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Electron transfer in cytochrome *c*. role of the polypeptide chain

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SUMMARY

Nuclear magnetic resonance relaxation studies on partially reduced solutions of *Candida krusei* and horse heart cytochrome *c* reveal diversity in their functional behavior. Electron transfer between the two oxidation states *in vitro* proceeds at a rate nearly an order of magnitude slower in *Candida krusei* than in the horse heart protein. This difference is interpreted in terms of an active role of the polypeptide chain in the electron transfer process since the hemes in the two proteins are identical in chemical structure and similar electronically. In both cases, electrostatic repulsion between positively charged protein molecules contributes significantly to the observed free energy of activation for the electron transfer process.

It is of biological interest to understand the mechanism of electron transfer in and out of cytochrome *c*, a component of the mitochondrial oxidative phosphorylation electron transport chain. There is an appreciable change in the conformation of the polypeptide chain wrapped around the heme in cytochrome *c* upon electron transfer[1]. Whether this means that the protein moiety is the carrier for the electron during transfer is not known. The conformational changes observed could merely reflect the variation in requirements for the minimum energy condition for the two oxidation states. However, it is interesting to ask if the extent of conformational change regulates the transfer process.

The technique of nuclear magnetic resonance (NMR), in suitable cases, offers the possibility of examining some dynamic aspects of the electron transfer problem. We have

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used this technique to study structure and function of cytochrome *c* in our laboratory[2-7]. In mixed samples of oxidized and reduced cytochrome *c*, electrons are continuously transferred from one protein molecule to another and, as a consequence, the protein molecules are exchanging oxidation states[2,4,7,8]. The rate of this transfer process may be taken as a measure of the rapidity with which cytochrome *c* is able to transport electrons. Since we study transfer between protein molecules, our measurements ought to be superior to ascorbat or ferrocyanide reducibility studies in providing an *in vitro* means of analyzing cytochrome *c* function[9-11].

Experimentally we measure the proton spin-lattice relaxation rate (T_1^{-1}) of the methionine methyl group nuclear magnetic moment. This rate is 5 s^{-1} and 500 s^{-1} in the completely reduced and oxidized state, respectively. In mixed solutions, the apparent T_1 of the reduced state methionine methyl group is dependent, among other things, on the redox state of the mixed solution. From the observed value of this apparent T_1, we can determine the rate of exchange. This is possible only if the rate of exchange is comparable to, or greater than, the spin lattice relaxation rate of methionine methyl protons in the absence of exchange[7]. Our results, published earlier[7], showed that the electron exchange between cytochrome *c* molecules involves binary collisions and yielded a second-order rate constant for the transfer process in the range $1 \cdot 10^3 - 1 \cdot 10^5$ $mole^{-1} \cdot s^{-1}$ for horse heart cytochrome *c*, depending on buffer variables and temperature. In this communication we present data which assigns an active role to the polypeptide chain in the electron transfer process.

Our experimental results are summarized below. For convenience, we have included, where needed, relevant previous results[7] from our laboratory. Horse heart and *Candida krusei* proteins were purified preparations obtained from Sigma Chemical Co. (type VI) and Calbiochem, respectively. The cytochrome *c* concentrations in solutions used were in the range 5–15 mM. These were partially reduced with dithionite and electrodialyzed overnight to free them from ionic impurities. The only ions then used were $Tris^+$, $cacodylate^-$ or $succinate^{2-}$, which provided a non-binding buffer medium for the protein[12]. A home-built pulsed Fourier transform NMR spectrometer operating at 100 MHz was used for our measurements[13,14].

(1) The rate of electron transfer near neutral pH is dependent on the ionic strength of the solution, increasing with increase in ionic strength, in contrast to the rate at the isoelectric point (pH $\simeq$ 10) which is unaffected by ionic strength.

(2) The activation energy for the transfer process measured for horse heart cytochrome *c* at neutral pH is dependent on ionic strength. It is 13(±1) kcal at low ionic strength ($\simeq$ 0.1 M) and 7(±1) kcal at high ($\simeq$ 1 M) ionic strength.

(3) The observed electron transfer rate constants measured at 40°C are approximately $1 \cdot 10^3$ $mole^{-1} \cdot s^{-1}$ and $1 \cdot 10^4$ $mole^{-1} \cdot s^{-1}$ for horse heart and $1 \cdot 10^2$ $mole^{-1} \cdot s^{-1}$ and $1 \cdot 10^3$ $mole^{-1} \cdot s^{-1}$ for *Candida krusei* cytochrome *c* at low (0.1 M) and high (1 M) ionic strength, respectively. At higher ionic strengths, the rate for well-screened horse heart protein molecules at neutral pH approaches the isoelectric pH rate of $2 \cdot 10^4$ $mole^{-1} \cdot s^{-1}$.

(4) Acidic and basic pH forms of cytochrome *c* are incapable of transferring electrons with moderate rapidity[7] and also the transfer between neutral pH forms is slowed down by the presence of imidazole, cyanide, guanidine hydrochloric and urea in concentrations sufficient to perturb oxidized state conformation.

The experimental results in (1) and (2) above can be understood by invoking a dependence of the transfer rate on electrostatic charges on the protein molecules, repulsion between like charges limiting their distance of closest approach. This limitation results in a drop in the transfer rate apparently because, for transfer to take place, the protein molecules must approach close enough for the molecular electron clouds of an oxidized and a reduced molecule to overlap appreciably. The electrostatic repulsion also manifests itself in the energy of activation. The protein molecules involved in the electron exchange must overcome this repulsion barrier in order to transfer electrons. This contribution to the free energy of activation, which is absent at high ionic strengths, can be estimated by coulomb law as $(Z_O Z_R e^2/\epsilon r_{OR})$ where Z_O, Z_R are the effective electrostatic charges on oxidation state O and R, ϵ is the dielectric constant of the intervening medium, r_{OR} is the classical separation at which electron transfer occurs and may be taken to be the diameter of the protein molecule. Assuming that the effective charge equals the net protein charge (approx. +8 electron units at pH 7 (ref.15)), this theoretical estimate agrees fairly well with the observed difference of 6 kcal in activation energy at low and high ionic strength.

The observed difference between the transfer rates for horse heart and *Candida krusei* cytochrome *c* of nearly an order of magnitude is somewhat surprising. This difference seems to contradict the earlier experiments showing that cytochrome *c* from an entire range of species are interchangeable in maintaining the rate of electron transport in mitochondria[15]. To reconcile these observations with ours, assuming, of course, that the *in vitro* electron transfer that we observe resembles that of the *in vivo* process, we must conclude that the rate-determining step for mitochondrial electron transport in mammalian-type systems is not transfer through cytochrome *c*.

Since the rate of electron exchange is dependent on the charge state of the protein, it is conceivable that the difference between horse heart and *Candida krusei* proteins arises from different charge density distributions on the surface near the transfer site. However, this is ruled out in the present case since the difference in rates persists even when all charges are well screened by the presence of 1 M ions. One might also argue that the intermolecular electron transfer site is on the heme and that the heme is differently exposed to the outside of the molecule in the two cases. However, this does not seem to be the case at hand since it is known that the heme moiety is well exposed[16] in the low pH forms of cytochrome *c*, though such forms are ineffective in electron transfer. Thus the variation in rates between horse heart and *Candida krusei* could not be arising due to variation in the exposure of the heme.

The hemes in horse and *Candida krusei* cytochromes have identical chemical structures and are similar electronically. The assertion regarding electronic equivalence is based on the observed hyperfine contact shifts of the heme ring methyl and the methionine methyl

proton resonances which show little variation (less than 10%) between the two cytochromes[4,7]. However, the primary sequence of the two polypeptide chains differ by 40–50% in amino acid residues[15]. Our observation of a difference of nearly an order of magnitude in rates in (4) above must then mean that there are significant differences in the tertiary structure of the polypeptide chain of cytochrome *c* from the two species and/or the variable residues are directly participating in the electron transfer process. Moreover, the differences in the tertiary structure in our case will be reflected in the transfer rate only if the protein moiety were the carrier for the electron during transfer and/or if the redox conformational changes in the protein were limiting (regulating) the rate of the electron transfer. This we believe is the first evidence for the involvement of the polypeptide portion of the protein molecule in the electron transfer process.

It is not possible to propose a mechanism of electron transfer on the basis of our limited data. More species need to be examined. If an aromatic ring like tyrosine-67 or phenylalanine-82 removes electrons from the heme directly *via* overlap of π-electron clouds, then a small change in the position of the aromatic ring relative to the heme arising due to variation in amino acid residues could cause the observed change in the transfer rate. Similarly, replacement of isoleucine in horse by alanine in *Candida krusei* at position 81 could disturb the energy balance critically maintained between this residue and phenylalanine-82 (one is embedded into hydrophobic environment at the cost of the other which points into water (ref. 1)). Since these residues interchange their positions following the change in oxidation state, the difference in energy gained or lost by positioning one in hydrophobic environment and the other in water could contribute to the activation barrier and thus influence the transfer process.

According to the Frank–Condon principle, the probability of an electron exchange taking place with an appreciable instantaneous change in the nuclear positions is small. This means that the nuclei must be placed in the correct positions before electron exchange can take place[17]. Presumably then, before electron transfer can occur, the two oxidation states distort themselves to a similar conformation and then the electron transfers from one molecule to the other requiring little energy to do so. Following the transfer the two states recover their equilibrium conformations. In the light of this model, we can understand the observed absence of electron transfer between the basic pH forms of ferricytochrome *c* and ferrocytochrome *c*. Since the basic pH form of ferricytochrome *c* has a ligand on the heme (very likely a lysine) at its sixth coordination site different from that on ferrocytochrome *c* (methionine-80), it will have to overcome a large energy barrier involving ligand reorganization to approach a polypeptide chain conformation similar to that of the reduced state. This requirement will retard the electron transfer. On the other hand, the acidic pH forms of cytochrome *c* which are more open than the neutral pH forms and have no sixth ligand on the heme other than a fast exchanging water molecule or an anion[5], should be able to distort themselves with lesser energy than the neutral pH forms. The predicted absence of moderately rapid exchange of electrons between the acidic forms must then mean that somehow the presence of methionine on the heme is essential for the transfer. Imidazole and cyanide, which replace methionine, inhibit exchange. Guanidine

hydrochloride and urea which perturb the oxidized state conformation also have an inhibitory effect on the exchange. The presence of these agents tends to induce a high spin state lacking methionine coordination. Unfortunately, we still can not conclude that electron flows *via* methionine sulfur. It could well be that the residue directly involved in transferring electrons is capable of doing so only when methionine is coordinated, since the latter holds the former in the spatial position such that it interacts with the heme.

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