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PURIFICATION AND SOME PROPERTIES OF DIFFERENT FORMS OF HYDROGEN DEHYDROGENASE

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SUMMARY

Hydrogen dehydrogenase has been purified 300-fold using $MnCl_2$ to stabilize the enzyme. The presence of $MnCl_2$ produced an enzyme with the following properties: (a) heat stability, (b) diminished sensitivity to *p*-chloromercuribenzoate, (c) smaller K_m values. The reaction of $MnCl_2$ with the protein was rapid and reversible. High concentrations of NaCl and Tris buffer cause an irreversible denaturation. The hydrogen dehydrogenase reaction can be reversed to produce H_2 from DPNH.

INTRODUCTION

Some autotrophically grown hydrogen bacteria contain enzyme systems which reduce DPN with hydrogen[1-3]. In two cases, *Pseudomonas saccharophila*[4] and *Hydrogemonas ruhlandii*[5] an enzyme has been purified and shown not to require additional cofactors for the reduction of DPN.

This communication is concerned with the purification and some properties of this enzyme in *H. ruhlandii*. Although extensive purification was obtained no evidence was found that indicated a multiprotein system. This protein will accordingly be called hydrogen dehydrogenase as proposed by BERNSTEIN AND VISHNIAC[5]. Further, the presence of two types of hydrogen-activating enzymes in *P. saccharophila*[3,4], one a particulate hydrogenase and the other a DPN-specific enzyme warrants the use of the name hydrogen dehydrogenase to describe the enzyme that catalyzes the following reaction:

$$H_2 + DPN^+ \rightleftharpoons DPNH + H^+$$

Abbreviations: PCMB, *p*-chloromercuribenzoate; PEM, 0.05 M potasium phosphate (pH 7.8)–0.001 M EDTA–0.0007 M $MnCl_2$.

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MATERIALS AND METHODS

H. ruhlandii[6] was grown on a medium containing KH_2PO_4, 1.0 g; $MgSO_4 \cdot 7\ H_2O$, 0.5 g; $CaCl_2 \cdot 2\ H_2O$, 0.01 g; NH_4Cl, 1.0 g; $NaHCO_3$, 0.5 g; $Fe(NH_4)_2(SO_4)_2 \cdot 6\ H_2O$, 10 mg in 1 l of distilled water. The pH was adjusted to 7.4 with 1 N NaOH and trace elements were supplied as recommended by COHEN AND BURRIS[7]. 22 l H_2–CO_2–O_2–N_2 (68 : 15 : 8 : 9) were circulated through 1 l of medium at the rate of 10 l of gas/min with a Dyna-Pump (model 4 K, Neptune Products, Inc., Dover, N.J. (U.S.A.)). The gas was distributed through an 8-cm glass sparger. With 100 ml of a fast growing culture as an inoculum, 2 l of medium yielded, after 16 h of growth at room temperature, 6–8 g wet weight of cells. These cells were stored at −20° until ready for enzyme purification.

For assay, the enzyme, dissolved in 3 ml of 0.1 M Tris–HCl buffer (pH 7.8) was gassed for 4 min with hydrogen, and the reaction started by adding 2 μmoles DPN. The progress of the reaction was followed by recording the absorbancy at 340 mμ in a Cary spectrophotometer model No. 11 at 25°. The initial rate was proportional to the enzyme concentration up to an absorbancy change of 0.2/min. A unit of enzyme activity is defined as 1.0 μmole DPNH formed/min, and specific activity as μmole DPNH/min/ mg protein. Protein was estimated by the LOWRY method[8].

Ammonium sulfate used in enzyme purification was recrystalized from 0.001 M EDTA. Dialysis tubing used in purification procedure B was soaked in 0.05 M potassium phosphate buffer (pH 7.8)–0.001 M EDTA–0.0007 M $MnCl_2$ (PEM) for 30 min before use. DEAE-cellulose, type 20 was a product of Brown Co., Berlin, N.H., U.S.A. and TEAE-celluose was obtained from California Foundation for Biochemical Research.

RESULTS

Enzyme purification

Procedure A: This procedure has been described briefly[5] and is now given in outline. Cells (8 g wet wt.) were disrupted in the Hughes press and then extracted with 200 ml of 0.02 M potassium phosphate buffer (pH 7.8) 10 μg DNAase and 0.5 ml of 0.1 M $MgCl_2$ for 30 min at 25°. The extract was centrifuged and then treated with 0.2 volume of 10% streptomycin sulfate to remove nucleic acids. After centrifugation, the supernatant was fractionated with saturated $(NH_4)_2SO_4$ solution and the fraction between 40–65% saturated $(NH_4)_2SO_4$, was collected and dissolved in 0.02 M phosphate buffer (pH 7.8). This fraction was dialysed against 30 volumes of 0.02 M phosphate buffer (pH 7.8) overnight and then applied to a 2 × 20-cm column of DEAE-cellulose. Protein was eluted by a linear sodium chloride gradient between 0.01 M and 1.0 M NaCl. The enzyme is present in fractions containing 0.44–0.68 M NaCl and the most active fractions were combined and dialysed against phosphate buffer overnight. This fraction was then chromatographed on a second DEAE-cellulose column as described for the first column. Fractions containing enzyme were immediately dialysed and then stored at −20°. Details of purification are given in Table I and this dehydrogenase preparation will be referred to as enzyme A.

Procedure B: The cells after being crushed in the Hughes press, were suspended

in 32 ml of PEM buffer. This gelatinous suspension was sonicated for 4 min in a 10 kC Raytheon sonicator, followed by centrifuging at 35 000 × *g* for 20 min to give a cell-free extract. Sufficient KCl was added to the extract to make a 0.4 M solution of which a small sample was titrated with a solution containing 1% protamine sulfate in 0.4 M KCl until the enzyme just started to precipitate. Using this titration value (0.2–0.3 volume), the protamine sulfate solution was added slowly to the extract and then stirred for 15 min. The precipitate was spun down at 10 000 × *g*

TABLE I

PURIFICATION OF HYDROGEN DEHYDROGENASE

	Total activity (units)	*Specific activity*
Procedure A		
Crude extract	903	1.47
Streptomycin supernatant	863	1.54
Ammonium sulfate (40–65%) dialysed	784	4.39
DEAE-cellulose eluate, fraction 22	376	15.84
fraction 23	243	12.78
Fraction 22 + 23 rechromatographed on DEAE-cellulose, fraction 23	161	27.45
fraction 24	93	19.44
Procedure B		
Crude extract	519	0.84
Protamine supernatant	420	1.32
Ammonium sulfate (40–60%)	350	3.51
Heat treatment supernatant dialysed	310	12.1
TEAE-cellulose eluate, fraction 37	47	49.2
fraction 38	111	264
fraction 39	97	153
fraction 40	43	123
fraction 41	18	90

for 10 min and the supernatant was made 40% saturated with $(NH_4)_2SO_4$ solution, followed by stirring for 15 min. The supernatant solution collected after centrifugation, was made 60% saturated with $(NH_4)_2SO_4$. The precipitate collected by centrifugation was taken up in 10 ml of PEM buffer, and divided into two portions. Each portion was pipetted into a 50 ml cellulose nitrate centrifuge tube which was immersed in water at 50°. The tube was shaken by hand for 4 min and then placed in an ice bath. After the heat treatment the solution was centrifuged and the supernatant was dialysed for 15 h against 1 l of PEM buffer. The dialysed solution was applied to a 2 × 22-cm column of TEAE-cellulose which had been equilibrated with PEM buffer. Protein was eluted from the column with a linear gradient between 200 ml of PEM buffer and 200 ml of 0.5 M potassium phosphate buffer (pH 7.4)–0.001 M EDTA–0.0007 M $MnCl_2$. The flow rate was approx. 15 ml/h and 5–6-ml fractions were collected. Fractions were concentrated 5–10-fold by placing a dialysis tubing containing 30 parts Carbowax 4000 and 1 part Sephadex G-25 into the fractions. These concentrated fractions were dialysed against 100 volumes of PEM buffer. Table I summarizes a typical purification procedure, with a yield of 21%

and 300-fold purification. The enzyme is stable for 6 months if kept frozen at −20° but loses activity slowly when frozen and thawed many times. Some preparations were made without added $MnCl_2$ and in this case the heat treatment step was omitted.

Diaphorase and reduction of dyes

Enzyme preparations A and B contain a diaphorase activity assayed with methylene blue, which is a small contaminant in the case of enzyme B. That hydrogen dehydrogenase and diaphorase are two different enzymes is suggested by an enzyme prepared using purification procedure B without added $MnCl_2$. This preparation on heating to 60° loses all the dehydrogenase activity in the first minute of heating whereas diaphorase retains 50% of the activity after 3 min of heating. Also enzyme fractions eluted off the TEAE-cellulose column do not have a constant diaphorase to dehydrogenase activity ratio. Hydrogen does not reduce methylene blue using enzyme A or B. The only dye reduced was benzyl viologen and the ratio of activity for benzyl viologen to DPN is 0.17 for enzyme A.

Effect of sulfhydryl compounds and inhibitors on hydrogen dehydrogenase

The effect of cysteine on enzymes A and B is different in that enzyme A is activated by cysteine and DPN, whereas, in contrast, enzyme B is not affected by cysteine and DPN (Table II). BAL also enhances the activity of enzyme A.

TABLE II

EFFECT OF CYSTEINE AND DPN ON HYDROGEN DEHYDROGENASE

Preincubation mixture contained in 3 ml: 300 μmoles Tris–HCl (pH 7.8) and hydrogen dehydrogenase and was incubated with additions for 10 min, then H_2 gas bubbled rapidly for 1 min. Reaction started by adding 2 μmoles DPN.

Additions	Enzyme A		Enzyme B	
	lag (min)	rate (A/min)	lag (min)	rate (A/min)
None	1	0.04	0	0.05
Cysteine (10^{-2} M)	0.5	0.06	0	0.05
Cysteine (10^{-2} M) + DPN ($6 \cdot 10^{-4}$ M)	0	0.10	0	0.05

When comparing the effect of PCMB on enzymes A and B, enzyme A is shown to be very sensitive to lower concentrations of PCMB (Table III). PCMB inhibition can be partially reversed by sulfhydryl compounds and the preincubation of enzyme A with DPN prevents complete inhibition by PCMB. Other sulfhydryl reagents, iodoacetate (10^{-3} M) and iodoacetamide (10^{-3} M) do not inhibit these enzymes.

K_m and pH optimum

The Michaelis constants for enzyme A are $K_m^{H_2}$ $4.8 \cdot 10^{-4}$ M, K_m^{DPN} $5.7 \cdot 10^{-4}$ M and for enzyme B $K_m^{H_2}$ $7.6 \cdot 10^{-4}$ M, K_m^{DPN} $6.6 \cdot 10^{-4}$ M. To determine the Michaelis con-

stant for H_2 of enzyme A, various concentrations of H_2 and He were used as the atmosphere in a Warburg vessel with excess DPN as the hydrogen acceptor. $K_m^{H_2}$ for enzyme B was determined spectrophotometrically by varying the amount of H_2 added as a H_2-saturated solution in the presence of excess DPN. Michaelis con-

TABLE III

EFFECT OF PCMB ON HYDROGEN DEHYDROGENASE

Preincubation mixture contained in 3 ml: 300 μmoles Tris–HCl (pH 7.8) and hydrogen dehydrogenase. Incubation time 5 min with additions followed by 1 min of rapid H_2 gassing. The second addition was made after 5 min preincubation. Reaction started by adding 2 μmoles DPN.

Additions	Enzyme A inhibition (%)	Enzyme B inhibition (%)
PCMB (10^{-4} M)	34	0
PCMB ($5 \cdot 10^{-4}$ M)	77	0
PCMB (10^{-3} M)	96	11
PCMB (10^{-3} M) then BAL (10^{-2} M)	00	5
PCMB (10^{-3} M) then cysteine (10^{-2} M)	85	6
PCMB (10^{-3} M) + DPN ($6 \cdot 10^{-4}$ M)	72	9

stants for DPN were determined with the buffer saturated with H_2. The pH optimum for both enzymes in Tris buffer is 7.8.

Reversibility

Using the DPN-specific glucose-6-phosphate dehydrogenase of *Leuconostoc mesenteroides*[9] as the generator of DPNH, the evolution of 2.5 μmoles/h of alkali insoluble gas can be demonstrated with 105 μg of enzyme A (specific activity 19.4) at pH 7.0. The pH optimum for this reaction is 6.8–7.0. The disappearance of DPNH can also be followed spectrophotometrically. Michaelis constant for enzyme B is $8.1 \cdot 10^{-5}$ DPNH and the forward reaction is 450 times as fast as the reverse reaction. H_2 can also be generated by the dehydrogenase from reduced methyl viologen and the ratio of activities of H_2 produced from reduced methyl viologen to the reduction of DPN by H_2 remains constant at 0.7 throughout purification scheme A.

Effect of salts

Table IV shows that enzyme B is sensitive to high concentrations of NaCl. This was also noticed during purification procedure A when it was necessary to remove NaCl as rapidly as possible after chromatography on DEAE-cellulose. The effect of other salts are not so marked except for the case of KNO_3. Recently REPASKE[10] reported DPN reduction by hydrogen in extracts of *H. eutropha* was sensitive to high concentrations of salts.

When investigating possible metal activation of this enzyme, a non-specific activation was found for enzyme B purified in the absence of $MnCl_2$. This confirmed

TABLE IV

EFFECT OF SALTS ON ENZYME B

Hydrogen dehydrogenase preincubated with 20 μmoles Tris·HCl buffer (pH 7.8) and 125 μmoles of salt except in the case of K_2SO_4 75 μmoles, in a volume of 0.2 ml for 10 min at 30°. Reaction started by adding 2.8 ml of solution containing 280 μmoles Tris–HCl buffer (pH 7.8), 2 μmoles DPN and saturated with H_2.

Salt	Relative activity (%)
None	100
NaCl	46
LiCl	79
KCl	82
Potassium phosphate (pH 7.8)	91
KF	90
KBr	86
KNO_3	64
K_2SO_4	78
$KHCO_3$	78

TABLE V

EFFECT OF DIVALENT METAL SALTS ON HYDROGEN DEHYDROGENASE

Hydrogen dehydrogenase used in these experiments was purified by procedure B without $MnCl_2$ and had a specific activity of 51. Experiment A: Reaction started by adding the enzyme to 2.8 ml of solution containing 280 μmoles Tris–HCl buffer (pH 7.8), 2 μmoles DPN, 0.20 μmole ferrous ammonium sulfate or $MnCl_2$ or $CoCl_2$ and saturated with H_2. Experiment B: Hydrogen dehydrogenase preincubated with 150 μmoles Tris HCl buffer (pH 7.8), 0.20 μmole ferrous ammonium sulfate or $MnCl_2$ or $NiCl_2$ in 0.4 ml for 10 min at 30°. Reaction started by adding 2.6 ml of solution containing 200 μmoles Tris HCl buffer (pH 7.8), 2 μmoles DPN and saturated with H_2. Experiment C: Hydrogen dehydrogenase heated at 50° for 4 min with 0.6 ml of PEM buffer and in the case of the metal salts of Fe^{2+}, Mg^{2+}, Ni^{2+}, Co^{2+} replaced Mn^{2+} in PEM buffer. Reaction started as in Experiment B using 0.2 ml of supernatant after heat treatment.

Treatment	Rate (ΔA/min.)
Experiment A	
No metal salt	0.084
Fe^{2+}	0.104
Mn^{2+}	0.124
Co^{2+}	0.124
Experiment B	
No metal salt	0.022
Fe^{2+}	0.134
Mn^{2+}	0.130
Ni^{2+}	0.074
Experiment C	
No heating + Mn^{2+}	0.22
Heated, no metal salt	0
Fe^{2+}	0.22
Mg^{2+}	0.19
Mn^{2+}	0.22
Ni^{2+}	0.145
Co^{2+}	0.22

earlier observations with a crude enzyme preparation[11]. Enzyme activity was stimulated 47% by the addition of $MnCl_2$ (Table V, Expt. A).

Further investigation of this effect showed that these metals could protect the enzyme against high Tris concentrations for short periods of time (Table V, Expt. B). Storage of the enzyme B in 0.1 M Tris–HCl buffer (pH 7.8) at −20° for 3 days results in complete loss of activity. Also, $MnCl_2$ was only effective against denaturation by low concentrations of NaCl (0.1 M) for up to 30 min at room temperature.

Expt. C, Table V, indicates that the effect of the metal is to stabilize the enzyme against heat treatment. From these results the divalent cation seems to stabilize the enzyme rather than activate the enzyme. This is a reversible process as enzyme B can be dialysed free of manganese and the resulting enzyme preparation then becomes sensitive to inactivation by heat. To study the rate of $MnCl_2$ stabilization, enzyme B was prepared without added $MnCl_2$ (A). The two controls were this enzyme B preparation without the addition of $MnCl_2$ (B) and enzyme B prepared in the normal way (C). To (A) $MnCl_2$ added just before the heating of (A), (B), (C) as in Expt. C, Table V. There was no difference in activity of preparations (A) and (C), whereas (B) was completely inactive. Another method to establish the rate of stabilization is to use the same conditions as Expt. B, Table V and measure the activity of enzyme B to which $MnCl_2$ was just added prior to incubation with Tris buffer. In this experiment there was no difference in activity between enzyme B whether prepared in the presence of $MnCl_2$ or with $MnCl_2$ added before incubation. It was concluded that the stabilization process occurs within 5 min.

During purification, the most effective ratio of $MnCl_2$ to EDTA was found to be 0.7, values higher than this resulted in an enzyme requiring cysteine for maximum activity.

DISCUSSION

Manganese has been shown to stabilize a series of proteins against irreversible denaturation[12]. ROSENBERG[13] has extensively investigated the stabilization of carnosinase by Mn^{2+} and has concluded that the enzyme exists in several forms which are in equilibrium, the rate of inactivation being different for each form and the Mn^{2+}-form being the most stable. Other schemes have been proposed which include a Mn^{2+}-enzyme in equilibrium with different forms of the enzyme[12]. A similar scheme can account for the effect of Mn^{2+} on hydrogen dehydrogenase. Enzyme B or Mn^{2+}-enzyme has the following properties: (a) increased heat stability, (b) insensitivity to low concentrations of PCMB, (c) smaller K_m values, when compared to enzyme A. From these changes in properties of hydrogen dehydrogenase on the addition of $MnCl_2$, the protein is assumed to rearrange or form aggregates. Of particular interest is the change in the K_m values for both substrates and we can assume that the active site configuration has been altered in some unknown manner. The reduction in sensitivity to PCMB and heat stabilization could be part of the same phenomenon, *i.e.* on addition of $MnCl_2$ the protein involutes and hides the thiol groups within the molecule away from oxygen and sulfhydryl reagents. Manganese stabilization of hydrogen dehydrogenase is a rapid process when compared to the formation of Mn^{2+}-stabilized carnosinase[13]. The reversal of the Mn^{2+} effect by NaCl and Tris buffer is interpreted as the displacement of Mn^{2+} by Na^+ and Tris to give an unstable protein.

The effect of DPN on enzyme A (Table II) appears to be that of protecting the enzyme against Tris buffer in a similar manner to the protection of DPN-linked dehydrogenases against urea and heat denaturation[14]. DPN also prevents the complete inhibition by PCMB (Table III) which is consistent with the hypothesis that DPN combines with thiol groups of DPN-linked dehydrogenase[14].

The reversibility of hydrogen dehydrogenase has little physiological significance for the hydrogen bacteria and the presence of this type of H_2-producing enzyme in anaerobic bacteria has yet to be demonstrated.

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