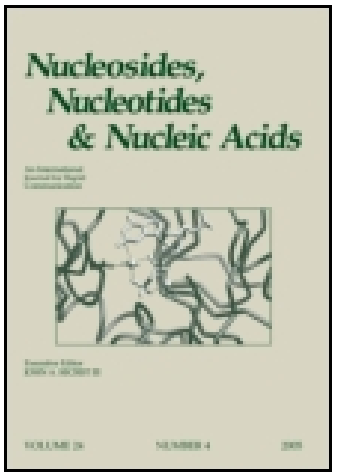

Nucleosides, Nucleotides and Nucleic Acids

Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/lncn20

Nucleoside Phosphorylases from Clostridium Perfringens in the Synthesis of 2′,3′-Dideoxyinosine

S. Christoffersen [a] , I. Serra [b] , M. Terreni [b] & J. Piškur [a]

[a] Department of Cell and Organism Biology , Lund University , Lund, Sweden

[b] Dipartimento di Chimica Farmaceutica and Italian Biocatalysis Center , University of Pavia , Pavia, Italy

Published online: 11 Jun 2010.

To cite this article: S. Christoffersen , I. Serra , M. Terreni & J. Piškur (2010) Nucleoside Phosphorylases from Clostridium Perfringens in the Synthesis of 2′,3′-Dideoxyinosine, Nucleosides, Nucleotides and Nucleic Acids, 29:4-6, 445-448, DOI: 10.1080/15257771003741422

To link to this article: http://dx.doi.org/10.1080/15257771003741422

Nucleosides, Nucleotides and Nucleic Acids, 29:445–448, 2010

ISSN: 1525-7770 print / 1532-2335 online
DOI: 10.1080/15257771003741422

NUCLEOSIDE PHOSPHORYLASES FROM *CLOSTRIDIUM PERFRINGENS* IN THE SYNTHESIS OF 2′,3′-DIDEOXYINOSINE

S. Christoffersen,[1] I. Serra,[2] M. Terreni,[2] and J. Piškur[1]
[1]*Department of Cell and Organism Biology, Lund University, Lund, Sweden*
[2]*Dipartimento di Chimica Farmaceutica and Italian Biocatalysis Center, University of Pavia, Pavia, Italy*

□ *Four* Clostridium perfringens *phosphorylases were subcloned, overexpressed and analyzed for their substrate specificity. DeoD(1) and PunA could use a variety of purine substrates, including an antiviral drug 2′,3′-dideoxyinosine (ddI). In one-pot synthesis using* Clostridium *phosphorylases, 2′,3′-dideoxyuridine and hypoxanthine were converted to ddI at yield of about 30%.*

Keywords Nucleoside phosphorylase; *Clostridium perfringens*; biocatalysis; 2′,3′-dideoxyinosine; anti-viral drugs

INTRODUCTION

Nucleoside phosphorylases (NPs) catalyze the cleavage of (deoxy) ribonucleosides to give the free base and ribose 1-phosphate or deoxyribose 1-phosphate, respectively.[1] Apart from its biomedical application,[2] this class of enzymes is of interest because they can be used as biocatalysts for the enzymatic synthesis of nucleoside analogues. In fact, NPs catalyze both the phosphorolytic and synthetic reactions, and in the presence of a second nucleobase (sugar acceptor) catalyze the synthesis of a new nucleoside. Such transglycosylation reaction has been used for the one-pot synthesis of several nucleosides starting from cheap, commercially available nucleosides ("sugar donors") and modified bases ("acceptor bases").[3,4] In this work, we explored the *Clostridium perfringens* NCTC8449 genome, identified five putative genes and subsequently subcloned and overexpressed four NPs. The recombinant enzymes were subsequently used to synthesize the nucleoside analogue 2′,3′-dideoxyinosine (ddI), a reverse transcriptase inhibitor which

Address correspondence to J. Piškur, Department of Cell and Organism Biology, Lund University, Sölvegatan 35, 22362 Lund, Sweden. E-mail: Jure.Piskur@cob.lu.se

is used in combination with other drugs in the highly active antiretroviral therapy (HAART).

MATERIALS AND METHODS

Cloning and Purification

Three putative NPs genes encoding DeoD(1) (Gene ID 989708), DeoD(2) (Gene ID 988651), and PunA (Gene ID 990873) were subcloned from genomic DNA (kindly provided by A.R. Clausen) into TOPO 151 vector (Invitrogen Paisley, UK) giving the following plasmids, P967, P969, and P968, respectively. The plasmids were overexpressed as fusion proteins with a N-terminal His_6 tag, and purified as reported by Knecht et al.[5] The total protein content of the soluble fraction was measured by the Bradford method.

Purine NP Activity Assays

Xanthine oxidase coupled assay was used to test NPs towards inosine and 2′-deoxyinosine. The assay was optimized from a previously reported assay.[6] The activity was calculated from the increase in absorbance at 293 nm using 11.5 as millimolar extinction coefficient. The activity on guanine and adenine nucleosides was assayed following the increase in absorbance at 260 nm. The amount of free base formed was deduced by using a millimolar extinction coefficient of 1.316 and 5.4 for adenine and guanine, respectively.

HPLC-Based Assays

The reaction took place in 50 mM potassium buffer pH 7.5 (10 ml) containing 5 mM of nucleoside substrate, and was started by the addition of the enzyme, kept under mechanical stirring and stopped by ultra filtration of the enzymes with the 10 kDa MWCO filter devices. The reaction was monitored by HPLC. The column was a RP18 Lichrocart 250 × 4.6, 5 μm (Merck, Darmstadt, Germany); eluent, 0.01 M potassium phosphate buffer pH 4.6 and methanol 90% (97:3); flow, 1 ml/min; T = 35°C; λ = 260 nm.

One-Pot Synthesis of ddI

A solution of 10 mM potassium buffer pH 7.5 (1.5 ml) containing 5 mM of 2′,3′-dideoxyuridine (ddU) and 5 mM of hypoxanthine was prepared. Reaction was started by the addition of the enzymes (2.4 U of UP and 40 U of either DeoD (1) or PunA). The reaction was kept under mechanical stirring and stopped by ultra filtration of the enzymes with 10 kDa MWCO filter devices. The reaction was monitored by HPLC as described above.

TABLE 1 Kinetic parameters of *Clostridium* Deod (1) and PunA

Enzyme	Substrate	V_{max} (U/mg)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/K_m ($M^{-1}s^{-1}$)
DeoD (1)	Inosine	16	7.9	900	8777
	2′-deoxyinosine	29.5	14.5	1300	10769
	Guanosine*	8	3.9	400	9750
	2′-deoxyguanosine*	6.6	3.2	400	8000
	Adenosine*	12	6.1	200	3050
PunA	Inosine	4.8	2.7	279	9677
	2′-deoxyinosine	4.3	2.4	400	5988
	Guanosine*	41.5	23.1	249	92771
	2′-deoxyguanosine*	31.1	17.4	174	100000
	Adenosine*	n.d.	n.d.	n.d.	n.d.

U: μmol min^{-1}.
*Double reciprocal plot calculation.
n.d. = not detected.

RESULTS AND DISCUSSION

The *C. perfringens* JCTC8449 genome codes for five putative NPs. We cloned, overexpressed and characterized four of them with the exception of pyrimidine nucleoside phosphorylase (DeoA). DeoD (1) and PunA belong to purine specific NPases (Table 1). One NP, DeoD (2), showed very poor activity towards all the substrates tested and was not subjected to further investigations. *Clostridium* uridine phosphorylase (UP, Gene ID 988640, overexpressed from plasmid P966) is described elsewhere (in preparation). For DeoD(1) K_m values were 400 μM for guanosine and 2′-deoxyguanosine and 200 μM for adenosine, while for inosine and 2′-deoxyinosine K_m values were higher: 900 and 1300 μM, respectively (Table 1). However, the latter compounds had higher k_{cat} values. Therefore, all substrates exhibited similar catalytic efficiency (k_{cat}/K_m). PunA was specific for nucleosides based on guanine or hypoxanthine as base, the K_m values for all these compounds were in the range of 174 to 400 μM, but phosphorolysis of hypoxanthine nucleosides occurred to almost one tenth of guanine nucleosides (Table 1). The enzymes were tested for their activity also towards ddI and showed a specific activity of 0.09 and 0.02 U/mg for DeoD(1) and PunA, respectively (Table 2). One pot bi-enzymatic synthesis of 2′,3′-dideoxyinosine was carried

TABLE 2 Specific activities of *Clostridium* NPases on 2′,3′-dideoxyriboside substrates (as determined by the HPLC assay)

Enzyme	ddU (U/mg)	ddI (U/mg)
UP	1.9	—
DeoD(1)	—	0.09
PunA	—	0.02

U: μmol min^{-1}.

out with these two NPs and with UP that accepts 2′,3′-dideoxyuridine. Combining *Clostridium* UP with either PunA or DeoD(1), we achieved after 24 hours about 30% yield of ddI.

In conclusion, we successfully cloned and overexpressed four *C. perfringens* NPs, and showed that two of them accept different native purine substrates, including ddI (Tables 1 and 2). We could run a one-pot synthesis approach [3,4] with two pairs of enzymes, PunA and UP, and DeoD(1) and UP, using ddU and hypoxanthine as substrates and converting them into ddI. In future, the yields could easily be optimized, for example in terms of reagents concentrations, pH, temperature and by using immobilized biocatalysts.[6]

REFERENCES

1. Pugmire, M.J.; Ealick, S.E. Structural analyses reveal two distinct families of nucleoside phosphorylases. *Biochem. J.* **2002**, 361, 1–25.
2. Zhang, Y.; Parker, W.B.; Sorscher, E.J.; Ealick, S.E. PNP anti-cancer gene therapy. *Curr. Top. Med. Chem.* **2005**, 5, 1259–1274.
3. Prasad, A.K.; Trikha, S.; Parmar, V.S. Nucleoside synthesis mediated by glycosyl transferring enzymes. *Bioorg. Chem.* **1999**, 27, 135–154.
4. Terreni, M.; Ubiali, D.; Bavaro, T.; Cecchini, D.A.; Serra, I.; Pregnolato, M. Bioprocesses for the synthesis of nucleosides and nucleotides. In: *Biocatalysis Research Progress* (Columbus, F., Ed.) Nova Science Publishers, Hauppauge, 2008, pp. 47–94.
5. Knecht, W.; Sandrini, M.P.B.; Johansson, K.; Eklund, H.; Munch-Petersen, B.; Piškur, J. A few amino acid substitutions can convert deoxyribonucleoside kinase specificity from pyrimidines to purines. *EMBO J.* **2002**, 21, 1873–1880.
6. Rocchietti, S.; Ubiali, D.; Terreni, M.; Albertini, A.M.; Fernandez-Lafuente, R.; Guisàn, J.M.; Pregnolato, M. Immobilization and stabilization of recombinant multimeric uridine and purine nucleoside phosphorylases from *Bacillus subtilis*. *Biomacromolecules* **2004**, 5, 2195–2200.