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Flavodoxin–Cytochrome *c* Interactions: Circular Dichroism and Nuclear Magnetic Resonance Studies†

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ABSTRACT: Circular dichroism and ^1H and ^{31}P nuclear magnetic resonance spectroscopy have been used to investigate complex formation between cytochrome *c* and the flavodoxins from *Azotobacter vinelandii* and *Clostridium pasteurianum*. Such complexes are known to be involved in the mechanism of electron transfer between these two redox proteins. A large increase in ellipticity in the Soret band of the cytochrome heme was observed upon formation of the *Clostridium* flavodoxin complex, whereas much smaller changes were found for the complexes with either *Azotobacter* flavodoxin or an 8α-imidazolyl-FMN-substituted *Clostridium* flavodoxin analogue. Similarly, the magnitudes of the perturbations of the contact-shifted heme proton resonances obtained upon complexation of cytochrome *c* by *Azotobacter* flavodoxin were much smaller than those previously shown for *Clostridium* flavodoxin [Hazzard, J. T., & Tollin, G. (1985) *Biochem. Biophys. Res. Commun. 130*, 1281-1286]. ^{31}P nuclear magnetic resonance measurements were also consistent with differences in the interactions between the components in the complexes of the two flavodoxins with cytochrome *c*. It is suggested that these spectral changes are due to a loosening or opening of the heme crevice upon *Clostridium* flavodoxin binding, which allows closer contact between the heme and flavin prosthetic groups and results in a faster rate of electron transfer. The implications of these observations for biological oxidation–reduction processes are considered.

The electron-transfer reactions from flavodoxin semiquinones to cytochrome *c* have been extensively studied as a model to probe factors that are important in the determination of electron-transfer rates in redox proteins (Simondsen et al., 1982; Simondsen & Tollin, 1983; Tollin et al., 1984; Cheddar et al., 1986; De Francesco et al., 1987). The observation of saturation kinetics at high cytochrome *c* concentrations and the marked influence of ionic strength on reaction rates have demonstrated the involvement of electrostatistically stabilized complexes of the two proteins in the electron-transfer mechanism. Complex formation between *Clostridium pasteurianum* flavodoxin and horse heart cytochrome *c* has also been observed by NMR spectroscopy (Hazzard & Tollin, 1985). Electrostatic free energy calculations (Weber & Tollin, 1985), based on a computer-generated model of the structure of the complex (Simondsen et al., 1982) in which the exposed redox centers are in van der Waals contact, have shown that the ionic strength dependence of the kinetic rate constants for the electron-transfer reaction is dominated by those charged amino acid side chains which are localized predominantly in the vicinity of the FMN and heme prosthetic groups (lysine residues in the case of cytochrome *c* and aspartate and glutamate residues in the case of flavodoxin).

A previous comparative study (Cheddar et al., 1986) of *Clostridium pasteurianum* and *Azotobacter vinelandii* flavodoxins has shown the latter to be considerably less reactive than the former, despite the significantly lower redox potential for the oxidized/semiquinone couple of the *Azotobacter* protein, which would provide a larger thermodynamic driving force resulting in an increased rate of electron transfer. Cross-linking studies have shown that *Azotobacter* flavodoxin forms a tight 1:1 complex with cytochrome *c*, with an estimated association constant of 4×10^4 M^{-1} at 88 mM ionic strength (Dickerson et al., 1985), which compares favorably with the kinetically determined value for cytochrome *c* and *C. pasteurianum* flavodoxin semiquinone (18×10^4 M^{-1})

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(Simondsen et al., 1982). More recent studies in this laboratory have shown that the kinetically determined K_a for *Clostridium* flavodoxin is approximately the same as that for *Azotobacter* flavodoxin (De Francesco et al., 1977), whereas the rate constant for electron transfer is approximately 4-fold greater for *Clostridium* than for *Azotobacter* flavodoxin. Furthermore, substitution of the native FMN coenzyme in each of the flavodoxins with 8α-*N*-imidazolyl-FMN results in a dramatic decrease in the rate of electron transfer (at 45 mM ionic strength both substituted flavodoxins give a limiting rate constant of 7 s^{-1}, as compared with 236 s^{-1} for *Clostridium* and 62 s^{-1} for *Azotobacter* flavodoxin), whereas the K_a values vary by less than a factor of 2 (De Francesco et al., 1987).

These observations suggest that, although the thermodynamics of complex formation are not substantially different either for the two native flavodoxins or for the flavodoxins after modification of the bound flavin cofactor, the specific structural features of the complexes could vary considerably for these systems, which would be expected to alter the observed rate constants for electron transfer. Since *Azotobacter* flavodoxin is unique among the flavodoxins thus far studies in that it has a disubstituted phosphorus residue covalently linked to the protein (Edmondson & James, 1979), it was of interest to determine whether this moiety might be involved in the formation of a complex and thus influence its geometric properties.

CD[1] spectral perturbations have previously been shown to occur upon complex formation between the redox flavoprotein ferredoxin:NADPH oxidoreductase and either ferredoxin or rubredoxin [cf. Zanetti and Curti (1984) and Przysiecki et al. (1985)]. In this paper, we report the occurrence of large changes in the CD spectral properties of the Soret band of cytochrome *c* upon binding *C. pasteurianum* flavodoxin. This perturbation is substantially decreased in magnitude upon substitution of the FMN moiety with a bulky 8α-substituent or upon binding *Azotobacter* flavodoxin. ^{1}H NMR spectral data also show major differences in the interaction of *Azotobacter* flavodoxin with cytochrome *c* when compared with previous observations using *C. pasteurianum* flavodoxin (Hazzard & Tollin, 1985). ^{31}P NMR experiments show that the resonances due to the FMN phosphate and to the disubstituted, covalently bound, phosphate in *Azotobacter* flavodoxin are relatively unperturbed upon cytochrome *c* binding with respect to chemical shift and to susceptibility to paramagnetic line broadening on the addition of Mn(II). These findings demonstrate that specific structural and geometric features of the complex between flavodoxin and cytochrome *c* are important factors in determining the rate of electron transfer.

Materials and Methods

Materials. *Azotobacter* and *Clostridium* flavodoxins were purified according to published procedures (Hinkson & Bulen, 1967; Mayhew, 1971) and the apoproteins prepared by trichloroacetic acid precipitation as described previously (Edmondson & Tollin, 1971a). Horse heart cytochrome *c* (type VI) was purchased from Sigma Chemical Co.

8α-*N*-Imidazolylriboflavin was synthesized by published procedures (Williamson & Edmondson, 1985) and was phosphorylated to the FMN level according to Flexser and Farkas (1952). The 5′-phosphate isomer was purified by preparative HPLC (R. De Francesco and D. E. Edmondson, manuscript in preparation) before reconstitution with the apoflavodoxin. All CD and ^{1}H NMR spectral experiments were carried out at ambient temperatures in 5 mM phosphate buffer, containing 0.1 mM EDTA, at pH 7.0.

Methods. Circular dichroism spectra were measured on an AVIV 60DS instrument interfaced to an AT&T Model 6300 computer. Quartz cells used include a split cell with two compartments of 0.45-cm path length each or a 0.1-cm path length cell for higher concentrations.

^{1}H NMR spectra were measured by using a Nicolet 360-MHz spectrometer with 5-mm tubes. Samples were lyophilized twice from 99.8% D_2O and dissolved in "100%" D_2O for spectral analysis. Quadrature phase detection was used, and chemical shifts were referenced to an external standard of 0.35% TSP. Instrument settings were 32-kHz spectral width, 32K data points, 3-s recycle time, and 2000–3000 acquisitions.

^{31}P NMR spectra were obtained at 81 MHz on an IBM/Bruker WP-200SY spectrometer. All spectra were measured by using 10-mm NMR tubes from Wilmad, and chemical shifts were determined relative to an external standard of 85% phosphoric acid. Broad-band proton decoupling was employed at low power (1 W) to ensure that no sample heating occurred. Samples were dissolved in 50 mM Tris–acetate, pH 8.0, and were treated with Chelex to remove any contaminating paramagnetic metal ions. The following instrumental settings were used: 2-kHz spectral width, 14.8-μs pulse width (45° flip angle), 4K data points, and 1.024-s acquisition time.

Results

Figure 1 shows the visible CD spectra of *C. pasteurianum* flavodoxin, both native and 8α-*N*-imidazolyl-FMN- (8-IMF) substituted, and horse heart cytochrome *c*, obtained in a low ionic strength medium (5 mM phosphate buffer, pH 7.0). These spectra are in good agreement with previously published results under higher ionic strength conditions (Edmondson & Tollin, 1971b; Myer, 1968) and further show that the environment of the bound 8-IMF is quite similar to that of the bound FMN in the native protein. Analogous results were obtained with the *Azotobacter* protein (data not shown). The similarity between the CD spectra of the native and 8-IMF proteins is in agreement with expectation based on the available X-ray data on flavodoxins (Mayhew & Ludwig, 1975; Ludwig et al., 1982), which show that the 8-position is exposed to solvent. Thus, no major alteration in the flavin–protein interaction would be anticipated upon the addition of a bulky 8α-substituent to the flavin ring.

Figure 2A shows a comparison between the CD spectrum of a 1:1 mole ratio mixture of native *C. pasteurianum* flavodoxin and cytochrome *c* (measured under the same experimental conditions as in Figure 1) and the sum of the individual spectra of the components. It is evident that a large perturbation has occurred upon mixing of the two proteins. That this change is associated with complex formation is shown by a control experiment in which the ionic strength of the medium was increased to 90 mM by addition of KCl. Under these conditions, the electrostatically stabilized complex would be expected to be largely dissociated (Simondsen et al., 1982; Hazzard & Tollin, 1985). This ionic strength increase resulted in the CD spectra of the mixture becoming identical within experimental uncertainty to that of the sum of the individual proteins (data not shown). In Figure 2B is plotted the difference CD, obtained by computer subtraction of the spectra shown in Figure 2A. It is apparent that the major spectral effect occurs at approximately 402 nm (i.e., in the Soret band of the cytochrome heme) and thus reflects a change in the

[1] Abbreviations: CD, circular dichroism; NADPH, reduced nicotinamide adenine dinucleotide phosphate; EDTA, ethylenediaminetetraacetic acid; TSP, sodium 3-(trimethylsilyl)tetradeuteriopropionate; Tris, tris(hydroxymethyl)aminomethane; 8-IMF, 8α-*N*-imidazolyl-FMN; FMN, flavin mononucleotide.

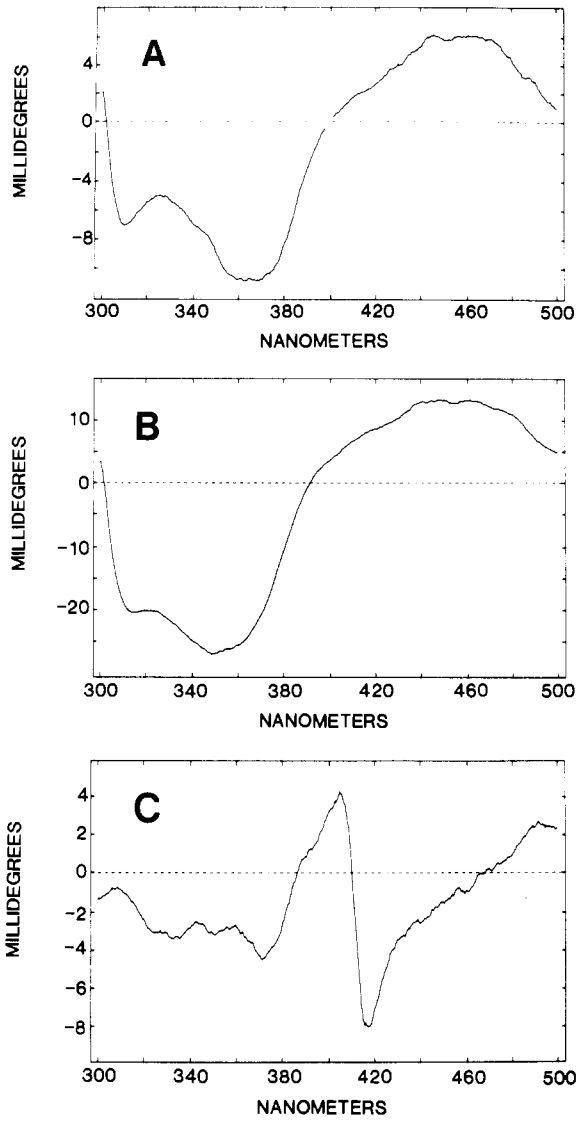

FIGURE 1: CD spectra of flavodoxins and cytochrome *c* in 5 mM phosphate buffer and 0.1 mM EDTA, pH 7.0: (A) native *C. pasteurianum* flavodoxin (50 μM); (B) 8-IMF-substituted *C. pasteurianum* flavodoxin (96 μM); (C) horse heart cytochrome *c* (20 μM).

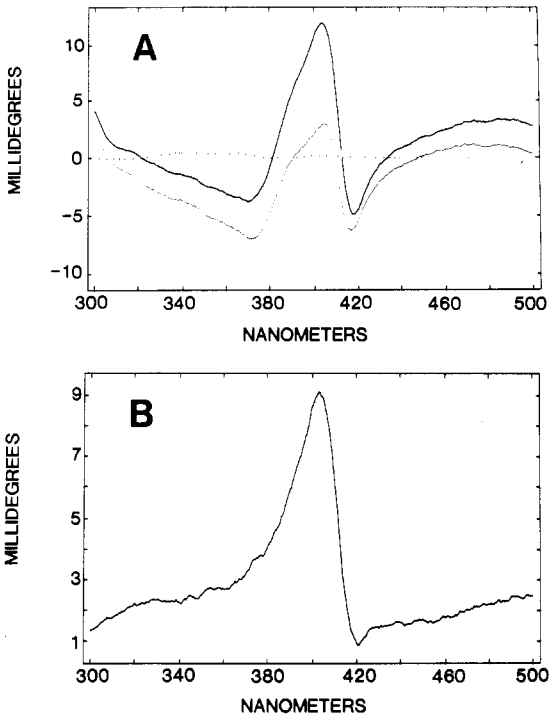

FIGURE 2: CD spectra of *C. pasteurianum* flavodoxin/cytochrome *c* mixtures. Buffer conditions as in Figure 1. Protein concentrations were 33 μM each; cell path length was 0.45 cm. (A) Thin line, sum of individual spectra; thick line, spectrum of 1:1 mixture. (B) Difference spectrum (mixture minus sum).

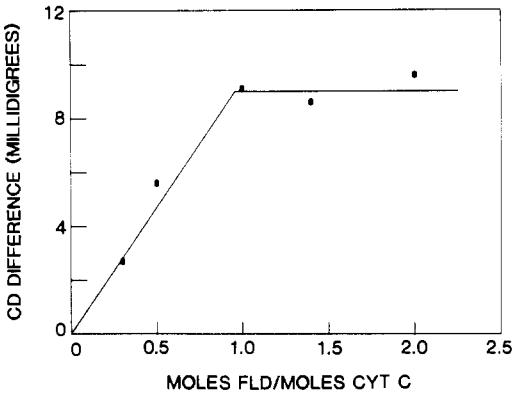

FIGURE 3: Plot of magnitude of CD difference vs. mole ratio of *C. pasteurianum* flavodoxin to cytochrome *c*.

environment of the heme prosthetic group on complex formation with *C. pasteurianum* flavodoxin (see below for further comments).

Figure 3 shows the results of an experiment in which the magnitude of the CD spectral perturbation was measured as a function of the concentration of added *C. pasteurianum* flavodoxin (the cytochrome *c* concentration was kept constant at 27 μM). As is evident, the size of the difference CD signal is proportional to the amount of added flavodoxin, up to a ratio of 1 mol per mole of cytochrome *c*. This supports the above interpretation of the change as being due to 1:1 complex formation between *C. pasteurianum* flavodoxin and cytochrome *c*. The sharp break at a 1:1 mixture is consistent with the value of the kinetically measured equilibrium formation constant, which is 8×10^5 M^{-1} at 50 mM ionic strength (Simondsen & Tollin, 1982) and which should be even larger at the ionic strength used in the CD titration.

Figure 4 demonstrates that a much smaller CD difference spectrum is obtained with *Azotobacter* flavodoxin. That this was not due to a smaller association constant was shown by an experiment in which 215 μM flavodoxin was mixed with 145 μM cytochrome *c* in a 0.1-cm path length cell (i.e., approximately a 5-fold increase in concentration). The difference CD spectrum under these conditions was the same as that in Figure 4B (data not shown).

Previous ^{1}H NMR studies (Hazzard & Tollin, 1985) have shown that complex formation between *Clostridium* flavodoxin and cytochrome *c* results in extensive broadening of the contact-shifted heme, Met-80 and Cys-17 thioether Me resonances. Of particular interest is the observation that some of the resonances were broadened to a much greater extent than others. In view of the presently observed differences in the magnitude of the CD spectral perturbations upon binding of the two flavodoxins to cytochrome *c*, analogous ^{1}H NMR studies were carried out on the *Azotobacter* flavodoxin–cytochrome *c* system. Figure 5 shows the NMR spectra of free cytochrome *c* and of a 1:1 mixture of *Azotobacter* flavodoxin and cytochrome *c*. Only the low-field and high-field resonances are plotted. A broadening of the spectral lines is observed in the mixture, as would be expected simply on the basis of the decreased rotational correlation time (τ_R) of the complex, without the occurrence of any significant changes in chemical shift. The extent of the line broadening is similar in magnitude

Table I: Proton NMR Spectral Band Parameters for Cytochrome *c* Alone and in 1:1 Mixtures with Flavodoxins

cyt *c* alone		1:1 mixtures: *Azotobacter*		1:1 mixtures: *Clostridium*[a]		
δ	$\Delta\nu^b$ (Hz)	δ	$\Delta\nu^b$ (Hz)	δ	$\Delta\nu^b$ (Hz)	assignment
35.5	31	35.4	45	35.2	190	heme 8-Me[c,d]
32.6	39	32.6	52	32.4	160	heme 3-Me[c,d]
19.1	54	19.1	72	19.2	219	heme 7-propionate[c]
14.6	35	14.6	55	14.3	190	heme 7-propionate[d] or His-18 CH[e]
12.7	55	12.7	72	12.7	88	heme meso CH[d]
−2.5	23	−2.5	32	−2.5	*f*	Cys-17 thioether Me[d,g]
−2.8	23	−2.8	31	−2.9	*f*	polypeptide H[g]
−4.3	49	−4.3	65	−4.3	*f*	heme meso CH[d,g]
−6.5	51	−6.5	63	−6.5	*f*	heme meso CH[d,g]
−24.4	102	−24.4	132	−24.2	350	Met-80 Me[c,d]

[a] Values from Hazzard and Tollin (1985). [b] Calculated from the full width at half-band intensity. Values are ±2 Hz. [c] Redfield and Gupta (1971). [d] McDonald and Phillips (1971). [e] Moore and Williams (1984). [f] Broadening too extensive to measure accurately. [g] Keller and Wuthrich (1978).

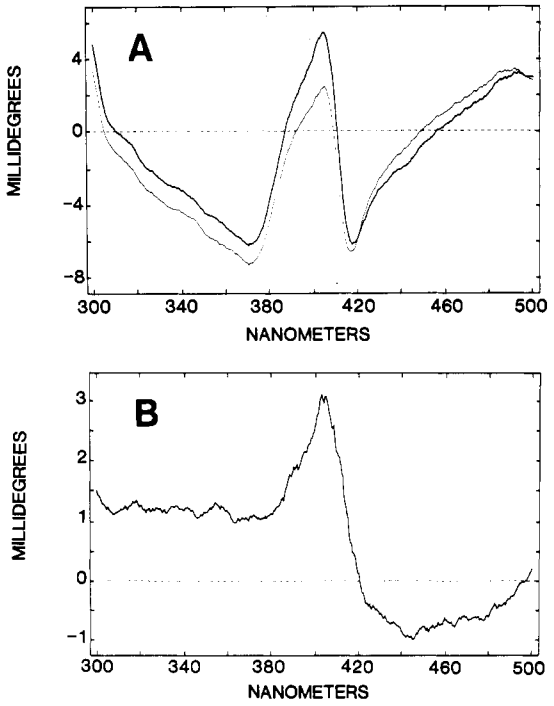

FIGURE 4: CD spectra of *A. vinelandii* flavodoxin/cytochrome *c* mixtures. Buffer conditions as in Figure 1. Protein concentrations were 33 μM each; cell path length was 0.45 cm. (A) Thin line, sum of individual spectra; thick line, spectrum of 1:1 mixture. (B) Difference spectrum (mixture minus sum).

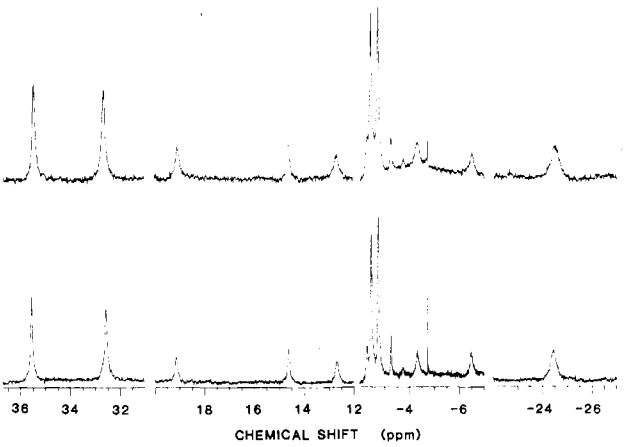

FIGURE 5: 360-MHz ^{1}H NMR spectra of cytochrome *c* and cytochrome *c*/flavodoxin mixtures. Buffer conditions as in Figure 1. (Upper) *A. vinelandii* flavodoxin plus cytochrome *c* (2 mM in each); (lower) cytochrome *c* alone (2 mM).

to that observed upon complexation of cytochrome *c* with cytochrome b_5 (Eley & Moore, 1983).

The spectral parameters measured from these resonance lines are listed in Table I, along with the previously published results obtained with *Clostridium* flavodoxin (Hazzard & Tollin, 1985). In all cases, the extent of the line broadening is substantially smaller for the *Azotobacter* system. This is in spite of the fact that the τ_R of the *Azotobacter* flavodoxin–cytochrome complex should be greater since the molecular weight of *Azotobacter* flavodoxin is larger than that of the *Clostridium* protein (21 vs. 14.5 kDa, respectively). However, it should be kept in mind that in the absence of hydrodynamic information on the complexes the τ_R values cannot be accurately assessed. The observation that no measurable chemical shift changes occur with the *Azotobacter* complex suggests a slower exchange rate relative to that of the *Clostridium* complex (Hazzard & Tollin, 1985), which is in agreement with stopped-flow kinetic data (De Francesco et al., 1987). The fact that four of the cytochrome resonances are broadened extensively by complexation with *Clostridium* flavodoxin, but not with *Azotobacter* flavodoxin, is analogous to the results of the CD experiments. Thus, the alterations in the heme–protein interaction caused by *Clostridium* flavodoxin binding that lead to the CD perturbation could also produce changes in the NMR relaxation properties, resulting in substantial line broadening by a mechanism which is suggested to involve Curie spin relaxation (see Discussion).

Since substitution of the FMN moiety in the flavodoxins by 8-IMF results in a substantial decrease in the electron-transfer rate constants (De Francesco et al., 1987), without a major change in the K_a value, it was of interest to determine whether the large CD perturbation of the heme of cytochrome *c* observed with the native *Clostridium* flavodoxin (cf. Figure 2) is also observed with the 8-IMF-substituted protein. The rationale behind this experiment is as follows. Since *Azotobacter* flavodoxin produces a smaller CD perturbation and also has a slower electron-transfer rate than does *Clostridium* flavodoxin, it is possible that a correlation exists between the extent of heme perturbation upon flavodoxin binding and the kinetics of electron transfer. Figure 6A shows that substitution of the 8α-position of FMN in the *Clostridium* flavodoxin results in the virtual disappearance of the CD spectral perturbation observed with the native protein. That this was not due to a diminished association constant was shown by control experiments at higher protein concentrations (up to 145 μM in each component), in which the same results were obtained (data not shown). Thus, we conclude that specific interactions that occur within the native *Clostridium* flavodoxin–cytochrome *c* complex and which produce alterations in the heme

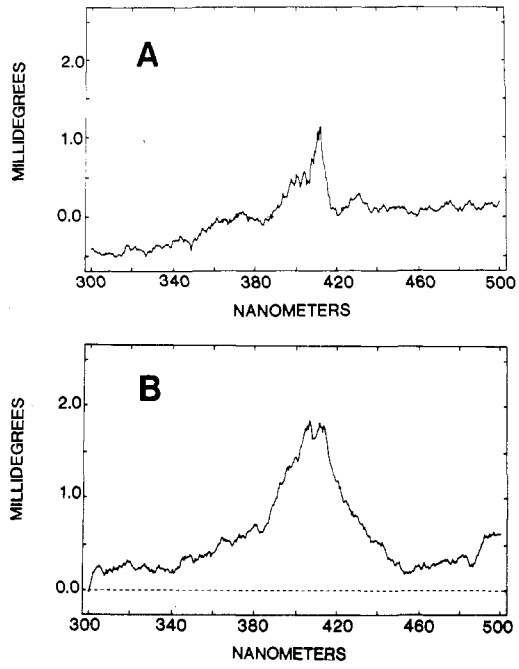

FIGURE 6: Difference CD spectra (mixture minus sum) of cytochrome *c*/8-IMF flavodoxin mixtures. Buffer conditions as in Figure 1. Protein concentrations were 33 μM each; cell path length was 0.45 cm. (A) 8-IMF *C. pasteurianum* flavodoxin; (B) 8-IMF *A. vinelandii* flavodoxin.

environment play an important role in determining the rate constant for electron transfer. On this basis, one would predict that the appreciable heme NMR line broadening observed with the native *Clostridium* flavodoxin would not be found with the 8-IMF-substituted flavodoxin. Unfortunately, lack of sufficient quantities of the latter precluded testing this prediction. In this context, however, it is noteworthy that a similar CD experiment with *Azotobacter* flavodoxin showed a much smaller effect of 8α-substitution on the difference spectrum obtained with the native protein (Figure 6B; compare with Figure 4B). Again, control experiments at higher concentrations showed that this was not due to a decrease in association constant (data not shown).

A unique feature of *Azotobacter* flavodoxin, which is not shown by other flavodoxins examined to date, is the presence of a disubstituted covalently bound phosphorus residue (Edmondson & James, 1979, 1982). This residue has been shown to be on the surface of the protein, whereas the FMN phosphate is "buried" in the interior of the protein, on the basis of paramagnetic line broadening induced by the addition of Mn(II) to protein solutions at concentrations that are $^1/_{10}$ that of the protein (Edmondson & James, 1982). It was thus of interest to determine whether the binding of cytochrome *c* to *Azotobacter* flavodoxin would influence the ^{31}P resonances of either the FMN or the covalent phosphorus group. Figure 7A shows the ^{31}P NMR spectra of *Azotobacter* flavodoxin, free and in the presence of an equal concentration of cytochrome *c* (1.5 mM) at low ionic strength. The resonance at 6.3 ppm is due to the FMN phosphate, and the resonance at 1.3 ppm is due to the covalent phosphate. It should be noted that these samples of flavodoxin did not contain the "labile" phosphate group present in the samples used in the earlier studies (Edmondson & James, 1979, 1982). The observed chemical shift of the FMN resonances is in good agreement with a recently published value (Vervoort et al., 1986), whereas our value for the covalent phosphate is 0.4 ppm downfield. No alterations in chemical shift are observed upon cytochrome binding (Figure 7A), and thus the magnetic environments of the two

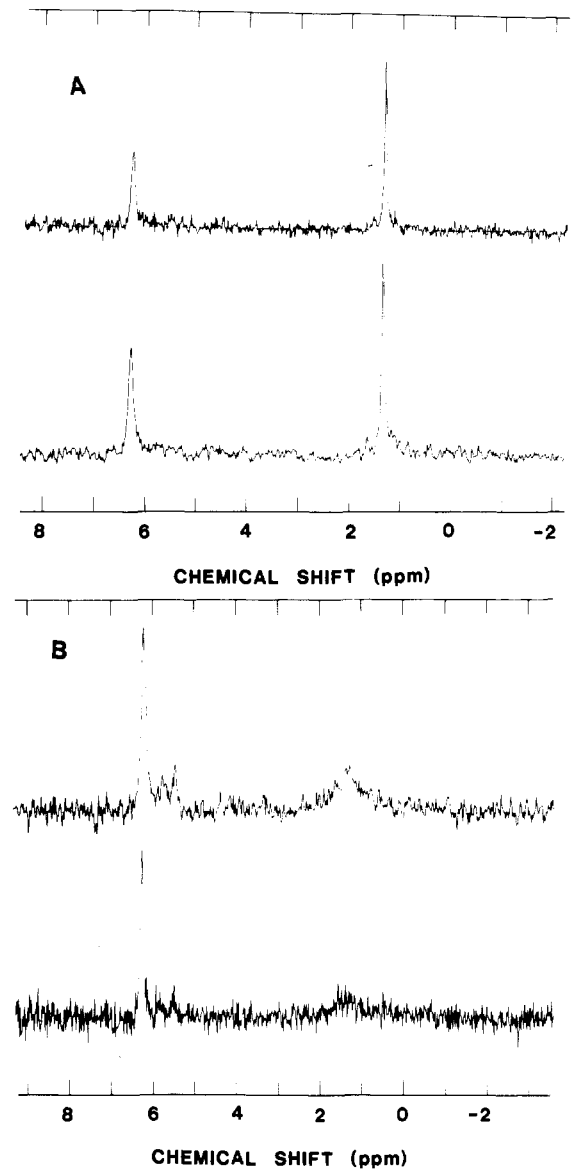

FIGURE 7: ^{31}P NMR spectra of *Azotobacter* flavodoxin (2 mM). (A) Bottom spectrum, no additions; top spectrum, in the presence of 2 mM cytochrome *c*. (B) Bottom spectrum, in the presence of 0.2 mM $MnCl_2$; top spectrum, in the presence of 2 mM cytochrome *c* and 0.2 mM $MnCl_2$. An exponentional line-broadening parameter of 1 Hz was applied before Fourier transformation of each spectrum. See Materials and Methods for instrumental settings.

residues are unperturbed by the paramagnetic heme group. However, the line widths of the two resonances are broadened (by 1.5 Hz for the FMN and by 1.9 Hz for the covalent phosphate, which is in the range expected for an increase in τ_R upon complex formation).

If the covalent phosphorus group were involved in the binding interface between the two proteins, one might expect to observe a differential line-broadening effect on the addition of Mn(II) to free and bound flavodoxin. The results shown in Figure 7B demonstrate that extensive broadening of the covalent phosphate resonance is obtained for both free and complexed *Azotobacter* flavodoxin. Although the effect appears to be larger for the free flavodoxin, this is probably the result of a decreased amount of free Mn(II) in solution as a consequence of nonspecific binding to the cytochrome *c*.

Only a small broadening of the FMN resonance of the free flavodoxin (2 Hz) is observed on Mn(II) addition. This is decreased to 0.9 Hz in the flavodoxin–cytochrome complex, thus indicating a small paramagnetic contribution to the line

width of the buried FMN resonance. These data show that there are no major alterations in the environment of the FMN phosphate group upon *Azotobacter* flavodoxin binding to cytochrome *c*.

^{31}P NMR studies on *Clostridium* flavodoxin (data not shown) show no effect of cytochrome *c* binding on the chemical shift of the FMN phosphate (the only phosphorus group present in this protein). These data suggest that no significant alteration in the FMN conformation occurs on complex formation. The FMN phosphate resonance is weakly influenced by the paramagnetic heme moiety since the line width of the FMN phosphorus resonance ($\Delta\nu_{1/2}$ = 4.9 Hz) is *decreased* by ~0.5 Hz on cytochrome *c* binding rather than the increase of 1.5 Hz observed for the FMN phosphorus resonance on binding cytochrome *c* to *Azotobacter* flavodoxin. These results again demonstrate the differences in binding geometries of the two flavodoxins with cytochrome *c* and suggest that in the *Clostridium* flavodoxin–cytochrome *c* complex the flavin–heme distance is smaller than it is in the *Azotobacter* flavodoxin–cytochrome *c* complex.

Discussion

Protein–protein complexation as a required step prior to electron transfer is a well-known phenomenon in the field of biological oxidation–reduction processes. Detailed spectroscopic studies of the complexes formed between various flavodoxins and cytochromes can facilitate the attainment of a better understanding of the factors involved in determining the rates of electron transfer within such intermediates, inasmuch as extensive information is available on the reaction kinetics of these model systems and a large body of structural information exists as well. In the accompanying paper (De Francesco et al., 1987), it was shown that *Clostridium* and *Azotobacter* flavodoxins exhibit quite different rates of electron transfer (opposite to that expected on the basis of oxidation–reduction potential), even though the equilibrium constants for complex formation are quite similar. These results can only be explained by differences in the structural and geometric features of the intermediate complexes that modulate the electron-transfer rates.

The results presented in the present paper demonstrate that appreciable changes in the CD spectrum in the Soret region of the heme of cytochrome *c* result from the binding of *Clostridium* flavodoxin, but not from binding either *Azotobacter* flavodoxin or an 8α-substituted FMN analogue of *Clostridium* flavodoxin. Furthermore, the magnitude of these changes appears to be correlated with the rate of electron transfer. It is thus of some interest to ascertain whether any specific structural changes can be associated with these spectral effects. From this point of view, it is quite striking that CD spectral perturbations very similar to those seen here have been observed with horse heart cytochrome *c* (Myer, 1968; Myer et al., 1980; Aviram et al., 1981) upon urea treatment, thermal denaturation, and stepwise maleylation of the lysine residues. These spectral effects have been interpreted as resulting from a decrease in the negative dichroism associated with transitions at 440, 417, and 372 nm, caused by uncoupling of the dipole interactions between segments of the polypeptide chain and the heme chromophore upon loosening of the heme crevice. It seems quite reasonable then to conclude that a similar opening of the heme binding site is occurring upon complex formation with native *Clostridium* flavodoxin.

The observed differences in the ^{1}H NMR spectral perturbations of the contact-shifted heme resonances between the cytochrome *c* complexes of *Clostridium* and *Azotobacter* flavodoxins (Table I) are completely consistent with the conclusions reached from the CD spectral studies. Thus, the extensive broadening caused by *Clostridium* flavodoxin binding (Hazzard & Tollin, 1985) can be ascribed to a perturbation of the heme environment that apparently does not occur upon *Azotobacter* flavodoxin binding. Since the extent of the broadening is much greater than that predicted simply from an increase in τ_R, another relaxation mechanism must be dominant. A reasonable possibility is the phenomenon of Curie spin relaxation, which is directly applicable to hemoproteins (Gueron, 1975). Briefly stated, this relaxation process involves either the dipolar or scalar interaction of a nuclear spin with the thermal average of the electron spin (Curie spin). Modulation of this interaction can occur by molecular motion, which involves the rotational correlation time of the complex for dipolar relaxation and internal molecular motion for scalar relaxation. Since the heme resonances are broadened unequally (Table I), this suggests that internal motions are dominating the relaxation process for at least some of the lines. The opening of the heme crevice, which is indicated by the CD spectral effects, would reasonably lead to the types of nuclear motions required for the Curie spin relaxation mechanism. Further NMR studies are necessary in order to substantiate these conclusions and to provide more quantitative information about the changes in the motional properties of the heme.

The question of what kinds of alterations in the heme environment might act to increase the rate of electron transfer can only be addressed in general terms at present. Recent studies (Tollin et al., 1986) have suggested that the exposed region of the heme of *c*-type cytochromes in the vicinity of ring C, especially the sulfur of the thioether bridge, may play a direct role in electron transfer. Thus, changes in the spin density distribution in this area could affect redox rates. However, changes in spin density do not appear to be occurring upon *Clostridium* flavodoxin binding to cytochrome *c*, as evidenced by the lack of appreciable changes in the chemical shift values of the contact-shifted resonances. On the other hand, it is noteworthy that the resonances associated with the thioether bridge methyl group and two mesocarbon protons (one of which could be exposed at the surface around ring C) show much more extensive broadening than other heme proton resonances (cf. Table I). On this basis, it would appear that the most likely explanation for the increased rate of electron transfer for *Clostridium* flavodoxin as compared to that of *Azotobacter* flavodoxin is that the opening of the heme crevice increases the exposure of the ring C region of the heme, which allows the exposed dimethylbenzene ring of the FMN cofactor of flavodoxin to approach more closely to the heme edge than would otherwise be possible. We estimate a distance of 7.7 Å between the 8-methyl position and the 5′-phosphate of the bound FMN of *Clostridium* flavodoxin from computer graphics analysis of the 3-dimensional structure; thus, the ^{31}P NMR data are also consistent with the view that binding of *Clostridium* flavodoxin to cytochrome *c* results in a perturbation of the heme crevice, which allows a closer proximity of the benzenoid portion of the FMN to the heme. In contrast, the smaller spectral perturbations observed with either *Azotobacter* flavodoxin or 8-IMF-substituted *Clostridium* flavodoxin indicate less structural rearrangement of the heme crevice and thus presumably a larger average distance between the flavin and heme prosthetic groups, resulting in a slower rate of electron transfer.

Inasmuch as the crystal structure of *Azotobacter* flavodoxin has not been determined, it is difficult at this time to specify the nature of the structural differences between *Azotobacter*

and *Clostridium* flavodoxins that result in the differing abilities of these two proteins to perturb the heme binding site of cytochrome *c* upon complexation. However, it is interesting to note that Drummond (1986), on the basis of sequence alignment with the structurally characterized *Anacystis nidulans* flavodoxin, has suggested the presence of a positively charged helix near the flavin binding site and the occurrence of Tyr and Leu residues flanking the isoalloxazine ring (as opposed to Trp and Met in *Clostridium* flavodoxin). The extent to which these apparent differences have functional significance will have to await further study.

The possibility exists that binding-site changes of the kind described here are a *necessary* concomitant of effective electron transfer within protein–protein complexes. Other examples of this do in fact exist. For both cytochrome *c* oxidase (Ahmad et al., 1982; Bickar et al., 1985) and cytochrome *c* peroxidase (Hazzard et al., 1987), reduction by small electron donors is greatly facilitated by complexation with cytochrome *c*. In the latter case, Poulos and Finzel (1984) have proposed that complex formation involves the formation of an effective electron-transfer pathway from the surface of the peroxidase molecule to its buried heme prosthetic group, which does not exist in the free peroxidase. If such structural modifications accompanying protein–protein interacts are found to be of more general occurrence, they may represent an important modus operandi of biological electron-transfer systems. This is worthy of further investigation.

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