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Formation and Functioning of Fused Cholesterol Side-Chain Cleavage Enzymes

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ABSTRACT

We studied the properties of various fused combinations of the components of the mitochondrial cholesterol side-chain cleavage system including cytochrome P450scc, adrenodoxin (Adx), and adrenodoxin reductase (AdR). When recombinant DNAs encoding these constructs were expressed in *Escherichia coli*, both cholesterol side-chain cleavage activity and sensitivity to intracellular proteolysis of the three-component fusions depended on the species of origin and the arrangement of the constituents. To understand the assembly of the catalytic domains in the fused molecules, we analyzed the catalytic properties of three two-component fusions: P450scc–Adx, Adx–P450scc, and AdR–Adx. We examined the ability of each fusion to carry out the side-chain cleavage reaction in the presence of the corresponding missing component of the whole system and examined the dependence of this reaction on the presence of exogenously added individual components of the double fusions. This analysis indicated that the active centers in the double fusions are either unable to interact or are misfolded; in some cases they were inaccessible to exogenous partners. Our data suggest that when fusion proteins containing P450scc, Adx, and AdR undergo protein folding, the corresponding catalytic domains are not formed independently of each other. Thus, the correct folding and catalytic activity of each domain is determined interactively and not independently.

INTRODUCTION

Narhi and Fulco (1986) discovered a 119-kDa protein of *Bacillus megaterium*, termed cytochrome P450 BM-3 or CYP102, with an extremely high turnover number exceeding 1500. This protein consists of two catalytic domains, one of which resembles cytochrome P450 and contains heme, while the another includes the binding sites for FAD and FMN, similar to NADPH:cytochrome P450 reductases. A mammalian nitrite synthase has also been described that contains two domains, one of which binds a cytochrome P450-like heme and with the other binding a flavin group (McMillan *et al.*, 1992; Geller *et al.*, 1993). These findings stimulated work on design and heterologous expression of fused enzymes built of various microsomal forms of cytochrome P450 and NADPH:cytochrome P450 reductase (Yabusaki *et al.*, 1988; Sakaki *et al.*, 1990, 1994; Shibata *et al.*, 1990; Fisher *et al.*, 1992; Chaurasia *et al.*, 1995; Wilks *et al.*, 1995; Chun *et al.*, 1996; Estabrook *et al.*, 1996; Shet *et al.*, 1996, 1997; Parikh and Guengerich, 1997; Helvig and Capdevila, 2000). General conclusions from these investigations were: (1) the fused proteins show high monooxygenase activities with turnover numbers varying from 1 (Chun *et al.*, 1996) to 200 (Sakaki *et al.*, 1990); (2) monooxygenase activities of microsomal fractions containing fused proteins are several times higher than those of mixtures of the fractions containing separately expressed cytochrome P450s and reductases (Shibata *et al.*, 1990; Sakaki *et al.*, 1994); (3) the activities of some isolated fusion proteins are higher than those of the systems reconstituted from individual components (Wilks *et al.*, 1995; Shet *et al.*, 1996); (4) in some cases the fused proteins can be activated by adding the reductase component (Chaurasia *et al.*, 1995; Shet *et al.*, 1996; Helvig and Capdevila, 2000), while in others such activation does not occur (Chun *et al.*, 1996). These observations suggest that the fused proteins act as self-sufficient systems ensuring effective intramolecular electron transfer between the catalytic domains.

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There are fewer studies concerning design and expression of three-component mitochondrial monooxygenase systems. In the first of these, COS-1 cells expressing the P450scc–AdR–Adx recombinant protein produced pregnenolone from 22(R)-hydroxycholesterol five times more effectively than cells simultaneously expressing equimolar amounts of P450scc, AdR, and Adx (Harikrishna *et al.*, 1993; Black *et al.*, 1994). Similar results were obtained for the P450c27–AdR–Adx fusion protein (Dilworth *et al.*, 1996). However, fusion proteins composed of P45011β, Adx, and AdR were less active than cytochrome P45011β expressed in COS-1 cells and functioning in combination with endogenous mitochondrial electron carriers (Cao *et al.*, 2000). The qualitative nature of these data did not allow one to estimate the specific activities of the fused proteins, because the intracellular contents of the expressed proteins were not estimated (Harikrishna *et al.*, 1993; Black *et al.*, 1994; Dilworth *et al.*, 1996; Cao *et al.*, 2000), and because the activities of these fusion proteins were estimated indirectly by the rate of accumulation of reaction products in the cell growth medium. Nevertheless, the data obtained for P450scc indicated that this protein acts as a self-sufficient multienzyme system (Black *et al.*, 1994).

The soluble nature of bacterial cytochrome P450 enzymes permitted expression of fusion proteins composed of cytochrome $P450_{CAM}$, putidaredoxin (Pd), and putidaredoxin reductase (PdR) from *Pseudomonas putida* expressed in the *Escherichia coli* (Sibbesen *et al.*, 1996). The activity of the isolated fusion PdR-Pd-$P450_{CAM}$ was comparable to that of a system reconstituted from its individual proteins. These data confirmed that such fusions of three-component cytochrome P450 systems can function as a self-sufficient system. Furthermore, fusion proteins of rat microsomal cytochrome P4501A1 fused to maize ferredoxin I and pea ferredoxin:$NADP^+$ reductase from plant chloroplasts yielded the same conclusion (Lacour and Ohkawa, 1999).

We used similar approaches for the three-component fusions of cytochrome P450scc, Adx, and AdR expressed in *E. coli*. Both the P450scc-AdR-Adx hybrid (termed F2) and P450scc catalyze cholesterol side-chain cleavage of cholesterol in mitochondria but not in the endoplasmic reticulum (Black *et al.*, 1994). By contrast, all the components of cholesterol side-chain cleavage system synthesized in the *E. coli* cells have normal catalytic activities (Wada *et al.*, 1991; Palin *et al.*, 1992; Iwahashi *et al.*, 1992; Uhlmann *et al.*, 1992; Vonrhein *et al.*, 1999). Thus, *E. coli* should be a suitable host for expressing the corresponding triple fusions. We now describe the expression and characterization of two or three components of the cholesterol side chain cleavage system in *E. coli*.

MATERIALS AND METHODS

Bacterial strains and plasmids

We used *E. coli* strain JM109 (Promega, Madison, WI) and pTrc99A vector with the hybrid *trp/lac(trc)* promoter (Amann *et al.*, 1988). The pTrc99A/P450scc plasmid containing DNA encoding the mature form of bovine cytochrome P450scc was a generous gift of Dr. M.R. Waterman (Vanderbildt University, USA) (Wada *et al.*, 1991). pYeDP/pre-CoxIV-F2 and pYeDP/F2 were constructed as earlier (Novikova *et al.*, 2000). The pYeDP/pre-CoxIV-F2 plasmid contains the nucleotide sequence, encoding fusion protein NH_2-pre-CoxIV-P450scc (bovine)–AdR(human)–Adx(human)–COOH. The fused human protein termed F2 (NH_2-pre-P450scc–AdR–Adx–COOH) was constructed from the cDNAs for human P450scc (Chung *et al.*, 1986), human Adx (Picado-Leonard *et al.*, 1988), and human AdR (Solish *et al.*, 1988), and cloned in the pECE vector as previously described in (Harikrishna *et al.*, 1993; Black *et al.*, 1994).

Design of recombinant DNA encoding fusion proteins

The recombinant proteins shown in Figure 1 were constructed using corresponding recombinant plasmids based on the pTrc99A vector. The pTrc99A/P–AdR–Adx(b–h) plasmid for expression of the NH_2–P450scc(bovine)–AdR(human)–Adx(human)–COOH fusion protein, designated as P–AdR–Adx(b–h), in *E. coli* was prepared by modification of pTrc99A/P450scc (Wada *et al.*, 1991). The DNA fragment with the stop codon was excised from pTrc99A/P450scc with *Bal*I and *Sma*I. The resulting vector encoding the N-terminal region of mature P450scc was then used for cloning a DNA fragment excised from pYeDP-pre-CoxIV-F2 (Novikova *et al.*, 2000) by treatment with *Eco*RI (unique site at the 3′-end of the F2 stop codon), Klenow fragment, and *Bal*I. The fused fragment inserted into pTrc99A contained the nucleotide sequence for the fragment of bovine cytochrome P450scc followed by the sequence for the (human) AdR–Adx segment of F2. The second codon in DNA encoding mature P450scc was modified to substitute Val for Ile.

The pTrc99A/P–AdR–Adx(h) plasmid was constructed using pTrc99A/P450scc, which, to remove its P450scc-encoding sequence, was consecutively treated with *Nco*I and *Kpn*I, completed with T4 DNA polymerase, and dephosphorylated with calf intestinal alkaline phosphatase (CIAP). The PCR-amplified pYeDP/F2 fragment (Novikova *et al.*, 2000) encoding the human F2 protein without the N-terminal presequence of its P450scc moiety [P-AdR–Adx(h)], was prepared using phosphorylated primers 1 and 2 (Table 1) and cloned into the pTrc99A fragment of the initial plasmid. Silent substitutions were made in the first four codons to enrich this region for A+T, which enhances expression of cytochromes P450 in *E. coli* (Wada and Waterman, 1992). The changes in the nucleotide sequence resulted in substitution of Val for Ile downstream of initiating Met in the mature form of P450scc.

The pTrc99A/AdR–Adx–P(h) plasmid capable of expressing the human AdR–Adx–P450scc fusion protein (Fig. 1) was prepared in two steps. First, the DNA fragment for AdR–Adx without a stop codon was inserted into pTrc99A, resulting in pTrc99A/AdR–Ad; the P450scc cDNA was then added to this vector. To prepare pTrc99A/AdR–Adx, the pTrc99A/P450scc plasmid was consecutively treated with *Nco*I and *Sal*I to remove the P450scc sequence, completed with T4 DNA polymerase, and dephosphorylated with CIAP. The fragment of pYeDP/F2 encoding its AdR–Adx moiety was amplified by PCR using phosphorylated primers 3 and 4 (Table 1) and cloned into pTrc99A. *Nco*I and *Bcu*I sites were formed upstream and downstream of the inserted DNA fragment; the resulting pTrc99A/AdR–Adx was cut with *Bcu*I, completed with T4

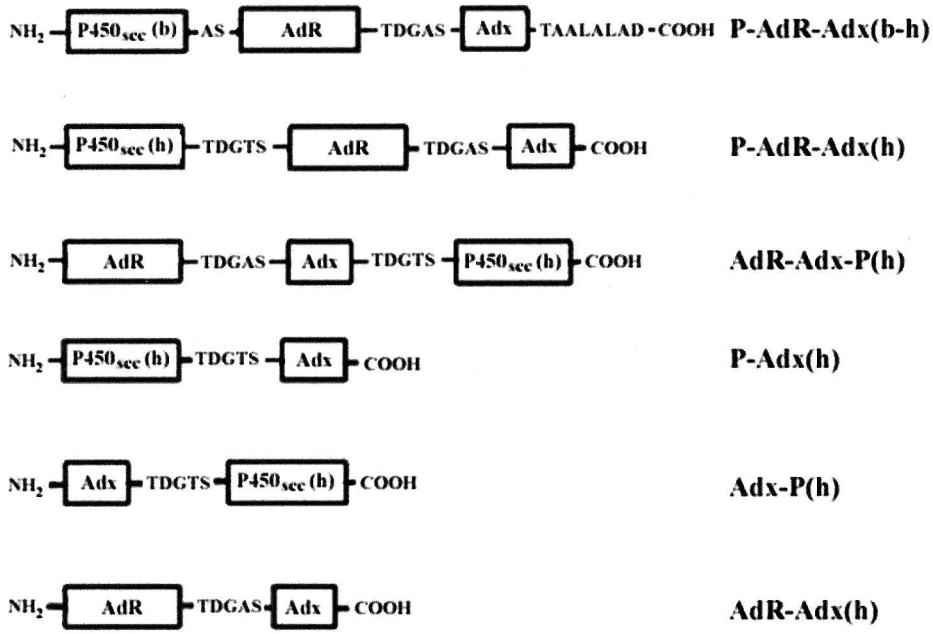

FIG. 1. Schematic representation of the fusion protein constructs. DNA encoding mature forms of human AdR and Adx were linked in frame to the C terminus of mature bovine cytochrome P450scc to obtain the P450scc–AdR–Adx(b–h) chimeric protein. All other fused proteins are composed of human P450scc, AdR, and Adx. The diagrams show the linkers between protein domains in each fusion protein with the amino acids in the linkers given in the single-letter code.

DNA polymerase, and dephosphorylated The pYeDP/F2 fragment encoding mature human P450scc was amplified by PCR using phosphorylated primers 5 and 6 (Table 1) and cloned into pTrc99A/AdR–Adx to obtain pTrc99A/AdR–AdX–P(h). Construction of pTrc99A/AdR–Adx(h) was started with pTrc99A/P450scc. The pTrc99A fragment of the initial plasmid was obtained as it was for pTrc99A/P–AdR–Adx(h) and joined with the sequence for human AdR–Adx that had been prepared by PCR amplification of the corresponding region of pYeDP/F2 with phosphorylated primers 7 and 8 (Table 1). In AdR–Adx–P(h) and AdR–Adx(h), the additional Val codon was downstream from the Met translational initiation codon of the AdR moiety.

The pTrc99A/P–Adx(h) plasmid was prepared using pTrc99A/P–AdR–Adx(h) that was sequentially treated with *Nhe*I and *Bcu*I, which ensured removal of the AdR-encoding sequence. The resulting vector was ligated afterwards.

The pTrc99A/Adx–P(h) plasmid was constructed from pTrc99A/P450scc by cleavage with *Nco*I and *Kpn*I, completion with T4 DNA polymerase, and dephosphorylation The large pTrc99A fragment was isolated and joined to the Adx–P(h)-encoding sequence prepared by PCR amplification of the corresponding region of pTrc99A/AdR–Adx–P(h) with phosphorylated primers 9 and 10 (Table 1).

To avoid possible errors during PCR amplification, we used a thermostable DNA polymerase with correcting activity (LR polymerase, Silex M, Moscow), and amplifications were performed for only five to eight cycles. The PCR products and the pTrc99A fragment of pTrc99A/P450scc were purified using

TABLE 1. OLIGONUCLEOTIDE PRIMERS FOR FUSION CONSTRUCTION

Oligo No.	*Used to construct*[a]	*Fragment, generated by PCR*	*Sequence*
1	P–AdR–Adx(h)	P450scc–AdR–Adx	GTCTCCACAAGAAGTCCTCGCCCCTTCAATGA
2		3233 bp	GTACCTCAGGAGGTCTTGCCCACATCA
3	AdR–Adx–P(h)	AdR–Adx	GTCTCCACACAGGAGAAGA
4		1781 bp	CTAGTGCCGTCGGTGGAG
5	AdR–Adx–P(h)	P450scc	TACTTCCACCCGCAGT
6		1451 bp	ATCACTGCTGGGTTGCTTC
7	AdR–Adx(h)	AdR–Adx	GTCTCCACACAGGAGAAGA
8		1775 bp	GTACCTCAGGAGGTCTTGCCCACATCA
9	Adx–P(h)	Adx–P450scc	GCAAGCAGCTCAGAAGATAAAAT
10		1802 bp	ATCACTGCTGGGTTGCTTC

[a]See Figure 1 for schematic representation of the constructs.

0.6% LGT agarose. The structures of all recombinant plasmids were confirmed by sequencing.

Expression of genes encoding recombinant proteins

Competent bacteria were transformed with the recombinant plasmids by standard procedures. A colony was transferred from a Petri dish into 5 ml of LB liquid medium containing ampicillin (100 μg per ml), and cultivated overnight at 37°C. The culture was diluted 1:200 with TB medium (ampicillin 100 μg per ml). Gene expression was induced by adding IPTG (0.5 mM) into the growth medium supplemented with δ-aminolevulinic acid (0.5 mM). The culture was then incubated for 48 h at 22–28°C with shaking at 140 rpm.

Preparation of bacterial homogenates

Bacteria (1 liter culture of $OD_{600} \approx 6$–7) were harvested by centrifugation (7500 × *g*, 10 min), washed with buffer (150 mM NaCl, 5 mM $MgSO_4$, 10 mM HEPES, pH 7.0), and centrifuged for 15 min at 3500 × *g*. The sediment was suspended in 12 ml of cooled buffer (5 mM $MgSO_4$, 20 mM potassium phosphate buffer pH 7.4, and 4 mM DTT), and the bacteria were disrupted using a French press (maximum pressure of 8 t per cm^2). Debris was removed by centrifugation for 15 min at 15,000 × *g* and the resulting supernatant was centrifuged for 15 min at 39,000 × *g* to sediment inclusion bodies that could have formed during synthesis of the recombinant proteins. The supernatant containing soluble proteins and the membrane vesicle fraction was supplemented with glycerol to a final concentration of 20% and stored at −70°C.

Spectral characteristics of recombinant proteins

Reduced CO difference spectra were recorded from cell homogenates after removing of bacterial debris and the 39,000 g sediment. Spectra were registered with an Aminco spectrophotometer.

Estimation of enzymatic activity

Cholesterol side-chain cleavage activity was measured in a mixture containing 0.5–5.0 mg of bacterial protein and 25 nmol of 22(R)-hydroxycholesterol in 0.5 ml of 30 mM phosphate buffer (pH 7.5) supplemented with 0.05% Tween 20. When measuring the activities of P450scc or double fusion proteins, the corresponding missing components (purified bovine Adx, AdR, and P450scc) of the cholesterol side-chain cleavage system were added to the reaction mixture in saturating amounts (0.2 nmol each). When measuring the activation of double fusion proteins by extra amounts of their constituents, these amounts were also 0.2 nmol. The reaction was started by adding an NADPH-generating system. The resulting pregnenolone was then converted to progesterone with cholesterol oxidase, the steroids were extracted, evaporated to dryness, and the residue was dissolved in buffer, as described (Novikova *et al.*, 2000). Progesterone was then determined with an ELISA kit (antiprogesterone antibodies and progesterone–peroxidase conjugate), generously provided by Dr. A.G. Pryadko (Institute of Bioorganic Chemistry, Minsk, Belarus).

Other methods

P450scc, AdR–Adx(h), P–Adx(h), Adx–P(h), P–AdR–Adx(b–h), P–AdR–Adx(h), and AdR–Adx–P(h) were visualized by Western blotting after $NaDodSO_4$-PAGE (10% acrylamide gel) of bacterial homogenates using IgG fractions of antisera against P450scc, Adx, and AdR. Bovine cytochrome P450scc, Adx, and AdR were prepared as described in (Akhrem *et al.*, 1979; Chashchin *et al.*, 1984).

RESULTS

Synthesis and characteristics of the P–AdR–Adx(b–h), P–AdR–Adx(h), and AdR–Adx–P(h) fusion proteins

E. coli were transformed with pTrc99A/P–AdR–Adx(b–h), pTrc99A/P–AdR–Adx(h), or pTrc99A/AdR–Adx–P(h) and grown at 22–28°C, which allowed the proteins to accumulate in reasonable amounts without affecting bacterial growth. Immunoblotting of *E. coli* homogenates with anti-Adx, anti-P450scc, and anti-AdR antibodies revealed an immunoreactive protein of about 120 kDa (Fig. 2); this is the expected size for fusion proteins consisting of P450scc, Adx, and AdR. In addition, some smaller proteins were detected by immunoblotting that were not found in control *E. coli* homogenates, probably representing proteolysis of the fusion protein.

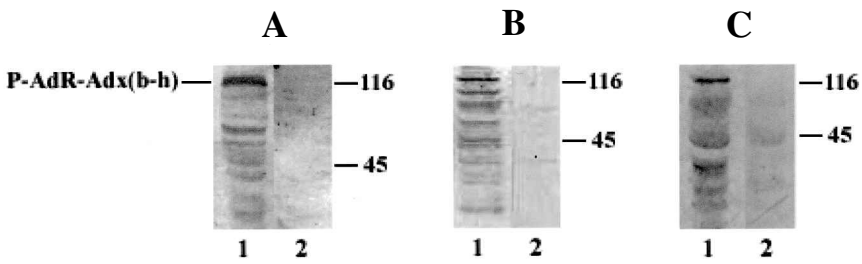

FIG. 2. Western blot analysis of homogenates from transformed *E. coli* containing P–AdR–Adx(b–h). The homogenates (100 μg protein each) were subjected to $NaDodSO_4$-PAGE (10% acrylamide). The fusion protein (about 120 kDa) was detected with antisera to Adx (**A**), P450scc (**B**), and AdR (**C**). In each case, lane 1 corresponds to homogenate with P–AdR–Adx(b–h), and lane 2 corresponds to homogenate of nontransformed cells.

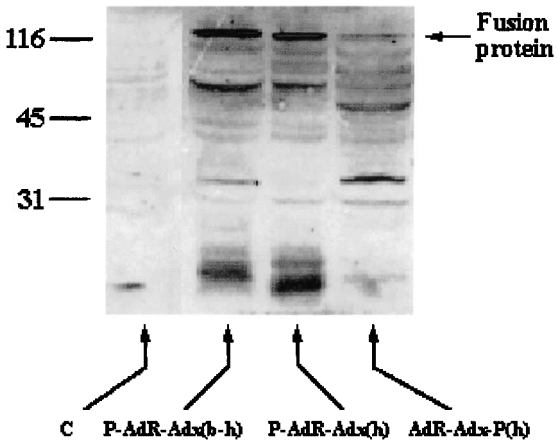

FIG. 3. Western blot analysis of homogenates from transformed *E. coli* containing P–AdR–Adx(b–h), P–AdR–Adx(h), and AdR–Adx–P(h). The homogenates (100 μg protein each) were subjected to $NaDodSO_4$-PAGE (10% acrylamide). The recombinant proteins were detected with anti-Adx. The molecular mass of the fusion was about 120 kDa (markers are shown on the left). Homogenate of nontransformed cell (C) is on the left lane.

When the P–AdR–Adx(b–h), P–AdR–Adx(h), and AdR–Adx–P(h) triple fusion proteins were expressed in *E. coli*, levels of expression of the full-sized products differed (Fig. 3). Although P–AdR–Adx(h) and AdR–Adx–P(h) differ only in the arrangement of the three protein domains, the population of cleavage products detected with anti-Adx antibodies were different. Expression of P–AdR–Adx(h) yielded a 65-kDa polypeptide, while expression of AdR–Adx–P(h) yielded a 33-kDa polypeptide as the main product (Fig. 3). Hence, different sites in the P–AdR–Adx(h) and AdR–Adx–P(h) fusion proteins are preferentially cleaved by bacterial proteases. The expression levels of P–AdR–Adx(b–h) and P–AdR–Adx(h), which differ only in containing bovine or human cytochrome P450scc moieties, were also different. This may reflect different rates of synthesis, as the levels of expression of bovine (Palin *et al.*, 1992) and human (Woods *et al.*, 1998) P450scc in the same vector in *E. coli* are 40–60 and 10–35 nmol per liter of culture, respectively.

The homogenates containing P–AdR–Adx(b–h), P–AdR–Adx(h), and AdR–Adx–P(h) catalyzed side-chain cleavage activity with 22(R)-hydroxycholesterol as a substrate, but the homogenates of control bacteria did not (Table 2). The average activity P–AdR–Adx(b–h) in 15 independent experiments was 0.9 pmol pregnenolone per min per mg cell homogenate protein, 30-fold higher than for the P–AdR–Adx(h), construct, and 14-fold higher for the AdR–Adx–P(h) construct. To examine whether *E. coli* homogenates were free of endogenous factors that could interact with components cholesterol side-chain cleavage system and contribute to the side-chain cleavage reaction, we assayed homogenates containing recombinant P450scc supplemented with isolated bovine Adx, AdR, or both. In the absence of exogenously added Adx or AdR, the homogenates containing had no detectable side-chain cleavage activity (Table 2).

Expression of the mature form of cytochrome P450scc in *E. coli* results in the formation of an integral membrane protein with characteristic reduced CO difference spectra characteristic of all native cytochrome P450s (Wada *et al.*, 1991). Similarly, fusions of cytochrome P450s with NADPH:cytochrome P450 reductases also yield classical difference spectra (Yabusaki *et al.*, 1988; Sakaki *et al.*, 1990; Shibata *et al.*, 1990; Chaurasia *et al.*, 1995; Wilks *et al.*, 1995; Chun *et al.*, 1996; Parikh and Guengerich, 1997). However, the CO-difference spectra of *E. coli* expressing P–AdR–Adx(b–h) does not show the presence of normally folded cytochrome P450scc moiety, although a typical spectrum is seen in the homogenates containing recombinant P450scc (Fig. 4). These data suggest that the number of P–AdR–Adx molecules having a normally folded P450scc moiety is too small to be detected spectroscopicly.

Synthesis and characteristics of Adx–P(h), P–Adx(h), and AdR–Adx(h) fusion proteins

The results with P–Adx–AdR(h) and AdR–Adx–P(h) suggest that their characteristics depend on the arrangement of their three constituents. To elucidate the principles of formation of multicomponent fusion proteins, we analyzed some properties of simpler two-component constructions. When plasmids encoding P–Adx, Adx–P, and AdR–Adx, were expressed in *E. coli*, immunoblotting with anti-Adx (Fig. 5), or anti-P450scc (not shown) revealed the presence of a single protein product

TABLE 2. SIDE-CHAIN CLEAVAGE ACTIVITY OF FUSION PROTEINS IN HOMOGENATES OF RECOMBINANT *E. COLI*

	Activity, pmol pregnenolone-min^{-1}·mg^{-1} homogenate protein			
	Additives			
Protein	*AdR*	*Adx*	*AdR+Adx*	*Free*
P–AdR–Adx(p–h)	—	—	—	0.898 ± 0.523
P–AdR–Adx(h)	—	—	—	0.029 ± 0.020
AdR–Adx–P(h)	—	—	—	0.065 ± 0.010
P450scc	0.002 ± 0.001	0.003 ± 0.001	36.99 ± 12.99	—

The values (mean ± SD) from at least three experiments are given. The activity of P450scc in bacterial homogenates was measured in the presence of Adx, AdR, or Adx+AdR. For the measurements, individual bovine proteins (AdR and Adx) were taken at a rate of 0.2 nmol per a sample.

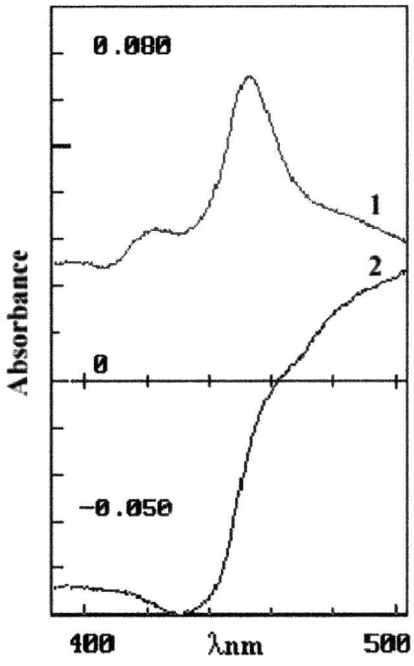

FIG. 4. Reduced CO difference spectra of *E. coli* homogenates containing recombinant bovine P450scc (1) and P–AdR–Adx(b–h) (2). The contents of samples (1) and (2) upon spectra registration were 3 and 17 mg cell homogenate protein in 0.7 ml of 50 mM K-phosphate buffer containing 0.5 M sucrose, pH 7.4, respectively. An approximate estimation showed a five-fold excess of the intracellular content of P450scc over that of P–AdR–Adx(b–h).

in the homogenates. To assess the correct folding of these double fusions, we assayed their side-chain cleavage activity by adding the missing component of the whole system, i.e., AdR for P–Adx and Adx–P, P450scc for AdR–Adx. We also examined their ability to be activated by adding extra amounts of isolated bovine P450scc, Adx, or AdR, depending on the composition of double fusion proteins. In all cases, the bacterial homogenates containing double fusion proteins exerted minimal side-chain cleavage activity in the absence of corresponding missing component of the cholesterol hydroxylase/lyase system.

Homogenates of bacteria expressing P–Adx(h), supplemented with exogenously added AdR, had minimal side-chain cleavage activity, with or without adding Adx to the reaction mixture; by contrast, adding P450scc increased the activity 20-fold (Fig. 6A). These data suggest that the P450scc moiety is the limiting component of the P–Adx(h) fusion protein. Consistent with this, the reduced CO difference spectrum of the homogenate containing P–Adx(h) showed a maximum at 420 nm (Fig. 7A), which is characteristic of a denatured cytochrome P450. By contrast, the Adx moiety is fully active and its substrate-binding site is accessible for both exogenous AdR, an obligatory component of the reaction mixture, and P450scc, an activating component, because an excess of exogenous Adx does not increase the activity of P–Adx(h) significantly (Fig. 6A). Results with the converse construct, Adx–P(h) were qualitatively similar to the results seen with P–Adx(h), but the overall level of activity was 10-fold greater (note differences on the y-axis scales) (Fig. 6B). Hence, similarly to P–Adx(h), the P450scc moiety is the rate-limiting component of Adx–P(h), whereas the Adx moiety is active and accessible for both exogenous P450scc and AdR. Consistent with the 10-fold greater activity of Adx–P(h) compared to P–Adx(h), Adx–P(h) had a normal reduced CO difference spectrum (Fig. 7B). However, the P450scc moiety, although native, must interact with the Adx moiety poorly, explaining the low activity of Adx–P(h) without added P450scc.

When supplemented with exogenously added P450scc, the AdR–Adx(h) fusion has very little activity, even when supplemented with AdR (Fig. 6C). However, addition of Adx raises activity 200-fold. This suggests either that the Adx moiety is improperly folded or that its substrate-binding site is not accessible to either endogenous or exogenous AdR. By contrast, the AdR moiety in the fusion can easily interact with exogenous Adx, yielding effective side-chain cleavage activity with added P450scc.

DISCUSSION

Although numerous fused microsomal monooxygenases have been constructed and studied (Yabusaki *et al.*, 1988; Shibata *et al.*, 1990; Sakaki *et al.*, 1990, 1994; Fisher *et al.*, 1992; Chaurasia *et al.*, 1995; Wilks *et al.*, 1995; Chun *et al.*, 1996; Shet *et al.*, 1996, 1997; Estabrook *et al.*, 1996; Parikh and Guengerich, 1997; Helvig and Capdevila, 2000), only a few fusions of three-component cytochrome P450-containing enzymes have been built (Harikrishna *et al.*, 1993; Black *et al.*, 1994; Dilworth *et al.*, 1996; Cao *et al.*, 2000; Sibbesen *et al.*, 1996; Lacour and Ohkawa, 1999; Novikova *et al.*, 2000). Two of them, with bacterial $P450_{CAM}$ (Sibbesen *et al.*, 1996) and microsomal P4501A1 (Lacour and Ohkawa, 1999), were studied *in vitro* by conventional enzymatic approaches. These data supported the conclusion from molecular biologic studies (Black *et al.*, 1994) that these fusions represent self-sufficient catalytic units effectively functioning through intramolecular electron transfer. The activity of the recombinant protein containing $P450_{CAM}$ was comparable to that of a system reconstituted from individ-

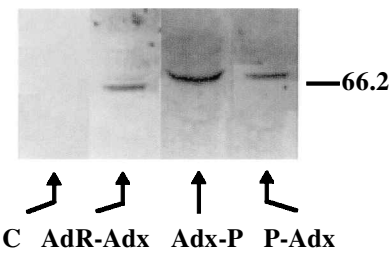

FIG. 5. Western blot analysis of bacterial homogenates (200 μg protein) containing P–Adx(h), Adx–P(h), and AdR–Adx(h), detected with anti-Adx. Homogenate of nontransformed bacteria (200 μg) (C) is in the left lane. Molecular mass marker is on the right.

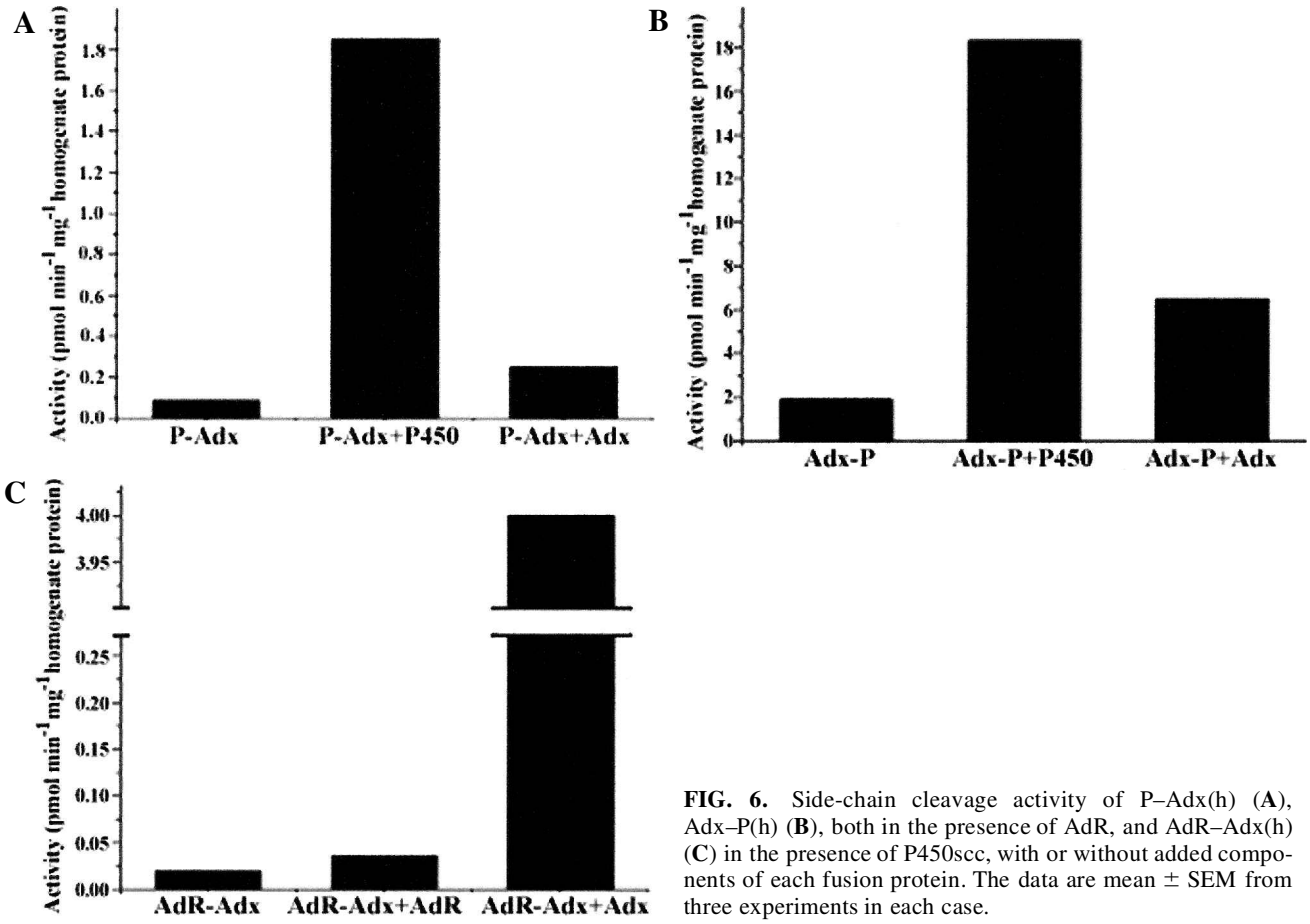

FIG. 6. Side-chain cleavage activity of P–Adx(h) (**A**), Adx–P(h) (**B**), both in the presence of AdR, and AdR–Adx(h) (**C**) in the presence of P450scc, with or without added components of each fusion protein. The data are mean ± SEM from three experiments in each case.

ual components (Sibbesen *et al.*, 1996). The effective functioning of the triple fusions implies that each has three catalytically active domains. To achieve intramolecular electron transfer, the P450 domain and the reductase domain would need to be positioned either so that they could form a temporary triple complex with the ferredoxin electron carrier, or the P450 and reductase moieties might interact sequentially with ferrodoxin. The former case admits a fixed structure of the fused molecules, while the latter requires a flexible structure. It is not yet clear which of these mechanisms is operative.

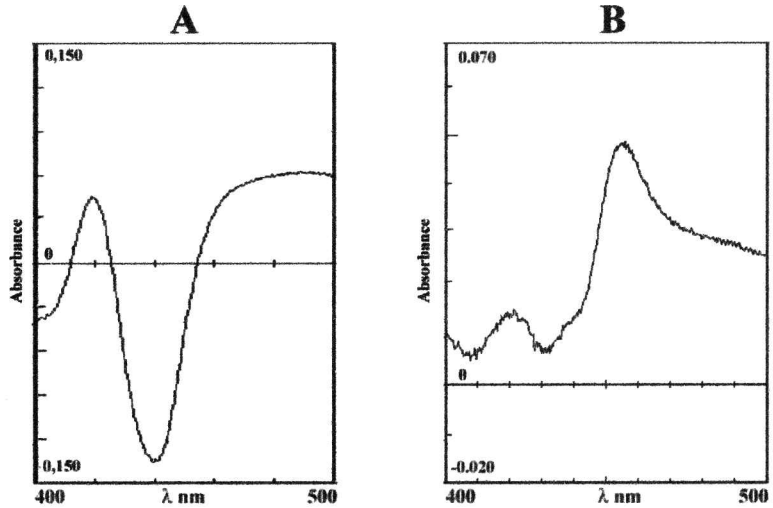

FIG. 7. Reduced CO difference spectra of bacterial homogenates containing the P–Adx(h) (**A**) and Adx–P(h) (**B**) fusion proteins. The samples contained 10 mg protein in 0.7 ml of 50 mM K-phosphate buffer containing 0.5 M sucrose, pH 7.4.

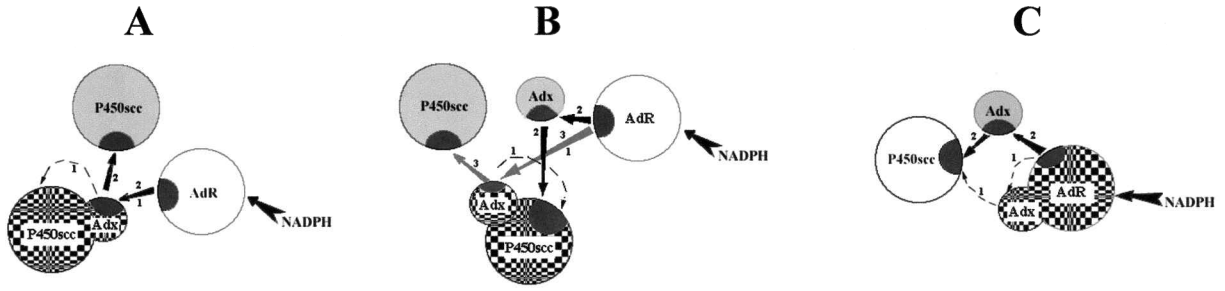

FIG. 8. Proposed schemes for the interactions of the components of double fusion proteins (checkered figures) and corresponding missing components of the whole system (empty circles) as well as added activating components of fusion proteins (shaded circles). In black are binding sites of P450scc, AdR, and Adx. Thick and thin arrows show effective and ineffective ways of interaction, respectively. (**A**) P–Adx(h) in combination with AdR (1) or AdR + P450scc (2); (**B**) Adx–P(h) in combination with AdR (1), AdR + Adx (2) and AdR + P450scc (3); (**C**) AdR–Adx(h) in combination with P450scc (1) and P450scc + Adx (2).

Previous studies show that the P450scc–AdR–Adx (Harikrishna *et al.*, 1993), P450c27–AdR–Adx (Dilworth *et al.*, 1996), and P45011β–AdR–Adx (Cao *et al.*, 2000) fusion proteins expressed in COS-1 cells confer the expected catalytic activities, but the specific activities, spectral characteristics and the mechanisms of interaction among the catalytic domains of these fusions were not investigated. We sought to clarify the principles of formation and functioning of fused systems containing P450scc, Adx, and AdR. Because P450scc and AdR bind to the same site on Adx (Lambeth *et al.*, 1984) it has been thought that P450scc and AdR attach to Adx sequentially (the "shuttle" mechanism) (Tuls *et al.*, 1987; Vickery, 1997; Muller *et al.*, 2001). Alternatively, it has been suggested that a temporary triple complex may form (Turko *et al.*, 1988; Hara and Kimura, 1989), although the arrangement of such a complex is currently difficult to envisage.

Assuming the shuttle mechanism for the cholesterol side-chain cleavage system, one could consider whether the activity of P–AdR–Adx results from flexibility of the fused molecule. Presumably, the higher the flexibility, the higher the activity; however, this feature of the fused molecule makes it highly sensitive to proteinases. This would suggest that the AdR–Adx–P version would be a better catalyst than others, because it is almost completely cleaved in *E. coli.* In fact, the activity of AdR–Adx–P(h) normalized to the cell homogenate protein is higher than that of its P–AdR–Adx(h) homologous version (Table 2), although the intracellular context of AdR–Adx–P(h) is much lower than that of P–AdR–Adx(h) (see Fig. 3). Harikrishna *et al.* (1993) initially proposed that the key element for activity was that the Adx moiety be on a flexible "tether" so that the same site on Adx could alternatively interact with the P450scc and AdR moieties. However, the P450scc–Adx–AdR (F3) construct expressed in COS-1 cells also had high but variable activity and low intracellular content (Harikrishna *et al.*, 1993). Thus, it appears that increased flexibility enhances catalytic activity of a fused enzyme but also decreases the stability of the fusion. Thus, optimal fusion design is a balance between needed flexibility and increased susceptibility to proteolysis.

The principles of folding of the P–AdR–Adx protein were elucidated by assaying the activities of the two-component P–Adx(h), Adx–P(h), and AdR–Adx(h) fusion proteins when the third component of the system was added exogenously. Examining the effects of exogenous P450scc, Adx, and AdR on the catalytic activity of these fusion proteins identified the rate-limiting component in each. In P–Adx(h) and Adx–P(h), the Adx moiety was active and easily accessible to both exogenous P450scc and AdR (Fig. 8A). The position of the P450scc moiety relative to the Adx moiety determines whether the P450 was either native or denatured. However, even native P450scc did not interact effectively with added Adx, indicating the Adx-binding site of P450scc was shielded in the fused molecule (Fig. 8B). Similarly, the Adx moiety was shielded (or disrupted) in the AdR–Adx(h) fusion protein, whereas the AdR moiety was fully active and accessible to exogenous Adx (Fig. 8C). These results indicate that the catalytic domains of these fusion proteins are not folded independently of each other. Because of this, the activity of the fused molecules depends on the arrangement of individual catalytic domains, as indicated by earlier results (Sakaki *et al.*, 1990; Harikrishna *et al.*, 1993; Cao *et al.*, 2000; Sibbesen *et al.*, 1996; Lacour and Ohkawa, 1999).

The three-component fusions of the cholesterol side-chain cleavage system are very susceptible to proteolysis in *E. coli*, similar to their proteolytic sensitivity when expressed in mammalian cells (Harikrishna *et al.*, 1993) or in yeast (Novikova *et al.*, 2000). By contrast, the two-component constructs are resistant to proteases. This probably reflects the greater flexibility of the three-component systems, which appears to be necessary to facilitate contacts between the active centers. A more detailed understanding of the formation and functioning of these fusion proteins will require catalytic and biophysical studies with isolated proteins.

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