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STRUCTURE AND EVOLUTION OF
FERREDOXINS

A Doctoral Thesis

by

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I. GENERAL CONSIDERATION

Ferredoxin, a non-heme iron-containing protein is the name given by Mortenson, Valentine, and Carnahan (1). They first isolated this protein from a non-photosynthetic anaerobic bacterium, Clostridium pasteurianum in 1962 (1). This protein functioned as an electron carrier in the phosphoroclastic reaction. Tagawa and Arnon (2) in 1962 crystallized C. pasteurianum ferredoxin and demonstrated that this ferredoxin could be replaced by a chloroplast protein, known as methohemoglobin-reducing factor (3), TPN-reducing factor (4), or photosynthetic pyridine nucleotide reductase (5) in a system of photoreduction of NADP^+ by spinach chloroplast. They renamed the chloroplast protein chloroplast ferredoxin and proposed that the term ferredoxin extended to include other non-heme iron proteins with low oxidation-reduction potentials. Since then, ferredoxins have subsequently been found in many organisms, anaerobic bacteria, aerobic bacteria, photosynthetic organisms, and animals. With the increase of information about biological activity (6), chemical structure (7), biosynthesis (8), immunochemical property (9), and evolutionary implication (10) of ferredoxins, our understanding of biological role and molecular properties of ferredoxins has grown rapidly.

Physical and chemical studies have established that

the active centers of various ferredoxins consist of iron and sulfur clusters which capable of exchanging electron during the biological reaction. Depending on the number of iron and sulfur per molecule ferredoxins are classified as 2Fe-2S, 4Fe-4S, and 8Fe-8S ferredoxins.

Chloroplast-type ferredoxins are found in all oxygenic photosynthetic organisms from blue-green algae to higher plants. They contain 2Fe-2S atoms and have a molecular weight of about 12,000. Bacterial ferredoxins are a very diverse group. They contain 2Fe-2S, 4Fe-4S, and 8Fe-8S atoms and have a molecular weight of ranging from 6,000 to 14,000. Study of the primary structure of these ferredoxins is one of most active field of investigation of ferredoxins. The first amino acid sequence of ferredoxin from C. pasteurianum was reported by Tanaka et al. (11) in 1964. It appeared that the clostridial ferredoxin was composed of two homologous halves, each containing 28 and 27 amino acid residues. The first sequence of plant ferredoxin was determined by Matsubara et al. in 1967 (12), and it was found that the structure was different from those of clostridial ferredoxins, but some similarities were found between those proteins of different origins (13), and it was suggested that these two different ferredoxins were derived from a common ancestor which appeared in an early evolutionary era. Since then, many sequences have been established for other ferredoxins from anaerobic bacteria, photosynthetic bacteria, aerobic bacteria,

plants, and algae.

Ferredoxins with interesting and unique properties have recently been isolated from various organisms and many efforts in structural studies are required in obtaining a better understanding of their structure and function relationship as well as their evolutionary relationship. For example, an extreme halophilic bacterium, Halobacterium halobium has 2Fe-2S ferredoxin which is considered to be similar to chloroplast-type ferredoxin from optical measurements (14). A group of ferredoxins represented by Azotobacter vinelandii ferredoxin (15, 16) completely different from clostridial and photosynthetic bacterial ferredoxins are found in a wide range of bacteria. In order to clarify the relationship among these ferredoxin, I felt that it is necessary to purify ferredoxin from various organisms and to determine their complete amino acid sequences to relate them to the three dimensional structures of representative ferredoxins some of which are already available in our hand as shown later..

II. PURIFICATION OF FERREDOXINS

1. Introduction

Ferredoxins are a group of low molecular weight, strongly acidic proteins containing both non-heme iron and inorganic sulfur. They are now known to be present in a wide variety of organisms, non-photosynthetic anaerobic bacteria, aerobic bacteria, photosynthetic bacteria, algae including blue-green algae, mosses, ferns, higher plants and animals. Ferredoxin was first isolated from Clostridium pasteurianum by Mortenson et al. (1) in 1962. Since then, different procedures for the purification of ferredoxin have been described (17, 18). Most of the procedures are based on several unique properties of ferredoxin; it is relatively stable in acetone-water mixture (19), strongly adsorbed on DEAE-cellulose due to its acidic nature, adsorbed on DEAE-cellulose or Sepharose equilibrated with 60 - 70 % ammonium sulfate (20, 21), and soluble in highly concentrated solution of ammonium sulfate. In this chapter, I describe the procedures for isolation of ferredoxins from three organisms; a blue-green alga: Aphanothece sacrum, a horsetail: Equisetum arvense, and an extreme halophilic bacterium: Halobacterium halobium. The procedures of the purification of ferredoxins include the following steps, (1) preparation of a cell extract

- (2) adsorption of crude ferredoxin on a DEAE-cellulose
- (3) fractionation by ammonium sulfate
- (4) chromatography on a DEAE-cellulose column
- (5) gel filtration on a Sephadex G-75 column

2. Materials and Methods

Tris-HCl buffer, pH 7.5 containing NaCl: Two stock solutions, 1 M Tris-HCl buffer, pH 7.5, and 5 M NaCl, were mixed and diluted to make an appropriate concentration necessary. All buffers used for preparation of ferredoxins were made up in this manner unless otherwise specified.

Concentration of Ferredoxin: A dilute ferredoxin solution was concentrated on a DEAE-cellulose column as follows. The ferredoxin solution after chromatography was saturated at 70 % with solid ammonium sulfate and passed through a small DEAE-cellulose column equilibrated with 70 % ammonium sulfate without dialysis or dilution according to the method of Mayhew (20). The 70 % saturated ammonium sulfate solution was prepared by adding 472 g of solid ammonium sulfate to one liter of 0.01 M Tris-HCl buffer, pH 7.5. The ferredoxin was eluted with 0.1 M buffer containing 0.7 M NaCl to a small volume.

Aphanothece sacrum Ferredoxins: The Aphanothece sacrum cells were collected from a river bed on Amagi, Fukuoka and purchased from Endo Kanagawa-Do.

Step 1. About 18 kg of frozen algal cells were thawed and allowed to stand overnight in a cold room. The pH of the supernatant of the cell suspension was kept 7 - 8 with adding Tris powder.

Step 2. The supernatant was filtrated through a fine mesh of cloth and DEAE-cellulose (about one liter of thick suspension) equilibrated with 0.05 M buffer was added to it. The suspension with DEAE-cellulose, whose color changed to violet due to the adsorption of phycobiliproteins together with ferredoxin, was collected and packed into a column. The dark fluorescent, violet solution was eluted with 0.1 M buffer containing 0.7 M NaCl.

Step 3. Solid ammonium sulfate was added to the eluate to give a final concentration of 70 % saturation. The solution was centrifuged for 20 min at 9,000 rpm and the supernatant was directly applied to a DEAE-cellulose column equilibrated with 70 % ammonium sulfate. Crude ferredoxin was eluted with 0.1 M buffer containing 0.7 M NaCl and the eluate was dialyzed against 0.1 M buffer.

Step 4. The dialysate was applied to a DEAE-cellulose column (3.6 x 35 cm) equilibrated with 0.1 M buffer. Ferredoxin was eluted with 0.05 M buffer containing 0.35 M NaCl. The ferredoxin was separated into two bands on the column and the fractions containing ferredoxin in majority corresponding to each band were pooled and concentrated.

Step 5. Each of the two ferredoxins was separately chromatographed on a Sephadex G-75 column (4 x 75 cm)

equilibrated with 0.05 M buffer containing 0.35 M NaCl and eluted with the same buffer. The two ferredoxins were highly purified by these procedures.

Equisetum arvense Ferredoxins: Horsetail leaves

(about 40 kg) were collected on our campus in spring and stored in a deep freezer for several days before preparation.

Step 1. One Kg portions of frozen leaves were blended with 2 liters of water and 2 g of Tris powder. The pH of the homogenate was kept between 7 and 8.

Step 2. The blend was filtered through a fine mesh of cloth and DEAE-cellulose equilibrated with 0.05 M buffer was added to the green juice. The mixture was allowed to stand for a few hours with occasionally stirring, the supernatant was decanted off, and the DEAE-cellulose was washed with water three times and packed into column. The dark green solution eluted with 0.07 M buffer containing 0.44 M NaCl was diluted with 5 volumes of water and readsorbed on a DEAE-cellulose column. Ferredoxin was eluted with 0.05 M buffer containing 0.35 M NaCl. This procedure was repeated twice.

Step 3. Ferredoxin solution was fractionated with 70 % ammonium sulfate and the supernatant after centrifugation for 20 min at 9,000 rpm was concentrated on a DEAE-cellulose column equilibrated with 70 % ammonium sulfate. The crude ferredoxin obtained from 40 Kg of leaves was combined and dialyzed against 0.1 M buffer.

Step 4. The crude ferredoxin was further purified by

chromatography on a DEAE-cellulose column (2.5 x 35 cm) equilibrated with 0.1 M buffer. Elution was performed with 0.05 M buffer containing 0.3 M NaCl. Two ferredoxins separated on this column were concentrated.

Step 5. The two ferredoxins were separately chromatographed on a Sephadex G-75 column as described in A. sacrum ferredoxin. At final step, each was rechromatographed on the same column.

Halobacterium halobium Ferredoxin: H. halobium was grown at 37° in a medium composed of 10 g peptone, 250 g NaCl, 20 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, 2 g KCl, and 3 g trisodium citrate per one liter adjusted to pH 7.0 with 3 N NaOH (22). After 1 week of shaking, the bacterial cells were harvested by centrifugation.

Step 1. The packed bacteria (150 g) were suspended in 1.5 liters of 0.01 M buffer containing 5 mM MgCl_2 and stirred with adding 2 mg of DNase until a homogeneous suspension was obtained.

Step 2. Solid ammonium sulfate was added to the suspension to give a final concentration of 50 % saturation. The solution was allowed to stand overnight with stirring. After removal of the precipitate by centrifugation for 20 min at 9,000 rpm, solid ammonium sulfate was further added to a concentration of 70 %. After stirring for 30 min the supernatant was collected by centrifugation as described above.

Step 3. The supernatant was applied to a DEAE-cellulose

column equilibrated with 70 % ammonium sulfate. After the column was washed the same solution, ferredoxin was eluted with 0.1 M buffer containing 0.7 M NaCl. This procedure was repeated twice and the crude ferredoxin was dialyzed against 0.1 M buffer.

Step 4. The dialysate was applied to a DEAE-cellulose column (2.5 x 35 cm) equilibrated with 0.1 M buffer and ferredoxin was eluted a linear gradient system in 0.1 M buffer increasing the content of NaCl from 0.1 to 0.4 M (one liter in each reservoir). The ferredoxin solution obtained was concentrated as described above.

Step 5. The ferredoxin was further purified by gel-filtration on a Sephadex G-75 as described in A. sacrum ferredoxin.

Activity of Ferredoxins: The activity of ferredoxins was determined by the photoreduction of NADP^+ by spinach, Rumex sp., or Phytolacca americana chloroplasts according to the method of Buchanan and Arnon (17). Spinach leaves were from a local market and other leaves were collected in our campus. Broken chloroplasts were prepared essentially by the procedure described by Whatley and Arnon (23). The reaction mixture (3 ml) contained broken chloroplasts (about 50 μg chlorophyll), 300 μmoles of Tris-HCl buffer, pH 7.5, 1 μmole of NADP^+ , 0.2 μmole of 2,6-dichlorophenolindophenol, 20 μmoles of sodium ascorbate, and 100 -200 μg of ferredoxin. The reaction was initiated in 3 ml cuvettes with 1 cm light pass by illumination with

a projector lamp. The exogeneous ferredoxin was omitted in the blank cuvette. Reduced NADP⁺ was determined by the increase in absorbance at 340 nm.

Amino Acid Compositions and Terminal Sequences:

These analytical procedures were all the same as described in the next chapter.

3. Results and Discussion

Two Ferredoxins from *A. sacrum*: Wada et al. (24)

first isolated a ferredoxin from *A. sacrum* and described some chemical and structural properties of the ferredoxin. During the course of our study of ferredoxin from the same organism, two components of ferredoxin were detected. They were separated on a DEAE-cellulose column developed with 0.05 M Tris-HCl buffer, pH 7.5 containing 0.35 M NaCl. The elution pattern of these ferredoxins from the column is shown in Fig. 1. The minor component, which was eluted faster was designated as ferredoxin II and the major one as ferredoxin I. After successive chromatographies of these components on a Sephadex G-75 column, highly purified ferredoxins were obtained. Ferredoxin I showed the same chemical and physical properties as those previously reported (24). Ferredoxin II showed several properties different from those of ferredoxin I. The absorbance ratio, $R = A_{422}/A_{275}$ of ferredoxin II was 0.54, but that of fer-

redoxin I was 0.525. The absorption spectra of these two

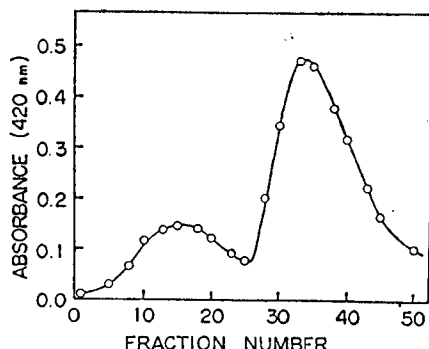


Fig. 1. Elution pattern of two ferredoxin components of A. sacrum cells from a DEAE-cellulose column. The column (3.6 x 35 cm) was developed with 0.05 M Tris-HCl buffer, pH 7.5 containing 0.35 M NaCl. Collection of 10 ml fractions was started just before the elution of ferredoxin II.

ferredoxins are shown in Fig. 2. It is evident that the ferredoxin II has a shoulder at around 290 nm and a minimum at around 302nm, which suggests the presence of tryptophan, as will be shown later. The molar extinction coefficient of ferredoxin II at 422 nm was 10.9×10^3 based on the amino acid composition, which gave a molecular weight of 10,649. The coefficient of ferredoxin I was 10.4×10^3 (24).

Ferredoxin II was always obtainable from cells collected at different seasons, February, April, and October in this case, and always corresponded to about 20 % of the total ferredoxin extracted, as shown in Table 1.

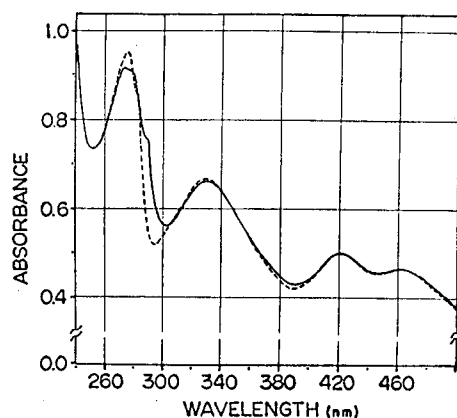


Fig. 2. The absorption spectra of two components.

The Aphanothece ferredoxins were dissolved in 0.1 M Tris-HCl buffer, pH 7.5: Ferredoxin I, - - - ; Ferredoxin II ——— .

Table 1. Yields of two ferredoxin components isolated from Aphanothece cells collected in different seasons. The preparation of ferredoxins was carried out on 18 Kg cells collected in February, April, and October. The yield is expressed as mg based on the molar extinction coefficient of each ferredoxin, as described in the text.

Ferredoxin (mg)				
	February cells	April cells		October cells
Ferredoxin I	14	10	8	8
Ferredoxin II	95	56	55	41

Table 2 shows the amino acid composition of ferredoxin II together with that of ferredoxin I (24). It is clear from the table that the two compositions are quite different

and it is particularly interesting to note that ferredoxin II has one tryptophan and two methionine residues. Other remarkable differences include the contents of lysine, proline, and tyrosine residues. The low content of lysine

Table 2. Amino acid composition of Aphanothece ferredoxins I and II.

Amino acid	Average values obtained after hydrolysis for 20 and 72 hr	Nearest integer	Composition of the fer- redoxin I (24)
Lysine	1.95	2	4
Histidine	0.96	1	1
Arginine	0.98	1	1
Aspartic acid	13.2	13	14
Threonine	8.06 ^a	8	7
Serine	5.17 ^a	5	6
Glutamic acid	14.3	14	12
Proline	3.32	3	6
Glycine	8.49	8	7
Alanine	13.0	13	9
Cysteine	4.08 ^b	4 - 5	4
Valine	5.97	6	6
Methionine	2.11	2	0
Isoleucine	5.40	5	5
Leucine	9.03	9	8
Tyrosine	1.89	2	6
Phenylalanine	0.96	1	1
Tryptophan	0.88 ^c	1	0
Total residues		98 - 99	97

^a Values extrapolated to zero time of hydrolysis.

^b Cysteine was determined with a performic acid-oxidized sample. ^c Based on spectrophotometric analysis and acid hydrolysis with 4 % thioglycolic acid.

and the high content of acidic amino acid residues in ferredoxin II should mean that it will have a more acidic nature than ferredoxin I, but ferredoxin II showed actually a less acidic nature than ferredoxin I on a DEAE-cellulose column, as shown Fig. 1. This suggests a high contents of amide derivatives of the acidic residues in ferredoxin II.

The N-terminal sequence of ferredoxin II was determined by the Edman degradation procedure on a trichloroacetic acid-treated sample to be Ala-Thr-Tyr-. That of ferredoxin I is Ala-Ser-Tyr- (24). Determination of the C-terminal sequence of ferredoxin II was performed by carboxypeptidase A digestion, which gave the sequence -Glu-Val-Leu, as shown in Table 3. This sequence is completely different from that of ferredoxin I, -Glu-Ala-Leu-Tyr (24).

Table 3. The digestion profile of carboxyl-terminal residues of the ferredoxin II with carboxypeptidase A. About 1 mg of trichloroacetic acid-treated ferredoxin II was digested with 0.04 mg of enzyme in 0.1 M Tris-HCl buffer, pH 8.0. The amount of amino acid released was calculated as a molar ratio to the protein used.

Amino acid released	Molar ratio of the amino acid released to protein digested		
	Digestion time		
	1 h	3 h	16 h
Leucine	1.00	1.00	1.00
Valine	0.50	0.86	0.91
Glutamic acid	trace	0.24	0.61

The biological activity of ferredoxin II was compared with that of spinach ferredoxin and ferredoxin I in the catalytic system of NADP^+ -photoreduction by spinach and Rumex chloroplasts. Figure 3 compares these three ferredoxin activities in a system of spinach chloroplast. Ferredoxin II had a lower activity than the others. Similar results were also observed with a system using Rumex chloroplasts.

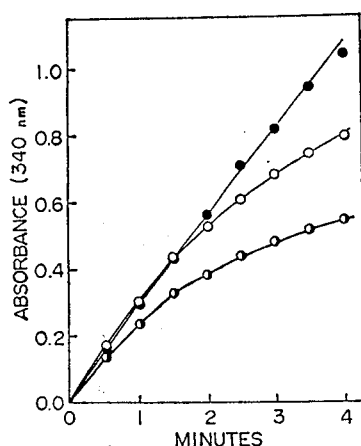


Fig. 3. Comparison of ferredoxin activities. The complete reaction mixture (3 ml) contained spinach chloroplasts with about 50 μg chlorophyll, 300 μmoles of Tris-HCl buffer, pH 7.5, 1 μmole of NADP^+ , 0.2 μmole of 2,6-dichlorophenol-indophenol, 20 μmole of sodium ascorbate, and 200 μg of ferredoxin. spinach ferredoxin, ● ; Aphanothece ferredoxin I, ○ ; Aphanothece ferredoxin II, ◐ .

Two Ferredoxins from E. arvense: The purification of ferredoxin from E. arvense was rather difficult because of its low content and interference from polyphenolic compounds

(25). In this study, adsorption of ferredoxin on successive beds of DEAE-cellulose and elution with various concentrations of Tris-HCl buffer containing NaCl gave a good result to remove a dark brown material from ferredoxin solution as described by Keresztes-Nagy and Margoliash (26). Two ferredoxins were separated by chromatography on a DEAE-cellulose column. The faster moving ferredoxin was designated as ferredoxin II and the slower one ferredoxin I. The yields of ferredoxin I and II were approximately 44 mg and 26 mg per 40 Kg of leaves, respectively. The absorption spectra of these two ferredoxins are shown in Fig. 4.

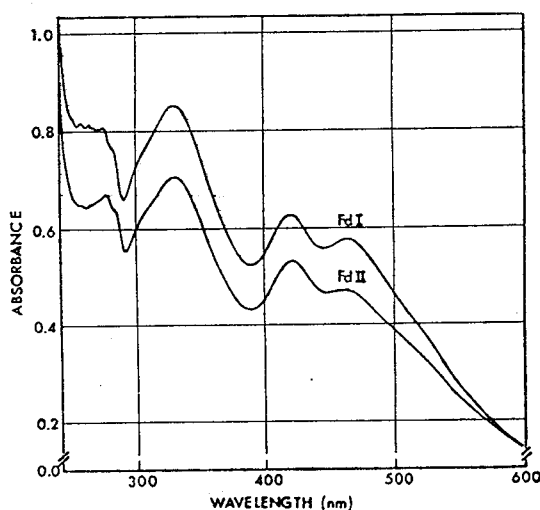


Fig. 4. Absorption spectra of *E. arvense* ferredoxin I and II. Ferredoxins were dissolved in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.35 M NaCl.

These spectra are typical of chloroplast-type ferredoxins and similar to those of E. telmateia ferredoxins (27, 28). A shoulder - more like a bulge - is noted at around 300 - 310 nm, which was not described in other ferredoxins. The absorbance ratios, A_{422}/A_{276} , were 0.79 for ferredoxin I and 0.81 for II. The amino acid compositions of the two ferredoxins are given in Table 4. The amino acid composition of ferredoxin I is similar to that of ferredoxin from E. telmateia reported previously (27).

Table 4. Amino acid compositions of the two ferredoxins from E. arvense

	Ferredoxin I		Ferredoxin II	
	From acid hydrolysate ^a	From sequence study	From acid hydrolysate ^a	From sequence study
Lysine	4.02	4	4.85	5
Histidine	1.01	1	1.08	1
Arginine	0.99	1	0.98	1
Cm-Cysteine	3.22	4	3.38	4
Aspartic acid	7.95	7 ^b	10.5	10 ^b
Threonine	7.00	7	6.52	7
Serine	8.39	9	6.37	7
Glutamic acid	13.9	14 ^b	13.8	14 ^b
Proline	4.27	4	4.40	4
Glycine	8.97	9	8.28	8
Alanine	6.47	7	5.34	5
Valine	7.22	7	6.76	7
Methionine	0.99	1	0.00	0
Isoleucine	4.56	4	8.03	8
Leucine	8.96	9	8.10	8
Tyrosine	1.88	2	1.79	2
Phenylalanine	4.90	5	2.05	2
Total residues		95		93

^a Acid hydrolyses were carried out on native ferredoxins for 24 and 72 h and on Cm-ferredoxins for 24 h. The values of threonine and serine were obtained by extrapolations to zero time of hydrolysis. Values of valine and isoleucine were of 72 hr-hydrolysates. ^b Sum of acid and amide forms.

Ferredoxin from H. halobium: The method of purification described above is simple and rapid it compared to that of Kersher et al. (14). Ammonium sulfate fractionation was very effective to remove many impurities such as red particles which were trapped on the DEAE-cellulose column and disturbed the chromatography of ferredoxin. After successive chromatography on a DEAE-cellulose and a Sephadex G-75 column, highly purified ferredoxin was obtained. The yield was about 60 mg from 150 g cell paste. The ferredoxin obtained by this procedure gave an A_{422}/A_{275} ratio of 0.31 which showed a high degree of purity it compared to 0.25 for the same protein obtained by Kersher et al. (14). The absorption spectrum of H. halobium ferredoxin is shown in Fig. 5. Absorption maxima are at 275, 330, 422, and 465 nm. The shape of the absorption spectrum is the same as those of plants and algal ferredoxin.

Table 5 shows the amino acid composition of this ferredoxin together with that deduced from the sequence studies of the ferredoxin prepared by Kersher et al. (14) as shown later. N- and C-terminal sequences of this ferredoxin are Pro-Thr-Val-Glu- and -Val-Ile, respectively. From the amino acid composition and terminal sequences, our ferredoxin is identical with that of Kersher et al. (14).

Despite of the structural similarity to chloroplast-type ferredoxins, H. halobium ferredoxin was unable to be substituted for chloroplast-type ferredoxin in the catalytic

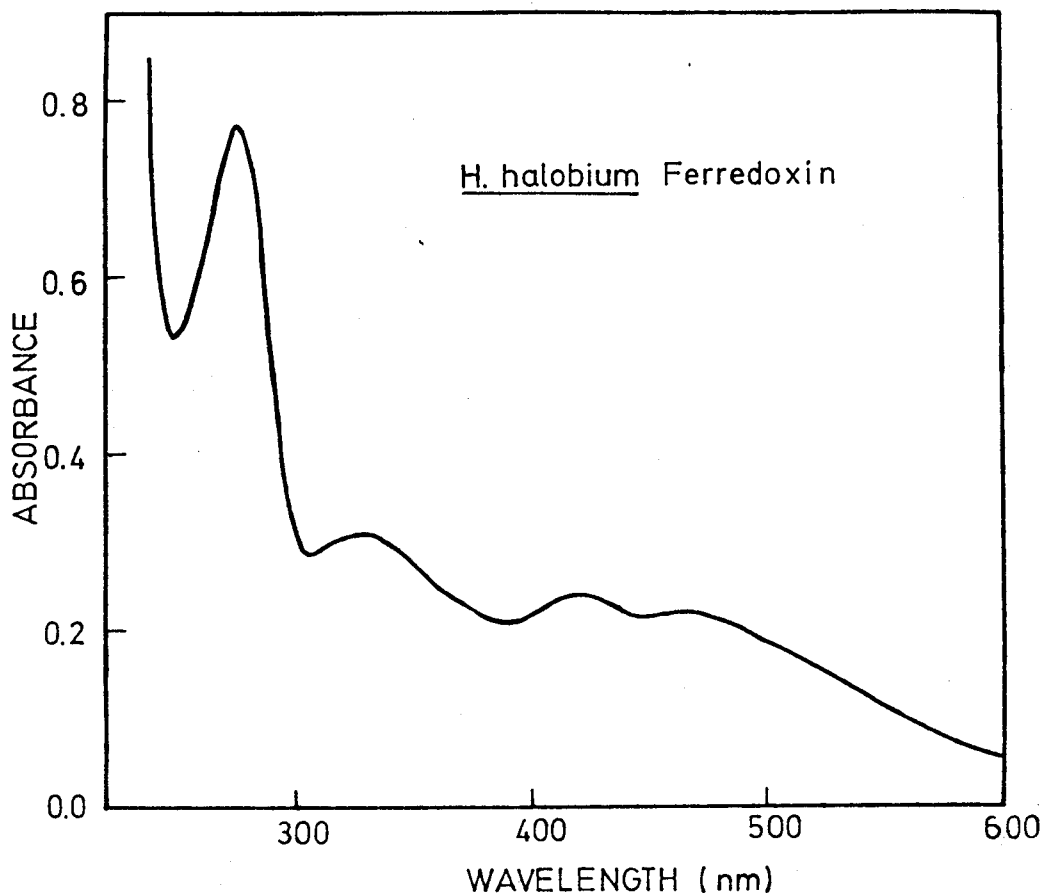


Fig. 5. Absorption spectrum of H. halobium ferredoxin. Ferredoxin was dissolved in 0.05 M Tris-HCl buffer containing 0.35 M NaCl.

system of NADP^+ -photoreduction by chloroplasts as shown in Fig. 6. Werber et al. (29) have reported that Halobacterium ferredoxin could be reduced by illuminated chloroplasts and that its failure to serve as cofactor in the photoreduction of NADP^+ could be due to the fact that it is not recognized by the plant ferredoxin:NADP oxidoreductase.

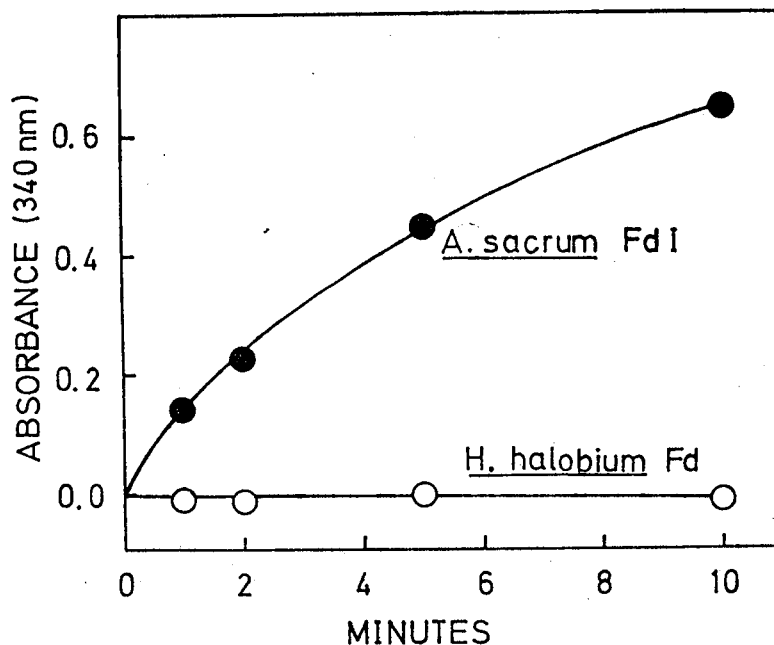


Fig. 6. Photoreduction of NADP^+ by P. americana chloroplasts. The complete reaction mixture (3 ml) contained P. americana chloroplasts with about 50 μg chlorophyll, 300 μmoles of Tris-HCl buffer, pH 7.5, 1 μmole of NADP^+ , 0.2 μmole of 2,6-dichlorophenol-indophenol, 20 μmole of sodium ascorbate, and 180 μg of A. sacrum ferredoxin or 140 μg of H. halobium ferredoxin.

Table 5. Amino acid composition of H. halobium ferredoxin.

Amino acid	From acid hydrolysate ^a	From sequence studies ^b
Lysine	5.00	5
Histidine	1.17	1
Arginine	2.90	3
Cm-cysteine	4.06	4
Aspartic acid	20.5	21.1
Threonine	4.11	4
Serine	3.90	4
Glutamic acid	21.0	21
Proline	2.90	3
Glycine	9.16	9
Alanine	13.4	14
Valine	7.75	8
Methionine	3.78	4
Isoleucine	6.45	7
Leucine	9.11	9
Tyrosine	6.74	7
Phenylalanine	2.08	2
Tryptophan	N. D.	2

^a Acid hydrolysis were carried out on Cm-ferredoxin for 24 h. ^b See the text in chapter III. ^c Sum of acid and amide forms.

III. AMINO ACID SEQUENCE DETERMINATION OF FERREDOXINS

1. Introduction

Since Sanger and his coworkers established the amino acid sequences of two chains of insulin in the early 1950s (30, 31), the number of proteins sequenced has increased explosively. The amino acid sequences of proteins have been used for elucidation of structure-function relationship of proteins which are involved in many important biological functions, for the description on genetic basis of protein biosynthesis which is a result of the translation of a particular gene, and for the reconstruction of evolutionary history of proteins and organisms. Because of such multifarious information contained in primary structure of protein, many efforts were poured in establishing the rapid, accurate and microscale procedures for completing the sequence.

The first ferredoxins of different types sequenced were from a bacterium, C. pasteurianum, and a plant, spinach, which were reported by Tanaka et al. (11, 32) and Matsubara et al. (12, 33, 34), respectively. The characteristic features of their structures acted as a stimulus to further investigations of various ferredoxins. In these sequence studies, a large quantities of sample (about 140 μ mol for C. pasteurianum ferredoxin and about 50 μ mol for spinach

ferredoxin) were used. But it is considerably difficult to obtain such quantities of ferredoxins from bacterial and plant sources except for a special case. For example, to purify 50 μmol of ferredoxin from E. arvense, about 500 Kg of its leaves would be needed.

Today the strategies for completing amino acid sequence of only a small amount of sample have been well developed, although the principle employed in the sequence studies is almost the same as that of Sanger, that is, fragmentation of protein, separation of peptides, sequence determination of peptides, and reconstruction of primary structure by overlapping fragments. In this study, only several μmol of ferredoxins were available and I chose the following methods as a routine procedure: (1) fragmentation of protein; peptide bond hydrolysis was performed with enzymes having very high specificities such as trypsin and staphylococcal protease. Since the basic amino acid residues occurred only in a small number in ferredoxins, large polypeptides fragments were obtainable by trypsin digestion, (2) separation of peptides; gel filtration was mainly used which was useful for separating few large peptides derived from the fragmentation described above, (3) sequence determination; sequence analysis was performed using Edman degradation and carboxypeptidase digestions. PTH-derivatives obtained on successive steps of degradation were directly analyzed with a thin layer chromatography, and (4) reconstruction of primary structure; not only overlapping

peptides obtained from different methods of fragmentation, but also alignment of peptides by homology could give an useful information in constructing whole protein chain.

In this chapter, I describe the amino acid sequence determination of ferredoxins from 13 organisms, blue-green algae: Aphanothece sacrum, Nostoc muscorum, Mastigocladus laminosus, a red alga: Cyanidium caldarium, horsetails: Equisetum telmateia, E. arvense, a green alga: Dunaliella sorlina, and various bacteria: Halobacterium halobium, Bacillus stearothermophilus, Pseudomonas ovaris, Mycobacterium smegmatis, Chlorobium thiosulfatophilum Tassajara, Chromatium vinosum.

2. Materials and General Methods

Chemicals and Enzymes: Chemicals were analytical or sequanal grade purchased from Wako Pure Chemical Industries, Japan and Nakarai Chemical Co., Japan. Enzymes used in these experiments were obtained as described below. TPCK-trypsin, chymotrypsin, and carboxypeptidase A, carboxypeptidase B, and leucine aminopeptidase treated with DFP were purchased from Worthington Biochemical Corp., U.S.A. and carboxypeptidase Y from Oriental Yeast Co. Ltd., Japan. Thermolysin and staphylococcal protease were gifts from Dr. S. Endo (Daiwa Kasei Co., Ltd., Japan) and Dr. R. P. Ambler (Department of Molecular Biology, University of

Edinburgh, U.K.), respectively.

Ferredoxins: A. sacrum ferredoxin I and II, E. arvense ferredoxins I and II were isolated according to the methods described in previous chapter. Other ferredoxins were provided as follows: N. muscorum ferredoxin I and E. telmateia ferredoxins I and II were from Dr. K. Wada (University of Osaka, Japan), M. laminosus ferredoxin, D. sorlina ferredoxins I and II, C. caldarium ferredoxin, H. halobium ferredoxin, and B. stearothermophilus ferredoxin were from Dr. K. K. Rao (University of London King's College, U.K.) and his coworkers, P. ovalis ferredoxin from Dr. Ohmori (University of Juntendo, Japan), M. smegmatis ferredoxin from Dr. Tobari (University of Rikkyo, Japan), and C. thiosulfatophilum ferredoxin and C. vinosum ferredoxin from Dr. M. C. W. Evans (University College London, U.K.)

Trichloroacetic Acid Treatment: In order to remove iron and inorganic sulfur, native ferredoxin was treated with trichloroacetic acid according to the method of Matsubara et al. (34).

Cm-ferredoxin and Oxidized Ferredoxin: The apo-ferredoxin after TCA treatment was reduced with 2-mercaptoethanol and carboxymethylated with iodoacetic acid according to the method of Crestfield et al. (35). Performic acid oxidized ferredoxin was prepared as in ref. (34).

Enzymic Digestion of Ferredoxin Derivatives: The detailed conditions for digestions are presented in each case of sequence studies of various ferredoxins. Brief

conditions with respect to enzyme to substrate ratios, temperature, time of digestion, and buffers are as follows. Trypsin, chymotrypsin, and staphylococcal protease were usually employed at the level of 1 to 5 % by weight of substrate in Tris-HCl buffer, pH 8.0 at 40° for 2 h to overnight. Thermolysin digestion was carried out in Tris-HCl buffer, pH 8.0 containing 2 mM CaCl_2 . Hydrolysis was terminated by adding several drops of 30 % acetic acid or by freezing.

Purification of Peptides: Peptides were separated by ion exchange chromatography, gel filtration, paper chromatography, and paper electrophoresis. Ion exchange chromatography was carried out using two anion exchange resins, Dowex 1 (Bio-Rad AG 1 x 2) and DEAE-cellulose (Whatman DE-52). Buffers used for elution were pyridine-acetate and ammonium bicarbonate for Dowex 1 and DEAE-cellulose, respectively. Gel filtration was carried out on a Bio-Gel column (Bio-Gel P-2, P-4, P-6, and P-10) developed by 0.2 M ammonium bicarbonate, pH 9.0, or 0.05 M triethylamine-acetate buffer, pH 8.5. Peptide fractions from column effluent were monitored by the ninhydrin method (36) without alkaline hydrolysis or measuring absorbance at 220, 230, and 280 nm.

Preparative paper electrophoresis and chromatography were also employed for fractionation of peptide mixtures. Paper electrophoresis was carried out at pH 3.6 (pyridine/acetic acid/water, 1:10:189, v/v) or at pH 6.5 (pyridine/

acetic acid/water, 10:0.4:180, v/v). Paper chromatography was carried out with n-butanol/pyridine/acetic acid/water (15:10:3:12, v/v, BPAW) or pyridine/3-methyl-1-butanol/0.1 M NH_4OH (6:3:5, v/v, PIN). Location of peptides separated on paper was detected by the ninhydrin method (36) and fluorescamine method (37) followed by specific staining for tryptophan (38), histidine (39), tyrosine (39), and arginine (40). Peptides were eluted from paper with 0.5 N NH_4OH .

Amino Acid Analysis: Amino acid compositions of protein and peptides were determined with an amino acid analyzer (Beckman model 120 B) according to the method of Spackman et al. (41). The sample was hydrolyzed in an evacuated, sealed tube for 20-24 h or 72 h at 110° with 6 N HCl containing a small amount of thioglycolic acid. Tryptophan was analyzed after hydrolysis with 6 N HCl containing 4 % thioglycolic acid (42) or 3 N mercaptoethanesulphonic acid (43). The basis for the calculation of the content of each amino acids was the average value obtained from the stable amino acids in the peptides.

Sequence Analysis: A manual Edman degradation procedure (44) was applied to the Cm-ferredoxin and peptides to determine their N-terminal sequences. The amount of sample for degradation was usually about 0.1 μmol . Phenylthiohydantoin derivatives (PTH) were identified by TLC on Merck silica-gel plates using mainly two solvent systems, II; methanol/chloroform (10:90, v/v) (45) and V; n-propanol/

ethylene-dichloride/propionic acid (58:25:17, v/v) (45). PTH-histidine and PTH-arginine were identified by paper electrophoresis at pH 6.5 (46). Some tryptic peptides containing a lysine residue at the C-terminus were coupled to aminopolystyrene or aminopropylglass using p-phenylene-diisothiocyanate (DITC) (47) and sequenced with a solid phase Edman degradation procedure (48) using an LKB 4020 Solid-Phase Sequencer, Sweden.

The C-terminal sequences of the protein and peptides were determined by digestion them with carboxypeptidase A, B, and/or Y (49, 50). The sample was dissolved in 0.1 M Tris-HCl or borate buffer, pH 8.0, or in 0.1 M Na-acetate buffer, pH 5.5, for carboxypeptidase A, in 0.1 M borate buffer, pH 8.0 for carboxypeptidase B, and in 0.1 M pyridine-acetate buffer, pH 5.5 for caboxyptidase Y. After the reaction, aliquots were inactivated by the addition of Na-citrate-HCl buffer, pH 2.2 and subjected to amino acid analysis.

Nomenclature of Peptides: Peptides obtained from tryptic, chymotryptic, thermolysin, and staphylococcal protease digests were referred to T-, C-, Th-, and S-, respectively and peptides obtained from CNBr treatment were referred to CN-, and all peptides were numbered consecutively according to their positions in the polypeptide chain from N-terminus.

All values are expressed in moles per mole of peptide or protein.

3. Amino Acid Sequence of Halobacterium halobium Ferredoxin

3.1. Experimental

Enzymic Hydrolysis of Cm-Ferredoxin: One μmol of Cm-ferredoxin was digested with 0.36 mg of trypsin in 1 ml of 0.05 M Tris-HCl buffer, pH 8.0, at 40° for 3 h. Each 0.7 μmol of Cm-ferredoxin was digested with 0.3 mg of staphylococcal protease in 1 ml of Tris-HCl buffer, pH 8.0, and with 0.25 mg of chymotrypsin in 0.5 ml of Tris-HCl buffer, pH 8.0, at 40° for 3 h, respectively.

Purification of Peptides: Tryptic and staphylococcal protease digests were separately fractionated by gel filtration on a Bio-Gel P-4 column (1.6 x 190 cm) developed by 0.2 M ammonium bicarbonate. Each fraction (2.2 ml) was monitored in terms of the absorbance at 220 or 230 nm and at 280 nm. Pooled fractions were lyophilized and subjected to analyses or further purifications by paper electrophoresis at pH 3.6 or pH 6.5 and paper chromatography with PIN or BPAW. The peptides eluted at the void volume of the Bio-Gel column were further separated by ion exchange chromatography on a DEAE-cellulose column (1.2 x 24 cm) using a gradient system from 0.1 M to 1 M ammonium bicarbonate, pH 8.0, 200 ml each, and each eluate fraction (2.2 ml) was monitored as mentioned above.

Chymotryptic digest was separated by paper electrophoresis at pH 3.6 and chromatography with BPAW.

Identification of N^ε - Acetyllysine: An unusual amino acid was present at residue 118 and in order to identify it four authentic lysine derivatives were compared with this residue. They were N^ε-mono-, di-, and tri-methyllysine and N^ε-acetyllysine. N^ε-acetyllysine was semi-synthesized as follows. About 1 μmol of a peptide, CM-C-10 (34) isolated from spinach ferredoxin, which contained a lysine residue, was acetylated with 3 μl of acetic anhydride in 0.2 M sodium bicarbonate at 0° for 20 min. The acetylated peptide was digested with staphylococcal protease and a peptide, Thr-His-Lys-Glu-Glu-Glu, was purified by paper chromatography using the BPAW system. The amino acid composition of this peptide was Thr₁, His₁, Lys₁, and Glu₃ after acid hydrolysis. However, an amino-peptidase M digest of this peptide did not give free lysine at all and instead gave a peak between proline and glycine on the analyzer. This component was assumed to be N^ε-acetyllysine. Three steps of Edman degradation of this peptide gave PTH-Thr, PTH-His, and PTH-Lys, respectively, and PTH-Lys moved with thin-layer chromatography between PTH-Gln (R_f=0.24) and PTH-Tyr (R_f=0.50), at R_f=0.38, using solvent system II (45), but did not move at all using system V (45). It was easily extracted into the ethylacetate under acidic conditions.

To confirm the presence of the acetyl group further

both the spinach and halobacterial peptides were subjected to hydrazinolysis and the acetylhydrazide produced was identified as a dansyl derivative by two-dimensional silica gel chromatography (51).

3.2. Results and Discussion

Amino Acid Composition and Terminal Sequences of

Cm-Ferredoxin: The amino acid composition of Cm-ferredoxin of H. halobium is shown in Table 1. This ferredoxin contained a large number of acidic amino acids, as commonly observed in ferredoxins from other organisms. Only four cysteine residues were obtained and they must fulfill the minimum cysteine requirement for chelating two iron atoms to construct the active center of this protein. The total number of residues was 128, which agreed well with that deduced from the sequence study, as shown later.

A manual Edman degradation of 90 nmol of Cm-ferredoxin revealed the N-terminal sequence up to 15 residues without any ambiguous identification to be as follows: Pro-Thr-Val-Glu-Tyr-Leu-Asn-Tyr-Glu-Thr-Leu-Asp-Asp-Gln-Gly-. Carboxypeptidase A (0.02 mg) released two major amino acids, valine (0.71) and isoleucine (0.72), at 1 h from Cm-ferredoxin (35 nmol): the order of these residues could not be decided, but as shown later the C-terminal sequence was found to be -Val-Ile.

Table 1.. Amino acid composition of Cm-ferredoxin.^a

Amino acid	Analysis	Sequence
Lysine	4.93	5
Histidine	0.87	1
Arginine	2.78	3
Cm-cysteine	4.19	4
Aspartic acid	20.6	21
Threonine	4.06	4
Serine	4.05	4
Glutamic acid	20.6	21
Proline	2.77	3
Glycine	9.44	9
Alanine	14.0	14
Valine	6.77	8
Methionine	3.70	4
Isoleucine	5.96	7
Leucine	8.95	9
Tyrosine	6.71	7
Phenylalanine	1.86	2
Tryptophan	N.D. ^b	2
Total		128

^a Values are given as moles of residue per mole of Cm-ferredoxin for 24 h hydrolysate. ^b N.D., not determined.

Tryptic Peptides: The tryptic digest was first fractionated by gel filtration on a Bio-Gel P-4 column (Fig. 1a) and the peptides eluted in the first peak were further separated on a DEAE-cellulose column (Fig. 1b). Peptides thus fractionated were further purified on paper. The amino acid compositions and characteristics of the isolated peptides are given in Table 2 and amino acid sequence data for the tryptic peptides are summarized in Table 3. The sequences of Peptides T-3, T-5, T-6, T-7, T-8, and T-9 were completely elucidated by Edman degradation.

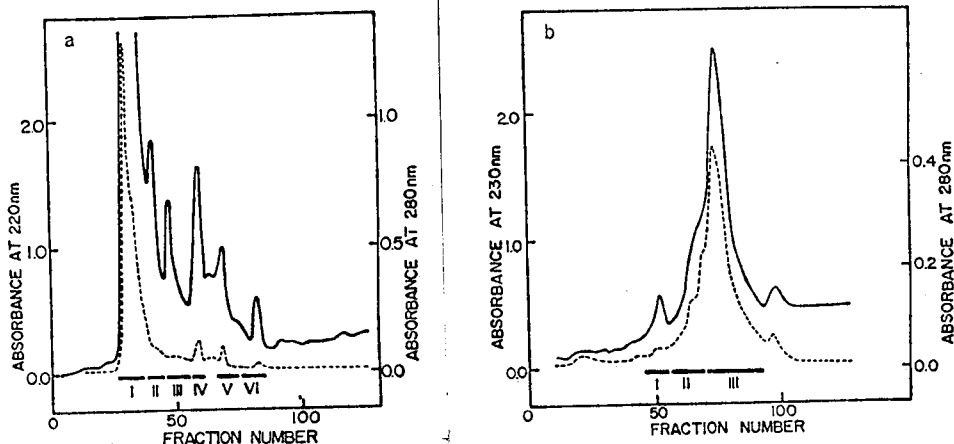


Fig. 1 (a) Elution pattern of tryptic peptides of Cm-ferredoxin. Peptides were chromatographed on a Bio-Gel P-4 column with 0.2 M NH_4HCO_3 , pH 8. The fraction (2.2 ml each) were monitored by following the absorbances at 220 nm (—) and 280 nm (---). (b) Elution pattern of the first peak eluted from the Bio-Gel column shown in Fig. 1a. Peptides were chromatographed on a DEAE-cellulose (DE-52) column using a gradient system from 0.1 M to 1 M NH_4HCO_3 , pH 8, 200 ml each. Each fraction (2.2 ml) was monitored by following the absorbances at 230 nm (—) and 280 nm (---).

Peptide T-9 must be the C-terminal peptide, Val-Ile, and this was consistent with the results obtained by carboxypeptidase A digestion of Cm-ferredoxin as described above.

Partial sequences of Peptides T-1, T-2, and T-4 were determined by Edman degradation. Peptide T-2 was probably derived from an unspecific cleavage by trypsin. Tyrosine was present at the C-terminus of this peptide as identified by carboxypeptidase A digestion. Digestion of Peptide T-4 by carboxypeptidase B for 1.5 h followed by carboxypeptidase A gave the following results:

Table 2. Amino acid compositions of tryptic peptides of Cm-ferredoxin. Values are from 20-24 h hydrolysate without corrections for incomplete hydrolysis or destruction.

Amino acid	T-1	T-2	T-3	T-4	T-5	T-6	T-7	T-8	T-9
Lysine	0.92(1)			0.97(1)	1.16(1)		1.06(1)	1.11(1)	
Histidine								0.94(1)	
Arginine			0.83(1)			1.03(1)		0.74(1)	
Cm-cysteine			1.09(1)	2.15(2)			1.10(1)		
Aspartic acid	8.20(8)	3.87(3) ^a	1.00(1)	1.02(1)	3.34(3)	0.99(1)	1.02(1)	3.33(3)	
Threonine	1.83(2)	0.94(1)					0.85(1)		
Serine			1.28(1)	0.85(1)	1.05(1)		0.88(1)		
Glutamic acid	3.89(4)	6.85(7)			7.56(8)		0.98(1)	0.99(1)	
Proline	0.96(1)		0.79(1)				1.13(1)		
Glycine	1.15(1)	4.91(5)		1.08(1)	1.24(1)		1.09(1)		
Alanine	0.21	6.55(7)		3.51(4)	0.28		2.03(2)	1.10(1)	
Valine	1.02(1)	1.08(1)		0.69(1)	1.16(1)	0.98(1)	0.97(1)	0.45(1) ^a	0.97(1)
Methionine	1.05(1)	0.89(1)			1.76(2)				
Isoleucine		0.92(1)		0.68(1)	2.02(2)		0.83(1)	0.52(1)	1.03(1)
Leucine	3.00(3)	2.02(2)			1.04(1)		0.85(1)	2.17(2)	
Tyrosine	1.82(2)	2.56(3)						1.81(2)	
Phenylalanine	0.93(1)		1.01(1)						
Tryptophan	+(1) ^a		+(1) ^a						
Total residues	26	31	7	12	20	3	13	14	2
Yield (%)	17	16	10	53	79	39	77	19	17
Color reaction ^b	P,E	P	E					P	
Bio-Gel P-4 fractions	I	I	V	III	I	VI	II	IV	VI
DE-52 fractions	III	II			I				
Purification ^c	PIN (R_f 0.41 -0.51)	PIN (R_f 0.14 -0.26)	PE ₁ (m -1.6)	PE ₁ (m -2.5)		BPAW (R_f 0.29)	PE ₁ (m -4)	PE ₂ (m +3)	BPAW (R_f 0.82)

^aFrom the sequence studies. ^bP, Pauly reaction positive (39); E, Ehrlich reaction positive (38). ^cPIN, paper chromatography in pyridine/3-methyl-1-butanol/0.1 M NH₃. BPAW, paper chromatography in butan-1-ol/pyridine/acetic acid/water. PE₁ and PE₂, paper electrophoresis at pH 3.6 and pH 6.5, respectively. R_f, mobility on paper chromatography in the solvent system used for purification. m, mobility in cm from the origin toward the anode (+) or cathode (-) after electrophoresis for 1 h at 43 V/cm.

Table 3. Amino acid sequences of the tryptic peptides of Cm-ferredoxin.

Peptide	Residues	Sequence
T-1	1-26	<u>Pro-Thr-Val-Glu-Tyr-Leu-Asn-Tyr-Glu-Thr-Leu-Asp-Asp-</u> <u>Gln-Gly-Trp-Asp</u> (Met, Asp, Asp, Asp, Asp, Leu, Phe, Glu, Lys)
T-2	27-57	<u>Ala-Ala-Asp-Ala-Gly-Leu-Asp-Gly-Glu-Asp-Tyr-Gly-Thr-</u> <u>Met-Glu-Val-Ala-Glu-Gly-Glu-Tyr-Ile-Leu-Glu-Ala-Ala-</u> <u>Glu-Ala</u> (Gln, Gly) <u>Tyr</u>
T-3	58-64	<u>Asp-Trp-Pro-Phe-Ser-Cys-Arg</u>
T-4	65-76	<u>Ala-Gly-Ala-Cys-Ala-Asn-Cys-Ala-Ser-Ile-Val-Lys</u>
T-4-Th-1	65-68	(Ala, Gly, Ala, Cys)
T-4-Th-2	69-71	(Ala, Asn, Cys)
T-4-Th-3	72-73	(Ala, Ser)
T-4-Th-4	74-76	<u>Ile-Val-Lys</u>
T-5	77-96	<u>Glu-Gly-Glu-Ile-Asp-Met-Asp-Met-Gln-Gln-Ile-Leu-Ser-</u> <u>Asp-Glu-Glu-Val-Glu-Glu-Lys</u>
T-6	97-99	<u>Asp-Val-Arg</u>
T-7	100-112	<u>Leu-Thr-Cys-Ile-Gly-Ser-Pro-Ala-Ala-Asp-Glu-Val-Lys</u>
T-8	113-126	<u>Ile-Val-Tyr-Asn-Ala-Lys-His-Leu-Asp-Tyr-Leu-Gln-Asn-</u> <u>Arg</u> <u>Val-Ile</u>
T-9	127-128	<u>Val-Ile</u>

The arrow (→) denotes a degradation step by the Edman procedure and (←) carboxypeptidase digestion. A dotted arrow indicates uncertain identification.

Table 4. Amino acid compositions of thermolysin peptides of Peptide T-4

Amino acid	T-4-Th-1	T-4-Th-2	T-4-Th-3	T-4-Th-4
Lysine				1.00 (1)
Cm-cysteine	0.94 (1)	0.87 (1)		
Aspartic acid		0.83 (1)		
Serine			0.91 (1)	
Glycine	1.17 (1)			
Alanine	1.80 (2)	1.29 (1)	1.09 (1)	
Valine				0.59 (1)
Isoleucine				0.59 (1)
Total	4	3	2	3

	Lys	Cmc	Ser	Ala	Val	Ile
30 min	0.77					
2.0 h	N.D.				0.45	0.48
4.5 h	0.77	0.24	0.27	0.20	0.63	0.64
N.D.: not determined						

The results showed that lysine was present at the C-terminus, and valine and isoleucine were near the C-terminus. Peptide T-4 (0.24 μ mol) was further digested with thermolysin (0.01 mg) in 0.1 M Tris-HCl buffer, pH 8.0, containing 10 mM CaCl_2 . Four peptides, T-4-Th-1 to T-4-Th-4, were purified by paper electrophoresis at pH 3.6 and their amino acid compositions are shown in Table 4. Three steps of Edman degradation of Peptide T-4-Th-4 were performed. Although no sequence study of other thermolysin peptides was carried out, the complete amino acid sequence of Peptide T-4 was thus established.

Staphylococcal Protease Peptides: The result of exclusion chromatography of the staphylococcal protease digest on a Bio-Gel P-4 column is shown in Fig. 2a. The first fraction was further divided into several peptides by ion exchange chromatography on a DEAE-cellulose column as shown in Fig. 2b. The peptides thus fractionated were purified by paper chromatography as described above. Table 5 and 6 give the characteristics of the isolated peptides and their sequence data, respectively. Complete sequences of all peptides except for Peptide S-3 and S-4 were determined

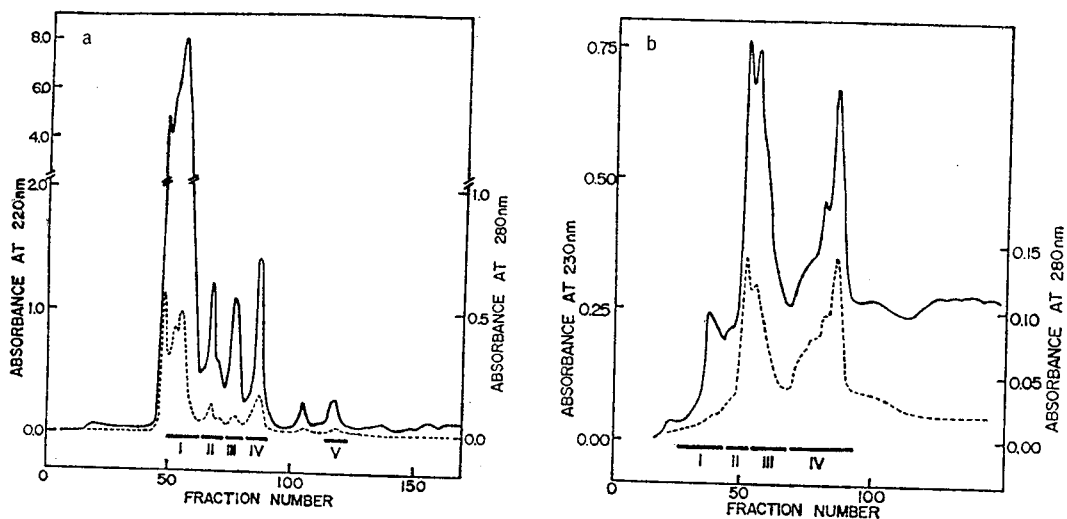


Fig. 2. (a) Elution pattern of staphylococcal protease peptides of Cm-ferredoxin. Peptides were chromatographed on a Bio-Gel P-4 column and fractions were monitored as described in Fig. 1a. (b) Elution pattern of the first fraction eluted from the Bio-Gel column shown in Fig. 2a. Peptides were chromatographed on a DEAE-cellulose column using a gradient system and fractions were monitored as described in Fig. 1b.

by Edman degradations. After 10 steps of degradation on Peptide S-3, carboxypeptidase Y digestion of the residue at pH 5.5 was performed, releasing glutamic acid (0.23), leucine (0.31), and phenylalanine (0.22) at 1 h. Glutamic acid must be at the C-terminus of Peptide S-3 from the protease specificity.

Chymotryptic Peptides: The chymotryptic digest was subjected to paper electrophoresis and chromatography as described in "Experimental." Five peptides were recovered, and their compositions and sequence data are shown in Table 7 and 8, respectively. Tryptophan residues were present in Peptide C-1, C-2, and C-4 as detected by the

Table 5. Amino acid compositions of staphylococcal protease peptides of Cm-ferredoxin. Abbreviation are as in Table 2.

	S-1	S-2	S-3	S-4	S-5	S-6	S-7
Lysine				0.92 (1)			
Cm-cysteine				0.17			
Aspartic acid		1.02 (1)	6.71 (7)	3.06 (3)			
Threonine	0.83 (1)		0.90 (1)	0.89 (1)			
Serine				0.33			
Glutamic acid	1.13 (1)	1.09 (1)	2.18 (2)	2.30 (2)	1.01 (1)	2.12 (2)	1.03 (1)
Proline	0.89 (1)						
Glycine			1.09 (1)	3.07 (3)		0.94 (1)	
Alanine				3.14 (3)	1.03 (1)		1.97 (2)
Valine	1.13 (1)				0.97 (1)		
Methionine			1.03 (1)	0.99 (1)			
Isoleucine						1.01 (1)	
Leucine		1.01 (1)	2.01 (2)	0.94 (1)		0.98 (1)	
Tyrosine		1.88 (2)		0.91 (1)		0.94 (1)	
Phenylalanine			0.94 (1)				
Tryptophan			+ (1) ^a				
Total residues	4	5	16	16	3	6	3
Yield (%)	65	67	28	28	70	75	73
Color reaction ^b		P	E	P		P	
Bio-Gel P-4 fraction	III	IV	I	I	IV	II	IV
DE-52 fraction			IV	III			
Purification ^c	PE ₂ (^m ₊₅)	PE ₂ (^m ₊₄)	BPAW (R _f 0.4)	PE ₂ (^m ₊₅)	PE ₂ (^m ₊₇) BPAW (R _f 0.35)	PE ₂ (^m ₊₆)	PE ₂ (^m ₊₇) BPAW (R _f 0.24)
	S-8	S-9	S-10	S-11	S-12	S-13	S-14
Lysine	0.98 (1)				0.89 (1)	1.80 (2)	
Histidine						0.99 (1)	
Arginine	1.03 (1)				0.93 (1)		0.92 (1)
Cm-cysteine	3.24 (3)				1.12 (1)		
Aspartic acid	2.36 (2)	1.96 (2)	3.12 (3)		1.97 (2)	2.03 (2)	1.05 (1)
Threonine					0.93 (1)		
Serine	1.91 (2)		0.88 (1)		0.96 (1)	0.40	
Glutamic acid	1.68 (2)	1.16 (1)	5.24 (5)	2.02 (2)	1.01 (1)	0.50	1.08 (1)
Proline	1.09 (1)				1.00 (1)		
Glycine	2.15 (2)	1.12 (1)	1.09 (1)		1.12 (1)		
Alanine	5.15 (5)		0.31		2.07 (2)	1.00 (1)	
Valine	0.81 (1)		0.42	0.97 (1)	1.01 (1)	1.34 (2) ^a	0.91 (1)
Methionine		0.88 (1)	1.37 (2) ^a				
Isoleucine	0.72 (1)	0.89 (1)	1.73 (2)		1.03 (1)	0.72 (1)	0.82 (1)
Leucine	0.24		0.96 (1)		0.99 (1)	1.13 (1)	1.04 (1)
Tyrosine	0.81 (1)					0.95 (1)	0.90 (1)
Phenylalanine	0.95 (1)						
Tryptophan	+ (1) ^a						
Total residues	24	6	15	3	15	11	7
Yield (%)	23	21	21	14	47	23	18
Color reaction ^b	P,E					P	P
Bio-Gel P-4 fraction	I	II	I	II	I	III	V
DE-52 fraction	II		III		I		
Purification ^c	BPAW (R _f 0.33)	PE ₂ (^m _{+9.5}) BPAW (R _f 0.19)	PE ₂ (^m ₊₈)	PE ₂ (^m _{+9.5}) BPAW (R _f 0.28)	PE ₂ (^m ₊₄)	PE ₂ (^m ₋₃)	PE ₂ (^m ₋₅)

Table 6. Amino acid sequence of the staphylococcal protease peptides of Cm-ferredoxin.

Peptide	Residues	Sequence
S-1	1-4	<u>Pro-Thr-Val-Glu</u>
S-2	5-9	<u>Tyr-Leu-Asn-Tyr-Glu</u>
S-3	10-25	<u>Thr-Leu-Asp-Asp-Gln-Gly-Trp-Asp-Met-Asp</u> (Asp, Asp) <u>Asp-Leu-Phe-Glu</u>
S-4	26-41	<u>Lys-Ala-Ala-Asp-Ala-Gly-Leu-Asp-Gly-Glu-Asp-Tyr-</u> <u>Gly-Thr-Met-Glu</u>
S-5	42-44	<u>Val-Ala-Glu</u>
S-6	45-50	<u>Gly-Glu-Tyr-Ile-Leu-Glu</u>
S-7	51-53	<u>Ala-Ala-Glu</u>
S-8	54-77	<u>Ala-Gln-Gly-Tyr-Asp-Trp-Pro-Phe-Ser-Cys-Arg-Ala-</u> <u>Gly-Ala-Cys</u> (Ala, Asn, Cys, Ala, Ser, Ile, Val, Lys, Glu)
S-9	78-83	<u>Gly-Glu-Ile-Asp-Met-Asp</u>
S-10	78-92	<u>Gly-Glu-Ile-Asp-Met-Asp-Met-Gln-Gln-Ile-Leu-Ser-</u> <u>Asp-Glu-Glu</u>
S-11	93-95	<u>Val-Glu-Glu</u>
S-12	96-110	<u>Lys-Asp-Val-Arg-Leu-Thr-Cys-Ile-Gly-Ser-Pro-Ala-</u> <u>Ala-Asp-Glu</u>
S-13	111-121	<u>Val-Lys-Ile-Val-Tyr-Asn-Ala-Lys-His-Leu-Asp</u>
S-14	122-128	<u>Tyr-Leu-Gln-Asn-Arg-Val-Ile</u> Ac

Ehrlich reaction on paper. The C-terminal residue of Peptide C-2 was tryptophan, which was the sole residue released by carboxypeptidase A. The compositions and sequences of Peptides C-1, C-2, and C-3 suggested that the latter two peptides were derived from peptide C-1 by cleavage with chymotrypsin at the tryptophan residue. Six steps of Edman degradation on Peptide C-3 were carried out and carboxypeptidase A released leucine (0.15) and phenylalanine

Table 7. Amino acid compositions of chymotryptic peptides of Cm-ferredoxin. Meanings of abbreviations are as in Table 2.

Amino acid	C-1	C-2	C-3	C-4	C-5	C-5*
Lysine					0.96 (1)	
Histidine					0.90 (1)	0.91 (1)
N ^ε -Acetyllysine						0.91 (1)
Asparagine						0.77 (1)
Aspartic acid	6.90 (7)	1.92 (2)	5.21 (5)	0.90 (1)	1.95 (2)	1.00 (1)
Threonine	0.98 (1)	0.80 (1)				
Serine	0.18	0.28				
Glutamic acid	2.10 (2)	2.08 (2)				
Proline				1.11 (1)		
Glycine	1.20 (1)	1.13 (1)				
Alanine	0.25				1.02 (1)	1.08 (1)
Methionine	0.93 (1)		0.93 (1)			
Leucine	1.82 (2)	0.88 (1)	0.94 (1)		1.35 (1)	1.30 (1)
Tyrosine	0.25				0.83 (1)	0.95 (1)
Phenylalanine	0.92 (1)		0.96 (1)	1.00 (1)		
Tryptophan	+ (1) ^a	+ (1) ^a		+ (1) ^a		
Total residues	16	8	8	4	7	
Yield (%)	9	13	31	19	51	
Color reaction ^b	E	E		E	P	
Purification ^c	PE ₁ (^m _{+0.5}) BPAW(^{R_f} _{0.32})	PE ₁ (^m _{-0.5}) BPAW(^{R_f} _{0.27})	PE ₁ (^m _{-4.5})	PE ₁ (^m _{-0.5}) BPAW(^{R_f} _{0.51})	PE ₁ (^m _{-5.5})	

* Aminopeptidase M digest of Peptide C-5. The color value of N^ε-acetyllysine was the average of those of other amino acids.

Table 8. Amino acid sequences of the chymotryptic peptides of Cm-ferredoxin.

Peptide	Residues	Sequence
C-1	9-24	<u>Glu-Thr-Leu-Asp-Asp</u> (Gln,Gly,Trp,Asp,Met,Asp,Asp,Asp,Asp,Leu,Phe)
C-2	9-16	(Glu,Thr;Leu,Asp,Asp,Gln,Gly) <u>Trp</u>
C-3	17-24	<u>Asp-Met-Asp-Asp-Asp-Asp-Leu-Phe</u>
C-4	58-64	<u>Asp</u> (Trp,Pro,Phe)
C-5	116-122	<u>Asn-Ala-Lys</u> (His,Leu,Asp,Tyr) Ac

(0.96) from this peptide. Thus, the sequence of Peptide C-3 was completely determined and the partial sequences of Peptide T-1 and S-3 were supplemented by these completed sequences of C-3.

Detection of N^ε-Acetyllysine: In the present studies an unusual PTH-derivatives was identified on thin-layer chromatography at the sixth, eighth, and third steps of Edman degradation of Peptides T-8, S-13, and C-4, respectively. This derivative was easily extracted into ethylacetate under acidic conditions. It moved between PTH-glutamine and PTH-tyrosine with solvent II and did not move with solvent V, as described in "Experimental." Such behavior on chromatography was completely identical with that of PTH-N^ε-acetyllysine semi-synthesized as described in "Experimental."

The acid hydrolysates of Peptides T-8, S-13, and C-5 gave one, two, and one lysine residues, respectively, as shown in Table 2, 5, and 7. Only Peptide C-5 was chosen for a detailed compositional study. When Peptide C-5 was hydrolyzed with aminopeptidase M overnight, the composition was the same as that obtained after acid hydrolysis except for the lysine, which was missing, and a new component which emerged between proline and glycine on the analyzer. Also one out of two aspartic acids moved to the position of asparagine. Analysis of the aminopeptidase M hydrolysate of the semi-synthesized peptide containing N^ε-acetyllysine derived from spinach ferredoxin gave the same elution.

pattern as that observed for the Peptide C-5 digest on the analyzer. The composition of the aminopeptidase digest of Peptide C-5 is included in Table 7. N^ε-mono-, di-, and tri- methyllysine were compared with N^ε-acetyllysine on the analyzer and by thin-layer chromatography after conversion to PTH-derivatives, and they behaved differently from N^ε-acetyllysine. Hydrazinolysis of Peptide C-5 yield acethydrazide which was identified as a dansyl derivative by a silica gel chromatography using an authentic derivative of acethydrazide for comparison (51). These studies strongly indicated that the lysine residue was acetylated.

Complete Amino Acid Sequence: The complete amino acid sequence of Cm-ferredoxin of H. halobium is shown in Fig. 3. Edman degradation of the original protein allowed the alignment of Peptide T-1, S-1, S-2, and S-3. Peptides T-9 and S-14 were the C-terminal peptides of the protein judging from their C-terminal sequence, Val-Ile. Other peptides derived by tryptic and staphylococcal protease digestions overlapped each other and confirmed the alignments. Peptide C-3 gave the sequence of unknown regions of Peptides T-1 and S-3. The positions of tryptophan residues were confirmed by studies of Peptides C-2 and C-4. Thus, the complete amino acid sequence of this ferredoxin was established; the total number of residues was 128 including one N^ε-acetyllysine residue. The molecular weight was calculated to be 14,330 excluding iron and sulfur atoms.

Comment on Sequence Study: A cluster of aspartic acid

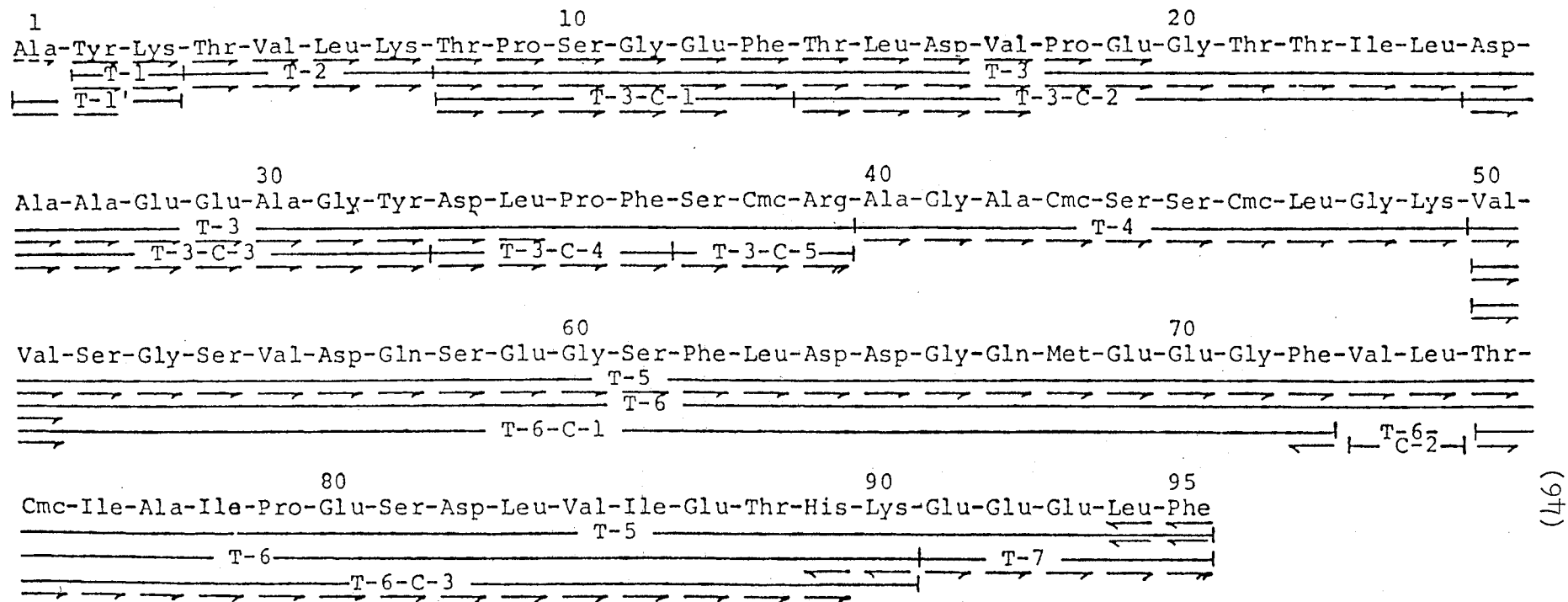
sufficient to hydrolyze these bonds completely.

The presence of N^ε-acetyllysine in H. halobium ferredoxin is the first reported occurrence of this amino acid in an iron-sulfur protein, although other proteins such as histone f2a1 (53) have been shown to contain this derivative. This ferredoxin contained five lysine residues at positions 26, 76, 96, 112, and 118. Comparatively high yields of peptides containing N^ε-acetyllysine (T-8; 14 %, S-13; 11 %, C-5; 51 %) suggested that only lysine 118 was completely acetylated, because the modified lysine could not be detected during sequence studies of any other peptide, and in addition the other four lysine residues were identified as PTH-lysine by thin-layer chromatography. The finding that this specific site of H. halobium ferredoxin was acetylated suggests the presence of an acetylating enzyme and raises questions as to the possible biological significance of acetylation for ferredoxin activity.

4. Sequence Studies of Other 14 Ferredoxins

The amino acid sequence determinations of other ferredoxins are described only in brief on account of limited space. Summaries of sequence studies of each ferredoxin are shown in following figures.

Figure	Ferredoxin
Fig. 4.	<u>E. telmateia</u> ferredoxin I
Fig. 5.	<u>E. telmateia</u> ferredoxin II
Fig. 6.	<u>E. arvense</u> ferredoxin I
Fig. 7.	<u>E. arvense</u> ferredoxin II
Fig. 8.	<u>D. sorlina</u> ferredoxin I
Fig. 9.	<u>D. sorlina</u> ferredoxin II
Fig. 10.	<u>C. caldarium</u> ferredoxin
Fig. 11.	<u>A. sacrum</u> ferredoxin I
Fig. 12.	<u>A. sacrum</u> ferredoxin II
Fig. 13.	<u>N. muscorum</u> ferredoxin I
Fig. 14.	<u>M. laminosus</u> ferredoxin
Fig. 15.	<u>B. stearrowthermophilus</u> ferredoxin
Fig. 16.	<u>C. thiosulfatophilum</u> ferredoxin
Fig. 17.	<u>P. ovalis</u> ferredoxin
Fig. 18.	<u>M. smegmatis</u> ferredoxin



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Fig. 4. Summary of the sequence studies of *E. telmateia* ferredoxin I. T- and C- refer to peptides derived by tryptic digestion of Cm-ferredoxin and chymotryptic digestion of tryptic peptides, respectively. Arrows, (→) and (←), below the sequence show, respectively, Edman degradation by use of a sequence analyzer, which was previously reported(25) and carboxypeptidase A digestion of Cm-ferredoxin. Arrows, (→) and (←), below the peptide fragments show, respectively, manual Edman degradation and carboxypeptidase A or B digestion. Arrow, (→), shows the direct identification of amino acids on the analyzer after completion of Edman degradation.

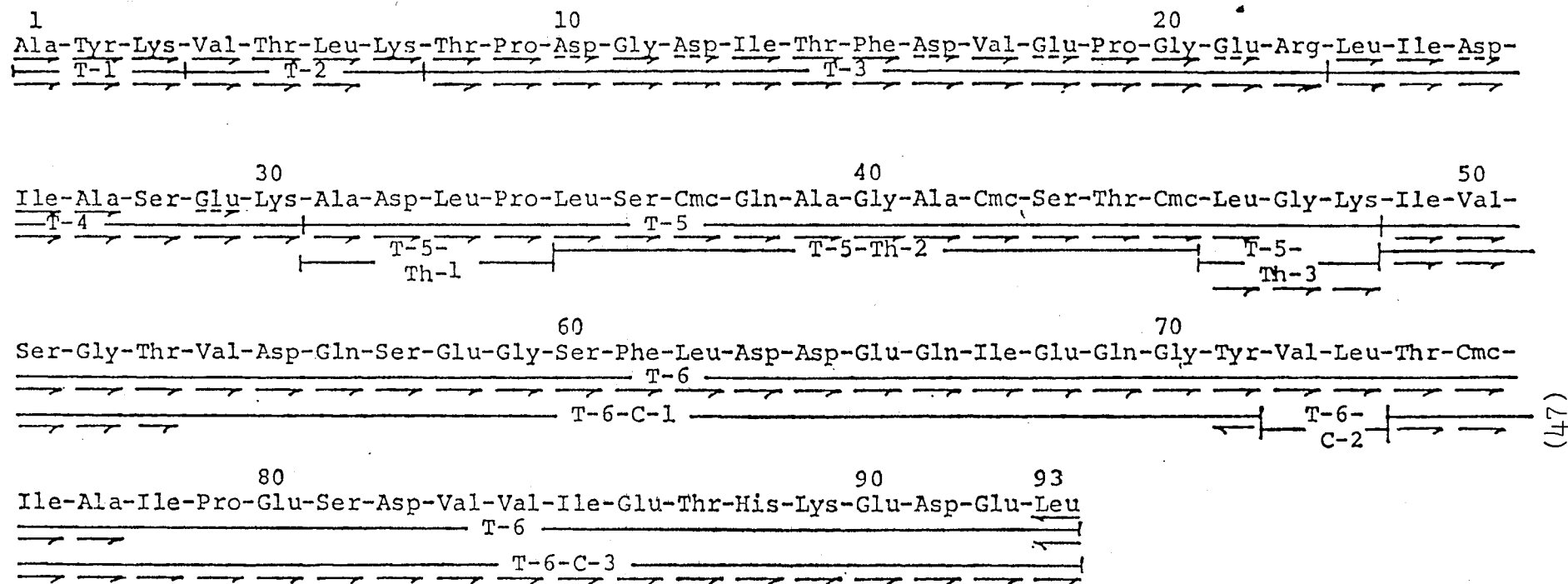
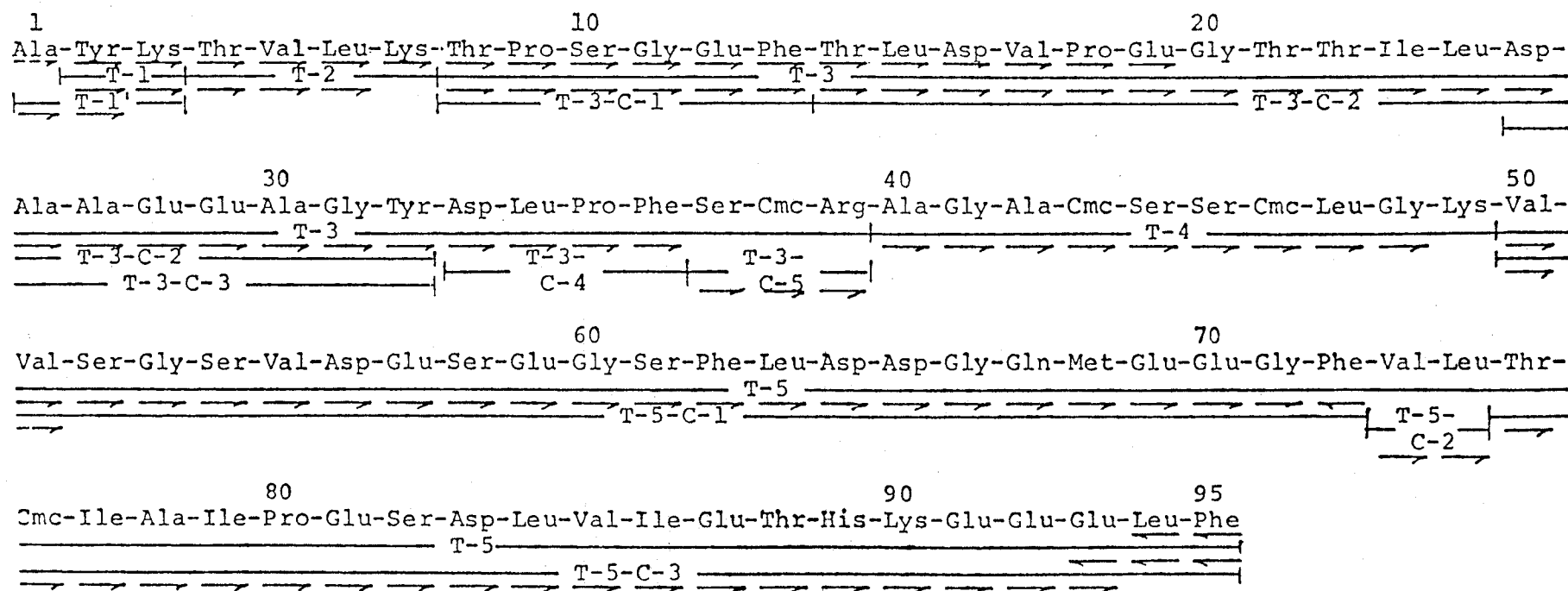


Fig. 5. Summary of the sequence studies of *E. telmateia* ferredoxin II. T-, C-, and Th- represent peptides derived by tryptic digestion of Cm-ferredoxin, and chymotryptic and thermolysin digestion of tryptic peptides, respectively. Various arrows show Edman degradations and carboxypeptidase A digestion as described in Fig. 4. The arrow, (--->), indicates ambiguous identification by the sequence analyzer.



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Fig. 6. Summary of the sequence studies of *E. arvense* Cm-ferredoxin I. T- and C- represent peptides produced by tryptic digestion of Cm-ferredoxin and chymotryptic digestion of the tryptic peptides, respectively. Arrows, (→) and (←), show, respectively, manual Edman degradation and carboxypeptidase A digestion of Cm-ferredoxin and peptide fragments. Cmc refers carboxymethylcysteine.

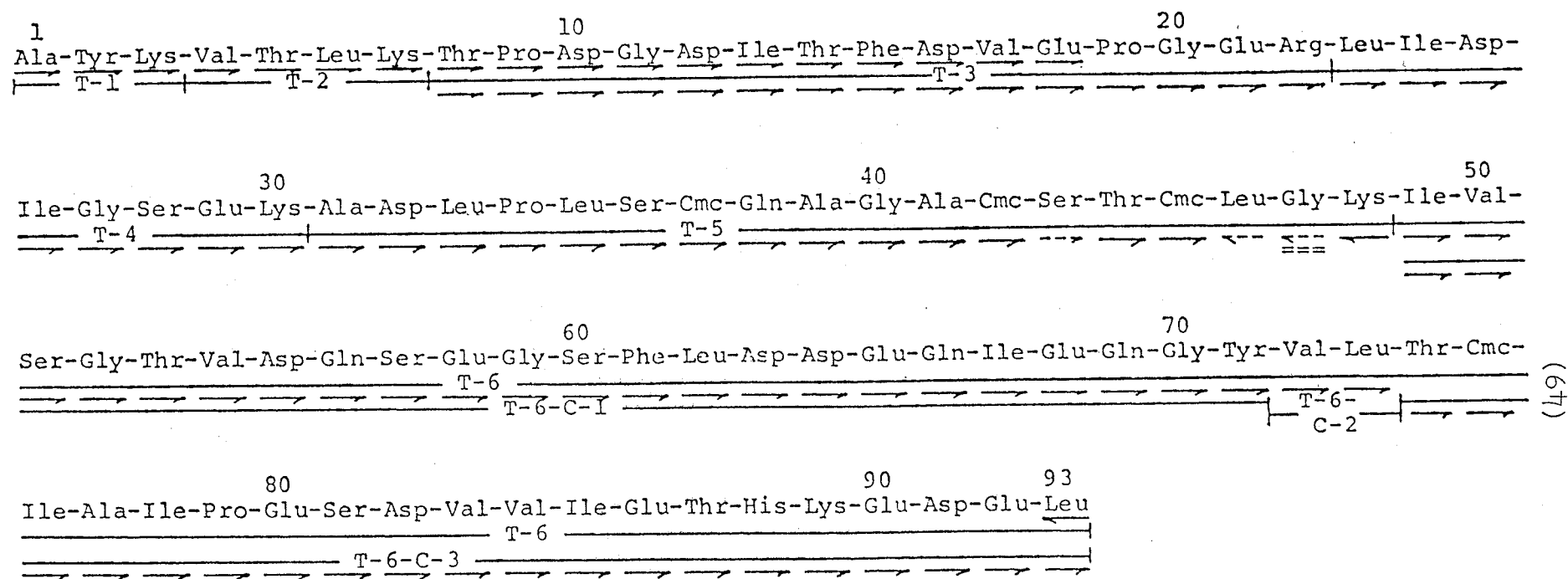


Fig. 7. Summary of the sequence studies of *E. arvense* Cm-ferredoxin II. All notations are as mentioned in Fig. except that the dotted arrows, (∇ ---), refer to the residues released by carboxypeptidase digestion which could not decide the sequence and (===) identification by hydrazinolysis.

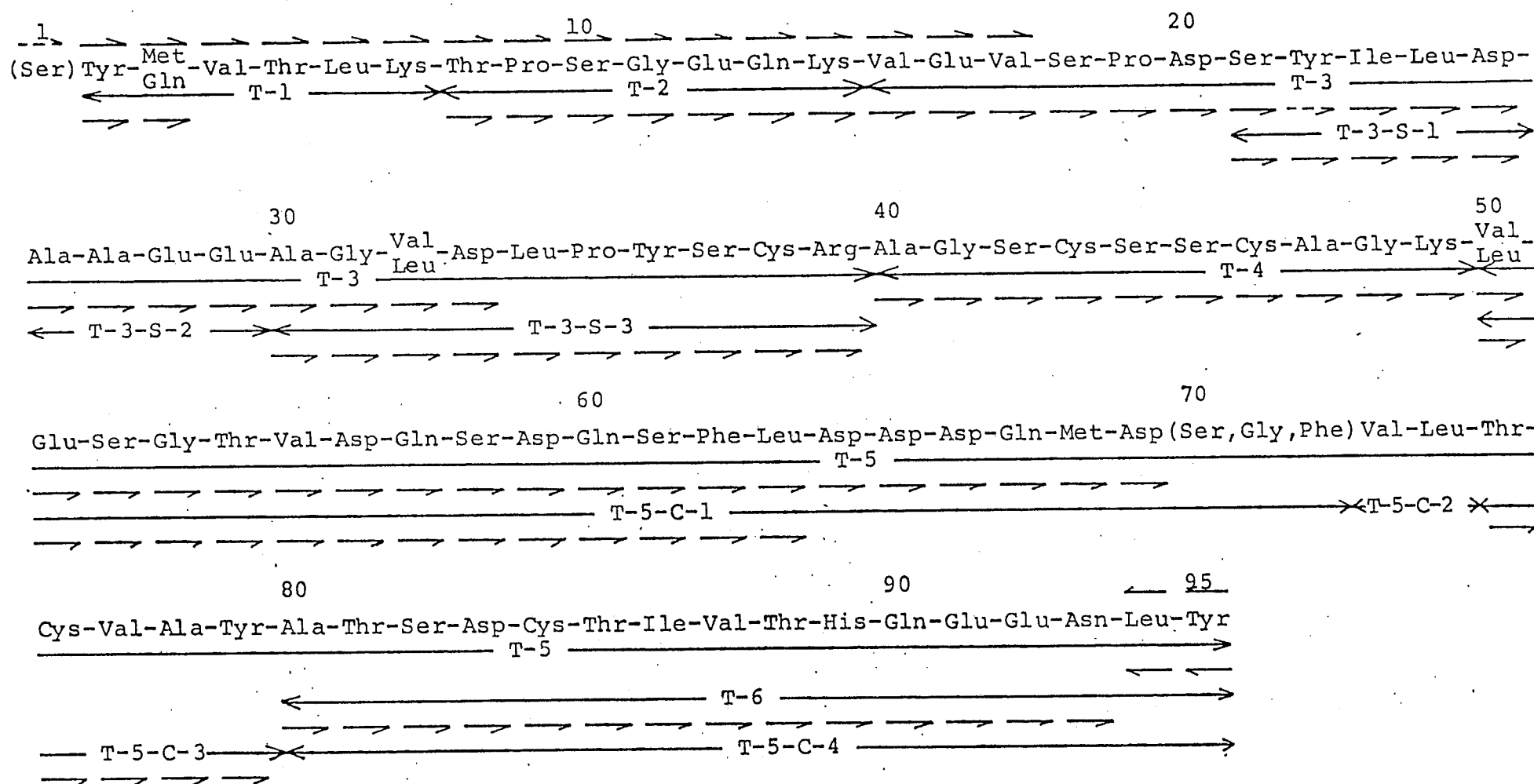


Fig. 8. Summary of the sequence studies of *Dnaliella* Cm-ferredoxin I. T, C, and S refer to tryptic, chymotryptic, and staphylococcal protease peptides, respectively, and Cys refer to Cm-cysteine. Arrows, (→) and (←), above the sequences represent Edman degradation and carboxypeptidase A digestion on Cm-ferredoxin I, respectively. Arrows, (→) and (←), below the sequence represent Edman degradation and carboxypeptidase Y digestion on peptides.

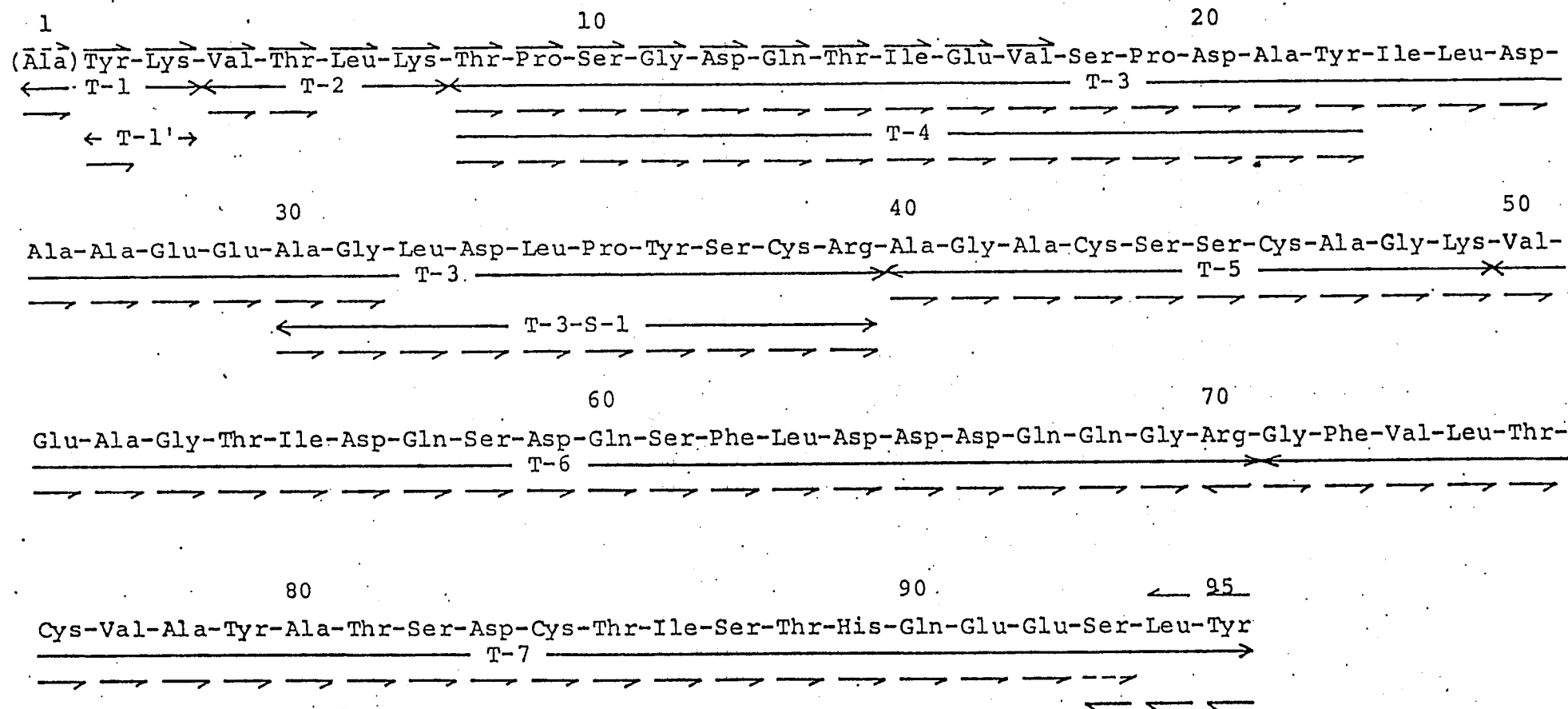


Fig. 9. Summary of the sequence studies of *Dunaliella* Cm-ferredoxin II. T and S refer to tryptic and staphylococcal protease peptides and Cys refers to Cm-cysteine. Arrows () and (), above the sequence represent Edman degradation and carboxypeptidase A digestion on Cm-ferredoxin II, respectively. Arrows () and (), below the sequence represent Edman degradation and carboxypeptidase B or Y digestion on peptides.

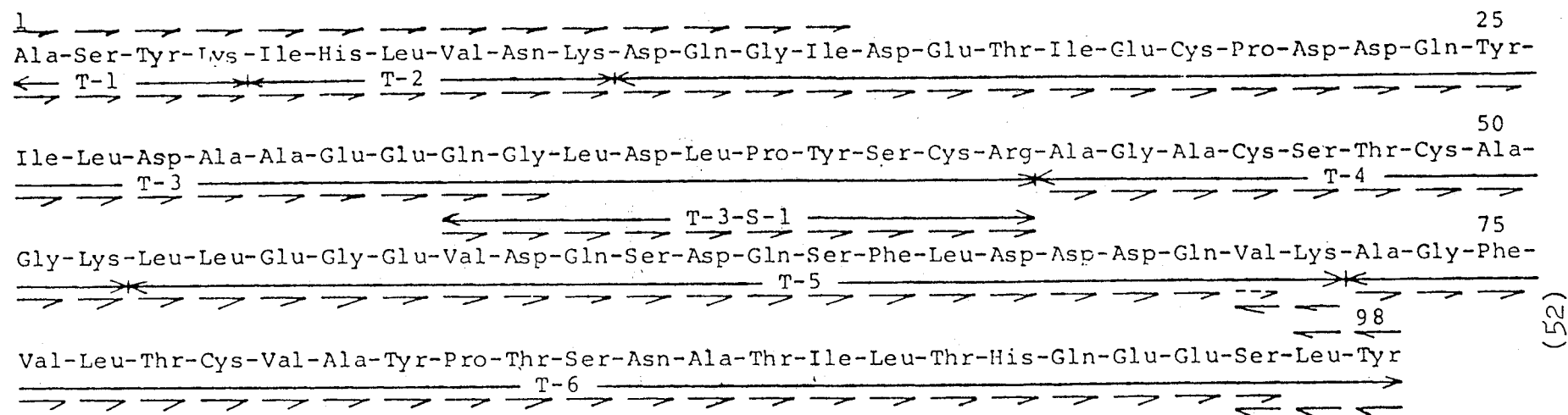


Fig. 10. Summary of the sequence studies of *Cyanidium* ferredoxin. T- and S- refer, respectively, to peptides derived by tryptic digestion of Cm-ferredoxin and staphylococcal protease digestion of peptide T-3. Arrows (→) above the sequence and below the sequences of peptides show, respectively, Edman degradations for Cm-ferredoxin and peptides. Arrows (←) above and below the sequence show, respectively, carboxypeptidase A digestion on Cm-ferredoxin and peptides. A dotted arrow indicates an ambiguous identification.

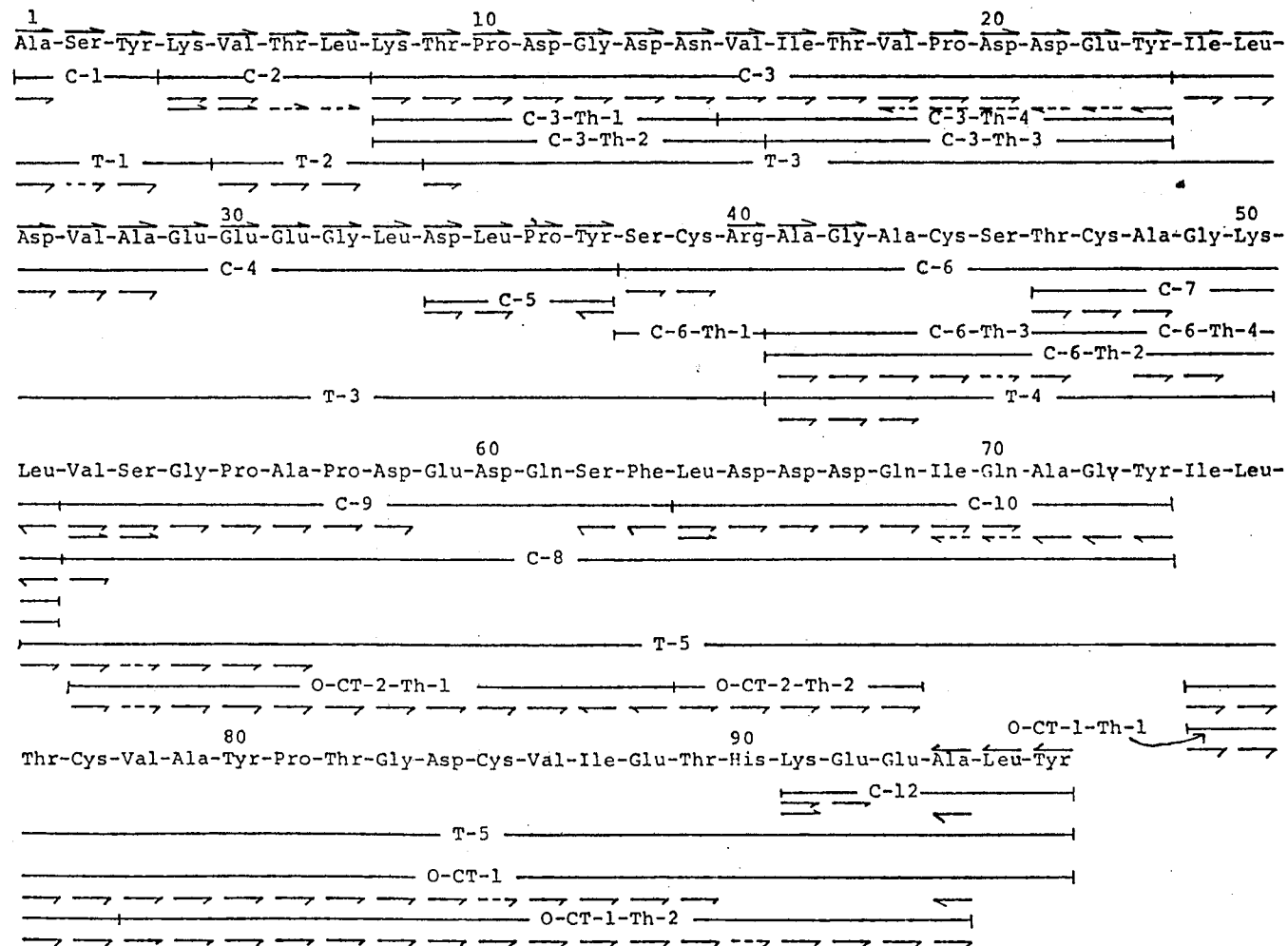


Fig. 11. Summary of the amino acid sequence studies of *Aphanothece sacrum* ferredoxin I. Each step of Edman degradation on ferredoxin, using a sequence analyzer (24), is shown by an arrow (→) just above the sequence. An arrow (←) just above the sequence represents carboxypeptidase A digestion of ferredoxin (24). C and T represent the peptides derived by chymotryptic and tryptic digestion of Cm-ferredoxin and O-CT represents the peptides derived by the digestion of O-ferredoxin by chymotrypsin. Arrows (→), (⇨), and (←) below the sequence show, respectively, manual Edman degradation, leucine aminopeptidase digestion, and carboxypeptidase digestion of peptides. The dotted arrows indicate uncertain identification by each procedure.

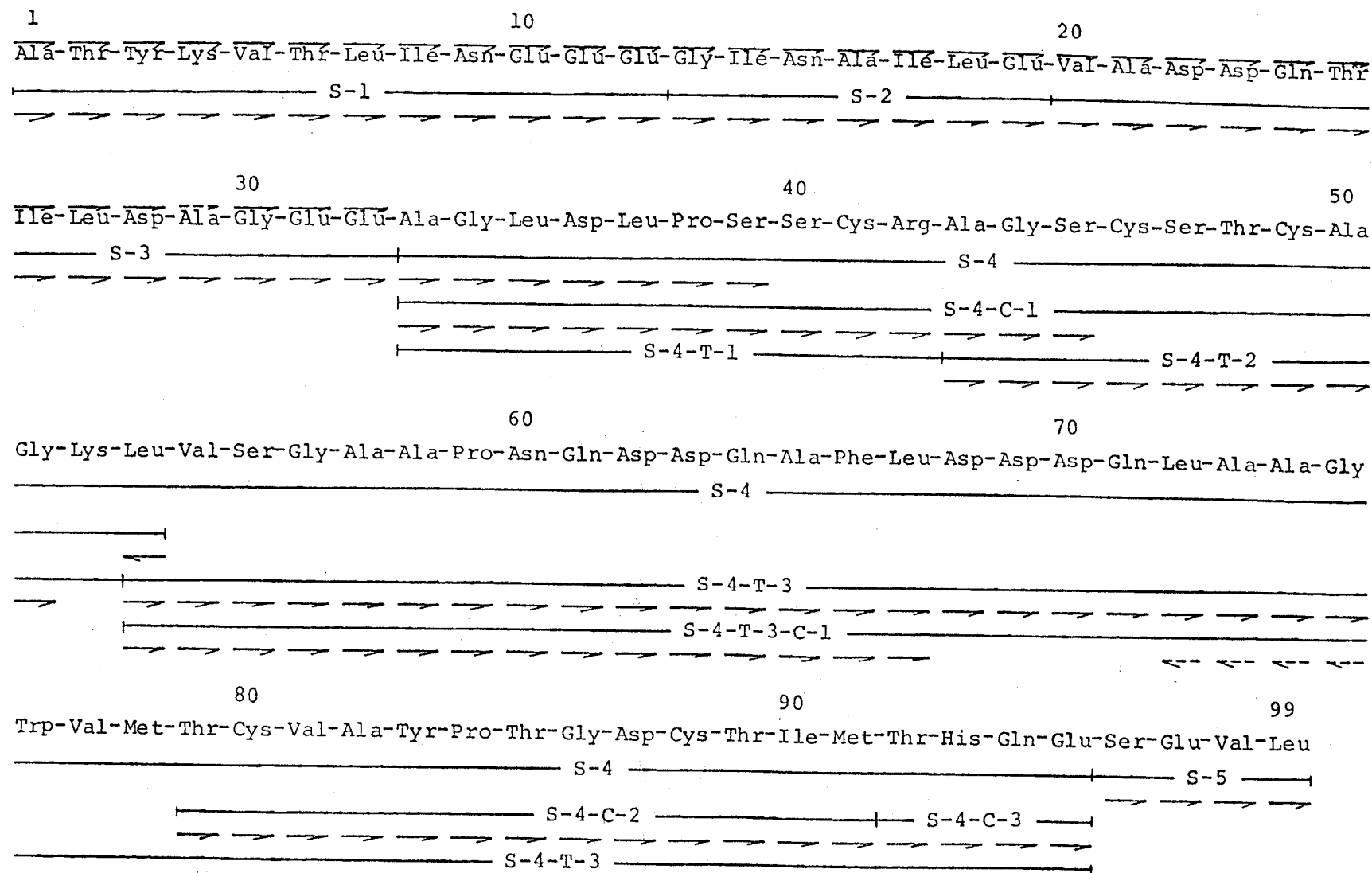


Fig. 12. Summary of the sequence studies of *A. sacrum* ferredoxin II. S- refers to peptides derived by staphylococcal protease digestion of Cm-ferredoxin, and T- and C- to peptides derived by tryptic digestion of S- peptides and chymotryptic digestion of either S- peptides or S-T- peptides, respectively. Arrows (→) above the sequence and below the sequences of peptides show, respectively, Edman degradation using a sequence analyzer for Cm-ferredoxin and manual Edman degradation of various peptide fragments. Arrows (↖) and (↗), below the peptide sequences show, respectively, carboxypeptidase A digestion with clear and with ambiguous identification. Cys refers to carboxymethylcysteine.



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Fig. 13. Summary of the amino acid sequence studies of *Nostoc muscorum* ferredoxin. T- and C- represent peptides obtained by tryptic digestion of Cm-ferredoxin and chymotryptic digestion of tryptic peptides, respectively. Arrows, (↗) and (↖), below the sequence show, respectively, manual Edman degradation and carboxypeptidase digestion of peptides. Arrows, (↗↗), show the direct identification of amino acids on the analyzer at the last step of Edman degradation. The dotted arrows indicate uncertain identification by carboxypeptidase A digestion. Cys was identified as PTH-Cm-cysteine.

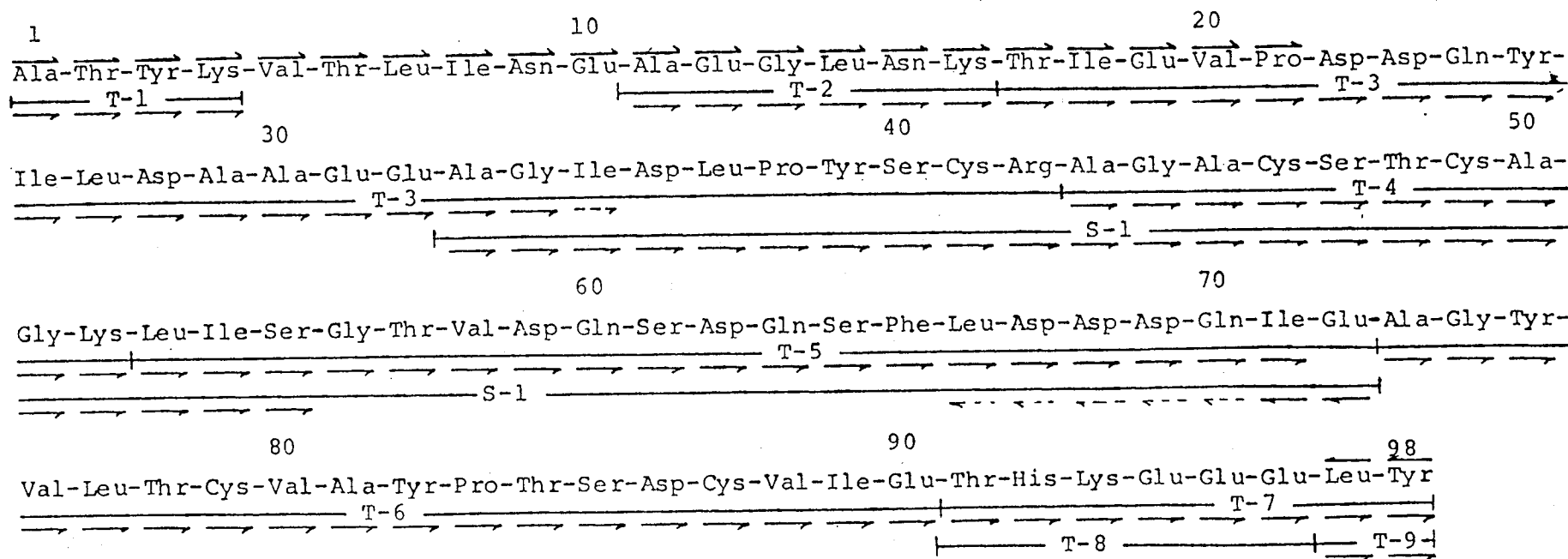


Fig. 1. Summary of sequence studies of *Mastigocladus* ferredoxin. T and S refer to tryptic and staphylococcal protease peptides, and Cys refers to Cm-cysteine. Arrows, (→) and (←), above the sequence represent Edman degradation and carboxypeptidase A digestion on Cm-ferredoxin, respectively. Arrows, (↗) and (↖), below the sequence represent Edman degradations and carboxypeptidase digestion on peptide fragments. Dotted arrows indicate amino acids released by carboxypeptidase without decision of sequence.

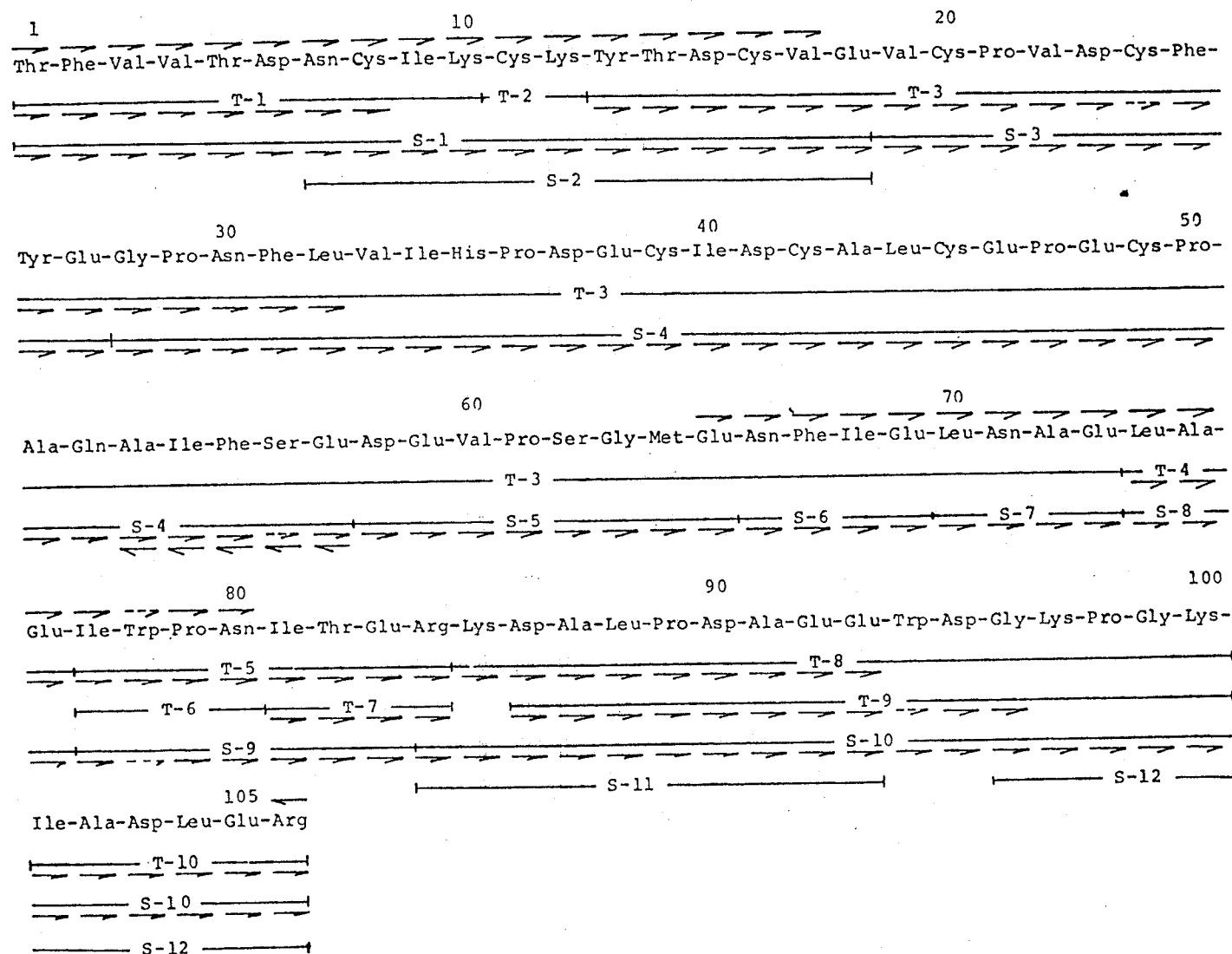
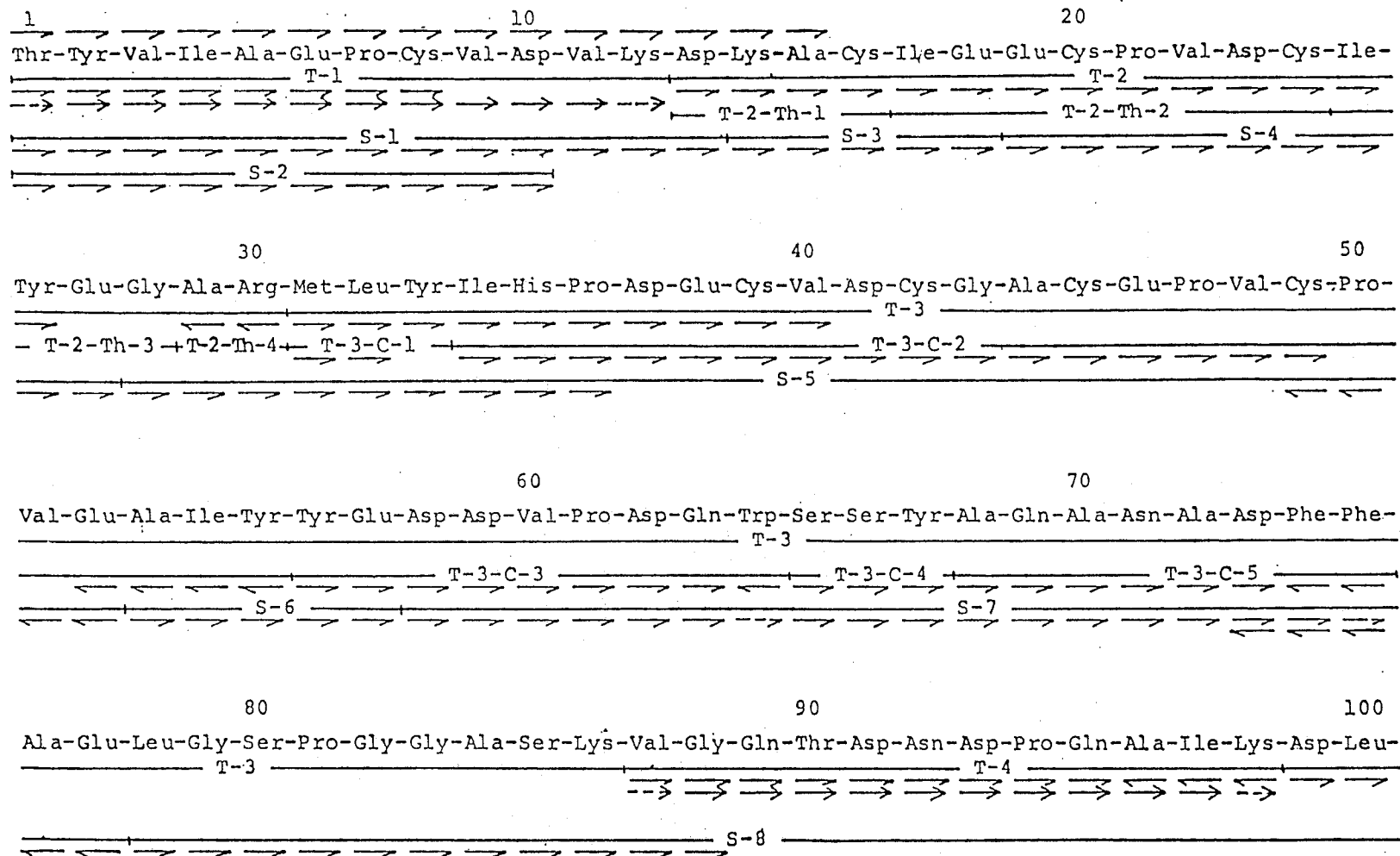


Fig. 17. Summary of the sequence studies of *Ps. ovalis* ferredoxin I. The arrows (→) and (←) above the sequence indicate a manual Edman degradation and carboxypeptidase B digestion, respectively, on Cm-ferredoxin. T- and S- represent the peptides obtained by trypsin and staphylococcal protease digestion, respectively, of Cm-ferredoxin. Arrows, (→) and (←) below the peptide sequences indicate Edman degradation and carboxypeptidase Y digestion, respectively. Dotted arrows indicate ambiguous identifications.



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Fig. 18. Summary of the sequence studies of *M. smegmatis* ferredoxin.

Pro-Pro-Gln-Gly-Glu-Asp
 T-5

T-, S-, C-, and Th- refer respectively, to peptides derived by tryptic and staphylococcal protease digestion of Cm-ferredoxin and cymotryptic digestion of Peptide T-3 and thermolysin digestion of Peptide T-2.

IV. STRUCTURAL CHARACTERISTICS OF FERREDOXINS

1. Introduction

From the content of iron and sulfur, it seems likely that most of ferredoxins so far characterized fall into three groups, those with 2Fe-2S, 4Fe-4S, and 8Fe-8S. Ferredoxins with 2Fe-2S have a binuclear iron cluster with two bridging inorganic sulfurs and coordinated with 4 cysteine residues in protein and ferredoxins with 4Fe-4S or 8Fe-8S have one or two tetranuclear iron clusters with 4 bridging inorganic sulfurs and coordinated with 4 cysteine residues in protein as shown in Fig. 1. At present, 24 amino acid sequences of chloroplast-type ferredoxins from various plants and algae, 2 amino acid sequences of Halobacterium ferredoxins, and 16 amino acid sequences of various bacterial ferredoxins are available. Table 1 lists the various ferredoxins which have been sequenced. Chloroplast-type and Halobacterium ferredoxins contain 2Fe-2S, Desulfovibrio gigas and Bacillus stearothermophilus ferredoxins 4Fe-4S, and other bacterial ferredoxins 8Fe-8S. From the comparison of sequences of these ferredoxins, various interesting characteristics are recognized. In this chapter, I describe the structural characteristics of the ferredoxins whose sequences are determined in chapter III.

Table 1. The ferredoxins whose sequences are being determined.

2Fe-2S	
Angiosperms	<u>Spinach</u> (12, 33, 34), <u>Alfalfa</u> (54), <u>Koa</u> (55), <u>Taro</u> (56), <u>P. americana</u> (I, II) (57) <u>P. esculenta</u> (I, II) (58)
Fern	<u>G. japonica</u> (59)
Horsetails	<u>E. telmateia</u> (I, II) (60), <u>E. arvense</u> (I, II) (61)
Green algae	<u>S. quadricauda</u> (62), <u>D. sorlina</u> (I, II) (63)
Red algae	<u>P. umbilicalis</u> (64), <u>C. caldarium</u> (65)
Blue-green algae	<u>S. maxima</u> (66), <u>S. platensis</u> (67, 68) <u>A. sacrum</u> (I, II) (69, 70), <u>N. muscorum</u> (65) <u>M. laminosus</u> (72)
Halophilic bacteria	<u>H. halobium</u> (73, 74), <u>H. The Dead Sea</u> (75)
4Fe-4S	
Sulfate reducing bacteria	<u>D. gigas</u> (76)
Aerobic bacteria	<u>B. stearothermophilus</u> (77)
8Fe-8S	
Anaerobic bacteria	<u>C. pasteurianum</u> (11, 32), <u>C. butyricum</u> (78), <u>P. aerogenes</u> (79), <u>C. aciduri</u> (80), <u>C. tartarivorum</u> (81), <u>M. elsdenii</u> (82), <u>C. thermosaccharolyticum</u> (83), <u>C. M-E</u> (84)
Photosynthetic bacteria	<u>C. vinosum</u> (85, 86), <u>C. limicola</u> (I, II) (87, 88) <u>C. thiosulfatophilum</u> (89)
Aerobic bacteria	<u>P. ovalis</u> (90), <u>M. smegmatis</u> (91)

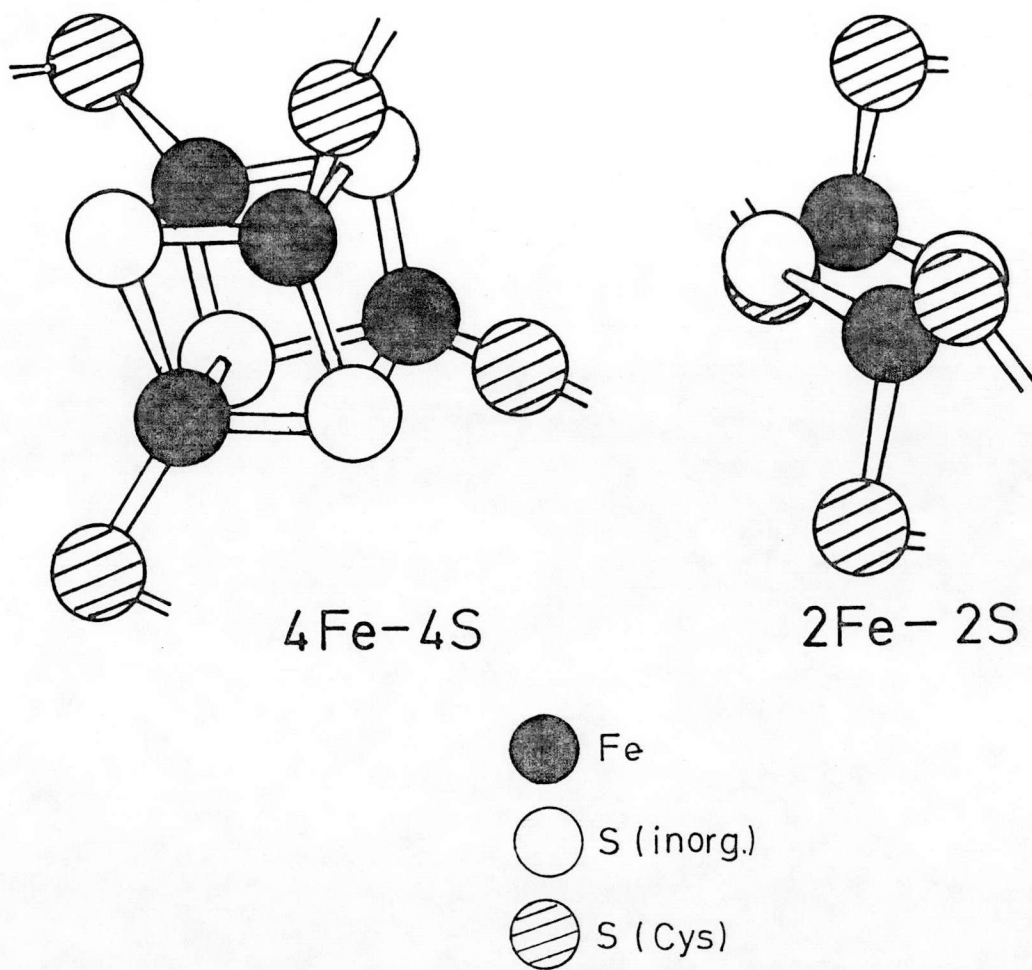


Fig. 1. Active centers of 2Fe-2S and 4Fe-4S

2. 2Fe-2S Ferredoxins

2.1. Distribution of Amino Acid Residues of Chloroplast-Type Ferredoxins

Twenty four amino acid sequences of chloroplast-type ferredoxins are shown in Fig. 2, together with 2 sequences of Halobacterium ferredoxins. Several gaps are placed to make all sequences maximally homologous. The amino acid compositions of these ferredoxins are also

	10	20	30	40
(1) Koa	-AFKVKLLT-PDG	-PKEFECPDDVYIL	DQAEELGIDL	PPYSCRAGS
(2) Spinach	AAYKVTLLVT-PTG	-NVEFQCPDDVYIL	DAEEEEGIDL	PPYSCRAGS
(3) Alfalfa	ASYKVKLLVT-PEG	-TQEFQCPDDVYIL	DHAEEEGIVL	PPYSCRAGS
(4) Taro	ATYKVKLLVT-PSG	-QQEFQCPDDVYIL	DQAEEEVGLD	PPYSCRAGS
(5) <i>P. americana</i> I	ATYKVTLLVT-PSG	-TQTIDCPDDTYVL	DAEEEEAGLD	PPYSCRAGS
(6) <i>P. americana</i> II	AASYKVTFFVT-PSG	-TNTITCPCADTYVL	DAEEESGLDL	PPYSCRAGA
(7) <i>P. esculenta</i> I	ATYKVTLLVT-PSG	-TQTIDCPDDTYVL	DAEEEEAGLD	PPYSCRAGS
(8) <i>P. esculenta</i> II	AASYKVTFFVT-PSG	-TKTITCPCADTYVL	DAAEEDTGLD	PPYSCRAGA
(9) <i>G. japonica</i>	AIFKVKFLT-PDG	-ERTIEVPDDKFI	LDAGEEEAGLD	PPYSCRAGA
(10) <i>E. telmateia</i> I	-AYKTVLKT-PSG	-EFTLDVPEGTTI	LDAAEEAGYD	LPFSCRAGA
(11) <i>E. telmateia</i> II	-AYKTVLKT-PSG	-DITFDVEPGERLI	DIASEKA-DL	PLSCQAGA
(12) <i>E. arvense</i> I	-AYKTVLKT-PSG	-EFTLDVPEGTTI	LDAAEEAGYD	LPFSCRAGA
(13) <i>E. arvense</i> II	-AYKTVLKT-PSG	-DITFDVEPGERLI	DIASEKA-DL	PLSCQAGA
(14) <i>S. quadricauda</i>	ATYKVTLLKT-PSG	-DQTIIECPDDTYI	LDAAEEAGLD	PPYSCRAGA
(15) <i>D. sorlina</i> I	-SYMVTLLKT-PSG	-EEKVEVSPDSYI	LDAAEEAGVD	LPYSCRAGS
(16) <i>D. sorlina</i> II	-AYKVTLLKT-PSG	-DQTIIEVSPDAYI	LDAAEEAGLD	PPYSCRAGA
(17) <i>P. umbilicalis</i>	ADYKIHLLVSKKEGI	DVTFDCSEDTYIL	DAEEEGGIEL	PPYSCRAGA
(18) <i>C. caldarium</i>	ASYKIHLLVNKDDQGI	DETEIECPDDQYI	LDAAEEQGDL	PPYSCRAGA
(19) <i>S. platensis</i>	ATYKVTLLINEAEGIN	ETIDCDDDTYIL	DAEEEGAGLD	PPYSCRAGA
(20) <i>S. maxima</i>	ATYKVTLLISEAEGIN	ETIDCDDDTYIL	DAEEEGAGLD	PPYSCRAGA
(21) <i>N. muscorum</i> I	ATFKVTLLINEAEGTK	HEIEVPDDQYIL	DAEEEGYDLP	FPSCRAGA
(22) <i>M. laminosus</i>	ATYKVTLLINEAEGLN	KTIIEVPDDQYIL	DAEEEGAGLD	PPYSCRAGA
(23) <i>A. sacrum</i> I	ASYKVTLLKT-PDG	-DNVITVPDDEYI	LDVAAEEEGLD	PPYSCRAGA
(24) <i>A. sacrum</i> II	ATYKVTLLINEEEGIN	AILLEVADDDQTI	LDAGEEAGLD	LPSSCRAGS
(25) <i>H. halobium</i>	LF EKAAADAGLDGEDY	GTMEVVAEGEYILE	AAEAQGYDWP	FPSCRAGA
(26) <i>H. Dead Sea</i>	VFG EASDMDLDEDY	GSLEVN EGEYILE	AAEAQGYDWP	FPSCRAGA

(1) CSSCAGKLV	EGDL-DQSDQSF	LDDEQIIEEGWV	-LTC	AAAYPRSDVVI	ETHKKEEEL	LTG
(2) CSSCAGKLV	KTGSL-NQDDQSF	FLDDDDQIIEEGWV	-LTC	AAAYPVSDVTI	ETHKKEEEL	LTG
(3) CSSCAGKLV	AAGEV-NQSDQSF	FLDDDDQIIEEGWV	-LTC	AAAYPVSDGTI	ETHKKEEEL	LTG
(4) CSSCAGKLV	KVGVGV-DQSDQSF	FLDDDDQIIEAGFV	-LTC	AAAYPVKGDVTI	ETHKKEEEL	IV-
(5) CSSCTGKVT	AGTV-DQEDQSF	FLDDDDQIIEAGFV	-LTC	AAAYPVKGDVTI	ETHKKEEEL	IA-
(6) CSSCAGKVT	AGAV-NQEDQSF	FLDEEQMEAGWV	-LTC	AAAYPVSDVTI	ETHKKEEEL	LSA
(7) CSSCTGKVT	AGTV-DQEDQSF	FLDDDDQIIEAGFV	-LTC	AAAYPVKGDVTI	ETHKKEEEL	IA-
(8) CSSCAGKVT	AGSV-NQEDQSF	FLDEEQMEAGWV	-LTC	AAAYPVSDVTI	ETHKKEEEL	LSA
(9) CSSCTGKVT	AGRV-DQSEQSF	FLDDDDQMAEGFV	-LTC	AAAYPVAGDITI	ETHKKEEEL	KL--
(10) CSSCLGKVV	SGSV-DQSEGSF	FLDDDDQMEEGFV	-LTC	AAAYPVSDLVIE	ETHKKEEEL	FL--
(11) CSTCLGKIV	SGTV-DQSEGSF	FLDDDEQIEEGYV	-LTC	AAAYPVSDLVIE	ETHKKEEEL	FL--
(12) CSSCLGKVV	SGSV-DESEGSF	FLDDDDQMEEGFV	-LTC	AAAYPVSDLVIE	ETHKKEEEL	FL--
(13) CSTCLGKIV	SGTV-DQSEGSF	FLDDDEQIEEGYV	-LTC	AAAYPVSDLVIE	ETHKKEEEL	FL--
(14) CSSCAGKVE	AGTV-DQSDQSF	FLDDDDQMDGGFV	-LTC	AAAYPVSDCTI	ATHKKEEEL	DLF-
(15) CSSCAGKVE	AGTV-DQSDQSF	FLDDDDQM(D,S,G,F)V	-LTC	AAAYPVSDCTI	IVTHKKEEEL	LY-
(16) CSSCAGKVE	AGTI-DQSDQSF	FLDDDDQGRGFV	-LTC	AAAYPVSDCTI	STHKEEESLY	-
(17) CSTCAGKVT	EGTV-DQSDQSF	FLDDDEQMLKGYV	-LTC	AAAYPVSDCTI	ILTHKKEEEL	LY-
(18) CSTCAGKLV	EGEV-DQSDQSF	FLDDDDQVKAAGFV	-LTC	AAAYPVSDCTI	SNATHKKEEEL	LY-
(19) CSTCAGTIT	SGTI-DQSDQSF	FLDDDDQIEAGYV	-LTC	AAAYPVSDCTI	IKTHKKEEEL	LY-
(20) CSTCAGKIT	SGSI-DQSDQSF	FLDDDDQIEAGYV	-LTC	AAAYPVSDCTI	IQTHKKEEEL	LY-
(21) CSTCAGKLV	SGTV-DQSDQSF	FLDDDDQIEAGYV	-LTC	AAAYPVSDCTI	IVTHKKEEEL	LY-
(22) CSTCAGKLV	SGTV-NQSDQSF	FLDDDDQIEAGYV	-LTC	AAAYPVSDCTI	CVIETHKKEEEL	LY-
(23) CSTCAGKLV	SGPAPD-EDQSF	FLDDDDQIQAGYI	-LTC	AAAYPVSDCTI	GVIEETHKKEEEL	LY-
(24) CSTCAGKLV	SGAAPNQDQAF	LDDQLAAGWV-MTC	AAAYPVSDCTI	MTIMTHKKEEEL	SEVL-	
(25) CANCASI	VKEGEI-DMDMQQ	ILSDEEVEEKDVR	LTCIGSPA	ADEVKIVYN	AKHLDY	
(26) CANCAAI	VLEGGDI-DMDMQQ	ILSDEEVEDKNVR	LTCIGSPA	ADEVKIVYN	AKHLDY	

Fig. 2. Amino acid sequences of 24 chloroplast-type and 2 *Halo-*
bacterium ferredoxins. The references for the sequences are
as in Table 1. The numbering starts from the N-terminus of spinach
ferredoxin and therefore, the first residue of *P. americana*
ferredoxin II should be counted as -1. Several gaps shown by -
are inserted to make the sequence homologous alignments.

Table 2. The amino acid compositions of chloroplast-type ferredoxins

^a	(1)	(2)	(3)	(4)	(5)	(6)	(7)	(8)	(9)	(10)	(11)	(12)	(13)	(14)	(15)	(16)	(17)	(18)	(19)	(20)	(21)	(22)	(23)	(24)	(25)	(26)
Lys	5	4	5	5	4	3	4	4	5	4	5	4	5	4	3	3	4	4	2	2	4	4	4	2	5	3
His	1	1	2	1	1	1	1	1	1	1	1	1	1	1	1	1	2	2	1	1	2	1	1	1	1	1
Arg	2	1	1	1	1	1	1	1	3	1	1	1	1	1	1	2	1	1	1	1	1	1	1	1	3	3
Asp	11	11	8	10	12	6	10	8	11	7	10	7	10	12	10	10	10	11	12	12	11	10	12	10	17	21
Asn	0	2	1	0	0	2	0	1	0	0	0	0	0	0	1	0	0	2	2	1	1	2	1	3	4	6
Thr	4	8	6	6	12	12	13	12	6	7	7	7	7	10	7	8	8	6	12	10	8	8	7	8	4	2
Ser	7	7	8	8	6	8	6	9	5	9	7	9	7	8	13	10	7	7	6	8	6	6	5	6	4	5
Glu	12	9	13	9	7	10	7	8	9	12	10	13	10	6	7	6	12	8	8	8	11	10	8	8	16	14
Gln	4	4	3	6	4	2	4	2	3	2	4	1	4	4	5	6	4	7	4	5	4	4	3	5	5	4
Pro	5	4	3	4	4	4	4	4	4	4	4	4	4	4	3	3	2	3	2	2	3	3	6	3	3	3
Gly	7	6	7	9	7	7	7	7	8	9	7	9	8	7	6	7	6	6	7	7	6	6	7	8	9	8
Ala	6	9	9	6	8	13	9	12	9	7	6	7	5	10	7	11	7	9	10	10	9	10	9	13	14	11
Cys	5	5	5	5	5	5	5	5	4	4	4	4	4	6	5	5	6	5	6	6	4	5	5	5	4	4
Val	6	7	9	10	9	8	8	8	5	7	7	7	7	5	8	5	6	5	3	3	8	6	7	6	8	11
Met	0	0	0	0	0	1	0	1	1	1	0	1	0	1	2	0	1	0	0	0	0	0	0	2	14	4
Ile	4	4	4	4	4	2	4	2	5	4	8	4	8	3	2	4	6	5	8	8	5	7	5	5	7	7
Leu	10	8	6	6	6	6	6	6	9	9	8	9	8	7	7	7	8	10	7	7	7	8	8	9	9	9
Tyr	3	4	4	4	3	4	4	4	2	2	2	2	2	4	5	5	6	5	6	6	5	6	6	2	7	8
Phe	3	2	2	2	3	2	2	2	5	5	2	5	2	3	2	2	2	2	1	1	3	1	1	1	2	2
Trp	1	1	1	1	0	1	0	1	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	1	2	2
Total	96	97	97	97	96	98	96	98	95	95	93	95	93	96	95	95	98	98	98	98	98	98	96	99	128	128

^a The numbers in parentheses represent ferredoxins as described in Fig. 2.

(65)

presented in Table 2.

Cysteine residues: Chloroplast-type ferredoxins contain a cluster with two iron and two inorganic sulfur atoms and sulfhydryl groups of 4 cysteine residues chelating two iron atoms as shown in Fig. 1. It is important to establish the location of the cysteine residues in the protein to form a 2Fe-2S cluster. There is a total of 4 to 6 cysteine residues in the various ferredoxins so far studied. From the comparison of 24 sequences of chloroplast-type ferredoxins, 4 types of cysteine distribution are found as shown in Table 3. All types of ferredoxins have substantially similar spectroscopic properties and the common 4 cysteine residues are considered to be involved in the formation of the cluster.

Table 3. The distribution of cystein residues of chloroplast-type ferredoxins

Type	number of cysteine residues	position of cysteine residues ^a
1	6	20, 41, 46, 49, 79, 87
2	5	20, 41, 46, 49, 79
3	5	41, 46, 49, 79, 87
4	4	41, 46, 49, 79

^aThe numbering of cysteine residues is that used for S. platensis ferredoxin

S. platensis ferredoxin is representative of type 1, spinach ferredoxin type 2, and A. sacrum ferredoxin I type 3. Ferredoxins having only 4 cysteine residues (type 4) have

been completely sequenced for the first time in this study, and they are from E. telmateia (60), E. arvense (61), and N. muscorum (71) and the 4 cysteine residues are located at positions 41, 46, 49, and 79. On several occasions, the 4 cysteine residues required to chelate two iron atoms were suggested to be located at the same positions as described above (24, 25, 27, 71, 92). This proposal has been recently confirmed by an X-ray crystallographic analyses of S. platensis ferredoxin and A. sacrum ferredoxin I showing that the clusters of both ferredoxins are located near the molecular surface (93, 94) and that the sulfur atoms of Cys 41 and Cys 46 are coordinated to an iron atom and those of Cys 49 and Cys 79 to the other in S. platensis ferredoxin (95). Therefore, it is concluded that the distribution of cysteine residues required for the formation of a 2Fe-2S cluster in chloroplast-type ferredoxins are those in a -Cys-X-X-X-X-Cys-X-X-Cys- segment in the middle of molecule and a -Cys- far from this segment.

Invariant and Semi-invariant residues: In comparison of 24 available sequences of chloroplast-type ferredoxins, there are 24 invariant residues and 22 semi-invariant residues. These residues are considered to occupy structurally and functionally essential portions of the molecule.

is shown in Fig. 3, residues, Gly 13, Asp 28, Leu 37, Pro 38, Ser 40, Cys 41, Ala 43, Gly 44, Cys 46, Ser 47, Cys 49, Gly 51, Gly 56, Phe 65, Leu 66, Gln 70, Gly 74, Thr 78, Cys 79, Ala 81, Ile 89, Thr 91, His 92, and Glu 94

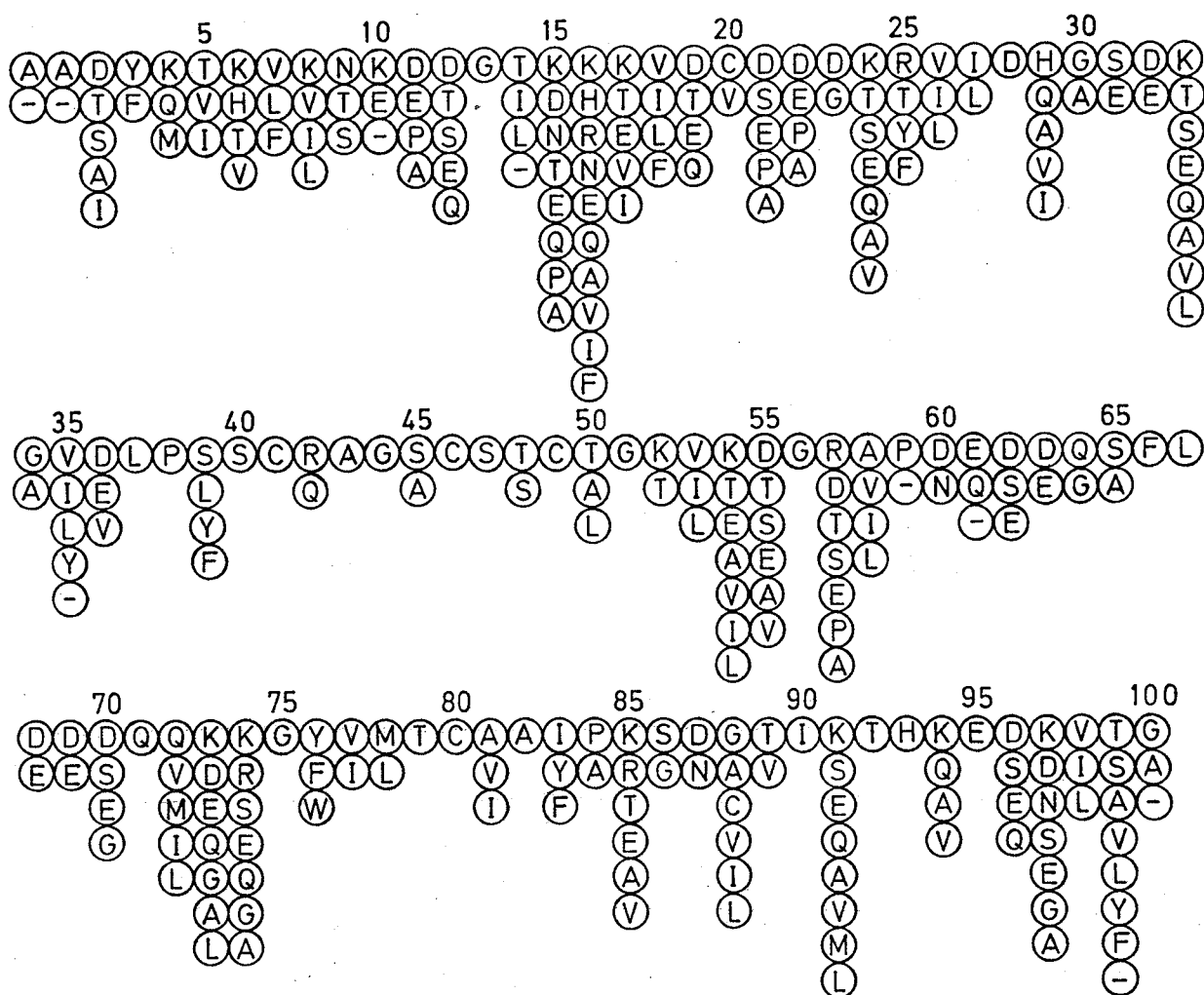


Fig. 3. Distribution of various amino acids in the chloroplast-type ferredoxins as shown in Fig. 2 except for Halobacterium ferredoxins. - represents deletions.

are invariant and those, Tyr or Phe 3, Val, Leu, or Phe 7, Asn, Ser, or Thr 9, Val, Ile, Leu, or Phe 18, Val, Ile, or Leu 26, Ile or Leu 27, Gly or Ala 30, Asp or Glu 32, Gly or Ala 34, Ser or Ala 45, Thr or Ser 48, Val, Ile, or Leu 53, Asp or Glu 62, Ser or Ala 64, Asp or Glu 67, Asp or Glu 68, Tyr, Phe, or Trp 75, Val or Ile 76, Met or Leu 77, Ile, Tyr, or Phe 82, Ser or Gly 85, and Val, Ile, or Leu 97 are semi-invariant (or conservative). The residue numbers correspond to those of S. platensis ferredoxin which have a deletion at position 59 as shown in Fig. 2.

The three dimensional structure of S. platensis ferredoxin has been elucidated recently (95). The molecule has approximate dimensions 40 x 35 x 25 Å and the center of the 2Fe-2S cluster is about 8 Å from the molecular surface. Folding of the main chain represented by α -carbons is shown in Fig. 4, although the fine structures of side chains are not available. It is of interest to trace the locations of the invariant and semi-invariant residues on the three dimensional structure of S. platensis ferredoxin. Invariant residues are marked in the three dimensional structure as shown in Fig. 5. Four cysteine residues, Cys 41, Cys 46, Cys 49, and Cys 79, binding the two iron atoms are, of course, invariant and all located at the top of the figure. Seven invariant residues, Pro 38, Ser 40, Ala 43, Gly 44, Ser 47, Thr 78, and Ala 81 and 3 semi-invariant residues, Ser or Ala 45, Ser or Thr 48, and Ser or Ala 64 surround the cluster. It is clear that these

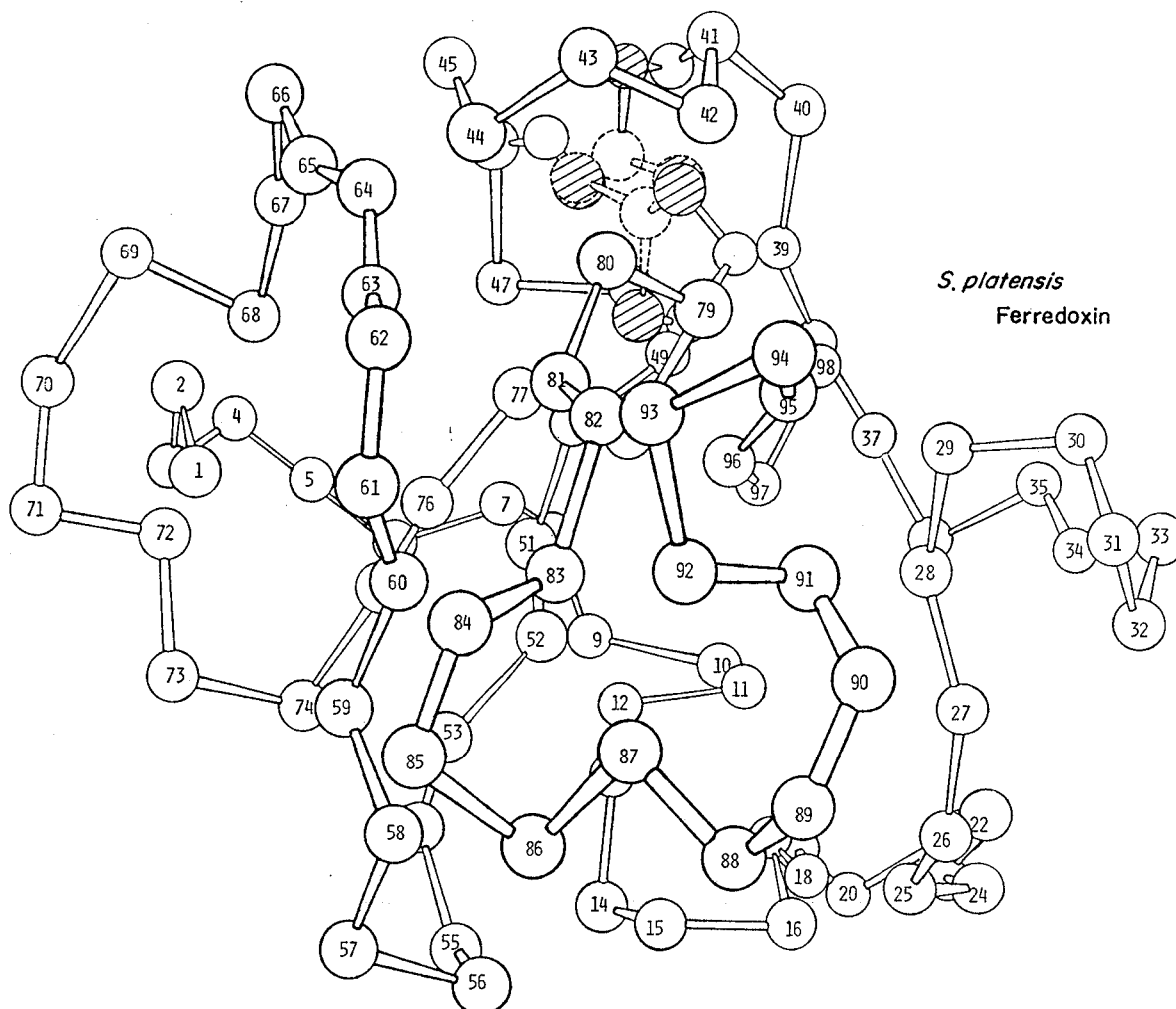


Fig. 4. Three dimensional structure of S. platensis ferredoxin. Amino acid sequence of this protein is as follows (67),

The cluster is located at the top of the molecule and only the chain from the Cys 41 to Cys 46 covers the top-front side of the cluster. The chain around the cluster folds relatively closely, while that at lower part of the molecule fold loosely. There is no α -helix and the structure of 3_{10} turn is found; Asn 9 - Glu 12, Ile 14 - Thr 17, Asp 19 - Asp 22, Ala 30 - Ala 33, Tyr 39 - Arg 42, and Thr 84 - Cys 87 (95).

sponds to Tyr 75 in S. platensis ferredoxin was suggested to be near the cluster from the fluorescence measurements (96) and if the side chain of tryptophan points to the cluster region, the quenching effect of the cluster to tryptophan fluorescence might be reasonably explained by the close distance of these two chromophores revealed by X-ray.

From these observations, it is suggested that the cluster cavity is surrounded mainly by invariant or semi-invariant residues and that these evolutionary conserved residues make a common environment for 2Fe-2S clusters of various ferredoxins with completely different amino acid sequences.

Other invariant or semi-invariant residues are located apart from the cluster region. Some special features are further found. Four glycine residues at positions 13, 51, 56, and 74 are invariant and may be necessary for the peptide chain folding. There is a unique portion where several hydrophobic residues, Ile 89, Val, Ile, Leu, or Phe 18, Cys or Val 20, Val, Ile, or Leu 26, and Val, Ile, or Leu 27 occur close together on the three dimensional structure. It has been reported that Cys 20 in spinach and S. platensis ferredoxins were not reacted with modification reagent in native form, and but reacted with them in the presence of denaturant (92, 97). These hydrophobic residues are considered to be buried in the interior of the molecule to aid in constructing an stable environment for the cluster. Hydrophilic residues, Asp 28, His 92, Glu 94, and Asp or Glu at positions 32, 62, 67, and 68 are located at the exterior

of the molecule. In contrast to the residues forming the cluster cavity there is no special feature on the surface such as charged side chains conserved, even though some of these residues might be involved in a specific interaction with ferredoxin linked enzymes.

Variant Residues: Variant residues are defined as positions in which more than 6 different amino acid residues are found in this case and 14 positions, 15 (8 residues), 16 (10 residues), 26 (7 residues), 33 (8 residues), 54 (7 residues), 55 (6 residues), 57 (7 residues), 72 (7 residues), 73 (7 residues), 84 (6 residues), 87 (6 residues), 90 (8 residues), 96 (7 residues), and 98 (7 residues) are found. The numbering of positions correspond to that for S. platensis ferredoxin. The distribution of these residues in the primary structure does not give any special feature except for the fact that they are not found near the functionary important cysteine residues. But it is obvious from the three dimensional structure as shown in Fig. 5 that variant residues occur at the lower and surface region of the molecule and that a remarkable compartmentation between variant and invariant residue are recognized. It is possible to exclude these variant residues from the region thought to participate in the oxidation-reduction mechanism of ferredoxin.

2. 2. Halobacterium Ferredoxin- A New Chloroplast-Type
Ferredoxin

The 2Fe-2S ferredoxins were isolated from H. halobium (this study and ref. 14) and H. The Dead Sea (21). Both ferredoxins are composed of 128 amino acid residues including only four cysteine residues. These two sequences are compared to chloroplast-type ferredoxins as shown in Fig. 2 and in Fig. 6.

1 10 20

(A) P T V E Y L N Y E T L D D Q G W D M D D D L F E

(B) P T V E Y L N Y E V V D D N G W D M Y D D D V F G

(C) - - - - - - - - - - - - - - - - - A T F

30 40 50

(A) K A A D A G L D G E D Y G T M E V A E G E Y I L E

(B) E A S D M D L D D E D Y G S L E V N E G E Y I L E

(C) K V T L I N E A E G T K H E I E V P D D E Y I L D

60 70

(A) A A E A Q G Y D W P F S C R A G A C A N C A S I V

(B) A A E A Q G Y D W P F S C R A G A C A N C A A I V

(C) A A E E E G Y D L P F S C R A G A C S T C A G K L

100

(A) K E G E I D M D M Q Q I L S D E E V E E K D V R L

(B) L E G D I D M D M Q Q I L S D E E V E D L N V R L

(C) V S G T V D Q S D Q S F L D D D Q I E A G Y V - L

110 120

(A) T C I G S P A A D E V K I V Y N A K H L D Y L Q N

(B) T C I G S P D A D E V K I V Y N A K H L D Y L Q N

(C) T C V A Y P T S D V V - I - Q T H K E E D L Y - -

128

(A) R V I

(B) R V I

(C) - - -

Fig. 6. Comparison of Halobacterium ferredoxins and N. muscorum ferredoxin I. Among (A): H. halobium ferredoxin, (B): H. The Dead Sea ferredoxin, and (C): N. muscorum ferredoxin I, 38 identical residues and 16 similar residues are found.

Halobacterium ferredoxins are 22 residue longer than chloroplast-type ferredoxins at the N-terminal region. There are only 4 cysteine residues in these two Halobacterium ferredoxins, which should be involved in the binding of the two iron atoms at the active center. The relative positions of these 4 cysteine residues at positions 63, 68, 71, and 102, are the same as those in chloroplast-type ferredoxins. Out of 128 residues of Halobacterium ferredoxins about 50 residues are identical with one of those of other chloroplast-type ferredoxins in the alignment as shown in Fig. 2. These residues are mainly distributed in a region from residue 40 to residue 110, which contains the functionary important cysteine residues. In addition to the sequence homology, similarities have been observed in the optical properties between Halobacterium and chloroplast-type ferredoxins, e. g. absorption spectra (as shown in Fig. 5 in chapter II), ORD and CD spectra (14). These results show that polypeptide chain structures surrounding the 2Fe-2S cluster must be very similar in both ferredoxins.

Despite of this structural similarities, Halobacterium ferredoxins were functionally different from chloroplast-type ferredoxins. Halobacterium ferredoxins were unable to be substituted for plant ferredoxins either in the photo-reduction system of NADP^+ (Fig. 6 in chapter II, 14) or in reduction system of cytochrome c by NADPH in the presence of ferredoxin: NADP^+ reductase (98). Recently, it has been found that H. halobium ferredoxin functions as

the physiological electron acceptor in the oxidation of α -ketoacids in this organism catalyzed by α -ketoacid ferredoxin oxidoreductases (99) and that H. The Dead Sea ferredoxin serves as an electron donor for nitrite reduction by nitrite reductase (100). These two functions have been reported for the chloroplast-type ferredoxins (101, 102). Thus, Halobacterium ferredoxins are not only structurally but in some aspects functionally similar to chloroplast-type ferredoxins, and therefore, they represent a new class of chloroplast-type ferredoxins.

In general halophilic proteins require high concentration of salt for their stability (103). In fact the ferredoxin from H. halobium is not precipitated in saturated ammonium sulfate solution and relatively stable even at 70° when 4 M NaCl is present. One of remarkable nature of halophilic proteins is that they contain rather high content of acidic amino acid residues than non-halophilic ones (103). In fact Halobacterium ferredoxins have more acidic residues than other chloroplast-type ferredoxins as shown in Table 2, and especially the long extra N-terminal regions contain 40 % of acidic residues and no basic residues, which must be the result of adaptation of these bacteria to survive on concentrated salt solutions.

3. 4Fe-4S and 8Fe-8S Ferredoxins

Contrary to chloroplast-type ferredoxins, bacterial ferredoxins are considered to be very diverse group.

From the nature of (4Fe-4S)₁₋₂ cluster(s) and the amino acid sequences, 16 bacterial ferredoxins with known sequences are classified into 4 groups as shown below. Their amino acid sequences and amino acid compositions are shown in Fig. 7 and Table 4, respectively.

Clostridium ferredoxins: The complete amino acid sequences of 8Fe-8S ferredoxins from 8 anaerobic bacteria have been determined. These ferredoxins are from C. pasteurianum (11, 32), C. butyricum (78), C. acidi-urichi (80), C. M-E (84), Peptococcus aerogenes (79), C. tartarivorum (81), C. thermosaccharolyticum (83), and Megasphaera elsdenii (82). They consist of 54 - 55 amino acid residues, 8 of which are cysteine residues, and are homologous each other to high degree as shown in Fig. 7. The invariant residues in all clostridial-type ferredoxins are found in 19 positions in the sequences and the variant residues in 13 positions which has more than 5 residues in a position. These sequences show a characteristic structure: i. e. a high similarity is found between the first half of the sequences and the second, each containing 4 invariant cysteine residues distributed symmetrically (11), which suggests the present-day clostridial-type ferredoxins to be arisen by gene duplication (104).

	1	10	20	30	40	50
(A)	A-YKI--ADSCVSCG--ACASECPVNAISQGDSIFVI----					
(B)	A-FVI--NDSCVSCG--ACAGECPVSAITQGDTQFVI----					
(C)	A-YVI--NEACISCG--ACDPECPVDAISQGDSRYVI----					
(D)	A-YKI--TDGCINCG--ACEPECPVEAISESDAVRVI----					
(E)	A-YVI--NDSCIACG--ACKPECPVN-IQQG-SIYAI----					
(F)	AHI-I--TDECISCG--ACAAECPVEAIHEGTGKYQV----					
(G)	AHI-I--TDECISCG--ACAAECPVEAIHEGTGKYEV----					
(H)	MH-VI--SDECVKCG--ACASTCPTGAIEGETKYVV-----TDSCI-DC-----GACEAVCPGTAISAE					

	1	10	20	30	40	50	60
(I)	ALY-I--TEECTYCG--ACEPECPVTAISAGDDIYVI----						
(J)	AH-RI--TEECTYCA--ACEPECPVNAISAGDEIYIV----DES VCT-DC--EGYDEP---ACVAVCPVDCIIKV						
(K)	ALY-I--TEECTYCG--ACEPECPVTAISAGSEIYVI----DAAGCT-EC--VGFADAP---ACAAVCPAECIVQG						
(L)	ALM-I--TDQCINCN--VCQPECPNGAISQGDETYVI----EPSLCT-EC--VGHYETS---QCVEVCPVDCIIKDPSHEETEDELRAKYERTGEG						

	1	10	20	30	40	50	60	70
(M)	T-FVV--TDNCIKCKYTDCVEVCPVDCFYEGPNFLVI----HPDECI-DC-----A--LCEPECPAQAI FSEDEVPSGMENFIELNAELAEIW PNI TERKDALPDAEWDGKPGKIADLER							
(N)	T-YVI--AEPDVDKDKACIEECPVDCIYEGARMLYI----HPDECV-DC-----G--ACEPVCPVEAIYEDDVPDQWSSYAQANADFFAEL GSPGGASKVGQTDNDPQAIKDLPPQGED							

	1	10	20	30	40	50
(O)	PI-QV---DNCMACQ--ACINECPVDVF-QMDEQGDKAVNI-----PNSNLDDQC-VEAIQSCPA-AIRS					
(P)	PKYTI VDKETCIACG--ACGAAAP-DIY-DYDEDGIAYVTLLDNQGIVEVPD-ILIDDM-MDAFEGCPTESIKVA---DEPFDGDPNKFD					

	1	10	20	30	40	50	60	70	80
--	---	----	----	----	----	----	----	----	----

Fig. 7. Amino acid sequences of 16 bacterial ferredoxins.

The references for the sequences are as in Table 1.

(A): C. pasteurianum, (B): C. butyricum, (C): C. acidu-urici,
 (D): C. M-E, (E): P. aerogenes, (F): C. tartarivorum, (G): C.
thermosaccharolyticum, (H): M. elsdenii, (I): C. limicola I,
 (J): C. limicola II, (K): C. thiosulfatophilum, (L): C. vinosum,
 (M): P. ovalis, (N): M. smegmatis, (O): D. gigas, (P): B. stearo-
thermophilus.

Table 4. The amino acid compositions of bacterial ferredoxins

^a	(A)	(B)	(C)	(D)	(E)	(F)	(G)	(H)	(I)	(J)	(K)	(L)	(M)	(N)	(O)	(P)
Lys	1	0	0	2	1	2	2	2	0	1	0	2	5	4	1	4
His	0	0	0	0	0	2	2	1	0	1	0	2	1	1	0	0
Arg	0	0	1	1	0	0	0	0	0	1	0	2	2	1	1	0
Asp	5	5	7	6	5	4	4	3	4	5	2	5	10	13	6	15
Asn	3	4	1	2	3	0	0	0	2	1	1	3	5	2	5	2
Thr	1	3	1	2	0	4	4	5	4	3	4	6	4	2	0	4
Ser	5	3	3	2	5	1	1	4	1	2	2	4	2	4	3	1
Glu	2	2	2	5	2	5	7	6	7	8	7	12	15	10	3	6
Gln	2	3	2	0	2	2	0	0	2	0	1	4	1	5	6	1
Pro	3	3	4	3	5	2	2	2	3	4	4	5	9	10	4	6
Gly	4	5	4	4	4	5	5	5	4	2	5	5	4	7	1	6
Ala	8	7	9	8	7	10	10	7	9	7	12	3	8	11	6	8
Cys	8	8	8	8	8	8	8	8	9	9	9	9	9	8	6	4
Val	6	6	6	5	4	4	4	5	5	7	4	6	7	9	5	5
Met	0	0	0	0	0	0	0	1	0	0	0	1	1	1	2	2
Ile	5	4	5	6	6	5	5	4	5	6	5	7	8	6	5	8
Leu	0	0	0	0	0	0	0	0	2	0	1	3	6	3	1	2
Tyr	1	0	2	1	2	1	1	1	3	4	3	3	2	6	0	4
Phe	1	2	0	0	0	0	0	0	0	0	1	0	5	2	1	3
Trp	0	0	0	0	0	0	0	0	0	0	0	0	2	1	0	0
Total	55	55	55	55	54	55	55	54	60	61	61	82	106	106	56	81

^a The alphabet in parentheses represents ferredoxins as described in Fig. 7.

The X-ray crystal structure of the protein from *P. aerogenes* has been determined (105) as shown in Fig. 8. The iron-sulfur chromophore in this molecule exists as two almost identical cubical cluster, and 4 iron atoms in each cluster linked to 4 cysteine sulfur atoms of the polypeptide chain and 4 atoms of inorganic sulfur. The 4 iron atoms of one of the clusters are coordinated with cysteines at positions 8, 11, 14, and 45 and the 4 iron atoms of the other cluster with cysteines at positions 35, 38, 41, and 18. The invariant and variant residues as

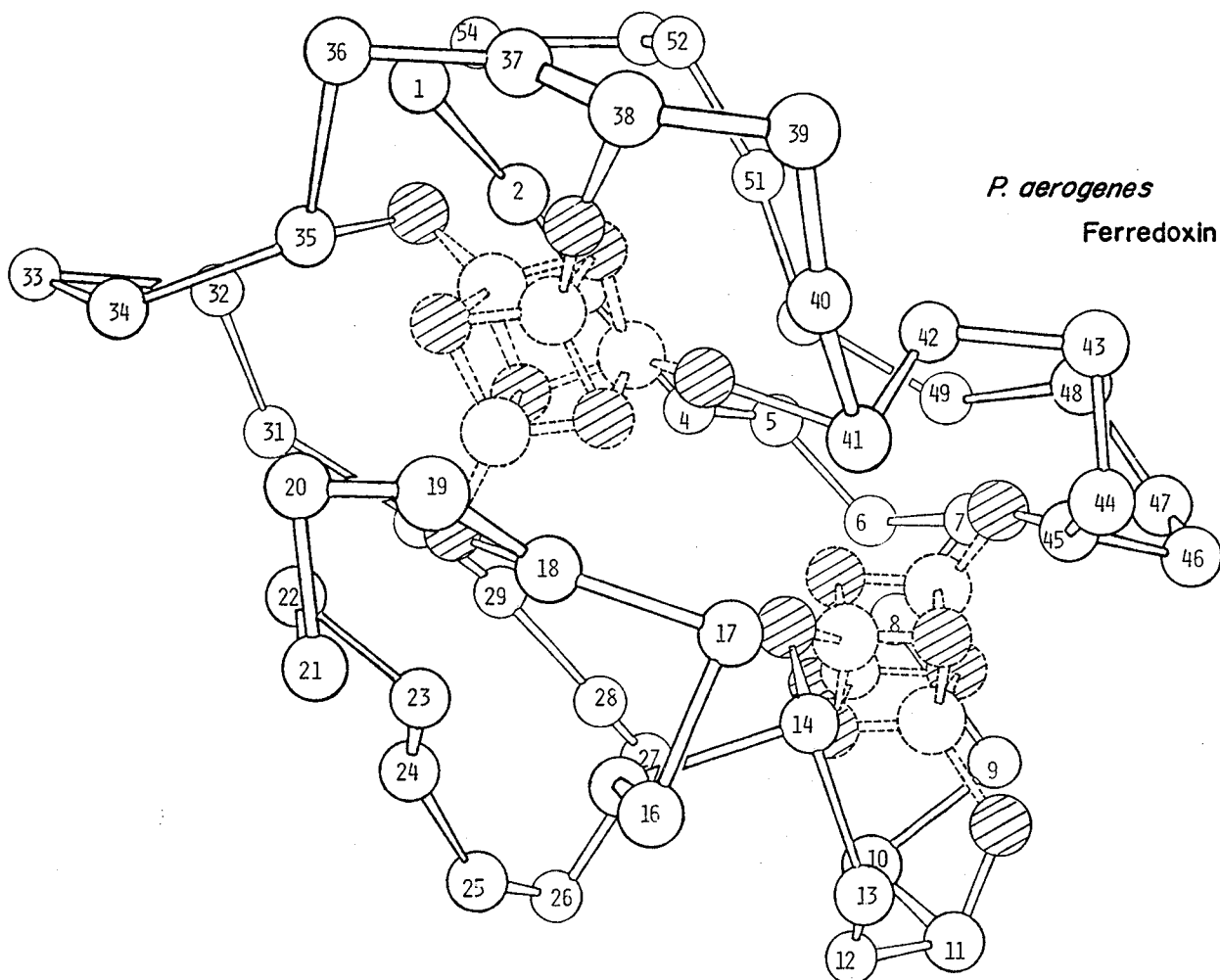


Fig. 8. Three dimensional structure of *P. aerogenes* ferredoxin.
Amino acid sequence of this protein is as follows (79),

1	5	10	15	20	25	30
A	Y	V	I	N	D	S
C	I	A	C	G	A	C
K	P	E	C	P	V	N
I	Q	Q	G	S	I	Y
A	I	D	A	D		
35	40	45	50			
S	C	I	D	C	G	S
C	A	S	V	C	P	V
G	A	P	N	P	E	D

○ Fe, ▨ S(inorganic), ▩ S(Cys).

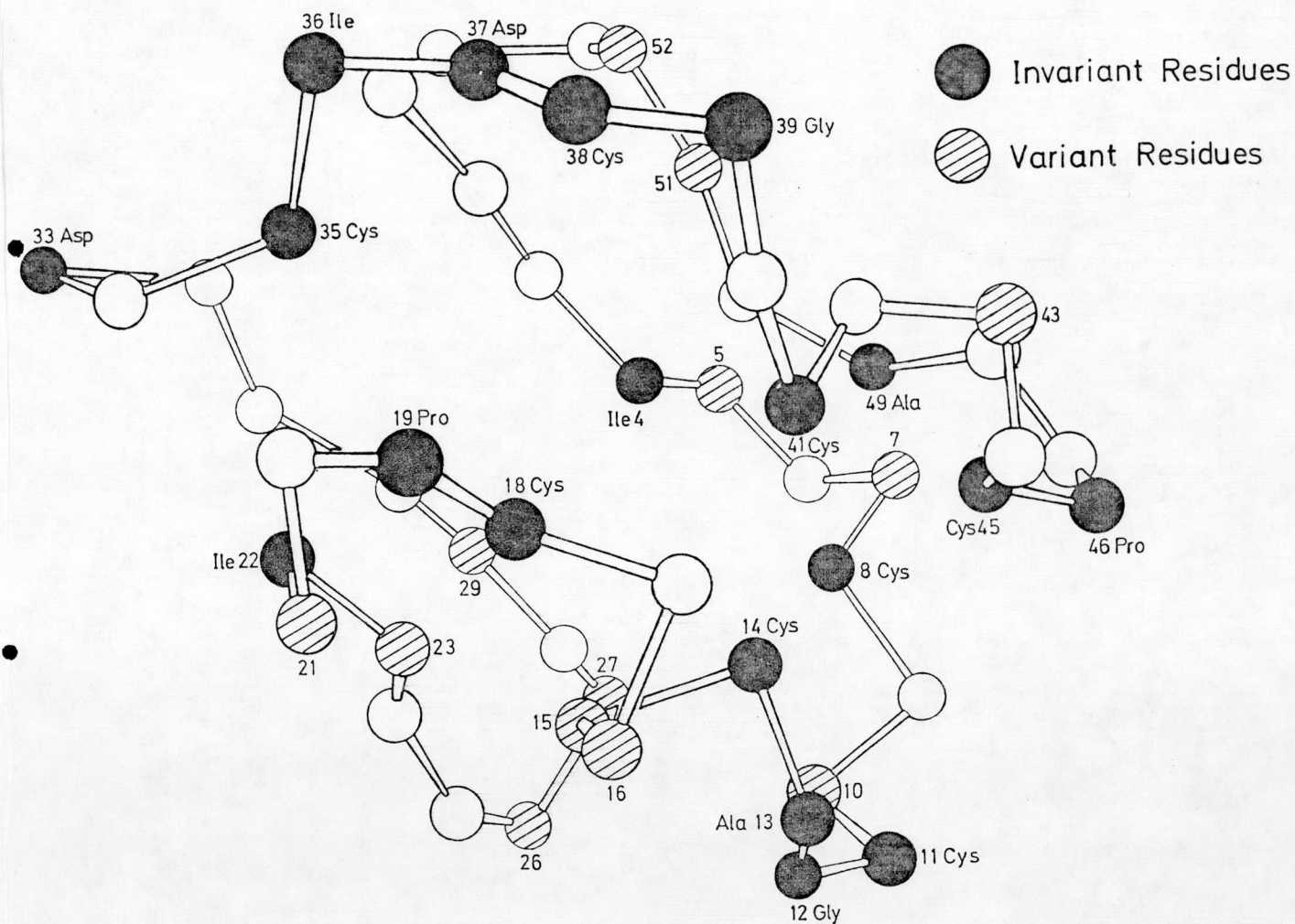


Fig. 9. The distribution of invariant and variant residues on the three dimensional structure of P. aerogenes ferredoxin.

described above are marked on the three dimensional structure as shown in Fig. 9. The clusters are surrounded by invariant residues which give essentially a hydrophobic environment (106).

Photosynthetic Bacterial (Chromatium, Chlorobium)

Ferredoxins: The amino acid sequences of 8Fe-8S ferredoxins from 3 photosynthetic bacteria, 2 green sulfur bacteria; Chlorobium limicola (87,88), C. thiosulfatophilum (89), and a purple sulfur bacterium; Chromatium vinosum (85, 86)

have been determined. These ferredoxins have larger molecular size than clostridial-type ferredoxins. From the comparison with clostridial-type ferredoxins as shown in Fig. 7, it is noted that Chlorobium ferredoxins consist of 60-61 amino acid residues with an insertion of 5 - 6 residues between residues 39 and 40 in the P. aerogenes ferredoxin and that Chromatium ferredoxin consists of 82 amino acid residues with two extra residues, one is the same insertion as described above and the other 21 residues at the C-terminus. The common insertion seems to loop out of the molecule, if the three dimensional structure of this group of ferredoxin is essentially the same as that of P. aerogenes ferredoxin (10). Eleven invariant residues are found among all clostridial and photosynthetic bacterial ferredoxins. Photosynthetic bacterial ferredoxins have 9 cysteine residues, 8 of which belong to the invariant residues and these are assumed to be involved in iron chelation.

Desulfovibrio gigas and Bacillus stearothermophilus ferredoxins: D. gigas (107) and Bacillus stearothermophilus (108) ferredoxins contain a single (4Fe-4S) cluster with varying molecular sizes. B. stearothermophilus ferredoxin consists of a total of 81 amino acid residues with 4 cysteine residues (77) and D. gigas ferredoxin 56 residues with 6 cysteine residues (76). The amino acid sequences of these two ferredoxins show that the N-terminal halves of these ferredoxins resemble closely to these of clostridial-

type and photosynthetic bacterial ferredoxins as shown in Fig. 7. The most valuable information derived from the present sequence study of B. stearothermophilus ferredoxin is the location of the 4 cysteine residues forming a 4Fe-4S chromophore. They are located at position 11, 14, 17, and 61. The isolated cysteine residue at position 61 is followed by a proline, a sequence found also in clostridial-type and photosynthetic bacterial ferredoxin. Therefore, it is concluded that the requirement of formation of the 4Fe-4S cluster is the presence of a -Cys-X-X-Cys-X-X-Cys- segment and a -Cys-Pro- sequence at far distance from the segment in the molecule in agreement with the result of the relative positions of the cysteine residues derived by the X-ray crystallographic studies on a 8Fe-8S ferredoxin having two 4Fe-4S clusters. (105). Not only by analogy with above conclusion, but by aligning the sequence of D. gigas ferredoxin in such a way that the -Cys-Pro- segment in the C-terminal half of molecule is matched with the -Cys-Pro- segment in the other ferredoxins as shown in Fig. 7, it seems likely that the cysteine residue at position 50 of D. gigas ferredoxin is the 4th cysteine residue involved in the 4Fe-4S cluster formation and that the cysteine residue at position 43 as another candidate was suggested by Yasunobu and Tanaka (7) is not.

Ferredoxin containing a single 4Fe-4S cluster with varying molecular sizes and amino acid compositions occur in many diverse species of bacteria. B. polymyxa fer-

redoxins I and II (109), both have a molecular weight of about 9,000 and 4 cysteine residues and resemble the ferredoxin of B. stearothermophilus. A similar ferredoxin was also purified from B. thermoproteolyticus Rokko (110). Several 4Fe-4S ferredoxins have been purified from D. desulfuricans (111), Clostridium thermoaceticum (112), and C. perfringens (113) and they have smaller molecular weights of 6,000 - 7,000 similar to that of D. gigas ferredoxin (107). Rhodospirillum rubrum ferredoxin II (114) and Mycobacterium flavum ferredoxin II (115) have comparatively larger molecular weights of 14,500 and 13,500, respectively, and show different EPR signal as shown below.

Pseudomonas ovalis and Mycobacterium smegmatis
Ferredoxins: Some bacteria contain 8Fe-8S ferredoxin with two 4Fe-4S clusters of widely different midpoint potentials (about 700 mV apart); e. g. Azotobacter vinelandii ferredoxin I (16), R. rubrum ferredoxin IV (116), and M. flavum ferredoxin I (115). These ferredoxins have common characteristics of molecular weight, amino acid composition, absorption spectrum, and EPR signal. For example, A. vinelandii ferredoxin I contained two 4Fe-4S clusters, both of which appeared to function between the same pair of oxidation states as the single 4Fe-4S cluster in Chromatium high-potential-iron-protein (117), but the midpoint reduction potentials of two clusters was 760 mV different; one was with E_0 -420 mV and the other with E_0 +350 mV.

Recently P. ovalis ferredoxin I (118) and M. smegmatis

ferredoxin (119) have been isolated and shown to belong to the same class of these ferredoxins. P. ovalis ferredoxin (90) is composed of 106 amino acid residues with 9 cysteine residues and M. smegmatis ferredoxin (91) the same number of amino acid residues with 8 cysteine residues. The molecules are much larger than those of clostridial-type ferredoxins, although a homologous nature of sequences is evident among these ferredoxins. Comparison of the two ferredoxins from P. ovalis and M. smegmatis as shown in Fig. 7. gives an interesting feature, 37 residues are identical between the two sequences and most of these residues are located at the N-terminal half (55 % and 15 % are identical in the N- and C-terminal halves, respectively). The comparison of C. vinosum and P. ovalis ferredoxins is also interesting as shown in Fig. 10. Cysteine cluster regions at the N- and C-terminal segments of P. ovalis ferredoxin I shows a close similarity to those at the C- and N-terminal segments of C. vinosum ferredoxin, respectively. Especially the sequence of residues 16 - 24 of P. ovalis ferredoxin I is completely identical with that of residues 49 - 57 of C. vinosum ferredoxin. An insertion after residues 12 in P. ovalis ferredoxin I is also found in C. vinosum ferredoxin after residue 41, although the length of insertion is different.

The N-terminal sequence of A. vinelandii ferredoxin I (120) is surprisingly similar to that of P. ovalis ferredoxin I. Only the N-terminal residue is clearly

different as shown in Fig. 10. The two bacteria seem to be distantly related organisms based on their metabolic systems; A. vinelandii is an aerobic nitrogen-fixer and P. ovalis an aerobic non-nitrogen-fixer, which was grown on glucose to use in this experiment (118). At present, the implication of this anomalous similarity of the two sequences is unknown.

P. ovalis ferredoxin I has 9 cysteine residues at positions 8, 11, 16, 20, 24, 39, 42, 45, and 49 and 8 residues excluding the residue at position 24 are located at the same positions as those found in clostridial-type ferredoxins as shown in Fig. 7. There is no substantial difference in cysteine distribution between P. ovalis ferredoxin I and other bacterial ferredoxins which have two low potential 4Fe-4S clusters. M. smegmatis ferredoxin, however, has only 8 cysteine residues and notable change in cysteine distribution is found in comparison with P. ovalis ferredoxin I. They are located at positions 8, 16, 20, 24, 39, 42, 45, and 49, and the residue at position 11 was substituted to valine in M. smegmatis ferredoxin. Therefore, it is concluded that the 8 cysteine residues required for the formation of two clusters are at positions 8, 16, 20, 24, 39, 42, 45, and 49 in both the ferredoxins from P. ovalis and M. smegmatis. It is hard to speculate for the present which 4 cysteine residues participate in the constitution of one cluster and the other, although residues at positions 20, 39, 42, and 45 are located

(A) 1 10 20 30
T-F-V-V--T-D-N-C-I-K-C-K D-C-V-E-V-C-P-V-D-C-F-Y-E-G-P-N-
Y-T

(B) A-F-V-V--T-D-N-C-I-K-C-K D-C-V-E-V-C-P-V-D-C-F-Y-E-G-P-{S}
Y T {T}

(C) 1 10 20
A-L-M-I--T-D-Q-C-I-N-C-N-V-C-Q-P-E-C-P-N-G-A-I-S-Q-G-D-E-

(A) 40 50
F-L-V-I-H-P-D-E-C-I-D-C-A-L-C-E-P-E-C-P-A-Q-A-I-F-S-E-D-E-

(B) F-L-V-I-{S}-P-D-E-C-I-D-C-A-L-{S}
{Q}

(C) 30 40 50 60
T-Y-V-I-E-P-S-L-C-T-E-C-V Q-C-V-E-V-C-P-V-D-C-I-I-K-D-P-S-
E S
H-Y-E-T

(A) 60 70 80
V-P-S-G-M-E-N-F-I-E-L-N-A-E-L-A-E-I-W-P-N-I-T-E-R-K-D-A-L-

(C) 70 80
H-E-E-T-E-D-E-L-R-A-K-Y-E-R-I-T-G-E-G

(A) 90 100
P-D-A-E-E-W-D-G-K-P-G-K-I-A-D-L-E-R

Fig. 10. Sequence comparison of ferredoxins from (A) P. ovalis, (B) A. vinelandii (120) and C. vinosum. { } in A. vinelandii ferredoxin were not yet unambiguously established (120). Gaps and insertions are placed into the sequences in order to produce a higher homology.

at the homologous position with those constituting one cluster of P. aerogenes ferredoxin. M. flavum ferredoxin II (115) with 4 cysteine residues had only one HIPIP-type cluster of midpoint potential at about -420 mV. Therefore, the sequence of this ferredoxin may give some insights to above discussion.

V. MOLECULAR EVOLUTION OF FERREDOXINS

1. Introduction

Proteins are unique among the chemical components of organisms because they are macromolecules containing functional, genetic, and evolutionary information. The mechanisms of protein biosynthesis appear to be common to all living organisms and amino acid sequences are translated directly from genetic code involved in DNA. All organisms that now live on earth seem to contain the evolutionary history of a species in the structure of their own proteins. Therefore, by comparing the amino acid sequences of the homologous proteins of organisms with those of others, the evolutionary relationship of these organisms could be estimated, as exemplified by cytochrome c (121) and hemoglobin (121).

Ferredoxins, an ubiquitous group of proteins in animals, plants, and bacteria, are suitable for studying protein structure in terms of evolution. The amino acid sequences of ferredoxins are now available for various plants, algae, and bacteria and the evolutionary stability and variability in their structure give valuable insights to define which parts of the molecule are essential for function as described in chapter IV. In this chapter, I describe the molecular evolution of chloroplast-type and bacterial-type ferredoxins and phylogenetic relationship of organisms having those ferredoxins.

2. Chloroplast-Type Ferredoxins

Chloroplast-type ferredoxins have been found in many organisms ranging from photosynthetic eukaryotes to prokaryotes such as blue-green algae and an extreme halophile, Halobacterium. The amino acid sequences of these ferredoxins are quite similar to one another as discussed in chapter IV. This similarity cannot be attributed to convergent evolution among these ferredoxins. They must be derived from a common ancestor. This suggests that the evolution of ferredoxins in diverse organisms can be quantitatively traced by comparative studies of the amino acid sequences of the chloroplast-type ferredoxins.

Phylogenetic Tree of Chloroplast-type Ferredoxins:

A matrix method (123) is used to construct a phylogenetic tree of chloroplast-type ferredoxins. The calculations are based on the number of differences in amino acids at corresponding loci in the various ferredoxins shown in Fig. 2 in chapter IV. A deletion or insertion is counted as one difference. The calculated numbers of amino acid differences are shown in Table 1 and a phylogenetic tree of chloroplast-type ferredoxins including Halobacterium ferredoxins is shown in Fig. 1. From this tree, some valuable information are obtainable for phylogenetic relationships of each ferredoxins as follows.

1. The algal ferredoxins are evolutionally a very diverse group (67, 124). Among blue-green algal ferredoxins,

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25	26
1. Koa		0.31	0.26	0.25	0.46	0.46	0.43	0.48	0.46	0.61	0.59	0.62	0.60	0.46	0.55	0.55	0.58	0.49	0.49	0.51	0.40	0.40	0.49	0.62	0.99	0.96
2. Spinach	20		0.28	0.26	0.38	0.38	0.35	0.38	0.49	0.60	0.65	0.60	0.66	0.41	0.47	0.49	0.51	0.51	0.45	0.42	0.42	0.42	0.47	0.51	0.98	1.04
3. Alfalfa	23	19		0.21	0.36	0.35	0.35	0.36	0.48	0.59	0.61	0.60	0.62	0.42	0.47	0.49	0.52	0.47	0.46	0.45	0.41	0.44	0.51	0.56	1.03	1.05
4. Taro	20	18	16		0.40	0.38	0.38	0.39	0.47	0.58	0.65	0.59	0.66	0.38	0.47	0.48	0.52	0.52	0.49	0.48	0.47	0.44	0.56	0.61	1.01	1.04
5. <i>P. americana</i> I	35	30	27	28		0.27	0.02	0.26	0.40	0.51	0.63	0.52	0.65	0.26	0.41	0.41	0.51	0.46	0.32	0.32	0.41	0.35	0.46	0.52	0.96	1.00
6. <i>P. americana</i> II	37	31	27	29	23		0.25	0.06	0.43	0.56	0.67	0.57	0.67	0.35	0.58	0.48	0.56	0.49	0.43	0.42	0.47	0.44	0.47	0.58	0.97	1.02
7. <i>P. esculenta</i> I	34	29	26	27	2	22		0.24	0.39	0.53	0.65	0.54	0.66	0.25	0.40	0.40	0.50	0.45	0.31	0.31	0.40	0.34	0.43	0.53	0.95	1.00
8. <i>P. esculenta</i> II	37	31	28	30	23	6	22		0.47	0.55	0.67	0.56	0.68	0.33	0.45	0.46	0.55	0.48	0.41	0.39	0.47	0.42	0.46	0.58	0.98	1.03
9. <i>G. japonica</i>	34	37	37	36	31	38	30	38		0.50	0.65	0.51	0.63	0.37	0.45	0.45	0.53	0.45	0.46	0.45	0.42	0.37	0.46	0.53	0.94	0.94
10. <i>E. telmateia</i> I	36	39	39	36	35	39	35	38	35		0.39	0.01	0.40	0.44	0.52	0.53	0.56	0.65	0.59	0.57	0.48	0.49	0.59	0.68	0.91	0.95
11. <i>E. telmateia</i> II	39	45	45	43	43	47	43	47	46	29		0.40	0.01	0.62	0.63	0.60	0.65	0.73	0.60	0.60	0.52	0.53	0.55	0.73	0.99	0.98
12. <i>E. arvense</i> I	37	40	40	37	36	40	36	39	36	1	30		0.41	0.45	0.49	0.55	0.57	0.66	0.60	0.58	0.49	0.51	0.57	0.71	0.91	0.94
13. <i>E. arvense</i> II	40	46	46	44	44	48	44	48	45	30	1	31		0.63	0.65	0.61	0.66	0.74	0.62	0.61	0.53	0.54	0.57	0.72	1.00	1.00
14. <i>S. quadricauda</i>	33	30	29	26	19	25	18	25	28	30	42	31	43		0.24	0.21	0.44	0.40	0.29	0.29	0.38	0.30	0.41	0.51	0.96	1.00
15. <i>D. sorlina</i> I	37	35	33	34	32	37	31	37	34	34	44	35	45	21		0.19	0.51	0.45	0.39	0.39	0.42	0.37	0.44	0.53	0.97	1.04
16. <i>D. sorlina</i> II	36	35	33	33	28	34	27	34	32	34	41	35	42	16	16		0.47	0.42	0.35	0.35	0.44	0.37	0.43	0.55	0.93	0.98
17. <i>P. umbilicalis</i>	40	37	37	36	38	40	37	40	44	39	43	40	44	31	37	35		0.39	0.39	0.37	0.53	0.44	0.58	0.61	0.93	0.96
18. <i>C. caldarium</i>	39	37	36	37	34	37	33	37	34	44	51	45	52	27	33	29	28		0.35	0.34	0.40	0.35	0.45	0.50	0.89	0.90
19. <i>S. platensis</i>	40	36	38	37	28	36	27	36	38	42	44	43	45	25	31	27	29	24		0.04	0.31	0.19	0.41	0.40	0.95	0.96
20. <i>S. maxima</i>	39	34	37	36	28	35	27	34	37	40	44	41	45	25	31	27	28	25	4		0.31	0.21	0.40	0.41	0.95	0.98
21. <i>N. muscorum</i> I	32	34	34	36	32	37	31	36	36	38	40	39	41	28	34	33	36	29	21	21		0.18	0.31	0.45	0.88	0.86
22. <i>M. laminosus</i>	31	31	32	32	29	36	28	35	33	36	39	37	40	24	30	28	31	25	15	16	13		0.26	0.37	0.92	0.92
23. <i>A. sacrum</i> I	36	34	37	38	32	35	31	36	35	42	41	42	42	30	34	31	41	33	32	31	27	25		0.48	0.93	0.92
24. <i>A. sacrum</i> II	47	40	45	46	42	48	41	49	42	50	56	51	55	40	41	41	45	38	31	31	34	29	37		1.03	1.02
25. <i>H. halobium</i>	91	94	96	94	96	95	95	95	93	87	96	87	97	92	95	91	90	89	92	92	87	90	95	98		0.20
26. <i>H. Dead Sea</i>	91	98	99	96	98	97	98	97	95	90	97	89	98	94	96	93	92	91	94	94	88	92	95	99	20	

Table 1. The matrix of mutation distance required to interrelate pair of chloroplast-type ferredoxins. Values in the lower left half of the table show the amino acid differences among ferredoxins and those in the upper right half of the table show the minimum base differences per codon among ferredoxins. The calculations are based on the alignment of sequences as shown in Fig. 2. in chapter IV.

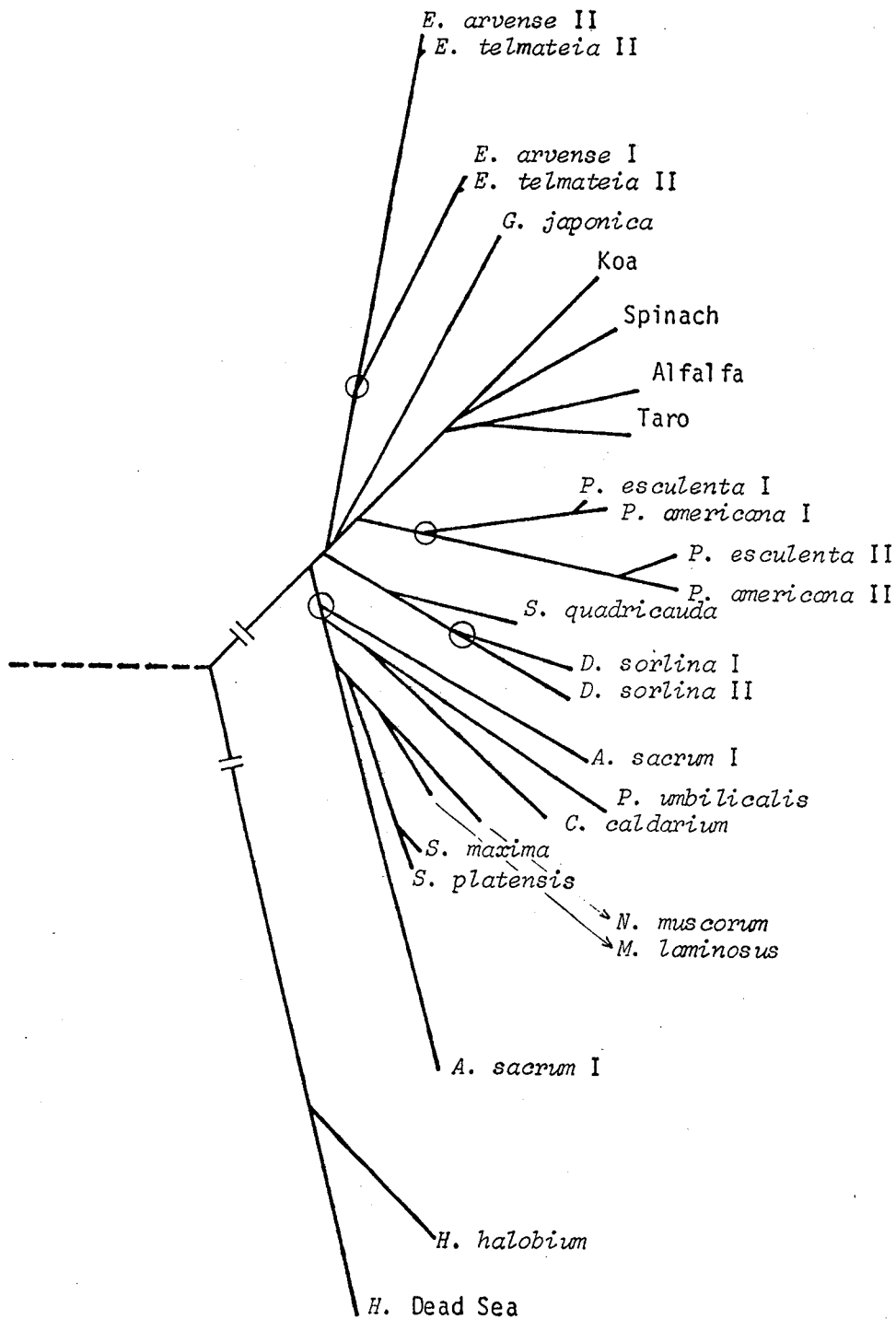


Fig. 1. Phylogenetic tree of chloroplast-type ferredoxins. This tree is constructed from the amino acid differences shown in Table 1 by the matrix method (123). Solid circles show the point at which gene duplications occurred.

A. sacrum ferredoxins I and II are far from other ferredoxins in accordance with their cellular organization: A. sacrum in the Chroococcales order belongs to a unicellular group, and Spirulina and Nostoc in the Nostocales order and Mastigocladus in the Stigonematales order belong to a filamentous group.

The ferredoxin of a red alga, P. umbilicalis, is diverged from the blue-green algal ferredoxin line as shown in Fig. 1. P. umbilicalis ferredoxin has two insertions at positions 10 and 14 and these insertions are unique for all blue-green algal ferredoxins except for A. sacrum ferredoxin I. In terms of the presence of these insertions, P. umbilicalis seems to be more closely related to blue-green algae than green algae. The presence of phycobili-proteins in both blue-green and red algae also suggests a close connection between the both algae.

2. Cyanidium caldarium is a unicellular eukaryotic algae of uncertain classification in morphology. But from the tree, this algal ferredoxin shows closer similarity to P. umbilicalis ferredoxin than other algal ferredoxins. In the sequence comparison as shown in Fig. 2 in chapter IV, unique amino acid residues occupy several positions in Cyanidium and Porphyra ferredoxins, such as Ile 5, His 6, and Lys 10, which also support the close relationship between these two organisms. These data suggest C. caldarium to be a member of red algae. However, more extensive sequence study of red algal ferredoxins is required to

confirm that Cyanidium forms an evolutionary link between the blue-green and red algae.

3. Halobacterium ferredoxins are homologous to chloroplast-type ferredoxins. From the tree, it seems that Halobacterium ferredoxins and chloroplast-type ferredoxins could have evolved independently from a common ancestral form. Halobacteria are strictly aerobic and they must have evolved after the appearance of blue-green algae. Recently, comparative studies of 16 S rRNA (125) have shown that H. halobium did not arise, as a halophilic adaptation from typical bacteria, "Eubacteria", and that H. halobium is rather a member of "Archaeobacteria" known to contain only the methanogenic bacteria, which appear to be no more related to bacteria than they are to eukaryotic cytoplasm (126). And more recently the structural comparison of 5 S rRNA and ribosomal proteins (127, 128) suggests that Halobacterium is more related to eukaryotes than to prokaryotes. These studies and our ferredoxin data strongly suggest that there must be close relations between Halobacterium and eukaryotes which diverged in very old evolutionary time.

Gene Duplications: Two molecular species of ferredoxins in one organism in higher plants and algae are known.

A comparative studies of these molecules may give important data about gene duplication of ferredoxins. Two ferredoxins have been reported to occur in the following organisms, A. sacrum (129), N. MAC (130, 131), N. muscorum (65),

N. verrucosum (132), S. maxima (133), and D. sorlina (63) in algae and P. americana (134), P. esculenta (135), E. telmateia (61), E. arvense (62), two Petunia species (136), and Pisum sativum (137) in higher plants. In every case studied two molecular species of one organism showed large number of amino acid substitutions, 37 residues between A. sacrum ferredoxins I and II, 16 residues between D. sorlina ferredoxin I and II, 29 residues between E. telmateia ferredoxins I and II, 31 residues between E. arvense ferredoxins I and II, 23 residues between P. americana ferredoxins I and II, and 22 residues between P. esculenta ferredoxins I and II. The similar large number of amino acid substitutions are also predictable for ferredoxins of N. MAC (131), N. verrucosum (132), and two Petunia species (136). These phenomena probably reflect gene duplications that occurred at early evolutionary stages rather than allelic type ferredoxins that occur in the ferredoxins from a single tree of Koa (55).

The points where gene duplications occurred are shown in Fig. 1. In the cases of Equisetum, D. sorlina, and Phytolacca, the duplications of ferredoxin genes have occurred within each phyletic lines after divergence from other plant phyla. These phenomena are shown diagrammatically in Fig. 2. Two Equisetum species, E. telmateia and E. arvense, and two Phytolacca species, P. americana and P. esculenta, evolved relatively recently from the ancestral species with keeping two ferredoxin genes.

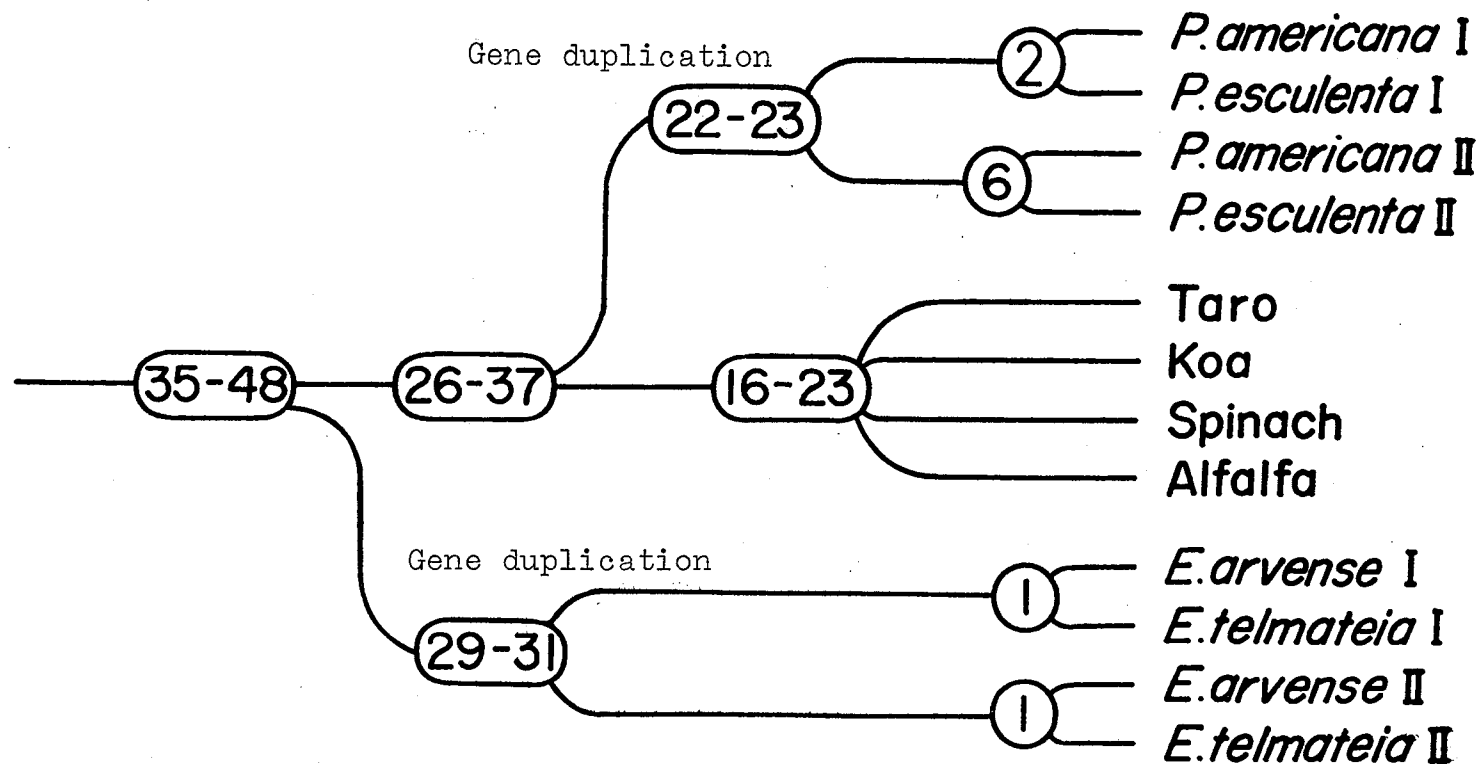


Fig. 2. A diagram representing phyletic relations among ferredoxins with special reference to gene duplication. The numbers show amino acid differences.

In the case of A. sacrum, the gene duplication took place within blue-green and red algal line before A. sacrum diverged from other algae. An interesting feature in comparison of the sequences of these algal ferredoxins is that A. sacrum ferredoxin I as well as higher plants and algal ferredoxins has no extra residues at positions 10 and 14 as shown in Fig. 2. in chapter IV. In terms of the absence of these two residues, A. sacrum ferredoxin I is similar to those of green-algal and higher plant ferredoxins. If this is diagrammatically expressed as individual genes (Fig. 3), a ferredoxin gene corresponding to A. sacrum ferredoxin I might be present in other blue-green and red algae. In fact, N-terminal sequences of two ferredoxins from N. MAC have been reported (131) and ferredoxin II of N. MAC without any gap corresponds to ferredoxin II of A. sacrum and ferredoxin I of N. MAC to ferredoxin I of A. sacrum, although only one gap at position 10 is observed and instead an insertion between positions 2 and 3 is recognized. Therefore, Nostoc MAC ferredoxin I seems to represent of a transient form between A. sacrum ferredoxin II and eukaryotic ferredoxins.

There is no clear evidence suggesting any functional difference in the two ferredoxins in one organism although it has been suggested that they might be physiologically differentiated in the organism (131, 132).

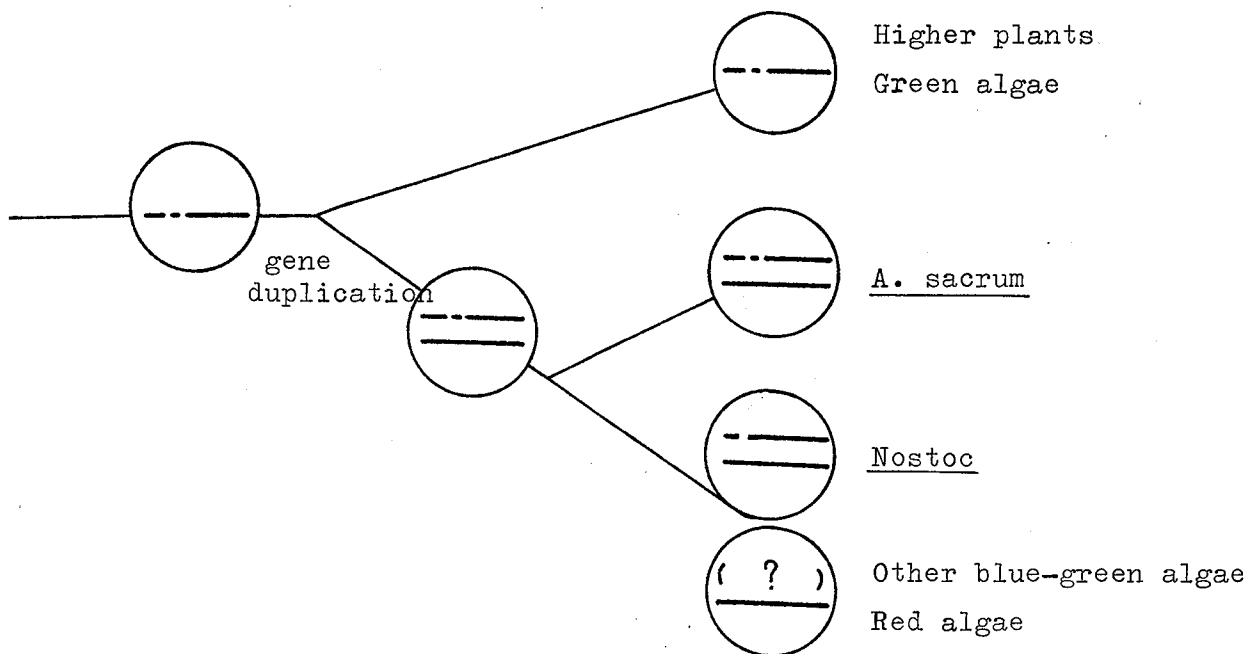


Fig. 3. A diagram representing phyletic relations among algal ferredoxins with special reference to gene duplication. The broken bars (---) represent the genes corresponding to A. sacrum ferredoxin I and higher plant ferredoxins, other broken bar (---) that corresponding to N. MAC ferredoxin I, and the solid bars (—) those corresponding to A. sacrum ferredoxin II and other blue-green and red algal ferredoxins.

3. Bacterial Ferredoxins

Contrary to chloroplast-type ferredoxins which are very closely related each other, the bacterial ferredoxins are a very diverse group. As shown in Fig. 7 in chapter IV, many gaps are placed to align bacterial ferredoxins. The similarity of amino acid sequence of the two halves of the clostridial-type ferredoxins (104) have been adopted

for the strong evidence of internal gene duplication. In other bacterial ferredoxins, there have been also such a similarity of two halves (138). Therefore, it seems that all contemporary bacterial ferredoxins come from duplication of an ancestral ferredoxin gene which was about a half size of clostridial-type ferredoxin and that variation of molecular size found in bacterial ferredoxins may be attributed to insertion, deletion, and recombination in the ferredoxin genes. A quite obvious insertion is found in comparison between several clostridial and three photosynthetic bacterial ferredoxins (85). Long extra segments at the C-terminal region are found in C. vinosum, P. ovalis, M. smegmatis, and B. stearothermophilus ferredoxins.

A phylogenetic tree of bacterial ferredoxins are constructed by using a matrix method (123) as described in the previous section. The matrix of amino acid differences based on the sequence alignment shown in Fig. 7 in chapter IV is shown in Table 2 and a phylogenetic tree in Fig. 4. The divergent point of ferredoxins from the ancestral one is uncertain, and therefore, this is shown with dashed lines. From the tree, 4 large branches are observed. There is high homology among ferredoxins from Clostridium, Megasphaera, and Peptococcus: they are little change in structure of these ferredoxins for a long time, because the trace of gene duplication is well conserved. Generally, anaerobic organisms such as Clostridium, which is only capable to obtain energy from fermentation, are believed

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16
1. <i>C. pasteurianum</i>		9	14	19	18	23	23	27	31	37	33	60	87	82	55	70
2. <i>C. butyricum</i>	9		16	23	18	23	23	26	35	40	38	61	86	82	55	71
3. <i>C. acid-urici</i>	14	16		18	18	25	24	30	28	34	31	57	86	81	55	67
4. <i>C. M-E</i>	19	23	18		26	25	25	31	30	35	32	57	85	84	55	68
5. <i>P. aerogenes</i>	18	18	18	26		26	26	30	37	40	38	61	85	82	56	70
6. <i>C. tartarivorum</i>	23	23	25	25	26		2	21	33	35	36	62	86	86	57	69
7. <i>C. thermosaccharolyticum</i>	23	23	24	25	26	2		19	33	35	36	62	86	86	57	68
8. <i>M. elsdenii</i>	27	26	30	31	30	21	19		39	38	39	64	85	84	56	71
9. <i>C. limicola</i> I	31	35	28	30	37	33	33	39		24	14	51	96	92	53	66
10. <i>C. limicola</i> II	37	40	34	35	40	35	35	38	24		22	49	98	92	54	69
11. <i>C. thiosulfatophilum</i>	33	38	31	32	38	36	36	39	14	22		51	97	93	56	67
12. <i>C. vinosum</i>	60	61	57	57	61	62	62	64	51	49	51		95	95	77	77
13. <i>P. ovalis</i>	87	86	86	85	85	86	86	85	96	98	97	95		69	103	112
14. <i>M. smegmatis</i>	82	82	81	84	82	86	86	84	92	92	93	95	69		107	110
15. <i>D. gigas</i>	55	55	55	55	56	57	57	56	53	54	56	77	103	107		64
16. <i>B. stearothermophilus</i>	70	71	67	69	70	68	68	71	66	69	67	77	112	110	64	

Table 2. Amino acid difference among bacterial ferredoxins as shown
Fig. 7 in chapter IV.

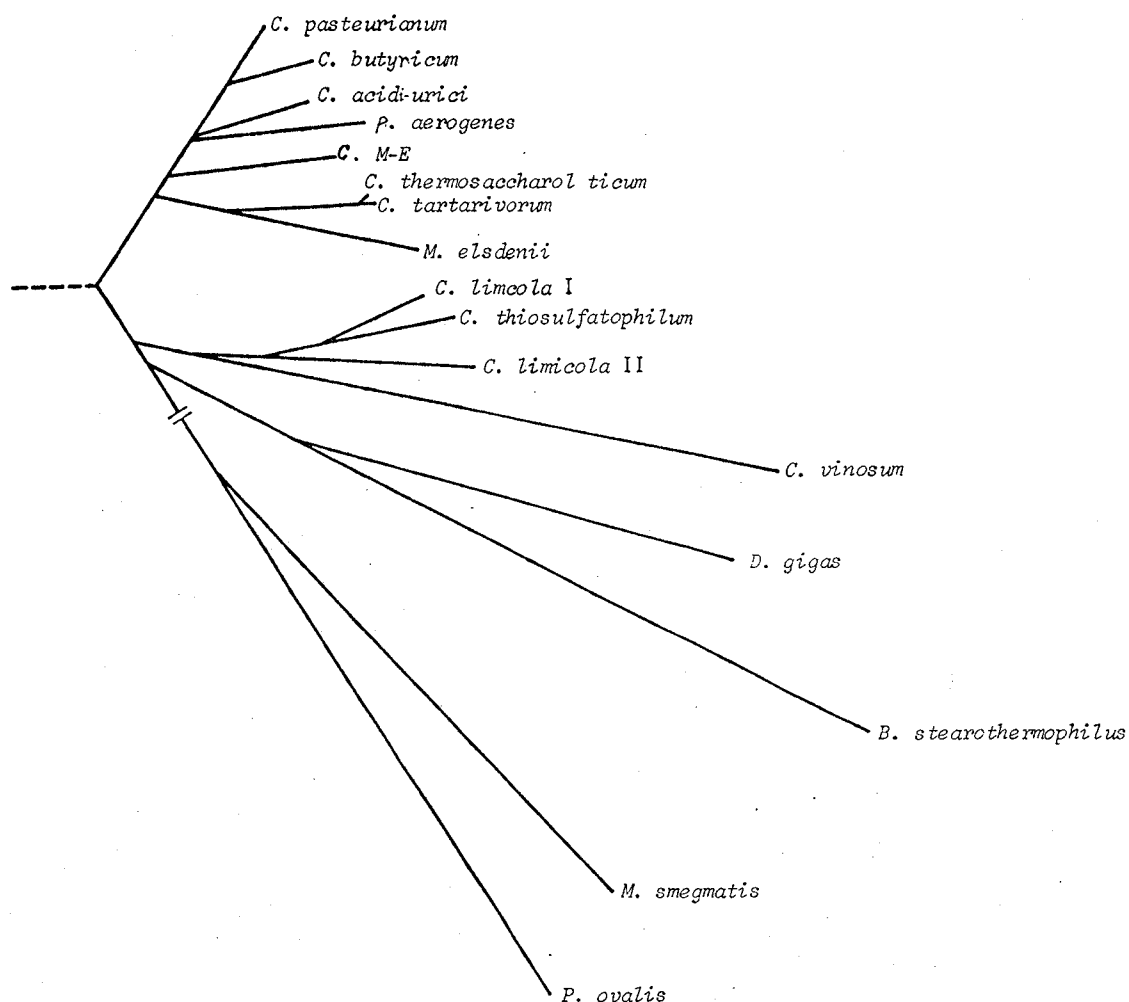


Fig. 4. Phylogenetic tree of bacterial ferredoxins.

to be the oldest living form. Chromatium and Chlorobium, photosynthetic bacteria using H_2S as an electron donor, form another group of ferredoxins on the tree. C. limicola ferredoxins I and II may be derived from a gene duplication, which is a similar event as discussed in chloroplast-type ferredoxins such as horsetail ferredoxins I and II. From the tree, C. thiosulfatophilum ferredoxin is more resemble to C. limicola ferredoxin I than ferredoxin II and the

ferredoxin corresponding to C. limicola ferredoxin II may exist in C. thiosulfatophilum. The non-sulfur purple bacteria of Rhodospirillum rubrum whose ferredoxins are considered to be quite different from those of Chromatium and Chlorobium as described in chapter IV, may not closely related with these photosynthetic bacteria. Other ferredoxins on the tree are divided into two group; one includes D. gigas and B. stearrowthermophilus ferredoxins and the other P. ovalis and M. smegmatis ferredoxins. B. stearrowthermophilus, P. ovalis, and M. smegmatis are aerobic bacteria having respiratory chains and D. gigas is an anaerobic sulfate reducing bacterium. It is obvious that these 4 ferredoxins changed more rapidly than Clostridial ferredoxins, and it is impossible to conclude whether sulfate reducing bacteria originate after or before the appearance of Clostridial and photosynthetic bacteria. It is interesting to note that M. smegmatis and P. ovalis ferredoxins are very similar to that of A. vinelandii as shown in Fig. 10 in chapter IV, although it is not clear at present how these bacterial ferredoxins relate phylogenetically to each other.

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REFERENCES

1. Mortenson, L. E., Valentine, R. C., and Carnahan, J. E.
(1962) Biochem. Biophys. Res. Commun. 7, 448
2. Tagawa, K. and Arnon, D. I. (1962) Nature, 195, 537
3. Davenport, H. E., Hill, R., and Whatley, F. R. (1952)
Proc. Roy. Soc. Ser. B, 139, 346
4. Arnon, D. I., Whatley, F. R., and Allen, M. B. (1957)
Nature, 180, 182, 1325
5. San Pietro, A. and Lang, H. M. (1958) J. Biol. Chem.
231, 211
6. Buchanan, B. B. and Arnon, D. I. (1970) Advan. Enzymol.
33, 119
7. Yasunobu, K. T. and Tanaka, M. (1973) in Iron-Sulfur
Proteins (W. Lovenberg, ed.) Vol. 2, pp.27
8. Brodrick, J. W. and Rabinowitz, J. C. (1977) in Iron-
Sulfur Proteins (W. Lovenberg, ed.) Vol. 3, pp. 101
9. Tel-Or, E., Cammack, R., Rao, K. K., Rogers, L. J.,
Stewart, W. D., and Hall, D. O. (1977) Biochim. Biophys.
Acta, 490, 120
10. Matsubara, H. (1972) in Aspect of Cellular and Molecular
Physiology (K. Hamaguchi, ed.) Vol. 19, pp. 31
11. Tanaka, M., Nakashima, T., Benson, A., Mower, H. F.,
and Yasunobu, K. T. (1964) Biochem. Biophys. Res. Commun.
16, 422
12. Matsubara, H., Sasaki, R. M., and Chain, R. K. (1967)
Proc. Nat. Acad. Sci. U.S.A. 57, 439

13. Matsubara, H., Jukes, T. H., and Cantor, C. R. (1968) Structure, Function, and Evolution in Proteins,
Brookhaven Symp. Biol. 21, 201
14. Kerscher, L., Oesterhelt, D., Cammack, R., and Hall, D. O. (1976) Eur. J. Biochem. 71, 101
15. Yoch, D. C. and Arnon, D. I. (1972) J. Biol. Chem. 247, 4514
16. Sweeny, W. V., Rabinowitz, J. C., and Yoch, D. C. (1975) J. Biol. Chem. 250, 7842
17. Buchanan, B. B. and Arnon, D. I. (1971) in Methods in Enzymology (A. San Pietro, ed.) Vol. 23, pp. 413
18. Rabinowitz, J. C. (1972) in Methods in Enzymology (A. San Pietro, ed.) Vol. 24, pp. 431
19. Mortenson, L. E. (1963) Biochim. Biophys. Acta, 81, 71
20. Mayhew, S. G. (1971) Anal. Biochem. 42, 191
21. Werber, M. M. and Mervarech, M. (1978) Arch. Biochem. Biophys. 187, 447
22. Oesterhelt, D., Meentzen, M., and Scumann, L. (1973) Eur. J. Biochem. 40, 453
23. Whatley, F. R. and Arnon, D. I. (1963) in Methods in Enzymology (S. P. Colowick and N. O. Kaplan, eds.) Vol 6, pp. 308
24. Wada, K., Kagamiyama, H., Shin, M., and Matsubara, H. (1974) J. Biochem. 76, 1217
25. Kagamiyama, H., Rao, K. K., Hall, D. O., and Matsubara, H. (1975) Biochem. J. 79, 329
26. Keresztes-Nagy, S. and Margoliash, E. (1966) J. Biol. Chem. 241, 5955

27. Aggarwal, S. J., Rao, K. K., and Matsubara, H. (1971)
J. Biochem. 69, 601
28. Wada, K. unpublished results
29. Werber, M. M., Mevarech, M., Leicht, W., and Eisenberg, H. (1978) in Energetics and Structure of Halophilic Microorganisms (S. R. Caplan and M. Ginzburg, eds.)
30. Sanger, F. and Tuppy, H. (1951) Biochem. J. 49, 481
31. Sanger, F. and Thompson, E. O. P. (1953) Biochem. J. 53, 366
32. Tanaka, M., Nakashima, T., Benson, A., Mower, H., and Yasunobu, K. T. (1966) Biochemistry, 5, 1666
33. Matsubara, H., Sasaki, R. M., and Chain, R. K. (1968)
J. Biol. Chem. 243, 1725
34. Matsubara, H. and Sasaki, R. M. (1968) J. Biol. Chem. 243, 1732
35. Crestfield, A. M., Moore, S., and Stein, W. H. (1963)
J. Biol. Chem. 238, 622
36. Bennett, J. C. (1967) in Methods in Enzymology (C. H. W. Hirs, ed.) Vol. 11, pp. 330
37. Vunderkerckhove, J. and Van Montagu, M. (1974) Eur. J. Biochem. 44, 279
38. Dalgliesh, C. E. (1952) Biochem. J. 52, 3
39. Bray, H. G., Thorpe, W. V., and White, K. (1950)
Biochem. J. 46, 271
40. Sakaguchi, S. (1950) J. Biochem. 37, 231
41. Spackman, D. H., Stein, W. H., and Moore, S. (1958)
Anal. Chem. 30, 1190

42. Matsubara, H. and Sasaki, R. M. (1969) Biochem. Biophys. Res. Commun. 35, 175
43. Penke, B., Ferenczi, R., and Kovács, K. (1974) Anal. Biochem. 60, 45
44. Blombäck, B., Blombäck, M., Edman, P., and Hessel, B. (1966) Biochim. Biophys. Acta, 115, 371
45. Niederwieser, A. (1972) in Methods in Enzymology (C. H. W. Hirs and S. N. Timasheff, eds.) Vol. 25, pp. 60
46. Edman, P. and Henshen, a. (1975) in Protein Sequence Determination (S. B. Needleman, ed.) pp. 245
47. Laursen, R. A. (1977) in Methods in Enzymology (C. H. W. Hirs and S. N. Timasheff, eds.) Vol. 47, pp. 277
48. Laursen, R. A. (1971) Eur. J. Biochem. 20, 89
49. Ambler, R. P. (1972) in Methods in Enzymology (C.H. W. Hirs and S. N. Timasheff, eds.) Vol. 25, pp. 262
50. Hayashi, R. (1977) in Methods Enzymology (C. H. W. Hirs and S. N. Timasheff, eds.) Vol. 47, pp. 84
51. Schmer, G. and Kreil, G. (1969) Anal. Biochem. 29, 186
52. Houmard, J. and Prepean, G. R. (1972) Proc. Natl. Acad. Sci. U.S.A. 69, 3506
53. Delange, R. J., Fambrough, D. M., Smith, E. L., and Bonner, J. (1969) J. Biol. Chem. 244, 319
54. Keresztes-Nagy, F., Perini, F., and Margoliash, E. (1969) J. Biol. Chem. 244, 891
55. Benson, A, M. and Yasunobu, K. T. (1969) J. Biol. Chem. 244, 955
56. Rao, K. K. and Matsubara, H. (1970) Biochem. Biophys. Res. Commun. 38, 500

57. Wakabayashi, S., Hase, T., Wada, K., Matsubara, H.,
Suzuki, K., and Takaichi, S. (1978) J. Biochem. 83,
1305
58. Wakabayashi, S., Hase, T., Wada, K., Matsubara, H.,
and Suzuki, K. (1979) J. Biochem. in preparation
59. Hase, T., Yamanashi, H., and Matsubara, H. (1979)
J. Biochem. in preparation
60. Hase, T., Wada, K., and Matsubara, H. (1977) J. Biochem.
82, 267
61. Hase, T., Wada, K., and Matsubara, H. (1977) J. Biochem.
82, 277
62. Sugeno, K. and Matsubara, H. (1969) J. Biol. Chem.
244, 2979
63. Hase, T., Matsubara, H., Rao, K. K., Hall, D. O.,
and Ben-Amotz, A. (1979) Phytochemistry, in preparation
64. Takruri, I., Haslett, B. G., Boulter, D., Andrew, P. W.,
and Rogers, L. J. (1978) Biochem. J. 173, 459
65. Hase, T., Wakabayashi, S., Wada, K., Matsubara, H.,
Jüttner, F., Rao, K. K., Fry, I., and Hall, D. O.
(1978) FEBS Lett. 96, 41
66. Tanaka, M., Haniu, M., Zeitlin, S., Yasunobu, K. T.,
Rao, K. K., and Hall, D. O. (1975) Biochemistry, 14, 5535
67. Wada, K., Hase, T., Tokunaga, H., and Matsubara, H.
(1975) FEBS Lett. 55, 102
68. Tanaka, M., Haniu, M., Yasunobu, K. T., Rao, K. K.,
and Hall, D. O. (1976) Biochem. Biophys. Res. Commun.
69, 759

69. Hase, T., Wada, K., and Matsubara, H. (1976) J. Biochem.
79, 329
70. Hase, T., Wakabayashi, S., Wada, K., and Matsubara, H.
(1978) J. Biochem. 83, 761
71. Hase, T., Wada, K., Ohmiya, M., and Matsubara, H.
(1976) J. Biochem. 80, 993
72. Hase, T., Wakabayashi, S., Matsubara, H., Rao, K. K.,
Hall, D. O., Windmer, H., Gysi, J., and Zuber, H.
(1978) Phytochemistry, 17, 1863
73. Hase, T., Wakabayashi, S., Matsubara, H., Kerscher, L.,
Oesterhelt, D., Rao, K. K., and Hall, D. O. (1977)
FEBS Lett. 77, 308
74. Hase, T., Wakabayashi, S., Matsubara, H., Kerscher, L.,
Oesterhelt, D., Rao, K. K., and Hall, D. O. (1978)
J. Biochem. 83, 1657
75. Hase, T., Wakabayashi, S., Matsubara, H., and Werber,
M. M. (1979) in preparation
76. Travis, J., Newman, D. J., Le Gall, J., and Peck, H. D.,
Jr. (1971) Biochem. Biophys. Res. Commun. 45, 452
77. Hase, T., Ohmiya, M., Matsubara, H., Mullinger, R. N.,
Rao, K. K., and Hall, D. O. (1976) Biochem. J. 159, 55
78. Benson, A. M., Mower, H. F., and Yasunobu, K. T. (1967)
Arch. Biochim. Biophys. 121, 563
79. Tsunoda, J. N., Yasunobu, K. T., and Whiteley, H. R.
(1968) J. Biol. Chem. 243, 6262
80. Rall, S. C., Bolinger, R. E., and Cole, R. D. (1969)
Biochemistry, 8, 2486

81. Tanaka, M., Haniu, M., Matsueda, G., Yasunobu, K. T., Himes, R. H., Akagi, J. M., Barnes, E. M., and Devanathan, T. (1971) J. Biol. Chem. 246, 3953
82. Azari, P., Tsunoda, J., Glanz, M., Mayhew, S., and Yasunobu, K. T. (1970) unpublished data cited in ref. 7
83. Tanaka, M., Haniu, M., Yasunobu, K. T., Himes, R., and Akagi, J. (1973) J. Biol. Chem. 248, 5215
84. Tanaka, M., Haniu, M., Yasunobu, K. T., Jones, J. B., and Stadman, T. C. (1974) Biochemistry, 13, 5284
85. Matsubara, H., Sasaki, R. M., Tsuchiya, D. K., and Evans, M. C. W. (1970) J. Biol. Chem. 245, 2121
86. Hase, T., Matsubara, H., and Evans, M. C. W. (1977) J. Biochem. 81, 1745
87. Tanaka, M., Haniu, M., Yasunobu, K. T., Evans, M. C. W., and Rao, K. K. (1974) Biochemistry, 13, 2953
88. Tanaka, M., Haniu, M., Yasunobu, K. T., Evans, M. C. W., and Rao, K. K. (1975) Biochemistry, 14, 1938
89. Hase, T., Wakabayashi, S., Matsubara, H., Evans, M. C. W., and Jennings, J. V. (1978) J. Biochem. 83, 1321
90. Hase, T., Wakabayashi, S., Matsubara, H., Ohmori, D., and Suzuki, K. (1978) FEBS Lett. 91, 315
91. Hase, T., Wakabayashi, S., Matsubara, H., Imai, T., Matsumoto, T., and Tobari, J. (1979) FEBS Lett. in press
92. Matsubara, H., Wada, K., and Masaki, R. (1976) in Iron and Copper Proteins (K. T. Yasunobu, H. F. Mower, and O. Hayaishi, eds) pp. 1
93. Ogawa, K., Tsukihara, T., Tahara, H., Katsube, Y.,

- Matsuura, Y., Tanaka, N., Kakudo, M., Wada, K., and Matsubara, H. (1977) J. Biochem. 81, 529
94. Kunita, A., Koshibe, M., Nishikawa, Y., Fukuyama, K., Tsukihara, T., Katsube, Y., Matsuura, Y., Tanaka, N., Kakudo, M., Hase, T., and Matsubara, H. (1978) J. Biochem. 84, 989
95. Tsukihara, T., Fukuyama, K., Tahara, H., Katsube, Y., Matsuura, Y., Tanaka, N., Kakudo, M., Wada, K., and Matsubara, H. (1978) J. Biochem. 64, 1645; Fukuyama, K. (1979) in Doctoral Thesis at Faculty of Science, Osaka Univ.
96. Masaki, R., Wada, K., and Matsubara, H. (1977) J. Biochem. 81, 1
97. Petering, D. H. and Palmer, G. (1970) Arch. Biochem. Biophys. 141, 456
98. Geiger, B., Mevarech, M., and Werber, M. M. (1978) Eur. J. Biochem. 84, 449
99. Kersher, L. and Oesterhelt, D. (1977) FEBS Lett. 83, 197
100. Werber, M. M. and Mevarech, M. (1978) Arch. Biochem. Biophys. 186, 60
101. Leach, C. K. and Carr, N. G. (1971) Biochim. Biophys. Acta, 245, 165
102. Hewitt, E. J. (1975) Ann. Rev. Plant Physiol. 26, 73
103. Lanyi, J. K. (1974) Bacteriol. Rev. 38, 272
104. Fitch, W. M. (1966) J. Mol. Biol. 16, 17

105. Adman, E. T., Sieker, L. C., and Jensen, L. H. (1973)
J. Biol. Chem. 248, 3987
106. Carter, C. W., Jr. (1977) in Iron Sulfur Proteins
(W. Lovenberg, ed.) Vol. 3, pp. 157
107. Le Gall, J. and Dragoni, N. (1966) Biochem. Biophys.
Res. Commun. 23, 145
108. Mullinger, R. N., Cammack, R., Rao, K. K., and
Hall, D. O. (1975) Biochem. J. 151, 75
109. Shethna, Y. I., Stombaugh, N. A., and Burnis, R. H.
(1971) Biochem. Biophys. Res. Commun. 42, 1108
110. Hase, T. and Matsubara, H. unpublished results
111. Zubieta, J. A., Mason, R., and Postgate, J. R.
(1973) Biochem. J. 133, 851
112. Yang, S. S., Ljungdahl, L. G., and Le Gall, J. (1977)
J. Bacteriol. 130, 1084
113. Seki, S., Hagiwara, M., Kudo, K., and Ishimoto, M.
(1979) J. Biochem. in press
114. Yoch, D. C. and Arnon, D. I. (1975) J. Biol. Chem.
250, 8330
115. Yates, M. G., O'Donnell, M. J., Lowe, D. J., and
Bothe, H. (1978) Eur. J. Biochem. 85, 291
116. Yoch, D. C., Carithers, R. P., and Arnon, D. I. (1977)
J. Biol. Chem. 252, 7453
117. Dus, K., DeKlerk, H., Sletten, K., and Bartsch, R. G.
(1967) Biochim. Biophys. Acta, 140, 291
118. Ohmori, D. (1976) Biochem. Biophys. Res. Commun.
72, 566

119. Imai, T., Matsumoto, T., Ohta, S., and Tobari, J.
(1979) Eur. J. Biochem. submitted
120. Howard, J. B., Lorsback, T., and Que, L. (1976)
Biochem. Biophys. Res. Commun. 70, 582
121. Fitch (1976) J. Mol. Evol. 8, 13
122. Goodman, M. Moore, G. W., and Barnabes, J. (1974)
J. Mol. Evol. 3, 1
123. Fitch, W. M. and Margoliash, E. (1967) Science, 155, 279
124. Andrew, P. W., Rogers, L. J., Boulter, D., and Haslett,
B. G. (1976) Eur. J. Biochem. 69, 243
125. Magrum, L. J., Luehrsen, K. R., and Woese, C. R.
(1978) J. Mol. Evol. 11, 1
126. Woese, C. R. and Fox, G. E. (1977) Proc. Natl. Acad.
Sci. U.S.A. 74, 5088
127. Hori, H. and Osawa, S. (1979) Proc. Natl. Acad. Sci.
U.S.A. 76, 381
128. Matheson, A. T., Yaguchi, H., Nazas, R. N., Visentin,
L. P., and Willick, G. E. (1978) in Energetics and
Structure of Halophilic Microorganisms (S. R. Caplan
and Ginzberg, M. eds.) cited in ref. 127
129. Hase, T., Wada, K., and Matsubara, H. (1975) J. Biochem.
78, 605
130. Hutson, K. G. and Rogers, L. J. (1975) Biochem. Soc.
Trans. 3, 377
131. Hutson, K. G., Rogers, L. J., Haslett, B. G., Boulter,
D., and Cammack, R. (1978) Biochem. J. 172, 465
132. Shin, M., Sukenobu, M., Oshino, R., and Kitazume, Y.
(1977) Biochim. Biophys. Acta, 460, 85

133. Cammack, R., Rao, K. K., Barger, C. P., Hutson, K. G., Andrew, P. W., and Rogers, L. J. (1977) Biochem. J. 168, 205
134. Takaichi, S., Makino, K., Iwasaki, A., and Suzuki, K. (1978) J. Biochem. 83, 1151
135. Suzuki, K. (1976) Abstract in Ann. Meeting of Japanese Plant Physiol. pp. 9
136. Huisman, J. G., Stapel, S., and Muijsers, A. O. (1978) FEBS Lett. 85, 198
137. Mukhin, E. N., Neznaiko, N. F., Chugunov, V. A., and Erokhin, Yu, E. (1975) Dokl. Akad. Nauk SSSR 221, 228
138. Barker, W. C., Ketcham, L. K., and Dayhoff, M. O. (1978) J. Mol. Evol. 10, 265