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Developing a Collection of Immobilized Nucleoside Phosphorylases for the Preparation of Nucleoside Analogues: Enzymatic Synthesis of Arabinosyladenine and 2′,3′-Dideoxyinosine

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The use of nucleoside phosphorylases (NPs; EC 2.4.2.n) represents a convenient alternative to the chemical route for the synthesis of natural and modified nucleosides. We purified four recombinantly expressed nucleoside phosphorylases from the bacterial pathogens *Citrobacter koseri*, *Clostridium perfringens*, and *Streptococcus pyogenes* (*Ck*PNPI, *Ck*PNPII, *Cp*UP, *Sp*UP) and their substrate specificity was investigated towards either natural pyrimidine or purine nucleosides and some analogues, namely, arabinosyladenine (araA) and 2′,3′-dideoxyinosine (ddI). A 2–3% activity towards these latter compounds (compared to the natural substrates) was observed. Enzyme activities were compared to the specificities obtained for the enzymes pyrimidine nucleoside phosphorylase from *Bacillus subtilis* (*Bs*PyNP) and purine nucleoside phosphorylase from *Aeromonas hydrophila* (*Ah*PNPII) previously reported by some of the authors. The enzymes displaying the suitable specificity for the synthesis of araA and ddI were immobilized on aldehyde–agarose. The immobilized preparations were highly stable at alkaline pH and in the presence of methanol or acetonitrile as cosolvent. They were used in the synthesis of araA and ddI by a one-pot, bienzymatic transglycosylation achieving 74 and 44% conversion, respectively.

Introduction

Nucleoside phosphorylases (NPs; EC 2.4.2.n) are important intracellular enzymes distributed widely in eukaryotes and prokaryotes. They act in the salvage of nucleobases and catalyze the reversible conversion of (deoxy)ribonucleosides to their corresponding free base and alfa-D-(deoxy)ribose-1-phosphate in the presence of inorganic orthophosphate (phosphorolysis).

NPs are divided into two main structural classes: NPI and NPII. Enzymes of the NPI family share a conserved structural fold and include both the trimeric purine nucleoside phosphorylase (PNPI) and the hexameric purine nucleoside phosphorylase (PNPII), and uridine phosphorylase (UP). Trimeric PNPI is specific for guanine and hypoxanthine (2′-deoxy)ribonucleosides, whereas hexameric PNPII also accepts adenine nucleosides.[1] The members of the NPII family (thymidine phosphorylase, TP, and pyrimidine nucleoside phosphorylase, PyNP) are structurally distinct from the NPI class and are specific for pyrimidine nucleosides.[2]

The potency of NPs for the biocatalytic synthesis of nucleoside analogues is primarily due to the advantages that these enzymes provide over the commonly used complex and multistage chemical methods. In fact, the reaction reversibility of NPs can be exploited to perform a transglycosylation reaction: if a second nucleobase (sugar acceptor), either natural or not, is added to the reaction medium, the formation of a new nucleoside can result (Scheme 1).[3] Most examples of nucleosides synthesized through biocatalytic routes employ whole cells as catalysts.[3–7] However, it has been demonstrated that isolated and immobilized UP/PyNP and PNP, simultaneously used in a so-called one-pot cascade reaction, show such an efficiency that it could form the basis of nucleoside-production strategies.[8,9]

Despite the efforts made so far extending the application of NPs to large-scale production, nucleoside analogues are still largely synthesized by "conventional" chemical methods. In particular, limitations of NPs are currently related to their strict substrate specificity (that narrows the number of possible applications),[10] but protein stability is also a crucial issue: reaction productivity greatly improves upon high temperatures, high pH, and the presence of cosolvents ensuring solubilization of a large amount of substrates and products.[9] For these reasons, the research in this field focuses mainly on the iden-

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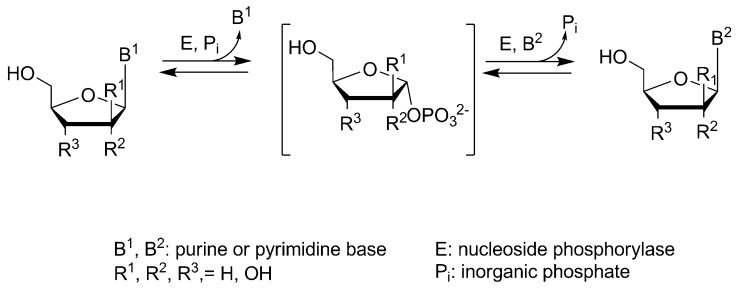


Scheme 1. General scheme of the transglycosylation reaction catalyzed by NPs.

tification of new enzymes with suitable specificity and their subsequent stabilization.

Generally speaking, microorganisms are preferred to mammalian NP sources because of their higher substrate promiscuity and, in recent years, a number of microbial NPs have been identified, cloned, and expressed.[1,11,12] In this context, microorganisms from extreme environments are also being sought; it is hardly surprising, for example, that thermostable enzymes are attractive alternatives to their thermolabile counterparts in many biocatalytic applications.[13–16]

However, the most widespread procedure for enzyme stabilization is immobilization on solid supports. Immobilization of biocatalysts also allows many other enzyme limitations to be addressed, such as their solubility and reuse. In addition, when properly designed, immobilization may be effective in modulating activity or selectivity.[17]

Only a few examples of the immobilization of NPs have been reported so far. One of the most efficient techniques for NP immobilization is ionic adsorption on carriers coated with polyethylenimine (PEI) followed by cross-linking with aldehyde dextran. This approach, which prevents enzyme desorption, was successful in the case of thymidine phosphorylase from *Escherichia coli* (*Ec*TP) and pyrimidine nucleoside phosphorylase from *Bacillus subtilis* (*Bs*PyNP), both belonging to the NPII family.[18,19] The adsorption on the highly flexible PEI-coated carrier does not hamper the domain movement required during catalysis and the multimeric quaternary structure is preserved by the final cross-linking with oxidized dextran.[18,20] Immobilized *Ec*TP and *Bs*PyNP have been conveniently assayed in enzymatic transglycosylation.[9,19]

For many years, our group has been involved in research aimed at developing a collection of solid and stable biocatalysts endowed with substrate specificities suitable for the synthesis of nucleoside analogues. In this vein, the use of a PNP from *Aeromonas hydrophila* (*Ah*PNPII) in the preparative synthesis of 6-modified purine ribonucleosides has been recently reported.[21]

In the present study, the specificity of *Bs*PyNP and *Ah*PNPII has been investigated further by considering nucleosides bearing unnatural sugars useful for the synthesis of some chemotherapeutic drugs. In addition, the search for new enzymes active on modified nucleosides has been considered. Thus, four more microbial NP genes have been isolated, cloned, and the recombinant enzymes expressed and studied. In particular, the following enzymes were prepared: *Clostridium perfringens* uridine phosphorylase (*Cp*UP), *Streptococcus pyogenes* uridine phosphorylase (*Sp*UP), *Citrobacter koseri* purine nucleoside phosphorylase I (*Ck*PNPI), and *Citrobacter koseri* purine nucleoside phosphorylase II (*Ck*PNPII).

The immobilization on solid carriers of the most promising enzymes has been investigated to obtain stable biocatalysts suitable for easy and convenient use in preparative processes. The immobilized biocatalysts were then exploited for the synthesis of the two antiviral drugs arabinosyladenine (araA) and 2′,3′-dideoxyinosine (ddI) through one-pot transglycosylation.

Conventional chemical syntheses of araA and ddI require several protection and deprotection steps that result in tedious procedures and moderate to poor yields of the target compounds.[22,23] In the case of ddI, for example, this nucleoside can be synthesized by direct 5′-*O*-benzoylation of 2′-deoxyinosine, followed by deoxygenation at the 3′-position and benzoyl-group removal in about 20% yield overall.[22] Alternative synthetic routes have been developed over the years with the aim to achieve more straightforward methods as well as safer and cleaner reaction conditions.[24–26] In spite of a few advancements, ddI is still synthesized through laborious schemes in moderate to good yields (30–60%).

Results and Discussion

Enzyme production and substrate specificity

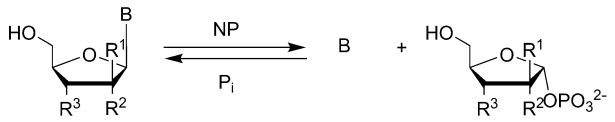
Hypothetic genes coding four new nucleoside phosphorylases from bacterial sources (*Clostridium perfringens*, *Streptococcus pyogenes*, and *Citrobacter koseri*) were amplified from the genomic DNA using primers designed on the basis of the corresponding genome sequences reported in the National Center for Biotechnology Information (NCBI) database. After expression, all the proteins were purified by affinity chromatography. Enzyme purity (>90%) was estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

The four recombinant enzymatic activities were evaluated versus a range of nucleoside compounds (Scheme 2). Table 1 shows the enzymatic activities of the *Clostridium perfringens*

Table 1. The activity of *Cp*UP, *Sp*UP, and *Bs*PyNP towards pyrimidine substrates.[a] The percentage of conversion relative to the values for 2′-deoxyuridine are given in parentheses.

Substrate	*Cp*UP [IU mg^{-1}] (%)	*Sp*UP [IU mg^{-1}] (%)	*Bs*PyNP [IU mg^{-1}] (%)
uridine (**1**)	60 (88)	11 (50)	20 (133)
2′-deoxyuridine (**2**)	68 (100)	22 (100)	15 (100)
thymidine (**3**)	31 (46)	18 (82)	6 (40)
arabinosyluracil (**4**)	**3.0** (**4.0**)	**0.4** (**1.8**)	n.a.
2′,3′-dideoxyuridine (**5**)	**2.0** (**3.0**)	**1.7** (**7.8**)	n.a.
cytidine (**6**)	n.a.	n.a	n.a.

[a] IU: μmol min^{-1}. Experimental conditions: 50 mM phosphate buffer pH 7.5, RT, 10 mL, [substrate] 5 mM; n.a.: not active.



Nucleoside	R^1	R^2	R^3	B
1 (uridine)	H	OH	OH	uracil
2 (2′-deoxyuridine)	H	H	OH	uracil
3 (thymidine)	H	H	OH	thymine
4 (arabinosyluracil)	OH	H	OH	uracil
5 (2′,3′-dideoxyuridine)	H	H	H	uracil
6 (cytidine)	H	OH	OH	cytosine
7 (adenosine)	H	OH	OH	adenine
8 (2′-deoxyadenosine)	H	H	OH	adenine
9 (arabinosyladenine)	OH	H	OH	adenine
10 (guanosine)	H	OH	OH	guanine
11 (2′-deoxyguanosine)	H	H	OH	guanine
12 (inosine)	H	OH	OH	hypoxanthine
13 (2′-deoxyinosine)	H	H	OH	hypoxanthine
14 (2′,3′-dideoxyinosine)	H	H	H	hypoxanthine

Scheme 2. Substrates (**1**–**14**) submitted to phosphorolysis catalyzed by NPs.

and the *Streptococcus pyogenes* UPs together with the values obtained for the *Bacillus subtilis* PyNP enzyme.

As expected, none of the phosphorylases tested could convert cytidine (**6**). The *Cp*UP and *Sp*UP enzymes accepted all the remaining naturally available pyrimidine nucleoside substrates. It is worth noting that these enzymes also converted 2′,3′-dideoxyuridine (**5**) and arabinosyluracil (**4**), which bear unnatural sugars. In comparison with 2′-deoxyuridine (**2**), the activity decreased by thirtyfold presumably as a consequence of the inversion of the configuration at the C-2′ position or of the lack of a hydroxyl group in C-3′ (Table 1).

In Table 2, the activities obtained for the purine NPs relative to inosine (**12**) conversion are shown together with the values for *Aeromonas hydrophila* PNPII. All PNPs showed high activities with ribo- and 2′-deoxyribonucleosides. PNPII from *A. hydrophila* and *C. koseri* presented very similar profiles for their activities towards all the purine substrates tested and both enzymes accepted arabinosyladenine (**9**). These activities were about forty times less than the activity obtained for adenosine (**7**). The highest turnover for these enzymes was observed using 2′-deoxyinosine (**13**).

Table 2. The activity of *Ah*PNPII, *Ck*PNPII, and *Ck*PNPI towards purine substrates.[a] The percentage of conversion relative to the values for inosine are given in parentheses.

Substrate	*Ah*PNPII [IU mg^{-1}] (%)	*Ck*PNPII [IU mg^{-1}] (%)	*Ck*PNPI [IU mg^{-1}] (%)
adenosine (**7**)	35 (46)	15 (45)	n.a.
2′-deoxyadenosine (**8**)	28 (37)	21 (63)	n.a.
arabinosyladenine (**9**)	**0.9** (**1.2**)	**0.8** (**2.4**)	n.a.
guanosine[b] (**10**)	26 (35)	11 (33)	14 (32)
2′-deoxyguanosine[b] (**11**)	28 (37)	46 (139)	23 (50)
inosine (**12**)	75 (100)	33 (100)	46 (100)
2′-deoxyinosine (**13**)	137 (182)	108 (327)	36 (78)
2′,3′-dideoxyinosine (**14**)	0.02 (0.03)	0.16 (0.5)	**1.6** (**3.4**)

[a] IU: μmol min^{-1}. n.a.: not active. Experimental conditions: 50 mM phosphate buffer pH 7.5, RT, 10 mL, [substrate] 5 mM. [b] 1 mM.

*Ck*PNPI can be described as specific towards inosine/guanosine as it was inactive with adenine nucleosides. This enzyme showed the highest activity in the phosphorolysis of inosine (**12**) and a progressive reduction of its activity was registered when first the hydroxyl group in the C-2′ and then in the C-3′ positions were removed. However, this enzyme retains a certain activity also towards 2′,3′-dideoxyinosine (**14**; Table 2).

Immobilization of AhPNPII: A key study

Since *Ah*PNPII had been proven already to have an outstanding potential for synthetic purposes owing to its activity towards modified purine ribonucleosides,[21] and herein towards arabino- and dideoxynucleoside analogues, it was selected for the first immobilization trial. The immobilization techniques resulting in a balance between activity and stability were then applied also to *Ck*PNPI and *Cp*UP.

Stability of the soluble enzyme

The stability of the soluble enzyme was determined under several conditions useful for immobilization, including the presence of stabilizing agents.

In the case of immobilization on aldehyde carriers, alkaline conditions (pH 10) are required to promote an efficient enzyme-carrier interaction.[27] This procedure relies on the formation of Schiff bases between the enzyme amino groups (that is mostly surface lysine (Lys)) and the support aldehydes, followed by reduction of the weak imino bonds with $NaBH_4$ into stable C—N bonds. When working at high pH, Lys residues are not ionized and can thus react with the aldehyde groups of the carrier. The results obtained are shown in Figure 1: at pH 10 and room temperature the soluble enzyme loses 100% of its activity after 1 h but, by lowering the temperature to 4 °C, an increase of stability was achieved.

The influence of glycerol, known as a stabilizing and preservative compound,[28,29] was evaluated. The enzyme stability improved in the presence of an increasing concentration of this cosolvent (Figure 1). The presence of 20% glycerol (v/v) ensured the almost complete stability of *Ah*PNPII at pH 10 and room temperature.

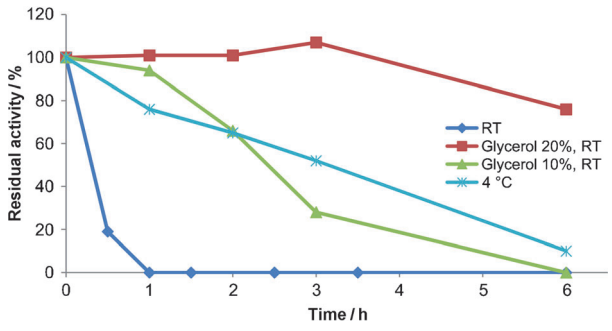


Figure 1. Stability of soluble *Ah*PNPII at pH 10: influence of the temperature and glycerol. [*Ah*PNPII] = 0.06 mg mL^{-1} (corresponding to 0.3 mg of enzyme in the assay and 22.5 IU).

Immobilization

We explored different immobilization carriers and techniques, while bearing in mind the multimeric structure expected for *Ah*PNPII. In fact, other NPs characterized by the same substrate specificity (for adenine, hypoxanthine, and guanine nucleosides) are reported to be hexameric.[2] Both ionic adsorption and covalent binding were taken into account for immobilization. Moreover, post-immobilization cross-linking was considered to induce the stabilization of the multimeric structure of the protein.

Adsorption on an ionic support followed by cross-linking with aldehyde polymers was considered first, according to the results previously obtained on the stabilization of other multimeric enzymes.[18,20] The ionic interaction requires mild immobilization conditions (5 mM phosphate buffer, pH 7.5) that do not affect the enzyme activity. In particular, Sepabeads coated with polyethylenimine (PEI) were selected as the ionic support to combine very mild reaction conditions with the strong ionic interaction exerted by the polyamine.[18] As reported in Table 3, about 80% of the enzyme was adsorbed and the recovered activity after ionic adsorption was complete (results not shown). The post-immobilization cross-linking was then performed with aldehyde dextran. To ensure a full coating of all the enzyme subunits we studied dextran with different molecular weights. The best results were obtained with 100 kDa dextran that afforded a 20% recovered activity after cross-linking (entry 2, Table 3), whereas with higher and lower molecular weights the immobilization yields decreased (13 and 12% with 20 and 500 kDa dextran, respectively; entries 1 and 3, Table 3).

Among covalent linkages, the attention was focused on aldehyde activation since it was shown to be suitable for the immobilization/stabilization even of multimeric enzymes[27,30] including trimeric PNPI from *Bacillus subtilis*.[18] Both hydrophobic and hydrophilic matrices were considered. When using the hydrophobic aldehyde–Sepabeads, 93% of the protein was immobilized after 1.5 h at 4 °C (to ensure suitable enzyme stability), but the expressed activity was very poor (7% yield; entry 4, Table 3).

Aldehyde–agarose was considered as the support and different immobilization conditions, such as temperature and the presence of stabilizing cosolvents, were assayed (Table 3). In all cases, immobilization proceeds very fast and after 1.5 h almost 100% of the protein was immobilized. When immobilization was performed at 4 °C for 1.5 h, a yield of 21% was achieved (entry 5, Table 3). This value was threefold higher than that obtained after immobilization on aldehyde–Sepabeads under the same conditions. The carrier activation is equal, which highlights the detrimental effect of the hydrophobic nature of Sepabeads on the enzyme stability.

To enhance the formation of a high number of bonds between the support and the enzyme, two strategies were pursued. First, the contact time at 4 °C was increased up to 3 h, but afforded only 13% yield (entry 6, Table 3). This result can be reasonably ascribed to the poor stability of the soluble enzyme under these conditions, retaining the 60% of its initial activity (Figure 1).

Second, the immobilization was performed at room temperature in the presence of 20% glycerol. This choice was addressed by the stability investigation described above (see Figure 1): the enzyme is completely stable after 3 h of incubation (that is, the immobilization time) and retains 80% of its initial activity after 6 h. Following the latter approach, a 20% immobilization yield was achieved (entry 7, Table 3). However, the crucial step is represented by the final reduction with $NaBH_4$ that is necessary, at the end of the process, to convert the imino bonds into stable C–N bonds. In fact, the enzyme retained 45% of the initial activity after 3 h of interaction with the activated carrier (Figure 1 in the Supporting Information) but chemical reduction decreased the final yield by half (Table 3).

Owing to the promising results achieved with aldehyde–agarose, this immobilization protocol was subjected to a further optimization. It is known that the addition of an inhibitor or a substrate to the immobilization suspension can prevent possible distortions of the enzyme structure upon interaction with the activated carrier. In fact, the ligand can shield the active site by binding to it during immobilization. In this case, when hypoxanthine was added to the immobilization medium, after 3 h, the yield increased to 30% (entry 8, Table 3). The cross-linking of the resulting preparation with oxidized 100 kDa dextran was not beneficial to the outcome of the process and the yield was very low (8%; entry 9, Table 3).

Table 3. Immobilization of *Ah*PNPII.[a]

Entry	Support	Activation	*T* [°C]	pH	Time	Cross-linking [kDa] (h)	Additives	Immobilized protein [%][b]	Yield [%][c]
1	Sepabeads	PEI	RT	7.5	20 min	20 (1)	–	78	13
2	Sepabeads	PEI	RT	7.5	20 min	100 (1)	–	83	21
3	Sepabeads	PEI	RT	7.5	20 min	500 (1)	–	74	12
4	Sepabeads	aldehyde	4	10	1.5 h	–	–	93	7
5	agarose	aldehyde	4	10	1.5 h	–	–	100	21
6	agarose	aldehyde	4	10	3 h	–	–	96	13
7	agarose	aldehyde	RT	10	3 h	–	glycerol	100	20
8	agarose	aldehyde	RT	10	3 h	–	glycerol, hpx[d]	100	30
9	agarose	aldehyde	RT	10	3 h	100 (6)	glycerol, hpx[d]	100	8
10	agarose	glutaraldehyde	RT	8.5	3 h	–	–	100	2.5
11	agarose	glutaraldehyde	RT	7	3 h	–	–	96	0
12	agarose	glutaraldehyde	RT	8.5	3 h	–	glycerol	100	3
13	agarose	glutaraldehyde	4	8.5	3 h	–	–	100	17

[a] Load: 1 mg g^{-1}. [b] Determined by the Bradford method. [c] On the basis of the expressed activity considering inosine as the reference substrate. [d] 5 mM.

As a final approach, agarose activated with glutaraldehyde, which relies on a very mild immobilization procedure, was considered. Although the soluble enzyme is completely stable in the immobilization buffer (data not shown), only the immobilization performed at 4 °C gave positive results, with an immobilization yield of 17% (entry 13, Table 3). Differently from aldehyde–agarose, the activity of the suspension, monitored during immobilization on glutaraldehyde–agarose, drastically decreased within the first hour of immobilization (Figure 1 in the Supporting Information). This fact leads us to hypothesize that the contact with the glutaraldehyde–agarose somehow induces the enzyme inactivation.

Stability of the immobilized preparations

The immobilized preparations were tested for their stability at pH 10 (see Figure 2 and Table 3). In spite of what was expected, *Ah*PNPII immobilized on Sepabeads–PEI (entry 2, Table 3) and covered with 100 kDa dextran showed very poor stability,

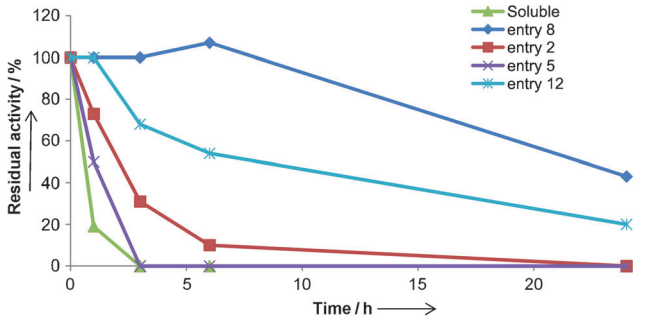


Figure 2. Stability at pH 10 of the soluble and immobilized *Ah*PNPII (referring to Table 3 entries).

slightly higher than that of the soluble enzyme; the aldehyde–agarose derivative immobilized at 4 °C for 1.5 h (entry 5, Table 3) displayed the same behavior. On the contrary, the same derivative immobilized at room temperature for 3 h in the presence of 20% glycerol (entry 8, Table 3) showed very high stability, retaining completely its activity after 6 h.

These data confirm that the length of time of contact and the reaction temperature are important parameters that need to be considered in the design of a covalent immobilization. In fact, by increasing the temperature and reaction time a multipoint attachment is induced, which leads to an increase in stability of the immobilized preparation.

Moreover, the stability of the best aldehyde–agarose preparation (see entry 8 in Table 3) was tested also in the presence of organic solvents, 20% acetonitrile (MeCN) or methanol (MeOH), and compared with that of the soluble counterpart. The immobilized enzyme was found to be several times more stable than the native one, in particular when incubated in the presence of MeCN (Figure 2 in the Supporting Information). However, in general, MeOH was a better solvent than MeCN for enzyme stability.

Immobilization of CkPNPI and CpUP

The aldehyde–agarose was found to be the best matrix so its applicability for the immobilization of other multimeric NPs (*Cp*UP and *Ck*PNPI) was studied. Both enzymes in the native form were stable, even in the absence of stabilizing agents, at pH higher than 10 up to 24 h (data not shown). For *Cp*UP, the immobilization yield was 33% at room temperature and pH 10. The presence of either uracil or 20% glycerol in the immobilization medium did not have any effect on the immobilization outcome. Also for this enzyme the glutaraldehyde activation afforded very poor results (Table 4). Immobilization of *Ck*PNPI provided higher yields than the other NPs, affording 51 and 35% of recovered activity for aldehyde and glutaraldehyde activation, respectively.

Table 4. Immobilization of *Cp*UP and *Ck*PNPI on aldehyde- and glutaraldehyde-activated agarose.

Enzyme	Activation	Load [mg g^{-1}]	Additives	Immobilized protein [%][a]	Yield [%][b]
*Cp*UP	aldehyde	1.4	glycerol	94	23
*Cp*UP	aldehyde	1.5	–	100	33
*Cp*UP	aldehyde	1.2	uracil[c]	100	35
*Cp*UP	glutaraldehyde[d]	1.0	–	100	4
*Ck*PNPI	aldehyde	0.9	glycerol	100	51
*Ck*PNPI	glutaraldehyde[d]	1.2	–	98	35

[a] Determined by the Bradford method. [b] On the basis of the expressed activity considering as reference substrates inosine and 2′-deoxyuridine for PNP and UP, respectively. [c] 5 mM. [d] Experimental conditions: 100 mM phosphate buffer, pH 8.5 and 4 °C.

The results obtained with aldehyde–agarose are consistent with those of *Ah*PNPII and agree with those previously reported for *Bs*PNPI.[18]

In terms of the stability in organic solvents, both aldehyde preparations of *Ck*PNPI and *Cp*UP maintained 100% of their initial activity after incubation for 24 h in the presence of 20% MeCN and MeOH (results not shown).

The higher immobilization yields obtained for PNPI, relative to *Cp*UP and *Ah*PNPII, may be ascribed to the simpler quaternary structure of the enzymes belonging to this class.[1]

Overall, the high performance of agarose as immobilization carrier for NPs regardless of the complexity of their quaternary structure can be explained by invoking the internal morphology of agarose beads. It can be assumed that this support is formed by fibers with a diameter thicker than a protein and, thus, it is able to offer both large surfaces for protein-support multi-interactions and to enhance a better geometric congruence between a large multimeric enzyme and the support.[27]

The different strategies developed for NPI and NPII can be tentatively explained by considering the diversity of catalytic requirements between these classes. In fact, NPsII have been successfully immobilized on a flexible ionic carrier followed by cross-linking with aldehyde dextran.[18,19] This result may be in-

terpreted as being a consequence of the peculiar catalytic mechanism of the enzymes belonging to this class. For instance, the crystal structure of *Ec*TP reveals an asymmetric dimer in which one subunit is in an open conformation and the second is in a closed substrate-bound form. The reaction mechanism is reported to involve significant movement of structural domains for the opening and closing of each subunit. The immobilization approach on Sepabeads–PEI likely allows the preservation of this domain movement (and the catalytic activity thereof). On the other hand, NPsI, which do not require any domain movement, were efficiently immobilized and stabilized through the more constraining covalent bonds on aldehyde agarose.

We also wondered whether the His6 tag might influence the immobilization yield and, toward this aim, new experiments were carried out. The His6 tag was cleaved from the purified *Ah*PNPII with Ac-TEV protease (see the Experimental Section) and the recovered wild-type protein (which had a specific activity comparable with that of the tagged enzyme) was immobilized on aldehyde–agarose. The cleaved protein showed similar percentages of immobilized protein (determined by Bradford method) and final yield (percentage of expressed activity) to the tagged enzyme (Table 5).

Table 5. Optimization of the immobilization on aldehyde–agarose.

Enzyme	Load [mg g^{-1}]	Additives	Immobilized protein [%][a]	Yield [%][b]
*Ah*PNPII non-tagged	0.1	–	77	17
*Ah*PNPII	0.9	–	100	21
*Ah*PNPII	1.0	glycerol, hpx	100	30
*Ah*PNPII	5.0	glycerol, hpx	100	17
*Cp*UP non-tagged	1.5	–	100	33
*Cp*UP	1.0	–	100	41
*Cp*UP	5.0	–	100	19
*Ck*PNPI	1.0	glycerol	100	51
*Ck*PNPI	5.0	glycerol	96	43

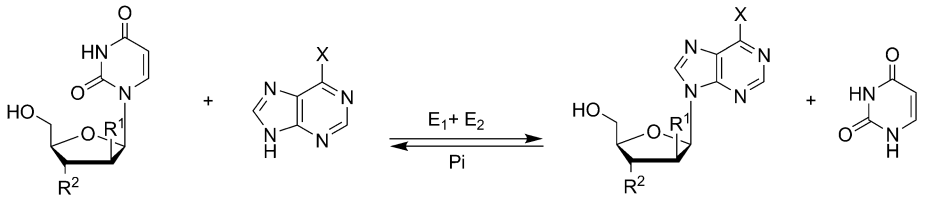
[a] Determined by the Bradford method. [b] On the basis of the expressed activity considering as reference substrates inosine and 2′-deoxyuridine for PNP and UP, respectively.

Similar outcomes were obtained with the native *Cp*UP obtained by cleavage of the glutathione S-transferase (GST) tag with thrombin. We can thus conclude that the presence of the tag does not influence the immobilization process on aldehyde–agarose.

Afterwards, the load of protein per gram of support was increased from 1 to 5 mg. As expected, in all cases, the expressed activity was sensitively reduced when a high amount of protein was offered per each gram of carrier (Table 5).

Synthesis of araA (9) and ddI (14)

By selecting the enzymes with the suitable substrate specificity, the one-pot syntheses of araA (**9**) and ddI (**14**) were carried out using araU and ddU as sugar sources, respectively (Scheme 3 and Table 6). The effect of a cosolvent on the maximum conversion and reaction rate was evaluated: the addition of organic solvents is indeed often necessary to ensure the complete solubilization of high concentrations of reagents and products. Thus, according to the stability of the different enzymes, MeOH and MeCN were tested; the former showed no relevant influence on the percentage of product formed, whereas MeCN induced a slight increase of the conversion, which was more evident for ddI. Under these conditions, and increasing the substrate concentration, the conversions achieved were 74 and 44% of araA and ddI, respectively (Table 6).



4 $R^1 = R^2 = OH$ X = NH_2 **adenine** **9** $R^1 = R^2 = OH$; X = NH_2
5 $R^1 = R^2 = H$ X = OH **hypoxanthine** **14** $R^1 = R^2 = H$; X = OH

E_1 = *Cp*UP
E_2 = *Ah*PNPII (araA) or *Ck*PNPI (ddI)

Scheme 3. Synthesis of araA and ddI by transglycosylation catalyzed by NPs.

Table 6. One-pot bienzymatic transglycosylation reaction for the synthesis of araA and ddI.[a]

Donor (mM)	Acceptor (mM)	Solvent[b]	Product	% Conv. (h)
4 (4)	Ade (2)	–	**9**	63 (48)
4 (4)	Ade (2)	MeOH	**9**	63 (48)
4 (4)	Ade (2)	MeCN	**9**	69 (48)
4 (10)	Ade (5)	MeOH	**9**	71 (48)
4 (10)	Ade (5)	MeCN	**9**	74 (48)
5 (4)	Hpx (2)	–	**14**	26 (24)
5 (4)	Hpx (2)	MeOH	**14**	30 (24)
5 (4)	Hpx (2)	MeCN	**14**	34 (24)
5 (10)	Hpx (5)	MeCN	**14**	44 (24)

[a] Experimental conditions: 50 mM phosphate buffer, pH 7.5, RT, 10 mL; *Cp*UP (6 IU): in the synthesis of araA and ddI *Ah*PNPII (12 IU) and *Ck*PNPI (12 IU) were used, respectively; one IU is referred to inosine for *Ah*PNPII and *Ck*PNPI and to 2′-deoxyuridine for UP. [b] 10% v/v.

Conclusion

New nucleoside phosphorylases have been prepared, partly characterized, and tested for their use in the syntheses of araA and ddI. Uridine phosphorylases from the bacterial pathogens

C. perfringens and *S. pyogenes* were shown to convert arabinosyluracil and 2′,3′-dideoxyuridine. In addition, purine nucleoside phosphorylases from *A. hydrophila* and *C. koseri* showed some activity for arabino- and 2′,3′-dideoxypurine nucleosides, which indicates that transglycosylation reactions for the formation of nucleoside analogues can be performed by a mixture of *Cp*UP or *Sp*UP and any of these PNPs. Pyrimidine nucleoside phosphorylase from *B. subtilis* did not show activity on pyrimidine nucleosides bearing arabinose and 2′,3′-dideoxyribose as sugar moieties. The studied enzymes (with the exception of *Ck*PNPII and *Sp*UP) were successfully immobilized and stabilized by covalent attachment on aldehyde-activated agarose. The immobilization protocol described here allowed the stabilization of all the studied enzymes regardless of their multimeric asset. Immobilization of NPI on aldehyde–agarose can be considered, therefore, a technique of general applicability within this class of enzymes. The results reported in this paper, in addition to our previous work,[18,19,21] provide a collection of stable solid biocatalysts for the enzymatic synthesis of modified nucleosides by transglycosylation. By selecting an appropriate enzyme within this collection (depending on the structure of the substrates) sugar- or base-modified nucleosides can be prepared. In the case of araA and ddI, the reaction optimization, scale-up, and product isolation are in progress.

Experimental Section

General

All nucleosides, heterocyclic bases, solvents, polyethylenimine (PEI), glutaraldehyde 25% (v/v), ethylendiamine (EDA), and dextran were purchased from Sigma Aldrich and/or VWR International (Milano, Italy). Sepabeads were a gift from Resindion (Mitsubishi, Binasco, Italy). Agarose CL6B was purchased from Pharmacia Biotech AB (Uppsala, Sweden). All solvents were HPLC grade. Enzymatic reactions were monitored by using a HPLC Merck Hitachi L-7100 instrument equipped with a UV detector L-7400 and column oven L-7300 (Darmstadt, Germany). PfuUltra DNA polymerase was from Stratagene (Agilent Technologies, Life Sciences and Chemical Analysis, Waldbronn, Germany). The pET/TOPO 151 vector was purchased from Invitrogen (San Giuliano Milanese, Italy) and the recombinant proteins were purified using Protino Ni-TED 1000 packed columns (MACHEREY-NAGEL, Düren, Germany) and a glutathione (GSH) column (Amersham Pharmacia, Uppsala, Sweden). The protein-concentration assay was performed on a Shimadzu spectrophotometer UV 1601 by using the Bradford method[31] using bovine serum albumin as standard. PyNP from *Bacillus subtilis* and *Ah*PNPII were prepared as previously reported.[18,21] All experiments were performed at least in duplicate. See the Supporting Information for details of the bacterial strains. *C. koseri* genomic DNA was extracted according to the protocol previously used for *A. hydrophila*.[21]

Cloning, expression and purification of the recombinant proteins

*Ah*PNPII, *Cp*UP, *Sp*UP, *Ck*PNPI, and *Ck*PNPII were prepared as fusion proteins with an N-terminal His6 tag; *Cp*UP was obtained also as a GST fusion protein. In addition, *Ah*PNPII and *Cp*UP were prepared as native proteins by cleavage of the tags with Ac-TEV or thrombin proteases, respectively. The putative NP genes were identified by using the basic local sequence alignment tool (BLAST).[32] The corresponding open reading frames were amplified from the genomic DNA by the polymerase chain reaction (PCR) using PfuUltra DNA polymerase (Stratagene) and primers (listed in the Supporting Information) with overhangs specific for cloning into the pET/TOPO 151 vector (Invitrogen).

The N-terminal His6 tag fusion proteins were purified according to the procedure reported by Ubiali et al.[21] The final concentration of the protein preparation determined by means of the Bradford method was in the range of 2–5 mg mL^{-1}.

In addition, *Cp*UP was also cloned into a pGEX-2T vector (GE Healthcare). The primers used are reported in the Supporting Information.

Purification and cleavage with thrombin of GST-*Cp*UP for preparation of native *Cp*UP

*Cp*UP over-expressed as the fusion protein with an N-terminal GST fusion tag was purified by glutathione–Sepharose affinity chromatography followed by thrombin cleavage. Purification was performed by slightly modifying the procedure reported by Knecht et al.[33] Briefly, the crude extract was loaded onto a 1 mL GSH column pre-equilibrated in binding buffer (20 mM Na_3PO_4, pH 7.3; 150 mM NaCl; 10% glycerol and 0.1% polyethylene glycol *p*-(1,1,3,3-tetramethylbutyl)phenyl ether (Triton X-100)); then the column was washed with binding buffer (20 mL). Thrombin cleavage was performed by applying binding buffer (1 mL) containing thrombin (50 IU) on the column and recirculating O/N at 4 °C. The native *Cp*UP was eluted from the column with binding buffer.

Cleavage with Ac-TEV™ protease for preparation of native *Ah*PNPII

In a total volume of 1 mL, the tagged protein (0.6 mg) was suspended in tris(hydroxymethyl)aminomethane hydrochloride (Tris HCl; 50 mM), pH 8.0, containing ethylenediaminetetraacetic acid (EDTA; 0.5 mM) and DL-dithiothreitol (DTT; 1 mM). Then Ac-TEV protease (20 IU) was added; the mixture was incubated under O/N with gentle stirring at 4 °C. The His6-tag fragment and the Ac-TEV protease were removed by loading the mixture on Protino Ni-TED 1000 packed columns. The cleaved native protein was in the flow-through fractions. The collected fractions were analyzed by SDS-PAGE and the protein concentration was determined by the Bradford method.

HPLC standard activity assay (phosphorolysis)

A solution of potassium buffer pH 7.5 (50 mM, 10 mL) containing the desired concentration of nucleoside substrate was prepared. The reaction was started by the addition of the enzyme (soluble or immobilized) and kept under mechanical stirring. At different times, samples were withdrawn, filtered (either by centrifugation with 10 kDa molecular weight cutoff (MWCO) Nanosep at 4 °C and 13200 rpm for 2 min for the soluble enzymes, or by using pipette-filter devices for the immobilized enzymes), and analyzed by HPLC. The column was a Gemini 250×4.6 mm, 5 μm (Phenomenex, Cernusco sul Naviglio, Italy); eluent: 0.01 M potassium phosphate buffer pH 4.6 and methanol 90% (95:5); flow: 1 mL min^{-1}; T = 35 °C; λ = 260 nm.

One international unit (IU) of the enzymatic activity corresponds to the amount of enzyme that hydrolyzes 1 μmol of substrate per

minute at room temperature. The specific activity is defined as units of enzyme activity per milligram of protein.

Preparation of the activated carriers

Preparation of aldehyde carriers: Aldehyde–agarose was prepared as previously reported.[34] Aldehyde–Sepabeads were prepared by oxidation of Sepabeads EC-HG following the previously reported protocol.[35]

Activation of agarose with glutaraldehyde: The amination of aldehyde–agarose with ethylendiamine (EDA) was performed as previously reported.[36] The EDA-activated agarose was then suspended in phosphate buffer pH 7 (0.2 M, 3.4 mL) and a solution of 25% (v/v) glutaraldehyde (5.1 mL) was added. The mixture was kept under stirring for 16 h at room temperature in the darkness. The activated support was washed with deionized water and used immediately.

Preparation of Sepabeads–PEI: Activation of epoxy Sepabeads with PEI was performed as previously reported.[18]

Immobilization of enzymes

Immobilization on aldehyde-activated carriers: Immobilization on carriers activated with aldehyde groups was performed by slightly modifying the procedure previously reported.[19,35] Briefly, the aldehyde support (1.4 mL) was suspended in potassium phosphate/carbonate buffer (50 mM) at pH 10.05. After the addition of the desired amount of protein, the suspension (14 mL) was kept under mechanical stirring. Chemical reduction of Schiff bases was carried out over 30 min by adding $NaBH_4$ (14 mg) to the mixture. The immobilized enzyme was then filtered and washed with 10 mM potassium phosphate buffer pH 5 and deionized water.

Immobilization on glutaraldehyde–agarose: The glutaraldehyde–agarose gel (1 g), prepared as described above, was suspended in potassium phosphate buffer (100 mM) at the desired pH. After the addition of the desired amount of protein, the solution (7.1 mL) was kept under mechanical stirring; after 3 h the pH was increased to 10.05 and $NaBH_4$ (7 mg) was then added. The reduction time was 30 min. Finally, the immobilized enzyme was filtered and washed with 10 mM potassium phosphate buffer pH 5 and deionized water.

Immobilization on Sepabeads–PEI and cross-linking with aldehyde dextran: Immobilization on Sepabeads–PEI and stabilization by cross-linking with 20% oxidized dextran (aldehyde dextran) was performed according to the procedure previously described.[19]

Enzyme-stability assay

Following a general procedure, the enzyme was added to buffer (5 mL; under the desired conditions) and kept under mechanical stirring at room temperature. At different times, samples were withdrawn and the residual enzymatic activity was monitored by a standard activity assay towards inosine.

General procedure of enzymatic transglycosylation

A solution of phosphate buffer (10 mL, 50 mM) with the appropriate amount of solvent (v/v) at pH 7.5 containing araU or ddU (**4** and **5**) and the heterocyclic base at the desired concentration was prepared. The enzyme preparations were added to the reaction and the mixture was kept at room temperature under mechanical stirring until the highest conversion was achieved. The reaction was monitored by HPLC ($\lambda = 260$ nm), which was used to identify the products by comparing their retention times with those of authentic samples. Mobile phase: 0.01 M KH_2PO_4 buffer pH 4.6/methanol 90% (90:10). Uracil, $t_R = 3.85$ min; hypoxanthine, $t_R = 4.68$ min; adenine, $t_R = 7.42$ min; arabinosyluracil (**4**), $t_R = 5.62$ min; 2′,3′-dideoxyuridine (**5**), $t_R = 11.55$ min; arabinosyladenine (**9**), $t_R = 12.33$ min; 2′,3′-dideoxyinosine (**14**), $t_R = 16.35$ min. The percentage of conversion was calculated on the basis of the depletion of the sugar acceptor compound (heterocyclic base) and monitoring the formation of the nucleoside products: Conversion [%] = [product area/(product area + base area)] × 100. The reaction was stopped by sub vacuum filtration of the immobilized biocatalyst.

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Keywords: biocatalysis · enzymes · immobilization · nucleosides · transglycosylation

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