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A COBALT CONTAINING PROTEIN ISOLATED FROM *Desulfovibrio gigas*, A SULFATE REDUCER

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SUMMARY

A protein which contains a cobalt porphyrin was isolated from the sulfate reducer *Desulfovibrio gigas*. This protein has a molecular weight of approximately 16,700 daltons and is acidic, having an iso-electric point at 3.7. The N-terminal residue was shown to be threonine, and a cobalt analysis gave 0.8 cobalt atoms/molecule, suggesting the presence of a single prosthetic group. The protein has a violet color with absorption bands typical of a metal porphyrin center with maxima at 420 nm, 580 nm with a shoulder at 550 nm. The ratio $A_{420}(\gamma)/A_{588}(\alpha)$ is 2.1. The protein has no electron paramagnetic resonance (e.p.r.) spectrum, and as the visible spectrum suggests, it probably contains diamagnetic Co^{III} porphyrin. However the cobalt centre appears to be protected from reduction by sodium dithionite or sodium borohydride. Attempts at ligand substitution with strong nucleophiles such as CN^-, causes a slight spectral shift to higher wavelenghts. The cobalt porphyrin can be extracted from the protein with an acidified acetone solution, indicating that it is not covalently bound to the protein.

INTRODUCTION

Sulfate reducing bacteria are representative of a group of organisms which are considered primitive on the evolution scale (1,2). However, many of the proteins involved in sulfate "respiration" are complex, and from the electron transfer point of view are highly sophisticated. For example, a number of complex electron carrier proteins have been isolated from *Desulfovibrio gigas* e.g. the four-iron four sulfur containing proteins, ferredoxin (3), hydrogenase (4), the rubredoxin type protein (5,6), the multi-heme proteins (7,8) and a complex

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protein containing molybdenum, iron and sulfur (9). In this communication we report the isolation of a protein which contains a cobalt-porphyrin. This violet colored protein was isolated from acidic protein fractions of extracts of *D.gigas*

MATERIALS AND METHODS

Organism and growth conditions - *D.gigas* (NCIB 9332) was grown on a lactate sulphate medium as previously described (7).

Acrylamide gel electroforesis and electrofocussing - Analytical gel electroforesis was performed according to the method of Davis (10) on 7% (v/v) gels at pH 8.8. Analytical thin layer electrofocussing in polyacrylamide gel was used to estimate the isoelectric point and purity of the Co-protein on a LKB Multiphor apparatus (11). A pH gradient between 2.5 and 6.0 was achieved by using anpholines.

Molecular weight determinations - 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: chymotripsin (M_r 24,000) soybean trypsin inhibitor (M_r 21,000), horse heart cytochrome (M_r 12,500) cytochrome *c* 553 (M_r 9,000) and *D.vulgaris* rubredoxin (M_r 6,000).

Protein determination - Protein was measured by the Lowry method (13) using bovine serum albumin as a standard or determined from amino acid analysis (see below).

Metal content determination - Iron, copper, manganese and cobalt were assayed on the purified protein by atomic absorption spectroscopy using a Unicam model SP 1900 spectrometer.

Amino acid analysis and identification of the N-terminal residue - - Amino acid analysis were carried out on a LKB amino acid analyser. Protein samples were hydrolyzed in 6 M HCl at 110°C for 20 h in evacuated, sealed tubes according to the method of Moore and Stein (14). Cysteine and methionine were analysed after performic oxidation as cysteic acid and methionine sulfone respectively, according to Hirs (15). The NH_2-terminal residue of the purified protein was identified as a fluorescent derivative of 5-dimethylamino-1-naphtalene sulphonyl chloride (dansyl chloride) according to the procedure of Gray and Hartley (16). The dansyl amino acids were identified by thin layer chromotography on polyamide layers (F 1700 Schleicher and Schull).

Optical spectra and molar extinction coefficients - Ultraviolet and visible spectra were recorded on a Cary 14 spectrophotometer (Varian Associates, Palo Alto, California, USA). The molar extinction coefficients were obtained by measuring the values of optical densities at the absorption maxima of a solution of known protein concentration. The molarity of the protein solutions was determined from the amino acid analysis.

Reactivity and extraction of the metal center - The reactivity of the metal center was tested against the reducing agents (sodium dithionite, and borohydride) and external ligands (mercuric acetate, methyl mercury, CO, iodine and cyanide). The metal center was extracted with 9 volumes acidified acetone (5%(v/v)) per volume of protein solution at 4°C (17). 100 M protein solutions were used in the extraction procedures. The precipitated apo-protein was removed by centrifugation and the extracted solution was concentrated by evaporation under vacuum.

E.p.r. spectroscopy - E.p.r. measurements were carried out on a Bruker spectrometer, model 200 TT, equiped with an Oxford Instruments cryostate.

RESULTS

A. Isolation of *D.gigas* Co-protein (purification scheme)

A frozen cell past (2.5 Kg wet weight) was treated as previously described in order to obtain an acidic protein extract (7). All the operations were carried out at +4°C unless otherwise stated. Tris-HCl and phosphate buffers, pH 7.6, of the appropriate molarity were used. Gradients were accomplished using NaCl in 10 mM Tris-HCl buffer medium.

Step I - The acidic protein extract was dialysed overnight against 20 l of distilled water and adsorbed onto two DEAE--cellulose columns (4x30 cm) (Whatman DEAE 23) previously treated according to the manufacturers indications and equilibrated with 10 mM Tris-HCl and eluted with a discontinuous gradient of 200 l of 0.10, 0.15, 0.20 ... up to 0.50 M NaCl in Tris-HCl 10 mM. A fraction containing mainly the Co-protein, rubredoxin (5), desulforedoxin (6), cytochrome c_3 (MW 26,000) (7) and traces of desulfoviridin (18) was eluted at 0.25-0.30 M and collected in a final volume of 1400 ml.

Step II - This fraction was dialysed overnight against 10 l of distilled water and adsorbed on the same DEAE-23 column previously washed with 1 M Tris-HCl and equilibrated with 10 mM Tris-HCl. A fine NaCl gradient was performed using 200 ml of the

following NaCl molarities: 0.050, 0.100, 0.125, 0.150, 0.175 ... up to 0.500. During elution at 0.150-0.200 M the Co-protein was separated from the more acidic rubredoxin type proteins. This fraction was collected in a volume of 410 ml.

Step III - The fraction containing the Co-protein was diluted twice with distilled water and adsorbed on a DEAE-52 cellulose column (2.5x27 cm). During elution with a fine gradient of Tris-HCl (0.050, 0.075, 0.100 ...) a good separation between traces of desulfoviridin present and the Co-protein was obtained. This last fraction was collected in a volume of 280 ml.

Step IV - The fraction from step III was applied on a hydroxylapatite (Bio-Rad) column (2.5x5 cm) previously equilibrated with 0.175 M Tris-HCl and the adsorbed protein was eluted with 10-50 mM phosphate buffer in a volume of 20 ml.

Step V - The violet colored Co-protein was applied on a Sephadex G-50 column (5x100 cm). The main band was obtained in a final volume of 100 ml and presents an absorbance ratio (purity index) of $A_{278}/A_{588} = 0.68$. The final yield was approx. 20 mg.

B. Properties of the Co-protein

Homogeneity of the Co-protein - The homogeneity of the Co-protein was cheked by polyacrylamide gel electrophoresis (a single band being obtained) and electrofocussing (isoelectric point 3.7). Also a single N-terminal residue was identified as threonine.

Cobalt content - Cobalt was the only metal that was detected by atomic absorption spectroscopy which was present in significant amounts. Several samples from different protein purifications were tested and contents ranging between 0.68-0.80 cobalt atoms per molecule could be detected based in protein

TABLE I

Physico-Chemical Characteristics of the *D.gigas* Co-Protein

Amino acid composition			
Lys	7	Ala	13
His	2	Cys(Half)	4
Arg	5	Val	15
Asp	9	Met	6
Thr	8	Ile	4
Ser	9	Leu	9
Glu	11	Tyr	2
Pro	8	Phe	8
Gly	16		136

Molecular weight	16.699[a] 14,000[b]
Isoelectric point	3.7
N-terminal residue	Thr
Nº of cobalt atoms/molecule	0.68-0.80

Spectral data

Molar extinction coefficients ($M^{-1}cm$) (at the indicated wavelengths)

22,000 (588)
8,835 (550)
46,280 (420) $A_{420}/A_{588} = 2.10$
14,900 (278) $A_{278}/A_{588} = 0.68$

a) Minimum molecular weight assuming the presence of two histidines

b) Molecular weight determined by gel filtration

concentrations determined by both the Lowry method and total amino acid composition (Table I).

Molecular weight - The molecular weight of the purified Co-protein was estimated to be 14,000 daltons, by gel filtration on Sephadex G-50.

Amino acid composition - In Table I the amino acid composition of the Co-protein based on stable amino acid residues is reported. Assuming two molecules of histidine per molecule of proteins, the protein was estimated to contain 136 stable amino acid residues giving a minimum molecular weight of 16,699 daltons.

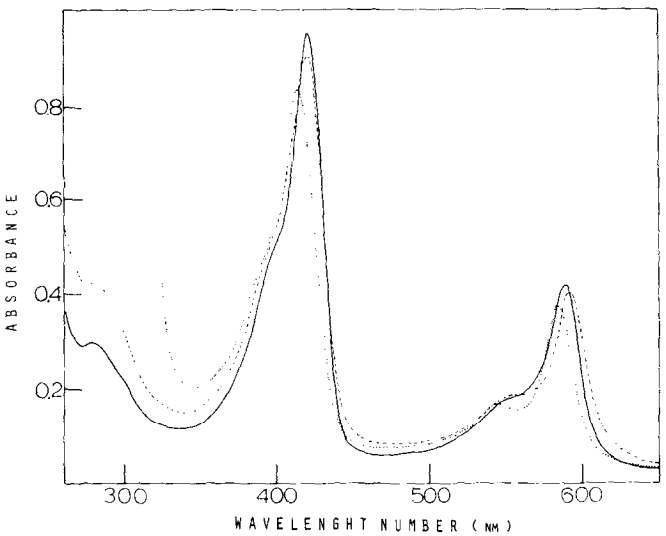

Figure 1. Visible-ultraviolet absorption spectra of the Co-protein. (——) as isolated, 18.6 μM in protein, in 10 mM Tris-HCl, pH 7.6; (---) after reaction with cyanide in basic medium heating at 60°C for 5 minutes; (...) extracted solution obtained after treatment of the Co-protein with acidified acetone solution as described in Materials and Methods.

Electronic spectral data - The ultraviolet-visible absorption spectrum of the purified Co-protein is presented in Figure 1. The cromophores has absorption maxima at 588 and 420 nm (with shoulders at 550, 395 and 300 nm). The protein peak appears at 278 nm. The molar extinction coefficients are reported in Table I. The optical spectra of the Co-protein with a band at 420 nm, is typical of metalloporphyrin proteins (17,20). The protein was not reduced by dithionite or borohydride. Attempts to form complexes with cyanide shifted the visible band at 588 nm to 592 nm, when the reaction was performed at high pH, and after heating the protein at 60°C for 5 minutes (Figure 1). In acidic medium, the band at 588 nm shifts in the opposite direction to 584 nm. Other attempts to react the metal center with external ligands are described in Table II. The absorption spectrum of the extracted solution obtained after treatment of the protein

TABLE II

Reactivity of the Metal Center in the *D.gigas* Co-protein

reactif	effect observed on the visible spectrum at 588 nm
urea 8 M	none
dithionite	none
borohydride	none
CO	none
acidification (HCl, pH 3.5)	588 → 584
methylmercury*	none
mercury acetate*	none
CN^-	none
CN^- + OH^- (at 60°C, 5 min.)	588 → 593
I_2	none

* (I.Moura, J.J.G.Moura and J.M.Wood, unpublished results).

with acidified acetone as described before is shown in Figure 1. No fluorescence could be detected in this solution. Examination of the protein by e.p.r. spectroscopy showed that the Co-porphyrin is diamagnetic, suggesting an oxidation state of Co^{III}.

DISCUSSION

The visible absorption spectrum of this Co-containing protein from *D.gigas* is characteristic of a metalloporphyrin with α, β and γ bands. This conclusion is supported further by magnetic circular dichroism studies on the protein which clearly show that the Co-chromophore is a porphyrin and not a corrinoid complex (19). The Co-porphyrin is extractable from the protein by acidified acetone solutions, and therefore is not covalently bound to the protein through thioether linkages. The small shift to the red, which is observed in the visible spectrum when the protein is reacted with CN^-, would indicate that the axial ligands are strong bases; a good candidate would be the coordination of cystenyl thiolate groups. Since the protein is e.p.r. silent

it is likely that the Co-porphyrin has the oxidation state Co^{III} which is diamagnetic. The small change observed in the spectrum upon acidification, could be due to protonation of the thiolate ligands. A cobalt porphyrin was shown to accumulate in appreciably amounts by hydrocarbon-utilizing bacterium, *Corynebacterium simplex* and the porphyrin was identified as coproporphyrin III (20). The formation of cobalt-porphyrin has also been observed in culture filtrates of *Propionibacterium arabinosum* but the role of these pigments is not understood (21,22). Generally they are expected to be antimetabolites of vitamin B_{12} and iron-porphyrins. Work is now in progress to identify the porphyrin present in the *D.gigas* Co-protein. To our knowledge no other natural occurring cobalt-containing proteins have been reported.

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