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THE PROPERTIES OF HYDROGENASE FROM *THIOCAPSA ROSEOPERSICINA*

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Summary

The soluble and chromatophore-bound hydrogenases from the purple sulphur bacterium *Thiocapsa roseopersicina* strain BBS were purified up to homogeneity and the properties studied. The amino acid composition of hydrogenase preparations from different fractions of *T. roseopersicina* is identical, glycine and alanine as N-terminal amino acid residues.

In comparison with other hydrogenases, especially in the immobilized state, the preparations obtained are shown to be more stable to O_2 during storage and they are characterized by high thermal stability. Inactivation is observed above 78—80°C and the optimal temperature for enzyme action is 70°C.

The homogeneous enzyme preparations catalyse the exchange reaction between $^2H_2$ and H_2O and reversible redox reactions of methyl viologen and benzyl viologen as well as H_2 formation from reduced ferredoxin. According to our data, the hydrogenase of *T. roseopersicina* bound with chromatophores is identical to the soluble one.

Introduction

Great attention has been directed recently to the investigation of properties of hydrogenases from different microorganisms [1,2]. Highly purified and homogeneous preparations of hydrogenase from dry cells of *Clostridium pasteurianum* [3] as well as soluble fractions of *Desulfovibrio vulgaris* [4], *Thiocapsa roseopersicina* [5] and *Alcaligenes eutrophus* [6] have been recently obtained. There are also data about isolation of bound hydrogenase from the cells of purple bacteria *Chromatium sp.* [7] and *T. roseopersicina* [8].

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This work is a comparative study of properties of homogeneous hydrogenase preparations isolated from the soluble fraction and from membrane (chromatophore) fractions of the purple sulphur bacterium *T. roseopersicina*.

Materials and Methods

Chemicals. The chemicals used were: *o*-phenanthroline, benzyl viologen, NAD, NADP, FAD and FMN from Serva; acrylamide, *N,N'*-methylenebisacrylamide, *N,N,N',N'*-tetramethylenediamine from Reanal; sodium dithionite and bathophenanthroline from Merck; Sepharose 4B, Ampholines, Sephadex G-25, G-100 and G-150 from Pharmacia; Ultragel AcA 44 from LKB; Commassie Blue and Dextran Blue from Ferack; alcohol dehydrogenase (yeast) from Calbiochem; acetone, Phenosafranine and Safranine T from Chemapol; all other chemicals were from Reachim.

Organism and cultivation. T. roseopersicina strain BBS [9] was grown in Pfennig's medium [10] with 0.2% sodium acetate, 0.2% thiosulphate and 0.1% NH_4Cl according to the method described previously [11].

Enzyme purification. The hydrogenases were purified from the soluble fraction and chromatophores to a homogeneous state by the previous method [5,8]. The main purification stages were as follows: disruption of cell suspension by sonication, heating at 75°C, $(NH_4)_2SO_4$ fractionation, Ultragel AcA 44 chromatography and preparative polyacrylamide gel electrophoresis. The chromatophore-bound hydrogenase was solubilized by treatment with Triton X-100 or acetone.

Enzyme assays. The hydrogenase activity was routinely measured by the gas chromatography method [12], using H_2 evolution from the reduced methyl viologen, by measuring the reduction of methyl viologen or benzyl viologen spectrophotometrically in 1 cm anaerobic cuvettes at 560—600 nm in the presence of H_2; also, manometrically, by following the uptake of H_2 at 30°C in the presence of different acceptors [13]. Hydrogenase activity was also measured by the gas chromatography using the exchange reaction between $^2H_2$ and H_2O [14].

When measuring the hydrogenase activity by H_2 evolution, the reduced ferredoxin (1 mg protein) from *Spirulina platensis* [15] as well as methyl viologen and benzyl viologen (1 μmol/ml) with dithionite (10 mg/ml) were used as H-donors. To measure hydrogenase activity spectrophotometrically, the following reaction mixture (2 ml) was used: 1.8 ml 0.02 M phosphate buffer (pH 7.3), 1 μmol methyl viologen or benzyl viologen, 0.1 ml hydrogenase sample, and H_2 in the gas phase. Azocarmine, methylene blue, methyl viologen, benzyl viologen, ferricyanide, NAD, NADP, FAD and FMN (5—10 μmol/ml) were used as acceptors during H_2 uptake measured manometrically. The hydrogenase activity was expressed as μmol H_2 evolved, or uptake during 1 h per mg protein or in μmol $^2H_2 \cdot h^{-1} \cdot mg^{-1}$.

Purity of the enzyme. The purity of hydrogenase preparations was checked by analytical polyacrylamide gel electropjoresis at pH 8.9 and 7 and 14% gel concentrations and by N-terminal amino acid determination.

Isoelectric focusing. The isoelectric point of hydrogenases was estimated by isoelectric focusing (pH range 4.0—6.0) of the enzyme preparation, obtained after

purification in the column with Ultragel [5].

Determination of molecular weight. The molecular weight of hydrogenases was determined by gel filtration [16] on a Sephadex G-150 column (1.8 × 44 cm) and also by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis [17] using the appropriate protein markers [5].

Metal and acid-labile sulfide determination. Iron of hydrogenase was determined with bathophenanthroline [18]. Total iron was estimated by atomic absorption spectrophotometry using a Hitachi spectrophotometer [19]. Determination of acid-labile sulfide was carried out with *N,N'*-dimethylphenylene diamine [20].

Subunit composition. The hydrogenase subunit composition and their molecular weight were determined by SDS polyacrylamide gel electrophoresis using the appropriate protein standards [17].

Amino acid analysis. The amino acid composition of hydrogenases was determined after hydrolysis of lyophilized samples in 6.5 M HCl (24 h, 110°C) using the automatic amino acid analyzer (Durrum, U.S.A.). Tryptophan was estimated by a spectroscopic technique [21].

Spectral studies. The absorption spectra of hydrogenases were measured with a Specord recording spectrophotometer (G.D.R.).

E'_0 determination. The redox potential of hydrogenases was calculated by the following equation [22]:

$$E_h = E_0(\text{dye}) + \frac{RT}{nF} \ln \frac{(\text{oxidized dye})}{(\text{reduced dye})} = E_0(\text{enzyme}) + \frac{RT}{nF} \ln \frac{(\text{oxidized enzyme})}{(\text{reduced enzyme})}$$

Concentrations of the oxidized and reduced hydrogenase and dye under redox titration were determined spectrophotometrically in anaerobic 1.8-cm cuvettes. The dithionite or buffer solution (saturated with O_2) was added by means of a syringe. The ratio between the oxidized and reduced forms of the enzyme was evaluated by the change in absorbance at 410—420 nm. Reduction of phenosafranine, safranine T and benzyl viologen was measured at 515, 520 and 560 nm, respectively.

Protein determination. Protein was determined by the method of Lowry et al. [23] using bovine serum albumin as standard.

Results

Purity of the enzyme

The results of isoelectric focusing of hydrogenase preparations from the soluble and membrane fractions of cells (obtained after purification in Ultragel AcA 44) indicate the presence of a single protein band with hydrogenase activity. The greatest hydrogenase activity has been shown in the fraction at pH 4.15 (for chromatophore-bound enzyme) and pH 4.20 (for soluble enzyme).

Electrophoresis in 7 and 14% polyacrylamide gel gave only one band in the hydrogenase preparations, after the preparative gel electrophoresis step. This can be interpreted as representing the electrophoretic homogeneity of the isolated enzymes.

Absorption spectrum

The absorption spectrum of homogeneous preparations of both the soluble and chromatophore-bound hydrogenases has a maximum at 280 nm as well as

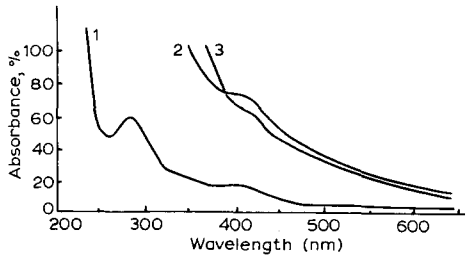


Fig. 1. Absorption spectrum of hydrogenase. Curve 1, general view of spectrum at low concentration of preparation; curve 2, spectrum of oxidized hydrogenase; curve 3, spectrum of reduced hydrogenase.

between 400 and 410 nm (Fig. 1) which is typical for the iron-sulfur proteins. When adding dithionite, the 15% decrease in absorbance has been shown in this region, confirming the presence of non-haem iron in the hydrogenase molecule.

Molecular weight

The molecular weight of hydrogenase preparations when examined by gel filtration on a Sephadex G-150 column (1.8 × 14 cm) is about 68 000 for both forms of the enzyme (soluble and chromatophore-bound).

Homogeneous preparations of soluble hydrogenase and of chromatophore-bound enzyme on SDS polyacrylamide gel electrophoresis showed three protein bands and the molecular weights were estimated at 25 000, 47 000 and 68 000.

Content of iron and sulfur

The content of iron and acid-labile sulfur in the soluble hydrogenase corresponds to 3.9 ± 0.5 and 3.7 ± 0.8 mol/mol protein, respectively. Since the amount of non-haem and total iron in the isolated soluble hydrogenase is nearly the same (3.8 and 3.9 mol/mol protein, respectively) this can be regarded as further proof of the high purity of this enzyme preparation. The content of iron and acid-labile sulfur in the preparation of chromatophore-bound hydrogenase is similar to that of the soluble hydrogenase.

Amino acid composition and N-terminal amino acids

The amino acid composition of soluble and chromatophore-bound hydrogenase is given in Table I. Each value represents the average of 6 separate determi nations on 3 different enzyme preparations. In general, both the enzymes had the same amino acid composition, which suggests identity. Both soluble and chromatophore-bound hydrogenases are characterized by the presence of a large number of acidic amino acids, which is in agreement with the observed isoelectric point.

N-terminal amino acid analysis of both soluble and chromatophore-bound hydrogenases showed the presence of two amino acids (alanine and glycine).

Oxidation-reduction potential

The titration of the reduced and oxidized forms of enzyme and dyes with low redox potentials (Table II) has shown that the average values of redox

TABLE I

AMINO ACID COMPOSITIONS OF HYDROGENASE FROM *T. ROSEOPERSICINA* (mol/mol)

The amino acid composition was calculated using 68 000 as the molecular weight of the enzyme.

Amino acid	Soluble	Chromatophore-bound
Asx	56	58
Thr	30	29
Ser	32	33
Glx	59	57
Pro	39	39
Gly	53	53
Ala	62	60
Val	31	32
Cys	7	8
Met	10	9
Ile	25	26
Leu	48	48
Tyr	18	17
Phe	20	20
Lys	15	16
His	17	18
Arg	24	26
Trp	15	16

potential at pH 7.0 (calculated for the soluble and chromatophore-bound hydrogenases) are similar.

Catalytic properties of homogeneous hydrogenase

The homogeneous preparations of soluble hydrogenase were able to catalyse the reduction of methyl viologen and benzyl viologen with H_2 continuing for 10 min with methyl viologen and about 30 min with benzyl viologen. Unlike unpurified preparations homogeneous enzyme has not reduced azocarmine, methylene blue, ferricyanide, NAD, NADP, FAD and FMN. The ability of soluble hydrogenase to catalyse exchange reaction between $^2H_2$ and H_2O is lost during the enzyme purification, but can be restored with the addition of dithionite. Under such conditions the homogeneous preparations of soluble hydrogenase catalyse the exchange of about 265 μmol $^2H_2 \cdot h^{-1} \cdot mg^{-1}$ protein.

Homogeneous preparations of the bound hydrogenase are also capable of H_2 uptake catalysis in the presence of methyl viologen and benzyl viologen.

TABLE II

OXIDATION-REDUCTION POTENTIAL OF THE SOLUBLE AND CHROMATOPHORE-BOUND HYDROGENASES FROM *T. ROSEOPERSICINA*

Indicator	E_0', mV		
	Indicator	Soluble	Chromatophore-bound
Phenosafranine	−252	−276 ± 8	−272 ± 10
Safranine T	−289	−269 ± 12	−276 ± 8
Benzyl viologen	−359	−302 ± 20	—

The reaction rate in the presence of benzyl viologen is about 15 times higher than that with methyl viologen (1.2 and 19.0 relative units, respectively). Both chromatophore suspension and homogeneous preparations of hydrogenase isolated from them are capable of exchange reaction between $^2H_2$ and H_2O with the addition of dithionite to the reaction mixture. Under such conditions the exchange of 255 μmol $^2H_2 \cdot h^{-1} \cdot mg^{-1}$ protein is catalysed by the homogeneous preparation of bound hydrogenase and 0.5 μmol $^2H_2 \cdot h^{-1} \cdot mg^{-1}$ protein exchange by the chromatophore suspension.

The homogeneous preparations of both soluble and bound hydrogenases are able to catalyse the reaction of H_2 evolution from the reduced methyl viologen (300 μmol $H_2 \cdot h^{-1} \cdot mg^{-1}$ protein) or ferredoxin (14.5 μmol $H_2 \cdot h^{-1} \cdot mg^{-1}$ protein).

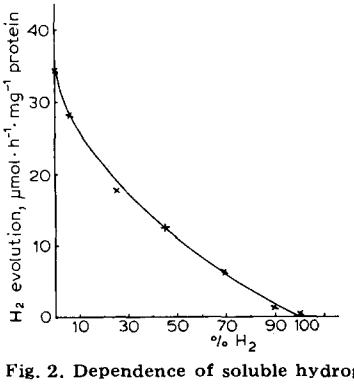
Formation of H_2 by hydrogenase preparations from the reduced methyl viologen is greatly influenced by H_2 content in the gas phase (Fig. 2). The rate of H_2 evolution gradually decreased with increasing in H_2 concentration in the gas phase. Complete inhibition of H_2 formation was shown to occur with a 100% H_2 gas phase.

Stability of the enzyme

According to the study of thermal denaturation of the soluble and bound hydrogenases obtained after purification at Ultragel AcA 44 stage and preincubated at different temperatures for 10 min (Fig. 3, curve 1) considerable inactivation of the enzyme is observed above 78—80°C. At 100°C, 3% of the initial enzyme activity was measured.

Comparing the dependence of the soluble and chromatophore-bound hydrogenases on temperature (Fig. 3, curve 2), H_2 evolution has been shown to occur at a greater rate at 70—80°C. The activation energy of the limiting catalysis stage of H_2 evolution from the reduced methyl viologen, calculated by the Arrhenius equation, is 16.0 ± 2 kcal/mol for both forms of enzyme.

To elucidate the influence of O_2 on hydrogenase stability, the kinetics of enzyme inactivation were studied using both intact cells and purified prepara-



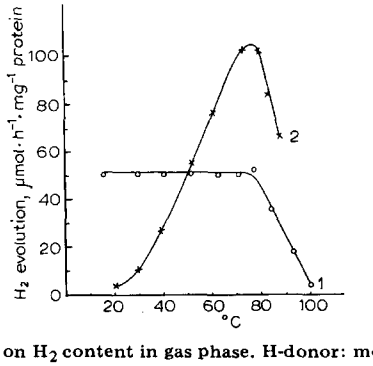


Fig. 2. Dependence of soluble hydrogenase activity on H_2 content in gas phase. H-donor: methyl viologen + $Na_2S_2O_4$.

Fig. 3. Thermal denaturation of hydrogenase (1) and dependence of enzyme activity on temperature (2). H-donor: methyl viologen + $Na_2S_2O_4$.

tions of the soluble fraction and incubating in different gas phases (Fig. 4). In an argon atmosphere the process of hydrogenase inactivation seems to consist of the thermal denaturation of protein. The first steps of denaturation are characterized by some decrease (25—50%) of the enzyme activity. This initial stage is described by the kinetics of first order reaction with the semi-conversion period about 6 days. Then the level of hydrogenase activity remains unchanged. It means that the cells and partially purified hydrogenase contain two forms of enzyme which differ in their stability under inert atmospheric conditions. If the preparations are stored under air (Fig. 4, curves 3 and 4) the hydrogenase activity is lost completely after some period of time. But even in such a case the period of enzyme activity semiconversion is about 6 days.

The hydrogenase activity of the cells and purified enzyme is less affected by oxygen after immobilization (Fig. 5). Only 15% decrease in hydrogenase activity of the cells immobilized in 20% polyacrylamide gel has been shown during the 6-month experiment. The immobilized purified preparations and especially homogeneous hydrogenase have been less resistant to O_2.

The study of oxygen influence on enzyme activity, measured by exchange reaction between $^2H_2$ and H_2O, shows that in the absence of O_2 under addition of dithionite the rate of exchange reaction is constant (Fig. 6, curve 1). In the absence of dithionite the exchange reaction has been shown only after some lag period, the duration of which depends on the time of enzyme kept under air. 1% O_2 being introduced in the gas phase results in complete inhibition of hydrogenase activity (Fig. 6, curve 2). A similar effect of oxygen has been shown under enzyme incubation at the beginning of gas phase containing 1% O_2. The activity has not been shown under introduction into a reaction vessel of 1% O_2 in the gas phase, neither. The enzyme activity has been completely restored after addition of 0.2 ml of 1% dithionite solution to the reaction mixture (Fig. 6, curve 3).

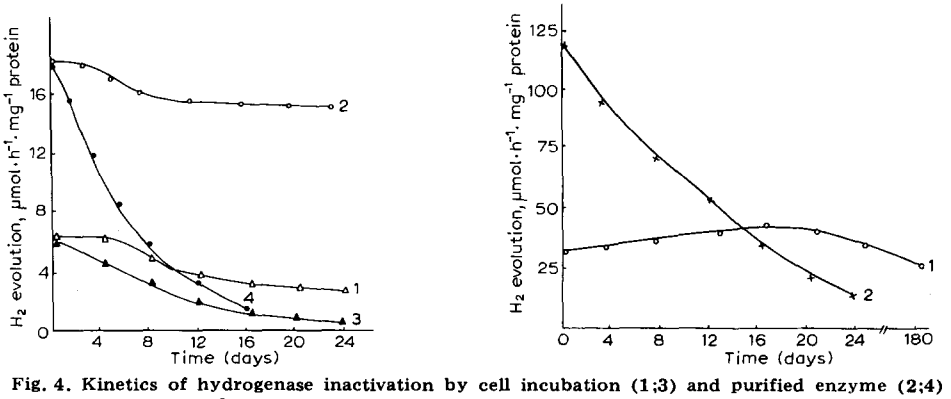


Fig. 4. Kinetics of hydrogenase inactivation by cell incubation (1;3) and purified enzyme (2;4) under different gas phases (4°C). Curves 1, 2: Ar; curves 3, 4: air. H-donor: methyl viologen + $Na_2S_2O_4$.

Fig. 5. Kinetics of hydrogenase inactivation by incubation of immobilized cells (1) and purified immobilized enzyme (2) under air. H-donor: methyl viologen + $Na_2S_2O_4$.

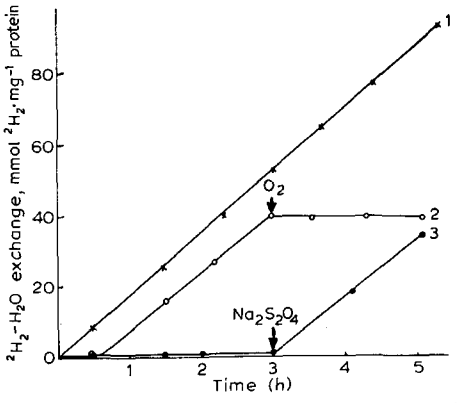


Fig. 6. Effect of O_2 (1%) and dithionite on the ability of highly purified hydrogenase preparations to catalyse the exchange reaction between $^2H_2$ and H_2O.

Discussion

During cell destruction the hydrogenase is shown both in soluble and membrane-bound fractions of a great number of microorganisms [3]. Those enzymes are assumed to perform different functions in the cell [24]. But there are no data on the comparative study of physico-chemical properties of homogeneous preparations of soluble and membrane-bound hydrogenase of phototrophic bacteria prooving their similarity or distinction.

No considerable distinctions have been observed in the preparations of soluble and membrane-bound hydrogenases of *T. rosepersicina* in some of their physico-chemical properties (absorption spectrum, p*I*, E'_0, amino acid composition, content of iron and acid-labile sulphur, subunit structure and N-terminal amino acid residues). So it is more probable that they are identical and the only difference is in their ability to pass to the soluble fraction during the cell destruction. But it is not improbable that despite the similar physico-chemical properties, the soluble and membrane-bound hydrogenases can perform in vivo different functions.

Like the highly purified preparations of hydrogenase from *Chromatium* sp. [7] the enzyme from *T. roseopersicina* is capable of catalysing both oxidation and reduction of corresponding methyl viologen form by H_2. But it goes with incomplete reduction of acceptors which is apparently accounted for by the establishment of equilibrium between the H_2 uptake and evolution reactions due to the reversible action of hydrogenase. Such an action is also shown by the ability of enzyme of homogeneous preparations to catalyse exchange reaction between $^2H_2$ and H_2O as well as by the inhibited H_2 formation in hydrogen atmosphere (Fig. 2).

According to the absorption spectrum, content of iron and acid-labile sulphur the enzyme from *T. roseopersicina* is similar to hydrogenases from *D. vulgaris* [4] and *Chromatium* sp. [7]. But it slightly differs from them as to molecular weight, amino acid composition and, especially, stability.

Unlike the hydrogenases from some other microorganisms [3], the enzyme from *T. roseopersicina* shows high thermal stability (Fig. 4). It is also more

stable when stored under air (the period of semi-inactivation is about 6 days). But despite the low oxidation rate in the air the mechanism of oxygen inactivation is apparently similar to that of other hydrogenases consisting in the oxidation of enzyme active center. This is proved by hydrogenase reactivation in a hydrogen atmosphere [25] as well as by the reduction of activity of the oxidized hydrogenase from *T. roseopersicina* under dithionite addition (Fig. 6). The regeneration of hydrogenase activity by dithionite can be apparently accounted for by the transformation of inactive oxidized form of an enzyme into the active reduced one. But the degree of temperature and O_2 inhibiting influence of the hydrogenase can be dependent on the structural peculiarities of the enzyme and its active center. In this aspect the hydrogenase *T. roseopersicina* is apparently more stable than the enzymes from the other investigated microorganisms.

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