Electrostatic Control of the Substrate Access Channel in Cytochrome P-450$_{cam}$[†]

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ABSTRACT: Camphor binding to ferric cytochrome P-450$_{cam}$ is a two-step process. The first step corresponds to the diffusion of camphor into the heme pocket, and the second one corresponds to an observable spin transition of the heme iron. In this paper, electrostatic interactions that may control the opening of the structure to allow substrate access to the buried and not solvent-exposed active site were examined. The electrostatic interactions occurring at the protein surface were weakened by increasing the ionic strength of the medium with sodium salts and strengthened by decreasing the dielectric constant of the medium with ethylene glycol as a cosolvent. The results obtained with the wild-type protein were compared to those obtained with the site-directed mutant of cytochrome P-450$_{cam}$ in which the Arg 186−Asp 251 and Lys 178−Asp 251 salt bridges, located at the entrance of the proposed access channel, were suppressed by replacing Asp 251 with an asparagine residue. Over a range of sodium chloride concentrations from 0 to 400 mM, camphor binding is favored, as seen in the variation in the first step dissociation equilibrium constant, K_{1d}, which decreases from 49.5 to 24 μM, respectively. Addition of ethylene glycol favors the dissociation of the substrate-bound complex. The addition of sodium to the ethylene glycol-containing samples reverses the effect of the cosolvent. Removal of the Arg 186−Asp 251 and Lys 178−Asp 251 salt bridges results in an alteration in camphor binding in which K_{1d} is equal to 34 μM without sodium. The thermodynamic analysis of the overall binding step shows that the thermodynamic parameters for the diffusion step are equal when salt bridges are disrupted by the addition of sodium to the wild-type protein sample or by complete removal as in the D251N mutant. These results suggest a model in which the Arg 186−Asp 251−Lys 178 bifurcated salt bridge plays a key role in the control of camphor access to the buried active site of cytochrome P-450$_{cam}$.

Cytochrome P-450$_{cam}$[1] is a monooxygenase from *Pseudomonas putida* that catalyzes the stereospecific hydroxylation of camphor at the 5-exo position. The three-dimensional X-ray structures of the substrate-free and substrate-bound proteins have been established (Poulos et al., 1986, 1987). The comparison of the two refined structures does not illuminate any important conformational changes when camphor binds to the buried active site, which is located above the heme with no direct access to the solvent. There is, at the very most, a small repositioning of the Phe 87 side chain, and the three regions centered on Tyr 96, Thr 185, and Asp 251 show decreased temperature factors. The six water molecules present in the substrate-free form are displayed from the heme pocket to the solvent, and the spin state of the heme iron shifts from low-spin to high-spin (Tsai et al., 1970; Sharrock et al., 1976; Sligar, 1976; Harris & Loew, 1993).

One challenge in cytochrome P-450$_{cam}$ catalysis is to understand how the substrate reaches its binding site. Besides a depression at the surface of the protein that could correspond to the access channel, there is no apparent pathway for camphor entry. Clearly the protein undergoes important conformational changes that allow the entrance of the substrate or the exit of the product. The mechanism of control of this gating process is not understood presently and is the subject of this investigation.

The substrate binding step has been the subject of numerous investigations using T-jump (Fisher & Sligar, 1987) and P-jump techniques (Marden & Hui Bon Hoa, 1982; Douzou & Hui Bon Hoa, 1987; Hui Bon Hoa et al., 1991). The results have shown that the variations in the observed rates of spin transition as a function of increasing camphor concentrations exhibit saturation behavior, which suggests that camphor binding is a two-step process. The first step corresponds to the entry of the substrate into the oxidized form of the protein, giving a low-spin substrate-bound form. The second step is the well-known spin transition. While the latter has been investigated extensively (Sligar, 1976; Fisher & Sligar, 1987; Atkins & Sligar, 1990; Di Primo et al., 1990, 1992), little is known about the initial substrate diffusion step. The X-ray structure suggests that salt bridges could control the opening of the structure. Of particular interest are those formed between Arg 186, Lys 178, and Asp 251, which are located at the entrance of the proposed access channel (Poulos et al., 1985).

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[1] Abbreviations: cytochrome P-450$_{cam}$, soluble hemoprotein from *Pseudomonas putida* that catalyzes the hydroxylation of camphor when utilized as the sole carbon source (EC 1.14.15.1); D251N, mutant of cytochrome P-450$_{cam}$ in which aspartate 251 was replaced by an asparagine residue.

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To test whether these ionic pairs are involved in the control of the diffusion step of substrate entry, we have altered the force of the electrostatic interactions by changing the ionic strength and the dielectric constant of the medium, and we measured the influence of theses changes on the actual binding step of the substrate. The results obtained with the wild-type protein were compared to those obtained with the site-directed mutant of cytochrome P-450$_{cam}$, in which the Arg 186−Asp 251 and Lys 178−Asp 251 salt bridges were suppressed by replacing Asp 251 with an asparagine residue. The thermodynamics of the two-step process was also examined.

The results presented in this paper support a view in which the bifurcated salt bridge located at the entrance of the proposed access channel plays a key role in the control of the opening of the structure.

MATERIALS AND METHODS

Cytochrome P-450$_{cam}$ wild type and the D251N mutant protein were generated and purified as previously described (Gunsalus & Wagner, 1978; Gerber, 1993) and stored at −80 °C in the presence of a saturating concentration of camphor. Substrate-free protein was obtained by passage of a pure sample through a Sephadex G-25 column previously equilibrated with the buffer used for the experiments: 100 mM Tris-HCl (pH 7) at 20 °C. All chemicals used were of the highest purity commercially available.

As suggested by T-jump and P-jump experiments, the proposed theoretical four-step model for camphor binding (Sligar, 1976) can be simplified to a two-step model as follows:

$$\text{P-450}^{o}_{LS} + S \overset{K_{1d}}{\leftrightarrow} \text{P-450}^{os}_{LS} \overset{K_2}{\leftrightarrow} \text{P-450}^{os}_{HS}$$

where o, LS, S, and HS refer to oxidized, low spin, substrate, and high spin, respectively. $K_{1d} = [\text{P-450}^{o}_{LS}][S]/[\text{P-450}^{os}_{LS}]$ is the equilibrium constant for the actual binding step of the substrate, the diffusion step, and $K_2 = [\text{P-450}^{os}_{HS}]/[\text{P-450}^{os}_{LS}]$ is the spin equilibrium constant.

K_{1d} was calculated by using thermodynamic equalities in a cycle of spin and substrate binding equilibria (Sligar, 1976; Gould et al., 1981; Fisher & Sligar, 1987):

$$K_{1d} = K_d(1 + K_2) \quad (1)$$

where $K_d = [\text{P-450}^{o}_{LS}][S]/([\text{P-450}^{os}_{HS}] + [\text{P-450}^{os}_{LS}])$ is the apparent dissociation constant for camphor binding. This relation was obtained utilizing the fact that the spin equilibrium constant without substrate is very small (Sligar, 1976). Eadie−Hofstee plots were used to determine K_d by optical titrations at two wavelengths, 417 and 392 nm, which correspond to the absorption peaks of the low-spin and high-spin oxidized forms of cytochrome P-450$_{cam}$, respectively (only fits with a correlation coefficient greater than 0.99 were considered). The concentration of bound high-spin form, [P-450$^{os}_{HS}$], was measured directly from this observable transition. The concentration of bound low-spin form, [P-450$^{os}_{LS}$], was then deduced using K_2. K_2 was determined at saturating concentration of camphor, 400 μM in aqueous medium and 1 mM in water/ethylene glycol mixtures, as described (Jung et al., 1991). Binding titrations were performed with 5−8 μM cytochrome P-450$_{cam}$ on a Uvikon 940 spectrophotometer thermostated with a Huber HS 40 circulating bath to allow determination of the enthalpies according to the van't Hoff law.

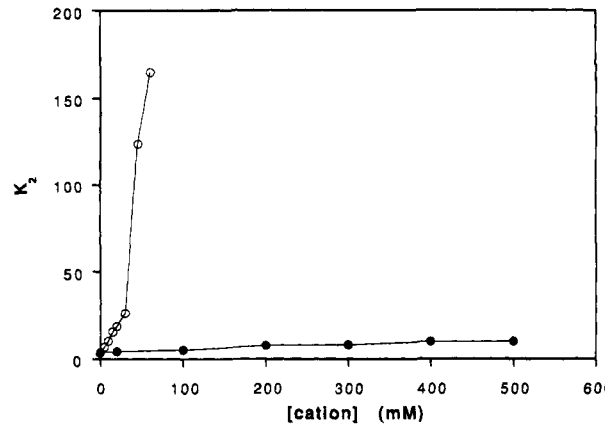

FIGURE 1: Effect of cations on the spin equilibrium constant, K_2: (○) potassium; (●) sodium. The experiments were performed at 20 °C in 100 mM Tris-HCl (pH 7). Similar results were obtained with the D251N mutant.

To modulate the electrostatic interactions by changing the ionic strength of the medium, it was crucial to use a cation that alters mainly the diffusion step. For this purpose, sodium salts are preferred over potassium due to the presence of a potassium-specific cation binding site in cytochrome P-450$_{cam}$ (Lange & Debey, 1979; Lange et al., 1979; Poulos et al., 1987; Di Primo et al., 1990; Deprez et al., 1994), which strongly influences the spin equilibrium (Figure 1). Similar results were obtained for the D251N protein. The effect of potassium corresponds to an almost complete transition to the substrate-bound high-spin form and therefore would not allow investigation of the diffusion step. The small effect of sodium on K_2 compared to that of potassium is taken into account to calculate K_{1d} according to eq 1.

Ethylene glycol was used to modulate the force of the ionic pair by changing the dielectric constant of the medium. The concentrations of water/ethylene glycol mixtures were varied from 0 to 50% (by volume), which corresponds to a change in the dielectric constant from 80.4 to 64.5 at 20 °C, respectively. In this range, the protonic activity and the viscosity of the medium are nearly the same (Douzou et al., 1976), and no inactivated (cytochrome P-420) or denaturated form of the protein was detected. In ethylene glycol solutions up to 60%, the extinction coefficients of all cytochrome P-450$_{cam}$ forms are unchanged, and ethylene glycol was not found to significantly influence the spin equilibrium (see supplementary material Table 2). Since the spin equilibrium is strongly sensitive to variations in pH (Lange & Debey, 1979), this physicochemical parameter was not used to alter the electrostatic interactions.

RESULTS

The interaction force between two charged partners follows the Coulomb law according to the equation $F = qq'/\epsilon d^2$, where F is the interaction force between two charges, q and q', separated by a distance d, in a medium where ϵ is the average dielectric constant. By increasing the ionic strength of the medium, which induces a screening effect on the Coulomb charges, the interaction force F decreases. By lowering the dielectric constant of the medium, the interaction force F increases. These two effects were induced by

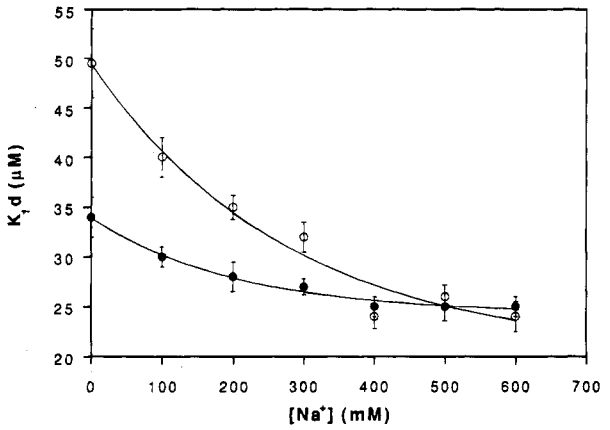

FIGURE 2: Effect of sodium on the first step equilibrium constant, K_{1d}: (○) wild-type cytochrome P-450$_{cam}$; (●) D251N mutant. Experimental conditions are those of Figure 1. K_{1d} was calculated according to eq 1 using measured K_d and K_2 values which are reported in Table 1 in the supplementary material.

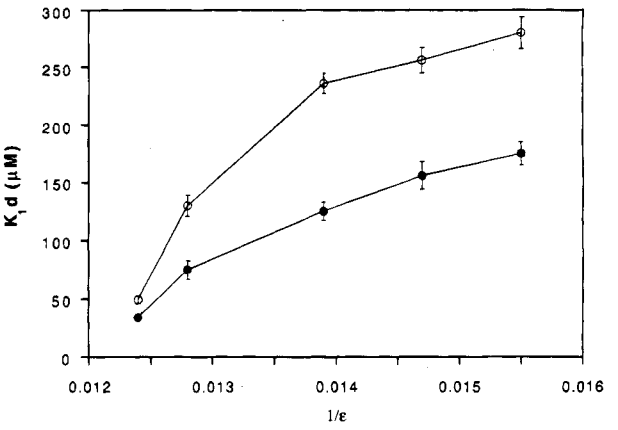

FIGURE 3: Effect of a change in dielectric constant on the first step equilibrium constant, K_{1d}: (○) wild-type cytochrome P-450$_{cam}$; (●) D251N mutant. Ethylene glycol was added to change the dielectric constant, as described in Materials and Methods. The experiments were performed at 20 °C in a water/ethylene glycol mixture, the protonic activity of which is equal to 7. See also Table 2 in the supplementary material for detailed values of K_d, K_{1d}, and K_2 as a function of increasing concentration of ethylene glycol.

using increasing concentrations of monovalent cation (Na^+) and polyols (ethylene glycol), respectively.

Figure 2 illustrates the ionic strength dependence of the dissociation constant for the diffusion step of the substrate, K_{1d}, for both wild-type cytochrome P-450$_{cam}$ and the D251N mutant. When the charges of the protein are neutralized by increasing the concentration of sodium, K_{1d} varies from 49.5 ± 3.5 to 24 ± 1.5 μM for the wild-type protein. The camphor-bound state of the protein is favored. A plateau is reached, which suggests that all electrostatic interactions involved in the binding process have been neutralized. Removal of the bifurcated salt bridge significantly alters K_{1d}, which is equal to 34 ± 2 μM in the absence of sodium salt. When the concentration of the cation is increased, K_{1d} decreases and reaches the plateau obtained with the wild-type protein, 25 ± 1 μM. These results suggest that the Arg 186−Asp 251 and Lys 178−Asp 251 salt bridges play a role in camphor binding, but these electrostatic interactions do not appear to be the only ones operating. The value obtained in the absence of sodium, 34 μM, is not equal to that expected if only the Arg 186−Asp 251−Lys 178 bifurcated salt bridge is involved in camphor binding, 25 μM. The mutant protein is still sensitive to changes in the ionic strength, as seen in the 9 μM variation in K_{1d} between samples without sodium and samples with 400 mM sodium. This also indicates that other electrostatic interactions, the nature of which is unknown, contribute to the control of camphor access.

In Figure 3, we present the variations in K_{1d} when the dielectric constant of the medium is decreased. The variation of $1/\epsilon$ is proportional to the Coulomb force. When increasing concentrations of ethylene glycol are added, K_{1d} increases for both wild-type cytochrome P-450$_{cam}$ and the D251N mutant. The dissociation of the substrate-bound complex is favored. The effect is more pronounced for the wild-type protein. K_{1d} varies from 49.5 ± 3.5 to 280 ± 14 μM in aqueous medium and 50% ethylene glycol, respectively. Again the effect on the mutant protein indicates that the Arg 186−Asp 251 and Lys 178−Asp 251 electrostatic interactions are not the only ones implicated in the diffusion step of the substrate. The large effect of the dielectric constant on K_{1d}, compared with the effect of the ionic strength, could be the result of an increased solubility of camphor in water/ethylene glycol mixtures, which then would contribute to increase K_{1d}. This point will be discussed in the next section.

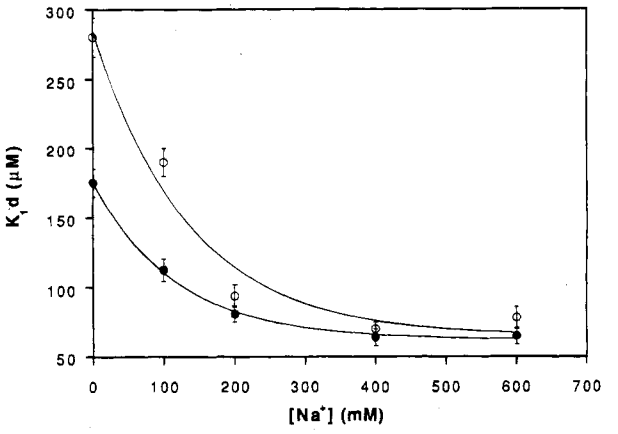

FIGURE 4: Effect of sodium on the first step equilibrium constant, K_{1d}, in 50% ethylene glycol-containing samples at 20 °C: (○) wild-type cytochrome P-450$_{cam}$; (●) D251N mutant. See also Table 3 in the supplementary material for detailed values of K_d, K_{1d}, and K_2 as a function of increasing concentration of sodium.

The effect of increasing concentrations of sodium on 50% ethylene glycol-containing samples is presented in Figure 4. The effect on K_{1d} induced by the dielectric constant is reversed by changes in the ionic strength. This result supports the model of electrostatic control of the substrate access channel in cytochrome P-450$_{cam}$. It should be noticed that the value of K_{1d} obtained at high concentrations of sodium, roughly 70 μM for both wild-type cytochrome P-450$_{cam}$ and its mutant, does not correspond to the value obtained in aqueous medium at the same concentration of sodium, 25 μM. The reasons for this discrepancy could be attributed to an increased solubility of camphor in water/ethylene glycol mixtures.

The thermodynamics of the two-step process was examined for both the wild-type protein and the D251N mutant. The results are presented in Tables 1 and 2 for two conditions of ionic strength: 0 and 400 mM sodium salt. An increasing ionic strength results in an increase in both the enthalpy and entropy of the wild-type protein for the diffusion step. Within the experimental errors, the diffusion step thermo-

Table 1: Thermodynamic Parameters for Camphor Binding to Wild-Type Cytochrome P-450$_{cam}$[a]

	first step	second step	overall reaction
(a) 100 mM Tris-HCl, pH 7, 20 °C			
ΔG (kcal mol^{-1})	-5.81 ± 0.06	-0.73 ± 0.03	-6.69 ± 0.05
ΔH (kcal mol^{-1})	$+3.5 \pm 0.2$	$+8.5 \pm 0.4$	$+10.0 \pm 0.6$
ΔS (cal mol^{-1} K^{-1})	$+32 \pm 1$	$+32 \pm 1$	$+57 \pm 2$
(b) 100 mM Tris-HCl, pH 7, 20 °C, 400 mM Sodium			
ΔG (kcal mol^{-1})	-6.23 ± 0.07	-1.35 ± 0.02	-7.63 ± 0.06
ΔH (kcal mol^{-1})	$+6.4 \pm 0.3$	$+8.1 \pm 0.5$	$+10.2 \pm 0.6$
ΔS (cal mol^{-1} K^{-1})	$+43 \pm 1$	$+32 \pm 1$	$+61 \pm 3$

[a] Thermodynamic parameters are determined by using van't Hoff plots between 25 and 11 °C (see supplementary material Figure 1). The parameters for the first step and the second step are deduced from the variations in $1/K_{1d}$ and K_2, respectively, as a function of temperature. The parameters for the overall reaction (apparent association reaction) are deduced from the variation in $1/K_d$, the apparent association constant, as a function of temperature.

Table 2: Thermodynamic Parameters for Camphor Binding to D251N Mutant[a]

	first step	second step	overall reaction
(a) 100 mM Tris-HCl, pH 7, 20 °C			
ΔG (kcal mol^{-1})	-6.03 ± 0.05	-0.78 ± 0.03	-6.95 ± 0.04
ΔH (kcal mol^{-1})	$+7.0 \pm 0.4$	$+3.6 \pm 0.4$	$+11.0 \pm 0.7$
ΔS (cal mol^{-1} K^{-1})	$+44 \pm 2$	$+15 \pm 1$	$+61 \pm 3$
(b) 100 mM Tris-HCl, pH 7, 20 °C, 400 mM Sodium			
ΔG (kcal mol^{-1})	-6.20 ± 0.03	-1.28 ± 0.04	-7.56 ± 0.04
ΔH (kcal mol^{-1})	$+7.4 \pm 0.3$	$+2.3 \pm 0.4$	$+9.8 \pm 0.5$
ΔS (cal mol^{-1} K^{-1})	$+46 \pm 2$	$+12 \pm 2$	$+59 \pm 4$

[a] Thermodynamic parameters were determined by using van't Hoff plots between 4 and 25 °C (see supplementary material Figure 2).

dynamic parameters for the mutant are not sensitive to ionic strength changes and are similar to those obtained with the wild-type protein in the presence of 400 mM sodium. The small effect of sodium on the spin equilibrium reported in Figure 1 is also observed for the corresponding thermodynamic parameters.

DISCUSSION

Electrostatic interactions play a key role in the formation of intermolecular complexes. The formation of salt linkages between cytochrome b_5 and cytochrome c (Rodgers et al., 1988; Stayton et al., 1988) and between putidaredoxin and cytochrome P-450$_{cam}$ (Stayton et al., 1989; Stayton & Sligar, 1990) allows appropriate orientation of the electron donor and acceptor partners. In hemoglobin, salt bridges between subunits are involved in the allosteric transition (Baldwin & Chotia, 1979). In sperm whale myoglobin, intramolecular electrostatic interactions are implicated in the dynamics of ligand diffusion through the protein matrix (Westrick et al., 1990). In this work, we present another example where intramolecular electrostatic interactions regulate the diffusion of a substrate from the solvent to a buried active site.

In cytochrome P-450$_{cam}$, the Arg 186−Asp 251−Lys 178 bifurcated salt bridge connects the F−G loop to the I helix in a depression formed by residues Phe 87, Thr 185, and Ile 395. This was proposed to correspond to the access channel for camphor (Poulos et al., 1985). Due to the size of the substrate molecule and the absence of an apparent pathway, this region needs to open, the dynamics of which could be controlled by the Arg 186−Asp 251 and Lys 178−Asp 251 salt linkages located at the entrance. Further X-ray results (Poulos & Howard, 1987; Raag et al., 1993) and molecular dynamics simulations on substrate-bound cytochrome P-450$_{cam}$ (Paulsen & Ornstein, 1991) suggest that this region undergoes important conformational changes that could be related to the opening of the structure.

In order to examine the role of the Arg 186−Asp 251−Lys 178 salt bridge, the electrostatic interactions within cytochrome P-450$_{cam}$ have been modified: first weakened by increasing the ionic strength of the medium with sodium salts and then strengthened by decreasing the dielectric constant of the medium with ethylene glycol. As seen in Figures 2 and 3, the effects of these changes on K_{1d} are opposite and are more pronounced for the wild-type protein than for the D251N mutant. The increased interactions induced by ethylene glycol were reversed by the addition of sodium directly to the ethylene glycol-containing samples. However, there is a discrepancy between the value reached at the plateau ($K_{1d} = 70$ μM) and the value expected if the reversion is complete ($K_{1d} = 25$ μM), for both wild-type cytochrome P-450$_{cam}$ and the D251N mutant. This result could be due to an effect of ethylene glycol on the solubility of camphor, thus partitioning the substrate over the solvent/protein phases.

The measured binding free energy of the diffusion step would be equal to ΔG_1, which is the actual binding free energy, plus the phase transition free energy of camphor, ΔG_{trans}, from a water/ethylene glycol mixture to aqueous medium. To determine ΔG_{trans}, we measured, at 20 °C, the solubility of camphor in 100 mM Tris-HCl, Sol$_T$, and in 50% ethylene glycol, Sol$_{EGOH}$. ΔG_{trans} is then equal to RT ln Sol$_{EGOH}$/Sol$_T$ and was found to be equal to 516 cal/mol. ΔG_1 is then equal to −6.12 and −6.18 kcal/mol for wild-type cytochrome P-450$_{cam}$ and the D251N mutant, respectively, giving values of K_{1d} equal to 29 μM for the wild-type and 26 μM for the D251N mutant at 20 °C. Within experimental errors, these corrected values correspond to the one reached in aqueous medium with sodium at 400 mM (Figure 2). Similarly, the corrected values of K_{1d} in 50% ethylene glycol without sodium (plateau of Figure 3 and starting point of Figure 4) are equal to 116 and 72 μM for wild-type cytochrome P-450$_{cam}$ and the D251N mutant, respectively.

The analysis of the thermodynamic data shows that the second step of the binding process is slightly sensitive to changes in the ionic strength of the medium, while the first step is affected significantly (Table 1a,b) with ΔH and ΔS increasing. When the Arg 186−Asp 251 and Lys 178−Asp251 salt bridges are removed (Table 2a,b), the second step is unchanged when sodium is added compared to the wild-type protein, but the thermodynamic parameters for the diffusion step do not vary with sodium concentration. This emphasizes the key role played by the Arg 186−Asp 251 and Lys 178−Asp 251 interactions in the thermodynamic control of the entry of camphor in cytochrome P-450$_{cam}$.

In the charge interaction model of Bjerrum's theory (Bjerrum, 1926), developed to interpret thermodynamic data (Fuoss & Krauss, 1933; Schellman, 1953), the signs of ΔH and ΔS depend on the opposite of $d(\epsilon T)/dT$ and $d\epsilon/dT$, respectively. These derivatives are generally negative, particularly in water, and ΔH and ΔS are both positive when two charges associate. Experimentally, when two charges dissociate, ΔH is expected to be negative due to the release of heat by increased solvation of charges, and ΔS decreases due to electrostriction around solvent-exposed charges

(Schellman, 1953; Kauzmann, 1959; Cantor & Schimmel, 1980). This was observed by photoacoustic calorimetry studies of sperm whale myoglobin and its Arg 45−heme salt bridge mutant (Westrick et al., 1990). In wild-type cytochrome P-450$_{cam}$, the increase in ΔH and ΔS for the first step can be attributed to a decreased negative contribution of these parameters when the charges are neutralized by the addition of sodium. The effect of neutralization of charges by sodium on ΔH and ΔS is not observed for the D251N mutant, the charge−charge interaction being disrupted by the mutation. These thermodynamic parameters are then identical to those obtained with the wild-type protein when the salt bridges are opened by the addition of sodium cation.

It is interesting to note that the break observed in the van't Hoff plots at 11 °C (see supplementary material Figure 1) for K_{1d} and K_d, in agreement with previous work (Griffin & Peterson, 1972), is not seen for the mutant protein (see supplementary material Figure 2). The origin of the nonlinearity of these plots below 11 °C is not known. However, it suggests that the Arg 186−Asp 251−Lys 178 bifurcated salt bridge is implicated in the overall dynamics of the protein related to the diffusion of camphor through the protein matrix.

In conclusion, the results presented in this paper suggest a model in which the diffusion step of the substrate is controlled by electrostatic interactions, with a key role attributed to the Arg 186−Asp 251 and Lys 178−Asp 251 salt bridges located above the heme pocket. These salt linkages would couple the entry of camphor and a charge relay from the solvent to the heme, where Asp 251 would be implicated in a general acid catalytic mechanism of O−O cleavage and camphor hydroxylation (Gerber & Sligar, 1992).

SUPPLEMENTARY MATERIAL AVAILABLE

Two tables showing experimental data of camphor dissociation constants (K_d) and the spin equilibrium constant (K_2) versus sodium concentration in either Tris-HCl buffer or Tris-HCl/ethylene glycol (50%/50%) mixed buffer (pH 7) at 20 °C. One table showing K_d and K_2 versus dielectric constant (varied by adding various volumes of ethylene glycol to Tris buffer). Each table compares K_d and K_2 for wild-type and D251N (salt bridge mutant) proteins. Two figures of van't Hoff plots of K_d, K_{1d} and K_2 for wild-type cytochrome P-450$_{cam}$ and D251N mutant, showing breaks in the van't Hoff plots of K_d and K_{1d} for the wild-type cytochrome P-450$_{cam}$ (4 pages). Ordering information is given on any current masthead page.

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