Electron microscopy of native and artificial methylreductase high-molecular-weight complexes in strain Gö 1 and *Methanococcus voltae*

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The preparation of inside-out vesicles from methanogenic bacteria with protein cell walls was improved with regard to the preservation of structure and localization of membrane-bound proteins Complexes similar to the methanoreductosome in the methanogenic bacterium Gö 1 [1] were also found attached to the inner aspect of the cytoplasmic membrane of *Methanococcus voltae*. Methanoreductosomes were purified from crude extracts of Gö 1-cells by affinity chromatography. Under specific conditions at high protein concentrations methyl-CoM-methylreductase molecules isolated from Gö 1-cells could be reassociated to spherical complexes of various sizes, with an appearance similar to that of methanoreductosomes isolated from strain Gö 1.

Methanogenic bacterium; Inside-out vesicle, Methyl-CoM methylreductase (component C); Methanoreductosome; Partial reconstitution, Electron microscopy

1. INTRODUCTION

The methyl-CoM-methylreductase system catalyzes the reduction of methyl-coenzyme M to methane with hydrogen as electron donor:

$$CH_3\text{-S-CoM} + H_2 \longrightarrow CH_4 + \text{HS-CoM}$$

Studies of the effects of uncouplers and artificial proton gradients on methanogenesis and the cellular ATP-level indicating ATP synthesis by a chemiosmotic mechanism prove the participation of the methylreductase system in proton translocation [2].

The methyl-CoM-methylreductase (MCR) of *Methanococcus voltae*, and of *Methanobacterium thermoautotrophicum* grown under conditions limiting the amount of factor 430 (the prosthetic group of the MCR) could be localized at the cell periphery as revealed by immunoelectron microscopy [3,4].

Recently, a high-molecular-weight complex bound to the inner aspect of the cytoplasmic membrane of the methanogenic bacterium strain Gö 1 could be visualized by use of electron microscopy of inside-out vesicles [1]. The complex was shown to consist of several substructures; it was called methanoreductosome [1]. Its spherelike head-part contains MCR as revealed by immunoelectron microscopy [1]. A good preservation of membrane-bound reductosomes is one of the prerequisites for the investigation of the fine structural organization of this enzyme complex. Therefore, the method of preparation of inside-out vesicles from Gö 1 was improved and applied on cells of *Methanococcus voltae*. Additional experiments were performed in order to further substantiate the interrelationship described earlier [1] to exist between the methanoreductosome and the MCR-molecule.

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2. MATERIALS AND METHODS

2.1. *Bacterial strains*

Strain Go 1 was grown under heterotrophic conditions in N_2/CO_2 atmosphere (80%/20%, v/v) as described previously [5]. *Methanococcus voltae* was grown autotrophically under H_2/CO_2 atmosphere (20%/80%, v/v) as described previously [3].

2.2. *Protoplast formation*

Protoplasts from whole cells were obtained as described previously [5], but instead of pronase K, trypsin (bovine pancreas) was used as proteolytic enzyme for protoplast formation (see section 3). Protoplast formation was terminated by the addition of the double amount of trypsin inhibitor.

2.3. *Vesicle preparation*

Inside-out vesicles were obtained by vigorous homogenization of the washed protoplast suspension in a glass homogenizer, or by osmotic lysis of protoplasts as described for protoplasts of *E. coli* [6].

2.4. *Protein purification*

The purification of MCR from Go 1 was performed as described in [7] for the purification of MCR from *Methanobacterium thermoautotrophicum*.

2.5. *Affinity chromatography*

The antibody fraction of the serum directed against the MCR of *Methanosarcina barkeri* [3] was coupled to a Protein-A-Sepharose column as described [8]. MCR-containing components (e.g. head parts of the methanoreductosome) of 300 μl crude extract were allowed to bind to the coupled antibodies for about 4 h. Afterwards, unbound protein was eluted with 100 mM potassium phosphate buffer.

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Finally, bound antibody-MCR complexes were eluted with 0.1 M glycine-HCl buffer [8].

2.6. *Identification and characterization of MCR*

Antibodies raised against the MCR of *Methanosarcina barkeri* [3] were used for immunological identification of the protein as described [9,10].

Polyacrylamide gel-electrophoreses were performed according to the methods of Jovin [11] and Laemmli [12]. The gels were stained with a silver-staining method [13] or used for Western-blotting. Molecular-weight determinations were performed according to [14].

2.7. *Electron microscopy*

Vesicle samples and samples of the purified MCR were negatively stained as described by Valentine et al. [15].

Crystalline, air-dried samples obtained from a highly concentrated protein solution were prepared for electron microscopy as described [17]. Alternatively, samples mounted in the same way were contrasted by unidirectional shadow-casting at an angle of 30° with platinum-iridium (80%/20%, w/w).

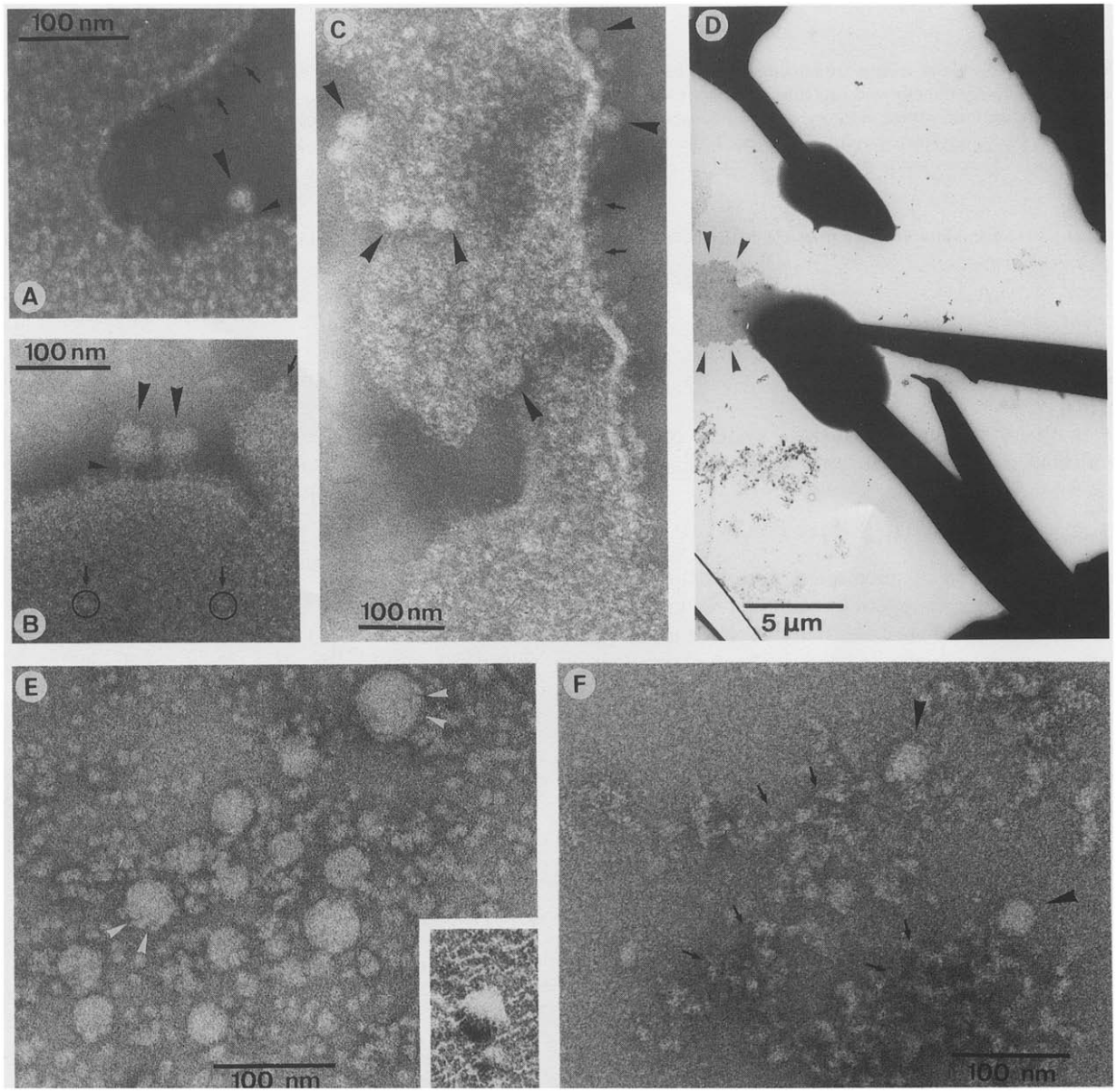

Fig. 1. (A) Spherical particle (large arrowhead) attached to a vesicle-membrane of Go 1 (small arrowhead: attachment site); small arrows mark ATPase. (B, C) ATPase (small arrows) and larger particles (large arrowheads) attached to the inside-out vesicle membrane of *Methanococcus voltae*. Small arrowhead: stick-like connection to the membrane surface. The samples were negatively stained with 3% (w/v) phosphotungstic acid (pH 7.0). (D) Needle-like crystals of MCR attached to the electron microscopic support film. The arrowheads depict a 'micro-pool' brought about by wetting the grid with negative staining solution which caused partial desintegration of the crystal tips. (E) Spherical aggregates (see text). The composition of the aggregates of single native MCR-particles as indicated by small arrowheads is visible. Inset: metal-shadowed aggregate (same magnification as in Fig. E). (F) Spherical complexes obtained by affinity chromatography (see text). Arrowheads depict sphere-like aggregates. The irregular complexes (small arrows) are assumed to represent aggregates of IgG antibodies (compare with the SDS-PAGE of the sample shown in Fig. 2D). The samples were negatively stained with 4% (w/v) uranyl acetate (pH 4.8).

3. RESULTS

3.1. *Inside-out vesicles*

Incubation of Gö 1 cells with trypsin at a concentration of 0.5 mg/ml and an incubation time of 15 min turned out to be the condition for optimal protoplasting; for *Methanococcus voltae* 1.0 mg trypsin and an incubation time of 15 min were optimal, though not all cells had reached the state of protoplasts afterwards. Vesicles from Gö 1 and *Methanococcus voltae* obtained by osmotic lysis and homogenization showed a dense population of F_0F_1-like ATPase particles [16] and larger spherelike particles connected to the vesicle surface. In the case of Gö 1 vesicles few large particles, with a diameter of about 25 nm, were membrane-associated (Fig. 1A); in contrast, membranes of *Methanococcus voltae* exhibited numerous large particles (Fig. 1B, C) with a diameter between 19 and 36 nm. In some cases, a stick-like connection to the vesicle membrane could be visualized (Fig. 1B). Size and shape of these large particles were similar to those already described for Gö 1 [1].

3.2. *Protein purification*

Purified native MCR as well as the three subunits reacted with antibodies directed against the MCR of *Methanosarcina barkeri* as shown by Western blotting (Fig. 2). SDS polyacrylamide gel electrophoresis revealed that the protein consists of three subunits (α, β and γ) with molecular weights of 66000, 44000 and 30000 Da. Using gradient gel electrophoresis and high-performance liquid chromatography a molecular weight for the native protein of 182000 Da was determined. The absorption spectrum showed a peak at 424 nm and a shoulder at about 445 nm.

3.3. *Electron microscopy of MCR*

Some small fragments obtained by air-drying from highly concentrated protein solutions appeared to be needle-shaped crystals with sharp edges (Fig. 1D). Parts of these crystals, when mounted for electron microscopy, had contact with the moistured surface of the grid, and dissolved to set MCR enzyme particles free. These 'micro-pools' contained single molecules, irregular aggregates and spherelike aggregates with diameters from 19 to 64 nm (Fig. 1E). The most abundant spheres exhibited a diameter of about 32.6 nm, others measured 27.5, 35.6, 37.8, 44.1, 50.2 and 61 nm (Fig. 3A). A total of 19.5 ± 2.3 enzyme molecules could be accommodated in the 'wall' of a spherelike particle with 32.6 nm diameter (calculated on the basis of the dimensions of enzyme molecules). Spheres with a diameter of 61 nm would contain about 70 enzyme molecules in their 'wall'. Thus, the data indicate that

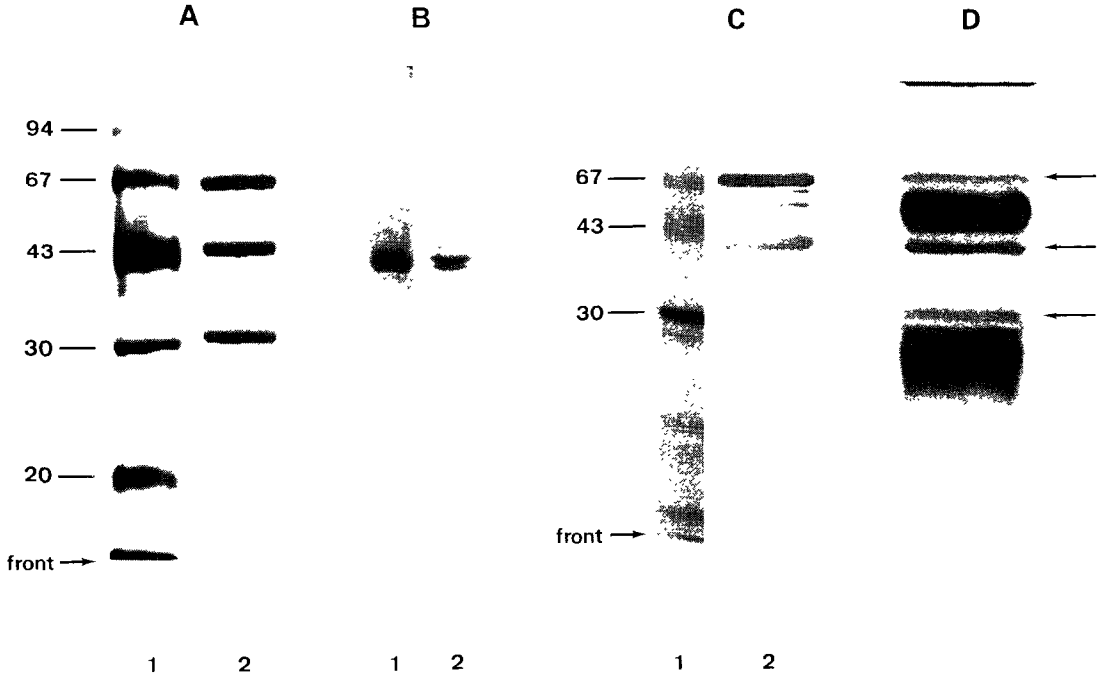

Fig. 2. SDS-PAGE (15% acrylamide gels) and Western blotting of MCR; the numbers indicate the molecular weights of the standard proteins in kDa. (A) SDS-PAGE of the purified MCR (10 μg protein). Protein bands were visualized by silver staining. 1, Standards; 2, denatured MCR exhibiting the three subunits. α, 66000 Da; β, 44000 Da; γ, 30000 Da (average molecular weights determined according to [14]). (B) Western blot of native MCR of Go 1. 1, Purified MCR; 2, crude extract. (C) Western blot of purified denatured MCR of Go 1. 1, Standards; 2, denatured MCR (25 μg protein); weaker bands near the α and γ subunit represent the product of a partial decomposition of the subunits. (D) SDS-PAGE of the MCR purified by affinity chromatography (see text); arrows indicate the subunits of MCR, large bands represent the α and β subunits of the IgG antibodies present in the sample.

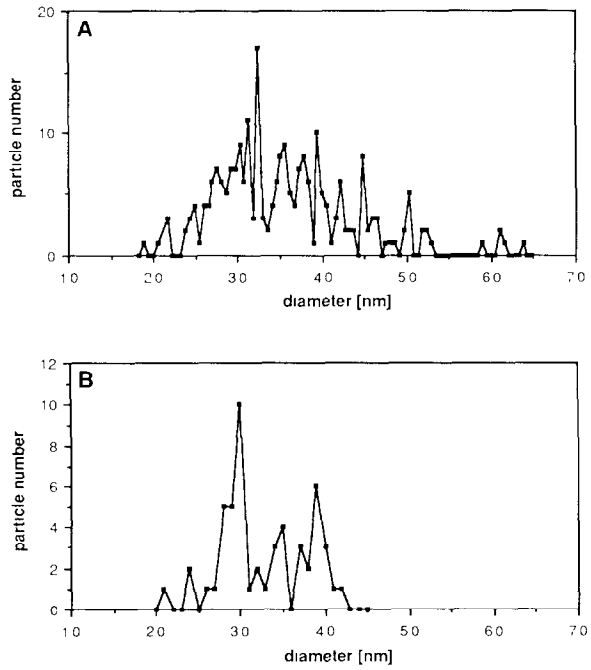

Fig. 3. (A) Frequency distribution curve of the diameters of negatively stained spherical MCR-aggregates formed artificially from the purified enzyme (see Fig. 1E) (B) Curve obtained for particles prepared by affinity chromatography (see Fig. 1F).

the most abundant complex (diameter 32.6 nm) has a molecular weight around 3.6×10^6 Da.

4. DISCUSSION

Protoplasts of cells of strain Gö 1 obtained according to Jussofie et al. [5] have the ability to form methane and can be used for the preparation of inside-out vesicles [1,16]. However, these vesicles usually lose a part of their membrane-bound F1-particles of ATP-ase; loosely associated reductosomes [1] may be lost quantitatively. Crude vesicle preparations obtained from Gö 1 convert methyl-coenzyme M and H_2 to methane [18], whereas washed vesicles only show methylreductase activity after addition of the cytoplasmic fraction. This result indicates that the methylreductase is completely detached from the membrane in these preparations.

Cell wall degradation experiments performed with smaller amounts of proteolytic enzyme did not lead to complete degradation of the wall of all cells; not all vesicles prepared from such a preparation by homogenization or osmotic lysis were inside-out. This kind of preparation, due to inhomogeneity, would not be useful for energetic investigations as described by Deppenmeier et al. [18]. However, vesicles obtained from this kind of partial protoplasting showed sufficient preservation of ultrastructure and preserved attachment of membrane-bound or membrane-associated enzyme complexes. It has been demonstrated previously by immunoelectron microscopy [1] that one type of these complexes, a large spherelike particle, contains methylreductase.

The usually measured molecular mass of native MCR from various methanogenic bacteria is close to 300000 Da [19]. In the case of Gö 1 the molecular weight of the isolated protein was only about 60% of this value (182000 Da). A molecular weight of 130000 Da was described for the MCR from *Methanobacterium thermoautotrophicum* [20]. However, other authors described a molecular weight of 300000 Da for this MCR [17,19]. It might be assumed that in the case of Gö 1 and *Methanobacterium thermoautotrophicum* isolated MCR contains only one of each of the 3 types of subunits in the enzyme molecule instead of the otherwise usual subunit stoichiometry ($\alpha_2\beta_2\gamma_2$).

A formation of globular aggregates was first observed for the MCR from *Methanobacterium thermoautotrophicum* after dilution of highly concentrated MCR solutions [17].

High amounts of spherical aggregates were only formed by dissolving small crystals of MCR which had been obtained under certain conditions (see above). The most abundant spherelike aggregate had a diameter of 32.6 nm. This is similar to the value obtained for spherical MCR complexes isolated from crude extracts by affinity chromatography. A theoretically possible composition of pentagonal and hexagonal segments (for larger aggregates) as found for some bacteriophages [21] could explain the occurrence of defined size-classes in our reconstitution experiments (Fig. 3A). For other protein complexes consisting of 20 identical units the form of a pentagonal dodecahedron has been reported [22,23].

It is obvious that the range of diameters measured for spherelike enzyme particles isolated from crude cell extracts (Figs 1F and 3B) was considerably smaller as that for the spheres formed in our reconstitution experiments. This might indicate a size-determining role of other components also present in the native methanoreductosome which were missing in the spheres formed, in vitro, from purified MCR protein.

REFERENCES

[1] Mayer, F., Rohde, M., Salzmann, M., Jussofie, A. and Gottschalk, G. (1988) J. Bacteriol. 170, 1438–1444.
[2] Blaut, M. and Gottschalk, G. (1984) Eur. J. Biochem. 141, 217–222.
[3] Ossmer, R., Mund, T., Hartzell, P.L., Konheiser, U., Kohring, G.W., Klein, A., Wolfe, R.S., Gottschalk, G. and Mayer, F. (1986) Proc. Natl. Acad. Sci. USA 83, 5789–5792.
[4] Aldrich, H.C., Beimborn, D.B., Bokranz, M. and Schonheit, P. (1987) Arch. Microbiol. 147, 190–194.
[5] Jussofie, A., Mayer, F. and Gottschalk, G. (1986) Arch. Microbiol. 146, 245–249.

[6] Kaback, H.R. (1971) in: Enzyme Purification and Related Techniques, Methods in Enzymology, Vol. 22 (Colowick, S.P. and Kaplan, N.O. eds) pp. 99–120, Academic Press, New York.
[7] Hausinger, R.P., Orme-Johnson, W.H. and Walsh, C.T. (1984) Biochemistry 23, 801–804.
[8] Ey, P.L., Prowse, S.J. and Jenkin, C.R. (1978) Immunocytochemistry 15, 429–436.
[9] Towbin, H., Staehelin, T. and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350–4354.
[10] Taketa, K. (1987) Electrophoresis 8, 409–414.
[11] Jovin, T., Chrambach, A. and Naughton, M.A. (1964) Anal. Biochem. 9, 351–369.
[12] Laemmli, U.K. (1970) Nature 227, 680–685.
[13] Blum, H., Beier, H. and Gross, H.J. (1987) Electrophoresis 8, 93–99.
[14] Shapiro, A.L., Vinuela, E. and Maizel, J.V., Jr. (1967) Biochem. Biophys. Res. Commun. 59, 815–820.
[15] Valentine, R.C., Shapiro, B.M. and Stadtman, E.R. (1968) Biochem. J. 7, 2143–2152.
[16] Mayer, F., Jussofie, A., Salzmann, M., Lubben, M., Rohde, M. and Gottschalk, G. (1987) J. Bacteriol. 169, 2307–2309.
[17] Wackett, L.P., Hartwieg, E.A., King, J.A., Orme-Johnson, W.H. and Walsh, C.T. (1987) J. Bacteriol. 169, 718–727.
[18] Deppenmeier, U., Blaut, M., Jussofie, A. and Gottschalk, G. (1988) FEBS Lett. 241, 60–64.
[19] Hartzell, P.L. and Wolfe, R.S. (1986) System. Appl. Microbiol. 7, 376–382.
[20] Gunsalus, R.P. and Wolfe, R.S. (1980) J. Biol. Chem. 255, 1891–1895.
[21] Casjens, S. and King, J. (1975) Annu. Rev. Biochem. 44, 555–611.
[22] Johannssen, W., Schutte, H., Mayer, F. and Mayer, H. (1979) J. Mol. Biol. 134, 707–726.
[23] Harrison, P.M. (1963) J. Mol. Biol. 6, 404–422.