ISOLATION OF THE MEMBRANE-BOUND HYDROGENASE FROM RHODOSPIRILLUM RUBRUM

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Summary: The hydrogenase of the non-sulphur purple bacterium Rhodospirillum rubrum is located in the particulate fraction. Incubation with deoxycholate yielded a partially solubilized "membrane-bound" enzyme of high molecular weight. Complete solubilization, using pancreatin and high salt concentrations, gave a "membrane-free" enzyme that has been further purified to homogeneity by G-100 and DEAE cellulose chromatography. The hydrogenase is a thermostable iron-sulphur protein, relatively insensitive to oxygen, capable of catalysing both the oxidation and evolution of H_2 with suitable mediators. The "membrane-bound" and "membrane-free" enzymes differed in thermal stability and oxygen sensitivity. The temperature dependence of the reaction and the apparent Km for methyl viologen was the same for both enzyme preparations.

Introduction: The enzyme hydrogenase catalyses the reversible activation of molecular H_2. The enzyme has been found in many bacteria and algae (1). In the last few years, there has been much effort to purify hydrogenases from bacterial sources and the soluble enzyme has been purified from Clostridium pasteurianum (2,3), Desulfovibrio vulgaris (4,5) and D. gigas (6). The membrane associated enzyme has been solubilised and purified from Chromatium (7), Alcaligenes eutrophus (8), Thiocapsa roseopersicina (9) and D. vulgaris (10).

In photosynthetic bacteria, the evolution and consumption of H_2 is thought to be catalysed by different hydrogenases (11-13). Photoproduction of hydrogen by Rhodospirillum rubrum (14) and other purple non-sulphur bacteria (1,13,15) is well documented and is thought to be mediated by the nitrogenase enzyme acting as an ATP-dependent hydrogenase (16). Bose et al (17) demonstrated the presence of a non-ATP-dependent hydrogenase in R. rubrum, and Ormerod and Gest (18) showed that this bacterium is able to use hydrogen as the sole source of reducing power. This probably represents the true physiological role of the hydrogenase in R. rubrum.

The hydrogenase of R. rubrum is associated with the particulate fraction (19). Peck and Gest (20) prepared cell extracts of R. rubrum and demonstrated the reduction of methylene blue by molecular H_2 although the extracts were unable to liberate H_2 from reduced methyl viologen, nor

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reduced benzyl viologen. Gogotov (21) has reported that cell extracts of R. rubrum are able to evolve H_2 from reduced methyl viologen. We present here the solubilization and purification of the hydrogenase from R. rubrum membranes.

Materials and Methods. Rhodospirillum rubrum cells (strain: 1761-la, Göttingen) were a generous gift from Dr. Gudin, SF BP. France. The cells were cultivated, under natural daylight, in a N_2-atmosphere containing 1% CO_2 in a mineral medium with ammonia as the N. source. The chemicals used were of the highest available purity and obtained from: Sigma Chemical Co., deoxycholate, Triton X-100, CTAB and pancreatin (P.1750); from Boehringer, albumin, aldolase, catalase, cytochrome c; from Pharmacia, Sephadex G-100; from Whatman, DEAE-cellulose (DE52); from BDH Ltd., Molecular Weight Markers (14,300-85,800) and all other chemicals.

Chromatium, Clostridium and Spirulina ferredoxins were isolated by the methods of Rao et al (22), and R. rubrum ferredoxins (I + II) according to the method of Yoch et al (23).

Hydrogenase activity was routinely assayed by the rate of H_2 evolution using sodium dithionite as the electron donor and methyl viologen as mediator. The reaction mixture (final volume 2 mls) in 15 ml sealed vials contained 20 mM phosphate buffer pH 7.0 and methyl viologen (2.5 mM). After flushing with N_2, the enzyme was added. The vials were shaken at 30^0 and the reaction started by the addition of fresh sodium dithionite to a final concentration of 10 mM. The hydrogen evolved was determined by gas chromatography as described in Rao, Rosa and Hall (24). Analytical disc electrophoresis was performed according to the method of Weber, Pringle and Osborn (25) using a 2.5% stacking gel (pH 6.9) and a 10% analyser gel (pH 8.8). Hydrogenase activity was located by the method of Ackrell et al (26). The gels were immersed in H_2-saturated 20 mM phosphate buffer pH 7.0 containing methyl viologen (0.25%). Sodium dithionite (0.1%) was added to remove any traces of O_2, a blue tinge indicating anaerobicity of the solution. Blue bands due to reduced methyl viologen corresponding to hydrogenase activity, appeared on the gel within 20 mins. Controls were run in an H_2-free system. The hydrogenase could not be detected by an NAD-linked staining procedure (8). Molecular weight determination was performed on polyacrylamide gels in the presence of SDS and 2-mercapto-ethanol according to Weber and Osborn (27). All gels were stained for protein with Coomassie Blue and destained electrophoretically. Total iron was determined using bathophenanthroline (28). Protein was determined by the Lowry method (29).

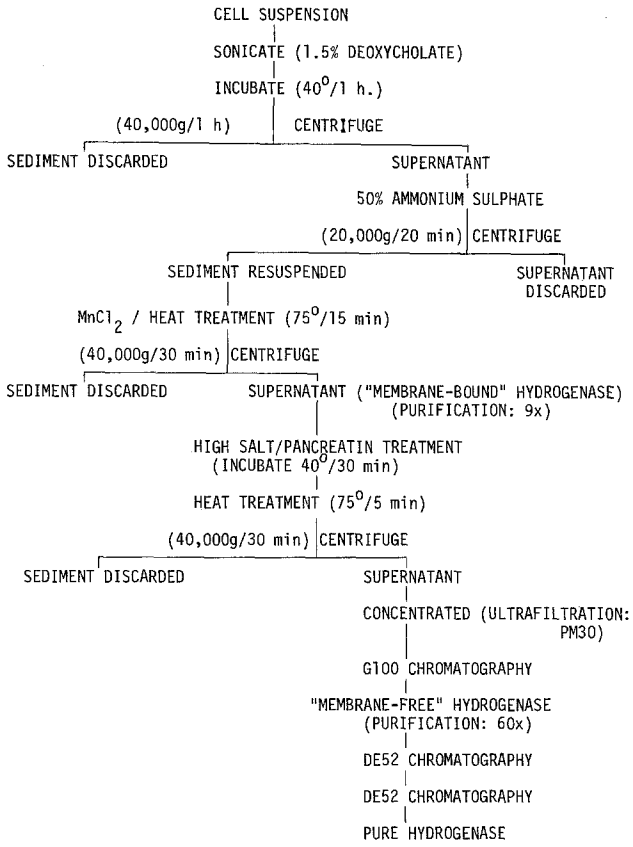

Figure 1 Purification procedure of the hydrogenase of R. rubrum. Recovery of activity 5%, Recovery of protein 2.5×10^{-2}%. Final purification: 200-fold.

Results and Discussion. The purification procedure is given in Fig. 1. All steps were performed under anaerobic conditions at 4° unless otherwise stated. The cells were suspended in 50 mM Tris-Cl buffer pH = 8.0, sonicated for 15 mins in the presence of deoxycholate (1.5% $^{w}/v$) and incubated at 40° for 1 hr. The 50% ammonium sulphate precipitate (containing deoxycholate) was resuspended in 50 mM phosphate buffer pH 7.0. Addition of manganese chloride (7) produced a precipitate which on heating, removed much pigment and protein but the hydrogenase was not affected: considerable purification was achieved. Incubation in the presence of pancreatin (pancreatin : protein, 1 : 10) and a high salt concentration (1.0M KCl, 0.25M sucrose) solubilized the hydrogenase as assayed by polyacrylamide gel electrophoresis. All chromatography steps were carried out in 50mM Tris-Cl buffer, pH 8.0. In the first DE52 chromatography step,

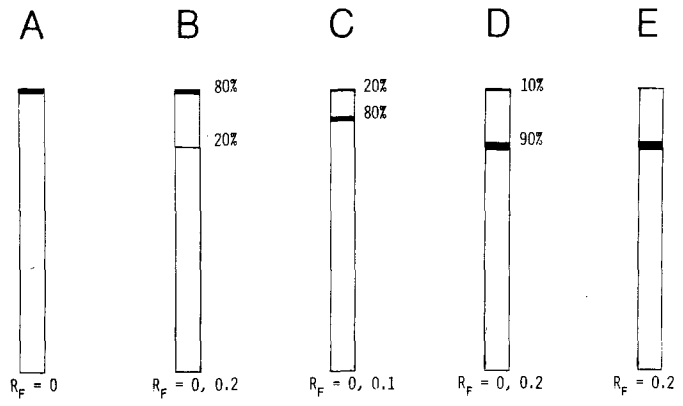

Figure 2 Electrophoretograms of R. rubrum hydrogenase activity in polyacrylamide gels. Electrophoresis and staining carried out as in Materials and Methods. % indicates the approximate intensity of the stain. The preparations used to give the indicated electrophoretic patterns were:-

a) Crude supernatant. The same pattern was obtained with the "membrane-bound" enzyme (purification: 9-fold).
b) After treatment of the "membrane-bound" enzyme with high salt.
c) After treatment of the "membrane-bound" enzyme with pancreatin.
d) After treatment of the "membrane-bound" enzyme with pancreatin and high salt.
e) "Membrane-free" enzyme (purification: 60-fold) after separation from the "membrane-bound" enzyme by G-100 chromatography.

100mM MCl eluted the activity. In the second step, the column was washed with 30mM KCl and the activity eluted with 50mM KCl. The final yield of the hydrogenase was 5% as judged by activity.

Preliminary experiments had indicated that the hydrogenase was associated with the particulate fraction. After sonication of a cell suspension and centrifugation (40,000g, 1 hr) only 20% of the total activity was left in the supernatant fraction. Various treatments were tried to solubilise and release the enzyme from the membranes. After such treatments the amounts of the "membrane-bound" and "solubilised" enzymes were determined by gel electrophoresis. The use of the non-ionic detergent Triton X-100 (0-5%) did not increase the activity of the supernatant. However, deoxycholate (1.5%) increased this activity to 80% of the total. Electrophoresis of this crude supernatant in 10% polyacrylamide gels revealed no migration of the enzyme activity (Fig. 2(a)). Further, the activity was eluted with the void volume from a Sephadex G-100 column indicating a high particle weight of greater than 150,000 (30). Incubation in the presence of the cationic detergent CTAB, or the chaotropic agent

$NaClO_4$, had no effect. The crude supernatant was further purified by ammonium sulphate precipiation, a $MnCl_2$-heat treatment step and centrifugation, but the migration on the gels and elution from the G-100 column remained the same (Fig. 2(a)).

The ability of deoxycholate to disrupt membranes is enhanced by high salt concentrations (31). After incubation in the presence of 1M KCl in 0.25M sucrose, electrophoresis revealed the migration of approximately 20% of the activity (relative stain intensity) with an R_f value of 0.2 (Fig.2(b)). It thus seemed the enzyme could be further solubilised by this treatment. Incubation in the presence of pancreatin (a crude enzyme preparation containing lipase) was also investigated. Here 80% of the activity migrated, but gave an R_f value of only 0.1 (Fig. 2(c)). It was thought that the pancreatin only partially solubilised the enzyme compared to the high salt treatment. Accordingly, a combination of both treatments was carried out. Electrophoresis revealed that 90% of the activity had migrated with an R_f of 0.2 (Fig. 2(d)). The assumption that the increased migration was due to a decrease in particle size rather than a charge effect was supported by the separation of the two activities in Fig. 2(d) on a size basis by G-100 chromatography. Some activity (10%) was eluted with the void volume (yellow green fraction), this did not migrate on the gels. The remainder of the activity was included in the gel (colourless fraction), suggesting a Molecular Weight of 60-70,000 and gave a single activity band on the gels corresponding to an R_f value of 0.2 as before (Fig. 2(e)). This high activity fraction represented a 60-fold purification over the crude sonicate and was termed the "membrane-free" preparation. The 9-fold purified supernatant was termed the "membrane-bound" preparation (Fig. 1).

The "membrane-free" preparation gave 6-7 minor protein contaminant bands in polyacrylamide gels. After two further chromatography steps (DEAE-cellulose) the preparation gave a single protein band, coincidental with a single activity band. A single band was also obtained on SDS gels corresponding to a molecular weight of approximately 65,000 when compared to standard molecular weight markers and standard proteins.

Thermal denaturation results are given in Table 1. The "free" enzyme is stable at 70^0 with 60% of the original activity remaining after 2 hrs. At 80^0 30% remained after 1 hr. The "bound" enzyme is more stable, irreversible denaturation only occurring after 20 mins at 80^0. It is remarkable that the membrane fragments stabilise the enzyme at these temperatures. Incubation at 75^0 for 15 mins did not "release" the "bound" enzyme as assayed by the migration of activity in the gels.

The effect of temperature on the rate of H_2 evolution was studied.

TABLE 1 Thermal denaturation of R. rubrum hydrogenase. The incubations were carried out under anaerobic conditions. The activity was measured by the liberation of H_2 from reduced methyl viologen at 30^0.

Incubation Time (min)	% OF ZERO TIME ACTIVITY			
	"MEMBRANE-FREE" ENZYME		"MEMBRANE-BOUND" ENZYME	
	70^0	80^0	70^0	80^0
20	100	50	100	100
60	65	30	100	65
120	60	0	100	40

TABLE 2 Oxygen sensitivity of R. rubrum hydrogenase. After 14 days incubation at various temperatures, the activity was measured by the liberation of H_2 from reduced methyl viologen at 30^0.

Temperature (^{0}C)	% OF ZERO TIME ACTIVITY AFTER 14 DAYS			
	"MEMBRANE-FREE" ENZYME		"MEMBRANE-BOUND" ENZYME	
	Anaerobic	Aerobic	Anaerobic	Aerobic
20^0	40	20	100	0
4^0	95	50	20	0
-20^0	88	83	88	82
-186^0	100		100	

There was no difference between the "free" and "bound" enzymes between 20^0 and 60^0. A three-fold increase in rate was observed from 30^0 to 50^0. The temperature optimum over a 1 hr incubation was 55^0. An activation energy of 57 KJ.mol^{-1} was calculated. The temperature optimum is a function of the reaction rather than mediator stability since the optimum temperature for H_2 evolution from reduced methyl viologen by Thiocapsa hydrogenase is 70^0 (9).

The pH optima for the reaction at 30^0 was 6.2 with a rate increase of some three-fold between pH 8 and pH 6. To what extent this increase is due to the increase in substrate (proton) concentration is unknown. The apparent K_m for reduced methyl viologen was the same for both the "bound" and "free" enzyme (1.35mM) indicating the active centres are equally accessible.

The oxygen sensitivity of the enzyme was investigated. The enzyme was found to be less sensitive to oxygen compared to some other hydrogenases (2,4-6,8). Differences in stability were found between the "free" and "bound" enzymes. The activities remaining after 14 days incubation under various oxygen and temperature regimes are given in Table 2. The "membrane-free" enzyme under aerobic conditions had a half-life of 7 days compared to

12 days when stored under N_2. At 4^0 it is more stable and once frozen, most or all of the activity is retained. In contrast, the "membrane-bound" enzyme appears to be cold-labile. It retained all its activity under anaerobic conditions at 20^0 but only 20% remained at 4^0 after the incubation period. Similarly, exposed to O_2, the half-life at 20^0 was 6 days, but only 2 days at 4^0. It would appear the"membrane-bound" enzyme is more O_2-sensitive and also cold labile compared to the "free" enzyme. Again most or all of the activity is retained in the frozen state.

Preliminary work on the purified enzyme indicated it is an iron-sulphur protein. EPR studies at liquid helium temperature showed that the oxidised protein had a spectra characteristic of a 4Fe-4S protein with g-values similar to that exhibited by HiPIP (32). The iron content was 4 g-atoms per 65,000g of protein. The hydrogenase also catalyses the evolution of H_2 from reduced benzyl viologen, methylene blue, Janus Green, FMN and R. rubrum ferredoxins (I + II) but at much lower rates than from reduced methyl viologen. No H_2 evolution was detected from Chromatium, Spirulina and Clostridium ferredoxins, nor from NADH when used as substitutes for methyl viologen. Hydrogen uptake can also be demonstrated with methyl and benzyl viologens, DCPIP, ferricyanide and mammalian cytochrome c as electron acceptors.

The ability of the membrane fragments, as isolated, to confer greater thermal stability, greater oxygen sensitivity and cold lability on the hydrogenase, compared to the "free" enzyme, is an interesting phenomenon. However the catalytic reaction, i.e. H_2 evolution from reduced methyl viologen, was unaffected, as regards temperature dependence and substrate affinity, by the conferred properties. The preliminary studies on the "membrane-free" enzyme reveal an iron-sulphur protein with characteristic properties, similar to those associated with hydrogenases from other photosynthetic bacteria (7,9).

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