from formyl-CoA. Then, decarboxylation of oxalyl-CoA by the soluble thiamine pyrophosphate-dependent oxalyl-CoA decarboxylase (Baetz and Allison, 1989) consumes a proton inside the cell and therefore generates a $\Delta \rho \phi$. Liberation of formate from formyl-CoA in the next reaction cycle finishes the decarboxylation reaction. The electrogenic oxalate$^2$:formate$^+$ antiporter is a further key element for energy conservation in this bacterium (Anantharam et al., 1989). Since a component with a single negative charge is exchanged for a double negatively charged molecule, an electrical potential ($\Delta \psi$) is generated. Together, the membrane potential $\Delta \psi$ and the pH-gradient provide a proton motive force $\Delta \mu H^+$, that can be used to drive ATP-synthesis (for a review see Dimroth and Schink, 1998). The decarboxylation consumes a scalar proton and liberates CO$_2$, which diffuses directly across the membrane or after hydration to carbonic acid. The subsequent equilibration between carbonic acid and bicarbonate liberates protons, and as a result, an inwardly directed proton gradient is formed. The combined activities of the vectorial antiport reaction and the decarboxylation step with subsequent carbonic acid diffusion comprise an electrogenic proton pump. The electrochemical proton potential is then used for ATP synthesis.
A similar mechanism is valid for *Lactococcus lactis* subsp. *lactis* biovar diacetylactis. During growth on citrate a soluble, non-biotin-dependent oxaloacetate decarboxylase catalyzes the conversion of oxaloacetate$^{2-}$ to pyruvate$^{1-}$ and generates $\Delta p$H. The electrical component of $\Delta \mu$H$^+$ in this case is established by the electrogenic exchange of H-citrate$^{2-}$ for the final fermentation products acetate$^{1-}$ or pyruvate$^{1-}$ (Hugenholtz et al., 1993). In malolactic fermentation of *Lactobacillus sp.* the electrogenic malat$^{\text{-}}$/lactate$^{2-}$ transporter operates together with the malolactic enzyme for a similar energy conservation pathway (Poolman et al., 1991).

![Diagram](image)

Figure 2: Reactions involved in oxalate fermentation by *Oxalobacter formigenes*. (A) oxalate/formate antiporter, (D) complex of formyl CoA:oxalate CoA transferase and oxalyl CoA decarboxylase, (F) H$^+$-translocating $F_1F_0$ ATP synthase (adopted from Dimroth and Schink, 1998).

### 1.2.3 Establishing $\Delta \mu$Na$^+$ by primary Na$^+$ pumping decarboxylases

As already mentioned, some anaerobes have developed a different mechanism for the conservation of decarboxylation energy. They actively pump Na$^+$ to the outer side of the membrane upon decarboxylation of certain carboxylic acids, like, oxaloacetate, malonate, methylmalonyl-CoA, or glutaconyl-CoA (Dimroth, 1987; Dimroth, 1993; Dimroth, 1997; Dimroth and Schink, 1998). In these cases, a sodium motive force ($\Delta \mu$Na$^+$) is established directly by the action of membrane-bound decarboxylases. The
generated Δ]$\text{Na}^+$ can either be converted into Δ]$\text{H}^+$ by Na$^+/H^+$ antiport, and subsequently Δ]$\text{H}^+$ can be used for ATP synthesis by a F$_{1}$F$_{0}$ ATPase. In Propionigenium modestum, both a Na$^+$-dependent methylmalonyl-CoA decarboxylase and a Na$^+$-dependent F$_{1}$F$_{0}$ ATP synthase are active. This means, that this organism is capable of performing a completely Na$^+$-based energy conservation (Hilpert et al., 1984; Laubinger and Dimroth, 1988). The enzyme family of the Na$^+$-pumping decarboxylases includes the methylmalonyl-CoA decarboxylases from Veillonella parvula (Huder and Dimroth, 1993) and Propionigenium modestum (Bott et al., 1997), the glutaconyl-CoA decarboxylase from Acidaminococcus fermentans (Buckel and Semmler, 1983), the malonate decarboxylases from Malonomonas rubra (Hilbi et al., 1992), and Rhodobacter capsulatus and the oxaloacetate decarboxylases from Klebsiella pneumoniae (Stern, 1967), Salmonella typhimurium (Wifling and Dimroth, 1989), Treponema pallidum (Fraser et al., 1998), and Archaeoglobus fulgidus (Klenk et al., 1997).

<table>
<thead>
<tr>
<th>membrane-bound decarboxylases</th>
<th>bacterium</th>
</tr>
</thead>
<tbody>
<tr>
<td>malonate decarboxylase</td>
<td>Malonomonas rubra</td>
</tr>
<tr>
<td></td>
<td>Rhodobacter capsulatus</td>
</tr>
<tr>
<td>methylmalonyl-CoA decarboxylase</td>
<td>Veillonella parvula</td>
</tr>
<tr>
<td></td>
<td>Propionigenium modestum</td>
</tr>
<tr>
<td></td>
<td>Peptostreptococcus, strain 9 succ 1</td>
</tr>
<tr>
<td>glutaconyl-CoA decarboxylase</td>
<td>Acidaminococcus fermentans</td>
</tr>
<tr>
<td></td>
<td>Peptostreptococcus asaccharolyticus</td>
</tr>
<tr>
<td></td>
<td>Fusobacterium nucleatum</td>
</tr>
<tr>
<td></td>
<td>Clostridium symbiosum</td>
</tr>
<tr>
<td>oxaloacetate decarboxylases</td>
<td>Klebsiella pneumoniae</td>
</tr>
<tr>
<td></td>
<td>Salmonella typhimurium</td>
</tr>
<tr>
<td></td>
<td>Archaeoglobus fulgidus</td>
</tr>
<tr>
<td></td>
<td>Treponema pallidum</td>
</tr>
</tbody>
</table>

Table 1: Membrane-bound decarboxylases.
a) Malonate decarboxylases

The malonate decarboxylase enzyme system of *Malonomonas rubra* (Dimroth and Hilbi, 1997) involves several proteins, that catalyze the individual reactions leading to malonate decarboxylation (Figure 3). Malonate is activated for decarboxylation by forming a thioester with a protein-bound thiol cofactor (Hilbi et al., 1992). The inactive SH-form of the enzyme is converted into the catalytically active acetyl-S enzyme by post-translational acetylation of the acyl carrier protein (ACP) subunit with a specific ligase, and ATP and acetate as substrates (Berg et al., 1996; Hilbi et al., 1992). The acetyl-S-ACP is then converted to malonyl-S-ACP by an acyl carrier protein transferase. Subsequently, the free carboxyl group of the malonyl thioester with the enzyme is transferred to a small biotin protein, thereby regenerating the acetyl-S-ACP. The carboxybiotin protein is believed to diffuse to the membrane, where it is decarboxylated by an integral membrane protein that couples this exergonic reaction to Na⁺ pumping across the membrane (Hilbi and Dimroth, 1994).

![Figure 3: Reaction mechanism proposed for malonate decarboxylase of *Malonomonas rubra* including the anticipated functions of the mad gene products. (1) deacetyl acyl carrier protein:acetate ligase (MadH), (2) acetyl-S-acyl carrier protein:malonate acyl carrier protein-SII transferase (MadA), (3) carboxyltransferase (MadC,D), (4) carboxybiotin decarboxylase (MadB), (ACP) acyl carrier protein (MadE) (Berg et al., 1997).](image)

A substrate activation mechanism, as described for the malonate decarboxylase of *M. rubra* (see above), is also used by the soluble, non-biotin-dependent malonate decarboxylase of *K. pneumoniae* (Schmid et al., 1996). The enzyme also catalyzes the
turnover of malonate and acetate as enzyme-bound thioesters, and the essential acetyl thioester of the enzyme can be removed similarly.

b) Methylmalonyl-CoA decarboxylases
The methylmalonyl-CoA decarboxylase of *P. modestum* consists of four different subunits. The α subunit contains the carboxyltransferase activity, the β subunit functions as a carboxybiotin-carrier protein decarboxylase, the γ subunit is the biotin-carrier protein, and the δ subunit has a function in the assembly of the complex (Bott et al., 1997). The very similar methylmalonyl-CoA decarboxylase of *Veillonella parvula* contains a fifth subunit. This ε subunit has no catalytic function, but increases the stability of the complex (Huder and Dimroth, 1995).

\[
\begin{align*}
\text{Na}^+/\text{H}^+ & \\
\text{ADP} + \text{P} & \\
\text{methylmalonyl-CoA} & \\
\text{Na}^+ & \\
\text{propionyl-CoA} & \\
\end{align*}
\]

Figure 4: Energy metabolism in *Propionigenium modestum* with a Na⁺ cycle coupling the exergonic decarboxylation of (S)-methylmalonyl-CoA to endergonic ATP synthesis. (D) methylmalonyl-CoA decarboxylase, (F) Na⁺ translocating F₁F₀ ATP synthase.

c) Glutaconyl-CoA decarboxylases
The glutaconyl-CoA decarboxylase of the strict anaerobic bacterium *Acidaminococcus fermentans* uses the free energy of decarboxylation in order to translocate Na⁺ ions across the membrane (Buckel and Semmler, 1983). The enzyme consists of four subunits. The largest subunit GcdA (carboxyltransferase) catalyzes the transfer of CO₂ from glutaconyl-CoA to biotin covalently attached to GcdC (biotin carrier). GcdB (decarboxylase) is responsible for the decarboxylation of the carboxybiotin, which drives Na⁺ translocation. GcdD is a protein of unknown function. Unlike the related
carboxyltransferases, GdcA catalyzes carboxyltransfer not only with the native substrate, but also with free biotin (Berger et al., 1996). In the case of *Peptostreptococcus asaccharolyticus*, it has been shown, that the sodium pump contributes to energy conservation (Wohlfarth and Buckel, 1985).

d) Oxaloacetate decarboxylases

This enzyme was the first example for the new energy conservation concept. The oxaloacetate decarboxylase from *Klebsiella pneumoniae* (Stern, 1967) is the best studied member of this enzyme family and is now the paradigm of Na⁺-pumping decarboxylases. The enzyme consists of three subunits, the water-soluble α subunit and the membrane-bound β and γ subunits. The γ subunit mediates the association of the peripheral α subunit with the membrane-bound β subunit, and the system forms a stable complex at the membrane (Laußermair et al., 1989; Schwarz and Oesterhelt, 1985; Schwarz et al., 1988). A very similar oxaloacetate decarboxylase was isolated from *Salmonella typhimurium* (Wifling and Dimroth, 1989). The genes encoding this enzyme were sequenced as they were in the case of *K. pneumoniae*, and a comparison of the deduced amino acid sequence revealed 71-93 % identical amino acids, depending on the respective subunits (Woehlke et al., 1992b). Also, the catalytic properties of the *S. typhimurium* enzyme very much resemble those of the *K. pneumoniae* decarboxylase. Very recently, two further examples of membrane-bound oxaloacetate decarboxylases were identified in *Archaeoglobus fulgidus* (Klenk et al., 1997), and *Treponema pallidum* (Fraser et al., 1998) during genome sequencing projects of these organisms. In case of *A. fulgidus*, genes for proteins similar to the α and β subunits were found, whereby the α subunit seems to split into two subunits. Interestingly, no gene for a γ subunit was identified, which questions the existence of a stable complex in this case. In the case of *T. pallidum* genes for all three subunits were found, whereby the α subunit is missing the extended alanine/proline linker between the carboxyltransferase- and the biotin binding-domain. Further, the β subunit at its N-terminus is about 80 amino acids longer than that of all other known Na⁺-pumping decarboxylases.
1.3 Oxaloacetate decarboxylase from *Klebsiella pneumoniae* and its role in citrate fermentation

The oxaloacetate decarboxylase plays an essential role in citrate fermentation by *K. pneumoniae* which is dependent on Na\(^+\) for anaerobic growth on this substrate (O'Brien, 1975a, b; O'Brien and Stern, 1969). The anaerobic citrate fermentation pathway of *K. pneumoniae* including the generation of Δ\(\Delta\)Na\(^+\), ATP and NAD(P)H\(^+\) is shown in Figure 5. Citrate is transported into the cell in symport with Na\(^+\) and H\(^+\) (Dimroth and Thomer, 1986; Dimroth and Thomer, 1990; van der Rest et al., 1992; Pos and Dimroth, 1996). Citrate lyase cleaves citrate to acetate and oxaloacetate. The latter is decarboxylated by the oxaloacetate decarboxylase Na\(^+\) pump to pyruvate and concomitantly Δ\(\Delta\)Na\(^+\) is generated, which can be used for further citrate uptake. Pyruvate is degraded by pyruvate formate lyase to formate and acetyl-CoA. The latter is converted into acetyl-phosphate by phosphotransacetylase and subsequently to acetate with ATP generation by acetate kinase. Formate is cleaved by the formate hydrogen lyase into CO\(_2\) and H\(_2\), which serves as electron donor for the reduction of NAD(P)\(^+\) by a membrane-bound NAD(P)\(^+\)-dependent hydrogenase (Steuber et al., 1999). Since the fermentation of citrate to acetate does not involve redox reactions that lead to the formation of reducing equivalents, *K. pneumoniae* must use an alternative pathway to synthesize NADH, which includes the membrane-bound hydrogenase.

![Figure 5: Citrate fermentation pathway in *K. pneumoniae*. (CC) citrate carrier, (CL) citrate lyase, (OAD) oxaloacetate decarboxylase, (PFL) pyruvate formate lyase, (PTA) phosphotransacetylase, (AK) acetate kinase, (FHL) formate hydrogen lyase, (H) NAD(P)\(^+\)-reducing hydrogenase.](image-url)
The genes encoding enzymes specifically needed for anaerobic citrate fermentation are organized in a regulon on the *K. pneumoniae* genome (see Figure 6). Within this regulon, the genes for the oxaloacetate decarboxylase are located consecutively in the order *oadGAB*. Directly upstream of *oadG*, the gene for the citrate transporter, *citS*, is located. In downstream direction, *oadB* is followed by *citAB*, which encode a two component regulatory system with CitA being the sensor kinase and CitB the response regulator (Bott et al., 1995). Also part of the citrate regulon are the genes for the citrate lyase ligase (*citC*), the citrate lyase (*citDEF*) and CitG, which is involved in the biosynthesis of enzymatically active citrate lyase. These genes are orientated in the opposite direction at the 5'-end of *citS*. The expression of the enzymes for citrate fermentation is induced by citrate, Na⁺ ions and anaerobic conditions (O'Brien, 1975a, b) and is regulated by the CitAB system (Bott et al., 1995).

![Figure 6: Citrate fermention genes of *K. pneumoniae*. The function of the genes is indicated as far as known.](image)

Because of its unique role for energy conservation during citrate fermentation, the oxaloacetate decarboxylase of *Klebsiella pneumoniae* is described in detail.

### 1.3.1 The oxaloacetate decarboxylase Na⁺ pump of *Klebsiella pneumoniae*

The oxaloacetate decarboxylase of *Klebsiella pneumoniae* consists of three different subunits, forming a stable complex, the membrane-bound β and γ subunits of 44.9 and 8.9 kDa, to which the peripheral α subunit of 63.6 kDa is attached (Laußermair et al., 1989; Schwarz et al., 1988; Wochlke et al., 1992a) via the γ subunit, as shown schematically in Figure 7 (Di Berardino and Dimroth, 1995). The α subunit contains two different domains, which are connected by an extended alanine/proline rich linker peptide. The larger N-terminal domain (~ 51 kDa) harbours the carboxyltransferase activity, while the biotin-binding site resides on the smaller C-terminal domain. The
biotin prosthetic group is attached via amide linkage to the ε-group of a lysine residue of the C-terminal domain. The biotinylated lysine residue resides within the conserved tetrapeptide, A-M-K-M, 35 amino acid residues from the C-terminus apart. The oxaloacetate decarboxylase γ subunit contains at its C-terminus a remarkable motif of three (K. pneumoniae) or four (S. typhimurium) histidines in series which could provide a binding site for the Zn$^{2+}$ metal ion, that is present in this enzyme (Dimroth and Thomer 1992). The high conservation of membrane domains of the β subunit may imply the presence of structural constraints, which could be important for Na$^+$ translocation.

The catalytic reaction cycle starts with the carboxyltransfer from oxaloacetate to the prosthetic biotin group. This reaction is catalyzed by the α subunit alone at a low rate. In presence of the Zn$^{2+}$ containing γ subunit, the rate increases, probably because the metal ion polarizes the carbonyl oxygen bond of oxaloacetate, facilitating the carboxyltransfer to the prosthetic biotin group (Dimroth and Thomer, 1983; Di Berardino and Dimroth, 1995). The carboxybiotin moves from the carboxyltransfer site at the α subunit to the decarboxylase site at the β subunit. This movement is facilitated by the extended alanine/proline linker in the region between the carboxyltransferase domain and the biotin binding domain in the α subunit. The free energy of the subsequent decarboxylation of the carboxybiotin is used to translocate one to two Na$^+$ ions across the membrane to the periplasm and one H$^+$ into the opposite direction. This proton is consumed during the release of CO$_2$ from the biotin carboxylate.
Figure 7: Model of the oxaloacetate decarboxylase Na\(^+\) pump (adopted from Di Berardino and Dimroth, 1995). The \(\beta\) and \(\gamma\) subunits are integral membrane proteins to which the peripheral \(\alpha\) subunit is attached. The catalytic cycle involves carboxyltransfer from oxaloacetate to the prosthetic biotin group (B-H), movement of the carboxybiotin (B-C(O)\_), and decarboxylation with H\(^+\) as the second substrate that is coupled to Na\(^+\) translocation across the membrane.

### 1.3.2 The membrane-bound \(\beta\) subunit of the oxaloacetate decarboxylase Na\(^+\) pump of *Klebsiella pneumoniae*

The \(\beta\) subunit (OadB) is a very hydrophobic protein, which is thought to traverse the membrane in a zigzag fashion in nine hydrophobic rods (Woehlke et al., 1992a, b). The original topology model was based entirely on hydropathy plots (Woehlke, 1994). The model has been revised during this work by taking into account data from fusion analyses and probing the sidedness of engineered cysteine residues (see Chapter 2). OadB is responsible for decarboxylation of carboxybiotin, as mentioned above. The strict requirement of Na\(^+\) ions for the decarboxylation reaction suggests that this step is coupled to Na\(^+\) translocation. The \(\beta\) subunit is specifically protected from proteolysis by Na\(^+\) ions, which is further evidence for a Na\(^+\) binding site on this subunit (Dimroth and Thomer, 1983; Dimroth and Thomer, 1992). The sequence of the OadB was compared with those from other members of the Na\(^+\) translocating decarboxylase family, i.e. methylmalonyl-CoA decarboxylases of *Veillonella parvula* (Huder and Dimroth, 1993) and *Propionigenium modestum* (Bott et al., 1997), malonate decarboxylases from *Malonomonas rubra* (Berg et al., 1997) and *Rhodobacter capsulatus*, the oxaloacetate decarboxylases from *Archaeoglobus fulgidus* (Klenk et al., 1997), *Salmonella typhimurium* (Woehlke et al., 1992b) and *Treponema pallidum* (Fraser et al., 1998), and glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* (Braune et al., 1999). The \(\beta\) subunits from all these complexes contain extended stretches of sequence identity in the putative membrane-spanning segments. Recently, it was suggested that the \(\beta\) subunit of these decarboxylases spans the membrane eleven times (Braune et al., 1999), using glutaconyl-CoA decarboxylase as an example.

Site-directed mutagenesis of the highly conserved aspartate residue Asp 203 in one of the putative transmembrane helices to asparagine or glutamate completely abolished Na\(^+\) transport and decarboxylase activities, whereas the carboxyltransfer activity was retained (Di Berardino and Dimroth, 1996; Dimroth, 1997). Based on these and other results, a direct coupling mechanism has been proposed, in which Asp 203 plays an
essential role in both, the vectorial and the chemical reaction (see Figure 8). In the proposed mechanism, the carboxybiotin binds together with a Na\(^+\) ion to a binding pocket of OadB. Subsequently, the Na\(^+\) ion is envisaged to switch to Asp 203, and simultaneously the proton, originally bound to this residue, moves to the biotin carboxylate, where it catalyzes the immediate decarboxylation of this acid-labile compound. This exergonic decarboxylation is coupled to the release of bound Na\(^+\) ions to the positive side of the membrane (Di Berardino and Dimroth, 1996; Dimroth, 1997). In the following, this model for the decarboxylation of enzyme-bound carboxybiotin and the coupled transport of Na\(^+\) is described in more detail. As already mentioned, two different Na\(^+\) binding sites are envisaged. One Na\(^+\) binding site with high affinity is located at the β subunit-bound carboxybiotin and one binding site with low affinity at Asp 203 of this subunit. The high affinity binding site is accessible from the cytoplasm (under physiological conditions) and must be occupied with Na\(^+\), if decarboxylation shall occur. The low affinity binding site is accessible from the periplasm and may be occupied with Na\(^+\), H\(^+\), or remain empty. In this model only one of the two binding sites can be occupied with Na\(^+\), but protonation of Asp 203 does not prevent Na\(^+\) binding at the high affinity binding site. The catalytic cycle proceeds as follows: As soon as the biotin prosthetic group on the α subunit becomes carboxylated by carboxyltransfer from oxaloacetate (E→A), the negatively charged carboxybiotin can move into a binding pocket on the β subunit, where it becomes part of the Na\(^+\) binding site (A→B). With Na\(^+\) bound to this site, the enzyme appears in a closed conformation, where an exchange of Na\(^+\) is only possible between the two binding sites (B). The Na\(^+\) transfer may be facilitated by releasing the carboxybiotin from the binding pocket into the hydrophobic environment of the β subunit (B→C). The associated conformational change of the protein exposes the Na\(^+\) binding site at Asp 203 to the outside of the membrane, where the Na\(^+\) ion can be exchanged with another Na\(^+\) or by a H\(^+\) (C). Only the latter substitution leads to decarboxylation of the carboxybiotin through steps D to E and leads to a net Na\(^+\) translocation. The protein regains a closed conformation with concomitant regeneration of the high affinity site and Na\(^+\) binding to it (D). In this state, an exchange of Na\(^+\) and H\(^+\) between the two binding sites is possible. Acceptance of the H\(^+\) from Asp 203 by the carboxybiotin leads to decarboxylation, which opens the conformation, so that the Na\(^+\) ion can be released from its low affinity site to the periplasmic side of the membrane (E) (Di Berardino and Dimroth, 1996).
Suggestions of a second, lower affinity binding site for Na⁺ of glutaryl-CoA decarboxylase from *Acidaminococcus fermentans* were made, including the second highly conserved aspartate residue in the β subunit (Asp 149 of the oxaloacetate decarboxylase of *K. pneumoniae* or Asp 97 of the glutaryl-CoA decarboxylase of *Acidaminococcus fermentans*; Braune et al., 1999).

### 1.4 Topological studies of integral membrane proteins

The amino acid sequence of cytoplasmic proteins frequently reveals little or no information about how the polypeptide is folded into its three-dimensional structure. Integral membrane proteins, in contrast, are known to contain easily identifiable long continuous stretches of hydrophobic residues, which generally correspond to transmembrane α helical domains. With a plot of the average hydrophobicity along the sequence of a membrane protein, a possible two-dimensional model of its membrane topology can be drawn (Kyte and Doolittle, 1982; Rao and Argos, 1986). Further information about the protein can be obtained, for example, by probing protease susceptibility, location of glycosylation sites, antibody recognition, fusion to reporter proteins and electron X-ray diffraction (reviewed by Jennings, 1989).
The use of gene fusions to study membrane protein topology is now a well established technique. Following the generation of one or more topological models with hydrophathy plots and the "positive-inside rule" (Fasman and Gilpert, 1990; von Heijne, 1992; Hofmann and Stoffel, 1993; Cserzo et al., 1997), gene fusions can be used to confirm or to discriminate between such models. Various reporter proteins have been used to determine the membrane topology of integral membrane proteins, but the most commonly employed are alkaline phosphatase, β-galactosidase and β-lactamase (Prinz and Beckwith, 1994).

Alkaline phosphatase is a non-specific phosphomonoesterase that hydrolyses phosphate ester bonds from a variety of compounds at low pH concentrations. The enzyme consists of a homodimer with two intramolecular disulfide bridges per monomer (Kim and Wyckoff, 1990). This means, the enzyme remains inactive as long it is located inside the cytoplasm (Derman and Beckwith, 1991). The reducing conditions of the cytoplasm of E. coli and thioredoxin maintain the thiol groups protonated (Derman and Beckwith, 1991, 1995), and thus prevent disulfide bridge formation. However, the wild-type protein has a signal peptide that allows export to the periplasm. Here, the cysteine residues become oxidised and the active conformation of the enzyme is achieved.

Alkaline phosphatase hydrolyses 5-bromo-4-chloro-3-indolyl phosphate to give a product, that upon further oxidation in air gives a blue color. p-Nitrophenol phosphate is also hydrolysed by alkaline phosphatase. The product of this reaction, p-nitrophenol, absorbs light at 420 nm. Assaying the enzyme in the presence of this compound shows the activity of the alkaline phosphatase. If the enzyme is periplasmic it will be active in contrast to the enzyme found in the cytoplasm, which will be inactive.

β-Lactamase, the enzyme that confers ampicillin resistance, is another protein which is active in the periplasm and that has been used to characterize several membrane protein topologies through gene fusions (Boyd et al., 1987).

β-Galactosidase, like alkaline phosphatase and β-lactamase, can be fused to membrane-bound proteins to elucidate their topology. The enzyme is, in contrast to alkaline phosphatase, active in the cytoplasm only, where it converts lactose to galactose and glucose. The enzyme can hydrolyse the phosphorylated sugar derivative 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside, and the product is the same blue component, that is created by alkaline phosphatase (see above). As β-galactosidase is a cytoplasmic protein, its gene does not carry a signal sequence. Therefore, fusions of the target
protein to the start of the gene are carried out. As the fusions will be cytoplasmic, this technique is complementary to the alkaline phosphatase method of topology study.

Another approach to determine the topology of integral membrane proteins is the genetic introduction of cysteine residues and subsequent chemical labeling. This method has been used to study the topology, e.g., of subunit a in the *Escherichia coli* F$_1$F$_0$ ATP synthase (Valiyaveetil and Fillingame, 1998) and of the oxalate/formate transporter of *Oxalobacter formigenes* (Fu and Maloney, 1998). For this approach, impermeant and fluorescent probes were used to localize cytoplasmic and periplasmic loops. Recently, the membrane topology of the sodium ion-dependent citrate carrier of *Klebsiella pneumoniae* (CitS) was investigated by insertion into the endoplasmatic reticulum membrane (van Geest et al., 1999).

Further investigations on membrane protein topology were made by alanine insertion scanning mutagenesis of transmembrane helices (Braun et al., 1997). Single residue insertions into transmembrane helices are expected to be highly disruptive to protein structure and function. Insertions of amino acids into transmembrane helices of polytopic membrane proteins disrupt helix-helix interactions with loss of function, while insertions into loops have little effect on transmembrane helices and, therefore, little effect on activity. The inverse approach, amino acid deletion, was utilized systematically to approximate loop-helix boundaries in the lactose permease of *Escherichia coli* (Wolin and Kaback, 1999).

As the crystallization of integral membrane proteins is very difficult, there are other methods for gaining structural information. A perfect example how to get an idea of the structure without crystals is the lactose permease of *Escherichia coli*.

### 1.4.1 Helix packing in polytopic membrane proteins: the lactose permease of *Escherichia coli*

The lactose permease of *Escherichia coli*, encoded by the lacY gene, is a polytopic integral membrane protein and a paradigm for proteins that transduce free energy stored in electrochemical ion gradients into solute concentration gradients (Kaback, 1976; Kaback, 1983; Poolman and Konings, 1993). This hydrophobic, polytopic membrane protein catalyzes the coupled stoichiometric translocation of β-galactosides and H$^+$ and has been found solely responsible for β-galactoside transport as monomer (Viitanen et al., 1986; Sahin-Toth et al., 1994). All available experimental evidence indicates, that the protein is composed of 12 α-helical rods that traverse the membrane in a zigzag
fashion with N and C termini on the cytoplasmic face (for a review see Kaback, 1994; Figure 9). The entire lactose permease has been subjected to cysteine-scanning mutagenesis in order to determine which residues play an obligatory role in the mechanism. Analysis of the mutants has led to the following: 1) only six residues play an irreplaceable role in the transport mechanism; 2) the permease is a highly flexible molecule; 3) a helix packing model of the enzyme, 4) a working model that explains coupling between β-galactoside and H⁺ translocation (Frillingos et al., 1998).

**Figure 9**: Secondary structure model of the lactose permease (adopted from Frillingos et al., 1998). The one letter amino acid code is used, and putative transmembrane helices are shown in boxes. Residues irreplaceable with respect to active transport are enlarged: those involved in substrate translocation are shown in black; those involved in H⁺ translocation and coupling are shown in grey. Charge pairs are colored grey. Non-essential residues, thought to be involved in substrate translocation, are encircled.

Hydrophobic membrane proteins are notoriously difficult to crystallize, and a high resolution structure of the lactose permease is not available; therefore, the development of alternative methods for obtaining tertiary structure information is absolutely essential. Some noncrystallographic approaches to helix packing in the lactose permease are listed here:

**Second site suppressor analysis**

This classical genetic technique gives frequently important clues to proximity relationships. A primary mutation, which leads to loss of activity can be suppressed by a second mutation and partial or full activity is regained (King et al., 1991; Lee et al., 1992). It is assumed that the sites of primary and second mutation are in close contact.
If the two mutation sites reside on different helices, conclusions can be made on helix packing.

**Excimer fluorescence**

Two pyrene moieties can form an exited-state dimer (excimer) that exhibits an emission maximum at longer wavelengths (~ 470 nm) than the monomer (~ 380 - 420 nm) if the conjugated ring systems are within about 3.5 Å and in the correct orientation (Kinnunen et al., 1993). A lactose permease containing two cysteine replacements at desired positions could be labeled with the thiol-reactive probe N-(1-pyrenyl)maleimide and could be tested for excimer fluorescence (Jung, K. et al., 1993). In a number of other proteins proximity relationships have also been delineated by this method, e. g., rabbit skeletal α-tropomyosin (Betcher-Lange and Lehrer, 1978; Ishii and Lehrer, 1987), sarcoplasmic reticulum ATPase (Ludi and Hasselbach, 1987), lens proteins (Sen and Chakrabarti, 1990), troponin C (Wang et al., 1992) and the transmembrane domain of diphteria toxin (Zhan et al., 1994).

**Engineered divalent metal binding sites**

The fact, that bis-histidine residues are able to chelate divalent metal ions, is used for the design of metal-binding sites (Higaki et al., 1992). Mutants of the lactose permease were constructed, in which two positions, assumed to be close to each other, were replaced with histidine residues. Such mutants were analyzed using electron paramagnetic resonance (EPR) and the mutant proteins could be shown to bind the transition metal Mn²⁺ (Jung, K. et al., 1995; He et al., 1995a, b).

**Site-directed chemical cleavage**

Metal chelates bound to protein are effective reagents for the localized cleavage of polypeptide chains by reactive oxygen species (Ermacora et al., 1992; Rana and Meares, 1991). When EDTA-Fe is attached covalently to a protein, cleavage occurs close to the attachment site in the 3D structure. It was suggested, that the backbone segments, that are close to the EDTA-Fe attachment site, can be identified. Diffusible hydroxyl radicals and reagent-bound metal-peroxide species have been proposed to explain the reactions, as these highly reactive species attack the polypeptide backbone and sidechains leading to the chemical modification and protein fragmentation (Bateman et al., 1985; Rana and Meares, 1991).

**EPR of double nitroxide labeled mutants**

The magnetic dipolar interaction between two spin-labeled cysteinyI sidechains can be used to determine the proximity of domains within the protein (Rabenstein and Shin,
1995; Wu et al., 1996; Farrens et al., 1996; Mchaourab et al., 1997). The interspin
distance is proportional to spectral broadening due to the presence of a second label.
The technique is highly suitable for studying proximity relationships, because it is
devoid of many of the background problems.

**Metal-spin label interactions**

To provide inter-residue distance information in lactose permease, the magnetic dipolar
interaction between a site-directed nitroxide spin label and a paramagnetic metal ion
bound to an engineered site could be used (Voss et al., 1995).

**Site-directed cross-linking**

Expression of lactose permease in two contiguous, nonoverlapping fragments leads to
functional complementation (Zen et al., 1994; Bibi and Kaback, 1990; Wrubel et al.,
1990; Wrubel et al., 1994). A general method to study helix packing in lactose permease
in situ, is assessing the helix proximity by using site-directed disulfide or chemical
cross-linking of coexpressed lactose permease fragments (Wu and Kaback, 1996).

**Identification of discontinuous monoclonal antibody (mAB) epitopes**

Most interesting mABs against lactose permease are directed against conformational
epitopes. A technique has been developed, which involves binding studies with "right-
side-out" and "inside-out" membrane vesicles to determine the topology of the epitope
(Sun et al., 1996; Sun et al., 1997a, b).

By the combined use of these approaches a helix packing model of the lactose permease
has been determined (Figure 10), which was recently confirmed by two-dimensional
crystallization (Zhuang et al., 1999).
Figure 10: Helix packing in the lactose permease viewed from the cytoplasmic surface (adopted from Frillingos et al., 1998). Positions of the four irreplaceable residues for coupling are enlarged in black. Positions of the two charge pairs are enlarged in grey. Residues involved in substrate binding are shown in white.

Applying all these methods to the lactose permease even allowed to propose a model of the substrate binding site of the enzyme (see Figure 11).

Replacement of either Glu 126 or Arg 144 with various neutral amino acids completely abolishes active transport (Frillingos et al., 1997). Taken together with observations regarding the roles of Cys 148 and Met 145 in substrate binding (Jung, H. et al., 1994; Wu and Kaback, 1994), the results are consistent with the model.

The major points are the following. 1) One of the guanidino N atoms of Arg 144 H-bonds to the hydroxyl at the fourth and possibly the third position of the galactosyl moiety of the substrate, an interaction that plays a key role in the substrate specificity of the lactose permease. Substrate binds with low affinity, but has all the properties of any substrate of the lactose permease; glucose, which differs only in the orientation of the hydroxyl group at the fourth position of the pyranose ring, has no affinity (Wu and Kaback, 1994). 2) The other guanidino N atom is salt bridged with Glu 126, and the interaction holds Arg 144 and Cys 148 in the proper conformation to interact with the galactosyl moiety. One of the oxygen atoms of the carboxylate could also act as an H-bond acceptor from the hydroxyl at the sixth position of the galactosyl moiety. 3) Cys 148 interacts weakly and hydrophobically with the galactosyl moiety of lactose and
other galactosides (Jung, H. et al., 1994; Wu and Kaback, 1994). 4) Met 145 interacts more weakly with the glucosyl moiety of lactose.

![Image of lactose permease](image)

Figure 11: Substrate binding site of the lactose permease (adopted from Frillingos et al., 1998).

Maybe it could be possible to study the structure of the oxaloacetate decarboxylase by applying some of these methods to get further insight into the coupling mechanism, the structure of the Na" binding site, and the structure of the ion translocation pathway, located on the β subunit.

### 1.5 Aims of this work

a) The first aim of this work was to obtain a topological model of OadB, as the oadB gene encodes a very hydrophobic protein of 433 amino acid residues constituting the β subunit of the oxaloacetate decarboxylase Na" pump from Klebsiella pneumoniae. To study the mechanism of Na"-translocation across the membrane it is necessary to have a topology model, which is supported by experimental data similar to that of the lactose permease of *E. coli*.

b) Based on this, the next aim was to find polar amino acid residues involved into Na" binding and translocation or being part of the coordination sphere of Na" ions by site-directed mutagenesis studies. As previous experiments already denoted a specific protection of the β subunit by Na" ions from proteolytic digestion, a Na" binding site on
this subunit was suggested. Further evidence for the presence of a Na$^+$ binding site on
the \(\beta\) subunit has been obtained with sequence alignments with the related Na$^+$ pumps
glutaconyl-CoA decarboxylase and methylmalonyl-CoA decarboxylase. Conspicuous
residues, Asp 149 and Asp 203 within hydrophobic domains of the \(\beta\)-subunit have been
replaced. Mutants with Asp 203 to Glu or Asn substitution were completely inactive not
only in Na$^+$ transport but also in the decarboxylation of oxaloacetate. The
carboxyltransferase activity was not affected by these mutations. As one can imagine,
Asp 203 can not be the only residue essential for ion translocation, there must be other
amino acid residues involved in Na$^+$ and H$^+$ translocation.
c) Based on the results from b), the third aim of this work was to study the mechanism
of Na$^+$ translocation and to improve a model for the coupling mechanism of the
chemical and vectorial reaction, suggested before. It was proposed that Asp 203 not
only contributes to the Na$^+$ binding site for the transport of this alkali ion but is also
involved in H$^+$ transport in the opposite direction that leads to the decarboxylation of the
acid-labile carboxybiotin.
1.6 References


He, M. M., Voss, J., Hubbell, W. L., and Kaback, H. R. (1995a) Use of designed metal-binding sites to study helix proximity in the lactose permease of Escherichia coli. 1. Proximity of helix VII (Asp237 and Asp240) with helices X (Lys319) and XI (Lys358), Biochemistry 34, 15661-15666.

He, M. M., Voss, J., Hubbell, W. L., and Kaback, H. R. (1995b) Use of designed metal-binding sites to study helix proximity in the lactose permease of Escherichia coli. 2. Proximity of the helix IX (Arg302) with helix X (His322 and Glu325), Biochemistry 34, 15667-15670.


Wochlke, G., Laußermair, E., Schwarz, E., Oesterhelt, D., Reinke, H., Beyreuther, K., and Dimroth, P. (1992a) Sequence of the β-subunit of oxaloacetate decarboxylase


CHAPTER 2

Membrane Topology of the β-Subunit of the Oxaloacetate Decarboxylase Na⁺ Pump from Klebsiella pneumoniae.

PETRA JOCKEL, MARCO DI BERARDINO AND PETER DIMROTH

Mikrobiologisches Institut der Eidgenössischen Technischen Hochschule,
ETH-Zentrum, CH-8092 Zürich, Switzerland

Biochemistry (1999), in press.

Running title: Topology of the oxaloacetate decarboxylase β-subunit
2 Membrane Topology of the β-Subunit of the Oxaloacetate Decarboxylase Na⁺ Pump from Klebsiella pneumoniae

2.1 Abstract

The topology of the β-subunit of the oxaloacetate Na⁺ pump (OadB) was probed with the alkaline phosphatase (PhoA) and β-galactosidase (lacZ) fusion technique. Additional evidence for the topology derived from amino acid alignments and comparative hydropathy profiles of OadB with related proteins. Consistent results were obtained for the three N-terminal and the six C-terminal membrane-spanning α-helices. However, the two additional helices that were predicted by hydropathy analyses between the N-terminal and C-terminal blocks did not conform with the fusion results. The analyses were therefore extended by probing the sidedness of various engineered cysteine residues with the membrane-impermeant reagent 4-acetamido-4′maleimidylstilbene-2,2′-disulfonate. The results were in accord with those of the fusion analyses, suggesting that the protein folds within the membrane by a block of three N-terminal transmembrane segments and another one with six C-terminal transmembrane segments. The mainly hydrophobic connecting segment is predicted not to traverse the membrane fully, but to insert in an undefined manner from the periplasmic face. According to our model the N-terminus is at the cytoplasmic face and the C-terminus is at the periplasmic face of the membrane.

Abbreviations

AP, alkaline phosphatase; IASD, 4-acetamido-4′maleimidylstilbene-2,2′-disulfonic acid, disodium salt; IPTG, isopropyl-β-D-thiogalactopyranoside; LacZ, β-galactosidase; LB, Luria Bertani; MTSET, [2-(trimethylammonium)ethyl]methanethiosulfonate bromide; PhoA, alkaline phosphatase; SDS, sodium-dodecyl-sulfate; SDS-PAGE, sodium-dodecyl-sulfate-polyacrylamidegel-electrophoresis; TM, transmembrane
2.2 Introduction

Oxaloacetate decarboxylase of *Klebsiella pneumoniae*, whose overall geometry and function is shown in Figure 1, consists of three different subunits, α, β and γ with molecular masses of 63.5 kDa, 44.9 kDa and 8.9 kDa, respectively (Woehlke et al., 1992a; Laußermair et al., 1989; Schwarz et al., 1988; Dimroth, 1997; Dimroth and Schink, 1998). The cytoplasmic biotin-containing α-subunit is bound to the membrane-embedded βγ-subunits, which were shown to be closely associated (Di Berardino and Dimroth, 1995; Dimroth and Thomer, 1983; Dimroth and Thomer, 1988). The catalytic reaction cycle starts with the carboxyltransfer from oxaloacetate to the prosthetic biotin group that is attached to the C-terminal part of the α-subunit. This reaction is catalyzed at a low rate by the α-subunit alone (Dimroth and Thomer, 1983). In the additional presence of the Zn$^{2+}$-containing γ-subunit the rate increases approximately 1000-fold, probably because the metal ion polarizes the carbonyl oxygen bond of oxaloacetate, thereby facilitating the carboxyltransfer to biotin (Di Berardino and Dimroth, 1995). The carboxybiotin now moves from the carboxyltransfer site at the α-subunit to the decarboxylase site at the β-subunit. The mobility of the biotin residue is facilitated by an extended alanine/proline linker in the interdomain region between the carboxyltransferase and the biotin domain (Schwarz et al., 1988). In the course of the subsequent decarboxylation reaction, one to two Na$^+$ ions are pumped across the membrane into the periplasm, and one H$^+$ traverses the membrane in the opposite direction. This proton is consumed during the release of CO$_2$ from the biotin carboxylate (Dimroth and Thomer, 1983; Di Berardino and Dimroth, 1996).
The oadGAB genes encoding the γ, α and β-subunits of oxaloacetate decarboxylase have been cloned, sequenced and functionally expressed in *Escherichia coli* (Woehlke et al., 1992a; Laüßermair et al., 1989; Schwarz et al., 1988; Di Berardino and Dimroth, 1995). These genes are clustered on the chromosome together with other genes of the fermentative citrate metabolism (Bott and Dimroth, 1994). Previous experiments already denoted that the β-subunit (OadB) is specifically protected by Na\(^+\) ions from proteolytic digestion, suggesting a Na\(^+\) binding site on this protein (Dimroth and Thomer, 1983; Dimroth and Thomer, 1992). Complementary evidence for the presence of a Na\(^+\) binding site on the β-subunit has been obtained with the related Na\(^+\) pumps glutaconyl-CoA decarboxylase (Braune et al., 1999) and methylmalonyl-CoA decarboxylase (Hilpert and Dimroth, 1983). Conspicuous residues for such a site are two conserved aspartate residues (D149 and D203) within hydrophobic domains of the β-subunit. While mutagenesis of D149 to E or N did not yield a new phenotype, mutants with D203E or N substitution were completely inactive, not only in Na\(^+\) transport, but also in the decarboxylation of oxaloacetate, whereas the carboxyltransferase activity was retained (Di Berardino and Dimroth, 1996). Based on these and other results, a direct coupling mechanism has been proposed, in which D203 plays an essential role in both the vectorial and the chemical reaction. In the proposed mechanism, the carboxybiotin binds together with a Na\(^+\) ion close to D203 of the β-subunit. Subsequently, the Na\(^+\) ion is envisaged to switch to D203, and simultaneously, the proton originally bound to this residue moves to the biotin carboxylate, where it catalyzes immediate decarboxylation of this acid-labile compound. This exergonic reaction is coupled to the release of the bound Na\(^+\) ion to the positive side of the membrane (Di Berardino and Dimroth, 1996).

To select further amino acids for mutational analyses, we have investigated the topology of OadB by gene fusion analyses and by determining the sidedness of several engineered cysteine residues with a membrane-impermeable probe. The results indicate a topology for OadB, consisting of nine transmembrane helices with interconnecting cytoplasmic and periplasmic loops. Interestingly, a hydrophobic and highly conserved domain (segment IIIa, Figure 6B), linking helices III and IV that includes the catalytically
essential D203 residue does not appear to fold into membrane-spanning α-helices, as suggested from hydrophobic profiles. Part of this region rather seems to be oriented to the outer surface of the membrane, while other parts reside within the membrane but do not penetrate through it. Based on these results, a new topology model for OadB is proposed. Numbering of the hydrophobic segments and the connecting loops in the following text is according to this model (Figure 6B).

2.3 Experimental Procedures

Bacterial strains
The bacterial strains used in this study are Escherichia coli DH5α (Bethesda Research Laboratories), Escherichia coli JM110 (Yanish-Perron et al., 1995) and Escherichia coli CC118 (Manoil and Beckwith, 1985).

Media and reagents
The screening of active alkaline phosphatase (AP) and β-galactosidase fusion proteins was performed on Luria Bertani (LB) plates containing 100 μg/ml ampicillin, 40 μg/ml 5-bromo-4-chloro-3-indolyl-phosphate or 40 μg/ml 5-bromo-3-indolyl-β-d-galactopyranoside and 1mM isopropyl-β-D-thiogalactopyranoside (IPTG).

Recombinant DNA techniques
Standard recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). Polymerase chain reactions (PCRs) were performed using Vent-Polymerase from New England Biolabs (Beverly, MA, USA). DNA sequencing was carried out according to the dideoxynucleotide chain-termination method (Sanger et al., 1977) using a Taq DyeDeoxy terminator cycle sequencing kit and the ABI Prism 310 genetic analyzer from Applied Biosystems.

Construction of oadB-'phoA gene fusions
Nested deletions of the oadB gene from its C-terminus: The ‘phoA gene lacking its leader sequence was removed as PstI/NlaIV fragment from pCH2 (Hoffman and Wright, 1985) and cloned into pKAB (downstream of the β-subunit), which was digested with the compatible enzymes NsiI and NruI, resulting in plasmid pKAB-'PhoA. pKAB was constructed by replacing the BclI/SacI fragment of pSK-BXY (Di Berardino and Dimroth, 1995) with the corresponding fragment obtained by PCR technology, using primer A (5’-ACGCGCTGATCAACATCGTC-3’) on the BclI site (bold letters) of the β-subunit and primer KAB-fus (5’-
Membrane Topology of the β subunit

cgatcggggtccgatagatgcagcctcgcggcCTACATCGCCAGCACGT ACT-3') at the C-terminus of the β-subunit (upper case letters represent complementary bases at the C-terminus, the bases in lower case letters introduced the following restriction sites: Smal in italics, NruI in bold, NsiI underlined, SacI underlined in italics and NotI underlined in bold). Exonuclease III was used to progressively digest oadB from its 3' end via the 5' overhang created by NotI digestion of pKAB-'PhoA, essentially according to Henikoff (Henikoff, 1984). The 'phoA gene was protected from Exonuclease III digestion by the 3' overhang generating SacI site. After treatment with S1 nuclease and Klenow fragment, the ends were ligated together and the resulting plasmids transformed into competent E. coli CC118 cells. The screening of in-frame fusions was performed on LB plates, containing 5-bromo-4-chloro-3-indolyl-phosphate, IPTG and ampicillin (100 μg/ml). Plasmid DNA of blue and pale blue colonies was further examined by restriction analysis to approximately localize the fusion site and subjected to sequence analysis.

Site-directed phoA-fusions to predicted cytoplasmic and periplasmic loops of the β-subunit: The primers used for the synthesis of PCR products, containing the N-terminal region of oadB to which the 'phoA gene had to be fused and the resulting fusion sites are listed in Table 1 in Appendix. Fusions to the predicted periplasmic loop 4 (E217) as well as to the putative cytoplasmic loops 2 (V80, G134), 5 (E257), 7 (Q338) and 9 (A398) were obtained as follows: The PCR product resulting from primer BNH2 and the corresponding primer for a specific fusion site (E1, 4, 5 and 8, see Appendix) was digested with Kpnl and used to replace the oadB-containing KpnI/Sacl fragment from pKAB-'PhoA. In order to obtain an in-frame fusion, pKAB-'PhoA was first linearized with SacI, Mung Bean Nuclease treated to remove the 3' protruding termini and digested with Kpnl. The thus resulting plasmids were transformed into CC118 cells to determine their phosphatase activity. Further oadB-'phoA-fusions were constructed by amplifying PCR products from pKAB, using primer BNH2 for the N-terminal sequence of oadB and primers prP41, prP57, prV80, prP103, prN156, prT179, prG184, prT189, prT245, prL329 and prN365 which define the phoA-fusion site. The PCR products were digested with Kpnl and SacI and cloned into pKAB-'PhoA, from which the oadB-containing Kpnl/Sacl fragment was removed. For the localization of the C-terminus of the β-subunit, an in-frame PhoA-fusion to the C-terminal Met 433 was constructed. For this purpose, the PCR product, obtained with primer A and primer PhoA-C_term (see Appendix) using pKS-BXY (Di Berardino and Dimroth, 1995) as template DNA, was
digested with \textit{BclI} and \textit{NruI} and moved into pKAB, replacing the corresponding fragment to yield pKAB2. The \textit{PstI/NlaIV} fragment of pCH2 containing \textit{phoA} was cloned into pKAB2, which was digested with \textit{NsiI} and \textit{NruI} (\textit{NsiI} and \textit{PstI} produce compatible ends), yielding pKAB-PhoA\textit{end}. To make sure that no mutation occurred during the amplification and cloning steps, all PCR products as well as the fusion sites were sequenced.

\textit{Construction of oadB-\textit{lacZ} fusions}

\textit{Site-directed} \textit{lacZ}-fusions to predicted cytoplasmic loops and the C-terminus of the \textit{\beta}-subunit: The primers used for the synthesis of PCR products containing the N-terminal region of \textit{oadB} to which the \textit{lacZ} gene had to be fused and the resulting fusion sites are listed in Table A in Appendix. Fusions to the predicted cytoplasmic loops 2 (H72, V80) and to the hydrophobic segment IIIa (Q167, L180, G184, T189) and to the C-terminus were obtained as follows. The \textit{oadB-\textit{lacZ}}-fusions were constructed by amplifying PCR products from pKAB, using primer \textit{BNH2} for the N-terminal sequence of \textit{oadB} and primers \textit{prH72lac}, \textit{prV80lac}, \textit{prQ167lac}, \textit{prL180lac}, \textit{prG184lac} and \textit{prT189lac}, which define the \textit{lacZ}-fusion site. The PCR products were digested with \textit{KpnI} and \textit{ApaI} and cloned into pKAB-\textit{lacZ}, from which the \textit{oadB}-containing \textit{KpnI/NotI} fragment was removed. pKAB-\textit{lacZ} was constructed by removing the \textit{lacZ} gene as \textit{Dral/PstI} fragment from pNM480 (Minton, 1984) and cloned into pKAB (downstream of the \textit{\beta}-subunit), which was digested with the compatible enzymes \textit{NsiI} and \textit{NruI}. For the localisation of the C-terminus of the \textit{\beta}-subunit, an additional in-frame \textit{LacZ} fusion to the C-terminal Met 433 was constructed. For this purpose, the PCR product, obtained with primer \textit{A} and primer \textit{PhoA-C_{term}} (see Appendix) using pKS-BXY (Di Berardino and Dimroth, 1995) as template DNA, was digested with \textit{BclI} and \textit{NruI} and moved into pKAB, replacing the corresponding fragment to yield pKAB2. The \textit{PstI/DraI} fragment of pNM 481 (Minton, 1984), containing \textit{lacZ}, was cloned into pKAB2, which was digested with \textit{NsiI} and \textit{NruI} (\textit{NsiI} and \textit{PstI} produce compatible ends), yielding pKAB-\textit{LacZ\textit{end}}. To make sure that no mutation occurred during the amplification and cloning steps, all PCR products as well as the fusion sites were sequenced.

\textit{Site-directed cysteine mutants of the \textit{\beta}-subunit}

The primers used for the site-directed mutagenesis are listed in Table B in Appendix. Site-directed cysteine mutants, placed into the predicted cytoplasmic loop 2 and into hydrophobic segment IIIa, were obtained as follows: The PCR fragments, containing the mutation, were constructed in a two step protocol. For the N-terminal part of the
PCR fragments of the β-subunit, primer prN and the primers prV129Crev, prL178Crev, prY182Crev, prS187Crev, prP191Crev, prA194Crev, prI198Crev and prP205Crev were used, pSK-GABC87A served as template. For the corresponding PCR fragments for the C-terminal part of the β-subunit, primer prC and the primers prV129Cfor, prL178Cfor, prY182Cfor, prS187Cfor, prP191Cfor, prA194Cfor, prI198Cfor and prP205Cfor were used, pSK-GABC87A served as template. After purification, those PCR fragments served as templates for yielding the PCR products from primers prN and prC. The PCR products from this second step contained the mutation. PCR fragments were digested with KpnII and BclI and cloned into pSK-GAB (Di Berardino and Dimroth, 1995). From pSK-GAB, which was isolated from JM110 cells, the KpnII/BclI fragment was removed. Site-directed cysteine mutants, placed into the N- and C-terminus, were obtained as follows: The PCR fragments, containing the mutation, were constructed in a two step protocol, as mentioned above. For the mutation at the N-terminus of the β-subunit, primers prNruIfor and prL7Crev were used for the N-terminal part of the PCR fragment. For the C-terminal part of the PCR fragment, primers prNrev and prL7Cfor were used. Plasmid pSK-GAB served as template. After purification, those PCR fragments served as templates for yielding the PCR products from primers prNruIfor and prNrev. For the mutation at the C-terminus of the β-subunit, primers prBN and prV430Crev were used for the N-terminal part of the PCR fragment and primers prBC and prV430Cfor were used for the C-terminal part. Plasmid pSK-GAB served as template. After purification those PCR fragments served as templates for yielding the PCR products from primers prBN and prBC. The PCR products from this second step contained the mutation. PCR fragments were digested with NruI and KpnII (L7C mutation) or BclI and BstI107I (V430C mutation) and cloned into pSK-GABC87A. From pSK-GABC87A, which was isolated from E. coli JM110 cells, the NruI/KpnII (L7C mutation) or BclI/BstI107I (V430C mutation) fragment was removed. Plasmid pSK-GABC87A was obtained the same way, using primers prC87Arev and prC87Afor, primerN and primerC and pSK-GAB (Di Berardino and Dimroth, 1995) as template.

Enzyme assays
Assays of alkaline phosphatase and β-galactosidase activities. Precultures of Escherichia coli CC118 cells, harboring the oadB-‘phoA fusion plasmids, were grown overnight in LB medium, containing 100 μg/ml ampicillin and used to inoculate 5 ml LB cultures, containing 100 μg/ml ampicillin and 10 mM IPTG. Cultures were grown
for two hours to reach an $A_{600}$ of 0.5-0.8. The cells were centrifuged, resuspended in 1M Tris/HCl pH 8.0 and washed twice. The cells were resuspended in 3 ml 1M Tris/HCl pH 8.0 to obtain an $A_{600}$ of 0.8 to 1.0, permeabilized with sodium-dodecyl-sulfate (SDS) and methylene chloride and assayed for alkaline phosphatase activity, as described by Miller (1972) and Pourcher et al. (1996). Each assay was performed at 37°C in duplicate on two separate occasions. The units of enzyme activity were calculated according to the references mentioned above. Assays of $\beta$-galactosidase activity were performed as described by Miller (1972). Each assay was performed in duplicate on separate occasions. Oxaloacetate decarboxylase activities of site-directed cysteine mutants were determined as described (Dimroth, 1986).

**Verification of the expression of the $\beta$-subunit**

To be sure that the $\beta$-subunit was functionally expressed from the plasmids, which were used as targets for the 'phoA and 'lacZ fusion constructs, *E. coli* cells, carrying pKS-BXY (Di Berardino and Dimroth, 1995) as well as pKAB and pKAB2, were assayed for their ability to reconstitute the decarboxylation activity after incubation with the missing $\gamma$-subunits. For this purpose, membrane vesicles of the mentioned *E. coli* clones were prepared, solubilized with 2% Triton X-100 and incubated with isolated $\gamma$-complexes as described (Di Berardino and Dimroth, 1995). The decarboxylation activity was subsequently determined (Dimroth, 1986).

**Western immunoblotting analysis**

Cultures were grown under the same conditions described for the phosphatase assay and $\beta$-galactosidase assay experiments and adjusted to an $A_{600}$ of 0.8 to 1.0. The sedimented cells were resuspended in SDS-PAGE loading buffer, boiled for 5 min and electrophoresed on 8% (PhoA fusions) and 10% (LacZ fusions) SDS-polyacrylamide gels, according to Schägger and von Jagow (1987). The proteins were blotted onto a nitrocellulose membrane (Amersham Life Science, Buckinghamshire, UK), which was subsequently incubated with antiserum against bacterial alkaline phosphatase or $\beta$-galactosidase. Bound antibody was visualized with goat anti-rabbit IgG (PhoA fusions) or goat anti-mouse IgG (LacZ fusions) fused to alkaline phosphatase (AP) (Bio-Rad Laboratories, Richmond, CA, USA and Sigma-Aldrich) and using nitrotetrazolium blue and 5-bromo-4-chloro-3-indolyl phosphate for color development.
**Computer analyses**

Hydropathy plots were carried out with the TMpred server of the bioinformatics group of the ISREC (Swiss Institute for Cancer Research) (Hofmann and Stoffel, 1993).

**Thiol-specific fluorescence labeling**

Site-specific fluorescence labeling was designed to selectively alkylate cysteines exposed either to the periplasmic or to the cytoplasmic face of the membrane (Fu and Maloney, 1998). To label cysteines exposed to the periplasm, intact cells (5 - 6 g wet weight) were harvested by centrifugation (4,000 g x 30 min) and resuspended in 200 ml Buffer A (100 mM sodium sulfate, 50 mM potassium phosphate, pH 8.0), containing 40 μM freshly prepared IASD. After a 20-min incubation at 23°C, the labeling reaction was quenched by addition of 2 mM β-mercaptoethanol, followed immediately by three cycles of centrifugation and washing, using Buffer A alone. Labeled cells were resuspended in 10 ml 20 mM potassium phosphate pH 7.5, 0.5 M NaCl, 1 mM Mg-ethylenediaminetetraacetic acid, 0.02 mM diisopropyl fluorophosphatc and DNase I. Oxaloacetate decarboxylase was purified by affinity chromatography of a solubilized (Triton X-100) membrane extract on a SoftLink monomeric avidin-Sepharose column (Promega), as described (Dimroth, 1986). The eluate of the avidin-Sepharose column was concentrated in a Biomax centrifugal filter 10K (Millipore) up to 50 to 60 μl.

Cysteines exposed to the cytoplasm were identified by a two-step protocol. External cysteines were blocked by an initial 5-min preincubation of intact cells (5 - 6 g wet weight) in 200 ml Buffer A, containing 200 μM MTSET (freshly dissolved), a nonfluorescent, membrane-impermeable thiol specific reagent. Excess MTSET was removed by three cycles of centrifugation and washing with Buffer A. Cells were resuspended in 10 ml 20 mM potassium phosphate pH 7.5, 0.5 M NaCl, 1 mM Mg-ethylenediaminetetraacetic acid, 0.02 mM diisopropyl fluorophosphatc and DNase I. After preparing inside-out vesicles (Dimroth, 1986), these were dissolved in Buffer C (20 mM sodium phosphate pH 8.0). The exposed unmodified cysteines, which had faced the cytoplasm, were labeled by adding 40 μM IASD (freshly dissolved) and incubated for 20 min at 23°C. The reaction was quenched with 2 mM β-mercaptoethanol, followed immediately by one cycle of centrifugation and washing with Buffer C alone. Vesicles were resuspended in 20 mM potassium phosphate pH 7.5, containing 0.5 M NaCl. Oxaloacetate decarboxylase was purified, as described above and subjected to SDS-PAGE (20 μg protein per sample, βC87A/L7C variant 6.8 μg protein per sample), using 10% SDS-polyacrylamide gels according to Schägger and
von Jagow (1987). After electrophoresis, the gel was rinsed briefly with a destaining solution (10% glacial acetic acid, 15% methanol), and fluorescence was monitored at an excitation wavelength of 312 nm. The gels were subsequently stained with silver.

2.4 Results

Hydropathy profile and sequence alignments of the *K. pneumoniae* oxaloacetate decarboxylase β-subunit (OadB) with related proteins

Based on hydropathy plots, a topology for OadB with nine membrane-spanning α-helical segments was proposed previously (Woehlke et al., 1992b). Nine to eleven membrane-spanning α-helices were envisaged for the related β-subunits of the methylmalonyl-CoA decarboxylase (MmdB) Na⁺ pumps from *Veillonella parvula* (Huder and Dimroth, 1993) and *Propionigenium modestum* (Bott et al., 1997), for the glutaconyl-CoA decarboxylase from *Acidaminococcus fermentans* (Braune et al., 1999) and for the malonate decarboxylase from *Malonomonas rubra* (MadB) (Berg et al., 1997). Additional members of this family have been identified in genome sequencing projects: a MadB homologue in *Rhodobacter capsulatus* and OadB homologues in *Archaeoglobus fulgidus* (Klenk et al., 1997) and *Treponema pallidum* (Fraser et al., 1998). Hydropathy profiles of a selection of related proteins are shown in Figure 2. Clearly, the profiles reveal a considerable degree of similarity. Characteristic is a very hydrophilic region near the middle of the molecules that is flanked on both sides by a number of hydrophobic stretches. These are mostly connected by only short hydrophilic loops. Ten hydrophobic portions are found in all proteins and have been numbered I, II, III, IIIa, IV, V, VI, VII, VIII and IX. The most clearly defined hydrophobic stretches in OadB and MmdB are the segments I, III-VII and IX. These are regions predicted to be membrane-spanning domains in previous models. (Woehlke et al., 1992b; Huder and Dimroth, 1993). The profile of MadB shows that stretch II is clearly an additional hydrophobic domain that must be considered as a membrane-spanning α-helical segment. The less pronounced hydrophobicity apparent for OadB and MmdB in this region is due to the presence of either glutamate or arginine in these sequences (Figure 3). The other region in question of forming a membrane integral domain of these proteins is area VIII. Due to a conserved arginine or lysine residue in this segment, the hydrophobicity is not very pronounced, and with the profile alone one cannot predict, whether area IX spans the membrane.
The alignment of the sequences of the related proteins, shown in Figure 3, provides additional information on the putative membrane-spanning domains. The most similar regions of these proteins fall into the ten hydrophobic stretches. Next to segment IIIa, which contains the catalytically essential aspartic acid residue (Di Berardino and Dimroth, 1996), the most highly conserved portion is segment VIII. One should notice that the length of nine of the hydrophobic areas of the proteins is conserved, comprising between 20 and 28 amino acid residues, which is in the range expected for membrane-spanning α-helices. The hydrophobic segment IIIa, however, is much longer, which may be due to an unusual folding (see below). Gaps of more than one residue can be noticed in the N- and C-terminal portions and in the segment connecting the hydrophobic domains II and III and in the hydrophobic segment IIIa, respectively.

From all these results, the structure of OadB and the related proteins could include ten transmembrane helical segments. However, no information is available from these data.
on the orientation of the protein within the membrane. In order to fill this gap and to get more confidence in the predicted membrane topology of OadB, the gene fusion technology with alkaline phosphatase and β-galactosidase was applied (Manoil, 1990; Manoil et al., 1990; Manoil, 1991; Traxler et al., 1993).

**Generation of oadB-‘phoA and oadB-‘lacZ gene fusions**

Two different approaches were used to fuse OadB, truncated to various degrees from the C-terminus, to alkaline phosphatase (AP). One set of fusions was obtained by the nested-deletion method with Exonuclease III (Henikoff, 1984; Sugiyama et al., 1991), using plasmid pKAB-‘PhoA. Gene fusions, encoding hybrid proteins with decreasing length of OadB at its C-terminus joined to AP, were thus generated. Clones with positive AP activities (transformants of various blue color intensities on XP plates) were selected and subjected to restriction and sequence analyses, as described under Experimental Procedures. In the second approach, PCR technology was applied to cover those areas of the protein within the predicted loop structures and at the C-terminal end that were not already obtained by the first method. Thus, a total of 24 different uniformly distributed OadB-PhoA fusions were obtained by the two different approaches and used for the determination of AP activity. A total of 7 different OadB-LacZ fusions were constructed by PCR technology and used to get complementary evidence for the folding of OadB within the membrane by measuring β-galactosidase activities.

**Alkaline phosphatase (AP) and β-galactosidase activities of OadB-PhoA and OadB-LacZ fusion products**

The AP activities of the fusion proteins, listed in Table 1, vary over the range from 0 to 93 Miller units. Colonies with AP activities > 5 units were blue, while those with activities < 5 units were white. Candidates of the first category were therefore regarded as positive and those of the second category as negative PhoA fusions. In-frame PhoA fusions to the cytoplasmic domain of the protein show 20 to 40 times less activity than fusions at periplasmic domain sites (Manoil and Beckwith, 1986). In previous topology analyses for melibiose permease (Pourcher et al., 1996) or Mtr permease (Sarsero and Pittard, 1995) of *Escherichia coli*, the AP activities of fusion proteins varied between 1 and 137 Miller units. The authors defined activities below 20 units as negative, between 20 and 60 units as intermediate, and above 60 units as positive.
FIGURE 3. Sequence alignment of Mr-MadB with Rc-MadB (related subunit from R. capsulatus), Kp-OadB, St-OadB (related subunit from S. typhimurium), Af-OadB (related subunit from A. fulgidus), Pm-MmdB, Vp-MmdB and Tp-OadB (related subunit from T. pallidum) (for other abbreviations see Figure 2). The putative membrane domains are boxed and numbered as in Figure 6B. Identical residues are indicated by an asterisk, conservative exchanges by a dot. Sequence of Kp-OadB in bold and underlined.
The β-galactosidase activities of the fusion proteins, listed in Table 2, vary over a range from 0 to 463 Miller units. LacZ fusion proteins with activities < 50 Miller units were regarded as negative and fusions with activities > 350 Miller units as positive. Sarsero and Pittard (Sarsero and Pittard, 1995) found activities between 6 and 246 Miller units and distributed them into categories below 20, between 20 and 60, and over 60 Miller units. In the topological models of OadB (Figure 6), we have marked fusion sites yielding positive AP with blackk circles and those yielding negative AP with white circles. Positive LacZ fusions are shown in grey circles and negative LacZ fusions are shown in white circles with grey outline.

In the following, we will go through the models by describing the results of the fusion analyses, starting from the C-terminus. In our new model (Figure 6B), we place the C-terminal methionine on the periplasmic surface which contrasts the model proposed previously (Woehlke et al., 1992b; Figure 6A). The PhoA fusion at this side was considered positive by the blue colony color and the alkaline phosphatase activity of 7 Miller units. This comparatively low activity is explainable by low stability of the fusion construct (Table 1). The complementary LacZ fusion was with 29 Miller units negative, according to our definition, especially as the fusion protein was strongly expressed. It will be shown below that an engineered cysteine at position 430, three residues before the C-terminus, was specifically accessible from the periplasm. Hence, the C-terminus is located at the periplasmic side of the membrane. This defines the orientation of helix IX, which is further supported by the low AP activity (2 Miller units, white colonies) of the A398-fused product. The membrane topology between residues 360 and 217 is the same in the two different models and therefore consistent with the results from hydropathy profile and fusion analyses. As both N365 and the C-terminus are periplasmically located, whereas A398 faces the cytoplasm, we need to define an additional membrane spanning helix (VIII, in Figure 6B) within the region between N365 and A398.

The information on the membrane topology of OadB obtained from the PhoA and LacZ fusion approach was also valuable for the N-terminal part of the molecule, although the results were not as clear as for the C-terminal part. The positive PhoA fusions at position 41 and 156 might indicate that these residues are in periplasmic loops, as drawn in the model shown in Figure 6B. This would indicate three transmembrane helices in the N-terminal region before N156 and a cytoplasmic location of the N-terminus. The outgoing helix I is very hydrophobic and is followed by a short hydrophilic loop and the
ingoing membrane helix II (not predicted in the previous model). The polypeptide chain continues with the extended cytoplasmic loop 2 and the outgoing helix III, running from I135 to A155. The orientation of the latter helix is the same in both models and is supported by the negative PhoA fusions at P103 and G134. The two very strong LacZ fusions at H72 and V80 further support the topology of OadB, shown in Figure 6B. However, the PhoA fusions at P57, H72 and V80 were also positive and, therefore, apparently contradicting the proposed topology. These results indicate that the short N-terminal peptides probably do not adopt unequivocal topologies, but may flip between both surfaces. Therefore, the topology cannot be predicted for the N-terminal part of the protein by fusion analyses alone. To further analyze the topology, the location of C87 and the mutants βC87A/L7C and βC87A/V129C was determined. The cytoplasmic location of all three cysteine residues confirms the topology model of Figure 6B (see below).

The hydrophobic region, flanked by residues T159 and S211, linking the N-terminal block of three membrane helices and the C-terminal block of six helices could fold into two additional membrane-spanning segments, connected by a short loop, as proposed previously (Figure 6A). However, three positive PhoA fusions (T179, G184, T189) and three negative LacZ fusions (L180, G184, T189) in the suggested cytoplasmic loop argue against this model, and rather indicate that the region around residues T179-T189 is exposed to the periplasm.
<table>
<thead>
<tr>
<th>Fusion position $^a$</th>
<th>Alkaline phosphatase activity $^b$</th>
<th>Anticipated location $^c$</th>
<th>Colony color $^d$</th>
<th>Signal strength in Western blots</th>
</tr>
</thead>
<tbody>
<tr>
<td>P41</td>
<td>36 +/- 7</td>
<td>Loop 1 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>P57</td>
<td>56 +/- 2</td>
<td>Helix II</td>
<td>blue</td>
<td>strong</td>
</tr>
<tr>
<td>H72</td>
<td>17 +/- 1</td>
<td>Loop 2 C</td>
<td>pale blue</td>
<td>weak</td>
</tr>
<tr>
<td>V80</td>
<td>63 +/- 4</td>
<td>Loop 2 C</td>
<td>blue</td>
<td>strong</td>
</tr>
<tr>
<td>P103</td>
<td>2 +/- 1</td>
<td>Loop 2 C</td>
<td>white</td>
<td>strong</td>
</tr>
<tr>
<td>G134</td>
<td>0</td>
<td>Loop 2 C</td>
<td>white</td>
<td>weak</td>
</tr>
<tr>
<td>N156</td>
<td>9 +/- 2</td>
<td>Loop 3 P</td>
<td>blue</td>
<td>strong</td>
</tr>
<tr>
<td>T179</td>
<td>93 +/- 2</td>
<td>Loop 4 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>G184</td>
<td>33 +/- 3</td>
<td>Loop 4 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>T189</td>
<td>21 +/- 4</td>
<td>Loop 4 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>E217</td>
<td>12 +/- 2</td>
<td>Loop 5 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>T245</td>
<td>5 +/- 2</td>
<td>Loop 6 C</td>
<td>white</td>
<td>average</td>
</tr>
<tr>
<td>L257</td>
<td>0</td>
<td>Loop 6 C</td>
<td>white</td>
<td>instable</td>
</tr>
<tr>
<td>L286</td>
<td>37 +/- 3</td>
<td>Helix V</td>
<td>blue</td>
<td>weak</td>
</tr>
<tr>
<td>M289</td>
<td>39 +/- 4</td>
<td>Helix V</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>V301</td>
<td>10 +/- 1</td>
<td>Loop 7 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>L329</td>
<td>2 +/- 0</td>
<td>Loop 8 C</td>
<td>white</td>
<td>average</td>
</tr>
<tr>
<td>Q338</td>
<td>3 +/- 2</td>
<td>Loop 8 C</td>
<td>pale blue</td>
<td>average</td>
</tr>
<tr>
<td>V344</td>
<td>11 +/- 3</td>
<td>Helix VII</td>
<td>blue</td>
<td>weak</td>
</tr>
<tr>
<td>G357</td>
<td>30 +/- 4</td>
<td>Helix VII</td>
<td>blue</td>
<td>instable</td>
</tr>
<tr>
<td>V358</td>
<td>30 +/- 7</td>
<td>Helix VII</td>
<td>blue</td>
<td>instable</td>
</tr>
<tr>
<td>N365</td>
<td>46 +/- 15</td>
<td>Loop 9 P</td>
<td>blue</td>
<td>average</td>
</tr>
<tr>
<td>A398</td>
<td>2 +/- 0</td>
<td>Loop 10 C</td>
<td>white</td>
<td>weak</td>
</tr>
<tr>
<td>M433</td>
<td>7 +/- 3</td>
<td>C-Terminus</td>
<td>blue</td>
<td>weak</td>
</tr>
</tbody>
</table>

Table 1. Properties of OadB-PhoA fusions. $^a$ Amino acid in OadB after which alkaline phosphatase is fused. $^b$ Units of alkaline phosphatase activity calculated according to Miller (1972) and Pourcher et al. (1996). $^c$ according to the new model (Figure 6B). $^d$ Color of colonies of Escherichia coli CC118 on LB Amp100X'O'plates.
Membrane Topology of the β subunit

<table>
<thead>
<tr>
<th>Fusion position</th>
<th>β-galactosidase activity</th>
<th>Anticipated location</th>
<th>Signal strength in Western blots</th>
</tr>
</thead>
<tbody>
<tr>
<td>I172</td>
<td>463 +/- 21</td>
<td>Loop 2 C</td>
<td>strong</td>
</tr>
<tr>
<td>V80</td>
<td>399 +/- 3</td>
<td>Loop 2 C</td>
<td>strong</td>
</tr>
<tr>
<td>Q167</td>
<td>436 1/- 56</td>
<td>Region IIIa</td>
<td>strong</td>
</tr>
<tr>
<td>L180</td>
<td>15 +/- 1</td>
<td>Loop 4 P</td>
<td>average</td>
</tr>
<tr>
<td>G184</td>
<td>0</td>
<td>Loop 4 P</td>
<td>average</td>
</tr>
<tr>
<td>T189</td>
<td>0</td>
<td>Loop 4 P</td>
<td>average</td>
</tr>
<tr>
<td>M433</td>
<td>29 +/- 23</td>
<td>C-Terminus</td>
<td>strong</td>
</tr>
</tbody>
</table>

Table 2. Properties of OadB-LacZ fusions. a Amino acid in OadB after which β-galactosidase is fused. b Units of β-galactosidase activity calculated according to Miller (1972). c according to the new model (Figure 6B).

Stability of fusion proteins

To investigate whether the results obtained from the AP and β-galactosidase activity measurements correlated with the stability of the fusion proteins, Western immunoblot analyses of the OadB-PhoA and OadB-LacZ fusions were performed. Figure 4 shows a selection of immunoblots with OadB-PhoA fusions, for which cell lysates were used. In most cases, the expected fusion products are visible, although they are accompanied by a degradation product that corresponds to the native AP and in part by additional degradation products. The approximate intensities of all OadB-PhoA fusion bands are compiled in Table 1 and of the OadB-LacZ fusion bands in Table 2. In accord with previous studies, the alkaline phosphatase fusions to periplasmic loops were usually more stable, yielding stronger bands than those to cytoplasmic loops or to amino acids within helices (Boyd et al., 1987; Derman and Beckwith, 1991; Derman and Beckwith, 1995). Exceptions are the fusions at V80 and P103 within loop 2, which yielded strong signals on the immunoblot. The variance of alkaline phosphatase activities is therefore partly due to different stabilities of the fusion products. However, as alkaline phosphatase, cleaved from the fusion sites, may contribute to the measured AP activities, we did not attempt to correct these activities for the different expressions of
the fusion products, as observed by the Western analyses. Nevertheless, the correlation of white colonies with very low to zero AP activities and of blue colonies with middle to high AP activities allows with reasonable confidence to place the OadB-PhoA fusion to the cytoplasmic or periplasmic side of the membrane.

![Immunoblot analysis of OadB-PhoA fusion products. Lysates of logarithmically grown cells were electrophoresed on a 8% SDS-polyacrylamide gel and blotted onto a nitrocellulose membrane. Anti-PhoA antiserum was used to detect OadB-PhoA fusion proteins. The lanes are labeled after the amino acid residue of the β-subunit, to which PhoA was fused. (M), molecular mass marker; (AP), purified alkaline phosphatase from E. coli, marked with an arrow. Upper band in the immunoblot represents the OadB-PhoA fusion protein. Bands between AP and the fusion proteins represent degradation products. M, P41, P57, G134, N156, G184 and T189 resulted from trial A; AP and P103 from trial B; E217 and T245 from trial C; L286, M289 and L329 from trial D; N365 from trial E, and A398 and M433 from trial F.](image)

**Construction of OadB cysteine variants and sidedness of cysteine residues**

To construct cysteine OadB variants, we first removed the cytoplasmic cysteine 87 and changed it against alanine. This mutation did not interfere with catalytic activity (Table 3). Membrane-located cysteines 291 and 351 did not have to be removed, because they were not accessible for IASD. Ten new OadB cysteine variants were obtained, which are listed in Table 3 together with their specific oxaloacetate decarboxylase activities, their sidedness and their anticipated location. All double mutants exhibited specific oxaloacetate decarboxylase activities between 15 and 60 U/mg protein, except for
cysteine mutants in the N-terminal tail and for two proline mutants in the hydrophobic region IIIa, which were inactive. All mutant proteins were synthesized at nearly identical levels as the wild-type enzyme, except for the mutants with P/C substitutions and the cysteine mutant in the N-terminal tail, which were only poorly synthesized. Accessibility of cysteine residues was determined with the membrane-impermeable, thiol specific fluorescent probe IASD (Fu and Maloney, 1998). Labeling of periplasmically oriented cysteines was achieved by direct exposure of intact cells to the probe, while labeling of cytoplasmically oriented cysteines was performed with inside-out membrane vesicles, after external targets had been alkylated by a nonfluorescent membrane-impermeable blocking agent (MTSET). After the labeling reaction, the mutant oxaloacetate decarboxylases were purified by affinity chromatography on avidin-Sepharose, subjected to SDS-PAGE and inspected for protein bands, containing the fluorescent label. The data of Figure 5A show that the α-subunit of all decarboxylase variants investigated was specifically labeled from the cytoplasmic side of the membrane. In all samples, the intensities of the fluorescence of the α-subunit was comparable, as were the intensities of the silver-stained bands of the α- as well as of the β-subunits (Figure 5B). An exception is the βC87/L7C variant, where less protein was applied to the gel. The data further show that βC87 of the wild-type was exposed to the cytoplasm. Cysteine variants which became labeled from the cytoplasmic surface were βC87A/L7C and βC87A/V129C. The βC87A/E7C mutant deserves attention, because the oxaloacetate decarboxylase containing the βE7C substitution was inactive. Similar results were obtained with another cysteine mutant near the N-terminus (βC87A/L4C) and after insertion of the FLAG epitope after position M1 of the protein. Hence, an unmodified N-terminal region of OadB seems to be critical for the function of the enzyme, and therefore, the mutants in this region do not provide reliable information for assessment of the topology. A periplasmic location was on the other hand found for the cysteine variants βC87A/S187C and βC87A/V430C. These results confirm exposure of the segment of region IIIa around S187 to the periplasm and further strengthen the periplasmic exposure of the C-terminal tail. The other cysteine mutants investigated were not labeled with the fluorescent probe applied from either side of the membrane, indicating that residues L178, Y182, P191, A194, I198 and P205 are located within the membrane or buried within a pocket of the protein. This also applies to the inaccessible cysteine residues of the wild-type β subunit C291 and C351.
<table>
<thead>
<tr>
<th>OadB cysteine variant</th>
<th>specific activity (U/mg)</th>
<th>sidedness(^a)</th>
<th>anticipated location(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type (C87)</td>
<td>26</td>
<td>cytoplasm</td>
<td>Loop 2 C</td>
</tr>
<tr>
<td>C87A</td>
<td>32</td>
<td>not accessible</td>
<td>Loop 2 C</td>
</tr>
<tr>
<td>C87A/L7C</td>
<td>inactive</td>
<td>cytoplasm</td>
<td>N-terminal tail</td>
</tr>
<tr>
<td>C87A/V129C</td>
<td>60</td>
<td>cytoplasm</td>
<td>Loop 2 C</td>
</tr>
<tr>
<td>C87A/L178C</td>
<td>15</td>
<td>not accessible</td>
<td>Region IIIa</td>
</tr>
<tr>
<td>C87A/Y182C</td>
<td>33</td>
<td>not accessible</td>
<td>Loop 4 P</td>
</tr>
<tr>
<td>C87A/S187C</td>
<td>27</td>
<td>periplasm</td>
<td>Loop 4 P</td>
</tr>
<tr>
<td>C87A/P191C</td>
<td>inactive</td>
<td>not accessible</td>
<td>Region IIIa</td>
</tr>
<tr>
<td>C87A/A194C</td>
<td>28</td>
<td>not accessible</td>
<td>Region IIIa</td>
</tr>
<tr>
<td>C87A/I198C</td>
<td>25</td>
<td>not accessible</td>
<td>Region IIIa</td>
</tr>
<tr>
<td>C87A/P205C</td>
<td>0.7</td>
<td>not accessible</td>
<td>Region IIIa</td>
</tr>
<tr>
<td>C87A/V430C</td>
<td>29</td>
<td>periplasm</td>
<td>C-terminal tail</td>
</tr>
</tbody>
</table>

Table 3. Features of cysteine variants of OadB. \(^a\) probed with the membrane-impermeant agent IASD (Fu and Maloney, 1998). \(^b\) according to the new model (Figure 6B).
Membrane Topology of the β subunit

FIGURE 5. SDS-PAGE of purified oxaloacetate decarboxylases after site-specific labeling of cysteine residues with the fluorescent probe TASD and corresponding silver-stained SDS-gels. (A) Cysteine residues accessible from the periplasm (P) and cytoplasm (C) and (B) corresponding silver-stained SDS-gels. The soluble α-subunit serves as positive control for accessibility from the cytoplasm, as it contains 4 cysteine residues. Wildtype and cysteine variants βC87A, βC87A/L178C, βC87A/Y182C, βC87A/S187C, βC87A/P191C, βC87A/A194C, βC87A/I198C, βC87A/P205C resulted from trial A, βC87A/L7C from trial B and βC87A/V129C and βC87A/V430C from trial C. 20 µg protein were applied per sample, except for the βC87A/L7C variant, where 6.8 µg were applied.

2.5 Discussion

We report here on a new model for the membrane topology of OadB (Figure 6B) which differs in several important aspects from a previous one (Woehlke et al., 1992b; Figure 6A), which was based on predictions from hydropathy plots only. The hydropathy plots of OadB indicate eight to eleven different hydrophobic segments of sufficient extension to span the membrane (Figure 2) and nine of these were predicted to be the only membrane-spanning domains of the previous model. Two less pronounced hydrophobic areas of OadB can be identified, following the first and preceding the last membrane domain of the previous model. Hydropathy profile alignments indicate that the area following TM-helix I is clearly more hydrophobic in several related proteins, e. g. MadB, because E59 leading to a depression of hydrophobicity in OadB is replaced by V...
In MadB (Berg et al., 1997). In all related proteins, the hydrophobicity of the segment preceding the last membrane domain is clearly lower than those of the other segments, in this case being caused by a conserved arginine or lysine residue. This segment comprises the most highly conserved region of the protein besides that part of the hydrophobic segment IIIa that includes the catalytically essential D203 residue (DiBerardino and Dimroth, 1996). It has therefore already been speculated that the area preceding the C-terminal helix might also have an integral membrane location (Huder and Dimroth, 1993).

In the following discussion, we want to go through the protein from the N-terminus to the C-terminus, describing the evidence from the various techniques used in this study for the folding of OadB within the membrane. OadB has a blocked N-terminal methionine, which was defined by sequencing of the appropriate cyanogen bromide peptide (Laußermair et al., 1989). We propose in our new model (Figure 6B) that a tail of approximately 15 N-terminal amino acids is exposed to the cytoplasm. OadB with an L7C mutation was specifically alkylated from the cytoplasmic side of the membrane. Unfortunately, the above mentioned mutant did not exhibit oxaloacetate decarboxylase activity and, therefore, does not provide valuable information for assessment of the topology. This phenotype was also observed with another cysteine mutant (βC87A/L4C) or by inserting a FLAG epitope into the N-terminal tail. Therefore, this part of the molecule seems to be critical for the functional integrity of the decarboxylase. Such a critical role would be more consistent with an internal location, perhaps at the interface with the α-subunit, rather than an external (periplasmic) location.

The first TM helix (I) is very hydrophobic and runs from L16 to A36. This is followed by the first periplasmic loop, comprising amino acids K37KFEP41 and the less hydrophobic helix II, which runs from L42 to L63. The positive PhoA fusion at position P41 within the periplasmic loop is in accord with this folding. However, a positive PhoA fusion at P57 within helix II and at H72 and V80 in the adjacent cytoplasmic loop indicate that within this short N-terminal region of OadB the PhoA fusion technique fails to make reliable predictions of the topology. Anomalously high levels of alkaline phosphatase activity near the N-terminus of cytoplasmic loops of membrane proteins is a well recognized phenomenon (Prinz and Beckwith, 1994). Therefore, the topology of this area has also been analyzed by LacZ fusions at H72 and V80, and both had very high β-galactosidase activities. The cytoplasmic location of the extended loop
Membrane Topology of the β subunit connecting helices II and III was supported by the negative PhoA fusions at P103 and G134 in the C-terminal part of this loop. Furthermore, the wild-type cysteine 87 and cysteine replacing valine at position 129 were both exclusively alkylated from the cytoplasm. It should also be noted that the extended cytoplasmic loop 2 is a specific property of OadB from *K. pneumoniae* and *S. typhimurium*, whereas the related β subunit of methylmalonyl-CoA decarboxylase of *V. parvula* has only 15 amino acid residues in this region with no apparent similarity to its relatives. Hence, folding of the OadB region between amino acids 64 and 134 into an additional TM helix is unlikely.

The next TM helix runs from I135 to A155 and is defined by the results just mentioned and the AP-positive fusion at N156.

Following the short peptide N₁₅₆P₁₅₉T including a conserved proline residue, the OadB sequence continues with a hydrophobic region between L160 and L210 that has been termed region IIIa. The topology analysis of this area yielded a positive LacZ fusion at Q167, positive PhoA fusions at T179, G184 and T189 and negative LacZ fusions at L180, G184 and T189. In addition, S187 has been mutagenized into a cysteine residue, and this was shown to be periplasmically exposed. A cysteine introduced at position 182 was not accessible to the alkylating probe and may therefore have an integral membrane location or may be buried within a pocket of the protein. These results strongly indicate that the peptide comprizing T179 to T189 extends into the periplasm. Based on hydrophobicity of the sequence between L160 and L178, this portion of the protein should insert into the membrane. However, it is too short to span the membrane as two α-helices. We propose, therefore, that the polypeptide chain following L160 inserts into the membrane from the periplasm and folds backwards to emerge from its surface again with T179. Such very short membrane-integral α-helical structures are known from the pore helices of the potassium channel from *Streptomyces lividans*, whose structure was resolved recently by X-ray analysis at 3.2 Å (Doyle et al., 1998). It may be worth mentioning that defining the region between T179 and T189 as a periplasmic loop rather than an integral membrane portion is more compatible with the gaps of 5 - 6 residues found here in the various related proteins. For the region between L190 and L210, we propose again that it inserts from the periplasm into the membrane, but not as far as to reach the cytoplasmic surface. Rather, the polypeptide chain turns the direction and emerges from the periplasmic surface with S21E. The integral membrane location of this area is likely from inaccessibility of four cysteine mutants (βC87A/P191C, βC87A/A194C, βC87A/I198C and βC87A/P205C) by the polar
fluorescent alkylating agent IASD. The putative role of D203 as a binding site for Na\(^+\) or H\(^+\) during the transport of these cations across the membrane (Di Berardino and Dimroth, 1996) would hardly be compatible with a location of this residue outside the membrane. The critical role of this area is further indicated by containing seven invariant residues around the universal and functionally indispensable D203. The enzymatic activity was completely abolished even with the most conservative exchange of D203 to E (Di Berardino and Dimroth, 1996). In the βC87A/P191C or βC87A/P205C mutants, the oxaloacetate decarboxylase activity was either severely reduced or extinguished. Likewise, the insertion of FLAG epitopes (DYKDDDK) between amino acids L178 and Y182 led to inactive oxaloacetate decarboxylases. Hence, several mutations in this part of the molecule have drastic effects on the catalytic activity of the enzyme, corroborating its functional significance.

It is envisaged that the polypeptide emerges from the membrane again with the periplasmic peptide S\(_{211}\)GKLAP\(_{217}\). The next TM-helix (IV) runs from L218 to Q237 and is continued by a highly charged cytoplasmic loop between P238 and K265. This folding is in accord with the positive PhoA fusion at E217 and two negative PhoA fusions at T245 and L257, respectively. The protein curls again into a TM helix (V) between I266 and G293 and forms the periplasmic loop between N294 and T308. The folding is supported by three positive PhoA fusions at L286, M289 and V301 and the inaccessibility of C291 by the charged alkylating agent IASD. The next TM helix (VI) starts with V309 and ends with A327. The following cytoplasmic loop is supported by the two negative PhoA fusions at L329 and Q338. As pale blue colonies were obtained at the latter fusion site, Q338 may alternatively be located within the next outgoing TM helix (VII), which could start with F334 already. Within this helix and the adjacent periplasmic loop, positive PhoA fusions were obtained at V344, G357, V358 and N365. The positive PhoA fusions within the helix are in accord with the notion that five or more residues of an outgoing TM helix could promote the export of AP (Boyd et al., 1987; Calamia and Manoil, 1990). The folding of OadB in the region comprising TM helices IV to VII is the same in the previous and our present model. The old model predicted a large periplasmic loop and an ingoing TM helix for the C-terminal part of the molecule. This folding, however, is not in accord with a negative PhoA fusion at A398 and the positive PhoA and negative LacZ fusion at the C-terminal methionine. Furthermore, cysteine introduced at position 430 instead of valine within the C-terminal tail was specifically alkylated by IASD from the periplasmic surface. Hence, the
periplasmic loop is shorter than assumed previously and the highly conserved segment, flanked by I372 and K393, forms another TM helix (VIII). This is succeeded by the cytoplasmic loop between K393 and N402 and the outgoing TM helix IX, placing the C-terminus into the periplasm.

In summary, the new topology of OadB described here predicts two additional TM helices (II and VIII) with respect to the previous one and also places the N-terminus to the cytoplasm and the C-terminus to the periplasm, opposite to their orientation proposed previously. In addition, the previously predicted folding of the region between amino acids 159 and 211 into two membrane-spanning helices and a cytoplasmic loop could not be confirmed. This region is now being considered to fold into two pairs of membrane-inserted segments, which are connected by a periplasmic loop.

One of the criteria in predicting the orientation of helices within the membrane is the “positive-inside-rule”, introduced by von Heijne (1992). Regarding the topology of OadB, there appear to be major exceptions: the cytoplasmic N-terminus carries a negative charge, the first, the third and the ninth periplasmic loops are positively charged, and the charges in the second large cytoplasmic loop are essentially balanced. The rule clearly applies to the sixth cytoplasmic loop, which is the most hydrophilic part of the molecule and contains nine positively charged R and K residues and two negatively charged D and E residues. The rule is valid also for the cytoplasmic loop 8, which contains two K and one D residues. However, the cytoplasmic loop 10 contains one positive and two negative charges, and the periplasmic loop 9 has three positively charged and no negatively charged amino acids. It is possible, therefore, that the main determinant for the proper insertion of OadB into the membrane is the highly positively charged cytoplasmic loop 6. Overall, OadB has a net charge of +4 in the cytoplasmic loops and a net charge of +4 in periplasmic loops.

Taken together, we provide here information with confidence on the folding of the oxaloacetate decarboxylase β-subunit within the membrane. This topology is a suitable point of departure for functional studies of the Na⁺ pump by site-directed mutagenesis. We will report elsewhere that specific mutations within the newly recognized helix VIII abolish the oxaloacetate decarboxylase activity, thus emphasizing the functional significance of amino acids in this region.
FIGURE 6. Topological models of the oxaloacetate decarboxylase ß-subunit. The amino acid residues, to which the signal-sequence-depleted alkaline phosphatase (AP) or ß-galactosidase was fused, are marked by circles. Fusion sites, yielding clones with positive AP activities (> 5 units, blue colonies), are shown in black, those with negative AP activities (< 5 units, white colonies) are shown in white. Fusion sites, yielding clones with positive ß-galactosidase activities, are shown in grey (> 350 units), those with negative ß-galactosidase activities (< 50 units) are shown in white with grey outline. The black circles with grey outline mean white with grey outline and black circles, e.g. negative LacZ and positive PhoA fusion. The amino acid residues, which had been changed to cysteines, are marked by squares. Cysteines, which are labeled from the cytoplasmic side, are shown in grey, those, which are labeled from the periplasmic side, are shown in black and those which are not labeled from either side, are shown in white. (A) previous model (27). (B) new model.

Acknowledgements
We are grateful to Dr. L. Hederstedt (Department of Microbiology, University of Lund, Sweden) for providing the polyclonal AP antibody. Kathryn Adcock and Günther Woehlke did preliminary work in constructing PhoA-OadB fusion proteins.
2.6 References


CHAPTER 3

A molecular Mechanism for Energy Coupling in the Oxaloacetate Decarboxylase Na$^+$ pump.

PETRA JOCKEL, MARKUS SCHMID, JULIA STEUBER, AND PETER DIMROTH

Mikrobiologisches Institut der Eidgenössischen Technischen Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland

Biochemistry (1999), submitted

Running title: Energy coupling in the oxaloacetate decarboxylase
3 A molecular Mechanism for Energy Coupling in the Oxaloacetate Decarboxylase Na\(^+\) pump

3.1 Abstract

The oxaloacetate decarboxylase Na\(^+\) pump consists of subunits \(\alpha, \beta\) and \(\gamma\), and contains biotin as the prosthetic group. Membrane-bound subunit \(\beta\) catalyzes the decarboxylation of carboxybiotin coupled to Na\(^+\) translocation, and consumes a periplasmically derived proton. Site-directed mutagenesis of conserved amino acids of transmembrane helix VIII indicated that residues N373, G377, S382 and R389 are functionally important. The polar side chains of these amino acids may constitute together with D203 a network of ionizable groups which promotes the translocation of Na\(^+\) and the oppositely oriented H\(^+\) translocation across the membrane. Evidence is presented that two Na\(^+\) ions are bound simultaneously to subunit \(\beta\) during transport with D203 and S382 acting as binding sites. Sodium ion binding from the cytoplasm to both sites elicits decarboxylation of carboxybiotin, and a conformational switch exposes the bound Na\(^+\) ions towards the periplasm. After dissociation of Na\(^+\) and binding of H\(^+\), the cytoplasmically exposed conformation is regained.

Keywords: oxaloacetate decarboxylase/Na\(^+\) pump mechanism/direct coupling/Na\(^+\) binding sites
3.2 Introduction

Oxaloacetate decarboxylase of *Klebsiella pneumoniae* is the prototype for the sodium ion transport decarboxylase family of enzymes, which also includes methylmalonyl-CoA decarboxylases, malonate decarboxylase and glutaconyl-CoA decarboxylases from various anaerobic bacteria. These enzymes use the decarboxylation energy to pump Na$^+$ ions across the membrane (Dimroth, 1997; Dimroth and Schink, 1998; Braune et al., 1999). The thus generated ΔVNa$^+$ drives active transport reactions or the synthesis of ATP. The latter process has been termed decarboxylation phosphorylation and is the only ATP-generating mechanism in *Propionigenium modestum* or *Malonomonas rubra*, which grow from the decarboxylation of succinate to propionate or malonate to acetate, respectively (Hilpert et al., 1984; Dimroth and Hilbì, 1997).

The oxaloacetate decarboxylase Na$^+$ pump of *K. pneumoniae*, whose overall geometry and function is shown in Figure 1A, is composed of three different subunits α, β, and γ (OadA, B, and G), with molecular masses of 63.5 kDa, 44.9 kDa, and 8.9 kDa, respectively (Dimroth, 1997; Dimroth and Schink, 1998; Woehlke et al., 1992; Laußermair et al., 1989; Schwarz et al., 1988). The peripheral α-subunit consists of two domains, the N-terminal carboxyltransferase domain and the C-terminal biotin-binding domain, which are connected by a flexible proline/alanine linker (Schwarz et al., 1988). The β-subunit comprizes the main membrane integral portion of the enzyme complex. It folds into an N-terminal block of three membrane-spanning α-helices and a C-terminal block of six membrane-spanning α-helices. The connecting fragment consists of two loops that insert from the periplasm into the membrane, but do not reach the cytoplasmic surface (Figure 1B; Jockei et al., 1999; see Chapter 2). The second of these membrane-integral loops carries the invariant D203 residue, which is crucial for catalytic activity and resides within the most highly conserved area of OadB (Di Berardino and Dimroth, 1996). The γ-subunit is anchored in the membrane with a single N-terminal α-helix. This is succeeded on the cytoplasmic surface by a proline/alanine linker and a short hydrophilic domain harboring three conservative histidines near the C-terminus. These histidines are likely ligands for the Zn$^{2+}$ ion associated with it. The β- and γ-subunits form a subcomplex to which the α-subunit associates (Di Berardino and Dimroth, 1995; Dimroth and Thomer, 1988, 1992, 1993).

The catalytic reaction cycle starts with the carboxyltransfer from oxaloacetate to the prosthetic biotin group. This reaction step is catalyzed by the α-subunit alone, but its
rate is enhanced approximately 1000-fold in the additional presence of the γ-subunit, probably because the Zn²⁺ metal ion polarizes the carbonyl oxygen bond of oxaloacetate, thereby promoting the carboxyl transfer to biotin (Di Berardino and Dimroth, 1995; Dimroth and Thomer, 1993). Facilitated by the flexible proline/alanine linker, the carboxybiotin switches from the carboxyltransfer site at the α-subunit to the decarboxylase site at the ß-subunit (Schwarz et al., 1988). In the course of the subsequent decarboxylation reaction, one to two Na⁺ ions are pumped into the periplasm, and one H⁺ traverses the membrane in the opposite direction. This proton is consumed during the release of CO₂ from the biotin carboxylate. Switching the biotin back to the carboxyltransferase site completes the cycle (Di Berardino and Dimroth, 1996).

Previous site-directed mutagenesis studies indicated that the universally conserved D203 residue of OadB is absolutely essential for the Na⁺ transport and decarboxylase activities, but not for the carboxyltransferase activity. Based on these and other results, a direct coupling mechanism was proposed, in which D203 plays an essential role in both the vectorial and the chemical reaction. In the proposed mechanism, the protonated D203 residue takes up a Na⁺ ion with the proton moving simultaneously to the carboxybiotin and catalyzing the immediate decarboxylation of this acid-labile compound (Di Berardino and Dimroth, 1996).

Next to the region around ßD203, the most highly conserved area is within a hydrophobic stretch near the C-terminus, which was shown by topology analysis to form transmembrane (TM) helix VIII (Figure 1B; Jockel et al., 1999; see Chapter 2). We have used an *Escherichia coli* expression clone harboring the three genes for the oxaloacetate decarboxylase on plasmid pSK-GAB (Di Berardino and Dimroth, 1995) to replace highly conserved amino acids within TM-helix VIII of OadB. The phenotypic characterization of the derived mutants confirmed that this region of OadB is essential for catalytic activity, identifying N373, G377, S382 and R389 as amino acids with primary importance for function.
FIGURE 1. (A) Cartoon showing the overall geometry of the oxaloacetate decarboxylase and features of the catalytic events. B-H, biotin; B-CO$_2^-$, carboxylbiotin; Lys, biotin-binding lysine residue; 1 carboxyltransferase reaction and 2 decarboxylase reaction, for details see text. (B) Topology model for OadB emphasizing functionally important amino acid residues.
3.3 Results

Selection of amino acids for site-directed mutagenesis

Sequence alignments of OadB with related proteins reveal two highly conserved areas. One of these is the region surrounding D203 and the other is transmembrane helix VIII (Figure 1B; Jockel et al., 1999; see Chapter 2). An alignment of the latter segment, shown in Figure 2A, indicates the presence of nine invariant amino acid residues. We selected the polar amino acids N373, S382, R389 and N392 of this domain for mutational analyses, because these residues could participate in the translocation of Na⁺ or H⁺ across the membrane. In addition, the role of the uniquely conserved glycines at position 377 and 380 was investigated. In this study, the highly conserved proline residues at positions 374 and 385 were not mutagenized, because prolines are considered to be important structural determinants within hydrophobic segments (Nilsson and von Heijne, 1998).

Synthesis of mutant oxaloacetate decarboxylase in Escherichia coli

To synthesize the mutant oxaloacetate decarboxylases, mutated DNA fragments were cloned into pSK-GAB (Di Berardino and Dimroth, 1995) using appropriate restriction sites and used to transform E. coli DH5α, as described in Materials and methods. Grown cells were disrupted, and mutant oxaloacetate decarboxylases were affinity purified from the solubilized membrane fractions (Dimroth, 1986). The synthesis of the three polypeptide chains of the decarboxylase complexes was verified for all mutants described here by sodium-dodecyl-sulfate-polyacrylamide-gel-electrophoresis (SDS-PAGE), and a selection of these analyses is shown in Figure 3. The expression of mutant enzyme complexes was quantified by protein determination, and the data of Table 1 show that between 0.03 and 1 mg of the mutant enzymes could be isolated from 2 - 3 g wet packed cells. Also shown in Table 1 are the specific oxaloacetate decarboxylase activities of the isolated mutant enzymes.

By mutating G377 to A or S382 to A, C, E, N or Q, the decarboxylase activity was completely abolished, indicating that G377 and S382 are of functional significance. The drastic effect of the G377A mutation on activity is unique to this residue, because several other glycine/alanine mutants including G380A retained significant oxaloacetate decarboxylase activities (Table 1, data not shown in part). The S382T mutant retained about 10 % of the oxaloacetate decarboxylase activity, and a similar activity was found for the S382D mutant. Hence, a protonable group on a side chain with proper length
might be required at position 382 and might cycle between the protonated and unprotonated state (see Discussion). Replacement of R389 by the neutral amino acids A or L reduced the oxaloacetate decarboxylase activity to about 10%. With the conservative R389K exchange, the activity was not significantly affected, but in the mutant with a R389D substitution, the activity dropped below 1%. Other polar residues of helix VIII are N373 and N392, located near the periplasmic and cytoplasmic border, respectively. The N373L mutant enzyme was nearly inactive, indicating an important functional role for the asparagine residue at position 373. In contrast, decarboxylase with the N392L substitution only showed 30% reduction of activity and therefore, this asparagine residue is not considered to be of functional significance.

**Formation of stable carboxybiotin enzyme derivatives with mutants in OadB which are inactive in oxaloacetate decarboxylation**

We assumed that enzymes that had been mutated in OadB and yielded an oxaloacetate decarboxylase negative phenotype were not affected in the first partial reaction, i.e. the carboxyltransfer from oxaloacetate to the prosthetic biotin group. The enzymes with the G377A, S382A, S382C, S382E, S382N or S382Q mutations became rapidly labeled upon incubation with [4-14C] oxaloacetate, which indicates transfer of the labeled carboxylate to the biotin. The amount of radioactive label acquired by the proteins was approximately 100% of the expected value for all these OadB mutants except for the S382C mutant, where the labeling reached 60% (data not shown). As the subsequent decarboxylation of the carboxybiotin-enzyme was impaired, Na+ ions were without effect on the labeling of the enzyme. In contrast, the carboxybiotin-containing protein of the wild-type was only obtained, if the presence of Na+ was carefully excluded (Dimroth, 1982). We conclude therefore, that in the OadB mutations listed above the carboxybiotin decarboxylase activity was specifically impaired, whereas the carboxyltransferase activity was retained (Figure 1A).
FIGURE 2. (A) Sequence alignment of transmembrane helix VIII (boxed) of the β-subunits of oxaloacetate decarboxylases from K. pneumoniae (Kp-OadB), S. typhimurium (St-OadB), A. fulgidus (Af-OadB) T. pallidum (Tp-OadB), and the related membrane subunits of the Na⁺ pumping methylmalonyl-CoA-decarboxylases from P. modestum (Pm-MmdB) and V. parvula (Vp-MmdB) and the malonate decarboxylases from M. rubra (Mr-MadB) and R. capsulatus (Re-MadB). Identical residues are indicated by an asterisk, conservative exchanges by a dot. Sequence of Kp-OadB in bold and underlined. (B) Helical wheel model of helix VIII. Conserved residues are marked by an asterisk. Functionally important residues are boxed. Small numbers mark the position in the helix.
FIGURE 3. Expression of mutant oxaloacetate decarboxylases as evidenced from SDS-PAGE after purification of the enzymes. Mutations in OadB are indicated; Wt = wild-type enzyme; marker = marker proteins with molecular masses shown (in kDa). α, β, γ denote the three subunits of oxaloacetate decarboxylase.
<table>
<thead>
<tr>
<th>mutation</th>
<th>amount of oxaloacetate decarboxylase isolated (mg)</th>
<th>specific activity of isolated oxaloacetate decarboxylase at pH 7.5 (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>1.0</td>
<td>45.4</td>
</tr>
<tr>
<td>D203N</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>N373L</td>
<td>0.03</td>
<td>0.3</td>
</tr>
<tr>
<td>G377A</td>
<td>0.3</td>
<td>0</td>
</tr>
<tr>
<td>G380A</td>
<td>0.4</td>
<td>14.6</td>
</tr>
<tr>
<td>S382A</td>
<td>0.1</td>
<td>0</td>
</tr>
<tr>
<td>S382C</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>S382D</td>
<td>1.0</td>
<td>4.2</td>
</tr>
<tr>
<td>S382E</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>S382N</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>S382Q</td>
<td>0.6</td>
<td>0</td>
</tr>
<tr>
<td>S382T</td>
<td>0.9</td>
<td>3.4</td>
</tr>
<tr>
<td>R389A</td>
<td>0.5</td>
<td>3.4</td>
</tr>
<tr>
<td>R389D</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>R389K</td>
<td>1.3</td>
<td>35.8</td>
</tr>
<tr>
<td>R389L</td>
<td>0.9</td>
<td>4.4</td>
</tr>
<tr>
<td>N392L</td>
<td>0.3</td>
<td>31.7</td>
</tr>
</tbody>
</table>

Table 1. Synthesis of mutant oxaloacetate decarboxylases from OadB variants and specific activities of isolated enzymes. * from 2l LB culture (2 - 3 g wet packed cells).

_Tryptic hydrolysis of mutant oxaloacetate decarboxylases_

It has been noted previously that at Na⁺ concentrations of 20 - 50 mM, OadB was specifically protected from tryptic hydrolysis (Dimroth and Thomer 1983, 1992). Two different Na⁺ binding sites were envisaged, one with high affinity reflected by a halfmaximal activation at 0.5 mM and one with an approximately two orders of magnitude lower binding affinity (Di Berardino and Dimroth, 1996; Braune et al., 1999). Occupation of these sites with Na⁺ apparently results in a conformational change of OadB, which makes the protein more resistant to tryptic hydrolysis. Under our conditions, the halftime for tryptic digestion of wild-type OadB was 12 h in the absence of Na⁺ and > 24 h in the presence of 50 mM NaCl. The halftime in presence of Na⁺
dropped to about 1 h for the mutants G377A, S382C, S382E, S382N and S382Q (see Table 2), indicating that these mutant OadB's adopt conformations that are more susceptible to proteolysis. In contrast, OadB's with S382D or R389A mutations exhibited the wild-type properties with respect to tryptic hydrolysis. Hence, these variants not only adopt a rather rigid structure, so that cleavage by trypsin is slow, but they also retain the Na⁺ binding sites, which, when occupied, further protect OadB from tryptic digestion. Of particular interest are the catalytically inactive mutants D203N and S382A. In these enzymes, the halftime for tryptic digestion was with 10 h or 11 h close to that of the wild-type, but the protective effect of Na⁺ ions was missing. Based on these data, we propose two Na⁺ binding sites in OadB, located at D203 and S382, respectively. Only if both of these sites are present and occupied by Na⁺, OadB changes into its rigid conformation and becomes highly resistant to proteolysis.

<table>
<thead>
<tr>
<th>mutation</th>
<th>halfmaximal activation by Na⁺ (mM)</th>
<th>pH-optimum</th>
<th>half-time for OadB digestion (Na⁺-Na⁺) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.5</td>
<td>7.0</td>
<td>&gt;24 / 12</td>
</tr>
<tr>
<td>D203N</td>
<td></td>
<td></td>
<td>10 / 10</td>
</tr>
<tr>
<td>N373L</td>
<td>0.7</td>
<td>8.7</td>
<td>n. d.</td>
</tr>
<tr>
<td>G377A</td>
<td></td>
<td></td>
<td>&lt;1 / &lt;1</td>
</tr>
<tr>
<td>G380A</td>
<td>0.08</td>
<td>6.5 - 7.0</td>
<td>n. d.</td>
</tr>
<tr>
<td>S382A</td>
<td></td>
<td></td>
<td>11 / 11</td>
</tr>
<tr>
<td>S382C</td>
<td></td>
<td></td>
<td>&lt;1 / n. d.</td>
</tr>
<tr>
<td>S382D</td>
<td>0.8</td>
<td>6.4</td>
<td>&gt;24 / 12</td>
</tr>
<tr>
<td>S382E</td>
<td></td>
<td></td>
<td>&lt;1 / n. d.</td>
</tr>
<tr>
<td>S382N</td>
<td></td>
<td></td>
<td>&lt;1 / n. d.</td>
</tr>
<tr>
<td>S382Q</td>
<td></td>
<td></td>
<td>&lt;1 / n. d.</td>
</tr>
<tr>
<td>S382T</td>
<td>0.7</td>
<td>8.0</td>
<td>n. d.</td>
</tr>
<tr>
<td>R389A</td>
<td>1.0</td>
<td>8.5</td>
<td>&gt;24 h / 12</td>
</tr>
<tr>
<td>R389D</td>
<td>n. d.</td>
<td>6.5</td>
<td>n. d.</td>
</tr>
<tr>
<td>R389K</td>
<td>1.3</td>
<td>6.5</td>
<td>n. d.</td>
</tr>
<tr>
<td>R389L</td>
<td>2.6</td>
<td>9.2</td>
<td>n. d.</td>
</tr>
<tr>
<td>N392L</td>
<td>0.6</td>
<td>6.5</td>
<td>n. d.</td>
</tr>
</tbody>
</table>
Effect of OadB mutations on Na\(^+\) binding characteristics and pH profiles

The oxaloacetate decarboxylase is specifically activated by Na\(^+\), with a halfmaximal activity at about 0.5 mM. For several OadB mutants, Na\(^+\) activation profiles were determined to check for variations in the Na\(^+\) binding affinity. The results, shown in Table 2, indicate that in the N373L, S382D, S382T and N392L mutants the halfmaximal activation is achieved at slightly higher Na\(^+\) concentrations than in the wild-type enzyme. The affinity for Na\(^+\) decreased 2-fold, 2.5-fold and 5-fold in the R389A, R389K, and R389L mutants, respectively. In the mutant enzyme with G380A substitution, the halfmaximal activation was observed at Na\(^+\) concentrations about 5-times lower than in the wild-type decarboxylase and Na\(^+\) inhibition was observed at lower concentrations (> 2 mM) than usual (> 20 mM) (Di Berardino and Dimroth, 1996). For most mutant decarboxylases, the pH optimum was between 6.5 and 7.0 and thus similar to that of the wild-type enzyme. Significantly higher pH optima of 8.5 and above were found for the N373L and the R389A or R389L mutants. The significance of these results with respect to the ion translocation mechanism will be described in the Discussion section.

The mutants were further characterized by their Na\(^+\) pumping activity. For this purpose, we took advantage of the fact that growth of E. coli EP432 is impaired by elevated NaCl concentrations due to deletions of both Na\(^+\)/H\(^+\) antiporters (Pinner et al., 1993). We reasoned that the Na\(^+\) toxicity could be overcome by transforming the cells with plasmid pSK-GAB (Di Berardino and Dimroth, 1995), from which Na\(^+\) pumping oxaloacetate decarboxylase is expressed. The results of Figure 4 show that this is indeed the case. E. coli EP432, transformed with plasmid pSK-GAB, resumed growth on glucose minimal medium, containing 260 or 360 mM NaCl, after a lag phase of approximately 20 h. In contrast, E. coli EP432 transformed with the vector (pSK\(^-\)) alone or with mutant plasmids, expressing catalytically inactive oxaloacetate decarboxylases, were unable to grow at the elevated NaCl concentrations (Figure 4; Table 3). It has been noticed that on prolonged incubation of E. coli EP432 on LB medium containing elevated NaCl concentrations, spontaneous mutants arise that start growing under these conditions (Harel-Bronstein et al., 1995). In our experiments, however, growth was clearly
dependent on the expression of the oxaloacetate decarboxylase Na$^+$ pump from plasmid pSK-GAB (see above). The results of Table 1 and 3 indicate that *E. coli* EP432 expressing a mutated oxaloacetate decarboxylase with specific activity > 3 U/mg protein (after purification) is generally able to grow in the presence of 360 mM NaCl. Exceptions are the R389A and R389L mutants, which are unable to grow, although their isolated oxaloacetate decarboxylases have specific activities of about 4 U/mg protein at pH 7.5. Please note that in these mutants, the pH optimum is shifted into the alkaline area (Table 2) and that the growth experiments were performed at pH 5.9, where the specific activities for the R389A and R389L mutants are reduced to 1.8 and 2.6 U/mg protein, respectively. Diminished growth is observed for the R389K mutant, despite of good expression and near wild-type specific oxaloacetate decarboxylase activity of the isolated enzyme. Reduced growth is also observed for the N392L mutant, from which enzyme with high specific activity was isolated. In this case, however, the growth reduction might be due to the diminished expression of the enzyme (Table 1).

<table>
<thead>
<tr>
<th>mutation</th>
<th>optical density at 600 nm $^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.45</td>
</tr>
<tr>
<td>G380A</td>
<td>0.15</td>
</tr>
<tr>
<td>S382D</td>
<td>0.22</td>
</tr>
<tr>
<td>S382T</td>
<td>0.16</td>
</tr>
<tr>
<td>R389K</td>
<td>0.15</td>
</tr>
<tr>
<td>N392L</td>
<td>0.10</td>
</tr>
</tbody>
</table>

Table 3. Growth of *E. coli* EP432 transformed with plasmids expressing mutant oxaloacetate decarboxylases in the presence of 360 mM NaCl. No growth was observed for mutants N373L, G377A, S382A, S382C, S382E, S382N, S382Q, R389A, R389D and R389L. $^a$ The optical density was determined after 28 h growth at 30°C.
FIGURE 4. Complementation of the Na\(^+\)/H\(^+\) antiporter-deficient *E. coli* mutant EP432 with the oxaloacetate decarboxylase from *K. pneumoniae*. Growth was followed at 260 mM NaCl (closed symbols) or 360 mM NaCl (open symbols) with *E. coli* EP432 transformed with plasmid pSK-GAB encoding the oxaloacetate decarboxylase (circles) or plasmid pSK\(^+\) as a control (triangles).
Dependence of oxaloacetate decarboxylase activity on Na⁺ concentration

The results on site-directed mutagenesis of OadB described above suggested that S382 could act as a binding site for Na⁺ during the translocation of the alkali ion across the membrane. From previous studies, D203 and the carboxybiotin were inferred putative Na⁺ binding sites of OadB (Di Berardino and Dimroth, 1996). Two of these sites could be occupied simultaneously, because one to two Na⁺ ions are translocated per decarboxylation event (Dimroth and Thomer, 1993). We have therefore reinvestigated the dependence of oxaloacetate decarboxylase activity on Na⁺ concentration with special attention to cooperativity. The results of Figure 5 clearly show a sigmoid shape of the Na⁺ activation profile for oxaloacetate decarboxylase. The experimental data follow the line calculated by the Hill equation with $n_H = 1.8$, but not that calculated according to Michaelis/Menten. The results indicate that 2 Na⁺ ions have to bind simultaneously to achieve the maximum activity of the enzyme. The simultaneous binding of 2 Na⁺ ions to OadB is in accord with the observation that the two putative Na⁺ binding residues D203 and S382 must be present to achieve Na⁺-dependent protection of the protein from tryptic hydrolysis. We therefore propose that D203 and S382 act as two different Na⁺ binding sites in OadB.
3.4 Discussion

Recent topological analyses of OadB have indicated that the highly conserved area comprizing residues 372 to 393 folds into transmembrane helix VIII (Jockel et al., 1999; Figure 1B and 2; see Chapter 2). Our present mutational studies show that this region harbors several amino acids with functional significance. Inactive or nearly inactive enzyme specimens were obtained by mutating βN373 to L, βG377 to A or βS382 to A, C, E, N or Q. These OadB mutations specifically affect the carboxybiotin decarboxylase activity of the enzyme, but not the carboxyltransferase activity which is intrinsic to OadA (Di Berardino and Dimroth, 1995; Dimroth and Thomer, 1983; Figure 1A). We assume that the carboxybiotin binds near the cytoplasmic surface in the vicinity of helix VIII. The proton, which is consumed during the decarboxylation reaction, stems from
the periplasmic reservoir and two Na\textsuperscript{+} ions are pumped into this reservoir from the cytoplasmic compartment (Figure 1A; Di Berardino and Dimroth, 1996). Hence, protons and Na\textsuperscript{+} ions traverse the membrane into opposite directions, for which they might use the same network of ionizable amino acid residues. Previously, an important role in both Na\textsuperscript{+} and H\textsuperscript{+} transport has been assigned to D203, which according to the OadB topology is located in the vicinity of the periplasmic surface (Di Berardino and Dimroth, 1996).

We assign a similar role as to D203 to S382, which is located within helix VIII, close to the center of the membrane. This assumption is based on the fact that for retention of decarboxylase activity, S, T, or D are the only amino acids tolerated at position 382. It is intriguing that the enzyme with the S382D substitution retained approximately 10\% of the wild-type activity, whereas that with the S382N mutation was completely inactive. As D or N might be considered to be about equally suitable as Na\textsuperscript{+} binding ligands, we attribute the preference of D over N at position 382 to the protonation and deprotonation of this site during the catalytic cycle. However, if the only role of S382 would be that of a proton carrier, it is difficult to explain, why the S382C or S382E mutants are completely inactive. For these reasons we propose a dual function for S382, serving as a Na\textsuperscript{+} binding site during the transport of this alkali ion into the periplasm and as H\textsuperscript{+} binding site for guiding protons in opposite direction through the membrane into the catalytic site. Hence, S382 is likely to perform a similar role as D203 (Di Berardino and Dimroth, 1996), and both residues could be part of a Na\textsuperscript{+} and H\textsuperscript{+} translocation network within the membrane.

To function in proton conduction, the side chain hydroxyl of S382 has to switch between the protonated and deprotonated state. In aqueous environments, the hydroxyl group of serine has a pK \textgreater 13 and therefore does not deprotonate under physiological conditions. However, as exemplified for the serine proteases, abstraction of the proton from serine is feasible with histidine acting as a base within a hydrophobic pocket of the enzyme (for review see Paetzel and Dalbey, 1997). A candidate to take over a proton from S382 in OadB is the pair R389/carboxybiotin. The R389 residue is located two helical turns apart from S382 towards the cytoplasm, and both residues have their side chain exposed to the same side (Figure 2B). Furthermore, R389 is conserved among species, except for the conservative R389K exchanges in the malonate decarboxylase β-subunits (Figure 2A). Mutational analyses reported here strengthen the proposal of an intimate interaction between S382 and R389 in the proton-conducting pathway. While
the R389K mutant exhibited almost wild-type oxaloacetate decarboxylase activity and was a functional Na\(^+\) pump, all other investigated mutants had severely reduced decarboxylase and impaired Na\(^+\) pumping activities as judged from growth assays with the Na\(^+\)-sensitive *E. coli* EP432 strain. An increase of the pH-optimum of the decarboxylase by about 2 units in the R389A or R389L mutants compared to the wild-type (Table 2) is further in accord with the proposed proton conduction from S382 via R389 to carboxybiotin, where it initiates the decarboxylation of this acid-labile compound.

In the following, we would like to propose a model for the coupling mechanism of the oxaloacetate decarboxylase Na\(^+\) pump that takes present and previous results into account. The model, shown in Figure 6, is significantly distinct from a previous one, in which the simultaneous binding of two Na\(^+\) ions to OadB was not yet recognized (Di Berardino and Dimroth, 1996). Mutational analyses have identified transmembrane helix VIII and that part of region IIIa surrounding D203 as essential for the decarboxylation of carboxybiotin and for the translocation of Na\(^+\) and H\(^+\) across the membrane that is coupled to it. It is proposed that unliganded OadB switches between conformation 1, in which the Na\(^+\) binding sites are open to the cytoplasm and conformation 2, in which these sites are open to the periplasm. The catalytic cycle starts by the binding of carboxybiotin, which stabilizes conformation 1. This binding may be supported by charge pairing of the biotin carboxylate with R389, and the biotin carboxylate would therefore penetrate from the cytoplasmic surface into its binding pocket that might be created by the unusual folding of region IIIa (Figure 1B; Jockel et al., 1999; see Chapter 2; step D to A). Further progress of the reaction requires the binding of two Na\(^+\) ions from the cytoplasm to the appropriate sites at the interface between helix VIII and region IIIa. The first Na\(^+\) ion penetrates the membrane almost completely and binds to D203 close to the periplasmic surface. The conserved N373 of helix VIII near the periplasmic surface could be an additional ligand of this site. This would be in accord with an almost complete inactivation of oxaloacetate decarboxylase activity in the N373L mutant (Table 1). We further envisage that the conserved G377 one helical turn further up on helix VIII is at a critical narrow position of the channel, because the G377A mutant was completely inactive. Possibly, binding of the first Na\(^+\) ion elicits some interhelical rearrangements, by which the binding of the second Na\(^+\) ion to the S382 including site becomes feasible. Upon Na\(^+\) binding to this site, the proton from S382 is abstracted, facilitated by the pair R389/carboxybiotin, and upon
protonation of the latter residue, this acid-labile compound undergoes immediate
decarboxylation (step B). The reaction is accompanied by the change to conformation 2
that closes the channel to the cytoplasmic surface and opens it towards the periplasm
(step B to C). Once open, the Na⁺ dissociates from the D203/N373 pair into the
periplasmic reservoir, and a proton enters the channel and displaces the Na⁺ from S382.
The D203/N373 pair resides near the membrane surface, where the pK of D203 may not
be significantly higher than in water (3.9) and H⁺ binding is not required for Na⁺
release. Delocalization of the charge by hydrogen bonding between D203 and N373 will
also contribute to the dissociation of Na⁺. In contrast, the Na⁺ bound to S382 near the
center of the membrane undoubtedly will have to be displaced by the protonation of the
side chain oxygen anion, because a negative charge would not be tolerated here for
electrostatic reasons (step D). After these events, carboxybiotin formed by OadA by
carboxyltransfer with oxaloacetate binds to OadB (step D to A). This stabilizes
conformation 1 and a new reaction cycle begins.

FIGURE 6. Model for coupling of Na⁺ and H⁺ movements across the membrane to the decarboxylation of
carboxybiotin. The model shows the approximate location of important residues of helix VIII and region
IIIa of the β-subunit. Also shown is the participation of these residues in the vectorial and chemical
events of the Na⁺ pump. For details see text.
Observations in accord with the model

In the following, we will give a critical evaluation of experimental data collected over the years with respect to the proposed model.

(i) In the absence of Δ\(\mu\)Na\(^+\), the oxaloacetate decarboxylase Na\(^+\) pump operates at a Na\(^+\) to oxaloacetate stoichiometry of \(\sim 2\) and one H\(^+\) moves into the opposite direction (Dimroth and Thomer, 1993; Di Berardino and Dimroth, 1996). This observation is in accord with the sequence of events as formulated in Figure 6 (complete reaction cycle from steps A to D).

(ii) At high Δ\(\mu\)Na\(^+\), the Na\(^+\) to oxaloacetate stoichiometry drops and the pump catalyze an exchange of internal and external Na\(^+\) ions (Dimroth and Thomer, 1993). This observation can be explained, if Na\(^+\) is not completely dissociated from D203/N373 to the periplasmic reservoir, due to the high Na\(^+\) concentration at this side. If the conformation changes from 2 to 1 (step D to A) with Na\(^+\) still bound to this site, the Na\(^+\) to oxaloacetate stoichiometry will be reduced. In conformations 1 or 2, the Na\(^+\) at this site may equilibrate with Na\(^+\) ions present in the cytoplasmic and periplasmic compartment, respectively, resulting in the observed exchange of internal and external Na\(^+\) ions.

(iii) Oxaloacetate decarboxylation is inhibited by high Na\(^+\) concentrations, especially at high pH values (Di Berardino and Dimroth, 1996). With respect to our model, it is reasonable to assume that these conditions are unfavorable for the H\(^+\)-dependent displacement of Na\(^+\) from S382 to the periplasmic surface (step C). Reduction of the reaction velocity by these conditions is therefore expected.

(iv) The pump can be reversed catalyzing pyruvate carboxylation in the presence of large Δ\(\mu\)Na\(^+\) (Dimroth and Hilpert, 1984). The high periplasmic Na\(^+\) concentration triggers Na\(^+\) binding to S382 with exchange for H\(^+\), for which D203 may be required as a base. After binding of the second Na\(^+\), also favored by these conditions, the conformation changes from 2 to 1 and biotin becomes carboxylated. During this reaction, the proton moves from biotin to S382, thereby releasing the bound Na\(^+\) into the cytoplasm. After dissociation of the second Na\(^+\) into the cytoplasmic reservoir, the carboxybiotin switches from OadB to OadA, where transfer of the carboxyl group to pyruvate yields oxaloacetate (reverse reaction cycle from steps D via C and B to A).

(v) The decarboxylation is strictly dependent on Na\(^+\) ions (Dimroth and Thomer, 1986). In the model, the essential proton that is consumed in the decarboxylation of carboxybiotin stems from S382, from which it is only released in an exchange with Na\(^+\).
and after the second Na\(^+\) binding site on D203/N373 has been occupied with the alkali ion (step C). Accordingly, mutants S382A, C, E, N or Q, D203E or N or N373L that affect the Na\(^+\) coordination geometry are inactive or nearly inactive.

In summary, we present here a molecular model for the coupling of the chemical reaction and the vectorial ion movements across the membrane by the oxaloacetate decarboxylase Na\(^+\) pump. The central feature for this mechanism is to use membrane-buried amino acid residues (S382; D203/N373) as binding sites for the oppositely oriented movements of Na\(^+\) and H\(^+\). The two sites take up Na\(^+\) ions from the cytoplasm and deliver them to the periplasm. Simultaneously, a proton is translocated across the membrane, following the opposite route towards carboxybiotin, where it is consumed in catalyzing the decarboxylation of this acid-labile compound. This is a very elegant example for a direct coupling mechanism, in which the Na\(^+\) movement triggers the oppositely oriented translocation of H\(^+\) across the membrane and in which these vectorial protons are directly involved in the chemical events of the catalysis.

3.5 Materials and methods

**Bacterial strains and plasmids**

The bacterial strains used in this study are *Escherichia coli* DH5\(\alpha\) (Bethesda Research Laboratories), *Escherichia coli* JM110 (Yannish-Perron et al., 1995), *Escherichia coli* EP432 (Pinner et al., 1993) and *Escherichia coli* BL21(DE3)pLysS (Dunn and Studier, 1983). All strains, except *E. coli* EP432, were routinely grown at 37°C in Luria Bertani (LB) medium (Sambrook et al., 1989). *E. coli* EP432 was grown in 50 mM potassium phosphate pH 5.9, 18.7 mM ammonium chloride, 43 mM potassium sulfate, 1 mM magnesium sulfate, 0.1 mM calcium chloride, 0.1 mg/ml threonine, 2.5 \(\mu\)g/ml thiamin and either 50 mM, 260 mM or 360 mM sodium chloride at 30°C. Plasmid-containing strains were supplemented with the selective antibiotics ampicillin (100 \(\mu\)g/ml) and/or chloramphenicol (40 \(\mu\)g/ml) or kanamycinsulfate (50 \(\mu\)g/ml). Plasmid pSK-GAB was prepared as described (Di Berardino and Dimroth, 1995). Plasmid pSK\(^+\) was purchased from MBI Fermentas.
Recombinant DNA techniques

Standard recombinant DNA techniques were performed essentially as described (Sambrook et al., 1989). Polymerase chain reactions (PCRs) were performed using Vent-DNA-Polymerase from New England Biolabs (Beverly, MA, USA). DNA sequencing was carried out according to the dideoxynucleotide chain-termination method (Sanger et al., 1977) using a Taq DyeDeoxy terminator cycle kit and the ABI Prism 310 genetic analyzer from Applied Biosystems.

Construction of site-directed mutants of the β-subunit

The primers used for site-directed mutagenesis are listed in Table 4. Site-directed mutants were obtained as follows: The PCR fragments containing the mutations were constructed in a two step protocol. For the N-terminal part of the PCR fragments of the β-subunit, primer prBN and primers with the affix rev were used, and pSK-GAB (Di Berardino and Dimroth, 1995) served as template. For the corresponding C-terminal part of the PCR, primer prBC and primers with the affix for were used, and pSK-GAB (Di Berardino and Dimroth, 1995) served as template. After purification, those PCR fragments were used as template for the PCR products from primer prBN and prBC, which contained the mutation. PCR products were digested with BclI and Bst1107I and cloned into pSK-GAB. From pSK-GAB, which was isolated from JM110 cells, the BclI/Bst1107I fragment was removed.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Restriction sites/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>prBN</td>
<td>CAGAACGCAGCTGATCAACATC GTCACCATCTTC</td>
<td>BclI site in bold</td>
</tr>
<tr>
<td>prBC</td>
<td>GATTTTTTCGGGTATACATTGGG TAGATAGA</td>
<td>Bst1107I site in bold</td>
</tr>
<tr>
<td>prN373Lrev</td>
<td>CGAGCCGATCAGCGGTAAAAATT TTGTGCGGC</td>
<td>mutation underlined, ApoI site in bold</td>
</tr>
<tr>
<td>prN373Lfor</td>
<td>GCCGGCACAAAATTTACCCGCT GATCGGCTCG</td>
<td>mutation underlined, ApoI site in bold</td>
</tr>
<tr>
<td>prG377Arev</td>
<td>CCCCGCCGAGGGCGATACGGCG</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG377Afor</td>
<td>CCGCTGATCCGGCTCGGCGGGG</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG380Arev</td>
<td>CGCCGACACGCCGCCGGAGGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG380Afor</td>
<td>GGCTCGGCGGGCCGTGCAGCG</td>
<td>mutation underlined</td>
</tr>
</tbody>
</table>
Mechanism of Energy Coupling

<table>
<thead>
<tr>
<th>Mutation</th>
<th>Sequence</th>
<th>Underlined Sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>prS382Arev</td>
<td>CGCCATCGGCACCGCCGCACC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCCGCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Afor</td>
<td>TCGGCGGGGTTGCGCGGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATGGCG</td>
<td></td>
</tr>
<tr>
<td>prS382Crev</td>
<td>CGCCATCGGCACCGCGCACACC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCCGCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Cfor</td>
<td>TCGGCGGGGTTGCGCGGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATGGCG</td>
<td></td>
</tr>
<tr>
<td>prS382Drev</td>
<td>CATCGGCACCAGCGTCCACCC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>GCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Dfor</td>
<td>TCGGCGGGGTTGACGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATG</td>
<td></td>
</tr>
<tr>
<td>prS382Erev</td>
<td>CGCCATCGGCACCGCTCCACCC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCCGCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Efor</td>
<td>TCGGCGGGGTTGACGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATGGCG</td>
<td></td>
</tr>
<tr>
<td>prS382Nrev</td>
<td>CGCCATCGGCACCGTTCACC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCCGCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Nfor</td>
<td>TCGGCGGGGTTGACGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATGGCG</td>
<td></td>
</tr>
<tr>
<td>prS382Qrev</td>
<td>CGCCATCGGCACCGCTGCAACC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCCGCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Qfor</td>
<td>TCGGCGGGGTTGACGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATGGCG</td>
<td></td>
</tr>
<tr>
<td>prS382Trev</td>
<td>CATCGGCACCAGCGGTCAACCC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>GCCGA</td>
<td></td>
</tr>
<tr>
<td>prS382Tfor</td>
<td>TCGGCGGGGTTGACGCGGTGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGATG</td>
<td></td>
</tr>
<tr>
<td>prR389Arev</td>
<td>GTTCGACACCACCACGCCGCACCATC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>GCCACCACGC</td>
<td></td>
</tr>
<tr>
<td>prR389Afor</td>
<td>GCGGTTGCGATGGCGGCCGGCGGG</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>TGTCGAAC</td>
<td></td>
</tr>
<tr>
<td>prR389Drev</td>
<td>CCCACCTGTTCGACACATCCG</td>
<td>mutation underlined, SacII site in bold</td>
</tr>
<tr>
<td></td>
<td>CGGCCATCGGCACCGG</td>
<td></td>
</tr>
</tbody>
</table>
Table 4. Primers used for mutagenesis of the $\beta$-subunit.

**Purification of oxaloacetate decarboxylase mutants and enzyme assays**

Oxaloacetate decarboxylase mutants were purified from DH5a/pSK-GAB variants by affinity chromatography of a solubilized membrane extract on a SoftLink monomeric avidin-Sepharose column (Promega) (Dimroth, 1986). Large-scale purification was performed according to Di Berardino and Dimroth (1996). The decarboxylation activity was determined with the simple spectrophotometric assay at 265 nm as described (Dimroth, 1986).

**Screening of oxaloacetate decarboxylase activity of mutant clones**

Before a large-scale purification of a mutant protein was performed, a small-scale culture of the respective clone was used to measure oxaloacetate decarboxylase activity (Di Berardino and Dimroth, 1996). Another method used was transformation of the mutant plasmid into *E. coli* EP432, which lacks both Na⁺/H⁺ antiporters and is therefore unable to grow in the presence of 360 mM NaCl (Pinner et al., 1993). In the presence of an active oxaloacetate decarboxylase Na⁺ pump, however, the strain resumes to grow under these conditions (see Figure 4). This is therefore a convenient assay for the *in vivo* function of the Na⁺ pump.
**Labeling of oxaloacetate decarboxylase and mutant enzymes with $^{14}$CO$_2$ from [4-$^{14}$C]oxaloacetate**

[4-$^{14}$C]oxaloacetate, prepared from [4-$^{14}$C]L-aspartate and α-oxoglutarate with glutamate:oxaloacetate transaminase was used to measure the transfer of the radioactive carboxyl residue to biotin bound to OadA as described (Di Berardino and Dimroth, 1995). The radiolabeled protein was separated after 10 min incubation from excess substrates by Sephadex G-25 column chromatography in 100 mM Tris/HCl buffer, pH 8.5. Fractions of 400 μl were collected and the radioactivity was determined by liquid scintillation counting.

**Determination of oxaloacetate decarboxylase activity at various Na$^+$ concentrations and pH values**

The decarboxylation activity of wild-type (DH5α/pSK-GAB) and mutant oxaloacetate decarboxylases was measured at different pH values in the range between pH 5.5 and pH 8.8 in 40 mM Mes/Tris buffer containing variable Na$^+$ concentrations with the simple spectrophotometric assay according to Dimroth (1986).

**Analytical procedures**

The protein content of samples was determined according to Bradford (1979) or by the bicinchoninic acid method (Smith et al., 1985).

**Effect of Na$^+$ on tryptic hydrolysis of the oxaloacetate decarboxylase β-subunit**

The incubation mixtures contained in 60 μl or 84 μl at 25°C: (A) 20 mM potassium phosphate buffer, pH 7.5, 50 mM potassium chloride, purified wild-type or mutant oxaloacetate decarboxylase (25 μg or 35 μg) and 3 μg trypsin. A parallel incubation mixture (B) contained 50 mM sodium chloride instead of potassium chloride. Samples (12 μl) were transferred after 0 (before addition of trypsin), 2, 4, 7.5 and 24 h or 0 (before adding of trypsin), 15, 30, 45, 60, 90 and 120 min incubation into 1 μl 50 mM phenylmethanesulfonyl fluoride to inactivate the trypsin and 12 μl SDS sample buffer. After heating the samples to 95°C for 5 min, they were subjected to 10% SDS-PAGE. The gels were immediately stained with silver. Quantification of the β-subunit bands was performed with the program ImageMaster 1D Prime purchased from Pharmacia/Amersham. Halftimes of the β-subunit in the presence of trypsin and + / - Na$^+$ were obtained with the program Sigmaplot.
**Determination of Na\(^+\) uptake into membrane vesicles**

Na\(^+\) uptake was followed by atomic absorption spectroscopy as described by Krebs et al. (1999). Incubation mixtures contained in 150 µl: membrane vesicles (1.3 to 1.4 mg protein) prepared as described (Dimroth, 1986), 50 mM potassium phosphate buffer, pH 7.5, 20 mM NaCl, and 7 µM valinomycin. The reaction was started by adding 1 mM oxaloacetate (final concentration). At different times (10 and 30 s), samples of 70 µl were applied to a 1 ml plastic syringe containing 0.6 ml Dowex 50 (K\(^+\)), equilibrated with assay buffer without NaCl. The eluate was collected in plastic tubes, and the Na\(^+\) entrapped in the vesicles was determined. Controls were performed without oxaloacetate. The results reported are averages from three independent experiments.

**Acknowledgements**

We are grateful to Prof. Lutz Nover (Department of Molecular Cell Biology, Goethe-University, Frankfurt a. M., Germany) for providing the program ImageMaster 1D Prime. This work was supported by Swiss National Science Foundation.
3.6 References


CHAPTER 4

Essential Role of Tyrosine 229 of the Oxaloacetate Decarboxylase β Subunit in the Energy Coupling Mechanism of the Na⁺ Pump.

PETRA JOCKEL¹, THOMAS CHOINOWSKI², AND PETER DIMROTH¹

¹Mikrobiologisches Institut der Eidgenössischen Technischen Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland

²Institut für Biochemie der Eidgenössischen Technischen Hochschule, ETH-Zentrum, CH-8092 Zürich, Switzerland

Running title: Energy coupling mechanism of oxaloacetate decarboxylase
4 Essential Role of Tyrosine 229 of the Oxaloacetate Decarboxylase β Subunit in the Energy Coupling Mechanism of the Na⁺ Pump

4.1 Summary

The membrane-bound β-subunit of oxaloacetate decarboxylase catalyzes the decarboxylation of carboxybiotin, which is coupled to Na⁺ translocation and consumes a periplasmically derived proton. Upon site-directed mutagenesis of twenty polar and/or conserved residues within putative membrane-integral regions, the specific oxaloacetate decarboxylase activities were reduced to various extents, but only the enzyme with a Y229F mutation was completely inactive. We propose that Y229 is part of the network by which the proton of S382 is delivered to carboxybiotin, where it is consumed upon catalyzing the immediate decarboxylation of this acid-labile compound. Y229 is not a Na⁺ binding site residue like S382 or D203, because protection of the β-subunit from tryptic digestion by 50 mM NaCl was maintained in the Y229F or Y229A mutants. The enzyme with a βC291A mutation was unstable in the absence of Na⁺ and dissociated into an α-γ subcomplex, indicating a Na⁺-induced conformational change, which stabilizes the assembly of the complex.
4.2 Introduction

Oxaloacetate decarboxylase is the paradigm for the sodium ion translocating decarboxylases, an enzyme family that also includes methylmalonyl-CoA decarboxylase, glutaconyl-CoA decarboxylase, and malonate decarboxylase (Dimroth, 1997; Dimroth and Schink, 1998; Braune et al., 1999). The oadGAB genes encode subunits γ, α, and β (OadG, A, and B) of oxaloacetate decarboxylase (Schwarz et al., 1988; Laußerinair et al., 1989; Woehlke et al., 1992) and are part of the citrate fermentation gene cluster. This cluster, which has been thoroughly characterized in Klebsiella pneumoniae, also includes the genes for citrate lyase, a Na⁺-dependent citrate carrier and a two component regulatory system (Bott and Dimroth, 1994; Bott et al., 1995; Bott, 1997). The derived proteins are specifically required for the anaerobic growth on citrate.

Organization and function of the three subunits of oxaloacetate decarboxylase are illustrated in Figure 1A. The α subunit is a peripheral membrane protein, harboring the carboxyltransferase activity in the N-terminal domain and the biotin-carrier-function in the C-terminal domain (Dimroth and Thomer, 1986; Schwarz et al., 1988). The β subunit is an integral membrane protein, composed of nine transmembrane (TM) α-helices, a hydrophobic segment inserting into the membrane from the periplasmic surface in an undefined manner (region IIIa) and connecting loops of various lengths (Figure 1B; Jockel et al., 1999; see Chapter 2). The β subunit catalyzes the decarboxylation of carboxybiotin bound to OadA, which is coupled to Na⁺ pumping and consumes a periplasmically derived proton (Di Berardino and Dimroth, 1996; Dimroth, 1997). The γ subunit has a membrane anchor in its N-terminal part and a hydrophilic C-terminal portion with an attached Zn²⁺ metal ion (Di Berardino and Dimroth, 1995; Dimroth and Thomer, 1992). The γ subunit keeps the complex together, and it accelerates the carboxyltransfer reaction, presumably by polarizing the carbonyl oxygen bond of oxaloacetate with its Zn²⁺ ion (Di Berardino and Dimroth, 1995).

The most highly conserved portions of OadB are region IIIa in the vicinity of D203 and transmembrane helix VIII. Mutational analyses indicated that D203, S382 and G377 are essential not only for Na⁺ translocation but also for the decarboxylation of carboxybiotin (Di Berardino and Dimroth, 1996; Chapter 3). Other residues of functional significance are N373 and R389. It was proposed that the D203/N373 pair forms a Na⁺ binding site near the periplasmic surface and that a second Na⁺ binding site
is located at S382 in the center of the bilayer (see Chapter 3). A possible mechanism involves binding of carboxybiotin near R389 and of two cytoplasmically derived Na\(^+\) ions to the two binding sites. Binding of Na\(^+\) to S382 displaces a proton, which moves via R389 to the carboxybiotin, thereby catalyzing the decarboxylation of this acid-labile compound. This triggers a conformational change, by which the Na\(^+\) ions become exposed and dissociate towards the periplasmic surface. Simultaneously, a proton entering the channel reforms the hydroxyl group of S382. Rebinding of carboxybiotin switches OadB back to the original conformation, and a new catalytic cycle begins (see Chapter 3).

In this study, we have selected additional conserved amino acid residues of OadB for site-specific mutagenesis. In all of these mutants, the oxaloacetate decarboxylase activity was reduced to various extents. The most conspicuous results were obtained with mutants of Y229, defining this residue as crucial for the catalytic mechanism.
FIGURE 1. A) Cartoon showing the overall geometry of the oxaloacetate decarboxylase and features of the catalytic events. B-H, biotin; B-CO₂, carboxybiotin; Lys, biotin-binding lysine residue; (1) carboxyltransferase reaction and (2) decarboxylase reaction. B) Topology model of the β subunit of the oxaloacetate decarboxylase showing functionally important amino acid residues in grey. Those mutagenized residues which led to functionally intact oxaloacetate decarboxylases are indicated in black.
4.3 Results

Selection of amino acids for site-directed mutagenesis

The oxaloacetate decarboxylase β-subunit consists of 433 residues, of which 59 are conserved among eight related proteins. These and a few others with conservative exchanges are the only ones that could be of functional relevance. Site-directed mutagenesis could therefore be restricted to these residues. We further reasoned that conserved polar residues in transmembrane helices are the key candidates to participate in ion binding and translocation, and we therefore selected them for mutagenesis first. Conserved glycines were included, since they might be well suited to align channel portions within the molecule due to their lacking side chains. With this approach, we have already identified D203 of region IIIa and N373, G377, S382 and R389 of helix VIII as residues with functional importance for decarboxylation-coupled Na⁺ translocation across the membrane (Di Berardino and Dimroth, 1996; Chapter 3). Additional amino acids of the category described above have now been mutagenized and characterized. All mutated amino acids are highlighted in Figure 1B.

Synthesis of mutant oxaloacetate decarboxylases in Escherichia coli and oxaloacetate decarboxylase activities

To synthesize the mutant oxaloacetate decarboxylases, mutated DNA fragments were cloned into pSK-GAB (Di Berardino and Dimroth, 1995) using appropriate restriction sites, and used to transform E. coli DH5α, as described in Experimental procedures. Grown cells were disrupted, and the oxaloacetate decarboxylases were purified by affinity chromatography of solubilized membrane fractions (Dimroth, 1986). Synthesis of the three subunits of the decarboxylases was verified for all mutants described here by sodium-dodecyl-sulphate-polyacrylamide-gel-electrophoresis (SDS-PAGE), and a selection of these results is shown in Figure 2. Expression of mutant enzymes was quantified by protein determination. These results are given in Table 1 together with the specific activities of the isolated mutant enzymes. Between 0.05 and 0.6 mg/g cells of the oxaloacetate decarboxylase variants could be isolated. In most of these mutants, the specific oxaloacetate decarboxylase activity dropped to a range between 5 and 54 % of the wild-type enzyme. The corresponding amino acids contribute therefore to a high specific activity of the decarboxylase, but are not absolutely essential for function. The most severe effects on activity were observed by mutating tyrosines 227 and 229 in transmembrane helix IV. In the Y227A mutant, the specific activity dropped to 0.5
U/mg protein, but the Y227C or Y227F variants had specific activities of around 10 U/mg protein. Hence, a tyrosine at position 227 assures high specific activity of the oxaloacetate decarboxylase but is not essential for the function of the enzyme. The oxaloacetate decarboxylase activity was completely abolished upon mutating Y229 to F and below 0.1 % of the wild-type activity was found in the Y229A mutant. Therefore, for Y229 we envisage a crucial role in the decarboxylation mechanism (see Discussion).

Oxaloacetate decarboxylase with a βC291A mutation retained 8 % of the wild-type specific activity. An interesting phenotype of this mutant was a severe reduction of the stability of the enzyme complex in the absence of Na+. Under these conditions, an α–γ subcomplex was purified by avidin-Sepharose affinity chromatography, whereas isolation of the α–β–γ complex required the presence of 0.5 M NaCl (Figure 2).
FIGURE 2. Expression of mutant oxaloacetate decarboxylases as evidenced from SDS-PAGE after purification of the proteins. Mutations in OadB are indicated; Wt = wild-type; marker = marker proteins with molecular masses shown (in kDa). α, β, γ denote the three subunits of oxaloacetate decarboxylase. Oxaloacetate decarboxylase with the C291A mutation was isolated in the absence of Na⁺ (C291A - Na⁺) or in the presence of 0.5 M NaCl (C291A + Na⁺).
<table>
<thead>
<tr>
<th>mutation</th>
<th>amount of oxaloacetate decarboxylase isolated (mg/g wet packed cells)</th>
<th>specific activity (U/mg)</th>
<th>optical density at 600 nm</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.5</td>
<td>45</td>
<td>0.5</td>
</tr>
<tr>
<td>N55A</td>
<td>0.6</td>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>T148A</td>
<td>0.5</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td>T189A</td>
<td>0.3</td>
<td>18</td>
<td>0.4</td>
</tr>
<tr>
<td>Q192L</td>
<td>0.1</td>
<td>2</td>
<td>0.4</td>
</tr>
<tr>
<td>G197A</td>
<td>0.3</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>G200A</td>
<td>0.3</td>
<td>5</td>
<td>0.4</td>
</tr>
<tr>
<td>G201A</td>
<td>0.3</td>
<td>10</td>
<td>0.4</td>
</tr>
<tr>
<td>G204A</td>
<td>0.2</td>
<td>7</td>
<td>0.4</td>
</tr>
<tr>
<td>T206A</td>
<td>0.3</td>
<td>20</td>
<td>0.4</td>
</tr>
<tr>
<td>Y209A</td>
<td>0.05</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>E217A</td>
<td>0.4</td>
<td>6</td>
<td>0.4</td>
</tr>
<tr>
<td>Y227A</td>
<td>0.4</td>
<td>0.5</td>
<td>0</td>
</tr>
<tr>
<td>Y227C</td>
<td>0.3</td>
<td>12</td>
<td>0.3</td>
</tr>
<tr>
<td>Y227F</td>
<td>0.1</td>
<td>9</td>
<td>0.4</td>
</tr>
<tr>
<td>Y229A</td>
<td>0.2</td>
<td>0.02</td>
<td>0</td>
</tr>
<tr>
<td>Y229F</td>
<td>0.1</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Q237A</td>
<td>0.2</td>
<td>8</td>
<td>0.5</td>
</tr>
<tr>
<td>D282A</td>
<td>0.3</td>
<td>24</td>
<td>0.4</td>
</tr>
<tr>
<td>C291A</td>
<td>0.2</td>
<td>4</td>
<td>0.4</td>
</tr>
<tr>
<td>T318A</td>
<td>0.5</td>
<td>12</td>
<td>0.4</td>
</tr>
<tr>
<td>C351A</td>
<td>0.2</td>
<td>3</td>
<td>0.3</td>
</tr>
<tr>
<td>T354A</td>
<td>0.2</td>
<td>3</td>
<td>0.4</td>
</tr>
<tr>
<td>S419A</td>
<td>0.1</td>
<td>4</td>
<td>0.4</td>
</tr>
</tbody>
</table>

Table 1. Synthesis of mutant oxaloacetate decarboxylases from OadB variants and specific activities of isolated enzymes and growth of E. coli EP432 transformed with plasmids expressing mutant oxaloacetate decarboxylases in the presence of 360 mM NaCl. The optical density was determined after 28 h growth at 30°C.
Formation of stable carboxybiotin enzyme derivatives with mutants in OadB, which are inactive in oxaloacetate decarboxylation

The first step in oxaloacetate decarboxylation is the transfer of the carboxyl group from oxaloacetate to the biotin prosthetic group (Dimroth and Thomer, 1988). This reaction is catalyzed by OadA and was therefore not affected by the Y229F mutation, as shown by the formation of the $[^{14}C]$carboxybiotin enzyme derivative upon incubation of the mutant with [$^{4-14}$C]oxaloacetate (Table 2). As the subsequent decarboxylation was impaired, sodium ions were without effect on the labeling of the enzyme. In contrast, stable carboxybiotin enzyme derivatives were not obtained for the Y227A or Y229A mutants, which have low residual oxaloacetate decarboxylase activities (Table 1). We conclude therefore that the carboxybiotin decarboxylase activity was abolished in the Y229F mutant and that this activity was significantly affected but not impaired in the Y227A or Y229A mutants.

<table>
<thead>
<tr>
<th>mutation</th>
<th>amount of enzyme (µg)</th>
<th>protein-bound radioactivity expected at 100 % labeling (cpm)</th>
<th>protein-bound radioactivity found (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Y227A</td>
<td>12</td>
<td>1100</td>
<td>95</td>
</tr>
<tr>
<td>Y229A</td>
<td>24</td>
<td>2200</td>
<td>200</td>
</tr>
<tr>
<td>Y229F</td>
<td>24</td>
<td>2200</td>
<td>2800</td>
</tr>
<tr>
<td>wild-type</td>
<td>15</td>
<td>1380</td>
<td>100</td>
</tr>
</tbody>
</table>

Table 2. Formation of $[^{14}$C]carboxybiotin enzyme derivatives by carboxyltransfer from [$^{4-14}$C]oxaloacetate to mutants in OadB, which are inactive or nearly inactive in oxaloacetate decarboxylation. The reactions were performed in the presence of 10 mM NaCl.

Effect of OadB mutations on Na$^+$ binding characteristics and pH profiles

As reported previously, OadB is specifically protected from tryptic hydrolysis at 20 - 50 mM Na$^+$ (Dimroth and Thomer, 1983, 1992). Under our conditions, the halftime for tryptic digestion of wild-type OadB was 12 h in the absence of Na$^+$ and > 24 h in the presence of 50 mM NaCl. It has also been noticed that the catalytically inactive OadB mutants G377A, S382C, S382E, S382N or S382Q yielded halftimes for tryptic digestion of about 1 h, indicating less rigid structures for these OadB variants (see Chapter 3). The characteristics of the OadB mutants Y227A, Y227F, Y229A, Y229F, and C291A with respect to tryptic hydrolysis in the presence or absence of Na$^+$ ions.
were similar to the wild-type enzyme. Hence, these variants not only adopt a rather rigid structure, so that cleavage by trypsin is slow, but they also retain the Na\(^+\) binding sites which, when occupied, further protect OadB from tryptic digestion.

The Na\(^+\) binding affinities were also analyzed by the Na\(^+\) activation profiles for oxaloacetate decarboxylation. The results of Table 3 indicate that in all mutants the Na\(^+\) concentration yielding half maximal activation increased to various extents compared to the wild-type, most significantly in the Q192L, G201A and T318A mutants, where the increase was between seven and five-fold. For most mutant decarboxylases, the pH optimum was between 6.5 and 7.5 and thus similar to the wild-type enzyme. Exceptions are the Q192L and Y209A mutants, which have a pH optimum of 5.5.

The mutants were further characterized by their Na\(^+\) pumping activity using an in vivo assay with the Na\(^+\)/H\(^+\) antiporter deletion mutant E. coli EP432 (Pinner et al., 1993). These cells are unable to grow in the presence of elevated Na\(^+\) concentrations, but after transformation with plasmids encoding an oxaloacetate decarboxylase Na\(^+\) pump, growth in the presence of 360 mM NaCl is observed (see Chapter 3). The results given in Table 1 show that all OadB mutants investigated in this study with a specific oxaloacetate decarboxylase activity of more than 2 U/mg protein were functional Na\(^+\) pumps. The Y227A, Y229A and Y229F mutants, on the other hand, having specific activities of less than 0.5 U/mg protein, were unable to complement E. coli EP432, which indicates that the Na\(^+\) pump function is either impaired or very low.
<table>
<thead>
<tr>
<th>mutation</th>
<th>halfmaximal activation by Na$^+$ (mM)</th>
<th>pH-optimum</th>
<th>half-time for OadB digestion $^a$ (+Na$^+$ / - Na$^+$) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>0.5</td>
<td>7.0</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>N55A</td>
<td>1.2</td>
<td>6.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>T148A</td>
<td>0.7</td>
<td>6.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>T189A</td>
<td>1.2</td>
<td>6.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>Q192L</td>
<td>3.6</td>
<td>5.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>G197A</td>
<td>0.8</td>
<td>6.5-7.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>G200A</td>
<td>0.7</td>
<td>-</td>
<td>n.d.</td>
</tr>
<tr>
<td>G201A</td>
<td>2.3</td>
<td>6.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>G204A</td>
<td>1.9</td>
<td>6.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>T206A</td>
<td>0.8</td>
<td>6.0-6.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>Y209A</td>
<td>1.7</td>
<td>5.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>E217A</td>
<td>0.9</td>
<td>6.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>Y227A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Y227C</td>
<td>1.1</td>
<td>7.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>Y227F</td>
<td>1.0</td>
<td>6.5-7.0</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Y229A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Y229F</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Q237A</td>
<td>0.7</td>
<td>6.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>D282A</td>
<td>0.8</td>
<td>7.0</td>
<td>n.d.</td>
</tr>
<tr>
<td>C291A</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>T318A</td>
<td>2.7</td>
<td>6.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>T354A</td>
<td>0.9</td>
<td>6.5</td>
<td>n.d.</td>
</tr>
<tr>
<td>S419A</td>
<td>1.1</td>
<td>7.5</td>
<td>n.d.</td>
</tr>
</tbody>
</table>

Table 3. Effect of OadB mutations on Na$^+$ binding characteristics and pH profiles. $^a$ Half-time for digestion of OadB by trypsin in presence of 50 mM NaCl (+Na$^+$) or in the absence of Na$^+$ (-Na$^+$). n. d. = not determined
4.4 Discussion

To determine all functionally important amino acid residues of OadB, we have now mutagenized another twenty polar and/or conserved amino acid residues within putative integral membrane regions. The mutant proteins were synthesized in E. coli and were purified as the three-subunit complexes, irrespective of whether Na\(^+\) ions were present or absent. Hence, these mutations do not affect the assembly or stability of the enzyme complex. The only exception is the βC291A mutant, from which the αβγ complex could only be isolated in the presence of 0.5 M NaCl, while in the absence of Na\(^+\), the enzyme dissociated into the α-γ subcomplex and the free β subunit during chromatography on the monomeric avidin-Sepharose column. According to recent topology analysis, C291 is located within putative transmembrane helix V near the periplasmic surface (Jockel et al., 1999; see Chapter 2). As C291 is not conserved, it is unlikely to play an universal role in complex formation among the related enzymes. We rather assume a conformational change in OadB due to the C291A mutation through which the three subunit complex becomes destabilized. It has already been noted that in presence of elevated Na\(^+\) concentrations (> 20 mM) OadB changes its conformation and becomes more resistant to tryptic digestion (Dimroth and Thomer, 1983, 1992). It is possible that the more compact conformation of OadB, adopted at elevated Na\(^+\) concentrations, contributes to the stability of the complex. The more labile complex generated by the C291A mutation, therefore, dissociates in the absence of Na\(^+\), but keeps together in the presence of 0.5 M NaCl. The glutaconyl-CoA decarboxylase β subunit from *Acidaminococcus fermentans* contains a cysteine residue at position 299 (OadB numbering) in the loop connecting helices V and VI. This particular cysteine residue was modified by N-ethylmaleimide and NaCl (> 10 mM) specifically protected from the modification, indicating a Na\(^+\)-induced conformational change within this area of the molecule (Braune et al., 1999). However, as the loop between helices V and VI is located in the periplasm, where Na\(^+\) binding is not expected to take place, it is likely that the Na\(^+\)-induced burying of C299 marks a more global conformational change, including other parts of the protein as well.

In most of the OadB mutants investigated here, the specific oxaloacetate decarboxylase activity was significantly affected. As we have mutated conserved residues that may contribute to the optimal function of these proteins, this result is not unexpected. It is noteworthy that the low catalytic activities of the mutated enzyme specimens were
paralleled by inferior Na\(^+\) binding properties, as revealed from increases of the Na\(^+\) concentrations required for half maximal activation. It is also noteworthy that all mutant decarboxylases except those with the tyrosine mutations described below were functional Na\(^+\) pumps. Hence, none of the mutations investigated here led to an uncoupled phenotype of the decarboxylase. Based on our recent proposal for the coupling mechanism, mutations leading to an uncoupled oxaloacetate decarboxylase are not to be expected: in this proposal, the proton consumed in the decarboxylation of carboxybiotin and the Na\(^+\) ions pumped traverse the membrane within opposite directions. Furthermore, S382 in the center of the membrane acts as a binding site for Na\(^+\) and H\(^+\). For the hydroxyl proton of S382 to be displaced and consumed in the decarboxylation reaction, Na\(^+\) binding is mandatory (see Chapter 3). Hence, any mutation that affects the Na\(^+\) binding characteristics of this site or the Na\(^+\) access channels to this site will automatically affect the decarboxylation of carboxybiotin, and the decarboxylation event cannot be uncoupled from Na\(^+\) translocation.

Transmembrane helix IV contains two universally conserved tyrosine residues close to the center of the membrane, which are separated by a single serine or leucine residue. Decarboxylase with a Y227A mutation was nearly inactive, but with the Y227F or Y227C mutations, approximately 25% of the wild-type decarboxylase activity was retained, thus indicating that Y227 is not an indispensable residue for function. In contrast, enzyme with a Y229F mutation was completely inactive and that with a Y229A mutation retained only traces of activity. Therefore, Y229 appears to be an important residue for the function of the enzyme. Unlike the Na\(^+\) binding residues D203 and S382 (Di Berardino and Dimroth, 1996; Chapter 3), neither of these tyrosine residues appear to participate in Na\(^+\) binding, since Na\(^+\) protection from trypptic hydrolysis was similar in the wild-type OadB and in the Y227A, Y227F, Y229A or Y229F mutants. We do not anticipate a mere structural role for Y229, because this would not be compatible with the complete knock-out of catalytic activity upon replacing the phenol by a phenyl ring. It is more likely that the hydroxyl group of Y229, which is inevitable for function, participates in proton translocation through OadB into the catalytic site of carboxybiotin decarboxylation. Tyrosine 229 could be involved in a hydrogen-bonded network allowing efficient proton transfer from S382 to carboxybiotin, where the proton is consumed in the decarboxylation reaction. R389 and bound water molecules might also be involved in this network. In the Y229A mutant, the smaller alanine side chain may allow a water molecule to come close to S382 and to
participate in the translocation network instead of Y229. This would explain the low oxaloacetate decarboxylase activity of the Y229A mutant. However, replacement of Y229 with the more similar but bulkier F is not expected to provide enough space for an extra water molecule and, therefore, this mutant was completely inactive. Precedence for a hydrogen-bonded network containing tyrosines was found in the crystal structure of bacteriorhodopsin (Luecke et al., 1998). Here, the network includes an aspartate, an arginine, two tyrosines and a water molecule.

A potential catalytic mechanism, in which Na\(^+\) binding at S382 and binding of carboxybiotin near R389 initiates a proton translocating cascade involving Y229 and water is shown in Figure 3. We anticipate that with Na\(^+\) approaching S382 through the cytoplasmic channel, the proton from the hydroxyl side chain of S382 is displaced. This induces the rearrangement of hydrogen bonding within the network consisting of S382, Y229, H\(_2\)O, R389 and carboxybiotin, with the consequence that the proton delivered to carboxybiotin catalyzes the immediate decarboxylation of this acid-labile compound. The proton is consumed in the decarboxylation reaction and this leads to a conformational change by which the cytoplasmic channel closes and a periplasmic channel opens. To displace the Na\(^+\) from S382 and deliver it to the periplasmic surface, a periplasmic proton penetrating through this channel must restore the hydroxyl group of S382. A hydrogen bonded network including D203 and bound H\(_2\)O molecules could be involved in the proton pathway connecting the periplasmic reservoir with S382.
FIGURE 3. Stereoscopic view of the model of two helical segments (IV and VIII) of the active site domain of the oxaloacetate decarboxylase β subunit generated using the program GRASP (Nicholls et al., 1991). The Cα-backbone of the helical model is depicted in blue and amino acids Y229, S382 and R389, being relevant for the coupling mechanism, are shown as ball-and-stick models with the hydrogen bonds being drawn as yellow lines. The model of the energy coupling mechanism of OadB emphasizes details of the proton translocation network within the cytoplasmic channel, linking S382 with carboxybiotin. S382 is in hydrogen bonding distance with Y229, which is hydrogen bonded via a water molecule with R389, and this is hydrogen bonded to the ring carbonyl oxygen of carboxybiotin. Na⁺ entering through a part of the channel, consisting of helix IV and VIII, displaces a proton from the hydroxyl side chain of S382. This leads to the rearrangement of hydrogen bonds in the network, delivering a proton to the ring carbonyl oxygen of carboxybiotin, where it is consumed in the decarboxylation reaction. Please note that for simplification biotin was drawn as the free cofactor. In the complex, it is bound with its side-chain carboxyl group to a specific lysine residue of OadA.
4.5 Experimental procedures

**Bacterial strains**
The bacterial strains *Escherichia coli* DH5α (Bethesda Research Laboratories), *Escherichia coli* JM110 (Yanish-Perron et al., 1995), *Escherichia coli* EP432 (Pinner et al., 1993) and *Escherichia coli* BL21(DE3)pLysS (Dunn and Studier, 1983) were used in this study. All strains, except *E. coli* EP432, were grown at 37°C in Luria Bertani (LB) medium (Sambrook et al., 1989). *E. coli* EP432 was grown as described (see Chapter 3). Plasmid-containing strains were supplemented with the selective antibiotics ampicillin (100 μg/ml) and/or chloramphenicol (40 μg/ml) or kanamycinsulfate (50 μg/ml).

**Recombinant DNA techniques**
Standard recombinant DNA techniques were performed essentially as described by Sambrook et al. (1989). Polymerase chain reactions (PCRs) were performed using Vent-DNA-Polymerase from New England Biolabs (Beverly, MA, USA). DNA sequencing was carried out according to the dideoxynucleotide chain-termination method (Sanger et al., 1977) using a *Taq* DyeDeoxy terminator cycle kit and the ABI Prism 310 genetic analyzer from Applied Biosystems.

**Construction of site-directed mutants of the β subunit**
The primers used for site-directed mutagenesis are listed in Table 4. Site-directed mutants were obtained as follows: The PCR fragments, containing the mutations were constructed in a two step protocol. For the N-terminal part of the PCR fragments of the β subunit, primer prN or prBN and primers with the affix rev were used, and pSK-GAB (Di Berardino and Dimroth, 1995) served as template. For the corresponding PCR fragments of the C-terminal part of the β subunit, primers prC or prBC and primers with the affix for were used, and pSK-GAB (Di Berardino and Dimroth, 1995) served as template. After purification, those PCR fragments were used as template for the PCR products from primers prN and prC or primers prBN and prBC, which contained the mutation. PCR fragments were digested with *Kpn*2I and *Bcl*I, in case of fragments from primers prN and prC, or with *Bcl*I and *Bst*1107I, in case of fragments from primers prBN and prBC and cloned into pSK-GAB. From pSK-GAB, which was isolated from JM110 cells, the *Kpn*2I/Bcl*I or Bcl*I/Bst*1107I fragment was removed.
<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Restriction sites/comments</th>
</tr>
</thead>
<tbody>
<tr>
<td>prN</td>
<td>CTCTCCAATATTTCCGGAGGGCG</td>
<td>Kpn2I site in bold</td>
</tr>
<tr>
<td></td>
<td>GGCTGGCG</td>
<td></td>
</tr>
<tr>
<td>prC</td>
<td>GAAGATGGTGACGATGTTGATC</td>
<td>BclI site in bold</td>
</tr>
<tr>
<td></td>
<td>AGCCGCCTTCTCG</td>
<td></td>
</tr>
<tr>
<td>prBN</td>
<td>CAGAACGCCTGATCAACATC</td>
<td>BclI site in bold</td>
</tr>
<tr>
<td></td>
<td>GTCACCATCTTTC</td>
<td></td>
</tr>
<tr>
<td>prBC</td>
<td>GATTTTTTCCGGGTATACATTGGG</td>
<td>Bst1107I site in bold</td>
</tr>
<tr>
<td></td>
<td>TAGATAGA</td>
<td></td>
</tr>
<tr>
<td>prN55Arev</td>
<td>GCCCTCCGGAATAGCGGAGAGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>AGGCCGCAGGAA</td>
<td></td>
</tr>
<tr>
<td>prN55Afor</td>
<td>TTCGGCGGCCTGCTCTCCGCTAT</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>TCCGGAGGCCC</td>
<td></td>
</tr>
<tr>
<td>prT148Arev</td>
<td>GAAATCCGCCATCGCTCCGCTAT</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCCATAAAA</td>
<td></td>
</tr>
<tr>
<td>prT148Afor</td>
<td>TTTATGGGCCTCGGAGCGATGGG</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CCGATTTTC</td>
<td></td>
</tr>
<tr>
<td>prT189Arev</td>
<td>GCCCGCCGCCCTGCGGAGAGC</td>
<td>mutation underlined, SapI</td>
</tr>
<tr>
<td></td>
<td>GAAGCTGATGATGCC</td>
<td>site in bold</td>
</tr>
<tr>
<td>prT189Afor</td>
<td>GCCATCATCAGCTTCGGGTCTTC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>CGCAGGCCAGGGCGCGGCC</td>
<td>site in bold</td>
</tr>
<tr>
<td>prQ192Lrev</td>
<td>GCCGATGCGCGCCCGCTAGCGG</td>
<td>mutation underlined, NheI</td>
</tr>
<tr>
<td></td>
<td>CAGGCTGAAGGC</td>
<td>site in bold</td>
</tr>
<tr>
<td>prQ192Lfor</td>
<td>GCTTCAACCCTGCCGCTAGCGGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td></td>
<td>GGCCATCGGCC</td>
<td>site in bold</td>
</tr>
<tr>
<td>prG197Arev</td>
<td>GCCGATGCGGCATGCGGCCGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG197Afor</td>
<td>GCCGCCATCGGCCATCGGCC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG200Arev</td>
<td>GTCCGCGCGCGCGATGATGCC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG200Afor</td>
<td>GCCATCATCGCGCGCGCGCGAC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG201Arev</td>
<td>GCCGTCGCGCGCCCGCGATGAT</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG201Afor</td>
<td>ATCATCGCGCGCGCGCGACGC</td>
<td>mutation underlined</td>
</tr>
<tr>
<td>prG204Arev</td>
<td>CGCCGTCGCGCGCGCGCGC</td>
<td>mutation underlined</td>
</tr>
</tbody>
</table>
Essential Role of Tyrosine 229 of OadB in Energy Coupling

\[
\begin{align*}
\text{prG204A for} & \quad \text{GGCGCCGACGC\ldots \text{mutation underlined}} \\
\text{prY227A rev} & \quad \text{CGCCATATAGGACGC\ldots \text{mutation underlined}} \\
\text{prY227A for} & \quad \text{ATCGCGGTGGGCGCG\ldots \text{mutation underlined}} \\
\text{prY227C rev} & \quad \text{GCACCAGCGC\ldots \text{mutation underlined, } PstI} \\
\text{prY227C for} & \quad \text{GCCATCGCGG\ldots \text{mutation underlined, } PstI} \\
\text{prY227F rev} & \quad \text{CGCCATATAGGAGA\ldots \text{mutation underlined}} \\
\text{prY227F for} & \quad \text{ATCGCGGTGGGCGCGT\ldots \text{mutation underlined}} \\
\text{prY229A rev} & \quad \text{CGCCAT\ldots \text{mutation underlined}} \\
\text{prY229A for} & \quad \text{ATCGCGGTGGGCGCGT\ldots \text{mutation underlined}} \\
\text{prY229F rev} & \quad \text{CGGC\ldots \text{mutation underlined}} \\
\text{prQ237A rev} & \quad \text{CTTCATGATCG\ldots \text{mutation underlined}} \\
\text{prQ237A for} & \quad \text{CTGCTGCCGCTG\ldots \text{mutation underlined}} \\
\text{prD282A rev} & \quad \text{CAGCGGC\ldots \text{mutation underlined}} \\
\text{prD282A for} & \quad \text{CTCCTGCTG\ldots \text{mutation underlined}} \\
\text{prC291A rev} & \quad \text{GC\ldots \text{mutation underlined}} \\
\text{prC291A for} & \quad \text{AATTC\ldots \text{mutation underlined}}
\end{align*}
\]
prBNT318A  CAGAACGCGCTGATCAACATC  mutation underlined, BclI
     GTCGCCATCTTC  site in bold
prC351Arev  CCCGGCGGCGGTCCCCACTTCG  mutation underlined
     AAGGCGATCACCACCCAGCAC
prC351Afor  GTGCTGGGGGTGATCGCTTCG  mutation underlined
     AAGTGCGGACCAGCCGCCCGG
prT354Arev  CAGCACCCCCGCGGCTGACCC  mutation underlined, BsgI
     CACGCAGAAGGGCG  site in bold
prT354Afor  CGCCTTCTGGCTGAGGCTGACCC  mutation underlined, BsgI
     GCCGGGGTGCCTG  site in bold
prS419Arev  GCGGGCGATCGCCCGCGGGATC  mutation underlined
     ACCCCCGGC
prS419Afor  GCGGGGGGTGATCGCGCGATCG  mutation underlined
     CGGCC

Table 4. Primers used for site-directed mutagenesis studies of the β-subunit.

Purification of oxaloacetate decarboxylase variants and enzyme assays
Mutant oxaloacetate decarboxylases were purified from DH5α/pSK-GAB variants by
affinity chromatography of a solubilized membrane extract on a SoftLink monomeric
avidin-Sepharose column (Promega) (Dimroth, 1986). Large-scale purification was
performed as described previously (Di Berardino and Dimroth, 1996). The
decarboxylation activity was determined with the simple spectrophotometric assay at
265 nm (Dimroth, 1986).

Screening of oxaloacetate decarboxylase activity from mutant clones
Before a large-scale purification of the mutant protein was performed, a small-scale
culture of the respective clone was used to measure oxaloacetate decarboxylase activity
(Di Berardino and Dimroth, 1996). To check for an active Na⁺ pump, the mutant
plasmid was transformed into E. coli EP432, which lacks both Na⁺/H⁺ antiporters, and
the growth was determined in the presence of 360 mM NaCl. Such growth is only
observed, if the Na⁺ pump is functional (see Chapter 3).
Labeling of oxaloacetate decarboxylase and mutant enzymes with $^{14}$CO$_2$ from [4-$^{14}$C]oxaloacetate

[4-$^{14}$C]oxaloacetate, prepared from [4-$^{14}$C]L-aspartate and 2-oxoglutarate with glutamate:oxaloacetate transaminase, was used to measure the transfer of the radioactive carboxyl residue from [4-$^{14}$C]oxaloacetate to the biotin bound to OadA, as described (see Chapter 3).

Determination of oxaloacetate decarboxylase activity at various Na$^+$ concentrations and pH values

The decarboxylation activities of wild-type (DH5α/pSK-GAB) and mutant oxaloacetate decarboxylases were measured at different pH values, in the range between pH 5.5 and pH 8.8, in 40 mM Mes/Tris buffer, containing variable Na$^+$ concentrations, utilizing the simple spectrophotometric assay (Dimroth, 1986).

Analytical procedures

The protein content of samples was determined according to Bradford (1979) or by the bicinchoninic acid method (Smith et al., 1985).

Effect of Na$^+$ on tryptic hydrolysis of the oxaloacetate decarboxylase β subunit

Protection from proteolytic digestion of the β subunit by Na$^+$ ions was determined for the mutants Y227A, Y227F, Y229A, Y229F and C291A. The incubation mixtures contained in 60 µl at 25°C: (A) 20 mM potassium phosphate buffer, pH 7.5, 50 mM potassium chloride, purified wild-type or mutant oxaloacetate decarboxylase (25 µg) and 3 µg trypsin. A parallel incubation mixture (B) contained 50 mM sodium chloride instead of potassium chloride. Samples (12 µl) were transferred after 0 (before adding of trypsin), 2, 4, 7.5, and 24 h incubation into 1 µl 50 mM phenylmethanesulfonyl fluoride and 12 µl SDS sample buffer to stop proteolysis. The samples were subsequently analyzed by SDS-PAGE (see Chapter 3).

Molecular modeling

Molecular modelling of the active site domain in the β subunit of oxaloacetate decarboxylase was performed on a Silicon Graphics INDIGO 2 workstation with the program CHAIN (Sack, 1988). The three-dimensional model of the two transmembrane helices IV and VIII was build using α-helical polyalanine segments consisting of 20 residues. These alanines were then transformed into the corresponding side chains according to the primary sequence (Laußermair et al., 1989; Woehlke et al., 1992). Helices IV and VIII, having an extension of about 29 Å, were positioned within van der Waals contact distances parallel to each other. The atomic coordinates of a biotin from
the biotin-binding protein avidin (Brookhaven Protein Data Bank code 1AVD; Pugliese et al., 1993) were used as a template to generate the carboxybiotin.
4.6 References


CHAPTER 5

General Discussion

5 Mechanisms of ion translocation through bacterial membrane proteins

Bacteria conserve and transduce metabolic energy by means of an electrochemical gradient of protons across the cytoplasmic membrane in accordance with the chemiosmotic theory of Mitchell (Mitchell, 1961, 1968, 1973, 1976). This electrochemical gradient is created by different types of primary active transport mechanisms in bacteria, ATPases (ion translocation is coupled to the hydrolysis of ATP), redox enzymes (complexes of the respiratory chain or photosynthesis couple the ion translocation to electron transfer reactions), light-driven ion pumps of halophilic bacteria, and the Na\(^+\) pumping decarboxylases of some fermenting bacteria. The different types of ion pumps show diversity in size, structure and the catalysis and nature of the transported ion (for a review see Rosen and Silver, 1987). In the following, the ion translocation mechanisms through primary ion pumps, sodium ion translocation of the F\(_{1}F_{0}\) ATPase of *Propionigenium modestum* and ion translocation and ion selectivity of the K\(^+\) channel of *Streptomyces lividans* are described. Further, the mechanisms of ion translocation through secondary transporters (examples for transducing the free energy stored in the electrochemical gradient into a solute concentration gradient), like ion translocation and ion selectivity of the melibiose permease of *Escherichia coli*, and the mechanism for energy coupling in the lactose permease of *Escherichia coli* are circumstantiated. Finally the coupling between Na\(^+\) translocation and decarboxylation of Na\(^+\) pumping decarboxylases, which comprize primary ion pumps, will be described.

5.1 Mechanism of ion translocation through F\(_{1}F_{0}\)-ATPases

As already mentioned in Chapter 1, F\(_{1}F_{0}\) ATP synthases consist of the cytoplasmic F\(_{1}\)-part (subunit composition \(\alpha_3\beta_3\gamma_8\delta\varepsilon\)), which contains three catalytic sites, and the membrane-bound F\(_{0}\)-part, which comprizes an ion channel. In 1997 Boyer and Walker received the Nobel prize for chemistry for their studies on the mechanism of ATP-synthesis by the H\(^+\)-translocating F\(_{1}F_{0}\) ATP synthase. They described the enzyme as a
rotary machine driven by an electrochemical potential. A catalytic mechanism of ATP hydrolysis, which involves the rotation of the extended γ-subunit within the central cavity of the α₃β₃ headpiece, was suggested and proven by experimental evidence (Duncan et al., 1995; Sabbert et al., 1996; Noji et al., 1997). The membrane-embedded F₀-component is responsible for the translocation of coupling ions across the membrane. In bacteria, the subunit composition is ab₂c₉₁₂. The 9 - 12 c subunits are assembled into a ring that is flanked at the periphery by the a and the two b subunits (Birkenhäger et al., 1995; Singh et al., 1996; Takeyasu et al., 1996). Additionally the γ and c subunit are attached to the transmembrane disk of c subunits. A rotational mechanism, as proven for the F₁-part, was also suggested for the F₀-part of the ATPase of Propionigenium modestum (Kaim et al., 1998). In this rotational model for the coupling ion movement via the a and c subunits across the membrane, the c subunits are accessible from the cytoplasm and are able to bind/release the Na⁺ ions in a reversible manner. After occupation of the Na⁺ binding sites on the multiple c subunits from the cytoplasm, ATP hydrolysis moves a Na⁺ loaded c subunit into close contact with the release channel on the a subunit. Simultaneously, an empty c subunit is displaced from this position. The Na⁺ ion of the c subunit, making the new contact with subunit a, is subsequently removed from its binding site and released to the periplasm via the ion-selective a subunit channel (see Figure 1). The Na⁺ binding site on the c subunit is Glu 65 (Kluge and Dimroth, 1993), and Gln 32 and Ser 66 are necessary for coordination of this ion (Kaim et al., 1997), which is in accord to the structural information of the P. modestum subunit c (Matthey et al., 1999). The critical charged residues in the a subunit of P. modestum are Arg 227 and possibly Asp 259. Together with Glu 65 of the c subunit they may be essential to drive rotation of the c subunits by the attraction and repulsion of electrical charges (Kaim et al., 1998). The Na⁺ (or Li⁺ or H⁺) ion translocation that is connected to this rotation probably contributes to the electrical charge forces, e. g. by transiently neutralizing the negative charge of Glu 65 at the coupling ion binding site of the c subunit. A mechanochmical model for the transduction of transmembrane sodium-motive force into rotary torque has been recently presented for the P. modestum ATP synthase, which is likely to operate in other F₁F₀-ATPases with the same mechanism (Dimroth et al., 1999). Oster and Wang described the ATP synthase as an enzyme with two motors and two fuels. The enzyme comprizes two reversible rotary motors: F₀ is either an ion "turbine" or an ion pump, and F₁ is either a hydrolysis motor or an ATP synthesizer (Oster and Wang, 1999).
5.2 The $K^+$ channel of *Streptomyces lividans*

Potassium ions diffuse rapidly across cell membranes through proteins called $K^+$ channels. Although $K^+$ channels are best known for their role in neuronal signaling, they are found in almost all biological organisms. The structure of the $K^+$ channel (KcsA) from *Streptomyces lividans* led to a more detailed understanding of the ion translocation mechanism and additional, KcsA represents an example for ion specificity. The channel is an integral membrane protein with sequence similarity to all known $K^+$ channels, particularly the pore region (Schrempf et al., 1995; Heginbotham et al., 1997; Cortes and Perozo, 1997). The KcsA channel is formed by four identical subunits. Each subunit is composed of 158 residues. All $K^+$ channels exhibit very similar ion permeability and selectivity characteristics (Hille, 1992), i.e., $K^+$ ions are 10,000 times more permeant than $Na^+$ ions. This extreme ion selectivity implies strong energetic interactions between $K^+$ and the pore. It is known from the structure of this protein, that the channel contains three sites for $K^+$ location at three different positions of the molecule (Doyle et al., 1998), in agreement with the fact that $K^+$ channels are multi-ion
channels. K⁺ channels are classified as "long pore channels", invoking the notion that multiple ions queue inside a long narrow pore in single file. Two distinct ion binding sites in the tetramer are formed by the loops connecting the pore helix with the outer helix, and the third location site consists of a central water-filled, hydrophobic cavity in the middle of the membrane. It is supposed, that the ion within this cavity is stabilized by the polarity of four pore helices facing toward the center of the cavity. The large water-filled cavity and helix dipoles are positioned so as to overcome electrostatic destabilization of an ion in the pore at the center of the bilayer. The selectivity filter, located between the first and the second binding site, is a narrow funnel with a diameter of 3 Å, lined with backbone carboxylic oxygen atoms (Armstrong, 1998). Four identical subunits create an inverted cone, cradling the selectivity filter of the pore in its outer end, as it reveals from X-ray analysis, with data to 3.2 Å. The selectivity is maintained by this filter, which consists of two ion binding sites about 7.5 Å apart.

Figure 2: Two mechanisms by which the K⁺ channel stabilizes a cation in the middle of the membrane. First, a large aqueous cavity (1) stabilizes an ion (black) in the otherwise hydrophobic membrane interior. Second, oriented helices point their partial negative charge (carboxyl end, 2) towards the cavity where a cation is located (adopted from Doyle et al., 1998).

The structure of the filter is inflexible as suggested by X-ray analysis, whereby the rigidity is maintained by interactions between tryptophane and tyrosine residues located on different subunits of the tetramer. The narrow filter is only 12 Å long, whereas the remainder of the pore is wider and lined with hydrophobic amino acids. Main chain carbonyl oxygen atoms from the K⁺ channel signature sequence line the selectivity filter, which is held open by structural constraints to coordinate K⁺ ions but no smaller
Na⁺ ions. Na⁺ binding is prevented, because it obtains no precise fit to the filter. This configuration promotes ion conduction by exploiting electrostatic repulsive forces to overcome attractive forces between K⁺ ions and the selectivity filter. The architecture of the pore establishes the physical principles underlying selective K⁺ conduction. Binding of K⁺ ions appears as follows: K⁺ dehydrates completely by entering the selectivity filter. The carbonyl oxygen atoms of the binding motif V-G-Y-G come in very close contact to the K⁺ ions to compensate the energetic cost of the dehydration (Doyle et al., 1998). Molecular dynamics simulations, based on the X-ray structure led to several conclusions: (1) The channel is engineered so as to repel anions and attract cations, i. e., K⁺ and Na⁺, into the first binding site within the pore. This valence selectivity is due to the specific channel conformation, with negative charges located near to the channel axis. (2) When the channel is not yet occupied by a cation, the charge configuration of the channel is able to strip most of the water molecules from a hydrated cation, thus efficiently catalyzing dehydration. (3) A major difference between Na⁺ and K⁺ in the channel pore is that Na⁺ keeps more water molecules attached than K⁺ (Guidoni et al., 1999).

5.3 The melibiose permease of Escherichia coli

The melibiose permease of Escherichia coli is a hydrophobic protein, which consists of 472 amino acid residues and uses for melibiose transport Na⁺, Li⁺ and H⁺ as coupling ions. In contrast the Klebsiella pneumoniae enzyme uses exclusively H⁺ for melibiose transport, and the Salmonella typhimurium enzyme cannot use H⁺, but Na⁺ and Li⁺ ions (Poolman et al., 1996). The primary sequence of the melibiose permease is barely homolog to the lactose permease (see Chapter 1), although these carriers share several sugars as substrates (melibiose, methyl-1-thio-β-D-galactopyranoside, p-nitrophenyl-α-galactopyranoside). Topological studies suggest that the melibiose permease spans the membrane twelve times with N- and C-terminus located in the cytoplasm (Pourcher et al., 1996). By constructing chimeras of the melibiose permease of Escherichia coli and Klebsiella pneumoniae it could be shown, that the N-terminal 81 amino acids of the E. coli enzyme provided the Na⁺ coupling ability (Hama and Wilson, 1993, 1994). There are only five amino acid residues in this region, which are not conserved within these two bacteria. Site-directed mutagenesis studies revealed, that Asn 58 of the E. coli permease (Ala 58 in K. pneumoniae) is responsible for the specificity of the coupling ion. Substitution of Ala 58 in the K. pneumoniae permease with Asn provided Na⁺ coupled
melibiose transport activity. Replacing Asn 58 in the *E. coli* enzyme with Ala resulted in only a two-fold stimulation of melibiose transport with Na⁺, whereas the wild-type enzyme shows a 17-fold stimulation (Franco and Wilson, 1996). The efficiency of H⁺ coupled melibiose transport in the *E. coli* N58A mutant could be increased by the mutation W54L (corresponding residue in the *K. pneumoniae* permease), whereas the stimulation of Na⁺ was not affected by this second mutation. Further mutagenesis studies of five Asp residues in the first four putative transmembrane helices of the *E. coli* melibiose permease (Asp 19, Asp 35, Asp 55, Asp 59, and Asp 124) led to the speculation, that for the coordination of Na⁺, Asn 58 and the Asp residues are essential. For the binding of Li⁺ and H⁺ a more flexible binding pocket is possible (Poolman et al., 1996). Recently, structural studies of the melibiose permease of *Escherichia coli* by fluorescence resonance energy transfer gave evidence for an ion induced conformational change of the structure of the sugar binding site or of its immediate vicinity (Maehrel et al., 1998). Data of these studies provide additional support, that the sugar binding site is localized in the C-terminal part of the transporter and that the N-terminal domain, particularly putative cytoplasmic loop 2-3, is close to the sugar binding site (Cordat et al., 1998). In the melibiose permease, ion specificity is represented by different amino acid residues at distinct positions in the enzyme. It should be mentioned, that only permeases of the galactosidc-pentose-hexuronide (GPH) transporter family, which have an Asn residue at position 58, are able to couple substrate transport with Na⁺ (Poolman et al., 1996).

5.4 A molecular mechanism for energy coupling in a membrane transport protein, the lactose permease of *Escherichia coli*

Experimental observations from structural and extensive mutational analysis have led to a proposed mechanism for energy coupling between sugar and H⁺ transport (Frillingos et al., 1998; Kaback, 1997). Site-directed and cysteine-scanning mutagenesis revealed that 6 of 417 residues in the lactose permease are irreplaceable. These are, with respect to coupling between lactose and H⁺ translocation: Glu 269 (helix VIII), Arg 302 (helix IX), His 322 (helix X), and Glu 325 (helix X). For substrate binding Glu 126 (helix IV) and Arg 144 (helix V) are indispensable (Kaback and Wu, 1997; Frillingos et al., 1998; Venkatesan and Kaback, 1998; Sahin-Toth et al., 1999).

The model is based on four propositions: (1) in the absence of substrate, the lactose permease does not catalyze significant H⁺ translocation (Viitanen et al., 1983); (2) a
substrate concentration gradient generates $\Delta \mu H^+$, the polarity of which depends on the direction of the substrate concentration gradient (Viitanen et al., 1983); (3) Glu 325 is the primary and possibly the only residue directly involved in $H^+$ translocation (Frillingos and Kaback, 1996); and (4) the catalytic cycle starts with protonated Glu 325, because it faces the low dielectric interior of the membrane (Venkatesan, P., and Kaback, H. R., unpublished observations; Frillingos and Kaback, 1996; Frillingos et al., 1998).

Figure 3: Proposed mechanism for energy coupling in lactose permease (adopted from Frillingos et al., 1998). Cytoplasmic view of the six helices which play a central role in the mechanism.
Figure 3 shows a cytoplasmic view of the six helices that play a central role in the mechanism. Between helices IX and X a water-filled crevice is suggested. The postulated mechanism for influx is as follows.

In the ‘outward-facing’ conformation, the crevice between helices IX and X is opened to the periplasm and Glu 325 (helix X) is protonated. Glu 269 (helix VIII), Arg 302 (helix IX), and His 322 (helix X) form a stable uncharged triad (step A). Ligand binding at the interface between helices IV and V induces a conformational change that disrupts the triad via a network of interacting residues, where helix VIII is involved (step B). With saturating substrate concentrations at both surfaces of the membrane, the protonated form of Glu 325 is stabilized and the permease can oscillate between ‘outward’- and ‘inward-facing’ conformations, thereby catalyzing exchange and countercflow with no $\text{H}^+$ translocation. In the presence of a substrate concentration gradient $\Delta \mu \text{lac}$ or $\Delta \mu \text{H}^+$, the changes associated with substrate binding lead to a marked decrease in the pK_a of Glu 325 and to its deprotonation, by bringing Arg 302 into proximity with the carboxylic acid (step C), and the crevice is opened to the cytoplasm. The order of release is always sugar first and proton second. When the $\text{H}^+$ is released from Glu 325 between helices IX and X, it can be acted upon equally by either the electrical potential or the pH gradient across the membrane. The stable, uncharged triad between Glu 269, His 322, and Arg 302 is reformed in the ‘outward-facing’ conformation, crevice opened to the periplasm, but Glu 325 is negatively charged and embedded in the low dielectric of the membrane, which is thermodynamically unfavorable (step D). Finally, Glu 325 is reprotonated and the cycle can be repeated (Frillingos et al., 1998). The changes described are accompanied by appropriate changes in helix tilt, resulting in the reciprocal opening and closing of crevices on respective sides of the membrane with transient accessibility from both sides of the membrane. Arg 302, His 322 and Glu 269 are not directly involved in $\text{H}^+$ translocation, but Arg 302 is important for decreasing the pK_a of protonated Glu 325, and Glu 269 and His 322 ly close to the interface between helices IV and V where substrate binding occurs, and they may be important for stabilization of this interface.

One aspect of the mechanism is the ability of the permease to catalyze $\text{H}^$/lactose symport in both directions across the membrane, with a stoichiometry of 1:1 for influx, but less than unity for efflux. Efflux could only be measured in vitro (membrane vesicles). The permease functions more efficiently in the influx direction than in the efflux direction. The rigid body movement that takes place between the helices during
turnover may involve changes in tilt that results in reciprocal opening and closing of partial pathways between helices, thereby allowing for movement of substrate and H³ in either direction across the membrane via protonation and deprotonation of Glu 325.

### 5.5 Mechanism of Na⁺ translocation by oxaloacetate decarboxylase of Klebsiella pneumoniae

Figure 4 shows the model of the energy coupling mechanism and the proton translocation network. In this model two different Na⁺-binding sites are envisaged. One binding site is represented by the Asp 203 (Di Berardino and Dimroth, 1996), located in the highly conserved region IIIa, the second is assigned to the deprotonated Ser 382, located in the highly conserved transmembrane helix VIII, where the proton is abstracted by the pair carboxybiotin/Arg 389 (Figure 4). As already mentioned in Chapter 3, mutational analysis indicated functional significance of N373, G377, S382 and R389 in helix VIII. Strong support for the presence of two Na⁺ binding sites in OadB derives from kinetic studies, which indicated that the activation of oxaloacetate decarboxylation by Na⁺ is clearly cooperative with a Hill coefficient of 1.8. This was further supported by studies on tryptic digestion of the β subunit. Protection by Na⁺ ions is only observed if both Na⁺ binding sites are present, indicating that the protein adopts a more compact conformation in the presence of Na⁺ ions.

The new model for the energy coupling mechanism is significantly distinct from a previous one, in which the simultaneous binding of two Na⁺ ions to OadB was not yet recognized (Di Berardino and Dimroth, 1996). Transmembrane helix VIII and the part of region IIIa, surrounding D203, have been identified as essential for the decarboxylation of carboxybiotin and for the coupled translocation of Na⁺ and H⁺ across the membrane. According to topology analysis (Chapter 2), the IIIa region inserts from the periplasm into the membrane and builds two hairpin structures. This folding creates a binding pocket for carboxybiotin. Unliganded OadB switches between conformation 1, in which the Na⁺ binding sites are open to the cytoplasm, and conformation 2, in which these sites are open to the periplasm (see Figure 6 in Chapter 3). The catalytic cycle starts by the binding of carboxybiotin, which stabilizes conformation 1. This binding is supported by charge pairing of the biotin carboxylate with R389. The biotin carboxylate penetrates from the cytoplasmic surface into the binding pocket, created by the two hairpin structures of region IIIa. Further progress of the reaction requires the binding of two Na⁺ ions from the cytoplasm to the appropriate sites at the interface.
between helix VIII and region IIIa. The first Na\(^+\) ion traverses the membrane almost completely and binds to D203 close to the periplasmic surface. The conserved N373 of helix VIII near the periplasmic surface is an additional ligand of this site. Binding of the first Na\(^+\) ion elicits some interhelical rearrangements, by which the binding of the second Na\(^+\) ion to the S382 including site becomes facilitated. Upon Na\(^+\) binding to this site, the proton from S382 is abstracted via a network of hydrogen bonds, where the hydroxyl group of Y229 is essential, by the pair R389/carboxybiotin, and upon protonation of the latter residue, this acid-labile compound undergoes immediate decarboxylation. A potential mechanism in which Na\(^+\) binding at S382 and binding of carboxybiotin near R389 initiates a proton translocating cascade involving Y229 and water is shown in Figure 3 in Chapter 4. The proton translocation network allows fast transfer of the proton from S382 to carboxybiotin, where it catalyzes the immediate decarboxylation. Decarboxylation is accompanied by a conformational change, giving the bound Na\(^+\) ions access to the periplasmic surface. Once open, the Na\(^+\) dissociates from the D203/N373 pair into the periplasmic reservoir and a proton enters the channel and displaces the Na\(^+\) from S382. The D203/N373 pair resides near the membrane surface, where the pK of D203 may not be significantly higher than in water (3.9) and H\(^+\) binding is not required for Na\(^+\) release. Delocalization of the charge by hydrogen bonding between D203 and N373 will also contribute to the dissociation of Na\(^+\). In contrast, the Na\(^+\) bound to S382 near the center of the membrane undoubtedly will have to be displaced by the protonation of the side chain oxygen anion, because a negative charge would not be tolerated here for electrostatic reasons. D203 may help the proton to pass through the channel to S382, especially, because the reverse reaction must also be possible. The deprotonation of the hydroxyl group of S382 requires a strong base, for which D203 is certainly a candidate. After these events, carboxybiotin, formed by OadA by carboxyltransfer with oxaloacetate, binds to OadB. This stabilizes conformation 1 and a new reaction cycle begins.

In Figures 5 and 6 helix packing models of the oxaloacetate decarboxylase β subunit are shown. These models derived from helical wheel and mutational analyses. Helical wheel analyses revealed conserved regions and additional hydrophilic and hydrophobic regions which are considered in Figure 5. Functional important residues are located in hydrophilic areas of the helices within the membrane, maybe a water-filled channel. These residues were placed in vicinity to each other as inferred from data of Chapter 3 and 4.
Figure 4: Model of the energy coupling mechanism and the proton translocation network in OadB. The model emphasizes details of the proton translocation network. Na⁺ binding at S382 and binding of carboxybiotin near R389 initiates a proton translocating cascade involving Y229. The proton delivered to carboxybiotin catalyzes the immediate decarboxylation of this acid-labile compound. To displace the Na⁺ from S382 and deliver it to the periplasmic surface, a periplasmic proton penetrating through the channel must restore the hydroxyl group of S382. A hydrogen bonded network including D203 could be involved in the proton pathway connecting the periplasmic reservoir with S382.
Figure 5: Helix packing model of the β subunit as inferred from helical wheel analysis of OadB. Hydrophobic regions are marked in black and hydrophobic regions are marked in grey. Residues which are involved in Na⁺ and H⁺ translocation are emphasized.

Figure 6: Three-dimensional helix packing model of the β subunit. Amino acid residues with functional significance are emphasized. Helices VI and IX are not shown, because they are located in front of helix VIII.
5.6 Conclusions and outlook

As one can see from the above chapter, there is a great diversity in coupling mechanism between ion translocation and enzymic activity. The common theme in all cases is the participation of negative charges located within membrane regions. This is also true for the oxaloacetate decarboxylase, as further outlined in this thesis. During this work, the topology of the β subunit of the oxaloacetate decarboxylase was investigated, and a new topological model was proposed. This model is supported by experimental data of three different approaches, and based on it, a number of site-directed mutants was created to investigate the mechanism of coupling between Na⁺ translocation and decarboxylation of oxaloacetate. Indeed, a new model for the coupling mechanism was elucidated, which is an elegant example for a direct coupling mechanism, in which the vectorial protons perform the chemical catalysis. Additionally a helix packing model for the β subunit, based on site-directed mutagenesis studies and helical wheel analyses, was suggested, which shows the hypothetical proton transfer pathway through the β subunit. This work represents a further step in understanding the mechanism of ion translocation through bacterial membrane proteins. A crystal structure of the oxaloacetate decarboxylase is highly desireable, but before this would be possible, it would be helpful to apply all those methods used for the lactose permease, to obtain an idea of the structure. A well known tool to study conformational changes due to ligand binding is to investigate fluorescence of native or introduced Trp residues in presence or absence of substrate. This would be a further step for understanding the reaction mechanism of the oxaloacetate decarboxylase. All in all, the ultimate information about function and structure could be obtained by a crystal structure, only.
5.7 References


predicted transmembrane segments from Streptomyces lividans, EMBO J. 14, 5170-
5178.

Topographical structure of membrane-bound Escherichia coli F_{i}F_{0} ATP synthase in
aqueous buffer, FEBS Lett. 397, 30-34.

Takeyasu, K., Omote, H., Nettikadan, S., Tokumasu, F., Iwamoto-Kihara, A., and
Futai, M. (1996) Molecular imaging of Escherichia coli F_{0}F_{1}-ATPase in reconstituted


(1983) Mechanism of lactose translocation in proteoliposomes reconstituted with lac
carrier protein purified from Escherichia coli. 2. Deuterium solvent isotope effects,
Biochemistry 22, 2531-2536.
6 Appendix

6.1 Appendix to Chapter 2

Table A lists the primers used for generation of OadB-PhoA and OadB-LacZ fusions. Table B lists the primers used for construction of site-specific cysteine mutants in OadB.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5’-3’)</th>
<th>Restriction sites/comments</th>
<th>Fusion site</th>
</tr>
</thead>
<tbody>
<tr>
<td>B_{Nhf}</td>
<td>ggaaaaggtaccGAAATGGA</td>
<td>KpnI site in bold, ATG start codon underlined</td>
<td></td>
</tr>
<tr>
<td></td>
<td>AAGTCTGAACGCCCTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L1</td>
<td>GCCCGAGCCCAATCGCC</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ACCTTA</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L4</td>
<td>CTCCGGCGCCAGCTTG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>CCCGAC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L5</td>
<td>CAGCTGCACCATGCAGG</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>ATCTTT</td>
<td></td>
<td></td>
</tr>
<tr>
<td>L8</td>
<td>CGCTTCCGACCCACCT</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGTTC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>prP41</td>
<td>CAGCAGAGCTCCCGG</td>
<td>SacI site in bold</td>
<td></td>
</tr>
<tr>
<td></td>
<td>CTCGAACCTTCTTCGCA</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>AT</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Restriction sites in Table A are followed by the corresponding restriction enzyme name (KpnI, NcoI, XhoI). The underlined codons indicate mutations introduced by site-directed mutagenesis. The fusion site is indicated by the first three codons of the fusion protein.
Appendix

prP57
CGCCAGCCGAGCTCCC  SacI site in bold
GGAATATTTGGAGAGCA
G

prV80
GAGCTTCCCCGAGCTCCC  SacI site in bold
ACCGCCAGCTGCGC

prP103
CTGCCCCCGAGCTCCC  SacI site in bold
GGCAAGGGCCAG

prN156
CAGCAGCCGAGCTCCC  SacI site in bold
GACAAGCGCTGCGC

prT179
GCCGAAGCGAGCTCCC  SacI site in bold
CGTCAGCGCCCCCGAG

prG184
GGTGAACCGAGCTCCC  SacI site in bold
GCCGAAGTAGTTCA

prT189
CGCCGCCGAGCTCCG  SacI site in bold
GTGAAGCTGATGAT

prT245
CCGCTCCCCGAGCTCCG  SacI site in bold
GTCAGCGCCCTTCAT

prL329
GAACTTCCCCGAGCTCCC  SacI site in bold
AGCTTGGCCCCCAG

AAT CCG GGA GCT CAT
Ile  Pro Gly Ala His
GCA GCT CAG
Ala  Ala Gln

GGC CGT GGA GCT CAT
Arg  Val Gly Ala His
GCA GCT CAG
Ala  Ala Gln

CTG CCG GGA GCT CAT
Leu  Pro Gly Ala His
GCA GCT CAG
Ala  Ala Gln

GCT AAC GGA GCT CAT
Ala  Asn Gly Ala His
GCA GCT CAG
Ala  Ala Gln

CTG ACG GGA GCT CAT
Leu  Thr Gly Ala His
GCA GCT CAG
Ala  Ala Gln

TTC GGC GGA GCT CAT
Phe  Gly Gly Ala His
GCA GCT CAG
Ala  Ala Gln

TTC ACC GGA GCT CAT
Phe  Thr Gly Ala His
GCA GCT CAG
Ala  Ala Gln

CTG ACC GGA GCT CAT
Leu  Thr Gly Ala His
GCA GCT CAG
Ala  Ala Gln

AAG CTG GGA GCT CAT
Lys  Leu Gly Ala His
GCA GCT CAG
Ala  Ala Gln
CAGGATCCGAGCTCCC \textit{Sau}I site in bold
TGGGCGTCGAGGAAGCT

CAGCAGAGCTCCGTTCC \textit{Sau}I site in bold
ATTCAGCTCTGCCCATCA

G

cggtcgcgtgcgatgca\textsc{TAC}A \textit{Nru}I site in bold, \textit{Nsi}I site underlined
TCGCCAGCAGCTACTT

GCTCATGGGCCCACTG \textit{Apa}I site in bold
TGGGCCAGCAGGCTCT

C

GCTCATGGGCCCACTC \textit{Apa}I site in bold
ACCGCCAGCTGCGCCG

G

GCTCATGGGCCCACTC \textit{Apa}I site in bold
TGCGCCGCCGCCCA

G

GCTCATGGGCCCACTC \textit{Apa}I site in bold
AGCGTCAGCGCCCG

G

CCG CAG GGA GCT CAT
Pro \textbf{Gln} Gly Ala His
GCA GCT \textit{CAG}
Ala Ala \textbf{Gln}

ATG AAC GGA GCT CAT
\textit{Ile} \textbf{Asn} Gly Ala His
GCA GCT \textit{CAG}
Ala Ala \textbf{Gln}

CGG ATG TAT GCA GCT
\textbf{Ala} Met Tyr Ala Ala
CAG
\textit{Gln}

GCC CAC AGT GGG CC
\textit{Ala} His Ser Gly Pro
CTG AGC TCA TGC AGC
Leu Ser Ser Cys Ser
CAA GCT TGC GAT CCC
Gln Ala Cys Asp Pro

CGG GTG AGT GGG CC
\textbf{Ala} Val Ser Gly Pro
CTG AGC TCA TGC AGC
Leu Ser Ser Cys Ser
CAA GCT TGC GAT CCC
Gln Ala Cys Asp Pro

CGG CAG AGT GGG CC
\textbf{Ala} \textit{Gln} Ser Gly Pro
CTG AGC TCA TGC AGC
Leu Ser Ser Cys Ser
CAA GCT TGC GAT CCC
Gln Ala Cys Asp Pro

ACG CTG AGT GGG CC
\textbf{Thr} Leu Ser Gly Pro
CTG AGC TCA TGC AGC
Leu Ser Ser Cys Ser
CAA GCT TGC GAT CCC
Gln Ala Cys Asp Pro
<table>
<thead>
<tr>
<th>prL7For</th>
<th>GGAAAGTCTGAACGCTGAC</th>
<th>Mutations in bold, NsiI site underlined</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ATTCAGGGACTGGGGCTGA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TGC</td>
<td></td>
</tr>
<tr>
<td>prL7Rev</td>
<td>GCATCAGCCCCAGTCCCTG</td>
<td>Mutations in bold, NsiI site underlined</td>
</tr>
<tr>
<td></td>
<td>AATGCAATGCGTTTCAGACTT</td>
<td></td>
</tr>
<tr>
<td></td>
<td>TCC</td>
<td></td>
</tr>
<tr>
<td>prC87For</td>
<td>GCCGGCAAGCTCCACGCCG</td>
<td>Mutations in bold</td>
</tr>
<tr>
<td></td>
<td>CGCCGGACGGTCCACGCC</td>
<td></td>
</tr>
<tr>
<td>prC87Rev</td>
<td>GGCGTTGGACGTCCGGCGCG</td>
<td>Mutations in bold</td>
</tr>
<tr>
<td></td>
<td>GCCTGGAGTCGGCGGC</td>
<td></td>
</tr>
<tr>
<td>prV129For</td>
<td>GCCATTCTCTATAAGTGCG</td>
<td>Mutations in bold</td>
</tr>
<tr>
<td></td>
<td>CGATTGGCTCCGCGC</td>
<td></td>
</tr>
<tr>
<td>prV129Rev</td>
<td>GCCCGAGCCTCGCGC</td>
<td>Mutations in bold</td>
</tr>
<tr>
<td></td>
<td>TTATAGAAGATGCC</td>
<td></td>
</tr>
<tr>
<td>prL178For</td>
<td>GCCACCGTGCTCGGGGCTCT</td>
<td>Mutations in bold, PstI site underlined</td>
</tr>
<tr>
<td></td>
<td>GCAGCGCTGAACGTCTCGGC</td>
<td></td>
</tr>
<tr>
<td>prL178Rev</td>
<td>GCCGAACTAGTTCAAGGCTG</td>
<td>Mutations in bold, PstI site underlined</td>
</tr>
<tr>
<td></td>
<td>CAGGCCCGAGACGGAGGC</td>
<td></td>
</tr>
<tr>
<td>prY182For</td>
<td>GCCTCGGCGCTGACGCTTC</td>
<td>Mutations in bold, Muni site underlined</td>
</tr>
<tr>
<td></td>
<td>AATTGCTTCGGCATCATCA</td>
<td></td>
</tr>
<tr>
<td>prY182Rev</td>
<td>GCTGATGATGCCGAGGCAA</td>
<td>Mutations in bold, Muni site underlined</td>
</tr>
<tr>
<td></td>
<td>TTGGACGTTCAGCCGCCCAG</td>
<td></td>
</tr>
<tr>
<td>prS187For</td>
<td>CGCTGAACTACCTCGCGCATC</td>
<td>Mutations in bold, NdeI site underlined</td>
</tr>
<tr>
<td></td>
<td>ATATGCTTCACCCGCGCA</td>
<td></td>
</tr>
<tr>
<td></td>
<td>GCGG</td>
<td></td>
</tr>
</tbody>
</table>
Table B. Primers used for the construction of site-directed oadB-"cysteine" mutants.
6.2 Appendix to Chapter 3 and 4

Further experimental data, which are not published in Chapters 3 and 4, are listed below, including the tryptic hydrolysis, Na\(^+\) activation profiles, and pH profiles of OadB variants.

6.2.1 Effect of Na\(^+\) on tryptic hydrolysis of the oxaloacetate decarboxylase β-subunit

The incubation mixtures contained in 60 µl or 84 µl at 25°C: (A) 20 mM potassium phosphate buffer, pH 7.5, 50 mM potassium chloride, purified wild-type or mutant oxaloacetate decarboxylase (25 µg or 35 µg) and 3 µg trypsin. A parallel incubation mixture (B) contained 50 mM sodium chloride instead of potassium chloride. Samples (12 µl) were transferred after 0 (before adding of trypsin), 2, 4, 7.5 (or 6 or 8) and 24 (or 19) h or 0 (before adding of trypsin), 15, 30, 45, 60, 90 and 120 min incubation into 1 µl 50 mM phenylmethanesulfonyl fluoride to inactivate the trypsin and 12 µl SDS probe mixture. After heating the samples to 95°C for 5 min, they were subjected to 10% SDS-PAGE. The gels were immediately stained with silver. Quantification of the β-subunit bands was performed with the program ImageMaster 1D Prime purchased from Pharmacia/Amersham. Half-times of the β-subunit in the presence of trypsin and + / - Na\(^+\) were obtained with the program Sigmaplot.
FIGURE1: Tryptic digestions of oxaloacetate decarboxylases in presence of 50 mM NaCl or 50 mM KCl. Digestions analyzed by SDS-PAGE are shown for mutants \( \beta Y227A, \beta Y229A, \beta Y229F, \beta D203N, \beta R389A, \beta S382N.\)
<table>
<thead>
<tr>
<th>mutation</th>
<th>half-time for OadB digestion&lt;sup&gt;a&lt;/sup&gt; (+Na&lt;sup&gt;+&lt;/sup&gt; / - Na&lt;sup&gt;+&lt;/sup&gt;) (h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild-type</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>D203N</td>
<td>10 / 10</td>
</tr>
<tr>
<td>Y227A</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Y227F</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Y229A</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>Y229F</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>C291A</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>G377A</td>
<td>&lt; 1 / &lt; 1</td>
</tr>
<tr>
<td>S382A</td>
<td>11 / 11</td>
</tr>
<tr>
<td>S382C</td>
<td>&lt; 1 / &lt; 1</td>
</tr>
<tr>
<td>S382D</td>
<td>&gt; 24 / 12</td>
</tr>
<tr>
<td>S382E</td>
<td>&lt; 1 / &lt; 1</td>
</tr>
<tr>
<td>S382N</td>
<td>&lt; 1 / &lt; 1</td>
</tr>
<tr>
<td>S382Q</td>
<td>&lt; 1 / &lt; 1</td>
</tr>
<tr>
<td>R389A</td>
<td>&gt; 24 / 12</td>
</tr>
</tbody>
</table>

Table 1: Effect of OadB mutations on protection by Na<sup>+</sup> ions from tryptic digestion. <sup>a</sup> Half-time for digestion of OadB by trypsin in presence of 50 mM NaCl (+Na<sup>+</sup>) or in the absence of Na<sup>+</sup> (-Na<sup>+</sup>). n. d. = not determined.
6.2.2 Determination of oxaloacetate decarboxylase activity at various Na$^+$ concentrations and pH values

The decarboxylation activity of wild-type (DH5α/pSK-GAB) and mutant oxaloacetate decarboxylases was measured at different pH values in the range between pH 5.5 and pH 8.8 in 40 mM Mes/Tris buffer containing variable Na$^+$ concentrations with the simple spectrophotometric assay according to Dimroth (1986).

FIGURE 2. Activation profiles of oxaloacetate decarboxylase variants by Na$^+$ ions at pH 7.5. Wild-type enzyme (■); βG380A mutant (▲); βS382D mutant (●); βR389A mutant (○).
FIGURE 3. Activation of oxaloacetate decarboxylase variants by Na\(^+\) ions at pH 7.5. Wild-type enzyme (●); βT189A mutant (▼); βY227C mutant (♦); βG197A mutant (■).
FIGURE 4: pH profiles (20 mM NaCl) of oxaloacetate decarboxylase variants. Wild-type (-); βR389L mutant (---); βR389A mutant (....).
6.3 References

Selte Leer / Blank leaf
Curriculum Vitae

Petra Jockel

born February 20th, 1970 in Giessen, Germany

1976 - 1980 Primary education in Grundschule Garbenteich (D)

1980 - 1982 Ludwig-Uhland-Schule Giessen (D)

1982 -1989 Liebigschule Giessen (D)
   School leaving exame and university entrance qualification (Abitur)

1989 -1994 Studies of Biology, Justus-Liebig-University, Giessen (D)

1993 -1994 Diploma Student, Hoechst AG, Frankfurt am Main (D)

1995 -1999 Assistant researcher at the Institute of Microbiology ETH, Zürich (CH)
   Ph. D. Thesis
LIST OF PUBLICATIONS