Identification of a Non-Magnetic Iron Centre and an Iron-Storage or -Transport Material in Blue-Green Algal Membranes by Mössbauer Spectroscopy

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At least two species of iron were identified in crude membrane preparations of the blue-green algae *Chlorogloea fritschii* and *Anacystis nidulans* by Mössbauer spectroscopy. One species was non-magnetic with chemical shift more characteristic of a ferredoxin-like centre than of any known low-spin Fe(II) biological compounds. Studies on cells grown under depleted-iron conditions show the other iron species to be part of the system for iron metabolic transport and/or storage.

In addition to haemoproteins, biologically important iron-containing molecules occur as iron-sulphur proteins, storage materials and transport proteins. The latter comprise at least two types, these being siderochromes from micro-organisms (see Neilands, 1974) and, for example, transferrin isolated from higher organisms (Oosterhuis & Spartalian, 1976). The storage of iron in an inorganic form in association with protein was observed some years ago by Granick (1946) and the structure of the iron-protein complex is a subject of continuing investigation (Crichton, 1973). The observation of the iron-storage material, ferritin, in chloroplasts (Hyde *et al.*, 1963) and in the mould *Phycomyces* (David, 1974; Spartalian *et al.*, 1975) extends the distribution of such storage material throughout the major classes of eukaryotic cells. It has been proposed on the basis of e.p.r. signals that a membrane-bound iron-sulphur protein is associated with the primary reaction of Photosystem I (Malkin & Bearden, 1971). A similar e.p.r. signal has been observed in membrane preparations of *Chlorogloea fritschii* (Evans *et al.*, 1976). Several species of blue-green algae, including *C. fritschii* and *Anacystis nidulans*, have been shown to accumulate large amounts of iron (Jones *et al.*, 1977) well in excess of that which could be contained in cytochromes and ferredoxins. The present paper describes the observation, by ^{57}Fe Mössbauer spectroscopy, of two types of iron species present in membrane preparations of the prokaryotes *C. fritschii* and *A. nidulans*.

Experimental

Organisms and growth conditions

Chlorogloea fritschii (Culture Collection of Algae and Protozoa no. 1411/1; Department of Botany, Downing Street, Cambridge, U.K.) and *Anacystis nidulans* (Collection of Algae; U.T.E.X. 625; University of Texas, Austin, TX, U.S.A.) were grown on medium C of Kratz & Myers (1955) as previously described (Mann & Carr, 1974). This medium contains 6.3 mg of iron/litre, supplied as $\text{Fe}(\text{SO}_4)_2 \cdot 6\text{H}_2\text{O}$. Organisms were enriched with ^{57}Fe by conversion of ^{57}Fe into $^{57}\text{FeSO}_4$ and use of this in the, $6\text{H}_2\text{O}$ otherwise normal growth medium. AnalaR chemicals, and water that had been deionized and glass-distilled, were used throughout. Organisms were harvested, washed with buffer (0.05 M-Tris/HCl/0.05 M-mannitol/0.03 M-EDTA, pH 7.8) and disrupted in a French Pressure Cell at 150 MPa (10 tonnes/in²) at 2°C. Unbroken organisms were sedimented (3000 g for 15 min) and the supernatant centrifuged (144000 g for 2 h) to yield a pellet, which was washed with buffer and sedimented as before. This pellet contained the photosynthetic pigments, except the soluble phycocyanin, which remained with the cytochromes in the supernatant. The membrane-containing pellet was immediately frozen in a Mössbauer sample cell by immersion in liquid N₂.

For studies on the iron-storage material, *C. fritschii* and *A. nidulans* were grown on lower iron concentrations than normal. Both organisms grew when the iron in the growth medium was decreased to 10 and 1% of that available under the standard conditions described above. Neither organism showed significantly decreased mean generation times

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‡ Abbreviation: e.p.r., electron paramagnetic resonance.

when supplied with 10% of the standard iron concentration; 1% iron decreased the mean generation time of *C. fritschii* by 40% and that of *A. nidulans* by less than 10%.

Mössbauer spectroscopy

The samples were held in Perspex cells (approx. 1 ml volume) which could be immersed in a liquid-He bath for experiments at 4.2 and 1.4 K, or held in thermal contact with a liquid-N₂ bath for those at 77 K. The ⁵⁷Fe Mössbauer absorption spectra were taken by using a conventional constant-acceleration spectrometer, the counts from a ⁵⁷Co-in-rhodium source being collected by an argon/methane-filled proportional counter after transmission through the sample and fed into a multichannel analyser operating in the multiscaler mode. A superconducting solenoid was used to provide the 6 T external applied magnetic field. An absorber of pure iron was used for velocity calibrations: chemical shifts are quoted relative to the centre of gravity of the pure iron spectrum at 290 K, and all the spectra are plotted with this centre of gravity as the zero of the velocity scale.

Results and Discussion

Mössbauer spectra of *C. fritschii* membranes after growth in complete medium are shown in Figs. 1 and 2. The spectra in Figs. 1(a) and 2(a) are at 77 K, in Figs. 1(b) and 2(b) at 4.2 K and in Figs. 1(c) and 2(c) at 1.4 K. The spectra in Fig. 2 were taken on a much smaller velocity scale than those in Fig. 1, to investigate more closely the central region.

At 77 K there was clearly a superposition of more than one broad quadrupole doublet (Fig. 2a) with little or no absorption at higher velocities (Fig. 1a). The parameters (in units of mm/s) of a least-squares computer fit at 77 K to two quadrupole doublets ('component I' and 'component II') were: component I, $\delta = 0.45$, $\Delta E_Q = 0.66$; component II, $\delta = 0.44$, $\Delta E_Q = 1.16$. Where δ is the chemical shift relative to metallic iron at 290 K, and $\Delta E_Q (\equiv \frac{1}{2}e^2qQ)$ is the quadrupole splitting. Errors were typically ± 0.02 mm/s. The relative peak areas corresponding to the two components were equal within experimental error.

At 4.2 K the spectrum (see Fig. 2) appears to consist of only one broad quadrupole doublet (II in Fig. 2) and a six-line pattern due to magnetic hyperfine splitting (I in Fig. 1). The two centre lines of the hyperfine component coincide with the two lines of the doublet. The doublet is identified as originating from component II, because its parameters at 4.2 K are almost identical [$\delta(4.2\text{ K}) = 0.44$ mm/s, $\Delta E_Q(4.2\text{ K}) = 1.20$ mm/s] with those given above at 77 K.

The changes in the spectrum of component I may be explained by the increase in its spin-relaxation time at the lower temperature. The hyperfine field at 4.2 K is approx. 49 T. The apparent quadrupole splitting of this component at 4.2 K is less than that expected from the 77 K spectrum and its linewidths are clearly much broader at 4.2 K than at 77 K. On further cooling to 1.4 K (Figs. 1c and 2c) the spectra show no significant changes from those obtained at 4.2 K.

The temperature-dependence of component I can be explained by two distinct hypotheses. (a) If this species of iron was an iron(III) oxy-hydroxide complex similar to ferritin, then the changes would

Fig. 1. Mössbauer spectra of *C. fritschii* membranes on large velocity range (a) 77 K; (b) 4.2 K; (c) 1.4 K. 'Stick' spectra represent approximate absorption line positions of components I and II. For discussion see the text.

Fig. 2. Mössbauer spectra of *C. fritschii* membranes on smaller velocity range than those shown in Fig. 1. (a) 77 K; (b) 4.2 K; (c) 1.4 K. For discussion see the text.

be explained by superparamagnetic behaviour of the antiferromagnetically ordered iron cores of the ferritin-like molecules (Spartalian *et al.*, 1975). This type of material generally has an inhomogeneous, amorphous nature and in this case the increased linewidth and apparent decrease in quadrupole splitting would be due to variations in the hyperfine interactions at different iron sites. (b) If the iron(III) was far more dilutely dispersed throughout the protein material to which it is attached it would show paramagnetic behaviour. In general, if sufficiently dilute, iron(III) paramagnets show magnetic hyperfine splitting at low temperatures. In this case it is not possible to predict the line-shape of the spectra expected until one has fairly detailed information about the hyperfine interactions present, but there is no feature of component I that is inconsistent with a paramagnetic origin. Of course, there is the possibility that component I could be due to a mixture of both of the above forms of iron.

In principle the application of an external field to the sample should distinguish between the above two hypotheses. Fig. 3 shows spectra of a normally grown sample of *C. fritschii* obtained at 4.2 K in

Fig. 3. Mössbauer spectra of *C. fritschii* membranes at 4.2 K (a) In zero applied field; (b) in a magnetic field of 6 T applied perpendicularly to the γ -ray direction; (c) in a magnetic field of 6 T applied parallel to the γ -ray direction ($\Delta m = 0$ transitions are marked by arrows). For discussion see the text.

zero field (a), 6.0 T applied perpendicular to the γ -ray direction (b) and 6.0 T applied parallel to the γ -ray direction (c).

The applied-field-dependence of component II shows it to be a non-magnetic species of iron, such as low-spin iron(II) or a spin-coupled (to total spin zero) state of a polyatomic centre similar to the oxidized state of two- or four-iron ferredoxins. We cannot conclusively distinguish between these alternatives at this stage, but we believe the latter to be more likely for the following reasons. The chemical shift, 0.44 mm/s at 77 K, is at the extreme high end of the range of chemical shifts observed for low-spin Fe(II) compounds (see, e.g., Greenwood & Gibb, 1971). The only well-characterized low-spin iron(II) biological compound with chemical shift greater than 0.25 mm/s is ferrocyanide (Lang, 1970), which has parameters very similar

Fig. 4. Mössbauer spectra of *A. nidulans* membranes at 4.2 K after growth under three different iron concentrations in the medium

(a) Membranes from cells grown on the normal iron concentration in the medium; (b) membranes from cells grown on medium containing 10% of normal iron concentration; (c) membranes from cells grown on medium containing 1% of normal iron concentration. Note the change of vertical scale for the lower spectrum, resulting in the exaggerated background dip which is a purely geometric effect of the spectrometer and should not be confused with absorption. For further discussion see the text.

to component II. This can be excluded as a possibility, because no cytochrome was detected in the membrane fraction by optical spectroscopy. The observed parameters, however, are very similar to those of known ferredoxins [e.g. oxidized *Bacillus stearothermophilus* ferredoxin $\delta(77\text{ K}) = 0.42 \pm 0.1\text{ mm/s}$, $\Delta E_Q(77\text{ K}) = 0.98 \pm 0.02\text{ mm/s}$ (Mullinger *et al.*, 1975)]. The assignment of a ferredoxin-like nature to component II is also attractive in view of the ferredoxin-like e.p.r. signals observed in the same preparations when reduced (Evans *et al.*, 1976).

It is noteworthy that for component I there is a net movement inwards of the absorption lines as

the field strength is increased. Furthermore the $\Delta m = 0$ transitions (arrowed in Fig. 3c) are decreased in intensity, as the relevant selection rules would imply if the effective magnetic hyperfine field acting on the nuclei is aligned parallel to the γ -rays. Both of these observations are typical of the application of an external magnetic field to a paramagnetic material. The fact that the $\Delta m = 0$ transitions were still weakly present could be an indication of either the paramagnetic species not being a simple free-spin paramagnet or that there was some proportion of antiferromagnetically coupled iron present, whose effective hyperfine field was not aligned parallel to the γ -ray direction by the applied field. The behaviour of component I at 4.2 K is not consistent with that of any of the haemoproteins thus far investigated by Mössbauer spectroscopy (see, e.g., Lang, 1970).

The results obtained from *A. nidulans* membranes after growth in complete medium were in general agreement with the conclusions drawn from the above data for *C. fritschii*.

Membranes from *A. nidulans* grown under three regimes of iron nutrition showed considerable differences in their Mössbauer spectra (Fig. 4). Note the change of vertical (percentage absorption) scale for Fig. 4(c), which makes the curved background effect more prominent. This background effect is a purely geometric effect of the spectrometer, and hence the exaggerated curved dip in Fig. 4(c) must not be confused with a broad band of absorption. The ratio of component I to component II was of the order 1:1 under normal growth conditions, whereas the ratio is zero, or very nearly zero, under the iron-deficient growth conditions, which may easily be seen by the lack of the outer absorption lines in Figs. 4(b) and 4(c). It is on this basis, coupled with the observation that the mean generation time was not significantly decreased during growth in medium containing 10% of the normal iron concentration that component I is provisionally ascribed to a form of iron concerned in the processes of iron storage and/or transport present in the membrane preparation. Similar results were obtained with *C. fritschii*.

Thus two sorts of iron species in crude membrane preparations of *C. fritschii* and *A. nidulans* have been identified, a non-magnetic species and one whose concentration is related to the availability of iron to the algae. This latter species has characteristics that are consistent neither with ferritin nor with siderochromes so far characterized. It must be noted that it is the relative iron concentration that is observed and not that of the protein to which it may be bound. Thus, although it is thought that siderochrome concentration rises in blue-green algal cultures in times of iron starvation (C. Van Baalen, personal communication), the amount of iron chelated by the siderochrome may actually fall. However,

all siderochromes so far characterized are soluble, but component I of *C. fritschii* and *A. nidulans* is possibly membrane bound. (The presence of cytochromes in the supernatant fraction makes estimation of any soluble component I uncertain.)

The presence of a ferredoxin-like centre in these preparations is consistent with the finding of ferredoxin-like e.p.r. signals of an iron-containing species on chemical or light-reduction (Evans *et al.*, 1976).

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