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A COBALT PORPHYRIN CONTAINING PROTEIN REDUCIBLE BY HYDROGENASE ISOLATED FROM DESULFOVIBRIO DESULFURICANS (Norway)

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SUMMARY

A cobalt-porphyrin containing protein has been isolated from the sulfate-reducer Desulfovibrio desulfuricans (Norway). This violet-colored protein has a molecular weight of approx. 13,000 daltons and contains 1 cobalt atom/molecule. The apo-protein was estimated to contain 104 amino-acid residues giving a molecular weight of 11,000 daltons. The UV-visible absorption spectrum of the protein exhibiting maxima at 588,418 and 280 nm with a shoulder at 550 nm is characteristic of metalloporphyrin proteins. The molar extinction coefficients of the cobalt-protein at 588, 418 and 280 nm are 31,330 , 64,670 and 17,200 respectively and its absorbance ratio A_{280}/A_{588} is 0.54 . The protein is reduced by dithionite giving a blue-colored reduced form. Important spectral modifications of the chromophore occurred during the reduction including a shift of the Soret peak from 418 to 381 nm and a shift of the α band in the opposite direction from 588 to 593.5 nm. The Co-protein was slowly reduced by the hydrogenase from D.desulfuricans under hydrogen in the presence of cytochrome c_3 . The reported data suggest that the redox states of the cobalt center of this new electron carrier correspond to the Co(III) and Co(II) states.

INTRODUCTION

Sulfate-reducing bacteria are present day representatives of a very ancient process : the dissimilatory reduction of sulfates. However, they exhibit an intricate electron-transfer system constituted by highly complex redox proteins (1,2). Several c-type cytochromes, different flavoproteins as well as proteins with different types of iron-sulfur clusters have been isolated from Desulfovibrio species (2,3). Physico-chemical characterization of these electron transfer proteins have allowed a better understanding of their redox properties (2).

Desulfovibrio desulfuricans Norway strain lacks desulfoviridin (4) which is replaced by another pigment (desulforubidin) catalyzing the dissimilatory

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reduction of bisulfite (5). In addition to sulfate, this microorganism is able to use elemental sulfur as electron acceptor (6). Several electron transfer proteins including c-type cytochromes (7,8), ferredoxins (7,9) and rubredoxin (7) have been characterized from D.desulfuricans (Norway). The present work deals with the isolation and the characterization of a protein containing a cobalt-porphyrin from this microorganism. This violet-colored protein which appears to be similar to the cobalt-containing protein recently isolated from D.gigas (10) is reducible by the hydrogenase-cytochrome c_3 system under hydrogen atmosphere .

MATERIALS AND METHODS

Analytical methods. D.desulfuricans Norway 4 (NCIB 8310) was grown at 35°C on lactate-sulfate medium as reported previously (7).

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 (11). The molecular weight of the protein was determined by gel filtration on a Sephadex G-75 column following the procedure of Whitaker (12). It was also estimated by the method of Weber and Osborn (11) on SDS-gel electrophoresis using the following standards : ovalbumin (43,000), chymotrypsinogen (25,000) soybean trypsin inhibitor (20,100) and horse heart cytochrome c (12,500).

Protein was determined according to the procedure of Lowry (13) or estimated from amino acid analysis.

Amino acid analysis were performed on a LKB 3201 amino acid analyzer. Protein samples were hydrolyzed for 18 , 24 and 48 h at 110°C in 6 N HCl according to the method of Moore and Stein (14). The average was calculated from several analysis. Cysteine and methionine were analyzed after performic acid oxydation as cysteic acid and methionine sulfone, respectively, according to Hirs (15) .

Cobalt and iron in the protein were estimated by atomic absorption spectrometry using an Unicam model SP 1900 spectrometer.

Absorption spectra were measured on a Cary 14 spectrophotometer. Molar extinction coefficients of the protein were obtained by measuring the values of the optical densities at its absorption maxima using a solution of known protein concentration calculated from amino acid analysis. The reduction of the cobalt-containing protein has been carried out under anaerobic conditions using suitable cuvettes with rubber stoppers. The protein and buffer have been bubbled with argon. The reaction started after injection of dithionite. The reduction of the Co-protein by the hydrogenase-cytochrome c_3 system has been performed under similar anaerobic conditions. The reaction started after flushing the cuvette with hydrogen as described previously (16). A pure hydrogenase preparation isolated from D.desulfuricans (Norway) and exhibiting a specific activity of 1830 µmoles H_2 evolved/min/mg protein (Hatchikian E.C., unpublished results) has been utilized in the electron transfer reactions. The cytochrome c_3 used in the redox studies was purified from D.desulfuricans (Norway) as previously reported (7).

Isolation of the Cobalt-protein from D.desulfuricans. The bacterial paste (1,500 g wet weight) was suspended in 600 ml of 10 mM Tris-HCl buffer con-

taining a few deoxyribonuclease crystals and the cell suspension was passed once through a French pressure cell. The crude extract was prepared by centrifuging the resulting extract at 38,000 x g for 30 min. The acidic protein extract which contains the Co-protein and the hydrogenase activity in addition to ferredoxins, rubredoxin and desulforubidin was subsequently obtained as previously reported (7). The acidic protein extract (990 ml) was dialysed overnight and adsorbed onto two DEAE-cellulose (Whatman DEAE 52) columns (4 x 24 cm) previously equilibrated with 10 mM Tris-HCl and eluted with a discontinuous gradient of the same buffer (50 → 600 mM). A fraction containing the Co-protein with desulforubidin and hydrogenase was eluted with 200-260 mM Tris-HCl buffer. After dialysis, this fraction was adsorbed on a second DEAE-cellulose column (4.2 x 18 cm) and eluted with a discontinuous Tris-HCl gradient from 50 to 350 mM using 150 ml of each Tris-HCl molarity. The fraction containing the Co-protein as well as the hydrogenase was eluted with 200-280 mM and collected in a volume of 340 ml. It was then concentrated to 120 ml in a 350 ml ultrafiltration cell with a PM-10 filter (Amicon). The subsequent fraction was divided in two parts of 60 ml and each filtered through an Ultrogel AcA 44 column (5 x 100 cm). At this stage, the Co-protein which appears on the column as a violet-colored band was separated from hydrogenase and desulforubidin. The protein was then applied on a HA-Ultrogel (IBF) column (2.5 x 8 cm) equilibrated with 20 mM Tris-HCl. The Co-protein was not adsorbed on the column and collected in a volume of 150 ml. The resulting fraction was concentrated on a small DEAE-cellulose column and subsequently passed through an Ultrogel AcA 54 column (2.5 x 100 cm). Finally the Co-protein was adsorbed on a hydroxylapatite (HTP, Bio-Rad) column (2 x 6 cm) and eluted with 10 mM phosphate buffer. At this stage, the protein which exhibited an intense violet colour, was judged to be pure both from its spectrum (A_{280}/A_{588} = 0.54) and its amino acid composition. The yield was approx. 10 mg.

RESULTS

Homogeneity of the protein - When subjected to polyacrylamide gel electrophoresis, the purified Co-protein shows before staining the presence of a violet-colored band. After staining the gels, a small band appears in addition to the first. The possibility that this second band could be the apo-protein was checked with SDS-gel electrophoresis. The results showed the same electrophoretic pattern, indicating that the second band has a molecular weight slightly lower than that of the main band. When the Co-protein was treated with 2 N HCl and heated at 80° for 10 min, its SDS-gel electrophoresis showed the presence of a single band corresponding to the apo-protein.

Molecular weight - The molecular weight of the Co-protein was estimated to be 13,000 daltons by gel filtration on Sephadex G-75. The SDS-gel electrophoresis indicated molecular weights of 13,500 and 12,000 for the native protein and the apo-protein respectively. The minimum molecular weight determined from the amino acid composition was 11,001 without the prosthetic group.

Amino acid composition - The amino acid composition of the Co-protein is reported in Table I. The protein was estimated to contain 104 residues. The comparison of its amino acid composition with that of D. gigas Co-protein shows that the two proteins contain a number of acidic residues higher than the basic ones. They exhibit both a high amount of methionine but differ clearly by their content in cysteine, valine, leucine and aromatic residues.

Metal analysis - The protein was analyzed for iron and cobalt by atomic absorption spectrometry. Cobalt was the only metal that was detected. An average value of 0.9 cobalt atoms per molecule was obtained from three samples of the protein.

Absorption spectrum and extinction coefficients - The UV-visible absorption spectrum of D. desulfuricans Co-protein is shown in Figure 1. It exhibits absorption maxima at 588, 418 and 280 nm with shoulders at about 550, 396 and 296 nm. The absorbance ratios A_{280}/A_{588} and A_{418}/A_{588} are 0.54 and 2.06 respectively. The molar extinction coefficients of the Co-protein at 588, 418 and 280 nm are 31,330 , 64,670 and 17,200 respectively (Table I). The absorption spectrum of D. desulfuricans cobalt-protein with α (588 nm), β (550 nm) and Soret (418 nm) bands is typical of metalloporphyrin proteins (17).Although the spectra of D. desulfuricans and D. gigas Co-proteins are similar, differences are apparent on the wavelenghts of maxima absorption (Soret peak and protein peak) as well as on the values of the extinction coefficients (Table I). The reduction of D. desulfuricans Co-protein by dithionite has been performed under anaerobic conditions. The results are reported in Figure 1. The protein reacted slowly with dithionite, the reduction being complete in approx. 3 hours. The reduced protein exhibits a blue color. Important spectral modifications of the chromophore occurred during the reduction : the α band shifted from 588 to 593.5 nm whereas the Soret peak shifted in the opposite direction from 418 to 381 nm with an important absorption decrease. The spectra recorded at different stages of reduction exhibited four isosbestic points occurring at 593.5 , 532 , 437 and 392 nm (Figure 1). The spectra of

Table I. Comparison of the physico-chemical characteristics of D.desulfuricans (Norway) and D.gigas Co-proteins.

Amino acid composition	D.desulfuricans	D.gigas[a]
Lys	5	7
His	2	2
Arg	3	5
Asp	10	9
Thr	7	8
Ser	5	9
Glu	10	11
Pro	6	8
Gly	11	16
Ala	13	13
Cys(Half)	0	4
Val	8	15
Met	8	6
Ile	5	4
Leu	5	9
Tyr	1	2
Phe	5	8
Total residues	104	136
Molecular weight	11,001[b]	16,699[b]
Cobalt atoms/molecule	1	1
Spectral data		
Molar extinction coefficients ($M^{-1}cm^{-1}$, at the indicated wavelenghts)		
588	31,330	22,000
550	12,130	8,830
418	64,670	
420		42,280
280	17,200	
278		14,900

a) From Moura et al. (10)

b) Minimum molecular weight calculated from amino acid composition.

the oxidized and reduced forms of the protein are indicative of the presence of cobalt as chelated metal since the Soret peak of cobalt porphyrin is known to shift to the blue upon reduction (18). The reversibility of the reduction could be observed after anaerobic addition of ferricyanide to the reduced

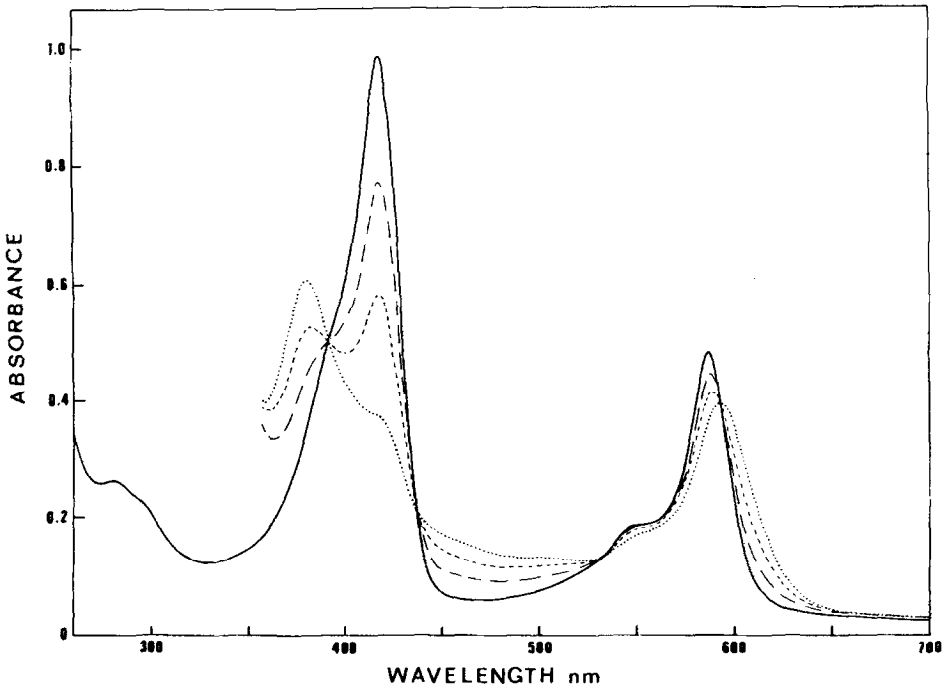

Figure 1 - Absorption spectra of oxidized and reduced forms of D.desulfuricans Co-protein. A 1cm light path stoppered cuvette (total volume, 1ml) contained 15 µM of Co-protein in 20 mM Tris-HCl buffer (pH 7.6) under an atmosphere of argon. After addition of 200 nmoles of sodium dithionite the spectrum of the solution was recorded at regular intervals on a Cary 14 spectrophotometer. (——) spectrum of the oxidized protein. (-·-) spectrum of the protein 5 min after addition of dithionite. The following spectra (---) , (...) were recorded respectively 1 and 3 hours after addition of dithionite.

protein. The native Co-protein reacted also with ferricyanide ; the only detected spectral modification is the shift of the α band to 586 nm.

Reduction of the Co-protein by the hydrogenase-cytochrome c_3 system

Hydrogenase from D. desulfuricans failed to reduce directly the Co-protein under hydrogen atmosphere. As reported for other electron carriers (19,16) cytochrome c_3 was required for the reduction of the Co-protein by hydrogenase. The results are shown in Figure 2. When the spectrum of the Co-protein reduced by hydrogenase is compared to that of the protein reduced by dithionite, a difference is observed on the wavelenght of the Soret peak which has its maximum at 386 nm instead of 381 nm. Further spectral features appeared in the UV region including notably a shoulder at about 360 nm and

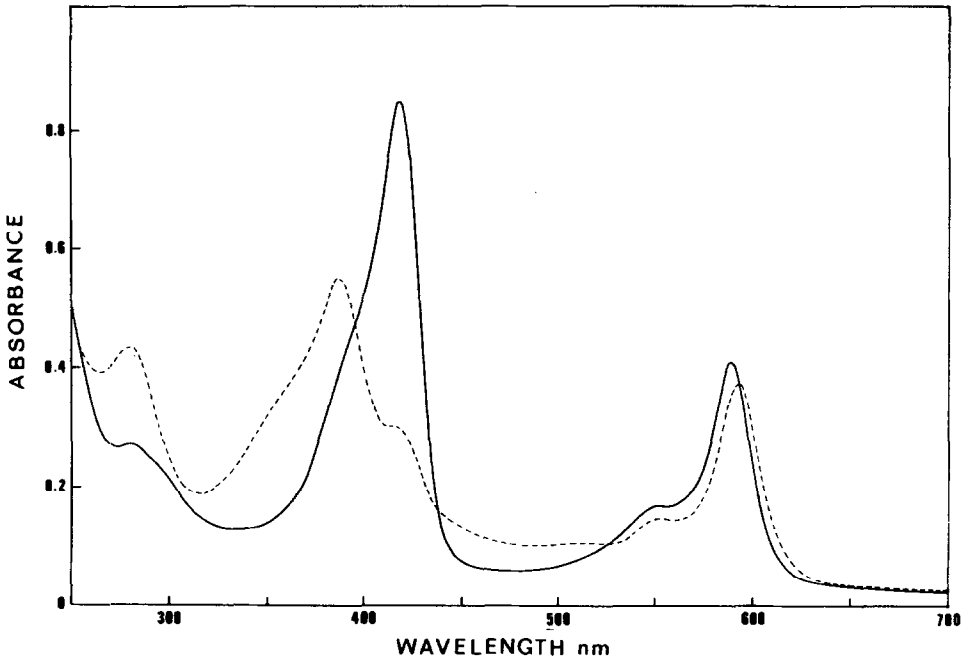

Figure 2 - Reduction of D.desulfuricans Co-protein by the hydrogenase-cytochrome c_3 system under hydrogen. A 1cm light path stoppered cuvette (total volume 1 ml) contained 13 µM of Co-protein, 0.12 µM of hydrogenase and 0.04 µM of cytochrome c_3 in 20 mM Tris-HCl buffer (pH 7.6). (——) spectrum under argon. (...) same sample under hydrogen (1 atm.) after 6 hours.

an increase of absorbance in the 280 nm region. In our experimental conditions, the cobalt-porphyrin containing protein showed low reactivity towards the hydrogenase-cytochrome c_3 system since the complete reduction occurred after approx. 6 hours. The rate of the electron transfer reaction both with dithionite and hydrogenase appeared to be very low suggesting that the cobalt center is protected.

DISCUSSION

The cobalt-containing protein isolated from D. desulfuricans is an acidic protein of low molecular weight which exhibits a visible absorption spectrum with α , β and γ bands typical of a metalloporphyrin. The cobalt-porphyrin which could be dissociated from the protein by acidification and heating is not covalently bound to the protein. The position of the α band in the 588 - 593 nm range of the spectrum, its high extinction coefficient and the

very small absorbance ratio A_{418} (γ)/A_{588} (α) suggest very strongly that the porphyrin to which the cobalt atom is bound is similar to a dihydro- or tetrahydroporphyrin, like in heme d or siroheme or to a porphyrin a (17, 20-22). However we could not find in the literature any spectroscopic data about Co-porphyrin complexes of these types. On the other hand, we can exclude the prosthetic group examined here to be a Co-porphyrin of the deutero- , meso- , proto- (23) or copro- types (18, 24, 25). Indeed, in such cases α and β bands have comparable intensities and lie in the 520-570 nm range and the γ/α absorbance ratio lies between 10 and 20. Although this violet-colored protein appears to be similar to the Co-protein recently isolated from D. gigas (10) it shows significant differences when compared to this homologous protein. The two proteins clearly differ by their amino acid composition, spectral data and redox properties. It is noteworthy that the values of the extinction coefficient of D.desulfuricans Co-protein at 588, 550 and 418 nm are approximately 30 per cent higher than those of D. gigas protein. The most important difference between the two proteins concerns their redox behaviour. In contrast to the Co-protein of D.gigas, the protein isolated from D.desulfuricans is reduced by dithionite and the reaction is reversed by ferricyanide. Furthermore, this Co-protein could be reduced by hydrogenase under hydrogen, however, this electron transfer reaction requires catalytic amounts of cytochrome c_3 as reported for the other non-heme containing electron carriers of Desulfovibrio (16, 19). The Co-porphyrin containing protein from D.desulfuricans appears thus to function as an electron carrier, however, the redox state of the cobalt center in the protein needs more investigation. Although cobalt in its Co(II) state (d^7) is expected to give rise to an EPR spectrum (26, 27) the redox state of the cobalt in the protein is still undetermined since no EPR spectrum could be detected at 77 K, 9.1 GHz from 90 μM samples of the oxidized or reduced protein (Y. Henry, personnal communication). Of the three possible oxidation states of the cobalt atom (Co(I) , Co(II) , Co(III)), the

Co(I) state (d^8) is very unusual and unstable, like in the vitamin B_{12s} complex (28). So, despite the lack of evidence from an EPR spectrum expected for the Co(II) (d^7) state, it is suggested that the oxidized and reduced protein correspond to the Co(III) and Co(II) states. Studies are in progress to elucidate the redox state of the cobalt center and to identify the porphyrin present in the protein.

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REFERENCES

1. Le gall, J., Bruschi, M. and Hatchikian, E.C. (1975) Proc. FEBS Meet. 10th, 40, 277-285.
2. Le Gall, J., Der Vartanian, D.V. and H.D. Peck, Jr. (1979) Current Topics in Bioenergetics, 9, 237-265.
3. Xavier, A.V., Moura, J.J.G. and Moura, I. (1981) Structure and Bonding 43, 187-213.
4. Miller, J.D.A. and Saleh, A.M. (1964) J. Gen. Microbiol. 37, 419-423.
5. Lee, J.P., Yi, C., Le Gall, J. and H.D. Peck, Jr. (1973) J. Bacteriol. 115, 453-455.
6. Biebl, H. and Pfenning, N. (1977) Arch. Microbiol. 112, 115-117.
7. Bruschi, M., Hatchikian, E.C., Golovleva, L.A. and Le Gall, J. (1977) J. Bacteriol. 129, 30-38.
8. Fauque, G., Bruschi, M. and Le Gall, J. (1979) Biochem. Biophys. Res. Commun. 86, 1020-1029.
9. Guerlesquin, F., Bruschi, M., Bovier-Lapierre, G. and Fauque, G. (1980) Biochim. Biophys. Acta 626, 127-135.
10. Moura, J.J.G., Moura, I., Bruschi, M., Le Gall, J. and Xavier, A.V. (1980) Biochem. Biophys. Res. Commun. 92, 962-970.
11. Weber, K. and Osborn, M. (1969) J. Biol. Chem., 244, 4406-4412.
12. Whitaker, J.R. (1963) Anal. Chem., 35, 1950-1953.
13. Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Bio. Chem., 193, 265-275.
14. Moore, S. and Stein, W.H. (1963) In Methods in Enzymology (Colowick, S.P. and Kaplan, NO., eds.) vol. 6, pp. 819-831, Academic Press.
15. Hirs, C.H.W. (1976) In Methods in Enzymology (Hirs, C.H.W., ed.) vol. 11 pp. 167-199, Academic Press, New York.
16. R'zaigui, M., Hatchikian, E.C. and Benlian, D. (1980) Biochem. Biophys. Res. Commun. 92, 1258-1265.
17. Falk, J.E. (1964) In Porphyrins and Metalloporphyrins, Elsevier, New York.
18. Tsutsui, M., Velapoldi, R.A., Hoffman, L., Suzuki, K. and Ferrari, A. (1969) J. Am. Chem. Soc. 91, 3337-3341.

19. Bell, G.R., Lee, J-P., Peck, H.D., Jr. and Le Gall, J. (1978) Biochimie 60, 315-320.
20. Lemberg, R. and Barret, J. (1973) In Cytochromes, Academic Press.
21. Murphy, M.J., Siegel, L.M., Tove, S.R. and Kamin, H. (1974) Proc. Nat. Acad. Sci. USA 71, 612-616.
22. Smith, K.M. (1975) In Porphyrins and metalloporphyrins (Smith, K.M. ed.)
23. Yonetani, T., Yamamoto, H. and Woodrow, G.V., III (1974) J. Biol. Chem. 249, 682-690.
24. Sone, N. (1971) J. Biochem. 69, 753-760.
25. Fujii, K., Tanaka, A. and Fukui, S. (1974) Arch. Microbiol. 97, 259-271.
26. Lin, W.C. (1979) In the Porphyrins IV B, pp. 355-377 (D. Dolphin, ed.) Academic Press.
27. Subramanian, J. (1975) In Porphyrins and Metalloporphyrins, pp. 553-589 (K.M. Smith, ed.) Elsevier.
28. Schrauzer, G.N. (1969) Ann. N.Y. Acad. Sci. 158, 526-539.