The Common I172N Mutation Causes Conformational Change of Cytochrome P450c21 Revealed by Systematic Mutation, Kinetic, and Structural Studies*

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We have investigated the structure and function of P450c21 with regard to a conserved site around Ile-172 by site-directed mutagenesis making single amino acid substitutions of residues 169–173. Substitutions of Ile-171 and -172 resulted in production of mutant proteins with dramatic reductions in enzymatic activities, indicating the importance of these two residues in maintaining the structure and function of P450c21. The I172N protein was present at a slightly lower level, due to a decreased rate of protein synthesis. The I172N apoprotein was synthesized at the normal rate, but its heme-bound P450 form was present at a much lower level. This I172N protein was tightly integrated into the membrane of endoplasmic reticulum, similar to the wild type P450c21, as shown by immunofluorescence detection, alkaline extraction, and cellular fractionation. Kinetic studies indicated that I172N had a lower Vmax value. In addition, the I172N protein was more sensitive to protease K digestion, indicating a possible alteration of conformation. This conformational change may result in the lower yield of the I172N hemoprotein and the reduced catalytic activity.

Cytochrome P450 is a protein superfamily of more than 200 members (1). Every member in the superfamily binds heme molecules at their active site to carry out mixed-function monooxygenation reactions at the end of the electron transport chain (2, 3). These proteins are termed cytochromes P450, because the reduced heme in the protein shows characteristic absorption at 450 nm upon binding to carbon monoxide (4).

In contrast to the bacterial P450s, eukaryotic P450s are associated with the membrane of the endoplasmic reticulum (ER) or mitochondria. The topology of P450s relative to the membrane has not been elucidated due to a lack of knowledge about the three-dimensional structure. It is generally believed that the bulk of the P450s have a structure similar to that of the bacterial P450s, with an N-terminal hydrophobic domain integrated into the membrane. This N-terminal hydrophobic domain not only serves as the signal for ER membrane targeting and integration (5, 6), it also participates in the determination of the overall structure and stability of P450c21 (7). The rest of the polypeptide probably forms a globular structure located at the cytoplasmic phase of the membrane (8–11), although a part of the polypeptide might interact with the membrane (8).

Cytochrome P450c21 as a microsomal protein is integrated into the membrane of the smooth ER. It catalyzes the conversion of progesterone and 17-hydroxyprogesterone (17-OHP) into deoxycorticosterone and 11-deoxycortisol, two essential steps of steroid hormone synthesis (3). Its deficiency is the main cause of congenital adrenal hyperplasia, due to decreased cortisol synthesis, leading to virilization and sometimes salt loss. 21-Hydroxylase deficiency is mainly caused by mutations of the CYP21A2 (c21B) gene that encodes P450c21 (12, 13). c21B and its neighboring CYP21A1P (c21A) genes are more than 98% identical in sequence, but the c21A gene does not encode an active protein due to multiple mutations throughout the gene (14, 15). Because of their proximity, these two genes exchange their sequences frequently through gene conversion events. This gene conversion is the main cause for the mutations found in the c21B gene (16) and can be used as the basis for the detection of known mutations in patients (17).

The loss of enzymatic activity as a result of mutations of the c21B gene is shown by the expression of mutant proteins corresponding to each mutation in various cell types in mammalian (18), vaccinia (19), or yeast vectors (20). Some mutations cause the complete loss of enzymatic activity, resulting in the severe salt-wasting type of the disease. Other mutations have less deleterious effects, resulting in mutant proteins with some residual activities, causing the milder form of the disease (21–22). The availability of the expression systems enables the examination of parameters affecting the structure and function of wild type and mutated cytochromes P450. One common mutation is the Ile-172 to Asn substitution (23, 24). This mutation causes 100-fold loss of enzymatic activity resulting in the simple virilizing form of the disease (24). In this report, we investigated the structural perturbation caused by the substitution. Despite the 100-fold loss of enzymatic activity causing the simple virilizing form of the disease, the Ile-172 mutation did not grossly affect the integration of the protein into the endoplasmic reticulum. Instead, the mutant protein became more sensitive to protease K digestion. This result indicated that the mutant protein had an altered conformation.

EXPERIMENTAL PROCEDURES

Plasmid Construction—Site-directed mutagenesis of the P450c21 cDNA cloned in M13 using a kit was performed as described previously.
RESULTS

Rationale for Site-directed Mutagenesis of P450c21 at Residues 169–173—To understand the functional importance of the domain surrounding the site of the common Ile-172 to Asn mutation of P450c21, sequence alignment of many microsomal P450s was made (Fig. 1). Ile-172 is invariant, implying its functional importance. Site-directed mutagenesis was performed substituting Ile-172 with either hydrophobic (Leu, Val) or polar (Gln, His) amino acids. Residue 171 is either Ile or Val. It was replaced by Val as found in some P450s or Asn to study the degree of changes that can be tolerated. Other residues in the vicinity are more variable, either hydrophobic or polar. Conservative changes replacing Cys-169 by Thr, Ser-170 by Thr, and Cys-173 by Ser were made separately to avoid dramatic changes of the protein structure. All mutant proteins were expressed in yeast and the levels of protein production, enzymatic activities, and kinetic properties were assayed individually.

Amount of Mutant Proteins—The steady state levels of wild type and mutant P450c21 protein in the cells were detected by immunoblotting (Fig. 2). Antiserum against yeast hsp60 protein was added at the same time to serve as an internal control for the amount of protein loading in each well (32). Most of the mutant proteins were present at levels similar to that of the wild type P450c21, except the I171N mutant protein, which appeared to be present at a lower amount in many independent experiments. Reduced amounts of proteins in the cell usually indicate that either the synthesis of the protein is decreased or that the newly synthesized protein is very rapidly degraded.

To determine synthetic rates of proteins, mutant proteins were pulse-labeled with [35S]Met for 5 min followed by immunoprecipitation. Fig. 3 showed that most of the mutant proteins were synthesized at normal rate except the I171N mutant. Scanning of protein intensities from four separate experiments showed that the I171N protein was produced at 70 ± 16% of the normal rate.

Function and Kinetic Properties of Mutant Proteins—The enzymatic activities of wild type and mutant proteins toward both substrates, progesterone and 17-OHP, were assayed. As shown in Fig. 4A, most substitutions did not greatly affect the enzymatic activity of P450c21, except the mutations changing the conserved residues at 171 and 172 from Ile to Asn. When replacing Ile-172 with four other amino acids, the resulting enzymatic activities were all less than 5% of wild type (Fig. 4B). The enzymatic activities for both substrates paralleled each other, indicating that there is no preferential loss of utilization of one particular substrate. The extremely low activity of all 172 mutants expressed in yeast was also observed in the proteins expressed in COS-1 cells (18), indicating the requirement for Ile at this conserved site.

The kinetic properties of the mutated and wild type enzymes in the yeast microsomes were determined further (Table I). Some P420 forms, which usually represent denatured proteins with altered interaction with heme (30), were associated especially with the mutant protein during purification after cells were broken. Similar to other P450s like P450cam (33) and P450c11 (34), adding the substrate 17-OHP during purification stabilized P450c21 and increased yield of the P450 form for
We obtained similar amounts of P450s (about 2 nmol/liter) for most of the mutant forms, except the I171N and I172N mutant proteins. The yield for the I171N mutant (1.26 nmol/liter) was about 50% of the wild type protein (Table I), consistent with the decreased abundance of the apoprotein in the cell. The yield for the I172N P450 (0.29 nmol/liter) was about 10% of that of the wild type. Since P450 content is a measure of the amount of hemoprotein (4), it indicated that there is less hemoprotein although the I172N apoprotein was produced at the normal amount (Fig. 2).

The $K_m$ values of all mutants were similar to that of the wild type protein with a slight elevation of the I171N protein (Table I). This result indicates similar affinity of the mutant proteins toward the substrate. As the I172N mutation does not drastically affect the ability of the protein to bind substrate, the Ile-172 residue of P450c21 does not seem to be involved directly in substrate binding. The $V_{\text{max}}$ value of the I172N mutant protein, however, was decreased to about 1/6 to 1/10 that of the normal protein when equal amounts of the P450 were compared. This together with about 10% yield of the I172N P450 results in overall low enzymatic activity.

The I172N Mutant Protein Integrates into the Membrane of ER Normally—

The cause of the reduced overall enzymatic activity created by the I172N mutation was investigated further. Amino acids 167–185 of P450b (CYP2B1) could serve as a stop transfer signal and membrane anchoring domain under a certain experimental condition, implying the ability of this motif to traverse the membrane (6). There was also the notion that the Ile-172 to Asn mutation might affect the ability of P450c21 to localize in the ER (19). We therefore tested whether the I172N protein is impaired in ER targeting. Immunofluorescence detection, either under the conventional light microscope or a confocal microscope, showed that the wild type P450c21 and the I172N protein expressed in Rat-1 cells were both distributed in the perinuclear space in a ladelike structure typical of ER location (Fig. 5). Therefore, ER localization did not seem to be affected by the mutation.

In addition to cytological examination, we also used a biochemical method to examine cellular localization of the I172N mutant protein. Cellular proteins were fractionated into the microsomal and soluble portions. As shown in Fig. 6A, wild type P450c21 sedimented in the pellet portion, indicative of its membrane association. Likewise, the I172N protein was also fractionated into the pellet portion. Therefore, the I172N mutant was associated with the membrane as well. There was no immunoreactivity from the yeast strain harboring vector pYE8 only.

### Table I

| Mutant | P450 yield | $K_m$  | $V_{\text{max}}$ |
|--------|------------|-------|-----------------|
| WT     | 2.43       | 0.15 ± 0.08 | 645 ± 77 |
| C169S  | 1.99       | 0.08 ± 0.02 | 579 ± 124 |
| S170T  | 2.16       | 0.09 ± 0.03 | 402 ± 25 |
| I171N  | 1.26       | 0.59 ± 0.31 | 436 ± 197 |
| I172N  | 0.29       | 0.12 ± 0.03 | 158 ± 31 |
| C173S  | 2.17       | 0.10 ± 0.04 | 743 ± 202 |

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**Fig. 3.** Amounts of wild type and mutant P450c21 proteins in yeast detected by immunoprecipitation. Yeast cells harboring vector pYE8, wild type (WT), or mutant P450c21 cDNA plasmid as indicated on top of each lane were labeled with $^{35}$S]Met for 5 min, lysed, and immunoprecipitated with anti-P450c21 and anti-hsp60 antisera. The precipitated proteins were analyzed by gel electrophoresis. The bands corresponding to P450c21 and hsp60 are marked.

**Fig. 4.** Activities of P450c21 and mutant proteins expressed in yeast cells. A, serial mutations. B, mutations at 171 and 172. Yeast cells containing P450c21 and mutant proteins were incubated with $^{14}$C-labeled 17-OHP for 30 min or progesterone (Prog) for 2 h. The amount of enzymatic activity of each mutant relative to that of the wild type P450c21 is shown.

**Fig. 5.** Cellular location of wild type P450c21 and its I172N mutant protein by immunofluorescence detection. Rat-1 cells expressing P450c21 or the I172N mutant were incubated with anti-P450c21 antibodies followed by fluorescein isothiocyanate-conjugated anti-rabbit IgG before observing the immunofluorescence under the microscope.

**Fig. 6.**
Some peripheral membrane proteins can associate with the membrane not by direct membrane integration, but by charge interaction. These proteins can be extracted into the soluble membrane not by direct membrane integration, but by charge proteins from yeast cells harboring wild type P450c21 (15). Assay. Plasmids harboring wild type (WT-c21), I172N, or truncated soluble portions (Δ52) forms of P450c21 cDNA were transcribed and translated in vitro in the presence of membranes. The total translation products (T) were extracted with alkali and centrifuged to separate into the supernatant (S) and pellet (P) forms. The full-length translation products are indicated by arrows.

FIG. 6. Membrane association of P450c21. A, fractionation. Yeast microsomes from yeast cells harboring wild type P450c21 (15 μg) or its I172N mutant (60 μg) were fractionated into the particulate (P) and soluble portions (S) as described under "Experimental Procedures." Electrophoresed, and reacted with anti-P450c21 antisera. Lane 1 contains lysate from cells harboring vector pYE8. B, membrane integration assay. Plasmids harboring wild type (WT-c21), I172N, or truncated (Δ52) forms of P450c21 cDNA were transcribed and translated in vitro in the presence of membranes. The total translation products (T) were extracted with alkali and centrifuged to separate into the supernatant (S) and pellet (P) forms. The full-length translation products are indicated by arrows.

Proteinase K Digestion Showed Altered Conformation of the I172N Mutant Protein—To probe the structure of mutant P450s, we digested P450c21 from yeast microsomes with increasing concentrations of proteinase K (Fig. 7). Wild type P450c21 was first digested into three major fragments at low proteinase K concentration (0.2 to 0.4 mg/ml). Then it was completely degraded at higher proteinase K concentration (1 mg/ml). This result is similar to that obtained from digesting purified bovine P450c21 from lipid vesicles by trypsin (35, 36). It indicated that wild type P450c21 was folded into a structure with limited protease entry sites at its surface. Initial digestion at the surface of the protein generates a few proteolytic fragments, which were then further digested to completion after the disruption of the tertiary structure of the protein.

The I172N mutant protein isolated from yeast microsomes was present at a slightly lower amount (lane 6, Fig. 7). This protein was more sensitive to proteinase K digestion. It was digested to completion at a low concentration (0.2 mg/ml) when proteinase K just began to digest the wild type P450c21 into three fragments. The lack of visible bands in lane 8 of Fig. 7 was not due to the lower level of the I172N protein, as overexposure of the gel did not show any band. The complete sensitivity of the I172N protein toward proteinase K digestion indicated that it was partially unfolded so as to allow penetration of the protease into the protein. The I172N mutant expressed in COS-1 cells was also more sensitive to trypsin digestion than the wild type P450c21 (data not shown). These results indicate that the I172N mutant protein had partially unfolded conformation which could be detected by higher sensitivity toward proteinase K digestion, irrespective of the cell types which expressed the proteins.

Fig. 7. Proteinase K digestion of wild type P450c21 and its I172N mutant expressed in yeast. Yeast microsomes (120 μg of proteins) containing either wild type P450c21 or the I172N mutant protein was digested with various concentrations of proteinase K (from 0.02 to 1 mg/ml) as indicated on top of each lane at room temperature for 30 min. The digestion products were separated by gel electrophoresis before immunoblotting.
The combined effects of lower hemoprotein content and reduced catalytic function resulted in a defective protein with about 100-fold reduction in enzymatic activity.

There has been an earlier suggestion that the I172N mutant protein might be impaired in ER targeting (19). We showed that both normal P450c21 and its I172N mutant are localized to the ER membrane by a combination of methods including immunofluorescence (Fig. 5) and cellular fractionation (Fig. 6). This result is expected since the N-terminal membrane-targeting and anchoring domain of the I172N mutant is intact. There is, however, speculation that the Ile-172 motif might be associated with the ER membrane, probably not by spanning the membrane but by forming a loop structure (8). Our data do not rule out the possibility that the Ile-172 motif might be involved in other types of membrane interaction. In addition to spanning the membrane by an α-helical domain, a protein can be associated with the lipid bilayer by direct fatty acylation (41, 42), through a glycosylphosphatidylinositol anchor (43), or by noncovalent interaction with other membrane proteins. The exact nature of the interaction of the microsomal P450s with the ER membrane besides the N-terminal crossing is not known yet and should be investigated further.

A different mutant protein, I171N, also had lower enzymatic activity. This protein had a lower steady state level (Fig. 2), which was probably due to its lower synthetic rate (Fig. 3). This result is expected since the N-terminal membrane-targeting domain of the I171N mutant is intact. There has been an earlier suggestion that the I172N mutant is associated with the lipid bilayer by direct fatty acylation (41, 42), through a glycosylphosphatidylinositol anchor (43), or by noncovalent interaction with other membrane proteins. The exact nature of the interaction of the microsomal P450s with the ER membrane besides the N-terminal crossing is not known yet and should be investigated further.

We used mammalian and yeast cells as two separate expression systems to obtain wild type P450c21 and mutant proteins for our study. Most of the kinetic parameters presented in this report were obtained from proteins expressed in yeast, as yeast cells are easier to grow and higher amounts of P450c21 can be obtained more easily for the study. Some of the data were also confirmed using mammalian expression systems. Since similar results were obtained regarding the structure and function of the protein in spite of the expression system, different expression systems provide a cross-check for the experimental results. We are certain we were measuring the property of the protein, not any artifact resulting from the expression system.

To probe the structure of P450c21, we investigated the differential sensitivity of the wild type and mutant proteins toward proteinase K digestion. Proteinase digestion has been widely used to study the conformational change of a protein (44–46). It, however, does not provide detailed structural information of the protein, which can be obtained only through crystallization and x-ray diffraction. What are the specific structural changes caused by a single substitution? How do these structural changes affect heme-binding capacity and catalytic activity? These are important questions which await further investigation.

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