Bacterial Synthesis of Nucleotides

Part X. Phosphorylation of 5-Amino-4-imidazole Carboxamide Riboside

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5'-Phosphoribosyl 5-amino-4-imidazole carboxamide was prepared by incubating 5-amino-4-imidazole carboxamide riboside and a phosphate compound with the bacteria characterized to phosphorylate at C_{5'} via the phosphoryl transfer reaction. Aromatic phosphate compounds and 5'-nucleotides were able to act as the phosphate donor. This material was isolated chromatographically and its properties were studied. The other bacteria characterized to phosphorylate at C_{3'} (or 2') also phosphorylated a little probably at C_{3'} (or 2') of 5-amino-4-imidazole carboxamide riboside.

5'-Phosphoribosyl-5-amino-4-imidazole carboxamide (AICAR-5'-P) is well known to be an intermediate both in the de novo synthesis of 5'-IMP¹⁾ and in the conversion of 5-amino-4-imidazolecarboxamide (AICA) to IMP.²⁾ One of the latter involves the conversion of AICA to 5-amino-4-imidazolecarboxamide riboside (AICAR) mediated by a nucleoside phosphorylase³⁾ and the concomitant conversion of AICAR to its monophosphate by a kinase enzyme present in both yeast and pigeon liver.⁴⁾ The second mechanism was studied by Flaks et al.²⁾; in their experiments, AICA and 5-phosphoribosyl pyrophosphate were condensed directly by a nucleotide pyrophosphorylase.

More recently Huang prepared AICAR-5'-P by incubating AICAR with actively fermenting yeast.⁵⁾ On the other hand, this was

also prepared by the chemical degradation⁶⁾ of a derivative of IMP or by the chemical synthesis.⁷⁾

In the course of studies on the acceptor specificity of bacterial nucleoside phosphotransferases, as described in the preceding papers,^{8~10)} AICAR was found to be also phosphorylated as well as purine and pyrimidine ribonucleosides. This paper deals with the details of the bacterial preparation of AICAR-5'-P via a phosphoryl transfer reaction.

MATERIALS AND METHODS

Material.

Enzyme Source: Cultivation of bacteria was carried out as described in the preceding paper.¹¹⁾

Biochemicals.

AICAR was found to be accumulated in the cultured medium of a purine-requiring mutant of *B. subtilis*

Abbreviations used in this work include AICAR: 5-Amino-4-imidazolecarboxamide- β -D-ribose, AICAR-P: Phosphoribosyl-5-amino-4-imidazolecarboxamide, *p*-NPP: *p*-Nitrophenylphosphate, and *p*-NP: *p*-Nitrophenol.

1) S. C. Hartman and J. M. Buchanan, *Ann. Rev. Biochem.*, **28**, 365 (1956).

2) J. G. Flaks, M. J. Erwin and J. M. Buchanan, *J. Biol. Chem.*, **228**, 201 (1957).

3) E. D. Korn and J. M. Buchanan, *ibid.*, **217**, 183 (1955).

4) G. R. Greenberg, *ibid.*, **219**, 423 (1956).

5) H. T. Huang, *Biochemistry*, **4**, 58 (1965).

6) E. Shaw, *J. Am. Chem. Soc.*, **83**, 4770 (1961).

7) G. Shaw, D. V. Wilson and C. P. Green, *J. Chem. Soc.*, **2650** (1964).

8) K. Mitsugi, A. Kamimura, E. Nakazawa and S. Okumura, *This Journal*, **28**, 828 (1964).

9) K. Mitsugi, E. Nakazawa and S. Okumura, *ibid.*, **28**, 838 (1964).

10) K. Mitsugi, A. Kamimura, S. Okumura and N. Katsuya, *ibid.*, **28**, 849 (1964).

11) K. Mitsugi, K. Komagata, M. Takahashi, H. Iizuka and H. Katagiri, *ibid.*, **28**, 586 (1964).

and isolated by one of the authors and his colleagues.¹²⁾ Nucleotides and various phosphate compounds were commercial products.

Methods.

The composition of assay mixture and the detection of reaction products on the paper chromatogram were the same as mentioned in the preceding paper.⁸⁾ Detection of AICAR-5'-P was carried out by estimating the absorbancy at 268 m μ and by calculating from the molar extinction coefficient of AICAR, 9,400 at pH 1.0.²⁾ Diazotizable amine was determined by the method of Bratton-Marshall¹³⁾ as follows; one drop of concentrated acetic anhydride was added to 1 ml of sample, and the mixture was shaken and placed for 30 min at room temperature. Four ml of 0.2 N nitric acid was added to the solution, followed by adding 0.5 ml of 0.1% sodium nitrite. After 1 min, 0.5% ammonium sulfamate was added. Three min later, 0.5 ml of 0.1% N-(1-naphthyl)-ethylene-diamine dihydrochloride was added, and, after 30 min, the color was measured with the spectrophotometer at 540 m μ (molar extinction coefficient of the chromophore: 26,400). Other chemical or biochemical methods for the identification of AICAR-5'-P synthesized were achieved as described previously.^{8,10)}

RESULTS

1. Phosphorylation of AICAR

Various kinds of naturally occurring ribo-⁸⁾ and deoxyribo-⁹⁾ nucleosides and their ribosyl derivatives¹⁰⁾ were observed to be phosphorylated by the bacterial nucleoside phosphotransferase.

In the similar reaction system, in which AICAR was employed as an acceptor, the phosphorus-containing material, of which the U.V. absorption spectrum was identical with that of AICAR, was formed as given in Table I. Both U.V.-absorbing and phosphorus-containing materials formed by *Ps. trifolii* and *Ser. marcescens* were oxidized by metaperiodate on the paper chromatogram, while no phosphorus-containing material was detected in the reaction system from which *p*-NPP, a phosphate

TABLE I. PHOSPHORYLATION OF AICAR

Strains	Phosphorylated material	<i>p</i> -NP liberated
	μ moles per ml	
<i>Ps. trifolii</i> (IAM 1555)	12.0	30.6
<i>Ser. marcescens</i> (IFM F420)	7.4	16.4

Reaction mixture containing 80 μ moles of AICAR, 280 μ moles of *p*-NPP, 4 μ moles of CuSO₄, 1.6 mmoles of acetate buffer (pH 4.0) and 8.0 mg of intact cells of bacterium as dry matter was incubated in a final volume of 4 ml at 37°C for 20 hr.

donor, was eliminated. This fact indicates that AICAR was not transformed to its phosphate in the absence of phosphate donor, but was phosphorylated by the nucleoside phosphotransferase activity in the presence of a phosphate donor. The material newly formed by *Ps. trifolii* was separated by the anion exchanger, as shown in Fig. 1, and its U.V. absorption spectrum was similar to that of AICAR (Fig. 2).

FIG. 1. Anion Exchange Chromatogram of A Reaction Mixture, Subjected to *Ps. trifolii*.

2. Isolation and Identification of AICAR-P

In order to isolate and identify the phosphorylated material, a reaction mixture containing, in a volume of 5 ml, 100 μ moles of AICAR, 350 μ moles of *p*-NPP, 5 μ moles of CuSO₄, 2 mmoles of acetate buffer (pH 4.0), and intact cells, 10 mg as dry matter, of *Ps. trifolii* (IAM 1555) was incubated at 37°C for 20 hr, and the reaction was terminated by adding 1 ml of 10% solution of trichloroacetic

12) T. Shiro, A. Yamanoi, S. Konishi, S. Okumura and M. Takahashi, This Journal, 26, 785 (1962).

13) A. C. Bratton and E. K. Marshall, J. Biol. Chem., 128, 537 (1939).

acid, followed by centrifugation to remove cells or proteins. The supernatant fluid containing 60 μ moles of the phosphorylated material was adjusted to pH 9.0 with 4N NH_4OH and was extracted by ethylacetate (4 ml $\times$ 5) in order to remove *p*-NP liberated. The resulting solution was applied on the top of Dowex-1 $\times$ 4-formate column (0.8 $\times$ 12 cm, 200~400 mesh). After water wash (10 volume of column bed), which removed 37 μ moles of unreacted AICAR, 0.1N formic acid was passed through the column at a flow rate of 0.7 ml per min, and fractions of 10 ml were collected. AICAR-P was eluted between 50 and 82 fraction. It was characterized in the eluate (at pH 1.8) by the U. V. absorption spectra and the ratio of optical density at 280 $\text{m}\mu$ /270 $\text{m}\mu$, 0.70, and at 240 $\text{m}\mu$ /270 $\text{m}\mu$, 0.71. These fractions containing AICAR-P were pooled, and pH was brought to 7.0 with 4N NH_4OH . The mixture was evaporated to 10 ml in vacuo and freeze-dried. The freeze-dried solid was dissolved in 5 ml of water, and 0.8 ml of 0.1M barium acetate was added together with 40 ml of abs. ethanol. The precipitate formed was permitted to stand at 0°C overnight and then was filtered. The sodium salt of this material in aqueous solution which was prepared by decomposing with sodium sulfate was used for identification.

FIG. 2. U. V. Absorption Spectra of AICAR-P at Various pH Values and of AICAR.

AICAR-P in 0.1N HCl (—), in H_2O (— —), and in 0.1N NaOH (— · —) or AICAR in 0.1N HCl (— · — · —)

This solution was confirmed to have an identical U. V. absorption spectrum with that of AICAR (Fig. 2) and to show the same R_F value (Fig. 3) and mobility (Fig. 4) as observed in the reaction mixture (Figs. 3 and 4). The contents of diazotizable amine, ribose,

FIG. 3. Paper Chromatogram of AICAR-P.

1) AICAR-P isolated, 2) Reaction Mixture, 3) AICAR. These materials were developed in an ammonium isobutyrate-isobutyric acid system.

FIG. 4. Anodic Migration of AICAR-P Isolated

A) AICAR-P isolated, B) Reaction Mixture minus enzyme solution, C) Complete reaction mixture, D) Authentic AICAR, E) *p*-NPP and *p*-NP.

TABLE II. ANALYSIS OF AICAR-P SYNTHESIZED BY *Ps. trifolii*

Analysis	Contents per mole of AICAR-P* ¹)
	moles
Ribose* ²)	0.95
Organic phosphorus	0.98
Diazotizable amine* ³)	1.01
Metaperiodate consumed* ⁴)	0.99
AICAR-P hydrolyzed	
by 5'-Ntase* ⁵)	0.95
by 3'-Ntase* ⁶)	0.00

*) Amounts of AICAR-P were determined spectrophotometrically assuming its molar extinction coefficient as 12,800 at pH 7.0.

*²) Amounts of ribose were determined by calculating from the quantity of ribose contained in the known concentration of 5'-AMP as reference standard.

*³) Details are described in the text.

*⁴) Residual metaperiodate was determined at 222.5 m μ in an assay mixture containing 50 μ moles of metaperiodate and 18.7 μ moles of sample.

*⁵) Assay mixture described previously¹¹⁾ was incubated at 37°C for 35 min. and the amount of Pi liberated from 2.0 μ moles of sample was determined.

*⁶) 3.0 μ moles of sample was treated with bacterial 3'-nucleotidase at 37°C for 5 hr. Under the same condition, 3'-AMP was hydrolyzed completely, but 5'- and 2'-AMP were not attacked.

organic phosphorus, and the amounts of metaperiodate consumed were analyzed as given in Table II. It is apparent that ribose, phosphorus, and diazotizable amine were contained in the ratio of approximately 1 : 1 : 1 per mole of this material, calculated as AICAR-derivative from the absorbancy at 268 m μ . This material which reacted readily with the same amount of metaperiodate was hydrolyzed by snake venom 5'-nucleotidase but was not attacked by bacterial 3'-nucleotidase. These results indicate that this material, is AICAR-5'-monophosphate.

3. Donor Specificity

An ability of various phosphate compounds as a donor was studied. The data shown in Table III indicate that the aromatic phosphates and nucleotides were available as a donor as observed in the phosphorylation of nucleosides. Among these phosphate compounds, *p*-NPP was the most excellent donor and 5'-nucleotides were also useful, so far as *Ps. trifolii* was employed as enzyme source. This reac-

TABLE III. DONOR SPECIFICITY (I)

Phosphate compound	AICAR-P synthesized μ moles per ml	Pi liberated μ moles per ml
<i>p</i> -NPP	10.8	16.5
<i>o</i> -Nitrophenyl phosphate	1.1	12.5
Phenyl phosphate	2.6	27.7
5'-AMP	2.8	15.1
Glucose-1-phosphate	0.0	30.5
Glucose-6-phosphate	0.0	34.0
Ribose-5-phosphate	0.0	7.8
Fructose-1,6-diphosphate	0.0	24.7
Creatine phosphate	0.0	34.2
Carbamyl phosphate	0.0	30.5

Reaction mixture containing, per ml, 20 μ moles of AICAR, 40 μ moles of phosphate compound, 1 μ mole of CuSO₄, 100 μ moles of acetate buffer (pH 4.0), and 2 mg of intact cell of *Ps. trifolii* was incubated at 37°C for 20 hr.

TABLE IV. DONOR SPECIFICITY (II)

Phosphoryl Transfer from Mononucleotides

Strain	CMP Isomer as Donor	AICAR-P syn- thesized μ moles per ml	Pi liberated μ moles per ml
<i>Flav. harrisonii</i> (NO. 1161)	5'-	5.2	8.0
"	3'-	0.0	3.7
<i>Ps. trifolii</i> (IAM 1555)	5'-	4.6	5.3
"	3'-	0.0	3.7
<i>Ser. marcescens</i> (IFM F 420)	5'-	4.9	7.4
"	3'-	0.0	3.5

Reaction mixture containing, per ml, 20 μ moles of AICAR, 40 μ moles of CMP isomer, 1 μ mole of CuSO₄, 500 μ moles of acetate buffer (pH 4.0) and 2 mg of intact cells was incubated at 37°C for 20 hr.

tion was also catalyzed by the bacteria characterized to phosphorylate at C_{5'}, but 3'- isomer was unable to be a phosphate donor (Table IV), as observed previously.¹⁴⁾

4. Phosphorylation of AICAR by Various Bacteria

The ability to phosphorylate AICAR was studied, using the typical strains of bacteria demonstrated previously.¹¹⁾ As given in Table V, the bacteria characterized to synthesize 5'-isomer phosphorylated at C_{5'}, whereas rather small amount of AICAR-P, most of which may be considered to be 3'(& 2')-isomer

14) K. Mitsugi, This Journal, 28, 659 (1964).

15) K. Mitsugi, unpublished observation,

TABLE V. PHOSPHORYLATION OF AICAR BY BACTERIA

Strains	AICAR-P synthesized	Pi liberated
	μ moles per ml	
<i>Ps. trifolii</i> (ATCC 12287)	14.3*	24.2
<i>Ps. perluxida</i> (IAM 1600)	13.3*	39.8
<i>Ser. marcescens</i> (IAM 1161)	7.8*	11.6
<i>Flav. harrisonii</i> (No. 1161)	13.6*	20.5
<i>Ach. liquidum</i> (IAM 1667)	9.6*	12.9
<i>Ach. superficialis</i> (IAM 1058)	15.2*	37.9
<i>E. coli</i> (IFM I-17)	0.8	20.2
<i>E. freundii</i> (IFM T-36)	0.4	20.2
<i>E. intermedia</i> (IFM A-21)	0.7	12.9
<i>Aerobact. aerogenes</i> (ATCC 8329)	0.6	11.1
<i>Aerom. hydrophila</i> (IAM 1018)	0.3	7.2
<i>Aerom. punctata</i> (IAM 1163)	1.2	17.3
<i>Prot. vulgaris</i> (IFM OR-1)	1.3	5.3
<i>Prot. mirabilis</i> (IFM OM-9)	3.8	17.5

Assay mixture containing, per ml, 20 μ moles of AICAR, 70 μ moles of *p*-NPP, 1 μ mole of CuSO₄, 400 μ moles of acetate buffer (pH 4.0) and 2.5 mg of intact cells of bacteria, as dry matter, was incubated at 37°C for 20 hr.

*: Metaperiodate oxidizable spot.

judging from their non-oxidizable characters in the metaperiodate oxidation reaction, was formed by the other group of bacteria characterized to phosphorylate at C_{3'} or 2'.

DISCUSSION

These results described here indicate that the nucleoside phosphotransferase of bacteria characterized to phosphorylate at C_{5'} may also participate in the synthesis of AICAR-5'-P, or of an important intermediate of purine nucleotides, as well as nucleoside phosphorylase-kinase system and the nucleotide pyrophosphorylase, in addition to the de novo synthesis.

The fact that the phosphoryl transfer reaction between AICAR and 5'-ribonucleotide was observed to be carried out at even physiological pH ranges¹⁵⁾ other than pH 4.0 seems to be very interesting in view of the biological significance of this activity.

In addition to several methods for the preparation of AICAR-5'-P, not only biochemically but also chemically, this method described here is also a useful procedure. AICAR-5'-P isolated was observed to have a flavour-enhancing activity, as reported recently by Huang.⁶⁾

SUMMARY

5'-Phosphoribosyl 5-amino-4-imidazole carb-oxamide was synthesized in a reaction mixture containing 5-amino-4-imidazolecarboxamide riboside, *p*-nitrophenylphosphate, and the bacteria characterized to phosphorylate at C_{5'}, just as the nucleosides were phosphorylated. This phosphorylated material was isolated chromatographically and identified chemically or enzymatically. The other bacteria synthesizing 3'-or 2'-nucleotides also phosphorylated a little probably at C_{3'} (or 2').

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