rier sites as the uptake, since it can be inhibited by lanthanides and enhanced by butacaine.

Acknowledgments

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References

Chance, B. (1951), *Rev. Sci. Instr. 22*, 634.
Chance, B. (1965), *J. Biol. Chem. 240*, 2729.
Chance, B., and Mela, L. (1966a), *J. Biol. Chem. 241*, 4588.
Chance, B., and Mela, L. (1966b), *Biochemistry 5*, 3220.
Chance, B., Mela, L., and Harris, E. J. (1968), *Federation Proc. 27*, 902.
Cotton, F. A., and Wilkinson, G. (1967), Advanced Inorganic Chemistry, New York, N. Y., Interscience, p 1052.
Dixon, M., and Webb, E. C. (1964), The Enzymes, New York, N. Y., Academic, p 315.
Ernster, L., Hollander, P., Nakazawa, T., and Nordenbrand, K. (1968), *in* Mitochondria-Structure and Function, Drahota, Z., and Ernster, L., Ed., London, Academic (in press).
Mela, L. (1968a), *Arch. Biochem. Biophys. 123*, 286.
Mela, L. (1968b), *Trans. N. Y. Acad. Sci.* (in press).
Mela, L., and Chance, B. (1968), *Biochemistry 7*, 4059.
Rossi, C., Carafoli, E., and Lehninger, A. L. (1968), *in* Mitochondria—Structure and Function, Drahota, Z., and Ernster, L., Ed., London, Academic (in press).
Scarpa, A., and Azzi, A. (1968), *Biochim. Biophys. Acta 150*, 473.
Scarpa, A., and Azzone, G. F. (1968), *J. Biol. Chem. 243*, 5132.

The Amino Acid Sequence of Ferredoxin from *Clostridium acidi-urici**

S. C. Rall, Robert E. Bolinger,† and R. David Cole

ABSTRACT: The sequence of amino acids in *Clostridium acidi-urici* ferredoxin was determined using two derivatives, *S*-β-aminoethylated ferredoxin and oxidized ferredoxin. Peptides derived from tryptic, papain, and partial acid hydrolyses were used to construct the total sequence. The sequence of amino acids in the individual peptides was determined by the Edman degradation and the exopeptidases leucine aminopeptidase, carboxypeptidase A, and carboxypeptidase B. Like the other two clostridial ferredoxins previously sequenced, *C. acidi-urici* ferredoxin has an amino terminal alanine. Out of a total of 55, 37 amino acids are found in identical positions in all three species, including 8 cysteine and 3 proline residues.

Ferredoxin, an iron-containing polypeptide of low potential, has been found in plants and bacteria. The structure of the ferredoxin isolated from spinach contains five cysteinyl residues and seems distantly related in structure to the bacterial ferredoxins (Matsubara *et al.*, 1967). Structural studies on the bacterial ferredoxins from *Clostridium pasteurianum* (Tanaka *et al.*, 1966) and *Clostridium butyricum* (Benson *et al.*, 1966) have shown that these polypeptides are very closely related, each consisting of two nearly homologous halves. Within each half there are four cysteinyl residues, the spacing of which is probably significant in the binding of iron and inorganic sulfide (Malkin and Rabinowitz, 1966) as required for biological function. From the evolutionary standpoint, these similarities between plant and bacterial ferredoxin and between the two halves of an individual bacterial ferredoxin are currently of concern in comparative biochemistry (Eck and Dayhoff, 1966).

There are differences in the properties of ferredoxin from *Clostridium acidi-urici* on the one hand, and those from *C. pasteurianum* and *C. butyricum* on the other. Ferredoxin from *C. acidi-urici* crystallizes more readily and has a lower activity in the phosphoroclastic assay than the other two (Lovenberg *et al.*, 1963). As a step in correlating these variations in properties with structural differences, and also to aid in the comparative study, the amino acid sequence of ferredoxin from *C. acidi-urici* was determined.

Methods

Purification of Ferredoxin. Ferredoxin from *C. acidi-urici* was isolated and purified according to the method of Lovenberg *et al.* (1963), and was the generous gift of Dr. R. Malkin and Dr. J. C. Rabinowitz. The purified protein had an $A_{390}/A_{280} = 0.78$.

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Preparation of Derivatives of Ferredoxin. AE-Fd.[1] Ferredoxin (15 mg, *ca.* 2.5 μmol) in 90% $(NH_4)_2SO_4$, was dialyzed against water until sulfate could not be detected in the dialysate with $BaCl_2$. The solution was taken to dryness in a 50-ml round-bottomed flask, to which was then added 1.25 ml of 8 M urea, 0.25 ml of 4 M Tris buffer (pH 8.6), and 25 mg of 1,10-phenanthroline. The flask was purged with N_2 gas; 100 μl of 2-mercaptoethanol was added and the stoppered flask was allowed to stand 6 hr at room temperature in a nitrogen atmosphere. At the end of this time, 375 μl of ethylenimine was added in three equal portions at 10-min intervals (Raftery and Cole, 1963). The reaction mixture was allowed to stand an additional 30 min and then was acidified to pH 3 ((HCl). The red solution was exhaustively dialyzed against 0.01 N acetic acid or passed through a Sephadex G-25 column (1.2 × 44 cm) equilibrated with 0.01 N acetic acid to remove the iron–phenanthroline complex. The aminoethylated apoprotein was then lyophilized. Recovery was *ca.* 10 mg.

OFd. The procedure was the same as above through the 6-hr treatment with mercaptoethanol. At this point, the solution was acidified to pH 3 (HCl), dialyzed, and lyophilized. The lyophilized protein (20 mg) was then oxidized according to Moore (1963). The yield of OFd was 18 mg.

Fragmentation Procedures. TRYPTIC DIGESTION OF AE-Fd. In one experiment (A), AE-Fd (24 mg in 5 ml of H_2O) was digested with 1% (0.24 mg) L-1-(*p*-toluenesulfonyl)amide-2-phenylethyl chloromethyl ketone treated trypsin (Wang and Carpenter, 1965) at 25° for 12 hr at pH 8.0, maintained by addition of 0.1 N NaOH in a pH-Stat. After 6 hr, 1% trypsin was added again.

In another experiment (B), AE-Fd (20 mg in 6 ml of H_2O) was digested with 2% L-1-(*p*-toluenesulfonyl)amide-2-phenylethyl chloromethyl ketone treated trypsin at 31° for 12 hr at pH 8.0. After 1.5 hr, 2% trypsin was added again.

PAPAIN DIGESTION OF OFd. OFd (18 mg) was digested with papain (Worthington Biochemical Corp.) according to the method of Konigsberg and Hill (1962).

PARTIAL ACID HYDROLYSIS OF AE-Fd. AE-Fd (15 mg in 3.0 ml of 0.03 N HCl, pH 1.7) was hydrolyzed at 100° for 12 hr in a sealed, evacuated tube (Schultz *et al.*, 1962).

PEPSIN DIGESTION OF TR-D1. To the tryptic peptide TR-D1 (0.3 μmol in 0.2 ml of 0.01 N HCl) was added 100 μl of pepsin (Worthington, twice crystallized, 2 mg/ml in 0.01 N HCl). The reaction was carried out at 30° for 8 hr. The digestion mixture was applied to a Sephadex G-25 column (1.2 × 47 cm) and eluted with H_2O at a flow rate of 11 ml/cm²-hr. Fractions of 2.7 ml were collected. The fractions contained in each peak were pooled, concentrated, and stored in H_2O at −10°.

Isolation of Peptide Fragments. TRYPTIC PEPTIDES. The tryptic digestion mixture was lyophilized, dissolved in 3 ml of 0.2 M pyridinium–acetate (pH 2.8) and applied to a column (0.9 × 19 cm) of Dowex 50-X8 (expt A) or Dowex 50-X2 (expt B) and developed at a flow rate of 30 ml/hr at 51° with a linear gradient of 500 ml from 0.2 M pyridinium–acetate (pH 2.8) to 2.0 M pyridinium–acetate (pH 5.2). Fractions of 3 ml were collected. Ninhydrin analysis (Moore and Stein, 1954) on 0.3 ml of each fraction was used to locate the peptides.

Fractions contained in each peak were combined, taken to dryness *in vacuo*, and stored at −10° in vials containing 3 ml of H_2O. Peaks were checked for purity by paper electrophoresis at pH 3.5 or 6.4 in a pyridine–HOAc–H_2O system (pH 3.5, 1:10:280; pH 6.4, 25:1:235). Impure fractions were then further purified by preparative paper electrophoresis.

PEPTIDES FROM PARTIAL ACID HYDROLYSIS. Isolation and purification were essentially the same as the above, except that the starting buffer for the Dowex 50-X2 column was pH 2.5. Impure fractions were all purified by paper electrophoresis at pH 3.5, 16 V/cm, for 2 hr and eluted with H_2O.

PAPAIN PEPTIDES. The papain digest was lyophilized, dissolved in 3 ml of *N*-ethylmorpholine–picoline–pyridine–HOAc–H_2O buffer (pH 9.4) (Schroeder *et al.*, 1962), and applied to a Dowex (AG) 1-X2 column (0.9 × 22 cm). The column was developed at 35°, with a flow rate of 40 ml/hr and with an exponential gradient (Rombauts and Raftery, 1965). Acidic fractions were neutralized with 1 N NaOH to prevent hydrolysis. Further procedures were as above.

Analysis for Compositions and Sequences. AMINO ACID COMPOSITIONS OF PEPTIDES. Amino acid compositions were determined on a Beckman Model 120 B automatic amino acid analyzer, after hydrolysis of samples in 6 N HCl for 22 hr at 110° in a sealed, evacuated tube (Moore and Stein, 1963).

EDMAN DEGRADATION. The procedure for thiocarbamylation was essentially that of Eriksson and Sjöquist (1960). The cyclization reaction was according to Konigsberg and Hill (1962). The phenylthiohydantoin derivative was determined as the free amino acid by the method of Africa and Carpenter (1966). In order to conserve peptide materials, subtractive analysis was rarely done.

CARBOXYPEPTIDASES A AND B. Carboxypeptidase A and carboxypeptidase B (Worthington, DFP treated) were dissolved in 10% LiCl (1 mg/ml) before use. Digestions were carried out at various times with 10 μl of enzyme in 0.025 M Tris-HCl–0.1 M NaCl (pH 7.5) at 25, 30, or 37°. Aliquots were analyzed directly on the amino acid analyzer.

LEUCINE AMINOPEPTIDASE. Crystalline leucine aminopeptidase was prepared from bovine lens according to Hanson *et al.* (1965), and was the gift of Miss Susanne Warner and Dr. F. H. Carpenter. Leucine aminopeptidase (0.5–1 mg in 0.1 ml) was activated at 40° for 90 min in 2.0 ml of 0.05 M Tris–0.01 M $MgCl_2$ (pH 9.1). Peptide digestions were carried out using 100 μl of activated enzyme solution at 40° in 0.05 M Tris (pH 9.1) for various times.

AMIDE DETERMINATIONS. Amides were determined by the method of Offord (1966) and by digestion of peptides with leucine aminopeptidase and carboxypeptidase A.

Results

Amino Acid Composition of Ferredoxin. The amino acid composition of *C. acidi-urici* ferredoxin has been reported (Malkin and Rabinowitz, 1966). Amino acid analysis of AE-Fd was in agreement with the earlier report and contained eight half-cystine residues, determined as *S*-β-aminoethylcysteine.

N-Terminal Sequence of Ferredoxin. Two steps of the Edman degradation on 0.045 μmol of whole AE-Fd showed the N-terminal sequence to be Ala-Tyr. The yield at each step was *ca.* 20% (see Tanaka *et al.*, 1964).

Sequence Determination of the Tryptic Peptides of Experiment A. The elution profile of the tryptic peptides from expt A

[1] Abbreviations used are: OFd, oxidized ferredoxin; AE-Fd, S-β-amino-ethylated ferredoxin.

TABLE I: Amino Acid Compositions of Tryptic Peptides of AE-Fd.

Peptide	TR-A	TR-B1	TR-B2	TR-C	TR-D1	TR-D2	TR-E	TR-F	TR-G	Total Residues[a]
Aspartic acid	1.10 (1)	2.24 (2)	1.11 (1)	3.00 (3)	2.92 (3)	1.03 (1)			1.06 (1)	8
Threonine		0.82 (1)	0.10							1
Serine		0.34	0.14	1.83 (2)	1.78 (2)		0.90 (1)			3
Glutamic acid	1.00 (1)	0.15	1.00(1)	1.84 (2)	2.05 (2)					4
Proline	1.87 (2)			2.07 (2)	1.72 (2)					4
Glycine	1.12 (1)	0.27		2.11 (2)	1.08 (1)			1.04 (1)	1.04 (1)	4
Alanine	2.94 (3)	1.00 (1)	1.96 (2)	2.35 (2)	0.99 (1)			1.00 (1)	1.00 (1)	9
Valine	2.74 (3)	0.85 (1)	0.86 (1)	1.38 (1)	1.00 (1)					6
Isoleucine		0.82 (1)	0.90 (1)	0.96 (1)	0.91 (1)	1.00 (1)	1.13 (1)		0.86 (1)	5
Tyrosine		0.82 (1)	0.93 (1)							2
Aminoethyl-cysteine	1.00 (1)	0.91 (1)	0.98 (1)	1.92 (2)	0.89 (1)	0.98 (1)	0.96 (1)	0.98 (1)	1.75 (2)	8
Arginine				0.93 (1)	1.08 (1)					1
Total residues	12	8	8	18	15	3	3	3	6	55
Mobility, pH 6.4	Neutral	Acidic	Neutral		Acidic	Neutral	Basic	Basic	Basic	
Expt A, yield[b] (%)	67	←——164[d]——→		38	Not detected	Not detected	39	29	33	
Expt B, yield[c] (%)	32	30	25	Not detected	21	22	21	42	Not detected	

[a] TR-C and TR-G not included, TR-F counted twice. [b] Yield calculated from ninhydrin analysis (leucine equivalents) in Dowex 50 chromatography. [c] Yield calculated from amino acid analyses after final purification. [d] Combined yield of TR-B1 and TR-B2.

is presented in Figure 1. Only fraction TR-B was impure and it was further purified by paper electrophoresis at pH 3.5, 16 V/cm, for 2 hr and finally eluted from paper with H_2O. The peak marked TR-D was ammonia.

The amino acid compositions of the tryptic peptides are summarized in Table I. The color value of *S*-β-aminoethylcysteine was taken to be 91% that of lysine (Plapp *et al.*, 1967). All values are uncorrected for hydrolytic loss or slow release.

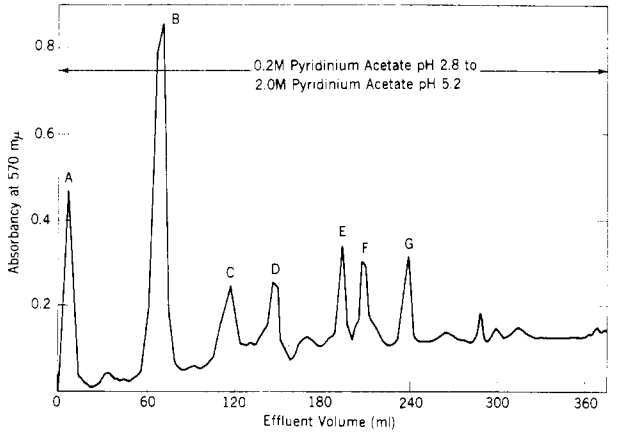

FIGURE 1: Dowex 50-X8 chromatography of the tryptic peptides of AE-Fd, expt A. The digest was applied to a Dowex 50-X8 column (0.9 × 19 cm) and eluted with a linear gradient from 0.2 M pyridinium–acetate (pH 2.8) to 2.0 M pyridinium–acetate (pH 5.2).

PEPTIDE TR-A (RESIDUES 44–55). Ala-Gly-Val-*S*-β-aminoethylcysteine-Pro-Val-(Asp,Ala,Pro,Val)-Gln-Ala. Edman degradation results are presented in Table II. Numbers are μmol of amino acid recovered without any corrections. Yields are calculated from the amount of original peptide without any corrections.

After carboxypeptidase A digestion of TR-A, aliquots were analyzed directly on the amino acid analyzer. The results showed (in micromol): 10 min, 37°; Ala 0.0188, Gln 0.0136, Val 0.0052: 60 min, 37°; Ala 0.0473, Gln 0.0369, Val 0.0062.

Glutamine chromatographed in the serine position on the analyzer and was only tentatively identified as Gln. Later studies on related peptides showed it to be Gln. Thus, the C-terminal sequence of TR-A was deduced to be Gln-Ala. Carboxypeptidase A digestion of whole ferredoxin releases 1 mol each of Ala and Gln (J.-S. Hong and J. C. Rabinowitz, personal communication). Since TR-A is the only tryptic peptide with a C-terminal sequence matching that of whole ferredoxin, it must be the C-terminal tryptic peptide of ferredoxin. Paper electrophoresis at pH 6.4 showed this peptide to be neutral, indicating one amide (Gln) and one carboxyl group (Asp) in the side chains. Leucine aminopeptidase digestion released Ala (100%), Gly (100%), and Val (93%), consistent with the N-terminal sequence of Ala-Gly-Val and with the implications of the Edman degradation that the next residues were X-Pro-

TABLE II: Edman Degradation of Peptide TR-A.

Amino Acid (μmol)	Step 1	2	3	4	5	6
Aspartic acid					0.0118	
Glutamic acid						
Proline					0.0554	
Glycine		0.1177	0.0403	0.0424	0.0394	0.0443
Alanine	0.1091			0.0343	0.0220	
Valine			0.0585	0.0147		0.0489
Sequence	Ala	Gly	Val	*S*-β-Aminoethylcysteine[b]	Pro	Val
Yield[a] (%)	84	90	45		43	38

[a] Yield calculated from amount of original peptide, without regard to losses. [b] *S*-β-Aminoethylcysteine is not observed because of its conversion to the ω-phenylthiocarbamyl derivative.

TABLE III: Edman Degradation of Peptide TR-B1.

Amino Acid (μmol)	Step 1	2	3	4
Aspartic acid				0.0226
Alanine			0.0104	
Valine		0.0594	0.0174	
Isoleucine			0.0878	
Tyrosine	0.0732			
Sequence	Tyr	Val	Ile	Asp
Yield[a] (%)	49	40	59	15

[a] See footnote *a* of Table II.

TABLE IV: Edman Degradation of Peptide TR-B2.

Amino acid (μmol)	Step 1	2	3	4	5
Aspartic acid					0.0214
Alanine	0.2100				0.0123
Valine			0.1272		
Iosleucine				0.0434	0.0076
Tyrosine		0.0656			
Sequence	Ala	Tyr	Val	Ile	Asp
Yield[a] (%)	123	39	75	26	13

[a] See footnote *a* of Table II.

Y. The presence of an *S*-β-aminoethylcysteine-Pro linkage is supported by the fact that there is a trypsin-resistant *S*-B-aminoethylcysteine bond in TR-A.

PEPTIDE-TR-B1 (RESIDUES 30–37). Tyr-Val-Ile-Asp-Ala-Asp-Thr-*S*-β-aminoethylcysteine. The results of the Edman degradation are presented in Table III.

Partial acid hydrolysis in 0.03 N HCl (pH 1.7), 100° 12 hr (Schultz *et al.*, 1962), of 0.05 μmole of TR-Bl and application of the digest directly to the amino acid analyzer showed only Asp (0.1135 μmol, (2.27 residues) and Ala (0.0393 μmol, 0.77 residue), suggesting an internal Asp-Ala-Asp sequence. Carboxypeptidase A + B digestion for 24 hr at 25° released *S*-β-aminoethylcysteine (81%), Thr (62%), Asp (49%), and Ala (17%), establishing the C-terminal sequence as Asp-Thr-*S*-β-aminoethylcysteine. Paper electrophoresis at pH 6.4 showed this peptide to be acidic and to have a charge of −0.9 by the method of Offord (1966), so that 2 carboxyl groups (Asp) were revealed.

PEPTIDE TR-B2 (RESIDUES 1–8). Ala-Tyr-Val-Ile-Asn-Glu-Ala-*S*-β-aminoethylcysteine. The results of the Edman degradation are shown in Table IV.

Treatment of this peptide for 5 hr at 25° with carboxypeptidase A + B released *S*-β-aminoethylcysteine (85%), Ala (70%), Glu (23%), Asn (16%), Ile (14%), and Val (17%). Therefore, the C-terminal sequence of TR-B2 is Glu-Ala-*S*-β-aminoethylcysteine.

Electrophoresis of this peptide at pH 6.4 showed it was neutral and so one carboxyl and one amide group must occur in the side chains. Complete digestion of the peptide with leucine aminopeptidase for 3 and 20 hr showed peaks in the serine and glutamic acid positions on the analyzer but no aspartic acid, suggesting the composition Asn, Glu. The N-terminal sequence of Ala-Tyr establishes that TR-B2 is the N-terminal tryptic peptide of ferredoxin.

PEPTIDE TR-C (RESIDUES 12–29). Gly-Ala-(*S*-β-aminoethylcysteine, Asp, Pro, Glu, *S*-β-aminoethylcysteine, Pro, Val, Asp, Ala, Ile, Ser, Gln, Gly, Asp, Ser, Arg). Two steps of the Edman degradation gave the following result (in micromoles): step 1, Glu 0.0107, Pro 0.0068, **Gly 0.0726 (26%)**,[2] Ala 0.0333, Val 0.0038, and Ile 0.0055; step 2, Asp 0.0045, Ser 0.0100, Glu 0.0054, Pro 0.0042, Gly 0.0097, and **Ala 0.0201 (14%)**.

[2] Boldface indicates amino acid removed.

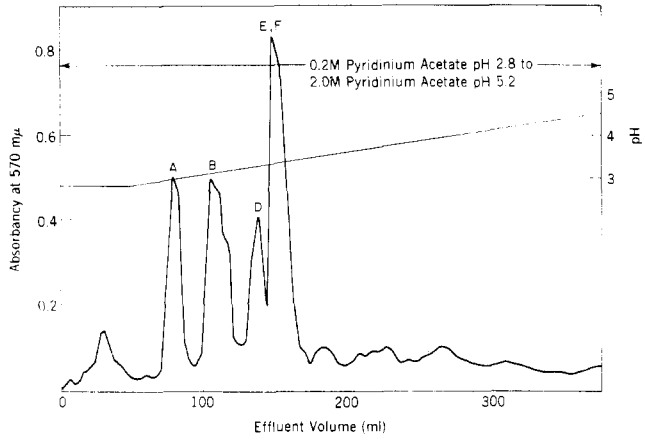

FIGURE 2: Dowex 50-X2 chromatography of the tryptic peptides AE-Fd, expt B. The digest was applied to a Dowex 50-X2 column (0.9 × 19 cm) and eluted with a gradient from 0.2 M pyridinium–acetate (pH 2.8) to 2.0 M pyridinium–acetate (pH 5.2).

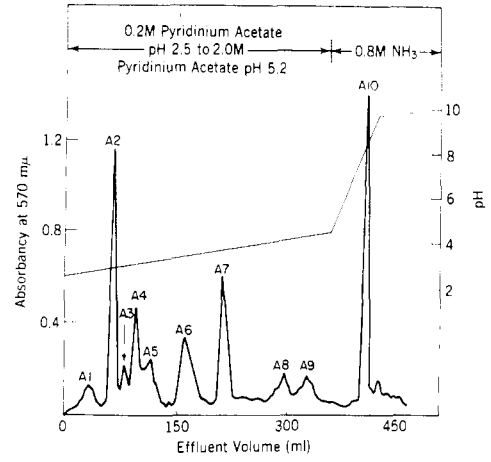

FIGURE 4: Dowex 50-X2 chromatography of peptides derived from partial acid hydrolysis of AE-Fd. The digest was applied to a Dowex 50-X2 column (0.9 × 19 cm) and eluted with a gradient from 0.2 M pyridinium–acetate (pH 2.5) to 2.0 M pyridinium–acetate (pH 5.2). At 350 ml, a stepwise change to 0.8 M NH_3 was made.

PEPTIDE TR-E (RESIDUES 9–11). Ile-Ser-*S*-β-aminoethylcysteine. Only Ile was detected as the end group by the Edman degradation (0.0297 μmol, 40%). Carboxypeptidase B treatment of TR-E for 2 hr at 25° released *S*-β-aminoethylcysteine (89%). Serine can be placed by difference and thus the sequence is Ile-Ser-*S*-β-aminoethylcysteine.

PEPTIDE TR-F (RESIDUES 12–14 AND 41–43). Gly-Ala-*S*-β-aminoethylcysteine. Two steps of the Edman degradation gave the following results (in micromol); Step 1, **Gly 0.0975**, Ala 0.0187; and step 2, Gly 0.0276, **Ala 0.0572**. Yields were not calculated. Carboxypeptidase B treatment for 2 hr at 25° released *S*-β-aminoethylcysteine (103%). Thus the sequence is Gly-Ala-*S*-β-aminoethylcysteine.

PEPTIDE TR-G (RESIDUES 38–43). Ile-Asp-(*S*-β-aminoethylcysteine, Gly, Ala, *S*-β-aminoethylcysteine). The first step of degradation showed Ile to be the end group (in micromol): Step 1, Asp 0.0104, Gly0.0 194, Ala 0.0134, **Ile 0.0592**. The yield was not determined. Subsequent steps failed. Partial acid hydrolysis of a sample of TR-G and application of the digest directly to the analyzer showed only Asp (71%) and Ile (62%), suggesting an Ile-Asp N-terminal sequence.

TRYPTIC PEPTIDES OF EXPERIMENT B. The elution profile of the tryptic peptides from expt B is presented in Figure 2. In this experiment, only TR-A was pure. The others were further purified by preparative electrophoresis at pH 6.4, 22 V/cm, for 2 hr. Water was used to elute the peptides from the paper. The compositions of the peptides are included in Table 1. TR-Dl from expt B is the same as TR-C from expt A, except it lacks Gly, Ala, and *S*-β-aminoethylcysteine in the N-terminal region. Also, TR-G from expt A seems to be a combination of TR-D2 and TR-F from expt B. A major cause of the low yields in expt B is presumably due to incomplete recovery from the electrophoretic paper.

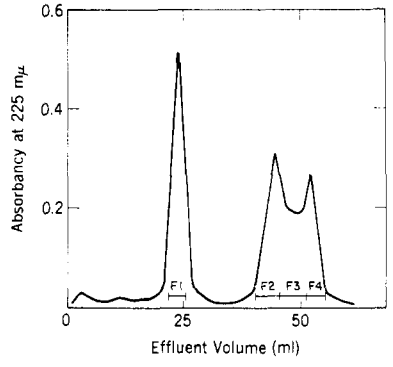

FIGURE 3: Sephadex G-25 chromatography of the peptic digest of peptide TR-D1. The digest was applied to a Sephadex G-25 column (1.2 × 47 cm) and eluted with water.

PEPTIDES TR-E (RESIDUES 9–11) AND TR-F (RESIDUES 12–14 AND 41–43). Ile-Ser-*S*-β-aminoethylcysteine. and Gly-Ala-*S*-β-aminoethylcysteine. These peptides eluted together on Dowex 50-X2 and were separated electrophoretically at pH 6.4 and further treated in identical fashion. The compositions are given in Table I. TR-E had a charge of +1.0 and TR-F a charge of +1.0 by the method of Offord (1966).

The yield of TR-F was exactly twice that of TR-E, which suggested that the peptide Gly-Ala-*S*-β-aminoethylcysteine occurred twice in the molecule. Since the peptides preceding and following positions 12–14 (TR-E, 9-11, and TR-D1, 15–29) and 41–43 (TR-D2, 38–40, and TR-A, 44–55) all were recovered in approximately equal amounts, then the peptide TR-F would be expected (see, for example, Plapp *et al.*, 1967) in twice that yield if it occurred twice. Further evidence that the Gly-Ala-*S*-β-aminoethylcysteine sequence occurred twice is that only 52 of 55 amino acids can be accounted for in expt B, counting each peptide once. The missing amino acids are Gly, Ala, and *S*-β-aminoethylcysteine.

To rule out the possibility of a mixture of Gly-Ala-*S*-β-aminoethylcysteine and Ala-Gly-*S*-β-aminoethylcysteine, a single step of the Edman degradation was performed on TR-F. The recovery of Gly and Ala from the **residue**, after acid hydrolysis, was Gly (28%) and Ala (119%). This result, together with the direct identification of the released terminal amino acids of TR-F, seemed to rule out the possibility of an Ala-Gly-*S*-β-aminoethylcysteine peptide. Had TR-F been such a mixture, the Edman degradation would have been expected to

TABLE V: Amino Acid Compositions of Partial Acid Peptides of AE-Fd.

Peptide	A1A	A1B	A2	A3	A4	A5	A6	A8	A9
Aspartic acid			++++						
Threonine								0.90 (1)	
Serine				0.91 (1)					0.22
Glutamic acid	1.00 (1)		+				1.04 (1)		
Proline	0.97 (1)						1.94 (2)		
Glycine						++			
Alanine	1.00 (1)	1.00 (1)		1.00 (1)	+++				
Valine	1.02 (1)	0.83 (1)					0.96 (1)		0.85 (1)
Isoleucine		0.70 (1)		1.06 (1)				1.11 (1)	0.82 (1)
Tyrosine		0.52 (1)							0.88 (1)
Aminoethyl-cysteine							1.00 (1)	0.99 (1)	
Arginine									1.00 (1)
Total residues	4	4	–	3	–	–	5	3	4
Yield (%)	36	24	–	5	–	–	9	28	30
Ninhydrin color	Purple	Purple		Purple	–	–	Yellow	Gray	Purple

show an almost equal reduction in Gly and Ala at the first step; it did not.

Apparently TR-C from expt A contained both the TR-F and the TR-D1 peptides of expt B, and TR-G from expt A contained both the TR-F and TR-D2 peptides of expt B. The increase in both trypsin concentration and temperature in B over those in expt A seems to have completely hydrolyzed two slowly cleaved *S*-β-aminoethylcysteine bonds, which were hydrolyzed only slightly in expt A. In expt A, peptides corresponding to TR-D1 and TR-D2 were not found, and in expt B, peptides corresponding to TR-C and TR-G were not detected.

PEPTIDE TR-D2 (RESIDUES 38–40). Ile-Asp-*S*-β-aminoethylcysteine. Digestion of this peptide with leucine aminopeptidase for 3 hours released Ile (110%), Asp (62%), and *S*-β-aminoethylcysteine (70%). Carboxypeptidase B treatment of TR-D2 for 2 hr at 25° released *S*-β-aminoethylcysteine (90%). Therefore, this peptide has the sequence Ile-Asp-*S*-β-aminoethylcysteine and is the N-terminal portion of the peptide TR-G from expt A. This peptide was neutral upon electrophoresis at pH 6.4, confirming the Asp assignment which was derived from leucine aminopeptidase digestion.

PEPTIDE TR-D1 (RESIDUES 15–29). Asp-(Pro, Glu, *S*-β-aminoethylcysteine, Pro, Val, Asp, Ala, Ile, Ser, Gln)-Gly-Asp-Ser-Arg. This peptide was acidic upon electrophoresis at pH 6.4 and had a charge of −2.0 by the method of Offord (1966). From this it can be concluded that TR-D1 has only one side-chain amide. Leucine aminopeptidase did not digest TR-D1 at all, even after 20-hr incubation; this is consistent with an X-Pro-Y N-terminal sequence. Carboxypeptidase B released arginine (99%) in 2 hr at 25°.

Peptic Digestion of TR-D1. The elution profile of the peptic digest of TR-D1 is shown in Figure 3. Pepsin appeared in the holdup peak (fraction 1). Fraction 4 was the salt peak and contained no peptides. Fraction 3 contained some of fraction 2 and the two components were separated by pH 3.5 electrophoresis. Fraction 3 (TR-D1-F3) had the composition, Asp 1.00, Ser 0.90, Gly 0.54, Ile 0.47, and Arg 0.80. No further separation was possible. End-group analysis by the dansyl-chloride method of Gray (1967) showed Asp, Gly, and Ile in equal amounts using solvent II of Woods and Wang (1967). Isoleucine cannot be derived from TR-D1, as is shown from the composition of fraction 2 given below, and was considered to be a contaminant of unknown origin, perhaps from autolysis of pepsin. There appeared to be, then, two peptides in nearly equivalent amounts: Gly-(Asp, Ser, Arg) and Asp-(Ser, Arg). Arginine is the C-terminal amino acid of TR-D1, so that these two peptides originate from the C-terminal region of TR-D1 and establish its C-terminal sequence as Gly-Asp-Ser-Arg. Electrophoresis of fraction 3 at pH 6.4 showed only a neutral spot, indicating the presence of Asp rather than Asn.

Fraction 2 (TR-D1-F2) had the following composition: Asp 1.87, Ser 0.95, Glu 2.21, Pro 1.87, Gly 0.64, Ala 1.04, Val 0.99, Ile 1.00, *S*-β-aminoethylcysteine 0.97, and Arg 0.39. This composition is very close to one calculated assuming approximately equivalent amounts of peptides 15–29, 15–26, and 15–25. This mixture did not resolve when submitted to electrophoresis at pH 6.4 and 3.5 or when subjected to chromatography on Sephadex G-25. End-group analysis of fraction 2 by the dansyl-chloride method showed only aspartic acid (solvent II). This fraction was not used in further deductions of the sequence.

Partial Acid Peptides of AE-Fd. The elution profile of peptides derived from partial acid hydrolysis is shown in Figure 4. A summary of the amino acid compositions of these peptides is given in Table V. The peak marked A2 contained free glutamic and aspartic acids; A3 contained free serine in addition to a peptide; A4 contained free alanine; and A5 free glycine. A7 was ammonia.

PEPTIDE A1A (RESIDUES 51–54). Ala-Pro-Val-Glu. The first three steps of the Edman degradation are shown in Table VI. After step 3, application of the residue to the amino acid analyzer without further hydrolysis showed **Glu 0.0327 μmol**

TABLE VI: Edman Degradation of Peptide A1A.

	Step		
Amino Acid (μmol)	1	2	3
Glutamic acid	0.0211	0.0051	0.0159
Proline		0.0277	
Alanine	0.0496	0.0165	0.0144
Valine	0.0058	0.0046	0.0235
Sequence	Ala	Pro	Val
Yield[a] (%)	94	53	45

[a] See footnote *a* of Table II.

TABLE VII: Edman Degradation of Peptide A6.

	Step			
Amino acid (μmol)	1	2	3	4
Glutamic acid	0.0067	0.0242	0.0128	0
Proline	0.0297	0.0297	0.0121	0.0181
Valine	0.0059	0.0046	0.0086	0.0028
Sequence	Pro	Glu	S-β-amino-ethyl-cysteine[b]	Pro
Yield[a] (%)	62	50		38

[a] See footnote *a* of Table II. [b] See footnote *b* of Table II.

TABLE VIII: Edman Degradation of Peptide A8.

	Step		
Amino acid (μmole)	1	2	3
Threonine	0.0249		0.0037
Isoleucine	0.0073		0.0192
Sequence	Thr	*S*-β-Amino-ethylcysteine[b]	Ile
Yield[a] (%)	27		21

[a] See footnote *a* of Table II. [b] See footnote *b* of Table II.

(62%) and Ala 0.0031 μmol. This peptide overlaps the unsequenced region of TR-A and thus establishes the total sequence of TR-A as Ala-Gly-Val-*S*-β-aminoethylcysteine-Pro-Val-Asp-Ala-Pro-Val-Gln-Ala. The Glu of peptide A1A corresponds to the Gln of TR-A. In the conditions employed for the partial acid hydrolysis, side-chain amides were converted to carboxylic acids, with loss of NH_3.

PEPTIDE A1B (RESIDUES 1–4). Ala-(Tyr, Val, Ile). One step of the Edman degradation revealed **Ala 0.0704 μmol (88%)**, Val 0.0035 μmol, and Ile 0.0054 μmol. This peptide corresponds to the N-terminal peptide of ferredoxin and is contained in TR-B2. No further sequence work was necessary.

PEPTIDE A3 (RESIDUES 22–24). (Ala, Ile, Ser). This is the same as the papain peptide P4. Peptide A3 eluted from Dowex 50 with free serine and was separated from it electrophoretically at pH 3.5.

PEPTIDE A6 (RESIDUES 16–20). Pro-Glu-*S*-β-aminoethylcysteine-Pro-Val. The results of the Edman degradation are presented in Table VII. At step 1, it appeared the peptide was incompletely carbamylated, and in subsequent steps, two species of peptide were being sequenced. In this case, the following amino acids should appear: step 1, Pro; step 2, Pro + Glu; step 3, Glu + *S*-β-aminoethylcysteine; and step 4, *S*-β-aminoethylcysteine + Pro. The results are consistent with this hypothesis. *S*-β-Aminoethylcysteine is not observed (see footnote *b* of Table II). After step 4, application of the residue to the amino acid analyzer without further hydrolysis showed only free valine.

PEPTIDE A8 (RESIDUES 36–38). Thr-*S*-β-aminoethylcysteine-Ile. Results of the Edman degradation are shown in Table VIII. Since no amino acid appeared at step 2, this was thought to be *S*-β-aminoethylcysteine, especially since step 3 clearly showed Ile.

PEPTIDE A9 (RESIDUES 29–32). (Arg, Tyr, Val, Ile). This peptide overlaps TR-D1 and TR-C on one side and TR-B1 on the other. Furthermore, it establishes that arginine directly precedes peptide TR-B1, as is consistent with the known action of trypsin and the results of carboxypeptidase B digestion of peptide TR-D1. No sequence work was done, since peptide A9 can only have the sequence Arg-Tyr-Val-Ile.

PEPTIDE A10. This fraction probably contained the peptides derived from residues 6–14 and 40–49, each of which contain 3 *S*-β-aminoethylcysteine residues. The mixture smeared on paper electrophoresis and on paper chromatography in butanol–pyridine–HOAc–H_2O (15:10:3:12) and failed to separate on Dowex AG 1-X2. No separation was possible and no further work was done on the mixture.

PAPAIN PEPTIDES OF OFD. The elution profile of the papain digest of OFd on Dowex AG 1-X2 is shown in Figure 5. A summary of the amino acid compositions is presented in Table IX. Pl contained ammonia only.

PEPTIDE P2 (RESIDUES 1–4). (Ala, Tyr, Val, Ile). This was the same as peptide A1B, derived from partial acid hydrolysis.

PEPTIDE P3 (RESIDUES 51–54). (Ala, Pro, Val)-Gln. This was the same as peptide A1A. The peptide was neutral upon electrophoresis at pH 6.4 and carboxypeptidase A digestion for 1 hr at 25° showed only a peak in the serine position on the amino acid analyzer (Gln, 45%), thus confirming that the Glu of peptide A1A was Gln in native ferredoxin.

PEPTIDE P4 (RESIDUES 22–24). Ala-Ile-Ser. The Edman degradation gave the following results (in micromol): Step 1, Ser 0.0107, **Ala 0.0597 (40%)**, and Ile 0.0131; step 2, Ser 0.0119, Ala 0.0046, and **Ile 0.0367 (25%)**. The residue after step 2 was applied to the amino acid analyzer directly and showed **serine 0.0864 (58%)** and alanine 0.0033. Therefore, the sequence is Ala-Ile-Ser.

PEPTIDE P6 (RESIDUES 5–7). (Asn, Glu, Ala). This sequence is contained in TR-B2. The Edman degradation failed and carboxypeptidase A did not digest the peptide appreciably.

TABLE IX: Amino Acid Compositions of Papain Peptides.

Peptide	P2	P3	P4	P6	P7	P8A	P8B	P9
Cysteic acid						1.03 (1)	1.14 (1)	3.21 (3)
Aspartic acid		0.05	0.25	0.82 (1)	3.04 (3)	1.07 (1)	0.91 (1)	1.84 (2)
Threonine				0.15	0.1			0.82 (1)
Serine		0.10	0.91 (1)	0.2	0.90 (1)	0.5	0.3	0.32
Glutamic acid		1.02 (1)		1.01 (1)	0.97 (1)	0.47	0.88 (1)	0.20
Proline		1.02 (1)				1.00 (1)	1.02 (1)	
Glycine		0.15	0.26	0.22	1.18 (1)	0.49	0.31	1.90 (2)
Alanine	+	0.97 (1)	0.91 (1)	1.16 (1)	0.83 (1)	0.25	0.41	2.28 (2)
Valine	+	0.98 (1)	0.28	0.05	0.93 (1)	1.67 (2)	1.03 (1)	
Isoleucine	+		1.01 (1)		0.78 (1)		0.2	1.19 (1)
Tyrosine	+[a]				>0.3[a] (1)			
Arginine					>0.52 (1)			
Total residues	4	4	3	3	11	5	5	11
Yield (%)		17	20	7	3	←——39[b]——→		8
Ninhydrin color	Purple	Purple	Purple	Purple	Negative	Purple	Purple	Blue
pH 6.4 mobility	Neutral	Neutral	Neutral	Acidic		Acidic	Acidic	Acidic

[a] Chlorotyrosine. [b] Combined yield of P8A and P8B before separation.

Digestion with leucine aminopeptidase for 48 hr released Asn (28%), Glu (30%), and Ala (38%). This peptide may have undergone a partial transformation to the β-aspartyl linkage (see Weber and Konigsberg, 1967), which would account for the incomplete release of amino acids by leucine aminopeptidase.

PEPTIDE P7 (RESIDUES 25–35). Gln-(Gly, Asp, Ser, Arg, Cl-Tyr, Val, Ile, Asp, Ala, Asp). This peptide was ninhydrin negative, and it did not react with the Edman reagent. Furthermore, leucine aminopeptidase did not release any amino acids. After mild hydrolysis employing conditions known to break pyrollidonecarboxylic acid rings without seriously affecting peptide bonds, leucine aminopeptidase completely digested this peptide. It was concluded that glutamine was the N-terminal amino acid and had cyclized to the pyrollidonecarboxylic acid derivative during papain digestion or subsequent chromatography of the digest. This result further suggests that the amidated group of TR-D1 is Gln, since P7 overlaps TR-D1 and TR-B1. Also, these data indicate that the C-terminal sequence of TR-D1 is Gln-Gly-Asp-Ser-Arg.

PEPTIDE P8A (RESIDUES 46–50). Val-($CySO_3H$, Pro, Val)-Asp. Peptides P8A and P8B were separated from one another by paper electrophoresis, first at pH 3.5 and then at pH 6.4. During this purification process, a significant amount of contaminating material was picked up, as is reflected in the amino acid composition. Analysis of the mixture before separation showed only 0.15 mol of serine/mol of cysteic acid, but after purification, this ratio increased to *ca.* 0.4.

One step of the Edman degradation revealed valine (22%), and carboxypeptidase A digestion released a small amount of aspartic acid (36% in 24 hr at 30°). The sequence of P8A is contained in TR-A.

PEPTIDE P8B (RESIDUES 17–21). (Glu, $CySO_3H$, Pro, Val)-Asp. Carboxypeptidase A digestion released a small amount of aspartic acid. Because it migrated faster toward the anode at pH 6.4 than P8A did, the Glu is probably not amidated. This peptide overlaps A6.

PEPTIDE P9 (RESIDUES 35–45). Asx-(Thr, $CySO_3H$, Ile, Asp, $CySO_3H$, Gly, Ala, $CySO_3H$)-Ala-Gly. P9 runs very fast to-

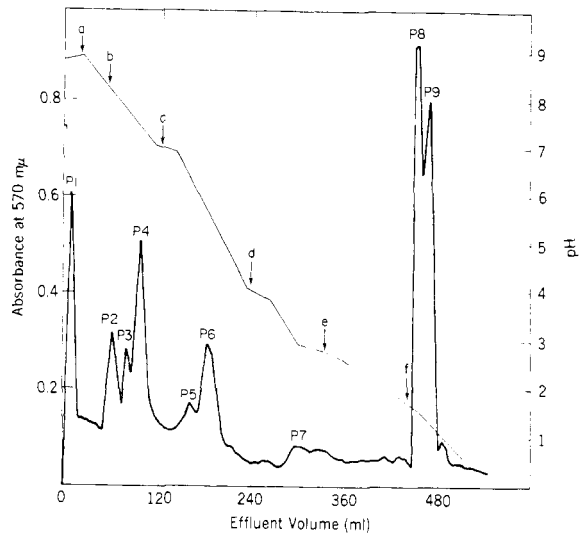

FIGURE 5: Dowex AG 1-X2 chromatography of the papain digest of OFd. The digest was applied to an AG 1-X2 column (0.9 × 22 cm) and was eluted with an exponential gradient. In the mixing chamber was 40 ml of *N*-ethylmorpholine–picoline–pyridine–HOAc–H_2O (pH 9.4). Additions to the upper chamber were made in the following manner and as indicated in the figure: (a) 90 ml of morpholine–picoline–pyridine–HOAc–H_2O, pH 8.4; (b) 120 ml of morpholine–picoline–pyridine–HOAc–H_2O, pH 6.5; (c) 90 ml of 0.5 N HOAc; (d) 100 ml of 2.0 N HOAc; (e) 100 ml of 0.1 N HCl; and (f) 1.0 N HCl.

TABLE X: Summary of Peptides Derived fromTryptic, Papain, and Partial Acid Hydrolyses of *C. acidi-urici* Ferredoxin.

Position	Peptide	Sequence[a]
1–8	TR-B2	Ala-Tyr-Val-Ile-Asn-Glu-Ala-*S*-β-Aminoethylcysteine
1–4	A1B	Ala-Tyr-Val-Ile
5–7	P6	Asn-Glu-Ala
9–11	TR-E	Ile-Ser-*S*-β-aminoethylcysteine
12–14	TR-F	Gly-Ala-*S*-β-aminoethylcysteine
12–29	TR-C	Gly-Ala-*S*-β-aminoethylcysteine-Asp-Pro-Glu-*S*-β-aminoethylcysteine-Pro-Val-Asp-Ala-Ile-Ser-Gln-Gly-Asp-Ser-Arg
15–29	TR-D1	Asp-Pro-Glu-*S*-β-aminoethylcysteine-Pro-Val-Asp-Ala-Ile-Ser-Gln-Gly-Asp-Ser-Arg
16–20	A6	Pro-Glu-*S*-β-aminoethylcysteine-Pro-Val
17–21	P8B	Glu-CySO$_3$H-Pro-Val-Asp
22–24	P4	Ala-Ile-Ser
22–24	A4	Ala-Ile-Ser
25–35	P7	Gln-Gly-Asp-Ser-Arg-Tyr-Val-Ile-Asp-Ala-Asp
26–29	TR-D1-F3	Gly-Asp-Ser-Arg
27–29	TR-D1-F3	Asp-Ser-Arg
29–32	A9	Arg-Tyr-Val-Ile
30–37	TR-B1	Tyr-Val-Ile-Asp-Ala-Asp-Thr-*S*-β-aminoethylcysteine
35–45	P9	Asp-Thr-CySO$_3$H-Ile-Asp-CySO$_3$H-Gly-Ala-CySO$_3$H-Ala-Gly
36–38	A8	Thr-*S*-β-aminoethylcysteine-Ile
38–40	TR-D2	Ile-Asp-*S*-β-aminoethylcysteine
38–43	TR-G	Ile-Asp-*S*-β-aminoethylcysteine-Gly-Ala-*S*-β-aminoethylcysteine
41–43	TR-F	Gly-Ala-*S*-β-aminoethylcysteine
44–55	TR-A	Ala-Gly-Val-*S*-β-aminoethylcysteine-Pro-Val-Asp-Ala-Pro-Val-Gln-Ala
46–50	P8A	Val-CySO$_3$H-Pro-Val-Asp
51–54	A1A	Ala-Pro-Val-Glu
51–54	P3	Ala-Pro-Val-Gln

[a] →, Edman degradation; ⇒, dansyl chloride; ⇢, leucine aminopeptidase; ←, carboxypeptidase A or B.

ward the anode in electrophoresis at pH 6.4 in spite of its size, suggesting a high negative charge. Carboxypeptidase A released only Gly (++) and Ala (+). Repeated attempts at Edman degradation failed, by both direct and subtractive approaches. Again, a β-aspartyl or imide transformation was suspected. It was concluded that the N-terminal residue was likely Asp or Asn. This peptide contains the C-terminal region of TR-B1, all of TR-G, and the N-terminal region of TR-A.

Discussion

Construction of the Total Sequence. A summary of all the peptides studied is presented in Table X. From these peptides the total sequence of *C. acidi-urici* ferredoxin was reconstructed as shown in Figure 6. TR-A must be the C-terminal peptide, since carboxypeptidase A digestion of whole *C. acidi-urici* ferredoxin releases 1 mol of Ala and Gln (J.-S. Hong and J. C. Rabinowitz, personal communication). TR-B2 is the N-terminal peptide, since it has the Ala-Tyr sequence found to be the N-terminal sequence of whole ferredoxin. TR-G precedes TR-A and TR-B1 precedes TR-G, because of the way in which P9 and A8 overlap those sequences. TR-D1 and TR-C precede TR-B1 since arginine must directly precede TR-B1, as deduced from peptide A9. TR-E can then be placed before TR-C and after TR-B2 by difference. The order of peptides A6 and P4 contained in TR-D1 must be as written or leucine aminopeptidase would have digested TR-D1. The C-terminal portion of TR-D1 is Gln-Gly-Asp-Ser-Arg, as deduced from the peptide mixture TR-D1-F3 and peptide P7.

Trypsin Specificity. There are two *S*-β-aminoethylcysteine-Pro linkages in AE-Fd which are completely resistant to trypsin, as expected (Tanaka *et al.*, 1966). There are also two slowly cleaved linkages in *C. acidi-urici* ferredoxin, Ala-*S*-β-aminoethylcysteine-Asp (residues 13–15) and Asp-*S*-β-aminoethylcysteine-Gly (residues 39–41). In contrast to Tanaka *et al.* (1966), we find that both these bonds can be hydrolyzed extensively in 12 hr under conditions comparable with theirs. Peptide bonds of the type X-Arg-Y, X-Lys-Y, or in this case X-*S*-β-aminoethylcysteine-Y, where X or Y is an acidic residue, may be cleaved only slowly by trypsin (Crestfield *et al.*, 1963). The presence of acidic residues at positions 15 and 39 could be responsible for the slow cleavage observed.

Selective Cleavage at Aspartyl Residues. The attempt to use the partial acid hydrolysis method of Schultz *et al.* (1962) to cleave selectively aspartyl residues out of ferredoxin had sev-

eral advantages. First, 8 of 55 residues were Asp or Asn, which theoretically would yield an intermediate number of moderate-sized peptides, an ideal situation for sequence work. Second, the unusual composition of ferredoxin limits the utility of proteases with a known high degree of specificity, *i.e.*, trypsin and chymotrypsin, so that a specific chemical method is advantageous.

In previous work (W. Lovenberg, M. A. Raftery, and R. D. Cole, unpublished observations), partial acid hydrolysis of ferredoxin from *C. pasteurianum* was found to be highly selective for cleavage at aspartyl residues but in the present case, the selectivity was only moderate. In addition to cleavage at aspartyl residues and release of side-chain amide groups, the following bonds were split significantly: The C-terminal Gln–Ala (54–55) bond, the Ser–Gln (24–25) bond, the Gln–Gly (25–26) bond, and the Ser–Arg (28–29) bond, which along with the Asp-Ala-Asp sequence (33–35) caused the release of the free amino acids, Glu, Ala, Gly, and Ser. Still, the method is useful and has somewhat more predictability than proteases such as papain, pepsin, or subtilisin.

Problems with the Edman Degradation. There were several problems with the Edman method employed. The small amounts of peptide material available necessitated the use of direct identification of the amino acid in order to preserve as much peptide as possible for succeeding steps. One difficulty encountered was that artifacts were consistently observed in the glycine and alanine positions on the amino acid analyzer, whether or not glycine and alanine were present in the peptide. The amount of these artifacts was not proportional to the amount of starting peptide material. To a lesser and more erratic extent, artifacts in the serine and glutamic acid position were also observed, even though serine or glutamic acid may not have been in the peptide.

Another problem was the inability to observe those amino acids which are destroyed in alkaline hydrolysis (Ser, Thr, Arg) and those which do not extract into the organic phase or are otherwise converted to other derivatives (Arg, *S*-β-aminoethylcysteine).

Finally, there was the difficulty of continuing stepwise degradation through Asx residues. This has been discussed by Weber and Konigsberg (1967). In most cases, the Edman degradation failed at or just after the appearance of Asx in the peptide, particularly in the case of papain peptides, where the relatively harsh acidic conditions used for column chromatography likely accelerated the formation of β-aspartyl linkages.

Choice of Ion-Exchange Resin. The separation achieved on Dowex 50 with the tryptic peptides was much more satisfactory with 50-X8 than 50-X2. Although Dowex 50-X8 is not generally recommended for peptide separations because the peptides tend to elute earlier than on Dowex 50-X2 under the same conditions (Schroeder, 1967), we find that although the larger peptides do indeed elute earlier, the smaller ones are retarded and separated from each other on Dowex 50-X8, without loss of resolving power on the larger peptides. Obviously, the ideal resin for separating any peptide mixture must be determined experimentally.

Point Mutations in Ferredoxin. It is of interest to consider the amino acid differences among the bacterial ferredoxins in terms of the required base changes in the mRNA for ferredoxin. Of the minimum of 29 base changes necessary to account for all the amino acid changes among the three clostridial ferredoxins, 27 involve the base adenine. If the base changes were random, the percentages would reflect the base composition of the combined mRNAs, which is approximately 35% guanine, 21% adenine, 23% uracil, and 21% cytosine. However, the real percentages are 29% guanine, 43% adenine, 14% uracil, and 14% cytosine. The mutation rate of adenine is twice that expected from random considerations. Since a change involving adenine in the messenger means a point mutation of thymine in DNA, this observation is significant in regard to the relative mutability of thymine in the ferredoxin gene.

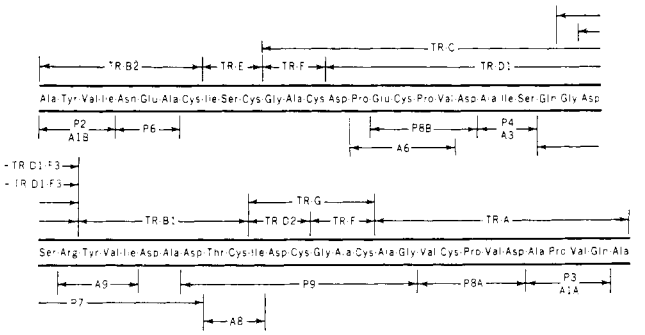

FIGURE 6: Construction of the total sequence of *C. acidi-urici* ferredoxin.

Comments on Structure. The residues of one-half of the ferredoxin molecule are nearly all paired with matching residues in the other half, as has been pointed out by Tanaka *et al.* (1966). *C. acidi-urici* ferredoxin has 15 such pairs, not counting residue 33, while *C. pasteurianum* ferredoxin has 13 and *C. butyricum* 14. The four cysteine residues in each half are separated by runs of two, two, and three amino acids, and each cysteine grouping terminates in a Pro-Val-Asp-Ala sequence. In addition, there are two paired Tyr-Val-Ile sequences, two Cys-Gly-Ala-Cys sequences, and two Cys-Ile sequences. Matsubara *et al.* (1968) have suggested a prototype of 29 amino acid residues which would be common to the two halves.

The positions of the 8 cysteines and 3 of 4 prolines are identical in all three clostridial ferredoxins; 37 of the 55 residues are identical in the three species, and alanine is the N-terminal amino in all three. In addition, the positions of the aromatic residues and most of those with hydrophobic character are the same.

The positions of the prolines and acidic residues in *C. acidi-urici* are not identical with those of *C. pasteurianum* and *C. butyricum* ferredoxins. This may have some significance in the observed differences in properties of the three ferredoxins. *C. acidi-urici* is the only clostridial species with four prolines; the others have only three. The position of the extra proline is curious, since it occurs in the cysteine region, which is involved in forming the prosthetic group with iron and sulfide. It may be that the presence of this proline is responsible for the very sharp crystalline formation of *C. acidi-urici* ferredoxin a property which other clostridial ferredoxins do not share (see Valentine, 1964). This proline and the unique acidic residues at positions 15, 21, and 50 may also explain the low activity of *C. acidi-urici* ferredoxin in the phosphoroclastic assay (Lovenberg *et al.*, 1963). The validity of such conclusions must await the X-ray determination of ferredoxin structures.

References

Africa, B., and Carpenter, F. H. (1966), *Biochem. Biophys. Res. Commun. 24*, 113.
Benson, A. M., Mower, H. F., and Yasunobu, K. T. (1966), *Proc. Natl. Acad. Sci. U. S. 55*, 1532.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 622.
Eck, R. V., and Dayhoff, M. O. (1966), *Science 152*, 363.
Eriksson, S., and Sjöquist, J. (1960), *Biochim. Biophys. Acta 45*, 290.
Gray, W. R. (1967), *Methods Enzymol. 11*, 139.
Hanson, H., Glässer, D., and Kirschke, H. (1965), *Z. Physiol. Chem. 340*, 107.
Konigsberg, W., and Hill, R. J. (1962), *J. Biol. Chem. 237*, 2547.
Lovenberg, W., Buchanan, B. B., and Rabinowitz, J. C. (1963), *J. Biol. Chem. 238*, 3899.
Malkin, R., and Rabinowitz, J. C. (1966), *Biochemistry 5*, 1262.
Matsubara, H., Jukes, T. H., and Cantor, C. R. (1968), *Brookhaven Symp. Biol.* (in press).
Matsubara, H., Sasaki, R. M., and Chain, R. K. (1967), *Proc. Natl. Sci. U. S. 57*, 439.
Moore, S. (1963), *J. Biol. Chem. 238*, 235.
Moore, S., and Stein, W. H. (1954), *J. Biol. Chem. 211*, 907.
Moore, S., and Stein, W. H. (1963), *Methods Enzymol. 6*, 819.
Offord, R. E. (1966), *Nature 211*, 591.
Plapp, B. V., Raftery, M. A., and Cole, R. D. (1967), *J. Biol. Chem. 242*, 265.
Raftery, M. A., and Cole, R. D. (1963), *Biochem. Biophys. Res. Commun. 10*, 467.
Rombauts, W. A., and Raftery, M. A. (1965), *Anal. Chem. 36*, 1611.
Schroeder, W. A. (1967), *Methods Enzymol. 11*, 358.
Schroeder, W. A., Jones, R. T., Cormick, J., and McCalla, K. (1962), *Anal. Chem. 34*, 1570.
Schultz, J., Allison, H., and Grice, M. (1962), *Biochemistry 1*, 694.
Tanaka, M., Nakashima, T., Benson, A., Mower, H., and Yasunobu, K. T. (1966), *Biochemistry 5*, 1666.
Tanaka, M., Nakashima, T., Mower, H. F., and Yasunobu, K. T. (1964), *Arch. Biochem. Biophys. 105*, 570.
Valentine, R. C. (1964), *Bacteriol. Rev. 28*, 497.
Wang, S.-S., and Carpenter, F. H. (1965), *J. Biol. Chem. 240*, 1619.
Weber, K., and Konigsberg, W. (1967), *J. Biol. Chem. 242*, 3563.
Woods, K. R., and Wang, K.-T. (1967), *Biochim. Biophys. Acta 133*, 369.

Quantitative Appraisals of Possible Catalytic Intermediates in the Succinyl Coenzyme A Synthetase Reaction*

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ABSTRACT: Previous data have suggested participation of an enzyme-bound form of coenzyme A in the succinyl coenzyme A synthetase reaction. The proposed mechanism requires participation of an oxygen atom from the enzyme or the coenzyme A.

Quantitative ^{18}O studies demonstrate a lack of participation of enzyme or of coenzyme A oxygens and a retention of substrate-^{18}O during catalysis of the succinate $\rightleftarrows$ succinyl coenzyme A, phosphate $\rightleftarrows$ phosphoryl enzyme and phosphate $\rightleftarrows$ adenosine triphosphate exchanges. These results together with a lack of detection of covalently bound coenzyme A and other findings provide compelling evidence against participation of an enzyme-bound coenzyme A form in the catalysis.

An enzyme-bound form of CoA has been suggested as possible catalytic intermediate in the succinyl-CoA synthetase reaction with the enzyme from *Escherichia coli* (Upper, 1964; Moyer and Smith, 1966; Moyer *et al.*, 1967) and from heart tissue (Cha *et al.*, 1965, 1967). The bound CoA was presumably released upon reaction with P_i with concomitant formation of the phosphorylated enzyme, E-P, or was converted into succinyl-CoA by reaction with succinate. Such reactions would require participation of an oxygen atom of either the enzyme or the CoA.

In related studies, Hersh and Jencks (1967) have presented convincing evidence that an enzyme-bound form of CoA participates in the succinyl-CoA–acetoacetate CoA transferase reaction. Further, Benson and Boyer (1969) established that with this transferase ^{18}O from substrates passes through a group on the enzyme in the catalytic cycle. In contrast, however, Moyer *et al.* (1967) did not detect any ^{18}O-labeling of succinyl-CoA synthetase during catalysis with ^{18}O-labeled

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