STRUCTURE/FUNCTION RELATIONSHIPS IN BIOLOGICAL ELECTRON TRANSPORT PROTEINS[1]

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I. INTRODUCTION

With few exceptions, all organisms are primarily dependent upon transducing electron transport chains for the production of adenosine triphosphate, the common high-energy chemical intermediate of living cells (Figure 1). Such biological electron transport chains are generally composed of two different types of electron carriers; those capable of conserving a change in the electromotive potential of a reducing electron as a chemically useful energized state (non-isopotential electron transfer proteins), and others which serve to transfer electrons between sites of energy conservation (isopotential electron carriers). Very little information is currently available concerning the structure of any of the non-isopotential energy conserving proteins, owing to the fact that they are generally integrally bound membrane proteins which do not lend themselves to crystallographic structure analysis. In contrast, there exists a wealth of structural information about many classes of the isopotential electron transport proteins, which are generally freely soluble molecules which undergo reversible binding interactions with their physiological oxidoreductases.

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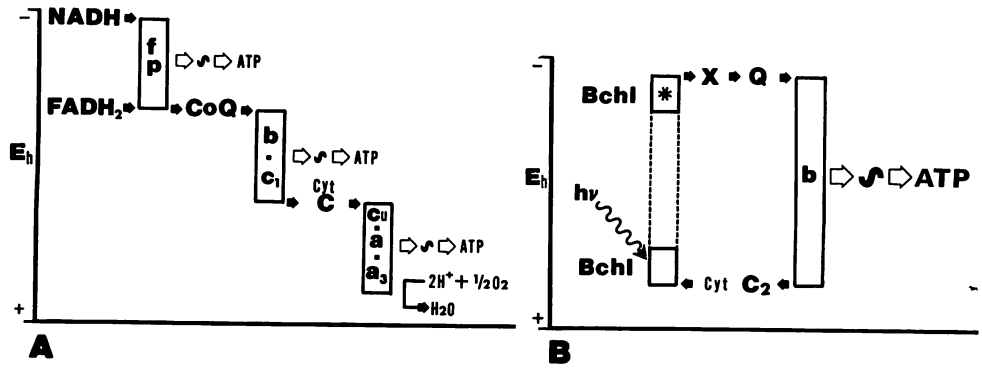

FIGURE 1. Energy-transducing electron transport chains.
A. The mitochondrial electron transport chain, in which reducing equivalents derived from the oxidation of carbohydrates are finally accepted by oxygen to form water.
B. A bacterial cyclic photophosphorylating electron transport chain, in which reducing equivalents are generated by the direct input of light energy. Solid arrows show sites of isopotential electron transfer events between isopotential electron carriers and non-isopotential energy conserving proteins.

Before dealing with the structures themselves, it is first appropriate to ask why biological organisms have evolved these enormously complex structures, consisting of thousands of atoms, for the purpose of transporting and transferring what is generally a single electron, when this function might appear to be equally well served by some small inorganic complex. One answer is certainly that the associations made between the protein polypeptide chain and a relatively small number of naturally occurring biological prosthetic groups lead to a functional diversity and evolutionary flexibility which would be extremely difficult to attain if only small inorganic or organic molecules were utilized for this purpose. As will be shown, it is now apparent that there are a variety of ways in which proteins interact with their prosthetic groups which result in a modification of prosthetic group physicochemical properties. These range from well understood electronic effects arising from the substitution of different protein contributed ligands to otherwise similar metal prosthetic groups to much less well-understood "environmental" effects.

A second, at least equally important, consideration arises from the observation that biological electron transfer processes, in common with most other protein mediated biological interactions, evidence exceptional specificity. The existence of such reactive specificity between reversibly interacting protein molecules clearly indicates the predominant role played by the polypeptide chain in furnishing the specific structurally complementary interactions required for the formation of a productive protein electron transfer complex. Although it might be argued that these specific complementary interactions are by themselves sufficient to promote efficient electron transfer, since they can serve to locate the reacting prosthetic groups both in close proximity and in the proper relative orientation, the true situation may be much more complex. For example, it is now apparent that for most catalytically active proteins (e.g. enzymes), the specific binding interactions between the protein and its substrate serve to distort the substrate towards the transition state, thus in effect utilizing the energy of the specific substrate binding interactions to lower the activation energy to product formation. Consequently, an electron transport protein should not be viewed as merely an appropriately "adjusted" prosthetic group surrounded by a polypeptide chain which confers specificity by virtue of its surface interactions, but rather as an integrated whole whose specific interactions with its physiological oxidoreductants may directly facilitate the electron transfer event.

From the preceeding discussion, it is apparent that the dependence of reactive specificity upon direct intermolecular interaction places upper limits on the range of the actual electron transfer process between reversibly binding electron carriers. In fact, as will be shown below, the available structural data indicate that for the majority (but not all) of known electron transport protein structures, the reactive prosthetic group is disposed to allow more or less direct interaction with the external environment. This would suggest that, in most cases, these molecules react by mechanisms involving intimate interactions between the prosthetic

groups in the transition state. However, it is by no means clear whether these intimate interactions are sufficient to allow electron transfer to take place by classical outer-sphere reaction mechanisms which require direct orbital overlap of the reacting species (1) or are better described as short-range tunneling processes (2).

We now turn to a consideration of several known electron transport structures which will serve to illustrate the preceding points.

II. ELECTRON TRANSPORT PROTEIN STRUCTURES

A. Flavin Containing Proteins

The only family of flavin containing electron transport proteins of known structure are the bacterial flavodoxins. These molecules are composed of a single polypeptide chain of ∿138 amino acid residues and a single non-covalently bound flavin mononucleotide (FMN) prosthetic group (Figure 2). Two related molecular species have been structurally investigated by research groups headed by L. Jensen at Seattle [*Desulfovibrio vulgaris* flavodoxin (3)], and M. Ludwig at Ann Arbor [*Clostridium MP* flavodoxin (4)]. This molecule presents an interesting example of how non-covalent interactions made between the protein polypeptide chain and the prosthetic group can serve to alter prosthetic group oxidoreduction potential.

Free FMN is a prosthetic group capable of sequentially taking up two reducing electrons. The reduction potential for the addition of the first electron, which leads to the production of the free radical semiquinone form of the flavin, is -238 mV. Addition of a second electron to form the fully reduced dihydroquinone requires a reducing potential of -172 mV. In contrast, measured values for the successive reduction steps in the intact protein are -92 mV and -399 mV, respectively. Consequently, it can be seen that in the protein, the first FMN reduction is easier than for free FMN, while the second reduction is substantially more difficult.

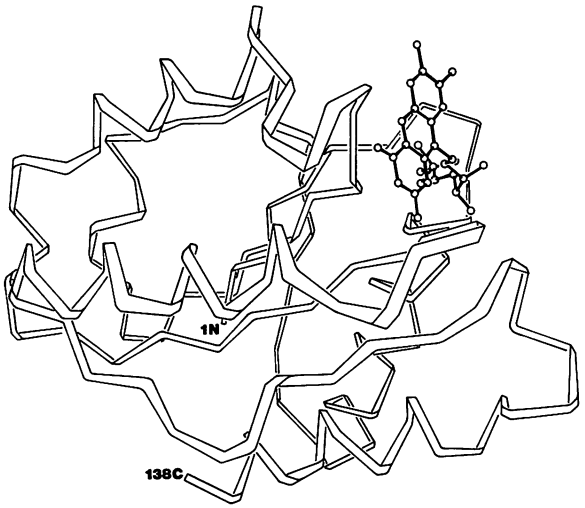

FIGURE 2. A ribbon backbone representation of the flavodoxin molecule, showing the situation of the non-covalently bound FMN prothetic group.

Crystallographic studies of flavodoxin having the FMN prosthetic group in the oxidized, semiquinone, and fully reduced form provide an explanation for these phenomena as follows (5): reduction of the oxidized flavin to the semiquinone in the protein is relatively facile because the polypeptide chain can make more favorable hydrogen-bonded interactions with the semiquinone radical isoalloxazine ring than with the fully oxidized flavin. This effect also manifests itself in an apparent increase in the binding constant of the protein for the semiquinone FMN relative to the fully oxidized flavin. Further, it is known from structural studies of model compounds that full reduction of free flavin to the dihydroquinone is accompanied by a sizable deviation of the isoalloxazine conjugated ring system from planarity. This distortion does not appear to take place in the protein-bound FMN, due to the intimate interactions between the polypeptide chain and the flavin ring. Indeed, these interactions appear to constrain the isoalloxazine ring in an approximately planar configuration even when it is fully reduced, resulting in a much lower reduction potential for this structurally constrained group in the protein relative to that seen for an unconstrained flavin in solution.

B. Non-heme Iron Proteins

These proteins generally contain one or more tetrahedrally coordinated iron atoms ligated by sulfur atoms contributed from protein cysteine side chains and/or inorganic sulfur. Three distinct members of this family have been structurally elucidated.

1. Rubredoxin. This is a small 54 residue protein, derived from the bacterium *Clostridium MP*, whose structure determination was carried out by L. Jensen and co-workers in Seattle (6). Rubredoxin is among the simplest of electron transfer proteins since it contains a single iron atom bound in tetrahedral coordination to four sulfur atoms furnished by protein cysteine side chains (Figure 3). The $E_{m,7}$ for this protein is 0.0 mV.

2. Ferredoxin. This is another small (54 residue) bacterial non-heme iron protein whose structure was determined by the Seattle group (7). The molecule contains two non-heme iron clusters, each composed of an approximately cubic array of four iron atoms and four inorganic sulfur atoms occupying alternate corners of the cube (i.e., the iron and inorganic sulfur atoms form interdigitating tetrahedra) (Figure 4). Each cluster is bound to the poly-

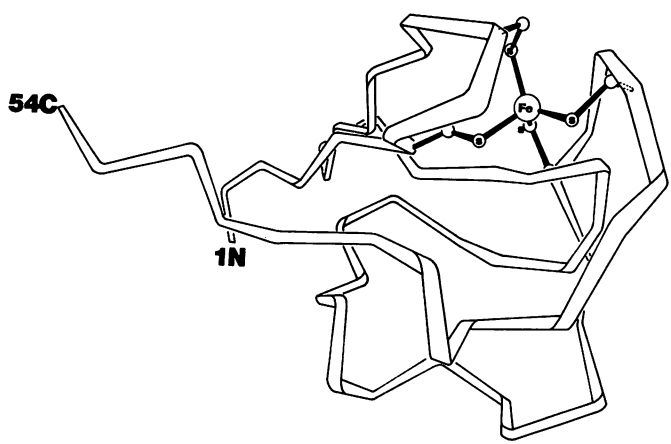

FIGURE 3 The bacterial non-heme iron protein, rubredoxin.

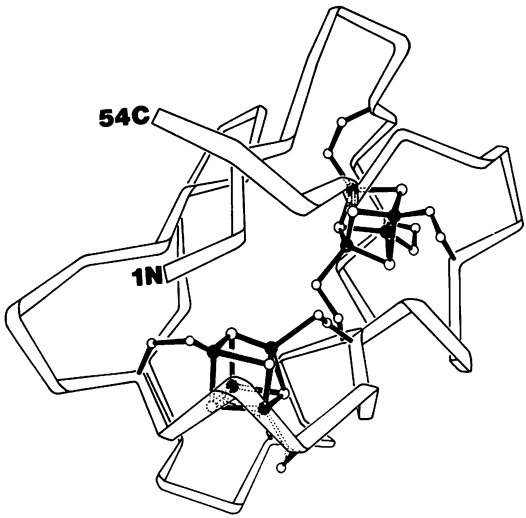

FIGURE 4. The bacterial non-heme iron protein, ferredoxin, containing two four-iron - four sulfur clusters.

peptide chain by four additional bonds made between the iron atoms and cysteine sulfur atoms, so that each iron atom is approximately tetrahedrally coordinated to three inorganic sulfur atoms and a sulfur ligand contributed by the protein. The reduction potential for these groups is -400 mV.

3. Chromatium *High Potential Iron Protein (HIPIP).* This is yet another bacterial non-heme iron protein, the structure of which was determined by C. Carter and J. Kraut at La Jolla (8). This protein contains a four-iron - four-sulfur prosthetic complex which appears to be essentially identical to that found in ferredoxin (Figure 5). However, as its name implies, the midpoint potential of the cluster in this protein is +350 mV, in contrast to the much lower potential observed in ferredoxin. A variety of spectroscopic and model studies now indicate that, in fact, the four-iron - four-sulfur cluster can accomodate two reducing electrons and that HIPIP oscillates between a fully oxidized and a singly reduced state, whereas ferredoxin oscillates between the singly and doubly reduced cluster oxidation states (9). At present it is unclear what specific structural differences in the cluster environments of these two proteins result in their completely different oxidoreduction properties.

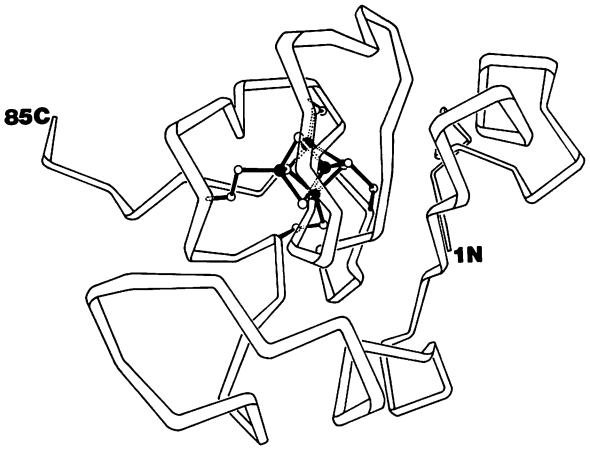

FIGURE 5. Chromatium *HIPIP, a high-potential non-heme iron protein. The prosthetic group of this protein is, in contrast to most other known electron transport proteins, shielded from direct contact with the external environment.*

C. Heme Proteins

The iron protoporphyrin complex (heme) is a prosthetic group found in many variations in biological macromolecules where it serves diverse functions in catalytic (e.g., peroxidases), transport (e.g., hemoglobin), and electron transfer (e.g., cytochromes) processes. Several heme-containing electron transfer protein structures are now known, including one member of the cytochrome *b* class and five members of the cytochrome *c* class. These two cytochrome classes are structurally distinguished according to the nature of the interactions made between the polypeptide chain and the protoheme IX prosthetic group. Specifically, the heme group in *b*-type cytochromes is not (exclusive of axial iron ligands furnished by the protein) covalently bound to the polypeptide chain. Cytochromes *c*, in contrast, contain the same protoheme IX prosthetic group, which is covalently attached to the polypeptide chain via thioether linkages formed by condensation of the heme IX vinyl groups with cysteine side chains of the protein.

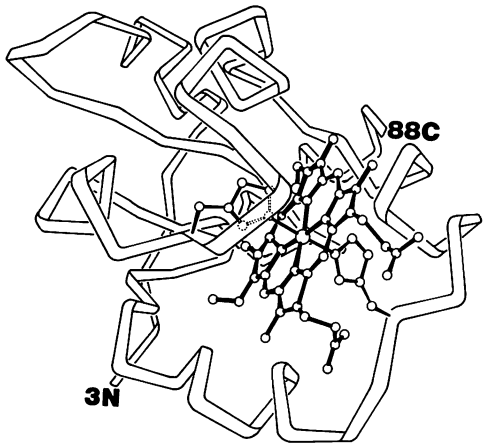

FIGURE 6. Microsomal cytochrome b_5*: a protein having a non-covalently bound heme prosthetic group and histidine imidazole nitrogen atoms as fifth and sixth heme iron ligands.*

1. Cytochrome b_5. This cytochrome is found in the microsomal membrane fraction of eucaryotic cells, to which it is quite firmly anchored by means of hydrophobic interactions furnished by the carboxy terminus of the polypeptide chain. However, a functionally intact, soluble form of the protein may be liberated by proteolytic cleavage of the hydrophobic anchoring peptide from the remainder of the molecule. The structure of this latter fragment, comprising about 90% of the total sequence, has been determined by Scott Mathews and co-workers at St. Louis (10) (Figure 6). The non-covalently bound heme is essentially completely enveloped by the polypeptide chain, leaving only one edge relatively solvent accessible. Strong field, axial coordinate heme iron ligands are furnished by two histidine imidazole nitrogen atoms, resulting in low spin heme complex in both oxidized and reduced states. The $E_{m,7}$ of the protein is +10 mV.

2. Cytochromes c. Representatives of this class of heme proteins have been isolated from virtually all organisms utilizing photosynthetic or oxidative electron transport chains for the production of ATP. Although the designation "cytochrome *c*" strictly

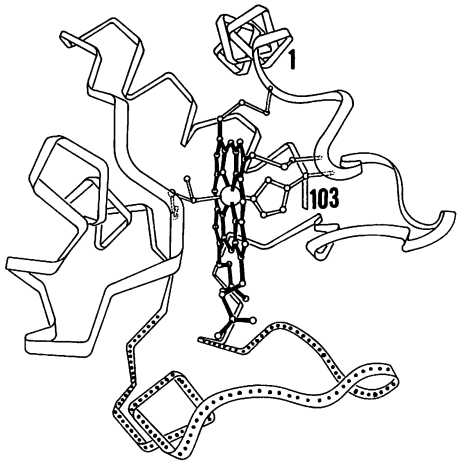

FIGURE 7. Mitochondrial cytochrome c. *This structure contains a covalently bound heme IX prosthetic group. Axial heme iron ligands are furnished by a histidine imidazole nitrogen and a methionine sulfur atom. Molecules derived from various photosynthetic and denitrifying bacteria show extensive overall similarity to the eucaryotic mitochondrial protein. Dotted section shows the region structurally deleted in the smaller cytochromes.*

refers to any protein having a covalently bound heme prosthetic group, structural studies have thus far been limited to those soluble proteins which appear to belong to a single evolutionary family, and share common physiological function as electron donors to the most oxidizing carrier of the electron transport chain in which they function. All members of this soluble class of *c*-type cytochromes share similar structural features, and are low-spin complexes having a histidine imidazole nitrogen and a methionine sulfur atom as axial heme iron ligands (10,11). Figure 7 shows a ribbon representation of mitochondrial cytochrome *c* as determined by Dickerson and co-workers at the California Institute of Technology (12). This structure is strikingly similar to those of two bacterial species, *R. rubrum* cytochrome c_2 (14) and *Paracoccus denitrificians* cytochrome c_{550} (15), despite the fact that *R. rubrum* cytochrome c_2 functions in a photosynthetic electron transfer chain.

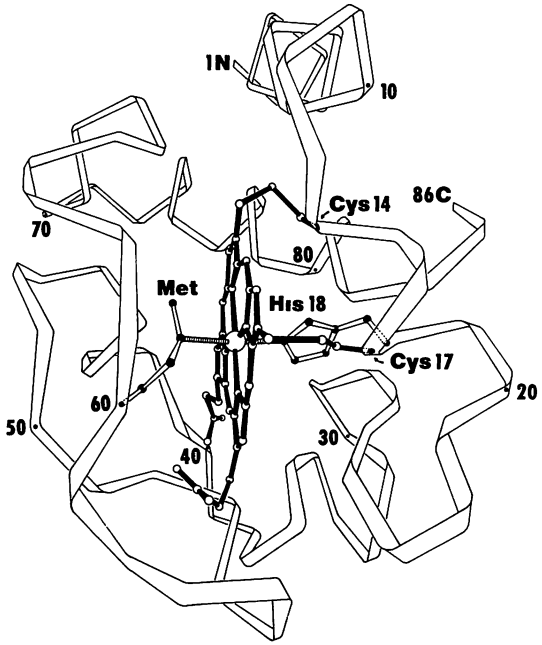

FIGURE 8. A representative small c-type cytochrome: cytochrome c_{555} *of* Chl. thiosulfatophilum. *This molecule principally differs from that shown in Figure 7 by a large deletion (shown dotted in Figure 7), forming the bottom of the larger molecules. However, rearrangement of the left side of the smaller molecule preserves the hydrophobic heme environment found in the larger molecules.*

Recently two additional bacterial cytochrome *c* structures have been determined, *Pseudomonas* cytochrome c_{551} (13) and *C. thiosulfatophilum* cytochrome c_{555} (14), which appear to constitute a structural subclass of the cytochrome *c* family as elaborated in the caption of Figure 8.

A structural characteristic common to all of these molecules is the large number of interactions of both covalent and hydrogen bonded character which constrain the heme so that only one relatively hydrophobic edge of the heme is solvent exposed, despite the fact that this necessitates an extensive set of internal interactions to stabilize the buried heme propionic acid groups (Figure 9).

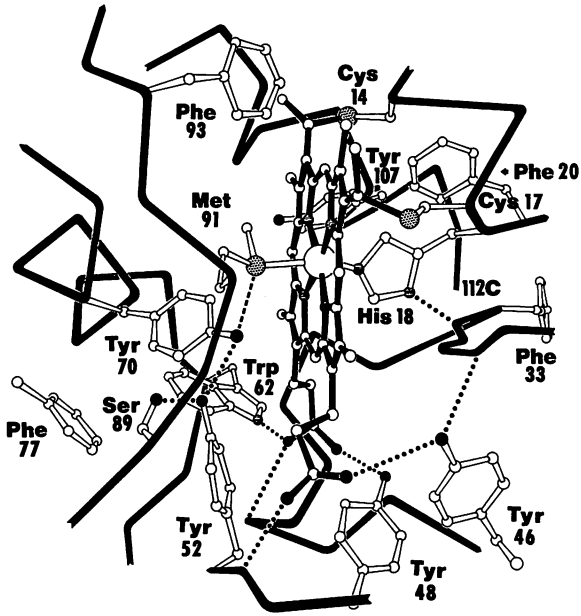

FIGURE 9. A close-up of the heme environment in R. rubrum *cytochrome* c_2*, showing the extensive set of covalent and hydrogen bonded interactions serving to orient the heme such that one hydrophobic edge is solvent-exposed.*

A further interesting point which emerges from this study of these structurally related molecules concerns the means by which the heme oxidoreduction potential is regulated by its interaction with the protein. The midpoint potential of *C. thiosulfatophilum* cytochrome c_{555} is +145 mV, some 100 to 200 mV lower than the other cytochromes *c* of known structure, and 140 mV lower than its closest known structural relative, *Pseudomonas* cytochrome c_{551}. This observation is of particular interest since it has been argued by Kassner (18,19) that the principal factor responsible for the regulation of heme prosthetic group potential is the hydrophobic nature of the heme environment. Basically, it is suggested that the difficulty of accommodating a positive charge on the oxidized heme iron, relative to the uncharged reduced heme state, is dependent upon the dielectric constant of the local heme environment.

Thus, heme prosthetic groups buried in the low dielectric hydrophobic interior of proteins (as they are in cytochromes *c*) will be more stable in the uncharged reduced state, and will consequently exhibit higher oxidoreduction potentials than otherwise identical prosthetic groups situated in an aqueous environment of high dielectric constant. While this effect undoubtedly accounts for much of the observed oxidoreduction potential difference between aqueous heme complexes and cytochromes *c*, it does not appear that it can account for the observed potential differences between cytochrome c_{555} and other cytochromes *c* of higher potential, which have shown no quantifiable differences in heme exposure or hydrophobicity of their heme environments.

Instead, it is possible that the observed differences in oxidoreduction potential among closely related cytochromes may be better interpreted in terms of differences in their dynamic, rather than their static structural properties. In this context, it is of particular interest to note that protein structures are in large part held together by weak forces (e.g. hydrogen bonded and van der Waals interactions), and that the polypeptide chain itself structurally admits considerable torsional freedom about single bonds. Structures of this sort would be expected to exhibit a continuum of vibrational states throughout which the ambient thermal energy of the environment could be partitioned. In fact, there exists a considerable body of experimental data indicating that the oxidized form of cytochrome *c* is considerably less rigid and thermally stable than the reduced form of the molecule (11). Since several crystallographic studies have shown the oxidized and reduced conformations to be essentially identical, it seems reasonable to conclude that the redox-coupled "conformational change" really manifests differences in what are probably small amplitude vibrational states of the cytochrome molecule. This sort of dynamic structural difference would, of course, be undetectable in the time-averaged view of the molecule obtained crystallographically.

In the case of cytochromes *c*, the most likely means by which heme reduction is directly coupled to the remainder of the molecular structure is through the sixth ligand methionine sulfur-heme iron bond. Indeed, several studies indicate that this bond is 100 to 1000 times stronger in the reduced form of the molecule than in the oxidized form (10), and as can be seen from Figure 7, this bond forms one of the principal covalent interactions serving to hold the left and right sides of the cytochrome *c* molecule together.

The salient point which emerges from this discussion is that the free energy change which the molecule undergoes upon reduction, which is directly proportional to the observed oxidoreduction potential, manifests itself as change in the vibrational properties of the molecule as a whole. It is consequently clear that if different cytochrome molecules have different vibrational properties (i.e. different relative thermal stabilities in their oxidized and reduced states), this will directly manifest itself in the observed redox potential of that protein. Indeed, typical potential differences among related cytochromes reflect energetic differences of 1 to 5 Kcal mol^{-1} for structurally similar prosthetic groups, values which appear most reasonable for small amplitude coupled vibrations of an extended polypeptide structure. From this standpoint, it is apparent that one advantage gained by wrapping a prosthetic group in a complicated polypeptide structure is that the prosthetic group potential can literally be adaptively tuned during evolution, due to the inherent low energy vibrational properties of the surrounding polypeptide structure.

III. MECHANISMS

Traditionally, the principal advantage of X-ray structural studies with respect to the elucidation of biological mechanisms is that it has been possible, by some subterfuge, to trap the molecules in the act. Unfortunately, this has not turned out to be technically feasible for interacting electron transport proteins, and since there generally appears to be little structural difference in the oxidized

and reduced molecular conformations, we are limited to observations of the isolated molecules and some inspired guessing.

By comparing structurally related molecules, however, it is possible to define some minimal structural requirements which might be important in the interactions and mechanism of a given class of electron transport proteins. In the case of the larger cytochromes *c* for example, it is apparent that they share a sequentially and/or structurally conserved ring of positively charged side chains about the perimeter of their heme crevices (11). Considerable evidence suggests that this positive charge ring is important in mediating the interactions between cytochromes *c* and both their physiological oxidases and reductases, and implies that heme oxidoreduction takes place by some mechanism involving essentially direct insertion or withdrawal of the electron at the exposed heme edge (11). Indeed, with the exception of HIPIP, all of the molecules described above appear to show at least some degree of prosthetic group exposure. Nevertheless, the picture presented by these skeletal drawings is misleading, and more realistic representations of the molecular surfaces, as shown in Figure 10, suggest that despite the apparent prosthetic group exposure, it is by no means clear that direct orbital overlap between prosthetic groups could be attained in an intermolecular electron transfer complex. Figure 11 shows a model for what might be best described as a pre-transition state complex between cytochromes *c* and b_5, which react readily although they are not natural physiological partners (21). Although there are many features of this hypothetical complex which are of mechanistic interest (11,21), the important point in the present context is that the closest approach between resonant atoms of the two heme groups is about 8.4 Å. This is very close to the range suggested by Hopfield for a thermally-activated tunneling process. However, it is possible that some degree of structural distortion (such as an integral feature of the Marcus theory) could accompany formation of the transition state in such a complex. This might allow direct orbital overlap between the prosthetic groups to occur,

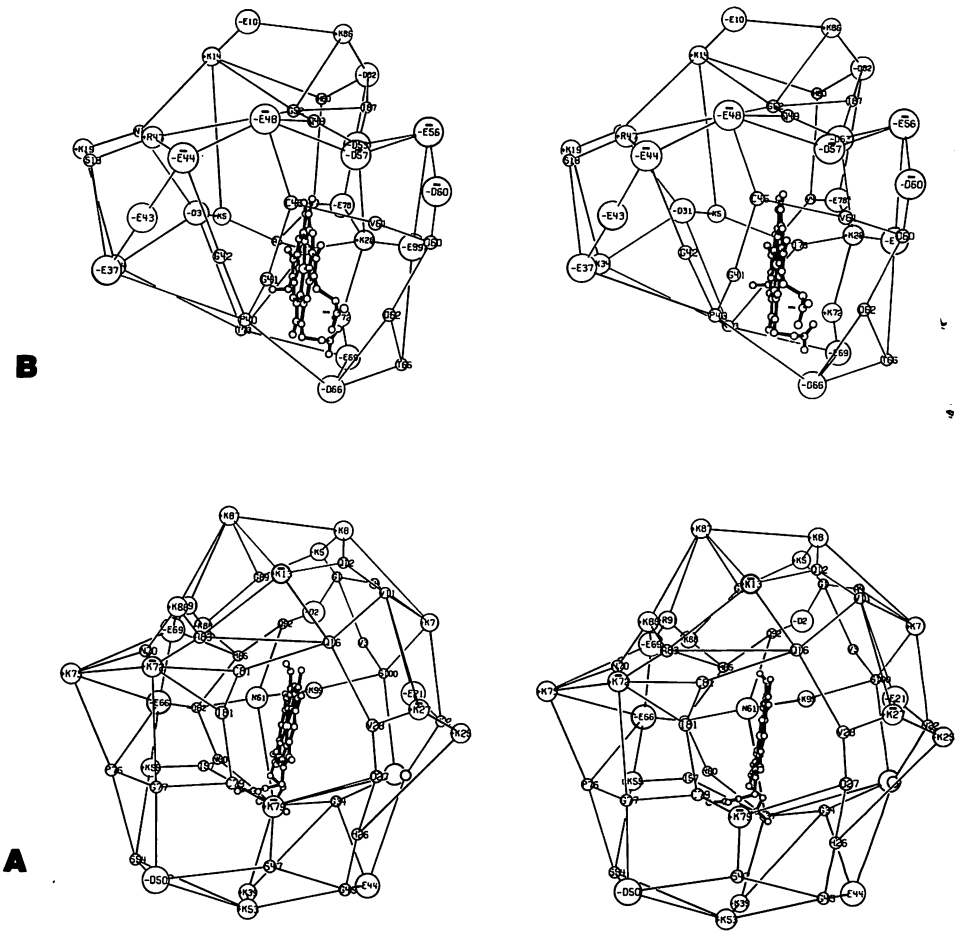

FIGURE 10. Polyhedral surface maps of A) cytochrome c and B) cytochrome b_5 *generated by connection of surface, solvent-exposed side chain atoms. Orientations are similar to Figures 6 and 7. This representation gives a more realistic picture of prosthetic group accessibility.*

and consequently allow electron transfer to proceed by classical outer-sphere reaction mechanisms. Irrespective of which model is used to describe the electron transfer process, a requirement for efficient transfer is that the reacting species be appropriately coupled vibrationally in the transition state. From the preceding

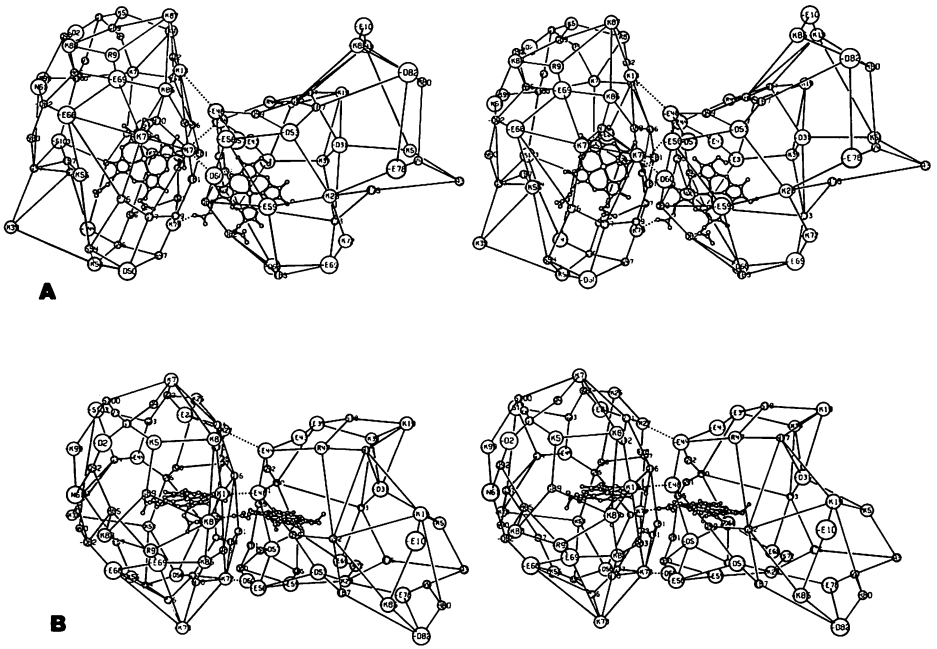

FIGURE 11. Stereoscopic view of a computer-generated complex of cytochromes c *and* b_5*, illustrating the nature and extent of interactions possible between electron transport proteins. Cytochrome* c *is on the left in these figures.*

discussion of the importance of dynamical structural factors in the regulation of the cytochrome heme potential, it is apparent that the maintenance of particular vibrational modes of the interacting molecules could play a critical role in the facilitation of the electron transfer process itself. Thus, the specificity of interaction manifest in biological electron transfer reactions may not only reflect the complementary specificity of surface interactions serving to juxtapose the reacting prosthetic groups in the proper proximity and orientation, but may also reflect similarities in the fundamental dynamic properties of the interacting proteins as a whole.

ACKNOWLEDGMENTS

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DISCUSSION

BOGOMOLNI: Can you give us an estimate of how much the solution conformation of all these molecules differs from their conformation in the crystals used for your molecular structure analysis?

SALEMME: Probably very little, since the crystals are generally 30% to 50% by volume water, so that the protein molecules in the crystal are really in an environment quite similar to aqueous solution. In addition, there is little cause to expect that the observed tenuous crystal packing interactions would be of sufficient energy to distort the molecules from their solution equilibrium conformations.

FEINBERG: The cytochrome *c* - b_5 couple appears to be an excellent model for both theoreticians and experimentalists. Has a binding constant been determined for the interaction of these two cytochromes?

SALEMME: Dr. Cusanovich has carried out some experiments aimed at answering this question, but at present about all that can be said is that the interaction appears to be weak.

BENNETT: What occupies the 8.4 Å separation between heme rings in the cytochrome *c* - cytochrome *b* complex?

SALEMME: Primarily surface amino acid side chains of the two molecules. In the hypothetical complex, there is essentially complete exclusion of bulk water from the intermolecular interface region. It is possible, however, that isolated water molecules might be accomodated.

FEINBERG: What is the redox potential difference between cytochrome c_{551} and c_{555}?

SALEMME: This is approximately 200 mV, and probably results from a difference in their vibrational modes, in one or the other of their oxidation states.