Cytochrome P450 is anchored to the endoplasmic reticulum membrane by an N-terminal transmembrane sequence with the catalytic domain facing the cytoplasmic side. Within the peptide sequence linking these two domains is a highly conserved proline-rich region. In cytochrome P450 2C2, this region has the sequence \( ^{30} \text{PPG}^37 \). To examine the structural requirements at these proline residues, each proline was replaced with alanine, glycine, valine, or an acidic amino acid, and the activities of the mutated proteins were determined in transfected COS-1 cells. Lauric acid 1\(,\omega\)-hydroxylase activities of Pro\(^{30}\) and Pro\(^{33}\) mutants were less than 10% of wild type for each substitution except for alanine, which was 25–30%. In striking contrast, substitutions at Pro\(^{31}\), including an acidic residue, did not substantially alter activity. At positions 35 and 37, acidic amino acid substitutions reduced activity to less than 10% of wild type while substitution of the other three amino acids had little effect. The tolerance of substitutions of charged residues at Pro\(^{31}\) suggests that the side chain at this position is exposed to a polar environment; conversely, the reduced activity with charged substitutions, but not with uncharged substitutions at positions 35 and 37, suggests that these residues are exposed to a hydrophobic environment, presumably within the folded protein. The loss of activity with substitutions at Pro\(^{30}\) and Pro\(^{33}\) implies that the motif PXXP is important for the formation of a functional cytochrome P450 and that this sequence might have a helical structure with a repeat of three, as in the left-handed poly-L-proline II helix. Insertion of alanine between positions 29 and 30 did not substantially affect activity, but insertions between either 33 and 34 or 37 and 38 resulted in activity less than 25% of wild type. These data indicate that the position of PXXP, relative to the sequence flanking it on the C-terminal side, may be important for its function.

**Different Structural Requirements at Specific Proline Residue Positions in the Conserved Proline-rich Region of Cytochrome P450 2C2**

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EXPERIMENTAL PROCEDURES

Plasmid Construction—Construction of plasmid pc2A containing P450 2C2 cDNA in pTZ18R has been described (14). Single strand DNA mutagenesis was performed as described (14). The following oligonucleotides were used as primers, and uracil-containing single-stranded DNA from plasmid pc2A grown in E. coli strain CJ236 was used as template: Pro29 mutants, 5′-GGGGAGGGAAGCTTGTTCCTGGC-3′; Pro31 mutants, 5′-GGAGGGAAGCTTCTGTGCGG-3′; Pro33 mutants, 5′-AAGCTTCTCCGCGG-GAGGGAAGCTTGT-3′; Pro35 mutants, 5′-CCTCGGGGCCCAC-TGTTCCTGGCGGTGA-3′; Pro37 mutants, 5′-GGCGCGCACTCCGGCGGTGA-3′; Pro30A/T34P, 5′-GGGGAGGGAAGCTTGTTCCTGGC-GTTGGGAAGMTTCCCTGCG-3′; wild-type DNA from plasmid pc2A grown in Escherichia coli strain CJ236 was used as template: Pro29 mutants, 5′-GGGGGAGGGAAGCTTGTTCCTGGCCCGGAC-3′; Pro31 mutants, 5′-GGAGGGAAGCTTCTGTGCGG-GAGGGAAGCTTGT-3′; Pro33 mutants, 5′-AAGCTTCTCCGCGG-GAGGGAAGCTTGT-3′; Pro35 mutants, 5′-CCTCGGGGCCCAC-TGTTCCTGGCGGTGA-3′; Pro37 mutants, 5′-GGCGCGCACTCCGGCGGTGA-3′; Pro30A/T34P, 5′-GGGGAGGGAAGCTTGTTCCTGGC-GTTGGGAAGMTTCCCTGCG-3′.

In order to construct Pro mutants in the pCMV5 mammalian expression vector, each pc2A-pTZ mutant was digested with KpnI and ApaI, and the isolated fragment containing a mutation in the Pro-rich region was ligated to the pc2A-pCMV vector cut with KpnI and ApaI. The mutations were confirmed by sequencing the KpnI-ApaI segment.

Cell Culture and Assay of Laurate Hydroxylase Activity—COS-1 cells were cultured and transfected with plasmid DNA as described (15). Laurate hydroxylase activity was assayed in whole cell lysates of transfected COS-1 cells, and lauric acid metabolites were separated by high performance liquid chromatography as described (15) except that the reaction was incubated for 30 min.

Immunoprecipitation of Expressed Proteins—Forty-eight h after transfection, cells were incubated for 4 h with 50 μCi/ml Tran35S-label and the isolated fragment containing a mutation in the Pro-rich region was ligated to the pc2A-pCMV vector cut with KpnI and ApaI. The mutations were confirmed by sequencing the KpnI-ApaI segment.

RESULTS

Effect of Mutations on the Level of Expression in Transfected Cells—The Pro-rich region of P450 2C2 contains five Pro residues in the sequence from amino acids 30–37 (Fig. 1). In order to examine the functional importance of the Pro residues in this region, each Pro was replaced with Ala, Gly, Val, and either Asp or Glu depending on the third base of the codon in the cDNA sequence. The four amino acid residues were chosen for the following reasons. Ala has a small side chain and is uncharged, properties that make it a “neutral” substitution in mutagenesis; Gly, like Pro, is often present in turns rather than α-helical structures but results in less stable structures; Val is a small hydrophobic amino acid with a tendency to β-carbon, which decreases the flexibility of the peptide backbone; and Asp and Glu are charged amino acids that dramatically change the hydrophobicity of the side chain. Each of the mutants was transfected into COS-1 cells, and lauric acid hydroxylase activity determined. The results demonstrate different structural requirements at specific Pro residue positions and suggest that a PXXP motif may be important for the function of a functional P450 2C2.

Any of the mutations could alter activity of the P450 expressed in COS-1 cells by affecting the rate of synthesis or degradation of the protein. To examine the levels of the proteins expressed in the COS-1 cells, transfected cells were labeled for 4 h with Tran35S-label, and P450 2C2 and the mutant proteins were immunoprecipitated and analyzed by SDS-PAGE. In mock transfected cells, only a weak band comigrating with P450 was detected; and a second protein migrating more rapidly was present, which served as a useful internal control for labeling and immunoprecipitation (Fig. 2). Since the half-life of the P450 expressed in COS-1 cells is less than 1 h (15), labeling the cells for 4 h provides a reasonable measure of the steady-state levels of the protein. Differences of any two-fold or less in the amount of radioactive protein immunoprecipitated for any of the mutants and wild-type P450 2C2 were detected in 2 or 3 independent experiments. Therefore, the changes in activity of the mutant P450s cannot be explained by different levels of protein expressed in COS-1 cells. While these experiments detected P450 apoprotein, it is possible that the folding, heme incorporation, or stability of functional forms of the protein might be affected by the mutations. Attempts to express the P30A variant, which has decreased activity in COS-1 cells, in E. coli in order to distinguish among these possibilities have been unsuccessful using conditions that allow expression of wild-type P450 2C2 and other P450 2C2 mutants that appear to have reduced stability.

Laurate 1α-Hydroxylase Activity of Single Pro Mutations in the Pro-rich Region—Within the highly conserved PPGP sequence, the effects of the mutations at Pro30 and Pro37 on lauric acid hydroxylase activity were similar and were remarkably different from effects of mutations at Pro31 (Fig. 3). At Pro30, no activity was detected with Gly, Val, or Asp substitutions, and less than 10% activity compared with wild type was detected with these substitutions at Pro31. Ala substitutions at these two positions were tolerated slightly better with 25–30% activity of wild type. These results indicate that the structural requirements for Pro at these positions are very stringent since the Gly substitution, which should be tolerated if only a turn structure is required, reduced activity. In striking contrast, all of the substitutions at Pro31 were well tolerated including the acidic residue, Asp, with a relatively large charged side chain, suggesting that the side chains at this position are on the surface and pointing toward the aqueous environment. In contrast, the side chains of positions 30 and 33 may be involved in packing of the PPGP peptide against another peptide structure.

Substitutions at Pro30 and Pro37 exhibited a third pattern, with Ala, Gly, or Val mutations being well tolerated and Asp or Glu mutations reducing activity to 15% or undetectable for...
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FIG. 3. Effect of substitutions for proline on laurate hydroxylase activity in COS-1 cells transfected with wild-type (WT) and mutated P450 2C2. Cell lysates from COS-1 cells, transfected with the indicated mutants, were incubated with 20 mM [1-14C]lauric acid at 37 °C. Lauric acid and its hydroxylated derivatives were extracted with ethyl acetate and separated by high performance liquid chromatography as described under "Experimental Procedures." The means of three to eight independent transfections for each protein with the standard errors are shown. Values for the individual hydroxylase activities were normalized against activity of the wild-type P450 2C2.

Pro35 and Pro37, respectively. The reduced activity with charged substitutions, but not with uncharged substitutions, suggests that these Pro residues are in a relatively hydrophobic environment, very likely buried inside the molecule. Alternatively, the size of Glu may sterically alter the structure, but this would also suggest that the side chain is packed against other parts of the protein structure.

Laurate 1ω-Hydroxylase Activity of a Secondary Mutant Restoring a PXXP Motif—The effects of substitutions at Pro30, Pro31, and Pro33 suggest that a PXXP motif is important in the structure-function relationship of P450 2C2. If the PXXP motif itself is important for activity, then re-creating a PXXP motif in the proteins with mutations at Pro30 or Pro33 might restore activity. To accomplish this, a double mutant, P30A/T34P was constructed in which Pro at positions 31 and 34 provide a PXXP motif (Fig. 1). The activity of this double mutant, however, was similar to the P30A mutation alone so that activity was not restored by the second mutation (Fig. 4). This result might be explained either by the fact that the position of the PXXP was moved one residue relative to its flanking regions or by some restrictions on the amino acids between or immediately flanking the two Pro residues.

Laurate 1ω-Hydroxylase Activity of Ala Insertion Mutants Flanking the PXXP Motif—In order to determine whether the position of the PXXP motif was critical, Ala was inserted on either the N- or C-terminal sides of the PXXP motif (Fig. 1). Insertion of an Ala between residues 29 and 30 on the N-terminal side of the PXXP only slightly reduced activity to about 75% of wild type (Fig. 4). On the other hand, insertions of Ala between residues 33 and 34 or 37 and 38 reduced activity to less than 25% of the activity of wild type, similar to the Ala substitutions at Pro30 and Pro33. These results suggest that the position of the PXXP motif relative to flanking sequence on the C-terminal side is important for the formation of functional P450 2C2, while the position relative to N-terminal sequences is not.

FIG. 4. Effect of PXXP position on laurate hydroxylase activity in COS-1 cells transfected with wild-type (WT) and mutated P450 2C2. Transfection of COS-1 cells and assay for laurate hydroxylase were the same as in Fig. 2. The sequences of the mutated P450 2C2 were shown in Fig. 1, and the designations of the mutants are as described in the legend of Fig. 1.

Natural Variants of the PXXP motif in Microsomal P450—The sequence PPGP is present in P450s of widely divergent organisms, ranging from plants to mammals as shown in the alignment of selected sequences in Table I. PPGP is completely conserved in family 2 P450s, and PPGP, or a variant PXXP, is present in most other mammalian P450s (Table II). Another variant, I/LPGP, is also commonly present, particularly in P450s 1A2, 3A, and 19. To examine whether Ile or Leu can substitute for the first Pro in the PPGP motif, mutants P30I and P30L were constructed. Replacing Pro30 with either Ile or Leu resulted in less than 10% activity of wild type (Fig. 4). Similarly an Leu mutation at this position in human P450 21A2 resulted in activity about 30% of normal for progesterone hydroxylation and represents a potential nonclassical steroid 21-hydroxylase deficiency allele (10). These data suggest that the first Pro in the PXXP motif of P450s cannot be replaced by amino acids present at this position in other P450s without substantial loss of activity and provide further evidence that Pro30 is important for formation of a fully functional P450 2C2. If the PXXP motif packs against other peptide segments of the P450, then the requirement for Pro at position 30 may be altered by different sequences in the interacting regions of those P450s with Ile or Leu at the first Pro position.

DISCUSSION

Mutations of the Pro residues in the Pro-rich region of P450 2C2 indicate that these residues can be divided into two structural regions, the highly conserved PPGP sequence and the more C-terminal Pro35 and Pro37. The latter Pro tolerated uncharged amino acid substitutions, but not charged substitutions, which is consistent with their location in a hydrophobic environment within the folded protein. In contrast, the PPGP sequence appears to be exposed on the surface of the protein or as a linker between the membrane anchor and catalytic domain since the second Pro could be replaced by both hydrophobic and charged amino acids without loss of activity. The presence of Pro and Gly residues in this sequence suggests a loop or turn structure for this region, which might be required for the proper orientation of the catalytic cytoplasmic domain relative to the membrane anchor. However, while PG is a common

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a PP3P sequence in an interleukin-5 receptor showed that signal transduction mediated by this receptor required the first and last Pro, but not the second (22), exactly the requirements of the P450 2C2 PP3P sequence for activity. Interactions of a synthetic Pro-rich polypeptide, containing a PP3P sequence, with SH3 ligands were critically dependent on the two end position Pro. In addition, flanking sequences outside of the Pro-rich core increased binding affinity and specificity (23). The distance of the PP3P motif from these flanking amino acids was fixed so that PP3P motifs incorrectly spaced did not mediate high affinity binding. Similarly, in the present study, insertion of Ala to the C-terminal side of the PP3P sequence resulted in reduced activity suggesting that the position of the PP3P motif relative to a flanking sequence on the C-terminal side was important for its function.

The requirements for a PP3P motif are consistent with a helical structure for this sequence with three amino acids/helical turn as is present in right-handed 310 or left-handed PPII helices (24). The 310 helix is stabilized by hydrogen bonding between residues 1 and 4, which is prevented if these two residues are Pro as in PP3P (25). In contrast, the structures of Pro-rich polypeptides, with PP3P sequences, complexed with SH3 domains have been shown to be PPII helices (26), and a PPII helical structure is present in the HIV viral protein Nef before interaction with a SH3 domain (27). Thus, if the PP3P requirement reflects a helical structure for PP3P in P450 2C2, it is likely a left-handed PPII helix as illustrated in Fig. 5. Most of the PPII helices are a maximum of four residues, and this structure is compatible with immediate transitions to α-helical or β-sheet structures at either end (24). If PP3P assumes this structure, the side chains of Pro30 and Pro33 would point in the same direction and that of Pro31 would point 120° in the opposite direction (Fig. 5). The effects of the mutations indicate that the Pro31 helix face would be exposed to the aqueous environment while the Pro30 and Pro33 face would interact with or pack against other peptide structures (Fig. 5).

The functional significance of a PP3P requirement is not clear but could involve intra or interprotein interactions that are important for folding of newly synthesized P450, for heme incorporation, or for stability or catalytic activity of the folded protein. Mutations at the first or last Pro positions in the PP3P motif of P450 2C11 either alone or in combination with mutations at other Pro positions in the Pro-rich region resulted in mutant proteins that were expressed in yeast but were inactive (15). None of the proteins exhibited the characteristic reduced CO difference spectrum at 420 or 450 nm of the P450 heme-protein, indicating that heme was not incorporated into the protein, and it was concluded that these Pro residues were

## Table I

| Species                  | P450 | Sequence                  |
|--------------------------|------|---------------------------|
| Rabbit                   | 2C2  | QSHGGGKLPP3PFPFLGN        |
| Human                    | 1A1  | QVQKLKLPQPPGWLIGH         |
| Monkey                   | 2C20 | QSSGRKLPP3PFLIGN          |
| Guinea pig               | 1A1  | QVPKGLKPPGWLIGH           |
| Rat                      | 2E1  | QYNSWNLPP3PFLIGN          |
| Mouse                    | 2F2  | SGRKQLPQPPGWLIGH          |
| Hamster                  | 2A8  | ORRLLKMRPLPFLIGN          |
| Dog                      | 2B11 | HPKAYGLHP3PFLIGN          |
| Bovine                   | 2D14 | RSRNPRPP3PFLNGL          |
| Chicken                  | 2H1  | STSRKQKLPP3PFPFLG        |
| Trout                    | 2K1  | SSEEQQKPP3PFLNGL         |
| C. elegans               | CEL10E1 | LYYKPRNLPP3PFLPMGN      |
| Eggplant                 | 7TA1 | PKTKSPNLPP3PFP15VG       |
| Arabidopsis              | T14112 | LKPSWKLPP3PFLPFLTIN     |

## Table II

| Species                  | P450 | Sequence                  |
|--------------------------|------|---------------------------|
| Rabbit                   | 2C2  | QSHGGGKLPP3PFPFLGN        |
| Human                    | 1A1  | QVQKLKLPQPPGWLIGH         |
| Monkey                   | 2C20 | QSSGRKLPP3PFLIGN          |
| Guinea pig               | 1A1  | QVPKGLKPPGWLIGH           |
| Rat                      | 2E1  | QYNSWNLPP3PFLIGN          |
| Mouse                    | 2F2  | SGRKQLPQPPGWLIGH          |
| Hamster                  | 2A8  | ORRLLKMRPLPFLIGN          |
| Dog                      | 2B11 | HPKAYGLHP3PFLIGN          |
| Bovine                   | 2D14 | RSRNPRPP3PFLNGL          |
| Chicken                  | 2H1  | STSRKQKLPP3PFPFLG        |
| Trout                    | 2K1  | SSEEQQKPP3PFLNGL         |
| C. elegans               | CEL10E1 | LYYKPRNLPP3PFLPMGN      |
| Eggplant                 | 7TA1 | PKTKSPNLPP3PFP15VG       |
| Arabidopsis              | T14112 | LKPSWKLPP3PFLPFLTIN     |

Frequency of occurrence of the Pro-rich sequence in mammalian P450s from different families

One hundred and fifty-eight mammalian P450 sequences available from sequences compiled by D. R. Nelson at http://drnelson.utmem.edu/nelsonhomepage.html (3/29/95) were examined for the occurrence of Pro-rich sequences. The family 2 P450s are shown separately from other families to illustrate the absolute conservation of PP3P in this family and to remove the bias for this sequence overall since the family 2 proteins represent 50% of sequenced P450s in the 14 known mammalian families. PXXP indicates sequences with amino acids other than Pro and Gly in the second and third positions. ILPPG indicates sequences with Ile and Leu in the first position. APXX indicates sequences with amino acids other than Pro and Gly in the first and third position. XXXX indicates either no Pro-rich sequences or the absence of a motif matching those described above.

| Sequence | Family | Total |
|----------|--------|-------|
| PP3P     | 75     | 89    |
| XP3P     | 0      | 31    |
| XLP3P    | 0      | 26    |
| XP3XP    | 0      | 5     |
| XXXX     | 0      | 7     |
| Total    | 75     | 158   |
important in folding of the molecule. Further support for mal-
folding was the observation that the inactive proteins segre-
gated in yeast is not conclusive since folding and assembly in the envi-
ronment of the yeast cell may be different from that in the mammalian cell. Alternatively, the PXXP requirement could reflect the necessity of this sequence to pack against the P450 molecule, and mutations might affect the stability or local conformation of the folded protein. For example, according to the alignment by Hasemann et al. (8), the PXXP motif is located adjacent to the Aα-helix that contains amino acids that are part of a postulated site for the initial interaction of the protein with its substrate (29–31). Alteration of the PXXP motif could indirectly alter the position of the Aα-helix and affect substrate interaction. Additional experiments will be needed to distinguish definitively among the possible effects of these mutations.

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FIG. 5. The PPGP motif modeled as a left-handed PPII helix. A, structure with the axis of the helix parallel to the paper; B, structure with the helical axis nearly perpendicular to the paper. The gray and black shading are to aid in visual interpretation only. C, schematic representation of the PFP GP PPII helix. A PPII helical structure is consistent with the requirement for Pro at 30 and 33 and the tolerance for substitutions of amino acids, including acidic residues, at 31. The PPII helix is a left-handed helix with a repeat of three amino acids per turn and, thus, forms a triangular structure with side chains pointing in three directions as indicated by the arrows in panel B. The critical Pro30 and Pro33 are on one side of the helix and form a hydrophobic protrusion that might provide specificity or steric surfaces important for formation of functional P450. Alternatively, packing along the surface formed by Gly32, Pro30, and Pro33, as illustrated in panel C, might occur.