Amino Acid Sequence Determination of the *Clostridium* M-E Ferredoxin and a Comment on the Role of the Aromatic Residues in the Clostridial Ferredoxins†

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ABSTRACT: The amino acid sequence of the ferredoxin from *Clostridium* M-E has been shown to be: Ala-Tyr-Lys-Ile-Thr-Asp-Gly-Cys-Ile-Asn-Cys-Gly-Ala-Cys-Glu-Pro-Glu-Cys-Pro-Val-Glu-Ala-Ile-Ser-Glu-Ser-Asp-Ala-Val-Arg-Val-Ile-Asp-Ala-Asp-Lys-Cys-Ile-Asp-Cys-Gly-Ala-Cys-Ala-Asn-Thr-Cys-Pro-Val-Asp-Ala-Ile-Val-Glu-Gly. This ferredoxin contains only one aromatic amino acid residue and lacks the second aromatic residue normally found in position 30 in other clostridial ferredoxins, which has been suggested previously to play a role in electron transfer.

Ferredoxin isolated from a lysine-fermenting anaerobic organism designated *Clostridium* strain M-E[1] was used for the sequence studies reported in the present communication. Biochemical studies carried out with this microorganism have dealt primarily with the series of reactions involved in the conversion of lysine to acetate, butyrate, and ammonia (Stadtman, 1963, 1964, 1973; Tsai and Stadtman, 1968) and it was established in studies with extracts of *Clostridium* M-E (Stadtman, 1962) that a cobamide coenzyme participates in the process. Large scale isolation procedures designed to obtain the cobamide coenzyme-dependent β-lysine and D-α-lysine mutases resulted in the concomitant separation of the prominant dark brown, acidic protein fraction which contained ferredoxin. This was recovered as a by-product from each batch of extract that was processed and after concentration with ammonium sulfate was stored in the frozen state for later use.

Materials and Methods

Culture of Clostridum M-E. Clostridium M-E was cultured in a 400-l. fermentor in a medium containing lysine and glucose as the principal fermentable substrates. The medium consisted of four different solutions that were autoclaved separately and mixed aseptically after cooling. Solution A, prepared in the fermentor tank in about 310 l. of distilled water, contained L-lysine · HCl, 1700 g; Difco yeast extract, 1700 g; KH_2PO_4, 460 g; and a concentrated metal salts solution consisting of $CaCl_2 \cdot 2H_2O$, 3.4 g; $MgSO_4 \cdot 7H_2O$, 34.0 g; $FeSO_4 \cdot 7H_2O$, 1.7 g; $CoCl_2 \cdot 6H_2O$, 2.04 g; and disodium ethylenediaminetetraacetate, 3.162 g disolved in 2 l. of distilled water and adjusted to pH 7.5 with NaOH. Solution B contained 680 g of D-glucose in 10 l. of distilled water. Solution C contained Na_2CO_3, 510 g, and $Na_2S \cdot 9H_2O$, 102 g, in 10 l. of distilled water. Solution D was prepared by adding 85 ml of concentrated HCl to 10 l. of sterile distilled water. Solution A was autoclaved and cooled under nitrogen or argon in the fermentor tank and solutions B and C were sterilized in 20-l. glass carboys. The cooled sterile solutions were added to the tank immediately after solution A was cool. The pH of the completed medium should be about 7.5. If further adjustment was required, this was achieved by addition of more sterile sodium carbonate to raise the pH or by bubbling carbon dioxide through the medium to lower the pH. The inoculum, consisting of 40 l. of an actively fermenting culture of *Clostridium* M-E prepared in the same medium, was added at once under a stream of nitrogen or argon. The temperature was maintained at 30–32°. When it was necessary to stir the culture to maintain constant temperature this was done at as slow a rate as possible and a gas phase of 5% carbon dioxide in nitrogen was introduced above the liquid to prevent loss of carbon dioxide from the medium. The cells were collected by centrifugation in a Sharples supercentrifuge after 16–20 hr of growth under the above conditions. A cell yield of about 1 g of wet packed cells per l. of medium was usually obtained. The cell paste was dropped in small aliquots into liquid nitrogen and the frozen pellets were stored at −80° until needed.

Preparation of extracts. Frozen cells were thawed in a buffer solution containing 20 mM Tris · HCl (pH 8.2), 20 mM K_2HPO_4, and 1 mM MgK_2EDTA (Titraplex, E. Merck, Darmstadt, Germany). For each 100 g of frozen cells about 150 ml of buffer solution was added. The cells were ruptured by sonication in a Bronson sonifier (two 1-min bursts at maximum power output) and the cellular debris was removed by centrifugation at 20,000*g*.

Separation of Ferredoxin from Crude Extracts. A rapid

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[1] The exact taxonomic relation of this organism to other members of the genus *Clostridium* has not been determined. The source of this organism was a sample of black mud from the left bank of the Rio Daule, Guayas Province, Ecuador, collected by Drs. Betty Meggers and Clifford Evans, Department of Archeology, Smithsonian Institution, Washington, D.C. *Clostridium* M-E was isolated from the mud in 1959 (Tarantola and Stadtman) by the enrichment culture technique using a mineral salts medium that contained lysine as fermentable substrate. Growth of the organism occurs only under strictly anaerobic conditions. It is a straight to slightly bent rod-shaped, motile organism, 0.5–0.75 μ in width × 1–3 μ in length.

chromatographic step in which the acidic proteins of *Clostridium* M-E crude extracts are adsorbed to coarse DEAE-cellulose was used. The procedure which was originally designed for isolation of β-lysine mutase (Stadtman and Renz, 1968; Stadtman and Grant, 1971) allows the stepwise elution first of the orange colored cobamide coenzyme linked mutase and finally the brown ferredoxin fraction. A representative fractionation is as follows. A crude extract, 675 ml containing 44 g of protein, was passed over a column of coarse floc DEAE-cellulose (8 cm × 23 cm bed volume) that had been equilibrated with pH 8 Tris · HCl buffer. The column was washed with 20 mM Tris · HCl–1 mM MgK_2EDTA buffer (pH 8) until the washings were free of protein. The effluent and wash combined contained 27.7 g of protein (63% of the total protein applied). After elution of an orange colored protein fraction (12.2 g of protein) with 0.25 M potassium phosphate (pH 7.2) the brown ferredoxin band remaining on the top of the column was eluted with buffer containing 1 M NaCl in 20 mM potassium phosphate (pH 7.2). This dark brown colored eluate, 580 ml containing 950 mg of protein, was adjusted to 0.9 saturation in ammonium sulfate and the precipitated protein was collected by centrifugation. The precipitate was dissolved in a small volume of water and then passed through a Sephadex G-25 column to desalt the solution. It was further purified by DEAE-cellulose chromatography as described by Lovenberg *et al.* (1963) to yield about 52 mg of ferredoxin with a 390/280 nm ratio of about 0.78.

Tryptic Digestion of Cm-ferredoxin[2] *and Anion Exchange Column Chromatography of Peptides.* The *Clostridium* M-E ferredoxin was converted to its apoprotein by treating the native protein with trichloroacetic acid and then to the Cm derivative by reaction with iodoacetic acid as described in a previous report (Tanaka *et al.*, 1971). About 1.6 μmol of the Cm-ferredoxin was incubated with Tos-PheCH_2Cl-trypsin (enzyme to substrate ratio was 1:40 on a weight basis) at pH 8.0 in a total volume of 0.50 ml. Additional Tos-PheCH_2Cl-trypsin was added at 6 hr and the digestion was performed at 28° for 24 hr. The nitrogen-dried tryptic digest of Cm-ferredoxin was applied to a Dowex 1-X2 column (1.0 × 20 cm). The digestion mixture was eluted by gradient elution by mixing 100 ml of water in the mixing chamber and 100 ml of 6.0 M acetic acid in the reservoir. The flow rate was 60 ml/hr. The fractions of 4.5 ml were collected and an aliquot of each fraction was assayed by reaction with ninhydrin after alkaline hydrolysis of the samples (Crestfield *et al.*, 1963). The peptides after column chromatography were further purified by paper chromatography in the solvent system, 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v).

Thermolytic Hydrolysis of Peptide T-4 and Dowex 1-X2 Column Chromatography of Peptides. Peptide T-4 (0.83 μmol) was hydrolyzed with thermolysin (enzyme to substrate ratio was 1:20 on a weight basis) at pH 8.0 in a volume of 0.50 ml. The hydrolysis reaction was carried out at 40° for 20 hr. The nitrogen-dried thermolysin digest of peptide T-4 was fractionated by chromatography on Dowex 1-X2 and the peptides were further purified by paper chromatography exactly as described above for the tryptic digest.

[2] The abbreviations used are: Cm, carboxymethyl; Cys(Cm), S-β-carboxymethylcysteine; Pth, phenylthiohydantoin; BPAW, 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v); and Tos-PheCH_2Cl, L-1-tosylamido-2-phenylethyl chloromethyl ketone.

TABLE I: Amino Acid Composition of *Clostridium* M-E Ferredoxin.

Amino Acid	From Acid Hydrolysates[a]	From the Sequence
Asp	7.97 (8)	8[b]
Thr	1.97 (2)	2
Ser	1.84 (2)	2
Glu	5.09 (5)	5[c]
Pro	2.87 (3)	3
Gly	3.94 (4)	4
Ala	7.89 (8)	8
Cys[d]	7.86 (8)	8
Val	4.95 (5)	5
Ile	5.92 (6)	6
Tyr	1.03 (1)	1
Lys	2.00 (2)	2
Arg	0.91 (1)	1
Total residues	55	55

[a] Acid hydrolyses were performed on Cm-ferredoxin for 24, 48, and 72 hr at 110° with 6 N HCl. The amino acid residues were calculated on the basis of a lysine content of 2.00 mol/mol of protein. The values of threonine and serine were obtained by extrapolation to zero time. The values of valine, leucine, and isoleucine were the maximum values (72 hr). Numbers in parentheses indicate values rounded off to nearest whole number. [b] Sum of six aspartic acids and two asparagines. [c] No glutamine present. [d] Determined as Cys-(Cm).

Analytical Procedures. The amino acid composition of the protein and peptides was determined on acid hydrolysates in a Beckman-Spinco Model 120C automatic amino acid analyzer (Spackman *et al.*, 1958). The instrument was equipped with high sensitivity cuvets and a 4–5 mV full scale range card.

The amino-terminal sequences of the Cm-ferredoxin were determined by the Beckman-Spinco Model 890 protein/peptide sequencer utilizing the protein double cleavage program. Prior to the sequencer analysis, the Cm-ferredoxin was allowed to react with 4-sulfophenyl isothiocyanate (Braunitzer *et al.*, 1971). The sequencer run was carried out once with the 4-sulfophenylthiocarbamyl derivative of the Cm-ferredoxin and once with the underivatized Cm-ferredoxin. The amino-terminal sequences of all the other peptides were obtained by the usual manual Edman degradation method (Edman and Sjoquist, 1956). The amino acid phenylthiohydantoins were identified by gas chromatography in a Beckman GC-45 gas chromatograph (Pisano and Bronzert, 1969), or by thin-layer chromatography (Edman and Begg, 1967), or by 6 N HCl hydrolysis of the amino acid phenylthiohydantoin to the free amino acids (Van Horten and Carpenter, 1969).

The carboxyl-terminal amino acids were determined on the protein and peptides by hydrazinolysis (Bradbury, 1958).

Peptide Nomenclature. Peptides obtained from the hydrolysis of Cm-ferredoxin with trypsin are designated by the symbol T. Peptide fragments obtained from the thermolytic hydrolysis of peptide T-4 are denoted by the symbol Th.

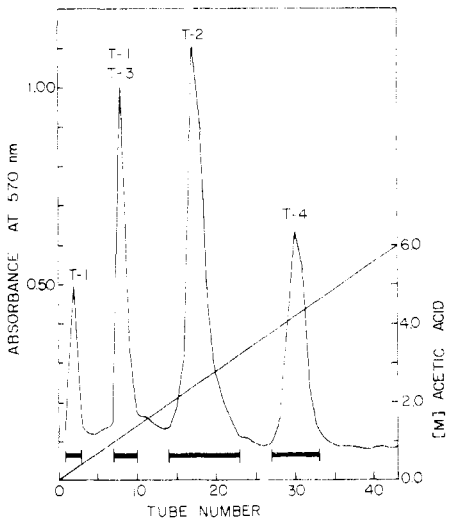

FIGURE 1: Dowex 1-X2 column chromatography of a tryptic digest of Cm-ferredoxin (about 1.6 μmol). See Materials and Methods for details of experimental conditions. Fractions under each peak which were pooled are shown by solid bars.

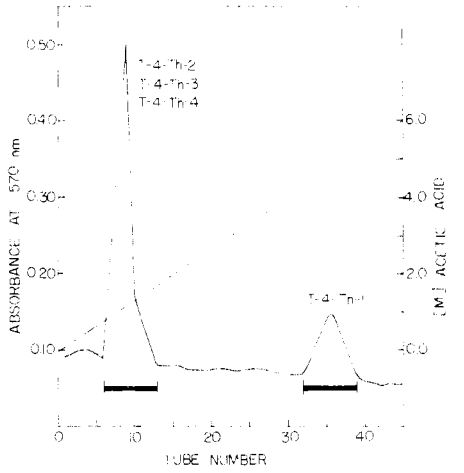

FIGURE 2: Dowex 1-X2 column chromatography of a thermolytic digest of peptide T-4 (0.83 μmol). See Materials and Methods for experimental details. Fractions under each peak which were pooled are indicated by solid bars.

Results

Amino Acid Composition and Amino- and Carboxyl-Terminal Amino Acid Analyses. The amino acid composition of the *Clostridium* M-E ferredoxin was obtained from 24-, 48-, and 72-hr hydrolysates of the Cm-ferredoxin. The results are summarized in Table I. Two steps of the manual Edman degradation of the Cm-ferredoxin showed that the amino-terminal sequence was Ala-Tyr. Hydrazinolysis yielded glycine in 76% yield. No carboxypeptidase A action was observed on Cm-ferredoxin at reaction times of 1, 3, and 6 hr.

Separation of Tryptic Peptides. Figure 1 shows the chromatogram obtained for the separation of tryptic peptides of Cm-ferredoxin on a Dowex 1 column. The first peak contained only peptide T-1. The second peak contained peptide T-3 together with about half the amount of peptide T-1 found in peak 1. Peptide T-1 from the second peak was combined, after purification by paper chromatography, with peptide T-1 from the first peak. The third and fourth peaks contained peptides T-2 and T-4, respectively. The method for the further purification of the peptide fractions, their amino acid compositions, and properties are summarized in Table II.

Isolation and Purification of Thermolytic Peptides from

TABLE II: Amino Acid Composition[a] and Properties of Tryptic Peptides of Cm-ferredoxin.

Amino Acid	T-1	T-2	T-3	T-4	Total Residues
Cys(Cm)		3.92 (4)		3.98 (4)	8
Asp		3.09 (3)	1.93 (2)	3.10 (3)	8
Thr		1.01 (1)		1.02 (1)	2
Ser		1.78 (2)			2
Glu		4.02 (4)		1.07 (1)	5
Pro		1.95 (2)		0.98 (1)	3
Gly		2.00 (2)		2.00 (2)	4
Ala	1.00 (1)	2.96 (3)	0.98 (1)	3.05 (3)	8
Val		2.02 (2)	0.94 (1)	1.98 (2)	5
Ile		3.01 (3)	0.92 (1)	1.96 (2)	6
Tyr	0.98 (1)				1
Lys	1.04 (1)		1.00 (1)		2
Arg		0.91 (1)			1
Total residues	3	27	6	19	55
Recovery (%)	88	93	91	80	
R_F[b]	0.40	0.22	0.32	0.26	
Color reaction with ninhydrin	Violet	Violet	Violet	Yellow to violet	
Purification method[b]	BPAW	BPAW	BPAW	BPAW	

[a] Results from 6 N HCl hydrolyses for 24 and 48 hr. The numbers in parentheses refer to the assumed stoichiometric number of residues per molecule of pure peptide. [b] Paper chromatography in the solvent system, 1-butanol-pyridine-acetic acid-water (60:40:12:48, v/v).

TABLE III: Amino Acid Composition[a] and Properties of Thermolytic Peptides of Peptide T-4.

Amino Acid	T-4-Th-1	T-4-Th-2	T-4-Th-3	T-4-Th-4	Total Residues
Cys(Cm)	2.84 (3)	0.86 (1)			4
Asp	1.03 (1)	1.02 (1)	1.01 (1)		3
Thr		0.86 (1)			1
Glu				0.99 (1)	1
Pro		0.99 (1)			1
Gly	0.96 (1)			1.00 (1)	2
Ala	1.00 (1)	1.00 (1)	1.00 (1)		3
Val			0.98 (1)	0.98 (1)	2
Ile	0.95 (1)			0.94 (1)	2
Total residues	7	5	3	4	19
Recovery (%)	60	50	74	75	
R_F[b]	0.20	0.23	0.33	0.56	
Color reaction with ninhydrin	Violet	Violet	Violet	Violet	
Purification method[b]	BPAW	BPAW	BPAW	BPAW	

[a,b] See footnotes *a* and *b* in Table II, respectively.

Peptide T-4. Shown in Figure 2 is the chromatographic pattern of the thermolytic digest of peptide T-4 on a column of Dowex 1. Two peaks were detected by the ninhydrin assay method after alkaline hydrolysis. The first peak contained three peptides, T-4-Th-2, T-4-Th-3, and T-4-Th-4, and these could be separated by paper chromatography in 1-butanol–pyridine–acetic acid–water. The second peak contained only peptide T-4-Th-1. The amino acid compositions and properties of these peptides are summarized in Table III.

Sequence Investigations. Only the sequence data necessary to establish the total sequence are presented here, even though additional sequence studies were performed.

THE SEQUENCER RESULTS OF Cm-FERREDOXIN. From the combined analyses on the 4-sulfophenylthiocarbamyl derivative of the Cm-ferredoxin and the underivatized Cm-ferredoxin, the first 34 amino acid residues from the amino terminus of the protein were determined and are shown in Figure 3.

The amino acid sequence of the peptide T-3 (residues 31–36) was established by manual Edman degradation as shown in Table IV.

THE PARTIAL SEQUENCE OF PEPTIDE T-4 (RESIDUES 37–55). Sixteen steps of the manual Edman degradation were carried out on this peptide. The Edman degradation results as well as hydrazinolysis data are summarized in Table V.

Two steps of the Edman degradation of peptide T-4-Th-3 yielded the following results: first step, Pth-valine in 94% yield; second step: Pth-aspartic acid in 90% yield. Pth-aspartic acid was also identified by thin-layer chromatography of the Pth-amino acid and aspartic acid was detected by amino acid analysis after 6 N HCl hydrolysis of the Pth derivative. After the second step of the Edman degradation,

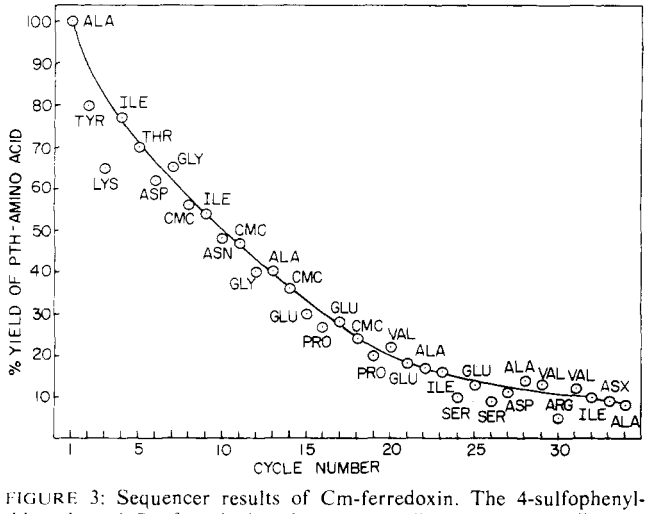

FIGURE 3: Sequencer results of Cm-ferredoxin. The 4-sulfophenylthiocarbamyl Cm-ferredoxin (about 0.4 μmol) was automatically analyzed in the Beckman-Spinco Model 890 protein sequencer. See Materials and Methods for details of analytical procedures. In the figure, CMC is the abbreviation for S-β-carboxymethylcysteine.

TABLE IV: Sequence Determination of Peptide T-3; Sequence: Val-Ile-Asp-Ala-Asp-Lys.

Step No. in Edman Degradation	Amino Acid	Yield (%)	Identification Method[a]
1	Val	95	Gc, tlc, hyd
2	Ile	90	Gc, tlc, hyd
3	Asp	80	Gc, tlc, hyd
4	Ala	72	Gc, tlc, hyd
5	Asp	70	Gc, tlc, hyd
5D[b]	Lys	50	

[a] The abbreviations used are: Gc, gas chromatography of Pth-amino acid; tlc, thin-layer chromatography of Pth-amino acid; and hyd, 6 N HCl hydrolysis of Pth-amino acid to the free amino acid. [b] Determined quantitatively as ε-phenylthiocarbamyllysine without acid hydrolysis.

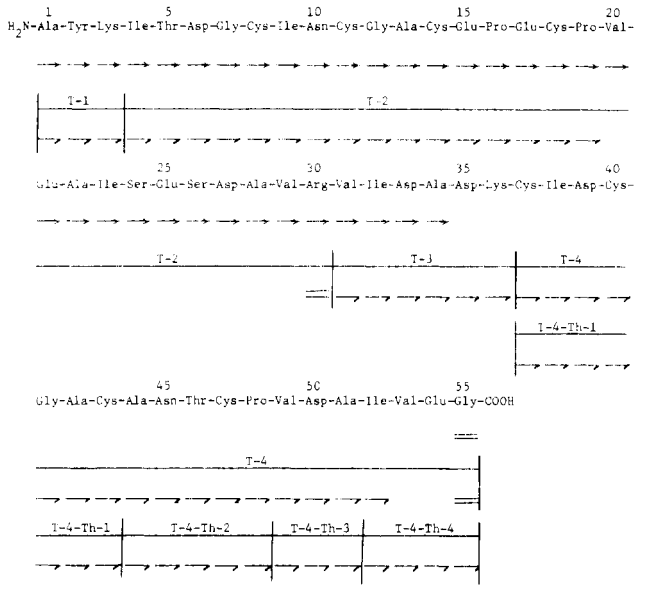

FIGURE 4: Reconstruction of the complete sequence of *Clostridium* M-E ferredoxin and the sequence data of the peptide fragments. In the figure, the symbols →, ⇀, and = represent sequences determined by the use of the Beckman-Spinco protein sequencer, by direct manual Edman degradation and by hydrazinolysis experiments, respectively.

direct amino acid analysis without acid hydrolysis yielded alanine in 75% yield.

The amino acid sequence of the peptide T-4-Th-4 was established by manual Edman degradation. The results obtained were as follows: first step, Pth-isoleucine in 96% yield; second step, Pth-valine in 85% yield; and third step, Pth-glutamic acid in 64% yield. Pth-glutamic acid was also identified by thin-layer chromatography as well as by amino acid analyses of the acid hydrolysate of the Pth-glutamic acid. After the third step of the Edman degradation, glycine was recovered in 60% yield by direct amino acid analysis without acid hydrolysis.

Complete Sequence. Amino-terminal analysis of the *Clostridium* M-E ferredoxin by the manual and automatic Edman degradation showed that alanine was the amino-terminal residue. The sequencer analysis of the protein showed that peptide T-1 was the amino-terminal peptide, peptide T-2 was next, and then peptide T-3 followed. Peptide T-4 is the carboxyl-terminal peptide based on the carboxyl-terminal analysis of the protein. The sequences of the individual fragments of the tryptic and thermolytic peptides necessary to establish the total sequence have been covered in the previous paragraph and are summarized in Figure 4.

TABLE V: Sequence Analyses of Peptide T-4; Sequence: Cys(Cm)-Ile-Asp-Cys(Cm)-Gly-Ala-Cys(Cm)-Ala-Asn-Thr-Cys(Cm)-Pro-Val-Asx-Ala-Ile(Val,Glx)GLY.[c]

Step No. in Edman Degradation	Amino Acid	Yield (%)	Identification Method[a]
1	Cys(Cm)	100	Gc, tlc
2	Ile	90	Gc, tlc, hyd
3	Asp	75	Gc, tlc, hyd
4	Cys(Cm)	70	Gc, tlc
5	Gly	56	Gc, tlc, hyd
6	Ala	50	Gc, tlc, hyd
7	Cys(Cm)	44	Gc, tlc
8	Ala	40	Gc, tlc, hyd
9	Asn	33	Gc, tlc, hyd
10	Thr	27	Gc, tlc
11	Cys(Cm)	22	Gc, tlc
12	Pro	12	Gc, tlc, hyd
13	Val	10	Gc, hyd
14	Asx[b]	7	Gc, hyd
15	Ala	8	Gc, hyd
16	Ile	5	Gc, hyd

[a] See footnote *a* in Table IV. [b] "Asx" means aspartic acid or asparagine. From the sequence analyses of the peptide T-4-Th-3, this "Asx" was determined to be aspartic acid. [c] Hydrazinolysis, glycine in 73% yield.

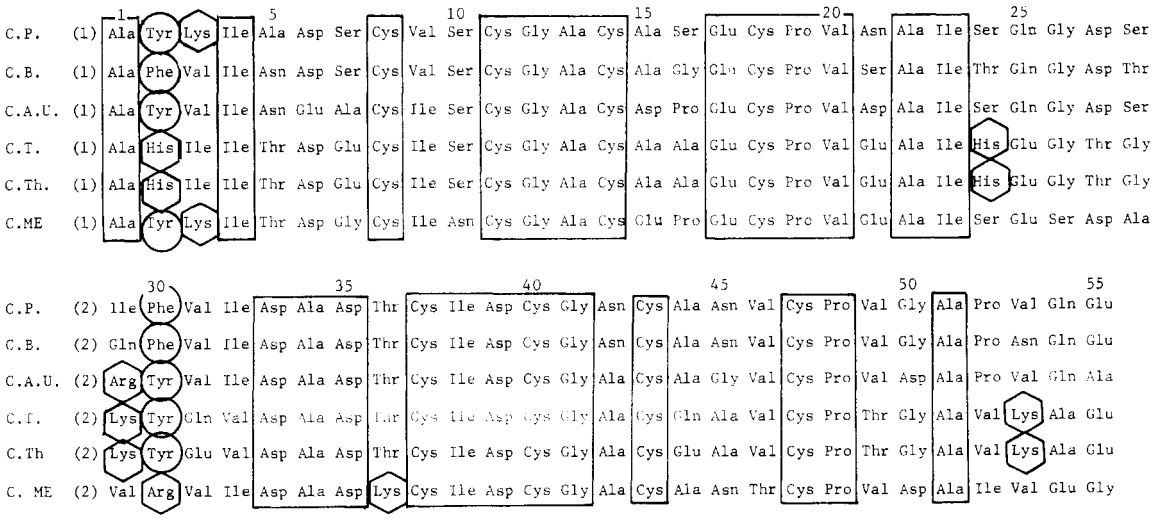

FIGURE 5: Comparison of the amino acid sequences of clostridial ferredoxins whose sequences are completed up to date. In the figure, the abbreviations used are: C.P., *Clostridium pasteurianum* (Tanaka *et al.*, 1966); C.B., *Clostridium butyricum* (Benson *et al.*, 1967); C.A.U., *Clostridium acidi-urici* (Rall *et al.*, 1969); C.T., *Clostridium tartarivorum* (Tanaka *et al.*, 1971); C.Th., *Clostridium thermosaccharolyticum* (Tanaka *et al.*, 1973), and C. ME, *Clostridium* M-E (this work). The squared off sequences indicate identical residues. The circles and the hexagons stand for aromatic amino acids (Tyr or Phe) and the basic amino acids (Lys, His, or Arg), respectively.

Discussion

The primary structures of ferredoxin from the following anaerobic bacteria have been determined: *Clostridium pasteurianum* (Tanaka *et al.*, 1966), *Clostridium butyricum* (Benson *et al.*, 1967), *Peptococcus aerogenes* (Tsunoda *et al.*, 1968), *Clostridium acidi-urici* (Rall *et al.*, 1969), *Peptostreptococcus elsdenii* (Azari *et al.*, 1970), *Clostridium tartarivorum* (Tanaka *et al.*, 1971), *Desulfovibrio gigas* (Travis *et al.*, 1971), and *Clostridium thermosaccharolyticum* (Tanaka *et al.*, 1973). Only for the *P. aerogenes* ferredoxin has the three-dimensional structure been resolved by the crystal X-ray diffraction technique (Adman *et al.*, 1973). In this ferredoxin the amino acids at positions 2 and 28 are both tyrosine residues and there are two apparently identical iron sulfide clusters consisting of eight atoms of iron and eight atoms of acid-labile inorganic sulfur. The X-ray data showed that the orientation of each of the tyrosine residues is such that it is parallel to one of the faces of the nearest distorted cube-like sulfide cluster, and the hydroxyl edge of the aromatic ring is in contact with the solvent. From a consideration of the results of the sequence and X-ray studies on the *P. aerogenes* ferredoxin, and, in view of the fact that analogous positions (2 and 30) in some of the other anaerobic bacterial ferredoxins also are aromatic amino acid residues, it was suggested by Adman *et al.* (1973) that these aromatic residues participate in the electron transfer process.

During the amino acid analysis of the *Clostridium* M-E ferredoxin, however, it was noted that the protein contains only one aromatic residue and no histidine despite the fact that it has the normal complement of eight cysteine residues which is indicative of two active sites each consisting of four Fe and four S^{2-}. Amino acid sequence determination of the *Clostridium* M-E ferredoxin disclosed that the single aromatic residue (tyrosine) is present in position 2 of the protein. Instead of an aromatic residue in position 30, there is an arginine residue at this position in the *Clostridium* M-E ferredoxin.

Recently Lode *et al.* (1974) removed the N-terminal amino acid residue and residue 2, the aromatic residue, from the *C. acidi-urici* apoferredoxin by Edman degradation and replaced these two residues with other amino acids by chemical synthesis. An altered ferredoxin containing leucine in position 2 and an N-terminal alanine residue was reactivated by the addition of iron and sulfide. This reconstituted ferredoxin containing only one aromatic residue was fully active as an electron carrier although it was less stable than the native ferredoxin.

The two aforementioned experimental findings show that biological activity does not depend on the presence of an aromatic residue in both positions 2 and 30 and suggest that these residues do not participate directly in the electron transfer process as postulated by Adman *et al.* (1973). Since the *Clostridium* M-E ferredoxin already contains arginine in place of an aromatic amino acid residue at position 30, it would be of interest to modify this protein chemically by the procedure of Lode *et al.* (1974). Substitution of leucine for the tyrosine residue at position 2 would yield a ferredoxin free of aromatic amino acids that could be used to rigorously test the theory regarding the importance of aromatic residues in the conserved positions of the ferredoxin polypeptide chain.

Summarized in Figure 5 are the constant and variable amino acid residues of the various clostridial ferredoxins that have been sequenced to date. Among these six ferredoxins, there are 13 identical amino acid residues in the amino-terminal half (residues 1–28) and 12 identical residues in the lower half (residues 29–55). Thus, 25 residues are constant in these clostridial ferredoxins.

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