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Reviews

Electron transfer in biological systems: an overview

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Introduction

Electron transfer is a central process in chemistry and biology, yet the functions of electron transfer proteins of biological systems are not understood in any detail. Recent developments of ideas and experimental observations concerning electron flow in biological systems have raised significant problems on the nature of the electron transfer components and on the mechanism of electron transfer that achieves a maximal efficiency of energy coupling. This review discusses some aspects of these advances which come from studies using a large number of small molecules and model reactions to investigate inter- and intramolecular electron transfer with either natural or artificial reaction partners. We examine currently proposed models and theories, as well as recent data of kinetic, thermodynamic, spectroscopic and theoretical studies on the four main classes of proteins involved in this essential role of electron transfer, namely the flavoproteins, the iron-sulphur proteins, the hemoproteins and the copper proteins. Some features are common to all four, namely their prosthetic groups (be it flavin, iron-sulphur cluster, heme or distorted tetragonal copper) appear to be constructed so that there is a minimum of local structural change concomitant with electron transfer. This review is focused on the effects of these microenvironments on the prosthetic groups. Other topics of electron transfer have been reviewed elsewhere already, e.g. the overall structure of electron transferring components[127,275,276,277], the protein disposition within complex structures[222,269,270,272] or its relationship to energy transduction[207,209,273,274].

Many of the most fundamental metabolic processes in biological systems involve the transfer of electrons between macromolecules, and, generally, between metal atoms or aromatic structures of the macromolecules. The intrinsic mechanisms by which such a transfer occurs and the detailed role of the protein moiety in mediating the electron transfer between redox centers, has become the matter of extensive physical and biochemical investigation.

All presently proferred models for the mechanism of electron transfer in biology may be traced to the pioneering work of Marcus[1], Forster[2] and Dexter[3] in energy transfer. However, the importance of some very general principles (as e.g. the well known Frank-London principle) in thermally activated electron transport in protein systems has only been recently realized[4–6] in spite of the fact that the chemists have known of their importance for quite some time.

Much of the literature pertinent to electron transfer in biological systems is concerned with the mechanism of electron transfer between proteins. This is generally accepted to proceed by an 'outer-sphere' mechanism, although other proposals have been made. Intramolecular electron transfer e.g. in multicenter redox-proteins have also been studied extensively, along with theoretical advances that required detailed knowledge of individual centers and of their parameters (e.g. their electron exchange integral), in order to distinguish between proposed mechanism. The theoretical model of Marcus[7,7a] considers the case of 2 weakly coupled redox systems, where there is very little spatial overlap of the electronic orbitals of the 2 reacting molecules. A reasonable wavefunction describing the weakly interacting particles is just a linear combination of the wavefunctions describing the reacting particles and product particles respectively. As can be seen from the Franck-London principle, the transport process must be adiabatic: when electrons make a transition from one configuration to another, the electronic motion is so rapid that the nuclei (including ligands or solvent molecules) do not have time to move during the electronic jump. In other words, the electron transfer thus proceeds by way of 2 successive intermediate states, both having the same nuclear configuration but one having the electronic configuration of the reactants (X*) and the other one the electronic configuration of the products (X). Conservation of energy demands that the total energy of these 2 intermediate states must be the same; therefore these intermediate states must be activated in some way from the initial reactant or final product states. It has been pointed out[8–10] that a complete quantum mechanical treatment of electron-transport (including the effects of both the low-frequency modes of the 'medium' and the high-frequency molecular modes) shows that the latter are important and, in fact, dominant in

determining the transfer rate. In particular, the assumption of Marcus is thus not applicable to the calculation of intramolecular electron transport in proteins.

In an effort to take the effects of these high-frequency molecular modes into account, Hopfield[11] developed a theory of electron transfer in biological systems which is analogous to the Forster-Dexter theory of electronic energy transfer[2,3] in condensed phases. A semi-classical rate equation is proposed to account for the transport of an electron from cytochrome to a hole made available by the earliest stages of the reaction going on at the photosynthetic reaction center of Chromatium, according to the experimental observation by Chance and DeVault[12]. This observation and others[12a] showed that the velocity of electron transfer remains practically constant in a wide temperature interval ($T < 130°K$) and immediately suggested an idea of tunneling, as developed by Hopfield later on.

Concepts in quantum-mechanical tunneling in systems of biochemical interest have been discussed[219] in context with the validity of traditional models of the influence of nuclear motions on electrochemical electron transfer reactions based on the modulation by these motions of the energies of localized initial and final electronic states. The same phenomena which raise electron transfer tend to delocalize the initial and final electronic states; furthermore in the non-adiabatic limit, vibration-induced modulations of the electronic hopping integrals (often referred to as dynamic off-diagonal disorder) can exert a strong influence on electron transfer rates although their consequences have been neglected in traditional models of these processes; however, attempts to mitigate these shortcomings of models have been presented[219].

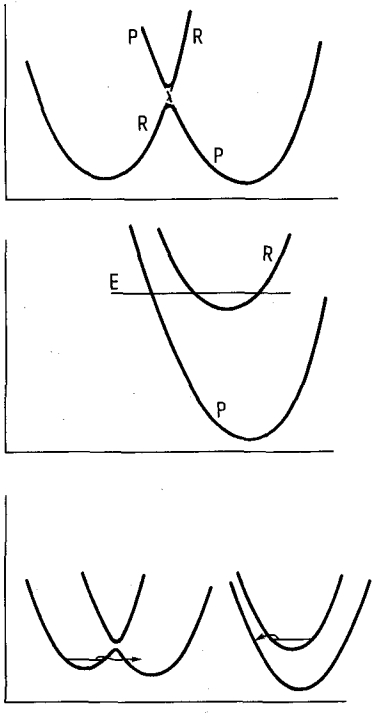

Figure 1. Plots of nuclear configuration versus potential energy of electron transfer reactions. Top: profile of a plot of the potential energy of the system of reactants (R) and products plus environment (P) versus configuration of the nuclei of the entire system for an approximately thermoneutral reaction in case of no electronic interaction between reactants (dotted lines) and of finite electronic interaction (solid lines at intersection). Middle: abnormal reaction where R and P do not intersect at readily accessible thermal reaction (very exothermic reaction). Bottom: nuclear tunneling through the barrier[7].

Hopfield[11,11a] proposes the following equation for the rate of electron transfer between site a and site b, assuming vibronic coupling:

$$W_{ab} = \frac{2\pi}{h} T_{ab}^2 \quad D_a(E)D_b'(E)dE$$

where W_{ab} is the electron transfer rate, T_{ab} is the 1-electron exchange integral between the sites, D_a (E) is the distribution of states for the electron in site a and D_b' (E) is the distribution of final states for the electron in site b, both relative to the energy for the electron at infinite. Other factors influencing electron transfer rates, such as the dependence of the rate on separation distance of the reactants, on standard free energy of reaction $\Delta G°'$ of each step, and on the 'intrinsic barrier' have been discussed[13]; it was shown that very negative $\Delta G°'$ s (very large thermodynamic driving forces) can actually have an inhibiting effect on a reaction rate, as may occur for instance in the back reaction in bacterial photosynthesis[218,234,235].

A modified form of Hopfield's equation relating the rate constants to electron transfer distances was applied to a series of metalloprotein redox reactions[226]. For proteins containing redox centers with minimal inner-sphere reorganisation barriers, the relation between half the intersite distance (R_p, Å) and the self-exchange rate constant at infinite ionic strength has been estimated. Calculated R values based on redox reactions of heme c, blue copper and Fe-S proteins with inorganic complexes support the conclusion that hydrophobic, π-containing ligands are able to penetrate into protein interiors, thereby reducing the distance over which electron transfer occurs. Recently, Jortner[14] pointed out that studies of non-adiabatic electron transport within the framework of multiphonon processes bear a close analogy to the formalism of electronic relaxation in large molecules and in solds[15,16], vibrational relaxation in condensed phases[17], electronic energy transfer[18] and small polaron motion in solids[19]. He thus developed the formalism of non-adiabatic multiphonon non-radiative decay in dense media in a form suitable for the study of temperature dependance of the electron transfer rate. He estimates the molecular properties with an Einstein-type approximation for the medium and for the molecular modes. The results of model calculations and the effects of medium have been studied recently by Jortner. The effects of both polar medium phonons and of molecular vibrations of the electron donors and the acceptor center were incorporated within the framework of nonadiabatic multiphonon description of the electron transfer process[260]. The nuclear parameters required to account for the temperature dependence are in accord with the available physical and chemical information regarding this system, while the large electronic coupling indicates the possible role of intermediate states in the electron transfer process[249]. Several biologi-

cal electron transfer reactions can be attributed to activationless electron transfer processes where the nuclear potential surfaces for the initial and for the final states cross at the minimum of the initial state. The theory of nonadiabatic multiphonon electron processes was used to characterize such activationless electron transfer processes (AETP). AETP correspond to exoergic processes. The configurational nuclear changes involved are optimal to ensure the fastest reaction rate, whose magnitude is restricted only by the electronic coupling[237]. The rate constant is temperature independent over a broad temperature range. The lowest temperature rate is determined essentially by the electronic coupling and not by nuclear contributions, whereas at higher temperatures, these processes exhibit an apparent negative activation energy.

Fundamental requirements of an electron transfer protein are that it provide the appropriate redox potential and an appropriate path for transfer, which both depends on its structure[232,251–255]. The structure of a great number of electron transferring proteins has therefore been studied by X-ray diffraction methods[127,20]. These studies have shown common factors which characterize an electron transfer protein: a) possession of a cofactor which acts as an electron sink and is placed near enough to the surface of the protein to allow an electron to enter; b) existence of a hydrophobic shell adjacent to, but perhaps not always entirely surrounding, the cofactor; c) presence of a flexible architecture which permits expansion or contraction in a preferred direction upon oxidation or reduction but without important structural changes or rearrangement of side chains upon transferring an electron. Furthermore the electron transfer system must be directed, and in many cases the system has evolved ways of controlling, increasing and decreasing the transfer rate, depending upon demand. For example, with several proteins electrons can be transferred intramolecularly from a tyrosine residue to electron deficient tryptophan units[21,208]. Rates of such transfer vary from about 10^2 per sec in lysozyme to about 2×10^4 per sec in trypsin with an activation energy of 45 kJ per mole; charge conduction along the polypeptide chain has been postulated. The electron transfer then proceeds directly between the aromatic groups, ruling out any mechanism that involves temperature labile hydrogen bonds as the main path. Electron transfer has been discussed in terms of biological evolution[245,246].

The influence of amino acid side chains on the electronic properties of proteins has been studied[233], particularly in the case of polypeptides containing 2 or 3 amino acid residues[23]. Due to the different potentials of different side-chain groups, the original valence and conduction bands of homopolypeptides are split into narrow bands. Comparison of the densities of electronic states in simple homopolypeptides and in composite polymers shows that new forbidden regions in the energy spectrum of proteins develop that affect the semiconductive properties of the proteins. Studies on electron transfer in proteins should also take into account the possible role of transition metal ions in variable oxidation state present in low concentration as impurities[22], where intramolecular electron hopping may be working together with intermolecular electron transfer[22].

Thin-layer spectro-electrochemical methods have been used to determine the formal oxidoreduction potentials of electron-transferring metalloproteins at 5–40°. Electron transfer reaction entropies were derived[24]. The values are more negative than corresponding ones for inorganic complexes with low inner-sphere electron-transfer barriers; this has been discussed in terms of changes in protein-solvent interaction accompagnying reduction by increased solvent ordering in the protein interior or a more compact protein structure in which solvent is excluded[24]. Furthermore, methods have been developed to express the flux of an electron transfer reaction as a function of the conjugate force, the redox potential throughout the pathway. The flux can be expressed by a product of the hyperbolic sine of the force, a factor (redox-poising parameter) determined by the redox potentials of subsystems (local pH's and pK's values) and some constants. This is analogous to the expression of the flux of a diffusion process by the product of its force and the concentration of the diffusing species. The expression has been applied to redox chains in which electron transfer processes are coupled to vectorial processes such as proton translocation or electrical current. There are several ways in which electron transfer can be induced and the rate internal to the protein measured: a) an electron can be 'injected' or 'released' by a photoactivation process and the subsequent electron transfer monitored as a function of time[212]. b) an electron can be injected by pulse radiolysis and the electron transfer is monitored spectrophotometrically. (Application of pulse radiolysis to studies of redox reactions in proteins and electron movement between and within proteins have been reviewed in detail[25] and will not be mentioned here.) c) Steady-state exchange rates can be determined from indirect effects such as the broadening of energy levels in a magnetic resonance experiment or e.g. isotope effects. For example, the effect of D_2O substitution on the oxydation of cytochrome c has been studied[26] and discussed in terms of the multiphonon radiationless transition theory. On the basis of these studies, a molecular mechanism for cytochrome c oxidation has been proposed. Isotope effects were also observed in the intramolecular binding of CO to myoglobin observed with time-resolved Fourrier-transformed IR spectroscopy; large isotope effects confirmed earlier evidence for molecular tunneling. A faster binding of $^{12}C^{18}O$ over $^{13}C^{16}O$ implies that structure effects are significant in molecular tunneling.

Investigation of electron transfer reactions by electrochemical methods has been reviewed[27]. Two new electrochemical techniques (thin-layer pulse and thin-layer staircase coulometry) were evaluated recently for determining $E^{0\prime}$ and n values of redox couples and proved useful for determination of redox potentials and electron transfer stoechiometries for biological redox species. Because these techniques do not require optical monitoring they may complement existing spectroelectrochemical techniques for studying biological redox systems[28,212,214,223–225,247].

Some complex electron transferring systems are embedded within a rigid membrane matrix, as e.g. in the

mitochondria or in photosynthetic particles: this allows a better organization of the system for intermolecular transfer with the consequence that the rate of electron transfer need not be diffusion controlled. Membrane proteins within a single system are usually organized such that the proteins act in parallel and the difference in redox potential between adjacent proteins is small (80 mV or less). This close association, however, makes extraction and purification of individual electron transfer proteins a most difficult task. The study of electron-transferring function of membranes[215] – particularly bacterial membranes – is further complicated by other facts, since the membrane is not an organelle of a single more or less specialized function, but usually offers low concentration of specific redox components, with a background of various other enzymatic reactions which could interfere[221], like transport functions of the membrane. Moreover, owing to their great adaptability to various environmental conditions, many bacteria may lack constitutive electron transport chains and synthetize individual redox components or even entire chains in response to external changes. All these draw-backs obviously necessitate the study of purified redox components to ascertain their discrete functions. As an alternative, several studies have concentrated on reconstituted protein-carrying membranes in the form of either small vesicles or planar bilayers that separate two aqueous phases[207, 209]. For example, photo-induced electron transport across protein-containing membranes has been studied, e.g. in lecithin lipsosomes loaded with an electron acceptor (Methylviologen or ferricyanide), and it has been shown that only the acceptor outside the liposome undergoes photoreduction, the phospholipid membrane being poorly permeable to the reducing equivalents; however, when liposomes were formed so that the membranes incorporated protein components, (either bacterial rhodopsins or mitochondrial ATPase), there was an increase in photoreduced methylviologen, the main portion of it being reduced inside the liposomes[29]. These observations and similar experiments with erythrocytes indicate that the protein in the membrane mediates donor to acceptor electron transport. Similar enhancement of electron transfer was also achieved with ionophores[30]. The rate of photoinduced electron transfer across vesicles walls in the absence of ion carriers is limited by co-transport of cations and is influenced further by generating transmembrane potentials of K^+ gradients in the presence of valinomycin. Laser flash photolysis of an asymetric lipid bilayer vesicle system containing chlorophyl, valinomycin and phosphatidylcholine has also been reported[31].

Specific electron transfer proteins

1. Flavoproteins

Despite the ubiquity of flavoproteins in nature, the working mechanisms of flavins are not well understood at a molecular level. The complications arise from the great variety of functions that can be assumed by flavins bound to suitable apoenzymes. Basically 3 modes of 'redox-equivalent transport' occur in biological oxido-reduction systems: hydride, carbanion and electron transport. While the first 2 types are assumed by nicotinamide derivatives, flavin is interconverting carbanion and electron equivalents. It is now widely recognized that biological dehydrogenation starts with a 2 electron transfer, i.e. a hydride equivalent is transformed to yield 2 electron equivalents and a proton and this process as well as its reversal is uniquely flavin-dependent all over the range of biochemistry. The transformation of the σ-pair of e.g. the CH-bond to be dehydrogenated into single π-electrons is mediated by $(4n + 2)$ π electrons (aromatic) systems such as NAD(P), which yields a homoaromatic reduced form and finally flavoquinone. Upon reduction of the latter, an extensively delocalized 4 $n\pi$-system (flavohydroquinone) is formed[76]. Alcohol dehydration in biological oxidation thus implies 2 modes, namely the nicotinamide-dependent mode, splitting the substrate CH-bond into hydride plus carbocation[32], and the flavin-dependent mode[33] splitting it into proton and carbanion. In flavin-dependent oxidoreduction, regiospecific hydrogen bridges between flavin and apoprotein decide the actual transport modes. Thus flavin-dependent 1-electron transfer in enzymes and chemical model systems can be differentiated from 2-electrons activities (i.e. [de-]hydrogenation and oxygen activation) by chemical structure and dynamics of the flavin molecule. For 1-electron transfers, 2 types of contacts are possible, namely outer-sphere for interflavin and flavin-heme and inner-sphere for flavin-ferredoxin contacts. Flavin is the indispensable mediator between 2-electrons and 1-electron in all biological redox chains[34] and there is a minimal requirement of 3 cooperating redox-active sites for this activity. The switch between 2-electron and 1-electron transfer is caused by apoprotein-dependent proton transfer on the flavin molecule; the effect of hy-

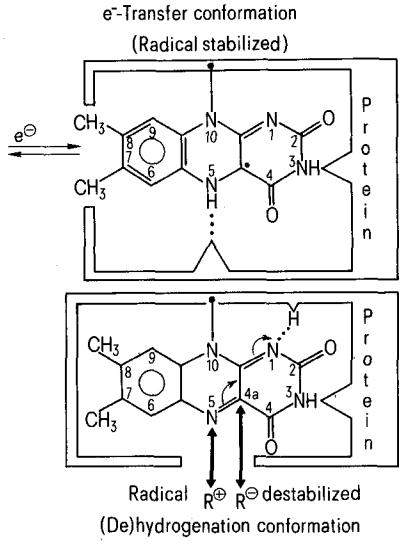

Figure 2. 1e –/2e -alternative of flavin-dependent redox transfer as regulated by regio-specific hydrogen bonds or positive charges from the apoprotein towards the lone pairs of either the N(1)/O(2)-region (2e -transfer, lower part) or the N(5) lone pair (1e -transfer, upper part). In the latter case the input and output of electrons is presumably through the C(8)-edge while in the former case the substrate (carbanion) access is through 4, 5. Blocking and deblocking of lone pairs is thus accompanied by opening and closing of the access site.

drogen bonding on the electronic structure and spectra of the flavin nucleus has been described in terms of the self-consistent field molecular orbital method[35]. Among the possible hydrogen bondings examined, characteristic spectral shifts were found for the hydrogen bondings at N(1) and N(5) of the nucleus. The spectral characteristics of the flavin chromophore in *Clostridium* MP and *Desulfovibrio vulgaris* flavodoxins coincided with the calculations; these calculated results, when applied to D-amino-acid oxidase, led to the conclusion that hydrogen bonding occurs at O(12), N(3)H, O(14) and N(5) of the isolloxazine nucleus. The occurence of hydrogen bondings at O(12), N(3) and O(14) is favorable for N(5) (fig. 2) of the isoalloxazine nucleus to accept electrons from an electron donor[35]. Hydrogen bonding is considered to occur with increasing concentrations of the proton donor, first at N(1), then at O(12), O(14) and N(3)H and finally at N(5)[36]. The major effect of the hydrogen bonding at the N(1), N(3)H and oxygen atoms of the flavin nucleus is to facilitate the electrophilicity of the N(5) position, as predicited by molecular orbital calculations and supported by the observations that the hydrogen-bonded flavin in its triplet state abstracts hydrogen from a donor at a faster rate than do the non-hydrogen-bonded species[36]. If the N(5)-lone pair is blocked, the system is switched for 1-electron transport. If the N(1)/O(2) region of the flavin molecule is blocked, the system is ready for 2-electron-(i.e. carbanion)-transport[34]. This behavior can be simulated with suitable model compounds. It also turns out that electron-transporting flavoproteins provoke one-electron activation of dioxygen, yielding superoxide, while true oxidases catalyze 2-electrons transport and direct formation of H_2O_2[37]. Combination of the 3 main types of flavin activities (dehydrogenation, electron transfer and dioxygen activation) lead to the further distinction of 5 flavoenzyme classes with specific experimental parameters (table 1). Some yeast methanol oxidases, however, show unusual properties[38].

Over the last years, a number of studies on flavoenzymes have been reported in which the native flavin coenzyme has been replaced by a 5-deazaflavin. The similarity in chemical properties of deazaflavins as compared to normal flavins has provided the basis for using these analogues to study both non-enzymatic and flavoenzyme reactions[39]. However, the chemistry of deazaflavins resembles much more that of pyridine nucleotides than of flavins (lower rate of reduction by substrate, no stabilization of the radical by the apoprotein; unability to participate in O_2-activation or electron transfer, i.e. only hydride transfer is feasable). These facts allow important mechanistic conclusions about flavoprotein catalysis. Another analog N'-ethyl-4a-(hydroperoxy)-3-methyllumiflavin served to define the mechanism for the microsomal flavoenzyme monooxygenase responsible for N- and S-oxygenation. The monooxygenase reactions responsible for the chemiluminescent oxidation of aldehydes (bacterial luciferases) have also been modeled. The minimal structure for light production has been estabished and it has been shown that 2 excited species are formed. The anion 4a-Fl Et-O-O^- transfers dioxygen moiety to ambident anion substrates (S^-) to yield reduced flavin and SOO^-. Dioxygen transfer from 4a-Fl-EtOO^- has its counterpart in the flavin dioxygenase enzymes[41,256]. The role of flavins in electron transfer between 2-electron donors and 1-electron acceptors has been discussed[44,213,220,231] in connection with the photochemistry of flavins[46] or with electron transfer in NAD molecules[240,242].

The biochemical properties of some electron transfer favoproteins from mitochondria have been reviewed recently[42,43,222,250]. Thermodynamic parameters of succinate dehydrogenase flavin were determined potentiometrically from the analysis of free-radical signal-levels as a function of the oxidation-reduction potential. Midpoint redox potentials of consecutive 1-electron transfer steps were found to correspond to a stability constant of intermediate stability (2.5×10^{-2}), which suggests also that flavin itself may be a converter from n = 2 to n = 1 electron transfer steps[47].

The effects of solvent environments on the rates of several flavin redox reactions (either electron transfer to the flavin triplet state – as a measure of oxidized flavin electrophilicity – or oxidation of flavin semiquinone by oxidized flavin radical) has shown that semiquinone yield, due to flavin reduction during the quenching reaction, was linearly dependent on the solvent dielectric constant, thus implying the existence of a polar or charged intermediate. A similar effect of solvent dielectric was found for the self-quenching reaction (which produces semiquinone and an oxidized flavin radical). The observation of a biphasic dependence on solvent dielectric was interpreted in terms of a change in mechanism with solvent polarity, i.e. a neutral transition state and hydrogen atom transfer in low dielectric media and a dipolar transition state and electron transfer in high dielectric media were postulated[48]. However, no specific effects of hydrogen-bonding interaction between flavin and solvent could be observed for any of these processes. NMR data also showed that the flavin molecule seems to exist in intramolecularly folded conformations that are extremely favorable for charge transfer interactions, the interaction being appreciably stronger than e.g. in NAD[49]. Stopped-flow and laser photolysis methods were used to investigate the rates of electron transfer reactions of fully reduced riboflavin and the 3 oxydation states of *Clostridium pasteurianum* flavodoxin. Dichloro substitution in the flavin considerably decreases the rate of flavodoxin semiquinone oxidation. Considered in conjunction with the redox potential shift of the flavoprotein produced by chlorine

Table 1. Combination of the 3 main types of flavin activities (dehydrogenation, electron transfer and dioxygen activation) lead to the further distinction of 5 flavoenzyme classes with specific experimental parameters

Flavoprotein type	Radical stability	Radical color	O_2-affinity	Product of O_2-reduction
I. Transhydrogenases	Low	–	Low	O_2^-
II. Dehydrogenases Oxidases	Interm.	Red	High	H_2O
III. Dehydrogenases Oxygenases	Low	–	High	$H_2O + (0)$
IV. Dehydrogenases e^--transferases	Stable	Blue	Low	O_2^-
V. Pure e^--transferases	Very stable	Blue	Low	O_2^-

substitution, these results support the hypothesis that electron transfer to and from the semiquinone form involves direct participation of the dimethylbenzene ring of the flavin, whereas it seems that the protein environment does not markedly influence coenzyme reactivity, as deduced from a comparison of oxidation rate constants for free and protein-bound fully reduced flavins in flavodoxins[41].

Flavodoxins are electron carrier proteins of small relative molecular mass that act in low oxidation-reduction reactions and are often interchangeable with the iron-sulfur proteins ferredoxins. They can be reduced in 2 distinct 1-electron steps with the formation of relatively oxygen-stable flavosemiquinone as an intermediate[32]. A report on a ^{31}P-NMR study on the binding of the FMN prosthetic group by *M. elsdenii* apoflavodoxin yielded information on the interaction of the phosphate group of the prosthetic group with the apoprotein together with the distance between the phosphorus atom and the isoalloxazine moiety and the rate of electron exchange between the molecules in the different redox states[33]. The electron exchange between the oxidized and semiquinone form of *M. elsdenii* flavodoxin is rather slow whereas that between the semiquinone and hydroquinone form is much more favored; thus the activation energy for the transition between the semiquinone and hydroquinone states must be smaller than that for the transition between the oxidized and semiquinone states. These results offer a reasonable explanation for the 1-electron transfer reaction of flavodoxins, a protein conformational change being inferred during the quinone-semiquinone transition and not during the semiquinone-hydroquinone transition[33].

The structure of flavodoxins from 3 species *(Anacystis nidulans, Clotridium* MP and *Desulfovibrio vulgaris)* have been determined crystallographically[127]. In D. vulgaris, the isolloxazine ring is sandwiched by hydrophobic residues and nearly all those portions of the ring which are able to hydrogen-bound to the protein do so. It is also assumed that N1 is not protonated but ionized in the fully reduced form[50]. Furthermore, 2 nearest neighbors of the alloxazine ring also display a hydrophobic patch that may be significant for electron transfer.

2. *Fe-S proteins*

The kinetics of oxidation and reduction of the various Fe-S proteins studied do not correlate with their oxidation-reduction potentials[53]. The electrostatic interactions between a particular high-potential-iron sulphur proteins (HiPIP), for example, and the Fe-hexacyanide is influenced by the charge on the Fe-S cluster, with specific amino acid side chains playing a significant but limited role in the interaction leading to electron transfer. The Fe-S cluster charge is apparently distributed on the surface of the HiPIP molecule through a network of hydrogen bonds. A structural region at which the Fe-S cluster is near to the protein surface has been defined for HiPIP. Somewhat similar studies on electron transfer reactions have been reported on spinach ferredoxins, using organic and inorganic mediators[51]. Introducing several electrochemical methods (like chronoamperometry, pulse and differential pulse polarography and rotating diskelectrode technique), the rate of reduction was determined by indirect electron transfer. It appears that for small molecules-protein interaction, electron transfer follows the Marcus' outer-sphere mechanism when the difference in electrode potential between the protein and the mediator is large, and the electron tunneling mechanism of Hopfield and Jortner when the difference is small[51].

Cusanovich et al.[52] studied the interaction of 2 samples of HiPIPs with mitochondrial cytochrome c from horse heart and bacterial cytochrome from *Rhodospirillum rubrum* and from other microorganisms. On the basis of the kinetics of electron transfer between the various HiPIPs and cytochromes, it appears that the interactions are more complex than those observed with non-physiological reactants. Evidently specific sites on both the HiPIPs and the cytochromes mediate electron transfer. The effect of ionic strength was markedly different from that expected on the basis of the interaction of the various proteins with e.g. Fe-hexacyanide. The interaction of HiPIP with some of the cytochromes investigated is heterogeneous, resulting from different possible orientations for interaction leading to electron transfer. No long-lived complexes between the HiPIPs and the cytochromes are formed. The second-order rate constant was measured which may in fact be the product of the association constant for any HiPIP and a particular cytochrome and a first order rate constant from the rate limiting step leading to products. Similar studies with non-physiological reactants (instead of cytochromes) as a function of pH and temperature allowed some conclusions concerning the mechanism of electron transfer by HiPIPs[53].

The electron transfer mechanism of tetranuclear clusters was also explored via a stopped-flow spectrometer kinetic study of the reduction of *Chromatium vinosum* and *Rhodopseudomonas gelatinosa* HiPIPs, by both native and trinitrophenyl-lysine-13 horse cytochrome c[54]. The influence of electrostatic effects was also effectively partitioned from the redox process per se. Despite the fact that the modified cytochrome c is orders of magnitude less reactive with its physiological reaction partners, as compared to the native cytochrome c, the correlation rates were somewhat faster for the modified cytochrome c over native cytochrome c, either because modified cytochrome c reacts more quickly – since modification of the lysine-13 residue destabilizes the heme crevice – or, in light of the hydrophobic nature of the TNP-group, because this group facilitates electron transfer by interacting with a hydrophobic region of the HiPIP molecule surface, e.g. the region about the S_γ atom which is the most exposed and the most accessible hydrophobic region of the HiPIP surface, in addition to being the point of easiest approach of the cluster to the external environment.

Heterogeneous electron transfer rate parameters for soluble spinach ferredoxins have been reported[55] using a recently developed single potential step spectroelectrochemical technique. The reductive kinetics were measured by monitoring the decrease of absorption as a function of time for several 1-potential steps at methylviologen modified Au minigrid electrodes. These measurements yielded an average formal heterogeneous

electron transfer rate constant $k = 6.5 \times 10^{-5}$ cm/sec and electrochemical transfer coefficient $a \sim 0.60$ at pH 7.5[205]. Photo-induced electron transfer of tetranuclear Fe-S analogues has been described, along with its dependance on the pH and wavelength of the light used. The reaction, carried out in presence of methylviologen, acenaphtenequinone or upon the addition of cysteine-molybdenum complexes as co-catalyst, showed the possibility of reversible intramolecular electron transfer of the cluster core to the ligand $SC_6H_4NO_2$-p[56].

The intrinsic self-exchange rate constants of tetranuclear clusters and model compounds were determined by general line-shape analysis of exchanged broadened ^{1}H-NMR spectra in CH_3CN solution[57]. Reactions are second order, first order each in oxydized and reduced clusters and around 300 °K, rate constants fall in the intervall 10^6–10^7 M^{-1} sec^{-1}, placing them among the faster inorganic self-exchange systems. The Fe-Moessbauer, magnetic susceptibility, magnetization and EPR properties of an extensive series of model compounds were examined in the solid state and in frozen acetonitrile solutions[57a]. The cluster trianions serve as analogs of reduced ferredoxin proteins. Based on similarities and differences in their properties in the solid state, the compounds divide into 2 categories: those whose $(Fe_4S_4(SR)_4)^{3-}$ clusters contain tetragonal or non-tetragonal Fe_4S_4 core structures. The lack of core structural uniformity is attributed to perturbing influences in the solid state. Irrespective of their solid-state category, all compounds in frozen solution exhibited essentially coincident properties (mostly documented by EPR and Moessbauer spectral results), which indicate a single core structure of a set of closely related core structures. From a previous demonstration of the similarities of properties of $[Fe_4S_4(SPh)_4]^{3-}$ salts in the solid and solution state, and the X-ray structure of this cluster, the solution core structure of the set of cluster trianions is identified as elongated tetragonal. These findings and others provide substantial experimental support for 2 proposals: 1) an elongated D_{2d} core structure is the intrinsically stable configuration of tetranuclear trianions; 2) the unconstrained, idealized core structural change accompanying electron transfer is $[Fe_4S_4(SR_4)]^{2-}$ (compressed D_{2d})$\rightleftharpoons$$[Fe_4S_4(SR)_4]^{3-}$ (elongated D_{2d}). The structural reorganization energy of Fe_4S_4 cores upon passing from a compressed tetragonal to an elongated tetragonal geometry via a proposed T_d geometry in an outer-sphere process is estimated to be about 1.4 kcal/mol[111]. This process serves as a representation of protein site structural changes in a $ferredoxin_{ox}$/$ferredoxin_{red}$ electron transfer couple in the absence of intrinsic constraints such as might be imposed by protein structural features[57a]. The 'slow' electron self-exchange rates on the NMR time scale between oxidized and reduced forms of proteins containing one 4-Fe site, can now be primarily attributed to kinetically retarding steric influences of protein structure rather than intrinsically slow reaction of the sites themselves.

An attempt to better understand the nature of the spin-coupling in iron-sulphur proteins has been made by measuring the electronic spin-lattice relaxation time T_1 of several binuclear ferredoxins[58] and more recently for tetranuclear clusters also[59]. The relaxation broadening of the EPR spectrum is interpreted by an Orbach process involving the first excited level which provides a simple method to measure the position of this level and the exchange interaction between the iron atoms of the cluster. The rapid relaxation broadening is very likely induced by a 2 phonon resonant process involving the low lying excited levels. The frequencies of the vibrational modes which are efficient in these 2 phonon resonant processes are relatively high and could hardly propagate through the protein molecule. The variations of the exchange coupling constant J which are observed within the group of 2Fe-2S ferredoxins have been discussed by these authors within a model involving the structural properties of the active site[60]. The J value is mainly dependent on the geometry of the bridge sulphur atoms and weakly on the nature of the other ligands.

Iron-sulphur proteins for which crystal structures are known all contain NH ... S hydrogen bonds between backbone amide groups and sulfur atom in the iron-sulfur cluster. Moreover there is an approximate correlation between the number of such NH ... S hydrogen bonds in the environment of a given (Cys-S)Fe_4S_4 cluster and its oxidation reduction potential[61]. In high potential Fe-S protein (HiPIP) these hydrogen bonds shorten and become more linear in the reduced state of the molecule[62]. The possibility that cluster oxidation states and hydrogen bond geometries may exhibit reciprocal influences one on the other – a characteristic of coupled equilibria – has been examined[63]. Ab initio molecular orbital calculations on model systems showed that hydrogen bond geometries in reduced HiPIP may be differentially stabilized relative to those in oxidized HiPIP by increased negative charge on the iron-sulfur cluster. A significant coupling between the hydrogen bond geometry and charge effects was found. It has also been shown that deuteration of the NH ... SH-bonds in ferredoxin from *C. pasteurianum* causes a small lowering of the oxidation-reduction potential of the molecule[64]. All iron-sulfur proteins studied so far by crystallography contain backbone NH groups with suit-

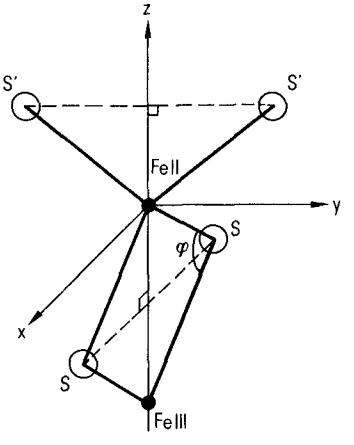

Figure 3. Representation of the Fe(II) site of the reduced [2Fe-2S] proteins. S and S' are respectively the labile and the cysteine sulfurs atoms. A C_{2v} symmetry is assumed for this site, as observed in synthetic analogs. Variations of the bridging angle induces structural distortion of the Fe(II) site[60].

able geometries to form hydrogen bonds to cysteine sulfurs and inorganic sulfurs in the cluster. In contrast to heme proteins[65] where the metal ligand is a proton donor, the ligand sulfurs in iron-sulfur proteins act as electron donors to these presumptive hydrogen bonds which therefore should stabilize more negative (more reduced) oxidation states. However, other factors related to the protein tertiary structure are also of importance in electron transfer[238] as exemplified by the various observations about the intermolecular electron-transfer of flavodoxins and ferredoxins used as mediators in systems for hydrogen evolution[66] or photosynthesis[228,243,259]. In electron transfer from dithionite to hydrogenase, of *Clostridium pasteurianum,* highest activity was shown by the ferredoxin from *Chlorogloeopsis fritschii* and a flavodoxin from *Megasphera elsdenii.* The latter was about 20-fold as active as comparable concentrations of methylviologen. Ferredoxins from *Anacystis nidulans* and *Porphyra umbilicalis* also showed high activity. In mediating electron transfer from chloroplast membranes to *Chromatium pasteurianum* hydrogenase, the flavodoxin from *A. nidulans* proved the most active with a *Nostoc* flavodoxin and *P. umbilicalis* ferredoxin also being more active than other cyanobacterial and higher plants ferredoxins. In this system, the ferredoxin and flavodoxin from *Chondrus crispus* and the ferredoxin from *gigurtina stellata* showed very low electron-transferring activity. Efficiency in supporting the electron-tranferring activity in these systems does not appear to be correlated with the mid-point redox potential of the ferredoxins or flavodoxins used as mediator in supporting either NADP photoreduction by chloroplasts or pyruvate oxidation by *C. pasteurianum* systems. Thus activity of the mediators in these systems primarily reflects differences in tertiary structure conferring different affinities for other components of the system[66].

Nitrogenase

Nitrogenase is an enzyme system (reduced carrier: N_2 oxidoreductase) that consists of 2 proteins and converts N_2, ATP-dependently, into NH_3. The nitrogenase system is composed of 2 dissociating protein components. One, called the Fe protein, contains 4 iron and 4 acid-labile sulphur atoms; the other, the Mo-Fe protein, contains 2 molybdenum, 28–32 iron, and about 28 acid-labile sulphur atoms. The properties of nitrogenases from bacterial sources were recently reviewed by Mortenson and Thorneley[268]. The nitrogenase reaction needs a low-potential electron donor and at least 2 molecules of MgATP are hydrolyzed to MgADP and P_i per electron transferred to the substrates. Electrons are donated to the Fe protein and pass to the Mo-Fe protein, ATP-dependently. The Mo-Fe protein acts as a storage sink for electrons and passes the electrons in multiples of 2 to the substrates. The electron donors in vivo are thought to be either a ferredoxin or a flavodoxin. The recent elucidation of the nitrogenase cofactors have enabled a better understanding of the mechanism of electron transfer in this exceedingly complex system. The environments of iron in nitrogenase fall mainly into 3 major categories: 1) the iron-protein which is thought to play a protective role against dioxygen, and shows iron-sulfur cores that may be quantitatively displaced and identified as 4 Fe-4 S centers[67]; 2) the Mo-Fe-protein which shows 2 types of electron-transferring centers, the so-called M-centers, made up of 2 clusters which account for 12 Fe atoms (out of 30) and, 3) the so-called P-clusters present in 4 covalently bound [4Fe-4S] cores in a low oxidation state[68]. These P-clusters are formed by 2 components[69] of a total of 16 iron atoms and are called iron D and Fe^{2+}; they undergo 4-electron oxidation and are associated in a structure of a novel type, namely a spin-coupled cluster which, by oxidation, gives an $S = 3/2$ species. The intensity ratio of 3:1 observed between the D and Fe^{2+} groups suggests the presence of $4 \times$ [4Fe-4S] units, but these centers have magnetic properties (e.g. Moessbauer isomeric shift) distinct from typical [4Fe-4S] centers. On the other hand, the M-clusters seem to involve 1 Mo and 6-Fe and labile sulfur and can be stabilized in 3 oxidation states, the 6 iron atoms being associated with a spin coupled structure that might have direct implication in the catalytic active site of the enzyme[69]. Model compounds that mimic the properties of this structure have been studied[71]. It seems that the Mo-Fe-protein alone is responsible for the reduction of substrates, whereas the Fe-protein (or dinitrogenase reductase) participates only indirectly. The nitrogenase reaction needs a low-potential electron donor and at least 2 molecules of MgATP which are hydrolyzed to MgADP and Pi per electron transferred to the substrates. Electrons are donated to the Fe-protein, ATP-dependently. The Mo-Fe protein thus acts as a storage sink for electrons and passes electrons in multiples of 2 to the substrates. A model for the steps in electron transfer from dithionite to dinitrogenase (the Mo-Fe-protein) has been discussed[72]. The experiments supporting this model involved determination of steady-state and pre-steady-state kinetics.

Furthermore, in the Mg-ATP dependent reaction catalyzed by nitrogenase, there is a rapid burst of ATP-hydrolysis in the pre-steady state reaction, that occurs on the same time scale as the electron transfer from dinitrogenase reductase to dinitrogenase. This burst corresponds to 2 ATP's hydrolyzed per electron transferred between the 2 proteins. Two Mg-ATP molecules are bound to dinitrogenase reductase with dissociation constants around 430 μM and 220 μM[73]. The effect of Mg-ATP concentrations on the pre-steady-state kinetics of electron transfer from dinitrogenase reductase to dinitrogenase showed that there are 2 Mg-ATP's required for that reaction with Km values very similar to the dissociation constants for Mg-ATP from dinitrogenase reductase. This indicates that electron transfer between the 2 proteins is substantially slower than the binding and dissociation of Mg-ATP from the dinitrogenase reductase. It was possible to predict quantitatively the steady-state kinetics from the pre-steady-state kinetics. This showed that the Mg-ATP dependance of electron transfer is sufficient to account for effects of Mg-ATP concentration on the steady-state hydrogenase reaction catalyzed by dinitrogenase. Thus the hydrolysis of 2 ATP molecules when an electron is transferred between the 2 proteins of the system is sufficient to account for

all the ATP hydrolysis occuring in the steady-state reaction, and the only role of Mg-ATP seems to be in supporting the electron-transfer from dinitrogenase reductase to dinitrogenase[73]. The distribution of electrons among substrates when 3 substrates are simultaneously available for reduction has been studied[74]. The electron allocation between NH_3, H_2 and C_2H_4, when N_2, H_2 or C_2H_4 were all used as substrates has been studied in response to altering the total electron flux through dinitrogenase (the Mo-Fe-protein) by variation of Mg-ATP or dithionite concentrations or the ratio between the dinitrogenase component proteins. The electron allocation between substrates is controlled specifically by the electron flux and not by Mg-ATP or dithionite concentration. At the lowest electron flux, dinitrogen does poorly, H_2 very well and C_2H_4 intermediately in the competition for electrons. At the highest electron flux, dinitrogen does very well, C_2H_4 rather poorly and H_2 intermediately, with the electron partitioning and electron transfer being associated with ATP-hydrolysis. In the absence of the Fe-protein (dinitrogenase reductase) or Mg-ATP the 2 classes of electron-transferring centers (i.e. the M centers and the P-clusters) of dinitrogenase are no more in rapid electron exchange equilibrium in the resting state[75]. Another study followed spectrophotometrically the oxidation of photochemically reduced low-potential electron carriers by nitrogenase with simultaneous activity measurements[76]. When the Mo-Fe protein is in redox equilibrium with the electron donor, the activity is maximum and independent of the redox potential up to −440 mV. At higher potentials the nitrogenase activity declines and no significant activity is detectable at potentials above −350 mV. The effect of the redox potential on the oxidation-reduction state of the [4Fe-4S] cluster of the Mo-Fe-protein as studied by EPR showed that the reaction is characterized by the transfer of 2 electrons per redox step in the absence of adenine nucleotides, with the cluster showing a mid-point potential of about −393 mV. In the presence of MgATP the mid-point potential undergoes a negative shift of 42 mV and the electron transfer still occurs in a 2 electron redox step. In contrast in the presence of MgADP, the results are characterized by a 1-electron step with a mid-point potential of −473 mV. Thus under redox potential limitation, catalysis might proceed by 2-electron transfer. It is not necessary that the 2 electrons are donated simultaneously: 1 electron can be donated rapidly, the second electron then must be donated more slowly. In this case the redox behavior of the Mo-Fe-protein-MgADP complex might explain the strong inhibition of the nitrogenase reaction by MgADP. The absence of the second electron prevents catalysis rather than competition with bound MgATP. A sequential reaction of the reduced Fe-protein with the Mo-Fe-protein has been proposed[76] (fig. 4).

Model studies involving the nitrogenase system has been reported. Reduction of N_2 in aqueous solution by BH_4^- in the presence of MoO_4^{--} and cystein or other thiols have been observed[77]; more recently the complex $MoO(CN)_4(H_2O)^{2-}$ has been used to effect this reaction with great success. It appears that the active species in the reduction is an Mo(IV) complex of unknown structure and the mechanism involved is rather obscure[78]. Better models of course are Mo-Fe-S cluster complexes[79] whose EXAFS spectra have quite similar properties to those of the Mo-Fe-protein.

3. *Hemoproteins*

Cytochrome c

Cytochrome c is a relatively small metalloprotein (with a molecular weight of about 12,400) that acts as an electron carrier in the respiratory chain of all aerobic organisms. X-ray studies[80–82] have revealed that the heme group is located in a crevice of the essentially globular protein. The iron lies in the plane of the porphyrin ring and the fifth and sixth coordination positions are respectively occupied by a nitrogen atom of the imidazole ring of His-18 and the sulfur atom of Met-80. In aqueous solutions the coordination environment of the heme depends on the pH, the ionic strength and the anionic composition of the medium[83]. At physiological pH, the coordination environment of iron is believed to be the same as in the solid. However at low pH, the Fe-S and Fe-N bonds are both broken and these coordination positions are probably occupied by water[84]. At pH 2 at high ionic strength in a chloride medium, spectral changes, particularly an increase in absorbance at the 695 nm peak, suggest that the Fe-N (His-18) bond is broken[83]. These 3 coordination situations around the ferric ion in the heme are associated with cytochrome c 'electronic' isomers that are called the low-spin, high-spin and mixed-spin species with the iron possessing 1, 5 and 3 unpaired electrons, respectively[85]. A striking feature of the structure of oxidized horse

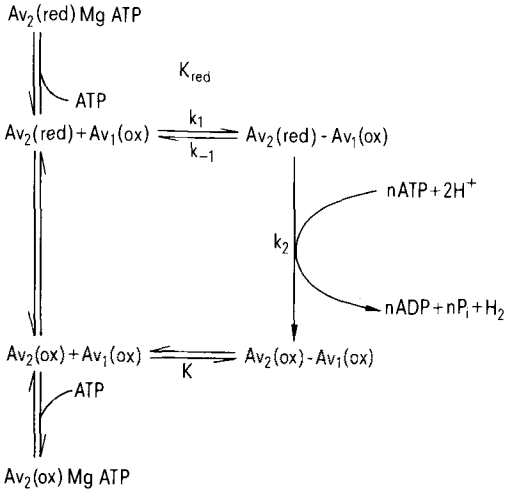

Figure 4. Basic steps for electron transfer in nitrogenase of *Azotobacter vinelandii*. The transfer of electrons in catalysis is irreversible and the rate-limiting step of the reaction occurs probably through Kox. This minimal hypothesis assumes fast equilibrium steps as compared with the rate of catalysis[76]. The following facts and assumptions were taken into account. a) The concentration of the Av_2(red) Av_1(ox) complex determines the rate of the raction, because of the 1000-fold higher affinity of the Fe protein for the Mo-Fe protein than for MgATP. b) The transfer of electrons in catalysis is irreversible and the rate-limiting step of the reaction occurs somewhere in the whole sequence of events. This is the rate of dissociation of Av_2(ox) from Av_2(ox) Av_1(ox). c) All equilibrium steps are fast compared with the rate of catalysis.

cytochrome c is a channel leading to the heme group from the molecular surface[81]. This channel (so called the right channel) is so characterized as there is a hydrophobic hole in the right-hand side of the protein (fig. 5)[80]. It is lined with the side-chains of hydrophobic amino acids; however, as the area in the center of the channel is not completely taken up with these groups, there is room for a small group in the molecule. Another hydrophobic region lies on the left hand side of the molecule, which is not solvent accessible and extends to the molecular surface from the heme, and is called the left channel. It has been suggested that the latter function is a charge transfer pathway for an electron into the protein[81], whereas the right channel would function as a pathway out of the protein. This older mechanism described the following hop series:

reductase phe/tyr-74 trp 59 phe/tyr 67 heme

This mechanism, however, has been reconsidered with respect to physiological reaction partners of cytochrome c[81a]. An alternative mechanism has been proposed based upon the electron entering the protein through the exposed edge of the heme[99]. The mechanism of transfer may involve in any case a channel of hydrophobic residues for tunnelling in a preferred direction[80]. As additional sequences appeared, however, it became apparent that alternative mechanisms are more satisfactory. A marked similarity in the distribution of evolutionarily conserved lysine side chains surrounding the heme crevice in cytochrome c and cytochrome c_3 was observed.

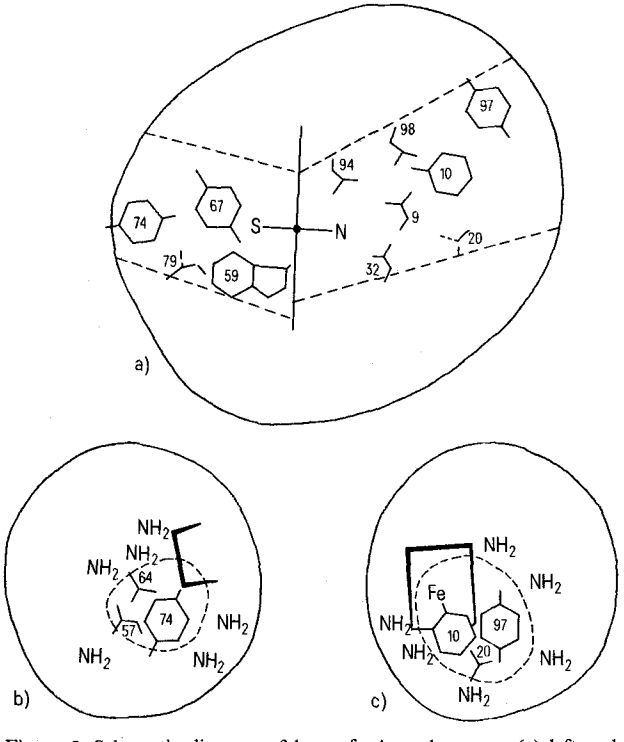

Figure 5. Schematic diagram of horse ferricytochrome c: (a) left and right channels viewed from the front of the molecule; (b) left channel viewed from the left side of the molecule; (c) right channel viewed from the right side of the molecule. Dotted lines: boundaries of the channels[81].

All cytochrome c molecules possess many common features including the common folding and predominance of positive charges in the vicinity of the heme edge. This leads to the idea that electron transfer would take place in both directions via the exposed heme edge, which must be properly positioned by interaction between the surrounding positively charged lysine side chains and complementary negatively charged groups on the electron donor or receptor molecule. A specific fit between the donor and acceptor molecule has, therefore, been postulated to define the functional role of cytochrome c and the mechanism of electron transfer between them. The case of the interaction between cytochrome c and its peroxidase is particularly interesting: a model[86, 86a] based on individual crystal structures of both proteins (yet not on crystal structure of the complex itself) suggested that aspartate 37, 79 and 219 on cytochrome c peroxidase appears to form a triangle of negative charges which neatly fits together with the positive charges of lysine 13, 27 and 72 on cytochrome c. Furthermore, 4 additional coulombic and hydrogen-bonded side-chain interactions are involved in this complex formation. The combined structure contains no empty spaces or gaps between the 2 component molecules, and the molecular interface contains a number of aromatic and conjugated groups whose planes are parallel to the heme-heme plane, i.e. the side chains of His-80, Trp-51 and Arg-48 in cytochrome c peroxidase and of Phe-82 in cytochrome c. The heme edges of the 2 proteins are certainly not in contact but are separated by a distance of 16.5 Å. A similar study with cytochrome b_5 and cytochrome c showed that they fit together reasonably well, with the 2 heme groups also almost parallel. The juxtaposition of parallel aromatic or conjugated systems in the interacting domains, including their hemes, permits overlap of adjacent orbitals and thus creates a low-lying 'supramolecular' conduction orbital extending over both protein molecules[87].

Ascorbate reduction of cytochrome c supports this view[88]: the reaction occurs in a 3-step process described as the loosening of the heme crevice opening, the solvent exposure of the polypeptide backbone and the disruption of the tryptophane-prophyrin interaction, respectively. The ascorbate reduction of the protein is independent of the state of the heme crevice opening and of the polypeptide organized structure. Instead, it is determined by the integrity of the tryptophan-indole-porphyrin interaction. It has been suggested that Arg-38 is the binding site and that the electronic interaction between the indole of Trp-59 and the porphyrin moiety must constitute, at least in part, the electron transfer path to the heme iron. Reaction of cytochrome c with Cr(III)-ions showed possible electron transfer path via spatial overlap of electron orbitals of chromous ions with the d-orbitals of S in the thioester bridge of Cys-17, but not directly from chromium (III) to heme group, so that reductive electron transfer occurs via the protein moiety[89].

As a means of understanding the in vivo mechanism of electron transfer by cytochrome c, the mechanisms of its redox reactions with inorganic reagents have been investigated in aqueous solutions[90-96]. The results of

such investigations show that 2 modes of electron transfer mechanism are possible with cytochrome c, viz., the adjacent attack mechanism typified by the reduction of the metalloprotein by chromous and dithionite ions. In this mechanism, the electron transfer is governed by a rate-limiting event in the protein such as the opening of the crevice or substitution on the iron center. The alternate mechanism is the remote attack in which electron transfer occurs via the exposed edge of the heme to the metal ion. The latter path has been suggested for homogeneous reactions of ferricytochrome c and simple inorganic reagents. A characteristic feature of the redox reactions of ferri- and ferrocytochrome c is the complexity of the hydrogen ion dependence for both oxidation and reduction rates[94,97,98,224]. For instance recent studies of the chromium (II)-reduction of cytochrome c in acidic media, led to the conclusion that the reactivity sequence for the adjacent mode of electron transfer for the 3 identified electronic isomers of this protein is low-spin > high-spin > mixed-spin[97]. It was also speculated that the relative rates for the mixed- and high-spin species may be inverted in electron-transfer by the remote attack mechanism. Reduction of cytochrome c by $Ru(NH_3)_6^{2+}$ showed that electron transfer from this ion proceeds by the remote attack mechanism[90]. The reaction rates of the different electronic isomers of the protein have been measured[114] and the same complex dependance on pH was observed. The electron transfer from the reductant to the ferric atom in the protein is supposed to proceed by the heme-edge mechanism. This study suggested that the reactivity for the reduction of the acid-induced 'electronic' isomers of cytochrome c by the heme-edge mechanism is low-spin > mixed-spin > high-spin, (as opposed to that observed in the reduction by chromous ions, a reaction that proceeds by the adjacent attack mechanism). For all reactions in aqueous solutions, an optimum pH of reactivity exists between pH 3 and 4. This is interpreted as implicating a single protonated cytochrome c as the most reactive form of the protein. Physiological reactions of this protein thus might be preceded by reductase/oxidase-sponsored pH-induced conformational change in order that electron transfer to and from cytochrome c might proceed via a path of minimum activation barrier. It has been proposed that the physiological reduction of cytochrome c could be facilitated by protonation of Tyr-78 by an acidic function on the reductase[99].

An important approach in the study of electron transfer in cytochrome c is the use of chemical derivatives of the protein. In some derivatives of cytochrome c the modification does not change the overall conformation net charge and reactivity of the protein but in some cases these changes can be significant[100]. Thus the reduction of acetylated, fully succinylated and dicarboxymethylated cytochrome c by the radicals $CO_2^{\cdot-}$, $O_2^{\cdot-}$ and e_{aq}^- have been reported, together with the oxidation of the reduced cytochrome c derivatives with $K_3Fe(CN)_6^{3-}$, as studied by the pulse radiolysis technique as a function of ionic strength[102]. The observed order of reactivity shows a diminished rate constant where the redox potential differences between the protein and reducing agent become larger, thus demonstrating the overriding influence of progressively greater heme edge exposure upon reactivity, since the redox potential of the derivative is an indicator of an open conformation in the region of the heme crevice or edge. According to Marcus theory, an increase in the reaction rate is expected as the difference in redox potential between the reactants increases[101]. Thus it seems that conformational effects play an important role upon the observed kinetics[102]. The positive charge of cytochrome c also plays a major role in this interaction with biological redox donors or acceptors[103], particularly certain positively charged lysine groups necessary for the binding of cytochrome c to cytochrome c oxidase[104] or cytochrome c reductase (Complex III)[266,267].

The reaction of cytochrome c with Fe-hexacyanides has been investigated by many workers[105–110]; however, the rate constants of the internal electron transfer has only recently be measured directly[111] and a method for calculating the rate constants of the interconversion between ferricytochrome c-ferrocyanide and ferrocytochrome c-ferricyanide has been developed. The dynamic behavior of various types of cytochrome c in the redox reaction with Fe-hexacyanide has been studied by the temperature-jump method in order to elucidate the molecular mechanism of the redox reaction of cytochromes with their oxidoreductants. Comparison of various cytochromes showed that the environment of the electronic pathway is not necessarily conserved throughout the molecular evolution[111]. It also appears that the Fe-hexacyanide-cytochrome c complex must be formed before electron transfer takes place[112,113].

The binding and electron transfer processes have been reported, giving both the oxidation electron transfer rate and the binding rate parameters. A deuterium effect of similar magnitude to that found in the cytochrome reactions in photosynthetic bacteria was detected also[114], suggesting that water rotation may be involved in both the binding of ferricyanide to the reduced cytochrome c and the subsequent oxidation electron transfer[115].

A near-zero activation energy in oxidation of reduced cytochrome c by ferricyanide has been firmly established[116]. Thus the energy for release or uptake of an electron at the iron center can come from the vibrational energy of the protein self and it seems quite probable that the conformational mobility of the protein may assist not only in the electron-transfer process itself but in the control of the process (gating) as well. Modification of lysines residues – which removes the sites for binding ferricyamide and eliminates surface positive charges – does not affect the activation energy that still remains near zero, nor is the electron transfer rate affected upon treatment of the protein with 4 M guanidine hydrochloride (which relaxes the binding of ferrocyanide to the reduced molecule without completely denaturing the cytochrome c). These facts support the notion that conformational changes are important in the electron transfer function of cytochrome c. The influence of temperature on the reduction of ferricyanide by cytochrome c shows that the apparent rate constant of the bimolecular reaction is pH dependent over a large range of temperatures and is unaffected by addition of dioxygen radicals scavenagers in the solution. Ionization of the His-residue nearby the heme during

electron transfer has been ruled out from calculation of the heat of activation of reduction, together with the acativation entropy and the free activation energy in relation to pH[117].

The reactivity of cytochromes with a variety of oxidants and reductants has shown that the requirements of the Marcus theory for outer sphere electron transfer are not met by most biological redox systems[118, 236], and that there is a substantial measure of reactivity which emanates from the specificity of interaction between oxidant and reductant. The microscopic details of the process of electron transfer in these proteins are unclear; there are frequent suggestions for a special role of the protein in affecting electron transfer though there does not appear to be any unequivocal evidence for this viewpoint[118]. Furthermore, in most systems the redox active center is completely insulated from the environment of the protein and thus one wonders whether 'long range' electron transfer can occur, and whether or not the protein per se can have any role in mediating the transfer process[119].

Some attempts have been made[120, 204] to study the properties of cytochrome c in systems where oxidant and reductant are separated by a lipophilic barrier in order to mimic a more physiological situation. Resealed erythrocyte ghosts have been prepared in which the only discernible pigment is cytochrome c. The internal cytochrome c participates in redox reactions with both soluble and insolubilized cytochrome c present externally and with external cytochrome b_5. However, no reaction is observed with plastocyanin, cytochrome oxidase or NADPH-cytochrome c reductase. When reductants of low molecular weight, such as ascorbate or glutathione, were used, complex kinetics were observed. It appears that ascorbate either binds weakly or is transported by a carrier into the erythrocyte; the external ascorbate is in rapid equilibrium with a species in the membrane that seems able to dissociate a dimeric membrane protein into its monomeric form by means of cleavage of a disulphide linkage. Thus there is a possibility that undefined species are present in the membrane that are capable of transferring reducing equivalents across the bilayer. These components react with certain metalloenzymes such as cytochrome c and cytochrome b_5 and not at all with others (plastocyanin). In order to examine the influence of protein perturbations on the prosthetic group and on the rate of electron transfer, Raman difference spectroscopy has been useful[121] in determining whether such perturbations are caused by binding to enzymic reaction partners or small ions or by changes in primary, secondary, tertiary or quaternary structure. In heme protein this technique is most sensitive to the electronic structure of the heme which has a direct bearing on reduction potentials, ligand affinities and electron transport properties. For cytochrome c it has been shown that aromatic and highly electronegative residues near the heme are important for determining the orbital electron density of the prosthetic group. The mechanism by which such residues modify the electronic structure could be some heme-amino acid side chain interactions such as charge transfer, Van der Waals and electrostatic interactions, that may have some regulatory effect on the function of the protein[121]. Such a control of the electronic properties of the heme by the protein may be important for the function of cytochrome c.

A heme undecapeptide from horse cytochrome c has been proposed as a model complex for comparison with cytochromes[257]. Molecular models indicate that the heme undecapeptide can shield no more than a quarter of the heme from the solvent. At high dilution, low-spin forms are stabilized and redox properties have been studied long ago[122]. Electron exchange has recently been observed and compared to self-exchange rates of different cytochromes[123, 124].

A fundamental question that has frequently been raised concerns how a single prosthetic group, like heme, can exhibit such a wide range of redox potentials and diversity of functions. Essentially the same heme group occurs, first of all, in the b-type cytochromes and cytochrome c_1 of Complex III, in the a-type cytochromes of Complex IV and in cytochrome c itself, all part of the electron transport chain. Then, in addition, the heme group is involved in oxygen binding by the globins, in peroxidation reactions by peroxydase and catalase and in hydroxylations by cytochrome P-450 and in numerous electron transport systems in bacterial organisms. The cytochromes c are by far the most thoroughly characterized components of the biological electron transport chain, and possibly the most thoroughly studied class of proteins of any kind. The X-ray structures of 6 distinct kinds of cytochrome c have recently been reported[125, 126]. These studies show that the c-type cytochromes, according to the obvious similarities of their detailed architecture, constitute a highly conservative class of proteins[127].

Hemoglobin and myoglobin

These oxygen transport and storage proteins fulfill most requirements for electron transport capability. As with other cytochromes (cytochrome a_3, b_5, P-450 and c') the heme groups of metmyoglobin and methemoglobin are in equilibrium between high- and low-spin forms, a fact relevant to electron transfer[128]. Kinetic studies of spin interconversions in ferric mixed-spin derivatives, performed by the coaxial-cable, temperature-jump method, showed a single relaxation, observed around 3 μs (except for the fluoride and aquo-derivatives)[258]. An estimate of the rate constants and activation parameters for the spin interconversion can be correlated with synthetic iron complexes and data on other hemoproteins. Upon binding of CO to hemoglobin, electron spin tunneling has been observed[129]. A nonadiabatic quantum tunneling process has been investigated as the mechanism for effecting the electronic spin change of the hemoglobin's iron upon the binding of carbon monoxide. As the carbon monoxide approaches, there is a spin state change in the Fe^{2+} from $S = 2$ to $S = 0$. The Born-Oppenheimer approximation was used to separate the recombination of the CO to the iron in the heme into a nuclear tunneling and an electronic tunneling term. A non-adiabatic Landau-Zener state-to-state tunneling was assumed based upon the energy splitting of the 2 states in the transition region and the size of the tunneling matrix elements. The tunneling rate can be changed

by changing the spin magnetic sublevel of the initially CO-unbound iron, e.g. by applying a strong magnetic field. This theory for a magnetic field dependence to the recombination rate of carbon monoxide to hemoglobin has been tested[129]. Binding of ligands to heme proteins is regulated by a series of barriers. A CO molecule, for instance, encounters 4 different barriers on moving from the solvent to the binding site at the heme iron. The innermost barrier has been described by an activation energy spectrum, rather than by a single activation energy; it thus appears that the heme proteins exist in many different conformation states with different activation energies, characteristic for the proteins. At low temperatures, the heme molecule is into a particular conformational state and the energy spectrum gives rise to nonexponential kinetics with rebinding becoming temperature independent at the lowest temperatures as a consequence of molecular tunneling through the barrier at the active center[134]. This quantum-mechanical molecular tunneling seems to correlated with a possible electron-transfer pathway on the protein[129,134]. The involvement of histidine-GH1 in the process of oxidation of sperm whale myoglobin has been recently reconsidered and a mechanism for electron transfer from myoglobin to an external acceptor has been presented[131]. By means of using measurements over the widest possible range of temperatures and times, unexpected and interesting results showed that the kinetics of electron transfer and substrate binding extends over many orders of magnitude in time[132].

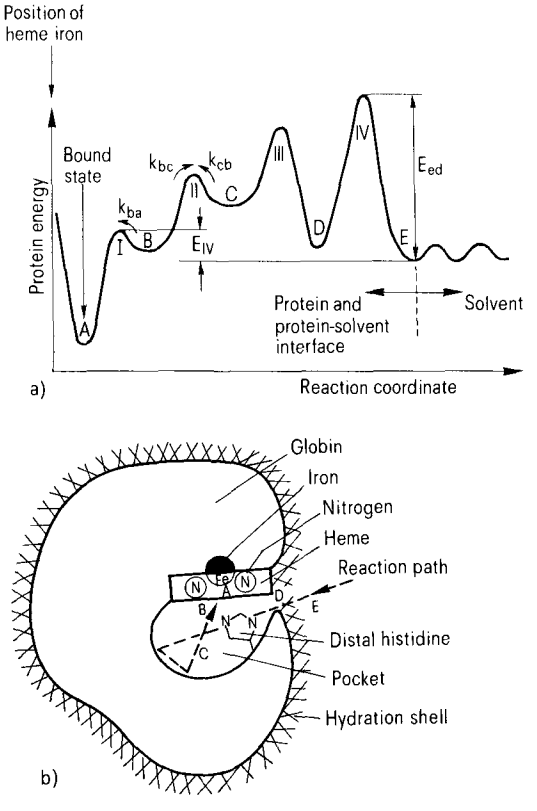

Figure 6. *a* Barrier which carbon monoxide molecule encounters on way from solvent to ferrous ion binding site. *b* Pictorial representation of structural features responsible for barrier of (a)[133].

This was explained by postulating the heme protein to exist in many different conformational states, with different activation energies. Activation energy spectra have been obtained for different heme proteins and are characteristic of the proteins[134]. Furthermore, at low temperatures, each heme protein molecule is 'frozen' into a particular conformational state and the energy spectrum gives rise to nonexponential kinetics, whereas at high temperature, each molecule changes rapidly from one conformational state to another (conformational relaxation) and binding becomes exponential (fig. 6). At lowest temperatures, rebinding becomes temperature independent, resulting from molecular tunneling through the barrier at the active center. The barrier width, estimated from the observed tunneling rate, agrees with the displacement of the iron upon ligand binding as measured by x-ray crystallography and neutron scattering[134]. Intermolecular electron transfer in hemoproteins upon interaction of hemoglobin with cytochrome b_5 showed direct involvement of lysyl residues in the electron transfer process. The 2 proteins, methemoglobin and cytochrome b_5, are believed to form a complex whose bonding is principally determined by complementary charge interactions between acidic groups of cytochrome b_5 and basic groups of hemoglobin. This binding then favors the electron transfer reaction between the two heme moieties via lysine residues β-66 and β-95 of the hemoglobin[135].

Tryptophan pyrrolase

Another well-characterized hemoprotein, tryptophan-2,3-dioxygenase or pyrrolase catalyzes a rupture of the pyrrole ring of tryptophan. This hemoprotein contains protoporphyrin IX as its sole prosthetic group[229]. Iron-protoporphyrin IX (the prosthetic group of hemoglobin and myoglobin) plays an important role in dioxygenation, mixed-function oxydation and peroxidatic or catalatic oxidation. The current hypotheses, based on the recent kinetic experiments, is presented in figure 7[226]. The figure is obviously an oversimplification but may be useful as a point of orientation for discussing the reaction mechanism. First, the native ferric enzyme binds with the substrate which is probably a very fast

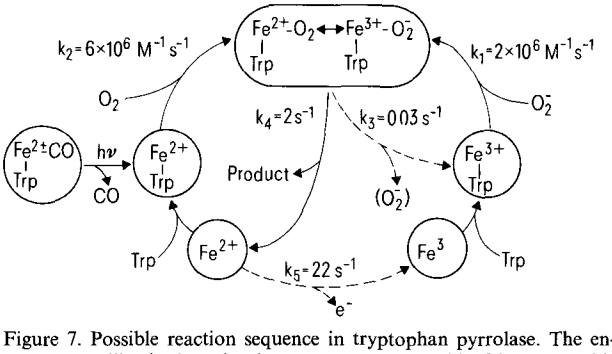

Figure 7. Possible reaction sequence in tryptophan pyrrolase. The enzyme can utilize both molecular oxygen or superoxide. The superoxide anion is absolutely essential in order to maintain the steady state of the reaction. The charge transfer complex of heme-oxygen is envisaged as the 'activated molecular oxygen' in these reactions[229]. See text for explanations on the reaction mechanism.

process; then the ES complex reacts with superoxide to form a ternary complex (identical with the ferrous oxygen substrate complex). This charge transfer complex is also produced by the binding of molecular oxygen with the ferrous form of enzyme and substrate. Once the ternary complex is produced, it decomposes to yield the reaction product, generating a ferrous form of enzyme. The first order rate constant for this reaction is estimated to be 2.0 s^{-1}. Thus, during the steady state of the reaction cycle, K_4 is the rate determining step and is in good agreement with the turnover number of the enzyme. During the catalytic cycle, however, the enzyme is slowly oxidized to the ferric form which, in the absence of the superoxide anion, remains inactive. Thus the enzyme can utilize both molecular oxygen and the superoxide anion. In order to maintain the steady state of the reaction, the superoxide anion is absolutely essential, and the supply of O_2 is the rate determining step in the overall process.

Fe(II)porphyrin has been shown to be a good model-system of the active-site of L-tryptophan pyrrolase[136]. This model complex effectively catalyzes the oxygenation of 3-substituted indole to form products corresponding to formylkynurenine (the oxygenation product of L-tryptophan). EPR analysis of the system indicates that *cooperative* electron transfer occurs from the substituted indole anion to the oxygen in the ternary system (indole anion-Fe(II)-porphyrin-dioxygen).It appears that no electron transfer occurs between a skatole anion and Fe(III)-porphyrin in the binary system at room temperature; however, under the same conditions, electron transfer from skatole anion to oxygen occurs in the ternary complex. Thus a specific activation mechanism of molecular oxygen in the Fe(II)-porphyrin-catalyzed oxygenation of skatole takes place. When the electron affinity of the acceptor is less than that of molecular oxygen ($E'' = 0.43$ eV), the electron donating power (ionization potential) of the donor must be as large as that of skatole anion for cooperative electron transfer to occur. This type of electron transfer is a new concept that should occur in any strong donor-Fe(II)-porphyrin acceptor system. Experimental data of the oxygenation mechanism of tryptophane and skatole has been discussed in terms of *cooperative* electron transfer[136]. It has been suggested that oxygen activation in cytochrome P-450 and the mechanism of action of bleomycin (an anticancer agent) might be explained by a similar concept of cooperative electron transfer (fig. 8).

Cytochrome P-450

Cytochrome P-450 is an unique heme protein due to its ability to hydroxylate organic substrates containing either carbon, nitrogen or sulfur atoms and to its optical properties, and was discovered because of the unusual Soret peak position of its CO-complex which appears at around 450 nm, red-shifted by about 30 nm from those of other heme proteins[137, 138]. The spectral features of P-450 have been attributed to the coordination of a cysteine thiolate axial ligand to the heme iron[139]. The presence of a cysteinate axial ligand has been established for ferric P-450 in the substrate-free, 6-coordinate, low spin[140, 141] and the substrate-bound, 5-coordinate, high-spin forms[142] and for ferrous P-450 in the CO-bound form[143, 144]. Anionic ligands coordinating to the P-450 heme iron form exclusively low spin complexes probably due to the large ligand field strenght of the cysteine thiolate such that all 6-coordinate P-450 adducts are enforced to be low spin regardless of how weak the ligand field strength of the trans ligand[145, 239], in strong contrast to the results with myoglobin where spin mixture varying from 100% to 0% low spin are seen. Factors controlling the steady-state concentration of high-spin ferric cytochrome P-450 and oxy-cytochrome P-450 have been studied by varying the pH of the medium. The oxygenated hemoprotein exhibits an enhanced negativity of its redox potential with increasing pH relative to that of the electron donor and this results in an interference with its further reduction to peroxy-cytochrome P-450. An attenuation of the turnover of cytochrome P-450 below the pH optimum for its monooxygenase and oxidase function is paralleled by an increase in ferric-cytochrome P-450 concentration in its substrate-complexed state. The reactions associated with the transfer of the first and second electrons of this system thus are controlled differentially in an opposing pH-dependent manner, involving a 'conterpoise'-regulation of the electron transfer reactions required for the activation of dioxygen[145]. Flash photolysis has been used to initiate electron transfer from ferredoxin to cytochrome P-450, in order to study the kinetics of electron transfer and product formation

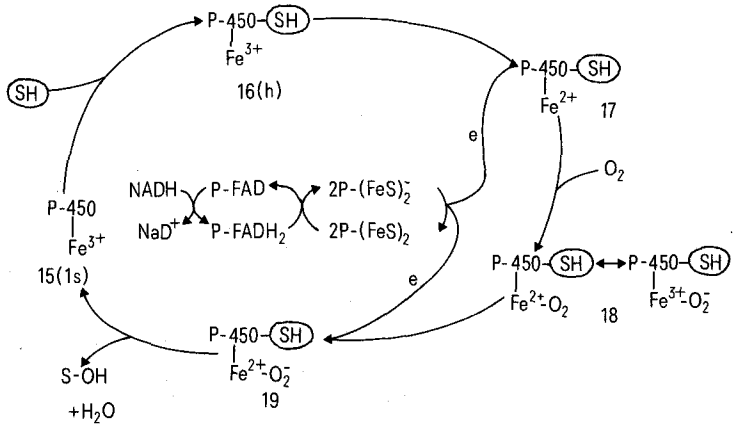

Figure 8. Reaction cycle in cytochrome P-450 (SH = camphor), as a model for cooperative electron transfer[136]. The membrane enzyme P-450-Fe^{3+} (15) is a low-spin (hexacoordinated) complex; through the incorporation of camphor as the substrate (SH), the spin state of P-450-Fe^{3+} SH (16) becomes high-spin. Its one-electron reduction with reductase systems gives the reduced form, P-450-Fe^{2+} SH (17), which is easily oxygenated with molecular oxygen to form P-450 Fe^{2+} O_2 SH (18). Further one-electron reduction of 18 should yield P-450-Fe^{2+}-O_2-SH (19), which should give the hydroxylated camphor (S-OH) and 15.

in the camphor hydroxylase system of *Ps.putida*[147]. Cytochrome P-450 has been shown to transfer electrons to cytochrome b_5. When incorporated into phospholipid vesicles containing NADPH cytochrome P-450 reductase and cytochrome P-450, cytochrome b_5 enhances about 5-fold the rate of hydroxylation of substrates, although it does not affect the rate of oxidation of NADPH oxidation itself. The cytochrome b_5-mediated increase in product formation strongly indicates that cytochrome b_5 is an efficient donor of the second electron to cytochrome P-450[148]. Electron transfer between cytochrome P-450 and cytochrome b_5 was investigated by difference and stopped-flow spectrophotometry. Both cytochromes were reduced photochemically, thus avoiding interference by the corresponding reductases or pyridine nucleotides. Formation of a 1:1 complex between ferrous cytochrome P-450 and cytochrome b_5 was a prerequisite for electron transfer between the two. However, formation of a 1:1 complex was observed only when both cytochromes were incorporated into micelles of phosphatidylcholine. The kinetics of reoxidation of either one of the cytochromes) showed that the rate of electron transfer from cytochome b_5 to oxyferrous cytochrome P-450 was increased about twice in the presence of benzphetamine. This study provides the first quantitative data on electron transfer between cytochrome b_5 and cytochrome P-450[149]. Reduction of cytochrome P-450 requires the presence of its reductase; a flavoprotein FMN-depleted cytochrome P-450 reductase does not catalyze rapid electron transfer to cytochrome P-450[30]. However, this activity could be restored when the enzyme is reconstituted either with FMN or with artificial flavins (with the exeption of 5-deazaflavin). The rate of cytochrome P-450 reduction by its reductase reconstituted with functional analogs such as $8\text{-}Cl^-$ and iso-FMN differs from that of the native enzyme such that a role of FMN in the transfer of electron to oxidized cytochrome P-450 via the reaction $FMNH_2$-FMNH has been proposed[150]. The investigation of the routes of electron transfer from NADH or NADPH to cytochrome p-450 have been attempted through immunological studies[206]. It now seems that stabilized radicals, like lipido-soluble imido-oxile radicals, are substrates of cytochrome P-450. The electron transfer of reduced cytochrome P-450 to such radical substrates together with the distance between a thiol-group bound radical and the Fe^{2+} in the active center has been reported[151].

Cytochrome c_3

Among the multitheme proteins, the cytochrome c_3 form a unique class of electron-carriers functioning in the anaerobic sulfate-reducing bacteria of *Desulfovibrio* species. In the electron transfer from the initial electron donor (H_2) to the final acceptor (SO_4^{2-}), cytochrome c_3 plays a key role in accepting electrons from hydrogenase and transferring them to the redox partners of the chain, such as rubredoxin or ferredoxin. The protein contains 4 c-type heme groups (with iron-axial ligands being histidyl side chains as in cytochrome b_5) with several non-identical, very low mid-point potentials[152]. A number of spectroscopic studies have been undertaken[153-156] in order to gain information about the function of these proteins and the electron pathways. These studies suggest that electron-transfer within cytochrome c_3 is governed by intra- and inter-molecular heme-heme interactions, involving close contacts between pyrrole rings of neighboring heme groups to give rise to an overlapping system of π-orbitals that favors direct electron transfer between some of the redox centers[157]. The folding of the molecule brings aromatic side chains close and between the hemes which promotes π-π interactions with adjacent porphyrin moieties and phenylalanine or cysteine residues, according to the following scheme:

heme 1 → Phe 88 → heme 4 → cys 61 → heme 3
→ Phe 34 → heme 2 → cys 114 → heme 1 - ...

The suggested pathway is quite similar to those already made for mono-heme cytochrome c[158,159]. Detailed kinetic studies[160] on cytochrome c_3 showed that the 4 hemes are equivalent two by two with regard to intramolecular electron transfer, in contrast to structural and NMR studies. The network of interlocked heme clusters in cytochrome c_3 might well be responsible for their high conductivity and their strong reactivity towards hydrogenase[161].

Cytochrome c-552

This protein is an unusual heme protein in that it contains 2 hemes and 1 flavin per molecule[162]. The question as to whether intramolecular electron transfer occurs in this protein was investigated by using pulsed-laser exci-

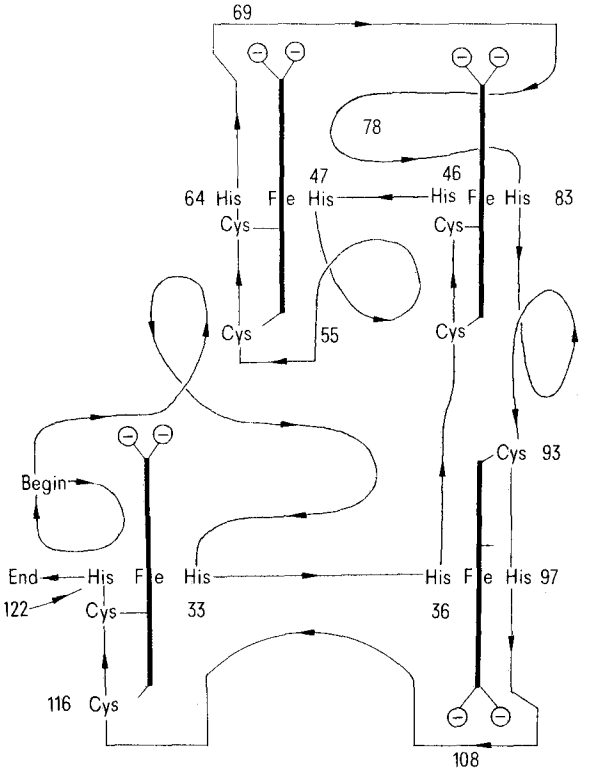

Figure 9. Outline structure of *Desulfovibrio vulgaris* cytochrome c_3[154].

tation and by measuring reduction kinetics on the microsecond time scale. Both the flavin and heme moieties of cytochrome c-552 are reduced simultaneously on a millisecond time scale with the transient formation of a protein-bound flavin anion radical, probably due to rapid intramolecular electron transfer. Steric restrictions plays an important role in the reduction reaction. The redox process following photolysis of CO-ferrocytochrome c-552 in which the flavin has been partly oxidized facilitated resolving the kinetics of intramolecular transfer from ferrous heme to oxidized flavin[163]. Studies indicated that the heme and flavin moieties are oriented to permit interactions between them, thus allowing rapid electron transfer between chromophores[164]. The electron transfer between the heme and flavin moieties reported for that system is among the most rapid of any biological systems in which electron transfer occurs between the normal ground-state forms of electron carriers. However, in the absence of any structural information nothing can be said concerning the distance or relative orientation of the chromophores[203].

Cytochrome b_2

The mechanism of electron transfer in this very interesting system has been the matter of controversy[165-167]. This protein which contains at least 2 domains (a heme-binding and a flavin-binding domain) transfers electrons directly to cytochrome c after abstracting a hydrogen from the substrate[167]. Three kinds of electron transfer have been postulated: 1) substrate (i.e. L-(+)-lactate) to FMN, 2) FMN to protoheme IX of the same monomer and 3) FMN to FMN between 2 promoters, with the first one being rate limiting[168]. A high isotope effect enabled the study of electron transfer between prosthetic groups at a very low rate of electron entry. On the basis of these data, a new scheme has been proposed for the intramolecular electron transfer[169] that postulates that the limiting step of the slow phases of heme and flavin reduction is a slow interprotomer electron exchange between a heme pair, a flavin pair or heme and flavin, and that fast conformational change controlled by the redox state of heme or flavin of one protomer can modulate the rate of electron transfer in another protomer. The rate of intra- and interprotomer electron transfer has been determined[169]. The reactivity with electron acceptors of reduced cytochrome b_2 has been presented[170]. Cytochrome c can withdraw an electron from the reduced (3-electron donor) flavo-cytochrome b_2 only at the heme site. The donor heme site is then supplied with 1 of the 2 electrons pertaining to the hydroquinone from flavin site by means of an intramolecular electron transfer from flavin to heme b_2, such that a second, then a third molecule of cytochrome c may come in turn, taking electrons one by one. Additional cytochrome c molecules can react only if new rotations of the enzyme have occured, at the expense of the reducing substrate[170].

Cytochrome b-563

The redox behavior of cytochrome b-563 in chloroplast is suggestive of the cytochrome participating in a protonmotive Q-cycle[171]. The role of cytochrome b-563 in chloroplast electron transfer has been investigated recently by a kinetic study of the electrochromic shift, proton release and the redox changes of cytochrome f and b-563 upon inducing cyclic electron flow by addition of NADH and ferredoxin[172]. Under these conditions, the observed kinetics seems to be consistent with a linear arrangement of the electron pathway[173].

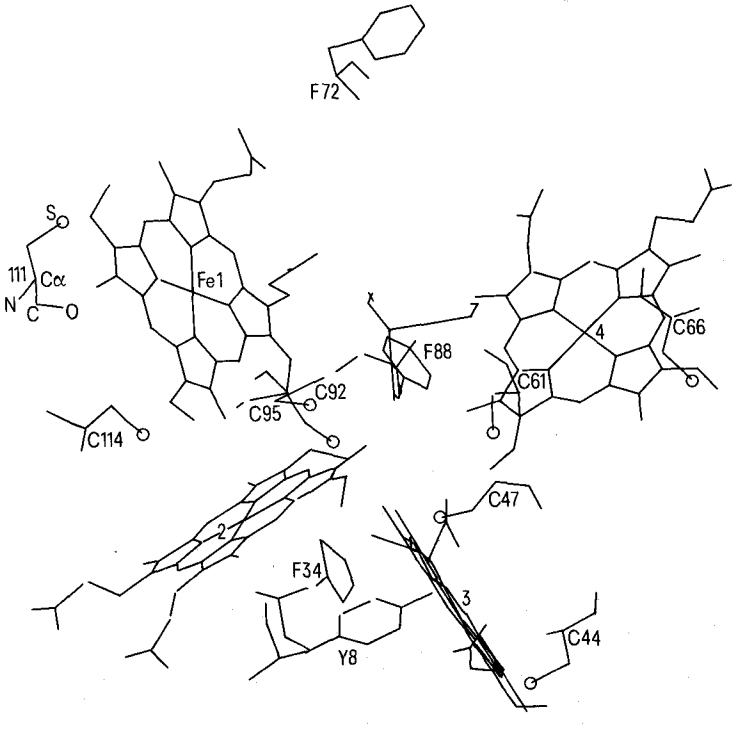

Figure 10. The heme cluster with the nearby aromatic side chains and cysteine ligands in multiheme cytochrome c_3. (His-Fe ligands have been omitted)[157].

Ferredoxin → b-563 → plastoquinone → f

and tends to favor a scheme in which cytochrome b-563 participates in a proton-motive Q-cycle. However, since there is no evidence for the release of more than one proton per electron transferred, other explanations have been proposed[173] suggesting that cytochrome b-563 acts as a plastoquinone dismutase instead of a vectorial transport system of protons. The rate of electron transfer of this reaction is slightly higher for monomeric cytochrome f than with the oligomeric cytochrome form of the same protein.

4. *Cu proteins*

All of the copper proteins, apart from the storage proteins called thioneins, are involved, either directly or indirectly, in the electron transfer to dioxygen. Table 2 includes the functional classes of these proteins with reference to this specialized biological role. The precise mechanism by which this general function is fulfilled and the mechanism of electron transfer, however, have to be defined in every copper protein. Some aspects of these studies have been recently reviewed[174, 175] and critically discussed with respect to biological electron transfer between metalloproteins.

Stellacyanin

The electron transfer reactions between *Rhus vernicifera* stellacyanin and either horse cytochrome c or *Pseudomonas aeroginosa* cytochrome c-551 were investigated by rapid reaction techniques. The time course of electron transfer was monophasic under all conditions and thus consistent with a simple formulation of the reactions[176]. Both stopped-flow and temperature-jump experiments yielded equilibrium constants in agreement with values calculated from the redox potentials. These results could be understood within the framework of the Marcus theory. Investigation of the structure of this protein by proton NMR spectroscopy showed little conformational change on reduction of the protein or on removal of the copper. In the aromatic region of the spectra of the holoprotein, resonance associated with 2 freely titrating histidine-residues have been observed[177], together with 2 additional sharp resonances (assigned to 2 additional histidine residues). This requires that no more than 2 histidine residues can be ligands, since there are only 4 such residues in the protein. Methionine is absent[177] and this may be one of the possible causes for the difference between stellacyanin and other blue-copper proteins. A structure for the copper-site in stellacyanin has been proposed. Similar NMR studies were reported on the interaction of plastocyanin with Cr(III) analogs of inorganic electron-transfer reagents[178]. A high degree of specificity in the binding of these complexes (e.g. hexaminechromium (III)-nitrate, tris-(1-10-phenantroline)-Cr(III)-perchlorate and potassium hexacyanochromate (III)) with 2 binding sites of the protein was observed; 1 site close to the copper-atom was clearly suited for outer-sphere electron transfer through one of the histidine-residues. The other binding site was more distant from the copper-atom and this mechanism could not be operative. Electron transfer via hydrophobic channels or electron-tunneling were thus suggested possible mechanisms[178].

Azurin

Azurin as isolated e.g. from *Paracoccus denitrificans* is a single polypeptide of molecular weight 13,790 which contains 1 copper molecule per mole and shows spectrum typical of type I blue-copper proteins with an intense band at 595 nm. Recent studies on that protein showed 5-fold greater electron transfer activity with membrane fragments than with the solubilized nitrate reductase[179]. This fact argues against the latter as being the primary physiological oxidase system for azurin (as previously proposed). The peculiar ESR spectrum of this protein (with a low A_{II} value and several effects contributing to the hyperfine interaction) has been correlated with optical and NMR results[180] and suggests the presence of distorted tetrahedral binding site with a strongly reducing ligand (possibly cysteine) while the

Table 2. Functional classes of copper proteins

Classes of functions	Cu proteins	Analogous Fe proteins Heme	Non-heme
I. Electron transfer from protein to O_2 without separation of charge (oxygen binding)	Hemocyanin	Hemoglobin	Hemerythrin
II. Electron transfer from protein to O_2 with separation of charge (oxygen reduction)			
1. with liberation of all O_2 as:			
1a. H_2O_2(H_2O_2-forming oxidases)	Amine oxidase, galactose oxidase		
1b. H_2O(H_2O-forming oxidases)	Laccase, coeruloplasmin, ascorbate oxidase, cytochrome oxidase		
2. with insertion of at least 1 atom of O_2 into an organic molecule (oxygenases)	Tyrosinase, dopamine-hydroxylase	Tri-oxygenase, cyt P-450	Many
III. Electron transfer between reduced O_2 species (O_2^- or H_2O_2-dismutases, peroxidases)	Superoxide dismutase	Hydroperoxidases	Superoxide dismutase
IV. Electron transfer between proteins (non-autoxidizable e^--transferase, involved in e^--transport chains with O_2 at one end)	Azurin, plastocyanin, stellacyanin, umecyanin, rusticyanin	Cytochromes	Ferredoxins

NMR work implicates 2 histidine ligands in the copper binding site[180]. Assignment of resonances in the ^{1}H-NMR spectra of copper-(I)-azurin to proton group in the protein has been attempted. Comparisons drawn between copper(I), copper(II), apoHg(II) and cobalt(II) azurin samples and redox titration of copper(I) azurin with potassium ferricyanate were used to correlate the NMR-spectal features. Observed-line broadenings derived from copper(II)-paramagnetic effects were used to deduce the distances of assigned proton groups from the copper-center. Also histidine-residues were characterized, together with rates of acid-base exchange near the pK and rates of C-^{2}H exchange with solvent ^{2}H. Thus a small number of NH-protons distinctively inert to ^{2}H with solvent ^2H_2O in the copper(I) protein showed increased lability on removal of the metal[181]. Azurin and plastocyanin[215] might have similar tertiary folding, with some exceptions[127]. The copper atoms are coordinated in a similar way through 2 histidine residues, 1 cysteine and 1 methionine[182]. An outer-sphere mechanism of electron transfer has been postulated[183] by studies of the reactivity of several blue-copper proteins with $[Ru(NH_3)_6py]^{3+}$. It seems that pyridine penetrates the protein by contact with a phenylalanine residue. However, a hydrophobic region in the protein has also been suggested as a cadidate for a tunneling mechanism[184], leading a channel that starts at the surface near a tyrosine residue and permits access to the copper.

Laccase

Laccase is an oxidase that catalyzes the single-electron oxidation of diphenols or arylamines at the expense of the 4-electron reduction of dioxygen to water. Three different types of copper redox sites, characterized by different optical and EPR properties are involved in the catalytic mechanism of the enzyme[185]. The type-I Cu^{2+}, absorbing near 610 nm, was recognized as the primary electron-acceptor from the reducing substrate; the type 3 copper site, absorbing around 330 nm, was suggested to interact in its reduced Cu^+-Cu^+ state with dioxygen. Reduced type-1 and -2 sites contribute 1 electron each to the reduction of the 2-electron accepting type-3 site[186]; H_2O_2 can interact with type-2 copper in fungal laccase and with type-3 copper of the lacquer-tree enzyme, whereas type-2 Cu^{2+} can bind at least 1 water molecule. The role of the type 2 copper is rationalized in figure 12. Pecht et al.[187] have shown that the solvated electron, e^-_{aq}, a potent reducer, interacts primarily with the polypeptide backbone and amino acid residues (aromatic and disulfides) to form adducts. In a subsequent step, electron transfer takes place from these radicals to type-1 Cu^{2+} leading to the reduction of this copper atom. Another study[188] has investigated whether these findings for e^-_{aq} would apply to other radicals such as CO^{2+} (a reducer), O_2^- (either a reducer or an oxidant), or $OH^{\cdot}$ (a strong oxidant). It appears that the reduction of type-1 copper can be described as proceeding either by intramolecular electron transfer from the polypepti-

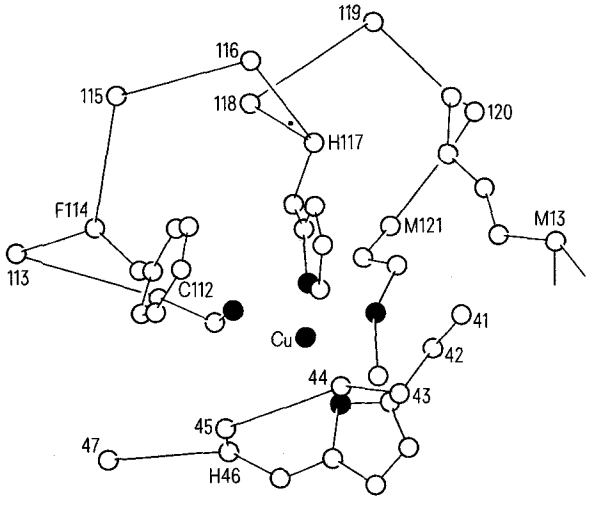

Figure 11. The Cu site in azurin seen from above the molecule.

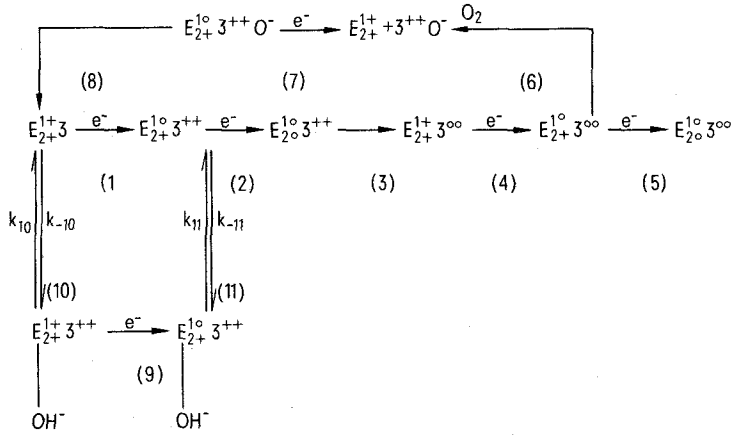

Figure 12. Proposed mechanism of electron transfer in laccase-catalyzed reactions (from Andreasson and Reinhammer[186]). In the scheme the oxidized states are denoted by '+', the reduced by 'o'. The type 3 site is represented by 2 symbols to emphasize its function as a 2-electron acceptor. At pH 6.0 and 6.5 the experimental results are consistent with the reaction sequence 1–5, with the reduction of the type 1 Cu^{2+} limiting the rate of electron transfer. As the pH is increased, however, the reduction of the 2-electron acceptor in a growing fraction of the molecules is rate-limited by a slow intramolecular pH-dependent reaction, while the type 1 copper is readily reduced. These results imply that the laccases can exist in at least 2 interconvertible states with relative concentrations regulated by the pH of the medium and the pK of some titrable group in the enzyme. The additional reactions of the 'inactive' high-pH form are represented by reactions 9–11 (lower pathway).

dic sites scavenging the radical to the type-1 copper site. Electron migration could proceed by some site hopping between aromatic amino acid residues or along the polypeptide backbone or possibly by electron tunnelling between the radical-adduct site to the type 1 copper site[188]. A limited number of amino acid residues are involved as primary sites of the enzyme to be reduced, some of them being part of the substrate binding-site on the enzyme. The 2 unstable intermediates $OH^{\cdot}$ and O_2^{-}, could be produced at the type-3 or type-2 sites in the oxidase enzymatic cycle. Due to its very short life-time in the presence of laccase, $OH^{\cdot}$ can only act in an intramolecular electron transfer process in the redox cycle of the enzyme[188]. pH-dependence of redox properties of the type-2 copper-depleted tree laccase are consistent with that viewpoint[202]. Model studies for the concerted electron transfer reactions of laccase have been reported, using several binuclear Cu^{2+}-complexes and 1 electron donors such as N,N,N′,N′-tetramethyl-p-phenylenediamine[189].

Hemocyanine and tyrosinase

The electron uptake and donation by tyrosinase from *Neurospora* shows similarities with hemocyanins. Treatment of the EPR-silent binuclear copper-site with excess ascorbic acid and NO_2^{-} gives rise to an EPR-detectable half-met-NO_2^{-} derivative that undergoes ligand substitution reactions with different anions[191] in a way very similar to those reported for arthropods and molluscs hemocyanins[192]. Upon addition of an o-diphenol substrate, this EPR signal completely disappears whereas in the presence of the strong enzyme inhibitor L-mimosine a new half-met derivative is formed. It seems that direct binding of the mono- and o-diphenolic oxygens to the binuclear copper-site is involved in tyrosinase.

The binding of CO to tyrosinase is associated with an intense visible emission[261]. The same observation has been made on the copper-containing proteins hemocyanins[262] and this property seems to be uniquely related to the structure of the binuclear copper-center of these proteins, since the addition of CO to laccase[263] and ascorbate oxidase did not give rise to a specific visible emission. The nature of the emitting species is not known. It is impossible to establish whether CO plays a direct role or simply acts through a perturbation of the ligand-field of copper atoms. However, CO was found to bind to only 1 of the 2 coppers of each binuclear center. EXAFS experiments suggest the presence of a Cu(I)-CU(II) pair in CO-hemocyanin[193], while a Cu(II)-Cu(II) pair with a strongly bound peroxide molecule was proposed for oxyhemocyanin[194]. In both derivatives a charge delocalization involving 1 to 2 electrons from the copper-center seems to take place, and is probably associated with the presence of a charge-transfer band in the electronic absorption spectrum[194]. Two singlet-excited states can populate the triplet state responsible for the observed emission (with other spin-states being capable to give similar transitions). An energy-transfer pathway for sensitized emission, through excitation of protein aromatic residues, has been proposed[195] with energy transfer causing a decrease of the intrinsic fluorescence quantum yield.

Cu-Zn superoxide dismutase

Bovine superoxide dismutase is a dimeric protein, binding 1 Zn(II) and 1 Cu(II) per identical 16,000-dalton subunit. Each Cu(II)-ion is directly coordinated to the imine-nitrogen atom of 4 histidine-residues, with one of these histidine-residues being fully deprotonated and binding to both the Cu(II) and the Zn(II). The Cu-coordinated atoms of the 4 histidine-residues and the Zn atom form an approximate geometric plane, with one side accessible to the solvent via a crevice in the protein structure while the other side projects toward the interior of the protein[196]. Furthermore, 1 water molecule serves as an axial ligand on the solvent side of the plane, making the Cu(II) pentacoordinate[197], whereas the Zn(II)-ion is additionnally coordinated to 2 other His- and 1 Asp-residues in approximately tetrahedral geometry. Monoclinic crystals of the native enzyme and its monocyano derivative were studied by means of EPR spectroscopy[264]. The distinctive EPR spectrum of the Cu(II) metal-binding site is sensitive to certain anions which bind to the Cu(II), and act as inhibitors of the dismutase activity, with cyanide showing a well defined EPR spectrum as a result of its association with the Cu(II). ENDOR results suggested that the Cu(II)-complex is liganded in a planar array of 3 histidines and 1 cyanide, with the two N-atoms *cis* to the CN^{-} having a larger coupling constant to the unpaired electron than the single *trans* N-atom[265]. The principal values of the magnetic tensors from the Cu(II)-metal-binding site change from rhombic to axially symetric upon binding CN^{-}. The superhyperfine structure, which changes also, changes from anisotropic to nearly isotropic upon cyanide binding, whereas the Cu(II)-Im-Zn(II) bridge remains unbroken under these conditions[40].

Ascorbate oxidase

The mechanism of ascorbate oxidation by dopamine-monooxygenase has been studied[198] in order to better establish the mechanism of electron transfer in this system. The path of electron transfer is believed to involve enzyme-bound copper[199]. Reduction by ascorbate of 1 copper per active site is thought to be the first step, followed by transfer of this first electron to bound dioxygen before the same copper accepts a second electron[200]. Alternative mechanisms involving 2 coppers per active site, and thus more in line with the mechanism of tyrosinase (a 2-electron transfer system) have been the matter of controversy[198], as evidenced from studies using the oxidized cytochrome c as scavenger. In this method, ascorbate free-radicals were found to reduce oxidized cytochrome c more rapidly than fully reduced ascorbate, hence accelerating the reduction of cytochrome c on addition of the oxidized enzyme. Addition of dopamine-monooxygenase markedly increased the reduction of cytochrome c in the presence of ascorbate. Similar results have been obtained with ascorbate oxidase[201], which also oxidizes ascorbate to the free radical in a 1-electron transfer step. The reduction site of the cytochrome c-ascorbate reaction has been studied together with a theoretical discussion of the possible electron-transfer pathway[202]. A correlation of kinetic and

thermodynamic observations led to the conclusion that the ascorbate reduction of the protein is independent of the state of the heme crevice opening and of the polypeptide organized structures; instead it is determined by the integrity of the tryptophan-indole-porphyrin interaction. Structural considerations suggested that Arg-38 is the ascorbate binding site and that the one-electron interaction between the indole of tryptophane-59 and the porphyrin moiety must constitute, at least in part, the electron transfer path to the heme iron of cytochrome c in this system[203].

Concluding remarks

The influence of the microenvironments near the prosthetic groups in proteins has become better understood thanks to the numerous investigations which have been undertaken, and which have been partly described here. It is certainly not possible in the present framework to do justice to all contributions in this exceedingly broad field, and no doubt much lies undiscovered in this area. These investigations have involved studies on the kinetics of electron transfer (with either natural or artificial reaction partners), studies on the effects of chemical modification and the comparison of proteins having deletions or replacements in their primary structures. On such a complex matter like electron transfer in biological systems, it is a most difficult task to attempt to draw any conclusion since it will be either too general to be of any benefit or it will be challenged by some of the numerous pieces of evidence scattered among the many observations made to date. Some pictures emerge anyway: with the involvement of complex ligands such as proteins, electron transfer centers are organized with respect to one another in space in such a way that outersphere collisional processes are possible with usually no direct collision occuring between them as is possible in solution. The electron transfer is then very much linked to hopping pathways within the protein structure, which are affected by conformational changes. Cooperative order-disorder transformations induced by these conformational changes in liquid crystal structures such as proteins or protein complexes will assist the electron-transfer steps or control the energies for electron flow. In turn, the microenvironment of the electron transfer centers most dramatically affects the kinetics of the flow process. This electron flow usually occurs within hydrophobic channels, (particularly when the prosthetic group is deeply imbedded in the protein structures as is the case in hemoproteins) where the electron moves in a vacuum and is affected by the dielectric of the solvent. A specific fit between the donor and acceptor molecule is usually observed although Nature shows greatest adaptability in each specific case, and the environment of the electronic pathway itself may not necessarily be conserved throughout molecular evolution (as shown on cytochromes and ferredoxins). This whole situation is even more complex in terminal oxidases and larger structures (mitochondrial or photosynthetic electron transfer chains) where electron transfer is also linked to proton transfer and energy transduction. Be it organized in complex or in relatively simpler structures, electron transfer in biological systems is modulated in the finest possible ways through the biological requirements of metabolic processes as a whole, thanks to the various influences on the environments of the prosthetic groups induced upon the protein conformational changes.

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