A Role for Nicotinamide Adenine Dinucleotide Glycohydrolase in the Control of Glycerate-3-phosphate Dehydrogenase Activity

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SUMMARY

Tumor nicotinamide adenine dinucleotide (NAD) glycohydrolase (EC.3.2.2.5) was purified 500-fold, and this preparation was used to study the influence of NAD glycohydrolase on the NAD+ content and activity of crystalline glycerate-3-phosphate dehydrogenase. The pseudomonomolecular velocity constant, k, for the hydrolysis of free NAD+ was 5-fold that of the constant for the enzyme-bound NAD+.

Hydrolysis of 90 percent or more of the bound NAD+ did not result in a shift in the absorption spectrum of the dehydrogenase, which has a maximum at 276 m/μ, or in a loss in enzyme activity. Native rabbit muscle glycerate-3-phosphate dehydrogenase was resistant to tryptic digestion but was rapidly inactivated by trypsin after hydrolysis of the bound NAD+ by NAD glycohydrolase. Complete protection against such inactivation in the simultaneous presence of NAD glycohydrolase and trypsin was conferred by excess NAD+. It is suggested that increased NAD glycohydrolase activity may influence the level of NAD-dependent dehydrogenases in tissues containing proteolytic enzymes.

INTRODUCTION

Ehrlich ascites tumor cell nicotinamide adenine dinucleotide (NAD) glycohydrolase activity is increased when mice bearing this tumor are injected with 1,3-bis(2-chloroethyl)-1-nitrosourea or after implantation of tumor cells which have been treated in vitro with this alkylating agent (10, 13). The level of NAD+, as affected by the action of NAD glycohydrolase, has been shown to be associated with the rate of Ehrlich ascites tumor cell proliferation (13), and with the toxic effects of trehalose-6,6′-dimycolic acid from virulent T. bacilli (cord factor) after injection into animals of different species (1, 3, 4).

The possibility exists that increased levels of NAD glycohydrolase activity may influence the activity of dehydrogenases in tissues containing proteolytic enzymes.

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RESULTS

The Kinetics of Hydrolysis of Free NAD+ and of NAD+ bound to Glyceradehyde-3-phosphate Dehydrogenase by NAD Glycohydrolase

Solutions of crystalline rabbit muscle glyceradehyde-3-phosphate dehydrogenase were prepared fresh each day by dissolving 3.00 to 3.50 mg in 10 ml of Tris-EDTA. In general the mean specific activity of the enzyme in such a solution was about 26 units per mg of protein. The NAD+ content (20) was about 2.2 μmoles per μmole of glyceradehyde-3-phosphate dehydrogenase, on the basis of a molecular weight of 140,000 gm per mole (9, 19). The mean value of the ratio of the absorbance at 280 μm to the absorbance at 260 μm was 1.17.

The reaction was started by adding varying amounts of NAD glycohydrolase to 5.0 ml of the glyceradehyde-3-phosphate dehydrogenase solution at 37°C. At periodic intervals an aliquot of the reaction mixture was removed and the NAD+ content determined. Decreases in NAD+ in the presence of NAD glycohydrolase activities ranging from 0.0 to 0.60 units per ml of reaction mixture are shown in Chart 2.

The equation for a first order reaction is \( k = \frac{1}{t} \ln \frac{A}{A-x} \), where \( k \) is the velocity constant, \( t \) is time in minutes, \( A \) is the initial NAD+ concentration, and \( x \) is the NAD+ hydrolyzed in time \( t \). This equation was applied to the data, and \( k \) was found to have a constant value, within experimental error, for the disappearance of 90 to 95% of the initial NAD+ concentration at each concentration of NAD glycohydrolase tested. The values of \( k \) for 0.20, 0.30, 0.40, 0.50, and 0.60 units of NAD glycohydrolase per ml of reaction mixture were proportional to the concentration of NAD glycohydrolase. The mean value of \( k \) per unit of NAD glycohydrolase was 0.091 min⁻¹.

The equation for a first order reaction was also applied to the time course of the action of NAD glycohydrolase on NAD+ not bound to glyceradehyde-3-phosphate dehydrogenase, i.e., free NAD+. Each of the reaction mixtures contained 2.2 × 10⁻⁶ M NAD+ and 0.05% bovine serum albumin to stabilize the NAD glycohydrolase. Over 90% of the NAD+ was hydrolyzed during 15 min at 37°C. The values of \( k \) were constant within experimental error throughout the time course of the reaction; they were 0.048 min⁻¹, 0.098 min⁻¹, and 0.140 min⁻¹ for 0.10, 0.20, and 0.30 units of NAD glycohydrolase per ml of reaction mixture respectively. The mean value of \( k \) per unit of NAD glycohydrolase was 0.48 min⁻¹, and it was about 5 times as large as the constant for the hydrolysis of NAD+ when bound to glyceradehyde-3-phosphate dehydrogenase, i.e., \( k = 0.091 \) min⁻¹.

The relationship between the initial concentration of free NAD+ and \( k \) was studied over a 20-fold range of substrate concentration, from 8.6 × 10⁻⁷ M to 15.6 × 10⁻⁶ M NAD+. In each of these reactions 85–95% of the initial NAD+ was hydrolyzed during 15 min. The velocity parameter for a first order reaction was constant throughout the time course of each reaction. The mean value for \( k \) per unit of NAD glycohydrolase was 0.54 min⁻¹. The pseudomonomolecular nature of the reaction held throughout the twenty-fold range in the concentration of free NAD+, a range which was, however, within the essentially straight-line portion of the substrate velocity relationship (11).
The Effect of NAD Glycohydrolase on Glyceraldehyde-3-phosphate Dehydrogenase

Glyceraldehyde-3-phosphate dehydrogenase-bound NAD\(^+\) can be removed from the enzyme by activated charcoal (7). Such a preparation of glyceraldehyde-3-phosphate dehydrogenase shows a shift from its characteristic absorption maximum of 276 nm to one at 280–282 nm (30). The effects of NAD glycohydrolase on the absorption spectrum, enzyme activity, and NAD\(^+\) content of glyceraldehyde-3-phosphate dehydrogenase were determined. Charcoal treatment of a reaction mixture containing glyceraldehyde-3-phosphate dehydrogenase and NAD glycohydrolase (at zero time) resulted in the removal of more than 90% of the dehydrogenase-bound NAD\(^+\) and shifted its absorption maximum from 276 nm to 282 nm with no loss in dehydrogenase activity (Table 1, lines 1 and 2). When the reaction mixture was allowed to incubate for 60 min at 25°C (Table 1, line 3), bound NAD\(^+\) was decreased to less than 10% of its original value, but there was no shift in the absorption maximum. Charcoal treatment of this 60-min sample resulted in a shift in the absorption maximum to 280–282 nm (Table 2, line 4).

The question whether, after incubation with NAD glycohydrolase, adenosine diphosphoribose (ADPR) and nicotinamide (NAM) were still on the glyceraldehyde-3-phosphate dehydrogenase molecule or were free in the reaction mixture was investigated.

A Sephadex G-25 gel which retards compounds with a molecular weight of 5000 or less was prepared in Tris-EDTA buffer and placed into a column. The following solutions were prepared in Tris-EDTA buffer: glyceraldehyde-3-phosphate dehydrogenase, 3.0 mg protein per ml; NAD\(^+\), 0.04 mM; NAM, 0.04 mM; ADPR, 0.04 mM. One ml of each was passed through the gel, and the effluent, collected in 3.0 ml aliquots, was read at 260 nm. At this wave length ADPR and NAM have maximal absorbancy (16, 28), and glyceraldehyde-3-phosphate dehydrogenase is about 80% of that at its maximum at 276 nm. Chart 3A shows that over 90% of the glyceraldehyde-3-phosphate dehydrogenase came through between Tubes 5 and 8. Similarly, NAD\(^+\) (Chart 3B) and ADPR (Chart 3C) came through almost completely between Tubes 12 and 16 and NAM (Chart 3D) between 13 and 16. When glyceraldehyde-3-phosphate dehydrogenase, which was incubated with 3.0 units NAD glycohydrolase for 1 hour at 25°C (see Table 1, line 3) was filtered through the Sephadex column, 260 nm absorbing material (Chart 3E) came through between Tubes 5 and 8, but no material appeared in Tubes 12 to 17, the region where NAD\(^+\), NAM, or ADPR would be expected.

The complete absorption spectrum, NAD\(^+\) content, and dehydrogenase activity were measured in the Sephadex G-25 effluent collected in Tube 7 from the experiments described in Chart 3A and 3E. Passage through the gel did not result in any significant change in the parameters reported previously in Table 1, lines 1 and 3.

The Effect of NAD Glycohydrolase on the Susceptibility of Glyceraldehyde-3-phosphate Dehydrogenase to Tryptic Digestion

NAD\(^+\) is required for the stabilization of the secondary structure of glyceraldehyde-3-phosphate dehydrogenase and protects it from digestion by trypsin (8, 29). The possibility of increased susceptibility of glyceraldehyde-3-phosphate dehydrogenase to tryptic digestion after treatment with NAD glycohydrolase was investigated (Table 2). As shown earlier in this study, NAD glycohydrolase caused the destruction of glyceraldehyde-3-phosphate dehydrogenase-bound NAD\(^+\) but did not decrease the enzymatic activity (Table 2, B). Crystalline trypsin had no effect on the level of bound NAD\(^+\) and very little effect on the activity of the glyceraldehyde-3-phosphate dehydrogenase, i.e., 8% loss in 1 hour at 10 μg trypsin, 19% in 1 hour at 30 μg trypsin, and 39% (not shown) in 1 hour at 50 μg trypsin (Table 2, C and D). However, the presence of both NAD glycohydrolase and trypsin caused a substantial increase in the rate of inactivation of glyceraldehyde-3-phosphate dehydrogenase, i.e., 37% in 1 hour at 10 μg trypsin, 53% in 1 hour at 30 μg trypsin, and 77% in 1 hour at 50 μg trypsin (Table 2, E and F).

The effect of the simultaneous presence of NAD glycohydrolase and trypsin on the inactivation of glyceraldehyde-3-
Control of Glyceraldehyde-3-P Dehydrogenase Activity

Table 1

<table>
<thead>
<tr>
<th>Incubation with NAD glycohydrolase (min)</th>
<th>Glyceraldehyde-3-P dehydrogenase activity (units)</th>
<th>NAD⁺ (µmoles)</th>
<th>Absorption maximum (µm)</th>
<th>280 µm</th>
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<tr>
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The effect of nicotinamide adenine dinucleotide (NAD) glycohydrolase on the NAD⁺ content, enzymatic activity, and ultraviolet absorption characteristics of glyceraldehyde-3-phosphate (P) dehydrogenase. The reaction mixture contained 3.3 mg glyceraldehyde-3-P dehydrogenase and 3.0 units of NAD glycohydrolase in 10 ml of Tris-EDTA buffer, pH 7.4. Incubation was for 60 min at 25°C was for 60 min at 25°C.

a The quantity is expressed per µmole of glyceraldehyde-3-P dehydrogenase protein (9, 19): Each value is the average of results from two experiments. Determinations were done in duplicate.

b Charcoal treatment of glyceraldehyde-3-P dehydrogenase (see Materials and Methods) after incubation with NAD glycohydrolase for the time shown.

Table 2

<table>
<thead>
<tr>
<th>Contents of incubation mixture</th>
<th>Time at 37°C (min)</th>
<th>Relative glyceraldehyde-3-P dehydrogenase activity (%)</th>
<th>Relative NAD⁺ content (%)</th>
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Susceptibility of glyceraldehyde-3-phosphate (P) dehydrogenase to trypsin digestion after incubation with nicotinamide adenine dinucleotide (NAD) glycohydrolase. Reaction mixtures contained 3.0 mg of glyceraldehyde-3-P dehydrogenase and 0, 10, or 30 µg crystalline trypsin in a final volume of 10 ml of Tris-EDTA buffer, pH 7.4, at 37°C. Three units of NAD glycohydrolase were added to start the reaction. Samples of the mixture were taken for assay at the time of mixing (zero time) and at 10-min intervals during 1 hour. All experiments were done at least in duplicate, and the activities checked each other within 0 to 3%. For convenience and brevity only the values at 20-min intervals during a typical run are shown. In each experiment glyceraldehyde-3-P dehydrogenase activity (7.00 units/ml) and the enzyme-bound NAD⁺ concentration (4.75 µmoles/ml) at zero time were set at 100%, and subsequent enzyme velocities and NAD⁺ concentrations were expressed as relative activities.

Chart 3. Separation of glyceraldehyde-3-phosphate (P) dehydrogenase from NAD⁺ adenosine diphosphoribose (ADPR), and nicotinamide (NAM) on a Sephadex G-25 gel. The bed volume was 25 ml; void volume was 15 ml and the flow rate was 0.5 ml per min under a head of hydrostatic pressure produced by a reservoir of buffer hung 20 cm above the surface of the gel. One-ml samples were placed on the gel and, 3.0 ml aliquots of effluent were collected. All steps were carried out at 4°C. NAD, nicotinamide adenine dinucleotide.
phosphate dehydrogenase was studied further (Chart 4). As noted above, 50 μg trypsin inactivated glyceraldehyde-3-phosphate dehydrogenase to the extent of 39% in 1 hour. The additional presence of NAD glycohydrolase increased the inactivation to 77%. The inclusion of 100 μg of soybean trypsin inhibitor in the incubation mixture completely abolished the inactivation of the glyceraldehyde-3-phosphate dehydrogenase. Similarly, when 250 μg NAD+ was substituted for the soybean trypsin inhibitor, tryptic inactivation of glyceraldehyde-3-phosphate dehydrogenase was reduced to less than 5% in 60 min.

The velocity constant k, described earlier in the present paper, was calculated for the hydrolysis of NAD+ by NAD glycohydrolase in the reaction mixtures containing trypsin as described in Table 2. The values for k per unit of NAD glycohydrolase in the presence of 10 or 30 μg of trypsin were 0.081 min⁻¹ and 0.087 min⁻¹ respectively, and it did not differ significantly from the value 0.087 min⁻¹ for the constant in the absence of trypsin.

**DISCUSSION**

In the present study we have shown that essentially all of the NAD+ which is bound to active glyceraldehyde-3-phosphate dehydrogenase is rapidly hydrolyzed at pH 7.4 by a highly purified soluble mammalian NAD glycohydrolase. These results differ from those of Astrachan et al. (2), who reported that, in the pH range of 7.4—8.0, a small fraction of the NAD+ bound to active glyceraldehyde-3-phosphate dehydrogenase was hydrolyzed by crude NAD glycohydrolase from Neurospora (2). Our finding that the time course of the hydrolysis of free NAD+ or NAD+ bound to crystalline rabbit muscle glyceraldehyde-3-phosphate dehydrogenase by purified NAD glycohydrolase was pseudomonomolecular enabled us to determine and compare the rates of hydrolysis of the free and enzyme-bound NAD+ with some precision. The value of the velocity constant k for the hydrolysis of the free NAD+ was 5-fold that of the enzyme-bound NAD+. Bock et al. (5), among others, have suggested that the intracellular localization of NAD glycohydrolase limits the action of this enzyme on NAD+. Astrachan et al. (2) have considered the availability of protein-bound NAD+ as a cofactor for dehydrogenase enzymes. Our kinetic findings suggest that the nature of a given NAD+-protein complex in the cell may be a factor in determining the rate at which NAD+ is catabolized in the cell.

Several investigators have suggested that the NAD+ molecule is attached to the glyceraldehyde-3-phosphate dehydrogenase through at least two linkages (2, 25, 29). In the present study, hydrolysis of bound NAD+ by NAD glycohydrolase did not result in a shift of the absorption maximum. The observation that no measurable ADPR or NAM was separated from the NAD glycohydrolase-treated enzyme by passage through a G-25 Sephadex gel suggests the possibility that the end products of the reaction, NAM and ADPR, remain attached to the enzyme. This supports the concept that NAD+ is bound to glyceraldehyde-3-phosphate dehydrogenase by at least two linkages.

Racker and Krinsky (21) reported that two inhibitors of glycolysis were present in normal mouse brain homogenates and identified one of them as being the enzyme responsible for the destruction of NAD+. In a subsequent series of reports (21—26), these authors suggested that the inhibition of glycolysis in mouse brain homogenates was due to the inactivation of endogenous glyceraldehyde-3-phosphate dehydrogenase by a proteolytic enzyme which was present in the homogenate. Addition of nicotinamide or NAD+ to the homogenate prevented this inhibition. We have shown in the present study that the hydrolysis of NAD+, bound to glyceraldehyde-3-phosphate dehydrogenase by NAD glycohydrolase, markedly increases the susceptibility of the dehydrogenase to tryptic digestion.

These findings are consistent with the formulation that an increase in the NAD glycohydrolase activity of Ehrlich ascites tumor cells (1, 3, 10, 13) could decrease the level of active glyceraldehyde-3-phosphate dehydrogenase or other dehydrogenase enzymes which require NAD+ for stability and protection against proteolytic inactivation. This formulation assumes that proteolytic enzymes are active in ascites cells with increased NAD glycohydrolase. To date no precise definition of the nature, intracellular localization, or concentration of proteolytic enzymes in Ehrlich ascites cells has been made. Studies are currently in progress to measure the activity of glyceraldehyde-3-phosphate dehydrogenase as well as several other NAD+-dependent dehydrogenases in ascites cells with...
increased NAD glycohydrolase. The activity of proteolytic en-
zymes in these cells will also be determined.

REFERENCES


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