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ELECTRON-TRANSFER PROTEINS*

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CONTENTS

A. INTRODUCTION

The functions of the electron-transfer proteins of biological systems are not understood in any detail. This is a long-standing problem, for the existence of such proteins was clearly recognised some fifty years ago. In this article we shall re-appraise the facts about their nature before indulging in any speculation as to their mode of action. This re-appraisal is necessary for the accumulation of new data even in the last two or three years has been very rapid. The article starts with a summary of the existing ideas about electron-adsorbing sites and their relative energies (redox potentials) and

* The substance of this review was given at the Harden Conference, England, 1974.

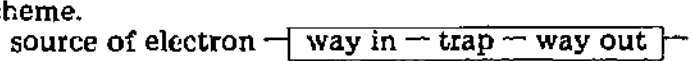
then describes in a general way the theories of electron mobility which physicists have found useful. A few chosen systems are mentioned showing to which systems the particular theories have been applied. The evidence is mostly from small "model" systems.

In subsequent sections there is a description of the different types of electron-transfer proteins and in these sections reference is made to the above facts and theories. The first electron-transfer proteins to be described are the cytochromes. Major weight is put on those of known structure but, in view of the effects of minor changes in amino acid sequence, the structural data will be considered critically in the light of a large number of sequences. Another family of proteins which is becoming well known is that of the iron—sulphur proteins. Here again we shall start from structures but we shall also keep the variations in sequences strongly in the forefront of our description. Turning to less well-known proteins we tackle the copper "blue" centres. Structures of these proteins are not known and even the coordination site is in doubt. Finally recent NMR work indicates that proteins quite generally may well be much more dynamic than is commonly conceded.

Given this limited knowledge of the character of the different groups of electron-transfer proteins the obvious property that we shall try to explain first is their redox potentials. Thermodynamic data should be understood more easily than kinetic data. We shall show in each section that our understanding remains relatively poor. The difficulty of discussion is not so much the immediate surrounds of the metal in the protein, that is the ligands and the "solvent" in which the complex might be thought to be dissolved, but arises from the interaction of the metal coordination sphere with the rest of the protein. There is no doubt that the redox potential of a metal in a protein can be controlled by a large number of factors involving large parts of the structure.

It is against the background of structure, structural dynamics and redox potentials that we must then examine the electron-transfer processess in metallo-proteins. Again in each section an attempt is made to highlight the best ideas (and their difficulties) we have. The ideas are generated from a study of a large number of small molecule (model) and solid-state systems. More cannot be done at the present time.

The problem of electron transfer in biology cannot be left here. For a biological system the transfer must be directed and in many cases the system has evolved ways of controlling, increasing and decreasing, the transfer rate depending upon demand. Direction can be understood from the following scheme.



The enclosed block represents the electron-transfer protein and at least two channels are needed. This is very different from a model (solution) electron-transfer problem as it provides directed electron transfer. The exercise of control can be achieved by a mechanism which "gates" either the entering or leaving channel.

B. GENERAL REVIEW OF ONE-ELECTRON REDOX ENERGIES AND REACTIONS

(i) Redox potentials

(a) Simple molecular systems in water

The redox potential at 25°C and at pH = 7.0 of a biological redox couple in vivo lies for the most part between −0.5 volts (at which potential $H_2O \rightarrow H_2$) and +0.85 volts (at which potential $H_2O \rightarrow O_2$). Most biological and model complexes have been studied over a range of pH values in water, i.e. in vitro. This has allowed a clear description of the factors affecting redox potentials of the complexes in this solvent. The factors which are separable in theory though not always clearly in practice are as follows:

(i) Electrostatic (negative) charge on the ligand which favours the higher oxidation state; the higher the negative charge, the lower the redox potential.

(ii) The donor power of the ligand, using the pK_a values as a guide to donor power of a series of closely related ligands. A higher pK_a gives rise to a lower redox potential.

(iii) The acceptor power of the ligand using unsaturation as a guide. The greater the π-acceptor power of the ligand the higher the redox potential.

(iv) Changes of spin state which can alter the importance of (ii) and (iii) radically.

(v) Steric factors which can be chosen so that one or other oxidation or spin state is favoured when (ii), (iii) and (iv) could be overridden.

TABLE 1

Redox potentials for some biochemical and chemical couples

System	E^0 (volts)
Cu (2,9-Me_2 1,10 phen)$_2^{2+}$—Cu (2,9-Me_2 1,10 phen)$_2^{+}$	+0.59
Cu (2,Cl-1,10 phen)$_2^{2+}$—Cu (2,Cl-1,10 phen)$_2^{+}$	+0.40
Cu (imidazole)$_2^{2+}$—Cu (imidazole)$_2^{+}$	+0.35
Cu $(NH_3)_2^{2+}$—Cu $(NH_3)_2^{+}$	+0.34
Cu (pyridine)$_2^{2+}$—Cu (pyridine)$_2^{+}$	+0.27
Cu (imidazole)$^{2+}$—Cu (imidazole)$^{+}$	+0.26
Cu (pyridine)$^{2+}$—Cu (pyridine)$^{+}$	+0.197
Cu (1,10 phen)$_2^{2+}$—Cu (1,10 phen)$_2^{+}$	+0.174
Cu^{2+} (aq)—Cu^{+} (aq)	+0.167
Cu(ala)$_2^{2+}$—Cu(ala)$_2^{+}$	−0.130
Cu(gly)$_2^{2+}$—Cu(gly)$_2^{+}$	−0.160
Laccase Cu^{2+}—Cu^{+}	+0.415
Ceruloplasmin Cu^{2+}—Cu^{+}	+0.390
Azurin Cu^{2+}—Cu^{+}	+0.380
Plastocyanin Cu^{2+}—Cu^{+}	+0.370

phen, phenanthroline.

The literature contains many reviews of model systems where these points are described [1—3]. However, the effect of geometric or steric constraints is not often stressed sufficiently. This factor takes on special significance in biology.

Geometric (steric) factors. If a model ligand is designed so that it cannot give the stereochemistry most favoured by a metal cation, then the redox potential reflects the resulting loss of stability of one of the oxidation states. The clear example of sterically hindered orthophenanthrolines, which give high E^0 values with the copper(I)/copper(II) couple, is shown in Table 1 [4]. The case of iron is made more complex by spin-state changes.

Spin-state changes. The iron(II)/iron(III) potential is controlled partly by the spin states of the two oxidation states [5]. Normally the ligand field strength required to drive iron(II) to low spin is lower than that required to drive iron(III) to low spin, for the spin-pairing energy is much greater for iron(III).

A clear series of examples are the phenanthroline complexes where iron(II) goes low spin in the tris-ortho-phenanthroline complexes (E^0 = 1.00 volts) with a much greater stabilisation energy than the iron(III) complexes. This example is a case of the use of neutral ligands to produce low-spin systems. A negatively charged ligand even when unsaturated, such as cyanide, makes both oxidation states low spin but stabilises the iron(III) state most. When we turn to the porphyrin ligands the situation is far more complicated, although the most immediately obvious effect is that the unsaturated anion, porphyrin, has a similar effect to cyanide.

However, porphyrin is itself a stereochemically constrained ring. The stability of complexes based upon ring ligands is known to depend upon the ionic radius of the metal in a critical fashion, for the best binding must be given by a metal ion which is not too large to go into the ring, and yet is not so small that it cannot form good bonds to all the donor groups of the ring simultaneously [6,7]. A ligand such as porphyrin offers a hole which fits the cations better in the order low-spin Fe^{III}>low-spin Fe^{II}>high-spin Fe^{III}>high-spin Fe^{II}. The oxidation state which gains most from a spin-state change cannot be predicted. Once again the biological ligands, both porphyrin and protein, are too complicated for us to be able to make simple statements about the cause of a given effect.

(b) Simple molecular systems in other solvents [8]

The redox potential in a solvent other than water can be treated by reference to partition coefficients.

$$E^0_S = E^0_{H_2O} + \frac{RT}{F}\ln\frac{p_1}{p_2}$$

where p_1 and p_2 refer to the partition coefficients from water into the solvent S of the reduced, p_1, and oxidised, p_2, forms respectively. p_1/p_2 is also depen-

dent upon well-known factors:

(i) The higher the overall charge on a complex the less the extraction into non-polar media. Thus the absolute charges, the sign of the charge difference (i.e. whether it increases or decreases with increase in redox state of the central metal), decide the direction of change $E^0_S - E^0_{H_2O}$ going from water to solvent S.

(ii) The nature of the complexes of the two states is such that they can differ in number of ligands bound or in stereochemistry. It should be remembered that water is a coordinating solvent but not all other solvents can coordinate. Some solvent molecules are ligands.

Generally extraction coefficients are very dependent upon the nature of the second solvent (S) but no simple rules, other than the above general statements, appear to be valid.

(c) Thermodynamic properties of complicated ligands

A ligand such as a protein is not easy to understand for it is not a rigid body. Thus the energy of a metal atom inside a protein can be adjusted by changes remote from the atom which force changes in the environment of the metal. Just as the affinity of hemoglobin for oxygen is under all sorts of control associated with the adjustment of bond length and bond angle [6,9,10] owing to distortions of the protein, so a redox potential is under similar control [5]. The two redox states of a metal ion demand different geometries, bond lengths and/or bond angles, and it is a matter of major concern how well the protein accommodates the two different oxidation states. Strain built into the active site in this way has been tentatively discussed by different authors for several years but it was only given general and definite consideration by Vallee and Williams in their paper on the entatic state [11]. If such control over redox potentials can be exerted by the protein, then there should be little surprise if we fail to understand these potentials in detail.

Apart from the problem of strain at the metal the protein provides a pocket of ill-defined chemical structure and physical properties. Clearly, as we shall see, some pockets are like organic solvents, others less so. In some the metal ion is surrounded very closely by organic groups while in others the site is more open. Definite knowledge of the effect of these factors on redox potentials cannot be claimed and we must look for large changes associated with particular structural features. The essence of a biological molecule is its function and we must expect a priori that any observed property has a functional significance, i.e. it may have arisen through evolutionary pressure, rather than that it was the obvious result of stabilisation by chemical and physical interactions. Evolution is a drive which can go against a thermodynamic free energy gradient.

Despite these caveats some general trends in the redox potentials of biological molecules can be discerned.

(d) Biological examples

This section summarises our knowledge of the effect of charge, σ-donors and π-acceptors upon the redox potentials of metallo-proteins plus small ligands as well as of the proteins themselves. Obviously if one oxidation state is known to bind a small ligand L with a binding constant K while the other oxidation state cannot be shown to bind that ligand, then we can calculate a limiting value of the redox potential of the complex ML from the minimum redox potential change

$$E = E^0 + \frac{RT}{nF} \ln K$$

where E^0 is the potential in the absence of the ligand L. Illustrating the point with myoglobin we have the following:

(i) Anions such as OH^-, F^-, acetate$^-$, N_3^-, SCN^- and CNO^- only bind to the high-spin oxidised state (III) and therefore they lower the redox potential. This suggests that low-potential high-spin heme proteins such as catalase and peroxidase, see Table 2, may well have an anion as a ligand (catalase) or at least as a next-nearest neighbour (peroxidase).

(ii) Neutral molecules such as imidazole, ammonia or pyridine cause rather little change in redox potential of myoglobin (imidazole, water ligands) as they bind nearly equally to both oxidation states of heme proteins. Thus cytochromes *b* (two imidazole ligands) have a redox potential quite close to myoglobins.

(iii) π-acceptors such as CO, NO, RNC and to a lesser degree R_2S (thioethers) bind the lower oxidation state much more strongly. Thus cyto-

TABLE 2

Redox potentials for some biochemical and chemical couples

System	E^0 (volts)
$Fe^{3+}(phen)_3$—$Fe^{2+}(phen)_3$	+1.10
$Fe^{3+}(dipy)_3$—$Fe^{2+}(dipy)_3$	+0.96
$Fe^{3+}(aq)$—$Fe^{2+}(aq)$	+0.77
$Fe(CN)_6^{3-}$—$Fe(CN)_6^{4-}$	+0.22
$Fe^{3+}(oxalate)_3$—$Fe^{2+}(oxalate)_2$	+0.02
$Fe^{3+}(oxime)_3$—$Fe^{2+}(oxime)_3$	—0.25
Cytochrome *c* Fe^{3+}—Fe^{2+}	+0.25
Hemoglobin Fe^{3+}—Fe^{2+}	+0.17
Myoglobin Fe^{3+}—Fe^{2+}	+0.05
Rubredoxin Fe^{3+}—Fe^{2+}	—0.06
Horseradish peroxidase Fe^{3+}—Fe^{2+}	—0.17
Clostridial ferredoxin (cluster)	—0.42
Catalase Fe^{3+}—Fe^{2+}	<—0.42

phen, phenanthroline; dipy, dipyridyl.

chromes *c* (imidazole thioether ligands) have a higher redox potential than myoglobins.

Another way of changing the heme redox potential involves change in polarity of the environment. A comparison between the heme proteins myoglobin, cytochrome *b* and cytochrome *c* does not show a pronounced difference between them. In fact all these redox centres are in a "hydrophobic" medium. In itself this means that the redox potentials will be higher than in water as nearly all redox potential active proteins have a neutral lower oxidation state, though catalase does not. One way of seeing the importance of a given amino-acid side-chain in contrast to the constant environment is to study the redox potential of point mutations affecting either the distant neighbours or the actual donor ligands. In hemoglobins it is known that the introduction of an anion, either carboxylate or phenolate, very close to the heme depresses the redox potential by as much as —0.5 volts. The method of using point mutations could also be used to assess environmental effects.

A much harder feature to estimate is the steric factor. In general as the lower oxidation state has longer bonds and often a less rigid stereochemical demand steric factors are thought to raise metallo-enzyme redox potentials [5]. Fortunately the effect of steric hindrance can often be seen by examining the splittings of the d—d absorption spectra of metallo-enzymes by absorption, EPR or other forms of spectroscopy. It is easy to show that amongst copper proteins Cu^{II} is rarely in a site of high symmetry. Usually it is in a distorted tetrahedron or tetragonal environment [11,12]. This would appear to be a major factor raising the copper(II)/copper(I) potential in proteins above that of the potential of the aquo couple, see Table 1.

The steric factor also shows its importance in the iron—sulphur proteins of the rubredoxin type where one Fe—S bond length differs from the other three in the $Fe—S_4$ tetrahedron [13]. The exact distortion is not known but is clearly seen in the split d—d absorption spectrum of the reduced state [14] and the rhombic EPR spectrum of the iron(III) state. The distortions in the Fe_2S_2 dimers and the Fe_4S_4 cubes of ferredoxins cannot be described as yet — see Sect. C although they too have been observed by spectroscopy.

The iron—sulphur proteins raise the general problem of the acceptance of more than one electron. Thus Carter et al. [15] proposed the series of two one-electron steps

$$Fe_x^{(n-2)+} \rightleftharpoons Fe_x^{(n-1)+} \rightleftharpoons Fe_x^{n+}$$

$$E^0 = -0.5 \qquad E^0 = +0.2$$

Although the absolute redox energies of these clusters can be readily controlled by environment, spectroscopic differences, given no change in spin state as revealed by magnetic evidence, should define the overall oxidation states clearly enough [16]. The redox potential of this particular series of complexes returns the discussion to the functional significance of E^0 values in proteins.

The factors controlling the redox potentials in the series

$$[Fe_4S_4]^0 \overset{-0.5}{\rightleftharpoons} [Fe_4S_4]^- \overset{+0.2}{\rightleftharpoons} [Fe_4S_4]^{2-}$$

ferredoxins HiPIP

must be partly ones of the medium, the higher the charge the less stable the state. However, whatever the causes it must be noted that the adjustment of redox potential is to a biological need (i.e. evolutionary pressures demand increase in usefulness). The potential —0.5 may well be designed to be the lowest energy trap possible which will avoid the danger of running reduction to generate H_2 gas. Thus in nitrogenases if N_2 reduction is blocked H_2 is generated slowly but this is a non-physiological reaction. Normally when reducing power builds up in a biological system there is a controlled switch-off to avoid H_2 evolution. We return later to the problem of the controls noting that the range of biological redox potentials is as in Table 3.

(ii) Electron-transfer mechanisms

There are two well-described solid-state electron-transfer mechanisms, the delocalised band model and the localised hop model [17]. In the first, electron wave functions are lattice-constrained, activation energy is associated with the gap between filled and unfilled lattice states, and mobility is high. In the second, electron wave functions are constrained to atoms, excitation is to a high vibronic state of the atoms and mobility is low. This second model is closely related to solution electron-transfer models. Both solid-state models are really limiting approximations, and polaron theory allows extension of the atom model to local groups of atoms which can pick up lattice rather than site vibrational energy. Tunnelling is common to all mechanisms where there is an energy barrier, for an electron will always tunnel so long as the distance between sites of minimum potential energy is not too great (i.e. $<\sim$15 Å). Before we look at some elaborations upon such ideas the new factors acting in solution systems should also be enumerated. In solution there are additional considerations due both to thermal collisions and exchange of partners [18—20]. Obviously an electron can pass from one atom to another

TABLE 3
Biological redox potentials E^0 (volts)

—0.5		0.0	+0.4	+0.8
Ferredoxin	Flavin	b-heme HiPIP	c,a_3-hemes Cu(blue)	$[Cu]_2$
Rubredoxin $(Fe/S)_n$	c_3-heme	$(Fe/S)_n$		Mn(?)
$[Mo]_2$?		Hb		
$H_2O \rightarrow H_2$		Myoglobin		$H_2O \rightarrow O_2$

in an atomic collision in the gas phase or in an inert solvent with little energy loss or gain except the thermodynamic redox potential energy. Orbital overlap then gives the electron-transfer probability but even a small overlap of orbitals gives a high transition probability.

When the process is examined in solution then the atoms, to and from which electron transfer is to occur, are surrounded by other atoms and direct orbital overlap is very small or requires too large a rearrangement of the coordination sphere. This rearrangement is likely to be energetically unfavourable and two other electron-transfer mechanisms are then found to be overwhelmingly important. The first requires the removal of the coordinating atoms from one reacting atom, which may still be quite energetically expensive, when electron transfer occurs through one bridging group, while in the second electron transfer occurs across both coordination spheres. If the atoms are separated by one "insulating" atom, $M_1 \cdot X \cdot M_2$ (inner sphere complex) then not only is the overlap, direct or through X, much reduced but relaxation of M_1X and M_2X to new bond lengths is required during reaction. This would seem to restrict electron transfer to the probability of a vibrational frequency multiplied by an exponential term $e^{-\Delta E/RT}$ where $-\Delta E$ is the energy of the vibration. This is the "Franck—Condon" energy of activation first discussed by Marcus (for a review of this work, see refs. 18 and 20). Obviously the greater the relative charge difference between M_1 and M_2 the higher is the activation energy while the greater the ligand covalence the better the electron-transfer possibility. X should be I^- rather than F^- and S^{2-} rather than O^{2-}. In fact in all circumstances ligands which are able to distribute the electron (which is to be transferred) or the hole (to which it is to be transferred) are advantageous for electron transfer. The size of the ligand gives the electron-transfer distance. Note that I^- (4.6 Å) has nearly twice the diameter of O^{2-} (2.8 Å). In cytochromes $[porphyrin]^{2-}$ is an ideal ligand for the spread of charge away from the central metal, and orbital overlap with a neighbour may be reasonable with such a large conducting ligand over at least 15 Å diameter (in plane). In the other biological electron-transfer systems, which we shall describe, either RS^-, S^{2-} or an organic radical allows the electron charge to spread over a large distance. We note that this has the following advantages for electron transfer:

(a) Smaller local charge changes on transfer.

(b) Smaller bond length changes on redox change.

(c) Less requirement for close approach of reacting metal atoms.

(d) As a consequence of (a) and (b) the electron lies in a broad potential energy minimum (see later) rather than a very steep one.

(e) The centres can provide an ~15 Å path by the inner sphere electron-transfer mechanism, i.e. the need to provide electron channels through a protein is already partially overcome.

The next stage of separation of the reacting metal atoms (outer sphere) is the interaction $M_1X—YM_2$. There is no change in the general argument from the $M_1 \cdot X \cdot M_2$ electron-transfer situation. The points made above are simply

exaggerated:

(a) Distance between M_1 and M_2 is increased so that direct overlap is reduced.

(b) Indirect overlap as it involves both X and Y orbitals may be reduced, but this depends on the nature of X and Y. If both are sulphur the situation is very different from the case where X = Y = oxide.

(c) The ligands of biology, porphyrin and RS^- are more obviously required and again low-spin states rather than high-spin states are preferred as overlap in them is increased.

(d) The major activation energy may well be associated with the vibronic energies needed to "equilibrate" oxidation states in the two coordination spheres.

Some model examples make it clear that exchange of electrons can still be very fast, e.g. between low-spin phenanthroline, cyanide, thiol ligand and porphyrin complexes in solution.

Given the rates of electron transfer required by biological systems either of the above two mechanisms are adequate if collision is possible. Many metal complex systems in both classes have electron-transfer rate constants in the range $10^8 \rightarrow 10^{10}$. Thus biological electron transfer can be by diffusion. It could well be that this is the biological function of freely diffusing entities such as quinones which are well-known in vitro mediators of electron transfer between electron-transfer proteins. The real problem in biology is that diffusion of one molecule to another is not always possible and it is necessary to look at other types of model in glasses, ordered liquids and the solid state. We shall not resort to a delocalised model as the properties of proteins do not make this a feasible mechanism. Thus it is the hop and polaron ideas to which we turn.

(a) Electron transfer over long distances

Solution electron transfer is a random collision process. Solid-state transfer is essentially directional. Biological transfer must be directional to some degree but it also is at a controlled rate. (We shall see that biological electron transfer is not strictly a solid-state nor a solution problem, however.) The three solution cases discussed above, i.e. $M_1 \rightarrow M_2$ (no control possible), $M_1 \cdot X \cdot M_2$, $M_1 \cdot X{-}Y \cdot M_2$ would provide very little opportunity for control of electron transfer. Clearly more complicated ligands which restrict collisions are required for this reason alone. Making the ligand large, e.g. a protein, is not adequate if the coordination sphere is at all exposed, for given the ligands of iron and copper observed in the proteins we would expect fast transfer of either the inner or outer sphere type. In fact we shall show that electron transfer between pairs of centres is fast even in non-"electron-transfer" proteins, e.g. between myoglobin and cytochrome *c*, when collisional processes provide the necessary random pathways, e.g. in vitro. In vivo the situation is very different for the packing of large protein molecules usually prohibits the pair-wise collisions. By some mechanism

electrons must be transferred over considerable distances, say 10 Å, from a metal through organic matrices, when overlap is very small, so that electrons go to and from centres far apart. It is this separation which generates the possibility of control. Recent experiments in model systems show in fact that electron transfer between metal atoms in frozen matrices need not be very slow. Unfortunately we have too little evidence to generalise but the following points deserve notice.

(b) Media in which electrons travel

Ice. Horne [19] showed in 1963 that the rates of electron transfer between hydrated Fe^{II} and Fe^{III} were as fast in ice as in water. This indicated that electron transfer could occur over considerable distances by a mechanism not unlike that of proton transfer. Thus it is conceivable that electron transfer in biology utilises a net of hydrogen bonds. The description of the electron-transfer proteins below shows that a proton or hydrogen atom migration is improbable, however, for the channels to the electron-transfer centres in proteins are hydrophobic.

Ionic media: channels. Small charged particles are able to move in a lattice such as that of sodium chloride. The particles that have been examined include the proton, the α-particle and the electron [21]. The particles do not migrate randomly but move selectively down lines in the structure following certain well-defined crystal directions. This effect has been called channelling. In a channel the particle avoids all regions of high electron and positive charge density. It is obvious that this helps motion as the repulsions and attractions between fixed charges hinder movement of a charge in a given direction. Channelling by electrons is not well studied and in particular we lack studies on the channels in hydrocarbon matrices. Note that most of these studies are of highly energised particles and are at unlikely energies for electron transfer in biology.

Hydrophobic media. A number of electron-transfer steps have been observed in low-temperature matrices. It is now known that electron transfer can occur in helium, argon, carbon dioxide and methane at temperatures well below 100°K and at reasonable rates. Reactions such as

$$Fe(CO)_4 + Na \rightarrow Na^+ + Fe(CO)_4^-$$

have been examined [22]. The distance of transfer can be as large as 5—10 Å. Note that the reactants have large, highly polarisable orbitals, see also refs. 23—27.

Recently a number of papers have appeared which discuss the mobility of electrons in such very inert (very clean) media [28,29]. It appears from the results of these studies that electron mobility is high in a material such as a short-chain hydrocarbon or benzene and that there is only a very small activation energy associated with electron movement within the solvent. Corres-

ponding studies in more polar media indicate that once again mobility can be high — see liquid ammonia (below) or water. These are important observations when we come to discuss proteins for it is not usual to consider a hydrocarbon or a simple polar medium as a possible redox path. The conventional treatment looks for "hopping" redox media.

From the above it is seen that electron transfer may not be inhibited by solvents as strongly as one might think and the real problem for such transfer then becomes the release of an electron from the hop or polaron centre; compare the injection energy from an electrode.

Redox media. It is possible to consider electron motion over a long-distance via a rapid series of hops. Thus transfer from metal atom to metal atom would involve the jumping of an electron from one organic group to another along a chain of molecules such as quinones. The hop mechanism is well known and it is obviously related to the mechanism of Dickerson et al. [30,31] for cytochrome *c*. The essential features of the hop are that the "hopping electron" must be in a fairly weak potential energy though for otherwise its probability of movement is too low for conduction. Table 4 gives possible(?) electron hop centres in proteins. We return to the validity of this concept later.

Liquid ammonia. The case of liquid ammonia deserves attention as electrons are highly mobile in this medium [32]. The reason liquid ammonia is not a good electron exchange medium is, however, not one of electron mobility but of getting the electron into the medium. The potential required to introduce the electron is about −2.0 volts (as for the free electron in water). At this potential the electron attacks benzene! In fact this is a general problem with most of the solvents. It would seem that electrons must be injected into them at considerable voltages. As this is the case a path must be present in the internal medium of proteins at much lower potentials, i.e. some injection method is required so that the electron is not trapped on the metal sites. Yet on leaving the site there must be a preferred path to the next centre.

TABLE 4

Classification of amino acid residues for electron-transfer purposes [37]

Donor/acceptors	Neutral	Electron repelling
Tyrosine (phenol)	Alkyl chains	Hydroxyl
Thiol	Phenylalanine	Amino
—S—S—	Ether residues	Carboxylate
Tryptophan (indole)		

(c) Injection

It would seem that a pathway has to be described by either (i) a sufficient ease of excitation of an electron into the solvent matrix or (ii) a discreet series of well-placed low-lying traps which the electron hops along.

Now in either case the electron must enter the system, leaving its metal redox centre. Experimentally in order to study electron mobility in the different media described above electrons are injected into the media. This process usually requires very considerable energy (volts) and we must ask how is this barrier overcome in biology? In biological systems the original source of electrons is usually an organic reducing agent (H—R) and the reaction is

$$H - R \rightarrow R^{\cdot} + H^{\cdot}(\text{bound}); H^{\cdot} \rightarrow H^{+} + e$$

or

$$H - R \rightarrow R^{+} + H^{-}(\text{bound}); H^{-} \rightarrow H^{+} + 2e$$

Thus injection is by chemical reaction at a site [33]. Now once on a specific metal or other site the electron flows downhill in the thermodynamic sense so that it is usually going to a site of higher redox potential (more positive) and is eventually picked up by molecules such as oxygen. The minimum energy opposing electron transfer is the thermodynamic redox energy of transfer and in biology this is generally favourable (actually often close to zero) and represents no problem, see the electron-transfer chains below. Thus the energy of injection is the activation energy required to free the electron from its trap.

A trap can be of any depth. The reason that NiO for example is a poor conductor is that Ni^{II} is a very good trap relative to the ions $Ni^{III} + Ni^{I}$. Pure FeO and Fe_2O_3 are also good insulators for the same reason while Fe_3O_4 is a very good semiconductor as the trap Fe^{II} is able to "see" the hole Fe^{III} [34]. Exchange of hole and electron where there is some direct overlap as here gives relatively low-energy mobility. However, if these ions are placed far apart in a silicate lattice or in a mixed $Cr^{III} \cdot Fe^{III} \cdot Mg^{II} \cdot Fe^{II}$ oxide the activation energy for conduction becomes very high for the oxide anions do not provide any suitable acceptor/donor energy levels, nor a possible channel [35]. The combinations of the ligands and the channels in a protein must supply a much easier path and it could well be necessary to have both easier electron release from the metal and a better pathway. The problem can be illustrated by a further comparison of solution and solid-state electron transfer.

(d) Activation energy of electron transfer

The activation energy for electron transfer in solution reactions must be considerably less than 20 kcal if the reaction rate for electron transfer is to be fast assuming that the entropy term is not exceedingly favourable. For small molecule reactions many cases of lower activation energies have been

observed [18]. In those solid-state conducting lattices which most resemble solutions, those to which modified atomic wave functions apply, there is a large density of charge carriers and it is the activation energy of mobility which controls conductivity. For a typical example such as Fe_3O_4 the activation energy for charge carrier mobility is low, around 5 kcal, and Fe_3O_4 has a high room-temperature conductivity [34] of about 10^2 mho cm. When the iron atoms are diluted in the lattice the transfer is that of an outer sphere conductor $Fe^{II} \cdot O_n Fe^{III}$. Despite considerable effort using this and a variety of other types of materials Williams and his colleagues failed to produce a good solid-state conductor using compounds in which only outer-sphere transfer was possible [34]. Conductivities usually fell well below 10^{-10} mho cm with activation energies of >15 kcal. The conclusion was drawn that electron transfer in a rigid matrix of outer-sphere complexes could not account for the electron-transfer rates and activation energies in biological systems. Proteins in biologically organised systems must be different from these ordered systems. The obvious difficulty in a lattice is that atoms in a lattice must lie in deep potential wells. It is at this point that we must return to the nature of a protein and why it can be said to be unlike both a solid or an ideal solution. It has limited cooperativity which is to be contrasted with total cooperativity (perfect crystal) and non-cooperativity (gas or perfect solution). The inside of a protein is probably closer to an ordered liquid.

(e) The nature of proteins — kinetic properties

Many studies of proteins have suggested that the protein has a high degree of internal fluctuations in structure. The work of Linderstrøm—Lang on deuterium exchange was outstanding in this respect [36]. Recently we have been able to study structural fluctuations by observing the spin-state changes of heme proteins, and the rates of rotation of tyrosine and phenylalanine rings about their long axes. In both cases considerable fluctations in structure of the protein would appear to occur and to be of the order [38] of $>10^4\ s^{-1}$. Such fluctuations are of the right order to influence electron-transfer probabilities. They suggest that the general internal motions of a protein could alter bond lengths of, say, Fe—N (histidine) in a protein very rapidly. Thus the equalisation of energy states of two oxidation states — a major barrier to electron-transfer rates (see above) — may be achieved. Again there is a variety of evidence which suggests that in catalytic proteins the lowest energy state of the protein may be that which places the surrounds of the metal close to the geometry of the transition state for electron transfer. Some insight can be had into this postulate by examination of enzyme action.

For a long time it has been considered that a protein in an enzyme was of such a structure that it matched the transition state of the substrate. This means in effect that the binding of the transition state is greater than the ground state of the substrate. This is Haldane's and Pauling's view of substrate activation. In the case of metal ions in enzymes a very different pos-

tulate has been made which is applicable to non-metal atoms too — that the enzymes groups are in the transition state for attack on the substrate before substrate enters [11]. The folding of the protein generates the heightened attacking power of the protein group in a "purposeful" manner. This is demonstrated in the hemoglobin structure by the preparation of five-coordinate Fe^{II} [10]. However, a very remarkable example has been discovered recently in the chemistry of the trypsin inhibitors [39]. The inhibitor is a protein which we shall look upon as carrying the attacking group; an electrophilic carbon, $-C(=O)-NH-$, attacking a serine hydroxyl of the substrate, here the protein trypsin. The attack is energised in part by a good match of the overall geometry of the proteins, but it is observed that the geometry of the $-C(=O)-NH-$ group is already close to that of its unimolecular transition state $C^{+}(-O^{-})-NH-$ as seen by its considerable distortion. Protein folding can therefore distort normal geometries of local groups so as to lower activation energies. There is hardly a better example of an activated (entatic) group [11] (Fig. 1). Now here the cooperative energy provides a "designed" ground state for activity but it is also possible that the protein is "designed" to supply cooperative energy by lattice changes. This is obviously true for an enzyme which catalyses a multi-step process. Electron transfer may require either bond length or bond angle changes to lower the activation energy of reaction as well as to adjust E^0. Thus the interest in electron proteins is that they must provide the following:

(1) A site of low injection energy for ready donation and acceptance of an electron.

(2) A source of vibronic energy which may be cooperative in the lattice.

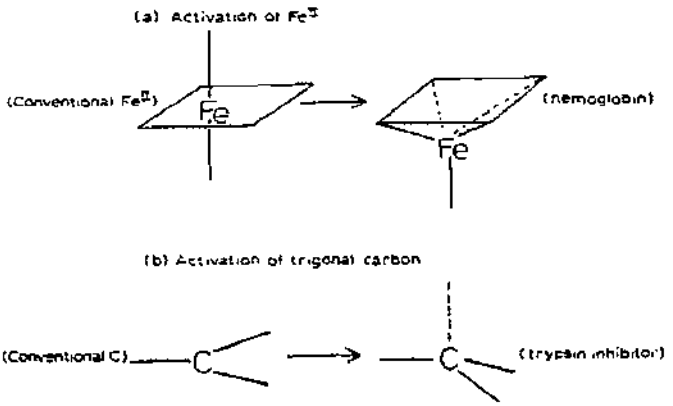


Fig. 1. Activated nature of functional groups in proteins: (a) hemoglobin; (b) trypsin inhibitor protein.

(3) A path for the electron which is directional and open to control.

It is the structure of the protein that has supplied these features, for the electron, unlike other substrates, has no structure.

We conclude this introductory section with the following summary statements about biological electron transfer.

(1) The path from metal to metal may involve organic amino acid side-chains.

(2) The state of a metal may be such that it is activated, energised, so as to be near in geometry to the transition state for electron transfer. Such an effect will be reflected in redox potentials as well as electron-transfer rates.

(3) The state of organic groups may be energised too.

(4) The protein is not to be viewed as a cooperative lattice in which cooperativity inevitably opposes changes of the coordinates of individual atoms (as in a perfect crystal) but is more related to a liquid in which fluctations can aid reactivity and these fluctuations need not be of high energy [40].

Before turning to the organic groups in proteins and their possible role in electron transfer there is a further point about the nature of electron conductivity in solids which is relevant to the discussion of mechanisms. It is frequently observed in the study of semiconductors that the electrical conductivity of a material undergoes a sudden change both in absolute value and in activation energy at a phase transition temperature. The transition temperature corresponds to either a first-order phase change when there is a change in structure in the solid lattice or to a second-order phase change when there is only a change in lattice order. The first-order phase change would correspond in protein chemistry to a considerable structural change and clearly we might look for control of electron-transfer rates by such a mechanism. The structure change need be no greater than that observed in going from a monoclinic to a rutile form of, say, VO_2 which is associated with the centring of the metal atom in the roughly octahedral unit. A parallel movement is that from high-spin to low-spin Fe^{III} in heme proteins, which generates a well-known "phase-change" in many heme proteins. A more probable parallel between inorganic semiconductors and proteins can be drawn from systems showing second-order phase changes. Such changes can be brought about by the introduction of cooperative (lattice) modes which may be based upon rotational movements locally or vibrations. The latter have been called mode-softening transitions. A protein is a small cooperative unit and could well show parallel cooperative effects which can greatly influence electron transfer and its control. (We thank Prof. C. N. Rao for drawing our attention to these observations.)

(f) Hop electron-transfer centres in biology

The electron-transfer centres with which this article is concerned are metal ions. They form the overwhelming majority of such centres and occur as single atoms, clusters and atoms linked through non-metal atoms. The only other electron-transfer centres which are known to be present in biological

the following way. The redox potential of Fe^{II}/Fe^{III} in aqueous acidic media is around +0.7 volts. There are good measurements of the redox potentials of phenols giving values of around +1.1 volts [41]. Thus, as observed experimentally, Fe^{III} will not oxidise phenol in acid media. In alkali media the couple $[Fe(CN)_6]^{4-}/[Fe(CN)_6]^{3-}$ has a potential of about +0.5 volts and is capable of producing some of the tris-t-butyl phenol radical [42]. Let us assume that the redox potential of tris-t-butyl phenolate (in alkali) is around +0.5 volts. The redox potential at pH 7.0 will then be $0.4 + (RT/\mathcal{F}) \ln(K + [H^+])/K$. For K (the dissociation constant of the phenol) is 10^{-11} and $[H^+]$ is 10^{-7} M, the redox potential of a simple phenol, e.g. tyrosine, will be >+0.75 volts. We note that the Fe^{II}/Fe^{III} potentials of cytochromes range from —0.3 to 0.4 volts. Thus reaction of phenols and Fe^{III} cytochromes is opposed by a thermodynamic barrier which could be very considerable. In order to get high rates of electron transfer through the phenol side-chain of tyrosine a minimum redox potential of the metal couple could be set at +0.6 volts.

Turning back to Table 1 we see that in fact the Cu^I/Cu^{II} potential can be as high as +0.7 volts in proteins so that here the oxidation of phenols both inside and outside (substrates) proteins is possible by Cu^{II}. Iron on the other hand has couples of sufficient oxidising power only in the Fe^{III}/Fe^{IV} and Fe^{IV}/Fe^{V} states. These are states produced by oxygen or hydrogen peroxide on addition to iron enzymes. The idea that electrons could be transported through proteins by phenol, indole and benzene is now seen to have arisen from a confused comparison of electron transfer by Fe^{III} and oxidation by higher oxidation states. We have inspected these ideas recently by an experimental examination [43] of a series of situations in which elec-

TABLE 5

Inspection of possible electron-transfer centres [43]

Oxidised centre	Reduced centre	Observation
Fe^{III} heme peroxidase	Phenols	No reaction
Fe^{III} heme peroxidase	Indoles	No reaction
Fe^{III} in oxygenases	Phenols	No reaction
Cu^{II} in oxidases	Phenols	Reaction
Phenol radicals	Indoles	Little reaction
Fe^{IV} in peroxidases	{Phenol / Indole}	Reaction
Fe^{V} in peroxidases	{Phenol / Indole}	Reaction
Fe^{IV} or Fe^{V} in peroxidases	Benzene	No reaction
Fe^{IV} or Fe^{VI} ? in P-450 cytochromes	{Alkyl chains / Benzene}	Reaction

systems are flavins, quinones and thiol groups. The properties of these organic centres which make them suitable for electron transfer are very similar to those of the metal complexes. Thus the metals are bound to either sulphur, porphyrin or even peptide nitrogen and are therefore "unsaturated organic" systems in that they have low-lying acceptor states in the oxidised form. This is also true of flavin$^+$, quinone and disulphide —S—S—. Again they, like the metal centres, have rather unstable electrons in their reduced states. The two groups of compounds resemble one another further in that they have extended molecular orbitals which are highly polarisable. Thus the centres provide a path of 5—15 Å through a protein without further assistance. Finally the redox potentials fall in the range —0.5 ↔ +0.4 volts which makes them ideally suited to the exchange of electrons with one another. It is very noticeable that an electron-transfer centre is usually "chosen" in biology so that it closely matches the mean potential of its donor and acceptor centre

$$\begin{array}{ccccc} \text{donor} & \rightleftharpoons & \underset{\text{unit}}{\text{transfer}} & \rightleftharpoons & \text{acceptor} \\ E^0_D & & \dfrac{E^0_D + E^0_A}{2} & & E^0_A \end{array}$$

This ensures that there is little thermodynamic barrier to electron transfer in either direction and many electron-transfer systems are almost reversible, e.g. in oxidative phosphorylation.

It will be noticed that none of these centres except —S—S— are side-chains of amino acids. In the next section we point to the problems of carrying out redox reactions with any such side-chains. The most likely side-chains as candidates for a role in electron transfer are phenol, indole and benzene groups of tyrosine, tryptophan and phenylalanine. We shall refer to these groups only and ignore the other common side-chains of amino acids.

Possible electron transfer to/from phenol, indole and benzene. As we shall see the channels in the electron-transfer proteins are hydrophobic and often have several aromatic amino acids associated with them. The possibility of electron transfer to or from these groups must be considered. It is known that in aqueous solution Fe^{III} will oxidise phenol provided that the phenol is ionised as phenolate, i.e. the Fe^{III} phenolate complex is required. However, in proteins this oxidation is not known. High spin Fe^{III} in hemoglobin-H mutants and in conalbumin does not oxidise its phenolate ligands. Presumably trapping in the protein prevents dissociation of the phenolate radical from the metal. (Note that even RS^- is not oxidised in the RS^- complexes of iron in ferredoxins.) Phenolate is of course a much better electron donor than phenol itself, indole or benzene so that the very fact that phenolate is not readily oxidised makes it unlikely that these other groups will be attacked.

The thermodynamic potentials of these redox reactions can be estimated in

tron transfer from Fe^{III} or Cu^{II} might have been supposed to occur on the basis of the mechanism proposed by Winfield [31] and Morpugo and Williams [37] and strongly supported by the interpretation of the structural studies on cytochrome *c* [30]. Table 5 lists some of these systems where the supposed electron-transfer path has been generated and which are described fully in the research literature [43]. In no case is there any evidence for electron-transfer. In line with knowledge of the relative redox potentials it must be assumed that electron transfer to and from these groups is not possible. Only in the higher oxidation states of Fe^{IV} and Fe^{V} or of high potential Cu^{II} is there a possibility of electron acceptance from organic groups. We stress this point by a short digression to the peroxidases and oxygenases.

The peroxidases can oxidise their substrates, phenols and indoles, to the free radicals. The reaction mechanism is known to be [44]

$$Fe^{II} + O_2 \rightarrow Fe^{II}\,O_2 \xrightarrow{e} Fe^{III}\,O_2^{2-} \xrightarrow{e} Fe^{IV}$$

$$Fe^{III} + H_2O_2 \rightarrow Fe^{III}\,O_2^{2-} \xrightarrow{e} Fe^{IV} \xrightarrow{e} Fe^{III} \xrightarrow{e} Fe^{II}$$

$$\text{substrate} \rightarrow \text{substrate}^{\cdot+} + e$$

The redox potentials of the Fe couples in peroxidases are [45]

$$Fe^{II}\,O_2 \rightarrow Fe^{III}\,O_2^{2-} \rightarrow FeO \rightarrow Fe^{III} \rightarrow Fe^{II}$$

(? high) $> +0.8 > +0.8$ $-0.2 \leftrightarrow -0.4$

phenol and indole oxidation | no reaction

Thus Fe^{III} in the peroxidases produces no measurable amount of phenol free radicals. We shall present NMR evidence elsewhere [46] as to the binding location of the phenol substrate relative to the iron porphyrin showing it to be just as near to the heme (~10 Å from the iron) as are the phenols (tyrosine) and indoles (tryptophan) of amino acid side-chains to heme in cytochromes. We note also that the copper(II)/copper(I) potentials in the copper proteins which oxidise phenol substrates are very high. Now none of these proteins even in the high potential states can readily oxidise benzene (compare phenylalanine) which requires very special activation as in cytochrome P-450 in a high oxidation state. Indole is intermediate between phenol and benzene. We conclude that in all cases of phenol, benzene or indole oxidation a high potential is essential and it would appear that even in the best case in biological molecules Fe^{III} cannot be brought to this potential.

Another very interesting demonstration of these facts is in cytochrome *c* peroxidase where cytochrome *c* can be oxidised by the peroxidase [47]. The action of the hydrogen peroxide is to generate FeO^{IV} plus a free radical in

the protein (probably phenol of tyrosine) from an initial Fe^{III}. The reaction requires an intermediate Fe^{V} state. Cytochrome *c* reacts with this "tyrosine" radical. This proves that radicals (probably tyrosine) can be produced in proteins and hence electron transfer via such radicals is possible at high redox potentials [48]. The Fe^{III} form of the enzyme does not oxidise the side-chain to a radical. It is therefore a good mechanism for the electron transfer from $M \cdot O_2$ or $M \cdot H_2O_2$ complexes or from other high potential redox centres in proteins of $E^0 > +0.6$ volts. Moreover once the radical is formed it can transfer electrons through to a second heme (cytochrome *c* peroxidase to cytochrome *c*) readily. No such reactions would appear possible with Fe^{III} inside proteins, however.

Thus phenol is inadequate as an electron donor and indole and benzene are progressively poorer. In the reverse reaction as electron acceptors from iron(II), these groups must be exceedingly poor. Any electron which can escape from Fe^{II} must have such an energy that it can have negligible resting time on benzene, indole or phenol. Since these views were generated and in keeping with the above points new sequences of cytochromes, ferredoxins and other electron-transfer proteins show that the electron channels of proteins do not require such aromatic groups to be conserved even though they are favoured over aliphatic side-chains.

The contrast between phenol, indole and benzene and the organic groups which do transfer electrons in biology is also informative. They are all two-electron systems which have stabilised intermediate radicals.

$$Q + e \rightleftharpoons Q^{\cdot -} + e \rightleftharpoons QH_2$$

$$\text{—S—S—} + e \rightleftharpoons \text{—S—S}^{\cdot} - + e \rightleftharpoons 2SH$$

$$\text{flavin} + e \rightleftharpoons \text{flavin}^{\cdot} + e \rightleftharpoons \text{flavin} \cdot H$$

The potentials of the two steps are closely matched so that reactions backwards or forwards are possible; E^0 is from −0.2 to +0.2 volts.

We conclude that the thermodynamics of the redox potentials of phenol, indole and benzene argue against a possible use in electron transfer for Fe^{III}/Fe^{II} reactions. Again the kinetic barrier especially in the case of benzene must be very high. At the same time we note that electron transfer is possible via RS^-, quinones or flavins.

In the second part of the article we shall turn to a detailed description of the electron-transfer proteins. The point of interest which we shall stress is the expression of evolutionary drive in the physicochemical properties of the protein. Evolution is expressed in the choice of metal, its coordinating atoms and the interaction of the coordinating centre with the whole protein. In the course of the description we shall look for explanations of thermodynamic properties (redox potentials) and kinetic properties (activation energies and channels for electron movement) and for possible modes of ejecting electrons from the metal ions which act as their traps. Increasingly it appears that the

special feature of proteins is their internal cooperativity which allows both static and dynamic features to be generated which are not possible in much smaller molecules. Our thinking about protein activities requires knowledge of ideal gas and solution processes but additionally we are forced to examine these large molecules in the context of properties of condensed phases.

C. SPECIFIC ELECTRON-TRANSFER PROTEINS

(i) Cytochromes c

Cytochromes *c* are characterised by possession of a heme group covalently bound to protein through thioether linkages [49]. Most cytochromes *c* are low spin, the fifth and sixth iron ligands being histidine (his) and methionine (met) (e.g. *c* and c_2) [49] or his and his (e.g. c_3) [50], but a few high-spin cytochromes *c* are also known (e.g. *c'*) [49]. Their fifth and sixth iron ligands are not known although one of them is probably histidine.

A large number of cytochromes *c* have been isolated and purified. They possess a wide range of properties (Table 6) and function in a large number of different redox processes. Owing to limitations of space we shall confine ourselves to a discussion of a small number of these, mainly those for which detailed structural analysis is available, and accordingly first turn our attention to cytochrome *c* itself.

(a) Cytochrome c

Cytochrome *c*s from about sixty different sources have been sequenced and comparisons of the proteins from eukaryotic sources highlight the conservative evolutionary nature of this protein [51]. Bacterial cytochrome *c*s are more variable in composition but there have been successful attempts to demonstrate homology between them and eukaryotic cytochromes *c* [52]. We are more concerned, however, with what these comparisons can tell us about the structure and functioning of cytochrome *c* rather than its evolution.

Almost invariably cytochromes *c* perform the role of electron-transfer agents, though it has been suggested that they may also act in ion transport [53]. They are the connecting link in a chain

$$\text{reduction centre} \rightarrow e \rightarrow \text{cytochrome C} \rightarrow e \rightarrow \text{oxidising centre}$$

The reduction centre can be hydrogen gas or a hydride and the oxidising centre can be oxygen or a group such as nitrate. The other electron-transfer proteins to be described below have a very similar role though sometimes in very different oxidation/reduction chains. Clearly one interesting test of the functional specialisation of the electron carriers both within the class and between classes is their ability to replace one another in a given electron-transfer chain. As we shall see it is found that replacement of one cytochrome

TABLE 6

Physical properties of cytochromes

Source	Molecular weight	No. of hemes	Redox potentinl (mV)	Ref.
Cytochrome *c*				
Ox	13500	1	+255	119
Horse	13500	1	+255	119
Yeast	13500	1	+260	120
Turkey	13500	1	+260	121
Tuna	13500	1	+265	121
Cytochrome c_1				
Beef	n.d. [a]	1	+220	122
Cytochrome c_2				
Rhodospirillum rubrum	13000	1	+320	123
Rhodospirillum molischianum	13400	1	+290	124
Rhodomicrobium vannieli	n.d.	n.d.	+305	125
Cytochrome c_3				
Desulphovibrio vulgaris	14700	4	—205	126
Desulphovibrio vulgaris	26000	8	n.d.	93
Cytochrome c_4				
Azotobacter vinelandii	24000	2	+300	127
Cytochrome c_5				
Azotobacter vinelandii	12100	1	+320	127
Cytochrome c_6 (= f)				
Pea	110000	2	+350	128
Cytochrome c'				
Rhodopseudomonas palustris	14800	1	+105	129
Bacterial [b] cytochrome *c*'s				
Bacillus subtilus c-550	13000	1	+210	130
Bacillus subtilus c-554	14300	1	—80	130
Pseudomonas fluorescens c-551	9000	1	+286	131
Pseudomonas denitrificans c-552	n.d.	1	+300	132
Bordatella pertusis c-550	n.d.	n.d.	+260	133
Bordatella pertusis c-553	n.d.	n.d.	+190	133
E. coli str. McElroy c-552	n.d.	n.d.	—150	134
Chlorobium thiosulfatophilum c-551	45000	2	+135	135
Cytochrome b_5				
Rabbit liver	11500	1	+20	136
Cytochrome b_{562}				
Escherichia coli	11000	1	+113	137
Cytochrome P 450				
Rabbit liver	45000	1	—400	138

[a] n.d., not determined.

[b] This classification system is based on the position of the band of the reduced protein. Some of these cytochromes will belong to the *c* and c_2 classes.

c by another is often possible [54,55]. Thus specificity of action must not be over-stressed. A favourite test system is the electron transfer system of oxidative phosphorylation where the oxidation of hydrides (from substrates such as malate) by molecular oxygen is coupled with the reaction [56]

adenosine diphosphate(ADP) + phosphate(P) → adenosine triphosphate(ATP)

All cytochrome *c*s from eukaryotic sources so far investigated are capable of restoring oxidative phosphorylation to cytochrome *c* depleted mitochondria from another source and they are indistinguishable, in terms of rate and maximum activity [54]. This latter point may be because cytochrome *c* is not involved in the rate determining step of mitochondrial electron transport. Recently ultra-pure preparations of different eukaryotic cytochromes *c* have been oxidised in vitro with a cytochrome oxidase preparation and shown to be indistinguishable here as well [57]. Thus, in spite of different amino acid sequences these eukaryotic cytochrome *c*s are functionally the same. With prokaryotic cytochrome *c*s the situation is more complex. Few bacterial cytochromes are functional in the eukaryotic mitochondrial system [58]. This is partly because eukaryotic cytochrome *c* is basic, whilst most bacterial cytochromes *c* are acidic and the interaction with cytochrome oxidase is an electrostatic one. For a similar reason eukaryotic cytochrome *c* is not oxidised by the basic *Pseudomonas aeruginosa* cytochrome oxidase in isolated preparations of the proteins [59].

Structure. The structures of various cytochromes *c* in different oxidation states have been elucidated by a combination of X-ray crystallography and NMR spectroscopy.

The heme group and crevice. The porphyrin group is attached to the protein via two thioether linkages in all cytochromes *c* sequenced to date apart from those from the protozoa *Crithidia oncopeltii* [60] and *Euglena gracilus* [61]. In these, the previously invariant residue cysteine (cys) 14, which provides one thioether linkage, is replaced by alanine. This loss of a thioether bond does not appear to materially affect these proteins: *Crithidia oncopeltii* cytochrome *c* is functional in the electron transport chain of beef heart mitochondria [62]. NMR spectroscopy shows [63] that the spin density distribution in the heme and axial ligands, and the bond angles and lengths of the iron axial ligands, must also be virtually the same as for mammalian cytochrome *c*.

X-ray analysis of horse [30], tuna [65] and bonito [66] cytochromes *c* have shown that the fifth and sixth iron ligands his 18 and met 80 [64] are axial in both oxidation states. The X-ray data for the closely related *Rhodospirullum rubrum* cytochrome c_2, however, have been interpreted [67] as showing that the Fe—S—Met bond is not axial in the oxidised protein but is displaced by about 0.5 Å from an axial position. In the reduced form NMR spectroscopy shows [68] that this bond is axial; the $Fe—S—CH_3$ PMR resonance is ring current shifted to the same position as the $Fe—S—CH_3$

resonance of horse cytochrome *c* indicating that both methyl groups possess the same alignment with respect to the porphyrin ring. Comparison of NMR spectra of oxidised horse cytochrome *c* and oxidised *R. rubrum* cytochrome c_2 are not helpful in this context because all the contact shifted PMR resonances are in different positions [69] — probably reflecting a difference in the electronic structures of their heme groups. It is not yet possible to associate this difference with any structural feature of the protein. One apparently conflicting result is that the X-ray data indicate [67] there can be but a very small conformational change upon reduction of cytochrome c_2. If the Fe—S—Met bond were to become axial some other changes in structure might be expected.

In both oxidised (horse and tuna) [70] and reduced (tuna [65] and bonito [66]) cytochrome *c* the heme crevice is blocked by a phenylalanine, phe 82. Previously [30] the heme crevice had been thought to be open in horse ferricytochrome *c* with phe 82 pointing into the solvent. The heme crevice of *R. rubrum* cytochrome c_2 is also blocked in both oxidation states [67] by phe 91.

Left and right "channels". A striking feature of the structure of oxidised horse cytochrome c is a "channel" leading to the heme group from the

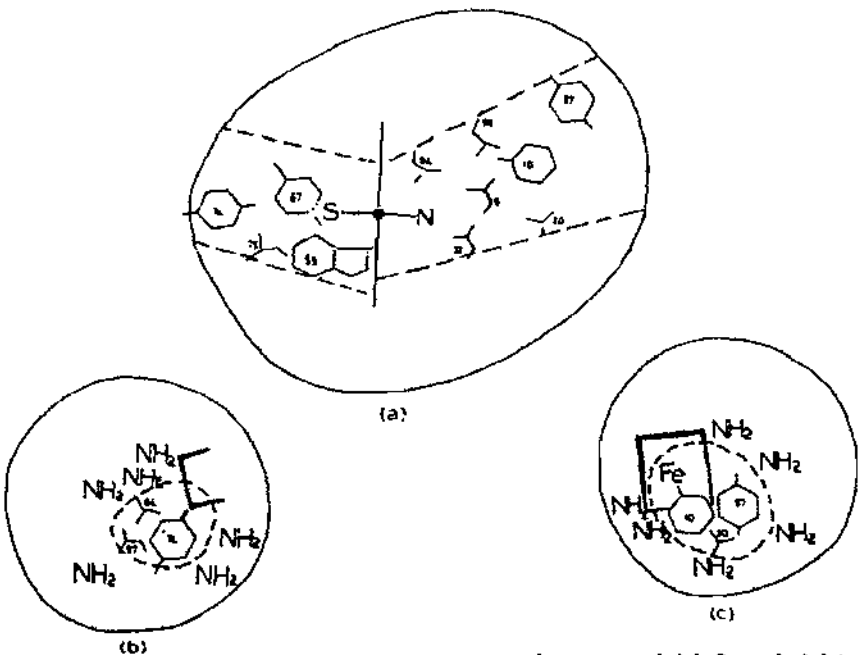


Fig. 2. Schematic diagram of horse ferricytochrome *c*: (a) left and right channels viewed from the front of the molecule; (b) left channel viewed from the left side of the molecule; (c) right channel viewed from the right side of the molecule. (– – – –), boundaries of channels. S is methionine, N is histidine.

TABLE 7

Amino acid side chains forming the right channel of cytochrome *c*

Source	9 [a]	10	11	20	32	35	36	65	94	95	97	98
Horse	I [b]	F	V	V	L	L	F	M	L	V	Y	L
Human	I	F	I	V	L	L	F	M	L	I	Y	L
Snapping turtle	I	F	V	V	L	L	I	M	L	I	Y	L
Bullfrog	I	F	V	C	L	L	I	M	L	I	Y	L
Carp	V	F	V	V	L	L	F	M	L	I	Y	L
Dog fish	V	F	V	V	L	L	F	R	L	I	Y	L
Lamprey	V	F	V	V	L	L	F	F	L	I	Y	L
Tuna	T	F	V	V	L	L	F	M	L	V	Y	L
Fruitfly	L	F	V	V	L	L	I	F	L	I	Y	L
Silkworm moth	I	F	V	V	L	F	Y	F	L	I	Y	L
Tobacco horn worm moth	I	F	V	V	L	F	F	F	L	I	Y	L
Mung bean	I	F	K	V	L	L	F	Y	L	I	Y	L
Candida krusei	L	F	K	I	L	I	F	S	L	V	Y	M
*Debaryomyces kloecker*i [117]	L	F	K	V	L	V	V	S	L	I	Y	L
Euglena gracilus [61]	L	F	E	A	L	V	Y	H	I	I	Y	M
Crithidia oncopeltii [60]	I	F	K	G	L	I	V	D	L	I	Y	L
Neurospora crassa	L	F	K	E	L	L	F	F	I	I	F	M
Humicola languinosa [118]	L	F	K	E	L	L	F	F	L	I	Y	L
Alternatives	I	F	V	V	L	L	F	M	L	V	Y	L
	V		I	C		F	I	R	I	I	F	M
	T		E	I		I	Y	F				
	L		K	A		V	V	Y				
				G				S				
				E				H				
								D				

[a] Numbering for horse cytochrome *c*

[b] One-letter amino acid code recommended by the IUPAC–IUB International Commission on Biochemical Nomenclature.

At least 50 other cytochrome *c*s possess one of the above right channels.

Ref. 116 apart from those prokaryotic proteins marked.

molecular surface [30]. This channel, the right channel, is so characterised as there is a hydrophobic "hole" in the right-hand side of the protein (Fig. 2). The channel itself is lined with the side-chains of hydrophobic amino acids (Table 7) but as the area in the centre of the channel is not completely taken up with these groups, there is room for a small group in the channel [30]. On the left-hand side of the molecule (Fig. 2) is another hydrophobic region extending to the molecular surface from the heme. This has been termed the left channel (Table 8) although it is not solvent accessible but is

TABLE 8

Amino acid side-chains forming the left channel of cytochrome *c*

Source	40[a]	49	51	52	57	59	64	67	74	75	78
Horse	T[b]	T	A	N	I	W	L	Y	Y	I	T
Silkworm moth	T	S	A	N	I	W	L	Y	Y	I	T
Mung bean	S	S	A	N	V	W	L	Y	Y	I	T
Bakers' yeast	S	T	A	N	V	W	M	Y	Y	I	T
Humicola languinosa [118]	T	T	A	N	I	W	L	Y	F	I	T
Euglena gracilus	S	S	A	N	I	W	L	F	Y	V	T
Crithidia oncopeltii [60]	S	S	A	N	V	W	L	Y	F	M	T
Alternatives	T	T	A	N	I	W	L	Y	Y	I	T
	S	S			V		M	F	F	V	
										M	

[a] Numbering for horse cytochrome *c*.
[b] One-letter amino acid code recommended by IUPAC—IUB International Commission on Biochemical Nomenclature.
At least 50 other cytochrome *c*s possess one of the above left channels.
Ref. 116 apart from those prokaryotic proteins marked.

completely filled in with hydrophobic side-chains [30]. The hydrophobic nature of these channels is conserved throughout all mitochondrial and some bacterial (e.g. *R. Rubrum* cytochrome c_2 and *Micrococcus denitrificans* cytochrome c_{550}) cytochromes *c*s (Tables 7 and 8).

Conformational differences of cytochromes c. A comparison of the X-ray structures of oxidised horse [30] and reduced tuna [65] cytochromes *c* has revealed that there are extensive conformational differences between them. This difference has been attributed [71] to oxidation state rather than sequence differences because oxidised tuna, bonito and horse cytochromes *c* are isomorphous. However, reduced tuna [65] and reduced bonito [66] cytochromes *c* also have different conformations. All three proteins, i.e. oxidised horse [30], reduced tuna [65], and reduced bonito [66] cytochromes *c*, crystallise in different space groups ($P4_3$, $P2_12_12_1$ and $P2_12_12$ respectively) and it is probable that crystal packing forces are partly responsible for these differences in conformation. Amino acid sequence changes could also be responsible for some of the changes observed between oxidised horse and reduced tuna cytochromes *c*. The amino acid sequences of these proteins are given in Table 9. Tuna cytochrome *c* differs from horse cytochrome *c* in 19 residues and from bonito in only 2. Also tuna and bonito cytochrome *c* are 103 residues long whereas horse cytochrome *c* is 104 residues long.

Comparing oxidised horse [30] and reduced tuna [65] cytochromes *c*, the most extensive conformational difference is in the region of the right channel; residues 18—29 move so as to block partly the channel in the re-

TABLE 9

Amino acid sequence of cytochrome *c*s

	1																																40
Horse	G	D	V	E	K	G	K	K	I	F	V	Q	K	C	A	Q	C	H	T	V	E	K	G	G	K	H	K	T	G	P	N	L	H	G	L	F	G	R	K	T
Tuna	G	D	V	A	K	G	K	K	T	F	V	Q	K	C	A	Q	C	H	T	V	E	N	G	G	K	H	K	V	G	P	N	L	W	G	L	F	G	R	K	T
Bonito	G	D	V	A	K	G	K	K	T	F	V	Q	K	C	A	Q	C	H	T	V	E	N	G	G	K	H	K	V	G	P	N	L	W	G	L	F	G	R	R	T
	41																																80
Horse	G	Q	A	P	G	F	T	Y	T	D	A	N	K	N	K	G	I	T	W	K	E	E	T	L	M	E	Y	L	E	N	P	K	K	Y	I	P	G	T	K	M
Tuna	G	Q	A	E	G	Y	S	Y	T	D	A	N	K	S	K	G	I	V	W	N	N	D	T	L	M	E	Y	L	E	N	P	K	K	Y	I	P	G	T	K	M
Bonito	G	Q	A	E	G	Y	S	Y	T	D	A	N	K	S	K	G	I	V	W	N	E	N	T	L	M	E	Y	L	E	N	P	K	K	Y	I	P	G	T	K	M
	81																																104
Horse	I	F	A	G	I	K	K	K	T	E	R	E	D	L	I	A	Y	L	K	K	A	T	N	E									
Tuna	I	F	A	G	I	K	K	K	G	E	R	Q	D	L	V	A	Y	L	K	S	A	T	S									
Bonito	I	F	A	G	I	K	K	K	G	E	R	Q	D	L	V	A	Y	L	K	S	A	T	S									

From ref. 116. Differences in sequence underlined.

TABLE 10

Hydrogen bonding pattern of some amino acid residues in cytochrome *c*

	Horse ferri-cytochrome *c* [a]	Bonito ferri-cytochrome *c* [a]	Bonito ferro-cytochrome *c* [b]	Tuna ferro-cytochrome *c* [c]
His 26	Pro 44	Gly 45	Glu 44	Gly 45
Asn 52	ip	ip	op	op
Trp 59	ip	ip	ip	ip

ip, inner propionic acid group of the heme; op, outer propionic acid group of the heme.
[a] Ref. 30.
[b] Ref. 66.
[c] Ref. 65.

duced tuna cytochrome *c*. In electron density difference maps between oxidised bonito and horse cytochromes *c*, a different hydrogen bonding pattern was observable for [30] residue 44. This is glutamate (glu) in both bonito and tuna cytochromes *c* but proline (pro) in horse cytochrome *c*. The amino acid substitution at this position affects the hydrogen bonding pattern [72] of his 26, shown in Table 10. The different hydrogen bonding pattern within the oxidised molecules causes a perturbation along the chain which involves [30] residues 37—43. A different hydrogen bonding pattern is observed [72] in comparisons of reduced tuna and oxidised horse cytochrome *c* and it could be the cause of the difference in conformation between oxidised horse and reduced tuna cytochrome *c* which involves residues 18—29. However, the oxidised horse cytochrome *c* hydrogen bonding pattern of his 26 is re-established in [66] reduced bonito cytochrome *c*. The structure of the reduced bonito molecule in this region resembles the oxidised horse molecule rather than the reduced tuna molecule. Thus a combination of crystal packing forces and sequence differences appears to be responsible for these differences in conformation.

Another region of conformational difference between oxidised horse and reduced tuna cytochrome *c* involves the left channel. A tryptophan residue, trp 59, which is in the centre of this channel, is 5.0 Å away from the rear methyl group of the heme in horse ferricytochrome *c* but only 3.5 Å away from this methyl group [65] in tuna ferrocytochrome *c*. Again, there is a different hydrogen bonding pattern (Table 10) involving trp 59. The importance of the trp 59 hydrogen bond to the heme propionic acid group can be judged from the facts that if trp is replaced [73] by phe or the trp indole nitrogen formylated [74], so that the trp 59—heme propionate hydrogen bond cannot form, the Fe—S—Met bond breaks. Thus, a different hydrogen bonding pattern involving trp 59 might be expected to affect the heme group. In fact, the heme has a different orientation with respect to the protein chain in these two proteins [65]. The alignment of trp 59 in reduced bonitc cytochrome *c* is midway between its alignment [66] in oxidised

horse and reduced tuna cytochromes *c*. The hydrogen bonding pattern (Table 10) for the reduced bonito trp 59 is the same as for the oxidised horse trp 59 but whether the different alignment of trp 59 is an effect of oxidation state or crystal packing forces cannot yet be decided.

Recent NMR studies [78] of horse and tuna cytochrome *c* confirm that there is some movement of the trp 59 group upon oxidation state change. These studies [38,78] also suggest that to a limited degree cytochrome c is a flexible protein undergoing rapid fluctuations in solution of some parts of its structure. Some perturbation of the tertiary structure of cytochrome *c* by crystal packing forces can now be readily understood.

Again, a comparison of cytochrome c with *R. rubrum* cytochrome c_2 is interesting. Cytochrome c_2 does not appear to undergo a conformational change upon reduction [75]. In the crystal state bonito cytochrome *c* can be oxidised without a major conformational change [76] but crystals of cytochrome *c* cannot be reduced because they shatter on contact with reducing agents [77].

Redox potentials. The redox potentials for a number of cytochromes *c* are given in Table 6. The hydrophobic environment of the heme group and the methionine ligand has been considered to be a major factor in determining the redox potential [79,80]. This has been discussed in Sect. B in relation to other redox proteins. It is difficult to assess the affect of other factors upon the redox potentials but a comparison of cytochrome *c* and cytochrome c_2 can be made.

The structural studies indicate that the heme group of both cytochromes *c* and c_2 have a similar environment. However, in ferricytochrome c_2 a hydrogen bond network involving the non-axial Fe—S bond is operative [67]. If the Fe—S bond of c_2 becomes axial upon reduction, then the reduced form should be more favoured than the oxidised form with respect to cytochrome *c*. The relative redox potentials support this.

Mechanism of electron transfer. A mechanism of action for cytochrome *c* has been suggested based on the X-ray studies of horse and tuna cytochrome *c*. The aromatic rings in the left channel have been suggested to function as a charge transfer pathway for an electron into the protein [65] and the aromatic rings of the right channel to function as a pathway out of the protein [81]. Both channels are surrounded by a region of amine groups which could serve as a binding site for the reductase and oxidase complexes, Fig. 2. Chemical modification experiments have shown that the oxidase activity can be reduced, without affecting the reductase activity, by blocking amine groups on the right side of the molecule [72]. Other blocking experiments, e.g. with antibodies [82] and pyridoxal phosphate [83], have also shown that the reductase and oxidase act at different sites on the molecule.

The charge transfer mechanism [30] for reduction is then said to involve

the following hop series:

reductase $\xrightarrow{e}$ phe/tyr 74 $\xrightarrow{e}$ trp 59 $\xrightarrow{e}$ phe/tyr 67 $\xrightarrow{e}$ heme

Exact coplanarity of the aromatic rings is necessary for this and X-ray data for horse and tuna cytochromes show that they can be correctly aligned but not all in the same oxidation state [65]. When X-ray data for the reduced bonito protein [66] are also considered the alignment of the rings is not suitable for this charge transfer, however. Tyrosine (tyr) 74 and trp 59 are coplanar, as are tyr 67 and the heme group in oxidised horse cytochrome *c*, and trp 59 and tyr 67 are coplanar in reduced tuna cytochrome *c*. However, trp 59 and tyr 67 are not coplanar in reduced bonito cytochrome *c*. Preliminary X-ray data for *Micrococcus denitrificans* cytochrome c_{550} also provide evidence against this mechanism [70]. The corresponding group to phe/tyr 74 is a leu residue. It is also important to note that recent NMR spectroscopic data for horse cytochrome *c* show that a number of aromatic groups are flipping [38]. This should be contrasted with the static picture presented by X-ray crystallography.

An alternative mechanism for cytochrome *c* and cytochrome c_2 has been proposed based upon the electron entering the protein through the exposed edge of the heme [75]. However, in a cytochrome *c*—cytochrome reductase complex the cytochrome *c* heme is still exposed to solvent [84]. We would emphasize the hydrophobic nature of the left channel and suggest that this generates an effective conduction path. We have discussed this in more detail in Sect. B and will return later to the general point that it is a path or channel of hydrophobic residues which is required for electron transfer and not a path of aromatic groups. The mechanism of transfer is not by hopping through the channel but by tunnelling in a preferred direction.

Before passing to the next group of proteins we draw attention to the other important features of the protein from a mechanistic point of view. The energy for release or uptake of an electron at the iron centre can come from the vibrational energy of the protein and it may well be that there is very little activation energy for the process owing to the compromised bond lengths to the iron generated by the protein. Finally the conformational mobility may assist not only in the electron-transfer process itself but in the control of that process, so-called gating.

(b) Cytochromes c' and cc'

These are cytochromes which possess similar visible absorption spectra, at room temperature and pH = 7.0, to myoglobin and hemoglobin [49]. Depending upon the isolation procedure proteins containing one heme each have been isolated, with a molecular weight of 10—15000 (cytochromes *c'*) or two hemes each with a molecular weight of 20—30000 (cytochromes *cc'*). Partly because the diheme proteins can be dissociated into identical monoheme subunits, the *c'—cc'* nomenclature system is no longer favoured [49]. We shall name all such proteins cytochrome *c'* and make it clear in the

text of how many units the protein consisted for the particular experiment being described.

The biological function of these proteins is not yet known.

Structure. The only data we have concerning the structure of cytochromes *c'* come from spectroscopic measurements and chemical investigations. A cys—X—X—cys—his sequence provides the heme binding site [85]. There is no complete sequence available for a cytochrome *c'* but the amino acid composition of some are known. The one heme (137 amino acids) cytochrome *c'* from *Rhodopseudomonas palustris* contains few aromatic amino acids; one tyr, five phe and no trp [86]. The diheme (molecular weight 29000) cytochrome *c'* from *Rhodospirrillum rubrum* on the other hand, contains seven tyr (?), eight phe and two trp residues [87].

The similarity of the visible spectrum of cytochrome *c'* to myoglobin indicates that the iron has a great deal of high-spin character [88]. However, EPR measurements [88] and resonance Raman spectroscopy [89] have shown that there are major differences in electronic structure between the heme groups of myoglobin and cytochrome *c'*. Both ferrocytochrome *c'* and ferricytochrome c' of *Chromatium* (diheme protein) exist in different molecular states. Transitions between the different states can be induced by changing the pH of a solution of the protein [88]. Four states were characterised by EPR spectroscopy for the diheme (molecular weight 24 000) *Chromatium* ferricytochrome *c'*, the transitions between these states being completely reversible [88].

The magnetic properties of the heme groups in two of these states, the extreme pH forms (pH = 1 state B_1 and pH = 11 state B_2), are similar to those of acid metmyoglobin and methemoglobin. However, the pH stability and EPR spectral properties of state B_2 make it unlikely that either H_2O or OH^- is the sixth iron ligand [88].

At pH = 7.0 EPR signals are observed for two species, states A_1 and A_2. An interpretation [88] of these signals concludes that there is a quantum-mechanical admixture of spin states of the heme iron electronic states. The iron electronic state corresponding to signal A_1 consists of an admixture of 65% of the intermediate-spin state ($S = 3/2$) and 35% of the high-spin state ($S = 5/2$), whilst the iron electronic state corresponding to signal A_2 consists of an admixture of 65% of the high-spin state and 35% of the intermediate-spin state. The protein state giving rise to signal A_1 has the spin quartet lying below the spin sextet whilst for the state A_2 the splitting is reversed. EPR studies of other ferricytochromes *c'* at pH = 7.0 and 100°K all show a similar pattern suggesting that this quantum-mechanical admixture of spin states is a common feature [88] of cytochromes *c'*. The monoheme protein from *Rhodopseudomonas palustris* also exhibits this mixing of spin states indicating that the mixing is not a result of dimerisation [88]. That states A_1 and A_2 represent different conformational states of the protein has been confirmed by visible [90] and CD spectroscopy [91].

Although both monoheme and diheme cytochromes *c'* from a wide variety of sources bind NO and CO in the reduced form they do not bind F^-, CN^- or N_3^- in the oxidized form [92]. It seems more likely that the environment of the heme prevents anion binding rather than lack of access because NO binds [92] to ferricytochrome *c'*. This is in agreement with solvent perturbation studies which indicate that there is a hydrophobic region surrounding the heme [88].

Mechanism of electron transfer. The low content [86] of aromatic amino acids of these proteins is significant in view of proposed mechanisms of electron transfer based upon charge transfer [65].

It is not possible to make two electron-transfer chains of hopping centres based upon tyr and trp for there is only one tyr and no trp in the sequence [86] of *R. palustris* cytochrome *c'*. The peculiarly poised electronic states (a clear example of an entatic state [11] of a heme moiety) has advantages for the ejection of electrons into the protein matrix, as discussed in the introductory sections.

(c) Cytochromes c_3

Cytochromes c_3 are a class of hemoproteins containing several covalently bound mesohemes and having a negative redox potential [93] at pH = 7. Such proteins have been isolated with a molecular weight of 13000 containing four hemes and with a molecular weight of 26000 containing eight hemes from the *Desulfovibrio* species of bacteria [93]. The amino acid composition of the 26000 molecular weight proteins shows that they are not merely dimers of the 13000 molecular weight proteins. In addition cytochrome [94] c_7, which contains three hemes, from *Chloropseudomonas ethylicum* is considered to be a member of this group [93].

The four heme cytochromes c_3 function as both an electron donor to, and an electron acceptor from, hydrogenase [93].

Structure. There are at least three redox states of the protein; fully reduced, fully oxidised and an intermediate state [50], with the protein being reduced in two two-electron steps separated in midpoint potential by about 50 mV.

From a combination of sequence comparisons and NMR spectroscopy a secondary structure has been proposed [50] for the 13000 molecular weight cytochrome c_3 from *D. vulgaris*, Fig. 3. The primary structures of all the cytochromes c_3 are similar, containing two cys—X—X—cys—his and two cys—X—X—X—X—cys—his regions which provide the heme binding sites. There are also four invariant his residues which, from the EPR and NMR data, have been assigned [50] as the sixth iron ligands. The hemes are therefore low spin.

The main feature of the proposed model is the four heme groups which form a box with two pairs of hemes roughly planar to each other and about 10 Å apart. Each heme has at least one aromatic amino acid associated with

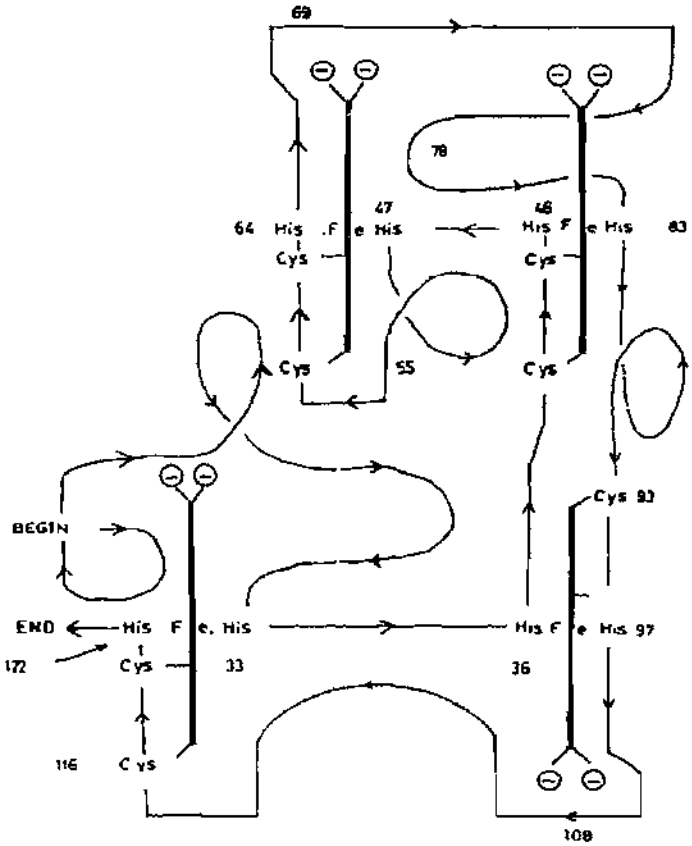


Fig. 3. Outline structure of *Desulfovibrio vulgaris* cytochrome c_3.

it. As there are only five (out of 84) aromatic amino acids there clearly cannot be a cytochrome *c* type alignment of them. Only three of these aromatic amino acids are conservatively replaced (Table 11).

All of the hemes are relatively exposed at the surface of the protein for

TABLE 11

Aromatic amino acids of cytochromes c_3

	20	31	49	55	78	79	82	89
D. gigas	F	F	P	Y	W	Y	V	A
D. vulgaris	—	F	V	Y	Y	Y	M	F
D. desulfuricans	—	F	W	I	F	Y	F	—
D. salexigens	—	F	W	V	F	Y	F	—

To maximise homology deletions (—) have been invoked.
From Ref. 50.

there are insufficient amino acids to completely cover them. There are 17 lys residues which are grouped on the molecular surface where two of the hemes are exposed.

Mechanism of electron transfer. An interesting feature of the protein is that despite the fact that the heme groups are only 10 Å apart there is relatively slow electron exchange between them [50]. The model shows that the hemes are only separated by a single polypeptide chain. In fact it is impossible to get a greater number of chains between them, and therefore a peptide chain per se cannot provide a path for rapid electron transfer.

(ii) Cytochromes b

Proteins of this group contain the prosthetic group protoheme, which is not covalently bound to the protein via heme substituents [49]. Such proteins function as electron carriers (e.g. mitochondrial cytochrome *b*) and terminal oxidases (e.g. cytochrome P-450). Cytochrome *b* of the mitochondrial respiratory chain was the first protein of this type to be reported but because of the difficulties in its preparation and purification little is known of its structure. This point also applies to most cytochromes b which have been reported and we shall therefore confine ourselves to a discussion of cytochromes b_5 and b_2 which have been extensively studied [49].

(a) Cytochrome b_5

Cytochrome b_5, a membrane bound protein, functions as an electron-transfer protein in a number of electron-transport systems, including those for steroid hydroxylation [95], fatty acid desaturation [96] and methemoglobin reduction [97]. The method for purification of this protein, involving treatment of a crude preparation with proteolytic enzymes, yields a water-soluble protein, fully active with cytochrome-b_5-reductase, in which approximately 44 amino acid residues have been lost from the N-terminus of the protein [98].

Structure.

The heme group. X-ray structural studies [98,99] have been carried out with the protein from calf-liver treated in the manner described. The modified protein consists of 93 amino acids and one heme which is attached to the protein via two Fe—his bonds in the axial position. The hydrophilic edge of the heme is exposed to the solvent, rather than one of the hydrophobic edges as in cytochrome *c*, Fig. 4.

EPR and absorption spectral data show that the heme is readily brought into an equilibrium between high-and low-spin states on minor changes of pH even though the iron atom has six nitrogen ligands [100]. The environment of the heme group is hydrophobic [99], a recurring feature of heme proteins. The heme group is situated in a box whose base is provided by a β-pleated sheet with sides made of α-helices [99] (Fig. 4).

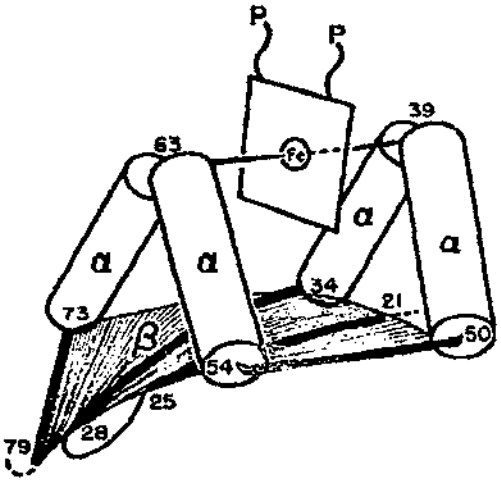


Fig. 4. Schematic diagram of liver microsomal cytochrome b_5 (from ref. 262). Cylinders are α-helices, wavy lines with P are propionic acid groups from the heme, and the four heavy rods with shading below are the β-pleated sheets.

Channels. There is a narrow hydrophobic groove on the surface of the molecule leading to the heme group [98]. The residues lining this groove are essentially hydrophobic and are conserved throughout the cytochromes b_5 of known sequence (see ref. 116).

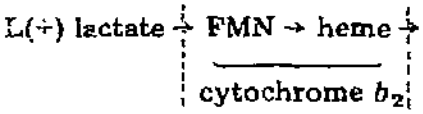
Conformation. NMR spectral data indicate that there is not an extensive conformation change upon reduction of the protein [101]. This is in agreement with preliminary X-ray data.

Mechanism of electron transfer. The spin-state equilibrium of the heme group has been taken to suggest that the iron ligands can alter their bond distances very rapidly [100]. Such a situation would facilitate electron transfer.

The hydrophobic groove [98] leading to the heme could function as a track for the transfer of electrons into and out of the protein. Such a model requires some degree of movement of the cytochrome b_5 molecule so that it can interact with both a reductase and an oxidase. There is some evidence to show that cytochrome b_5 has a high degree of mobility when membrane bound and that the hydrophobic tail, which binds cytochrome b_5 to the membrane, is hinged at its point of attachment to the b_5 hydrophilic portion [102]. This model also requires cytochrome b_5 reductase to bind to cytochrome b_5 reduce it and the complex to dissociate. The oxidase could then bind. Such a ping-pong kinetic mechanism has been observed for the NADH-cytochrome-c-reductase activity [103] of cytochrome b_5. A region of acidic groups by the mouth of the channel and around the heme group could then act as a binding site [99].

(b) Cytochrome b_2

Yeast cytochrome b_2, L(+) lactate-cytochrome-c-oxidoreductase, functions in the system [49]



The enzyme is specific for L(+) α-hydroxy-acids; it will not oxidise D(−) lactate, but its biological role is unknown.

Structure. Cytochrome b_2 is a tetrameric double-headed enzyme of molecular weight about 230000, containing one heme group and one FMN group per subunit [49]. Little is known of the structure of the intact enzyme. Digestion of cytochrome b_2 with trypsin yields a flavin-free peptide, cytochrome b_2 core, of molecular weight [104] 10000. The amino acid sequence of this peptide is homologous with the sequence [104] of cytochrome b_5. The EPR, absorption, fluorescence and NMR spectral data also indicate that the molecular conformation of cytochrome b_5 and cytochrome b_2 core are very similar [105]. In view of these findings it has been suggested that the cytochrome b_2 corresponds to the cytochrome b_5/cytochrome-b_5-reductase system [104].

(iii) Hemoglobin and myoglobin

Hemoglobin and myoglobin are usually only considered as oxygen transport and storage proteins respectively [106]. However, they fulfil some of our requirements for electron-transport capability. In fact both proteins, which bind molecular oxygen in the ferrous state, form stable ferric proteins, methemoglobin and metmyoglobin, whose respective E^0 values [107] are +170 mV and +46 mV. Their capacity for redox activity is demonstrated by the fact that plant ferredoxins can reduce both methemoglobin and metmyoglobin [108]. Myoglobin can also be oxidised [109] by ferricytochrome c.

The redox capability of hemoglobin is physiologically important. In red blood cells ~3% of the hemoglobin of a normal adult male becomes oxidised to methemoglobin during the course of a day. To reduce this methemoglobin red blood cells contain a methemoglobin reductase system. This redox chain has been demonstrated [97] to be

NADH → cytochrome-b_5 → cytochrome → methemoglobin

A redox activity for myoglobin in vivo has also been suggested based on various spectroscopic studies which indicate that myoglobin can attach itself to mitochondrial membranes and, when succinate is added, be converted to metmyoglobin [110]. It is interesting to note in this context that a

bacterial electron-transfer protein, *E. coli* cytochrome b_{562}, is considered to have an homologous amino acid sequence to myoglobin [111]. In addition chemical and spectral studies suggest that it has a similar structure, although it is much smaller [112].

Structure. Myoglobin is a monomer of molecular weight 16000 whilst hemoglobin is a tetramer ($\alpha_2\beta_2$) of molecular weight 64000. The individual hemoglobin subunits resemble myoglobin; they are in fact homologous proteins [106] and we shall therefore only discuss the structure of myoglobin.

Heme group. The iron is five coordinate in myoglobin, the ligands being the porphyrin nitrogens and a his, and six coordinate in metmyoglobin, the additional ligand being H_2O, OH^-, etc. [113]. Deoxymyoglobin is high spin whilst oxymyoglobin is low spin. There is also a thermal equilibrium between high- and low-spin forms of metmyoglobin at room temperature [114].

The heme group is located in a hydrophobic pocket of the protein which extends to the molecular surface. The polar edge of the heme group is exposed at the surface. This is a very open structure allowing O_2, CO, NO, etc., to enter and coordinate to the iron in myoglobin, and N_3^-, CN^-, SCN^-, OH^- to enter and bind with metmyoglobin. No ionic ligands bind to myoglobin, partly reflecting the requirement for electro-neutrality in the hydrophobic pocket [114]. The heme crevice can also bind small organic molecules, e.g. cyclopropane. Large ligands cannot enter the pocket and it is very unlikely that large water-soluble reagents such as those used in making electron-transfer rate measurements could reach within bond distance of the iron atom.

Structure of the protein. The protein chain provides a box for the heme group consisting of eight stretches of α-helix [106]. About 80% of the amino acid residues are involved in this. There are no hydrophobic or aromatic channels of the cytochrome *c* type leading to the heme and no regions of high positive or negative charge on the surface of the molecule.

Mechanism of electron transfer. As with cytochrome b_5 the heme groups of metmyoglobin and methemoglobin are in equilibrium between high- and low-spin forms. The relevance of this to electron transfer has already been discussed (Sect. B). Table 12 lists those heme proteins exhibiting such an equilibrium.

The interaction of myoglobin with cytochrome *c* is interesting [109]. Cytochrome *c* oxidises deoxymyoglobin; O_2 is an inhibitor of the reaction. Ferricytochrome *c* also oxidises milk xanthine oxidase [115] and for this reaction both myoglobin and myoglobin globin, although not hemoglobin, are potent competitive inhibitors. Denaturation of the globin removes the inhibition. Thus there appears to be a specific region of the cytochrome *c* molecule which reacts with both milk xanthine oxidase and myoglobin. By analogy with the proposed route for reduction of cytochrome *c* this could include the left channel of cytochrome *c*. However, a complex between

TABLE 12

Spin equilibria in hemoproteins [a]

Protein	Spin states			Ref.
	$s = 1/2$	$s = 3/2$	$s = 5/2$	
Cytochrome a_3	√		√	140
Cytochrome b_5	√		√	100
Cytochrome P 450	√		√	141
Myoglobin	√		√	114
Hemoglobin	√		√	114
Peroxidase	√	(mixing)?	√	139
Cytochrome *c'*	√	← mixed →		88

[a] Some NMR resonances of the heme groups of these proteins possess unusual temperature dependencies (chemical shift increases with temperature rather than decreases). This behaviour is not possessed by 'model' low-spin heme compounds and is possibly a consequence of the spin equilibria. Similar behaviour is exhibited by some of the heme NMR resonances of cytochrome *c* indicating that this protein may also possess a spin equilibration.

cytochrome *c* and myoglobin has been sought using spectroscopic, gel filtration and sedimentation methods [109]. All experiments failed to demonstrate the existence of a complex.

(iv) Iron—Sulphur Proteins

Iron—sulphur proteins have been defined as proteins containing non-heme iron bonded to sulphur [143]. We shall concentrate on the bacterial and plant type ferredoxins, and rubredoxins but we note that some iron—sulphur proteins do not readily fall into these classes, e.g. the Fe_6S_6 protein from *Rhodospirillum rubrum* [144].

Iron—sulphur centres are not restricted to the above proteins; many proteins contain iron—sulphur centres in association with other prosthetic groups: such as heme, Mo (ref. 142), FMN and FAD (ref. 145). Little is known of the structure of these multi-headed proteins and we shall therefore not consider them here. A review of these proteins has appeared in *Coordination Chemistry Reviews* [145] and we shall present only a summary of points relevant to the general theme of this article.

(a) Bacterial ferredoxins

The characteristic features of these proteins are a negative E^0 (Table 13) associated with a cubane type Fe/S* centre (Fig. 5). This differentiates them from the high-potential iron proteins (HiPIPs) which also contain a cubane Fe/S* centre but have a positive E^0 (Table 13).

Bacterial ferredoxins function as electron-transfer proteins carrying one electron per cubane centre in a wide range of reactions [146].

TABLE 13

Physical properties of iron—sulphur proteins

Protein source	No. of Fe atoms	No. of S* atoms	Molecular weight	Redox potential (mV)	Ref.
Rubredoxins					
Clostridium pasteurianum	1	—	6000	−57	197
Chlorobium thiosulfatophilum	1	—	6000	−61	198
Pseudomonas oleovarans	2	—	19000	−40	173
Ferredoxins					
Clostridium pasteurianum	8	8	6000	−415	199
Azotobacter vinelandii I	8	8	14400	−420	200
Azotobacter vinelandii II	8	8	n.d.	−460	200
Bacillus polymyxa I	4	4	9000	−390	201
Bacillus polymyxa II	4	4	9000	−420	202
Chromatium	8	8	8600	−490	203
Spinach	2	2	11600	−420	199
Parsley	2	2	n.d.	−420	204
Pseudomonas putida	2	2	12000	−260	143
Escherichia coli	2	2	12500	n.d.	205
Beef adrenals	2	2	15000	−270	143
Beef mitochondrial	2	2	26000	+230	164
Rhodospirillum rubrum I	6	6	6700	n.d.	144
Rhodospirillum rubrum II	2	2	7500	n.d.	144
High-potential iron proteins					
Chromatium vinosum	4	4	10000	+350	206
Rhodopseudomonas gelatinosa	4	4	9600	+330	206

n.d., not determined; S*, inorganic sulphur.

Structure. An X-ray crystallographic study of the oxidised form of the 8Fe/8S* ferredoxin from *Peptococcus aerogenes* (Fig. 6) has been carried out [147].

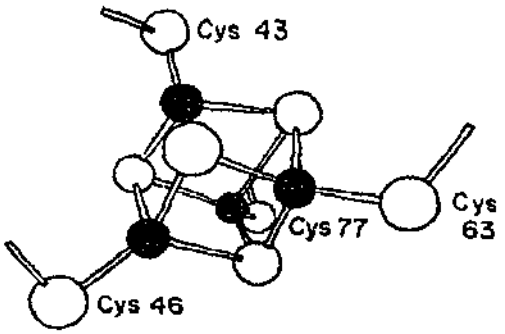


Fig. 5. Structure of the Fe/S* cluster of HiPIP and bacterial ferredoxin. The iron atoms, represented by the filled spheres, are coordinated each to one cysteinyl and three inorganic sulfides (empty spheres) in a tetrahedral manner (from ref. 166).

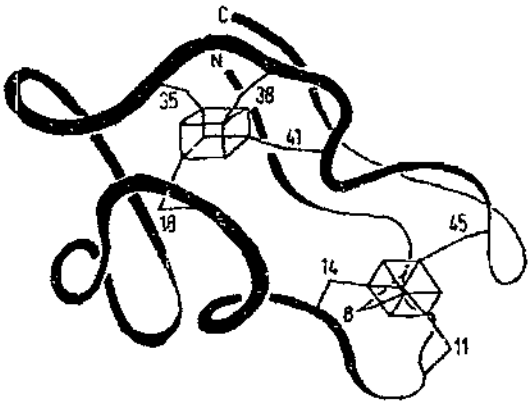


Fig. 6. Structure of *P.aerogenes* ferredoxin showing the relationship between the two iron clusters (from ref. 13).

Redox centre. The Fe and inorganic S (S^*) atoms form two redox centres each consisting of a cube of 4Fe and 4S^* at alternate corners (Fig. 7). These cubes are attached to the protein via four cysteine (cys) residues per cube which provide a sulphur ligand to each iron [147].

The cubes are placed at opposite ends of the protein (10—12 Å apart), just below the molecular surface, with the end face of each centre incompletely covered by the protein. The immediate environment of each centre is hydrophobic (Table 14) with a tyr residue 3.5 Å away from one face of the cubes [147]. The Fe/S^* centres of bacterial ferredoxins resemble those of the plant ferredoxins and oxidised HiPIPs; in the reduced form the electron is not delocalised over the entire centre. NMR spectroscopy shows that, for the oxidized ferredoxin, the contact shifts of the cys β-CH_2 resonances increase with temperature [148]. This is because of the antiferromagnetic coupling between the iron atoms. With the reduced 8Fe/8S^* proteins, however, the con-

TABLE 14

Environment of the Fe—S^* centre of *Peptococcus aerogenes* ferredoxin

Complex	Face	Residues "seen"
I	8-11-14	Tyr 28, Gly 12
	8-11-45	Ile 9, Val 47
	8-14-45	Ala 9, Ile 4 (Pro 50)
	11-14-45	Val 44, Ala 13, Pro 46
II	35-38-41	Tyr 2, Gly 39
	35-38-18	Ile 36, Val 20
	35-41-18	Ile 22, Ile 38
	38-41-18	(Pro 19)

From ref. 147.

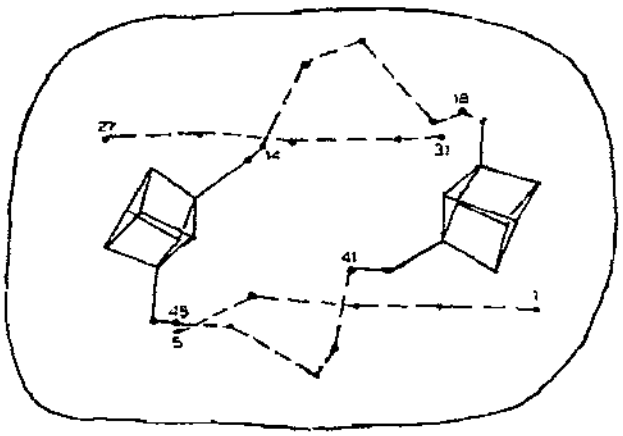


Fig. 7. Schematic diagram of *P.aerogenes* ferredoxin showing the channel between the Fe/S* centres: (– – – –), peptide chain; (•———•), Fe/S* cubes and cysteine ligands.

tact shifts of four of the β-CH_2 resonances increase with temperature whilst those of the remaining four decrease with temperature. A similar study with the 4Fe/4S* *Bacillus polymyxa* ferredoxin and oxidised *Chromatium* HiPIP reveals that two of the four β-CH_2s of each group are associated with each centre. This can only be rationalised if the additional electron is unevenly shared by the iron atoms [148].

Channel. There is a channel running between the two centres (Fig. 7). This channel is bounded by the two Fe/S* centres and the peptide chain, and contains the side groups of proline (pro) 50, isoleucine (ile) 4 and ile 9 as well as some β-CH_2 groups of the cys residues. The channel continues on to the molecular surface after the end face of each Fe/S* cube, where it terminates in a cluster of hydrophobic residues. For Fe/S* centre I these are pro 16, tyr 28, ile 9, valine (val) 47, pro 46 and val 44. There are two regions of surface negative charge to either side of the hydrophobic patch of centre I. One region is composed of four carboxyl groups (glu 23, glu 24, aspartate (asp) 31 and asp 33), and the other is composed of three carboxyl groups (glu 52, asp 53 and the C terminus). These acidic groups, as well as the hydrophobic groups, are present in all 8Fe/8S* ferredoxins whose sequence is available [116].

Conformation. The amino acid sequences of a number of 8Fe/8S* bacterial ferredoxins have been determined and there is considerable homology between them (Table 15), especially among those residues placed near the Fe—S* centres [116]. Homology is also evident between the two halves of the protein [149]. Each half contains four cys residues and instead of the structure (Fig. 8) which might have been expected crossover occurs (Fig. 8). One result of this is that there is an approximate two-fold axis of symmetry within the molecule. It has been suggested that this crossover occurs to maximise any conformational change between the two oxidation states

TABLE 16

Bacterial ferredoxin amino acid sequences [a]

	1																																35
Peptococcus aerogenes	A	Y	V	I	D	D	S	C	I	A	C	G	A	C	K	P	E	C	P	V	N	–	I	Q	Q	G	–	S	I	Y	A	I	D	A	D
Clostridium pasteurianum	A	Y	K	I	A	E	S	C	V	S	C	G	A	C	A	S	E	C	P	V	N	A	I	S	Q	G	D	S	I	F	V	I	D	A	D
Clostridium acidi-urici	A	Y	V	I	D	D	A	C	I	S	C	G	A	C	D	P	E	C	P	V	D	A	I	S	Q	G	D	S	R	Y	V	I	D	A	D
Chromatium str-D [b]	A	L	M	I	T	D	Q	C	I	N	C	N	V	C	Q	P	E	C	P	N	G	A	I	S	Q	G	D	E	T	Y	V	I	E	P	S
	36																					56												
Peptococcus aerogenes	S	C	I	D	C	G	S	C	A	S	V	C	P	V	G	A	P	N	P	E	D													
Clostridium pasteurianum	T	C	I	D	C	G	N	C	A	N	V	C	P	V	G	A	P	V	Q	E														
Clostridium acidi-urici	T	C	I	D	C	G	A	C	A	G	V	C	P	V	D	A	P	V	Q	A														
Chromatium str-D	L	C	T	E	C	V	D	C	Y	E	V	C	P	I	K	D	P	S	H	E	E	–												

[a] The one-letter amino acid code recommended by the IUPAC–IUB International Commission on Biochemical Nomenclature.
[b] The loop, 42-50, and the C-terminus, 57-71, have been omitted from the *Chromatium* sequence.
Differences in sequence underlined.
From ref. 116.

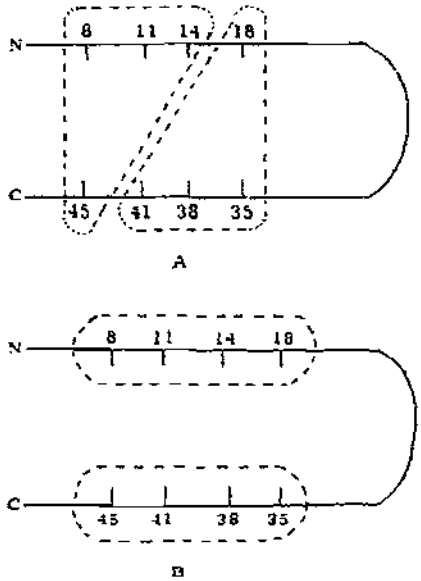


Fig. 8. Structure of *P.aerogenes* ferredoxin. The cysteine ligands are numbered The cross-over structure A occurs rather than the linear structure B (from ref. 166).

[150]. There is some evidence, in the form of T—H equilibration experiments with *Clostridium acidi-urici* ferredoxin, to suggest that a conformational change does occur on oxidation—reduction [151].

Mechanism of electron transfer. A possible role for the two tyr residues of *Clostridium acidi-urici* ferredoxin in electron transfer has been suggested based on NMR data. These data show that for the *Clostridium acidi-urici* ferredoxin the tyr ring protons 2′ and 6′, as well as 3′ and 5′, are equivalent in both oxidation states and are shifted from their resonance positions in N-acetyl tyr by a smaller amount than the corresponding C^{13} resonances, which are also equivalent. To account for the relative C^{13}—H^1 shifts some kind of interaction with the Fe/S* centre was postulated [152]. However, the relative magnitudes of the shifts could be due to the environment of the tyr residue. The equivalence of the 2′ and 6′, and the 3′ and 5′, positions indicates that, as with cytochrome c [38], these aromatic rings are able to rotate with a frequency $\geq 10^4\ s^{-1}$.

It should also be remembered that these proteins can function with phe in place of tyr 28 (ref. 153) and with both phe (ref. 154) and his (ref. 155) in place of tyr 2. If *Chromatium* ferredoxin is also considered to be homologous leucine (leu) can replace tyr 2 as well (Table 15). Recent experiments with modified *Peptococcus aerogenes* ferredoxin (leu instead of tyr 2) show that a tyr in this position is not essential for activity [156]. This modified protein was indistinguishable from the native protein in enzymic assays. With

this ferredoxin, however, it was not possible to exchange both tyr for leu. However, a ferredoxin from *Clostridium M-E* has been obtained in which position 28 is occupied by arginine (arg). There is only one aromatic residue, tyr 2, in the molecule. After modification (tyr—leu) this protein was fully active in the phosphoroclastic reactions [157]. Aromatic residues are therefore not essential for electron transfer, in ferredoxins at least.

Bacterial ferredoxins form 1 : 1 complexes with ferredoxin-NADP reductases and these complexes are inactivated by high ionic strength [158]. Thus, it appears as though there is an electrostatic interaction between the two proteins. The two regions of surface negative charge near cluster I could therefore provide for a binding site for ferredoxin-NADP reductase.

From the crystallographic study [147] we have a picture of *Peptococcus aerogenes* ferredoxin which is essentially 2Fe/S* centres separated by a hydrophobic channel (Fig. 7). This can also be viewed as a hydrophobic channel running through the protein with 2Fe/S* centres placed in it. Where the channel meets the molecular surface the electron injection/ejection sites are formed, probably at cluster I. This channel could function as a very effective conduction path. Both *Clostridium pasteurianum* and *Clostridium acidi-urici* are in fast exchange between oxidised and reduced forms on the NMR time scale (i.e. $\geqslant 10^5\ s^{-1}$) whereas the 4Fe/4S* *Bacillus polymyxi* ferredoxin and *Chromatium* HiPIP are in slow exchange [148]. Thus, the 8Fe/8S* ferredoxins are probably in fast exchange via an intramolecular transfer [143].

(b) High-potential iron proteins

These proteins, known as HiPIPs, derive their name from their high redox potentials [143] (Table 13). They possess similar cubane Fe/S* centres to the bacterial ferredoxins. In fact bond angles and lengths are nearly the same (Table 16) [15,147]. There is no homology between their sequences, how-

TABLE 16

Dimensions of Fe—S* clusters

	Oxidised *Chromatium* HiPIP	Reduced *Chromatium* HiPIP	Oxidised *P. Aerogenes* ferredoxin
Bond lengths (Å)			
Fe—Fe	2.68—2.78	2.74—2.87	2.77—2.93
Fe—S*	2.10—2.39	2.18—2.45	2.24—2.34
Fe—S	2.17—2.22	2.19—2.26	2.05—2.31
Bond angles (deg)			
Fe—S*—Fe	72—76	72—80	75—79
S*—Fe—S*	101—109	99—107	98—104
S*—Fe—S	107—120	106—126	112—120

S*, inorganic sulphur; S, cysteinyl sulphur.

ever [116]. Despite being one of the most abundant proteins in *Chromatium* cells the function of HiPIP is not known.

Structure. X-ray analysis [159,160] of the HiPIP from *Chromatium* in both oxidised and reduced forms (states C^+ and C) reveal that the redox centre is not accessible to solvent; it is bound in a hydrophobic pocket (Table 17). Upon reduction of the protein ($C^+ \rightarrow C$) there is a small expansion of the Fe/S* cube [161]. Although this does not cause a gross change in the protein conformation there may be small changes in the arrangement of the immediate side-chains [161].

The overall structure of the HiPIP molecule does have some gross similarities to the *Peptococcus aerogenes* ferredoxin. The molecules appear to be constructed of two halves, one region binding the Fe/S* centre, the other shielding it. In *Chromatium* HiPIP there is an aromatic group, tyr 19, located near the cube as well [160].

Redox potential. The fact that the Fe/S* centres of HiPIP and bacterial ferredoxin are similar appeared to pose a problem. Why do HiPIP and bacterial ferredoxin have redox potentials differing by 0.75 V (Table 13) when they possess similar redox centres? The paradox was resolved when it was established that the proteins were being compared for different redox reactions; the oxidation of the spin paired state (C) for HiPIP and the reduction

TABLE 17

Environment of the Fe—S* centre of HiPIPs

Chromatium	*Thiocapsa pfennigii*	*Rhodopseudomonas gelatinosa*
Leu 17	Leu	Leu
Tyr 19	Tyr	Tyr
Cys 43	Cys	Cys
Cys 46	Cys	Cys
Phe 48	Phe	Leu
Met 49	Ile	Phe
Trp 60	Trp	—
Cys 63	Cys	Cys
Leu 65	Leu	Leu
Phe 66	Tyr	Phe
Gly 68	Gly	Gly
Ile 71	Val	Val
Gly 75	Gly	Gly
Trp 76	Trp	Trp
Cys 77	Cys	Cys
Trp 80	Trp	Trp

From ref. 162.

of this state for bacterial ferredoxins [15]:

HiPIP $C^+ + e \rightleftharpoons C \quad E^0 = +350$ mV

ferredoxin $C + e \rightleftharpoons C^- \quad E^0 = -400$ mV

HiPIP can be obtained in the C^- state when it possesses similar spectral properties to bacterial ferredoxin in the C^- state [162].

Mechanism of electron transfer. Tyr 19 has been implicated in a mechanism of electron transfer [161]. Near to the tyr are two trp residues which extend the region of aromaticity to the surface of the protein. However, peptide chain is located between the tyr and trp residues and it is difficult to envisage any charge transfer pathways being operative. On the other hand there are no well-defined regions of the protein corresponding to the channels observable in bacterial ferredoxin and cytochrome *c*. There is, however, one face of the hydrophobic pocket exposed at the molecular surface. This is bounded by leu 17 and leu 65. The peptide chain does not block this approach to the redox centre. As the Fe/S* cube (S* 2 and S* 4) is only 4.5 Å away from the molecular surface [161], this region could represent a possible track for electron flow. There is no region of the molecular surface abundant in either amine groups or carboxylate groups. Any site for interaction with reductases and oxidases must therefore have a more complex recognition mechanism.

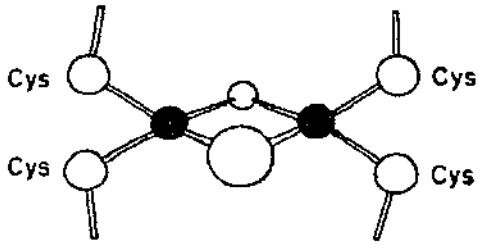
(c) Plant type ferredoxins

As their name suggests plant type ferredoxins were first isolated from plants but since then such proteins have been obtained from bacteria and animals as well [143] (Table 13). In addition, Fe_2S_2* proteins have recently been isolated with spectral properties and redox potentials characteristic of the HiPIPs. We shall not discuss these further. These proteins are classed together because they possess 2Fe and 2S* atoms per molecule with similar absorption and ORD spectra. They function as electron-transfer proteins carrying one electron per molecule [143].

Structure.

Redox centre. Plant type ferredoxins have not yet been characterised by X-ray crystallography but on the basis of spectroscopic measurements a structure for the redox centre has been proposed [164] (Fig. 9). Support for this proposal comes from an iron–sulphur compound which possesses similar spectroscopic properties to the proteins [165] and possesses the structure of Fig. 9.

The nature of the redox centre in both oxidation states has been described in terms of the proposed model [164]. In the oxidized protein the redox centre consists of two high-spin ferric ions antiferromagnetically coupled whilst in the reduced state it consists of one high-spin ferrous ion and one high-spin ferric ion antiferromagnetically coupled. The ligand symmetry



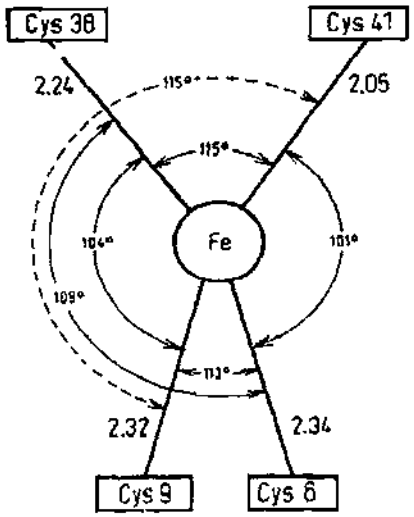


Fig. 9. Proposed model for the Fe/S* centre of plant ferredoxins. The iron atoms are represented by the filled spheres (from ref. 166).

Fig. 10. Geometry of the Fe/S centre of *C.pasteurianum* rubredoxin (from ref. 13). The irregularities of the structure are obvious.

around the iron atoms does not appear to be affected by reduction [164].

Conformation. Measurements of *g*-values have been taken, in conjunction with ORD and CD data and the effect of denaturants, to suggest that adrenodoxin and spinach ferredoxin have different conformations [166]. These data point to adrenodoxin possessing the same protein conformation in both oxidation states whilst spinach ferredoxin undergoes a conformation change. The amino acid sequences of these proteins show that, whereas the ferredoxins from plants form an homologous group, adrenodoxin bears little similarity to it [116,117].

Mechanism of action. As no detailed structural analysis has yet been carried out for these proteins it is not possible to discuss possible mechanisms in any detail. Of the five plant ferredoxins which have been sequenced (Spinach, Alfalfa, *Scenedesmus*, *Leucena glauca* and Taro) all possess six aromatic groups in the same position (out of 97 residues), apart from *Scenedesmus* which possesses five aromatic groups, the sixth position being occupied by leu [116].

In accord with the suggestion that adrenodoxin has a different structure to spinach ferredoxin are the results of replacement experiments; spinach ferredoxin cannot replace adrenodoxin in the adrenal hydroxylation system. A 2Fe/2S* protein from pig testes can, however, substitute for adrenodoxin [168]. This specificity, which is not apparent with bacterial ferredoxins, extends to the putidaredoxin hydroxylase system as well; adrenodoxin will not

substitute for putidaredoxin, even though they appear to be homologous proteins [169].

(d) Rubredoxins

These proteins, characterised by possession of Fe-$(SCH_2)_4$ centres (Fig. 10) can be divided into two classes: those of molecular weight 6000 containing one centre (type I) and those of molecular weight 19000 containing two centres (type II).

Type I. Proteins of this class have been isolated from a number of bacterial sources. Their biological function is not known although they can replace ferredoxin in some reactions [143].

Structure.

Redox centre. The crystal structure of the oxidised *Clostridium pasteurianum* rubredoxin has been carried through to a resolution [170] of 1.5 Å. This work shows that the redox centre is a distorted tetrahedron (Fig. 10). Spectroscopic data confirm that this distortion is maintained in solution [14]. The geometry of this redox centre, in terms of angular distortions, is intermediate between that which would be expected for isolated ferrous and ferric complexes [150]. Preliminary X-ray data indicate that upon reduction the coordinates of the Fe/S centre are practically the same as for the oxidized protein [37]. This is in agreement with a large body of chemical evidence showing that the oxidation and reduction of rubredoxin is not accompanied by a large conformational change [172].

The redox centre is exposed to the solvent at one end of the molecule [170] (Fig. 11). Hydrophobic amino acids are placed around it and there is some degree of conservation of these residues among rubredoxins of this group [116] (Table 18).

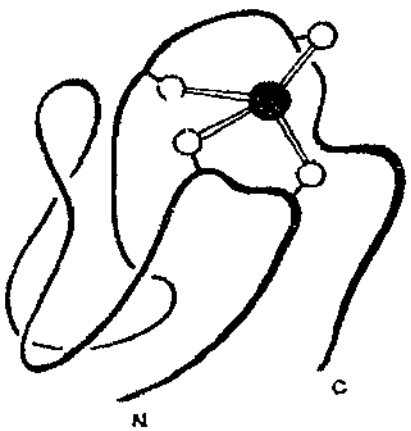


Fig. 11. Structure of the *C.pasteurianum* rubredoxin (from ref. 166).

TABLE 18

Rubredoxin amino acid sequences [a]

	1 … 32
Peptostreptococcus elsdenii	(M) D L Y E C S I C G Y I Y D — E A E G D D G N V A A G T K — F A D L —
	1
Peptococcus aerogenes	(M) Q L F E C T L C G Y I Y D — P A L V G P D T P D Q D G A — F E D V —
Pseudomonas oleovorans	1 … 32
N. terminus fragment [b]	A S Y K C P D C N Y V Y D — E S A G N V H E G F S P G T — P W H L I
Pseudomonas oleovorans	119 … 151
C-terminus fragment	L L W I C I T C G H I Y D W E A L G D E A E G F T P G T R F E D I —
Peptostreptococcus	33 … 52
elsdenii	P A D W V C P T C G A D K D A F — V K M D
Peptococcus aerogenes	S E N W V C P L C G A G K E D F E V Y E A
Pseudomonas oleovorans	33 … 52
N-terminus fragment	P E D W D C P C C — A V R D K L D F M L I
Pseudomonas oleovorans	152 … 174
C-terminus fragment	P — D W D C C W C B P G A T K E N Y V L Y E E K

[a] One-letter amino acid code recommended by the IUPAC—IUB International Commission on Biochemical Nomenclature.
[b] The central portion of the *P. oleovorans* rubredoxin (53—118) is not given.
From ref. 116.
(M), formylmethionine.

Type II. Proteins of this class have only been found in *Pseudomonads*, where they function as one-electron carriers in the electron-transport chain for ω-hydroxylation of alkanes [173]. The rubredoxin from *P. oleovorans* has been most studied [174].

Structure. No X-ray crystallographic study has been carried out with these proteins but we can discuss their structure in terms of the type I rubredoxin structure.

As isolated the *P. oleovorans* rubredoxin only contains one iron atom but it readily takes up a second [174]. The environment of these two iron atoms appears to be similar to that of the type rubredoxin; EPR and absorption spectra are identical [174]. Chemical studies have revealed that the basic structure is that in Fig. 12, i.e. crossover does not occur [174].

Mechanism of electron transfer. The nature of the Fe/S redox centre is again well suited for rapid electron transfer. We discuss electron transfer to and from this site in the section dealing with flavodoxins.

Although the type II rubredoxin can have two redox centres it only uses one. The protein can be cleaved to yield an N-terminal fragment (50 amino acids) and a C-terminal fragment (124 amino acids), each possessing one redox centre [175]. Only the C-terminal fragment is active with the ω-hydroxylase. This is the redox centre the protein possesses when isolated [174]. All the type I rubredoxins are also inactive with the ω-hydroxylase but they are active with the NADH-rubredoxin reductase [175]. (*Note:* In view of the similarity in the type I amino acid sequence to the N-terminal and C-terminal

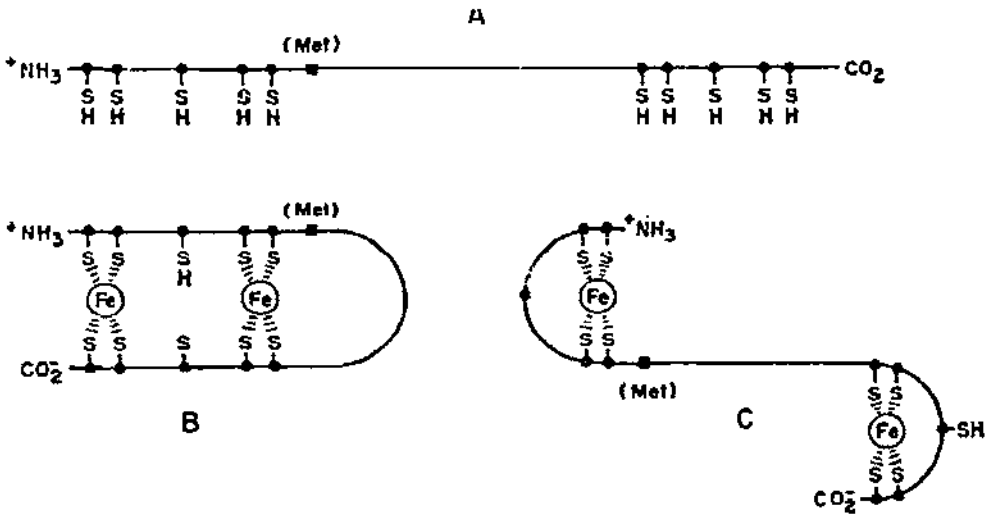


Fig. 12. Structure of the *P.oleovorans* rubredoxin. A is a schematic representation of the polypeptide chain showing the positions of the cysteinyl and methionyl residues. Structure C rather than B appears to be adopted by the protein (from ref. 14).

54 amino acids [176] of *P. oleovorans* rubredoxin (Table 18) the possibility that type I rubredoxins are degradation products of larger proteins has to be considered.)

(v) Flavodoxins

Flavodoxins are proteins of molecular weight about 15000 containing one FMN group (Table 19) [177,178]. They function in vivo as replacements for bacterial ferrodoxins and reaction II is therefore likely to be the biologically important reaction [177]:

I fully oxidised flavodoxin → semiquinone $E^0 = -115$ mV

II semiquinone → fully reduced flavodoxin $E^0 = -370$ mV

Bacterial ferredoxin possesses an $E^0 = -400$ mV.

Such flavoproteins have been isolated from algae and bacteria [178].

(a) Classification

On the basis of chemical and spectroscopic data flavodoxins have been divided into two classes [179]; one for which the *Desulfovibrio vulgaris* protein is characteristic and the other for which the *Clostridium MP* protein is typical [178]. X-ray data show that these two proteins have similar overall structures (Fig. 13) despite the difference in sizes (Table 19), but that they differ in the amino acid composition of the FMN binding region [180,182].

Comparisons of their amino acid sequences confirm that these proteins are homologous [183]. Thus, the different properties exhibited by these proteins appear to be associated with the redox centre.

The flavoprotein from *Azotobacter vinelandii*, azotoflavin, is placed in a separate group (Table 19) from the flavodoxins because it is inactive in the

TABLE 19

Physical properties of flavodoxins and related proteins

Source	Molecular weight	Redox potential (mV)[a]	Ref.
Clostridium pasteurianum flavodoxin	14600	−132; −419	178
Clostridium M.P. flavodoxin	13800	−90; −399	178
Peptostreptococcus elsdenii flavodoxin	15000	−115; −373	207
Escherichia coli K 12 flavodoxin	14500	−285; −455	205
Desulfovibrio gigas flavodoxin	16000	n.d.[b]	185
Azotobacter vinelandii "azotoflavin"	23000	−270; −460	178
Anacystis nidulans "phytoflavin"	12000	n.d.	208

[a] First potential is for fully oxidised ⇌ semiquinone and the second potential is for semiquinone ⇌ fully reduced.

[b] n.d., not determined.

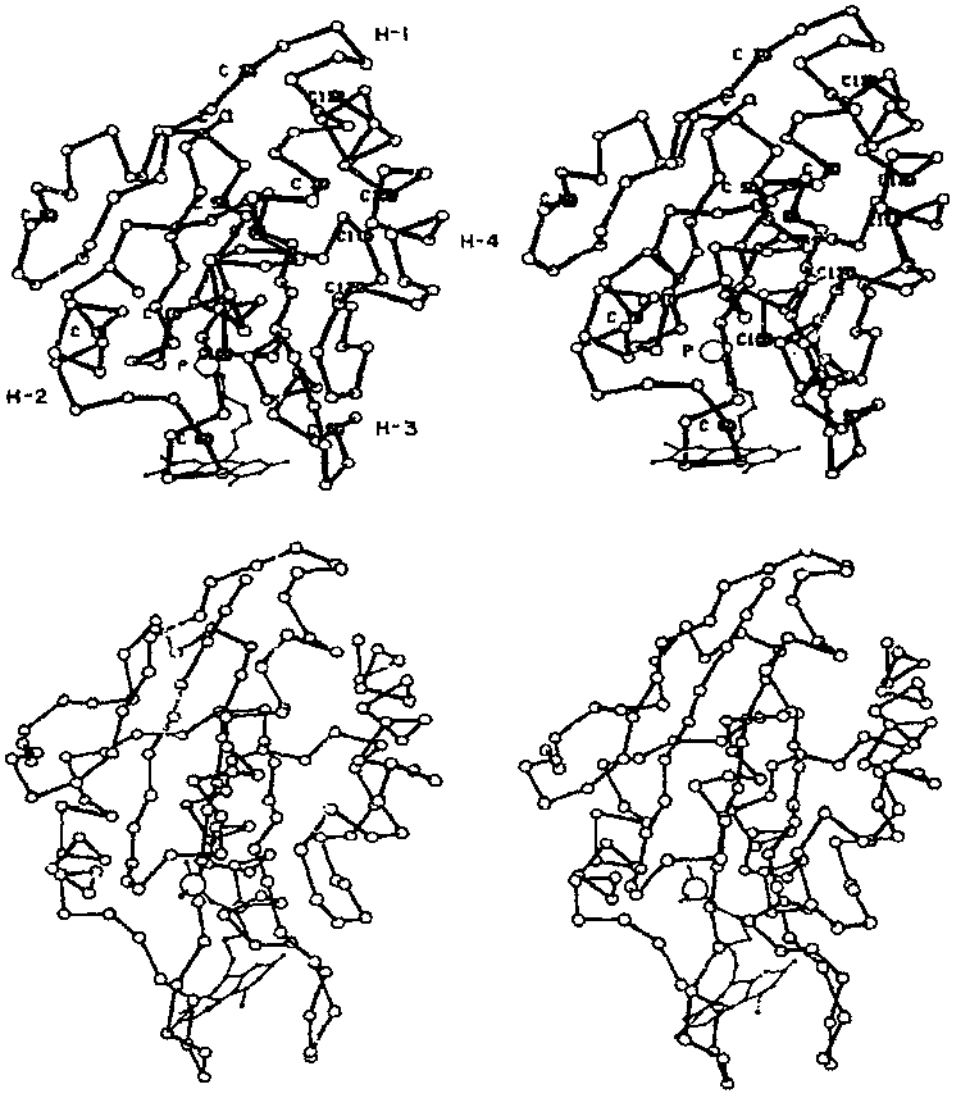


Fig. 13. Stereo diagrams of flavodoxin. (a) The radical form of *Clostridium M.P.* flavodoxin. The helices are labelled (H-1, etc.) from the N-terminus. (b) The oxidized form of *D. vulgaris* flavodoxin (from ref. 181).

phosphoroclastic reactions and NADP reduction, although it is active in N_2 fixation [178]. The electron-transport chain for *A. vinelandii* N_2 fixation is different to other N_2 fixation schemes; azotoflavin appears to be an essential component [179]. This protein also differs from the flavodoxins in the stability of its free radical to O_2; half-life $t_{1/2}$ = 2000 min as opposed to $t_{1/2}$ = 80 and 115 min for the *C. pasteurianum* and *P. elsdenii* flavodoxins [184].

A. vinelandii is an aerobic bacterium and this difference in stability may reflect the fact that it lives in an O_2-rich environment [178]. The other bacteria are fermentative bacteria.

(b) Flavin group

A number of experiments have shown that these flavoproteins can function with flavin groups other than FMN. Apoflavodoxins have been prepared and flavoproteins reconstituted from them. The flavodoxin from *D. gigas* reconstituted with FAD possesses 28% of the initial activity (as compared with 75% in controls with FMN) of flavodoxin [185], whilst the flavodoxin from *P. elsdenii* reconstituted with iso-FMN possesses 31% of the initial activity of flavodoxin in enzymic assays [186]. Part of the decrease in activity in the latter case was attributed to the higher redox potential of iso-FMN flavodoxin; −260 mV for semiquinone reduced form as opposed to −370 mV for the native protein. *P. elsdenii* flavodoxin reconstituted with 3,4-dihydro FMN was inactive, probably because 3,4-dihydro FMN cannot undergo one-electron reduction [186].

Structure. The FMN groups in both the *D. gigas* [180] and *Clostridium M.P.* [181,182] proteins are planar in all three oxidation states, and almost buried near the surface of the molecule. Note that planarity indicates a highly strained conformation of at least one oxidation state. For the *Clostridium* protein the methyl groups of the flavin, one edge of the flavin and the ribityl group are exposed at the surface [181,182]. The FMN is bound non-covalently via hydrogen bonds between the pyrimidine carbonyls and the protein as well as between the phosphate group, which is extended into the molecule, and the protein. A met residue is located on one side of the flavin whilst a trp residue is placed on the other side [182].

The FMN of the *D. vulgaris* protein is buried in a pocket extending into the protein [180]. On one side of the FMN, nearly coplanar with it, tyr 98 is located whilst on the other side trp 60 is situated (Fig. 14). The pyrimidine ring of the FMN is buried most deeply in the protein and hydrogen bonds between it and the peptide amide and carbonyl functions are formed. The 2'-hydroxyl of the ribityl group is also buried in the protein where it is hydrogen bonded, as is the phosphate group. The remainder of the ribityl group lies at the surface of the protein. The two methyl groups of the flavin also lie on the surface [180].

NMR spectroscopy [187] and X-ray crystallography [179] show that there are no major conformational differences between the three stable flavodoxin oxidation states.

Mechanism of electron transfer. The location of the redox centre of these flavoproteins [180,182] is similar to that of rubredoxin [170]; they are positioned at one end of the protein near the surface. Both rubredoxin and flavodoxins bind to ferredoxin-NADP reductase (FNR) [158], possibly at

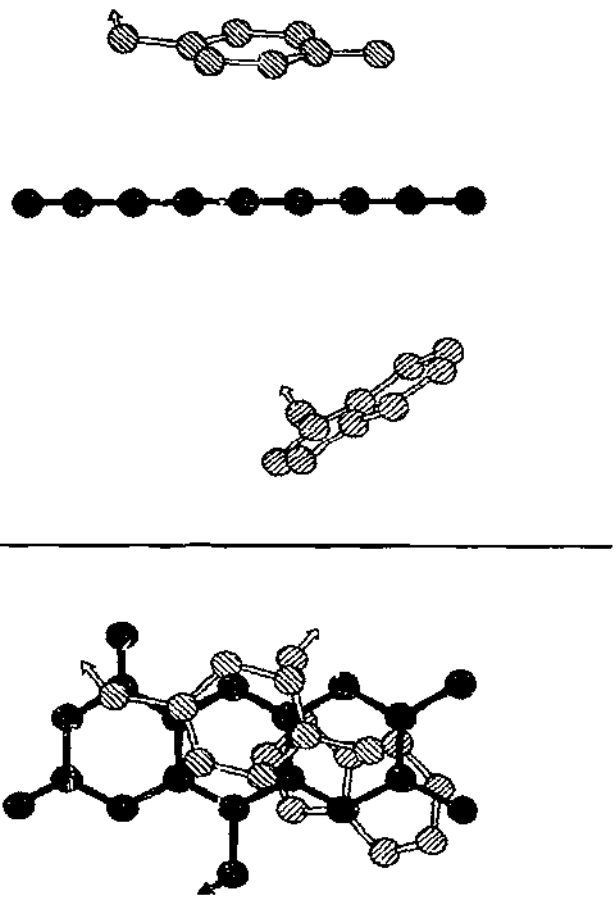


Fig. 14. *D.vulgaris* flavodoxin: orientation of Trp 60 and Tyr 98 relative to the flavin plane. Lower projection is rotated 90° relative to the upper (from ref. 183).

the same site as bacterial ferredoxins. All three complexes, electron-transfer protein plus FNR, are dissociated by high ionic strength and the changes in the absorption spectrum of FNR on complex formation are rather similar in each case [158]. We suggest that flavodoxin and rubredoxin bind at the mouth of a channel leading to the redox centre of FNR. Such binding would totally enclose the FMN group and Fe-$(SCH_2)_4$ centre by protein.

(vi) Type I copper proteins

The copper-containing electron-transfer proteins we shall be considering (Table 20) only contain type I Cu^{II} centres. They possess a characteristic blue colour which is associated with an intense charge transfer band [189] in the absorption spectrum, ~600 nm.

TABLE 20

Physical properties of type I copper proteins

Protein	Molecular weight	No. of Cu atoms	Redox potential (mV)	Ref.
Bordetella pertusis azurin	14600	1	+395	209
Pseudomonas aeruginosa azurin	14600	1	+300	210
Chlorella ellipsoidea plastocyanin	n.d.[a]	2	+390	211
Spinach plastocyanin	21000	2	+370	212
French bean plastocyanin	11000	1	n.d.	213
Pseudomonas denitrificans blue protein	16300	1	+230	214
Rhus vernicifera stellacyanin	n.d.	1	+184	215
Horseradish umecyanin[b]	14600	1	n.d.	216

[a] n.d., not determined.
[b] Also contains three glucosamine residues per molecule.

Structure. No crystallographic data are available for these proteins so we shall briefly discuss relevant spectroscopic and chemical data. In view of the experiments with thiol blocking agents there appears to be a thiol ligand to the Cu^{II} ion [190]. This has been confirmed from spectral studies of Co^{II} stellacyanin (Co^{II} instead of Cu^{II}). The electronic absorption spectrum resembled that of model Co^{II} thiol complexes [191].

Measurements of water spin–lattice relaxation times show that these Cu^{II} centres, unlike the type II Cu^{II} centres, are not accessible to solvent [192]. Azurin contains six phe and two tyr residues but only one trp [116]. The fluorescence emission associated with this trp residue is not only partly quenched by the Cu^{II} ion but is also anomalous in terms of emission wavelength [193]. This anomalous behaviour has been shown to be due to the trp being in a hydrophobic environment. Fluorescence perturbation studies are consistent with this; the trp is not accessible to the solvent [194]. Studies with the apoenzyme and heavy metal ions place this trp near the Cu^{II} ion [195]. Stellacyanin contains three trp residues [196] but only two of these are solvent accessible; the third is located near the Cu^{II} ion [194]. The two Cu^{II} ion containing plastocyanins (e.g. spinach) possess three tyr but no trp residues [194]. The tyr residues are not solvent accessible [194].

The EPR and absorption spectra indicate that these type I Cu^{II} centres are of low symmetry [100]. A stereochemistry intermediate between tetrahedral (preferred for four coordinate Cu^{I}) and square planar (preferred for Cu^{II}) has been suggested for them. The high redox potentials are in accord with this [100] (Table 20).

Mechanism of electron transfer. The evidence points to these proteins possessing a redox centre in a strained configuration located in a hydrophobic environment. This is similar to the heme and Fe/S proteins.

Sequence comparisons of azurin [116] (*Bordetella bronchiseptica* and *Pseudomonas fluorescens*) and the one Cu^{II} ion containing plastocyanins (French bean [217], *chlorella fusca* [218] and *Anabaene variabilis* [219]) show that these are related proteins. There is some homology between azurin and plastocyanin, particularly around the free cys residue, 85 in plastocyanin and 112 in azurin. Although there are nine aromatic residues common to both azurins and six aromatic residues common to the plastocyanins, comparisons of the sequences indicate there are only five conserved aromatic residues for azurin and three for plastocyanin.

D. ORGANISATION OF ELECTRON-TRANSFER CHAINS

Extraction and purification of electron-transfer proteins is a formidable task because most of these proteins are membrane bound. It is for this reason that the majority of proteins in electron-transfer chains have not been isolated in a pure form. The major purification problem is obtaining an active preparation free of extraneous protein and contaminating phospholipid. This is a difficult operation as many of these proteins are lipo-proteins. The proteins we consider in this article are, in most cases, small and fairly readily purified.

It is known that membrane binding does not greatly alter the protein conformation; the visible spectra of most cytochromes [49] are the same whether the proteins are membrane bound or in aqueous solution. Thus the inner core of the protein is not perturbed although little can be said about the surface. Again these proteins function with their respective oxidases and reductases in aqueous solution, although usually at much lower rates of reaction (e.g. cytochrome-*c*-reductase activity of cytochrome b_5) [103]. It is possible that membrane binding affects their relative redox potentials, however [221].

There are a number of possible ways for proteins to be bound into or onto membranes, for example as follows:

(1) "Free" protein can bind to membrane-bound proteins.

(2) A protein can have a large proportion of its molecular surface taken up with hydrophobic amino acids and so become soluble in lipids.

(3) A protein can have a hydrophobic peptide 'tail' which binds to the fatty regions of membranes (e.g. cytochrome b_5) [102].

(4) Proteins can form complexes with phospholipids, based on electrostatic interactions, and such complexes then interact with membrane structural proteins and/or the lipophilic tails of phospholipids.

Ferredoxin has a large amount of its surface occupied by hydrophobic groups and cytochrome *c* forms complexes [222] such as those described in (4), but it is not known whether these are the mechanisms by which they are bound by membranes.

The structure of the different possible positions of electron-transfer proteins in membranes is shown in Fig. 15. Given any one of these locations and knowing the electron transfer is between components X and Y which can

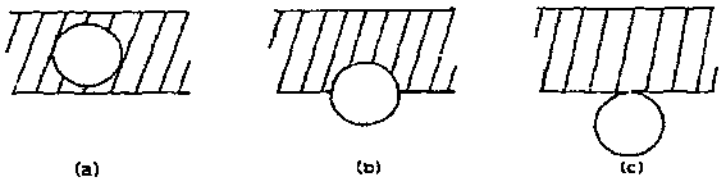


Fig. 15. Protein binding to membranes. Hatched region represents membrane, circles represents protein: (a) cytochrome oxidase; (b) cytochrome *c*; (c) cytochrome b_5.

also bind to the carrier or which are also fixed in the membrane, it is highly probable that there are two channels; one for electron entry from X and one from Y. Thus each protein must be examined for minimally two channels. In some cases an impression of the relative location of the two entrances has been obtained, e.g. in the cytochrome *c*—cytochrome c_1 complex [84] (Fig. 16).

Why are these systems involving electron transfer usually integrated with membranes? There appear to be a number of reasons for this:

(1) Many of the reactions coupled to electron transfer (e.g. ATP synthesis) are dehydration reactions and would be best carried out in a non-aqueous environment.

(2) The proteins constituting the redox chain will be held in the correct juxtaposition for reaction. A lipid may be highly organised resembling a liquid crystal rather than a simple liquid.

This organisational facet (2) of membrane binding is very important. It means that the rate of electron transfer need not be diffusion controlled. Also it prevents short-circuiting of the chain by one protein A_1, transferring its electrons to protein A_n instead of A_2. In the case of oxidative phosphorylation pathways the phosphorylation centres must not be bypassed.

Electron-transfer chains appear to have evolved so that there is a small difference in redox potential, ~80 mV, between adjacent proteins. This may be for reasons of control or efficiency but as a consequence there are often a large number of redox centres in an electron-transport chain, approximately fifty in the mitochondrial oxidative phosphorylation chain [56].

In order to separate the primary electron donors from the terminal electron acceptors some redox chains are compartmentalised; the mitochondrial

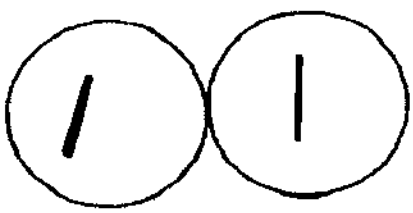


Fig. 16. The cytochrome c_1—cytochrome *c* complex.

oxidative phosphorylation chain spans a membrane [56]. In cases such as this it appears that a large number of redox centres are necessary to enable the electrons to be transferred across the large distances (50—70 Å) involved. This is because the rate of electron transfer, no matter what the mechanism, depends upon a degree of orbital overlap which is inversely proportional to the distance the electrons have to travel. The greater the distance, all other factors being equal, the lower is the rate of transfer. Rapid electron transfer can take place over 10—15 Å (e.g. between $Fe(CO)_4$ and Na in argon matrices) [22] but the only measurements available for biological systems refer to a *Chromatium* [225] electron-transfer system where the electron travels [226] about 8 Å.

Another way that electron-transfer systems have been organised is in the "multi-headed" proteins, proteins containing more than one redox centre. Often such proteins have an enzymic capability as well, e.g. laccase (ref. 189) and cytochrome b_2 (ref. 49). The same points about organisation that applied to membrane-bound systems apply to multi-headed proteins as well. In some cases electron transfer between redox centres within a multi-headed protein is not very fast yet the centres can only be 10 Å apart at most (e.g. cytochrome c_3) [50]. Thus multi-headed proteins can function as electron storage units. Taking the membrane-bound and multi-headed systems together the concentration of electron-transfer centres is usually such that they can hardly be more than 15—20 Å apart, i.e. one centre per 15—20000 molecular weight. Systems in which the electron-transfer centres are more than ~25 Å apart cannot operate by the described mechanisms.

E. SPECIFICITY OF ELECTRON-TRANSFER PROTEINS

In the past the specificity of enzyme-catalysed reactions has often been confused with their mechanism of action. We hope to have avoided this pitfall, but we must comment upon the apparent lack of specificity of most rapid electron-transfer reactions between proteins. It seems to us that this lack of specificity argues strongly for a mechanism of rapid electron transfer which does not involve specific bimolecular chemical intermediates (e.g. charge transfer complexes, free radicals, etc.) for the transfer of electrons between proteins. Specific electron-transfer reactions do occur, but their specificity can be understood on the basis that the proteins which fail to carry out rapid electron transfer do not fulfil one or more of the following requirements necessary for such electron transfer:

(1) The redox potentials of the two proteins must be poised.

(2) The two proteins must form a complex with an electron conducting track open between their redox centres.

(3) The distance between the redox centres must be such that some coupling between them can occur.

We have attempted to formulate a general mechanism for electron transfer which we believe is biologically feasible and we shall show that its lack of

specificity is an advantage to biological systems. The presence of cytochrome b_5 in the electron-transfer chains involving cytochrome P-450 [95], fatty acid desaturase [96] and methemoglobin [97] has already been mentioned, as has the involvement of cytochrome *c* in many electron-transfer processes. We therefore use the ferredoxin—flavodoxin replacement as an example for this discussion.

Certain bacterial species grown on an iron-containing medium contain low levels of flavodoxin and utilise ferredoxin for many processes involving electron transfer. When these species are grown on an iron-deficient medium flavodoxin is produced and utilised in those processes formerly using ferredoxin [227]. As flavodoxin can replace ferredoxin in cell-free extracts of bacteria grown on an iron-containing media for some activities (such as NADP reduction) [228], it seems as though the ferredoxin oxido-reductases are not specific for ferredoxin. In fact complexes between such proteins and flavodoxin have been isolated (Table 21) [158].

Proteins capable of transferring electrons to each other include HiPIP and cytochrome *c* (ref. 235) adrenodoxin and cytochrome *c*, (ref. 234) ferredoxin and xanthine oxidase, and rubredoxin, flavodoxin and ferredoxin with ferredoxin NADP reductase (see also Table 22). The exceptional examples of "specific" electron-transfer reactions are the reaction of rubredoxins with the alkane ω-hydroxylases [176], the reactions of plant type ferredoxins [177,178] and the reaction of cytochrome-*c*-peroxidase [188,229].

There is a built-in specificity for all protein—protein interactions, namely will the proteins form a complex? If they can, then, providing the requirements previously mentioned are fulfilled, reaction can take place. Examples

TABLE 21

Electron-transfer protein complexes

		K_d (M) at μ	Ref.
Spinach ferredoxin NADP-reductase	Spinach ferredoxin	5×10^{-8}; 0	158
		7.6×10^{-6}; 0.08	158
	C. pasteurianum ferredoxin	n.d.	158
	P. elsdenii flavodoxin	5×10^{-8}; 0	158
	P. elsdenii rubredoxin	5×10^{-8}; 0	158
	Adrenodoxin	n.d.	158
P. oleovorans ferredoxin NADH reductase	*P. oleovorans* rubredoxin	2.1×10^{-7}; n.d.	175
Cytochrome *c*	Cytochrome-*c*-oxidase	$0.1-1 \times 10^{-6}$; n.d.	233
	Cytochrome-*c*-peroxidase	$1-2 \times 10^{-6}$; n.d.	233
	Adrenodoxin	n.d.	234
	Cytochrome c_1	n.d.	84

n.d., not determined. μ = ionic strength.

TABLE 22

Rates of electron transfer between proteins and small inorganic redox agents

System	Rate (M^{-1} s^{-1})	pH	Temp (°C)	Ref.
(a) Protein oxidation				
Myoglobin + $[Fe(CN)_6]^{3-}$	2×10^6	6.0	19	238
	1.4×10^6	9.2	19	238
Hemoglobin + $[Fe(CN)_6]^{3-}$	7×10^4	6.0	19	238
	8×10^3	9.2	19	238
Beef cytochrome c_1 + $[Fe(CN)_6]^{3-}$	3.6×10^4	7.4	23	242
Horse cytochrome *c* + $[Fe(CN)_6]^{3-}$	1.6×10^7			243
	8.4×10^6	7.0		244
Pseudomonas aeruginosa cytochrome c-551 + $[Fe(CN)_6]^{3-}$	8×10^4			248
Rhodospirillum diheme cytochrome *c'* + $[Fe(CN)_6]^{3-}$	$\sim 10^9$	7.4	26	247
Chromatium diheme cytochrome *c'* + $[Fe(CN)_6]^{3-}$	$\sim 10^9$	7.4	26	247
Pseudomonas aeruginosa azurin + $[Fe(CN)_6]^{3-}$	1.2×10^4			248
Fungal laccase type 1 Cu^2 centre + $[Fe(CN)_6]^{3-}$	1.1×10^6			245
Chromatium HiPIP red + $[Fe(CN)_6]^{3-}$	4.2×10^3		25	235
Horse cytochrome *c* + $[Co(phen)_3]^{3+}$	1.5×10^5	7.0	25	246
Candida krusei cytochrome *c* + $[Co(phen)_3]^{3+}$	2.7×10^3	7.0	25	246
(b) Protein reduction				
Horse cytochrome *c* + $[Fe(CN)_6]^{4-}$	2.6×10^4	7.0		244
Bean leaf plastocyanin + $[Fe(CN)_6]^{4-}$	1.6×10^4	6.0	20	235
Spinach leaf plastocyanin + $[Fe(CN)_6]^{4-}$	2.1×10^4	6.0	20	235
Chromatium HiPIPox + $[Fe(CN)_6]^{4-}$	1.8×10^2		25	235
Clostridium pasteurianum rubredoxin + $[Ru(NH_3)_6]^{2+}$	9.5×10^4	6.3—7.0	25	251
Horse cytochrome *c* + $[Cr(H_2O)_6]^{2+}$	1.9×10^4	4.0	25	236
Horse cytochrome *c* + $[Ru(NH_3)_6]^{2+}$	4.2×10^4	7.0	25	250
Chromatium HiPIPox + $[Ru(NH_3)_6]^{2+}$	3.1×10^5	7.0	25	235
Clostridium pasteurianum rubredoxin + $[Cr(H_2O)_6]^{2+}$	1.2×10^3	3.5—4.0	25	251
Clostridium pasteurianum rubredoxin + $[V(H_2O)_6]^{2+}$	1.6×10^4	3.5—4.5	25	251
Horse cytochrome *c* + $[Fe(edta)]^{2-}$	2.8×10^4	3.5—4.5	25	249
(c) Controls				
$[Fe(bipy)_3]^{2+}$ + $[Fe(bipy)_3]^{3+}$	$\sim 10^6$		25	252
$[Mn(CN)_6]^{3-}$ + $[Mn(CN)_6]^{4-}$	$\sim 10^6$		25	252
$[Fe(CN)_6]^{3-}$ + $[Fe(CN)_6]^{4-}$	$\sim 10^5$		25	252
$[Ru(NH_3)_6]^{3+}$ + $[V(H_2O)_6]^{2+}$	1.5×10^3			235
$[Fe(H_2O)_6]^{3+}$ + $[V(H_2O)_6]^{2+}$	1.8×10^4			235
$[Ru(NH_3)_6]^{3+}$ + $[Cr(H_2O)_6]^{2+}$	2×10^2			235

of complexes of electron-transfer proteins which have been obtained are given in Table 24. The data in this table show that the interaction between two proteins is inhibited by high ionic strength. The rates of reaction are also affected by ionic strength. This strongly suggests that the interaction between two proteins is electrostatic in origin. In the case of cytochrome *c* there is a substantial body of chemical evidence to support this.

Cytochrome *c* (Sect. C) possesses two lysine-rich regions on its molecular surface [30]. Succinylation, introducing a negative charge in place of the positive charges, results in a protein inactive with cytochrome oxidase whereas guanidation, introducing an additional positive charge, enhances the rate of reaction with cytochrome oxidase [230]. More specifically, if lys 13 is triphenylated the rate of reaction with cytochrome oxidase is reduced [231] by 50%. These modification experiments implicate the amine groups of cytochrome *c* in its mechanism of action. The reaction of cytochrome *c* with cytochrome oxidase and cytochrome reductase is also inhibited by polylysine [232].

This simple approach to specificity ignores differences in electron transfer between proteins shown by the kinetics of electron transfer. We discuss the kinetics of electron transfer in the next section.

F. KINETICS OF ELECTRON-TRANSFER REACTIONS OF ELECTRON-TRANSFER PROTEINS

Some factors affecting rates of electron transfer, such as the spin state, stereochemistry and environment of metal centres, have been described in Sect. B. The purpose of this section is to show that electron transfer between proteins can be rapid. In addition we shall use the kinetic data to clarify some points concerning biological electron transfer.

Results of kinetic experiments with electron-transfer proteins and small inorganic reagents are summarised in Tables 22 and 23. Although such experiments are not biologically significant, a number of interesting points emerge from them.

(1) Reactions between electron-transfer proteins and small inorganic redox agents are, in many cases, as fast as reaction between the inorganic redox agents themselves. The majority of such reactions can be described in terms of inner- and outer-sphere mechanisms [235].

(2) In the majority of cases the oxidised and reduced proteins produced via these reactions are identical with those produced from reaction with protein oxidases and reductases (Cr^{II} reactions, see below, are an exception). Apparently, if a conformation change occurs upon reaction, it is caused solely by the electron entering or leaving the protein and not by some physical interaction with other proteins.

(3) There is more than one route for electrons to enter and leave the protein. The reactions of cytochrome *c* have been most studied in this respect and therefore we shall confine ourselves to a discussion of this protein. On

TABLE 23

Rates of reaction between electron-transfer proteins

Oxidase	Reductase	Rate (M^{-1} s^{-1})	Ref.
Parsley plastocyanin	Parsley cytochrome *f*	3.6×10^7	253
	Red algal cytochrome *c*-553	5×10^5	253
	Pseudomonas cytochrome *c*-551	7.5×10^5	253
	Mammalian cytochrome *c*	1.0×10^6	253
Azurin	Parsley cytochrome *f*	6×10^6	253
	Red algal cytochrome *c*-553	2×10^7	253
Fungal laccase	Horse cytochrome *c*	1.7×10^4	254
	Pseudomonas aeruginosa cytochrome *c*-551	5.7×10^4	254
	Pseudomonas aeruginosa azurin	8.2×10^4	
Pseudomonas aeruginosa azurin	*Pseudomonas aeruginosa* cytochrome *c*-551	$\sim 10^6$	248
	Horse cytochrome *c*	4.9×10^4	255
Bovine cytochrome *c*	Bovine cytochrome c_1	3.3×10^6	242
Moles of acceptor reduced per mole of reductase per minute			175
O_2	*Pseudomonas oleovorans*	<0.1	
O_2 + *Pseudomonas oleovorans* rubredoxin	NADH-rubredoxin reductase	41000	
Horse cytochrome *c*		0	
Pseudomonas oleovorans rubredoxin		12900	
Moles of xanthine oxidised per mole of flavin per minute			256
Ferredoxin	*Micrococcus lactilyticus*	1210	
O_2	Xanthine dehydrogenase	150	
Cytochrome *c*	(two flavin groups per molecule)	43	
Moles of cytochrome reduced (oxidised) per minute per milligram of reductase (oxidase)			
Horse cytochrome *c*	Bovine mitochondrial complexes I—III	24.6	232
Rhodospirillum rubrum cytochrome c_2		13.6	
Euglena gracilus cytochrome *c*-558		16.7	
Clorobium thiosulfatophilum cytochrome *c*-555		0.5	
Porphyra tenera cytochrome *f*		0.4	
Rhodospirillum rubrum cytochrome *c'*		0	
Rhodopseudomonas cytochrome *c'*		0	
Horse cytochrome *c*	Bovine DPNH-dehydrogenase	36.3	232
Rhodospirillum rubrum cytochrome c_2		19.5	
Rhodospirillum rubrum cytochrome *c'*		0.5	
Rhodopseudomonas cytochrome *c'*		0.2	

TABLE 23 *(continued)*

Oxidase	Reductase	Rate ($M^{-1}s^{-1}$)	Ref.
Moles of cytochrome reduced (oxidised) per minute per milligram of reductase (oxidase)			
Bovine cytochrome oxidase	Horse cytochrome *c*	16.6	232
	Rhodospirillum rubrum cytochrome c_2	0.9	
	Porphyra tenera cytochrome *f*	0	

reduction of ferricytochrome *c* with Cr^{II} some of the ferrocytochrome *c* produced has a Cr^{III} ion bound to it [236,237]. All of the ferrocytochrome *c* produced can be reoxidised with $K_3(Fe(CN)_6)$ and then reduced again with sodium dithionite, but not all the reoxidised cytochrome *c* can be completely reduced again with Cr^{II} [236]. The cytochrome *c* with Cr^{III} bound to it is not reduced. These findings suggest that, as expected, $K_3Fe(CN)_6$ and sodium dithionite do not act at the same site as Cr^{III}.

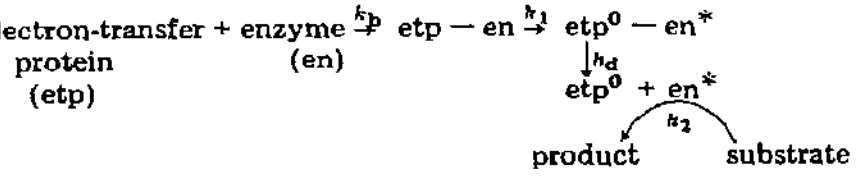
Biological oxidases and reductases will use different routes but even with these there may be more than one feasible biological route for electron transfer in a protein.

(4) Myoglobin and hemoglobin, proteins not normally thought of as electron-transfer proteins, are oxidised by $K_3Fe(CN)_6$ at comparable rates [238] to cytochrome *c*.

Pulse radiolysis techniques have also been used to study electron-transfer proteins. Proteins with a solvent accessible redox centre (e.g. cytochrome *c* (ref. 239)) are reduced in a direct reaction which is diffusion controlled. Where the redox centre is not solvent accessible the proteins are either not reduced (e.g. xanthine oxidase [240]) or reduction occurs with the electron entering the protein via functional groups (e.g. histidyl radicals for ceruloplasmin [241]). It should be emphasised that these reactions with high-energy electrons are not biologically significant.

The majority of studies of reactions between electron-transfer proteins have been carried out by biochemists and the results presented (Table 23) in the form of turnover numbers. Such data have usually been obtained from reactions in which enzymes were involved as this yielded a readily measurable factor to follow reaction. However, for our purposes, there are a number of drawbacks to this approach.

If the reaction is



We are interested in k_1 but are measuring k_2. If $k_2 < k_1$ the data obtained will not be a true measure of the rate of electron transfer although it will represent a minimum value of k_1. Also k_a and/or k_b could represent rate-determining steps.

Despite these comments the data do demonstrate that reaction between electron-transfer proteins can be rapid.

G. LONG-RANGE METAL—METAL INTERACTIONS

If the electron transfer between metal centres is direct over a fairly long distance, then the evidence for such a path will be hard to find. Examination of the physical properties of the groupings which line the path is not likely to be profitable. One possibility is to look for effects, small perturbations, of the presence of one metal centre upon another in the same multi-headed metallo-enzyme. In a few proteins such interactions have been seen but as yet we are insufficiently sure of their origin or even of the distance between the centres to say what these effects mean in terms of electron-transfer mechanisms. Table 24 gives one or two examples where some of the metals are now known to be in a simple cluster.

H. CONCLUSIONS

We are now able to make some conclusions about biological electron-transfer processes:

(1) The process of electron transfer can be via ligands in the coordination spheres in direct outer-sphere collisional processes. An example is the hemoglobin/cytochrome b reductase system. Both the proteins contain exposed heme groups and both are soluble in the cytoplasm of red cells.

(2) In many cases, either in single polypeptide chains or in multi-chain units, electron-transfer centres are organised with respect to one another in space and there is no possibility of direct collison between the coordination spheres in the way it is possible in solution.

(3) Electron transfer can occur via intermediate organic groups such as tyrosine and tryptophan. Possible examples are the high potential Cu^{II} blue

TABLE 24

Metal—metal interactions in proteins

Protein	Metals	Distance Å	Ref.
Cytochrome oxidase	Cu . . . Fe	10?	257
Eight iron ferredoxins	Fe . . . Fe	10 — 12	148
Laccase	Cu . . . Cu	10?	189
Xanthine oxidase	Mo . . . Fe	20 — 30	258
Hemerythrin	Fe . . . Fe	5 — 10?	259

proteins, cytochrome oxidase and cytochrome *c* peroxidase. It is highly unlikely that flavins, quinones or Fe^{III} proteins undergo such reactions. Some electron-transfer proteins do not have such aromatic residues.

(4) Electrons cannot travel across peptide chains very easily. Cytochromes c_3 provide examples.

(5) Electrons probably can pass through hydrophobic regions of proteins. This is exceedingly likely in the $(Fe_4S_4^*)_2$ proteins and appears to be the likely path in cytochromes *b*, and *c*, especially where these proteins have fixed positions in membranes. All these proteins possess hydrophobic channels where the electron moves in a vacuum effectively.

(6) The distance between centres for electron transfer postulated in (5) must be small. A maximum distance between metal atoms, even given the large conducting ligands of the metal ions, could be ~15 Å. This may explain why electron transfer chains are so rich in electron-transfer centres. Electron-transfer proteins are usually small and in general we would suppose that one metal (or other transfer centre) is necessary per 20000 molecular weight.

(7) The thermodynamic barriers to reversible electron flow are kept small by arranging organised redox centres in thermodynamic sequence.

(8) The uptake and loss of electrons by the metal centres is made of very low activation energy by the protein—metal ion interaction which is in two parts, thermodynamic and kinetic: (a) a controlled compromise coordination sphere to overcome the Franck—Condon energy of electron transfer; (b) a bulk vibronic term which permits electron movement through coupling of phonon energies with electron movement. The phonon energies need not be conventional localised vibrations as in small molecules. The motions of residues in both cytochromes and ferredoxins have been observed. The coupling of particular "lattice modes", vibrations of the protein as a unit and not bond vibrations, could generate a condition of the sites between which electron transfer was to occur, such that they had the same potential. Such a condition is required for tunnelling. It is very probable that tunnelling will be favoured in a medium or low rather than high dielectric constant. Together these features generate an unusual condition of the electron-transfer centres when compared with simple models. One of the many points which remain unexplained in the spectroscopy of the electronic ground states of these proteins is the temperature dependence of the contact-shifted resonances. Even in cytochromes *c* where the evidence for deviation from a pure low-spin state is weakest this temperature dependence is quite different from that seen in model porphyrins.

(9) A feature of the structures of proteins is that they are more like liquid crystals than true crystals and cooperative order/disorder transformations may well extend through the molecule. The energy of the disorder/order "phase" changes can assist the electron-transfer step. This is shown by the relative ease of small conformation changes mentioned above.

(10) The presumed channels of electron transfer and the injection energies of electrons from metal sites can be controlled by conformation changes in obvious ways.

If the above points are correct there is considerable danger in attempting to understand electron transfer in biology from small molecule behaviour in solution. Thus we may finish by stressing the message of the introduction. Electron transfer is a biologically required process and inspection of a given protein must keep in mind the evolutionary drive which can result in exquisite balance of physical—chemical interactions so as to optimalise a protein for a process.

Note. We have not discussed photo-activated electron transfer. In such biological reactions the electrons are elevated to a high energy by the absorbed photon. Thus the problem of the injection energy has been overcome by a supply of energy in excess of 25 kcal. The situation is now similar to that in an electron pulse experiment or to that in the liquid ammonia or hydrocarbon systems, Sect. B. The electron has high mobility in such solvents. In effect there is only a very low barrier to electron movement. The major problem is to supply a guide for the electron so that it does go to the correct electron trap, i.e. the trap at lowest E^0. This path could well be a hydrophobic channel. It is reasonable to suppose that after this first step the photon energy will have been dissipated. Our article is concerned with the subsequent movements of the electron in the biological system, see for example ref. 260. In this system and in certain oxidases [261] there are iron proteins of an unknown coordination acting in electron transfer.

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