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Microsomal P450s Use Specific Proline–Rich Sequences for Efficient Folding, but Not for Maintenance of the Folded Structure[1]

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The amino-terminal region of microsomal P450s contains three distinct sequence motifs, the signal-anchor sequence (SA), the basic sequence (BS), and the proline-rich sequence (PR). Studies with two P450s of the CYP2C subfamily, P4502C11 (CYP2C11) and P4502C2 (CYP2C2), have indicated that upon expression in eukaryotic cells (yeast, COS cells, and insect cells), specific proline residues in PR are important for proper folding. In the present study, we have established that the PR region in a very different CYP gene family, P450c17 (CYP17), is also important for efficient folding. These studies have been carried out using expression in *Escherichia coli*. Using P4502C11, we have established that the folding requirements for P450s in bacteria are very similar to those in eukaryotic cells. Interestingly, when the PR from P450c17 is swapped for that of P4502C11 and *visa versa*, complete misfolding is observed. However, both the BS and SA can be swapped between these P450s without affecting folding. After proper folding of P450c17, removal of the PR by factor Xa protease has no effect on the maintenance of the P450 structure. Inspection of the sequences of many different CYP gene families indicates that the PR sequence is conserved within a gene family but varies considerably between families. We conclude that PR is important for directing the folding pathway leading to the functional P450, but not for maintaining the functional form.

Key words: *Escherichia coli*, folding, microsomal P450, proline-rich sequence.

Cytochromes P450 (CYP, P450) are an ever expanding superfamily of hemoproteins with an absorption maximum at 450 nm in the reduced CO-difference spectrum. P450 members catalyze the oxidative metabolism of a wide variety of endogenous and xenobiotic compounds. In eukaryotes, all P450s are membrane bound in either the endoplasmic reticulum (ER) or the mitochondrial inner membrane. The former are synthesized on membrane-bound ribosomes of the ER and cotranslationally integrated into the membrane (*1, 2*). Their amino-terminal hydrophobic segments function as ER-targeting signals, which are recognized by signal recognition particles (*2, 3*). These sequences also act as type I membrane anchors so that the major portion of microsomal P450s, including the heme binding and catalytic sites, resides on the cytoplasmic side of the membrane (*3, 4*).

Following the amino-terminal signal-anchor sequence (SA) of microsomal P450s, there is a region enriched in proline residues, termed the proline-rich sequence (PR). Between SA and PR, a short sequence containing several basic amino acid residues (BS) is present. There are two reports that describe naturally occurring mutations within the PRs of P450c21 (CYP21) and P4502D6 (CYP2D6) (*5, 6*). Mutation at the first proline within the PR of P4502D6 (PPGPXPXP) diminishes its activity, causing poor metabolism of certain drugs (*6*), while mutation within the PR of P450c21 (PPLAP) also reduces activity leading to congenital adrenal hyperplasia (*5*). Substitutions of alanine for the first proline within the PR of recombinant P4502C11 (CYP2C11) (also PPGPXPXP), or for combinations of two or three proline residues within the PR, result in the loss of the CO-difference spectrum in yeast (*7*) or in low catalytic activity in COS-7 cells (*8*), although the protein is readily detected by immunotechniques. In P4502C2 (CYP2C2), deletion of the sequence PPGP from the PR (PPGPXPXP) results in a loss of activity without a change in subcellular location of P450 in COS-1 cells (*9*). Mutation studies within the PR of P4502C2 also indicate that the PPGP motif is important for the formation of functional P450 (*10, 11*). These studies have demonstrated that the PR is important for the correct folding of members of the CYP2C subfamily in

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Abbreviations: δ-ALA, δ-aminolevulinic acid; BS, basic sequence; CYP, cytochrome P450 (P450); Cm, chloramphenicol; ER, endoplasmic reticulum; mod-2C11, modified P450 2C11; mod-c17, modified P450c17; P450c17 (CYP17), steroid 17α-hydroxylase/17,20-lyase cytochrome P450; SA, signal-anchor sequence; trunc-2C11, signal-anchor truncated P450 2C11 (CYP2C11); trunc-c17, signal-anchor-truncated P450c17; PR, proline-rich sequence.

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eukaryotic cells. However, the highly conserved PR sequence of the CYP2C subfamily is found only in members of the CYP2 gene family, although a PR sequence is present in all microsomal P450s. Furthermore, even in the CYP2C subfamily, the function of the PR in folding is not clearly known. Possibilities for this function include: (i) orientation of the elongating peptide relative to the membrane, (ii) folding through interaction with other protein(s), (iii) folding through interaction with another part of the emerging P450 molecule, and (iv) stability or catalytic activity of the folded protein.

In this study, to examine whether other microsomal P450s also require PR for correct folding, bovine P450c17 (CYP17), which has a quite different PR sequence (PSLPLVGSLPFLP) from that of the CYP2C subfamily, was used, along with rat P4502C11 in expression studies in *Escherichia coli*. Effects of various modifications in the amino-terminal region on correct folding have been studied. Furthermore, to establish more clearly the function of PR, the effect of proteolytic removal of the PR from the correctly folded P450 and the dependency on heme for the expression of spectrally normal P450s in *E. coli* were investigated. The results demonstrate that correct folding of each P450 involves its own gene family-specific PR, and that the role of the PR is independent of the SA or the BS, suggesting that the PR may be important for correct folding of all microsomal P450s. The PR may interact with another segment of the emerging P450 molecule during protein synthesis to stabilize the nascent peptide, forming an intermediate structure on ribosomes, facilitating the correct folding pathway and subsequent efficient heme incorporation. But the PR is not essential for maintaining the structure of a properly folded microsomal P450.

MATERIALS AND METHODS

Materials—Competent DH5αF'IQ *E. coli* cells were purchased from GIBCO-BRL. Reagents for bacterial growth were from Difco. Chloramphenicol (Cm) and δ-aminolevulinic acid (δ-ALA) were from Sigma. Factor Xa was from Boehringer Mannheim. All other chemicals were of the highest grade commercially available.

Construction of Expression Plasmids—The expression constructs for modified (mod-c17) and truncated bovine P450c17 (trunc-c17), and modified (mod-2C11) and truncated rat P450 2C11 (trunc-2C11) have been described previously (*12–14*). Site-directed mutagenesis was performed to prepare proline mutants of P450c17 using a QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to supplier's instructions. The *Nde*I–*Eco*RI fragment from pCW containing the cDNA of mod-c17 (pCW-mc17) or trunc-c17 (pCW-tc17) was ligated into pET17b vector (Promega), which had been treated with *Nde*I and *Eco*RI, and resultant plasmids (pET-mc17 or pET-tc17) were used as templates for site-directed mutagenesis. The following primers were used: P28A mutant, 5′-CACTCTGGTGCCAAGTACGCGCGCAGCCTCCCATCCCTGCCC and 5′-GTGAGACCACGGTTCATGCGCGCGTCGGAGGGTAGGGACGGG; P32A mutant, 5′-AAGTTACCCCAGGAGCCTCGCTAGCCTGCCCCTGGTGGGCAGC and 5′-GCTGCCCACCAGGGGCAGGCTAGCGAGGCTCCTGGGGTACTT; P35A mutant, 5′-CAGGAGCCTCCCATCCCTAGCGCTGGTGGGCAGCCTGCCG and 5′-CGGCAGGCTGCCCACCAGCGCTAGGGATGGGAGGCTCCTG; P41A mutant, 5′-TCCCTGCCCCTGGTGGGCTCGCTAGCGTTCCTCCCCAGACGTG and 5′-CACGTCTGGGGAGGAACGCTAGCGAGCCCACCAGGGGCAGGGA; P44A mutant, 5′-GGGCAGCCTGCCGTTCCTGGCCAGACGTGGCCAGCAGC and 5′-GCTGCTGGCCACGTCTGGCCAGGAACGGCAGGCTGCCC; P32,35A mutant, 5′-AAGTACCCCAGGAGCCTCGCATCCCTAGCGCTGGTGGGCAGCCTGCCG and 5′-CGGCAGGCTGCCCACCAGCGCTAGGGATGCGAGGCTCCTGGGGTACTT. *Nde*I-*Eco*RI fragments encoding a mutation and an *Eco*RI-*Hin*dIII fragment from pCW-mc17 containing the remainder of the coding sequence were ligated into the pCWori+ bacterial expression vector (15) which had been treated with *Nde*I and *Hin*dIII.

Further truncation of mod-c17 at the amino-terminus was carried out by PCR using *Pfu* polymerase (Stratagene). The pCW-mc17 was used as a template, and the following oligonucleotides were used as the forward primers: d3-31, 5′-GGAATTCCATATGGCTCCATCCCTGCCCCTGGTG; d3-44, 5′-GGAATTCCATATGGCTAGACGTGGCCAGCAGCAC. The sequence of the reverse primer was 5′-GGCCACTTTGGGACGCCCAGA. The PCR products were blunt-ended by treatment with the Klenow enzyme and subcloned into the *Eco*RV site of pBluescript (pBS-PCR1, Stratagene). The *Nde*I–*Eco*RI fragments from each pBS-PCR1, which encodes the further truncated sequence of the P450c17 amino-terminus and the *Eco*RI-*Hin*dIII fragment from pCW-mc17, were ligated into pCWori+, which was treated with *Nde*I and *Hin*dIII.

The construction of proline mutants of P4502C11 (P30A, P30,31A, P30,31,33A, and P33,35,37A) has been described previously (*7*). Further truncated mutants (d3-27, d3-29, and d3-37) were also amplified by PCR using pSGM1 (*7*) as a template. DNA fragments were digested with *Nde*I and *Sal*I, and then subcloned between the *Nde*I and *Sal*I sites of pCWori+. The following forward primers were used to introduce a *Nde*I site and alanine at the second position: d3-27, 5′-GCATCATATGGCTGGGAAGCTCCCTCCTGGTC; d3-29, 5′-GCATCATATGGCTCTCCCTCCTGGTCCAACAC; d3-37, 5′-GCATCATATGGCTATCATTGGAAACACCCTTC. The sequence of the reverse primer used for each construct to introduce a *Sal*I site was 5′-AGAAGTCGACTTACAGATGAGAGCTTAGAG.

Two-stage PCR (*16*) was used to make swap-constructs at the amino-terminal regions between P450c17 and P4502C11 as shown in Fig. 1. PCR was performed with *Pfu* polymerase. Three sets of primers, a specific forward primer (SF), a reverse linker primer (RL), and a specific reverse primer (SR), were used; their oligonucleotide sequences are shown in Table I. The first PCR employed SF and RL using Template 1 as a DNA template, and the resulting intermediate PCR product was then used at limiting concentration in a second PCR employing SF and SR. The DNA template in this case was Template 2. After the 2nd PCR, all products were blunt-ended by treatment with the Klenow enzyme and subcloned into the *Eco*RV site of pBluescript (pBS-PCR2). To prepare swap-constructs of P450c17, the *Nde*I–*Eco*RI fragments from each pBS-PCR2 and *Eco*RI–*Hin*dIII fragment from pCW-mc17 were ligated into pCWori+, which had been treated with *Nde*I and *Hin*dIII. In the case of swap-constructs of P4502C11, the *Nde*I–*Nco*I fragments from each pBS-PCR2 and *Nco*I–*Sal*I frag-

ment from pCW-t2C11 were ligated into pCWori+, which was treated with *Nde*I and *Sal*I.

The expression construct containing a factor Xa cleavage site (Xa-tc17) was also prepared by site-directed mutagenesis using pET-tc17 as a template. A factor Xa cleavage sequence was introduced between amino acid positions 42 to 45 using the following primers: 5′-GGTGGGCAGCCTGCCGATCGAGGGACGTCGTGGCCAGCAGCACAA and 5′-TTGTGCTGCTGGCCACGACGTCCCTCGATCGGCAGGCTGCCCACC. The *Nde*I–*Eco*RI fragment encoding the Factor Xa cleavage site and the *Eco*RI–*Hin*dIII fragment from pCW-mc17 were ligated into pCWori+ treated with *Nde*I and *Hin*dIII. The nucleotide sequences of all PCR fragments described in this section were confirmed by automated sequencing (Applied Biosystems).

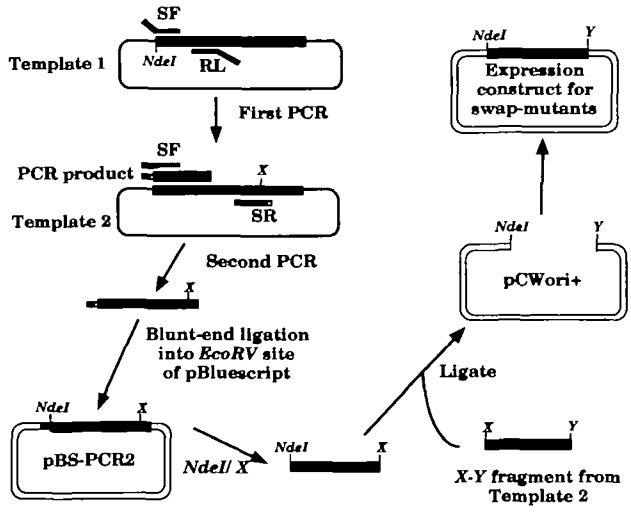

Fig. 1. **General scheme for the construction of amino-terminal swapping mutants between P450c17 and P4502C11.** The sequences of all primers used are shown in Table I. First PCR employed SF and RL using DNA Template 1. The resulting PCR product was then used at a limiting concentration in the second PCR employing SF and SR. The DNA template in the second PCR was Template 2. X and Y show the restriction sites as indicated in Table I.

Purification and Factor Xa Treatment of Xa-tc17—The expression of Xa-tc17, which has a factor Xa cleavage site after the PR, was carried out by the method described previously (*14*) except that the cells were harvested 24 h after induction. The suspension of spheroplasts was sonicated in 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.5 mM PMSF (buffer A), and then centrifuged at 1,800 ×*g* for 15 min at 4°C to remove cell debris. The supernatant was further centrifuged at 250,000 ×*g* for 60 min at 4°C in a Beckman TL-100. The pellet (membrane fraction) was resuspended in buffer A containing 1 M NaCl, and centrifuged at 250,000 ×*g* for 60 min at 4°C. The supernatant was used for the purification of Xa-tc17. The supernatant was diluted two-fold with buffer A, and nonidet P40 (NP40) was added to a final concentration of 0.5%. This solution was applied to a Ni-NTA column (Qiagen) and the column was washed with 3 column volumes of 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.5% NP40 (buffer B). Bound Xa-tc17 was eluted by buffer B containing 40 mM histidine. The eluted fraction was diluted (1:4, v/v) with 1 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.5% NP40 and then applied onto a hydroxylapatite column. After washing with 5 column volumes of 10 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, and 0.5% NP40, Xa-tc17 was eluted with 200 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA and 0.5% NP40. The purified Xa-tc17 was stored at –80°C until use.

Factor Xa treatment was carried out at 4°C for 16 h using 400 pmol Xa-tc17 (32 μg) and 150 μg factor Xa in 1 ml of 50 mM potassium phosphate buffer, pH 7.4, containing 20% glycerol, 0.1 mM DTT, 0.1 mM EDTA, 100 mM NaCl, 1 mM $CaCl_2$, and 2% NP40.

In Vitro Transcription / Translation Assay—*In vitro* transcription/translation of the plasmid DNAs was carried out using the *E. coli* S30 Extract System for Circular Templates Kit (Promega) according to supplier's instructions using [^{35}S]methionone. The reaction mixtures (15 μl) containing 300 ng of each expression plasmid were incubated at 37°C for 60 min, and then a 7.5 μl aliquot was added to

TABLE I. **Primers and restriction sites used for the preparation of swap-constructs.**

Constructs	Primers	Template 1	Template 2	X	Y
Swap1-c17	SF 5′-GCATCATATGGCTAGACAGAGCTTTGGGAGAG				
	SR 5′-GGCCACTTTGGGACGCCCAGA	pCW-t2C11	pCW-tc17	*EcoRI*	*HindIII*
	RL 5′-CTTGTGCTGCTGGCCACGTCTTGGGAGAGGTGTTGGACCAGG				
Swap2-c17	SF 5′-GCATCATATGGCTAGACAGAGCTTTGGGAGAG				
	SR 5′-GGCCACTTTGGGACGCCCAGA	pCW-t2C11	pCW-tc17	*EcoRI*	*HindIII*
	RL 5′-CTTGTGCTGCTGGCCACGTCTGGTGTTTCCAATGATTGGGAG				
Swap3-c17	SF 5′-GCATCATATGGCTAGACAGAGCTTTGGGAGAG				
	SR 5′-GGCCACTTTGGGACGCCCAGA	pCW-t2C11	pCW-tc17	*EcoRI*	*HindIII*
	RL 5′-GAACGGCAGGCTGCCCACCAGTGGACCAGGAGGGAGCTTCCC				
Swap4-c17	SF 5′-GCATCATATGGCTAGACAGAGCTTTGGGAGAG				
	SR 5′-GGCCACTTTGGGACGCCCAGA	pCW-t2C11	pCW-tc17	*EcoRI*	*HindIII*
	RL 5′-GCCCACCAGGGGCAGGGATGGGAGCTTCCCTCTCCCAAAGCT				
Swap1-2C11	SF 5′-GGGCATATGGCTAAAACCAAGCAC				
	SR 5′-TAATCAATAATGGCAGGGAAAGTA	pCW-tc17	pCW-t2C11	*NcoI*	*SalI*
	RL 5′-GAGAGGTGTTGGACCAGGAGGGAGGCTCCTGGGGTACTTGGC				
Swap2-2C11	SF 5′-GGGCATATGGCTAAAACCAAGCAC				
	SR 5′-TAATCAATAATGGCAGGGAAAGTA	pCW-tc17	pCW-t2C11	*NcoI*	*SalI*
	RL 5′-TCCAATGATTGGGAGAGGTGTGGGCAGGGATGGGAGGCTCCT				

30 μl of ice-cold acetone and incubated at 4°C for 10 min. The pellet obtained by centrifugation was resuspended in 30 μl of Laemmli buffer, placed in a boiling water bath for 5 min, and subjected to electrophoresis in an 8% SDS–polyacrylamide gel to analyze the products. The gel was dried and exposed to X-ray film (Kodak). The radioactivities of the products were determined with a liquid scintillation counter.

Miscellaneous Methods—The expression of P450s and measurement of the reduced CO-difference spectra were performed as described previously (*14*). *E. coli* proteins (5 μg total cell lysate) were size fractionated in 8% SDS–polyacrylamide gels and transferred to PVDF membranes. Immunoblot analyses were performed using rabbit antisera against human P450c17 (*17*) and rat P450 2C11 (*8*), with HRP-conjugated protein G as a secondary antibody. Immunoreactive signals were visualized by ECL detection (Amersham). All experiments were performed three times or more and representative data are presented.

RESULTS

Effect of Mutations within Proline-Rich Sequences on the Expression of Spectrally Normal P450s in E. coli—When full-length P4502C11 (mod-2C11), which has an alanine as the second amino acid, is expressed in *E. coli*, the expression level is ~80 nmol/g cell protein (240 nmol/liter culture) in the presence of 0.5 mM δ-ALA and 1 μg/ml Cm. Members of the CYP2C subfamily have a highly conserved PR sequence, PPGPXPXP. Substitutions of alanine for the first proline, or for combinations of two or three prolines within

TABLE II. **Proline-rich sequence of animal microsomal P450s.** Proline-rich sequences were saught among reported amino acid sequences. All P450s belonging to the same family usually have identical PR, with a few exceptions. The concerved PR of each P450 family is shown in the table. P and denote proline and glycine, respectively. X indicates an amino acid other than proline and glycine.

P450 families	PR sequences
1	P P G P X X X P
2	P P G P X P X P
3	P G P X P X P
4	P X P P
5	P X P X P
6	P X X X P
7, 8, 51	P P X X X X X X P
17	P X X X X P X X P
19	P G P G
21	P P X X P G
26	P P G X X X X P

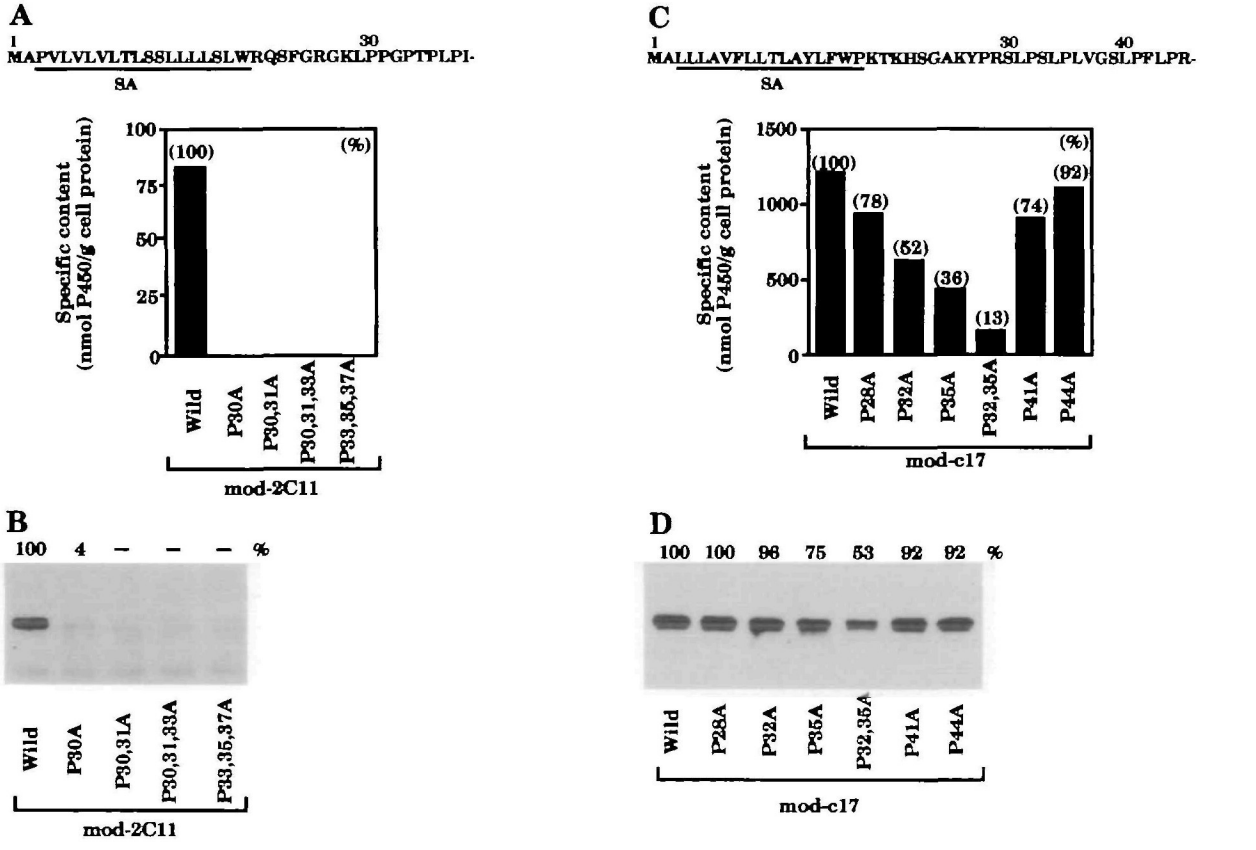

Fig. 2. **Effect of substitutions of alanine for proline residues within the PR on the expression of full-length P4502C11 and P450c17 in *E. coli*.** Transformed *E. coli* DH5αF'IQ was cultured at 29°C in the presence of Cm (1 μg/ml) and δ-ALA (0.5 mM). After 48 h incubation, cells were harvested and solubilized for spectral analysis. Specific contents (nmol P450/g cell protein) of mod-2C11 and its proline mutants (A), and those of mod-c17 and its proline mutants (C) are shown. The figures in parentheses show the ratio of the specific content of mutants to that of each original form (100%). Immunoblot analysis of mod-2C11 and its proline mutants (B), and that of mod-c17 and its proline mutants (D) expressed in DH5αF'IQ (5 μg) are shown. The ratios of band-intensity of mutants to that of each original form (100%) calculated using NIH image software are shown above each panel.

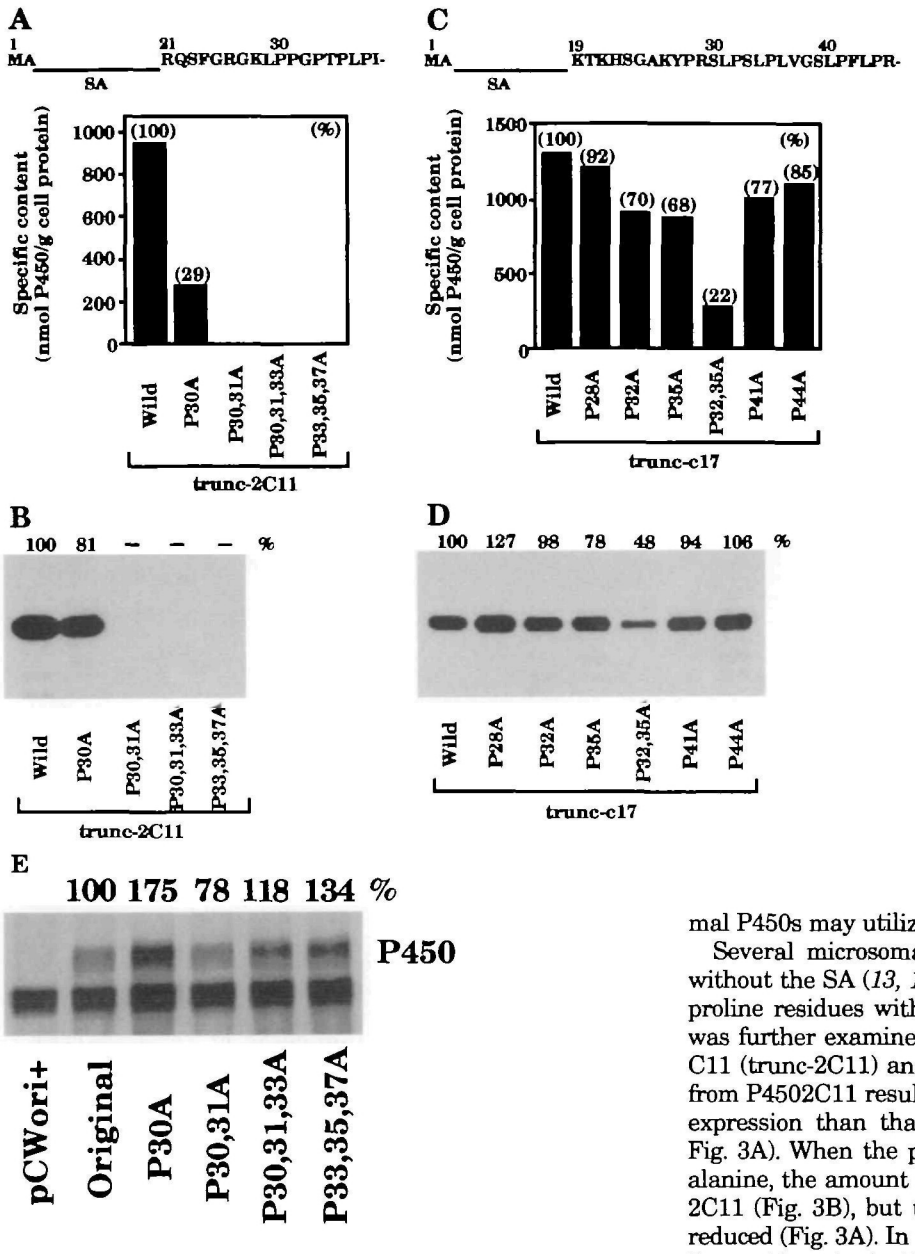

Fig. 3. **Effect of substitutions of alanine for proline residues within the PR on the expression of signal-anchor truncated P4502C11 and P450c17 in *E. coli*.** Specific contents (nmol P450/g cell protein) of trunc-2C11 and its proline mutants (A), and those of trunc-c17 and its proline mutants (C). Culture conditions are the same as in Fig. 2. The figures in parentheses show the ratio of specific content of mutants to that of each original form (100%). Immunoblot analysis of trunc-2C11 and its proline mutants (B), and that of trunc-c17 and its mutants (D) expressed in DH-5αF'IQ (5 μg) are shown. The ratios of the band-intensity of mutants to that of each original form (100%) calculated using NIH image software are shown above each panel. (E) An *in vitro* transcription/translation study of trunc-2C11 ("original") and its proline mutants is shown. Figures show the ratios of the radioactivity of proline mutants to that of trunc-2C11 (100%).

the PR result in a complete loss of both the detectable CO-difference spectrum and immunoreactive protein as shown in Fig. 2, A and B. Therefore, PR of P4502C11 is important for the correct folding of newly synthesized P4502C11 in *E. coli*, just as previously shown for its expression in yeast and COS-7 cells (*7, 8*). P450c17, which belongs to the CYP17 family, also has a PR containing four proline residues, but its sequence (PSLPLVGSLPFLP) is quite different from that of the CYP2 family members (Table II). We substituted alanine for proline residues within the PR of P450c17, and found that the mutations also affect folding (Fig. 2, C and D). Proline residues at positions 32 and 35 seem to be the most important, and a double proline mutant (P32,35A) has only 13% of the CO-difference spectrum obtained for mod-c17 (Fig. 2C), although about 50% of the protein is detected by immunoblot analysis (Fig. 2D). These results indicate that the PR of P450c17 is also important for efficient folding in *E. coli*, and suggest that all microsomal P450s may utilize the PR (Table II) for efficient folding.

Several microsomal P450s can be expressed in *E. coli* without the SA (*13, 18–25*). Thus, the effect of mutations of proline residues within the PR on the expression of P450 was further examined using SA-truncated forms of P4502C11 (trunc-2C11) and P450c17 (trunc-c17). Removal of SA from P4502C11 results in an approximately ten-fold higher expression than that of the full-length form (mod-2C11, Fig. 3A). When the proline at position 30 is replaced with alanine, the amount of expressed protein is ~80% of trunc-2C11 (Fig. 3B), but the CO-difference spectrum is greatly reduced (Fig. 3A). In other mutants, when two or three proline residues in the PR are replaced with alanine, neither a CO-difference spectrum nor protein are detected, as observed with mod-2C11 (compare Figs. 2 and 3). In the case of SA-truncated P450c17 (trunc-c17), almost the same results as with full-length P450c17 (mod-c17, Fig. 2, C and D) were obtained, showing the proline residues at positions 32 and 35 to be the most important for efficient folding (Fig. 3, C and D). These results indicate that in both CYP gene families the role of the PR in correct folding is independent of the SA which anchors the peptide to the inner aspect of the inner membrane in *E. coli* cells. When trunc-2C11 and its proline mutants are synthesized *in vitro* using an *E. coli* transcription/translation system, almost the same or even higher levels of products are detected (Fig. 3E). However, the three proline mutant proteins are not detected nor are partially degraded proteins when expressed in *E. coli* (Fig. 3B). Thus, it is possible that translation *in vivo* may be arrested because the proline mutant peptides of P4502C11 are unable to form stable intermediate structures on ribo-

somes. Rapid degradation of misfolded proteins in *E. coli* is another possible explanation for the difference between the *in vivo* and *in vitro* results.

Effect of Further Truncation at the Amino-Terminus on the Expression of Spectrally Normal P450s in E. coli—Microsomal P450s also have a short highly basic sequence

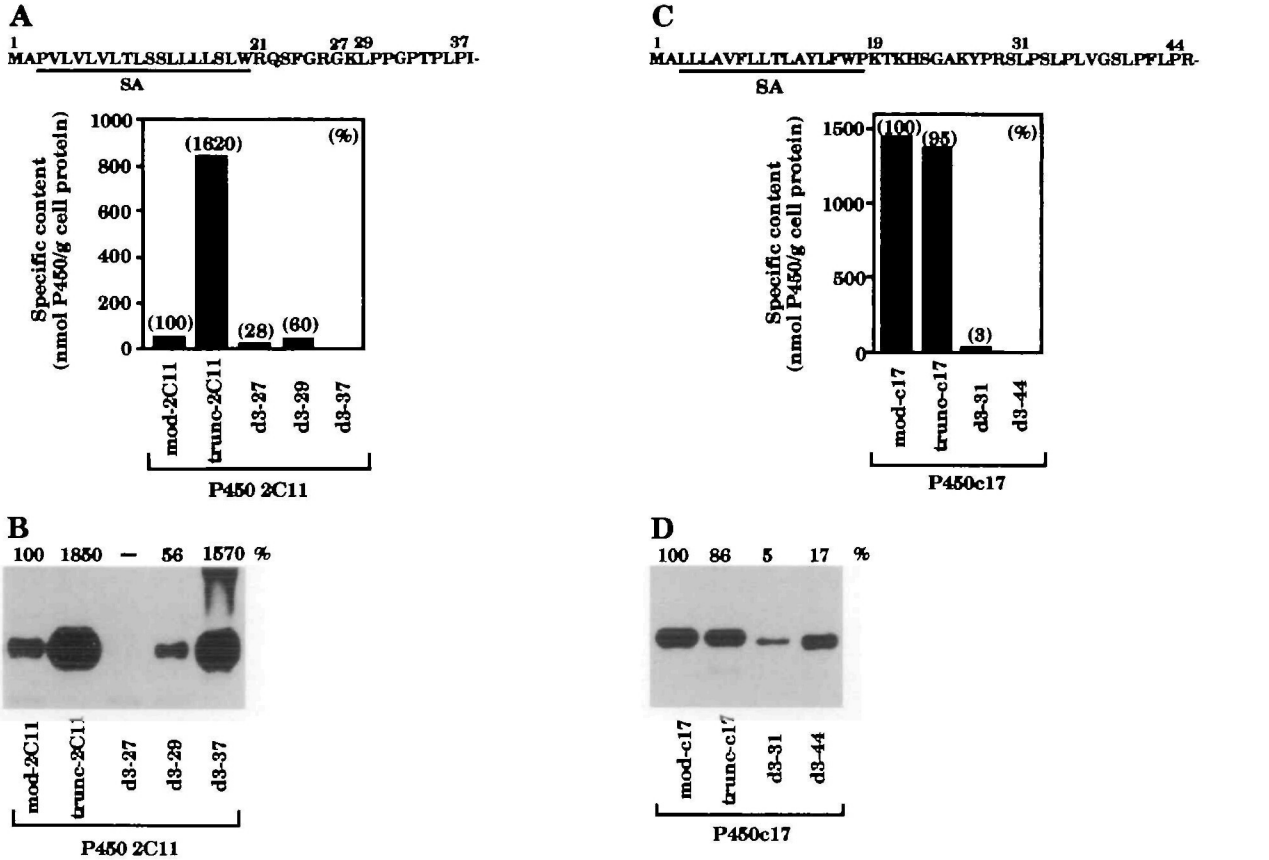

Fig. 4. **Effect of amino-terminal deletions on the expression of P4502C11 and P450c17 in *E. coli*.** Specific contents (nmol P450/g cell protein) of mod-2C11 and its deleted mutants (A) and those of mod-c17 and its deleted mutants (C). Culture conditions are the same as in Fig. 2. The figures in parentheses show the ratio of specific content of mutants to that of each original form (100%). Immunoblot analysis of mod-2C11 and its deleted mutants (B) and that of mod-c17 and its deleted mutants (D) expressed in DH5αF'IQ (5 μg) are shown. The ratios of band-intensity of mutants to that of each original form (100%) calculated using NIH image software are shown above each panel. (E) An *in vitro* transcription/translation study of mod-2C11 ("original") and its deleted mutants. Figures show the ratios of radioactivity of deleted mutants to that of mod-2C11 (100%).

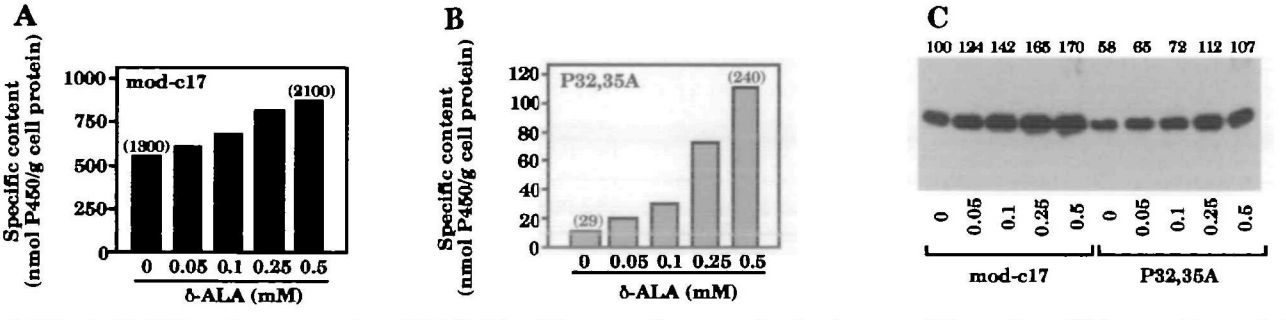

Fig. 5. **Effect of δ-ALA on the expression of P450c17 and its proline mutants in *E. coli*.** Transformed *E. coli* DH5αF'IQ was cultured at 29°C in the presence (0.05 to 0.5 mM) or absence of δ-ALA, and Cm (1 μg/ml). Specific contents (nmol P450/g cell protein) of mod-c17 (A) and its P32,35A mutant (B). The figures in parentheses show the expression levels as nmol/liter culture. (C) Immunoblot analysis of mod-c17 and its P32,35A mutants expressed in DH5αF'IQ (5 μg). Figures show the ratios of band-intensity to that of mod-c17 without δ-ALA.

(BS) between the SA and PR. To investigate the importance of and relationship among these conserved sequence motifs in correct folding, stepwise truncations from the amino-termini of both P4502C11 and P450c17 were carried out, and the truncated mutants were expressed in *E. coli*. For P4502C11, four truncated mutants were prepared (trunc-2C11, SA-truncated form; d3-27, SA and partial BS-truncated form; d3-29, SA and BS-truncated form; d3-37, SA, BS, and PR-truncated form); three truncated mutants were prepared in the case of P450c17 (trunc-c17, SA-truncated form; d3-31, SA and BS-truncated form; d3-44, SA, BS, and PR-truncated form). For both P450s, deletion of the SA sequence (trunc-2C11 and trunc-c17) has no effect on correct folding (Figs. 3 and 4), although removal of the SA greatly enhances the expression of trunc-2C11 in *E. coli*. When the BS of each P450 is also deleted (d3-27 and d3-29 in P4502C11 and d3-31 in P450c17), large decreases in the CO-difference spectra and protein for both P450s are observed. It appears, however, from the spectra/protein ratio, that BS has little effect on folding, even though the expression levels are greatly decreased by its deletion (Fig. 4). Deletion of PR (d3-37 in P4502C11 and d3-44 in P450c17), on the other hand, completely abolishes the CO-difference spectra of both P450s (Fig. 4), although significant protein can be detected by immunoblot analysis. These results indicate that neither SA nor BS is essential, but the PR could be indispensable for the expression of spectrally normal microsomal P450s in *E. coli*. *In vitro* translation of mod-2C11, trunc-2C11 and further truncated forms (d3-27, d3-29, and d3-37) was also carried out (Fig. 4E). The amounts of *in vitro* translation products correspond well to the levels of expressed protein in vivo (Fig. 4, B and E). *In vitro* translation seems to be affected greatly by the amino-terminal sequence of the peptides.

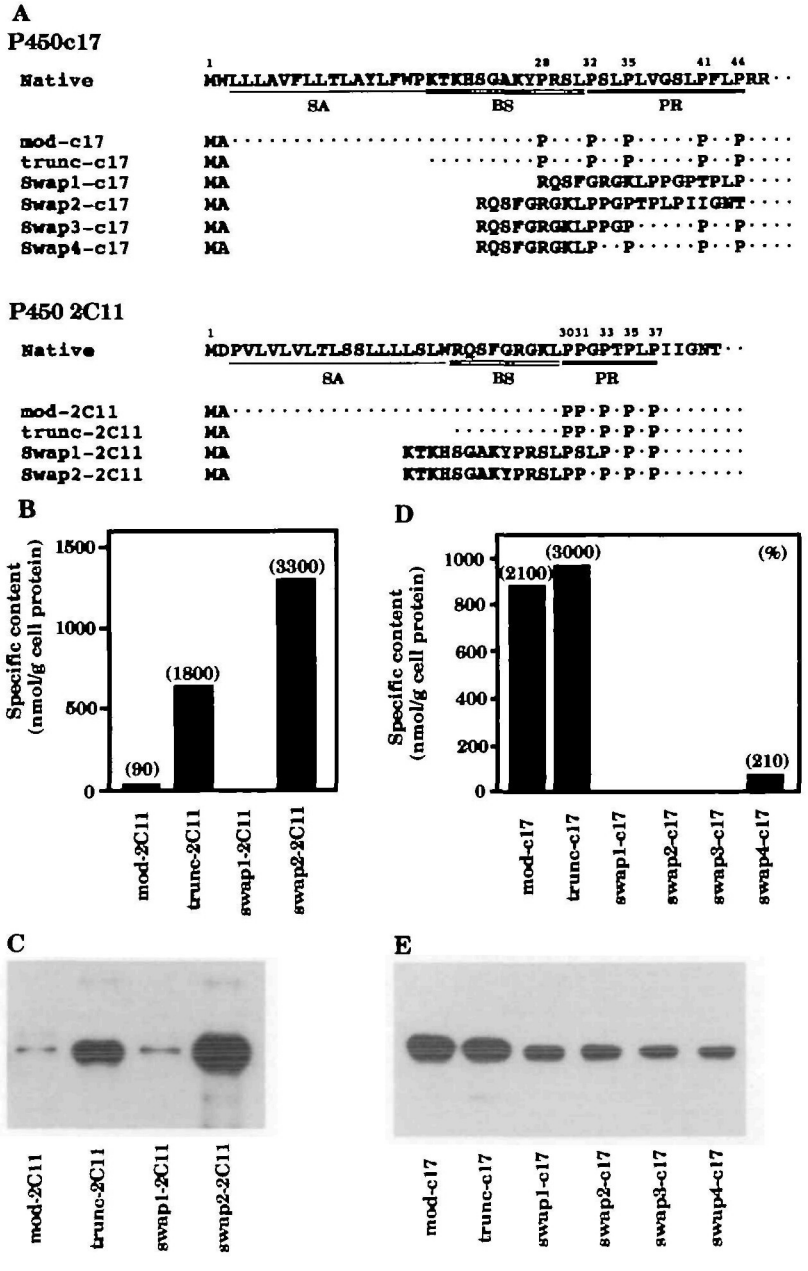

Fig. 6. **Effect of amino-terminal swapping between P4502C11 and P450c17 on the expression of P450s in *E. coli*.** (A) Amino-terminal sequences of the originals and their swap-mutants are shown. Swap-mutants were prepared by replacing amino-terminal sequence motifs of P450c17 with those of P4502C11 and vice versa. Swap1-c17 has the BS and PR (PPGPTPLP) of P4502C11 instead of the corresponding sequences of P450c17. In swap2-c17, five amino acids of P4502C11 (IIGNT) are added to swap1-c17 to align the important proline residues at the same positions. Swap3-c17 has the BS and a part of PR (PPGP) of P4502C11 instead of the BS and PSLP sequence of P450c17. In swap4-c17, only the BS of P450c17 is replaced with that of P4502C11. Swap1-2C11 has the BS and PSLP sequence of P450c17 instead of the BS and PPGP sequence of P4502C11. In swap2-2C11, only the BS of P4502C11 was replaced with that of P450c17. Specific contents (nmol P450/g cell protein) of P4502C11 and its swap-mutants (B) and those of P450c17 and its swap-mutants (D) are shown. Culture conditions are the same as in Fig. 2. The figures in parentheses show the expression level as nmol/liter culture. Immunoblot analysis of P4502C11 and its swap-mutants (C) and that of P450c17 and its swap-mutants (E) expressed in DH5αF'IQ (5 μg) are also shown.

Effect of the Addition of δ-ALA on the Expression of Spectrally Normal P450 in E. coli—To examine the effect of heme availability on the expression of P450s in *E. coli*, different concentrations of the heme precursor δ-ALA were added to the culture medium. When mod-c17 and its PR mutant (P32,35A) are expressed, CO-difference spectra and protein levels increase according to the δ-ALA concentration, as shown in Fig. 5. However, the ratio of enhancement of the expression of the PR mutant by δ-ALA is much higher than that of mod-c17. The expressed protein level of the PR mutant is always higher than the level estimated from the CO-difference spectrum (Fig. 5, B and C), suggesting the formation of large amounts of apo-protein, which we believe to be protein that misfolds before it can bind heme. Essentially the same result is obtained when trunc-2C11 and one of its PR mutants (P30A) are expressed in *E. coli* (not shown). These results indicate that heme incorporation is less efficient in PR mutants than in the original forms. However, increasing the supply of heme by adding δ-ALA to the culture medium can promote the correct folding of both the originals and their PR mutants, presumably by enhancing heme incorporation.

Exchange of Amino-Terminal Sequence Motifs between P450c17 and P4502C11—We exchanged the amino-terminal sequence motifs, BS and PR, between P4502C11 and P450c17, and examined the expression of the swap-mutants in *E. coli* (Fig. 6). In the case of P4502C11, the BS of P450c17 is functional, producing a CO-difference spectrum that corresponds to the level of the expressed P450 protein (swap2-2C11, Fig. 6, B and C). However, when the PPGP sequence is also replaced with the PSLP sequence of P450c17, no CO-difference spectrum is detected, although the expressed protein level is almost the same with mod-2C11 (swap1-2C11, Fig. 6, B and C). Essentially the same results are obtained with P450c17 (Fig. 6, D and E). Replacement of the BS with that of P4502C11 (swap4-c17) does not affect folding, but even partial exchange of the PR with that of P4502C11 (swap1-, swap2-, and swap3-c17) results in a complete loss of the CO-difference spectra, although essentially unchanged protein levels among swap-mutants are detected. These results indicate that the BS is exchangeable among different forms of microsomal P450s, but the specific PR is required for each CYP or CYP family to facilitate the correct folding of P450 in *E. coli*.

Proteolytic Removal of the PR from Correctly Folded P450c17 by the Introduction of a Factor Xa Cleavage Site—In order to study whether the PR is needed to maintain the conformation of the P450 molecule even after folding, a factor Xa cleavage site was introduced on the C-terminal side of the PR of trunc-c17 (Fig. 7A). Expressed Xa-tc17 exists almost equally in the cytosol and membrane fractions. The membrane-bound form is completely released by treatment with 1 M NaCl (lower NaCl concentrations were not tested). Purification of Xa-tc17 from the cytosol fraction by two column chromatography steps (Ni-NTA and hydroxylapatite columns) was not complete as estimated by SDS-polyacrylamide gel electrophoresis (not shown). However, using the membrane fraction supernatant treated with 1 M NaCl as a starting material, Xa-tc17 can be purified by two column chromatography (Ni-NTA and hydroxylapatite) steps (Fig. 7B), to a specific P450 content of 12.5 nmol/mg of protein. When purified Xa-tc17 (400 pmol) is incubated at 4°C for 16 h without factor Xa, 95% of the original P450 is recovered (Fig. 7D), indicating that Xa-tc17 is stable. When purified Xa-tc17 is treated with factor Xa under the same conditions, two bands (in a ratio of roughly 4:6) are detected by SDS-PAGE (Fig. 7C, with FXa), the lower band corresponding to the cleaved form, and a total of 360 pmol P450 (90%) is recovered (Fig. 7D). Since the 450 nm CO-difference spectrum was not affected by factor Xa treatment, which cleaves the PR from as much as 60% of the total protein, PR does not seem to be essential for the maintenance of the correctly folded conformation of P450 molecules.

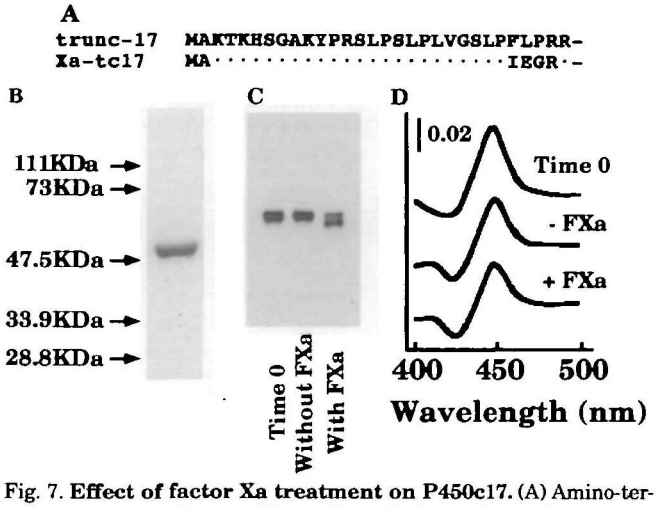

Fig. 7. **Effect of factor Xa treatment on P450c17.** (A) Amino-terminal sequence of trunc-c17 and Xa-tc17. Xa-tc17 contains a factor Xa cleavage site (IEGR) following the PR of trunc-c17. (B) SDS-polyacrylamide gel electrophoresis of purified Xa-tc17 (2.5 μg). (C) Immunoblot analysis of Xa-tc17 before and after the treatment. Purified Xa-tc17 was incubated at 4°C for 0 h (time 0), or 16 h with (with FXa) and without factor Xa (without FXa). Each sample (0.25 μg) was loaded on an 8% SDS-polyacrylamide gel and analyzed by immunoblot using antibody against P450c17. (D) CO-difference spectra of Xa-tc17 before (time 0) and after the incubation at 4°C for 16 h with (+FXa) and without factor Xa (-Fxa).

DISCUSSION

All microsomal P450s have a PR following the amino-terminal SA, and the sequence of the PR seems to be specific for each CYP gene family (Table II). Thus, it can be speculated that the amino acid sequence of the PR has been more conserved during evolution and diversification of the CYP gene superfamily than most other regions of the P450 sequence.

Members of the CYP2 gene family have a characteristic PR sequence, PPGPXPXP, and the importance of the PR for correct folding has been confirmed for two P450s, P4502C11, and P4502C2, belonging to the CYP2C subfamily (*7–11*). Here, we show that the PR of P450c17, which has a very different sequence (PSLPLVGSLPFLP) from that of CYP2 family members, is also important for efficient folding of this P450 when expressed in *E. coli*. We conclude that all microsomal P450s may require their PRs for correct folding.

One possibility for the function of the PR in the folding of the P450 peptide is to act as a rigid hinge connecting the membrane-anchored amino-terminal SA and the large catalytic domain of microsomal P450 molecules that lies on

the cytoplasmic side of the ER membrane, thus directing the orientation of the peptide after the amino-terminus is anchored to the membrane (*7, 10*). To examine this possibility, we constructed proline mutants using SA-truncated forms of P450s (trunc-2C11 and trunc-c17), and expressed them in *E. coli*. We found that the PR is also important for the correct folding of P450 peptides missing the SA sequence, indicating that the role of PR in P450 folding is independent of membrane anchoring *via* the SA.

A BS lies between the SA and PR in the amino-terminal region of microsomal P450s. Examination of the importance of BS for correct folding of P4502C11 and P450c17 shows that the BS is not essential since its deletion along with SA did not abolish the formation of spectrally normal P450s. However, the expression levels of these P450s were very much reduced. On the other hand, deletion of the PR in addition to the SA and BS resulted in complete loss of the CO-difference spectra of both P450s, although misfolded proteins were detectable. *In vitro* transcription/translation assays using P4502C11 indicated that the different expression levels among full-length and various truncated forms are related to differences in the efficiency of translation. Deletion at the 5′-end changes the secondary structure of mRNAs and possibly affects the initiation of translation. Particularly, BS rather than SA seems to be important for translation.

Proline-rich motifs are often involved in protein–protein interactions in intracellular signaling, and the PXXP sequence adopts a polyproline II (PPII) structure found in SH3 domain ligands (*26–30*). Thus, a possible role of the PR in P450 folding could be interaction with the chaperone-like protein(s) required for the folding of nascent peptides in the cytosol of *E. coli*. If this is true, perhaps the PR can be replaced between P4502C11 and P450c17 without affecting proper folding. Exchange of the BS between SA-truncated P4502C11 and P450c17 had no adverse effect on folding, spectrally normal P450s being expressed in each case. Furthermore, swapping SA sequences has been shown not to prevent the formation of P450s with normal enzymatic activities in COS cells (*31*). However, the replacement of the PPGP sequence of P4502C11 with PSLP from P450c17 resulted in the complete loss of the CO-difference spectrum, although high levels of protein expression were observed. The reverse was also true, replacement of the PR of P450c17 with that of P4502C11 also led to a complete loss of the CO-difference spectrum. These results indicate that the role of PR is specific for each CYP or CYP family, which suggests that PR does not interact with common external factors such as molecular chaperones, but rather interacts with some other segment of each folding P450 polypeptide to trigger correct folding.

When PR-deleted mutants of P4502C11 (d3-37) and P450c17 (d3-44) were expressed in *E. coli*, CO-difference spectra at 450 nm or 420 nm were not detected. This indicates that these PR-deleted mutants are unable to incorporate heme, suggesting that the role played by PR in proper folding may be important for efficient heme incorporation. Thus, we examined the effect of the addition of δ-ALA, a heme precursor, to the culture media on the expression of P450s using proline mutants of P4502C11 and P450c17. The expression level of mod-c17 was elevated as the δ-ALA concentration increased, reaching more than 2,000 nmol/liter culture in the presence of 0.5 mM δ-ALA, about 1.6 times higher than in the absence of δ-ALA. Immunoreactive protein levels corresponded well to the CO-difference spectra, indicating that virtually all the expressed protein was spectrally normal P450. On the other hand, the expression level of the P450c17 P32,35A mutant in the absence of δ-ALA was extremely low (29 nmol/liter culture), only about 2% of mod-c17. However, the expression level of the P32,35A mutant increased greatly with increasing δ-ALA concentration, attaining a level 8-times higher in the presence of 0.5 mM δ-ALA. The protein level of expressed P32,35A was significantly higher than the amount estimated from the CO-difference spectrum, indicating significant formation of misfolded apo-protein. However, a much greater increase in spectrally normal P450 compared to total protein was observed with the P32,35A mutant at higher δ-ALA levels. This indicates that the insertion of heme is possibly one of the critical steps in the folding process of P450, and increasing the availability of heme in *E. coli* by adding δ-ALA to the culture medium promotes correct folding. The same conclusion was also obtained with P4502C11. Therefore, mutation(s) within the PR seem to result in lowered efficiency of heme incorporation during the folding of the P450 molecule, because misfolding occurs more frequently.

Since evidence suggesting cotranslational protein folding has come from several experiments using eukaryotic and prokaryotic translation systems (*32–35*), *in vivo* folding of microsomal P450s may also proceed cotranslationally. Rabbit α-globin is capable of binding heme during its synthesis on ribosomes and heme binding begins when the emerging polypeptide achieves a length of 86 residues (*36, 37*). Perhaps microsomal P450s also incorporate heme cotranslationally when the peptides are still attached to the ribosomes. Therefore, excess amounts of heme may assist this folding process, resulting in the acceleration of translation. This may explain why the expression levels of various P450s in *E. coli* are greatly elevated by the addition of δ-ALA. In P450s, the heme-binding site is in the C-terminal portion of the polypeptide, while the PR is in the N-terminal portion, emphasizing the putative role of the PR in heme binding.

When three PR mutants of trunc-2C11 were expressed in *E. coli*, the levels of expressed protein were below the detection limit. However, the *in vitro* transcription/translation assay using an *E. coli* S30 lysate showed almost the same level of products of the mutants as with trunc-2C11. Translation of P450 peptides may be regulated at certain folding steps *in vivo*, but not *in vitro*. In the proline mutants of P4502C11, a misfolded intermediate may be formed because of the mutation, and the translation is arrested, resulting in no protein. *In vitro*, no such arrest in translation is observed. An alternative explanation for the difference between the *in vivo* and *in vitro* results is that correctly folded protein with heme is not so easily accessible to the cellular proteolytic degradation system as the misfolded apo-protein. If certain misfolded forms aggregate rapidly, however, they may escape proteolysis by forming insoluble aggregates of apo-protein, such as inclusion bodies. In this case, expressed misfolded protein is detected by immunotechniques.

PR-deleted mutants (P450c17; d3-44, P450 2C11; d3-37) did not form spectrally normal P450s, although high levels of expressed protein were detected. We wondered whether

PR plays an important role after the synthesis of the P450 molecule. Introduction of a factor Xa cleavage site into trunc-c17 (Xa-tc17) permitted the removal of PR after synthesis of the functional P450 molecule. After purification of the expressed P450 from *E. coli*, factor Xa treatment cleaved more than 60% of Xa-tc17, but the CO-difference spectrum was essentially unaffected. It is clear from the specific content (12.5 nmol/mg protein) that all the P450 is in the completely folded form. These data indicate that the removal of PR after synthesis has little effect on the conformation of the P450 molecule and that the PR has a specific requirement during synthesis and folding, but not afterwards.

Taking into account all the facts and considerations mentioned above, we conclude that the PR is important for correct folding of all microsomal P450s when expressed in *E. coli* and no doubt in eukaryotic cells as well. The function of PR in protein folding is independent of the membrane anchoring of the peptide by the SA. The PR probably interacts transiently with some other segment(s) of the P450 molecule during the elongation of the peptide to stabilize the nascent peptide, leading to the correct intermediate structure(s) on ribosomes, which directs a correct folding pathway for efficient heme incorporation. The sequence of the PR is highly variable among animal microsomal P450s, but the members of each CYP gene family usually or always have the same PR sequence (Table II). Therefore, the specific peptide segment that interacts with the PR during protein folding may also be conserved in the same gene family.

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