Equisetum (Horsetail) Ferredoxin:
Characterization of the Active Centre and Position of
the Four Cysteine Residues in this 2Fe-2S Protein

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Analysis of the ferredoxin of the primitive vascular plant *Equisetum* indicates that the cysteine residue normally found at position 18 of plant-type ferredoxins is replaced by a valine, although the spectroscopic properties of the ferredoxins are unaffected. It is concluded that the iron-sulphur cluster in plant-type ferredoxins is attached to cysteine residues 39, 44, 47 and 77.

Plant- or chloroplast-type ferredoxins contain two atoms of non-haem iron and of labile sulphur per molecular weight of about 10500. There are a total of four to six cysteine residues in the ferredoxins so far studied, but probably only four residues are involved in the binding of the iron to the protein (see Rao *et al.*, 1971; Matsubara, 1972; Orme-Johnson, 1973; Hall *et al.*, 1973; Yasunobu & Tanaka, 1973). It would be of interest in the study of the structure-function relationship of ferredoxins to locate those four cysteine residues which are essential for the formation of the active centre. The primary structures of ferredoxins from four different higher plants and one green alga are known but all of them contain five cysteines, at positions 18, 39, 44, 47 and 77. Attempts to determine the structure of chloroplast-type ferredoxins by X-ray analysis have not been successful so far.

Equisetum belongs to the Sphenopsida group of plants which thrived in the Carboniferous age. Today only the genus *Equisetum* remains of the Sphenopsida and 'it may not have undergone any significant change in the course of time. If this is so, *Equisetum* may be one of the oldest living genera of vascular plants in the world to-day' (Foster & Gifford, 1959). From an evolutionary point of view *Equisetum* provides important evidence on the origin of the Pteridophytes (ferns and higher plants).

Our earlier work on horsetail ferredoxin (Aggarwal *et al.*, 1971) indicated that the protein contained only four cysteines, and in the peptides obtained by tryptic and chymotryptic digestion no cysteine was present in the *N*-terminal segment; however, none of the peptides were sequenced in this previous study. We now report (a) the sequence of the protein from the *N*-terminus up to residue 18 (homologous with residue 20 of plant-type ferredoxins) and confirm the absence of cysteine from this segment; and (b) the

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sequence of a peptide containing three cysteine residues; the *C*-terminal peptide was shown to contain the fourth cysteine residue; (c) the spectroscopic properties of the protein show that the absence of cysteine-18 does not affect the active centre.

Experimental

Horsetail (*Equisetum telmateia*) green 'leaves' (in reality branches) were collected in the Fish Ranch Road area of Berkeley, Calif., U.S.A., and ferredoxin was purified as previously described (Aggarwal *et al.*, 1971). Two English varieties (*E. telmateia* and *Equisetum arvense*) proved unsuitable for the extraction of ferredoxin. The ferredoxin preparation showed the same visible-absorption spectrum as that reported previously.

For sequence determinations, about 5 mg of ferredoxin was either oxidized by performic acid (Hirs, 1967) or carboxymethylated by iodoacetic acid (Gurd, 1967) to modify the cysteine residues. The analyses of the *N*-terminal sequences of carboxymethyl and oxidized ferredoxin were performed by the Edman procedure (Edman & Begg, 1967) by using a Jeol sequence analyser, model JAS-47K (Jeol Ltd., Tokyo 196, Japan). All reagents were Sequenal grade purchased from Wako Pure Chemical Ind. Ltd., Osaka, Japan, and used without further purification. The programmes described in the Jeol Instruction Manual were partially modified in that coupling was for 30 min and cleavage was for 10 min. After extraction, the thiazolinone derivatives of the amino acids were converted into their phenylthiohydantoin derivatives, which were identified by the following three independent methods. (i) Gas chromatography of phenylthiohydantoin amino acid derivatives was carried out with a Jeol JGC-20K gas-chromatographic apparatus equipped with a hydrogen-flame-ionization detector. (ii) Thin-layer chromatography

of phenylthiohydantoin amino acid derivatives was performed as described by Jeppsson & Sjöquist (1967). (iii) Regeneration of amino acids was carried out by hydrolysing thiazolinone derivatives by the method of Smithies *et al.* (1971) with some modifications. After being dried in a small test tube the thiazolinone derivatives were hydrolysed for 20h at 127°C with 0.25 ml of HI (57%, w/v) containing 1% (v/v) thioglycolic acid under vacuum. The hydrolysate was dried in a desiccator and analysed with a Jeol JLC-6AH amino acid analyser.

The C-terminal sequence was determined by the carboxypeptidase A procedure (Matsubara *et al.*, 1968).

Circular-dichroism (c.d.) spectra were recorded on a Spectropol I spectropolarimeter (Sofica, St. Denis, France). Electron-paramagnetic-resonance (e.p.r.) spectra were recorded on a Varian E4 spectrometer (Varian Associates, Palo Alto, Calif., U.S.A.) with a flow of cold He gas as coolant.

Results and discussion

Amino acid sequence. The sequence analyser revealed 18 residues from the N-terminus. The first step gave the phenylthiohydantoin of tyrosine together with a small amount of the phenylthiohydantoin of alanine. The third and fourth steps gave the phenylthiohydantoin of threonine and valine respectively. These results were supported by the sequence studies of two tryptic peptides, Tyr-Lys and Thr-Val-Leu-Lys, which were unique and were assumed to be from

the N-terminal region. Digestion of the ferredoxin with carboxypeptidase A (EC 3.4.12.2) showed the C-terminal sequence Leu-Phe-OH, which is the same as the C-terminal of ferredoxin from the green alga *Scenedesmus* (Sugeno & Matsubara, 1969).

Table 1 shows a comparison of the sequences of two peptides of *Equisetum* ferredoxin with the sequence of spinach (*Spinacea oleracea*) ferredoxin. The N-terminal region of *Equisetum* ferredoxin is clearly homologous with those of other chloroplast ferredoxins except that two residues from the terminus are deleted and the sequence Thr-Val is commonly found as Val-Thr in other ferredoxins. The most interesting feature in this segment is the absence of the cysteine residue from position 16 which corresponds to cysteine-18 in other ferredoxins (see Hall *et al.*, 1973). It is replaced by valine in *Equisetum* ferredoxin.

The other peptide, which contains three cysteine residues, is homologous with residues 38–50 of spinach ferredoxin and shows that the cysteines are in equivalent positions in the two ferredoxins.

Analysis of the C-terminal peptide obtained after digestion with trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) showed that the fourth cysteine residue is in this peptide, as would be expected if the protein were homologous with spinach ferredoxin (Aggarwal *et al.*, 1971). However, shortage of material prevented the determination of the sequence of this region.

Physical properties. The plant ferredoxins have characteristic c.d. spectra in the region 300–600 nm, due to the two-iron-two-labile-sulphide chromophore (Garbett *et al.*, 1967). Fig. 1 shows a comparison

Spinach	1	Ala-Ala-Tyr-Lys-Val-Thr-Leu-Val-Thr-Pro-	10
<i>Equisetum</i>	-	- Tyr-Lys-Thr-Val-Leu-Lys-Thr-Pro-	
Spinach	11	Thr-Gly-Asn-Val-Glu-Phe-Gln-Cys-Pro-Asp-	20
<i>Equisetum</i>		Ser-Gly-Glu-Phe-Thr-Leu-Asp-Val-Pro-Glu	
Spinach	21	Asp-Val-Tyr-Ile-Leu-Asp-Ala-Ala-Glu-Glu-	30
Spinach	31	Glu-Gly-Ile-Asp-Leu-Pro-Tyr-Ser-Cys-Arg-	40
<i>Equisetum</i>		Ser-Cys-Arg-	
Spinach	41	Ala-Gly-Ser-Cys-Ser-Ser-Cys-Ala-Gly-Lys-	50
<i>Equisetum</i>		Ala-Gly-Ala-Cys-Ser-Ser-Cys-Leu-Gly-Lys-	

Table 1. Comparison of amino acid sequences (residues 1–50) of ferredoxins of spinach and *Equisetum*

Fig. 1. Circular-dichroism spectra of ferredoxins

—, *Equisetum*; ----, spinach. Spectra (a) are for the oxidized ferredoxins, and spectra (b) for ferredoxins reduced with sodium dithionite.

of the c.d. spectra of oxidized and reduced *Equisetum* ferredoxin with those of spinach ferredoxins. We have measured the c.d. spectra of four higher-plant and five algal ferredoxins, and found that, although the spectra are all substantially similar, there are subtle differences in shape which presumably reflect minor changes in the environment or conformation of the active site, as a result of amino acid substitution in the peptide chain. For example, it may be noted from Fig. 1 that in oxidized *Equisetum* ferredoxin there is a more pronounced shoulder at 450 nm than in spinach ferredoxin. This feature was observed in the algal ferredoxins. On the basis of this and other features, the c.d. spectrum of *Equisetum* ferredoxin resembles more closely those of the algae than of the higher plants.

Reduced *Equisetum* ferredoxin gave an e.p.r. spectrum which was similar in lineshape and temperature to those of the 2Fe ferredoxin from algae and higher plants. At 22°K the principal g values were measured as $g_x = 1.896$, $g_y = 1.966$, $g_z = 2.057$ (± 0.005).

The spectroscopic properties of *Equisetum* ferredoxin therefore indicate that it has a normal two-iron chromophore and that a cysteine at position 18 in the amino acid sequence is not an essential component of plant-type ferredoxins.

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