Mössbauer spectroscopy of haemoglobins

Study of the relationship of Fe^{2+} electronic and molecular structure of the active site

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Human normal, adult and foetal oxyhaemoglobins and oxyhaemoglobins from leukaemic patients were studied, by Mössbauer spectroscopy. The estimations of quadrupole splitting ΔE_Q and isomer shift δ allow one to distinguish between adult, foetal and leukaemic oxyhaemoglobins. The differences in the Fe^{2+} electronic structure and active site molecular structure of foetal and leukaemic oxyhaemoglobins were analyzed.

Fe^{2+} Electronic structure Molecular structure (Adult, Fetal, Leukemic) Oxyhemoglobin Mössbauer spectroscopy

1. INTRODUCTION

The problem of the relationship between molecular structure and biological function of haemoglobin stimulated many investigations that contributed to an understanding the molecular basis of biological function [1]. The study of the role of the Fe^{2+} electronic structure in the relationship of structure and function is of interest. The Mössbauer effect is one of the techniques most sensitive to the electronic structure of iron complexes and is widely used for studies on haemoglobin [2,3]. Investigations of the electronic structure of Fe complexes in normal and anomalous haemoglobins are of special interest. In the present paper the three oxyhaemoglobins studied were human adult $HbAO_2$, foetal $HbFO_2$ and $HbLO_2$ from leukaemic patients. The first two haemoglobins differ in molecular structure ($2\alpha 2\beta$ and $2\alpha 2\gamma$ subunits, respectively) and oxygen affinity [4]. The features of leukaemic haemoglobin have not been studied enough, although there are indications about structural changes in haemoglobin and other haem-proteins during the malignant process [5–7].

2. MATERIALS AND METHODS

Erythrocytes were prepared from adult venous blood (~97% HbA), from newborn children's umbilical-cord blood (~85% HbF), and from venous blood of patients with acute and chronic leukaemia of the myeloid and lymphocytic variety. A 5% Na citrate solution was used to prevent blood coagulation. The red blood cells were washed twice by 10 min centrifugation at 1500 rpm with 0.9% NaCl solution. The concentrated erythrocytes were saturated with oxygen and immediately frozen by liquid nitrogen. Red blood cell samples had an effective thickness ranging from 0.3 to 0.8 mg Fe/cm^2. For measurements samples were placed into the cryostat at a temperature of 87 ± 3 K. Mössbauer spectra of oxyhaemoglobin in erythrocytes with natural abundance of ^{57}Fe (~2.19%) were measured with the standard constant acceleration spectrometer using a 511 channel analyser. The velocity resolution values for all experiments were in the range 0.011–0.014 mm/s per channel. Their variation was due to tuning in the spectrometer driving system before the experiment began. The deviation

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of the velocity resolution during a single measurement time (~100 h) was ≤0.8%. The drift of zero point velocity was not more than half of the velocity resolution value. The 5×10^9 Bq ^{57}Co(Cr) source was used at room temperature. The linewidth Γ of the Mössbauer spectrum of sodium nitroprusside (5 mg Fe/cm^2) measured during 100 h was ≤0.26 mm/s. The Mössbauer spectra of the oxyhaemoglobins were fitted by least squares method using Lorentzian line shape. The spectral parabolic distortion due to geometrical factors was first corrected. The contribution of ^{57}Fe nuclei of the scintillator detector beryllium window to the spectrum was taken into account.

In addition, the haemoglobin oxygen saturation curves were measured by the Radiometer (Copenhagen) equipment (oxygen saturation meter and blood micro analyser system). The samples of venous blood were taken with heparin. Erythrocytes were washed as mentioned above and stored at room temperature during 24 h for oxyhaemoglobin deoxygenation. After this period the red blood cell samples were hemolyzed by freezing at −10°C. The oxygen saturation measurements were made at ~36.6°C. The results were fitted by least squares method using the Hill equation to determine the Hill parameter n and the oxygen affinity (P_{50}).

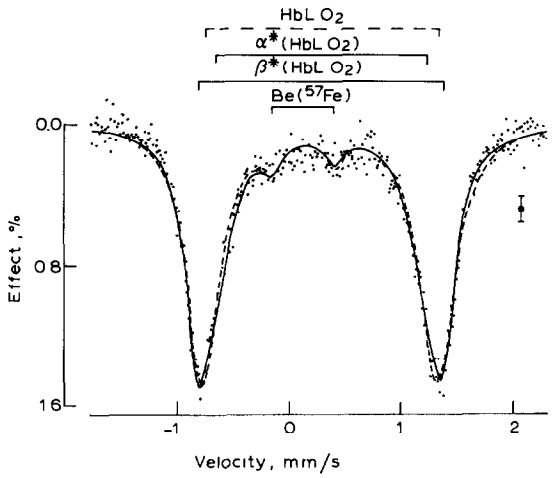

Fig.1. Mössbauer spectrum of oxyhaemoglobin from patients with acute myeloblastic leukaemia at 87 K. The dashed and continuous lines result from least squares fitting in model 1 and model 2, respectively.

3. RESULTS

Mössbauer spectra of oxyhaemoglobins (fig.1) were approximated by two methods. Firstly, we considered it as a usual quadrupole splitting doublet with Lorentzian fitting of each absorption peak. There are no differences of the Fe^{2+} electronic structure of non-identical haemoglobin subunits in this model [1]. Secondly, we took into account the Fe^{2+} electronic structure non-equivalence in non-identical haemoglobin subunits (model 2). Therefore Mössbauer spectra were fitted by superposition of two quadrupole doublets with Lorentzian line shapes.

3.1. *Model 1*

Quadrupole splitting ΔE_Q and isomer shift δ (with respect to metallic iron at 295 K) were estimated for all HbO_2 groups. The distinctions in these parameters were clearly seen when the result was presented in ΔE_Q and δ coordinates (fig.2). It was possible to find a region with a radius ~0.022 mm/s and with a center point ΔE_Q = 2.074 mm/s and δ = 0.253 mm/s where $HbAO_2$

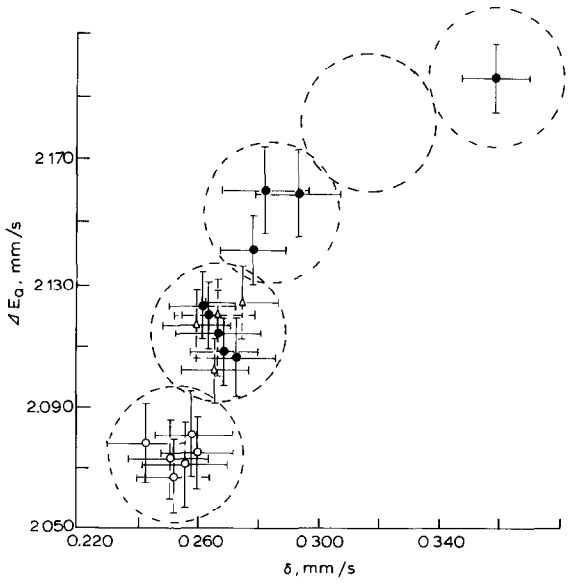

Fig.2. The regions of quadrupole splittings and isomer shifts of human oxyhaemoglobins obtained in model 1: (○) normal adult; (△) normal foetal; (●) leukaemic patient. The dashed circles show the conventional borders of both adult and foetal parameter regions and leukaemia parameter subregions.

parameter values of the Mössbauer spectra were enclosed (R_A). The region of $HbLO_2$ parameters could be conditionally divided into several subregions with the same radii and center points: ΔE_Q = 2.114 mm/s and δ = 0.267 mm/s (R_{L1}); ΔE_Q = 2.153 mm/s and δ = 0.285 mm/s (R_{L2}); ΔE_Q = 2.182 mm/s and δ = 0.317 mm/s (R_{L3}); ΔE_Q = 2.196 mm/s and δ = 0.359 mm/s (R_{L4}). R_{L4} was determined in one leukaemia case only and R_{L3} had no measured $HbLO_2$ Mössbauer parameters. With increasing numbers of $HbLO_2$ samples the region of $HbLO_2$ parameters would be expected to be more distinct. We point out that there is no correlation between leukaemia forms and subregions of the given parameters. The region of $HbFO_2$ parameters (R_F) appeared to differ from R_A. This fact distinguishes foetal from adult haemoglobins and the electronic structures of their complexed iron. At the same time R_F coincided with R_{L1}.

To determine the quantitative relationship of Mössbauer parameters ΔE_Q and δ, the electronic structure of the iron complex, and the molecular structure of the active site in haemoglobin, quantum-chemical calculations are needed which are unavailable. Therefore a qualitative analysis was carried out using the results of [8]. The energy spectrum of Fe^{2+} electronic states obtained for $HbAO_2$ is the following: the ground singlet state $^1A_1$ and low-lying excited triplet states $^3B_2$ and $^3B_1$ arising from ^3E term splitting in the rhombic field and lying at 140 cm^{-1} and 300 cm^{-1} above the ground state, respectively. (The low-lying excited triplet state energies are not clear yet. Hoenig and Gersonde [9] indicated the existence of an excited triplet state at about 17 cm^{-1}. Herman and Loew [10] calculated the lowest triplet state to be at 129 cm^{-1} above the ground state. By contrast, other calculations [11] indicated the lowest triplet state to be in the order of 4000–8000 cm^{-1} above the ground state. Recently Philo et al. [12] showed that any low-lying triplet states had to lie at least 900 cm^{-1} above the ground state. However we chose the results of Bacci et al. [8] which allow us to describe both Mössbauer and magnetic susceptibility data.) Since the contribution to the electric field gradient (EFG) on the ^{57}Fe nuclei from the $^1A_1$ term is zero [13], the ΔE_Q increase may be caused by decrease of the excited triplet state energies because $^3B_2$ and $^3B_1$ terms contribute to EFG with the same sign. The decrease of $^3B_2$ and $^3B_1$ state energies with respect to $^1A_1$ in $HbFO_2$ and $HbLO_2$ resulted from the weakness of the Fe^{2+}-ligand bonds, first of all of the axial ligands.

Table 1

Mössbauer spectral parameters of oxyhaemoglobins in model 2

Sample	mm/s						Relative areas of doublets (%)	
	Γ_1[a]	δ_1[b]	ΔE_{Q1}[b]	Γ_2[a]	δ_2[b]	ΔE_{Q2}[b]	1st	2nd
$HbAO_2$	0.254	0.263	2.174	0.410	0.261	1.819	52	48
$HbAO_2$	0.248[c]	0.258	2.161	0.401[c]	0.260	1.841	52	48
$HbFO_2$	0.247	0.261	2.209	0.395	0.262	1.873	53	47
$HbFO_2$	0.250[c]	0.269	2.194	0.413	0.269	1.827	55	45
$HbLO_2$	0.262[c]	0.294	2.274	0.423	0.295	1.915	49	51
$HbLO_2$	0.251[c]	0.359	2.295	0.431	0.357	1.931	51	49
$HbLO_2$	0.268	0.272	2.206	0.402	0.265	1.901	52	48
$HbLO_2$	0.250[c]	0.280	2.219	0.400	0.283	1.924	54	46
$HbLO_2$	0.270	0.290	2.288	0.429	0.274	1.959	44	56
$HbLO_2$	0.260	0.269	2.193	0.366	0.266	1.838	58	42

[a] Maximal experimental error ± 0.028 mm/s
[b] Maximal experimental error ± 0.014 mm/s
[c] Fixed parameter

Isomer shifts are given relative to metallic iron at 295 K

On the other hand, a weakness of the iron σ- and π-bonds leads to δ increase [14]. The changes of the Fe^{2+} bonds with axial ligands in comparison with those of $HbAO_2$ may be due to differences in the structure of the ligand pocket and in the proximal histidine region of $HbFO_2$ and $HbLO_2$. For instance, calculations [15] showed that ΔE_Q varied slightly as a function of Fe-O-O angle variation or O_2 rotation about the Fe-O bond. We note that changing the ground spin state of $HbLO_2$ from R_{L4} is possible, because the δ value is close to that of tetraphenylporphyrin-Fe^{2+} with an intermediate spin state $S = 1$ [16]. In this case Fe^{2+} in $HbLO_2$ (R_{L4}) may be out of the haem plane unlike the complex with four coordinate bonds.

3.2. *Model 2*

A number of Mössbauer investigations on oxyhaemoglobins discovered some special features of the spectra such as non-Lorentzian line shapes and temperature dependence of line shape and linewidth [17–28]. In [17,18,20] non-Lorentzian Mössbauer line shape was explained by relaxation effects. On the other hand, an approximation of Mössbauer spectra of oxyhaemoglobins by superposition of two quadrupole doublets appeared to be also acceptable [19,24,26,28]. However the interpretations of this approximation were made in different ways. Our explanation of the asymmetry of the absorption line shapes is based on the supposition that the Fe^{2+} electronic structure differs in non-equivalent oxyhaemoglobin subunits [21,22]. Thus, the fitting of the Mössbauer spectra of oxyhaemoglobins was made using two quadrupole doublets. Since each doublet was connected with ^{57}Fe nuclei in the identical oxyhaemoglobin subunits the areas of both doublets had to be equal and the parameters had to be reasonable. However the fitting with free variation of parameters was suitable for several spectra only. Sometimes a positive result was reached by the fixing of one or two parameters. The acceptable parameters of the 1st and 2nd doublets of the oxyhaemoglobin Mössbauer spectra are given in table 1. To interpret these results we analyzed $HbAO_2$ X-ray structure data [29,30]. Based on the structural differences of the active site in α and β subunits in $HbAO_2$, we suppose that the 2nd doublet is related to the ^{57}Fe nuclei in α subunits while the 1st doublet is attributed to the ^{57}Fe nuclei in β subunits [28]. The possibility of Fe^{2+} ground spin state change (low $\longrightarrow$ intermediate) in $\alpha(HbAO_2)$ subunits due to iron out of the haem plane position was also considered. We assume that Mössbauer spectra of $HbFO_2$ and $HbLO_2$ can be interpreted in the same way. The ΔE_{Q1} increase may be caused by the decrease of the overlap of π- and d-orbitals in $\gamma(HbFO_2)$ and $\beta^*(HbLO_2)$ subunits due to changes in the orientation of His F8 imidazole plane relative to the haem. It could be caused by the change of Fe^{2+}-N_ϵ(His F8) and Fe^{2+}-O_2 bonds also. The slight ΔE_{Q2} differences between $HbAO_2$ and $HbFO_2$ indicate that the conformation of α subunits may be slightly modified by the association with β and γ subunits. The increase of ΔE_{Q2} for $\alpha^*(HbLO_2)$ subunits could be explained as ΔE_{Q1}. In addition, the Fe^{2+} out of the haem plane displacement could be changed in $\alpha^*(HbLO_2)$ subunits. Moreover, we could assume that in the case of $HbLO_2$ with an anomalously large δ in both

Table 2

Oxygenation properties of normal adult and leukaemic haemoglobins

Sample	pH	n	P_{50} (mmHg)	Standard deviation
Donor 1	7.14	2.83	29.0	0.181
Donor 2	7.12	2.63	28.9	0.090
Donor 3	6.88	2.64	31.1	0.135
Chronic lympholeukaemia	7.10	2.87	28.1	0.108
Chronic myeloleukaemia	7.00	2.77	38.9	0.037
Acute myeloblastic leukaemia	6.36	2.81	39.0	0.051

α^* and β^* subunits the Fe^{2+} were out of the haem plane with intermediate ground spin state.

4. DISCUSSION

The relationship of Fe^{2+} electronic structure and stereochemistry of haemoglobin active sites could play a specific role in the ability of Fe^{2+} to bind oxygen [31,32]. HbF is known to have a higher oxygen affinity than HbA, however the affinity of $HbA(O_2)_3$ to the fourth oxygen is higher than that of $HbF(O_2)_3$ to its oxygen. This is in agreement with the assumption of a weaker Fe^{2+}-ligand bond in $HbFO_2$ (see also [33] where weaker bonds were associated with lower affinity). Since the biochemical properties of $HbLO_2$ are unknown, we suppose a change of the affinity to the fourth oxygen according to the ΔE_Q and δ parameters as the reason for our results. We present here the preliminary results of the oxygen saturation measurements for normal adult and leukaemic haemoglobins (table 2). In two samples of leukaemic haemoglobin P_{50} appeared to be shifted to greater values while the other leukaemic sample indicated very slight shift of P_{50} to lower values with respect to corresponding normal ones. More detailed analysis would be possible with 2,3-DPG and Bohr effect determination. The changes in oxygen affinity (as a referee has pointed out) are not likely to be one of the causes of anaemia in the case of leukaemia.

The observation that R_F and R_{L1} coincide (as well as ΔE_{Q1} and ΔE_{Q2} of $HbFO_2$ and those of some $HbLO_2$ samples) is reminiscent of α-foetoprotein [34] which is synthesized in embryonic as well as in some malignant cells. On the other hand, increases of HbF in leukaemia have been supposed due to decreases of Val and increases of Ile [7]. However, the point mutations in Val codons only in the β-globin gene leading to increases of Ile could not produce γ-chains. Therefore we conclude that coincidence of $HbFO_2$ and $HbLO_2$ hyperfine parameters does not explain its molecular structure and amino acid sequence identity. The similarity in Fe^{2+} stereochemistry and functional properties of these proteins can be assumed. However, Mössbauer spectroscopy alone is not enough for clarification, biochemical methods are also required.

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