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Isolation of a hydrogenase-cytochrome *b* complex from cytoplasmic membranes of *Xanthobacter autotrophicus* GZ 29

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1. INTRODUCTION

The aerobic nitrogen-fixing hydrogen bacterium *Xanthobacter autotrophicus* strain GZ29 [1] can grow autotrophically with hydrogen, oxygen, carbon dioxide, and molecular nitrogen as sole sources of electrons, energy, carbon, and nitrogen. Carbon dioxide is fixed via the Calvin cycle, and for activation of hydrogen only a membrane-bound hydrogenase activity was detected which does not reduce NAD [2]. This is true also for many other aerobic hydrogen bacteria [2,3], and it is assumed that in these organisms the membrane-bound hydrogenase has to provide electrons for NAD reduction in a reversed electron transport as well as for energy conservation. In *X. autotrophicus* the hydrogenase beyond it has to supply electrons for reduction of nitrogen by nitrogenase at the redox potential of ferredoxin (around −420 mV). For this reason the hydrogenase of *X. autotrophicus* should differ from the type of membrane-bound hydrogenase found in *Alcaligenes eutrophus* which reduces electron acceptors only at the redox potential of quinones [4]. It was also considered that even two different hydrogenases might be present, one reducing ferredoxin and one providing electrons for the respiratory chain. The involvement of ferredoxin in the nitrogen fixation process of *X. autotrophicus* GZ29 has recently been shown by electron transfer and regulation studies [5]. In the present study the membrane-bound hydrogenase system of *X. autotrophicus* is shown to be associated with a cytochrome *b* complex, and catalytic as well as molecular properties are described.

2. MATERIALS AND METHODS

2.1 Growth of the organism and preparation of membrane extracts

X. autotrophicus GZ29 (DSM1393) was grown autotrophically in 10-l batch cultures with 0.15 bar H_2, 0.03 bar O_2, 0.10 bar CO_2 and 0.72 bar N_2 in mineral medium [6]. The dissolved oxygen tension was measured polarographically and regulated by an automatic oxygen supply system [7]. Cells were harvested at $A_{546} \approx 10.0$, washed in 50 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer to a concentration of 1 g wet weight/4 ml buffer. Cells were disintegrated in a French pressure cell at 1875 bar. After removal of cell debris at $10000 \times g$ (10 min) membranes were sedimented at $100000 \times g$ for 50 min (Christ Omikron centrifuge, Heraeus-Christ, Osterode, F.R.G.), washed once with 50 mM potassium phosphate buffer, pH 7.0, and resuspended in the same buffer to a protein content of 8–10 mg/ml. The membrane suspension was mixed with additives from stock solutions to give final concentrations of 10% sucrose, 10 mM EDTA, 0.1% sodium deoxycholate, and 0.5% Triton X-100. The suspen-

sion was stirred at room temperature for 2 h and centrifuged at $100000 \times g$. The supernatant was stored overnight under air at 4°C.

2.2. Fractionation of the membrane extract

Ammonium sulfate was added to the membrane extract at 0°C to give a 25% saturated solution. After adding a thin layer of light petroleum the suspension was centrifuged for 20 min at $4000 \times g$ in a swing-vessel centrifuge. The lower aqueous phase was subjected to further fractionation and hydrogenase was precipitated between 35 and 55% ammonium sulfate saturation. The precipitate was dissolved in 50 mM potassium phosphate buffer, pH 7.0, and dialysed overnight at 4°C against the same buffer. During a second ammonium sulfate fractionation most of the hydrogenase was precipitated between 40 and 55% saturation.

This precipitate was dissolved in 20 mM Tris–HCl buffer, pH 8.0, containing 0.2% Genapol X-080, and, after dialysis, applied to a 2.5×45 cm diethylaminoethyl cellulose (Whatman, DE 52) column preequilibrated with the same buffer. The protein was eluted by a linear potassium chloride gradient (400 ml, 0.1–0.4 M) at a flow rate of about 25 ml/h. Fractions 85 and 90 were pooled separately, and the rest of the hydrogenase peak (fractions 82–95) was separated from detergent by stepwise precipitations with ammonium sulfate to 30% and to 60% saturation.

2.3. Biochemical methods

Hydrogenase was assayed either spectrophotometrically with methylene blue as electron acceptor [4] or, as also with other electron acceptors, manometrically [8]. Electrophoretic analysis of enzyme purity was performed in 0.5×10 cm glass tubes with gels containing 5, 7.5, or 10% acrylamide, at pH 8.9 [9]. Protein bands were stained with Coomassie brilliant blue G 250. Activity bands of hydrogenase on the gel slabs were identified according to [10] without addition of NAD. Sodium dodecyl sulfate electrophoresis was performed after [11]. Molecular weight was determined by gel filtration on a Sephadex G-200 column [12] and also by sucrose gradient centrifugation [13] both in the presence and absence of 0.2% Genapol X-080. Isoelectric focusing was carried out as recommended in the LKB manual using a 110 ml column (LKB) with ampholytes pH 3.5–10.0.

Flavin was extracted with trichloroacetic acid [10]. Absorption spectra were taken by a Zeiss DMR 21 spectrophotometer. Contents of iron and acid-labile sulfur were determined according to [14] and [15], respectively. Protein was determined by the method given in [16].

2.4. Chemicals

All chemicals were of reagent grade quality. Triton X-100 was obtained from Serva, Heidelberg, F.R.G., and Genapol X-080 was a gift of Farbwerke Hoechst, Frankfurt, F.R.G.

3. RESULTS

3.1. Solubilization and enzyme stability

Considerable amounts of hydrogenase activity of *X. autotrophicus* could only be solubilized by application of detergents. EDTA, KCl, KNO_3, NaCl, and sucrose failed. Optimal solubilization (78% of total activity) was achieved by combined application of Triton X-100 and sodium deoxycholate in the presence of EDTA and sucrose after washing with potassium phosphate buffer containing 0.25 M sucrose and 0.15 M NaCl. These findings indicate that this hydrogenase is an integral membrane protein. The membrane suspension as well as the detergent-solubilized membrane extract were relatively stable under air (Fig. 1). Oxygen even stabilized the enzyme to a certain extent whereas storage under oxygen-free hydrogen enhanced enzyme activity nearly fourfold. During spectrophotometric measurement of enzyme activity always a lag phase was observed which could be shortened by addition of glucose and glucose oxidase as an oxygen trap [4]. Inhibition of the enzyme reaction by oxygen was, therefore, reversible. Reducing agents (mercaptoethanol, dithioerythritol) inactivated the enzyme rapidly.

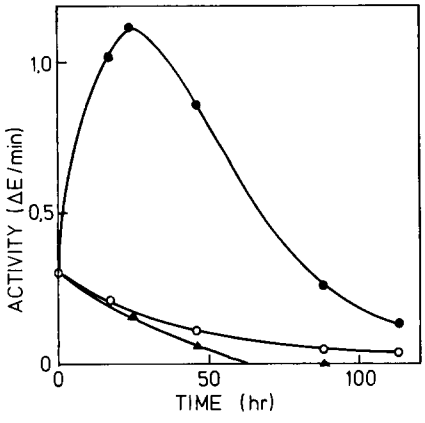

Fig. 1. Stability of solubilized membrane-bound hydrogenase of *X. autotrophicus* GZ 29. The enzyme solution (about 2 mg protein/ml in 50 mM phosphate buffer, pH 7.0) was stored at 4°C under air (○) under oxygen-free nitrogen (▲) or under oxygen-free hydrogen (●), and activity was assayed spectrophotometrically.

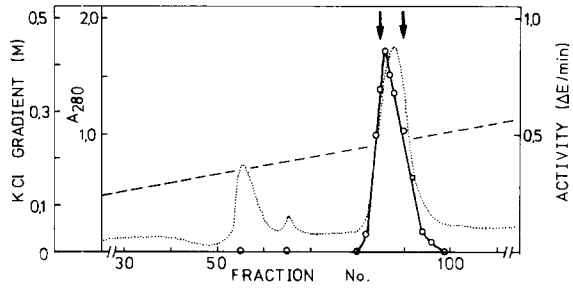

Fig. 2. Elution profile of *X. autotrophicus* hydrogenase on a diethylaminoethyl cellulose column in the presence of 0.2% genapol X-080. Fractions of 2 ml vol. were collected and assayed for protein content (A_{280}, ...) and hydrogenase activity (○——○). Arrows: Fractions 85 and 90.

3.2. Purification and molecular properties

The hydrogenase solution could be separated from lipids and detergents without any loss of activity by raising the ionic strength with ammonium sulfate. During the second ammonium sulfate fractionation a cytochrome-containing enzyme fraction was obtained. Electrophoretic as well as gel filtration and sedimentation analysis of this fraction revealed that the enzyme in the detergent-free state tended to form aggregates with itself and with at least one additional protein. The smallest complex active in methylene blue reduction was found to have an M_r of 100000 to 120000. Aggregates were found in discrete steps up to an M_r of 820000. These aggregates could be dissolved by addition of detergent. In the presence of 0.2% Genapol X-080 as well as in the crude membrane extract containing Triton X-100 and deoxycholate only one hydrogenase activity band could be identified. For further purification on DEAE cellulose the buffer was mixed with 0.2% Genapol X-080, a neutral Triton-like detergent (polyethyleneglycol isotridecanolether) not absorbing in ultraviolet light. During chromatography one hydrogenase activity peak appeared which was not totally identical with the accompanying protein peak (Fig. 2). Samples typical of either side of the protein peak, one more and one less active in methylene blue reduction (fractions 85 and 90), were analysed electrophoretically and spectrophotometrically. Both turned out to be electrophoretically homogeneous and to have the same subunit composition, one subunit with an M_r of 72000 and one of 37000 in a 1:1 ratio. Both contained a *b*-type cytochrome; the more active fraction 85 in a 1:1 ratio and the fraction 90 only in a 0.55:1 ratio per enzyme molecule as calculated from an extinction coefficient of 20.0 $cm^2/\mu mol$ of the wavelength pair 562 and 575 nm (Fig. 3). The ratio of relative methylene blue reducing activities of both enzyme preparations (1:0.6) corresponded to this component ratio. Similar elution patterns were found in six different chromatograms run with varying detergent or ion concentrations. In any case, the correlation between cytochrome content and methylene blue-reducing hydrogenase activity was the same. Benzyl viologen-reducing hydrogenase activity was independent of cytochrome content; however, a hydrogenase fraction totally free of cytochrome was not obtained. During the total purification procedure, the specific hydrogenase activity was raised from 4.2 U/mg protein in the membrane fraction to 43.5 U/mg protein in the pooled fractions 82–95 with a yield of 28.3%.

Isoelectric focusing revealed the isoelectric point at pH 5.5. The enzyme preparation contained 11.6 ± 0.3 mol iron and 10.0 ± 0.2 mol acid-labile sulfur per mol enzyme, as calculated from an M_r of

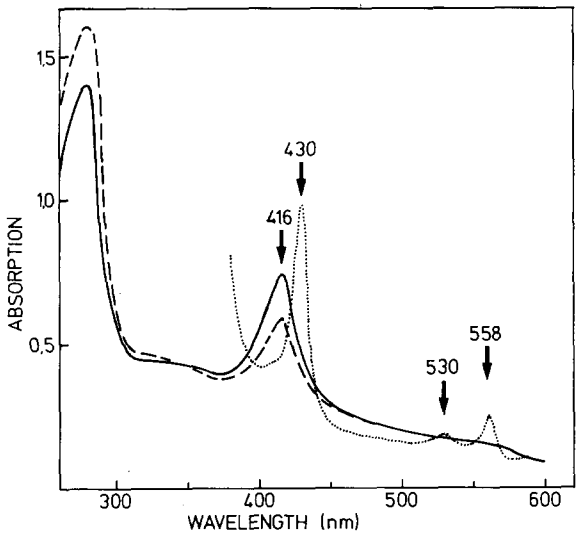

Fig. 3. Absorption spectra of two hydrogenase fractions from the ion exchange chromatogram shown in Fig. 2. Absorption was measured in a Zeiss DMR 21 spectrophotometer against phosphate buffer. (— — —) fraction 90, air-oxidized; (————) fraction 85, air-oxidized; (·····) fraction 85, dithionite-reduced.

110000. No flavins could be detected by optical spectroscopy of trichloroacetic acid extracts.

3.3. Catalytic properties

The detergent-free enzyme preparation reduced only few electron acceptors including methylene blue (set 100%), menadione (46%), phenazine methosulfate (35%), potassium hexacyanoferrate (9%), benzyl viologen (12%) and methyl viologen (3%). No reaction was found with dichlorophenol indophenol, flavinmononucleotide, FAD, NAD, ubiquinone Q_6, or cytochrome *c* (horse heart). Addition of lecithin or Triton X-100 to the reaction mixture had no remarkable effect on the measurable activities. No effects were found either with addition of $FeCl_3$, brenzcatechin disulfonic acid (Tiron), 1.10-phenanthrolin, $MgCl_2$, or EDTA, in the range of 1–10 mM.

Optimal methylene blue reduction was found in 50 mM potassium phosphate buffer at pH 7.0–7.5 and at 57°C. The activity was the same in 50 mM Tris–HCl buffer, and about 10% less in 50 mM triethanolamine–HCl buffer.

4. DISCUSSION

The membrane-bound hydrogenase of *X. autotrophicus* GZ29 differed from the membrane-bound hydrogenase of both *A. eutrophus* H16 [4] and *Pseudomonas pseudoflava* GA3 [17] with respect to molecular weight, subunit composition, isoelectric point, iron and sulfur content, and the tendency to form aggregates by lipophilic interactions. In immunological comparisons among hydrogen-oxidizing bacteria the hydrogenase of *X. autotrophicus* GZ29 proved to be related to the hydrogenase of some *Pseudomonas* and *Alcaligenes* species including those mentioned above but not to any other *X. autotrophicus* strains or heterotrophic nitrogen-fixing bacteria [18]. The membrane-bound hydrogenase of *Paracoccus denitrificans* did not show any relationship to all of these hydrogenases but tended to form lipophilic aggregates during the purification procedure just as did the *X. autotrophicus* enzyme [19]. In contrast to the hydrogenase of *A. eutrophus,* the enzymes of *P. pseudoflava, P. denitrificans,* and *X. autotrophicus* are able to reduce benzyl viologen. This capability may be of physiological importance for *X. autotrophicus* since the redox potential of benzyl viologen is comparable to that of ferredoxins and an electron transfer from hydrogen via ferredoxin to nitrogenase during autotrophic nitrogen fixation must be assumed. However, no direct reduction of *X. autotrophicus* ferredoxin by the membrane-bound hydrogenase could so far be demonstrated [5]. It cannot be excluded yet that there might be a further hydrogenase enzyme present in this organism which is able to reduce ferredoxin: however, in crude membrane extracts in the presence of detergents only one hydrogenase activity band could be identified and no second hydrogenase enzyme was detected during metronidazole assays [5].

Another property of physiological interest is the association of the *X. autotrophicus* hydrogenase with a *b*-type cytochrome which was identified by absorption maxima in its reduced form [20,21]. Electron transfer from hydrogenase to cytochrome *b* either directly or via an intermediate quinone has been demonstrated with *P. denitrificans* [22,23], *Escherichia coli* [24] and with Cyanobacteria [25].

The hydrogenase of soybean root nodules reduces cytochrome c_3, a low-potential electron carrier at about −200 mV [26]. The physiological electron acceptor of the membrane-bound hydrogenases of other aerobic hydrogen-oxidizing bacteria is so far unknown. The tight association of the *X. autotrophicus* hydrogenase with a *b*-type cytochrome and the involvement of the cytochrome in electron transport from hydrogen to methylene blue suggests that in this bacterium hydrogenase couples to cytochrome *b* either directly or via a hidden quinone compound. This coupling would allow at least one phosphorylation step between hydrogen and cytochrome *c* by transmembrane electron transport, a type of reaction in which *b*-type cytochromes usually are involved [27]. Two phosphorylation steps were detected with the oxyhydrogen reaction of *P. denitrificans* [23] and also with other hydrogen-oxidizing bacteria. Direct reduction of a high-potential cytochrome *c* by hydrogenase, as suggested for *A. eutrophus* [28], woud only allow one phosphorylation (during cytochrome *c* oxidation) and is, therefore, unlikely to occur. The *b*-type cytochromes should be further kept in mind when discussing electron acceptors of hydrogenases in aerobic hydrogen-oxidizing bacteria.

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