The Primary Structure of the Clostridium tartarivorum Ferredoxin, a Heat-stable Ferredoxin*

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SUMMARY

The amino acid sequence of the Clostridium tartarivorum ferredoxin was determined to be: Ala-His-Ile-Thr-Asp-Glu-Cys-Ile-Ser-Cys-Gly-Ala-Glu-Cys-Val-Glu-Ala-Ile-Olu-Gly-Thr-Gly-Lys-Tyr-Glu-Val-Asp-Ala-Thr-Cys-Ile-Asp-Cys-Gly-Ala-Cys-Gln-Ala-Val-Cys-Pro-Thr-Gly-Ala-Val-Lys-Ala-Glu. The first 23 residues were determined in the protein sequenator while the remainder of the sequences were determined by conventional methods. The primary structure of the heat-stable ferredoxin is compared with the ferredoxins from the mesophilic organisms.

EXPERIMENTAL PROCEDURES

Materials

Ferredoxin was isolated from C. tartarvorum as described in a previous report (1). The absorbance ratio at A280: A80 was about 0.78 which is an indication of the high purity of the sample used in the present investigation. Trypsin, chymotrypsin, and pepsin were obtained from Worthington. Thermolysin was purchased from Calbiochem and was recrystallized before use. Trypsin was treated with TPCK (2) before use. Chymotrypsin was treated with TLCK (3). The source of the other reagents used were described in previous reports from our laboratory (4–6).

Methods

The method used for the preparation of the aminoethylcysteine derivative of ferredoxin (Ae-ferredoxin) was published in previous reports (4–6). The carboxymethylcysteine derivative of ferredoxin (Cm-ferredoxin) was prepared as follows. The apoprotein was obtained by treating the protein with triethylammonium acid by the previously published procedure (4). The protein (20 mg) was converted to the Cm-derivative as described by Crestfield, Moore, and Stein (7).

Amino Acid Composition—The model 120C Beckman automatic amino acid analyzer was used to determine the amino acid composition of the samples as described by Spackman, Moore, and Stein (8) after acid hydrolysis. The sensitivity of the instrument was increased by the insertion of a range card in such a way that full scale on the recorder corresponded to 1.0 mv and by the use of high sensitivity cuvettes.

Amino and Carboxyl Terminus Analyses—The procedures for determining the NH₂-terminal residue by reaction with 2,4-dinitrofluorobenzene have been described in a previous report and was performed according to Fraenkel-Conrat, Harris, and Levy (9) with slight modifications. Carboxypeptidase A and B reactions were performed as described by Ambler (10). Hydroxylation reactions of samples were based on the procedure of Bradbury (11).

Sequence Studies—Most of the sequence was determined by the subtractive method (12) of Edman degradation (13). In order to determine the NH₂-terminal structure of the protein, the Beckman-Spinco protein-peptide sequencer, model 890, a commercial model of the instrument developed by Edman and
Begg was utilized (14). The ethyl acetate-soluble PTH-amino acids were identified by gas-liquid chromatography in the Beckman model GC-45 gas chromatograph. The two columns used and the experimental conditions for separating the PTH-amino acids or the trimethylsilyl derivative of the PTH-amino acids have been described by Pisano and Bronzert (15, 16). The ethyl acetate-soluble PTH-amino acids were identified by gas-liquid and thin layer chromatography. The two columns used for the separation were as follows. (a) Tubes 1 to 118, the gradient elution was obtained by mixing 100 ml of 0.1 M pyridine-acetate buffer (pH 5.0) with 30 ml of 0.1 M pyridine-acetate buffer, pH 3.0. (b) Tubes 119 to 221, the gradient elution was obtained by mixing 30 ml of 0.1 M pyridine-acetate buffer, pH 3.0, with 100 ml of 0.1 M pyridine-acetate buffer, pH 5.0. The columns were monitored by the ninhydrin assay method (17).

Nomenclature—Peptides obtained hydrolysis with trypsin and chymotrypsin are denoted by “T” and “C,” respectively. Products of further hydrolysis of these peptides with thermolysin and pepsin are denoted by “Th” and “P,” respectively.

RESULTS

Determination of NH2-terminal Sequence

The NH2-terminal sequence of the Cm-ferredoxin from C. tartaricorum was determined in a Beckman protein sequencer. Four separate runs were made in which 0.2 to 0.7 pmole of protein was taken for analysis. The amount of protein did not have much effect on the number of sequences which could be determined in the instrument. A typical result from one of the runs is shown in Fig. 1 which indicates the amino acid species and the yield of the PTH-amino acid liberated at each of the steps of the Edman degradation of the protein. Because of the solubility of the Cm-ferredoxin in the organic solvents used in...
Tryptic peptides of *Ae* ferredoxin (*Clostridium* tartarivorum)

In this and the following tables, PC and BPAW-PC represent paper chromatography and paper chromatography in 1-butanol-pyridine-acetic acid-water (60:40:12:48), respectively.

| Amino acid          | T-1 | T-2 | T-3 | T-4 | T-5 | T-5 | T-7 | T-8 | Total residues |
|---------------------|-----|-----|-----|-----|-----|-----|-----|-----|---------------|
| Aspartic acid       | 1.07(1) |     |     | 0.99(1) | 1.96(2) | 0.97(1) |     |     | 4             |
| Threonine           | 1.00(1) | 0.79(1) |     |     | 0.94(1) | 0.89(1) |     |     | 4             |
| Serine              |     |     |     |     |     |     |     |     | 1             |
| Glutamic acid       | 1.02(1) |     |     | 3.06(3) | 1.09(1) | 0.89(1) | 1.00(1) |     | 7             |
| Proline             | 0.91(1) |     |     | 0.92(1) |     |     |     |     | 2             |
| Glycine             |     |     | 0.96(1) | 1.97(2) | 1.00(1) | 1.02(1) |     |     | 5             |
| Alanine             | 1.00(1) | 1.00(1) |     | 2.98(3) | 0.98(1) | 1.07(1) | 2.01(2) | 0.98(1) | 10            |
| Valine              |     |     |     | 1.02(1) | 1.00(1) |     |     |     | 4             |
| Isoleucine          | 1.95(2) | 1.00(1) |     |     | 0.96(1) |     |     |     | 5             |
| Tyrosine            |     |     |     |     |     |     |     |     | 1             |
| Lysine              | 0.82(1) | 0.97(1) | 1.00(1) | 0.82(1) | 0.97(1) | 1.00(1) |     |     | 2             |
| Histidine           |     |     |     | 0.83(1) | 1.00(1) |     |     |     | 2             |
| S-Aminoethylcysteine|     |     |     | 0.82(1) | 0.97(1) | 1.00(1) |     |     | 5             |
| Total residues      | 8   | 3   | 3   | 15  | 8   | 6   | 10  | 2   | 55            |
| Total moles         | 1.04 | 0.88 | 0.80 |     | 1.20 | 0.76 | 0.60 | 1.28 | 1.54          |
| RP<sup>b</sup>       | 0.28 | 0.27 | 0.17 |     | 0.16 | 0.23 | 0.08 | 0.26 | 0.29          |
| Ninhydrin color reaction |     |     |     | Violet | Purple | Yellow to Violet |     |     |     |
| Separation of Peptides

In order to determine the sequence of the remainder of the *C. tartarivorum* ferredoxin, a tryptic hydrolysis of the *Ae*-ferredoxin was performed. The chromatographic pattern of the tryptic peptides is shown in Fig. 2. The amino acid compositions of the various peptides are summarized in Table I.

Separation of Chymotryptic Peptides

The chromatographic pattern of the chymotryptic peptides on DEAE-Sephadex is shown in Fig. 3 and the amino acid composition of the purified peptides is shown in Table II.

Sequence of Peptides

As mentioned earlier, the first 23 residues from the NH<sub>2</sub>-terminal end of the protein were determined by the protein sequencer. This left 32 residues the sequence of which remained undetermined. Although additional sequence studies were performed on fragments from the first 23-residue portion, they will be omitted here.

Peptide T-4: Ala-Ala-Glu-Cys*(Ac)*-Pro-Val-Glu-Ala-Ile-His-Glu-Gly-Thr-Gly-Lys (Residues 16 to 29)—The sequence through isoleucine was established by the protein sequencer.

In order to complete the sequence, the peptide was hydrolyzed with thermolysin. Two peptides (T-4-Th-1 and T-4-Th-2) were isolated by column (Fig. 4) chromatography of the digest and the amino acid composition and other properties of the peptides are summarized in Table III. The sequence data of Peptide T-4-Th-2 (Ile-His-Glu-Gly-Thr-Gly-Lys) is summarized in Table IV.

Peptide T-5: Tyr-Gln-Val-Asp-Ala-Asp-Thr-Cys*(Ac)* (Residues 30 to 37)—The sequence data is summarized in Table V. In order to complete the sequence of Peptide T-5, it was hy-
TABLE II

Chymotryptic peptides of Ae-ferredoxin (Clostridium tartarivorum)

| Amino acid | C-1 + C-7 | C-2 | C-3 | C-4 | C-5 | C-6 | C-8 |
|------------|------------|-----|-----|-----|-----|-----|-----|
| Aspartic acid | 1.05(1) | 1.00(1) | 2.08(2) | 1.88(3) | 0.06(1) |
| Threonine | 1.91(2) | 1.06(1) | 1.25(1) | 1.00(1) | 0.38 |
| Serine | 0.78(1) | 1.84(2) | 1.12(1) | 1.00(1) |
| Glutamic acid | 2.95(3) | 1.00(1) | 1.02(1) | 0.40 |
| Proline | 1.06(1) | 0.96(1) | 0.90(1) | 1.09(1) |
| Glycine | 1.14(1) | 0.06(1) | 1.27(1) | 1.02(1) | 1.00(1) |
| Alanine | 4.25(4) | 1.00(1) | 1.00(1) | 0.36 |
| Valine | 2.14(2) | 0.83(1) | 0.84(1) |
| Isoleucine | 2.83(3) | 1.00(1) |
| Tyrosine | 0.70(1) |
| Lysine | 1.00(1) |
| Histidine | 0.70(1) |
| β-Aminoethylcysteine | 2.64(3) | 1.00(1) | 2.02(3) | 0.85(1) | 1.84(2) |
| Total residues | 23 | 3 | 16 | 13 | 7 | 6 | 2 |
| Total pmoles | 2.15 | 0.72 | 1.26 | 1.20 | 0.26 | 0.38 | 0.07 |
| Rp* | 0.25-0.4 |
| Electrophoretic property | Basic | Basic | Neutral | Neutral | Acidic | Basic | Acidic |
| Ninhydrin color reaction | Violet | Yellow to violet | Violet | Violet | Violet | Purple |
| Purification method | PC | PC | PC |

* 44-hour hydrolysis.
* By BPAW-PC.
* At pH 6.50.
* BPAW-PC.

Fig. 4. AG 1-X2 column chromatography of the thermolysin digest of Peptide T-4. The details are described in the experimental section.

Dried by pepsin and two fragments were obtained by paper chromatography. The amino acid composition and properties of T-5-P-1 and T-5-P-2 are summarized in Table VI. Hydrolysis was performed on Peptide T-5-P-1 (Tyr-Gln-Val-Asp) and aspartic acid was obtained in 80% yield which in combination with sequence data on T-5 itself, completed the sequence of this peptide. Table VII summarizes the sequence data on Peptide T-5-P-2 which was shown to be Ala-Asp-Thr-Cys(Ae) (residues 34 to 37).

TABLE III

Properties of the thermolysin peptides of T-4

| Amino acid | T-4-Th-1 | T-4-Th-2 |
|------------|----------|----------|
| Threonine | 2.07(2) | 0.90(1) |
| Glutamic acid | 0.98(1) | 1.05(1) |
| Proline | 0.25-0.4 |
| Glycine | 1.80(2) |
| Alanine | 3.02(3) |
| Valine | 1.00(1) |
| Isoleucine | 1.00(1) |
| Lysine | 0.89(1) |
| Histidine | 0.79(1) |
| β-Aminoethylcysteine | 0.97(1) |
| Total residues | 8 | 7 |
| Yield (%) | 66 | 86 |
| Rp* | 0.25 | 0.24 |
| Electrophoretic property | Acidic | Basic |
| Ninhydrin color reaction | Violet | Violet |
| Purification method | PC | PC |

* Paper chromatography in BPAW.
* Paper electrophoresis at pH 6.50.

Peptide T-6: Ile-Asp-Cys(Ae)-Gly-Ala-Cys(Ae) (Residues 58 to 68)—The sequence data of this peptide are summarized in Table VIII.

Peptide T-7: Gln-Ala-Val-Cys(Ae)-Pro-Thr-Gly-Ala-Val-Lys (Residues 44 to 56)—The sequence data obtained on Peptide T-7 are summarized in Table IX.

Peptide T-8: Ala-Glu (Residues 54 to 56)—One step of the
TABLE IV  
Sequence results of Peptide T-1-Th-2  
Sequence: Ile-His-Glu-Gly-Thr-Gly-Lys  

| Amino acid      | Steps in Edman degradation |
|-----------------|----------------------------|
|                 | 0  | 1  | 2  | 3  | 4  | 5  | 6  |
| Threonine       | 0.99| 0.92| 1.00| 1.00| 0.16| 0.18|
| Glutamic acid   | 1.05| 1.06| 1.02| 0.14| 0.17| 0.11| 0.13|
| Glycine         | 1.86| 1.92| 1.95| 1.89| 1.05| 1.00| 0.26|
| Isoleucine      | 1.00| 0.00|     |     |     |     |     |
| Lysine          | 0.89| 0.97| 0.92| 0.93| 0.86| 0.88| 1.00|
| Histidine       | 0.79| 1.00| 0.19| 0.11| 0.00|     |     |

Thin layer chromatography:  
Yield (%)  
Ile 82  Glu 75  Gly 90  Thr 88

* In the various tables, arrows pointing to the right represent sequences determined by Edman degradation and arrows pointing to the left represent sequences determined by the use of carboxypeptidase.

TABLE V  
Partial sequence of Peptide T-6  
Sequence: Tyr-Gln-Val-Asx-Ala-Asx-Thr-Cys(Ae).  
Carboxypeptidase B digestion (1 hour): Cys(Ae) 93%.

| Amino acid      | Steps in Edman degradation |
|-----------------|----------------------------|
|                 | 0  | 1  | 2  | 3  | 4  | 5  | 6  |
| Aspartic acid   | 1.96| 2.08| 2.07| 1.92|     |     |     |
| Threonine       | 0.94| 1.00| 1.00| 0.84|     |     |     |
| Glutamic acid   | 1.09| 1.07| 0.26| 0.20|     |     |     |
| Alanine         | 0.98| 1.06| 1.06| 1.00|     |     |     |
| Valine          | 1.00| 1.11| 1.03| 0.30|     |     |     |
| Tyrosine        | 0.96| 0.00|     |     |     |     |     |
| Cys(Ae)         | 1.00| 0.83| 0.92| 0.75|     |     |     |

Thin layer chromatography:  
Yield (%)  
Tyr 95  Glu 89  Val 85

TABLE VI  
Properties of the peptic peptides from T-5  

| Amino acid      | T-5-P-1  | T-5-P-2  |
|-----------------|----------|----------|
| Aspartic acid   | 0.99(1)  | 1.05(1)  |
| Threonine       | 1.00(1)  | 1.00(1)  |
| Glutamic acid   | 1.05(1)  | 0.92(1)  |
| Alanine         | 1.00(1)  | 0.90(1)  |
| Valine          | 0.99(1)  | 0.88(1)  |
| Tyrosine        |          |          |
| β-Aminoethylcysteine | | |
| Total residues  | 4        | 4        |
| Yield (%)       | 87       | 81       |
| Rf*             | 0.53     | 0.19     |
| Electrophoretic property | Acidic | Neutral |
| Ninhydrin color reaction | Purple | Violet |
| Purification method | PC     | PC      |

* Paper chromatography in BPAW.
* Electrophoresis at pH 8.50.

Edman degradation disclosed that 0.08 residue of alanine remained while 1 residue of glutamic acid remained. PTH-alanine was identified by thin layer chromatography. The second step of the subtractive Edman degradation disclosed no amino acid in significant quantity. The PTH-glutamic acid was identified by thin layer chromatography.

Chymotryptic Peptides

The end groups of the chymotryptic peptides were determined in order to identify the end groups and to check the purity of the samples. Since it is possible to place all the tryptic peptides by analogy to the sequence of the ferredoxin which have been determined, the details will be omitted here.

Complete Sequence of Protein

Dinitrophenylation of native ferredoxin demonstrated that the NH₂-terminal residue was alanine since only DNP-alanine was detected among the possible NH₂-terminal amino acids.
The protein sequencer established the first 23 residues of the protein to be Ala-His-Ile-Ile-Thr-Asp-Glu-Cys(Cm)-Ile-Ser-Cys(Cm)-Gly-Ala-Cys(Cm)-Ala-Ala-Glu-Cys(Cm)-Pro-Val-Glu-Ala-Ile. Peptides T-1, T-2, T-3, and T-4 have the amino acid composition which corresponds to the sequence. Peptides C-1, C-2, and C-3 also have the amino acid composition which corresponds to this sequence. Peptide T-5 is adjacent to T-4 since Peptide C-3 overlaps these two peptides. Peptide T-6 is adjacent to Peptide T-5 since Peptide C-4 overlaps these two peptides. Peptide T-7 is adjacent to Peptide T-6 since Peptide C-7 contains Peptides T-7 and T-8 and since C-7 and the protein itself have been shown by hydrazinolysis to contain COOH-terminal glutamic acid residues. The yield of glutamic acid from Peptide C-7 was about 90%. Of course, it would have been also possible to place the tryptic peptides by analogy to the sequences of other ferredoxins which have been already determined. The complete sequence of the heat-stable ferredoxin from C. tartarivorum is shown in Fig. 5.

The amino acid composition, heat stability, optical rotatory dispersion curves, molecular weight, and other properties of the ferredoxin from C. tartarivorum and C. thermosaccharolyticum were previously reported (1). Two striking features pertinent to the present study are the heat stability and the occurrence of histidine for the first time in the bacterial ferredoxins. It was suggested that heat stability might arise from a more stable chelate structure than is found in mesophilic ferredoxin molecules. The present study has revealed that with the exception of several point mutations the over-all sequence of the thermophilic C. tartarivorum ferredoxin compares well with the sequence of the mesophilic ferredoxins. No gross changes in the primary structure have accompanied the acquisition of thermostability by the C. tartarivorum ferredoxin molecule. The cysteinyl residues which anchor the chelate structure can be superimposed upon the cysteinyl residues of the bacterial ferredoxins sequenced to date (4, 5, 6, 18) (see Fig. 6).

We have looked for amino acid replacements which might account for the heat stability especially around the cysteinyl residues which are involved in the chelation of iron. Two such replacements were found: serine which usually precedes cysteine was replaced by glutamic acid and alanine which usually occurs after cysteine was replaced by glutamine. Other drastic changes are also shown in Fig. 6. It should be noted that the basic residues are not located in the vicinity of the cysteinyl residues in the primary structure. The two histidines are found at positions 2 and 24 and the two lysines are found at positions 29 and 53.

Summarized in Table X are the types of residues found in the various ferredoxins. The classification of residues is based on definitions adopted by Dickerson and Geis (19). It should be noted that again the C. tartarivorum ferredoxin has the greatest content of basic amino acid residues of any ferredoxin sequenced to date and therefore has the least negative net charge.

Admittedly without the knowledge of the three-dimensional structure of the protein it is difficult to tell which residues are important for thermophilidy. Further studies are needed to elucidate the heat-stable nature of the C. tartarivorum ferredoxin.

### Table IX

**Sequence results of Peptide T-7**

| Amino acid | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 15 | 16 | 17 | 18 | 19 | 20 | 21 | 22 | 23 |
|------------|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Threonine  | 0.89 | 0.94 | 1.00 | 0.99 | 0.98 | 0.94 | 0.10 | 0.07 |
| Glutamic acid | 0.89 | 0.07 |
| Proline    | 0.82 | 0.82 | 0.97 | 0.88 | 0.94 | 0.06 | 0.02 | 0.01 |
| Glycine    | 1.00 | 0.00 | 0.96 | 0.95 | 1.00 | 1.00 | 1.00 | 1.00 |
| Alanine    | 1.85 | 1.81 | 1.83 | 0.97 | 0.91 | 1.05 | 0.59 | 1.00 |
| Valine     | 1.00 | 1.00 | 0.98 | 0.95 | 0.98 | 0.82 | 0.86 | 0.86 |
| Lysine     | 1.00 | 0.99 | 0.91 | 0.88 | 0.84 |

**Thin layer chromatography**

| Steps in Edman degradation | 01 | 02 | 03 | 04 | 05 | 06 | 07 | 08 | 09 |
|---------------------------|---|---|---|---|---|---|---|---|---|
| Gln                       | 98 | 100 | 95 | 85 | 88 | 82 | 88 | 80 | 100 |
| Ala                       | 100 |
| Val                       | 95 | 85 |
| Pro                       | 100 |
| Thr                       | 99 |
| Gly                       | 100 |

**Fig. 5. Reconstruction of the amino acid sequence of Clostridium tartarivorum ferredoxin from the sequenator, tryptic peptide, and chymotryptic peptide data.**
FIG. 6. A comparison of the primary structures of the ferredoxins sequenced to date. M.A., C.B., C.P., C.A., and C.T. represent *Micrococcus aerogenes*, *Clostridium butyricum*, *Clostridium pasteurianum*, *Clostridium acidiurici*, and *Clostridium tartarivorum* ferredoxins, respectively. The numbers refer to the residue number starting from the amino-terminal residue. Constant residues are blocked off while the amino acid replacements which may possibly account for the heat stability of the *C. tartarivorum* ferredoxin are starred.

TABLE X
Types of residues in ferredoxins from anaerobes

| Bacteria                   | Hydrophobic | Hydrophilic | Acidic | Basic | Net change |
|----------------------------|-------------|-------------|--------|-------|------------|
| *Micrococcus aerogenes*     | 26          | 28          | 8      | 2     | -6         |
| *Clostridium butyricum*     | 27          | 28          | 8      | 1     | -7         |
| *Clostridium pasteurianum*  | 27          | 28          | 8      | 2     | -6         |
| *Clostridium acidurici*     | 28          | 27          | 10     | 2     | -8         |
| *Clostridium tartarivorum*  | 26          | 29          | 10     | 5     | -5         |
| *Peptostreptococcus eldenii*| 32          | 22          | 8      | 4     | -4         |

*Includes carboxyl group of carboxy-terminal amino acids.

*b Amino group of amino-terminal residues included.

TABLE XI
Similarities in the amino acid sequences of bacterial ferredoxins

| Species                  | C. p | C. b | M. a | C. a | C. t |
|--------------------------|------|------|------|------|------|
| *Clostridium pasteurianum*| 46   | 39   | 41   | 33   |      |
| *C. butyricum*           | 46   | 38   | 39   | 33   |      |
| *M. aerogenes*           | 39   | 36   | 38   | 31   |      |
| *C. acidurici*           | 41   | 39   | 38   | 32   |      |
| *C. tartarivorum*        | 33   | 33   | 31   | 32   |      |

In the hope that a more meaningful comparison between thermophilic ferredoxins and the mesophilic ferredoxins can be made, sequence studies on the thermostable ferredoxin from *C. thermosaccharolyticum* have been initiated.

Let us now examine the similarities in the sequences of the various bacterial ferredoxins available. The results, expressed as the number of residues shared in common, are presented in Table XI. The procedure for aligning the various ferredoxins has been discussed in a previous report from our laboratory (4). A possible phylogenetic relationship based upon the ferredoxins is shown in Fig. 7. Although the results are based on a single protein, the validity of the phylogenetic tree can be further tested by structural studies on other sets of proteins, the rubredoxins and flavodoxins, which are also being sequenced.

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