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Function and membrane topology of wild-type and mutated cytochrome *P*-450c21

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We have studied membrane topology of cytochrome *P*-450c21 (P450c21) using the approaches of mutagenesis and protease digestion. P450c21 is located at the cytoplasm with an N-terminal hydrophobic domain integrated into microsomal membranes. When this hydrophobic domain was replaced by a secretory signal peptide, P450c21 was translocated into the lumen and lost enzymic activity. No other topogenic sequence was detected in the bulk of the P450c21 peptide. A mutant protein with Pro-30 replaced by Leu (L30) corresponding to the mutation found in the diseased state was created. L30 protein lost 90% of enzymic activity, while a double mutant (L30R32) with an additional Leu-32 to Arg mutation had slightly higher residual enzymic activity. Apart from lower activity, L30 was also present in the cell at a lower level than wild-type P450c21. This lower level is probably due to increased degradation, as L30 is synthesized at a normal rate. Both L30 and L30R32 proteins, however, were integrated into membranes normally. Therefore the Pro-30 → Leu mutation did not affect membrane integration, but affected the abundance and enzymic activity of P450c21.

INTRODUCTION

Cytochromes *P*-450 are haem-containing proteins which absorb light at 450 nm upon binding to carbon monoxide in the reduced state [1]. This superfamily of proteins have close to 500 members from animals, plants, fungi and bacteria [2]. They carry out oxidation-reduction reactions during detoxification of xenobiotics, synthesis of steroids and flavonoids, metabolism of bile acid, prostaglandins and fatty acids, etc.

Bacterial cytochromes *P*-450 are usually soluble, whereas those found in eukaryotes are integrated into either mitochondria or the endoplasmic reticulum (ER). The mitochondrial cytochromes *P*-450 combine with NADPH-ferredoxin-dependent reductase and ferredoxin to form an electron transport chain, whereas the cytochromes *P*-450 in the ER use only NADPH-dependent reductase to transport electrons [3]. Because of their membrane-binding property, eukaryotic cytochromes *P*-450 have been difficult to purify and crystallize. This may explain why there has been no tertiary structural determination of any eukaryotic cytochrome *P*-450. The only available cytochrome *P*-450 structures, cytochrome *P*-450cam, cytochrome *P*-450terp, and cytochrome *P*-450BM-3, are of bacterial origin [4–6].

The structures of eukaryotic cytochromes *P*-450 have been studied largely by sequence alignment [7–9], antibody probing [10], protease digestion [11–13] and chemical modifications [14,15]. These data point to a hypothesis that all the microsomal cytochromes *P*-450 probably share similar three-dimensional structures with some variations. The microsomal cytochromes *P*-450 integrate into the ER membrane using the N-terminal hydrophobic sequence as the membrane targeting, anchoring and stop transfer signal [16,17]. The rest of the protein is located at the cytoplasmic side of the ER membrane [16,18,19].

Microsomal cytochrome *P*-450c21 (P450c21) catalyses the conversion of progesterone and 17α-hydroxyprogesterone into deoxycorticosterone and 11-deoxycortisol, two important steps in the synthesis of steroids [3]. Deficiency of this enzyme causes a common genetic disease, congenital adrenal hyperplasia, affecting about one in 15000 newborn babies with symptoms of virilization, infertility, and/or salt loss [20]. The *P450c21* gene is located at the short arm of human chromosome 6, with a pseudogene situated next to the active gene [21,22]. Frequent gene conversion changing part of the active *P450c21* into the neighbouring mutated pseudogene is the main cause of the disease [23].

Many missense mutations which lead to amino acid substitutions of P450c21 have been characterized [24–26]. These amino acid substitutions affect enzymic activity of P450c21 and the effects vary depending on the site of amino acid replacement [27–29]. The availability of natural mutations in the *P450c21* gene found in the disease offers clues to important residues which support the structure and function of the protein. Mutation of the proline residue to leucine at position 30 causes the non-classical form of P450c21 deficiency [29,30]. The mutant protein has lower enzymic activity and patients are usually presented with clitoromegaly [29,30]. In this report we studied the membrane topology of P450c21 and its Pro-30 → Leu mutant protein (L30) by site-directed mutagenesis, *in vitro* translation, and protease digestion. We showed that the Pro-30 → Leu mutation caused loss of enzymic activity but not membrane insertion. We also showed that this Pro-30 → Leu protein was less abundant in the cell. These results increased our understanding of the effect of mutations on production, processing and function of enzymes.

MATERIALS AND METHODS

Construction of plasmids

Plasmid pgh21 (pg2151) was constructed by fusing porcine growth hormone cDNA [31], encoding the signal peptide from amino acids −26 to +3, to truncated *P450c21* cDNA encoding amino acids 51–495. Each DNA fragment was generated by PCR with a *Hin*dIII site at the 3′- or 5′-end of the PCR products respectively. This *Hin*dIII site encodes two amino acids in frame at the fusion point. The final product, encoding $GH_{1-29}P450_{51-495}$, was cloned into a pBluescript-based under the control of the bacteriophage T3 promoter.

Abbreviations used: ER, endoplasmic reticulum; P450c21, cytochrome *P*-450c21; L30, mutant of P450c21 containing a Pro-30 to Leu substitution; L30R32, mutant of P450c21 containing a double mutation Pro-30 to Leu and Leu-32 to Arg.

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pBg2151Δ166–380 was constructed by digesting pgh21 with *Eco*RI and *Mst*II, followed by Klenow treatment and blunt-end ligation. The construction of the N-terminal deletion plasmids Δ4 to Δ23 and pYE-c21 have been described previously [19,32].

Mutagenesis of DNA

The L30 and L30R32 (a double mutation: Pro-30 → Leu and Leu-32 → Arg) mutants were generated by a two-step PCR procedure [33]. Two sets of primers, ClaI (31-mer, 5′-UT to nt 13, with *Cla*I site at initiation codon, sense, AAAGCTGCTG-CAGGATCGATGCTGCTCCTGG), L30R (GGCAAGAGG-CAGGAGGTGGAG, 21-mer, nt 82–102 with L30 mutation, antisense), L30 (CTCCACCTCCTGCCTCTTGCC, 21-mer, nt 82–102 with L30 mutation, sense) and 21E (AGGAGAGAGA-ATTCCTCCTCAA, 22-mer, nt 482–503, antisense) were used to amplify DNA segments encoding amino acids 1–30 and 30–168 separately. The PCR products then served as templates for the amplification of a DNA fragment encoding amino acids 1–168 using the two end primers (ClaI and 21E). This DNA was digested with *Eco*RI/*Cla*I and subcloned into p3′c21, a plasmid with the 3′-half of the *P450c21* cDNA cloned into a pKS-based vector, to form a full-length *P450c21* cDNA with the specific mutations in it. Two clones, pL30, which contains the Pro-30 to Leu mutation, and pL30R32, which has an additional fortuitous mutation replacing Leu-32 with Arg, were identified by sequencing. For expression in COS-1 cells, the cDNA was released from pL30, pL30R32 and pgh21 by *Bam*HI/*Xho*I digestion, and cloned into pCDBam [34] under the control of the simian virus 40 (SV40) early promoter and enhancer, to form pCDL30, pCDL32R32 and pCDgh21. For yeast expression, cDNA was released from pL30 by *Cla*I/*Not*I double digestion, followed by Klenow fill-in and blunt-end ligation into pYE8 at a blunt-ended *Eco*RI site. The resulting plasmid was named pYE-L30. The sequence of every plasmid was verified by sequencing through its junction points and complete sequencing of the portion derived from PCR amplification.

In vitro protein analysis

Plasmids were linearized with *Bam*HI and transcribed *in vitro* using T3 RNA polymerase to produce RNA for *in vitro* translation in rabbit reticulocyte lysate and dog pancreas microsome membranes (Promega, Madison, WI, U.S.A.). Translation products were extracted in 0.1 M sodium carbonate, pH 11.5, as described before [19] or treated with 0.1 mg/ml proteinase K in 10 mM Tris/HCl, pH 8.0, 10 mM $CaCl_2$, or 0.1 mg/ml Pronase in 50 mM Tris/HCl, pH 8.0, 10 mM $CaCl_2$, 150 mM NaCl at room temperature for 30 min unless otherwise stated. Digestion was stopped in PMSF (1 mg/ml) and gel loading buffer. For endoglycosidase F digestion, translation products were mixed with 1 μl of 2 % (w/v) SDS and boiled for 1 min, before the addition of 18 μl of 0.1 M sodium phosphate, pH 6.1, 50 mM EDTA, 1 % Nonidet P-40, 1 % 2-mercaptoethanol, 1 unit/ml endoglycosidase F followed by incubation at 37 °C overnight. All the proteases were purchased from Boehringer Mannheim except trypsin (Sigma) and endoglycosidase F (New England Nuclear).

In vivo protein analysis

Expression of P450c21 by transfection into COS-1 cells, determination of 21-hydroxylase activity and immunoblotting procedures have been described previously [34]. The preparations of the polyclonal antisera against P450c21 and heat shock protein 60 (hsp60) are described elsewhere [34,35].

Manipulation of yeast cells

Expression plasmids were transformed into yeast, expressed, and recombinant proteins analysed according to published procedures [32]. In brief, yeast cells were made competent using 0.1 M lithium acetate, mixed with 10 μg of plasmid DNA, and incubated for 30 min before treatment with 20 % poly(ethylene glycol) and heat shock at 42 °C for 5 min. Yeast cells harbouring recombinant plasmids were grown on minimal medium (SD) supplemented with all amino acids except tryptophan. For pulse-labelling experiments, yeast cells grown to $A = 0.6$ were labelled with 50 μCi/ml [^{35}S]methionine for 5 min in SD medium lacking tryptophan and methionine. After cells were broken with glass beads and vortexing, P450c21 protein was immunoprecipitated as described before [19,36].

RESULTS

Replacing the N-terminal domain of P450c21 with a secretory peptide

We used an *in vitro* transcription/translation/translocation system to investigate the topology of P450c21 relative to the membrane. The N-terminal hydrophobic sequence has been shown to be the membrane targeting and anchoring signal [18]. Besides this N-terminal membrane targeting signal, the possible existence of other topogenic signals in the rest of the P450c21 peptide was further investigated. Codons 1–51 of *P450c21* were replaced by a DNA segment encoding the secretory signal peptide (amino acids 1–29) of porcine growth hormone. When this fusion protein (gh21) was synthesized *in vitro* in the presence of membranes; an extra protein band with a slower mobility was detected in addition to the nascent peptide (lanes 3 and 5, Figure 1A). This band resisted proteinase K digestion, indicating that it was fully translocated into the lumenal side of the membrane and was protected from protease digestion (Figure 1A, lane 4). The bigger molecule returned to the original size after endoglycosidase

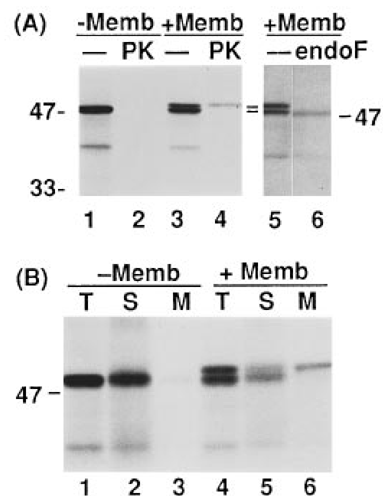

Figure 1 Membrane localization of the gh21 protein

The gh21 protein was translated *in vitro* in the absence or presence of membranes. (**A**) Proteinase K digestion. The translation products were treated with proteinase K (PK, lanes 2 and 4) or nothing (—, lanes 1 and 3) before gel electrophoresis. The total translation products (lane 5) were also treated with endoglycosidase F (endoF) before electrophoresis (lane 6). (**B**) Alkaline extraction. Total translation products (T) were extracted with alkaline sodium carbonate, followed by centrifugation to separate the membranous pellet fraction (M) and the soluble portion (S). Size markers are shown at the side of each gel.

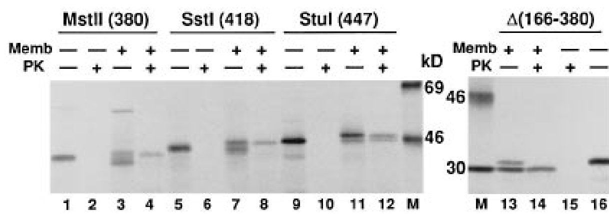

Figure 2 Translation of deletion clones of the gh21 protein

pgh21 was linearized by digestion with *Stu*I (447), *Sst*I (418), or *Mst*II (380), followed by *in vitro* transcription, and translation in the presence or absence of membranes. Proteinase K (PK) was added as indicated on top of each lane. pBg2151Δ(166–380) was also linearized before transcription and translation. The resulting protein products were separated on a 10% polyacrylamide gel.

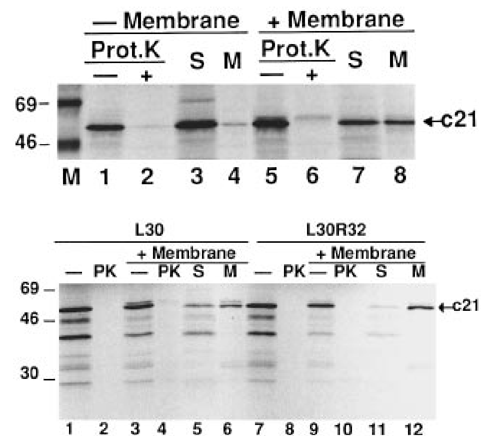

Figure 3 Membrane binding property of normal (upper panel) and L30 and L30R32 (lower panel) mutants of P450c21

Wild-type, L30 and L30R32 forms of the P450c21 (c21) RNA were translated in rabbit reticulocyte lysate in the absence or presence of dog pancreas microsomal membranes. The translation products were either digested with proteinase K (Prot.K or PK), or separated into the supernatant (S) and the membrane (M) portions after alkaline extraction and centrifugation. Lane M is size markers with the sizes shown in kDa. The full-length translation product is indicated by an arrow.

F digestion (lane 6). Since endoglycosidase F removes the carbohydrate moiety of the protein, this result showed that the increased size was due to glycosylation. P450c21 contains a putative glycosylation site with the sequence Asn-Ala-Thr at residues 346–348. This site is usually not used, as P450c21 normally faces the cytoplasm. Only when the bulk of the protein was flipped to the lumen under artificial conditions was the site used for glycosylation.

Figure 1(B) showed that while the nascent gh21 fusion protein remained soluble (lane 5), the translocated peptide was tightly associated with the membrane (lane 6).

Deletion analysis of the gh21 protein

Figure 1 showed that the body of the P450c21 peptide was fully translocated into the lumen after its N-terminal signal was replaced by a secretory peptide. To investigate this process further, we linearized the parent plasmid of gh21 by *Mst*II, *Sst*I or *Stu*I enzymes before *in vitro* transcription/translation. This procedure produced truncated proteins of 380, 418 and 447 amino acids respectively (Figure 2). When these peptides were synthesized in the absence of membranes, all of them were fully sensitive to proteinase K digestion as expected (Figure 2, lanes 2, 6 and 10). In the presence of membranes, in addition to the nascent peptide, one additional protein band with slower migration was observed in each case (Figure 2, lanes 3, 7 and 11). These larger protein bands were resistant to proteinase K digestion (lanes 4, 8 and 12), indicating that they were located in the lumen. The increased size was due to glycosylation, as endoglycosidase F treatment shifted these protein bands to the original size (results not shown). These results showed that the body of the P450c21 peptide was translocated to the lumen through the secretory signal at the N-terminus, and there was no other observable topogenic signal in the rest of the P450c21 polypeptide.

In addition to serial truncations from the C-terminal end, we also generated an internal deletion removing amino acids 166–380 of P450c21 from the gh21 fusion protein. In this construct, the putative glycosylation site located in residues 346–348 of P450c21 is no longer present and hence will not interfere with data interpretation. When synthesized in the presence of membranes, a mature protein (Figure 2, lane 13) with a size smaller than the nascent peptide (lane 16) was detected. This mature protein was resistant to proteinase K digestion, which is indicative of its luminal location. The reduced size of the mature peptide indicates that the secretory peptide of the fusion protein was cleaved during translocation. This pattern of protein translocation is typical of secretory proteins. The observation also suggested the lack of other topogenic sequences in the body of P450c21. This result is consistent with that of cytochrome *P*-450IIC2 [36] but is in contrast to that of cytochrome *P*-450b, whose hidden stop transfer signal at residues 167–185 could re-insert the protein back to the membrane under similar experimental conditions [16].

The L30 mutant of P450c21

When wild-type P450c21 was translated in reticulocyte lysate, it was integrated into membranes with the bulk of the protein located at the cytoplasmic side, as shown by its sensitivity to proteinase K digestion and sedimentation to the membrane portion after alkaline extraction (Figure 3, upper panel).

In addition to the wild-type P450c21, topologies of two mutant proteins were studied. One of them has a Pro-30 to Leu substitution (L30) and the other is a double mutant with an additional Leu-32 to Arg (L30R32) mutation. Pro-30 is located immediately following the N-terminal membrane targeting signal and is conserved among many microsomal cytochromes *P*-450. Figure 3 (lower panel) showed that translation products of L30 and L30R32 were sensitive to proteinase K digestion, demonstrating that both proteins were located at the cytoplasmic side of the membrane. The majority of the full-length 50 kDa translation products sedimented to the membranous pellet fractions after alkaline extraction and centrifugation, indicating their tight association with the microsomal membrane. The bands remaining in the soluble fractions represented minor untranslocated polypeptides. This result showed that substitution at Pro-30 did not appear to affect the integration of P450c21 into membranes. Both L30 and L30R32 mutant proteins have the same overall membrane topology as the wild-type P450c21, which is integrated into the microsomal membrane from the cytoplasmic side.

Function of wild-type and mutant protein

The effect of Pro-30 substitution on the function of protein, in

Table 1 Activity of L30, L30R32, gh21 and normal 21-hydroxylase

After transfection of COS-1 cells with pCDL30, pCDL30R32, pCDgh21, or pCD21 together with pRSV-β-gal as an internal control, 17α-hydroxy[^{14}C]progesterone was added to the cell culture and incubated for 1 h. Product conversion was calculated after separating the substrate and product by TLC. 21-Hydroxylase activity relative to that of the wild type was calculated after normalization with β-galactosidase activity. The mean values from six independent transfections were shown with standard deviations.

Plasmid	Activity (%)
pCD21	100.0 ± 0.0
pCDL30	11.2 ± 3.5
pCDL30R32	30.0 ± 9.0
pCDgh21	1.0 ± 1.9

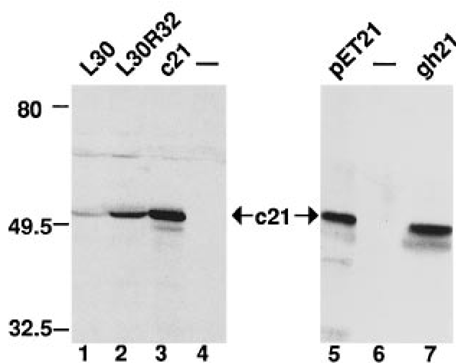

Figure 4 Analysis of the amount of mutant and normal proteins by immunoblot

After transfection of plasmids encoding wild-type P450c21 (c21) (lane 3), L30 (lane 1), L30R32 (lane 2), gh21 (lane 7), or nothing (lanes 4 and 6) into COS-1 cells, one-third of the cell extract was electrophoresed, transferred to nitrocellulose membrane, and reacted with anti-P450c21 sera. The protein bands were detected by ^{35}S-Protein A. An aliquot of the bacterially expressed c21 protein encoded by pET21 [34] was loaded in lane 5 as a control.

addition to membrane topology, was tested (Table 1). Mutant plasmids were recloned into a mammalian expression vector followed by co-transfection into COS-1 cells with a plasmid RSV-β-gal as an internal control. After normalization of transfection efficiency with β-galactosidase activity used as an internal control, L30 protein had about 11 % of wild-type activity. The double mutant L30R32, although enzymically less active than the wild-type protein, had twice the residual activity (about 23 %) of L30. The fusion protein gh21 had no activity at all, suggesting that this protein cannot perform its enzymic function *in vivo*.

The amount of P450c21 expression in COS-1 cells was examined by immunoblotting using polyclonal antisera directed against P450c21. This polyclonal antibody detected epitopes mostly in the central and C-terminal portions of P450c21 and therefore should react well with both wild-type P450c21 and its L30 mutant protein [37]. The same amounts of total proteins were loaded into each lane, while the result was calibrated after quantification with β-galactosidase activity derived from the co-transfected plasmid (Figure 4). In control transfection without

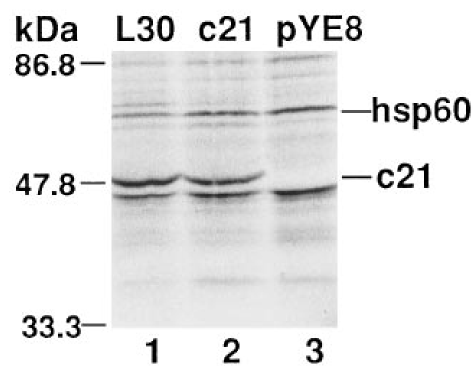

Figure 5 Rate of synthesis of wild-type and L30 mutant of P450c21

Yeast strains harbouring wild-type P450c21 (c21), L30 mutant, or the vector pYE8 alone were pulse-labelled with [^{35}S]methionine for 5 min in SD medium without methionine and tryptophan. After cells were broken by vortexing in the presence of glass beads, proteins in the cell lysate were immunoprecipitated with antisera against P450c21 and hsp60 followed by separation by PAGE and autoradiography. Protein bands corresponding to hsp60 and P450c21 are marked.

plasmid DNA, no P450c21 protein was detected (Figure 4, lanes 4 and 6). Ample amounts of the P450c21 protein were found in cells transfected with wild-type P450c21 (Figure 4, lane 3) or gh21 (Figure 4, lane 7). The abundance of gh21 protein indicated that this protein is very stable inside the ER lumen. L30R32 protein was present in a moderate amount, while a lower amount of the L30 protein was detected. The same result was confirmed when wild-type P450c21 and L30 mutant proteins were expressed from yeast (results not shown).

The lower amount of L30 protein could be due to either decreased translation or increased degradation. To test for synthesis rate of proteins, yeast cells harbouring either wild-type P450c21 or the L30 protein were pulse-labelled for 5 min. The amount of newly synthesized P450c21 was detected by immunoprecipitation followed by autoradiography (Figure 5). Antiserum against hsp60 [35] was used as an internal control to calibrate for the amount of protein loaded in each lane. It shows that wild-type P450c21 and L30 were synthesized at the same rate. This result is expected because both proteins were expressed from the same vector. It also implies that the lower amount of the L30 protein is probably due to increased degradation in the cell.

DISCUSSION

In this report we describe the use of mutagenesis and protease digestion to probe the topology of P450c21. All microsomal cytochromes *P*-450 have an N-terminal hydrophobic domain followed by positively charged amino acids serving as a membrane targeting and anchoring signal (Figure 6). This N-terminal hydrophobic segment is also sufficient for retention of cytochromes *P*-450 in the endoplasmic reticulum [38]. We searched for other topogenic sequences in P450c21 but found none. This is probably a common situation for microsomal cytochromes *P*-450, as cytochrome *P*-450IIC2 also lacks any additional topogenic sequence [35].

We studied the membrane topology and function of P450c21 with a Pro-30 to Leu substitution. The Pro-30 to Leu mutation was identified in patients suffering from the non-classical type of P450c21 deficiency [29,39]. The lower activity of L30 is consistent with the phenotype observed in the non-classical type of the disease due to P450c21 deficiency. We showed that the L30 and L30R32 mutant proteins were integrated into membranes with

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21A2 human        MLLLGLLLLLPLLAGARLLWNWWKLRSLHL PP LA P G
17   human           MWELVALLLLTLAYLFWPKRRC P GAKY P KSLLSL P
7    human       MMTTSLIWGIAIAACCCLWLLLGIRRRQTGE PP LENGLI P
2B4  rabbit       MEPSILLLLALLVGFLLLLVRGHPKSRGNF PP G P R P L P LL
2A1  rat        MLDTGLLLVVILASLSVMLLVSLWQQKIRGRL PP G P T P L P
2C11 rat            MDPVLVLVLTLSSLLLLSLWRQSFGRGKL PP G P T P L P
2D6  human     MGLEALVPLAVIVAIFLLLVDLMHRRQRWAARY PP G P L P L P
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Figure 6 Alignment of cytochrome *P*-450 sequences at the N-terminus

Various microsomal cytochromes *P*-450 from different species are aligned at the N-terminus. The N-terminal hydrophobic sequences are underlined. The charged residues are italicized and underlined. The conserved Pro residues are boxed.

the bulk of the protein at the cytoplasm, a situation similar to wild-type P450c21. Since the N-terminal membrane targeting and anchoring signal is intact in both L30 and L30R32 mutants, it is reasonable that both mutant proteins have normal membrane integration.

Pro-30 to Leu mutation, however, may affect the structure of the protein in a subtle way. As Pro-30 is part of the Pro-rich sequence immediately following the N-terminal hydrophobic domain and positive charges of microsomal cytochromes *P*-450 (Figure 6), it was proposed that these Pro residues may form a hinge between the transmembrane and the following domains [40,41]. Replacing one Pro residue by Leu has a deleterious effect on the structure and function of the protein, as shown by the decrease in enzymic activity. Another natural mutation, Pro-34 to Ser substitution of cytochrome *P*-4502D6, similarly abolished its bufuralol-hydroxylation activity [42]. The L30R32 mutant, which has an additional charged residue at Arg-32, had higher residual activity than L30, probably because the Arg-32 residue was more tolerable than Leu at this place, although this mutant still had much lower enzymic activity than the wild-type protein.

Another indicator of the perturbed structure of the L30 mutant was its reduced steady-state level. Usually denatured proteins are more prone to degradation [43,44]. Since the mutant and the wild-type P450c21 were synthesized off the same promoter from the same plasmid vector, they probably are synthesized at the same rate. The lower abundance of the L30 protein in the cell may reflect its faster degradation probably because of its structural disturbance.

During our study, we created a gh21 protein which contains the bulk of P450c21 protein fused to the secretory peptide of growth hormone. This fusion protein resides in the lumen as shown by the *in vitro* translation/translocation assay. The gh21 protein is tightly integrated into the membrane. How this protein is attached to the membranes is not clear. Although containing the bulk of the P450c21 peptide, the gh21 protein lacks enzymic activity, probably because the electrons are transported through NADPH reductase only from the cytoplasmic side of the membrane [45,46]. It is also possible that the protein has a wrong conformation after translocation into the lumen, as haem is not available from the lumenal side of the membrane. Without haem incorporation, gh21 protein is probably folded in an incorrect conformation to exert its enzymic activity. This gh21 protein, however, is very stable as evidenced by its high level in the cell.

In summary, the Pro-30 to Leu mutation did not seem to affect the integration of P450c21 into membranes. It does, however, cause a decrease in enzymic activity and lower abundance in the cell. This decreased abundance and activity underlie the basis of the non-classical form of P450c21 deficiency.

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REFERENCES

1 Omura, T. and Sato, R. (1964) J. Biol. Chem. **239**, 2370–2378
2 Nelson, D. R., Kamataki, T., Waxman, D. J., Guengerich, P., Estabrook, R. W., Feyereisen, R., Gonzalez, F. J., Coon, M. J., Gunsalus, I. C., Gotoh, O., Okuda, K. and Nebert, D. W. (1993) DNA Cell Biol. **12**, 1–51
3 Miller, W. L. (1988) Endocr. Rev. **9**, 295–318
4 Hasemann, C., Ravichandran, K., Peterson, J. and Deisenhofer, J. (1994) J. Mol. Biol. **236**, 1169–1185
5 Poulos, T. L., Finzel, B. C. and Howard, A. J. (1987) J. Mol. Biol. **195**, 687–700
6 Ravichandran, K. G., Boddupalli, S. S., Hasemann, C. A., Peterson, J. A. and Deisenhofer, J. (1993) Science **261**, 731–736
7 Nelson, D. R. and Strobel, H. W. (1988) J. Biol. Chem. **263**, 6038–6050
8 Nelson, D. R. and Strobel, H. W. (1989) Biochemistry **28**, 656–660
9 Edwards, R. J., Murray, B. P., Boobis, A. R. and Davis, D. S. (1989) Biochemistry **28**, 3762–3770
10 De Lemos-Chiarandini, C., Frey, A. B., Sabatini, D. D. and Kreibich, G. (1987) J. Cell Biol. **104**, 209–219
11 Churchill, P. F. and Kimura, T. (1979) J. Biol. Chem. **254**, 10443–10448
12 Brown, C. A. and Black, S. D. (1989) J. Biol. Chem. **264**, 4442–4449
13 Tsokos, D. C., Omata, Y., Robinson, R. C., Krutzsch, H. C., Gelboin, H. V. and Friedman, F. K. (1992) Biochemistry **31**, 7155–7159
14 Jänig, G.-R., Kraft, R., Blanck, J., Ristau, O., Rabe, H. and Ruckpaul, K. (1987) Biochim. Biophys. Acta **916**, 512–523
15 Bernhardt, R., Makower, A., Jänig, G.-R. and Ruckpaul, K. (1984) Biochim. Biophys. Acta **785**, 186–190
16 Monier, S., Van Luc, P., Kreibich, G., Sabatini, D. D. and Adesnik, M. (1988) J. Cell Biol. **107**, 457–470
17 Szczesna-Skorupa, E. and Kemper, B. (1993) J. Biol. Chem. **268**, 1757–1762
18 Sakaguchi, M., Mihara, K. and Sato, R. (1987) EMBO J. **6**, 2425–2431
19 Hsu, L.-C., Hu, M.-C., Cheng, H.-C., Lu, J.-C. and Chung, B.-c. (1993) J. Biol. Chem. **268**, 14682–14686
20 Miller, W. L. and Levine, L. S. (1988) J. Pediatr. **111**, 1–17
21 White, P. C., Grossberge, R., Onuffer, B. J., Chaplin, D. D., New, M. I., Dupont, B. and Strominger, J. L. (1985) Proc. Natl. Acad. Sci. U.S.A. **82**, 1089–1093
22 Higashi, Y., Yoshioka, H., Yamane, M., Gotoh, O. and Fuji-Kuriyama, Y. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 2841–2845
23 Miller, W. L. and Morel, Y. (1989) Annu. Rev. Genet. **23**, 371–393
24 Amor, M., Parker, K. L., Globerman, H., New, M. I. and White, P. C. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 1600–1604
25 Speiser, P. W., New, M. I. and White, P. C. (1988) N. Engl. J. Med. **319**, 19–23
26 Owerbach, D., Crawford, Y. M. and Draznin, M. B. (1990) Mol. Endocrinol. **4**, 125–131
27 Chiou, S.-H., Hu, M.-C. and Chung, B.-c. (1990) J. Biol. Chem. **265**, 3549–3552
28 Helmberg, A., Tusie-Luna, M.-T., Tabarelli, M., Kofler, R. and White, P. C. (1992) Mol. Endocrinol. **6**, 1318–1322
29 Tusie-Luna, M. T., Speiser, P. W., Dumic, M., New, M. I. and White, P. C. (1991) Mol. Endocrinol. **5**, 685–692
30 Owerbach, D., Sherman, L., Ballard, A.-L. and Azziz, R. (1992) Mol. Endocrinol. **6**, 1211–1215
31 Jou, Y. S. and Chang, W. C. (1986) ICSU Short Reports **6**, 176–177
32 Wu, D.-A., Hu, M.-C. and Chung, B.-c. (1991) DNA Cell Biol. **10**, 201–209
33 Ho, S. N., Hunt, H. D., Horton, R. M., Pullen, J. K. and Pease, L. R. (1989) Gene **77**, 51–59
34 Hu, M.-C. and Chung, B.-c. (1990) Mol. Endocrinol. **4**, 893–898
35 Szczesna-Skorupa, E. and Kemper, B. (1989) J. Cell Biol. **108**, 1237–1243
36 Hsu, L.-C., Hsu, N.-C., Guzova, J. A., Guzov, V. M., Chang, S.-F. and Chung, B.-c. (1996) J. Biol. Chem. **271**, 3306–3310
37 Wedlock, M., Asawa, T., Baumann-Antczak, A., Smith, B. R. and Furmaniak, J. (1993) FEBS Lett. **332**, 123–126
38 Murakami, K., Mihara, K. and Omura, T. (1994) J. Biochem. (Tokyo) **116**, 164–175
39 Chung, B.-c., Hu, M.-C., Guzov, V. M. and Wu, D.-A. (1995) Endocr. Res. **21**, 343–352
40 Yamazaki, S., Sato, K., Suhara, K., Sakaguchi, M., Mihara, K. and Omura, T. (1993) J. Biochem. (Tokyo) **114**, 652–657
41 Szczesna-Skorupa, E., Straub, P. and Kemper, B. (1993) Arch. Biochem. Biophys. **304**, 170–175
42 Kagimoto, M., Heim, M., Kagimoto, K., Zeugin, T. and Meyer, U. A. (1990) J. Biol. Chem. **265**, 17209–17214
43 Pakula, A. A., Young, V. B. and Sauer, R. T. (1986) Proc. Natl. Acad. Sci. U.S.A. **83**, 8829–8833
44 Pakula, A. A. and Sauer, R. T. (1989) Annu. Rev. Genet. **23**, 289–310
45 Morimoto, T., Matsuura, S., Sasaki, S., Tashiro, Y. and Omura, T. (1976) J. Cell Biol. **68**, 189–201
46 Cooper, M. B., Craft, J. A., Estall, M. R. and Rabin, B. R. (1980) Biochem. J. **190**, 737–746