ELECTRON PARAMAGNETIC RESONANCE OF NITRIC OXIDE CYTOCHROME C

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A virtually identical electron paramagnetic resonance (epr) spectrum of NO-ferrocytochrome c was obtained in solution at pH 12, pH 7 and in powdered form. The spectrum is entirely different from the one reported by previous authors. There are two sets of N^{14} hyperfine structure, one from $N^{14}O$ and the other due to the imidazole nitrogen of the axial histidine residue. Cytochrome c powder (ferri or ferro) which does not yield NO-ferrocytochrome c when dissolved in pH 7 buffer, was found to react with NO to give an identical epr pattern, indicating some structural change around the heme group in the dry state.

The electron paramagnetic resonance (epr) absorption of cytochrome c (oxidation state unspecified) in powdered form which was allowed to react with nitric oxide (NO) has been reported by Gordy and Rexroad.[1] They observed at 77°K a fairly well-resolved triplet with splitting of 18 G, which they interpreted to belong to the NO-cytochrome c complex, the valence of Fe not being clearly specified. However, our observation of NO-ferrocytochrome c prepared[2] in aqueous solution at pH 12 shows an entirely different spectrum at 77°K as illustrated in Fig. 1. The formation of NO-ferrocytochrome c under the same conditions has been previously demonstrated by optical absorption, and NO-ferricytochrome c was shown to be diamagnetic by magnetic susceptibility measurement.[3]

The epr spectral shape as well as its intensity remains unchanged when the pH is brought to neutrality. Not only is the triplet not so well-resolved, but the splitting is much larger (24 G) than in the result of Gordy and Rexroad. Furthermore, each component of the triplet is superimposed by what appears to be another triplet of about 6.8 G splitting. That the larger triplet is due to $N^{14}O$ and that the superimposed hyperfine structure is in fact a triplet was confirmed by the N^{15}O-cytochrome c spectrum (Fig. 1) where the resolution is better.

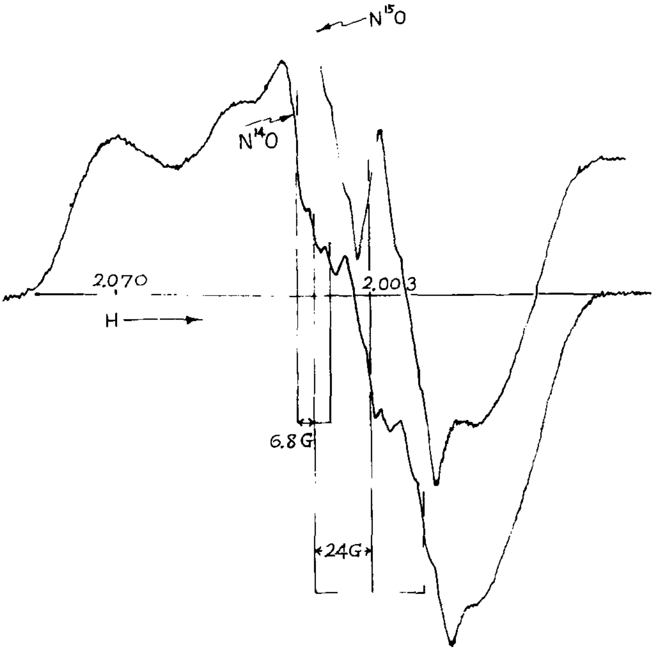

Fig. 1. Epr absorption of NO-ferrocytochrome c in powdered form. Virtually identical spectra are obtained in solution at pH 7 and pH 12.

It can be shown that the spectrum represents a system with rhombic symmetry and that the particular part of the spectrum described above corresponds to the so-called parallel absorption in which the heme normal is parallel to the external magnetic field and also that the unpaired electron is in an antibonding out-of-plane σ^* orbital having the symmetry of d_{z^2} rather than being localized in the π^* orbital of the NO group. These conclusions are based upon the results of the study of a number of model systems consisting of the heme-NO complex with various nitrogenous bases at one of the axial positions.[4]

The extra hyperfine structure is evidently due to another N^{14} nucleus which is in an axial position of the heme group. Of the two amino acid residues associated with the axial positions of native cytochrome c, one is known to be a histidine,[5,6] and the other probably a methionine.[7,8,9] Thus the present result demonstrates that a NO molecule replaces the methionine while histidine remains coordinated to Fe through the imidazole nitrogen.

As to the difference between the present epr spectrum and the one observed by Gordy and Rexroad, it was considered possible that the latter pattern may be characteristic of the powdered NO-cytochrome c. However, the reaction of NO gas with solid cytochrome c evacuated for two hours at 10^{-5} torr. results in the same spectrum as the one shown in Fig. 1, not the type reported by Gordy and Rexroad. They also reported a similar spectrum (a well-resolved triplet with 18 G split) with NO-hemoglobin powder. Recently the latter spectrum has been shown by the present writer to belong to the dehydrated form, i.e., the one obtained by dehydrating NO-ferrohemoglobin by evacuation.[2] In the case of NO-cytochrome c, however, an exhaustive evacuation at 85-90°C which would convert NO-hemoglobin completely to the dehydrated form results in hardly any change in the spectral shape. So far we have been unable to reproduce, with native cytochrome c, the result obtained by Gordy and Rexroad with the cytochrome c powder-NO system.

It may be worth noting the fact that powdered cytochrome c does react with NO, whereas the same material in solution around neutral pH does not give rise to epr absorption. It is well known[3] that the reduced form is quite inert to NO in neutral solution and that while the oxidized form combines with NO the reduction to form the paramagnetic NO-cytochrome c takes place only in an alkaline (pH = 12) medium. The present observation indicates that there is in the solid state some change in the structure around the heme group so as to allow one of the above processes to take place. Whether it is the reduced or the oxidized form that reacts to form eventually the paramagnetic species in the solid state can not be decided at the present stage, although the epr intensity is consistently much higher in the reduced, lyophilized material, which contains 50-70% reduced form, than in the oxidized, lyophilized material where the signal intensity is quite low. The integrated intensity, however, corresponds only to 30-52% of the reduced form. The CO absorption test[10] carried out immediately before use for epr observation showed less than 3% denaturation in each preparation. In order to see if some modified molecular species[6] of cytochrome c (e.g. dimer) is showing the reactivity to NO in powdered form but not in solution, the following observation was made. Crystalline horse heart cytochrome c was washed with 90% saturated $(NH_4)_2SO_4$ solution until the crystals showed clean surface when examined under a microscope. The reduced, lyophilized material prepared from the crystals in which there is no contamination from artifactual species,* gave an identical epr pattern with intensity per mg of reduced form even slightly higher than in the commercial specimen. Thus NO appears to react with native form of cytochrome c.

In any event there is observed only one kind of epr pattern in aqueous solution at pH 12, pH 7 and in the powdered form, and we believe that this spectrum represents NO-ferrocytochrome c complex.

Acknowledgment

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References

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*The conformation sensitive 695 mμ band[10] of ferricytochrome c has the same extinction coefficient in the crystals as in solution.[11]

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