Purification and Properties of Yeast Nicotinamide Adenine Dinucleotide Synthetase*

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SUMMARY

Yeast NAD synthetase was purified 2,000-fold. The purified enzyme gave one protein band on disc gel electrophoresis and was found to be monodisperse on high speed equilibrium ultracentrifugation with an apparent molecular weight for the "native" enzyme of 630,000. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis showed the presence of apparent nonidentical subunits with molecular weights of 80,000 and 65,000. The amino acid composition of "native" enzyme revealed no unusual amino acid residues.

The specificity of the substrates, nucleoside triphosphate requirement, and divalent and monovalent metal ion requirements were studied. Nicotinate adenine dinucleotide, but not nicotinate mononucleotide, is the amide acceptor, and glutamine or free ammonia is the amide donor. The enzyme is specific for ATP, exhibiting a stoichiometric cleavage into AMP and PPi, during the amidation. Mg2+ and K+ are required for enzymatic activity. Mn2+ or Co2+ can replace Mg2+ to a certain extent. NH4+ is as effective as K+ in stimulating enzymatic activity. The enzyme displays a broad peak of activity between pH 6.2 and 8.4, with maximal activity being observed around pH 7.6 when L-glutamine is used as the amide donor. However, when ammonium chloride is employed as the substrate, the enzymatic activity exhibits a rather narrow peak between pH 8.4 and 8.8. The apparent Km values for nicotinate adenine dinucleotide, ATP, and L-glutamine at pH 7.6 were found to be 1.9 \times 10^{-4}, 1.7 \times 10^{-4}, and 5 \times 10^{-4} M, respectively. The Km for NH4Cl at pH 8.6 is 6.4 \times 10^{-3} M. The catalytic constant was calculated to be approximately 1,260 moles of NAD produced per min per mole of enzyme, based on a molecular weight of 630,000.

When the enzyme was previously incubated with azaserine, nicotinate adenine dinucleotide, and ATP, glutamine-dependent activity was progressively inhibited as a function of prior incubation time. However, whereas NH4Cl as the amide donor there was an initial 20% inhibition and then NH4Cl-dependent activity remained constant. p-Chloromercuribenzoate and heavy metals inhibit both glutamine- and NH4Cl-dependent activity.

Hydroxamate analogues were formed when (nicotinate adenine dinucleotide + ATP) and/or glutamine were incubated with enzyme and hydroxylamine. Hydroxamate analogues were not formed from nicotinate adenine dinucleotide unless ATP was also present. Hydroxamate analogue formation was additive when glutamine, nicotinate adenine dinucleotide, and ATP were added.

In 1958, Preiss and Handler (1) demonstrated the existence of NAD synthetase in yeast and partially purified the enzyme. NAD synthetase catalyzes the amidation of nicotinate adenine dinucleotide (1, 2) as shown in the reaction:

Nicotinate adenine dinucleotide + glutamine or NH3 + ATP \rightarrow NAD + glutamate + AMP + PPi

The reported apparent K_m for NAD is in the order of 10^{-4} M. In contrast, the intracellular NAD concentration determined from various sources, such as Ehrlich ascites cells (3), Escherichia coli (4), and rat liver (5) varies from 10^{-8} to 10^{-4} M. Although NAD concentration in yeast has not been measured, the indications are that it is low (6). These discrepancies may indicate that the enzyme is under some type of regulatory control or is present in a great excess over the rate-limiting reactions in NAD synthesis. NAD is preferentially utilized in reactions involving L-glutamine and NH4Cl as substrates at pH 7.5 and pH 8.6, respectively. The pK of NH4+ is 9.2 (8), and the rate of reaction is not enhanced by the concomitant presence of saturated concentrations of NH4Cl and L-glutamine at pH 7.4. Studies on the physicochemical properties of yeast NAD synthetase and reinvestigations of its catalytic properties in the homogeneous state may provide possible clues to answer the above problems and also provide a way leading to further explorations of the nature of the enzyme at the molecular level. It is for these reasons that studies on NAD synthetase from yeast were initiated.

EXPERIMENTAL PROCEDURE

Materials—NAD, nicotinic acid, and ATP were obtained from Sigma Chemical Company, St. Louis, Missouri; alcohol de-
hydrogenase was from P-L Biochemicals, Milwaukee, Wisconsin. L-Glutamine and ammonium sulfate, enzyme grade, were from Nutritional Biochemicals, Cleveland, Ohio; bovine serum albumin was from Nutritional Biochemicals or Gallard-Schilding Chemical Manufacturing Corporation, Carle Place, New York. β-Mercaptoethanol and Cleland's reagent were obtained from Calbiochem, Los Angeles, California; crystalline inorganic pyrophosphatase was from Worthington Biochemical Corporation, Freehold, New Jersey. EDTA was from Matheson Coleman and Bell, Ohio; azaserine was obtained from the National Cancer Institute. 6-DiazO-5-oxo-L-norleucine was from Parke, Davis and Company, Detroit, Michigan; [8-14C]ATP and [8-14C]-AMP were from New England Nuclear Corporation, Boston; [32P]P, was from Amersham/Seaple Corporation, Arlington Heights, Illinois. [14C]NAD was a gift of Dr. Laphalle Fuller. SDS, reagent grade, was recrystallized from hot water, rinsed with acetone and ether, and allowed to air dry overnight. DEAE-cellulose, hydroxylapatite, and AG1-X8 (formate form) were purchased from Bio-Rad Laboratories, Richmond, California. Sepharose 4B was obtained from Pharmacia Chemical Company, Piscataway, New Jersey.

**Assay of Enzymatic Activity—NAD synthetase activity was determined by measuring the rate of NAD formation at 37°C. The reaction mixture for standard assays, except when otherwise stated, contained 1 mM NAD, 2 mM ATP, 20 mM L-glutamine, 5 mM MgCl₂, 56 mM KCl, (0.04%, BSA when needed), 50 mM Tris-Cl buffer, pH 8.0, and enzyme in a total volume of 0.25 to 0.5 ml. The reaction mixture was incubated for 30 or 60 min. After incubation, the reaction was terminated by heating in a boiling water bath for 30 to 60 s and the reaction mixture was centrifuged at 800 × g for 2 min. NAD was determined on the clear supernatant material by employing alcohol dehydrogenase (9) or fluorescence (10).

**Synthesis of Substrate—N⁵AD was synthesized by the direct exchange reaction of the nicotinamide moiety of NAD with free nicotinate catalyzed by fresh beef spleen microsomal NAD glycohydrolase (NADase) (EC 3.2.2.5) (11). The incubation procedure and isolation of N⁵AD were those described by Hontjo et al. (12). To obtain maximum N⁵AD formation from the NAD-nicotinate exchange reaction, it is critical to determine the optimum amount of enzyme needed as well as length of incubation time. This can best be done by performing small scale pilot experiments. By taking these precautions, it is possible to obtain N⁵AD with a yield as high as 70%.

**Disc Electrophoresis—For ordinary polyacrylamide gel electrophoresis in the absence of SDS, the Canalco gel system was used except that the porosity of the gels was adjusted to 5.6% or 4%. The gels contained 1 x 10⁶ units NAD synthetase, 56 mM KCl, 0.04% BSA. After incubation, the reaction was terminated by heating in a boiling water bath for 30 to 60 s and the reaction mixture was centrifuged at 800 × g for 2 min. NAD was determined on the clear supernatant material by employing alcohol dehydrogenase (9) or fluorescence (10).

**Two-dimensional paper chromatographic techniques were employed to identify and quantitate [4C]NAD, [4C]NMN, [4C]N⁵AD, and [4C]NAD. Solvent I was used in the first dimension and Solvent III (0.1 M phosphate, pH 6.8-ammonium sulfate-1-propanol, 100:60:2) in the second dimension. Descending technique was used throughout. The radioactive spots identified as N⁵MN, NMN, N⁵AD, and NAD were cut out and quantitated as described above.

**PP₃ was quantitated by measuring the orthophosphate produced by the hydrolysis of pyrophosphate in the presence of crystalline inorganic pyrophosphatase. Inorganic phosphate was determined by the method of Fiske and SubbaRow (14).

**ATP-32PP₃ and ATP-3¹⁴CAMP Exchange—In the ATP-3²PP₃ exchange studies, 3²PP₃ at a final concentration of 10⁻³, 2 x 10⁻³, or 10⁻⁴ M was added to the following sets of reaction vessels: +N⁵AD - GluNH₂, +N⁵AD - GluNH₂ - K⁺, +N⁵AD + GluNH₂, - N⁵AD + GluNH₂ or -N⁵AD - GluNH₂. The concentrations of all reactants were the same as described in the NAD synthetase assay. The reactions took place at pH 7.6 or 8.6. In other experiments, NAD synthetase was previously incubated with azaserine (2 x 10⁻⁴ M) in the presence of ATP, N⁵AD, and cations at pH 8.0 for 1 hour to inhibit glutaminase-dependency activity before 3²PP₃ (2 x 10⁻³ M) was added. In the case of the ATP-3¹⁴CAMP exchange studies, 3¹⁴CAMP (10⁻³ M) was added to a series of reaction vessels similar to those employed in the ATP-3²PP₃ exchange studies. Activity was measured in the presence or absence of PP₃ (10⁻⁴ M) at pH 7.6. Paper chromatography was employed to separate radioactive PP₃ and ATP with Solvent III in the descending technique. Separation and quantitation of radioactive ATP and AMP was performed as described previously.

**Protein was measured by the method of Lowry et al. (15), with crystalline BSA as a standard. Amounts of Tris-Cl, pH 7.4, Cleland's reagent, EDTA, and KCl equal to those present in samples being assayed were added to the BSA solutions before the BSA standard curve was run. Protein was also determined by measuring the absorbance at 280 nm.

**Molecular Weight Determination—The molecular weight of native NAD synthetase was determined according to the high speed equilibrium technique of Yphantis (16). Ultracentrifugation was carried out with a Spinco model E analytical ultracentrifuge equipped with an electronic speed control and Rayleigh interference optical system. During the centrifugation, the temperature was maintained at 281.4°C. Centrifuge runs were performed in a six-channel centerpiece at initial protein concentrations of 0.65 to 0.33 mg per ml and column heights were 3 mm. The fringe patterns were measured with a Nikon micro-comparator. Data were processed by computer programs developed by Small and Resnick (17). A partial specific volume
of 0.74 ml per mg was assumed for the calculation of molecular weight.

For molecular weight determinations of the subunits, 10% polyacrylamide gel in the presence of 0.1% SDS (as described above) was used. Proteins of known molecular weight were used to construct a molecular weight standard curve. The mobility of each species of protein was calculated according to Weber and Osborn (13). The molecular weights of the subunits of the native enzyme were determined by a comparison with the molecular weight standard curve.

**Amino Acid Analyses**—Yeast NAD synthetase was suspended in constant boiling hydrochloric acid (approximately 6 N) in a hydrolysis tube. The tube was thoroughly evacuated. After evacuation, the tube was sealed and heated to 100° C for 24 hours. The hydrolysate was taken rapidly to dryness with a rotary evaporator. Amino acid analysis was performed by the method of Spackman et al. (18) with a Beckman amino acid analyzer, model 120 C, having a recorder equipped with an expanded range card so that the full scale on the recorder is 0.1 absorbance.

**Formation of Hydroxamate Analogues**—Yeast NAD synthetase was incubated with 200 mM NH₄OH, 5 mM MgCl₂, 56 mM KCl, 50 mM Tris, pH 8.0, and the various substrates for 60 min at 37° C in a total volume of 0.55 ml. The reaction was terminated by the addition of 0.15 ml of 8% trichloroacetic acid containing 3.3% FeCl₃ and 2 N HCl. The hydroxamate analogues formed were measured as the ferric complex according to the procedure of Hartman (19).

### RESULTS

**Purification of NAD Synthetase**—All purification steps were carried out at 4° C. All of the buffer solutions used in the enzyme purifications contained 1 mM Cleland’s reagent and 1 mM EDTA. Cleland’s reagent could not be replaced by 20% glycerol.

Fleischmann bakers’ yeast cake purchased locally (2.7 kg) was washed twice with 0.9% NaCl solution. The yeast cells, suspended in 50 mM Tris, pH 7.4 (1 g wet weight per ml of buffer), were disrupted by employing a French press at a pressure of 16,000 p.s.i., 4,900 ml of the disrupted cells being obtained by this treatment. The broken cell suspension was centrifuged at 14,000 × g for 40 min and the pellet was washed once with the same buffer. The resultant turbid supernatant material was recentrifuged at 40,000 × g for 1 hour in a Spinco ultracentrifuge. NAD synthetase activity was recovered in the slightly turbid supernatant material.

To the supernatant material, solid ammonium sulfate (28.2 g per 100 ml) was added slowly with constant stirring. After the last addition of the salt, the suspension was stirred for 30 min and then kept at 4° C overnight. The precipitate was collected by centrifugation at 27,000 × g for 30 min with a Sorval centrifuge and dissolved in 0.05 M Tris-Cl, pH 7.4. The insoluble material was removed by centrifugation. Most of the NAD synthetase activity was recovered in this fraction (0 to 40% saturation of ammonium sulfate). The specific activity of the enzyme is highest in the fraction obtained at 0° C to 30% saturation, only a residual enzymatic activity being observed in fractions higher than 40% saturation. Our experience is that the same degree of final purity can be obtained using fractions from either 20 to 30% or 0 to 40% saturation. The preparation obtained from ammonium sulfate fractionation was dialyzed against 40 volumes of 0.04 M Tris-Cl, pH 7.2, containing 0.04 M KCl, overnight with one buffer change. This material was then subjected to ion exchange chromatography on DEAE-cellulose equilibrated with 40 mM Tris-Cl, pH 7.2, containing 40 mM KCl, Cleland’s reagent, and EDTA. The column (2.5 × 56 cm) was developed with a linear gradient of KCl (0.04 to 0.25 M). A single peak of enzymatic activity was obtained. The active fractions were pooled and concentrated to 20 ml using an Amicon Diaflo cell with a PM-30 or PM-10 membrane. The active material was dialyzed against 100 volumes of the buffer containing 25 mM potassium phosphate, pH 7.5, for 3 to 4 hours. The insoluble inactive materials were removed by centrifugation before the enzyme preparation was applied to the next column.

The active material was then applied to a hydroxylapatite column which had been equilibrated with 0.1 M potassium phosphate, pH 7.4, containing Cleland’s reagent and EDTA. After the proteins were adsorbed on the hydroxylapatite, the column was further washed with 200 ml of washing buffer. The column (1.6 × 15 cm) was developed with a linear gradient of potassium phosphate (0.025 to 0.3 M). The active fractions were pooled and concentrated to 4 ml using an Amicon Diaflo cell with a PM-10 membrane. This material was then dialyzed against 250 volumes of the buffer containing 50 mM Tris-Cl, pH 7.6, and 100 or 350 mM KCl for 4 hours. The insoluble inactive material was removed by centrifugation.

The active material from hydroxylapatite fractionation was applied to a Sepharose 4B column which had been equilibrated with 50 mM Tris-Cl, pH 7.6, and 100 or 350 mM KCl. Elution was carried out with the same buffer. Two-milliliter fractions were collected at a flow rate of approximately 10 ml per hour. The fractions which contained a single protein band coincident with NAD synthetase activity on disc gel electrophoresis at pH 9 were pooled. The pooled material was concentrated using an Amicon Diaflo cell with a PM-10 membrane. A single enzymatic peak was usually observed and a Vₑ/Vₑₑ = 2 to 2.2 was usually obtained. Attempts to locate another enzymatic peak at higher elution volumes were unsuccessful.

A summary of the purification of NAD synthetase from bakers’ yeast is presented in Table I. This enzyme was purified 2000-fold with a specific activity of 2 μmoles per min per mg of protein when freshly prepared.

### Table I

**Summary of purification of NAD synthetase**

| Step            | Volume | Protein | Specific activity | Fold | Yield |
|-----------------|--------|---------|-------------------|------|-------|
| Broken cell suspension | 1155   | 83.0    | 0.6               | 100  |
| Cell extract    | 1226   | 56.4    |                   | 1    | 144   |
| 20 to 30% (NH₄)₂SO₄ | 100    | 40.8    | 12                 | 11   | 90    |
| DEAE-cellulose column | 20    | 14.7    | 111                | 103  | 69    |
| Hydroxylapatite column | 4     | 6.7     | 430               | 400  | 21    |
| Sepharose 4B column | 1.2    | 0.95    | 2100              | 1945 | 5     |
plot of $\ln C$ versus $(\text{radius})^2$ obtained from high speed equilibrium ultracentrifugation according to Yphantis (16) (Fig. 2).

**Physicochemical Properties of NAD Synthetase**

**Stability**—The stability of NAD synthetase is dependent upon the degree of purity and protein concentration. NAD synthetase from ammonium sulfate fractionation with a protein concentration of 40 mg per ml can be stored at $-70^\circ$ for more than 3 months with loss of only about 30% of the enzymatic activity. The homogeneous enzyme with a protein concentration of 0.2 mg per ml loses 60% activity during 10 days of storage at $4^\circ$. This inactivation cannot be prevented by storing at $-70^\circ$ or $-20^\circ$. However, the rate of inactivation can be greatly decreased by the addition of BSA at a concentration greater than 10 mg per ml. Repeated freezing and thawing accelerate the enzymatic inactivation. Attempts to prevent inactivation of the pure enzyme by drying, changing the ionic strength, pH, addition of 1 mM Cleland's reagent, storing the enzyme in 30% glycerol, in 70% ammonium sulfate at neutral pH, or in 1 M urea were unsuccessful. Partially inactivated enzyme can be reactivated to the original activity by the addition of 1 to 2% mercaptoethanol. However, once enzymatic activity is totally lost, mercaptoethanol addition has no effect.

Exposure of NAD synthetase to various pH levels at $4^\circ$ overnight demonstrated that no loss of activity occurred between pH 6 and 10. At pH 5, 50% of the initial activity was lost. This also occurred when the enzyme preparation was incubated at pH 5 for 5 to 10 min at $37^\circ$.

**Molecular Weight**—The molecular weight of active, pure NAD synthetase obtained by the high speed equilibrium method of Yphantis (16) is summarized in Table II. Three expressions of molecular weight, i.e. weight average, number average, and $Z$ average, are almost identical. It is not known at present whether a molecular weight of 630,000 represents a molecular weight of a minimum active unit or a molecular weight of an aggregate from minimum active units.

One milligram of homogeneous yeast NAD synthetase catalyzes the formation of 2 μmoles of NAD per min when NAD and L-glutamine are used as the substrates at pH 7.4 at $37^\circ$. Since the molecular weight of NAD synthetase as isolated is approximately 630,000 (Table II), the minimum catalytic constant is calculated to be approximately 1,260 moles of NAD produced per min per mole of NAD synthetase under the assay conditions described. The rate of enzyme catalysis is not affected by using either 50 mM Tris-Cl or 10 mM potassium phosphate as the buffer in the incubation system at pH 7.4.

SDS-treated NAD synthetase, run on a disc gel (5.6%) containing SDS, was resolved into two bands which were stained in equal intensity by Coomassie blue (Fig. 3A). The SDS-treated protein was incubated at $37^\circ$ for 3 hours in 0.01 M sodium

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**Table II**

| Experiments                  | Enzyme | Apparent molecular weight |
|------------------------------|--------|---------------------------|
| High speed equilibrium      | "Native" | 634,000 ($M_w$)          |
|                              |        | 635,000 ($M_n$)          |
|                              |        | 622,000 ($M_z$)          |
| SDS gel electrophoresis     | Subunits |                          |
| A                            |        | 80,000                   |
| B                            |        | 65,000                   |
Amino acid composition of yeast NAD synthetase

Figures in parentheses are results obtained employing a high concentration (0.5 mg of protein in 2 ml) of hydrolysate.

| Amino acid       | Calculated residues based on a molecular weight of 630,000 |
|------------------|----------------------------------------------------------|
| Lysine           | 333 (383)                                                |
| Histidine        | 150 (144)                                                |
| Arginine         | 216 (286)                                                |
| Aspartic acid    | 532                                                       |
| Threonine        | 220 (244)                                                |
| Serine           | 314                                                       |
| Glutamic acid    | 423                                                       |
| Proline          | 223 (258)                                                |
| Glycine          | 348                                                       |
| Alanine          | 300 (318)                                                |
| Half-cystine     | 115 (88)                                                  |
| Valine           | 223 (263)                                                |
| Methionine       | 123 (131)                                                |
| Isoleucine       | 305 (329)                                                |
| Leucine          | 509                                                       |
| Tyrosine         | 139 (156)                                                |
| Phenylalanine    | 198 (202)                                                |

The condition was as described by Weber and Osborn (13).

**Amino Acid Composition**—Table III shows the amino acid composition of NAD synthetase. Because of the scarcity of pure enzyme, only the results obtained after hydrolysis for 24 hours in acid are presented. No correction was made on unstable residues such as serine and threonine. Tryptophan was not determined. No unusual amino acids were detected in this analysis. A rather high percentage of hydrophobic residues such as leucine, isoleucine, alanine, and lysine were noted. In addition, high amounts of aspartate and glutamate were observed. Asparagine and glutamine may have contributed to the high levels of glutamate and aspartate since a high percentage of ammonia was recovered after hydrolysis. Since the amount of ammonia formed was not quantitated, no estimate of the amount of asparagine and glutamine can be made.

**Catalytic Properties of NAD Synthetase**

pH optimum studies are presented in Fig. 5. NAD synthetase activity displays a broad peak of activity between pH 6.2 and 8.4, with maximal activity being observed around pH 7.6 when L-glutamine is used as the amide donor. However, when ammonium chloride is employed as the substrate, the enzymatic activity exhibits a rather narrow peak between pH 8.4 and 8.8. Enzymatic activity between pH 7 and 8 is directly correlated.
with the calculated concentration of free NH₃, indicating that free ammonia instead of the ammonium ion is the substrate. The decrease in activity above pH 9.0 is not due to the inactivation of the enzyme by alkali since pH stability studies indicate that the enzyme is stable at pH 10. The decrease in activity at low pH could be partly due to the irreversible inactivation of the enzyme since the enzyme is acid-labile.

**Stoichiometry of ATP Cleavage and NAD Formation**—The data presented in Table IV show the requirements for ATP cleavage. NAD synthetase does not exhibit ATPase activity. Omission of NAD from the complete incubation mixture results in no formation of ADP or AMP. However, when L-glutamine is omitted from the incubation mixture, small amounts of AMP are formed. When the complete incubation mixture is incubated with yeast NAD synthetase, a significant amount of AMP is formed. As shown in Table V, 1 pmole of [14C]AMP is produced per pmole of NAD synthesized. No trace of inorganic phosphate is found during the reaction under the standard assay conditions. This is in agreement with the report of Press and Handler (1).

NAD synthetase: ATP stoichiometry

Materials spotted | Incubation time (hr) | ATP (cpm/50 μl incubation mixture) | ADP (μmol/pmol NAD) | AMP (μmol/pmol NAD)
--- | --- | --- | --- | ---
Complete reaction mixture | 3 | 31,362 | 3.505 | 152
Complete reaction mixture | 1 | 30,376 | 3.930 | 199
Complete reaction mixture | 3 | 29,312 | 4.032 | 515
Complete reaction mixture | 3 | 28,123 | 3.621 | 179
Complete reaction mixture | 1 | 20,820 | 1.848 | 9,245

NAD synthetase: ATP stoichiometry

A homogeneous enzyme preparation was employed. The reaction mixture contained 1 X 10⁻¹⁰ M NAD, 2 X 10⁻¹⁰ M GluNH₄, 2 X 10⁻¹⁰ M ATP, 5.6 X 10⁻¹⁰ M KCl, 5 X 10⁻¹⁰ M MgCl₂, 5 X 10⁻¹⁰ M Tris-Cl, pH 8.0, and enzyme. The reaction was terminated by immersion in a boiling water bath for 1 min. [8-¹⁴C]ATP, having a specific activity of 3.5 x 10⁶ cpm/μmole, was used in this experiment. Fifty-microliter aliquots were applied to Whatman No. 1 paper. The compounds separated by descending paper chromatography with isobutyrate-ammonium hydroxide-water (66:1:33) was the solvent system. The spots corresponding to the markers ATP, ADP, and AMP were cut out and counted in a scintillation spectrometer.

**Metal Ion Requirements**—The amidation of NAD catalyzed by NAD synthetase requires Mg²⁺. Co²⁺ can replace Mg²⁺ to a certain extent (about 30% of the activity observed with Mg²⁺)

**Table IV**

NAD synthetase: requirements for ATP cleavage

A homogeneous enzyme preparation was employed. The reaction mixture contained 1 X 10⁻¹⁰ M NAD, 2 X 10⁻¹⁰ M GluNH₄, 2 X 10⁻¹⁰ M ATP, 5.6 X 10⁻¹⁰ M KCl, 5 X 10⁻¹⁰ M MgCl₂, 5 X 10⁻¹⁰ M Tris-Cl, pH 8.0, and enzyme. The reaction was terminated by immersion in a boiling water bath for 1 min. [8-¹⁴C]ATP, having a specific activity of 3.5 x 10⁶ cpm/μmole, was used in this experiment. Fifty-microliter aliquots were applied to Whatman No. 1 paper. The compounds separated by descending paper chromatography with isobutyrate-ammonium hydroxide-water (66:1:33) was the solvent system. The spots corresponding to the markers ATP, ADP, and AMP were cut out and counted in a scintillation spectrometer.

**Table V**

NAD synthetase: ATP stoichiometry

Aliquots were taken at the appropriate time from the reaction vessels of the experiment reported in Table IV (complete mixture). NAD and AMP were assayed as described in Table IV. The amount of AMP formed was calculated from the percentage of total radioactivity found in the AMP spot. NAD was assayed using ADH-PP; was determined by hydrolysis of inorganic pyrophosphate to inorganic orthophosphate with crystalline inorganic pyrophosphatase. Inorganic phosphate was measured by the method of Fiske and SubbaRow (14).

**Figure 5**

Effect of pH on NAD synthetase activity. Material obtained from a Sepharose 4B column (Table I) (specific activity 0.9 μmole per min per mg of protein) was used. For incubation at pH 5.0 to 5.6, potassium phosphate-acetate acid buffer was used; at pH 6 to 7.5, potassium phosphate buffer; at pH 8 to 8.5, Tris-Cl; and at pH 9 to 9.5, glycine-NaOH. After 30 min incubation at 37°C, the reaction mixtures were chilled in an ice bath and the NAD formed was immediately measured by the fluorescence technique.

- ☓ activity in the presence of glutamine; X-X, activity in the presence of NH₄Cl; O-O, calculated concentration of free NH₃ at the pH levels indicated.
The material obtained from the Sepharose 4B step (Table I) was used as the enzyme source. The reaction mixture contained 5 mM MgCl₂, 50 mM KCl, 50 mM Tris-Cl, pH 8.0, 200 mM NH₄OH, enzyme, and component(s) as follows: NAD (1 × 10⁻³ M), ATP (2 × 10⁻³ M), glutamine (20 mM), p-chloromercuribenzoate (1 × 10⁻⁴ M) to a total volume of 0.55 ml. The incubation was carried out at 37° for 60 min. The reaction was terminated by the addition of 0.15 ml of 8% trichloracetic acid containing 2 n HCl and 3.3% FeCl₃. The hydroxamate analogues were formed as a ferric complex by the procedure of Hartman (19).
RESULTS

Approximately 40% of the enzymatic activity is inhibited at a concentration of 10^{-5} M p-chloromercuribenzoic acid at pH 7.4 and 8.6, respectively. The tubes were incubated at 37°C for 60 min. The reaction was terminated by boiling for 1 min in a water bath. NAD formation was measured employing alcohol dehydrogenase as described in the text. X-X, + 6-diazo-5-oxo-n-norleucine (1 × 10^{-4} M); ○-○ control (- 6-diazo-5-oxo-n-norleucine).

DISCUSSION

NAD synthetase has been investigated in yeast (1), E. coli (2), and rat liver (1). However, a purification procedure resulting in a homogeneous NAD synthetase has not previously been reported. In the present study, this enzyme was purified from yeast approximately 2000-fold to a state of apparent homogeneity.

Physicochemical studies demonstrated that the apparent molecular weight of the active enzyme is 630,000 and the molecular weights of its subunits are 80,000 and 65,000. An enzyme having a molecular weight of that value is rather unusual although there are some examples, e.g. glutamine synthetase (27), β-galactosidase (28), and RNA polymerase (29). Whether the molecular weight obtained from equilibrium studies represents a state of aggregation of the minimum active units or a minimum unit is not yet clear. Gel filtration studies do not contradict these observations.

It was clearly demonstrated that the amidation of NAD requires ATP, Mg^{2+}, and K^{+} and is associated with ATP cleavage and AMP formation. A small amount of AMP was obtained during the incubation in the presence of NAD, ATP, Mg^{2+}, and K^{+}. Both ATP and NAD are necessary for the amidation of L-glutamine when L-glutamine was employed as the amide donor. ATP is also required for the amidation of NAD. ATP's role in the amidation of hydroxylamine which is not formed in the absence of ATP and Mg^{2+}. All of these findings taken together indicate that an enzyme-bound activated substrate is probably formed preceding the amidation step. This postulated enzyme-bound activated substrate complex may be analogous to those observed in the activation of acetate and amino acids in which the enzyme acyl AMP or enzyme-amino acyl-AMP complexes have been isolated (30-32). ATP-PPi exchange reactions, however, have been demonstrated in these cases (31-33).

On the other hand, Lagerkvist (34) reported that XMP aminase (pigeon liver) in the presence of the amino donor catalyzes the amidation of NAD to GMP with a stoichiometric cleavage of ATP to AMP and PPi. Experiments employing ^32P-XMP showed that ^32P is incorporated into the AMP in the presence of the amino donor.

We have not been able to demonstrate a significant exchange reaction between ATP and PPi or ATP and AMP. A small exchange reaction has been detected. However, since the purified NAD synthetase still contains a trace of NMN adenylytransferase activity, it is not known whether this very slight exchange between ATP and PPi is catalyzed by NMN adenylytransferase or by NAD synthetase.

The flux of materials through the various pathways of metabolism proceeds in a precise way according to the needs of the organism. For this to occur, metabolic pathways must be controlled. Regulation can be exerted at many levels. Studies on the individual enzymes catalyzing a series of consecutive reactions are one of the means to understand the metabolic regulation. The 2,000-fold purified NAD synthetase has a specific activity of 2 μmoles per min per mg of protein (Table I). The catalytic constant based on the specific activity and a molecular weight of 630,000 was found to be 1,260 μmoles per min per mg of enzyme. The K_m values for NAD and L-glutamine were determined to be 1.9 × 10^{-4} and 5 × 10^{-3} μm, respectively. The intracellular NAD concentration in yeast is about 5 × 10^{-4} μm.
(36), which is approximately 0.5 μmole per g wet weight tissue. The turnover time of hepatic NAD is about 9 hours (37). Utilizing these data and assuming that yeast cells contain 70% water and 30% soluble proteins, one concludes that yeast NAD synthetase is capable in vivo of producing approximately 216 μmoles of NAD per g wet weight of yeast per day. Furthermore, if one assumes that the NAD turnover time in resting yeast cells is similar to that observed in hepatic tissue (2 hours), then yeast cells must synthesize about 6 μmoles per g wet weight per day in order to maintain the above intracellular NAD concentrations. Since yeast NAD synthetase can synthesize 216 μmoles per g wet weight per day under optimum assay conditions, the yeast cell contains more than 30 times the amount of NAD synthetase needed to maintain normal levels of NAD. The intracellular NaAD concentration is 10^(-6) to 10^(-7) M (3, 38, 39). From the measured rate and K_m values, it can be calculated that the expected activity of NAD synthetase at endogenous substrate concentrations would be close to the NAD turnover rate. Thus, we feel that there is no need to postulate that NAD synthetase is under regulatory control. On the basis of the present study, yeast NAD synthetase activity could be regulated simply by the substrate concentrations.

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