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Metal as a novel type of the enzyme substrate

Metallic cadmium photogenerated in the system CdS-formate as a substrate of the NAD-dependent hydrogenase

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The process of NAD^+ photoreduction under the coupled action of CdS semiconductor and NAD-dependent hydrogenase from hydrogen-oxidizing bacterium *Alcaligenes eutrophus* may be divided into light and dark stages. At the first stage, illumination of the system leads to the photooxidation of the sacrificial electron donor and results in the reduction of the semiconductor surface. At the second dark stage NAD^+ is reduced to NADH in the presence of hydrogenase. Atoms of metallic Cd(0) are shown to be the true substrate of the enzymatic reaction The prerequisite for the electron transfer from Cd(0) to hydrogenase is enzyme adsorption on the semiconductor surface. the redox center of the hydrogenase reacting with Cd(0) atoms resides on the flavin-containing heterodimer of the protein. The activity of the hydrogenase immobilized on CdS in the reaction of NAD^+ reduction by metallic Cd is close to the enzyme activity with the physiological substrates in solution. Thus, the first example of a metal being the substrate of the enzymatic process is presented

NAD^+ reduction; Hydrogenase; *Alcaligenes eutrophus*; CdS semiconductor; Metallic cadmium

1. INTRODUCTION

A broad variety of substances ranging from the simplest inorganic molecules (H_2, O_2, water, etc.) to complex macromolecular compounds (proteins, DNA, RNA) may act as substrates of enzymatic reactions. Direct electron exchange between the enzyme active centers and the bulk of the solids, e.g. electrodes or semiconductors has also been experimentally verified. However, until now there was a group of potential enzyme substrates for which no documented evidence for their participation in the enzymatic reactions existed. These are metals in their zero redox state, in particular transition metals, which easily undergo redox transitions and are constituents of the active sites of a number of enzyme systems.

Hydrogenases, hydrogen activating enzymes, differ significantly in their structural organization, catalytic properties, stability and physiological function. Only for a few enzymes of this class physiological substrates are known (cytochromes, ferredoxins, flavodoxins, rubredoxins, factor F420, NAD). Hydrogenases are also known to catalyze the reactions of H_2 uptake with a number of artificial one- and two-electron acceptors and H_2 evolution from various electron donors (e.g. reduced viologens).

NAD-dependent hydrogenases found in some strains of hydrogen bacteria constitute a closely related family of one of the most complex types of hydrogenases characterized up to date. They are composed of four nonidentical subunits and are organized in two structural blocks each comprising one smaller and one larger protein subunit [1,2]. On of these heterodimers contains the center of H_2 activation residing on the Ni ion, while the other comprises the NAD-binding site, associated with FMN. The binding sites of these physiological substrates are connected via an electron transport chain composed of a number of iron–sulfur clusters of several types.

Direct coupling of some hydrogenases with inorganic semiconductors [3–7] or carbon electrodes [8] results in hydrogen activation due to electron exchange between the solid support and the enzyme electron transport chain. NAD-dependent hydrogenase grafted on the electrode [9–11] surface was shown to reduce NAD^+ to NADH in the absence of exogenious electron mediators. Recently we have provided the first example of the feasibility of photogeneration of NADH under mediatorless coupled action of inorganic semiconductor CdS and NAD-dependent hydrogenase from *Alcaligenes eutrophus* [12].

In the present paper evidence is presented that the NAD-dependent hydrogenase from *A. eutrophus* H16 is

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†With deep regret we inform the readers of the decease of academician A.A. Krasnovsky.

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able to utilize a novel type of substrate, metallic Cd(0), for NAD$^+$ reduction.

2. MATERIALS AND METHODS

CdS (99.999%, Aldrich), NAD$^+$ (Serva), MES, NADH (Reanal), sodium formate, potassium phosphate, potasium ferricyanide (Reachim) were used NAD-dependent hydrogenase from *A eutrophus* H16 was purified as described elsewhere [13]. Enzyme preparations with a specific activity of 8–15 U/mg in the NAD$^+$ reduction assay with hydrogen were used for the experiments. The enzyme was more than 90% pure according to electrophoretic tests. The procedure for obtaining and separation of active hydrogenase heterodimers will be described elsewhere The enzymatic activity of the flavin-containing heterodimer was assayed in the reaction of NADH (0.2 mM) oxidation by ferricyanide (0 5 mM).

Illumination experiments were performed in a special glass vial fitted with rubber septum Samples (1 ml) were deoxygenated by flushing with argon and then irradiated with a 250-W Hg lamp in the 400–800 nm range (5 10^5 erg·cm^{-2} s^{-1}) under stirring. Light was passed through cut-off glass filters. The amount of photooxidized formate was estimated by the amount of CO_2 formed in the photoreaction. In NAD$^+$ reduction experiments the formate-CdS system was first illuminated with light, then after termination of the illumination hydrogenase and NAD$^+$ were added to the vial constantly flushed with argon. NADH concentration was determined spectrophotometrically at 365 nm (ε = 3.4 l·mmol^{-1}·cm^{-1}) Absorption spectra were recorded with a Hitachi 557 spectrophotometer.

Hydrogenase adsorbtion on CdS was studied under the same conditions as NAD$^+$ reduction. The amount of the adsorbed protein was measured by the decrease in the activity and absorbance at 280 nm of the supernatant solution

Gas chromatographic analysis of CO_2 was performed with a Chrom-4 gas chromatograph using Porapack T column with helium as carrier gas.

3. RESULTS AND DISCUSSION

The process of NAD$^+$ photoreduction to NADH in the system formate-CdS semiconductor-NAD-dependent hydrogenase described in a previous publication [12] may be subdivided into the light and the dark stages. At the first stage of the process the electron donor, formate, is photooxidized by the semiconductor. The photoreaction does not require the presence of the enzyme or NAD$^+$ and stops with the termination of illumination. At the second, dark stage, the enzymatic NAD$^+$ reduction occurs at the account of the reducing agent formed during the light phase.

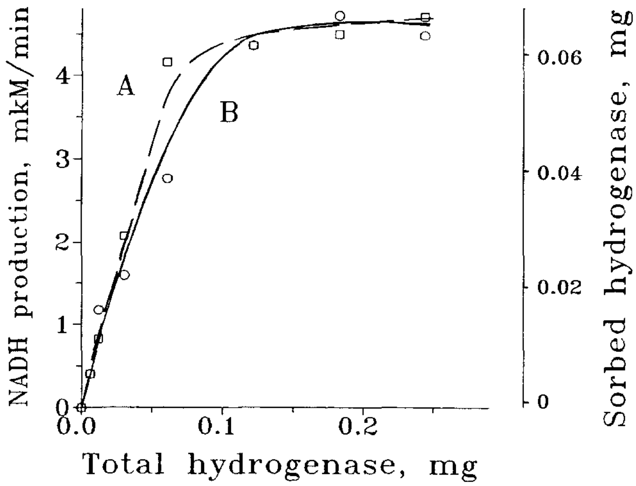

Fig. 1 Dependence of hydrogenase adsorption on CdS (A) and NAD$^+$ reduction (B) upon the amount of hydrogenase. NAD$^+$ reduction: 5 mg CdS is illuminated for 5 min in 1 ml 0 05 M MES pH 6.5 with 2 M HCOONa, then, illumination is switched off, hydrogenase and NAD$^+$ (1 5·10^{-3} M) are added.

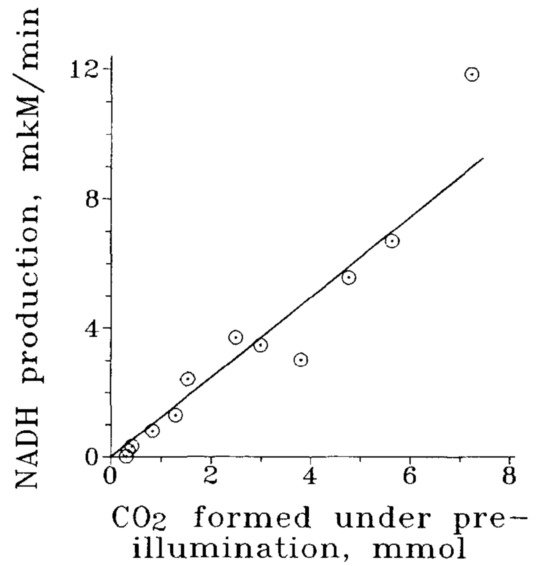

Fig. 2. Dependence of NAD$^+$ reduction upon the amount of CO_2 formed during pre-illumination of the system formate-CdS semiconductor 5 mg CdS are illuminated in 1 ml 0.05 M MES pH 6.5 with 2 M HCOONa, then illumination is switched off and hydrogenase (25 mg) and NAD$^+$ (1.5·10^{-3} M) are added.

Addition of hydrogenase and NAD$^+$ to the pre-illuminated system formate-CdS results in NAD formation in the dark. NAD$^+$ reduction proceeds at a constant rate without any noticeable lag-period. The reaction rate is proportional to the amount of hydrogenase adsorbed by the semiconductor, but not to the total amount of enzyme in the system (Fig. 1). The rate of NAD$^+$ reduction is enhanced with the increase of the illumination period of the formate-CdS system and is directly proportional to the amount of the donor photooxidized in the system (Fig. 2). The maximum rate of NAD$^+$ reduction obtained after 2 h of pre-illumination is 3-fold higher than the rate of NAD$^+$ reduction by hydrogen, catalyzed by hydrogenase immobilized on CdS, and is close to the rate of NAD$^+$ reduction by hydrogen catalyzed by the soluble enzyme under the same experimental conditions.

The time interval up to 20 min between the termination of illumination of the formate-CdS system and the addition of hydrogenase and NAD$^+$ has no effect on the rate of NADH formation in the dark. Moreover, the exposure of the system to oxygen for 10 min in the dark

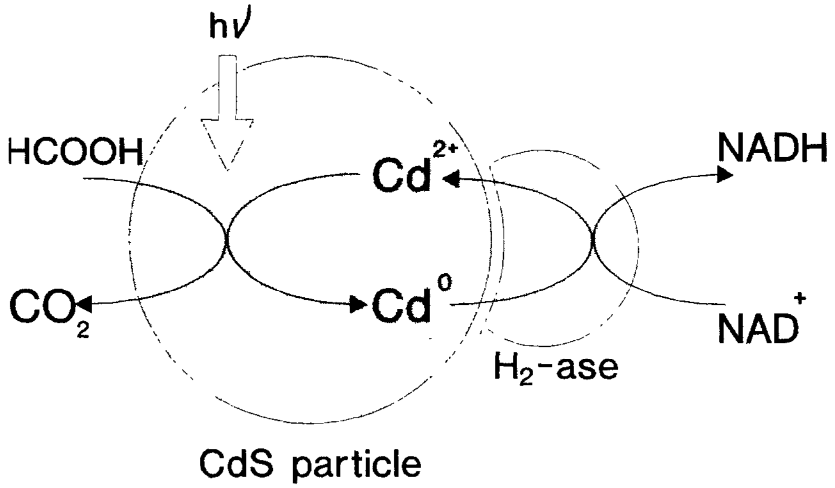

Fig 3. Scheme of hydrogenase (H_2-ase) catalyzed NAD^+ reduction with metallic cadmium photogenerated in the system formate-CdS semiconductor.

prior to addition of the enzyme and coenzyme also does not affect the rate of NAD^+ reduction.

Thus, NADH formation on the addition of hydrogenase and NAD^+ to the pre-illuminated formate-CdS system occurs due to utilization of the reductant which is stable for a long time both under anaerobic and aerobic conditions and is accumulated in the course of formate photooxidation by the semiconductor.

Four types of reducing agents are formed in the photochemical formate-CdS system [14,15]. These are electrons of the CdS conductivity zone, $CO_2^{\bullet-}$ anion-radicals, hydrogen and metallic Cd(0) formed in CdS photoreduction. The termination of illumination stops both formate oxidation by the semiconductor and the formation of all types of reductants.

Anion radicals $CO_2^{\bullet-}$ are readily oxidized by oxygen and undergo rapid dimerization in the absence of oxidants ($2k = 1.5 \cdot 10^9\ l \cdot mol^{-1} \cdot s^{-1}$) [16]. Thus, $CO_2^{\bullet-}$ radicals will disappear shortly after the end of illumination both under aerobic or anaerobic conditions and may not account for NAD^+ reduction by hydrogenase added to the pre-illuminated formate-CdS system.

In spite of the sufficiently low redox potential (−0.66 V [17]), the electrons of the CdS conductivity zone do not participate in the enzymatic reduction of NAD^+ either. Detailed studies of the mechanism of formate photooxidation by CdS revealed that the electrons of the conductivity zone rapidly disappear after termination of illumination due to chemical reactions [15].

Participation of molecular hydrogen in NAD^+ reduction seems rather unlikely because NADH production proceeds at the same rate after flushing of the pre-illuminated system with argon. Hydrogen participation in NAD^+ reduction was unequivocally ruled out by the experiments employing the flavin-containing hetero dimer of the hydrogenase. The flavin-containing fragment (heterodimer) of the enzyme, isolated under special conditions, catalyzes reactions of nicotinamide coenzymes (diaphorase activity with one- and two-electron acceptors, transhydrogenase activity) but is devoid of the H_2-activation center and is unable to use hydrogen as a substrate. Nevertheless, the rate of NAD^+ reduction by the system formate-CdS-hydrogenase is unaffected when native hydrogenase is replaced by its flavin-containing heterodimer, if the overall diaphorase activity in the system is preserved (not shown).

After a prolonged illumination of CdS in the presence of formate, the originally yellow semiconductor particles turn black. Darkening of the semiconductor particles is caused by the formation of cadmium atoms due to photoreduction of Cd^{2+} cations on the CdS surface. According to studies of the mechanism of formate photodecomposition by the CdS semiconductor, metallic Cd(0) is formed upon the reduction of Cd^{2+} cations of the semiconductor lattice as a result of the concerted action of electrons of the conductivity zone and anion radicals $CO_2^{\bullet-}$. The amount of the Cd(0) formed is nearly equal to that of the formate photooxidized by the semiconductor. Thus, metallic cadmium Cd(0) may accumulate in significant amounts in the system after illumination and appears to be stable under experimental conditions. Metallic cadmium is relatively stable under oxygen in the absence of catalysts, e.g. methyl viologen. The redox potential of the Cd(0) atoms formed on the surface of CdS is assumed to be within the range of −1.175 V (reaction $CdS + 2e = Cd(0) + S^{2-}$) to −0.403 V (reaction in solution $Cd^{2+} + 2e = Cd(0)$) and thus favors NAD^+ reduction.

These results altogether allow to conclude that the atoms of metallic Cd(0) are the true substrate of the hydrogenase absorbed on the surface of the semiconductor in the process of NAD^+ reduction (Fig. 3). The fact that the activity of immobilized hydrogenase at NAD^+ reduction with cadmium is close to the activity of the soluble hydrogenase in the assay with NAD$^+$ and hydrogen indicates a high efficiency of the electron transfer from Cd(0) to the enzyme reaction center.

The ability of the flavin-containing heterodimer of NAD-dependent hydrogenase to reduce NAD$^+$ when absorbed on the semiconductor allows to assume that the gateway for the electrons transferred from Cd(0) resides either on the α- or δ-subunit of the hydrogenase protein. Thus, electrons may be transferred from Cd(0) to the iron-sulphur clusters [4Fe-4S] located on the δ-subunit, or to [2Fe-2S] or FMN residing on the α-subunit [1].

Simple calculations show that the number of Cd(0) atoms participating in the process of NAD$^+$ reduction is far in excess of the number of metal atoms accessible to the adsorbed hydrogenase, even assuming the monomolecular coverage of the semiconductor surface by the protein. Actually, up to 20% of cadmium ions may be reduced to Cd(0) in the lattice of the semiconductor on illumination. It may be hypothesized that the lattice of the semiconductor acts as an 'electron pump' constantly regenerating Cd(0) located in the vicinity of hydrogenase reaction centers. These 'terminal' Cd(0) atoms might be regarded as semiconductor 'reaction centers' oscillating between the ionic and neutral forms and injecting electrons into the hydrogenase electron transport chain in the course of enzymatic process.

The important difference between the phenomenon observed in the present paper and the previously reported mediator-less coupling of hydrogenases and other enzymes with the electrodes is that in the latter cases the electrode material acts solely as transducer of the electrons, channelling them into the enzyme active sites, while the change of the redox state of cadmium in its interaction with the hydrogenase occurs. Thus, metallic cadmium may be classified as a substrate of NAD-dependent hydrogenase. As far as we know, this is the first example of the metal being a substrate of an enzyme.

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