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Identification and characterization of the iron compounds in bone marrow by means of Mössbauer spectrometry

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In order to determine and to demonstrate the cellular iron molecular states in hematopoietic bone marrow, direct investigations were performed by means of different and complementary spectroscopic techniques: optical absorption, electron spin resonance and Mössbauer spectrometry. In fact, the latter appears to have been the most informative. In addition to the hemoglobin forms, five- and six-coordination ligand protoporphyrins IX (monomeric and polymeric stacking, respectively) were observed. A small amount of non-hemic high-spin iron III storage component (ferritin) was measured. No diferric transferrin was detected. A ferrous compound was also observed and attributed to the mitochondrial iron pool.

Keywords: bone marrow, hematopoietic cell, heme iron pathway, iron storage, Mössbauer spectroscopy

Introduction

Studies carried out on hematopoietic cells have shown that the reticulocyte iron uptake from transferrin occurs according to the receptor-mediated endocytosis model (Dautry-Varsat 1986). This model is described by the transferrin binding on its receptors followed by endocytosis. The iron, fixed to transferrin, is reduced when it is delivered to the cell. A difference related to the mode of iron release from transferrin seems to appear with the hepatocyte iron uptake mechanism, i.e. a reduction process at the cell surface (Thorstensen 1988).

This iron II has two fates. It is well-known that this iron goes into the mitochondria for heme synthesis for the major part, while the remainder (about 20%) is the source of iron deposited in ferritin as intracellular iron storage (Zittoun & Sigaux 1984, Theil 1990). Between transferrin release and mitochondrial entry the iron is probably first incorporated in a small intracellular iron labile pool. This pool is made up of the so-called low molecular weight and maybe cytosolic non-heme proteins (Nunez *et al.* 1980, Zhan *et al.* 1990). This low molecular weight pool is kept constant. Its assumed function is to deliver iron to mitochondria (Weaver *et al.* 1989).

Spectroscopic techniques can be used to investigate the molecular states of iron in cells. In particular, electron spin resonance (ESR) spectroscopy and Mössbauer spectroscopy give complementary information from direct measurements carried out on entire tissues (without any biochemical perturbation process). The former, which is more sensitive, enables us to observe, under standard conditions, only iron III compounds. Mössbauer spectroscopy, as a fingerprint technique, allows us to identify and characterize all the chemical environments of the iron atoms.

The purpose of the present work is to apply the Mössbauer technique to the study of intracellular iron metabolism in erythroid cells. From absorption Mössbauer spectra, directly performed on bone marrow samples, we have been able to identify and characterize the main iron components which appear during the successive steps of hemoglobin synthesis in red blood cells maturing within bone marrow.

Materials and methods

The bone marrows studied were sampled from the posterior iliac medullar level of healthy donors. During the

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sampling course, an amount of circulating blood was unavoidably taken with bone marrow. This blood amounts from about 20% of the sample volume at the beginning of the sampling up to 50% at the end of suction. Our samples were taken from the beginning of sampling. Before any spectroscopic studies, bone marrow was well-severed from the circulating blood and, consequently, no blood contamination was taken into account.

All spectroscopic measurements were carried out directly on the bone marrow samples, except for optical spectra, for which a dilution in physiological serum (NaCl 0.9 g 100 ml^{-1}) was required.

Visible and UV absorption (300–700 nm) 37 °C spectra were recorded by means of an Uvikon 860-Kontron Instruments spectrophotometer.

ESR measurements (77 K) were performed with a Brucker device (9.65 GHz) with 40 mW microwave power, 100 kHz modulation and 1500 G average field with a 10 G amplitude modulation.

Using a conventional constant acceleration spectrometer with a 3.7 GBq ^{57}Co source in a rhodium matrix, the absorption Mössbauer measurements were carried out at 77 and 4.2 K. The spectra data were fitted by Lorentzian profiles by least squares analysis (doublet and sextet components) using a hyperfine interaction Hamiltonian. The isomer shifts are given relative to natural metal iron at room temperature. In cases of good statistic counting rates, the quantitative sensitivity threshold for separating the different components was better than 0.5%.

Results

First, the absorption spectra, performed on bone marrow samples, showed only oxyhemoglobin (HbO_2) [characterized by Soret band (416 nm) and by both α and β bands (541.5 and 576.2 nm)]. No deformation of the doublet due to deoxyhemoglobin (Hb) or protoporphyrin was observed. On one hand, this may be explained by the high affinity of Hb for O_2. Consequently, during sampling and absorption running times, Hb is completely transformed into HbO_2. On the other hand, the low energy resolution of absorption spectroscopy prevents us from distinguishing the protoporphyrin bands (553 and 574 nm) from those of HbO_2. Moreover, the protoporphyrin molecular extinction coefficient ($\varepsilon = 6400\ M^{-1}\ cm^{-1}$) is low compared with that of HbO_2 ($\varepsilon = 44000\ M^{-1}\ cm^{-1}$; Silver & Lucas 1983). Therefore, absorption spectroscopy is not very informative for studying bone marrow.

The 77 K ESR spectra are extremely broad and expanded over a g range of 3–6. These results indicate the presence of ferritin in bone marrow samples (Weir *et al.* 1985). With regard to the large ESR sensibility, the low intensity signal indicates that only a low amount of ferritin is present. Moreover, the $g = 2$ isotropic band, typical of hemosiderin, is not observed, implying that there is no hemosiderin in bone marrow. Considering that the presence of transferrin gives a narrow resonance near $g = 4.3$, absence of this signal suggests that transferrin must only be present in very small quantities.

Figure 1 shows the Mössbauer spectra of two different bone marrow samples measured at 77 K. The spectra are well-fitted by using six symmetric or asymmetric Mössbauer doublets represented by line lorentzian profiles. The full curve is the least-squares fit of the experimental points. The Mössbauer parameters (quadrupole splitting Δ and chemical isomer shift δ) are listed in Table 1. Component 1 is well-defined in Figure 1 and so can be easily attributed. Its parameters ($\Delta = 2.20\ mm\,s^{-1}$ and $\delta = 0.15\ mm\,s^{-1}$) are typical of HbO_2. Components 2 and 3 in Figure 1(B) exhibit comparable intensity and are well-distinguishable, therefore the line positions are derived from this figure. However, in Figure 1(A), component 2 exhibits a symmetric doublet and a higher absorption than component 3 which is asymmetric. Thus, the lines can be associated and the corresponding components can be assigned. Parameters of doublet 2 ($\Delta = 2.35\ mm\,s^{-1}$ and $\delta = 0.95\ mm\,s^{-1}$) are well-matched to Hb and its desoxygenated isolated α and β chains. The α and β subunits show Mössbauer parameters similar to Hb (Trautwein *et al.* 1976). Parameters of doublet 3 ($\Delta = 2.40\ mm\,s^{-1}$ and $\delta = 1.10\ mm\,s^{-1}$) are typical of those of monomeric protoporphyrin IX iron II with five-coordination ligands (Silver *et al.* 1987).

Parameters of the fourth doublet (4) ($\Delta = 0.70\ mm\,s^{-1}$ and $\delta = 0.45\ mm\,s^{-1}$) are in good agreement with those of ferritin (Williams *et al.* 1978, Rimbert *et al.* 1986) which is a high-spin paramagnetic ferric compound. The relative amount of this component is small, just above the quantitative sensitivity limit of the technique (the signal: noise ratio is about 1). Taking into account that the 77 K f-factor (recoil-free efficiency) values for hemoglobin and ferritin are nearly equal (Bauminger *et al.* 1979), the ferritin amount is about 0.5%. This ferritin component is confirmed by ESR results and by 4.2 K Mössbauer measurements (see below).

The assignment of components 5 and 6 is made in similar fashion as for components 2 and 3. Doublet 5 is slightly asymmetric and has parameters ($\Delta = 1.50\ mm\,s^{-1}$ and $\delta = 0.30\ mm\,s^{-1}$) identical to protoporphyrin IX in a polymeric form where iron is embedded in a six-coordinate ligand geometry. This

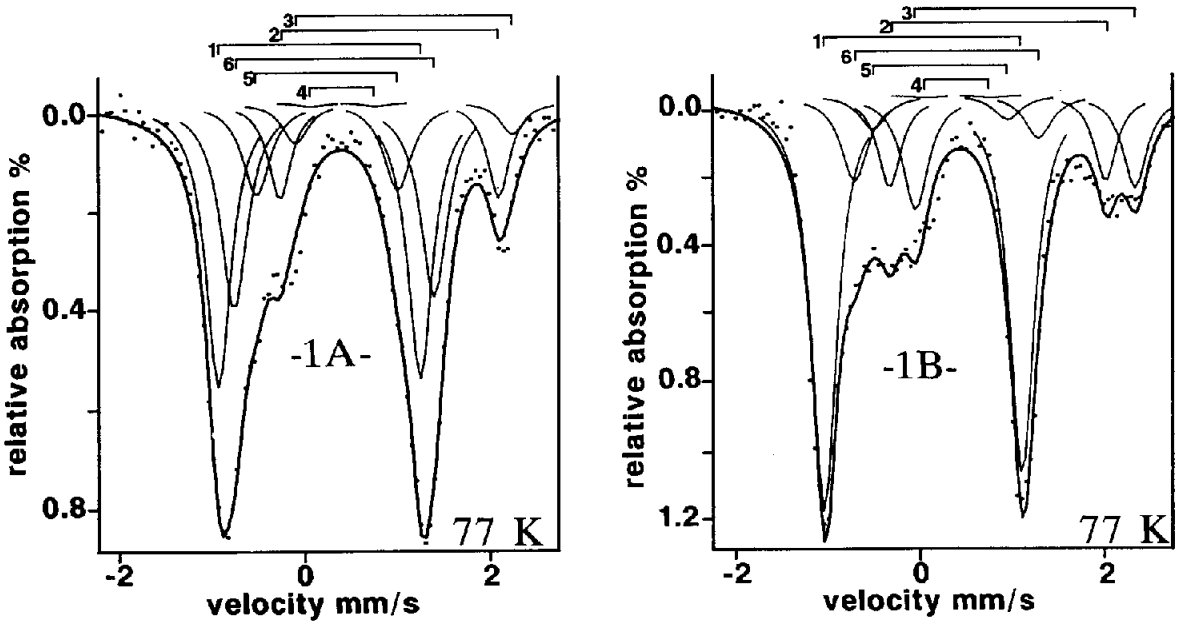

Figure 1. Bone marrow 77 K Mössbauer spectra. The spectra showed in (**A**) and (**B**) proceed from two different samples. The full curve is the least-squares fit of the experimental points with six qaudrupole doublets represented by line lorentzian profiles. HbO_2, 1; Hb and α and β hemoglobin subunits, 2; monomeric protoporphyrin IX, 3; ferritin, 4; polymeric protoporphyrin IX, 5; mitochondrial iron pool, 6.

Table 1. Experimental Mössbauer parameters and characteristics (charge, spin, amount) of the different components.

Component	77 K		4.2 K		H_b^{0eff} (kG)	Iron charge ion	Spin[c]	Relative ratio (%)
	Δ[a] (mm/s)	δ[a] (mm/s)	Δ[a] (mm/s)	δ[a] (mm/s)				
1	2.20	0.15	2.45	0.25	—	2+	0	39–58
2	2.35	0.95	2.30	1.00	—	2+	2	12
3	2.40	1.10	2.35	1.10	—	2+	2	5–15
4	0.70	0.45	−0.01	0.47	487	3+	5/2	0.5
5	1.50	0.30	1.90	0.30	—	2+	1	13.5–4.5
6	2.10	0.35	2.35	0.40	—	2+	1[d]	30–10

[a]Error: ± 0.05 mm/s^{-1}.
[b]Error: ± 5 kG.
[c]Spin assignments are based on literature data (Greenwood & Gibb 1971, pp. 352–362, Dickson 1984, Silver *et al.* 1987, Drabent *et al.* 1989).
[d]Presumed value (see text).
The component numbers are given in the legend of Figure 1.

protoporphyrin IX polymerization usually occurs at physiological pH (Silver *et al.* 1987). Doublet 6 has been added so as to obtain a good fitting analysis and varies from 10 to 30% of the total iron amount in the bone marrow, depending on the sample. Its parameters ($\Delta = 2.10$ mm s^{-1} and $\delta = 0.35$ mm s^{-1}) are in agreement with an iron II environment but do not match with any known hemoprotein or porphyric compound (Maeda 1979). The isomer shift data do not allow us to determine precisely the iron molecular spin ($S = 0$ or 1) (Greenwood & Gibb 1971, pp. 90–91). However, it must be noted that dichelated protoheme and some selected iron porphyrins, with a low spin ferric state ($S = 1/2$), have been observed with similar parameters (Straub & Connor 1973, Mielczarek *et al.* 1979).

As shown in Table 1, the spins of all other compounds are known: HbO_2, diamagnetic ferrous low spin $S = 0$; Hb, paramagnetic ferrous high spin $S = 2$; ferritin, ferric high spin $S = 5/2$; monomeric

protoporphyrin IX, ferrous high spin $S = 2$; and polymeric protoporphyrin IX, ferrous intermediate spin $S = 1$ (Silver *et al.* 1987, Drabent *et al.* 1989).

Furthermore, as shown in Figure 1, the overall shape of the Mössbauer spectra performed on several samples is rather different. This points out not only the variability but also the relationship for the relative ratios of several components. While an increase in HbO_2 is observed (in a 20% range), variations of the quantities of three other compounds are recorded. In particular, doublet 6 shows a decrease with the same amplitude. The monomeric and polymeric protoporphyrins IX exhibit opposing variations (a 10% increase for the former and a 9% decrease for the latter). Given that the total protoporphyrin IX pool is nearly constant (about 19%), this suggests that there exists an 'exchange equilibrium' mechanism between these two forms.

The amount of doublet 2, composed of Hb and the α and β chains, is kept at a constant value throughout the bone marrow samples studied. According to the O_2 partial pressure at the bone marrow level, hemoglobin, made in bone marrow, is strongly oxygenated and yields the following relative proportions: 95% HbO_2 and 5% Hb. Thus, the Hb amount contained in the doublet 2 is small (a few per cent), and consequently the isolated α and β subunits constitute the main part of this doublet. This α–β subunit pool seems to be nearly constant and to have reached its upper threshold (about 10%). All the proportions and their variations are summarized in Table 1.

In order to confirm the presence of ferritin and to study the temperature behavior of the hyperfine Mössbauer parameters of the other components, low temperature bone marrow Mössbauer spectral analysis has been performed. In the 4.2 K Mössbauer spectrum, the appearing magnetic sextet bears out the iron storage component but in small quantity (Figure 2). This sextet is due to an antiferromagnetic ordering taken by the iron core particles at low temperature (below 35 K) (Williams *et al.* 1986). The magnetic hyperfine field is measured as $H_{eff} = 487$ kG, in good agreement with previous ferritin measurements ($H_{eff} = 493$ kG) (Rimbert *et al.* 1986). Otherwise, the quadrupole splitting of the polymeric protoporphyrin IX is strongly enhanced by a decrease in temperature. Therefore, this hexacoordinate iron II form presents low rhombicity. Finally, the Mössbauer parameters of the sixth doublet are also temperature dependent. The large quadrupole splitting value associated with this temperature dependence describes a ferrous state and bears out a non-heme intermediate-spin iron II

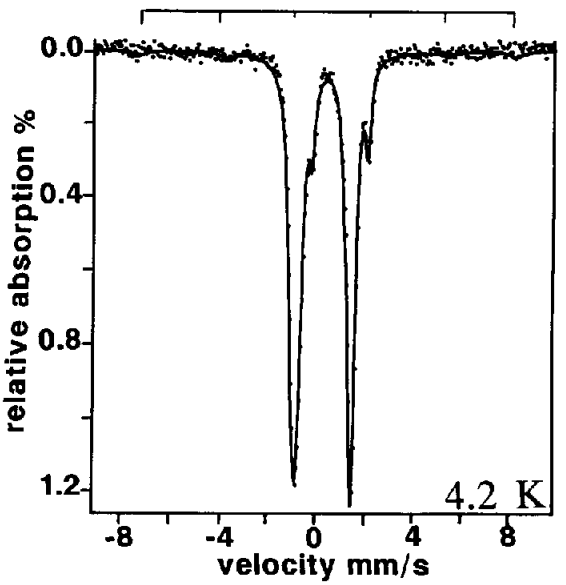

Figure 2. Bone marrow 4.2K spectrum. The full curve is the least-squares fit of the experimental points with a sextet for ferritin and five doublets corresponding to HbO_2, Hb and the α/β subunits, monomeric protoporphyrin IX, polymeric protoporphyrin IX and mitochondrial iron pool. Only the ferritin sextet is shown as a line pattern.

chemical environment for this yet to be named component 6, as was presumed.

Discussion

By means of Mössbauer spectroscopy, performed at 77 or 4.2 K, six iron components have been directly observed in bone marrow. They are presumed to be related to the iron pathway, from transferrin to hemoglobin, in the erythroid bone marrow cells.

The entrance of plasmatic iron III into the cell is accomplished according to the so-called transferrin cell cycle. The Mössbauer spectra, performed on transferrin at different temperatures (between 2 and 77 K), exhibit a well-resolved paramagnetic hyperfine structure (Spartalian & Oosterhuis 1973, Tsang *et al.* 1973). As the temperature is raised, however, a small increase in the width of the absorption lines is observed. This is due to the spin-lattice relaxation effects. However, since the iron transferrin magnetic spectrum can be seen as high as 77 K, the electronic relaxation times must be rather long. On all our 77 K bone marrow Mössbauer spectra, no paramagnetic hyperfine structure typical of transferrin was detected. Because of the fast transferrin turn-over into and in hematopoietic cells (Irie & Tavassoli 1987), the life-time of iron transferrin in the cells is short (below 15 min; Dautry-Varsat 1986). Consequently,

the amount of iron bound to transferrin in cells is small, below the sensitivity threshold of the spectroscopic technique.

Once inside the endocytic vesicle, the iron, released by transferrin, is reduced by means of a protonic pump and then released into the cytoplasm. This iron II is distributed according to a partition function between storage iron, in the ferritin form, and a utilization iron pool for heme synthesis (Crichton 1985, Klausner *et al.* 1985).

As soon as this iron II enters inside the apoferritin pocket, it is sequestered and stored as hydrated iron III oxide/phosphate mineral core (Rohrer *et al.* 1990, Fatemi *et al.* 1991, Harrison *et al.* 1991). Our results have demonstrated that ferritin is a minor compound in bone marrow. Thus, the majority of the iron, captured by the erythroid cells, is embedded in the hemoglobin synthesis pathway. In the first step, iron belongs to the form usually called low molecular weight or labile pool which has been found in low and constant quantities (Zhan *et al.* 1990). This pool has not been detected, probably due to the technique sensitivity threshold.

This iron is then delivered over to the mitochondria for heme synthesis. If hemoglobin is required, this iron will be used. Since the Mössbauer doublet 6 amount fluctuates, this doublet may be attributed to the mitochondrial iron fraction. During the maturation process, the expected protoporphyrin IX form appears. Our measurements have demonstrated that the protoporphyrin IX is observed as two different states in equilibrium: a monomeric form and a stacked form. According to the amount of HbO_2 needed, this equilibrium will be shifted towards one form or the other. As indicated in the ratio value column of Table 1, when HbO_2 synthesis is weak, protoporphyrin IX is mainly observed in its polymeric form. On the contrary, as in the case of high HbO_2 synthesis, the monomeric form prevails. It may thus be assumed that the polymeric form is an intermediate buffer state in which hemes are momentarily reserved while that of the monomeric form (direct precursor of hemoglobin) is ready to associate with globin to yield the α and β isolated chains. The presence of an upper threshold for the α–β subunit pool suggests that hemoglobin formation from four subunits comes about as soon as this threshold is attained.

These results allow us to define more accurately the intracellular iron metabolism of erythroid cells by emphasizing the major components (with regard to their quantity). These components can be arranged in an iron pathway scheme, summarized in the Figure 3.

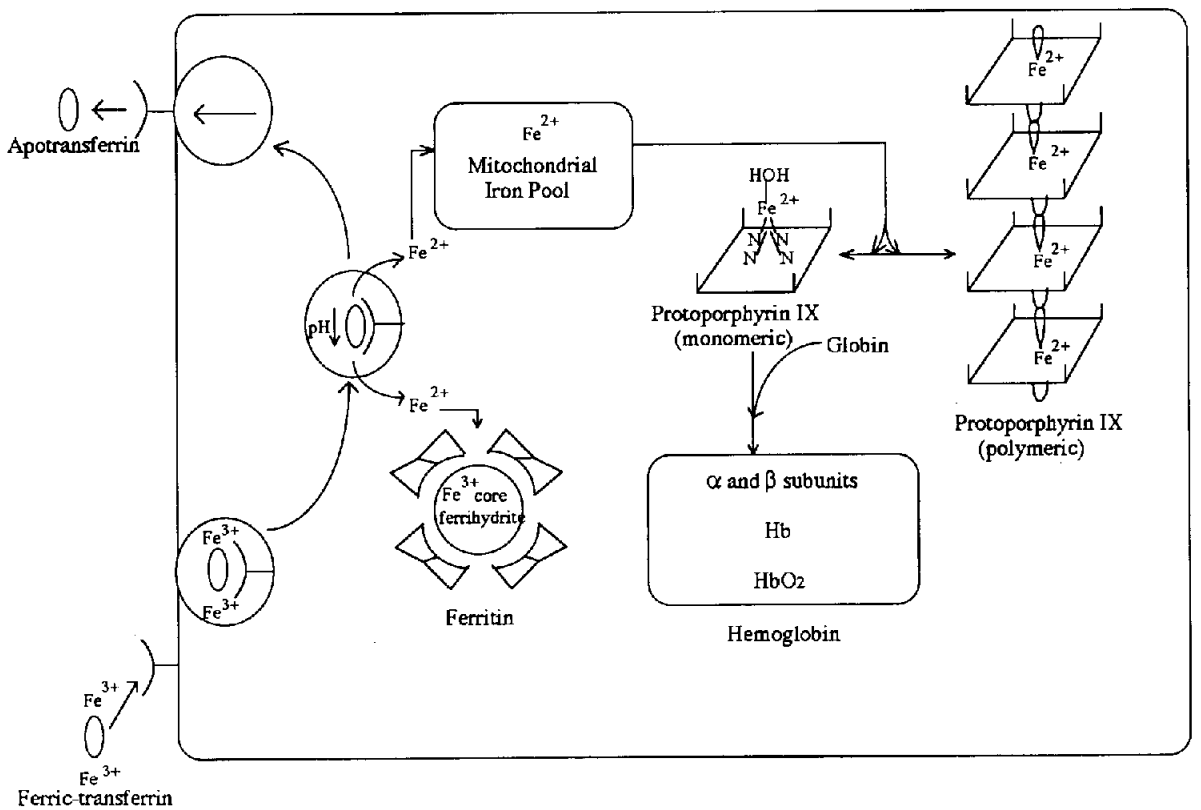

Figure 3. Hematopoietic cells iron pathway scheme. The transferrin cycle imaging is taken from Crichton (1985).

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