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Amino Acid Sequence of Ferredoxin from a Photosynthetic Green Bacterium, *Chlorobium limicola*†

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ABSTRACT: The amino acid sequence of ferredoxin I from the photosynthetic green sulfur-reducing bacteria, *Chlorobium limicola,* was deduced to be: Ala-Leu-Tyr-Ile-Thr-Glu-Glu-Cys-Thr-Tyr-Cys-Gly-Ala-Cys-Glu-Pro-Glu-Cys-Pro-Val-Thr-Ala-Ile-Ser-Ala-Gly-Asp-Asp-Ile-Tyr-Val-Ile-Asp-Ala-Asn-Thr-Cys-Asn-Glu-Cys-Ala-Gly-Leu-Asp-Glu-Gln-Ala-Cys-Val-Ala-Val-Cys-Pro-Ala-Glu-Cys-Ile-Val-Gln-Gly. The protein consists of 60 amino acid residues and the molecular weight of the native ferredoxin was calculated to be 6923. A comparison of the sequences was made between *Chlorobium limicola* ferredoxin and the other ferredoxins which have already been sequenced. The photosynthetic bacterial ferredoxins appear to be intermediate in size when compared with the clostridial and plant ferredoxins and therefore the sequences of these ferredoxins are useful for extracting evolutionary data.

The amino acid sequences of ferredoxins from seven anaerobic fermentative bacteria (Tanaka *et al.,* 1966, 1971, 1973; Benson *et al.,* 1967; Tsunoda *et al.,* 1968; Rall *et al.,* 1969; Travis *et al.,* 1971) are known and these sequences are extremely homologous except for a species from *Desulfovibrio gigas* (Travis *et al.,* 1971). The amino acid sequence of ferredoxin from the purple sulfur photosynthetic bacterium *Chromatium,* although 26 amino acids longer than the clostridial ferredoxins, shows enough homology with the latter group to suggest a common ancestor for the two types of ferredoxins. The amino acid sequence of a ferredoxin from a green photosynthetic bacterium will be very useful in tracing the evolutionary history of anaerobic bacteria. We have now determined the sequence of the ferredoxin I, one of the two ferredoxins from *Chlorobium limicola* which was purified from the extracts of *Chloropseudomonas ethylicum. C. ethylicum* is now considered to be a mixed culture of *C. limicola* and a nonphotosynthetic bacterium (Gray *et al.,* 1972). We have therefore also prepared ferredoxin from a pure culture of *C. limicola* kindly supplied to us by Dr. J. Olson. The amino acid composition and the amino acid sequence of amino-terminal region and carboxyl-terminus of a ferredoxin from *C. limicola* and of a ferredoxin from *C. ethylicum* whose sequence we are reporting are the same.

Experimental Section

Materials. The bacteria was grown and the ferredoxin extracted as described by Rao *et al.* (1969). The ferredoxin was further purified by DEAE-cellulose column chromatography and gel filtration on Sephadex G-50. The purified protein had an A_{390}/A_{280} ratio of 0.77. Reagent grade chemicals were used and their sources have been described in previous publications (Tanaka *et al.,* 1971). Chymotrypsin was obtained from the Worthington Biochemical Corporation as three times crystallized preparation. Prior to the use, chymotrypsin was treated with L-1-tosylamido-2-lysylethyl chloromethyl ketone (Mares-Guia and Shaw, 1963). Thermolysin was purchased from Calbiochem.

Methods. Non-Heme Iron, Labile Sulfur, and Amino Acid Composition. Iron and inorganic sulfide content was determined by standard methods (Harvey *et al.,* 1955; Fogo and Popowsky, 1949; Lovenberg *et al.,* 1963) and was found to be 8 atoms each per molecule of ferredoxin assuming E_{390} = 30,000 mol^{-1} cm^{-1}. The amino acid composition of the protein and peptides was determined on acid hydrolysates in a Beckman-Spinco Model 120C automatic amino acid analyzer as described by Spackman *et al.* (1958). The instrument was equipped with high sensitivity cuvets and a 4–5 mV full scale range card.

NH_2- and COOH-terminal Residues and Sequence Determinations. The NH_2-terminal sequences of the Cm[1]-ferredoxin were determined by the Beckman-Spinco Model 890 protein/peptide sequencer utilizing the Protein Double Cleavage Pro-

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[1] The abbreviations used are: Cm-, *S*-β-carboxymethylcysteinyl-; Cys(Cm), *S*-β-carboxymethylcysteine; PTH, phenylthiohydantoin; BPAW, 1-butanol-pyridine-acetic acid-water (60:40:12:48, v/v); BPW, 1-butanol-pyridine-water (50:50:50, v/v); and TLCK, L-1-tosyl- amido-2-lysylethyl chloromethyl ketone.

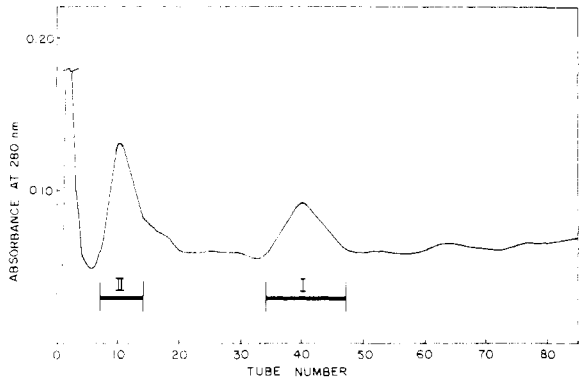

FIGURE 1: Dowex 1-X2 column chromatography of *Chlorobium limicola* Cm-ferredoxin (about 9 mg). See Procedure for experimental details. Fractions under each peak which were pooled are shown by a solid bar.

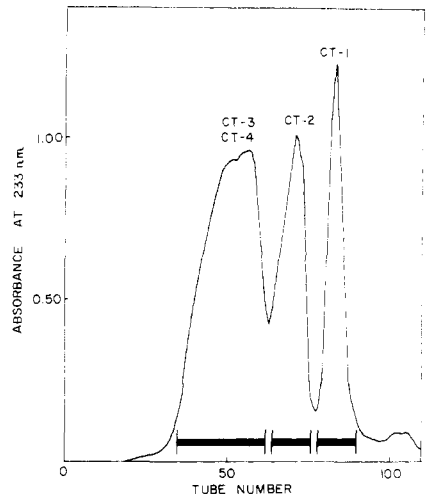

FIGURE 2: Molecular sieving of chymotryptic digest of Cm-ferredoxin (1.75 μmol). See Procedure for details. Fractions under each peak which were pooled are indicated by solid bars.

gram. The NH_2-terminal sequences of all the other peptides were achieved 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 as described by Pisano and Bronzert (1969), or by thin-layer chromatography as described by 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 COOH-terminal amino acids were determined by the use of carboxypeptidase A (Ambler, 1967). Hydrazinolysis was performed on the protein and peptides as described by Bradbury (1958).

Procedures. Preparation and Chromatography of Cm-ferredoxin. The *C. limicola* 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 previous reports (Tanaka *et al.*, 1971). In a typical experiment, the Cm-ferredoxin preparation (about 9 mg) was applied to a Dowex 1-X2 column (1 × 20 cm). Linear gradient elution was performed by addition of 200 ml of 8 M urea–2 M acetic acid in the mixing chamber and 200 ml of 8 M urea–6 M acetic acid in the reservoir. The flow rate was 51 ml/hr and each fraction volume was 5.1 ml. The fractions were detected by measuring absorbance at 280 nm.

Chymotrypsin Digestion, Chromatography of the Digest, and Further Purification of the Peptides. About 3.5 μmol of Cm-ferredoxin (ferredoxin I) was incubated with TLCK-chymotrypsin (enzyme to substrate was 1:30) at pH 8.0 in a total volume of 1.7 ml. Additional TLCK-chymotrypsin was added at 4 hr and the digestion was performed at 28° for 16 hr. Chymotryptic digest of Cm-ferredoxin (1.75 μmol) was applied to a Sephadex G-75 column (1.5 × 115 cm). The elution buffer was 0.1 M ammonia and the flow rate was 43 ml/hr. The fractions of 2.0 ml were collected and were detected by the absorbance of the samples at 233 nm. Peptides were further purified by paper chromatography in the solvent systems, 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v) or 1-butanol–pyridine–water (50:50:50, v/v).

Thermolytic Digestion of Peptide CT-4 and the Subsequent Chromatography of the Products. Peptide CT-4 (0.9 μmol) was hydrolyzed with thermolysin (enzyme to substrate was 1:20) at pH 8.0 in a volume of 0.5 ml. The hydrolysis reaction was carried out at 40° for 20 hr. The thermolysin digest of peptide CT-4 (0.9 μmol) was applied to a Dowex 1-X2 column (0.7 × 20 cm). The digestion mixture was eluted by gradient elution by mixing 100 ml of 0.1 M pyridine in the mixing chamber and 100 ml of 6 M acetic acid in the reservoir. The flow rate was 60 ml/hr. The fractions of 2.75 ml were collected and an aliquot in each fraction was assayed by ninhydrin procedure after alkaline hydrolysis of the samples (Crestfield *et al.*, 1963).

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

Results

Column Chromatography of Cm-ferredoxin Preparation from Chlorobium limicola. Figure 1 shows the chromatogram of the Cm-ferredoxin preparation from *C. limicola* on Dowex 1-X2. Three main peaks were observed. The fractions under each peak were pooled, dialyzed, lyophilized, and chromatographed on a column of Sephadex G-50 gel. The weight ratios of the proteins from peaks 1, 2, and 3 were 16:40:44, respectively. The ferredoxin fraction was about 84% pure on a weight basis. The presence of impurity would raise slightly the 390/280 nm absorbance ratio over the observed value of 0.77. The impurity was not adsorbed to the Dowex 1 column like the ferredoxin fractions.

The amino acid composition of ferredoxin I is presented in this report. However, detailed amino acid analyses have not yet been performed in ferredoxin II. However, the preliminary composition data on pure ferredoxin II showed that ferredoxin II like ferredoxin I contained nine cysteinyl residues. However, it differed from ferredoxin I in that it contained a residue of histidine, arginine, and lysine, none of which are present in ferredoxin I. Ferredoxin I contained 60 amino acid residues while ferredoxin II contained 62 amino acid residues. Since the sequence determination of ferredoxin II will be soon initiated, further comparisons of the two ferredoxins will be postponed at the present time. We have designated the protein present in peak 2, ferredoxin II, and the ferredoxin present in peak 3, ferredoxin I. The remainder of this publication is concerned with the structure determination of *C. limicola* ferredoxin I.

Amino Acid Composition and NH_2- and COOH-terminal Amino Acid Analyses. The amino acid composition of the *C. limicola* ferredoxin (ferredoxin I) was obtained from 24-, 48-, and 72-hr hydrolysates of the Cm-ferredoxin. The results are summarized in Table I along with the preliminary amino acid composition data previously reported by Rao *et al.* (1969). Three steps of the manual Edman degradation of the Cm-ferredoxin showed that the NH_2-terminal sequence was Ala-Leu-Tyr. Hydrazinolysis yielded glycine in 75% yield. Carboxypep-

TABLE I: Amino Acid Composition of *Chlorobium limicola* Ferredoxin.

Amino Acid	From Present Study[a]	From the Sequence	Values of Rao *et al.* (9)
Aspartic acid	5.87 (6)	6[b]	6 ~ 7
Threonine	3.70 (4)	4	4
Serine	0.90 (1)	1	2
Glutamic acid	9.02 (9)	9[c]	9
Proline	3.04 (3)	3	3
Glycine	3.94 (4)	4	4
Alanine	9.01 (9)	9	8
Half-cystine	8.76[d] (9)	9	8
Valine	4.99 (5)	5	5
Isoleucine	5.03 (5)	5	4
Leucine	1.95 (2)	2	2
Tyrosine	3.00 (3)	3	3
Total residues	60	60	58 ~ 59

[a] Acid hydrolyses were performed on Cys(Cm)-ferredoxin for 24, 48, and 72 hr at 100° with 6 N HCl. The amino acid residues were calculated on the basis of a tyrosine content of 3.00 mol/mol of protein. Extrapolations were made for threonine and serine. Values for valine, isoleucine, and leucine were taken from 72-hr hydrolysates. Values in parentheses indicate values rounded off to nearest whole number. [b] Sum of 4 aspartic acid and 2 asparagine. [c] Sum of 7 glutamic acid and 2 glutamine. [d] Determined as Cys(Cm).

tidase A alone and combined carboxypeptidase A–hydrazinolysis experiments on the ferredoxin showed that the COOH-terminal sequence was Cys(Cm)-Ile-Val-Gln-Gly. The results of these NH_2- and COOH-terminal amino acid sequences are identical with the results reported by Rao *et al.* (1969).

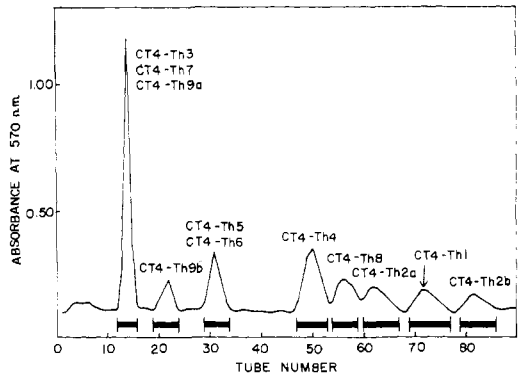

FIGURE 3: Dowex 1-X2 column chromatography of the thermolytic peptides of peptide CT-4. See Procedure for details of experimental conditions. Fractions under each peak which were pooled are indicated by solid bars.

Separation of Chymotryptic Peptides. Figure 2 shows the chromatogram obtained for the separation of the chymotryptic peptides of Cm-ferredoxin on Sephadex G-75. The first peak contained two long peptides, CT-3 and CT-4, together with a small amount of undegraded protein. The combinations of rechromatography with Sephadex G-75 column using 5 M ammonia as solvent and paper chromatography in the two solvent systems, 1-butanol–pyridine–acetic acid–water or 1-butanol–pyridine–water, were used to separate CT-3 and CT-4. The second and third peaks contained peptide CT-2 and CT-1, 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 Peptide CT-4. The thermolytic digest of peptide CT-4 was chromatographed on a column of Dowex 1-X2 as shown in Figure 3. A total of eight peaks were detected by the ninhydrin assay method after alkaline hydrolysis. The first peak con-

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

Amino Acid	CT-1	CT-2	CT-3	CT-4	Total Residues
Cysteine (Cm)		0.86 (1)	2.86 (3)	4.90 (5)	9
Aspartic acid			1.98 (2)	3.88 (4)	6
Threonine		1.89 (2)	0.96 (1)	1.01 (1)	4
Serine			0.86 (1)		1
Glutamic acid		1.96 (2)	2.02 (2)	5.05 (5)	9
Proline			1.99 (2)	1.04 (1)	3
Glycine			1.95 (2)	2.01 (2)	4
Alanine	1.00 (1)		2.97 (3)	4.96 (5)	9
Valine			1.03 (1)	3.80 (4)	5
Isoleucine		1.08 (1)	1.97 (2)	1.89 (2)	5
Leucine	0.99 (1)			0.98 (1)	2
Tyrosine	0.97 (1)	1.00 (1)	0.98 (1)		3
Total residues	3	7	20	30	60
Recovery (%)	90	60	66	43	
R_F[b]	0.76	0.50	0.45	0.19	
Color reaction with ninhydrin	Violet	Violet	Violet	Violet	
Purification method[c]	BPAW	S-G-75 BPAW	S-G-75 BPW	S-G-75 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 with 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v). [c] The abbreviations used are: BPAW, paper chromatography in the solvent system, 1-butanol–pyridine–acetic acid–water (60:40:12:48, v/v); BPW, paper chromatography with 1-butanol–pyridine–water (50:50:50, v/v); and S-G-75, rechromatography with Sephadex G-75 column using 5 M ammonia as solvent.

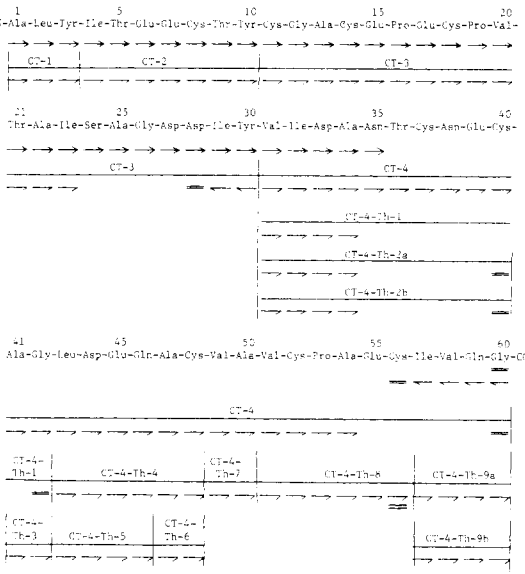

FIGURE 4: Construction of the complete sequence of *C. limicola* ferredoxin and sequence data of peptide fragments. In the figure, the symbols →, ⇀, ↽, and ═ represent sequences determined by use of the Beckman-Spinco protein sequencer, direct manual Edman degradation, carboxypeptidase A, and hydrazinolysis experiments, respectively.

tained three peptides, CT-4-Th-3, CT-4-Th-7, and CT-4-Th-9a. Paper chromatography in 1-butanol–pyridine–acetic acid–water separated CT-4-Th-3 but CT-4-Th-7 and CT-4-Th-9a were not separated. From the third peak, peptides CT-4-Th-5 and CT-4-Th-6 were isolated by paper chromatography. All of the other peptide fractions also were further purified by paper chromatography. 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.

Sequenator Results of Cm-ferredoxin. Ala-Leu-Tyr-Ile-Thr-Glu-Glu-Cys(Cm)-Thr-Tyr-Cys(Cm)-Gly-Ala-Cys-(Cm)-Glu-Pro-Glu-Cys(Cm)-Pro-Val-Thr-Ala-Ile-Ser-Ala-Gly-Asp-Asp-Ile-Tyr-Val-Ile-Asx-Ala-Asx- (Residues 1–35). The sequence analysis of the *C. limicola* Cm-ferredoxin I was determined twice in the Beckman-Spinco protein sequencer with the aid of the Protein Double Cleavage Program. It was possible to determine the first 35 residues from the amino-terminal end of the protein. The protein sequenator results of the Cm-ferredoxin are summarized in Table IV.

Peptide CT-4. Val-Ile-Asp-Ala-Asn-Thr-Cys(Cm)-Asn-Glu-Cys(Cm)-Ala-Gly-Leu-Asp-Glu-Gln-Ala-Cys(Cm)-Val-Ala-Val-Cys(Cm)-Pro-Ala[Glx,Cys(Cm),Ile,Val,Glx]Gly (Residues 31–60). Twenty-four steps of the manual Edman degradation were carried out on the peptide. The results are summarized in Table V. Hydrazinolysis of this peptide yielded glycine in 80% yield.

Peptide CT-4-Th-8. Val-Cys(Cm)-Pro-Ala-Glu-Cys(Cm) (Residues 51–56). Five steps of the manual Edman degradation and hydrazinolysis established the sequence of this peptide. The results are summarized in Table VI.

Peptide CT-4-Th-7 + CT-4-Th-9a. Val-Ala + Ile-Val-Gln-Gly (Residues 49 and 50 and 57–60). These two peptides could not be separated by ion exchange column chromatography and paper chromatography. Three steps of the manual Edman degradation on the peptide mixture yielded the following results. First step: PTH-isoleucine in 100% yield and PTH-valine in 95% yield. After the first step of degradation, free alanine (70% yield) was detected in the remainder by the direct amino acid analyses without acid hydrolysis. Second step: PTH-valine (85% yield) and PTH-alanine (50% yield). Third step: Only PTH-glutamine (74%) was detected. PTH-glutamine was also identified by thin-layer chromatography of the PTH-amino acid and glutamic acid was detected by amino acid analysis after 6 N HCl hydrolysis of the PTH derivative. After the third step of the Edman degradation, direct amino acid analysis without acid hydrolysis yielded glycine in 64% yield.

In order to verify the sequence from residues 57–60, manual Edman degradation was also performed on the peptide CT-4-Th-9b. This peptide was acidic and contained glutamic acid, glycine, valine and isoleucine. Three steps of the Edman degradation yielded the following results: (1) PTH-isoleucine in 100% yield; (2) PTH-valine in 96% yield; (3) PTH-glutamic acid in 81% yield. After the third step of the Edman degradation, direct amino acid analysis without acid hydrolysis yielded glycine in 67% yield. From the above results, it was concluded that peptide CT-4-Th-9b was a deaminated product of peptide CT-4-Th-9a, in which a glutamine had been converted to a glutamic acid.

Comments on Peptide CT-4-Th-2a and CT-4-Th-2b. Val-Ile-Asp-Ala[Asx,Thr,Cys(Cm),Asx,Glx]Cys(Cm) (Residues 31–40). The amino acid compositions of both of these peptides were identical but peptide CT-4-Th-2b was more negatively charged than CT-4-Th-2a as evidenced by the elution behavior on Dowex 1. Four steps of the manual Edman degradation on both of the peptides showed the same results, namely the sequence of Val-ile-Asp-Ala. Also, hydrazinolysis of each peptide yielded Cys(Cm) in good yields. Thus, it was assumed that peptide CT-4-Th-2b was a deaminated derivative of peptide CT-4-Th-2a in which the asparagine residue(s) had been deaminated during the purification of the peptides.

Complete Sequence. NH_2-terminal analyses of the *C. limicola* ferredoxin I by the manual and automatic Edman degradation showed that alanine was the NH_2-terminal residue. The sequenator analysis of the protein showed that peptide CT-1 was the NH_2-terminal peptide; that peptide CT-2 is next in order; and is followed by peptide CT-3. Finally peptide CT-4 is COOH-terminal peptide based on the COOH-terminal analysis of the protein. The sequences of the individual fragments of the chymotryptic and thermolytic peptides necessary to establish the sequence have been covered in the previous section and are summarized in Figure 4.

Discussion

Among the photosynthetic bacteria, the only organism from which ferredoxin has been sequenced to date is *Chromatium*, a purple sulfur-reducing bacteria (Matsubara *et al.*, 1970). Recently, Shanmugan *et al.* (1972) have demonstrated that *Rhodospirillum rubrum*, a purple non-sulfur reducing bacteria, contain two types of ferredoxins, one which contains 65–67 amino acid residues and the other with 73–76 amino acid residues. The larger of these ferredoxins was synthesized under light conditions while the smaller ferredoxin was biosynthesized either under dark or light conditions. No report has appeared on the sequence of the *R. rubrum* ferredoxins. From this report and from other publications, it is obvious that the size of the ferredoxins from the different photosynthetic bacteria differ. Thus, an investigation of the sequences of the photosynthetic bacterial ferredoxins should provide some interesting evolutionary data about the evolution of the ferredoxin containing microbes. Therefore, in the present investigation, the sequence of the ferredoxin from *C. limicola*, a photosynthetic green bacterium, was investigated. The isolation procedure

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

Amino Acid	CT-4-Th-1	CT-4-Th-2a	CT-4-Th-2b[d]	CT-4-Th-3	CT-4-Th-4	CT-4-Th-5	CT-4-Th-6	CT-4-Th-7 CT-4-Th-9a[g]	CT-4-Th-8	CT-4-Th-9b[f]	Total Residues[e]
Cysteine (Cm)	1.95 (2)	1.93 (2)	1.94 (2)		0.97 (1)		0.92 (1)		1.97 (2)		5
Aspartic acid	2.98 (3)	3.01 (3)	2.89 (3)		1.00 (1)	1.00 (1)					4
Threonine	0.95 (1)	0.90 (1)	0.92 (1)								1
Glutamic acid	1.05 (1)	0.95 (1)	0.96 (1)		1.99 (2)	1.95 (2)		1.00 (1)	0.98 (1)	1.00 (1)	5
Proline									0.94 (1)		1
Glycine	0.98 (1)			1.00 (1)				1.00 (1)		0.96 (1)	2
Alanine	2.00 (2)	1.00 (1)	1.00 (1)	0.95 (1)	1.00 (1)		1.00 (1)	1.10 (1)	1.00 (1)		5
Valine	0.93 (1)	0.96 (1)	0.95 (1)					2.05 (2)	0.95 (1)	1.01 (1)	4
Isoleucine	0.91 (1)	0.94 (1)	0.91 (1)					0.96 (1)		0.99 (1)	2
Leucine					0.98 (1)	0.96 (1)					1
Total residues	12	10	10	2	6	4	2	6	6	4	30
Recovery (%)	16	16	15	33	58	18	18	58	71	16	
R_F[b]	0.16	0.19	0.16	0.34	0.23	0.31	0.25	0.55	0.21	0.57	
Color reaction with ninhydrin	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	Violet	
Purification method[c]	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	BPAW	

[a–c] See footnotes *a–c* in Table II, respectively. [d] Peptide CT-4-Th-2b has same amino acid composition as peptide CT-4-Th-2a, but shows more negative charge(s) because of deamination of asparagine(s) during enzyme digestion followed by treatments of the digestion products. [e] Sum of the peptides, CT-4-Th-1, CT-4-Th-4, CT-4-Th-7, CT-4-Th-8, and CT-4-Th-9a. [f] Peptide CT-4-Th-9b is deaminated product of a glutamine in peptide CT-4-Th-9a. [g] Could not be separated by paper chromatography with BPAW, but manual Edman degradation results showed to be mixture of two peptides, Val-Ala (Peptide CT-4-Th-7) and Ile-Val-Gln-Gly (Peptide CT-4-Th-9a).

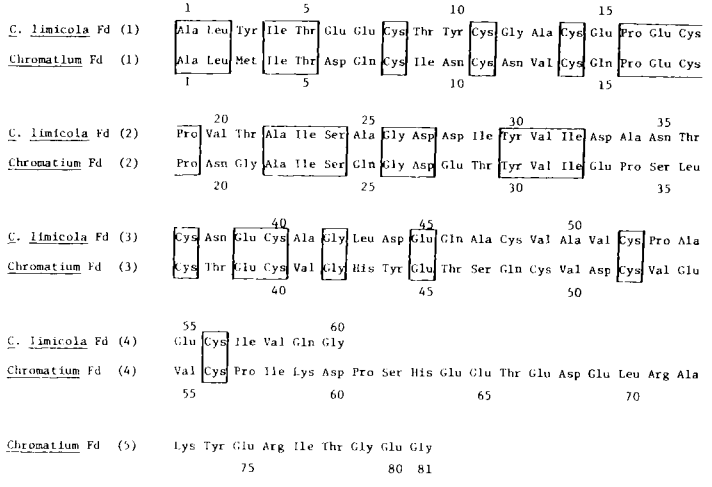

FIGURE 5: Comparison of the amino acid sequences of *C. limicola* ferredoxin and *Chromatium* ferredoxin. Amino acid sequence homology between *C. limicola* ferredoxin and *Chromatium* ferredoxin was calculated to be 43.3%.

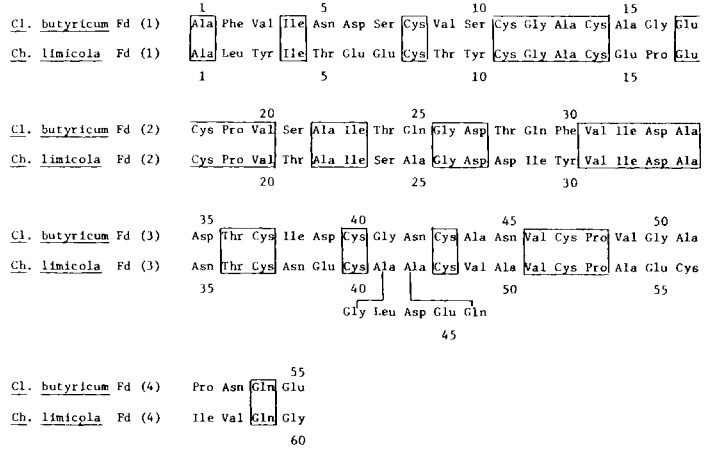

FIGURE 6: Comparison of the amino acid sequences of *Clostridium butyricum* ferredoxin and *C. limicola* ferredoxin. For maximum homology it was assumed that an insertion of five residues occurred after residue 41 in the *C. limicola* ferredoxin. A deletion of the five residues in the *C. butyricum* is possible but considered less likely.

TABLE IV: Automatic Sequenator Results of Cm-ferredoxin.

Step No.	Amino Acid	Yield (%)	Identification Method[a]		
1	Alanine	95	GC	TLC	HYD
2	Leucine	92	GC	TLC	HYD
3	Tyrosine	72	GC	TLC	HYD
4	Isoleucine	76	GC	TLC	HYD
5	Threonine	60	GC	TLC	
6	Glutamic acid	72	GC	TLC	HYD
7	Glutamic acid	54	GC	TLC	HYD
8	Cysteine (Cm)	46	GC	TLC	
9	Threonine	40	GC	TLC	
10	Tyrosine	38	GC	TLC	HYD
11	Cysteine (Cm)	40	GC	TLC	
12	Glycine	44	GC	TLC	HYD
13	Alanine	32	GC	TLC	HYD
14	Cysteine (Cm)	29	GC	TLC	
15	Glutamic acid	28	GC	TLC	HYD
16	Proline	22	GC	TLC	HYD
17	Glutamic acid	25	GC	TLC	HYD
18	Cysteine (Cm)	22	GC	TLC	
19	Proline	20	GC	TLC	HYD
20	Valine	20	GC	TLC	HYD
21	Threonine	17	GC	TLC	
22	Alanine	18	GC	TLC	HYD
23	Isoleucine	15	GC	TLC	HYD
24	Serine	10	GC	TLC	
25	Alanine	13	GC	TLC	HYD
26	Glycine	12	GC	TLC	HYD
27	Aspartic acid	10	GC	TLC	HYD
28	Aspartic acid	11	GC	TLC	HYD
29	Isoleucine	8	GC		HYD
30	Tyrosine	5	GC		HYD
31	Valine	7	GC		HYD
32	Isoleucine	7	GC		HYD
33	Asx	6	GC		HYD
34	Alanine	5	GC		HYD
35	Asx	5	GC		HYD

[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.

TABLE V: Manual Edman Degradation Results of Peptide CT-4.

Step No.	Amino Acid	Yield (%)	Identification Method[a]		
1	Valine	96	GC	TLC	HYD
2	Isoleucine	86	GC	TLC	HYD
3	Aspartic acid	67	GC	TLC	HYD
4	Alanine	60	GC	TLC	HYD
5	Asparagine	53	GC	TLC	HYD
6	Threonine	43	GC	TLC	
7	Cysteine (Cm)	36	GC	TLC	
8	Asparagine	35	GC	TLC	HYD
9	Glutamic acid	32	GC	TLC	HYD
10	Cysteine (Cm)	26	GC	TLC	
11	Alanine	28	GC	TLC	HYD
12	Glycine	20	GC	TLC	HYD
13	Leucine	22	GC	TLC	HYD
14	Aspartic acid	19	GC	TLC	HYD
15	Glutamic acid	19	GC	TLC	HYD
16	Glutamine	14	GC	TLC	HYD
17	Alanine	16	GC	TLC	HYD
18	Cysteine (Cm)	10	GC	TLC	
19	Valine	12	GC	TLC	HYD
20	Alanine	11	GC	TLC	HYD
21	Valine	10	GC		HYD
22	Cysteine (Cm)	6	GC		
23	Proline	5	GC		HYD
24	Alanine	5	GC		HYD

[a] See footnote *a* in Table IV.

and the amino acid composition of the ferredoxin from this bacteria have already appeared (Rao *et al.*, 1969). In their investigations the ferredoxin was isolated from what was believed to be *C. ethylicum*. However, it was found that the culture was not pure and in reality contained *C. limicola* and a nonphotosynthetic bacterium (Gray *et al.*, 1972). In the present investigation, a pure culture of *C. limicola* was used for growing the cells. It was interesting to note that two similar ferredoxins were present which were designated ferredoxins I and II. The present report is concerned only with the amino acid sequence of ferredoxin I.

The amino acid composition of ferredoxin I showed first of all that it contained 60 amino acid residues. Like the *Chromatium* ferredoxin, it contained nine cysteinyl residues per mole of protein. However the *C. limicola* ferredoxin is lacking in methionyl, phenylalanyl, tryptophyl, lysyl, histidyl, and arginyl residues. Like the other iron–sulfur proteins, *C. limicola* ferredoxin has an acidic isoelectric point since it contains four aspartyl and seven glutamyl residues. On the basis of the amino acid content and the sequence data, the molecular weight of ferredoxin I is 6227 for the apoprotein and 6923 for the native protein which contains 8 mol of sulfide and 8 g-atoms of iron/mol of protein.

The sequence investigations established that the *C. limicola* ferredoxin I is a protein which contains 60 amino acid residues arranged to form a single polypeptide chain. In general, the sequence determination did not offer any obstacles. However, a partial deamidation of the protein appears to have occurred but sufficient amounts of the amidated protein remained so that the assignment of the acidic residues as either the amide or the free acid was possible.

TABLE VI: Sequence Determination of Peptide CT-4-Th-8.

Sequence: Val-Cys(Cm)-Pro-Ala-Glu-Cys(Cm)

Hydrazinolysis: Cys(Cm) in 77% yield

Step No. in Edman Degradation	Amino Acid	Yield (%)	Identification Method[a]		
1	Valine	90	GC	TLC	HYD
2	Cysteine (Cm)	80	GC	TLC	
3	Proline	72	GC	TLC	HYD
4	Alanine	70	GC	TLC	HYD
5	Glutamic acid	63	GC	TLC	HYD
5D[b]	Cysteine (Cm)	60			

[a] See footnote *a* in Table IV. [b] Determined directly without acid hydrolysis.

TABLE VII: Properties of Various Ferredoxins.

Origin	Example	Amino Acid Residues	Molecular Weight
Green plant	Spinach	97	11,500
Photosynthetic purple bacteria	Chromatium	81	10,000
Photosynthetic green bacteria	Chlorobium	60	7,000
Nonphotosynthetic anaerobic bacteria	Clostridium	55	6,000

The present investigation has brought up some interesting points concerning the proposed amino acid sequence of the *Chromatium* ferredoxin. Residues 50–57 in this ferredoxin were reported to be Val-Asp-Cys-Val-Glu-Val-Cys-Pro by Matsubara *et al.* (1970). However, in their investigations, they also isolated the peptide Val-Glu-Val-Cys-Pro-Cys-Asn (peptide Th-X) which they were not able to place in the ferredoxin molecule and was ignored in the sequence determination. In addition, peptides Th-12 and Th-15, isolated by Matsubara *et al.* (1970), were obviously impure. If peptide Th-X was in reality the peptide with the sequence Val-Glu-Val-Cys-Pro-Val-Asp-Cys, while peptide Th-12 had the amino acid composition His_1, Thr_2, Ser_1, Glu_4, Gly_1, Val_2, Leu_1, Tyr_1, and $Cm(Cys)_3$ and peptide Th-15 had the composition Lys_1, His_1, Asp_3, Thr_1, Ser_1, Glu_4, Pro_2, Val_2, Ile_1, and $Cm(Cys)_2$, then the reported sequence of the *Chromatium* is wrong. If Val-Glu-Val-Cys-Pro-Val-Asp-Cys is considered to occupy residues 50–57, a better homology (52.5%) is observed between the ferredoxin sequences from *Chromatium* and *C. limicola*, both of which are from photosynthetic bacteria as shown in Figure 5. Thus, additional sequence studies on the *Chromatium* ferredoxin are desired in order to confirm the proposed amino acid sequence.

The homology of the amino acid sequences of the clostridial type (anaerobic bacterial type) and the photosynthetic bacterial ferredoxins will be considered. To simplify matters, we have selected the *Clostridium butyricum* ferredoxin since like the *C. limicola* ferredoxin, it does not contain any basic amino acid residues. The comparison is shown in Figure 6 in which the two ferredoxins are aligned with the NH_2-terminal residues in register. It is quite likely that the two ferredoxins have arisen from the same precursor although they are unequal in the number of total amino acid residues present in the proteins. In order to obtain the best homology, we have assumed that there was an insertion of five residues (Gly-Leu-Asp-Glu-Gln) after residue 41 in the *C. limicola* ferredoxin. It is equally possible that the five residues were deleted from the *C. butyricum* ferredoxin. It is interesting to note that this type of protein alteration was observed in the *Chromatium* ferredoxin but in this case, the loop (insertion) amounted to nine amino acid residues after residue 41 (Matsubara *et al.*, 1970). This type of protein alteration can best be rationalized in terms of the transposition of a short segment of DNA by recombination during evolution. Since this genetic change has now been observed in the ferredoxins from the purple and green photosynthetic bacteria, it would be interesting to see if this change is observed in other types of photosynthetic bacteria. Furthermore, all of the ferredoxins regardless of their source have arisen from a common ancestor. It is interesting to note that there is a gradual lengthening of the ferredoxin genome as one proceeds from the anaerobic bacteria to the photosynthetic green bacteria, to the photosynthetic purple bacteria and finally to the algae-plant species (Table VII). The best sequence homology is obtained if the NH_2-terminal regions of these various ferredoxins are aligned. Thus, the major changes in the sequences of the various ferredoxins have occurred in the C-terminal region. Finally, the sequence and the absorption spectrum of the *Chlorobium* ferredoxin I are more similar to the clostridial type of ferredoxin than the *Chromatium* ferredoxin although both are from photosynthetic bacteria.

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