ISSN 0003-6838, Applied Biochemistry and Microbiology, 2018, Vol. 54, No. 3, pp. 316–319. © Pleiades Publishing, Inc., 2018.
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Reactions of NADH Oxidation by Tetrazolium and Ubiquinone Catalyzed by Yeast Alcohol Dehydrogenase

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Received November 3, 2017

Abstract—The processes of NADH oxidation by *p*-NTF violet and ubiquinone catalyzed by isolated yeast alcohol dehydrogenase in aqueous and water-alcohol buffer solutions were studied. In the presence of *p*-NTF in aqueous solution at a pH of 6–7, NADH oxidation was extremely slow due to inhibition of the enzyme by the remaining enzyme-bound hydrophobic product, formazan, which forms during the reduction of *p*-Nitrotetrazolium. However, when the medium was alkalinized to a pH of 8–9 or when alcohol (ethanol or isopropanol) was added, formazan was desorbed from the enzyme, leading to an increase in the NADH oxidation rate. It was assumed that this redox reaction can be used as the basis for colorimetric measurement of the activity of different alcohol dehydrogenases. Tetrazolium reduction by alcohol was not observed at any value within the entire pH range. NADH oxidation in the presence of the enzyme and ubiquinone was also slow, even with the addition of alcohol, but its rate increased when the medium was acidified to a pH of 5.5–6. When a Tris-phosphate buffer was replaced with HEPES, a quasi-vibrational process was observed: NADH oxidization with ubiquinone to NAD^+ and its subsequent reverse recovery with alcohol to NADH.

Keywords: alcohol dehydrogenase, NADH, ubiquinone, electron transfer, *p*-Nitrotetrazolium, formazan

DOI: 10.1134/S0003683818030067

INTRODUCTION

Alcohol dehydrogenase (ADH) is a water-soluble dimeric (sometimes tetrameric) enzyme catalyzing the oxidation of alcohols and acetals to aldehydes and ketones [1]. NAD^+ is used as electron acceptor:

$$C_2H_5OH + NAD^+ \leftrightarrow C_2H_4O + NADH + H^+.$$

The reaction is accompanied by the release of a proton. The equilibrium of the reaction can be shifted by changing the pH and substrate concentration.

Since ADH is a key enzyme in the metabolism of alcohols in mammals, fish, insects, bacteria, yeasts, etc., the study of its properties is very important [1]. The use of ADH as a high sensitive sensor makes it possible to measure small concentrations of alcohol to solve applied problems [2].

The natural activity of ADH and other NAD-dependent dehydrogenases is usually determined photometrically by via measurement of the change in absorbance at a wavelength of 340 nm, at which NADH has absorption but NAD^+ does not [3]. Another method of dehydrogenase activity measurement is based on the photometric determination of dyed formazans, the products of tetrazolium salt reduction [4, 5]. Tetrazolium salts are often used to determine the activity of mitochondrial membrane dehydrogenases, but they are rarely used to measure the activity of ADH and other water-soluble dehydrogenases due to the low reaction rate [6, 7]. Dimethyl-thiazol-diphenyl-tetrazolium bromide (MTT) is usually used as the tetrazolium [4], whereas phenazine methosulfate is used to accelerate the reaction with MTT [5]. The monograph [6] considered various tetrazolia and found that only p-nitrotetrazolium violet (*p*-NTF) does not violate the metabolism of living cells. Phenazine methosulfate is not required to accelerate its action. In this regard, it was of interest to study the possible use of this tetrazolium in the absence of phenazine methosulfate to determine ADH activity.

Some enzymes of the ADH family use retinol but not ethanol as a substrate [8]. Their ability to oxidize retinol is competitively inhibited by high ethanol concentrations. There is an ADH family that prefers retinal as substrate instead of acetaldehyde [8]. Alcohol : quinone oxidoreductase activity, which was attributed to quinone-dependent ADH, was detected in the respiratory chain of some bacteria [9, 10]. However, this conclusion should be confirmed by experiments with isolated ADH.

The goal of the present work was to determine the possibility of NADH oxidation in aqueous and water-alcohol solutions catalyzed by isolated yeast ADH in the presence of *p*-NTF or ubiquinone and to reveal the optimal conditions.

Table 1. Rate of NADH oxidation (over the first hour) in ADH solutions differing in composition and pH

Solution composition	NADH:tetrazolium- reductase, μM/h/mg of protein	NADH:ubiquinone reductase, μM/h/mg of protein
15% ethanol + 1.25 μM ADH + 500 μM NADH + 200 μM *p*-NTF, pH 7	625	–
15% isopropanol + 1.25 μM ADH + 500 μM NADH + 200 μM *p*-NTF, pH 7	136.4	–
30% isopropanol + 1.25 μM ADH + 500 μM NADH + 200 μM *p*-NTF, pH 6	9.6	–
30% isopropanol + 1.25 μM ADH + 500 μM NADH + 200 μM ubiquinone, pH 6	–	1250
30% isopropanol + 1.25 μM ADH + 500 μM NADH + 200 μM *p*-NTF, pH 9	35.9	–
30% isopropanol + 1.25 μM ADH + 500 μM NADH + 200 μM ubiquinone, pH 9	–	0

MATERIALS AND METHODS

The following reagents were used in the work: purified yeast ADH (Mannheim Boehringer, Germany), *p*-nitrotetrazolium violet (*p*-NTF) (Reanal, Hungary), NADH and NAD^+ (Reanal, Hungary), Tris(hydroxymethyl)aminomethane phosphate (Reanal, Hungary), HEPES (Dia-M, Russia), ubiquinone Q2 (Ferak Berlin, Germany), and ethanol and isopropanol (chemically pure).

Two types of buffer solutions were used: 10 mM Tris-phosphate buffer and 10 mM HEPES. To accelerate the enzymatic reaction, 15 to 30% ethanol or isopropanol were added.

An ADH solution (0.04 mg/mL) was placed in the quartz cuvette (1 cm) of a 5400UF spectrophotometer (PromEkoLab, Russia) at 20°C. The NADH and formazan concentrations were determined at 340 nm and 540 nm, respectively. The enzyme activity was determined by measuring the oxidation and reduction rates for 1 h.

RESULTS AND DISCUSSION

NADH Oxidation and p-NTF Reduction

In 10 mM tris-phosphate buffer at neutral pH values of 6–7, ADH was almost unable to oxidize NADH or reduce *p*-NTF to formazan. NADH oxidation was blocked, since the water-insoluble formazan that formed in the first stage could not desorb from the enzyme, inhibiting the product reaction. This corresponded to the well-known fact that product desorption from the active site is a rate-limiting step of many enzymatic reactions, including those catalyzed by dehydrogenases [11, 12]. It was found earlier that NADH desorption from ADH is accelerated by UV irradiation due to instantaneous heating of the active site [11, 12].

After the addition of 15–20% ethanol or isopropanol to the reation mixture, ADH easily oxidized NADH and reduced p-NTF to formazan (Table 1):

$$\text{NADH} + p\text{-NTF} \rightarrow \text{NAD}^+ + \text{formazan}$$

This reaction was virtually irreversible at neutral pH values, because formazan is not soluble in water. It was accompanied by the appearance of an formazan absorption band at 540 nm. The addition of alcohol led to a decrease in the polarity of the ADH environment, which helped to desorb formazan from the enzyme. Under these conditions, the alcohol was also able to reduce the formed NAD^+ to NADH, which allowed the process to proceed without any inhibition for many hours (Fig. 1). In the presence of 15% ethanol, the NADH oxidation rate was 625 μM/h/mg of protein (Table 1).

It should be noted that Tris(hydroxymethyl) aminomethane (Tris) contains an –OH group and, like many alcohols, can be a substrate for ADH. However, in the controls without NADH, coloration at 540 nm was not observed, i.e., tetrazolium was not reduced to formazan. In a second control without tetrazolium, coloration was also not observed, and the rate of catalytic NADH oxidation by oxygen dissolved in water, as measured by the decrease in absorbance at 340 nm, did not exceed 119 μM/h/mg of protein.

The presence of ethanol or isopropanol and alkalization of the medium sharply activated the NADH : *p*-NTF-reductase reaction (Fig. 2). This was due to the fact that formazan could desorb from the active enzyme site under alkaline conditions, since it dissolves in alkaline media [6]. At a pH of 6–8, the reaction proceeded faster in the presence of ethanol but not isopropanol. This was due to the fact that ethanol binds to the enzyme better than polyols.

Analysis of the dependence of the rate of enzymatic NADH oxidation by tetrazolium in the presence of

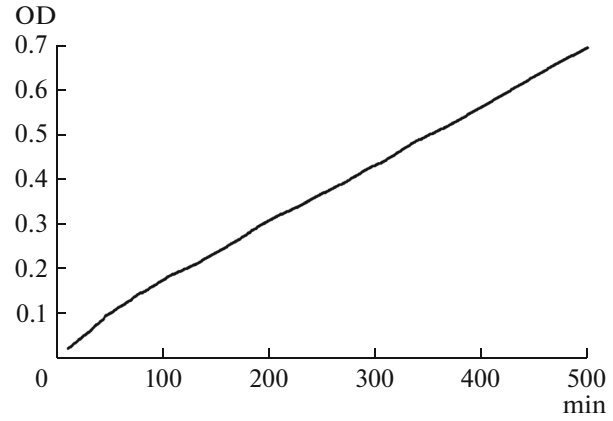

Fig. 1. Kinetics of change in absorbance at 540 nm during reduction of p-NTF (200 μM) to formazan by the enzymatic oxidation of NADH (500 μM) by ADH in 10 mM Tris-phosphate buffer, pH 7, containing 15% ethanol.

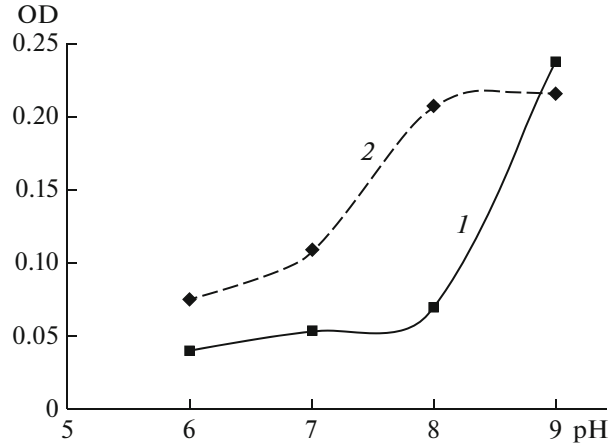

Fig. 2. Dependence of NADH : tetrazolium reductase reaction rate on the pH value in 10 mM Tris-phosphate buffer, pH 7, in the presence of 15% isopropanol (*1*) or 15% ethanol (*2*). Optical density was measured 1 h after the reaction.

alcohols on the pH value demonstrated that its optimal pH was in the range of 8–9 (Fig. 2). The differences between the curves demonstrating the dependence of the reaction of ethanol and isopropanol on the pH values indicated that it is not only the polarity of the alcohol but also its effect on enzyme conformation was important. XRD [13] and fluorescence analysis [14] demonstrated that ADH undergoes conformational changes caused by the binding of NAD^+ or NADH, including the rotation of the catalytic domain relative to the coenzyme-binding domain and reorganization of the active center to form the catalytically active form of the enzyme. It can be assumed that the addition of alcohol to the reaction mixture not only reduces the polarity of the surrounding of the enzyme active center but also changes its conformation.

Tetrazolium contacts with NADH on the enzyme surface without intermediate carriers (the zinc in the active ADH site is not a electron carrier) and accepts electrons.

In comparison with NADH (standard electrode potential –0.32 eV), ethanol is a poor reducing agent (standard electrode potential +0.19 eV) and has a lower affinity to the positively charged *p*-NTF [3]. NADH carries a negative charge on the phosphate groups, which bind to the enzyme by electrostatic interactions. Formazan formation was not observed during incubation of the enzyme with ethanol or isopropanol for the entire physiological pH range. Thus, alcohols were not able to reduce *p*-NTF.

The dependence of the rate of NADH oxidation by tetrazolium on the alcohol concentration was complex, and the optimal alcohol concentration was 15–30% (Table 1). The activation of NADH:*p*-NTF-reductase reactions by alcohols is associated with the release of formazan from the enzyme in the medium, as well as (which cannot be excluded) with its increase in steric accessibility to the active center for tetrazolium and NADH. An increase in the alcohol concentration from 15 to 30% has little effect on the reaction rate, whereas an increase up to 40% or higher inhibits the enzyme, which is probably due to its inactivation.

NADH Oxidation and Ubiquinone Reduction

The catalysis of NADH enzyme oxidation in the presence of ubiquinone was not observed at pH values of 6–7. Slow oxidation of NADH by ubiquinone catalyzed by ADH occurred after acidification of the medium to a pH of 5.5. In reality, the oxidation process requires a proton content in the acidic environment that is much higher than that in an alkaline one:

$$NADH + H^+ + Q2 \rightarrow NAD^+ + Q2H_2$$

This reaction was sharply activated in the presence of 30% alcohol. This could be explained by the fact that the enzyme was not inhibited by the reaction product, ubiquinone, in the presence of significant amounts of alcohol due to its release in water-alcohol phase. The rate of NADH:ubiquinone reductase reaction under these conditions reached 131 μM/h/mg of protein and exceeded the rate of NADH autoxidation on the enzyme with oxygen by 17.5 times. The reaction kinetics in Tris-phosphate buffer in the presence of 30% ethanol at different pH values is shown in Fig. 3.

The maximum rate of enzymatic NADH oxidation by ubiquinone in the presence of ethanol was reached via replacement of the Tris-phosphate buffer with HEPES. It reached 1250 μM/h/mg of protein at a pH of 6 (Table 1). This indicated the importance of not only the pH of the buffer solution but also its composition. It can be assumed that Tris(hydroxymethyl)aminomethane could inhibit the enzyme, since it contains a hydroxyl group and is able to bind to ADH.

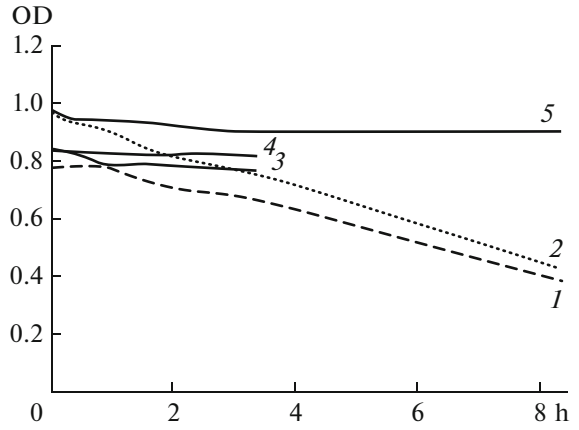

Fig. 3. Kinetics of the change in absorbance at 340 nm during the oxidation of NADH (500 μM) by ubiquinone (200 μM) in 10 mM tris-phosphate buffer in the presence of 30% ethanol at pH values of 5.5 (*1*), 6 (*2*), 8 (*3*), 9 (*4*), and 7 (*5*).

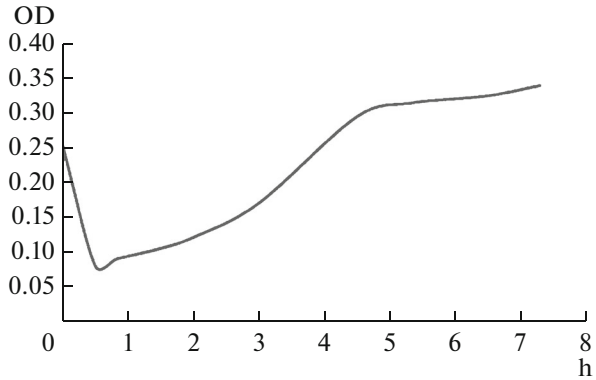

Fig. 4. Kinetics of the change in absorbance at 340 nm during the oxidation of NADH (500 μM) and ubiquinone (200 μM) in 10 mM HEPES in the presence of 30% ethanol at a pH of 6.

A quasi-vibrational process was detected with the use of HEPES buffer in the presence of alcohol: NADH was rapidly oxidized to NAD^+ by ubiquinone and NAD^+ was then slowly reduced to NADH by the alcohol (Fig. 4). This indicated that the NADH:ubiquinone- and alcohol:NAD^+-reductase reactions was partially separated in time. It can be assumed that this was caused by considerable differences in the values of binding constants of NADH (10^5 M^{-1}) and NAD^+ (10^4 M^{-1}) molecules. Otherwise, it was impossible to explain the fact that the NADH in the solution initially oxidized to NAD^+; then, after about 0.5 hour, NAD^+ began to reduce "spontaneously." Ubiquinone reduced to ubiquinol transfers electrons to the dissolved oxygen. It can be assumed that the differences in the order of magnitude between the values of binding constants of NADH and NAD^+ to the active site of the enzyme determined that all ADH molecules initially bound NADH and only after sufficient oxidation, which resulted in the formation of significant amounts of NAD^+. This, in turn, contacted with the enzyme, which led to its reduction by alcohol to NADH.

No quasi-vibrational process was observed in Tris buffer. This was probably due to the fact that Tris(hydroxymethyl)aminomethane containing an OH group, like many alcohols, is able to interact with the active ADH site and alter the binding constant with NAD^+ and NADH.

Thus, it can be assumed that the conjugated NADH:ubihinon- and alcohol:NAD^+-reductase reactions catalyzed by ADH may play an important physiological role in the cells.

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Translated by A. Bulaev