Biochimica et Biophysica Acta, 548 (1979) 267–275

BBA 47761

EPR SPECTRA OF PHOTOSYSTEM I AND OTHER IRON PROTEIN COMPONENTS IN INTACT CELLS OF CYANOBACTERIA

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(Received April 23rd, 1979)

Key words: Cyanobacteria; ESR; Photosystem I; Iron-protein; (Nostoc muscorum; Anabaena cylindrica)

Summary

Electron paramagnetic resonance (EPR) spectra were recorded of whole filaments of the cyanobacteria *Nostoc muscorum* and *Anabaena cylindrica*. Signals due to manganese were removed by freezing and thawing the cells in EDTA. EPR spectra were assigned on the basis of their g values, linewidths, temperature dependence and response to dithionite and light treatments. The principal components identified were: (i) rhombic Fe^{3+} (signal at $g = 4.3$), probably a soluble storage form of iron; (ii) iron-sulfur centers A and B of Photosystem I; (iii) the photochemical electron acceptor 'X' of Photosystem I; this component was also observed for the first time in isolated heterocysts; (iv) soluble ferredoxin which was present at a concentration of 1 molecule per 140 ± 20 chlorophyll molecules; (v) a membrane-bound iron-sulfur protein ($g = 1.92$). A signal $g = 6$ in the oxidized state was probably due to an unidentified heme compound. During deprivation of iron the rhombic Fe^{3+}, centers A, B and X of Photosystem I, and soluble ferredoxin were all observed to decrease.

Introduction

Low-temperature EPR spectroscopy can be used to observe metalloproteins in paramagnetic states, such as those containing Fe(III), Cu(II), and Mo(V) and the reduced states of iron-sulfur proteins [1,2]. We present here a study of EPR

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Abbreviation: Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid.

signals from intact filaments and purified heterocysts of the cyanobacteria (blue-green algae) *Nostoc muscorum* and *Anabaena cylindrica*. Because of the selectivity of EPR spectroscopy it was possible to observe a number of different electron transport components, in suitably prepared samples. EPR measurements on cyanobacteria have normally been carried out on cell-free preparations (e.g. Ref. 3), although Visser et al. [4] used EPR spectroscopy to observe the oxidized copper protein plastocyanin in whole cells of various algae including the cyanobacterium *Anacystis nidulans*, after treatment with far-red light.

The use of whole cells would be expected to avoid losses inevitably incurred during the isolation of components, though the selective permeability of the membrane presents some problems. In the present work, sample preparation was designed to produce quantitative results.

The principal difficulties are concerned with interferences due to metals such as manganese, and with the relatively low concentration of the proteins in cyanobacterial cells. Visser et al. [4] used growth on manganese-free medium. We have used another technique based on the complexing of manganese with EDTA which can be used on cells cultured on normal medium, and concentrated the cells by centrifugation into EPR tubes.

Experimental

Cells and cultures

N. muscorum strain 7119, kindly made available to us from Dr. D.I. Arnon, and *A. cylindrica* Lemn, from the Cambridge culture collection, were grown in an Allen and Arnon medium [5] continuously illuminated by fluorescent cool light (3000 lux) on a shaker and gassed with 95% air plus 5% CO_2 at 25°C. Considerable care was taken to keep the cultures and apparatus free from dust, since metal-containing particles would be concentrated with the cells, and give strong EPR signals.

Heterocysts were separated from filaments by Yeda press treatment [6], and the culture was harvested by centrifugation at 4000 × *g* for 10 min. The pellet was resuspended in half the original volume of 30 mM Hepes/6 mM EDTA, pH 7.5, and recentrifuged, then resuspended in one-tenth of the original volume of the same buffer in a centrifuge tube, and routinely frozen by immersion in liquid N_2. Samples could be stored at this stage in liquid N_2. The freezing in the presence of EDTA caused the loss of signals due to Mn^{2+} which overlap those of Photosystem I (see Results). For preparation of EPR samples, the cells were thawed, centrifuged and resuspended in 30 mM Hepes/6 mM EDTA to a concentration of approx. 0.2 mg chlorophyll/ml. Chlorophyll was measured by extraction of a sample with acetone, and measuring the absorbance at 663 nm [7].

Samples of concentrated cells for EPR spectroscopy, were prepared by centrifugation in specially constructed holders shaped to fit the 8 × 50 rotor of a Sorval RC2-B centrifuge, and each bored to fit four quartz tubes of length 95 mm. In order to prevent breakage of the tubes, the sockets in the holders were first filled with water. The EPR tubes were calibrated for internal diameter so the sample volumes could be calculated from the length. Algal cells

were sedimented at 17 000 X g for 10 min. The supernatant was removed from the tubes, leaving a total length (pellet + solution) of exactly 25 mm. This length was sufficient to fill the sensitive region of the EPR cavity. The pellet was evenly suspended by stirring with a stainless steel wire. Typically the concentrated samples contained 3 mg chlorophyll/ml.

Reduced samples were treated with 10 μl 0.1 M $Na_2S_2O_4$/0.2 M Tris-HCl, pH 9.0, under argon atmosphere, and stirred vigorously for 5 min. Samples were prepared either in complete darkness, in tubes wrapped in aluminium foil, or illuminated with white light, after dithionite addition, by a 300 W projector with a convex lens to focus the beam on the sample region. To prevent overheating, the tubes were immersed in water in a test tube with aluminum foil around the reverse side, which served to concentrate the beam on the sample. The tube was also illuminated during freezing in liquid nitrogen.

Oxidation-reduction titrations monitored by EPR spectroscopy were carried out in an apparatus similar to that described by Dutton [8] using the mediator system described in Ref. 9.

Results and Discussion

EPR signals due to manganese

Low-temperature EPR spectra of intact filaments of *Nostoc* and *Anabaena* are dominated by a series of lines around $g = 2$ (Fig. 1) and $g = 4.3$ (Fig. 2) typical of Mn^{2+} and Fe^{3+}, respectively. In agreement with these assignments,

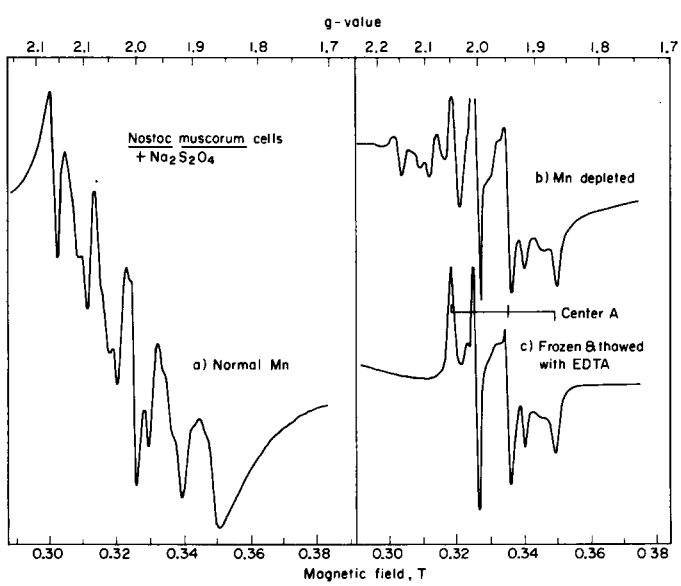

Fig. 1. EPR spectrum of whole *Nostoc* cells in the region around $g = 2$, showing iron-sulfur center A, oxidized *P*-700, and the overlaying spectrum of Mn^{2+}. Samples were frozen in 5 mM dithionite in the dark, and illuminated at 15 K. Sample (a) cells grown in normal medium; (b) depleted of Mn^{2+} by growth for 1 month on manganese-free medium; (c) cells grown on normal medium, but rapidly frozen and thawed in the presence of 6 mM EDTA. EPR spectra were recorded on a Varian E 109 spectrometer, with an Air Products liquid helium transfer system. Instrument settings: temperature 15 K, microwave power 10 mW, frequency 9.14 GHz, modulation amplitude 1 mT, frequency 100 kHz.

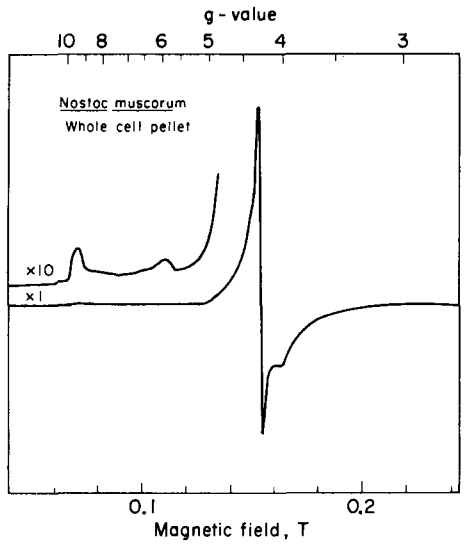

Fig. 2. Spectrum of a concentrated pellet of *Nostoc* cells, showing the intense signal around $g = 4.3$. Instrument settings: temperature 15 K, microwave power 10 mW, frequency 9.14 GHz, modulation amplitude 1 mT, frequency 100 kHz. The inset spectrum was at ten-fold increased gain.

these signals diminished in cells deprived of manganese and iron. To remove the intense Mn^{2+} signal which obscures radicals and Fe-S proteins, cells were grown on a medium having limiting manganese, thus permitting signals due to Photosystem I center A to be observed above a background of Mn^{2+} hyperfine lines (Fig. 1b). Manganese is required for Photosystem II activity and can therefore not be excluded completely. Mn^{2+} signals could be depleted more effectively if the cells were alternatively frozen and thawed in the presence of 6 mM EDTA before finally concentrating the preparation by centrifugation. Although membranes were presumably made more permeable by this process, there was little leakage of protein constituents such as phycocyanin if cell suspensions were frozen within a few seconds in liquid nitrogen. However, slow freezing at −15°C caused more extensive damage and considerable leaching of phycocyanin.

The results shown are for *Nostoc*. Essentially similar results were found for *Anabaena*.

Ferric iron

The signal around $g = 4.3$ is due to high-spin Fe^{3+} in a 'rhombic' invironment and arises from the middle Kramer's doublet of the $S = 5/2$ system [10]. The ground state of this system gives rise to a signal with a derivative peak near $g = 10$ (Fig. 2). Also visible in this spectrum is a small signal at $g = 6$, probably due to high-spin ferric heme such as a cytochrome. All of these signals disappeared on reduction with dithionite. Similar signals have been observed in animal and bacterial cells [11]. A variety of iron-containing proteins, such as transferrin, give signals around $g = 4.3$ [12,13]. It is probable that the spectrum at $g = 4.3$ (Fig. 2) represents a stored form of iron. It was diminished by more

than 80% during growth on a limiting concentration of iron.

After ultrasonic treatment and centrifugation at 100 000 × g for 60 min, the $g = 4.3$ signal was principally in the supernatant fraction, with only a small, narrow signal at $g = 4.3$ associated with the membrane pellet. Mössbauer spectroscopy of the membrane fractions of the cyanobacteria *Chlorogloea fritschii* and *A. nidulans* by Evans and coworkers [14] indicated the presence of a considerable amount of an iron storage component. This might be an EPR-silent form such as Fe (II) or ferritin. It seems likely that in cyanobacteria there are at least two forms of stored iron.

Cyanobacteria are known to produce siderochromes [15], the iron complexes of which have a signal at $g = 4.3$ [10]. However, these are mainly produced under conditions of iron limitation and are therefore unlikely to be present in large amounts in cells grown on normal medium. The 'soluble' iron might be bound to proteins, or metabolites such as citrate.

Photosystem I and ferredoxin

The membrane-bound primary acceptor complex of Photosystem I in plants contains the primary photochemical pigment *P*-700 (observed by EPR in the oxidized state as a radical at $g = 2.00$), a component 'X' (signal at $g = 1.78$, 1.88, 2.08 in the reduced state) which has been proposed as the primary electron acceptor [16] and two iron-sulfur centers A and B, ($g = 1.86$, 1.94, 2.05, when A is reduced, and $g = 1.89$, 1.92, 1.94, 2.05, when both are reduced) which are secondary electron acceptors [17]. All of these components have been observed in purified preparations from the cyanobacterium *C. fritschii* [18]. Their function is to transfer electrons to the soluble ferredoxin [19]. The membrane-bound components can be reduced chemically in the dark by dithionite, in the sequence of decreasing midpoint potential, A, B then X. Reduction of X is slower and requires an alkaline pH to achieve a sufficiently low applied potential [17]. The components can also be reduced photochemically. At 10 K photoreduction of A and B is irreversible, but photoreduction of X (in samples where A and B have already been chemically reduced) is reversible [18].

The most reliable method of obtaining samples in which signals from A, B and X could be observed was by reducing photochemically in the presence of dithionite at room temperature, and freezing the reduced samples in liquid nitrogen in continuous illumination, as described in Experimental. In whole cells the reduction of A and B was complete in a few seconds but development of the signal due to reduced X took several minutes.

The signals from reduced iron-sulfur centers of Photosystem I could readily be distinguished on the basis of their g values and temperature dependence. Fig. 3 shows spectra of a sample of *Nostoc* cells, treated with EDTA and illuminated in the presence of dithionite before freezing. The soluble ferredoxin has a [2Fe-2S] cluster and can be observed at higher temperatures than centers A and B which have [4Fe-4S] clusters [20]. At 48 K the spectrum at $g = 1.89$, 1.96, 2.05 is principally due to the soluble ferredoxin, though measurements of washed membrane preparations showed that a broadened spectrum due to Photosystem I centers A and B can be seen at this temperature. At 22 K the spectrum of ferredoxin is saturated with microwave power, and obscured by the sharper signals due to centers A and B. At 10 K, additional

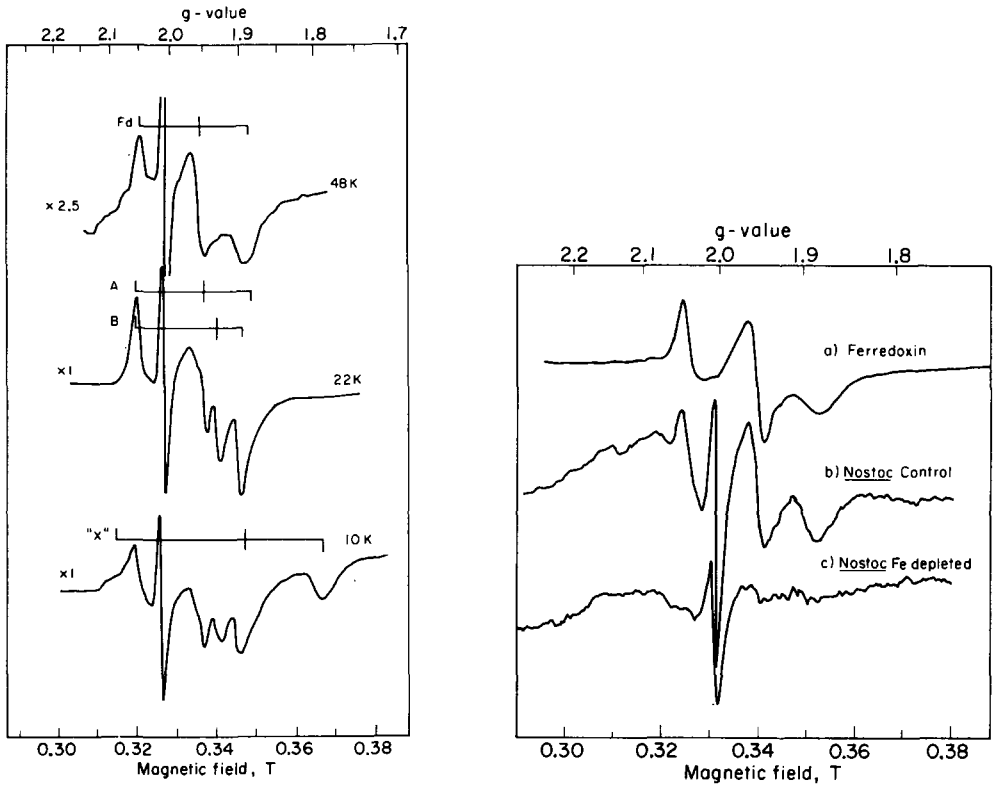

Fig. 3. Spectra of a pellet of *N. muscorum* cells, treated with 10 mM $Na_2S_2O_4$ and illuminated during freezing, (a) at 48 K, (b) at 22 K, (c) at 10 K. The gain setting for (a) is 2.5 times that for (b) and (c). Stick spectra indicate assignments of features due to soluble ferredoxin, and centers A, B and X of Photosystem I. Instrument settings: microwave power 10 mW, frequency 9.14 GHz.

Fig. 4. EPR spectra of dithionite-reduced soluble ferredoxin, measured at 62 K. (a) Purified *S. maxima* ferredoxin; (b) *Nostoc* cells, grown on normal medium, 2.68 mg chlorophyll/ml, reduced with dithionite in the dark, and (c) *Nostoc* cells grown on a medium lacking iron for 65 days, 1.0 mg chlorophyll/ml, reduced as for (b). (b) and (c) were recorded at a gain setting six-fold higher than for (a). Other instrument settings: microwave power 20 mW, frequency 9.29 GHz.

signals due to 'X' can be seen. The positions of the principal g values of these components are also indicated by stick spectra in Fig. 3.

In order to measure the concentration of ferredoxin in the absence of signals due to Photosystem I, samples were treated with dithionite in the dark for 10 min before freezing, and transferring to the EPR spectrometer in the dark. In this way the spectrum of reduced ferredoxin could be observed (Fig. 4b), and the concentration determined by comparing the amplitude of the $g = 1.96$ signal with that of a standard sample of reduced *Spirulina maxima* ferredoxin (Fig. 4a). The small amount of center A reduced under these conditions did not have any significant effect, since further photoreduction of A [21] by illumination in the cavity of the EPR spectrometer (monitored at 20 K) had negligible effect on the size of the $g = 1.96$ signal at 50 K.

The concentration of ferredoxin in various samples of *Nostoc* cells was estimated to be 1 per 140 ± 20 chlorophyll molecules. This is higher than the

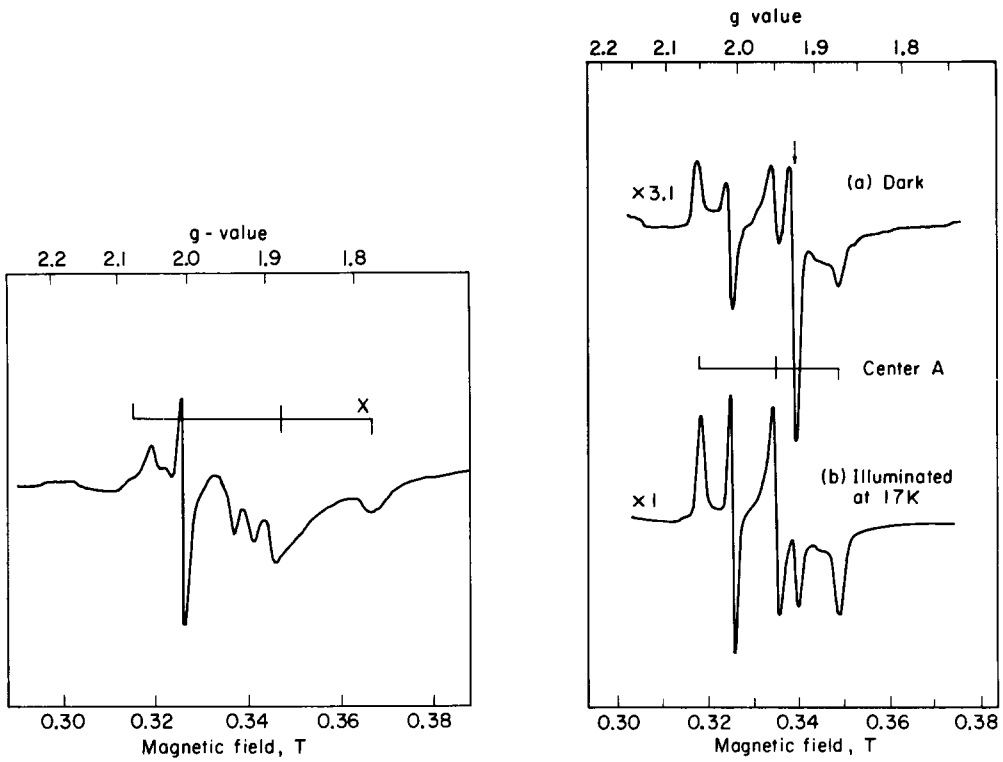

Fig. 5. Spectrum of Photosystem I in *Nostoc* heterocysts (0.8 mg chlorophyll/ml) showing signals due to 'X'. Sample was illuminated before freezing. Instrument settings: temperature 10 K, microwave power 10 mW, frequency 9.14 HGz, modulation amplitude 1 mT, frequency 100 kHz.

Fig. 6. Spectra of *Nostoc* cells, treated with 10 mM $Na_2S_2O_4$ in the dark before freezing, (a) Dark, showing prominent signal at $g = 1.92$; (b) illuminated at 17 K (gain decreased by a factor of 3.1) showing the increase in signals due to Photosystem I center A. Instrument settings: temperature 17 K, microwave power 20 mW, frequency 9.14 GHz, modulation amplitude 1 mT, frequency 100 kHz.

ratio of 1 : 400 seen for some other photosynthetic electron transport components [4]. It is possible that *N. muscorum* may contain two types of soluble ferredoxin, like those in *Nostoc* MAC [22]. (These have similar EPR spectra which would not be distinguished by this method [23].) Ferredoxin has a number of functions in addition to photosynthetic electron transport [23].

Photosystem I components in heterocysts

Fig. 5 shows the spectrum of 'X' in a concentrated preparation of heterocysts. The presence of this component in heterocysts of *A. cylindrica* was inferred from reversible photochemical oxidation of *P*-700 [24] but this is the first time that this Photosystem I component has been directly observed in heterocysts.

$g = 1.92$ signal

An additional signal at $g = 1.92$ was observed at 10—20 K in samples of cells reduced in the dark. This is shown arrowed in Fig. 6a. The g value and the fact

that it is a reduced component, suggest that it is due to an iron-sulfur center. One would expect another, higher g value for this spectrum, but this may be too broad to be detected, or else obscured under other features of the spectrum. The spectrum of reduced center A was observed in samples reduced in the dark, though Fig. 6b (recorded at lower gain) shows that it was small in comparison with that produced on illumination at 17 K, i.e. it represented only a small fraction of the total centers A.

The optimum temperature for observing the $g = 1.92$ signal was 10—20 K. It was observed in *N. muscorum* and *A. cylindrica*, in heterocysts and vegetative cells, in the membrane rather than the soluble fraction.

The midpoint potential of the component giving the $g = 1.92$ signal was estimated by potentiometric titration of membrane fragments. All EPR samples were prepared in the dark. The value obtained for the midpoint potential was -270 ± 25 mV.

The function of the '$g = 1.92$ component' is not yet known though it might be a respiratory component. In membrane preparations, it was not reduced by hydrogen or NADH, though in whole cells it was reduced by hydrogen gas.

Effects of iron deficiency on Photosystem I components

Nostoc cultures were grown in a medium from which iron (normally 6 ppm) was omitted. Fig. 7 shows the effect on the levels of Photosystem I centers A

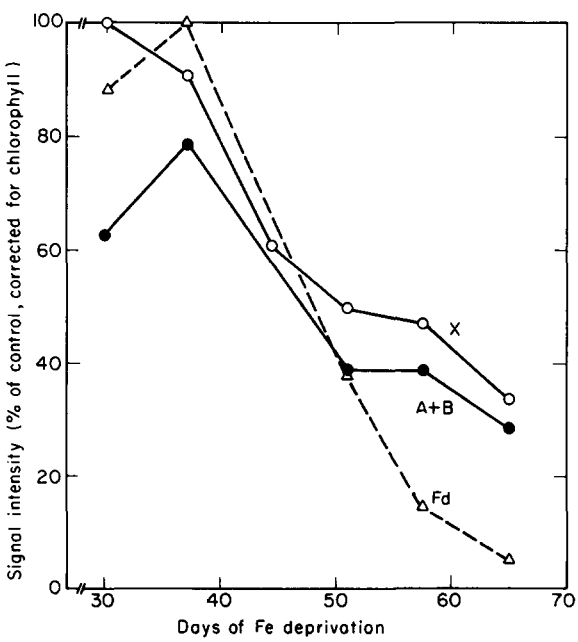

Fig. 7. Size of the EPR signals due to Photosystem I components, and ferredoxin (Fd), in a culture of *Nostoc* grown on iron-free medium. Centers A and B (●) determined from the size of the downward feature at $g = 1.89$ in the spectrum at 20 K (Fig. 3b); X (○) was estimated from the feature at $g = 1.78$ at 10 K (Fig. 3c) and ferredoxin (△) from the feature at $g = 1.96$ at 60 K (Fig. 4b). Signals were corrected for the chlorophyll concentration of the cells and expressed as a percentage of the values for control cells grown on normal medium.

and B (which appeared always in the same proportion), the primary acceptor X, and soluble ferredoxin, determined from the size of the EPR signals as described previously. The levels are expressed relative to chlorophyll concentration. For the first 30 days there was little effect, as the cells used up their reserves of stored iron; after 70 days the culture ceased growing. During the period 30—65 days, there was a dramatic decrease in soluble ferredoxin (Fig. 4c). It is known that in iron deficiency, flavodoxin can substitute for ferredoxin [22]. There was also a decrease in centers A and B, which, being [4Fe-4S] centers require considerable quantities of iron for their synthesis. It was found in other experiments (not shown) that addition of iron to the culture medium restored centers A, B, X and ferredoxin to their normal levels.

It is of interest that, in parallel with the decrease in centers A and B, the primary acceptor X also decreases on deprivation of iron. This may be taken as evidence that X is an iron protein. However, this conclusion should be taken with caution, since it may be that centers A and B are required before it can be reduced and observed by EPR spectroscopy. Alternatively in the presence of smaller amounts of centers A and B, the synthesis of X may also be repressed.

Acknowledgement

This work was supported by the Energy and Environment Division of the U.S. Department of Energy under contract No. W-7405-ENG-48.

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