BBA 63263

ATP-independent nicotinic acid mononucleotide pyrophosphorylase of *Astasia longa*

Nicotinic acid mononucleotide pyrophosphorylase (nicotinamidenucleotide: pyrophosphate phosphoribosyltransferase, EC 2.4.2.11) is a Mg^{2+}-dependent enzyme which catalyzes the conversion of nicotinic acid to nicotinic acid mononucleotide (deamido-NMN). Studies with this enzyme obtained from a variety of cell types have indicated a requirement for ATP as well as for 5-phospho-α-D-ribosyl pyrophosphate[1-3]. A nicotinamide monucleotide pyrophosphorylase isolated from rat liver has also been shown to require ATP[4]. That ATP may not always be involved in the reaction is indicated by the observation that in the formation of deamido-NMN from nicotinic acid and 5-phospho-α-D-ribosyl pyrophosphate in acetone powder extracts of human erythrocytes there was no requirement for ATP[5]. Results obtained with an extensively purified nicotinic acid mononucleotide pyrophosphorylase from beef liver led IMSANDE AND HANDLER[6] to suggest that an ATP–Mg^{2+} complex might stabilize the enzyme at the active site. More recently, IMSANDE has suggested that ATP activates the nicotinic acid mononucleotide pyrophosphorylase of *Escherichia coli*, perhaps by lowering the energy of activation of the enzyme–substrate complex[1]. Although IMSANDE could not demonstrate a stoichiometric relation between ATP consumption and deamido-NMN synthesis in *E. coli* preparations, HONJO *et al.*[3] recently demonstrated a stoiometric utilization of ATP in the synthesis of deamido-NMN by a partially purified enzyme obtained from yeast. Thus in all cases so far studied except the human erythrocyte, this enzyme appears to require ATP. In this report we present data demonstrating that the nicotinic acid mononucleotide pyrophosphorylase of Astasia does not require ATP.

Astasia longa (Jahn strain) were grown, harvested, and sonicated by routine procedures[7]. Although the experiments will not be reported in detail below, we have also used cultures of a streptomycin-bleached strain of *Euglena gracilis* var. bacillaris, and obtained results similar to those reported here with Astasia. [7-^{14}C]Nicotinic acid was purchased from Nuclear Chicago and was used at a specific activity of $8.4 \cdot 10^6$ counts/min per μmole. 5-phospho-α-D-ribosyl pyrophosphate and ATP were purchased from Pabst; hexokinase (type C-120, from yeast) from Sigma; ribose 5-phosphate from Boehringer and Soehne.

The reaction mixture used for assay of nicotinic acid mononucleotide pyrophosphorylase activity is described in the tables. At known times 0.1-ml aliquots were withdrawn, deproteinized by boiling for 1 min, and analyzed by ascending paper chromatography using 1 M ammonium acetate (pH 5.0)–95% ethanol (3:7, v/v) as the solvent system[7]. The percentage of nicotinic acid converted to deamido-NMN and nicotinic acid–adenine dinucleotide (deamido-NAD) was calculated as the sum of the counts associated with these products divided by the total counts found on the paper strips.

The products of the reaction were identified by paper chromatography in three solvent systems[8] and, in addition, the reaction products of large scale incuba-

Abbreviations: deamido-NMN, nicotinic acid mononucleotide; deamido-NAD, nicotinic acid–adenine dinucleotide.

tion mixtures were applied to a Dowex 1 (formate) column and eluted with a concave gradient of formic acid as described previously[9]. When ATP was omitted from the reaction mixture, two radioactive peaks were eluted at positions expected for nicotinic acid and for deamido-NMN. When ATP was added to the reaction mixture, an additional labelled compound was eluted at a position corresponding to deamido-NAD. These peaks were separately pooled and freed of formic acid by lyophilization. Their spectra after equilibration with 2 M KCN agreed with the known spectral properties of these compounds[10]. It was further shown that the deamido-NMN thus isolated could serve as a substrate for nicotinic acid–adenine dinucleotide pyrophosphorylase and that the deamido-NAD isolated could serve as a substrate for NAD synthetase upon the addition of appropriate cofactors and an aliquot of a freshly prepared cell-free extract of Astasia.

The presence of nicotinic acid mononucleotide pyrophosphorylase in cell-free extracts of Astasia is demonstrated in Table I. The requirement for 5-phospho-α-

TABLE I

NICOTINIC ACID MONONUCLEOTIDE PYROPHOSPHORYLASE IN CELL-FREE EXTRACTS OF ASTASIA

The complete reaction mixture included, in a total volume of 1.0 ml: 3 μmoles of 5-phospho-α-D-ribosyl pyrophosphate, 5.4 μmoles $MgCl_2$, 12.5 μmoles Tris–HCl (pH 7.4), 0.15 ml of cell-free homogenate (corresponding to $0.56 \cdot 10^6$ cells and containing 150 μg protein), and 0.5 μmole [7-^{14}C]nicotinic acid. Ribose 5-phosphate and ATP were added as indicated. Incubation was carried out at 30°. The rates of nicotinic acid conversion to the ribonucleotide were computed from data obtained during 60 min of incubation. During this period, the amount of deamido-NMN and deamido-NAD formed increased linearly with time.

Tube No.	*Omission*	*Addition (mM)*		*Activity (mμmoles/h)*		
				Deamido-NMN	*Deamido-NAD*	*Total*
1	None	none		42.9	none	42.9
2	5-Phospho-α-D-ribosyl pyrophosphate	none		< 1	none	< 1
3	5-Phospho-α-D-ribosyl pyrophosphate	Rib-5-*P*,	5.0	< 1	none	< 1
4	5-Phospho-α-D-ribosyl	Rib-5-*P*, ATP,	5.0 + 2.5	< 1	none	< 1
5	5-Phospho-α-D-ribosyl pyrophosphate	ATP,	2.5	< 1	none	< 1
6	None	ATP,	0.025	42.0	2.0	44.0
7	None	ATP,	0.25	31.0	12.2	43.2

D-ribosyl pyrophosphate could not be circumvented by the addition of Rib-5-*P* and ATP, and the reaction rate was not affected by the addition of ATP (Tables I and II). In the absence of ATP the only radioactive product detected was deamido-NMN. The formation of deamido-NAD, however, could be detected within 10 min if $2.5 \cdot 10^{-5}$ M ATP was added to the reaction mixture. Increasing the ATP concentration to 5 mM did not affect the rate of nicotinic acid conversion but increased the rate of conversion of deamido-NMN to deamido-NAD by nicotinic acid–adenine dinucleotide pyrophosphorylase.

The activity of the nicotinic acid mononucleotide pyrophosphorylase of the crude cell-free extracts of Astasia is much higher than the activities for crude en-

zyme preparations from a variety of sources[1], and is comparable to that of a purified preparation obtained from baker's yeast by HONJO *et al.*[3].

The observation that added ATP did not alter the rate of nicotinic acid conversion would be misleading if ATP was present in significant quantities in the cell-free extract, or if ATP was being generated enzymatically during the incubation, or if ATP was present as a contaminant of the 5-phospho-α-D-ribosyl pyrophosphate. The amount of ATP likely to be present in the cell-free extract is of the order of 0.1 mμmole /10^6 cells or about 0.1 mμmole /300 μg protein and is therefore insignificant[9]. To test for the presence and the possible generation of ATP during the reaction, an ATP-trapping system consisting of hexokinase and glucose was used. As shown in Table II, the trapping reagents did not affect the rate of deamido-NMN production

TABLE II

NICOTINIC ACID MONONUCLEOTIDE PYROPHOSPHORYLASE ACTIVITY IN THE PRESENCE OF AN ATP-TRAPPING SYSTEM

The control reaction mixture contained in a total volume of 1.0 ml: 3 μmoles of 5-phospho-α-D-ribosyl pyrophosphate, 5.4 μmoles $MgCl_2$, 12.5 μmoles Tris–HCl (pH 7.4), and 0.15 ml of cell-free homogenate containing 150 μg protein. In addition, Tubes 2, 3, and 4 contained 20 μmoles glucose and 0.03 ml of hexokinase (with activity corresponding to conversion of 3.2 μmoles ATP per min to ADP). ATP was added to Tubes 4 and 5 in the amounts indicated. In Tubes 3 and 4, the mixtures were preincubated for 23 min before the addition of 0.5 μmole labelled nicotinic acid, while in Tubes 1, 2, and 5 labelled nicotinic acid was added at zero time. Activity measurements obtained in Tubes 3 and 4 were corrected by a small factor to take into account the preincubation period.

Tube No.	*Addition*	*Treatment*	*Deamido-NMN (mμmoles)*			*Deamido-NAD (mμmoles)*		
		Incubation (min)	*10*	*30*	*90*	*10*	*30*	*90*
1	Control	none	12.0	32.0	72.0	none		
2	Hexokinase + glucose	none	11.0	31.5	67.0	none		
3	Hexokinase + glucose	preincubated	11.0	35.0	80.0	none		
4	Hexokinase + glucose + 0.13 mM ATP	preinbubated	10.5	31.0	72.0	none		
5	ATP, $2.5 \cdot 10^{-5}$ M	none	11.2	31.2	68.5	0.34	1.0	3.0

even if the trapping reagents were preincubated with the reaction mixture prior to the addition of labelled nicotinic acid. That the hexokinase was active is demonstrated by the observation that although $2.5 \cdot 10^{-5}$ M ATP produced detectable amounts of deamido-NAD within 10 min in the absence of hexokinase, $13.0 \cdot 10^{-5}$ M ATP did not lead to the formation of deamido-NAD in the presence of the hexokinase. The sensitivity of the assay was such that 1 μmμmole of deamido-NAD could have been detected.

It is concluded that, contrary to most other systems examined, the nicotinic acid mononucleotide pyrophosphorylase of Astasia is neither stimulated by nor requires ATP for the enzymatic conversion of nicotinic acid to deamido-NMN.

This work was supported by grant 5-Ro 1-HD-01269 from the National Institutes of Health. Dr. BLUM acknowledges the support of a Research Career Development award (5-K3-GM-2341) from the National Institutes of Health.

Department of Physiology and Pharmacology, VARDA KAHN
Duke University Medical School, J. J. BLUM
Durham, N.C. (U.S.A.)

1 J. IMSANDE, *Méchanismes de Régulation des Activités Cellulaires chez les Microorganismes*, Editions du Centre National de la Recherche Scientifique, Paris, 1965, 417.
2 N. OGASAWARA AND R. R. GHOLSON, *Biochim. Biophys. Acta*, 118 (1966) 422.
3 T. HONJO, S. NAKAMURA, Y. NISHIZUKA AND O. HAYAISHI, *Biochem. Biophys. Res. Commun.*, 25 (1966) 199.
4 L. S. DIETRICH, L. FULLER, I. L. YERO AND L. MARTINEZ, *J. Biol. Chem.*, 241 (1966) 188.
5 J. PREISS AND P. HANDLER, *J. Biol. Chem.*, 233 (1958) 493.
6 J. IMSANDE AND P. HANDLER, *J. Biol. Chem.*, 236 (1961) 525.
7 V. KAHN AND J. J. BLUM, *J. Biol. Chem.*, 240 (1965) 4435.
8 Y. NISHIZUKA AND O. HAYAISHI, *J. Biol. Chem.*, 238 (1963) *PC* 483.
9 V. KAHN AND J. J. BLUM, *Arch. Biochem. Biophys.*, 113 (1966) 315.
10 J. PREISS AND P. HANDLER, *J. Biol. Chem.*, 233 (1958) 488.

Received May 12th, 1967

Biochim. Biophys. Acta, 146 (1967) 305–308