The specificity of a diphosphopyridine nucleotide-linked hydrogenase*

A diphosphopyridine nucleotide (DPN)-linked hydrogenase[1] which appears to be a manganoflavoprotein was purified from *Hydrogenomnas ruhlandii*[2]. The reduction of DPN by molecular hydrogen depended on the presence of catalytic amounts of $MnCl_2$, cysteine, and flavin mononucleotide (FMN), and was stimulated by inorganic phosphate[3]. With crude hydrogenase preparations, a variety of compounds such as methylene blue (MB), 2,6-dichlorophenol indophenol, phenosafranin, benzylviologen (BV), riboflavin, FMN, flavin adenine dinucleotide, cytochrome *c*, and oxygen, but not triphosphopyridine nucleotide (TPN), could readily serve as H-acceptors in addition to DPN. The purification of hydrogenase with respect to its ability to effect the reaction,

$$H_2 + DPN \rightleftharpoons DPN + H^+ \qquad (1)$$

resulted in a progressive loss in ability to catalyze the reduction of all the other H-acceptors. Highest purity preparation catalyzed only DPN reduction and the D_2O exchange reaction**. Reaction (1) was shown to be reversible by demonstrating manometrically H_2 evolution from reduced DPN, which was generated by coupling purified hydrogenase to glucose-6-phosphate and glucose-6-phosphate dehydrogenase***. Thus, it is possible that the D_2O exchange reaction observed may simply be a consequence of the reverse reaction taking place. The place of the proton liberated in solution upon DPN reduction being taken by the D^+ of D_2O, resulting in HD formation.

The low 280 mμ/260 mμ absorption ratios obtained from spectrophotometric protein determinations during purification of the enzyme suggested that some DPN may have remained attached to the enzyme. Incubation of a partially purified hydrogenase preparation under H_2 showed an increased absorption at 340 mμ from which it was calculated that at least 2 moles of DPN/100,000 g protein were present. This same partially purified preparation could catalyze a slow reduction of BV but no oxygen reduction, under conditions when DPN reduction proceeded rapidly (Fig. 1). The addition of a catalytic amount of DPN, but not TPN, could spark the rate of reduction of these compounds (not shown for O_2), suggesting that their reduction was mediated by the prior pyridine nucleotide reduction. The reduction of O_2 by this metallo-flavoprotein may be similar to DPNH oxidase, which has been shown able to carry out the reaction,

$$DPNH + \tfrac{1}{2}\,O_2 \longrightarrow DPN^+ + H_2O \qquad (2)$$

without requiring additional enzymes[4].

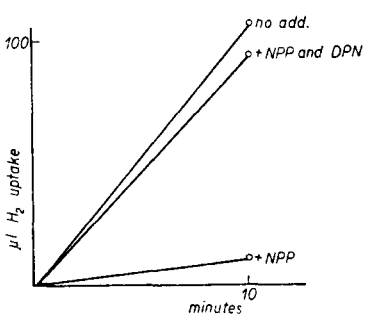

Fig. 1. The influence of DPN on the reduction of benzylviologen by H_2. The reaction mixture (3.0 ml) in Warburg flasks contained 450 μM tris (hydroxy) aminomethane buffer at pH 7.6, 3 μM cysteine, 5 μM $MnCl_2$, 0.005 μM FMN, 0.17 mg hydrogenase preparation, the additions shown (10 μM DPN and BV as H-acceptors and 0.1 μM DPN when used catalytically), and an atmosphere of 100% H_2 at 29° C.

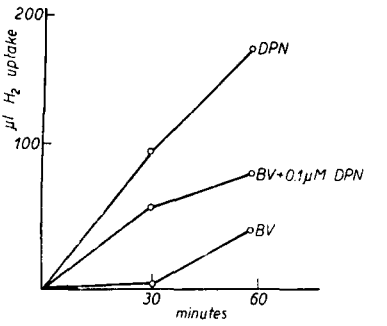

Fig. 2. The influence of nucleotide pyrophosphatase and DPN on methylene blue reduction by H_2. The reaction mixture (2.0 ml) in Warburg flasks contained 1.6 mg hydrogenase preparation, 30 μM phosphate buffer at pH 7.5, 1 μM $CoCl_2$, 10 μM MB, 0.1 mg NPP and 0.5 μM DPN as indicated, and an atmosphere of 100% H_2 at 30° C. The center-wells contained 200 μM KOH.

The reduction of TPN could be shown with a partially purified hydrogenase preparation if a catalytic amount of BV was added. This effect has been previously reported by PECK AND GEST for a soluble hydrogenase from *Clostridium pasteuranium*. The reduction of TPN under our conditions

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may be the result of a non-enzymic electron transfer from reduced DPN to TPN via BV. This contention is further supported by the observation that catalytic amounts of BV do not stimulate the rate of DPN reduction.

Further support for the contention that a pyridine nucleotide mediates a secondary reduction of these other H-acceptors is given by the effect of nucleotide pyrophosphatase (NPP)* on the reduction of MB by a crude hydrogenase preparation (Fig. 2). This crude enzyme preparation catalyzes a rapid MB reduction. The addition of NPP markedly inhibited the rate of dye reduction, and the inhibition was reversed by a catalytic amount of DPN.

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Isolation and properties of succinic dehydrogenase*

In previous communications the authors have described the solubilization, assay, and partial purification of succinic dehydrogenase[1-3]. The purpose of the present note is to outline the preparation of the enzyme in an essentially homogeneous state and to designate its salient properties. This enzyme is the primary dehydrogenase in the chain of proteins which link the oxidation of succinate to the reduction of cytochrome *c* in mitochondrial or purified particulate preparations[4]. Since this dehydrogenase catalyzes the one-step oxidation of succinate to fumarate in the presence of a suitable dye (phenazine methosulfate) and shares all the important characteristics usually associated with succinic dehydrogenase action, the demonstration that this reaction is catalyzed by a single, discrete protein molecule permits the identification of this protein as succinic dehydrogenase. A corollary of this conclusion is that the other components (protein or otherwise) which functionally link the oxidation of succinate to ferricyanide, cytochrome, methylene blue, *etc.* are members of an electron transport chain in the original particle.

The enzyme has been isolated from beef heart mitochondrial acetone powders[1,2]. Following extraction with 0.06 *M* tris(hydroxymethyl)aminomethane buffer, pH 8.9 and the removal of impurities by precipitation with protamine sulfate, the enzyme was precipitated by 0.5 saturated $(NH_4)_2SO_4$. After a brief dialysis the protamine and $(NH_4)_2SO_4$ precipitation steps were repeated; the enzyme was again dialyzed, and then subjected to two cycles of fractionation with calcium phosphate gel. The precipitate obtained between 0.3 and 0.40 saturated $(NH_4)_2SO_4$ was ultracentrifuged for 4 hours at 144,000 × *g* and the brown pellet was diluted, yielding a clear, amber solution of the dehydrogenase.

Examination in the analytical ultracentrifuge revealed the presence of a single component (Fig. 1). Electrophoretic analysis at pH 7.6 confirmed the above conclusion; only a trace of impurity was detectable. The sedimentation velocity (S_{20} = 6.5 S, determined at several protein concentrations) and a preliminary estimation of the diffusion constant indicate a molecular weight of the order of 110,000 to 140,000. The Q_{O_2} of the best preparations, isolated from fresh acetone powders, is 14,000 under the conditions of assay outlined elsewhere[5], and such preparations contain two atoms of Fe per mole of enzyme (1 atom Fe per 68,000 g protein). On the other hand a number of preparations have been obtained from somewhat older acetone powders with a Q_{O_2} of 7,500 and containing 1 atom

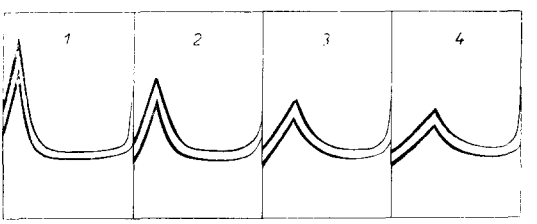

Fig. 1. Sedimentation pattern of succinic dehydrogenase in the ultracentrifuge. Protein concentration, 9.1 mg per ml; buffer, 0.1 *M* NaCl — 0.005 *M* phosphate, pH 7.6; temperature, 4.65°; speed, 59,771 r.p.m.; bar angle, 45°. Centrifugation was continued until the sedimenting peak reached the bottom of the cell. Each of the four exposures was spaced 16 minutes apart.

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