Biochemistry (Moscow), Vol. 70, No. 3, 2005, pp. 357-365. Translated from Biokhimiya, Vol. 70, No. 3, 2005, pp. 438-446.

Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM04-114, October 10, 2004.

Engineering of Proteolytically Stable NADPH-Cytochrome P450 Reductase

T. A. Bonina[1], A. A. Gilep[1], R. W. Estabrook[2], and S. A. Usanov[1]*

[1]Institute of Bioorganic Chemistry, National Academy of Sciences of Belarus, ul. Kuprevicha 5, 220141 Minsk, Belarus; fax: 375 (172) 63-7274; E-mail: usanov@iboch.bas-net.by
[2]Department of Biochemistry, University of Texas Southwestern Medical Center at Dallas, 5323 Harry Hines Blvd., Dallas, TX, 75235-9038, USA; E-mail: RonaldEstabrook@UTSouthwestern.edu

Received May 11, 2004
Revision received June 11, 2004

Abstract—NADPH-cytochrome P450 reductase (CPR) is a membrane-bound flavoprotein that interacts with the membrane via its N-terminal hydrophobic sequence (residues 1-56). CPR is the main electron transfer component of hydroxylation reactions catalyzed by microsomal cytochrome P450s. The membrane-bound hydrophobic domain of NADPH-cytochrome P450 reductase is easily removed during limited proteolysis and is the subject of spontaneous digestion of membrane-binding fragment at the site Lys56–Ile57 by intracellular trypsin-like proteases that makes the flavoprotein very unstable during purification or expression in *E. coli*. The removal of the N-terminal hydrophobic sequence of NADPH-cytochrome P450 reductase results in loss of the ability of the flavoprotein to interact and transfer electrons to cytochrome P450. In the present work, by replacement of the lysine residue (Lys56) with Gln using site directed mutagenesis, we prepared the full-length flavoprotein mutant Lys56Gln stable to spontaneous proteolysis but possessing spectral and catalytic properties of the wild type flavoprotein. Limited proteolysis with trypsin and protease from *Staphylococcus aureus* of highly purified and membrane-bound Lys56Gln mutant of the flavoprotein as well as wild type NADPH-cytochrome P450 reductase allowed localization of some amino acids of the linker fragment of NADPH-cytochrome P450 reductase relative to the membrane. During prolong incubation or with increased trypsin ratio, the mutant form showed an alternative limited proteolysis pattern, indicating the partial accessibility of another site. Nevertheless, the membrane-bound mutant form is stable to trypsinolysis. Truncated forms of the flavoprotein (residues 46-676 of the mutant or 57-676 of wild type NADPH-cytochrome P450 reductase) are unable to transfer electrons to cytochrome P450c17 or P4503A4, confirming the importance of the N-terminal sequence for catalysis. Based on the results obtained in the present work, we suggest a scheme of structural topology of the N-terminal hydrophobic sequence of NADPH-cytochrome P450 reductase in the membrane.

Key words: cytochrome P450, NADPH-cytochrome P450 reductase, site-directed mutagenesis, heterologous expression in *E. coli*, affinity chromatography, electron transfer

NADPH-cytochrome P450 reductase (CPR) is a membrane-bound flavoprotein localized in endoplasmic reticulum membranes; it participates in electron transfer from NADPH to cytochrome P450, as well as other microsomal enzymes and various artificial electron acceptors. The flavoprotein contains one molecule of both FAD and FMN as prosthetic groups, and electron transfer proceeds from NADPH to FAD, then to FMN and finally to the heme of cytochrome P450 [1-3]. The NADPH-cytochrome P450 reductase has at least two structurally organized domains: a hydrophobic N-terminal membrane-binding domain (6 kD), which serves to anchor the flavoprotein to the endoplasmic reticulum membrane and is necessary for reduction of cytochrome P450, and functional hydrophilic C-terminal catalytic domain involved in binding of FMN, FAD, and NADPH [4]. The N-terminal hydrophobic fragment of NADPH-cytochrome P450 reductase includes hydrophilic (Met1-Asp27) and hydrophobic (Met28-Phe44) regions, followed by a cluster of six charged amino acid residues containing stop-transfer signal [5], and a flexible, protease-sensitive region (Lys47-Lys56). According to earlier published data [6], the N-terminal hydrophilic fragment of NADPH-cytochrome P450 reductase is faced on the inner side of the endoplasmic reticulum membrane, while the hydrophobic fragment spans the phospholipid bilayer to form α-helix. At the same time, the C-terminal structural domain of NADPH-cytochrome P450 reductase is localized on the outer side of the endoplasmic reticulum membrane and is faced to the cytosol. The linker frag-

* To whom correspondence should be addressed.

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ment is localized on the border of the membrane bilayer and cytosol. The functional role and membrane organization of amino acid residues of this fragment are still not clearly understood.

The membrane-bound NADPH-cytochrome P450 reductase can be solubilized from endoplasmic reticulum membrane by limited proteolysis with pancreatic steapsin or trypsin, releasing the C-terminal hydrophilic 72-kD domain capable of reducing non-physiological, artificial electron acceptors such as cytochrome *c*, but incapable of transferring electrons to cytochromes b_5 and P450 [7-9]. The preferential site of trypsin attack on the rat NADPH-cytochrome P450 reductase was shown to be at Lys56–Ile57 [10]. During expression of NADPH-cytochrome P450 reductase in *E. coli*, purification, or long storage, intracellular trypsin-like proteases cleave the enzyme to form the truncated form of the flavoprotein by removing from NADPH-cytochrome P450 reductase the N-terminal membrane-binding domain. Therefore, final preparations of the purified flavoprotein usually besides full-length flavoprotein contain N-terminally truncated NADPH-cytochrome P450 reductase [11]. The site of spontaneous digestion of rat NADPH-cytochrome P450 reductase was found to be Lys56–Ile57, which makes the flavoprotein very unstable [10]. The low stability of the NADPH-cytochrome P450 reductase molecule is the reason for many unsuccessful attempts to study structure and function of full-length protein by using such methods as crystallography and expression of fusion proteins. The aim of the present work was site-directed substitution of amino acid residue Lys56 in an attempt to engineer proteolytically stable to endogenous bacterial proteases full-length NADPH-cytochrome P450 reductase for further structure-functional studies, preparation of fusion proteins, and crystallization of full-length flavoprotein.

At the present time, the most studied and characterized is the hydrophilic domain of NADPH-cytochrome P450 reductase, while the questions of the role of some amino acid residues of the hydrophobic domain as well as their localization in the membrane are still open. Earlier it was shown [10] that the linker sequence has specific sites for trypsin and protease from *St. aureus*. In the present work, using limited proteolysis we determined localization of these specific sites relative to the membrane and compared the catalytic properties of truncated forms of Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase. The data obtained indicate the direct participation of the N-terminal sequence (residues 1-46) in interaction with cytochrome P450.

MATERIALS AND METHODS

Chemicals. In the present study we used Emulgen 913 from Kao Atlas (Japan), restriction endonucleases and other modifying enzymes from New England Biolabs (USA), tryptone, peptone, and yeast extract from Difco (USA), and some other chemicals from Sigma (USA).

Site-directed mutagenesis and construction of expression plasmid for mutant form of NADPH-cytochrome P450 reductase. The site of protease attack (Lys56 of rat NADPH-cytochrome P450 reductase) was substituted by Gln. Site-directed mutagenesis was carried out using the Quick Change kit (Stratagene, USA) and the following primers:

K56Qd – GAGATACCGGAGTTCAGCCAGATCCAA-ACAACGGCCCCACCCG,

K56Qr – CGGGTGGGGCCGTTGTTTGGATCTGG-CTGAACTCCGGTATCTC.

Escherichia coli JM109 cells were transformed with the plasmid pRatCPR (Lys56Gln). The correctness of the site-directed modification and the absence of undesired modifications were confirmed by automatic sequencing using an A310 DNA sequencer (Applied Biosystems, USA).

Expression of recombinant proteins in *E. coli* and protein purification. Wild type reductase and its Lys56Gln mutant were purified from *E. coli* membranes using a combination of anion exchange and 2′,5′-ADP-Sepharose affinity chromatography [7]. Bacterial membranes obtained from two liters of culture were suspended in buffer A (50 mM Tris, pH 7.5, 0.5 mM EDTA, 0.5 mM PMSF, 20% glycerol) and solubilized with Triton X-100 (final concentration 1%). Insoluble material was removed by centrifugation at 100,000*g* for 60 min at 4°C, and the supernatant fraction was applied to a DE-52 column equilibrated with buffer B (50 mM Tris-HCl, pH 7.5, containing 20% glycerol, 0.5 mM EDTA, 0.5 mM PMSF, 0.1 mM DTT, 1 μM FMN, and 0.1% Triton X-100). The column was washed with 10 volumes of buffer B containing 0.13 M NaCl and then the protein was eluted using a linear gradient of NaCl (0.13-0.5 M NaCl) in buffer B. Fractions containing the flavoprotein were pooled, diluted to final concentration of 0.05 mM NaCl with buffer B, and applied to a 2′,5′-ADP-Sepharose 4B (Pharmacia, Sweden) affinity column equilibrated with the buffer B. The column was washed with buffer B and then with buffer B containing 0.1 M NaCl. The protein was then eluted with buffer B containing 0.25 M NaCl and 5 mM 2′-AMP. Fractions containing flavoprotein were pooled and dialyzed overnight against 50 mM Tris-HCl buffer, pH 7.5, containing 20% glycerol, 0.1 mM EDTA, and 0.1 mM DTT to remove 2′-AMP. The purified protein was stored frozen at −80°C. Recombinant goat cytochrome P450c17 and recombinant cytochrome P4503A4 were expressed and purified from *E. coli* as described previously [12, 13].

The concentration of cytochrome P450 was determined on a Shimadzu 3000 spectrophotometer using mil-

limolar extinction coefficient ε 91 $mM^{-1}·cm^{-1}$ at 450-490 nm [14]. Flavoprotein concentration was estimated based on the flavin content, using millimolar extinction coefficient of 21.4 $mM^{-1}·cm^{-1}$ at 456 nm [15].

Spectral analysis. Absorption spectra for re-oxidation of reduced purified wild type and Lys56Gln mutant form of NADPH-cytochrome P450 reductase were recorded using a Specord M40 (Specord, Germany). The reductase was diluted to a concentration of 7 μM using 0.1 M potassium phosphate (pH 7.7) containing 10% glycerol and 0.1 mM EDTA. An aliquot of NADPH (18 μM final concentration) was added, and the spectra were scanned at the indicated time periods.

Cytochrome *c* reductase assay. Enzymatic reduction of cytochrome *c* was determined using the Shimadzu UV-3000 (Shimadzu, Japan) spectrophotometer. The kinetics assay system contained 0.3 M sodium phosphate, pH 7.7, 50 μM cytochrome *c*, and 250 μM NADPH. Reactions were initiated with NADPH. Kinetics assays relied on the change in absorbance at 550 nm with an extinction coefficient of 21.0 $mM^{-1}·cm^{-1}$ [15]. Rates were linear with respect to time and reductase concentration. Measurements were made at room temperature (24°C). Activities are expressed as nanomoles of acceptor reduced in 1 min per 1 mg protein.

Determination of progesterone 17α-hydroxylase activity of recombinant goat cytochrome P450c17. Steroid hydroxylation in the reconstituted system by recombinant cytochrome P450c17 was carried out at 37°C in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ [12]. Highly purified recombinant goat cytochrome P450c17 was used at the final concentration 0.5 μM. Wild type or mutant NADPH-cytochrome P450 reductase was added to the incubation mixture at the final concentration 1 μM. Progesterone was dissolved in ethanol and used at final concentration 50 μM. The reaction was started by addition of NADPH at final concentration 0.5 mM. Samples (0.5 ml) were taken from the incubation mixture at different time intervals and rapidly mixed with 5 ml of methylene chloride. The layers were separated by centrifugation. The organic layer was removed and dried under a stream of nitrogen. The residue was dissolved in 100 μl of methanol, and the product formation was analyzed by HPLC on a C_{18} Bondapak column (3.9 × 300 mm) (Waters, USA). Steroid metabolites were identified based on the retention time of known standards.

6β-Hydroxylation of testosterone by recombinant cytochrome P4503A4. A mixture of recombinant cytochrome P4503A4 and NADPH-cytochrome P450 reductase was added to DOPC and CHAPS in 50 mM Tris-HCl buffer, pH 7.4, containing 10 mM $MgCl_2$ and incubated 10 min at 37°C with stirring. Then the samples were diluted to a final volume of 1.4 ml with buffer containing testosterone and an NADPH regenerating system. Final concentrations in the reaction mixture were 0.5 μM for cytochrome P4503A4, 1 μM NADPH-cytochrome P450 reductase, 0.15 mg/ml DOPC, 0.25 mg/ml CHAPS, 100 μM testosterone, 8 mM sodium isocitrate, and 0.1 unit/ml isocitrate dehydrogenase.

Samples were incubated for 5 min at 37°C, and the reaction was initiated by the adding of 100 μl NADPH (1 mM final concentration). Aliquots (0.5 ml) were removed after 10 and 30 min and rapidly mixed with 5 ml of methylene chloride. The methylene chloride layer was carefully removed and dried under a nitrogen stream. The residue was dissolved in 100 μl of methanol, and product formation was analyzed as described above.

Analytical methods. Analysis of protein composition of the bacterial cells and control of the purity of purified protein preparations was carried out by using SDS-PAGE according to the general procedure [16] using a Bio-Rad Mini Protean II apparatus (Bio-Rad, USA) using 10% separating gel. Proteins were stained with Coomassie Brilliant Blue R-250.

Limited proteolysis of NADPH-cytochrome P450 reductase. Highly purified NADPH-cytochrome P450 reductase (120 μg) in 1.0 ml of 50 mM Tris-HCl, pH 7.5, was incubated at 37°C with 0.6 μg of trypsin (trypsin/protein ratio 1 : 200). At the indicated times, 40 μl aliquots were transferred to tubes containing 10 μl of a 4-fold excess of soybean trypsin inhibitor (Sigma). Then one volume of 2× electrophoresis sample buffer was added. Samples (10 μl) were then directly loaded on SDS-PAGE gels. Membrane-bound reductase was incubated with trypsin at the trypsin/protein ratio of 1 : 100 (w/w) in 50 mM Tris-HCl, pH 7.5. The gels were of 10% polyacrylamide.

Purified or membrane-bound flavoproteins were incubated with protease from *St. aureus* in 10 mM sodium phosphate buffer, pH 7.0, during 2 h at 37°C using enzyme/substrate ratio 1 : 50 or 1 : 25 (w/w), respectively. Proteolysis was stopped by adding of one volume of 2× sample buffer containing 0.3% trichloroacetic acid, which preserves the sample from further proteolysis during electrophoresis.

N-Terminal sequencing of the limited proteolysis products was done using a sequencer (Applied Biosystems).

RESULTS AND DISCUSSION

NADPH-cytochrome P450 reductase is a unique universal donor of electrons to practically all known microsomal cytochrome P450s and one of the most important components of the monooxygenase system. However, it is still not clear how the single NADPH-cytochrome P450 reductase can interact with different cytochrome P450s and what is the role of the N-terminal membrane-bound fragment in this interaction. The membrane-binding hydrophobic segment of the NADPH-cytochrome P450 reductase is rather conserva-

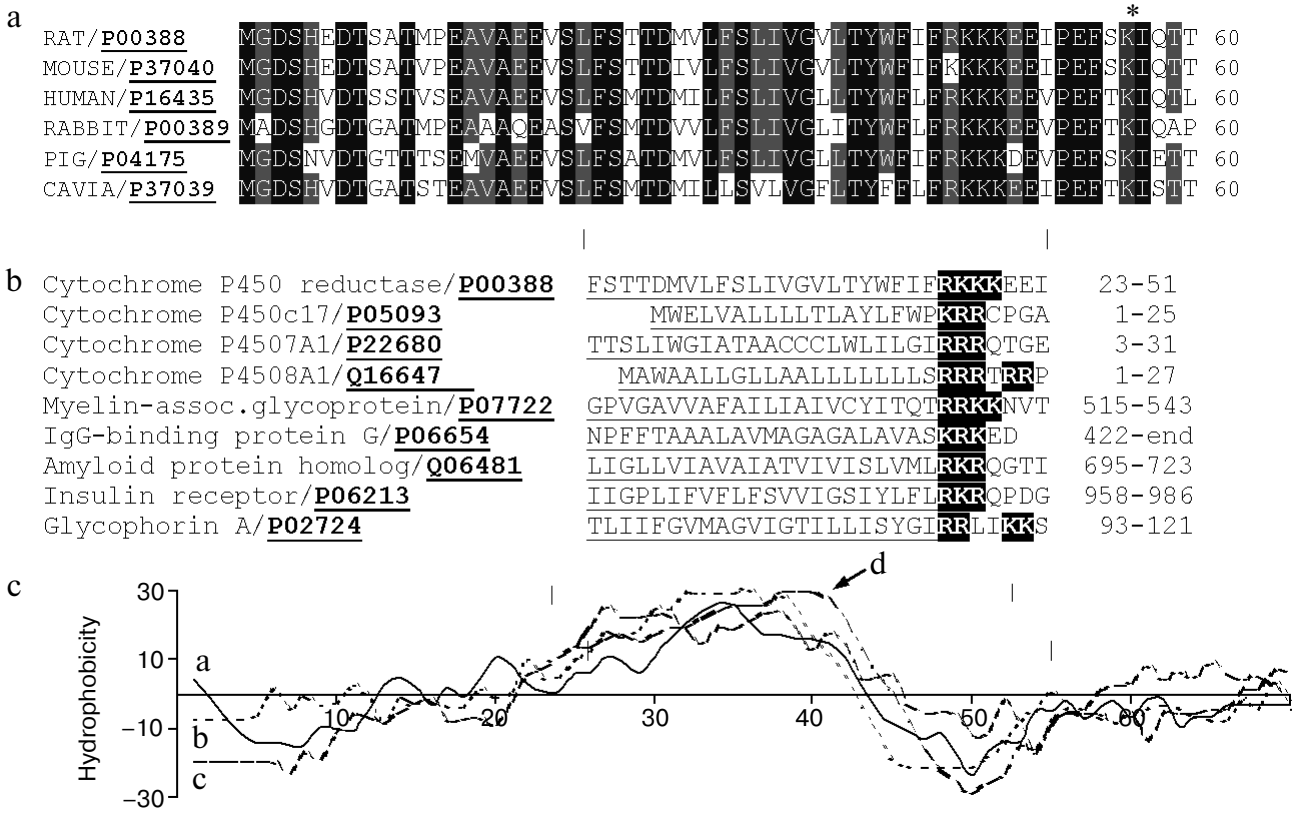

Fig. 1. N-Terminal membrane-binding sequences of NADPH cytochrome P450 reductases (a), amino acid sequences (b), and hydrophobic profiles (c) of membrane-bound proteins. The conservative residues are marked in black. Proteins are marked in accordance with their SWISS-PROT numbers. Proposed membrane fragments are underlined. Hydrophobic profiles of membrane-binding domains of proteins are calculated using the Protean program (DnaStar, USA): NADPH cytochrome P450 reductase (*a*, residues 1-60), myelin-associated glycoprotein (*b*, residues 491-551), glycophorin A (*c*, residues 71-131), and cytochrome P4508A1 (*d*, residues 1-36). The asterisk marks the site of trypsin attack.

tive among different representatives (Fig. 1) and contains unique and specific information essential for protein–protein recognition to realize the reactions of intermolecular electron transfer. We recently showed that fragments of the polypeptide chain of the proteins interacting with endoplasmic reticulum membrane independent of their localization either in N- or C-terminal sequence are rather conservative. Thus, the hydrophobic part of NADPH-cytochrome P450 reductase can be replaced by another hydrophobic region of another protein. The N-terminal membrane-binding segment of NADPH-cytochrome P450 reductase can be replaced by the C-terminal membrane-binding sequence of cytochrome b_5 to form a chimeric flavoprotein that is enzymatically active when reconstituted with some cytochrome P450s [17, 18].

The understanding of the molecular mechanisms by which these interactions occur is still an important biological goal. To solve this problem, structural studies of NADPH-cytochrome P450 reductase using spectral, electrochemical, and spectroscopic methods and kinetic analysis and site-specific mutagenesis are essential. Crystals of hydrophilic fragments of NADPH-cytochrome P450 reductase and some other domains of flavoprotein have been prepared [19, 20]. However, no three-dimensional structure of full-length NADPH-cytochrome P450 reductase has yet been reported, although the crystallization of a N-terminal truncated rat NADPH-cytochrome P450 reductase and separate domain of human NADPH-cytochrome P450 reductase has been described [19, 20]. Also, it was not possible to express full-length NADPH-cytochrome P450 reductase as a fusion protein with cytochrome P450 [21].

The fact that the full-length NADPH-cytochrome P450 reductase contains hydrophobic fragments responsible for interaction with membrane, which are highly conserved for different species (Fig. 1a) and can be easily removed during flavoprotein purification, storage, or crystallization, suggested that the removal of a protease-sensitive site by a single amino acid substitution (Lys56Gln) might lead to significant protein stabilization. Since rat NADPH-cytochrome P450 reductase, as well as

reductases from other species, has a conservative trypsin-sensitive site at position Lys56–Ile57, we introduced a single mutation (Lys56Gln) in order to prevent digestion and further degradation of the membrane-binding domain. An amino acid similar to Lys, such as Gln, was chosen in order to prevent potential negative effects on the folding and/or stability of the protein fragment. Similarly, the whole α-dystroglycan molecule was crystallized after mutation of a protease-susceptible site due to increase in protein stability [22].

Expression, purification, and characterization of Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase. Both wild type and Lys56Gln mutant of NADPH-cytochrome P450 reductase were successfully expressed in *E. coli*. The NADPH-cytochrome P450 reductase content was determined using the cytochrome *c* reduction assay. The specific contents, catalytic activities, and expression levels for the different flavoproteins are shown in the table. As expected, the Lys56Gln mutant expressed at a slightly higher level, approximately 1.4 μmol/liter compared to the wild type NADPH-cytochrome P450 reductase of 0.96 μmol/liter. This appears to be due to better stability of the mutant to proteolytic modification. The mutant and wild type NADPH-cytochrome P450 reductase after detergent solubilization from *E. coli* membranes were purified by ion-exchange chromatography and 2′,5′-ADP-Sepharose affinity chromatography. Figure 2 shows the results of SDS-PAGE of the Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase after storage at 4°C for 48 h. The wild type NADPH-cytochrome P450 reductase together with full-length also contains truncated flavoprotein, while the Lys56Gln mutant has only one band corresponding to the full-length NADPH-cytochrome P450 reductase. These data indicate the much greater stability of the Lys56Gln mutant to spontaneous proteolysis that is confirmed by higher expression level of Lys56Gln mutant of NADPH-cytochrome P450 reductase as compared to wild type flavoprotein.

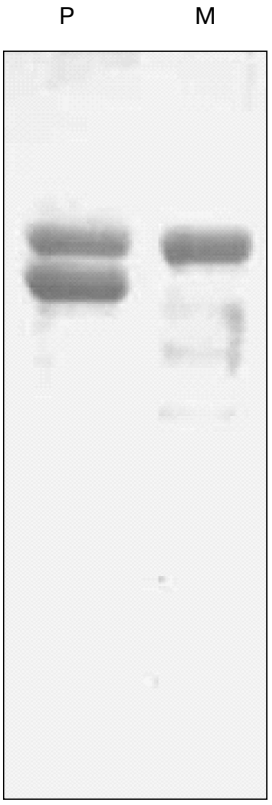

Fig. 2. SDS-PAGE of highly purified wild type NADPH cytochrome P450 reductase (P) and Lys56Gln mutant flavoprotein (M) after storage at 4°C for 48 h.

Both flavoproteins were further assayed by their ability to reduce cytochrome *c* and were found to have similar catalytic parameters (table). They also showed no significant differences in activity as tested in reconstituted system using cytochrome P450c17 as electron acceptor. A similar result was obtained when measuring the ability of Lys56Gln mutant and wild type NADPH-cytochrome

Expression level and catalytic activity of highly purified Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase

P450 reductase	Expression level, μmol/liter*	Yield, μmol/liter	Activity, min^{-1}		
			cytochrome *c***	cytochrome P450c17***	cytochrome P4503A4***
Wild type	0.96	0.23	2580	6.9	4.7
Lys56Gln	1.4	0.34	2770	7.4	5.2

Note: Conditions for reconstitution of monooxygenase activity are described above in "Materials and Methods". The results presented are means of at least two experiments and standard deviations do not exceed 10% of the average value.

* Liter of culture.

** In nanomoles of reduced cytochrome *c* in 1 min per 1 nmol of flavoprotein.

*** In nanomoles of product in 1 min per 1 nmol of cytochrome P450.

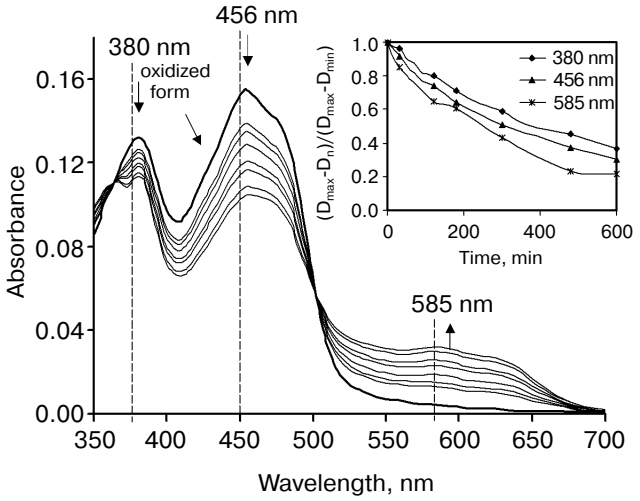

Fig. 3. Kinetics of oxidation of reduced wild type NADPH cytochrome P450 reductase and the Lys56Gln mutant. Absorbance spectra were obtained after addition of 2.5-fold molar excess of NADPH. Absorbance spectrum of oxidized NADPH cytochrome P450 reductase is shown by the bold line. The other spectra were recorded at definite time intervals after addition of NADPH. Spectral changes at a definite wavelength during oxidation were normalized using the equation, where D_{max} is an absorbance of oxidized form of flavoprotein, D_{min} is an absorbance on addition of NADPH, and D_n is an absorbance after definite time intervals.

P450 reductase to support the 6β-hydroxylation of testosterone by cytochrome P4503A4 (table). These data indicate that replacement of Lys56 with Gln does not have any significant effect on the ability of NADPH-cytochrome P450 reductase to interact and supply electrons to redox partners.

Both the wild type NADPH-cytochrome P450 reductase and Lys56Gln mutant are characterized by the characteristic spectrum with maximal absorption at 455 and 380 nm [14] (Fig. 3). Addition of NADPH to purified NADPH-cytochrome P450 reductase followed by re-oxidation (by incubating at room temperature for 10 min) results in decrease in the absorbance at 450 nm and the appearance of the "air-stable" neutral flavin semiquinone form of flavoprotein, characterized by the broad absorbance peak at 585 nm (Fig. 3). After reduction the flavoprotein becomes slowly oxidized in the presence of molecular oxygen, resulting in the complete oxidation of the proteins, as evidenced from repetitive scan spectrophotometric measurements of changes in the absorption spectrum. Measurements of the kinetics of re-oxidation of both NADPH-cytochrome P450 reductases were indistinguishable. Thus, the data indicate that replacement of Lys56 with Gln does not result in any changes in spectral properties of the flavoprotein and does not change protein folding and flavin binding.

Proteolysis of Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase. The treatment of highly purified Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase with trypsin under the conditions described above results in the time-dependent appearance of two bands corresponding to truncated flavoproteins with different molecular weights of about 74 and 72 kD, respectively. The SDS-PAGE of these proteolytic products is shown in Fig. 4a. As follows from Fig. 4, although Lys56Gln mutant of NADPH-cytochrome P450 reductase is much more stable to proteolysis with trypsin as compared to the wild type flavoprotein, increase in the trypsin concentration nevertheless results in proteolytic modification of the mutant flavoprotein. Truncated forms of the Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase have different molecular masses, 74 and 72 kD, respectively (Fig. 4). This also indicates that proteolytic modification of the two flavoproteins takes place at different proteolytic sites. Marked differences are also observed in kinetics of limited proteolysis of Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase. The wild type NADPH-cytochrome P450 reductase after 1 min incubation is completely converted to the truncated form, while the formation of the truncated form of Lys56Gln mutant is seen only after 10 min incubation (Fig. 4). It was shown earlier by Black and Coon [10] that after elimination of the hydrophobic fragment of the flavoprotein at Lys56, a

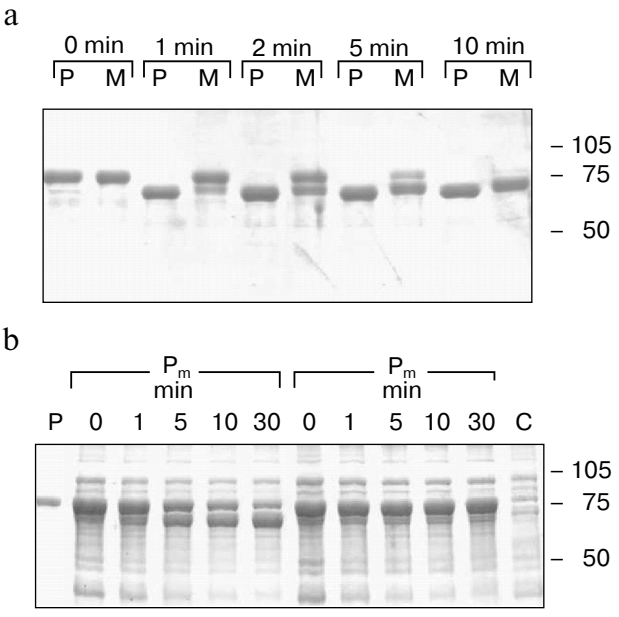

Fig. 4. SDS-PAGE of the products of limited proteolysis of purified flavoproteins (a) (P, wild type NADPH cytochrome P450 reductase; M, Lys56Gln mutant) and membrane-bound forms (b) (P_m, wild type NADPH cytochrome P450 reductases (membrane-bound form); M_m, mutant form of NADPH cytochrome P450 reductase (membrane-bound form); C, bacterial membranes not containing recombinant NADPH cytochrome P450 reductase).

second sensitive to trypsin site in NADPH-cytochrome P450 reductase appeared in the by Lys46–Lys47–Lys48 sequence, localized in the linker sequence connecting two structural domains. These results indicate that the sites sensitive to the trypsin cleavage are different in wild type NADPH-cytochrome P450 reductase and mutant form and have distinct limited proteolysis patterns. The replacement of the single amino acid at position Lys56 results in the dramatic increase in the stability against trypsin attack. However, prolong incubation or increasing of trypsin ratio results in proteolytic modification of the Lys56Gln mutant via limited proteolysis through the second proteolytic site with formation of truncated form with higher molecular weight.

To confirm our conclusion, we did a series of experiments on limited proteolysis of membrane-bound form of Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase to understand how membrane affects the proteolytic digestion of the mutant. For that purpose, membrane fractions of recombinant *E. coli* expressing Lys56Gln mutant and wild type NADPH-cytochrome P450 reductase were analyzed by SDS-PAGE after incubation with trypsin for the indicated times (Fig. 4b). The results of these experiments indicate that membrane-bound wild type NADPH-cytochrome P450 reductase is progressively digested as a result of trypsin treatment, while the membrane-bound Lys56Gln mutant of NADPH-cytochrome P450 reductase (lanes marked M_m) is absolutely stable to trypsinolysis. Thus, the interaction with membrane or the close proximity of the region corresponding to second recognition site to the membrane surface may mask residues 46-48 from tryptic attack. These results show that the fragment of the polypeptide chain including the sequence Lys46–Lys47–Lys48 that becomes accessible to proteolysis with high trypsin concentration in the Lys56Gln mutant is not accessible to trypsin when using membrane-bound Lys56Gln mutant. This indicates that this fragment of polypeptide chain is either localized on the surface of the membrane or even is inserted into the endoplasmic reticulum membrane. It is necessary to stress that limited proteolysis with proteases and in particular with trypsin of specific amino acid residues of membrane-bound proteins is a commonly used approach for determination of topology of these residues in the membrane [23, 24] and facilitated further building of topological models for trans-membrane domain of these proteins. It is suggested that amino acid residues localized inside the membrane are usually not accessible to proteases, while residues exposed on the surface of the membrane are subjected to limited proteolysis.

The fragment of polypeptide chain corresponding to the second recognition site is absolutely conserved for NADPH-cytochrome P450 reductases from different species and contains a cluster of positively charged residues flanked by membrane-binding hydrophobic segments, which is very typical for proteins integrated into the endoplasmic reticulum (Fig. 1, a and b). However, different membrane-bound proteins have short amino acid sequences enriched with positively charged residues (arginine and lysine) close to the extended hydrophobic fragments localized in the phospholipid bilayer (Fig. 1b) and their hydrophobic profiles are very similar (Fig. 1c). These structural elements in proteins appear to carry information on signal sequence and/or stop-transfer sequence, which are determinants involved in topogenesis of integral membrane proteins targeted to and across the endoplasmic reticulum membrane [25, 26]. In the case of NADPH-cytochrome P450 reductase, such membrane domain is responsible not only for membrane anchoring of the protein but also it is an obligatory component for interaction with redox partners. The amino acids of NADPH-cytochrome P450 reductase membrane domain responsible in intermolecular interaction are still unknown. Therefore, these fragments have a great importance. Moreover, it has not been excluded that the cluster of the positively charged residues due to its high polarity may be involved in this process.

It was shown earlier that Glu53 of NADPH-cytochrome P450 reductase is the specific site for cleavage of the flavoprotein with protease from *St. aureus* [10]. To localize this residue relative to the membrane, we performed limited proteolysis of NADPH-cytochrome P450 reductase with protease from *St. aureus* using both purified and membrane-bound flavoprotein. SDS-PAGE analysis of proteolytic modification products is shown in Fig. 5. The molecular weight of the fragment formed during proteolytic modification of purified NADPH-cytochrome P450 reductase coincide with the molecular weight of the fragment formed on limited proteolysis of membrane-bound Lys56Gln mutant and wild type flavoprotein, indicating that Glu53 is accessible to proteolytic attack in membrane-bound flavoprotein and, therefore, is exposed to the cytosol. Summarizing the data obtained in the present work, we suggest a scheme of the structural organization of the linker connecting two domains of NADPH-cytochrome P450 reductase in membrane (Fig. 6).

Catalytic activity of NADPH-cytochrome P450 reductase after limited trypsinolysis. Progesterone 17α-hydroxylation activity of P450c17 and testosterone 6β-hydroxylase activity of cytochrome P4503A4 in a reconstituted system were not detected in the presence of truncated forms of either wild type NADPH-cytochrome P450 reductase or Lys56Gln mutant obtained by adding of trypsin to corresponding full-length NADPH-cytochrome P450 reductases. This indicates that the truncated form of the mutant containing hydrophilic domain and linker region of NADPH-cytochrome P450 reductase also is unable to transfer electrons to cytochrome P450. As a control, we used treatment of preparation of full-length NADPH-cytochrome P450 reductase with

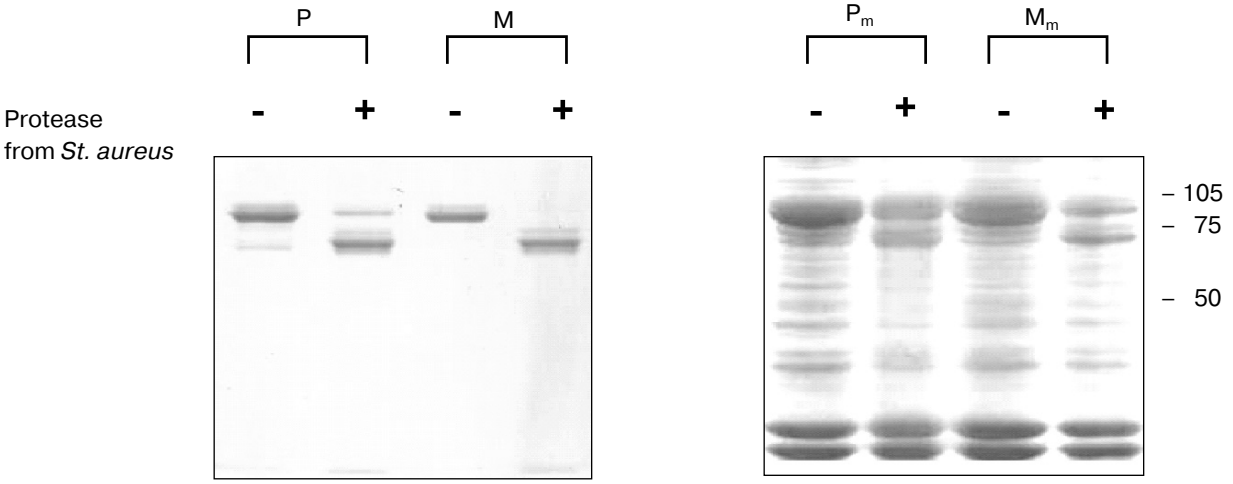

Fig. 5. SDS-PAGE of the products of limited proteolysis with protease from *St. aureus* of purified wild type NADPH cytochrome P450 reductase (P) and Lys56Gln mutant (M) and their membrane-bound forms, P_m and M_m, respectively.

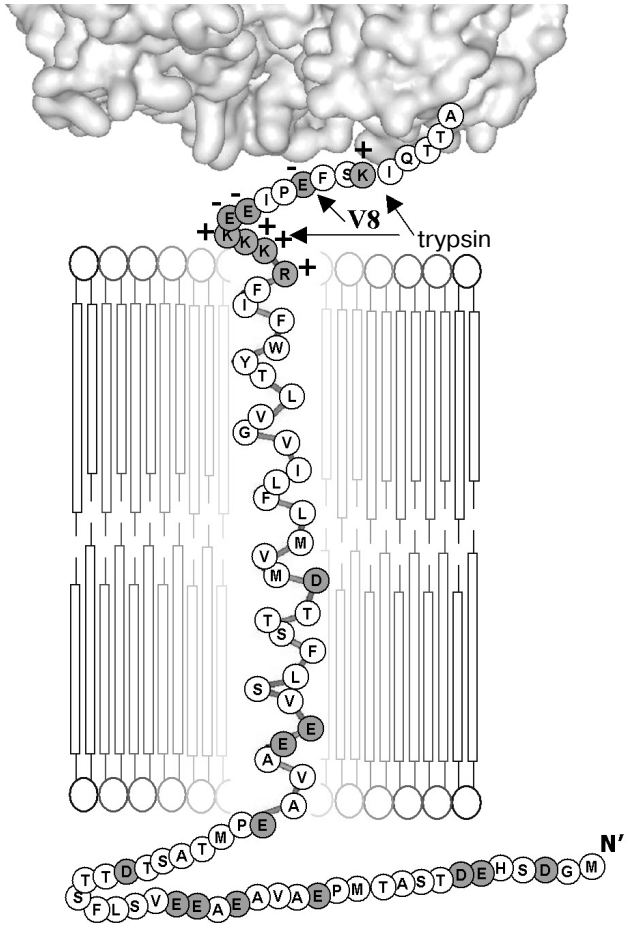

Fig. 6. Structural model of molecular organization of NADPH-cytochrome P450 reductase in the membrane.

trypsin in the presence of soybean inhibitor, which does not affect the enzyme activity of flavoproteins.

From these results, it follows that the presence of the extra highly charged region Lys48-Lys56 does not significantly improve the catalytic activity of truncated form of wild type NADPH-cytochrome P450 reductase.

The fact that the truncated form of NADPH-cytochrome P450 reductase is unable to transfer electrons to cytochrome P450c17 in the presence of hydrophobic fragment suggested that even the mixture of hydrophobic and hydrophilic domains of flavoprotein cannot replace the full-length molecule of NADPH-cytochrome P450 reductase in the hydroxylation reaction in the reconstituted system as catalyzed by recombinant cytochrome P450c17. Similar conclusions were obtained for another cytochrome P450 reconstituted system [8]. Since membrane anchoring of NADPH-cytochrome P450 reductase is essential for its electron transfer to the cytochrome P450, structural and topological information of the membrane-binding domain should be critical to understand the reaction mechanism.

In conclusion, in the present work we engineered, expressed, and purified proteolytically stable full-length NADPH-cytochrome P450 reductase retaining similar catalytic and spectral properties to wild type flavoprotein, which can serve an excellent model protein for further studies of the functional role of the hydrophobic domain and its interaction with membrane and use for possible crystallization of full-length flavoprotein. It is shown that truncated forms of flavoprotein (residues 46-676 of the mutant and residues 57-676 of wild type NADPH-cytochrome P450 reductase) are not able to transfer electrons to cytochrome P450c17 and P4503A4, i.e., addition to the hydrophilic fragment of an extra linker

sequence does not restore activity of the truncated forms, confirming direct participation of the hydrophobic N-terminal sequence of NADPH-cytochrome P450 reductase (residues 1-46) in the interaction with cytochrome P450.

This work was supported in part by grants from INTAS and BRFFI grant B02M-087.

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