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Evidence for a Ternary Complex Formed between Flavodoxin and Cytochrome c_3 : $^1\text{H-NMR}$ and Molecular Modeling Studies[†]

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Received July 19, 1993; Revised Manuscript Received September 29, 1993*

ABSTRACT: Small electron-transfer proteins such as flavodoxin (16 kDa) and the tetraheme cytochrome c_3 (13 kDa) have been used to mimic, *in vitro*, part of the complex electron-transfer chain operating between substrate electron donors and respiratory electron acceptors, in sulfate-reducing bacteria (*Desulfovibrio* species). The nature and properties of the complex formed between these proteins are revealed by $^1\text{H-NMR}$ and molecular modeling approaches. Our previous study with the *Desulfovibrio vulgaris* proteins [Moura, I., Moura, J. J. G., Santos, M. H., & Xavier, A. V. (1980) *Cienc. Biol. (Portugal)* 5, 195–197; Stewart, D. E., LeGall, J., Moura, I., Moura, J. J. G., Peck, H. D., Jr., Xavier, A. V., Weiner, P. K., & Wampler, J. E. (1988) *Biochemistry* 27, 2444–2450] indicated that the complex between cytochrome c_3 and flavodoxin could be monitored by changes in the NMR signals of the heme methyl groups of the cytochrome and that the electrostatic surface charge (Coulomb's law) on the two proteins favored interaction between one unique heme of the cytochrome with flavodoxin. If the interaction is indeed driven by the electrostatic complementarity between the acidic flavodoxin and a unique positive region of the cytochrome c_3 , other homologous proteins from these two families of proteins might be expected to interact similarly. In this study, three homologous *Desulfovibrio* cytochromes c_3 were used, which show a remarkable variation in their individual isoelectric points (ranging from 5.5 to 9.5). On the basis of data obtained from protein–protein titrations followed at specific proton NMR signals (*i.e.*, heme methyl resonances), a binding model for this complex has been developed with evaluation of stoichiometry and binding constants. This binding model involves one site on the cytochromes c_3 and two sites on the flavodoxin, with formation of a ternary complex at saturation. In order to understand the potential chemical form of the binding model, a structural model for the hypothetical ternary complex, formed between one molecule of *Desulfovibrio salexigens* flavodoxin and two molecules of cytochrome c_3 , is proposed. These molecular models of the complexes were constructed on the basis of complementarity of Coulombic electrostatic surface potentials, using the available X-ray structures of the isolated proteins and, when required, model structures (*D. salexigens* flavodoxin and *Desulfovibrio desulfuricans* ATCC 27774 cytochrome c_3) predicted by homology modeling.

Long-range electron-transfer reactions are critical for biological energy conservation. In sulfate-reducing bacteria (SRB),¹ this function is partially accomplished by low molecular weight electron transport proteins, like cytochromes, ferredoxin, rubredoxin, and flavodoxin, for which a large amount of information is available concerning both structural and functional aspects.

The formation of a specific complex between the interacting electron carriers is believed to be an important step in the process of electron transfer. A significant effort has been put on the study and characterization of the nature and properties

of several electron-transfer complexes: cytochrome c and cytochrome b_5 (Salemme et al., 1976; Stonehuerer et al., 1979; Mauk et al., 1982; Eley & Moore, 1983; Wendoloski et al., 1987; Nothrup et al., 1987, 1988, 1993; Burch et al., 1990; Whitford et al., 1990; Eltis et al., 1991; Willie et al., 1992), cytochrome c with cytochrome c peroxidase (Poulos & Kraut, 1980; Pelletier & Kraut, 1992), cytochrome c with flavodoxin (Simonsen et al., 1982; Simonsen & Tollin, 1983; Tollin et al., 1984, 1987; Weber & Tollin, 1985; Dickerson et al., 1985; Hazzard et al., 1986), cytochrome c with plastocyanin (Zhou & Kostic, 1992), hydrogenase with cytochrome c_3 , rubredoxin, ferredoxin, and flavodoxin (Bell et al., 1978), cytochrome c_3 with ferredoxin (Moura et al., 1977; Xavier & Moura, 1978; Xavier et al., 1979; Guerlesquin et al., 1984, 1985, 1987; Capeillère-Blandin et al., 1986; Cambillau et al., 1988; Park et al., 1991), cytochrome c_3 with rubredoxin (Moura et al., 1980a; Stewart et al., 1989), cytochrome c_3 with flavodoxin (Moura et al., 1980a; Stewart et al., 1988), and a macro inorganic anion (Mus-Veteau et al., 1992).

A number of experimental approaches have been used to study these protein complexes, such as kinetics experiments of electron transfer, chemical and genetic modifications of specific amino acids, and cross-linking and spectroscopic experiments (NMR, EPR, CD). However, except for the

* The authors acknowledge support received from JNICT, NATO PO FOOD, NATO CRG, NATO Fellowship, NIH, and Belgium Fund for Joint Basic Research (Contract 2.0018.91).

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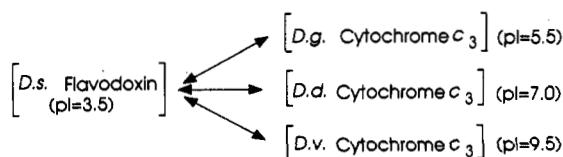
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^{*} Abstract published in *Advance ACS Abstracts*, May 1, 1994.

[†] Abbreviations: *D.*, *Desulfovibrio*; SRB, sulfate-reducing bacteria; FMN, flavin mononucleotide; cyt, cytochrome; $^1\text{H-NMR}$, proton nuclear magnetic resonance spectroscopy; EPR, electron paramagnetic resonance spectroscopy; CD, circular dichroism.

Scheme 1



photosynthetic redox center (Deisenhofer et al., 1984, 1985) and for the cytochrome *c* peroxidase–cytochrome *c* complex (Pelletier & Kraut, 1992), no three-dimensional structures of biological electron-transfer complexes have yet been elucidated by X-ray diffraction. However, computational chemistry and computational methods have aided this effort by allowing plausible models for protein–protein complexes to be built. Indeed, one outcome of these efforts has been the simulation of the dynamics of interactions in three of these cases: the cytochrome *c* and cytochrome *b*₅ interaction (Wendoloski et al., 1987), the cytochrome *c*₃ and rubredoxin interaction (Stewart et al., 1991), and the cytochrome *c*₃ and flavodoxin interaction (D. E. Stewart and J. E. Wampler, unpublished results).

This theoretical approach not only has allowed the explanation of several experimental results but has given as well an important contribution to the elucidation of some general rules that apply to the formation of complexes between most of these electron-transfer partners. In many cases, the observed dipole moment resulting from the asymmetrical distribution of its charged amino acids seems to facilitate the correct orientation of the redox functional groups (Northrup et al., 1987, 1988), in a step prior to protein complexation. For instance, it seems to be a common scheme for the interactions between *c*-type cytochromes and their redox partners that these complexes are mainly driven by electrostatic interactions between positively charged lysine and arginine residues surrounding the heme crevice and acidic residues surrounding the redox group of their partner (Stewart et al., 1988, 1989).

In this work, we extended the previous results (Stewart et al., 1988) on the interaction between cytochrome *c*₃ and flavodoxin from the sulfate-reducing bacteria of the genus *Desulfovibrio*. Since electrostatic interactions are believed to be an important driving force for the formation of complexes between these classes of redox proteins, we studied, in a comparative way (Scheme 1), the interaction between the acidic flavodoxin isolated from *Desulfovibrio salexigens* (isoelectric point around 3.5) and each of the cytochromes *c*₃ purified from *Desulfovibrio vulgaris* Hildenborough (*pI* = 9.5), *Desulfovibrio desulfuricans* ATCC 27774 (*pI* = 7.0), and *Desulfovibrio gigas* NCIB 9332 (*pI* = 5.5).

¹H-NMR and molecular modeling approaches were employed to probe and characterize the different flavodoxin–cytochrome *c*₃ complexes and to develop plausible stoichiometry and structural models of them. X-ray-determined atomic coordinates are available for some of the studied proteins, but when required, homology modeling was used to predict some of the needed structures (*D. salexigens* flavodoxin and *D. desulfuricans* ATCC 27774 cytochrome *c*₃).

Cytochrome *c*₃ is a small (*M_r*, ca. 13 kDa) monomeric and soluble protein located in the periplasmic side of the cell membrane (LeGall et al., 1965; Badziong & Thauer, 1980). This cytochrome is characterized by the presence of four hemes of low redox potentials inserted in a relatively short polypeptide chain of about 110 residues. Although each heme is covalently bound to cysteine residues in a characteristic arrangement of the type -Cys-x-x-Cys-His- or -Cys-x-x-x-Cys-His- and axially coordinated by two histidine residues, they present different solvent exposure and are surrounded by distinct

protein environments, as shown by spectroscopic and X-ray data. Despite the low sequence homology found among the several sequenced cytochromes *c*₃ (about 30%), the X-ray structures, now available for the proteins isolated from *D. vulgaris* Miyazaki F (Higushi et al., 1984), *D. gigas* (Sieker et al., 1986; Kissinger, 1989), and *D. baculatus* Norway 4² (Haser et al., 1979; Pierrot et al., 1982), reveal surprisingly conserved secondary and tertiary structures (60–90%). Recently, this last structure was reevaluated by 2D-NMR methods and a correction to the heme orientations was proposed (Coutinho et al., 1992). The X-ray structure of the *D. desulfuricans* ATCC 27774 cytochrome *c*₃ is also in progress (C. Frazão et al., manuscript in preparation).

Physiologically, the cytochrome *c*₃ is considered to mediate the electron transfer between hydrogenase and other electron carriers, like ferredoxin (Bell et al., 1978) in the sulfate-reducing pathway and the phosphorolytic reaction (Akaji, 1967; Suh & Akaji, 1969). It seems also to have a stimulatory effect in the reduction of sulfite (Postgate, 1956) and thiosulfate (LeGall, 1967).

Flavodoxin, on the other hand, is a small flavoprotein (*M_r*, ca. 16 kDa) containing an FMN group as the redox cofactor. Flavodoxin is not present in all *Desulfovibrio* species (LeGall & Hatchikian, 1967; Dubourdieu & LeGall, 1970; Fauque et al., 1987), and in some cases it is synthesized only under iron deficiency, where it can substitute ferredoxin (Knight & Hardy, 1966). This interfunctionality was also observed in the reactions of production and utilization of hydrogen (LeGall & Hatchikian, 1967; LeGall et al., 1979, 1982). The surprising similarity of the redox potentials of flavodoxin and ferredoxins I and II from *D. gigas* is consistent with the suggested interfunctionality between these two electron-transfer proteins (Peck & LeGall, 1982; Moura et al., 1978; Fauque et al., 1991).

In *D. vulgaris* Hildenborough, flavodoxin seems to be constitutive and is found in the cytoplasmic fraction (Dubourdieu & LeGall, 1970; LeGall & Peck, 1987). Flavodoxin has been characterized and sequenced from a number of species, such as *D. vulgaris* Hildenborough (Dubourdieu et al., 1973), *D. salexigens* (Moura et al., 1980c; Helms et al., 1990), *D. gigas* (ATCC 29464 and ATCC 19364) (Helms & Swenson, 1992), *Clostridium MP* (Tanaka et al., 1974a), *Chondrus crispus* (Wakabayashi et al., 1989), *Megasphaera elsdenii* (Tanaka et al., 1973, 1974b), and *D. desulfuricans* (ATCC 27774) (Caldeira et al., manuscript in preparation). The three-dimensional structures have been determined for flavodoxins from *D. vulgaris* Hildenborough [X-ray (Watenpaugh et al., 1972, 1973) and multidimensional NMR (Peelen & Vervoort, 1992; Lohr et al., 1992)], *Clostridium MP* (X-ray) (Andersen et al., 1972; Burnett et al., 1974; Ludwic et al., 1976), *Megasphaera elsdenii* (2D NMR) (Mierlo et al., 1990a,b), *Anacystis nidulans* (X-ray) (Smith et al., 1983; Ludwig et al., 1984; Laudenbach et al., 1987), and *Chondrus crispus* (X-ray) (Fukuyama et al., 1989, 1990). The crystallization of the protein from *D. desulfuricans* ATCC 27774 is also in progress (M. J. Romão and A. Romero, unpublished results).

The formation of a complex between cytochrome *c*₃ and flavodoxin may not be of physiological significance, due to their different compartmentalization in the cell. However, it is known that these two proteins can interact and form a specific complex *in vitro* (Moura et al., 1977; Bell et al., 1978) and transfer electrons within them (Moura et al., 1977; Barata et

² *Desulfovibrio* (*D.*) *desulfuricans* Norway 4 has been renamed *D. baculatus* Norway 4 and recently reclassified as *Desulfomicrobium* (*Dm.*) *baculatus* Norway 4 (Devreux et al., 1990).

al., 1992). Therefore, the aim of this study is to gain further insight into the understanding of the mechanism of electron transfer between heme and flavin groups in general and into the particular role of the individual hemes of such multi-heme cytochromes. As a consequence of these studies, some information has also been revealed pertinent to the general understanding of the relevant rules of protein recognition and complex formation.

METHODS

NMR Titrations. Flavodoxin and tetraheme cytochromes c_3 were purified by previously reported methods (Moura et al., 1980c; Liu et al., 1988; LeGall et al., 1965; DerVartanian & LeGall, 1974). The cytochromes were extensively dialyzed against distilled water at 4 °C and then lyophilized three times and redissolved in a small amount of D₂O. Flavodoxin was dialyzed against H₂O, lyophilized just once to avoid loss of FMN, and washed extensively against D₂O. The pH of the solutions was adjusted to 7.5 with NaOD and DCl. Protein concentrations were determined from the corresponding extinction coefficients.

Aliquots of the interacting protein (flavodoxin) were added to the NMR tube containing a solution of cytochrome c_3 , increasing the molar ratio of [flavodoxin]/[cytochrome c_3] in small steps until a convenient value. The ¹H-NMR spectra were obtained at 298 K on a CXP-300 Bruker NMR spectrometer equipped with an ASPECT 2000 computer. Chemical shifts are quoted in parts per million (ppm) from the methyl resonance of sodium 3-trimethylsilyl[2,2,3,3-²H₄]-propionate and the positive values referred to low-field shifts.

The chemical shifts of selected resonances were recorded and plotted (relative to the initial values, corresponding to the pure cytochrome c_3 solution) against the protein/protein molar ratio. Several models of the interaction equilibrium were developed and tested for their capacity of fitting the NMR data obtained (see below).

Binding Models and Fitting. The chemical shifts of selected resonances were tabulated versus the concentrations of both protein species and their molar ratio. For convenience, plots show the absolute shifts versus the protein/protein molar ratio. However, binding isotherms were fit with absolute concentrations of both protein species explicitly represented. Several binding models for the interaction equilibrium were developed and tested by nonlinear least squares fitting, including the following: (1) one site on each of the two proteins (1:1 model) with or without a variable degree of effect on the chemical shifts of individual protons; (2) two sites on one of the proteins and one site on the other (1:2 model), again with equal or variable effects on the individual chemical shifts.

In case 2, three binding models were tested, considering (i) independent equivalent sites, (ii) independent nonequivalent sites, and (iii) dependent nonequivalent sites. In each case, the full set of binding equilibria was represented mathematically along with chemical shift and mass conservation equations. For example, consider the simplest model (1:1 model); the pertinent equations would be

$$K_a = [\text{complex}] / [\text{flavodoxin}][\text{cytochrome}]$$

$$[\text{flavodoxin}]_{\text{initial}} = [\text{flavodoxin}] + [\text{complex}]$$

$$[\text{cytochrome}]_{\text{initial}} = [\text{cytochrome}] + [\text{complex}]$$

$$\Delta\delta_i = x\Delta\delta_{i,\max}$$

where the change in chemical shift of any resonance i ($\Delta\delta_i$)

is related to the molar fraction (x) of bound cytochrome molecules and to the chemical shift difference at saturation ($\Delta\delta_{i,\max}$).

Analysis of the model involves nonlinear least squares fitting where the knowns are the chemical shift changes ($\Delta\delta_i$) and the initial concentrations. The fit variables are the K_a and the maximum chemical shift differences ($\Delta\delta_{i,\max}$). The fits were carried out globally, i.e., fitting all data sets (different methyl resonances in each titration) simultaneously, generating one set of best fit values.

In the case of 1:2 models, i.e., where two cytochrome c_3 molecules bind to two distinct sites on flavodoxin, the change in chemical shift of each methyl group was considered to be composed of two components, one for the effect of each of the flavodoxin sites on that resonance, according to the equation

$$\Delta\delta_i = (x_\alpha f_{i,\alpha} + x_\beta f_{i,\beta})\Delta\delta_{i,\max} \quad (1)$$

where x_α and x_β are the molar fractions of cytochrome molecules bound to the flavodoxin interaction sites α and β , respectively, $f_{i,\alpha}$ and $f_{i,\beta}$ are the chemical shift sensitivity coefficients of cytochrome's resonance i , for sites α and β , of flavodoxin, and $\Delta\delta_{i,\max}$ is the chemical shift difference at saturation. Obviously, this representation for the chemical shift difference has three parameters where only two are needed, and the parameters, themselves, are interdependent. The purpose of this representation is to define the relative sensitivity of each resonance to the two flavodoxin sites. Thus, the interdependency of the three parameters can be resolved by normalizing the sensitivity parameters.

Molecular Modeling. Molecular graphics and force field calculations were performed on a Personal Iris-Silicon Graphics workstation, running the operating system UNIX and the molecular modeling software SYBYL v. 5.3 to 5.5 (Tripos Associates) and AMBER (Weiner & Kollman, 1981).

Atomic coordinates of flavodoxin (*D. vulgaris* Hildenborough) and of cytochromes c_3 (*D. vulgaris* Miyazaki F and *D. baculatus* Norway 4) were obtained from the Brookhaven Protein Data Bank at resolutions of 0.2, 0.18 and 0.25 nm, respectively. The coordinates of the cytochrome c_3 of *D. gigas* (0.25-nm resolution) were a courtesy of L. C. Sieker (University of Washington, Seattle).

Prediction of Three-Dimensional Structures by Homology Modeling. Homology modeling is based on the observation that functionally related proteins usually share the same class of tertiary structures, in particular, around functional domains (Browne et al., 1969; Sweet, 1986; Lesk et al., 1986). In this way, the primary structure of the target protein was first aligned with the sequence of a selected homologous protein, which served as a starting structural model. Once aligned, all the necessary insertions and deletions were made, and all the nonconserved residues were conveniently substituted in the structure, in order to superimpose the sequence of the target protein onto the tertiary structure of the model one. In this process, only the side-chain atoms were changed without changing the coordinates of the main-chain atoms. The orientation of the side chains relative to the α -carbons was also maintained.

The mutated structure was finally refined by energy minimization, using the AMBER force field with all hydrogen atoms explicit. These energy minimization calculations were done in four steps, as proposed before (Stewart et al., 1987): first, a strong constrain was imposed to the main-chain atoms, allowing the accommodation of side chains under a nonelectrostatic force field (i.e., not including electrostatic interactions). This step allows a soft reorientation of the side chains, relieving major steric conflicts introduced by the amino acid

substitutions. In the second step, the calculations were repeated with the same constraints, but including electrostatics, and finally, the third and fourth steps were done in the same way as the two first steps, but without any constraint applied, i.e., allowing all atoms to accommodate.

We have built predictive model structures of the *D. salexigens* flavodoxin and *D. desulfuricans* ATCC 27774 cytochrome *c*₃. The *D. salexigens* flavodoxin model was based on the known X-ray structure of its *D. vulgaris* Hildenborough homologous protein, for which the sequence homology is 55%. We have used the sequence alignment as proposed by Helms et al. (1990). A model for the *D. desulfuricans* cytochrome *c*₃ was generated using its amino acid sequence, recently determined by J. Van Beeumen (University of Ghent, unpublished results), and the structure of the *D. vulgaris* Miyazaki F protein as a template.

Surface Electrostatic Potential. The solvent-accessible surface of the protein was defined by the "tracing" of the center of a spherically ($r = 0.14$ nm) idealized solvent molecule over the van der Waals shell of the surface atoms. The electrostatic potential (V) was calculated over the solvent-accessible surface of the protein, considered *in vacuo*, with a cutoff radius of 2.0 nm and a distance-dependent dielectric constant.

Protein Docking. Model building of the complex between flavodoxin and cytochrome *c*₃ was done on the basis of computational and interactive computer graphics methods. In order to elect possible docking surfaces and complex conformations, the docked proteins were interactively (manually) translated and rotated around each other, while the interaction energy was monitored using the AMBER molecular mechanics force field. Additionally, four main criteria were applied, in order to reduce the searching space for docking surfaces: (i) the stoichiometry of the complex was as suggested by the NMR experimental data; (ii) in order to generate a physical model consistent with the observed electron transfer between the two proteins, a preferential model of the complex predicts a suitable proximity and relative orientation of the redox groups of both proteins. The interacting molecules were independently rotated and translated in an interactive way, in order to (iii) maximize the number of favorable electrostatic interactions between charged groups on the surface of each protein and to (iv) maximize topological complementary and eliminate steric conflicts between the interacting surfaces. Each eligible model was finally subjected to energy minimization *in vacuo* to optimize distances and angles.

RESULTS

NMR Protein Titrations. Figure 1a shows the low-field region of the ¹H-NMR spectrum of *D. gigas* ferricytochrome *c*₃, exhibiting a number of well-resolved signals, with chemical shifts quite distinct from the ones in the protein's main envelope. These resonances, which are not seen in the spectra of the reduced cytochrome, are due to protons located in the vicinity of the heme paramagnetic irons, and some of them have been assigned to the heme methyl groups, based on uni- and bidimensional NMR studies (Moura et al., 1977, 1980a,b, 1982; Xavier & Moura, 1978; Santos et al., 1984; Guerlesquin et al., 1985a; Fan et al., 1990). These resonances have been shown to serve as excellent intrinsic probes for the structure and function studies of cytochromes, revealing subtle changes in the chemical environment of the corresponding protons. In particular, they have been used for monitoring the formation of protein complexes involving cytochrome *c*₃ (Moura et al., 1977, 1980a; Guerlesquin et al., 1985b; Stewart et al., 1988, 1989; Park et al., 1991; Mus-Veteau et al., 1992).

Figure 1b shows the effect of increasing the [flavodoxin]/[cytochrome *c*₃] molar ratio on the low-field region of the spectrum of *D. gigas* cytochrome *c*₃, revealing progressive modifications of the chemical shift of selected resonances. This effect is better illustrated in Figure 2, where the changes in the chemical shifts of the affected resonances are plotted as a function of the ratio of *D. salexigens* flavodoxin and cytochrome *c*₃ for titrations involving three different cytochromes. Note that, in Figure 1, there is a progressive loss in intensity of the resonances and a slight increase in line width along the titration. These effects are due to an unspecific dilution effect and to the increase of the tumbling time of cytochrome *c*₃ upon complex formation with flavodoxin. Moreover, the shift of the resonances, without a change of the corresponding integrated areas, indicates that this complex is involved in a relatively fast equilibrium exchange with the uncomplexed proteins.

Without a full assignment of the affected methyl resonances to the corresponding hemes in the structure of the cytochromes, it is not possible to unequivocally identify the interaction surfaces. However, the fact that, in *D. desulfuricans* cytochrome *c*₃, at least six methyl resonances are affected by the binding of flavodoxin suggests that more than one heme group may be close enough to the interacting surface. The same conclusion can be drawn from the titration of *D. gigas* cytochrome *c*₃, for which it is known that methyl resonances 5 and 9 belong to the same heme group, while methyl resonances 4 and 6 are assigned to other hemes (Moura et al., 1980a; Santos et al., 1984; J. J. G. Moura, unpublished results). Two different hypotheses must be considered: (i) cytochrome *c*₃ may have more than one interaction site with affinity for flavodoxin or (ii) the cytochrome binds to the flavoprotein through one specific interaction site, suffering local conformational changes that would affect, to different extents, methyl resonances belonging to more than one adjacent heme.

It should be noted that in all the reported studies of the interactions between cytochrome *c*₃ and different acidic redox partners, such as ferredoxin, rubredoxin, flavodoxin, and even a macro inorganic anion, a heteropolytungstate (Mus-Veteau et al., 1992), the same heme appears to be involved. Analysis of the distribution of surface electrostatic potentials (Coulombic) [see Stewart et al. (1988, 1989) and Figures 9 and 10] indicates that one of the four hemes exhibits a unique region of positive potential. This heme is structurally equivalent in all three cytochromes *c*₃, suggesting a specific and conserved role for this heme in this interaction. In some cases, this assignment is also supported by cross-linking (Dolla & Bruschi, 1988; Dolla et al., 1991) and extended NMR (Guerlesquin et al., 1985b; Park et al., 1991; Mus-Veteau et al., 1992) experiments. Finally, in a very recent set of experiments, *D. salexigens* flavodoxin, reconstituted with a ¹³C-labeled FMN, was titrated with *D. gigas* cytochrome *c*₃, and further evidence was obtained supporting the hypothesis that the cytochrome moiety shows a unique site for binding flavodoxin (P. N. Palma, J. Caldeira, J. R. Ascenso, J. LeGall, A. Bacher, I. Moura, J. Wampler, and J. J. G. Moura, unpublished results).

The titrations shown in Figure 2 and those reported earlier (Stewart et al., 1988, 1989) are relatively sharp for reversible interactions. However, it is important to remember that the experimental conditions of these experiments make interpretation more difficult than in the more normal case where the variable component is in great excess and the other component is held to a constant concentration. In those cases the binding isotherm generally simplifies into a function of the varied concentration of the excess component. Under the conditions

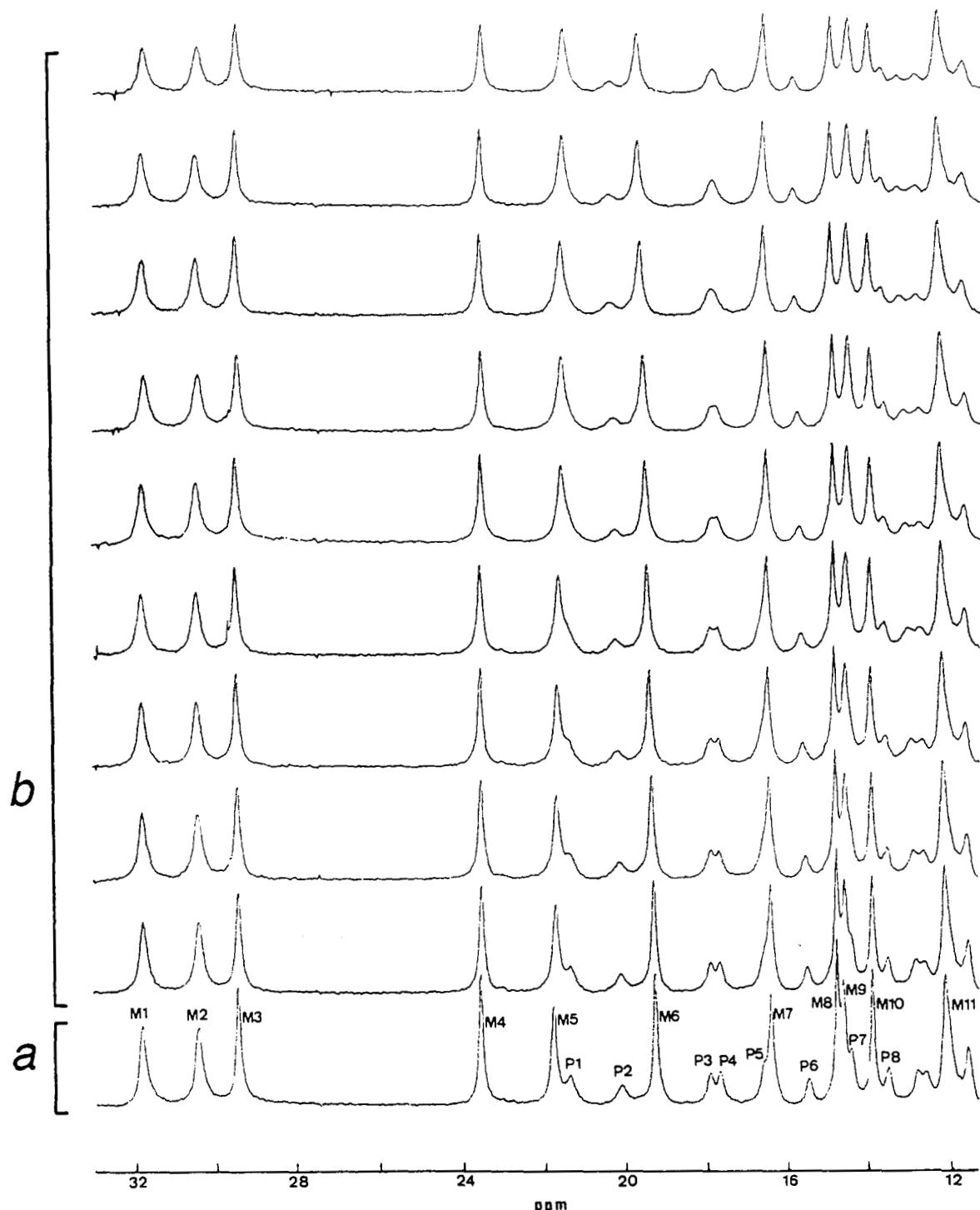


FIGURE 1: (a) Low-field region of the ¹H-NMR spectrum of a solution of 1 mM *D. gigas* ferricytochrome *c*₃ at pH 7.5, *T* = 298 K, and low ionic strength. Methyl resonances (*M*_i) are labeled upfield. (b) Effect of addition of *D. salexigens* flavodoxin on the low-field resonances of the same solution of cytochrome *c*₃. The titration was done at pH 7.5 and *T* = 298 K, using a stock 3 mM flavodoxin solution. The spectra shown were recorded for the following molar ratios of [flavodoxin]/[cytochrome *c*₃] (from bottom to top): 0.05, 0.10, 0.15, 0.25, 0.40, 0.60, 0.80, 1.1, and 2.6.

of these experiments, when significant binding occurs, the concentrations of free (and bound) components are changed significantly from the initial concentrations introduced into the mixture, and binding is dependent on the concentration of both components (cytochrome *c*₃ and flavodoxin). In addition, the experiment involves changes in initial concentrations of both components. Thus, while presentation of the data as a function of the ratio of the initial concentrations of the two components is convenient, the actual isotherm involves three variables (the chemical shift and the initial concentrations of cytochrome *c*₃ and flavodoxin). Our previous efforts to fit

these data (Stewart et al., 1988) indicated that only one site on the cytochrome *c*₃ molecule was involved; however, closer examination of the data and the expanded information of the data on Figure 2 suggested that the binding involves at minimum more than one site on the flavodoxin. This unexpected result prompted detailed analysis of a number of more complex models.

In Figure 2, all the curves show saturation with an equivalent point near to a [flavodoxin]/[cytochrome *c*₃] molar ratio around 0.5, and the shapes of a number of the curves are clearly complex. This behavior is not consistent with a simple

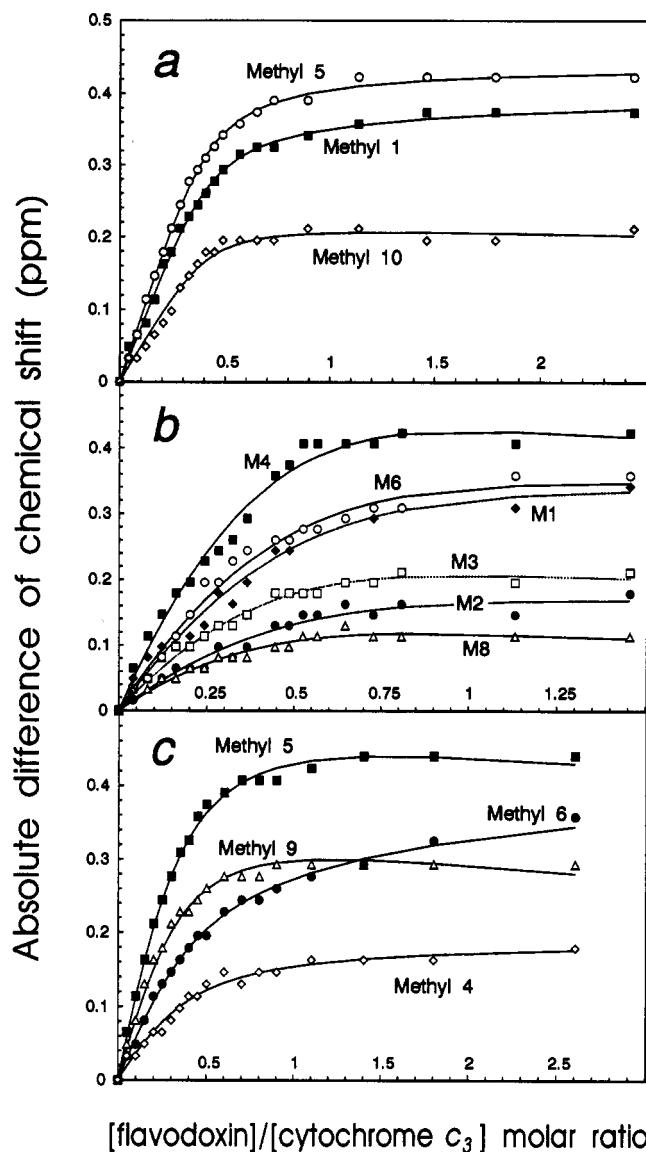


FIGURE 2: Comparison of the titrations of cytochrome c_3 from different species of *Desulfovibrio* with *D. salexigens* flavodoxin. Effect of increasing protein/protein molar ratios $R=[\text{flavodoxin}]/[\text{cytochrome}]$ on the chemical shifts of selected resonances associated to particular heme methyl groups. Cytochromes c_3 are from (a) *D. vulgaris* Hildenborough, (b) *D. desulfuricans* ATCC 27774, and (c) *D. gigas*. Experimental conditions are as described in Figure 1. The plotted values represent the absolute values of the difference to the initial chemical shift. The curves represent a fitting effort with the developed model of interaction described in Figure 4, using the parameters shown in Table 2.

stoichiometry of 1:1. Thus, several models for a 1:2 (flavodoxin–cytochrome c_3) interaction were also tested and compared to the 1:1 model. Figure 3 shows a schematic representation of the general model for the 1:2 process, where k_1 to k_4 are the microscopic association constants. We will call $K_1 = k_1k_2$ and $K_2 = k_3k_4$ the macroscopic constants for the formation of the binary and ternary complexes, respectively.

Figure 4 (curves A) shows that the simplest 1:1 model clearly fails to fit the experimental results. The simplest 1:2 case is when all four microscopic binding constants are equal, *i.e.*, $k_1 = k_4 = k_2 = k_3$, the model with independent and equivalent sites. Although this drastically improves the fit (Figure 4, curves B), as compared to the 1:1 model, it is far from being a good fit. Clearly, the different methyl resonances are not only affected to different extents (different values of the $\Delta\delta$ at saturation) but also change with quite distinct curvatures,

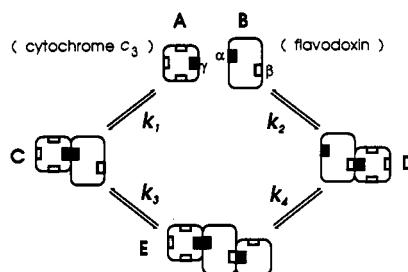


FIGURE 3: Developed model for the cytochrome c_3 –flavodoxin interaction used to fit experimental data: A, cytochrome c_3 ; B, flavodoxin; C–E, different associated forms. This model assumes a 1:2 (flavodoxin:cytochrome c_3) stoichiometry with two distinct but dependent microscopic association constants, corresponding to the two binding sites on the flavoprotein.

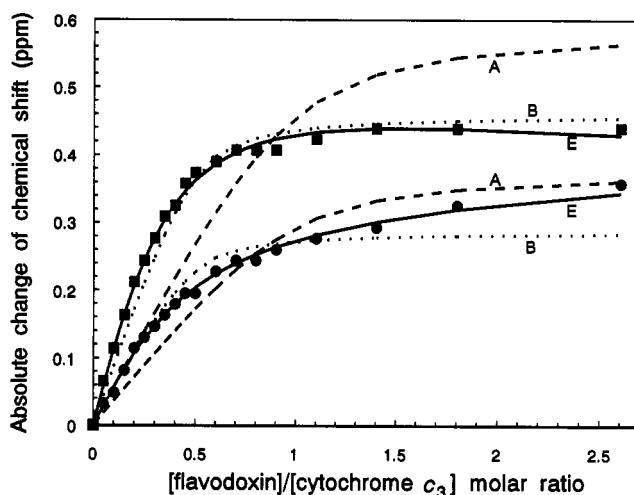


FIGURE 4: Illustrative simulation of the titration curves for signals M5 and M6 of *D. gigas* cytochrome c_3 (see Figure 2c). Each pair of curves represents the best possible fit of the two sets of data with each of the tested models (see Table 1). Curves A (dashed), B (dotted), and E (solid) correspond to models A, B, and C, respectively.

Table 1: Comparison of the Fitness of Several Interaction Models to the Experimental NMR Data

model ^a	χ^2/d^b		
	DvH	Dd	Dg
A ($k_2 = k_3 = k_4 = 0$)			338×10^{-5}
B ($k_1 = k_2 = k_3 = k_4$)	13.8×10^{-5}	26.9×10^{-5}	43.6×10^{-5}
C ($k_2 = 0, k_4 \rightarrow \infty$)	9.26×10^{-5}	16.7×10^{-5}	13.2×10^{-5}
D ($k_1 = k_4 \neq k_2 = k_3$)	8.70×10^{-5}	16.6×10^{-5}	10.8×10^{-5}
E ($k_1 \neq k_2 \neq k_3 \neq k_4$)	6.42×10^{-5}	16.6×10^{-5}	5.86×10^{-5}

^a Refer to Figure 4 for the significance of k_1 to k_4 . Models: A, binary complex; C, sequential ordered ternary complex. ^b The reduced chi squared (χ^2/d) is defined as the sum of the squares of the residuals divided by the number of degrees of freedom ($d = \text{no. of data points} - \text{no. of parameters} - 1$). The values are referred to the titrations of *D. vulgaris* Hildenborough (DvH), *D. desulfuricans* ATCC 27774 (Dd), and *D. gigas* (Dg) cytochromes c_3 .

and the simpler models are not flexible enough to fit them simultaneously. Of the 1:2 models tested, the best fit using the criterion of the smallest value of the reduced χ^2 (Table 1) is the one where all four constants are fitted. This corresponds to a model with nonidentical, dependent sites (Figure 4, curves E).

Table 2 gives the values of the association constants and the sensitivity coefficients corresponding to a global, least squares solution for each of the three sets of data. These best fits are represented in the form of predicted signal versus ratio by the solid lines in Figure 2. Examining the fit values of Table 2 shows that the sites exhibit positive cooperativity in all cases; *i.e.*, binding at one site increases the affinity to the other.

Table 2: Association Constants and Sensitivity Coefficients Derived from the Best Fitting of Data Shown in Figure 2 with the Interaction Model Proposed in Figure 4^a

	<i>D. vulgaris</i> M			<i>D. desulfuricans</i>				<i>D. gigas</i>			
	1.8E3 2.7E3	1.3E3b 2.7E3	1.0E2 7.5E2	1.0E2 7.5E2	5.0E1 7.5E2	3.0E3 5.0E3	1.0E3 3.0E4	1.0E3 1.0E3	3.0E1 3.6E1	2.3E1 3.6E1	3.0E1 3.6E1
k_1 (M^{-1})											
k_2 (M^{-1})											
k_3 (M^{-1})											
k_4 (M^{-1})											
$K_1 = k_1 k_2$ (M^{-2})											
$K_2 = k_3 k_4$ (M^{-2})											
	<i>D. vulgaris</i> M			<i>D. desulfuricans</i>				<i>D. gigas</i>			
	<i>M1</i>	<i>M5</i>	<i>M10</i>	<i>M1</i>	<i>M2</i>	<i>M3</i>	<i>M4</i>	<i>M6</i>	<i>M8</i>	<i>M4</i>	<i>M5</i>
f_α	1	1	0.14	1	1	1	1	1	1	0.91	1
f_β	0.48	0.91	1	0.21	0.18	0.11	0.11	0.18	0.08	1	0.14
$\Delta\delta_{\max}$ (ppm)	0.5332	0.4935	0.4550	1.584	0.9265	1.548	3.195	1.904	1.036	0.2363	1.330
											0.6600
											1.130

^a Symbols: K_j , macroscopic association constants; k_j , microscopic association constants; M_i , methyl resonances; f_α and f_β , sensitivity coefficients of methyl i resonance of cytochrome c_3 upon binding to sites α or β on flavodoxin. ^b Best fit values (shown as powers of 10) and upper and lower limits assuming a 5% change in the overall fit criteria.

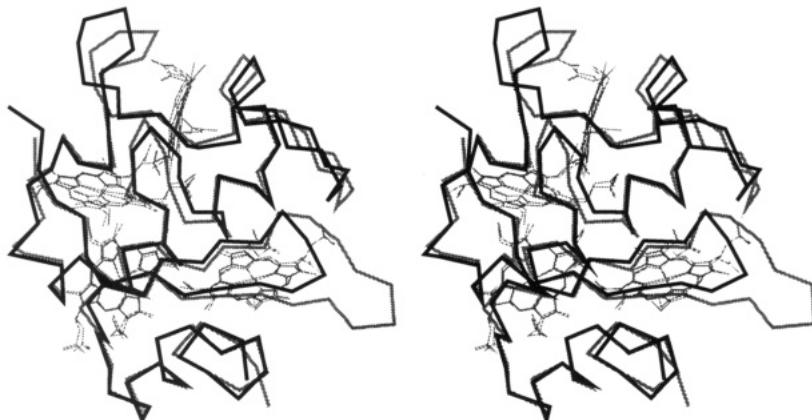


FIGURE 5: Superposition of the tertiary structures of cytochromes c_3 from *D. vulgaris* Miyazaki (thick black lines) and *D. gigas* (thick gray lines). Only main-chain α -carbon atoms are shown, along with the four hemes (thin lines).

However, it should be noted that, due to the noise in experimental data and to the number of parameters to which the data were fitted, the actual values of the constants presented in Table 2 must be handled with care in view of the error associated with their determination.

With enough parameters and a sufficiently complex model, we could conceivably fit every datum point exactly. This, of course, would be an incorrect model, since we know that some of the variation is experimental error. The reduced χ^2 measures the improvement in fit, adjusted for the addition of parameters. There are obviously many more complex models than those tested here, and they cannot be completely excluded without testing. However, examination of the structures and predicted structures (*D. desulfuricans* ATCC 27774 cytochrome c_3 and *D. salexigens* flavodoxin) of these proteins lends some support for the 1:2 model as described and discussed below.

Modeling the Structure of *D. desulfuricans* (ATCC 27774) Cytochrome c_3 . There are two critical steps in predicting a protein structure by homology modeling: (1) the choice of a homologous protein that will serve as the structural template and (2) the correct alignment of the corresponding amino acid sequences. The problem is particularly difficult if there is a large number of insertions and deletions needed in order to align the sequences.

The amino acid sequences of three homologous cytochromes c_3 (*D. vulgaris* Miyazaki F, *D. gigas*, and *D. baculatus* Norway 4) were aligned by mapping the direct residue to residue correspondence after superimposition of the their three-dimensional structures (see Figure 5). Figure 6 shows the

result of such alignment, where the structurally conserved regions are represented linearly at the base and the nonconserved regions are represented as outstanding loops. Three important observations can be made on these figures: (i) cysteine and histidine residues responsible for the heme binding are all conserved, as well as the relative position of the hemes themselves; (ii) regions with organized secondary structure tend to be conserved; (iii) even so, there is no simple criterion for alignment, since low amino acid homology is observed in some regions where the structural homology is obvious. Thus, homology of these proteins can be expressed not only in terms of conservation of amino acid sequence but also in terms of conservation of secondary and tertiary structures (Table 3).

The representation of Figure 6 is very useful for distinguishing the variable from the structurally conserved regions that may be important for the stability of the overall structure and function, and the complete comparison can be used to guide alignment of sequences where the structure is not known. In this way, the sequence of the cytochrome c_3 from *D. desulfuricans* ATCC 27774 was aligned with the other sequences on the basis of the following criteria: (i) strong emphasis was given to the conservation of the positions of the cysteines and histidines responsible for binding the hemes; (ii) the same global structural pattern of the protein was conserved by positioning insertions or deletions as necessary in the regions of low structural homology. The result of this alignment is also represented in Figure 6, and it suggests that *D. vulgaris* cytochrome is the closest homologue (Table 3) and should serve as the structural model for the *D. desulfuricans* protein.

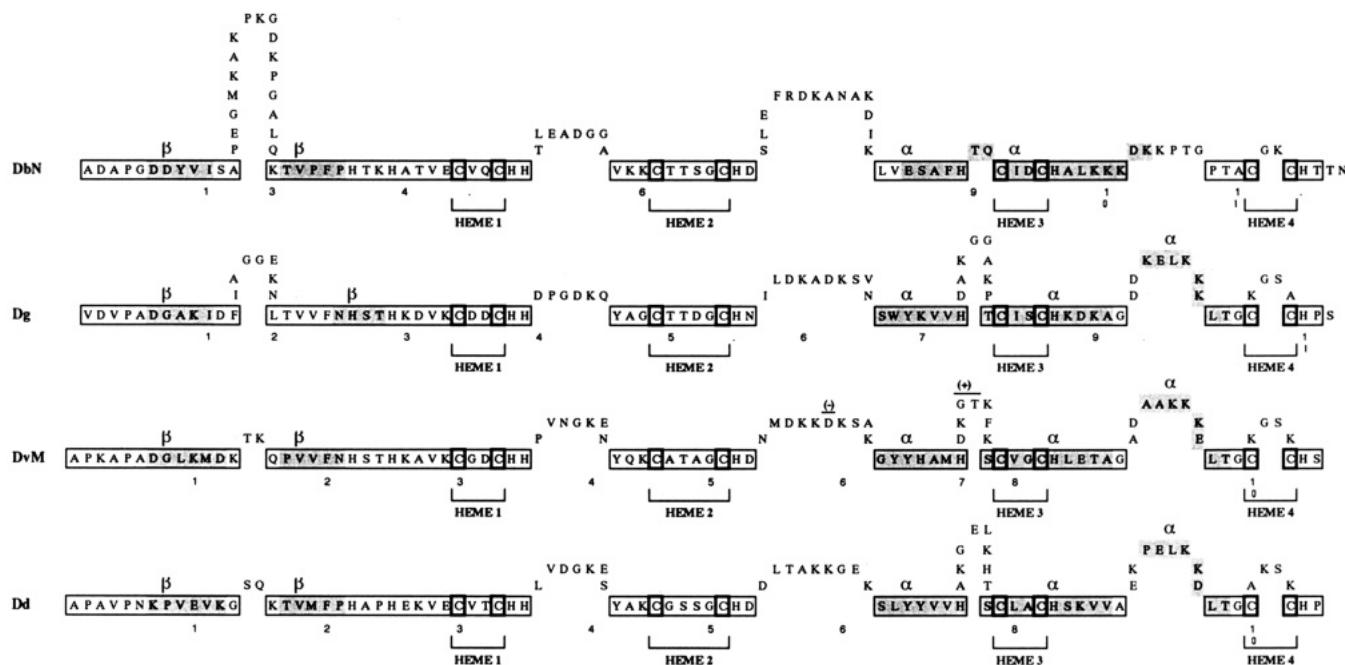


FIGURE 6: Alignment of the amino acid sequences of different cytochromes *c*₃, on the basis of structural criteria, upon superposition of the respective tertiary structures. Horizontal portions inside the boxes represent structurally conserved regions, while nonconserved regions in the 3D structures are represented as loops. Regions of organized secondary structure are shaded, and the heme anchoring sites are indicated. Heme labels are ordered from the N-terminus.

Table 3: Percentage of Homology between the Amino Acid Sequences and Tertiary Structures of Cytochromes *c*₃ from *D. vulgaris* Miyazaki F (*DvM*), *D. gigas* (*Dg*), *D. baculatus* Norway 4 (*DbN*), and *D. desulfuricans* ATCC 27774 (*Dd*)

	sequence homology (%)			structural homology (%)		
	<i>DvM</i>	<i>Dg</i>	<i>Dd</i>	<i>DvM</i>	<i>Dg</i>	<i>Dd</i>
<i>DbN</i>	28	28	29	<i>DbN</i>	63	61
<i>Dd</i>	44	38		<i>Dd</i>	96	79
<i>Dg</i>	50			<i>Dg</i>	89	

Following the same homology modeling procedures previously described (Stewart et al., 1987; Wampler et al., 1993), the predicted structure of the *D. desulfuricans* ATCC 27774 cytochrome *c*₃ presents a high similarity to the X-ray structure of the parent protein from *D. vulgaris* Miyazaki F. This result is not surprising, since the two molecules have the same number of amino acids and a significant degree of homology (40%), and the minimization process used will not change conformations dramatically, in any case. Since the relative position and orientation of these four hemes are strongly conserved among the known structures, it seems reasonable to form the homology model by such a procedure.

Indeed, for the propose this model structure was made, *i.e.*, the analysis of the protein surface topology and especially the electrostatic potential distribution, it should serve as a useful and reliable model in the absence of the X-ray structure of this cytochrome *c*₃.

Modeling the Structure of *D. salexigens* Flavodoxin. The sequence of *D. salexigens* flavodoxin was aligned with that of *D. vulgaris* Hildenborough, as suggested by Helms et al. (1990), and a homology model was built from the structure of the *D. vulgaris* protein (Watenpaugh et al., 1972, 1973), following the methodology described above. Figure 7 shows that most of the sequence differences are located in regions far from the FMN binding site. Since this site is the most likely region for interactions with other redox proteins (Weber & Tollin, 1985; Stewart et al., 1988) and since such differences tend to be compensated locally (Chothia & Lesk, 1986; Stackhouse et al., 1988), the model should be particularly

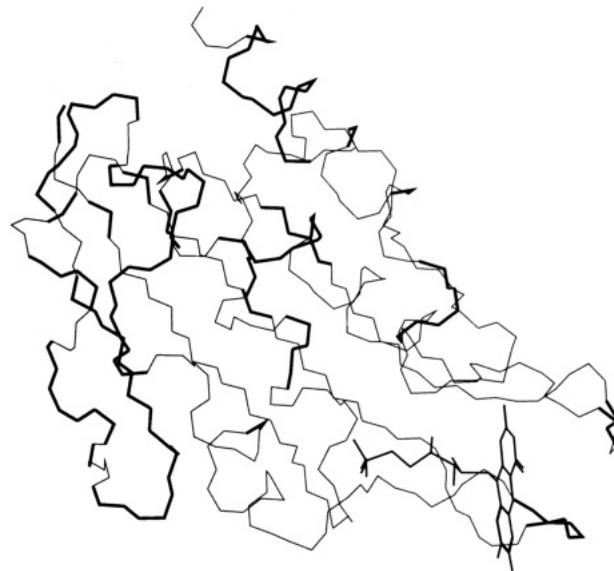


FIGURE 7: Three-dimensional structure of *D. vulgaris* Hildenborough flavodoxin, showing the location of amino acid differences between this flavodoxin and its homologous structure from *D. salexigens*. Conserved residues among the two proteins are represented as thin lines while nonconserved residues are shown as thick lines. Only main-chain carbon atoms and the FMN group are shown.

good for the purposes of this particular study. An interesting observation that arises from the comparison of the modeled and X-ray structures is the spatial conservation of the pattern of aromatic side-chain positions. Figure 8 shows that 10 of the 13 aromatic residues are conserved in both proteins (with tyrosines replacing both Phe 50 and Phe 75). Indeed, the three other aromatic residues, though shifted by 4, 5, and 8 residues in the amino acid sequence alignment (Helms et al., 1990), end up positioned to fill in the same spatial region (see arrows in Figure 8). Thus, as in rubredoxin (Meyer et al., 1990; Blake et al., 1991), the core of aromatic residues seems to be highly conserved within these two flavodoxins. In addition, this observation reinforces the idea that structural homology often transcends simple linear sequence alignment



FIGURE 8: Distribution of the aromatic residues in *D. vulgaris* and *D. salexigens* flavodoxins. The backbone of the polypeptide is represented by the α -carbon atoms (thin). The side-chain atoms were omitted except the ones belonging to the aromatic residues, which are represented as black thick lines in the *D. vulgaris* protein and as gray thick lines in the *D. salexigens* protein. Arrows indicate aromatic residues that are not conserved in the primary structures of the two proteins.

with three-dimensional positioning conserved by substitutions, shifts, transpositions, insertions, and deletions that cannot be interpreted in a linear comparison.

Analysis of the Surface Electrostatic Potentials. In Figure 11B, *D. salexigens* flavodoxin, in the center, is shown surrounded by its solvent-accessible surface color-coded according to the Coulombic electrostatic potential, calculated using a distance-dependent dielectric constant. The preponderance of negatively charged surface area correlates with the low measured isoelectric point of the protein ($pI = 3.5$). However, a kind of information that is not given by a macroscopic property such as the isoelectric point is the way the electrostatic potential is distributed along the protein surface. While the Coulombic electrostatic potential is probably not a good quantitative indication of this (Gilson & Honig, 1988), the qualitative information should be valid. Indeed, Figure 11B shows that this distribution is rather asymmetric and that the surface surrounding the FMN is the most uniformly negative. This observation, already mentioned by other authors (Simmondsen et al., 1982; Weber & Tollin, 1985; Stewart et al., 1988), supports the hypothesis that this negatively charged surface should be responsible for a favorable preorientation of the flavodoxin in the process of approximation and interaction with positively charged regions of redox partners.

All of the flavodoxins isolated from several species of *Desulfovibrio* are acidic, with low isoelectric points; however, the tetrahemic cytochromes c_3 are characterized by a wide range of pI values. As mentioned below, the isoelectric points of the three cytochromes c_3 studied in this work, *D. vulgaris* Miyazaki F,³ *D. desulfuricans* ATCC 27774, and *D. gigas*, are 9.5, 7.0, and 5.5, respectively. It is therefore interesting to observe the distribution of the electrostatic potential over

the surface of these proteins (Figure 9). The asymmetry of the surface charges was already emphasized in the case of *D. vulgaris* cytochrome c_3 (Stewart et al., 1988) and was used as a basis to predict which of the four hemes should preferentially interact with *D. vulgaris* flavodoxin.

In the case of the pairing considered here, this view of interaction between a uniquely positive heme on the cytochrome c_3 surface with the negative region around the FMN site of flavodoxin is also supported. The calculations indicate even greater asymmetry in the surface potential distribution for *D. gigas* cytochrome c_3 than for the *D. vulgaris* protein with the *D. desulfuricans* protein in an intermediate situation (Figures 9 and 10). In addition, it is interesting to note that, despite this variability, it is always the same equivalent heme (heme 4)⁴ that is consistently and uniformly surrounded by a positive electrostatic potential (Figure 10). Note that this is also true for the cytochrome c_3 from *D. baculatus* Norway 4 (not shown). Hemes 1 and 2, on the other hand, are located in a portion of the protein where the surface is mostly negatively charged, while heme 3 is in the most mixed charged environment.

The role of a protein dipolar moment has been previously indicated as a driving force for a favorable orientation during complex formation involving *c*-type cytochromes (Weber & Tollin, 1985; Zhou & Kostic, 1992). In particular, in previous studies on the interaction of cytochrome c_3 with several redox partners, a consistent emphasis has been given to the possible stabilization of the complexes due to the formation of salt bridges between the positively charged residues that surround one of the four hemes and acidic residues on the interacting protein (Cambillau et al., 1988; Stewart et al., 1988, 1989).

Computer Graphics Modeling of the Complex. Figure 11 shows two tentative structural models of a ternary complex between *D. salexigens* flavodoxin and *D. gigas* cytochrome c_3 based on the electrostatic complementarity. Flavodoxin was interactively docked to each of the four hemes, and in all cases, the most favorable models were the ones in which flavodoxin was facing heme 4. Hence, these results support the hypothesis in which cytochrome c_3 should have one preferential interacting site for flavodoxin.

In contrast, the results discussed above indicate that flavodoxin, on the other hand, contains two sites capable of binding a cytochrome c_3 molecule. Since *D. salexigens* flavodoxin is a larger molecule than cytochrome c_3 and possesses a broad surface with a negative electrostatic potential, it can accommodate two molecules of cytochrome c_3 with appropriate complementarity of potential.

To build the two models shown in Figure 11, we started with the previously proposed model for a binary complex between *D. vulgaris* cytochrome c_3 and flavodoxin (Stewart et al., 1988), in which heme 4 and the FMN groups are facing each other in a close proximity and coplanar orientation. This seems, in fact, to be one of the best interaction conformations, not only in terms of topological complementarity but also in terms of favorable energetics. A similar model has been proposed for the interactions between *Clostridium pasteurianum* flavodoxin and different *c*-type cytochromes (Weber & Tollin, 1985). This model is also consistent with experimental observations of ionic strength dependent electron transfer between the two proteins.

³ The cytochrome c_3 used in the experimental approach of this work was isolated from *D. vulgaris* strain Hildenborough, while the X-ray coordinates of the *D. vulgaris* Miyazaki F were used as a structural model for the prediction and interpretation of the experimental results. However, the high sequence homology (90%) found between these two proteins makes this approximation reasonable.

⁴ To avoid inconsistency in labeling the four hemes, we used a nomenclature to number them according to the position, in the amino acid sequence, of the cysteines they are bound to. It should be noted, however, that this nomenclature is different from the one used by other authors and in the *D. vulgaris* Miyazaki X-ray structure, hemes 1, 2, 3, and 4 are referred to as hemes 3, 2, 4, and 1, respectively.

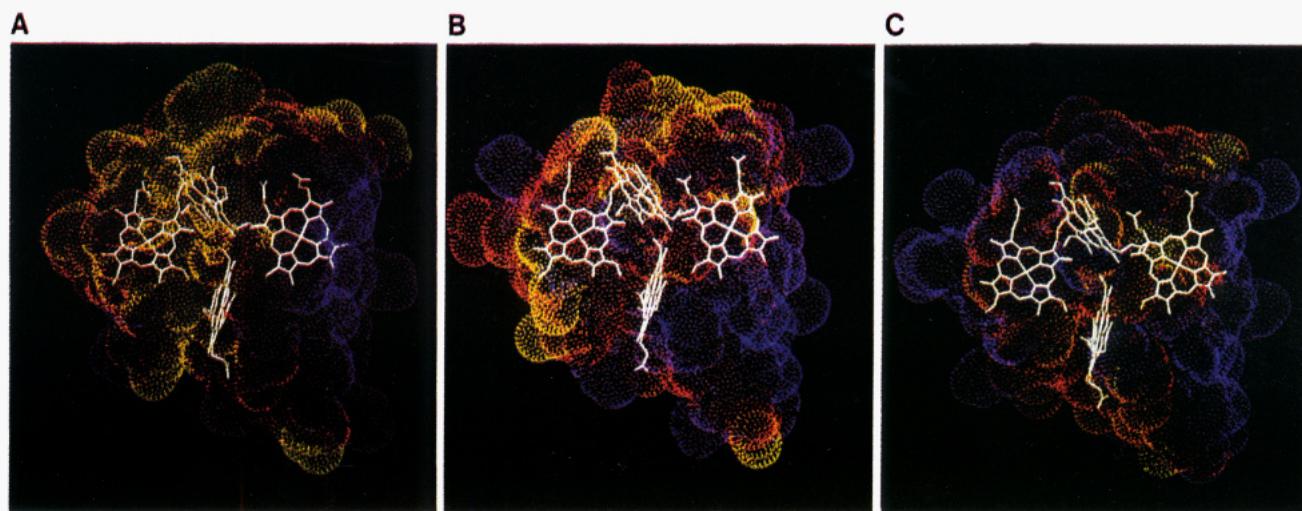


FIGURE 9: Representation of the electrostatic potential along the solvent-accessible surface of cytochrome *c*₃ from (A) *D. gigas*, (B) *D. desulfuricans* ATCC 27774, and (C) *D. vulgaris* Miyazaki. Color code: yellow ($V \leq -25$ kcal/mol), orange ($-25 < V \leq -10$ kcal/mol), red ($-10 < V \leq 0$ kcal/mol), violet ($0 < V \leq 10$ kcal/mol), and blue ($V > 25$ kcal/mol).

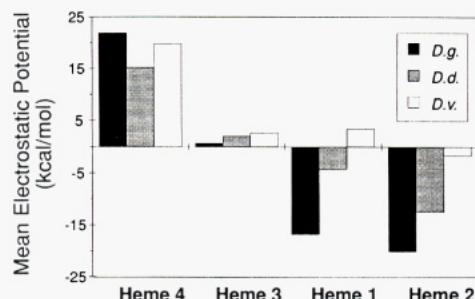


FIGURE 10: Mean electrostatic potential values around ($r = 1.2\text{--}1.3$ nm, centered on the iron) the four hemes of each cytochrome *c*₃ molecule. Heme labels are ordered from the N-terminus.

Our goal was then to investigate alternative sites on flavodoxin for the binding of a second cytochrome *c*₃ molecule. For this propose, the surface of the flavodoxin moiety of such a binary complex was interactively scanned with the surface of cytochrome *c*₃ that exposes heme 4, with the DOCKING facility of the program SYBYL (Tripos Associates). Figure 11 shows two alternative solutions for the ternary complex that represents low-energy conformations based on the AMBER (Weiner & Kollman, 1981) molecular mechanics force field, as implemented in SYBYL. In both cases, protein interactions can be stabilized by several salt bridges (yellow lines) between acidic residues on the surface of flavodoxin and the positively charged residues that surround the heme crevice, on cytochrome *c*₃ (Table 4). Both complexes have comparable interaction energies (*in vacuo*).

Unfortunately, we have no experimental evidence for any conformational change in flavodoxin resulting from binding the first molecule of cytochrome *c*₃, which would explain the observed increase in either k_3 or k_4 . However, it is interesting to note the possibility of forming two salt bridges between the two molecules of cytochrome *c*₃ (Figure 11A), which could also be invoked as the origin of the binding cooperativity suggested by the calculated values of the microscopic constants.

DISCUSSION

Molecular recognition is a key step in the regulation of the biological activity of proteins. The understanding of the mechanisms that drive interaction specificity is of great importance to the overall understanding of structure/function relationships. With electron-transfer proteins, an understanding of recognition and binding is a large part of the story,

since electron transfer is itself fast and the energetics are controlled by redox potentials, which are, of course, themselves determined in large part by structure. Development of different models for electron-transfer protein–protein complexes reveals some factors that may determine and orient these interactions, suggesting some general hypotheses for experimental testing. Numerous studies have been developed to understand the factors that govern the rate and specificity of the electron-transfer process between proteins. Different factors relevant to the control of the overall process are being considered in detail, including distance and orientation between the redox centers, solution ionic strength, and the nature of the interacting forces (such as electrostatics).

Several hypothetical complexes, based on experimental and computational molecular modeling, have been described, considering cytochrome *c*₃ and other small electron carriers isolated from sulfate-reducing bacteria (Cambillau et al., 1988; Stewart et al., 1988, 1989). The interaction between *D. desulfuricans* Norway 4 cytochrome *c*₃ and ferredoxin I from the same organism was detailed by NMR and cross-linking studies, indicating a binding constant of $6 \times 10^6 \text{ M}^{-1}$ (Guerlesquin et al., 1985, 1987; Capeillère-Blandin et al., 1986; Dolla & Bruschi, 1988). A polyanion (heteropolytungstate) was used to probe the binding process to the *D. desulfuricans* tetraheme protein with advantage in probing the charge and dimension of the docking site, and a similar binding constant was determined ($2 \times 10^6 \text{ M}^{-1}$) (Mus-Veteau et al., 1992). *D. vulgaris* Hildenborough cytochrome *c*₃ also interacts with rubredoxin and flavodoxin with binding constants of 10^4 and 10^3 M^{-1} , respectively (Moura et al., 1980a; Stewart et al., 1988, 1989). In all these cases, the profile of the NMR spectral parameters (chemical shifts) along the protein–protein titration was interpreted assuming a 1:1 stoichiometry. However, Park et al. (1991) studied the complex formed by ferricytochrome *c*₃ isolated from *D. vulgaris* Miyazaki F and ferredoxin I from the same organism, and, on the basis of 2D-NMR assignments of the heme resonances of the cytochrome *c*₃ and the NMR profiles on protein–protein titrations, they suggested a different stoichiometry, involving the binding of two cytochrome *c*₃ molecules per monomer of ferredoxin, with a binding constant of 10^8 M^{-2} .

In this work, the data collected on the interaction between *D. salexigens* flavodoxin and different cytochromes *c*₃, once more, consistently indicate a 1:2 stoichiometry involving the binding of two cytochrome *c*₃ molecules per one molecule of

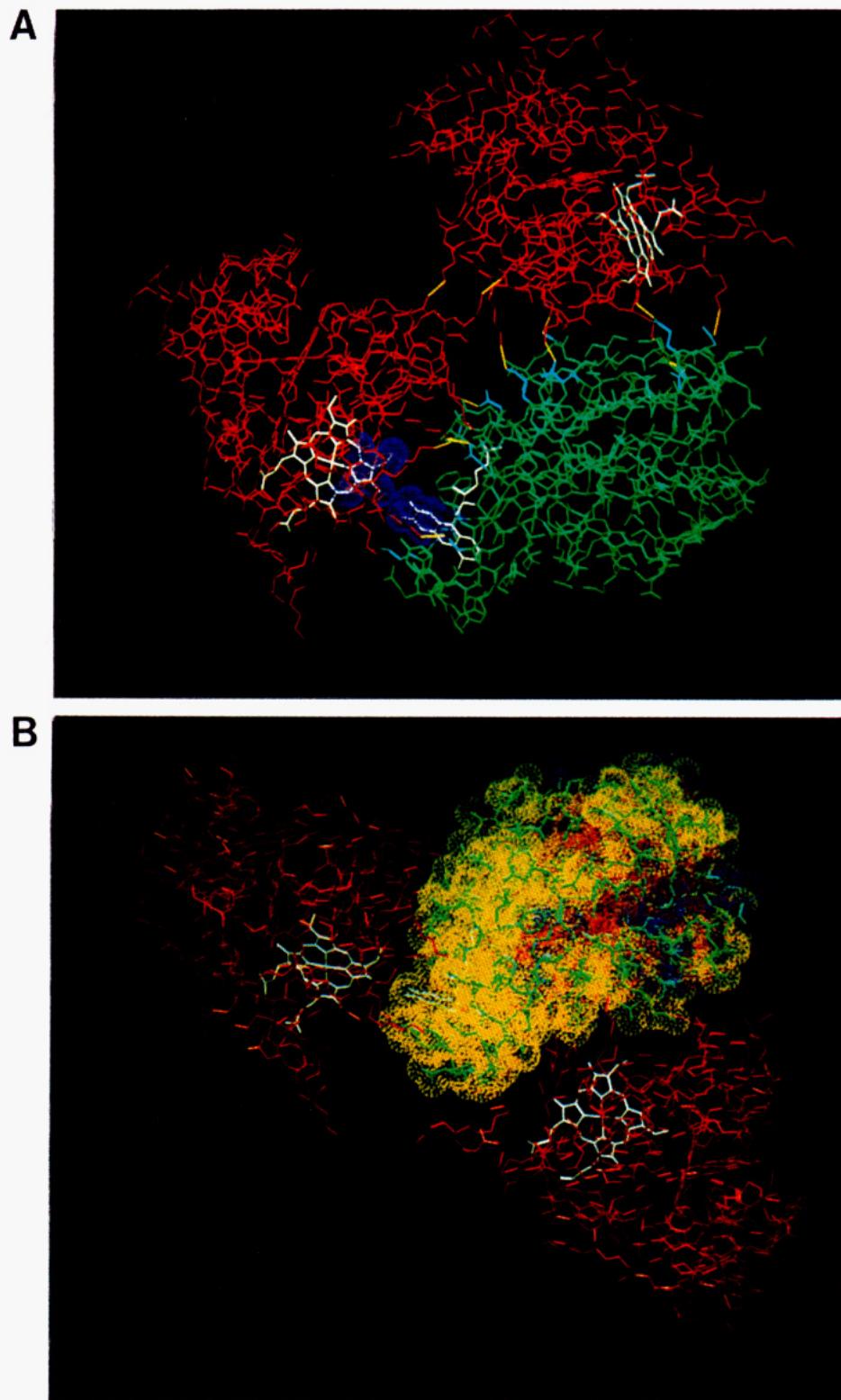


FIGURE 11: Two alternative structural models of the ternary complex between one molecule of *D. salexigens* flavodoxin and two molecules of *D. gigas* cytochrome *c*₃. Color code: green, flavodoxin; red, cytochrome *c*₃; white, interacting heme and FMN groups; yellow, intermolecular salt bridges. In (A), the solvent-exposed portions of the van der Waals surface of the two interacting redox groups are shown in purple. In (B), the solvent-accessible surface of flavodoxin is shown and colored according to the electrostatic potential, as in Figure 9.

flavodoxin. The self-coherence of the NMR profiles also enabled us to distinguish between different alternative models using global fitting. The proposed model for the interaction, where flavodoxin presents two docking sites with two distinct but dependent (cooperative) microscopic association constants, represents a reasonable compromise between physical and mathematical simplicity and the capacity of predicting the measurable properties. Although the values of the macroscopic binding constants, determined in this work, are significantly

different from the values based on a simple 1:1 model for the *D. vulgaris* flavodoxin–cytochrome *c*₃ interaction (Stewart et al., 1989), a review of this work (in progress) suggests that the 2:1 model also provides a better fit to these data as well.

Protein–protein titrations followed at specific proton NMR signals have been widely used to study the properties of protein complexes, such as stoichiometry and association constants. However, the analysis and interpretation of the NMR titration profiles may not be a straightforward process, since one of the

Table 4: Close Electrostatic Interactions Formed between Interacting Proteins in the Two Proposed Ternary Complexes Shown in Figure 11

model A ^c			model B ^c		
cyt <i>c</i> ₃ ^a	flavo-doxin	distance (nm)	cyt <i>c</i> ₃ ^a	flavo-doxin	distance (nm)
Lys 60	Glu 63	0.25	Lys 60	Glu 63	0.25
Lys 60	Glu 62	0.26	Lys 60	Glu 62	0.26
Lys 63	Asp 69	0.25	Lys 63	Asp 69	0.25
Lys 76	Asp 69	0.26	Lys 76	Asp 69	0.26
Lys 76	Asp 70	0.26	Lys 76	Asp 70	0.26
Lys 95	Asp 129	0.26	Lys 95	Asp 129	0.26
Lys 95	Asp 129	0.27	Lys 95	Asp 129	0.27
Lys 105	Asp 95	0.26	Lys 105	Asp 95	0.26
Lys 105	Asp 95	0.26	Lys 105	Asp 95	0.26
Asp 89	Lys 80	0.27	Asp 106	Lys 105	0.26
Asp 93	Lys 70	0.26	Glu 110	Lys 99	0.26
Glu 27	Lys 60	0.26	Glu 113	Lys 98	0.26
Glu 20	Lys 63	0.25	Asp 122	Lys 88	0.26
Glu 16	Lys 76	0.26	Asp 135	Lys 63	0.26
Asp 40	Lys 88	0.26	Glu 136	Lys 60	0.26
Asp 45	Lys 95	0.26	Asp 146	Lys 76	0.26
Asp 32	Lys 105	0.26			

^a Refer to Figure 11: cytochrome *c*₃ facing the FMN group.

^b Cytochrome *c*₃ bound to flavodoxin apart from the FMN. ^c Model A refers to Figure 11A and model B to Figure 11B.

experimental constraints typically used to simplify interpretation of titration data, namely, large excess in concentration of the ligand, is typically not met. This places greater demand on the number and quality of the experimental data points, in order to obtain accurate fits with complex, multiparameter binding isotherms.

Despite their various differences, some general outcomes have emerged from these studies of interactions with cytochromes *c*₃: (i) the complex formation affects the spectral parameters of more than one heme; (ii) the major interacting site on cytochrome *c*₃ was identified with the heme with the highest redox potential. In the models presented here, the site is also associated with the highest density, most uniform positive surface potential. Even with different tetraheme cytochromes *c*₃ that cover a wide range of isoelectric points, interaction with flavodoxin can be modeled using the same driving force and site complementarity.

The role of electrostatic interactions in the formation of specific protein-protein complexes has been widely emphasized. In fact, there is a good body of experimental and theoretical evidence for the dependence of either the association constants or the electron-transfer rate constants on the solution ionic strength, such as in the cases of the interactions between flavodoxins and a wide range of *c*-type cytochromes (Simonsen et al., 1982; Tollin et al., 1984; Weber & Tollin, 1985; Dickerson et al., 1985; Cheddar et al., 1986; Hazzard et al., 1986) or between cytochrome *c* and cytochrome *b*₅ (Mauk et al., 1986; Northrup et al., 1993).

In this work, we have also been tempted to interpret the nature and specificity of the complex formation between flavodoxin and cytochrome *c*₃, as well as its efficiency in electron transfer, mainly in terms of charge complementarity. Although it is generally accepted that electrostatic forces may be considered as selective guidelines for identifying interaction sites, we must keep in mind that these types of interactions are not necessarily specific, and additional forces must always be considered. Northrup and co-workers elegantly demonstrated (Northrup et al., 1987, 1988) with a computational study of the simulation of the Brownian movements associated with the interaction of cytochrome *c* with cytochrome *c* peroxidase, that several regions of a broad negatively charged surface of cytochrome *c* peroxidase interact with the positively

charged heme crevice of cytochrome *c*, as also previously suggested (Kang et al., 1977; Kornblatt & English, 1986). However, in order to predict the experimentally measured second-order rate constant of electron transfer, it was necessary to consider that not all the interactions are, in fact, productive in terms of electron transfer, due to improper positioning and orientation of the intervening redox groups.

In fact, it is not clear whether there must be a parallel relationship between the association constants of the complex formation and the kinetic constants of the electron-transfer process. This question may be of further complexity, when one considers electron-transfer complexes such as the one proposed in this work, where more than one associated molecular species are competing in solution and not all of them may be productive in terms of electron transfer. In fact, in the case of the flavodoxin-cytochrome *c*₃ interaction, the broad negatively charged surface of the flavoprotein actually can accommodate more than one cytochrome *c*₃. However, the two proposed models for the ternary complex (Figure 11) show that only one of the cytochrome molecules can bring one of its heme groups into close contact to the FMN redox group. Additional experimental work is being performed in order to determine the influence of the medium ionic strength on the thermodynamics and kinetics of the complex formation and the electron-transfer process, respectively.

Finally, two main questions should be considered about the results herein: first of all, although not likely, we cannot exclude the hypothesis of this ternary complex being an over-association artifact, due to the high protein concentration and low ionic strength solutions used in the NMR experiments. Moreover, it should be emphasized that the structural models proposed for the ternary complex represent the result of a partial and discontinuous search of docking possibilities. Indeed, it is impractical to cover and analyze the entire range of interaction possibilities, with such manual and interactive searching methods as the ones followed in this work. A full investigation of interaction possibilities that includes a broader range of interaction criteria would be required to complete this investigation. For instance, several more elaborated approaches have recently been developed (Jiang & Kim, 1991; Bacon & Moult, 1992; Katchalski-Katzir et al., 1992; Guida et al., 1992; Leach & Kuntz, 1992) for automatic scanning of molecular surfaces in multidimensional space, in order to identify interaction sites and conformations that optimize intermolecular interactions, such as size and shape complementarity, hydrogen bonding, and electrostatic and van der Waals interactions. Correspondence between such computational approaches and the growing body of experimental work should lead to a full explanation of recognition and binding in these cases.

The flavodoxin/cytochrome *c*₃ complex has been implicated in the electron transfer between donor and acceptor substrates in sulfate-reducing bacteria, namely, the electron transfer between pyruvate reduction and hydrogen production (phosphorolytic reaction) and hydrogen consumption and sulfite reduction (Moura et al., 1978, 1984). It is clear that a reversible electron transfer takes place between flavodoxin and cytochrome *c*₃.

Also recently it was shown that *in vitro* reconstitution of complex electron-transfer chains can be accomplished using soluble proteins from *D. gigas*, and these results point to the physiological relevance of our interactive model. In the first example (Chen et al., 1993) it was demonstrated that molecular hydrogen can be utilized for the reduction of sulfite, whereas in the second (Barata et al., 1993) it was shown that H₂ can be formed from aldehydes via a molybdenum-containing

aldehyde dehydrogenase. Both chains require cytochrome c_3 and flavodoxin, although the range of potentials involved is different: -420 to -110 mV in the first case and from -600 to -420 mV in the second. At this point of time, it is not possible to tell which of the two partners in the couple flavodoxin/cytochrome c_3 is the reductant or the oxidant. The following observation will demonstrate the complexity of the problem (Barata et al., 1993): aldehydes and the aldehyde dehydrogenase can only reduce cytochrome c_3 to 20% of the maximum, and flavodoxin is reduced only to the semiquinone level. So, in both situations, electrons cannot flow to hydrogenase. However, when cytochrome c_3 and flavodoxin are added together, flavodoxin transfers electrons to the cytochrome and both become fully reduced, and H₂ is formed in the complete system. This indicates that the couple flavodoxin/cytochrome c_3 works as a redox entity, whose properties are not the simple sum of the properties of the individual partners. The exploration of the kinetics of this complex should lead to fascinating discoveries in the field of the control of recognition and function of redox proteins.

ACKNOWLEDGMENT

We thank Dr. H. Demol for his participation in the determination of the *D. desulfuricans* ATCC 27774 cytochrome c_3 amino acid sequence.

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