ON THE INFLUENCE OF ATP ON THE ELECTRON PARAMAGNETIC RESONANCE SPECTRUM OF METHEMOGLOBIN

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1. Introduction

Benesch and Benesch [1] have observed that the binding of organic phosphates such as 2,3-diphosphoglycerate and ATP to hemoglobin decreases the O_2 affinity of hemoglobin. This decrease could be caused by a conformational change of the protein, in particular of the heme environment, involving the prosthetic group. Chanutin and Hermann [2] have found that organic phosphates also interact with methemoglobin. The paramagnetism of methemoglobin allows EPR (Electron Paramagnetic Resonance) measurements. Characteristic changes in the EPR spectrum reveal information concerning the electronic structure of the iron porphyrin complex at which structural changes of the protein in the vicinity of the heme influence the electronic configuration of the heme. The EPR spectrum of methemoglobin is specifically influenced by ligand binding [3], pH [4], and in the presence of inorganic phosphate [5]. This study shows that ATP causes a specific effect on the EPR spectrum of methemoglobin.

2. Materials and methods

Red blood cells were prepared in the usual manner by washing freshly drawn human blood with physiological saline. The intracellular oxidation was performed by sodium nitrite. After hemolysis with distilled water the stromata were removed by centrifugation. The hemolysate was stripped by incubation with charcoal and DEAE-Sephadex A-50 and a 24 hr dialysis against distilled water at 4°C. The amount of total phosphate was about 1.5 mole phosphate per mole hemoglobin. In some cases methemoglobin was deionized with a mixed-bed exchanger (Biorad AG 501–X 8 (D)). This preparation containing 0.04 mole phosphate per mole hemoglobin shows the same EPR character as methemoglobin with 1.5 mole phosphate per mole hemoglobin. EPR measurements were made with a laboratory-type X-band Superheterodyn spectrometer [6, 7] at temperatures of 77° and 20°K. The spectra were recorded as the first derivation of the resonance absorptions.

3. Results

As can be seen from fig. 1a, stripped hemoglobin shows strong absorption in the region of *g*-factor 2 which indicates a considerable low-spin portion. This low-spin portion is markedly reduced in the presence of 20 mM ATP at which the high-spin absorption with a *g*-value of 5.9 is correspondingly increased (fig. 1b), since the high-spin and low-spin portions possess a temperature-dependent equilibrium [8]. Measurements at 20°K (fig. 2a) show that the low-spin portion is made up of several superposed absorption lines suggesting that stripped methemoglobin has at least 2 low-spin conformations. Recently two conformeres of horse methemoglobin were detected by EPR studies [9]. The EPR spectrum recorded at 20°K in the presence of 20 mM ATP revealed complete conversion of the low-spin portion into the high-spin form at this temperature. Fig. 2b shows the typical absorptions of a high-spin complex with $g_\perp = 5.9$ and

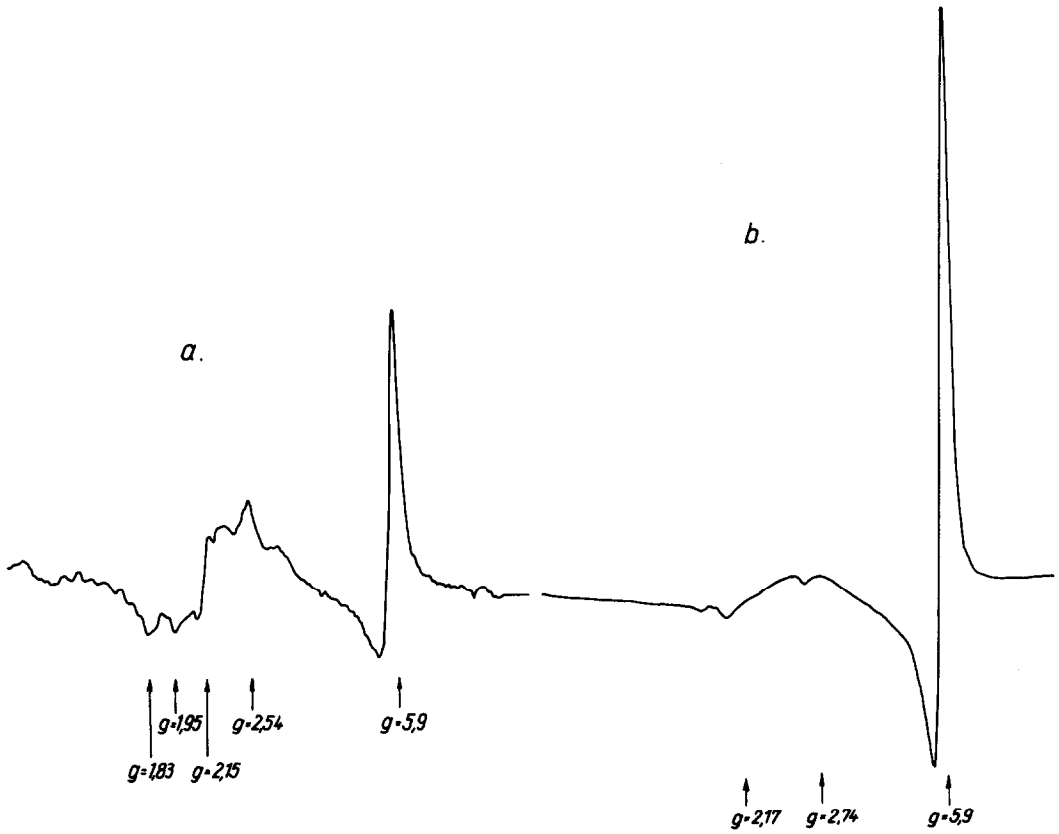

Fig. 1. Electron paramagnetic spectra of methemoglobin at 77°K. (a) stripped; (b) in presence of 20 mM ATP, pH 6.8; hemoglobin concentration: 2 mM per heme. (The spectra were taken with the same amplifier gain and the same microwave power, figs. 1 and 2).

$g_{\parallel} = 2$. Addition of salts (KCl, $K_3Fe(CN)_6$) at the same concentration as ATP to the stripped methemoglobin did not show the same effect as ATP. A tenfold excess of ATP was used in order to achieve complete saturation of all binding sites including those with lower affinity [10].

4. Discussion

Hemoproteins with trivalent heme iron are known to exist in two electronic configurations in the basic state [8]. The configuration with 5 unpaired electrons is the high-spin form, and that with one unpaired electron, the low-spin form. In the EPR spectrum the high-spin absorption is marked by an intensive band at $g = 6$; the low-spin form of the hemoproteins shows a broad absorption in the region of the g-factor 2, usually with 3 distinct g-factors. EPR measurement of stripped methemoglobin at pH 6.8 shows that at 77°K and 20°K both configurations are present. As ATP is bound to the protein far from the prosthetic group, where the binding sites are not yet known [11, 12], the effect of ATP upon the EPR spectrum of methemoglobin is conceivable only by a conformation change of the protein. This alters the geometry of the heme pocket in such a way that the distance between the proximal histidine (F 8) and the iron increases. Hoard [13] was able to demonstrate different binding lengths of the heme iron for high–low-spin complexes. The altered protein structure of the heme pocket diminished the interaction between the δ-electrons of the imidazole of the proximal histidine and the d_z2 orbitals of the iron so as to cause weakening

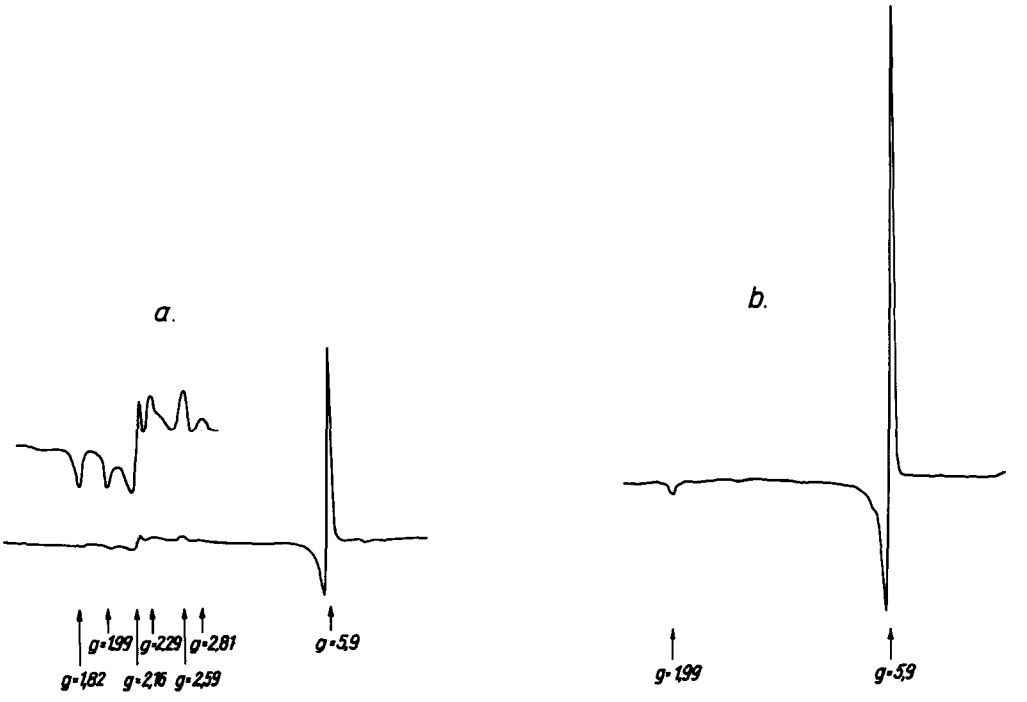

Fig. 2. Electron paramagnetic spectra of methemoglobin at 20°K. (a) stripped; above curve: tenfold amplifier gain. (b) in presence of 20 mM ATP. pH 6.8; hemoglobin concentration 2 mM per heme.

of its ligand field, i.e., the high-spin form increases. This weakening of the ligand field of iron caused by the protein conformation probably also occurs with bivalent hemoglobin in the native state under the influence of ATP, which is the cause of the lower oxygen affinity of hemoglobin in the presence of organic phosphates.

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