INHIBITION OF PIGEON LIVER NAD KINASE BY A NON-PHOSPHORYLATIBLE ANALOGUE OF NAD

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1. Introduction

Nicotinamide-2′-deoxy-adenosine dinucleotide (NdAD) has previously been prepared enzymatically [1] and chemically [2], and its interactions with dehydrogenases were studied. Since this analogue of NAD differs from the natural coenzyme in lacking the 2′-hydroxyl on the adenosine ribose, it cannot be phosphorylated by NAD kinase (ATP:NAD 2′-phosphotransferase, EC 2.7.1.23). In the present work, both $NdAD^+$ and NdADH have been found to inhibit NAD kinase; the modes of inhibition suggest that the K_m for NAD^+ equals K_s, the dissociation constant of the enzyme–NAD^+ complex; that equilibrium binding of $ATP.Mg^{2-}$also occurs; and that the 2′-hydroxyl is unimportant in the binding of NAD^+ and NADH to NAD kinase. A method for the preparation of NMN in high yield, by the cleavage of NAD^+ with crude rattlesnake venom, is also described.

2. Materials and methods

NAD^+, ATP, glucose-6-phosphate, yeast alcohol dehydrogenase and glucose-6-phosphate dehydrogenase (specific activity 350 units/mg) were obtained from Boehringer. AMP and deoxy AMP (free acids) and *Crotalus adamanteus* venom were supplied by Sigma. Dicyclohexyl carbodiimide was from BDH.

2.1. *Preparation and assay of NAD kinase*

NAD kinase was purified from fresh pigeon livers to a specific activity of 0.3 units/mg, essentially as described previously [3]. Reaction rates were measured in a medium containing 0.1 M triethanolamine-Cl buffer, pH 7.4; 5 mM glucose-6-phosphate; 3.5 units of glucose-6-phosphate dehydrogenase; 0.03 units of NAD kinase, and various concentrations of substrates and inhibitors, in a total volume of 1.0 ml. $NADP^+$ formed by the NAD kinase reaction was reduced by the dehydrogenase to NADPH, the overall reaction being followed at 340 nm in a Unicam SP800 spectrophotometer, equipped with scale expander and a cell compartment water-jacketed at 30°. With this high concentration of glucose-6-phosphate, inhibition of glucose-6-phosphate dehydrogenase by ATP [4] was negligible. The ratio of Mg^{2+} to ATP was 3:1 [3].

2.2. *Preparation of NMN^+*

Previously published [5, 6] methods for the preparation of NMN^+ have involved the separation of nucleotide pyrophosphatase from contaminating enzymes, notably 5′-nucleotidase. In the present work, the latter enzyme was inhibited by Zn^{2+} [7], and degradation of NMN^+ to nicotinamide riboside was further reduced by inclusion of 5′-AMP in the reaction mixture. Under these conditions, near-theoretical yields of NMN^+ from NAD^+ were obtained, using crude snake venom.

400 mg NAD^+ and 200 mg AMP were dissolved in 10 ml 2 mM $ZnSO_4$, and the pH adjusted to 9.5 with 3 M ammonium hydroxide. 15 mg *Crotalus adamanteus* venom was then added, and the mixture stirred at 30°. The pH was maintained at 9.5 by further addition of ammonia during the reaction, and 10 μl samples of the mixture were taken for assay of NAD^+ with alcohol dehydrogenase [8]. After 80 min over 90% of the NAD^+ had been destroyed, and the mixture

was applied to a 30 cm × 12.5 cm^2 column of Whatman DE32, equilibrated with 10 mM ammonium bicarbonate, pH 7.5. Elution was performed with a 1 litre linear gradient of 10–80 mM NH_4HCO_3. The peak of NMN^+ (140 ml) emerged after those of nicotinamide riboside and adenosine, and before residual NAD^+ and AMP. The NMN^+ was lyophilised; the yield was 450 μmoles, as judged by the absorbance of the nucleotide and its cyanide complex at 265 and 325 nm respectively [9]. The ammonium NMN^+ was then converted to the pyridinium salt, by passage through a column of pyridinium Dowex 50.

2.3. *Preparation of $NdAD^+$*

This was as described by Hughes et al. [10]. 500 μmoles NMN^+ and 800 moles dAMP were dissolved in 7.5 ml water, and 30 ml redistilled pyridine added. 10 g of dicyclohexyl carbodiimide were added over 5 days. The worked-up product was applied to a 30 cm × 12.5 cm^2 DE32 column in 20 mM NH_4HCO_3, pH 7.5, and eluted with a linear 1 litre gradient of this buffer, 20–150 mM. 80 μmoles $NdAD^+$ were eluted following peaks of pyridine, nicotinamide and NMN^+, and were lyophilised.

2.4. *Preparation of NdADH*

25 μmoles $NdAD^+$ were reduced with yeast alcohol dehydrogenase in an ethanol/pyrophosphate/semicarbazide medium [8]. The equilibrium mixture was applied to a 25 cm × 3.5 cm^2. DE32 column equilibrated with 0.2 M NH_4HCO_3 pH 7.5, and eluted with the same buffer. NdADH emerged after passage of 3 column volumes, and was lyophilised, with a yield of 22 μmoles.

The coenzyme analogues were assayed in the same way as the natural coenzymes [8]. Neither $NdAD^+$ nor NdADH gave any $NADP^+$ when substituted for NAD^+ in the NAD kinase assay system.

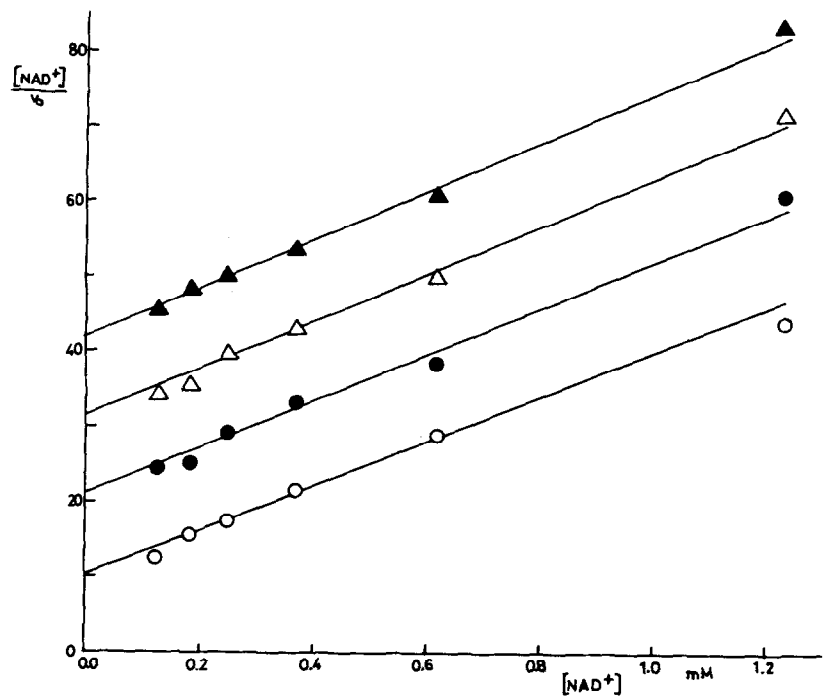

Fig. 1. Inhibition by $NdAD^+$, with NAD^+ as the variable substrate. [$NdAD^+$] = 0.0 mM 0; 0.48 mM •; 0.96 mM △; 1.44 mM ▲. [$ATP.Mg^{2-}$] = 5.0 mM. Initial velocities (V_0) in μmoles $NADP^+$ min^{-1}.

3. Results and discussion

Kinetic plots were of $[S]/v_0$ versus $[S]$, where $[S]$ is the substrate concentration and v_0 the initial velocity of the reaction; such plots have slope $1/V_{max}$ and abscissa intercept $-K_m$ [11].

Inhibition of NAD kinase by $NdAD^+$ was competitive with NAD^+ (fig. 1) and noncompetitive with $ATP.Mg^{2-}$ (fig. 2). The mean value of K_i, calculated from both plots, was 0.39 mM.

Inhibition by NdADH was again competitive with NAD^+ and noncompetitive with $ATP.Mg^{2-}$, and plots were qualitatively similar to figs. 1 and 2. The K_i was 0.105 mM.

Kinetic studies of NAD kinase [3, 12] suggested a mechanism of action in which the substrates bound in random order to the enzyme, the rate-limiting step of the reaction being interconversion of ternary complexes; binding of one substrate to the enzyme did not affect its affinity for the other. In such a mechanism, the K_m for each substrate should equal K_s, the dissociation constant of the appropriate binary complex. As pointed out previously [3], the finding of noncompetitive inhibition toward a given substrate indicates equilibrium binding of this substrate, since V_{max}, related to the catalytic rate constant, is reduced without affecting K_m, which must therefore be a simple dissociation constant, rather than a kinetic quantity. The noncompetitive inhibitions by $NdAD^+$ and NdADH with respect to $ATP.Mg^{2-}$ thus confirm equilibrium binding of this substrate.

The similarity of the K_i for $NdAD^+$ to the K_m for NAD^+, which is 0.30 mM [3], suggests that NAD^+ is also bound in equilibrium, since K_i is the dissociation constant of the enzyme-inhibitor complex; however it is possible that lack of the 2′-hydroxyl in $NdAD^+$ makes its K_i coincidentally similar to the K_m for NAD^+, for which $K_m \neq K_s$. This is rendered less likely by the close similarity of K_i for NdADH to that for NADH, which is 0.090 mM [3]; this suggests that the alteration in structure has little effect on the binding of the coenzyme to NAD kinase, and therefore that the 2′-hydroxyl is unimportant in this respect.

Although the dissociation constants for the 2′-deoxy coenzymes are slightly higher than for the natural forms, the differences are probably within experimen-

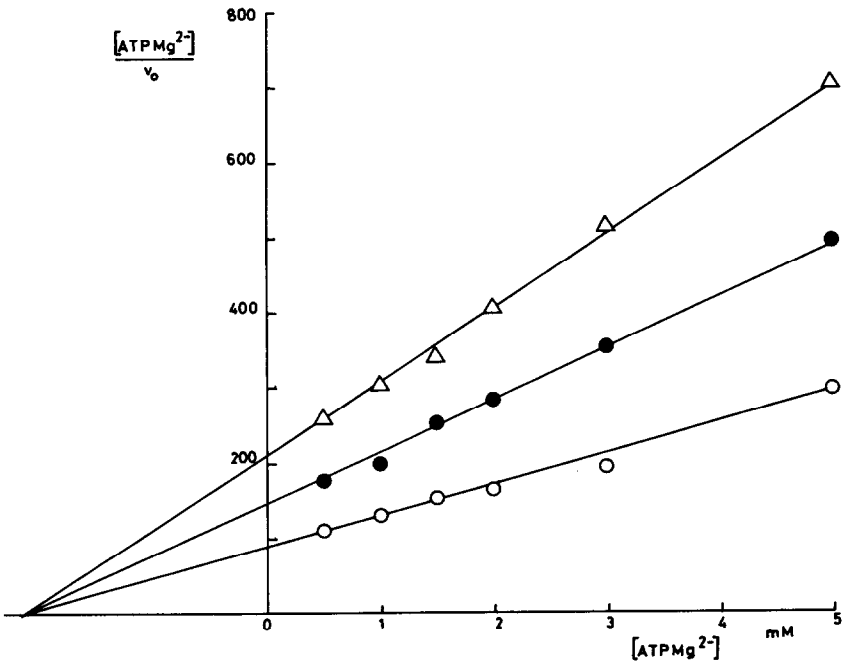

Fig. 2. Inhibition by $NdAD^+$, with $ATP.Mg^{2-}$ as the variable substrate. [$NdAD^+$] = 0.0 mM 0; 0.49 mM •; 0.98 mM △. [NAD^+] = 0.32 mM.

tal error. In this context, NdADH is apparently bound more weakly to horse liver alcohol dehydrogenase than is NADH [2].

Acknowledgement

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References

[1] H. Klenow and B. Andersen, Biochim. Biophys. Acta 23 (1956) 92.
[2] C.V. Fawcett and N.O. Kaplan, J. Biol. Chem. 237 (1962) 1709.
[3] D.K. Apps, European J. Biochem. 5 (1968) 444.
[4] G. Avigad, Proc. Natl. Acad. Sci. U.S. 56 (1966) 1543.
[5] S. Takei, Agr. Biol. Chem. (Tokyo) 34 (1970) 23.
[6] N.O. Kaplan and F.E. Stolzenbach, in: Methods in Enzymology, Vol. 3, eds. S.P. Colowick and N.O. Kaplan (Academic Press, London, 1957) p. 899.
[7] M.A.G. Kaye, Biochim. Biophys. Acta 18 (1955) 456.
[8] H.U. Bergmeyer, Methods of Enzymatic Analysis (Academic Press, London, 1965).
[9] Pabst Laboratories Circular OR-18.
[10] N.A. Hughes, G.W. Kenner and A. Todd, J. Chem. Soc. 735 (1957) 3783.
[11] C.S. Hanes, Biochem. J. 26 (1932) 1406.
[12] D.K. Apps, European, J. Biochim. 7 (1969) 260.