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THE AMINO ACID SEQUENCE OF DESULFOREDOXIN, A NEW TYPE OF NON HEME IRON PROTEIN FROM DESULFOVIBRIO GIGAS

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SUMMARY

A new type of non heme iron protein called desulforedoxin has been isolated from the sulfate reducing bacterium, Desulfovibrio gigas. The complete amino acid sequence has been established. The 36 amino acid residues of the sequence are aligned with the aid of peptides obtained by cyanogen bromide cleavage and by hydrolysis with a peptidase isolated from Staphylococcus aureus. Desulforedoxin has been described as a non heme iron protein of molecular weight 7,600 with 2 iron atoms linked to eight cysteine residues. In fact, sequence elucidation shows that it consists of a dimer of a peptide containing 36 amino-acids. We do not know whether if each monomer contains 1 iron atom linked to 4 cysteine residues or whether the two iron cross link the two monomers. Additional studies on the elucidation of the structure of this new cluster are presently under study.

The isolation and characterization of electron carrier proteins in sulfate reducing bacteria is far from being completed and the function of these different electron carriers is not well understood (1). Several types of non haem iron proteins have been isolated from Desulfovibrio gigas : a rubredoxin with one iron atom linked in a tetrahedral arrangement to the sulfur of cysteine residues and without labile sulfide (2,3), a ferredoxin containing one four iron-four-sulfur-cluster (2,4) and a molybdenum protein containing two-iron-two-sulfur clusters (5). A new type of non-haem iron protein which we have called desulforedoxin by reference to Desulfovibrio was recently isolated from

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D. gigas (6). It contains two iron atoms supposedly linked to the sulfur of eight cysteine residues and no labile sulfide for a molecular weight of 7,600. Seven amino acids are absent in the amino acid composition (histidine, arginine, tryptophane, serine, proline, isoleucine and phenylalanine). A comparative spectroscopic study of this new protein and of the rubredoxin from D. gigas has been published (7). The optical spectrum of the oxidized form of desulforedoxin is different from that of the rubredoxin. The redox potential is close to that of rubredoxin (- 35 mV). Upon reduction with dithionite there is no contribution to the visible region. The oxidation-reduction process is reversible but the protein is sensitive to repeated redox cycles.

The measurements of the magnetic susceptibility of the new protein suggested the existence of a spin-spin interaction between the two iron centres. The EPR spectrum is much more complex than those of the rubredoxins. Further work is needed to determine how similar the iron sites in the desulforedoxin are to those in rubredoxin.

Sequence determination of this new non haem iron protein allowed a better understanding of the configuration of the iron site and the possible residues involved in the binding of the Fe atom. Comparison of the amino acid sequence desulforedoxin with those of different non haem iron proteins like rubredoxins and ferredoxins could be utilized for the establishment of their phylogenetic relationships (8).

MATERIALS AND METHODS

Purification of D. gigas desulforedoxin. The methods used for growing the bacteria and for the isolation of desulforedoxin have been described previously (2, 6).

Apodesulforedoxin was obtained by precipitating native desulforedoxin with 3 % HCl at 80° C for 10 min.

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 (9). 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 theninhydrine-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 (10) is 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. (11) as modified by Niu and Fraenkel-Conrat (12) and by the use of carboxypeptidase A according to the conditions of Ambler (13, 14).

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. (15). 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 GF 254, Merck) as described by Edman (16, 17) particularly for the identification of Glu/Gln and Asp/Asn residues (18). In some instances, PTH derivatives have been characterized by amino acid analysis after conversion to the parent amino acid by hydrolysis with chlorhydric acid (19) or hydriodic acid (20) for serine residues.

Solid phase Edman degradation. The degradation was performed according to the method of Laursen (21). 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

N terminal sequence analysis. Two sequencer analysis of the desulforedoxin were performed. Oxidized desulforedoxin was subjected to sequential Edman degradation on the Socosi automatic sequencer. In DMBA buffer 32 amino acids were identified.

With the solid phase technique oxidized rubredoxin was coupled through the lysine residues to phenylene diisothiocyanate (DITC). 30 amino acids were

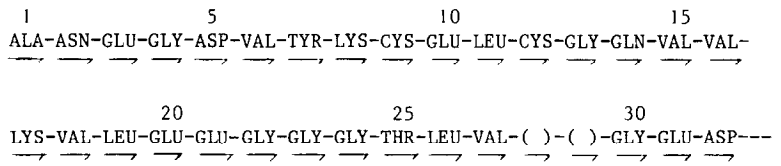

Fig. 1 - N terminal sequence of oxidized desulforedoxin. Parentheses indicate that the residues in these positions could not be identified.

identified from 94 nanomoles of protein. The results are reported in figure 1.

Digestion of protein with carboxypeptidase A. The experimental conditions for carboxypeptidase action are those described by Ambler (13, 14). For reaction with carboxypeptidase, the protein (0.2 µMole) was dissolved in 0.2 ml of 0.2 M N-ethyl morpholine (pH 8.4). After addition of a solution of carboxypeptidase (0.01 ml), the mixture was incubated at 37° C for 3 h with increasing time of incubation and then dried under vacuum.

The amino acids liberated were identified by paper electrophoresis at pH 2.0 or with an amino acid analyzer ; blank digestions were always run simultaneously.

The results are consistent with the following C terminal sequence

...Val-Lys-Gln

Peptides obtained by cyanogen bromide cleavage of desulforedoxin. Desulforedoxin contains only one methionine per chain. The peptides obtained were fractionated by gel filtration on G-25 Sephadex and paper electrophoresis and oxidized by performic acid.

Amino acid compositions of the two CNBr fragments of the protein are presented in Table I and their sequences elucidated by Edman degradation (figure 2).

The peptide CNBr F II was identified as the C terminal region of the molecule, since carboxypeptide released Val-Lys-Gln.

Sequence determination of peptides obtained by hydrolysis with Staphylococcus aureus protease. The peptides obtained by digestion of desul-

TABLE I

Amino acid composition of peptides formed by cyanogen bromide treatment of desulforedoxin

Peptide	Amino acid composition	Total residues	Yield
CnBr F I	Lys_2, Asp_3, H Ser_1, $Thr_{0.9}$, Glu_6, Gly_6, Ala_1, $Cys_{3.8}$, Val_5, Leu_3, $Tyr_{0.8}$	33	45 %
CnBr F II	Val_1, Lys_1, Glu_1	3	27 %

CNBr-F I

1 5 10 15
ALA-ASN-GLU-GLY-ASP-VAL-TYR-LYS-CYS-GLU-LEU-CYS-GLY-GLN-VAL-

19
VAL-LYS-VAL-LEU....

CNBr-F II

VAL-LYS-GLN

Fig. 2 - Amino acid sequence of peptides obtained by cyanogen bromide cleavage of desulforedoxin.

foredoxin with Staphylococcus aureus protease were separated and analyzed by the finger print technique. They are oxidized by the performic acid reagent on paper.

The electrophoretic mobility "m" was measured at pH 6.5 relative to lysine with a positive sign for the basic peptides and relative to aspartic acid with a negative sign for the acidic peptides.

The amino acid analysis of these peptides are presented in Table II. Their sequences were obtained by solid phase method as described under methods or by liquid phase method with DMBA buffer. Sequence studies are summarized in Table III.

TABLE II

Amino acid composition of peptides obtained by hydrolysis with Staphylococcus aureus protease

Amino acid	G-3	G-4a	G-4b	G-4c	G-4d
Lysine	1		1		1
Histidine					
Arginine					
Tryptophane					
Aspartic acid		1	1		1
Threonine				1	1
Serine					
Glutamic acid	2	1	1		3
Proline					
Glycine	1		1	4	4
Alanine		1			
Cystine (Half)*	1		1		2
Valine	3		1	1	2
Methionine*					1
Isoleucine					
Leucine	2			1	1
Tyrosine			1		
Phenylalanine					
Total	10	3	7	15	16
Yield %	53 %	52 %	49 %	17 %	26 %
Purification**	pH 6.5, pH 3.5 and pH 2.0	pH 6.5 and pH 3.5	pH 6.5 and pH 3.5	pH 6.5 and pH 3.5	pH 6.5, pH 3.5 and pH 2.0

Peptides G-4a (residues 1-3) and G-4b (residues 4-10) are the N terminal part of the protein. The order of peptides G-3, G-4d and G-4c was unequivocally established by the fact that the sequence is known up to the 32nd residue by use of the automatic Sequencer. However some residues which had not been identified in the whole protein were characterized. For example the presence of two residues of cysteine in positions 28 and 29 and the sequence of

TABLE III

Amino acid sequence of peptides obtained by hydrolysis with Staphylococcus aureus protease

Peptide	Position in the peptide chain	Sequence
G-3	11-20	Leu-Cys-Gly-Gln-Val-Val-Lys-Val-Leu-Glu
G-4a	1-3	Ala-Asn-Glu
G-4b	4-10	Gly-Asp-Val-Tyr-Lys-Cys-Glu
G-4c	22-36	Gly-Gly-Gly-Thr-Leu-Val-Cys-Cys-Gly-Glu-Asp-Met-Val-Lys-(Gln)
G-4d	21-36	Glu-Gly-Gly-Gly-Thr-Leu-Val-Cys-Cys-Gly-Glu-Asp-Met-Val-Lys-Gln

The half-arrows show the residues identified by using the Protein Sequencer. Parentheses indicate that the residues in these positions could not be identified.

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 1               5                  10                  15
ALA-ASN-GLU-GLY-ASP-VAL-TYR-LYS-CYS-GLU-LEU-CYS-GLY-GLN-VAL-VAL-

              20                  25                  30
LYS-VAL-LEU-GLU-GLU-GLY-GLY-GLY-THR-LEU-VAL-CYS-CYS-GLY-GLU-ASP-

         35
MET-VAL-LYS-GLN
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Fig. 3 - Amino acid sequence of D. gigas desulforedoxin

the C terminal part of the protein (residues 32-36) was established in peptides G-4c and G-4d

DISCUSSION

The total sequence of D. gigas desulforedoxin was elucidated from degradation of the whole protein by the protein sequencer and by elucidation of the sequence of the peptides obtained from the S. aureus protease (figure 3).

However desulforedoxin was described as a non heme iron protein of molecular weight 7,600 and 73 amino acids but the minimal amino acid composition is consistent with 36 amino acids. The molecular weight of desulforedoxin was estimated to be 7,900 by gel filtration on Sephadex G-50. Sequence elucidation shows that desulforedoxin consists of two identical chains of a peptide containing 36 amino acids. In fact cyanogen bromide cleavage, hydrolysis by the peptidase isolated from S. aureus and action of the carboxypeptidase have shown that desulforedoxin is composed of two identical chains of 36 amino acids. Tryptic peptides of oxidized desulforedoxin have been studied with the same result.

When compared to the sequence of rubredoxins, desulforedoxin shows a small amount of homology, only the sequence Tyr-X-Cys-X-X-Cys-Gly is conserved in the N terminal sequence.

The single chain has four cysteines at positions 9, 12, 28 and 29. The various rubredoxins sequenced thus far (22,23,24,25,26) have cysteines at positions 6, 9, 39 and 42 which have been shown to be ligands to the single iron atom. By analogy it can be assumed that Cys 9 and Cys 12 of one chain are possible ligands to one of the iron atoms, as the cysteine residues 28 and 29 are adjacent, it is possible to imagine that the two iron atoms link the two polypeptide chains together as in the manner of the ferredoxins (27). Indeed, being more speculative, it is interesting to note that if cysteines 9, 12 and 28 of the first chain act as ligands to the first iron atom then cysteines 9', 12' and 28' of the second cahin act as ligands to the second iron atom leaving Cys 28 to be the fourth to the second iron atom and Cys 28' to take the remaining fourth position of the first iron atom in direct correspondance to the two 4 (Fe S) cluster liganding arrangement in clostridial ferredoxins. This analogy can be carried further in noting that like most of the clostridial ferredoxins, desulforedoxin has only two aromatic residues and if the chains are arranged antiparallel desulforedoxin can have two fold symmetry in the same manner as the ferredoxin molecule has pseudo two fold symmetry. The cross linking of the

the two iron by the adjacent cysteines of each chain can also position the two iron centers close enough for the spin-spin interaction which has been observed.

The elucidation of the structure of the cluster and the interaction spin-spin between the two clusters of the dimer are presently in study. D. gigas desulforedoxin has been also crystallized by Sieker and an X-ray study of this protein is in progress.

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