Antigenic Determinants of the Membrane-Bound Hydrogenase in *Alcaligenes eutrophus* Are Exposed toward the Periplasm

KATHARINA EISMANN, KLAUS MLEJNEK, DANIELA ZIPPRich, MICHAEL HOPPERT, HOLGER GERBERDING, AND FRANK MAYER*

Institut für Mikrobiologie der Georg-August-Universität zu Göttingen, D-37077 Göttingen, Germany

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Electronic microscopic immunogold labeling experiments were performed with ultrathin sections of plasmolyzed cells of *Alcaligenes eutrophus* and “whole-mount” samples of spheroplasts and protoplasts. They demonstrated that antigenic determinants of the membrane-bound hydrogenase are exposed, at the outside of the cytoplasmic membrane, to the periplasm.

*Corresponding author. Mailing address: Georg-August-Universität Göttingen, Institut für Mikrobiologie, Grisebachstrasse 8, D-37077 Göttingen, Germany. Phone: 49-551-393829, Fax: 49-551-393793.

*Alcaligenes eutrophus* is a hydrogen-oxidizing, obligate aerobic, facultatively chemolithoautotrophic eubacterium. The key enzymes for autotrophic growth (hydrogen activation) are the NADH-reducing soluble hydrogenase (SH) and the membrane-bound hydrogenase (MBH) that feeds electrons to the respiratory chain (26, 28). Genetic analyses (4, 8, 14, 18, 19) have revealed that the gene locus for MBH (*hoxP*) located on megaplasmid pHG1 is about 100 kb long and contains at least two structural genes and at least eight accessory genes. The gene *hoxG* codes for the large subunit, and *hoxK* codes for the small subunit (SSU) of the enzyme. The enzyme-subunits undergo maturation (17) to be rendered catalytically active. When *A. eutrophus* is grown heterotrophically, SH and MBH may be more or less completely derepressed, depending on the carbon source (9, 10).

The macromolecular organization of SH (15) and MBH (12) was examined by electron microscopy. In immunochemical localization studies, SH was found in the cytoplasm (23). For the MBH (11), a preferentially peripheral location of the enzyme was observed when cells were harvested in the exponential phase of growth. The enzyme-specific label was distributed along the cell periphery and over the cytoplasm in cells harvested in the stationary phase of growth.

The gene product HoxM seems to be important for membrane association and activity of MBH (19). Sequence data (19) and biochemical findings (2, 6, 11, 17) indicate that at least part of the active MBH complex may be exposed toward the periplasm. However, this notion has not been verified. Therefore, in the present investigation an approach suitable for this kind of analysis, immunoelectron microscopy performed on plasmolyzed cells, spheroplasts, and protoplasts, was used to further support this assumption. A polyhydroxybutyrate-negative mutant, *A. eutrophus* H16 PHB− *4* (DSM 541), was chosen to simplify electron microscopic examination. The cells were grown either autotrophically as previously described (27) on 80% H2–10% O2–10% CO2 or heterotrophically (7, 11) on mineral medium containing fructose. Cells were harvested for electron microscopy during the exponential phase of growth by centrifugation. They were washed twice with phosphate-buffered saline (PBS) prior to further treatment.

For plasmolysis, 0.5 g (wet weight) of cells was resuspended in 1.0 M potassium phosphate buffer, pH 7.0, and incubated for 30 min at room temperature with continuous stirring. For preparation of spheroplasts and protoplasts, the cells were washed twice in 10 mM Tris-HCl buffer, pH 8.0. One gram (wet weight) of cells was resuspended at room temperature in 40 ml of 0.5 M sucrose in 30 mM Tris-HCl buffer, pH 8.0. After addition of 4.5 ml of 0.1 M EDTA, pH 8.0, the sample was incubated for 5 min at 30°C with continuous shaking. Then, 44.5 mg of lysozyme (1 mg/ml final concentration) was added, and the sample was further incubated on the shaker for 30 min. The ratio of protoplasts was increased by addition of 45 ml of double-distilled water 5 to 10 min after addition of lysozyme (see above). The spheroplasts and protoplasts obtained were collected by centrifugation (2,600 × g, 10 min, 4°C), washed twice with a 0.5 M sucrose solution, and stabilized by resuspension in 10 ml of a 0.5 M sucrose solution containing 10 mM MgCl2. The sample was stored at 6°C or on ice for several hours.

Samples containing untreated or plasmolyzed cells were chemically fixed by incubation in PBS-glycine buffer (10 mM glycine in PBS, pH 7.0) containing 0.2% (wt/vol) formaldehyde and 0.3% glutaraldehyde at 4°C for 45 min (24). Embedding was done with Lowieryl K4M resin (11, 24). Ultrathin sections were prepared with an Ultracut E (Reichert-Jung, Vienna, Austria) ultramicrotome. Sections mounted on nickel grids were prepared with an Ultracut E (Reichert-Jung, Vienna, Austria) ultramicrotome. Sections mounted on nickel grids were kept ready for further treatment (immunolabeling), face down, on 35 μl drops of PBS buffer (see above). For labeling, the protein A-collodion gold technique (1, 24, 25) was applied. Poststaining of sections was done with a 4% (wt/vol) aqueous uranyl acetate solution, pH 4.8.

In addition, “whole-mount” labeling combined with negative staining was performed. This was done as previously described (21), on carbon-reinforced Formvar grids. Nonspecific labeling was reduced by application of casein (5). After labeling, the grids were blotted dry on filter paper prior to negative staining with a 2% (wt/vol) neutralized aqueous solution of phosphotungstic acid (29).

The samples were observed in an EM 301 electron microscope (Philips, Eindhoven, The Netherlands) or an EM 902 microscope (Zeiss, Oberkochen, Germany) at calibrated magnifications. Quantitative evaluation of the distribution of colloidal gold particles was done by analysis of ultrathin sections of untreated *A. eutrophus* cells and plasmolyzed cells immunolabeled with the postembedding procedure and of negatively stained whole-mount samples. Four poststaining experiments...
were done. Forty cells per experiment were evaluated. Five whole-mount experiments were performed; 53 standardized cell areas taken at random from 25 electron micrographs were evaluated and compared with micrographs taken from control samples treated in the same way as the samples but without the primary immunoglobulin G antibody.

Postembedding labeling of sections prepared from untreated cells confirmed a predominantly peripheral location of MBH (11; Fig. 1a), provided cells were harvested in the exponential phase of growth. Cells harvested in the stationary phase of growth exhibited enzyme-specific label also distributed over the cytoplasm as expected (11), Table 1 presents the results of quantitative evaluations of label distribution. Postembedding labeling performed on sections prepared from plasmolyzed cells harvested in the exponential phase of growth revealed the location of the enzyme-specific label along the cytoplasmic membrane, within the wide gap between the cytoplasmic and outer membranes, and along the cell periphery (Fig. 1b and Table 1). Negatively stained whole-mount samples exhibited enzyme-specific label at the outside of protoplasts (Fig. 1d to f) and in those regions of the spheroplast surface where the cytoplasmic membrane was not covered by the outer membrane (Figs. 1c and d; Table 1 presents quantitative results). Minor amounts of label were found in the background; occasionally, this label appeared to be connected to small pieces of cell debris (Fig. 1e, e, and f).

Understanding of the physiological role of specific hydrogenses in eubacteria and archaea (3, 13, 16, 20, 22, 23) was improved by immunocytochemical approaches aimed at the cellular localization of these enzymes. It was no surprise that the NAD⁺-reducing SH in A. eutrophus was found in the cytoplasm (23). MBH-specific label found in the cytoplasm of cells harvested in the stationary phase of growth was surprising at first sight. After all, sequence studies (19) clearly indicated a membrane association of the enzyme by the finding that the SSU contains a leader sequence. Ultrastructural enzyme studies had revealed intimate contact between the SSU and the large subunit of the enzyme (12). This indicates that the large subunit should be located very close to the SSU, positioned within or outside of the cytoplasmic membrane because of the function of the leader sequence. A possible explanation for the surprising localization results may be that antigenic determinants of the enzyme complex are in fact located in the cytoplasm as long as processing and activation by maturation of the two subunits (17) have not occurred. This notion is supported by the finding that in a mutant of A. eutrophus lacking a specific component of the gene cluster coding for MBH, application of an enzyme-specific antiserum demonstrated the presence of enzyme-specific antigenic determinants in the cytoplasm. Upon complementation of this component, the distribution of the label was drastically changed: the enzyme-specific antigenic sites were found at the cell periphery (19).

The present investigation established that once MBH is mature, antigenic determinants of the enzyme are exposed toward the periplasm. This finding was obtained with a polyclonal antiserum raised against the holoenzyme. It contained immunoglobulin G antibodies directed against antigenic determinants located on the large subunit and, to a much smaller extent (data not shown), immunoglobulin G antibodies directed against determinants on the SSU. As soon as defined subunit-specific antibody preparations are available, obtained by preparation of either subunit-specific or oligopeptide-specific antibody samples, further discrimination and identification of the exposed antigenic determinants of MBH will be possible.

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**REFERENCES**


