Denaturation of Cytochrome P450 2B1 by Guanidine Hydrochloride and Urea: Evidence for a Metastable Intermediate State of the Active Site†

Xuan-Chuan Yu,‡ Sijiu Shen, and Henry W. Strobel*

Department of Biochemistry and Molecular Biology, The University of Texas Medical School at Houston, Houston, Texas 77225

Received October 6, 1994; Revised Manuscript Received January 9, 1995⊗

ABSTRACT: A metastable intermediate was found in the course of the denaturation of purified cytochrome P450 2B1 by increasing concentrations of guanidine hydrochloride (GuHCl). The metastable intermediate has no or low absorbance at 450 nm in the reduced carbon monoxide difference spectrum and has no absorbance at 420 nm. The intermediate is easily converted to P420 by increasing concentrations of GuHCl. Before it becomes P420, the cytochrome can be completely reconverted to native P450 by dilution and incubation at 4 °C. Cytochrome P420 resulting from exposure to higher concentrations of GuHCl (>3 M) failed to be reconverted to P450 by dilution. Denaturation of P450 2B1 by exposure to low concentrations of urea (<2 M) is also completely reversible but no obvious intermediate is detectable. An intermediate is observed, however, when the urea denaturation is conducted in the presence of 1 M NaCl. As is the case with higher concentrations of GuHCl, cytochrome P450 denatured by exposure to 5 M or higher concentrations of urea is not reversible. The failure of reconversion of P420 denatured by exposure of cytochrome P450 to high concentrations of GuHCl or urea is probably attributable to the extensive unfolding of the apoprotein, which favors aggregation, rather than to heme loss. Our results also suggest that the active site is more sensitive to denaturants than other regions of the protein.

Cytochrome P450 is a superfamily of hemoproteins, which catalyze monooxygenation reactions of a variety of hydrophobic substrates. Unlike other hemoproteins, the heme in P450[1] is deeply buried in a hydrophobic pocket, formed by a distal helix I, a proximal helix L, and a β-sheet region (Poulos et al., 1987). The name of P450 comes from a specific spectral characteristic, namely, the protein displays an intense peak around 450 nm in the reduced carbon monoxide difference spectrum. The existence of the 450-nm absorption peak was assumed to be a probe of the native form of cytochrome P450 and was used to determine P450 content (Omura & Sato, 1964). On the other hand, the heme in P450 is also a sensitive probe for the study of conformational changes in the active site. The 450-nm absorption peak shifts to 420 nm when the cytochrome P450 in microsomes is treated with some detergents (Omura & Sato, 1964), sulfhydryl reagents (Murakami & Mason, 1967), organic solvents (Imai & Sato, 1966), neutral salts (Imai & Sato, 1967), proteases (Omura & Sato, 1964), hydrostatic pressure (Fisher et al., 1985; Marden & Hui Bon Hoa, 1986; Hui Bon Hoa et al., 1989), and denaturants such as GuHCl (Imai & Sato, 1966) or urea (Mason et al., 1965). Cytochrome P420 was designated as an altered form of cytochrome P450 and appeared to be composed of several magnetically distinguishable species (Peisach et al., 1973). When one of the six ligands of heme iron is substituted by an artificial chemical compound or other amino acid residue of the protein in the course of denaturation, cytochrome P450 is converted to cytochrome P420. Some cytochromes P420 in microsomes treated with sodium deoxycholate or sulfhydryl reagents could be partially or completely reconverted to P450 (Ichikawa & Yamano, 1967a,b), and bacterial cytochrome P450cam was reported to be reconverted from pressure-denatured P420 (Hui Bon Hoa et al., 1990), but no success in reconversion of eukaryotic microsomal P420 denatured by denaturant treatment or in the conversion of purified eukaryotic cytochrome P420 was reported. Cytochrome P450 in microsomes is anchored in membrane and is thereby protected by lipid and possibly by other proteins from disturbance by effectors. When the cytochrome P450 in microsomes is converted to P420 by certain detergents, it is possible that the detergents have no or slight effect on the conformation of the apoprotein, so the P420 can be more easily reconverted to P450 after removal of the effectors. That GuHCl and urea have stronger effects on the conformation of apoprotein could explain the unsuccessful reactivation of P420 generated by exposure to GuHCl or urea. The fate of purified cytochrome P450 exposed to GuHCl or urea might be very different without the protection of lipid and other proteins. Thus, we are interested in the conformational changes of purified P450 in the course of denaturation with GuHCl or urea.

Because the heme is an internal conformational probe of the active site, it enables us to compare the conformational changes of the active site with those of the other parts of the protein. Relatively faster inactivation and slower observable conformational changes of the whole protein were reported in denaturation of creatine kinase (Yao et al., 1984; Zhou et al., 1993), D-glyceraldehyde-3-phosphate dehydrogenase (Tsou, 1986; Lin et al., 1990), ribonuclease A (Tsou, 1986), lactate dehydrogenase (Ma & Tsou, 1991), and papain (Xiao et al., 1993). The conformational flexibility of

† This research was partly supported by Grant CA53191 from the National Cancer Institute, DHHW.

* Correspondence should be addressed to this author.

‡ Permanent address: National Laboratory of Biomacromolecules, Institute of Biophysics, Academia Sinica, Beijing 100101, P. R. China.

⊗ Abstract published in *Advance ACS Abstracts,* April 1, 1995.

[1] Abbreviations: CD, circular dichroism; DTT, dithiothreitol; GuHCl, guanidine hydrochloride; P450, cytochrome P450.

0006-2960/95/0434-5511$09.00/0

enzymes was proposed and extensively discussed by Tsou (1986, 1993). It is usually not easy, however, to compare the conformational changes of the active site with those of the whole protein, because no convenient technique to detect the conformational changes of the active site directly is available. The only two pieces of direct evidence for conformational changes in the active sites reported by Tsou's laboratory were obtained by the introduction of fluorescent probes into the active sites (Ho & Tsou, 1979; Zhou et al., 1993). Introduction of a foreign fluorescent group in the active site is often accompanied by changes of the active site and makes the situation more complicated. The heme in cytochrome P450 active site, on the other hand, provides a good internal probe for the study of denaturation of P450.

Uvarov et al. (1990) reported the successful reconstitution of heme into the apoprotein of cytochrome P450 LM2(2B4), with a yield of 50%. The authors attributed the loss of the other 50% of the activity to the denaturation of the apoprotein during the preparation procedure, though they did not present any data concerning the denaturation process.

In this communication, we report the denaturation of purified cytochrome P450 2B1 by GuHCl and urea. The existence of a metastable intermediate in the course of converting cytochrome P450 2B1 to P420 by GuHCl was an interesting finding in these studies. Under appropriate conditions before the absorption peak at 420 nm appears, the 450-nm peak decreases significantly. The metastable intermediate is easily converted to P420 or P450.

MATERIALS AND METHODS

Materials. Benzphetamine was kindly provided by the research laboratory of the Upjohn Company. GuHCl and urea of ultrapure grade were obtained from Sigma, and the solutions were freshly made before use. Other chemicals were purchased from Sigma and were of analytical grade or better.

NADPH−cytochrome P450 reductase was purified from rat liver microsomes with a modified method as described previously (Dignam & Strobel, 1977). Reductase eluted from a DEAE A-25 column was directly applied to an affinity column of 2′,5′-ADP−agarose. The impurities and nonionic detergent Renex 690 were removed by extensively washing with 50 mM potassium phosphate (pH 7.5) containing 20% glycerol, 0.1 mM DTT, and 1 mM EDTA. Cytochrome P450 2B1 was purified from phenobarbital-induced rat liver microsomes by the method of Saito and Strobel (1981). Sodium cholate and nonionic detergent Renex 690 used in the purification procedure were removed by a hydroxylapatite column (Saito & Strobel, 1981).

Denaturation and Reactivation of P450 2B1 by Urea or GuHCl. Unless otherwise specified, P450 2B1 was incubated with denaturants (at the indicated concentrations) in 0.1 M potassium phosphate buffer (pH 7.5) at 4 °C for 24 h to ensure the establishment of denaturation equilibrium. For reactivation of the denatured P450, unfolded proteins were diluted 200−500-fold in 50 mM potassium phosphate buffer (pH 7.5) and incubated for 24 h at 4 °C.

Analytical Methods. Protein concentration was determined by bicinchoninic acid (Smith et al., 1985) using bovine serum albumin as standard. Benzphetamine demethylation activity of cytochrome P450 2B1 was assayed by fluorometry as described previously (Ryan et al., 1979) using a Perkin-Elmer LS-5 spectrofluorometer. The reconstitution assays were conducted with 50 pmol of P450, 0.4 unit of reductase, 0.5 mM dilauroylphosphatidylcholine, 0.5 mM NADPH, and 1 mM benzphetamine in 1 mL of 50 mM potassium phosphate buffer (pH 7.7). The reaction was initiated by addition of NADPH. The reaction supported by cumene hydroperoxide was carried out by replacing the reductase and NADPH with 2 mM cumene hydroperoxide (Nordblom et al., 1976). In the denaturation studies, the indicated concentrations of denaturants were added to the assay buffer and incubated for 24 h at 4 °C. Reduced carbon monoxide difference spectra of cytochrome P450 2B1 were recorded in 0.1 M potassium phosphate buffer (pH 7.5) containing 2% glycerol using an extinction coefficient of 91 mM^{-1} cm^{-1} (Omura & Sato, 1964). Protein intrinsic fluorescence spectra were taken in 50 mM potassium phosphate (pH 7.5) buffer on a Perkin-Elmer LS-5 spectrofluorometer. CD spectra were recorded in 10 mM potassium phosphate buffer (pH 7.5) on a Jasco 500C CD spectropolarimeter.

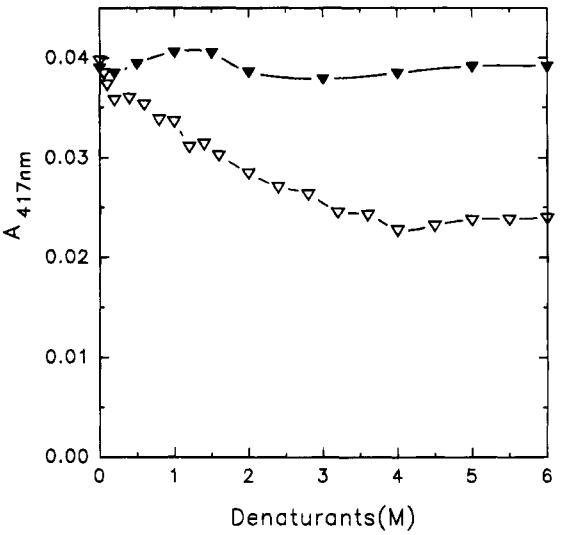

FIGURE 1: Effects of GuHCl and urea on the Soret peak of cytochrome P450 2B1. P450 (0.9 μM) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, with GuHCl (▽) or urea (▼) at the concentrations indicated for 24 h at 4 °C.

RESULTS

Effect of GuHCl and Urea on the Absorption Soret Peak of P450 2B1. The oxidized P450 2B1 shows an intense Soret absorption peak at 417 nm due to uniquely coordinated protoheme. The changes of Soret peak of P450 2B1 after incubation with GuHCl and urea are shown in Figure 1. The absorbance at 417 nm decreases as the concentration of GuHCl increases and reaches a maximal decrease (about 55% of the control) at 3 M GuHCl. No further decrease occurs when the GuHCl increases to 6 M, while the maximum absorbance peak shifts from 417 to 412 nm (data not shown). On the other hand, urea has no or only a slight effect on Soret absorption, as shown in Figure 1. Unlike cytochrome *b*5, in which the heme is completely detached when GuHCl reaches 4 M (Tajima et al., 1976), at the concentration of 6 M GuHCl or 8 M urea the heme of P450 2B1 seems to remain attached to the apoprotein. This was confirmed by reduced carbon monoxide difference spectra as shown in Figure 2. After incubation with 8 M urea, the absorption peak at 450 nm was completely shifted to 420 nm. The same result occurs with 6 M GuHCl (data not shown). Cyto-

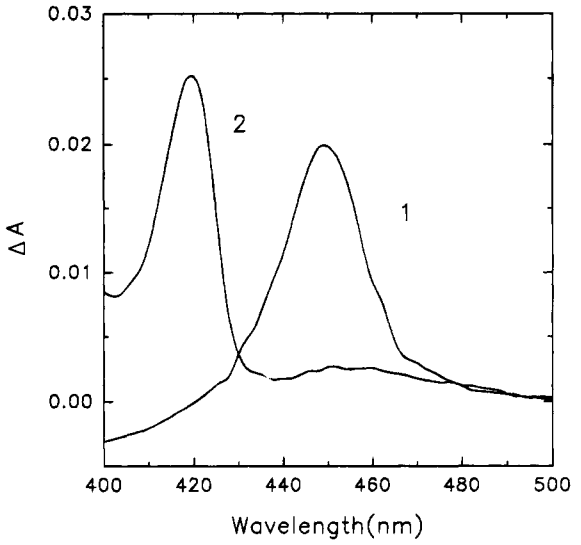

FIGURE 2: Reduced carbon monoxide difference spectra of cytochrome P450 2B1 before or after treatment with urea. P450 (0.4 μM) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, with 0 (curve 1) and 8 M (curve 2) urea for 24 h at 4 °C. Reduced carbon monoxide difference spectra were recorded according to the method of Omura and Sato (1964).

chrome P420 is an altered form of P450, in which one of the six ligands is replaced by another chemical compound or amino acid residue, but the heme is still attached to the apoprotein. Free heme incubated instead of cytochrome P450 with GuHCl or urea under the same conditions shows no peak at 420 nm. Although the heme is noncovalently bound to the protein, it is located in a deep hydrophobic pocket, surrounded by two α-helices. Thus, it is easier to understand that no heme seems to detach at lower concentrations of denaturants. But at higher concentrations of urea or GuHCl, the apoprotein is extensively unfolded, and the helical structure is significantly reduced. How the heme can still remain attached to P450 is a very interesting question. The reduced carbon monoxide difference spectra were recorded after the cytochrome proteins were incubated with denaturants for 24 h, so the heme attachment is not due to the protection of carbon monoxide and reductants. One explanation could be the broad hydrophobic interactions between the heme rings and the amino acid residues shown in the crystal structures (Ravichandran et al., 1993; Poulos et al., 1985).

Inactivation of P450 2B1 Prior to Detectable Conformational Change of Active Site. The data of Figure 3 present the decrease in benzphetamine demethylation activity and in the P450 content caused by treatment with GuHCl or urea. GuHCl is a more effective denaturant in inactivation of P450 than urea. At lower concentrations of GuHCl (<0.2 M), the activities supported by cumene hydroperoxide and NADPH−cytochrome P450 reductase decrease simultaneously, but the activity reconstituted with reductase becomes more sensitive to denaturant concentration than the cumene hydroperoxide-supported P450 activity when the concentration of GuHCl increases. The P450 activities supported by cumene hydroperoxide and reconstituted with reductase are completely lost at 1 and 0.5 M GuHCl, respectively. The loss of reconstituted system activity is not due to inactivation of reductase, because at 0.5 M GuHCl, reductase retains 50% of its activity, and complete inactivation of reductase occurs only at 1 M and higher concentration of GuHCl (Yu and

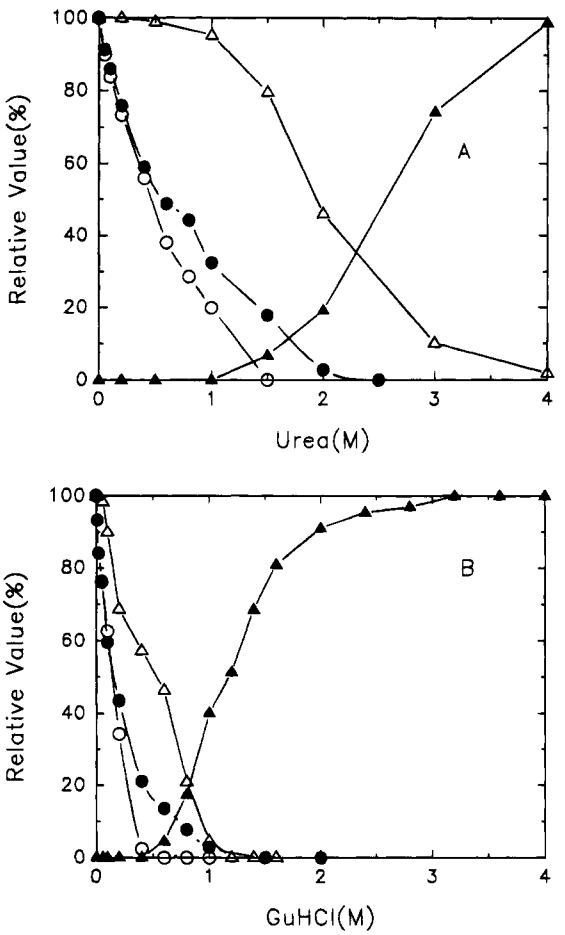

FIGURE 3: Inactivation of benzephetamine N-demethylation activity and conversion of P450 2B1 to P420 by urea (A) and GuHCl (B). Benzephetamine N-demethylation of P450 reconstituted with NADPH−cytochrome P450 reductase (○) or supported by cumene hydroperoxide (●) were assayed as described under Materials and Methods. P450 (△) and P420 (▲) content were determined as described in the legend to Figure 2 except with different concentrations of urea (A) and GuHCl (B) as indicated.

Strobel, unpublished data). Compromise of electrostatic interactions between P450 and reductase was suggested by our previous work (Strobel et al., 1989; Nadler & Strobel, 1991; Shen & Strobel, 1993) and by that of others (Stayton & Sligar, 1990) as a possible reason for the inactivation of the reconstituted system activity. That the reconstituted system is more sensitive to GuHCl than P450 or reductase alone could be explained by the disruption of the electron transfer from reductase to P450 by GuHCl. In addition to being a strong denaturant, GuHCl is also a strong electrolytic salt and thus can destroy the electrostatic interactions between proteins as shown in a recent paper (Yu et al., 1994). In the case of urea, the change of activity and P450 content were very similar to GuHCl denaturation except higher concentrations of urea are required to achieve the same degree of change. Complete inactivation of NAPDH−P450 reductase-supported and cumene hydroxyperoxide-supported activity occurs at 1.5 and 2.5 M urea, respectively. When the concentration of urea is greater than 0.5 M, the activity of the reconstituted system composed of P450 and reductase is also more sensitive to urea than the activity of the cumene hydroperoxide-supported P450 system, suggesting that other forces in addition to electrostatic interaction also exist between P450 and reductase as proposed by Voznesensky and Schenkman (1992a,b). In both the denaturants, P450

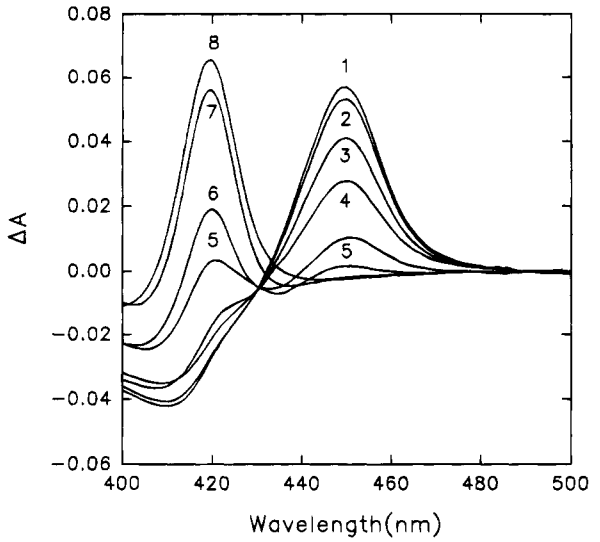

FIGURE 4: Effect of GuHCl on cytochrome P450 reduced CO difference spectra. P450 (0.65 μM) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, containing (1) 0, (2) 0.1, (3) 0.4, (4) 0.6, (5) 0.8, (6) 1.0, (7) 1.6, or (8) 3 M GuHCl for 24 h at 4 °C. Reduced carbon monoxide difference spectra were recorded as described in the legend to Figure 2.

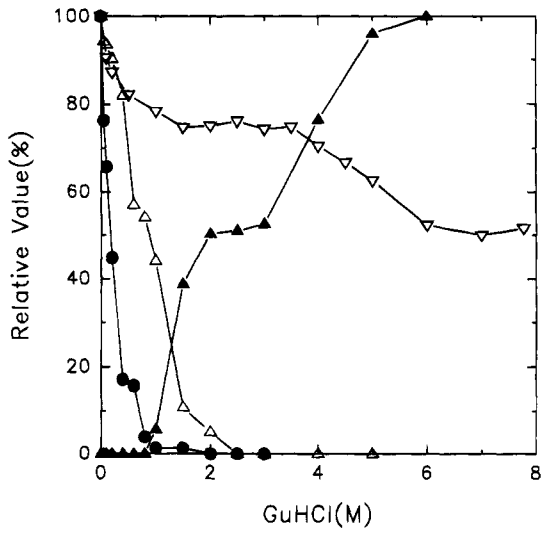

FIGURE 5: Conversion of cytochrome P450 to P420 by GuHCl. Experimental conditions were the same as described for Figure 3, except that the incubation time was shortened to 30 min. (●) Activity of P450 supported by cumene hydroperoxide, (△) P450 content, (▲) P420 content, (▽) absorbance at 417 nm.

contents decrease at higher concentrations of denaturants than do the activities. This is especially true of urea. At 1 M urea, the activity decreases significantly but P450 content only decreases slightly (Figure 3A). This is consistent with the results of Ichikawa et al. (1969) with microsomal P450 treated with sodium cholate but is in disagreement with the findings of parallel change between benzpyrene hydroxylation activity and P450 content (Silverman & Talalay, 1967). In microsomes, cytochrome P450 is probably protected by lipid and (or) other proteins, and it is possible that no or slight conformational changes result from treatment with lower concentration of denaturants. In the case of purified P450s, the protection by lipid and (or) other proteins is eliminated and the inactivation occurs at lower concentration of denaturants than does the loss of the 450-nm absorption peak. Comparing Figure 3 with Figure 1 reveals that the decrease in the Soret peak at 417 nm requires a much higher concentration of denaturants than does the loss of the absorbance peak in the reduced carbon monoxide difference spectrum at 450 nm. These results show that the presence of the Soret peak absorbance, even the presence of the P450 absorbance, does not guarantee that cytochrome P450 is catalytically competent. In other words, slight changes in the active center which have no effect on heme binding and heme ligands could result in the disruption of electron transfer and (or) the reduction of catalytic activity.

Intermediate State of Cytochrome P450 2B1. When cytochrome P450 was incubated with urea, the disappearance of P450 absorbance was accompanied by the appearance of P420 absorbance in the reduced carbon monoxide difference spectrum. In other words, cytochrome P450 was almost fully converted to P420 in one step (Figure 3A). The case is different with GuHCl as denaturant, in which the P450 absorbance decreases at low GuHCl concentration without the appearance of P420 absorbance (Figure 4). At 0.6 M GuHCl, for an example, P450 decreases to 50% but no obvious P420 peak appears at this point. At lower concentrations of GuHCl, along with the decrease of the P450 peak, an isosbestic point at 432 nm was observed, but at higher GuHCl concentrations, no obvious isosbestic point exists because the P450 peak disappears completely and only P420 absorbance increases under these conditions. The most likely explanation of these results is that there exists a metastable intermediate state of heme conformation, which does not display absorption at 420 nm and has no or low absorption at 450 nm. The case becomes more clearly demonstrable when the incubation time was shortened to 30 min (Figure 5) and before the denaturation reaches final equilibrium. The loss of benzphetamine demethylation activity after incubation for 30 min is almost the same as that after incubation for 24 h. The appearance of P420 requires much higher concentrations of GuHCl. Conversion to P420 begins at 1 M GuHCl (compared to 0.6 M in the case of a 24-h incubation) and is completed at 5 M GuHCl rather than at 2 M GuHCl when the incubation time was 24 h. As is true of the native P450, the intermediate and the fully denatured P450 are reducible by dithionite. The reductions result in a shift of the Soret absorbance peak from 417 to 422 nm and changes in the amplitudes of the absorbance peaks (data not shown).

As shown in Figure 3A, no obvious intermediate is observed upon the denaturation with urea. One of the major differences between urea and GuHCl is that urea does not affect the ionic strength of the solution. Therefore we examined the effect of NaCl on the urea denaturation. An intermediate state of denaturation was observed as shown in Figure 6. The intermediate state of the active site seems to be stabilized by NaCl and becomes detectable in the presence of 1 M NaCl.

Unfolding of the Whole Protein by GuHCl and Urea. The data of Figure 7 show the observable conformational change of the whole protein upon exposure to urea. At low concentrations of urea (<1.5 M), no obvious change is observed either in the CD spectra or in the intrinsic fluorescence spectra. The negative peak in the CD spectrum at 222 nm begins to decrease at 1.5 M urea and reaches maximal change at 3 M urea, where the molar ellipicity at 222 nm becomes almost 0, suggesting that all α-helices were unfolded at this point. On the other hand the intrinsic fluorescence begins to increase at 2 M urea and continues

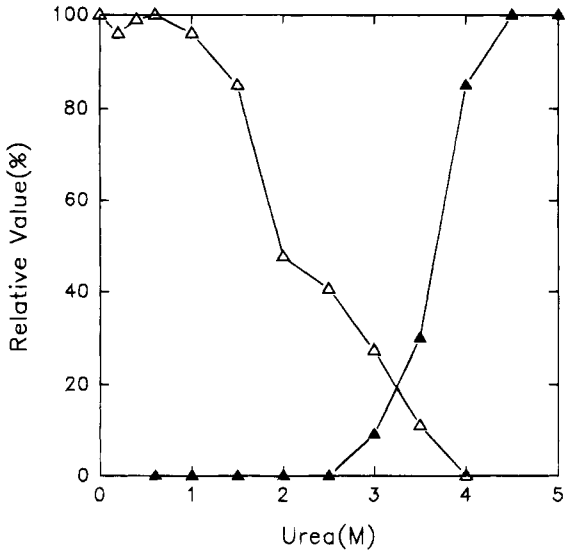

FIGURE 6: Effect of NaCl on urea denaturation. P450 (0.65 μM) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, containing 1 M NaCl and indicated concentrations of urea for 24 h at 4 °C. P450 (△) and P420 (▲) were determined as described in the legend to Figure 2.

increasing until the concentration of urea reaches 8 M, indicating that extensive unfolding is far behind the collapse of the α-helices. The situation observed upon GuHCl treatment is almost the same as that with urea treatment except that the changes occur at a lower concentration of GuHCl. The molar ellipicity at 222 nm of P450 begins to decrease at 1 M GuHCl and reaches a maximal change at 2 M GuHCl in CD spectra. The intrinsic fluorescence begins to increase at 1 M GuHCl and continues increasing until a maximal level of change is reached at 6 M GuHCl (Figure 8). There is no obvious difference between the CD spectra of P450 incubated with or without 1 M GuHCl.

When comparing the inactivation of P450 and the decrease of P450 content with the unfolding of the whole protein, it is clear that the inactivation of P450 occurs at much lower concentrations of denaturants than does the loss of the P450 carbon monoxide difference spectral absorbance peak, and the latter occurs at a lower concentration of denaturants than observable changes of protein conformation. In other words, the active site is more sensitive to denaturants than are other regions of the apoprotein.

Reactivation of P450 2B1. The possibility of reactivation of denatured cytochrome P450 2B1 was also investigated. The protein denatured by treatment with 6 M GuHCl or 8 M urea tends to aggregate and form a visible precipitate when the denaturants are removed by dialysis. An orange-yellow color is observed in the protein precipitate, and the supernatant is very clear without any absorbance at 412 nm. These observations strongly support the notion that the heme is in some way attached to and can be coprecipitated with the apoprotein.

Reactivation of P450 denatured by treatment with low concentrations of denaturants was possible (Figure 9). Cytochrome P450 inactivated by 1 M GuHCl or 2 M urea could be completely renatured by extensive dilution. When the P450 denatured by concentrations higher than 2 M GuHCl or 5 M urea, no reactivation could be observed on dilution (200−500-fold). The results are consistent with the explanations that either the reactivation of denatured P450 requires some residual native structure of the apoprotein as a refolding guide or that extensive unfolding favors aggregation of the protein.

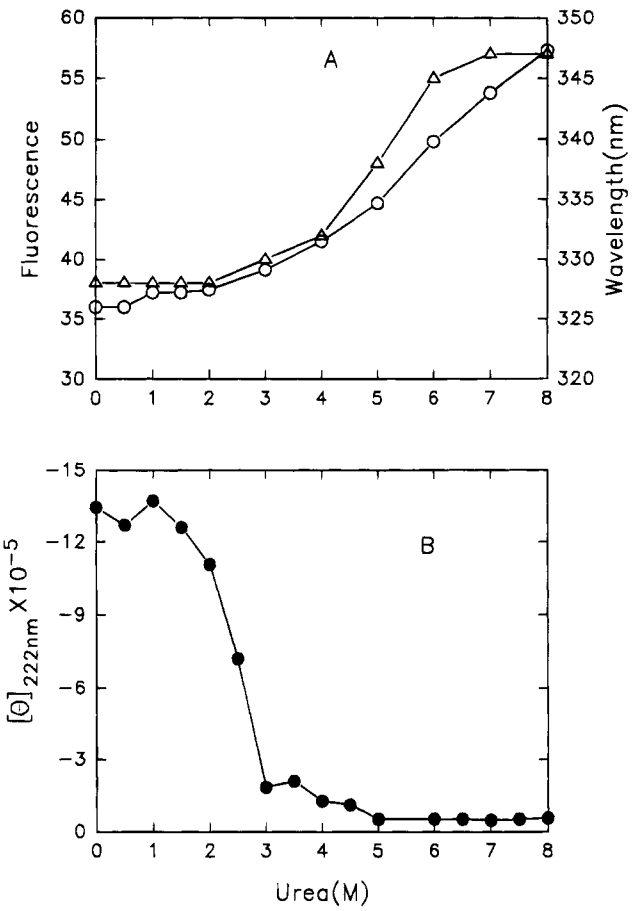

FIGURE 7: Conformation changes in cytochrome P450 2B1 by urea treatment. (A) Effect of urea on the intrinsic fluorescence of cytochrome P450 2B1. P450 (0.65 μM) was incubated in 0.1 M potassium phosphate buffer, pH 7.5, containing the indicated concentrations of urea for 24 h at 4 °C. (○) Increase of intrinsic fluorescence; (△) shift of the maximal emission fluorescence peak. Excitation wavelength is 278 nm. (B) Effect of urea on the molar ellipicity of P450 2B1 at 222 nm in the CD spectra. P450 (0.65 μM) was incubated in 0.01 M potassium phosphate buffer, pH 7.5, containing the indicated concentrations of urea for 24 h at 4 °C.

DISCUSSION

The three-dimensional structures of P450cam from *Pseudomonas putida* and P450 BM-3 from *Bacillus megaterium* show the heme binding and the active sites of these two P450s are in a quite similar pattern in spite of low sequence homology (Poulos et al., 1985; Ravichandran et al., 1993). Thus it is possible that many kinds of P450s share a common three-dimensional structural theme for heme binding. Unlike other hemoproteins, the heme of cytochrome P450 is not directly accessible from the surface of the protein. It is located in a heme pocket, of which the heme edge is within about 8 Å of the protein surface (Poulos et al., 1985). Although the conversion of P450 in microsomes to P420 by detergents, organic solvents, sulfhydryl reagents, and denaturants is extensively studied, the denaturation course for purified cytochrome P450 protein could be quite different. Since the cytochrome P450 in microsomes is anchored in membrane, it may be protected by lipid and possibly by other proteins from the disturbance of denaturing agents. The

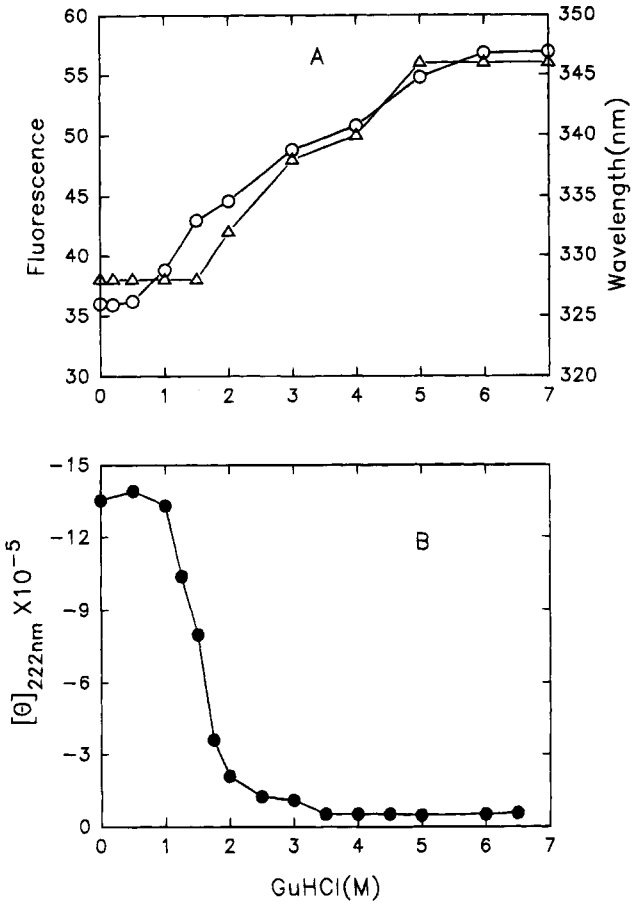

FIGURE 8: Conformation changes in cytochrome P450 2B1 by GuHCl treatment. Experimental conditions were the same as Figure 7 except GuHCl was used as a denaturant.

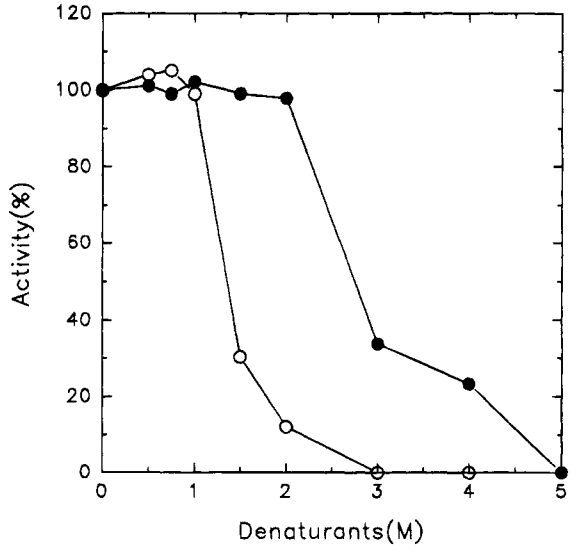

FIGURE 9: Reactivation of denatured cytochrome P450 2B1. Cytochrome P450 2B1 (0.5 μM) denatured by GuHCl (○) or urea (●) at the concentration indicated was diluted 200-fold with 50 mM potassium phosphate buffer (pH 7.5) and incubated at 4 °C for 24 h. Other experimental conditions were the same as described for Figure 3.

results presented here suggest that there are two phases of change in the active site of P450 following treatment with GuHCl or urea. First, an undefined change in the active center causes inactivation of P450. Second, the disturbance of heme ligands results in conversion of P450 to P420. In the case of GuHCl, one metastable intermediate is strongly suggested in the second step. In the metastable state, the manner of heme binding to apoprotein fits neither the spectral requirements for P450 nor those for P420. The metastable intermediate has no or low absorption at 450 nm and has no absorption at 420 nm in the reduced carbon monoxide difference spectra. The metastable intermediate seems to exist in the range of 0−1 M GuHCl, where the P450 peak decreases rapidly and only a slight increase of P420 peak appears by a concentration of 0.6 M GuHCl. At 1 M GuHCl or 2 M urea, almost no obvious conformational change of whole protein is observed, and the inactivation (denaturation) is completely reversible. This suggests that the only difference between the metastable intermediate and the native P450 is a slight change in the active site. This result is consistent with the previous report that there is a reversible transition state in the pressure denaturation of cytochrome P450cam (Hui Bon Hoa et al., 1989). The fact that this intermediate does not appear upon urea denaturation but becomes detectable in the presence of 1 M NaCl suggested that the intermediate seems to be stabilized by NaCl. That P450 protein unfolded at 3 M GuHCl or 5 M urea failed to be renaturable is consistent with the idea that the helices around the heme are probably necessary for the reactivation of the protein.

The relatively faster inactivation of the active site and slower change of the whole protein conformation have been found in many enzymes (Ho & Tsou, 1979; Yao et al., 1984; Tsou, 1986; Lin et al., 1990; Ma & Tsou, 1991; Zhou et al., 1993; Xiao et al. 1993). Tsou suggested that the interactions forming the active site are relatively weaker and the structural conformation in the active site is more flexible than the other parts of the enzyme (Tsou, 1986, 1993). But it is usually not easy to detect changes of the active-site conformation directly. In this study, heme proves to be a sensitive probe of the active site of P450. The results described above provide good evidence supporting Tsou's assertion that the active site of enzyme is more sensitive to denaturants than other parts of the protein.

ACKNOWLEDGMENT

We express our appreciation to Drs. Palmer and Kamensky of Rice University for assistance in recording the CD spectra and to Dr. Chyau Liang for his thoughtful reading of our manuscript. We owe our thanks to Laura Bankey for her technical assistance.

REFERENCES

Dignam, J. D., & Strobel, H. W. (1977) *Biochemistry 16*, 1116−1123.

Fisher, M. T., Scarlata, S. F., & Sligar, S. G. (1985) *Arch. Biochem. Biophys. 240*, 456−463.

Ho, Y.-S., & Tsou, C.-L. (1979) *Nature 277*, 245−246.

Hui Bon Hoa, G., Di Primo, C., Dondaine, I., Sligar, S. G., Gunsalus, I. C., & Douzou, P. (1989) *Biochemistry 28*, 651−656.

Hui Bon Hoa, G., Di Primo, C., Geze, M., Douzou, P., Kornblatt, J. A., & Sligar, S. G. (1990) *Biochemistry 29*, 6810−6815.

Ichikawa, Y., & Yamano, T. (1967a) *Biochim. Biophys. Acta 147*, 518−528.

Ichikawa, Y., & Yamano, T. (1967b) *Biochim. Biophys. Acta 131*, 490−497.

Ichikawa, Y., Yamano, T., & Fujishima, H. (1969) *Biochim. Biophys. Acta 171*, 32−46.

Imai, Y., & Sato, R. (1966) *Biochem. Biophys. Res. Commun. 22*, 620−626.

Imai, Y , & Sato, R. (1967) *Eur. J. Biochem. 1*, 419−426.
Lin, Y.-Z., Liang, S.-J., Zhou, J.-M., Tsou, C.-L., Wu, P.-Q., & Zhou, Z.-K. (1990) *Biochim. Biophys. Acta 1038*, 240−246.
Ma, Y.-Z., & Tsou, C. L. (1991) *Biochem. J. 277*, 207−211.
Marden, M. C., & Hui Bon Hoa, G. (1986) *Biophys. J. 49*, 619−627.
Mason, H. S., North, J. C., & Vanneste, M. (1965) *Fed. Proc. 24*, 1172−1180.
Murakami, K., & Mason, H. S. (1967) *J. Biol. Chem. 242*, 1102−1110.
Nadler, S. G., & Strobel, H. M. (1991) *Arch. Biochem. Biophys. 290*, 277−284.
Nordblom, G. D., White, R. E., & Coon, M. J. (1976) *Arch. Biochem. Biophys. 175*, 524−533.
Omura, T., & Sato, R. (1964) *J. Biol. Chem. 239*, 2379−2385.
Peisach, J., Stern, J. O., & Blumberg, W. E. (1973) *Drug. Metab. Disp. 1*, 45−57.
Poulos, T. L., Finzel, B. C., Gunsalus, I. C., Wagner, G. C., & Kraut, J. (1985) *J. Biol. Chem. 260*, 16122−16130.
Poulos, T. L., Finzel, B. C., & Howard, A. J. (1987) *J. Mol. Biol. 195*, 687−700.
Ravichandran, K. G., Boddupalli, S. S., Hasemann, C. A., Peterson, J. A., & Deisenhofer, J. (1993) *Science 261*, 731−736.
Ryan, D. E., Thomas, P. E., Korzeniowski, D., & Levin, W. (1979) *J. Biol. Chem. 254*, 1365−1374.
Saito, T., & Strobel, H. W. (1981) *J. Biol. Chem. 256*, 984−988.
Shen, S., & Strobel, H. M. (1993) *Arch. Biochem. Biophys. 304*, 257−256.
Silverman, D. A., & Talalay, P. (1967) *Mol. Pharmacol. 3*, 90−101.
Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Coeke, N. M., Olson, B. J., & Klenk, D. C. (1985) *Anal. Biochem. 150*, 76−85.
Stayton, P. S., & Sligar, S. G. (1990) *Biochemistry 29*, 7381−7386.
Strobel, H. M., Nadler, S. G., & Nelson, D. R. (1989) *Drug. Metab. Rev. 20*, 519−533.
Tajima, S., Enomoto, K.-I., & Sato, R. (1976) *Arch. Biochem. Biophys. 172*, 90−97.
Tsou, C.-L. (1986) *Trends. Biochem. Sci. 111*, 426−429.
Tsou, C.-L. (1993) *Science 262*, 380−381.
Uvarov, V. Y., Tretiakov, V. E., & Archakov, A. I. (1990) *FEBS Lett. 260*, 309−312.
Voznesensky, A. I., & Schenkman, J. B. (1992a) *J. Biol. Chem. 267*, 14669−14676.
Voznesensky, A. I., & Schenkman, J. B. (1992b) *Eur. J. Biochem. 210*, 741−746.
Xiao, J., Liang, S.-J., & Tsou, C.-L. (1993) *Biochim. Biophys. Acta 1164*, 54−60.
Yao, Q.-Z., Tian, M., & Tsou, C.-L. (1984) *Biochemistry 23*, 2740−2744.
Yu, X.-C., Wang, C.-C., & Tsou, C.-L. (1994) *Biochim. Biophys. Acta 1207*, 109−113.
Zhou, H.-M., Zhang, X.-H., Yin, Y., & Tsou, C.-L. (1993) *Biochem. J. 291*, 103−107.

BI9423502