Volume 306, number 2,3, 125–128 FEBS 11272 July 1992

Photogeneration of NADH under coupled action of CdS semiconductor and hydrogenase from *Alcaligenes eutrophus* without exogenous mediators

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Received 12 May 1992

Photoreduction of NAD has been accomplished by a system consisting of the NAD-dependent hydrogenase from *Alcaligenes eutrophus* immobilized on CdS particles with formate as artificial electron donor. Enzymatically active NADH is formed under illumination of this system by visible light. Accumulation of the coenzyme dimer $(NAD)_2$ was not detected. NAD photoreduction is supposed to proceed via the direct electron transfer from the semiconductor to the enzyme electron transport chain. However, NADH formation as a result of hydrogenase interaction with anion-radicals ($CO_2^{\cdot -}$) formed in the course of formate photooxidation cannot at present be excluded.

Photocatalysis; Electron transfer; NAD photoreduction; CdS semiconductor; Hydrogenase; *Alcaligenes eutrophus*

1. INTRODUCTION

The possibility of the realization of some enzymatic reactions by the coupled action of inorganic semiconductor photocatalysts and enzymes has been described earlier [1–4]. This coupling is readily achieved with the help of external electron carriers (e.g. viologens). Additionally, the enzymatic reaction may be induced via a direct electron transfer from the semiconductor conductivity zone to the enzyme reaction center. An efficient electron exchange in a system consisting of an inorganic semiconductor (TiO_2, CdS) and the hydrogenase from *Thiocapsa roseopersicina*, was shown to result in hydrogen photogeneration [5,6].

Numerous attempts have been made to develop NADH regeneration systems which could be employed in biocatalytic transformations [7,8]. A number of enzymatic, chemical, electrochemical and photochemical methods of NADH regeneration have been proposed. One of the possible photochemical approaches might be a combined action of inorganic semiconductors and NAD-dependent enzymes. In the systems studied heterogeneous powders or colloids (TiO_2, CdS) were used to accomplish the photoreduction of a soluble electron mediator, methyl viologen. Subsequent NADH regeneration in the presence of lipoamide dehydrogenase and electron donors (mercaptoethanol, formate) was observed [4,7]. No NADH formation occurred in the absence of methyl viologen in these systems.

Direct coupling of an inorganic semiconductor with the active center of a NAD-dependent enzyme seems especially interesting, because this would be a premise for the creation of a photobiocatalyst of NADH regeneration which would be driven solely by light energy and would not require any exogenous mediators. The correct choice of the enzyme employed for the realization of the mediatorless cofactor regeneration system will be decisive. Recently we have provided the first example of the feasibility of electron transfer between the active site of NAD-dependent dehydrogenase and a solid support. NAD-dependent hydrogenase was grafted on to the surface of a carbon electrode enabling direct electron exchange between the electrode and the enzyme active center [9].

NAD-dependent hydrogenases found in some strains of hydrogen bacteria comprise a closely related family of one of the most complex types of hydrogenases characterized. They are composed of four non-identical subunits and contain at least five types of prosthetic groups – FMN, Ni, and 3 types of iron–sulphur clusters [10]. The NAD-dependent hydrogenases are organized in two more or less tightly associated two-subunit structural blocks, hydrogenase and diaphorase. The hydrogenase part accommodates the H_2-activating site, which is assumed to reside on the Ni atom, while the diaphorase part of the enzyme comprises the NAD-binding site, which is associated with FMN.

In the present paper the photoreduction of NAD to NADH achieved by coupled action of the inorganic semiconductor, CdS, and the NAD-dependent hydrogenase from *Alcaligenes eutrophus*, in the absence of any exogenous electron carriers, is described.

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2. MATERIALS AND METHODS

Gas chromatographic analysis was performed with a Chrom-4 gas chromatograph using a molecular sieve 5 Å column. Absorption spectra were recorded with an Hitachi 557 spectrophotometer. Illumination experiments were performed in a glass vial fitted with a rubber septum. Samples (1 ml) were deoxygenated by flushing with argon and then irradiated with a 250 W Hg lamp in the region 400–800 nm ($5 \cdot 10^5$ $erg \cdot cm^{-2} \cdot s^{-1}$) under stirring. Light was filtered through cut-off glass filters. All the experiments were carried out at light saturation ($>3 \cdot 10^5$ $erg \cdot cm^{-2} \cdot s^{-1}$) and at saturating concentrations of the sacrificial electron donor, formate (2 M).

CdS (99.999%), potassium 2-ketoglutarate (Aldrich), NAD (Serva), L-glutamic dehydrogenase from bovine liver, bis-tris propane (Sigma), MES, Tris (Reanal), sodium formate, potassium phosphate, and ammonium phosphate (Reachim) were used. Hydrogenase from the hydrogen-oxidizing bacterium *A. eutrophus* H16 was purified as described elsewhere [11]. The enzyme preparations with a specific activity of 8–15 U/mg in a NAD reduction assay with hydrogen were used for the experiments. The enzyme was more than 90% pure according to electrophoretic tests.

The NADH concentration was determined spectrophotometrically at 340 nm ($\varepsilon = 6.220 \, l \cdot mol^{-1} \cdot cm^{-1}$). Enzymatic identification of NADH was performed by L-glutamic dehydrogenase in the presence of 0.01 M 2-ketoglutarate and 0.08 M ammonium phosphate [12].

3. RESULTS AND DISCUSSION

During illumination of the CdS suspension containing NAD-dependent hydrogenase from *A. eutrophus* and formate as electron donor NAD photoreduction occurs (Fig. 1). The product formed has the absorption spectrum characteristic of NADH and is completely reoxidized in the course of L-glutamic dehydrogenase-catalyzed reductive amination of 2-ketoglutarate. Thus, as a result of the photoreaction NAD is specifically transformed to NADH without formation of the dimer, $(NAD)_2$, the product of one-electron reduction. In the absence of formate, or semiconductor, or hydrogenase, NAD photoreduction does not occur.

The rate of NADH photogeneration in the system is proportional to the hydrogenase concentration up to an enzyme loading of 0.03 U hydrogenase/mg CdS, and is saturated in the range 0.07–0.13 U/mg CdS. Hydrogenase in the amounts used was completely absorbed on the surface of the semiconductor and was not desorbed in the course of the photoreaction.

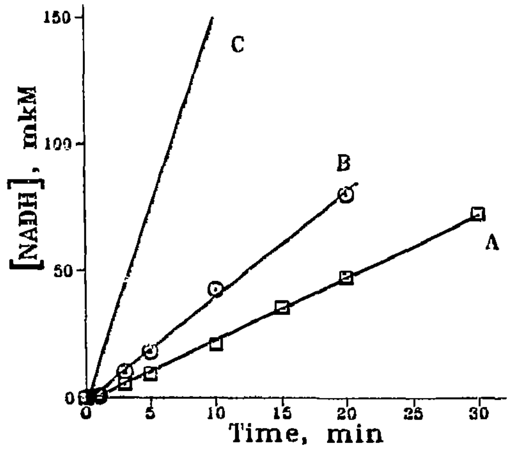

Fig. 1. Kinetic curves of photoreduction of NAD (A) and reduction of NAD by hydrogen (B,C): hydrogenase 0.064 U; NAD $1.5 \cdot 10^{-3}$ M; HCOONa 2 M; $CdCl_2$ 10^{-2} M; 0.05 M MES (pH 6.5). (A,B) hydrogenase, immobilized on 5 mg CdS; (C) hydrogenase in solution.

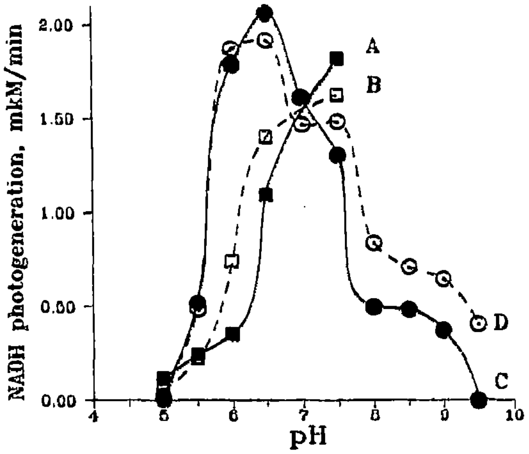

Fig. 2. pH-dependence of NAD photoreduction. Hydrogenase 0.13 U; CdS 5 mg; NAD $1.5 \cdot 10^{-3}$ M; HCOONa 2 M; 0.1 M MES (A,B); Tris 0.1 M (C,D). Detection of products of photoreduction by absorption at 340 nm (A,C) and detection of NADH by L-glutamic dehydrogenase (B,D).

MES, Tris, and bis-tris propane buffers provide the highest rates of the reaction. In the presence of multicharged anions (phosphate, borate) NADH formation is inhibited. The pH-optimum for NADH photoreduction in Tris and MES buffers (Fig. 2) is shifted towards the acidic side, as compared to the optimal pH for NAD reduction by hydrogen, catalyzed by hydrogenase in solution (pH 7.5–9.0). This may be accounted for, at least in part, by a local change of the surface charge of the semiconductor particles ($pK_{CdS} \approx 6$ [13]). It should be noted that at basic pH's NAD phototransformation occurs in the presence, as well as in the absence, of the hydrogenase. The product thus formed has an absorption maximum at 320–330 nm and fails to act as substrate for glutamate dehydrogenase.

Comparison of the rates of NAD photoreduction and NAD reduction by hydrogen in the dark, catalyzed by hydrogenase under the conditions optimal for the photoreaction (0.05 M MES, pH 6.5, 2 M formate), is presented in Fig. 1. Under these conditions the rate of photoreduction corresponds to 50% of the rate of NAD reduction by hydrogen catalyzed by the immobilized enzyme, and to 16% of that catalyzed by the soluble one. This makes 4% of the NAD reduction by hydrogen catalyzed by the soluble enzyme under optimal conditions (0.1 M phosphate buffer, pH 8.0, without formate). The K_m for NAD in the photoreaction ($2.2 \cdot 10^{-4}$ M) is close to the corresponding value for the process of NAD reduction by hydrogen by soluble enzyme ($5.6 \cdot 10^{-4}$ M [14]).

While discussing the mechanism of NAD photoreduction by hydrogenase the specific photochemical properties of the system, formate–CdS, should be taken into consideration. It was shown [15] that in a CdS suspension containing formate, the surface of the semiconductor under illumination is reduced and hydrogen is evolved. This is accounted for by formation of $CO_2^{\bullet-}$ anion radical ($E_0 \approx -0.97$ V) which reduces cadmium ions on the surface of the particles to $Cd^{\bullet}$. The metallic surface of the semiconductor catalyzes hydrogen generation by the reaction, $H^+ + e^- = \frac{1}{2}H_2$, utilizing electrons photogenerated in the conductivity zone of the semiconductor. Thus, in the system, formate–CdS–hydrogenase–NAD, the enzyme could use H_2 or $CO_2^{\bullet-}$ as the primary reducing agents, as well as the electrons photogenerated in the conductivity zone of the semiconductor for NAD photoreduction.

Hydrogen photoevolution in the system, formate–CdS, under our experimental conditions started only 15–30 min after the onset of illumination. Addition of 0.01 M $CdCl_2$ increased the lag-period before hydrogen evolution up to 50–70 min. The presence of hydrogenase or NAD produced no effect on H_2 photoevolution. On the other hand, NADH formation proceeded without any pronounced induction period (both in the absence and presence of $CdCl_2$). Therefore, participation of hydrogen in the process of enzymatic NAD photoreduction to NADH may be excluded.

It is known that the anion radical, $CO_2^{\bullet-}$, generated in the course of radiolysis of aqueous solutions of formate is able to reduce NAD to $NAD^{\bullet}$ which undergoes subsequent dimerization to $(NAD)_2$ [16]. As already mentioned above, during illumination of CdS suspensions containing formate and NAD in the absence of the enzyme the NAD dimers were not detected. Thus, some other route of radical quenching seems to be functioning. Rapid oxidation of the anion radical $CO_2^{\bullet-}$ or $NAD^{\bullet}$ radical by the semiconductor might occur. This is confirmed by the fact that at a low ratio of enzyme/ semiconductor darkening of the particles of CdS occurs due to the reduction of cadmium ions to $Cd^{\bullet}$ by $CO_2^{\bullet-}$ in the course of photoreduction. Also, at hydrogenase/ semiconductor ratios higher than 0.1 U/mg, CdS particles preserve intact their yellow color. Probably, in this case during the photoreaction, all the $CO_2^{\bullet-}$ radicals are used for NAD reduction. The data obtained do not allow a decision on the exact mechanism of $CO_2^{\bullet-}$ participation in NAD photoreduction. One can propose a direct interaction of the anion radicals with hydrogenase as well as their oxidation by photogenerated holes.

At high ratios of hydrogenase/semiconductor the enzyme utilizes all the reducing equivalents available in the system: the anion radical, $CO_2^{\bullet-}$, as well as electrons of the conductivity zone of the semiconductor. This is indicated by the fact that any further increase in the ratio of hydrogenase/semiconductor higher than 0.1 U/ mg CdS has hardly any influence on NADH synthesis (though all the protein is quantitatively absorbed on the semiconductor surface).

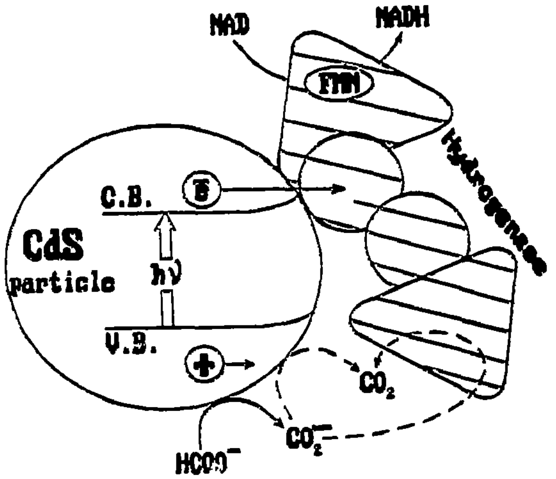

Fig. 3. Hypothetical scheme of NAD photoreduction by the system, formate–CdS semiconductor–hydrogenase from *Alcaligenes eutrophus*.

Thus, photogeneration of NADH in the system, formate–CdS–hydrogenase–NAD, can proceed either via a direct transfer of electrons from the semiconductor to the enzyme electron transport chain through multiple coupling points provided by NAD-dependent hydrogenase (e.g. iron-sulphur clusters located close to the protein surface) or as a result of hydrogenase interaction with $CO_2^{\bullet-}$ formed in the course of formate photooxidation, or due to both processes (Fig. 3). It should be noted that NAD photoreduction in a similar system with lipoamide dehydrogenase was obtained only in the presence of external electron carrier (methyl viologen). The photogeneration of NADH described in the present paper which occurs without any exogenous electron mediators may be due to a combination of some specific properties of NAD-dependent hydrogenase from *A. eutrophus*, favorable enzyme binding with semiconductor surface, and optimal photoreaction conditions ensuring the effective direct electron exchange between the semiconductor and the enzyme electron transport chain. However, participation of carriers, e.g. formed as a result of partial denaturation of the enzyme, cannot be completely excluded.

Thus, the data obtained testify to the possibility of NAD photoreduction occurring under coupled action of the inorganic semiconductor CdS and the NAD- dependent hydrogenase from *Alcaligenes eutrophus* in the absence of exogenous electron carriers.

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