The Equilibrium Constant and the Reversibility of the Reaction Catalysed by Nicotinamide–Adenine Dinucleotide Kinase from Pigeon Liver

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The reversibility of the NAD^+ kinase reaction was established, and the kinetic parameters of the rate equation in the reverse direction were determined. The equilibrium constant of the reaction was determined by using the purified pigeon liver enzyme and radioactively labelled nicotinamide nucleotides. The relationship between kinetic parameters of the forward and reverse reactions is in reasonable agreement with the measured equilibrium constant. As expected from the proposed mechanism of action, the enzyme does not catalyse isotope exchange between NAD^+ and $NADP^+$ in the absence of ADP and ATP. Although homogeneous as judged by polyacrylamide-gel electrophoresis, the enzyme preparation exhibits ADP/ATP isotope-exchange activity which could not be separated from NAD^+ kinase activity, but kinetic evidence suggests that this is probably due to a contaminant.

Steady-state kinetic studies with purified pigeon liver NAD^+ kinase (EC 2.7.1.23) suggested (Apps, 1968, 1969, 1971) that the enzyme acts by a random-addition rapid-equilibrium mechanism in the forward direction of the reaction:

$$NAD^+ + MgATP^{2-} \rightleftharpoons NADP^+ + MgADP^- + H^+ \quad (1)$$

In such a mechanism either substrate can bind to the free enzyme, the resultant binary complex then binding the second substrate to form a ternary complex. Since the rate-limiting step of the reaction appears to occur after formation of the ternary complex, there is a binding equilibrium between each substrate and its complexes with the enzyme; thus for each substrate the kinetic constant K_m equals K_s, the dissociation constant of the enzyme–substrate complex. In NAD^+ kinase the two types of substrate-binding site are independent, so that the apparent K_m for each substrate is independent of the concentration of the other.

However, deviations from this ideal behaviour were found to occur at very low concentrations of NAD^+, suggesting that this mechanism does not necessarily apply under these conditions (Apps, 1975), and prompting investigation of the mechanism by other means.

The study of isotope exchange between pairs of substrates at equilibrium can provide valuable confirmatory evidence for such a mechanism (Cleland, 1970). If NAD^+ kinase obeys the above mechanism, there should be no isotope exchange

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between NAD^+ and $NADP^+$, in the absence of ADP and ATP, nor exchange between ADP and ATP, in the absence of NAD^+ and $NADP^+$. Since all exchanges have a common rate-limiting step, they should have the same maximum velocity, and since the binding order is random, high substrate inhibition should not be apparent.

Before such studies could be performed it was necessary to investigate the kinetics of the reaction in the reverse direction, and to determine the equilibrium constant of the reaction. These results are reported in the present paper, but it was not possible to investigate the mechanism further because the NAD^+ kinase preparation was found to catalyse ADP/ATP exchange in the absence of NAD^+ and $NADP^+$. Although the enzyme appeared homogeneous by polyacrylamide-gel electrophoresis, and although this activity could not be separated from NAD^+ kinase, its kinetic properties suggested that it must be due to a contaminant.

Materials and Methods

Reagents

NAD^+, $NADP^+$, NADH, NADPH, ADP, ATP, CTP, GTP, 2′-deoxy-GTP, ITP, UTP, phosphoenol-pyruvate, 3-phosphoglyceric acid, alcohol dehydrogenase, glyceraldehyde 3-phosphate dehydrogenase, glucose 6-phosphate dehydrogenase, lactate dehydrogenase, adenylate kinase, 3-phosphoglycerate kinase and pyruvate kinase were supplied by Boehringer Corp. (London) Ltd., London W5 2TZ, U.K.

For kinetic experiments $NADP^+$ was purified by chromatography on DEAE-cellulose, eluted with a linear gradient of KCl (Dalziel & Dickinson, 1966).

[*Carbonyl*-^{14}C]NAD$^+$ (59 Ci/mol), [*carbonyl*-^{14}C]-NADP$^+$ (53 Ci/mol), [8-^{14}C]ADP (55 Ci/mol) and [^{32}P]P$_i$ (355 Ci/mol) were products of The Radio-chemical Centre, Amersham, Bucks., U.K.

NAD$^+$ kinase was purified from pigeon liver as described elsewhere (Apps, 1968, 1975) and showed a single band on polyacrylamide-gel electrophoresis (Apps, 1975). [γ-^{32}P]ATP was synthesized by exchange of [^{32}P]P$_i$ into ATP (Glynn & Chappell, 1964), and purified on a column (3.0 cm × 0.5 cm^2) of Dowex 1 (X8; 400 mesh).

Nucleotide separations

Separation of nucleotides was performed by ascending chromatography on strips (3 cm × 22 cm) of DEAE-cellulose paper (Whatman DE-81) at room temperature (22°C). For separation of [^{14}C]NAD$^+$ and [^{14}C]NADP$^+$, the solvent was ammonium formate/10 mM-EDTA, pH 3.1, the total concentration of formic acid plus formate being 0.12 M. For separation of [^{14}C]ADP and [^{14}C]ATP, the same system was used, with the concentration increased to 0.6 M. The sample (10 μl) was applied in a streak parallel to, and 3 cm from, the end of the strip; after chromatography and oven-drying, the strip was cut into pieces (1 cm × 3 cm), each of which was counted for radioactivity in 4 ml of toluene-based scintillator, containing 0.5% (w/v) 2,5-diphenyloxazole (PPO) and 0.03% 1,4-bis-(5-phenyloxazol-2-yl)benzene (POPOP).

Assays

NAD$^+$ kinase was assayed in the direction of NADP$^+$ synthesis as described elsewhere (Apps, 1968, 1975). For assays in the reverse direction incubation mixtures (100 μl) contained, in addition to the required concentrations of NADP$^+$ and ADP, 0.05 μCi of [^{14}C]NADP$^+$; the reaction was started by addition of enzyme, and stopped by applying a 10 μl sample to DEAE-cellulose paper for separation and determination of the products. Alternatively, the reaction was carried out with unlabelled substrates, the reaction stopped by boiling, and NAD$^+$ determined by the recycling procedure of Bernofsky & Swan (1973). Reactions were carried out at 30°C in 0.1 M-triethanolamine chloride, pH 7.0 at 30°C, with a free Mg^{2+} concentration of 10 mM. ATPase* activity was measured in 1 mM-ATP/10 mM-MgCl$_2$/0.1 M-triethanolamine chloride, pH 7.0, containing 0.25 μCi of [γ-^{32}P]ATP in a total volume of 300 μl. After incubation for 15 min at 30°C the reaction was stopped with 0.3 M-HClO$_4$, and liberated phosphate extracted as phosphomolybdate into butyl acetate (Post & Sen, 1967) and counted for radioactivity in toluene-based

* Abbreviations: ATPase, adenosine triphosphatase; Mops, 4-morpholinepropanesulphonic acid.

scintillator (0.5% PPO, 0.03% POPOP). [^{14}C]ADP/ATP exchange was assayed in 0.5 mM-ADP/5 mM-ATP/10 mM-MgCl$_2$/0.1 M-triethanolamine chloride, pH 7.0, containing 0.25 μCi of [^{14}C]ADP in a total volume of 100 μl. After incubation at 30°C the reaction was stopped by applying a 10 μl sample to DEAE-cellulose paper for separation and radioactivity counting of the adenine nucleotides. Kinetic studies of ADP/ATP exchange were made in a similar system, various components of the mixture being varied or replaced.

[^{14}C]NAD$^+$/NADP$^+$ exchange was measured in 1 mM-NAD$^+$/1 mM-NADP$^+$/10 mM-MgCl$_2$/0.1 M-triethanolamine chloride, pH 7.0, containing 0.2 μCi of [^{14}C]NAD$^+$ in a total volume of 100 μl, at 30°C.

Determinations of binding constants

The association constant for complex-formation between NADP$^+$ and Mg^{2+} was determined at 30°C, at I 0.1 and various pH values, by the resin competition method of Schubert (1952), with DEAE-Sephadex A-25. The details of this procedure have been published elsewhere (Apps, 1973).

The acid dissociation constant of NADP$^+$ was determined by titration of a 2 mM solution in 0.1 M-NaCl at room temperature (22°C), by using a Vibret 46A pH-meter (Electronic Instruments, Richmond, Surrey, U.K.) coupled to a Unicam AR-25 chart recorder, and a Gilson peristaltic pump to deliver acid or alkali.

Determination of the equilibrium constant of the NAD$^+$ kinase reaction

The concentrations of reactants at equilibrium were determined after approach to equilibrium from either NAD$^+$ and ATP, or NADP$^+$ and ADP. The reactants at the start of these experiments were 1 mM-NAD$^+$, 2 mM-ATP, 10 mM-MgCl$_2$ and 2.4 μCi of [^{14}C]NAD$^+$/ml or 1 mM-NADP$^+$, 5 mM-ADP, 0.5 mM-ATP, 10 mM-MgCl$_2$ and 1.0 μCi of [^{14}C]NADP$^+$/ml respectively. In each case the buffer was 0.1 M-sodium cacodylate, pH 6.1, containing 0.01% NaN$_3$. I was initially 0.1, and the volume 250 μl.

The mixtures were brought to equilibrium at 30°C by addition of 0.1 unit of NAD$^+$ kinase. After various times the ratio of NAD$^+$ to NADP$^+$ was determined chromatographically, and ATP, ADP and AMP were measured by enzymic assays (Jaworek et al., 1975, pp. 2097–2101, 2127–2131).

Experimental and Results

Reversal of the NAD$^+$ kinase reaction

Reversibility was established by following the formation of [^{14}C]NAD$^+$ from [^{14}C]NADP$^+$, or

[^{14}C]ATP from [^{14}C]ADP. The former method was used for kinetic studies because of the high K_m for ADP, and because under the experimental conditions used dephosphorylation of NADP$^+$ was negligible in the absence of ADP, whereas the enzyme preparation had detectable ATPase activity (see below). With 0.5mM-NADP$^+$, 5mM-ADP and 5m-units of NAD$^+$ kinase the reaction rate was constant for at least 20min; in kinetic studies the conversion of any substrate was not allowed to exceed 5%.

Fig. 1 shows Hanes (1932) plots for the reverse reaction, with MgADP$^-$ and NADP$^+$ as the varied substrates. These data fit the rate equation:

$$v_0' = V_{max.}' \cdot \left(1 + \frac{K_p}{[\text{MgADP}^-]} + \frac{K_q}{[\text{NADP}^+]} + \frac{K_{pq}}{[\text{MgADP}^-][\text{NADP}^+]}\right)^{-1} \qquad (2)$$

where v_0' is the initial reaction velocity, $V_{max.}'$ the maximum velocity and K_p and K_q are Michaelis constants, and the SIMPLEX program (Nelder & Mead, 1964) was used to obtain the kinetic parameters shown in Table 1. For comparison the kinetic parameters of the forward reaction according to the rate equation:

$$v_0 = V_{max.} \cdot \left(1 + \frac{K_a}{[\text{MgATP}^{2-}]} + \frac{K_b}{[\text{NAD}^+]} + \frac{K_{ab}}{[\text{MgATP}^{2-}][\text{NAD}^+]}\right)^{-1} \qquad (3)$$

are also shown. As pointed out by Alberty (1953), for the mechanism postulated there is a relationship between the parameters of the forward and reverse rate equations, and the equilibrium constant of the reaction:

$$K_{eq.} = \frac{V_{max.}.K_{pq}}{V_{max.}'.K_{ab}} \qquad (4)$$

Since $K_{ab} = K_a K_b$ (Table 1), this could also be written:

$$K_{eq.} = \frac{V_{max.}.K_{pq}}{V_{max.}'.K_a K_b} \qquad (5)$$

Substituting the measured kinetic parameters, $K_{eq.} = 40.4 \times 10^{-7}$M under the experimental conditions, from eqn. (4), or 33.9×10^{-7}M from eqn. (5), the pH of the assay media at 30°C being 7.0±0.05. The K_m for NADP$^+$ is somewhat lower than that for NAD$^+$; there is a similar relationship between the K_i values for NADH and NADPH (Apps, 1968), and the K_m for MgADP$^-$ is similar to the K_i found for ADP^{3-}. It is noteworthy that in the reverse reaction, $K_{pq} > K_p K_q$, so that the apparent K_m for each substrate is dependent on the concentration of the other (Fig. 1). This contrasts with the forward reaction,

where the relationship $K_{ab} = K_a K_b$ is at least approximately true.

Calculation of the equilibrium constant of the NAD$^+$ kinase reaction
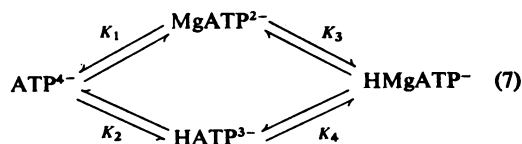
The equilibrium constant may be defined in terms of eqn. (1) as follows:

$$K_{eq.} = \frac{[\text{NADP}^+][\text{MgADP}^-][\text{H}^+]}{[\text{NAD}^+][\text{MgATP}^{2-}]} \qquad (6)$$

The species used in this expression are those thought to be the substrates of pigeon liver NAD$^+$ kinase;

evidence has been presented that NAD$^+$ (rather than, say, NAD$^+$Mg) and MgATP^{2-} (rather than ATP^{4-}) are substrates for the forward reaction (Apps, 1968; Apps & Marsh, 1972), but there is no direct evidence relating to NADP$^+$ and MgADP$^-$. Each of the nucleotides can exist in at least four states: free; magnesium complex; protonated; protonated magnesium complex; thus for ATP:

$$\begin{array}{ccc} & \overset{K_1}{\nearrow} \text{MgATP}^{2-} \overset{K_3}{\searrow} & \\ \text{ATP}^{4-} & & \text{HMgATP}^- \\ & \overset{K_2}{\searrow} \text{HATP}^{3-} \overset{K_4}{\nearrow} & \end{array} \qquad (7)$$

Here K_1 is the association constant of the complex MgATP^{2-}, and K_2, K_3 and K_4 are defined in similar ways as the association constants for the binding of protons or Mg^{2+}. Similarly, K_5–K_8 are defined for the corresponding reactions of ADP^{3-}, K_9–K_{12} for NAD$^+$ and K_{13}–K_{16} for NADP$^+$. In each of these sets there are only three independent constants, since in scheme (7)

$$K_1 K_3 = K_2 K_4 \qquad (8)$$

To calculate $K_{eq.}$ from the measured total concentrations of ATP, ADP, NAD$^+$ and NADP$^+$ at equilibrium, 12 independent binding constants had to be known (Table 2). Two of the most important, K_{13} and K_{16}, were not available in the literature, and were measured; in addition, the value of K_{14} was checked
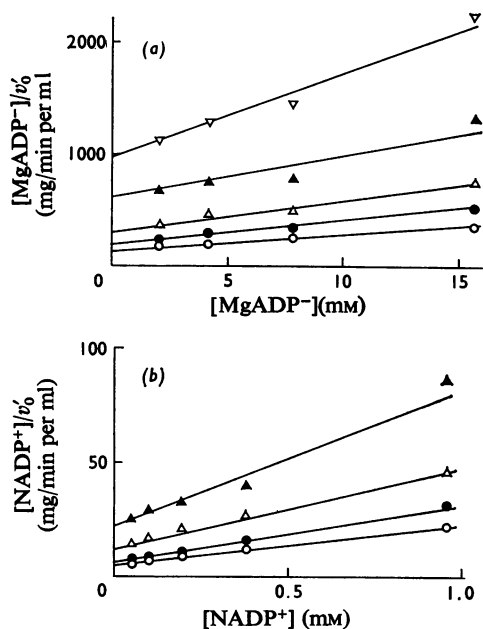
Fig. 1. *Reciprocal plots for the reverse NAD$^+$ kinase reaction*

(a) MgADP$^-$ varied; NADP$^+$: $\triangledown$, 48 μM; $\blacktriangle$, 96 μM; $\triangle$, 191 μM; $\bullet$, 382 μM; $\bigcirc$, 956 μM: (b) NADP$^+$ varied; MgADP$^-$: $\blacktriangle$, 2.09 mM; $\triangle$, 4.18 mM; $\bullet$, 7.85 mM; $\bigcirc$, 15.69 mM. The lines fit the kinetic parameters for eqn. (2), given in Table 1.

Table 1. *Kinetic parameters of the forward (eqn. 7) and reverse (eqn. 6) reactions of NAD$^+$ kinase*

In the forward direction, kinetic parameters were determined with [NAD$^+$] 0.1–2.0 mM and 36 velocity measurements. Weighted s.d. was 4.8%. In the reverse direction, kinetic parameters were determined with [NADP$^+$] 0.05–1.0 mM, [MgADP$^-$] 2–20 mM and 25 velocity measurements. Weighted s.d. was 6.2%.

K_a	(mM)	2.30
K_b	(mM)	0.58
K_{ab}	(mM^2)	1.12
$V_{max.}$	(μmol/min per mg)	1.08
K_p	(mM)	9.26
K_q	(mM)	0.28
K_{pq}	(mM^2)	3.73
$V'_{max.}$	(μmol/min per mg)	0.089

by titration of NADP$^+$. The value of $K_{eq.}$, as defined in eqn. (6), was determined by using a computer program which solved the simultaneous equations for the required form of each nucleotide, given the total concentration of each, the total Mg^{2+} concentration, the pH and the values of the constants listed in Table 2. The calculated value of $K_{eq.}$ was found to be constant after 16 h incubation. The enzyme retains at least 80% of its activity under these conditions, and

$K_{eq.}$ was not affected by addition of a further 0.1 unit of NAD$^+$ kinase after 20 h incubation. The degradation of nicotinamide nucleotides, sometimes detected by a labelled species (presumably nicotinamide) running at the solvent front, did not exceed 0.4% at pH 6.1.

A possible source of error was the presence of ATPase activity in the NAD$^+$ kinase preparation: this amounted to approx. 0.2% of $V_{max.}$, the maximum NAD$^+$ kinase activity in the forward direction, or 2.5% of $V'_{max.}$, the activity in the reverse direction, at pH 6.1. At equilibrium, there was a constant flux through the kinase due to hydrolysis of ATP, and the [NAD$^+$]/[NADP$^+$] ratio increased, the calculated value of $K_{eq.}$ remaining constant. Under these circumstances a steady state had arisen, increase in [ADP]/[ATP] (due to ATPase activity) being balanced by NAD$^+$ kinase activity in the reverse direction; to produce the NAD$^+$ kinase flux, there obviously had to be an increase in [ADP]/[ATP] over the equilibrium value, resulting in an overestimate of $K_{eq.}$. Although the contaminating ATPase activity is slight, the NAD$^+$ kinase activity close to equilibrium is obviously low, but it is not possible to assess the seriousness of the possible error without knowing all the kinetic parameters of both enzymes. For example, ATPase activity might well be inhibited by ADP. However, inclusion of adenylate kinase in the equilibrium mixture, which converted some of the adenine nucleotides into AMP but decreased the net ATPase flux, had no effect on $K_{eq.}$, and $K_{eq.}$ was not altered by doubling the amount of NAD$^+$ kinase added.

The values of $K_{eq.}$ obtained are given in Table 3.

Isotope exchange

The original purpose of determining the equilibrium constant was as a necessary preliminary to studying isotope exchange at equilibrium. Isotope exchange between the substrate pairs NAD$^+$/NADP$^+$ and ADP/ATP was first investigated in the absence of other substrates. No significant NAD$^+$/NADP$^+$ exchange was found, but there was a rapid equilibration of ^{14}C between ADP and ATP. Since the purification procedure did not include an exclusion-chromatography step, a sample of the purified enzyme was passed through an Ultrogel AcA34 column at 6°C. NAD$^+$ kinase activity was eluted at 1.83 × void volume, but most of the ADP/ATP-exchange and ATPase activities were eluted in parallel with it (Fig. 2). Such contaminants as adenylate kinase (EC 2.7.4.3) and nucleoside diphosphate kinase (EC 2.7.4.6) should have been removed by this procedure, and no adenylate kinase activity was detectable, but a number of experiments suggest that the ADP/ATP exchange activity was due to a contaminant, rather than NAD$^+$ kinase itself.

Table 2. *Sources and values of binding constants used in calculating the NAD^+ kinase equilibrium constant*

HNAD⁺ indicates NAD⁺ protonated at adenine-N_1 (net charge 0); HNADP⁺ indicates NADP⁺ protonated at 2'-phosphate (net charge -2); NAD⁺Mg has net charge $+1$, HNAD⁺Mg net charge $+2$, NADP⁺Mg net charge -1 and HNADP⁺Mg net charge 0.

Constant	Definition	Value (M^{-1})	Source
K_1	$\dfrac{[\text{MgATP}^{2-}]}{[\text{ATP}^{4-}][\text{Mg}^{2+}]}$	8.0×10^4	O'Sullivan & Perrin (1964)
K_2	$\dfrac{[\text{HATP}^{3-}]}{[\text{ATP}^{4-}][\text{H}^+]}$	9.3×10^6	O'Sullivan & Perrin (1964)
K_4	$\dfrac{[\text{HMgATP}^-]}{[\text{HATP}^{3-}][\text{Mg}^{2+}]}$	700	O'Sullivan & Perrin (1964)
K_5	$\dfrac{[\text{MgADP}^-]}{[\text{ADP}^{3-}][\text{Mg}^{2+}]}$	1.25×10^3	Martell & Schwarzenbach (1956)
K_6	$\dfrac{[\text{HADP}^{2-}]}{[\text{ADP}^{3-}][\text{H}^+]}$	4.0×10^6	Melchior (1954)
K_8	$\dfrac{[\text{HMgADP}]}{[\text{HADP}^{2-}][\text{Mg}^{2+}]}$	89	Phillips *et al.* (1966)
K_9	$\dfrac{[\text{NAD}^+\text{Mg}]}{[\text{NAD}^+][\text{Mg}^{2+}]}$	50	Apps (1973)
K_{10}	$\dfrac{[\text{HNAD}^+]}{[\text{NAD}^+][\text{H}^+]}$	7.9×10^3	Lamborg *et al.* (1958)
K_{12}	$\dfrac{[\text{HNAD}^+\text{Mg}]}{[\text{HNAD}^+][\text{Mg}^{2+}]}$	<50	Assumed
K_{13}	$\dfrac{[\text{NADP}^+\text{Mg}]}{[\text{NADP}^+][\text{Mg}^{2+}]}$	191 ± 12 (mean$\pm$S.E.M.; 4 expts.)	This paper
K_{14}	$\dfrac{[\text{HNADP}^+]}{[\text{NADP}^+][\text{H}^+]}$	$2.0 \pm 0.2 \times 10^6$ (mean$\pm$S.E.M.; 4 expts.)	This paper
K_{16}	$\dfrac{[\text{HNADP}^+\text{Mg}]}{[\text{HNADP}^+][\text{Mg}^{2+}]}$	60 ± 3 (mean$\pm$S.E.M.; 4 expts.)	This paper

Table 3. *Values of the equilibrium constant of the NAD^+ kinase reaction determined by direct measurement*

Approach from	Mean $K_{eq.}$ (M)	S.E.M. (M)	No. of experiments
NAD⁺, MgATP²⁻	28.5×10^{-7}	4.7×10^{-7}	6
NADP⁺, MgADP²⁻	30.1×10^{-7}	2.4×10^{-7}	4
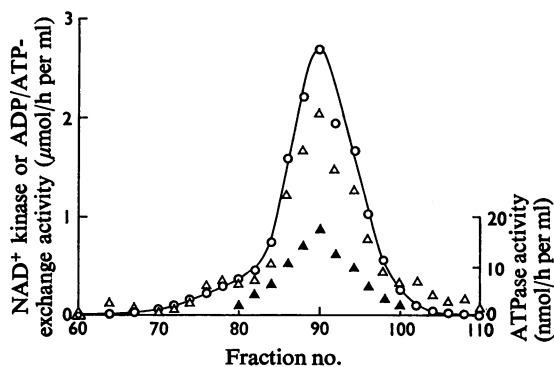
Fig. 2. *Elution of NAD^+ kinase, ADP/ATP exchange and ATPase activities from Ultrogel AcA34*

The column ($85\,\text{cm} \times 5.3\,\text{cm}^2$) was run in 0.1 M-Tris/HCl/0.2 M-NaCl/1 mM-EDTA, pH7.4 at 6°C, and fractions (3.2 ml) were collected. ○, NAD⁺ kinase; △, ADP/ATP exchange; ▲, ATPase.

(1) The exchange activity measured under the standard conditions (see the Materials and Methods section) was inhibited by only 11% by 2.0 mM-NAD⁺, 6% by 2.0 mM-NADP⁺, and not at all by 2.0 mM-NADH or -NADPH.

(2) The nucleoside triphosphate specificity differed from that of NAD⁺ kinase, as shown in Table 4.

(3) Thermal denaturation, in 0.1 M-Mops/NaOH buffer, pH7.0 at 71°C, caused destruction of both NAD⁺ kinase and ATP/ADP-exchange activity, with a first-order rate constant of $2.85 \times 10^{-3} \pm 0.11 \times 10^{-3}\,\text{min}^{-1}$ (four experiments); 5 mM-NAD⁺ afforded considerable protection to NAD⁺ kinase, decreasing the rate constant of inactivation to $0.57 \times 10^{-3} \pm 0.04 \times 10^{-3}\,\text{min}^{-1}$ (four experiments), but had no effect on the rate of inactivation of ATP/ADP-exchange activity.

(4) The K_m for MgADP⁻, with saturating (20 mM) MgATP²⁻, was 0.33 mM, with substrate inhibition apparent above 0.5 mM; the K_m of NAD⁺ kinase for

Table 4. *Nucleoside triphosphate specificity of NAD$^+$ kinase and ADP/ATP isotope exchange reactions*
The activities were determined at 30°C with 2.0 mM-nucleoside triphosphate, 10 mM-MgCl$_2$, 0.1 M-triethanolamine chloride, pH 7.3, and either 1.0 mM-NAD$^+$ or 0.5 mM-ADP.

Nucleoside triphosphate	NAD$^+$ kinase activity (μmol/min per mg)	ADP/ATP exchange (μmol/min per mg)
ATP	0.538	0.387
CTP	0.055	0.050
2′-Deoxy-GTP	0.056	0.077
GTP	0.029	0.089
ITP	0.014	0.093
UTP	0.017	0.070

MgADP$^-$ is higher (Table 1), and there is no substrate inhibition at high concentration.

Discussion

The measured value of $K_{eq.}$ yields a free-energy change of -8.4 ± 1.4 kJ/mol for the reaction in eqn. (1), and hence -29.6 kJ/mol for the standard free energy of hydrolysis of NADP$^+$ to NAD$^+$, taking $\Delta G_0' = -38.0$ kJ/mol for ATP hydrolysis under these conditions (Phillips *et al.*, 1969). This value of $K_{eq.}$ is in reasonable agreement with that obtained from kinetic parameters, which was not subject to errors of calculation arising from the existence of several states of each nucleotide. To assess the importance of the constants listed in Table 2 in calculating $K_{eq.}$, a calculation was made with each constant first doubled and then halved. Variation of K_1, K_2, K_4, K_5, K_6, K_{10}, K_{12} and K_{14} in this way produced less than 0.3% variation in the calculated value of $K_{eq.}$: doubling K_8 and K_{16} in turn decreased $K_{eq.}$ by 12% and 25% respectively; and the calculated value of $K_{eq.}$ was almost directly proportional to K_9 (98% increase on doubling) and inversely proportional to K_{13} (99% increase on halving). Thus $K_{eq.}$ depends largely on the magnitude of the binding constants for Mg^{2+} to the nicotinamide nucleotides. The validity of the identity given in eqn. (4) suggests that the postulated random-addition rapid-equilibrium mechanism probably applies under the conditions of these experiments, despite the kinetic discrepancies noted elsewhere (Apps, 1975), and deviations of the reverse reaction from Michaelis–Menten behaviour were not found with NADP$^+$ varied over the range 0.02–2.0 mM.

The catalysis of ADP/ATP exchange by the NAD$^+$ kinase preparation was an unexpected finding, but it is likely that this is due to contamination by small amounts of an enzyme with a high molecular activity; that of NAD$^+$ kinase is very low (Apps, 1975). It is clear that the NAD$^+$ kinase reaction cannot proceed by a substitution mechanism involving a free phosphorylated form of the enzyme, since no NAD$^+$/NADP$^+$ exchange was detected; this does not exclude the possibility of transfer of the γ-phosphoryl group

of ATP to the protein, within a ternary complex. The association of ADP/ATP-exchange activity with NAD$^+$ kinase makes the study of isotope exchange at equilibrium unprofitable, unless it can be separated from the NAD$^+$ kinase activity. NAD$^+$ kinase purified by affinity chromatography on immobilized Cibacron Blue F3GA (Apps & Gleed, 1976) has similar ADP/ATP-exchange and ATPase activities to the conventionally purified enzyme.

Purified NAD$^+$ kinase of *Azotobacter vinelandii* also catalyses ADP/ATP exchange (Orringer & Chung, 1971), which appears to reflect a genuine substitution mechanism for this enzyme, since the initial-velocity pattern is consistent with this mechanism, and the exchange is strongly inhibited by NAD$^+$.

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