Structural alteration of mouse P450coh by mutation of glycine-207 to proline: spin equilibrium, enzyme kinetics, and heat sensitivity

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Mouse cytochrome P450coh is a high-spin haem protein which specifically catalyses coumarin 7-hydroxylase activity. A mutation of Gly-207 to Pro shifts the P450coh completely to the low-spin form, indicating that the sixth axial position of the haem is hexaco-ordinated with a water molecule in the mutant G207P. Moreover, the G207P mutation increases the K_m value for coumarin 7-hydroxylase activity 100-fold and the K_d value for coumarin binding 200-fold. Conversely, the mutation decreases the K_i and K_a values 10- and 20-fold respectively when testosterone, a larger molecule, is used as a substrate. The results, therefore, are consistent with an idea that the substrate pocket may be larger in the mutant G207P than in the wild-type cytochrome P-450. A Gly-207 to Ala mutation (G207A) of P450coh (G207A), on the other hand, affects neither the spectral

nor the enzymic properties of P450coh. Pro-207, through *cis/trans* isomerization or formation of a kink, may confer on the G207P a structural alteration of its substrate-haem pocket. Our previous studies [Iwasaki, Juvonen, Lindberg and Negishi (1991) *J. Biol. Chem.* **266**, 3380–3382; Juvonen, Iwasaki and Negishi (1991) *J. Biol. Chem.* **266**, 16431–16435] show that the residue at position 209 in P450coh resides close to the sixth axial position of the haem, and the spin equilibrium of the cytochrome P-450 shifts toward the high-spin state as residue 209 becomes more hydrophobic and larger. A Gly-207 to Pro mutation, therefore, results in the creation of a larger substrate pocket in the mutant cytochrome P-450 by altering the protein structure around residue 209 so that a water molecule and testosterone can be accommodated.

INTRODUCTION

As a family of structurally related enzymes, cytochrome P-450s exhibit an extreme diversity in their hydroxylase activities. Understanding the structural basis for the diversity, therefore, is a major objective in cytochrome P-450 research.

Residue 209 in coumarin 7-hydroxylase P450coh (P4502A5) and steroid 15 α -hydroxylase P45015 α (P4502A4) plays a key role in determining the substrate specificity of the cytochrome P-450s. The K_m values of P450coh for coumarin 7-hydroxylase activity vary 200-fold depending on the type of residue 209 (Juvonen et al., 1991). Moreover, the mutations of residue 209 alter the substrate specificity of P450coh from coumarin 7- to 11-deoxy-steroid 15 α -hydroxylation, and then to 11 β -hydroxysteroid 15 α -hydroxylation (Lindberg and Negishi, 1989; Iwasaki et al., 1993). Wild-type P450coh is a complete high-spin form and shifts its spin equilibrium toward the low-spin state as residue 209 becomes smaller and more hydrophobic (Iwasaki et al., 1991). The results therefore indicate that residue 209 is critical in controlling the activity and substrate specificity because of its proximity to the sixth axial position of the haem in the cytochrome P-450.

Since the three-dimensional structure of mammalian cytochrome P-450s is not available, we used the bacterial P450cam structure (Poulos et al., 1987) as the model to examine the topology of residue 209 in P450coh (Iwasaki et al., 1993). An earlier sequence alignment (Nelson and Strobel, 1989) assigned residue 209 of P450coh in the region corresponding to helix E in P450cam. Therefore, the importance of residue 209 has been viewed as a mystery (Poulos, 1991), since this helix does not directly contact either the haem or the substrate in the P450cam structure. Our sequence alignments, however, map residue 209 of P450coh to Met-184 or Met-191 in the C-terminal region of

helix F or in the F–G loop of P450cam (Iwasaki et al., 1993). Recently, Gotoh also assigned residue 209 to similar regions (1992). These methionine residues constitute part of the substrate-haem pocket and/or the substrate-access channel. These homology alignments and modelling have provided therefore the structural basis for understanding the functional importance of residue 209.

This paper focuses on a mutation of Gly-207 altering the structure of the substrate pocket of P450coh. Like Phe-209, Gly-207 appears to be located in the C-terminal portion of the F helix or in the F–G loop (Iwasaki et al., 1993; Gotoh, 1992). A Gly-207 mutation may result therefore in a topological alteration of Phe-209 relative to the sixth axial position of the haem. To test this hypothesis, Gly-207 of P450coh was mutated to alanine or proline, and the mutant enzymes' spectral and enzyme-kinetic properties examined. Here we propose a role for Gly-207 in controlling the structure of the substrate pocket of P450coh.

EXPERIMENTAL

Construction of P450coh mutants and expression in yeast

We mutated P450coh cDNA using a pSELECT™-1 vector (Promega, Madison, WI, U.S.A.) and primers 5'-CGAAT-GATGCTGGCTAGCTTCCAGTTCAGTCTACC and CA-GATGATGCTGCCAAGCTTCCAGTTCAGTCTACC for G207A and G209P respectively, as previously described (Juvonen et al., 1991). The recombinant plasmids containing the mutated cDNAs were grown in *Escherichia coli* JM109. Then the cDNAs were digested with *Hind*III, ligated to pAAH5 vector, and finally transfected to *Saccharomyces cerevisiae* AH22 (Oeda et al., 1985).

Abbreviation used: DTT, dithiothreitol.

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Purification of cytochrome *P*-450 and hydroxylase activity

The wild-type and mutant P450cohs were purified from yeast microsomes as described previously (Juvonen et al., 1991), except that a hydroxyapatite column was included to remove further an endogenous yeast cytochrome *P*-450. For this, cytochrome *P*-450-enriched fractions from an ocytamino-Sepharose 4B column were applied to a hydroxyapatite column equilibrated with 10 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 1 mM dithiothreitol (DTT), and 0.2% sodium cholate. The column was washed first by 10 mM and then by 100 mM potassium phosphate buffer, and the cytochrome *P*-450 was eluted by increasing the buffer concentration to 400 mM. The reconstitution system for the hydroxylase activity consisted of the purified cytochrome *P*-450 (10 nmol), rat NADPH-cytochrome *P*-450 reductase (30 pmol), NADPH (0.5 mM), MgCl₂ (5 mM), didodecanoylphosphatidylcholine (6 nmol), and substrate (100 mM of coumarin or [4-¹⁴C]testosterone) in a final volume of 0.5 ml. After the incubation at 37 °C for 10 min, the hydroxylated products were extracted and coumarin 7-hydroxylase activity measured by the Aitio method (1978) and steroid hydroxylase activity by the method of Harada and Negishi (1984).

Substrate-induced difference spectrum analysis

To measure the substrate-induced difference spectrum, we used the methods previously reported (Juvonen et al., 1991). For the wild-type P450coh and mutant G207A, the corresponding yeast microsomes in 100 mM potassium phosphate buffer, pH 7.25, containing 20% (v/v) glycerol, 0.1 mM EDTA and 0.1 mM DTT were divided into two 1-cm-pathlength cuvettes. After addition of coumarin (0.5–100 µM) to the sample cuvette and addition of equivalent amounts of the solvent to the reference cuvette, the spectra were recorded from 460 nm to 360 nm and quantified using $\Delta(A_{392} - A_{418})$ with an extinction coefficient of 130 mM⁻¹·cm⁻¹ (Jefcoat, 1978). The spectra and K_d value of mutant G207P were also obtained in a similar manner using the purified cytochrome *P*-450. To measure K_d for testosterone binding, five different concentrations of the steroid were used to produce the substrate-induced spectra: 10–200 µM for mutant G207P and 40–600 µM for the wild-type P450coh and mutant G207A.

RESULTS

Alteration of spin equilibrium

Figure 1 shows the absolute spectra of the oxidized forms of the P450coh mutants in which Gly-207 was substituted by alanine or proline. As previously reported (Iwasaki et al., 1991), wild-type P450coh is a high-spin form which has absorption peaks at 389 nm and 647 nm. Based on the A_{390}/A_{417} ratio of 1.8, approx. 95% of the wild-type P450coh is in the high-spin form. A Gly-207 to Pro mutation (G207P), however, shifts the P450coh completely to a low-spin form which exhibits its absorption peaks at 417, 534 and 570 nm. More than 95% of G207P is in the low-spin form, as estimated by its A_{390}/A_{417} ratio. More than 90% of G207A, on the other hand, remains in the high-spin form, as indicated by the A_{390}/A_{417} ratio of 1.9.

Alteration of coumarin 7-hydroxylase activity and binding

As reported previously (Juvonen et al., 1991), and K_m the V_{max} values of wild-type P450coh for coumarin 7-hydroxylase activity

Figure 1 Absolute absorption spectra of P450coh mutants

Each of the purified G207A and G207P mutants (approx. 1.5 µM) was dissolved in 400 mM potassium phosphate buffer, pH 7.25, containing 20% (w/v) glycerol, 0.1 mM DTT, 0.1 mM EDTA and 0.2% sodium cholate. The spectra were obtained using a Simazu 2000 spectrophotometer.

Table 1 Enzyme-kinetic properties of wild-type and mutant forms of P450coh

As described in the Experimental section, for (a) we measured coumarin 7-hydroxylase activity and the coumarin-induced spectrum at various coumarin concentrations and calculated the K_m and V_{max} values by Lineweaver–Burk analysis and the K_d values by reciprocal correlation of coumarin concentration with ΔA between 392 and 418 nm. The number in parentheses is the percentage spin change. For (b), we used testosterone as a substrate as well as an inhibitor of coumarin 7-hydroxylase activity. Substrate-induced spectra were measured at five different testosterone concentrations from 10 µM to 190 µM for G207P and from 30 µM to 580 µM for wild-type and G207A and the K_i values were calculated. To obtain the K_i value, coumarin 7-hydroxylase activities (at a coumarin concentration of 100 µM) were measured in the presence of four different concentrations (from 0 to 25 µM for G207P and from 0 to 2000 µM for wild-type and G207A) of testosterone. The specific testosterone 15 α - and 7 α -hydroxylase activities in this table were determined at a substrate concentration of 100 µM.

	K_d (µM)	Type of spectrum	K_m (µM)	V_{max} (nmol/min per nmol of cytochrome <i>P</i> -450)	
				15 α	7 α
Wild type	1.5 ± 0.3	Type 1 (25)	2.6 ± 0.3	4.0 ± 0.2	
G207P	280 ± 23	Type 1 (28)	250 ± 50	2.9 ± 0.4	
G207A	4.1 ± 0.5	Type 1 (7)	2.9 ± 0.3	9.3 ± 0.4	

	K_d (µM)	Type of spectrum	K_i (µM)	Hydroxylase activity (nmol/min per nmol of cytochrome <i>P</i> -450)	
				15 α	7 α
Wild type	340	Reverse type I (63)	85 ± 9	1.1	0.14
G207P	16	Type I (7)	9.6 ± 1.7	< 0.05	< 0.05
G207A	450	Reverse type I (45)	65 ± 9	1.5	0.13

Figure 2 Heat sensitivity of coumarin 7-hydroxylase activity in wild-type and mutant forms of P450coh

Purified wild-type P450coh (○), and mutant forms G207A (X) and G207P (▲) were incubated in the reconstitution buffer without NADPH at room temperature for various time periods (days). Then coumarin 7-hydroxylase activity was initiated by adding NADPH as described in the Experimental section. Within a day, the activity was increased 1.5–3-fold depending on the type of cytochrome *P*-450. The decreases of activity were expressed by taking the highest activity of each cytochrome *P*-450 as 100%.

are approx. 2 μ M and 4 nmol/min per nmol of cytochrome *P*-450 respectively. G207A exhibits a similar K_m value to the wild-type P450coh, while the K_m value of G207P is 100-fold higher (Table 1). The V_{max} value, on the other hand, is higher in mutant G207A and lower in mutant G207P, but only by 2-fold when compared with the wild-type P450coh. Consistent with the increase in the K_m values, G207P also increases the K_d value for coumarin binding by 200-fold. The K_d value of G207A, on the other hand, is only 2-fold higher than that of the wild-type P450coh.

Alteration of steroid–P450coh interaction

The wild-type P450coh catalyses testosterone 15 α -hydroxylase activity at a 50-fold lower level than the other subfamily-member, P45015 α (P4502A4), does. Moreover, testosterone is a poor inhibitor for the P450coh-dependent coumarin 7-hydroxylase activity, although coumarin efficiently inhibits the 15 α -hydroxylase activity of P45015 α (Lindberg and Negishi, 1989). These results suggest that the substrate pocket of the wild-type P450coh is not large enough for testosterone to bind efficiently, because testosterone is a much larger molecule than coumarin. Consistent with this hypothesis, the wild-type P450coh exhibits a high K_d value (340 μ M) for testosterone binding and a high K_i value (85 μ M) for a competitive inhibition by testosterone of coumarin 7-hydroxylase activity (Table 1). Mutant G207P has approx. 20-fold and 10-fold lower K_d and K_i values respectively, compared with the wild-type P450coh. The mutation of Gly-207 to proline, however, abolishes the already low testosterone hydroxylase activity of P450coh (Table 1). As expected, a

mutation of Gly-207 to alanine does not change the K_d and K_i values.

Alteration of heat stability

The stabilities of the wild-type and mutant P450cohs were examined by the incubation of these cytochrome *P*-450s at room temperature for 1–4 days before determination of their coumarin 7-hydroxylase activities. Over 4 days, the wild-type P450coh and G207A maintained their original activities. G207P, on the other hand, lost more than 80 % of its activity within 2 days (Figure 2). In addition, the activity of G207P is inhibited by Emulgen 913 more efficiently than that of the wild-type and G207A cytochromes *P*-450: 0.6 % of Emulgen 913 completely inhibits coumarin 7-hydroxylase activity of G207P, while this concentration of the detergent does not inhibit the wild-type P450coh or G207A (data not shown). The results indicate, therefore, that a mutation of Gly-207 to Pro leaves the substrate-haem pocket in the cytochrome *P*-450 more sensitive to structural alterations by heat and detergent.

DISCUSSION

We demonstrate here that a mutation of Gly-207 to Pro, but not to Ala, results in structural alteration of the substrate-haem pocket in P450coh. This mutation shifts the spin equilibrium of the cytochrome *P*-450 completely from high to low. Presuming the natural sixth ligand of the haem is a water molecule (Yoshida et al., 1982; White and Coon, 1982; Dawson et al., 1982) and the removal of the aqua ligand shifts the spin state of cytochrome *P*-450 from low to high (Poulos et al., 1987), the high-to-low-spin shift in G207P cytochrome implies an enlargement of the substrate-haem pocket, at least locally, to create a space, an environment for a water molecule to co-ordinate at the sixth axial position. Consistent with this hypothesis, G207P has dramatically lower K_d and K_i values for testosterone. Despite the lower K_d and K_i values, however, mutant G207P enzyme exhibits no activity for testosterone hydroxylation. This indicates that G207P can accommodate testosterone but cannot metabolize it. Further study is needed to understand how the steroid-binding orientation is modified in the substrate pocket of G207P.

Gly-207 of P450coh appears not to be a helix-breaker, because a Gly-207 to Ala mutation does not alter the spectral and enzymic properties of the cytochrome *P*-450. Ala is the most favoured amino acid to form a helical structure, while Gly and Pro are strong helix-breakers (O'Neil and DeGrado, 1990; Padmanabhan et al., 1990). Regarding the localization of Gly-207 in P450coh, our amino-acid-sequence alignment maps this residue in the region corresponding to the C-terminal portion of helix F in the P450cam or in the F-G loop (Gotoh, 1992; Iwasaki et al., 1993). Presuming that this alignment is correct, it appears that Gly-207 does not break the helical structure, even if this residue is located in helix F. In fact, a glycine residue at either of the end positions of a general helix has been shown to exhibit little or no effect on the helical structure (Strehlow and Baldwin, 1989). Rat P4502B1 alters its product selectivity according to the type of residue 478; Gly-478 favours production of 16 β -hydroxyandrostenedione, while Ala-478 produces more 16 α -hydroxysteroid (Kedzie et al., 1991). This mutation also does not seem to affect the helical structure of the cytochrome *P*-450, because the corresponding residue in other mammalian cytochrome *P*-450s is located in a conserved region of a β -sheet in P450cam (Poulos, 1991).

Recently, Strehlow et al. (1991) have shown that proline, in a similar way to glycine, also exhibits little effect on overall helix content if it is located at either of the end positions of the helix.

In fact, proline residues in helices are found frequently in the first four residues of the structure (Richardson and Richardson, 1988). Like glycine, therefore, a proline to alanine mutation in a helix often results in little effect on the activity and helix content of many proteins and enzymes (Alber et al., 1988; Consler et al., 1991). Although both proline and glycine are similar in their property of breaking helical structure, proline has an unique characteristic not seen with glycine. Proline can undergo *cis/trans* isomerization more frequently than the other amino acids and can thereby induce a structural kink (Brandts et al., 1975; Piela et al., 1987). The spectral and enzymic alterations of mutant cytochrome G207P may result therefore from the isomerization of the proline residue at position 207.

Our previous work indicates that Phe-209 of P450coh resides close to the sixth axial position of the haem (Iwasaki et al., 1991, 1993; Juvonen et al., 1991; Negishi et al., 1992). As residue 209 becomes more hydrophobic and larger the spin equilibrium of the cytochrome P-450 shifts more toward the high-spin state by removing the natural sixth ligand water molecule. The identity of residue 209 of P450coh directly affects the hexacoordination of a water molecule. The mutations of the residue at position 207 in P450coh, on the other hand, exhibit no such correlation between the spin equilibrium of the cytochrome P-450 and the identity of residue 207. On the contrary, a mutation of Gly-207 to Pro, a larger hydrophobic amino acid, converts the P450coh into a low-spin form. Residue 207 in P450coh therefore appears to be located far from the sixth axial position of the haem and cannot control the hexacoordination directly. One possible explanation for the spin alteration of G207P is that Pro-207 causes an extensive structural modification of the substrate pocket so that Phe-209 can become positioned at a greater distance from the sixth axial ligand of haem. As a result, a water molecule can be co-ordinated at the sixth position of the haem in G207P.

Our sincere appreciation is due to Rickie Moore for his excellent technical assistance.

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