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The Amino Acid Sequence of Ferredoxin from the Red Alga *Porphyra umbilicalis*

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The amino acid sequence of the ferrodoxin of *Porphyra umbilicalis* was determined by the dansyl–phenyl isothiocyanate method, on peptides obtained by tryptic, chymotryptic and thermolytic digestion of the protein or its CNBr-cleavage fragments. The molecule consists of 98 residues, has an unblocked *N*-terminus and shows considerable similarity with other plant-type ferredoxins. It is the first reported sequence of a red-algal ferredoxin.

The ferredoxins are a group of iron–sulphur proteins present in photosynthetic organisms and in non-photosynthetic anaerobic bacteria. They participate in a number of electron-transfer reactions, including photosynthetic electron transport and nitrogen fixation. Several ferredoxins have been isolated and characterized from various higher plants, bacteria and algae. These proteins exhibit characteristic u.v.- and visible-absorption spectra, have negative redox potentials and show a characteristic e.p.r. signal in the reduced state. Those from higher plants, eukaryotic algae and blue–green bacteria (blue–green algae) possess the 2Fe–2S active centre, have a mol. wt. of approx. 11000 and accept a single electron on reduction. In general, bacterial ferredoxins contain two 4Fe–4S active centres, accept two electrons on reduction and have mol.wts. of between 6000 and 15000 depending on the source.

The amino acid sequences of ferredoxin from several higher plants (e.g. Matsubara & Sasaki, 1968; Keresztes-Nagy *et al.*, 1969), from the green alga *Scenedesmus* (Sugeno & Matsubara, 1969) and from the blue–green bacteria *Spirulina maxima* (Tanaka *et al.*, 1975), *Aphanothece sacrum* (Wada *et al.*, 1975*a*), *Spirulina platensis* (Wada *et al.*, 1975*b*) and *Nostoc muscorum* (Hase *et al.*, 1976) are known.

The present paper reports the first amino acid sequence of a ferredoxin obtained from a red alga. The protein has been characterized (Andrew *et al.*, 1976) and shown to be of the plant type, with a mol.wt. of approx. 11000 and a 2Fe–2S active centre.

Experimental

Materials

Porphyra umbilicalis was harvested from the seashore at Wallog, Wales (Ordnance Survey grid reference SN 589858) and if not used immediately was stored at −20°C. Trypsin (EC 3.4.21.4) (1-chloro-4-phenyl-3-L-tosylamidobutan-2-one-treated) and α-chymotrypsin (EC 3.4.21.1) were from Worthington Biochemical Corp., Freehold, NJ, U.S.A. Thermolysin (EC 3.4.24.4) (crystalline) was from Daiwa Kasei K.K., Osaka, Japan. Carboxypeptidase A (di-isopropyl phosphorofluoridate-treated) (EC 3.4.12.2) and carboxypeptidase C were from Sigma (London) Chemical Co., London S.W.6, U.K.

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Methods

Ferredoxin was extracted and purified as described by Andrew *et al.* (1976).

Carboxymethylation of ferredoxin

Ferredoxin was denatured in 6M-guanidine hydrochloride/1M-Tris/HCl, pH 8.5, and carboxymethylated as described by Milne & Wells (1970). Carboxymethylated protein was desalted by passage through a column (1.5cm×30cm) of Sephadex G-10 equilibrated with water. Eluted protein was subsequently freeze-dried.

Amino acid analysis

Amino acid analyses were carried out with a Locarte amino acid analyser. Samples were hydrolysed in 6M-HCl at 105°C in sealed evacuated tubes for 24, 48 and 72h (protein), 24 and 72h (CNBr fragments) or for 24h only (all peptides). Duplication hydrolyses were carried out for each hydrolysis time.

Cysteine content was measured as cysteic acid after performic acid oxidation before hydrolysis (Hirs, 1956).

Cleavage methods

CNBr cleavage was carried out by the method of Steers *et al.* (1965), in which the protein was

dissolved in 85% (v/v) formic acid and an 8-fold excess (w/w) of CNBr was added.

Digestion of carboxymethylated protein by trypsin or chymotrypsin was with 2% (w/w) ratio of enzyme to substrate at 37°C for 1–2h in 0.2M-*N*-ethylmorpholine/acetate buffer, pH8.5. Digestion with thermolysin was carried out in the same way except that the buffer also contained 5mM-$CaCl_2$. Carboxypeptidase A was made soluble by method 1 of Ambler (1972) and digestion was with a 2% (w/w) ratio of enzyme to substrate for periods of up to 1h in 0.2M-*N*-ethylmorpholine acetate buffer, pH8.5. Carboxypeptidase C was used to determine the *C*-terminus of peptide X1T3, digestion being carried out as with carboxypeptidase A. All enzymic digestions were terminated by the addition of an equal volume of acetic acid.

Purification of peptides

Peptides resulting from CNBr cleavage, trypsin digestion or chymotrypsin digestion were subjected to gel chromatography on a column (1.5cm×130cm) of Sephadex G-50 (superfine grade) equilibrated with 85% (v/v) formic acid. Elution profiles were followed by measurement of A_{280} and A_{206} with an LKB Uvicord III instrument, and by *N*-terminal analysis by the dansyl technique (Gray & Hartley, 1963). Tryptic and chymotryptic peptides that remained impure after this purification step and peptides produced by digestion with thermolysin were subjected to high-voltage paper electrophoresis at pH6.5 (pyridine/acetic acid/water, 25:1:225, by vol.) and at pH1.9 (acetic acid/formic acid/water, 4:1:45, by vol.) as described by Thompson *et al.* (1970). Peptides were detected on paper by using cadmium/ninhydrin reagent (Heilman *et al.*, 1957). Electrophoretic mobilities were determined as described by Thompson *et al.* (1970).

Amino acid sequence methods

Peptide sequences were determined by using the method of Gray & Hartley (1963) as described by Thompson *et al.* (1970). Separation of dansyl-amino acids on polyamide sheets (Woods & Wang, 1967) was performed by using the solvent systems described by Ramshaw *et al.* (1970). After digestion with carboxypeptidase A or C, released *C*-terminal amino acids were identified as their dansyl derivatives.

Nomenclature

CNBr fragments and peptides derived from digestion of the whole protein are numbered on the basis of their order within the complete sequence. Peptides derived from digestion of a larger peptide are numbered on the basis of their order within the parent fragment. The following abbreviations are used: X, CNBr fragment; H, thermolytic peptides; C, chymotryptic peptides; T, tryptic peptides.

Results

The amino acid composition of *Porphyra* ferredoxin together with that of the CNBr fragments is shown in Table 1. Composition of the fragments shows good agreement with that of the protein, which, however, shows single-residue differences in the contents of cysteine, tyrosine, isoleucine, glycine, proline and alanine from that reported by Andrew *et al.* (1976). The *C*-terminal sequence of the protein was found to be -Leu-Tyr.

As expected from the presence of a single methionine in the protein, CNBr cleavage produced two fragments, which were purified by gel chromatography. The larger fragment, X1, had the same *N*-terminal residue as the protein and homoserine at the *C*-terminus. Fragment X2 contained no homoserine and a *C*-terminus identical with that of the intact protein. The order of the fragments in the intact protein could therefore be unequivocally established. The amino acid analyses of the two fragments of the protein, together with the overlap of the two CNBr fragments with peptide C7, shows that the whole sequence has been accounted for.

Digestion of the X1 fragment with trypsin produced five peptides (see Fig. 1 and Table 2), of which the largest, T3, was purified by gel chromatography of the mixture and the others by paper electrophoresis. The *N*-terminal 40 residues of the protein have been determined previously by means of an automatic sequencer (Andrew *et al.*, 1976). The order of tryptic peptides in the parent fragment X1 could be partially established from these data, since peptides T1, T2 and T3 were overlapped by that *N*-terminal sequence. The two remaining tryptic peptides, T4 and T5, could be positioned in the X1 fragment, since peptide T5 contained *C*-terminal homoserine. Furthermore, all these tryptic peptides were overlapped by chymotryptic peptides (see below). The sequences of peptides T1, T2, T4 and T5 were established by direct Edman degradation. The sequence of peptide T3 was determined after digestion of the peptide by thermolysin, which produced six peptides, which were separated by paper electrophoresis and their sequences established by Edman degradation. Their order was established from overlap by chymotryptic peptides. Peptide T3H4b showed the presence of serine at position 40 of the full sequence and not alanine as reported earlier from automatic sequencing of the protein (Andrew *et al.*, 1976), although it had been stated in that

Table 1. *Amino acid compositions of ferredoxin of Porphyra umbilicalis and its CNBr fragments*
The results are expressed as residues/molecule and cysteine was measured as cysteic acid. Values for serine and threonine are extrapolated to zero time where possible, assuming first-order rate of destruction (Moore & Stein, 1963). For valine and isoleucine maximal values (72h hydrolysis) were taken. Abbreviations: n.d., not determined; Hse, homoserine; Seq., values from sequence determination.

	Total protein					X1 fragment				X2 fragment			
	24h	48h	72h	Average	Seq.	24h	72h	Average	Seq.	24h	72h	Average	Seq.
Asp	9.71	10.20	10.63	10.18	10	9.31	9.46	9.39	9	1.38	0.70	1.05	1
Thr	8.38	8.32	8.31	8.41	8	4.64	5.13	4.89	5	3.22	2.77	3.47	3
Ser	6.32	6.87	6.90	6.70	7	5.70	5.59	5.76	6	1.24	1.14	1.29	1
Hse	0	0	0	0	0	0.52	0.7	0.61	1	0	0	0	0
Glu	16.51	16.43	15.38	16.11	16	11.95	12.00	11.98	12	4.08	3.89	3.99	4
Pro	1.67	1.94	1.70	1.77	2	0.56	0.92	0.74	1	0.84	1.08	0.96	1
Gly	6.32	6.19	6.24	6.25	6	5.38	5.42	5.40	5	1.38	1.26	1.32	1
Ala	6.93	6.85	6.81	6.86	7	5.64	6.34	5.99	6	1.21	1.27	1.24	1
Cys	6.11	n.d.	n.d.	6.11	6	n.d.	n.d.	n.d.	(4)	n.d.	n.d.	n.d.	(2)
Val	5.27	5.57	5.72	5.72	6	3.74	3.96	3.96	4	1.99	2.00	2.00	2
Met	0.64	0.51	0.63	0.59	1	0	0	0	0	0	0	0	0
Ile	5.65	5.68	5.72	5.72	6	3.51	4.14	4.14	4	1.90	1.91	1.91	2
Leu	7.67	7.82	7.81	7.77	8	4.22	4.05	4.14	4	3.85	3.90	3.88	4
Tyr	5.56	5.65	5.72	5.64	6	3.39	2.44	2.92	3	2.54	2.63	2.59	3
Phe	1.85	2.03	1.95	1.94	2	1.91	1.77	1.84	2	0	0	0	0
His	1.64	1.81	1.80	1.75	2	1.38	0.78	1.08	1	0.82	0.89	0.86	1
Lys	3.61	3.84	3.76	3.74	4	3.38	2.84	3.12	3	0.95	1.17	1.06	1
Arg	1.02	1.02	1.14	1.06	1	1.37	1.33	1.35	1	0	0	0	0
Total residues					98				71				27

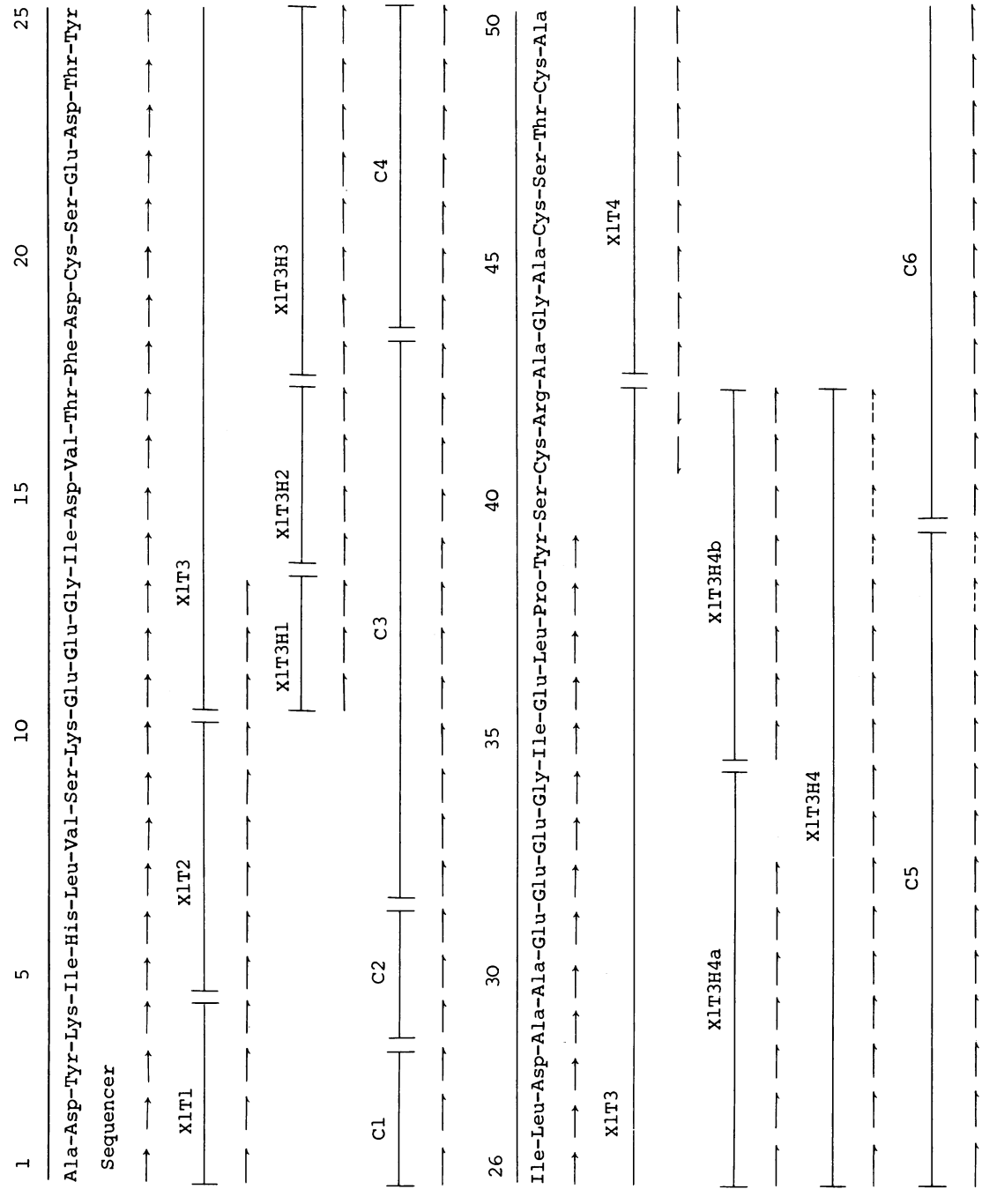
paper that evidence for the last three residues, 38, 39 and 40, was less firm. All the acidic residues of fragment X1 except those of the T5 tryptic peptide were established as such by direct identification of phenylthiohydantoins derivatives. These assignments agreed with the electrophoretic mobility of the constituent peptides (see Table 2). To determine the amide residue in peptide T5, the latter was digested with thermolysin and the electrophoretic mobility of the three peptides so formed determined (see Table 2). The mobility of peptide X1T5H1 showed no amides to be present. The positions of amides (see Fig. 1) within peptide X1T5H2 were established from changes in its electrophoretic mobility after successive cycles of Edman degradation. The amide position in peptide X1T5H3 was determined by carboxypeptidase A digestion of peptide T5, since the mobility of this peptide indicated the presence of only one amide (see Table 2).

The X2 fragment was cleaved into six peptides by thermolysin. The order of peptides X2H1, X2H2 and X2H3 was established from the sequence of the first 17 residues determined by direct Edman degradation of fragment X2. Peptides X2H4, X2H5 and X2H6 were overlapped by peptides C10 and C11. The dipeptide X2H6 had the same sequence as that determined by carboxypeptidase digestion for the two *C*-terminal residues of the protein. The electrophoretic mobilities of the thermolytic peptides (Table 2) suggested that peptide X2H5 was the only peptide to contain amide residues. Its charge was determined as −2 from the mobility at pH 6.5 and the molecular weight plot, so it contained a single amide position, since three acidic and no basic residues were present. The position of the amide (see Fig. 1) was established from the changes in electrophoretic mobility after successive cycles of Edman degradation.

Digestion of the whole protein with chymotrypsin gave rise to 11 peptides (Fig. 1 and Table 2), of which the largest, C6, was obtained pure after gel chromatography of the mixture, the remaining peptides being purified by paper electrophoresis. The order of peptides C1–C7 was determined by comparison with the sequence of fragment X1, and peptides C8–C10 were positioned by comparison with the *N*-terminal 17 residues of fragment X2, determined by direct Edman degradation. Peptide C11 was placed at the *C*-terminal end of the protein, since the sequence of the two *C*-terminal residues of the peptide was identical with those of the protein and of fragment X2.

Discussion

The amino acid sequence of ferredoxin of *Porphyra umbilicalis* contains cysteine residues at positions 18 and 85 in addition to the four invariant cysteine


1
5
10
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20
25
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Sequencer
X1T1
X1T2
X1T3
X1T3H1
X1T3H2
X1T3H3
C1
C2
C3
C4
26
30
35
40
45
50
Ile-Leu-Asp-Ala-Ala-Glu-Glu-Glu-Gly-Ile-Glu-Leu-Pro-Tyr-Ser-Cys-Arg-Ala-Gly-Ala-Cys-Ser-Thr-Cys-Ala
X1T3
X1T4
X1T3H4a
X1T3H4b
X1T3H4
C5
C6

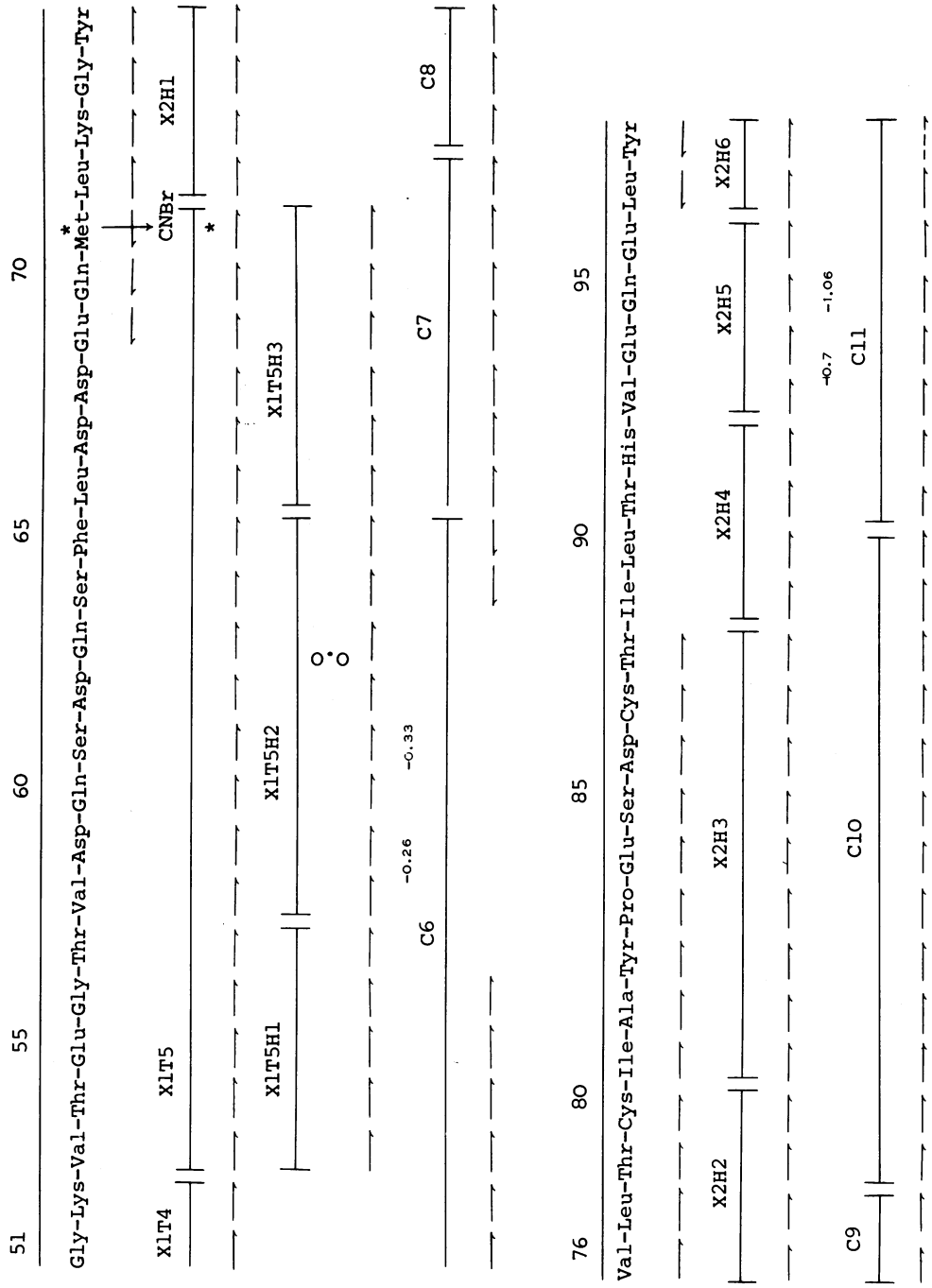

Fig. 1. *Amino acid sequence of ferredoxin of Porphyra umbilicalis*

X1 and X2 represent the CNBr fragments; * indicates homoserine was identified as the derivative from CNBr cleavage. T, C and H represent peptides obtained by tryptic, chymotryptic and thermolytic digestion of carboxymethylated ferredoxin respectively. Arrows (→, ⟶ and ←) represent automatic sequencing, manual Edman degradation and carboxypeptidase digestion respectively. Residues that were unambiguously identified are indicated with solid arrows, other with broken arrows. For peptides X1T5H$_2$ and X2H5 the mobilities at pH6.5 of the peptides after some degradation cycles are indicated.

Table 2. *Mobilities and amino acid compositions of peptides from Porphyra ferredoxin*

The mobilities of peptides during electrophoresis at pH6.5 were measured from dansyl-arginine relative to aspartic acid and at pH1.9 from 5-dimethylaminonaphthalene-1-sulphonic acid relative to dansyl-arginine. Amino acids present in less than 0.3 residue were disregarded. This indicates that some peptides were not completely pure. Abbreviations: Hse, homoserine; CmCys, carboxymethylated cysteine; Dns, 5-dimethylaminonaphthalene-1-sulphonyl; ——, value not determined.

	Mobility																			
Peptides	pH6.5	pH1.9	Asp	Thr	Ser	Glu	Pro	Gly	Ala	CmCys	Val	Met	Ile	Leu	Tyr	Phe	His	Lys	Arg	Hse
X1T1	0	0.9	0.61						0.60						0.75			0.70		
X1T2	+0.61	——			0.72						0.65		0.85	0.87			0.55	0.67		
X1T3	−0.63	——	3.83	2.40	1.52	7.00	0.36	2.27	2.30	——	1.36		2.37	2.25	1.54	0.78			0.80	
X1T3H1	——	0.50																		
X1T3H2	——	0.39																		
X1T3H3	——	0.25																		
X1T3H4	——	0.29																		
X1T3H4a	——	0.34																		
X1T3H4b	——	0.50																		
X1T4	−0.42	——		0.95	0.61	0.62		1.90	2.30	——								0.98		
X1T5	−1.12	——	4.10	1.70	1.63	5.40		1.20			2.10			0.86		0.83				0.52
X1T5H1	−0.37	0.4																		
X1T5H2	−0.44																			
X1T5H3	−0.86	0.34																		
X2H1	+0.44	1.00						0.71						0.88	0.72			1.17		
X2H2	−0.49	0.45		0.64						0.53	0.83			0.83						
X2H3	−0.79	0.35	0.87	0.97	0.78	0.99	0.70		0.79	0.65			0.62		0.78					
X2H4	+0.44	0.80		0.97									0.83	0.88			1.03			
X2H5	−0.82	0.45				2.94					0.76									
X2H6	0	0.50												0.89	0.85					
C1	−0.42	0.45	0.85						0.72						0.69					
C2	+0.95	——											0.76				0.56	0.76		
C3	−0.37	0.45	0.51	1.34	1.20	2.00		1.5			2.10		0.91	0.52		0.72		0.72		
C4	−1.06	——	2.22	1.04	1.04	1.32				——					0.60					
C5	−1.23	——	1.47			4.21	0.68	1.09	1.56				1.86	1.78	0.64					
C6	——	——	1.75	2.65	3.83	3.03		2.93	2.41	——	1.65					0.63		0.70	0.93	
C7	−0·82	0.34	1.58			1.87						0.51		1.64						
C8	+0.52							1.21							0.70			0.50		
C9	0	0.68									1.23			1.30						
C10	−0·72	——																		
C11	−0.28	0.59		0.79		2.46					1.46			0.48	0.40		0.44			

Table 3. *Matrix of amino acid-sequence differences for representative plant-type ferredoxins*

	Porphyra umbilicalis	*Scenedesmus*	*Spirulina maxima*	*Aphanothece sacrum*	*Nostoc muscorum*	Spinach	Alfalfa
Porphyra umbilicalis							
Scenedesmus	31						
Spirulina maxima	28	25					
Aphanothece sacrum	41	29	30				
Nostoc muscorum	37	28	21	26			
Spinach	37	30	34	33	34		
Alfalfa	37	29	38	34	35	19	

residues involved at the active site. It also has the unique presence of a histidine residue at position 6, compared with other ferredoxin sequences that have been determined. The molecular weight of *Porphyra* ferredoxin deduced from the sequence is 11443, including the active centre. This value shows good agreement with that reported earlier from sedimentation-equilibrium studies (Andrew *et al.*, 1976).

Table 3 gives the number of differences between various plant-type ferredoxins. *Porphyra* ferredoxin shows the greatest similarity to that from *Spirulina* (blue–green bacterium) and *Scenedesmus* (green alga) and the most difference from ferredoxin from *Aphanothece* (blue–green bacterium). However, the blue–green bacteria appear to be a very diverse group; for example, Ambler & Bartsch (1975) showed that cytochromes *f* of blue–green bacteria are as similar to all algal cytochromes *f* as they are to each other, although that from *Euglena* stood apart from the rest. Cross-reactivity of blue–green-bacterial ferredoxins to antibody raised to Swiss-chard ferredoxin suggests a greater degree of divergence among this group than among higher plants (Tel-Or *et al.*, 1977). The diversity among blue–green bacteria is also suggested by the high degree of variation found in base content of DNA from different taxa (Stanier *et al.*, 1971). Comparison of the *Porphyra* sequence with preliminary sequence data for ferredoxins from other red algae such as *Porphyridium* and *Rhodymenia* (B. G. Haslett, unpublished work) suggests that a similar diversity exists among the Rhodophyta. Thus it is not possible to draw any firm conclusions as to the phylogenetic relationships of the algae on the basis of the existing limited ferredoxin sequence data.

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