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ISOLATION AND CHARACTERIZATION OF A MOLYBDENUM IRON-SULFUR PROTEIN FROM DESULFOVIBRIO AFRICANUS

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SUMMARY

A molybdenum-containing iron-sulfur protein has been isolated from the sulfate reducer Desulfovibrio africanus. The protein appears to be a complex protein of high molecular weight (112,000) composed of 10 subunits (mol. wt. 11,500) and containing a high amount of molybdenum (5-6 atoms/mole) with approx. 20 atoms each of iron and labile sulfide. The spectrum shows peaks at around 615, 410 and 325 nm with a protein peak at 280 nm. Its millimolar extinction coefficients at 615, 410 and 280 nm are 48.4, 64.4 and 141 respectively. The protein contains 106 amino-acid residues per subunit of mol. wt. 11,262 and the number of cysteine residues is 2 per subunit. The N-terminal sequence which has been determined up to 26 residues is characterized by its high degree of hydrophobicity.

INTRODUCTION

The sulfate-reducing bacteria belonging to the genus Desulfovibrio include a great number of non-sporulating microorganisms which exhibit a strictly anaerobic mode of growth based on reduction of sulfate as the terminal electron acceptor (1). Desulfovibrio africanus strain Benghazi has been reported by Campbell et al. (2) to be in most respects typical of a Desulfovibrio. In conflict with the original description of this species, Jones (3) has claimed that the flagellation of D. africanus is bipolar and has found that in addition to cytochrome c_3 and desulfoviridin it contains b- and d-type cytochromes. No further studies on the electron carriers of D. africanus have been reported so far. It was thus of biochemical interest to investigate its electron carriers to see if differences

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could be found between D. africanus and other strains of Desulfovibrio. During the purification of the acidic electron carriers (ferredoxin and rubredoxin) and the c-type cytochromes (4) from this organism, we separated a blue-green protein which proved to contain molybdenum and iron-sulfur centers. In this study we report the isolation and some physicochemical properties of the protein as well as the sequence data of its N-terminal end.

MATERIALS AND METHODS

D. africanus strain Benghazi, NCIB 8401, was grown at 32° C with the medium of Starkey (5).

Protein determination. Protein was measured by the Lowry method (6) using dried bovine serum albumin as a standard.

Determination of molecular weight. The molecular weight of the protein was determined by gel filtration on a Sephadex G-150 column following the procedure of Whitaker (7). The minimum molecular weight of the protein was determined from the amino-acid composition.

Acrylamide gel electrophoresis. Analytical gel electrophoresis was performed in 7 per cent polyacrylamide gel with Tris-HCl glycine buffer at pH 8.8. SDS-polyacrylamide gel electrophoresis was carried out using the method of Weber and Osborn (8).

Spectrophotometric studies. The absorption spectra were recorded with a Cary 14 spectrophotometer.

Metal and acid-labile sulfide determination. Non-heme iron in the protein was measured by atomic absorption spectrometry using an Unicam model SP spectrometer as well as by the o-phenanthroline method (9). Molybdenum was estimated by the dithiol method (10) whereas the copper content was investigated by the procedure of Peterson and Bollier (11). Labile sulfide was assayed by the method of Fogo and Popowsky (12) as modified by Lovenberg et al. (13).

Amino acid analysis. Amino-acid analysis were performed on the Mo-Fe-S protein samples hydrolyzed for 20 h, 48 h and 70 h at 110° C in 6 M HCl in evacuated sealed tubes according to the method of Moore and Stein (14). The average was calculated from several analysis. The amino-acid composition was determined with a LKB 3201 amino acid analyzer. Cysteine and methionine were analyzed after performic acid oxidation as cysteic acid and methionine sulfone, respectively, according to Hirs (15).

N-terminal sequence determination. Sequence analysis by automated Edman degradation were performed both in a Socosi Protein Sequencer (P.S. 100) and in a Beckman sequencer (890 C) using the standard quadrol double-cleavage program (16). Dimethylbenzylamine buffer was also used (17). The quantitative determination of the PTH derivatives was done relative to known amounts of the appropriate standards on gas-chromatography GC45 using SP400 as stationary phase according to Pisano et al. (18). An ana-

lysis of the silylated PTH amino acids was always performed. PTH amino-acids were also characterized by thin-layer chromatography on silica-gel containing an ultraviolet fluorescent indicator (Silica Gel GF254, Merck) as described by Edman (19-21). In some instances, PTH derivatives have been characterized by amino-acid analysis after conversion to the parent amino-acid by hydrolysis with chlorhydric acid (22) or hydriodic acid (23) for serine and threonine residues.

After denaturation of the protein in 8M urea under nitrogen at + 4° C, the cysteine residues of the protein were converted to S-amino-ethylated cysteines after reduction with dithiothreitol and reaction with ethyleneimine according to Keresztes-Nagy and Margoliash (24). The content of S-aminoethylated cysteine was determined by automatic amino-acid analysis.

RESULTS

1 - Isolation of the Mo-Fe-S protein

In this purification procedure all operations were carried out at 0-4° C unless otherwise stated and Tris-HCl and phosphate buffers pH 7.6 of appropriate molarity were used.

For preparation of the crude extract, 1,500 g (wet weight) of bacterial paste were suspended in 950 ml of 10 mM Tris-HCl. After addition of a few deoxyribonuclease crystals, the cell suspension was passed once through a French pressure cell. The resulting extract was centrifuged at 38,000 x g for 30 min. and the pellet was discarded. The crude extract (1,620 ml) was subsequently centrifuged for 2 h at 140,000 x g and the supernantant which contained the soluble protein was collected. The resulting soluble protein fraction (1,180 ml, 39 mg/protein/ml) was passed through a calcinated alumina column (5 x 10 cm) equilibrated with 10 mM Tris-HCl to remove the c-type cytochromes. A blue-green protein band corresponding to the Mo-Fe-S protein was adsorbed at the top of the column with the cytochromes. The column was washed with 200 ml of 10 mM Tris-HCl and the Mo-Fe-S protein was then eluted together with the cytochromes with 1 M phosphate buffer. The resulting fraction (376 ml, 21 mg protein/ml) was dialyzed overnight against distilled water and after centrifugation, the dialysate (724 ml) was passed through a column of DEAE-cellulose (4 cm x 12 cm) previously equilibrated with 10 mM Tris-HCl. This step allowed the separation of the Mo-Fe-S protein from an acidic cytochrome. The fraction containing the

protein (836 ml, 8 mg protein/ml) exhibits at this stage a purity ratio A_{280}/A_{615} close to 10.5. It was then concentrated to 250 ml in a 350 ml ultrafiltration cell with a PM-10 filter (Amicon). Further purification of the protein was obtained from 50 ml of the concentrated fraction by filtration on Ultrogel AcA 4/4 column (5 x 100 cm) equilibrated with 20 mM Tris-HCl buffer. During this step the Mo-Fe-S protein was separated from a basic cytochrome c of low molecular weight. The fraction containing the protein (170 ml, 0.5 mg/ml) which has a purity ratio of 4.4 was then passed successively through a DEAE-cellulose column (2 x 8 cm) and a small silica gel column (2 x 3 cm). After chromatography on silica gel which was used to remove traces of the basic cytochrome still present, the Mo-Fe-S protein fraction (160 ml, 0.2 mg protein/ml) was finally adsorbed on a calcinated alumina column (3 x 5 cm) equilibrated with 20 mM Tris-HCl. After a non-linear gradient of phosphate buffer (20 → 100 mM) the protein was eluted from the column with 1 M of the same buffer. At this stage the Mo-Fe-S protein (14 ml, 2.1 mg protein/ml) which exhibits an intense blue-green colour, was judged to be pure both from its spectrum (A_{280}/A_{615} = 2.93) and by determination of an unique N-terminal amino-acid by dansylation and Edman degradation.

In order to remove the salt from the protein solution for further studies, it was dialyzed against 2 liters of distilled water and stored at - 20° C. This purification procedure gives a yield of 30 mg of protein from 300 g (wet weight) bacteria.

2 - Properties of the Mo-Fe-S protein

Polyacrylamide gel analysis : When subjected to polyacrylamide gel electrophoresis, the purified Mo-Fe-S protein shows before staining the presence of two blue protein bands which are close to each other. No other protein bands appear after staining the gels by Coomassie blue.

SDS-gel electrophoresis of the Mo-Fe-S protein was performed in the presence and absence of β-mercapto-ethanol. In these conditions and after

different times of incubation with 1 % SDS and 1 % β-mercapto-ethano, we always observed three bands of proteins of molecular weights approximatively 11,500, 24,000 and 47,000. These results suggest that the protein consists of several subunits probably of the same size (mol. wt. 11,500) and that in our experimental conditions the dissociation of the Mo-Fe-S protein is not complete.

Molecular weight : The molecular weight of the purified Mo-Fe-S protein was estimated to be 112,000 by gel filtration on Sephadex G-150. From the value of the molecular weight, the amino-acid composition and the data of SDS-gel electrophoresis we can postulate that the Mo-Fe-S protein of D. africanus probably consists of about 10 subunits of molecular weight 11,500.

Molybdenum, iron and labile sulfide analysis : The protein obtained in the final step of purification contained 5-6 atoms of molybdenum per molecule. The iron content of the protein has been estimated to be 21-23 atoms per molecule both by atomic absorption spectroscopy and by the o-phenanthroline method, while the amount of acid-labile sulfide in the protein has been found to be 21-22 atoms per molecule. The composition of the Mo-Fe-S protein is summarized in Table 1.

Other components : Assays for copper and manganese were performed with negative results. The spectrum of the Mo-Fe-S protein and the fluorescence assay of its acid-treated form did not indicate the presence of a flavin.

Absorption spectrum : The UV-visible absorption spectrum of the purified Mo-Fe-S protein is presented in Figure 1. The protein exhibits absorption peaks at around 615 nm, 410 nm and 325 nm with a small absorption band at around 525 nm. In the ultraviolet region, it has a protein peak at 280 nm with a shoulder at 290 nm. Its millimolar extinction coefficients at 615, 410 and 280 nm are 48.4, 64.4 and 141 respectively. The absorption peaks at 410 and 325 nm suggest the presence of 4 Fe-4 S type centers in

TABLE 1 - Amino-acid composition of the Mo-Fe-S protein from D. africanus

Lysine	9
Histidine	1
Arginine	6
Tryptophan	ND
Aspartic acid	9
Threonine	5
Serine	3
Glutamic acid	15
Proline	7
Glycine	11
Alanine	10
Cystine (half)[a]	2
Valine	4
Methionine[a]	4
Isoleucine	4
Leucine	9
Tyrosine	3
Phenylalanine	4
Total residues	106
Molecular weight	11,262[b] 112,000[c]
Number of iron atoms/molecule	21-23
Number of labile-sulfide groups/molecule	21-22
Number of Molybdenum atoms/molecule	5-6

[a]Cysteine and methionine were determined after performic oxidation
[b]Minimum molecular weight
[c]Molecular weight determined by gel filtration.

the protein. No spectral change occurs after addition of dithionite or ferricyanide.

Amino-acid composition : Table 1 shows the amino-acid composition of the Mo-Fe-S protein. It contains all the amino-acid residues. The number of cysteine residues which is 2 per subunit of mol. wt. 11,262 is similar to the number of iron atoms and acid-labile sulfide groups per molecule. The

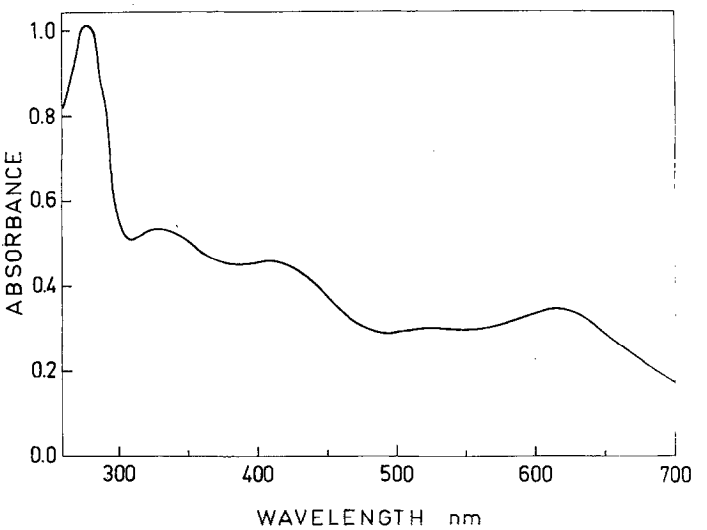

Figure 1 - Absorption spectrum of D. africanus Mo-Fe-S protein, 0.8 mg/ml, in 50 mM phosphate buffer (pH 7.6).

calculations for threonine, serine and tyrosine residues were extrapolated to zero time hydrolysis after several hydrolysis times of increasing length.

N-terminal sequence analysis : Several runs were performed on the apoprotein after removal of the Fe-S clusters by precipitation with 3 % HCl during 10 minutes at 80° C, or on the oxidized Mo-Fe-S protein or on the S amino ethyl derivative of the protein. The most satisfactory results were obtained with the apoprotein. In quadrol buffer, twenty six residues were identified ; the results are reported in Figure 2. A characteristic of this N-terminal sequence is its high degree of hydrophobicity.

Physiological role : The purified molybdenum-containing iron-sulfur protein was tested for different enzymatic activities. The assays for formate dehydrogenase, pyruvate dehydrogenase and thiosulfate reductase activities were performed with negative results. The function of the protein therefore remains unknown.

DISCUSSION

The molybdenum-iron-sulfur protein from D. africanus appears to be a complex protein of high molecular weight (112,000) consisting of a number

5 10 15
Gly-Lys-Ile-Glu-Asp-Ile-Lys-Lys-Arg-Gly-Val-Leu-Met-Ala-Gly

20 25
Val-Lys-Asp-( )-Glx-Glx-( )-Phe-Gly-Phe-Val

Figure 2 - N-terminal sequence of D. africanus Mo-Fe-S protein. Brackets indicate that the residues in these position could not be identified.

of subunits (mol. wt. 11,500) and containing a high amount of molybdenum (5-6 atoms/mole). The molar ratio Mo/Fe-S in the protein is approximatively 1/4. The optical spectrum of the protein which exhibits absorption peaks at 400 and 325 nm is characteristic of an iron-sulfur protein containing probably four iron-four sulfur type centers however the protein appears not to be reducible by dithionite. On the other hand, the absorption peak in the 615 nm region and the small absorption band around 525 nm have not been reported so far to be related to iron-sulfur proteins or to molybdenum-containing iron-sulfur proteins. It is to point out that a freshly prepared solution obtained by mixing a ferrous salt with an excess of sodium sulfide (molar ratio Na_2S/Fe^{++} = 60) gives spectral properties very similar to that of the Mo-Fe-S protein from D. africanus but with a slight shift of the maxima absorption to the red (630, 525 and 430 nm). This mixture which do not contain β-mercaptoethanol differs from the complex used by Hong and Rabinowitz (25) for the reconstitution of clostridial ferredoxin from apoferredoxin. On the other hand, the absorption spectrum of the Mo-Fe-S protein differs from that of the iron-sulfide chelates of some sulfur-containing peptides (26) by the presence of an absorption peak at 615 nm with a small absorption band in the 525 nm region. These data and the non reducibility of the protein by dithionite could indicate that the iron of the Fe-S centers is stabilized in the ferrous state.

The main characteristic found by the N-terminal amino-acid analysis of the protein is its high content of hydrophobic amino-acids. Sufficient

data are not available to conclude whether this is a new type of iron-sulfur cluster or not since the ratio of cysteine/iron per molecule is similar to the four iron-four sulfur cluster of Clostridial ferredoxins. However, it is to be noted that the Mo-Fe-S protein isolated from D. africanus differs from the other molybdenum-containing iron-sulfur proteins previously reported (27, 30). It differs also from the Mo-Fe-S protein isolated recently from D. gigas (31) by its spectral properties and its high content of molybdenum. The protein reported here is present in high amounts in D. africanus and has not been reported to be present in the other species of Desulfovibrio except Desulfovibrio salexigens which appears to contain a similar Mo-Fe-S protein (J. Le Gall, personal communication).

The physiological role of the Mo-Fe-S protein remains unknown. However, we can postulate from the oxido-reduction behaviour of the protein and from its spectral similarity with the iron-sulfide inorganic compound prepared by mixing a ferrous salt with an excess of sodium sulfide that it could function in the cell as an active iron-sulfur donor for the biosynthesis of ferredoxin or other iron-sulfur proteins. Further studies are required to clarify the structure of the active sites of this protein as well as its biological activity.

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