Double Nuclear Magnetic Resonance Observation of Electron Exchange between Ferri- and Ferrocytochrome c

Abstract. *Cyanide-inhibited electron exchange between ferri- and ferrocytochrome c molecules has been observed by nuclear magnetic resonance. Irradiation of a partially reduced protein solution at resonance frequencies arising from protons of the oxidized state results in a decrease in the absorption due to the corresponding protons of the reduced state. The experiment quantitates the hyperfine shifts observed in this system and can be used to identify hitherto unassignable resonances. Analysis of the shifts suggests that an aromatic side chain ring lies near the edge of the heme ring.*

Cytochrome c is an electron-transporting enzyme of the respiratory chain, responsible for the transfer of electrons from cytochrome b to cytochrome oxidase (*1*). The mechanism of this transfer, however, is not understood. Our experiments show direct evidence for the presence of electron exchange (via a direct or an indirect mechanism) between ferro- and ferricytochrome c molecules in aqueous solution, and show that the rate of exchange falls in a range suitable for observation by the nuclear magnetic resonance (NMR) technique. We observe this exchange by a double resonance variation of the NMR method. Detailed study along similar lines may be useful in elucidating the mechanism of electron transfer along the chain of cytochromes in the mitochondrion. In addition, the experiments enable us to assign several of the hyperfine shifted resonances in the NMR spectrum of ferricytochrome c. The analysis of the NMR data yields novel information on the tertiary structure of the protein near the heme group. The exchange of electrons between cytochrome c molecules in aqueous solution is seen to be inhibited by the presence of potassium cyanide, which is well known to poison electron transfer in the mitochondrion.

The experiment is simple in principle, and can be understood without specialized knowledge. The sample is a 10 percent solution of protein in D_2O, of which approximately half is reduced with ascorbate (*2*). The NMR absorption spectrum of this mixture is a superposition of the spectra observed in fully oxidized and fully reduced cytochrome c. Because the heme group in the oxidized form has an unpaired electron spin, a few resonances of groups on or near the heme ring are separated from the main part of the NMR spectrum by hyperfine interaction. Of these, methyl groups are most readily observed (*3*), and are identified as methyl by their intensity (Fig. 1). The reduced form, on the other hand, shows only one resolved methyl resonance (Fig. 2a, indicated by arrow) shifted upfield by the diamagnetic currents which are induced to flow around the heme ring by the applied field (*4*). Other methyl resonances in the diamagnetic state also are shifted by the same mechanism, but not sufficiently to pull them out of the confused central region.

We first apply a long pulse (0.1 second) of radio frequency power to the sample at the known frequency of one of the oxidized-state resonances indicated in Fig. 1. Such a pulse will momentarily bleach the absorption at this frequency, because the spins in resonance with it become saturated with energy (*5*). Just after this long pulse, we monitor the NMR absorption spectrum (*6*), and find, as expected, that the previously irradiated line is indeed bleached.

If the intensity of the long pulse is fixed at a level no greater than that needed to bleach the oxidized-state resonance, the absorption in the mixed sample also decreases at another corresponding point in the spectrum, which we believe to be the resonance frequency of the same methyl group in the reduced state. Apparently, a given protein molecule alternates between the oxidized and the reduced state in a time of the order of one quarter second, and a given methyl group likewise alternates in NMR frequency, transferring the bleaching effect from the oxidized to the reduced state (*7*, *8*). From a knowledge of the rate at which the reduced state recovers from bleaching (*5*) we can make a precise estimate of this rate. A study of the rate as a function of concentration and temperature is now in progress.

This picture is supported by the observation (Fig. 2) that in one case the transferred bleaching occurs at the one resolved reduced-state methyl resonance. The two resonances thus connected have been previously (*3*, *4*) identified with the methyl group of methionine coordinated at the face of the heme.

Table 1 gives the positions at which bleaching is induced by prepulsing at various oxidized-state resonances. The information in Table 1 can be used to identify these resonances with known methyl groups, or to infer, in suitable cases, other structural information when an assignment has previously been made. Besides the upfield resonances at-

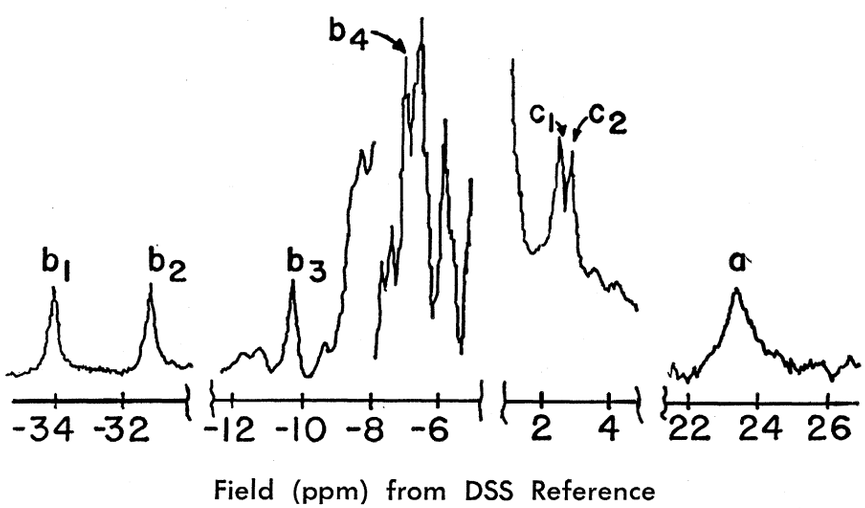

Fig. 1. Portions of the NMR absorption spectrum of oxidized cytochrome c, plotted as a function of magnetic field deviation, in parts per million (ppm), from the reference compound DSS (sodium 2,2-dimethyl-2-silopentane-5 sulfonate). The vertical baseline has been shifted at −8 ppm, and the vertical gain is greater by a factor of 3 at 22 to 26 ppm. A few single-spin resonances are found between −12 and −20 ppm, and between +4 and +8 ppm, and the vast majority of the spins resonate in the unshifted region from −5 to +1 ppm.

Table 1. Summary of methyl resonance cross-bleaching experiments. Positions are parts per million from DSS (see Fig. 1 legend) reference in both states and were obtained at room temperature (27°C). The oxidized state resonance was always preirradiated; cross-bleaching occurred at the frequency indicated under the reduced state. Except for the first resonance tabulated (Fig. 2), all the diamagnetic resonance positions inferred here occur in places in the spectrum where they are swamped by other overlapping resonances, if studied conventionally. Their observation was facilitated by automatically taking a difference spectrum with and without the prebleaching. Identification: *a*, methionine methyl; *b*, porphyrin ring methyl; *c*, porphyrin side chain methyl.

Identification	Oxidized state position (ppm)	Reduced state position (ppm)
a	23.4	3.3
b_1	−34.0	−2.1
b_2	−31.3	−3.8
b_3	−10.3	−3.5
b_4	− 7.2	−3.4
c_1	2.2	−1.4
c_2	2.6	−0.3

tributed to methionine, the two lowest field resonances in the oxidized state, b_1 and b_2 (Fig. 1), have been previously assigned (*3*) to two of the four methyl groups directly attached to the porphyrin ring; it is not known which two of the four. In model heme compounds (separated from protein) and most other heme proteins, resonances of all four of these groups are conspicuous in the range from about −25 to −10 parts per million (ppm). In cytochrome c, these four resonances are spread out even more than in other heme proteins. Wuthrich (*3*) suggested that the skewness of the unpaired electron's spin density distribution thus implied might be related to the electron transfer function.

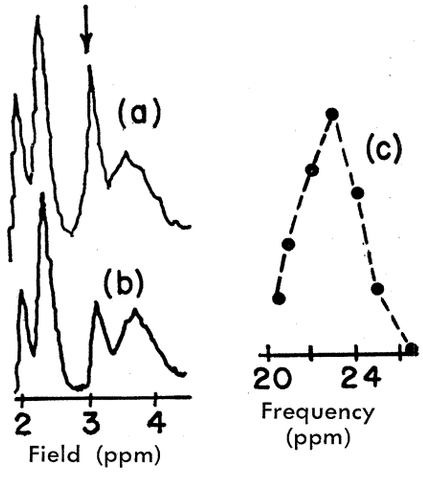

Fig. 2. (a) Part of the spectrum of the mixed sample, showing the resolved methyl peak from the reduced state (arrow) and two methyl peaks downfield from it, which are resonances c_1 and c_2 of Fig. 1. (b) The spectrum just after a bleaching pulse applied to the resonance at +23.4 ppm. (c) The size of the decrease in the 3.3 ppm resonance, as a function of bleaching frequency.

We find that three other methyl resonances, b_3, b_4, and c_1, are shifted, in the diamagnetic state, to positions appreciably downfield from that expected for an aliphatic methyl group. They are, therefore, likely candidates for either ring methyls, or else groups not directly attached to the porphyrin ring but located near its edge, because such groups will be shifted downfield by the same porphyrin ring current which shifts methionine methyl resonance upfield (*9*) in the diamagnetic form. In the paramagnetic form a porphyrin ring methyl resonance is hyperfine-shifted by contact interaction with the wave function of the unpaired electron on the ring (*3*); this shift can conceivably be in either direction, depending on the sign of the unpaired spin density on the ring carbon to which the methyl is attached. A group not directly on the ring will experience little or no shift from this mechanism (*10*), but both types of groups will experience a "pseudocontact" shift as a result of direct magnetic dipolar interaction with the electron spin. It is unlikely that this shift is negative (*11*); therefore since b_3 and b_4 shift downfield on oxidation, they are likely to be ring methyls, an assignment strengthened by the fact that their position in the reduced protein is exactly where ring methyl resonances are found in model hemes (*9*).

We tentatively assign the remaining resonances c_1 and c_2 to the porphyrin side chain methyls. However, it is possible that the porphyrin side chain methyl resonances are not much shifted, and that c_1 and c_2 are amino acid side chain methyls near the edge of the heme plane. One of these, c_1, is shifted on reduction to nearly a position where porphyrin side chain methyl resonances are found in model diamagnetic hemes (*9*).

The ring methyl resonance b_1 found most downfield in the oxidized state is shifted appreciably upfield relative to the other ring methyl resonances on reduction. This shift could be explained by assuming that this group is near the face of an aromatic amino acid side chain ring. Williams (*12*) has suggested that tyrosine may help transfer electrons to the heme group, and tyrosine could give such a shift. Resonance c_2 also appears shifted upfield in the diamagnetic state, perhaps by the same, or a similar, group.

We also investigated the effect of adding potassium cyanide on electron exchange between cytochrome c molecules. On adding potassium cyanide to a partially reduced solution of cytochrome c, an NMR spectrum showing the superposition of resonances arising from cyanoferricytochrome c and ferrocytochrome c was observed. Exchange seems to be blocked, as indicated by the absence of any cross-bleaching effects expected on saturating the hyperfine shifted methyl resonances of cyanoferricytochrome c if the exchange were present.

The cross-bleaching experiments can be done with several of the single spin resonances which are resolved in the oxidized state, but are more difficult because of their weaker intensity. Unfortunately, this type of experiment sheds no light on possible conformation shift on oxidation.

RAJ K. GUPTA
ALFRED G. REDFIELD
IBM Watson Laboratory,
Columbia University, New York 10025

References and Notes

1. E. Margoliash and A. Schejter, *Advan. Protein Chem.* **21**, 113 (1966); see also R. E. Dickerson and I. Geis, *The Structure and Action of Proteins* (Harper and Row, New York, 1969), and references therein.
2. Horse heart cytochrome c (Sigma Chemical Co., type VI) was purified by running it through a Chelex column equilibrated to *p*H 5.5 in 0.05*M* sodium acetate buffer to remove any possible metal ion impurities. It was then half-reduced with ascorbic acid and passed twice through a Sephadex G-25 column equilibrated with distilled water to remove any remaining traces of impurities and ascorbic acid. The sample was lyophilized and redissolved in D_2O. An unbuffered 10 percent solution of the protein ($pD \simeq 7$) was then used for the final experiments.
3. K. Wuthrich, *Proc. Nat. Acad. Sci. U.S.* **63**, 1071 (1969), and references therein. This paper describes the "contact shift" mechanism which shifts the porphyrin ring methyl resonances.
4. C. C. McDonald, W. D. Phillips, S. N. Vinogradov, *Biochem. Biophys. Res. Commun.* **36**, 442 (1969).
5. We use "bleach" in place of the NMR term "saturate." It occurs because the rate of absorption is equal to the difference between the rates of stimulated absorption and emission, and thus is proportional to the population difference between the two nuclear spin states. This population difference, normally present at thermal equilibrium because of the small energy difference between states, is destroyed by the bleaching pulse. It persists after the pulse for a spin-lattice relaxation time, which is the inverse of the transition probability for a nuclear spin flip as a result of random interactions with other spins. The mechanism for spin-lattice relaxation is unimportant here; the relaxation time is approximately 0.2 second for nuclei in the reduced state, and 0.002 to 0.2 second for different resolved lines in the oxidized state. Because the oxidized state relaxation times are shorter than those in the reduced state, it is harder to cross-bleach an oxidized state resonance by irradiating the corresponding reduced state resonance.

6. Rapid observation of the absorption spectrum is possible because we use the pulsed-Fourier-transform method of spectroscopy. See R. R. Ernst and W. A. Anderson, *Rev. Sci. Instr.* **37**, 93 (1966). We used a Jeolco 100-Mhz spectrometer converted to pulsed operation; a detailed report describing this instrument is available from us on request. In principle, the same experiment could be performed on a conventional swept spectrometer by steady-state double irradiation.
7. Transfer of energy from one methyl group to a neighboring group in the same protein ("cross relaxation") would produce a similar effect. The cross bleaching due to cross relaxation and exchange can, however, be distinguished by a comparison of the results on mixed and unmixed samples.
8. A. Kowalsky [*Biochemistry* **4**, 2382 (1965)] reports a broadening of the NMR lines in a mixed oxidized and reduced sample. He attributes this broadening to electron interchange as in the present experiment.
9. W. H. Caughey, J. L. York, P. K. Iber, in *Magnetic Resonances in Biological Systems*, A. Ehrenberg, B. G. Malmstrom, T. Vanngard, Eds. (Pergamon, New York, 1967), p. 26.
10. Such a group, isolated by an extra C-C single bond from the porphyrin ring, might have a contact shift of about 0.05 times that of a ring methyl, or around ±1 ppm. See, for example, M. K. Carter and G. Vincow, *J. Chem. Phys.* **47**, 302 (1967).
11. The dipolar interaction produces a "pseudo-contact" shift if the electron's g-tensor is anisotropic; see G. N. LaMar, *J. Chem. Phys.* **43**, 1085 (1965). The principal g-values have been found to be 1.24, 2.24, and 3.06 at 4°K by I. Salmeen and G. Palmer, *J. Chem. Phys.* **48**, 2049 (1968). If the $g = 3.06$ principal axis is roughly perpendicular to the heme plane, then a spin at the distance of a ring methyl group is pseudocontact-shifted zero to +5 ppm, depending on the orientation of the other two g-tensor axes. In myoglobin azide the largest g axis is perpendicular to the heme plane; see D. J. E. Ingram, *Biological and Biochemical Applications of E.S.R.* (Hilger, London, 1969), p. 255.
12. R. J. P. Williams in *Chemistry of Hemes and Heme Proteins*, B. Chance, R. W. Estabrook, T. Yonetani, Eds. (Academic Press, New York, 1966), p. 585.
13. We thank Mrs. H. Shlank for preparing our samples, and Dr. T. Fabry for suggesting the study of half-reduced mixtures to us.

4 May 1970; revised 1 July 1970 ■

Oxidation of a Polymer Surface with Gas-Phase Singlet ($^1\Delta_g$) Oxygen

Abstract. *Singlet molecular oxygen ($^1\Delta_g$) produced by the microwave discharge of ground state oxygen in a flow system was passed over a film of* cis*-polybutadiene. This treatment resulted in the formation, at the surface, of hydroperoxides which were detected by internal reflection infrared spectroscopy.*

Electronically excited molecular oxygen in its singlet state ($^1\Delta_g$) has been frequently discussed as an agent that in polluted air may be responsible for the oxidative degradation of various naturally occurring and synthetic substances (*1*). There has also been speculation on the role of singlet oxygen in producing cancer on the skins of irradiated animals treated with photosensitizing substances (*2*) and in promoting the photooxidation of polyethylene (*3*) and a variety of other substances (*4*). An important question that must be answered before one invokes a singlet oxygen mechanism to account for such surface effects is whether metastable singlet oxygen ($^1\Delta_g$) molecules can indeed survive long enough to chemically react with an environmental surface.

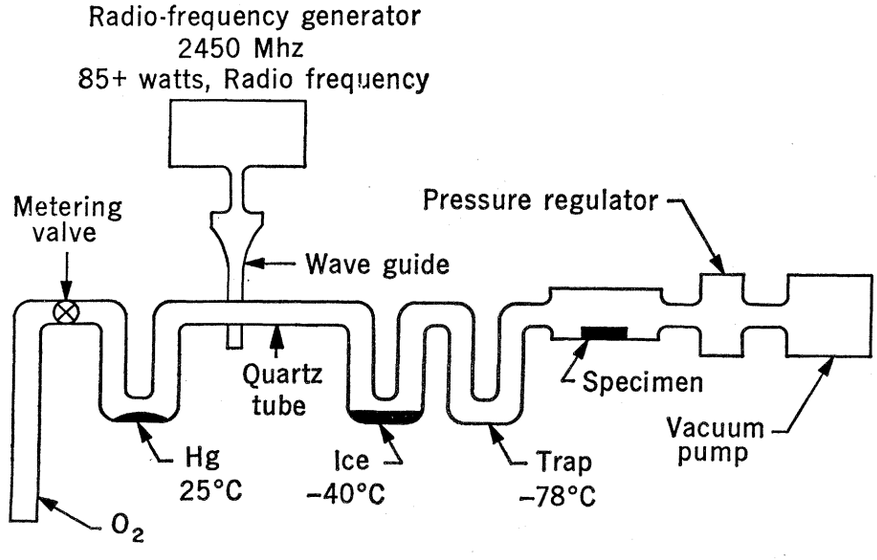

Fig. 1. Diagrammatic representation of the flow system used for polymer oxidation.

Our results show conclusively that gaseous singlet oxygen impinges on the surface of *cis*-polybutadiene films causing the formation of the expected hydroperoxides.

The electrodeless discharge of oxygen either by radio-frequency or microwave energy is known to produce a variety of active species including atomic oxygen, excited molecular oxygen ($^1\Delta_g$ and $^1\Sigma_g$), ozone, and some ionic components (*5*). The reaction of such a complex plasma with a large number of bulk polymers has been used to improve adhesion and alter other surface-related properties (*6*). The primary effects in those cases were attributed to atomic oxygen. Similar experiments were performed on thin films of methyl linoleate with the observation of an increase in the absorbance at 234 nm, which was interpreted as indicating the formation of conjugated hydroperoxides [R–C=C–C=C–C(OOH)R] from the attack of singlet oxygen on the 1,4-diene structure (*7*). Although singlet ($^1\Delta_g$) oxygen was produced by photosensitization and not electrodeless discharge, the mean diffusion path for excited molecular oxygen through stearate films has been determined to be 115 Å (*8*). It was estimated that in traversing that distance in the film one-half of the diffusing molecules had been deactivated.

The flow system used in our work (Fig. 1) has the advantage that all active species except singlet oxygen ($^1\Delta_g$) are removed from the gas stream before contact with the polymer surface. Tank oxygen is passed over mercury (25°C) and into a quartz tube. A wave guide focuses microwave power (2450 Mhz) so that energy couples with the gas at the center of the quartz tube. The pumping speed based on the capacity of the vacuum pump is 33.4 liter/min. The mercury vapor in the stream is oxidized, forming a mercuric oxide ring both upstream and downstream from the plasma glow. This mercuric oxide is effective in removing oxygen atoms and ozone and in enhancing the singlet oxygen ($^1\Delta_g$) concentration (*9*). Ionic species deactivate a few millimeters from the glow region (*5*). The stream is then passed over ice at −40°C to quench the singlet oxygen in its $^1\Sigma_g$ state (*10*) and then through a −78°C trap to remove water and mercury vapor carried over by the gas flow. The distance traveled by the excited molecular oxygen before it reaches the sample chamber is about 100 cm. We esti-