THE PYRIDINE NUCLEOTIDE CYCLE: PRESENCE OF A NICOTINAMIDE MONONUCLEOTIDE-SPECIFIC AMIDOHYDROLASE IN PROPIONIBACTERIUM SHERMANII

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SUMMARY

A specific nicotinamide mononucleotide amidohydrolase which catalyzes the stoichiometric conversion of NMN to nicotinate mononucleotide and ammonia has been partially purified from an extract of Propionibacterium shermanii. The reaction has optimum activity at pH 5.6, a K_m of 70 μM, and an experimental activation energy of 14.5 Kcal/mole. The enzyme appears to be highly specific for NMN. Neither free nicotinamide nor NAD, NADH, NADP, NADPH compete with NMN. Numerous substances such as isonicotinic acid hydrazide and quinolinic acid are also without effect. It can be stored at -15° in 12% glycerol, but is somewhat unstable in the absence of this solvent. The enzyme is composed of a heat-stable and a heat-sensitive subunit. This enzyme considerably simplifies the pyridine nucleotide cycle, and may, besides this salvage function for NAD, play a role in B_{12} biosynthesis and in the bacterial DNA ligase reaction.

INTRODUCTION

In a recent communication (1) the role of a newly discovered specific and membrane-associated NMN glycohydrolase in the pyridine nucleotide cycle (2) of Escherichia coli was postulated. In this pathway three enzymatic steps are required to transform NMN to nicotinate mononucleotide (N_aMN), namely the above glycohydrolase to yield free nicotinamide, followed by deamidation by nicotinamide amidohydrolase to yield nicotinate, which in turn is converted by nicotinate phosphoribosyltransferase to N_aMN in a reaction requiring the participation of PRPP. We wish to report here the characterization of a highly specific soluble NMN amidohydrolase which carries out the transformation of NMN to N_aMN in one step, and whose activity, in addition, does not require the participation of a high energy compound. This enzyme has been mentioned in a number of publications from this laboratory (3,4) and has, moreover, been used for a simple enzymatic preparation of N_aMN from NMN (5).

MATERIALS AND METHODS

NMN-carbonyl-^{14}C, which is not available commercially, was purified via the

corresponding ^{14}C-labeled NAD obtained from an extract of intact P. shermanii (ATCC 9614) which had been incubated with nicotinic acid-carboxyl-^{14}C (6).

For assay of the reaction a mixture of 5 μmoles of succinic acid-NaOH buffer, pH 5.6, was incubated at 30° with 10-15 mμmoles of NMN-carbonyl-^{14}C, 3×10^5 cpm/μmole, and enzyme in a total volume of 25 μl. Test tubes, 6 x 50 mm capped with parafilm, were convenient. After suitable intervals, usually 15-30 min, the reaction was interrupted by the addition of 5 μl of 6 N formic acid. The N_aMN formed was separated from unchanged NMN by means of ionophoresis on Whatman No. 1 paper in 0.5 or 1.0 N acetic acid as described previously (5). An aliquot, usually 25 μl, was streaked half-way between the electrodes in the apparatus described before (7,8). After a potential of 26 volts/cm had been applied for about 2 hr, the N_aMN had migrated about 40 mm to the anode, and the NMN about 10 mm to the cathode. The radioactivity corresponding to these two substances was determined in the dried strips by the use of a Nuclear Chicago Actigraph III strip counter provided with a Model 8735 digital integrator (see ref. 8).

The growth, harvesting and disruption of P. shermanii with glass beads, followed by purification with protamine sulfate and ammonium sulfate were described previously (5). After the ammonium sulfate step the preparation was thoroughly dialyzed against 0.02 M Tris-HCl, pH 8.6, in 0.05 M NaCl and 12% glycerol, and applied to a column of DEAE-Sephadex A-50 which had been equilibrated against the same buffer. For bacterial extract from 200 g packed cells a refrigerated column of dimensions 2.5 x 15 cm was convenient. A wash with about 200 ml of the above buffer removes considerable protein but no enzyme. The same buffer, with the NaCl concentration increased to 0.09 M, was used to elute the enzyme. A shallow peak with a gradually tailing shoulder, as monitored by absorbance at 254 nm, was obtained. Enzyme activity began to appear just beyond the maximum of the peak. About 240 ml of solution were eluted beyond this point, and pooled. Ultrafiltration through an Amicon PM-10 membrane under nitrogen pressure with stirring was used for concentration to a volume of about 5 ml and a protein concentration of about 6 mg/ml.

RESULTS

Stability of the enzyme.--In the presence of 12% glycerol the enzyme at a protein concentration of about 0.5 mg/ml could be stored at -15° for at least a month without significant loss of activity. It could be stored at 4° provided that the glycerol concentration was increased to at least 24%.

Reaction products.--Ionophoretic, chromatographic and spectrophotometric properties of the product isolated by means of a Dowex 1 column (5) showed it to be identical with an authentic chemically prepared sample of N_aMN (3,9). This was confirmed by acid hydrolysis

(10,11) to free nicotinic acid (5). Strict stoichiometry was observed between N_aMN and ammonia formed. The ammonia was assayed by a micro-modification of a method (12), using absorption on Dowex 50, followed by elution with Nessler's reagent.

pH optimum.--Maximum activity was observed at a pH value of 5.6. Activity dropped more rapidly on the acid than on the alkaline side. Only 21% of the maximum activity was obtained at pH 3.8, while 48% and 25% respectively of the maximum activity were retained at pH values 7.4 and 10.

Kinetic properties.--The reaction rate varied linearly with time up to 50% conversion of product and deviated only little from linearity up to about 90% conversion. The rate was proportional to enzyme concentration under the standard assay conditions. The substrate saturation curve was hyperbolic, with a half-maximal reaction rate obtained at a substrate concentration of $7.4 \pm 1.3 \times 10^{-5}$ M.

Effect of temperature on reaction rate.--A linear plot of reaction rate against the reciprocal of the absolute temperature was obtained between 0° and 36°. An experimental activation energy of 14.5 Kcal/mole was calculated.

Specificity.--The P. shermanii extracts through the ammonium sulfate purification step show nicotinamide deamidating activity, but this is removed on the anion exchanger. To date no substrate for the reaction besides NMN has been found with the purified enzyme. By direct assay neither free nicotinamide nor NAD are attacked, while the rate of the reaction with NMN is not affected by these two substances at a final concentration of 4 mM by NADP, NADPH, NADH, α-NAD, α-NADH, or by nicotinamide plus ribose-5-phosphate. In addition, none of the following substances was inhibitory at 6.7 mM final concentration: AMP, GMP, IMP, UMP, CMP (all 5'), 3-cyanopyridine, nicotinic acid, nicotinic acid methyl ester, isonicotinic acid, isonicotinamide, isonicotinic acid hydrazide, iproniazid phosphate, N,N-diethylnicotinamide, quinolinic acid. Neither EDTA at 13.4 mM nor 5'-deoxyadenosylcobalamin or cyanocobalamin between 0.05 and 5 mM had any effect on the reaction rate. It is of interest that the purified nicotinamide amidohydrolase of Torula cremoris does not decompose NMN (13).

DISCUSSION

The present enzyme appears to be quite widely distributed. It was first demonstrated in extracts of Clostridium sticklandii (3). It also occurs in Azotobacter vinelandii (14) and in E. coli (15). The deamidation of NMN rather than of nicotinamide enables one to simplify the pyridine nucleotide cycle from the one, with dashed lines, suggested before (1,2, 16) to that with the solid lines (Fig. 1). Clearly this shortened cycle is far more economical

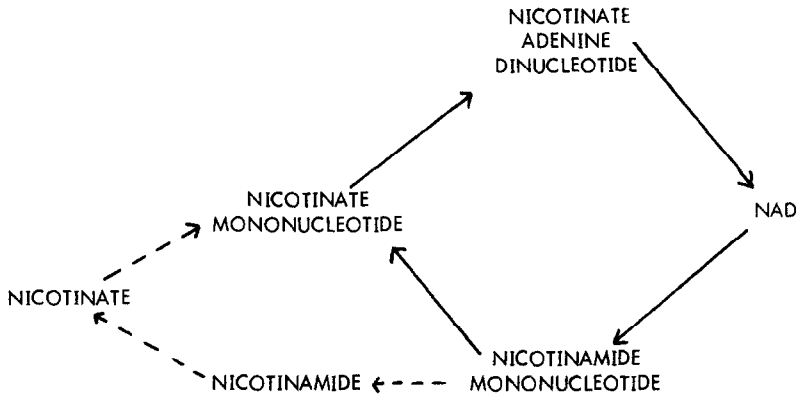

Fig. 1. Pyridine nucleotide cycles.

as to the number of enzymes and the energy supply required. Moreover, it constitutes a nucleotide cycle proper, since free nicotinate or nicotinamide do not participate.

It might be argued that the larger cycle is possibly more subject to control. It is, hence, noteworthy that the P. shermanii NMN amidohydrolase reveals some very interesting properties when further purification is instituted on a phosphono-cellulose column (unpublished observations). Under these conditions two protein fractions are eluted, each of which is only slightly active by itself. The first fraction is stable to heat, while the second is not. When aliquots of the two fractions are mixed, the resulting activity is at least four-fold higher than the sum of the two separate activities. The optimum conditions for reactivation, the implications for mechanism and control of these observations, and the possible participation of the enzyme,not only in the pyridine nucleotide cycle but also in the bacterial DNA ligase reaction and as a source of N_aMN in B_{12} (see refs. 3,4) are under investigation.

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