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Oxidative damage to creatine kinase possible links to neurodegeneration

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OXIDATIVE DAMAGE TO CREATINE KINASE: POSSIBLE LINKS TO NEURODEGENERATION

A thesis submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY ZURICH

for the degree of
DOCTOR OF NATURAL SCIENCES

presented by
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2001
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>ATP</td>
<td>adenosine triphosphate</td>
</tr>
<tr>
<td>ADP</td>
<td>adenosine diphosphate</td>
</tr>
<tr>
<td>P$_i$</td>
<td>inorganic phosphate</td>
</tr>
<tr>
<td>PCr</td>
<td>phosphocreatine</td>
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<tr>
<td>CK</td>
<td>creatine kinase</td>
</tr>
<tr>
<td>Cr</td>
<td>creatine</td>
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<tr>
<td>MtCK</td>
<td>mitochondrial creatine kinase</td>
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<tr>
<td>uMtCK</td>
<td>ubiquitous mitochondrial creatine kinase</td>
</tr>
<tr>
<td>sMtCK</td>
<td>sarcomeric mitochondrial creatine kinase</td>
</tr>
<tr>
<td>M-CK</td>
<td>muscle type creatine kinase</td>
</tr>
<tr>
<td>B-CK</td>
<td>brain type creatine kinase</td>
</tr>
<tr>
<td>kD</td>
<td>kilo Dalton</td>
</tr>
<tr>
<td>ROS</td>
<td>reactive oxygen species</td>
</tr>
<tr>
<td>RNS</td>
<td>reactive nitrogen species</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NOS</td>
<td>nitric oxide synthase</td>
</tr>
<tr>
<td>mtNOS</td>
<td>mitochondrial nitric oxide synthase</td>
</tr>
<tr>
<td>ONOO$^-</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>O$_2$^-</td>
<td>superoxide anion</td>
</tr>
<tr>
<td>SOD</td>
<td>superoxide dismutase</td>
</tr>
<tr>
<td>CuZnSOD</td>
<td>copper zinc superoxide dismutase</td>
</tr>
<tr>
<td>MnSOD</td>
<td>manganese superoxide dismutase</td>
</tr>
<tr>
<td>RR</td>
<td>ruthenium red</td>
</tr>
<tr>
<td>ALS</td>
<td>Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>FALS</td>
<td>familial Amyotrophic Lateral Sclerosis</td>
</tr>
<tr>
<td>BH$_4$</td>
<td>tetrahydrobiopterin</td>
</tr>
</tbody>
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1 Summary

Creatine Kinase (CK) is a key enzyme for maintaining energy homeostasis and is present in cells with high and fluctuating energy demands, like skeletal and cardiac muscle, as well as brain and other neuronal tissues, where a mitochondrial and a cytosolic isoform are coexpressed. CKs are known to be very susceptible to free radical damage and are inactivated by superoxide radicals ($O_2^-$) and peroxynitrite ($ONOO^-$).

In the first part of this work, we compared the susceptibility of the two human mitochondrial creatine kinase (MtCK) isoenzymes, ubiquitous (uMtCK) and sarcomeric (sMtCK), towards $ONOO^-$ with respect to activity and octamer stability. Both isoforms were irreversibly inactivated by $ONOO^-$ in a similar concentration dependency. $ONOO^-$ did not lower the residual activity of an active site cysteine (C278G) MtCK mutant, strongly indicating that CK is indeed inactivated by $ONOO^-$ via oxidation of this C278 residue in wild type enzyme, which is a very important residue for catalytic activity of MtCK. Treatment of MtCK with $ONOO^-$, additionally, led to a dissociation of MtCK octamers into dimers. uMtCK was, however, much more stable towards $ONOO^-$-induced dissociation than sMtCK. Mass spectrometric analysis revealed chemical modification of residues in peptide G263-R271 located at the dimer/dimer interfaces, most likely corresponding to nitration of the dimer/dimer interface tryptophan W264, an important residue for octamer stability. As shown by anti-dinitrophenyl-antibody labeling, protein carbonyls in MtCK increased dose dependently with $ONOO^-$ administration, correlating with octamer dissociation, whereas inactivation of CK was preceding carbonyl formation. Our data provide an explanation how oxidative damage can lead to inactivation and to a decreased octamer/dimer ratio of MtCK, as seen in neurodegenerative diseases and heart pathology, respectively.

In the second part, we addressed the question, of whether mitochondria from heart cells produce nitric oxide (NO), and whether overproduction of NO leads to $ONOO^-$ formation and consequently to inactivation of $ONOO^-$ sensitive enzymes. Using the newly introduced fluorescent NO probe, 4,5-diaminofluorescein diacetate (DAF-2 DA), we provide evidence for the presence of a mtNOS in isolated bovine cardiac muscle mitochondria. Heart mitochondrial NOS (mtNOS) continuously produces NO and its activity is stimulated when mitochondria are loaded with $Ca^{2+}$. Stimulation of heart mtNOS hampers mitochondrial oxygen consumption and both the formation of NO and the suppression of oxygen consumption are blocked by conventional NOS inhibitors or by the mitochondrial $Ca^{2+}$ uptake blocker, ruthenium red (RR). Prolonged loading of heart mitochondria with $Ca^{2+}$ induces oxidative damage to mitochondrial enzymes. Mitochondrial aconitase is inactivated in a manner prevented by
conventional NOS inhibitors, RR, a superoxide dismutase (SOD) mimic, or a ONOO\textsuperscript{-} scavenger. Mitochondrial SOD inactivation is prevented by NOS inhibitors or RR, and MtCK inhibition is prevented by RR or a SOD mimic. Mitochondrial adenylate kinase remains unaffected under the same conditions, arguing against a none-specific damage induced by Ca\textsuperscript{2+}. We propose that under mitochondrial basal Ca\textsuperscript{2+} homeostasis mtNOS physiologically regulates heart mitochondrial function by regulating respiration, whereas its overstimulation by overloading the organelles with Ca\textsuperscript{2+} induces oxidative damage to mitochondrial enzymes via distinct nitrosative/oxidative stress pathways.

The third part of this work focusses on the beneficial effects of Creatine (Cr) supplementation in neurodegeneration. Cr was shown to be neuroprotective in several models of neurodegeneration, including familial amyotrophic lateral sclerosis (FALS). In order to investigate mechanistic features of this beneficial effect, we determined CK activities and mitochondrial respiration rates in tissues from transgenic mice, overexpressing a mutant form of human SOD (G93A), which develop FALS-like symptoms. Respiration rates of mitochondria from FALS or control mice isolated from spinal cord showed no difference. However, CK activity was decreased to 49\% in homogenates and to 67\% in mitochondrial fractions from spinal cord of FALS mice compared to CK activities in control mice. In contrast, no such decrease could be detected in cortex of FALS mice. In homogenates or mitochondrial fractions from FALS mice supplemented with 2\% Cr, a slight but not significant increase of CK activity levels could be detected compared to regular fed FALS animals. Experiments with isolated mitochondria showed that in the presence of Cr and ATP mitochondrial CK activity was protected against ONOO\textsuperscript{-}-induced inactivation, which is believed to be responsible for tissue damage in neurodegeneration. Our data provide further evidence for a role of ONOO\textsuperscript{-} in neurodegenerative diseases like FALS and corroborate an involvement of oxidative damage to the CK system in the manifestation of disturbed cellular and mitochondrial energetics in neurodegeneration. Possible mechanisms for the neuroprotective effect of Cr are suggested.
ZUSammenfassung

Kreatin Kinase (CK) ist ein Schlüsselenzym für die Regulation der zellulären Energetik und kommt in Zellen mit hohem und wechselndem Energiebedarf vor, wie Muskel- und Herzzellen, aber auch Hirn- und anderen neuronalen Geweben, wo jeweils eine mitochondriale und eine cytosolische Isoform koexprimiert werden. Kreatin Kinasen sind bekanntermaßen sehr empfindlich gegenüber Schädigungen durch freie Radikale und werden durch Superoxidanion ($O_2^-$) und Peroxynitrit inaktiviert (ONOO$^-$).

Im ersten Teil dieser Arbeit wurden die Empfindlichkeit der beiden humanen mitochondrialen CK-Isoenzyme (MtCK), ubiquitäre (uMtCK) und sarkomere (sMtCK), gegenüber ONOO$^-$ in Bezug auf Aktivität und Oktamer-Stabilität verglichen. Beide Isoformen wurden durch ONOO$^-$ in ähnlicher Konzentrationsabhängigkeit irreversibel inaktiviert. ONOO$^-$ reduzierte nicht die Restaktivität einer MtCK-Mutante, deren Cysteinrest im aktiven Zentrum gegen Glycin ausgetauscht wurde (C278G), was stark auf eine Inaktivierung durch ONOO$^-$ durch Oxidation eben dieses Cysteinrests im Wildtyp-Enzym hindeutet, der sehr wichtig für die katalytische Aktivität ist. Die Behandlung von MtCK mit ONOO$^-$ führte auch zu einer Dissoziation des MtCK-Oktamers in Dimere. uMtCK war jedoch sehr viel stabiler als sMtCK. Durch massenspektrometrische Analyse konnte gezeigt werden, dass Aminosäurereste im Peptid G263-R271, das in den Kontaktstellen der Dimere liegt, chemisch modifiziert wurden, wahrscheinlich durch Nitrierung des Tryptophanrests W264, einem für die Oktamerstabilität wichtigen Aminosäurerest. Wie durch Bindung eines Antikörper gegen Dinitrophenylreste gezeigt wurde, stieg die Proteincarbonyl-Konzentration in MtCK mit der Konzentration des zugegebenen ONOO$^-$, entsprechend der Dissoziation in Dimere, während die Inaktivierung der CK der Proteincarbonyl-Bildung vorausging. Diese Ergebnisse bieten eine Erklärung, wie eine oxidative Schädigung zu einer Inaktivierung und einem reduzierten Oktamer/Dimer-Verhältnis führen können, wie es in neurodegenerativen Krankheiten bzw. bei Herzsymptomatik beobachtet wird.

Im zweiten Teil gingen wir der Frage nach, ob Mitochondrien aus Herzzellen Stickstoffmonoxid (NO) produzieren und ob Ueberproduktion von NO zu der Bildung von ONOO$^-$ und nachfolgend zur Inaktivierung von ONOO$^-$-empfindlichen Enzymen führt. Unter Verwendung des neu eingeführten NO-sensitive Fluoreszenzfärber, 4,5-diaminofluorescein diacetate (DAF-2 DA), liefern wir Hinweise für die Gegenwart einer NO-Synthase in Mitochondrien, die aus Rinderherz isoliert wurden. Mitochondriale NOS (mtNOS) produziert kontinuierlich NO und seine Aktivität wird stimuliert, wenn Mitochondrien mit Kalzium geladen werden. Stimulierung von mtNOS aus Herz beeinträchtigt den
Zusammenfassung

mitochondrialen Sauerstoffverbrauch, aber sowohl die Bildung von NO als auch die Unterdrückung des Sauerstoffverbrauchs werden von herkömmlichen NOS-Inhibitoren oder durch Blockierung der Aufnahme von Kalzium in die Mitochondrien durch Ruthenium Rot (RR) verhindert. Anhaltend erhöhte Konzentration an Kalzium in Mitochondrien ruft oxidative Schädigungen von mitochondrialen Enzymen hervor. Mitochondriale Aconitase wird inaktiviert, was durch herkömmliche NOS Inhibitoren, RR, einem Superoxid Dismutase (SOD)-Analog oder durch einen ONOO⁻-Fänger verhindert werden kann. Mitochondriale SOD-Inaktivierung kann durch NOS Inhibitoren oder RR, und MtCK Inaktivierung durch RR oder einem SOD-Analog verhindert werden. Mitochondriale Adenylat Kinase bleibt unter den selben Bedingungen unbeeinträchtigt, was gegen eine unspezifische Schädigung der Mitochondrien durch Kalzium spricht. Wir schlagen vor, dass unter normaler mitochondrialer Kalzium-Homeostase mtNOS die Herzfunktion durch die Regulierung der Atmung physiologisch reguliert, während die Ueberstimulierung durch erhöhte Konzentration an Kalzium in den Organellen eine oxidative Schädigung der mitochondrialen Enzyme hervorrufst, die durch verschiedene oxidative Reaktionen entstehen.

3 Introduction

3.1 General Features of Creatine Kinases

Living cells require a continuous supply of energy to perform the multitude of processes, necessary to sustain life. In all biological systems the primary source of this free energy is adenosine triphosphate (ATP), which is generated by metabolic breakdown of carbohydrates and fatty acids or by photosynthesis in the case of plants. ATP is hydrolyzed to adenosine diphosphate (ADP) and inorganic phosphate (P$_i$), giving a yield of 7.3 kcal per mole of ATP. The major pathways used in higher organisms to generate cellular ATP are (1) glycolysis, converting carbohydrates into pyruvate, (2) mitochondrial oxidative phosphorylation, involving the citric acid cycle and the respiratory chain for the aerobic breakdown of pyruvate and fatty acids and (3) anaerobic ATP production by converting pyruvate into lactate. The contribution of each of these systems depend largely on the functional properties of the cell. Cellular energy demand and supply are generally highly balanced and tightly regulated for economy and efficiency of energy use. A principle problem faced especially by excitable cells and tissues, such as skeletal and cardiac muscle, brain, retinal photoreceptor cells and spermatozoa, which have high and fluctuating energy demand, is the need to continuously adapt their rate of ATP synthesis to meet the variations of ATP demand. Since the local concentrations of adenine nucleotides and the ATP/ADP ratio are known to be key regulatory parameters that influence many fundamental processes, these parameters have to be kept relatively constant within their physiological range. Even though the cellular pools of ATP are rather small (typically 3-5 mM) which would be only sufficient to sustain muscle contraction for a few seconds (Infante et al. 1965), no significant decrease in the ATP concentration can be detected during cell activation (Mommaerts et al. 1967). Instead, large quantities of the metabolically inert “high-energy” phosphagen phosphocreatine (PCr) are accumulated with intracellular concentrations of about 20-35 mM (Fitch 1977). ATP is continuously and efficiently replenished from the large pool of PCr by the action of the essential enzyme creatine kinase (CK; EC 2.7.3.2) (for reviews see Bessman et al. 1981; Wallimann et al. 1992)). CK catalyses the reversible reaction in which the high-energy phosphoryl groups of PCr and ATP are rapidly exchanged (Fig. 3.1)(for review see Watts 1973; Kenyon et al. 1983)) which was discovered in 1934 (Lohmann 1934).

The favoured direction of the CK reaction depends on substrate concentrations, pH, the isoform involved and its subcellular localization. Under in vivo conditions, cytosolic isoforms catalyze preferentially the reaction in direction of ATP production (reverse reaction) (Meyer et
3 Creatine Kinase

Fig. 3.1: The Creatine Kinase reaction

The reversible production of phosphocreatine from creatine and MgATP catalyzed by CK

al. 1985; Rees et al. 1989), whereas the mitochondrial isoforms (MtCK) seems to preferentially catalyze PCr production (forward reaction) (Jacobus 1985; Tombes et al. 1985).

The CK/PCr is thought to be a key component of a metabolic network to keep up with the cellular demand for ATP (Wallimann 1999).

3.1.1 THE CREATINE KINASE/PHOSPHOCREATINE-SHUTTLE

In recent years the obvious importance of the CK isoenzymes for cellular energetics has attracted some attention. Cytosolic CK isoenzymes are always coexpressed with mitochondrial CKs and maintain, together with easy diffusible Cr and PCr, a unique cellular energy buffer and energy transport system - the CK/PCr circuit (Wallimann et al. 1992; Wallimann 1999). Three main functions have been assigned to the CK/PCr system.

First, the PCr circuit serves as a temporal energy buffer (Meyer et al. 1984), by keeping the concentrations of ATP and ADP constant and prevent a rapid decrease in the intracellular ATP concentrations during cellular work. This temporal buffer function also prevents an intracellular acidification caused by the hydrolysis of ATP in activated cells (Balaban et al. 1986). In resting cells, effective concentration of ATP and ADP are typically 3-5 mM and 10-30 μM respectively. During high workload, the cell can immediately regenerate hydrolyzed ATP from the PCr pool (20-35 mM) via the CK system. This has been confirmed by a transgenic animal approach where mice express significant levels of brain type CK (BB-CK) in the liver, where normally no or only minor CK activity is found (Brosnan et al. 1990; Koretsky et al. 1990). The liver of the transgenics show a strong buffering effect on the ATP levels and intracellular pH accompanied by an improved ability of the liver to regenerate as well as generally enforced hepatic energy metabolism (Satoh et al. 1996).
Second, the CK/PCr system is also thought to fulfill the role of a spatial energy buffer system, connecting sites of energy production, e.g. mitochondrial oxidative phosphorylation, with cellular sites of energy consumption, i.e. various ATPases. In the proposed model ATP, synthesized in mitochondria, is preferentially used by MtCK for the synthesis of PCr (Jacobus et al. 1973; Saks et al. 1985; Schlattner et al. 1998; Kay et al. 2000) which is then exported out of the mitochondria into the cytosol. In a similar way ATP, generated by glycolysis is used by the cytosolic CKs to produce PCr and thus, high-energy phosphate pools (Wallimann et al. 1989). The PCr diffuses then to various cellular ATPases, where it is used by nearby located cytosolic CKs for the regeneration of ATP. Creatine (Cr), produced during this process, subsequently diffuses back to the sites of energy production and completes the cycle. PCr and Cr are thought to act as the primary “energy carrier” in the cells because of their higher concentration together with the higher diffusion coefficients based on their smaller size and lower negative charge in contrast to ATP and ADP (Jacobus 1985; Yoshizaki et al. 1987; Hubley et al. 1995; Nicolay et al. 1995). The transport function is especially important in highly polar cells such as photoreceptor cells (Hemmer et al. 1993) or spermatozoa (Wallimann et al. 1994) where diffusion limits of ATP and ADP can be overcome by the CK system.

Third, the PCr-circuit represents a regulatory system for the physiologically required maintenance and adjustment of intracellular ATP/ADP ratios (Kammermeier 1987). The concentrations of ATP and ADP as well as their local ratio in the cell control many metabolic processes by allosteric interactions and the phosphorylation potential. By keeping the intracellular ADP concentration low and preserving high ATP/ADP ratios at subcellular sites where the CK system is functionally coupled to ATPases, the efficiency of intracellular energy utilization is increased (Wallimann et al. 1994). So is CK, associated with the sarcoplasmatic reticulum (SR) (Baskin et al. 1970), thermodynamically important for efficient \( \text{Ca}^{2+} \)-pump activity, as shown in situ with skinned muscle fibers (Duke et al. 1999; Duke et al. 2000) as well as \textit{in vitro} with isolated SR vesicles (Rossi et al. 1990; Korge et al. 1993). For the SR \( \text{Ca}^{2+} \)-pump this function is important, since it operates close to the thermodynamic equilibrium and depends very much on high local ATP/ADP ratios (Kammermeier 1987). The role of CK in the energetics of \( \text{Ca}^{2+} \) homeostasis in excitable cells probably represents the most important role of the CK system in general. This has become evident from analysis of the muscle phenotype of knock-out mice lacking the expression of both, cytosolic muscle type CK (MM-CK) and sarcomeric MtCK (Steeghs et al. 1997). Skeletal muscle of these animals show problems with \( \text{Ca}^{2+} \) release and reuptake which led to significantly prolonged relaxing times (Duke et al. 1999). These three important functions are summarized in the model in Fig. 3.2.
Additionally, the CK system may prevent a net loss of adenine nucleotides from muscle cells by preventing a significant rise in intracellular ADP concentration (Iyengar 1984).

However, there are still discussions related to the spatial buffer transport function via PCr shuttling. Results obtained by in situ $^{31}$P-NMR saturation transfer measurements, investigating the CK mediated reaction flux at different work loads of muscle (McFarland et al. 1994), or under Cr depletion (Wiseman et al. 1995), have been interpreted as evidence against the PCr shuttle. On the other hand, transgenic mice, expressing graded CK levels (van Deursen et al. 1994) showed an unexpected anomalous behavior in $^{31}$P-NMR terms, suggesting that some of the CK-flux, measured in situ by saturation may evade detection by this method (Wallimann 1996). Furthermore, some ATP may be NMR-invisible as well, most probably due to the compartmentation and/or binding to cellular components of adenine nucleotides in vivo (Joubert et al. 2000). Thus a revision of earlier NMR data might be necessary with respect of the concept of “hidden” energy metabolite pool. Recent $^{31}$P-NMR studies comparing CK-mediated fluxes of inactive versus active sea-urchin sperm corroborate the existence of a PCr-shuttle within these cells (Dorsten et al. 1997).

### 3.1.2 The Creatine Kinase Isoenzymes

Since the first purification of CK from rabbit muscle (Kuby 1954) and its kinetic characterization (Kuby 1963) extensive biochemical studies have shown that at least five distinct CK isoenzymes are expressed in vertebrates in a tissue specific manner (Eppenberger et al. 1967; Wallimann et al. 1992) and localized in different intracellular compartments.

**The Cytosolic CK Isoforms**

Cytosolic CK isoenzymes exist as exclusively dimeric enzymes composed of the M (for muscle) and B (for brain) subunits (Dawson et al. 1967; Eppenberger et al. 1967). They assemble to homodimeric MM- and BB-CK, or the heterodimeric MB-CK isoenzymes, with molecular weights varying from 80-86 kD. MM-CK is rather specific for differentiated skeletal and cardiac muscle. The BB-CK isoform is mainly found in brain and neuronal tissues, developing cardiac muscle and a large variety of specialized tissues, such as retina, kidney, spermatozoa, placenta, uterus, endothelial cells, bone cells, macrophages, tumor and cancer cells (for review see (Wallimann et al. 1994)). The heterodimeric MB-CK appears as a minor fraction in adult mammalian heart and during muscle cell differentiation, when a transition from BB-CK via the transitory MB-CK hybrid to MM-CK homodimer occurs (Caravatti et al. 1979; Trask et al. 1990).
Fig. 3.2: The PCr-shuttle

Summary of the transport function according to the CK/PCr-circuit model. Cellular ATP is derived from two major synthetic pathways - from oxidative phosphorylation in the mitochondria and from glycolysis in the cytosol. The depicted model illustrates the distribution of CK activity among four main compartments, which represent the cellular basis for the CK/PCr-circuit model: (1) strictly soluble CK (CK_c) which catalyses the free equilibrium of the cytosolic ratios of phosphocreatine/creatine and ATP/ADP. (2) cytosolic CK which is associated with subcellular sites (CK_d) of high ATP consumption, like myofibrils or the sarcoplasmic reticulum. (3) cytosolic CK (CK_g) which couples glycolytic ATP production to PCr synthesis and (4) mitochondrial CK (MtCK) in the mitochondrial intermembrane space, which is functionally coupled to oxidative phosphorylation. For the latter function, the octameric structure of MtCK is crucial since the dimeric form can no longer bind to mitochondrial membranes. Therefore the ADP, derived from the CK reaction, is no longer produced in the vicinity of oxidative phosphorylation and is not stimulating mitochondrial respiration (red arrow).
The vertebrate genome codes for two distinct and tissue-specific MtCK isoforms sharing 82-85% amino acid sequence identity (Mühlebach et al. 1994). Ubiquitous uMtCK is found in several non muscle organs like kidney or brain whereas expression of sarcomeric sMtCK is restricted to sarcomeric muscle tissue. Both MtCK isoforms form dimeric, as well as highly ordered octameric structures with a molecular weight of approx. 86 and 340 kD, respectively (Schlegel et al. 1988a; Wyss et al. 1992). The octamer/dimer ratio of MtCK in vitro was shown to be influenced by enzyme concentration, pH, ionic strength and the presence of substrates (Gross et al. 1993). The prevalent species of MtCK under normal conditions in vivo is the octamer (Schlegel et al. 1988b), which is localized in the mitochondrial intermembrane space and the cristae and binds to mitochondrial contact sites (Rojo et al. 1991). Human MtCK isoenzymes from heart and brain have recently been shown to differ in octamer stability and membrane binding (Schlattner et al. 2000). The direct transphosphorylation of intramitochondrially produced ATP into PCr is probably the primary function of MtCK (Wallimann et al. 1992; Wyss et al. 1992; Schlattner et al. 1998). The functional coupling of MtCK to oxidative phosphorylation via the adenine nucleotide translocator (ANT) has been reported (Jacobus 1985; Saks et al. 1985). The stimulating effect of extramitochondrial Cr on the oxidative phosphorylation also suggests a functional coupling and a possible structural interaction with porin (reviewed by (Stachowiak et al. 1998b)). Such a tight functional coupling of CK to oxidative phosphorylation has recently been shown directly (Kay et al. 2000). Experiments with rat neonatal cardiomyocytes revealed the importance of the octameric structure of MtCK in mitochondria for this stimulating effect of extramitochondrial Cr on respiration. Respiration of mitochondria isolated from cells transfected with wild type MtCK could be stimulated by the addition of Cr whereas this effect was lost in mitochondria, isolated from cells transfected with a mutant MtCK which had a decreased octamer stability but full catalytic activity (Khuchua et al. 1998) (see also Fig. 3.2). The physiological relevance of a dynamic octamer/dimer equilibrium in vivo is still unclear. It has been proposed that the octamer/dimer ratio plays a role in medium or long-term regulation of the MtCK system (Schlegel et al. 1990). There is, however, evidence that the octamer/dimer transition plays a role in pathological situations, since increased levels of the dimeric form were detected in animal models of ischemic and infarcted heart (Soboll et al. 1999).
3.2 **THE MITOCHONDRIAL RESPIRATORY CHAIN**

Mitochondria are the main ATP production sites in the cell. When glucose is converted by glycolysis to pyruvate, only a small fraction of the total free energy potentially available from the glucose is released. Pyruvate is then imported into mitochondria where it is broken down to acetyl CoA, which is metabolized by the citric acid cycle to NADH and FADH$_2$. From these two electron carriers high-energy electrons are passed to the mitochondrial respiratory chain, which uses the energy that is liberated by transporting electrons from a higher to a lower energy state to pump protons out of the mitochondrial matrix and thus, build up a electrochemical proton gradient. Through this chemiosmotic coupling energy is stored in a membrane potential which is the driving force for ATP-synthesis. In detail, NADH is the substrate of NADH-ubiquinone oxidoreductase complex (complex I), which accepts electrons from NADH and passes them through a flavin and at least five iron-sulfur centers to ubiquinone and simultaneously pumps protons out of the mitochondrial matrix into the intermembrane space. Ubiquinone or coenzyme Q is a small hydrophobic molecule dissolved in the lipid bilayer of the inner membrane and can pick up one or two electrons and transfer them to the next protein complex in the respiratory chain, cytochrome bc$_1$ complex or complex III. Ubiquinone can also accept electrons from succinate-ubiquinone reductase (complex II) which upon conversion of succinate to fumarate, a reaction step in the citric acid cycle, transfers hydrogens to FAD resulting in the formation of FADH$_2$. Electrons are passed through an iron-sulfur center to ubiquinone. In contrast to all other reactions of the protein complexes of the respiratory chain, this does not result in a transport of protons through the inner membrane. Complex III accepts electrons from ubiquinone and passes them on to a further small electron carrier molecule, cytochrome c. Cytochrome c is a small protein of about 100 amino acids and is held loosely to the membrane by ionic interactions. The iron atom in the bound heme can carry a single electron, which is transferred to cytochrome oxidase complex (complex IV) where it is transferred in a final step to reduce oxygen to water. One oxygen molecule needs four electrons to reduce to water. Complex IV binds oxygen tightly until it is completely reduced to avoid the release of oxygen radicals which would contribute to cellular oxidative stress. The protons transported out of the mitochondrial matrix into the intermembrane space by the respiratory complexes I, III and IV build up an electrochemical gradient over the inner mitochondrial membrane. The flow of protons back into the matrix along the pH gradient fuels the ATP synthase (complex V) and leads finally to the synthesis of ATP from ADP. The membrane potential over the inner mitochondrial membrane is the driving force for this reaction. The main reactions of the mitochondrial respiratory chain are summarized in Fig. 3.3.
Fig. 3.3: The mitochondrial respiratory chain

Schematic representation of the mitochondrial respiratory chain and the transport of the electrons down the chain. Green arrows represent the way electrons are transported down the chain to finally reduce oxygen to water. A detailed description is given in the text (Chapter 3.2).
3.3 Reactive Oxygen and Nitrogen Species

The intensive research surrounding nitric oxide (NO) and its metabolites has given rise to a new term, the ‘reactive nitrogen species’ (RNS), which are derivatives from NO. Besides the important physiological functions of NO and other derivatives, reactive oxygen species (ROS) and RNS have been implicated in a variety of myocardial diseases, especially ischemia/reperfusion injury as well as in a number of neurodegenerative disorders, when produced by the cells in excess. The most important of them are superoxide anion (O$_2^-$), produced mainly by the mitochondrial respiration chain as a result of incomplete reduction of oxygen to water. NO is produced \textit{in vivo} by the family of nitric oxide synthases (NOS). Peroxynitrite (ONOO\(^-\)) is the reaction product of the latter two species and is considered to be an extremely powerful oxidant which is thought to be responsible for apoptotic and necrotic cell death and thus for tissue damage. In the following these ROS and RNS are discussed, since CK is known to be very susceptible to oxidative damage.

3.3.1 Nitric Oxide

Nitric oxide (NO) is a unique messenger molecule involved in the regulation of diverse physiological processes including smooth muscle contractility, platelet reactivity, central and peripheral neurotransmission, and the cytotoxic actions of immune cells. An inappropriate release of NO, however, has been linked to a number of pathologies like ischemia/reperfusion damage or inflammatory response. The paradox of NO acting both as a physiological regulator and a cytotoxic agent (Hibbs et al. 1987; Palmer 1987; Hibbs et al. 1988) was thought to be resolved after finding that NO reacts with O$_2^-$ to produce ONOO\(^-\), which is a potent oxidant and cytotoxic agent. Thus, it was suggested that NO is mostly responsible for the physiological regulation, while its congener ONOO\(^-\) is responsible for the cytotoxic properties (Beckman et al. 1996). The latter will be discussed in Chapter 3.3.3.

NO is synthesized by members of the NO synthase family (NOS, EC 1.14.13.39). There are at least four distinct isoforms discovered so far. The first enzyme to be purified (Bredt et al. 1990) and cloned (Bredt et al. 1991) was the rat neuronal NOS (nNOS). This enzyme is Ca$^{2+}$- and calmodulin-dependent (Bredt et al. 1990) and is constitutively expressed in brain, peripheral and central neural system and, despite its name, also in skeletal muscle (Nakane et al. 1993). A second, Ca$^{2+}$- and calmodulin-dependent, constitutive enzyme (eNOS) was discovered in vascular endothelial cells, but is also expressed in a variety of neuronal populations in brain. The third isoform is clearly distinct from the two constitutive enzymes and is induced in a variety of cells by exposing them to inflammatory mediators and bacterial products. Recently, a fourth isoform was discovered, that is, mitochondrial nitric oxide synthase (mtNOS),
3 Reactive Oxygen and Nitrogen Species

**Fig. 3.4:** The nitric oxide synthase reaction

The nitric oxide synthesis pathway by NO synthases

localized in mitochondria at the inner mitochondrial membrane and being Ca$^{2+}$-dependent, as well (Ghafourifar et al. 1997; Giulivi et al. 1998). The role of this enzyme will be discussed in Chapter 3.3.5. MtNOS, eNOS and nNOS produce NO in small and highly regulated bursts that are well suited for the molecular messenger function of NO (Bredt et al. 1990), whereas iNOS produces large amounts of NO continuously for long periods, a feature that is responsible for the cytotoxicity of NO. Only in pathological states, for example ischemia, in which intracellular Ca$^{2+}$ concentrations remains persistently elevated, eNOS and nNOS and probably also mtNOS become continuously activated and produce potentially toxic amounts of NO (Iadecola 1997). Because the concentration of SOD and O$_2^-$ in a given tissue are relatively constant, the primary driving force for ONOO$^-$ formation is NO concentration (Torreilles et al. 1999).

### 3.3.2 Superoxide Anion

Mitochondria are the main source of O$_2^-$ as the respiratory chain generates them by incomplete reduction of oxygen to water (Schapira 1997). Since oxygen in the ground state is in a triplet configuration with two unpaired electrons in the outer shell its reduction to water must occur in four consecutive one-electron steps. Some of the partially reduced oxygen intermediates generated in this process are very stable, among them O$_2^-$, but cytochrome oxidase, complex IV of the respiratory chain, is able to retain them until all electrons are transferred. However, a small proportion of the oxygen molecules are converted to O$_2^-$ by other respiratory components. The question of which fraction of O$_2$ is turned into O$_2^-$ is still a matter of debate (Forman et al. 1997). There are two main sites in the respiratory chain where these reactions
occur: Complex I (Turrens et al. 1980) and Complex III (Boveris et al. 1976; Cadenas et al. 1977; Turrens et al. 1985), the latter producing about 80% of O$_2^\cdot$.

Another source of O$_2^\cdot$ might be NOS itself. It has been reported that NOS under certain circumstances can produce O$_2^\cdot$ enzymatically by ‘uncoupling’ the electron transfer between NAD(P)H in NOS and arginine (Vasquez-Vivar et al. 1997; Vasquez-Vivar et al. 1998). At very low tetrahydrobiopterin (BH$_4$) concentrations (< 10$^{-9}$M), no BH$_4$ molecules bind nNOS and O$_2^\cdot$ is produced, whereas at high BH$_4$ concentrations (> 10$^{-6}$M), two BH$_4$ molecules bind the enzyme and NO is produced. For BH$_4$ concentrations in between the two dissociation constant values, only one BH$_4$ molecule binds the enzyme, both subunits function independently and ONOO$^-$ is produced (Gorren 1998).

Under normal physiological conditions, O$_2^\cdot$ is metabolized by superoxide dismutase (SOD) as a detoxifying mechanism. There is a cytosolic copper/zinc-containing SOD (CuZnSOD) and a mitochondrial manganese-containing SOD (MnSOD). The importance of these enzymes, especially the mitochondrial MnSOD isoform, is demonstrated by knock-out mice. CuZnSOD knock-out mice appear normal but exhibit differences after traumatic injury, whereas MnSOD knock-outs do not survive past three weeks of age (Li et al. 1995; Reaume et al. 1996). Since O$_2^\cdot$ reacts more than three times faster with NO than with MnSOD (Hsu et al. 1996), this makes formation of ONOO$^-$ in vivo highly probable, especially in pathophysiological situations like ischemia-reperfusion, where an initial burst of O$_2^\cdot$ is produced, which is sustained at lower levels during later phases of reperfusion (Bolli 1991). In addition, MnSOD itself is inactivated by ONOO$^-$ leading to a vicious cycle of increased oxidative damage and simultaneously to a weakening of the cell’s defence strategy. It has been shown that O$_2^\cdot$ production together with NO and consequently ONOO$^-$ production, is increased in myocardial ischemia-reperfusion injury (Liu et al. 1997).

### 3.3.3 NO AND O$_2^\cdot$ COMBINE TO PEROXYNITRITE

As mentioned before, NO reacts with O$_2^\cdot$ to form ONOO$^-$ (Beckman et al. 1990) at a nearly diffusion-controlled rate. ONOO$^-$ is a highly reactive, powerful oxidating and nitrating agent which can react with a variety of biological molecules. Lipid peroxidation by ONOO$^-$ (Radi et al. 1991b), which leads to a disruption of cell membranes, as well as DNA oxidation (King 1992) have been reported. In addition, ONOO$^-$ inactivates proteins either via oxidation of a protein bound metal, needed for enzyme activity (Castro et al. 1994) or via reaction with amino acid side chains e.g. nitration of tyrosine (Ischiropoulos et al. 1992; Ischiropoulos et al. 1995), nitration (Alvarez et al. 1996) and oxidative ring-opening (Kato et al. 1997) of
tryptophan residues and oxidation of cysteine (Radi et al. 1991a) and methionine (Pryor et al. 1994; Perrin et al. 2000), as well as selenocysteine residues (Padmaja et al. 1998). In Fig. 3.5 some of these reactions are depicted with their chemical structure.

These rather specific reactions can lead to protein inactivation, if a residue susceptible to ONOO$^{-}$ is essential or important for the catalytic activity of a protein. This is the case for CuZnSOD (Ischiropoulos et al. 1992), as well as for MnSOD, the mitochondrial form of SOD, which are inactivated by ONOO$^{-}$ (MacMillan-Crow et al. 1996). These authors could show that only three of a total of nine tyrosine residues in the MnSOD protein were nitrated whereas the one located in the active site seemed to be the most susceptible (MacMillan-Crow et al. 1998). Others, however, found the active site tyrosine to be the only one nitrated in the sequence (Yamakura et al. 1998). Cytochrome c is nitrated at a specific tyrosine residue upon ONOO$^{-}$ exposure, leading to increased peroxidatic activity, resistance to reduction by ascorbate and impaired support of state 4-dependent respiration in intact rat heart mitochondria (Cassina et al. 2000). Examples for inactivation via cysteine oxidation are tryptophan hydroxylase, where the inactivation is due to sulphydryl oxidation beyond sulfenic acid, although tyrosine nitration was detected but considered to play a relatively minor role (Kuhn et al. 1999), SH-oxidation at glyceraldehyde-3-phosphate Dehydrogenase (Souza et al. 1998) and inactivation of CK, which will be discussed in detail in Chapter 3.3.4. Metal oxidation includes the inactivation of aconitase by disrupting the iron-sulfur cluster (Castro et al. 1994) and respiratory chain complexes I, II, and III (Radi et al. 1994).

ONOO$^{-}$ has also been linked to apoptosis. Some of the first evidence consistent with ONOO$^{-}$-induced apoptosis has recently been obtained in motor neurons and is thought to be relevant to amyotrophic lateral sclerosis (Estevez et al. 1998). The mechanism involved in ONOO$^{-}$-induced apoptosis is so far unclear although subsequent formation of ROS has been suggested (Lin et al. 1997). A mechanism independent from mitochondrial permeability transition pore was proposed, where stimulation of mtNOS and subsequent ONOO$^{-}$-formation lead to cytochrome c-release (Ghafourifar et al. 1999c).

In isolated rat hearts ONOO$^{-}$ induced ventricular dysfunction manifested as significantly decreased left ventricular developed pressure (Lopez et al. 1997). Similar results have been obtained with infusion of ONOO$^{-}$ into the isolated heart, which causes a concentration and time-dependent loss in contractile function (Schulz et al. 1997).

It has been argued that the reaction of ONOO$^{-}$ with glutathion represents a major protection mechanism against ONOO$^{-}$-induced oxidative damage (Karoui et al. 1996). Another detoxifying mechanism might involve the peroxidase enzyme glutathion peroxidase, which after classical view catalyses the glutathion-dependent reduction of hydrogen peroxide and
1) Tyrosine

![Tyrosine Reaction]

\[
\text{Rv-O-0H} + \text{ONOO}^- \rightarrow \text{3-Nitrotyrosine}\;
\Delta m = 45 D
\]

2) Tryptophan

![Tryptophan Reaction]

\[
\text{R} - \text{H} + \text{ONOO}^- \rightarrow \text{N-Formylkynurenine}\;
\Delta m = 32 D
\]

\[
\text{R} - \text{H} + \text{ONOO}^- \rightarrow \text{5-Nitro-tryptophan}\;
\Delta m = 45
\]

3) Cysteine

![Cysteine Reaction]

\[
\text{R' SH} + \text{ONOO}^- \rightarrow \text{S-Nitroso-cysteine}\;
\Delta m = 29
\]

\[
\text{R' SH} + \text{ONOO}^- \rightarrow \text{Sulfonic acid}\;
\Delta m = 48
\]

4) Methionine

![Methionine Reaction]

\[
\text{R' S} + \text{ONOO}^- \rightarrow \text{Sulfonic acid}\;
\Delta m = 16
\]

**Fig. 3.5:** Reactions of Peroxynitrite

Possible reactions of ONOO\(^{-}\) with different amino acid side chains and their products. \(\Delta m\) represents the increased mass after reaction of the amino acid which is important for mass spectrometrical analysis.
thus represents an important antioxidant defense mechanism. It was shown that glutathion peroxidase rapidly reduces ONOO\(^-\) to nitrite and protects against formation of 3-nitrotyrosine (Sies et al. 1997).

Despite all its deleterious effects it should be mentioned that ONOO\(^-\) possesses also physiological properties, which are similar to those of NO (reviewed in (Ronson et al. 1999)). However, since this thesis focuses on the detrimental effects of ONOO\(^-\), this subject shall not be discussed in detail.

### 3.3.4 Sensitivity of Creatine Kinase to Reactive Oxygen Species

MtCK, located in the mitochondrial compartment and therefore in close proximity of the production sites of ROS, as explained before, is permanently exposed to damage by these compounds. Several studies show that CK activity is very sensitive to \(H_2O_2\), \(O_2^-\), NO and ONOO\(^-\). For example mitochondrial membranes were incubated with a xanthine/xanthine oxidase system producing \(O_2^-\) which reduced MtCK activity in a time- and dose-dependent manner. Xanthine (0.133 mM) and xanthine oxidase (0.002 U/ml) decreased CK activity by 60\%, whereas 1 mM DTT or 10 mM cysteine could completely block this inhibition by \(O_2^-\) (Yuan et al. 1992), suggesting that oxidation of sulfhydryl groups is involved in the inactivation of the enzyme. Inactivation of purified muscle type CK was shown to be enhanced by adding \(Fe^{2+}\) in micromolar concentrations (Thomas et al. 1994), but CK activity could also be protected by reduced glutathion (GSH), corroborating the above conclusions.

It has been shown that different NO-donors, like SNAP or SNAC, can inactivate cytosolic rabbit muscle CK in a time- and concentration-dependent manner. This inactivation was reversible by addition of 10 mM DTT (Gross et al. 1996; Arstall et al. 1998), reducing the S-nitrosation which is responsible for the inactivation of CK. A similar effect of NO on CK was observed in an isolated rat heart perfused with SNAC (Gross et al. 1996), in adult rat ventricular myocytes (Arstall et al. 1998), in CK preparations solubilized from mitochondria, in isolated mitochondria and in saponin-skinned muscle fibres (Kaasik et al. 1999). In all these cases CK activity was decreased and could be recovered by adding DTT.

In contrast to these findings, inactivation due to ONOO\(^-\) is irreversible which is the case for cytosolic muscle type CK (Konorev et al. 1998), as well as for MtCK (Stachowiak et al. 1998a). The latter study showed that a 350 \(\mu\)M addition of ONOO\(^-\) (which is well within the physiological range (Denicola et al. 1993)) to purified MtCK reduced the enzyme activity to 14\% of control. With a full set of substrates of the forward reaction (Cr and MgATP) the remaining activity was increased to 28\%, whereas only one substrate (either Cr or MgATP) showed no or little protection. There was an even more pronounced protective effect with the
addition of the substrates of the reverse reaction (MgADP and PCr) resulting in a residual activity of 50\%. At the level of mitochondria, inhibition of MtCK occurred at doses of ONOO\(^-\) where other components of the respiratory chain and oxidative phosphorylation system were not yet affected, showing that MtCK is extremely sensitive to ONOO\(^-\)-induced damage (Stachowiak et al. 1998a).

Due to different behavior in reversibility of CK activity, the cause of inactivation can be distinguished: inactivation by NO is due to S-nitrosation of Cys 278 (MtCK numbering) and is reversible by DTT, inactivation by ONOO\(^-\) is probably due to oxidation of the same active site cysteine, but is irreversible. It remains an open question, whether reversible inhibition of cytosolic and mitochondrial CKs by NO may be of physiological significance in regulation of energy metabolism in vivo and whether this adds to the multitude of cellular processes controlled by NO. On the other hand, it is to be expected that irreversible inhibition of the CK-system severely impairs cellular energy homeostasis. The latter would also be the case if the octamer/dimer equilibrium of MtCK is affected by reaction of the enzyme with radicals, in particular ONOO\(^-\), as it was shown that MtCK loses its ability to stimulate mitochondrial respiration upon losing its ability to form octameric molecules (Khuchua et al. 1998). Therefore, one objective of this thesis was a careful investigation of the modification sites of ONOO\(^-\) on the MtCK sequence and their correlation with structural and functional changes, the results of which are presented in Chapter 4.

### 3.3.5 The role of Mitochondrial Nitric Oxide Synthase

The presence of a NOS isoform in mitochondria sheds a new light on the role of NO on mitochondrial functions. It has been reported that very low (nanomolar) levels of NO cause a completely reversible inhibition of mitochondrial respiration at cytochrome oxidase (complex IV) in competition with oxygen (Brown et al. 1994; Cleeter et al. 1994; Schweizer et al. 1994). This raised the exciting possibility that NO was a physiological regulator of mitochondrial respiration, in fact the only known direct regulator of respiration (Brown 1992). With a mtNOS in close vicinity to the mitochondrial respiratory chain this function of NO becomes even more likely. However, an overproduction of NO by mtNOS due to Ca\(^{2+}\) overload which for example takes place in ischemia/reperfusion injury, would lead to a stronger inhibition of mitochondrial respiration. It is well known (Turrens 1997) that this specific inhibition leads to an increased production of O\(_2^-\) which then can combine with the NO, present in excess from the overstimulated mtNOS, to form highly reactive ONOO\(^-\), leading to irreversible damage to the mitochondrial respiratory chain, as well as certain other enzymes and to all the consequences described in Chapter 3.3.3.
The production of NO by mitochondria has so far been shown in cells of liver (Ghafourifar et al. 1997; Giulivi et al. 1998; Tatoyan et al. 1998; Ghafourifar et al. 1999c), brain (Arnaiz et al. 1999) and thymus (Bustamante et al. 2000). No reports are known about NO production in heart cells. It has been shown, however, that overstimulation of mtNOS leads to ONOO⁻ production (Ghafourifar et al. 1999c). Direct evidence for mitochondrially produced NO in heart, and protein inactivation due to subsequently produced ONOO⁻ is given in Chapter 5 of this thesis.
3.4 NEURODEGENERATIVE DISORDERS

Neurodegenerative diseases are a heterogeneous group of illnesses with distinct clinical phenotypes and genetic etiologies. They include Alzheimer’s disease, Huntington’s disease and Amyotrophic Lateral Sclerosis (ALS) as the most known and widespread of these diseases. Despite the presence of genetic defects in widely varying proteins substantial evidence points to mitochondrial dysfunction as a unifying fundamental mechanism involved in neuronal degeneration. Mitochondrial dysfunction leads to impaired energy production, impaired cellular Ca$^{2+}$-buffering, activation of proteases and phospholipases, activation of NOS and generation of free radicals.

3.4.1 BENEFICIAL EFFECT OF CREATINE SUPPLEMENTATION IN NEURODEGENERATION

The fact that mitochondrial dysfunction is one of the common features in neurodegeneration lead to the idea that Cr supplementation could be beneficial in those pathophysiologic situations (Klivenyi et al. 1999). Since mitochondrial dysfunction leads to impaired energy production, buffering intracellular energy levels could exert neuroprotective effects. This, in fact, is one of the most important functions of the CK system as it was pointed out in Chapter 3.1.1. Therefore, Cr administration was tested in various systems to provide evidence whether there is indeed a beneficial effect of Cr on neurons.

In cell cultures Cr protected neurons from glutamate toxicity, even if it was administrated two hours after glutamate was added. The same effects could be observed when β-amyloid, the toxic peptide found in Alzheimer brains, was added to the culture (Brewer et al. 2000). By feeding mice or rats with Cr, the extent of cortical damage after traumatic brain injury could be reduced. The authors suggested a Cr-induced maintenance of mitochondrial bioenergetics as reason for protection (Sullivan et al. 2000). In different animal models of Huntington’s disease chronic supplementation of Cr exerted neuroprotective effects as well. In Huntington’s disease there is reduced mitochondrial complex II-III activity in postmortem tissue (Gu et al. 1996; Browne et al. 1997). Malonate and 3-nitropropionic acid are reversible and irreversible inhibitors of mitochondrial complex II, respectively, which produce striatal lesions similar to those of Huntington’s disease (Beal et al. 1993; Brouillet et al. 1993). Oral supplementation of Cr to rats could protect the animals of malonate- or 3-nitropropionic acid-induced lesions (Matthews et al. 1998). A similar effect could be seen in a transgenic mouse model of Huntington’s disease. Here, Cr supplementation significantly slowed down the development of brain atrophy while survival, as well as motor performance, and body weight were improved (Ferrante et al. 2000).
Corresponding results have been found with a transgenic mouse model for ALS. A mouse line, overexpressing a human mutant SOD (G93A) and thus developing ALS-like symptoms (Gurney et al. 1994), was fed with a 1% or 2% Cr diet from the age of four weeks on. This resulted in an increased life span from about 144 days (normal diet) to about 157 days (1% Cr) and 170 days (2% Cr), respectively. The latter corresponds to an increased life span of 17%. Additionally, Cr protected against motor neuron loss, reduced increase of 3-nitrytyrosine levels and improved significantly motor performance (Klivenyi et al. 1999). As this was a suitable model to us to investigate the mechanism, how Cr could exert its neuroprotective effects, ALS will be introduced in detail in the following chapter.

3.4.2 **AMYOTROPHIC LATERAL SCLEROSIS**

ALS is a prototypical neurodegenerative disease of late life characterized by progressive muscle weakness, atrophy and spasticity. It leads to paralysis and death, usually from respiratory failure, within 3-5 years after onset. Characteristic neuropathologic features are loss of anterior horn motor neurons, as well as degeneration of the corticospinal tracts. 90% of ALS cases are apparently sporadic with no identifiable genetic or environmental risk factors. The remaining 10% of cases show familial autosomal dominant inheritance (familial ALS, FALS). A major advance in the understanding of ALS was the identification of point mutations in the SOD gene in approximately 25% of patients who have familial ALS (Rosen 1993). More than 60 mutations have now been associated with the disease. The observation that mutations in SOD cause familial ALS suggested that oxidative injury might be playing a role in its pathogenesis. Several studies investigated catalytic properties of different mutations found in ALS patients. Six different mutant enzymes, which were expressed in COS-1 cells, were tested for activity. Five of this six mutants, which are A4V, G37R, G41D, G93C and 1113T, retain 30 to 100% of wild type activity, whereas only one of them, G85R, is inactive. All of the mutants showed a reduced half-live of the molecules. Taken together, these results of SOD1 mutant activity revealed a continuum bounded by G85R, which has no activity, and G37R, which possesses full specific activity but reduced half-live, resulting in about 60% of wild type activity (Borchelt et al. 1994). Although there is a decrease in SOD activity in familial ALS patients, these results lead to the suggestion that not a loss-of-function but a gain-of-function is responsible for the neurodegeneration in ALS. This is corroborated by the dominant inheritance pattern of familial ALS, the lack of correlation between enzyme activity and disease severity (Bowling et al. 1995), and the observation that overexpressing of the mutant enzyme G93A in transgenic mice leads to motor neuron degeneration (Gurney et al. 1994). In order to clarify the nature of this gain-of-function, the hypothesis of increased catalytic oxidation of substrates by H₂O₂ of FALS-associated mutations was raised. To test this
hypothsis, wild type as well as mutant proteins A4V and G93A were expressed in yeast and purified to homogeneity. Both mutants catalyzed the oxidation of a model substrate by H$_2$O$_2$ at a higher rate than the wild type enzyme, and catalysis of this reaction by the mutants was more sensitive to inhibition by copper chelators (Wiedau-Pazos et al. 1996). Another mutant, D90A, isolated from erythrocytes of patients, showed only small differences in stability and minor changes in hydroxyl radical formation and no difference in activity compared to the wild type enzyme (Marklund et al. 1997). Probably the best characterized mutant protein is the G93A mutant, since this is the protein which is overexpressed in a mouse line, which develops FALS like symptoms and is the most used FALS mouse model (Gurney et al. 1994). The G93A mutant as well as the wild type protein were expressed in Sf9 insect cells and purified. Both enzymes contain one copper per subunit and showed identical dismutation activity. However, the free radical generating function of the G93A mutant is enhanced, compared to the wild type enzyme, particularly at low H$_2$O$_2$ concentrations, which is due to a small but reproducible decrease in the value of K$_m$ for H$_2$O$_2$ (Yim et al. 1996). The mutations of SOD1 seen in ALS may relax the conformation of the active channel thereby allowing increased access of hydrogen peroxide or ONOO$^-$ to the active site copper. It has been shown that a broad range of FALS-linked SOD1 mutants (A4V, G37R, G41D, H46R, H48Q, G85R, G93C and 1113T) do bind copper in vivo under physiological conditions, which supports a mechanism of SOD1 mutant-mediated disease arising from aberrant copper mediated chemistry catalyzed by less tightly folded and hence less constrained mutant enzymes (Corson et al. 1998). This is predicted to increase generation of hydroxyl radicals or nitronium ions which can then nitrate proteins. Furthermore, there is evidence that in two different strains of transgenic ALS mice 3-nitrotyrosine concentrations increases (Bruijn et al. 1997). In addition, increased complex I activity in both postmortem brain material of patients with the A4V SOD mutation, as well as in transgenic mice with G93A SOD mutation, was found (Bowling et al. 1993). It is possible that increased generation of free radicals may damage the inner mitochondrial membrane. This could lead to a proton leak which would therefore have to be compensated by increased activity of the mitochondrial electron transport complexes responsible for proton transport.

Nevertheless, reports concerning the radical generation ability of FALS-associated mutants are not fully consistent and not a common feature of all mutant proteins. Similarly, the role of ONOO$^-$ in the progression of the disease is discussed contradictory. Whereas most of the studies suggest a role of ONOO$^-$ in ALS, there is one report on the lack of involvement of neuronal nitric oxide synthase in the pathogenesis of ALS. These authors found that transgenic mice expressing the G93A SOD1, but no nNOS, do not survive significantly longer than G93A mice, which lead them to the conclusion, that ONOO$^-$ formation is not involved in ALS.
Although it is well accepted that radicals play a role in the progression of the disease, the actual cause of the disease might be unrelated to radical generation and the latter just a consequence of a different mechanism, which was overlooked so far.

In order to investigate the beneficial effects of Cr in the ALS model described above (Chapter 3.4.1), we analyzed brains from these transgenic animals fed with or without Cr in a more biochemical manner. The results are presented in Chapter 6.
4 DIFFERENTIAL EFFECTS OF PEROXYNITRITE ON HUMAN MITOCHONDRIAL CREATINE KINASE ISOENZYMES: INACTIVATION AND OCTAMER DESTABILIZATION

4.1 INTRODUCTION

Creatine kinases (CK) are key enzymes in energy metabolism by catalyzing the reversible transphosphorylation of ATP to phosphocreatine (PCr). The CK system is present in cells with high and fluctuating energy demands, like skeletal and cardiac muscle, as well as brain and other neuronal tissues, where a mitochondrial and a cytosolic isoform are coexpressed (for reviews see (Wallimann et al. 1992; Wyss et al. 1992; Wallimann et al. 1998)). In muscle tissue, cytosolic MM-Cr is coexpressed together with sarcomeric mitochondrial CK (sMtCK), while in brain tissues cytosolic BB-Cr is found together with ubiquitous mitochondrial CK (uMtCK). In contrast to the cytosolic isoforms, which exist exclusively as dimers, the mitochondrial isoenzymes (MtCK) can form cube-like octameric molecules (Schnyder et al. 1991). The MtCK enzyme is bound on the outer side of the inner mitochondrial membrane and located in the intermembrane space, where it cross-links both mitochondrial membranes forming contact sites (Adams et al. 1989; Brdiczka et al. 1990), as well as along the cristae membranes (Wegmann et al. 1991). MtCK forms functional complexes with the adenylate translocator of the inner and porin of the outer mitochondrial membrane as evidenced experimentally by createin(Cr)-stimulation of mitochondrial respiration (Kay et al. 2000). This arrangement facilitates the vectorial transport of high-energy phosphate from the sites of energy production in the mitochondrial matrix to the sites of energy consumption in the cytosol via the formation of PCr from mitochondrial ATP by MtCK (for review see (Brdiczka et al. 1994)). PCr thus generated is transported into the cytosol where it can be transphosphorylated to ATP in situ by cytosolic CK located in the vicinity of cellular ATPases. The octameric structure of MtCK was shown to be crucial for this transport function of the "PCr-shuttle". Rat neonatal cardiomyocytes were transfected with MtCK mutated at the N-terminus, showing a decreased octamer stability (Kaldis et al. 1994), which resulted in a reduction of Cr-stimulated respiration (Khuchua et al. 1998).

MtCK is known to be very susceptible to oxygen radical damage (Koufen et al. 1999). Nitric oxide (NO) inhibits CK activity in solution, as well as in adult rat ventricular myocytes. This inhibition could be reversed by the addition of dithiothreitol (Arstall et al. 1998). In contrast, the inhibition of CK by superoxide anion (O$_2^-$) (Yuan et al. 1992; Thomas et al. 1994), as well
as by peroxynitrite (ONOO\textsuperscript{-}), is irreversible (Konorev et al. 1998; Stachowiak et al. 1998a). Inactivation of cytosolic and mitochondrial CK would interrupt the "PCr-shuttle" and would have severe effects on the energetics of work performance and Ca\textsuperscript{2+}-homeostasis in muscle similar to those seen in double-knockout mice, lacking both the cytosolic and mitochondrial CK (Steeghs et al. 1998).

ONOO\textsuperscript{-} is the product of the nearly diffusion-controlled reaction between NO and O\textsubscript{2}\textsuperscript{-}, both of these compounds being produced by mitochondria (Ghafourifar et al. 1997). As MtCK is located in close proximity to the production site of ONOO\textsuperscript{-}, a powerful oxidant, it is feasible that MtCK is a prime target for ONOO\textsuperscript{-}-induced damage. ONOO\textsuperscript{-} is known to play a role in cells under oxidative stress, as is the case in heart disease and after ischemia/reperfusion (Liu et al. 1997; Yasmin et al. 1997), as well as in certain neurodegenerative diseases like ALS, Huntington's and Alzheimer's Disease (for review see (Beal 2000)). Several reactions of ONOO\textsuperscript{-} with amino acid side chains are described in the literature, including nitration of tyrosine (Ischiropoulos et al. 1992; Ischiropoulos et al. 1995) and tryptophan (Ischiropoulos et al. 1995; Alvarez et al. 1996), as well as oxidation of methionine (Pryor et al. 1994; Perrin et al. 2000) and cysteine (Radi et al. 1991a). Nitrotyrosine serves as a marker for ONOO\textsuperscript{-}-induced damage in tissues (for review see (Herce-Pagliai et al. 1998)).

In the present study, we compared the kinetics of enzyme inactivation by ONOO\textsuperscript{-}, as well as the destabilization of MtCK octamers of both human MtCK isoenzymes after ONOO\textsuperscript{-} administration \textit{in vitro}. We were able to pinpoint the modification sites of ONOO\textsuperscript{-} on the MtCK amino acid sequence by mass spectrometry. These target sites for ONOO\textsuperscript{-}, affecting MtCK activity (active site cysteine C278) or octamer stability (tryptophan residues at the dimer/dimer interfaces), are fully consistent with the respective functional domains of the MtCK isoenzymes, as evident from their X-ray structures (Fritz-Wolf et al. 1996; Eder et al. 2000a)
4.2 RESULTS

The sensitivity of human sMtCK and uMtCK towards ONOO⁻ was compared with respect to enzyme activity and octamer stability. Activity was determined immediately after ONOO⁻ administration. Both human MtCK isoforms were inactivated in a dose dependent fashion already at very low concentrations of ONOO⁻ with an IC₅₀ of about 8 μM (Fig. 4.1). This inactivation was neither reversed by subsequent addition of 20 mM dithiothreitol, which reduces disulfides and reverses S-nitrosylation (Arstall et al. 1998), nor by 37 mM sodium arsenite, which reduces sulfenic acid to cysteine (Torchinsky 1981) (data not shown). To test whether the inactivation was due to oxidation of cysteine C278, which is known to be a crucial active site residue in all CK's, a C278G mutant of chicken sMtCK (Furter et al. 1993), as well as the wild type enzyme, was treated with ONOO⁻ in the same manner. This mutant shows, depending on the assay conditions, a residual CK activity of about 3-5% of that of the wild type enzyme, and this residual enzymatic activity was completely unaffected by ONOO⁻ (Fig. 4.1), while the chicken wild-type form is inactivated in exactly the same manner as both human isoforms (data not shown). Taken together, these findings strongly suggest that inactivation of MtCK by ONOO⁻ is due to oxidation of this active site cysteine C278 in wild type enzyme, either to sulfenic or to sulfonic acid.

In parallel experiments, protein solutions of either sMtCK or uMtCK, with an initial octamer content of more than 80% were treated with increasing concentrations of ONOO⁻ and kept overnight at room temperature to reach a new octamer-dimer equilibrium. Subsequent gel permeation chromatography was performed to determine the oligomeric state of the isoenzymes as a function of ONOO⁻ concentration (Fig. 4.2). It is obvious that upon ONOO⁻ administration, both MtCK isoforms got destabilized and tended to dissociate into dimers. However, ubiquitous MtCK was significantly more stable to ONOO⁻-induced dimerization than sarcomeric MtCK. While for sMtCK a concentration of only about 240 μM ONOO⁻ was sufficient to dissociate 50% of the initial octamer concentration into dimers (C₅₀ value), this concentration was about 790 μM for the brain-type uMtCK (Fig. 4.2).

Protein carbonyl formation through oxidation can be detected by derivation of carbonyls with dinitrophenylhydrazine (DNPH) and subsequent Western blot analysis using an anti-dinitrophenyl (DNP) antibody (Levine 1994). ONOO⁻-treated chicken sMtCK showed carbonyl formation at concentrations of 50 μM ONOO⁻ and higher, whereas at lower concentrations no signal could be detected (Fig. 4.3). Similarly, nitrotyrosine formation could be detected at 50 μM ONOO⁻ and higher but not at 10 μM ONOO⁻ as determined by Western blot analysis using a monoclonal antibody against nitrotyrosine (data not shown). This suggests that carbonyl formation as well as tyrosine nitration is rather accompanied by octamer
Fig. 4.1: MtCK activity after Peroxynitrite treatment

Inactivation of human uMtCK (black squares ■), human sMtCK (open squares □) and chicken sMtCK C278G mutant (black triangles ▲) by ONOO⁻. Activities are expressed in percent of control activity to allow better comparison of the inactivation patterns. In absolute terms mutant C278G showed a residual CK activity of approximately 3-5% compared to the wild type enzyme. Each data point is the mean value of four independent experiments, error bars within boundaries of symbols. Note the very similar inactivation kinetics by peroxynitrite of both native human MtCK isoenzymes, as well as the robustness of the C278G active site cysteine mutant towards the same reagent.

dissociation than by inactivation, as there was neither an anti-DNP signal nor an anti-nitrotyrosine signal at 10 µM ONOO⁻, where the enzyme is already mostly inactivated. This is confirmed by the finding that mutant MtCK C278G, whose activity was not changed upon ONOO⁻ administration, showed exactly the same extent of DNP-antibody labeling as a function of ONOO⁻ (data not shown).

To further identify the exact modification sites by ONOO⁻ in the MtCK sequence, chicken sMtCK was treated with ONOO⁻ in the same manner and, after exhaustive digestion by trypsin, subjected to mass spectrometric analysis. From the tryptic digest, peptides could be identified by their molecular mass. Chemically modified peptides resulting from ONOO⁻-treated MtCK should show a mass shift towards a higher mass compared to peptides derived from the native unmodified protein. Fig. 4.4 illustrates all the peptides that could be unambiguously identified by mass spectrometry in the native, as well as the ONOO⁻-treated
Fig. 4.2: Octamer content of MtCK after Peroxynitrite treatment

Differential dissociation of octamers of human MtCK isoenzymes due to peroxynitrite-induced damage \( u \text{MtCK} \) (black squares ■) and \( s \text{MtCK} \) (open squares □). Each data point represents the mean value of three independent experiments, error bars are SEM. Note the significantly higher susceptibility of \( s \text{MtCK} \), compared to \( u \text{MtCK} \), towards \( \text{ONOO}^- \) in terms of octamer destabilization.

Fig. 4.3: Protein carbonyl content of MtCK after Peroxynitrite treatment

Protein carbonyl formation as detected by DNPH in chicken wt \( s \text{MtCK} \) treated with different \( \text{ONOO}^- \) concentrations. A: Ponceau S staining, 0 μM (lane 1), 1 μM (lane 2), 5 μM (lane 3), 10 μM (lane 4), 50 μM (lane 5), 100 μM (lane 6), 500 μM (lane 7). B: Western blot of \( \text{ONOO}^- \)-treated chicken wt \( s \text{MtCK} \), derivatized with DNPH (for details see Materials and Methods).
chicken MtCK, aligned with its amino acid sequence. These stretches along the sequence are highlighted in grey. Only one single peptide comprising amino acids G263-R271 (marked in black in Fig. 4.4) was found to be chemically modified by ONOO⁻ treatment. The peptide L272-R287, which includes the active site cysteine C278 (marked light grey in Fig. 4.4), was detected in the digest of the native protein but not in that of the ONOO⁻-treated one. Potentially, there would be more ONOO⁻-sensitive residues in the MtCK sequence (big letters in Fig. 4.4) that could possibly be modified by ONOO⁻, since not all the theoretically possible fragments have been covered by the mass spectrometric analysis. However, as these fragments do not seem to have a known function in terms of structure/function relationships of the enzyme, the search for these particular fragments was not pursued. Similar results, concerning the covering of the sequence and the modification pattern were obtained with both human MtCK isoforms.

In the X-ray structures of both, sMtCK (Fritz-Wolf et al. 1996), as well as uMtCK (Eder et al. 2000a), the modified peptide G263-R271 is located at the dimer/dimer interfaces of the protein octamer. It contains two tryptophan residues, one of which (W264) has been identified by site-directed mutagenesis to be crucial for octamer stability (Gross et al. 1994).

Most of the mass differences detected in the G263-R271 peptide by MALDI (see Fig. 4.5) can be explained by well-known reactions of ONOO⁻ with certain amino acid side chains. A difference of 16D would point to oxidation of methionine M267, a 32D-difference is compatible with an oxidative ring opening at tryptophan resulting in N-formyl-kynurenine and
the observed 45D-shift would correspond to a nitration of either tryptophan W264 or W268. All these modifications began to appear at a concentration of 100 μM ONOO⁻ (Fig. 4.5), which was exactly the concentration where significant octamer dissociation started, as well (Fig. 4.2).

**Fig. 4.5:** Modification of the dimer/dimer interface peptide after Peroxynitrite treatment

MALDI mass spectra of the peptide G263-R271 treated with different amounts of peroxynitrite. Numbers indicate the molecular mass species of this fragment arising from modification by peroxynitrite. The unmodified peak from each spectrum was standardized to a relative intensity of 1.0 to show the increase of additional peaks relative to the unmodified one.
4.3 DISCUSSION

MtCK enzymes are highly conserved proteins. Sarcomeric MtCK sequences among each other share 89-96% identity, ubiquitous isoforms even 91-98% (Mühlebach et al. 1994). Therefore, results for chicken and results for human enzymes can be compared and assumed that results would be the same for both chicken and human MtCK.

In this study, we show that human MtCK isoenzymes are very sensitive to ONOO\(^-\)-induced damage. Both sMtCK and uMtCK were inactivated at very low concentrations of ONOO\(^-\). Already at 8 \(\mu\)M ONOO\(^-\), which would correspond to a ONOO\(^-\):MtCK monomer-ratio of 0.7:1, the enzyme was inactivated to 50%. This is in line with earlier results, showing that chicken sMtCK was inactivated with an IC\(_{50}\) of 35 \(\mu\)M (Stachowiak et al. 1998a). The slightly decreased IC\(_{50}\) value in this study could be due to the stronger buffering capacity used in the present study. Cytosolic MM-CK was reported to be inactivated with an IC\(_{50}\) of 2.5 \(\mu\)M ONOO\(^-\) (Konorev et al. 1998). However, in this latter study the authors used much lower protein concentrations (0.4 \(\mu\)g/ml, compared to the 0.5 mg/ml used here), resulting in a significantly higher ONOO\(^-\):protein ratio of about 50:1 at the IC\(_{50}\) value. It is well known that ONOO\(^-\) added by one single bolus addition can decompose very quickly in water without reacting with the highly diluted protein.

Both studies, however, suggest an oxidation of the highly reactive active site cysteine (C278 in MtCK and C283 in cytosolic CK). Site-directed mutagenesis of this cysteine residue leads to significant but not complete inactivation of the enzyme, but most of all to a complete loss of substrate synergism (Furter et al. 1993; Fritz-Wolf et al. 1996). The homologous cysteine in the transition state structure of arginine kinase is in fact in direct contact with the reactive guanidino group of the arginine substrate (Zhou et al. 1998). Since the residual enzyme activity of the C287G mutant MtCK was not affected at all by ONOO\(^-\) (Fig. 4.1), we take this result as a formal proof that it is indeed the very specific modification of the highly reactive C278 that leads to inactivation of the enzyme and that modifications by ONOO\(^-\) of other targets do not markedly affect enzyme activity. Interestingly, the peptide L272-R287, containing the active site cysteine, was detected by MALDI-MS only in the tryptic digest of the native protein, but escaped detection in the ONOO\(^-\)-modified homologue. This suggests that some modification of this oligopeptide took place, changing its chemical properties in such a way that it was no longer detected by our MALDI methods. Such a phenomenon may be explained by the poor ionization/protonation potential of a highly acidic peptide containing a sulfinic or sulfonic acid.
The tryptophan residue W223 is known from site directed mutagenesis, as well as from the X-ray structure of CK, to be located in the active site. Replacement of this residue by phenylalanine leads to a loss of CK activity by more than 96% (Gross et al. 1994). The peptide containing tryptophan W223 (G211-R231) showed no mass shift after ONOO' exposure, indicating that W223 is not modified by ONOO' which is fully in line with an earlier study using radiation inactivation (Koufen et al. 1999).

It has been shown recently that the octamer is the physiological form of MtCK and that reduced octamer stability leads to reduced Cr stimulated respiration (Khuchua et al. 1998). In addition to inactivation of CK, ONOO' affects octamer stability of MtCK by destabilizing the octameric structure and preventing the formation of octamers from dimers (Soboll et al. 1999). Interestingly enough, in this study we can show that the extent of octamer destabilization by ONOO' depends very much on the MtCK isoenzyme. In our hands, human uMtCK was markedly more stable to ONOO'-induced dimerization than human sMtCK. Evidence for an intrinsically higher octamer stability of human uMtCK compared to human sMtCK has recently been demonstrated (Schlattner et al. 2000) by using transition state analogue complex, which is known to lead to a dimerization of MtCK octamers (Milner-White et al. 1971).

The contact regions of the dimer/dimer interface are well defined (Fritz-Wolf et al. 1996) and include the residues listed in Tab. 4.1. Most of the peptides containing residues important for octamer stability could be detected by MALDI-MS. These peptides showed no mass shift upon ONOO' treatment, indicating, that no modification took place. Residues that are not detected by MALDI-MS include amino acids asparagine (N44), glycine (G45), serine (S47), leucine (L48), aspartic acid (D49), glutamine (Q50) and threonine (T172), none of which is known to be susceptible to ONOO', therefore a chemical modification of one of these residues is very unlikely. Thus, the mass spectrometric results presented here with chicken sMtCK suggest that the destabilization of MtCK octamers, upon ONOO' exposure, can be explained by chemical modification of the peptide G263-R271 which is located in the dimer/dimer interface and is responsible for hydrophobic stabilization of the MtCK octamer (Fritz-Wolf et al. 1996; Eder et al. 2000a).

Such a modification is most probably involving a nitration of tryptophan W264, the replacement of which by site-directed mutagenesis leads to octamer destabilization (Gross et al. 1994). By comparing the X-ray structures of sMtCK and that of the recently solved uMtCK, specifically the dimer/dimer interfaces, it became obvious that uMtCK has a much stronger participation of polar interactions at the dimer/dimer interface than it is the case for sMtCK (Fritz-Wolf et al. 1996; Eder et al. 2000a). This could recently be confirmed by biophysical methods studying the temperature dependence of the octamer/dimer equilibrium of the two
MtCK isoforms (Schlattner et al. 2000). Therefore, chemical damage to tryptophan, which mediates hydrophobic interactions, would have stronger effects on the stability of sMtCK compared to uMtCK, since the polar interactions at the uMtCK dimer/dimer interface can at least partly compensate for the loss of W264-mediated interactions. A modification of two tyrosine residues, Y15 and Y34, which are also located at the dimer/dimer interface (Fritz-Wolf et al. 1996), could be excluded as the peptides containing these residues (L8-R19 and K20-K36, respectively) did not show any mass shift upon ONOO\(^-\) treatment. Thus, the occurrence of tyrosine nitration seen in Western blot has its origin rather in a nitration of tyrosine residues that may not be as important in terms of structure/function relationships.

The CK system plays an important physiological role for cellular energetics in health and disease (for review see (Wallimann 1999)). A compromised CK system, caused among others by oxygen radical damage to the enzyme, has been implied in heart disease (Nascimben et al. 1996), as well as in many neurodegenerative diseases like Huntington’s Disease (Klivenyi et al. 1999), ALS (Ferrante et al. 2000) and Alzheimer’s Disease (for review see (Beal 2000)). In Alzheimer brains, it was found that CK activity is decreased while the mRNA levels did not
change significantly (Aksenov et al. 1997; Aksenova et al. 1999). The same authors could show recently that cytosolic brain-type CK (BB-CK) is a target for oxidative modification in Alzheimer brains and that the content of protein carbonyls in BB-CK is significantly increased in diseased brains (Aksenov et al. 2000). The present study demonstrates that with purified MtCK, after treatment with ONOO' in vitro, the content of protein carbonyls is increased, as well. However, this reaction was preceded by inactivation of the enzyme, which was already complete before a first signal by the anti-DNP-antibody could be detected. The active site, including the cysteine that is highly reactive and exquisitely sensitive to oxidative damage, is highly conserved not only in mitochondrial and cytosolic CK’s, but also throughout the evolution of guanidino kinases (Zhou et al. 1998; Eder et al. 1999). Therefore it can be tacitly assumed that the modification responsible for CK inactivation is the same in all CK isoforms and that the active site cysteine is involved as the prime target, demonstrated here with the C278G sMtCK mutant. Thus, the increase in protein carbonyl formation is rather correlated with oxidation of tryptophan W264, which would lead to a destabilization of the octamer, or additionally with oxidation of other amino acid side chains, not yet identified, which do not seem to have a crucial function for activity and/or structure of the MtCK octamer. The increase of protein carbonyls in CK exposed to ONOO' and CK in Alzheimer brains could further confirm the role of ONOO' in neurodegenerative diseases in general. For example, in spinal cord tissues of animal models of ALS, nitrotyrosine was detected (Klivenyi et al. 1999). This clearly points to an involvement of ONOO' in this disease.

Beside the integrity of the active site, the octameric state of MtCK seems also to be crucial for efficient vectorial channeling of high-energy phosphates from mitochondria to the cytosol (Schlattner et al. 1998). In animal models of acute and chronic ischemia-reperfusion damage, as well as in samples from human patients with heart disease, a significant shift of the octamer/dimer equilibrium to 40% dimers compared to 17% dimers in the control animals was observed (Soboll et al. 1999). Since it is known that some of the main pathological symptoms of these cardiomyopathies are caused by reactive oxygen and nitrogen species (Schulz et al. 1997) our results, showing that chemical modification of tryptophan W264 by ONOO' causes a dimerization of MtCK octamers, make it highly probable that this very modification together with an increase in dimeric MtCK occurs also in vivo. A similar specific mechanism of radical-induced damage to the dimer/dimer interface of MtCK via W264 could also take place in neurodegenerative diseases. However, since uMtCK, at least in vitro, is significantly more stable, this shift towards a propensity of dimeric uMtCK in brain and neuronal tissue is likely to happen at a more advanced stage of neurodegeneration. In addition, since octameric MtCK has been shown to stabilize mitochondria from going into permeability transition (O'Gorman
et al. 1997), decrease in MtCK octamer content, caused by ONOO$, could also lead to early events of apoptosis (Beutner et al. 1998).

In summary, this study shows that ONOO$ has severe effects on CK enzyme activity, as well as on the stability of MtCK octamers. In vivo this would lead to an interruption of the Cr/PCr-shuttle and therefore to a lower cellular energy state with all its far-reaching consequences.
4 Peroxynitrite and mitochondrial Creatine Kinase

4.4 **EXPERIMENTAL PROCEDURES**

*Chemicals* – All enzymes and coenzymes were obtained from Roche Molecular Biochemicals (Rotkreuz, Switzerland), α-cyano-4-hydroxycinnamic acid was from Aldrich (Buchs, Switzerland) 2,5-dihydroxybenzoic acid and 2,4-dinitrophenylhydrazine (DNPH) was from Fluka (Buchs, Switzerland). Further chemicals were purchased from standard suppliers and were of the highest purity commercially available. Human MtCK isoforms (Schlattner et al. 2000) and chicken sMtCK were heterologously expressed according to a protocol described earlier (Furter et al. 1992). ONOO⁻ was a generous gift from Prof. W. Koppenol, Laboratorium für Anorganische Chemie, ETH Zürich, Switzerland

*ONOO⁻ administration* – The concentration of ONOO⁻ stock solution, synthesized from the reaction of gaseous NO with solid potassium O₂⁻ (Koppenol 1996), was determined photometrically at 302 nm in 10 mM NaOH (ε₉₇ = 1.67) prior to use. ONOO⁻, in a volume of 20 μl of 10mM NaOH, was added to 200 μl protein solution containing 0.5 mg/ml MtCK in 100 mM Na₃PO₄, 150 mM NaCl, pH 7.2 to reach final ONOO⁻-concentrations between 1 and 1000 μM. To the control, 20 μl NaOH but no ONOO⁻ was added, which led to a pH-shift in the protein solution of < 0.05 pH-units.

*Determination of octamer content* – The oligomeric state of MtCK was determined by gel permeation chromatography on a Superose 12 FPLC column (Pharmacia) at room temperature in running-buffer (50 mM Na₃PO₄, 150 mM NaCl, 2 mM BME, 0.2 mM EDTA, pH 7.0) at a flow rate of 0.8 ml/min. Peak areas in the elution profile were quantified by graphical integration.

*CK-activity determination* – The specific CK activities were assayed photometrically in the reverse reaction, using the glucose-6-phosphate-dehydrogenase/hexokinase–coupled enzyme assay as described (Eder et al. 2000b) at room temperature (22°C).

*Derivatization of protein carbonyls* – 10 μl DNPH (10 mM in 2N HCl) were added to 50 μl of protein solution and incubated for 1h at room temperature. For protein precipitation, 60 μl 20% TCA were added and incubated for at least 10 min on ice, then centrifuged for 10 min at 13000 rpm (14000 g). Pellets were washed twice with ethylacetate:ethanol 1:1 and subsequently resuspended in 50 μl Na₃PO₄ (10 mM, pH 7).

*Oxyblot* – SDS-gel chromatography was performed according to Laemmli (Laemmli 1970) loading 10 μg of protein per lane. Following electrophoresis, proteins were transferred onto nitrocellulose membrane (Schleicher&Schuell, Dassel, Germany). Membranes were blocked with 4% fat free milk powder in phosphate buffered saline (PBS) for 1h at room temperature, then incubated with rabbit anti-DNP antibody (Sigma, Buchs, Switzerland), diluted 1:1000 in
PBS/milk powder for 1h at room temperature. After 3 washing steps with PBS, membranes were incubated with anti-rabbit-antibody, horseradish peroxidase-conjugated (Nordic Immunological Laboratories, Tilburg, Netherlands), diluted 1:5000 in PBS/milk powder for 1h at room temperature, then washed three times and developed using enhanced Luminol reagent from NEN (Boston, USA).

_Tryptic digest of MtCK — ONOO⁻ treated chicken MtCK, as well as native chicken MtCK, were dialyzed against 50 mM NH₄HCO₃, 20 mM methylamin, pH 8.0, then urea to the final concentration of 1 M was added. The proteins were digested for 16h at 37°C by adding trypsin in an enzyme to protein ratio of 1:100. The reaction was stopped by freezing the samples._

_Mass spectrometric analysis — Mass spectra were performed on a Voyager-DE Elite MALDI-TOF (PerSeptive Biosystems) in positive ion reflector mode, using either a-cyano-4-hydroxycinnamic acid or 2,5-dihydroxybenzoic acid in a 2:1 mixture of acetonitril:0.1% trifluoroacetetic acid in H₂O. For sample preparation, 0.5 µl of the digested protein solution was mixed without further preparation with 0.5 µl of the matrix solution directly on target and the solution was air-dried._
5 Heart mitochondrial Nitric Oxide Synthase

5 Heart mitochondria carry a Ca\(^{2+}\)-dependent Nitric Oxide Synthase which induces Oxidative Stress upon Overstimulation

5.1 Introduction

The discovery of the obligatory role of endothelium in acetylcholine-induced vasodilation (Furchgott et al. 1980) which led to uncovering nitric oxide (NO) as the endothelium-derived relaxing factor (Ignarro et al. 1987; Palmer 1987) opened new windows in biomedical research. Nitric oxide is synthesized by the NO synthase (NOS) isoforms and exerts a wide range of biological activities, many of them are mediated via its interaction with mitochondria (reviewed in Ref. (Ghafourifar 2000)). Between 1995 and 1996 immunohistochemical studies (Bates et al. 1996; Frandsen et al. 1996) provided evidence for the presence of a NOS-like protein in mitochondria of different organs. In 1997 for the first time the presence of a constitutively expressed and continuously active NOS in rat liver mitochondria (mitochondrial NOS, mtNOS), its localization in the inner mitochondrial membrane, and determination of its activity was reported by Ghafourifar et al. (1997). It was shown that mtNOS is Ca\(^{2+}\)-dependent and that the enzyme substantially controls respiration and mitochondrial transmembrane potential (ΔΨ). The presence of mtNOS and its association with the inner mitochondrial membrane were thereafter confirmed (Giulivi 1998; Giulivi et al. 1998; Tatoyan et al. 1998; Arnaiz et al. 1999; Ghafourifar et al. 1999b; Ghafourifar et al. 1999c; Bustamante et al. 2000; Lopez-Figueroa et al. 2000) and the enzyme was purified (Tatoyan et al. 1998). It has been shown that mtNOS regulates mitochondrial O\(_2\) consumption and ΔΨ formation (Ghafourifar et al. 1997; Ghafourifar et al. 1999c), ATP synthesis (Giulivi 1998), Ca\(^{2+}\) homeostasis (Ghafourifar et al. 1999b; Bringold et al. 2000), and matrix pH (Ghafourifar et al. 1999b). The formation of NO by mitochondria has been demonstrated in cells of liver (Ghafourifar et al. 1997; Giulivi 1998; Giulivi et al. 1998; Tatoyan et al. 1998; Ghafourifar et al. 1999c), brain (Arnaiz et al. 1999) and thymus (Bustamante et al. 2000). Very recently mitochondrial NO formation by PC12 and COS1 cells was also reported (Lopez-Figueroa et al. 2000). Recently it was shown that prolonged stimulation of mtNOS in liver mitochondria results in increase of lipid peroxides and the release of mitochondrial cytochrome c, probably as a result of peroxynitrite (ONOO\(^{-}\)) formation (Ghafourifar et al. 1999c). Several mitochondrial enzymes are known to be inactivated by ONOO\(^{-}\). Mitochondrial aconitase, a Krebs cycle enzyme containing an iron sulphur cluster in the active site, was reported to be extremely sensitive to reactive nitrogen species (RNS), especially ONOO\(^{-}\), which inactivates the enzyme by
Fig. 5.2: Heart mtNOS activity.

Basal unstimulated formation of NO in heart mitochondria in the absence (blue, control) or presence of 1 mol/L ruthenium red (red, RR) is expressed as the fluorescent intensities of DAF-2 DA (5 mol/l). Stimulation of mtNOS was achieved by loading mitochondria with 40 mol/l Ca\(^{2+}\) (pink, Ca\(^{2+}\)). When indicated, 1 mol/l RR (green, RR + Ca\(^{2+}\)) or 10 mmol/l L-NMMA (yellow, L-NMMA + Ca\(^{2+}\)) were added prior to Ca\(^{2+}\) as described in "Materials and Methods". The fluorescence intensity was recorded every second.

Muscle mitochondria carry a Ca\(^{2+}\)-sensitive NOS. Supplementation of mitochondria with 1 mmol/l L-arginine and/or 12 mol/l tetrahydrobiopterin did not increase NO formation. Mitochondria contain all the substrates and cofactors needed for a NOS activity in excess concentrations (reviewed in (Ghafourifar 2000)) and Ca\(^{2+}\) per se seems sufficient to trigger mtNOS stimulation (Ghafourifar et al. 1999b; Ghafourifar et al. 1999c; Bringold et al. 2000). Fig. 5.2 shows that blockade of mitochondrial Ca\(^{2+}\) uptake by RR prevents the stimulation of mtNOS (green, RR + Ca\(^{2+}\)) compared to RR alone (red, RR), which indicates that the observed NOS activity requires the uptake of Ca\(^{2+}\) into mitochondria and argues against a significant contamination of our preparation with non-mitochondrial, e.g., cytosolic NOS. The DAF-2 DA fluorescent signal was not altered when RR was added to a mitochondrial suspension after Ca\(^{2+}\) uptake was completed or when NO was generated by the donor 1-hydroxy-2-oxo-3-(3-aminopropyl)-3-isopropyl-1-triazene (NOC-5) in the absence of mitochondria (not shown). In our preparations of bovine heart mitochondria the endogenous Ca\(^{2+}\) level varies between 2-10 nmol/mg mitochondrial protein (measured as in (Ghafourifar et al. 1999a)), which is sufficient to support a basal mtNOS activity. We also could show, that mitochondria take up Ca\(^{2+}\), when
added exogenously, but they start to release it again after about 200 sec. and the release is completed after about 250 sec. (Fig. 5.3). This corresponds to the time point, where the curve of NO formation, detected with DAF-2 DA (Fig. 5.2) flattens and NO formation rate is decreased. Thus we conclude, that this decreasing NO formation rate after 200 sec. is rather dependent on the release of Ca$^{2+}$ and that mitochondria do not run out of substrates.

Addition of exogenous respiratory substrates, e. g., succinate or pyruvate plus malate, did not increase the formation of NO. A collapse of Δψ decreases mtNOS activity in liver mitochondria (Ghafourifar et al. 1999c). Inhibition of mitochondrial respiratory chain complex I by rotenone, which deenergizes mitochondria and allows Ca$^{2+}$ to leak out, prevents mtNOS stimulation (not shown). Neither of the respiratory inhibitors decreased the activity of purified commercially available NOS (cNOS, Calbiochem) determined by the NO-electrode (ISO-NO, Mark II, World Precision Instruments) or citrulline assay (as described in (Richter et al. 1999)). To further confirm our present and previous reports (Ghafourifar et al. 1997; Ghafourifar et al. 1999a; Richter et al. 1999), we measured the mtNOS activity of rat liver mitochondria with the same DAF-2 DA fluorescent setup and also observed the endogenous and Ca$^{2+}$-stimulated NOS activity sensitive to L-NMMA, RR, EGTA or rotenone.

**Fig. 5.3:** Ca$^{2+}$ uptake of mitochondria.

Mitochondria were incubated with arsenazo and Ca$^{2+}$ was added. The signal increases as arsenazo builds a complex with Ca$^{2+}$. Ca$^{2+}$ uptake of mitochondria leads to a decrease in the signal and the following Ca$^{2+}$-release, starting at about 200 sec., again to an increase. Mitochondria, where no exogenous Ca$^{2+}$ was added, released endogenous Ca$^{2+}$ after about 400 sec.
Nitric oxide, added exogenously or produced by mitochondria, inhibits mitochondrial respiration by competing O$_2$ at the level of cytochrome oxidase (reviewed in (Ghafourifar 1999)). It has been suggested that Ca$^{2+}$ per se is sufficient to stimulate mtNOS and consequently decrease the oxygen consumption of rat liver mitochondria (Ghafourifar et al. 1997; Giulivi et al. 1998; Ghafourifar et al. 1999c; Richter et al. 1999). Here we demonstrate that also loading heart mitochondria with Ca$^{2+}$ per se is adequate to hamper the consumption of oxygen in an L-NMMA- and RR-sensitive manner (Fig. 5.4). The same sensitivity was observed to 1400W (not shown). As shown in Fig. 5.4, uptake of Ca$^{2+}$ per se seems sufficient to decrease the consumption of oxygen by heart mitochondria. Although the importance of mitochondria in the phasic cellular Ca$^{2+}$ homeostasis was overlooked, recent studies show the crucial role of these organelles as physiologically relevant intracellular Ca$^{2+}$ buffers that continually participate in intracellular Ca$^{2+}$ spikes in excitable tissues (Rizzuto et al. 1999). Stimulation of mtNOS by mitochondrial Ca$^{2+}$ uptake and impairing respiration and respiration-dependent mechanisms such as synthesis of ATP (Giulivi 1998), might play important roles in heart bioenergetics especially when oxygen or ATP is limited such as in hypoxia or heart failure.

**Fig. 5.4:** *Regulation by mtNOS of heart mitochondrial oxygen consumption.*

Mitochondrial oxygen consumption without addition of exogenous respiratory substrates was measured under the same conditions as in Fig. 5.2 in the absence (blue, control) or presence of RR (red, RR). Stimulation of mtNOS was achieved by loading mitochondria with 40 mol/l Ca$^{2+}$ (pink, Ca$^{2+}$) and its inhibition with 10 mmol/l L-NMMA (yellow, L-NMMA + Ca$^{2+}$). Ca$^{2+}$ uptake was prevented by 1 mol/l RR (green, RR + Ca$^{2+}$).
Mitochondria are one of the main cellular sources of ROS and, thus, are equipped with several redox defense mechanisms. Overproduction of ROS or downregulation of the redox barriers cause oxidative stress (reviewed in (Ghafourifar 2000)). Increased ROS formation is observed in a wide variety of experimental and clinical cardiac and vascular abnormalities and the existing evidence suggest that oxidative stress plays a crucial role in these conditions (Dhalla et al. 2000). Many of these pathophysiological conditions, such as ischemia/reperfusion injury (Ylitalo et al. 2000) impaired myocyte contractile activity (Tatsumi et al. 2000) and cardiomyopathy (Doliba et al. 1999) are associated with prolonged elevated cytosolic Ca\(^{2+}\), formation of ROS and RNS, and mitochondrial malfunction (Kristian et al. 1998).

Mitochondrial aconitase, an important Krebs cycle enzyme with an iron-sulfur center at the active site, is very susceptible to oxidative/nitrosative damage (Castro et al. 1998; Konorev et al. 1999). Fig. 5.5 shows that prolonged loading heart mitochondria with Ca\(^{2+}\) (pink, Ca\(^{2+}\)) causes a drastic loss of mitochondrial aconitase activity which is prevented when mitochondrial Ca\(^{2+}\) uptake is blocked by RR (green, Ca\(^{2+}\) + RR) or when mtNOS activity is inhibited by L-NMMA (yellow, Ca\(^{2+}\) + L-NMMA). The same effects were observed with

![Graph showing changes in OD at 240 nm over time for different conditions.](image_url)

**Fig. 5.5:** *Prolonged stimulation of mtNOS causes inactivation of aconitase in heart mitochondria.*

Aconitase activity of heart mitochondrial MIF (blue, control) is decreased upon loading mitochondria with Ca\(^{2+}\) (pink, Ca\(^{2+}\)). Effect of prevention of mitochondrial Ca\(^{2+}\) uptake by RR (green, Ca\(^{2+}\) + RR) or inhibition of mtNOS by L-NMMA (yellow, Ca\(^{2+}\) + L-NMMA) or scavenging ONOO\(^-\) with urate (brown, Ca\(^{2+}\) + urate) is shown.
1400W or with EGTA (not shown). Also, a SOD mimic, MnTBAP or an ONOO\textsuperscript{−} scavenger, urate (brown, Ca\textsuperscript{2+} + urate), largely prevent the Ca\textsuperscript{2+}-induced aconitase inactivation. This strongly suggests that the observed inactivation is induced by overstimulation of mtNOS and the consequent intramitochondrial formation of ONOO\textsuperscript{−} (Ghafourifar et al. 1999c; Bringold et al. 2000). Accordingly, mtNOS overstimulation and generation of ONOO\textsuperscript{−} (Ghafourifar et al. 1999c) might be the underlying mechanism for the Ca\textsuperscript{2+}-induced oxidative damage to mitochondrial aconitase which is prevented by lessening ROS/RNS and is seen in a variety of cells such as cardiomyocytes (Konorev et al. 1999) or fibroblasts (Castro et al. 1998).

Mitochondria utilize more than 90% of the total cellular oxygen content. Although most of the O\textsubscript{2} is completely reduced to water at the level of cytochrome oxidase, a fraction is incompletely reduced to O\textsubscript{2}\textsuperscript{−} at the level of other mitochondrial respiratory complexes. Presently, there is no general consensus on which fraction of O\textsubscript{2} is turned into O\textsubscript{2}\textsuperscript{−} (Forman et al. 1997), nevertheless mitochondria contain their own SOD. The critical protective role of MnSOD as the major mitochondrial detoxifying mechanism in heart pathophysiology is broadly investigated. Overexpression of MnSOD protects the myocardium against oxidative injury induced by ischemia/reperfusion (Chen et al. 1998) or drugs e. g., adriamycin (Yen et al. 1999) and adriamycin cardiomyopathy is associated with MnSOD downregulation (Li et al. 2000). MnSOD gene-knockout mice (Sod2\textsuperscript{−/−}) exhibit extensive mitochondrial injury and die shortly after birth (Lebovitz et al. 1996). Fig. 5.6 shows that MnSOD activity of untreated heart mitochondria (blue, control) is decreased upon loading the organelles with Ca\textsuperscript{2+} (pink, Ca\textsuperscript{2+}) in a manner prevented when mitochondrial Ca\textsuperscript{2+} uptake is blocked (green, Ca\textsuperscript{2+} + RR) or when mtNOS activity is inhibited (yellow, Ca\textsuperscript{2+} + L-NMMA). The same prevention was observed with 1400W or with EGTA (not shown). This finding shows that mitochondrial Ca\textsuperscript{2+} overload causes MnSOD inactivation via a mtNOS-dependent mechanism. It suggests that by downregulation of MnSOD, the mitochondrial major detoxifying molecule, the newly described mtNOS may play undetected roles in mitochondria-originated oxidative stress. We did not observe prevention of MnSOD inactivation by the ONOO\textsuperscript{−} scavenger, urate. Although MnSOD is very susceptible to ONOO\textsuperscript{−} (MacMillan-Crow et al. 1996), this finding suggests that RNS other than ONOO\textsuperscript{−} are mediating the mtNOS-induced MnSOD inactivation, and that different mitochondrial enzymes are distinctly susceptible to RNS/ROS formed intramitochondrially upon mtNOS overstimulation.

MtCK is another mitochondrial enzyme susceptible to RNS and ROS. The octameric structure of MtCK (Fritz-Wolf et al. 1996; Soboll et al. 1999), as well as its catalytic function are important for mitochondrial energy channeling (Khuchua et al. 1998; Schlattner et al. 1998; Kay et al. 2000). Inactivation of MtCK and a marked decrease in its octamer/dimer ratio are
Fig. 5.6: **Loading heart mitochondria with Ca\textsuperscript{2+} inactivates MnSOD.**

Mitochondria were incubated in the absence of Ca\textsuperscript{2+} (blue, control) with RR (red, RR), or with Ca\textsuperscript{2+} (pink, Ca\textsuperscript{2+}) in the presence of the mitochondrial Ca\textsuperscript{2+} uptake blocker (green, Ca\textsuperscript{2+} + RR) or mtNOS inhibitor (yellow, Ca\textsuperscript{2+} + L-NMMA) as described in "Materials and Methods". 30 g MIF was added to a 1 ml cuvette containing 50 mmol/l phosphate buffer, pH 7.8, 50 mol/l xanthine, 100 mol/l EDTA, 2 mmol/l KCN and 30 mmol/l xanthine oxidase. After 1 min cytochrome c (10 mol/l) was added (time = 0) and its reduction was measured at 550 nm.

observed in animal models of myocardial infarction and ischemia/reperfusion injury (Soboll et al. 1999). Fig. 5.7A shows that MtCK (blue) is inactivated when mitochondria are loaded with Ca\textsuperscript{2+} (pink) and that RR prevents this inactivation (green). An increased intracellular Ca\textsuperscript{2+} along with a failure of oxidative capacities, e. g., consumption of oxygen (Davydov et al. 1999) and a drop in ∆ψ (Ylitalo et al. 2000), as well as MtCK inactivation (Bakker et al. 1994; Davydov et al. 1999; De Sousa et al. 1999) are observed in failing ischemic myocardium, old animals are more susceptible (Allen et al. 1993) and RR prevents these damages (Leperre et al. 1995). Present findings suggest that the MtCK inactivation observed in failing ischemic myocardium is due to the overload of mitochondria with Ca\textsuperscript{2+}. Surprisingly, L-NMMA does not prevent Ca\textsuperscript{2+}-induced MtCK inactivation (yellow). We studied the sensitivity of MtCK to O\textsubscript{2}• by subjecting control MIF (Fig. 5.7B, blue, control) to the O\textsubscript{2}•-generating xanthine/xanthine oxidase system (XA/XAO) (Fig. 5.7B, pink, treated) and observed a strong MtCK inactivation. Although MtCK is strongly inhibited by exogenously added ONOO• (Stachowiak et al. 1998a) the ineffectiveness of L-NMMA in preventing Ca\textsuperscript{2+}-induced MtCK damage suggests that this inactivation is not directly induced by NO or its congeners such as ONOO•.
but by other ROS (Yuan et al. 1992) such as $O_2^-$. Observing that the membrane permeable SOD mimic, MnTBAP (Ghafourifar et al. 1999c) prevents the Ca$^{2+}$-induced MtCK inactivation (not shown) supports this suggestion. How loading heart mitochondria with Ca$^{2+}$

**Fig. 5.7:** Effect of prolonged loading heart mitochondria with Ca$^{2+}$ on MtCK and adenylate kinase activities.

*Fig. 5.7 (A):* MtCK activity of MIF of control mitochondria (blue), with RR (red), or loaded with Ca$^{2+}$ (pink), in the presence of L-NMMA (yellow) or RR (green). *Fig. 5.7 (B):* creatine kinase activity of MIF prior to (blue, control) or after subjected to the $O_2^-$ generating system (pink, treated), 50 mol/l xanthine and 30 nmol/l xanthine oxidase. *Fig. 5.7 (C):* adenylate kinase activity of the samples of panel A. *Fig. 5.7 (D):* adenylate kinase activity of the samples of panel B.

increases the formation of $O_2^-$ is not fully clear to us. However, overloading mitochondria with Ca$^{2+}$ induces cytochrome c release (Ghafourifar et al. 1999c) and from the elegant report of
Cai and Jones (Cai et al. 1998) and from our studies (Ghafourifar et al. 1999a; Ghafourifar et al. 1999c) it seems that $O_2^-$ formation is increased as the consequence, and not the cause, of cytochrome c loss. Displacement of cytochrome c from mitochondria produces a gap in the hierarchically arranged respiratory complexes III and IV, and therefore a site for electron leakage. Another possible mechanism would be the inhibition of mitochondrial respiration by NO, produced as a consequence of Ca$^{2+}$ overload and thus, stimulation of mtNOS. It has been suggested, that this inhibition of mitochondrial respiration at the level of complex IV could lead to increased production of $O_2^-$ at complexes I (Turrens et al. 1980) and II (Turrens et al. 1985) of the respiratory chain, thus increasing the $O_2^-$ concentration. Inactivation of MnSOD, reported here, can uphold the formation of $O_2^-$, provoke mitochondrial redox barrier and extend MtCK inactivation. The inactivated MtCK was not recovered by 10 mmol/l β-mercaptoethanol, therefore a direct inactivation of MtCK by NO seems very unlikely because such an inactivation, probably due to S-nitrosylation, is reversible (Arstall et al. 1998).

Adenylate kinase activity is not affected by loading mitochondria with Ca$^{2+}$ (Fig. 5.7C) nor by the $O_2^-$ generating system, XA/XAO (Fig. 5.7D). This shows that the observed Ca$^{2+}$-induced mitochondrial enzyme inactivation is not due to a non-specific damage induced by Ca$^{2+}$ followed by a general mitochondrial weakening, e.g., the so-called "Ca$^{2+}$ cycling".

Altogether present results show that heart mitochondria carry a Ca$^{2+}$-sensitive NOS that regulates mitochondrial oxygen consumption. It also suggests that overloading mitochondria with Ca$^{2+}$ causes oxidative stress by formation of different RNS/ROS that affect mitochondrial enzymes distinctly. Mitochondrial aconitase is inactivated by overstimulation of mtNOS and intramitochondrial formation of $\text{ONOO}^-$, MnSOD inactivation requires mtNOS stimulation and formation of RNS rather than $\text{ONOO}^-$, and MtCK is inactivated by mechanisms resembling elevation of $O_2^-$ formation. Different compartmentalization and sub-organelle microenvironments, such as pH or the presence of redox molecules, can influence the distribution and the deleterious effects of very short lasting RNS or ROS. Inactivation of aconitase, an enzyme required in the Krebs cycle, and MnSOD, thus elevating the $O_2^-$ levels, can constitute a negative loop leading to a drastic failure of mitochondrial bioenergetics. MtCK is functionally coupled to oxidative phosphorylation (Schlattner et al. 1998) and ADP produced by MtCK during phosphorylation of creatine is transported back into the mitochondrial matrix (Kay et al. 2000). Oxidative phosphorylation and Cr-induced stimulation of respiration (Kay et al. 2000), which is diminished upon inactivation of MtCK, may explain the significant decrease in the oxygen consumption and ATP-production observed in the pathophysiological conditions with prolonged elevated Ca$^{2+}$ levels, such as in ischemia/reperfusion injury (Lebovitz et al. 1996; Chen et al. 1998; Yen et al. 1999).
5.3 EXPERIMENTAL PROCEDURES

Isolation of heart mitochondria - Intact mitochondria were isolated from bovine heart muscle as described (Schlegel et al. 1990) with an additional Percoll purification step. Briefly, bovine heart muscle was minced and homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in 220 mmol/l mannitol, 70 mmol/l sucrose, 10 mmol/l HEPES, 0.2 mmol/l EDTA, 0.1% fatty acid free bovine serum albumin, pH 7.4 (MSH). The homogenate was centrifuged at 600 g for 5 min and the supernatant was re-centrifuged at 10'000 g for 10 min. The pellet was resuspended in MSH pH 7.4 and purified with a 25% Percoll gradient centrifugation at 100'000 g, and washed twice in MSH (10'000 g, 10 min) to yield highly enriched purified mitochondria. Protein contents of mitochondria or mitochondrial subfractions were determined according to Bradford using the commercially available Bio-Rad protein assay reagents. All the isolation steps were carefully carried out at 4°C and all the measurements were performed in 0.1 mol/l HEPES pH 7.1 at room temperature with 0.2 mg mitochondrial protein/ml.

Detection of NO formation by heart mitochondria - Basal endogenous formation of NO by freshly isolated intact heart mitochondria (0.2mg/ml) was detected with a Perkin Elmer Luminescence spectrometer (LS50B) at the excitation wavelength of 492 nm and the emission wavelength of 515 nm at room temperature in the presence of 5 μmol/l DAF-2 DA (Kojima et al. 1998). Stimulation of nNOS was performed by loading mitochondria with 40 μmol/l Ca²⁺. No exogenous mitochondrial respiratory substrates were added.

Determination of mitochondrial Ca²⁺ uptake - Mitochondrial Ca²⁺ uptake and release was measured in 0.1 M HEPES buffer, pH 7.0, containing 10 μM CaCl₂ (10 nmol of Ca²⁺/mg of mitochondrial protein) in the presence of 50 μM Arsenazo III and the absorbance change of arsenazo at 685-675 nm was monitored as described (Frei et al. 1985).

Oxygen consumption measurements - Endogenous mitochondrial oxygen consumption was measured at room temperature with a two-channel high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria). The compounds in study were present in the buffer prior to the addition of mitochondria (0.2mg/ml) and no exogenous respiratory substrates were added.

Determination of mitochondrial enzyme activities - Freshly isolated mitochondria (0.2mg/ml) were incubated at 4°C in 0.1 mol/l HEPES buffer pH 7.1 and NOS inhibitors, L-NMMA (10 mmol/l) or N-(3-(aminomethyl)benzyl)acetamidine (1400W) (100 μmol/l), ONOO⁻ scavenger, urate (100 μmol/l) or SOD mimic, MnTBAP (10 μmol/l) 10 were present in the buffer prior to the addition of mitochondria. After 10 min incubation 100 μmol/l Ca²⁺ was added.
(Ghafourifar et al. 1999c; Bringold et al. 2000). Samples were incubated at room temperature for 10 min and then spun at 10'000 g for 10 min at 4°C. Pellets were resuspended in a small volume of 0.1 mol/l HEPES pH 7.1, and 4 times the volume of ice cold H$_2$O was added to rupture mitochondrial membranes (Ghafourifar et al. 1997). Then 0.05 mol/l phosphate buffer pH 8.5 containing 1% Triton X-100 was added and the suspensions were mixed and spun at 100'000 g for 20 min. The supernatant of this centrifugation, referred to mitochondrial matrix and intermembrane space fractions (MIF), was split in aliquots and kept at -80°C. EGTA (1 mmol/l) or RR (1 μmol/l) were added before Ca$^{2+}$.

Aconitase activity of 50 μg MIF was determined by following the optical density of 0.2 mmol/l cis-aconitate as the substrate at 240 nm as described (Henson et al. 1967). The SOD activity of 30 μg MIF was determined by the Fridovich method (McCord et al. 1968) with minor modification in the presence of 2 mmol/l KCN to block possible contamination with CuZnSOD. CK activity of 2.5 μg MIF was determined by adding 10 mmol/l phosphocreatine to the coupled enzyme system for adenylate kinase and by subtracting adenylate kinase activity from the result obtained (Wallimann et al. 1977). Adenylate kinase activity of 2.5 μg MIF was assayed spectrophotometrically at 340 nm in a reaction mixture containing 1 mmol/l ADP, 20 mmol/l glucose, 0.5 mmol/l NADP$^+$, 2.5 mmol/l MgCl$_2$, 7.2 units G6PDH, and 6.3 units hexokinase as described (Wallimann et al. 1977).

Data presentation - Each figure represents 3 to 5 independent experiments.
6 Reduced Creatine Kinase Activity in the Spinal Cord but not in Cortex of Transgenic Amyotrophic Lateral Sclerosis Mice

6.1 Introduction

Amyotrophic lateral sclerosis (ALS) is a severe neurodegenerative disorder characterized by motor neuron loss, rapidly progressive motor weakness and early death (Brown 1995). The disease exists in a sporadic and in an autosomal dominant familial form (FALS), the latter constitute 10% of all ALS cases. The finding that about 20% of the patients with FALS carry a point mutation in the SOD1 gene, which encodes for the cytosolic copper- and zinc-dependent SOD (Rosen 1993), has focused interest on the involvement of oxidative damage in the pathogenesis of the disease, and lead to the development of several animal models of FALS (Elliott 1999).

Interestingly, most of the mutations, found in FALS patients, retain full or partial specific SOD activity (Borchelt et al. 1994). This, together with the dominant inheritance pattern in FALS, suggests that the onset of the disease is rather dependent on a gain-of-function of SOD than a loss-of-function. Evidence supporting this includes i) the finding that SOD-knockout mice do not develop symptoms of ALS, ii) the report of a lack of correlation between disease severity and enzyme activity, and iii) the finding that overexpression of the mutant enzyme in transgenic mice leads to motor neuron degeneration (Gurney et al. 1994; Ripps et al. 1995; Reaume et al. 1996). An increased ability in generating $O_2^-$ radicals from hydrogen peroxide found in a mutant SOD form, where glycine 93 has been replaced by alanine corresponding to one mutation found in FALS patients, may play a major role in the pathogenesis of ALS (Yim et al. 1996). Transgenic mice overexpressing this G93A SOD mutant (G93A FALS mice) develop symptoms similar to those seen in ALS patients (Gurney et al. 1994). An elevated level of $O_2^-$ will contribute to enhanced oxidative stress, which is known to play a role in neurodegenerative diseases (for review see (Torreilles et al. 1999)). Among the toxic agents contributing to this oxidative stress, peroxynitrite (ONOO$^-$), the reaction product of $O_2^-$ and nitric oxide (NO), is considered to lead to significant protein and lipid damage (Radi et al. 1991a; Radi et al. 1991b). The mechanisms underlying the development of ALS, however, may be an interplay between oxidative stress and mitochondrial dysfunction, the latter having been shown to lead to ATP depletion and selective motoneuron death (Kaal et al. 2000). Therefore, buffering intracellular energy levels could prevent ATP depletion and thus help the cell to be able to cope with energy deficiency.
The creatine kinase (CK) system, consisting of a cytosolic and a mitochondrial isoform together with their substrates creatine (Cr) and phosphocreatine (PCr), is the cell's most important immediate energy buffering and transport system (Wallimann et al. 1992; Wallimann et al. 1998), especially in muscle and neuronal tissues (Wallimann et al. 1994). Cr is phosphorylated to PCr in the intermembrane space of mitochondria, where mitochondrial creatine kinase (MtCK) is located, and then transported into the cytosol (Schlattner et al. 1998). There, the energy pool can be regenerated by transphosphorylation of PCr to ATP, which is catalyzed by cytosolic CK (B-CK in brain) located in close vicinity of cellular ATPases. Increased Cr levels in the cell, paralleled by increased PCr levels, can enhance ATP regeneration and thus improve the cellular energy status of brain and muscle cells. Indeed, Cr protects against neuronal death in vitro (Brewer et al. 2000), and supplementation with Cr was shown to be neuroprotective in several animal models of neurodegenerative diseases (Matthews et al. 1998; Ferrante et al. 2000), including the transgenic G93A FALS mouse model of ALS (Klivenyi et al. 1999). This study showed that life span, as well as the motor performance of G93A FALS mice fed with Cr diet, was significantly improved and that motor neuron loss was decreased.

Cytosolic, as well as MtCK isoenzymes, are prime targets for oxidative damage leading to inactivation of CK, which can be partly protected if both substrates, Cr and MgATP, are present (Yuan et al. 1992; Konorev et al. 1998; Stachowiak et al. 1998a). Additionally, reactive oxygen species lead to dimerization of MtCK (Soboll et al. 1999). Inactivation of CK and dimerization of MtCK are both expected to contribute to a deterioration of the cellular energy state (Stachowiak et al. 1998b). Considering this fact, we examined in the present study whether CK activity in these same G93A FALS mice was altered in the spinal cord or cortex, and whether succinate-stimulated respiration of mitochondria isolated from these regions of diseased brains was affected. Furthermore, we investigated the effect of Cr supplementation on these parameters.
6.2 RESULTS

Mitochondrial respiration was measured using mitochondria isolated from spinal cord or cortex of 120 days old G93A FALS mice and their littermate controls, both either fed a regular or a Cr-supplemented diet. At 120 days of age, the FALS mice had already developed full symptoms of ALS. First, succinate, the substrate of succinate-ubiquinone reductase (complex II) was added to induce state 4 respiration. By adding Rotenone, NADH-ubiquinone oxidoreductase (complex I) was inhibited so that only complex II-mediated oxygen consumption was measured. The respiration rates thus measured were 22.7±4.6 and 20.2±7.6 nmol O$_2$/min mg) for regularly fed control and FALS animals, respectively, and 28.6±12 and 23.0±3.4 nmol O$_2$/min mg) for Cr-fed control and FALS animals, respectively. To stimulate respiration to maximal state 3 levels, ADP was added to a final concentration of 1 mM. The values obtained were 41.8±6.0 and 43.8±10.8 for regularly fed control and FALS, respectively, and 63.6±18 and 44.3±3.9 for Cr-fed control and FALS animals, respectively. Atractyloside, a specific inhibitor of the adenine nucleotide translocator (ANT), reduced the respiration rates again to state 4 levels, indicating that in these mitochondria respiration was still coupled to ATP production. Mitochondria from regularly fed mice, both control and FALS did not show any difference in oxygen consumption rates at any state (Fig. 6.1). In contrast, mitochondria from control mice fed with Cr showed increased oxygen consumption in ADP-stimulated state 3 respiration. Mitochondria from diseased spinal cord of G93A FALS mice fed with Cr did no longer show this increase in oxygen consumption. All respiration rates were expressed as mean values with corresponding standard deviations of 4 independent experiments, each of them representing 5-7 animals at the age of 120 days.

To investigate the question of whether CK may be involved in the development of ALS, CK activities were measured as well. In homogenates from cortex of 120 days old mice, total CK activity levels did not differ between control and FALS brains (0.71±0.11 U/mg protein vs. 0.72±0.14), but these values were decreased by 50% in the homogenates of spinal cord from FALS mice (0.85±0.12 vs. 0.42±0.10 U/mg protein). Feeding these animals with 2% Cr in the diet from the age of 4 weeks on, showed a tendency to slightly higher CK activity values indicating some protection by Cr against CK inactivation (0.47±0.08 U/mg protein or 56% of control activity) which, however, were not statistically significant (Fig. 6.2A). In mitochondrial fractions, a similar picture was noted. MtCK activity in cortex was not changed from control to FALS brains (3.46±0.30 for controls vs. 3.25±0.30 U/mg protein for FALS), whereas MtCK activity in spinal cord from FALS mice was significantly decreased to 64% of control values (3.3±0.46 in controls vs. 2.22±0.65 U/mg protein in FALS). When G93A FALS mice were fed with Cr, CK activity decreases to only 70% of control (2.52±0.54 U/mg protein)
Fig. 6.1: Mitochondrial respiration rates

A: regular fed mice and B: creatine fed mice. The dark bars represent wild type, the light bars FALS animals. Values are the mean from 4 independent experiments with 5-7 animals. No differences in respiration rates between wild type and FALS animals could be detected. Error bars represent standard errors.

although this difference was statistically also not significant (Fig. 6.2B). All CK activities were expressed as mean values and standard deviations of 4-5 independent experiments, each of them consisting of 5-7 animals at age of 120 days.
Fig. 6.2: Creatine kinase activities

A: homogenates and B: mitochondrial fractions. The dark bars represent wild type, the light bars FALS animals. Values are the mean from 4-5 independent experiments with 5-7 animals. Note the decreased CK activities in both homogenates and mitochondrial fractions prepared from spinal cord. Error bars represent standard errors.
One experiment with 5-7 G93A FALS mice of age of 80 days, which is a time point preceding the development of ALS symptoms, revealed no decreased CK activity, neither in homogenates nor mitochondrial fractions from spinal cord or cortex of (data not shown).

The decrease in MtCK activity was not due to decreased expression levels of MtCK protein as Western blot analysis of mitochondrial fractions revealed similar amounts of immunoreactive CK in spinal cord of control and FALS animals (Fig. 6.3).

![Fig. 6.3: Determination of MtCK expression level](image)

**Fig. 6.3:** **Determination of MtCK expression level**
Western Blot of mitochondrial fractions of spinal cord from regularly fed control animals (lane 1) and regularly fed FALS animals (lane 2) against mitochondrial creatine kinase. The same amount of protein (20 mg) was loaded on each lane. The lower band is due to protein degradation after freezing and thawing the samples. Note the similar expression levels of MtCK in wild type and FALS animals.

The enzymatic activity of purified MtCK is partly protected against ONOO\(^-\)-induced oxidative damage by the presence of the full set of substrates (Stachowiak et al. 1998a). Possibly, this may be one of the reasons why MtCK activities tend to be elevated in Cr-fed as compared to unsupplemented FALS animals. To further corroborate this hypothesis, ONOO\(^-\) was administrated to isolated bovine heart mitochondria at different states of respiration in the presence or absence of exogenous Cr. Thereafter, CK activities were measured in the mitochondrial fractions (Fig. 6.4). If ONOO\(^-\) was added to non-respiring mitochondria, MtCK was almost fully inactivated with a remaining 5% of control activity. Under these conditions MtCK was not working since the substrates were present in very low or zero concentrations.
and therefore no or little substrates were bound to the active site of the CK enzyme. Adding ONOO· to mitochondria respiring on succinate, a protective effect could only be seen if additional Cr was added (17.4% of control activity). With increasing amounts of added ATP the protective effect on MtCK increased, as well. This effect was much more pronounced in the presence of Cr, resulting in residual CK activity of 47% of control values. The conditions with high concentrations of ATP (1mM) plus 20mM Cr, under which maximal protection was obtained *in vitro*, correspond to the conditions *in vivo* where the full set of substrates is present.

![Bar chart showing MtCK activity in bovine heart mitochondria after peroxynitrite administration.](image)

**Fig. 6.4:** MtCK-activity *in bovine heart mitochondria after peroxynitrite administration.*

Peroxynitrite was added at different states of respiration, which is to inactive mitochondria (PN), to mitochondria respiring on succinate (PN after succinate), mitochondria respiring on succinate, stimulated with small amounts of ATP (PN after 25µM ATP) and mitochondria, respiring on succinate, stimulated with high amount of ATP (PN after 1mM ATP), in the absence (dark bars) or presence (light bars) of 20 mM creatine. Activities are expressed in percent of control activity, which was measured in mitochondria under the same conditions but not exposed to peroxynitrite. Note the protective effect of Creatine on peroxynitrite-induced inactivation of MtCK.

Our results indicate that in G93A FALS animals displaying FALS symptoms, total CK activity as well as MtCK activity was significantly reduced and that chronic Cr supplementation
seemed to tend to reduce CK inactivation, which is in line with a number of reports that demonstrated marked neuroprotective effects of Cr (Matthews et al. 1998; Brewer et al. 2000; Ferrante et al. 2000; Sullivan et al. 2000).
6.3 DISCUSSION

There is substantial evidence implicating mitochondrial dysfunction and free radical generation as major mechanisms of neuronal death in neurodegenerative diseases including ALS (Beal 1998). We therefore investigated the involvement of the mitochondrial enzyme MtCK in the development of disease in a transgenic mouse model of FALS.

For the first time, we here report a reduced activity of CK in the spinal cord of the G93A FALS transgenic mice. This strongly supports the involvement of mitochondrial dysfunction in the development of motor neuron disease. Earlier studies have shown increased complex I activity in FALS patients (Bowling et al. 1993), as well as in G93A FALS mice (Beal 1998). It has been suggested that increased generation of free radicals may damage the inner mitochondrial membrane leading to a proton leak. This would have to be compensated by increased complex I activity to maintain the proton gradient over the inner membrane and thus, maintain ATP generation by ATP synthase (Beal 1998). CK inactivation by free radicals, which may be the reason for decreased CK activity in FALS spinal cord shown in this study, could additionally contribute to impaired ATP production and therefore be an additional reason for increased complex I activities.

However, we found the oxygen consumption rate in mitochondria isolated from spinal cord of G93A FALS mice was unchanged from age-matched control animals (Fig. 6.1). As it is known that complex I activity in FALS patients as well as in G93A FALS mice is increased (Bowling et al. 1993), we measured mitochondrial oxygen consumption mediated by succinate only, the substrate of complex II. Apparently, succinate-stimulated mitochondrial respiration is not irreversibly affected in spinal cord of G93A FALS mice and therefore no difference in respiration rates could be detected in our experimental setup. We cannot, however, exclude a reversible inhibition of mitochondrial respiration under in vivo conditions. Interestingly, feeding control mice with Cr lead to an increase of mainly state 3 respiration, which was not seen in G93A FALS mice after they were fed with Cr.

There is strong evidence that neurodegeneration in ALS is at least partly mediated by oxidative stress. It is also well known that CK, cytosolic as well as mitochondrial isoforms, are exquisitely susceptible to oxidative damage, especially when exposed to ONOO⁻ (Konorev et al. 1998; Stachowiak et al. 1998a). Here we show that CK activity was clearly decreased in FALS spinal cord at a time point when mitochondrial respiration chain was not yet affected. This CK inactivation is related to the duration of the disease since presymptomatic 80 days old mice did not show decreased CK activity levels. If neurodegeneration is indeed highly dependent on oxidative damage then the results in this study would confirm our hypothesis
that CK is a prime target of oxygen radical species, particularly of ONOO\(^-\) (Stachowiak et al. 1998a).

To rule out that the decreased CK activity is due to decreased protein levels, which could be a consequence of the motor neuron loss in FALS animals, we performed Western blot analysis which showed similar expression levels of MtCK in mitochondrial fractions of wild type and FALS animals. Together with the unchanged respiration rates in isolated mitochondria, these results suggest a radical-induced inactivation of CK rather than a decreased expression level or changes in the amount of mitochondria present in the cells.

It was earlier shown that feeding G93A FALS mice with 2\% Cr in the diet protected against motor neuron loss and increased life span with 15-18\% (Klivenyi et al. 1999). In the present study supplementation with 2\% Cr non-significantly increased the CK activity in spinal cord of G93A FALS mice with 12-13\% in homogenates and mitochondrial fractions, respectively. This increase is within the range of the effect of Cr on survival, but due to the variations of CK levels between animals or different preparations, it did not reach significance.

Support for a protective effect of Cr supplementation on the CK activity level comes from the present in vitro experiments, which also provide a possible mechanism of action. An increased level of cellular Cr can lead to higher saturation and/or substrate turnover of the enzyme and therefore, binding of substrates to CK at a given time point seems statistically more probable. If both substrates are bound to CK, the active site cysteine is no longer accessible to radical damage and thus protected (Stachowiak et al. 1998a). This could be directly shown with isolated mitochondria where, with increasing concentrations of ATP, the inactivation of MtCK upon ONOO\(^-\) administration could be reduced. The presence of 20mM Cr additionally improved the protective effect on MtCK seen with 1mM ATP alone, from 25\% with ATP to almost 50\% with ATP and Cr of control activity (see Fig. 6.4). We therefore propose that Cr supplementation of G93A FALS mice contributes to the observed neuroprotective effects of Cr by protecting MtCK, as well as cytosolic CK, from inactivation by oxygen radicals.

In addition, improved PCr/ATP ratios in brain, reached by Cr supplementation (Dechent et al. 1999), improves cellular energetics, especially for Ca\(^{2+}\)-handling (Wallimann 1999), which is disturbed in neurodegenerative diseases where one observes chronic Ca\(^{2+}\)-overload (Mattson 1992). Elevated total Cr levels stimulate mitochondrial respiration (Kay et al. 2000), which may be a reason for the maintenance of ATP levels in cells with chronic Cr (Sullivan et al. 2000). Finally, Cr together with MtCK prevents the opening of the mitochondrial permeability transition pore (O'Gorman et al. 1997), with MtCK representing a key component of the permeability transition pore multi-enzyme complex (Beutner et al. 1998). These in vitro results have recently been confirmed in an in vivo model of traumatic brain injury, where Cr
supplementation showed a marked neuroprotective effect in the brain. This was due to Cr-induced maintenance of cellular ATP levels and inhibition of mitochondrial permeability transition resulting in increased mitochondrial membrane potential and decreased intramitochondrial levels of calcium and ROS (Sullivan et al. 2000).

In conclusion, the present results show a decrease in CK activity in G93A FALS mice. Furthermore, Cr supplementation attenuated the decrease in CK activity, and Cr together with their co-substrates ATP directly protected CK isoenzymes from inactivation by oxidative damage in vitro. This suggests that both impaired energy production and oxidative damage contribute to the development of ALS, and that Cr might exert its neuroprotective effect on both of these mechanisms by enhancing cellular energetics and protecting CK isoenzymes from radical-induced inactivation, especially by ONOO⁻.
6.4 MATERIALS AND METHODS

*Mice* - Transgenic male mice with the G93A human SOD1 (G1H+/+) mutation (B6SJL-TgN (SOD1-G93A)1 Gur; Jackson Laboratories, Bar Harbor, ME, USA) were bred with female B6SJL mice (Jackson Laboratories, Bar Harbor, ME, USA). The F1 generations were genotyped with polymerase chain reaction (PCR) on tail DNA and used in the experiments. All animal experiments were carried out in accordance with the NIH Guide lines for the Care and Use of Laboratory Animals and were approved by the local animal care committee.

*Treatment/Protocol* - Creatine (Avicena Group, Cambridge, MA, USA) was mixed into the mouse food (Purina Test Diet, Richmond, IN, USA) at 2% (w/w) concentration. Treatment started at four weeks of age. At 84 or 120 days of age, 5-7 animals in each group were decapitated, the spinal cord and brain were rapidly removed by two persons simultaneously, and the cortex was immediately dissected on a chilled surface. The tissue was placed in special preservation medium (mixture of 1 unit B27 and 49 units Hibernate A; Life Technologies, USA) for transport purposes and kept cold for 24h until it was processed for biochemical assays.

*Isolation of mitochondria from mouse brain* - Mitochondria were isolated from spinal cord and cortex by differential centrifugation according to a protocol described earlier (Rehnerona et al. 1979). Briefly, brain sections form 5-8 mice were washed with buffer A (225 mM mannitol, 75 mM sucrose, 10 mM HEPES, 1 mM EGTA, pH 7.1) and incubated 2.5 ml of buffer C (0.6 mg/ml subtilisin and 3 mg/ml BSA, dissolved in buffer A) for 1 minute on ice. Then, 7.5 ml of buffer B (1 mg/ml fatty acid-free BSA in buffer A) was added and the brains sections were homogenized with a glass/teflon potter. The resulting homogenate was centrifuged for 3 min at 2500g and the supernatant was centrifuged for 8 minutes at 12000g, resulting in a crude mitochondrial fraction, which was further purified by ultracentrifugation (35 min at 100 000g) on a 20% Percoll gradient. Percoll was removed from the purified mitochondrial fractions by washing twice with buffer A.

*Isolation of mitochondria from bovine heart* - Intact mitochondria were isolated from bovine heart muscle as described (Schlegel et al. 1990) with an additional Percoll purification step. Briefly, bovine heart muscle was minced and homogenized with a Polytron homogenizer (Kinematica, Luzern, Switzerland) in 220 mM mannitol, 70 mM sucrose, 10 mM HEPES (MSH), and 0.2 mM EDTA, 0.1% fatty acid free bovine serum albumin, pH 7.4. The homogenate was centrifuged at 600 g for 5 min and the supernatant was re-centrifuged at 10'000 g for 10 min. All further steps were carried out as above.
Protein concentration - Protein contents of homogenates or mitochondria were determined according to Bradford using the commercially available Bio-Rad protein assay with BSA as a standard.

CK-Activity determination - CK activity was assayed photometrically in the reverse reaction (ATP production from PCr), using the glucose-6-phosphate-dehydrogenase/hexokinase-coupled enzyme assay as described (Eder et al. 2000b) at room temperature (22°C). For the calculation of activity, a blank of each sample, measured without PCr, was subtracted from the determined CK activity values. Specific CK activity was expressed as IU/mg protein whereby 1 IU corresponds to 1 μmol PCr hydrolyzed per min at 22°C.

Mitochondrial respiration measurements - Mitochondrial respiration was measured at room temperature by using the two-channel high resolution respirometer (Oroboros Oxygraph, Paar KG, Graz, Austria) in 1 ml buffer A containing 5 mM MgCl₂, 10 mM NaPi and 5 μM Rotenone and a final mitochondrial protein content of 0.2 mg/ml. Succinate was added (5 mM final concentration) to initiate state 4 respiration, followed by administration of ADP (1 mM final concentration) to stimulate oxidative phosphorylation to the maximum state 3 respiration. Subsequently, atractyloside was added to a final concentration of 25 μM to check whether the mitochondria were still coupled to ATP-production.
7 Concluding Remarks

The main objective of this work was the role of the reaction of reactive oxygen species (ROS) with creatine kinase, and in particular with mitochondrial creatine kinase. To establish the importance of a ROS-induced CK-inactivation in vivo, we carried out three studies with different levels of complexity. First, we reacted purified MtCK with ONOO⁻ and could determine the modification sites on the MtCK sequence. We could show, that inactivation by ONOO⁻ is due to oxidation of the active site cysteine 278 and that destabilization of the octamer is induced by modification of a peptide located in the dimer/dimer interface, most probably by nitration of tryptophan 264.

It has been shown in a previous study from our laboratory that MtCK is also inactivated by ONOO⁻ when the latter is added to isolated mitochondria (Stachowiak et al. 1998a), which represents a situation closer to physiological conditions. However, there might be a difference between the reaction of exogenous added ONOO⁻ or endogenous formed ONOO⁻. Therefore, we tested the effects of overproduction of NO in mitochondria. NO produced in excess is believed to lead to formation of ONOO⁻, since NO inhibits mitochondrial respiration which then leads to O₂⁻ production from the respiratory chain. The recently discovered NOS present in mitochondria (mtNOS) makes this mechanism of O₂⁻ formation even more likely. If NO and O₂⁻ are present together, they form ONOO⁻ through a reaction at nearly diffusion limited rate. We showed for the first time NO formation by mitochondria isolated from heart muscle. By overstimulating mtNOS we could induce an inactivation of mitochondrial enzymes, susceptible to ONOO⁻, including MtCK. However, this inactivation was not prevented by the NOS inhibitor L-NMMA. Since MtCK is not only inactivated by ONOO⁻ but also by other ROS like O₂⁻ the reduction in activity could also be due to reaction with other ROS. In addition, although aconitase and MnSOD are inactivated as well, which makes the involvement of ONOO⁻ very likely, the last proof for the formation of ONOO⁻ in this system is lacking. The exact mechanism of MtCK inactivation under these conditions is not clear and has to be further elucidated.

ONOO⁻ is believed to be involved in neurodegenerative diseases like ALS. For the familial form of this disease an transgenic animal model exists. We could show that in these animal models cytosolic, as well as mitochondrial CK activity was significantly decrease. Since the level of protein expression is not significantly changed, this reduced activity is rather due to inactivation of the enzyme than decreased protein levels. The results in the first part of this work support the hypothesis, that CK is inactivated by the reaction of ROS, especially ONOO⁻, with the active site cysteine 278 (in MtCK) or 283 (in M-CK), which is an important residue.
for enzyme activity. The finding that Cr supplementation of the animals can keep total CK activities at a higher level suggests a new mechanism for the observed neuroprotective effect of Cr. In addition to improved cellular energetics, Cr also protects CK from ROS-induced inactivation, which would lead to an additional impairment of energy metabolism.

This work shows that ROS can inactivate CK in vitro, in isolated mitochondria and in pathophysiological situations in vivo. The in vitro observed octamer dissociation was neither observed in isolated mitochondria while overstimulating mtNOS, nor in vivo in FALS mice. However, since the octamer dissociation in vitro only starts after the inactivation reaction is complete, we expect this phenomenon only at very high ONOO⁻-levels, which are probably not reached in heart mitochondria, when mtNOS is overstimulated, because MtCK is not fully inactivated under these conditions. The same holds true for the in vivo situation in the FALS mice. Additionally, we showed that ubiquitous MtCK, which is expressed in brain, is much more stable against ONOO⁻-induced octamer dissociation, making the latter unlikely to occur in brain. In contrast, significant octamer dissociation has already been shown in heart (Soboll et al. 1999), where the sarcomeric isoform is expressed, which is much more susceptible to ONOO⁻-induced octamer dissociation.

Taken together, this thesis confirms the susceptibility of CK to ROS and specifies in detail the reaction of ONOO⁻ with specific amino acid side chains and their influence of the structure and function of the enzyme. Further evidence is provided for the involvement of ROS in vivo in neurodegenerative diseases and an additional mechanism for the neuroprotective effect of Cr is suggested.
8 References


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**Abstracts**

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