The Effect of Nicotinamide Adenine Dinucleotide (NAD) and Nicotinamide Adenine Dinucleotide Phosphate (NADP) on the NAD Glycohydrolase-inhibitor Complex of *Pseudomonas putida* KB1

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Abstract

Crude cell extracts of *Pseudomonas putida* KB1 contain a heat-stable NADase (nicotinamide adenine dinucleotide glycohydrolase). Extracts also contain a heat-labile protein inhibitor of the enzyme. Enzyme and inhibitor are present as a complex in cell-free extracts.

The enzyme-inhibitor complex was purified by ammonium sulphate precipitation and chromatography on DEAE-cellulose (diethylaminoethyl-cellulose). Purified preparations were essentially free of unbound inhibitor.

Incubation of purified enzyme-inhibitor complex with NAD (nicotinamide adenine dinucleotide) (1-10 millimolar) leads to NAD hydrolysis. The effect is more pronounced at higher NAD concentrations. Approximately 4 per cent of the total enzyme activity present in the preparations was released by 10 millimolar NAD. This partial activation of the enzyme-inhibitor complex was also observed in the presence of NADP (nicotinamide adenine dinucleotide phosphate).

Introduction

NADases catalyse the reaction:

$$NAD^+ + H_2O \rightarrow \text{Nicotinamide} + \text{ADP-ribose} + H^+$$

The enzyme has been isolated and purified from several micro-organisms including *Mycobacterium butyricum* (9), *M. tuberculosis* (5), *Bacillus subtilis* (3), *Neurospora crassa* (3, 8) and *Pseudomonas putida* (16, 17). Some of these NADases are remarkably heat-stable—the *P. putida* enzyme loses only 30% of its activity after 30 min at 100°C. Heat-labile protein inhibitors are often associated with the enzymes in crude cell-free extracts. Enzyme activity is therefore only detected in extracts after inactivation of the inhibitor by heat treatment. The *P. putida* enzyme (23,500 daltons) is combined with a heat labile protein inhibitor (15,000 daltons) in cell extracts. The binding of inhibitor to enzyme is mole/mole. Inhibitor is present in excess over enzyme (16, 17) in cell extracts.

Gholson (4) and others (7, 21) have proposed that NAD(P)ases in both bacterial and mammalian cells are physiologically important in the process of NAD(P) turnover. Nicotinate, formed by deamination of the nicotinamide released from NAD is incorporated into nicotinic acid mononucleotide by nicotinate phosphoribosyltransferase (E.C.2.4.2.11) in the presence of 5-phosphoribosyl-1-pyrophosphate (Fig. 1). NAD can then be resynthesised via the Preiss-Handler path-

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way (19, 20). Pyridine nucleotide turnover in *Escherichia coli* recently has been investigated in detail (12, 13, 15, 18). NAD in this organism appears to be partly recycled by formation of nicotinamide.

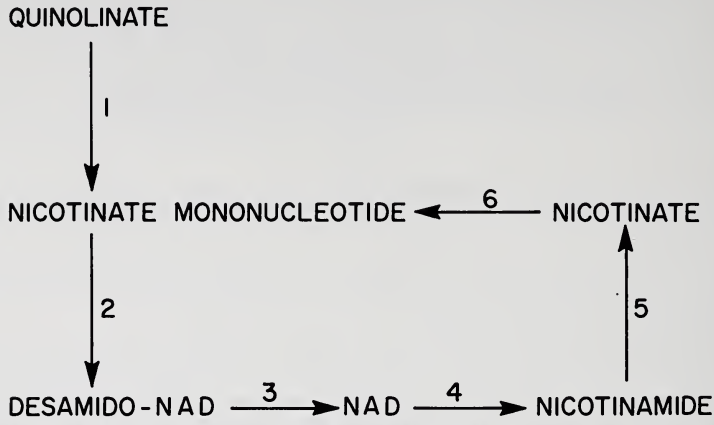

FIGURE 1. *The pyridine nucleotide cycle; enzymes of the pathway are: 1) quinolinate phosphoribosyl transferase; 2) adenosine triphosphate: nicotinatemononucleotide adenylyltransferase; 3) NAD synthetase; 4) NAD glycohydrolase; 5) nicotinamide deamidase; 6) nicotinate phosphoribosyl transferase.*

Preliminary results obtained by Griffin (cited by Kaplan) (7) indicate some dissociation of the NADase-inhibitor complex of *Bacillus subtilis* in the presence of 'high concentrations of NAD.' Dissociation of the complex leads to NAD hydrolysis. The exact concentration of NAD used was not stated.

In this present project the NADase-inhibitor complex from *Pseudomonas putida* KB1 has been partially purified and the effect of NAD and NADP on the association of enzyme with inhibitor studied.

Materials and Methods

Growth of the Organism

Pseudomonas putida KB1 (10, 17) was grown on succinate (0.5%, w/v) in minimal media containing (g/l): $(NH_4)_2SO_4$, 0.5; KHO_2, 10.0; nitrilotriacetic acid, 1.0; and trace element solution (1) (1.0 ml/l). The pH of the medium was adjusted to 7.0 with NaOH (10%, w/v).

Culture medium (10 l) was inoculated from two overnight starter cultures (300 ml) and grown at 30°C until the end of the exponential phase. The cells were harvested in a Sorvall continuous centrifuge (12,000 *g*). Yields of approximately 2.5 g cells, wet weight/l were routinely obtained.

Preparation of Cell-free Extracts

Frozen stored cells were thawed, suspended at 4°C in 0.1 M phosphate buffer, pH 7.0 (prepared as a KH_2PO_4–KOH mixture) and

broken in 10 ml portions for 5 min, by exposure to the full output of an M.S.E. 100 W ultrasonic generator. The suspension was kept cool by surrounding it with an ice-salt freezing mixture. Cell debris was removed by centrifugation at 0°C (38,000 *g*; 2.0 hours).

Assays

1) Enzyme activity: Enzyme activity was assayed by the cyanide addition method described by Colowick, Kaplan & Ciotti (2). One unit of enzyme activity is defined as the amount of enzyme which will hydrolyze 1 u mole NAD/min.
2) Enzyme as enzyme-inhibitor complex: Enzyme present as enzyme-inhibitor complex was assayed as above after a standard heat treatment (10 min, 100°C) followed by immediate cooling in a crushed ice-salt mixture.
3) Inhibitor activity: Free inhibitor was assayed by measuring residual enzyme activity after 0.1 ml of test solution had been incubated for 30 min at 25°C with 0.1 ml of a standard enzyme solution (0.05 units).

   One unit of inhibitor activity is defined as that amount of inhibitor which will cause 50% inhibition of 1 unit of enzyme after a 30 min preincubation.
4) Protein determination: Protein was determined by the biuret reaction (6), using bovine serum albumin, fraction V (Sigma, London Ltd.) as protein standard.

Partial Purification of Enzyme-inhibitor Complex

All operations were carried out at 4°C.

1) Fractionation with ammonium sulphate: Crude cell extract (35 mg protein/ml) prepared as described above was fractionated into eight fractions (%, w/v); 0-25, 25-35, 35-45, 45-55, 55-65, 65-75, 75-85 and greater than 85, by the addition of solid ammonium sulphate. After dialysis against 5 l of 0.1 M phosphate buffer, pH 7.0, overnight, each fraction was assayed for enzyme activity after a standard heat treatment (Table 1). Over 80% of the enzyme-inhibitor complex was recoverable between 25-45% saturation. For routine preparations the extracts were fractionated into three portions (%, w/v); 0-20, 20-50 and greater than 50 and the 20-50% fraction was retained.
2) Chromatography on DEAE-cellulose: Preswollen DEAE-cellulose (Whatman DE-52) was equilibrated for 30 min in 0.1 M potassium phosphate buffer, pH 7.0 and packed into a column (35 x 3 cm). After application of the protein sample, the initial buffer solution was passed through the column for one hour before applying a linear gradient (400 ml, total volume) of KCl (0-0.5 M) in 0.1 M phosphate buffer, pH 7.0. Fractions (5.0 ml) were collected with an L.K.B. fraction collector (Ultrorac, type 700) and assayed for enzyme-inhibitor complex and unbound inhibitor. Those fractions containing enzyme-inhibitor complex free of unbound inhibitor were collected and the complex pre-

cipitated with ammonium sulphate (20-50%, w/v). The purified enzyme-inhibitor complex (approximately 25 units purified/column) was redissolved in 0.1 M phosphate buffer, pH 7.0 and dialyzed overnight against the same buffer (5 l).

TABLE 1. *Fractionation of crude cell-extract with ammonium sulphate.*

Fraction (%) saturation	Activity (units)	Activity recovered (%)
0-25	4.6	7.3
25-35	22.0	35.6
35-45	29.5	47.7
45-55	5.0	8.1
55-65	0.8	1.3
65-75	0	0
75-85	0	0
85	0	0

Chemicals

NAD and NADP were obtained from Sigma (London) Ltd. The NAD was Grade III purity.

Results

Purified enzyme-inhibitor complex from *Pseudomonas putida*, prepared as described in Materials and Methods, was incubated with NAD and separately with NADP (1-10 mM). The amount of enzyme bound to inhibitor present in the samples was determined after a standard heat treatment (see Materials and Methods). Enzyme activity, which increased with increasing NAD or NADP concentration, was detected within the range of concentrations tested (Fig. 2). NAD at the lowest concentration (1 mM), was almost completely hydrolyzed during the time of incubation. The enzyme activity in this sample was therefore separately determined from a time course experiment.

As a control experiment enzyme separated from inhibitor by heat treatment was incubated with NAD over a similar range of concentrations. Enzyme was added at the level detected on incubating enzyme-inhibitor complex in the presence of 1 mM NAD. There was an approximately two-fold increase in enzyme activity in the control samples as the substrate concentration was increased from 1.0 to 10.0 mM. This is surprising at these concentrations of NAD, since the Km of the enzyme is 0.85 mM (17) and the enzyme in purified extracts displays classical Michaelis-Menten kinetics. However, we have previously shown that heat treatment of enzyme-inhibitor complex does not lead to complete removal of inhibitor from the enzyme (17). Fragments of inhibitor appear to remain associated with enzyme until after chromatography on DEAE-cellulose. The increase in enzyme activity in the control samples is therefore probably due to further activation of the enzyme in the presence of substrate.

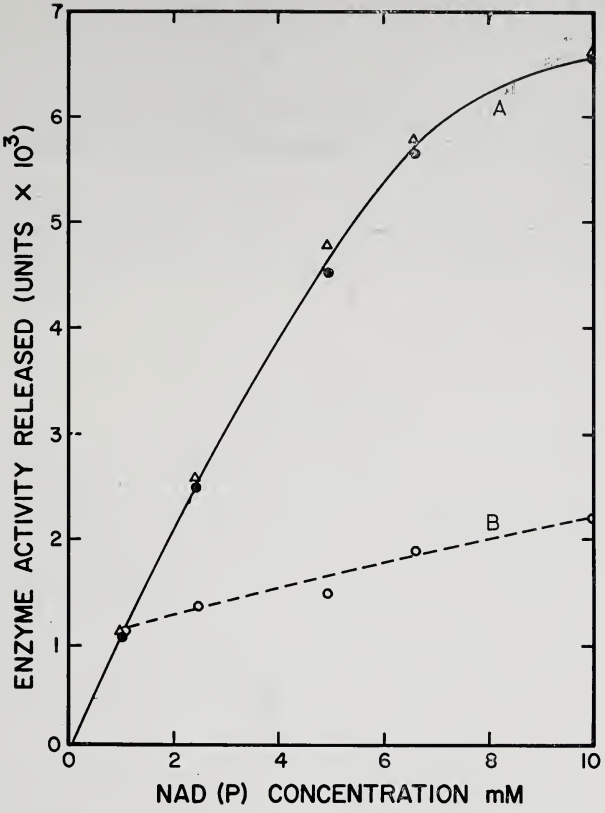

FIGURE 2. *Enzyme activity associated with the NADase-inhibitor complex as a function of NAD concentration. Incubation mixtures contained in a final volume of 0.2 ml, either enzyme-inhibitor complex (A) (0.162 units enzyme) or free enzyme (B) (0.0011 units); NAD (O), (0.2 μmole-2.0 μmole) or NADP (Δ) (0.2 μmole-2.0 μmole) and phosphate (10 μmole). The temperature was 25°C. Enzyme activity was assayed by the cyanide method (2) after 3.0 hours incubation.*

Release of enzyme activity from enzyme-inhibitor complex appears to be rapid since incubation of enzyme-inhibitor complex with NAD (2.5 mM) leads to a linear decrease in substrate concentration over a period of 3.0 hours (Fig. 3). Attainment of equilibrium over a long time period would be excepted to lead to departure from linearity.

Discussion

Addition of NAD or NADP to purified NADase-inhibitor complex leads to the release of some enzyme activity. At an NAD concentration of 10 mM this release amounts to 4% of the total amount of enzyme present in solution and bound to inhibitor. Previous work has also shown that the binding of inhibitor to free enzyme is reversibly inhibited by NAD (17). Removal of NAD by dialysis leads to combination of the inhibitor with enzyme. These observations tend to suggest control

mechanisms for maintaining a constant level of NAD in the cell. If enzyme and inhibitor are physically separated within the cell, high concentrations of NAD will reduce the possibility of enzyme-inhibitor interaction and lead to a lowering in the level of NAD. This present study indicates that preformed enzyme-inhibitor complex can be partially activated by high NAD concentrations, again leading to lowered pyridine nucleotide levels.

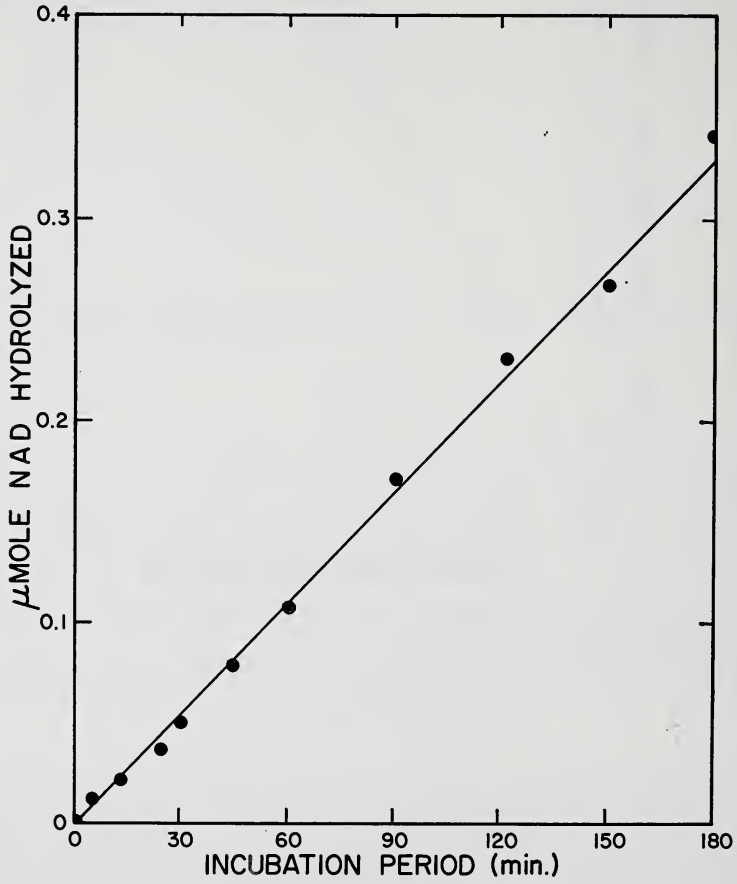

FIGURE 3. *Time course of enzyme-inhibitor complex activation. Incubation mixtures contained in a final volume of 0.2 ml; enzyme-inhibitor complex, NAD (0.5 μmole) and phosphate buffer pH 7.0 (10 μmole). The solutions were incubated for the times indicated in the figure at 25° C, and the enzyme activity assayed by the cyanide addition method (2).*

For an enzyme to function under physiological conditions the Km should be of the same order as the concentration of its substrate *in vivo*. Calculation from values reported by London & Knight (11) and Luria (14) indicates that the intracellular NAD concentration in *Pseudomonas putida* KB1 is approximately 0.7 mM. This is of the same order as the

value (1.3 mM) reported for certain nicotinate auxotrophs of *Escherichia coli* (12). These values are close to the Km of the NADase (0.85 mM) (17) in *P. putida*.

Pyridine nucleotide turnover in *E. coli* is thought to involve at least two cycles, the cycle ascribed to Gholson (4) (Fig. 1) and a second pathway which involves nicotinamide mononucleotide as an intermediate, possibly formed by NAD dependent DNA-ligase (15). Turnover studies have not been undertaken with *P. putida* although the presence of an NADase-inhibitor system in the organism which is partly influenced by NAD levels suggests that at least the first pathway may be present in this organism.

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