Synthesis of 2′-Deoxynucleosides by Transglycosylation with New Immobilized and Stabilized Uridine Phosphorylase and Purine Nucleoside Phosphorylase

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Abstract: Multimeric uridine phosphorylase (UP) and purine nucleoside phosphorylase (PNP) of *Bacillus subtilis* have been expressed from genes cloned in *Escherichia coli*, purified, characterized, immobilized and stabilized on solid support. A new immobilization strategy has been developed for UP onto Sepabeads coated with polyethyleneamine followed by cross-linking with aldehyde-dextran. PNP has been immobilized onto glyoxyl-agarose. At pH 10 and 45 °C these derivatives catalyzed the transglycosylation of 2′-deoxyuridine to 2′-deoxyguanosine in high yield (92%). Under the same conditions the not immobilized enzymes were promptly inactivated.

Keywords: 2′-deoxynucleosides; enzyme catalysis; immobilization; purine nucleoside phosphorylase; uridine phosphorylase

Introduction

Natural nucleosides and their modified derivatives are commonly used for the synthesis of antiviral and antitumour agents as well as for the preparation of oligonucleotides.[1,2] Nucleosides are largely synthesized by multi-step chemical procedures which, however, are plagued by low yields and the formation of undesired by-products.[3] Alternatively, nucleosides can be prepared by a biosynthetic process through a transglycosylation reaction between a pyrimidine nucleoside and a purine base in aqueous solution catalyzed by nucleoside phosphorylases or microorganisms which produce them.[4] Specifically, the coupling of uridine phosphorylase (UP; E.C. 2.4.2.3) and purine nucleoside phosphorylase (PNP; E.C. 2.4.2.1) efficiently transfers a sugar moiety from a donor nucleoside to an acceptor base.[1,4–7]

Despite the advantages of the bioconversions performed by enzymes, stability limitations of the biocatalysts may play a pivotal role when drastic reaction conditions are required, for example, to solubilize substrates and products at the high concentrations necessary to develop preparative processes. Specifically, the transglycosylation with guanine requires the use of alkaline pH and/or high temperatures in order to solubilize suitable concentrations of this substrate. The enzymatic synthesis of 2′-deoxyguanosine (2′-dG) has never been optimized mainly because of the poor water solubility of guanine in the mild conditions optimal for the native enzymes.

The immobilization of UP and PNP from *Bacillus stearothermophilus* on an anionic exchange resin has been developed.[8] Nevertheless, this approach does not generally afford sufficiently stable biocatalysts under extreme conditions of pH and temperature necessary for the synthesis of 2′-dG. Desorption of the enzyme may, in fact, occur due to the reversibility of this immobilization technique. Besides, using multimeric enzymes, an extensive study is necessary to efficiently immobilize and stabilize these enzymes. The stabilization of the quaternary structure[9–11] is indispensable in order to prevent any dissociation of the protein which would cause the loss of activity. This dissociative process is usually favoured by low ionic strength, extreme pH and high temperature, conditions often necessary to successfully perform the transglycosylation reactions with poorly water-soluble substrates. Indeed, despite several efforts

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and the potential advantages of enzyme catalysts, nucleosides are still preferably prepared by procedures not involving enzymes. This reflects the relative inefficiency of current enzymatic methods indicating the need for improvement in the enzymatic approach to nucleoside synthesis. In this work, we report the cloning, expression and purification of PNP and UP from *Bacillus subtilis*[12] and the characterization of their multimeric structures. The enzymes have been stabilized by immobilization on solid support and successfully used for the preparative synthesis of purine 2′-deoxynucleosides such as 2′-deoxyinosine (2′-dI) and 2′-dG. The main goal of this research has been, indeed, the study and the development of a highly efficient catalytic process based on enzymes with the aim to provide a competitive synthetic approach for 2′-deoxynucleosides. The extensive study about the enzyme immobilization-stabilization will be reported elsewhere.

Results and Discussion

Cloning, Expression and Purification of the Enzymes

PNP (PnpI/PupG) is coded by the *pup*G (*punA*) gene, part of the *drm-pup*G operon located at 2446.40 kb of the *B. subtilis* chromosome,[13,14] which can complement the *E. coli deoD* mutation. This enzyme is one of the two purine nucleoside phosphorylases of *B. subtilis*. PNP specifically catalyzes the cleavage of the glycosidic bond of ribo- and deoxyribo-nucleoside in the presence of inorganic orthophosphate as a second substrate, to generate the purine base and ribose or deoxyribose 1-phosphate. This reaction is reversible for the natural substrates. The other purine phosphorylase activity PnpII/PuPII of this bacterium, coded by the *pupA (punB)* gene at 2135.40 kb of the chromosome, is specific for adenosine and deoxyadenosine.

UP (Up/Pdp) is the only pyrimidine nucleoside phosphorylase of *B. subtilis*. This enzyme is coded from the *pdp* gene of the *dra-nupC-pdp* operon at 4049.30 kb of the chromosome. UP uses uridine, thymidine and deoxyuridine as pyrimidine source in the salvage pathway[15] catalyzing the phosphorolytic cleavage of pyrimidine nucleoside to the pyrimidine base and deoxyribose or ribose 1-phosphate.

By using gene specific primers, we have amplified the *pup*G and *pdp* genes by PCR; the amplified fragments were cloned into the pTrcHis vector (Invitrogen), and the proteins were expressed in *E. coli* DH5α. This strategy of cloning allows the IPTG (isopropylthio-β-D-galactoside) inducible transcription of the gene from the P_{trc} promoter and the translation of the *pup*G and *pdp* CDS without the fusion to the N-terminal His-tag. The PNP protein expressed in *E. coli*, due to the cloning strategy, starts with the sequence MEDR at the N terminus instead of the native MKDR. For the same reason, the UP protein expressed in the same *E. coli* strain starts with the sequence MGMV at the N terminus, instead of MRMV. We verified that the methionine at the amino terminus of UP was removed in the mature protein. Both the enzymes were purified from the crude extracts by FPLC ion exchange column chromatography (Pharmacia 26/10 Q Sepharose) after a preliminary cut with 60% ammonium sulphate followed by precipitation with 90–95% ammonium sulphate and dialysis. The elution profiles gave reasonably pure fractions for both the enzymes as verified by SDS-PAGE electrophoresis. The PNP and UP activities were determined as reported in the Experimental Section.

The subunit composition of both enzymes was confirmed by aldehyde-dextran cross-linking,[16] followed by SDS-PAGE of the complex and by gel filtration, showing that PNP was a tetramer according to Bzowska et al.[17] and suggesting a trimeric organization for UP. Aldehyde-dextran has been previously used to stabilize the quaternary structure of some soluble enzymes[18–20] showing a high efficiency as a cross-linking reagent.[21] Using an aldehyde-dextran significantly smaller than the protein, the molecular weight of a protein checked by SDS-PAGE is not sensitively affected.[21] In our case, the experiment was performed using two dextran sizes (MW 20000 or 6000) and, in both cases, the SDS-PAGE supported a tetrameric structure for PNP and a trimeric structure for UP, according to the resulting bands of 120 kDa and 135 kDa, respectively.

Synthesis of 2′-dI and 2′-dG

To immobilize and stabilize UP and PNP, we have used different immobilization strategies. In particular, for UP, a novel immobilization and stabilization approach has been developed and it will be described in detail in a forthcoming paper. This procedure includes a first strong adsorption of the enzyme on a flexible ionic support obtained by derivatization of the epoxide resin Sepabeads EC-EP with high molecular weight polyethyleneamine (Sepabeads-PEI).[22] Successively, the adsorbed enzyme has been cross-linked with 20% oxidized dextran affording, after reduction, the covalent immobilization of the protein (Figure 1).

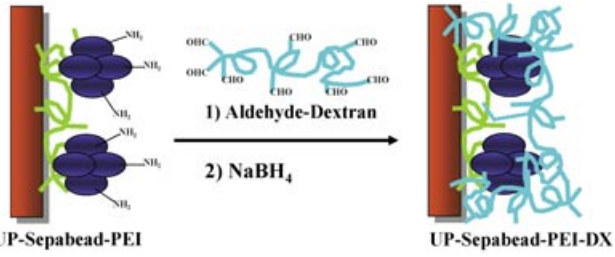
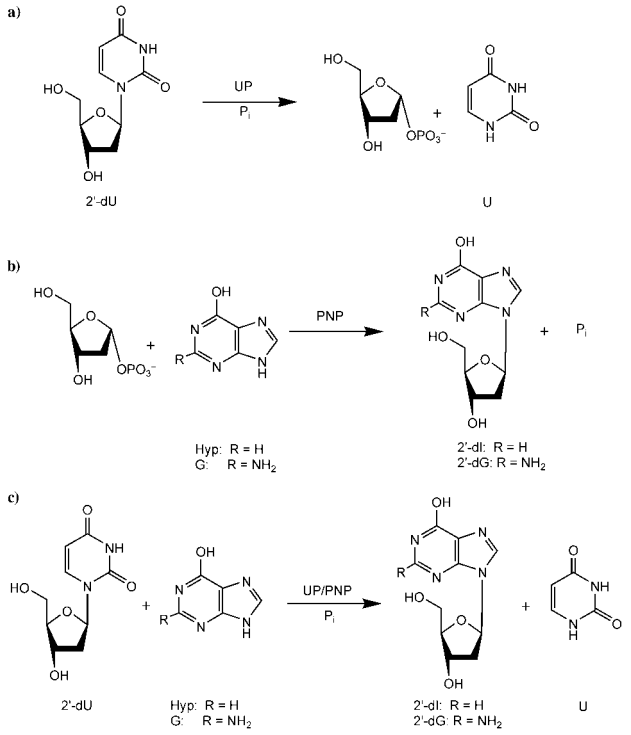


Figure 1.



Scheme 1.

Optimally stabilized and immobilized PNP has been obtained by multipoint covalent immobilization on agarose gel by modifying the standard procedure[23] by the addition of stabilizing ($MgCl_2$ and inosine) and adjuvant (Triton X-100) agents as reported in the Experimental Section. The immobilized enzymes were active and stable at pH 10–11 and 40–50 °C. No subunits dissociation was detected (data not shown).

Using the active and stable immobilized catalysts, we studied the influence of different experimental conditions. To this purpose, the synthesis of 2′-dI (Scheme 1, **c**) was selected as reference reaction because of the high water solubility of the purine base (hypoxanthine) unlike guanine.

As reported in Table 1, on decreasing the ionic strength the yield sensitively increased (reactions 1–3). At low buffer concentration (10 mM), with increasing pH and temperature, only a slight reduction of the yield was observed (Table 1, reactions 3 and 4). A further improvement of the yield was achieved, as expected, also by raising the amount of 2′-dU (sugar donor) and the substrates concentration (reactions 5–7, yield 81% to 85%). Similarly, the synthesis of 2′-dG starting from 5 mM guanine (G) afforded a 87% bioconversion (reaction 8). When a 5-fold higher concentration of 2′-dU (50 mM) and G (25 mM) was used (reaction 9) the yield of 2′-dG was 92%. After removal of the biocatalysts by filtration, 2′-dG was isolated as a white solid from the solution by precipitation at pH 5.5 and 4 °C (97% purity by HPLC analysis).

Conclusion

We have described the cloning, expression and purification of PNP and UP from *B. subtilis* and the characterization of their multimeric structures. A novel immobilization-stabilization technique for multimeric UP was developed affording an active and very stable biocatalyst. This modified enzyme was successfully used along with the PNP preparation (on glyoxyl-agarose gel) for the one-pot synthesis of 2′-dG in water, otherwise impossible to be performed by native, not stabilized enzymes in solution due to their inactivation in the extreme experimental conditions required (no 2′-dG formation was detected after 8 h). The immobilization-stabilization approach reported here can indeed be considered as a general strategy to overcome the limitations of multimeric enzymes, thus allowing the synthesis of poorly water soluble nucleosides, provided that the substrate specificity of the enzymes is properly taken into account in the selection of the microbial source.

Table 1. One-pot enzymatic synthesis of 2′-dI and 2′-dG.

Reaction	KH_2PO_4 [mM]	pH; T [°C]	[Purine base] [mM]	[2′-dU] [mM]	Time [h]	Product, Yield [%]
1	200	7; 25	[Hyp] 5	5	8	2′-dI, 37
2	20	7; 25	[Hyp] 5	5	8	2′-dI, 42
3	10	7; 25	[Hyp] 5	5	8	2′-dI, 63
4	10[a]	10; 45	[Hyp] 5	5	8	2′-dI, 56
5	10[a]	10; 45	[Hyp] 5	10	5	2′-dI, 81
6	10[a]	10; 45	[Hyp] 10	20	5	2′-dI, 82
7	10[a]	10; 45	[Hyp] 25	50	5	2′-dI, 85
8	10[a]	10; 45	[G] 5	10	4	2′-dG, 87
9	10[a]	10; 45	[G] 25	50	24	2′-dG, 92

Catalysts: UP-Sepabeads-PEI-DX (14 Units) and PNP-glyoxyl-agarose (30 Units); Hyp: hypoxanthine; G: guanine; 2′-dU: 2′-deoxyuridine.
[a] In K_2CO_3 10 mM buffer.

Experimental Section

General

Sepabeads FP-EC3 were kindly provided by Resindion Mitsubishi Chemical Co. (Milan, Italy). Agarose (Sepharose CL-4B) was purchased from Amersham Pharmacia Biotech (Uppsala, Sweden). Nucleosides, uracil, guanine and hypoxanthine were supplied by Pro.Bio.Sint. (Varese, Italy). Activity assays were performed on a Shimadzu spectrophotometer UV 1601 (UV-Probe 1.0 Shimadzu); reactions were monitored by HPLC Merck Hitachi (HPLC Multi HSM Manager Merck Hitachi D-7000); the pH values of the enzymatic reactions were kept constant by using an automatic titrator 718 Stat Tritino from Metrohm (Herisau, Switzerland); proteins were purified using the FPLC AKTA system (Pharmacia). All reagents and solvents were of commercial quality and were not further purified before use.

Cloning and Expression of the *B. subtilis* pupG and pdp Genes in *E. coli*

We used the following gene specific primers to amplify by PCR the coding region of both genes from chromosomal *B. subtilis* strain PB168 DNA:

PPN: 5′ CG*GGATCC*ATGTTTCGAATTTTGGGCATAA 3′;
PNP1: 5′ *CATG*CCATGG*AGGACAGAATTGAACGCGCAGC 3′*;
UP2: 5′ CG*GGATCC*AATATCCAGCCGATCACATCCTA 3′;
UP1/1: 5′ CATCATG*CCATGG*GAATGGTAGATATCATCATCAAAA 3′.

BamH1 and *NcoI* sites were engineered into the 5′ and 3′ ends of the fragments amplified by PCR. The amplified fragments of the *B. subtilis* genome have been digested with the restriction enzymes and ligated into the pTrcHis *E. coli* expression vector (Invitrogen) *Nco*I and *Bam*H1. The correct construction of the expression plasmids, pPnp and pUp, has been verified by restriction analysis and direct sequencing of the cloned coding regions.

The expression of the recombinants PNP and UP was obtained from cultures of DH5α/pPnp and DH5α/pUp grown in LB at 30 °C induced at 0.5 $OD_{600\,nm}$ with 0.5 mM IPTG (isopropylthio-β-D-galactoside) for 12 h at the same temperature. The elution of the purified enzymes from the Pharmacia 26/10 Q Sepharose column was obtained with 0–0.5 M NaCl gradients. The amino-terminal amino acid sequence of UP was determined by automated Edman degradation.

Purification of the Enzymes

The supernatant of the crude extract from DH5/pPNP or DH5/pPDP9 cells (20–25 g) grown at 30 °C in LB medium (3 L) and induced with 0.5 mM IPTG was first purified by two precipitations with ammonium sulfate. To the PNP crude extract 60% ammonium sulphate was added followed by centrifugation; to the resulting supernatant 95% ammonium sulphate was added. The same protocol was used for UP except for the second precipitation, made with 90% ammonium sulphate. In both cases, the precipitate was resuspended in 10 mM Tris HCl and 1 mM $MgCl_2$ and then purified by FPLC with an ion-exchange chromatography column (Pharmacia 26/10 Q Sepharose). Proteins were eluted with a KCl gradient (0 to 1 M) in 10 mM Tris HCl, pH 7.4 and 1 mM $MgCl_2$. The purity of the proteins (usually about 80–90%) was confirmed by SDS-PAGE electrophoresis (4–20%), where a single band of 30 kDa for PNP or 45 kDa for PDP was present. The pure fractions were dialyzed against 50 mM phosphate buffer, pH 7.5. The final concentration of the enzymes, determined by Bradford assay[24] was usually between 3 and 8 mg/mL. The PNP enzymatic activity was evaluated by the xanthine oxidase assay[25,26] and the PDP activity was determined optimizing the procedure previously reported.[27]

Confirmation of UP and PNP Multimeric Composition by SDS-PAGE

A solution of PNP (500 μL, conc. 7 mg/mL) was added to KH_2PO_4 buffer (0.5 M, pH 7.0; 90 μL) containing $MgCl_2$ (100 mM, 10 μL), oxidized dextran (400 μL, 20 kDa or 6 kDa) and trimethylaminoborane (30 mg). After 24 h, the pH was set at 10 by adding few drops of a solution of $NaHCO_3$ (1 M, pH 10). Then, $NaBH_4$ (10 mg) was added and the reduction was carried out for 30 min. A solution of SDS (120 μL) was added to 80 μL of the sample and boiled for 10 min. An aliquot (10 μL) was analyzed by electrophoresis showing a spot at 120 kDa. As the subunit is 29 kDa, we concluded that PNP was a tetramer.

The same procedure was followed for UP (500 μL, conc. 7 mg/mL) using 100 μL KH_2PO_4 buffer 0.5 M pH 7.0 in absence of $MgCl_2$. In this case, the electrophoresis showed a spot at 135 kDa and, as the subunit is 46 kDa, we concluded that UP might be a trimer.

Immobilization of PNP on Glyoxyl Agarose Gel

Glyoxyl agarose gel[23] (1.4 mL) was suspended in 50 mM KH_2PO_4 buffer (pH 10.05) containing 100 mM $MgCl_2$ (140 μL), inosine (38 mg) and triton X-100 (5% solution; 1.4 mL). A suitable amount of PNP solution was added (20 Units) and the suspension (14 mL; Vgel/Vtot = 1/10) was kept under mechanical stirring at room temperature for 2 h. Then, $NaBH_4$ (14 mg, 1 mg/mL suspension) was added and the reduction was carried out for 30 minutes. The derivative was filtered, washed with 10 mM KH_2PO_4 buffer (pH 5) and then with deionized water.

Immobilization of UP on Sepabead-PEI (Sep-PEI) and Stabilization with Oxidized Dextran (Sep-PEI-DX)

Sepabeads FP-EC3 (25 g) were added to a 10% solution of polyethyleneamine (MW = 20000) in 1 M NaCl (315 mL, pH 11) and the mixture was stirred for 24 h; then the support was filtered, washed with the NaCl solution and with deionized water. The activated support (1 g) was suspended in 5 mM KH_2PO_4 buffer (pH 7.5) with a suitable amount of UP solution (20 Units; final volume = 14 mL; Vgel/Vtot = 1/10) and kept under

mechanical stirring for 8 h. The derivative was filtered and washed with deionized water. Dextran (1.67 g, MW = 19500) was suspended in deionized water (50 mL) and sodium periodate (0.872 g) was added. The reaction was carried out for 2 h at room temperature and promptly dialyzed (3500 Daltons MWCO) with deionized water. Oxidized dextran dialyzed solution (1.4 mL) was added to the immobilization suspension of Sep-PEI (14 mL) and the mixture was stirred for 1 h. The pH was set to 10 with dilute NaOH and $NaBH_4$ (15.4 mg) was added (1 mg/mL suspension). The reduction was carried out for 30 minutes then the derivative was filtered and washed with 10 mM KH_2PO_4 buffer (pH 5) and with deionized water.

Activity Assay of PNP

The activity assay was carried out optimizing the procedure previously reported.[25,26] In a 1.5 mL quartz cuvette with a 1-cm light path, KH_2PO_4 buffer (0.5 M at pH 7.0; 400 μL), 20 mM inosine (100 μL), xanthine oxidase (0.2 U/mL, 100 μL) and 300 μL of deionized water were mixed to allow a final volume of 0.9 mL. The reaction was started by addition of a solution of PNP (100 μL). The activity was calculated from the increase in absorbency at 300 nm (ε = 8828).

Activity Assay of UP

The activity assay was carried out optimizing the procedure previously reported.[27] In a final volume of 830 μL, KH_2PO_4 buffer (0.5 M at pH 7.4, 230 μL), 2′-deoxyuridine (100 mM, 50 μL) and deionized water (550 μL) were mixed. After the addition of a solution of UP (100 μL), the mixture was incubated at 37 °C for 15 minutes. The reaction was stopped by addition of 10 M NaOH (70 μL). The test solution was read against its control at 297 nm in a cuvette of 1-cm light path (ε = 1912).

General Procedure for the Enzymatic Synthesis of 2′-Deoxyinosine (2′-dI)

A solution of 10 mM K_2CO_3 buffer at pH 10 (25 mL) containing 570.5 mg of 2′-deoxyuridine, 2.5 mL of hypoxanthine solution (50 mM) in the same buffer and 68 mg of KH_2PO_4 was kept at 45 °C under mechanical stirring. The final pH was set up to 10 with dilute NaOH. The Sep-PEI-DX derivative of UP (11 Units) and PNP immobilized on glyoxyl-agarose gel (19 Units) were added to the suspension. The remaining solution of hypoxanthine (22.5 mL) was automatically added dropwise to the reaction mixture at the rate of 0.166 mL/min. The reaction was monitored by HPLC. The column was an RP select B Lichrocart 60 C8 (Merck, Darmstadt, Germany); eluent: 0.02 M KH_2PO_4 buffer/90% MeOH (97:3); flow: 1 mL/min.; λ: 260 nm; T = 35 °C. The suspension was stirred for 24 h and then stopped by filtration of the immobilized enzymes under reduced pressure (2′-I, r.t. 10.77 min.); yield: 85%.

General Procedure for the Enzymatic Synthesis of 2′-Deoxyguanosine (2′-dG)

A solution of 10 mM K_2CO_3 buffer at pH 10 (25 mL) containing 570.5 mg of 2′-deoxyuridine, 0.375 mL of guanine solution (50 mM at pH 11) in the same buffer and 68 mg of KH_2PO_4 was kept at 45 °C under mechanical stirring. The final pH was set up to 10 with dilute NaOH. The Sep-PEI-DX derivative of UP (14 Units) and PNP immobilized on glyoxyl-agarose gel (30 Units) were added to the suspension. The remaining solution of guanine (24.625 mL) was automatically added dropwise to the reaction mixture over 8 h (rate: 0.02–0.04 mL/min). The pH was kept at 10 with 2 N HCl by automated titration. The reaction was monitored by HPLC. The column was an RP select B Lichrocart 60 C8 (Merck, Darmstadt, Germany); eluent: 0.02 M KH_2PO_4 buffer/90% MeOH (97:3); flow: 1 mL/min.; λ: 260 nm; T = 35 °C. The suspension was stirred for 24 h and then stopped by filtration of the immobilized enzymes under reduced pressure. After washing the derivatives with 10 mM K_2CO_3 buffer (pH 10), the solution was cooled to 4 °C and slowly acidified to pH 5.5 until the formation of a white precipitate. The solid was filtered and characterized by HPLC as 2′-deoxyguanosine (r.t. 12.06 min., purity 97%); yield: 92%.

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