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CHARACTERIZATION, AMINO ACID SEQUENCE AND PHYLOGENETIC CONSIDERATIONS REGARDING THE FERREDOXIN FROM *OCHROMONAS DANICA*

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Key Word Index—*Ochromonas danica*; Chrysophyceae; ferredoxin; redox potential; protein sequence; taxonomy.

Abstract—A [2Fe-2S] ferredoxin of M_r 11 100 was isolated from the Chrysophycean flagellate *Ochromonas danica*. In its oxidized form the ferredoxin had absorption maxima at 275, *sh.* 281, 330, 423, 458 nm. The midpoint potential of the ferredoxin was −380 mV; it was active in a NADPH-cytochrome *c* reductase assay. The amino acid sequence determination was based on Edman degradation of the Cm-ferredoxin and peptides obtained by protease treatments. Comparison with other ferredoxin sequences by constructing a phylogenetic tree showed that *Ochromonas danica* ferredoxin was more closely related to the ferredoxins from red algae and filamentous cyanobacteria than to those from green algae.

INTRODUCTION

Photosynthetic oxygen-evolving cells invariably contain a soluble ferredoxin, and sometimes two, possessing a single [2Fe-2S] cluster. Ferredoxins from a considerable number of cyanobacteria (blue-green algae) have been isolated and characterized to varying extents and approaching 20 have been sequenced [1, 2]. However, the data for other algae are less extensive and only 10 sequences have been reported: those for the ferredoxins from *Porphyra umbilicalis* and *Rhodymenia palmata* [3] in the Rhodophyceae, including also *Cyanidium caldarium* in this class; *Bryopsis maxima* [4], *Dunaliella salina* (Ferredoxins I and II) and *Scenedesmus quadricauda* (Chlorophyceae); *Euglenoid* LJ1 (Euglenophyceae); *Peridinium bipes* (Dinophyceae); and *Bumilleriopsis filiformis* (Xanthophyceae). Except where shown, sequences are summarized in [1] or are unpublished data.

Ochromonas danica is a member of the order Ochromonades (Suborder; Ochromonadineae) of the Class Chrysophyceae in the Division Chrysophyta [5]. It is a freshwater unicellular flagellate, capable of heterotrophic phototrophic and phagotrophic nutrition [6]. Aspects of its photosynthesis are of interest in both a comparative biochemical and an evolutionary context. Concerning the former, the lipids of *Ochromonas danica* have been characterized [5] and a high sulpholipid content [7] has proved of particular interest. However, since these molecules have detergent properties in cell extracts the studies of proteins from *Ochromonas* have been fraught with difficulty, though we can now report the isolation and characterization of the soluble ferredoxin from this alga.

RESULTS AND DISCUSSION

Isolation of the ferredoxin

There were a number of difficulties associated with isolation of electron transport proteins from the alga. Firstly, cultures maintained in our collection deteriorated gradually in terms of the yield of ferredoxin from bulk cultures, though cell growth was apparently normal. Secondly, the best yields of ferredoxin were obtained from acetone-dried cells rather than cells broken by French pressure-cell treatment; even so, the best yields came from acetone powders prepared from cells harvested in the log phase of growth and used within a week of preparation. This may be due to phenolic compounds accumulating progressively at the stationary phase, and the complexing of these with ferredoxin in extracts and even gradually in acetone powders. This may have been the cause of a darkening of the white/light green colour of acetone powders stored for several weeks. The best yield was some 15 mg ferredoxin from 65 g acetone powder used within a week of preparation from cells harvested in the late log phase of growth (cell density 3.1×10^6/ml) from 22 l of culture. This was about a fifth of the yield that would typically be obtained from a similar amount of a cyanobacterial powder [e.g. 8].

The ferredoxin could be obtained in a homogeneous state as demonstrated by analytical polyacrylamide gel electrophoresis on 10, 15 and 20% (w/v) gels stained with Coomassie Brilliant Blue. Homogeneity was also confirmed by isoelectric focusing on polyacrylamide gels, which gave a pI value of 3.4 for the ferredoxin.

Composition and physical properties

The absorption maxima in the UV-Vis spectrum of *Ochromonas danica* ferredoxin were at 275, sh. 281, 330,

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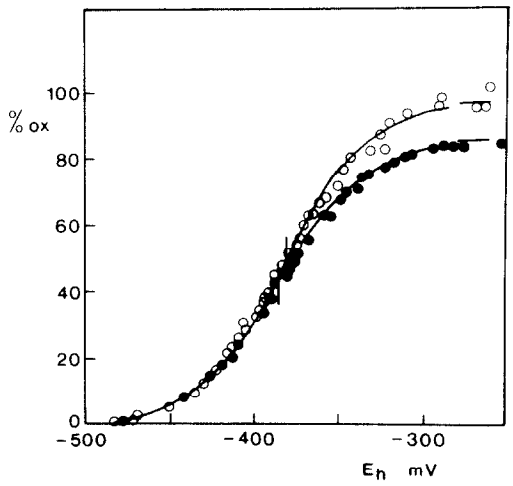

Fig. 1. Midpoint redox potential of *Ochromonas danica* ferredoxin. Experimental points are the absorbance change at 460 nm for the ferredoxin as a function of the redox potential at which the samples, in 0.15 M Tris–HCl, pH 8.0, were poised. Data for successive reductive (○) and oxidative (●) titrations are superimposed on theoretically derived plots for one-electron-transferring species.

423, 458 nm with A_{max}/A_{275} nm ratios of 0.80, 0.54 and 0.48, respectively, for the maxima. The spectrum was characteristic of [2Fe-2S] ferredoxins from other algae [2]; determination of non-haem iron content confirmed the nature of the ferredoxin. The molar absorption coefficient at 423 nm based on the spectrum and protein determinations was 5.2 l/mmol/cm, similar to a number of other algal ferredoxins. The midpoint redox potential of -381 mV± 3 ($n=6$) was based on monitoring ΔA_{460} nm during potentiometric titrations (Fig. 1). There was apparently some loss in protein integrity under the strongly reducing conditions at the end of the reductive titration and at the conclusion of the successive oxidative titration the initial A_{460} was decreased by some 10%; however, the experimental points still fitted closely the theoretical curve for a one-electron-transferring species. This E_m value is in the mid-range of those for algal ferredoxins [2].

The M_r of the ferredoxin was determined by meniscus-depletion sedimentation equilibrium to be 11 400; calculation subsequently from the sequence is 11 094, including the iron-sulphur cluster.

Biological activity

The biological activity of the ferredoxin was assessed by its ability to mediate in electron transfer from NADPH to cytochrome *c* in the presence of ferredoxin–$NADP^+$ oxidoreductase. In this assay, 7 μM ferredoxin supported a cytochrome *c* reduction of 60 nmol/min in a standard assay. This was *ca* 65% the activity shown in parallel experiments by the ferredoxins from the cyanobacteria *Nostoc* strain MAC (ferredoxin I) and *Aphanothece halophitica*, although these have similar E_m values, -350 and -380 mV, respectively.

Sequence determination

The strategy of the sequence determination is summarized in Fig. 2. The elucidation of the sequence provided some difficulty due to the distribution of the residues susceptible to cleavage by trypsin and chymotrypsin. This was circumvented by use also of lysylendopeptidase and supplementary cleavage of some of the resulting peptides with staphylococcal protease, with recourse to carboxypeptidase to resolve some remaining problems. Even so, there were no overlapping sequences at the peptides cleaved at residues 52, 55 and 73. However, for the first two the amino acid compositions for the small tryptic peptides T-6 and T-7 were unequivocal and no residue was unaccounted for in the sequence as shown. Even discounting homology with other ferredoxins, inspection of the data shows no alternative placement of T-7 within the sequence is possible. The same degree of confidence for the amino acid composition of T-8 would not be justified because of the larger size of this peptide. There was therefore an element of uncertainty remaining in the identification of residue 73 as glutamic acid. However, the great majority of two-iron ferredoxins have either glutamic acid or alanine at this position; the single alanine detected in the composition of T-8 could be allocated with certainty during sequence analysis to position 81 (84 aligned as in [1]) where, moreover, it is invariant in all the ferredoxins so far sequenced.

Some details of the sequence are of interest. These include the absence of deletions at positions 12 and 16 [cf. 1] introduced in the aligned sequences of green algal ferredoxins to give maximum homology to (most) red algal and cyanobacterial ferredoxins (Fig. 3). Of the 38 or so such ferredoxins so far sequenced [1–4; aligned as in 1], *Ochromonas danica* ferredoxin is the only one to have Lys-3, His-14, Asn-35 and Asn-36 while for a number of other positions the same residue occurs only in one other ferredoxin. These include Asn-15, Ser-93 and Asn-99 found only in *Bumilleriopsis filiformis*, and *Dunaliella salina* ferredoxins II and I, respectively; all these are eukaryotic algae as is *Ochromonas*.

As more sequences are available, attention can focus on the declining numbers of residues which are invariant in all [2Fe-2S] ferredoxins and which therefore may have critical structural or functional roles. Inspection of the sequences available, including unpublished work for the *Euglenoid* ferredoxin (R. Ambler, personal communication) limit such residues to the four cysteines which bind the iron-sulphur cluster and in addition glycine residues at positions 46, 53, 58 and 77, the last with one exception (Ser in *Rhodymenia palmata*), and Ala-84 (two exceptions; Thr in *Peridinium bipes* and *Euglenoid* st. LJ-1). Presumably these small residues must play important roles in stabilising the conformation of the molecule.

Construction of a phylogenetic tree for a representative selection of algal and cyanobacterial ferredoxins (Fig. 4) suggests that on the basis of its ferredoxin *Ochromonas danica* is closer to eukaryotic red algae, a yellow-green alga and filamentous cyanobacteria than to green algae. This conclusion is supported by a specific feature of its sequence (Fig. 3), in the absence of deletions at positions 12 and 16 necessary to maximize homology for higher plant and green algal ferredoxins.

EXPERIMENTAL

Ochromonas danica (Pringsheim) 933/2 was obtained from the Culture Collection of Algae and Protozoa, Cambridge, U.K. and was grown photoheterotrophically according to [6]. Cultures were grown at 25° in an 11 l fermentation vessel with agitation

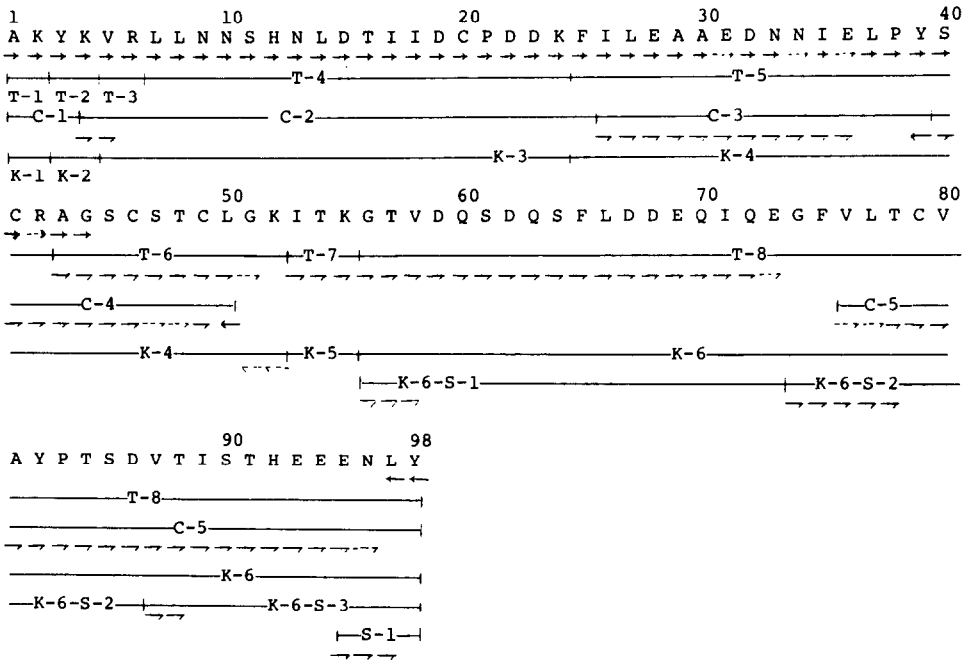

Fig. 2. Summary of the sequence studies with *Ochromonas danica* ferredoxin. T, C, K and S indicate peptides obtained by trypsin, chymotrypsin, lysylendopeptidase and staphylococcal protease treatment of the carboxymethylated protein, respectively. The amino acid sequence was determined by a combination of gas-phase Edman degradation of the intact Cm-ferredoxin (——→) and manual Edman degradations of peptide fragments (—→), and carboxypeptidase A (←——) and Y treatment (←—). Dotted arrows represent ambiguous identifications. Amino acids are shown by their single letter notation.

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   1        10
1 -AT-YKVTLKT-PSG-DQ
2 -AT-YSVKLIN-PDG-EV
3 ----FKVTLDT-PDG-KK
4 -AK-YKVRLLNNSHNLDT
5 -AT-YSVTLVNEEKNINA
6 -AD-YKIHLVSKEEGIDV
7 -AS-YKIHLVNKDQGIDE
8 -AT-YKVTLVR-PDGSET
9 -AT-YKVTLVNAAEGLNT
10-AT-YKVTLISEAEGINE
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Fig. 3. *N*-Terminal sequences of algal ferredoxins. Gaps are introduced as necessary to give maximum homology. Numbering of the residues corresponds to that shown in [1] for a range of [2Fe-2S] ferredoxins. The numbers of the sequences correspond to Fig. 4. The sequence for *Euglenoid* st. LJ-1 is from unpublished work (R. Ambler, personal communication).

and aeration; illumination at the vessel surface was 2000 lux. Cells were harvested in the late stage of logarithmic growth by centrifugation at 4000 *g* for 5 min. and a Me_2CO-powder prepared from them [2].

Routinely, the ferredoxin was isolated by homogenisation of 50 *g* Me_2CO-dried powder in 0.15 M Tris–HCl, pH 7.4; treatment with deoxyribonuclease and ribonuclease; and successive $(NH_4)_2SO_4$ treatment, anion-exchange chromatography on DEAE-cellulose, and gel filtration on Sephadex G-50. The protocol was essentially that adopted previously for the isolation of ferredoxins from *Nostoc* strain MAC [8] with only non-substantive modifications. The yield of ferredoxin was *ca* 10 mg per 50 g Me_2CO powder.

Other methods were as in [2] except for the following:

Midpoint redox potential was determined by potentiometric titration with $Na_2S_2O_4$ (reduction) and $K_3[Fe(CN)_6]$ (oxidation) in a 1 cm light-path cuvette whose contents could be maintained under anaerobic conditions by a flow of Ar gas purged of traces of O_2 by passage through Fieser's solution [9].

The cell oxidation–reduction potential at ambient temperature was measured with a Pt electrode by reference to a calomel electrode connected by a salt bridge to the cuvette contents. Reductant or oxidant, maintained under anaerobic conditions, were added by microsyringe as necessary.

The degree of reduction of the ferredoxin (75–150 μM) was monitored spectrophotometrically with a Hitachi–Perkin–Elmer dual-wavelength spectrophotometer (model 356) by following absorbance change at 460 nm relative to a reference wavelength of 730 nm. Choice of monitoring wavelength was to take advantage of a plateau in the spectrum at a wavelength free of interference by other components and where the decrease in absorbance during reduction (*ca* 56%) was maximal. For the reference wavelength there was no suitable isosbestic point and 730 nm was chosen as a wavelength where neither redox form of the ferredoxin absorbed. The potentials at equilibrium after each addition of reductant or oxidant were plotted against % oxidation ($100\Delta A/A_{ox} - A_{red}$) and the data fitted to theoretical curves

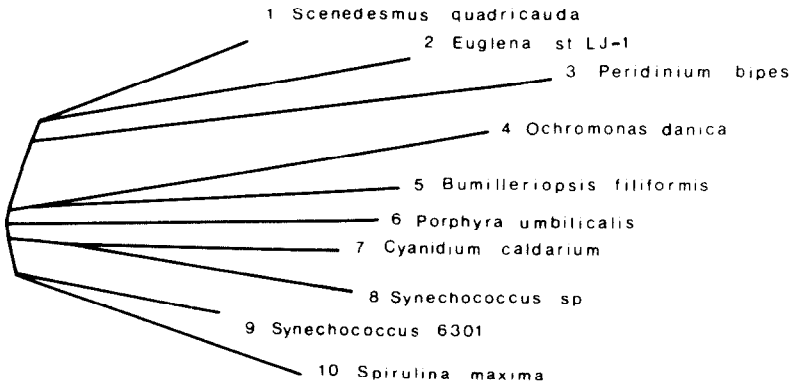

Fig. 4. Phylogenetic tree of representative algal ferredoxins, constructed according to [9]. A deletion has been counted as one difference.

based on the Nernst equation, enabling E_m and stoichiometry of electron transfer to be determined. The same information was obtained from plots of log (redox ratio) against cell oxidation–reduction potential.

The mediators used, with their $E_{m,7}$ values, were 1,1′-dimethyl 4,4′-bipyridylium dichloride, 1,1′-dibenzyl 4,4′-bipyridylium dichloride and 7,8-dihydro 6H-dipyrido[1,2*a*: 2′,1-*c*]diazepinedium dibromide, all at 10 μM.

Biological activity was assessed from the ability of the ferredoxin to support cytochrome *c* reduction from NADPH in the presence of ferredoxin-NADP$^+$ oxidoreductase. The reaction mixture contained in a total volume of 1 ml: 15 μmol of Tris–HCl, pH 7.8; 0.12 μmol NADPH; 0.05 μmol horseheart cytochrome *c*; 10 μg ferredoxin-NADP$^+$ reductase partially purified from *Chondrus crispus*; and in separate assays varying amounts (2–10 nmol) of ferredoxin. The rate of reduction of cytochrome *c* at 30° was followed at 550 nm, using an absorption coefficient of 19.1 l/mmol/cm.

The amino acid sequence determination of the carboxymethylated ferredoxin was based on gas-phase and manual Edman degradations of intact protein and of peptides produced by protease treatment and purified by HPLC. Peptides produced by treatment with trypsin, chymotrypsin or lysylendopeptidase, and others derived by staphylococcal protease digestion of the largest lysylendopeptidase peptide, formed the basis of the determination; some information was also obtained from carboxypeptidase A and Y treatment of the Cm-ferredoxin and derived peptides. The strategy has been described in previous publications [3, 4].

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