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Negative Modulation of *Escherichia coli* NAD Kinase by NADPH and NADH

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NAD kinase was purified 93-fold from *Escherichia coli*. The enzyme was found to have a pH optimum of 7.2 and an apparent K_m for NAD^+, ATP, and Mg^{2+} of 1.9, 2.1, and 4.1 mM, respectively. Several compounds including quinolinic acid, nicotinic acid, nicotinamide, nicotinamide mononucleotide, AMP, ADP, and $NADP^+$ did not affect NAD kinase activity. The enzyme was not affected by changes in the adenylate energy charge. In contrast, both NADH and NADPH were potent negative modulators of the enzyme, since their presence at micromolar concentrations resulted in a pronounced sigmoidal NAD^+ saturation curve. In addition, the presence of a range of concentrations of the reduced nucleotides resulted in an increase of the Hill slope (n_H) to 1.7 to 2.0 with NADH and to 1.8 to 2.1 with NADPH, suggesting that NAD kinase is an allosteric enzyme. These results indicate that NAD kinase activity is regulated by the availability of ATP, NAD^+, and Mg^{2+} and, more significantly, by changes in the $NADP^+$/NADPH and NAD^+/NADH ratios. Thus, NAD kinase probably plays a role in the regulation of NADP turnover and pool size in *E. coli*.

Few reports have been devoted to the study of NAD kinase from bacteria. NAD kinase has been studied in a limited manner in crude, cell-free preparations from *Escherichia coli* (10). The enzyme has been purified up to 500-fold from *Azotobacter vinelandii* and has been found to have apparent K_m values for NAD^+ and ATP of 0.4 and 1 mM, respectively (7). NAD kinase has also been detected in immobilized *Achromobacter aceris* (25) and *Brevibacterium ammoniagenes* (9).

Recently, we purified NAD kinase 180-fold from *Bacillus licheniformis* (31). Using this partially purified preparation, we have shown that this enzyme is subject to competitive inhibition by $NADP^+$ and that it plays a key role in the regulation of NADP turnover in this organism (31). In addition, we have also purified NAD kinase from *Bacillus subtilis* and demonstrated that it is negatively modified by $NADP^+$ and positively modified by NADPH (V. Lopez, E. Gomez, H. Kwong, and A. J. Andreoli, Fed. Proc. **44**:2649, 1985).

E. coli cells possess a functional NAD cycle that accounts for the turnover of NAD in this organism (2). In addition, *E. coli* cells also demonstrate NADP turnover (16; A. J. Andreoli, unpublished results). In the present investigation, we purified NAD kinase from *E. coli* to determine its role in the regulation of NADP turnover and pool size in this organism. Evidence is presented indicating that NADPH and NADH are potent negative modulators of this enzyme.

MATERIALS AND METHODS

Growth conditions. *E. coli* K-12 was grown at 37°C with forced aeration in the glycerol-minimal salts medium of Yates and Pardee (30). Cells were harvested at the late logarithmic phase by centrifugation at 4,000 × *g* for 10 min and washed with a solution containing 0.069 M KCl and 0.069 M NaCl.

NAD kinase purification. Unless otherwise indicated, all procedures were carried out at 2 to 4°C. Cells were suspended in 50 mM potassium phosphate (KPO_4) (pH 7.5) in the ratio of 1 g (wet weight) of cells to 5 ml of KPO_4. The cell suspension was divided into 30-ml batches, and each was treated with sonic oscillation in 15-s bursts for a total of 2 min at 200 W with a Bronwill Biosonic IV sonicator. Disrupted cells were stirred for 15 min before centrifugation at 29,000 × *g* for 30 min. To remove nucleic acids, we added a solution of 15.6% streptomycin sulfate dropwise to the supernatant to a final concentration of 2.6%. After 30 min of being stirred, the preparation was clarified by centrifugation at 12,000 × *g* for 10 min and dialyzed against 50 volumes of 0.1 M KPO_4 (pH 7.5) for 12 h. The dialysate was adjusted with solid ammonium sulfate to 30% saturation, stirred for 30 min, and centrifuged at 39,000 × *g* for 1 h. The supernatant was adjusted to a 50% ammonium sulfate saturation, stirred for 30 min, and centrifuged at 27,000 × *g* for 20 min. The resulting ammonium sulfate pellet could be stored at −20°C for up to 3 months without significant loss of NAD kinase activity.

The 30 to 50% ammonium sulate fraction was dissolved and diluted to a protein concentration of 20 mg/ml with 0.125 M KPO_4 (pH 6.5) and dialyzed for 4 to 7 h against 50 volumes of the same buffer. Approximately 160 mg of dialysate protein was loaded onto a Sephadex G-200 column (2.5 by 36 cm) and then eluted at a rate of 0.26 ml/min with 0.125 M KPO_4 (pH 6.5). After the collection of 60 ml of eluting buffer, NAD kinase appeared and was collected in the next 75 ml. The enzyme was concentrated by treating the eluate with ammonium sulfate to 60% saturation. The resulting ammonium sulfate pellet could be stored at −20°C for up to 1 month without significant loss of NAD kinase activity. This pellet was dissolved and diluted to a protein concentration of 20 to 30 mg/ml with 0.10 M KPO_4 (pH 6.5) and dialyzed for 4 to 7 h against 50 volumes of the same buffer. Approximately 200 mg of dialysate protein was then loaded onto a

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TABLE 1. Purification of NAD kinase from *E. coli*

Fraction	Protein (mg)	Total activity[a]	Sp act[b]
Crude extract	6,580	10.2	1.55
Streptomycin sulfate supernatant	10,590	10.0	0.94
Ammonium sulfate	2,200	8.30	3.77
Sephadex G-200	303	6.15	20.3
QAE-Sephadex	30	4.37	144

[a] Micromoles per minute.
[b] Nanomoles per minute per milligram of protein.

QAE-Sephadex column (1 by 27 cm) that was preequilibrated with 0.10 M KPO_4 (pH 6.5). The column was allowed to stand 1 h to ensure maximal binding of protein. Elution of the column was effected with a linear gradient produced with a Buchler gradient maker at a flow rate of 0.26 ml/min. Two chambers were used with 200 ml of 0.10 M KPO_4 (pH 6.5) in the mixing chamber and 200 ml of 0.50 M KPO_4 (pH 6.5) in the reservoir chamber. The enzyme appeared in the first 100 ml of eluate and was concentrated by treatment with ammonium sulfate to 60% saturation. The resulting precipitate could be stored at −20°C up to 3 weeks without significant loss of enzyme activity. Precipitate was diluted with 0.25 M KPO_4 (pH 6.5) to a final concentration of 0.1 IU/ml before use. In this preparation, NAD kinase was purified 93-fold with respect to crude extract. A summary of the purification is shown in Table 1. The apparent increase in the quantity of total protein after the streptomycin sulfate step was due to color development by streptomycin sulfate in the protein assay.

NAD kinase assay. NAD kinase activity was determined by continuous enzymatic detection of NADP as described previously (31). Because of the presence of an NAD-using glucose-6-phosphate dehydrogenase in preparations before the QAE-Sephadex step, the continuous NAD kinase assay could not be used (31). Such preparations were assayed by endpoint enzymatic detection of NADP (31). This assay method was also used to assay the enzyme in studies in which NADP was present in the reaction mixture. Reaction mixtures contained 10 μmol of NAD, 10 μmol of ATP, 14 μmol of $MgCl_2$, 100 μmol of 3-(*N*-morpholino)propane sulfonate, (pH 7.2), and enzyme in a total volume of 1.0 ml.

Protein determination. Protein concentration was determined by the method of Lowry et al. (15) with bovine serum albumin as a standard. Protein concentrations were approximated by a spectrophotometric method (13).

Determination of Hill slopes. Hill plots were prepared by plotting log $[v_0/(V_m - v_0)]$ versus log [NAD] with the assumption that the initial velocity (v_0) is proportional to the fractional saturation of NAD-binding sites (3). The maximal velocity (V_m) was determined from a Lineweaver-Burk plot (14) of the NAD^+ saturation data in the absence of NADH or NADPH, since these data followed Michaelis-Menten kinetics. The presence of NADH or NADPH did not alter the V_m (see below). Because Hill plots do not necessarily yield straight lines over the whole range of $v_0/(V_m - v_0)$ (5, 8), only datum points corresponding to the middle range of $v_0/(V_m - v_0)$ were used for calculations. Hill slopes (n_H) were calculated by linear regression analysis.

Other methods. Glucose-6-phosphate dehydrogenase was assayed as described by Sanwal (21). Nucleotide phosphotransferase was assayed by substituting adenosine-2′-phosphate (6) for ATP in the continuous NAD kinase assay system. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed as described by Laemmli (12).

RESULTS

Properties of NAD kinase. There was no detectable glucose-6-phosphate dehydrogenase or nucleotide phosphotransferase activity in the 93-fold-purified NAD kinase preparation. However, sodium dodecyl sulfate-polyacrylamide gel electrophoresis revealed that this preparation was not homogenous. The pH optimum of the partially purified enzyme was determined to be 7.2 and was relatively broad, with 90% of maximal NAD kinase activity occurring between pH 6.4 and 8.1. Plots of NAD kinase initial velocity versus substrate or cosubstrate concentration were in all cases hyperbolic; the apparent K_m values for ATP, NAD^+, and Mg^{2+} were 1.9, 2.1, and 4.1 mM, respectively. These kinetic constants were not significantly different from those determined with enzyme preparations of lesser purity.

Regulation of NAD kinase. Several compounds were tested to determine their effect on NAD kinase activity. Quinolinate, nicotinate, nicotinamide, nicotinamide mononucleotide, ADP, AMP, and $NADP^+$ had no significant effect on enzyme

TABLE 2. Effects of various compounds on NAD kinase activity[a]

Compound	Concn (mM)	Relative activity
No additions	0.00	100
Quinolinate	0.10	91
	0.50	96
Nicotinate	0.10	96
	0.50	98
	1.0	101
Nicotinamide	0.10	99
	0.50	97
	1.0	97
Nicotinamide mononucleotide	0.10	94
	0.50	96
ADP	0.50	91
	1.0	91
	2.0	82
AMP	1.0	100
	5.0	100
NADP	0.05	102
	0.10	96
	0.20	96
NADPH	0.010	68
	0.015	43
	0.020	33
NADH	0.050	84
	0.10	60
	0.15	50
NADPH + NADH	0.010 + 0.050	49
	0.015 + 0.100	29
	0.020 + 0.150	15

[a] Assays were performed as described in Materials and Methods, except that the NAD^+ concentration was 4.0 mM.

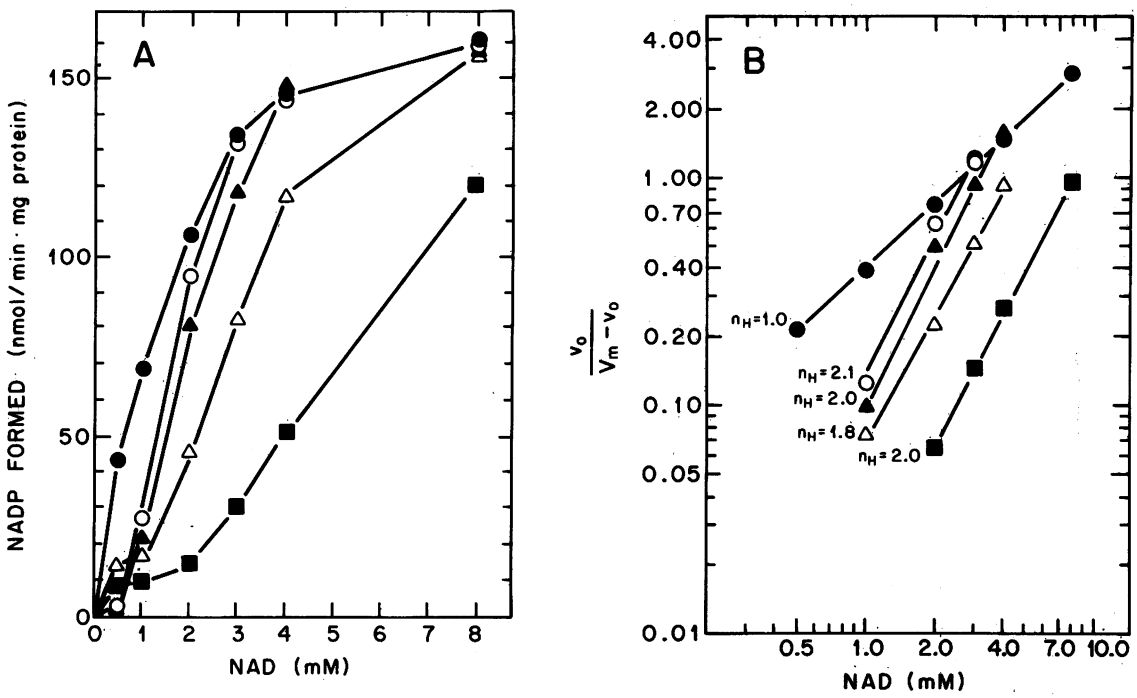

FIG. 1. (A) Effect of NADPH on the initial velocity of NAD kinase with NAD^+ as the varied substrate; (B) Hill plot of the data in panel A. Final concentrations (in micromolars) of NADPH added: 0 (●), 5.0 (○), 7.5 (▲), 15 (△), and 30 (■).

activity (Table 2). In contrast, NADPH was a potent inhibitor of the enzyme at 5 to 30 μM (Table 2; Fig. 1A), concentrations at the lower end of those found in logarithmic-phase cells of *E. coli* (16). Similarly, NAD kinase was also inhibited by NADH at 50 to 200 μM (Table 2; Fig. 2A), concentrations typically found in logarithmic-phase cells (16). Approximately 10 times more NADH was required to cause the same degree of inhibition caused by NADPH (Table 2). In addition, the inhibitory effects of NADH and NADPH were additive (Table 2), indicating that the effect of

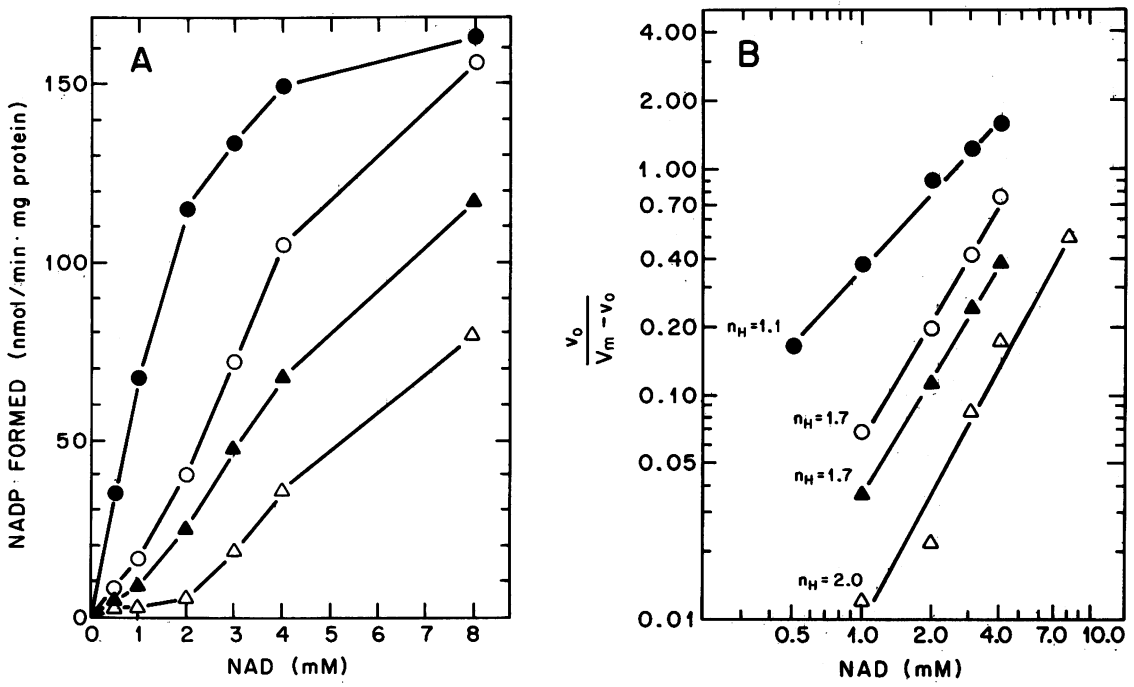

FIG. 2. (A) Effect of NADH on the initial velocity of NAD kinase with NAD^+ as the varied substrate; (B) Hill plot of the data in panel A. Final concentrations (in micromolars) of NADH added: 0 (●), 50 (○), 100 (▲), and 200 (△).

either compound on NAD kinase does not exclude the effect of the other. Inhibition by NADPH and NADH appeared to be competitive with respect to NAD^+ because of the ability of higher concentrations of NAD^+ to overcome the inhibition (Fig. 1A and 2A). More significantly, the presence of either NADPH or NADH transformed the NAD saturation curve from a hyperbolic to a sigmoidal shape (Fig. 1A and 2A). Hill plots of the data revealed that the slope (n_H) was 1.0 to 1.1 in the absence of NADPH and NADH (Fig. 1B and 2B). In contrast, n_H increased to 1.8 to 2.1 and to 1.7 to 2.0 in the presence of different concentrations of NADPH and NADH, respectively (Fig. 1B and 2B).

There was no significant effect on NAD kinase when the adenylate energy charge (4) was varied by holding the concentration of total adenine nucleotides (AMP plus ADP plus ATP) at 4.0 mM and adjusting the concentration of individual nucleotides to give energy charge values between 0 and 1. In addition, changes in the adenylate energy charge did not affect NAD kinase activity in the presence of 15 μM NADPH.

DISCUSSION

We have described the partial purification and kinetic properties of NAD kinase from *E. coli*. The enzyme was purified to the point when competing enzymes such as glucose-6-phosphate dehydrogenase and nucleotide phosphotransferase were removed. The latter enzyme catalyzes the phosphorylation of NAD at the 2′ position to give NADP (6). The optimal pH of 7.2 for NAD kinase is within physiological range. In contrast, the pH optima of NAD kinase from *B. licheniformis* (31) and *B. subtilis* (V. Lopez, E. Gomez, H. Kwong, and A. J. Andreoli, Fed. Proc. **44**:2649, 1985) were lower at 6.8 and 6.5, respectively. The Michaelis constants of the enzyme were all within the physiological concentration ranges of the substrates in *E. coli*: ATP is known to range up to 4.0 mM (5); NAD^+ in cells grown under aerobic conditions is 4.8 nmol/mg of dry cells (28), which is equivalent to 1.2 mM, assuming that 80% of the cell is water; and Mg^{2+} is known to range up to 5.0 mM (17).

In contrast to the *B. licheniformis* enzyme, which displayed a biphasic ATP saturation curve (31), *E. coli* NAD kinase had a hyperbolic ATP saturation curve. The *E. coli* enzyme was markedly inhibited by NADPH and NADH (Table 2). Because these compounds transformed the NAD saturation curve from a hyperbolic to a sigmoidal shape (Fig. 1A and 2A) and increased the Hill slope (Fig. 1B and 2B), we suggest that *E. coli* NAD kinase is an allosteric enzyme and that NADPH and NADH are its negative modifiers. Since the slope of a Hill plot (n_H) is an indication of the minimum number of substrate-binding sites (11), our data suggest that *E. coli* NAD kinase has more than one NAD-binding site. However, studies of NAD binding that involve a homogeneous preparation of this enzyme will be required to determine the exact number of NAD-binding sites.

The concentrations of NADPH and NADH in exponentially growing *E. coli* cells are 0.22 and 0.40 mM, respectively (16). Assuming that the NADPH and NADH pools have full access to NAD kinase, the enzyme would function at a diminished rate in vivo due to the marked inhibition exerted by these compounds. Because this is inconsistent with the rapid incorporation of [^{14}C]nicotinic acid into NAD and NADP (2, 16), *E. coli* NAD kinase probably has one or more positive modifiers in addition to the negative modifiers NADPH and NADH.

NAD kinase from *B. subtilis* also displayed sigmoidal kinetics in the presence of $NADP^+$ (V. Lopez, E. Gomez, H. Kwong, and A. J. Andreoli, Fed. Proc. **44**:2649, 1985). However, in this organism, $NADP^+$ was a negative modifier, whereas NADPH was a positive modifier of NAD kinase (V. Lopez, E. Gomez, H. Kwong, and A. J. Andreoli, Fed. Proc. **44**:2649, 1985). The marked negative modulation of *E. coli* NAD kinase by NADPH and NADH, coupled with the lack of effect of $NADP^+$ and NAD^+, suggests that the enzyme is also regulated by changes in the NADPH/$NADP^+$ and NADH/NAD^+ ratios. Both of these ratios have been shown to change with growth conditions (18). In addition, the NADH/NAD^+ ratio has been shown to change depending on carbon source (22) and exposure of cells to oxygen during growth (28). More recent studies demonstrate that the NADH/(NADH plus NAD^+) and NADPH/(NADPH plus $NADP^+$) ratios in exponential-phase *E. coli* cells are remarkably constant except for transient changes observed after interruption of growth (1). Therefore, since NAD kinase activity is regulated by the NADPH/$NADP^+$ and NADH/NAD^+ ratios, NADP formation in *E. coli* should be tightly coupled to the growth conditions of the medium, including carbon source and the presence or absence of oxygen.

The inhibition of NAD kinase by NADPH and NADH has been reported for the rat liver enzyme. Inhibition was competitive with respect to NAD^+, and the K_i values for NADPH and NADH were 50 and 100 μM, respectively (19). Pigeon liver NAD kinase was also found to be inhibited by NADH in a competitive manner with respect to NAD^+ (26). However, in both cases, NAD kinase kinetics followed the Michaelis-Menten equation and NAD saturation curves remained hyperbolic with the use of NADPH, NADH, or both (19, 26). The *E. coli* and *B. subtilis* enzymes appear to be the only known NAD kinases capable of transition to sigmoidal kinetics in the presence of NADPH, NADH, or $NADP^+$.

The transition to sigmoidal kinetics and inhibition of activity in the presence of NADH, NADPH, or both has been observed for other *E. coli* enzymes, such as citrate synthase (27), NADP-specific malic enzyme (23, 24), malate dehydrogenase (20), phosphoenolpyruvate carboxykinase, (29) and glucose-6-phosphate dehydrogenase (21). Since NADH is a negative modulator of both NADP-specific malic enzyme and glucose-6-phosphate dehydrogenase, Sanwal (22) pointed out that NADH controls all of the NADPH-generating pathways in *E. coli*. Therefore, since NADPH provides reducing equivalents in biosynthetic pathways, the level of NADH may play an indirect role in controlling enzymes that catalyze biosynthetic reactions. The negative modulation of *E. coli* NAD kinase by NADH is an example of direct regulation of a biosynthetic reaction by this compound. Furthermore, NADPH-generating reactions are dependent on the availability of $NADP^+$. Therefore, since NAD kinase catalyzes the formation of $NADP^+$, its negative modulation by NADH is consistent with the role this compound plays in regulating NADPH-generating pathways in *E. coli* (22).

Since the K_m values of NAD kinase for ATP, NAD^+, and Mg^{2+} are all within in vivo concentrations, NAD kinase activity is regulated by the availability of these substrates in *E. coli*. More significantly, NAD kinase activity is tightly coupled to the NADPH/$NADP^+$ and NADH/NAD^+ ratios. These results suggest that NAD kinase may play an impor-

tant role in the regulation of NADP turnover and pool size in *E. coli*.

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