NMR Studies of Cytochrome P-450$_{scc}$. Effects of Steroid Binding on Water Proton Access to the Active Site of the Ferric Enzyme†

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ABSTRACT: Water proton relaxation rates of various complexes of cholesterol side chain cleavage cytochrome P-450 (P-450$_{scc}$) were investigated to gain information about the structure and dynamics of the steroid binding site. In all cases bulk water protons were found to be in rapid exchange with protons near the paramagnetic Fe^{3+} center, and the long electron spin relaxation time of the heme iron, $\tau_s \sim 0.3$ ns, resulted in fast relaxation rates. For the steroid-free enzyme, the closest approach of exchangeable protons is ~2.5 Å, a distance consistent with a water molecule binding directly to the heme iron or rapidly exchanging with a coordinated ligand. When cholesterol was bound, the distance increased to ~4 Å, indicative of displacement of water from the immediate coordination sphere of the heme but still in close proximity to the active site. For the complex with (22*R*)-22-hydroxycholesterol, a distance of ~2.7 Å is observed, suggesting a reorganization of the active site when this intermediate is formed from cholesterol. Complexes of P-450$_{scc}$ with the competitive inhibitors (22*R*)-22-aminocholesterol, 22-amino-23,24-bisnor-5-cholen-3β-ol, or (20*R*)-20-phenyl-5-pregnene-3β,20-diol, also yielded distances of ~2.5 Å and reveal no effect of side chain size on access of protons to the heme. In the nitrogen-coordinated amino–steroid complexes, the distances observed indicate solvent proton exchange with the heme-bound nitrogen ligand. In contrast to cytochrome P-450$_{cam}$, in which water is excluded from the heme center in the substrate complex [Griffin, B. W., & Peterson, J. A. (1975) *J. Biol. Chem. 250*, 6445–6451; Philson, S. B., Debrunner, P. G., Schmidt, P. G., & Gunsalus, I. C. (1979) *J. Biol. Chem. 254*, 10173–10179], protons have rapid access to regions near the active site of several steroid complexes of P-450$_{scc}$. This suggests that the active site of P-450$_{scc}$ may be open to solvent and that solvent water molecules, rather than acid/base groups in the active site, may provide the protons required during the monooxygenation reaction cycle.

Cytochrome P-450 enzymes catalyze a variety of monooxygenation reactions on a number of physiological compounds as well as a diverse spectrum of foreign chemicals. The typical reaction in which cytochrome P-450 acts as the oxygenating catalyst is given in eq 1 where RH represents the substrate

$$RH + O_2 + NADPH + H^+ \rightarrow ROH + H_2O + NADP^+ \quad (1)$$

(White & Coon, 1980). It appears that most, if not all, cytochrome P-450 enzymes share a common mechanism in which molecular dioxygen is split to yield an activated form of iron-bound oxygen, which is then inserted into the substrate. A general feature of the compounds, which serve as substrates for cytochrome P-450 reactions, is a high degree of lipophilicity, suggesting that the substrate binding site is highly hydrophobic. A completely apolar active site, however, makes it difficult to explain the origin of the protons required for the splitting of dioxygen to generate an atom of active oxygen and a molecule of water. The required protons must originate either from acid/base groups provided by amino acid side chains within the active site or from water molecules with access to the active site.

NMR studies of relaxation rate enhancement of solvent water protons have been employed to investigate the accessibility of solvent to the heme iron of cytochrome P-450. For mammalian P-450 enzymes, conflicting results of slow (Grasdalen et al., 1978) and fast (Rein et al., 1976; Maricic et al., 1979) exchange of solvent water molecules have been reported; these studies, however, were complicated by the presence of multiple isozymes of P-450 in the preparations used and uncertain heme ligation state. The most extensively characterized system is the soluble bacterial enzyme, P-450$_{cam}$,[1] a camphor hydroxylase from *Pseudomonas putida*. Griffin and Peterson (1976) and Philson et al. 1979) showed that the heme center in ferri-P-450$_{cam}$ is freely accessible to solvent water molecules in the absence of camphor but that access is blocked upon substrate binding. The crystallographic structure of the camphor complex (Poulos et al., 1985, 1986) confirms this finding: the heme of P-450$_{cam}$ is buried within the protein, and the camphor is located nearby in a hydrophobic pocket inaccessible to solvent water molecules. Moreover, amino acid side chains capable of acid/base catalysis are absent from the active site, and no bound water molecules appear to occur in the near vicinity.[2] The findings with this system thus raise the question of how protons are provided in the transition state of reaction 1.

In the camphor complex of P-450$_{cam}$, the ferric heme exists in a predominantly high-spin ($S = {}^5/_2$) state as a result of the absence of a sixth ligand. For a number of mammalian P-450

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[1] Abbreviations: P-450$_{scc}$, cholesterol side chain cleavage cytochrome P-450; P-450$_{cam}$, camphor hydroxylase cytochrome P-450; 22-ABC, 22-amino-23,24-bisnor-5-cholen-3β-ol; 22-aminocholesterol, (22*R*)-22-amino-5-cholesten-3β-ol; 22-hydroxycholesterol, (22*R*)-22-hydroxy-5-cholesten-3β-ol; 20-PPD, (20*R*)-20-phenyl-5-pregnene-3β,20-diol.

[2] Recent refinements in the crystallographic structure of the camphor complex of ferri-P-450$_{cam}$ to 1.6 Å also reveal no bound water near the heme or camphor (T. L. Poulos, personal communication).

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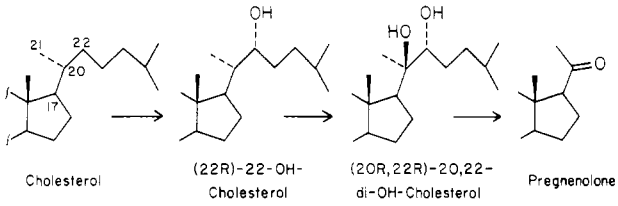

FIGURE 1: Intermediates in the cholesterol side chain cleavage reaction.

enzymes, however, this is not the case. Many remain low spin ($S = {}^1/_2$) even when substrate is bound. Stabilization of the low-spin form is presumed to require a sixth ligand such as the water in substrate-free P-450$_{cam}$. While the spectral properties of low-spin P-450–substrate complexes are consistent with an oxygen donor ligand, it is not known whether water has access to the active site or an amino acid residue provides the donor oxygen.

We report here the results of NMR studies on water proton relaxation rates in the presence of cholesterol side chain cleavage cytochrome P-450, designated P-450$_{scc}$. This enzyme catalyzes the conversion of cholesterol to pregnenolone in steroidogenic tissues and can be highly purified [cf. Simpson (1979)]. The side chain cleavage reaction is unusual in that is involves three separate, sequential monooxygenation steps and two intermediate substrates (Figure 1). It is of particular interest that each steroid produces a complex in which the heme differs in spin state: the complex with cholesterol is high spin, the complex with 22-hydroxycholesterol is low spin, and the complex with 20,22-dihydroxycholesterol is mixed spin [cf. Orme-Johnson et al. (1979)]. Thus, P-450$_{scc}$ provides an opportunity to compare water proton access to the active site of both high- and low-spin forms of substrate complexes in the same cytochrome P-450 enzyme. Also, the availability of different types of cholesterol analogues that act as competitive inhibitors makes it possible to determine the degree to which the steroid side chain affects proton access.

Materials and Methods

Sample Preparation. Cytochrome P-450$_{scc}$ was isolated as the cholesterol complex from bovine adrenocortical mitochondria (Duval & Vickery, 1980); samples contained 10–12 nmol of P-450/mg of protein [determined according to Omura and Sato (1964)], and similar results were obtained with several different preparations. The steroid derivatives investigated were prepared by published procedures: (22*R*)-22-hydroxycholesterol (Burrows et al., 1969); (22*R*)-22-aminocholesterol (Khuong-Huu et al., 1974); 22-amino-22,23-bisnor-5-cholen-3β-ol (Sheets & Vickery, 1983); (20*R*)-20-phenyl-5-pregnene-3β,20-diol (Vickery & Kellis, 1983).

Stock solutions of the enzyme as the cholesterol complex were stored in 1:1 mixture of glycerol and potassium phosphate buffer (100 mM, pH 7.1). Before each NMR measurement, an aliquot of the enzyme was concentrated and glycerol was removed by several cycles of dilution and concentration by a nitrogen-pressurized stirred cell and semipermeable membrane (Amicon Corp., Lexington, MA). In some cases an aliquot of the stock solution was first dialyzed extensively against buffer containing Chelex-100 and then concentrated. The same T_{1m} values were obtained after either procedure, indicating no significant paramagnetic metal ion impurities. Final concentrations of P-450$_{scc}$ ranged from 400 to 750 μM. A portion of the final filtrate from the Amicon stirred cell was used as the buffer reference sample in the NMR measurements.

Substrate-free P-450$_{scc}$ was formed by treatment of the high-spin cholesterol complex with 0.5% Tween-20; in this solvent, cholesterol dissociates and the heme is converted to a low-spin state (Sheets & Vickery, 1983). Complexes of P-450$_{scc}$ with steroid derivatives were obtained by depositing a thin film of the ligand in an ethanol solution on the bottom of a small vial and evaporating the alcohol; concentrated enzyme in buffer was added to the dried steroid, and the vial was gently swirled until the absorption spectrum indicated complete binding. The reduced CO complex of P-450$_{scc}$ was used for diamagnetic corrections (Fe^{2+}, $S = 0$); this was formed by the addition of a few crystals of sodium dithionite directly to samples in NMR tubes and exchanging the gas phase with carbon monoxide.

Formation of all P-450$_{scc}$–ligand complexes was verified by absorption spectroscopy. Before and/or after NMR measurements aliquots of samples were diluted into 50 mM sodium phosphate, pH 7.0, and spectra were recorded at ambient temperatures with 1 cm path length cuvettes in a Cary Model 17D spectrophotometer. Affinities for each of the steroid derivatives studied are sufficiently high that the enzyme was completely saturated under all conditions. In fact, the NMR samples (ca. 0.5 mM) could be diluted 100-fold without significant dissociation of the ligands.

NMR Measurements. ^{1}H NMR measurements were made at 500 and 250 MHz on Bruker WM series NMR spectrometers equipped with variable-temperature controllers. A typical sample contained ~25 μL of the enzyme in a microcell (Wilmad Glass Co., Buena, NJ) placed inside a 5-mm-o.d. NMR sample tube. $CDCl_3$ for field frequency lock was placed between the two tubes. T_1 measurements were made by the standard inversion–recovery sequence, 180°–τ–90°, and 12–15 time points were used in a three-parameter nonlinear least-squares analysis (Farar & Becker, 1971; Martin et al., 1980)

$$I_\tau = I_\infty[1 - A \exp(-\tau/T_1)] \qquad (2)$$

where I_τ is the signal intensity at pulse spacing τ, I_∞ the intensity at $\tau = \infty$, and $A = 2$ for an exact 180° pulse. On the basis of standard deviations from the least-squares analyses and deviations between independent measurements made with different samples, the random error in T_{1p} calculated through eq 3 (vide infra) is less than 20%, implying less than 4% error in the distances calculated from eq 4.

Theory

The Solomon–Bloembergen equations (Solomon, 1951; Solomon & Bloembergen, 1956; Bloembergen, 1957) describe the effect of a paramagnetic center on the spin relaxation of a ligand nucleus. Under a limited set of circumstances (vide infra), this effect can be used to determine paramagnet–ligand distances [for reviews see Mildvan and Gupta (1978) and Morris and Dwek (1977)]. A brief description of the theoretical aspects essential for the analysis of the data obtained here is given below.

Paramagnetic (T_{1p}^{-1}) and diamagnetic (T_{1d}^{-1}) terms contribute to the observed proton relaxation rate (T_{1o}^{-1}) in aqueous solutions of the P-450 enzyme

$$T_{1p}^{-1} = \frac{[H]}{[P\text{-}450]}(T_{1o}^{-1} - T_{1d}^{-1}) = \frac{n}{T_{1m} + \tau_m} \qquad (3)$$

where T_{1m}^{-1} and T_{1d}^{-1} are the relaxation rates in the paramagnetic and diamagnetic complexes, respectively, τ_m is the lifetime of the paramagnetic complex, n is the number of exchanging protons, and [H] and [P-450] are the proton and enzyme concentrations, respectively. Within experimental error, the same diamagnetic values, T_{1d}, were obtained with the reduced-CO enzyme and enzyme-free buffer indicating no significant outer-sphere contributions. Similar results were

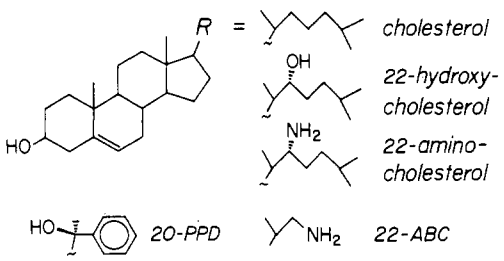

FIGURE 2: Structures of steroids used to form complexes with P-450$_{scc}$.

reported for the P-450$_{cam}$ system (Philson et al., 1979). We also found that T_{1p}^{-1} is independent of concentration in the range examined.

By making T_{1p} measurements as a function of temperature, it is possible to determine the relative contributions of T_{1m} and τ_m. While T_{1m} has a weak temperature dependence, the exchange rate, τ_m^{-1}, exhibits a dependence on temperature that follows the Arrhenius equation (McConnell, 1958). Plots of log T_{1p} vs. reciprocal temperature will reveal whether $\tau_m \ll T_{1m}$. In general, a negative slope in such plots implies that τ_m can be neglected in eq 3.

The distance between the paramagnetic center and the ligand nucleus (r) can be obtained from T_{1m}. For the special case of ferric heme proteins (Philson et al., 1979)

$$T_{1m}^{-1} = \frac{2}{15}\frac{S(S+1)\gamma_I^2\gamma_s^2\hbar^2}{r^6}\frac{3\tau_s}{1+\omega_I^2\tau_s^2} \qquad (4)$$

where τ_s is the electron spin relaxation time, ω_I is the proton Larmor frequency, and symbols characterizing the nuclear and electron moments have their customary meanings. The frequency dependence of T_{1m} provides an estimation of τ_s. In the heme iron system examined in this study, both spin states, $S = {}^1/_2$ and $S = {}^5/_2$, are important. From eq 4 we note that at equal τ_s and distances, T_{1m} ($S = {}^1/_2$) will be approximately 11.7 times T_{1m} ($S = {}^5/_2$). A less dramatic difference is to be expected when τ_s for the high state is less than that for the low-spin state. Thus, changes in spin state alone will have a significant effect on relaxation times.

Results

P-450$_{scc}$ was studied in its low-spin, substrate-free form and as a complex with each of the steroid derivatives shown in Figure 2. The cholesterol and 22-hydroxycholesterol complexes are high and low spin, respectively (Orme-Johnson et al., 1979), while the inhibitor complexes, 22-aminocholesterol (Nagahisa et al., 1985), 22-ABC (Sheets & Vickery, 1982), and 20-PPD (Vickery & Kellis, 1983) are low spin. Determinations of the paramagnetic contribution to the proton relaxation times for the substrate-free enzyme and the cholesterol complex at 500 and 250 MHz are presented in Figure 3. If the iron–proton distances in the substrate-free and cholesterol P-450$_{scc}$ complex were the same, there would be a significantly smaller T_{1p} relaxation time for the cholesterol complex due to its high-spin iron (see eq 4). This is clearly not the case, and the data indicate that the iron–proton distance in the cholesterol-bound complex is significantly greater than in the substrate-free form.

In order to calculate iron–proton distances from the T_{1p}^{-1} data, the lifetime of the paramagnetic complex (τ_m) and the electron spin relaxation time (τ_s) must be evaluated. The dependence of the water proton T_{1p} values on temperature was measured for each complex to assess τ_m. Figure 3 shows the results obtained for the substrate-free enzyme and the cholesterol complex at 500 and 250 MHz and for the 22-ABC complex at 500 MHz. Complexes of P-450$_{scc}$ with 22-

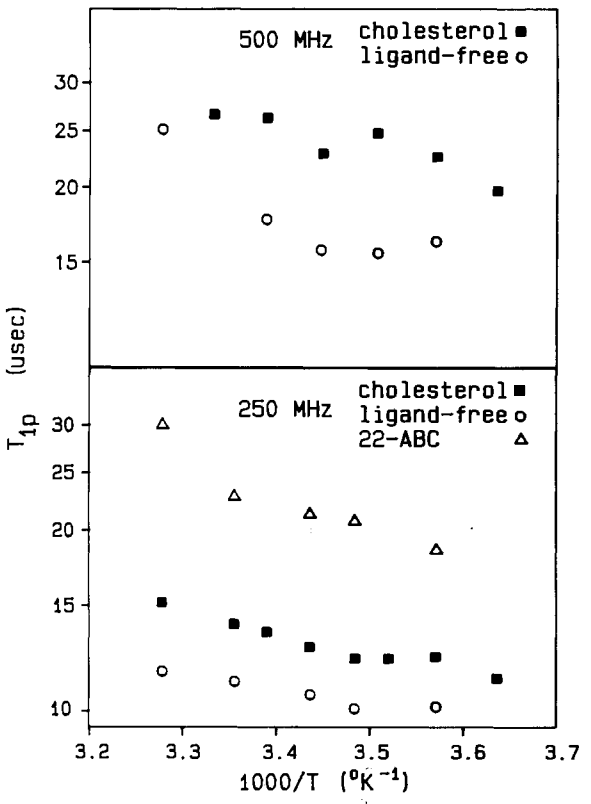

FIGURE 3: Temperature dependences of T_{1p} values for P-450$_{scc}$ complexes. Negative slopes indicate $T_{1m} \gg \tau_m$ and fast exchange.

hydroxycholesterol, 22-PPD, and 22-aminocholesterol yielded similar results with slopes intermediate between that of the 22-ABC complex and that of the ligand-free enzyme. For each complex, an increase in temperature caused an increase in T_{1p}, indicating fast exchange of protons between bulk solvent water and the P-450 paramagnetic site. Thus, τ_m in eq 3 can be neglected, and $T_{1m} = nT_{1p}$. It should be noted that in the event of a significant residence time for solvent protons near the paramagnetic site (i.e., τ_m not small compared to T_{1m}), the true distance would be *less* than those shown.

The frequency dependence of T_{1p} can be used to obtain an estimate for the electron spin relaxation time, τ_s, from eq 4. T_{1p} is significantly larger at 500 MHz than at 250 MHz, and an average obtained using several independent samples yields $\tau_s \sim 0.35$ ns. No significant difference was found between the high- and low-spin forms. Similar values, $\tau_s \sim 0.3$–0.5 ns, were found with P-450$_{cam}$ for the low-spin state by NMR water proton relaxation measurements (Philson et al., 1979) and for the high-spin state by ESR line-width measurements (Griffin & Peterson, 1975). Although only two frequencies were used to obtain τ_s in this study and in that of Philson and co-workers (1979), the general agreement between the P-450$_{cam}$ and P-450$_{scc}$ systems indicates that $\tau_s \sim 0.3$ ns is a reasonable estimate for these systems.

The experimentally determined values for T_{1p} and the calculated distances of closest approach of protons to the heme of P-450$_{scc}$ are summarized in Table I. Distances were calculated on the assumption of fast exchange ($\tau_m \ll T_{1m}$), a single exchangeable proton ($n = 1$), and $\tau_s = 0.35$ ns. If we assume exchange of both protons on a water molecule ($n = 2$), the distance values will be increased by a factor of 1.12. For the low-spin forms the proton–iron distances vary from 2.3 to 2.7 Å. Considering the assumptions and approximations that are involved in the distance calculation (Mildvan & Gupta, 1978), the ligand-free enzyme and each of the complexes can be considered to have an iron–proton distance of

Table I: Calculation of the Distance of Closest Approach of Protons to the Paramagnetic Heme Iron of Ferri-P-450$_{scc}$[a]

frequency (MHz)	enzyme	ligand	T_{1p} (μs)	distance (Å)
500	P-450$_{scc}$	cholesterol	26	3.9
		substrate free	18	2.5
		22-aminocholesterol	13	2.3
250		cholesterol	14	3.9
		substrate free	11	2.5
		22-ABC	12	2.5
		22-hydroxycholesterol	19	2.7
		22-PPD	11	2.5
220	P-450$_{cam}$	substrate free	11[b]	2.6
27.5		substrate free	8[c]	2.6

[a] Equations 3 and 4 were employed with the following conditions: $\tau_m \ll T_{1m}$, [H] = 111.1 M = 2[H_2O], n = 1, τ_s = 0.35 ns, T = 22 °C. Relaxation times and the distance estimate for cytochrome P-450$_{cam}$ in the native, substrate-free state (Fe^{3+}, $S = {}^1/_2$) are included for comparison (Philson et al., 1979). [b] Assumes ratio of T_{1p} at 220 MHz to T_{1p} at 27.5 MHz is 1.42 as stated by Philson et al. (1979). Agrees with T = 28 °C data in Table II of Philson et al. (1979). [c] From Figure 1 of Philson et al. (1979), T = 22 °C.

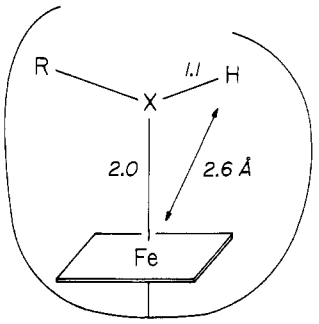

FIGURE 4: Approximate distances in ferric heme ligand complexes assuming tetrahedral bond angles about the X atom.

~2.5 Å. In the cholesterol-bound, high-spin state, the distance is increased to 3.9 Å.

Discussion

Significant enhancement of bulk solvent water proton relaxation rates was observed for each form of ferri-P-450$_{scc}$ studied. The magnitude and temperature dependence of the proton T_{1p} values indicate that the relaxation enhancement is due to rapid exchange of protons between the solvent and the substrate pocket containing the heme–iron. In the case of the substrate-free enzyme, solvent protons can approach to within approximately 2.5 Å of the paramagnetic heme center. This distance is similar to that observed with P-450$_{cam}$ (Griffin & Peterson, 1975; Philson et al., 1979) and is small enough for the exchangeable proton to be directly bonded to an axial heme ligand. As shown in Figure 4, the distance of a proton bonded to a ligand of a ferric heme center is approximately 2.6 Å. While optical spectroscopic data are consistent with an oxygen donor ligand to the heme, it is not known whether a water molecule or an amino acid side chain (Ser, Thr, Tyr) is the actual ligand.

When cholesterol is bound to the enzyme, the minimum distance of closest approach of solvent protons to the heme increases to ~4 Å. Binding of camphor to P-450$_{cam}$ also increases the distance of solvent approach to about 4–10 Å (Griffin & Peterson, 1975). These results suggest that substrate binding to these enzymes either directly blocks solvent access or results in a conformational change in the protein that excludes solvent from the immediate coordination sphere of the heme iron. In P-450$_{cam}$, the positioning of camphor near the heme and the folding of the protein both appear to contribute to the exclusion of water (Poulos et al., 1985, 1986). For P-450$_{scc}$, partial shielding of the heme by close proximity of the substrate may also occur, but the exact structure of the protein forming the active site is not known.

Unlike the cholesterol complex, other steroid complexes of P-450$_{scc}$ did not block proton access to the heme iron. The fast exchange observed agrees with that reported for crude preparations of hepatic microsomal P-450 enzymes (Rein et al., 1976; Maricic et al., 1979). For P-450$_{scc}$ bound with the intermediate substrate, 22-hydroxycholesterol, and three substrate analogues that act as competitive inhibitors, solvent protons can approach to within ~2.5 Å. This distance is similar to that observed for the substrate-free enzyme and indicates that steroid binding to the substrate site, per se, is not sufficient to block proton access. The distance is also consistent with direct bonding of the exchangeable proton with a heme axial ligand. In the cases of the complexes with 22-ABC and 22-aminocholesterol, optical spectra suggest that the nitrogen of the steroid coordinates directly to the heme of P-450$_{scc}$ (Sheets & Vickery, 1982, 1983; Nagahisa et al., 1985). The NMR results thus indicate that a proton on the coordinated nitrogen is in fast exchange with solvent protons. The fact that the longer side chain of 22-aminocholesterol does not affect this result also indicates that the side chain must not significantly block access to the active site. In the cases of the hydroxy steroid derivatives, the optical spectra suggest an oxygen donor ligand (Orme-Johnson et al., 1979; Vickery & Kellis, 1983). As with the substrate-free enzyme, however, it is not known whether the steroid hydroxyl, an amino acid side chain, or a water molecule is coordinated. In any case, the NMR data indicate that the hydroxyl proton is in fast exchange with solvent protons, and neither the alkyl side chain nor the large aromatic group appears to affect proton access.

Conclusion

The results with the cholesterol complex of P-450$_{scc}$ show that solvent protons have only limited access to the active site as was observed with P-450$_{cam}$. Binding of several related steroid derivatives, however, shows that proton access depends critically on the exact structure of the steroid. In the case of the 22-hydroxycholesterol complex, the increased proton access may reflect a somewhat different mode of binding for the two substrates. This altered interaction may be important in determining the sites of hydroxylation: C22 for cholesterol and C20 for 22-hydroxycholesterol. The close approach of protons in the case of the 22-hydroxycholesterol complex also shows that protons need not be excluded from the immediate coordination sphere of the heme iron even in the case of substrate complexes. This suggests that solvent water molecules may provide the protons required during the P-450 monooxygenation reaction cycle.

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Characterization of Interaction between DNA and 4′,6-Diamidino-2-phenylindole by Optical Spectroscopy†

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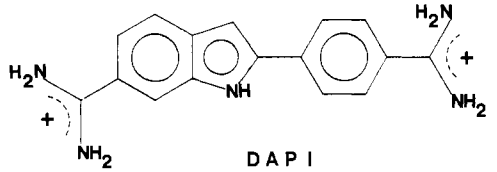
ABSTRACT: We have examined the interaction between 4′,6-diamidino-2-phenylindole (DAPI) and DNA using flow linear dichroism (LD), circular dichroism (CD), and fluorescence techniques. We show the presence of two spectroscopically distinct binding sites at low binding ratios with saturation values of 0.025 and 0.17, respectively. In both sites DAPI is bound with its long axis approximately parallel to the grooves of the DNA helix. Resolution of CD spectra shows that an exciton component is present at higher binding ratios, which we attribute to the interaction of two accidentally close-lying DAPI molecules. We also find evidence that DAPI, at least in the high-affinity site, binds preferentially to AT-rich regions. From the spectroscopic results, supported by structural considerations, we can completely exclude that DAPI is bound to DNA by intercalation. Binding geometries and site densities are consistent with a location of DAPI in the grooves of DNA, with the high-affinity site most probably in the minor groove.

The trypanocide 4′,6-diamidino-2-phenylindole (DAPI),[1] first



DAPI

synthesized by Dann et al. (1971), has been shown to bind reversibly to DNA as evidenced by fluorescence enhancement and sedimentation experiments in cytochemical studies (Williamson & Fennel, 1975) and by changes in absorption (Lin et al., 1977; Chandra & Mildner, 1979) and fluorescence spectra (Lin et al., 1977). A large increase in the fluorescence quantum yield of DAPI upon binding to nucleic acids has been reported (Williamson & Fennel, 1975; Kapuscinski & Skoczyklas, 1978). The increase seems to be specific for double-stranded DNA and has not been observed together with RNA or single-stranded DNA [poly(rI) being the only exception] (Kapuscinski & Szer, 1979), and it seems to be largest in AT-rich regions (Williamson & Fennel, 1975; Schweizer, 1976; Lin et al., 1977; Kapuscinski & Szer, 1979; Cavatorta et al., 1985). These properties have made DAPI very useful in cytochemical investigations (Williamson & Fennel, 1975; Schweizer, 1976; Langlois et al., 1980; Coleman et al., 1981; Tijssen et al., 1982; Lee et al., 1984), as a marker in DNA electrophoresis (Kapuscinski & Yanagi, 1979; Naimski et al., 1980), and in DNA–protein interaction studies (Kania & Fanning, 1976; Stepien et al., 1979; Mazus et al., 1986). DAPI has also been used in protein investigations, e.g., with tubulin (Bonnet et al., 1985).

Recent circular dichroism (CD) measurements show that DAPI binds to DNA in two spectroscopically distinct sites.

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[1] Abbreviations: DAPI, 4′,6-diamidino-2-phenylindole; LD, linear dichroism; CD, circular dichroism; LD′, reduced linear dichroism; A_{iso}, isotropic absorbance; EDTA, ethylenediaminetetraacetic acid; R, ratio of total amount of DAPI to DNA phosphate; r, ratio of bound DAPI to DNA phosphate; $\Delta\epsilon$, difference in molar absorptivity between left- and right-handed circularly polarized light.

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