An Electrostatically Driven Conformational Transition Is Involved in the Mechanisms of Substrate Binding and Cooperativity in Cytochrome P450eryF[†]

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ABSTRACT: The effect of ionic strength (*I*) on substrate-induced spin transitions and cooperativity in cytochrome P450eryF was studied. At a saturating concentration of 1-pyrenebutanol (1-PB) increasing ionic strength in the 0.06−1.2 M range promotes the formation of the high-spin state of P450, which fraction increases from 26% at 0.06 M to 75% at 1.2 M. This effect was associated with a considerable decrease in cooperativity as revealed in the 1-PB-induced spin shift. While P450eryF exhibits distinct positive cooperativity ($S_{50} = 8.3$ μM, $n = 2.4$) with this substrate at low ionic strength ($I = 0.06$ M), *n* decreases to 1.2 ($S_{50} = 3.2$ μM) at $I = 0.66$ M. Increasing ionic strength also increases the distance between the first (effector) molecule of 1-PB and the heme, as detected by the changes in the efficiency of FRET from 1-PB to the heme. The modification of Cys^{154} with 7-(diethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin (CPM) largely suppresses these effects of ionic strength and causes a prominent decrease in the cooperativity. The same effect was observed when Cys^{154} was substituted with isoleucine. Importantly, Cys^{154} is located at the C-terminal end of helix E and is surrounded by salt bridges formed by arginine, glutamate, and aspartate residues located in helices D, E, F, and G. Our results suggest that the binding of the first substrate molecule causes an important conformational transition in the P450eryF that facilitates the substrate-induced spin shift. This transition is apparently accompanied by dissociation or rearrangement of several salt bridges in the proximity of Cys^{154} and modulates accessibility and hydration of the heme pocket.

Cytochrome P450 is the terminal oxidase in multienzyme monooxygenase systems found in animals, plants, bacteria, and archaea. The membrane-bound liver microsomal monooxygenase system catalyzes the oxygenation of both endogenous substrates, such as steroids, and exogenous compounds, such as drugs. The reaction cycle requires the sequential input of two reducing equivalents (two electrons and two protons), which are necessary to activate molecular oxygen to the formal level of peroxide (recently reviewed in ref *1*). The P450 catalytic cycle has been thoroughly investigated for bacterial cytochrome P450cam from *Pseudomonas putida* (*1*−*8*). These studies demonstrated the crucial importance of heme pocket-bound water molecules in the function of the enzyme (*4*, *5*, *8*). In total, 19 water molecules were implicated in the interactions of P450cam with substrate (fenchone) and in the spin transition of the heme protein (*9*). The magnitude of this change is much higher than expected from the degree of hydration of the substrate-binding pocket near the heme moiety, where from six to nine water molecules were found in substrate-free P450cam (*10*). Thus, the interaction of P450cam with substrate and spin transitions of the heme protein were inferred to involve water bound at sites other than the substrate-binding site and the heme moiety (*9*), suggesting an important conformational change in the enzyme (*2*, *9*, *11*, *12*). Water expulsion is critical, since an excess of water in the heme pocket would lead to uncoupling of the enzyme and production of reactive oxygen species, such as superoxide anion and hydrogen peroxide (*4*, *5*). It appears that the solvent accessibility of the heme pocket of P450cam is controlled by a precise mechanism that coordinates the openness of the active site with discrete catalytic cycle events, such as substrate and oxygen binding and electron transfer. In P450cam this mechanism involves rearrangement of a charge-pairing system centered around Asp^{251} that also includes Lys^{178}, Arg^{186}, Arg^{97}, and Asp^{197} (*13*−*16*). Correspondingly, substrate binding and subsequent spin transitions in P450cam exhibit a strong ionic strength dependence (*6*, *15*). In addition to a K^+-specific effect associated with preferential binding of potassium cation in the proximity of Tyr^{96}, there is a general influence of ionic strength on substrate binding and spin equilibrium, which is mediated by dissociation of intramolecular salt bridges (*15*).

Precise regulation of heme pocket accessibility and hydration also appears to be of special importance for microsomal drug-metabolizing cytochromes P450. Being adapted to metabolize a broad spectrum of structurally diverse compounds, these enzymes often possess large substrate-binding pockets and are characterized by a marginal degree of coupling and low catalytic efficiency (*17*−*19*). As a loose fit of the substrate molecule into the binding pocket is rather common, the displacement of protein-bound water is highly dependent on the nature of the compound. Thus, regulation

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of heme pocket accessibility during the catalytic cycle appears to be essential to maintain catalytic efficiency and coupling within adequate limits.

It is reasonable to suggest that a charge-pairing-driven mechanism controlling heme pocket accessibility and hydration as in P450cam might also be involved in the catalytic mechanism of other P450 enzymes. A marked effect of ionic strength on the spin equilibrium was also reported for substrate-bound bacterial cytochrome P450lin (*15*), as well as for substrate-free P450 52A from *Candida tropicalis* (*20*) and mammalian P450 1A2 and 2B1 (*21*, *22*). Pronounced effects of ionic strength and pH on catalytic efficiency, stereospecificity, and coupling of testosterone oxidation by cytochromes P450 2A1, 2B1, 2C11, and 3A1 were reported by Gemzik and coauthors (*23*). An increase in catalytic efficiency and coupling of P450 2B4 at elevated ionic strength was also found by the laboratory of Schenkman (*24*). These experimental results together with the above analysis of the P450cam data demonstrate the importance of further detailed studies on the effect of ionic strength on structure and function of cytochromes P450.

Studies of the role of intramolecular ionic interactions might be of special importance in the case of cytochromes P450 that exhibit homo- and heterotropic cooperativity. Initially, the cooperativity in substrate binding and catalysis was considered as an unusual peculiarity of certain P450 enzymes, of which P450 3A4 is the best-known example (*25*−*29*). However, observation of allosteric phenomena in microsomal P450 1A2 (*30*−*32*), P450 1A1 (*33*), P450 2C9 (*33*−*35*), an N172H variant of P450 2B6 (*36*), and P450 2B4 (*37*), as well as in bacterial P450eryF[1] (*38*−*41*) shows that allostery has more general importance in the P450 superfamily. Importantly, in all known cytochromes P450 exhibiting homotropic cooperativity this phenomenon is observed only with some substrates, while with other ones (usually those of larger molecular size) the same P450 species exhibit simple Michaelis−Menten behavior. For instance, cytochrome P450eryF exhibits no cooperativity with its natural substrate 6-deoxyerythronolide (6-DEB), while showing sigmoidal substrate saturation curves with androstenedione, 9-aminophenanthrene, 1-pyrenebutanol (1-PB), and 7-benzyloxyquinoline (7-BQ) (*38*, *40*, *41*). The presence of multiple binding sites within the same substrate-binding pocket is believed to be a primary cause of allosteric effects in cytochromes P450 (*29*, *38*, *42*−*44*). Most concepts of the mechanisms of cooperativity in these enzymes are based on the assumption of a loose fit of a single substrate molecule in the large binding site (*29*, *44*, *45*), thus requiring binding of the second substrate molecule for efficient catalysis. Implicitly, these models suggest that the binding of multiple molecules of substrate ensures more efficient expulsion of water from the active site, hence improving the catalytic efficiency of the enzyme. The importance of water expulsion from the active site of P450 for efficient coupling of monooxygenation (*4*, *5*) led us to infer a connection between allosteric effects and coupling of monooxygenation. This conclusion is supported by a recent observation of Hutzler and coauthors with P450 2C9 that allosteric activation of flurbiprofen hydroxylation by dapsone and *N*-hydroxydapsone is associated with a considerable increase in the coupling of the reaction (*35*). Thus, the multiple binding-site model of P450 cooperativity has an important parallelism in P450cam with loosely fitting substrate analogues (*5*, *46*), where the control of the accessibility of the binding site by electrostatic tethering is especially evident (*15*, *46*). By analogy with P450cam we hypothesize that the ionic tethering mechanism (*47*) may play an important role in the modulation of substrate and water accessibility of the active site in cytochromes P450 exhibiting homo- and heterotropic cooperativity.

The present study was carried out as a first step toward the systematic investigation of the role of intramolecular electrostatic interactions in the mechanism of P450 cooperativity. Here we investigate the effect of ionic strength on substrate-induced spin transitions in P450eryF, the only bacterial cytochrome P450 known to possess homotropic cooperativity. In this study we use the fluorescent compound 1-pyrenebutanol (1-PB) as a substrate of P450eryF. Recently, we have shown that P450eryF exhibits high affinity and prominent cooperativity of binding and oxidation of 1-PB (*41*). Using FRET to monitor substrate binding, we also demonstrated that substrate-induced formation of the high-spin state of the heme protein is observed only in the complex of P450eryF with two molecules of 1-PB, while the binding of the first (effector) molecule of 1-PB is assumed to induce a conformational transition, facilitating the spin shift observed in the ternary complex (*41*). The aim of the present study was to probe the relevance of electrostatic tethering mechanisms to this conformational transition, which is proposed to be pivotal in the mechanisms of P450 cooperativity.

EXPERIMENTAL PROCEDURES

Materials. 1-PB and 7-(ethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin maleimide (CPM) were from Molecular Probes Inc. (Eugene, OR). 2-Hydroxypropyl-β-cyclodextrin (HPCD) powder was from Sigma Chemicals (St. Louis, MO). Oligonucleotide primers for PCR were obtained from Sigma Genosys (Woodlands, TX). The QuikChange site-directed mutagenesis kit was from Stratagene (La Jolla, CA). All other chemicals were of ACS grade and were used without further purification. P450eryF was expressed in *Escherichia coli* and purified on a metal-affinity column as described (*39*).

Construction of P450eryF Mutants. All mutants were generated using overlap extension PCR (*39*). His-tagged P450eryF served as the template. The following PCR primers were employed for the respective sites: C154T, forward, 5′-CCCATCAAGGTCATCACAGAGCTGCTCGGC-3′, and reverse, 5′-GCCGAGCAGCTCTGTGATGACCTTGATGGG-3′; C154M, forward, 5′-CCCATCAAGGTCATCATGGAG-CTGCTCGGC-3′, and reverse, 5′-GCCGAGCAGCTCCAT-GATGACCTTGATGGG-3′; C154W, forward, 5′-CCC-ATCAAGGTCATCTGGGAGCTGCTCGGC-3′, and reverse, 5′-GCCGAGCAGCTCCCAGATGACCTTGATGGG-3′; C154I, forward, 5′-CCCATCAAGGTCATCATTGAGCT-GCTCGGC-3′, and reverse, 5′-GCCGAGCAGCTC-

[1] Abbreviations: P450eryF, cytochrome P450eryF (CYP107A1) from *Saccharopolyspora erythraea*; Hepes, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; CPM, 7-(ethylamino)-3-(4′-maleimidylphenyl)-4-methylcoumarin; 1-PB, 1-pyrenebutanol; 7-BQ, 7-benzyloxyquinoline; 6-DEB, 6-deoxyerythronolide; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; FRET, fluorescence resonance energy transfer.

AATGATGACCTTGATGGG-3′; E155A, forward, 5′-CAAGGTCAT**A**TGCGCACTGCTCGGCGTC-3′, and reverse, 5′-GACGCCGAGCAGTGCGCA**T**ATGACCTTG-3′; E155Q, forward, 5′-CAAGGTCAT**A**TGCCAACTGCTCGGCGTC-3′, and reverse, 5′-GACGCCGAGCAGTTGGCA**T**ATGACCTTG-3′. The mutant codon is underlined, and nucleotides changed from the original sequence to engineer a restriction site without changing the amino acid sequence are shown in bold. All constructs were sequenced to ensure that only the desired mutation was present (Protein Chemistry Core Laboratory, The University of Texas Medical Branch, Galveston, TX).

Preparation and Characterization of CPM-Labeled P450eryF. Labeling of P450eryF was performed by incubation of a 4 μM solution in 0.1 M Na-Hepes buffer, pH 7.4, with a 5-fold molar excess of CPM added as a 2−3 mM solution in acetone−methanol (1:1). Dithiothreitol (DTT) present in the storage buffer at 1 mM was removed by dialysis against several changes of 0.1 M Na-Hepes buffer, pH 7.4, under argon prior to modification. The reaction with CPM was followed by the increase in fluorescence at 455 nm (excitation at 385 nm). The incubation time (about 20−30 h at 25 °C) was adjusted in each particular case to reach at least 95% of modification, as calculated from the fitting of the kinetic curve of modification to a multiexponential equation. The reaction was stopped by addition of 10 mM glutathione. Removal of the excess label was achieved by Sephadex G-25 gel filtration. The protein was then concentrated on a Centrisart I (MWCO 20 000) concentrator (Sartorius AG, Goettingen, Germany) and stored at −80 °C as a 20−50 μM solution. The extent of modification was determined from absorbance spectra in the 340−700 nm region using free P450eryF and CPM as spectral standards to approximate the spectra of the samples of P450eryF$_{CPM}$. For various samples of P450eryF$_{CPM}$ the molar ratio of bound CPM to P450 varied from 0.85:1 to 1:1.

Experimental. The absorbance and fluorescence spectra were measured with a MC2000-2 multichannel CCD rapid scanning spectrometer (Ocean Optics, Inc., Dunedin, FL) equipped with one absorbance and one fluorescence channel, a pulsed Xe-lamp PX-2 light source, and a custom-made thermostated cell chamber with a magnetic stirrer. A semimicro fluorescence quartz cell with a stirring compartment (10 × 4 mm light path) from Hellma GmbH (Müllheim, Germany) was used in the titration experiments. The excitation of fluorescence was achieved using a Solar Blind 250−330 nm band-pass filter (catalog no. 57860; Thermo Oriel, Stratford, CT). The setup used in our FRET experiments permitted simultaneous registration of the UV−vis transmittance/absorbance and fluorescence spectra of the same sample. This design allowed us to monitor the intensity and the spectral parameters of the excitation light during the experiment. The spectrum of transmittance of the sample was taken together with each emission spectrum, and the intensity of fluorescence was carefully corrected for the changes in the intensity of the excitation light.

All experiments were performed at 25 °C with continuous stirring in 0.1 M Na-Hepes buffer, pH 7.4, containing 1 mM DTT and 1 mM EDTA. The ionic strength (*I*) of this buffer was determined to be 0.058 M (*48*). The desired ionic strength of the medium was achieved by addition of an appropriate amount of KCl (as a 2.5 M stock solution), if not otherwise indicated. In this study HPCD, which was used in our previous experiments to solubilize 1-PB (*41*), was generally omitted, as the interactions of 1-PB with HPCD were affected by the increasing salt concentration. A 15−20 mM stock solution of 1-PB in acetone was used in all titration experiments so that the final concentration of acetone in the assay media did not exceed 0.3%. The concentration of 1-PB in acetone solutions was determined by the absorbance at 344 nm using the extinction coefficient of 41 mM^{-1} cm^{-1} (*49*). Fluorometric assays of H_2O_2-dependent activity of P450eryF were performed as previously described (*41*).

Circular dichroism (CD) measurements were done using an Aviv 62DS CD spectrophotometer (Aviv Instruments, Lakewood, NJ) at 25 °C in 0.1 cm path length optical cell. Samples containing 2 μM P450eryF were prepared in 25 mM potassium phosphate buffer. Ionic strength was varied by addition of KCl. Far-UV spectra were recorded from 190 to 250 nm. High absorbance of solvent at high salt concentrations prevented the collection of data below 190 nm. Spectra were acquired by averaging three scans. Buffer absorbances were recorded simultaneously and subtracted from each sample measurement. Determination of the secondary structure from the CD spectra was done by averaging the results obtained with the variable selection method of Johnson (*50*) and the method of Provencher and Glickner (*51*) implemented in CDSSTR and CONTIN/LL programs included in the CDPro package by Narasimha Sreerama (http://lamar.colostate.edu/∼sreeram/CDPro).

Data Processing. Series of spectra obtained by spectrophotometric titrations were interpreted with a mathematical method based on principal component analysis (PCA) as described earlier (*52*, *53*). This approach allowed us to significantly increase the signal-to-noise ratio and to eliminate the spectral perturbations due to the changes in turbidity of the system during the experiments. To interpret the spectral transitions in terms of the changes in the concentration of P450 species, we used a least-squares fitting of the spectra of principal components to the set of the spectral standards of pure high- and low-spin P450 species of the heme protein, as described (*52*, *53*). These standard spectra of the absorbance of low- and high-spin P450eryF were obtained from a series of spectra reflecting the temperature- and substrate-induced spin shift, as described earlier for P450BM-3 (*41*). All data treatment and fitting of the titration curves were performed with our SpectraLab software package (*52*).

RESULTS

Effect of Ionic Strength on Spin Transitions in P450eryF. A series of spectra of P450eryF obtained at increasing concentrations of KCl and at a nearly saturating concentration of 1-PB (40 μM) is shown in Figure 1. An increase in ionic strength results in a profound decrease in the absorbance band at 418 nm with a concomitant increase in the absorbance at 360−400 nm, which is indicative of the displacement of the spin equilibrium toward the high-spin state of the enzyme (Figure 1a). Figure 1b shows these changes in terms of the content of high-spin heme protein. Importantly, this effect was not cation-specific, as the changes in spin equilibrium induced by KCl, NaCl, and RbCl were identical (Figure 1b, open symbols). The effect of ionic strength on the spin state of the heme iron is likely to be a fundamental feature of

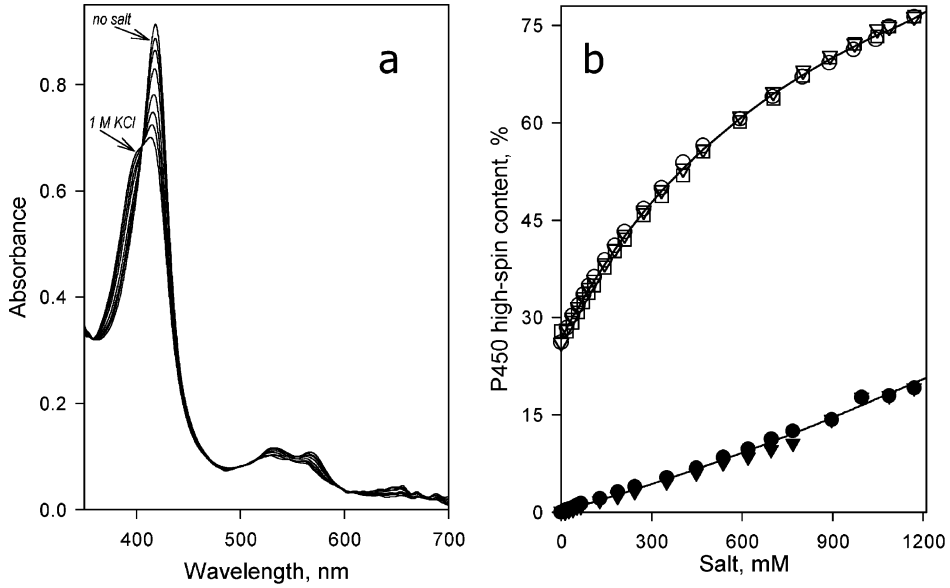

FIGURE 1: Effect of increasing salt concentration on the spin state of P450eryF. (a) A series of absorbance spectra of substrate-bound P450eryF obtained at no salt present and at 0.06, 0.11, 0.21, 0.4, 0.6, 0.8, and 1 M KCl. (b) Content of the high-spin state of the heme protein as a function of concentration of KCl (circles), NaCl (triangles), and RbCl (squares) at no substrate added (filled symbols) and at 40 μM 1-PB (open symbols). Conditions: 0.1 M Na-Hepes buffer, pH 7.4 (I = 0.058 M), 1 mM DTT, 1 mM EDTA, 9 μM heme protein, 25 °C.

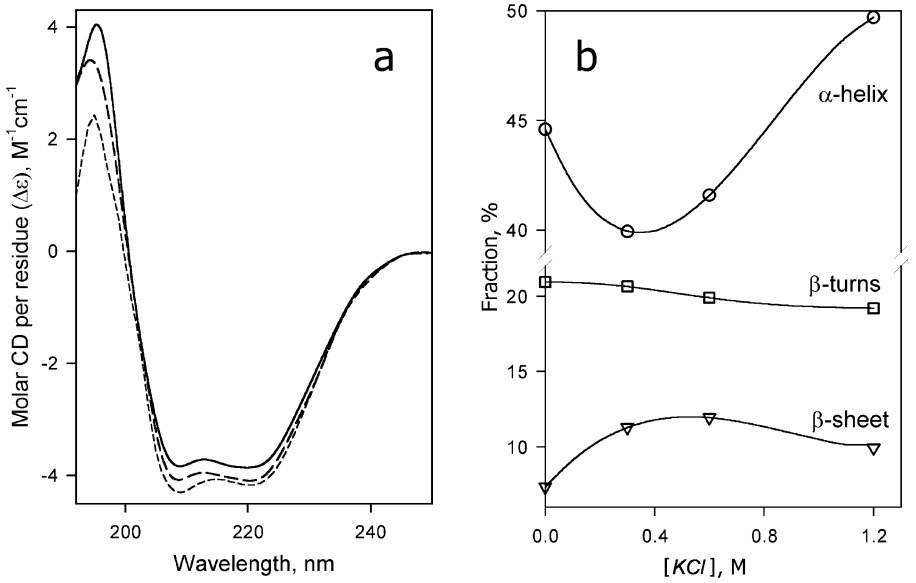

FIGURE 2: Effect of increasing concentration of KCl on the secondary structure of P450eryF determined by circular dichroism spectroscopy. (a) CD spectra of P450eryF at no salt added (solid line) and at 0.3 M (long dash) and 0.9 M (short dash) KCl. (b) Dependence of the fractions of α-helices (circles), β-turns (squares), and β-strands (triangles) on the concentration of KCl. Conditions: 25 mM potassium phosphate buffer, pH 7.4, 2 μM P450eryF, increasing concentrations of KCl, spectral path length 1 mm.

substrate-bound P450eryF, as it was observed in the enzyme complexed with another allosteric substrate, 7-benzyloxyquinoline (7-BQ) (data not shown). The effect of the addition of salt on the spin state of P450eryF in the absence of substrate was considerably lower than that observed in the substrate-bound enzyme (Figure 1b, closed symbols). These results suggest that the substrate-induced spin shift in P450eryF is associated with some conformational transition that involves rearrangement of intramolecular electrostatic interactions in the enzyme.

The magnitude of the effect of ionic strength on the spin state of 1-PB-bound P450eryF was close to that observed with P450cam with low-affinity substrates, such as camphoroxime, or with P450cam Y96F with camphor (*15*). Likewise, the effect of ionic strength on substrate-free P450eryF is comparable with that reported for substrate-free P450 1A2 (*54*) and P450 2B1 (*21*). Presumably, the control of the heme accessibility by electrostatic tethering may be a common feature of P450cam, P450eryF, P450 1A2, and P450 2B1.

Effect of Ionic Strength on the Secondary Structure. Determination of the secondary structure of P450eryF by circular dichroism spectra shows that the enzyme retains its native conformation in the whole range of salt concentration used in our studies (0−1.2 M) (Figure 2). Averaging the estimates obtained with CDSSTR (*50*) and CONTIN/LL (*51*) algorithms from a series of CD spectra taken at 0, 0.3, 0.6, and 1.2 M KCl shows that the fractions of α-helices and

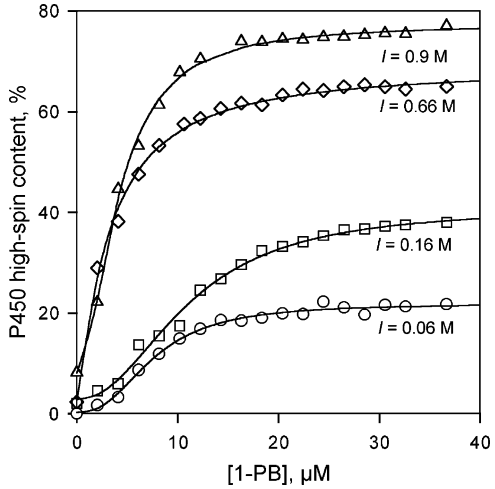

FIGURE 3: Effect of ionic strength on the interactions of P450eryF with 1-PB monitored by the substrate-induced spin shift. Titrations were performed in 0.1 M Na-Hepes buffer, pH 7.4, at no salt added ($I = 0.058$ M, circles) and at various concentrations of KCl: 100 mM ($I = 0.16$ M, squares), 600 mM ($I = 0.66$ M, diamonds), and 840 mM ($I = 0.9$ M, triangles). Solid lines correspond to the results of data fitting to the Hill equation with the following values of S_{50} and Hill coefficient (n) for each data set, respectively: $S_{50} = 8.3$ μM, $n = 2.4$; $S_{50} = 10$ μM, $n = 2.1$; $S_{50} = 3.2$ μM, $n = 1.2$; $S_{50} = 4.1$ μM, $n = 1.9$. The concentration of P450 eryF was 1.5 μM. Other conditions are as indicated in Figure 1.

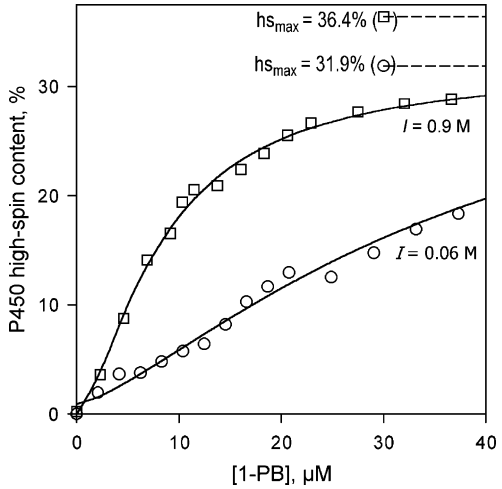

FIGURE 4: Effect of ionic strength on the interactions of CPM-modified P450eryF with 1-PB monitored by the substrate-induced spin shift. Titrations were performed in 0.1 M Na-Hepes buffer, pH 7.4, at no salt added ($I = 0.058$ M, circles) and in the presence of 840 mM KCl ($I = 0.9$ M, squares). Solid lines show the results of data fitting to the Hill equation with $S_{50} = 33$ μM, $n = 1.2$, and $S_{50} = 11$ μM, $n = 1.6$, respectively. The conditions are as indicated in Figure 3.

β-strands in 1-PB-bound P450eryF are 44 ± 4% and 10 ± 2%. These values are in good agreement with those determined from the X-ray structure of P450eryF complexed with 6-DEB, which exhibits 48% of α-helices and 10% of β-strands (*55*). Although the fold of the protein remains conserved over the whole range of salt concentrations, comparison of the estimates obtained at different ionic strength reveals some systematic trend in these values (Figure 2b). While the plots of the fractions of α-helices and β-strands versus ionic strength are given by concave and convex curves, respectively, with extremes around 0.4−0.5 M, the fraction of β-turns exhibits a continuous decrease over the whole range of ionic strength (Figure 2b). As will be shown below, this trend has an important parallelism in the ionic strength dependencies of the functional properties of the enzyme.

Effect of Ionic Strength on Allosteric Properties of P450eryF. As reported in our previous communication, the binding of 1-PB to P450eryF exhibits prominent homotropic cooperativity revealed in both the substrate-induced spin shift and H_2O_2-dependent oxidation (*41*). Figure 3 presents the results of spectrophotometric titrations of P450eryF with 1-PB at different ionic strength. This series reveals a profound decrease in cooperativity of 1-PB binding with increased ionic strength, which is concomitant with a prominent decrease in the S_{50} value that signifies an increase in the affinity of P450eryF for this substrate. While the binding of 1-PB in 0.1 M Na-Hepes buffer ($I = 0.058$ M) fits the Hill equation with $n = 2.4$ and $S_{50} = 8.3$ μM, upon addition of 0.6 M KCl the Hill coefficient drops to 1.2 and the S_{50} value to 3.2 μM. Therefore, the ionic strength-induced perturbation in the structure of P450eryF favors the substrate-induced spin transition and also eliminates cooperative behavior of the enzyme with 1-PB.

Effect of Modification of Cys^{154} on the Allosteric Properties of P450eryF. In a continued effort to find novel approaches for direct monitoring of substrate binding to P450eryF, we introduced a fluorescent probe into the enzyme by chemical modification of the single nonactive site cysteine residue (Cys^{154}) with CPM. The CPM probe was intended as an acceptor fluorophore in FRET-based monitoring of the interactions of 1-PB with P450eryF. Modification of the enzyme by CPM was found to be extremely slow, taking more than 20 h to complete at a 5-fold molar excess of the reagent, suggesting that Cys^{154} is poorly accessible from the surface of the enzyme. Most interestingly, this modification of P450eryF drastically decreases the cooperativity and largely suppresses the effect of ionic strength on the amplitude of the substrate-induced spin transition and cooperativity (Figure 4). Decrease in the cooperativity upon CPM modification is also revealed in the substrate dependence of the rate of H_2O_2-dependent oxidation of 1-PB by P450eryF. For the wild-type enzyme V vs S plots obey the Hill equation with $n = 1.7 \pm 0.2$, $S_{50} = 14.4 \pm 1.0$ μM, and $k_{cat} = 1.2 \pm 0.1$ min^{-1}. Modification of the enzyme with CPM decreases the value of the Hill coefficient to 1.2 ± 0.1, while increasing S_{50} and k_{cat} to 42.9 ± 1.6 μM and 3.7 ± 0.3 min^{-1}, respectively.

Analysis of the crystal structure of P450eryF (*56*, *57*) shows that Cys^{154}, which is located at the C-terminal end of helix E, is partially buried. This residue is surrounded by a system of salt links formed by charged residues located in helices D, E, and F. Modification of Cys^{154} by the bulky CPM molecule might disrupt this bonding system, leading to a loss of allostery and a decrease in the ionic strength dependence of P450eryF.

Effect of Replacement of Cys^{154} and Glu^{155} in P450eryF on Substrate Binding and Cooperativity. To test the above hypothesis, we studied the effect of substitution of Cys^{154} with larger amino acid residues on the function of the enzyme. For this purpose C154W, C154I, C154M, and C154T were generated. C154M and C154W failed to express, whereas the expression level of C154I and C154T (2.1 and 2.4 μM, respectively) was close to that of the wild type (3.1

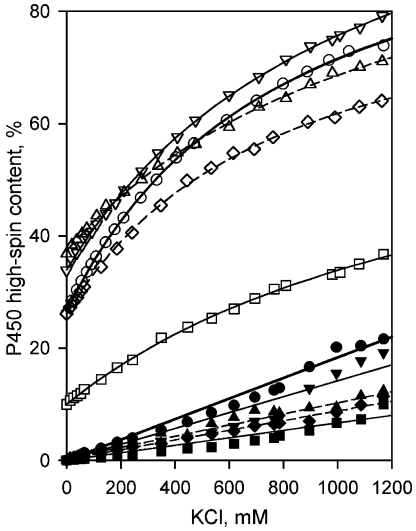

FIGURE 5: Effect of increasing concentrations of KCl on the spin state of P450eryF wild type (circles, bold lines) and its mutants: C154T (inverted triangles, thin lines), C154I (squares, thin lines), E155Q (diamonds, dashed lines), and E155A (triangles, dashed lines). Titrations were performed at no substrate added (filled symbols) and at 40 μM 1-PB (open symbols). The conditions are as indicated in Figure 3.

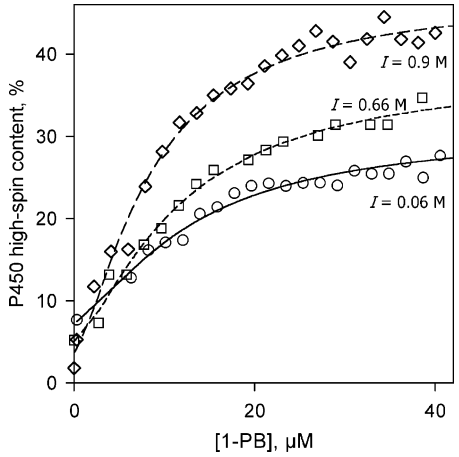

FIGURE 6: Effect of ionic strength on the interactions of P450eryF C154I with 1-PB monitored by the substrate-induced spin shift. Titrations of P450eryF with 1-PB were performed in 0.1 M Na-Hepes buffer, pH 7.4, at no salt added ($I = 0.058$ M, circles, solid line), as well as at 600 mM ($I = 0.66$, squares, short dash) and 840 mM KCl ($I = 0.9$ M, diamonds long dash). Lines show the results of data fitting to the Hill equation with the following values of S_{50} and Hill coefficient (n) for each data set, respectively: $S_{50} = 16$ μM, $n = 1.2$; $S_{50} = 12$ μM, $n = 1.4$; $S_{50} = 11$ μM, $n = 1.6$. The conditions are as indicated in Figure 3.

μM). We also investigated the effect of substitution of the neighboring glutamic acid residue with alanine (E155A) and glutamine (E155Q). Although the substitutions at this position decreased the expression level, it remained sufficient for purification of the enzyme (1.4 and 1.0 μM for E155A and E155Q, respectively).

Figure 5 illustrates the effect of the substitutions at positions 154 and 155 on the ionic strength-induced displacement of the spin equilibrium in substrate-free and 1-PB-bound enzyme. Among the four mutants analyzed, C154I exhibits the most prominent difference from the wild-type P450eryF. In this mutant the ionic strength-induced displacement of the spin equilibrium of substrate-bound enzyme is largely suppressed and the amplitude of the 1-PB-induced spin shift at low ionic strength decreased, similar to the CPM-modified enzyme. Replacement of Cys^{154} by threonine had no such effect on the enzyme.

Both mutants at position 155 (E155A and E155Q) also exhibit a considerably reduced effect of ionic strength on the spin equilibrium of the substrate complex (Figure 5, dashed lines) but no effect on the amplitude of the 1-PB-induced spin shift at low ionic strength. These results indicate involvement of Glu^{155} in the suggested ionic tethering system controlling spin transitions in P450eryF.

To explore the properties of C154I further, a series of spectrophotometric titrations was performed with 1-PB at various ionic strengths in the range of 0.06−1.26 M. As illustrated by the results obtained with C154I at $I = 0.06$ M, $I = 0.66$ M, and $I = 0.9$ M (Figure 6), the increase in ionic strength does not change the shape of the titration curves, in contrast to the wild-type enzyme (Figure 3). Analysis of the effect of ionic strength on the allosteric properties of P450eryF wild type and C154I in terms of the Hill equation is shown in Figure 7. In P450eryF wild type an increase in ionic strength from $I = 0.06$ M to $I = 0.6$ M results in a sharp decrease in S_{50}, while n drops from 2.4 to 1.2. A further increase in ionic strength partially reverses the change in S_{50} and increases the value of n back to 2.8. This reversal might be attributed to the effect of high osmotic pressure at high salt concentrations. It is notable that the profiles of salt-induced changes in the fractions of α-helices and β-strands in P450eryF detected in our circular dichroism experiments (Figure 2b) are close to the profile of the dependence of the Hill coefficient on ionic strength (Figure 7b, circles), exhibiting a break around $I = 0.5$ M (Figure 2). Thus, it appears likely that the changes in the secondary structure exhibited in Figure 2b reflect a conformational transition that is associated with a decrease in P450eryF cooperativity.

Most of these ionic strength-induced changes observed in the wild-type P450eryF were suppressed in C154I. The only significant effect of ionic strength found here was a continuous decrease in the S_{50}. Therefore, we may conclude that the modification of Cys^{154} by the bulky and hydrophobic CPM, as well as substitution with isoleucine, results in perturbation of a system of intramolecular electrostatic interactions that is involved in substrate-induced spin transitions and is crucial for the allosteric mechanism of P450eryF.

Effect of Ionic Strength on the Interactions of P450eryF with the First (Effector) Molecule of 1-PB Monitored by FRET from 1-PB to the Heme. Monitoring the interactions of P450eryF with 1-PB by FRET provides a direct approach to detect the binding at the higher affinity (effector) binding site (*41*). The selectivity of the method for the formation of a 1:1 complex is ensured by the experimental setup, which involves the titration of a low concentration of 1-PB with increasing enzyme, so that the latter is always present in molar excess over the ligand. Both wild-type P450eryF and, to a lesser extent, C154I exhibited an increase in the affinity for the first substrate molecule at increasing ionic strength (Figure 8). However, the effect of ionic strength on the efficiency of FRET (ΔF_{max}) reveals a contrast between wild-type P450eryF and the mutant. In the wild type an increase in ionic strength from $I = 0.06$ M to $I = 0.6$ M results in a sharp decrease in ΔF_{max} (Figure 9b), which apparently

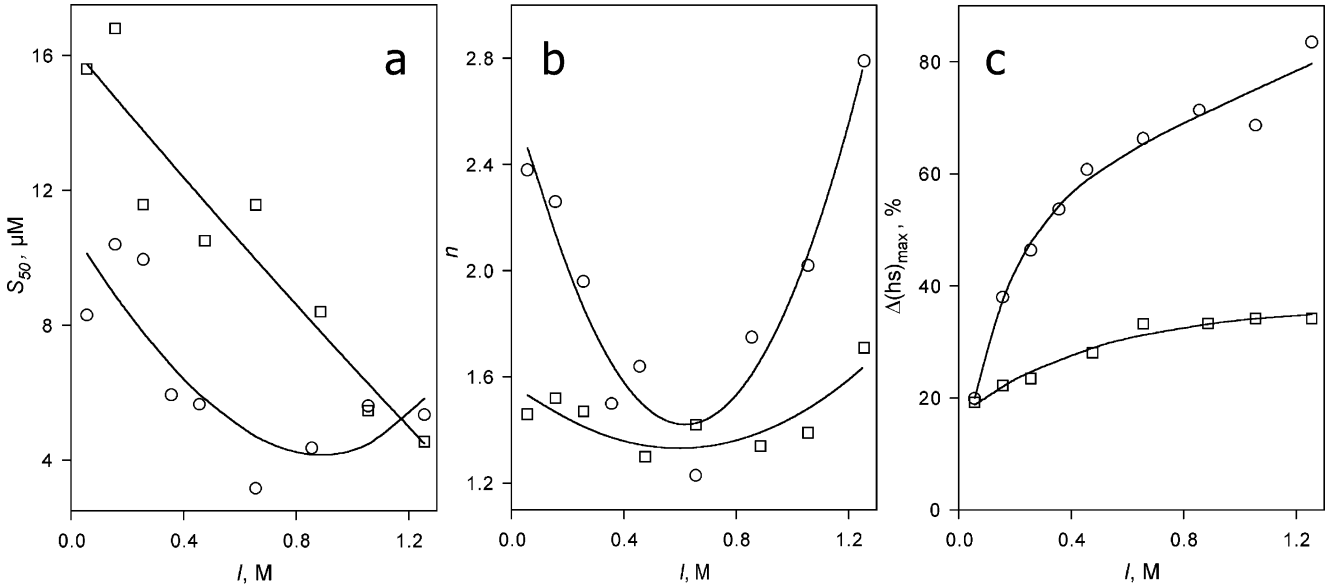

FIGURE 7: Effect of the Cys[154] to Ile substitution on the interactions of P450eryF with 1-PB. The parameters of the Hill equation, S_{50} (panel a), n (panel b), and the maximal amplitude of the spin shift, $\Delta(\text{hs})_{max}$ (panel c), are shown as a function of ionic strength. Titrations of P450eryF wild type (circles) and C154I mutant (squares) with 1-PB were performed at increasing KCl concentrations. The conditions are as indicated in Figure 2. The curves shown on these plots were obtained by the fitting of the data points by a biexponential equation. These curves are given solely to visualize the general trend of the points and have no conceptual meaning.

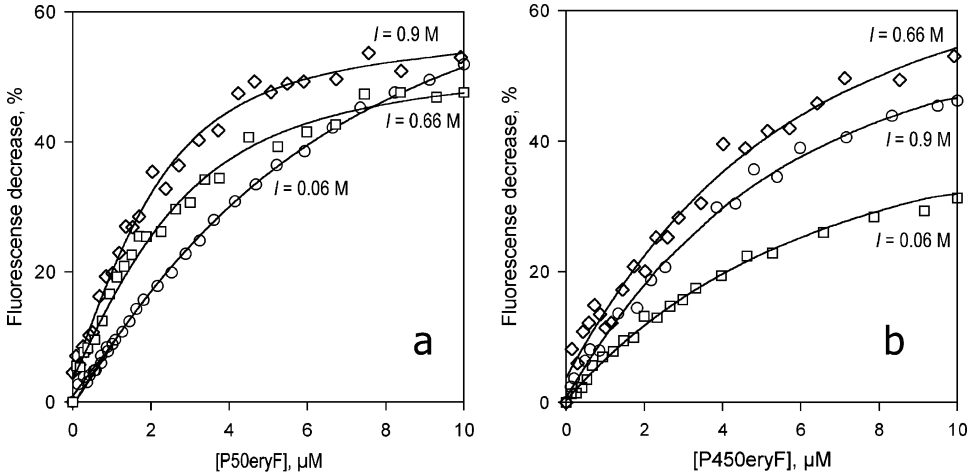

FIGURE 8: Effect of ionic strength on the interactions of P450eryF wild type (a) and its C154I mutant (b) with 1-PB monitored by FRET. Titrations were performed in 0.1 M Na-Hepes buffer, pH 7.4, at no salt added ($I = 0.058$ M, circles), as well as at 600 mM ($I = 0.66$ M, squares) and 840 mM KCl ($I = 0.9$ M, diamonds). Lines show the results of data fitting to the equation for the equilibrium of bimolecular association. The respective values of K_{D1} are equal to 3.1, 1.2, and 0.9 μM for P450eryF wild type and 4.9, 3.7, and 3.6 μM for the mutant. Conditions: 0.1 M Na-Hepes buffer, pH 7.4 ($I = 0.058$ M), 1 mM DTT, 1 mM EDTA, 1.7 μM 1-PB, 25 °C.

signifies an increase in the distance between the heme and the first (effector) molecule of substrate. Importantly, the dependencies of ΔF_{max} and the Hill coefficient on ionic strength were remarkably similar, suggesting that increasing ionic strength induces a conformational transition that distances the effector binding site from the heme and impedes cooperativity. On the other hand, this transition facilitates the spin shift induced by substrate binding at the second (catalytic) site. The opposite effect of ionic strength on ΔF_{max} for C154I (Figure 9) shows that this substitution impairs the conformational transition.

DISCUSSION

Our finding that the high-spin content in P450eryF complexes with 1-PB increases drastically at high salt concentration reveals an important ionic strength-dependent conformational transition that controls the spin equilibrium. Thus, the conversion of the low-spin substrate-bound cytochrome to the high-spin form apparently requires dissociation of some internal salt links in the protein. It is likely that the continuous decrease in the fraction of β-turns at increasing ionic strength observed in our circular dichroism experiments (Figure 2) is related to the ionic strength-induced conformational transition in causing the displacement of the spin equilibrium in the enzyme−substrate complex. The cooperativity of the enzyme also decreases at higher salt concentration in parallel with the increase in the content of the high-spin form of both substrate-bound and (to a lesser extent) substrate-free enzyme. Ionic strength-induced changes in the secondary structure observed in CD experiments are also consistent with the changes in P450eryF cooperativity. The decrease in cooperativity observed upon ionic strength increase in the 0.06−0.6 M range appears to be associated with a decrease of the fraction of α-helical structures from

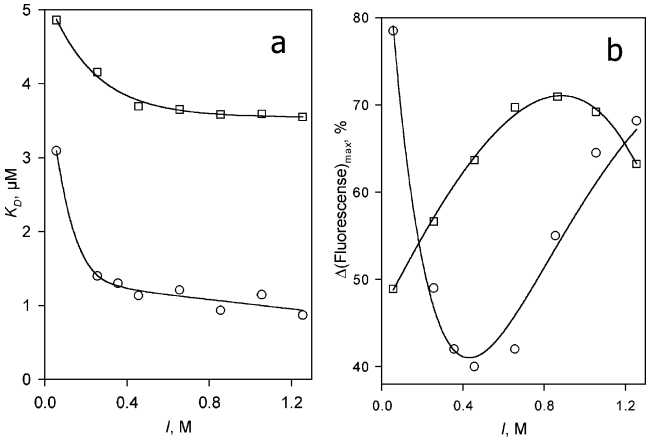

FIGURE 9: Effect of the Cys[154] to Ile substitution on the interactions of P450eryF with 1-PB monitored by FRET. The values of the dissociation constant (K_{D1}, panel a) and the amplitude of FRET in the enzyme−substrate complex (ΔF_{max}, panel b) are shown as a function of ionic strength. Titrations of 1-PB with P450eryF wild type (circles) and its C154I mutant (squares) were performed at various concentrations of KCl. The other conditions are as indicated for Figure 8. The curves shown on these plots were obtained by the fitting of the data points by a biexponential equation. These curves are given solely to visualize the general trend of the points and have no conceptual meaning.

48% to 40%. This conformational transition reverses upon further increase in the salt concentration concomitant with a reversal in the changes in the Hill coefficient (Figures 2b and 7b). Such a reversal is apparently due to the effect of high osmotic pressure, which is known to displace chemical equilibria toward the state with a minimal degree of solvation of the solute (*58*), thus causing dehydration of solvent-exposed charges in the protein by burying them and by formation of salt bridges. Thus, the direction of osmotic pressure-induced changes is usually opposite to that of the changes caused by increasing ionic strength. Notably, the effects of increased salt concentration on the spin equilibrium (Figure 5) and on the fraction of β-turns in the secondary structure (Figure 2b) are not reversed at 0.5−0.6 M, retaining the same direction over the whole range of salt concentrations studied. Therefore, we may suggest that the ionic strength-induced conformational transition consists of two distinct rearrangements with different sensitivity to osmotic pressure.

In our previous communication (*41*) we introduced a model of the mechanism of P450eryF cooperativity, where the binding of the first (effector) molecule of substrate induces a conformational transition, which is required for a substrate-induced spin shift observed only in the complex of the enzyme with two substrate molecules. Our present results suggest that the conformational transition caused by the binding of the first (effector) molecule of substrate is closely related to that caused by increasing ionic strength. Therefore, the ionic strength-induced rearrangement in the enzyme eliminates the requirement for saturation of the effector-binding site to elicit the substrate-induced spin transition. Following this logic, our initial model represents a simplification, which is adequate at low ionic strength only. Indeed, in good agreement with the above conclusion, the data obtained in this study at ionic strength higher than 0.3 M failed to fit the equation (see eq 3 in ref *41*) deduced for our initial model (data not shown).

In the following discussion, by analogy with classical models of allostery, the conformation with apparent dissociated salt links, which is predominant at high ionic strength, will be referred to as R ("relaxed"), while the conformation observed at low ionic strength is designated as T ("tense"). In the R-conformation binding of one substrate molecule to the heme protein is sufficient for its transition to the high-spin state. In this context it is important to note that increasing ionic strength induces a prominent decrease in the efficiency of FRET from the first (effector) molecule of 1-PB to the heme (Figure 9b), which is indicative of an increase in the distance between the effector binding site and the heme upon the transition from the T- to R-conformation.

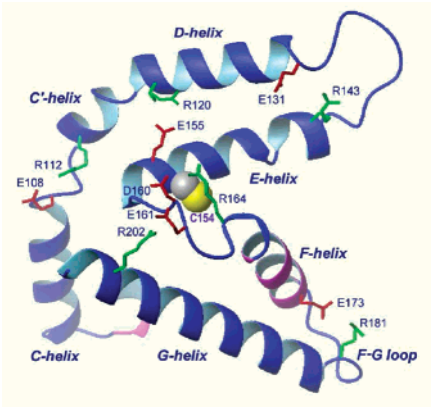

FIGURE 10: Model of part of the crystal structure of the complex of P450 with 6-DEB (*55*) showing putative charge-pairing contacts in the vicinity of Cys[154] whose side chain is shown as a space-filling model. Regions of the F-helix and B−C loop, which constitute part of the substrate binding site, are shown in magenta.

The observation that modification of Cys[154] with CPM or substitution with isoleucine suppresses the ionic strength dependence of the spin equilibrium and abolishes the allosteric properties suggests that these changes impair the transition between T- and R-conformations. Cys[154] is located at the C-terminal end of helix E and is surrounded by several salt bridges formed by neighboring arginine, glutamate, and aspartate residues located in helices E, F, G, and D (*55*) (Figure 10). This region is known to be of high functional importance in microsomal cytochromes P450. In particular, the neighboring C and C′ helices are known to participate in the interactions of cytochromes P450 with their redox partners: NADPH−cytochrome P450 reductase and cytochrome b_5 (see ref *59* for review). Residues in helix F and the B−C loop constitute part of the substrate recognition sites (SRS) in P450 proteins (*60*−*63*). The loop between helices F and G forms an entrance gate to the substrate-access channel to the heme moiety in some P450 enzymes (*10*, *62*).

On the basis of the above considerations we propose that rearrangement of the charge-pairing bundle among helices C, D, E, and G shown in Figure 10 may have a profound effect on the openness of the active site of P450eryF and the conformation of some SRS regions, as well as the region of C−C′ helices. Structural transitions in this latter region in mammalian P450 enzymes may underlie the displacement of the spin equilibrium upon interaction with P450 reductase and cytochrome b_5 (*64*−*66*) and the effect of substrate binding on the affinities for the reductase and b_5 (*64*). Thus, the region surrounding Cys[154] in P450eryF might be considered a strategic axle determining the mutual effects of the regions involved in substrate access and binding and those

involved in protein−protein interactions on the conformation of each other.

To probe the involvement of Glu^{155} in the suggested mechanism of ion tethering, we probed the effect of substitution with alanine or glutamine. As the properties of E155A and E155Q in terms of the ionic strength-induced spin shift in the substrate complex were considerably different from those of the wild-type enzyme (Figure 5), Glu^{155} appears to be involved in the proposed transition between the R- and T-conformations of P450eryF. Other possible candidates for salt links involved in this transition are D160−R164 and E161−R202 charge pairs. In addition, electrostatic interaction in the region of the F−G loop and helix G involving such residues as E173, R181, and R185 may also affect the position of that helix and affect the conformation around Cys^{154}. The role of residues 173, 181, and 185 is predicted by the results of simulations of substrate expulsion from P450eryF (*67*), showing that the hydrogen bonding of R185 in helix G with I174 and V176 in the F−G loop as well as with A74 and L76 in B−B′ loop might be an important determinant of the openness of the substrate access channel (*67*). These interactions seem to be affected by the degree of ionization of R185 and possible formation of the E173−R181 salt bridge. The importance of this region is also confirmed by our recent finding that substitution of I174 or I175 with phenylalanine alters significantly the affinity of P450eryF for testosterone and 7-benzoxyquinoline and the rates of oxidation of these substrates (*39*). The role of D160, R164, E161, R202, E173, R181, and R185 in the ionic strength-induced conformational transition in P450eryF will be probed in future experiments.

In general, a prominent effect of ionic strength on the spin equilibrium in substrate-bound cytochrome P450eryF indicates that the control of the active site accessibility by ion tethering proposed for cytochrome P450cam (*13*, *15*, *16*, *47*) may also take place in other cytochromes P450. This mechanism is tightly related to the modulation of the heme pocket hydration that plays a pivotal role in P450 catalysis. A water molecule of strategic importance serves as a sixth ligand of the heme iron in the low-spin state of the enzyme. Dissociation and apparent expulsion of this molecule caused by substrate binding are necessary for the transition of the heme iron to the high-spin state, which is believed to be a prerequisite for subsequent electron transfer in some P450 enzymes (*2*, *68*). In addition to this water molecule, which appears to be the only one that leaves the active site in the spin transition of the substrate-free cytochromes P450cam, P450BM-3, and P450 2B4 (*69*), several other water molecules are expelled during the spin transition of the substrate-bound enzymes (*69*). The openness of the active site to the solvent is an important factor for this expulsion. If solvent accessibility is controlled by an ion tethering mechanism, increasing ionic strength should facilitate water expulsion and subsequent displacement of the spin equilibrium. The linear relationship between the molar volume change (which reflects the changes in the protein hydration) and the change in Gibbs free energy ($\Delta G°$) in the low- to high-spin transition observed in substrate-bound cytochromes P450cam, CYP102 (P450BM-3), and rabbit liver microsomal P450 2B4 (*69*) suggests that substrate-induced water expulsion in evolutionarily distant cytochromes P450 may have common mechanism.

It is notable that ionic strength has a greater effect on the high-spin content of cytochrome P450cam complexes with substrate analogues characterized by looser interactions with the enzyme. These substrates have several additional water molecules in the P450 active site (*12*, *46*, *70*), which results in lower efficiency and greater uncoupling of catalysis (*5*). As stated in the introduction, much of the current interpretation of P450 cooperativity is based on the assumption that small substrate molecules that fit loosely in a large binding site of P450 proteins require binding of a second ligand for efficient catalysis (*29*, *35*, *44*). Implicitly, these models suggest that efficient expulsion of water from the active site might be achieved only upon the binding of multiple substrate molecules. On the basis of the effect of ionic strength on the spin transition and cooperativity in P450eryF demonstrated in this study, as well as by analogy with P450cam, we propose that "the ionic tethering and electrostatic steering" mechanisms (*47*) may play an important role in the modulation of substrate and water accessibility of the active site in cytochromes P450 exhibiting homo- and heterotropic cooperativity. Therefore, further studies on the role of intramolecular electrostatic interactions and dynamics of protein-bound water in substrate binding and spin transitions appear to be of crucial importance for the understanding of the mechanisms of P450 cooperativity. The detailed exploration of the mechanisms of the ionic strength-induced transitions in cytochromes P450 and related changes in heme pocket accessibility is also essential to define the main structural determinants of the catalytic efficiency and coupling in microsomal monooxygenases.

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