Pressure-Induced Deformation of the Cytochrome $P450_{cam}$ Active Site

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Abstract: The reaction of cytochrome $P450_{cam}$ with aryldiazenes (ArN═NH) yields σ-bonded iron−aryl (Fe−aryl) complexes. Oxidation of the complexes causes regioselective migration of the aryl group from the iron to the porphyrin nitrogens. The influence of high pressure on the formation and rearrangement of the Fe−aryl complexes is reported here. The natural logarithm of the rate of formation of the Fe−phenyl complex decreases linearly with pressure with a volume of activation of 38.2 mL mol^{-1}. The Fe−phenyl, Fe−(2-naphthyl), and Fe−(*p*-biphenyl) complexes are stable at atmospheric pressure but decompose in a pressure-dependent manner at pressures above 1500 (Fe−phenyl) or 2500 bar (Fe−(2-naphthyl) or Fe−(*p*-biphenyl)). This pressure-induced decomposition results in formation of the *N*-arylprotoporphyrin IX adducts. The *N*-aryl porphyrin regioisomer patterns obtained by pressure-induced migration of the aryl groups (N_B:N_A:N_C:N_D, Ph, 10:14:33:43; 2-naphthyl, 12:13:37:38; *p*-biphenyl, 15:15:33:37) differ from those obtained by oxidation of the $P450_{cam}$ Fe−aryl complexes at atmospheric pressure (Ph, 00:05:25:70; 2-naphthyl, 00:00:100:00; *p*-biphenyl, 00:14:40:46). Preincubation of $P450_{cam}$ at elevated pressure followed by decompression, Fe−phenyl complex formation, and oxidative shift yields the same N−phenyl regioisomer ratio as the pressure-induced shift. The three principal findings of this study are that (a) the iron-to-nitrogen migration of the aryl group can be promoted by pressure, (b) differential distortion of the $P450_{cam}$ active site by high pressure causes small displacements of the I-helix and residues associated with the substrate access channel, and (c) pressure causes a subtle structural change in the $P450_{cam}$ active site that persists at atmospheric pressure.

Cytochrome $P450_{cam}$ (CYP101) catalyzes the first step in the oxidation of camphor that allows this terpene to be used by *Pseudomonas putida* as its sole carbon source.[1] Crystal structures are available of $P450_{cam}$ in the ferric state with and without a bound camphor as well as with a variety of other ligands.[2−7] The structure of the ferrous−CO complex of the enzyme has also been determined.[8] These structures show that the catalytic site, as defined by the location of the heme iron atom and the bound ligand, is buried within the protein. An access channel of sufficient dimensions for substrates to diffuse into the active site is not seen in any of the crystal structures. The protein must therefore undergo a conformational rearrangement that opens a transient access channel. The exact nature of this access channel is not known, but the finding that Phe-193, Tyr-96, and Phe-87 are displaced in the structure of $P450_{cam}$ to which a large inhibitor is bound supports the earlier suggestion that the access channel is formed by motions that include residues Phe-193, Tyr-96, Phe-87, Tyr-29, Phe-98, and Ile-395.[3,6] Apart from the changes that relate to the access channel, only minor differences are observed in the active site of the $P450_{cam}$ crystal structures regardless of the iron oxidation state or the nature of the ligand in the active site.

The reaction of $P450_{cam}$ with aryldiazenes (ArN═NH) results in the formation of σ-bonded iron−aryl (Fe−aryl) complexes with absorption maxima at ∼480 nm. The structure of the $P450_{cam}$ Fe−phenyl complex has been unambiguously established by X-ray crystallography.[7] The Fe−aryl complexes of this protein are indefinitely stable in the absence of external oxidizing agents, but oxidation of the protein-bound complex with ferricyanide causes migration of the aryl group from the iron to the heme pyrrole nitrogens.[9,10] The four possible *N*-arylprotoporphyrin IX products thus formed can be isolated, separated by high-pressure liquid chromatography (HPLC), and quantitated,[11] and the regioisomer ratio can be used to construct a low-resolution topological model of the active site. Phenyl-, *p*-biphenyl-, and 2-naphthyldiazene have thus been used to investigate the active sites of several P450 enzymes,[12] the three isoforms of nitric oxide synthase,[13] and chloroperoxidase.[14]

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The effects of high pressure on proteins and their reactions derive from the reaction volume dependence ($\Delta V°$) of equilibria and the activation volume ($\Delta V^{\ddagger}$) dependence of reaction rates.[15,16] From Le Chatelier's principle, any equilibrium with a change in reaction volume shifts toward the more compact state when placed under hydrostatic pressure, and any reaction with a positive (negative) activation volume is slowed (accelerated) by pressure. In general, the volume changes are composite thermodynamic parameters that are not easily interpreted in molecular terms because of the number of bonding interactions that are perturbed by pressure. However, with protein models of known three-dimensional structure and interactions whose nature is defined, volume changes provide information that is useful in predicting how pressure changes the structure. It is well established that hydration plays a major role in these volume changes. In particular, ion pairs and hydrophobic groups are strongly destabilized by hydrostatic pressure. These effects have been attributed to the electrostrictive effect of water molecules on exposed charged groups[17,18] and the hydrophobic solvation of water around nonpolar residues.[19]

Elevated hydrostatic pressure favors both a spin state change in camphor-bound P450$_{cam}$[20] and the dissociation of camphor with the accompanying high-to-low shift in the iron spin state.[21,22] At pressures above 800 bar, P450$_{cam}$ is gradually converted to the inactive P420 form.[21] Transition to the P420 form is associated with an increase of the intrinsic tryptophan fluorescence and hydration of the active site. This process has been suggested to involve conformational changes that restrict substrate binding or alter the ligand access channel because a number of good P450$_{cam}$ ligands do not detectably bind to the P420 form.[23−25] The secondary structures of the cytochrome P420 forms generated by incubation of the enzyme at elevated temperature or by exposure to potassium thiocyanate (KSCN) were shown by FTIR not to be identical and to differ from that of native P450$_{cam}$.[26] The principal change in the secondary structure is the conversion of some α-helix structure to β-sheet. However, no information is available on the topological changes in the active site caused by high pressure. For pressures below that at which denaturation occurs, compression changes the protein interatomic distances and this effect is closely related to the so-called intrinsic protein compressibility estimated by sound velocity measurements.[27,28] Pressure-dependent protein compression has been investigated by fluorescence resonance energy transfer spectroscopy,[29] crystallography,[30] NMR,[31] hole burning measurements,[32] and molecular dynamics simulations.[33] Although hole burning experiments give the same results for intrinsic protein compressibility as sound velocity measurements, the fluorimetric determination of the distances between tryptophan and heme groups in different heme proteins has led to intrinsic compressibilities higher than that of water, i.e., 49×10^{-6} bar^{-1}. In contrast, crystallographic studies of lysozyme under pressure yield an overall compressibility of 47×10^{-5} bar^{-1}, a value one order of magnitude lower than that of water and one-third of that estimated from sound velocity measurements. Interestingly, the crystallographic studies indicate that the volume reductions in lysozyme occur exclusively in the α-helical domain, although only a few atoms move more than 0.1 nm. The β-domain was found to be essentially incompressible. Similar results were obtained by computer simulations which showed that, on the average, shorter distances compress less than longer ones. This nonuniform contraction underlines the peculiar nature of the structural changes due to pressure, in contrast to those mediated by temperature.

To examine the problem of asymmetric topological deformation of a protein by pressure, we have investigated the behavior of three P450$_{cam}$ Fe−aryl complexes under high pressure. We have unexpectedly found that high pressure promotes a shift of the aryl groups from the iron to the porphyrin nitrogens in the absence of external oxidizing agents. Differences in the observed regiochemistry of the aryl shifts at normal versus high pressure identify regions of the active site that undergo pressure-dependent conformational changes.

Experimental Section

Materials and Methods. Cytochrome P450$_{cam}$ was expressed heterologously in *Escherichia coli* and was purified as previously reported.[34] Methylphenyldiazenecarboxylate azo ester was obtained from Research Organics (Cleveland, OH). Methyl 2-naphthyldiazene carboxylate azo ester and methyl *p*-biphenyldiazenecarboxylate azo ester were prepared from the corresponding hydrazines according to the method of Huang and Kosower.[35] UV−vis spectra were recorded with either a Cary 3E or Cary 1 spectrophotometer mounted with a pressure chamber as described elsewhere.[36] The increase in the chamber pressure was produced by a manual 7 kbar hydraulic press through a custom-made flexible capillary. The fluid used in the hydraulic press was *n*-pentane. The hydrostatic pressure was measured with a Bourdon type manometer. HPLC analyses 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).

Aryl−Iron Complex Formation. The methyl aryldiazenecarboxylate azo esters (1−2 mg) were dissolved in 100 μL of methanol and the solutions were diluted with 100 μL of 2 N NaOH to hydrolyze the esters and release the corresponding aryldiazenes. Typically, 1−2 μL of the diazene solution was added to 500 μL of cytochrome P450$_{cam}$ (2−9 nM) in phosphate buffer (50 mM, pH 7.4). Formation of the Fe−aryl complex was followed by monitoring the change in the UV−vis spectrum of the solution. The addition of excess aryldiazene is to be avoided because it causes protein denaturation at high hydrostatic pressures.

Kinetics of Pressure-Induced Aryl−Iron Complex Decomposition. Kinetic measurements were obtained by pressure jump from 1800

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bar to the desired final pressure as described elsewhere.[37] Decomposition of the iron−aryl complexes was followed at 478 nm. The curves that were obtained were fitted with use of the following expression:

$$OD_t = (OD_0 - OD_f) \exp(-kt) - OD_f$$

where OD_0 and OD_f are the initial and final optical densities, respectively.

From the transition state theory of Eyring,[38] the rate constant k of a reaction is given by the equation:

$$k(P,T) = \left(k_B \frac{T}{h}\right) \exp\left(-\frac{\Delta G^{\ddagger}(P,T)}{RT}\right) \quad (1)$$

$$\Delta G^{\ddagger} = E_x - RT - T\Delta S^{\ddagger} + P\Delta V^{\ddagger} \quad (2)$$

where k_B is Bolzmann's constant, h Planck's constant, R the gas constant, and T the absolute temperature. $\Delta G^{\ddagger}$, E_x, and $\Delta S^{\ddagger}$ are the activation free energy (or activation barrier), activation energy, and entropy, respectively. $\Delta V^{\ddagger}$ is the activation volume, which is equal to the difference in the volumes of the activated ($V^{\ddagger}$) and initial (V_i) states and characterizes the dependence of the rate constant k on the pressure. The activation volume changes $\Delta V^{\ddagger}$ were calculated from the following equation:

$$k = k_0 \exp\left(-\frac{P\Delta V^{\ddagger}}{RT}\right) \quad (3)$$

where k and k_0 are respectively the rate constants of the reaction at pressure P and at the initial pressure, or

$$\ln k = -\frac{P\Delta V^{\ddagger}}{RT} + \ln k_0 \quad (4)$$

by determining the slope of the plot of $\ln(k) = f(P)$.

Extraction of the *N*-Arylprotoporphyrin IX Isomers. The samples (500 μL) were added to 8 mL of 5% aqueous H_2SO_4 and the mixtures were allowed to stand at 4 °C for ∼16 h before they were extracted with $CHCl_3$. The residue obtained after evaporation of the $CHCl_3$ was redissolved in 40 μL of 6:4:1 methanol/water/acetic acid and the solution was analyzed as described elsewhere.[39]

Results

Formation of the P450$_{cam}$ Fe−Phenyl Complex. The formation of the Fe−phenyl complex in the reaction of P450$_{cam}$ with phenyldiazene is inhibited by increasing hydrostatic pressure, whether the rate of the reaction is determined from the decrease in the Soret maximum of the camphor-free enzyme at 415 nm or the increase in the absorption of the Fe−phenyl complex at 480 nm. A straight line is obtained if a plot is made of the natural logarithm of the rate of the Soret band disappearance versus the applied pressure (Figure 1). In the pressure range utilized, no pressure-induced inactivation of cytochrome P450$_{cam}$ was detected. The volume of activation ($\Delta V^{\ddagger}$) for the reaction of P450$_{cam}$ with phenyldiazene calculated from the slope of the plot is +38.2 mL mol^{-1}. An inverse dependence of the rate of formation of the Fe−phenyl complex on pressure is expected because the reaction results in the release of a molecule of nitrogen gas.

Pressure Stability of the P450$_{cam}$ Fe−Aryl Complexes. The P450$_{cam}$ Fe−phenyl, Fe−(*p*-biphenyl), and Fe−(2-naphthyl) complexes were formed at atmospheric pressure as previously reported.[40,41] The complexes were then subjected to pressures rising from 1 to 4000 bar in 500 bar increments. The maximum at 478−480 nm of the Fe−aryl complex decreases as the pressure is increased without a concomitant recovery of the normal ferric Soret absorbance and/or formation of the inactive P420 form. A plot of the Fe−phenyl absorbance at 478 nm as a function of the pressure shows that the complex is stable up to approximately 2000 bar and then decomposes in a pressure-dependent manner (Figure 2). Similar plots are obtained for the Fe−(*p*-biphenyl) and Fe−(2-naphthyl) complexes, although these latter complexes are more stable than the Fe−phenyl complex and the decrease in the 478 nm absorbance commences at approximately 3000 rather than 2000 bar (Figure 2). Decompression of the solution followed by extraction with $CHCl_3$ shows that the loss of 478 nm absorbance is associated with formation of *N*-arylprotoporphyrin IX products with a characteristic absorbance maximum at 416 nm (Figure 3). Formation of the *N*-arylprotoporphyrin IX adducts was confirmed by HPLC analysis (vide infra). Thus, the iron-to-nitrogen shift of the P450$_{cam}$ Fe−aryl complexes, a reaction that normally requires exposure to an oxidizing agent such as ferricyanide, is brought about by high pressure in the absence of any exogenous oxidizing agent.

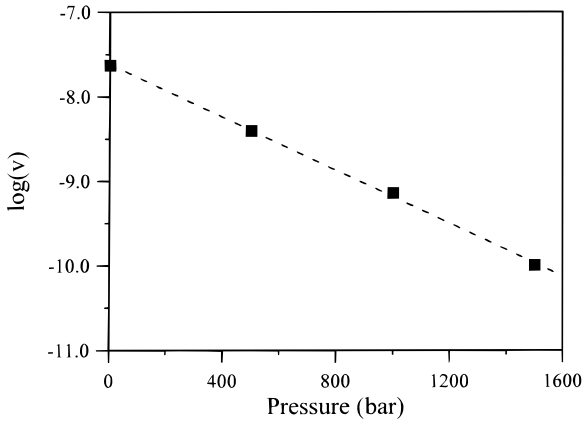

Figure 1. Plot of the natural log of the rate of the Soret band disappearance as a function of the pressure.

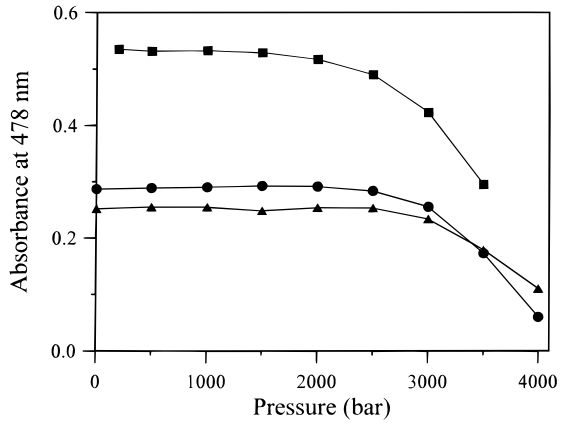

Figure 2. Plot of the absorbance at 478 nm as a function of the pressure applied to solutions of the Fe−phenyl (■), Fe−2-naphthyl (●), and Fe−*p*-biphenyl (▲) complexes. The shift of the aryl group is complete at the last point for each probe.

Kinetics of the Pressure-Induced Decomposition of Fe−Aryl Complexes. The rate of decomposition of the Fe−phenyl

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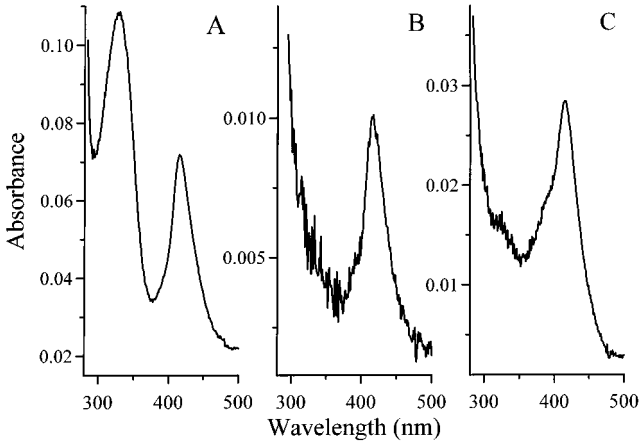

Figure 3. UV−vis spectra of the $CHCl_3$ extracts after pressure-induced decomposition of the (A) Fe−phenyl, (B) Fe−2-naphthyl, and (C) Fe−*p*-biphenyl complexes. The peak with an absorption maximum at 416 nm is that for an *N*-arylprotoporphyrin IX.[11] The heme extracted from unreacted P450$_{cam}$ under the same conditions has an absorption maximum at 388 nm.

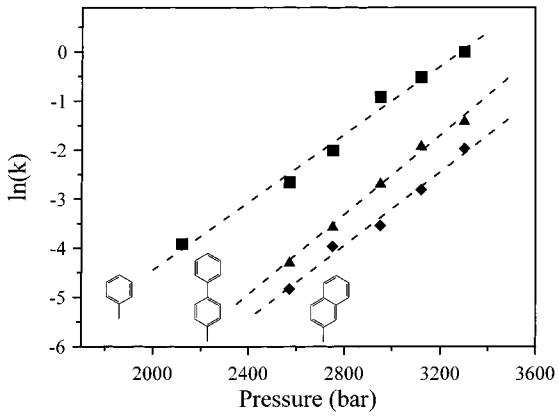

Figure 4. Plot of ln *k* as a function of the applied pressure for the Fe−phenyl (■), Fe−(2-naphthyl) (◆), and Fe−(*p*-biphenyl) (▲) complexes.

complex is pressure dependent and adheres to an exponential decay pattern. Furthermore, the UV−vis spectra of the reactions at the end of the kinetic experiments indicate that the reaction goes to completion at all pressures (not shown). The same results are observed for the Fe−(2-naphthyl) and Fe−(*p*-biphenyl) complexes, but the rates of decomposition are considerably slower (Figure 4). The stability of the Fe−aryl complexes toward hydrostatic pressure increases in the order phenyl ≪ *p*-biphenyl < 2-naphthyl. The plots of the natural log of the rate constant versus pressure are linear for each probe (Figure 4) and the volumes of activation determined from the slopes are negative: −84.3, −91.3, and −98.7 mL mol^{-1} for the Fe−phenyl, Fe−(2-naphthyl), and Fe−(*p*-biphenyl) complexes, respectively, indicating an acceleration of the rate of the transition by hydrostatic pressure**.**

***N*-Arylprotoporphyrin IX Regioisomer Distributions.** If the P450$_{cam}$ Fe−aryl complexes are oxidized by ferricyanide at normal atmospheric pressure, the *N*-arylprotoporphyrin IX regioisomers are formed in the following ratios (N_B:N_A:N_C:N_D, where the subscript denotes the pyrrole ring bearing the *N*-aryl group): phenyl, 00:05:25:70; 2-naphthyl, 00:00:100:00; and *p*-biphenyl, 00:14:40:46 (Table 1).[41] The phenyl shift pattern (Figure 5, trace a) is consistent with the topology of the active site near the heme group revealed by the crystal structure, which shows that the region above pyrrole ring D is open, that above

Table 1. *N*-Arylprotoporphyrin IX N_B:N_A:N_C:N_D[a] Isomer Ratios Obtained from the P450$_{cam}$ Fe−Aryl Complexes by Oxidation with Ferricyanide or Exposure to 4000 bars of Pressure[b]

aryl group	$K_3Fe(CN)_6$	4000 bars of pressure
phenyl	00:05:25:70[c]	10:14:33:43
2-naphthyl	00:00:100:00[c]	12:13:37:38
p-biphenyl	00:14:40:46[c]	15:15:33:37

[a] N_A, N_B, N_C, and N_D are the *N*-arylprotoporphyrin IX regioisomers with the aryl group on the nitrogens of pyrrole rings A, B, C, and D, respectively. [b] The error in the individual measurements, as determined from three independent experiments, is ±1. [c] This regioisomer ratio was reported previously.[42]

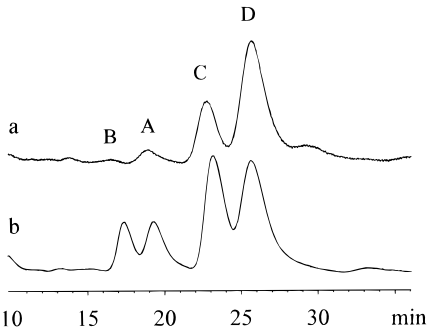

Figure 5. HPLC separation of the *N*-phenylprotoporphyrin IX regioisomers obtained from phenyldiazene-treated P450$_{cam}$: (a) the isomers obtained when the Fe−phenyl complex is oxidized with potassium ferricyanide at atmospheric pressure; (b) the isomers obtained when the Fe−phenyl complex is exposed to 4000 bar of pressure in the absence of an added oxidizing agent.

pyrrole ring C only partially open due to some masking by Leu-244, that immediately above pyrrole ring A almost completely occluded by Thr-252, and that above pyrrole ring B completely occupied by residues of the I-helix. The complete shift to pyrrole ring C with the 2-naphthyl probe is rationalized by the finding that at higher levels of the active site, the region above pyrrole ring C is relatively open whereas that above pyrrole ring D is blocked by Phe-87 (Figure 6). Thus, the observed shift of the 2-naphthyl group toward pyrrole ring C requires some degree of displacement of Leu-244, the residue immediately above pyrrole ring C. If the migration had occurred toward pyrrole ring D, it would have required displacement of Phe-87.

When the phenyl−iron shift is brought about by hydrostatic pressure, a somewhat different distribution of *N*-phenylprotoporphyrin IX isomers is obtained (Figure 5, trace b, and Table 1). The isomer with the *N*-phenyl on pyrrole ring D (N_D) remains favored, but less so than in the oxidative rearrangement at normal pressure. The decrease in the proportion of the N_D isomer is compensated for by increases of 10% in the proportions of the N_A and N_B isomers. In the case of the *p*-biphenyl probe, the proportions of the N_C and N_D isomers decrease and are compensated for by increases in the N_B isomer. Most dramatically, the 2-naphthyl probe shifts to give a 12:13:37:38 N_B:N_A:N_C:N_D ratio of isomers rather than the single N_C isomer obtained under atmospheric pressure (Table 1).

N-Phenylprotoporphyrin IX Regioisomer Distribution as a Function of Pressure. The distribution of the *N*-phenylprotoporphyrin IX regioisomers obtained as a function of the applied pressure is given in Table 2. The relative ratios observed are essentially pressure independent and indicate that deformation of the active site occurs at a pressure below 2000 bar. In addition this result suggests that topological deformation of the active site and complex decomposition may be independent events.

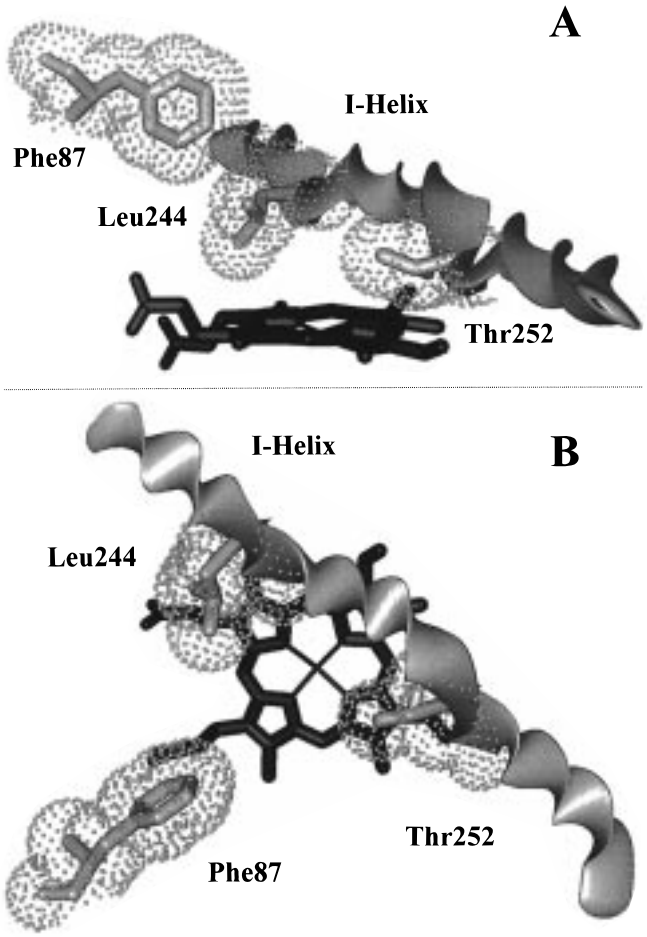

Figure 6. Model of the active site of $P450_{cam}$ based on the crystal structure of the Fe−phenyl complex with the phenyl group removed.[7] The I-helix backbone is shown as a twisted ribbon and the location of the residues Leu-244, Phe-87, and Thr-252 is indicated. In panel A, the relative orientation of the I-helix and the three amino acid side chains relative to the heme group is shown. Going counterclockwise from the bottom right corner the pyrrole rings are A, B, C, and D. In panel B, a sidewise view of the same structural elements is shown that emphasizes their relative height from the heme plane. The view in panel B is obtained by rotating the view in panel A 90° with the bottom edge coming toward the viewer.

Table 2. *N*-Phenylprotoporphyrin IX Regioisomer Distribution Obtained after Exposure of the Fe−Phenyl Complex of $P450_{cam}$ to Increasing Hydrostatic Pressure

incubation pressure, bars	regioisomer ratio N_B:N_A:N_C:N_D
2000	11:13:34:42
2500	17:16:34:33
3000	13:16:34:37
3500	14:16:35:35
4000	10:14:34:42

Fe−Phenyl Complex Formation and *N*-Phenylprotoporphyrin IX Regioisomer Distribution after Incubation of $P450_{cam}$ under Increasing Hydrostatic Pressure. To further determine the effect of the hydrostatic pressure on the active site of $P450_{cam}$, the camphor-free protein was preincubated for 15 min at various pressures and the protein was decompressed. The Fe−phenyl complex formed at atmospheric pressure immediately after protein decompression. The UV spectra of the resulting Fe−phenyl complexes (Figure 7) shows that the extent of Fe−phenyl complex formation decreases with increasing preincubation pressure and is almost nonexistent after preincubation at 2000 bar or higher. The decrease in complex formation correlates with the increase in the 420 nm absorbance observed with increasing preincubation pressure.

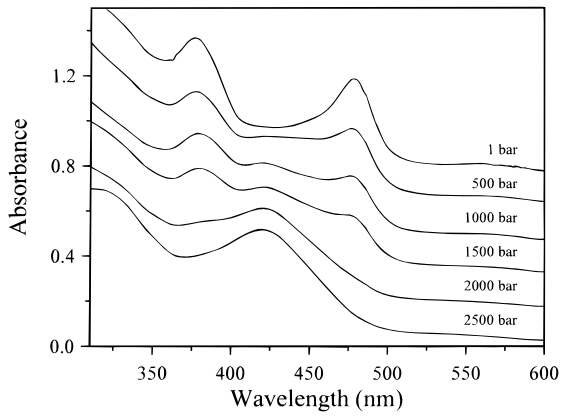

Figure 7. UV spectrum of the Fe−phenyl complex formed after preincubation of $P450_{cam}$.

Table 3. *N*-Phenylprotoporphyrin IX Regioisomer Distribution Obtained after Preincubation of $P450_{cam}$ at Increasing Hydrostatic Pressures

preincubation pressure, bars	regioisomer ratio N_B:N_A:N_C:N_D	rel yields of *N*-arylporphyrin isomers
1	01:05:26:68	
500	14:16:31:39	7.1
1000	17:17:30:36	6.3
1500	18:17:30:35	5.6
2000	15:17:31:37	1.5
2500	17:22:25:34	1.0

The *N*-phenylprotoporphyrin IX regioisomer distributions and the relative yields obtained after $K_3Fe(CN)_6$-mediated oxidative rearrangement at normal pressure of the complexes formed from pressure pretreated $P450_{cam}$ are given in Table 3. The isomer ratios obtained after preincubation of $P450_{cam}$ at elevated pressure are similar to the ratios obtained by pressure-induced decomposition of the Fe−phenyl complex (Table 2). The ratios are similar and are pressure independent except for the ratio obtained with the unpressurized protein. The altered regioisomer pattern is observed even if the decompressed protein is allowed to stand for up to 1 h before the phenyldiazene reaction is carried out (not shown). The decrease in the total yields of *N*-phenylprotoporphyrin IX regioisomers with increasing preincubation pressure parallels the pressure-mediated formation of P420 and the decrease in the absorbance of the Fe−phenyl complex at 478 nm, as expected if the remaining P450 fraction, and not the P420 component, is involved in the reaction (Figure 7).

Discussion

The presence of an Fe−aryl complex stabilizes the cytochrome $P450_{cam}$ active site with respect to conversion to cytochrome P420 or other species. Decomposition of the Fe−phenyl complex is observed at pressures above 1500 bar, and decomposition of the *p*-biphenyl and 2-naphthyl complexes is observed at pressures above 2500 bar, whereas conversion of substrate-bound $P450_{cam}$ to the P420 form, which is preceded by dissociation of the substrate, is observed at pressures as low as 800 bar.[21] Stabilization of the active site structure by the Fe−aryl complexes is due, at least in part, to the fact that the aryl groups are rigid structures that are covalently bound to the iron atom. The aryl moieties, unlike camphor, are not extruded from the active site as the pressure increases and thus are more

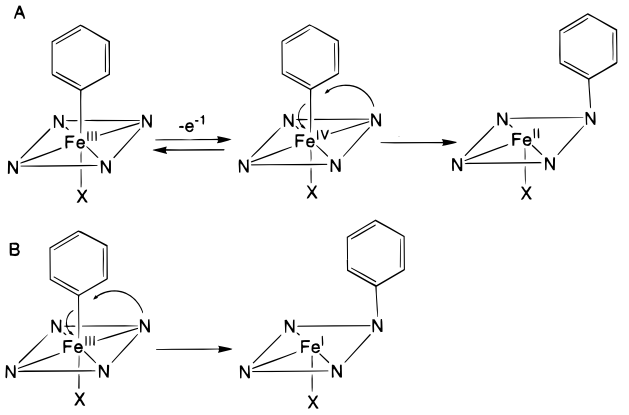

Figure 8. (A) Iron oxidation state changes in the iron-to-nitrogen migration of the phenyl group caused by reaction of the P450$_{cam}$ phenyl−iron complex with ferricyanide (B). The hypothetical changes in the iron oxidation state that may be caused by the pressure-induced migration of the phenyl group.

effective than camphor in protecting the active site against pressure-induced inactivation. The Fe−aryl complexes appear to function as pillars that anchor the active site structure with respect to the heme group. The larger *p*-biphenyl and 2-naphthyl groups, which fill a greater proportion of the active site cavity and extend to greater distances above the heme plane, introduce greater rigidity than the smaller phenyl moiety and consequently stabilize the active site toward higher hydrostatic pressures.

The finding that the Fe−aryl shifts are promoted by pressure is unexpected because the only known mechanism for inducing these shifts is oxidation of the iron to the Fe^{4+} state.[9,43−45] This oxidized complex undergoes a two-electron rearrangement that transfers the phenyl group to a pyrrole nitrogen with concomitant reduction of the iron to the Fe^{2+} state (Figure 8, reaction A). The final product is thus the ferrous *N*-arylprotoporphyrin IX. A key feature of the mechanism in the present context is the fact that the shift involves a two-electron change in the redox state of the iron atom. If a comparable two-electron mechanism holds for the pressure-mediated Fe−aryl shift, the reaction begins with the iron in the ferric state and therefore terminates with the iron in the Fe^{1+} state (Figure 8, reaction B). If so, the rearrangement might be reversible because model studies have shown that iron N-phenylporphyrins undergo a reverse nitrogen-to-iron shift of the phenyl group when the iron is reduced to the Fe^{1+} state.[43,44] However, no reversal of the rearrangement was observed when the pressure was released. One possibility is that the Fe^{1+} species is oxidized to the ferrous (or ferric) state by electron transfer to molecular oxygen. To test this possibility, the pressure-induced shift of the Fe−phenyl complex was carried out under an atmosphere of argon. The shift occurred normally but was again not reversed by release of the pressure (not shown). Thus, if the reaction terminates with the iron in the Fe^{1+} state, alterations of the structure of the heme or the active site due to migration of the aryl group to the iron rule out the reverse reaction. One possibility is that irreversible changes in the ligation state accompanying the aryl migration, analogous to those associated with P420 formation, preclude the reverse reaction.

The driving force for the shift is probably provided by lateral physical pressure on the aryl moiety as residues in contact with it are displaced inward by the hydrostatic pressure. In accord with this hypothesis, the rate of the aryl iron-to-nitrogen shift is linearly related to the pressure (Figure 4). Increasing pressure accelerates the rate of the shift indicating that the process is controlled by a high negative activation volume ($\Delta V^{\ddagger} = -84.3$ mL mol^{-1} for the Fe−phenyl complex). Thus, the transition state is formed with a considerable contraction in volume relative to the starting Fe−aryl complex. The apparent activation volume obtained in these studies is for a complex multistate reaction and cannot be interpreted in terms of a transition state structure and reaction mechanism without further characterization of all the reaction intermediates. Even for a simple two-state reaction, such as the redox interconversion of cytochrome *c*, the volume of activation is a composite term that can only be interpreted in mechanistic terms by dividing it into at least two components: chemical bond breaking and formation, $\Delta V^{\ddagger}$(bond), and solvent rearrangement associated with formation of a charged transition state, $\Delta V^{\ddagger}$(solvation). In the case of an outer-sphere electron-exchange reaction in which no bond formation or rupture occurs, the observed activation volume ($\Delta V^{\ddagger}$) should be negative and of the order of −11 to −24 mL mol^{-1}, in large part due to strong electrostrictive solvent effects due to charge redistribution. In addition, the formal potential of the ferri-ferrocytochrome *c* couple is very sensitive to pressure.[46] The potential shift at 5 kbar is as large as 75 mV, indicating that reduction of the protein is favored by the application of pressure and involves a net decrease of volume. The activation volume obtained for our pressure-induced shift is higher than that obtained for cytochrome *c*. It is likely to include a third component, the amplitude of which can only be evaluated when the different intermediates of the pressure-induced shift are characterized, that reflects the pressure-induced conformational changes in the active site. The overall activation volume of −84.3 mL mol^{-1} for the pressure-induced Fe−phenyl to N−phenyl shift is therefore compatible with some internal redistribution of electrons in progressing to the transition state and with an effect of local solvent rearrangement on formation of the charged transition state. A combination of both effects is consistent with the large amplitude of the activation volume changes. The shifts of the 2-naphthyl and *p*-biphenyl groups are slower and have larger volumes of activation (Figure 4) than the phenyl shift but are also pressure dependent. The activation free energies $\Delta G^{\ddagger}$ ($P = 0$) for these transitions, calculated from eq 1, are 26.2, 28, and 28.1 kcal mol^{-1} for the Fe−phenyl, Fe−(2-naphthyl), and Fe−(*p*-biphenyl) complexes, respectively. The higher activation barriers for the last two complexes could explain why the corresponding aryl shifts are slower than those for the Fe−phenyl complex. The shift is thus caused by a structural change that depends directly on the applied pressure, as might be expected for progressive deformation of the active site with increasing pressure. Increasing compression of the active site by pressure progressively decreases the energy barrier and favors the aryl shift ($\Delta G_P^{\ddagger} = \Delta G_0^{\ddagger} + P\Delta V^{\ddagger}$, in which $\Delta V^{\ddagger}$ is negative). If the shift is due to pressure on the aryl group due to deformation of the active site, the shift should be active site dependent. Some evidence that this may be true is provided by the finding that the Fe−phenyl complex of sperm whale myoglobin is stable to pressure and

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does not undergo the shift (unpublished results), although the result is not unambiguous because the ligand to the iron in myoglobin is a histidine rather than the cysteine thiolate found in the P450 enzyme. Regardless of the detailed mechanism, the pressure-induced shift is a novel phenomenon for which, to the best of our knowledge, there is no precedent.

Comparison of the regiospecificity of the aryl shifts under normal and high pressure provides evidence for regioselective distortion of the active site. At normal pressure, none of the three aryl groups shifts significantly to the nitrogen of pyrrole ring B, which is completely obstructed in the crystal structure of $P450_{cam}$ by the I-helix.[2] However, at 4000 bar the phenyl, 2-naphthyl, and *p*-biphenyl groups shift to the extent of 10, 12, and 15%, respectively, to the nitrogen of pyrrole ring B. The shift of the aryl group to the nitrogen of pyrrole ring A, which is obstructed in the $P450_{cam}$ crystal structure by residue Thr-252 of the I-helix, is also increased. Essentially the same increase in the proportion of the N_A and N_B regioisomers is observed at pressures ranging from 2000 to 4000 bar. The change in the isomer ratio is thus pressure dependent, but raising the pressure above a low threshold level, perhaps ~500 bar (see Table 3), does not further alter the pattern. The structural change that controls the isomer pattern thus differs from the pressure-dependent effect that determines the rate of the aryl shift.

The increases in the proportions of the *N*-phenyl N_A and N_B regioisomers suggest that pressure displaces components of the I-helix away from the heme iron atom, increasing the accessibility of the pyrrole ring A and B nitrogens. The structural alteration simultaneously causes a change in the proportions of the N_C and N_D regioisomers. The crystal structure of $P450_{cam}$ shows that pyrrole ring C is partially masked by Leu-244 and the region above pyrrole ring D is open immediately above the heme plane but is occluded at some distance from it by the phenyl of Phe-87 (Figure 6). Migration to pyrrole ring C decreases for the 2-naphthyl and *p*-biphenyl moieties (Table 1). Most impressively, the 2-naphthyl group, which shifts exclusively to pyrrole ring C at atmospheric pressure, shifts under pressure to all four pyrrole nitrogens and particularly to pyrrole ring D. As reported earlier,[40] the upper ring of the 2-naphthyl group fits into a groove above pyrrole ring C defined by Phe-87, Ile-395, and Thr-185. At the same height above the heme plane the space above pyrrole ring D is occupied by Phe-87. Increased migration of the 2-naphthyl moiety toward pyrrole ring D could be due to displacement of Phe-87 or to a hardening in the steric encumbrance above pyrrole ring C provided by Leu-244.

An intriguing finding emerges from the *N*-phenylporphyrin pattern obtained from $P450_{cam}$ after incubating it at various pressures before returning it to atmospheric pressure, forming the phenyl−iron complex, and promoting the phenyl migration with $K_3Fe(CN)_6$. The *N*-phenylprotoporphyrin IX regioisomer ratios thus obtained indicate that the active site deformation caused by high pressure is preserved when the protein is returned to atmospheric pressure. Preincubation at pressures from 500 to 2500 bar causes essentially the same increase in the proportions of the N_A and N_B isomers at the expense of the N_D isomer (Table 3). This result clearly indicates that the deformation that determines the regioisomer ratio occurs at a pressure below that (800 bar) at which even the wild-type protein begins to denature to the inactive P420 form.[21] After preincubation at pressures below 800−1000 bar, the absorption changes in the Soret region are reversible upon decompression. The *N*-phenylporphyrin yield decreases at elevated pressures due to the formation of P420 (Figure 7), but this does not interfere with the above observation because P420 binds ligands very poorly and does not form an Fe−phenyl complex (unpublished results).[23−25] Time-dependence experiments have shown that the deformation persists for a period of at least 60 min. The nature of the pressure-induced active site modification preserved after decompression of the protein is not known, but it is likely to involve a change in the hydration state or the hydrogen bond pattern of the protein structure[21,22] not detectable in the heme Soret band. A detailed examination of the pressure-modified protein at atmospheric pressure should prove of great interest because hydration and hydrogen bonding contribute to the extent of coupled versus uncoupled catalytic turnover.[47]

The results suggest that hydrostatic pressure specifically compresses the active site cavity above pyrrole rings C and D, the most open region of the active site, and causes an outward displacement of the I-helix. The changes above pyrrole ring C and D may involve dislocation of particularly mobile residues, notably Phe-87, Phe-193, and Tyr-96, thought to participate in reversible formation of a substrate access channel.[3,6] The binding of a large multifunctional inhibitor has been shown by X-ray crystallography to displace Phe-193 out of the proposed channel into the medium, of Tyr-96 out of the active site toward the medium, and of Phe-87 in a lateral direction.[6] This same region of the molecule undergoes the greatest changes in mean temperature factors when the crystal structures of camphor-bound and camphor-free $P450_{cam}$ are compared.[3] The residues that make up the putative access channel thus appear to be particularly mobile, and their mobility is restricted by substrate binding. Although the direction of their displacement under pressure is likely to differ from that observed on binding of a large ligand, their unusual mobility is consistent with the suggestion that the pressure-induced deformation is particularly severe in that region (Figure 6). Furthermore, the pressure on the Fe−aryl group due to compression of these residues into the active site may provide the driving force for the non-oxidative shift of the aryl group to the porphyrin nitrogens.

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