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UNUSUAL SPIN-STATE TRANSITIONS IN THE REDUCTION OF FERRYLMYOGLOBIN AT LOW TEMPERATURE

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SUMMARY: Ferrylmyoglobin is reduced in γ-irradiated aqueous ethylene glycol solutions at 77 K to form a novel ferric low-spin type compound.The transition of the low-temperature product to the final high-spin ferrimyoglobin at higher temperature involves several intermediate species appearing in a sequence as temperature increases.With manifestation of the transient low-spin forms and probably the mid-spin S=3/2 state,the process of ferrylmyoglobin reduction is found to display the spin-state transitions scarcely observed in ferric heme reactions.

INTRODUCTION: Ferrylmyoglobin is a peroxide-hemoprotein compound that contains iron in the ferryl (Fe(IV)) oxidation state (1).Since ferrylmyoglobin is formed in one-electron-equivalent oxidation of ferrimyoglobin (1,2),the reduction of the ferrylhemoprotein would convert it back into the ferric state. An approach to the mechanism of these redox reactions seems to be important in view of the crucial role of quadrivalent iron compounds played in biological peroxidations (3).To gain information on the details of the ferrylhemoprotein reduction, the low-temperature radiolysis method (4,5) which allows investigation of the nature of the transient species in the reaction by means of EPR and optical spectroscopies,has been applied in the present work.The data show that the reduction of ferrylmyoglobin to ferrimyoglobin proceeds by a pathway involving several intermediate spin-states of the ferric heme,

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where some of the spin-state transitions appear to be unusual in ferric heme magnetochemistry.

MATERIALS AND METHODS: Sperm whale ferrimyoglobin (Fe 0.31%, batch No 76186) was obtained from Koch Light Laboratories Ltd. Ferrylmyoglobin was formed in the reaction of ferrimyoglobin with a fourfold excess of hydrogen peroxide in 0.2 M sodium phosphate buffer solution at pH 8.0 (6).The reaction was stopped,within 1 to 2 min,by quenching of the solution in liquid nitrogen immediately after addition of ethylene glycol.All low-temperature spectra were determined for solutions in 50% ethylene glycol,frozen to form clear glasses.The ferrylmyoglobins in the glass-forming solvent were reduced by electrons produced upon irradiation at 77 K (4,5).The irradiation was carried out by Co-60 rays at a dose rate of 6 kGy per hour. For optical studies the solvent trapped electrons were removed by bleaching with visible light of the tungsten lamp.Optical spectra were determined at 77 K with a Beckman DK-2A spectrophotometer,while for measurements at higher temperatures a Beckman Acta MIV spectrophotometer was used.The irradiated samples were annealed in a cold nitrogen gas flow system.EPR spectra were recorded at 77 K with X-band microwave spectrometer SE-X/20 (Poland) provided with TE_{104} cavity,at 100 kHz magnetic field modulation.DPPH and Mn^{++} were used as field markers.

RESULTS: Fig.1 shows changes in optical absorption spectra associated with γ-radiation induced reduction of ferrylmyoglobin by electrons in ethylene glycol/water glass at 77 K.The

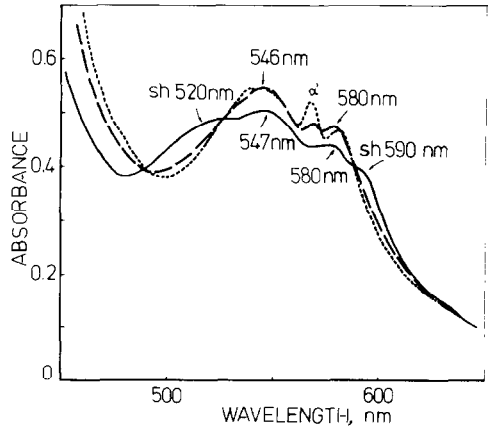

FIG.1. Optical absorption spectral changes associated with γ-irradiation of aqueous ethylene glycol solutions of ferrylmyoglobin at 77 K.The spectra were obtained before (solid line) and after irradiation with 24 kGy (dashed line) and 48 kGy (dotted line) of Co-60 rays at 77 K.Protein concentration approx.0.5 mM.Phosphate buffer,0.1 M,pH 8.0.Optical cells of 0.1 cm were used.For other details see text.

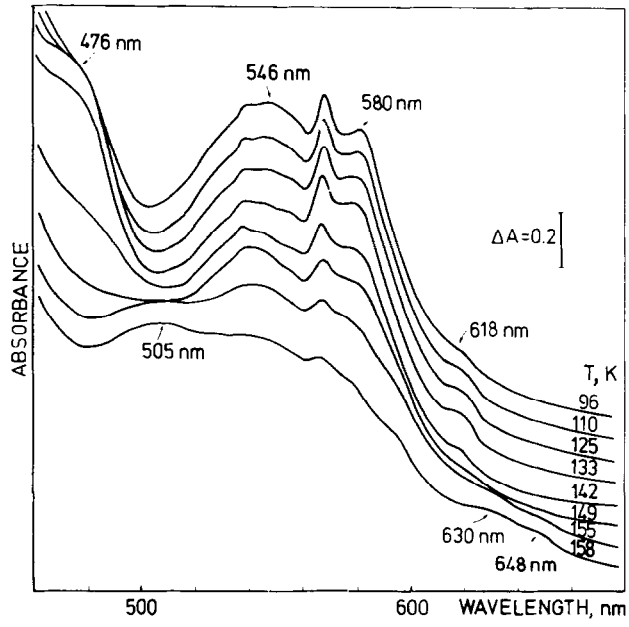

FIG.2. The effect of temperature on the optical absorption spectra of the irradiated frozen ethylene glycol/water solution containing ferrylmyoglobin.The spectra were taken successively at temperatures indicated in the figure.The γ-irradiation dose was 24 kGy at 77 K.Sample conditions were the same as in FIG.1,except that the protein concentration of approx. 1 mM was used.The spectra are displaced on the ordinate axis.

absorption spectrum of the reduced ferrylmyoglobin,with two bands in the visible region centered at 580 nm (α-band) and 546 nm (β-band),is characteristic of the low-spin state of the ferric heme.The additional small features seen in the spectrum (with major component of the structure marked α') are ascribable to low-spin ferrous heme (5) and can thus arise from two consecutive one-electron additions to the ferrylheme center at 77 K,since they become more intense on irradiation with larger doses.

The experiments with warming the irradiated solutions confirmed that the spectrum at 77 K reflected the transient state in one-electron reduction of ferrylmyoglobin.Fig.2 illustrates the change of the absorption spectra upon the successive increase of temperature in the range of 90 to 160 K.The

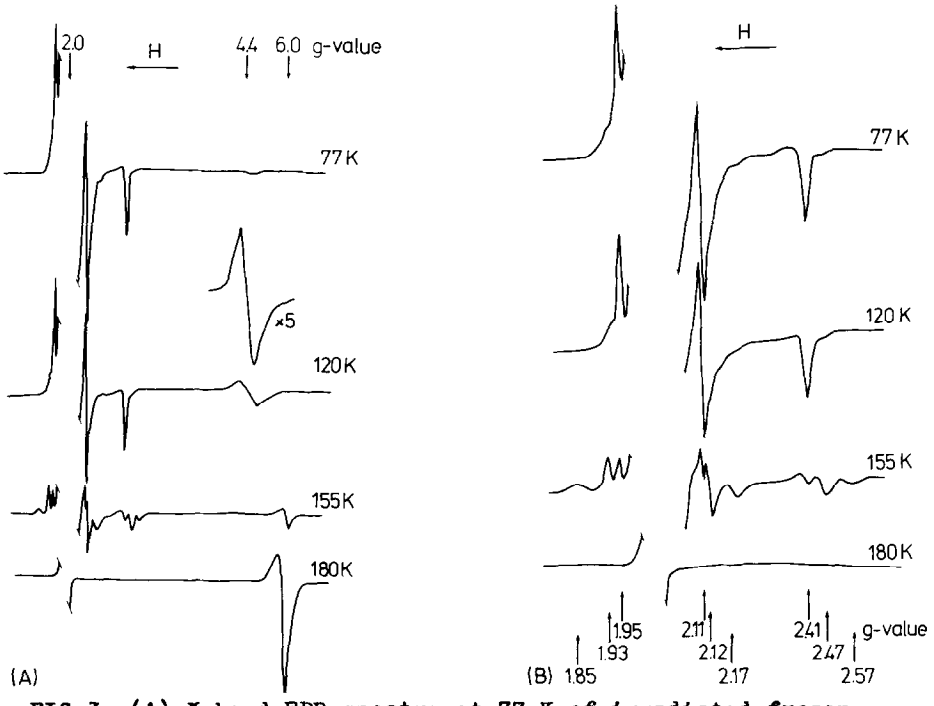

FIG.3. (A) X-band EPR spectra at 77 K of irradiated frozen ethylene glycol/water solutions of ferrylmyoglobin at 77 K and annealed for 10 min at different temperatures indicated in the figure. (B) Temperature dependence of the EPR spectra in the low-spin region.Protein concentration 2 mM.The γ-irradiation dose was 24 kGy.The solvent radicals signal at g = 2.0 is omitted.

transition from the low-spin state of the ferric heme to a novel,apparently high-spin state,characterized by absorption bands centered at 476 and 618 nm,is clearly seen with increasing temperature to about 125 K.The higher temperature spectrum is almost unchanged at 77 K,suggesting that the new compound,which is actually seen up to 140 K,is not basicly in thermal equilibrium between two spin-states.Above this temperature the spectrum is indicative of a mixture of the typical low- and high-spin states of the ferric heme.

Fig.3 shows the EPR spectra of the irradiated aqueous ethylene glycol solutions of ferrylmyoglobin at liquid nitrogen temperature.Irradiation at 77 K leads to the formation of an intense signal in the g = 2.0 region (not shown) due to ra-

dicals arising from solvent radiolysis,and a distinctive three-line pattern with principal values of g-tensor at g_x = 1.95, g_y = 2.11 and g_z = 2.41,which can be attributed to a low-spin ferric compound.The temperature dependence of the low-spin type EPR absorptions is shown in more detail in Fig.3B.The EPR spectra of samples annealed at about 160 K show two sets of other low-spin compounds.The species with g-values at 1.93 , 2.12 and 2.47 is a minority constituent of the low-spin type absorption,while that with g-values at 1.85 , 2.17 and 2.57 is characteristic of a ferrimyoglobin hydroxide complex (7). When irradiated samples are annealed in the range of 90 to about 140 K the EPR at 77 K reveals a novel resonance signal having g-value of 4.4 and the line-width of about 160 G (Fig.3A).

The EPR spectra of samples annealed at higher temperature clearly show the presence of the high-spin ferrimyoglobin complex,exhibiting the well known signal at g = 6.At 180 K all the low-spin type absorptions are lost;the purely high-spin state remains on recooling at 77 K (Fig.3A).The temperature induced change of EPR spectra is in agreement with the effect of temperature on the optical absorption spectra (Fig.2) showing that the spin-state transitions seen optically correspond to those detected by EPR.The results point also to an irreversible character of the transition from the initial low-spin state to the final high-spin state of the reduced ferrylmyoglobin.

DISCUSSION: The present experimental results demonstrate that γ-irradiation of low-temperature glassy solutions of ferrylmyoglobin results in effective one-electron reduction of the ferrylheme center.The reaction at 77 K leads to the formation

of ferric low-spin species which convert to high-spin ferrimyoglobin upon warming up.There exist several intermediate species in this transition appearing in a sequence,as shown by their optical absorption and EPR characteristics.The first transient species in the sequence are unusual if one compares their optical absorption and EPR spectra with those of the typical ferric heme compounds.The most likely explanation for the unusual g-value ($g_{\perp} \sim 4.4$ for this apparently high-spin state) is that the ground state heme-iron electronic configuration is a mid-spin S = 3/2 state,or that this state consists of a quantum mechanical admixture of the close-lying S = 3/2 spin quartet and the S = 5/2 spin sextet (8).

An alternative explanation would require the high-spin iron ion to reside in an environment of almost purely rhombic symmetry (9),which is less likely,because the amount of rhombicity observed in high-spin ferrihemoproteins does not exceed about 16% (10).Considering the optical and magnetic properties of the transient state it is of interest to assume that the iron's sixth ligands are different between the transient and the final high-spin states.From a number of works (11,12) it may be concluded that the iron ligand in the ferrylheme is O= (oxometal).Consequently,the properties of the low-temperature reduced ferrylmyoglobin can be envisaged as due to the exceptional ligand of the ferric heme iron.The product of ferrylmyoglobin reduction at 77 K can thus be considered as a Fe(III)O species.The EPR spectrum of this species resembles that of the $Fe(III)O_2H^-$ center formed from the electron addition compound of the heme-dioxygen unit (13,14).The apparent differences in the EPR spectra between the Fe(III)O and $Fe(III)O_2H^-$ species can be attributed to a variable rhombici-

ty of the crystal field in these ferric low-spin heme comlexes (15).The cryterion for obtaining the S = 3/2 spin state for the Fe(III)O is that the one-electron d_z2 orbital remains close in energy to t_{2g} orbitals,and that the energy difference between the $d_{x^2-y^2}$ and t_{2g} orbitals must be high enough allowing the former to be unoccupied.This requires strong bonding in the equatorial (x,y) directions and very weak bonding in the axial (z) direction,cf.(16).According to the valence bond theory,the Fe(III)O derivatives should thus fall into "covalent", Fe(III)=O, S = 1/2,or "ionic",$Fe(III)O^{2-}$,S = 3/2,categories. The positions of the optical absorption bands associated with the latter structure is then expected to arise from a configuration interaction between a charge transfer state and the porphyrin π states (17).The observed temperature dependence of the transition between the low-spin and mid-spin states, showing that they are not in a simple thermal equilibrium,can be accounted for by assuming that factors other than the spin degeneracy must also play a role.A plausible hypothesis is to suppose effects on the spin-state transition resulting from structural changes which are induced by a cooperative interaction between the porphyrin and the globin (18).This is consistent with a prerequisite for stalilization of the S = 3/2 state in a ferrihemoprotein,based mainly on steric constrains which lead to a change of the iron-heme configuration (8,19,20). The ferric heme-hydroxide complex can be easily formed as a result of protonation of the primary reduced species.A second protonation is required for conversion of the hydroxide complex to high-spin ferrimyoglobin,according to a pK value of 9.2 (7).The reason why the hydroxide complex is formed as a transient species at pH lower than the pK value,is probably

that the second protonation involves hydronium ions from the bulk of the solvent,and the proton transfer is inhibited at lower temperature.The proton transfer reactions can be postulated as a rate-determing factor in the reduction of quadrivalent iron compounds of peroxidase enzymes in biological processes,cf.(3).

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