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NICOTINAMIDE ADENINE DINUCLEOTIDE KINASE FROM *AZOTOBACTER VINELANDII* CELLS. A POSSIBLE MECHANISM FOR THE ENZYME REACTION

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SUMMARY

Nicotinamide adenine dinucleotide kinase (ATP:NAD 2'-phosphotransferase, EC 2.7.1.23) from *Azotobacter vinelandii* cells catalyzes an exchange reaction between ADP and ATP. This exchange reaction is inhibited by NAD^+. The nature of the relationship between the exchange reaction and the biosynthesis of $NADP^+$ suggests a common enzyme bound intermediate for both reactions. The most reasonable possibility for this intermediate is a phosphorylated enzyme. Steady state kinetic studies on the enzyme yield complex results which may be related to the regulation of the enzyme.

INTRODUCTION

NAD^+ kinase (NAD:ATP 2'-phosphotransferase, EC 2.7.1.23) catalyzes the synthesis of $NADP^+$ by the phosphorylation of NAD^+ (refs. 1–3). The enzyme plays a vital role in biological systems since it is the major pathway for generating $NADP^+$, the coenzyme for many biosynthetic reactions. Partial purification of the enzyme has been achieved from yeast cells[2,4], pigeon liver[3,5], rat liver[6], spinach[7], and *Azotobacter vinelandii* cells[8]. The mechanism of action of the pigeon liver enzyme has been investigated by APPS[5] who proposed a rapid equilibrium random mechanism for this enzyme from his kinetic studies. In this communication we will describe experiments which suggest the possibility that the Azotobacter enzyme catalyzes the formation of $NADP^+$ through a phosphorylated enzyme intermediate.

PROCEDURES AND MATERIALS

EXPERIMENTAL

Materials

NAD^+, $NADP^+$, ATP, ADP, 5'-AMP, *threo*-D_SL_S-isocitrate, sodium salt, and pig heart isocitrate dehydrogenase were obtained from Sigma Chemical Co., St. Louis,

Mo. 2-Mercaptoethanol was obtained from Eastman Organic Chemicals, Rochester, N.Y. DEAE-cellulose was obtained from Carl Schleicher and Schuell, Keene, N.H. Calcium phosphate gel was prepared according to the method of KEILEN AND HARTREE[9]. Bovine serum albumin was supplied by Mann Research Laboratories, New York, [8-^{14}C]ADP was obtained from New England Nuclear, Boston, Mass. Eastman Chromatogram sheets precoated with cellulose were obtained from Fisher Scientific Co., Pittsburgh.

Methods

Purification of NAD$^+$ kinase. NAD$^+$ kinase from *A. vinelandii* extracts was purified by a combination of ion-exchange cellulose chromatography, calcium phosphate gel fractionation, and ammonium sulfate precipitation as previously described[8].

Assay of enzyme. The enzyme activity was assayed by a modification of the method of KORNBERG[2] as previously described[8]. Absorbance measurements were made with a Gilford Model 2000 spectrophotometer or a Cary 1605 recording spectrophotometer at 340 nm in a standard cuvette with a 1-cm light path.

Protein determination. Protein determinations were routinely made by the procedure of LOWRY *et al.*[10].

Electrophoresis. High voltage paper electrophoresis was carried out with a Camag HVE system. The buffer system used for separating NAD$^+$, NADP$^+$, ADP, and ATP consisted of 0.05 M sodium citrate, pH 4.5, containing 0.003 M $MgSO_4$. The electrophoresis was carried out for 60 min on 20 cm $\times$ 40 cm sheets of Schleicher and Schuell 2040-B paper. The components were well separated and in order of increasing mobility were NAD$^+$, NADP$^+$, ADP, and ATP respectively. In those experiments in which it was necessary to resolve a mixture of AMP, ADP, and ATP the same buffer system was used and as expected AMP had the lowest mobility and was well separated from ADP and ATP.

Thin layer chromatography. An alternate method for the separation of AMP, ADP, and ATP was thin-layer chromatography on sheets of polyethylenimine cellulose (PEI-cellulose). Eastman chromatogram sheets precoated with cellulose were impregnated with a 2% solution of polyethylenimine, and dried at room temperature overnight. The sheets were developed in 2 M NaCl and then in water. These washed sheets were dried at room temperature overnight and then used for separating the nucleotides. For separating the nucleotides the solvent system was 1.6 M LiCl. Prior to development after the sample was applied, the chromatogram was dipped in anhydrous methanol to remove excess salt. The solvent system gave good resolution of the nucleotides. In this method ATP had the lowest R_F value.

Detection and quantitation of radioactivity. Autoradiography was carried out on the samples subjected to thin layer chromatography on PEI-cellulose. The chromatograms were exposed to Ilford Type G X-ray film for varying lengths of time, and the dark bands on the developed films were compared with those bands on the chromatograms which corresponded to the nucleotides. The nucleotides were detected by short wave ultraviolet light. The specific activities of the nucleotides could not be quantitated since the PEI-cellulose sheets yielded a high background of material which absorbed ultraviolet light. For quantitation the nucleotides and coenzymes were separated by high voltage electrophoresis and the bands or spots corresponding to each nucleotide or coenzyme eluted separately for radioactivity measurement and

spectrophotometric analysis. The elution was accomplished by cutting out the appropriate bands and allowing the paper to stand overnight in approximately 3 ml of water. Aliquots of the supernatant solution were mixed with 10 ml of a solution containing 5.5 g of 2,5-diphenyloxazole, 0.1 g 1,4-bis-2-(5-phenyloxazolyl)-benzene, 667 ml toluene and 333 ml Triton X-100, and the mixture counted in a Beckman LS-100 liquid scintillation counter. The background radioactivity from a section of the electrophoretogram treated in a similar manner as the sample was subtracted from the values obtained for the compounds which were being studied. The specific radioactivity of each compound was calculated from the recovered radioactivity and the net absorbance of the sample at 260 nm. The value used for ε_M for ATP, ADP, and AMP was $15.4 \cdot 10^6$ and for NAD^+ and $NADP^+$ $18 \cdot 10^6$. The recovery of radioactivity by the elution technique described was between 80 and 90%.

RESULTS

[8-^{14}C]ADP–ATP exchange reaction. The requirements for the exchange reaction between [8-^{14}C]ADP and ATP are summarized in Table I. It may be seen that the reaction is dependent on native enzyme and magnesium in addition to the nucleotides.

TABLE I

REQUIREMENTS FOR [^{14}C]ADP–ATP EXCHANGE

The complete system in a total volume of 1.14 ml contained: potassium phosphate buffer (pH 7.0), 57 μmoles: ATP, 2.2 μmoles; [^{14}C]ADP, 2.0 μmoles containing $1.1 \cdot 10^6$ disint./min; $MgSO_4$, 10 μmoles. The reaction was initiated with 0.02 ml enzyme containing 0.16 mg protein with a specific activity of 7 enzyme units/mg protein. Either Mg^{2+} or ATP was omitted from the incubation as indicated and in the experiment with boiled enzyme, the enzyme was heated at 100° for 8 min prior to addition of substrates. The reaction mixture was incubated at 37° for 1 h. At the end of this time the reaction was terminated by immersing the reaction tube in a boiling water bath for 1 min. In the experiment in which ATP was omitted 1.1 μmoles of ATP and 0.8 μmoles of AMP were added after the reaction had been terminated. Aliquots of each reaction mixture were removed and subjected to high voltage electrophoresis and subsequent analysis as described in EXPERIMENTAL PROCEDURES AND MATERIALS. The radioactivity recovered from the electrophoretograms were converted to total [^{14}C]ATP in the reaction mixtures. The recoveries of ATP from the electrophoretograms varied between 80 and 90%.

System	[^{14}C]*ATP recovered (disint./min)*
Complete	64 300
Minus Mg^{2+}	2 700
Minus ATP	6 800
Boiled enzyme	6 700

In the absence of ATP in the initial incubation mixture no AMP or ATP was generated. This latter experiment indicates that the enzyme preparation contained no myokinase activity which might have accounted for the incorporation of radioactivity into ATP.

The effect of NAD^+ on [8-^{14}C]ADP–ATP exchange reaction. In order to establish that the [^{14}C]ADP–ATP exchange reaction was related to the formation of $NADP^+$ from NAD^+, competition experiments were carried out in which the rate of the

TABLE II

COMPETITION BETWEEN [^{14}C]ADP–ATP EXCHANGE AND NADP$^+$ FORMATION

The reaction mixture contained the following components in a total volume of 1.17 ml; potassium phosphate buffer (pH 7.0), 68.5 μmoles; [^{14}C]ADP when added, 1.68 μmoles with a specific activity of $5.6 \cdot 10^5$ disint./min per μmole; ATP, 1.97 μmoles; $MgSO_4$, 10 μmoles; and NAD^+ as indicated. The reaction was initiated with 0.02 ml enzyme containing 0.16 mg protein with a specific activity of 7 enzyme units/mg protein. Incubation was carried out at 37° for 1 h. The reaction was terminated by heating for 1 min at 100°. An aliquot of the reaction mixture was analysed for NADP$^+$ formation by adding excess isocitrate and isocitrate dehydrogenase. From the increase in $A_{340\ nm}$ the NADP$^+$ content was determined. Another aliquot was subjected to high voltage electrophoresis as described previously in EXPERIMENTAL PROCEDURES AND MATERIALS, and the ATP band was eluted and monitored for radioactivity and concentration. The [^{14}C]ATP formed was calculated from the specific activity of the [^{14}C]ADP and the radioactivity found in the ATP band. No correction was made for the reconversion of [^{14}C]ATP back to [^{14}C]ADP since the [^{14}C]ATP was diluted with unlabeled ATP and was therefore trapped in the ATP pool. No detectable adenosine triphosphatase activity was detectable in separate experiments with enzyme of comparable purity under similar experimental conditions in the absence of ADP.

NAD	[^{14}C]*ADP*	*NADP+*		[^{14}C]*ATP formed*	[^{14}C]*ATP formed*
		Formed	*Inhibition*		*Inhibition of NAD+ formatiin*
(μmoles)		*(nmoles/h)*	*(nmoles/h)*	*(nmoles/h)*	
19.4	–	26.0	—	—	—
19.4	+	15.6	10.4	18.1	1.7
9.5	–	33.7	—	—	—
9.5	+	23.2	10.5	69.8	6.7
3.8	–	25.4	—	—	—
3.8	+	18.6	6.8	78.9	11.7
2.0	–	21.8	—	—	—
2.0	+	16.6	5.2	82.2	15.8
0.34	–	13.9	—	—	—
0.34	+	13.4	0.5	95.6	191.2

[^{14}C]ADP–ATP exchange was determined in the presence of varying concentrations of NAD^+. In Table II it may be seen that, at constant [^{14}C]ADP concentration as the concentration of NAD^+ is decreased from $2.2 \cdot 10^{-2}$ M to $4 \cdot 10^{-4}$ M, the [^{14}C]-ADP–ATP exchange rate increased from 18 nmoles to 96 nmoles [^{14}C]ATP formed/h. At the highest concentration of NAD^+ used, the ratio of exchange to inhibition of NADP$^+$ formation by ADP is 1.7; and, at the lowest concentration of NAD^+ used, this ratio increased to 191. These data strongly indicate that NAD^+ and ADP compete for a common enzyme bound intermediate.

DISCUSSION

The data presented indicate that ADP is an inhibitor of NADP$^+$ formation by NAD^+ kinase. The enzyme catalyzes an exchange reaction between [^{14}C]ADP and ATP. The exchange reaction requires magnesium ions and intact enzyme. Competition experiments with varying concentrations of NAD^+ and a constant concentration of ADP indicate that the exchange reaction relative to the biosynthesis of NADP$^+$ increases with decreasing concentration of NAD^+. At the highest concentration of NAD^+ used, the inhibition of formation of NADP$^+$ by ADP was close to the rate of

exchange of [^{14}C]ADP into [^{14}C]ATP. These observations indicate that a common enzyme-bound intermediate exists for both the exchange reaction and the biosynthesis of $NADP^+$. The rationale behind this conclusion is discussed extensively by JENCKS[11]. If a common enzyme-bound intermediate such as a phosphorylated enzyme exists, and if the formation of this intermediate is the rate limiting step in the reaction, then both NAD^+ and ADP will compete for this intermediate. In the presence of NAD^+ alone the intermediate will be converted exclusively to $NADP^+$. In the presence of ADP and saturating concentrations of NAD^+ the formation of $NADP^+$ will be decreased by the same amount as the formation of ATP from ADP, *i.e.* the exchange reaction will be equal to the inhibition of $NADP^+$ formation. This situation is approached with the kinase reaction. If on the other hand NAD^+ is at less than saturating levels, the formation of enzyme bound intermediate will no longer be the rate limiting step and in the presence of ADP and the inhibition of $NADP^+$ formation will be much smaller than the exchange reaction. This again is the result observed with the kinase. Here the ratio of exchange to synthesis of $NADP^+$ increases about 100-fold as the NAD^+ concentration is decreased.

The conclusion obtained from the exchange experiments is supported by steady state kinetic studies on the reaction. In Table III a summary of the effects of ATP

TABLE III

EFFECT OF ATP CONCENTRATION ON LINEWEAVER–BURK PLOTS OF INITIAL VELOCITY *vs.* NAD^+ CONCENTRATION

The initial velocity for the enzyme reaction was determined at 4 different concentrations of NAD^+ varying between $1.7 \cdot 10^{-4}$ and $1 \cdot 10^{-3}$ M for each concentration of ATP indicated. Plots were obtained by plotting reciprocal initial velocity *vs.* reciprocal NAD^+ concentration[13]. The plots were analyzed by the least squares method and the slopes and intercepts with their standard deviations obtained. Each point on each graph was done in duplicate.

ATP ($M \times 10^3$)	*Slope* ± *S.D.*	*Intercepts* ± *S.D.*
6.84	0.020 ± 0.002	0.041 ± 0.005
3.91	0.022 ± 0.003	0.042 ± 0.009
1.71	0.018 ± 0.001	0.053 ± 0.004
1.62	0.019 ± 0.002	0.063 ± 0.004

on the initial velocity of the enzyme reaction as a function of NAD^+ concentration is given. The results indicate within experimental error that parallel line kinetics are obtained, which suggest a ping-pong mechanism. The reciprocal experiment where the effects of NAD^+ on initial velocity with varying ATP concentration yielded complex results in which the primary plots did not intersect at any one point. A partial explanation could be that NAD^+ has a dual function in the catalytic reaction as previously suggested by CHUNG[12]. The Azotobacter enzyme probably exists in the native state as a dimer, and NAD^+ serves a structural role in maintaining the dimeric state in addition to its function as a substrate. Further studies on product inhibition yielded the following results, ADP was non-competitive with respect to ATP and competitive with respect to NAD^+ which supports a ping-pong mechanism. However, $NADP^+$ was non-competitive with respect to ATP, and with NAD^+ varying the primary plots intersected to the right of the ordinate. In a simple classical ping-pong mechanism it

would be expected that $NADP^+$ would be competitive with ATP and non-competitive with respect to NAD^+.

In summary, it is suggested that the mechanism of NAD^+ kinase involves an enzyme bound intermediate most probably a phosphorylated enzyme. The final proof of the existence of this suggested intermediate will be dependent on the isolation of the enzyme in a homogeneous state.

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