PROBING AXIAL LIGANDS IN FERRIC HAEMOPROTEINS: AN ESR STUDY OF MYOGLOBIN AND HORSERADISH PEROXIDASE IN $H_2{}^{17}O$

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SUMMARY

Substitution of $H_2{}^{16}O$ by $H_2{}^{17}O$ induces a substantial broadening of the high-field line in the electron-spin resonance spectrum of ferric myoglobin due to the presence of $H_2{}^{17}O$ at the axial ligand-site. Computer simulations of the experimental spectra yielded the values of the reciprocal relaxation time $T_2{}^{-1} = 7.8$ G and the ^{17}O-hyperfine coupling constant $A = 18 \pm 1$ G. Under identical experimental conditions no effect of $H_2{}^{17}O$ was observed in horseradish peroxidase. The latter finding excludes the possibility that a water molecule is liganded to the peroxidase haem-iron and supports either the idea that both axial ligands are amino acid residues or that the haem in ferric horseradish peroxidase is pentacoordinate.

INTRODUCTION

In order to explain the specificities of various reactions in which haemoproteins take part, it is necessary to obtain evidence for the identity of axial ligands to the haem-iron. In the case of peroxidases, catalysts in the oxidations of various aromatic compounds by hydrogen peroxide, much evidence exists for histidine as the fifth haem-ligand (1). The nature of the sixth ligand is less certain, but several hypotheses have been put forward: (i) Peroxidases are of the "close-crevice" type haemoproteins, i.e. both axial ligand sites of the ferric ion are occupied by atoms from neighbouring amino acids (2). (ii) Distal (sixth) ligand of the ferric enzyme is a water molecule (3) hydrogen-bounded to an amino acid (4). (iii) Recently also penta-coordinated haem in high-spin ferric enzyme has been proposed (5). Neither of these hypotheses has been decisively proved by direct observation of the sixth ligand.

The water molecule coordinated at the sixth ligand site of ferric haemoproteins may be observed by X-ray crystallography (6), but when probing its presence by spectroscopic methods, data are generally less decisive. It has been proposed recently that solvent-proton magnetic relaxation might be the technique capable to resolve whether a water molecule is coordinated at the

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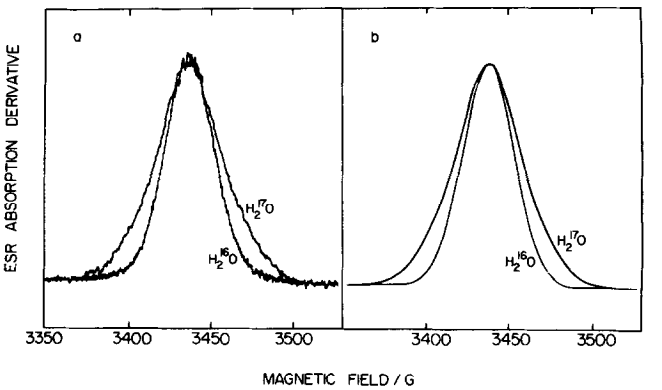

Fig. 1. High-field parts (g=2) of ESR spectra of aquo complex of ferric myoglobin in $H_2{}^{16}O$ and 50% $H_2{}^{17}O$. a) Experimental spectra taken at X-band, microwave power 5 mW, modulation amplitude 2.5 G, temperature 10 K, protein concentration 0.5 mM. The amplitude of the spectrum in $H_2{}^{17}O$ has been adjusted to the $H_2{}^{16}O$-spectrum by increasing receiver gain. b) Corresponding simulated ESR spectra. Best-fit parameters are T_2^{-1} = 7.8 G and A = 18 ± 1 G.

axial ligand-site (7), but this view has been subsequently criticized (8).

In order to probe spectroscopically the presence of the axially liganded water molecule we utilized the nuclear spin of oxygen-17 in $H_2{}^{17}O$. Similarly to fluoride in fluoro complexes (9), ^{17}O has an effect especially in the high-field part of the electron-spin resonance spectrum of aquo complexes of ferric haemoproteins. This part of the spectrum is broadened by the super-hyperfine interaction of ^{17}O nuclear spin and d^5 iron-electrons when $H_2{}^{17}O$ is present at the sixth coordination site. In this communication we present results of our study of $H_2{}^{17}O$ interaction in ferric myoglobin and horseradish peroxidase.

EXPERIMENTAL

Horse heart myoglobin (Sigma, Type III) and horseradish peroxidase (Sigma, Type VI, Lot No. 65C-9530-1) were used without further purification. Lyophilized proteins were dissolved in 100 mM sodium phosphate, pH 6.0, to a final concentration of 0.5 mM. These solutions were subsequently freeze-dried and redissolved in an equal amount of either $H_2{}^{16}O$ or H_2O 50% enriched in ^{17}O (Heavy Oxygen Plant, Weizmann Institute of Science). The ESR spectra were taken by a Varian E-12 spectrometer at X-band (9.35 GHz) operating under nonsaturating conditions of microwave irradiation. Measurements were performed below 10 K using a home-made liquid helium flow system.

RESULTS

Since the axially liganded water in ferric myoglobin has been observed by X-ray diffraction (6), this protein was chosen to investigate the effect of the presence of $H_2{}^{17}O$ at the axial ligand site. The powder spectrum of high-

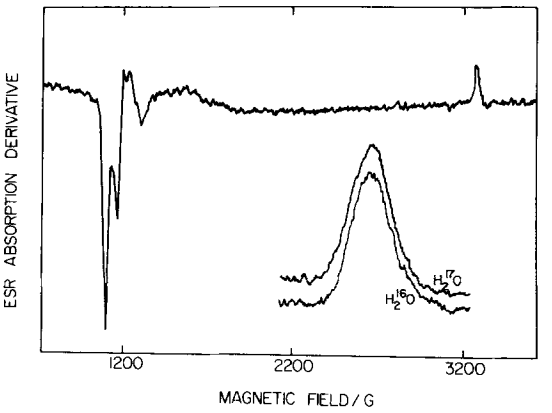

Fig. 2. ESR spectrum of horseradish peroxidase in $H_2{}^{16}O$ recorded under the same conditions as in Fig. 1. Insert: enlarged high-field parts of the spectra in $H_2{}^{16}O$ (lower curve) and 50% $H_2{}^{17}O$.

spin aquo complex of ferric myoglobin is highly anisotropic. This anisotropy arises from the difference in absorption when the magnetic field is applied in the haem-plane (g=6) and perpendicular it (g=2), and is due, in turn, to the large zero-field splitting parameters (10). The effect of ligands possessing non-zero nuclear spins is especially remarkable in the absorption around g=2, as exemplified by the superhyperfine interaction with the nuclear spin of ^{19}F (I=1/2) (9). Therefore, we expect this part of the spectrum to be sensitive also to the presence of $H_2{}^{17}O$.

The high-field part of the ESR absorption derivative (g=2) of the ferric aquo complex of myoglobin in $H_2{}^{16}O$ is shown in Fig. 1a. Substitution of $H_2{}^{16}O$ by water enriched 50% in ^{17}O produces a substantial broadening of this region of the spectrum accompanied by a decrease of amplitude (Fig. 1a). These experimental powder spectra were interpreted through computer simulation by choosing appropriate g-values and varying the line-width parameter until full correspondence to experimental spectra was achieved. The program includes the corrected transition probability, according to Aasa and Vänngård (11), for field swept spectra. Thus, a value of $T_2{}^{-1}$ = 7.8 G was obtained for ferric myoglobin in $H_2{}^{16}O$. The simulated high-field line is shown in Fig. 1b. The spectrum recorded in the presence of $H_2{}^{17}O$ was simulated by superposition of two different properly weighted spectra: (i) of the spectrum in $H_2{}^{16}O$ described above, and (ii) of the spectrum in $H_2{}^{17}O$ with the same parameters as the previous one, but split into a sextet due to the nuclear spin of ^{17}O (I = 5/2). In the case of ^{17}O-broadened spectrum the best correspondence

between the experimental and simulated spectra has been obtained using the value of the hyperfine coupling constant A = 18 ± 1 G (Fig. 1b). It is important to mention here that the width of the simulated high-field line is very sensitive to the value of A resulting in significant deviations from the observed spectra even upon minor changes of this parameter.

The spectrum of herseradish peroxidase in $H_2{}^{16}O$ is shown in Fig. 2. An expanded-scale high-field part of the spectra both in $H_2{}^{16}O$ and H_2O enriched 50% in ^{17}O are also depicted in Fig. 2. It is readily apparent that ^{17}O does not induce any pronounced broadening as in the case of myoglobin (Fig. 1a).

DISCUSSION

Judging from the profound effect in the parallel region of the spectrum in acquo complex of ferric myoglobin, isotopic substitution appears to be a sensitive method for the investigation of identity and/or reactions of oxygen-containing ligands in ferric haemoproteins. The lack of any similar effect in horseradish peroxidase can be interpreted in two different ways. The first possibility is the "closed-crevice" structure (2) where the sixth-ligand position would also be occupied by an amino acid residue. A tyrosine phenolate has been considered as a likely candidate (3,11), but in the recently proposed distal sequences of five plant peroxidases there is no tyrosine (1). In the case of "closed crevice" structure our data would be consistent also with a carboxylate involved in the distal contact with the iron, a possibility discussed by Ricard et al (3). The second possibility, raised recently on the basis of magnetic circular dichroism data (5), is the pentacoordinated haem. However, the present experiments cannot discriminate between the two possibilities. Obviously, more data are needed in order to reach a definite conclusion about the actual sixth ligand of the iron, but a water molecule as the axial ligand can be ruled out in favor of either of described alternatives.

Electron-spin resonance experiments using ^{17}O as a probe in the study of metalloproteins, similar to the present one, have been introduced only recently (12,13). Using higher enrichments of ^{17}O and even lower temperatures than in the present study, the method may be sensitive enough to monitor changes of the spin density at ^{17}O-nucleus. Such changes may result from direct or protein-mediated effects on the haem due, for instance, to the altered properties of the medium, the presence of allosteric effectors and/or substrates and inhibitors.

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