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The Amino Acid Sequence of *Clostridium pasteurianum* Ferredoxin*

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ABSTRACT: Four derivatives of the *Clostridium pasteurianum* ferredoxin were prepared from trichloroacetic acid treated ferredoxin. They were hydrolyzed with chymotrypsin, trypsin, or pepsin and the peptides were purified by chromatography on Dowex 50-×2 and AG 1-×2.

The peptides thus obtained were further purified by paper chromatography, paper electrophoresis, or column chromatography. The sequences of the purified peptides were determined by the Edman degradative method, dinitrophenylation, by the use of leucine aminopeptidase and carboxypeptidase A and B. From the sequences of the individual peptides and the overlaps between them, the total amino acid sequence of the 55 amino acid residues comprising the *C. pasteurianum* ferredoxin was deduced.

Ferredoxin, a nonheme iron containing protein was isolated from *Clostridium pasteurianum* by Mortenson *et al.* (1962, 1963). The electron transport protein has also been isolated from numerous anaerobic bacteria in crystalline form. A recent review article (Valentine, 1964) discusses the occurrence, structure, and function of the various bacterial ferredoxins.

The latest report on the physicochemical properties of *C. pasteurianum* ferredoxin indicates that the protein has a molecular weight of *ca.* 6000, contains about 50 amino acid residues, and has seven iron atoms per molecule of protein (Lovenberg *et al.*, 1963). The protein contains no tryptophan, methionine, or –S–S– bridges. Our laboratory has published a preliminary note on the amino acid sequence of the protein (Tanaka *et al.*, 1964b).

Experimental Section

Preparation of Ferredoxin. The procedure for isolating ferredoxin from *C. pasteurianum* was based upon a method devised by Mortenson *et al.* (1962, 1963) and has been described in a previous publication (Tanaka *et al.*, 1964b). For purity check, each preparation of ferredoxin was analyzed for the 390:280 mμ ratio (0.79 or greater), the amino acid composition, and the NH_2-terminal amino acid residue.

Derivatives of Ferredoxin. Iron and sulfide were removed from intact ferredoxin by trichloroacetic acid precipitation as previously described (Tanaka *et al.*, 1964b). This method is a simple and easy procedure for obtaining iron-removed and sulfide-free ferredoxin (TCAFd)[1] without cleavage of the peptide bonds.

Due to scarcity in the protein of peptide bonds which are hydrolyzed by trypsin or chymotrypsin, and due to the high content of cysteine residues, the ferredoxin molecule posed certain problems. To overcome these difficulties, four derivatives were prepared.

A. OXIDIZED FERREDOXIN (OFd).[1] This was made in the usual manner by performic acid oxidation of the trichloroacetic acid treated ferredoxin (TCAFd) (Schram *et al.*, 1954). After oxidation, the oxidized ferredoxin was passed through a column of Sephadex G-25 and then lyophilized. The oxidized ferredoxin obtained was a flocculent and white powder which was water soluble.

B. *S*-β-AMINOETHYLCYSTEINYL-FERREDOXIN (AE-

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[1] The following abbreviations were adopted: OFd, oxidized ferredoxin; AECFd, *S*-β-aminoethylcysteinyl-ferredoxin; TCAFd, trichloroacetic acid treated ferredoxin; CAMCFd, *S*-carboxamidomethylcysteinyl-ferredoxin; DECFd, *S*-β-dithiocarbaminoethylcysteinyl-ferredoxin; BAW, butanol–acetic acid–water; BPAW, butanol–pyridine–acetic acid–water; BPW, 1-butanol–pyridine–water; LAP, leucine aminopeptidase; CPase, carboxypeptidase; AEC, *S*-β-aminoethylcysteine; PTH, phenylthiohydantoin; and PTC-, phenylthiocarbamyl.

CFd).[1] TCAFd, 348 mg, was aminoethylated by the procedure described by Lindley (1956) as modified by Raftery and Cole (1963). The aminoethylated ferredoxin was precipitated by the addition of solid ammonium sulfate to 100% saturation and was left standing overnight in the cold room. The precipitate produced was centrifuged, washed twice with 20 ml of water, washed once with 20 ml of ethanol, once with 20 ml of ether, and then dried *in vacuo*. The aqueous phase obtained during the washing process was applied on a Sephadex G-25 column (6 × 41 cm) and eluted with water. (This step served to remove the ammonium sulfate from the solution.) The eluate was then lyophilized. The precipitate and the lyophilized materials showed the same amino acid composition. The total yield of AECFd was 98.8% and the protein was insoluble in water.

C. *S*-CARBOXAMIDOMETHYLCYSTEINYL-FERREDOXIN (CAMCFd).[1] TCAFd, 107 mg, was treated with iodoacetamide as described by Crestfield *et al.* (1963). The reaction mixture was treated with solid ammonium sulfate to 100% saturation and the precipitate was collected by centrifugation. The precipitate was washed with water, ethanol, and ether as described in the preparation of AECFd. The yield was almost quantitative and the product was water insoluble.

D. *S*-β-DITHIOCARBAMINOETHYLCYSTEINYL-FERREDOXIN (DECFd).[1] AECFd, 100 mg, was treated with excess carbon disulfide by the procedure described by Merigan *et al.* (1962). The solution was then bubbled with nitrogen to remove the excess carbon disulfide. The DECFd was then treated with chymotrypsin. Although the protein was at first in suspension, as hydrolysis proceeded, the products became water soluble.

Enzymatic Hydrolyses of the Ferredoxin Derivatives. A. TRYPTIC HYDROLYSIS OF AECFd. AECFd, 338 mg, was suspended in 15 ml of water and the solution was adjusted to pH 8.0 by the addition of 10% trimethylamine. The mixture was incubated with 2% trypsin by weight (Worthington Biochemical Corp.) which was pretreated with 0.06 N hydrochloric acid for 12 hr. The digestion was allowed to proceed for 55 hr at 36° during which time the solution was maintained at pH 8.0 by the manual addition of 10% trimethylamine. (An additional 2% of trypsin was added at 10 hr during the 55 hr reaction time.) The reaction mixture was centrifuged and a small amount of dark brown precipitate was obtained. The precipitate was washed with water and the combined supernatant fractions were lyophilized. A hygroscopic brownish white material was obtained which weighed 240 mg.

B. PEPSIN DIGESTION OF OFd. OFd, 300 mg, was dissolved in 30 ml of 0.02 N acetate buffer, pH 2.3, and incubated with 30 mg of pepsin (three times recrystallized product of the Nutritional Biochemical Corp.). The mixture was allowed to react for 14 hr at 27° and then lyophilized.

C. CHYMOTRYPTIC CLEAVAGE OF CAMCFd. CAMCFd, 104 mg, was suspended in 10 ml of water and the solution was adjusted to pH 8.0 with 10% trimethylamine. The suspension was incubated with 2% α-chymotrypsin (by weight) (three times recrystallized from the Worthington Biochemical Corp.). At 9 and 22 hr, additional α-chymotrypsin (2% by weight) was added to the reaction mixture. After 34 hr, the reaction mixture was lyophilized.

D. CHYMOTRYPTIC DIGESTION OF DECFd. A DECFd solution, 15 ml, at pH 9.4 was incubated with 2.0 mg of α-chymotrypsin for 2 hr at 30°. The reaction was terminated by lowering the pH to 2.0 with 3 N HCl. The solution was then vigorously bubbled with nitrogen in order to remove the carbon disulfide bound to the amino groups. The digestion mixture was then applied directly to a Dowex 50-×2 column as described in a later section.

Column Chromatography of Digestion Products. A. COLUMN CHROMATOGRAPHIC SEPARATION OF THE TRYPTIC PEPTIDES FROM AECFd. The reaction product was dissolved in 16.6 ml of the starting buffer and applied to a column of Dowex 50-×2 (2 × 108 cm) which has been equilibrated with the starting buffer. The peptides were purified by gradient elution using various pyridinium–acetate buffers as described by Margoliash and Smith (1962). The reservoir contained 2 l. of 2.0 M pyridinium–acetate buffer, pH 4.8, and the mixing chamber contained 2 l. of 0.2 M pyridinium-acetate buffer, pH 3.1. Fractions of 6 ml were collected and 0.05-ml aliquots of each fraction were analyzed by the ninhydrin reaction developed by Moore and Stein (1948). Ninhydrin-positive fractions were collected, dried *in vacuo*, dissolved in 10 ml of distilled, deionized water, and stored in the freezer. The peptide FT-4 became water insoluble after drying *in vacuo* and it was washed with water and ethanol several times and then dried *in vacuo* and stored in the freezer.

B. COLUMN CHROMATOGRAPHY OF THE PEPTIC PEPTIDES FROM OFd. The reaction mixture was dissolved in 15 ml of pyridinium–collidinium–acetate buffer (20:20:2.2 ml in 2 l. of water), pH 7.8. The solution was applied to a column of AG 1-×2 (2 × 100 cm) (Bio-Rad Laboratories) and eluted using the buffers described by Schroeder *et al.* (1958). The peptide fractions were located by ninhydrin analyses, pooled, and stored at −12°.

C. CHROMATOGRAPHIC SEPARATION OF THE CHYMOTRYPTIC PEPTIDES OF CAMCFd. The digestion mixture was dissolved in 3 ml of the starting buffer and applied to a column (1.2 × 58 cm) of DEAE-Sephadex A-25 (Pharmacia Co.) which had been equilibrated with the starting buffer. Gradient elution was used first, the solvents consisting of 150 ml of 0.1 M acetic acid, pH 3.1, and 150 ml of 0.1 M collidinium–acetate buffer, pH 8.65, followed by stepwise elution with 150 ml of 0.1 M acetic acid, pH 2.3, as described by Carnegie (1961). Fractions of 2 ml were collected. Peptide-containing fractions were pooled and stored at −12°.

D. SEPARATION OF CHYMOTRYPTIC PEPTIDES OF DECFd. The enzymatic digest was applied to a Dowex 50-×2 column (1.6 × 60 cm) which had been equilibrated with the starting buffer. Gradient elution was used where the reservoir contained 300 ml of 2.20 M pyridinium–acetate buffer, pH 5.26, and the mixing chamber contained 300 ml of 0.2 M pyridinium–acetate

buffer, pH 3.1. Fractions of 5 ml were collected and analyzed by the ninhydrin procedure except for fractions above 90 which were also analyzed after alkaline hydrolysis as described by Hirs *et al.* (1956). The peptide-containing fractions were collected, dried, dissolved in water, and stored at −12°.

Further Purification of Peptides. In all of the experiments, purity checks of each fraction were made on Whatman No. 3MM paper by electrophoresis using pyridinium–acetate buffer, pH 6.5, at a potential of 9 v/cm for 2 hr in the first dimension and chromatography (BPAW)[1] as described by Margoliash and Smith (1962) in the second dimension. In some cases, the solvent system 1-butanol–pyridine–water (BPW)[1] (1:1:1, by volume) or 1-butanol–acetic acid–water (BAW)[1] (4:1:5, by volume) was used in the second dimension. If further purification was required, each peptide was purified by paper chromatography, paper electrophoresis, or a combination of these procedures.

In some cases, the peptides were purified by column chromatography on Dowex 50-×2 or AG 1-×2 (acetate form). In the case of the chymotryptic peptides from CAMCFd, stepwise elution from DEAE-Sephadex A-25 followed by chromatography on Dowex 50-×2 was necessary to obtain the pure peptides.

Pepsin Digestion of Peptide FT-2. Peptide FT-2 from the tryptic digest of AECFd was further digested with pepsin. FT-2, 4.4 μmoles, was dissolved in 1.0 ml of 0.01 N HCl, pH 2.0, and 1.0 ml of a solution containing 2.0 mg of pepsin in 0.01 N HCl was added. The mixture was incubated for 7 hr at 28°. The digest was lyophilized, dissolved in 1 ml of the equilibrating buffer, and applied to a column of Dowex 50-×2 (1 × 41 cm) which had been equilibrated with 0.2 M pyridinium–acetate buffer, pH 3.0. Gradient elution was used where the mixing chamber contained 100 ml of 0.17 M pyridinium–acetate buffer, pH 4.6, and the reservoir contained 100 ml of 1.07 M pyridinium–acetate buffer, pH 5.45. Three peaks were detected by direct ninhydrin analysis and readings were taken at both 440 and 550 mμ.

Sequence Determination of Peptides. A. DINITROPHENYLATION. The usual dinitrophenylation procedure as described by Fraenkel-Conrat *et al.* (1955) was used. In some cases, an aliquot of the aqueous layer was concentrated to dryness, rehydrolyzed, and analyzed in the automatic amino acid analyzer.

B. EDMAN PROCEDURE. The sequence of each peptide was determined by the subtractive procedure (Konigsberg and Hill, 1962) and by the direct identification of the PTH–amino acid formed (Sjoquist, 1953; Fraenkel-Conrat *et al.*, 1955; Edman and Sjoquist, 1956). Solvent systems A and F were used almost exclusively. In the case of NH_2-terminal glycine peptides, cyclizations were performed in a glacial acetic acid–concentrated HCl mixture (5:1) instead of trifluoroacetic acid as described by Margoliash (1962).

C. LEUCINE AMINOPEPTIDASE (LAP).[1] A Worthington Biochemical Corp. preparation of LAP was used. The reactions were usually performed at pH 8.0 and at 40° for 22 hr in the presence of 0.005 M $MgCl_2$. Before use, the LAP was activated by incubation with 0.005 M $MgCl_2$ at pH 8.0 and 40° for 30 min. An aliquot of the digestion mixture was directly analyzed in the amino acid analyzer and the remainder was dried, dissolved in the starting buffer, and applied on a Dowex 50-×2 (equilibrated with 0.1 M pyridinium–acetate buffer, pH 3.1) column (0.6 × 12.0 cm). Stepwise or gradient elution with pyridinium–acetate buffers of different molarities was used. Ninhydrin-positive peaks were collected and hydrolyzed and the amino acid compositions were determined.

D. CARBOXYPEPTIDASE A AND B (CPASE A AND B).[1] DFP-treated CPase A (three times crystallized) and DFP-treated CPase B were purchased from the Worthington Biochemical Corp. CPase A reactions were usually performed at pH 8 for 12 hr at either 30 or 40°. CPase B reactions were carried out at pH 8.0 and 30° for 1 hr. The reaction mixtures were purified as described in the LAP experiments.

Amino Acid Composition of Peptides. Peptides from the enzymatic hydrolysates were hydrolyzed with 6 N HCl (triply distilled) at 110° for 22 hr in evacuated sealed tubes. FT-3 which was obtained from the tryptic digestion of AECFd was hydrolyzed for 44 hr because the peptide contained a valyl–isoleucyl bond which was hydrolyzed only *ca.* 60% after 22 hr of hydrolysis. The amino acid composition of the protein and peptides was determined in the Model 120 Beckman–Spinco automatic amino acid analyzer (Spackman *et al.*, 1958).

Results

Amino Acid Composition of Ferredoxin. Since the sequence of the protein gives the best amino acid composition and since other investigators have already published the amino acid composition of *C. pasteurianum* ferredoxin (Lovenberg *et al.*, 1963; Mortenson, 1964), detailed studies were not made to determine the amino acid composition. However, since there was a lack of agreement concerning the cysteine, serine, valine, and isoleucine residues in the protein, these were in-

TABLE I: Cysteic Acid, Serine, Valine, and Isoleucine Content of Oxidized Ferredoxin.[a]

	Residues/Mole (Ratio to Aspartic)				
	Hydrolysis Time (hr)				Extrapolated
Amino Acid	12	24	48	96	Values
Aspartic acid	8.00	8.00	8.00	8.00	8
Serine	4.5	4.2	3.7	3.37	5
Cysteic acid	7.73	7.84	7.77	7.71	8
Valine	5.24	5.64	5.67	5.81	6
Isoleucine	3.95	4.23	4.52	4.71	5

[a] The preparation of oxidized ferredoxin is given in the Experimental Section. [b] See Valentine *et al.* (1963) for the amino acid content of other amino acid residues.

TABLE II: Properties of the Tryptic Peptides from AECFd.

Peak No.	Total (μmoles) (Leu Equiv)	Electrophoretic Properties	Mobility[a] (μ), cm	R_F (BPAW)	Color with Ninhydrin	Method[c] of Purification
FT-1	162	...	...	...	...	...
FT-2	20	Neutral	−2.4	0.30	Blue	PC
FT-3	20.5	Acidic	+2.5	0.30	Blue	PC
FT-4	21					
FT-4-p	10	...	...	0.28[b]	Violet	PC
FT-4-a-1	2	Acidic	+2.5	0.67	Blue	PC and PE
FT-4-a-2	1.6	Neutral	−1.1	0.67	Blue	PC and PE
FT-4-c-1	5.6	Neutral	−1.0	0.25	Violet	PC and PE
FT-5	20	Neutral	−2.3	0.25	Blue	PC
FT-6	46.5					
FT-6-a	20	Basic	−10.2	0.25	Blue	PC
FT-6-b	20	Basic	−11.7	0.19	Yellow	PC
FT-7	6	...	...	...	...	PC
FT-8	15	Basic	−8.0	0.10	Blue	PC
FT-9	27	Basic	−9.8	0.38	Purple	PC

[a] 500 v, 2 hr in pyridine–acetic acid–water (100:4:900), pH 6.5. [b] 1-Butanol–pyridine–water (60:60:60) on Whatman No. 1 filter paper. [c] Abbreviations used are PC, paper chromatography and PE, paper electrophoresis.

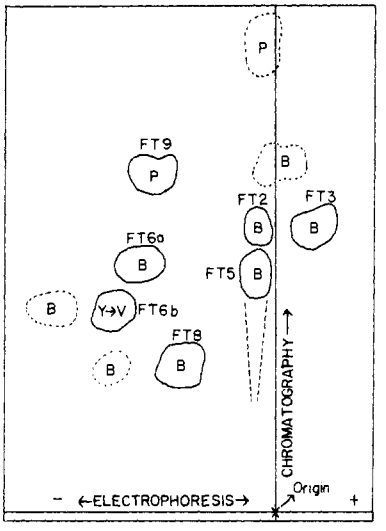

FIGURE 1: Fingerprint map of the trypsin digest of AECFd. The first dimension (paper electrophoresis) 500 v, 2 hr in pyridine–acetic acid–water (100:*ca.* 4:900), pH 6.5. The second dimension (paper chromatography) 1-butanol–pyridine–acetic acid–water (375:250:75:300), on Whatman No. 3MM filter paper. Abbreviations used are: B, blue; V, violet; P, purple; Y, yellow; and Y → V indicates color change from yellow to violet. The colors are those obtained after spraying with ninhydrin.

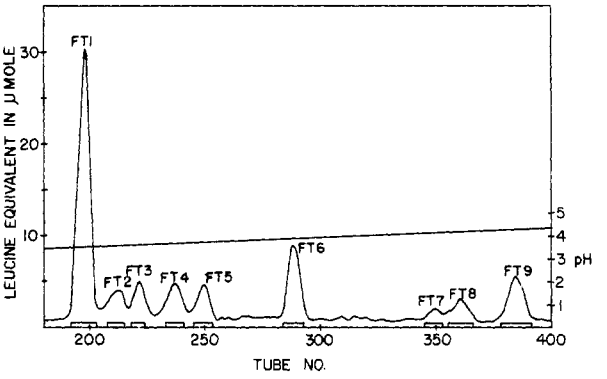

FIGURE 2: Dowex 50-X2 elution pattern of the tryptic digest of AECFd. The lyophilized digestion mixture (240 mg) was applied on a Dowex 50-×2 column (2 × 108 cm). Gradient elution was carried out from 0.2 M pyridine–acetate buffer, pH 3.1, to 2 M pyridine–acetate buffer, pH 4.8. Fractions of 6 ml were collected and aliquots of 0.05 ml were checked for ninhydrin reaction.

vestigated on a sample of performic acid oxidized ferredoxin. The results are summarized in Table I.

Hydrolysis of AECFd with Trypsin. After the trypsin hydrolysis of AECFd for 55 hr at pH 8.0 and 36°, the pH of the digest was lowered to pH 3.0 by the addition of 1 N hydrochloric acid and the hydrolysate was lyophilized. Figure 1 shows the fingerprint map of the tryptic digest of AECFd. Seven main spots were detected on the map.

Column Chromatography. The lyophilized digestion mixture was dissolved in 16.6 ml of 0.2 M pyridine–acetic acid buffer, pH 3.1, and applied on a Dowex 50-×2 column (2 × 108 cm). Gradient elution was used and the reservoir contained 2.0 l. of 2.0 M pyridine–

TABLE III: Amino Acid Composition of Tryptic Peptides from AECFd.

Amino Acid	FT-2	FT-3	FT-4-p	FT-4-a-1	FT-4-a-2	FT-4-c-1	FT-5	FT-6-a	FT-6-b	FT-8	FT-9	Total[a]
Lysine	...	...	...	...	...	...	...	...	...	...	0.95(1)	1
AEC	0.77(1)	0.87(1)	0.80(1)	...	..	0.57(1)	0.9 (1)	0.97(1)	0.95(1)	1.63(2)	...	8
Aspartic acid	1.08(1)	2.14(2)	2.2 (2)	1.11(1)	...	1.1 (1)	1.0 (1)	...	...	2.10(2)	...	8
Threonine	...	0.89(1)	...	...	...	...	...	...	...	...	...	1
Serine	...	...	2.7 (3)	1.83(2)	...	1.1 (1)	0.93(1)	0.95(1)	...	...	...	5
Glutamic acid	2.01(2)	...	2.1 (2)	1.14(1)	...	1.0 (1)	...	...	...	...	...	4
Proline	1.91(2)	...	1.0 (1)	...	...	1.0 (1)	...	...	...	...	...	3
Glycine	1.05(1)	...	1.1 (1)	1.09(1)	...	...	...	...	1.06(1)	1.01(1)	...	4
Alanine	1.93(2)	1.05(1)	2.0 (2)	...	1.0(1)	1.8 (2)	1.0 (1)	...	0.99(1)	...	1.0 (1)	8
Valine	3.05(3)	0.91(1)	1.0 (1)	...	...	0.8 (1)	...	1.08(1)	...	...	...	6
Isoleucine	...	0.83(1)	1.9 (2)	1.95(2)	...	0.3	1.0 (1)	...	...	1.0 (1)	...	5
Tyrosine	...	...	...	...	1.0(1)	...	...	...	...	...	1.05(1)	1
Phenylalanine	...	...	0.8 (1)	0.93(1)	...	...	...	...	...	...	...	1
Total residues	12	7	16	8	2	8	5	3	3	6	3	55

[a] The numbers of amino acid residues in FT-4-a-1, FT-4-a-2 and FT-4-c-1 are not included since FT-4-p contains FT-4-c-1 plus FT-4-a-1 and FT-4-a-2 is included in FT-9.

acetic acid buffer, pH 4.8, and the mixing chamber contained 2.0 l. of 0.2 M pyridine–acetic acid buffer, pH 3.1. Figure 2 shows the Dowex 50-×2 column chromatographic pattern of the trypsin digest of AECFd.

Purification of Fractions. Each fraction which was separated by the Dowex 50-×2 column chromatography was combined and lyophilized, and R_F values and mobilities were determined by paper electrophoresis and paper chromatography. Table II shows the properties of the tryptic peptides of AECFd.

Fractions FT-2, FT-3, FT-5, FT-8, and FT-9 were nearly pure peptides. Fraction FT-6 consisted of two peptides, FT-6-a and FT-6-b. Fraction FT-4 contained a water-insoluble material (FT-4-p) after lyophilization. Extraction of FT-4-p produced a water-soluble fraction which was resolved by paper chromatography using BPAW to yield two peptides, FT-4-a and FT-4-c. FT-4-a was further resolved by paper electrophoresis to yield two peptides, FT-4-a-1 and FT-4-a-2. Fraction FT-7 contained mainly peptide FT-8 plus impurity. Fraction FT-1 contained large amounts of ammonia along with small quantities of peptides.

FT-2, FT-3, FT-5, FT-6-a, FT-6-b, FT-8, and FT-9 were purified by paper chromatography in the solvent system BPAW. A combination of paper chromatography in BAW and paper electrophoresis was necessary to purify peptides FT-4-a-1, FT-4-a-2, and FT-4-c.

Amino Acid Composition of Purified Peptides. Table III shows the amino acid compositions of the tryptic peptides derived from AECFd. All hydrolyses were carried out at 110° for 22 hr using 6 N HCl except for FT-3 which was hydrolyzed for 44 hr. AEC values were often low because of the decomposition during hydrolysis. Authentic aminoethylcysteine (AEC)[1] was prepared from L-cysteine with ethylenimine by a modification of the technique of Raftery and Cole (1963). It chromatographed between lysine and ammonia on the automatic amino acid analyzer. The C_{HW} constant for aminoethylcysteine was calculated to be 22.6, equal to that of lysine.

Sequences of Peptides. PEPTIDE FT-2: ALA-ASPNH_2-VAL-AEC-PRO-VAL-GLY-ALA-PRO-VAL-GLUNH_2-GLU. Table IV shows the results of the Edman degradation of the peptide FT-2. Neither CPase A nor trypsin hydrolyzed this peptide. After the pepsin digestion of this peptide and Dowex 50-×2 column chromatography, two peptides, FT-2-P-1 and FT-2-P-2, were obtained.

Table V shows the properties of the peptides obtained from peptide FT-2. The sequence of FT-2-P-1 (Pro-Val-GluNH_2-Glu) was determined by Edman degradation. The PTH–amino acids were directly identified for the first three steps and when the residue after the third step was analyzed, glutamic acid was the only amino acid detected. The subtractive procedure yielded the following results: step 1, *Pro (0.08)*, Val (0.99), and Glu (2.0); step 2, Pro (0.0), *Val (0.18)*, and Glu (2.0); step 3, Pro (0.0), Val (0.0), and *Glu (1.0)*. LAP digestion of the residual peptide from FT-2-P-1 obtained after the first Edman degradation was carried out. Application of an aliquot directly to the automatic amino acid analyzer disclosed an amino acid chromatographing in

TABLE IV: Edman Degradation of Peptide FT-2.

Step	0[a]	1	2	3	4	5	6	7	8	9	10
Yield (%)[b]	...	88	77	78	60	37	25	86	70	59	45
PTH–amino acid found	...	Ala	$AspNH_2$	Val	AEC	...	Val	Gly	Ala	Pro	...
AEC	0.77	0.73	0.66	0.61	*0.11*[c]	0.07	...	...	...	...	...
Aspartic acid	1.08	1.1	*0.24*	0.20	0.20	0.20	0.19	0.18	0.19	0.08	0.09
Glutamic acid	2.01	2.1	2.2	2.1	2.0	1.99	1.94	2.11	2.10	1.95	2.00
Proline	1.91	1.9	1.9	1.8	1.8	*0.91*	0.98	0.93	0.96	*0.24*	0.14
Glycine	1.05	1.0	1.0	1.0	1.0	1.06	1.00	*0.48*	0.34	0.21	0.23
Alanine	1.93	*1.0*	1.0	1.0	1.0	1.01	1.03	1.00	*0.36*	0.15	0.16
Valine	3.05	3.0	3.0	*2.1*	2.05	2.03	*1.33*	1.34	1.22	1.07	*0.44*
Sequence	...	Ala	$AspNH_2$	Val	AEC	Pro	Val	Gly	Ala	Pro	Val

[a] Original peptide. [b] Values at each step. [c] Italic figures show the NH_2-terminal amino acids.

TABLE V: Pepsin Digestion of Peptide FT-2.[a]

Fraction No.	1	2	3
Peptide	FT-2-P-1	FT-2-P (recd)	FT-2-P-2
Yield, μmoles (%)	2.7 (61)	0.9 (20)	2.7 (61)
Electrophoretic properties	Acidic	Neutral	Basic
Mobility (μ), cm	+3.5	−1.6	−6.0
Color with ninhydrin	Yellow	Blue	Blue
Amino acid compn			
AEC	...	0.71 (1)	0.75 (1)
Aspartic acid	...	1.0 (1)	1.01 (1)
Glutamic acid	2.03 (2)	2.0 (2)	...
Proline	0.92 (1)	1.86 (2)	1.03 (1)
Glycine	...	1.0 (1)	0.97 (1)
Alanine	...	2.0 (2)	1.86 (2)
Valine	1.04 (1)	3.0 (3)	2.0 (2)
Total residues	4	12	8

[a] 4.4 μmoles of FT-2 was digested with pepsin in 0.01 M HCl (pH 2) for 7 hr at room temperature (28.3°) and the hydrolysate was purified by Dowex 50-×2 (1 × 41 cm) column chromatography using gradient elution in which the mixing chamber contained 100 ml of 0.17 M pyridine–acetate buffer (pH 4.6) and the reservoir contained 100 ml of 1.07 M pyridine–acetate buffer (pH 5.45). Three peaks were obtained of which peak 1 consisted of FT-2-P-1, peak 2 was original peptide, undigested, and peak 3 was FT-2-P-2.

the same position as serine ($GluNH_2$) in 97% yield as well as Glu (100% yield) and Val (100% yield). Acid hydrolysis of the digest followed by analysis resulted in the increase of the glutamic acid content to two residues and disappearance of the peak in the serine position.

FT-2-P-2: (Ala,$AspNH_2$,Val,AEC,Pro)-Val-Gly-Ala. Hydrolysis of this peptide with CPase A liberated Ala, Gly, and Val in the ratio of 1.0:0.4:0.1, respectively.

Peptide FT-3: Val-Ileu-Asp-Ala-Asp-Thr-AEC. Table VI shows the results of the Edman degradation of peptide FT-3. After the CPase B digestion of this peptide followed by Dowex 50-×2 column chromatography, two components, FT-3-CPB-1 and FT-3-CPB-2, were obtained. FT-3-CPB-1 showed the following amino acid composition: Asp (2.00), Thr (0.96), Ala (1.00), Val (1.00), and Ileu (1.00), and no AEC. FT-3-CPB-1 was not hydrolyzed by CPase A even after a 12-hr incubation at 40°. FT-3-CPB-2 was AEC.

After LAP digestion of FT-3 followed by Dowex 50-×2 column chromatography, the peptide FT-3-LAP-b (Asp-Thr-AEC) was obtained in 60% yield and the amino acids AEC (0.37), Asp (1.40), Thr (0.41), Ala (1.00), Val (1.00), and Ileu (1.00) were also recovered in the ratios indicated. FT-3-LAP-b [Asp (1.00), Thr (1.00), and AEC (0.77)]: The Edman reaction produced PTH-Asp and the amino acid composition of the

TABLE VI: Edman Degradation of FT-3.

Step	0	1	2	3	4	5
Yield (%)	...	95	88	66	59	63
PTH–amino acid found	...	Val	Ileu	Asp	Ala	...
Amino acid						
AEC	0.87	0.75	0.74	0.64	0.55	...
Aspartic acid	2.14	2.08	2.08	*1.11*[a]	0.98	*0.37*
Threonine	0.89	0.91	1.0	0.93	1.01	1.00
Alanine	1.05	1.0	1.0	0.96	*0.22*	0.19
Valine	0.91	*0.0*	...	...	...	...
Isoleucine	0.83	0.96	*0.03*	...	...	...
Sequence	...	Val	Ileu	Asp	Ala	Asp or $AspNH_2$

[a] See footnote *c* of Table IV.

TABLE VII: Edman Degradation of Peptide FT-4-p.

Step	0	1	2	3	4	5	6	7	8
Yield (%)	...	98	70	63	95	66	95	78	77
PTH–amino acid found	...	Ala		Glu	AEC		Val		Ala
Amino acid									
AEC	0.8	0.73	0.68	0.66	*0.21*[a]	0.13	...	...	...
Aspartic acid	2.2	2.0	2.0	2.0	1.9	2.1	1.9	*1.4*	1.4
Serine	2.7	2.7	*1.75*	1.68	1.6	1.7	1.6	1.7	1.6
Glutamic acid	2.1	2.0	2.0	*1.23*	1.2	1.2	1.2	1.2	1.2
Proline	1.0	0.92	1.0	1.0	0.91	*0.21*	0.2	0.31	0.25
Glycine	1.1	1.0	1.0	1.0	1.0	1.0	1.0	1.1	1.1
Alanine	2.0	*1.0*	1.0	1.0	1.0	1.0	1.0	0.9	*0.51*
Valine	1.0	1.0	1.06	1.0	1.0	1.1	*0.38*	0.33	0.30
Isoleucine	1.9	1.8	1.84	1.9	1.8	1.9	1.8	1.8	1.8
Phenylalanine	0.8	0.81	0.82	0.75	0.8	0.8	0.82	0.8	0.81
Sequence	...	Ala	Ser	Glu	AEC	Pro	Val	Asp or $AspNH_2$	Ala

[a] See footnote *c* of Table IV.

residual peptide was Thr (1.00) and AEC (0.69). PTH-Thr was identified by paper chromatography in the second step of the Edman procedure.

PEPTIDE FT-4-P: ALA-SER-GLU-AEC-PRO-VAL-ASP-NH_2-ALA(ILEU, SER, GLUNH_2, GLY, ASP, SER, ILEU, PHE). This peptide was obtained as yellowish-white crystals after drying the sample *in vacuo*. Peptide FT-4-p was insoluble in water, ethanol, BPAW, and PAW (pH 6.5) solvent systems and soluble in BPW. Accordingly this peptide was purified by paper chromatography using BPW. Table VII shows the results of the Edman degradation of peptide FT-4-p. In the Edman degradation, the products which were obtained after the first, second, and third steps were insoluble in 0.2 M acetic acid.

PEPTIDE FT-4-A-1: ILEU-SER-(GLUNH_2,GLY,ASP,SER)-(ILEU,PHE). The dinitrophenylation procedure yielded only DNP-Ileu. The Edman degradation gave the following results: step 1, *Ileu* (*0.8*), Ser (1.80), Glu (1.1), Gly (1.1), Asp (1.0), and Phe (0.8); step 2, Ileu (0.8), *Ser* (*0.9*), Glu (1.1), Gly (1.0), Asp (1.0), and Phe (0.8). After 2 hr of digestion of peptide FT-4-a-1 with CPase A, Ileu and Phe were liberated in equal quantity in 50% yield.

PEPTIDE FT-4-A-2: ALA-TYR. This peptide is derived from the NH_2-terminal part of the ferredoxin molecule and the sequence of this peptide is described in a previous publication (Tanaka *et al.*, 1964a).

PEPTIDE FT-4-C-1: ALA-SER-GLU-(AEC,PRO,VAL,-$AspNH_2$,ALA). Only DNP–Ala was found by the dinitrophenylation procedure. The results of the Edman degradation were as follows: Original peptide, Asp (1.1), Ser (1.1), Glu (1.0), Pro (1.0), Ala (1.8), Val (0.8), and AEC (0.75). After the first Edman degradative step, the residual peptide showed the following amino acid composition: Asp (1.0), Ser (1.0), Glu (1.0), Pro

(0.9), *Ala* (*1.0*), Val (0.9), and AEC (0.7). After the second step, the residual peptide had the following composition: Asp (1.1), *Ser* (*0.4*), Glu (1.0), Pro (1.1), Ala (0.9), Val (1.0), and AEC (0.65). After the third step, the amino acid composition of the residual peptide was: Asp (0.9), Ser (0.3), *Glu* (*0.4*), Pro (1.0), Ala (0.9), Val (0.9), and AEC (0.6).

CPase A digestion of FT-4-c-1 produced Asp (14%), Ala (90%), and $AspNH_2$ (76%). (This amino acid appeared in the serine position when analyzed in the amino acid analyzer.) After column chromatographic separation of this digest on Dowex 50-×2, two peaks, FT-4-c-1-CPA-1 and FT-4-c-1-CPA-2, were obtained. The hydrolysates of these fractions with 6 N hydrochloric acid showed the following amino acid compositions: FT-4-c-1-CPA-1: Asp (1.0) and Ala (1.0); FT-4-c-1-CPA-2: Asp (0.0), Ser (1.0), Glu (1.2), Pro (1.1), Ala (0.9), Val (0.8), and AEC (0.72).

PEPTIDE FT-5: ILEU-ALA-ASP-SER-AEC. The sequence was determined by the Edman procedure. The subtractive procedure yielded the following results: step 1; Asp (1.0), Ser (0.9), Ala (1.0), and *Ileu* (*0.05*); step 2; Asp (1.0), Ser (0.9), *Ala* (*0.2*), and Ileu (0.0); step 3; *Asp* (*0.3*), Ser (1.0), Ala (0.0), and Ileu (0.0). AEC was not determined in steps 1–3. At each step, the PTH–amino acid was directly identified.

CPase B digestion of this peptide and Dowex 50-×2 column chromatography of the reaction mixture yielded two fragments, FT-5-CPB-1 and FT-5-CPB-2. The amino acid composition of FT-5-CPB-1 was Asp (1.1), Ser (1.0), Ala (1.0), Ileu (1.0), and no AEC. FT-5-CPB-2 was free AEC.

PEPTIDE FT-6-A: VAL-SER-AEC. The Edman degradation gave the following results: step 1, Ser (1.00), *Val* (*0.03*), and AEC (0.60). CPase B digestion and Dowex 50-×2 column chromatography yielded two fractions. Fraction FT-6-a-CPB-1 showed the following amino acid composition: Ser (0.9), Val (1.0), and no AEC. Fraction FT-6-a-CPB-2 was free AEC.

PEPTIDE FT-6-B: GLY-ALA-AEC. The subtractive Edman degradative procedure yielded the following results: step 1, *Gly* (*0.1*), Ala (1.00), and AEC (0.60); step 2, PTH-Ala was identified by paper chromatography. CPase B digestion and Dowex 50-×2 column chromatography of the digest yielded FT-6-b-CPB-1 (84% yield), Gly (1.0), Ala (1.0), and no AEC; and FT-6-b-CPB-2 (84% yield), AEC.

PEPTIDE FT-8: ILEU-ASP-AEC-GLY-$AspNH_2$-AEC. Dinitrophenylation of this peptide followed by acid hydrolysis produced only DNP-Ileu in the ether layer and the amino acid composition of water layer was Asp (1.9), Gly (1.1), and Ileu (0.18). (Free AEC was not recovered because of its conversion to β-DNP-AEC.) The results of the Edman degradation were as follows: step 1, PTH-Ileu was directly identified. The amino acid composition of the residual peptide was Asp (2.00), Gly (1.00), *Ileu* (*0.0*), and AEC (1.50). step 2, PTH-Asp was directly identified. The residual peptide showed the composition *Asp* (*1.12*), Gly (1.00), Ileu (0.0), and AEC (1.30). Step 3, PTH-AEC was directly identified. The amino acid composition of the residual peptide was Asp (1.05), Gly (1.00), Ileu (0.0), and *AEC* (*0.62*). Step 4, PTH-Gly was identified by paper chromatography. The residual peptide was shown by analysis to be Asp (1.00), *Gly* (*0.33*), Ileu (0.0), and AEC (0.50). Step 5, PTH-$AspNH_2$ was identified directly. Step 6, PTH-AEC and PTH-$AspNH_2$ were identified by paper chromatography. With another sample, after the third Edman degradative step, the residue was digested with LAP and direct amino acid analysis of the digest showed the composition of the residual peptide to be Asp (13%), $AspNH_2$ (79%), and Gly (83%). (Free AEC was not detected because of its conversion to β-PTC-AEC in the Edman degradation.)

A prolonged trypsin digestion of the peptide FT-8 was tried. Aliquots of the digestion mixture were taken at intervals and checked by paper electrophoresis (500 v, 2 hr in PAW, pH 6.5). The results are summarized in Table VIII.

After 8-days digestion of peptide FT-8, the remainder of the reaction mixture was resolved by paper electrophoresis. FT-8-T-1 contained various fragments and no separation of the components was possible. FT-8-T-2 was the original undigested peptide. Peptide FT-8-T-3

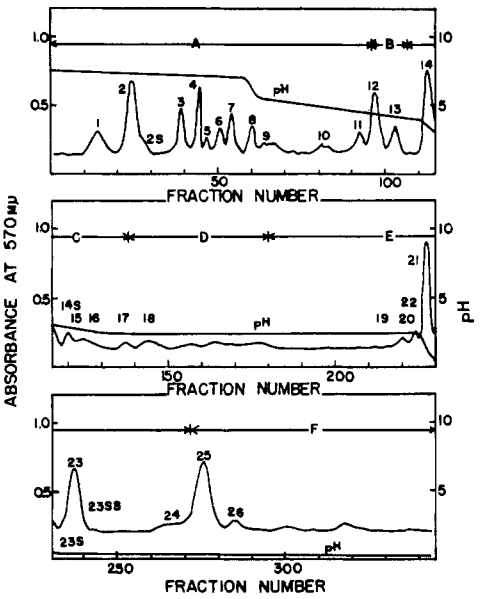

FIGURE 3: AG 1-×2 column chromatography of the pepsin digest of OFd. The peptic digest of OFd (280 mg) was applied to an AG 1-×2 column (acetate form), 2 × 100 cm, which was equilibrated with pyridine–acetic acid–2,4,6-collidine-water (20:2.2:20:1958, v/v; total of 2000 ml, pH 7.8) and eluted as follows. A, gradient elution where the mixing chamber contained 700 ml of starting buffer and the reservoir 700 ml of 0.4 M acetic acid; stepwise elution was used thereafter; B, 660 ml of 0.2 M acetic acid plus 660 ml of 0.4 M HCl; C, 300 ml of 0.44 M acetic acid; D, 700 ml of 0.44 M acetic acid plus 750 ml of 0.44 M HCl; E, 500 ml of 0.2 M HCl plus 500 ml of 2 M HCl; and F, 1.3 M HCl.

TABLE VIII: Properties of Tryptic Peptides of FT-8.

Peptide	FT-8-T-1	FT-8-T-2	FT-8-T-3
Electrophoretic migration (cm)	−3.0	−8.0	−10.6
Electrophoretic properties	Neutral	Basic	Very basic
Color with ninhydrin	Blue	Blue	Yellow
Relative yield			
0 time	None	All	None
2 days	+1	+5	+1
7 days	+1	+2	+1

showed the amino acid composition Asp (1.0), Gly (1.0), and AEC (0.61). CPase B digestion of FT-8-T-3 liberated only free AEC.

PEPTIDE FT-9: ALA-TYR-LYS. This peptide is the NH_2-terminal peptide of ferredoxin and the sequence of peptide FT-9 has been described in a previous paper (Tanaka *et al.*, 1964a).

Pepsin Digestion of OFd. The peptic digest of OFd was checked by the fingerprint technique, paper electrophoresis (460 v, 2 hr at pH 6.5) in the first dimension and paper chromatography (BPAW) in the second dimension. Eight spots were observed.

Figure 3 shows the AGl-×2 column chromatographic pattern of the peptic digest of OFd. Twenty-six peaks were separated by the column chromatography. The fractions which were obtained after no. 260 were neutralized with 1 N NaOH at once to avoid hydrolysis by hydrochloric acid. Each peak was checked for the mobility by paper electrophoresis (460 v for 2 hr) and R_F by paper chromatography (BPAW). The purification methods used were Dowex 50-×2 column chromatography, AGl-×2 (acetate form) column chromatography, paper chromatography (BPAW), and paper electrophoresis (PAW), or combinations of these methods. Eleven peaks were purified in this manner. Tables IX and X show the properties and amino acid compositions of the purified peptic peptides of OFd. FP-2 and FP-3 were Ala and Tyr, respectively. FP-6 and FP-7 showed the same amino acid composition and had the same sequence as shown in a later section of this paper.

Sequence of Peptic Peptides. FRACTION FP-2: ALA. After the first step of the Edman degradation, PTH-Ala[1] was directly identified.

FRACTION FP-3: TYR. The Edman degradation procedure when applied to FP-3 indicated that tyrosine was NH_2-terminal.

PEPTIDE FP-4: ALA-ILEU-SER-GLUNH_2-GLY. The Edman degradation of FT-4 gave the following result: step 1, PTH-Ala was found by direct identification and the residual peptide consisted of Ser (0.80), Glu (1.00), Gly (1.00), *Ala (0.00)*, and Ileu (0.90); step 2, PTH-Ileu was directly identified and the residual peptide characterized as Ser (0.80), Glu (1.10), Gly (1.00), Ala (0.00), and *Ileu (0.04)*; step 3, *Ser (0.16)*, Glu (1.00), Gly (1.10), Ala (0.00), and Ileu (0.00); step 4, PTH-Glu NH_2 was determined by direct identification and the residual peptide characterized as Ser (0.10), *Glu (0.23)*, Gly (1.00), Ala (0.00), and Ileu (0.00).

PEPTIDE FP-6: ILEU-SER-GLUNH_2-GLY. The results of the Edman degradation of this peptide were as follows: step 1, PTH-Ileu was identified by paper chromatography and the composition of the residual peptide was shown by analysis to be: Ser (0.70), Glu (1.00), Gly (1.00), and *Ileu (0.09)*; step 2, PTH-Ser was obtained by direct identification and the residual peptide characterized as having the composition *Ser (0.08)*, Glu (1.00), Gly (1.00), and Ileu (0.00); step 3, PTH-GluNH_2 was directly identified and the residual peptide was shown to contain Ser (0.08), *Glu (0.16)*, Gly (1.00), and Ileu (0.00).

PEPTIDE FP-7: ILEU-SER-GLUNH_2-GLY. The amino acid composition and sequence of this peptide were identical with peptide FP-6. The results of the Edman degradation of peptide FP-7 were as follows: step 1, Ser (0.80), Glu (1.10), Gly (1.00), and *Ileu (0.04)* by the subtractive method; step 2, PTH-Ser was identified by

TABLE IX: Properties of the Peptic Peptides of OFd.

Peptide No.	Yield (%)	Mobility (μ), cm	R_F (BPAW)	Color with Ninhydrin	Method of Purification
FP-2	19	−2.5	0.36	Purple to violet	...
FP-3	7	−2.5	0.62	Blue	...
FP-4	8	−2.5	0.39	Purple	...
FP-6	6	−2.5	0.38	Purple	...
FP-7	11	−2.5	0.38	Purple	...
FP-11	9	+8.7	0.23	Purple to blue	BPAW
FP-14-S	5	+2.6	0.23	Blue	Dowex 50-×2
FP-15	2	+1.2	0.48	Blue	Dowex 50-×2
FP-16	3	+4.7	0.45	Purple plus yellow	...
FP-18	5	+10.0	0.36	Violet	...
FP-23-II	24	+2.5	0.61	Violet	PE

TABLE X: The Amino Acid Composition of Peptic Peptides of OFd.

Peptide No.	FP-2	FP-3	FP-4	FP-6	FP-7	FP-11	FP-14-S	FP-15	FP-16	FP-18	FP-23-II
Aspartic acid	...	...	...	...	...	...	1.35(1)	1.27(1)	1.05(1)	0.15	1.08(1)
Threonine	...	...	...	...	...	...	...	...	...	0.04	...
Serine	...	...	0.91(1)	0.94(1)	0.89(1)	...	1.67(2)	1.73(2)	0.93(1)	0.07	0.92(1)
Glutamic acid	...	...	1.04(1)	1.08(1)	1.04(1)	2.31(2)	1.00(1)	1.08(1)	0.27	2.22(2)	...
Proline	...	...	...	...	...	1.00(1)	...	...	...	...	...
Glycine	...	...	1.00(1)	1.00(1)	1.00(1)	...	1.00(1)	1.00(1)	0.19	0.07	...
Alanine	1.00(1)	...	1.07(1)	...	...	...	0.33	0.33	0.17	0.15	...
Valine	...	...	...	...	...	0.97(1)	0.16	0.25	0.12	1.00(1)	...
Isoleucine	...	...	0.95(1)	1.02(1)	1.01(1)	...	0.98(1)	1.87(2)	1.00(1)	...	1.00(1)
Tyrosine	...	1.00(1)	...	...	...	...	...	...	0.03	...	...
Phenylalanine	...	...	...	...	...	...	...	...	0.07	...	0.91(1)
Total residues	1	1	5	4	4	4	6	7	3	3	4

paper chromatography and the residual peptide contained *Ser (0.23)*, Glu (1.00), Gly (1.00), and Ileu (0.00); step 3, Ser (0.16), *Glu (0.33)*, Gly (1.00), and Ileu (0.00) by the subtractive procedure.

PEPTIDE FP-11: PRO-VAL-(GLU,GLU). The amino acid composition of this peptide was identical with peptide FT-2-P-1. Accordingly, the Edman degradation of FP-11 was checked only by the subtractive procedure: step 1, Glu (2.20), *Pro (0.00)*, and Val (0.80); step 2, Glu (2.00), Pro (0.00), and *Val (0.38)*.

PEPTIDE FP-14-S: ILEU-SER-GLUNH_2-GLY-ASP-SER. The Edman procedure gave the following results: step 1, Asp (1.10), Ser (1.80), Glu (1.00), Gly (1.00), and *Ileu (0.31)*; step 2, PTH-Ser was directly identified and amino acid composition of the residual peptide was Asp (1.00), *Ser (0.90)*, Glu (1.00), Gly (1.00), and Ileu (0.14); step 3, PTH-GluNH_2 was identified by paper chromatography and the subtractive procedure yielded the result Asp (1.00), Ser (0.90), *Glu (0.30)*, Gly (1.10), and Ileu (0.00); step 4, the residual peptide was analyzed and shown to contain Asp (1.00), Ser (0.90), Glu (0.26), *Gly (0.40)*, and Ileu (0.00); step 5, PTH-Asp was identified by paper chromatography and the residual peptide was found to contain *Asp (0.50)*, Ser (1.00), Glu (0.30), Gly (0.40), and Ileu (0.00).

PEPTIDE FP-15: ILEU-SER-GLUNH_2-GLY-ASP-(SER, ILEU). The results of the Edman degradation are as shown below: step 1, PTH-Ileu was directly identified and the amino acid composition of the residual peptide was Asp (1.10), Ser (1.73), Glu (1.13), Gly (0.99), and *Ileu (1.06)*; step 2, PTH-Ser and Asp (1.03), *Ser (0.96)*, Glu (1.08), Gly (1.01), and Ileu (0.92); step 3, PTH-GluNH_2 and Asp (1.04), Ser (1.00), *Glu (0.43)*, Gly (1.07), and Ileu (0.86); step 4, Asp (1.09), Ser (1.03), Glu (0.44), *Gly (0.54)*, and Ileu (0.87); step 5, PTH-Asp and *Asp (0.54)*, Ser (0.98), Glu (0.49), Gly (0.40), and Ileu (1.02).

PEPTIDE FP-16: ASP-SER-ILEU. The results of the Edman reactions are summarized below: step 1, PTH-Asp by direct identification and amino composition of the residual peptide was *Asp (0.23)*, Ser (0.80), and Ileu (1.00); step 2, Asp (0.20), *Ser (0.20)*, and Ileu (1.00); step 3, PTH-Ileu was identified by paper chromatography.

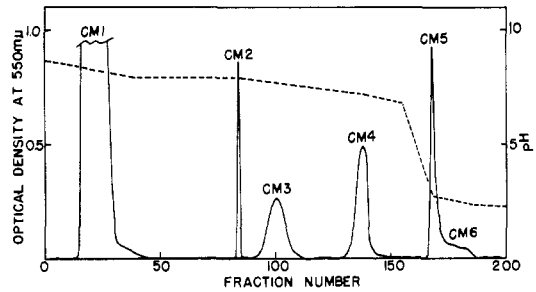

FIGURE 4: DEAE-Sephadex A-25 column chromatography of the chymotryptic digest of CAMCFd. The chymotryptic digest of CAMCFd (107 mg) was applied to a DEAE-Sephadex A-25 column (1.2 × 58 cm). Gradient elution was used initially. The mixing chamber contained 150 ml of 0.1 M collidine-acetic acid buffer (pH 8.65) and the reservoir 150 ml of 0.1 M acetic acid (pH 3.1). After fraction 156, 150 ml of 1 M acetic acid (pH 2.3) was passed through the column.

PEPTIDE FT-18: VAL-GLU-GLU. This peptide originated from COOH-terminal position of ferredoxin. However, the central amino acid (which should be glutamine) was identified as glutamic acid by paper chromatography of the PTH derivative. The results of the Edman procedure were: step 1, PTH-Val by direct identification and the subtractive procedure indicated Glu (2.00) and *Val (0.16)*; step 2, PTH-Glu was identified directly and the residual peptide was composed of *Glu (0.89)* and Val (0.00).

PEPTIDE FT-23-II: ASP-SER-(ILEU,PHE). The results from the Edman degradation of this peptide were as follows: step 1, *Asp (0.08)*, Ser (0.94), Ileu (1.00), and Phe (1.00); step 2, Asp (0.21), *Ser (0.27)*, Ileu (1.00), and Phe (1.00). The PTH–amino acids were also identified at each step.

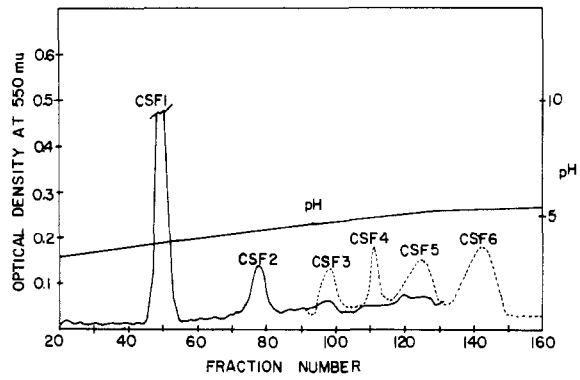

FIGURE 5: Dowex 50-×2 column chromatography of the chymotryptic digest of DECFd. The DECFd, which was prepared from AEC–Fd (100 mg) by reaction with carbon disulfide, was hydrolyzed by chymotrypsin and the digest was applied to a Dowex 50-×2 column (1.6 × 60 cm) and eluted with a linear gradient of 600 ml ranging from 0.2 M pyridine–acetic acid buffer (pH 3.06) to 2.2 M pyridine–acetic acid buffer (pH 5.26). The solid and broken lines represent values obtained by ninhydrin assay before and after alkaline hydrolysis, respectively.

TABLE XI: Amino Acid Composition and Properties of Chymotryptic Peptides of CAMCFd.

Peptide No.	CM-3-b	CM-6-b
Lysine	1.00(1)	...
CMCysteine	0.89(1)	2.10(2)
Aspartic acid	1.30(1)	4.00(4)
Threonine	0.16	0.81(1)
Serine	1.90(2)	0.19
Glutamic acid	0.10	0.32
Proline	0.00	0.27
Glycine	0.30	1.15(1)
Alanine	1.30(1)	1.31(1)
Valine	1.00(1)	0.94(1)
Isoleucine	1.00(1)	1.89(2)
Total residues	8	12
Mobility[a] (μ), cm	−1.1	+4.0
Electrophoretic properties	Neutral	Acidic
R_F (BPAW)	0.29	0.25
Color with ninhydrin	Violet	Violet

[a] 500 v, 2 hr, pH 6.5.

Chymotrypsin Digestion of CAMCFd. The fingerprint map of the chymotryptic digest of CAMCFd showed three main spots plus minor spots.

Column chromatography of the digest on DEAE-Sephadex A-25 yielded six components (CM-1, CM-2, CM-3, CM-4, CM-5, and CM-6) as shown in Figure 4. CM-1 contained free lysine and a lysyl peptide which were separable by paper electrophoresis. CM-2 was ammonia. CM-3 consisted of two different lysyl peptides which were separable by paper chromatography in BPAW. CM-4 was alanyltyrosine which originated from the NH_2-terminal position of native ferredoxin. CM-5 was further purified by using a second DEAE-Sephadex A-25 column chromatography step, a Dowex 50-×2 column chromatography step, and paper chromatography (BPAW). Several nonlysine-containing peptides were separated. CM-6 was purified by paper chromatography in BPAW. *Via* these purification steps, two interesting peptides, CM-3-b and CM-6-b, were isolated.

The amino acid composition and properties of CM-3-b and CM-6-b are shown in Table XI. The sequence of the two peptides was determined. The results of the subtractive Edman procedure when applied to CM-3-b (Lys-Ileu-Ala-Asp-Ser-CMCys)-(Val,Ser) are shown as follows: step 1, *Lys (0.14)*, CMCys (1.00), Asp (1.00), Ser (1.80), Ala (1.00), Val (0.90), and Ileu (0.98); step 2, Lys (0.00), CMCys (0.80), Asp (1.10), Ser (1.80), Ala (1.10), Val (0.90), and *Ileu (0.15)*; step 3, Lys (0.00), CMCys (0.86), Asp (1.00), Ser (1.80), *Ala (0.31)*, Val (1.00), and Ileu (0.00); step 4, Lys (0.00), CMCys (0.82), *Asp (0.44)*, Ser (1.80), Ala (0.33), Val (1.00), and Ileu (0.00), and PTH–aspartic acid was identified by paper chromatography.

CPase A digestion of peptide CM-3-b yielded at 2 hr CMCys (16%), Ser (28%), and Val (28%). At 12 hr the values were CMCys (46%), Ser (64%), and Val (66%).

PEPTIDE CM-6-B: [VAL-ILEU-ASP-(ALA,ASP,THR,CM-CYS,ILEU,ASP,CMCYS,GLY,ASPNH_2)]. The subtractive Edman degradation of this peptide gave the following results: step 1, CMCys (2.30), Asp (4.00), Thr (0.90), Gly (1.20), Ala (1.30), *Val (0.29)*, and Ileu (1.80); step 2, CMCys (2.10), Asp (3.80), Thr (0.90), Gly (1.20), Ala (1.20), Val (0.19), and *Ileu (1.00)*; step 3, CMCys (1.85), *Asp (3.00)*, Thr (0.81), Gly (1.10), Ala (1.20), Val (0.18), and Ileu (1.00).

Chymotrypsin Digestion of DECFd. Figure 5 shows the chromatographic pattern of the chymotryptic digest of DECFd on Dowex 50-×2. Fractions which were eluted after no. 90 were detected by the ninhydrin procedure with and without alkaline hydrolysis. Six fractions were separated. Peak CSF-1 was ammonia. Peaks CSF-2 and CSF-3 had the same amino acid composition and contained 25 amino acid residues (residues 31–55). Peaks CSF-4 and CSF-5 were intact undigested DECFd. Peak CSF-6 contained 30 amino acids (residues 1–30). Both peptides, CSF-2 and CSF-6, were purified by paper electrophoresis. Their amino acid composition and properties are summarized in Table XII.

PEPTIDE CSF-2: VAL-ILEU-(ASP,ALA,ASP,THR,AEC, ILEU, ASP, AEC, GLY, ASPNH_2, AEC, ALA, ASPNH_2, VAL, AEC,PRO,VAL,GLY,ALA,PRO,VAL,GLUNH_2,GLU). Dinitrophenylation of peptide CSF-2 yielded DNP-Val only. LAP digestion of this peptide gave Val (69%) and Ileu (60%) after incubation for 22 hr. CPase A did not hydrolyze the peptide.

PEPTIDE CSF-6: ALA-(TYR,LYS,ILEU,ALA,ASP,SER, AEC,VAL,SER,AEC,GLY,ALA,AEC,ALA,SER,GLU,AEC,

TABLE XII: Amino Acid Composition and Properties of Chymotryptic Peptides of DECFd.

Peptide No.	CSF-2		CSF-6
Hydrolysis time (hr)	22	44	22
Lysine	...	...	1.10(1)
AEC	3.67	3.40(4)	3.85(4)
Aspartic acid	5.08	5.00(5)	3.27(3)
Threonine	0.92	0.88(1)	...
Serine	0.12	0.12	4.32(5)
Glutamic acid	2.09	2.10(2)	1.96(2)
Proline	1.83	1.80(2)	1.02(1)
Glycine	2.02	2.00(2)	2.02(2)
Alanine	2.97	2.80(3)	5.00(5)
Valine	3.81	4.00(4)	1.94(2)
Isoleucine	1.69	1.80(2)	2.96(3)
Tyrosine	...	...	0.92(1)
Phenylalanine	...	...	0.90(1)
Total residues		25	30
Mobility[a] (μ), cm		−2.0	−4.5
Electrophoretic properties		Neutral	Basic
R_F (BPAW)		0.06	0.1
Color with ninhydrin		Blue	Blue

[a] 500 v, 2 hr, pH 6.5.

PRO,VAL,$AspNH_2$,ALA,ILEU,SER,$GluNH_2$,GLY,ASP,SER, ILEU,PHE). Only DNP-Ala was obtained by the dinitrophenylation procedure. LAP digestion and CPase A digestion of peptide CSF-6 were not possible since this peptide was insoluble at pH 8.

Discussion

Specificity of Trypsin. Even after prolonged digestion of AECFd with trypsin, the AEC-Gly linkage (residues 40 and 41) was not hydrolyzed. However, the Edman procedure clearly showed the AEC-Gly sequence. The failure of trypsin to hydrolyze this linkage may be due to hydrogen bonding between the COOH group of the aspartic acid (residue 39) and the NH_2 group of AEC (residue 40) since the AEC-Ala (residues 43 and 44) while preceded by $AspNH_2$ is hydrolyzed by trypsin.

According to the published reports, the Glu-Lys-X (residues 4–6) and the Glu-Lys-X (residues 21–23) which are found in human cytochrome *c* (Matsubara and Smith, 1963) and the Asp-Lys-X (residues 6–8) and the Asp-Lys-X (residues 126–128) in the α chain (Konigsberg and Hill, 1962) and the Glu-Lys-X (residues 7–9) in the β chain (Goldstein *et al.*, 1963) of human hemoglobin were reported to be cleaved by trypsin but the Asp-Lys-X (residues 94–96) in the β chain of human hemoglobin was not. Additional studies are necessary to clarify this point.

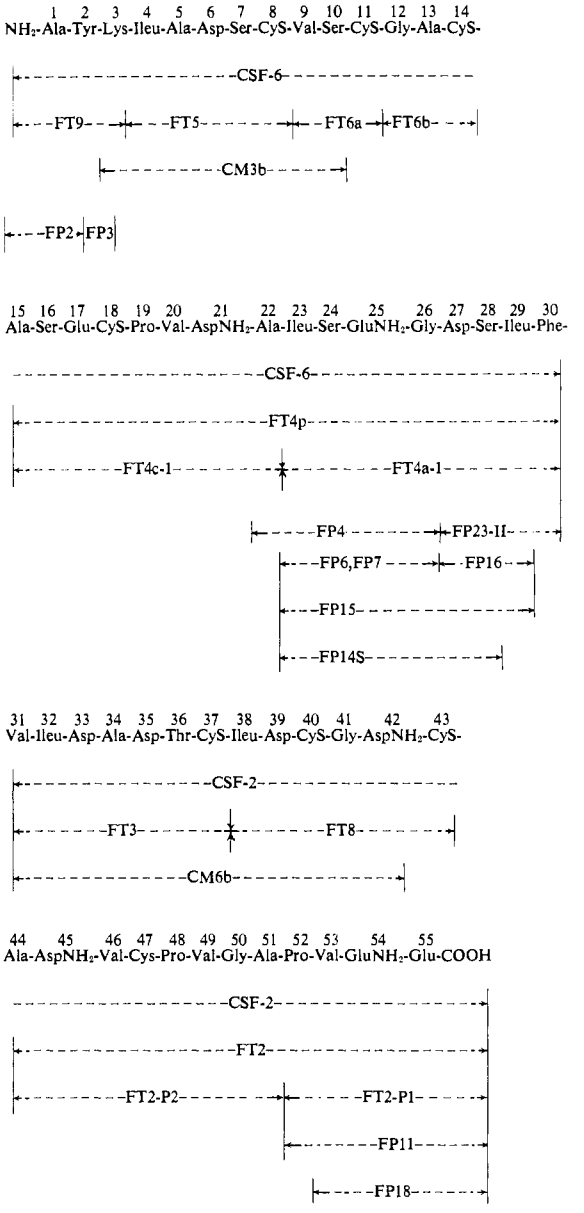

FIGURE 6: Construction of the total amino acid sequence of the *C. pasteurianum* ferredoxin.

From the tryptic digest of AECFd, peptides FT-4-a-2 and FT-4-p were isolated. These peptides appear to have been produced by chymotrypsin contamination of the trypsin preparation.

Peptide FT-4-p was partially hydrolyzed to give rise to peptides FT-4-c-1 and FT-4-a-1. This peptide was hydrolyzed sometime during the purification process.

The lysyl–prolyl bond has been shown to be a linkage which is not hydrolyzed by trypsin (Shepherd *et al.*, 1956; Hirs *et al.*, 1956), and in AECFd, the AEC-Pro (residues 18 and 19) and AEC-Pro (residues 47 and 48) were not hydrolyzed by trypsin.

Specificity of Chymotrypsin. During the chymotryptic

TABLE XIII: The Amino Acid Composition and Sequence of Peptides from Various Derivatives of *C. pasteurianum* Ferredoxin.

Sequence	Peptide No.	Amino acid sequence	Derivative
1	FP-2	Ala[a]	OFd
2	FP-3	Tyr	OFd
1–2	C-2	Ala-Tyr	OFd
1–2	FT-4-a-2	Ala-Tyr	AECFd
1–2	CM-4	Ala-Tyr	CAMCFd
1–3	T-2	Ala-Tyr-Lys	OFd
1–3	FT-9	Ala-Tyr-Lys	AECFd
1–30	CSF-6	(Ala)-Tyr-Lys-Ileu-Ala-Asp-Ser-Cys-Val-Ser-Cys-Gly-Ala-Cys-Ala-Ser-Glu-Cys-Pro-Val-AspNH_2-Ala-Ileu-Ser-GluNH_2-Gly-Asp-Ser-Ileu-Phe	DECFd
3–10	CM-3-b	Lys-Ileu-Ala-Asp-Ser-Cys-Val-Ser	CAMCFd
4–8	FT-5	Ileu-Ala-Asp-Ser-Cys	AECFd
9–11	FT-6-a	Val-Ser-Cys	AECFd
12–14	FT-6-b	Gly-Ala-Cys	AECFd
15–22	FT-4-c-1	(Ala)-Ser-Glu-Cys-Pro-Val-AspNH_2-Ala	AECFd
15–30	FT-4-p	Ala-Ser-Glu-Cys-Pro-Val-AspNH_2-Ala-Ileu-Ser-GluNH_2-Gly-Asp-Ser-Ileu-Phe	AECFd
22–26	FP-4	Ala-Ileu-Ser-GluNH_2-Gly	OFd
23–26	FP-6	Ileu-Ser-GluNH_2-Gly	OFd
23–26	FP-7	Ileu-Ser-GluNH_2-Gly	OFd
23–28	FP-14-S	Ileu-Ser-GluNH_2-Gly-Asp-Ser	OFd
23–29	FP-15	Ileu-Ser-GluNH_2-Gly-Asp-Ser-Ileu	OFd
23–30	FT-4-a-1	(Ileu)-Ser-GluNH_2-Gly-Asp-Ser-Ileu-Phe	AECFd
27–29	FP-16	Asp-Ser-Ileu	OFd
27–30	FP-23-II	Asp-Ser-Ileu-Phe	OFd
31–37	FT-3	Val-Ileu-Asp-Ala-Asp-Thr-Cys	AECFd
31–42	CM-6-b	Val-Ileu-Asp-Ala-Asp-Thr-Cys-Ileu-Asp-Cys-Gly-AspNH_2	CAMCFd
31–55	CSF-2	(Val)-Ileu-Asp-Ala-Asp-Thr-Cys-Ileu-Asp-Cys-Gly-AspNH_2-Cys-Ala-AspNH_2-Val-Cys-Pro-Val-Gly-Ala-Pro-Val-GluNH_2-Glu	DECFd
38–43	FT-8	(Ileu)-Asp-Cys-Gly-AspNH_2-Cys	AECFd
44–51	FT-2-P-2	Ala-AspNH_2-Val-Cys-Pro-Val-Gly-Ala	AECFd
44–55	FT-2	Ala-AspNH_2-Val-Cys-Pro-Val-Gly-Ala-Pro-Val-GluNH_2-Glu	AECFd
52–55	FT-2-P-1	Pro-Val-GluNH_2-Glu	AECFd
52–55	FP-11	Pro-Val-Glu-Glu	OFd
53–55	FP-18	Val-Glu-Glu	OFd

[a] Arrows to the right represent sequences determined by the Edman method. Dashed arrows pointing to the right indicate sequences determined by leucine aminopeptidase. Parentheses show sequences determined by dinitrophenylation. Arrows to the left represent sequences determined by carboxypeptidase A or B.

digestion of DECFd, the Tyr-Lys linkage was not hydrolyzed. Evidently the peptide bond composed of a Tyr-dithiocarbamino-Lys linkage is not hydrolyzed by chymotrypsin.

Miscellaneous. In peptides FP-11 and FP-18, which were obtained from peptic digest of OFd, the glutamine residue (no. 54) was deaminated but was intact in the case of peptide FT-2-P-1. These results were confirmed by the electrophoretic properties of the peptides.

CPase A digested peptides FT-4-a-1 (carboxyl-terminal residue -Phe), FT-4-c-1 (-Ala), FT-2-P-2 (-Ala), and CM-3-b (-Ser) but not peptides FT-2 (-Glu), CSF-2 (-Glu), and CM-6-b (-AspNH_2). CPase B hydrolyzed all of the peptides in which AEC was in the COOH-terminal position.

Total Amino Acid Sequence of C. pasteurianum Ferredoxin. Table XIII shows the structures of the various peptides which were isolated from the ferredoxin derivatives. From the results in Table XIII, the total amino acid sequence of *C. pasteurianum* ferredoxin can be reconstructed as shown in Figure 6.

The *C. pasteurianum* ferredoxin contains only one lysine and one tyrosine residue and the amino-terminal sequential analysis of the ferredoxin has shown that Ala-Tyr-Lys is the amino-terminal sequence (Tanaka *et al.*, 1964a) and, therefore, CSF-6 is the NH_2-terminal peptide and, therefore, CSF-2 must represent the COOH-terminal sequence.

As far as CSF-6 is concerned, peptides FT-9, FT-5, FT-6-a, FT-6-b, and FT-4-p are contained in it. FT-9 is of course the NH_2-terminal peptide. The presence of peptide CM-3-b shows that FT-5 must be adjacent to FT-9, and FT-6-a must follow FT-5. Native ferredoxin contains only one phenylalanine residue and since CSF-6 is a chymotryptic peptide of DECFd, FT-4-p must be the COOH-terminal peptide of CSF-6. FT-6-b occurs only once in the molecule and it should be located between FT-6-a and FT-4-p in order to satisfy the amino acid composition of CSF-6. Thus, peptide CSF-6 contains peptides FT-9, FT-5, FT-6-a, FT-6-b, and FT-4-p in that order. FT-4-p is composed of peptides FT-4-c-1 and FT-4-a-1 as well as peptides FP-4, FP-6, FP-7, FP-14-S, FP-15, FP-16, and FP-23-ii.

Concerning peptide CSF-2, it contains peptides FT-3, FT-8, and FT-2. Since FT-3 has an NH_2-terminal valyl-isoleucyl sequence, it must be the NH_2-terminal peptide of CSF-2. As shown in the studies on CM-6-b, FT-8 must be adjacent to FT-3 and therefore FT-2 represents the COOH-terminal portion of CSF-2. It has been reported already (Tanaka *et al.*, 1964a) that the COOH-terminal amino acid residue of native ferredoxin is glutamic acid and all the results are therefore accounted for. Thus, CSF-2 is composed of peptides FT-3, FT-8, and FT-2 in that order. FT-2 is composed of peptides FT-2-P-2 and FT-2-P-1 and the sequential studies of these peptides demonstrated that -Val-GluNH_2-Glu is COOH-terminal sequence of the ferredoxin molecule.

Comments on the Ferredoxin Structure. The structure of the ferredoxin molecule is almost symmetrical about the central phenylalanine. Four cysteine residues are in the COOH-terminal half and four cysteine residues are present in the NH_2-terminal half of the molecule. These cysteine residues are separated by two, two, and three amino acid residues in each half of the molecule. Each run of cysteine residues ends with a Pro-Val sequence. By properly aligning the molecule as shown in Figure 7,

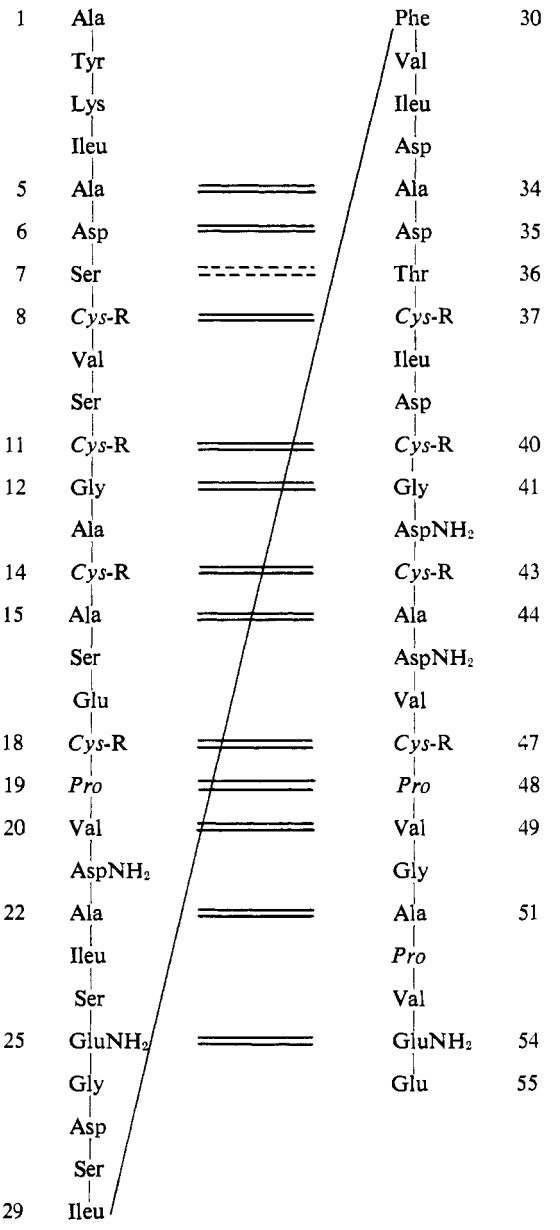

FIGURE 7: The symmetry in the positioning of amino acid residues and the probable nonhelical nature of the ferredoxin molecule. The residue numbers and matching amino acid residues about a central plane in the molecule are indicated in the figure. Amino acid residues which prevent formation of an α helix are italicized. It is assumed that the R groups (iron and sulfide attached perhaps in the manner as shown in reference of Tanaka *et al.*, 1965) joined to the cysteine residues would prevent helix formation.

24 of the residues are identical in both halves of the molecule and two residues are very similar.

Chelate Structure of Ferredoxin. Recently Blomstrom *et al.* (1964) have reported on the location of iron in ferredoxin. They indicated that all seven iron atoms in ferredoxin are strong-field ferric and that these exist in two structurally nonequivalent environments. These nonequivalent ferric states are in a ratio of 2:5. These results were obtained from studies of the magnetic susceptibility and Mossbauer spectrum of ferredoxin. These authors proposed a model for the active site of ferredoxin, in which the seven iron atoms are arranged linearly and are bound together *via* sulfur bridges furnished by the seven cysteine residues and six inorganic sulfide atoms. The relation of this structure to our amino acid sequence has been discussed in a previous report (Tanaka *et al.*, 1965).

However, Sieker and Jensen (1965) have shown from X-ray studies of crystalline *Micrococcus aerogenes* ferredoxin that a structure based on the symmetrical distribution of Fe can be excluded. This might rule out the structure proposed by Blomstrom *et al.* (1964).

A recent report by Bayer *et al.* (1965) has proposed that there is no inorganic sulfide present in *C. pasteurianum* ferredoxin and that the iron is directly attached to the sulfhydryl groups of the cysteine residues. Experiments were conducted to remove the iron by the α,α'-bipyridyl method of Bayer *et al.* (1965) using exactly the conditions described by these investigators. Their preparation of apoferredoxin was claimed to contain the full complement of cysteine residues since the apoenzyme was converted to native ferredoxin by the addition of iron. The cysteine in the apoferredoxin prepared in our laboratory according to the method of Bayer *et al.* (1965) was converted to cysteic acid, hydrolyzed with 6 N HCl for 24 hr, and analyzed in the amino acid analyzer. According to the proposal of Bayer *et al.* (1965) the cysteine content would be expected to increase to 16 residues if it is assumed that our procedure for the removal of the iron converted the cysteine residues to pyruvic acid. However, the cysteic acid content of the apoferredoxin prepared according to Bayer *et al.* (1965) did not increase. Moreover, although quantitative measurements were not made, a strong hydrogen sulfide odor was detected when all of our preparations of ferredoxin were treated with trichloroacetic acid. The conclusions of Bayer *et al.* (1965) are based upon a base-catalyzed elimination of hydrogen sulfide from the cysteine derivatives and would not predict the evolution of hydrogen sulfide from ferredoxin under acidic conditions. Our results are, therefore, in agreement with the report of Lovenberg *et al.* (1963) that inorganic sulfide is present in ferredoxin. In view of the finding that our method of preparing apoferredoxin consistently yielded eight cysteic acid residues after performic acid oxidation it is difficult to accept the results of Bayer *et al.* (1965) that there is no inorganic sulfide present in ferredoxin and that the hydrogen sulfide arises solely from the decomposition of the cysteine residues.

The amino acid sequence of other ferredoxins is being investigated in our laboratory. The preliminary results with *C. butyricum* ferredoxin confirm the following facts: (1) Ferredoxin is composed of 55 amino acid residues. (2) There are eight half-cystine residues per mole of ferredoxin.

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Purification and Physical Characterization of Tyrosyl Ribonucleic Acid Synthetases from *Escherichia coli* and *Bacillus subtilis**

Richard Calendar and Paul Berg

ABSTRACT: Tyrosyl ribonucleic acid synthetases from *Escherichia coli* and *Bacillus subtilis* have been purified to near homogeneity. These enzymes have molecular weights of 95,000 and 88,000, respectively. They can be separated electrophoretically on starch or polyacrylamide gels. The *E. coli* enzyme contains 14 half-cystine residues/mole while the *B. subtilis* enzyme has 2–3.

Antiserum to the *E. coli* enzyme neutralizes and precipitates the *E. coli* enzyme but does not cross-react with the *B. subtilis* enzyme. Antiserum to the *B. subtilis* enzyme neutralizes and precipitates the *B. subtilis* enzyme and also shows partial cross-reaction with the *E. coli* enzyme.

Aminoacyl RNA[1] synthetases are one of a few types of known proteins which can discriminate between structurally similar nucleic acid molecules (Berg, 1961). How each aminoacyl RNA synthetase distinguishes the correct t-RNA from all others is not known; moreover, virtually nothing is known about the type of interactions which occur between the protein and the polynucleotide chain during the formation of an aminoacyl RNA.

Several examples are known in which aminoacyl RNA synthetases from one species fail to utilize the corresponding t-RNA's from another species; complete cross-reaction between enzymes and t-RNA's from different species is also well documented (Benzer and Weisblum, 1961; Yamane and Sueoka, 1963; Doctor and Mudd, 1963). Quite possibly, studies of one of the aminoacyl RNA synthetases, isolated from species which do and do not cross-react with their respective t-RNA's, could shed some light on the nature of the interaction between the enzyme and the t-RNA. Our first approach was to compare two enzymes from species which show complete cross-reaction with their corresponding t-RNA's (Calendar and Berg, 1966). Later studies will extend the comparison to the same enzyme from species which fail to react with the heterologous t-RNA's.

In this paper we report the isolation and a comparison of several physical and chemical properties of tyrosyl RNA synthetases from *Escherichia coli* and *Bacillus subtilis*, two enzymes which cross-react completely with their heterologous t-RNA's. Although the two enzymes have similar molecular weights, they are readily distinguishable by their electrophoretic mobility, amino acid composition, and immunological reaction. In the next paper (Calendar and Berg, 1966) the catalytic properties of the two enzymes are described.

Experimental Section

Materials. L-Tyrosine was purchased from California Corp. for Biochemical Research and gave only a single peak when chromatographed on an amino acid analyzer (Spackman *et al.*, 1958).

ATP and glutathione were purchased from Sigma Chemical Corp. ^{32}P-Labeled pyrophosphate ($^{32}PP_i$) was synthesized from ^{32}P-labeled orthophosphate (Oak Ridge National Laboratories) as described by Bergmann *et al.* (1961). Crystalline bovine plasma albumin was purchased from Armour Pharmaceutical Co., Kankakee, Ill. Sucrose, Baker analyzed grade, was recrystallized from ethanol and filtered through a 0.45 μ Millipore filter before use in sucrose-gradient centrifugation. Fluorochemical FC43 was obtained

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[1] Abbreviations used: RNA, ribonucleic acid; t-RNA, transfer RNA; ATP, adenosine triphosphate.