Biochemistry (Moscow), Vol. 65, No. 12, 2000, pp. 1362-1366. Translated from Biokhimiya, Vol. 65, No. 12, 2000, pp. 1618-1623.

Interaction of Catalytic Domains in Cytochrome P450scc–Adrenodoxin Reductase–Adrenodoxin Fusion Protein Imported into Yeast Mitochondria

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Received March 13, 2000
Revision received March 28, 2000

Abstract—We have constructed plasmids for yeast expression of the fusion protein pre-cytochrome P450scc–adrenodoxin reductase–adrenodoxin (F2) and a variant of F2 with the yeast CoxIV targeting presequence. Mitochondria isolated from transformed yeast cells contained the F2 fusion protein at about 0.5% of total protein and showed cholesterol hydroxylase activity with 22(R)-hydroxycholesterol. The activity increased 17- or 25-fold when sonicated mitochondria were supplemented with an excess of purified P450scc or a mixture of adrenodoxin (Adx) and adrenodoxin reductase (AdxRed), respectively. These data suggest that, at least in yeast mitochondria, the interactions of the catalytic domains of P450scc, Adx, and AdxRed in the common polypeptide chain are restricted.

Key words: cytochrome P450scc, adrenodoxin, NADPH:adrenodoxin reductase, fusion protein, enzymatic activity, yeast, mitochondria, proteolysis

Cytochrome P450 enzyme systems consist of a large family of enzymes receiving electrons from a generic electron donor. In the common microsomal (type II) enzymes, P450 receives electrons from NADPH: cytochrome P450 reductase. The report of a naturally occurring bacterial fusion protein of a cytochrome P450 and its reductase [1] led to the experimental creation of many P450:reductase fusion proteins [2-10]. Some of these fusions appeared to have greater catalytic efficiency than did mixtures of their component parts, suggesting that the fusions showed "more efficient electron transfer" [5-7]. The activity of a fusion protein appears to depend on the spatial relationships of the active centers of the P450 and reductase moieties, as activity can be changed 10-fold by changing the length and amino acid sequence of the hinge between the two moieties [3, 4].

Mitochondrial (type I) P450 systems are more complex—electrons from NADPH are taken up by NADPH:ferredoxin reductase (adrenodoxin reductase, AdxRed), a flavoprotein which donates them to adrenodoxin (Adx), an iron/sulfur protein which then donates them to the P450 moiety. The best-studied mitochondrial P450 is the cholesterol side-chain cleavage enzyme, P450scc (for review see [11]). Although some authors have suggested that the P450scc, AdxRed, and Adx form ternary or higher complexes during catalysis [12, 13], most investigators believe that catalysis can not involve a ternary complex because both P450scc and AdxRed interact with the same surface of the Adx molecule [14-16]. Nevertheless, Harikrishna et al. showed that the fusion protein H_2N-P450scc–AdxRed–Adx-COOH (termed F2) catalyzed the conversion of 22(R)-hydroxycholesterol to pregnenolone when expressed in COS-1 cells [17]. A similar result was obtained with the 25-hydroxylation of 1α-hydroxy vitamin D by the fusion protein H_2N-P450c27–AdxRed–Adx-COOH [18]. As the activity of cells transfected with the P450scc fusion protein was greater than that of cells triply transfected with plasmids encoding P450scc, Adx, and AdxRed, and as the apparent V_{max} of the fusion protein was six times greater than that of the triply transfected cells, Harikrishna et al. suggested that the fusion protein had increased enzymatic activity [17]. The role of the covalently linked Adx moiety in the F2 protein, rather than an interaction of the F2 fusion protein with Adx present in COS-1 cells, was shown by mutagenesis of the Fe/S cen-

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ter of the Adx moiety of the fusion [19]. This appeared to be consistent with the suggestion that the carboxy-terminal Adx moiety interacts with both P450scc and AdxRed in the F2 molecule.

Because the above ideas were based on experiments with whole cells, more detailed information about the catalytic properties of F2 was necessary. In principle, new insight into the problem can be achieved by dealing with the isolated fusion protein and mitochondria containing it. To this end, we have studied a human/bovine F2 fusion expressed in yeast.

MATERIALS AND METHODS

Yeast strain and plasmids. We used *Saccharomyces cerevisiae* strain 2805 (*MATα pep4::HIS3, prb1-δ, can1, GAL2, his 3δ, ura 3-52*) kindly provided by Dr. Sang-Ki Rhee (GERI, Korea) and vector pYeDP1-8/2 from Dr. D. Pompon (CNRS, France). Construction of the pYeDP/pre-CoxIV-P450scc (bovine) plasmid was carried out according to [20]. Recombinant DNA for the human F2 fusion in the pECE vector has been described [17].

Design of DNAs for F2 and pre-Cox IV-F2 fusion proteins. A plasmid for expression of the pre-CoxIV-F2 fusion protein in yeast was constructed from the pYeDP/pre-CoxIV-P450scc plasmid [20] encoding bovine cytochrome P450scc, in which the mitochondrial leader sequence was replaced with that of subunit IV of yeast cytochrome *c* oxidase for stimulating both the import of foreign protein and its processing in yeast mitochondria. In this plasmid, an AAA triplet was inserted just ahead of the initiating ATG codon to ensure a high level of expression of the recombinant protein. A new plasmid was constructed by deleting the terminating codon in the reading frame of the bovine P450scc cDNA, thus forming a unique *Kpn*I restriction site in its place; a DNA fragment for human AdxRed–Adx was cloned behind the bovine P450scc cDNA. This DNA fragment was excised from the plasmid constructed earlier for expression of the human F2 fusion protein in COS-1 cells [17]. This procedure included treatment of the plasmid with the *Spe*I endonuclease (the unique *Spe*I site is located ahead of the oligonucleotide sequence encoding AdxRed–Adx), filling in with T4 DNA polymerase, and treatment with *Eco*RI (the unique *Eco*RI site lies at the 3′-end of the TGA stop codon in the reading frame for the double fusion protein). The resulting construct had the following structure:

...ATG-pre-CoxIV-P450scc-GCTAGT-AdxRed-ACC-GACGGCGCTAGC-Adx-ACCGCGGCGCTAGCAC-TAGCTGAC-TGA...

We also recloned the DNA fragment encoding the human F2 fusion protein [17] from the pECE plasmid into the pYeDP-1/8 vector [21] using the unique *Kpn*I and *Eco*RI restriction sites. In this case, the activating AAA triplet also preceded the initiating ATG codon to ensure a high level of expression of this foreign protein in yeast.

Expression of recombinant DNAs. Yeast cells were transformed with pYeDP/F2, pYeDP/pre-CoxIV-F2, and pYeDP/pre-CoxIV-P450scc supplemented with the GAL10-CYC1 promoter. The cells were grown in a minimal synthetic SD medium containing 2% glucose, 0.67% YNB (without amino acids, Difco, USA), and 1% casamino acids (Difco). Growth was continued for 14-16 h to an optical density of 3 to 5 (A_{600}). Transcription of recombinant DNAs in yeast was induced by substituting galactose for glucose in the medium. To this end, the cells were washed three times with sterile water and transferred to fresh SG minimal synthetic medium containing 2% galactose as the sole carbon source (A_{600} = 1.0). The induction continued for 18-22 h at 30°C with intensive shaking.

Assays of cholesterol hydroxylase activity. Cholesterol hydroxylase activity of F2 was assayed in total sonicated mitochondria or submitochondrial particles (SMP) pelleted from sonicated mitochondrial suspension at 105,000*g*. The reaction medium contained mitochondria or SMP (2 mg protein) and 25 nmoles of 22(R)-hydroxycholesterol in 0.5 ml of 30 mM sodium phosphate buffer (pH 7.2) supplemented with 0.05% Tween 20. The same medium was used for sonication of the mitochondria. Purified bovine Adx (5 nmoles) and AdxRed (0.5 nmole) or P450scc (0.5 nmole) were added when necessary. The samples were preincubated for 20 min at 25°C, and the reaction was started by adding an NADPH-generating system including 0.1 mM NADPH, 5 mM glucose-6-phosphate, and G-6-P dehydrogenase (1 unit/ml). The reaction was stopped after 30 min by plunging the samples into boiling water for 15-20 sec. Then the samples were mixed with 3 ml of 100 mM sodium phosphate buffer (pH 7.2) with 0.05% Tween 20, and cholesterol oxidase was added in the amount of 0.1 unit per sample to convert any pregnenolone formed from 22(R)-hydroxycholesterol to progesterone. After 45-min incubation at 37°C, the samples were treated with ethyl acetate to extract the steroids. The extracts were evaporated to dryness and dissolved in 50 mM sodium phosphate (pH 7.4) with 0.2% BSA and 0.02% sodium azide. The content of progesterone was determined using [^{125}I]progesterone and anti-progesterone antibodies. Catalytic activity of recombinant P450scc synthesized and imported into yeast mitochondria *in vivo* [20] was measured in sonicated mitochondria supplemented with bovine Adx and AdxRed. All other procedures were as described above.

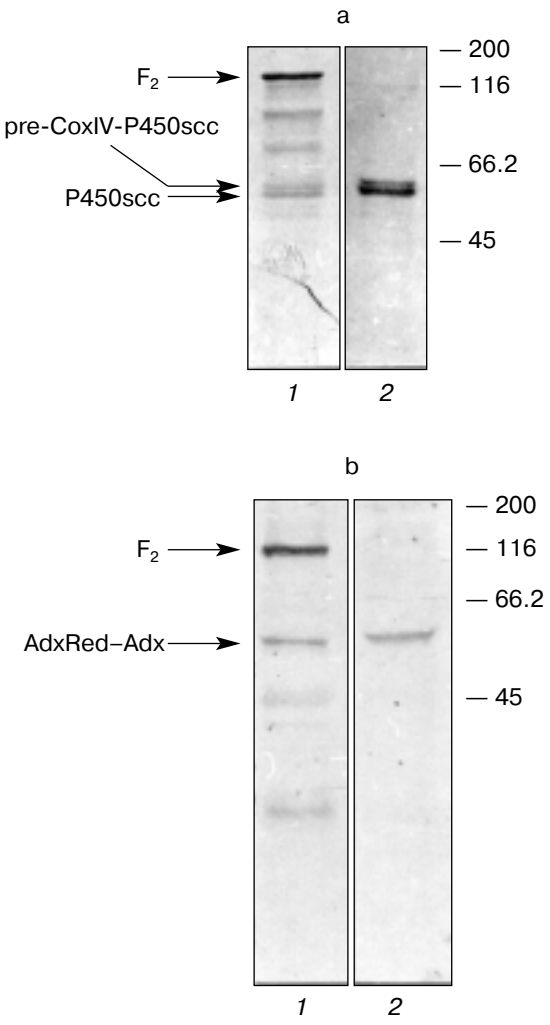
Other methods. Yeast cells were fractionated after Akiyoshi-Shibata et al. [22]; mitochondria were sub-fractionated as in [23], which yielded sonicated SMP. SDS-

PAGE was carried out in 10-12% polyacrylamide gels. Immunoblotting was performed using IgG fractions of anti-P450scc and anti-Adx antisera.

Recombinant DNAs were sequenced by Sanger's method, and site-directed mutagenesis was performed after Drutsa et al. [24].

RESULTS AND DISCUSSION

Expression of the fusion proteins in yeast. The plasmids described above were used for transformation of yeast cells. Synthesis of the fusion proteins was initiated by transferring the cells into medium containing galactose. After 18-22-h induction, the cells were lysed, mitochondria were isolated, and mitochondrial lysates were subjected to SDS-PAGE followed by immunoblotting with antibodies against either bovine P450scc or bovine Adx. Similar results were obtained for mitochondria isolated from the yeast strains expressing pre-CoxIV-F2 (figure) or F2. Immunoblotting detected several bands, the highest of which corresponded to the size of the F2 fusion protein. By comparison to an immunoblotted bovine P450scc standard, the intramitochondrial content of the fusion protein was estimated at 0.5% of total mitochondrial protein.



Immunoblotting of F2 (lane *1* in (a) and (b)) in mitochondrial preparations using anti-P450scc (a) or anti-Adx (b) antibodies. Mitochondria were used for SDS-PAGE in amount 10 μg (a) or 100 μg (b) per lane. Mitochondria isolated from yeast producing pre-CoxIV-P450scc [20] (lane *2* in (a)) or pre-AdxRed–Adx [25] (lane *2* in (b)) were used as a control. The recombinant proteins were detected using anti-P450scc or anti-Adx antibodies. a: *1*) F2; *2*) P450scc; b: *1*) F2; *2*) AdxRed–Adx.

Two of the other bands corresponded to P450scc (figure, panel (a)) and AdxRed–Adx (figure, panel (b)) proteins in their size and immunoreactivity with anti-P450scc and anti-Adx antibodies, respectively. These and other bands reflect proteolysis of the fusion protein in yeast, which seems to be much more intensive than in COS-1 cells [17, 19]. Treatment of the mitochondria with trypsin or pronase K prior to SDS-PAGE did not affect the pattern seen on the immunoblots, suggesting that either F2 was cleaved inside the mitochondria or the products of its cytoplasmic proteolysis were imported into mitochondria. However, at least some of such fragments should not be able to enter into mitochondria after proteolysis in the cytoplasm. This particularly holds for a truncated polypeptide corresponding to the AdxRed–Adx fragment, as such a polypeptide is unlikely to be imported into mitochondria because of the lack of the mitochondrial targeting signal. Therefore, proteolysis of F2 most likely occurred within the mitochondria.

The high proportion of proteolytic products of F2 suggests that some stages of topogenesis of this polypeptide are hampered in yeast mitochondria. As found earlier [20], only a small portion of bovine P450scc expressed in yeast cells is inserted into the mitochondrial inner membrane and exhibits cholesterol hydroxylase activity in a reconstituted system supplemented with purified Adx and AdxRed. Most of the foreign protein is either digested by the Pim1p protease or aggregates, the competition of these two processes being controlled by the mitochondrial mtHsp70 system including co-chaperones Mdj1p and Mge1p [26]. Probably something similar occurred with F2.

Cholesterol hydroxylase activity of isolated mitochondria and sonicated submitochondrial particles. Mitochondria and sonicated submitochondrial particles isolated from yeast cells expressing F2 or pre-CoxIV-F2 exhibited cholesterol hydroxylase activity with 22(R)-hydroxycholesterol as the substrate. The average activity observed in 10 independent experiments constituted about 0.03 pmole of pregnenolone formed per min per mg of mitochondrial protein (table). This value depended on the level of expression of the fusion protein. The fractional cholesterol hydroxylase activity of submitochondr-

ial particles was usually seven times higher than that of mitochondria. Mitochondria prepared from yeast expressing pre-CoxIV-P450scc exhibited almost 600 times higher cholesterol hydroxylase activity in the presence of added excess purified bovine Adx and AdxRed, although its intramitochondrial content was close to that of F2 (table). These data suggest that the normal topogenesis of the protein in yeast mitochondria is complicated by side-processes (proteolysis and aggregation).

Cholesterol hydroxylase activity of yeast mitochondria containing the fusion protein increased 17 or 25 times upon addition of excess amounts of purified P450scc or Adx with AdxRed, respectively (table). Such effects took place for mitochondria containing wholly human F2 or F2 in which the human P450scc moiety was replaced with bovine P450scc.

In a single molecule encompassing three catalytic domains, their interactions depend on the internal flexibility of the polypeptide chain. The activating action of added P450scc or the mixture of Adx and AdxRed in yeast mitochondria could thus suggest that cholesterol hydroxylase activity of F2 is limited by its restricted flexibility, which in part may be accounted for by inappropriate environment in the mitochondrial inner membrane.

This interpretation is at variance with the earlier views on the function of fusion proteins composed of microsomal cytochromes P450 and P450 reductases, wherein joining of these enzymes in a common polypeptide chain results in higher monooxygenase activity owing to more efficient electron transfer between the constituents (see, e.g., [5, 7]). Thus, added NADPH: cytochrome P450 reductase did not stimulate monooxygenase activity of the cytochrome P4501A1–P450 reductase fusion protein [10]. However, this was not the case with other constructs [6] or with F2 (see above) in yeast mitochondria.

While the idea that activation of F2 by P450scc or Adx plus AdxRed is a consequence of restricted flexibility of the fusion molecule is attractive, one should also consider some other possibilities. First, a portion of the F2 molecules may be lacking in FAD or may be folded improperly at the COOH-terminus. That is suggested by the higher activating effect of Adx plus AdxRed than that of P450scc. In fact, Akiyoshi-Shibata et al. [22] were not able to detect cytochrome *c* reductase activity in mitochondria isolated from transgenic yeast synthesizing bovine AdxRed with the pre-CoxIV presequence. It has been suggested that this resulted from amino acid sequence changes in the recombinant protein that occurred in the course of genetic engineering manipulations. However, this suggestion is unlikely, as the added P450scc significantly stimulated the cholesterol hydroxylase activity in yeast mitochondria. Furthermore, as follows from a recent publication by Duport et al. [27], AdxRed expressed together with P450scc and Adx in yeast is catalytically active. Second, one should consider

Cholesterol hydroxylase activities of mitochondria and submitochondrial particles prepared from yeast cells synthesizing pre-CoxIV-F2 or pre-CoxIV-P450scc

Preparation used	nmoles of pregnenolone/min per mg protein × 10^4		
	no additions	+ P450scc	+ Adx + AdxRed
F2 (mito)	0.32 ± 0.08	5.43 ± 3.21	7.64 ± 2.05
F2 (SMP)	2.16 ± 1.06	—	—
P450scc (mito)			191.26 ± 59.70

Note: Production of pregnenolone by mitochondria or submitochondrial particles prepared from transformed yeast cells. Data are from three separate transformations as mean ± SEM ($n = 3$). The values minus those obtained in control experiments (mitochondria isolated from cells transformed with the pYeDP vector) are given.

that we detected both the F2 fusion protein and also the products of its intramitochondrial proteolysis. As mentioned above, the two major proteolytic fragments corresponded to the P450scc and AdxRed–Adx fragments. If these fragments retained their catalytic activities, addition of a large quantity of complementary proteins would result in increased cholesterol hydroxylase activity of mitochondria isolated from the transformed yeast cells. However, the 20-fold increase in the cholesterol hydroxylase activity of yeast mitochondria would require a corresponding excess of the fragments over the fusion protein, which was not the case. Besides, highly selective proteolysis of F2 yielding catalytically active fragments does not seem very probable. Third, we cannot exclude that the active centers of the cytochrome P450scc, Adx, and AdxRed parts are somehow deformed in the fusion molecule, which leads to some decrease in their partial catalytic activities. In such case, the addition of excessive amounts of the native partners might stimulate the integral catalytic activity of the F2 fusion.

The present work together with other data [27] demonstrates the feasibility of constructing transgenic yeast with cholesterol hydroxylase activity. However, it also shows that the topogenesis of the P450scc–AdxRed–Adx fusion protein in yeast mitochondria is complicated. The effect of activation of this protein by its component parts provokes some new questions regarding the mechanism of functioning of the multienzyme protein.

This work was supported by the Russian Foundation for Basic Research (grants No. 99-04-48003 and 96-04-97814 to V. N. L.). The authors thank Drs. S. A. Usanov and A. A. Chernogolov (Institute of Bioorganic Chemistry, Minsk) for preparations of bovine P450scc, Adx, and AdxRed and corresponding antibodies, as well

as Dr. A. G. Pryadko for [^{125}I]progesterone and anti-progesterone antibodies. The authors also express gratitude to their colleague Dr. I. E. Kovaleva for a yeast clone producing the pre-AdxRed–Adx fusion protein.

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