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ISOLATION AND CHARACTERIZATION OF YEAST NICOTINAMIDE ADENINE DINUCLEOTIDE KINASE

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Summary

NAD$^+$ kinase (ATP:NAD$^+$ 2′-phosphotransferase, EC 2.7.1.23) from yeast has been purified utilizing ion-exchange and NAD$^+$-agarose affinity chromatography to give a 2100-fold purification. The apparent homogeneity of the enzyme preparation was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis and analytical ultracentrifugation. The enzyme has a subunit molecular weight of 31 000, and a native molecular weight of 124 000, and is, thus, probably a tetramer. The single form of the enzyme has an apparent isoelectric point of 5.85. Initial velocity studies in the forward direction with both substrates gave intersecting Lineweaver-Burk plots, and this suggests a sequential mechanism in which both substrates are bound before products are released. Replots of these data were linear and gave K_m values for NAD$^+$ and ATP of 0.68 mM and 2.3 mM, respectively.

Introduction

The cellular pyridine nucleotide pools are divided chemically and functionally between the NAD * nucleotides and the NADP nucleotides. NAD is utilized in many catabolic reactions involved in the generation of ATP and biosynthetic intermediates, while NADP is used in reactions that generate and consume biosynthetic reducing equivalents. NAD and NADP are rapidly interconverted in vivo [1,2]. The levels of NADP within cells are generally lower

* NAD and NADP are used to represent the sum total of both oxidized and reduced forms of these two classes of nucleotides.

than the lvels of NAD, although the size of the NADP pool varies in different cell types from less than 1% to over 30% the size of the NAD pool [3,4]. In yeast the size of the NADP pool relative to the NAD pool is reversibly adjusted in response to the carbon source in the growth medium [5]. The manner in which the levels of NADP relative to NAD are regulated is poorly understood. A potential key reaction in this regulation is the ATP-dependent phosphorylation of NAD^+ catalyzed by NAD^+ kinase (ATP:NAD^+ 2′-phosphotransferase, EC 2.7.1.23). This enzyme was partially purified for the first time by Kornberg [6] from brewers yeast. It has since been partially purifed from a number of sources [7—17] but has been purified to homogeneity only from pigeon liver [18]. Owing to a possible important role of NAD^+ kinase in the regulation of NADP levels in yeast, we report here a procedure for the isolation of the yeast enzyme. We also report preliminary characterization of the physical and kinetic properties of the enzyme.

Materials and Methods

Materials. NAD^+, NADPH, ATP, glucose 6-phosphate, bovine serum albumin, Trizma base, phospho*enol*pyruvate, Coomassie brilliant blue G, DEAE-cellulose, pyruvate kinase, and glucose-6-phosphate dehydrogenase were obtained from the Sigma Chemical Co. (St. Louis, MO). Sucrose, glucose, EDTA, KH_2PO_4, glycerol, NaOH, $MgCl_2$, $CaCl_2$, phosphoric acid, and KOH were obtained from Fisher Scientific Co. (Pittsburgh, PA). 2-Mercaptoethanol and ethanolamine were obtained from the Eastman Chemical Co. (Rochester, NY). Bacto-yeast extract and Bacto-peptone were obtained from the Difco Laboratory (Detroit, MI). $(NH_4)_2SO_4$ was from Baker Chemical Co. (Phillipsburg, NJ). Glass beads (0.45—0.50 mm) were from VWR Scientific (San Francisco, CA). NAD^+-agarose (AGNAD TYPE 1) was from P.L. Biochemicals (Milwaukee, WI). Sodium dodecyl sulfate, bisacrylamide (*N,N′*-methylene-bisacrylamide), ammonium persulfate, and acrylamide for gel electrophoresis were obtained from Bio-Rad Laboratories (Richmond, CA). Ampholine carrier ampholytes were purchased from LKB-Instruments (Rockville, MD).

Enzyme Assays. The reaction mixture consisted of 100 mM Tris-HCl, pH 9.0, 3 mM ATP, 2 mM NAD^+, 4 mM $MgCl_2$, 1 mg/ml bovine serum albumin, 1 mM glucose 6-phosphate, 2.5 units glucose-6-phosphate dehydrogenase and enzyme in a final volume of 1.0 ml. Assays were initiated by addition of enzyme and incubation was at 30° C. The increase in fluorescence at 460 nm with excitation at 340 nm was recorded with time with an Aminco-Bowman fluorimeter. For measurement of activity in crude extracts a modified assay was used. The reaction mixture contained an ATP-generating system (10 mM phospho*enol*pyruvate, 10 mM KCl, and 20 μg/ml pyruvate kinase), and glucose 6-phosphate and glucose-6-phosphate dehydrogenase were omitted. At different times portions of reaction were adjusted to 0.5 M $HClO_4$. The extracts were chilled and centrifuged at $1500 \times g$ for 10 min and the supernatants were adjusted to pH 7.0—7.5 by adding 1 M KOH, 0.33 M potassium phosphate, with rapid mixing. After standing on ice for at least 15 min, $KClO_4$ was removed by centrifugation. The $NADP^+$ was converted to NADPH by the addition of glucose 6-phosphate and glucose-6phosphate dehydrogenase, and fluor-

escence was measured as above. One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 nmol NAD^+ to $NADP^+$/min at 30°C.

Data processing. The initial velocity results were first plotted graphically as double-reciprocal plots with each data point an average of replicates. The individual data points were fitted to Eqn. 1 for an ordered bisubstrate reaction by the weighted least squares method of Wilkinson [19]. The fits to the equation were performed by a digital computer with the Fortran programs of Cleland [20].

$$v_0 = \frac{VAB}{K_{ia}K_b + K_aB + K_bA + AB} \quad (1)$$

Protein determinations. Protein was determined by the binding of Coomassie brilliant blue G to protein using bovine serum albumin as standard [21]. Protein concentrations of column eluants were determined by monitoring absorbance at 280 nm.

Growth of yeast. A strain * of *Saccharomyces cerevisiae* originally isolated from commercial yeast (Red Star Yeast and Products Co., Milwaukee, WI) was grown in a liquid complex medium which contained 5% glucose, 1% yeast extract. 0.5% Bacto-peptone, and salts: 0.9% KH_2PO_4, 0.6% $(NH_4)_2SO_4$, 0.03% $CaCl_2$, and 0.03% $MgSO_4$. Inoculum cultures were grown at 30°C on a rotary incubator for 24 h. The inoculum (120 ml) was transferred to a Microferm Fermentor containing 10.8 l of liquid complex medium and grown for 9 h at 30°C. Under these conditions, the cells grow logarithmically with a doubling time of approximately 70 min and are in late log phase at harvest. The growth yield is 30—35 g wet weight/l. The cells were harvested with a Sharples centrifuge (23 000 rev./min) and washed with ice-cold 100 mM Tris-HCl (pH 9.0), 100 mM sucrose, 1 mM EDTA, 0.5 mM 2-mercaptoethanol.

Preparation of crude extract (fraction I). Fresh cell pellets from an 11 l culture were suspended in 600 ml 100 mM Tris-HCl buffer, pH 9.0, 100 mM sucrose, 1 mM EDTA and 0.5 mM 2-mercaptoethanol, and an equal volume of glass beads was added. Cells were disrupted in a Waring blendor for a total of 5 min at 4°C. Intermittent cooling periods were used to prevent the extract from reaching temperatures above 10°C. The extract was subjected to centrifugation at 2740 × *g* for 5 min. The supernatant was then centrifuged at 20 700 × *g* for 1 h, and designated as fraction I.

Preparation of $(NH_4)_2SO_4$ fraction (fraction II). This fraction was brought to 70% (v/v) saturation with $(NH_4)_2SO_4$ by addition of a saturated solution with stirring at 4°C. After 30 min, the precipitate was collected by centrifugation for 30 min at 28 300 × *g*, and resuspended in 55% (v/v) saturated ammonium sulfate. After 15 min, the preparation was centrifuged at 28 300 × *g* for 30 min. The supernatant was devoid of NAD^+ kinase activity and was discarded. The precipitate was resuspended in 30% $(NH_4)_2SO_4$. After centrifugation at 28 300 × *g* for 30 min, the supernatant solution which contained the NAD^+ kinase activity was dialyzed overnight against 10 mM potassium phosphate

* Inocula of this strain can be obtained from the authors on request.

buffer (pH 7.5), 1 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol and designated fraction II. This fraction could be stored at −15°C for several months without appreciable loss of activity.

DEAE-cellulose chromatography (fraction III). DEAE-cellulose was equilibrated with 10 mM potassium phosphate buffer adjusted to pH 7.5. The column (2.5 × 61 cm) was washed with 10 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol in a cold room at 0—4°C. The eluant was tested to ensure that the desired pH had been attained before the same was applied. Fraction II (30 ml containing 405 mg protein) was thawed and applied and the column was eluted with buffer followed by a linear gradient generated by mixing 1800 ml starting buffer and 1800 ml buffer containing 0.3 M NaCl (25-ml fractions, flow rate, 1 ml/min). NAD^+ kinase activity was eluted at approx. 0.024 M NaCl. The fractions containing enzyme activity were pooled and concentrated to 0.5 ml by a negative pressure protein dialysis concentrator (ProDiCon) using a 10 000 molecular weight exclusion membrane. The concentrated solution was either used immediately or stored at −15°C. The NAD^+ kinase activity was unstable at this point. Inactivation could be minimized by concentrating the enzyme as rapidly as possible.

NAD^+-agarose column chromatography (fraction IV). Fraction III was applied to a NAD^+-agarose affinity column (bed volume = 1 ml) which was equilibrated in 10 mM potassium phosphate buffer (pH 7.5), 1 mM EDTA, 10% glycerol, 10 mM 2-mercaptoethanol. The column was first washed with buffer and then with buffer containing 5 mM NAD^+ (1 ml fractions). The fractions containing NAD^+ kinase activity were stored at −15°C. The purified enzyme was stable for several months when stored under these conditions.

Electrophoresis. Polyacrylamide gel electrophoresis in the presence of 1% sodium dodecyl sulfate (SDS) was carried out in 5.6% gels according to the method of Fairbanks et al. [22]. Electrophoresis was at 3 mA/tube until the tracking dye migrated into the gel. The current was then increased to 5 mA/tube and the gels were run for 3 h. Gels were stained for protein with 0.2% (w/v) Coomassie blue in methanol/acetic acid/H_2O (46 : 8 : 46, v/v) and destained in 10% acetic acid/45% methanol/45% H_2O.

Isoelectric focusing. Isoelectric focusing was carried out in a sucrose density gradient using 2.5% LKB ampholines in a 110 ml LKB column as described previously by Vesterberg and Svensson [23]. Electrofocusing was performed with pH 3—7 ampholines and at 300 V at 4°C for 72 h (1 ml fractions). The pH value of each fraction was determined immediately and NAD^+ kinase activity was measured as described above.

Analytical ultracentrifugation. Meniscus depletion sedimentation equilibrium ultracentrifugation was done with a Beckman-Spinco Model E analytical ultracentrifuged equipped with RTIC temperature regulation. An AN-D rotor and 12 mm double sector Yphantis cell with sapphire windows were used. The sample was exhaustively dialyzed against 50 mM Tris-HCl buffer (pH 8.0), 0.1 M NaCl, 10 mM 2-mercaptoethanol for 3 days. Centrifugation was at 18 000 rev./min for 19 h at 15.2°C. Rayleigh interference fringes were analyzed with Nikon Model 6 C microcomparator equipped with digital x-y encoders.

Results and Discussion

NAD kinases from most organisms so far examined catalyze the phosphorylation of NAD^+ but not NADH [7—15,17]. However, yeast contain a mitochondrial NAD kinase that is specific for NADH [16]. Previously studies have shown that the cytoplasmic NAD^+ kinase represents about 95% of the total NAD^+ and NADH kinase activities of the yeast cell [16]. In addition, the cells in the present study were grown under conditions where the synthesis of mitochondrial enzymes is glucose repressed. The activity purified in this study therefore represents cytoplasmic NAD^+ kinase.

Previous attempts to purify NAD^+ kinase from yeast have been hampered by severe instability of the enzyme during purification [14]. We have found that this problem can be largely overcome by including 10 mM 2-mercaptoethanol in all solutions used for purification. However, the presence of a high concentration of thiols precludes the use of sensitive enzymatic cycling assays that have been used to assay the enzyme [16,18]. Blomquist has described a stop assay for NAD^+ kinase using fluorescence detection of NADPH [17]. We have modified this assay to allow $NADP^+$ formed in the reaction mixture to be continuously monitored by including glucose 6-phosphate and glucose-6-phosphate dehydrogenase. We have found this assay to be reproducible and to be of sufficient sensitivity. It is also convenient for screening a large number of fractions for enzyme activity and for kinetic studies which require a large number of assays.

The four-step purification procedure for the isolation of yeast NAD^+ kinase described in Table I has been used successfully in five separate preparations. The ammonium sulfate precipitation/reverse extraction procedure resulted in a 16-fold increase in specific activity, however, much of this increase is due to a 6.5-fold increase in total enzyme activity. This must be due to factors in the crude extract that either inhibit the enzyme or interfere with its assay. The chromatographic pattern of fraction II on DEAE-cellulose is shown in Fig, 1. A single peak of activity is eluted at a salt concentration of approximately 0.024 M. Although this step results in a 32-fold purification, considerable losses of total activity are encountered as the enzyme is quite unstable at this point. Inactivation can be minimized by concentrating the enzyme as rapidly as possible.

TABLE I

PURIFICATION OF NAD^+ KINASE FROM *SACCHAROMYCES CEREVISIAE*

Fraction	Protein concn. (mg/ml)	Total volume (ml)	Total protein (mg)	Total activity (units *)	Spec. act. (units/mg)	Purification
I Crude extract	16.5	64	1056	1 626	1.54	1
II Ammonium sulfate	13.5	31	418	10 509	25.1	16
III DEAE-cellulose chromatography	6.1	0.6	3.66	2 909	795	516
IV NAD^+-agarose chromatography	0.26	3.0	0.78	2 496	3200	2078

* Units: 1 nmol $NADP^+$ formed from substrate/min.

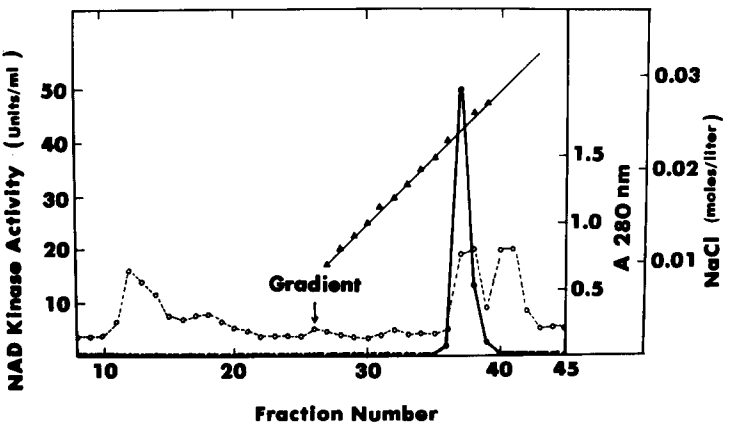

Fig. 1. Column chromatography of NAD^+ kinase on DEAE-cellulose. Fraction II (30 ml containing 405 mg protein) was applied to a column of DEAE-cellulose which was equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 10% glycerol and 10 mM 2-mercaptoethanol. After 625 ml of the same buffer was passed through the column, a linear NaCl gradient was applied to the column. Fractions were collected and assayed for NAD^+ kinase activity (●), protein concentration (○) and NaCl concentration (▲).

The chromatography of fraction III on an NAD^+-agarose affinity column is shown in Fig. 2. The enzymatic activity is quantitatively bound to the column although a flow-through peak of protein is observed. The enzyme can be eluted by the addition of buffer containing 5 mM NAD^+. This purification step results in a 3.9-fold purification with 86% recovery and yields a homogeneous preparation of enzyme (Fig. 3).

Although NAD^+-agarose affinity chromatography has been used in the purification of a number of enzymes [24—27], to our knowledge this is the

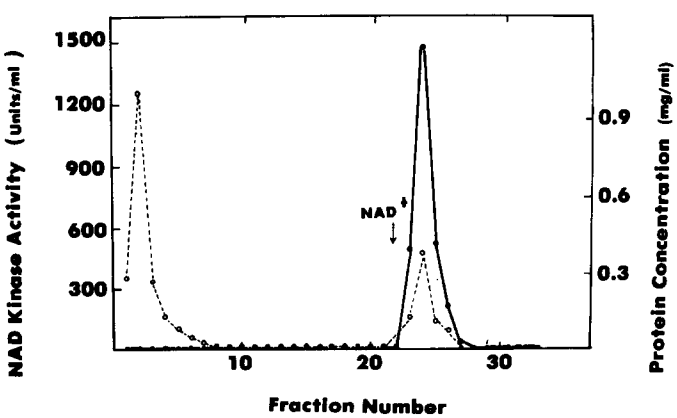

Fig. 2. Column chromatography of NAD^+ kinase on NAD^+-agarose. Fraction III (0.5 ml containing 3.11 mg protein) was applied to a column of NAD^+-agarose which was equilibrated with 10 mM potassium phosphate buffer, pH 7.5, containing 1 mM EDTA, 10% glycerol and 10 mM 2-mercaptoethanol. After elution with 22 ml of the same buffer, buffer containing 5 mM NAD^+ was applied. Fractions were collected and assayed for NAD^+ kinase activity (●) and protein concentration (○).

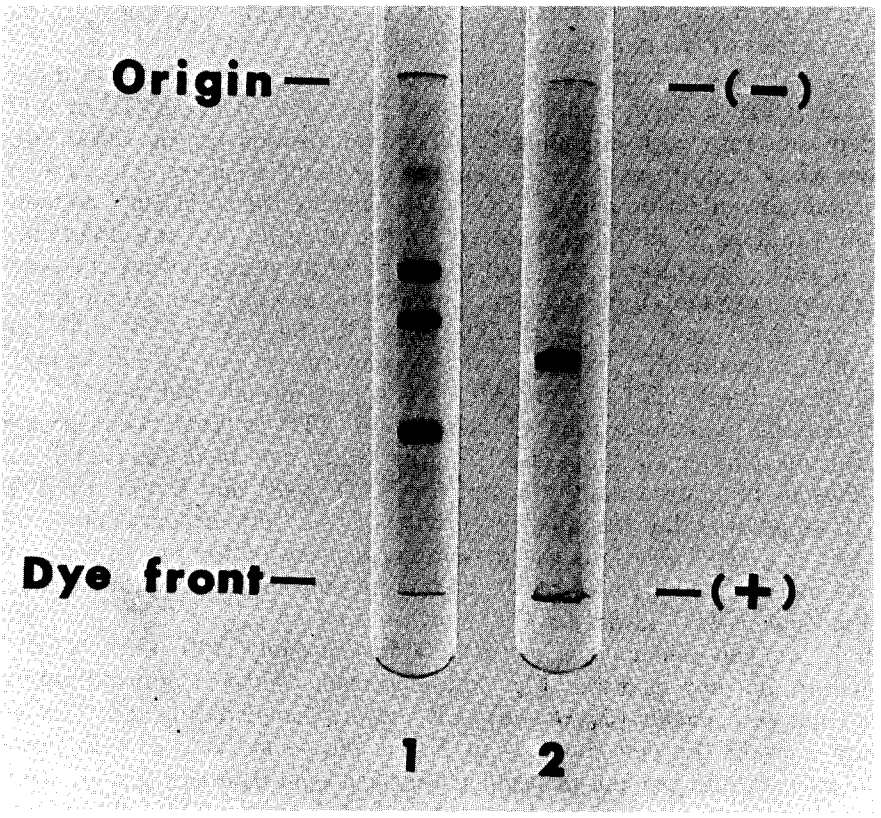

Fig. 3. SDS-polyacrylamide gel electrophoresis of purified NAD^+ kinase. Gel 1 contained (from top) bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), and ribonuclease A (M_r 13 700). Gel 2 contained 20 μg of purified NAD^+ kinase (fraction IV).

first time it has been used in the purification of NAD^+ kinase. Previously, NAD^+ kinase has been isolated from pigeon liver by a seven-step procedure that yielded a preparation of specific activity of approximately 800 units/mg. The purification procedure reported here achieves an overall purification of approximately 2100-fold in four steps and results in a homogenous preparation with a specific activity of 3200 units/mg.

The subunit molecular weight of NAD^+ kinase was determined by comparing the relative mobility of purified enzyme and standards on SDS-polyacrylamide gels (Fig. 3). A subunit molecular weight of 31 000 was determined. When the enzyme was subjected to equilibrium sedimentation under non-denaturing conditions, plots of log fringe displacement vs. r^2 were linear indicating a monodisperse species. From the slope of this line, a native molecular weight of 124 000 ± 6000 (four determinations) was estimated. Thus, the native enzyme is probably a tetramer, with identical or nearly identical subunits. The subunit molecular weight of the enzume is very similar to that of 34 000 reported for NAD^+ kinase from pigeon liver, however, the native molecular weight reported for pigeon liver was 270 000 [18]. Isoelectric focusing of the enzyme is shown

in Fig. 4. A p*I* of 5.85 was determined. This is consistent with the chromatographic properties of the enzyme on DEAE-cellulose.

The steady-state kinetics of the reaction were studied in the forward direction. The data of Fig. 5 shows double-reciprocal plots with either NAD^+ or ATP as the varied substrate with changing/fixed concentrations of the other substrate. Other assay conditions were the same as the standard assay. Assays were done at a total concentration of Mg^{2+} of 4 mM. Varying the total Mg^{2+} concentration from 4 mM to 20 mM had no effect on the reaction rate at a fixed concentration of ATP. The intersecting nature of the plots suggests that the reaction proceeds by a sequential mechanism. Linear replots were also obtained and K_m values for NAD^+ and ATP were determined to be 0.68 ± 0.16 mM and 2.30 ± 0.6 mM, respectively. The V_{max} of the preparation was 3.2 ± 0.4 μmol/min per mg. Thus, the $V/[E_t]$ for the enzyme is approximately 400 min^{-1}.

The intracellular concentrations of NAD^+ and ATP in yeast cells grown under conditions similar to those used in this study can be estimated from literature data to be approximately 1 mM for NAD^+ [28] and 2 mM for ATP [28–31]. If we assume that the specific activity of the crude preparation (1.54 nmol/min per mg, see Table I) is the maximum activity of the enzyme in the cell, then we can use the kinetics results of the enzyme to predict in vivo rates. By assuming that NAD^+ binds to the enzyme first ($K_{NAD^+} = K_{i(NAD^+)}$) and using Eqn. 1, we can calculate a value of approximately 400 pmol $NADP^+$ synthesized/min per mg protein. The crude extract contains approximately half of the total cell protein (Jacobson, M.K., unpublished data), and thus we can estimate that in the cell there is an NAD^+ kinase activity of 200 pmol $NADP^+$ synthesized/min per mg protein. Under the conditions of growth used in this study the yeast cells contain approximately 2.5 nmol of NADP/mg total protein and grow with a doubling time of 70 min [5]. The cells must therefore

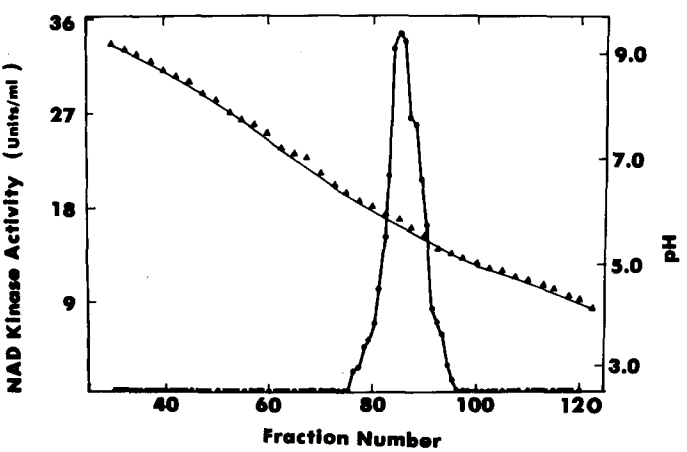

Fig. 4. Isoelectric focusing of NAD^+ kinase. Purified enzyme (0.17 mg) was subjected to isoelectric focusing with 2.5% ampholines (pH 3.0–10.0) as described in Materials and Methods. Fractions were collected and assayed for NAD^+ kinase activity (●) and pH (▲).

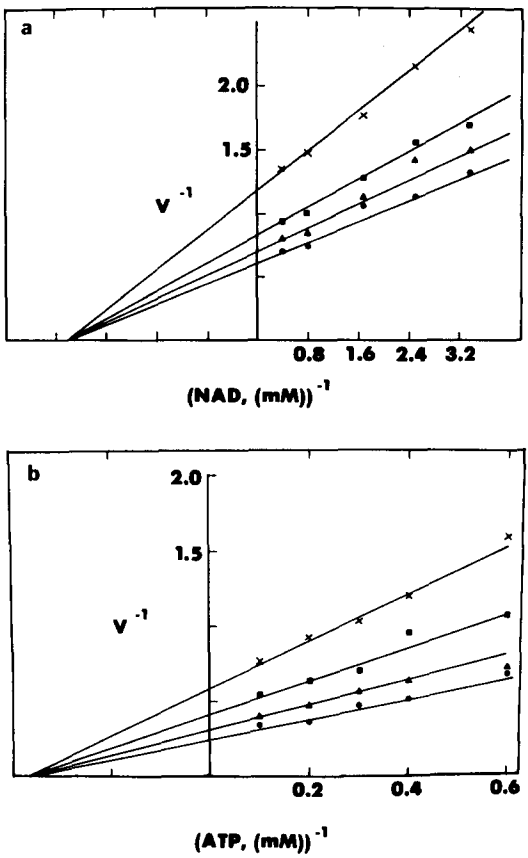

Fig. 5. Kinetic studies of NAD^+ kinase in the forward direction. *V* is expressed in nmol $NADP^+$ formed/min per μg protein. (a) Double-reciprocal plot for the reaction, with NAD^+ as the varied substrate and changing/fixed concentrations of ATP. The ATP concentrations were: X, 1.0 mM; ■, 2.0 mM; ▲, 3.0 mM; ●, 4.0 mM. (b) Double-reciprocal plot for the reaction, with ATP as the varied substrate and changing/fixed concentrations of NAD^+. The NAD^+ concentrations were: X, 0.33 mM; ■, 0.6 mM; ▲, 1 mM; ●, 2 mM.

achieve the net synthesis of approximately 36 pmol NADP/min per mg total protein in addition to maintaining the pool of NADP. Although the enzyme activity measurements were made at the unphysiological pH of 9.0, these calculations indicate that the apparent activity of NAD^+ kinase in crude extracts can easily account for the observed net synthesis of NADP. However, very little information is available on the in vivo turnover rate of NADP in yeast. Further, the increase in total activity after ammonium sulfate fractionation could indicate the presence of in vivo modulators of NAD kinase activity. Thus, while it appears that there is a more than adequate activity of NAD^+ kinase in cells to account for the net synthesis of NADP, many questions remain as to the possible regulation of this activity.

The steady-state levels of NADP in cells will reflect the steady-state levels of NAD in addition to the relative rate of conversion of NAD to NADP catalyzed

by NAD kinases and the rate of conversion of NADP to NAD catalyzed by NADP phosphatases or other catabolic reactions of NADP. In yeast, the levels of NADP relative to NAD are reversibly adjusted in response to changes in the carbon source of the growth medium [5]. Such a change must involve a change in the rate of NAD phosphorylation or NADP breakdown or both. The availability of homogeneous yeast NAD^+ kinase will allow the detailed study of factors that affect the catalytic activity of this enzyme. This should allow a more complete evaluation of the role of NAD^+ kinase in the regulation of cellular NADP levels in yeast.

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References

1 Stollar, V. and Kaplan, N.O. (1961) J. Biol. Chem. 236, 1863–1866
2 Lundquist, R. and Olivera, B.M. (1971) J. Biol. Chem. 246, 1107–1116
3 Glock, G.E. and McLean, P. (1955) Biochem. J. 61, 388–390
4 Kaplan, N.O. (1972) in Harvey Lectures 1970–1971, Series No. 66, pp. 105–133, Academic Press, New York
5 Ting, H.Y., Jacobson, E.L. and Jacobson, M.K. (1977) Arch. Biochem. Biophys. 1973, 89–104
6 Kornberg, A. (1950) J. Biol. Chem. 182, 805–813
7 Wang, T.P. and Kaplan, N.O. (1954) J. Biol. Chem. 206, 311–325
8 Yamomoto, Y. (1966) Plant Physiol. 41, 523–528
9 Chung, A.E. (1967) J. Biol. Chem. 242, 1182–1186
10 Apps, D.K. (1968) Eur. J. Biochem. 5, 444–449
11 Yero, I.L., Farinas, B. and Dietrich, L.S. (1968) J. Biol. Chem.. 243, 4885–4888
12 Oka, H. and Field, J.B. (1968) J. Biol. Chem. 243, 815–819
13 Apps, D.K. (1969) Eur. J. Biochem. 7, 260–266
14 Apps, D.K. (1970) Eur. J. Biochem. 13, 223–230
15 Fernandes, M. (1970) J. Neurochem. 17, 503–509
16 Griffiths, M.M. and Bernofsky, C. (1972) J. Biol. Chem. 247, 1473–1478
17 Blomquist, C.H. (1973) J. Biol. Chem. 248, 7044–7048
18 Apps, D.K. (1975) Eur. J. Biochem. 55, 475–483
19 Wilkinson, G.N. (1961) Biochem. J. 80, 324–332
20 Cleland, W.W. (1970) Adv. Enzymol. 29, 1–32
21 Bradford, M.M. (1976) Anal. Biochem. 72, 248–254
22 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
23 Vesterberg, O. and Svensson, H. (1966) Acta Chem. Scand 20, 820–834
24 Mosbach, K., Guilford, H., Ohlsson, R. and Scott, M. (1972) Biochem. J. 127, 625–631
25 Lowe, C. and Dean, P. (1973) Biochem. J. 133, 515–520
26 Grover, A.K. and Hammes, G.C. (1974) Biochim. Biophys. Acta 356, 309–318
27 Lowe, C., Harvey, M., Craven, D., Kerfoot, M., Hollows, M. and Dean, P. (1973) Biochem. J. 133, 507–513
28 Chapman, C. and Bartley, W. (1969) Biochem. J. 111, 609–613
29 Polakis, E.S. and Bartley, W. (1966) Biochem. J. 99, 521–533
30 Betz, A. and Moore, C. (1967) Arch. Biochem. Biophys. 120, 268–273
31 Chapman, A.G., Fall, L. and Atkinson, D.E. (1971) J. Bacteriol. 108, 1072–1086