Alteration of High and Low Spin Equilibrium by a Single Mutation of Amino Acid 209 in Mouse Cytochromes P450*

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The identities of the amino acid at position 209 are most critical in determining specific coumarin 7- and steroid 15α-hydroxylase activity in P450coh and P45015α, respectively. This system, therefore, provides us with an excellent model to study the structural basis for P450 specificity as a monooxygenase. We expressed in Saccharomyces cerevisiae a series of the mutated P450s in which residue 209 was substituted with various amino acids and characterized the spectral property and hydroxylase activity of these mutated P450s. The positioning of a hydrophobic residue including Phe, Leu, and Val at position 209 resulted in shifting the P450 to the high-spin state, while a charged amino acid such as Lys or Asp produced the low-spin form. Moreover, a P450 with Asn or Gly in this position exhibited spectra indicating a mixture of the high- and low-spin forms. This spin alteration, depending upon the hydrophobicity and size of residue at position 209, indicates that this position is likely to reside close to the sixth axial ligand on the distal surface of the heme in these P450s. This proximity of residue 209 to the ligand may explain the critical role of this residue in determining the hydroxylase specificity and activity of these P450s.

Although P450s in general exhibit broad substrate and product selectivity to metabolize a large number of endogenous and exogenous lipophilic compounds, many P450s show high specificity toward a particular substrate and product. Understanding the structural basis for this paradoxic characteristic of P450s as monooxygenases, therefore, is of major interest in P450 research. In this respect, the sixth ligand and the configuration around the ligand must play a crucial role in overall specification of the catalytic process, since a dioxygen molecule binds to heme iron on the same surface of the porphyrin as the sixth ligand.

Despite their divergent hydroxylase activities, mouse P450coh and P45015α differ only in 11 amino acids within their 494 residues (1–3). The activity, however, depends critically on the types of amino acids at positions 117, 209, and 365 (4). Moreover, a single mutation of P450coh from Phe-209 to Leu confers the steroid hydroxylase activity to the coumarin hydroxylase. These results indicate that residue 209 in the P450s plays the most important role in determining the specificity and, therefore, is likely to reside close to the enzymes’ catalytic sites.

In an attempt to further study the function of residue 209, we constructed a series of the P450coh and P45015α, mutants in which this residue was substituted with various amino acids and expressed these mutants in Saccharomyces cerevisiae. The mutated P450s were purified from the recombinant yeasts and then subjected to spectroscopic analysis. Alteration of spin equilibrium and activity by the substitution of residue 209 indicates the proximity of residue 209 to the sixth ligand.

EXPERIMENTAL PROCEDURES

Site-directed Mutagenesis—We performed mutagenesis reactions with the single-stranded cDNA templates in M13 vectors according to the oligonucleotide-directed in vitro mutagenesis kit (Amersham Corp.). The mutated oligonucleotide primers were synthesized using an automatic Beckman System 1 plus DNA synthesizer and purified through Sephadex G-25 (NAP-5 column). The primers used were: 5'AGAACCTGGAGCTCCTCCAC, 5'AGAACCTGGGTCCCTCCCCAC, 5'AGAACCTGGTGTCCCTCCCCAC, 5'AGAACCTGGTTCCTCCCCAC, 5'AGAACCTGGGCTCCCTCCCCAC, 5'AGAACCTGGGCTCCCTCCCCAC, and 5'AGAACCTGGGCTCCCTCCCCAC. The underlines show the codon mutated. The sequences of the mutated cDNAs were confirmed using the dideoxynucleotide termination method (5). Each of these mutated cDNAs was inserted into the yeast expression vector pAAH5 as described previously (6) and then transformed to S. cerevisiae AH2 cells using the LiCl method.

Partially, Purification of Mutated P450s—Microsomes were prepared from a 10-liter culture of each recombinant yeast using the method described by Oeda et al. (6). Purification was carried out by essentially the same procedures described previously (7). Yeast microsomes were solubilized in 300 ml of 100 mM potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.05% sodium cholate, 1 mM EDTA, 1 mM DTT, and 0.25 mM phenylmethylsulfonyl fluoride, and then centrifuged at 105,000 X g for 90 min. Subsequently, the supernatant was applied to an aminoethyl Sepharose 4B column (1.3 X 8.5 cm) which had been equilibrated with the solubilizing buffer. The column was washed with 100 ml potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.05% sodium cholate, 1 mM EDTA, and 1 mM DTT. Finally, the P450 was eluted from the column with 100 ml potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.4% sodium cholate, 0.2% Emulgen 913, 1 mM EDTA, and 1 mM DTT.

Analytical Methods—P450 contents were determined by the method of Omura and Sato (8). Coumarin 7-hydroxylase and steroid 15α-hydroxylase activities were measured by the method of Kaipainen et al. (9) and Harada and Negishi (7), respectively. The amount of the high-spin form was calculated from the absorption at 645 nm of the absolute spectrum of ferric form using an extinction coefficient of 1.4 mM cm−1. The absorbance at 407 nm (isosbestic point) was determined using absorption at 407 nm (isosbestic point) with an extinction coefficient of 90 mM cm−1.

RESULTS AND DISCUSSION

Fig. 1 shows the absolute absorption spectra of ferric forms of the purified P450s. Leu-mutated C6L displayed a high-spin spectrum with absorption peaks at 389 and 647 nm, whereas Lys-mutated C6K showed a low-spin spectrum with peaks at

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1 P450coh and P45015α, are members of subfamily IIA.

The abbreviations used are: CHAPS, 3-(3-cholamidopropyl)-dimethylammonio]-1-propanesulfonic acid; DTT, dithiothreitol.
The P450s used to measure the spectra were 1.25, 1.54, 1.48, and 1.80. Bic residue favored the high-spin form and a hydrophilic p~ for C6L (Leu-209), C6G (Gly-209), C6N (Asn-209), and C6K as the spin equilibrium of P-450I5, was concerned, wild-type Emulgen 913, potassium phosphate buffer, pH 7.25, containing 20% glycerol, 0.2% mutated C6D, wild-type P450coh (C6F), wild-type P450I5, mutated C6N: Val-mutated C6V, Met-mutated C6M, Asp-addition, the spin equilibria were measured for the other spin form, while more C6N was in the low-spin form. Consistent with the finding that a hydrophobicity and hydrophilicity of residue 209 are the primary factors in maintaining the P450s in the high- and low-spin forms, respectively. Furthermore, the comparison of the C6L with the C6V suggested that as the amino acid becomes smaller, the equilibrium is shifted toward the low-spin state, indicating that size is also a factor. Thus, it can be concluded that the spin equilibrium of the P450 is determined by a combination of the hydrophobicity and size of the residue 209.

Surprisingly, C6M was found to be in the low-spin form (Table I). Methionine, therefore, did not follow the principle that hydrophobicity and hydrophilicity determine the spin equilibrium of the P450s. In addition, C6M had little catalytic activity. It is intriguing, however, that the reduced CO difference spectrum of purified C6M detected only one-third of the total P450 content as measured from the absolute spectra of the oxidized and reduced C6M (data not shown). Taking into consideration that residue 209 must be localized very close to the sixth axial ligand of heme, this apparent inhibition of the CO binding to C6M may imply that the sulfur in the methionine’s thioether group directly coordinates to the sixth axial position of the P450s. Alternatively, the nonbonded electrons of the sulfur may play a role in keeping the field strength weak enough for water to remain at the sixth coordination site of the heme iron. Further work is in progress in our laboratory.

Yoshida et al. (11), White and Coon (12), and Dawson et al. (13) concluded, by analyzing the spectroscopic nature of rabbit P450s and bacterial P450cam, that the native sixth ligand of the ferric low-spin P450s is a water molecule. Subsequently, Poulos et al. (14) and Raag and Poulos (15) determined the x-ray crystal structures of P450cam and confirmed that the low-spin cytochrome remained hexacoordinated to an aqua molecule. Moreover, they proposed that the removal of an aqua ligand shifted the spin state of P450 from low to high. Our present finding, that the hydrophobicity and size of residue 209 determine the spin equilibrium, agrees with these conclusions about the sixth ligand: a hydrophobic and/or large amino acid at position 209 creates an environment in which the heme can no longer be hexacoordinated, and, therefore, the P450s are converted to the high-spin forms. Based on the x-ray crystal structure of bacterial P450cam and its sequence alignment to the mammalian P450s, Poulos (16) argued that residue 209 could not be situated close to the catalytic site, since helix E in which the residue 209 was assigned, was located far from heme in the predicted mammalian P450 structure. Our spectroscopic studies with the mutated P450s indicate, however, that residue 209 is likely to reside close to the sixth ligand, because the types of amino acid 209 determine the spin state of these P450s.

Threonine 252 in P450cam is located in helix I that runs over the distal surface of the heme and, therefore, plays a critical role in the proper formation of the oxygen binding pocket and, consequently, may be involved in determining the cytochrome’s catalytic activity (14, 17). Moreover, the sequence analysis of the mammalian P450s has suggested that the corresponding threonine and helix are conserved also in these cytochromes (18, 19). Numerous works have been reported to suggest that helix I in the mammalian P450s plays the same functional role as it does in the P450cam (20–22). Imai and Nakamura (21) characterized mutants of rabbit P450(ω-1) in which the conserved Thr-301 was substituted with Ser, Asn, Val, or Ile and found that the spin equilibrium

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of the P450 shifted more toward the high state as the hydrophobicity of residue 301 increased. The results were consistent in that the threonine was localized close to the sixth ligand in the P450(ω-1). The maximum spin shift observed, however, was only about 50%, which is in sharp contrast to the complete shifts in the spin state with mutations of residue 209 in the mouse P450s. The spectroscopic evidence suggests, therefore, that residue 209 resides as close or closer to the sixth ligand of P450coh and P45015α as Thr-301 does to the ligand in rabbit P450(ω-1).

If residue 209 resides close to the sixth ligand of the surface of the heme, this explains the fact that the identity of residue 209 determines hydroxylase specificity and activity of the P450coh and P45015α, as shown in our previous study (4) and in the present experiments (Table I). It appears that as the environment becomes more hydrophobic and tighter around the catalytic site, P450coh exhibits higher specificity and activity with regard to coumarin hydroxylation. Conversely, as seen in the C6N, C6K, C6D, and S6N, a hydrophilic environment results in decreases in the specificity and activity. Moreover, a negatively charged amino acid Asp (also Glu) abolished both coumarin 7-hydroxylase and steroid 15α-hydroxylase activity. The differences in the specificity and activity, however, cannot uniformly be explained by the types of residue 209. C6V and C6N, for instance, exhibit very similar activities toward coumarin as well as steroid. This is not surprising since they are determined not only by residue 209 but also by the residues of other positions including 117 and 365 (4). In conclusion, the high- and low-spin equilibrium of the P450 shifted more toward the high state as the hydrophobicity of residue 301 increased. The results were consistent in that the threonine was localized close to the sixth ligand in the P450(ω-1). The maximum spin shift observed, however, was only about 50%, which is in sharp contrast to the complete shifts in the spin state with mutations of residue 209 in the mouse P450s. The spectroscopic evidence suggests, therefore, that residue 209 resides as close or closer to the sixth ligand of P450coh and P45015α, as Thr-301 does to the ligand in rabbit P450(ω-1).

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3 M. Iwasaki, R. Juvonen, R. Lindberg, and M. Negishi, unpublished observation.