ISOLATION AND CHARACTERIZATION OF DESULFOREDOXIN, A NEW TYPE OF NON-HEME IRON PROTEIN FROM *DESULFOVIBRIO GIGAS*

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SUMMARY: *Desulfovibrio gigas* desulforedoxin is a new type of non-heme iron protein of molecular weight 7,900. It contains two iron atoms, no labile sulfide and eight cysteine residues per molecule. The optical spectrum of the oxidized form presents important differences from that of the rubredoxin type proteins. 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 protein contains 73 amino-acid residues. Like rubredoxin it does not contain histidine and arginine but it also lacks proline, isoleucine, phenylalanine, and tryptophan. The N-terminal sequence has been determined up to 35 residues and no evident homology was found with other non-heme iron proteins.

INTRODUCTION

The electron transfer proteins from *Desulfovibrio* species have been recently surveyed (1). The criterium used to see whether electron transfer proteins can be useful as models for the understanding of the bacterial electron transfer chain includes the understanding of their electron redox mechanism and the establishment of their phylogenetic relationships (1).

Proteins with different types of iron-sulfur clusters have been isolated from *D.gigas*: a rubredoxin with one iron atom complexed by four 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).

Physico-chemical characterization of these proteins allowed a better understanding of their redox properties (4,6,7). Structural studies of different oligomeric forms of ferredoxin have shown that the redox properties of the same cluster are regulated by their environment. These studies indicate how the clusters can be used in more complex iron-sulfur proteins

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(e.g., hydrogenase and nitrogenase) in order to accumulate the necessary number of electrons at the potentials needed for the reactions in which they are involved. Desulfovibriones rubredoxin and ferredoxin have been utilized for the establishment of their phylogenetical relationships (8).

In this article a new type of non-heme iron protein is characterized. Its physico-chemical properties are compared with those of other non-heme iron proteins, and in particular with rubredoxin.

EXPERIMENTALS

Isolation of *D.gigas* desulforedoxin - *D.gigas* (N.C.I.B. 9332) was grown in a lactate sulfate medium. A frozen paste (2.7Kg) of cells was treated as previously described in order to obtain an acidic protein extract (9). All the operations were carried out at 0-4°C unless otherwise stated. Tris-HCl and phosphate buffers pH 7.6 of appropriated molarity were used. Gradients were accomplished using NaCl in a 0.01M Tris-HCl buffer medium.

Step I - The acidic proteins extract was dialysed overnight against 20 l of distilled water and adsorbed on two DEAE--cellulose columns (40x350mm) (Whatman DEAE-23) previously treated according to the manufactor indications and equilibrated with 0.01M Tris-HCl. The adsorbed proteins were washed with 200ml of 0.01M Tris-HCl and eluted with a discontinuous gradient of 200ml of 0.10, 0.15, 0.20, 0.30, 0.35, 0.40, and 0.50 M NaCl in 0.01M Tris-HCl. A fraction containing mainly rubredoxin, desulforedoxin, cytochrome *c* (MW 26,000) and desulfoviridin was eluted at 0.25-0.30 NaCl and collected in a volume of 900ml.

Step II - This fraction was dialysed overnight against 10 l of distilled water and adsorbed in the same DEAE-23 columns, previously washed with 1M Tris-HCl and equilibrated with 0.01M Tris-HCl. A fine NaCl gradient was performed using 200ml of the following NaCl molarities in 0.01M Tris-HCl: 0.010, 0.025, 0.050, 0.075, 0.100.....up to 0.500. During elution at 0.25--0.30 NaCl a good separation was achieved between rubredoxin and the more acidic desulforedoxin. This protein fractions were collected in 170ml and 420ml respectively.

Step III - The fraction containing desulforedoxin was applied on a silica (Backer) column (25x270mm) equilibrated with 0.30M NaCl. The fraction not fixed contained the desulforedoxin and traces of desulfoviridin (300ml). It was subjected to another column (35x270mm) equilibrated with 0.30M NaCl.

Step IV - The fraction containing desulforedoxin (500ml) was concentrated in an alumina column (25x100mm) equilibrated with 0.30M NaCl and eluted with 0.01M phosphate buffer to a volume of 50ml.

Step V - Removal of traces of desulfoviridin was achieved by hydroxyapatite chromatography on a 25x50mm column equilibrated with 0.01M Tris-HCl and eluted with 0.01-0.05M phosphate buffer. The homogeneous protein (as judged by polyacrilamide gel electrophoresis) was obtained in a volume of 25ml and exhibits an absorbance ratio of $A_{278}/A_{507} = 1.41$.

Analytical procedures - Iron was determined by atomic absorption spectrophotometry using an Unicam model SP 1900 spectrometer. The inorganic sulfide content was determined by adaptation of

TABLE I: Comparison of molar extinction coefficients of desulforedoxin and rubredoxin from *D.gigas*

Oxidized proteins	(nm)	($M^{-1}cm^{-1}$)	U.V./visible ratio
desulforedoxin	278	9,934	$\frac{A_{278}}{A_{507}} = 1.41$
	285	9,437	
	310	6,540	
	370	11,960	
	507	7,029	
rubredoxin	278	17,210	$\frac{A_{278}}{A_{493}} = 2.47$
	355(s)	7,729	
	376	8,454	
	493	6,966	
	515(s)	3,149	

the Fogo and Popowsky (10) method by Lovenberg et al. (10). The molecular weight of the purified protein was determined by gel filtration on a G-50 Sephadex column, according to Whitaker(12) using the following standards: chymotrypsin (24,000), cytochrome *c* (12,500), *D.vulgaris* cytochrome c_{553} (9,100) and *D.vulgaris* rubredoxin (6,000). The visible and ultraviolet spectra were run on a Cary 14 spectrophotometer.

Amino-acid composition and sequence determination - Protein samples were hydrolyzed in 200 µl of 6M HCl at 110°C for 18 hours according to Moore and Stein (13) in sealed evacuated tubes. Amino acid analyses were carried out on a Beckman Multichrom amino acid analyser. The values of threonine, serine, and tyrosine were corrected after extrapolation to zero time hydrolysis. Cysteine and methionine were analysed after performic acid oxidation as cysteic acid and methionine sulphone respectively (14). Tryptophan was determined by the colorimetric method of Spies and Chambers (15). Sequence determinations were performed on a Socosi Protein Sequencer (SP100). Purified apocytochrome from horse heart is added as a protecting protein during protein degradation in the reaction cup (16). DMBA (N,N, dimethyl--benzylamine) buffer is used instead of quadrol as for other low molecular weight proteins. The concentration of the PTH derivatives was determined by comparison with the known amounts of the appropriate standards on gas chromatography (Beckmann gas chromatograph GC45 using SP400 as stationary phase) according to the technique of Pisano et al. (17). The silylated PTH derivatives were also analysed by thin layer chromatography on silica gel containing an ultraviolet fluorescent indicator (silica gel GF 254 from Merck) as described by Edman (18, 19), particularly for the identification of Lys, Glx, and Asx residues (20). Some residues have been characterized by amino-acid analysis after conversion to the parent amino acid by hydrolysis with HCL (21) or HI (22) for Ser residues.

TABLE II: Amino acid composition and constituentes of desulforedoxin and rubredoxin from *D.gigas*

	D.gigas desulforedoxin				*D.gigas* rubredoxin (3)
	Hydrolysis time			nearest integer	
	24h	48h	72h		
Lysine	6.0	6.0	6.0	6	5
Histidine	0	0	0	0	0
Arginine	0	0	0	0	0
Tryptophan[a]	0	0	0	0	1
Aspartic acid	6.1	6.0	6.1	6	8
Threonine	2.2	1.9	1.7	2	2
Serine	1.0	0.7	0.5	1	2
Glutamic acid	13.9	14.0	13.5	14	4
Proline	0	0	0	0	5
Glycine	10.7	11.5	11.4	11	5
Alanine	2.0	2.2	2.0	2	4
Cystine(Half)[b]				8	4
Valine	11.5	12.2	11.5	12	3
Methionine[b]				2	1
Isoleucine	0	0	0	0	2
Leucine	6.2	6.5	6.8	7	1
Tyrosine	1.8	1.4	1.1	2	3
Phenylalanine	0	0	0	0	2
TOTAL RESIDUES				73	52
MOLECULAR WEIGHT				7,631[c] 7,900[d]	6,567[c]
NUMBER OF IRON ATOMS/MOLECULE				2	1
NUMBER OF LABILE SULFUR ATOMS/MOLECULE				0	0

[a]determined by the colorimetric method of Spies and Chambers(15)
[b]cysteine and methionine were determined after performic oxidation.
[c]minimum molecular weight
[d]molecular weight determined by gel filtration

RESULTS

Molecular weight, iron and labile sulfide analysis - The molecular weight of the purified desulforedoxin was estimated to be 7,900 daltons, by gel filtration on Sephadex G-50. The same result

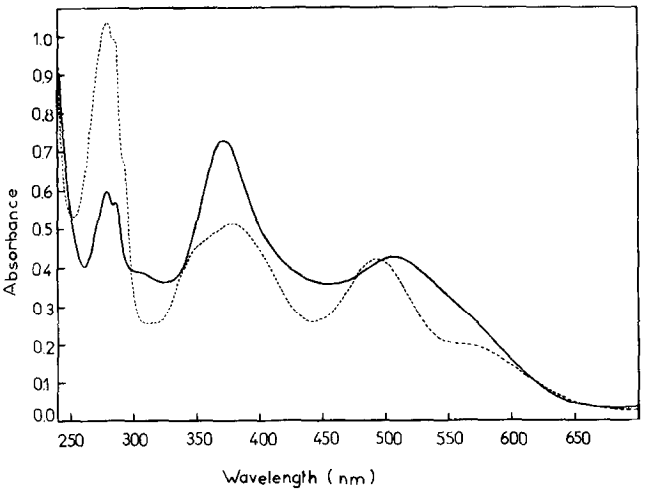

Fig. 1 Ultraviolet and visible spectra of oxidized *D.gigas* desulforedoxin (——) and oxidized *D.gigas* rubredoxin (-----). Spectra were recorded on a Cary 14 spectrometer at pH 7.6, buffered with 0.01M Tris-HCl. Protein concentrations were 6.04×10^{-5}M in both spectra.

is obtained for the apoprotein prepared by precipitation in 3% HCl. Atomic absorption spectroscopy gives 1.6 atoms of iron per molecule. Assays of acid-labile sulfide were performed with negative results.

Amino-acid composition - Table II shows the amino-acid composition of desulforedoxin. It does not contain histidine, arginine, proline, isoleucine, phenylalanine and tryptophan which is very unusual for a protein. It has four cysteine residues per iron atom and a remarkably high content of valine and glutamic residues. The calculations for threonine, serine and tyrosine residues were extrapolated to zero time hydrolysis after several hydrolyses of increasing lenght.

N-terminal sequence analysis - Oxidized desulforedoxin was subjected to sequential Edman degradation on the automatic sequencer. In DMBA buffer 35 of the 73 residues were identified fig. 2). Cys 9 was identified with the amino-acid analyser as cysteic acid by back hydrolysis with HCl.

Physiological activity - The presence of NADH rubredoxin oxido--reductase has been described in *D.gigas* (23). This enzyme shows no activity with desulforedoxin. No other physiological activity has been found for desulforedoxin.

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 1                   5                  10                  15
Ala-Asp-Glu-Gly-Asn-Val-Tyr-Lys-Cys-Glu-Leu-( )-Gly-Gln-Val

                   20                  25                  30
Val-Lys-Val-Leu-Glu-Glu-Gly-Gly-Gly-Thr-Leu-Val-( )-( )-Gly

                   35
Glu-Asp-( )-Lys-Val
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Fig. 2 N-terminal sequence of *D.gigas* desulforedoxin. Parentheses indicate that the residues in these positions could not be identified.

Electronic spectra data - Fig. 1 shows a comparison of the spectra of oxidized *D.gigas* desulforedoxin and rubredoxin at the same concentration. The maxima on visible spectra of desulforedoxin are shifted and the characteristic rubredoxin shoulder at 350nm is not observed in desulforedoxin. A very weak shoulder not shown on fig.1 (50 per ion atom cm^{-1}) is observed at 720nm for oxidized desulforedoxin. The protein absorption in the U.V. regions arises from the tyrosine content of the protein. The fine structure observed is not present in the apo-protein spectrum. The lower contribution to the U.V. absorption spectrum of desulforedoxin is due to the difference in aromatic residues content when compared with rubredoxin. Dithionite reduced desulforedoxin presents no contribution to the visible spectrum. Recovery of 90-95% of the colour was achieved by reoxidation of the protein by air. The reduction of the protein in the presence of ascorbic acid is very slow and the process stops when about 90% of the colour remains. The protein is unstable to repeated oxidation-reduction cycles. Table I shows the absorption bands and molar extinction coefficients and presents a comparison with *D.gigas* rubredoxin. The extinction coefficients were calculated using protein concentrations determined by amino-acid analysis.

DISCUSSION

The amino-acid composition of desulforedoxin (Table I) is very unusual. It does not contain histidine and arginine which are also absent in rubredoxin, nor does it contain proline, isoleucine, phenylalanine or tryptophan. Altogether six types of amino-acid residues are absent. Worth stressing is the absence of proline whose content as been related to the stability of

tertiary structure of proteins (24). Also no evident homology is observed when comparing the N-terminal sequence of previously isolated non-heme iron proteins.

The presence of eight cysteine residues and two iron atoms per molecule, the absence of labile sulfide, and the general features of the electronic spectra shows that the two iron centers should be of the rubredoxin type. However there are small shifts of the maxima of the visible spectra and the chromophore contribution does not account for the double content of iron. In contrast the spectrum of the two iron rubredoxin from *Pseudomonas oleovorans* is the simple addition of two one iron rubredoxin spectra (25). These results would suggest that there is an interaction between the two iron centers and this is now under study. Thus the weak shoulder observed at 720nm ($\varepsilon \simeq 50$ per iron atom cm^{-1}) is comparable to that observed in the case of hemerythrin and in model compounds with two interacting iron atoms and was assigned as a "magnon" d-d band (26). Also preliminary EPR measurements show that the spectrum is more complex than the spectrum of the two-iron rubredoxin (27).

The unusual amino-acid composition, the original N-terminal sequence and the presence of two interacting rubredoxin type iron centers indicate that desulforedoxin belongs to a new class of non-heme iron proteins, which may not be exclusive of *Desulfovibrio* sp.

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