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FUNCTION AND ROLE OF NAD+ IN MECHANISM OF ACTION OF RABBIT-MUSCLE GLYCERALDEHYDEPHOSPHATE DEHYDROGENASE

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SUMMARY

1. NAD+ is required for the oxidation of NADH by 1,3-diphosphoglycerate or acetyl phosphate.

2. The maximal rate of acetyl phosphate reduction by NADH or APADH is reached when about 3 moles NAD+ per mole enzyme are added. Larger amounts of NAD+ inhibit with a K_i (NADH as substrate) equal to 45 μM.

3. The NAD-binding site with the lowest affinity for NAD+ appears to be the most active in the dehydrogenase reaction. Under optimal conditions the E–$(NAD)_3$ complex is the catalytically active enzyme.

4. The E–$(NAD)_3$ complex appears also to be the form with maximal catalytic activity in the transferase reactions.

5. NAD+ can be replaced by APAD+ in both the dehydrogenase and arsenolysis reactions.

INTRODUCTION

Rabbit-muscle glyceraldehydephosphate dehydrogenase (glyceraldehyde-3-phosphate:NAD+ oxidoreductase (phosphorylating), EC 1.2.1.12) consists of four identical polypeptide chains[1], arranged at least on a 2-fold axis of symmetry[2]. The dissociation constants of the four NAD+ molecules are different. KOSHLAND and co-workers[3,4] and DE VIJLDER AND SLATER[5] showed that two molecules are bound very strongly, the third less strongly and the fourth relatively weakly.

Measurements made spectrophotometrically[5,6], fluorimetrically[7] and by means of optical rotatory dispersion and circular dichroism[8] give titration curves in which three NAD+ molecules contribute equally to the parameters measured while the fourth makes little, if any, contribution.

Abbreviations: APAD+ and APADH, oxidized and reduced acetylpyridine–adenine dinucleotide, respectively.

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Since it was shown by HILVERS and co-workers[9,10] that the oxidation of NADH by 1,3-diphosphoglycerate or acetyl phosphate, like the transferase reactions[11–13], requires NAD^+, the role of the differently bound NAD^+ molecules in these activities was investigated. The effect of $APAD^+$ was also studied.

RESULTS

Requirement for and inhibition by NAD^+ of the dehydrogenase activity

The reduction of 1,3-diphosphoglycerate by NADH was studied during the early stages of the reaction using a stopped-flow apparatus. As can be seen in Fig. 1, NAD^+ is required for this reaction. In the complete absence of NAD^+ (Curve C), obtained by addition of $NAD(P)^+$ nucleosidase (EC 3.2.2.6), no reaction could be observed, whereas in the presence of 2.4 moles NAD^+ per mole enzyme (Curve A), the reaction started immediately. The reaction catalysed by charcoal-treated enzyme, containing only traces of NAD^+, exhibited a lag phase (Curve B). The same results were obtained with the yeast enzyme.

When NADH was oxidized in the presence of acetyl phosphate the same result was obtained. HILVERS AND WEENEN[9] observed also that no oxidation of NADH by

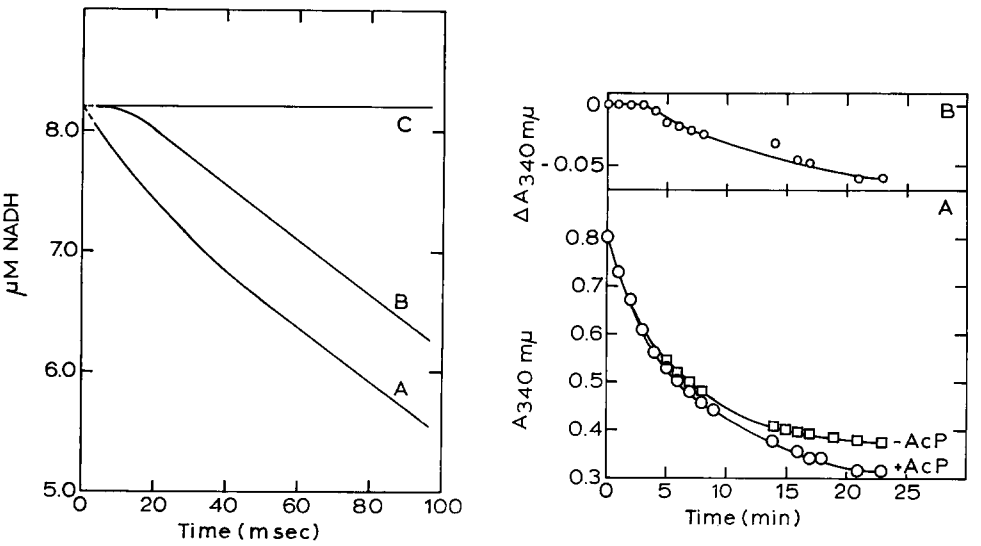

Fig. 1. NAD^+ requirement for the oxidation of NADH by 1,3-diphosphoglycerate. One of the two syringes of the stopped-flow apparatus contained 171 nM glyceraldehydephosphate dehydrogenase in 0.1 M Tris–HCl buffer (containing 5 mM EDTA and 0.5% serum albumin, final pH 7.3). The other syringe contained 16.4 μM NADH and 8 μM 1,3-diphosphoglycerate in the same Tris–EDTA mixture. Temp., 25°; light path, 2 cm; wavelength, 340 mμ. Curve A, reaction started in the presence of NAD^+ (2.4 moles/mole enzyme); Curve B, reaction started in the presence of a trace NAD^+; Curve C, same as Curve B, but in the presence of Neurospora $NAD(P)^+$ nucleosidase (530 units)[22].

Fig. 2. NAD^+ requirement for NADH oxidation by acetyl phosphate. 150 mM Tris–HCl buffer (pH 7.4), 27.6 μM enzyme, 130 μM NADH, 6300 units Neurospora $NAD(P)^+$ nucleosidase. Reaction started with 10 mM NAD^+. (A) Absorbance decrease in the presence and absence of 5 mM acetyl phosphate. (B) The difference in absorbance decrease in the presence and absence of acetyl phosphate.

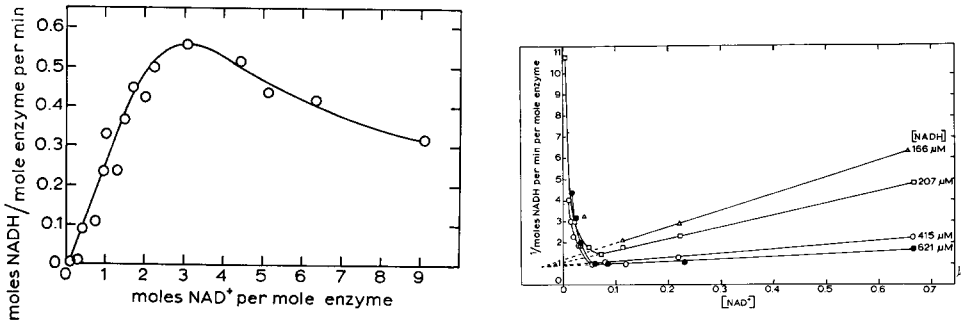

Fig. 3. Effect of NAD^+ on rate of NADH oxidation by acetyl phosphate. The medium contained 150 mM Tris–HCl buffer (pH 7.0), 22.1 μM charcoal-treated enzyme and 125 μM NADH. The reaction was started with 5 mM acetyl phosphate. Temp., 25°.

Fig. 4. Inhibition by NAD^+ of the NADH oxidation by acetyl phosphate. 100 mM Tris–HCl buffer (pH 7.4), 22.3 μM enzyme. The reaction was started with 10 mM acetyl phosphate. Temp., 20°.

acetyl phosphate occurred when $NAD(P)^+$ nucleosidase was present. They found a decrease in absorbance at 340 mμ when NAD^+ was added. During the present investigation, however, it became clear that this decrease was not only due to NADH oxidation but also to NAD^+ breakdown. The decrease at 340 mμ on NAD^+ addition in the presence or absence of acetyl phosphate (Fig. 2A) was compared. The difference in absorbance decrease (Fig. 2B) is due to NADH oxidation by acetyl phosphate. The triphasic kinetics can be explained in terms of (a) inhibition of the reaction by excess NAD^+ (0–3 min); (b) oxidation of NADH in the presence of NAD^+ (3–20 min), and (c) inhibition of the reaction due to the removal of NAD^+ after 20 min.

Stoicheiometry

The effect of different amounts of NAD^+ on the initial rate of acetyl phosphate reduction by NADH is shown in Fig. 3. Optimal stimulation was observed at about

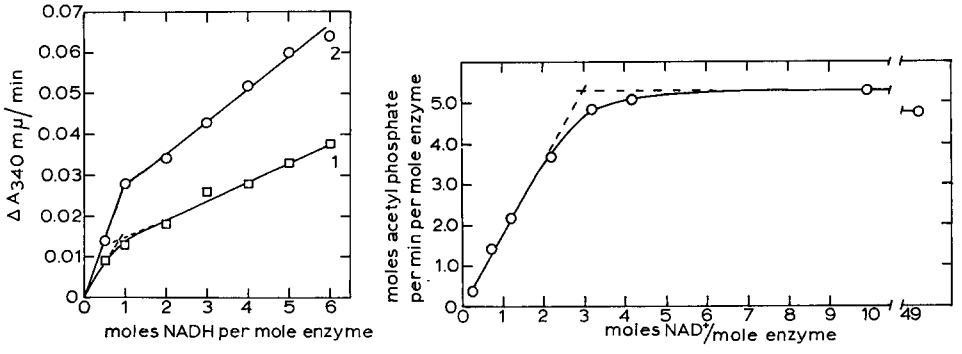

Fig. 5. Effect of NADH concentration on rate of oxidation of NADH by acetyl phosphate. 125 mM Tris–HCl buffer (pH 7.3), 23.8 μM charcoal-treated enzyme, temp., 20°. Curve 1, 1.0 moles NAD^+/mole enzyme; Curve 2, 3.0 moles NAD^+/mole enzyme. The reaction was started with 5 mM acetyl phosphate.

Fig. 6. Effect of NAD^+ concentration on arsenolysis of acetyl phosphate. The medium contained 50 mM Tris–HCl buffer, 0.5 mM EDTA, 20 mM Na_2HAsO_4 and 38.4 μM charcoal-treated enzyme. Temp., 25°. The reaction was started with 8.3 mM acetyl phosphate. Final pH, 7.3.

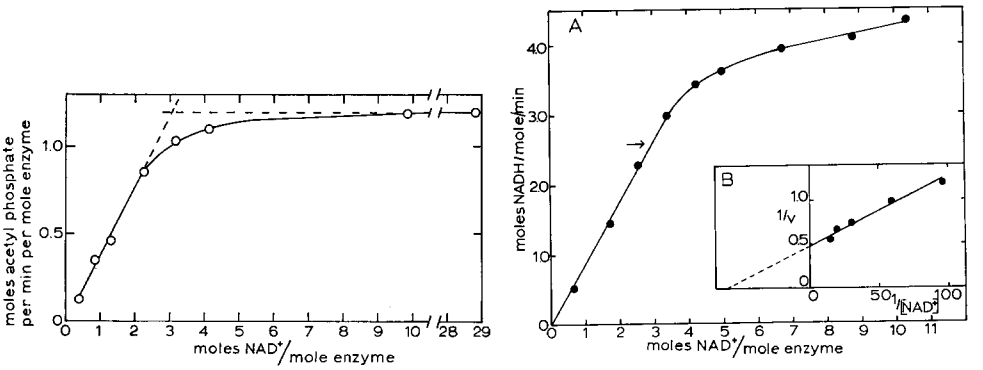

Fig. 7. Effect of NAD^+ concentration on hydrolysis of acetyl phosphate. The medium contained 50 mM Tris–HCl buffer, 0.5 mM EDTA, 72.5 μM charcoal-treated enzyme. Temp., 25°. The reaction was started with 10 mM acetyl phosphate. Final pH, 7.1.

Fig. 8. Effect of NAD^+ concentration on rate of NAD^+ reduction by glyceraldehyde. The medium contained 100 mM Tris–HCl buffer, 1 mM EDTA, 43 mM Na_2HAsO_4, 0.1% serum albumin and 10.1 μM charcoal-treated enzyme. The reaction was started with 8.6 mM DL-glyceraldehyde. Final pH, 8.8; temp., 25°. In insert Lineweaver–Burk plot for the fourth NAD^+ molecule. The rate is the measured rate *minus* the rate with 3 moles added NAD^+ (see arrow); the substrate concentration refers to free NAD^+ calculated from the amount added (4.25 moles NAD^+ per mole enzyme or more) and the dissociation constant of the fourth binding site (35 μM).

3 molecules NAD^+ added per molecule enzyme. At higher concentrations NAD^+ was inhibitory, competitive with NADH (Fig. 4). The inhibition constant was 45 μM. A similar stimulatory and inhibitory effect of NAD^+ was observed on the reduction of acetyl phosphate by APADH.

The effect of different amounts of NADH on the rate of reduction of acetyl phosphate in the presence of catalytic amounts of NAD^+ (1 or 3 molecules NAD^+ per molecule enzyme) is shown in Fig. 5. The curves show a clear break at about one molecule NADH per molecule enzyme. Experiments in the presence of 2 and 5 molecules NAD^+ per molecule enzyme gave the same result (not shown).

The stoicheiometry of the NAD^+ requirement for the arsenolysis and hydrolysis of acetyl phosphate is shown in Figs. 6 and 7, respectively. Both reactions gave a linear increment in activity up to 2 molecules NAD^+ per molecule enzyme, after which the increment per molecule NAD^+ declined. On extrapolation, an intersection point was found at about 3 molecules NAD^+, indicating that maximal activity is reached when 3 NAD^+ molecules are bound.

The rate of oxidation of glyceraldehyde by NAD^+, in the presence of arsenate, is plotted as a function of NAD^+ concentration in Fig. 8A. The rate is directly proportional to NAD^+ concentration up to 3 moles NAD^+ added. The slope of the curve declines above this point, and above 5 moles NAD^+ added per mole enzyme an almost linear curve of low slope is obtained. On extrapolation of this line, it intersects the initial steep line at about 4 moles per mole enzyme, as has been reported previously[14,15,5]. However, in reality the curve above 3 moles NAD^+ per mole enzyme is a rectangular hyperbola, as is shown in Fig. 8B. The K_m for free NAD^+, calculated for this part of the curve, is 17 μM, in close agreement with the value found by FAHIEN[15], using low concentrations of enzyme. The maximum velocity V, at infinite NAD^+ concentration,

TABLE I

COMPARISON OF NAD^+ AND $APAD^+$ AS STIMULATOR OF REDUCTION OR ARSENOLYSIS OF ACETYL PHOSPHATE

Reduction was measured as described in Fig. 4. Temp., 25°; pH 7.3. Extrapolated to infinite NADH concentration. Arsenolysis was measured as in Fig. 6. Optimal amounts of nucleotide.

	Rate (moles acetyl phosphate/min per mole enzyme)	
	NAD^+	*$APAD^+$*
Reduction	1.75	0.67
Arsenolysis	5.30	0.64

calculated from this plot is 0.035 mole·sec^{-1} per mole enzyme, compared with an average of 0.014 mole·sec^{-1} for the first three molecules.

Replacement of NAD^+ by $APAD^+$

KAPLAN *et al.*[16] showed that NAD^+ could be replaced by $APAD^+$ as oxidant for glyceraldehyde 3-phosphate. This was confirmed using charcoal-treated enzyme in the presence of $NAD(P)^+$ nucleosidase in order to eliminate a possible effect of residual NAD^+. The initial rate of oxidation was one-tenth that of the initial rate with NAD^+, in agreement with the observations of KAPLAN *et al.*[16].

The reduction of acetyl phosphate by NADH was also stimulated by $APAD^+$, and inhibited by more than 3 moles per mole enzyme ($K_i = 114\ \mu M$). These measurements were carried out in the presence of $NAD(P)^+$ nucleosidase in order to prevent stimulation by NAD^+ formed during NADH oxidation. $APAD^+$ can also satisfy the nucleotide requirement for the arsenolysis.

In Table I the reaction rates found for the reduction of acetyl phosphate by NADH and its arsenolysis in the presence of optimal amounts of NAD^+ or $APAD^+$ are compared.

DISCUSSION

The NAD^+ requirement of glyceraldehydephosphate dehydrogenase for the oxidation of NADH by 1,3-diphosphoglycerate and acetyl phosphate, as first reported by HILVERS *et al.*[9,10] but criticised by COLOWICK *et al.*[17], was confirmed. In this respect, there is no difference between the acyltransferase reactions and NADH oxidation (contrast ref. 17). Since no NADH oxidation could be shown in the complete absence of NAD^+, it seems evident that this stimulating effect of nucleotide cannot be provided by NADH, as was suggested by FURFINE AND VELICK[18]. The lag found with charcoal-treated enzyme is a consequence of NAD^+ production during the reaction.

The transfer reactions catalysed by the enzyme are useful for the study of the stimulating effects of NAD^+ since these reactions are little if at all inhibited by excess NAD^+. The reaction velocity is directly proportional to the NAD^+ concentration until a level of two molecules NAD^+ per molecule enzyme is reached, after which the increment per molecule NAD^+ added is less. The intersection point at about three molecules

NAD^+ per molecule enzyme suggests that under the conditions used maximal hydrolysis or arsenolysis takes place if three moles NAD^+ are bound per mole enzyme.

Spectrophotometric[5,6], fluorimetric[7], circular dichroism and optical rotatory dispersion[8] studies have shown that the fourth NAD^+ molecule bound behaves differently from the other three. It also appears to behave differently with respect to reduction by glyceraldehyde. In view of the very rapid reaction between enzyme and NAD^+ (refs. 5 and 19), and the slow catalysis by the enzyme of the oxidation of glyceraldehyde by NAD^+, it is clear that the rate-limiting step in the catalytic reaction (initial rates were measured) is the hydride transfer from glyceraldehyde to enzyme-bound NAD^+. For the first three molecules of enzyme bound, we may write

$$E\text{–}(NAD)_3 + 3H^- \xrightarrow{k} E\text{–}(NADH)_3$$

where k is 0.014 sec^{-1}, under the conditions of the experiment. Writing E–$(NAD)_3$ as E', we may write the reaction with the fourth molecule as

$$E' + NAD^+ \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E'\text{–}NAD^+$$

$$E'\text{–}NAD^+ + H^- \xrightarrow{k_2} E'\text{–}NADH$$

$$E'\text{–}NADH \overset{k_3}{\rightleftharpoons} E' + NADH$$

It was found that the K_m for NAD^+ for this reaction, 17 μM, is the same as that previously reported for the dissociation constant, K_D, for this molecule. Since $K_m = [k_3(k_{-1}+k_2)]/k_1(k_2+k_3)$ and $K_D = k_{-1}/k_1$, it follows that $k_{-1} \gg k_2$ and $k_3 \gg k_2$*. Since $k_1 = 3 \cdot 10^4\, k_{-1}$, the hydride transfer is the rate-limiting step in this reaction also. Thus, k_2 may be calculated from the velocity at infinite NAD^+ concentration, *viz.* 0.035 sec^{-1}. This is more than twice the corresponding k for the first three molecules. Thus, the fourth molecule of NAD^+ is catalytically more active in the oxidation of glyceraldehyde by NAD^+ than the other three. CONWAY AND KOSHLAND[4] showed also that the average turnover per site increases with increasing NAD^+ bound to the enzyme, and concluded that some or all the sites in the E–$(NAD)_4$ complex are more active than in the E–$(NAD)_2$ complex. Fig. 8 gives some indication that the third site might be more catalytically active than the first two in our experiments, but the difference is too small to be certain of this.

Since the K_i (45 μM) for the competitive inhibition by NAD^+ of the reduction of acetyl phosphate by NADH is about the same as the dissociation constant (35 μM) of the fourth molecule of NAD^+ bound to the enzyme, it is probable that NADH binds to the same site on the enzyme as the fourth NAD^+ molecule. This is supported by the sharp break at 1 molecule NADH per molecule enzyme in the curve describing the effect of NADH concentration on the reduction of acetyl phosphate, in the presence of NAD^+. The importance of loose and tight binding sites in enzyme kinetics have been stressed by FRIEDEN[20], FAHIEN[15] and CONWAY AND KOSHLAND[4].

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* See NOTE ADDED IN PROOF (p. 227).

EXPERIMENTAL

Glyceraldehydephosphate dehydrogenase was isolated from rabbit muscle by the method of CORI *et al.*[21], slightly modified by HILVERS[22]. NAD^+ was removed by stirring with a 20% charcoal suspension (*cf.* ref. 23). The enzyme concentration was calculated from absorbance measurements at 280 mμ, using the extinction coefficient reported by FOX AND DANDLIKER[6]. A molecular weight of 145 000 was assumed[1].

$NAD(P)^+$ nucleosidase [$NAD(P)^+$ glycohydrolase, EC 3.2.2.6] was isolated from *Neurospora crassa* or pig brain according to the method of KAPLAN[24].

$APAD^+$ was prepared according to KAPLAN AND STOLZENBACH[25]. Before isolation the $APAD^+$ solution was treated for 30 min with Neurospora $NAD(P)^+$ nucleosidase, in order to remove traces of NAD^+ still present. After isolation the $A_{365\ m\mu}$: $A_{340\ m\mu}$ ratio of the reduced product was 1.51. The absence of NAD^+ was checked by paper chromatography. The concentration was measured with alcohol dehydrogenase and ethanol, making use of $\varepsilon_M = 9.1 \cdot 10^3$ at 363 mμ (*cf.* ref. 26). APADH was made from $APAD^+$ and ethanol in the presence of alcohol dehydrogenase, traces of $APAD^+$ being destroyed by boiling the solution at pH 10 for 5 min. 1,3-Diphosphoglyceric acid was prepared from glyceraldehyde 3-phosphate as described by NEGELEIN AND BROMEL[27] as modified by FURFINE AND VELICK[18].

Acetyl phosphate was determined by the hydroxamate method of LIPMANN AND TUTTLE[28].

Stopped-flow measurements were performed on a Durrum stopped-flow apparatus (Durrum Instrument Corporation).

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Dr. K. DALZIEL has pointed out that K_m is also equal to K_D when $k_{-1} = k_3$. In this case, the velocity at infinite NAD^+ concentration equals $k_2k_3/(k_2 + k_3)$, and k_2 is either equal to or greater than 0.035 sec^{-1}. However, the conclusion that the fourth molecule is catalytically the more active remains.

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