Doctoral Thesis

Heterogeneity of cytochrome P-450 biochemical and biophysical studies

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HETEROGENEITY OF CYTOCHROME P-450

BIOCHEMICAL AND BIOPHYSICAL STUDIES

A dissertation submitted to the
SWISS FEDERAL INSTITUTE OF TECHNOLOGY

for the degree of
Doctor of Natural Sciences

presented by
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Dr. E.E. Di Iorio, co-examinator

Zurich 1986
My thanks go to:

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Prof. A. Fiechter for providing his expertise in evaluating this thesis.

Ernesto Di Iorio for scientific support and excellent guidance. I deeply appreciated his friendship, his moral support and the lively discussions which contributed to the excellent atmosphere in his laboratory.

Christoph Richter for his technical advice, for the very stimulating and fruitful collaboration.

Ueli Marti for carrying out the immunological part of this work.

Peter Wild for his help in all matters concerning the animals.
The first principle is that you must not fool yourself, and you are the easiest person to fool. So you have to be very careful about that. After you have not fooled yourself, it is easy not to fool other scientists. You just have to be honest in a conventional way after that.

Richard P. Feynman
PREFACE

During evolution living organisms had to adapt their metabolism to toxic agents. The industrial revolution polluting in the biosphere at an increasing pace made the problem more acute. In addition to environmental problems man has developed a tendency to ingest substances for purposes other than simple sustenance, which tax the physiological mechanisms of disposal.

The ability of organisms to endure these substances relies on the machinery of biotransformation which resides mostly in the cellular endoplasmic reticulum. These remarkable mechanisms, many of which involve the cytochrome P-450 monooxygenase system not only detoxify, but also activate and solubilize a wide variety of drugs and physiological substances.

Many reactions of biotransformation increase their activity to meet demand. After a chronic exposure to e.g. polycyclic hydrocarbons, alcohol or barbiturates, an increased hydroxylating capacity can be observed in several tissues. This phenomenon is called "induction". Groups of more or less specific liver cytochrome P-450 isozymes can be induced by injecting animals with different drugs (1-3).
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<th>Description</th>
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<tr>
<td>P-450&lt;sub&gt;b&lt;/sub&gt;</td>
<td>Major form of rat liver phenobarbital-inducible cytochrome P-450 isolated after the method described by Ryan et al. (102), corresponding to P-450PB-B and P-450&lt;sub&gt;PB-4&lt;/sub&gt; using the nomenclature of Guengerich et al. (51) or Waxmann and Walsh (103), respectively</td>
</tr>
<tr>
<td>P-450&lt;sub&gt;e&lt;/sub&gt;</td>
<td>Minor form of rat liver phenobarbital-inducible cytochrome P-450 isolated after the method of Ryan et al. (102), corresponding to P-450&lt;sub&gt;PB-5&lt;/sub&gt; of Waxmann and Walsh (103)</td>
</tr>
<tr>
<td>P-450&lt;sub&gt;C&lt;/sub&gt;</td>
<td>Major form of rat liver 3-methylcholanthrene-inducible cytochrome P-450 isolated after the method of Ryan et al. (102), corresponding to P-450 NF-B of Guengerich et al. (51)</td>
</tr>
<tr>
<td>CHAPS</td>
<td>3-(3-cholamidopropyl)-dimethylamino-1-propanesulfonate</td>
</tr>
<tr>
<td>IEF</td>
<td>Isoelectric focusing</td>
</tr>
<tr>
<td>HPLC</td>
<td>High performance liquid chromatography</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulfate</td>
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</table>
1. INTRODUCTION

1.1 Cytochrome P-450

In 1956 Garfinkel reported that although 50-90% of the cytochrome b₅ could be solubilized from microsomes, the sedimented microsomal pellet still contained much of the red colour of the original microsomes (4). Two years later Klingenberg reported for the first time on a carbon monoxide-binding pigment in the microsomes and published its light absorption spectra (5). They were obtained upon addition of carbon monoxide to microsomes, which were reduced either with dithionite or NADH. Their main characteristic was a maximum at 450 nm. Due to this unusual Soret-band the carbon monoxide-binding pigment was called "cytochrome P-450".

1.1.1 Physico-Chemical Properties

1.1.1.1. Spectral Properties

A number of different cytochrome P-450s isolated from all kinds of tissues and many organisms have been characterized. Their absorption spectra are all alike in the macroscopic sense but show significant differences upon
detailed comparison (tab. 1)

Electronic absorption properties of the oxidized, reduced and reduced carbon monoxide-bound P-450 form are given in tab. 1 and fig. 1. The oxidized form has prominent α-, β- and Soret-bands, which are in agreement with those of other low-spin ferric proto hemeproteins. In contrast to other heme-proteins, where the unliganded form has a Soret band blue shifted compared to the CO derivative, in P-450 a) the absolute position of the Soret band of its carbonyl form is very unusual and b) it is red shifted compared to that of the ferrous unligated derivative (tab. 1). Under various conditions, e.g. in the presence of deoxycholate or urea, at extreme pHs or at temperatures above 20°C the Soret maximum of the P-450-CO complex shifts to about 420 nm (6). This position is comparable with that of other heme-proteins (7).

Isocyanides, which are well known ligands for hemoglobin and myoglobin (7) also bind to reduced cytochrome P-450. The spectrum for the ethylisocyanide-complex with reduced cytochrome P-450 is given in fig. 1. The maximum in the Soret-region at 455 nm is again unusual for hemeproteins. In analogy with the reduced carbon monoxide-complex a conversion to P-420 can be obtained also with ethylisocyanide. This is accompanied by a blue shift of the Soret-maximum to 433 nm (9). Once more this wavelength
compares well to that of other ethylisocyanide bound heme proteins (7).

Tab. 1: Absorption Maxima for different P-450 isozymes in the ferric, ferrous and ferrous carbon monoxide-bound form. For comparison the maxima for sperm whale myoglobin (SW Mb) are also listed. P-450b and P-450 are phenobarbital-inducible, P-450 3-methylcholanthrene-inducible forms of rat liver microsomes. P-450 is isolated from Saccharomyces cerevisiae (8). P-450cam is isolated from Pseudomonas putida grown on camphor as the only carbon source.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oxid. State</th>
<th>Ligand</th>
<th>Soret</th>
<th>(\beta)</th>
<th>(\alpha)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450b</td>
<td>Ferric</td>
<td></td>
<td>417nm</td>
<td>533nm</td>
<td>567nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous</td>
<td></td>
<td>419nm</td>
<td>--</td>
<td>540nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous CO</td>
<td></td>
<td>450nm</td>
<td>--</td>
<td>548nm</td>
</tr>
<tr>
<td>P-450e</td>
<td>Ferric</td>
<td></td>
<td>415nm</td>
<td>536nm</td>
<td>570nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous</td>
<td></td>
<td>413nm</td>
<td>--</td>
<td>547nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous CO</td>
<td></td>
<td>450nm</td>
<td>--</td>
<td>554nm</td>
</tr>
<tr>
<td>P-450c</td>
<td>Ferric</td>
<td></td>
<td>417nm</td>
<td>533nm</td>
<td>567nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous</td>
<td></td>
<td>409nm</td>
<td>--</td>
<td>540nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous CO</td>
<td></td>
<td>447nm</td>
<td>--</td>
<td>546nm</td>
</tr>
<tr>
<td>P-450yeast</td>
<td>Ferric</td>
<td></td>
<td>418nm</td>
<td>540nm</td>
<td>575nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous</td>
<td></td>
<td>412nm</td>
<td>--</td>
<td>550nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous CO</td>
<td></td>
<td>447nm</td>
<td>--</td>
<td>555nm</td>
</tr>
<tr>
<td>P-450cam</td>
<td>Ferric</td>
<td></td>
<td>392nm</td>
<td>--</td>
<td>643nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous</td>
<td></td>
<td>409nm</td>
<td>--</td>
<td>545nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous CO</td>
<td></td>
<td>446nm</td>
<td>--</td>
<td>552nm</td>
</tr>
<tr>
<td>SW Mb</td>
<td>Ferric (pH = 11.5)</td>
<td></td>
<td>414nm</td>
<td>542nm</td>
<td>582nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous</td>
<td></td>
<td>434nm</td>
<td>--</td>
<td>556nm</td>
</tr>
<tr>
<td></td>
<td>Ferrous CO</td>
<td></td>
<td>423nm</td>
<td>542nm</td>
<td>579nm</td>
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</tbody>
</table>
While the light absorption spectra of cytochrome P-450 are difficult to interpret, the electron paramagnetic resonance (EPR) properties of this cytochrome are typical of a low-spin Fe$^{3+}$ b-type cytochrome. For microsomal suspensions Mason et al. (10) found a triplet with g-values of 1.92, 2.25 and 2.42, which they called "microsomal Fe$_x$" and which coincide with values obtained for low-spin denatured derivatives of ferric hemoglobin in which one of the axial ligand is a cysteine (11). Under anaerobic conditions, after enzymatic reduction of the microsomal suspension in the presence of carbon monoxide, the EPR signal decreases. Since ferrous heme has six 3d electrons and the carbon monoxide complex is low spin, this observation shows that "microsomal Fe$_x$" is indeed cytochrome P-450.

Fig. 1: Absolute Electronic Spectra of microsomal P-450 in 0.1 M K-phosphate buffer containing 50% glycerol (34).

- oxidized
- dithionite reduced
- reduced CO-bound
- reduced ethylisocyanide-bound

Absorbance

Wavelength (nm)
1.1.1.2. The Prosthetic Group

The light absorption spectra provide no clue to the nature of the carbon monoxide-binding pigment in the microsomes. In 1964 Omura and Sato (12,13) provided evidence that the pigment is an atypical hemeprotein. They were able to separate cytochrome b\textsubscript{5} and the carbon monoxide-binding pigment from each other by digestion of microsomes with steapsin. They determined, from the spectrum of alkaline pyridine hemochromogen, protoheme as the only heme present in the carbon monoxide-binding pigment. Yu and Gunsalus were able to reconstitute the spectral properties of cytochrome P-450 by the addition of equimolar amounts of ferriprotoporphyrin IX to bacterial apocytochrome (isolated from Pseudomonas putida) (13). Thus, the prosthetic group of cytochrome P-450 is the same as in hemoglobin: ferriprotoporphyrin IX. The unusual Soret-maxima of the reduced carbon monoxide- and ethyl-isocyanide-complexes can therefore not be attributed to the prosthetic group.

1.1.1.3. Axial Ligands

Based on ESR data Mason et al. (15) proposed in 1965 a sulfur atom as proximal ligand of the heme-iron in cytochrome P-450. Dawson et al. (16) concluded from magnetic circular dichroic spectra of the various oxidation
states of P-450 by comparison with appropriate model compounds, thiolate likely to be the fifth ligand in P-450, but not in P-420. For P-420, which spectroscopically behaves as described above like all other hemeproteins, a nitrogen atom provided by an imidazole is proposed as the most probable ligand.

Many groups since confirmed these findings by differing experimental approaches (17-19). Model peptides with the heme-iron linked via a cysteine confirmed that the unusual absorption spectra of P-450 are due to the sulfur atom ligated to the heme on the proximal side (20-22).

1.1.1.4. Molecular Weight

Tab. 2: Molecular Weights of different cytochromes P-450 (23,25).

PB = phenobarbital, MC = 3-methylcholanthrene

<table>
<thead>
<tr>
<th>source</th>
<th>molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>rat liver, PB-treated</td>
<td>P-450 \textsuperscript{b} 48,000</td>
</tr>
<tr>
<td>rat liver, PB treated</td>
<td>P-450 \textsuperscript{e} 49,000</td>
</tr>
<tr>
<td>rat liver, MC treated</td>
<td>P-450 \textsuperscript{c} 56,000</td>
</tr>
<tr>
<td>rat liver, MC treated</td>
<td>P-450 \textsuperscript{d} 51,000</td>
</tr>
<tr>
<td>yeast</td>
<td>P-450 \textsuperscript{y} 53,000-58,000</td>
</tr>
<tr>
<td>bacteria</td>
<td>P-450 \textsuperscript{cam} 46,000</td>
</tr>
</tbody>
</table>
The molecular weight of cytochrome P-450 was determined by SDS gel electrophoresis. For the various proteins isolated from different species and different tissues molecular weights between 43 kDa and 58 kDa were obtained (tab. 2). In aqueous solutions free of detergents cytochrome P-450 aggregates to form hexamers (24). The non-denaturing zwitterionic detergent CHAPS is able to monomerize certain cytochrome P-450s as shown by gel filtration experiments (25). Therefore, experiments in which the aggregation phenomenon could represent a complication must be performed in the presence of 1% CHAPS (25,26).

1.1.1.5. Amino Acid Composition and Topology of P-450

Microsomal cytochrome P-450s are integral membrane proteins. Thus the relatively high content of hydrophobic amino acids found in their sequence is not surprising (27). Since chromatographic separation of hydrophobic polypeptides is not an easy task, direct sequence determination of cytochrome P-450 is difficult. Most of the sequences known today are determined via cDNA (28-30). Based on the sequence of rabbit cytochrome P-450, Heinemann and Ozols (27) proposed a model for the topology of the protein in the membrane. Eight hydrophobic polypeptide segments are postulated to traverse the nonpolar portion of
the membrane in a helical conformation (fig.2).

Fig. 2: Suggested Topology of Microsomal Cytochrome P-450 in the Membranes. Most of the protein is embedded in the nonpolar region of the membrane. A large segment near the COOH-terminus is exposed on the membrane surface (27).
Comparisons of sequences of different P-450 revealed two homologous regions, one near the amino- one near the carboxyl-end of the polypeptide chain, each containing a cysteiny1 residue. They are therefore candidates as heme-binding site. Black and Coon (32) selectively modified the cysteine near the amino-end without loss of the native ferrous carbonyl spectrum. Thus, the cysteine residue near the carboxyl-end, which is in a hydrophylic region and not embedded into the lipid bilayer, was identified as the proximal ligand for the heme. The opposite conclusion was reached by Taniguchi et al. (33) who examined the binding of the hydrophobic substrate benzphetamine to liposome-bound P-450 at various temperatures. They found, that the substrate-binding site of P-450 directly faces the membrane lipid phase and that P-450 binds substrates dissolved in the membrane as shown in fig. 3.

Fig. 3: Substrate-binding Site
Proposed scheme describing the substrate binding reactions for hydrophobic substrates assuming that the substrate binding site faces the membrane (33).

\[ \text{Bph} \] = benzphetamine

\[ \text{Bph} \]
Kunz et al. (34) measured fluorescence lifetime of DPH (1,6-diphenyl-1,3,5,-hexatriene) in unilamellar vesicles containing cytochrome P-450. Their results indicate an average distance of about 60 Å between the heme and the DPH. This distance is consistent with the results of Black and Coon (32) and with the model proposed by Heinemann and Ozols (fig. 2), suggesting as heme binding site the hydrophilic part near the carboxyl end of the polypeptide chain which is also facing the cytoplasmic side of the microsomal membrane.

Since during the catalyzed reaction (see fig. 4) one oxygen atom from the heme-bound oxygen molecule must be transferred to the substrate, one could speculate that the bound substrate must lie in the proximity of the heme. This concept is supported by inactivation of cytochrome P-450 by suicide substrates (35). During the reaction, suicide substrates covalently bind to the P-450 heme and thus lead to heme-loss and concomitant loss of activity.

Studies concerning the structure and the topology of cytochrome P-450 provide so far controversial results and more work is needed to reach final conclusions.
1.1.2. Distribution and Function

1.1.2.1. Distribution

Cytochrome P-450 was first found in mammalian liver microsomes and was thought to be a curiosity occurring only in restricted biological systems (4,5). Subsequent studies, however, revealed that proteins with the same unique spectral properties are found in all forms of living organisms. In vertebrates various amounts of different cytochrome P-450s are found in the endoplasmic reticulum (microsomes) of liver, lung, kidney, small intestine, adrenal cortex, skin, testes, placenta and several other tissues. Mitochondria of mammalian glands such as the adrenal cortex, testes and corpus luteum contain P-450s which are responsible for steroid hormone synthesis (36,37). The available studies on the distribution of cytochrome P-450s in invertebrates are not extensive. However, studying insecticide metabolism, it has been shown that microsomes isolated from Drosophila melanogaster contain relatively large amounts of P-450 (38). In yeast P-450 is a component of a monooxygenase system involved in the ergosterol biosynthesis and hydrocarbon oxidation (23,39).

In all these cases the enzyme was found in the microsomal
fraction (36).

The heme protein is even found in bacteria. In these primitive organisms the enzyme is a soluble protein in the cytoplasm. Because of its easy access and its stability the cytochrome P-450_{cam} isolated from Pseudomonas putida, grown on camphor as the only carbon source, is the best characterized P-450 so far (8,40).

1.1.2.2. Function of P-450

Cytochrome P-450 is a major component of the microsomal polysubstrate monooxygenase system. A typical reaction, in which cytochrome P-450 serves as the oxygenating catalyst in the presence of one or more electron transfer proteins, is shown in the following equation:

\[
\text{RH} + \text{O}_2 + \text{NAD(P)H} + \text{H}^+ \rightarrow \text{ROH} + \text{H}_2\text{O} + \text{NAD(P)}^+
\]

where \(\text{RH}\) represents the substrate (41).

1.1.2.2.1. Components of the Monooxygenase System

In liver microsomes cytochrome P-450 reductase, a flavoprotein containing both FMN and FAD, catalyzes the transfer of electrons from reduced pyridine nucleotides to
the cytochrome. For certain types of P-450 and certain catalyzed reactions cytochrome b₅ possibly also supplies electrons (42-46). The role of b₅ in the catalytic cycle of the cytochrome P-450 activity is presently not understood. In Pseudomonas putida and in adrenal mitochondria an iron-sulfur protein, putidaredoxin and adrenodoxin respectively, serves as an intermediate between the FAD containing reductase and the cytochrome (47,48). An additional component required for full activity in the reconstituted liver microsomal system is phosphatidylcholine (49).

1.1.2.2.2. Substrates

Some P-450 isozymes participate in important metabolic transformations of lipids and also catalyze the alteration of xenobiotics in ways that usually lead to detoxification. In some instances they yield products with greater cytotoxic, mutagenic or cancerogenic properties (41). Usually, enzymes are named according to their function. This approach is not possible for cytochrome P-450 since the different types of P-450 catalyze many kinds of reactions including hydroxylation, N-oxidation, epoxidation, dealkylation, peroxidation, deamination, etc. For some P-450s a specific physiological substrate is not
yet known and with the commonly used "in vitro substrates" one finds an overlapping substrate specificity (50,51).

Fig. 4: Reaction Scheme.
Proposed scheme for the mechanism of action of cytochrome P-450 in hydroxylation reactions.
RH represents a substrate and ROH the corresponding product (41).
1.1.2.2.3. Reaction Scheme

Fig. 4 shows a scheme for the proposed reaction of cytochrome P-450.

A substrate, indicated with RH binds to the oxidized cytochrome. This substrate binding is accompanied by a low to high spin transition of the heme-iron (52). In a second step the enzyme-substrate complex is reduced, and subsequently binding of molecular oxygen to this complex takes place (41).

The following reaction steps and intermediates are presently only hypothetical, but some evidence for the proposed sequence is available (53-55,35). It is supposed that a second electron is being supplied to the iron-oxygen center leading to the splitting of the oxygen-oxygen bond. One oxygen atom combines with two protons to form water. The other oxygen atom, presumably now an "activated oxygen", is inserted into a carbon-hydrogen bond of the substrate to produce the corresponding alcohol, which is then released with regeneration of the ferric state of the enzyme and completion of the catalytic cycle (41).

The above reaction scheme suggests, that cytochrome P-450 must have at the same time properties of an electron transferring enzyme as other cytochromes (steps 2,4), and of a reversible ligand binding one (steps 3,7) such as hemoglobin and myoglobin.
1.1.3. The Hepatic Microsomal Monooxogenase System

1.1.3.1. Microsomes

Electron micrographs of the rat hepatocyte show the endoplasmic reticulum (ER) to be present throughout the cytoplasm as an extensive network of tubules, vesicles and lamellae (57,60). 60% of the surface of this organelle is covered with ribosomes and is called rough ER. The remaining area is called smooth ER. 19% of the total protein, 48% of the total phospholipid and 58% of the total RNA of the rat hepatocyte are associated with the ER (58). ER from rat hepatocyte can be isolated as microsomes by fractionated centrifugation of liver homogenate. Microsomes are vesicles of ER, which remain in the supernatant after centrifugation at 10'000 g but can be spun down at 100'000 g.

1.1.3.2. NADPH Cytochrome P-450 Reductase

The monooxygenase system of the liver consists of two major proteins: cytochrome P-450 and NADPH cytochrome P-450 reductase. Cytochrome P-450 is an integral membrane protein with a molecular weight of about 50 kDa (see 1.1.1.4.). Cytochrome P-450 reductase has a molecular weight of about
79 kDa and consists of a small hydrophobic tail of 6-10 kDa, embedded into the membrane, and a large hydrophilic part protruding from the cytoplasmic surface of the membrane. Each molecule of reductase contains one molecule of FAD and one of FMN. In microsomes one reductase molecule is present per 20-30 molecules of P-450. With rotational diffusion measurements Gut et al. (59,60) investigated the interactions of P-450 with its reductase in phospholipid vesicles with phospholipid compositions similar to that of the microsomal membrane. Although the situation in microsomes is expected to be more complex than in the model membranes used for their experiments, their results allowed the following conclusions concerning the interaction of P-450 with its reductase: a) Microsomal P-450 and reductase can form 1:1 heterodimeric complex. b) Excess cytochrome P-450 exists in a mixture of monomers and small aggregates. c) Electron transfer takes place in a mixture of monomolecular 1:1 complex and by collision with mobile cytochrome P-450.
1.1.3.3. Cytochrome $b_5$

Microsomal P-450 possibly also interacts with cytochrome $b_5$ and its reductase. The role of $b_5$ in P-450 dependent reactions is presently not fully understood. Some authors propose that the second electron in the reaction cycle (step 4 in fig. 4) is provided by NADH via $b_5$ (61). More recent reports about specific P-450 activities suggest that the addition of cytochrome $b_5$ to the NADPH dependent monooxygenase system results in some cases in stimulation, in other cases in inhibition of the specific activities, depending on the particular P-450 and substrate tested (42). A plausible explanation for this observation is that $b_5$ may induce structural changes in P-450 leading to more or less active configurations.

1.1.3.4. Induction

The initial observation of induction of the monooxygenase system was provided 1951 by Richardson and Cunningham (62). They found that after feeding rats an aminoazo dye the animals developed hepatomas. The carcinogenesis was considerably suppressed when small amounts of 3-methylcholanthrene were instilled intravaginally. In 1956 Conney et al. (63) suggested that 3-methylcholanthrene administration enhances the metabolism of aminoazo dyes by
increasing the amount of intracellular N-demethylase and azo dye reductase rather than reducing the level of an activating system. The N-demethylation and azo bond reduction reactions are important steps in the detoxification of the aminoazo dye.

A few years later, while studying the problem of tolerance to barbiturates in animal model systems, Remmer (64) became impressed by the marked alterations in both the morphology of the liver and the activity of the hepatic drug-metabolizing enzyme system.

Today, phenobarbital and 3-methylcholanthrene are used as "classical" inducers. Both cause a selective enhancement of specific cytochrome P-450s. It could be shown that after the treatment of rats with phenobarbital the amount of a specific P-450 increases by a factor of up to fifty (51). With other inducers such as 3-methylcholanthrene or β-naphtoflavone significant but smaller induction can be observed (51). Upon treatment with phenobarbital the NADPH cytochrome P-450 reductase is also induced.

Enhancement of protein synthesis, proliferation of coding mRNA and decreased protein degradation are all responsible for the elevated P-450 level after treatment with an inducer (1).
1.1.3.5. Metabolic Activities of Hepatic P-450

Many hydroxylation reactions are catalyzed by P-450 dependent monooxygenase systems. In mammalian liver cytochrome P-450 participates in the biosynthesis of cholesterol (65). Several important steps in the conversion of cholesterol to bile acids (e.g. cholesterol 7α-hydroxylation) are also catalyzed by cytochrome P-450 containing monooxygenase systems (36).

Vitamin D₃ in its normal state is physiologically not active. It has to be metabolically activated. This activation involves 25-hydroxylation in liver microsomes followed by 1-hydroxylation in kidney mitochondria. Both processes are P-450 dependent (36).

Medium and long chain saturated fatty acids are excreted as dicarboxylic acids. In 1962 Lu and Coon (66) reported the successful isolation of a hepatic cytochrome P-450 monooxygenase system that catalyzed the ω-hydroxylation of laurate. Prostaglandin ω-hydroxylation by a hepatic P-450 dependent monooxygenase has also been reported (67).
1.1.3.6. Cytochrome P-450 Multiplicity

Even if we consider the hepatic cytochrome P-450 dependent monooxygenase system the variety of substrates which can be metabolized is remarkable. The existence of many different P-450 isozymes is therefore not surprising. Recently, using monoclonal antibodies, different P-450 isozymes could be detected (50). However, considering the great degree of homology between the isozymes, antibodies may not be an adequate tool to clearly distinguish between them. Bansal et al. (68) used the high resolution properties of HPLC to demonstrate that various P-450 isozymes are present in microsomes.

In view of the development of increasingly sophisticated techniques for protein separation it is expected that many more different P-450s will eventually be found.
1.2. Kinetics

1.2.1. Pre-steady-state Kinetics

In principle, two types of approaches can be used for kinetic investigations (69):

a) Steady-state kinetic: When an enzyme is mixed with a large excess of substrate after an initial period, during which the concentrations of intermediates increase rapidly, a steady state situation is reached where such intermediate levels remain practically constant for quite some time. It is during this steady-state period that enzymatic activities are traditionally measured.

b) Pre-steady-state measurements provide information about the number and the nature of reaction steps and therefore also about the mechanism of the studied reaction.

If the spectroscopic properties of the educts and products are different changes can be recorded continuously, and quantitative kinetic analysis can be made.

Pre-steady-state reactions may be classified according to their reaction order:

In zero-order reactions the velocity of the reaction is independent on the concentration of the reactants.

First-order reactions are those which proceed at a rate proportional to the concentration of one reactant.
Second-order reactions are those in which the reaction-rate is proportional to the product of the concentrations of the two reactants or to the second power of a single reactant (70).

A summary of the equations used for the analysis of the various types of reactions is shown in tab. 3.

Tab. 3: Kinetics
Equations for the different reaction orders.

<table>
<thead>
<tr>
<th>reaction order</th>
<th>differential-equation</th>
<th>integrated equation</th>
</tr>
</thead>
<tbody>
<tr>
<td>0. A → B</td>
<td>(-\frac{dA}{dt} = k)</td>
<td>(k = \frac{1}{t} (A-A_o))</td>
</tr>
<tr>
<td>1. A → B</td>
<td>(-\frac{dA}{dt} = kA)</td>
<td>(k = \frac{1}{t} \ln \frac{A}{A_o})</td>
</tr>
<tr>
<td>2. A + B → C</td>
<td>(-\frac{dA}{dt} = kA B)</td>
<td>(k = \frac{1}{t} (\ln \frac{A B}{A_o B_o})/(A_o - B_o))</td>
</tr>
<tr>
<td>2A → C</td>
<td>(-\frac{dA}{dt} = kA^2)</td>
<td>(k = \frac{1}{t} (\frac{1}{A} - \frac{1}{A_o}))</td>
</tr>
</tbody>
</table>
As can be seen, the reaction-rates \( k \) for zero- or first-order reactions can easily be determined, since \( k \) is proportional to \( \frac{1}{t(A-A_o)} \) and \( \frac{1}{t(ln \ A_o/A)} \), respectively. For second-order reactions the analysis is more complex. If one reactant is in large excess over the other, a second-order reaction can be treated as first-order (so called pseudo-first-order reaction). The concentration of the reactant in excess is considered to remain constant with time and is inglobated in the reaction rate \( k \).

Measurements of pre-steady-state kinetics can be performed by any one of the following approaches:

a) rapid mixing methods: e.g. continuous-flow, stopped-flow. The reaction is detected after complete mixing of the reactants. The dead time of the instrument depends on the time needed for complete mixing and lies in the milliseconds range.

b) perturbation methods: e.g. T-jump, p-jump. Using these methods a system is perturbed from its equilibrium, and its rate of relaxation to a new equilibrium is measured. The dead time depends on the time required to alter the physical parameter (T,p) which influences the reaction equilibrium and can therefore be quite short (typically ranging between several ns and few us).

c) flash photolysis: In this case a pulse of light is used to activate a reactant or to split the bond between two
atoms. The reaction of the "photo-activated" species can be followed after the light pulse. With the use of LASERS the dead time of flash photolysis apparatuses can be as low as a few femtoseconds (71).

1.2.2. Kinetics of Ligand Binding to Heme Proteins

<table>
<thead>
<tr>
<th>protein</th>
<th>$l_{on}$</th>
<th>$l_{off}$</th>
<th>$k_{on}$</th>
<th>$k_{off}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>DD-Hb</td>
<td>12</td>
<td>0.27</td>
<td>30</td>
<td>10</td>
</tr>
<tr>
<td>$\alpha$-human</td>
<td>0.4</td>
<td>0.013</td>
<td>5</td>
<td>28</td>
</tr>
<tr>
<td>$\beta$-human</td>
<td>0.45</td>
<td>0.008</td>
<td>6</td>
<td>16</td>
</tr>
<tr>
<td>$\beta$ ZH</td>
<td>2.5</td>
<td>7</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>CTT-ery</td>
<td>2.7</td>
<td>0.095</td>
<td>30</td>
<td>218</td>
</tr>
<tr>
<td>HRP</td>
<td>0.00034</td>
<td>0.00016</td>
<td>0.0058</td>
<td>0.007</td>
</tr>
</tbody>
</table>
Ligand-binding to hemeproteins has been studied extensively using all three methods (76-78). Characteristic spectroscopic differences between the ligated and the unligated form of the hemeproteins exist, so that the kinetics of ligand-binding can easily be detected. Tab. 4 summarizes the kinetic rate constants for the reaction of a few heme proteins with oxygen and carbon monoxide.

Information about heme pocket geometry of DD-Hb (hemoglobin of Dicrocoelium dendriticum) was obtained by comparison of the kinetic parameters for carbon monoxide- and oxygen-binding with those of other well known heme proteins. Di Iorio et al. (76,77) ascribed the fast ligand-binding velocities of DD-Hb:

a) to a large cavity on the distal side of the heme (DD-Hb has a distal glycine instead of a distal histidine as usually found in hemoglobin chains) and

b) to the position of the iron atom referred to the heme plane (ESR data of DD-Hb-NO indicated an increased distance between the N-atom of the proximal histidine and the iron). The authors correlated the decreased distance between the iron and the heme plane with a reversed heme orientation (180° rotation around an axis going through the iron and between the two propionic acid side chains) (79).

This is just one example of how valuable structural
informations can be obtained from comparative kinetic investigations.

1.2.3. Pre-Steady-State Kinetic Investigation on P-450

1.2.3.1. Reduction Kinetics

In the reaction scheme proposed in fig. 4 two steps can be investigated by pre-steady-state kinetic methods: step 2, the reduction of ferric to ferrous P-450 and step 3, ligand-binding to ferrous P-450. It is possible to investigate the reduction of ferric cytochrome P-450 by anaerobically mixing in a stopped-flow carbon monoxide-saturated solutions of reductase/P-450-complex with NADPH. The appearance of the reduced carbon monoxyde-complex of P-450 can be observed spectrophotometrically. Several groups have conducted such experiments using purified P-450 and reductase. For microsomal systems biphasic reduction kinetics were reported. The rate constants for the two parallel first-order reactions with rate constants depend on the species of P-450, the presence of substrate and the method of reconstitution of the isolated proteins (25,80-83). A satisfactory explanation of the biphasicity of the reduction for microsomal systems has
not been found. Oprian et al. (80) suggested that a complicated intra- or inter-reductase redox equilibrium is responsible for the biphasicity. This is inconsistent with the finding of Davydov and Kurganov (84) who reported biphasic reduction kinetics even when dithionite instead of the reductase is used.

For P-450 \textsubscript{cam} single exponential reduction kinetics were reported for both, the substrate-free and the camphor-bound enzyme (85).

1.2.3.2 Ligand Binding

After reduction to the ferrous form the heme is able to bind an oxygen molecule (step 3 in fig. 4). This is the first step in oxygen activation, thus, understanding the mechanism of ligand-binding to reduced cytochrome-P450 is of importance.

The ferrous dioxygen complex is stable enough to be observed with P-450 \textsubscript{cam} (86,87) and adrenal mitochondrial P-450 (88), but not with liver microsomal P-450 (89). Ferrous cytochrome P-450 also binds other small ligands, such as isocyanides or carbon monoxide, which give stable complexes.

Due to the large differences in the absorption spectra between the ferrous P-450 and its carbon monoxide
derivative around 450 nm (fig. 1) a large signal upon carbon monoxide-binding to the reduced enzyme is expected. Kinetics of carbon monoxide-binding can be studied by both stopped-flow techniques and flash photolysis. Klingenberg (5) and Omura and Sato (12) were unable to obtain photodissociation of the reduced carbon monoxide P-450 complex and could therefore not study ligand-binding to the enzyme after flash photolysis. The earliest measurements of photodissociation of dithionite reduced carbon monoxide bound P-450 were made in 1965 by Omura et al. (90) Over the last decade, several groups have investigated carbon monoxide-binding to different cytochrome P-450s. Their results are not consistent with each other. Dolphin et al. (91) reported monophasic, single exponential kinetics for P-450 with \( k_{on} \) and \( k_{off} \) of \( 3.6 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) and \( 2.8 \text{ s}^{-1} \) respectively. Tuckey and Kamin (92) studied carbon monoxide-binding to adrenal mitochondrial P-450 under various conditions. For substrate-depleted, cholesterol-bound and 20α-hydroxycholesterol-bound P-450 they found monophasic reactions with association rates around \( 2 \times 10^5 \text{ M}^{-1} \text{s}^{-1} \) and dissociation rates around \( 0.1 \text{ s}^{-1} \). No changes in the reaction rates were observed if either aqueous buffer or phosphatidylcholine vesicles were used for the investigation. When 20,22-dihydroxy-
cholesterol-bound P-450 was used, triphasic carbon monoxide-binding was observed in aqueous solutions, while measurements with phosphatidylcholine vesicles yielded monophasic kinetics.

The kinetics of combination of carbon monoxide with phenobarbital-induced ferrous cytochrome P-450 from rat liver microsomes (P-450_b), both bound to microsomial membranes and in the solubilized form, were investigated by Douzou and coworkers (93-95), Roesen and Stier (96), the group of Brunori (97) and more recently by Gray (98-100). In rapid-mixing measurements on purified P-450_b the group of Douzou reported a single bimolecular process with a rate constant of 10^6 M^{-1}s^{-1} at 20°C. Brunori found two simultaneous processes, the first having a rate constant of 7 \times 10^5 M^{-1}s^{-1} at 20°C. Gray reported a three-step binding reaction, consisting of a bimolecular process (k_1 = 3 \times 10^6 M^{-1}s^{-1} at 20°C) followed by a second one whose velocity has a hyperbolic dependence on carbon monoxide concentration and a third monomolecular one. For carbon monoxide-binding to microsomal membranes, measured by flash photolysis, Douzou found two bimolecular steps (k_1 = 2.3 \times 10^6 M^{-1}s^{-1} and k_2 = 4 \times 10^5 M^{-1}s^{-1} at 40°C). The same results were obtained with purified P-450_b (95). Roesen and Stier described a single exponential process with association and dissociation rate constants of 1.3 \times 10^6 M^{-1}s^{-1} and 0.2 s^{-1}
at 25°C respectively (96). Gray (98) reported three bimolecular processes ($k_1 = 1.2 \times 10^7 \, M^{-1}s^{-1}$; $k_2 = 2.3 \times 10^6 \, M^{-1}s^{-1}$ and $k_3 = 2.3 \times 10^5 \, M^{-1}s^{-1}$ at 20°C) and a forth, very slow, monomolecular one ($k_4 = 0.3-0.6 \, s^{-1}$) when the measurements were done by stopped-flow techniques.

Based on these contradictory findings, it is impossible to propose a reaction mechanism for the binding of ligands to ferrous cytochrome P-450.
1.3. Aims of the Present Study

During the last decade a lot of work has been done to elucidate the mechanism of P-450 action. In its catalytic cycle, reduced P-450 needs to bind a molecule of oxygen in order to activate it, and subsequently perform the chemical modification of the substrate. It is thus of fundamental relevance to clarify the mechanism of ligand binding to reduced P-450. Since the ferrous P-450 dioxygen complex is not stable with liver microsomal P-450, carbon monoxide binding to the reduced enzyme serves as a model reaction. As stated above contradicting results have been reported on the kinetics of ligand binding to liver microsomal P-450. The diversity of the results suggests that the preparations used by the different groups varied in their protein composition.

We have undertaken the present study to clarify the situation and propose a possible mechanism of general validity for the reaction of ferrous P-450 with ligands. For this purpose we have concentrated our efforts on:

a) A comparative kinetic investigation of the reaction between P-450 and carbon monoxide.

b) The purification of liver microsomal P-450 by IEF and HPLC.
2. MATERIAL AND METHODS

2.1. Biochemical Methods

2.1.1. Isolation of Different Cytochrome P-450 Isozymes

Cytochromes P-450\textsubscript{p} and P-450\textsubscript{e} were induced in rat liver microsomes by injecting male Sprague Dawley rats (150-200 gr) intraperitoneally with 1 ml phenobarbital solution (100 mg/kg body weight dissolved in 0.9% NaCl (wt/v)) on four consecutive days. Before sacrificing, the animals were starved over night.

Cytochrome P-450\textsubscript{c} was induced either by 3-methylcholanthrene (40 mg/kg body weight dissolved in 0.5 ml corn oil) 48 hours before sacrifice or by β-naphtoflavone (40 mg/kg body weight dissolved in 0.5 ml corn oil) on three consecutive days before sacrificing the animals. The drugs were injected intraperitoneally.

The animals were killed by decapitation. The livers were perfused in situ with ice cold KCl-solution (1.15% wt/v) to remove blood, minced with scissors and homogenized in the same buffer (approx. 80 ml/2 livers) with a Potter-Elvelhjem. The homogenate was spun at 4\textdegree C at 18'000 g for 20 minutes. The pellet was resuspended and the centrifugation was repeated. Microsomes were isolated as a
pellet after spinning the combined supernatants at 4°C at 105'000 g for 1 hour. After washing the microsomal pellet with 1.15% KCl (wt/v) containing 10 mM EDTA and with 0.1 M K-pyrophosphate pH 7.4 the microsomes were finally suspended in 0.25 M sucrose (101).

Freshly prepared microsomes were diluted and solubilized with sodium cholate and cytochrome P-450 precipitated with polyethylene glycol as described by West et al. (101). The precipitated proteins were dissolved in 15 mM K-phosphate pH 7.4 containing 0.5% sodium cholate, 0.2% Emulgen 911 and 20% glycerol, and applied on a Whatmann DE-52 column equilibrated with the same buffer. The proteins were eluted and fractionated by applying a linear gradient from 0-0.3 M NaCl as described by Ryan et al. (102). The various eluted fractions were analyzed on SDS-gels. The fractions containing P-450_b and P-450_e (in the case of phenobarbital-induced microsomes) or P-450_c (in the case of 3-methylcholanthrene- or β-naphtoflavone-induced microsomes) were pooled and further purified. For each fraction of the b+e peak the absorbance at 417 nm was measured (see chromatogram in fig. 6). After that, they were analysed on IEF gels, which resulted for all tested fractions of the b+e peak in five focused protein bands, whose relative amounts were determined by gel scanning. Thus, the absorbance at 417 nm of each fraction can be
divided into the different contribution of each of the individual focused bands. The obtained values were plotted against the fraction number. This plot corresponds to a chromatogram of the DE-52 b+e peak for the individual focused bands.

P-450<sub>b</sub> and P-450<sub>e</sub> were separated and purified on hydroxylapatite according to Waxmann and Walsh (103). P-450<sub>c</sub> was purified as described by Ryan et al. (102).

2.1.2. Purification of Cytochrome P-450 by Isoelectric Focusing

2.1.2.1. Analytical Agarose Gels

For an isoelectric focusing (IEF) gel of 90 x 120 x 1 mm the following chemicals were mixed to form an IEF-stock solution: 0.36 g agarose IEF (Pharmacia), 3.6 g sorbitol (Fluka), 6 ml of 87% glycerol and 26.4 ml H<sub>2</sub>O. This stock solution was brought to approximately 75°C and 10.5 ml of it were added to a mixture of 0.76 ml Ampholyte pH 5-8 (LKB), 120 mg CHAPS (Sigma) and 240 mg Triton X-100 or alternatively 240 ul of a 10% Emulgen 911 solution. The detergents were dissolved by stirring very carefully with a glass rod to avoid the formation of air bubbles. The gel was cast on a preheated glass plate and allowed to harden.
at 4°C for one hour before the focusing experiment. Up to 3 µg protein were applied with a filter-paper near the anodic side of the gel. The electrode strips were soaked in 0.05 M H₂SO₄ for the anode and in 1 M NaOH for the cathode. Prefocusing was not required and at 10°C about 3000 volts x hours, with a maximal current of 7 mA, were needed to get well focused bands. Immediately after focusing, the gels were stained with benzidin for heme. Then the proteins were fixed with 10% TCA in 33% ethanol and stained with Coomassie-blue. The relative amounts of the different bands were determined either by scanning the Coomassie-blue stained gels or by cutting out the bands immediately after running the gel and eluting the heme proteins into 0.1 M K-phosphate buffer containing 20% glycerol and determining the absorption at 417 nm.

2.1.2.2. Preparative Agarose Gels
For a preparative IEF gel of 90 x 120 x 3 mm 0.16 g agarose IEF (Pharmacia), 3.6 g sorbitol, 6 ml of 87% glycerol and 26 ml H₂O were mixed and heated in a waterbath until the solution became clear. The hot solution was added to a mixture of 2.5 ml Ampholyte pH 5-8 (LKB), 720 mg Triton X-100, or 720 µl of a 10% Emulgen 911 solution, and 360 mg CHAPS (Sigma). The gel was cast on a preheated glass plate.
The sample, approximately 1.5 mg, was applied on five layers of filterpaper which were carefully placed into a cast trough near the anodic side of the gel. Gel was removed on both sides, so that preparative, 5 mm thick electrode strips (Pharmacia) could be put into place. The electrode solutions were the same as for the analytical gels. The proteins were focused at a maximal current of 8 mA and a maximal voltage of 2000 V at 10°C until the bands were well separated. This procedure took about five hours.

Immediately after focusing, the bands were cut out and put into 5 ml 0.1 M K-phosphate buffer containing 20% glycerol. The proteins were eluted into this buffer by diffusion over night at 4°C. Detergents were removed by stirring the protein solutions together with Amberlite XAD-2 (Fluka, Switzerland) for one hour at 4°C and then concentrated on an Amicon PM-30 membrane.
2.1.3. HPLC-Analysis of Cytochrome P-450

The HPLC analysis were carried out as described by Bansal et al. (104) with some minor modifications. The samples were applied to an Anpac anion exchange column (4.1 x 250 mm) (Anspec, Warrenville Ill. USA) equilibrated with buffer A (20 mM Tris/acetate pH 7.2 containing 20% glycerol and 0.2% Emulgen 911). The column was eluted at a flow-rate of 1.2 ml/min with a linear gradient of buffer B (0.8 M Na-acetate in buffer A) incorporating 40% buffer B into buffer A within 20 minutes. Sample detection was carried out at 417 nm.

2.1.4. Purification of NADPH Cytochrome P-450 Reductase

NADPH cytochrome P-450 reductase was isolated from phenobarbital-induced rat liver microsomes by affinity chromatography on agarose-hexane-adenosine 2'5'-diphosphate (PL Biochemicals) as described by Shephard et al. (105). The isolated protein was concentrated on an Amicon PM-30 membrane and stored in liquid nitrogen.
2.1.5. Measurements of Monooxygenase Activities with Different Substrates

Activities of the various P-450s were measured in reconstituted systems obtained as follows: purified cytochrome P-450 and cytochrome P-450 reductase (protein ratio P-450 : reductase = 5:1) were added to a clear solution of 300 μM L-α-dilauroyl-3-phosphatidylcholin (obtained by sonication) and incubated for five minutes at room temperature. The different substrates and a NADPH regenerating system, consisting of 10 mM glucose-6-phosphate and 5 ul glucose-6-phosphate dehydrogenase were added. By dilution with the appropriate buffer a total volume of 1 ml was obtained. Ethoxycoumarin deethylase activities were measured in a fluorimetric assay (106). Activities against aminopyrin were measured by determining the formation of formaldehyde by the method of Nash (107). p-Nitroanisol O-demethylation was measured in a dual wavelength spectrophotometer using 417 and 480 nm as measuring and reference wavelength to follow the formation of p-nitrophenol (108). For the measurements of activities against benzo(a)pyrene \(^3\)H-labelled substrate was used. The ratios of radioactivity found in a water phase (metabolized substrate) to the radioactivity found in a hexane phase (unreacted substrate) at different time points was used to calculate specific
activities (109).

2.1.6. Inactivation by Suicide Substrates

A reconstituted system consisting of the appropriate cytochrome P-450 (1 uM), reductase (1 uM) and L-α-dilauroyl-3-phosphatidylcholin (30 uM) was incubated with 5 μl 1-octin for 2 minutes at 37°C in the presence of a NADPH regenerating system (glucose-6-phosphate and glucose-6-phosphate dehydrogenase). The total volume was 3 ml and the buffer was 0.1 M K-phosphate pH 7.4 containing 150 mM KCl and 1.5 mM EDTA.

The reaction was started by the addition of 15 μl 200 mM NADPH. The P-450 contents, still active and not modified by 1-octin, were determined spectroscopically after 0, 10 and 30 minutes (110,111).

2.1.7. Other Methods

Protein concentrations were determined according to Lowry (112). Bovine serum albumine was used as a standard.

Cytochrome P-450 concentrations were determined from the carbon monoxide-reduced difference spectra according to Omura and Sato (12).

SDS gel-electrophoresis on polyacrylamide was performed
using the buffer system described by Laemmli (113) except,
that the concentrations of Tris and glycine in the running
buffer were doubled.
NADPH cytochrome P-450 reductase activities were measured
as described by Vermillion and Coon (114).
2.2. Flash Photolysis

Fig. 5: Scheme of a Flash Photolysis Apparatus. 
M = monochromator, W = waterbath, P = photomultiplier 
a)-e) see text

Ligated derivatives of heme proteins, such as hemoglobins and myoglobins (cf. tab. 4) are photodissociable. A visible light pulse is absorbed by the heme leading to the dissociation of the ligand from the heme iron. After the light pulse ligand rebinding occurs and the process can be
followed spectrophotometrically.

A flash photolysis apparatus for kinetic spectrophotometry consists of a) a pulsed light source to produce photodissociation of a liganded protein; b) a thermostated cell for the sample; c) a light source for the observation of absorbance changes in the sample; d) a monochromator for the selection of the monitoring wavelength; and e) a detection system to convert time dependent changes in the intensity of light transmitted by the sample following photolysis into voltages, which may then be recorded (see fig. 5) (115).

Carbon monoxide binding to reduced cytochrome P-450 was measured by flash photolysis. For the measurements two photolysis systems were used.

In one instrument a flash lamp pumped dye LASER (model 34 from Electro Photonics Ltd., Belfast, Northern Ireland, United Kingdom), equipped with a 1.4-microfarad capacitor (Maxwell Laboratories, Inc., San Diego, CA) was used. In place of the standard output coupler (a dielectric mirror) a broad band resonator plate (Phase-R Co., New Durham, NH) was mounted on the LASER. Rhodamine 6G (Lambda Physik, Goettingen, FRG) was used as dye, giving a light pulse centered around 585 nm. Its duration was about 4 us. Monitoring of the reaction was done by recording the percent transmission changes on a transient recorder.
(TM-109, R. Maurer, Lucerne, Switzerland) with a time resolution of 1 us per point. Synchronization of the data acquisition system with the LASER was performed by means of a photodyode which was triggering the transient memory when illuminated by the LASER pulse. The whole system was controlled by a MINC-11/23 computer (Digital Equipment Corporation, Maynard, MA) by means of an IEEE-488 interface and a digital to analog converter module. This allowed direct recording and averaging of several traces with considerable improvement of the signal to noise ratio.

The second instrument used is based on a Q-switched Nd:YAG LASER (YG-471 A-P from Quantel, Orsay-Cedex, France). This LASER gives much shorter light pulses (about 12 ns duration) as compared to the flash lamp pumped dye LASER. By frequency-doubling its fundamental emission (1064 nm) a light beam in the green (532 nm) is obtained, perfectly suitable for ligand photodissociation of a hemeprotein. Due to the shorter duration of the LASER pulse the acquisition of the data is also performed in a different way so that very fast (ns range) and quite slow (up to minutes) processes can be monitored at the same time.
2.2.2. Sample Preparation

2.2.2.1. Dithionite Reduced P-450/P-420

The measurements of carbon monoxide binding to reduced P-450 must be performed under strictly anaerobic conditions to avoid autooxidation and formation of superoxide anions. Buffers (0.1 M K-phosphate containing 20% glycerol) were saturated with argon. To obtain various carbon monoxide concentrations the appropriate amounts of carbon monoxide saturated buffer ([CO]=1 mM) were mixed with the argon saturated buffer. From concentrated P-450 solutions (50-100 uM) the appropriate amount was added to obtain final P-450 concentrations between 2 and 5 uM. The protein was reduced by adding a few grains of dithionite, which also served to remove the last traces of oxygen present. For P-450 the whole procedure was carried out on ice. The conversion of P-450 to P-420 was achieved by incubating the carbonyl-P-450 for one hour at 37°C in the presence of dithionite.
2.2.2.2. NADPH Reduced P-450

For measurements of carbon monoxide binding to P-450 reduced by NADPH via NADPH cytochrome P-450 reductase, equimolar amounts of P-450 and reductase (4 nmoles) were diluted with 2 ml buffer. The mixture was saturated with argon by flushing argon for two hours over the protein solution and carefully avoiding the formation of air bubbles. Carbon monoxide was added as described for the dithionite reduced proteins. Reduction was obtained by adding NADPH to a final concentration of 0.5 mM.

2.2.2.3. Dissociation of P-450/P-420 Aggregates

To dissociate P-450 oligomers into monomers, oxygen-free solutions of the carbonyl form of the proteins were incubated over night at 0°C with 1% CHAPS (25). The dissociation of P-450 aggregates was detected by gel-permeation chromatography on Sepharose CL-6B (Pharmacia fine Chemicals, Uppsala) (25).

2.2.3. Data Analysis

Kinetic data were analyzed by least squares fitting to the appropriate model equation (single exponential, double exponential, exponential and power law, etc.). For the non
linear models the procedure of Levenberg (116) and Marquart (117) were used to optimize the initial guesses of the parameters. The subroutine ZXSSQ (IMSL, Houston, TX) was applied for this purpose.

Fig. 6: Data Acquisition: Block diagram of a computer-assisted data acquisition system (119).
Fig. 6 shows the general features of the apparatus used by us. The signal from the detector D of the measuring instrument is pre-amplified (I) before it is sent through a BNC cable to the amplifier A. Here the signal is further amplified (II) and subsequently split to obtain two identical outputs, one of which is recorded by a fast transient digitizer (100 MHz) and the other directly by the computer (PDP-11/23 from Digital Equipment Corporation), via a relatively slow (38 kHz) digital to analog converter module (ADC). The resolution of the ADC module is sufficiently high (12 bits) so that the signal can be recorded, without further amplification, using the full voltage range of the module (10 V). On the contrary, the fast transient recorder has a low resolution (8 bits) and it is therefore very important that the trace covers as much as possible the full range of the instrument. For this purpose an offset (OFF) is built on the amplifier IV. This allows the output signal of IV to be placed always close to ground level and to be thus conveniently amplified at the input of the transient analyzer.

When the trigger from the flash photolysis apparatus arrives to the transient digitizer, this begins recording the trace and, simultaneously, starts the sequence of A/D conversions on the ADC module of the computer, at the frequency given by the programmable clock.
Recording a trace over a broad time range without losing time resolution is somewhat complex. One possibility is to build a transient analyser with logarithmic time base (not available on the market), as described by Austin et al. (118). Using the same principles applied by Austin in his TRANSLOG, we simulate a logarithmic time base by keeping constant the frequency of the A/D conversions performed by the ADC module and averaging a continuously increasing number of data points as time goes by (see appendix A). This software-simulated logarithmic time base is obtained by means of an assembler subroutine which performs the averaging while the A/D conversions are made. The advantage of using such a subroutine is that by dimensioning an array of say only 200 data points, signals can be recorded for up to several minutes with a resolution of a few microseconds. Our fast transient digitizer records 4096 data points per sweep with a linear time base. We reduce them to 200 by an averaging procedure analogous to the one just described. Needless to say that the sampling rates of the transient analyzer and of the ADC module have to be such that some overlapping exists between the two simultaneously recorded traces (119).
3. RESULTS

3.1. Isolation of Different P-450 Isozymes

The chromatograms of the DE-52 columns for both preparations, phenobarbital- and 3-methylcholanthrene-induced, are given in fig. 7. Peaks designated as b+e and c respectively show on SDS gel electrophoresis one major band contaminated with several minor ones. After further purification on hydroxylapatite, the two phenobarbital induced P-450s, P-450,b and P-450,e show a single band on SDS gels (fig. 8). The 3-methylcholanthrene-induced form, P-450,c, show one major band and two minor ones with higher molecular weight.

If analyzed by IEF on an agarose gel, the fractions corresponding to the b+e peaks display five bands, bI-bV with some contaminations (see below). The calculated chromatograms for the individual bands are given in fig. 9.
Fig. 7: Chromatograms for the DE-52 Columns. The columns were equilibrated with 10 mM K-phosphate buffer pH 7.4 containing 20% glycerol, 0.1 mM EDTA, 0.1 mM DTT, 0.2% Emulgen and 0.5% cholate. The proteins were eluted with a linear NaCl-gradient. The pooled fractions, b + e for phenobarbital treated rats and c for 3-methylcholanthrene treated rats, are marked.
Fig. 8: SDS-Polyacrylamide Gel Electrophoresis of different P-450 isozymes.
a) standards, b) P-450, c) P-450, d) P-450

Fig. 9: Calculated Chromatograms for the DE-52 Column for the different IEF-bands of the P-450 peak.

--- bI
----- bII
----- bIII
----- bIV
----- bV
3.2. Purification of P-450 by Isoelectric Focusing

On the isoelectric focusing gel the "SDS-homogeneous" P-450\(_b\) is resolved into five distinct bands, all detectable by both, benzidin and Coomassie-blue staining. They are designated P-450\(_{bI}\), P-450\(_{bII}\), P-450\(_{bIII}\), P-450\(_{bIV}\) and P-450\(_{bV}\) respectively and have isoelectric points between 7.4 and 7.8 (see fig.10).

About 60% of the applied heme-protein can be recovered from the agarose gels. The relative amounts of the individual bands are given in tab. 5. In all fractions, no conversion to P-420, as estimated from the carbon monoxide reduced difference spectra, is detected.

P-450\(_e\) could be focused into the bands bI-bIII with a slight contamination by bIV.

P-450\(_c\) could not be focused. Most of the applied protein did not enter the gel and precipitated at the application site.
Fig. 10: IEF Pattern of P-450. a) SDS gel of P-450, b) c) agarose IEF gel pH 5-8 of P-450, b) after benzidin staining, c) after Coomassie-blue staining.

Tab. 5: Subfractions of P-450 isolated after isoelectric focusing on agarose gels. The relative amount and the isoelectric points of the different subfractions are listed.

<table>
<thead>
<tr>
<th>subfraction</th>
<th>pH</th>
<th>% of eluted material</th>
</tr>
</thead>
<tbody>
<tr>
<td>b-I</td>
<td>7.43</td>
<td>23.8</td>
</tr>
<tr>
<td>b-II</td>
<td>7.50</td>
<td>18.8</td>
</tr>
<tr>
<td>b-III</td>
<td>7.56</td>
<td>26.0</td>
</tr>
<tr>
<td>b-IV</td>
<td>7.67</td>
<td>16.9</td>
</tr>
<tr>
<td>b-V</td>
<td>7.77</td>
<td>14.4</td>
</tr>
</tbody>
</table>
3.3. HPLC Profiles of P-450

Fig. 11: HPLC Profiles of P-450 and of its IEF purified subfractions. a) P-450, b) P-450 (I), c) P-450 (II), d) P-450 (III), e) P-450 (IV), f) P-450 (V).
The HPLC profiles of P-450\textsubscript{b} and of its IEF-purified subfractions P-450\textsubscript{b'I}-P-450\textsubscript{b'V} are given in fig. 11. For P-450\textsubscript{b'} three peaks are detected at 417 nm. For P-450\textsubscript{b'IV} and P-450\textsubscript{b'V} only one major peak is seen, while the other three subfractions show two or three peaks with different intensities.

3.4. Cytochrome P-450 Reductase

Cytochrome P-450 reductase, isolated from phenobarbital induced rat liver microsomes by affinity chromatography on a 2'5'ADP-agarose column, shows a homogeneous band on SDS gel indicating a molecular weight of 78 kd. Our preparations of reductase had an average specific activity of about 400 nmoles reduced cytochrome c per minute and mg reductase.

3.5. Monooxygenase Activities

Tab. 6 summarizes the activities of different P-450 isozymes with four different substrates
Tab. 6: Monooxygenase Activities of different P-450 isozymes of the IEF purified subfractions with various substrates at 25°C.
Activities are given in nmoles/min nmole P-450.
nd = not determined.

| Protein | 7-ethoxy-coumarin | p-nitro-anisol | amino-pyrin | benzo(a) pyrene |
|---------|------------------|----------------|-------------|----------------|----------------|
| P-450c  | 0.56             | 0.051          | 91.8        | 4.9            |
| P-450e  | --               | --             | 60.2        | 0.63           |
| P-450b  | 0.28             | 0.032          | 188.5       | 2.21           |
| P-450bI | 0.21             | 0.052          | nd          | nd             |
| P-450bII| 0.25             | 0.053          | nd          | nd             |
| P-450bIII| 0.21            | 0.023          | nd          | nd             |
| P-450bIV| 0.39             | 0.090          | nd          | nd             |
| P-450bV | 0.68             | 0.087          | nd          | nd             |
3.6. Inactivation of P-450 by Suicide Substrates

The relative amounts of P-450 after the incubation with the suicide substrate 1-octin are shown in fig. 12. While in P-450 and in its IEF-purified subfractions only about 20-30% of the initial P-450 could be detected after 30 minutes, P-450 cannot be inactivated by this substrate.

*Fig. 12: Suicide Inactivation: P-450, P-450, and its IEF-purified subfractions P-450, P-450, and P-450, are inactivated by 1-octin. The % remaining spectrosopically detectable P-450 is given after 10 and 30 minutes of 1-octin influence.*
3.7. Flash Photolysis

3.7.1. Carbon Monoxide Binding to Different P-450s and to P-420

Wavelength-dependent flash photolysis studies of carbon monoxide binding to P-420 show a quasi-isosbestic point around 455 nm with significant fluctuations from measurement to measurement. Thus, monitoring the combination of carbon monoxide with P-450, P-450 and P-450 at this wavelength (to be determined each time) eliminates contribution to the signal due to the presence of P-420 in the sample. Under these conditions, the kinetics of carbon monoxide binding to different P-450 isozymes can be represented by a two-exponential function. Fig. 13 depicts the time course of carbon monoxide combination with P-450. The same results were obtained in rapid-mixing experiments (data not shown). The data of Gray (98), a three step binding process, can be reproduced by following the reaction at the same monitoring wavelength reported in his papers, i.e. 448 nm which simultaneously observes carbon monoxide binding of P-450 and contaminating P-420.

Changing the monitoring wavelength reveals an isosbestic
Fig. 13: Flash Photolysis Progress Curves for the binding of carbon monoxide to P-450, (A) and to P-420, (B). Both, experimental points and fitted curves are given. Conditions were as follows: buffer 0.1 M K-phosphate pH 7.4 containing 20% glycerol, protein concentration 2 uM, CO concentration 20 uM, temperature 10°C.
Fig. 14: Plots of the Apparent Pseudo First Order Constants ($k_{\text{app}}$) for carbon monoxide binding constants to P-450, (A) and to P-420, (B), obtaining by least-squares fitting to the flash photolysis data, as a function of ligand concentration for fast (---) and slow (----) phases.
point between unliganded and carbon monoxide bound \( P-450_b \) around 418 nm. Thus, this wavelength was chosen to monitor carbon monoxide binding to \( P-420_b \). This reaction was also found to be biphasic (fig. 13). The ligand concentration linearly affects carbon monoxide binding to \( P-450_b \) and \( P-420_b \) as well as to \( P-450_e \) and \( P-450_c \). This is shown in fig. 14, where plots of the apparent pseudo-first order rate constants for \( P-450_b \) and \( P-420_b \) are plotted against the total carbon monoxide concentrations. Analogous results have been obtained for \( P-450_e \) and \( P-450_c \) (data not shown).

No appreciable differences in the rate constants could be detected between \( P-450s \) reduced by NADPH via NADPH cytochrome P-450 reductase or by dithionite except that the final extent of reduction with NADPH was only about 50%. A summary of the kinetic parameters obtained for the different P-450 isozymes reduced in both ways are given in tab. 7.

**Tab. 7: Rate Constants for Carbon Monoxide Binding to different isozymes.** Conditions were as follows: buffer 0.1 M K-phosphate pH 7.4 containing 20% glycerol, protein concentration 2 uM, temperature 10°C, CO concentrations 20-100 uM.

<table>
<thead>
<tr>
<th>protein</th>
<th>reductant</th>
<th>( f_1 )</th>
<th>( k_1 )</th>
<th>( k_2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( P-450_b )</td>
<td>dith.</td>
<td>0.68</td>
<td>1.23 ( 10^6 )</td>
<td>1.36 ( 10^5 )</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>0.70</td>
<td>1.10 ( 10^6 )</td>
<td>1.20 ( 10^5 )</td>
</tr>
<tr>
<td>( P-450_c )</td>
<td>dith.</td>
<td>0.40</td>
<td>0.93 ( 10^5 )</td>
<td>1.50 ( 10^4 )</td>
</tr>
<tr>
<td></td>
<td>NADPH</td>
<td>0.41</td>
<td>1.07 ( 10^5 )</td>
<td>1.46 ( 10^4 )</td>
</tr>
<tr>
<td>( P-450_e )</td>
<td>dith.</td>
<td>0.72</td>
<td>2.40 ( 10^6 )</td>
<td>3.48 ( 10^5 )</td>
</tr>
<tr>
<td>( P-420_b )</td>
<td>dith.</td>
<td>0.42</td>
<td>1.70 ( 10^7 )</td>
<td>1.50 ( 10^6 )</td>
</tr>
</tbody>
</table>
Lowering the intensity of the photodissociating light pulse does not affect the velocity of carbon monoxide binding, nor does it produce a change in the fraction of fast phase for either P-450\textsubscript{b} or P-420\textsubscript{b} (data not shown).

3.7.2. Temperature Effect

The rate constants for carbon monoxide binding to P-450\textsubscript{b}, P-420\textsubscript{b} and P-450\textsubscript{c} were determined at different temperatures between 10 and 30°C. Arrhenius plots (ln k vs. 1/T) were linear for all investigated processes. From the regression lines through the experimental points the overall activation energies listed in tab. 8 were calculated. No effect of temperature on the fraction of fast phase was observed. Fig. 15 shows Arrhenius plots for carbon monoxide binding to P-450\textsubscript{b} and P-420\textsubscript{b}.

Tab. 8: Overall Activation Energies: The overall activation energies for carbon monoxide binding to P-450\textsubscript{b}, P-420\textsubscript{b}, and P-450\textsubscript{c} are listed for both the fast and the slow phase.

<table>
<thead>
<tr>
<th>protein \ phase</th>
<th>activation energy (kJ/mole)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-450\textsubscript{b} fast</td>
<td>45.2</td>
</tr>
<tr>
<td>P-450\textsubscript{b} slow</td>
<td>51.2</td>
</tr>
<tr>
<td>P-420\textsubscript{b} fast</td>
<td>25.6</td>
</tr>
<tr>
<td>P-420\textsubscript{b} slow</td>
<td>43.2</td>
</tr>
<tr>
<td>P-450\textsubscript{c} fast</td>
<td>41.3</td>
</tr>
<tr>
<td>P-450\textsubscript{c} slow</td>
<td>54.4</td>
</tr>
</tbody>
</table>
Fig. 15: Arrhenius Plots for the binding of carbon monoxide to P-450$_b$ (A) and P-420$_b$ (B) for the fast (—) and the slow (---) phases. Protein concentrations and buffer were as described in the legend to fig. 12. CO concentrations were 70 uM for P-450 and 100 uM for P-420.
3.7.3. Solvent Effects

Changing the glycerol concentration in the buffer from 0 to 50% produces a significant increase in both reaction rates without affecting the fraction of fast phase (tab. 9). Furthermore, the conversion of P-450 to P-420 slows down when the glycerol concentration is increased.

In the pH range between 7.4 and 8.1 the reaction velocities, as well as the fraction of fast phase, remain constant. In contrast, lowering the pH from 7.4 to 6.1 produces significant increases in the reaction rates, but leaves the fraction of fast phase unchanged (tab. 9).

Tab. 9: Solvent Effects: The apparent pseudo first order constants (k app) and the fraction of fast phase (f1) for carbon monoxide binding to P-450 are given for different glycerol concentrations and for different pHs. Experimental conditions unless specified in the table were as described in the legend to fig. 14.

<table>
<thead>
<tr>
<th>glycerol concentration</th>
<th>pH</th>
<th>f1</th>
<th>k1 app</th>
<th>k2 app</th>
</tr>
</thead>
<tbody>
<tr>
<td>0%</td>
<td>7.4</td>
<td>0.68</td>
<td>39.1</td>
<td>3.8</td>
</tr>
<tr>
<td>20%</td>
<td>7.4</td>
<td>0.69</td>
<td>48.8</td>
<td>4.4</td>
</tr>
<tr>
<td>50%</td>
<td>7.4</td>
<td>0.69</td>
<td>65.2</td>
<td>5.8</td>
</tr>
<tr>
<td>20%</td>
<td>6.1</td>
<td>0.68</td>
<td>60.2</td>
<td>6.0</td>
</tr>
<tr>
<td>20%</td>
<td>8.1</td>
<td>0.69</td>
<td>46.0</td>
<td>3.8</td>
</tr>
</tbody>
</table>
3.7.4. Effects of Substrates

The addition of 1.25 mM phenobarbital to the reaction mixture does not influence the carbon monoxide binding process, whereas 1 mM benzphetamine or 7-ethoxycoumarin, which induce type I substrate spectra in ferric P-450\textsubscript{b} (120,121), produce appreciable effects on the reaction rates as well as on the fraction of fast phase (tab. 10). In the case of P-420\textsubscript{b}, the addition of 7-ethoxycoumarin produces no effect on the carbon monoxide binding kinetics (data not shown).

3.7.5. Effects of the Aggregation State

Overnight incubation with 1% CHAPS dissociates P-450\textsubscript{b} aggregates (23). Such disaggregation does not alter the velocities of the two processes, but slightly modifies their relative amplitudes (tab. 10).
Tab. 10: Substrate and Aggregation Effects: The apparent pseudo first order constants ($k_{\text{app}}$) and the fraction of fast phase ($f_1$) for carbon monoxide binding to P-450 under various conditions are listed. At least three parallel experiments were performed for each group in the table. The differences in the controls between different groups may be ascribed to experimental errors, mainly variations of the ligand concentrations. For each group 50% glycerol (wt/v) was used as control condition, except when the effect of ethoxycoumarin was studied, since 1% ethanol was added to dissolve the substrate, 50% glycerol and 1% ethanol was used as control in this case. Other conditions were as described in the legend to fig. 12.

PB = phenobarbital, BP = benzphetamine, EC = ethoxycoumarin

<table>
<thead>
<tr>
<th>exp. conditions</th>
<th>$f_1$</th>
<th>$k_1$ app</th>
<th>$k_2$ app</th>
</tr>
</thead>
<tbody>
<tr>
<td>control</td>
<td>0.64 ± 0.02</td>
<td>70.4 ± 4.2</td>
<td>9.2 ± 1.6</td>
</tr>
<tr>
<td>1.25 mM PB</td>
<td>0.65 ± 0.06</td>
<td>58.6 ± 8.0</td>
<td>10.4 ± 3.2</td>
</tr>
<tr>
<td>control</td>
<td>0.67 ± 0.01</td>
<td>67.2 ± 8.0</td>
<td>7.2 ± 2.0</td>
</tr>
<tr>
<td>1 mM BP</td>
<td>0.79 ± 0.05</td>
<td>102.2 ± 8.4</td>
<td>19.8 ± 8.0</td>
</tr>
<tr>
<td>control</td>
<td>0.68 ± 0.05</td>
<td>73.6 ± 11.2</td>
<td>6.4 ± 2.8</td>
</tr>
<tr>
<td>1 mM EC</td>
<td>0.72 ± 0.04</td>
<td>100.6 ± 14.2</td>
<td>8.0 ± 3.8</td>
</tr>
<tr>
<td>control</td>
<td>0.66 ± 0.03</td>
<td>57.0 ± 7.6</td>
<td>7.2 ± 1.2</td>
</tr>
<tr>
<td>1% CHAPS</td>
<td>0.72 ± 0.02</td>
<td>73.4 ± 5.4</td>
<td>7.4 ± 1.8</td>
</tr>
</tbody>
</table>
3.7.6. Carbon Monoxide Binding to IEF Purified Subfractions of P-450$_b$

Flash photolysis studies on the carbon monoxide binding to dithionite reduced subfractions of P-450$_b$ show for bIV and bV single exponential ligand binding curves. bI and bII exhibit biphasic carbon monoxide binding. While for bI the fast portion of the reaction is much larger than that of the slow one, the opposite applies for bII. No flash photolysis measurement could be made on bIII since its apparent quantum yield turned out to be practically zero. Apparent pseudo first order constants for the different subfractions are given in tab. 11. Representative flash photolysis progress curves for the binding of carbon monoxide to IEF purified subfractions of P-450$_b$ are given in fig. 16. It is worth mentioning that the stability of the purified P-450$_b$s is much lower than for the unfractionated protein. A rapid decrease of the signal during the measurements did not allow determinations of the kinetic constants as function of the carbon monoxide concentration.
Tab. 11: IEF Purified Subfractions: Apparent pseudo first order constants \((k_{app})\) for carbon monoxide binding to IEF purified subfractions of P-450. Conditions were as follows: buffer 0.1 M K-phosphate \(\text{pH} 7.4\) containing 20% glycerol, CO concentration 40 \(\mu\)M, temperature 10°C.

<table>
<thead>
<tr>
<th>subfraction</th>
<th>(k_1) app</th>
<th>(k_2) app</th>
<th>(f_1)</th>
</tr>
</thead>
<tbody>
<tr>
<td>bI</td>
<td>108.6</td>
<td>15.7</td>
<td>0.61</td>
</tr>
<tr>
<td>bII</td>
<td>163.5</td>
<td>29.7</td>
<td>0.28</td>
</tr>
<tr>
<td>bIII</td>
<td>not photodissociable</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>bIV</td>
<td>77.4</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td>bV</td>
<td>55.6</td>
<td>--</td>
<td>--</td>
</tr>
</tbody>
</table>
Fig. 16: Representative Flash Photolysis Progress Curves for the binding of carbon monoxide to bIV and bV. Both experimental points and fitted curves are given in a double logarithmic plot. Conditions were the same as described in legend to tab. 11.
Log (Delta OD) vs. Log (sec.)

P-450-bIV 35uL CO ~40 uM .1M phos. pH=7.4 20% gly 1cm cell
Original data in files: 170186.R 1 - 8.91
Lambda= 453.0 nm  T= 293.0 K

Rate const. 0.7744E+02[1/sec]
A0:  0.1023E-01
Correlation coeff. = 0.9751
4. DISCUSSION

4.1. Isolation of Different P-450 Isozymes

Cytochromes P-450\textsubscript{b} and P-450\textsubscript{e} isolated from phenobarbital induced rat liver microsomes according to a "standard procedure" described in the literature (102) give a single band on SDS gels, a method which separates according to molecular weight only (see fig. 8). On the contrary cytochrome P-450\textsubscript{c} isolated from 3-methylcholanthrene induced rat liver microsomes shows three bands on the same gel. Comparison with molecular weight standards indicates that the protein bands with higher molecular weight could represent dimeric and trimeric forms of the protein.

Our determinations of monooxygenase activities against 7-ethoxycoumarin, p-nitroanisol, aminopyrin and benzo(a)pyrene (see tab. 6) yield for all three isozymes values slightly lower than those obtained by Guengerich (51), but the relative values for the different enzymes and substrates are in agreement with the literature. It is well known that detergents can bind to proteins and influence monooxygenase activities (122,123). During the isolation procedure for all isozymes 0.2% Emulgen 911 is used as a detergent. This is removed before concentrating the isolated P-450s by stirring the protein solutions.
together with Amberlite XAD-2. It is conceivable that the discrepancy between our monooxygenase activities and those in the literature reflect incomplete detergent removal.

4.2. Isoelectric Focusing

Isoelectric focusing clearly shows that preparations of the major phenobarbital induced cytochrome P-450, P450\textsubscript{b}, apparently homogeneous on SDS gels, are indeed heterogeneous (see fig. 10). All five resolved proteins bI-bV react with monoclonal antibodies from 26 different clones against the unresolved P450\textsubscript{b} as antigen (data not shown). These results are consistent with those of Bansal et al. (68) who were able to resolve the P-450"peakB2" of Guengerich and Martin (124) into four enzymatically active and immunologically identical hemeproteins.

P450\textsubscript{e} elutes from the DE-52 column at a slightly higher salt concentration than P450\textsubscript{b} but the two isozymes cannot be completely separated on this column (see fig. 7, a slight shoulder of the b+e peak). Indeed, P450\textsubscript{e}, homogeneous on SDS gels is resolved into three bands bI-bIII when focused on agarose IEF gels. These results are in agreement with the calculated chromatogram (see fig.9). At lower salt concentrations mainly bIV and bV are found in the eluate, while at higher salt concentration only bI-bIII
are found. Cytochrome P-450\textsubscript{c}, induced by 3-methylcholanthrene in rat liver microsomes can not be focused at all. Most of the applied protein precipitates at the application site. One possible reason for this may be that this isozyme is too hydrophobic and forms aggregates which precipitate under the experimental conditions used. This possibility is supported by the aggregates found in the SDS gels (see fig. 8).

The anion exchange chromatography on the DE-52 column is carried out in the presence of 0.2\% Emulgen 911, while the IEF is performed in the presence of 1\% CHAPS. Unlike 1\% CHAPS 0.2\% Emulgen 911 is not able to monomerize P-450\textsubscript{b} (25). This is a plausible explanation for the fact that P-450\textsubscript{b} purified on an anion exchange column exhibits heterogeneity on IEF gels in the presence of 1\% CHAPS.

4.3. Characterization of the IEF Purified Subfractions of P-450\textsubscript{b}

Changing the application site from the anodic to the cathodic side of the gel does not alter the result of the IEF experiment. Therefore, it is unlikely that the multiple bands obtained reflect an artefact. This conclusion is further confirmed by the observation that refocusing of the
eluted subfractions exhibit single bands for bIV and bV. For bI, bII and bIII contamination with the neighbouring bands is observed (data not shown). Furthermore, HPLC analysis of the subfractions gives profiles with one major peak for P-450_{bIV} and P-450_{bV}, while the profiles for P-450_{bI}, P-450_{bII} and P-450_{bIII} show contaminations (see fig 11). Thus, the HPLC and IEF results are consistent with each other.

The contaminations of bI, bII and bIII can be ascribed to the very close isoelectric points for the three bands (see fig. 10). Carbon monoxide-reduced difference spectra of the different P-450_{b} subfractions, eluted from preparative agarose IEF gels, show absorption maxima at 450 nm indicating an intact heme binding site. Incubation of all subfractions with the suicide substrate 1-octin in the presence of reductase results in heme loss to about the same extent as for the unresolved P-450_{b}. In agreement with previous reports (35,110) for P-450_{c} no heme loss (fig. 12) can be observed after incubation with 1-octin. Since heme loss after the reaction with suicide substrates implies an interaction between P-450 and its reductase, it follows that the structural features responsible for this interaction are kept in all subfractions during isoelectric focusing. Furthermore, the various enzymes all show catalytic activities against 7-ethoxycoumarin and
p-nitroanisol (see tab. 6) indicating an intact substrate binding site. All these results provide a good evidence that the various P-450s are eluted from the gel in a native form.

P-450_{DIV} and P-450_{IV} exhibit significantly higher specific activities against both 7-ethoxycoumarin and p-nitroanisol than the others (tab. 6). This is in agreement with the finding that P-450_{e}, in which these two subfractions are not present, display very low activities against both substrates.

4.4. Possible Explanations for P-450_{b} Heterogeneity

The reason for the heterogeneity of P-450_{b} is presently not known. Several possibilities may be considered. First, the different bands could represent different isozymes and thus different gene products. Peptide mapping after BrCN cleavage of the different subfractions resulted in peptide patterns which differ only very slightly (data not shown). Bansal et al. (104) have also analyzed their subfractions of the major phenobarbital inducible P-450 by peptide mapping after digestion with Staphylococcus aureus V8 protease. Again no major differences could be detected. It is therefore unlikely that the various fractions differ in their aminoacid sequence. Post-translational modifications,
such as binding of specific phospholipids or metabolites, phosphorylation, carbamilation etc. may also be responsible for the formation of various derivatives differing in their isoelectric points, but not significantly in their molecular weights. Support to this hypothesis is provided by data in the literature. Imai et al. (125) were able to show that in P-450<sub>c</sub> preparations various amounts of the inducer 3-methylcholathrene are bound to the isolated protein. Pyerin et al. (126,127) provided evidence for a correlation between specific phosphorylation of P-450, isolated from rabbit liver microsomes after phenobarbital induction, and monooxygenase activity. At present, we can neither exclude nor prove any of the above possible explanations and more experiments are needed to give an answer to this problem.
4.5. Kinetics of Carbon Monoxide Binding to P-450

The results reported in tab. 7 and figs. 13 and 14 show that carbon monoxide binding to rat liver microsomal P-450 is a much simpler process than previously reported by some authors (94,98-100). It is clear that the three bimolecular processes observed by Gray (98), monitoring at 448 nm, result from the simultaneous combination of carbon monoxide to P-420 and P-450. In fact, the slow carbon monoxide binding process for P-420 and the fast one for P-450 have similar reaction rates (tab. 7). Gray found for the second process hyperbolic dependence of the reaction rate on the carbon monoxide concentration. In contrast, the independence of the rate constants on the carbon monoxide concentration found by Gray for his third phase does not find an immediate explanation in our results. The flash-photolysis data reported by the group of Douzou (95) are fully consistent with our findings. These investigators kept P-450 always at very low temperature, therefore largely preventing the conversion to P-420 (95). Our data are also in agreement with what found by Brunori (97).

The presence of denatured forms of the enzyme P-420 in P-450 preparations seems to have played a dominant role in producing inconsistency in the results reported in the literature. Once we determined the conditions under which the contribution of P-420 to the reaction of P-450 with
carbon monoxide can be eliminated, an analysis of this process aimed at the understanding of its mechanism was possible.

Tab. 7 summarizes the rate constants and the fraction of fast phase for different P-450 isozymes. The reaction rates for P-450\textsubscript{b} and P-450\textsubscript{e} are in the same order of magnitude, while for P-450\textsubscript{c} the rate constants for the fast phase is approximately the same as the rate constants for the slow phases for P-450\textsubscript{b} and P-450\textsubscript{e}. For P-420\textsubscript{b} the velocities for both processes are about ten times faster than those of P-450\textsubscript{b}.

From these results, some speculations on the geometry of the heme pocket in P-450 can be made. The value of the fast rate constant for carbon monoxide binding to P-450\textsubscript{b} and P-450\textsubscript{e} are consistent with an open heme crevice (see tab. 4). The same does neither apply to the slow phase of P-450\textsubscript{b} and P-450\textsubscript{e} nor to the fast phase of P-450\textsubscript{c}. This differences may be related to modifications of the structure of the protein at either the distal or the proximal side of the heme. Since in the case of P-450\textsubscript{c} 3-methylcholanthrene is known to be bound to the isolated protein (125), the slower ligand binding reaction rates compared to P-450\textsubscript{b} can be ascribed to steric hindrance at the distal side of the heme. On the other hand regulation of the heme reactivity from and through the proximal
ligand, as reported for other hemeproteins (77,78), is very likely to be prominent in P-450. Indeed, binding of the substrates 7-ethoxycoumarin and benzphetamine to this protein increases the velocity of its reaction with carbon monoxide as reported in tab. 10. 

The differences in the carbon monoxide association rates between P-450 and P-420 may also reflect different structural features at the proximal side of the heme. We presently do not know which molecular alterations are responsible for the formation of P-420 from P-450. The variability of the isosbestic wavelength between unliganded and carbonyl P-420 (see results p. 60) and the relatively poor fitting of the carbon monoxide binding kinetic data with multiple exponential models (see fig. 13) favour the hypothesis that P-420 is a mixture of different products of denaturation of P-450. The position of the Soret peak in the electronic spectrum of carbonyl P-420 may suggest the presence of an axial nitrogenous ligand bound to the iron. However, the very high rates of carbon monoxide dissociation obtained for P-420 (see fig. 14) are inconsistent with what is observed in other heme proteins which have a histidine as proximal ligand of the heme iron (see tab 4). Furthermore, for a heme model compound having a sulfur atom as axial ligand, a very low carbon monoxide affinity has been reported (128). A plausible explanation
for the big difference in carbon monoxide dissociation rates between P-450₀ and P-420₀ could be ascribed to a different position of the iron atom relative to the heme plane, resulting in a more or less loose bond with the proximal ligand. Such an explanation has been invoked for other heme proteins (78).

Fig. 17: P-450 to P-420 Conversion: Proposed situation on the distal and the proximal side of the heme.
We have formulated a plausible, although speculative explanation for the differences in ligand binding properties between P-450<sub>b</sub> and P-420<sub>b</sub>. Investigations by other authors have revealed the presence of a histidine in the distal area of the heme pocket of P-450 (129). Our hypothesis is that in the process of denaturation the bond between the heme iron and the proximal cysteine in P-450 is broken and the histidine present at the distal side in the native protein becomes the proximal ligand in the denatured material. This would imply that the orientation of the heme, as referred to its proximal ligand, is rotated by 180° along the axis going through the iron and between the two propionic acid side chains (see fig. 17) in P-420 as compared to P-450. This rotation is known to increase both the ligand association and dissociation velocities in other heme proteins (77,130) (see 1.2.2.). An indirect evidence supporting the above assumption is the very slow rate of conversion of P-450<sub>c</sub> to P-420. In this enzyme 3-methylcholanthrene is bound in the vicinity of the heme, most likely at the distal side, and the conversion to P-420 is therefore sterically hindered.
4.6. Kinetics of Carbon Monoxide Binding to P-450<sub>b</sub> and to the Isolated Subfractions

The progress curve depicted in fig. 13 shows that the process of carbon monoxide binding to P-450<sub>b</sub> can be reasonably well fitted to a two exponentials model. Under pseudo first order conditions both the fast and the slow phases are linearly influenced by the carbon monoxide concentration as can be seen in fig. 14. No differences in the carbon monoxide binding kinetics are observed if the reaction is investigated by rapid mixing or by flash photolysis and the progress curves are not influenced by the intensity of the photolyzing light pulse. All these findings show that a) the biphasic nature of the reaction cannot be ascribed to monomolecular structural rearrangements of the protein during ligand binding (99,131) and b) that both processes are bimolecular.

The kinetic investigations on the IEF purified subfractions unequivocally demonstrate the validity of the above conclusion. Bands bIV and bV, which by refocusing and HPLC analysis were homogeneous, display a single exponential carbon monoxide binding process. The apparent pseudo first order rate constants of carbon monoxide binding are practically the same. The biphasicity of the reaction of P-450<sub>bI</sub> and P-450<sub>bIII</sub> with carbon monoxide is consistent with the non homogeneity of these bands detected both by
IEF and HPLC. The different distribution between fast and slow processes in the time courses of carbon monoxide binding to the subfractions bI and bII well correlates with the relative amounts of the two proteins in the eluted bands.

The difference in apparent quantum yields observed for the various subfractions make it difficult to quantitatively correlate the kinetic data on the isolated proteins to those obtained with the unfractionated P-450. However, the fact that the various subfractions react differently to the photolysing light pulse is a further demonstration that they are not the result of an artefact in the isolation procedure. More than that, the finding that the apparent quantum yield varies from protein to protein may give some hints on the nature of the differences between them. In this context the multibarrier model for ligand binding to heme proteins (132) can be of great help. According to this model ligand binding can be described in terms of at least three steps: I) binding from inside the heme pocket, II) binding from inside the protein matrix and III) binding from the solvent. Process I is the fastest, while process III is the slowest of all. It has been seen for other heme proteins that a correlation exists between the activation energy for process I and the apparent quantum yield measured at room temperature (133,134). There is no logical reason
why the same principle should not apply to P-450. Along this line of reasoning it would therefore seem that the various P-450 subfractions are characterized by quite different ligand binding properties from inside the heme pocket. This implies that the intrinsic reactivity of the heme varies from subfraction to subfraction. Both proximal and distal side phenomena may account for such differences and at present we cannot even guess if one or the other may play a larger role in our enzymes. Clearly a deeper investigation of the problem, involving measurements at liquid helium temperatures and in the picosecond range are required to gain detailed information to explain the functional differences between the various subfractions of P-450.
5. CONCLUSION

We have shown that carbon monoxide binding to liver microsomal phenobarbital induced P-450, P-450_b, is a simple bimolecular process. The so far observed complexity of ligand binding to reduced P-450_b can clearly be ascribed to heterogeneity in the protein preparations. This implies that other ligands as the physiological one, oxygen, also bind according to a simple bimolecular mechanism. We could therefore, demonstrate that P-450 from rat liver microsomes behaves as other P-450s, e.g. bacterial P-450_cam.
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Appendix A

General principles (118) by which the software-simulated logarithmic time base described in the text is generated.

a) The ADC converts the input voltage at a constant frequency.

b) The digital data are averaged in groups whose size is doubled every R averaging cycles.

Thus, if the A/D conversions are done say every 50 microseconds, the number of linearly averaged points R is 10 and the doubling of the number of averaged points is done M=20 times, the resulting number of data points is \( R \times M = 200 \) and the total time spanned is 511.18 seconds equivalent to ca. \( 10^7 \) A/D conversions.
SUMMARY

The kinetics of carbon monoxide binding to phenobarbital induced rat liver microsomal P-450\textsubscript{b} and to its enzymatically inactive form P-420\textsubscript{b} have been investigated by flash photolysis spectrophotometry. When the simultaneous presence of both forms of the enzyme is taken into account, the binding of carbon monoxide to these two proteins can be described in terms of two bimolecular processes with the following rate constants:

- **P-450\textsubscript{b}**: $1.2 \times 10^6$ M\textsuperscript{-1}s\textsuperscript{-1}; $1.4 \times 10^5$ M\textsuperscript{-1}s\textsuperscript{-1}
- **P-420\textsubscript{b}**: $1.7 \times 10^7$ M\textsuperscript{-1}s\textsuperscript{-1}; $1.5 \times 10^6$ M\textsuperscript{-1}s\textsuperscript{-1}

From kinetic studies on the binding of carbon monoxide to P-450\textsubscript{b} under different experimental conditions and comparative kinetic investigations of P-450s from different sources, we concluded that carbon monoxide binding to reduced P-450\textsubscript{b} is a simple bimolecular process and that the observed biphasic traces are due to heterogeneity of the protein preparations. This conclusion contrasts with previous reports of complex reaction mechanisms for the binding of carbon monoxide to P-450\textsubscript{b}.

We were able to verify the above conclusion by analysing the kinetics of ligand binding to proteins purified by isoelectric focusing on agarose gels containing the non denaturing detergent CHAPS. P-450\textsubscript{b} can be resolved into
five subfractions which react with carbon monoxide according to a simple bimolecular mechanism. Each of the above fractions also exhibits different specific catalytic activities against 7-ethoxycoumarin and p-nitroanisole.
ZUSAMMENFASSUNG

Kinetische Untersuchungen über die Bindung von Kohlenmonoxid an phenobarbitalinduziertes Cytochrom P-450\textsubscript{b} aus Rattenleber Microsomen, sowie an P-420\textsubscript{b}, eine enzymatisch inaktive Form davon, wurden mit Hilfe von Blitzlicht-Photolyse durchgeführt. Unter Berücksichtigung der Tatsache, dass in einer Probe beide Formen vorhanden sind und gleichzeitig mit Kohlenmonoxid reagieren können, findet man zwei bimolekulare Prozesse, die durch folgende Geschwindigkeitskonstanten charakterisiert sind:

\begin{align*}
P-450\textsubscript{b}: & \; 1.2 \times 10^6 \text{M}^{-1}\text{s}^{-1} ; 1.4 \times 10^5 \text{M}^{-1}\text{s}^{-1} \\
P-420\textsubscript{b}: & \; 1.7 \times 10^7 \text{M}^{-1}\text{s}^{-1} ; 1.5 \times 10^6 \text{M}^{-1}\text{s}^{-1}
\end{align*}

Die Resultate von kinetischen Studien über das Binden von Kohlenmonoxid an P-450\textsubscript{b} unter verschiedenen experimentellen Bedingungen und Vergleiche von Resultaten mit unterschiedlichen P-450 Isozyemen führten zur Folgerung, dass das Binden von Kohlenmonoxid an reduziertes P-450\textsubscript{b} ein einfacher bimolekularer Prozess ist und dass die beobachtete Biphasizität Heterogenitäten innerhalb der Proteinpräparationen zugeschrieben werden muss. Diese Interpretation widerspricht anderen Veroeffentlichungen, in denen komplexe Mechanismen für das Binden von Kohlenmonoxid an P-450\textsubscript{b} vorgeschlagen werden.

Unsere obige Folgerung konnte durch kinetische Studien der
Kohlenmonoxidassoziation an P-450\textsubscript{b} Unterfraktionen, die auf Agarose IEF Gelen unter nicht denaturierenden Bedingungen mit CHAPS als Detergenz gereinigt wurden, verifiziert werden. P-450\textsubscript{b} kann in fünf Banden fokussiert werden. Diese Subfraktionen von P-450\textsubscript{b} reagieren mit Kohlenmonoxid in einem einfachen bimolekularen Mechanismus. Alle fünf zeigen unterschiedliche spezifische Aktivitäten gegenüber 7-Ethoxycoumarin und p-Nitroanisol.
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