Amino Acid Sequence of [2Fe-2S] Ferredoxin from *Clostridium pasteurianum*†

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ABSTRACT: The complete amino acid sequence of the [2Fe-2S] ferredoxin from the saccharolytic anaerobe *Clostridium pasteurianum* has been determined by automated Edman degradation of the whole protein and of peptides obtained by tryptic and by staphylococcal protease digestion. The polypeptide chain consists of 102 amino acids, including 5 cysteine residues in positions 11, 14, 24, 56, and 60. The sequence has been analyzed for hydrophilicity and for secondary structure predictions. In its native state the protein is a dimer, each subunit containing one [2Fe-2S] cluster, and it has a molecular weight of 23 174, including the four iron and inorganic sulfur atoms. The extinction coefficient of the native protein is 19 400 M^{-1} cm^{-1} at 463 nm. The positions of the cysteine residues, four of which are most probably the ligands of the [2Fe-2S] cluster, on the polypeptide chain of this protein are very different from those found in other [2Fe-2S] proteins, and in other ferredoxins in general. In addition, whole sequence comparisons of the [2Fe-2S] ferredoxin from *C. pasteurianum* with a number of other ferredoxins did not reveal any significant homologies. The likely occurrence of several phylogenetically unrelated ferredoxin families is discussed in the light of these observations.

The widespread occurrence of ferredoxins in living systems, together with their small size, has stimulated their extensive use for the derivation of evolutionary trees based on amino acid sequences. These investigations have focused on two main groups of ferredoxins: those containing [4Fe-4S] or [3Fe-*x*S] clusters have contributed to establishing or confirming phylogenetic relationships among a number of very diverse bacterial species (Dayhoff, 1983), whereas [2Fe-2S] ferredoxin sequences have allowed the construction of an evolutionary tree for oxygen-evolving phototrophs (Tsukihara et al., 1982).

[2Fe-2S] ferredoxins are not only found in algae and plants but also in a variety of other organisms, including the anaerobe *Clostridium pasteurianum* (Hardy et al., 1965; Cardenas et al., 1976; Meyer et al., 1984), several aerobic bacteria [Shethna et al., 1968; Tanaka et al., 1974; Knoell & Knappe, 1974; Robson, 1979; Subramanian et al. (1984) and references cited therein], halobacteria (Hase et al., 1978a, 1980), photosynthetic bacteria (Yamanaka & Kamen, 1967), anaerobic protozoa (Marczak et al., 1983; Gorrell et al., 1984), and vertebrates (Tanaka et al., 1973; Yoon & DeLuca, 1980, Maruya et al., 1983; Tuckey & Stevenson, 1984). [2Fe-2S] ferredoxins are thus unusually ubiquitous, and therefore a comprehensive set of amino acid sequences of these proteins is likely to provide useful evolutionary information. A number of such sequences are known to date, including those of the putidaredoxin from *Pseudomonas putida* (Tanaka et al., 1974), adrenodoxins from beef (Tanaka et al., 1973) and pig (Akhrem et al., 1978) adrenal cortex, and about thirty plant and algal (Tsukihara et al., 1982) and two halobacterial (Hase et al., 1978a, 1980) ferredoxins. However, these data do not nearly cover the complete range of organisms containing [2Fe-2S] ferredoxins.

Amino acid sequences have also essential bearings on the structure of the iron–sulfur chromophore: discrepancies between the primary structure of adrenodoxin (Tanaka et al., 1973) and those of plant ferredoxins (Tsukihara et al., 1982), for instance, are reflected in a number of spectroscopic differences (Sands & Dunham, 1975; Yachandra et al., 1983) between these two types of proteins.

We have determined and report here the complete amino acid sequence of the [2Fe-2S] ferredoxin from *C. pasteurianum*, which is unusual both by the spectroscopic properties of its prosthetic group (Hardy et al., 1965; Cardenas et al., 1976; Meyer et al., 1984) and by its presence in an anaerobic heterotroph which is phylogenetically well outside the range of organisms usually recognized as containing [2Fe-2S] ferredoxins.

Materials and Methods

C. pasteurianum W5 (ATCC 6013) cells were grown on N_2 (Rabinowitz, 1972) and processed in 200–300-g batches as described previously for the purification of nitrogenase (Tsö et al., 1972). During the last purification step of the iron protein of nitrogenase (Cp2), a Sephadex G-100 gel filtration, the [2Fe-2S] Fd was eluted as a pink fraction following Cp2, concentrated on a small (2-mL) DE-52 column, and further purified by ammonium sulfate fractionation between 50% and 80% of saturation. A total of 15–20 mg of [2Fe-2S] Cp Fd[1] was obtained per 200–300 g of cells (wet weight). The protein had an A_{463}:A_{280} absorption ratio of 0.49 and was pure according to its SDS–polyacrylamide electrophoresis pattern (Meyer et al., 1984).

For the preparation of apoferredoxin, Fd solutions (2–4 mg/mL in 0.05 M Tris-HCl, pH 8.0) were made 0.5 M in

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[1] Abbreviations: Fd, ferredoxin; Cp, *Clostridium pasteurianum*; CM, S-carboxymethylated; HPLC, high-performance liquid chromatography; CNBr, cyanogen bromide; TPCK–trypsin, trypsin pretreated with L-1-(tosylamido)-2-phenylethyl chloromethyl ketone; Quadrol, *N,N,N',N'*-tetrakis(2-hydroxypropyl)ethylenediamine; PTH, phenylthiohydantoin; TFA, trifluoroacetic acid; NBRF, National Biomedical Research Foundation (Washington, DC); SDS, sodium dodecyl sulfate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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Table I: Amino Acid Compositions[a] of [2Fe-2S] Cp Fd and of Peptides Isolated after Tryptic or Staphylococcal Protease Digestion of the S-Carboxymethylated Protein

	peptide									
	T-1	T-2	T-5	T-6	T-7	T-8	S-6	S-8	S-10	whole protein
residues covered[b]	1–5	6–15	20–27	28–43	44–98	76–98	49–71	72–88	96–102	1–102
Asp (D) + Asn (N)	1.0 (1)			2.2 (2)	6.6 (7)	3.6 (4)	2.0 (2)	2.5 (3)		11.0 (11)
Thr (T)		0.8 (1)		0.8 (1)	1.7 (2)	0.9 (1)	0.8 (1)	0.9 (1)		3.6 (4)
Ser (S)		0.8 (1)	0.8 (1)	1.6 (2)	3.6 (4)	0.6 (1)	0.9 (1)			6.4 (8)
Glu (E) + Gln (Q)			2.1 (2)	3.8 (4)	8.5 (8)	5.1 (5)	2.2 (2)	3.0 (3)	1.1 (1)	15.6 (15)
Pro (P)	1.0 (1)				2.0 (2)		1.8 (2)			2.8 (3)
Gly (G)			1.0 (1)		5.5 (6)	2.2 (2)	2.8 (3)	2.0 (2)		7.9 (8)
Ala (A)					1.3 (1)	1.0 (1)		1.0 (1)		1.0 (1)
Cys (C)[c]		1.6 (2)	0.7 (1)		1.8 (2)		1.8 (2)			5.1 (5)
Val (V)	0.9 (1)	0.9 (1)		1.8 (2)	10.5 (11)	4.6 (5)	4.5 (5)	3.3 (4)	1.4 (2)	14.7 (15)
Met (M)[d]	0.9 (1)			0.6 (1)	1.0 (1)		1.0 (1)			2.9 (3)
Ile (I)		0.9 (1)		0.9 (1)	3.6 (4)	1.8 (2)	1.8 (2)	0.9 (1)	0.9 (1)	6.9 (7)
Leu (L)				0.9 (1)	1.2 (1)				1.0 (1)	3.9 (4)
Tyr (Y)			0.8 (1)		1.5 (2)		0.9 (1)	0.8 (1)		2.9 (3)
Phe (F)		1.0 (1)	0.9 (1)	0.8 (1)	1.0 (1)		1.1 (1)			3.9 (4)
His (H)		1.7 (2)			0.8 (1)	1.1 (1)				3.0 (3)
Lys (K)	1.0 (1)		0.9 (1)		1.0 (1)	1.0 (1)			1.0 (1)	4.0 (4)
Arg (R)		0.8 (1)		0.7 (1)					1.0 (1)	3.0 (3)
Trp (W)[e]					nd[f] (1)			nd[f] (1)		1 (1)
yield (%)	58	12	22	9	8	11	31	12	45	

[a] By amino acid analysis (18, 24, or 48 h acid hydrolysis) or (in parenthesis) derived from the sequence (Figure 1). [b] See Figure 1 for residue numbering. [c] Analyzed as CM-Cys or as cysteic acid. [d] Analyzed as homoserine or as methionine sulfone. [e] Determined spectrophotometrically (Beaven & Holiday, 1952). [f] Not determined.

HCl, stirred 30 min at 0 °C, and centrifuged (10000*g*, 10 min). The pellet was dissolved in 0.1 M Tris-HCl, pH 8.0, and reprecipitated as described above. S-Carboxymethylated Fd (CM-Fd) was prepared by dissolving the apo-Fd pellet in 0.5 M Tris-HCl, pH 8.9, and 6 M guanidine hydrochloride (Pierce, Sequanal grade) and treating it with iodoacetic acid (Crestfield et al., 1963). CM-Fd was dialyzed for 48 h against 4 or 5 changes of distilled water and lyophilized.

Methionine peptides were produced by reacting CM-Fd in 70% formic acid with a 300-fold molar excess of CNBr for 24 h at room temperature in the dark.

For enzymatic digestions, CM-Fd was dissolved in 0.1 M NH_4HCO_3 and allowed to react at 37 °C for 3 h with a 1/50 enzyme to substrate ratio of TPCK–trypsin (Worthington) at pH 8.5, of α-chymotrypsin (Sigma) at pH 8.5, or of *Staphylococcus aureus* V8 protease (Miles) at pH 8.0.

Peptides were purified by reverse-phase HPLC on a Waters Associates system (Model 6000 pumps, M720 controller, M730 data module, and M481 spectrophotometer). The 0.46 × 25 cm μBondapak C_{18} (Waters) column was equilibrated with 0.1% TFA, and the peptides were eluted by increasing concentrations of acetonitrile containing 0.1% TFA. Absorbance was recorded at 220 nm. Peptides were obtained in amounts sufficient for sequence analysis by performing several (five to ten) chromatographic runs, each involving 50–100 nmol of protein digest.

Protein and peptide samples were hydrolyzed in 0.2 mL of 6 M HCl at 110 °C for 18–48 h in sealed evacuated tubes, and the amino acid compositions were determined with a LKB Model 4150 amino acid analyzer according to the method of Spackman et al. (1958). The values for serine, threonine, and tyrosine were corrected for decomposition during hydrolysis. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively (Hirs, 1967). The content in S-carboxymethylated cysteine was determined by amino acid analysis.

Liquid-phase Edman degradations were carried out in a modified Socosi P.S.100 or in a Beckman 890C sequencer, using the 0.1 M Quadrol program of Brauer et al. (1975) or of Tarr et al. (1978) in the presence of Polybrene. Amino acid sequence analyses were performed by degradation of 100–300 nmol of protein and 20–100 nmol of peptides. The quantitative determination of the PTH derivatives was done relative to known amounts of the appropriate standards by HPLC (Bonicel et al., 1981) and by amino acid analysis after conversion to the parent amino acids by hydrolysis with hydriodic acid (Smithies et al., 1971). Peptides T-8 and S-10 (see Results) were also sequenced in 1–5-nmol amounts on an Applied Biosystems A470 gas-phase sequencer (Hewick et al., 1981).

The carboxy-terminal amino acids of the protein were determined by using carboxypeptidases A, B (Sigma) (Ambler, 1967), and Y (Pierce) (Hayashi et al., 1973).

Iron was determined on wet ashed samples (Beinert, 1978) to which 0.75 mL of saturated sodium acetate solution, 1.7 mL of distilled water, 0.1 mL of sodium ascorbate (5% in water), and 0.4 mL of bathophenanthroline disulfonate (0.1% in water) were successively added. After 1 h the absorbance at 535 nm was measured and the concentration of iron calculated by using a molar extinction coefficient of 22 140 M^{-1} cm^{-1} for the iron–bathophenanthroline complex (Blair & Diehl, 1961).

The isoelectric point of the protein was determined by using the first dimension of the two-dimensional electrophoresis system of O'Farrell (1975).

Sequence comparisons (Dayhoff et al., 1983) and secondary structure predictions were performed on a PRIME computer connected to the Base Information sur les Séquences Acides Nucléiques pour les Chercheurs Européens (Centre Interuniversitaire de Traitement Informatique, Paris, France), which has access to several sequence data banks and data processing programs.

Results

The [2Fe-2S] Cp Fd is comprised of 102 amino acids, resulting in a molecular weight of 11 415 for the monomeric apoprotein. The amino acid composition derived from the sequence is consistent with that obtained by amino acid analysis, including the presence of five cysteine residues (Table I).

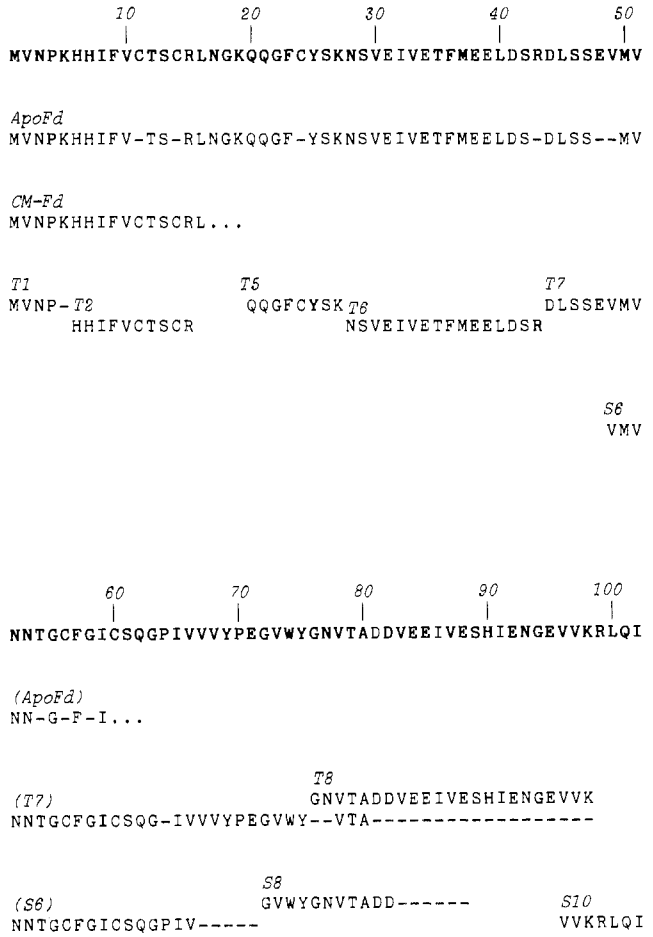

FIGURE 1: Summary proof of the sequence of [2Fe-2S] Cp Fd. The one-letter code (see Table I) designates amino acid residues unambiguously identified by Edman degradation. Those not identified are shown by dashes, except for apo-Fd and CM-Fd, where the continuation of the sequence is indicated by dots. Peptides generated by trypsin and by staphylococcal protease digestion are designated by the prefixes T and S, respectively. The final sequence derived is shown at the top in boldfaced letters.

The sequencing strategy is summarized in Figure 1. Automated Edman degradation of the apoprotein (Table II) yielded the amino-terminal sequence up to the 59th cycle, with only nine unidentified residues, four of which were subsequently shown to be cysteine. The sequencing of the CM-Fd afforded only 16 residues, but allowed the identification of CM-Cys-11 and CM-Cys-14.

The tryptic peptides isolated by HPLC (Figure 2) covered most of the sequence: only the C-terminal tetrapeptide was missing (Figure 1). Their automated Edman degradation (Table II) yielded the complete sequence of the protein, except for Pro-64 and the four C-terminal residues. Several of the more hydrophobic peptides were obtained in low yields (Table I). This is probably due in part to their being less easily released from the C_{18} column than hydrophilic peptides. In addition, partial cleavage at some sites, combined with the residual chymotryptic activity of TPCK–trypsin, led to the occurrence of overlapping peptides. This was the case, for instance, of the peptides T-7, T-8, and T-9 (the latter displayed the same amino acid composition as T-8 and was obtained with a yield of 16%), the cumulated yield of which was 35%. When similar corrections were made for the other peptides obtained in low yield (≤20%), the yields of peptide purification were found to occur in the 30–60% range. This is somewhat lower than the yields reported in the literature, which are most often in the 40–80% range. It should be pointed out, however, that

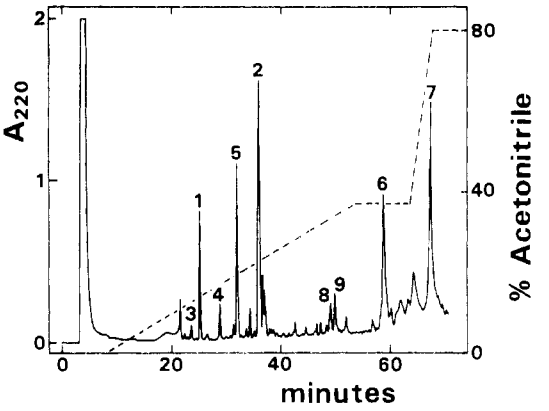

FIGURE 2: Fractionation of a tryptic digest of CM-Fd (55 nmol) on a μBondapak C_{18} column (0.46 × 25 cm) equilibrated with 0.1% TFA and eluted at a flow rate of 1 mL/min by a gradient of acetonitrile. The fractions were collected by hand and lyophilized. The numbers above the peaks refer to the T peptides subsequently identified in those peaks. Peptides T3 (16–19) and T4 (20–23), which are not described in Table I, were identified by amino acid analysis and were both obtained in 27% yield. Peptide T9 had the same amino acid composition as T8 and was obtained in 16% yield.

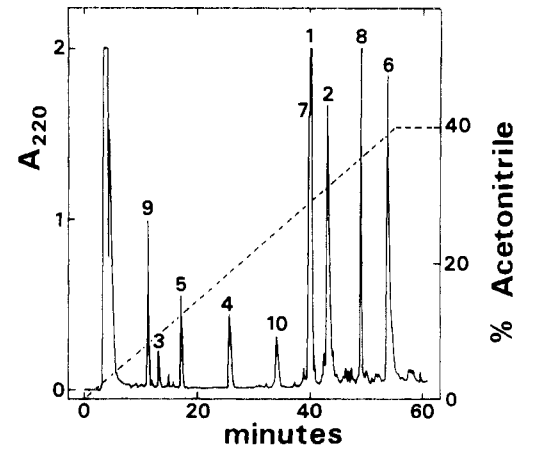

FIGURE 3: Fractionation of a staphylococcal protease digest of CM-Fd (65 nmol) on a μBondapak C_{18} column (0.46 × 25 cm) equilibrated with 0.1% TFA and eluted at a flow rate of 1 mL/min by a gradient of acetonitrile. The fractions were collected by hand and lyophilized. The numbers above the peaks refer to the S peptides subsequently identified in those peaks. The peptides not described in Table I are S1 (1–31), S2 (1–34, yield 18%), S3 (32–34 or 86–88, yield 42%), S4 (35–39, yield 43%), S5 (40–48, yield 43%), S7 (72–85), S9 (89–92, yield 43%). S1 and S7 were separated on the same column by rechromatography with a shallower gradient of acetonitrile.

the yields given here (Table I, Figures 2 and 3) were based on the amounts of native ferredoxin used as starting material, i.e., they represent the combined yields of the following sequence of reactions: apoprotein preparation, carboxymethylation, enzymic digestion, and peptide purification by HPLC.

Digestion with *S. aureus* protease V8 afforded another set of peptides (Figure 3) covering the complete sequence of Cp Fd, except for the 93–95 tripeptide. About 70 residues were identified by automated Edman degradation of these peptides. The data relative to only three of them are given in Table I: S-6 allowed unambiguous identification of Pro-64, S-8 confirmed the overlap of peptides T-7 and T-8, and S-10, which overlaps peptide T-8 by three residues, afforded the C-terminal sequence.

Two additional sets of peptides were obtained by CNBr cleavage at methionine residues and by α-chymotrypsin digestion (results not shown). In each case, the identification of more than 60 residues was performed by automated Edman

Table II: Repetitive Edman Degradation of *C. pasteurianum* [2Fe-2S] Apoferredoxin and of Some Significant Peptides

apoferredoxin[a]			T-5[b]			T-7[c]			T-8[d]			S-10[e]		
cycle	amino acid[f] residue	yield[g] (nmol)	cycle	amino acid[f] residue	yield[g] (nmol)	cycle	amino acid[f] residue	yield[g] (nmol)	cycle	amino acid[f] residue	yield[g] (nmol)	cycle	amino acid[f] residue	yield[g] (nmol)
1	Met	127	1	Gln*	50	1	Asp	4.9	1	Gly	1.9	1	Val	2.1
2	Val	175	2	Gln*	32	2	Leu	5.6	2	Asn	1.3	2	Val	1.9
3	Asn	98	3	Gly*	22	3	Ser	6.0	3	Val	2.4	3	Lys	2.5
4	Pro	30	4	Phe*	8	4	Ser	3.0	4	Thr		4	Arg	0.9
5	Lys	67	5	CM-Cys*		5	Glu	7.9	5	Ala	1.8	5	Leu	2.5
6	His*		6	Tyr*	8	6	Val	6.5	6	Asp	0.45	6	Gln	1.9
7	His*		7	Ser*	4	7	Met	3.5	7	Asp	1.0	7	Ile	1.5
8	Ile*	64	8	Lys*		8	Val	4.3	8	Val	1.3			
9	Phe*	80				9	Asn	5.1	9	Glu	0.27			
10	Val*	85				10	Asn	3.7	10	Glu	0.34			
11						11	Thr	1.8	11	Ile	0.80			
12	Thr	20				12	Gly	1.2	12	Val	0.87			
13	Ser	5.5				13	CM-Cys	4.8	13	Glu	0.32			
14						14	Phe	1.8	14	Ser				
15	Arg	34				15	Gly	1.6	15	His				
16	Leu	37				16	Ile	2.7	16	Ile	0.38			
17	Asn	14				17	CM-Cys	2.7	17	Glu	0.12			
18	Gly	15				18	Ser	1.2	18	Asn	0.08			
19	Lys	20				19	Gln	2.8	19	Gly	0.19			
20	Gln*	27				20	Gly	2.4	20	Glu				
21	Gln*	29				21			21	Val	0.17			
22	Gly*	7.5				22	Ile	1.5	22	Val	0.21			
23	Phe*	20				23	Val	1.5	23	Lys	0.04			
24						24	Val	1.4						
25	Tyr*	13.8				25	Val	0.92						
26	Ser	3.3				26	Tyr	1.8						
27	Lys*	4.1				27	Pro	0.39						
28	Asn*	7.8				28	Glu	0.83						
29	Ser	2.2				29	Gly	0.35						
30	Val*	4.5				30	Val	0.51						
31	Glu*	2.9				31	Trp	0.16						
32	Ile*	4.1				32	Tyr	0.93						
33	Val*	4.1				33								
34	Glu*	1.5				34								
35	Thr*	1.7				35	Val	0.64						
36	Phe*	3.0				36	Thr	0.18						
37	Met*	3.9				37	Ala	0.43						
38	Glu*	1.5												
39	Glu	1.5												
40	Leu*	2.9												
41	Asp	1.3												
42	Ser	0.47												
43														
44	Asp	0.91												
45	Leu	1.4												
46	Ser	0.33												
47	Ser	0.33												
48														
49														
50	Met	0.76												
51	Val	0.91												
52	Asn	0.51												
53	Asn	0.56												
54														
55	Gly	0.74												
56														
57	Phe	0.75												
58														
59	Ile	0.34												

[a] Protein amount, 250 nmol; initial yield, 44.5%; repetitive yield, 90.1%; sequencer, Beckman 890C. [b] Peptide amount, 130 nmol; initial yield, 38%; sequencer, Beckman 890C. [c] Peptide amount, 46 nmol; initial yield, 16.5%; repetitive yield, 91.9%; sequencer, Socosi P.S.100. [d] Peptide amount, 5 nmol; initial yield, 46%; repetitive yield, 86.6%; sequencer, Applied Biosystems A470. [e] Peptide amount, 4.4 nmol; initial yield, 48%; repetitive yield, 96%; sequencer, Applied Biosystems A470. [f] Identified by HPLC and (*) confirmed by amino acid analysis after acid hydrolysis of the PTH derivatives. [g] The yields were corrected for out-of-stepness (ca. 1.5%).

degradation. Thus, all of the residues in the sequence of [2Fe-2S] Cp Fd were identified at least 3 times.

Digestion of S-carboxymethylated Fd with a mixture of carboxypeptidases A and B (Table III) provided the six C-terminal residues, with an uncertainty concerning the order of appearance of Gln and Leu. Digestion with carboxypeptidase Y (Table IV) resolved this uncertainty. Thus, the combined results of Tables III and IV afforded the C-terminal sequence-Val-Lys-Arg-Leu-Gln-Ile-COOH and confirmed that the peptide S-10 is from the C-terminus of the sequence.

The sequence of [2Fe-2S] Cp Fd contains enough cysteine residues to accommodate one [2Fe-2S] chromophore per ca.

Table III: Amino Acids Released by Carboxypeptidases A and B from 90 nmol of CM-Fd

	nmol after digestion time				
amino acid	10 min	30 min	60 min	90 min	120 min
Ile	20	44	45	51	69
Gln		43	44	50	68
Leu		40	42	44	47
Arg			3	32	49
Lys			1	24	43
Val				23	29

Table IV: Amino Acids Released by Carboxypeptidase Y from 60 nmol of CM-Fd

	nmol after digestion time			
amino acid	1 min	2 min	4 min	6 min
Ile	5.1	8.1	13.8	17
Gln	4.4	6.8	12.4	16
Leu		2.7	4.8	6.6

12000 molecular weight. Previously determined iron contents of 2 Fe per 25000 molecular weight (Cardenas et al., 1976) have recently been shown to be underestimated (Meyer et al., 1984). We have here carried out iron assays and amino acid analyses on two different protein preparations and obtained an iron content of 2.1 ± 0.3 iron atoms (mean of six measurements) per monomeric polypeptide chain. Correlation of the iron assays with spectrophotometric measurements yielded an extinction coefficient of 9700 ± 400 M^{-1} cm^{-1} per [2Fe-2S] chromophore at 463 nm.

Discussion

The amino acid composition of [2Fe-2S] Cp Fd, as derived from its sequence of 102 residues (Table I), is similar to that reported previously, based on a molecular weight of 25000 (Cardenas et al., 1976), except for alanine, tyrosine, tryptophan, and cysteine. It is an acidic protein having an isoelectric point, as calculated from its amino acid sequence, of 4.8, in agreement with experimental values of 4.6 (Hinton & Mortenson, 1985) and 4.9 (our unpublished results). Isoelectric points of 4.8 have also been calculated for putidaredoxin and adrenodoxin, but significantly lower values (4.0–4.5) have been obtained for plant ferredoxins (our unpublished calculations). Cp Fd contains a large excess of glutamic acid over aspartic acid while the reverse is true for other [2Fe-2S] Fd. It is noteworthy that 14 of the 15 acidic residues of Cp Fd are concentrated in two segments (31–48 and 81–95) of the polypeptide chain. Similar clustering of the acidic residues is observed in plant Fd (Tsukihara et al., 1982), but neither in adrenodoxin (Tanaka et al., 1973) nor in putidaredoxin (Tanaka et al., 1974). Cp Fd also differs from most other [2Fe-2S] Fd by its containing fifteen valine residues and only one alanine. The numbers and positions of the cysteine residues will be discussed below.

According to the presently known three-dimensional structures (Stout, 1982), [2Fe-2S] ferredoxins display relatively more secondary structure than the other small iron–sulfur proteins. In *Spirulina platensis* [2Fe-2S] Fd, for instance, 25% of the polypeptide chain is folded in β-sheet and 10% in α-helix (Tsukihara et al., 1981). Circular dichroism measurements on [2Fe-2S] Cp Fd have led to the prediction that 14% of the residues belong to α-helical domains (Cardenas et al., 1976). We have analyzed the amino acid sequence of this protein using the algorithm of Chou and Fasman (1978) and found segments 31–41 and 81–90, i.e., 20% of the total sequence, to be potentially α-helical. While this figure is unusually high for a ferredoxin, it is only slightly in excess of the prediction based on circular dichroism data (Cardenas et al., 1976). The sequence has also been examined for other secondary structural features (Chou & Fasman, 1978): β-sheet structures may possibly occur in the 5–15 and 65–75 regions, whereas the 15–29, 41–45, and 53–63 segments display relatively high β-turn scores. A hydrophilicity profile (Hopp & Woods, 1981) has also been determined (not shown): the sequence contains a long hydrophobic region (48–78), whereas the C-terminus and the 36–47 segment are hydrophilic. The N-terminal part of the sequence (4–35) displays an alternation of short hydrophilic and hydrophobic segments. Among the cysteine residues, only Cys-14 occurs next to a hydrophilic region, which suggests that the corresponding side of the [2Fe-2S] cluster may be exposed to the solvent.

Previous measurements of the molecular weight of the native protein by ultracentrifugation and by gel filtration yielded values close to 25000 (Hardy et al., 1965; Cardenas et al., 1976; Meyer et al., 1984). We have recently shown, on the basis of electrophoretic evidence, that the protein is a dimer (Meyer et al., 1984). This is now strongly supported by the sequence data, which afforded a molecular weight of 22830 for the dimer, not including iron and inorganic sulfide. With respect to the chromophore content, former values of two iron atoms per molecule of native protein (Hardy et al., 1965; Cardenas et al., 1976) have been shown to be underestimated (Meyer et al., 1984). In the present paper we have correlated iron assays and amino acid analyses and obtained an iron content of two atoms per molecule of polypeptide. Although we have not carried out inorganic sulfide assays, all the spectroscopic properties of Cp Fd indicate that the iron atoms belong to [2Fe-2S] clusters (Cardenas et al., 1976; Meyer et al., 1984). The native protein thus contains two [2Fe-2S] clusters, resulting in a molecular weight of 23174.

The five cysteine residues present in the polypeptide chain exceed the minimum requirement for an all-sulfur ligation of the Fe_2S_2 core. Thus, there is no need for other ligands, unlike in the case of the Rieske protein from *Thermus thermophilus* (Fee et al., 1984), the Fe_2S_2 active sites of which are bonded to two cysteinyl sulfur and to two nitrogen ligands (Cline et al., 1985). Despite the presence of three histidine residues in positions 6, 7, and 90 in the sequence of [2Fe-2S] Cp Fd, the redox potential and the spectroscopic properties of the latter protein (Cardenas et al., 1976; Meyer et al., 1984) are much closer to those of plant ferredoxins than to those of the Rieske protein from *T. thermophilus* (Fee et al., 1984). It is therefore most likely that $Fe_2S_2(S\text{-}Cys)_4$ chromophores are involved in the [2Fe-2S] Cp Fd.

The positions of the cysteine residues along the polypeptide chain are known to have an important role in defining the structure and properties of the iron–sulfur chromophores (Stout, 1982). We have therefore compared the [2Fe-2S] Cp Fd with other [2Fe-2S] proteins from this point of view (Figure 4). Among plant, algal, and halobacterial ferredoxins, the four cysteine ligands of the active site have nearly invariant relative positions (Tsukihara et al., 1982), with the characteristic feature: Cys-(four amino acids)-Cys-X-Y-Cys, X-Y being S-S, S-T, or A-N. These three Cys residues are near the middle of the sequence, and the fourth one is some 30 residues further toward the C-terminal end. The positions of the cysteines in adrenodoxin (Tanaka et al., 1973) and in putidaredoxin (Tanaka et al., 1974) are homologous to each other but differ from those observed in plant Fd: the interval between the first two cysteines is increased from four to five residues, and the fourth cysteine is shifted eight residues

Table V: Sequence Homology Scores among Various Types of Ferredoxins[a]

	putidaredoxin	adrenodoxin	spinach Fd	*A. vinelandii* Fd I	*C. pasteurianum* 2[4Fe-4S] Fd
[2Fe-2S] Cp Fd	1.61	0.16	1.17	2.04	0.79
putidaredoxin		11.64	1.25	1.35	1.26
adrenodoxin			0.32	−0.72	0.28
spinach Fd				0.51	2.90
A. vinelandii Fd I					12.16

[a] The amino acid sequences of [2Fe-2S] Cp Fd (this work), putidaredoxin (Tanaka et al., 1974), adrenodoxin (Tanaka et al., 1973), spinach Fd (Matsubara et al., 1967), *A. vinelandii* Fd I (Howard et al., 1983), and 2[4Fe-4S] Cp Fd (Tanaka et al., 1966) were compared by using the program ALIGN with the Mutation Data Matrix, a gap penalty of 10, and 100 random comparisons (Dayhoff et al., 1983). Alignment scores among plant and algal Fd were close to 30, and those between plant and halobacterial Fd were in the 10–15 range (not shown in Table). The alignment scores of [2Fe-2S] Cp Fd with several plant and halobacterial Fd were all lower than 3. The results displayed in the table remained qualitatively similar when the Genetic Code Matrix was used with a gap penalty of 1 or when the program RELATE was used (Dayhoff et al., 1983). Underlined scores reveal significant homologies.

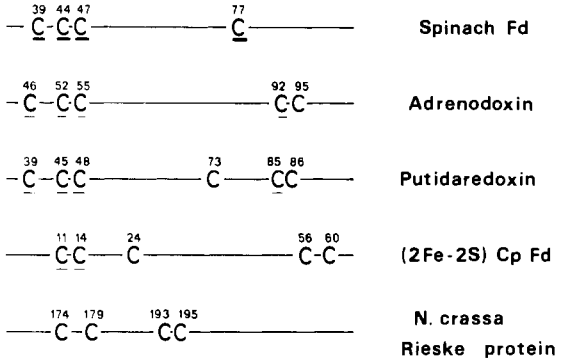

FIGURE 4: Relative positions of the cysteine residues in the amino acid sequences of spinach Fd (Matsubara et al., 1967), adrenodoxin (Tanaka et al., 1973), putidaredoxin (Tanaka et al., 1974), [2Fe-2S] Cp Fd (this work), and the precursor of *N. crassa* Rieske protein (Harnisch et al., 1985). Spinach Fd, as well as several other plant and algal ferredoxins, contains a cysteine residue in position 18 (not shown here), which is not a ligand of the [2Fe-2S] cluster. Thickly underlined cysteines are known, from the three-dimensional structure of *S. platensis* Fd (Tsukihara et al., 1981), to be the ligands of the [2Fe-2S] cluster. Thinly underlined cysteine residues are likely to be involved in the iron–sulfur chromophore for reasons of homology.

further toward the C-terminal end. The [2Fe-2S] Cp Fd displays considerable differences with the preceding proteins: the only homologous feature remaining, namely, the C-T-S-C fragment, now occurs near the N-terminus (residues 11–14), and the three other cysteines are found on the C-terminal side of this segment, in positions that are not homologous to those of the cysteine residues in the other polypeptide chains. By analogy with the other [2Fe-2S] proteins, it may be inferred that Cys-11 and Cys-14 are most probably ligands of the iron–sulfur cluster in Cp Fd. For the two other ligands, further investigations are wanted before a choice can be made among Cys-24, Cys-56, and Cys-60. The Rieske protein from *Neurospora crassa*, of which the gene has recently been sequenced (Harnisch et al., 1985), shows an even more atypical spacing of its four cysteine residues (Figure 4).

From the unusual positions of the cysteine residues along the polypeptide chain of [2Fe-2S] Cp Fd, it may be inferred that this protein bears little homology with other ferredoxins. In fact, a comparison of the sequence of [2Fe-2S] Cp Fd with the complete NBRF data bank using the program SEARCH with the Mutation Data Matrix (Dayhoff et al., 1983) failed to detect any homologous sequences. We have attempted to quantify the differences between the primary structures of [2Fe-2S] Cp Fd and of several benchmark ferredoxins containing 2 Fe, 3 Fe, and 4 Fe active sites (Table V), by using the programs ALIGN and RELATE (Dayhoff et al., 1983). Ferredoxins from sulfate reducers (Bruschi et al., 1985) and from the archaebacteria *Methanosarcina barkeri* (Hausinger et al., 1982), *Thermoplasma acidophilum* (Wakabayashi et al., 1983), and *Sulfolobus acidocaldarius* (Minami et al., 1985), which are all related to 2[4Fe-4S] clostridial Fd, were not included in these comparisons. Significant relationships were observed only between putidaredoxin and adrenodoxin and among the 3Fe and 4Fe bacterial ferredoxins. It is worth noticing that none of the [2Fe-2S] proteins seems to be related with the 3Fe and 4Fe bacterial Fd.

From the comparison of the [2Fe-2S] Fd sequences in Table V, the occurrence of three (not including the Rieske proteins) unrelated types of primary structures may be inferred: the first one is represented by plant Fd, the second one by adrenodoxin and putidaredoxin, and the third one by [2Fe-2S] Cp Fd. The latter conclusion, drawn from the comparison of whole sequences, is consistent with the comparison of the positions of the cysteine residues along the polypeptide chains (Figure 4): the three above-mentioned types of proteins display definitely distinct patterns of cysteine spacing, except for the C-X-Y-C fragment. It should be emphasized, however, that the latter segment cannot be considered as a characteristic feature of [2Fe-2S] proteins, since it also occurs in other FeS proteins (Stout, 1982), in *c*-type cytochromes (Ambler, 1982), and in numerous zinc-containing proteins (Berg, 1986). Thus, the assembly and stabilization of [2Fe-2S] clusters in ferredoxins is not achieved by a unique and characteristic pattern of cysteine ligands. Instead, several solutions seem to have been found in the course of evolution to meet the structural requirements for the stabilization of [2Fe-2S] active sites.

Although the physiological role of [2Fe-2S] Cp Fd remains undetermined, its synthesis is known to be associated with the expression of the *nif* genes (Hardy et al., 1965; Hinton & Mortenson, 1985; our unpublished results). The latter observation would suggest that it is a protein of ancient origin: it has indeed been shown recently that the *nif* genes may have evolved to a large extent similarly to the bacteria that carry them, i.e., they were not laterally distributed among microorganisms by a recent gene transfer (Hennecke et al., 1985). The latter authors did not reject the possibility of an early gene transfer between, for instance, cyanobacteria and *C. pasteurianum*. However, if the gene coding for [2Fe-2S] Cp Fd had undergone such a transfer, one would expect cyanobacterial Fd and Cp Fd to display some detectable relationships, which they do not (Table V). It is thus reasonable to assume an early occurrence of [2Fe-2S] Fd in saccharolytic anaerobes, probably in connection with nitrogen fixation. At this point it would be interesting to know if [2Fe-2S] ferredoxins are present in clostridia which do not fix nitrogen. [2Fe-2S] proteins involved in nitrogen fixation have also been found in aerobic bacteria of the genus *Azotobacter* (Haaker & Veeger, 1977; Robson, 1979). How closely these proteins are related to Cp Fd remains to be determined.

In summary, the comparisons carried out above (Table V) strongly suggest the occurrence of three (in the present state of knowledge) phylogenetically unrelated groups of [2Fe-2S] Fd. Furthermore, since the latter proteins do not display homology with the 4Fe and 3Fe Fd, it may be inferred that the various types of [2Fe-2S] Fd and the bacterial 4Fe and 3Fe Fd constitute four groups of proteins having appeared and evolved independently from each other. Multiple ferredoxin origins in the course of evolution may also be considered as likely in view of the large number and diversity of proteins having clustered cysteine residues (Stout, 1982; Ambler, 1982; Berg, 1986). If this hypothesis is confirmed by further analysis of the rapidly expanding body of Fd sequence data, it will imply the existence of several independent Fd evolutionary trees. As none of the latter is likely to encompass all bacteria and early eucaryotes, it will be essential, in order to correlate the phylogenetic information obtained from one group of Fd with that obtained from the others, to collect sequence data from organisms containing two or more unrelated Fd. Such data are now available for *C. pasteurianum*, from which a [2Fe-2S] Fd (this work) and a 2[4Fe-4S] Fd (Tanaka et al., 1966) have been sequenced, and for *P. putida*, from which the sequences of the [2Fe-2S] putidaredoxin (Tanaka et al., 1974) and of a 7Fe Fd (Hase et al., 1978b) related to *Azotobacter vinelandii* Fd I have been elucidated. Fd sequences are thus likely to become increasingly useful, concurrently with other protein sequences (Dayhoff, 1983) and with other features such as 16S ribosomal RNA sequences (Fox et al., 1980; Woese et al., 1985) and three-dimensional ribosome structure (Lake et al., 1985), to ultimately solve the much-debated problem of the evolution of the early forms of life.

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Bacterial Sarcosine Oxidase: Comparison of Two Multisubunit Enzymes Containing both Covalent and Noncovalent Flavin†

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ABSTRACT: Sarcosine oxidase was purified to homogeneity from *Corynebacterium* sp. P-1, a soil organism isolated by a serial enrichment technique. The enzyme contains 1 mol of noncovalently bound flavin [flavin adenine dinucleotide (FAD)] plus 1 mol of covalently bound flavin [8α-(N^3-histidyl)-FAD] per mole of enzyme (M_r 168 000). The two flavins appear to have different roles in catalysis. The enzyme has an unusual subunit composition, containing four dissimilar subunits (M_r 100 000, 42 000, 20 000, and 6000). The same subunits are detected in Western blot analysis of cell extracts prepared in the presence of trichloroacetic acid, indicating that the subunits are a genuine property of the enzyme as it exists in vivo. The presence of both covalent and noncovalent flavin in a single enzyme is extremely unusual and has previously been observed only with a sarcosine oxidase from a soil *Corynebacterium* isolated in Japan. The enzymes exhibit many similarities but are distinguishable in electrophoretic studies. Immunologically, the enzymes are cross-reactive but not identical. The results indicate that the synthesis of a sarcosine oxidase containing both covalent and noncovalent flavin is not a particularly unusual event in corynebacteria.

Sarcosine oxidase from *Corynebacterium* sp. U-96, a soil organism isolated in Japan, is the first example of a flavoprotein containing both covalently bound flavin [1 mol of 8α-(N^3-histidyl)-FAD[1]/mol of enzyme] and noncovalently bound flavin (1 mol of FAD/mol of enzyme) (Hayashi et al., 1980, 1982; Suzuki, 1981). The enzyme catalyzes the oxidative demethylation of sarcosine (eq 1). Recent studies by

$$CH_3NHCH_2COOH + O_2 + H_2O \rightarrow H_2CO + NH_2CH_2COOH + H_2O_2 \quad (1)$$

Jorns (1985) suggest that the two flavins in this enzyme have different roles in catalysis. The noncovalent flavin appears to function as a dehydrogenase flavin, accepting electrons from sarcosine and then transferring them to the covalent flavin. Oxygen is reduced to hydrogen peroxide by the covalent flavin

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[1] Abbreviations: FAD, flavin adenine dinucleotide; FMN, flavin mononucleotide; EDTA, ethylenediaminetetraacetic acid; sarcosine oxidase P, sarcosine oxidase from *Corynebacterium* sp. P-1; sarcosine oxidase J, sarcosine oxidase from *Corynebacterium* sp. U-96; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; ELISA, enzyme-linked immunosorbent assay; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; Tween 20, poly(oxyethylene) sorbitan monolaurate; TCA, trichloroacetic acid; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)-aminomethane.

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