Thermodynamic Reaction Control of Nucleoside Phosphorolysis

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Abstract: Nucleoside analogs represent a class of important drugs for cancer and antiviral treatments. Nucleoside phosphorylases (NPases) catalyze the phosphorolysis of nucleosides and are widely employed for the synthesis of pentose-1-phosphates and nucleoside analogs, which are difficult to access via conventional synthetic methods. However, for the vast majority of nucleosides, it has been observed that either no or incomplete conversion of the starting materials is achieved in NPase-catalyzed reactions. For some substrates, it has been shown that these reactions are reversible equilibrium reactions that adhere to the law of mass action. In this contribution, we broadly demonstrate that nucleoside phosphorolysis is a thermodynamically controlled endothermic reaction that proceeds to a reaction equilibrium dictated by the substrate-specific equilibrium constant of phosphorolysis, irrespective of the type or amount of NPase used, as shown by several examples. Furthermore, we explored the temperature-dependency of nucleoside phosphorolysis equilibrium states and provide the apparent transformed reaction enthalpy and apparent transformed reaction entropy for 24 nucleosides, confirming that these conversions are thermodynamically controlled endothermic reactions. This data allows calculation of the Gibbs free energy and, consequently, the equilibrium constant of phosphorolysis at any given reaction temperature. Overall, our investigations revealed that pyrimidine nucleosides are generally more susceptible to phosphorolysis than purine nucleosides. The data disclosed in this work allow the accurate prediction of phosphorolysis or transglycosylation yields for a range of pyrimidine and purine nucleosides and thus serve to empower further research in the field of nucleoside biocatalysis.

Keywords: nucleosides; nucleoside phosphorylase; nucleoside phosphorolysis; equilibrium constant; temperature

Introduction

Nucleosides serve as drugs against a variety of cancers and viral infections.1 Thus, their cost- and time-efficient preparation is of high interest. However, the synthesis of nucleosides and nucleoside analogs via conventional synthetic methods comes with several challenges posed by regio- and stereochemical complexity, functional group sensitivity and, consequently, heavy reliance on protecting groups.2 Biocatalytic methods are a valuable alternative as pyrimidine and purine nucleosides can be synthesized with high selectivity in sustainable enzyme-catalyzed processes.3 Here, the use of nucleoside phosphorylases (NPases) has been firmly established and these enzymes are widely applied for the synthesis of nucleosides and their analogues.3 NPases catalyze the phosphorolysis of nucleosides to pentose-1-phosphates, as well as the corresponding reverse reaction (Scheme 1). They are generally classified as either pyrimidine nucleoside phosphorylases
(Py-NPases) or purine nucleoside phosphorylases (Pu-NPases), depending on their substrate spectra[14].

The use of NPases as biocatalysts in organic chemistry offers the advantage of using mild aqueous reaction conditions, a broad substrate spectrum regarding sugar and base moieties, as well as perfect regio- and stereoselectivity at the C1’ position. Employing these enzymes, pentose-1-phosphates can be obtained as important synthetic intermediates from the accessible pool of nucleosides[5,6] which have been shown to be highly valuable precursors for the synthesis of base- and sugar-modified nucleosides.[7–9] Furthermore, NPases are widely used for the synthesis of nucleoside analogues in transglycosylation reactions.[10–12]

Current research activities mainly focus on kinetic aspects of individual NPase-catalyzed reactions. Hence, kinetic data for a variety of NPases are available. To increase the final yield of phosphorolysis, mainly the choice of enzyme,[13,14] enzyme immobilization[15] or enzyme engineering[16,17] have been investigated. Nonetheless, NPases generally fail to facilitate full conversion.[18] A recent report by Alexeev and coworkers has shed more light on the cause of this phenomenon and showed that the thermodynamic properties of the nucleosides involved in the reaction influence the yields of NPase-catalyzed reactions.[19]

Since nucleoside phosphorolysis is a reversible reaction (Scheme 1), there is a point, where the rates of the forward and the backward reactions are equal and no apparent change in concentrations is observed. In this equilibrium state, the reaction quotient defined as the quotient of the concentrations of the products and the substrates can be derived from the law of mass action and is generally referred to as the apparent equilibrium constant $K$.[20] The following expression results for nucleoside phosphorolysis reactions:

$$K' = \frac{[B][P1P]}{[N][P]} \quad (1)$$

where $K'$ is the apparent equilibrium constant of phosphorolysis, $[B]$ is the equilibrium concentration of the free nucleobase [mM], $[P1P]$ is the equilibrium concentration of the pentose-1-phosphate [mM], $[N]$ is the equilibrium concentration of the nucleoside [mM] and $[P]$ is the equilibrium concentration of inorganic phosphate [mM].

This equilibrium constant exists irrespective of the (bio)catalyst used and $K$ values for some substrates in NPase-catalyzed reactions have been reported.[19,21–26] Thus, according to the law of mass action, only the reaction conditions influence the final equilibrium concentrations and not the enzyme used for catalysis. Alexeev and colleagues[19] recently employed this dependency in their method for accurate yield prediction of nucleoside transglycosylation reactions based on the equilibrium constants of the individual reactions. Ultimately, this allows for thermodynamic reaction control by adjusting the concentrations of the starting materials to facilitate an optimal yield.

In this work we provide extensive evidence for the universal interpretation of nucleoside phosphorolysis reactions as thermodynamically controlled endothermic equilibrium reactions that adhere to the law of mass action. To this end, we performed and monitored several biocatalytic nucleoside phosphorolysis reactions. We demonstrate that nucleoside phosphorolysis proceeds to a reaction equilibrium dictated by the substrate-specific equilibrium constant of phosphorolysis, irrespective of the type or amount of NPase used. Furthermore, we show that the equilibrium constant is temperature dependent. Therefore, we determined the equilibrium constants of phosphorolysis of 24 nucleosides at different temperatures and derived the apparent transformed enthalpies and entropies. The resulting data show that pyrimidine nucleosides are generally more susceptible to phosphorolysis than purine nucleosides. Additionally, our enthalpy and entropy data allow calculation of equilibrium constants at different temperatures as well as prediction of phosphorolysis yields, as demonstrated herein.

Results and Discussion

Maximum Conversions of Nucleosides in Phosphorolysis Reactions Are Independent of the Applied Biocatalyst

Previous research activities have focused on the discovery of new enzymes[13,14] or enzyme immobilization strategies[15] to increase the obtained yield for
nucleoside phosphorolysis and transglycosylation reactions. Based on the thermodynamic characteristics of these reactions, however, we anticipated that the maximum conversion in phosphorolysis reactions would behave independently of the enzyme used.

To investigate the impact of different NPases on the equilibrium states of nucleoside phosphorolysis reactions, we initially performed the phosphorolysis of uridine (1) with *Escherichia coli* uridine phosphorylase (*E. coli* UP), *E. coli* thymidine phosphorylase (*E. coli* TP), *Bacillus subtilis* Py-NPase (*B. subtilis* Py-NPase) and two commercially available thermostable Py-NPases (Py-NPase Y01 and Py-NPase Y02). All enzymes readily accepted this substrate and reaction completion could be observed after 5 to 80 min using 10 μg·ml⁻¹ of the respective enzymes (Figure 1A). Using 5 equivalents (eq.) of phosphate with respect to the nucleoside substrate, a maximum conversion of 55% was achieved regardless of the enzyme used.

To exclude any concentration effects of the enzyme preparations, we additionally conducted the phosphorolysis of 1 with different concentrations of Py-NPase Y02 (Figure 1B). Higher enzyme concentrations led to faster reaction completion. However, irrespective of the amount of the enzyme used, the same equilibrium as previously observed was reached.

To eliminate the possibility of enzyme inactivation preventing the completion of the reaction, we performed the phosphorolysis of 1 with Py-NPase Y02 and added additional enzyme after apparent reaction completion (Figure 1C).

This experiment resulted in no change of reactant concentrations after addition of more enzyme, confirming that this reaction was not terminated by means of inhibition and/or enzyme inactivation.

This evidence confirms that the phosphorolysis of uridine (1) is a thermodynamically controlled equilibrium reaction which is consistent with previous reports.²⁻¹⁹,²¹–²⁴ From the data collected, the apparent equilibrium constant of phosphorolysis *K* under these conditions was 0.15, as calculated from equation (1).

To broadly confirm the thermodynamic reaction control of nucleoside phosphorolysis, we investigated the conversion of other nucleosides by NPases. We observed a similar behavior for the phosphorolysis of the pyrimidine nucleosides 2'-deoxyuridine (2), 5-fluorouridine (3) and 2'-deoxy-5-fluorouridine (4), employing the same Py-NPases (Figure 2A–2C). As observed for uridine (1), the choice of enzyme had no effect on the equilibrium conversions. Pyrimidines 2–4 displayed very similar apparent equilibrium constants to 1 (0.20 for 2, 0.14 for 3 and 4).

To extend this investigation to purine nucleosides, we performed the phosphorolysis of two natural (adenosine, 5, and 2'-deoxyadenosine, 6) and two modified purine nucleosides (2-chloroadenosine, 7, and 2-chloro-2'-deoxyadenosine, 8) with three different NPases (thermostable Pu-NPases N01 and N02 and *E. coli* Pu-NPase). Similar to the pyrimidine nucleosides mentioned above, the choice of the enzyme had no effect on the equilibrium concentrations of these reactions (Figure 2D–2G). Here, the equilibrium conversions with 5 eq. of phosphate were significantly lower, in the range of 20%. Calculation of the apparent equilibrium constants of phosphorolysis yielded values

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**Figure 1.** Enzymatic phosphorolysis of uridine (1). A Phosphorolysis of 1 with five different Py-NPases. B Phosphorolysis of 1 with Py-NPase Y02 using different enzyme concentrations. C Phosphorolysis of 1 with Py-NPase Y02 employing spiking of enzyme upon apparent reaction completion. All reactions reach the same equilibrium, regardless of the enzyme or its amount used for catalysis. Reactions were performed with 2 mM nucleoside substrate and 10 mM K₂HPO₄ in 50 mM MOPS buffer at pH 7.5 and 37°C in a total volume of 500 μL. 10 μg·ml⁻¹ of the respective enzyme were used in A and 10–40 μg·ml⁻¹ Py-NPase Y02 were used in B. The reaction in C was started with 40 μg·ml⁻¹ (20 μg total enzyme) Py-NPase Y02 and 10 μg of the enzyme were added at 10 min and 15 min each, indicated by the arrows. Reactions in A were performed in duplicate and the standard deviation (SD) is shown as error bars.
around 0.01, which is consistent with previous reports for 5.\textsuperscript{[19,28]}

Interestingly, Py-NPases Y01, Y02 and \textit{B. subtilis} Py-NPase performed roughly equally well with uridine (1) compared to 2'-deoxyuridine (2), whereas our data nicely reflect the inverse substrate specificity of \textit{E. coli} UP and TP, as reported previously.\textsuperscript{[29,30]} \textit{E. coli} UP showed excellent activity with uridine (1) but significantly diminished activity with the 2'-deoxy analogue 2. \textit{E. coli} TP, on the other hand, displayed only low activity with 1 but facilitated remarkably quick reaction completion with 2 (Figure 1A and 2A).

Notably, although it is commonly believed that Pu-NPases should be specific to purine nucleosides, they generally also catalyze the phosphorolysis of pyrimidine nucleosides. Compared to corresponding Py-NPases they usually exhibit > 2,000-fold lower turnover rates. Nonetheless, given the thermodynamic reaction control of nucleoside phosphorolysis, Pu-NPases should still be expected to complete the phosphorolysis of their unfavored substrates, despite

**Figure 2.** Enzymatic phosphorolysis of pyrimidine and purine nucleosides. The phosphorolysis of A 2'-deoxyuridine (2), B 5-fluorouridine (3), C 2'-deoxy-5-fluorouridine (4), D adenosine (5), E 2'-deoxyadenosine (6), F 2-chloroadenosine (7) and G 2-chloro-2'-deoxyadenosine (8) was performed in duplicate with 2 mM nucleoside substrate, 10 mM K$_2$HPO$_4$ and 10 μg·ml$^{-1}$ of the respective enzyme (except for the phosphorolysis of 3 with Pu-NPase N02, where 600 μg·ml$^{-1}$ were used) in 50 mM MOPS buffer at pH 7.5 and 37°C in a total volume of 500 μL. Note the different time scales. Error bars show the SD.
their kinetic handicap. Consequently, for the phosphor
olysis of the pyrimidine 5-fluorouridine (3), even the
use of the thermostable Pu-NPase N02 proved success-
ful in reaching the thermodynamic equilibrium (Fig-
ure 2B). In this case, a 60-fold higher amount of
zyme had to be employed and the reaction took
considerably longer than with any of the Py-NPases
under the same reaction conditions. Thus far, to the
best of our knowledge, only the phylogenetically pecu-
liar Plasmodium falciparum Pu-NPase has been
reported to perform the phosphorolysis of a pyrimidine
nucleoside.51 Here, we not only show for the first time
that the thermostable Pu-NPase N02 accepts pyrimi-
dine substrates such as 3, but also achieves reaction
completion given sufficient time. This strikingly
emphasizes the non-significance of the enzyme ki-
netics for the ultimate reaction outcome, as it is
possible to drive these phosphorolysis reactions into
their chemical equilibrium even with an extremely
small enzyme activity, in a range commonly consid-
ered negligible.

Thus, all nucleoside phosphorolysis reactions we
investigated behaved according to the law of mass
action and reached maximum product concentrations
after a variable run time. Despite differences in
reaction speed, the maximum conversion yields in the
equilibrium are dictated by a characteristic and
substrate-specific equilibrium constant which was
shown to be independent of the type and amount of
zyme used. It may be reasonable to assume that this
holds true for all nucleosides that are subjected to
phosphorolysis. Important exceptions from this are
nucleosides that are not converted by NPases because
of issues such as excessive steric demands or severely
unfavored transition states which prevent transforma-
tion.

Higher Reaction Temperature yields Higher Con-
version of Nucleosides by NPases

The absolute values of the equilibrium constants of
phosphorolysis of the nucleosides discussed above
were all found to be well below 1. This means that
they describe endothermic reactions, which are not
favored under standard conditions. Consequently, we
anticipated a temperature-dependence of \( K^e \) and set out
to investigate the effect of higher reaction temperatures
on the equilibrium states of nucleoside phosphorolysis.
Profiting from the use of the thermostable NPases Y02
and N02, we were able to perform the phosphorolysis
of 24 nucleosides, including 12 pyrimidine and 12
purine nucleosides, at temperatures of 40 to 70°C. To
prevent decomposition of the produced pentose-1-
phosphates, which is known to happen rapidly at high
temperatures and neutral pH values,52 we increased the
reaction pH to 9. Under these conditions, both enzymes
displayed excellent activity with all substrates 1–24
and allowed efficient reaction completion and monitor-
ing. Consistent with our initial assumptions, we
observed a trend towards higher conversions at higher
temperatures. For example, under these conditions the
phosphorolysis of 2'-deoxy-5-fluorouridine (4; Fig-
ure 3A) showed equilibrium conversions in the range
of 51% at 40°C and values around 55% at 70°C. A
similar behavior could be observed for all nucleosides
investigated which confirms that the phosphorolysis of
1–24 are thermodynamically controlled endothermic
reactions (Table S1).

The difference between the equilibrium conversions
determined at pH 9 (this section) and the ones
determined at pH 7.5 (see above) were insignificant
(compare Figure 1 and Figure 2 with Table S1). Although a difference between them might be expected
from the definition of the biochemical equilibrium, it
seems that pH only minorly influences the equilibrium
constant by changing the contribution of charged
species.

Figure 3. Examples of nucleoside phosphorolysis thermody-
namic data processing. A Raw data of the phosphorolysis of 4
at different temperatures. The reactions were performed with
2 mM nucleoside substrate, 10 mM \( \text{K}_2\text{HPO}_4 \) and 16 \( \mu \text{g} \cdot \text{ml}^{-1} \)
Py-NPase Y02 in 50 mM glycine buffer at pH 9 and 40–70°C
in a total volume of 500 \( \mu \text{L} \). B Transformed and fitted data for
the derivation of \( \Delta_{p}H^\circ \) and \( \Delta_{p}S^\circ \). Raw data were processed via
equation (1) and then fitted with equation (2).
Pyrimidine Nucleosides are Generally more Susceptible to Phosphorolysis than Purine Nucleosides

From the described data, we derived \( K' \) at various temperatures and determined the transformed apparent reaction enthalpy \( \Delta_{ph}H' \) and transformed apparent reaction entropy \( \Delta_{ph}S' \) via fitting of the data to:

\[
K' = e^{\frac{\Delta_{ph}G'}{RT}} = e^{\frac{\Delta_{ph}H' - T \Delta_{ph}S'}{RT}}
\]

(2)

where \( \Delta_{ph}G' \) is the transformed apparent Gibb’s free energy of phosphorolysis [J·mol\(^{-1}\)], \( T \) is the temperature [K], \( R \) is the universal gas constant (8.314 J·mol\(^{-1}\)·K\(^{-1}\)), \( \Delta_{ph}H' \) is the transformed apparent enthalpy of phosphorolysis [J·mol\(^{-1}\)], \( \Delta_{ph}S' \) is the transformed apparent entropy of phosphorolysis [J·mol\(^{-1}\)·K\(^{-1}\)], and the definitions from above.

Here, we carried out direct determinations through a non-linear fit to prevent error propagation by linearization (Figure 3A and 3B; see Table S1 for all derived values for \( \Delta_{ph}H' \) and \( \Delta_{ph}S' \); note that we communicate transformed thermodynamic values, as they were measured at constant pH and do not consider charged species, and explicitly abstain from referring to our results as “standard” values, but apparent ones, as they were not measured under standard biochemical conditions).

The knowledge of \( \Delta_{ph}H' \) and \( \Delta_{ph}S' \) then allowed access to the transformed apparent Gibb’s free energy \( \Delta_{ph}G' \) of these phosphorolysis reactions at different temperatures. The Gibb’s free energy of phosphorolysis \( \Delta_{ph}G' \) at 40°C of pyrimidine nucleosides was found to be in the range of 1.2–5.5 kJ·mol\(^{-1}\), which is notably lower than for the investigated purine nucleosides that display values of 5.7–12.6 kJ·mol\(^{-1}\) (Figure 4A). These values correspond to apparent equilibrium constants \( K' \) in the range of 0.12–0.62 for pyrimidine nucleosides and 0.01–0.11 for purine nucleosides. Noticeably, different equilibrium conversions can be observed across these nucleosides, with pyrimidines generally featuring higher equilibrium conversions than purines, as dictated by their respective equilibrium constants (Figure 4B).

The practical value of our collection of \( \Delta_{ph}H' \) and \( \Delta_{ph}S' \) data lies within their use for the prediction of the equilibrium phosphorolysis conversion of the nucleosides investigated here. Using equation (2), the equilibrium constants of phosphorolysis of any of the nucleosides investigated here can easily be obtained for any given temperature. Employing then:

\[
[B] = \frac{\ln([N]_0 + [P]_0)}{2(K' - 1)} - \frac{\sqrt{K'[N]_0 + K'[P]_0} - 4(K' - 1)K'[N]_0[P]_0}{2(K' - 1)}
\]

(3)

where \([N]_0\) is the initial concentration of the nucleoside [mM], \([P]_0\) is the initial concentration of phosphate [mM] and definitions from above yields the concentrations of the free nucleobase and pentose-1-phosphate with variable initial concentrations of nucleoside and phosphate. Thus, conversions of these nucleosides can be predicted. To ease these calculations, we provided an Excel spreadsheet that is freely available from the externally hosted online supplementary material.

To verify the predictions available through equation (3), we performed the NPase-catalyzed phosphorolysis of uridine (1) and adenosine (5) at pH 9 and 40°C with different concentrations of phosphate (Figure 5). For example, pyrimidine 1 reached a conversion of 80% with 20 eq. of phosphate whereas for purine 5 we only observed a conversion of 37% under the same conditions, which is consistent with the predictions obtained via our collection of \( \Delta_{ph}H' \) and \( \Delta_{ph}S' \) data (Table S1). This emphasizes the practical value of the knowledge of the equilibrium constants of phosphorolysis of these nucleosides, as they allow for accurate prediction of phosphorolysis yields to optimize the reaction conditions.

Conclusion

In the present work, we broadly demonstrated that nucleoside phosphorolysis catalyzed by NPases is a thermodynamically controlled endothermic reversible equilibrium reaction. Therefore, maximum yields for each substrate are independent of the NPase used, as demonstrated by several examples, and can be predicted via the substrate-specific apparent equilibrium constant \( K' \). We anticipate that this holds true for all nucleosides that can be subjected to phosphorolysis. Furthermore, we presented data on the temperature-dependency of the equilibrium constants of phosphorolysis of 24 nucleosides that display characteristic behavior. The available data allow for the calculation of \( K' \) at any given temperature and enable accurate prediction of phosphorolysis or transglycosylation yields for a range of pyrimidine and purine nucleosides. Subsequently, the equations described by Alexeev et al., the thermodynamic data reported in this study, as well as the tools provided herein (see external supplementary material) serve to facilitate streamlined reaction engineering of NPase-catalyzed reactions by in silico yield prediction and thermodynamic reaction control.

Since our findings show that maximum yields in NPase-catalyzed reactions can be achieved independently of the enzyme applied, we believe that efforts to influence the yield of these reactions by varying the enzyme are unlikely to bear fruit. Instead, extending the toolbox of available NPases to improve kinetic parameters to reach reaction equilibrium faster or to
convert challenging substrates such as arabinosyl nucleosides, fluorinated glycosides or bulky nucleobases will certainly prove beneficial.

Lastly, the quantification of effects of reaction conditions such as ionic strength, different salts, pH and chelating agents on the equilibrium states of nucleoside phosphorolysis may be explored experimentally by future studies.

Figure 4. Gibb’s free energy of phosphorolysis and conversion of nucleosides. A Gibb’s free energy of phosphorolysis determined via equations (1) and (2) from the equilibrium concentrations of nucleoside phosphorolysis reactions with 2 mM nucleoside substrate, 10 mM K₂HPO₄ and 16 μg·ml⁻¹ Py-NPase Y02 or 66 μg·ml⁻¹ Pu-NPase N02 in 50 mM glycine buffer at pH 9 in a total volume of 500 μL performed at 40–70 °C. Error bars quantify the uncertainty of the prediction at 25 °C calculated via equation (4).

B Equilibrium conversion of nucleosides in a phosphorolysis reaction at 40 °C and pH 9 employing 5 eq. of phosphate and equilibrium constant K (also see Table S1). [a] Rib = ribosyl, dRib = 2′-deoxyribosyl, [b] extrapolated from experimental data for Δ₍₀₎H* and Δ₍₀₎S*, [c] experimental data (conditions as above), [d] calculated from data for 50–70 °C since data for 40 °C were excluded from analysis (see Supplementary Material for full dataset).

Experimental Section

General Remarks

All chemicals were of analytical grade or higher and purchased, if not stated otherwise, from Sigma Aldrich (Steinheim, Germany), Carbosynth (Berkshire, UK), Carl Roth (Karlsruhe, Germany), TCI Deutschland (Eschborn, Germany) or VWR (Darmstadt, Germany). All nucleosides (Figure S1) and nucleobases were used without prior purification. Water deionized to 18.2 MΩ·cm with a Werner water purification system was used. For the preparation of NaOH solutions deionized water was used.
Enzymes

Enzymes were either purchased from BioNukleo GmbH (Berlin, Germany) or Sigma Aldrich (see Table S2). Enzymes provided by BioNukleo were E-PyNP-0001 (Py-NPase Y01; EC 2.4.2.2), E-PyNP-0002 (Py-NPase Y02; EC 2.4.2.2), E-PNP-0001 (Pu-NPase N01; EC 2.4.2.1), E-PNP-0002 (Pu-NPase N02; EC 2.4.2.1), E-UP-0001 (Escherichia coli uridine phosphorolysase; E. coli UP; EC 2.4.2.3), E-TP-0001 (E. coli thymidine phosphorolysase; E. coli TP; EC 2.4.2.4) and E-PNP-04 (E. coli pyrimidine nucleoside phosphorolysase; E. coli Pu-NPase; EC 2.4.2.1). Enzymes were desalted against 2 mM KH₂PO₄ (pH 7.0, measured at 25°C) buffer and stored at 4°C at concentrations listed in Table S2. Bacillus subtilis pyrimidine phosphorolysase (B. subtilis Py-NPase; EC 2.4.2.2) was obtained from Sigma Aldrich and prepared as a 1 mg·mL⁻¹ solution in 2 mM KH₂PO₄ buffer (pH 7.5, measured at 25°C) prior to use.

The activity of the stock solutions of Py-NPase Y02 and Pu-NPase N02 was assessed by the UV/Vis spectroscopy-based method described recently (also see below) [34]. Reactions were performed with 1 mM of nucleoside substrate in 50 mM glycine buffer at pH 9 and 40°C. One unit (U) is defined as the conversion of 1 μmol of substrate per minute. For Py-NPase Y02 and Pu-NPase N02 an activity of 40 U·mg⁻¹ (65 U·mL⁻¹) and 57 U·mg⁻¹ (379 U·mL⁻¹) for their natural substrates uridine (1) and adenosine (5), respectively, was determined.

Phosphorolysis of Pyrimidine and Purine Nucleosides

Enzymatic nucleoside phosphorolysis reactions were prepared from stock solutions of nucleoside, phosphate, buffer and water in 1.5 mL reaction tubes (Sarstedt, Nümbrecht, Germany) and started by the addition of the enzyme. Reactions were performed in duplicate with 2 mM nucleoside and 10 mM K₂HPO₄ in 50 mM MOPS buffer (pH 7.5, measured at 30°C) in a total volume of 500 μL. Prior to the addition of enzyme solution, reactions were preheated to 37°C. Unless stated otherwise, final enzyme concentrations of 10 μg·mL⁻¹ were used for all enzymes and substrates. For the phosphorolysis of 5-fluorouridine (3) with Pu-NPase N02, 600 μg·mL⁻¹ of enzyme were employed.

To investigate possible effects of the enzyme concentration, uridine (1) was subjected to phosphorolysis using 10–40 μg·mL⁻¹ of Py-NPase Y02. To exclude possible enzyme inactivation effects, 1 was also subjected to phosphorolysis with 40 μg·mL⁻¹ of Py-NPase Y02 (20 μg total enzyme) and 10 μg of the enzyme were added after apparent reaction completion at 10 min and 15 min each. To validate the predictions of phosphorolysis conversion with different phosphate concentrations, 2 mM of 1 and 5, respectively, were converted with 40 μg·mL⁻¹ Py-NPase Y02 or 40 μg·mL⁻¹ Pu-NPase N02 in 50 mM glycine buffer at pH 9 and 40°C with 5, 10, 20 or 40 mM (2.5, 5, 10 or 20 eq.) K₂HPO₄. The reactions were monitored, and equilibrium samples were taken after 10, 15 and 20 min.

Monitoring of Enzyme Reactions

Sampling, data collection and analysis were carried out as described previously [34]. Briefly, samples of 30 μL (for pyrimidine nucleosides) or 20 μL (for purine nucleosides) were withdrawn from the reaction mixture and pipetted into 100 mM NaOH solution (200 mM NaOH solution for 3, 4 and 13–16) to give a final volume of 500 μL of diluted alkaline sample. From this mixture, 200 μL were pipetted into wells of a UV/Vis-transparent 96-well plate (UV-STAR F-Bottom #655801, Greiner Bio-One). The UV/Vis absorption spectra of these alkaline samples were recorded from 250 to 350 nm to determine the nucleoside/nucleobase ratio via spectra unmixing. All data presented in this study can be obtained from an external online repository [32] along with the software for spectral unmixing and metadata treatment detailed in our previous work [34, 35].

Temperature Dependence of the Equilibrium Constant

Equilibrium constants for the phosphorolysis of 24 nucleosides (1–24) were determined at pH 9 and at 40, 50, 60 and 70°C. All reactions were performed with 2 mM nucleoside substrate and 10 mM K₂HPO₄ in 50 mM glycine buffer at pH 9. Unless stated otherwise, 16 μg·mL⁻¹ (0.6 U·mL⁻¹) Py-NPase Y02 (for pyrimidine nucleosides) or 66 μg·mL⁻¹ (3.7 U·mL⁻¹) Pu-NPase N02 (for purine nucleosides) were applied. For inosine (23) and 2′-deoxyinosine (24) 330 μg·mL⁻¹ of Pu-NPase N02 were used and for 5-iodouridine (13) and 2′-deoxy-5-iodouridine (14) 32 μg·mL⁻¹ of Py-NPase Y02. All reactions were performed in duplicate at temperatures from 40–70°C in steps of 10°C. Three samples were taken from each reaction once equilibrium was reached to confirm reaction completion. The time points used for sampling are given in Table S3. In total, 576 data points were recorded. Outliers that either displayed an elevated baseline due to UV absorption of the 96-well plate or differed...
more than 1.5 percentage points from the other two data points within a sample set of a given temperature and reaction mixture were excluded from data interpretation. Consequently, 519 data points were considered for further evaluation. Data treatment and fitting was carried out with Origin ProLab 9. All data, as well as all subsequent calculations can be inspected as freely available data from an external online repository.[32] For each data point, \( K \) was calculated via equation (1) and transformed apparent reaction enthalpy \( \Delta R H^* \) [J mol\(^{-1}\)] and apparent reaction entropy \( \Delta S R^* \) [J mol\(^{-1}\) K\(^{-1}\)] were fitted directly to all available datapoints for a substrate with equation (2). Similarly, \( \Delta R G^0 \) was calculated with equation (2) assuming that the apparent values \( \Delta R H^* \) and \( \Delta S R^* \) are independent of the reaction temperature in the considered range. The errors \( \Delta \Delta R H^* \) [J mol\(^{-1}\)] and \( \Delta \Delta S R^* \) [J mol\(^{-1}\) K\(^{-1}\)] were derived from the fit and \( \Delta R G^0 \) was calculated via Gaussian error propagation:

\[
\Delta R G^0 = \sqrt{\Delta \Delta R H^*}^2 + T^2 \Delta \Delta S R^*^2
\]

with definitions from above.

**Conflict of Interest**

A.W. is CEO of the biotech company BioNukleo GmbH. F.K. is a researcher at BioNukleo GmbH and P. N. is a member of the advisory board.

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**References**


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