Articles

The Highly Conserved Glu149 and Tyr190 Residues Contribute To Peroxynitrite-Mediated Nitrotyrosine Formation and the Catalytic Activity of Cytochrome P450 2B1

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Tyr190 in cytochrome P450 2B1 has previously been shown to be a prime target for nitration by peroxynitrite (PN) resulting in nitrotyrosine formation and the inactivation of this enzyme. Modeling studies have suggested that Tyr190 may play a structural role in maintaining the integrity of the protein for maximal activity through hydrogen bonding with Glu149. To elucidate the roles of Tyr190 and Glu149 hydrogen-bonding in maintaining the catalytically competent structure of P450 2B1, we have mutated Tyr190 to Phe or Ala and Glu149 to Gln or Ala to characterize the catalytic activities and the structural stabilities of mutated proteins. The results demonstrate that (a) the catalytic activities of all four mutants were decreased significantly compared to wild-type (WT); (b) nitration of Tyr190 by PN or mutation of Tyr190 to Phe did not alter the K_m of the reductase for P450; (c) PN decreases the catalytic activity of the heat-treated Y190A, E149Q, and E149A mutants to a much greater extent than the WT and Y190F; and (d) after exposure of the P450s to PN, the extent of nitrotyrosine formation and the inactivation of the catalytic activity of the E149Q and E149A mutants were markedly decreased when compared to WT. These findings suggest that (1) the hydrogen bond between Tyr190 and Glu149 stabilizes the protein for maximal activity; (2) the benzyl ring and hydroxyl groups of Tyr190 stabilize the protein structure when P450 is exposed to the temperatures higher than 45 °C; and (3) Glu149 may be critical in directing the site of nitration by PN. Since Glu149 and Tyr190 are both highly conserved in the P450 2 family, they may play an important role in the tertiary structure and functional properties of these P450s.

Introduction

Peroxynitrite (PN),[1] formed under physiological conditions by the rapid reaction of nitric oxide and superoxide, is a potent cellular oxidant and nitrating agent (*1*, *2*). The formation of nitrotyrosine represents a specific PN-mediated protein modification; thus, the detection of nitrotyrosine is considered to be a biomarker for endogenous PN formation (*3*−*5*). A strong correlation between tyrosine nitration and the inactivation of protein function as result of exposure to PN has been demonstrated for Mn superoxide dismutase, prostacyclin synthase, neurofilament-L, tyrosine hydroxylase, protein kinase C, and cytochrome P450 2B1 (*6*−*11*). Nitrotyrosine formation has been identified in diverse human diseases including acute lung injury, rheumatoid arthritis, chronic rejection of organ transplantation, atherosclerosis, Parkinson's disease, and Alzheimer's disease (*2*, *6*, *12*−*16*).

The cytochrome P450 enzymes, a family of heme-containing monooxygenases, play a central role in the metabolism of a variety of drugs, fatty acids, chemical carcinogens, pesticides, and steroids (*17*, *18*). It is of great interest to elucidate the structure and function of these enzymes for their potential in the industrial and drug discovery processes. We have previously reported that P450 2B1 was nitrated and inactivated by PN, and a peptide composed of residues 160−225 was identified as the major nitrotyrosine-containing peptide (*11*). Studies with P450s containing single mutations of Tyr190 or Tyr203 to Ala suggested that Tyr190, located at the end of the E−F-loop and only three residues distant from the F-helix, is a prime target for PN-mediated nitration and that nitration of this tyrosine is responsible for the inactivation of cytochrome P450 2B1 (*19*). Modeling studies suggest that Tyr190 may play a structural role

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[1] Abbreviations: PN, peroxynitrite; EFC, 7-ethoxy-4-(trifluoromethyl)coumarin; HFC, 7-hydroxy-4-(trifluoromethyl)coumarin; DLPC, dilauroyl-L-α-phosphotidylcholine; WT, wild-type recombinant P450 2B1; WT+PN, WT P450 that has been treated with PN; reductase, NADPH-cytochrome P450 reductase; Y190F, mutation of Tyr190 to Phe; Y190A, mutation of Tyr190 to Ala; E149Q, mutation of Glu149 to Gln; E149A, mutation of Glu149 to Ala.

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in maintaining the integrity of the protein for maximal activity through hydrogen bonding with the carboxylate side chain of Glu149, located on the D-helix, in P450 2B1 (*19*). On the basis of sequence alignments and homology modeling of members of P450 2 family, both Glu149 and Tyr190 are highly conserved in this family (*20*−*22*). To investigate the function of the Tyr190 side chain and the importance of hydrogen bonding between Glu149 and Tyr190 in the P450 2 family, the plasmid for wild-type P450 2B1 (WT) was used as the template for the mutation of Tyr190 to Phe (Y190F) or Ala (Y190A) and the mutation of Glu149 to Gln (E149Q) or Ala (E149A). The catalytic activities, interactions with reductase, and the structural stabilities at higher temperatures of the mutants were examined and compared to the WT enzyme. Previous studies with Cu,Zn superoxide dismutase and neurofilament-L have established that the presence of a neighboring glutamate may contribute to the selectivity of nitrotyrosine formation by PN (*1*, *8*). Therefore, the E149Q and E149A mutants were employed to test this hypothesis. After exposure of the P450s to PN, the loss of catalytic activity and the extents of nitrotyrosine formation were characterized. Finally, the effect of *n*-octylamine on the nitration process and the effect of PN on the reduced CO difference spectra of the P450s were characterized.

Our results demonstrate that (1) hydrogen bonding between Tyr190 and Glu149 contribute to catalysis; (2) the phenolic aromatic ring and the hydroxyl group of Tyr190 stabilize the protein for enzymatic activity when the temperature increases; and (3) Glu149 may play a role in directing the site of nitration by PN to a specific tyrosine.

Materials and Methods

Materials. Testosterone, benzphetamine, *n*-octylamine, NADPH, and dilauroyl-L-α-phosphotidyl choline (DLPC) were from Sigma-Aldrich Chemical Co. (St. Louis, Mo). 7-Ethoxy-4-(trifluoromethyl)coumarin (EFC) was from Invitrogen, Corp. (Carlsbad, CA). Peroxynitrite (PN) was purchased from Cayman Chemicals (Ann Arbor, MI) and stored in 0.3 M NaOH at −80 °C. Rabbit polyclonal antibody to P450 2B1 was prepared as described (*23*). Rabbit polyclonal antibody to nitrotyrosine was from Upstate Biotechonology, Inc. (Lake Placid, NY). Goat anti-rabbit IgG horseradish peroxidase conjugate was from Bio-Rad Laboratories, Inc. (Hercules, CA). All other chemicals and solvents used were of the highest purity available from commercial sources.

Construction of Expression Plasmids. The wild-type recombinant pCW2B1 was used as the template for the construction of the mutants at positions 149 and 190 (*24*). Mutations were carried out using an in vitro QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). The primer 5′-GGAGAGCGCTTTGACTTCCACAGACCGCCAGTTC was used for the conversion of Tyr190 to Phe. The primer 5′-GAACGGATTCAGGAGCAAGCCCAATGTTTGGTGG was used for the Glu149 to Gln conversion. The primer 5′-GAACGGATTCAGGAGGCAGCCCAATGTTTGGTGG was used for the Glu149 to Ala conversion. The construction of the Tyr190 to Ala mutant was described previously (*19*). All of the mutations were confirmed by DNA sequencing carried out at the University of Michigan Core Facility (Ann Arbor, MI).

Purification of the Expressed Enzymes. The WT and mutant P450s were expressed in *Escherichia coli* MV1304 cells. NADPH-cytochrome P450 reductase (reductase) was expressed in *E. coli* Topp 3 cells. All of the enzymes were purified according to methods described previously (*24*).

Determination of Catalytic Activity. The concentration of the P450s was determined from the reduced CO difference spectra (*25*). The same concentration of each P450 was used for all the reactions. The catalytic activity of the individual P450 for each of the substrates was determined utilizing a reconstituted system containing 20 pmol of P450, 20 pmol of reductase, and 10 μg of DLPC at 22 °C for 30 min. The assays for the metabolites of testosterone, benzphetamine, and EFC by P450s have been described previously (*19*).

Determination of Apparent K_m Values for the Reductase. The K_m values were determined by observing the rates of 7-hydroxy-4-(trifluoromethyl)coumarin (HFC) formation from the deethylation of EFC at a constant P450 concentration (0.1 μM) while varying the concentrations of reductase from 0.1 to 0.6 μM. The apparent K_m values of the WT and modified P450s for reductase were determined from the *x*-intercept of the plot of the double reciprocal of the rate of product formation as a function of reductase concentration (*26*).

Effect of Increased Temperatures on Enzyme Activity. Studies were carried out by incubating the P450s in 100 mM potassium phosphate buffer (pH 7.7) with 20% glycerol at different temperatures (37, 45, 50, and 55 °C) for 5 min. The structural stability at a higher temperature was investigated further by incubating the P450s at 45 °C for different time intervals (3, 6, 9, and 12 min). After treatment, the samples were removed, immediately cooled on ice, and kept there until the EFC deethylation activity was measured at 30 °C.

Treatment of P450s with PN. After exposing 25 pmol of P450 to 300 μM PN or 2 μL of 0.3 M NaOH (as control) in the presence of 10 μg DLPC in 200 μL of 100 mM potassium phosphate buffer (pH 7.7) at 22 °C for 3 min, 25 pmol of reductase was added to each P450-containing solution and incubated for 10 min. Samples containing 10 pmol of P450 were used to determine EFC deethylation activity. Aliquots containing 5 pmol of the PN-treated P450s were subjected to SDS−PAGE analysis using a 10% polyacryamide gel. The resolved proteins were transferred to a nitrocellulose membrane and probed with the primary antibodies. For detection of P450 2B1, the blot was incubated with rabbit polyclonal anti-P450 2B1 antibody. For detection of nitrotyrosine formation, the blot was incubated with rabbit polyclonal anti-nitrotyrosine antibody. The immuno-complexed membranes were then probed with a goat anti-rabbit horseradish peroxidase-conjugated secondary antibody, and the immunoreactive bands were detected using enhanced chemiluminescence as described by the manufacturer (Pierce, Rockford, IL).

Interaction of PN with the Heme Iron Center. The effect of *n*-octylamine, an axial ligand for the heme iron and a potent inhibitor of activity, on the nitration process was investigated first. P450 2B1 (1 μM) was pretreated with *n*-octylamine at concentrations up to 20 mM and exposed to 30 or 300 μM PN; the extent of nitrotyrosine formation was then determined. To investigate the effects of PN on the heme and its environment, the P450s were exposed to 30 or 300 μM PN followed by the addition of dithionite and CO, and the reduced CO difference spectra were determined (*25*).

Homology Modeling of the P450 2B1. The homology model of P450 2B1 was constructed as previously described, except that the crystal structure of the P450 2B4 complexed with 4-(4-chlorophenyl)imidazole was used and the inhibitor was removed from the modeling (*19*, *27*−*29*). Because of the high sequence identity between P450 2B1 and the template, the sequence of 2B1 fit to the template very well. In particular, the structure of the C-terminal region is highly conserved. The new P450 2B1 homology model is almost identical to that based on P450 2C5 in a previous study (*19*). The major variations in the new model occur in the F−G-loop region (209−222) that was absent from the crystal structure of P450 2C5 and in a six residue loop flanking Phe106 (103−108). Another noticeable variation is the orientation of Tyr190. In the new model, Tyr190 is predicted to be shifted 0.64 Å away from the heme and the benzyl ring rotates about 45° around the Cβ−Cγ axis to keep the phenolic oxygen within hydrogen-bonding distance to the carboxylate oxygen of Glu149 (2.72 Å). The conformation of Glu149 in both

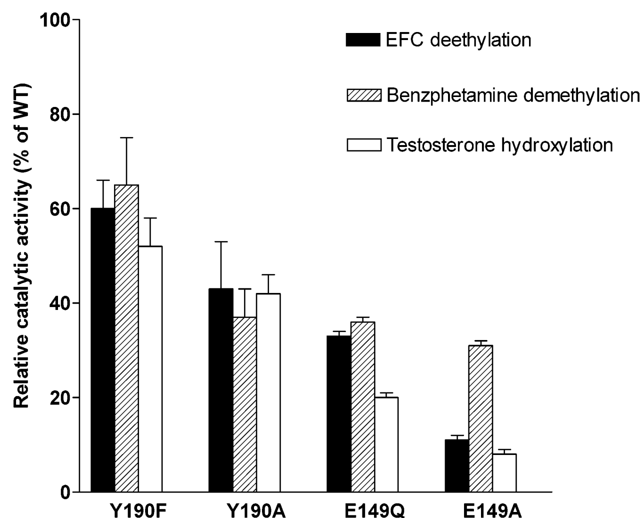

Figure 1. Comparison of the enzymatic activities of the WT and mutant P450s. EFC, benzphetamine, and testosterone were used as substrates to measure the enzymatic activities of all the proteins at the same P450 concentration using the conditions described in Material and Methods. The relative activities were assessed by comparison to WT protein. The experiments were performed four times in duplicate.

of the 2B1 models, based on either 2C5 or 2B4, is predicted to be identical.

Data Analysis. Results are given as the mean ± SD, and the statistical evaluations are based on the unpaired, two-tailed Student's *t* test.

Results

Catalytic Activity. The ability of the two Tyr190 mutants (Y190F and Y190A) and the two Glu149 mutants (E149Q and E149A) to metabolize EFC, benzphetamine, and testosterone was determined. In general, all four mutants exhibited diminished catalytic activity when compared to the WT enzyme (Figure 1). The order of catalytic activity of the four mutated P450s compared to the WT P450 is WT > Y190F > Y190A > E149Q > E149A. Benzphetamine demethylation activity was not decreased as drastically as testosterone hydroxylation activity in E149Q and E149A. In the E149A mutant, the EFC deethylation activity was also decreased markedly, indicating that substitution of the Glu by the alanine at this position may severely alter the protein structure. The ability of the residues at positions 190 and 149 to hydrogen-bond was removed effectively by the substitution of the Phe and Ala residues and the ability to hydrogen-bond was also altered by replacing the carboxyl group in Glu with the amide group in Gln. These results indicated that hydrogen bonding between Tyr190 and Glu149 plays an important structural role for catalysis by P450 2B1.

Interaction with Reductase. To investigate the possibility that the decreases in the catalytic activities of modified P450s might be due to the interference with reductase binding and the subsequent transfer of electrons, the P450s were incubated with various concentrations of reductase (0.1−0.6 μM), and then the EFC deethylation activity was measured. The results are shown in Figure 2A. A double reciprocal plot of these data is shown in Figure 2B. The apparent K_m values for the binding of the reductase to 0.1 μM P450 calculated from these data ranged from 0.18 to 0.23 μM for all three P450s. This result indicates that modification of Tyr190 to nitrotyrosine by PN or to Phe by site-directed mutagenesis did not significantly alter the binding of the reductase to the P450s. Thus, the loss of activity upon modification of Tyr190 is not due to the interference with reductase binding. This finding further supported the hypothesis that disruption of the hydrogen bonding between Tyr190 and Glu149 may be one of the major factors responsible for PN-mediated inactivation. Since Tyr190 is located on a distal surface of P450 2B1, our result is in agreement with previous studies on rabbit P450 2B4. In that study, the Tyr190 in P450 2B4 was chosen as a negative control and the results demonstrated that the binding sites for cytochrome b_5 and cytochrome P450 reductase are located on the proximal surface of P450 (*26*). In short, these results demonstrated that Tyr190 is not functionally involved in the interaction of P450 2B1 with the reductase.

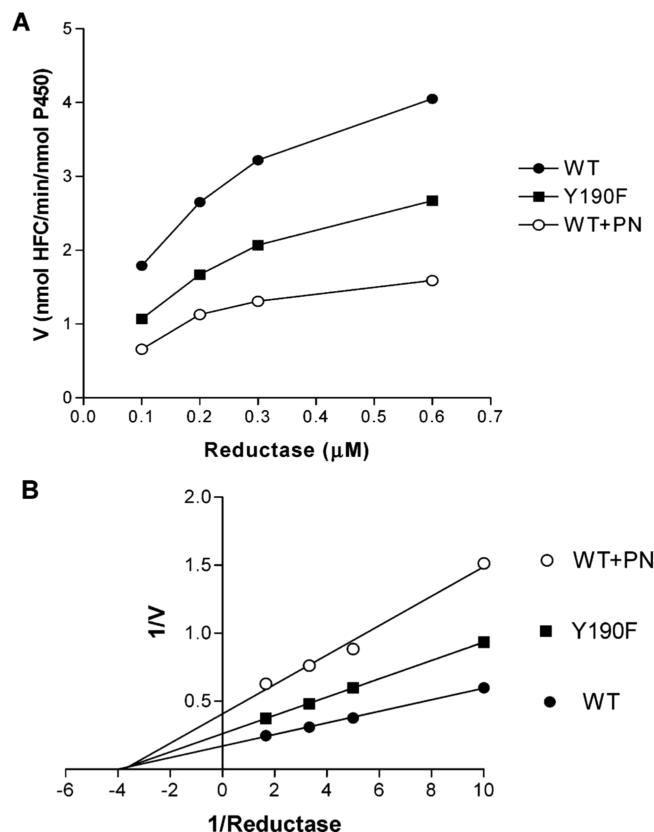

Figure 2. Determination of the apparent K_m values of the P450s for the reductase. (A) The EFC *O*-deethylation activity was measured at a constant concentration of P450 (0.1 μM) while varying the concentration of the reductase with WT, WT+PN, and Y190F proteins. (B) A plot of the reciprocal of the rate of product formation versus the reciprocal of the reductase concentration from the data in panel A. The value for the apparent K_m for the reductase with each P450 was determined from the *x*-intercept of the double reciprocal plot. The results are from two separate experiments done in duplicate.

Effect of Temperature on Enzyme Activity. To better understand the contributions of hydrogen bonding and the hydrophobic binding forces exerted by the side chain of Tyr190 to protein stability and catalysis, we investigated the effects of nitration and mutations of Tyr190 on the thermal stability of proteins. This was done by incubating the P450s at different temperatures (37, 45, 50, and 55 °C) for 5 min or at 45 °C for different time intervals (3, 6, 9, and 12 min) and then measuring the activity remaining. As shown in Figure 3A, the decrease in the catalytic activity with increasing tem-

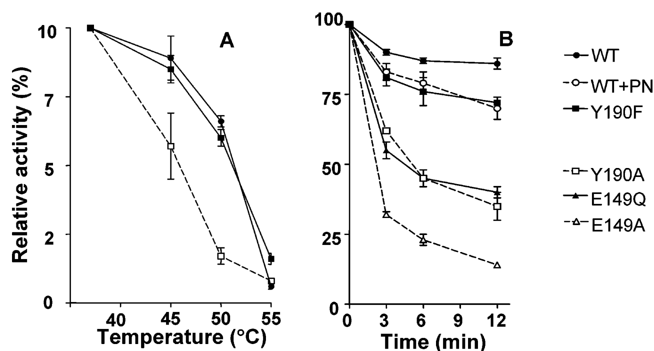

Figure 3. Effect of temperature on the EFC deethylation activities of the WT and mutant P450s. (A) Purified P450s were pretreated at the temperatures indicated for 5 min, then the activities were measured and expressed as percentages of the activities at 37 °C. (B) Purified P450s were pretreated at 45 °C for the times indicated before measuring the activity, and activities are expressed as percentages of the activities of the untreated samples. Each data point is the average of two separate experiments done in duplicate.

perature showed pronounced differences between WT or Y190F and Y190A. The Y190A exhibited a 50% reduction in activity at ~46 °C, whereas the WT and Y190F proteins exhibited a similar reduction at ~53 °C. As shown in Figure 3B, the enzyme activity decreased with incubation time for the WT+PN and Y190F proteins, but not as drastically as for the Y190A, E149Q, and E149A proteins. The mutation of Tyr to Phe removes the phenolic hydroxyl group but leaves the benzyl ring intact, whereas the mutation of Tyr to Ala effectively removes the phenolic hydroxyl group and the benzyl ring. The results from the three P450 variants of Tyr190 suggest that the benzyl ring may almost be able to compensate for the loss of hydrogen bond. The consequences of the increased temperatures on the WT+PN are almost identical to those seen with Y190F, indicating that in this case the nitrotyrosine behaves in a fashion very similar to the Phe. We have previously proposed that the addition of a nitro group may disrupt/alter the hydrogen bond resulting in some loss in the structural integrity of protein (*19*). Although the Tyr190 residue is still intact in the E149Q and E149A mutants, these two mutants are very sensitive to temperature change, suggesting the carboxylic group at position 149 may also play a critical role in maintaining the structure required for full enzyme function.

Exposure of the WT and Glu Mutants to PN. After exposure to PN, the residual catalytic activities of the WT, E149Q, and E149A enzymes are shown in Figure 4A. The WT protein had only 13%, the E149Q mutant had 52%, and the E149A mutant had 85% activity remaining. The PN-treated P450s were subjected to Western blot analysis and probed with anti-P450 2B1 antibody or anti-nitrotyrosine antibody. As shown in Figure 4B (top panel), the WT and E149Q mutant displayed approximately equal immunoreactivity for P450 2B1, whereas E149A displayed more than a 2-fold stronger signal for 2B1 apoprotein, suggesting that not all of the apoprotein in E149A was expressed as the holoprotein. As shown in Figure 4B (bottom panel), the extent of nitrotyrosine formation in E149Q and E149A was extremely low compared to WT protein. The extent of nitrotyrosine formation correlated with the loss of catalytic activity after the exposure of the P450s to PN. In particular, the ability of PN to nitrate tyrosine and

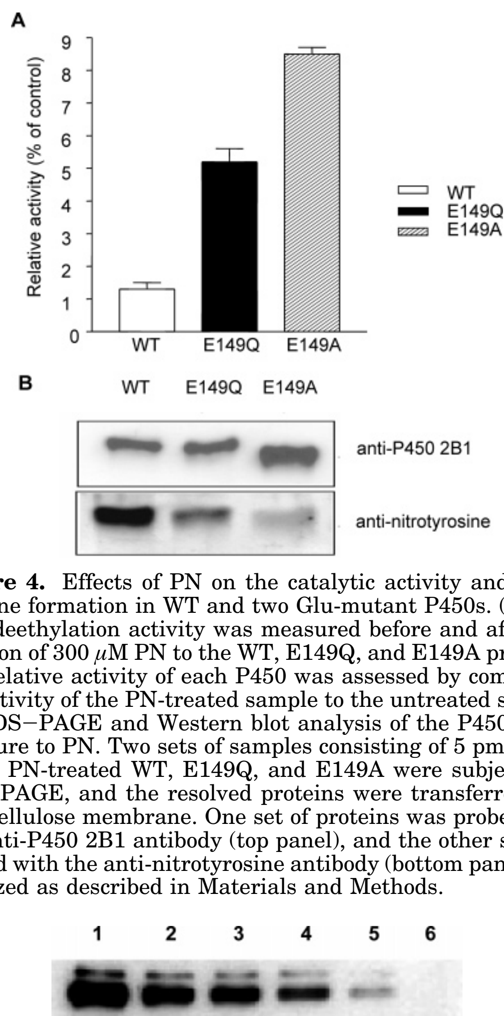

Figure 4. Effects of PN on the catalytic activity and nitrotyrosine formation in WT and two Glu-mutant P450s. (A) The EFC deethylation activity was measured before and after the addition of 300 μM PN to the WT, E149Q, and E149A proteins. The relative activity of each P450 was assessed by comparing the activity of the PN-treated sample to the untreated sample. (B) SDS−PAGE and Western blot analysis of the P450s after exposure to PN. Two sets of samples consisting of 5 pmol each of the PN-treated WT, E149Q, and E149A were subjected to SDS−PAGE, and the resolved proteins were transferred to a nitrocellulose membrane. One set of proteins was probed with the anti-P450 2B1 antibody (top panel), and the other set was probed with the anti-nitrotyrosine antibody (bottom panel) and analyzed as described in Materials and Methods.

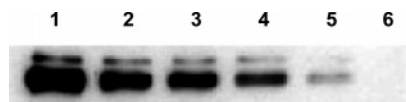

Figure 5. The effect of *n*-octylamine on nitrotyrosine formation in P450 2B1 by PN. P450 2B1 (1 μM) was incubated with (1) 0, (2) 1, (3) 3, (4) 5, (5) 10, and (6) 20 mM *n*-octylamine at 22 °C for 10 min prior to the addition of 30 μM PN. A total of 5 pmol of P450 from each incubation mixture was subjected to SDS−PAGE and Western blot analysis as described above.

inactivate enzyme activity in E149A was almost eliminated. These results clearly indicate that mutation of the glutamate at position 149 markedly abolished the formation of nitrotyrosine by PN treatment as well as the inactivation of the mutant P450s.

The Interaction of PN and the Heme Iron. Figure 5 shows that pretreatment of WT P450 with increasing concentrations of *n*-octylamine, which binds tightly to the heme iron, decreased the extent of nitrotyrosine formation following addition of PN at a concentration of 30 μM. However, *n*-octylamine did not block nitrotyrosine formation at a PN concentration of 300 μM (data not shown). When a non-heme protein such as bovine serum albumin was used to test whether *n*-octylamine would act as a scavenger of PN or nitrating intermediates, the extent of nitrotyrosine formation with or without the addition of 20 mM *n*-octylamine was not significantly different when PN at a final concentration of 30 μM was used. This result indicates that at the lower concentration of PN, the interaction of PN with the heme iron is required for the efficient nitration of tyrosine. We have previously demonstrated that the absolute spectrum of the P450 2B1 was not altered after incubation with 300 μM PN (*11*). However, as shown in Figure 6, after treatment with PN,

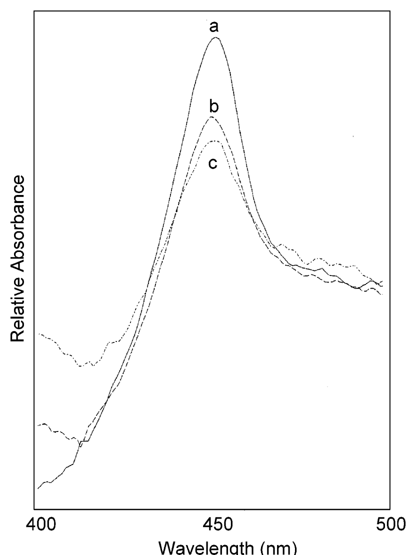

Figure 6. The effect of PN on the P450 reduced CO difference spectrum. P450 2B1 (1 μM) was exposed to (a) 0, (b) 30, and (c) 300 μM PN. Dithionite and CO were subsequently added to measure the CO-binding spectrum. Two separate experiments were performed.

the reduced CO difference spectrum of WT protein decreased to ~60% and ~45% with 30 μM PN and 300 μM PN, respectively. No detectable P420 was observed under these conditions. These results suggest that the heme−thiolate ligation is not destroyed by PN, but that the coordination of heme with the other axial ligand may be altered by PN modification of the tyrosine residue in P450 2B1.

Discussion

In this study, we have used site-directed mutagenesis to expand on previous studies of PN-mediated nitration of P450 2B1 and to test the structure−function relationships of Glu149 and Tyr190. When the ability of Tyr190 and Glu149 to form a hydrogen bond was eliminated or altered (in the case of E149Q), there was a decrease in the catalytic activity of all four mutant P450s (Y190F, Y190A, E149Q, and E149A) for the three substrates tested when compared to WT enzyme. Thus, hydrogen bonding between Tyr190 and Glu149 appears to play an important structural role for maximal activity. This finding supports our previous hypothesis that the nitration of tyrosine may disrupt/alter the hydrogen bonding and lead to some loss in the structural integrity and protein function (*19*). Furthermore, the hypothesis regarding the role of hydrogen bonding in the optimization of catalytic activity was further strenghtened by studies involving preincubation of the P450s at temperatures higher than 45 °C. Because the hydrogen-bonding capacity between Tyr190 and Glu149 was removed/altered in all the mutants, the greater decreases in the relative catalytic activities with increasing temperature when compared with WT enzyme suggest that hydrogen bonding between these two residues contributes significantly to the structural stability of P450 for the catalytic activity. Although the E149Q mutant can still form a hydrogen bond, our results suggest that the nature of the hydrogen bonding was altered with the conversion of COOH to $CONH_2$ and that the presence of the carboxylate seems to be important. To further interpret the experimental data, a homology model of P450 2B1 was constructed as previously described (*19*, *27*) except that the P450 2B4 crystal structure was used as a template in the present study (*29*). As expected, Tyr190 and Glu149 are close together and they are exposed to the surface. The detailed structure of the distal surface surrounding the Tyr190 and Glu149 residues was constructed, and it was found that the distance between the phenolic hydroxyl group of Tyr190 and the carboxylate side chain of Glu149 is only 2.72 Å (Figure 7). This is comparable to the distance between these two residues in P450 2C5 and P450 2B4, which is 2.39 Å and 2.74 Å, repectively (*21*, *29*).

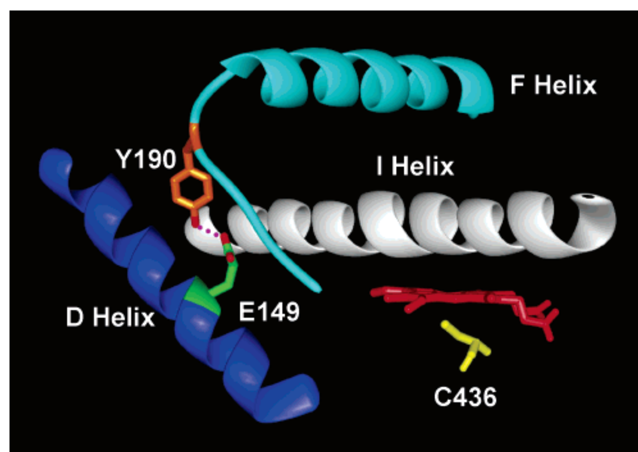

Figure 7. Distal surface of P450 2B1 showing the hydrogen bond between Tyr190 and Glu149. The distance between the hydroxyl group of Tyr190 and the carboxylate group of Glu149 is 2.72 Å.

The relative activity remaining after exposure to higher temperature (45 °C) was WT ≥ Y190F ≅ WT+PN > Y190A. These results demonstrate that (a) the benzyl ring can effectively, although not completely, compensate for the loss of hydrogen bonding and (b) the Tyr190 in the WT enzyme after exposure to PN functions similarly to Phe residue. Perhaps, the benzyl ring of the Tyr, the Phe, or the nitrotyrosine at position 190 is able to interact with the neighboring hydrophobic residues such as Phe188 and Phe195 and use hydrophobic forces to promote protein stability, whereas the methyl side chain of Ala at position 190 is unable to participate in hydrogen bonding. Thus, the retention of the hydrophobic interaction can compensate partially for the missing phenolic OH group involved in forming the hydrogen bond. In combination, it appears that both the hydrogen bonding and the hydrophobic interactions from the side chain of Tyr190 may contribute to P450 2B1 catalysis and structural stability. In this regard, the potential contributions of tyrosine residues to protein structure and function have been previously addressed by a number of investigators (*30*−*32*). On the other hand, although the Tyr190 residue is still intact in the E149Q and E149A mutants, these two P450s exhibited substantially lower catalytic activities and were very sensitive to the temperature effect, indicating the importance of the carboxylate side chain of Glu149 in maintaining the structure and function of the P450. Obviously, Tyr190 cannot function by itself without the support of Glu149 to form the hydrogen bond. The importance of hydrogen bonding between Tyr and Glu for the maintenance of the structural stability of an enzyme and its catalytic activity has been demonstrated for several proteins (*33*−*35*). Moreover, in the study of the inactivation and modification of yeast

Table 1. Sequence Alignments from −3 to +3 Relative to the Highly Conserved Glu149 and Tyr190 Residues of P450 2B1 (*20−22*, *41*)

P450	Glu149 (E)	Tyr190 (Y)
2A4	IQE(E)AGF 152	RFD(Y)EDK 193
2B1	IQE(E)AQC 149	RFD(Y)TDR 190
2C5	IQE(E)ARC 148	RFD(Y)KDE 189
2D6	VTE(E)AAC 156	RFE(Y)DDP 196
2E1	QRE(E)AHF 150	HFD(Y)NDE 191
2F2	ILE(E)ARF 151	RFD(Y)DDE 192
2G1	IQE(E)AGY 152	RFD(Y)EDQ 193
2J2	IQE(E)AQH 162	RFE(Y)QDS 203

hexokinase by carbodiimide and a nucleophile, the γ-carboxyl group of glutamyl residue was found to be modified. It was concluded that a glutamyl residue in the hydrophobic microenviroment was essential for enzyme activity (*36*).

When the two mutants at Glu149 were challenged by exposure to PN, the inactivation was markedly diminished and the extent of nitrotyrosine formation was decreased in parallel with the decrease in the inactivation. This finding strongly supports the hypothesis that a neighboring glutamate may guide PN to attack a specific tyrosine as was proposed in studies on bovine erythrocyte Cu,Zn superoxide dismutase, mouse neurofilament-L, and human Mn superoxide dismutase (*1*, *8*, *37*). Ischiropoulos and co-workers have studied the factors determining the selectivity for protein tyrosine nitration, and they hypothesized that the presence of a nearby negative charge within a few angstroms of the tyrosine residue may be critical in determining the site of nitration (*38*, *39*). It seems that the glutamate residue helps to make the tyrosine more likely to ionize and be more susceptible to radical attack. Alternatively, glutamate may help to remove the hydrogen at the 3-position to facilitate the attack of nitrogen dioxide on the ring of the tyrosine radical to yield 3-nitrotyrosine. However, it is not clear how the carboxylate side chain brings the PN to the tyrosine. It is possible that nitration might be enhanced by forming a nitrinium−caboxylate intermediate, and nitrocarboxylates have been proposed to function as nitrating agents for the formation of nitrophenols (*1*, *8*). The nitration of tyrosine residues may share similar requirements with phosphorylation and sulfation such as the requirement for a specific peptide sequence and a carboxylic acid rich microenvironment (*39*, *40*). In the P450 2 family, the Glu in position 149 is highly conserved and participates in hydrogen bonding to the Tyr at position 190, which is also highly conserved. These two residues play a key role in folding the E−F-loop and the D-helix together to form the tertiary structure required for protein function. The sequence alignments from −3 to +3 relative to the Glu149 and Tyr190 of P450 2B1 shown in Table 1 reveal the apparent sequence homologies and the presence of acidic residues within the same span of residues (*20−22*, *41*). Several other mammalian P450s (rabbit P450 2B4, human P450 2B6, and human P450 2E1), exhibited a marked loss of enzymatic activity concomitant with the formation of nitrotyrosine following treatment with 150 μM PN (data not shown).

The differential effect of *n*-octylamine on the levels of nitrotyrosine formation formed following the addition of 30 or 300 μM PN is in accordance with that observed with other P450s by Ullrich and co-workers (*7*, *42−45*). It appears that either the heme iron itself or metal catalysis may play a critical role in the nitration of the protein tyrosine by PN at low steady-state concentrations. However, in most studies, higher concentrations of PN were used to detect specific site(s) of nitration. Under these conditions, the nitration process may not be catalyzed by the metal or the heme-thiolate. The sensitivity and selectivity of PN-mediated tyrosine nitration in proteins may depend on a variety of factors including the total amount of tyrosine residues exposed on the surface of protein, the presence of specific peptide sequences, the hydrophobic environment, the absence of steric hindrance, the local electrostatic environment, and the presence of a neighboring negative charged residue, in particular the presence of hydrogen bonding between the tyrosine and glutamic acid (*1*, *8*, *19*, *37−40*, *45*, *46*).

Immunoreactive 3-nitrotyrosine residues have been detected in various proteins in the affected areas of Parkinson's disease and Alzheimer's disease, implicating the generation of PN in the brain lesions associated with these diseases (*15*, *16*, *47*). P450 2B6 and P450 2D6 are widely distributed in various regions of the brain and metabolize a wide variety central nervous system acting drugs (*48*). Thus, under a variety of pathogenic conditions, P450s in the brain may be inactivated by the generation of PN. As a consequence, decreases in the metabolism of drugs and endogenous compounds may affect drug function and toxicity in the brain. The use of therapeutic agents known to prevent the formation of the nitrating agent, as well as phenolic antioxidants such as sinapinic acid, caffeic acid, and ferulic acid to inhibit nitration of tyrosine (*49−51*), and the elimination of the factors responsible for the sensitivity or selectivity of nitration may protect P450 proteins in the brain from inactivation. The approaches may allow neuronal injury to be limited and the metabolism of xenobiotics/endobiotics to be maintained.

In conclusion, the identification of Tyr190 as a major site for nitration and its contribution to the loss of P450 2B1 enzyme function has resulted in additional studies of the relationship between the structure and function of members of P450 2 family. Our results suggest that the hydrogen bond formed between Tyr190 and Glu149 contributes to the tertiary structure of the protein and to protein stability and catalytic activity. This may well be a common feature for all members of P450 2 family since these two residues are highly conserved.

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