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Importance of a Proline-Rich Sequence in the Amino-Terminal Region for Correct Folding of Mitochondrial and Soluble Microbial P450s[1]

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All microsomal P450s have a proline-rich sequence (PR) in the amino-terminal region that is needed for proper folding [Kusano, K., Sakaguchi, M., Kagawa, N., Waterman, M.R. and Omura, T. (2001) *J. Biochem.*, 129, 259–269]. There are also multiple proline residues near the amino-termini of the mature forms of all mitochondrial P450s and the amino-termini of soluble microbial P450s. To examine the functional significance of the PR in mitochondrial P450s, we expressed human P450c27 (CYP27) and bovine P450scc (CYP11A1) in an *Escherichia coli* heterologous expression system, and found that in each one specific proline residue is important for correct folding. Deletions from the amino-terminus further indicated the importance of the PR for the expression of a spectrally normal P450c27. Essentially the same results were obtained with two soluble microbial P450s, P450cam (CYP101) and P450nor, in each of which a PR is important for proper folding. We conclude that in all P450s (mitochondrial, microbial and microsomal P450s), a proline-rich sequence located in the amino-terminal region is important for proper folding. Furthermore, we predict that the importance of the PR in P450 folding is to reduce the tendency of the polypeptide to misfold prior to heme binding.

Key words: *Escherichia coli*, folding, mitochondrial P450, proline-rich sequence, soluble microbial P450.

There are three broad classes of cytochrome P450, microsomal, mitochondrial and soluble microbial P450s. Microsomal P450s, which are synthesized on the membrane-bound ribosomes of the endoplasmic reticulum and cotranslationally integrated into the membrane (*1*, *2*), have three conserved amino-terminal sequence motifs, a signal-anchor sequence (SA), a basic sequence (BS), and a proline-rich sequence (PR). The role of the PR in the correct folding of microsomal P450s has been clearly established (*3–8*). Mitochondrial P450s are synthesized on cytoplasmic ribosomes as larger precursor proteins with a presequence at the amino-terminus (*9–11*) that contains the information for specific targeting to mitochondria, similar to most other mitochondrial proteins (*12*). After the precursor proteins are imported into the matrix space of mitochondria, the presequence is cleaved by the mitochondrial matrix processing peptidase to produce mature P450 peptides (*13*, *14*). Therefore, mitochondrial P450s fold post-translationally, the mature P450s being localized in the inner mitochondrial membrane facing the matrix. When the amino-terminal sequences of mature-type mitochondrial P450s are aligned, they are seen to have several proline residues highly conserved within each CYP gene family or subfamily (Fig. 1). Bacterial P450s and at least one fungal P450 are different from microsomal and mitochondrial P450s in that they are water soluble. As shown in Fig. 2, they also have several proline residues in the amino-terminal region. While well studied in microsomal P450s, the significance of these PRs in mitochondrial and soluble microbial P450s has not been examined.

In this study, we used two mitochondrial P450s, P450c27 and P450scc, and two soluble microbial P450s, P450cam and P450nor, to examine the effect of proline residue mutations in the amino-terminal region on the correct folding of the P450 proteins expressed in *Escherichia coli*. The effects of stepwise truncations from the amino-terminus were also studied. We have found that one specific proline residue is important for proper folding of each mitochondrial and soluble microbial P450 in *E. coli*, indicating that both classes of P450s use a PR in the amino-terminal region for correct folding. Therefore, we propose that the formation of functional forms of all types of cytochrome P450 depends on a

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Abbreviations: δ-ALA, δ-aminolevulinic acid; BS, basic sequence; CYP, cytochrome P450 (P450); Cm, chloramphenicol; P450c17 (CYP17), steroid 17α-hydroxylase/17,20-lyase cytochrome P450; P450c27 (CYP27), cholesterol 27-hydroxylase cytochrome P450; P450cam (CYP101), camphor 5-monooxygenase; P450nor, nitric-oxide reductase; P450scc (CYP11A1), cholesterol side chain cleavage cytochrome P450; SA, signal-anchor sequence; PR, proline-rich sequence.

P450s	Animal species	Aligned amino-terminal sequences
11A1	scc	bovine IS-TKTPRPYSEIPS-PGDNGWL
11A1	scc	human IS-TRSPRPFNEIPS-PGDNGWL
11A1	scc	rat ISSTNSPRSFNEIPS-PGDNGWI
11B1	11β	rat GTTAKVA--PKTLKPFEAIPQ-YSRNKWL
11B1	11β	bovine GTRGAAA--PKAVLPFEAMPR-CPGNKWM
11B1	11β	human GTRAARV--PRTVLPFEAMPR-RPGNRWL
11B2	aldo	rat GTTATLA--PKTLKPFEAIPQ-YSRNKWL
11B2	aldo	human GTRAARA--PRTVLPFEAMPQ-HPGNRWL
11B3		rat GSTATQA--PKTLKPFEAIPQ-YSRNKWL
27A1	c27	rat ALRDHESTEGPGTG-QDRPRLRSLAELPG-PGT----
27A1	c27	human ALPSDKATGAPGAGPGVRRRQRSLEEIPR-LGQ----
24	c24	rat RAPKEVPLCPMLTD-GETRN-VTSLPGPT-NWPLLG-
24	c24	human PQPREVPVCPLTAG-GETQN-AAALPGPT-SWPLLA-
2C11		rat SSLL--L-LSLWR--------QSFGRGKLPPGPIPLP

Fig. 1. **Amino-terminal sequences of the mature forms of selected mammalian mitochondrial P450s.** Each P450 is represented by both gene family name and conventional name including animal species. Sequence alignment was based on a search at the Homepage of D.R. Nelson at http://drnelson.utmem.edu/nelsonhomepage.html. Proline residues are boxed. The microsomal P450 rat 2C11 is included for comparison, with its PR underlined.

P450s	Amino-terminal sequences
P450cam	TTETIQSNANLAPLPPHVPKHLVFDFDM
P450nor	MASGAPSFPFSRASGPEPFAEFAXLRATH
P450terp	MDARATIPEHIARTVILPQGYADDEVIXP
P450BM-3	MTIKEMPQPKTFGELKNLPLLNTDKPVQA
P450BM-1	MNKEVIPVTEIPKFQSRAEEFFPIQWYKE
P450eryF	MTTVPDLESDDSFHVDWYRTYAELRETAP

Fig. 2. **Amino-terminal sequences of selected soluble microbial P450s.** P450cam, P450BM-1, P450BM-3, P450terp, and P450eryF are bacterial P450s, whereas P450nor is a fungal P450. Proline residues are boxed.

PR in the amino-terminal region for correct folding.

METHODS

Materials—Competent DH5αF'IQ *E. coli* were purchased from GIBCO-BRL and competent JM109 *E. coli* from Stratagene. Reagents for bacterial growth were from Difco. Chloramphenicol (Cm) and δ-aminolevulinic acid (δ-ALA) were from Sigma. The antibody and cDNA (pIBI25) for P450cam were kindly provided by Dr. Hideo Shimada (Keio University) and Dr. Julian A. Peterson (University of Texas, Southwestern Medical Center), respectively. The antibody and cDNA (pT7-nor) for P450nor were kind gifts from Dr. Hirofumi Shoun (University of Tsukuba). All other chemicals were of the highest grade commercially available.

Construction of Expression Plasmids—The expression constructs for P450c27 (pTrc-c27) and P450scc (pTrc-scc) have been described previously (*15*, *16*). Site-directed mutagenesis was performed to prepare proline mutants of P450-c27 and P450scc using pTrc-c27 and pTrc-scc as templates and the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene) according to the supplier's instructions. The primers used in this study are listed in Table I. To prepare proline mutants of P450c27, each *Nco*I–*Kpn*I fragment encoding a mutation and the *Kpn*I–*Sal*I fragment from pTrc-c27 containing the remainder of the coding sequence were ligated into the pTrc99A bacterial expression vector, which had been pretreated with *Nco*I and *Sal*I. In the case of

TABLE I. **Oligonucleotide sequences for mutagenesis study.** Substitution of alanine for each amino acid was performed as described in "MATERIALS AND METHODS." F and R indicate foward and reverse primer, respectively.

P450	Mutant		Sequence
P450c27	P3A	F	5'-AAACAGACCATGGCTCTCGCGAGTGATAAAGCCACCGGA
		R	5'-TCCGGTGGCTTTATCACTCGCGAGAGCCATGGTCTGTTT
	P11A	F	5'-AAAGCCACCGGAGCTGCCGGCGCCGGGCCTGGTGTC
		R	5'-GACACCAGGCCCGGCGCCGGCAGCTCCGGTGGCTTT
	P15A	F	5'-GCTCCCGGAGCCGGGGCCGGCGTCCGGCGGCGGCAA
		R	5'-TTGCCGCCGCCGGACGCCGGCCCCGGCTCCGGGAGC
	P28A	F	5'-CGGAGCTTAGAGGAGATCGCGAGACTAGGACAGCTGCGC
		R	5'-GCGCAGCTGTCCTAGTCTCGCGATCTCCTCTAAGCTCCG
P450scc	P6A	F	5'-GCTAGTACAAAAACTGCGCGCCCCTACAGTGAG
		R	5'-CTCACTGTAGGGGCGCGCAGTTTTTGTACTAGC
	P8A	F	5'-GCTAGTACAAAAACTCCGCGCGCCTACAGTGAGATCCCC
		R	5'-GGGGATCTCACTGTAGGCGCGCGGAGTTTTTGTACTAGC
	P13A	F	5'-CCCTACAGTGAGATCGCGAGCCCTGGTGACAATGGC
		R	5'-GCCATTGTCACCAGGGCTCGCGATCTCACTGTAGGG
	P15A	F	5'-AGTGAGATCCCCTCCGCCGGCGACAATGGCTGGCTT
		R	5'-AAGCCAGCCATTGTCGCCGGCGGAGGGGATCTCACT
P450cam	P13A	F	5'-CAACGCCAATCTTGCAGCGCTGCCACCCCATGTG
		R	5'-CACATGGGGTGGCAGCGCTGCAAGATTGGCGTTG
	L14A	F	5'-ACGCCAATCTTGCCCCTGCTCCTCCCCATGTGCCAGAG
		R	5'-CTCTGGCACATGGGGAGGAGCAGGGGCAAGATTGGCGT
	P15A	F	5'-AATCTTGCCCCTCTGGCGCCCCATGTGCCAGAG
		R	5'-CTCTGGCACATGGGGCGCCAGAGGGGCAAGATT
	P16A	F	5'-TCTTGCCCCTCTGCCTGCGCATGTGCCAGAGCAC
		R	5'-GTGCTCTGGCACATGCGCAGGCAGAGGGGCAAGA
	H17A	F	5'-TTGCCCCTCTGCCACCCGCGGTACCAGAGCACCTGGTA
		R	5'-TACCAGGTGCTCTGGTACCGCGGGTGGCAGAGGGGCAA
	V18A	F	5'-TTGCCCCTCTGCCACCGCATGCGCCAGAGCACCTGGTA
		R	5'-TACCAGGTGCTCTGGCGCATGCGGTGGCAGAGGGGCAA
	P19A	F	5'-TCTGCCACCCCATGTGGCAGAGCACCTGGTATT
		R	5'-AATACCAGGTGCTCTGCCACATGGGGTGGCAGA
	E20A	F	5'-CTGCCACCCCATGTGCCTGCGCACCTGGTATTCGAC
		R	5'-GTCGAATACCAGGTGCGCAGGCACATGGGGTGGCAG
P450nor	P6A	F	5'-ATATGGCTTCTGGTGCGGCGTCTTTCCCGTTCTC
		R	5'-GAGAACGGGAAAGACGCCGCACCAGAAGCCATAT
	P9A	F	5'-TGGTGCTCCGTCTTTCGCATTCTCGCGCGCCTCTG
		R	5'-CAGAGGCGCGCGAGAATGCGAAAGACGGAGCACCA
	P16A	F	5'-TCTCGCGCGCCTCTGGCGCCGAGCCACCCGCCGAGT
		R	5'-ACTCGGCGGGTGGCTCGGCGCCAGAGGCGCGCGAGA
	P18A	F	5'-CGCCTCTGGTCCTGAGGCGCCCGCCGAGTTCGCCA
		R	5'-TGGCGAACTCGGCGGGCGCCTCAGGACCAGAGGCG
	P19A	F	5'-CTCTGGTCCTGAGCCAGCTGCAGAGTTCGCCAAACTTC
		R	5'-GAAGTTTGGCGAACTCTGCAGCTGGCTCAGGACCAGAG

P450scc proline mutants, each *Nco*I–*Bam*HI fragment encoding a mutation and the *Bam*HI–*Kpn*I fragment from pTrc-scc were ligated into the pTrc99A bacterial expression vector previously treated with *Nco*I and *Kpn*I.

Stepwise truncations of P450c27 from the amino-terminus were carried out by PCR using *Pfu* polymerase (Stratagene). The pTrc-c27 was used as a template, and the primers used are listed in Table II. 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 *Nco*I–*Kpn*I fragment from each pBS-

TABLE II. **Oligonucleotide sequences for deletion mutants.** Deletions were performed by PCR as described in "MATERIALS AND METHOD." One forward primer and the reverse primer were used to prepare each deletion mutant.

P450	Mutant	Sequence
P450c27	d2-17	5'-AAAGACCATGGCTCGGCGGCGGCAACGGAGC
	d2-27	5'-AAAGACCATGGCTCCACGTCTAGGACAGCTG
	d2-28	5'-AAAGACCATGGCTCGTCTAGGACAGCTGCGC
	Reverse	5'-CGCTGGCTTCAGCAACCGCTGGTT
P450cam	d2-14	5'-AAGGCATATGACGCCACCCCATGTGCCAGAG
	d2-15	5'-AAGGCATATGACGCCCCATGTGCCAGAGCAC
	d2-17	5'-AAGGCATATGACGGTGCCAGAGCACCTGGTA
	d2-19	5'-AAGGCATATGACGGAGCACCTGGTATTCGAC
	Reverse	5'-GATCCGGTTCTCCAGCTTATC

PCR1, which encodes the amino-terminal truncated sequence of P450c27, and the *Kpn*I-*Sal*I fragment from pTrc-c27 were ligated into pTrc99A, which was pretreated with *Nco*I and *Sal*I.

The cDNA for P450cam in pIBI25 (pIBI25-cam) was used as a template for PCR to modify the 5′ portion of the P450cam cDNA. The 5′-primer, 5′-AAGGCATATGACGACTGAAACCATACAA, was designed to introduce the initiator codon ATG within the *Nde*I site. The 3′-primer, 5′-GATCCGGTTCTCCAGCTTATC, was template-specific. The PCR reactions were performed using *Pfu* polymerase, and the products blunt-ended by treatment with the Klenow enzyme were subcloned into the *Eco*RV site of pBluescript (pBS-PCR2). The *Nde*I–*Sph*I fragment, which encodes ATG within the *Nde*I site, and the *Sph*I–*Hin*dIII fragment from pIBI25-cam were ligated into pCWori+ (*17*), which had been pretreated with *Nde*I and *Hin*dIII (pCW-cam).

Site-directed mutagenesis to prepare proline mutants of P450cam used pBS-PCR2 as a template and the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). The sequences of the mutagenesis primers are listed in Table I. The *Nde*I–*Sph*I fragment containing the mutation and the *Sph*I–*Hin*dIII fragment from pIBI25-cam were ligated into pCWori+ previously treated with *Nde*I and *Hin*dIII. Stepwise truncations of P450cam from the amino-terminus were also carried out by PCR using *Pfu* polymerase. pCW-cam was the template, and the primers are listed in Table II. The PCR products were blunt-ended by treatment with the Klenow enzyme and subcloned into the *Eco*RV site of pBluescript (pBS-PCR3). The *Nde*I–*Sph*I fragment from each pBS-PCR3, encoding the amino-terminal truncated sequence of P450cam, and the *Sph*I–*Hin*dIII fragment from pIBI25-cam were ligated into pCWori+, which had been pretreated with *Nde*I and *Hin*dIII.

The *Nde*I–*Xba*I fragment of pT7-nor, which encodes the complete cDNA of P450nor, was ligated into pCWori+ previously treated with *Nde*I and *Xba*I. Site-directed mutagenesis was also performed to prepare proline mutants of P450nor using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene). The pT7-nor was used as a template and the sequences of the mutagenesis primers are listed in Table I. The *Nde*I–*Sal*I fragment, which encodes mutations, and the *Sal*I–*Xba*I fragment from pT7-nor were ligated into pCWori+ previously treated with *Nde*I and *Xba*I.

The nucleotide sequences of all PCR products were confirmed by automated sequencing (Applied Biosystems).

Other Methods—DH5αF′IQ *E. coli* were used for the expression of P450c27, P450cam and P450nor, and JM109 *E. coli* were used for P450scc. Culture conditions and the measurement of the reduced CO-difference spectra have been described previously (*18*). An *in vitro* transcription/translation assay with *E. coli* S30 lysate was carried out as described in the companion paper (*8*). *E. coli* proteins (5 μg total cell lysate) were size fractionated in 8% SDS–polyacrylamide gels and transferred onto PVDF membranes. Immunoblot analysis was performed using rabbit antisera against human P450c27, bovine P450scc, P450cam and P450nor, and 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 of Proline Residues in the Amino-Terminal Region on the Expression of Spectrally Normal Mitochondrial P450s in E. coli—Mature forms of mitochondrial P450s have several proline residues in the amino-terminal region, which are conserved for each CYP gene subfamily, as shown in Fig. 1. The mature form of human P450c27 is expressed in *E. coli* at a level of 650 nmol/g cell protein (1,300 nmol/liter culture) in the presence of 0.5 mM δ-ALA and 1 μg/ml Cm. Mature P450c27 contains four proline residues at positions 3, 11, 15, and 28 in the amino-terminal region. Substitutions of alanine for the residues at 3, 11, and 15 (P3A, P11A, and P15A) have little effect on the expression of spectrally normal P450. The intensities of the expressed protein and CO-difference spectra correlate well (Fig. 3, A and B). However, when Pro-28 is replaced by alanine (P28A), the CO-difference spectrum decreases greatly to only 12% of that of the wild type (Fig. 3A), although the level of the expressed protein detected by immunoblot analysis is same as that of the wild type (Fig. 3B). Thus, Pro-28 is important for the efficient folding of mature P450c27 in *E. coli*.

The mature form of bovine P450scc also has four proline residues, at positions 6, 8, 13, and 15 (Fig. 1). Wild-type P450scc shows an expression level of 150 nmol/g cell protein (490 nmol/liter culture) in the presence of 0.5 mM δ-ALA (Fig. 3C). When the proline at position 13 is replaced by alanine (P13A), the CO-difference spectrum is not detected although the amount of expressed protein is 57% that of the wild type (Fig. 3, C and D). The other proline mutants (P6A, P8A, and P15A) give CO-difference spectra that are at least 50% that of the wild type, indicating clearly that Pro-13 is most important for the correct folding of mature P450scc in *E. coli*.

These observations demonstrate that a specific proline residue in the amino-terminal region is important for correct folding of both mitochondrial P450s. Pro-28 in P450c27 and Pro-13 in P450scc are highly conserved within their respective subfamilies, and these proline residues are found at the same position in P450c27 and P450scc according to the alignment given on the Homepage of D.R. Nelson (http://drnelson.utmem.edu/nelsonhomepage.html). In fact, the mature forms of all mitochondrial P450s have a proline residue at this position (Fig. 1). It is highly likely that all mitochondrial P450s, regardless of gene family, require this specific proline residue for efficient folding of the functional enzyme.

Effect of N-Terminal Truncation on the Expression of

Spectrally Normal P450c27 in E. coli—To confirm further the importance of Pro-28 on the expression of mature P450-c27, stepwise truncations from the amino-terminus were performed, and the mutant P450s were expressed in *E. coli*.

Deletion mutant d2-17, which does not contain the first three proline residues (Pro-3, Pro-11, and Pro-15) shows about 30% of the CO-difference spectrum compared with the wild type (Fig. 4A), a level that corresponds well with the expressed protein level (Fig. 4B). This confirms that while proper folding is most efficient in the presence of these three proline residues, they are not essential for proper folding of expressed P450c27. Further truncations (d2-27, which still contains Pro-28, or d2-28, which lacks Pro-28) result in the complete loss of the CO-difference spectra and immunoreactive protein.

Effect of Mutations of Proline Residues in the Amino-Terminal Region on the Expression of Spectrally Normal Soluble Microbial P450s in E. coli—As shown in Fig. 2, soluble microbial P450s have several proline residues in their amino-terminal regions. P450cam has four clustered prolines within a narrow range from amino acid 13 to 19. Alanine scanning mutagenesis of this region was performed and the results are shown in Fig. 5. When wild-type P450-cam is expressed in *E. coli* in the absence of δ-ALA, the expression level is 1,200 nmol/g cell protein (3,300 nmol/liter culture). Replacement of Pro-15 with alanine (P15A) results in a large decrease in the CO-difference spectrum to less than half that of the wild type. However, the expressed protein level of P15A is almost the same as the wild type,

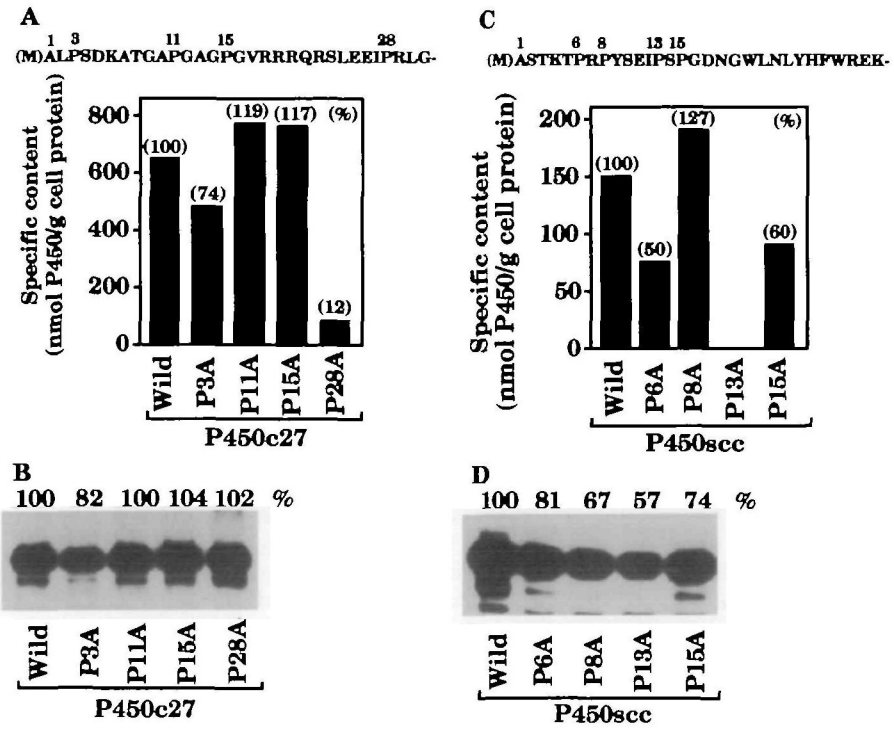

Fig. 3. **Effect of substituting of alanine for prolines on the expression of the mature forms of P450c27 and P450scc in *E. coli*.** *E. coli* DH5αF'IQ transformed with pTrc-c27 were cultured at 29°C in the presence 0.5 mM δ-ALA and 1 μg/ml Cm. Since the expression level of P450scc in *E. coli* JM109 is higher than that in DH5αF'IQ, JM109 was used in this study. *E. coli* JM109 with pTrc-scc were cultured at 27°C in the presence of 0.5 mM δ-ALA. After 48 h incubation, the cells were harvested and solubilized for spectral analysis. Specific contents (nmol P450/g cell protein) of P450c27 and its proline mutants (A) and those of P450scc and its mutants (C) are shown. Figures in parentheses are the ratios of the specific content of the mutants to that of each wild type (100%). The amino-terminal sequences of P450c27 and P450scc are shown above each figure. Immunoblot analyses of P450c27 and its proline mutants (B) and those of P450scc and its proline mutants (D) are shown (5 μg of cell protein per each lane). The ratios of the band-intensities of the mutants to that of each wild type (100%) were calculated using NIH image software, and are shown above panels B and D.

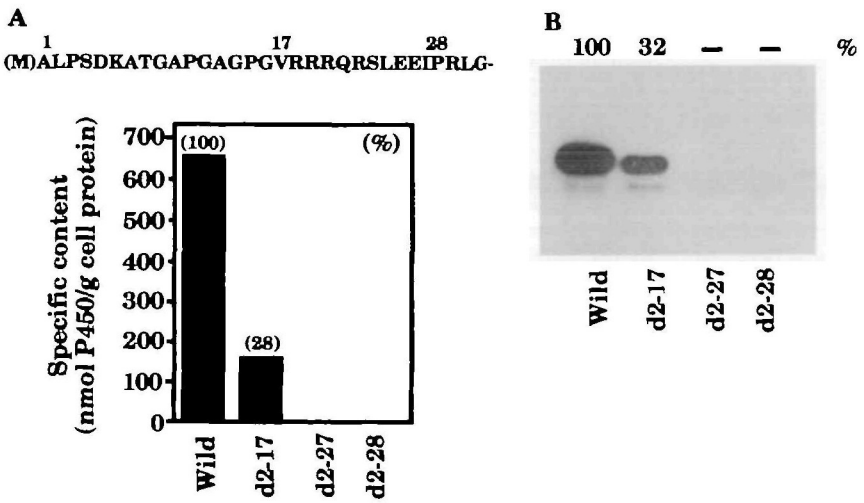

Fig. 4. **Effect of amino-terminal deletions on the expression of P450c27 in *E. coli*.** Transformed *E. coli* DH5αF'IQ were cultured at 29°C in the presence of 0.5 mM δ-ALA and 1 μg/ml Cm. After 48 h incubation, the cells were harvested and solubilized for spectral analysis. (A) Specific contents (nmol P450/g cell protein) of P450c27 and its deletion mutants. Figures in parentheses show the ratios of the specific contents of the mutants to that of the wild type (100%). The amino-terminal sequence of P450-c27 is shown above the panel. (B) Immunoblot analysis of P450c27 and its deletion mutants expressed in DH5αF'IQ (5 μg of cell protein per each lane). Figures above the panel show the ratios of the band-intensities of the mutants to that of the wild type (100%) calculated using NIH image software.

indicating that a significant portion of the expressed protein is misfolded (Fig. 5, A and B). Among other residues between Pro-13 and Glu-20, only mutation of His-17 to alanine (H17A) results in a significantly reduced CO-difference spectrum. Perhaps His-17 is also involved in proper folding, although to a lesser degree than Pro-15. When 0.5 mM δ-ALA is added to the culture medium, the expression level of wild-type P450cam increases to 2,000 nmol/g cell protein (about 1.6-fold), and the expression levels of all mutants are also enhanced (Fig. 5, C and D). The degree of enhancement of the P15A mutant is greater than that of the wild type (about 3-fold), although the level of the CO-difference spectrum remains significantly below that of the wild type. These results show that the incorporation of heme is less efficient in the P15A mutant than in wild-type P450cam, and the increased availability of heme by the addition of δ-ALA facilitates heme incorporation and proper folding of the mutant.

There are five proline residues in the amino-terminal region of P450nor, as shown in Fig. 2. When wild-type P450nor is expressed in *E. coli*, the expression level is 60 nmol/g cell protein (260 nmol/liter culture) in the presence of 0.5 mM δ-ALA and 1 μg/ml Cm (Fig. 6A). The substitution of alanine for Pro-16 (P16A) results in a large decrease in the CO-difference spectrum to only 25% that of the wild type (Fig. 6A). However, the expressed protein level of the P16A mutant is higher than that of the wild type (Fig. 6B), indicating that a large portion of the expressed P16A mutant protein is in a misfolded apo-form. Substitution of alanine for each of the other four proline residues does not

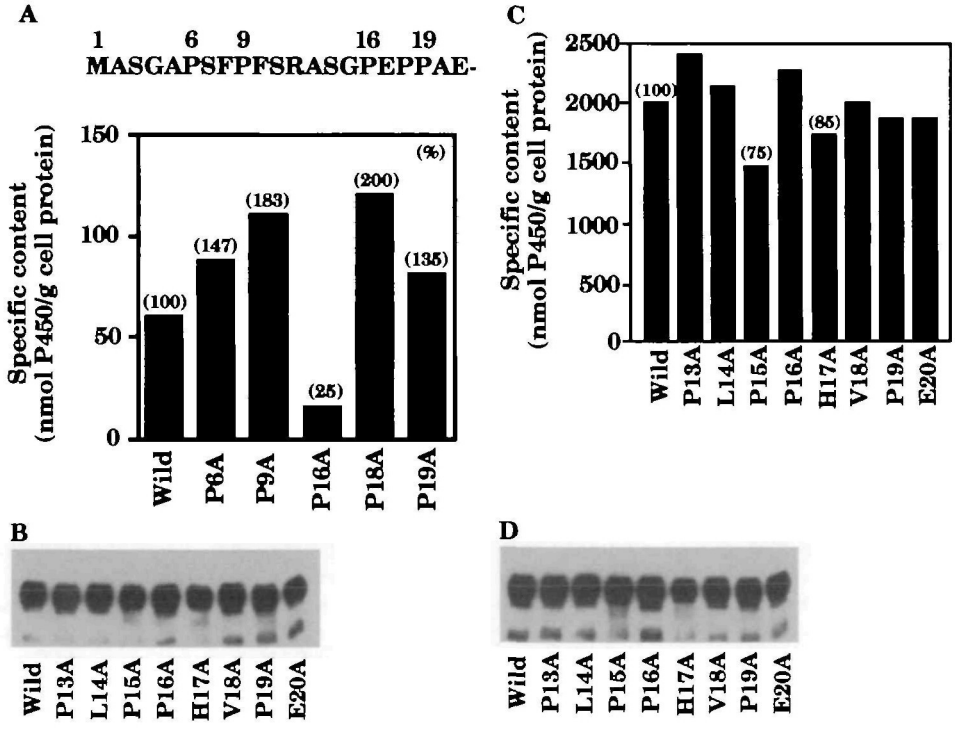

Fig. 5. **Alanine scanning mutagenesis of the proline-rich sequence of P450cam.** Transformed *E. coli* DH5αF'IQ were cultured at 29°C in the presence and absence of 0.5 mM δ-ALA. After 24 h incubation, the cells were harvested and solubilized for spectral analysis. Specific contents (nmol P450/g cell protein) of P450cam and its mutants expressed in the absence (A) and presence of 0.5 mM δ-ALA (C) are shown. Figures in parentheses show the ratios of the specific contents of mutants to that of the wild type (100%). The amino-terminal sequence of P450cam is shown above panel A. Immunoblot analyses of P450cam and its mutants expressed in DH5αF'IQ in the absence (B) and presence of 0.5 mM δ-ALA (D) are also shown (5 μg cell protein per each lane).

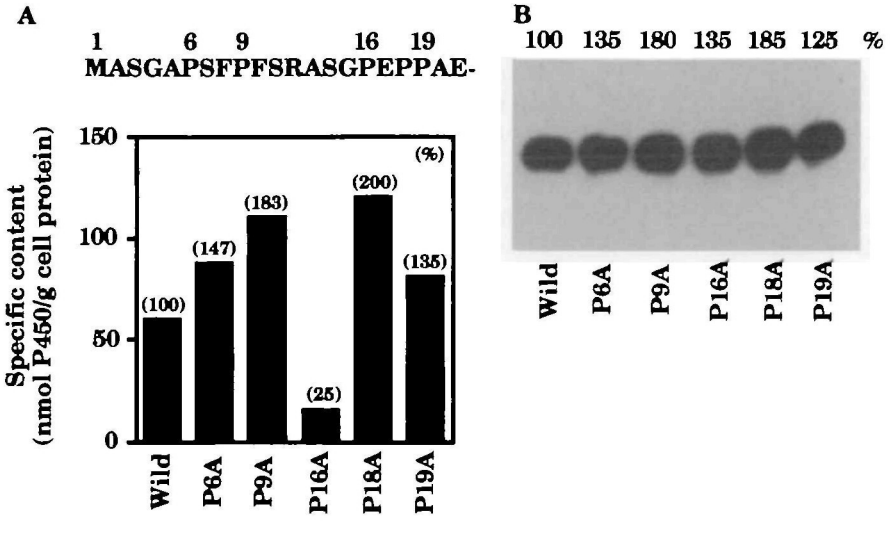

Fig. 6. **Effect of substituting alanine for prolines on the expression of P450nor in *E. coli*.** Transformed *E. coli* DH5αF'IQ were cultured at 29°C in the presence of 0.5 mM δ-ALA and 1 μg/ml Cm. After 48 h incubation, the cells were harvested and solubilized for spectral analysis. (A) Specific contents (nmol P450/g cell protein) of P450nor and its proline mutants. Figures in parentheses show the ratios of the specific contents of the mutants to that of the wild type (100%). The amino-terminal sequence of P450nor is shown above the panel. (B) Immunoblot analysis of P450nor and its proline mutants expressed in DH5αF'IQ (5 μg of cell protein per each lane). Figures show the ratios of the band-intensities of the mutants to that of the wild type (100%) calculated using NIH image software.

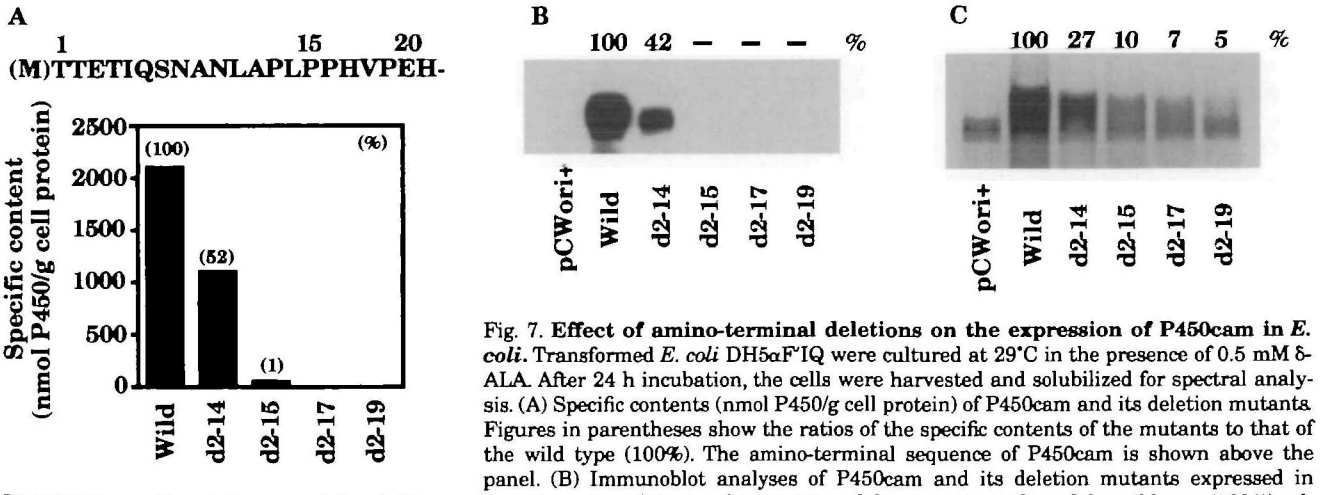

Fig. 7. **Effect of amino-terminal deletions on the expression of P450cam in *E. coli*.** Transformed *E. coli* DH5αF'IQ were cultured at 29°C in the presence of 0.5 mM δ-ALA. After 24 h incubation, the cells were harvested and solubilized for spectral analysis. (A) Specific contents (nmol P450/g cell protein) of P450cam and its deletion mutants. Figures in parentheses show the ratios of the specific contents of the mutants to that of the wild type (100%). The amino-terminal sequence of P450cam is shown above the panel. (B) Immunoblot analyses of P450cam and its deletion mutants expressed in DH5αF'IQ (5 μg cell protein per each lane). Figures show the ratios of the band-intensities of the mutants to that of the wild type (100%) calculated using NIH image software. The result obtained with empty vector (pCWori+) is also shown. (C) *In vitro* transcription/translation assays of P450cam and its deletion mutants. Figures above the panel show the ratios of radioactivities of the deletion mutants to that of the wild type (100%).

reduce the production of spectrally normal P450nor and, in fact, even increases it. The expressed protein levels correspond well with the CO-difference spectra (Fig 6, A and B). When wild type P450nor and its proline mutants are expressed in the absence of δ-ALA, the CO-difference spectrum of the P16A mutant is undetectable, whereas the wild type and other four proline mutants show about 50% of the CO-difference spectra found in the presence of δ-ALA (data not shown). As observed for P450cam, a specific proline residue in the amino-terminal region of P450nor is important for efficient heme binding and folding.

Effect of N-Terminal Truncation on the Expression of Spectrally Normal P450cam in E. coli—Stepwise truncation of P450cam from the amino-terminus was also performed to confirm further the importance of specific proline residues. The expression level of wild-type P450cam in *E. coli* is 2,100 nmol/g cell protein (5,300 nmol/liter culture) in the presence of 0.5 mM δ-ALA (Fig. 7A). Deletion of amino acid residues before Pro-15 (d2-14) results in a decrease in the CO-difference spectrum to about 50% that of the wild type (Fig. 7A). The immunoreactive protein level also decreases in a parallel fashion (Fig. 7B). The level of the *in vitro* translation product of the d2-14 deletion mutant is about 30% that of the wild type (Fig. 7C), indicating that the parallel decreases in the CO-difference spectrum and the expressed protein level are due to a lowered efficiency of translation upon truncation, and not to misfolding. When Pro-15 is also deleted (d2-15), only 1% of the CO-difference spectrum is found compared with the wild type, and the expressed protein is undetectable by immunoblot analysis (Fig. 7, A and B). Further truncations (d2-17 and d2-19) result in the complete loss of both the CO-difference spectrum and protein expression (Fig. 7, A and B). However, low levels of *in vitro* translation products of d2-15, d2-17, and d2-19 are detectable; 10, 7, and 5% of the wild type level, respectively (Fig. 7C), suggesting misfolding. Thus, the deletion of the amino-terminal sequence up to the key proline residue affects both folding and translation, the combination resulting in a great decrease or complete loss of the CO-difference spectrum.

DISCUSSION

Here, we demonstrate that the PR plays an important role in the folding of two mature mitochondrial P450s, P450c27 and P450scc, and two soluble microbial P450s, P450cam and P450nor, when they are expressed in *E. coli*. Both mitochondrial P450s have one specific proline residue in the amino-terminal region that is important for correct folding. These critical proline residues in P450c27 (Pro-28) and P450scc (Pro-13) align at the same position and are conserved within each subfamily. All other mammalian mitochondrial P450s have a proline residue at the corresponding position, suggesting a common role of the proline residue at this position for correct folding of the mature peptides. While the PRs are now clearly established to be extremely important in the folding of both mammalian microsomal and mitochondrial P450s, an interesting difference between microsomal and mitochondrial P450s emerges. As noted in the companion paper (*8*), each microsomal CYP gene family has its own unique PR sequence that is key to preventing misfolding of the polypeptide. For mature mitochondrial P450s, a single proline fills this role and the location of this residue is conserved among different gene families. However, it will be important to test this in additional mitochondrial P450s.

Linker scanning mutagenesis of the APLPPHVPE sequence near the amino-terminus of P450cam has shown that Pro-15 is important in preventing misfolding of the peptide. Similarly, Pro-16 is important for correct folding of another soluble microbial P450, P450nor. Stepwise truncations of P450cam from the amino-terminus further indicate the importance of Pro-15 in the folding of the expressed protein. This identification of a single important proline residue in P450cam and P450nor resembles the results observed in mitochondrial P450s, another example of similarity between mitochondrial and bacterial P450s.

When δ-ALA, a heme precursor, is added to the culture medium, the CO-difference spectra of P450cam and its mutants increase, with the increase in the CO-difference

spectrum of the P15A mutant being greater (about 3-fold) than that of the wild type (about 1.6-fold). This indicates that a key role played by proline in folding is to increase the efficiency of heme incorporation as observed with the PR mutants of microsomal P450s (*8*). P450nor is a very novel P450, a soluble fungal P450, which does not require an associated redox partner. It, too, requires a single proline residue for the prevention of misfolding. In this way it resembles mitochondrial P450s and P450cam. We suggest from these studies that other soluble P450s also require a PR near the amino-terminus for proper folding.

This study establishes that the PR is required for efficient folding of both mitochondrial and soluble microbial P450s in *E. coli*. Since the importance of the PR for correct folding of microsomal P450s is already known, we can now conclude that the PR in the amino-terminal region is important for correct folding of all classes of cytochrome P450. If we assume that the presequence of mitochondrial P450 precursors corresponds to the signal-anchor sequence (SA) of microsomal P450s, and that soluble microbial P450s are equivalent to SA-truncated forms of microsomal P450, the critical PR residue(s) in these three types of cytochrome P450 are aligned at nearly the same position (Fig. 1), suggesting that the roles played by different PR residues in the many different types of P450 enzymes are similar: to reduce the tendency for these polypeptides to misfold prior to heme binding.

The function of specific PR residues at the biochemical level in the folding process of P450 peptides is not yet clarified. P450s belonging to the same gene family or subfamily have identical proline-rich sequences (PR), and exchange of the PR between two microsomal P450s belonging to different gene families results in misfolding of the chimeric proteins (*8*). It is therefore likely that the PR interacts with some specific portion of the same P450 peptide on the carboxyl-terminal side of the PR, a region that is also conserved in each gene subfamily or family. Many plant microsomal P450s, which are very distantly related to animal P450s, have a PPGP sequence in the amino-terminal region, which is the characteristic PR motif of CYP2 family animal microsomal P450s. This unexpected connection in PRs between plant microsomal P450s and the CYP2 family may be utilized to predict the partner sequence that associates with critical PR residues in an early stage of folding of newly synthesized P450 peptides. A chimeric P450 of P450cam and P4502C9 (CYP2C9) has recently been reported (*19*). The amino-terminal region of the chimeric P450 comes from P450cam and the C-terminal half comes from P4502C9. This chimeric P450 is spectrally normal and catalytically active when expressed in *E. coli*, indicating that it is folded correctly. If the specific PR residue in P450cam, Pro-15, interacts with some other portion of the P450cam peptide in folding, the partner sequence lies in the amino-terminal half of P450cam.

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