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THE EFFECT OF NAD^+ ON THE CATALYTIC EFFICIENCY OF GLYCERALDEHYDE-3-PHOSPHATE DEHYDROGENASE FROM RABBIT MUSCLE

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SUMMARY

The catalytic center activity for the oxidation of glyceraldehyde, acetaldehyde and propionaldehyde by glyceraldehyde-3-phosphate dehydrogenase was not observed to be appreciably affected by the degree of saturation of the enzyme with NAD^+. A small increase in intrinsic rate on going from $E(NAD^+)_1$ to $E(NAD^+)_4$ was noted for glyceraldehyde (1.1 fold) and a small decrease in intrinsic rate for acetaldehyde (1.4 fold) and propionaldehyde (1.2 fold). These small changes, contrasted with the approx. $1 \cdot 10^7$-fold decrease in ligand affinity induced by the binding of NAD^+, are consistent with the induced fit model of allosterism.

The initial rate of oxidation of glyceraldehyde 3-phosphate by glyceraldehyde-3-phosphate dehydrogenase, in the presence of arsenate, decreased rapidly within the first few percent of the reaction. Investigation of the end product inhibition of this reaction suggests that the accumulation of 1-arseno-3-phosphoglycerate is partly responsible for the observed reduction in rate, and thus that the spontaneous hydrolysis of this mixed anhydride may not be rapid relative to the rate of catalysis.

INTRODUCTION

The catalytic activity of many enzymes has now been shown to be regulated by specific metabolites, usually referred to as allosteric effectors. Most of these studies reveal changes in ligand affinity induced by the effector molecules, although in some cases the intrinsic catalytic properties of the enzyme may also be affected[1]. Since proteins are composed of multiple subunits and there is increasing evidence that the conformations of these subunits often change in a sequential manner during ligand binding[2], it is of interest to evaluate the catalytic efficiencies of individual subunits in such hybrid molecules. Moreover, such a study would be helpful in elucidating the nature of the conformational changes at intermediate stages during the binding of substrates or effectors.

The difficulty in analyzing an enzyme showing positive cooperativity is considerable since it is difficult to isolate individual species except at very low saturation or

very high saturation of the protein. However, the observation that enzymes may also bind ligand in a negatively cooperative manner[3] makes this problem approachable. Glyceraldehyde-3-phosphate dehydrogenase (D-glyceraldehyde 3-phosphate: NAD oxidoreductase (phosphorylating), EC 1.2.1.12) from rabbit muscle, which possesses four NAD^+ binding sites, has been experimentally shown to display such cooperativity since the first NAD^+ bound makes it more difficult for the second NAD^+ to bind, the second more difficult for the third, *etc.*[4,5]. As a result of this stepwise binding of NAD^+ and a fairly wide separation of the constants, it is possible to examine species which are largely present as $E(NAD^+)_1$, $E(NAD^+)_2$, $E(NAD^+)_3$ and $E(NAD^+)_4$. To examine the kinetic properties of these species glyceraldehyde, acetaldehyde and propionaldehyde were chosen as substrates because (a) the slow rate of reaction of these compounds allows accurate assessment of initial velocities, and (b) being poor substrates they are less likely to affect appreciably NAD^+ binding or site-site interactions.

The rate of oxidation of glyceraldehyde by glyceraldehyde-3-phosphate dehydrogenase as a function of NAD^+ concentration was the subject of excellent studies by MURDOCK AND KOEPPE[6], FURFINE AND VELICK[7], FAHIEN[8], and DE VIJLDER AND SLATER[4]. At the time of these studies, however, the detailed binding of NAD^+ was not known and the intrinsic catalytic constants were not evaluated. In some preliminary kinetic studies CONWAY AND KOSHLAND[5] reported that the progressive binding of NAD^+ caused a 3-fold increase in the catalytic center activity with glyceraldehyde as substrate, and a substantially larger increase with glyceraldehyde 3-phosphate. In view of the significance of these intrinsic kinetic constants, the phenomenon has been reinvestigated and the findings are seen to be relevant to conformational changes within and between subunits.

MATERIALS

DL-Glyceraldehyde 3-phosphate was prepared from the barium salt of the diethylacetal (Sigma Chemical Co.) according to the procedure of RACKER *et al.*[9]. DL-Glyceraldehyde (Calbiochem), acetaldehyde (Eastman Chemical Co.), priopionaldehyde (Baker Chemical Co.), D(−)-3-phosphoglyceric acid and NAD^+ (Sigma Chemical Co.), D(−)-3-phosphoglyceric acid and NAD^+ (Sigma Chemical Co.) were used without further purification.

Glyceraldehyde-3-phosphate dehydrogenase was obtained from Calbiochem as a crystalline suspension in 2.5 M $(NH_4)_2SO_4$.

METHODS

Absorbance measurements were made on a Zeiss PM QII spectrophotometer and fluorescence measurements on a Hitachi MPF-2A recording fluorescence spectrophotometer. Glyceraldehyde-3-phosphate dehydrogenase containing 1.4–1.7 moles of NAD^+ bound per mole of enzyme was prepared by dialyzing the native enzyme (about 20 mg/ml) against a 500-fold excess of a 50 mM sodium pyrophosphate–2 mM EDTA (pH 7.5) solution, containing 3 g of acid washed charcoal (Norit A), for 24 h at 4°. Glyceraldehyde-3-phosphate dehydrogenase, after dialysis, possessed the same specific activity as the native enzyme, approx. $1.4 \cdot 10^4$ moles/mole per min when assayed as described earlier[4].

The concentration of native and dialyzed glyceraldehyde-3-phosphate dehydrogenase was determined spectrophotometrically as described by FOX AND DANDLIKER[10]. The moles of NAD^+ bound per mole of glyceraldehyde-3-phosphate dehydrogenase after dialysis was estimated both spectrophotometrically from the $A_{280\ m\mu}/A_{260\ m\mu}$ ratio of the enzyme[10], and by heat precipitation of the enzyme and determination of the amount of NAD^+ released by a fluorometric assay[11]. Results from the two methods agreed to within 4%.

The rate of reduction of NAD^+ by glyceraldehyde-3-phosphate dehydrogenase, in the presence of acetaldehyde, glyceraldehyde or propionaldehyde was measured fluorometrically. Assay solutions were excited at 340 mμ and the increase in fluorescence, due to the formation of NADH, monitored at 460 mμ. The rate of formation of as little as $5 \cdot 10^{-7}$ M NADH (less than 3% of the lowest initial NAD^+ concentration) could be measured with this assay. The rate of reduction of NAD^+ by glyceraldehyde-3-phosphate dehydrogenase, in presence of glyceraldehyde 3-phosphate, was determined spectrophotometrically at 340 mμ.

RESULTS

Glyceraldehyde, acetaldehyde and propionaldehyde

The catalytic activity of glyceraldehyde-3-phosphate dehydrogenase, initially containing 1.4–1.7 moles of NAD^+ per mole of enzyme, was measured as a function

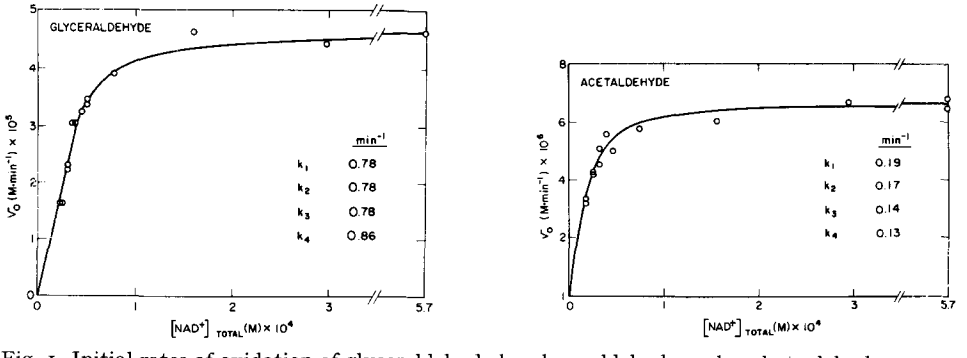


Fig. 1. Initial rates of oxidation of glyceraldehyde by glyceraldehyde-3-phosphate dehydrogenase as a function of NAD^+ concentration. In addition to NAD^+, assay mixtures contained $1.35 \cdot 10^{-5}$ M glyceraldehyde-3-phosphate dehydrogenase, $1 \cdot 10^{-2}$ M DL-glyceraldehyde, $1 \cdot 10^{-2}$ M sodium arsenate, $5 \cdot 10^{-3}$ M EDTA and $5 \cdot 10^{-2}$ M sodium pyrophosphate, at pH 8.5, 25°.

Fig. 2. Initial rates of oxidation of acetaldehyde by glyceraldehyde-3-phosphate dehydrogenase as a function of NAD^+ concentration. In addition to NAD^+, assay mixtures contained $1.3 \cdot 10^{-5}$ M glyceraldehyde-3-phosphate dehydrogenase, $1 \cdot 10^{-2}$ M acetaldehyde, $1 \cdot 10^{-2}$ M sodium arsenate, $5 \cdot 10^{-3}$ M EDTA and $5 \cdot 10^{-2}$ M sodium pyrophosphate, at pH 8.5, 25°.

of increasing NAD^+ concentration. Initial velocities of NADH formation, plotted *versus* total NAD^+ concentration, for the substrates glyceraldehyde, acetaldehyde and propionaldehyde are shown in Figs. 1, 2 and 3, respectively.

The initial formation of NADH, monitored fluorometrically, as described in the METHODS, was linear with time.

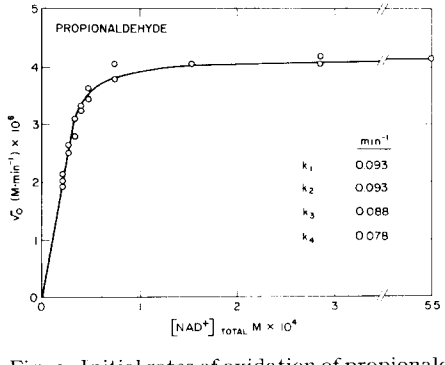
The solid curves through the experimental points in Figs. 1, 2 and 3 were,

generated from Eqn. 1 which describes initial velocity, v_0, as the sum of the individual turnover rates for each NAD^+–enzyme complex.

$$v_0 = k_1 \cdot E(NAD^+)_1 + 2k_2 \cdot E(NAD^+)_2 + 3k_3 \cdot E(NAD^+)_3 + 4k_4 \cdot E(NAD^+)_4 \quad (1)$$

The concentrations of $E(NAD^+)_1$, $E(NAD^+)_2$, $E(NAD^+)_3$ and $E(NAD^+)_4$, which represent molecular species with 1, 2, 3 and 4 moles of NAD^+ bound per mole of enzyme, respectively, were calculated from the dissociation constants for each of these complexes. (The concentration of the NAD^+–enzyme complexes could be calculated directly from the dissociation constants since the rates of equilibration of NAD^+ with the enzyme are rapid[4] relative to the rates of catalysis of glyceraldehyde, acetaldehyde and propionaldehyde.) The dissociation constants employed above were those determined by CONWAY AND KOSHLAND[5], $K_1 = < 1 \cdot 10^{-11}$, $K_2 = < 1 \cdot 10^{-9}$, $K_3 = 3 \cdot 10^{-7}$, $K_4 = 2.6 \cdot 10^{-4}$. These values are in substantial agreement with the parameters measured by DE VIJLDER AND SLATER[4]. The values of the intrinsic catalytic rate constants, k_1, k_2, k_3 and k_4, which represent the average catalytic center activity for a particular complex, were chosen so as to generate from Eqn. 1 theoretical curves which closely approximated the experimental data. The values of the rate constants which gave the best fit of the experimental data for each of the three substrates are given in Figs. 1, 2 and 3. The ratio of $k_1:k_2:k_3:k_4$ for glyceraldehyde is 0.9:0.9:0.9:1.0, for acetaldehyde, 1.4:1.3:1.1:1.0 and for propionaldehyde 1.2:1.2:1.1:1.0. The progressive binding of NAD^+ to glyceraldehyde-3-phosphate dehydrogenase thus causes a small increase in the average catalytic center activity for glyceraldehyde and a small decrease in the turnover numbers for acetaldehyde and propionaldehyde. These results are slightly different but are in good general agreement with the values reported by DE VIJLDER *et al.*[12].

In order to demonstrate that the function generated by Eqn. 1 is relatively sensitive to the values selected for k_1, k_2, k_3 and k_4, the best theoretical curve for the glyceraldehyde kinetic data is compared in Fig. 4 with two other theoretical curves



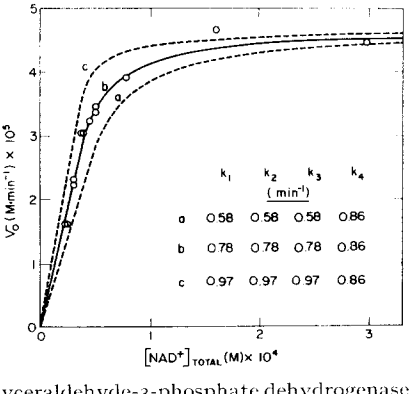


Fig. 3. Initial rates of oxidation of propionaldehyde by glyceraldehyde-3-phosphate dehydrogenase as a function of NAD^+ concentration. In addition to NAD^+, assay mixtures contained $1.33 \cdot 10^{-5}$ M glyceraldehyde-3-phosphate dehydrogenase, $1 \cdot 10^{-2}$ M propionaldehyde, $1 \cdot 10^{-2}$ M sodium arsenate, $5 \cdot 10^{-3}$ M EDTA and $5 \cdot 10^{-2}$ M sodium pyrophosphate, at pH 8.5, 25°.

Fig. 4. Comparison of best theoretical curve generated by Eqn. 1 for glyceraldehyde kinetic data (Curve b) with curves generated by assuming the values of k_1, k_2 and k_3 were 25% lower (Curve a) or 25% higher (Curve c) than the values of the rate constants giving the best fit.

which were generated assuming k_1, k_2 and k_3 were either 25% higher or 25% lower, relative to k_4, than the values giving the best fit. Although the relative values of the intrinsic catalytic rate constants might differ somewhat from those calculated above, due to experimental error, it appears from Fig. 4 that they are fairly close to these values.

Glyceraldehyde 3-phosphate

In Fig. 5 is shown the glyceraldehyde-3-phosphate dehydrogenase catalyzed reduction of NAD^+, in the presence of glyceraldehyde 3-phosphate, plotted as a function of time. It may be observed that starting with relatively high concentrations of NAD^+ ($1 \cdot 10^{-2}$ M) and glyceraldehyde 3-phosphate ($1 \cdot 10^{-2}$ M) the rate of formation of NADH drops to less than 50% of that initially measured after approx. 0.3% of the substrates are consumed. At lower levels of NAD^+ the rate of reaction decreased even more rapidly, prohibiting the measurements of true initial velocities by conventional assay procedures. Consequently, the effect of NAD^+ on intrinsic catalytic rates with glyceraldehyde 3-phosphate as substrate could not be determined over a suitable range of saturation.

In an effort to account for the rapid reduction in rate seen in Fig. 5, the final

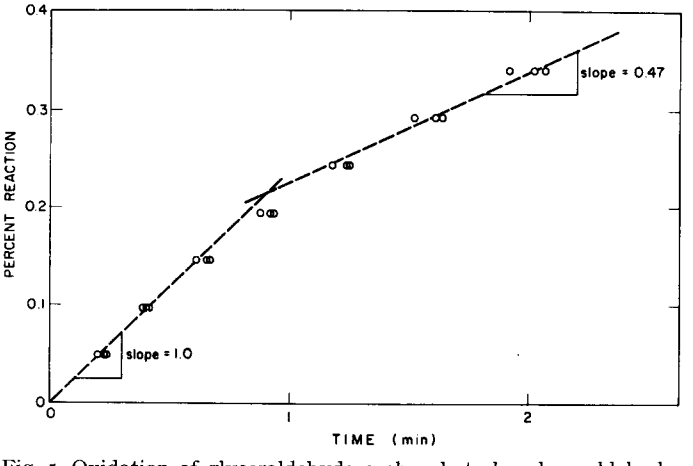


Fig. 5. Oxidation of glyceraldehyde 3-phosphate by glyceraldehyde-3-phosphate dehydrogenase plotted as a function of time. Assay mixture initially contained $1.5 \cdot 10^{-9}$ M glyceraldehyde-3-phosphate dehydrogenase, $1 \cdot 10^{-2}$ M DL-glyceraldehyde 3-phosphate, $1 \cdot 10^{-2}$ M NAD^+, $1 \cdot 10^{-2}$ M sodium arsenate, $5 \cdot 10^{-3}$ M 2-mercaptoethanol, $5 \cdot 10^{-3}$ M EDTA, and $5 \cdot 10^{-2}$ M sodium pyrophosphate at pH 7.0, 25°.

end products of the glyceraldehyde 3-phosphate reaction in the presence of arsenate (NADH and 3-phosphoglycerate) were investigated as reversible inhibitors of glyceraldehyde-3-phosphate dehydrogenase.

In Fig. 6 is shown the effect of NADH ($9.3 \cdot 10^{-5}$ M) on the initial rate of reduction of NAD^+ ($8.6 \cdot 10^{-3}$ M). It may be observed that when the concentration of NADH is approx. 1% that of the initial concentration of NAD^+, an 11% reduction in initial velocity is obtained. NADH thus has a greater affinity than NAD^+ for at least the enzyme's fourth binding site—a finding which is in agreement with an earlier ob-

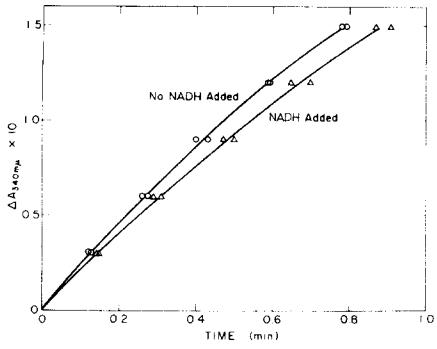


Fig. 6. Rate of oxidation of glyceraldehyde 3-phosphate by glyceraldehyde-3-phosphate dehydrogenase in the presence and absence of NADH. Assay mixtures contained $1 \cdot 10^{-2}$ M DL-glyceraldehyde 3-phosphate, $8.6 \cdot 10^{-3}$ M NAD^+, $1 \cdot 10^{-2}$ M sodium arsenate, $5 \cdot 10^{-3}$ M EDTA, $5 \cdot 10^{-2}$ M sodium pyrophosphate at pH 7.0 and 25°, with (△) and without (○) $9.3 \cdot 10^{-5}$ M NADH.

servation by VELICK AND FURFINE[13]. Although NADH binds effectively to glyceraldehyde-3-phosphate dehydrogenase, end product inhibition by this compound alone cannot account for the 50% reduction in initial rate after 0.3% reaction seen in Fig. 5.

3-Phosphoglycerate, the second end product in the arsenate catalyzed reaction, appears to possess little or no affinity for glyceraldehyde-3-phosphate dehydrogenase. The initial rate of NADH formation was measured in an assay medium containing $2 \cdot 10^{-5}$ M DL-glyceraldehyde 3-phosphate, $1 \cdot 10^{-3}$ M NAD^+, $5 \cdot 10^{-2}$ M sodium pyrophosphate, $1 \cdot 10^{-2}$ M sodium arsenate, and $5 \cdot 10^{-3}$ M EDTA, at pH 7.0 in the presence and absence of $1 \cdot 10^{-2}$ M D-3-phosphoglycerate. No detectable inhibition by 3-phosphoglycerate, present at 1000 times the concentration of the active isomer of glyceraldehyde 3-phosphate, was observed.

DISCUSSION

The studies of the initial rates of glyceraldehyde-3-phosphate dehydrogenase with various aldehydes as a function of NAD^+ concentration revealed, in general, that there was little change in the catalytic center activity for molecular species $E(NAD^+)_2$, $E(NAD^+)_3$ and $E(NAD^+)_4$. An overall 1.1-fold rate increase was observed with glyceraldehyde as a substrate, a 1.4-fold decrease with acetaldehyde and a 1.2-fold decrease with propionaldehyde. These small changes are to be contrasted with the approx. $1 \cdot 10^7$-fold decrease in ligand affinity induced by the binding of NAD^+ on going from $E(NAD^+)_1$ to $E(NAD^+)_4$ (ref. 5). It may well be asked how it is possible that allosteric interactions which cause such enormous changes in binding of NAD^+ can have so little effect on catalytic efficiency, when the binding and catalytic groups for substrates must be in close juxtaposition. This phenomenon may be explained by the induced fit reasoning in which ligand induced conformational changes, transmitted through subunit interactions, account for cooperative binding effects[3]. As an illustration, the binding of NAD^+ and glyceraldehyde to glyceraldehyde-3-phosphate dehydrogenase, at saturating concentrations of the aldehyde, is schematically represented in Fig. 7 by a simple case in which only two conformations for an individual subunit are shown. The affinity of the enzyme for NAD^+ is determined by the number of subunits which

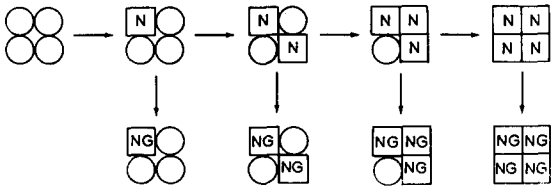


Fig. 7. Schematic illustration of the binding of NAD^+ and glyceraldehyde to glyceraldehyde-3-phosphate dehydrogenase at saturating concentrations of the aldehyde for the 'square' case of the induced fit model. The conformation of subunits without bound NAD^+ is denoted by circles, and the conformation of subunits with bound NAD^+ by squares. N = NAD^+; G = glyceraldehyde.

contain bound NAD^+ and the strengths of the interactions between subunits. The stability relations between subunits however, may not appreciably affect the glyceraldehyde portion of the site. Therefore, the molecular activity of glyceraldehyde, which only reacts at those sites containing bound NAD^+, will be affected little, if at all, by the subunit interactions which control NAD^+ affinity. It is quite conceivable that a conformational change is induced by the glyceraldehyde, but one may assume from these data that this change is fairly small or is localized and does not extend to the subunit boundaries.

The kinetic results reported here support previous investigations[4,6–8] which indicate a linear relationship between molecular activity and the first few equivalents of NAD^+ added. This study goes one step further, however, in relating catalytic rates to the NAD^+ bound using previously determined equilibrium values for NAD^+ binding. Since the theoretical curves were obtained from dissociation constants determined from equilibrium dialysis in the absence of an aldehyde substrate, the close agreement of theory and experiment not only supports the idea that NAD^+ binding is unaffected by the aldehyde but can be used in reverse, *i.e.*, the kinetic studies can provide a saturation plot which indicates extent of NAD^+ binding. Plots of v *vs.* $[S]$ frequently assume that v is proportional to substrate bound but this is not often verified by independent checks. The finding of a direct proportionality is important since it may allow one to explore the details of saturation curves at extreme values of low $[S]$ and high $[S]$ where equilibrium dialysis experiments are particularly inaccurate.

The general conclusion that the negatively cooperative binding interactions induced by NAD^+ have little effect on the intrinsic catalytic rates of glyceraldehyde-3-phosphate dehydrogenase must be tempered by two considerations. Firstly, the kinetic observations which support the above conclusion apply to glyceraldehyde, acetaldehyde and propionaldehyde, compounds which are oxidized by glyceraldehyde-3-phosphate dehydrogenase much more slowly (approx. 1000 fold) than its natural substrate, glyceraldehyde 3-phosphate. Since these aldehydes obviously do not form the most catalytically productive complex with glyceraldehyde-3-phosphate dehydrogenase, they may also be insensitive to subtle structural alterations induced by NAD^+ which do affect the molecular activities for the oxidation of glyceraldehyde 3-phosphate. Secondly, the equilibrium constants for the dissociation of the NAD^+–enzyme complexes ($E(NAD^+)_1$, $E(NAD^+)_2$, $E(NAD^+)_3$ and $E(NAD^+)_4$) were measured in the absence of an aldehyde substrate, and therefore may not be identical with the equilibrium constants for the dissociation of the NAD^+–acyl enzyme complexes.

The effect of NAD^+ on the intrinsic rates of catalysis of glyceraldehyde-3-phosphate dehydrogenase, with glyceraldehyde 3-phosphate as substrate, was not studied here, because the high molecular activity of this substrate prohibited initial velocity measurements, by conventional assay procedures, at low levels of NAD^+. It may be calculated, for example, that even in the absence of end product inhibition or an unfavorable reaction equilibrium, the rate of oxidation of glyceraldehyde 3-phosphate, in the presence of half saturating concentrations of NAD^+, will drop to 10% of its initial value about 0.02 sec after initiation of the reaction. This rapid reduction in rate is due to the depletion of NAD^+. The finding, however, that the rate of oxidation of glyceraldehyde 3-phosphate is, in fact, very much affected by end product inhibition or an unfavorable equilibrium (Fig. 5) indicates that the time in which true initial velocities must be measured, may be much less than that calculated above. Thus, if conventional assay procedures, which require at least 5 sec, are employed, the catalytic rates which are actually measured will be much slower than the true initial rates. More importantly, however, in relation to determining the effect of NAD^+ on intrinsic molecular activities, the difference between the pseudo rates and true rates will be greater at lower concentrations of NAD^+ than at higher levels of the nucleotide. These observations indicate that an earlier study of glyceraldehyde-3-phosphate dehydrogenase with glyceraldehyde-3 phosphate (ref. 5) in which intrinsic catalytic rates appeared to be substantially faster at the higher concentrations of NAD^+ is probably misleading.

The finding that the rate of oxidation of glyceraldehyde 3-phosphate by glyceraldehyde-3-phosphate dehydrogenase decreased rapidly even at relatively high concentrations of NAD^+ prompted further investigation of this phenomenon.

In the glyceraldehyde-3-phosphate dehydrogenase catalyzed oxidation of glyceraldehyde 3-phosphate in the presence of arsenate, the product initially formed is 1-arseno-3-phosphoglycerate, which spontaneously and irreversibly hydrolyses to 3-phosphoglycerate. If the rate of hydrolysis of 1-arseno-3-phosphoglycerate is rapid relative to the rate of enzyme catalysis, then NADH and 3-phosphoglycerate, the only end products in significant concentration, must be responsible for the observed decrease in initial rate. The finding that neither NADH nor 3-phosphoglycerate could fully account for this decrease suggests that 1-arseno-3-phosphoglycerate must be present in sufficient concentration to participate in the feedback inhibition of the enzyme. The accumulation of 1-arseno-3-phosphoglycerate, in turn, indicates that the hydrolysis of this product may not be rapid relative to the rate of catalysis.

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