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Ferredoxin from *Halobacterium* of the Dead Sea — Mössbauer and EPR Spectra and Comparison with Mössbauer Spectrum of Whole Cells

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Abstract. Recoil-free measurements were carried out on a 2 Fe-ferredoxin, which was isolated and purified from an extreme halophile, *Halobacterium* of the Dead Sea. The spectrum of this ferredoxin in the oxidized state at 82 K is a superposition of two quadrupole doublets, representing two non-equivalent Fe^{3+} sites of equal intensity. The spectrum of the reduced ferredoxin is consistent with the presence of two pure classes of iron atoms, ferric (lower isomer shift) and ferrous (higher isomer shift). Interpretations of the recoil-free spectra are discussed. Mössbauer measurements were also carried out on frozen whole bacterial cells and the resulting spectrum was found to be quite different from that observed in the isolated ferredoxin. Tentative conclusions are reached concerning the localization of this ferredoxin in the cytosol of the Halobacteria.

The EPR spectrum of the reduced ferredoxin obtained at 24 K exhibits rhombic symmetry with the following g values: 1.894, 1.984 and 2.07. These values are similar to those obtained with 2 Fe-ferredoxins of the plant type, except that the g_y and g_z values are somewhat higher. Both from the EPR and Mössbauer data, it is deduced that the spin relaxation times in reduced halophilic ferredoxins are faster than in the reduced plant ferredoxins.

Key words: Mössbauer spectra — Halophilic bacteria — 2 Fe-Ferredoxins — EPR — Localization of ferredoxin in the cell.

Introduction

Iron-sulfur proteins are electron transport proteins which are probably present in all living organisms. Many different iron-sulphur proteins are known and possess a wide range of different functions in mitochondrial respiration, photosynthesis, hydroxylation reaction, nitrate-nitrite reduction as well as in nitrogen fixation [6, 14].

Since these proteins are ubiquitously distributed, it was expected that some will also be found in halophilic bacteria. Indeed, a two-iron ferredoxin with interesting

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properties has recently been isolated from several *Halobacterium* species [8, 9, 17]. The purified ferredoxin from *Halobacterium* of the Dead Sea has a visible absorption spectrum characteristic of plant ferredoxins (with a ratio of absorbancies, between 420 and 277 nm of 0.33) and a minimal molecular weight (from sedimentation equilibrium and amino acid analysis) of 14,000 [17].

The technique of recoil-free absorption (Mössbauer effect) is an important spectroscopic tool yielding valuable information on the valence, spin state and local stereochemistry of the iron atoms in proteins [11]. In particular, characteristic Mössbauer spectra have been observed in isolated ferredoxins, both in oxidized and reduced states [15, 16]. EPR spectroscopy has also been used to detect the characteristic signals obtaining around $g = 1.94$ in ferredoxins in the reduced state only, the oxidized state being non-magnetic [6, 13].

In this communication we present and analyze the recoil-free (Mössbauer) and EPR spectra of pure ferredoxin from *Halobacterium* of the Dead Sea. Mössbauer measurements were also carried out on frozen whole bacterial cells. The resulting ^{57}Fe spectrum is compared to that obtaining in the isolated pure ferredoxin. Some tentative conclusions are drawn with respect to the localization of ferredoxin in *Halobacterium* of the Dead Sea.

Part of the present work has already been briefly reported at the recent International Conference on Applications of the Mössbauer Effect [1].

Materials and Methods

Bacteria. The bacteria (*Halobacterium* of the Dead Sea [3]) were grown in 6 l Erlenmeyer flasks containing 1.5 l of the following medium (concentrations in g/l): NaCl – 208; $MgSO_4$ (anhydrous) – 46.6; $CaCl_2$ – 0.05; $MnCl_2$ – 0.125; Difco yeast extract – 10; and ^{57}Fe (in the form of ferrous sulfate) – 0.002. The bacteria for the Mössbauer experiments were harvested by centrifugation at 15,000 rpm, washed twice with 4.3 M NaCl – 0.01 M phosphate buffer, pH 7.2 and kept as a pellet.

Ferredoxin. Ferredoxin enriched in ^{57}Fe was purified from the supernatant of the centrifuged sonicate, according to a procedure described elsewhere [17]. The purified ferredoxin was dialyzed against solutions of 2 M NaCl – 0.01 M phosphate buffer, pH 7.2. We wish to stress here the convenience and efficiency of producing the enriched protein directly by bacterial growth in an ^{57}Fe enriched nutrient medium, as compared to the introduction of ^{57}Fe into apoferredoxin.

Reduction of Ferredoxin. A sample (0.6 ml) of ferredoxin (6 mg/ml) in 2 M NaCl – 0.01 M phosphate buffer, pH 7.2, was reduced under a helium atmosphere by adding a large excess of sodium dithionite (12 mg) in the presence of sodium bicarbonate (12 mg).

Technique of Mössbauer Measurements. Recoilless absorption spectra of the 14.4 keV gamma ray of ^{57}Fe in pure 2 Fe-ferredoxin were measured at 4.1, 82 and 220 K. Since it was necessary, at times, to measure very small recoil-free effects ($\sim$ 0.3%)

the counting rates obtaining in the recoil-free measurements were maximalized by using as source 100 mCi ^{57}Co in rhodium and a Harwell proportional counter running at about 10^5 total counts per s as the detector of the 14.4 keV radiation. Measurements on the frozen whole bacterial cells were carried out at 82 K. The samples were held in perspex cells approximately 4 mm thick.

EPR *Spectra.* These were recorded on a Varian E4 spectrometer, with a flow of cold helium gas to cool the samples. A sample of ferredoxin (0.22 mg in 100 µl of 4.3 M NaCl – 0.01 M phosphate buffer, pH 7.2) was reduced before the experiment under a stream of argon by the addition of 5 µl of an oxygen-free solution of 100 mM sodium dithionite – 200 mM Tris buffer, pH 9.0. The ferredoxin used in these experiments contained iron of normal isotopic content.

Results

Mössbauer Spectra of Halophilic Bacteria and Oxidized Ferredoxin

The recoil-free absorption spectrum obtained at 82 K in isolated 2 Fe-ferredoxin in the oxidized state is shown in Figure 1. Similar spectra were obtained at 4.1 and 220 K. These spectra are evidently a superposition of two well-defined quadrupole doublets. The spectra are in a general way similar to those obtained in other 2 Fe-ferredoxins [15, 16] but show a much better resolution into two non-equivalent sites of equal intensity, each distinctly characterized by a quadrupole doublet. The computer-fitted values of the quadrupole splittings, isomer shifts and linewidths of these spectra are given in Table 1.

The identification of the peaks as belonging to two different sites as shown in the Figure (1 and 4 – site *a*; 2 and 3 – site *b*) is carried out on the assumption that the isomer shifts at the two sites *a* and *b* do not differ very much. If this assumption is

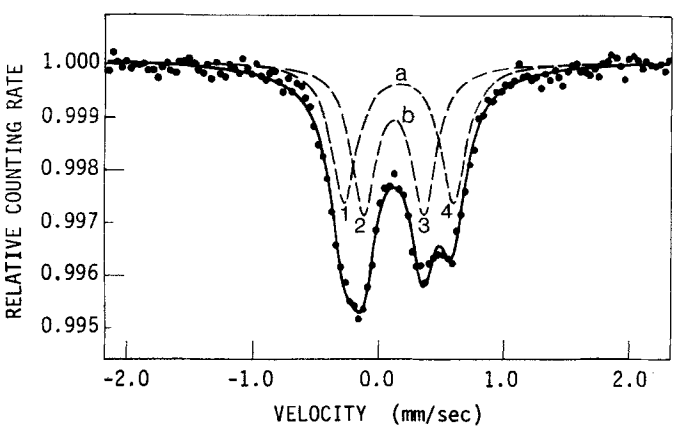

Fig. 1. Recoilless absorption spectrum of the 14.4 keV gamma ray of ^{57}Fe in isolated 2 Fe-ferredoxin from *Halobacterium* of the Dead Sea (in the oxidized state) at 82 K. The solid line is the least squares computer fit to the spectrum. It is the sum of the two quadrupole subspectra *a* and *b* (inner lines) of equal intensity with parameters as given in Table 1

Table 1. Parameters[a] of Mössbauer experiments performed on samples of ferredoxin in the oxidized and reduced states and of whole cells of *Halobacterium* of the Dead Sea

Oxidation state	Temperature (°K)	Site a		Site b		Linewidth (mm/s)
		Quadrupole splitting (mm/s)	Isomer[b] shift (mm/s)	Quadrupole splitting (mm/s)	Isomer[b] shift (mm/s)	
Oxidized FD[c]	4	0.87 (1)	0.290 (5)	0.472 (6)	0.250 (5)	0.30 (1)
Oxidized FD	82	0.87 (1)	0.272 (5)	0.480 (6)	0.232 (5)	0.25 (1)
Oxidized FD	220	0.90 (2)	0.225 (10)	0.50 (2)	0.19 (1)	0.29 (3)
Reduced FD	200	0.60 (2)	0.30 (1)	2.64 (2)	0.55 (2)	0.28 (3)
Oxidized FD (alternative)[d]	82	0.71 (1)	0.350 (4)	0.632 (4)	0.15 (1)	0.25 (1)
Whole cells	82	0.06 (4)	0.40 (2)	0.64 (4)	0.29 (2)	0.41 (3)

[a] Values obtained by least squares computer fits to the experimental spectra. The number in parentheses indicates the error in the last digit

[b] Isomer shifts given relative to metallic iron at room temperature

[c] FD-ferredoxin

[d] Alternative identification (see text)

dropped, the alternative identification of peaks 1 and 3 belonging to one site and peaks 2 and 4 to the other site – cannot be ruled out. The parameters for the spectrum obtained at 82 K, according to the latter assignment, are also given in Table 1 (fifth row). In both interpretations, no significant dependence of the quadrupole coupling on temperature is noticeable, whereas the isomer shifts show a small temperature-dependent decrease as expected. There is no evidence of any magnetic splitting even at 4.1 K. The linewidths obtained are close to the natural linewidth.

The recoil-free spectrum obtained at 82 K from a pellet of frozen halophilic bacteria in 4.3 M NaCl is shown in Figure 2. It is clear that the spectrum of the bacterial cells is quite different from that of the isolated ferredoxin. The width and shape of the lines observed in this spectrum suggest that it is of complex origin. Formally, a computer fit can be obtained again assuming a superposition of two quadrupole doublets of equal intensity. The result of such a computer fit is displayed in Figure 2 and the parameters obtained are given in Table 1. However, in this case, the linewidth of a single line turns out to be about 0.40 mm/s, considerably greater than the linewidth of 0.25 mm/s found in the resolved spectra of the isolated 2 Fe-ferredoxin. This suggests a complex situation produced by a larger number of components or the presence of other line-broadening factors.

Mössbauer and EPR *Spectra of Reduced Ferredoxin*

Recoil-free spectra of ferredoxin reduced with excess of sodium dithionite were also measured at various temperatures. Figure 3 displays the spectrum at 200 K. In the reduced samples about half of the iron in the protein appears in the form of a ferrous

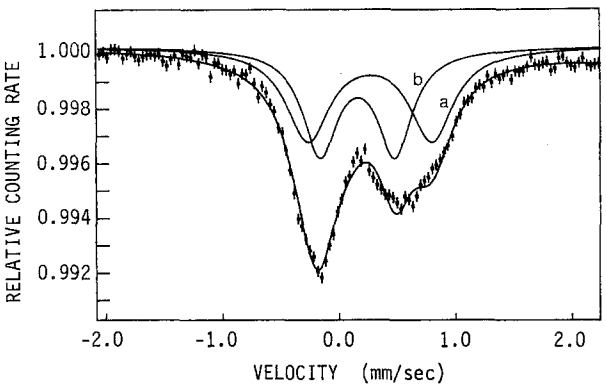

Fig. 2. Recoilless absorption spectrum of the 14.4 keV gamma ray of ^{57}Fe in a frozen sample of whole cells of *Halobacterium* of the Dead Sea at 82 K. The absorber contained about 300 mg/cm^2 packed cells (with 10 μg ^{57}Fe per gram of cells). The solid line is the least squares computer fit to the spectrum. It is the sum of the two quadrupole subspectra *a* and *b* (inner lines) with parameters as given in Table 1

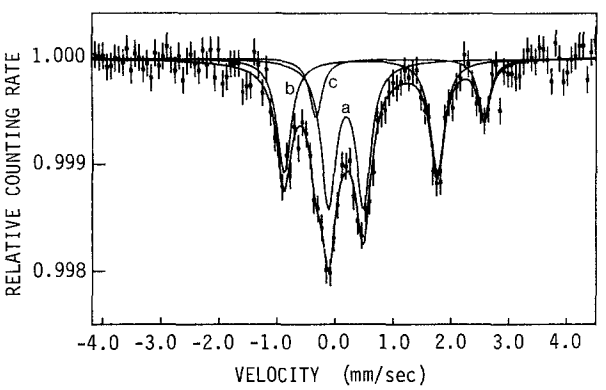

Fig. 3. Recoilless absorption spectrum of the 14.4 keV gamma ray of ^{57}Fe in isolated 2 Fe-ferredoxin from *Halobacterium* of the Dead Sea, in the reduced state at 200 K. The solid line is the least squares computer fit to the spectrum. It is the sum of the two quadrupole subspectra *a* and *b*, with parameters as given in Table 1, and a third subspectrum *c*, which is attributed to denatured ferredoxin

quadrupole doublet, the other half remaining in the form of a ferric quadrupole doublet. This phenomenon is again characteristic of 2 Fe-ferredoxins [15, 16]. The quadrupole splittings and isomer shifts obtaining at the two sites for the spectrum at 200 K are given in Table 1. As seen from Figure 3, one site only (site *b* in the Table) is reduced; the other site (site *a* in the Table) shows an isomer shift and a quadrupole splitting characteristic of Fe^{3+}.

The hyperfine parameters, however, are not identical to the parameters of any site in the original ferredoxin at the corresponding temperature, 200 K. This, perhaps, should not be too surprising, as the Fe^{3+} ion has, in the reduced state, a divalent neighbor – whereas in the oxidized state it has a trivalent neighbor. It is thus impossible at this stage to decide which of the two non-equivalent iron sites in

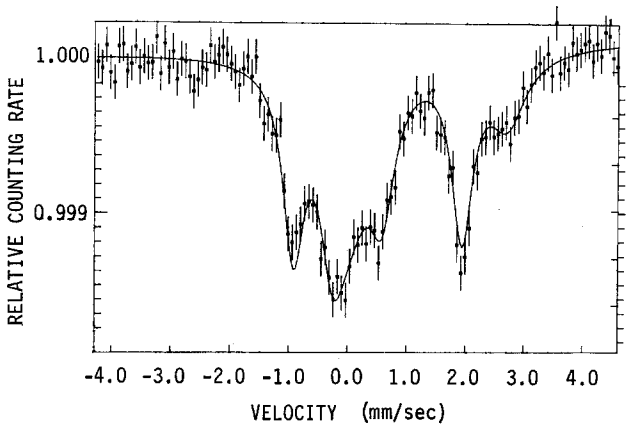

Fig. 4. Recoilless absorption spectrum of the 14.4 keV gamma ray of ^{57}Fe in reduced isolated 2 Fe-ferredoxin from *Halobacterium* of the Dead Sea at 82 K. Other conditions as under Figure 3

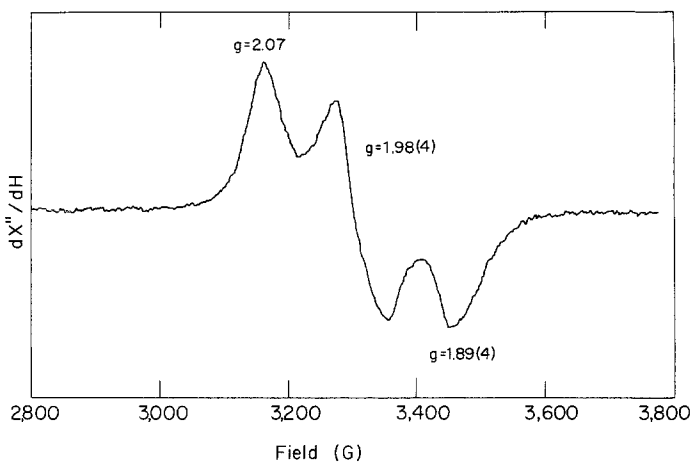

Fig. 5. EPR spectrum of reduced ferredoxin from *Halobacterium* of the Dead Sea. Ferredoxin (2.2 mg/ml) was reduced under a stream of argon by sodium dithionite and frozen. Conditions of measurement: microwave power, 1 mW; microwave frequency, 9.25 GHz; modulation amplitude, 10 G; temperature, 24 K

the oxidized state "goes over" to the reduced state in the reduced ferredoxin. The spectrum in the reduced ferredoxin at 82 K is shown in Figure 4. Although the general shape of this spectrum resembles that of the spectrum at 200 K, the individual lines are much broadened. This is almost certainly due to relaxation effects obtained in the paramagnetic molecule. In Figures 3 and 4, the small peak at ~ 0.27 cm/s is part of a quadrupole doublet, whose second partner is at -0.02 cm/s (site *c* in Fig. 3). We believe this doublet arises from a partial denaturation of the protein during the reducing process.

The EPR spectrum of the reduced ferredoxin obtained at 24 K (Fig. 5) is characteristic of 2 Fe-ferredoxins, exhibiting rhombic symmetry with the following *g* val-

ues: 1.894, 1.984, and 2.07. An EPR signal is obtained only from the reduced ferredoxin and is totally absent at 82 K (liquid nitrogen). The intensity of the EPR signal increases on lowering the temperatures below 50 K.

Discussion

The recoil-free spectra of ^{57}Fe observed in frozen halophilic bacteria and ferredoxin show different features (Table 1). The linewidth is much broader in the spectra obtaining in the whole cells and the isomer shifts of at least one of the sites are observed at higher velocities than in the ferredoxin (Table 1). Since the 2 Fe-ferredoxin was purified from the supernatant of the centrifuged sonicate, it seems likely that the 2 Fe-ferredoxin is present mainly in the cytosol. However, a loose association of the ferredoxin with the membranes, which is overcome by sonication cannot be excluded.

The spectrum observed in the whole cells indicates, that there exists in the halophilic bacteria in addition to the 2 Fe-ferredoxin, one or a number of other iron-containing proteins. The 2 Fe-ferredoxin represents probably only a small fraction of the total iron-proteins of the cell and its characteristic doublets are therefore not clearly distinguished in the spectrum obtained in the whole bacteria. A rough estimate of iron content indicates that this fraction is less than 10%. We notice that the parameters obtained by computer analysis for site *a* in the spectrum of the whole cells at 82 K (Fig. 2) are very similar to those of the 4 Fe-ferredoxin from *Bacillus stearothermophilus* [12]. This suggests that a significant fraction of the total iron-protein content might be in the form of a 4 Fe-ferredoxin.

The Mössbauer parameters obtained in reduced ferredoxin are consistent with the presence of two pure classes of iron atoms, ferric (lower isomer shift) and ferrous (higher isomer shift), rather than with electron delocalization over the 2 Fe atoms. In this feature as well, ferredoxin from *Halobacterium* of the Dead Sea resembles plant ferredoxins [15, 16]. Thus, similarly, to other 2 Fe-ferredoxins, this oxidized ferredoxin contains two antiferromagnetically coupled high-spin Fe^{3+} atoms [2]. Upon reduction, one electron is transferred to a particular iron atom – which specifically becomes reduced.

The EPR signal observed for the reduced ferredoxin resembles those of plant ferredoxins [4] in its rhombic symmetry. However, although the value of $g_x = 1.89$ is almost identical, the values of $g_y = 1.98$ and $g_z = 2.07$ are clearly higher than those of plant ferredoxins: $g_y = 1.95$ and $g_z = 2.04$. Similarly, the average g-value of *H. halobium* ferredoxin has been reported [9] to be unusually high, $g = 1.98$. The linewidth of the signal of *Halobacterium* of the Dead Sea ferredoxin at 24 K is rather broad, probably due to the effect of the high chloride concentration (4.3 M) in the medium [4]. On the other hand, the EPR spectrum of halophilic ferredoxin differs also from that of *E. coli* ferredoxin [10], which displays a virtually axial symmetry (i.e. $g_x = g_y$).

Halophilic ferredoxins differs also from plant ferredoxins, in requiring a lower temperature, < 50 K, for the appearance of a well-defined EPR signal. This difference is probably due to the more rapid electron-spin relaxation rate [5] in the halophilic ferredoxins. A similar conclusion may be deduced from the Mössbauer spec-

trum of reduced halophilic ferredoxin at 82 K (Fig. 4). This spectrum resembles that of *Euglena* ferredoxin [7] and is different from those of *Scenedesmus* [15] and plant [16] ferredoxins, measured at the same temperature. The linewidth in the latter ferredoxins is much broader, indicating that spin relaxation times are shorter in ferredoxins from *Euglena* and *Halobacterium* of the Dead Sea than in plant and algal ferredoxins. As pointed out in the case of the ferredoxin from *H. halobium* [9], an enhanced relaxation rate may reflect a weaker antiferromagnetic coupling between the two iron atoms of halophilic ferredoxins, when compared to plant ferredoxins.

In conclusion, it seems that although ferredoxin from *Halobacterium* of the Dead Sea resembles plant ferredoxins, with respect to the valence of the iron atoms and their stereochemistry, both the Mössbauer and EPR spectra display some differences, probably due to different protein environments of the active center.

Finally, we would like to stress that in the present work the technique of recoil-free absorption has been used as a spectroscopic tool to look both at an isolated iron-protein and at the total of iron-containing components in a whole bacterial cell. This combined approach can be expected to yield useful information on biological systems.

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