Paramagnetic Species in β-Thalassemic Sera: an ESR Study

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Homozygous beta thalassemic sera show an electron spin resonance (ESR) signal at $g \simeq 6$ which is not present in normal and heterozygous sera. The signal arises from high spin heme groups exhibiting a departure from tetragonal symmetry toward rhombic. Binding by a serum protein is very likely responsible for such a distortion. In contrast with other authors' findings, we report that heme-human albumin complex shows a similar rhombic distortion.

The observed ESR signal at $g \simeq 6$ is then attributed to this complex.

Introduction

Beta thalassemia is a hereditary pathological condition resulting from the deficient production of one of the two subunits of hemoglobin, the beta polypeptide chain. In homozygous beta thalassemia, excess unstable alpha chains precipitate in erythrocytes, forming inclusion bodies [1]. These inclusions, similar to the Heinz bodies, are believed to be responsible for the damage to the membrane of the erythrocytes and for their premature destruction [2].

It has been shown that the inclusion bodies found in beta thalassemia have the optical and electron spin resonance spectra characteristics of hemichromes [3, 4]. It is however, believed [5] that the formation of hemichromes may be only one intermediate step, probably followed by heme release, in a series of reactions resulting in the denaturation of the hemoglobin subunits and the subsequent destruction of the red cell by hemolysis. Although it is known that the inclusion bodies are eventually removed by the spleen, very little is known about the dynamics of these paramagnetic species.

In order to contribute to this knowledge we have undertaken an ESR study on homozygous beta thalassemic sera, where it is not unreasonable to imagine that some paramagnetic species could be found after hemolysis of red cells and before their removal from the blood stream.

We report here the results concerning the detection, in these sera, of an ESR signal at $g \simeq 6$, characteristic of high spin heme groups, in which, however, the symmetry of iron is rhombically distorted from tetragonal.

The signal has been attributed to the heme-human albumin complex after the ESR reinvestigation of the latter.

Materials and Methods

The sera analyzed were prepared by centrifugation after venipuncture of forty homozygous thalassemic patients (splenectomized or not) living in the south of Italy. The serum samples were placed in 4.8 mm i. d. precision class tubes open at both ends, frozen in liquid nitrogen and stored. To record the ESR spectrum the sample was removed from the tube by warming the surface just sufficiently to allow the cylindrical sample to be pushed from the tube. It was then placed in an E-246 Varian dewar filled with liquid nitrogen. All samples were large enough to fill completely the ESR sensitive volume in the resonant cavity. ESR spectra were recorded by a Varian spectrometer E-4 with 100 KHz modulation using a microwave frequency of 9,130 MHz. Magnetic field calibration was performed with a Magnion Precision NMR Gaussmeter, Model G-502, using the proton magnetic resonance as a standard. Hemin chloride was a Sigma Chemical Co. product (type III). Human albumin was purchased from two different commercial houses (Fluka and Sigma). The products were used without further purification.

Results and Discussion

When the ESR spectrum of a normal serum is recorded at 77 K two main multiplets are encoun-

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Abbreviation: ESR, Electron Spin Resonance.

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tered: one at $g \simeq 2.05$, due to copper ceruloplasmin, and the other at $g \simeq 4.3$, due to iron transferrin [6, 7].

Besides these resonances, all the homozygous beta thalassemic sera investigated show an additional signal at $g \simeq 6$ (Fig. 1) this not present in heterozygous sera. It has long been known that ESR resonances at this g value are due to high spin iron-containing proteins [8]. A detailed examination of this signal shows that the resonance is split into two

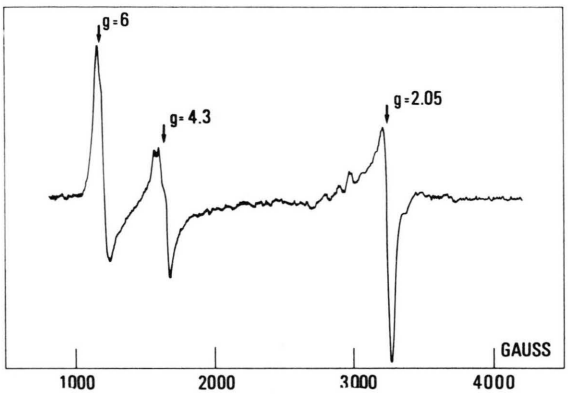

Fig. 1. ESR spectrum of homozygous beta thalassemic sera recorded at 77 K. Microwave power level: 20 mW. Magnetic field sweep rate: 4.000 gauss in 16 min. Time constant: 3 sec. Modulation amplitude: 5 gauss.

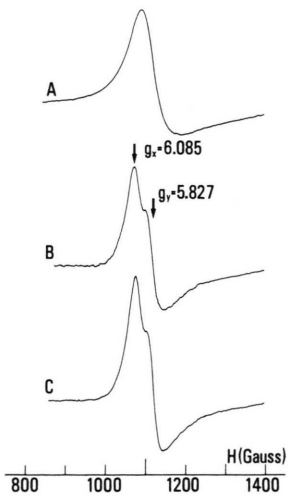

Fig. 2. ESR spectra in the region $g = 6$ of: A) isolated ferrihemoglobin from homozygous beta thalassemic sera in frozen aqueous solution or added to normal and heterozygous serum; B) homozygous beta thalassemic sera; C) hemin chloride added to normal or heterozygous sera.

resolvable g values $g_x = 6.085$ and $g_y = 5.827$ (Fig. 2 B). This splitting may be ascribed to a departure from tetragonal symmetry of heme iron toward rhombic [9]. In general, departure from tetragonality may be brought about by constraints imposed on the heme by the protein environment. A first approach led us to suppose that the signal could arise from traces of slightly altered ferrihemoglobin, the presence of which in the serum might be due to hemolysis which is a normal factor in beta thalassemia. However, such a hypothesis was not confirmed either by the ESR analysis of an aqueous solution of isolated ferrihemoglobin from the same patients, or by addition of such hemoglobin to normal or heterozygous thalassemic sera. In both cases the ESR spectra obtained in the region of $g \simeq 6$ were identical to that shown in Fig. 2 A, which is typical of normal ferrihemoglobin. Furthermore, one might imagine that the signal could arise from ferric alpha chains which tend to accumulate in the red cells of these patients and might pour into the serum after hemolysis. In effect, it has been reported [10] that the symmetry of the heme of isolated alpha chains is different from that of the heme of these same subunits when associated with ferric beta chains in the parent tetramer. Nevertheless the g_x, g_y values and the separation between derivative extrema reported by these authors for the ESR spectra of isolated alpha chains are quite different from these relative to our ESR signal. In addition, heating the sera up to 50 °C or increasing their pH up to 10.5 did not convert the high spin signal to a low spin one as should have occurred if the signal at $g \simeq 6$ were due to isolated alpha chains [10]. In particular, the additional ESR signal present in homozygous beta thalassemic sera remained unaltered, in shape and intensity, even at pH 11 or when the samples were exposed to the air at room temperature for a number of days.

In an attempt to identify this signal, we added 0.2 ml of an alkaline solution (pH = 8.6) containing 0.04 mg of hemin chloride to 2 ml of both normal and heterozygous serum and lowered the pH of the mixtures to the physiological value. The ESR spectrum recorded in the two cases was identical to that reported in Fig. 2 C. As can be seen, the shape and g value obtained are the same as those of the ESR spectrum from the patients' sera (Fig. 2 B). Even in this case the shape and intensity of the resonance at $g \simeq 6$ remained unaltered over a wide range of pH and temperature.

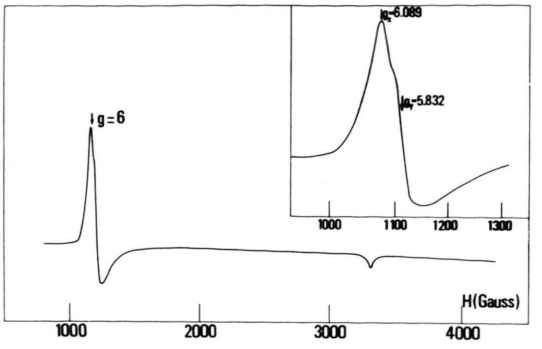

Fig. 3. ESR spectrum of aqueous solution of heme-human albumin complex, recorded at 77 K. Recording conditions as in Fig. 1. *Insert:* detailed scanning of the spectrum around the $g = 6$ value. Magnetic field sweep rate: 1.000 gauss in 8 min. Time constant: 1 sec.

On the basis of these results, it can be concluded that the ESR signal observed in the homozygous beta thalassemic sera is due to high spin heme groups. Moreover, since the ESR spectrum of a hemin chloride aqueous solution does not show any departure from tetragonal symmetry, it can be deduced that in serum the heme should be tightly bound by a protein so that the tetragonal symmetry of iron is distorted toward rhombic.

On the other hand, Bearden *et al.* [11] have reported that the two most generally accepted carriers of heme in human serum, hemopexin and albumin, give, when bound to heme *in vitro,* an ESR spectrum quite different from that obtained by us in serum. In particular, the complex heme-hemopexin displays a low spin ESR spectrum; whereas heme-albumin shows a typical high spin spectrum which does not indicate any rhombic distortion of the tetragonal symmetry of the heme iron.

However, since no high spin heme carriers, other than albumin, have been proposed up to now, we were led to reinvestigate the low field region of the ESR spectrum displayed by the complex heme-albumin. We have recorded the ESR spectrum of the complex at different pH values and at various heme to albumin molar ratios. The spectrum obtained at 77 K for the complex heme-albumin is shown in Fig. 3. In the insert of the same figure a detailed scanning of the spectrum around $g = 6$ region is reported. As observed, the ESR signal exhibits a departure from tetragonality with $g_x = 6.089$ and $g_y = 5.832$. Such a rhombic distortion was encountered for all the heme-albumin samples whose pH value ranged between 5 and 11 and whose molar ratio varied from 1 : 1 to 1 : 10. Both commercial albumin samples used showed a similar behaviour.

By comparing Figs. 2 and 3, it may be observed that the g_x and g_y values found for the heme-albumin complex are very similar to those obtained for the homozygous thalassemic sera or for normal sera with added hemin.

On the base of these results it is quite straightforward to attribute the additional signal present in the pathological sera to the complex heme-albumin.

Effectively, this complex, otherwise called methem-albumin, has been detected by optical absorption in the serum of patients with various hemolytic disorders [12].

From a molecular point of view, the rhombic distortion observed could provide a new insight into the elucidation of the interaction site of albumin with heme which still remains an open problem [13].

Little can be said about the origin of the heme groups in sera and how their release is related to the molecular dynamics of the pathological state. In keeping with the findings of some authors, especially those of Rachmilewitz and Thorell [3] and of Bunn [5], we can only suggest that the release of the heme occurs after the formation of hemichromes and probably during the attack of the erythrocyte membrane which leads to hemolysis.

We feel that the above observations could contribute to a new approach in the study of the molecular pathology of the beta thalassemias, also with respect to the aberrant catabolism [14] of the hemes in these pathological states.

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