Reversible Pressure Deformation of A Thermophilic Cytochrome P450 Enzyme (CYP119) and Its Active-Site Mutants

Richard A. Tschirret-Guth,[†] **Laura S. Koo,**[†] **Gaston Hui Bon Hoa,**[‡] **and Paul R. Ortiz de Montellano***[,†]

Contribution from the Department of Pharmaceutical Chemistry, School of Pharmacy, University of California, San Francisco, CA 94143-0446, and Unité 473, Institut National de la Santé et de la Recherche Médicale (INSERM), 84 rue du Général Leclerc, 94276 Le Kremlin Bicêtre Cedex France

Received November 13, 2000

Abstract: The pressure stability of the thermophilic CYP119 from *Sulfolobus solfataricus* and its active-site Thr213 and Thr214 mutants was investigated. At 20 °C and pH 6.5, the protein undergoes a reversible P450-to-P420 inactivation with a midpoint at 380 MPa and a reaction volume change of −28 mL/mol. The volume of activation of the process was −9.5 mL/mol. The inactivation transition was retarded, and the absolute reaction volume was decreased by increasing temperature or by mutations that decrease the size of the active-site cavity. High pressure affected the tryptophan fluorescence yield, which decreased by about 37% at 480 MPa. The effect was reversible and suggested considerable contraction of the protein. Aerobic decomposition of iron−aryl complexes of the CYP119 T213A mutant under increasing hydrostatic pressure resulted in variation of the *N*-arylprotoporphyrin-IX regioisomer (N_B:N_A:N_C:N_D) adduct pattern from 39:47:07:07 at 0.1 MPa to 23:36:14:27 at 400 MPa. Preincubation of the protein at 400 MPa followed by complex formation and decomposition gave the same regioisomer distribution as untreated protein. The results indicate that the protein is reversibly inactivated by pressure, in contrast to the irreversible inactivation of $P450_{cam}$ and other P450 enzymes, and that this inactivation process is modulated by changes in the active-site cavity dimensions.

Introduction

Cytochrome P450 enzymes catalyze a broad range of oxidative and reductive processes involved in fatty acid metabolism, sterol biosynthesis, and xenobiotic elimination.[1] This versatility, as well as the broad range of substrates, makes P450 enzymes potentially useful catalysts for bioreactors. Unfortunately, the P450 enzymes are, for the most part, relatively unstable, requiring that their thermal and piezostability be improved if they are to fulfill their promise as useful catalysts. The discovery of CYP119, the first cytochrome P450 enzyme isolated from an extremophilic organism, is therefore of great interest since it provides a starting point for the design of stable biocatalysts.

CYP119 was cloned from the acidothermophilic archaebacteria *Sulfolobus solfataricus,* a sulfur autotroph found in volcanic hot springs.[2] The optimal growth conditions for *S. solfataricus* were found to be 87 °C at pH 4.5.[3] Like most bacterial P450 enzymes, CYP119 is a soluble protein that lacks the hydrophobic tail generally found in mammalian isoforms. The recombinant protein heterologously expressed in *E. coli* was stable at pH 6.0 up to 85 °C [3,4] and withstood pressures up to 200 MPa (1 MPa = 9.872 atm) without forming the inactive P420 form.[3−5] In contrast, the mesophilic cytochrome $P450_{cam}$ precipitates above 60 °C and is inactivated at pressures above 130 MPa.[6]

A recent X-ray structure of CYP119 has shown that the overall fold of the protein is typical of a cytochrome P450 enzyme with two main differences: an unusual degree of flexibility in the loop connecting the F helix to the G helix and the presence of a large cluster of aromatic residues spanning ∼39 Å on the edge of the protein.[7]

It has been shown that high pressure destabilizes weak interactions such as hydrophobic bonds and salt-bridges that play important roles in maintaining the 3D protein structure.[8−11] The combination of high pressure with high temperature thus provides an interesting tool with which to probe the free energy differences of stabilization of protein structures, particularly the differences in the stabilities of the hyperthermophilic and barophilic versus mesophilic proteins.

Results

Effects of Pressure on the Absorption Spectrum of CYP119. The effects of hydrostatic pressure on the absorption spectrum of wild-type CYP119 at 20 °C are shown in Figure

† University of California.
‡ Institut National de la Santé et de la Recherche Médicale.

(1) Ortiz de Montellano, P. R. *Cytochrome P450: Structure, Mechanism and Biochemistry,* 2nd ed.; Ortiz de Montellano, P. R., Ed.; Plenum Press: New York, 1995; pp 473−574.
(2) Wright, R. L.; Harris, K.; Solow, B.; White, R. H.; Kennelly, P. J. *FEBS Lett.* **1996**, *384*, 235−239.
(3) McLean, M. A.; Maves, S. A.; Weiss, K. E.; Krepich, S.; Sligar, S. G. *Biochem. Biophys. Res. Commun.* **1998**, *252*, 166−172.
(4) Koo, L. S.; Tschirret-Guth, R. A.; Straub, W.; Moënne-Loccoz, P.; Loehr, T. M.; Ortiz de Montellano, P. R. *J. Biol. Chem.* **2000**, *275*, 14112−14123.
(5) Tschirret-Guth, R. A.; Koo, L.; Ortiz de Montellano, P. R. *J. Biol. Inorg. Chem.* **2000**, *5*, 204−212.
(6) Hui Bon Hoa, G.; Di Primo, C.; Dondaine, I.; Sligar, S, G.; Gunsalus, I. C.; Douzou, P. *Biochemistry* **1989**, *28*, 651−656.
(7) Yano, J. K.; Koo, L. S.; Schuller, D. J.; Li, H.; Ortiz de Montellano, P. R.; Poulos, T. L. *J. Biol. Chem.* **2000**, *275*, 31086−31092.
(8) Morild, E. *Adv. Protein Chem.* **1981**, *34*, 93−166.
(9) Jeanike R. *Eur. J. Biochem.* **1991**, *202*, 715−728.
(10) Weber G. *Protein Interactions*; Chapman and Hall: New York, London, 1992.
(11) Mozhaev, V. V.; Heremans, K.; Frank, J.; Masson, P.; Balny, C. *Proteins* **1996**, *24*, 81−91.

10.1021/ja003947+ CCC: $20.00 © 2001 American Chemical Society
Published on Web 03/27/2001

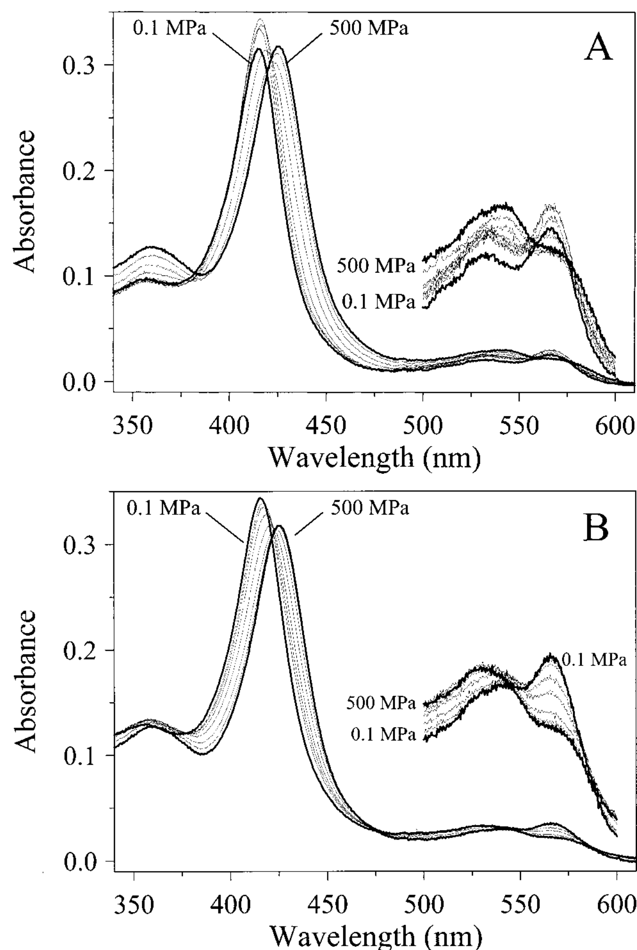

Figure 1. UV−vis spectra of CYP119 as a function of the hydrostatic pressure at 20 °C. Each spectrum was taken 2 min after pressure adjustment; (A) 0.1, 100, 200, 300, 350, 400, 450, 500 MPa; (B) 500, 450, 400, 350, 300, 200, 100, and 0.1 MPa.

1. Each spectrum was taken 2 min after pressure stabilization. At atmospheric pressure, the spectrum of CYP119 has a Soret absorbance at 415 nm with α and β bands at 530 and 565 nm, characteristic of a low-spin hexacoordinated heme iron. Somewhat unusual is the fact that the α band has a greater intensity than the β band. Increasing the pressure from 0.1 to 200 MPa induced an initial hyperchromic effect followed by a red-shift of the Soret absorbance peak to 425 nm (Figure 1A). The β band increases in intensity with pressure and then shifts to 540 nm, whereas the α band increases, then decreases, in intensity and shifts to 570 nm. The initial increases in absorbance of the α and β bands are due to an increase in sample concentration due to pressure-induced volume contraction. On the basis of previous reports for $P450_{cam}$,[6] $P450_{BM-3}$,[12] and CYP2B4[13] and the fact that similar changes are observed when the CYP119 P420 is obtained by incubating the enzyme at pH 10.8, these spectral changes have been attributed to conversion of the enzyme to the inactive P420 form. Assuming a simple P450-to-P420 transition without intermediate species, the fraction of P420 present as a function of the pressure is shown in Figure 2. The measured molar reaction volume change ($\Delta V^o = V_{P420} - V_{P450}$) and the pressure of half inactivation ($P_{1/2}$) were determined to be −28 mL/mol and 380 MPa respectively (Table 1). The rate of the inactivation transition could be determined by pressure-jump experiments following the changes in absorbance at 415 nm as a function of time. At all pressures single-exponential curves were obtained (data not shown), suggesting that no intermediates intervened in the P450-to-P420 transition. The plot of $\ln(k_{inact})$ as a function of pressure (Figure 2, insert) was linear, and the activation volume ($\Delta V^{\ddagger}$), determined using eq 2, was −9.5 mL/mol. At pressures above 550 MPa, there was an irreversible increase in the baseline accompanied by some irreversible heme bleaching.

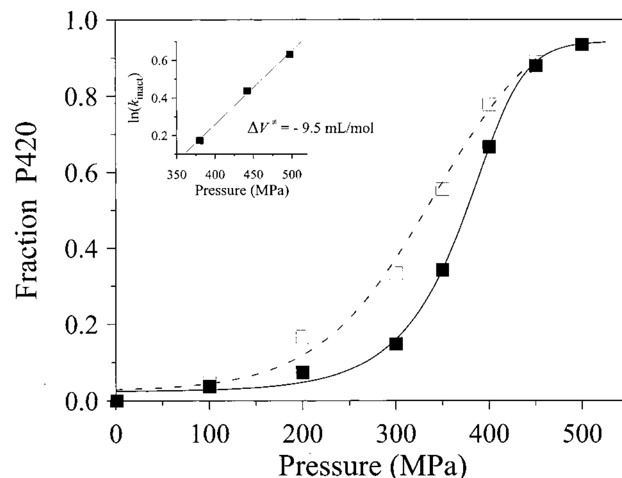

Figure 2. Inactivation profile of CYP119 as a function of pressure. The data were calculated from Figure 1. (■) Pressurization; (□) depressurization. Insert: Plot of $\ln(k_{inact})$ as a function of pressure at 20 °C.

Table 1. Half Inactivation Pressure and Reaction Volume for CYP119 and Several Mesophilic P450 Enzymes

	temperature (°C)	$P_{1/2}$ (Mpa)	ΔV^o (mL/mol)
CYP119 wild type[a]	5	320	−20
	20	380	−28
	35	430	−25
	50	480	−17
T213A	20	350	−19
T213S	20	350	−20
T213V	20	380	−28
T213F	20	550	−7
T213W	20	570	−6
T214A	20	390	−17
T214V	20	360	−21
T213A/T214A	20	480	−9
$P450_{cam}$[b]	4	110	−35
$P450_{cam}$[c]	4	140	−153
$P450_{lin}$[b]	4	>300	ND
$P450_{BM3}$[d]	25	210	−99
CYP 2B4[e]	25	150	−86

[a] This work. [b] Reference 6. [c] In the presence of 32 mM cysteine.[24] [d] Reference 32. [e] Reference 13.

Unexpectedly, upon decompression from 500 MPa, the protein rapidly and completely returned to its active P450 state (Figures 1B and 2). To confirm the reversibility of the pressure-induced inactivation, the ferrous-CO spectrum of CYP119 was obtained after depressurization and was found to have a maximum at 450 nm with no component at 420 nm (data not shown).

The stability of the ferrous-CO form of CYP119, which exhibited a maximum absorbance at 450 nm, was also examined (Figure 3). Only minor changes were observed from 0.1 to 300 MPa with a small increase at 420 and 450 nm that were attributed to solvent compression. At 400 MPa the peak at 420 nm became more prominent and was accompanied by an

(12) Davydov, D. R.; Hui Bon Hoa, G.; Peterson, J. A. *Biochemistry* **1999**, *38,* 751−761.
(13) Davydov, D. R.; Deprez, E.; Hui Bon Hoa, G.; Knyushko, T. V.; Kuznetsova, G. P.; Koen, Y. M.; Archakov, A. I. *Arch. Biochem. Biophys.* **1995**, *320,* 330−344.

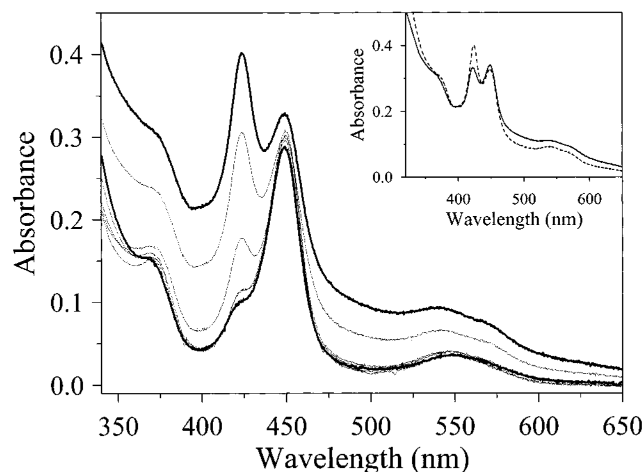

Figure 3. Pressure-induced changes in the absorbance spectrum of ferrous-CO CYP119 at 20 °C: P = 1, 100, 200, 300, 400, 500 MPa. Insert: (- - -) 500 MPa, (—) after return to 0.1 MPa.

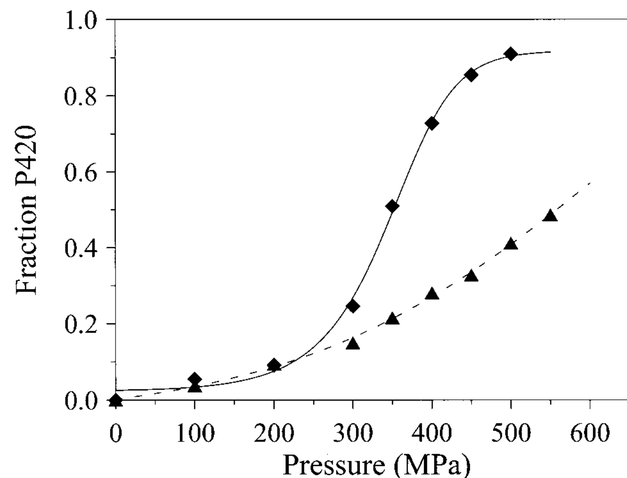

Figure 4. Inactivation profile for the ferric CYP119 T213S (◆) and T213W (▲) mutants at 20 °C.

increase in turbidity at higher pressure, which indicated that the P420 form was unfolding and exposing hydrophobic groups to the solvent. When the pressure was released to 0.1 Mpa, there was no return to the P450 form (Figure 3, Insert). The data suggest that at 400 MPa the P420−CO complex is highly unstable and readily denatures and thus that the reduced P420−CO complex is less flexible than the oxidized P420 form. There are few data in the literature concerning the relationship between pressure-induced inactivation, compressibility, and unfolding of cytochrome P450. The pressure-induced P420cam−CO complex was very stable under pressures up to 500 MPa, whereas the $P450_{lin}$−CO complex was converted concomitantly into $P420_{lin}$−CO and unfolded forms.[14] By analyzing the pressure-induced shift in the position of the Soret band it was shown that the active site of $P450_{cam}$−CO was very compressible.[15] Compressibility of the active site is apparently correlated with its capacity to resist hydrostatic compression and pressure-induced unfolding.

Effect of Temperature. CYP119 withstands temperatures up to 80 °C without conversion to P420, loss of activity, or significant changes in the overall fold of the protein.[3,4] The effects of temperature on the pressure-induced inactivation parameters ($P_{1/2}$ and ΔV^{o}) are shown in Table 1. High temperature stabilized CYP119, and $P_{1/2}$ increased linearly at 3.56 MPa/°C between 5 and 50 °C. This observation is in agreement with the general stabilizing effect of pressure on protein structure at elevated temperature.[16] Changes in the reaction volume as a function of temperature did not mirror the changes at $P_{1/2}$. There was an absolute maximum of ΔV^{o} at 20 °C and lower values at either lower or higher temperature. These results suggest that the effect of the temperature on ΔV^{o} is multifactorial.

Active-Site Mutants. Alignment of the sequence of CYP119 with the sequences of P450 enzymes of known crystal structure suggested that either Thr213 or Thr214 was in the vicinity of the heme, an inference subsequently confirmed by the crystal structure of the protein.[7] We have shown by mutation of these residues that Thr213 serves as a catalytic residue, whereas Thr214 is noncatalytic but influences the spin state of the protein.[4] In light of these observations we have also examined the effect of the mutations on the pressure-induced inactivation of CYP119 (Figure 4 and Table 1). All mutations at Thr213 and Thr214 caused a decrease in the absolute volume of the transition. However, the effect was more pronounced when Thr213 was mutated to a larger amino acid or when Thr213 and Thr214 were both replaced by alanines.

The replacement of Thr213 with residues that decreased the active-site steric encumbrance had little or no effect on the pressure of half inactivation but reduced the reaction volume. On the other hand, mutation to tryptophan or phenylalanine, which reduced the open volume of the active site, retarded considerably the pressure-induced inactivation. Mutations of Thr214 had practically no effect on the conversion to P420. Noteworthy, however, was the non-additive effect of the double mutation T213A/T214A on the reaction parameters with respect to the corresponding single mutations.

Fluorescence. CYP119 contains tryptophan residues at positions 4, 21, 147, 231, and 281. The recently determined crystal structure[7] indicates that they are distributed around the heme at distances of 23, 25, 20, 22, and 18 Å from the heme iron and that the respective angles between the Trp transition dipoles and the heme plane, θ, are 164, 32, 103, 110, and 89°. In heme proteins, the fluorescence of tryptophan can be greatly altered due to energy transfer to the heme group.[17] The energy-transfer rate is proportional to the inverse sixth power of the distance from the fluorophore to the heme and proportional to the orientation factor of the Trp residues. Therefore, it is expected that changes in protein conformation and compressibility will modify the overall tryptophan emission spectrum. The fluorescence spectrum of CYP119 at 0.1 MPa and 20 °C, showed an emission maximum at 338 nm with a very weak shoulder at 396 nm. This maximum corresponds to a 4 nm red-shift from tryptophan buried in a hydrophobic pocket and is in agreement with the structure, which shows that only Trp4 is partially solvent-exposed.[7] The tryptophan fluorescence yield of CYP 119 was low (0.003) compared to the yield observed for $P450_{cam}$ (0.008), $P450_{lin}$ (0.014), and free tryptophan (0.20). Single mutations to alanine had little effect on the fluorescence properties of CYP119 (Table 2). The T213W and T213A/T214A mutants, however, showed an important red-shift of their fluorescence maximum, along with an increased shoulder at 396 nm. These observations suggest that there was a significant alteration in the protein structure, causing some of the tryptophan

(14) Hui Bon Hoa, G.; Di Primo, C.; Dondaine, I.; Sligar, S. G.; Gunsalus, I. C.; Douzou, P. *Biochemistry* **1989**, *28*, 651−656.
(15) Jung, C.; Hui Bon Hoa, G.; Davydov, D.; Gill, E.; Heremans, K. *Eur. J. Biochem.* **1995**, *233*, 600−606.
(16) Zipp, A.; Kauzman, W. *Biochemistry* **1973**, *12*, 4217−4228.
(17) Marden, M. C.; Hui Bon Hoa, G.; Stetzkowski-Marden F. *Biophys. J.* **1986**, *49*, 619−627.

Table 2. Fluorescence Properties of Wild-Type CYP119 and Several Mutants

	emission max (nm)	shoulder (nm)	Φ_O (20 °C)	Φ_P/Φ_O (P) (50 °C)
$P450_{cam}$	335		0.0080	
CYP119 wt	338		0.0030	0.63 (480 MPa)
T213A	335		0.0036	
T214A	336		0.0031	
T213W	346	396	0.0026	0.80 (480 MPa)
T213A/T214A	347	396	0.0076	0.45 (400 MPa)

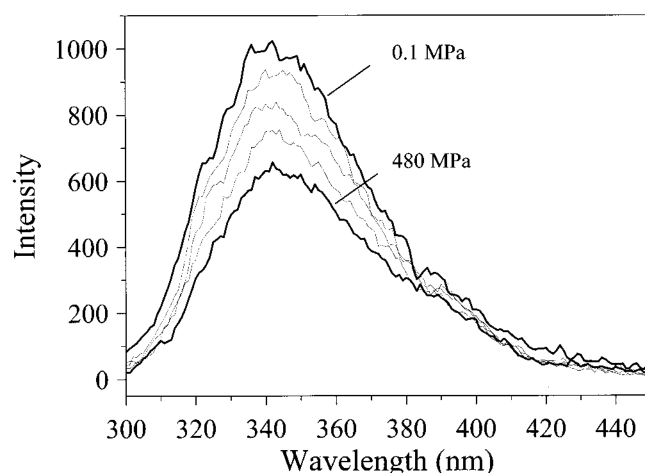

Figure 5. Tryptophan fluorescence spectra of ferric CYP119 as a function of hydrostatic pressure at 50 °C: $P = 1, 100, 200, 300, 480$ MPa.

residues to become more solvent accessible. The fluorescence yield of the T213W mutant was slightly lower than that of the wild type (0.0026). Topological studies have revealed that the additional tryptophan in this mutant is close to the heme, and therefore its contribution to the total fluorescence emission is most likely minor due to very efficient energy transfer to the porphyrin.[4,5] As a result, the reduced quenching observed for this mutant is due exclusively to the peripheral tryptophans.

The residual low tryptophan fluorescence yield makes fluorescence a particularly sensitive probe of small, pressure-induced conformational changes and pressure-induced compressibility changes. Upon increasing hydrostatic pressure from 0.1 to 480 MPa, there was a drop of approximately 37% in the total fluorescence yield of the wild-type CYP119 accompanied by a small (1−2 nm) red-shift in the maximum emission wavelength (Figure 5, Table 2). Such a small shift in the emission spectrum with increasing pressure indicates that there is little change in the environment of the tryptophans and strongly suggests that the protein does not undergo unfolding during the P450-to-P420 transition. The relative fluorescence yield of free tryptophan increases with pressure up to 1000 MPa.[18] The overall fluorescence quenching of CYP119 can be attributed to protein compression that brings the tryptophan fluorophores closer to the heme. These effects are reversible, and when the pressure was released, the fluorescence intensity was restored. Similar wavelength changes and reversibilities were observed when the T213W and T213A/T214A mutants were pressurized, but the extents of fluorescence quenching were 20% (at 480 MPa) and 55% (at 400 MPa), respectively (Table 2). These observations are readily rationalized by a direct effect of the steric encumbrance in the active site on the protein compressibility. In the T213W mutant, the tryptophan side chain likely acts as a pillar in the active site that attenuates protein compression, minimizing the change in the energy transfer from the peripheral tryptophans to the heme. Conversely, in the double mutant a greater cavity has been created that makes the protein less resistant to hydrostatic pressure.

Active-Site Topology. The reaction of aryldiazenes or arylhydrazines with cytochrome P450 enzymes generates iron-bound aryl complexes.[19] Decomposition of these complexes with ferricyanide leads to an iron-to-pyrrole nitrogen shift of the aryl ligand and formation of the four possible *N*-arylprotoporphyrin-IX (*N*-arylPPIX) regioisomers. These isomers can be separated and quantitated by HPLC. The relative distribution of the four *N*-arylPPIX adducts has been shown to be related to the active-site topology and has been used to probe the active-site topologies of P450 enzymes of unknown structure.[19] Recently, we have shown that the iron−aryl complexes of CYP119 are unusually oxygen-sensitive and decompose, in the absence of ferricyanide, to form the *N*-arylPPIX products.[4,5] The topology deduced from these studies indicated that the active site of CYP119 is closed above pyrrole nitrogens A and B but relatively open above pyrrole nitrogens C and D. This general topology is confirmed by the crystal structure of the protein.[7] We have examined the effect of hydrostatic pressure on the decomposition rate of the preformed complex as well as the ratio of the *N*-aryl regioisomers formed. Table 3 shows that hydrostatic pressure did not increase the rate of the aerobic decomposition of the *p*-trifluoromethylphenyl−iron complex of CYP119. Above 350 MPa, the rate could not be accurately measured due to rapid denaturation of the protein after complex decomposition. Isolation of the *N*-arylPPIX adducts after complex decomposition at 0.1 and 400 MPa revealed that high pressure caused a small decrease in the relative proportions of the A and B isomers, suggesting that pressure might cause a lateral motion of the I helix toward the center of the heme. To amplify and confirm these observations we used an active-site mutant (T213A) in which the shift was oriented mainly toward pyrrole nitrogens A and B (Table 3, Figure 6A). As the pressure was increased, there was a progressive change in the regioisomer ratio, with migration toward nitrogens C and D increasing from 07/07 to 14/27, respectively. These changes parallel the one observed with the wild-type protein in which migration to pyrrole nitrogens A and B was slightly reduced at elevated pressure. Incubation of the CYP119 T213A mutant at 400 MPa, followed by complex formation and aerobic decomposition of the complex at atmospheric pressure, showed no change in the active-site topology. This suggests that the protein undergoes reversible deformation around the active site at elevated hydrostatic pressure.

Discussion

The conversion of cytochrome P450 enzymes to the P420 form is important because it converts the protein into an inactive form. The molecular details of the transition, as well as the structural features of P420, remain unclear.[20−22] It has been suggested that it results from either protonation, or loss altogether, of the thiolate ligand. The transition to P420 is readily induced not only by cyanide and other agents but also by temperature or pressure. In the absence of stabilizing agents

(18) Li, T. M.; Hook, J. W.; Drickamer H. G.; Weber G. *Biochemistry* **1976**, *15*, 3205−3211.

(19) Ortiz de Montellano, P. R. *Biochimie* **1995**, *77*, 581−593.

(20) Blanke, S. R.; Martinis, S. A.; Sligar, S. G.; Hager, L. P.; Rux, J. J.; Dawson, J. H. *Biochemistry* **1996**, *35*, 14537−14543.

(21) Martinis, S. A.; Blanke, S. R.; Hager, L. P.; Sligar, S. G.; Hui Bon Hoa, G.; Rux, J. J., Dawson, J. H. *Biochemistry* **1996**, *35*, 14530−14536.

(22) Mouro, C.; Jung, C.; Bondon, A.; Simmoneaux, G. *Biochemistry* **1997**, *36*, 8125−8134.

Table 3. Aerobic Decomposition of Aryl−Iron Complexes of CYP119 and Its T213A Mutant

pressure (MPa)	CYP119 + *p*-CF_3−phenyldiazene		CYP119 T213A + *p*-Br-phenyldiazene	
	rate of complex decomposition at 20 °C s^{-1}	regioisomer ratio at 60 °C N_B:N_A:N_C:N_D	regioisomer ratio at 40 °C N_B:N_A:N_C:N_D	regioisomer ratio at 20 °C after protein preincubation N_B:N_A:N_C:N_D
0.1	1.0×10^{-4}	10:10:41:39	39:47:07:07	
100	1.1×10^{-4}		37:45:08:10	38:45:07:09
200	1.0×10^{-4}		33:35:15:17	
300	1.2×10^{-4}		27:34:16:23	
400		05:06:47:42	23:36:14:27	39:45:07:08

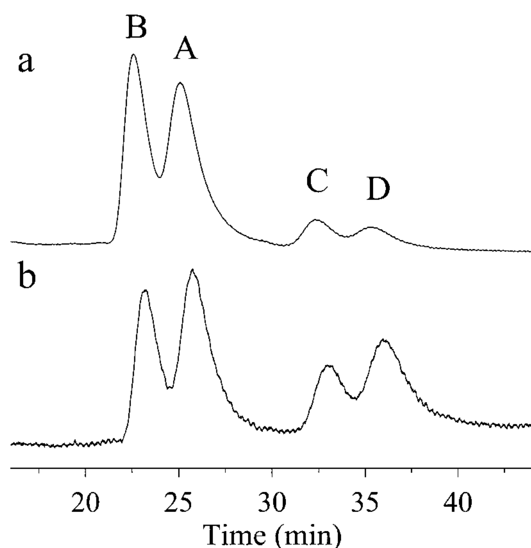

Figure 6. HPLC separation of the *N*-*p*-bromophenylprotoporphyrin IX regioisomers obtained from aerobic decomposition of the *p*-bromophenyl−iron complex of the CYP119 T214A mutant at 40 °C: (a) regioisomers obtained at 0.1 MPa, and (b) regioisomers obtained at 400 MPa.

such as sulfhydryl-containing compounds, the conversion to P420 is irreversible for cytochrome $P450_{cam}$ and $P450_{lin}$.[14,23] In the presence of DTT, the pressure-induced inactivation of $P450_{BM3}$ and CYP2B4 is partially or completely reversible with time.[12]. In this context, the thermal and barostatic behavior of CYP119 is unique. We and others have reported elsewhere that CYP119 is not converted to P420 even after incubation at 80 °C for 90 min,[3,4] and we demonstrate here that CYP119 is unusually stable under elevated hydrostatic pressure. The pressure of half inactivation for CYP119 was found to be 100−200 MPa higher than what has been observed for mesophilic isoforms (Table 1). The noticeable exceptions are $P450_{BM3}$,[24] which was done in the presence of 20% glycerol, a reagent that can stabilize the transition by up to 100 MPa,[23] and $P450_{lin}$, for which no transition was observed up to 300 MPa.[6] However $P450_{lin}$ underwent rapid conversion to $P420_{lin}$, and then unfolded and aggregated, at pressures above 300 MPa. By contrast, no unfolding of the oxidized CYP119 was observed up to 550 MPa. This differential behavior may be attributed to the presence of large clusters of aromatic amino acids in CYP119.[7] The stacking interactions of aromatic rings have negative volume changes and are therefore stabilized by pressure.[25] The increased number of Ile over Ala residues in CYP119 might also increase the packing and cause a resistance to water infiltration.[7,26] It has also been postulated that ion pairs may contribute to the thermal stability of CYP119. However, under pressure ionic pairs are destabilized due to the electrostrictive effect of charge separation[27] and do not contribute to piezostability.

The volume change and the volume of activation associated with the P450-to-P420 transition were found to be unusually small (−28 and −9 mL/mol, respectively) when compared to those for mesophilic enzymes. Such small volumes are usually associated with a high hydration content of the heme pocket[28] and, as a consequence, a higher protein compressibility[15] The significant tryptophan fluorescence quenching observed for CYP119 seems to argue for such a high compressibility of this protein. It appears that under pressure the protein undergoes, at least locally, an important contraction that causes the tryptophan residues to move closer to the heme, resulting in a larger energy transfer. Thus, our results suggest that the heme pocket of the protein, which is initially hydrated, undergoes some repacking under pressure but with only a small change in the extent of protein hydration of the protein. Interestingly, the reaction volume was sensitive to active-site mutations and replacement of Thr213 by a phenylalanine or tryptophan caused a drop in the overall volume change by about 18−20 mL/mol. The same change in the reaction volume was observed with the double-Ala mutant, suggesting that an additional water molecule might have filled the space left vacant by the mutations in this protein.

Another unique feature of CYP119 is that the pressure-induced conversion to P420 was completely reversible in the absence of sulfhydryl-containing compounds or other agents such as spermine.[29] To date, all other mesophilic P450, with the exception of $P450_{BM3}$ and CYP2B4, have been found to undergo irreversible inactivation in the absence of these stabilizers. The exact mechanism by which these stabilizing compounds reactivate the P420 back to P450 is not well understood, nor is it known whether the intimate structure of the resulting protein is identical to that of the original P450. In the case of CYP119, our fluorescence and topological investigations suggest that, even though the active site undergoes changes at high pressure, the native structure is restored upon depressurization.

We have found that the rate of the aerobic decomposition of the iron−aryl complex of CYP119 is not accelerated by hydrostatic pressure. This stands in stark contrast with observations made previously with $P450_{cam}$[30] for which aryl−iron complexes rapidly decomposed at pressure above 200 MPa. For $P450_{cam}$ it was suggested that the shift was caused by physical pressure on the probe, although it is possible that the pressure-

(23) Hui Bon Hoa, G.; Di Primo, C.; Geze, M.; Douzou, P.; Kornblatt, J. A.; Sligar, S. G. *Biochemistry* **1990**, *29,* 6810−6815.
(24) Anzenbacherova, E.; Bec, N.; Anzenbacher, P.; Hudecek, J.; Soucek, P.; Jung, C.; Munro, A. W.; Lange, R. *Eur. J. Biochem.* **2000**, 267, 2916−2920.
(25) Mozhaev, V. V.; Heremans, K.; Frank, J.; Masson, P.; Balny, C. *Proteins* **1996**, *24,* 81−91.
(26) Chang, Y.-T.; Loew G. *Biochemistry* **2000**, *39,* 2484−2498.
(27) Gross, M.; Jaenicke, R. *Eur. J. Biochem.* **1994**, *221*, 617−630.
(28) Di Primo, C.; Hui Bon Hoa, G.; Douzou, P.; Sligar, S. G. *Eur. J. Biochem.* **1992**, *209*, 583−588.
(29) Hui Bon Hoa, G.; Di Primo, C.; Geze, M.; Douzou, P.; Kornblatt, J. A.; Sligar, S. G. *Biochemistry* **1990**, *29,* 6810−6815.
(30) Tschirret-Guth, R. A.; Hui Bon Hoa, G.; Ortiz de Montellano, P. R. *J. Am. Chem. Soc.* **1998**, *120*, 3590−3596.

induced probe migration is caused by appearance of the P420 form. In the case of CYP119 at 60 °C, the transition to P420 would be around 520 MPa, based on the data in Table 1, and might be raised even higher in the presence of the active-site probe. A rate enhancement would therefore not be observed below 400 MPa if conversion to the P420 form is critical. This could explain the aryl-substituent effect observed with $P450_{cam}$[30] When compared to the phenyl group, the *p*-biphenyl and naphthyl groups provided additional stability to the protein by acting as pillars in the active site and raising the P450-to-P420 transition to higher pressures.

The fact that the rate of complex decomposition was pressure-insensitive also raises interesting considerations concerning the mechanism of the aerobic shift. We have found that the rate-limiting step of the aryl shift is oxidation of the heme iron by molecular oxygen.[5] One possible mechanism would involve rate-limiting outer-sphere electron transfer. However outer-sphere electron transfers have activation volumes in the order of −11 to −24 mL/mol and should be substantially accelerated by hydrostatic pressure, contrary to what is observed. An alternative mechanism might involve binding of oxygen to the *proximal* side, *trans* to the aryl group, followed by iron oxidation. However, our present findings suggest that oxygen binding is not part of the rate-determining step since such an event is expected to occur with a significant negative volume of reaction and should, therefore, be accelerated by elevated pressure. The nature of the rate-determining iron oxidation step therefore remains unclear.

Aerobic decomposition of the iron−aryl complex of CYP119 and its T213A mutant at high pressure provides evidence that the active site undergoes gradual, pressure-dependent deformation. As the pressure is increased, the I helix, which lies above pyrrole nitrogens A and B, appears to be displaced further toward the heme center. When the protein was incubated at high pressure before complex formation and aerobic decomposition, the active-site topology remained identical to that of the unpressurized sample. We therefore conclude that the pressure-induced active-site deformation is completely reversible. These results contrast with what we have observed with $P450_{cam}$.[30] $P450_{cam}$ undergoes an active-site deformation that appears to cause an outward motion of the I helix that facilitates probe access to pyrrole nitrogens A and B. These changes were complete at pressures below 50 MPa and were irreversible.

In conclusion, CYP119, the first cytochrome P450 enzyme to be isolated from an extremophilic organism, displays remarkable barostatic properties. The enzyme undergoes conversion to P420 at pressures above 300 MPa, and mutations of active-site residues can further shift the transition to higher pressures. Absorption, fluorescence, and topological studies all indicate that the pressure-induced structural changes are reversible. The unique pressure and thermal stabilities of CYP119 appear to be closely related to its unusual structural plasticity and reversible deformability.

Experimental Section

Materials and Methods. CYP119 and its mutants were heterologously expressed in *Escherichia coli* and were purified as reported elsewhere.[4] Ethyl *p*-bromophenyldiazene carboxylate azo ester and ethyl *p*-trifluoromethylphenyldiazene carboxylate azo ester were prepared from the corresponding hydrazines according to the method of Huang and Kosower.[31] UV−vis spectra were recorded using a Cary 1 or a Cary 3E spectrophotometer mounted with pressure chambers as described elsewhere.[32] Fluorescence experiments were done using an SLM 4800 spectrofluorimeter mounted with a similar pressure chamber.[32] The pressure increase in the chamber was generated by either a motor-controlled or a manual 700 MPa hydraulic press (the high-pressure fluid used was either *n*-pentane or *n*-heptane) through a flexible capillary tube. All experiments were done in 50 mM potassium phosphate buffer at pH 6.5. HPLC analysis were carried out on a Hewlett-Packard 1090 system using a Partisil ODS-3 column (5 μm × 4.6 mm × 250 mm) (Alltech, San Jose, CA) eluted at 1 mL/min with an isocratic mixture of 40% solvent B (MeOH:acetic acid, 10:1) into solvent A (MeOH:water:acetic acid, 6:4:1). The effluent was monitored at 416 nm.

Pressure-Induced Transition. The half pressure of inactivation ($P_{1/2}$) and the molar reaction volume ($\Delta V°$) were calculated using the equation:[10]

$$K_{eq}(P) = \frac{\exp[(P_{1/2} - P)\Delta V°]}{RT} \quad (1)$$

for each temperature.

The activation volume changes of inactivation ($\Delta V^{\ddagger}$) was calculated using the equation:

$$\ln k = -\frac{P\Delta V^{\ddagger}}{RT} + \ln k_0 \quad (2)$$

Fe−Aryl Complex Formation. The diazenes were prepared immediately prior to use by adding 1 μL of 2 N NaOH to 5 μL of a 100 mM stock solution of the corresponding alkyl aryldiazene carboxylate azo ester in methanol and diluting the resulting mixture with 44 μL of potassium phosphate buffer (50 mM, pH 7.3). Typically 5−20 μL of the diazene was added to a concentrated protein solution (~40 μM) in deoxygenated 50 mM potassium phosphate buffer, pH 8.0, at 40 °C, and the formation of the complex was monitored with a UV−vis spectrophotometer. After completion of the reaction, the protein sample was cooled to 4 °C and kept under an argon atmosphere to minimize complex decomposition. The complex solution (75 μL) was then diluted with 400 μL of air-saturated phosphate buffer at pH 6.0 and immediately transferred to the high-pressure cell. The pressure was adjusted and the reaction followed by UV−vis scanning at regular intervals.

***N*-ArylPPIX Analysis.** The heme products were isolated by pouring the protein mixtures into 8 mL of 5% H_2SO_4 in water and extracting with 3 × 1 mL of $CHCl_3$. After evaporation of the combined organic layers, the residue was redissolved in 50 μL of solvent A and was then injected into the HPLC.

Acknowledgment. These studies were supported by National Institutes of Health Grant GM25515 (P.R.O.M) and the Institut National de la Santé et de la Recherche Médicale (INSERM) (G.H.B.H.). G.H.B.H. thanks the Scientific and Administrative Councils of INSERM for his recent emeritus position and several international reviewers for their kind support.

JA003947+

(31) Huang, P. D.; Kosower, E. M. *J. Am. Chem. Soc.* **1968**, *90*, 2354−2362.

(32) Hui Bon Hoa, G.; Douzou, P.; Dahan, N.; Balny, C. *Anal. Biochem.* **1982**, *120*, 125−135.