THE PYRIDINE NUCLEOTIDE CYCLE: PRESENCE OF A NICOTINAMIDE MONONUCLEOTIDE-SPECIFIC GLYCOHYDROLASE IN ESCHERICHIA COLI[1]

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SUMMARY

Demonstration that a functional pyridine nucleotide cycle and NAD turnover occur in Escherichia coli suggested the need to identify the enzyme(s) responsible for nicotinamide formation from NAD. An NAD glycohydrolase has been assumed to be responsible for nicotinamide formation, despite the inability to demonstrate its presence in E. coli. A nicotinamide mononucleotide-specific glycohydrolase however, has now been demonstrated. The membrane localization, substrate specificity and other characteristics of the enzyme are described.

INTRODUCTION

Our recent demonstration of nicotinamide adenine dinucleotide (NAD) turnover and the presence of a functional pyridine nucleotide cycle in Escherichia coli (1) made it evident that intracellular nicotinamide, a turnover product of NAD, as well as exogenous NAD and nicotinamide (2) are utilized for the resynthesis of NAD.

The presence of NAD glycohydrolase (NADase) has been demonstrated in cell-free preparations from Mycobacterium butyricum (3), M. tuberculosis (4,5) and Bacillus subtilis (6) subsequent to heat, acid or analog activation of the enzyme. Presumably, the enzyme would account for the formation of nicotinamide from NAD, if in fact NAD turnover occurs as a normal cell function in all these species as is true in E. coli (1). In contrast, the presence of NADase has not been detected in preparations from E. coli, despite extensive efforts in this laboratory and elsewhere (7) to achieve activation and detection of the enzyme.

The significant rate at which NAD turnover occurs in E. coli during

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active growth (1) and the lack of information about the enzyme(s) responsible, suggested the need to re-examine the basis for nicotinamide formation from NAD. This report is concerned with the detection of a membrane localized nicotinamide mononucleotide-specific glycohydrolase (NMNase) which hydrolyzes NMN to yield nicotinamide and ribose-5-phosphate.

EXPERIMENTAL METHODS

Cell Preparations: E. coli, K-12 cells were grown at 37° in a New Brunswick fermentor with vigorous aeration, as previously described (8). Dried cells were prepared from freshly harvested cells by 1) treating with cold acetone, 2) drying over dessicant in vacuo or 3) by lyophilization, as described by Gunsalus (9).

Enzyme Preparations and Assays: Cell-free enzyme preparations were obtained either by disrupting cells with sonic oscillation as previously described (10), or by extracting with buffer acetone-dried, dessicant-dried or lyophilized cells as described in the text. NADase activity was determined spectrophotometrically by following the rate of NAD hydrolysis with a modification of the cyanide-addition method and the glucose dehydrogenase method described by Colowick et al. (11). NMNase was determined with the cyanide method with the exception that NMN was substituted for NAD as the substrate.

RESULTS AND DISCUSSION

Enzyme extracts prepared by disrupting E. coli cells with sonic oscillation were tested for the presence of NADase, with consistently negative results. Since previous reports (3,5) described the presence of heat or acid activated NAD hydrolyzing enzymes in other bacterial systems, a series of experiments utilizing similar methods of activation were conducted. Aliquots of cell extracts were heated at temperatures from 60° to 100° for periods ranging from 30 seconds to 20 minutes, in potassium phosphate, Tris HCl or Bicine buffers at pH values from 6.0 through 8.5 and assayed for NADase activity. In a second series of experiments, extracts were treated for intervals of 30 seconds

to 10 minutes at pH values of 1.0 to 4.0 and were assayed for NADase activity. No activity was detected in the heat or acid-treated enzyme preparations.

In yet another series of experiments, acetone-dried, dessicant-dried and lyophilized cells were extracted with phosphate, Tris HCl and Bicine buffers at pH values from 6.0 through 9.0 for periods ranging from 30 minutes through 24 hours, and at 4^{o}, 23^{o} and 37^{o}. When assayed for NADase activity prior or subsequent to heat or acid treatment (as described above), no detectable NADase activity was demonstrable in these preparations. These results, although not conclusive, indicated that a readily detectable or uninhibited form of NADase does not exist in E. coli. If present in an inhibited form, several previously successful methods of activation are not appropriate to manifest activity of the enzyme.

The only enzyme proved to be involved in the degradation of NAD in E. coli is the NAD-specific DNA ligase (12,13). In the absence of a detectable NADase our attention turned to an alternative mode for the formation of intracellular nicotinamide from NAD, i.e. the detection of an enzyme responsible for the hydrolysis of nicotinamide mononucleotide; the product of DNA ligase activity. In contrast to the negative results of the previous experiments, enzyme activity specific for the hydrolysis of NMN (NMNase) was readily detected in cell-free extracts prepared by sonic oscillation or extraction of acetone-dried, dessicant-dried and lyophilized cells. Identification of the reaction products of NMN degradation by a partially-purified preparation of the enzyme (14) was established with paper chromatography utilizing two solvent systems (15,16) and the stoichiometry of the nicotinamide and ribose-5-phosphate formed, by chemical methods (17,18).

In the course of attempted purification it became evident that the enzyme might well be associated with cell membrane components. In order to determine the cellular distribution of NMNase, stable spheroplasts were prepared (19), disrupted by osmotic lysis and cytoplasmic components and spheroplast membranes recovered (20). The results presented in Table I show

Table I

Cellular Distribution of
Nicotinamide Mononucleotide Glycohydrolase in E. coli

	Specific[2] Activity	Total Activity	Per cent of Activity
Spheroplast			
a. Membranes[1] (washed 5 to 8 times)	0.33	34.3	86.7
b. Cytoplasm	0.07	4.6	13.3
c. Membrane washes	--	none	--

[1] Membranes were prepared and washed as described by Kaback (20), with the exception that 20mM Bicine buffer, pH 8.0 containing 1mM $MgCl_2$ was used throughout.

[2] Enzyme activity was linear with protein concentration from 0.5 through 5.0 mg. Specific activity is expressed as micromoles of NMN hydrolyzed per hour per milligram of protein. Protein was determined according to Lowry et al. (21).

that approximately 87% of the total NMNase activity is associated with the membranes recovered and 13% with the cytoplasm of the spheroplasts. Also, when the membranes were washed in Bicine buffer even as many as eight times, no detectable release of the enzyme and little change in the specific activity of the membrane preparation was observed. Evaluation of the substrate specificity of the membrane-bound enzyme in terms of other nicotinamide containing nucleotides showed that it failed to hydrolyze NAD, NADP, NADH or NADPH, and indeed was specific for NMN.

To further clarify the apparent membrane localization of NMNase, E. coli cells harvested during exponential growth were subjected to the osmotic shock technique devised by Nossal and Heppel (22) for the selective release of pericytoplasmic enzymes. Evaluation of the shock medium showed that less than one per cent of the cellular NMNase was released by utilizing this technique. These findings are consistent with the view that NMNase is not located in the

periplasmic region of the cell nor loosely attached to the outer membrane surface, but firmly associated with the membrane.

Demonstration of an NMN-specific glycohydrolase in E. coli suggests its direct participation in the pyridine nucleotide cycle (1), as shown in Figure 1. NAD is degraded in E. coli by only one known enzyme, DNA ligase. The NMN formed by the ligase is then hydrolyzed by NMNase to yield nicotinamide, a product typical of NAD turnover. This sequence of known reactions may describe the only way in which NAD turnover occurs and turnover products are recovered by the pyridine nucleotide cycle in E. coli (1).

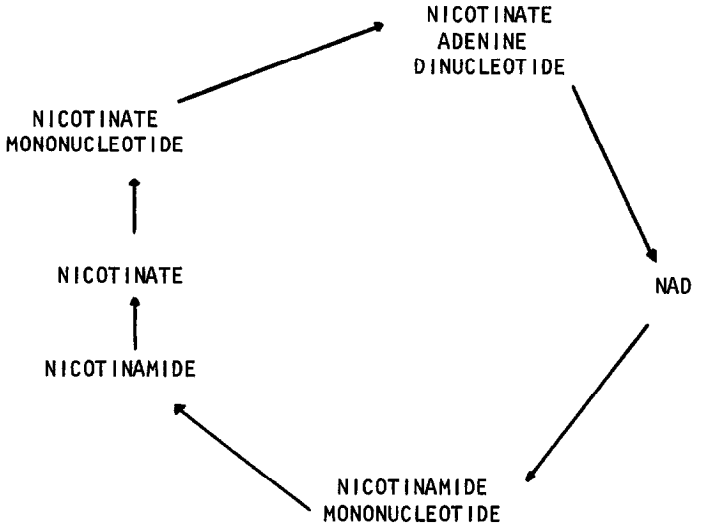

Figure 1. Pyridine nucleotide cycle in E. coli

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