

Complexation of Membrane-Bound Enzyme Systems

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Cytochrome P450, NADPH-Cytochrome P450 Reductase, Membrane-Binding Domains

The effect of changes in the N-terminal membrane-binding domain of cytochrome P450 forms and NADPH-cytochrome P450 reductase types on the cytochrome P450-dependent monooxygenase activities, has been examined. The nifedipine oxidase activity of two human P450 forms (CYP3A4, CYP3A4NF14) which differ only in their primary structure by ten amino acid residues in the N-terminal membrane-binding domain, yields nearly the same catalytic cycle time $\tau = 2.65 \pm 0.15$ s, due to their identical cytosolic catalytic protein structure. In contrast, the complex formation process ($[P450] + [reductase] \leftrightarrow [complex]$) described by the dissociation constant K_D at high substrate concentration ($[S] \gg K_S$) and low product concentration ($[P] \ll K_P$) is determined to be $K_D/[P450]_0 = 0.3$ and 2.0, respectively. These values reflect large differences in the affinity of both P450 forms for the same type of reductase which is only due to their modified membrane-binding domains. In the present work, it has been shown for the first time, that the membrane-binding domain of cytochrome P450 enzymes determines the complexation process of the binary P450:reductase system. Furthermore, the nifedipine oxidase activity of the human CYP3A4 form reconstituted with two different types of reductase from human and rabbit also has the same catalytic cycle time $\tau = 2.65 \pm 0.15$ s. This result is based on the similarity of the primary structure of the cytosolic catalytic domain of both reductase types. However, the complex was formed with different dissociation constants of $K_D/[P450]_0 = 0.3$ and 4.7, respectively. This different affinity of both reductase types to the same P450 form is interpreted as a consequence of the substantial alteration of the amino acids in the N-terminal primary structure of their membrane-binding domains. 7-Ethoxycoumarin O-deethylase activity of two rat P450 forms (CYP2B1 and CYP1A1) were reconstituted with the same rat reductase. The catalytic cycle time for each P450 form is $\tau = 1.8$ and 0.6 s, respectively. Correspondingly, the complex formation process controlled by the dissociation constant K_D has changed from $K_D/[P450]_0 = 2.3$ to 1.7, respectively. This is because both forms differ in their cytosolic as well as in their membrane-binding domains.

Introduction

Almost every chemical reaction in a cell is catalyzed either by a single enzyme or by an enzyme complex. An example is the membrane-bound monooxygenase system consisting of two functionally different proteins, the heme protein cytochrome P450 and the flavoprotein NADPH-cytochrome P450 reductase. Mammalian cytochrome P450 enzymes (P450) comprise a super family of membrane-bound hemoproteins (≈ 200 different types) involved in the oxidative biotransformation of a wide variety of endogenous and exogenous compounds, including steroids, therapeutic drugs and carcinogens (Guengerich, 1995; Gonzalez, 1992). The mayor part of both enzymes (≈ 480 and 680

amino acid residues, respectively) is present in the cytosol of the cell. It is generally accepted that this part of both enzymes is responsible to form a catalytically active P450 : reductase complex (Tamburini and Schenkman, 1986; Strobel *et al.*, 1989; Bernhardt *et al.*, 1988; Voznesensky and Schenkman, 1992). Both enzymes anchor with their relative short N-terminal membrane-binding domain (≈ 22 and 56 amino-acid residues, respectively) in the lipid bilayer of the endoplasmatic reticulum of the liver cell (Vergères *et al.*, 1989; Uvarov *et al.*, 1989; Black and Coon, 1982). This work addresses the following question: are the amino-terminal membrane-binding domains of both enzymes the recognition peptide for forming a catalytically active enzyme complex? To answer this question we mea-

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sured the enzyme activity of the binary system with proteins having different membrane-binding peptides.

Materials and methods

Chemicals

7-Ethoxycoumarin and 7-hydroxycoumarin were purchased from EGA-Chemie KG (Steinheim, Germany). Nifedipine (NF) (3,5-dimethoxycarbonyl-2,6-dimethyl-4-[2-nitrophenyl]-1,4-dihydropyridine) and its oxidized metabolite (3,5-dimethoxycarbonyl-2,6-dimethyl-4-[2-nitrophenyl]-pyridine (NF_{ox}) were purchased from RBJ (Natick, MA, USA). L- α -dilauryl-*sn*-glycero-3-phosphate and L- α -phosphatidyl-L-serine were purchased from Sigma (Deisenhofen, Germany).

Enzyme preparations

Native human cytochrome P4503A4 (CYP3A4) was purified to gel-electrophoretically homogeneous state as described by Guengerich *et al.* (1986) and by Müller-Enoch (1999) (CYP stands for cytochrome P450). Recombinant human CYP3A4NF14 and human NADPH-P450 reductase (reductase) were purchased from Oxford Biochemical Research, Inc., Oxford, MI (USA). Rat CYP2B1 and CYP1A1 and rat and rabbit NADPH-P450 reductase were purified to electrophoretic homogeneity using procedures described by Guengerich *et al.* (1982).

Vesicular reconstituted P450:reductase systems

The procedure utilized was a modification of the method described by Müller-Enoch *et al.* (1984). Phospholipid liposomes were prepared by sonication of a solution of 4 mg L- α -dilauryl-*sn*-glycero-3-phosphocholine and 4 mg L- α -phosphatidyl-L-serine in 1 ml of a 0.1 M potassium HEPES (N-[2-hydroxyethyl] piperazine-N'-[2-ethanesulfonic acid]) buffer (pH = 7.6) containing 20 % glycerol (v/v) until the reaction medium became completely clear. To this phospholipid preparation (PLP) aliquots of the cytochrome P450 forms (native CYP3A4 or recombinant CYP3A4NF14 or CYP2B1 or CYP1A1) and the reductase forms (human, rat and rabbit) were added in varying molar ratios of P450:reductase:PLP of 1:X:1100, respectively. These reconstituted vesicular systems were allowed to stand for

2–3 h at 25 °C (Müller-Enoch *et al.*, 1984). Aliquots of these reconstituted P450:reductase systems were used for measuring the P450 enzyme activity.

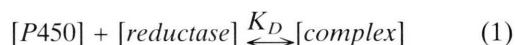
General assays

Nifedipine-oxidase assay: 8 μ l of each of the reconstituted vesicular CYP3A4 : reductase systems (native CYP3A4 or recombinant CYP3A4 NF14 with human or rabbit reductase) are incubated with 0.2 mM nifedipine, 5 mM MgCl₂ and 0.5 mM NADPH in 0.1 M potassium HEPES buffer (pH 7.6) to give a final volume of 0.2 ml. The reaction proceeded for 5 min at 37 °C. After stopping the reaction, the HPLC analysis of the oxidized nifedipine product NF_{ox} was performed as described by Müller-Enoch (1999).

The 7-ethoxycoumarin O-deethylase activities of rat CYP2B1 and CYP1A1 were assayed using the continuous fluorometric assay described by Ullrich and Weber (1972). N-terminal amino acid sequences of the enzymes were performed with gel-electrophoretically pure enzymes, using an Applied Biosystems 477A automated Edman Sequencer. Protein concentrations were estimated using the general method of Lowry *et al.* (1951).

Enzyme kinetics

The complexation of the P450 forms with one type of reductase can be described very generally by the mass action equation where the two enzymes form a binary complex (P450:reductase) (Müller-Enoch *et al.*, 1984; Müller-Enoch, 1993)



with the dissociation constant, K_D

$$K_D = \frac{[P450] \cdot [reductase]}{[complex]} \quad (2)$$

The reaction velocity, v , is the product formation rate, $[dP]/dt$ which can be factorized into three different terms:

$$v = [\text{complex}] \cdot k_E^0 \cdot \frac{[S]}{1 + \frac{[S]}{K_S} + \frac{[P]}{K_P}} \quad (3)$$

The reaction velocity is proportional to: (i) The protein complex concentration ($[\text{complex}]$). It approaches the initial P450 concentration ($[P450]_0$) for very high reductase concentration ($[Red]_0 \rightarrow \infty$). (ii) The catalytic rate constant, k_E^0 , which describes the action of the working enzyme complex under optimal conditions ($[S] \rightarrow \infty$ and $[Red]_0 \rightarrow \infty$). (iii) The loading of the enzyme with the substrate S where K_S and K_P are the dissociation constant of the substrate and the product molecules of the enzyme complex, respectively. All the experiments were performed under high substrate concentrations ($[S] \gg K_S$) and low product concentrations ($[P] \ll K_P$). Under such conditions the third term in Eqn.(3) becomes one.

$$v = [\text{complex}] \cdot k_E^0. \quad (4)$$

The maximal reaction velocity, v_{max} , is reached when each P450 enzyme is complexed with a reductase ($[Red]_0 \rightarrow \infty$) and each complex has a substrate molecule ($[S] \rightarrow \infty$).

In our investigations the enzyme activity, A , is used which is defined as the product formation rate, $d[P]/dt$, divided by the initial P450 concentration $[P450]_0$. Eqn.(4) changes to

$$A = \frac{[\text{complex}]}{[P450]_0} \cdot k_E^0. \quad (5)$$

The predicted enzyme activity is obtained from Eqns (2) and (5) and the protein conservation law ($[P450]_0 = [P450] + [\text{complex}]$ and $[Red]_0 = [Red] + [\text{complex}]$).

$$A = \frac{k_E^0}{2} \left(\left[1 + \frac{[Red]_0}{[P450]_0} + \frac{K_D}{[P450]_0} \right] - \sqrt{\left[\left(1 + \frac{[Red]_0}{[P450]_0} + \frac{K_D}{[P450]_0} \right)^2 - \frac{4[Red]_0}{[P450]_0} \right]} \right). \quad (6)$$

A straight line is obtained if the inverse of the enzyme activity is plotted versus $[P450]_0/[Red]_0$. The

catalytic rate constant, k_E^0 , is obtained by extrapolating $[P450]_0/[Red]_0 \rightarrow 0$ and the slope of the line defines the complexation constant, K_D , divided by $[P450]_0$.

Results and Discussion

In the following experiments the complexation of the two types of proteins is investigated by measuring the enzyme activity, A , for different initial P450 and reductase concentrations. It is only important to know the ratio of both enzymes, $[P450]_0/[Red]_0$.

Nifedipine oxidase activity

First, we investigated the enzyme activity (nifedipine oxidase activity) for two human cytochrome P450 forms which differs only in the length of their membrane-binding domains. Compared to the native form (CYP3A4) (Beaune *et al.*, 1986), the recombinant form (CYP3A4NF14) (Gillam *et al.*, 1993) is truncated by 10 amino acid residues. The results are shown in Fig. 1.

The cycle or turnover time, τ ($=1/k_E^0$) (Gruler and Müller-Enoch, 1991), can be obtained by extrapolating the enzyme activity for very high reductase concentrations. The catalytic cycle time of the native form is 2.5 ± 0.2 s and 2.8 ± 0.2 s of the recombinant form, respectively. The maximal enzyme activity is essentially the same for both forms (24 ± 2 and 21.5 ± 2 ($[\text{nmol}(\text{NF})_{ox}]$ $[\text{nmol} [P450]^{-1} \cdot \text{min}^{-1}]$, respectively). Such a result was expected because the primary structure of both cytosolic parts is the same (Beaune *et al.*, 1986; Gillam *et al.*, 1986). We have demonstrated that the action of the catalytic center where the chemical reaction takes place is not influenced by the membrane-binding peptide but that the complexation process is influenced by the membrane-binding domains. In case of the native CYP3A4 the dissociation constant divided by $[P450]_0$ is 0.3 ± 0.05 and for the recombinant CYP3A4NF14 the same ratio is 2.0 ± 0.2 . This shows that the affinity of the reductase to native CYP3A4 is much higher than for the recombinant CYP3A4NF14. This result demonstrates that the membrane-binding domains are essential in the complexation process because both P450 forms used differ only in the primary structure of their binding domain.

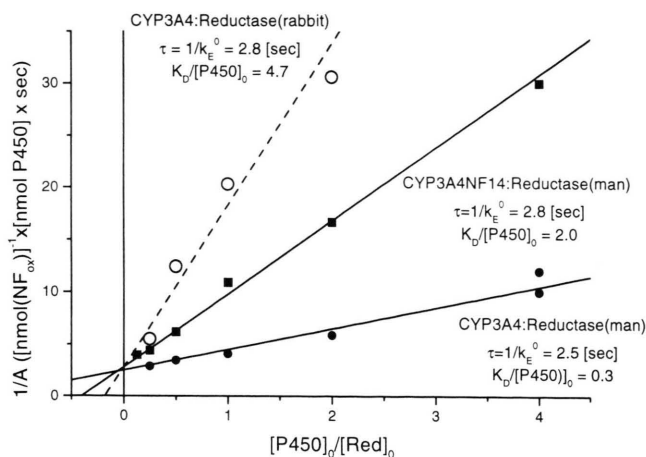


Fig. 1. The nifedipine oxidase activity of human cytochrome P450:reductase complexes is shown. The inverse enzyme activity, $1/A$, is plotted versus the P450/reductase ratio. In the experiment the amount of reductase was varied. Different types of experiments are performed: (i) Full circles (native human CYP3A4 and human reductase), (ii) full squares (recombinant human CYP3A4NF14 and human reductase), and (iii) open circles (native human CYP3A4 and rabbit reductase). The catalytic cycle times, $\tau = 1/k_E^0$, 2.5 s, 2.8 s and 2.8 s are obtained for $x = 0$, or the maximum enzyme activities are 24 ± 2 , 21.5 ± 2 and 21 ± 2 ($[\text{nmol}(\text{NF}_{\text{ox}})] [\text{nmol P450}]^{-1} \text{ min}^{-1}$), respectively. The normalized dissociation constant $K_D/[\text{P450}]_0$, 0.3, 2.0 and 4.7, respectively, are obtained from the slope of the straight lines.

In an additional experiment, the working enzyme complex is formed of proteins of different species using the human P450 form (CYP3A4) and the rabbit reductase. The catalytic cycle time is 2.8 ± 0.2 s with the rabbit reductase and 2.5 ± 0.1 s for the human reductase. Obviously, the catalytic cycle time is independent of the reductase used. However, complex formation of the monooxygenase system with enzymes of different species is different. The dissociation constant K_D divided by $[\text{P450}]_0$ is 4.7 ± 0.2 for the rabbit reductase, and 0.3 ± 0.05 for the human reductase. These results are important to understand the enzymes as molecular machines since the rabbit reductase differs significantly by 13 amino acid residues in its membrane-binding domain compared with that of the human reductase whereas the cytosolic part of both proteins have nearly the same primary structure (Porter and Kasper, 1985; Katagiri *et al.*, 1986; Hanui *et al.*, 1989; Shephard *et al.*, 1992). Now, the results of Müller-Enoch (1999) are understandable where he investigated the enzyme activity of one human P450 form (CYP3A4) with three different reductase forms (man, rabbit, and rat). The nifedipine oxidase enzyme activity was reduced by a factor of 5 using the animal reductase in the binary complex at a fixed reductase : P450 ratio of 2.

7-Ethoxycoumarin O-deethylase activity

In the last step, two different rat P450 forms (CYP2B1 and CYP1A1) are used in combination with the rat reductase. The results are shown for the 7-ethoxycoumarin O-deethylase activity in Fig. 2. The catalytic cycle time of the CYP1A1:reductase complex is 0.6 ± 0.05 s and of 1.8 ± 0.1 s. This shows that the maximal enzyme activity is different for both forms (100 ± 7 and 33 ± 2 ($[\text{nmol}(7\text{-hydroxycoumarin})] \cdot [\text{nmol P450}]^{-1} \cdot \text{min}^{-1}$), respectively). This can be explained by the different primary structure of both forms at the catalytic center. The complex formation for both P450 forms with the same reductase is different. The normalized dissociation constant $K_D/[\text{P450}]_0$ is 1.7 ± 0.2 (CYP1A1) and 2.3 ± 0.2 (CYP2B1). Again, the membrane-binding peptides of both forms differ significantly by 16 amino-acid residues.

Conclusions

The enzyme activity depends basically on two protein domains of the binary complex: The cytosolic catalytic domain and the membrane-binding domain: (i) The formation of the binary complex depends essentially on the membrane-binding do-

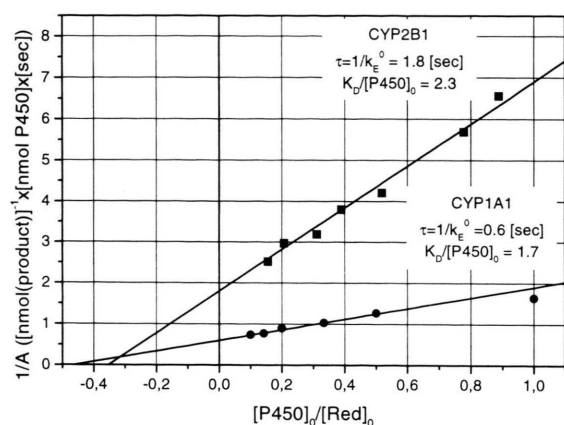


Fig. 2. The 7-ethoxycoumarin O-deethylase activity of rat cytochrome P450:reductase complexes are shown. The inverse enzyme activity, $1/A$, is plotted versus the P450/reductase ratio. In the experiment the amount of reductase was varied: The circles and the squares represent the activities with the form (CYP1A1) and the form (CYP2B1), respectively. Straight lines were fitted to the experimentally determined dots: The cycle times, $\tau = 1/k_E^0$, 0.6 s and 1.8 s are obtained for $x = 0$, corresponding to maximal enzyme activities of 100 ± 7 and 33 ± 2 ($[\text{nmol}(7\text{-hydroxycoumarin})] \cdot [\text{nmol P450}]^{-1} \cdot \text{min}^{-1}$), respectively. The normalized dissociation constant $K_D/[P450]_0$, 1.7 and 2.3 are obtained from the slope of the straight lines.

main. (ii) The catalytic cycle time for the catalytic reaction depends on the cytosolic domain. Hence, the enzyme activity can be optimized by two different ways: Either by changing the catalytic domain to make the chemical reaction very fast or by changing the membrane-binding domain to

produce a large number of working binary complexes.

Our results are of general interest for understanding the self-organization of complex membrane-bound molecular machines. The above discussed monooxygenase system is one example but other complex systems are the intracellular signal transduction chains where membrane-bound proteins are involved. In the case of the monooxygenase system the two enzymes form a working complex which has a life time of approximately three times the catalytic cycle time (Gruler and Müller-Enoch, 1991). But in the case of a signal transduction chain the lifetime of the binary complex formed must be very short. For example in the G-protein driven signal chain where one activated membrane-bound receptor activates one to two thousands membrane-attached G-proteins per second. Likewise, our results can be used to create tools for making artificial self-organized membrane-bound molecular machines. E.g. one has the task to bring the functional group like a catalytical active group of a polymer to a specific macromolecular target molecule. If the macromolecules are anchored in a lipid matrix then the anchor has to be constructed in such a way that the elastic membrane distortions lead to attractive forces (Gruler and Sackmann, 1977; Marcerou *et al.*, 1984; Leibler, 1986).

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