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PURIFICATION AND SEQUENCE DETERMINATION OF TWO FERREDOXINS FROM *DUNALIELLA SALINA*

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Key Word Index—*Dunaliella salina*; Chlorophyceae; green alga; protein sequence; ferredoxin; evolution; halophyte.

Abstract—A ferredoxin was purified from the halophyte *Dunaliella salina* by standard procedures. This preparation was found to be heterogeneous by amino-terminal analysis and was resolved into two fractions by chemical modification and chromatography. The primary structure of both ferredoxins consists of 95 amino acid residues which include five cysteines. There are 16 amino acid differences in the sequences of the two ferredoxins. The amino-terminal of one of the ferredoxins is serine whereas all other plant and algal ferredoxins so far sequenced have alanine at the amino-terminus. The sequences of the two ferredoxins from this halophyte resemble those of other chloroplast ferredoxins but do not show any special homology to *Halobacterium* ferredoxin.

INTRODUCTION

The green alga *Dunaliella* [1] is adapted to life in concentrated salt solutions. It grows and thrives in water too brackish to support any but a few strains of halophilic bacteria, and it is able to adjust to a broad range of salt concentrations from low salinity to almost saturated salt solutions. The mechanism used by the alga to withstand salinity differs radically from that observed in several halophilic and halotolerant bacteria. *Dunaliella* protects itself from dehydration normally brought about by immersion in high salt concentration through photosynthetic production of free glycerol [2]—under optimum conditions 80% of *Dunaliella*'s dry weight is glycerol. Ferredoxins are low molecular weight electron transfer proteins found in all living organisms; they are essential catalysts in photosynthetic CO_2 fixation and ATP production [3]. We have recently determined the sequence of a 2Fe–2S ferredoxin from *Halobacterium halobium* [4]. This bacterial ferredoxin was found to have homologous segments with plant and algal ferredoxins though it had about 30 extra amino acid residues in its sequence than the latter group of ferredoxins. The aims of this study were: to isolate and characterize the ferredoxin from *Dunaliella salina* and compare its properties with ferredoxins from nonhalophilic algae and *Halobacterium*; and to determine the sequence of this ferredoxin and examine the homology to other plant-type ferredoxins and with *Halobacterium* ferredoxins.

RESULTS AND DISCUSSION

The yield of ferredoxin from *D. salina* was very high—*ca* 150 mg of protein from 300 g wet cells. The catalytic activity of this ferredoxin in transferring electrons to NADP and to *Clostridium pasteurianum* hydrogenase was comparable to those of other algal and plant ferredoxins.

Separation of carboxymethyl (Cm)-ferredoxins

Chromatography of the Cm-ferredoxin on a DEAE-cellulose column using an NH_4HCO_3 gradient gave two major protein fractions. These fractions were pooled and each was rechromatographed. The slower moving component was designated as Cm-ferredoxin I and the faster one as Cm-ferredoxin II. The amino acid compositions of these components are shown in Table 1 which shows clearly that the two ferredoxins are different; particularly in the contents of arginine, alanine, valine, methionine and isoleucine.

Terminal sequences of Cm-ferredoxins I *and* II

N-Terminal sequences of Cm-ferredoxins I and II were determined by manual Edman degradation. At the first step for both Cm-ferredoxins, two PTH (phenylthiohydantoin)-amino acids were detected by TLC; PTH-Tyr as the major components, for both Cm-ferredoxins, and PTH-Ser and PTH-Ala as minor ones for Cm-ferredoxins I and II, respectively. All following steps showed two spots of PTH-amino acid and the minor ones corresponded to the major ones of the previous step. These results suggested that both ferredoxins had minor molecular species with one residue longer than the major ones at their *N*-termini. Similar phenomena have been observed in the ferredoxins from *Equisetum telmateia* and *E. arvense* [5,6]. *N*-Terminal sequences of two ferredoxins were deduced from the 16 steps of Edman degradation as follows:

Cm-ferredoxin I: (Ser)-Tyr-Met-Val-Thr-Leu-Lys-
Thr-Pro-Ser-Gly-Glu-Gln-Lys-Val-Glu-Val-

Cm-ferredoxin II: (Ala)-Tyr-Lys-Val-Thr-Leu-Lys-
Thr-Pro-Ser-Gly-Asp-Gln-Thr-Ile-Glu-Val-.

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Table 1. Amino acid composition of Cm-ferredoxins I and II of *Dunaliella salina*

	Cm-ferredoxin I		Cm-ferredoxin II	
	From acid hydrolysate	From sequence study	From acid hydrolysate	From sequence study
Lys	2.7	3	3.0	3
His	1.1	1	1.1	1
Arg	1.2	1	1.9	2
Cm-Cys	5.1	5	5.1	5
Asp	10.8	11	10.7	10
Thr	7.1	7	7.6	8
Ser	10.1	13	9.3	10
Glu	13.7	13*	12.2	12
Pro	3.2	3	3.1	3
Gly	6.5	6	7.3	7
Ala	7.5	7	9.8	11
Val	7.4	8*	5.4	5
Met	0.9	1*	trace	0
Ile	2.1	2	3.4	4
Leu	6.8	7*	6.9	7
Tyr	4.9	5	4.8	5
Phe	2.0	2	2.0	2

* Positions 3, 32 and 50 showed the heterogeneity. These values are based on an assumption that the major amino acid residues occupying the three positions are Gln, Leu and Val, respectively (see text for detail).

The amino acids in parentheses are the extra residues found in the minor components in each ferredoxin I and II.

Carboxypeptidase A released leucine (0.99) and tyrosine (1.02) from Cm-ferredoxin I and leucine (0.99) and tyrosine (0.98) from Cm-ferredoxin II after 30 min digestion at 40°. The order of leucine and tyrosine could not be detected, but as shown later, carboxyl (*C*)-terminal sequences of both the ferredoxins were found to be -Leu-Tyr.

Sequence studies of Cm-ferredoxin I

Six tryptic peptides, T-1 to T-6, were fractionated by Bio-Gel P-6 column chromatography. Their amino acid compositions are given in Table 2 and the summary of the sequence studies is shown in Fig. 1. Each peptide was

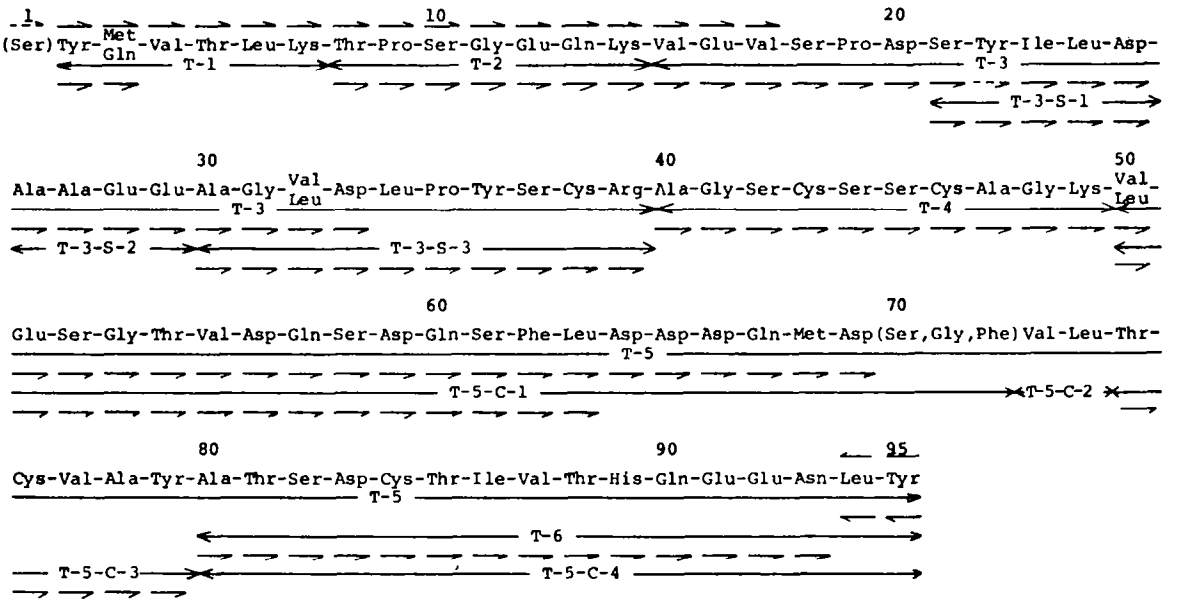

Fig. 1. Summary of the sequence study of *Dunaliella* Cm-ferredoxin I. T, C and S refer to tryptic, chymotryptic and staphylococcal protease peptides, respectively, and Cys refers to Cm-cysteine. Arrows, (→) and (←), above the sequence represent Edman degradation and carboxypeptidase A digestion on Cm-ferredoxin I, respectively. Arrows, (→) and (←), below the sequence represent Edman degradation and carboxypeptidase Y digestion on peptides.

Table 2. Amino acid compositions of tryptic peptides of Cm-ferredoxin I*

	T-1	T-2	T-3	T-4	T-5	T-6
Lys	1.1 (1)	1.1 (1)		1.0 (1)		
His					0.9 (1)	0.8 (1)
Arg			1.0 (1)			
Cm-cys			0.2 (1)†	2.1 (2)	2.0 (2)	0.9 (1)
Asp			3.0 (3)		7.8 (8)	1.8 (2)
Thr	1.0 (1)	0.9 (1)			4.7 (5)	2.7 (3)
Ser		1.0 (1)	2.2 (2)†	2.4 (3)†	4.6 (5)	1.5 (1)
Glu	0.4 (1)†	2.0 (2)	3.2 (3)		7.4 (7)	3.1 (3)
Pro		1.0 (1)	2.1 (2)			
Gly		1.0 (1)	1.2 (1)	2.1 (2)	2.2 (2)	0.2
Ala			3.3 (3)	2.3 (2)	2.3 (2)	1.0 (1)
Val	1.1 (1)		2.7 (3)‡		4.4 (5)‡	0.7 (1)
Met	0.7 (1)‡				0.5 (1)	
Ile			1.1 (1)		0.9 (1)	0.9 (1)
Leu	1.0 (1)		2.4 (3)‡		3.4 (4)‡	1.0 (1)
Tyr	1.0 (1)		1.4 (2)†		1.9 (2)	0.9 (1)
Phe					1.9 (2)	
Total	6‡	7	25‡	10	46‡	16
Yield (%)	45	64	31	84	36	20
Colour reaction§	P		P		P	P

* Impurities, the values below 0.20 are excluded from the table.
† From the sequence studies.
‡ From the sequence heterogeneity (see text).
§ P = Pauly reaction [22] positive.

subjected to Edman degradation (peptide T-1: 2 steps, peptide T-2: 7 steps, peptide T-3: 19 steps, peptide T-4: 10 steps, peptide T-5: 20 steps, peptide T-6: 14 steps). Sequences of peptides T-2 and T-4 were completely determined. In some cases, two definite spots of PTH-amino acid were detected; PTH-Met and PTH-Gln at the second step of peptide T-1, PTH-Val and PTH-Leu at the eighteenth step of peptide T-3 and PTH-Val and PTH-Leu at the first step of Peptide T-5. These heterogenieties were confirmed by further sequence studies. Of the amino acid composition of peptide T-1, the values of glutamic acid and methiomine were nonintegral and the sum of these values was 1.01. Sequence heterogeneity of peptide T-1 accounts for the low value of glutamic acid and methionine.

Peptide T-3 was further digested with staphylococcal protease and the digest was separated by paper electrophoresis at pH 3.6. Three peptides T-3-S-1 to T-3-S-3 were purified and their amino acid compositions are given in Table 3. The eighth step of Edman degradation of peptide T-3 gave an ambiguous result, but the residue at this position was found to be tyrosine from the sequence study of peptide T-3-S-1. Edman degradation of peptide T-3-S-3 gave the *C*-terminal sequence of peptide T-3. It is important to note that PTH-Leu and PTH-Val were detected at the third step of Edman degradation of peptide T-3-S-3 and that this step corresponded to the eighteenth step of the original peptide T-3 as described above.

Peptide T-5 was further digested with chymotrypsin. Four peptides T-5-C-1 to T-5-C-4 were purified by gel filtration on a Bio-Gel P-4 column followed by paper electrophoresis at pH 3.6. The amino acid compositions of these peptides are shown in Table 3. Fourteen steps of Edman degradation of peptide T-5-C-1 were performed and this sequence was in accordance with the *N*-terminal sequence of the original peptide T-5. At the first step of Edman degradation of peptide T-5-C-1, PTH-Val and PTH-Leu were detected. The amino acid sequence of peptide T-5-C-2 was considered to be Val-Leu from chymotrypsin specificity. Peptide T-5-C-3 was completely sequenced by Edman degradation. The amino acid composition of peptide T-5-C-4 was identical to that of peptide T-6, whose *N*-terminal sequence was determined by 14 steps of Edman degradation. Therefore, no sequence study of peptide T-5-C-4 was performed. Carboxypeptidase Y digestion of peptide T-5 released leucine (0.22) and tyrosine (0.86) after 5 min, and leucine (0.86) and tyrosine (0.91) after 3 hr (but no asparagine), which showed that the *C*-terminal sequence of peptide T-5 was Leu-Tyr in accordance with that of Cm-ferredoxin I. The alignment of peptides T-5-C-2 and T-5-C-3 was from the basis of homology to Cm-ferredoxin II and other chloroplast-type ferredoxins. The sequence from residue 69 to 71 could not be completed.

The *N*-terminal sequence of Cm-ferredoxin I gave the overlaps from peptides T-1 to T-3. Peptide T-5 was the *C*-terminal peptide of Cm-ferredoxin I. Peptide T-6 was also the *C*-terminal peptide which was probably derived from non-specific cleavage by trypsin. Peptide T-4 must be placed between peptides T-3 and T-5. From these results the partial sequence of *Dunaliella* Cm-ferredoxin I was deduced with sequence heterogeneities occurring in positions 3, 32 and 50.

Sequence studies of Cm-ferredoxin II

Seven peptides T-1 to T-7 were separated on a Bio-Gel P-6 column. Their amino acid compositions are given in

Table 3. Amino acid compositions of staphylococcal protease peptides of peptide T-3 and chymotryptic peptides of peptide T-5*

	T-3-S-1	T-3-S-2	T-3-S-3	T-5-C-1	T-5-C-2	T-5-C-3	T-5-C-4
His							0.9 (1)
Arg			0.9 (1)				
Cm-Cys			0.8 (1)			1.0 (1)	1.0 (1)
Asp	1.1 (1)		1.2 (1)	6.4 (6)			2.2 (2)
Thr				1.1 (1)		1.0 (1)	2.9 (3)
Ser	1.1 (1)		0.9 (1)	3.6 (4)			1.1 (1)
Gly		2.1 (2)		4.3 (4)			3.0 (3)
Pro			1.1 (1)				
Gly			1.1 (1)	1.9 (2)			
Ala		2.0 (2)	1.1 (1)			1.0 (1)	1.1 (1)
Val			0.5 (1)†	1.7 (2)†	1.1 (1)	1.0 (1)	0.8 (1)
Met				0.5 (1)			
Ile	0.9 (1)						0.8 (1)
Leu	1.1 (1)		1.7 (2)†	1.5 (2)†	0.9 (1)		1.0 (1)
Tyr	0.9 (1)		1.2 (1)			0.9 (1)	1.0 (1)
Phe				1.9 (2)			
Total	5	4	10*	23*	2	5	16

* Impurities, the values below 0.20 are excluded from the table.
† From the sequence heterogeneity (see text).

Table 4 and a summary of the sequence studies is shown in Fig. 2. Each peptide was subjected to Edman degradation (peptide T-1: 1 step, peptide T-1′: 1 step, peptide T-2: 2 steps, peptide T-3: 24 steps, peptide T-4: 15 steps, peptide T-5: 10 steps, peptide T-6: 20 steps, peptide T-7: 23 steps). The *N*-terminal residues of peptides T-1 and T-1′ were alanine and tyrosine, respectively, and these two peptides were considered to be from the *N*-terminal region of Cm-ferredoxin II whose *N*-terminal heterogeneity was as mentioned above. Peptide T-3 was digested with staphylococcal protease and the digest was separated by paper electrophoresis at pH 3.6. Peptide T-3-S-1 had an amino acid composition as follows, Arg; 0.87 (1), Cm-Cys: 0.56 (1), Asp: 1.15 (1), Ser: 0.88 (1), Pro: 1.04 (1), Gly: 0.94 (1), Ala: 1.12 (1), Leu: 1.92 (2), Tyr: 0.98 (1), suggesting that this peptide was derived from the *C*-terminal region of peptide T-3 and ten steps of Edman degradation completed the sequence. The amino acid

Table 4. Amino acid compositions of tryptic peptides of Cm-ferredoxin II*

	T-1	T-1′	T-2	T-3	T-4	T-5	T-6	T-7
Lys	1.2 (1)	1.2 (1)	1.1 (1)			1.1 (1)		
His								1.0 (1)
Arg				0.8 (1)			0.8 (1)	
Cm-Cys				1.1 (1)		1.7 (2)		1.8 (2)
Asp				4.3 (4)	2.0 (2)		4.6 (5)	1.2 (1)
Thr			0.9 (1)	1.9 (2)	1.9 (2)		1.0 (1)	3.5 (4)
Ser				2.9 (3)	1.9 (2)	1.8 (2)	1.9 (2)	2.5 (3)
Glu				4.3 (4)	2.1 (2)		5.3 (5)	3.1 (3)
Pro				2.5 (3)	1.9 (2)			
Gly				2.1 (2)	1.1 (1)	2.0 (2)	2.0 (2)	1.2 (1)
Ala	0.8 (1)			3.9 (4)	1.2 (1)	2.7 (3)	1.0 (1)	2.0 (2)
Val			1.1 (1)	1.3 (1)	0.9 (1)		1.4 (1)	2.0 (2)
Ile				1.6 (2)	0.9 (1)		0.7 (1)	1.0 (1)
Leu			0.9 (1)	2.8 (3)			1.0 (1)	2.0 (2)
Tyr	1.0 (1)	0.8 (1)		1.8 (2)	0.8 (1)			1.6 (2)
Phe				0.2			1.0 (1)	0.8 (1)
Total	3	2	4	32	15	10	21	25
Yield (%)	24	34	74	66	18	60	40	34
Colour reaction†	P	P		P	P			P

* Impurities, the values below 0.20 are excluded from the table.
† P = Pauly reaction [22] positive.

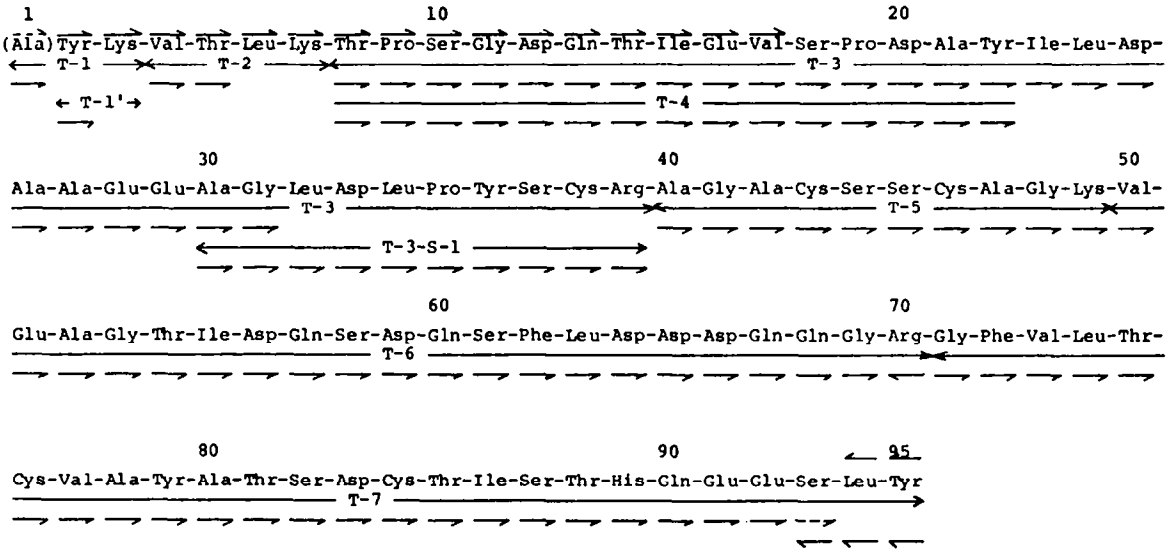

Fig. 2. Summary of the sequence study of *Dunaliella* Cm-ferredoxin II. T and S refer to tryptic and staphylococcal protease peptides and Cys refers to Cm-cysteine. Arrows, (→) and (←), above the sequence represent Edman degradation and carboxypeptidase A digestion on Cm-ferredoxin II, respectively. Arrows, (→) and (←), below the sequence represent Edman degradation and carboxypeptidase B or Y digestion on peptides.

sequence of peptide T-4 was identical to the *N*-terminal sequence of peptide T-3 and peptide T-4 was derived from T-3 by the cleavage of Tyr-Ile bond.

Carboxypeptidase digestions were performed on peptides T-6 and T-7 to obtain their *C*-terminal sequences. Carboxypeptidase B digestion of peptide T-6 released arginine (0.68) after 1-hr digestion and no other residue was released after further digestion with carboxypeptidase A. Carboxypeptidase Y digestion of peptide T-7 released leucine (0.63) and tyrosine (0.93) after 20-min digestion, and serine (0.56), leucine (0.85) and tyrosine (0.92) after 3-hr digestion. Thus, the *C*-terminal sequence of peptide T-7 was determined as -Ser-Leu-Tyr.

The *N*-terminal sequence of Cm-ferredoxin II gave the overlaps from peptides T-1 to T-3. No effort to overlap peptides T-5 and T-6 was made and they were aligned on the basis of homology to all other chloroplast-type ferredoxins. Peptide T-7 was the *C*-terminal peptide. These results gave the sequence of *Dunaliella* Cm-ferredoxin II and no sequence heterogeneity except at the *N*-terminus was observed.

Although there are 16 amino acid differences in the sequences of the two *Dunaliella* ferredoxins studied most of the substitutions are conservative in nature and hence there is very little difference in the total charge between the ferredoxins. This would explain why it was difficult to separate the two forms by ion-exchange chromatography of the native protein. Two ferredoxins have been isolated from *Equisetum telmateia*, *E. arvense*, *Nostoc muscorum*, *Nostoc* MAC, *N. verrucosum*, *Aphanothece sacrum*, *Phytolacca americana* and *Spirulina maxima* (see ref. [7]). In some species the two ferredoxins differ in their catalytic rate in donating electrons to various acceptors.

Both ferredoxin molecules in *D. salina* contain only 95 amino acid residues, the average chain length of ferredoxins being 97 amino acids. Ferredoxin II is unique in having a serine residue at the NH_2-terminus; all other plant and algal ferredoxin chains so far sequenced have alanine at the amino terminus. As can be expected, the positions of the four cysteines which chelate the [Fe–S] active centre are identical in these as in other ferredoxins. When compared to the complete sequences of 23 other ferredoxins known, there are 23 positions where the same amino acid residues occur. Matsubara *et al.* [7] have constructed an evolutionary tree of ferredoxins and have come to the conclusion that the algal ferredoxins are phylogenetically a diverse group and have diverged from higher plants early in evolutionary time. The ferredoxin from *Halobacterium halobium* resembles plant ferredoxins in spectral properties and in the nature of the active site and its co-ordination to the protein chain, but has 128 residues in the molecule [8]. A comparison of the sequence of *H. halobium* ferredoxin to the ferredoxins from the halophyte *D. salina* does not reveal any special homology between the two groups of ferredoxins.

EXPERIMENTAL

Dunaliella salina was grown in a medium containing 1.5 M NaCl [9]. For isolating ferredoxin, 300 g of cell paste containing about 50% dry alga was suspended in 600 ml 20 m M Tris–HCl, pH 8.0 (buffer) and the homogenate sonicated for 3 min. After centrifuging the sonicate for 1 hr at 15000*g* the pellet was discarded and 25 g DEAE-cellulose (Whatman DE23) was sitrred into the supernatant. The DE23, containing bound ferredoxin, was poured into a column and washed with 0.2 M NaCl in buffer. Ferredoxin was eluted from the column as a greenish brown band by passing 0.8 M NaCl in buffer. $(NH_4)_2SO_4$ (0.3 g per ml) was added to the eluate to 50% saturation to precipitate the green material. The mixture was centrifuged at 25000*g* for 1 hr and the supernatant containing ferredoxin dialysed in the buffer to remove $(NH_4)_2SO_4$. Further purification was achieved by chromatography of the ferredoxin on DE-52 using an NaCl gradient (0.2–0.8 M), gel filtration on Sephadex G-75 and finally chromatography on calcium phosphate gel [10].

Determination of ferredoxin activity. Photoreduction of NADP was assayed at 20° by measuring the rate of O_2 evolution from

spinach chloroplasts illuminated with saturating white light [11]. H_2 evolution was measured with *Clostridium pasteurianum* hydrogenase and 10 mM sodium dithionite as electron donor [12].

Separation of carboxymethyl (Cm)-ferredoxins. As the preparation of native ferredoxin was found to contain at least two forms of ferredoxin judged by the amino (*N*)-terminal analysis and as the resolution of these two was difficult in the native protein, we tried to separate them after chemical modification. The native ferredoxin was treated with trichloroacetic acid and converted to a Cm derivative according to the method of ref. [13]. The Cm-ferredoxin was applied to a DE-52 column (2.1 × 43 cm) equilibrated with 0.2 M NH_4HCO_3, pH 8.0 and chromatographed with a linear gradient system from 0.2 to 0.8 M NH_4HCO_3, pH 8.0 (500 ml in each reservoir). The flow rate was 20 ml/hr. Each fraction (5.5 ml) was monitored in terms of the absorbance at 280 nm. Rechromatography was performed on a DE-52 column (1.5 × 37 cm) with the same buffer system as described above (200 ml in each reservoir).

Enzymic digestion and peptide separation. Digestions of Cm-ferredoxins (0.64 μmol of Cm-ferredoxin I and 0.5 μmol of Cm-ferredoxin II) with trypsin (0.2 mg) were carried out in 1.0 ml of Tris–HCl, pH 8.0, at 40° for 3 hr. Each digest was fractionated by gel filtration on a Bio-Gel P-6 column (2 × 197 cm) as described in ref. [14]. Peptide fractions were subjected to analyses or further purification by paper electrophoresis at pH 3.6 or 6.5 and PC [15]. Further digestions were performed on some large peptides. Peptides resulting from chymotryptic digestion of peptide T-5 from CM-ferredoxin I was fractionated on a Bio-Gel P-4 column and purified by paper electrophoresis at pH 3.6. Staphylococcal protease digests of each peptide T-3 from Cm-ferredoxins I and II were separated by paper electrophoresis at pH 3.6.

Amino acid analysis and sequence determination. Amino acid compositions of Cm-ferredoxins and peptides were determined as usual after hydrolysis of proteins and peptides with 6 N HCl in an evacuated, sealed tube at 110° for 24 hr [16]. A manual Edman degradation procedure [17] was applied to the Cm-ferredoxins and peptides to determine their *N*-terminal sequences. The amount of sample for degradation was usually *ca* 0.1 μmol. Phenylthiohydantoin derivatives (PTH) were identified by thin layer chromatography on Si gel plates (Merck) using various solvent systems [18]. PTH-histidine and PTH-arginine were identified by paper electrophoresis at pH 6.5 [19]. The carboxyl (*C*)-terminal sequences of the proteins and peptides were determined by digesting them with carboxypeptidases A, B and Y [20, 21].

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