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Induction of nucleoside phosphorylase in *Enterobacter aerogenes* and enzymatic synthesis of adenine arabinoside*

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Abstract: Nucleoside phosphorylases (NPases) were found to be induced in *Enterobacter aerogenes* DGO-04, and cytidine and cytidine 5′-monophosphate (CMP) were the best inducers. Five mmol/L to fifteen mmol/L cytidine or CMP could distinctly increase the activities of purine nucleoside phosphorylase (PNPase), uridine phosphorylase (UPase) and thymidine phosphorylase (TPase) when they were added into medium from 0 to 8 h. In the process of enzymatic synthesis of adenine arabinoside from adenine and uracil arabinoside with wet cells of *Enterobacter aerogenes* DGO-04 induced by cytidine or CMP, the reaction time could be shortened from 36 to 6 h. After enzymatic reaction the activity of NPase in the cells induced remained higher than that in the cells uninduced.

Key words: Nucleoside phosphorylase (NPase), *Enterobacter aerogenes*, Cytidine, Cytidine 5′-monophosphate (CMP), Adenine arabinoside

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INTRODUCTION

Nucleoside phosphorylases (NPases) are widely distributed in mammalian cells and bacteria and play a central role in the nucleoside metabolism salvage pathway. Their transcriptional regulations, protein structures and catalytic mechanisms have been extensively studied (Bzowska *et al*., 2000; Zolotukhina *et al*., 2003; Caradoc-Davies *et al*., 2004). NPases can be divided into three main types on the basis of substrate specificity: purine nucleoside phosphorylase (PNPase), uridine phosphorylase (UPase) and thymidine phosphorylase (TPase). Each type catalyzes the reversible phosphorolysis of ribo- or deoxyribonucleosides to yield the corresponding free bases and a pentose-1-phosphate.

The reversible phosphorolysis reaction catalyzed by NPases has been exploited to synthesize nucleosides and their derivates, with either purified NPases or whole bacterial cells or immobilized cells as catalyst (Trelles *et al*., 2004; Jarkko *et al*., 2007). Although nucleosides can be synthesized chemically, enzymatic synthesis is advantageous because it does not require the use of protecting groups or separation of isomers.

PNPase and TPase are encoded by the *deo* D and the *deo* A genes, respectively. In *Escherichia coli* and *Salmonella typhimurium*, *deo* D and *deo* A are part of the *deo* operon, which consists of *deo* A (TPase; EC 2.4.2.1), *deo* B (phosphopentomutase; EC 2.7.5.6), *deo* C (deoxyriboaldolase; EC 4.1.2.28) and *deo* D (PNPase; EC 2.4.2.1) (Robertson *et al*., 1970; Hammer-Jespersen *et al*., 1971; Ling *et al*., 1994). Transcription of the *deo* operon is controlled by two regulator proteins, DeoR and CytR, which are en-

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coded by inducible genes responsive to the deoxyribonucleoside metabolite, deoxyribose-5-phosphate. The pathway leading to deoxyribose-5-phosphate generation involves cleavage of deoxyribonucleosides by the corresponding phosphorylase to give a free base and deoxyribose-1-phosphate that is then converted to deoxyribose-5-phosphate by phosphopentomutase. Deoxyribose-5-phosphate induces all four genes in the *deo* operon, whereas purine nucleosides induce only *deo* B and *deo* D, indicating that the *deo* operon may be regulated by two different mechanisms. The *udp* gene encoding UPase constitutes a type of CytR regulon; the cAMP-CRP (cAMP receptor protein) complex, which acts as a co-repressor for the CytR protein, transcriptionally activates the *udp* gene. In *E. coli*, UPase is induced by cytidine but not by uridine (Thomsen *et al*., 1999; Esipov *et al*., 2002; Zolotukhina *et al*., 2003). In contrast, both uridine and cytidine induce UPase in *S. typhimurium*. The difference between UPase regulation in *E. coli* and in *S. typhimurium* reflects organism-specific differences in CytR: *E. coli* CytR interacts only with cytidine but *S. typhimurium* CytR responds to both uridine and cytidine (Thomsen *et al*., 1999). In *Klebsiella* sp., UPase is induced by both adenosine and uridine (Ling *et al*., 1994).

Adenine arabinoside (Ara-A) has significant activity against almost all DNA viruses and some RNA viruses, both in vitro and in vivo, and has been used clinically to treat viral infections including herpes simplex, herpes zoster and hepatitis. The enzymatic synthesis of Ara-A has been extensively studied (Krenitsky *et al*., 1979; Utagawa *et al*., 1985; Ling *et al*., 1990; Giuseppina *et al*., 2000), and showed capability of producing high substrate conversion rates and a high yield of final product. However, the enzymatic approach used to produce Ara-A is slower compared to the rate of natural nucleosides production. In this paper, we used induction of NPases by nucleosides and nucleotides to improve enzyme activities, thereby increasing the speed and efficiency of Ara-A synthesis.

MATERIALS AND METHODS

Materials

Uracil arabinoside (Ara-U) and other nucleosides and nucleotides were generously provided by Nantong Qiuzhiyou Bioscience and Biotechnology Co., Ltd., China. Xanthine oxidase was purchased from Sigma, USA. Tryptone and yeast extract were purchased from Oxoid, UK. Other chemicals were of analytical grade. The *Enterobacter aerogenes* strain DGO-04 from our laboratory was used throughout.

Medium 1 (per 1 L): 1.32 g $K_2HPO_4{\cdot}3H_2O$, 5 g $(NH_4)_2SO_4$, 0.1 g $MnSO_4{\cdot}H_2O$, 0.8 g $MgSO_4$, 0.01 g $ZnSO_4$, 0.01 g $CuSO_4{\cdot}5H_2O$, 0.01 g $CaCl_2$, 0.001 g $FeSO_4{\cdot}7H_2O$ and 2 g yeast extract. The pH was adjusted to 7.0 with 4 mol/L NaOH. Medium 2 (per 1 L): 10 g meat extract, 10 g tryptone, 5 g yeast extract and 5 g NaCl. The pH was adjusted to 7.0 with 4 mol/L NaOH.

Bacteria culture and induction of NPases

The first seed was prepared by inoculating a 250 ml flask containing 25 ml Medium 2 with *E. aerogenes* DGO-04 obtained from a slant culture, and incubating at 36 °C with reciprocal shaking for 15 h. A 1 ml aliquot of the first seed was used at the same day to inoculate a 250 ml flask containing 25 ml Medium 1 or Medium 2, with or without inducers. The cells were then incubated at 36 °C for 16 h with reciprocal shaking.

Preparation of wet cell paste

The cells were collected by centrifugation for 20 min at 4000 r/min. The precipitated cell paste was washed twice with 100 mmol/L potassium phosphate buffer (pH 7.0), stored at 4 °C and used as the source of enzymes in subsequent assays.

Enzyme assays

UPase was assayed according to the method described by Saunders *et al*.(1969). Briefly, an assay solution containing 0.5% (w/v) wet cell paste, 10 mmol/L uridine and 100 mmol/L potassium phosphate buffer (pH 7.0) in a total volume of 2 ml was incubated at 60 °C for 20 min. The reaction was terminated by adding 1 mol/L ice-cold NaOH (2 ml). The reaction mixture was centrifuged and the supernatant was diluted 50-fold with NaOH (pH 12).

TPase assay was the same as that for UPase, except that 10 mmol/L uridine was replaced by 10 mmol/L thymidine, wet cells paste 0.5% (w/v) by 3% (w/v), and incubation time 20 min by 10 min.

PNPase assay following the method of Herman

(1947) were the same as that for UPase, except that 10 mmol/L uridine was replaced by 10 mmol/L inosine and the total incubation time was 10 min. The reaction mixture was centrifuged and the supernatant was diluted 50-fold with 100 mmol/L potassium phosphate buffer (pH 7.5). Xanthine oxidase was added to the diluted supernatant as described by Kalckar and Klenow (1948) and incubated for 1 h at room temperature.

All enzyme activities were determined spectrophotometrically by measuring absorption at 290 nm relative to a blank. The blank solution was the same as the assay solution except that cells were added after the addition of NaOH. The specific activities of enzymes were expressed as the change in the absorption of reaction mixture at 290 nm per concentration of wet cell paste.

The specific activity of wet cell paste towards Ara-U was measured as for UPase, except that the reaction mixture contained 10 mmol/L Ara-U instead of uridine, and the incubation time was 1 h.

Synthesis of Ara-A

The reaction mixture, containing 30 mmol/L Ara-U, 10 mmol/L adenine and 25 mmol/L potassium phosphate buffer (pH 7.0) in a total volume of 10 ml was incubated at 60 °C. Ara-A was analyzed by high performance liquid chromatography (HPLC) with ultraviolet (UV) detection at 258 nm using a Hypersil ODS2 5 μm column (4.6 mm×250 mm) with a solvent of 5% (v/v) acetonitrile and 95% (v/v) water and a flow rate of 0.70 ml/min.

RESULTS

Induction of NPases in *E. aerogenes*

Both Mediums 1 and 2 were used to test the induction of NPases in *E. aerogenes* by uridine, thymidine and inosine. In Medium 1, all nucleosides induced UPase, TPase and PNPase, with each nucleoside having the greatest effect on its respective NPase (Table 1). These results demonstrate that nucleosides induce increased production of NPases generally, as well as selectively, in Medium 1.

The induction experiments performed in Medium 2 yielded somewhat different results summarized in Table 2. Uridine, cytidine, CMP (cytidine 5′-monophosphate) and UMP (uridine 5′-monophosphate) substantially induced UPase. Adenosine, AMP (adenosine 5′-monophosphate) and dCMP (deoxycytidine 5′-monophosphate) also acted as inducers, but inosine added to Medium 2 did not induce UPase. In addition, adenine and hypoxanthine slightly induced UPase, which has not been previously reported. All nucleosides and nucleotides were tested to induce TPase; cytidine, thymidine, deoxynucleosides

Table 1 Induction of NPases by nucleosides in Medium 1

Induction	Relative specific activity*		
	UPase	TPase	PNPase
−	1.000	1.000	1.000
Inosine	1.325	2.000	5.590
Uridine	2.143	2.316	2.487
Thymidine	1.351	4.350	3.487

*Relative specific activity is expressed as the induced specific activity normalized to that obtained in the absence of induction; The uninduced specific activities of UPase, TPase and PNPase in Medium 1 were 15.4, 2.0 and 7.8 U/mg wet cells, respectively

Table 2 Induction of NPases by nucleosides in Medium 2

Induction	Relative specific activity*		
	UPase	TPase	PNPase
−	1.000	1.000	1.000
Inosine	0.725	2.031	0.919
Adenosine	1.308	2.000	1.694
Cytidine	2.275	4.750	1.774
Uridine	2.058	2.531	1.210
Thymidine	1.033	4.656	1.565
CMP	1.985	4.250	1.630
UMP	1.598	3.156	1.335
GMP	0.977	1.969	1.627
AMP	1.311	2.250	1.757
dCMP	1.364	4.188	1.405
dTMP	0.932	4.750	1.371
dGMP	0.864	3.469	1.390
dAMP	1.053	4.406	1.399
Cytosine	1.041	0.757	1.250
Uracil	1.033	1.000	1.264
Thymine	1.089	0.865	1.389
Adenine	1.396	0.892	2.403
Hypoxanthine	1.268	1.432	1.472
Ribose	0.730	1.324	0.955
Arabinose	0.874	0.973	1.042

*Relative specific activity is expressed as the induced specific activity normalized to that obtained in the absence of induction; The uninducted specific activities of UPase, TPase and PNPase in Medium 2 were 24.4, 1.2 and 14.6 U/mg wet cells, respectively. CMP: Cytidine 5′-monophosphate; UMP: Uridine 5′-monophosphate; GMP: Guanosine 5′-monophosphate; AMP: Adenosine 5′-monophosphate; dCMP: Deoxycytidine 5′-monophosphate; dTMP: Thymidine 5′-monophosphate; dGMP: Deoxyguanosine 5′-monophosphate; dAMP: Deoxyadenosine 5′-monophosphate

monophosphate and CMP were especially robust inducers of TPase activity. Almost all nucleosides and nucleotides were tested to induce PNPase. One exception was inosine, which had no effect in Medium 2, but greatly enhanced the activity of PNPase in Medium 1 (Table 1). The ability of nucleotides to act as inducers reflects their conversion to the corresponding nucleosides by the action of 5′-nucleotidases. Of the five bases that were tested in Medium 2 and shown to induce PNPase, only adenine, which was extremely effective in our assays, has been previously reported to induce PNPase (Ling *et al*., 1994).

Effects of cytidine and CMP concentrations and time of addition on NPase induction

Cytidine and CMP, which showed the most robust inductions of UPase, TPase and PNPase in Medium 2 (Table 2), were selected for further study. In experiments probing the dose dependence of cytidine and CMP, we found that both nucleosides induced evident increases in NPases beginning at concentrations between 1 and 5 mmol/L (Fig.1). NPase induction by cytidine and CMP tended to plateau at about 5 mmol/L, although TPase activity continued to trend upward at higher doses of inducers. The pattern was different for the specific activity of wet cell paste towards Ara-U, which continued to increase in a dose-dependent manner to concentrations of cytidine and CMP as high as 15~20 mmol/L.

We also tested the effects of time of cytidine or CMP addition on the levels of UPase, TPase and PNPase in Medium 2 (Fig.2). There was no effect of cellular growth phase on the induction of UPase, TPase or PNPase by cytidine and CMP. However, decreases in the specific activity of wet cells towards Ara-U were evident when the inducers were added at 14 h, possibly because cells were already in a decline phase at this time.

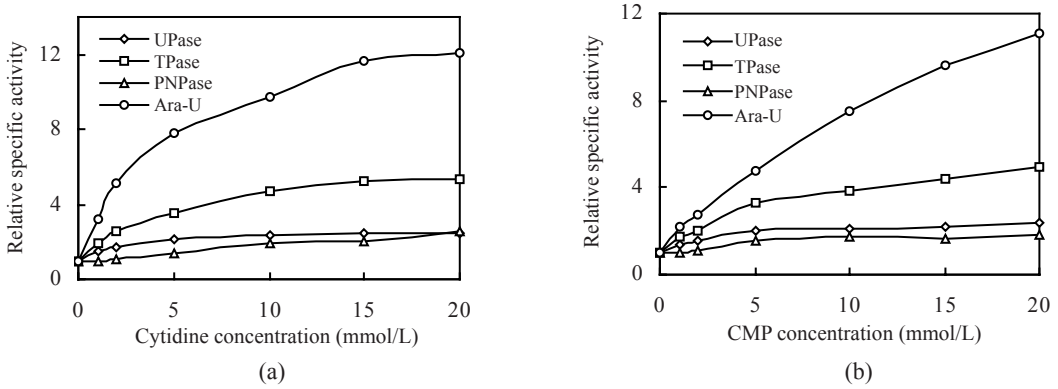

Fig.1 Effects of cytidine (a) and CMP (b) concentrations on the NPase induction

Relative specific activity is expressed as the induced specific activity normalized to that obtained in the absence of induction in Medium 2; The uninduced specific activities of UPase, TPase, PNPase and Ara-U in Medium 2 were 24.4, 1.2, 14.6 and 2.11 U/mg wet cells, respectively

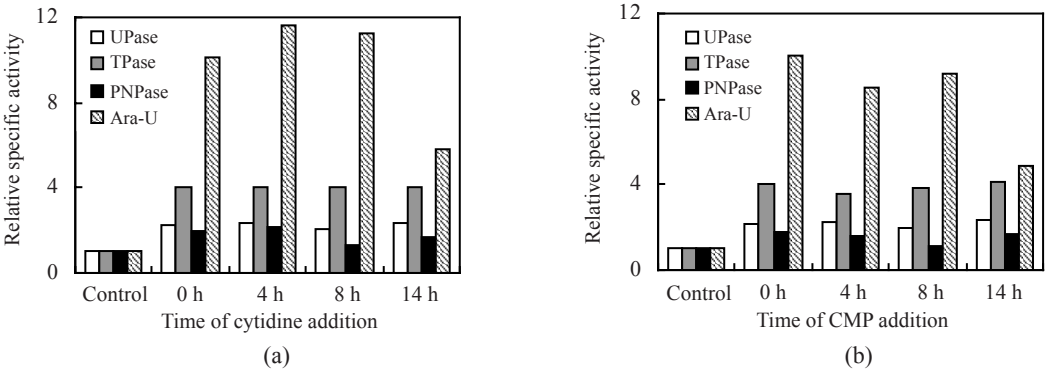

Fig.2 Effects of time of cytidine (a) and CMP (b) additions on NPase induction

Relative specific activity is expressed as the induced specific activity normalized to that obtained in the absence of induction in Medium 2; The uninduced specific activity of UPase, TPase, PNPase and Ara-U in Medium 2 were 24.4, 1.2, 14.6 and 2.11 U/mg wet cells, respectively; The concentrations of cytidine and CMP were 20 mmol/L

Effect of induced NPase activity on the synthesis of Ara-A and changes of enzymatic activities after reaction

When cells grown in Medium 2 supplemented with inducers were used to catalyze the synthesis of Ara-A, the efficiency of Ara-A enzymatic synthesis, measured as yield of adenine, was strikingly enhanced. As shown in Fig.3, the enzymatic reaction time was dramatically shortened from 36 h to 6 h.

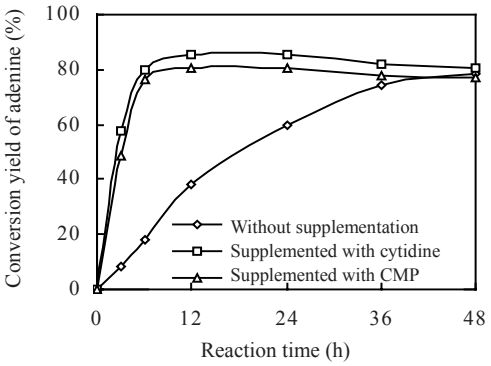

Fig.3 Effect of induction by cytidine and CMP on the enzymatic synthesis of Ara-A

The changes in UPase, TPase and PNPase activities after the enzymatic reaction, measured in three separate experiments (Fig.4), were similar. After the reaction, the specific activity of UPase increased slightly, the activity of TPase decreased slightly, and the activity of PNPase trended upward. However, after the reaction, the specific activity of wet cell paste towards Ara-U was substantially decreased. Although the specific activity towards Ara-U also decreased considerably after the reaction in wet cell paste from cells grown in Medium 2 supplemented with inducers (Fig.4), it was still relatively high compared to the activity in uninduced cell paste (Fig.4a).

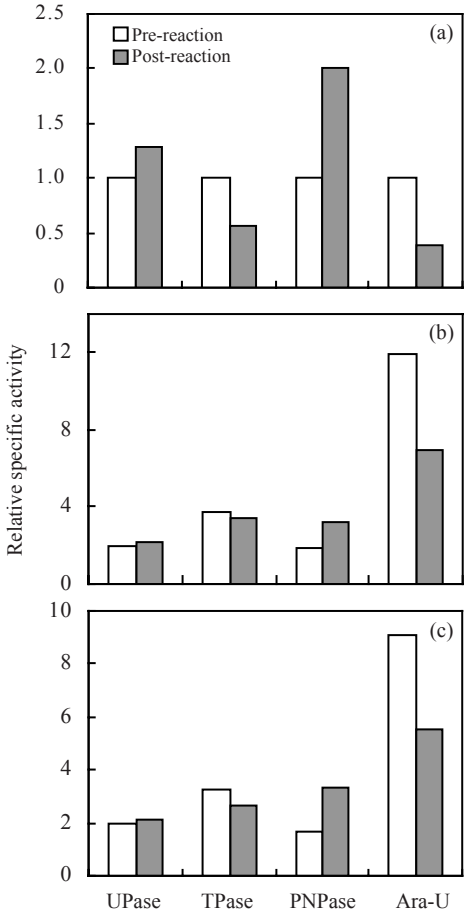

Fig.4 Pre-reaction and post-reaction activities of NPases in cells cultured in Medium 2. (a) Non-inducer; (b) Cytidine added; (c) CMP added

Relative specific activity is expressed as the induced specific activity normalized to that obtained in the absence of induction in Medium 2; The uninduced specific activities of UPase, TPase, PNPase and Ara-U in Medium 2 were 24.4, 1.2, 14.6 and 2.11 U/mg wet cells, respectively

DISCUSSION

The composition of Medium 1 (see MATERIALS AND METHODS) is suitable for studying the NPase induction, and much of the research in this field has been conducted with this simple media formulation. Our experiments on NPases of *E. aerogenes* have confirmed this. However, compared to the richer Medium 2, Medium 1 is poorly suited for use in enzyme production. Our results reveal a number of differences between the effects of inducers in Mediums 1 and 2. For example, inosine and thymidine modestly induced UPase in Medium 1, but had no effect in Medium 2. The difference in inosine induction was even more striking for PNPase, which was intensely induced by inosine in Medium 1 but not at all in Medium 2. This reduced effect of nucleosides is likely due to the fact that Medium 2 contains higher nucleoside levels, which are probably sufficient to provide near-maximal induction in the absence of added inducers.

NPases from different bacteria exhibit species-specific differences in their responses to nucleoside and nucleotide inducers. UPase from *E. coli* is

induced by cytidine, CMP and adenosine, but not by uridine (Robertson *et al*., 1970; Hammer-Jespersen *et al*., 1971; Vita *et al*., 1983), whereas in *Klebsiella* sp. (Ling *et al*., 1994) UPase is induced by both uridine and adenosine. In this study, we showed that UPase from *E. aerogenes* is induced by adenosine, cytidine, uridine and their corresponding nucleotides using Medium 2 (Munch-Petersen, 1968; Robertson *et al*., 1970; Hammer-Jespersen *et al*., 1971; Vita *et al*., 1983). TPases from *E. coli* and *S. typhimurium* are induced by deoxynucleosides and have also been reported to respond with cytidine (Hammer-Jespersen *et al*., 1971). Here, we found that TPase from *E. aerogenes* is induced by almost all nucleosides and nucleotides tested.

PNPase from *E. coli* is induced by deoxynucleosides, cytidine, purine nucleosides and their corresponding nucleotides (Vita *et al*., 1983), with the exception of AMP. We showed here that PNPase from *E. aerogenes* is induced by most nucleosides and nucleotides, except inosine. For the most part, bases have no effect on the levels of NPases of *E. coli* and *Bacillus cereus* (Tozzi *et al*., 1981; Vita *et al*., 1983), although adenine has been reported to induce the PNPase of *E. coli* strain K12 (Hammer-Jespersen *et al*., 1971). However, we found that the UPase of *E. aerogenes* was induced by adenine and hypoxanthine, TPase was induced by hypoxanthine, and PNPase was induced by all bases (guanine was not tested), with the effect of adenine being clearly the most prominent.

In Medium 2, cytidine and CMP effectively induced NPases of *E. aerogenes* within a concentration range of 5 to 20 mmol/L. The specific activity of cells towards Ara-U in Medium 2 supplemented with cytidine reached a high plateau value at concentrations of 15 to 20 mmol/L. In cells grown in Medium 2 with CMP, the activity towards Ara-U increased in a dose-dependent manner throughout the CMP concentration range.

PNPase activity induced by inosine in *B. cereus* cells has been shown to remain relatively high during the stationary phase (Tozzi *et al*., 1981). Our results indicate that, in *E. aerogenes*, the NPase induction by cytidine and CMP was largely independent of cell growth phase, although the specific activity of wet cell paste towards Ara-U was relatively low when inducers were added during the decline phase.

Studies on the NPase induction in nutrient medium are significant because such media supporting good bacterial growth may show enhanced effects of inducers on NPase activities. In the absence of induction, the specific activity of UPase from *E. aerogenes* towards Ara-U is only one-tenth that towards uridine, which may account for the longer time required for UPase and PNPase to catalyze the synthesis of Ara-A compared to the synthesis of natural nucleosides. The shortest synthesis time reported for Ara-A is about 15 h (Utagawa *et al*., 1985). Here, we show that induction of *E. aerogenes* by cytidine or CMP greatly enhances the efficiency of the Ara-A reaction, dramatically reducing the enzymatic reaction time from 36 h to 6 h. Thus, the induction of cells grown in a nutrient medium represents a simple and effective way to enhance the activities of NPases.

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