INTERMEDIATE SPIN-STATES IN ONE-ELECTRON REDUCTION OF OXYGEN–HEMOPROTEIN COMPLEXES AT LOW TEMPERATURE

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1. Introduction

The oxy–heme unit of oxygen–hemoprotein complexes contains four oxidizing equivalents in excess of the ferrous state. One-electron-equivalent reduction of the oxy–heme center would therefore convert it formally to the oxidation state of +5. This oxidation state is known to represent the catalytic intermediate compound I of peroxidase enzymes [1,2]. However, no such intermediate compound has been found in the case of the oxygen storage and transport hemoproteins such as myoglobin and hemoglobin. These results seem to be compatible with the difference in catalytic specificity and reactivity of oxy–heme complexes of heme proteins and enzymes.

Various theoretical and spectroscopic studies [3–7] have been recently undertaken to define stereochemical origins of the oxy–heme reactivity. The potential application of low-temperature radiolysis method [8,9] to produce one-electron-addition compounds of the oxygen–hemoprotein complexes in glassy matrices for spectroscopic analysis is demonstrated in the present work.

The data show the differences in the pathway of one-electron reduction of oxyperoxidase and oxymyoglobin, which can be correlated with basic functional properties of the hemoproteins.

2. Materials and methods

Sperm whale metmyoglobin was obtained from Koch Light Labs (pure, Fe, 0.31%). Horseradish peroxidase was purchased from ICN Pharmaceut. (RZ, 3.2, lot 0973).

Reduction of metmyoglobin was accomplished on deaerated solutions using a slight excess of sodium dithionite. Oxymyoglobin was prepared by stirring of the ferromyoglobin solution in the O_2 atmosphere. Peroxidase was transformed into the CO derivative by dithionite reduction under a CO atmosphere. The excess of dithionite and CO was removed by chromatography on a Sephadex G-25 column. Oxyperoxidase was prepared by photolysis of solution of the CO derivative in the presence of air. To check for dithionite effect, a radiation–chemical reduction system [10] ($CO_2^{\cdot -}$ generated by γ-irradiation of 0.1 M formate buffer solution saturated with N_2O) was also employed with identical results.

All low-temperature spectra were determined with buffered solutions in 50% ethylene glycol, frozen to form a clear glass. The oxygen– hemoprotein complexes in the solvent were irradiated by ^{60}Co γ-rays at the temperature of liquid nitrogen. The dose rate was 6 kGy/h. For optical studies, the solvent trapped electrons were removed by bleaching with $\lambda > 450$ nm light.

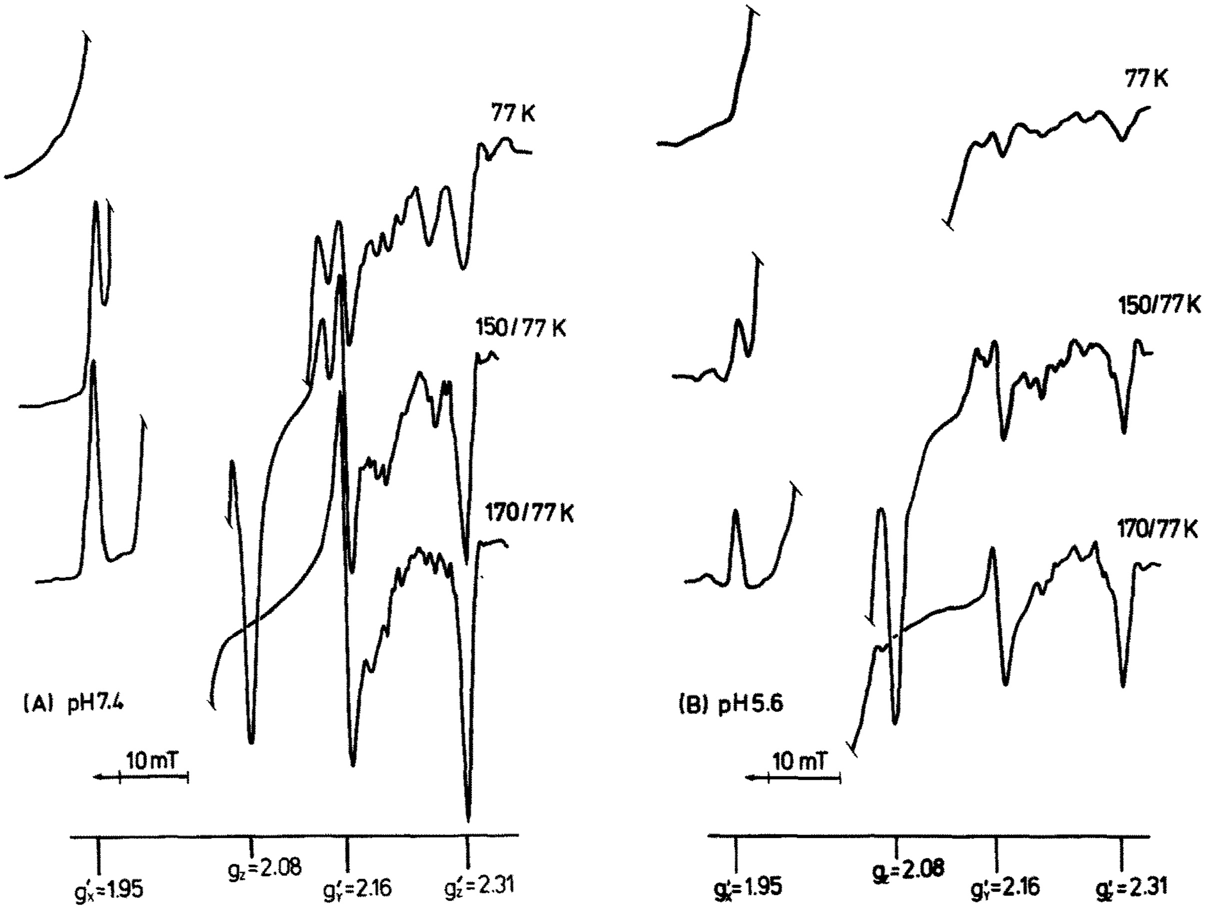
Optical absorption spectra were determined at 77 K with a Beckman DK-2A spectrophotometer. For measurements at higher temperatures a Beckman Acta MIV spectrophotometer equipped with a thermoregulated assembly was used. The cells of ~0.1 cm optical pathlength were employed. EPR measurements were made at 77 K with an X-band, SE/X-20 spectrometer (Poland) with 100 kHz field modulation. The samples were annealed by controlled warming in the Dewar insert. 1,1′-diphenyl-2-picrylhydrazyl radical and Mn^{2+} were used as field markers.

3. Results

Figure 1 shows the EPR spectra of irradiated aqueous ethylene glycol solutions containing oxyperoxidase. Irradiation at 77 K leads to the formation of an intense signal in the $g = 2.0$ region, due to radicals arising from the solvent radiolysis, and several weak lines in the $g = 2.1-2.3$ region. On careful annealing at >77 K and recooling to 77 K, the radicals signal decreases, and the additional signal, underlying the overall EPR spectrum, at $g = 2.08$ can be detected. When irradiated samples are annealed at 120–160 K, the signal at $g = 2.08$ decreases, being lost upon warming to >160 K. When the system is neutral or alkaline the loss of the signal at $g = 2.08$ results in a concomitant rise of intensity of a distinctive 3-line pattern with g-values of 1.95, 2.16 and 2.31 (fig.1A). The relative intensity of the two features changed with decreasing pH, and acidic ethylene glycol/water glass failed to give more of the 3-line pattern over the same temperature range (cf. fig.1A–C).

Exposure of aqueous ethylene glycol solutions containing oxymyoglobin to ^{60}Co rays at 77 K results in the formation of a primary paramagnetic product which exhibits 3 distinct g-values of 1.97, 2.11 and 2.21 (fig.2) as originally observed [11]. The higher-temperature product with g-values of 1.94, 2.16 and 2.30, resembles closely that of the corresponding oxyperoxidase species.

Figure 3A shows changes in optical absorption spectra of oxyperoxidase associated with γ-irradiation of the glassy solutions at 77 K. By irradiation, a decrease of the A_{577} (α-band) and A_{541} (β-band) characteristic to oxyperoxidase, is observed. The spectrum obtained after a complete transformation is characterized by the new β-band at 541 nm, larger than α-band at 573 nm, and is not altered by the pH



changes employed. The radiation-modified spectrum of oxyperoxidase in acidic ethylene glycol/water solution is changed markedly as temperature increases over 100–170 K (fig.4A). At 170 K it exhibits a unique set of the absorption bands centered at 530, 552, 628 and 656 nm, and the near-ultraviolet band at 418 nm (not shown).

The spectral changes which occur on irradiation of oxymyoglobin in aqueous ethylene glycol at 77 K resemble those of oxyperoxidase. Accordingly, in the visible region the spectrum of the product has two maxima, at 538 nm and ~580 nm (fig.3B). The feature α' at 568 nm arises from a small amount of ferrimyoglobin [9] present in irradiated samples. However, the spectrum undergoes only minor changes upon warming over 100–160 K (fig.4B).

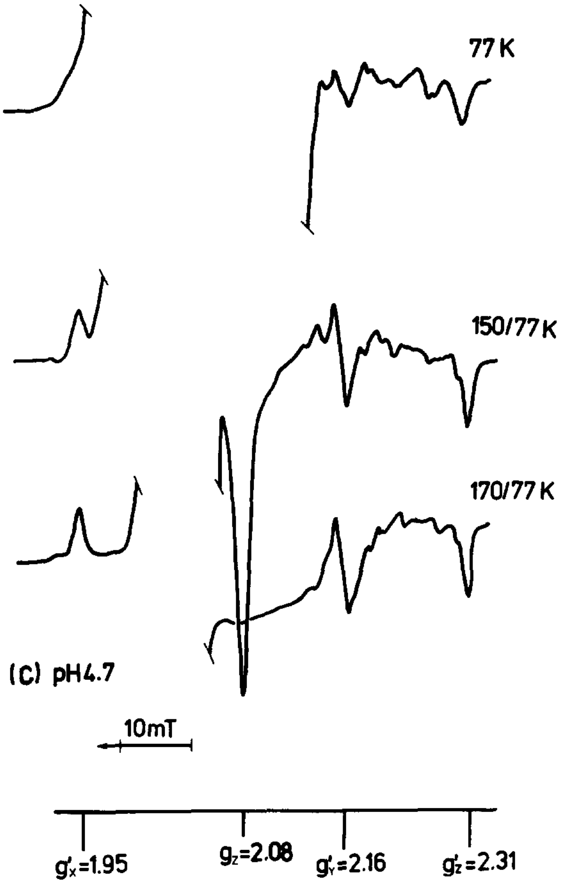


Fig.1. The EPR spectra of irradiated frozen ethylene glycol/ water solutions of oxyperoxidase at 77 K, containing: (A) 0.1 M phosphate buffer, (pH 7.4); (B) 0.1 M phosphate buffer (pH 5.6); (C) 0.1 M acetate buffer, (pH 4.7). Top: measured at 77 K after irradiation. Middle: annealed at 150 K for 10 min and recorded at 77 K. Bottom: annealed at 170 K for 10 min and recorded at 77 K. Protein concentration 0.5 mM. The γ-irradiation dose was 24 kGy. The solvent radical signal at g = 2.0 is omitted.

4. Discussion

It has been shown by several authors [9,11,12] that γ-irradiation of low-temperature glassy solutions of hemoprotein complexes results in efficient electron addition to the heme center, the probability of two-

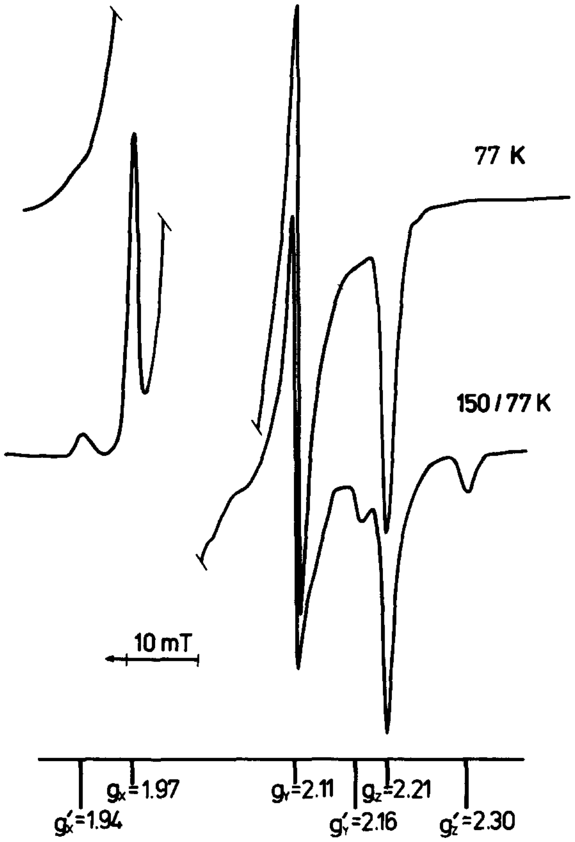


Fig.2. The EPR spectra of irradiated frozen ethylene glycol/ water solutions of oxymyoglobin at 77 K. Top: measured at 77 K after irradiation. Bottom: annealed at 150 K for 10 min and recorded at 77 K. Oxymyoglobin, 0.5 mM; 0.1 M Phosphate buffer (pH 7.0). The γ-irradiation dose was 24 kGy. The solvent radicals signal at g = 2.0 is omitted.

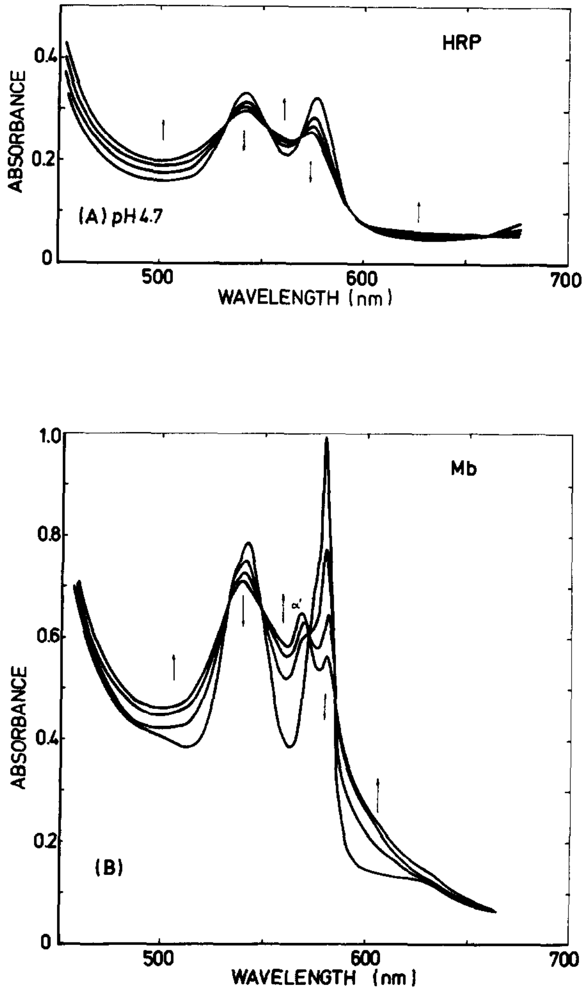


Fig.3. Optical absorption spectral changes associated with γ-irradiation of aqueous ethylene glycol solutions of oxy-hemoproteins at 77 K. (A) Oxyperoxidase. The spectra were obtained before and after irradiation with 24, 48 and 72 kGy of ^{60}Co rays at 77 K. Protein concentration ~0.2 mM; 0.1 M acetate buffer (pH 4.7). (B) Oxymyoglobin. The spectra were obtained before and after irradiation with 12, 24 and 36 kGy of ^{60}Co rays at 77 K. Protein concentration ~0.5 mM; 0.1 M phosphate buffer (pH 7.0). For other details see text.

electron capture at one center being negligibly small. The present experimental results demonstrate that oxygen–hemoprotein complexes are readily reducible at low temperature. The product of the one-electron reduction at 77 K can thus be considered as a $[\mathrm{Fe/II/O_2}]^-$ species. A simple picture of the

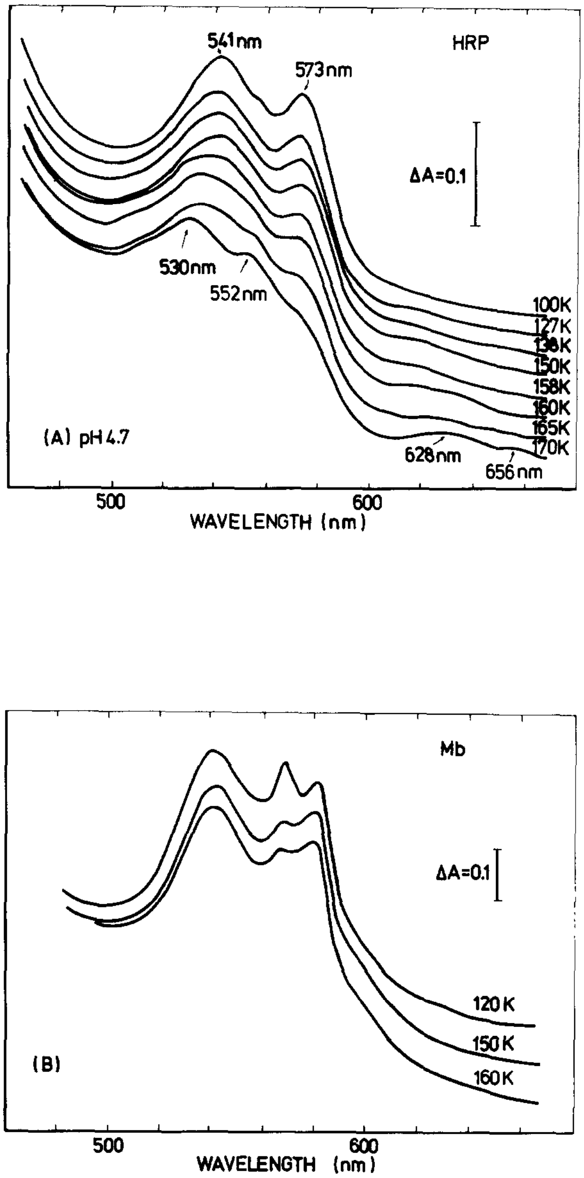


Fig.4. (A) The effect of temperature on the optical absorption spectra of the irradiated frozen ethylene glycol/water solution containing oxyperoxidase. The spectra were taken successively at temperatures indicated in the figure. The γ-irradiation dose was 72 kGy at 77 K. Sample conditions were the same as in fig.3A. The spectra are displaced on the ordinate axis. (B) The effect of temperature on the optical absorption spectra of the irradiated frozen ethylene glycol/water solution containing oxymyoglobin. The spectra were taken at temperatures indicated in the figure. The γ-irradiation dose was 36 kGy at 77 K. For sample conditions see fig.3B. The spectra are displaced on the ordinate axis.

paramagnetic $[Fe(II)O_2]^-$ center implies that the odd electron is located in a π^* molecular orbital having considerable density on iron and on oxygen [11]. The EPR spectrum of the reduced oxymyoglobin (fig.2) with 3 components of the g-tensor, resembles those of low-spin ferric derivatives, although it exhibits the reduced spread in g-values. However, it should be noted that the optical absorption spectrum of the $[Fe(II)O_2]^-$ is a typical low-spin ferric type (fig.3B) suggesting also $Fe(III)O_2^{2-}$ rather than $Fe(II)O_2^-$ for this species. In any case, the assignation of the low-spin ferric state to the $[Fe(II)O_2]^-$ may give the critical argument concerning a crystal field analysis [13] of EPR spectra of this species.

Oxyperoxidase gives the primary $[Fe(II)O_2]^-$ center which exhibits unusually small spread in g-values, as indicated by the only identified component with $g = 2.08$ (marked g_z only for correspondence). In attempting to account for the difference in the EPR spectra between the primary electron addition products of oxyhemoproteins one would consider the effect of Fe → O_2 charge transfer in these compounds. The unusually small spread in g-values in the case of oxyperoxidase, comparable with that of the isoelectronic oxy–cobalt heme derivatives [5,14], indicates the major unpaired spin density onto oxygen, resulting in a 'superoxide' $Fe(II)O_2^-$ structure. The extent of the Fe → O_2 charge transfer can be possibly controlled by the electron pair donation to the iron ion by the lone pair of the proximal imidazole ligand. This *trans*-effect is largely conformation-controlled, as shown with the nitrosyl heme derivatives [15]. Therefore, it seems that oxygen in oxymyoglobin and oxyperoxidase responds primarily to different steric constraints, leading to different electronic states of the $[Fe(II)O_2]^-$ species.

When warmed to >77 K the new paramagnetic center, exhibiting the larger spread in g-values, is formed as a result of protonation of the primary reduced species [11]. These centers in oxymyoglobin and oxyperoxidase are closely similar, and can be considered as $Fe(III)O_2H^-$ units. The second protonation at higher temperature is required for conversion of these species to high-spin Fe(III) with loss of peroxide. It has been noticed [16] that acidic glycol glasses favour to give high-spin Fe(III) in the case of myoglobin and hemoglobin. In the case of peroxidase, the optical absorption data indicate that in acidic solution a novel species is formed from the primary product upon softening of the rigid glass. The general features of the absorption spectrum of this species, with α, β and γ or Soret bands at 552, 530 and 418 nm, respectively (fig.4A) are ascribable to an Fe(IV) porphyrin [1]. Thus the electronic configuration of the species would be similar to that proposed for peroxidase compound I. The accepted electronic configuration of compound I has two unpaired electrons situated on the Fe(IV) and one electron located on a nearby radical. The almost unperturbed spectrum of Fe(IV) porphyrin obtained here, suggests that the radical is not located close to the porphyrin π-system [17]. Since this is expected to be an organic free radical, its EPR signal should be obscured by the solvent radicals'signal. Thus the new peroxidase species resembles both the cytochrome *c* peroxidase complex ES [18] and the photochemically produced species of peroxidase compound I at low temperature [19]. The reason why the reduced peroxidase species showed the different response to temperature increase in the low pH solution would be consistent with the necessity of the simultaneous participation of two proton sources in the reactions leading to the compound I-like structure:

$$[Fe(II)O_2]^- + 2\,H^+ \rightarrow \{Fe(IV)\ldots{}^{\cdot}OH\} + OH^- \text{ or}$$

$$Fe(IV)OH^- + {}^{\cdot}OH\ {}^{\cdot}OH + HR(\text{protein}) \rightarrow$$

$${}^{\cdot}R(\text{Protein}) + H_2O$$

Such a concerted proton transfer in the transient state, leading to heterolysis of O–O bonds, has been proposed [20] in the conversion of peroxidase into compound I with alkyl peroxides. There is much recent evidence for peroxidase showing the presence of potential proton sources near the heme center by virtue of pK values, essential also to peroxidase activity [21,22].

Finally it should be emphasized that the above results may give information connected to the unsolved problems of oxyperoxidase reactivity. It has been suggested that oxidase activity of peroxidase is attributed to its oxygenase-like function, where the oxygen binding to the ferro enzyme is an essential process. In the peroxidase–oxidase oscillatory reactions a considerable amount of the enzyme is converted into oxyperoxidase, i.e., compound III [23] but it is still an open question whether compound III is an active intermediate. In these reactions the formation of compound I from compound III has been proposed [24,25] as a result of a direct donation of electrons to compound III by substrate molecules. There are several substrates which have an effect on the breakdown of compound III [25]. On the other hand, of great interest is the possible role of compound III in reactions in which hydroxylation of aromatic molecules has occurred [1,26]. It has been proposed that ˙OH radical is required for this reaction but the mechanism of ˙OH radical generation is still obscure [26].

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