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Constrained water access to the active site of cytochrome P450 from the piezophilic bacterium *Photobacterium profundum*

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Abstract

Living species inhabiting ocean depths must adapt to high hydrostatic pressure. This adaptation, which must enable functioning under conditions of promoted protein hydration, is especially important for proteins such as cytochromes P450 that exhibit functionally important hydration-dehydration dynamics. Here we study the interactions of substrates with cytochrome P450-SS9, a putative fatty acid hydroxylase from the piezophilic bacterium *Photobacterium profundum* SS9, and characterize the protein's barotropic properties. Comparison of P450-SS9 with cytochrome P450BM-3, a mesophilic fatty acid hydroxylase, suggests that P450-SS9 is characterized by severely confined accessibility and low water occupancy of the active site. This feature may reveal a mechanism of structural adaptation of the piezophilic enzyme. We also demonstrate that saturated and unsaturated fatty acids exert opposite effects on solvent accessibility and hydration of the active site. Modulation of the protein conformation by fatty acids is hypothesized to have an important physiological function in the piezophile.

Keywords

pressure-perturbation spectroscopy; cytochrome P450; piezophilic organism; protein hydration; substrate binding

1. Introduction

Living species that inhabit ocean depths (piezophiles) exhibit a multi-level adaptation to high-hydrostatic pressure [1]. In particular, the structure of piezophilic proteins has to be adapted to permit their functioning under conditions of highly promoted protein hydration. The role of this adaptation is especially important in the case of proteins whose functional mechanisms involve hydration-dehydration dynamics, such as cytochromes P450 [2–4]. The structural and functional comparison of proteins from deep-sea organisms with their homologs from non-piezophilic species may help to elucidate the functional role of protein hydration and provide important information on the dynamics of protein-bound water in enzymatic mechanisms, ligand-induced conformational transitions, and protein-protein interactions.

The cytochromes P450 from piezophilic bacteria represent an excellent subject for the studies of pressure-sensitive conformational equilibrium and may provide clue to the mechanisms controlling the dynamics of protein-bound water in the enzyme. A search in the genomes of piezophilic microorganisms revealed putative cytochrome P450 proteins in *Moritella* sp. strain PE36 and in *Photobacterium profundum* strains SS9 [5]. The protein

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from *Moritella*, an obligatory piezophilic bacterium is similar to the *Photobacterium* protein (46% identity, 68% similarity). In addition, the genome of the shallow water strain *P. profundum* 3TCK also contains a putative P450 protein, which is 97% identical to the one from *P. profundum* SS9. The barotropic adaptation of the two strains of *P. profundum* is the most extensively studied [5], and the pair of putative P450 proteins from these strains provides a good example of closely related proteins with different degrees of adaptation to high hydrostatic pressure.

A BLAST search with the sequence of the P450 protein from *P. profundum* SS9 (P450-SS9) through the genome database showed that the protein has the closest homology to P450s known to be fatty acid hydroxylases, such as human CYP4X11, or P450BM-3 from *Bacillus megaterium*. Significant similarity of the amino acid sequence of P450-SS9 with the sequence of well-studied fatty acid hydroxylase P450BM-3 (22% identity, 44% similarity) allowed us to use the latter mesophilic protein as a reference in the present study, where we sought to probe the manifestations of the high pressure adaptation of the piezophilic protein.

2. Materials and methods

2.1 Plasmid construction

The gene (YP_133374.1) encoding P450-SS9 protein was amplified using add-on PCR by addition the *Nde*I and *Not*I restriction sites and was introduced into pET29(b) vector (Novagen). The resulting plasmid encodes a P450-SS9 protein with hexa-histidine tag at the C-terminus.

2.2 Protein purification

P450-SS9-His6 was expressed in *E. coli* HMS173 (DE3) using cells harboring the pET29-P450SS9 plasmid and purified using the combination of ion-exchange and Ni-NTA affinity chromatography.

2.3. Experimental

All experiments were performed at 25 °C in 0.1 M Na-Hepes, pH 7.4, 0.5 mM EDTA. Ambient absorbance measurements were done with continuous stirring. Myristic acid and arachidonoyl glycine (AraGly) were used as 40 mM stock solutions in acetone. The experiments with the reduced carbonyl complex of P450-SS9 were performed at 4 °C in the buffer saturated with CO. Reduction of P450 was achieved by adding sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) to a concentration of 12.5 mM. Absorbance titration studies were performed with a S2000 rapid scanning spectrometer (Ocean Optics Inc., Dunedin, Florida) equipped with a thermostated cell chamber, magnetic stirrer and a L7893 UV-Vis fiber optics light source. High-pressure spectroscopic studies were performed as described [6] using a high-pressure optical cell [7] connected to a pressure generator (High Pressure Equipment, Erie, PA) capable of generating a pressure of up to 6000 bar.

2.4 Data analysis

To interpret the changes in the absorbance spectra observed in pressure-perturbation experiments in terms of the changes in the concentration of P450 and P420 species, we used principal component analysis (PCA) [8]. The set of spectral standards used in experiments consisted of the prototypical spectra of the ferric high-spin, ferric low-spin and ferric P420 states of the heme protein [9]. The spectra obtained in pressure-perturbation experiments were corrected for solvent compression prior to the analysis.

Interpretation of the effect of pressure on spin equilibrium was based on the canonical equation for pressure dependence of the constant of chemical equilibrium [10], p. 212, eq. 9:

$$K_{eq} = K_{eq}^0 \cdot e^{-P\Delta V^0/RT} = e^{(P_{1/2}-P)\Delta V^0/RT} \quad (1)$$

Here K_{eq} is the equilibrium constants of the reaction at pressure P , $P_{1/2}$ is the pressure at which $K_{eq} = 1$ ("half pressure" of the conversion), ΔV^0 is the standard molar reaction volume, and K_{eq}^0 is the equilibrium constant extrapolated to zero pressure, $K_{eq}^0 = e^{P_{1/2}\Delta V^0/RT}$.

To find the exact position of the maximum of the Soret band in the analysis of the heme pocket compressibility we used GRAMS32/AI software (Thermo Fisher Scientific, Waltham, MA) to approximate the spectra with a combination of Gaussian and Lorentzian functions as described [11].

3. Results and discussion

3.1 Expression, purification and spectral properties of P450-SS9

P450-SS9 cDNA was amplified and incorporated into a pET29a plasmid, expressed in *E. coli*, and purified. The maximum of the Soret absorbance band of purified P450-SS9 protein was found at 393 nm (Figure 1a). This position of the band suggests that the penta-coordinated high-spin state predominates (>80%) in P450-SS9, in contrast to the majority of other cytochromes P450, which are in the hexa-coordinated (water-ligated) low-spin state in the absence of substrates.

A distinctive feature of cytochromes P450 is the position of the Soret band of the reduced carbonyl complexes of the heme protein, which is usually located at 448–451 nm. However, in the case of P450-SS9 this band was found at ~446 nm, which appears to be the most "blue-shifted" position of the P450 Soret band ever reported. This unusual short-wavelength position may indicate a very non-polar environment of the heme chromophore [12].

Both the position of the spin equilibrium and the blue shift of the Soret band P450-SS9 are consistent with non-polar environment of the heme chromophore and low water occupancy of the heme pocket of the piezophilic protein.

3.2 Ligand binding

Preliminary analysis showed that P450-SS9 reveals the closest homology to the P450 proteins known to be fatty acid hydroxylases, and cytochrome P450BM-3 in particular. Thus, we probed the interactions of P450-SS9 with a series of fatty acids and their derivatives. The enzyme binds both saturated and unsaturated alkyl chains with affinity similar to known fatty acid hydroxylases. In the present study we analyzed the interactions of the enzyme with myristic acid and arachidonoyl glycine (AraGly), as examples of high affinity ligands having saturated and unsaturated alkyl chains, respectively.

As shown in Figure 1a, addition of either myristic acid or AraGly to P450-SS9 result in pronounced spectral changes. Myristic acid increases the amplitude of the Soret band located at 393 nm, which is indicative of a further low-to-high shift in the spin equilibrium (type I spectrum). This behavior is typical of cytochromes P450, which exhibit an increase in the high-spin fraction in response to the binding of most substrates. In contrast, addition of AraGly resulted in the displacement of the maximum of the Soret band to ~415–416 nm, which reveals a transition of the enzyme to the low-spin state (reverse type I spectrum). Titration of the enzyme with myristic acid and AraGly yielded the binding constants of 1 μ M and 17 μ M respectively (Fig. 1b).

Studies with other fatty acids demonstrated that the direction of the substrate-induced spin shift depends on the degree of saturation of the alkyl chain (data not shown). Although the dependence of the spin shift amplitude on the bulkiness and geometry of a substrate molecule is well-documented [13–14], a switch between type I and reverse type I is unprecedented. The substances that produce reverse type I spectra with P450 enzymes often have hydroxyl groups and are suggested to replace water as the ligand to the heme iron and, therefore, stabilize the low-spin state [15]. Conversely, it was recently shown that some compounds having no hydroxyl or other possible iron-ligating groups may also induce this type of changes in cytochromes P450 [16]. However, the mechanism of this effect remains obscure.

3.3 Active site accessibility in P450-SS9

The ability of hydrogen peroxide to oxidize and destroy the heme moiety provides a simple means to probe the accessibility of the heme pocket to small molecules [17]. In Fig. 2 we compare the kinetics of H_2O_2 -dependent heme depletion in P450-SS9 and P450-BM3. The bleaching of P450BM-3 is very fast, so that the time at which the concentration of the heme protein is reduced by $1/2$ ($t_{1/2}$) is as short as 50 sec. However, in P450-SS9 the process of bleaching is much slower ($t_{1/2} \sim 1700$ sec). This finding is consistent with the above assumption of confined water accessibility to the active site of P450-SS9. Importantly, the two probed substrates have an opposite effect on the kinetics of heme depletion, which may reveal a modulatory mechanism of potential functional importance. Thus, myristic acid decreased the reaction rate ($t_{1/2} \sim 2300$ sec), whereas AraGly noticeably accelerated the bleaching ($t_{1/2} \sim 900$ sec). The confined water accessibility of the active site found in these experiments may reveal a mechanism of structural adaptation of P450-SS9 to a high pressure environment.

3.4 Compressibility of the active site of P450-SS9

Next, we explored compressibility of the heme pocket, which was assessed from the pressure-induced displacement of the Soret absorbance band of the CO complex of the ferrous heme protein. These studies were performed in the pressures not exceeding 200 MPa, where pressure-induced conversion to the P450 state did not exceed 15% (See Fig. 1S in the Supplement). An increase in hydrostatic pressure results in a displacement (“red-shift”) and broadening of the absorbance band, indicating a compression of the chromophore environment that results in tightening interactions of the excited state with adjacent polar groups and the solvent molecules [12]. The slope (α) of the dependence of the Soret band wave number (ν) on pressure may therefore be used as a measure of the compressibility of the heme pocket. The effect of pressure on the position of the Soret band in P450-SS9 and its substrate complexes is illustrated in Fig. 3 and Table 1. Compressibility of substrate free P450-SS9 ($\alpha = -0.28 \text{ cm}^{-1}/\text{MPa}$, Fig. 3a) falls within the range of other cytochrome P450 studied to date, (α in the range of -0.22 to $-0.39 \text{ cm}^{-1}/\text{MPa}$; see [11] for an overview), and myristic acid has little effect. However, binding of AraGly results in a remarkable increase in the slope of the pressure dependence of the Soret band wave number at pressures 1–50 MPa ($\alpha = -0.42 \text{ cm}^{-1}/\text{MPa}$). The dependence obtained in the presence of AraGly exhibits a break at ~ 40 MPa, after which compressibility decreases significantly (Fig. 3b). This break apparently signifies a conformational transition of the protein to a state with decreased compressibility of the heme pocket. The fact that this transition is observed only in the presence of AraGly may indicate a significant modulation of the protein conformation by this substrate. Another, equally possible explanation of the effect of AraGly may be that the conformational transition revealed in this break is a general feature of P450-SS9 even without substrate but that AraGly is necessary for this transition to affect the heme pocket compressibility. It is important that this apparent conformational switch in the protein takes place close to the physiological pressure of inhabitation of *P. profundum* SS9 (~ 30 MPa).

3.5 Pressure induced P450 → P420 transition of ferric P450-SS9

Pressure-induced changes have been studied extensively in a number of bacterial and eukaryotic cytochromes P450 [2,6,18]. In all those enzymes studied to date, increasing hydrostatic pressure results in two major processes 1) a displacement of the spin equilibrium towards the low spin state, which is coupled with dissociation of the enzyme-substrate complex and expulsion of at least one water molecule from the active site ($\Delta V^\circ \geq 18$ ml/mol), and 2) further conversion of the P450 heme protein into so-called cytochrome P420, an inactivated form of the enzyme. Similar to other cytochromes P450, P450-SS9 exhibits a displacement of the Soret band from 393 to 416 nm which takes place at the initial steps of compression and is completely reversible upon decompression from ≤ 150 MPa. This displacement suggests a pressure induced spin shift towards the low spin state, which is essentially incomplete either in the substrate free P450-SS9 or in its complexes with myristic acid or AraGly (see Supplement, Fig. 2S). Incompleteness of the pressure induced spin shift appears to be a distinctive feature of the piezophilic enzyme, which may be a sign of a conformational change that decreases the water accessibility of the heme pocket. The detailed analysis of the effect of pressure on the position of spin equilibrium will be provided in a forthcoming publication.

The P450→P420 transition in P450-SS9 takes place at pressures above 150 MPa and is reflected in a further red shift of the Soret band, which reaches approximately 427–428 nm at 450–500 MPa. The process is essentially irreversible, so that the heme protein was found completely in P420 state after decompression from 500 MPa. Similar to other P450 enzymes [9,19–20] the P450→P420 transition in P450-SS9 exhibits an important negative volume change that suggests that the enzyme inactivation is associated with considerable hydration of the protein. However, compared with P450-BM3 [9], the transition in P450-SS9 is displaced to higher pressures and is characterized by a smaller negative ΔV° value (Table 1, Fig.4a). Stabilization of the piezophilic enzyme against pressure-induced inactivation revealed in this difference is not surprising and may reflect the high pressure adaptation of the enzyme.

Similar to the complexes of P450BM-3 with palmitic acid [9], addition of myristate does not change ΔV° but displaces the inactivation to higher pressures in P450-SS9 (Fig. 4b, $P_{1/2}$). This finding is consistent with a model where the substrate molecule physically screens the water access to the active site and hinders the heme pocket hydration that is required for the P450→P420 transition. However, the effect of AraGly is opposite, in that the transition to P420 occurs at lower pressures, and ΔV° is increased considerably. The increase in ΔV° may signify an AraGly-induced conformational transition. This conclusion is in line with the results on the effect of AraGly on the kinetics of H_2O_2 -dependent heme depletion and heme pocket compressibility discussed above.

4. Conclusions

1. The piezophilic cytochrome P450-SS9 has decreased water accessibility and tightened control of heme pocket hydration, which is anticipated to be a result of high-pressure adaptation of the enzyme.
2. The opposite effect of saturated and unsaturated fatty acids on solvent accessibility of the heme pocket and the spin state of the heme iron suggests an important modulation of the enzyme conformation by substrate binding. This effect is especially important with AraGly, in which case the formation of the enzyme-substrate complex results in a sharp decrease in the compressibility of the heme pocket that takes place at pressures close to the pressure of inhabitation of *P. profundum* SS9.

3. Contrasting effects of saturated and unsaturated fatty acids on P450-SS9 may be important in modulation of the enzyme activity *in vivo*. Further studies are necessary to reveal the place of the enzyme in the metabolic pathways of *P. profundum* and to elucidate a possible physiological role of this regulatory mechanism.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Figure 1.

Interactions of P450-SS9 with substrates a) The spectra of the substrate-free ferric P450-SS9 and its complexes with myristic acid and arachidonoyl glycine. b) Spin changes observed upon titration of 0.8 μM P450-SS9 with myristic acid and AraGly. Solid lines represent the approximation of the experimental results with the equation for the equilibrium of binary association.

Figure 2.

Kinetics of hydrogen peroxide-dependent heme depletion. Conditions: 1 μM P450-SS9 or the heme domain of P450BM-3 and 100 mM H_2O_2 in 100 mM Na-HEPES buffer, pH 7.4, and 1 mM EDTA, and P450-SS9 in complexes with myristic acid (16 μM) and AraGly (32 μM). The kinetic curves were fitted to a multiexponential equation (solid lines).

Figure 3.

Pressure induced displacement of the Soret band of the ferrous carbonyl complexes of substrate free P450-SS9 (a) and the complexes of the enzyme with myristic-acid ($\circ$) or AraGly (∇). Parameters for the linear approximation of the data sets slope are given in the Table 1.

Figure 4.

Pressure induced P450→P420 transition in ferric (Fe^{3+}) P450-SS9 a) P450→P420 transition of the substrate-free ferric P450-SS9, its complexes with myristic acid (8 μM) and AraGly (16 μM), and the substrate-free heme domain of the cytochrome P450BM-3. Solid lines represent the fitting of the data sets with equation (1). Barotropic parameters for P450-SS9 deduced from this fitting may be found in Table 1. The dependence obtained with P450BM-3 is characterized by $\Delta V^\circ = -84 \text{ mL/mol}$ and $P_{1/2} = 204 \text{ MPa}$. The curves were scaled to 0–100% interval relative to the maximal amplitude determined from fitting. b) Half-pressure of inactivation ($P_{1/2}$) as a function of concentration of myristic acid and AraGly.

Table 1

Results of pressure perturbation studies of P450-SS9

	P450→P420 transition			Active site compressibility	
	$P_{1/2}$ (MPa)	$-\Delta V^\circ$ (ml/mol)	ΔG°_{P420} (kJ/mol)	α^b (cm ⁻¹ /MPa)	ν^b (cm ⁻¹)
No substrate	251.7 ± 19.1	41.4 ± 1.6	10.4 ± 0.3	-0.28 ^c	22372.0
Myristic acid	297.0 ± 8.4	42.2 ± 2.9	12.5 ± 1.2	-0.23 ^c	22371.8
Arachidonoyl glycine	209.8 ± 2.4	64.9 ± 1.8	13.6 ± 0.5	-0.42 ^d	22356.7
				-0.12 ^e	22343.7

The slope α and the offset ν° characterize the linear approximation of the pressure dependence of the Soret band:

^a Gibbs free energy of reaction calculated using the $-\Delta V$ and $P_{1/2}$ values.

^b As shown in the Figure 2.

^c in 0.1–200 MPa region.

^d in 0.1–60 MPa region

^e in 60–200 MPa region