ISSN 0006-2979, Biochemistry (Moscow), 2006, Vol. 71, No. 1, pp. 32-38. © Pleiades Publishing, Inc., 2006.
Original Russian Text © A. N. Minenko, V. N. Luzikov, I. E. Kovaleva, 2006, published in Biokhimiya, 2006, Vol. 71, No. 1, pp. 42-49.
Originally published in Biochemistry (Moscow) On-Line Papers in Press, as Manuscript BM05-075, July 3, 2005.

Use of the Addressing Sequence of Yeast D-Lactate Dehydrogenase for Insertion of CYP11A1p into the Inner Membrane of Yeast Mitochondria

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Received March 16, 2005
Revision received April 21, 2005

Abstract—Mammalian cytochrome P450scc (CYP11A1p) is a pseudointegral protein of the inner membrane of mitochondria with the active center exposed in the matrix. Upon import of the CYP11A1p precursor into yeast mitochondria, only a minor part was incorporated into the inner mitochondrial membrane and acquired catalytic activity (Kovaleva, I. E., Novikova, L. A., Nazarov, P. A., Grivennikov, S. I., and Luzikov, V. N. (2003) *Eur. J. Biochem.*, **270**, 222-229). The present work is an attempt to increase the efficiency of this process by substitution of the inherent N-terminal presequence of CYP11A1p by the addressing signal of D-lactate dehydrogenase (D-LD) of the yeast *Saccharomyces cerevisiae*. D-LD is known to be inserted into the inner membrane of mitochondria through its transmembrane domain located close to the N-terminus of the polypeptide chain in such a way that the protein globule is exposed in the intermembrane space. The hybrid protein D-LD(1-72)-mCYP11A1p synthesized in yeast cells was imported into yeast mitochondria, underwent processing, and was inserted into the inner membrane on the side of the intermembrane space. In the presence of adrenodoxin and adrenodoxin reductase, the hybrid protein exhibited cholesterol side-chain cleavage activity. Thus, CYP11A1p insertion into the inner membrane of mitochondria mediated by the D-LD topogenic signal resulted in the catalytically active mCYP11A1p domain in the hybrid protein.

DOI: 10.1134/S0006297906010044

Key words: CYP11A1, topogenic signal, D-lactate dehydrogenase, yeast mitochondria, membrane insertion

Cytochrome P450scc (CYP11A1p) is a starting enzyme in the synthesis of mammalian steroid hormones. It cleaves off the side chain of the cholesterol molecule that transforms it to pregnenolone. CYP11A1p, with a specific cleavable N-terminal presequence, is synthesized in the cytoplasm and upon import into mitochondria is incorporated into the mitochondrial inner membrane. The active center of the enzyme is exposed in the matrix, as well as the trypsin-sensitive site between the N- and C-terminal domains of the protein [1, 2]. The N-terminal domain of CYP11A1p is not extracted with carbonate from adrenocortical mitochondria; therefore, CYP11A1p can be considered to be an integral protein of the inner mitochondrial membrane [2]. However, data on the amino acid sequence of the protein show the absence of elongated hydrophobic regions that could play the role of transmembrane domains [3]. The F-G loop region and also the hydrophobic region in the N-terminal area of the mature protein are suggested [4, 5] to be responsible for its interaction with the membrane by providing for the insertion of the polypeptide chain into the lipid bilayer without its complete intersection.

It was suggested earlier that the CYP11A1p precursor should be imported only into mitochondria of steroid-producing organs [6]. But later *in vitro* synthesized CYP11A1p with its inherent presequence was shown to be imported into isolated plant [7] and yeast mitochondria [8]. Controlled fractionation of yeast mitochondria revealed accumulation of the protein mainly in the mitochondrial matrix, where it was subjected to degradation or aggregation [9]. Similarly, the expression of CYP11A1p which possessed the inherent presequence or the subunit IV presequence of yeast cytochrome *c* oxidase (preCoxIV-mCYP11A1p) was associated with incorpora-

Abbreviations: Ad) adrenodoxin; AdR) adrenodoxin reductase; D-LD) D-lactate dehydrogenase; PiC) inorganic phosphate carrier; AAC) adenine nucleotide translocase; PMSF) phenylmethylsulfonyl fluoride; SSQ1p) a thermal shock response protein of the mitochondrial matrix; Tom40p) a component of the outer membrane translocase.

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tion into the inner membrane of mitochondria of only a minor part of the imported protein, which became catalytically active [10]. A similar situation seemed to occur when the CoxVIp-containing mCYP11A1p was expressed in yeast cells [11].

Mechanism of the CYP11A1p insertion into the inner membrane of mitochondria is still unknown. It has been mentioned that the polypeptide chain of CYP11A1p contains no distinct transmembrane regions. Consequently, to increase efficiency of CYP11A1p insertion into the inner membrane of yeast mitochondria (which seems to be a prerequisite for generation of a catalytically active protein), it was reasonable to design its recombinant variants using known topogenic signals of integral proteins of the inner membrane of yeast mitochondria. In the work [10] the addressing signal of subunit 9 of ATPase of the yeast *Neurospora crassa* was used, which provided the insertion of reporter proteins into the membrane on the matrix side by the re-export mechanism [12]. Although the hybrid protein Su9(1-112)-mCYP11A1p effectively bound to the mitochondrial membrane, its catalytic activity was low [10]. Therefore, in the present work we attempted to increase the efficiency of the CYP11A1p insertion into the inner membrane using the target signal of D-lactate dehydrogenase (D-LD), which is an integral protein of the inner membrane of yeast mitochondria. The insertion of D-LD into the membrane is known to occur by a mechanism not associated with the initial complete transfer of the polypeptide chain into the matrix [13]. And upon cleavage of the signal sequence, the N-terminal region of the polypeptide chain is directed into the matrix, whereas the protein bulk resides in the intermembrane space as a compact folded domain. The N-terminal sequence of D-LD (first 72 amino acid residues presenting the cleavable presequence and a sorting signal) mediated insertion of the soluble protein dihydrofolate reductase into the inner mitochondrial membrane oriented in the same way as D-LD. In the present work, the sequence of 72 N-terminal residues of D-LD was used to promote CYP11A1p insertion into the inner membrane of yeast mitochondria on the side of the intermembrane space.

MATERIALS AND METHODS

22R-Hydroxycholesterol, δ-aminolevulinic acid, tetramethylbenzidine, secondary antibodies–horseradish peroxidase conjugate, glucose-6-phosphate dehydrogenase, cholesterol oxidase, and NADPH were from Sigma (USA); restriction endonucleases, the Klenow fragment, T4-DNA ligase, and a reagent kit for elution of DNA were from Fermentas (Latvia); zymolyase 20T was from ICN Biomedicals (USA). The liquid and agarized LB-media for bacterial cells and also SD and SG media were prepared from Difco (USA) reagents. The proteins were transferred using Hybond-C extra nitrocellulose filters (Amersham, USA).

Primary antibodies to adenine nucleotide translocase (AAC), a component of the outer membrane translocase (Tom40p), inorganic phosphate carrier (PiC), a thermal shock response protein of the mitochondrial matrix (SSQ1p), D-LD, and the soluble domain of D-LD were obtained from Prof. V. Neupert (Institute of Physiological Chemistry, Munich, Germany). Cytochrome P450scc, adrenodoxin reductase (AdR), and adrenodoxin (Ad) from bovine adrenal cortex and antibodies to P450scc were given by V. M. Shkumatov (Institute of Physico-Chemical Problems, Byelorussian State University, Minsk, Belarus). Progesterone was determined by enzyme immunoassay (ELISA) using a kit presented by A. G. Pryadko (Institute of Bioorganic Chemistry, Academy of Sciences of Belarus, Minsk, Belarus).

Cell strains and plasmids. *Escherichia coli* cells of the JM-109 strain (Promega, USA) and the yeast *Saccharomyces cerevisiae* strain 2805 [MATa pep4::HIS3, prbl-δ, can1, GAL2, his3δ, ura3-52] from S-K. Ri (Research Institute of Gene Engineering, South Korea) were used. Yeasts were cultured at 30°C on a LabLine shaker (USA) in selective SD media (0.67% yeast nitrogen bases, 2% α-D-glucose, 0.1% casamino acids) or in SG media (0.67% yeast nitrogen bases, 2% α-D-galactose, 0.1% casamino acids).

The expressing plasmid was designed using the plasmid pGEM-4Z derivatives (Promega) and the pYeDP/1-8/2 yeast shuttle-vector [14]. The plasmid pGEM-4Z/D-LD(1-72)-DHFR was a gift from Prof. V. Neupert (Munich). The plasmid pGEM-4Z/Su(1-112)-CYP11A1 was designed in our laboratory [10].

Designing plasmid pYeDP/D-LD(1-72)-mCYP11A1 to express hybrid protein D-LD(1-72)-mCYP11A1 in yeast cells. All manipulations with recombinant DNAs were performed by standard methods of gene engineering [15]. cDNA of the hybrid protein D-LD(1-72)-mCYP11A1 was designed in the bacterial plasmid pGEM-4Z and then cDNA of the hybrid was recloned into the yeast vector pYeDP/1-8/2 as follows. From the plasmid pGEM-4Z/D-LD(1-72)-DHFR a DNA fragment encoding the sequence D-LD(1-72) was excised by restriction sites *Eco*RI and *Bam*HI and inserted into the plasmid pGEM-4Z/Su9(1-112)-mCYP11A1 after detachment of the *Eco*RI-*Bam*HI fragment encoding the sequence Su9(1-112). The resulting plasmid pGEM-4Z/D-LD(1-72)-mCYP11A1 was treated successively with the restrictase *Eco*RI, Klenow fragment, and restrictase *Kpn*I to isolate cDNA of the hybrid protein D-LD(1-72)-mCYP11A1. The hybrid protein cDNA was ligated with the vector pYeDP/1-8/2, which was successively cleaved with the restrictase *Bam*HI, treated with the Klenow fragment, and cleaved with the restrictase *Kpn*I. The resulting plasmid YeDP/D-LD(1-72)-mCYP11A1 was used to transform the yeast strain 2805 as described in [16].

Isolation and fractionation of yeast mitochondria. Yeast mitochondria were isolated as described in [17]. For alkaline extraction of mitochondrial proteins, mitochondria were re-precipitated at 12,000 rpm for 12 min with an Eppendorf (Germany) centrifuge, suspended in a fresh solution of 100 mM Na_2CO_3, and incubated on ice for 30 min. The membrane fraction was precipitated by centrifugation at 125,000*g* for 30 min (SW50; Beckman, USA). Proteins of the supernatant were precipitated with 10% trichloroacetic acid (TCA).

Mitoplasts were prepared as described in [18]. Mitochondria were re-precipitated by centrifugation and resuspended in buffer SHKCl containing 0.6 M sorbitol, 50 mM HEPES-KOH (pH 7.4), and 80 mM KCl. An aliquot of mitochondria was subjected to osmotic shock with nine volumes of 20 mM HEPES-KOH (pH 7.4) followed by incubation on ice for 20 min. Then a fivefold excess volume of buffer SHKCl was added, the mitoplasts were centrifuged at 14,000 rpm for 12 min with an Eppendorf centrifuge, and the resulting precipitate was resuspended in SHKCl buffer.

The mitochondria and mitoplasts were treated on ice with proteinase K (50 μg/ml) in SHKCl buffer for 20 min. Proteinase was inactivated by incubation of the preparations with 1 mM phenylmethylsulfonyl fluoride (PMSF) for 10 min. The organelles were precipitated at 14,000 rpm for 12 min using an Eppendorf centrifuge, and the precipitates were analyzed by SDS-electrophoresis in polyacrylamide gel (SDS-PAGE) with subsequent immunoblotting.

Determination of enzymatic activity of recombinant protein. The reaction mixture for determination of the cholesterol side-chain cleavage activity of the hybrid protein contained 200-300 μg mitochondrial protein, purified bovine Ad (0.8 nmol), AdR (0.2 nmol), and 22R-hydroxycholesterol (25 nmol) in 0.5 ml of 30 mM sodium phosphate buffer (pH 7.5) supplemented with 0.05% Tween 20. The reaction was started by addition of a NADPH-regenerating system (0.1 mM NADPH, 5 mM glucose-6-phosphate, and glucose-6-phosphate dehydrogenase (1 mg/ml)) and performed according to the protocol presented in [10]. Pregnenolone was converted into progesterone by adding cholesterol oxidase. The end product (progesterone) was quantified by enzyme immunoassay with a commercial IFA-PROGESTERON kit containing antibodies to progesterone.

Other methods. SDS-PAGE and immunoblotting were performed using standard procedures [19]. Protein content was determined by the Lowry method [20].

RESULTS

Expression of hybrid protein D-LD(1-72)-mCYP11A1 in yeast cells. Yeast cells of the 2805 strain were transformed with the plasmid pYeDP/D-LD(1-72)-mCYP11A1. The transformed clones were grown for 16 h on minimal SD medium and then kept for 12 h in SG medium to induce the promoter. The cells were precipitated, and mitochondria were isolated from them. The hybrid protein was detected in the mitochondria by immunoblotting using antibodies to CYP11A1p and the full-size protein D-LD. Data of SDS-PAGE with subsequent immunoblotting indicated that the mitochondria contained the hybrid D-LD(1-72)-mCYP11A1p with molecular weight of 57.7 kD (Fig. 1). Moreover, the mitochondria contained a minor amount of the D-LD(1-72)-mCYP11A1 precursor, which differed from the mature protein by the presence of 25 amino acid residues of the detachable signal sequence [13].

To approximately assess expression of the hybrid, a protein-equal sample of mitochondria containing the hybrid Su9(1-112)-mCYP11A1p was also applied onto the gel. The hybrid D-LD(1-72)-mCYP11A1p content determined with the Scn Image program was about 25 times lower than that of the hybrid Su9(1-112)-mCYP11A1p.

Location of the hybrid protein in yeast mitochondria. To elucidate whether the topogenic signal from the first 72 amino acids of D-LD was responsible for the hybrid

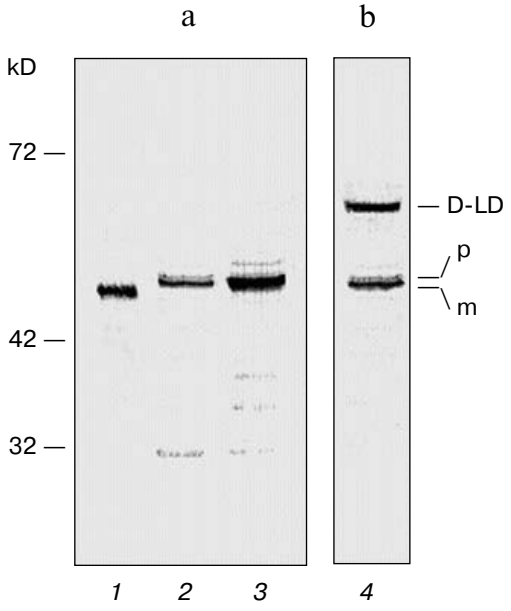

Fig. 1. Analysis by immunoblotting of mitochondria isolated from yeast strain expressing hybrid D-LD(1-72)-mCYP11A1p. Mitochondrial proteins were analyzed by SDS-PAGE followed by immunoblotting. Nitrocellulose filters were stained with antiserum to CYP11A1p (a) or with antiserum to the full-size protein D-LD (b). Lanes: *1*) mature CYP11A1p; *2*) lysate of mitochondria of the yeast cells transformed with the plasmid pYeDP/D-LD(1-72)-mCYP11A1; *3*) lysate of mitochondria of the yeast cells transformed with the plasmid pYeDP/Su9(1-112)-mCYP11A; *4*) lysate of mitochondria of the yeast cells transformed with the plasmid pYeDP/D-LD(1-72)-mCYP11A1. Designations: p, precursor; m, mature D-LD(1-72)-mCYP11A1p.

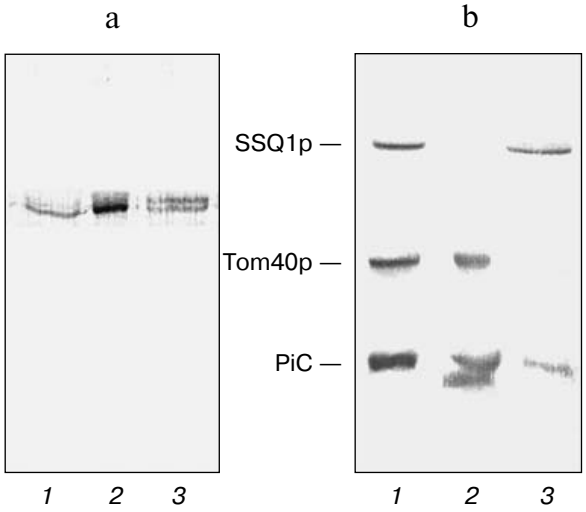

Fig. 2. Fractionation of mitochondria containing the hybrid protein D-LD(1-72)-mCYP11A1p by alkaline extraction. Proteins of the precipitate and soluble fraction were analyzed by SDS-PAGE (aliquots of the fractions prepared from 100 μg mitochondria were applied) followed by immunoblotting. The filter was stained with (a) polyclonal antibodies to CYP11A1p or with (b) polyclonal antibodies to yeast mitochondria proteins: SSQ1p (matrix), phosphate carrier PiC (an integral protein of the inner membrane), and Tom40p (an integral protein of the outer membrane). Lanes: *1*) lysate of mitochondria; *2*) precipitate; *3*) supernatant.

protein integration into the membrane, yeast mitochondria were treated with Na_2CO_3 at pH 11.5. Such a treatment is thought to induce the passage of soluble and membrane-associated proteins into the supernatant fraction, while integral membrane proteins are not extracted from the membrane. Upon the alkaline extraction of the mitochondria, the mature hybrid protein D-LD(1-72)-mCYP11A1p was found mainly in the fraction of insoluble proteins, similarly to marker proteins of the outer and inner membranes (Tom40p and PiC, respectively) (Fig. 2). As differentiated from them, the marker protein SSQ1p of the mitochondrial matrix was found only in the soluble fraction. Because the hybrid protein occurred mainly in the membrane fraction, it was concluded that the topogenic signal D-LD ensured the effective integration of the hybrid protein into the membrane. The bulk of the hybrid had undergone processing earlier. Because the processing was realized with involvement of the leader peptidase of the mitochondrial matrix, the hybrid protein was concluded to insert into the inner mitochondrial membrane with the N-terminal presequence exposed in the matrix.

Study on topology of hybrid protein D-LD(1-72)-mCYP11A1p. To study in more detail the protein D-LD(1-72)-mCYP11A1p, topology in yeast mitochondria, intact mitochondria, and mitochondria subjected to osmotic shock and transferred into isotonic medium were treated with exogenous proteinase. Later these mitochondria with breaks in the outer membrane will be named mitoplasts. The mitochondria were subjected to proteolysis under isotonic conditions. As shown in Fig. 3, the majority of hybrid molecules were protected against proteinase K. Partial proteolysis of the hybrid protein and D-LD seemed to be caused by the presence in the mitochondrial fraction of a number of organelles with broken outer membrane.

The presence of the transmembrane domain in the inner mitochondrial membrane of D-LD was associated with the bulk of the protein exposed in the intermembrane space as a compact folded domain resistant to proteinase K [13]. In mitoplasts, D-LD was a membrane-associated protein, but on treatment with proteinase K the soluble domain was separated from the membrane and found in the supernatant after the mitoplasts were precipitated by centrifugation.

Figure 3 presents results of the proteinase K treatment of the mitoplasts containing the hybrid protein D-LD(1-72)-mCYP11A1p. After incubation with proteinase K, the mitoplasts were precipitated by centrifugation, and immunoblotting was used to determine contents of the hybrid protein, the soluble domain D-LD, and marker proteins of the inner membrane (PiC) and matrix (SSQ1p). Figure 3 shows the D-LD coprecipitation with the mitoplasts, but on treatment with proteinase K, the mitoplasts completely lost D-LD but retained SSQ1p and PiC. These findings suggested that inner membrane integrity retained during the experiment, whereas the soluble domain D-LD was either subjected to complete proteolysis, or was separated from the membrane and entered the supernatant fraction. The hybrid D-LD(1-72)-mCYP11A1p was also co-precipitated with the mitoplasts, and the treatment with proteinase dramatically decreased its content in the mitoplasts with the accompa-

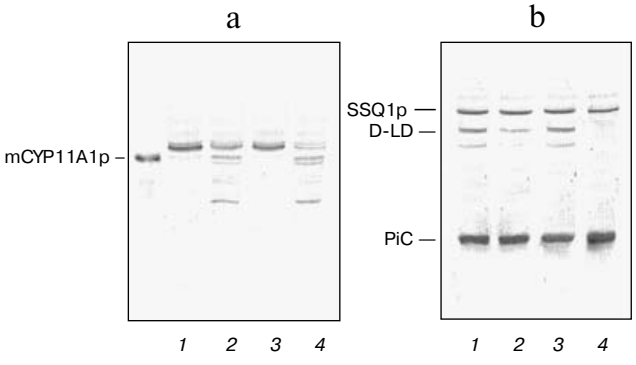

Fig. 3. Hybrid D-LD(1-72)-mCYP11A1p location in yeast mitochondria determined by limited proteolysis. Mitochondria and mitoplasts after treatment with proteinase K were analyzed by immunoblotting. Filter was stained with (a) antiserum to CYP11A1p or with (b) antibodies to SSQ1p, PiC, and to the soluble moiety of D-LD. Lanes: *1*) mitochondria; *2*) mitochondria treated with proteinase K; *3*) mitoplasts; *4*) mitoplasts treated with proteinase K.

nying appearance of a small number of membrane-associated fragments, one of which had molecular weight close to that of mCYP11A1p.

Thus, the hybrid D-LD(1-72)-mCYP11A1p was inserted into the inner mitochondrial membrane in such a way that it was accessible for proteinase K from the side of the intermembrane space, similarly to D-LD. But this similarity was incomplete because of different properties of the D-LD domain exposed in the intermembrane space and the mCYP11A1p domain of the hybrid protein.

On expression in yeast cells of the hybrid Su9(1-112)-mCYP11A1p possessing the topogenic signal responsible for the initial entrance of the hybrid molecules into the mitochondrial matrix, the majority of imported and processed molecules were aggregated, as they failed to pass into the soluble fraction on treatment of the mitochondria with 1% Triton X-100 [10]. To examine if the hybrid molecules D-LD(1-72)-mCYP11A1p were aggregated, the mitochondria were subjected to proteolysis in the presence of 1% Triton X-100, and the soluble fraction was analyzed. With antibodies to CYP11A1p this fraction was found to contain a fragment of the hybrid with the molecular weight of the domain mCYP11A1p, whereas PiC, Tom40p, and SSQ1p were virtually fully degraded under these conditions (Fig. 4). Consequently, the domain mCYP11A1p was relatively resistant to proteolysis, and the hybrid molecules D-LD(1-72)-mCYP11A1p were not prone to aggregation because the specific separation of only the soluble fragment from the aggregated protein was unlikely.

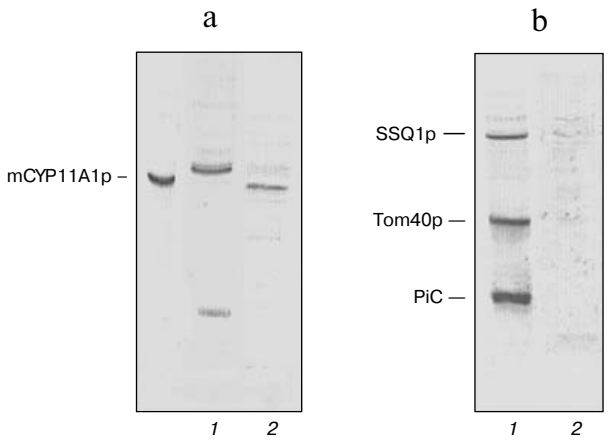

Fig. 4. Study on the hybrid protein D-LD(1-72)-mCYP11A1p aggregation in mitochondria. Mitochondria were treated on ice with proteinase K (50 μg/ml) in the presence of 1% Triton X-100 for 20 min, the proteinase was inactivated with 1 mM PMSF, and then the sample was centrifuged with an Eppendorf centrifuge at 12,000*g* for 15 min. An aliquot of untreated mitochondria and supernatant were analyzed by immunoblotting. The filter was stained with (a) antiserum to CYP11A1p or with (b) antibodies to SSQ1p, Tom40p, and PiC. Lanes: *1*) initial mitochondria; *2*) supernatant after precipitation of aggregated proteins.

Cholesterol side-chain cleavage activity of mitochondria containing the hybrid proteins D-LD(1-72)-mCYP11A1p and Su9(1-112)-mCYP11A1p

Mitochondrial fraction containing the hybrid molecules	Activity, a.u.
D-LD(1-72)-mCYP11A1p minus Ad, AdR	3
D-LD(1-72)-mCYP11A1p	39
Su9(1-112)-mCYP11A1p	60

Determination of cholesterol side-chain cleavage activity of D-LD(1-72)-mCYP11A1p. The activity of the hybrid protein was determined in mitochondria on addition of bovine Ad and AdR and also of the NADPH-regenerating system. 22R-Hydroxycholesterol was used as a substrate. The activity was expressed in 10^{-4} nmol pregnenolone produced for 1 min per mg protein of the mitochondrial fraction (activity units, or a.u.). The results are shown in the table. A sample of mitochondria without Ad and AdR was used as a control. For comparison, the cholesterol side-chain cleavage activity was determined in parallel in the mitochondria containing the hybrid Su9(1-112)-mCYP11A1p after the processing. The activities of the hybrids D-LD(1-72)-mCYP11A1p and Su9(1-112)-mCYP11A1p per mg mitochondrial protein were about 39 and 60 a.u., respectively. However, taking into account the significant difference in the expression of these hybrid proteins and, respectively, in their specific content per mg mitochondrial protein (Fig. 1), the specific activity of the hybrid D-LD(1-72)-mCYP11A1p occurs to be markedly higher than that of the hybrid Su9(1-112)-mCYP11A1p.

DISCUSSION

Similarly to other mitochondrial cytochromes of the P450 family, CYP11A1p possessing a polypeptide chain free of a specific transmembrane region is incorporated into the membrane [3]. At present, interaction of this protein with the membrane and also the mode of its incorporation into the membrane are obscure. It is also unknown during what stage of the protein topogenesis the heme is adjoined and its active conformation is acquired.

Integral proteins of the inner mitochondrial membrane, which are synthesized in the cytoplasm with a cleavable N-terminal presequence, are known to contain transmembrane regions (topogenic or sorting signals) which are required for the incorporation of the protein into the membrane [21]. Such topogenic signals either arrest translocation of the polypeptide chain across the translocon of the inner membrane (the TIM complex), or

promote polypeptide chain insertion into the membrane already after its passage into the mitochondrial matrix (the re-export mechanism). Cytochrome CYP11A1p is characterized by the absence of specific topogenic signals capable of promoting its incorporation into the membrane by one of these mechanisms.

Therefore, in attempts to increase the efficiency of CYP11A1p incorporation into the membrane, we initially used the topogenic signal (1-112)Su9p responsible for the effective transfer of reporter proteins into the mitochondrial matrix and their subsequent incorporation into the inner membrane by the re-export mechanism [12]. The hybrid Su9(1-112)-mCYP11A1p efficiently bound to the inner mitochondrial membrane. But the enzymatic activity and solubility in 1% Triton X-100 of the hybrid molecules were low [10]. Based on these findings and also on data on the incorporation into yeast mitochondria of CYP11A1p with the inherent presequence or with the presequence CoxIV [9, 10], it was suggested that CYP11A1p inside the mitochondrial matrix should be unable to adopt an adequate conformation and, therefore, should undergo proteolysis or aggregation.

To achieve the incorporation of cytochrome CYP11A1p into the inner membrane of yeast mitochondria, in the present work we used the topogenic signal of D-LD, which is an integral protein of the inner membrane of yeast mitochondria. D-LD is synthesized as a precursor with the N-terminal cleavable presequence and imported into mitochondria depending on potential but without involvement of the mitochondrial Hsp70 [13], as occurs in the case of soluble proteins of the mitochondrial matrix [22]. The D-LD incorporation into the inner membrane is directed by a composite sorting signal consisting of a hydrophobic transmembrane segment and a cluster of positively charged amino acid residues on the C-side. This region of the polypeptide chain located close to the signal sequence arrests the transmembrane translocation of the D-LD polypeptide chain preventing its penetration into the matrix. On processing of the signal sequence, the N-terminal region of the polypeptide chain is located in the matrix, whereas the bulk of the protein is exposed in the intermembrane space as a compact folded domain. In this work, the sequence of 72 N-terminal residues of D-LD (which represented the separable presequence and the sorting signal) was used to incorporate CYP11A1p into the mitochondrial inner membrane.

The hybrid protein D-LD(1-72)-mCYP11A1p was imported into yeast mitochondria where it was subjected to processing. The hybrid D-LD(1-72)-mCYP11A1p was effectively incorporated into the inner mitochondrial membrane, with the mCYP11A1p domain exposed in the intermembrane space. Because the bulk of the hybrid underwent processing, its N-terminal region seemed to be directed into the matrix. The treatment of "mitoplasts" with proteinase K digested the hybrid D-LD(1-72)-mCYP11A1p similarly to D-LD. But the CYP11A1p domain was more resistant to proteolysis than the globular domain D-LD exposed in the intermembrane space. This suggested either a partial submergence of the CYP11A1p domain into the inner mitochondrial membrane or specificity of its molecular organization.

The hybrid molecules D-LD(1-72)-mCYP11A1p displayed cholesterol side-chain cleavage activity that was significantly higher than that of the hybrid Su9(1-112)-mCYP11A1p. The processed forms of the hybrid proteins D-LD(1-72)-mCYP11A1p and Su9(1-112)-mCYP11A1p were different only in the short transmembrane segments which seemed unlikely to determine the mode of the mCYP11A1p domain folding, and the low activity of the hybrid Su9(1-112)-mCYP11A1p seemed to be due to the tendency of the mCYP11A1p domain for aggregation in the matrix [10]. Therefore, the mCYP11A1p domain in the D-LD(1-72)-mCYP11A1p folded inside the intermembrane space did not undergo aggregation. This was accompanied by binding of heme and taking an active conformation. Unfortunately, in the present work we failed to get a sufficiently high expression of the hybrid protein D-LD(1-72)-mCYP11A1p. A promising approach in this line seems to be provided by searching for topogenic signals responsible for an increased content of the active protein in the inner membrane of yeast mitochondria with the CYP11A1p domains preferentially oriented from the matrix side of the membrane, which corresponds to the natural location, i.e., in adrenocortical mitochondria.

This work was supported by the Russian Foundation for Basic Research (project Nos. 02-04-48204 and 02-04-81001) and INTAS (project No. 03-51-4366).

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