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Mechanism of Steroidogenic Electron Transport: Role of Conserved Glu429 in Destabilization of CYP11A1–Adrenodoxin Complex

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Abstract—In the present work the role of conserved residue E429 of cytochrome P45011A1 has been studied. The charge neutralization of E429Q results in 3-fold decrease of K_d as well as V_{max} compared to the wild type hemoprotein indicating tighter binding and, as the result, the impaired dissociation of oxidized adrenodoxin from the complex. As cytochrome P45011A1–adrenodoxin complex formation is driven primarily by electrostatic interactions, the low activity of E429Q mutant is completely restored to that of wild type hemoprotein by increasing of ionic strength. The charge neutralization of the corresponding residue of rat cytochrome P45011B2 has the same effect: the activity is 10-fold decreased but it is restored by increasing of ionic strength without effect on the ratio of products formed. Thus, this is the first report on identification of residues involved in modulation of dissociation of redox partner from the complex with cytochrome P450s.

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Mitochondrial cytochrome P450 dependent monooxygenases consist of at least three components—NADPH-ferredoxin reductase, an iron–sulfur protein (ferredoxin), and cytochrome P450 (CYP)—and play an important role in biosynthesis of many physiological bioregulators [1]. During a catalytic cycle, redox components of the cytochrome P450-dependent system form specific complexes inside of which electrons needed to activate molecular oxygen are transferred from NADPH to cytochrome P450 [2]. Recently many studies using different methodological approaches have been done in attempts to elucidate the intrinsic mechanism of electron transfer in mitochondrial cytochrome P450-dependent systems.

Adrenodoxin, a mammalian Fe_2–S_2 ferredoxin, was found to be a universal redox partner for all mitochondrial cytochrome P450s [3]. Electrostatic interactions play a crucial role in redox partner recognition and electron transfer from NADPH-adrenodoxin reductase (Adr) and adrenodoxin (Adx) to mitochondrial cytochrome P450s [3, 4]. The crystal structures of Adx, Adr, and cross-linked complex of two proteins have recently been solved [5-7], confirming that surface negatively charged residues of Adx are indeed involved in redox partner recognition and binding. Moreover, the overlapping sites on the surface of Adx involved in interaction with Adr and cytochrome P450 have been mapped by site-directed mutagenesis (for review see [3]). While the interactions of Adx and Adr are well studied [7], the mode of Adx binding to mitochondrial cytochrome P450s is still controversial since no crystal structure of any mitochondrial cytochrome P450 is yet available.

Since Adx is a one electron carrier, while for activation of molecular oxygen it is necessary to transfer at least two electrons, the involvement of two reduced adrenodoxins is evident, but the mode of second electron transfer by Adx to complete oxygen activation is still obscure. Several models have been proposed to explain the mechanism of electron transfer in mitochondrial cytochrome

Abbreviations: Adr, NADPH-adrenodoxin reductase; Adx, adrenodoxin; CPR, NADPH-cytochrome P450 reductase; CYP, cytochrome P450; 7-DHC, 7-dehydrocholesterol; DOC, 11-deoxycorticosterone; DTT, dithiothreitol; Pdx, putidaredoxin.

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P450-dependent systems. According to one of them, electron transfer between redox components is performed in an organized complex of proteins and there are two non-overlapping binding sites on the surface of adrenodoxin responsible for interaction with NADPH-adrenodoxin reductase and cytochrome P450, respectively [9]. Another one presumes only one binding site on the surface of adrenodoxin responsible for sequential binding of Adx with NADPH-adrenodoxin reductase and cytochrome P450, and to transfer two electrons to cytochrome P450 Adx should act like a shuttle to get electrons from NADPH-adrenodoxin reductase and transfer it to cytochrome P450 forming specific complexes first with one protein and then with the other one. This model does not exclude the electron transfer between free and cytochrome P450-bound adrenodoxin [10]. Finally, it has been suggested that binary complexes of electron transfer proteins might be involved in electron transfer [11].

Initially using protein chemistry, the number of positively charged lysine residues of the proximal surface of bovine cytochrome P45011A1 have been identified [12-15] as candidates for Adx binding with subsequent confirmation of their involvement by site-directed mutagenesis [16-18]. Charge neutralization of conserved Lys338 and Lys342 (here and further numeration for processed cytochrome P45011A1 without mitochondrial targeting sequence is used) results in compromised activity correlated with decreased affinity towards Adx [16]. Less dramatic effect has been observed in case of the CYP11 family-specific Lys110, 403, 405 residues [17]. Moreover, the recently identified Arg residues (425 and 426) of the meander region and the α-helix L in close vicinity to Cys422 involved in heme iron coordination have been recognized to be the most crucial in redox partner binding [18, 19]. Unlike replacements of the aforementioned residues, the mutant cytochrome P45011A1 R426Q fails to interact with immobilized Adx, to accept electrons from it, or to demonstrate any detectable activity [19]. Arg426 is a part of conserved sequence R/K-R-x-x-E specific for mitochondrial but not microsomal cytochrome P450s. According to the available models of tertiary structure of mitochondrial cytochrome P450, this charged residue of this conserved sequence is located on the solvent exposed surface of the α-helix L [18]. While the involvement of R426 in Adx binding is thought to be definitely established and recently confirmed by mutagenesis of corresponding residue of mouse cytochrome P45027B1 [20], the role of R425 is still obscure as no functional mutant protein has been expressed and characterized due to its instability. Thus, the numerous studies have not revealed the exact geometry or the number of Adx binding sites on the proximal surface of mitochondrial cytochrome P450s.

In the present work the role of a conserved glutamic acid residue (E429) of α-helix L signature motif of cytochrome P45011A1 in the interaction with redox partner has been clarified. The phenotype of cytochrome P45011A1 E429Q mutant is absolutely different from that of others described for Adx or cytochrome P450 mutants that affect the interactions of Adx and mitochondrial cytochrome P450s. The presented results suggest the role of E429 in dissociation of Adx from cytochrome P450. The data obtained in the present work indicate that residue E429 plays a critical role in regulation of affinity and dissociation of the electron transfer complex between cytochrome P45011A1 and Adx during the catalytic cycle.

MATERIALS AND METHODS

Analytical methods. Spectra were measured using a Shimadzu UV-3000 (Shimadzu, Japan) spectrophotometer. The concentration of Adr and Adx was determined spectrophotometrically using molar extinction coefficients 11 and 10 $mM^{-1}\cdot cm^{-1}$ at 450 and 414 nm, respectively [21, 22]. Concentration of cytochrome P45011A1 was determined from reduced carbon monoxide difference spectra using molar extinction coefficient 91 $mM^{-1}\cdot cm^{-1}$ at 450 nm [23].

Site-directed mutagenesis of cytochrome P45011A1 and cytochrome P45011B2. Cytochrome P45011A1 and P45011B2 were subjected to site-directed mutagenesis using a Quick Change kit (Stratagene, USA). The presence of a desired substitution in cDNAs coding cytochrome P45011A1 and P45011B2 was confirmed by automatic sequencing on an A3110 DNA sequencer (Applied Biosystems, USA).

Expression and purification of proteins. Recombinant bovine Adr and Adx were expressed in *Escherichia coli* and purified as described [24, 25]. Wild type cytochrome P45011A1 and its mutant were expressed and purified according to a scheme developed for purification of substrate-bound high-spin form of the hemoprotein [26, 27], comprising sequentially (i) ultrasonic treatment of *E. coli* cells, (ii) solubilization of sonicated lysate with Emulgen 913, (iii) precipitation of solubilized P45011A1 with polyethyleneglycol (6 kDa) at final concentration 20%, (iv) incubation of solubilized pellet with cholesterol, and (v) affinity chromatography on Adx-Sepharose-4B. Rat cytochrome P45011B2 and its mutant were expressed and purified as described previously [28].

Studies of the interaction of cytochrome P45011A1 with adrenodoxin. The spectral changes induced by adding of Adx to cytochrome P45011A1 reflecting the changes of spin state were recorded as previously described using $A_{390\text{-}470}/A_{416\text{-}470}$ difference in absorbance [26]. For the minimal ratio that corresponds to fully low-spin cytochrome P45011A1 we used the ratio 0.4, while for the maximal (100%) content of high-spin form of cytochrome P45011A1 we used the ratio 2.2.

For determination of the apparent dissociation constants for the complex of cytochrome P45011A1 with Adx

we used 1 μM cytochrome P45011A1 in 20 mM Hepes buffer, pH 7.2, containing 0.1% Tween-20, 0.1 mM EDTA, 0.1 mM dithiothreitol (DTT), 50 mM NaCl, and 20 μM cholesterol at room temperature. Different amounts of adrenodoxin were added to cytochrome P45011A1 sequentially increasing the concentration of Adx. The absence of denaturation during the spectral titration was confirmed by recording the carbon monoxide difference spectra of reduced cytochrome P45011A1.

Determination of enzymatic activity of cytochrome P45011A1 and cytochrome P45011B2. Cholesterol side-chain cleavage activity was measured in a reconstituted system with ratio Adr/Adx/CYP11A1 (1 : 8 : 4) in 25 mM Hepes buffer, pH 7.2, with 0.1 mM EDTA, 0.1 mM DTT, 0.15% sodium cholate, and variable NaCl concentrations (as specified) at 37°C. The concentrated protein mix was incubated at room temperature for 5 min and then diluted with buffer to final concentration of 1 μM cytochrome P45011A1, 2 μM Adx, and 0.25 μM Adr. 7-Dehydrocholesterol was used as the substrate. The steroid was dissolved in 45% 2-hydroxypropyl-β-cyclodextrin and added to the final concentration 100 μM. The reaction was started by adding 100 μM NADPH and a NADPH-regenerating system. The same reaction conditions were used for rat cytochrome P45011B2 with 11-deoxycorticosterone (DOC) as the substrate (stock solution in ethanol). After an indicated time of incubation, the reaction was stopped by heating in boiling water. Steroids were extracted with dichloromethane and dried under a stream of nitrogen. The samples were analyzed by reverse-phase HPLC with gradient solvent 80-100% methanol for cytochrome P45011A1 and 60% for cytochrome P45011B2 using a Shimadzu LC-10AD computerized liquid chromatograph equipped with μ-Bondapak C_{18} column (3.9 × 300 mm) and an SPD-10A UV spectrophotometric detector.

RESULTS

Bioinformatic analysis for finding targets for site-directed mutagenesis. Sequence comparison of all available mitochondrial cytochrome P450s in GenBank reveals a common pattern of charged amino acid distribution in the region corresponding to α-helix L in the known crystal structures of eukaryotic cytochrome P450s (Fig. 1a; see color insert). These residues as well as their relative position to the proximal heme ligand consensus sequence are highly conserved in cytochrome P450s of the mitochondrial clan but not in other eukaryotic cytochrome P450s. Deviations of the consensus sequence R-R-x-x-E have been observed only in a few cases. The arginine residue corresponding to bovine cytochrome P45011A1 R426 is invariable in mitochondrial cytochrome P450s except in *Urochordata* (ci0100143467 and ci0100147340), where lysine is present instead of arginine. At the same time, arginine corresponding to bovine cytochrome P45011A1 R425 is more frequently changed to lysine in invertebrate mitochondrial cytochrome P450s (e.g. cytochrome P450 12 and P450 314). Finally, the glutamate residue corresponding to cytochrome P45011A1 E429 follows the double arginines with two non-conserved residue gaps, implying that all these residues are located on the same site of α-helix L. The absence of negatively charged residue has been found only in golden hamster cytochrome P45011B (discussed later), insect CYP315A1, and the abovementioned mitochondrial cytochrome P450 of *Ciona intestinalis* (Urochordata). The next conserved glutamic acid residue corresponding to E431 of bovine CYP11A1 is not unique among mitochondrial cytochrome P450s and is semi-conserved in all eukaryotic cytochrome P450s. In known crystal structures of cytochrome P4502B4 (1PO5) [29], P4502R1 (3C6G) [30], P4502C9 (1R9O) [31], and P4503A4 (1TQN) [32], the corresponding to this glutamic acid residues are on the opposite site of α-helix L and deeply embedded under the surface, being at the interface of α-helixes E and I (Fig. 1b).

Analysis of available theoretical models of bovine cytochrome P45011A1 reveals that irrespective of the model used, the residues of α-helix L signature motif are surface exposed (Fig. 1a) [18, 33]. The relative position of positively charged residues involved in Adx binding as well as for E429 varies from model to model, but each model has been proved to be useful for prediction of the residue on the proximal surface implicated in redox partner recognition. The surface exposure of α-helix L motif residues is in agreement with the surface exposure of corresponding residues in known crystal structures of cytochrome P4502B4, P4502C9, and P4503A4. On the proximal surface of cytochrome P45011A1, the conserved E429 is surrounded by the positively charged residues that were proved by site-directed mutagenesis to be involved in Adx binding (Fig. 1a). Thus, based on the sequence analysis we suggested the direct participation of glutamic acid E429 as well as residues R425 and R426 in interactions of cytochrome P45011A1 with Adx.

Expression and purification of cytochrome P45011A1 mutant E429Q. Cytochrome P45011A1 E429Q mutant is expressed in *E. coli* at the same level as the wild-type hemoprotein, which allows the same protocol for purification to be applied. No difference in stability and properties of the mutant was noted upon purification. Unlike the cytochrome P45011A1 R426Q mutant, the E429Q mutant retains the ability to interact with immobilized Adx. Cytochrome P45011A1 E429Q mutant was purified in high-spin form. The highly purified cytochrome P45011A1 E429Q mutant displays the same spectral properties as wild type cytochrome P45011A1 (not shown).

The replacement of negatively charged E429 with the neutral glutamine residue does not affect the ability of

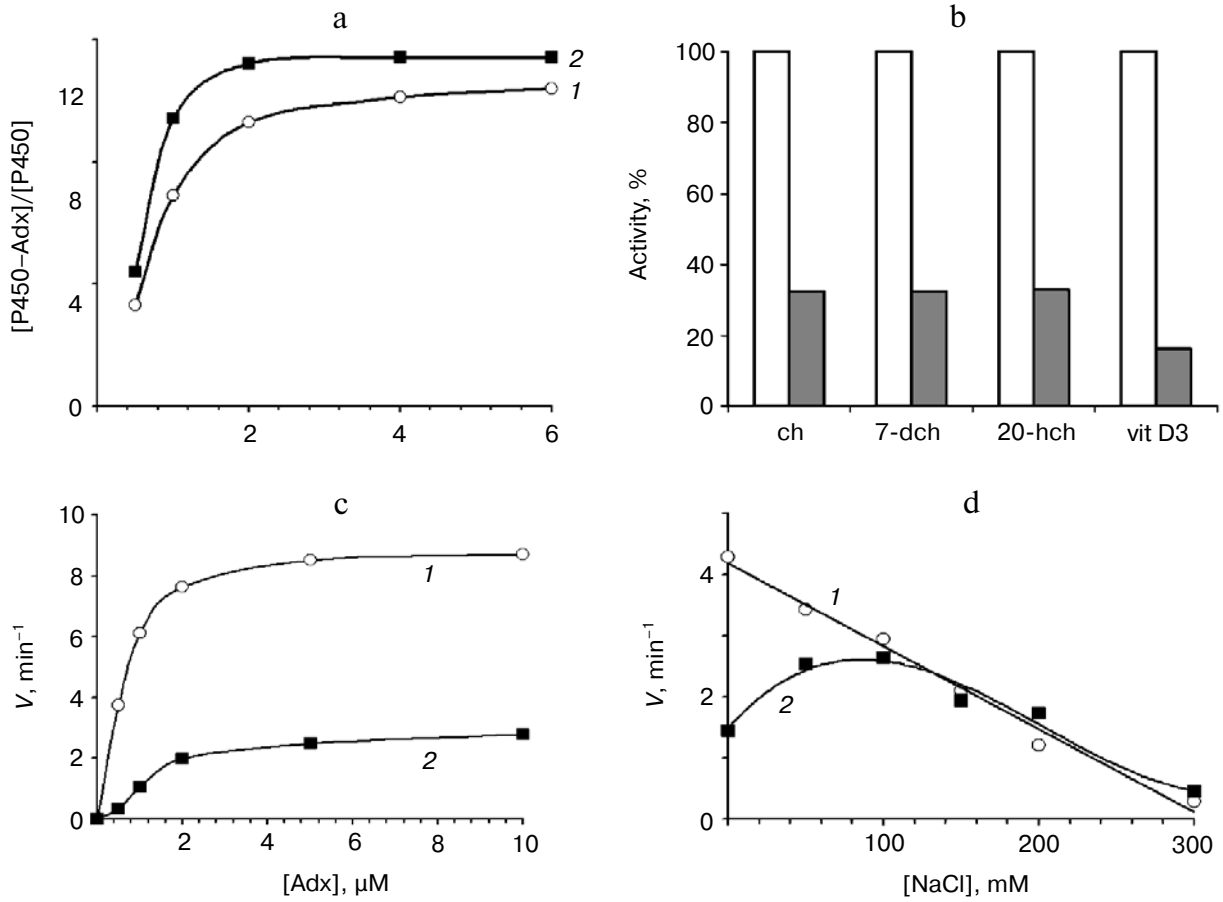

Fig. 2. a) Spectral titration of cytochrome P45011A1 with adrenodoxin. Dependence of the amplitude of spectral changes of wild type (WT) cytochrome P45011A1 (*1*) and E429Q mutant (*2*) on concentration of added adrenodoxin. The cytochromes were titrated in 20 mM Hepes buffer, pH 7.2, containing 0.1% Tween 20, 0.1 mM EDTA, 0.1 mM DTT, 50 mM NaCl, and 20 μM cholesterol. b) Catalytic activity of wild type cytochrome P45011A1 (light columns) and E429Q mutant (dark columns) with respect to different substrates (100 μM) in 25 mM Hepes buffer, pH 7.2, containing 0.1 mM EDTA, 0.1 mM DTT, 0.15% sodium cholate. Ch, cholesterol; 7-dch, 7-dehydrocholesterol; 20-hch, 20-hydroxycholesterol; vitD3, vitamin D3. c) Dependence of the rate of cholesterol side-chain cleavage reaction catalyzed by wild type cytochrome P45011A1 (*1*) and E429Q mutant (*2*) on concentration of added adrenodoxin. d) Effect of ionic strength on the rate of cholesterol side-chain cleavage reaction catalyzed by wild type cytochrome P45011A1 (*1*) and E429Q mutant (*2*).

the cytochrome P45011A1 mutant to be enzymatically reduced by NADPH in the presence of Adx and Adr. The reduction of cytochrome P45011A1 by Adx was monitored spectrally at 450 nm by CO-complex formation. In the presence of substrate, the reduction of wild type cytochrome P45011A1 and E429Q mutant reached the plateau within the dead time of measurements (30 sec). Therefore, the efficiency of wild type cytochrome P45011A1 and E429Q mutant interaction with reduced Adx has not been quantitatively characterized. However, the wild type cytochrome P45011A1 like the E429Q mutant retains the ability to undergo low-to-high-spin transition upon binding of Adx. The K_d values determined from spectral titration experiments for wild type cytochrome P45011A1 is about three times higher than for the mutant (Fig. 2a and Table 1). Thus, the decrease in K_d indirectly reflects the increased stability of cytochrome P45011A1–Adx_{ox} complex in the case of E429Q mutant due to the higher k_{+1} or lower k_{-1} or both:

$$\text{P450} + \text{Adx} \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} [\text{P450–Adx}].$$

Enzymatic activity of cytochrome P45011A1 E429Q mutant. The activity of cytochrome P45011A1 E429Q mutant was tested toward different known cytochrome P45011A1 substrates. The results in comparison with activity of wild type cytochrome P45011A1 are shown on Fig. 2b. The rate of product formation from cholesterol and its derivatives is reduced up to 25% as compare to wild type hemoprotein when the negative charge of E429 is neutralized. The conversion of cholesterol and 7-dehydrocholesterol to pregnenolone and 7-dehydropreg-

Table 1. Kinetic parameters of reaction of 7-dehydrocholesterol hydroxylation by cytochrome P45011A1 (WT) and its E429Q mutant in the presence of adrenodoxin and its mutant D76E

CYP11A1	K_d, mM	K_m, mM	V_{max}, min^{-1}	V_{max}/K_m
		Adx WT		
WT	0.368 ± 0.101	0.633 ± 0.109	9.554 ± 0.397	15.1
E429Q	0.129 ± 0.058	1.692 ± 0.584	3.271 ± 0.377	1.9
		Adx D76E		
WT	–	9.349 ± 3.197	11.199 ± 1.775	1.2
E429Q	–	5.620 ± 2.850	7.558 ± 1.479	1.3

nenolone, respectively, by cytochrome P45011A1 requires three subsequent hydroxylation steps, unlike two hydroxylation reactions in the case of 20-hydroxycholesterol. Cytochrome P45011A1 is a highly processive enzyme since no mono- and dihydroxylated intermediates accumulation was noted even at the low Adx/CYP11A1 ratio and substrate excess as well as for E429Q mutant despite overall reaction rate inhibition. Irrespective of the substrate used, the turnover for wild type hemoprotein and E429Q mutant catalyzed hydroxylation is 36 and 12 min^{-1}, respectively (Table 1). Unexpectedly, the activity towards vitamin D3 [34] is much more severely affected than for other substrates. Only the monohydroxylated product of vitamin D3 metabolism by cytochrome P45011A1 was detected in the case of the mutant.

Under conditions of substrate excess, the activity of cytochrome P45011A1 is limited primarily by electron transfer from NADPH via Adr and Adx. Since no difference in activity of wild type cytochrome P450 and E429Q mutant toward cholesterol and 7-dehydrocholesterol was noted, the kinetic parameters for Adx–CYP11A1 interactions were obtained using 7-dehydrocholesterol as the substrate (Fig. 2c). The K_m for cytochrome P45011A1 E429Q mutant for Adx is not significantly affected, while V_{max} is decreased to 30% compared to that of wild type hemoprotein (Fig. 2c and Table 1). The inability of cytochrome P45011A1 E429Q mutant to reach the V_{max} characteristic for wild type hemoprotein at high Adx concentration suggests that the electron transfer by reduced Adx is not the rate-limiting step in the E429Q mutant catalytic cycle and later steps are affected. The correlation of K_d and V_{max} for Adx suggests that the impairment of Adx shuttling between Adr and cytochrome P45011A1 could be due to high affinity of the E429Q mutant to oxidized Adx.

Effect of ionic strength on activity of cytochrome P45011A1 E429Q mutant. Electrostatic interactions play the crucial role in cytochrome P450–Adx complex formation [10] and as a result the activity of wild type cytochrome P45011A1 is inhibited proportionally to increase in ionic strength, which favors the disintegration of protein complexes stabilized by interactions between charged residues. Thus, to confirm the suggestion that the reduced activity of cytochrome P45011A1 E429Q mutant is attributed to the higher stability of cytochrome P450–Adx complex, the activity of the mutant was studied at increasing ionic strength (Fig. 2d). In contrast to wild type cytochrome P45011A1, the activity of the E429Q mutant gradually increased with increasing ionic strength, reaching that demonstrated by the wild type hemoprotein at NaCl concentration about 120 mM. At higher concentrations of NaCl the effect of the E429Q mutation is not evident and the activity is the same as that of wild type cytochrome P45011A1. Thus, increasing of ionic strength practically eliminates the inhibitory effect caused by replacement of negatively charged residue Glu with Gln.

Interactions of cytochrome P45011A1 E429Q mutant with Adx D76E mutant. The outstanding role of E429Q mutant in dissociation of cytochrome P45011A1–Adx is further supported by the studies of its interactions with the Adx D76E mutant. The D76E replacement in Adx does not change the overall surface charge distribution but disrupt specific interactions with mitochondrial cytochrome P450s resulting in dramatic decrease in affinity and the ability to support activity of cytochrome P45011A1. The activity of wild type cytochrome P45011A1 is restored at high concentrations of Adx D76E to that of wild type Adx (Figs. 2c and 3), unlike cytochrome P45011A1 E429Q mutant and wild type Adx. The same correlation of K_m and V_{max} has been demonstrated for a number of cytochrome P450 mutants with compromised ability to bind Adx. Therefore, the mutual effect of both mutants was analyzed. While K_m of cytochrome P45011A1 E429Q mutant for Adx D76E is not significantly affected, suggesting that the initial cytochrome P45011A1–Adx complex formation is still limited by D76E mutation of Adx, the reduced complex

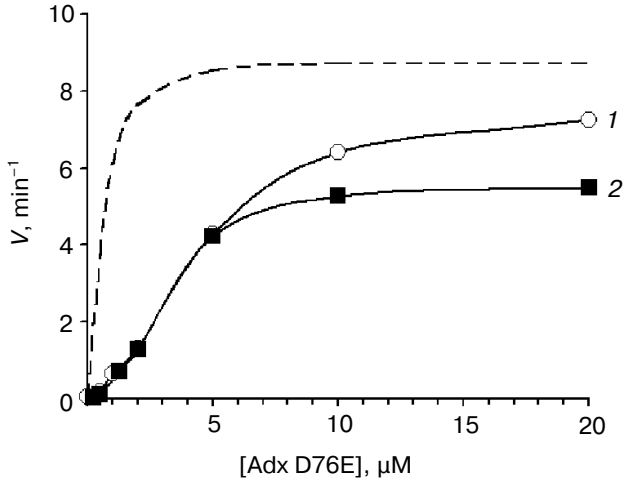

Fig. 3. Dependence of the rate of cholesterol side-chain cleavage reaction catalyzed by wild type cytochrome P45011A1 (*1*) and E429Q mutant (*2*) on concentration of adrenodoxin D76E mutant. The dashed line shows dependence of the rate of cholesterol side-chain cleavage reaction catalyzed by wild type cytochrome P45011A1 on concentration of wild type adrenodoxin for comparison.

stability due to D76E mutation results in partial recovery of low V_{max} of the E429Q mutant (Fig. 3 and Table 1). Thus the negative charge of E429 does not prevent the reduced Adx binding, otherwise higher activity of the E429Q mutant compared to the wild type cytochrome P45011A1 at low concentrations of Adx D76E mutant would have to be expected, but it is crucial for oxidized Adx dissociation from the complex due to electrostatic repulsion between E429 and the negatively charged interaction domain of Adx. It is obscure how this is realized at the molecular level. Anyway, aspects of mitochondrial cytochrome P450–Adx electron transfer, binding, and dissociation can be separated by site-directed mutagenesis, suggesting an essential role of conformational changes that occur upon electron transfer in Adx or cytochrome P45011A1 or more probably in both.

Effect of E438Q mutation on activity of cytochrome P45011B2. As is evident from the alignment in Fig. 1c, the E429 residue is highly conserved in all mitochondrial cytochrome P450s except insect cytochrome P450315A1, a poorly characterized 2-hydroxylase involved in the ecdysone biosynthesis pathway [35], and golden hamster cytochrome P45011Bs. Golden hamster is unique among rodents and mammals with respect to the composition of corticosteroids secreted into the blood. The capacity of the hamster CYP11B1 to hydroxylate at the 11β- and 19-positions in nearly equal ratio [36] is atypical since 19-OH-deoxycorticosterone in other species is the minor product, if it forms at all, and subsequently its decarboxylation results in 19-nor-deoxycorticosterone formation [37], a very potent mineralocorticoid probably involved in the development of essential hypertension [38]. The activity of hamster cytochrome P45011B2 is also shifted to formation of more hydroxylated products, the aldosterone/corticosterone ratio being the highest known [36], about 1 : 4, in contrast to 1 : 100 for rat CYP11B2 [38]. To clarify weather increased processivity of hamster cytochrome P45011B1 and cytochrome P45011B2 is due to the natural mutation of the residue corresponding to E429 of cytochrome P45011A1, the mutant of rat cytochrome P45011B2 has been constructed. As is evident from the data presented in Table 2, the activity of that mutant is reduced up to 10-fold compared to the wild type hemoprotein and is restored quite completely when ionic strength is increased. No difference in pattern and ratio of products formed has been observed. Much more profound effect of neutralization of the conserved Glu residue charge of cytochrome P45011B2 than of cytochrome P45011A1 might reflect tighter complex formation of cytochrome P45011B2 with Adx.

DISCUSSION

Despite of numerous studies, the molecular mechanisms and the nature of electron transfer complexes in mitochondrial cytochrome P450 dependent enzymes are

Table 2. Effect of replacement of E438Q and ionic strength on catalytic activity of cytochrome P45011B2

CYP11B2	Product/corticosteron ratio, %				Turnover number, min^{-1}
	B	18(OH)DOC	18(OH)B	Aldo	
WT	100	4.0	7.6	1.2	18.6
E438Q	100	n.d.	9.1	2.1	1.7
E438Q (100 mM NaCl)	100	3.5	8.4	1.6	14.5

Notes: B, corticosterone; 18(OH)DOC, 18-hydroxydeoxycorticosterone; 18(OH)B, 18-hydroxycorticosterone; Aldo, aldosterone; n.d., not determined.

still unknown. In the present work the intrinsic mechanism of protein–protein interactions between cytochrome P45011A1 and Adx has been studied by site-directed mutagenesis of highly conservative residue E429. The results indicate that residue E429 might play an essential role in modulation of the stability of the complex between the electron transfer proteins.

The regulation and molecular mechanisms of ferredoxin dissociation was unstudied for a long time. Only recently comparison of structures of reduced and oxidized putidaredoxin (Pdx) obtained by NMR and X-ray crystallography revealed large conformational changes at interaction site induced by the redox changes in the Fe_2-S_2-cluster [39]. The exposure of bulky amino acid side chains of Y33, R66, and W106 in the oxidized state is considered to prevent its tight binding to cytochrome P450101A1, ultimately leading to dissociation of oxidized Pdx [39, 40]. Unlike Pdx, where charged residues important for the interaction with P450101A1 (i.e. D34, D38, and W106) [40, 41] reside within the "core domain" in close proximity to the Fe_2-S_2-cluster, the charged residues of Adx involved in interaction of cytochrome P450s (i.e. D72, E73, D76, and D79) [8] are all located within the large flexible hairpin structure, the so-called "interaction domain" [5]. However, comparative NMR studies of reduced and oxidized Adx did not reveal similar conformational changes [42, 43].

The highly conservative nature of E429 in mitochondrial cytochrome P450s implies functional significance of the surface exposed negatively charged residue in close vicinity to residues involved in Adx binding. Since there are no positively charged residues on the surface of Adx around the negatively charged clusters proved to be involved in cytochrome P450 binding, a role of E429 in repulsion of Adx can be envisioned.

Charge neutralization by site-directed mutagenesis results in three-fold increase in the affinity of cytochrome $P45011A1_{ox}$ to Adx_{ox}, suggesting a modulatory role of E429 in redox partner binding (Fig. 1a). A similar decrease in K_d has been described for truncated Adx (4-108) [44] as well as for the Adx T54S mutant [45], and an inhibitory role of Adx C-terminal peptide in cytochrome P450 binding was suggested. The increased affinity of Adx (4-108) is accompanied by higher activity of cytochrome P45011A1 and cytochrome P45011B1 at low Adx concentrations, the K_m for mutant Adx being 3- and 6-fold lower, respectively. At the same time, V_{max} of cytochrome P45011A1 is not changed, while V_{max} of cytochrome P45011B1 is increased 3.5-fold as compared to full length Adx. The same would have to be expected for cytochrome P45011A1 E429Q mutant. But unlike Adx (4-108), kinetic parameters of cytochrome P45011A1 E429Q mutant for Adx are changed in the opposite way: K_m is 3-fold increased and V_{max} is 3-fold decreased compared to wild type cytochrome P45011A1. The discrepancies in these results suggest an active role of cytochrome P45011A1 residue E429 in regulation of affinity to reduced (Adx_{red}) or oxidized (Adx_{ox}) forms of Adx during the catalytic cycle. While the increased affinity of truncated Adx is due to an increase in k_{+1} in case of cytochrome P45011A1 E429Q mutant, the low K_d is determined by the decrease in k_{-1} and as a consequence the dissociation constant of the cytochrome P45011A1–Adx (k_2) complex, which becomes the rate-limiting step in the catalytic cycle:

$$P450_{ox} + Adx_r \underset{k_{-1}}{\overset{k_{+1}}{\leftrightarrow}} [P450_{ox}{-}Adx_r] \overset{k_2}{\rightarrow} [P450_r{-}Adx_{ox}] \rightarrow P450_r + Adx_{ox}\,.$$

This limiting step can not be overcome by increasing Adx concentration, which is reflected in three times lower V_{max}, but otherwise factors favoring the dissociation of the cytochrome P45011A1–Adx complex complement the effect of E429Q mutation, e.g. high ionic strength and low affinity Adx mutants.

At low ionic strength the cholesterol side-chain cleavage reaction catalyzed by wild type cytochrome P45011A1 reaches the K_m value observed at Adx concentrations below that of for cytochrome P450. Thus the 2-fold increase in K_m for cytochrome P45011A1 E429Q mutant for Adx could be readily explained by the fact that the concentration of available free Adx is below the threshold due to the fact that Adx_{ox} is in tight complex with the E429Q mutant. The same is not observed for mutant Adx with higher K_d and, as a consequence, K_m for wild type cytochrome P45011A1 is reached at Adx concentration 9-fold exceeding that of wild type cytochrome P45011A1. As a result no significant difference of K_m for Adx mutant and wild type or E429 mutant is observed. It has to be emphasized that the ability of Adx D76E mutant to support the cholesterol side-chain cleavage reaction is the same for wild type cytochrome P45011A1 and E429Q mutant at concentration of Adx D76E mutant below 5 μM, while at higher concentrations the effect of the E429 replacement becomes evident, suggesting that the negative charge of E429 does not prevent Adx binding.

Thus, the negatively charged residue E429 is located on the proximal surface of cytochrome P45011A1 (Fig. 4a; see color insert) in the surrounding of positively charged amino acid residues (K267, K403, K405, R426) participating in electrostatic interaction with ferredoxin. Figure 4b shows the theoretical model of tertiary structure of the complex between cytochrome P45011A1 and adrenodoxin [18] taking into account results obtained in the present work. The electron transfer complex of two proteins formed due to electrostatic interactions between negatively charged residues of ferredoxin (blue color) and positively charged residues of the hemoprotein (red color) is under control of residue E429 of cytochrome

P45011A1, which regulates the stability of the complex during reaction.

Three alternative models can be suggested to explain the role of E429 in dissociation of Adx (Fig. 5; see color insert). According to the first, residue E429 is permanently exposed on the cytochrome P45011A1–Adx complex interface and plays a passive role decreasing not only the cytochrome P45011A1–Adx complex stability but also the binding of Adx to cytochrome P45011A1 due to repulsion between E429 and negatively charged residues of the interaction domain of Adx (Fig. 5a). This model is the least probable since it cannot explain the conservative nature of residue E429 in mitochondrial cytochrome P450s as well as the small observed effect of E429Q replacement on K_m for Adx. The second model, like the first, implies the permanent exposure of residue E429 on the cytochrome P450 surface except that negatively charged residues of Adx_{red}, unlike Adx_{ox}, do not overlap with negative charge of residue E429. The electron transfer from Adx_{red} to cytochrome P450 induces conformational changes in Adx_{ox} that result in juxtaposition of negatively charged residues of the Adx interaction domain and residue E429, leading to destabilization and dissociation of the cytochrome P45011A1–Adx complex (Fig. 5b). The last model suggests temporary surface availability of E429 in the Adx binding site of cytochrome P45011A1 during the catalytic cycle either due to transient interactions with positively charged residues of the proximal surface or movements of α-helix L (Fig. 5c). These two models are in good agreement with experimental data presented in this article and currently it is not possible to discriminate between them.

It is of special interest to compare the redox-partner binding sites among mitochondrial and microsomal cytochrome P450s. As in the case of cytochrome CYP11A1, the positively charged residues of α-helices C and L and the meander region have been shown by site-directed mutagenesis to be involved in NADPH-cytochrome P450 reductase binding by cytochrome P4502B4 [46]. Moreover, there is an inversion of conserved positively and negatively charged residues of α-helix L exposed on the proximal surface of cytochrome P450s of the CYP2 subfamily, CYP1, CYP17, CYP21 as compared to mitochondrial cytochrome P450s: C-GE---R and C-GRR—E (Fig. 1c). Whether this conserved Glu residue of microsomal cytochrome P450s plays a similar role in interactions with NADPH-cytochrome P450 reductase as residue E429 does in mitochondrial cytochrome P450s in interaction with Adx is still left to be determined.

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