THE REACTION OF PIGEON LIVER NAD KINASE WITH BROMOACETYL PYRIDINE

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1. Introduction

In previous papers [1, 2, 3] the results of steady-state kinetic experiments with pigeon liver NAD kinase (ATP : NAD 2'-phosphotransferase, E.C.2.7.1.23) have been reported. From these data it was inferred that the enzyme acts by a mechanism in which four binary and two or more ternary complexes are in equilibrium with the substrates and the free enzyme, interconversion of the ternary complexes being the rate-limiting step of the reaction. The case for such a mechanism would be strengthened by an independent demonstration that either substrate could bind to the free enzyme. Such methods as equilibrium dialysis and protein fluorescence quenching measurements were unsuitable, since the enzyme was not available pure or in large quantities, and has a relatively high K_m for each substrate. However, it was possible to demonstrate that both NAD and ATP-metal complexes afforded partial protection from attack by a number of irreversible inhibitors. Of these, the alkylating agent 3-(bromoacetyl) pyridine (BAP) was selected for detailed study because the rate of reaction with NAD kinase was such as to produce extensive inactivation in a reasonable time, whilst using inhibitor concentrations such that the inhibitor: enzyme ratio was insignificantly altered throughout the reaction.

2. Materials and methods

ATP, NAD, NADH and supplementary enzymes and their substrates were obtained from the Boehringer Corporation Ltd. NAD kinase was purified from pigeon liver and assayed as previously described [2]. 3-(Bromoacetyl) pyridine hydrobromide was prepared by the action of pyridinium perbromide hydrobromide [4] on 3-acetyl pyridine (L.Light and Co.) as described by Wingfield [5], and recrystallized three times from glacial acetic acid.

Time courses of inhibition were constructed by incubating NAD kinase (50–100 μg/ml) with 1.0 mM BAP.HBr in 0.1M triethanolamine hydrochloride buffer, pH 7.4, at 30°, and removing 10 μl. aliquots for assay at selected times. Results were expressed as plots of $\log_{10}$% enzyme activity against time of incubation.

Partially inactivated NAD kinase was produced

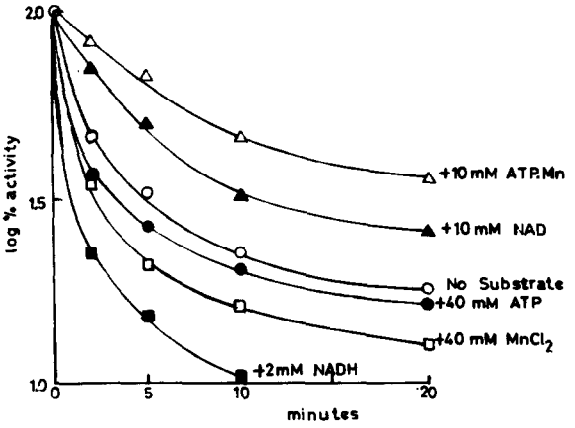

Fig. 1. Time courses of inactivation of NAD kinase by 1 mM BAP, pH 7.4, 30°.

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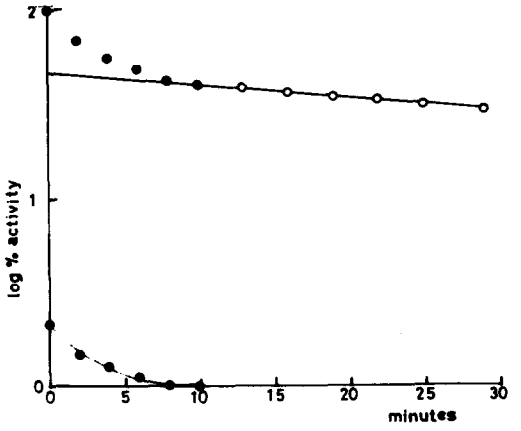

Fig. 2. Analysis of individual rate constants. Upper curve: slow pseudo-first-order reaction. Lowere curve: rapid modification of at least two groups.

by incubating 800 μg/ml enzyme with 1.0 mM BAP. HBr for 16 min at 30° under which conditions all the enzyme molecules were calculated to have been modified, with minimum total inactivation. After this time the mixture was made 10 mM in *N*-acetyl cysteine, and the mixture run through a 29-ml column of Sephadex G-25 equilibrated with 0.1 M KCl, 0.05M tris chloride pH 7.5. A control sample was treated similarly, but with omission of BAP.

3. Results and discussion

Semilogarithmic plots of the time course of inhibition appear in fig. 1. If enzyme inactivation occurs by reaction of a single group, with the concentration of inhibitor effectively constant throughout, the reaction obeys pseudo-first-order kinetics, and such plots should be linear [6]: the curvilinearity of these plots therefore indicates the modification of more than one aminoacid in NAD kinase. A more detailed plot (fig. 2) shows linearity at incubation times above 12 min. Ray and Koshland [6] show that for a reaction in which two groups are attacked at different rates, semilog plots have slopes which decrease with time, tending to become linear. Subtraction of the extrapolated linear part of the plot from the initial part can give the individual rate constants of modification. This treatment, applied to the reaction of NAD

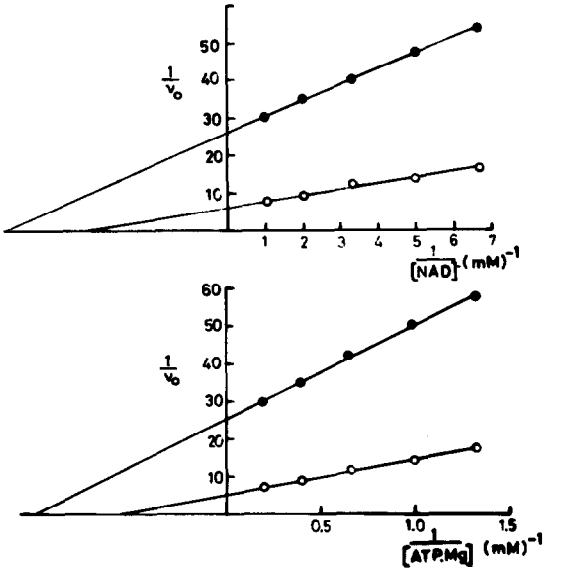

Fig. 3. (A). Determination of $K_m^{NAD^+}$.ATP.Mg$^=$ concentration is 2.50 mM.
(B). Determination of $K_m^{ATP.Mg^=}$.NAD$^+$ concentration is 0.50 mM.
Lower plots: native enzyme. Upper plots: modified enzyme. V_0 in μMoles NADP/mg protein/hr.

kinase with BAP (fig. 2) does not produce linearity, and the data are not precise enough for more detailed analysis. It is therefore concluded that the reaction involves initial modification of at least two groups, yielding a partially active species which is totally inactivated by the slower reaction of a third residue.

Fig. 1 shows that both NAD$^+$ and ATP.Mn$^=$ protect the enzyme from BAP, whilst NADH, Mn^{++} and ATP^{4-}, all of which are reversible inhibitors [3], increase the rate of inactivation. As expected [7], such effects were only encountered with substrate or inhibitor concentrations well above the K_m or K_i values. The complexity of the time courses precluded the calculation of dissociation constants, but the protection by both NAD$^+$ and ATP.Mn$^=$ confirms qualitatively that either substrate may bind to the free enzyme (although such protection could conceivably arise with ordered addition mechanisms, through formation of dead-end complexes with the "wrong" substrate). The alteration of the rate of inactivation may arise directly, as a result of interaction of the ligand with the reacting groups in the

Table 1
Kinetic constants of NAD kinase (forward reaction).

		Native	Modified
ϕ_0	$(\mu Moles/mg/hr)^{-1}$	5.75×10^{-2}	11.52×10^{-2}
ϕ_1	$(\mu Moles/mg/hr)^{-1}mM$	12.50×10^{-2}	17.29×10^{-2}
ϕ_2	$(\mu Moles/mg/hr)^{-1}mM$	1.78×10^{-2}	1.82×10^{-2}
ϕ_{12}	$(\mu Moles/mg/hr)^{-1}(mM)^2$	3.50×10^{-2}	3.10×10^{-2}
$\phi_1\phi_2/\phi_{12}$	$(\mu Moles/mg/hr)^{-1}$	6.36×10^{-2}	10.25×10^{-2}

(S_1 is $ATP.Mg^{=}$; S_2 is NAD^+.)

enzyme, or through a conformational change.

The partially active species had lost 41% of its activity, measured in the standard assay [2]. Investigation of

$$K_m^{ATP.Mg^{=}} \text{ and } K_m^{NAD^+}$$

at a single second substrate concentration showed that the value of each was decreased (fig. 3). Double reciprocal plots were then constructed with each substrate concentration varied independently [8]. Each K_m was found to be independent of the second substrate concentration, as with the native enzyme [2]. The kinetic constants of the rate equation [8]

$$\frac{\epsilon}{V_0} = \phi_0 + \frac{\phi_1}{S_1} + \frac{\phi_2}{S_2} + \frac{\phi_{12}}{S_1 S_2}$$

are shown in table 1. For the modified enzyme V_{max} was lowered from 17.4 to 8.7 μMoles NADP formed per mg protein per hour, and K_m values for NAD^+ and $ATP.Mg^{=}$ lowered from 0.30 and 2.10 to 0.17 and 1.52 mM respectively. As shown by Dalziel [8] the Haldane relationship

$$\phi_0 = \frac{\phi_1\phi_2}{\phi_{12}}$$

applies to enzymes acting by the random addition, rapid equilibrium mechanism. Since this relationship applies to the modified NAD kinase as well as the native form, equilibrium binding of both substrates probably occurs, and therefore the decrease in each K_m is not a necessary consequence of the decrease in the catalytic rate constant, V_{max}. This reduction in K_m (=K_s, the dissociation constant of a binary complex) thus indicates a real increase in the affinity of the enzyme for its substrates. The mechanism by which this occurs might be through the provision of a more hydrophobic environment at the substrate binding sites, or through a conformational change. Similarly, the nature of the groups attacked is at present uncertain; in the only previously reported use of this inhibitor, Woenckhaus and Pfleiderer [9] found that BAP reacted with a cysteine and a lysine in lactate dehydrogenase. Such reactions might also occur in NAD kinase; the presence of a sulphydryl group, concerned with the binding of $ATP.Mg^{=}$ was inferred from the pH-dependence of

$$K_m^{ATP.Mg^{=}}$$

[2] and the NAD^+ binding site may contain a positively charged residue, as NADH is bound more strongly [3]. It was also suggested that a histidine might be involved in catalysis [2] and this and other aminoacids could possibly react with BAO. The 3 or more groups which are apparently modified might owe their different rates of reaction to differences in environment rather than differences in identity. Experiments to establish the nature of these groups are currently in hand.

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