Organic & Biomolecular Chemistry

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The effect of bisphosphonate acidity on the activity of a thymidylyltransferase†

Cite this: *Org. Biomol. Chem.*, 2013, **11**, 5473

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Received 15th May 2013,
Accepted 3rd July 2013

DOI: 10.1039/c3ob41017j

www.rsc.org/obc

Thymidylyltransferases (thymidine diphospho pyrophosphorylases) are nucleotidylyltransferases that play key roles in the biosynthesis of carbohydrate components within bacterial cell walls and in the biosynthesis of glycosylated natural products. They catalyze the formation of sugar nucleotides concomitant with the release of pyrophosphate. Protein engineering of thymidylyltransferases has been an approach for the production of a variety of non-physiological sugar nucleotides. In this work, we have explored chemical approaches towards modifying the activity of the thymidylyltransferase (Cps2L) cloned from *S. pneumoniae*, through the use of chemically synthesized 'activated' nucleoside triphosphates with enhanced leaving groups, or by switching the metal ion co-factor specificity. Within a series of phosphonate-containing nucleoside triphosphate analogues, thymidylyltransferase activity is enhanced based on the acidity of the leaving group and a Brønsted-type analysis indicated that leaving group departure is rate limiting. We have also determined IC_{50} values for a series of bisphosphonates as inhibitors of thymidylyltransferases. No correlation between the acidity of the inhibitors (pK_a) and the magnitude of enzyme inhibition was found.

Introduction

Glycoconjugates responsible for many significant cellular interactions.[1] The large structural and functional diversity of mammalian glycosylated biomolecular structures arises from only nine different sugar nucleotides.[2,3] Other organisms, including plants, fungi and bacteria have substantially greater numbers of sugar nucleotides, reflecting a broader diversity in their biochemistry.[4] A long-standing challenge remains the requirement to furnish quantities of sugar nucleotides to initiate structure-function studies. Enzymatic approaches towards sugar nucleotide preparation have gained significant traction[5] due in part to the ease of scale-up and defined stereospecificity. Sugar nucleotides also play important roles as intermediates in the biosynthesis of glycosylated natural products, and many of these natural products are used clinically.[6–8] Thorson pioneered the use of protein engineering to modify the substrate specificity of a nucleotidylyltransferase[9] in order to provide access to novel sugar nucleotides for natural product glycodiversification.[10] The sugar nucleotides are subsequently consumed by glycosyltransferase-mediated glycosylation of acceptors. Active site engineering or directed evolution approaches of thymidylyltransferase RmlA (*Salmonella enterica typhimurium* LT2) has enhanced the scope of sugar phosphates accepted.[11] Thymidylyltransferases have high sequence, structure and mechanistic homology to other nucleotidylyltransferases in the NTP-transferase superfamily including bacterial thymidylyltransferases (RmlA[12]), uridylyltransferases (UDP pyrophosphorylases, GalF, GalU[13] or GlmU[14]), adenylyltransferases (GlgC[15]), guanidylyltransferases[16] and paralogous thymidylyltransferases (Rffh[17]). The enzymes adopt similar structural folds, have similar key catalytic residues and all require divalent magnesium for catalysis. A Bi–Bi ordered mechanism is common where nucleophilic attack of the sugar phosphate upon the alpha phosphorus of a nucleoside triphosphate results in the formation of a trigonal bipyrimidal phosphoryl ternary complex where pyrophosphate is released prior to the sugar nucleotide.[18–20] RmlA and its homologues are also recognized antibacterial targets.[21]

Phosphonates have long been recognized as important non-scissile mimics of phosphates, particularly in enzymatic systems where phosphate bond-breaking or forming is involved.[22,23] The concept of isosteric and isoelectronic

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†Electronic supplementary information (ESI) available: Michaelis–Menten and Lineweaver Burk plots, metal ion activities and IC_{50} data. See DOI: 10.1039/c3ob41017j

phosphonate analogues of phosphates was first developed by Blackburn,[24] wherein the methylene moiety of a phosphonate is substituted with electronegative functionality such as fluorine. This has the potential effect of more closely aligning the geometry of the phosphonate towards those of the phosphate, both in terms of bond angles and bond lengths about phosphorus,[25] and also by reducing the second ionization constant (pK_{a2}) towards that of the parent phosphate.[26] Several phosphoryl-transfer enzymes have been crystallized with difluoromethylene phosphonate analogues of their substrates including adenylate kinase,[27] glycerol kinase,[28] phosphoglycerate kinase,[29] and DNA polymerase beta.[30] Often, the resulting structure is different to the unliganded enzyme and a more closed form of the enzyme is observed with these isosteric, isoelectronic non-scissile substrate analogues bound. This provides insight into the enzyme motion necessary for catalysis. Monofluoromethylene phosphonate analogues are often viewed as potentially superior phosphate mimics than the difluoromethylene analogues due to the greater similarity between the second ionization constants at phosphorus (pK_{a2}),[31–33] however, the more challenging synthesis has limited their applicability. A synthesis and analysis by Berkowitz of individual diastereomers of monofluoromethylene phosphonate analogues of glucose 6-phosphate as G6PDH substrates demonstrated an eleven-fold difference in k_{cat}/K_m between the CHF diastereomers. This indicated the ability of G6PDH to preferentially select between them.[34] McKenna demonstrated stereospecific binding of the (*R*)-CHF diastereomer of dGMPPCHFP to DNA polymerase beta through X-ray analysis.[35,36]

Cps2L, a thymidylyltransferase cloned from *Streptococcus pneumoniae*,[37] is responsible for the first enzymatic step of the rhamnose biosynthetic pathway, catalyzing the coupling of α-D-glucose 1-phosphate (**1**) and dTTP (**2**) resulting in the release of inorganic pyrophosphate (**4**), and dTDP-α-D-glucose (**3**) (Fig. 1). Previously, Cps2L has been studied as a catalyst to synthesize sugar nucleotide analogues, including furanosyl nucleotides,[38] lipophilic sugar nucleotides,[39] and isosteric phosphono-analogues of sugar nucleotides.[40–42]

Herein, we report a study exploring two different approaches to improve the activity of thymidylyltransferase Cps2L: (a) with a series of phosphonate-containing analogues of nucleoside triphosphates with leaving groups of different acidity; (b) effects of metal co-factors upon the rate of reaction. Furthermore, we describe the inhibition of related bisphosphonates against Cps2L as a starting point for the development of inhibitors of this class of enzyme.

Results

Three analogues of uridine 5′-triphosphate (**5–7**) were used in a series of assays to evaluate the substrate specificity and assess the effects of leaving group acidity on the wild-type Cps2L enzyme. A summary of substrate specificity of the wild-type enzyme with analogues **5–7** and **1**, is presented in Fig. 2.

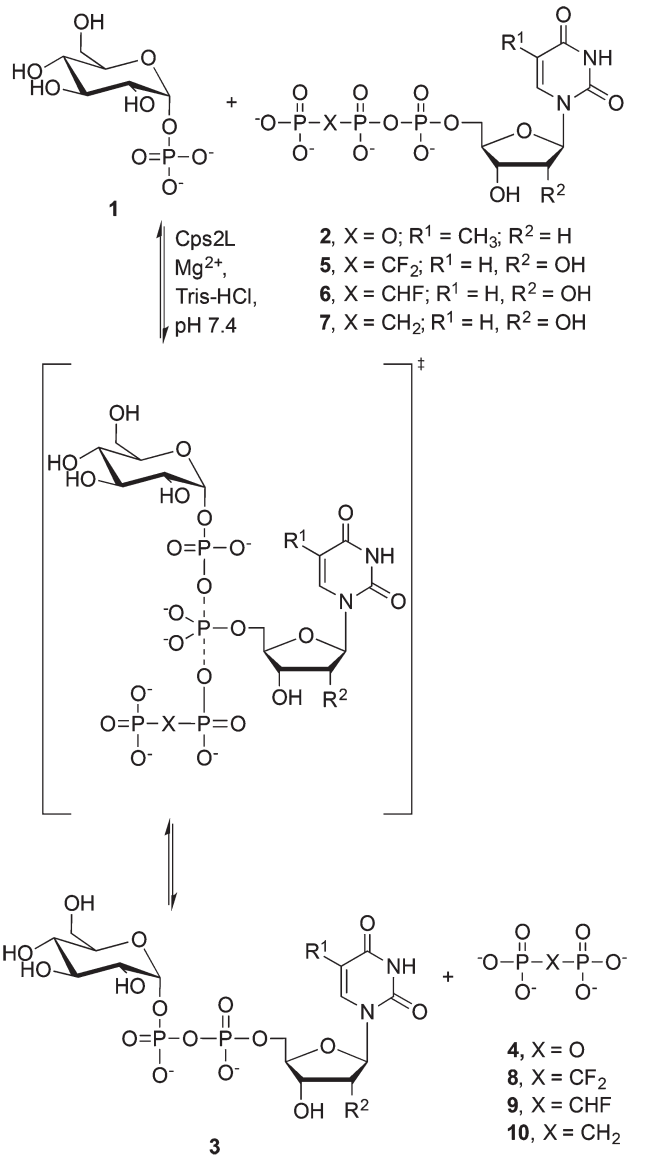

Fig. 1 Primary physiological reaction catalyzed by Cps2L (X = O, $R^1 = CH_3$, R^2 = H) and the structure of sugar nucleotide **3**.

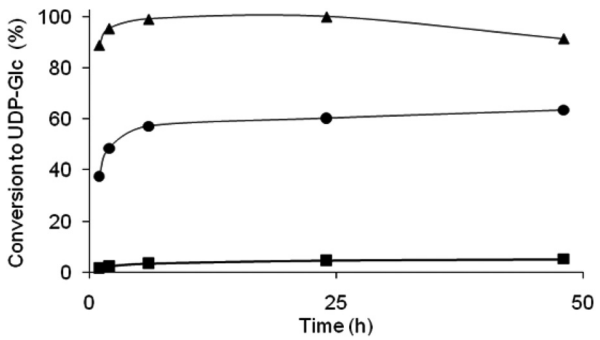

Fig. 2 Graphical summary of substrate conversions for the formation of UDP-α-D-Glc by Cps2L (10 EU) over 50 h by probing the enzyme with UTP analogues: **5** (▲), **6** (●), and **7** (■).

The incubation of **1** and **5** resulted in a quantitative conversion to the corresponding sugar nucleotide product, UDP-α-D-Glc, by the wild-type enzyme, indicating that **5** is readily accepted as alternative nucleoside triphosphate to **1**. Incubation of the wild-type enzyme with **1** and **6** gave approximately 50% conversion. When the reaction of **1** and **7** was investigated, negligible product was formed, demonstrating that a less acidic nucleoside triphosphate analogue results in decreasing yields of product. The very rapid consumption of **6** to ~50% led us to speculate that the thymidylyltransferase was able to consume only one of the diastereomeric forms of UMPPCHFP. Our attempts to perform ^{19}F NMR experiments to ascertain whether a single diastereomer of **6** remained upon incubation with the thymidylyltransferase were unsuccessful.

Residue Q24 in Cps2L is conserved as Q26 in RmlA from *Pseudomonas aeruginosa*. The position of Q26 in the nucleoside triphosphate binding site is represented in Fig. 3. Previously, we have shown that substituting a primary amide with a primary hydroxyl functionality within the active site of Cps2L in the form of a Q24S mutant broadened the substrate specificity of the enzyme from 2-deoxyribose toward ribose-configured nucleoside triphosphates.[43] The modified UTP analogues were anticipated to function more efficiently with this mutant than with the wild-type enzyme. This was evaluated for the wild-type enzyme and the mutant by measuring apparent kinetic parameters with respect to **2**, UTP, **5**, and **6** in the forward direction upon coupling with **1**, or with respect to **1** in the forward direction upon coupling with **2**, in order to establish whether the variation in the conversions observed with the UTP analogues was as a result of hindered binding affinity or rate limitations with the chemistry (Table 1). Initial reaction rates were monitored using discontinuous HPLC, and it was determined that the K_m, as well as the k_{cat} values, for **2** were similar with the wild-type or mutant Cps2L. This indicates that the formation of an activated Michaelis complex is accomplished approximately equally with either wild-type or mutant Cps2L. There were no significant differences to the k_{cat} for either the wild-type or mutant Cps2L. For both the wild-type and the mutant, the K_m values increased over an order of magnitude between UTP, **5**, and **6**; however, more substantial differences were observed in the magnitude of the changes to k_{cat}. In comparison to the physiological substrate **2**, UTP displayed a 1.6-fold increase in k_{cat}, **5** a 100 000-fold decrease, and **6** a 500 000-fold decrease with the wild-type enzyme as catalyst. Exploring the catalytic power of the mutant enzyme revealed that, with respect to **2**, UTP displayed a 4.5-fold decrease in k_{cat}, a 19 000-fold decrease for **5** and a 300 000-fold decrease for **6**. Thus, the differences between activity for both the wild-type and the mutant catalyzed reactions lies primarily within the k_{cat} value. Additionally, these results indicate that the Q24S mutation provides a small enhancement in the catalytic efficiency with respect to wild-type Cps2L and **2**. These data demonstrate that both Q24S and wild-type Cps2L bind nucleoside triphosphates similarly, as observed through the K_m values, but the nucleophilic attack of the phosphate group and the breaking of the phosphate ester become more hindered with the UTP-analogues containing less acidic pyrophosphonate leaving groups. Rates for **7** were not obtained as a result of difficulties associated with observing reproducible product peaks for integration.

Eighteen metal cations (both divalent and trivalent) were screened to explore the cofactor promiscuity of Cps2L. All nucleotidylyltransferases, such as Cps2L, require a metal cofactor, which plays a key role in stabilizing the departure of

Table 1 Apparent kinetic parameters of substrates with Cps2L WT and Q24S

Substrate	Cps2L	$K_{m,app}$/μM	k_{cat}/s^{-1}	k_{cat}/K_m/μM^{-1} s^{-1}	pK_{a4} [a]
2	WT	105	39	0.371	
2	Q24S	109	46	0.422	
UTP	WT	74.4	45	0.605	
UTP	Q24S	265	24	0.091	
5	WT	664	<0.003	$<4.5 \times 10^{-6}$	6.7
5	Q24S	302	<0.007	$<2.3 \times 10^{-5}$	6.7
6	WT	1020	<0.0007	$<6.7 \times 10^{-7}$	7.5
6	Q24S	1120	<0.002	$<1.8 \times 10^{-6}$	7.5
1	WT	237	50	0.211	
1	Q24S	66.2	43	0.650	

[a] pK_{a4} values based on literature measured values for adenosine triphosphate analogues. The pK_{a4} value for the methylene analogue (equivalent to compound **7**) was 8.4. The pK_{a4} for ATP was 7.1.[41]

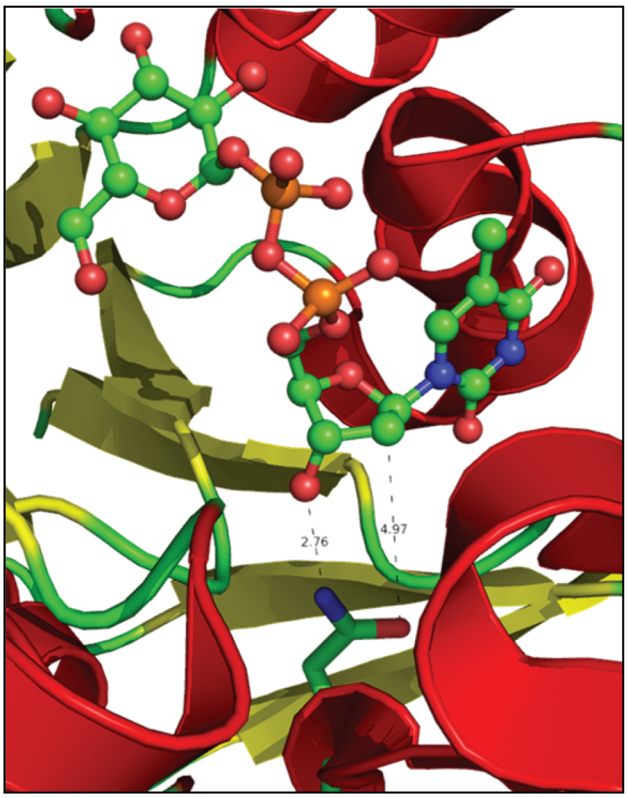

Fig. 3 Structure of *P. aeruginosa* RmlA [Protein Data Bank (PDB) entry 1g1 l] indicating the position of Q26 in the nucleoside triphosphate binding site. dTDP-α-D-Glc is represented as ball and stick. Residue Q26 is represented as stick. Site-directed mutagenesis was performed on Cps2L to yield the Q24S mutant.[31]

Table 2 Comparison of bisphosphonate inhibitory activity against the physiological reaction catalyzed by Cps2L

	Forward reaction			Reverse reaction		
Bisphosphonate	IC_{50}/mM	K_i wrt G1P/mM[a]	K_i wrt dTTP/mM[a]	IC_{50}/mM	pK_{a3} Reference	pK_{a4} Reference
8	1.4 ± 0.05	0.11	0.07	15.6 ± 1.4	5.8[26]	8.0[26]
9	3.6 ± 0.2	0.28	0.19	23.7 ± 1.0	6.2[26]	9.4[26]
10	2.6 ± 0.1	0.21	0.14	20.8 ± 0.1	7.5[26]	11.0[26]
11	4.7 ± 0.2	0.37	0.25	[b]	5.7[60]	8.3[60]
12	10.0 ± 0.1	0.79	0.53	[c]	5.8[61]	9.9[61]
13	10.1 ± 0.3	0.80	0.53	[c]	6.1[60]	10.4[60]

[a] Assuming competitive inhibition, K_i values have been calculated using the Cheng–Prusoff equation.[45] [b] Inhibition was not observed with inhibitor concentrations of 100 mM. [c] Bisphosphonates **12** and **13** were not tested as inhibitors for the reverse reaction due to the high IC_{50} values observed for those bisphosphonates which were better inhibitors for the forward reaction.

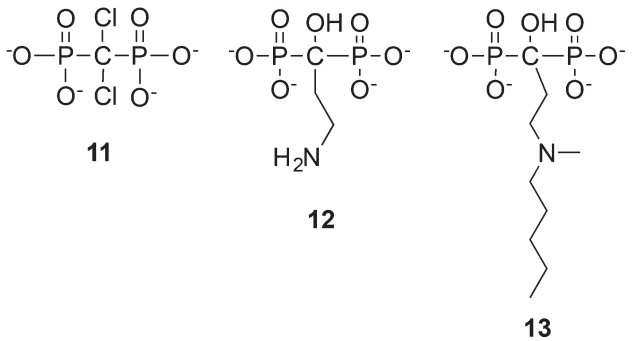

Fig. 4 Structures of commercial bisphosphonates clodronate (**11**), pamidronate (**12**), and ibandronate (**13**).

inorganic pyrophosphate.[17,44] A summary of the product formation using various metal cations is presented in the ESI.† Despite several metals obtaining quantitative conversion after 30 min, it was concluded that Mg^{2+} (the natural cofactor to Cps2L) was the most efficient.

The inhibition strength of a number of non-commercial (**8–10**) and commercial (**11–13**) bisphosphonates was investigated against wild-type Cps2L. IC_{50} values were determined for the six bisphosphonates in the forward physiological reaction catalyzed by Cps2L (ESI†). The IC_{50} and K_i values (as calculated using the Cheng–Prusoff equation[45]) are presented in Table 2. All inhibition values were spread within an order of magnitude and ranged from one to ten millimolar in the forward reaction. In an attempt to correlate the acidity of the bisphosphonate to the inhibition values, plots of IC_{50} values *versus* pK_{a3} and pK_{a4} (ESI†) of the bisphosphonates were constructed. No trends were observed correlating acidity of the pyrophosphate analogue to the IC_{50} (Fig. 4).

Discussion

The progress curves of wild-type Cps2L enzyme with synthetically prepared analogues of UTP indicated that the two most acidic synthetic nucleoside triphosphates were readily accepted by Cps2L as substrates. The difluoromethylene analogue (**5**) was most readily accepted followed by the monofluoromethylene analogue by both the wild-type and mutant enzymes. The monofluoromethylene analogue (**6**) was synthesized and assayed as a mixture of diastereomers at the CHF stereocentre. McKenna was able to distinguish between diastereomers using ^{19}F NMR spectroscopy of dGMPPCHFP.[36] However, the ^{19}F spectra of **6**[46] failed to show two sets of overlapping multiplets as observed by McKenna and co-workers for dGMPPCHFP; at the basic pH of the McKenna studies compound **6** broke down.[36] Nevertheless, we conducted a series of ^{19}F NMR analyses of reaction mixtures of **1** + **6** + Cps2L, varying concentrations, reaction times and pH. Unfortunately, none of these conditions delivered data that could support or refute the hypothesis that a single diastereomer was consumed by Cps2L. Assuming that 50% conversion with **1** + **6** + Cps2L is due to the consumption of a single diastereomer of **6**, this demonstrates that acidity alone is not the determining factor in the rate of these reactions. The methylene derivative (**7**) was, at best, a very poor substrate for both the wild-type and the Q24S mutant. In an effort to probe the mechanism of the nucleotidylyltransferase, we constructed a Brønsted-type plot to explore leaving group activity for compounds **5–7**. This is a common method of analysis for enzymes, particularly with a series of synthesized substrate analogues, as has been accomplished for many enzyme classes including bis(5′-nucleosidyl) tetraphosphatase,[47] and glycoside hydrolases.[48–51] We determined apparent kinetic parameters for the nucleoside triphosphate analogues, calculated $\log(k_{cat})$ and $\log(k_{cat}/K_m)$, values and plotted them as a function of leaving group ability which are defined by the pK_{a4} value of the conjugate acid of the bisphosphonate leaving group (ESI†). We used pK_{a4} values determined for corresponding adenosine triphosphate analogues since the pK_a trend between the fluorinated analogues is unequivocal.[52] The slopes from these plots (β_{lg}) provide a measure of the relative charge build up on the leaving group oxygen.[53] The negative gradient for all slopes indicated that the rate limiting step in the reaction shown in Fig. 1 is the departure of the bisphosphonate for the wild-type and the Q24S mutant using these modified substrates. The limitation in our experimental setup meant that we could not accurately measure rates for analogue **7**, limiting the Brønsted-type analysis to only two points. Nevertheless, the same trend to the β_{lg} values would likely be observed if we had been able

to measure rates with **7**, given a pK_{a4} value of ~8.4.[52] Since the only structural difference between these compounds is the nature of the linker between the β- and γ-phosphates, and **5** is more acidic than UTP and **6** and **7** less acidic, this suggests acidity is not the sole determinant of reactivity. The bond lengths and geometries of phosphonates vary to those of phosphates. The phosphonate C–P bond length is generally 10% longer than the phosphate O–P bond length, and the bond angles are marginally smaller (10%) in the phosphonate *versus* the phosphate.[54] Thus, the alteration in geometry and/or sterics associated with **5–7** may play important roles in modulating the reactivity. An additional factor may be how the interactions between the β- and γ-phosphonates in the ternary complex coordinate to the required Mg^{2+} cation, these interactions may not be optimal in comparison to the interactions observed with **2** or UTP.[55] Nevertheless, a bis-difluoromethylene dTTP analogue was determined to be an inhibitor of DNA polymerase beta,[30] and the X-ray data clearly indicates that there is significant complementarity when oxygen is substituted with a difluoromethylene substituent.

There were substantially greater rates observed with UTP than with the three UTP analogues **5–7**. The large difference between rates observed between UTP and the UTP analogues is consistent with the differences in rates observed for other enzyme systems including glycosidases with physiological and non-physiological substrates. For glycosidases where the physiological substrate is known,[51,56,57] there is often a similarly large difference in either or both K_m or k_{cat} for the physiological substrate *versus* the non-physiological aryl glycosides used for construction of free energy relationships.

The kinetic data provides quantitative insight into the effects of the Q24S mutation *versus* the wild-type enzyme. In terms of catalytic efficiency, the mutant is marginally more active than wild-type with respect to dTTP, but 7-fold less active with UTP. The mutant is 6-fold more active with the difluoro analogue **5**, but only 2-fold more active with the monofluoro analogue **4**. We initially foresaw that the Q24S mutant would benefit the production of UDP-α-D-Glc with respect to improved binding of the ribo-configured nucleotide, based on analysis of the crystal structure of a Cps2L homologue (Fig. 3[39]). Analysis of our kinetic data clearly demonstrated that the mutated residue did not improve binding (in terms of K_m), but instead increased the overall efficiency of the reaction by increasing k_{cat}. This demonstrates the difficulties in predicting changes to catalytic activity upon active site mutation based upon structural data, and demonstrates the importance of effective mutagenesis and screening approaches to modify enzyme substrate specificity.[58] In another effort to potentially improve the activity of Cps2L, we examined the effect of different metal ions present in the enzyme assay. Our premise was that by altering the metal ion cofactor, we could enhance UTP-metal interactions and potentially increase the rate of pyrophosphate release, thereby increasing production of the sugar nucleotide. One study on a homologous thermostable nucleotidylyltransferase from *S. tokodaii*, revealed improvements in the activity of the enzyme by utilizing different metal ions, and that the relative activity of Co^{2+} (243%) and Mn^{2+} (164%) was more effective than Mg^{2+} (100%).[59] We observed a wide range of activities with different metal ions, but Mg^{2+} was confirmed as being the most effective metal cofactor, followed by other biologically relevant metal ions, including Mn^{2+} and Fe^{2+}. The homologous enzyme RmlA from *E. coli* also possesses a similar requirement for Mg^{2+}, and its binding residues have been confirmed by structural analysis.[17]

One consideration with the UTP analogues (**5–7**) was the potential of them, or their pyrophosphonate group, to act as an inhibitor of Cps2L. Therefore, a series of bisphosphonates were evaluated as inhibitors of Cps2L in the forward and reverse directions by HPLC to determine IC_{50} values. Included in this study were three bisphosphonates prepared synthetically (**8–10**) and that corresponded to the leaving groups from compounds (**5–7**). In addition, commercially available bisphosphonates (**11–13**) that are used clinically for the treatment of bone diseases were also evaluated, the structural similarity between these bisphosphonates and pyrophosphate prompted us to use them in this study. All of the bisphosphonates showed millimolar IC_{50} values when the enzyme was monitored in the forward direction (Table 2). In the reverse reaction catalyzed by Cps2L, the IC_{50} values for **8–10** were approximately ten-fold higher than in the forward reaction, while **11** did not show any inhibition up to a 100 mM concentration; as a consequence, **12** and **13** were not evaluated in the reverse direction. These bisphosphonates presumably act as competitive inhibitors, therefore, K_i values were determined using the Cheng–Prusoff equation.[45] The most active compound was the difluoromethylene bisphosphonate **8**. This compound had micromolar K_i values with respect to both G1P and dTTP. The two amino-containing bisphosphonates, **11** and **12** were the least active. These two compounds are the most sterically encumbering, and also contain an amine functionality, these structural features result in a 5 to 10-fold reduction in inhibition, as observed from the calculated K_i values. There was no correlation between IC_{50} and either pK_{a3} or pK_{a4} (ESI†), indicating that charged bisphosphonates alone are not sufficient to generate inhibitors.

Conclusion

In summary, we have demonstrated, using a series of phosphonate analogues, that the mechanism of a nucleotidylyltransferase is subject to a rate limiting step comprising of the departure of the leaving group for a series of synthetic substrate analogues, but that this rate limiting step does not necessarily occur for natural nucleoside triphosphates. We have demonstrated that a series of halogenated bisphosphonates are able to inhibit the nucleotidylyltransferase and that no correlation exists between the degree of inhibition and acidity of the analogues. These insights may guide the design of more selective inhibitors of nucleotidylyltransferases.

Experimental procedures

The synthetic preparation of **5–7**, and **9–10** was based on literature methodology.[46] The expression and purification of Cps2L wild-type and Cps2L Q24S were accomplished as described previously.[37,43] Enzyme assays were performed as described previously[37] using 1.0 mM NTP, 2.0 mM sugar 1-phosphate, 2.2 mM $MgCl_2$, and 0.5 EU inorganic pyrophosphatase, and were initiated by the addition of 2 EU nucleotidylyltransferase in Tris-HCl buffer (20 mM final buffer concentration, 50 μL reaction volume), where 1 EU is defined as the amount of enzyme required to catalyze the conversion of 1 μmol of dTTP (**2**) and α-D-Glc-1-P (**1**) per minute at 37 °C. Reactions were monitored by HPLC at 254 nm using a linear gradient from 90/10 to 40/60 A/B over 8.0 min followed by a plateau at 40/60 A/B from 8.0 to 10.0 min at 1.0 mL min^{-1} where A is an aqueous buffer containing 12 mM *n*-Bu_4NBr, 10 mM KH_2PO_4, and 5% HPLC grade CH_3CN (pH 4.0) and B is 100% HPLC grade CH_3CN. Conversions were calculated by dividing the area of the apparent product peak by the total area of the product and substrate peaks combined. ESI-MS/MS was used to confirm the mass and fragmentation of the products, using previously described parameters.[38–40]

Kinetic assays were performed, as described previously,[38,40] for the wild-type and mutant enzymes at various concentrations of **2**, UTP, **5**, and **6** (20 μM, 50 μM, 100 μM, 200 μM or 400 μM) with 2 mM α-D-Glc-1-P. Each assay consisted of five dilutions of the nucleotide substrate run in separate reactions. Aliquots were removed from each reaction at 1, 2, 4, 6, and 10 min, and were quenched with an equal volume of methanol. The same HPLC method was used to determine conversions. Final concentrations of the sugar nucleotides were determined by comparing the peak area of the product to that of a uridine internal standard (100 μM final concentration). Initial rates were determined by plotting concentration of sugar nucleotide products *versus* time. Michaelis–Menten and Lineweaver–Burk plots were fitted to the Michaelis–Menten equation using GraFit 5.0 software. The overall efficiency of the enzyme for a substrate was determined by k_{cat}/K_m, where K_m indicated the binding affinity of the enzyme for the substrate and k_{cat} measured the rate of reaction.

Various soluble divalent and trivalent metal cations were added to the physiological reaction in the place of Mg^{2+} to test for reaction efficiency. A total of seventeen cations (Ca^{2+}, Zn^{2+}, Fe^{3+}, Cu^{2+}, Ce^{3+}, Ni^{2+}, Fe^{2+}, Co^{2+}, Mn^{2+}, La^{3+}, Cr^{2+}, Cr^{3+}, Gd^{3+}, Ga^{3+}, In^{3+}, Pb^{2+}, and Sn^{2+}) were compared to Mg^{2+}. Salt solutions of each of the cations of interest were prepared to a concentration of 10 mM (2.2 mM final). Identical enzymatic reactions were performed as described above, but by replacing Mg^{2+} with one of the seventeen cations listed.

IC_{50} values for **8–13** were determined through enzymatic assays of the physiological reaction catalyzed by Cps2L. Reaction mixtures were carried out in 50 mM Tris HCl buffer, pH 7.4. Reaction substrates included $MgCl_2$ (50 mM), α-D-Glc-1-P (100 mM), dTTP (50 mM), Cps2L (0.004 EU) recombinantly expressed in *E. coli* and bisphosphonate analogues ranging from 0.01 mM – 20 mM. Control reactions were performed in the absence of bisphosphonate analogues. Assays were incubated at 37 °C and quenched at 15 minutes with an equivalent volume of methanol. HPLC analysis of quenched aliquots was used to confirm product conversion at a wavelength of 254 nm, and IC_{50} values were determined by plotting changes in conversion and fitted using GraFit 5.0 software. K_i values were calculated using the Cheng–Prusoff equation.[45]

Abbreviations

ESI-MS/MS	Electrospray ionization tandem mass spectrometry
NTP	Nucleoside 5′-triphosphate
NDP	Nucleoside 5′-diphosphate
NMP	Nucleoside 5′-monophosphate
dTTP	Deoxythymidine 5′-triphosphate
UTP	Uridine 5′-triphosphate
ATP	Adenosine 5′-triphosphate
CTP	Cytidine 5′-triphosphate
GTP	Guanosine 5′-triphosphate
GDP	Guanosine diphosphate
α-D-Glc-1-P	α-D-Glucopyranose-1-phosphate
Glc*N*-1-P	α-D-Glucosamine-1-phosphate
Glc*N*Ac-1-P	*N*-Acetyl α-D-glucosamine-1-phosphate
α-D-Man-1-P	α-D-Mannose-1-phosphate
dTDP-α-D-Glc	Deoxythymidine α-D-glucopyranose
UDP-α-D-Glc	Uridine-α-D-glucopyranose
wrt	With respect to.

Acknowledgements

The Canadian Institutes of Health Research, the Natural Science and Engineering Research Council of Canada, and the Dalhousie College of Pharmacy Endowment Fund are thanked for funding. Dr Steve Bearne (Dalhousie University) is thanked for helpful discussions.

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