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NICOTINAMIDE ADENINE DINUCLEOTIDE KINASE FROM *AZOTOBACTER VINELANDII* CELLS. REVERSIBLE INACTIVATION OF THE ENZYME

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SUMMARY

NAD^+ kinase (EC 2.7.1.23) from *Azotobacter vinelandii* cells was partially inactivated by dialysis. The inactivated enzyme could be reactivated by incubation with NAD^+ and, to a greater extent, with a combination of NAD^+ and 2-mercaptoethanol. 2-Mercaptoethanol by itself did not reactivate the enzyme. Treatment of the enzyme with *o*-iodosobenzoate, which oxidizes protein sulfhydryl groups to the disulfide state, resulted in an almost complete loss of catalytic activity. The *o*-iodosobenzoate-treated enzyme could be reactivated partially by incubation with 2-mercaptoethanol and NAD^+. NAD^+ and 2-mercaptoethanol, when used separately, were only very slightly effective in restoring the activity of the *o*-iodosobenzoate-treated enzyme. The sedimentation behavior of a reduced and inactive enzyme preparation, obtained by dialysis against phosphate buffer and 2-mercaptoethanol, was compared with that of the same preparation which had been reactivated by incubation with NAD^+ and that of the original undialyzed enzyme. The data indicate that the reduced enzyme has a sedimentation constant of 4.6 S and that the reactivated enzyme and the original enzyme had sedimentation constants of 6.1 S and 5.9 S, respectively.

The experimental data suggest that NAD^+ kinase can exist in several forms: (a) An inactive reduced form, (b) an active form induced by NAD^+ and which contains intact sulfhydryl groups and (c) an inactive oxidized form which can be reactivated by incubation with 2-mercaptoethanol and NAD^+.

INTRODUCTION

NAD^+ kinase (EC 2.7.1.23) catalyzes the synthesis of $NADP^+$ by the phosphorylation of NAD^+(refs. 1–3). The enzyme from *Azotobacter vinelandii* (ATCC 9104) cells can utilize a variety of nucleoside triphosphates as the phosphate donor for $NADP^+$ synthesis[4]. It has been reported that the purified dialyzed enzyme from *A. vinelandii* cells was very labile to heat and that the enzyme could be stabilized to a remarkable

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degree by NAD+ (ref. 4). It was furthermore indicated that the dialyzed enzyme could be activated by preincubation of the enzyme with NAD+ (ref. 4) The central position of NAD+ kinase in the interconversion of two critically important coenzymes, NAD+, primarily involved in electron transport reactions, and NADP+, primarily involved in biosynthetic reactions, warranted a closer investigation of these observations. It was hoped that studies on the behavior of the purified enzyme would help to elucidate the delicate control mechanisms which maintain a critical balance between these coenzymes *in vivo*.

EXPERIMENTAL PROCEDURES AND MATERIALS

Materials

NAD+, NADP+, AcPyAD+, deamino-NAD+, ATP, 2'-AMP, DL-sodium isocitrate, pig heart isocitrate dehydrogenase and recrystallized yeast alcohol dehydrogenase were obtained from the 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 KEILIN AND HARTREE[5]. Bovine serum albumin was obtained from Mann Research Laboratories, New York. *o*-Iodosobenzoic acid was obtained from Dr. O. K. REISS.

Experimental

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[4]. In the last three steps of purification, the ammonium sulfate precipitation, second DEAE-cellulose chromatography and calcium phosphate gel fractionation, $5 \cdot 10^{-3}$ M 2-mercaptoethanol was included in the buffers. The enzyme used for the density gradient experiments was further purified by gel filtration on Sephadex G-200 to a specific activity of 60 enzyme units/mg protein. The eluting buffer was 0.1 M potassium phosphate, pH 7.0, which contained $1.5 \cdot 10^{-4}$ M NAD+ and 10^{-2} M 2-mercaptoethanol. The active enzyme fractions were concentrated by an Amicon diaflo apparatus (Amicon Corp., Boston, Mass.). The specific activity of this purified preparation was 1000–1500 times greater than that of the unfractionated enzyme from crude bacterial extracts.

Assay of enzyme: The enzyme activity was assayed by the methods previously described[4]. The direct spectrophotometric method was used for assay of the sucrose density gradient fractions and in the more refined kinetics measurements. Absorbance measurements were made with a Zeiss PMQ II spectrophotometer at 340 mμ in a standard cuvette with a 1-cm light path.

Protein determinations: Protein determinations were routinely made by the procedure of LOWRY *et al.*[6].

Sedimentation analysis of NAD+ kinase in sucrose density gradients: Sedimentation analysis of NAD+ kinase after various treatments was carried out on 5-ml columns of 4–20% sucrose, as described by MARTIN AND AMES[7]. The sucrose was dissolved in 0.1 M potassium phosphate buffer (pH 7.0) which contained 10^{-2} M 2-mercaptoethanol and $1.5 \cdot 10^{-4}$ M NAD+, except in the analysis of the reactivatable enzyme species in which case the NAD+ was omitted. The sedimentation pattern of

each sample was compared with yeast alcohol dehydrogenase as internal marker. After sedimentation, fractions of 10 drops each were collected and analyzed for both NAD^+ kinase activity and yeast alcohol dehydrogenase activity[8]. The reactivatable enzyme was located after activation of the collected fractions by incubation with $2.2 \cdot 10^{-2}$ M NAD^+ at room temperature for 3 h.

RESULTS AND DISCUSSION

It was observed that when a preparation of NAD^+ kinase, which had been purified in the presence of NAD^+, was dialyzed for 16 to 24 h against potassium phosphate buffer, there was a large decrease in the catalytic activity of the enzyme. For example, as much as 90% of the initial activity was often lost after dialysis for 20 h. However, in the presence of NAD^+ the enzyme was remarkably stable[4]. Since there was no loss of protein from the dialysis sac, these observations suggested that removal of NAD^+ from the enzyme solution resulted in a catalytically inactive protein molecule or a molecule which was readily inactivated by changes in its conformation or chemical structure or both. Experiments were undertaken in order to obtain a better understanding of the function of NAD^+ in stabilizing the enzyme and the changes in the protein molecule which occur upon its removal.

It was found that the catalytic activity which was lost upon dialysis could be partially restored by incubation of the enzyme with NAD^+ at 30°. As shown in Fig. 1, the reactivation of the enzyme by NAD^+ was time dependent. Moreover, the rate and extent of reactivation were greater when 2-mercaptoethanol was added together with NAD^+ than with NAD^+ alone. 2-Mercaptoethanol by itself had no effect on the enzyme activity. If neither 2-mercaptoethanol nor NAD^+ was added, the residual enzyme activity was further reduced. The failure to observe an increase in activity upon incubating the enzyme alone at 30° ruled out a possible reversible cold-inactivation of the enzyme as described for glucose-6-phosphate dehydrogenase[9–11] and other enzymes[11]. In the experiment described, the extent of reactivation in the presence of 2-mercaptoethanol and NAD^+ was approximately 60% of the original activity. The extent and rate of reactivation in other experiments was, however, dependent upon the preparation used.

The above results indicated that the removal of NAD^+ from the enzyme solution by itself could result in an inactivation of the enzyme without concomitant oxidation of the essential enzyme sulfhydryl groups. This conclusion was based upon the observation that NAD^+ alone could reactivate the enzyme. Since it had previously been shown[4] that the enzyme required intact sulfhydryl groups for activity, then the reactivation by NAD^+ alone indicated that the sulfhydryl groups in a portion of the enzyme molecules were still in the reduced state and that the activation effect of NAD^+ was on such molecules. The greater extent of activation observed in the presence of 2-mercaptoethanol and NAD^+ suggested that some of the enzyme sulfhydryl groups were oxidized during the dialysis procedures. The 2-mercaptoethanol presumably reduced these oxidized groups, probably disulfide groups, to a form of the enzyme which was capable of being activated by NAD^+.

Further evidence to support the validity of the conclusions above was obtained from studies on the effect of *o*-iodosobenzoate on the dialyzed NAD^+ kinase. *o*-Iodosobenzoate is known to oxidize protein sulfhydryl groups to the disulfide state[12]. As

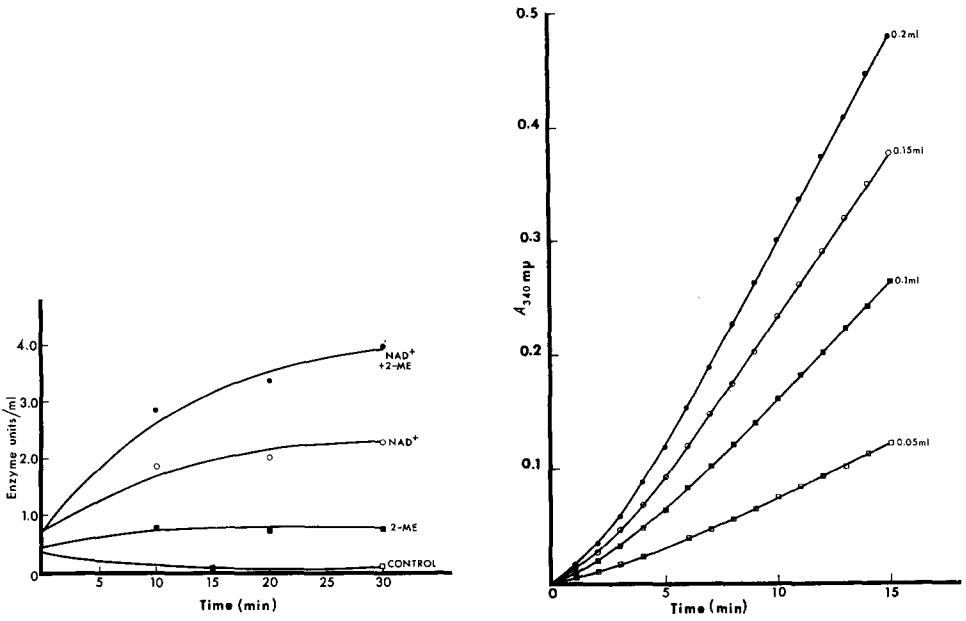

Fig. 1. Reactivation of partially inactivated NAD+ kinase. 4 ml of partially purified enzyme solution containing 0.6 mg protein/ml and 6.96 enzyme units/ml were dialyzed against 2 changes of 1000 ml each of 0.05 M potassium phosphate buffer, pH 7.0, for 24 h. The dialyzed solution contained 0.6 mg protein/ml. Incubation vessels were prepared which contained 0.5 ml enzyme solution with 0.1 ml NAD+ solution ($2 \cdot 10^{-2}$ M); 0.2 ml 2-mercaptoethanol (2-ME) solution (1 M); 0.1 ml NAD+ solution ($2 \cdot 10^{-2}$ M) and 0.1 ml 2-mercaptoethanol solution (1 M); or no additions, respectively. The vessels were incubated at 30° and at predetermined intervals 0.1-ml aliquots were removed and assayed for enzyme activity by the direct spectrophotometric assay previously described[4].

Fig. 2. Time course of NADP+ formation by reduced NAD+ kinase. Reduced NAD+ kinase was prepared by dialyzing 4 ml of purified enzyme solution against 2 changes of 1000-ml volumes of 0.1 M potassium phosphate buffer, pH 7.1, containing $5 \cdot 10^{-2}$ M 2-mercaptoethanol. The total dialysis time was 20 h. The specific activity of the partially purified enzyme before dialysis was 19 enzyme units/mg protein and the protein content was 0.58 mg/ml. The time course of the increase in absorbance at 340 mμ was determined with the volumes of enzyme solution indicated. The assay system was as previously described[4].

described in Table I, exposure of the dialyzed enzyme to this reagent resulted in an almost complete loss of catalytic activity. The activity was restored to a marked degree upon incubation of this oxidized enzyme with NAD+ and 2-mercaptoethanol. Neither NAD+ nor 2-mercaptoethanol alone was more than slightly effective in reactivating the enzyme. In contrast to these results with the oxidized enzyme, NAD+ alone and NAD+ and 2-mercaptoethanol to a greater extent, markedly increased the catalytic activity of the dialyzed but untreated kinase. These results clearly indicate that the kinase can exist in at least three states: (a) An oxidized inactive form; (b) a reduced inactive or slightly active form; and (c) an active form which was dependent upon the presence of intact sulfhydryl groups and NAD+.

It was difficult to demonstrate conclusively that the reduced form of the enzyme in the absence of NAD+ was completely inactive because of the nature of the system for assay of enzymatic activity. The assay system contained NAD+ and the time constant for the activation process was such that it was not small compared with the

TABLE I

REACTIVATION OF NAD^+ KINASE AFTER TREATMENT WITH *o*-IODOSOBENZOATE

The preparation used was obtained by dialysis of the enzyme against 0.05 M potassium phosphate buffer, pH 7, for a total time of 19 h, under an atmosphere of N_2. The dialyzed enzyme solution contained 0.58 mg protein/ml and 0.58 enzyme unit/ml. A portion of this enzyme solution was incubated with *o*-iodosobenzoate at a final concn. of 10^{-3} M, for 30 min at 25°. This treated enzyme was redialyzed against 0.05 M potassium phosphate buffer, pH 7.0, to remove excess reagent. The dialyzed treated enzyme contained approximately 0.04 enzyme unit/ml. Portions of both the untreated and treated enzymes were incubated at 30° for 60 min with the reagents indicated. At the end of the incubation the residual enzyme activity in each experiment was determined by the direct spectrophotometric assay[4]. The values reported in the table were corrected for dilution by the various reagents. 100% activity refers to the dialyzed untreated enzyme before incubation.

Additions and concentrations		*Untreated enzyme*		*Treated enzyme*	
		Enzyme units/ml	*% Original activity*	*Enzyme units/ml*	*% Original activity*
None		0.26	55	0.00	0
2-mercaptoethanol	10^{-1} M	0.59	126	0.11	24
NAD^+	$2 \cdot 10^{-3}$ M	1.19	257	0.07	16
2-mercaptoethanol + NAD^+	10^{-1} M $2 \cdot 10^{-3}$ M	1.45	312	0.50	108

time of assay. However, by decreasing the time of assay the extent of activation during the assay could be minimized. In Fig. 2, the time course of NADPH formation in the usual assay system with the reduced form of the kinase is shown. The reduced form of the enzyme was obtained by dialyzing the purified enzyme against potassium phosphate buffer containing 2-mercaptoethanol. The activation of the enzyme during the assay is quite clearly seen. The initial rate of reaction when 0.1 ml of enzyme was used was 0.007 absorbance unit change per min, but after 7 min, the rate had increased to an almost constant value of 0.021 absorbance unit change per min. Similar activation of the enzyme was observed at the other concentrations. This is in contrast to the time course of the assay with the activated enzyme prepared in the presence of NAD^+ reported earlier[4].

The nature of the reactivation process was more closely investigated. The reactivation could not be due to a mere reduction of oxidized sulfhydryl groups of the enzyme and a stabilization by NAD^+. NAD^+ must in some way have caused changes in the enzyme structure. The sedimentation behavior of the reduced reactivatable enzyme was compared with those of the original undialyzed enzyme and the reactivated dialyzed enzyme by sucrose density gradient sedimentation analysis. In these experiments the untreated enzyme had a specific activity of 60 enzyme units/mg protein. The reduced reactivatable enzyme was prepared by dialysis of the purified enzyme against 0.1 M potassium phosphate buffer containing 10^{-2} M 2-mercaptoethanol. The reactivated enzyme was prepared by incubating the dialyzed enzyme with $1.5 \cdot 10^{-3}$ M NAD^+ at room temperature for 2 h. The sedimentation constants for the untreated enzyme, the dialyzed reactivatable enzyme and the reactivated dialyzed enzyme were 5.9, 4.7 and 6.1 S, respectively, if one assumes that the sedimentation constant for yeast alcohol dehydrogenase is 6.7 S. If the shapes and partial specific volumes of the proteins are similar, the respective molecular weights of the enzyme species would be

125 000, 84 000 and 131 000, assuming the molecular weight of yeast alcohol dehydrogenase to be 150 000. These data indicate that the removal of NAD+ from the active enzyme species results in a reversible change in the structure of the enzyme which is concomitant with changes in the catalytic activity. The nature of the structural change remains unsettled. Among the possibilities which exist are (a) a change in the state of aggregation of the protein with concomitant changes in the folding of the subunits and (b) a gross change in the conformation but not the state of aggregation of the enzyme in the absence of NAD+ which is reflected by the change in sedimentation constant. The results, therefore, indicate that the catalytic activity of the kinase is dependent both on its state of oxidation and on its correct structural organization, which is dependent upon the presence of NAD+.

The specificity of the reactivating reagent in the presence of mercaptoethanol was determined under conditions similar to those described for Fig. 1. It was found that NAD+ was the most effective activator. At comparable concentration 2'-AMP and the NAD+ analogues, deamino-NAD+ and AcPyAD+ were ineffective, while ATP was able to reactivate and stabilize the enzyme to a slight extent. The specificity indicates some special physiological importance of the reactivation process by NAD+.

The properties of the purified kinase described offer many possibilities for the control of its activity *in vivo*. It is possible that the kinase is synthesized as an inactive protein which could subsequently be activated as the cell requirements for NADP+ increased. This protein may simply be the inactive reduced species or it may be an "oxidized" form of the enzyme capable of being reduced by an auxiliary enzyme and then activated by NAD+. After the enzyme is activated, its activity would be subject to the controlling effects of the various compounds related to its substrates[3,4]. These control mechanisms could account for the delicate balance between the levels of NAD+ kinase and the synthetic requirements of the cell for NADP+.

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