Spectroscopic Characterization of Ferredoxin from the Blue-Green Alga *Microcystis flos-aquae*

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Ferredoxins have been isolated as components of the photosynthetic systems of a very wide range of organisms from the anaerobic green and purple photosynthetic bacteria to the higher plants (see Hall & Evans, 1969; Buchanan & Arnon, 1970). All these ferredoxins have very low redox potentials close to that of hydrogen ($E' = 420\text{mV}$), and are interchangeable to some extent in biological activity. The ferredoxins from higher plants are the best characterized; they have molecular weights about 11000, contain two atoms each of Fe and labile sulphide/molecule, and accept one electron on reduction. The ferredoxins of photosynthetic bacteria are less well characterized, but seem to be similar to those of the anaerobic fermentative bacteria, which contain eight atoms each of Fe and labile sulphide/molecule and accept two electrons. In the photosynthetic bacteria the ferredoxins also appear to be involved in fixation of atmospheric N_2 (Evans & Smith, 1971).

The blue-green algae represent an intermediate stage in evolution between the photosynthetic bacteria and plants. The blue-green algae are prokaryotic organisms, as are the bacteria; they have no nuclear envelope and their photosynthetic apparatus is distributed throughout the cell. In contrast the green algae and higher plants are eukaryotic; they have a nuclear envelope and their photosynthetic apparatus is localized in the cell inside the chloroplasts.

It is now thought that the division of living organisms into prokaryotes and eukaryotes is a more basic one than into animals and plants (see Allsopp, 1969). Estimates of evolutionary distance, based on comparisons of protein amino acid sequences and of tRNA base sequences, indicate that the divergence of prokaryotes and eukaryotes was a much more remote event in evolution than the divergences of the eukaryotes into separate kingdoms (McLaughlin & Dayhoff, 1970).

However, the photosynthetic system of the blue-green algae is biochemically more similar to that of the plants than of the photosynthetic bacteria. In the blue-green algae and higher plants water is used as electron donor and O_2 is evolved, in contrast with photosynthetic bacteria, which use other electron

donors such as sulphide. In those blue-green algae that fix N_2 the ferredoxin also functions as electron donor to the nitrogenase system (Smith *et al.*, 1971).

Ferredoxins have been isolated from several species of blue-green algae, including *Nostoc* (Black *et al.*, 1963; Mitsui & Arnon, 1971), *Anacystis nidulans* (Smillie, 1965; Evans *et al.*, 1968; Yamanaka *et al.*, 1969), *Anabaena variabilis* (Susor & Krogmann, 1966) and *Anabaena cylindrica* (Smith & Evans, 1971). All these ferredoxins were similar in their biological activity and optical absorption spectra to those of the higher plants, rather than to those of the bacteria. However, little work has been reported on the spectroscopic properties of blue-green algal ferredoxins.

The present paper describes a detailed study of the ferredoxin from the planktonic blue-green alga *Microcystis flos-aquae*. It was possible to obtain large quantities of this alga from freshwater reservoirs in almost pure culture by exploiting the fact that, because of its high buoyancy, the alga forms virtually uni-algal blooms under certain weather conditions (see Fogg, 1969). The purpose of the study was to determine what structural differences, if any, exist between the ferredoxins of the blue-green algae and those of the higher plants. C.d. (circular-dichroism) and e.p.r. (electron-paramagnetic-resonance) spectra of the ferredoxins are capable of detecting structural differences, such as those induced by urea or guanidinium chloride, that cause very little change in the optical absorption (Garbett *et al.*, 1967; Padmanabhan & Kimura, 1970; Cammack *et al.*, 1971a).

^{57}Fe Mössbauer spectroscopy is capable of giving detailed information about the chemical state of the iron in these molecules; for example it was possible to show that the electronic structure of the Fe atoms in ferredoxin are similar to, but not identical with, those in adrenodoxin (Cammack *et al.*, 1971b; Dunham *et al.*, 1971).

Experimental and results

Preparation of *Microcystis* ferredoxin. *Microcystis* cells were collected from a local freshwater reservoir during August, through the courtesy of Mr. Steele, of the Limnology Laboratory, Metropolitan Water Board, London, U.K. As a result of the very low

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specific gravity of the *Microcystis* cells compared with the other algal material present in the reservoir, the cells had concentrated on the surface as a virtually uni-algal bloom. After collection the cells were concentrated by centrifugation and the purity of the samples was confirmed by microscopic investigation. The cells were stored at -15°C before use. Frozen cells (5 kg) were thawed overnight in 2 vol. of 20 mM-potassium phosphate buffer, pH 7.5, with stirring, and the suspension was passed once through a Manton-Gaulin homogenizer (APV Ltd., Crawley, Sussex, U.K.) at a setting of 8000 lbf/in² (55 MN/m²). The resulting suspension of broken cells was centrifuged at 2000 g for 10 min. Ferredoxin was extracted from the supernatant by batchwise treatment with DEAE-cellulose, and purified on columns of DEAE-cellulose and hydroxyapatite, as described for spinach ferredoxin (Rao *et al.*, 1971). The yield was 150 mg of ferredoxin with an E_{422}/E_{276} ratio 0.56. For measurements of the Mössbauer effect the ferredoxin was chemically reconstituted with ^{57}Fe isotope as described by Rao *et al.* (1971). Samples were stored in liquid N_2 before use.

Properties of the ferredoxin. The amino acid composition of *Microcystis* ferredoxin was determined for us by Professor K. T. Yasunobu and Dr. R. Chung, of the Department of Biochemistry and Biophysics, University of Hawaii, Honolulu, Hawaii, U.S.A. The composition was measured to be (Lys₃, His₁, Arg₁, Asx₁₃, Thr₇, Ser₆, Glx₁₃, Pro₄, Gly₁₂, Ala₉, Cys₅, Val₄, Met₁, Ile₆, Leu₉, Tyr₃, Phe₁) (Trp not determined). The total of 98 amino acids, with $2\text{Fe}+2\text{S}$, gives a minimum value for the molecular weight of 10356. These values should be regarded as somewhat tentative, since the values obtained from analysis of the hydrolysate of a whole protein rarely agree entirely with those from the determination of the amino acid sequence. However, the proportions of the different amino acids are, with the exception of a higher proportion of glycine, very similar to those of spinach ferredoxin (Matsubara & Sasaki, 1968), which has a formula molecular weight of 10646.

Chemical analysis of the native *Microcystis* ferredoxin gave values of 2.3 g-atoms of Fe and 2.3 g-atoms of labile sulphide/mol. The ^{57}Fe -reconstituted ferredoxin gave values of 2.1 g-atoms of Fe and 1.9 g-atoms of labile sulphide/mol. Spectrophotometric titration of *Microcystis* ferredoxin with $\text{Na}_2\text{S}_2\text{O}_4$ solution by the method of Dixon (1971) indicated that on reduction the ferredoxin molecule accepts one electron. This result is in agreement with the measurements made (by using other methods) on ferredoxin from *Anacystis nidulans* (Evans *et al.*, 1968).

The absorption spectrum of *Microcystis* ferredoxin was similar to the spectra of other blue-green-algal ferredoxins (see, e.g., Smillie, 1965; Susor & Krogmann, 1966; Mitsui & Arnon, 1971) and higher-plant ferredoxins, with absorption maxima at 276, 330,

422 and 464 nm. The highest E_{422}/E_{276} ratio obtained was 0.58. On removal of iron and sulphur the peaks at 330, 422 and 464 nm disappeared and the peak at 276 nm was decreased in intensity. Reconstitution with ^{57}Fe and inorganic sulphide restored the original spectrum.

The c.d. spectra of oxidized and of reduced *Microcystis* ferredoxin showed a complex series of bands in the region 250–600 nm. The spectra of the native and ^{57}Fe -reconstituted forms were essentially identical. The spectra were very similar to those of plant ferredoxins such as that from spinach (Garbett *et al.*, 1967), suggesting that the chromophoric groups in the ferredoxins have very similar configurations. However, small differences in the detailed shape of the spectra were observed that presumably reflect differences in the amino acid sequence of the proteins, and suggest that the shape of the c.d. spectra in the region 260–600 nm is a sensitive monitor of subtle changes in the tertiary structure of the ferredoxin protein around the chromophoric group.

Reduced *Microcystis* ferredoxin showed an e.p.r. signal centred around $g=1.96$. The shape of the signal and g values were very similar to those of reduced spinach ferredoxin at a number of different temperatures. Fig. 1 shows the signal at 26°K . The principal apparent g values of this signal were: $g_x=1.89$, $g_y=1.96$, $g_z=2.05$. The signal from reduced ^{57}Fe -reconstituted *Microcystis* ferredoxin (Fig. 1)

Fig. 1. E.p.r. spectra of reduced *Microcystis* ferredoxin at 26°K

—, Native ferredoxin; ----, ^{57}Fe -reconstituted ferredoxin. Samples (0.5 mm), in 0.3 cm-bore tubes, were reduced with 2 mM- $\text{Na}_2\text{S}_2\text{O}_4$ under argon at 20°C for 2 min before being frozen. The settings for measurement were: microwave frequency, 9.170 GHz; power, 4 mW; modulation amplitude, 4 G.

Fig. 2. Mössbauer spectra of reduced ferredoxins from *Microcystis* (0.37 mm), *Scenedesmus* (1.0 mm) and spinach (1.9 mm)

(a) *Microcystis* ferredoxin, 198°K; (b) *Scenedesmus* ferredoxin, 198°K; (c) spinach ferredoxin, 198°K; (d) *Microcystis* ferredoxin, 4.2°K; (e) *Scenedesmus* ferredoxin, 4.2°K; (f) spinach ferredoxin, 4.2°K. Spectra (d), (e) and (f) were all recorded with a field of 0.3 kG applied perpendicular to the γ -rays. The values of percentage absorption for the three ferredoxins are not directly comparable because of different cell geometries.

was somewhat broader than that of the native (^{56}Fe -containing) ferredoxin, as a result of nuclear hyperfine interaction (Thornley *et al.*, 1966), as observed in spinach ferredoxin (Palmer, 1967). No e.p.r. signal was detected from oxidized *Microcystis* ferredoxin.

Mössbauer spectra were recorded of *Microcystis* ferredoxin reconstituted with ^{57}Fe , in the oxidized and reduced forms, at temperatures between 4.2 and 198°K, in various applied magnetic fields. These spectra were identical with the corresponding spectra of spinach and *Scenedesmus* ferredoxins, to within experimental error. Fig. 2 compares the spectra of the reduced ferredoxins, at 198°K, showing the two quadrupole-split doublets due to the Fe^{3+} and Fe^{2+} atoms, and at 4.2°K in a small applied magnetic field, showing the hyperfine splitting due to the unpaired electron. The interpretation of these Mössbauer spectra has been reported elsewhere (Rao *et al.*, 1971; Cammack *et al.*, 1971b; Dunham *et al.*, 1971). Briefly, the results indicate that the oxidized ferredoxin contains two non-equivalent high-spin Fe^{3+} atoms that are in a non-magnetic state and that are most probably antiferromagnetically coupled together; the reduced ferredoxin contains a high-spin Fe^{2+} and a high-spin Fe^{3+} atom, antiferromagnetically coupled together to give a net spin of $S=\frac{1}{2}$. These results are in agreement with the model proposed by Gibson *et al.* (1966) and Thornley *et al.* (1966).

Discussion

This study confirms previous observations that the ferredoxin in the prokaryotic blue-green algae is strikingly similar to the ferredoxins of the eukaryotic algae and higher plants, and dissimilar to those of the green and purple photosynthetic bacteria (Buchanan *et al.*, 1969; Matsubara *et al.*, 1970). This observation is of considerable interest from the point of view of the evolution of photosynthesis. It indicates that the photosynthetic system of the blue-green algae is plant-like, not only in that water is oxidized to O_2 at one end of the electron-transport chain, but also that at the other end NADP^+ is reduced via a plant-type ferredoxin. This observation lends weight to the suggestion (see Echlin, 1970) that the chloroplasts of higher plants are the descendants of blue-green algae that came to live symbiotically inside the early plant cells.

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