STOICHIOMETRIC UTILIZATION OF ADENOSINE 5'-TRIPHOSPHATE IN NICOTINATE RIBONUCLEOTIDE SYNTHESIS FROM NICOTINATE AND 5-PHOSPHORIBOSYL-1-PYROPHOSPHATE*

Tasuku Honjo, Sigenobu Nakamura+, Yasutomi Nishizuka and Osamu Hayaishi

Department of Medical Chemistry
Kyoto University Faculty of Medicine
Kyoto, Japan

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The enzymic formation of nicotinate ribonucleotide from nicotinate and PP-ribose-P[1/] was described first in human erythrocytes (Preiss and Handler, 1958). The enzyme responsible for this reaction was subsequently purified from beef liver (Imsande and Handler, 1961) and from Bacillus subtilis (Imsande, 1964), and was shown to be markedly stimulated by ATP. Reports from this and other laboratories have proposed that ATP may act as a conformational modifier (Nakamura et al., 1964) or a stabilizer (Imsande and Handler, 1961) of the enzyme. Imsande (1964) has also suggested that ATP is consumed during the enzyme reaction. The exact

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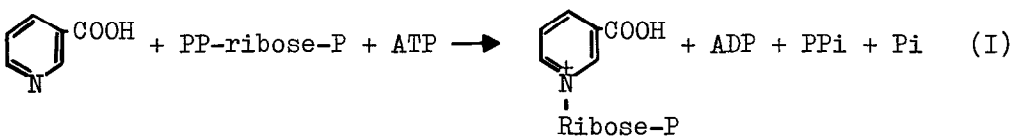
1/ PP-ribose-P; 5-phosphoribosyl-1-pyrophosphate.

role of ATP in this reaction, however, has remained obscure. The major obstacle to the elucidation of the reaction mechanism is the presence of abundant adenosine triphosphatase activity in the enzyme preparations available so far. With the use of the enzyme purified from Baker's yeast, unequivocal evidence is now obtained indicating that one mole of ATP is expended for each mole of nicotinate ribonucleotide synthesized (Reaction I). The present communication reports the stoichiometry of the reaction together with some aspects of the reaction mechanism. Purification and properties of the enzyme are also described briefly.



The enzyme was assayed by measuring the formation of radioactive nicotinate ribonucleotide from nicotinate-7-^{14}C in the presence of ATP and PP-ribose-P. The product was isolated by Dowex 50 column chromatography and the radioactivity was determined. Baker's yeast cells were ground with alumina and the enzyme was extracted with phosphate buffer, pH 7.4. The enzyme was subsequently purified about 150-fold by ammonium sulfate fractionation (45-70%), followed by DEAE-cellulose, CM-Sephadex and DEAE-Sephadex column chromatography. The preparation thus obtained was practically free of adenosine triphosphatase, adenylate kinase, 5'-nucleotidase and other phosphatases.

Table I shows the stoichiometry of the reaction. The amounts of nicotinate ribonucleotide, ADP and Pi produced well coincided with those of nicotinate and ATP utilized. Although PP-ribose-P decomposed even in the absence of one of the substrates because of its instability, the increment of PP-ribose-P breakdown in the

Table I. Stoichiometry of the Reaction

The reaction mixture (0.8 ml) containing 50 mμmoles of nicotinate-7-^{14}C (8,700 cpm/mμmole), 148 mμmoles of PP-ribose-P, 100 mμmoles of ATP, 7.5 μmoles of $MgCl_2$, 35 μmoles of Tris-HCl buffer, pH 7.4, and 0.07 mg of the purified enzyme was incubated at 37°. After 60 minutes, an aliquot (0.4 ml) of the reaction mixture was immediately applied to a Dowex 50 column (H^+ form, 200-400 mesh, diameter, 0.5 cm; length, 2 cm). The column was washed with 5 ml of water to wash out the ribonucleotide produced. Nicotinate was subsequently eluted from the column with 15 ml of 6 N formic acid containing 0.5 M ammonium formate. The radioactivity of the washing and eluate was determined with a Nuclear Chicago gas flow counter. PP-ribose-P was determined enzymatically with orotate-^{14}C according to the method of Kornberg et al. (1955a). In order to estimate the amounts of ATP utilized and ADP produced another set of incubation mixtures containing the same ingredients, except that ATP and nicotinate-7-^{14}C were replaced by ATP-8-^{14}C (10,000 cpm/mμmole) and nicotinate, were incubated under identical conditions. Radioactive ADP and ATP were separated by Dowex 1-formate columns. A third set of incubation mixtures containing ATP-γ-^{32}P and nicotinate were also run to determine radioactive Pi liberated as the phosphomolybdate complex after extraction into isobutanol-benzene mixture (Martin and Doty, 1949). ATP-γ-^{32}P was prepared using photophosphorylation of ADP by spinach chloroplasts according to the method of Jagendorf and Avron (1958).

All numbers are expressed in mμmoles.

	Produced			Utilized		
Omission	Nicotinate ribonucleotide	ADP	Pi	Nicotinate	ATP	PP-ribose-P
None	22.1	20.3	22.4	21.2	21.7	39.8
ATP	1.8	--	--	0	--	22.2
Nicotinate	--	0	1.1	--	0	--
PP-ribose-P	0	1.3	2.0	2.2	0	--

presence of all substrates was almost equivalent to the amount of nicotinate ribonucleotide produced. Essentially no nicotinate ribonucleotide was produced when either ATP or PP-ribose-P was omitted from the reaction mixture. Reproducible results could be obtained using different preparations of the enzyme. PP-ribose-P could not be replaced by ribose 5-phosphate. Nicotinate ribo-

nucleotide produced was isolated from a large scale incubation mixture, and identified by paper chromatography in three different solvent systems and by paper electrophoresis under the conditions specified earlier (Nishizuka and Hayaishi, 1963).

The reaction was specific for ATP. Other ribonucleotides were inert as substrates including ADP, GTP, UTP and CTP. The Km values for nicotinate, ATP and PP-ribose-P were 1.85×10^{-6} M, 1.2×10^{-4} M and 7.7×10^{-6} M, respectively. The maximal activity was observed between pH 8.0 and pH 9.0 with Tris-HCl buffer. Pi stabilized the enzyme against inactivation by heat or dialysis. In addition, Pi markedly stimulated the activity, the maximum activity being observed at 1.5×10^{-2} M Pi. PP-ribose-P was also a potent stabilizer of the enzyme against heat inactivation. The enzyme was specific for nicotinate. Nicotinamide, quinolinate, purines and pyrimidines were totally inert as substrates.

These results indicate that ATP is expended as a substrate giving rise to ADP and Pi[2/]. The PP-ribose-P dependent formation of purine and pyrimidine ribonucleotides from the respective free bases does not require any additional energy from ATP (Kornberg et al., 1955b; Lieberman et al., 1955). The synthesis of imida-

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2/ A preliminary report from this laboratory (Nakamura et al., 1964) described evidence suggesting that nicotinate ribonucleotide pyrophosphorylase purified from beef liver might be identical with an enzyme which hydrolyzes the 5'-phosphate ester of the ribonucleotide, and that ATP might act as a conformational modifier of the pyrophosphorylase. Further purification by DEAE-Sephadex column chromatography, however, has revealed that these two enzymes can be separated. Using the purified preparation of beef liver pyrophosphorylase, also one mole of ATP has been established to be expended for each mole of ribonucleotide produced. The presence of ATP did not change the sedimentation velocity of the enzyme protein. The details of the experimental results together with a reevaluation of the previous report will be described elsewhere. During the preparation of this manuscript, the nonidentity of these two enzyme activities in yeast was also described (Ogasawara et al., 1966).

zole-acetate ribonucleotide, however, has been shown to consume a stoichiometric quantity of ATP in addition to PP-ribose-P (Crowley, 1964). Nicotinamide ribonucleotide pyrophosphorylase recently purified from rat liver (Dietrich et al., 1966) and from Lactobacillus fructosus[3] has also been shown to be markedly stimulated by ATP. This somewhat unexpected consumption of ATP might be explained by considering the bond energy between pyridine-nitrogen and ribose. The free energy of hydrolysis of the bond has been calculated to be about -8,200 cal. per mole (Zatman et al., 1953), whereas those of purine and pyrimidine ribonucleosides are less than -4,800 cal. per mole (Kalckar, 1954). Although the free energy of hydrolysis of pyrophosphate from PP-ribose-P is unknown, it might not be so remote from that of glucose-1-phosphate which is approximately -4,700 cal. per mole (Colowick and Sutherland, 1942). Thus it might be plausible that ATP is expended for the formation of a high energy bond between pyridine-nitrogen and ribose[4].

The absence of ATP utilization when nicotinate or PP-ribose-P was omitted from the reaction mixture (Table I) makes it unlikely that dissociable intermediates are formed between ATP and one of the other substrates. In order to elucidate the reaction mechanism, possible exchange reactions were tested including ATP-ADP-^{14}C, ^{32}Pi-ATP, and nicotinate-^{14}C-nicotinate ribonucleotide exchanges. So far only negative results have been obtained except for the ATP-ADP-^{14}C exchange reaction. Though the incubation of ATP and

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3/ Ohtsu, E., in preparation.

4/ The PP-ribose-P dependent formation of nicotinate ribonucleotide from quinolinate does not require ATP as an additional substrate (Nakamura et al., 1963). In this case pyridine-nitrogen might be activated by the α-carboxyl group of quinolinate, which is decarboxylated concomitantly with the formation of the C-N bond.

ADP-^{14}C with the enzyme resulted in a slight incorporation of radioactivity into ATP, it is still uncertain whether this exchange reaction is due to phosphoryl enzyme formation or to a slight contamination by nucleoside diphosphokinase activity. The reaction was practically irreversible. Neither pyrophosphorolysis nor arsenolysis of the nicotinate ribonucleotide was detected. Nor did arsenate inhibit the reaction. Further studies are now in progress to elucidate the detailed mechanism whereby additional phosphate bond energy is transferred from ATP during the formation of the C-N bond between pyridine-nitrogen and ribose.

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