A Hypothetical Model of the Flavodoxin–Tetraheme Cytochrome c_3 Complex of Sulfate-Reducing Bacteria†

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ABSTRACT: A hypothetical model of the flavodoxin–tetraheme cytochrome c_3 electron-transfer complex from the sulfate-reducing bacterium *Desulfovibrio vulgaris* has been constructed by using interactive computer graphics based on electrostatic potential field calculations and previous NMR experiments. Features of the proposed complex are (1) van der Waals contact between the flavin mononucleotide prosthetic group of flavodoxin and one heme of the cytochrome, (2) unique complementarity of electrostatic fields between the region surrounding this heme and the region surrounding the exposed portion of the flavin mononucleotide group of flavodoxin, and (3) no steric interferences between the two polypeptide chains in the complex. This complex is consistent with all structural and spectroscopic data available.

The tetraheme cytochromes c_3 from sulfate-reducing bacteria are thought to play a pivotal role in electron transfer in these organisms (LeGall et al., 1982; Yagi et al., 1983). In vivo, they facilitate the electron transfer from hydrogenase to ferredoxin and are a necessary component of several different types of redox reactions in crude extracts. In vitro, specific interactions have been demonstrated between these cytochromes and a variety of low molecular weight, redox-active proteins including ferredoxin (Moura et al., 1977), flavodoxin (Bell et al., 1978), and rubredoxin (Bell et al., 1978). Using molecular modeling and computer graphics, we have been able to model specific protein–protein interactions between cytochrome c_3 and each of these small redox proteins (Stewart et al., unpublished results). In this study, the proposed complex between the cytochrome c_3 and the flavodoxin of *Desulfovibrio vulgaris* is described and discussed in detail.

Both cytochrome c_3 and flavodoxin are small proteins (M_r 13 000 and 15 000, respectively). As indicated above, the primary function of cytochrome c_3, mainly found in the periplasm, is as an electron carrier for the enzyme hydrogenase (Yagi, 1970). Flavodoxin, a cytosolic protein, contains a flavin mononucleotide (FMN)[1] prosthetic group and has been found to replace the iron–sulfur protein ferredoxin in some reactions (Odom & Peck, 1984) including electron transfer in vitro to cytochrome c_3 (Moura et al., 1977; J. J. G. Moura, unpublished results). Of all the *Desulfovibrio* species, *D. vulgaris* is unique in that large amounts of flavodoxin and only small amounts of ferredoxin are synthesized (Odom & Peck, 1984).

Although the complex formed between these two proteins may not be of direct physiological significance due to their compartmentalization, it should be an excellent model for the study of the mechanism of electron transport between hemes and flavin groups. In addition, these modeling studies provide insight into the roles of the four heme centers of this unique multiheme cytochrome. A similar, nonphysiological model complex between tuna cytochrome *c* and *Clostridium MP* flavodoxin has given insight into the physiological role and chemistry of the monoheme cytochrome *c* (Weber & Tollin, 1985).

The interactions between monoheme *c*-type cytochromes and their redox partners have been well studied (Capeillere-Blandin et al., 1986; Tollin et al., 1984; Kang et al., 1978), and several models have been proposed (Mauk et al., 1986; Weber & Tollin, 1985; Poulos & Kraut, 1980). A general scheme in these interactions involves the formation of intermolecular salt linkages between lysine residues surrounding the heme crevice of the cytochrome and acidic residues on the redox partner in a manner such that the two prosthetic groups are as close spatially as steric constraints will allow.

The FMN group of flavodoxin is partially exposed to the surface of the protein, as are the hemes of cytochrome c_3. Thus is is likely that electron transfer between flavodoxin and cytochrome c_3 occurs via a mechanism involving direct contact of the prosthetic groups (Simondsen et al., 1982). NMR studies and EPR titrations have shown that flavodoxin interacts preferentially in vitro with one heme of the tetraheme cytochrome c_3 (Moura et al., 1980; J. J. G. Moura, unpublished experiments). The purpose of this study is to use molecular modeling and electrostatic field calculations to identify the heme with which this interaction is most likely to occur and to elucidate the factors responsible for the specificity of the interaction.

Computer graphics molecular modeling has been used previously in the investigation of protein–protein interactions (Salemme, 1976; Mauk et al., 1986; Weiner et al., 1982; Simondsen et al., 1982; Poulos & Mauk, 1983). A nice example of how experiments may be designed on the basis of model building studies is that of studies of the cytochrome *c*/cytochrome *c* peroxidase (Poulos & Kraut, 1980; Liang et al., 1987). In the modeled complex, an aromatic and hydrogen-bonded network was proposed to facilitate electron transfer

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[1] Abbreviations: FMN, flavin mononucleotide; NMR, nuclear magnetic resonance spectroscopy; EPR, electron paramagnetic resonance spectroscopy.

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Table I: Atoms on Which the Spheres Are Centered in the Calculations of Electrostatic Potential Surfaces and Fields

protein	atom name[a]
cytochrome c_3	CHB (heme 1)
	C3D (heme 2)
	C2A (heme 3)
	O1A (heme 4)
flavodoxin	C7M (FMN)

[a] Naming of atoms is in accord with coordinate data obtained from Bernstein et al. (1977).

between the two hemes, which in the model are approximately 15 Å apart. Phenylalanine-87 of cytochrome *c*, which in the model was a critical part of this electron-transfer network, was changed by site-directed mutagenesis to tyrosine, serine, and glycine (Liang et al., 1987). It was found that in the serine and glycine mutants electron transfer occurred at a rate 10000 times less than those with an aromatic residue at that position.

Other computational methods have also been used to study the interactions of proteins. Scheraga et al. have utilized conformational energy calculations to study the interaction of enzymes with their substrates, such as α-chymotrypsin (Platzer et al., 1972) and lysozyme (Pincus et al., 1976; Smith-Gill et al., 1984), to understand the basis of molecular recognition and catalysis (Pincus & Scheraga, 1981).

Experimental Procedures

The coordinate data used for *D. vulgaris* Miyazaki cytochrome c_3 at 1.8-Å resolution (Higuchi et al., 1984) and for *D. vulgaris* Hildenborough flavodoxin at 2.0-Å resolution (Watenpaugh et al., 1976) were obtained from the Brookhaven Protein Data Bank (Bernstein et al., 1977). To build the hypothetical model, flavodoxin was docked to each of the four hemes of cytochrome c_3. The heme chosen was selected on the basis of (1) freedom to bring the two prosthetic groups into close proximity without steric interferences between the two polypeptide chains, (2) greatest number of potential intermolecular salt linkages formed, and (3) lack of electrostatic charge repulsion between protruding anionic heme propionates of the cytochrome and acidic residues on the surface of flavodoxin.

The electrostatic surfaces and fields were calculated by using the Mulliken net atomic charges from the AMBER data base (Weiner et al., 1984; Weiner & Kollman, 1981), which were calculated from ab initio SCF–LCAO–MO (self-consistent field–linear combination of atomic orbitals–molecular orbital) calculations using a STO-3G (three Gaussian functions per Slater-type orbital) basis set (Hayes & Kollman, 1976). All polar hydrogen atoms were included explicitly, while all nonpolar hydrogen atoms were included by using a united-atom representation. No solvent water molecules were included in the calculations.

In the equation

$$V = \sum_i [q_i/(D|r_i - r_0|)]$$

a distance-dependent dielectric constant $D|r_i - r_0|$ was used in calculating the electrostatic potential V at r_0, a point 1.4 Å above the contact surface. Each atom within a 20-Å radius located at position r_i with an atomic charge of q_i was included in the sum; 1.4 Å represents the center of a solvent sphere at its closest approach to the protein (Weiner et al., 1982). The method of neutral spheres (Adams, 1979) was utilized as previously described (Weiner et al., 1982).

The electrostatic field vectors were calculated at points on concentric spheres of radius of up to 14 Å centered on selected exposed atoms of the prosthetic groups (Table I). The fields were calculated from the partial derivatives of the potential with respect to the three coordinate axes. The vectors point in the direction a negative charge would move if placed in the field.

Table II: Intermolecular Salt Linkages Formed in the Proposed Complex

flavodoxin	cytochrome c_3	distance (Å)
Asp-69	Lys-60	2.90
Glu-66	Lys-58	2.91
Asp-62	Lys-15	2.93
Asp-95	Lys-101	2.91
Asp-129	Lys-95	3.00

Table III: Heme to FMN Distances[a]

FMN	heme	distance (Å)
C8M	C2B	4.18
C7M	C3A	5.04
C8	CHB	5.53
C7M	Fe	8.35
C8M	Fe	7.37

[a] Nomenclature is standard Brookhaven Protein Data Bank convention (Bernstein et al., 1977).

The solvent-accessible surfaces on which the electrostatic potentials were displayed were calculated by using the program MS (Connolly, 1983). Calculations were performed on a Digital Equipment Corp. VAX 11/780 running VMS version 4.5. Computer graphics modeling was performed on an Evans and Sutherland Corp. PS340 Graphics Workstation with the molecular graphics software MOGLI and SIMPLE (P. K. Weiner, unpublished software).

The heme iron atoms of cytochrome c_3 were each assigned a charge of +2 kcal/mol. The phosphate oxygens of FMN were each assigned a charge of −0.850 kcal/mol.

Results

The hypothetical model of the flavodoxin–cytochrome c_3 complex is shown in Figure 1A. On the basis of the above criteria for docking, heme 1 [numbering according to Higuchi et al., (1984)] was chosen as the site at which flavodoxin interacts. Intermolecular salt linkages formed between the basic lysine residues surrounding the heme crevice of the cytochrome and acidic residues near the FMN binding site of flavodoxin are all within acceptable bonding limits (Table II) and are uniformly distributed.

As can be seen from Figure 1A, the flavin macrocycle and the heme planes are nearly coplanar and parallel. The distance between the two prosthetic groups (Table III) is small enough to facilitate van der Waals contact of atoms of the two groups (Figure 1B). The closest approach of the two π-electron systems is 5.5 Å.

The electrostatic potential surface of flavodoxin (Figure 2) shows a very asymmetric charge distribution. The area about the FMN binding region appears to consist entirely of negative charges, while the opposite end of the molecule has regions of positive charge.

The electrostatic potential fields for each of the regions about the four hemes of cytochrome c_3 are shown in Figure 3. It can be seen that three of the four hemes are in similar electrostatic environments while the other is in a strikingly different environment. The exposed portion of this heme, heme 1, is surrounded by a positively charged electrostatic field in contrast to the other hemes, which are surrounded by electrostatic fields containing both negative and positive charges.

This hypothetical model is based upon the coordinates of proteins from two different strains of *D. vulgaris*. From the primary sequence alignment of the cytochromes (Higuchi et

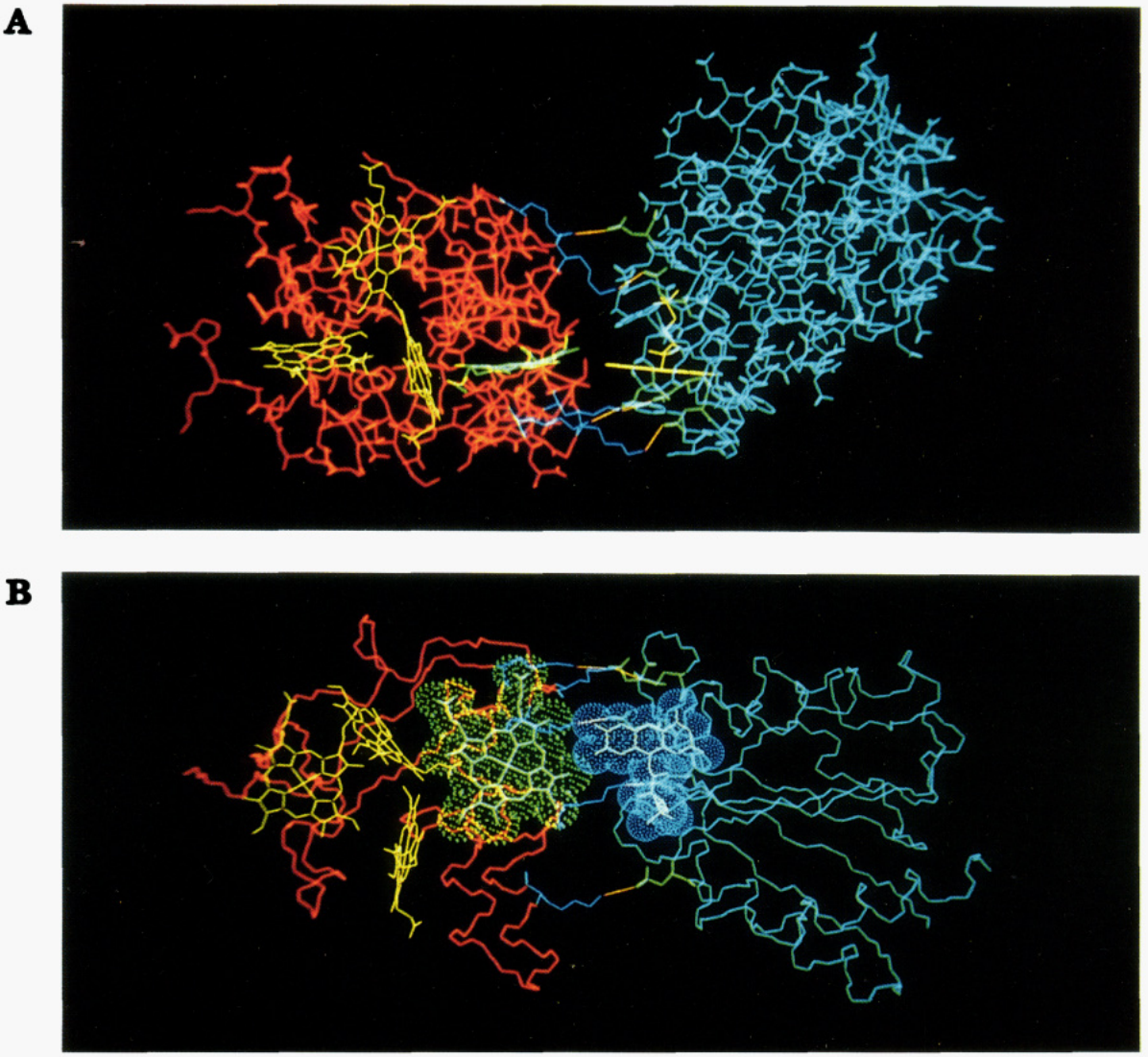

FIGURE 1: (A) Proposed complex formed between cytochrome c_3 (red) and flavodoxin (cyan). The FMN group of flavodoxin is shown in green, and the hemes are shown in yellow (except for heme 1 in green). The intermolecular salt linkages formed between the acidic residues (green) of flavodoxin and the lysine residues (blue) of cytochrome c_3 are represented as orange. This view shows the coplanarity and parallel alignment of the two prosthetic groups. (B) Coloring is as in (A). Only the main-chain atoms of the residues not involved in the complex are shown. In this view of the complex the two aromatic systems are viewed from the top and the van der Waals radii of the prosthetic groups are indicated by a dotted surface. Close interaction of the two groups is obvious.

al., 1984) it is seen that only 14 of the 107 residues differ between the Miyazaki and Hildenborough strains, none of which are involved in this complex. A method of predicting related structures developed in this laboratory (Stewart et al., 1987) has shown that the cytochromes from the two strains should have only subtle structural differences, which can be neglected for the purpose of this model.

Discussion

It is interesting to note that the model presented here is similar to that proposed for the interaction between *Clostridium MP* flavodoxin and tuna cytochrome *c* (Weber & Tollin, 1985). Both sets of molecules exhibit similarities in the distribution of charged groups—a heme crevice surrounded by lysine residues and a FMN binding site surrounded by acidic residues. Also, the two complexes are similar in that the flavin ring and the heme are nearly coplanar and parallel and within van der Waals contact distance.

The occurrence of coplanarity of aromatic prosthetic groups has been observed previously in electron-transfer complexes (Poulos & Kraut, 1980; Salemme, 1976). This allows maximum overlap of π-electron systems, thus facilitating intermolecular electron transfer. In the cytochrome c_3–flavodoxin complex proposed here, there is a preponderance of aromatic residues located around the prosthetic groups of both proteins, which may mediate electron transfer.

The distribution of surface charges on both of these molecules is likely to play an important role in the correct orientation of the two proteins prior to docking. Figure 2 shows that charge distribution on the surface of flavodoxin is asymmetric with the region about the flavin site uniformly negatively charged (negative is red; positive is blue). This has also been observed with clostridial flavodoxin (Matthew et al., 1983). The polarity of the molecule should aid correct alignment of the prosthetic groups of the docking proteins by orientating flavodoxin so that this anionic region about the FMN binding site approaches the positively charged cytochrome. Since the cationic region around heme 1 projects the strongest and most uniform attractive field (Figure 3), it should, therefore, be the favored docking site. This should serve to increase the efficiency of complex formation, since the electrostatic fields would preorient the molecules. Instead of random collisions, the two prosthetic groups would approach each other in an orientation favorable to complex formation.

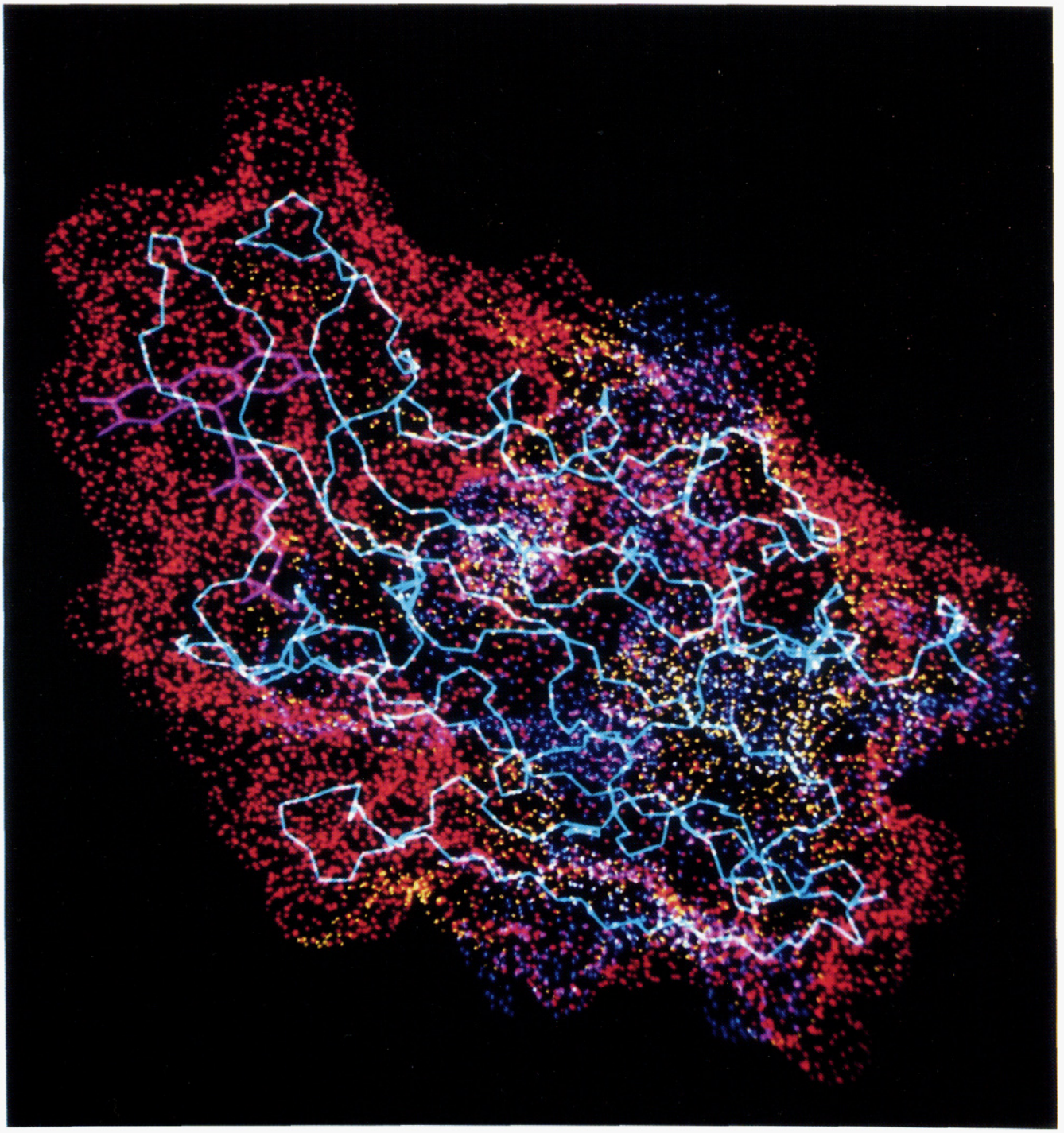

FIGURE 2: Electrostatic potential surface of *D. vulgaris* Hildenborough flavodoxin. The underlying structure is indicated by the main-chain atoms and the prosthetic group (in pink). The surface charge at the solvent-accessible surface is color coded as follows: red = <−5 kcal/mol; yellow = −5 to 5 kcal/mol; blue = >5 kcal/mol.

The lysine residues of cytochrome c_3 involved in this complex are largely conserved in spite of the very low homology of the known sequences. With the various sequence alignments suggested (Haser et al., 1979; Shinkai et al., 1980; Higuchi et al., 1981; Guerlesquin et al., 1984), there are lysines in or near (within one or two residues) these positions in five of the six known sequences. With these five and the Shinkai et al. alignment, only 3 of the 25 lysines potentially associated with this site are not perfectly aligned. These lysines are much more highly conserved than the bulk of lysines of the molecule. In the least homologous sequence, that from *Desulfovibrio desulfuricans* (Norway 4) (also known as *Desulfovibrio baculatus*), the conservation of these residues seems much lower; however, alignment of this sequence with the others is much more difficult. In addition, no flavodoxin has been found in this species which contains more ferredoxin than either *Desulfovibrio gigas* or *D. vulgaris* (Bruschi et al., 1977). Even so, when the two crystal structures are compared, every lysyl position involved in our proposed complex on the heme 1 surface of *D. vulgaris* (*miyazaki*) is matched by a lysyl residue on the surface of the corresponding heme of *D. desulfuricans*. The structural and sequence homology of these residues suggests that there may be an important physiological role for the protein–protein interaction described at this heme site.

Detailed NMR experiments have been performed on cytochrome c_3 from *D. gigas* (Santos et al., 1984; Moura et al.,

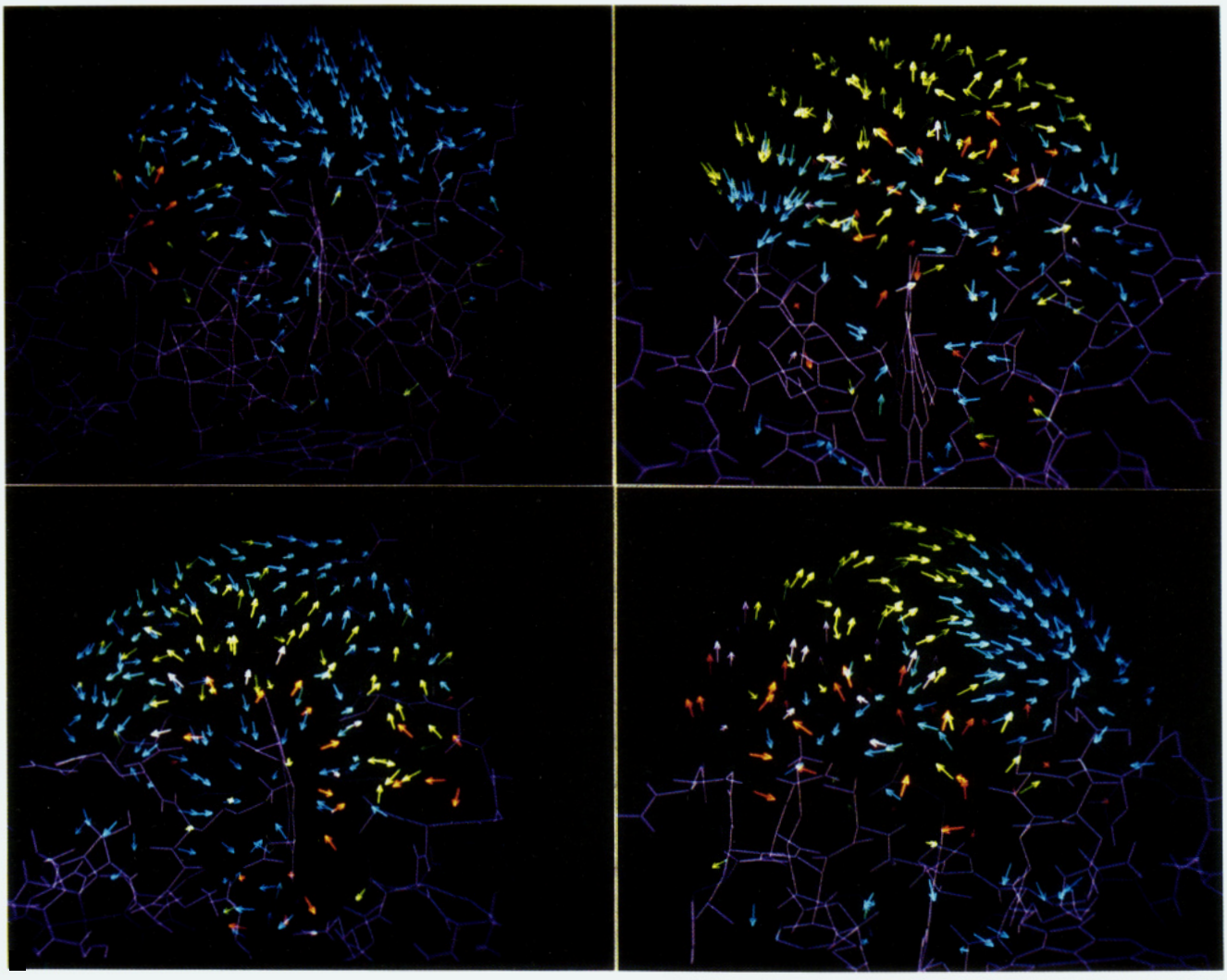

FIGURE 3: Electrostatic field vectors around the heme regions in *D. vulgaris* Miyazaki cytochrome c_3. The vectors point in the direction in which a negative charge would move if placed in the field and are colored according to magnitude as in Figure 2 (red = <−5 kcal/mol; yellow = −5 to 5 kcal/mol; blue = >5 kcal/mol). The field vectors were calculated at points on concentric spheres of radius up to 14 Å (see Table I). (Top left) heme 1; (top right) heme 2; (bottom left) heme 3; (bottom right) heme 4. The atoms of the protein and hemes are shown in purple. The hemes are shown edge on. Note: vectors that are located in the interior of the molecule are artifacts of the calculations and should be disregarded.

1977, 1980; McDonald et al., 1974). X-ray structure studies of this protein are in progress (Sieker et al., 1986), and preliminary results indicate that the structure is similar to that of the protein from *D. vulgaris* (L. C. Sieker and L. LeGall, personal communication). With this preliminary indication and the sequence and structural homology discussed above, the *D. vulgaris* protein may serve as a model for the interpretation of the NMR data obtained from experiments on the protein from *D. gigas*.

These *D. gigas* NMR studies have shown that the hemes are apparently specific for different redox partners. Of particular importance to the present studies are the resonances of the four methyl groups of each of the four hemes. Thirteen of the sixteen resonances are shifted downfield far enough to be assigned and examined experimentally, while the remaining three are lost in the aromatic region. The reoxidation patterns for cytochrome c_3 1:1 mixtures with rubredoxin (Moura et al., 1980), flavodoxin (Moura et al., 1980), and ferredoxin (Moura et al., 1977) indicate that each pairing gives unique effects. All increase the rate of electron exchange, but the complete reoxidation of all four hemes follows different time courses and may involve different reoxidation orders. The pattern of resonances affected for each redox partner is different. Santos et al. (1984) have assigned the most negative redox potential to a heme having a methyl group with the largest chemical shift (methyl resonance 1). In their studies of flavodoxin binding by NMR, Moura et al. (1980) also showed that this resonance is shifted by flavodoxin binding. While an earlier study (McDonald et al., 1974) comparing the NMR of the two cytochromes c_3 shows considerable differences in the behavior of the methyl resonances, the pH and temperature dependence of the most shifted resonances are similar. Thus, a working hypothesis would assign methyl resonance 1 to the heme involved in flavodoxin binding and identified structurally by this study.

Stellwagen (1978) examined the relationship of heme surface exposure to redox potential for a number of cytochromes having known three-dimensional structures. It was found that as heme solvent exposure decreased, the redox potential increased. With *D. vulgaris* cytochrome c_3, the least exposed heme is heme 1 (107.5 Å^2). Thus assignment of methyl resonance 1 to this heme is consistent with the *D. gigas* assignments of Santos et al. (1984). Recent NMR results with the proteins from *D. vulgaris* (J. J. G. Moura and I. Moura, unpublished results) also support the assignment of the site of interaction of flavodoxin with the heme assigned to methyl

resonance 1. However, these studies indicate that rubredoxin binds at the same site as flavodoxin in this case. Preliminary modeling studies indicate that the nonheme iron of *D. vulgaris* rubredoxin is located so that the nearby surface is electrostatically negative and can be docked to the heme 1 site of cytochrome c_3 with a match of ionic and hydrogen-bonding groups similar to that reported here. This results in a heme to nonheme iron distance of less than 7 Å and is consistent with broadening seen for NMR methyl resonance 1 upon formation of the 1:1 complex. A full report on modeling and NMR studies of the rubredoxin–cytochrome c_3 interaction is now in preparation.

An interesting point to note is that flavodoxin and ferredoxin are able to carry out the same function, at least in two physiologically important reactions—the reduction of sulfite by molecular hydrogen (LeGall & Hatchikian, 1967) and the evolution of hydrogen from pyruvate (Hatchikian & LeGall, 1970)—despite the fact that they contain different classes of prosthetic groups (Odom & Peck, 1984). It is expected that the FMN-containing protein would interact with a heme in such a way that the heme and flavin ring are in close contact (Weber & Tollin, 1985; Simondsen et al., 1982). Similarly, Wherland and Gray (1977) have argued that outer sphere electron-transfer mechanisms are favored in the interactions of hemes with proteins containing metal centers.

Previous NMR data (Moura et al., 1982) indicate that the methyl resonances of *D. vulgaris* cytochrome c_3 give similar reoxidation patterns to those of *D. gigas*. Thus, this study immediately suggests a set of NMR experiments examining the interactions of the *D. vulgaris* proteins that are currently in progress in our laboratories. We are currently investigating the interactions between the electron-transfer proteins from *D. vulgaris*, *D. gigas*, and *D. desulfuricans* by NMR, structure prediction, and modeling studies.

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Cytochrome *c* and c_2 Binding Dynamics and Electron Transfer with Photosynthetic Reaction Center Protein and Other Integral Membrane Redox Proteins†

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ABSTRACT: To further the understanding of the details of *c*-type cytochrome action as a redox carrier between major electron-transfer proteins, the single-turnover kinetics time course of cytochrome *c* and cytochrome c_2 oxidation by flash-activated photosynthetic reaction center (purified from the bacterium *Rhodobacter sphaeroides*) has been examined under a wide variety of conditions of concentration, ionic strength, and viscosity with reaction center present in detergent dispersion and phosphatidylcholine proteoliposomes. We find that the three-state model proposed by Overfield and Wraight [Overfield, R. E., & Wraight, C. A. (1980) *Biochemistry 19*, 3322–3327] is generally sufficient to model the kinetics time course; many similarities are found with the cytochrome *c*–cytochrome *c* oxidase interaction in mitochondria. Further, we find the following: (1) Significant "product inhibition" by oxidized cytochrome *c* (c_2) bound to the reaction center is apparent. (2) The viscosity sensitivity of the electron transfer into the reaction center from bound cytochrome *c* (c_2) suggests a physical interpretation of the distal state. (3) The exchange dynamics of oxidized and reduced cytochrome *c* (c_2) are similar regardless of the state of activation of the reaction center. (4) Preferential binding of the oxidized form of cytochrome *c* is revealed upon reconstitution of the reaction center into neutral lipid vesicles, permitting an independent confirmation of the binding suggested by the kinetics. (5) Flash-activated electron-transfer kinetics in reaction center hybrid protein systems have shown that diffusion and competitive binding characterize the behavior of cytochrome *c* as a redox carrier between the reaction center protein and either the cytochrome bc_1 complex or the cytochrome *c* oxidase.

Cytochromes of the *c* type have been intensively studied for many years. Since many are small, water soluble, and crystallizable, they have provided a convenient vehicle for study of a wide variety of questions of cytochrome structure–function using almost all biochemical and physical techniques. In physiological terms, their role is well established (Dutton, 1986). In prokaryotic and eukaryotic respiratory systems, a cytochrome (cyt)[1] *c* operates to shuttle electrons between the much larger integral membrane redox proteins, ubiquinol–cyt-*c* oxidoreductase (cyt bc_1 complex) and cytochrome *c* oxidase. In photosynthetic bacteria, they act similarly between analogous ubiquinol–cyt-c_2 oxidoreductase and the photochemical reaction center (RC) protein. In all these systems, the structural arrangement is the same: the cyt *c* is located in the aqueous periplasmic space outside the intracytoplasmic membrane and inside the outer membrane or cell wall. The subunits of the membrane redox proteins containing immediate electron-donating or -accepting redox components for cyt *c* are exposed to the periplasm; cyt *c* appears to diffuse between these reaction sites along the membrane–aqueous interface and through the aqueous solution (Hochman et al., 1985; Vanderkooi et al., 1985; Ferguson-Miller et al., 1986). At another level, investigations into the structure and dynamics of cyt *c* complexation with the membrane protein sites where electron transfer occurs have been fewer; nevertheless, useful models built upon early observations interpreted as multiple binding states of cyt *c* with cyt *c* oxidase (Ferguson-Miller et al., 1976) and reaction center proteins (Dutton et al., 1975; Dutton & Prince, 1978) have been presented.

A unique route to the understanding of the details of cyt *c* action as a redox carrier between major electron-transfer proteins is offered by photosynthetic bacteria, where the opportunity to initiate electron transfer by light provides far-reaching experimental benefits. The delivery of a brief flash of light that turns over the RC just once permits the electron-transfer reactions such as cyt *c* oxidation to be studied without the complications attendant with steady-state analysis; elementary first- and second-order reactions can be monitored as they progress and transient states are revealed. The approach has the potential to expose binding properties, stable and unstable reaction states, and also functional details that would be difficult or perhaps impossible to uncover with conventional forms of activation.

The first application of this approach with isolated RC preparations was by Ke et al. (1970). They used early, high molecular weight RC preparations in the detergent Triton and

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[1] Abbreviations: $[BChl]_2$ and $[BChl]_2^+$, normal and oxidized bacteriochlorophyll dimer, respectively; cyt, cytochrome; cyt bc_1 complex, ubiquinol–cytochrome-*c* (c_2) oxidoreductase; EDTA, ethylenediaminetetraacetic acid; K_D, dissociation constant; LDAO, lauryldimethylamine oxide; *Rb.*, *Rhodobacter*; RC, reaction center; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; SDS, sodium dodecyl sulfate.

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