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AMINO ACID SEQUENCE OF DESULFOVIBRIO GIGAS FERREDOXIN : REVISIONS

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SUMMARY

Reexamination of the amino acid sequence of Desulfovibrio gigas ferredoxin revealed that the sequence published in 1971 should be revised. This sequence was determined using automatic protein sequencer in liquid phase and in solid phase. Peptides derived from tryptic hydrolysis, Staphylococcus aureus protease hydrolysis, cyanogen bromide cleavage were used to construct the total sequence. This ferredoxin contains 6 cysteines per minimum molecular weight of 6,400. 4 cysteines are linked to a (4 Fe-4 S) cluster and the two others possibly participate in a disulfide bridge.

INTRODUCTION

Ferredoxins have been reported to be involved in a wide variety of reactions of photosynthesis, nitrogen fixation, sulfate reduction and other oxidation-reduction reactions. They are divided into photosynthetic bacterial, anaerobic bacterial and plant types which differ in the number of attached iron and sulfur atoms to the sulfur of cysteine (1). Three different forms of (4 Fe-4 S) ferredoxin from D. gigas Fd I, Fd I' and Fd II have been previously characterized. Their apoproteins and physiological activities are identical but they differ in their aggregation states, spectral properties, potential redox and thermal stabilities (2-5). Treatment with sodium dodecyl sulfate induces a depolymerisation into monomeric units of molecular weight 6,400. Fd I and Fd I' are different trimers and Fd II a tetramer of this same basic unit containing one (4 Fe-4 S) cluster. The observation (5) that Fd II is more efficient than Fd I in an enzymatic assay, coupling the electron transfer between hydrogenase and sulfite reduction and that Fd I is more active in the phosphoroclastic reaction, indicate that Fd I and II are probably not an artefact of isolation but have a physiological role in the organism. In our work to verify that there were no differences in the amino acid sequence between Fd I and Fd II, it became apparent that discrepancies existed when the published sequence (6) was compared with our results. This paper describes the revision of the reported sequence. Evolutionary relationships between D. gigas ferredoxin and plant and bacterial-type ferredoxins (7) will also require revision.

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MATERIALS AND METHODS

The cultivation of D. gigas and the purification of the ferredoxin have been previously described (8, 9). Apoferredoxin was obtained by precipitating native ferredoxin with 3 % HCl at 80° C for 10 min.

Preparation of S-β aminoethyl cysteinyl ferredoxin. The aminoethylated apoprotein was prepared according to Keresztes-Nagy et al. (10). The content of S-amino ethylated cysteine was determined by amino acid analysis.

Digestion of protein with proteolytic enzymes. Digestions were carried out at 37° C for 3 h by trypsin and by a peptidase isolated from Staphylococcus aureus (gift from Dr. R.P. Ambler) which is specific for hydrolysis of glutamic acid residues (11). The protein was dissolved in ammonium acetate solution pH 8.5 and a freshly prepared aqueous solution of the enzyme (10 mg/ml) was added. The enzyme to substrate ratio was 1:50. Digestion was stopped by lyophilization.

Purification of peptides. The peptides were purified by high voltage paper electrophoresis using a Gilson high voltage electrophorator at pH 1.9, 3.5 and 6.5. The following buffer systems were used : pyridine/acetic acid/water (25:1:225, by vol.) pH 6.5 ; pyridine/acetic acid/water (1:10:89, by vol.) pH 3.5 ; formic acid/acetic acid/water (1:4:45 by vol.) pH 1.9. Peptides were located on paper by means of the ninhydrine-collidine reagent.

Performic acid oxidation. Peptides were oxidized on paper by performic acid vapour (formic acid/30 % H_2O_2 (95:5, v/v))for 1.5 h in a dessicator under vacuum.

Cyanogen bromide cleavage. The procedure described by Gross and Witkop (12) was used. The protein was dissolved in 50 % formic acid and treated with an equal weight of CNBr for 20 h at 20° C. The reaction mixture was diluted with 10 volumes of water and lyophilized. The peptides were purified by gel filtration on Sephadex G-25 in 10 % formic acid.

C. terminal residue identification. The carboxy-terminal amino acid of the protein was determined both by the hydrazinolysis procedure of Akabori et al. (13) as modified by Niu and Fraenkel-Conrat (14) and by the use of carboxypeptidase A according to the conditions of Ambler (15, 16).

Amino acid composition. Protein and peptides samples were hydrolyzed in 200 µl of 6 M HCl at 110° C for 18 hours in sealed evacuated tubes. Amino acid analysis were performed on a LKB 3201 amino acid analyzer.

Liquid-phase Edman degradation. Sequence determinations were performed in the Socosi Protein Sequencer (p.S. 100). D.M.B.A. (N;N, dimethylbenzylamine) buffer was used. The quantitative determination of the PTH (phenyl thio hydantoïne) derivatives was done relative to known amounts of the appropriate standards on gas chromatography (Beckman gas chromatography GC45 using SP400 as stationary phase according to the technique of Pisano et al.(17). An analysis of the silylated PTH derivatives of the amino acids was always performed. PTH derivatives were also analyzed by thin layer chromatography on silica gel containing an ultraviolet fluorescent indicator (Silica Gel GF254, Merck) as described by Edman (18,19) particularly for the identification of Glu/Gln and Asp/Asn residues (20). In some instances, PTH derivatives have been characterized by amino acid analysis after conversion to the parent amino acid by hydrolysis with chlorhydric acid (21) or hydriodic acid (22) for serine residues

Solid phase Edman degradation. The degradation was performed according to the method of Laursen (23). In the solid phase procedure, the protein or the peptide is attached to the support (amino ethyl propyl glass) either through

the C terminal carboxyl group using carbodiimide or through a side-chain functional groups using p-phenylene diisothiocyanate (DITC) to cross link the NH_2 groups.

RESULTS

Amino acid composition and N terminal sequence analysis. The amino acid composition of D. gigas ferredoxin after hydrolysis of 24 h, 48 h and 72 h shows that the total number of residues is 57 instead of 56 originally counted (6). The valine content should be 6 instead of 5.

The N terminal sequence established by automatic Edman degradation on oxidized ferredoxin and on aminoethylated ferredoxin was determined up to 32 residues with some residues not identified :

1 5 10 15
Pro-Ile-Glu-Val-Asn-Asp-Asp-Cys-Met-Ala-Cys-Glu-Ala-Cys-Val-Glu-Ile-

20 25 30
Cys-Pro-Asp-Val-Phe-Glu-Met-( )-Glu-Glu-Gly-( )-Lys-Ala-Val-----

Some discrepancies were observed from the sequence previously established (6). The amide or acid forms of the aspartic and glutamic residues do not agree with the identification of the charge on silica gel chromatography. Another correction is the addition of one aspartic residue between Asp in position 6 and Cys in position 8.

Sequence determination of the tryptic peptides of oxidized D. gigas ferredoxin. As the ferredoxin contains only one residue of lysine and one residue of arginine we obtained 3 peptides, T-1, T-2 and T-3 with a yield of respectively 35 %, 70 % and 32 %. The sequence of 25 residues of peptide T-2 was obtained by the solid phase sequanator after coupling the C terminal carboxyl group to the amino ethyl propyl glass with carbodiimide. T-1 is the C terminal amino acid and T-3 was identified as the N terminal sequence of the protein (Table 1).

TABLE 1 - Amino acid sequences of the tryptic peptides of oxidized ferredoxin

Peptide	Position in the peptide chain	Sequence
T-1	57	Ser
T-2	31-56	Ala-Val-Val-Ile-Asn-Pro-Asp-Ser-Asp-Leu-Asp-Cys-Val-Glu-Glu-Ala-Ile-Asp-Ser-Cys-Pro-Ala-Glu-Ala-Ile-(Arg)
T-3	1-30	Pro-Ile-Glu-Val-Asn-Asp-Asp-Cys-Met-Ala-Cys-Glu-Ala-Cys-Val-Glu-Ile-Cys-Pro-Asp-Val-Phe-Glu-Met-Asn-Glu-Glu-Gly-Asp-Lys

Peptides obtained by cyanogen bromide cleavage of ferredoxin. D. gigas ferredoxin contains two methionine residues. The three peptides obtained after cyanogen bromide cleavage were fractionated by gel filtration and paper electrophoresis and oxidized by performic acid. The sequence of the peptide CnBr-1 (residues 25-27) elucidated by liquid phase sequenator permits overlapping between the N terminal sequence and the tryptic peptide T- 2 :

Asn-Glu-Glu-Gly-Asp-Lys-Ala-Val-Val-Ile-Asn-Pro-Asp-(Ser-Asp-Leu-Asp-Cys-Val-Glu-Glu-Ala-Ile-Asp-Ser-Cys-Pro-Ala-Glu-Ala-Ile-Arg-Ser)

The peptide CNbr-2 is located from Ala in position 10 to Met in position 24 and CNBr-1 contains the 9 first residues of the protein.

C terminal sequence of ferredoxin. Ser was identified as the COOH terminal residue by carboxypeptidase digestion. The C terminal peptide was purified after digestion of the protein with Staphylococcus aureus protease. The residue of glutamic acid in position 53 was split and the resulting C terminal peptide was purified and his sequence determined : Ala-Ile-Arg-Ser.

DISCUSSION

By a combination of N terminal sequence analysis with automatic sequanator, cyanogen bromide fragmentation, tryptic and Staphylococcus aureus protease digestion, it was possible to obtain overlapping peptides and to reconstruct the sequence shown in Fig. 1. The cysteines residues are located in positions 8, 11, 14, 18, 42 and 50 instead of 7, 10, 13, 17, 43 and 50 as previously published. From residues 34 to 57, some residues should be mutually displaced or some identification are incorrect. The total number of residues should be 57 instead of 56.

It is to be noted that we have verified that the three oligomeric forms of ferredoxin which have the same amino acid composition, have also the same sequence. The aggregation states in which the three forms were isolated may be due to tertiary or quaternary type interactions. These interactions have an

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 1                5                  10                 15
Pro-Ile-Glu-Val-Asn-Asp-Asp-Cys-Met-Ala-Cys-Glu-Ala-Cys-Val-Glu-Ile-Cys-Pro-
*Pro-Ile-Gln-Val-Asp-Asn-Cys-Met-Ala-Cys-Gln-Ala-Cys-Ile-Asn-Glu-Cys-Pro-Val-

 20                25                 30                 35
Asp-Val-Phe-Glu-Met-Asn-Glu-Glu-Gly-Asp-Lys-Ala-Val-Val-Ile-Asn-Pro-Asp-Ser-
Asp-Val-Phe-Gln-Met-Asp-Glu-Gln-Gly-Asp-Lys-Ala-Val-Asn-Ile-Pro-Asn-Ser-Asn-

     40                45                 50
Asp-Leu-Asp-Cys-Val-Glu-Glu-Ala-Ile-Asp-Ser-Cys-Pro-Ala-Glu-Ala-Ile-Arg-Ser
Leu-Asp-Asp-Gln-Cys-Val-Glu-Ala-Ile-Gln-Ser-Cys-Pro-Ala-Ala-Ile-Arg-Ser
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Fig. 1 - Reexamination of the sequence of D. gigas ferredoxin.
*from Travis et al. (6).

influence in the magnetic properties and the potential redox of the three forms (- 455 mV for Fd I and - 130 mV for Fd II) (4). One of the characteristics of D. gigas ferredoxin is the presence of two residues of cysteine which are not involved in the formation of the 4 Fe-4 S cluster. Some experiments using p-chloromercuribenzoate titrations (2) have shown that they are not free-SH groups. Attempts are being made to localize the cysteine residues that participate in intrachain disulfide bridge, by purification and identification of the peptides obtained by tryptic cleavage of the only lysine residue (number 30) of the molecule. The sequence of the resulting dipeptide attached by the disulfide bridge is

$$\overset{1'}{\text{Pro}}\text{-Ile---} \quad \overset{2'}{} \qquad \text{and} \qquad \overset{31'}{\text{Ala}}\text{-}\overset{32'}{\text{Val}}$$

which shows that the disulfide bridge is localized between the first half and the second half of the molecule. X-ray crystallographic studies on the eight iron ferredoxin from Peptostreptococcus aerogenes (24) have shown that the four iron atoms of one of the clusters are linked to cysteines 8, 11, 14 and 45 and the four iron atoms of the other cluster are linked to cysteines 35, 38, 41 and 18.

In all the sequences studies, it seems that the requirement for formation of a (4 Fe-4 S) cluster is the presence of a sequence Cys-2 a.a.-Cys-2 a.a.-Cys- in the N terminal part of the protein and a sequence Cys-Pro in the second half. If we compare the sequence of D. gigas ferredoxin it appears that by analogy, the cluster would be linked to cysteines 8, 11, 14 and the cysteine 50 which is followed by a proline residue. The disulfide bridge would be between Cysteine 18 and 42 and replaces the second cluster of the ferredoxin of Peptostreptococcus aerogenes.

Good crystals of Fd II have been obtained (L.C. Sieker, private communication) and a preliminary X-ray study is in progress.

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