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The Formation of Cytochrome P-450 from Cytochrome P-420 Is Promoted by Spermine†

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ABSTRACT: This paper is concerned with camphor-bound bacterial cytochrome P-450 and processes that alter its spin-state equilibrium and influence its transition to the nonactive form, cytochrome P-420, as well as its renaturation to the native camphor-bound cytochrome P-450. Spermine, a polycation carrying a charge of 4+, and potassium, a monovalent cation, were shown to differently cause an increase of high-spin content of camphor-bound cytochrome P-450. The spermine-induced spin transition saturates around 75% of the high spin; a further addition of KCl to the spermine-containing sample shifted the spin state to 95% of the high spin. The volume change of these spin transitions as measured by the use of high pressure indicated an excess of −40 mL/mol for the sample containing potassium as compared to that containing spermine. These results suggest that the proposed privileged site for potassium has not been occupied by spermine and that pressure forces both the camphor and the potassium ion from its sites, allowing solvent movement into the protein as well as ordering of solvent by the excluded camphor and potassium. Cytochrome P-420 was produced from cytochrome P-450 by hydrostatic pressure in the presence of potassium, spermine, and cysteine. Potassium cation shows a bigger effect on the stability of cytochrome P-450 than spermine or cysteine, as revealed by a higher value of the pressure of half-inactivation, $P_{1/2}$, and a bigger inactivation volume change. However, potassium cation did not promote renaturation of cytochrome P-420 to cytochrome P-450 while the presence of spermine did. The rate of renaturation to cytochrome P-450 was compared with that induced by cysteine, the only previously known effector of P-420 to P-450 interconversion. A probable electrostatic binding site for spermine is suggested and discussed.

Cytochrome P-450[1] is a monooxygenase that catalyzes the formation of 5-*exo*-hydroxycamphor from camphor, reduced putidaredoxin, and oxygen (Bradshaw et al., 1959; Hedegaard & Gunsalus, 1965). It has been shown that the overall electron-transfer and oxygenase activity of the enzyme is related to the percentage of the enzyme that is present as the high-spin form (Schwarze et al., 1985; Backes et al., 1982; Fisher & Sligar, 1985). The spin state of the substrate-bound enzyme is greatly modulated by cations, the most potent of which is potassium (Lange & Debey, 1979; Lange et al., 1979a, 1980). Sodium and spermidine also cause a shift of the high-spin state, but to a lesser extent, and appear to obey a general ionic strength dependence consistent with electrostatic shielding (Lange et al., 1979b). From the high-resolution X-ray structure of the substrate-bound and free enzyme, a cation binding site has been suggested to be located near the carboxy terminus of helix B′ (residues 86–96) with the cation coordinated to the amide carbonyls of glutamic acid-84 and tyrosine-96 (Poulos et al., 1987). Recent results using site-directed mutagenesis support this model (Di Primo et al., 1990).

The spin state of cytochrome P-450 with camphor bound is known to be influenced by pressure (Hui Bon Hoa & Marden, 1982; Marden & Hui Bon Hoa, 1982); this results from the pressure-induced displacement of the substrate camphor from the heme pocket (Fisher et al., 1985; Marden & Hui Bon Hoa, 1987). The resulting influx of water (Poulos et al., 1985, 1986; Fisher & Sligar, 1987) allows solvent coordination to the heme iron, thereby increasing the ligand field, favoring a low-spin, hexacoordinate configuration (Fisher & Sligar, 1985; Raag & Poulos, 1989). This solvation of the pocket is responsible for the spin-state change and a 170-mV shift in the redox potential (Sligar, 1976; Sligar & Gunsalus, 1976).

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[1] Abbreviations: P-450, bacterial cytochrome P-450 involved in camphor hydroxylation; spermine, *N*,*N*′-bis(3-aminopropyl)-1,4-butanediamine.

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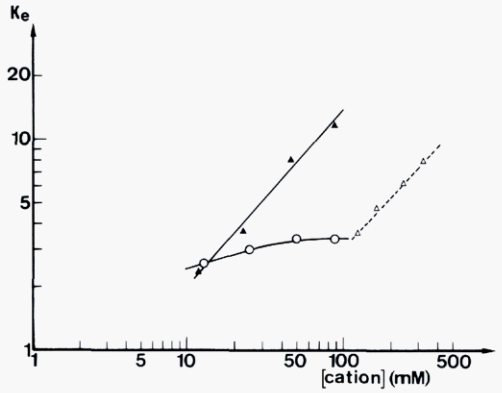

FIGURE 1: Log–log plot of the spin equilibrium constant, K_e [K_e = [P-450(HS)]/[P-450(LS)]], for the substrate-bound bacterial cytochrome P-450 as a function of total cation concentration. The buffer was 200 mM Tris, pH 7.2, and 400 μM camphor; T = 4 °C. Symbols: closed triangles, potassium monovalent cation; open circles, spermine tetravalent cation; open triangles, addition of potassium after saturation with spermine yields the same response as without the polycation except that the curve is displaced toward higher KCl concentrations.

Although high pressure has been a key tool in unraveling these coupled processes, there is a limit to which pressure can be used to influence the spin state. Above about 800 bar, in aqueous medium, the protein begins to convert to an inactive form of cytochrome P-450 termed P-420 (Marden & Hui Bon Hoa, 1986). Following the cytochrome P-420 conversion, additional pressure causes a slow, time-dependent denaturation of the enzyme.

Previous work has shown that cytochrome P-450 can be re-formed from inactive cytochrome P-420 by incubation of the latter with high concentrations of cysteine (Yu & Gunsalus, 1974) or cysteine plus glycerol; other compounds are totally without effect on the renaturation. The electronic structure of the heme iron and thiolate ligand that give rise to the unique ferrous carbonyl adduct with Soret maxima at 450 nm has been described (Hanson et al., 1976); it was also suggested that the inactive P-420 state may reflect loss or protonation of the axial thiolate.

A key goal in cytochrome P-450 research has been to elucidate the structural and electronic features that enable the P-450 class of *b*-type cytochromes to carry out facile carbon atom functionalization whereas the globin-type hemoproteins with histidine ligation are completely inactive. With the ability to reversibly interchange the P-450 active state to a myoglobin-like P-420 form by application of pressure, it becomes very interesting to examine the effects of cations and hydrostatic pressure on the spin and P-450/P-420 equilibria. In this paper we demonstrate that spermine, a polycation carrying a charge of 4+, causes an increase of high-spin state to a lesser extent than potassium and promotes re-formation of camphor-bound cytochrome P-450. The possible relation between the effects of cysteine and spermine is discussed as well as the possible mode of spermine action.

Experimental Procedures

Spermine tetrahydrochloride and cysteine hydrochloride were purchased from Janssen and Sigma, respectively. They were freshly prepared and neutralized with Trizma base before use. All other chemicals were of high quality and were used without further purification. Cytochrome P-450cam was purified from *Pseudomonas putida* (Gunsalus & Wagner, 1978). The purity index, determined by the ratio of absorbance at 392–280 nm, was always greater than 1.5. Spectra were obtained with a Cary 219 spectrophotometer interfaced to a high-pressure bomb as previously described (Hui Bon Hoa

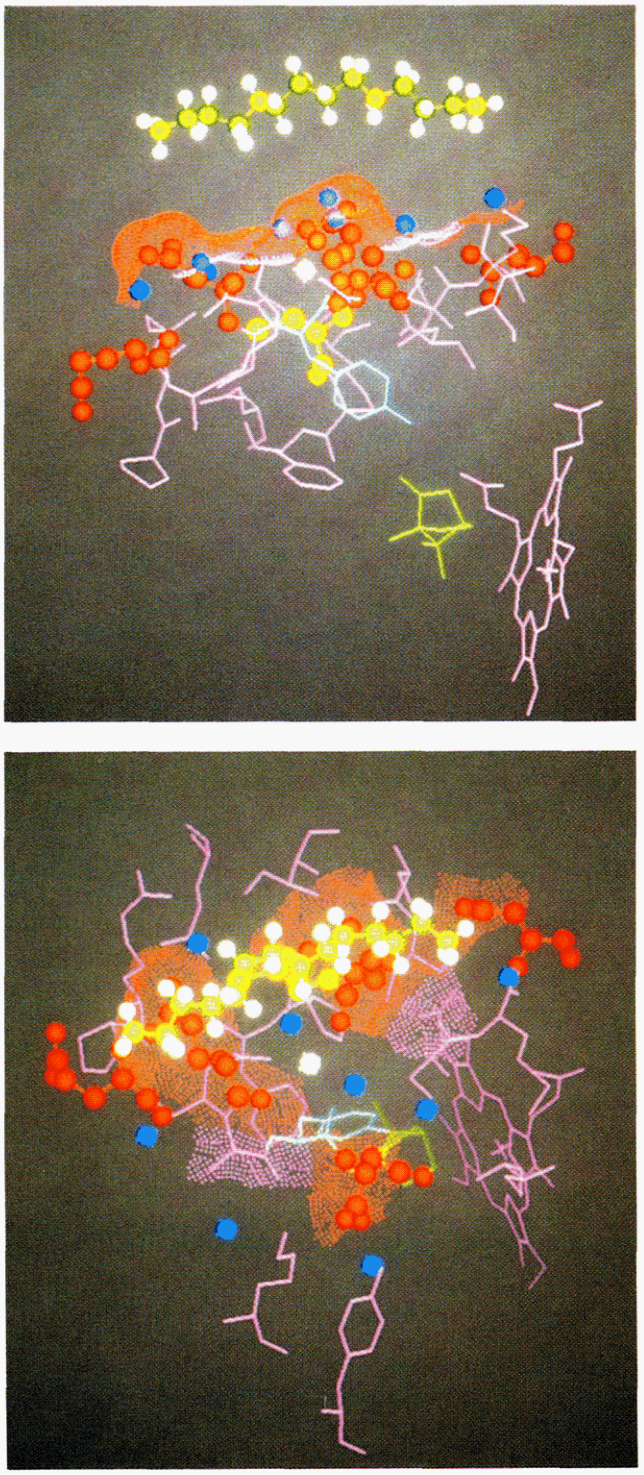

FIGURE 2: A model of the cytochrome P-450 structure (Poulos et al., 1987) showing only the proposed region of the molecule involving the potassium binding site as it interacts with spermine. (Top) The proposed cation binding site (water-515 in the crystal structure) is shown in white (Poulos et al., 1987). Spermine is docked 5 Å above the surface. The five negatively charged carboxylates (Asp-97 and -104; Glu-84, -91, and -94) are indicated by red CPK surfaces at half-scale. Cysteine-85 (yellow) is located behind a vertical plane that includes the potassium site; the interatomic distance, K^+–cysteine-85 sulfur, is 5.37 Å. Also shown are camphor (green) and heme (pink); the camphor carbonyl–K^+ distance is 8 Å and the iron–K^+ distance is 18 Å. The water-accessible surface is shown as a red net, and the immobilized water molecules from the X-ray structure are shown in blue. (Bottom) The model at the top has been rotated approximately 60° about the X-axis. Spermine lines up along an axis formed by four of the five carboxylates. Cysteine-85 and K^+ are completely covered by the spermine when the model is rotated such that the view is directly above the spermine.

et al., 1982). Thermal regulation was provided by a commercial thermostated circulating bath (Huber HS40) and controlled at 4 ± 0.5 °C for all experiments except where explicitly stated. All the absorbances as a function of pressure were corrected for compression of solvent (Bridgeman, 1931). For the experiments on the spin equilibrium of P-450, the concentrations of the high- and low-spin forms were calculated as previously described (Hui Bon Hoa & Marden, 1982). The reaction volume, or $\Delta V°$, for both the spin equilibrium and the interconversion of P-450 and P-420 was evaluated from

$$\Delta V° = -RT(\partial \ln Ke)/(\partial P)$$

as previously described (Hui Bon Hoa & Marden, 1982). The relative concentrations of cytochromes P-450 and P-420 were determined from the absorbance at 404 nm, the isosbestic point of the spin transition, by using the extinction coefficients ϵ_{404}(P-450) = 77 mM^{-1} cm^{-1} and ϵ_{404}(P-420) = 53 mM^{-1} cm^{-1} and A_{404} = [P-420] (53 mM^{-1}) + [[P-450 (initial)] − [P-420]] (77 mM^{-1}). Molecular graphics simulations were performed on a Silicon Graphics 4D/20 workstation running Biosym Insight/Discover.

Results

The camphor-bound cytochrome P-450 is mixed spin in the presence of buffer and most salts, with potassium ions exerting a rather specific effect to drive the complex to more than 95% high spin (Lange & Debey, 1979; Lange et al., 1979a,b, 1980). We have been interested in trying to separate cation effects that are due to general electrostatic influences at the surface of the P-450 molecule from those that result from ion ligation at specific sites. Spermine carries a charge of 4+ at pH 7 and has an approximate minimum radius of 11 Å. In its most compact form, it is at least 40% larger than camphor and highly charged and might reasonably be expected to be excluded from the interior of the camphor-bound protein matrix. Effects of this cation could arise primarily due to bulk electrostatics.

Figure 1 shows the effects of spermine and KCl on the spin transition of cytochrome P-450cam at saturated camphor concentration (400 μM). The KCl curve is similar to those reported previously (Lange & Debey, 1979; Lange et al., 1979a,b, 1980; Peterson, 1971). The effect of spermine has not been reported before. The effects of spermine on the spin state of the cytochrome P-450 with camphor bound are less pronounced than those of KCl. Between 0 and 50 mM spermine there is a 2.3-fold increase in the equilibrium constant for the high/low-spin transition, *Ke*. Above 50 mM spermine the curve saturates, indicating that the privileged site for potassium has not been occupied and that the increased *Ke* is not the result of contaminating potassium ions. Addition of KCl to the spermine-containing sample of Figure 1 caused the high-spin content of the P-450 to increase; the slope of the curve parallels that of the sample which does not contain spermine, but substantially higher concentrations of KCl are required. This implies that there is interaction between the two cations.

Figure 2 presents a graphic model of the portion of the P-450 molecule which contains a proposed potassium binding site (Poulos et al., 1987). The site is located within a few angstroms of the water-accessible surface and consists, among other things, of a cage of five carboxylate groups (Glu-84, -91, and -94 and Asp-97 and -104). The heme of the P-450 is also shown and its heme iron is 18 Å from the potassium site. Between the two are located the camphor and tyrosine-96. The current model (Di Primo et al., 1990) of potassium action is the following: Potassium binds to its site, displaces water, and

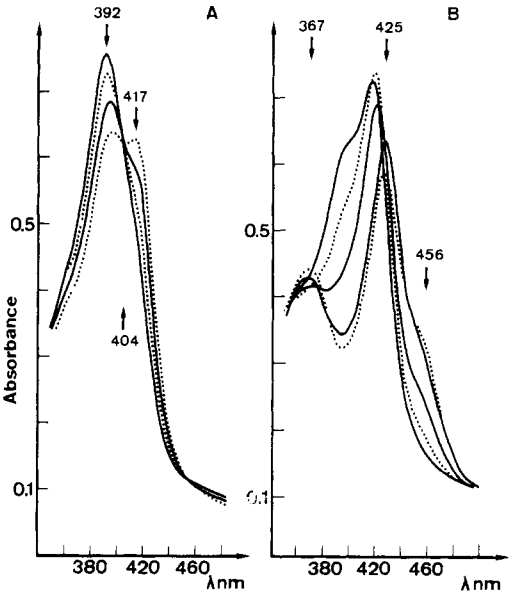

FIGURE 3: Optical spectra in the Soret region of substrate-bound P-450 as a function of hydrostatic pressure. Reaction mixtures contained 200 mM Tris, pH 7.2, 60 mM spermine, and 400 μM camphor and reactions were carried out at 4 °C. (A) Low-pressure-range spectral changes showing the spin transition of the camphor-bound protein toward low spin. Spectral intensities decreasing at 392 nm and increasing at 417 nm correspond to successive pressures of 1, 400, 600, and 800 bar. A clean isosbestic point at 404 nm is observed. (B) High-pressure-range spectral changes showing the transition from native cytochrome P-450 to inactive cytochrome P-420. At 2500 bar a clean spectrum of the oxidized cytochrome P-420 was obtained with optical maxima at 367, 425, and 456 nm. No precipitation of the sample was observed at the pressures indicated. Each spectrum was taken after a 6-min dwell time. Spectral intensities decreasing at 392 nm correspond to pressures of 1000, 1200, 1400, 1800, and 2500 bar.

forces the helix B′ carrying tyrosine-96 into a slightly new geometry. This propagates a change in the heme pocket and the substrate through a hydrogen bond between the camphor carbonyl and tyrosine-96. These effects force water, the sixth axial ligand of the iron of the low-spin structure, from the pocket. This later water loss is viewed as the cause of the spin-state change.

Spermine probably does not have access to the interior of the P-450. In Figure 2 (bottom) we present a graphic illustration of spermine docked onto the potassium site. The two opposing charged surfaces, 4+ for spermine and 4− for the potassium site, match up quite well and can easily be brought to within 5 Å of one another. There is no necessity to remove water-515 (shown as the white ball) that is proposed to occupy the potassium site. This suggests why the site can be completely saturated with spermine but still only 75% high spin.

As discussed in the introduction, hydrostatic pressure can shift the spin equilibrium and can also convert P-450 to P-420. Figure 3 shows a representative series of spectra of the substrate-bound cytochrome P-450 complex as it was subjected to pressure. Each spectrum was taken 10 min after the final pressure was stabilized. The final spectra of Figure 3A were the same, whether pressurizing or depressurizing, and equilibrium was reached slowly in about 6–10 min. The buffer for the experiment was 200 mM Tris containing 60 mM spermine, pH 7.2 at 4 °C. Two pressure ranges can be distinguished. In the low-pressure range (Figure 3A), below 800 bar, pressure induces a reversible spectral transition between Soret maxima at 392 and 417 mn, indicating a spin-state transition with a clear isosbestic point at 404 nm. The reaction volume for this first process

$$\Delta V° = V(\text{ls}) - V(\text{hs})$$

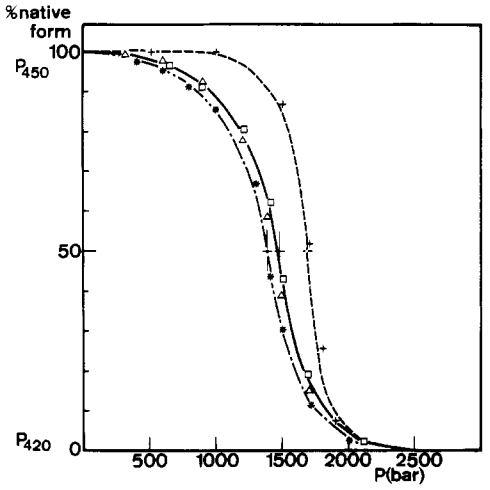

FIGURE 4: Transition profile of pressure-induced active cytochrome P-450 to inactive P-420 in the presence of KCl, spermine, and cysteine at 4 °C. Buffer conditions: 200 mM Tris, pH 7.2 (final), and 400 μM camphor, 4 °C, plus 240 mM KCl or 60 mM spermine or 32 mM cysteine or 60 mM spermine and 32 mM cysteine. The pressures at half-inactivation in the presence of cysteine, spermine, and KCl were 1400, 1500, and 1700 bar, respectively. The relative concentrations of P-450 and P-420 were determined from the absorbance at 404 nm, the isosbestic point of the spin transition. ϵ_{404}(P-450) = 77 mM^{-1} cm^{-1}; ϵ_{404}(P-420) = 53 mM^{-1} cm^{-1}. The pressure of half-inactivation, $P_{1/2}$, is indicated by a cross in the figure, with cysteine (*), cysteine plus spermine (▽), spermine (□), and KCl (+) bound states.

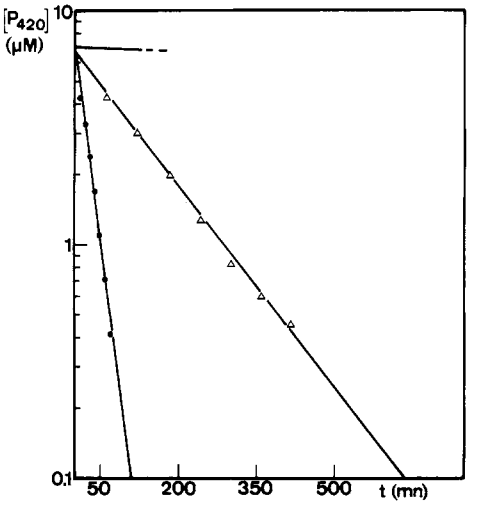

FIGURE 5: Renaturation kinetics of cytochrome P-420. Cytochrome P-450 was converted to cytochrome P-420 by application of 2500 bar for 10 min. Three solutions were studied; they contained, in addition to 200 mM Tris, pH 7.2 (final), and 400 μM camphor, 240 mM KCl or 60 mM spermine or 32 mM cysteine. The temperature was 20 °C. After complete conversion to cytochrome P-420, the pressure was brought back to 1 bar and the disappearance of cytochrome P-420 was followed by monitoring the increase in absorbance at 392 nm. Upper trace, KCl; middle trace, spermine; bottom trace, cysteine.

was found to be −44 mL/mol. This value should be compared to −48 mL/mol for $\Delta V°$ in the presence of Tris buffer (200 mM, pH 7.2) alone and −85 mL/mol when KCl (200 mM) is present.

The −40 mL/mol differences between the spermine, Tris, and the KCl samples could suggest that pressure forces the potassium ion from its site(s). This increase in volume is partially accounted for by electrostriction of the potassium itself (−0.5 mL/mol) (Pande & Wishnia, 1986) but, more importantly, by a differential solvation of the heme iron and by solvent movement into the potassium site thereby shielding charges exposed by the dissociation of potassium under pressure. Spermine probably does not occlude water from the potassium site. Additionally, spermine-containing samples do not show the larger volume change exhibited by potassium-containing samples. The small volume change is most readily explained by postulating that there is no water influx into the potassium site when, or if, spermine is dissociated by pressure.

In the high-pressure range, up to 3000 bar (Figure 3B), pressure causes a decrease in intensity and a red shift of the absorption maximum from 417 to 425 nm. This spectral change indicates formation of the so-called inactive "P-420" form (Marden & Hui Bon Hoa, 1986). The spectral characteristics of cytochrome P-420 are different at low and high pressure: at 1 bar this form absorbs at λ_{max} = 367 and 420 nm with corresponding extinction coefficients: ϵ_{367} = 46.8 mM^{-1} cm^{-1} and ϵ_{420} = 71.5 mM^{-1} cm^{-1}. Pressure shifts these absorption maxima toward longer wavelengths; for example, the extinction coefficients and peak positions at 2500 bar are ϵ_{367} = 43 mM^{-1} cm^{-1} and ϵ_{425} = 64.6 mM^{-1} cm^{-1}.

The high-pressure spectrum of cytochrome P-420 shows a supplementary shoulder around 456 nm. Between 2500 and 3000 bar the spectrum is invariant. The protein, at these pressures, is a stable form of P-420; protein solutions stay clear and the P-420 shows no tendency to undergo unfolding. In Figure 4, we plot the appearance of cytochrome P-420 in the presence of 400 μM camphor as a function of pressure for several different conditions. The pressure required to arrive at 50% of cytochrome P-420, $P_{1/2}$, is about 1400 bar with buffer alone (data not shown), 1500 bar in the presence of spermine, 1400 bar in the presence of cysteine, and 1700 bar in the presence of KCl.

The spermine and cysteine data are interesting in that, while the two show about the same midpoints for the P-450/P-420 transition, the two inactivation processes begin from entirely different percentages of low spin. The protein in the presence of spermine is initially only 41% low spin whereas in the presence of cysteine it is 64% low spin. In the presence of KCl it was only 20% low spin. We have calculated the volume change for the P-450/P-420 transition assuming that it is totally reversible and taking cognizance of the fact that there is a pressure-dependent red shift in the P-420 spectrum (Figure 3). These results are −210 mL/mol in the presence of KCl, −169 mL/mol with spermine, and −153 mL/mol with cysteine. It is interesting that the differences in $\Delta V°$ (spin equilibrium) between the sample with KCl and those without added cation are also reflected in the volume change for the P-450 → P-420 interconversion.

Cytochrome P-420 is not a denatured form of cytochrome P-450; there is no apparent change in the gross helical structure of the protein (Satake et al., 1976). Cytochrome P-420 has been called an "altered form" (Marden & Hui Bon Hoa, 1986; Yu & Gunsalus, 1974; Sato & Omura, 1978) and has been shown, under certain conditions, to convert back to cytochrome P-450 (Yu & Gunsalus, 1974; Ichikawa & Yamano, 1967). The structural nature of cytochrome P-420 is not certain. Disruption of the iron–cysteine-357 bond has been shown to lead to a cytochrome P-420 like structure (Sato & Omura, 1978; Lipscomb et al., 1978), and the pocket of the cytochrome P-420 is thought to be myoglobin-like (Yu & Gunsalus, 1974). It has been shown that cytochrome P-420 is not always a unique species but rather an ensemble of products that depends on the nature of formation. High pressure, on the other hand, has been shown to yield a clean reversible transition to the inactive form (Hui Bon Hoa et al., 1989).

In Figure 5, we show the kinetics of the P-420 → P-450cam transition in the presence of KCl, spermine, and cysteine. Cytochrome P-420 was generated by applying pressures

greater than 2000 bar. Spectra were recorded after the rapid release of pressure and were monitored as a function of time. They showed a clear isosbestic point at 407 nm, and the kinetic data were deduced from the observed spectral changes as described under Experimental Procedures.

The kinetic data in the presence of cysteine are similar to those reported (Yu & Gunsalus, 1974); first-order plots for the reaction showed good linearity, suggesting two-state behavior with a time constant for renaturation of about 30 min. In the presence of spermine, the time constant for conversion to P-450 was 120 min. Spermine–cysteine mixtures showed cysteine renaturation kinetics (data not shown). On the other hand, high concentrations of KCl did not promote renaturation. KCl and cysteine showed cysteine renaturation kinetics (data not shown). The renaturation of cytochrome P-420 to the active form, cytochrome P-450, was verified by the determination of the CO binding capacity. Reversibility was typically about 90% in the presence of cysteine and 75% in the presence of spermine at 20 °C.

The most significant aspect of these data is the indication that a multiply charged perturbant such as spermine, which, unlike cysteine, is not a facile reductant and which probably acts through bulk electrostatic contributions, can bring about effects similar to those of compounds that have entirely different properties. Cysteine, in contrast to spermine, is a much smaller molecule and was originally thought to act by keeping indigenous disulfides in their reduced states. When the X-ray structure of P-450 revealed no disulfide bridges, the mode of cysteine reversion of P-420 to P-450 became more obscure (Yu & Gunsalus, 1974).

Discussion

The crystal structures of both the free and camphor-bound cytochromes P-450 have been determined to very high resolution (Poulos et al., 1987, 1986, 1985). These structures show very few differences in the positions of the amino acid side chains and the heme upon association of substrate. The most pronounced differences between the two are found in the heme/camphor pocket. When camphor is present, the pocket is occupied by the hydrophobic substrate and water is excluded (Poulos et al., 1987). In the absence of camphor the pocket is occupied by several water molecules (Poulos et al., 1986). It was suggested that a cation binding site is located in a region where it does not interact directly with the camphor (Poulos et al., 1987) but stabilizes an unfavorable polypeptide conformation of the B′ helix. This coupling has been recently demonstrated to occur between the hydrogen bond of Tyr-96 to camphor (Di Primo et al., 1990). It also appears from previous work (Fisher et al., 1985; Marden & Hui Bon Hoa, 1987) that pressure acts to drive the camphor from the pocket. From the data presented here, it appears that pressure drives the potassium from its binding site(s), thereby favoring substrate release and active-site solvation. Exclusion of both species from the protein is accompanied by the transfer of substantial amounts of solvent into the protein (Fisher & Sligar, 1987) as well as ordering of solvent by the excluded camphor and potassium. The volume differences seen when we compare the effects of pressure on the system with spermine present as opposed to potassium are then representative of the differences between the cation site with solvent water present as opposed to exchange of potassium for water.

Structural information about cytochrome P-420 is much less certain. It is clear that this nonnative structure can arise from perturbing different aspects of the protein and, further, that the spectral entity that one observes is one in which the iron–thiolate bond is disrupted either completely or partially. The presence of KCl, which specifically alters the spin transition of the cytochrome P-450–camphor complex, does not favor the renaturation of cytochrome P-420. Conversion from cytochrome P-450 to cytochrome P-420 in the presence of high concentrations of potassium may expose and then disturb the hydrophobic environment around the heme moiety, thereby altering the coordination of the cysteine-357 anion to the heme. Earlier suggestions proposed that exogenously added cysteine acted to convert P-420 to the active P-450 form through effects on the redox potential of the system (Yu & Gunsalus, 1974). With spermine there are no direct redox effects and the compound is not acting directly at the active site in the heme, pocket; rather, the renaturation of cytochrome P-420 must be transmitted from the protein surface via the peptide backbone, amino acid side chains, and a network of ordered solvent molecules or through modulation of the protein's electrostatic potential.

Registry No. P-450, 9035-51-2; P-420, 9035-49-8; K, 7440-09-7; L-cysteine, 52-90-4; spermine, 71-44-3.

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^{13}C NMR Relaxation Times of Hepatic Glycogen in Vitro and in Vivo†

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ABSTRACT: The field dependence of relaxation times of the C-1 carbon of glycogen was studied in vitro by natural-abundance ^{13}C NMR. T_1 is strongly field dependent, while T_2 does not change significantly with magnetic field. T_1 and T_2 were also measured for rat hepatic glycogen enriched with [1-^{13}C]glucose in vivo at 4.7 T, and similar relaxation times were observed as those obtained in vitro at the same field. The in vitro values of T_1 were 65 ± 5 ms at 2.1 T, 142 ± 10 ms at 4.7 T, and 300 ± 10 ms at 8.4 T, while T_2 values were 6.7 ± 1 ms at 2.1 T, 9.4 ± 1 ms at 4.7 T, and 9.5 ± 1 ms at 8.4 T. Calculations based on the rigid-rotor nearest-neighbor model give qualitatively good agreement with the T_1 field dependence with a best-fit correlation time of 6.4×10^{-9} s, which is significantly smaller than τ_M, the estimated overall correlation time for the glycogen molecule (ca. 10^{-5} s). A more accurate fit of T_1 data using a modified Lipari and Szabo approach indicates that internal fast motions dominate the T_1 relaxation in glycogen. On the other hand, the T_2 relaxation is dominated by the overall correlation time τ_M while the internal motions are almost but not completely unrestricted.

Glycogen is the main storage form of glucose in mammalian cells. Although glycogen has a very high molecular weight (ca. 10^7) (Drochmans, 1962; Goldsmith et al., 1982), previous studies have shown that it is 100% ^{13}C NMR visible both in vitro and in vivo at ambient temperatures (Sillerud & Shulman, 1983; Hull et al., 1987; Shalwitz et al., 1987). This visibility has allowed many studies of synthesis and metabolism of glycogen in vivo by ^{13}C NMR spectroscopy (Jue et al., 1989; Laughlin et al., 1988; Reo et al., 1984; Shulman et al., 1988). Considering the high molecular weight, the estimated rotational correlation time (τ_M) for a rigid molecule with the size of glycogen would be ca. 10^{-5} s. This would lead to very short values of T_2, so that in the absence of any other motion the ^{13}C NMR lines of glycogen would be too broad to observe.

In order to understand the mechanism of NMR visibility of glycogen, we have studied the magnetic field dependence of the glycogen relaxation times in vitro. In addition, we have measured the T_1 and T_2 of hepatic glycogen directly in an intact living rat at 4.7 T. We have compared the experimental results with those of theoretical calculations based on the rigid-rotor nearest-neighbor model (RRNN)[1] and the modified Lipari and Szabo approach (MLSA).

Materials and Methods

In Vitro. Type III rabbit liver glycogen, prepared by hot water extraction, was purchased from Sigma Chemical Co. (St. Louis, MO). A solution of glycogen (30 mg/mL) in 50 mM phosphate buffer, pH 7, was prepared for natural-abundance ^{13}C NMR measurements at 2.1 and 4.7 T without further purification. For measurements at 8.4 T, D_2O was added to the solution to 10% by volume with a final glycogen concentration of 100 mg/mL.

Natural-abundance ^{13}C NMR measurements were performed on an 8.4-T Bruker Instrument AM superconducting spectrometer system, and on 2.1- and 4.7-T Biospec superconducting spectrometer systems, respectively. At 8.4 T, ^{13}C spectra of glycogen were taken at 90.5 MHz using a 5-mm probe. At 4.7 and 2.1 T, the spectra were taken at 50.4 and 22.89 MHz using 20-mm sample tubes in $^{13}C/^1H$ solenoidal probes. Spin–lattice relaxation times (T_1) and spin–spin relaxation times (T_2) were measured with the inversion recovery (180°–τ–90°) and spin-echo (90°–τ–180°–τ–echo) methods, respectively. Results were analyzed by using a three-parameter fitting program from the spectrometer computer (Bruker In-

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[1] Abbreviations: MFA, model-free approach; MLSA, modified Lipari and Szabo approach; RRNN, rigid-rotor nearest-neighbor.

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