THE AMINO ACID SEQUENCE OF RUBREDOXIN FROM THE SULFATE REDUCING BACTERIUM, DESULFOVIBRIO GIGAS

Mireille BRUSCHI
with the technical assistance of J. Bonicel,
G. Bovier-Lapierre and P. Couchoud
Laboratoire de Chimie Bactérienne, C.N.R.S.,
13274 Marseille Cedex 2, France

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SUMMARY

Determination of the complete amino acid sequence of the rubredoxin isolated from the sulfate reducing bacterium Desulfovibrio gigas showed that the molecule consists of a single polypeptide chain of 52 residues. The sequence of the first 42 residues was determined using an automatic Protein Sequencer. Peptides derived from tryptic hydrolysis and from specific cleavage at tryptophan residue were used to construct the total sequence. Compared with the sequence of Desulfovibrio vulgaris rubredoxin, 37 positions are identical, and with the sequences of Clostridium pasteurianum, Peptostreptococcus elsdenii, Micrococcus aerogenes and D. vulgaris rubredoxins, 20 matching residues occur. A crystallographic study of the D. gigas rubredoxin is in progress.

Rubredoxin, a non-heme iron containing protein was first isolated from Clostridium pasteurianum by Lovenberg et al. (1) and observed in many other anaerobic bacteria. Like bacterial ferredoxin, it has been described as a low molecular weight protein (6 000) but contains one iron atom per molecule and lacks acid-labile sulfide. Rubredoxins isolated from anaerobes have similar absorption spectra with an absorption maximum at 490 nm. The complete amino acid sequences of rubredoxins from several anaerobic bacteria : C. pasteurianum (2), Micrococcus aerogenes (3) and Peptostreptococcus elsdenii (4) have been reported. An NADH + H^+ rubredoxin oxydo reductase has been described by Le Gall in Desulfovibrio gigas (5) ; but the specific function of rubredoxin in the metabolism of anaerobic bacteria is not yet clear.

A 20,000 molecular weight rubredoxin isolated from the aerobic bacterium Pseudomonas oleovorans (6) was shown to be involved in the hydroxylation of hydrocarbons.

In the present communication, we report the complete amino acid sequence of D. gigas rubredoxin.

The sequence of the rubredoxin of another sulfato reducing bacterium D. vulgaris has been elucidated and a comparison of the amino acid sequences of these two homologuous proteins could give information on the evolution of this protein especially by comparison with the other rubredoxins so far studied. Additionally it is of use in the study of the configuration of the active site and the specific residues involved in the binding of the metal.

MATERIALS AND METHODS

The cultivation of D. gigas and the purification of the rubredoxin have been previously described (8, 9).

Preparation of carboxymethyl derivative of rubredoxin. The protein was reduced and denatured under N_2 in 1 M Tris-HCl pH 8.6 containing 8 M urea and mercaptoethanol according to the procedure of Crestfield et al. (10).

Enzymatic hydrolysis. Trypsin digestion was carried out at 37° C on the S-carboxymethylated protein during 3 hours. The protein was dissolved in ammonium acetate solution pH 8.5 and a freshly prepared aqueous solution of TPCK-trypsin (10 mg/ml) was added. The enzyme to substrate ratio was 1:50.

Specific cleavage of tryptophan residue. The single tryptophanyl bond of the protein was cleaved according to the method of Omenn et al. (11). 2 µmoles of the protein were dissolved in 80 % formic acid (20 mg protein/ml) and 50 equivalents of BNPS-Skatole purchased from Pierce Chemical Co. were added. After 30 min. at room temperature, the mixture was diluted with water to lower the formic acid concentration to 70 %. Mercaptoethanol (0.1 ml/ml solution) was added to destroy the excess reagent and to reduce methionine sulfoxide residues. After reaction overnight at room temperature the mixture was diluted twofold with water and extracted with 1 ml portions of ethyl acetate. The peptides in the aqueous layer were fractionated by gel filtration on Sephadex G-25 equilibrated with 50 % acetic acid.

Purification of peptides. Peptides were purified by high voltage paper electrophoresis 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 the paper by means of the ninhydrin reagent.

Amino acid composition and sequence analysis. Protein and peptide samples were hydrolyzed in 200 µl of 6 M HCl at 110° C for 18 hours in sealed evacuated tubes. A Beckman amino acid analyzer (Multichrom model) was employed. Sequence determinations were performed in the Socosi Protein Sequencer (P.S. 100). The reagents used were further purified. D.M.B.A. (N.,N., dimethylbenzylamine) buffer appeared to be suitable for peptides and low molecular weight proteins like rubredoxins. The quantitative determination of the PTH derivatives was done relative to known amounts of the appropriate standards on gas chromatography (Beckman gas chromatography GC 45 using SP 400 as stationary phase according to the technique of Pisano et al. (12). 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 (13, 14) particularly for the identification of lysine, Glu/Gln and Asp/Asn residues (15). In some instances, PTH derivatives have been characterized by amino acid analysis after conversion

to the parent amino acid by hydrolysis with chlorhydric acid (16) or iodhydric acid (17) for serine residues. The carboxy-terminal amino acid of the protein was determined by the hydrazinolysis procedure of Akabori (18) as modified by Niu and Fraenkel-Conrat (19) and by the use of carboxypeptidase A according to the conditions of Ambler (20, 21).

RESULTS

Amino acid composition and end groups. The amino acid composition of D. gigas rubredoxin was reported in 1969 by Laishley et al. (22). The amino acid composition of the S carboxymethylated rubredoxin prepared here was shown to contain 52 residues. The four half-cystine residues were determined as S-carboxymethylated cysteine.

Edman degradation of the protein suggested the presence of an N terminal blocking group. As the N terminal methionine of the C. pasteurianum rubredoxin is formylated (23), prior deformylation in 1 M HCl in methanol (24) was necessary before degradation could be initiated. Methionine was shown to be the N terminal residue. Glutamine was identified as the COOH terminal residue by carboxypeptidase A digestion.

N terminal sequence determined by using the automatic Protein Sequencer. The most satisfactory results were not obtained after deformylation of the N terminal residue or after preparation of an S carboxymethyl derivative of the protein but were obtained after cyanogen bromide cleavage of the N terminal methionine. The reaction was carried out in 70 % formic acid, overnight at room temperature, at a protein concentration of 10 mg/ml. In DMBA buffer, 42 of the 52 residues were identified (fig. 1) using an automatic Protein Sequencer. The cystein residues 6 and 9 in the sequence were identified with the amino acid analyzer as cysteic acid by back hydrolysis using a sample of oxidized rubredoxin. The serine residue in position 22 was recovered after hydrolysis with iodhydric acid.

Sequence determination of the tryptic peptides of reduced ans S carboxymethylated rubredoxin. The amino acid compositions of the tryptic peptides obtained after reduction and S carboxymethylation of the protein are summarized in Table I.

Peptide T-2b (residues 1-17), the N terminal peptide of the protein whose sequence was established using the automatic Sequencer, was only characterized by its amino acid composition. The order of peptides T-2b (residues 1-17), T-2a (residues 18-29), T-3b (residues 30-46) and T-4 (residues 47-51) was unequivocally established by the fact that the sequence is known up to the 42nd residue by use of the automatic Sequencer. However some residues which had not been identified on the whole protein were characterized in the corresponding peptides. For example the presence of the tryptophan

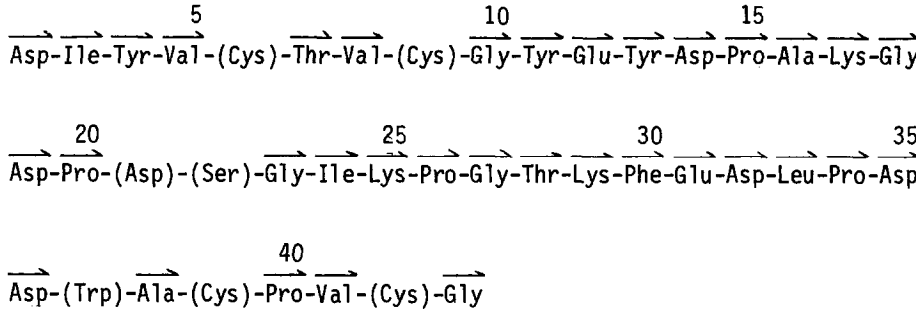

Figure 1. N terminal sequence of rubredoxin subjected to the CNBr cleavage reaction. The upper half-arrows show the residues identified by using the Protein Sequencer. Parentheses indicate that the residues in these positions could not be identified.

residue in position 37 was established by thin layer chromatography and by gas chromatography. Peptide T-4 is in the C terminal part of the protein. The sequence of the various peptides is given in Table II.

Specific cleavage of the tryptophan residue in position 37. The reaction with BNPS Skatole was used to cleave the only tryptophanyl peptide bond of the protein. The C terminal peptide (residues 38-52) was purified by filtration on Sephadex G-25 (50 % acetic acid) and high voltage paper electrophoresis. The yield of the purified peptide was 15 %. The sequence of the peptide, established by the Protein Sequencer, is given in Fig. 2.

DISCUSSION

The total sequence of *D. gigas* rubredoxin was elucidated from degradation of the protein by the Protein Sequencer which established the first 42 residues from the NH_2-terminal end and from the peptide (38-52) obtained from specific cleavage of the only tryptophan residue of the protein. However, the preparation of the tryptic peptides of the protein was necessary for characterization of some residues which could not be identified on the whole protein, particularly tryptophan in position 37, and for confirmation of threonine and serine residues.

The complete amino acid sequence is reported in fig. 3. The iron is attached to the cystein residues in positions 6, 9, 39 and 42. The 4 cystein residues involved in the binding of the iron in the various rubredoxins which have been sequenced thus far *D. vulgaris*, *C. pasteurianum*, *P. elsdenii* and *M. aerogenes*, are in identical positions. A comparison of the rubredoxins of the sulfate reducing bacteria *D. vulgaris* and *D. gigas* showed that

Table I : Effect of 300 µM SIBA on Con A and NWSM stimulated cells, when added after the mitogen

Time of addition* hrs	% inhibition			
	human cells		rabbit cells	
	Con A	NWSM	Con A	NWSM
3	-	-	98	84
6	-	-	98	73
12	-	-	97	66
24	88	37	93	66
48	83	24	90	58
72	75	47	81	0
96	56	63	-	-

* Delay between the addition of the mitogen and the inhibitor

bition was observed with both 100 and 300 µM SIBA when lymphocytes were stimulated by Con A, NWSM and PG whereas PHA treated cultures, were inhibited only with 300 µM SIBA (83 % inhibition). The effect of the compound on untreated cells was the same as in the case of human lymphocytes.

Inhibition of blastogenesis by 300 µM SIBA added after the mitogens

As shown in Table 1 Con A induced blast response was severely impaired even when SIBA was added 3 days after the lectin to the human or rabbit lymphocytes (75 % and 81 % inhibition respectively). NWSM induced blastogenesis of human lymphocytes was the most strongly inhibited when SIBA was added to the cells the third or fourth day of stimulation, while the action of the compound on NWSM stimulated rabbit cells decreased upon addition of the drug later than 48 hours after the mitogen.

Recovery of the mitogen response after preincubation with SIBA

Human rabbit or lymphocytes were treated for 24 hours with 300 µM SIBA, then washed three times to eliminate the inhibitor and

TABLE II

Amino acid sequences of tryptic peptides of reduced and S-carboxymethylated rubredoxin

Peptide	Position in the peptide chain	Sequence
T-2b	18-29	Gly-Asp-Pro-Asp-Ser-Gly-Ile-Lys-Pro-Gly-Thr-Lys
T-3b	30-46	Phe-Glu-Asp-Leu-Pro-Asp-Asp-Trp-Ala-(Cys)-Pro-Val-(Cys)-Gly-Ala-(Ser)-Lys
T-4	47-51	Asp-Ala-Phe-Glu-Lys

Ala-Cys-Pro-Val-Cys-Gly-Ala-Ser-Lys-Asp-Ala-Phe-Glu-Lys-(Gln)

Figure 2. Amino acid sequence of the C terminal peptide (residues 38-52) of the D. gigas rubredoxin. The upper 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
Met-Asp-Ile-Tyr-Val-Cys-Thr-Val-Cys-Gly-Tyr-Glu-Tyr-Asp-Pro-Ala-Lys-Gly-

     20                  25                  30                  35
Asp-Pro-Asp-Ser-Gly-Ile-Lys-Pro-Gly-Thr-Lys-Phe-Glu-Asp-Leu-Pro-Asp-Asp-

             40                  45                  50
Trp-Ala-Cys-Pro-Val-Cys-Gly-Ala-Ser-Lys-Asp-Ala-Phe-Glu-Lys-Gln-
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Figure 3. Amino acid sequence of D. gigas rubredoxin

sulfate reducing bacteria and those of C. pasteurianum, P. elsdenii and M. aerogenes will be presented elsewhere.

D. vulgaris and D. gigas rubredoxins have been crystallized and an X-Ray study of the two proteins is in progress. This will allow a comparison with the tertiary structure of C. pasteurianum rubredoxin and give further valuable information on this class of non-heme iron proteins.

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REFERENCES

1. Lovenberg, W. and Sobel, M. (1971) Proc. Nat. Acad. Sci. U.S., 54, 193-199.
2. Bornstein, P. (1969) Biochem. Biophys. Res. Commun., 36, 957-964.
3. Bachmayer, H., Benson, A.M., Yasunobu, K.T., Garrard, W.T. and Whiteley, H.R. (1968) Biochemistry, 7, 986-996.
4. Bachmayer, H., Yasunobu, K.T., Peel, J.L. and Mayhew, S. (1968) J. Biol. Chem., 243, 1022-1030.
5. Le Gall, J., (1968) Ann. Inst. Pasteur, 114, 109-115.
6. Peterson, J.A., Basu, D. and Coon, M.J. (1966) J. Biol. Chem., 241, 5162-5164.
7. Bruschi, M., Biochim. Biophys. Acta (submitted).
8. Le Gall, J., Mazza, G. and Dragoni, N. (1965) Biochim. Biophys. Acta, 99, 385-387.
9. Le Gall, J. and Dragoni, N. (1966) Biochem. Biophys. Res. Commun., 23, 145-149.
10. Crestfield, A.M., Moore, S. and Stein, W.H. (1963) J. Biol. Chem., 238, 622-627.
11. Omenn, G.S., Fontana, A. and Anfinsen, C.B. (1970) J. Biol. Chem., 245, 1895.
12. Pisano, J.J., Bronzert, T.J. and Brewer, H.B. Jr. (1972) Anal. Biochem., 45, 43-59.
13. Edman, P. and Sjoquist, J. (1956) Acta Chem. Scand., 10, 1507-1509.
14. Edman, P. and Begg, G. (1967) Europ. J. Biochemistry, 1, 80-91.
15. Edman, P. (1970) in "Protein Sequence Determination". S.B. Needleman, Ed., p. 211-255, Springer Verlag, Berlin.
16. Van Orden, H.O. and Carpenter, F.H. (1964) Biochem. Biophys. Res. Commun., 14, 399-403.
17. Smithies, O., Gibson, D., Fanning, E.M., Goodfliesh, R.M., Gilman, J.G. and Ballantyne, D.L., (1971) Biochemistry, 10, 4912-4921.
18. Akabori, S., Ohno, K. and Narita, K. (1952) Bull. Chem. Soc. Japan, 25, 214-218.
19. Niu, C.J. and Fraenkel-Conrat, H. (1955) Biochim. Biophys. Acta, 16, 597-598.
20. Ambler, R.P. (1963) Biochem. S., 89, 349-378.
21. Ambler, R.P. (1967) Methods in Enzymology, Vol. XI, 436-445.
22. Laishley, E.J., Travis, J. and Peck, H.D. Jr., (1969) J. Bacteriol., 98, 302-303.
23. Kenneth, F., Mc Carthy and Walter Lovenberg (1970) Biochem. Biophys. Res. Commun., 40, 1053-1057.
24. Sheehan, J.C. and Yang, D.D.H. (1958) J. Am. Chem. Soc., 80, 1154-1158.