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Synthesis of 2′-Deoxyuridine 5′-(α,β-Imido)triphosphate: A Substrate Analogue and Potent Inhibitor of dUTPase

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Abstract—The dUDP analogue, 2′-deoxyuridine 5′-(α,β-imido)diphosphate (dUPNP) was synthesized. The corresponding triphosphate analogue (dUPNPP) was prepared by enzymic phosphorylation of dUPNP using the enzyme pyruvate kinase and phosphoenolpyruvate as the phosphate donor. This method was successful in phosphorylating the imidodiphosphate analogue of 2′-deoxythymidine (dTPNP) to 2′-deoxythymidine 5′-(α,β-imido)triphosphate (dTPNPP), in contradiction to a previous report. The properties of dUPNPP have been tested using the enzyme dUTPase from *Escherichia coli*. This enzyme, having a crucial role in nucleotide metabolism, is strictly specific for its substrate (dUTP) and catalyzes the hydrolysis of the α,β-bridge, resulting in dUMP and pyrophosphate. Replacement of the α,β-bridging oxygen in dUTP with an imido group resulted in a nonhydrolyzable substrate analogue and a potent competitive inhibitor of dUTPase (K_i=5μM). The analogue prepared (dUPNPP) may be utilized in crystallographic studies of the active site of dUTPase to provide knowledge about specific interactions involved in substrate binding and as a parental compound in design of dUTPase inhibition for medical purposes. Copyright © 1996 Elsevier Science Ltd

Introduction

The interest in dUTPase has increased dramatically in recent years due to the crucial role of this enzyme in pyrimidine metabolism and DNA replication.[1,2] dUTPases are found in a variety of eukaryotic and prokaryotic organisms, as well as viruses like herpesviruses, poxviruses, and certain retroviruses.[3–8] The presence of dUTPase is important for the cell life cycle and essential for the viability of *E. coli*[9] and *Saccharomyces cerevisiae*.[10]

The specificity constant (k_{cat}/K_M) of *E. coli* dUTPase for dUTP is 10^5 times higher than that for any of the other common nucleotides (Larsson et al., personal communication) indicating an almost exclusive specificity of the enzyme for dUTP. Its active site discriminates between nucleotides with respect to base, sugar, and phosphate moieties. The crystal structure of native dUTPase reveals a trimeric arrangement of monomers.[11] The subunit is composed of 152 amino acid residues[12] of which the first 136 residues have been identified in the structure. The remaining 16 carboxy-terminal residues are invisible and believed to be flexible.[11] Primary structures of dUTPase from widely different sources have become available, and comparisons indicate interesting similarities in five regions scattered along the sequence.[13] Four of these motifs come together in the quaternary structure forming a complex interaction between the subunits, with the fifth homologous region positioned on the flexible part. The active site has been proposed to be located in this area.[11]

The purpose of the present study was to find a substrate analogue suitable for crystallographic and other investigations of the active site of dUTPase. An interesting compound, very closely related to the natural substrate, would be dUPNPP, having the hydrolyzable α,β-oxygen bridge in dUTP replaced by —NH—. In this paper, we describe the synthesis of dUPNPP and dTPNPP (Fig. 1) from their corresponding diphosphate analogues using pyruvate kinase as the enzyme and the phophoenolpyruvate as phosphorylating agent, according to Jaffe and Cohn.[14] In a recent paper, Ma et al.[15] claimed that dTPNP is not a substrate for kinases, such as pyruvate kinase, arginine kinase, and creatine kinase and the terminal phosphate of dTPNPP has to be incorporated chemically through activation of the pyrophosphate moiety of the nucleoside diphosphate by carbonyldiimidazole. However, both pyruvate kinase and creatine kinase could be successfully used in the preparation of

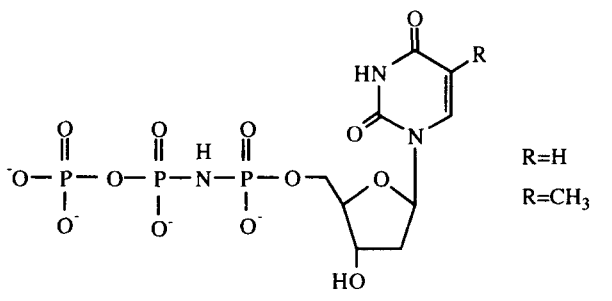

Figure 1. Structure of pyrimidine analogues (dUPNPP and dTPNPP) synthesized.

AMPNPP from its corresponding α,β-imidodiphosphate analogue, AMPNP.[16,17] The present investigation shows that enzymic phosphorylation has, in fact, a broader applicability and can be used for preparation of pyrimidine triphosphates from the corresponding diphosphates.

Results and Discussion

Synthesis of dUPNPP and dTPNPP

We have successfully synthesized dUPNPP enzymically from dUPNP by a method described by Jaffe and Cohn.[19] These authors used pyruvate kinase and phosphoenolpyruvate in transforming ADPβS to ATPβS. The natural substrate for pyruvate kinase is ADP, but AMPNP has also proven to be a substrate.[17]

In a first attempt to synthesize dUPNPP, we tried a chemical method to incorporate the terminal γ-phosphate.[15] However, a complex reaction mixture was obtained and the triphosphate analogue was difficult to isolate in satisfactory yield. An alternative way would be to use an enzyme to phosphorylate dUPNP. Enzymic specificity would diminish side reactions, thus facilitating the purification. By the method of Jaffe and Cohn,[14] we were able to obtain a pure triphosphate analogue in 92% yield, in 6 h at 33 °C. The reaction time is 10- to 15-fold longer than used for the adenosine thioanalogue (ADPβS).

As reported by Ma et al.,[15] the natural nucleotide dTDP, but not dTPNP, is a substrate for pyruvate kinase. Our finding above led us to investigate if the methyl group in the 5-position of the uracil ring would result in a poorer substrate compared to dUPNP. The phosphorylation would be very slow, demanding a prolonged reaction time. We found that dTPNP is a poor substrate with a 5-fold slower reaction rate than dUPNP and 60-fold compared to ADPβS under comparable conditions. Both dUPNPP and dTPNPP were stable under the reaction conditions and no breakdown products could be observed after 24 h at 33 °C.

Effect of dUPNPP on dUTPase

The true substrate for dUTPase is dUTP in complex with Mg^{2+} which in the neutral pH range is hydrolyzed with a K_M of 0.2 μM and a k_{cat} of 6 s^{-1} resulting in a specificity constant ($3 \times 10^7 s^{-1} M^{-1}$) which is 10^5 times higher than that for dCTP, the next best substrate for dUTPase among the common nucleotides. This remarkable specificity results from the ability of dUTPase to discriminate between different nucleotides, not only with respect to the base but also the sugar and phosphate moieties (Larsson et al., personal communication).

To determine if the imido analogue of dUTP is hydrolyzed by the enzyme, dUPNPP was incubated at 25 °C at neutral pH in the presence of Mg^{2+}. The stability of dUPNPP was tested under the same conditions, but without enzyme. The analogue was found to be resistant to hydrolysis, and no reaction or breakdown products could be detected after 30 h of incubation, even in the presence of enzyme. The hydrolysis of dUTP in the presence of varying concentrations of dUPNPP was measured, and the inhibition of the dUTPase reaction was calculated. dUPNPP was shown to be a potent competitive inhibitor with an inhibition constant, $K_i = 5$ μM at pH 7.9 (Fig. 2).

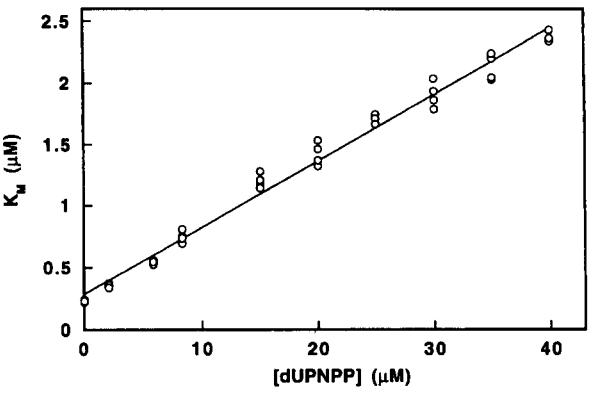

Figure 2. Inhibition of the enzymic activity of dUTPase by dUPNPP. The apparent K_M versus the concentration of inhibitor was plotted and the inhibition constant was estimated to 5 μM.

dUPNPP is probably retaining most of the properties of the parent nucleotide. It is expected to mimic closely the α,β-oxygen bridge in dUTP. In the crystal structures of pyrophosphate and imidodiphosphate, the bond angles and lengths of the P—O—P and P—N—P linkages are nearly identical.[18] An analogueous similarity between dUTP and dUPNPP is consistent with the relatively tight binding of the latter to dUTPase. It binds ~10^3 times better than dCTP and dTTP, which are slowly hydrolyzed, emphasizing at the same time the importance of an uracil base for tight binding and a relatively undistorted triphosphate moiety for hydrolysis. The differences between dUTP and dUPNPP may be expressed in charge distribution, metal-binding properties and to some degree in structure, differences that individually or taken together can explain the stability of enzyme-bound dUPNPP. This stability suggests that the inhibitor should be a potent analogue in crystallographic studies of the active site of dUTPase, allowing the identification of functional groups involved in the substrate binding. The importance of such a study is twofold. dUTPase may be a target for the design of inhibitory compounds for use in cancer and virus chemotherapy as supported by recent experiments using α,β-methylene derivatives of dUTP.[19] Secondly, the structural information of the active site would contribute to the knowledge and understanding of the mechanism of enzymic hydrolysis of phosphoric anhydrides, a fundamental process in biological systems.

We have recently been able to crystallize the dUTPase–dUPNPP complex and the crystallographic work is now in progress.

Experimental

Abbreviations

dUTPase, deoxyuridine 5′-triphosphate nucleotidohydrolase (EC 3.6.1.23); dUTP, 2′-deoxyuridine 5′-triphosphate; dUMP, 2′-deoxyuridine 5′-monophosphate; UTP, uridine 5′-triphosphate; dCTP, 2′-deoxycytidine 5′-triphosphate; dTTP, 2′-deoxythymidine 5′-triphosphate; dTDP, 2′-deoxythymidine 5′-diphosphate; dUPNP, 2′-deoxyuridine 5′-(α,β-imido)diphosphate; dUPNPP, 2′-deoxyuridine 5′-(α,β-imido)triphosphate; dTPNP, 2′-deoxythymidine 5′-(α,β-imido)-diphosphate; dTPNPP, 2′-deoxythymidine 5′-(α,β-imido)triphosphate; ADP, adenosine 5′-diphosphate; ADPβS, adenosine 5′-(β-thio)diphosphate; ATPβS, adenosine 5′-(β-thio)triphosphate; AMPNP, adenosine 5′-(α,β-imido)diphosphate; AMPNPP, adenosine 5′-(α,β-imido)triphosphate; *E. coli*, *Escherichia coli*; FPLC, fast protein liquid chromatography.

Chemistry

Nucleosides were dried in vacuo over P_2O_5 at 80 °C for 4 h. Triethylphosphate was distilled and stored over 4 Å molecular sieves. ^{31}P NMR was recorded on a Varian XL-300 spectrometer, and ^1H broadband decoupling was used. The chemical shifts were determined relative to 85% H_3PO_4 with positive shifts being downfield of the reference. ^1H NMR spectra were taken on a Varian XL-300 spectrometer. Mass spectra were recorded on a Jeol JMS-SX-102 spectrometer. Pyruvate kinase and phosphoenolpyruvate were purchased from Boehringer Mannheim. The deoxyuridine was from Sigma and deoxythymidine and dUTP were purchased from Pharmacia. For ion-exchange chromathography Q-Sepharose, MonoQ HR and MonoS HR, all from Pharmacia, were used.

Trichloro[(dichlorophosphoryl)imino]phosphorane. This was prepared according to Emsley et al.[20] and used for the preparation of 2′-deoxyuridine 5′-(α,β-imido)diphosphate (dUPNP) and 2′-deoxythymidine 5′-(α,β-imido)diphosphate (dTPNP).

2′-Deoxythymidine 5′-(α,β-imido)diphosphate. This was synthesized as previously described by Tomasz et al.[21] and Ma et al.[15]

2′-Deoxyuridine 5′-(α,β-imido)diphosphate. The dried nucleoside (0.5 g, 2.2 mmol) was dissolved in anhydrous triethylphosphate (11 mL) at −15 °C to −20 °C and 1.5 equivalents of trichloro[(dichlorophosphoryl)imino]phosphorane (0.89 g, 3.3 mmol) in 2.5 mL of anhydrous triethylphosphate was added dropwise. The reaction mixture was stirred for 1.5 h at −15 °C to 20 °C and then 100 mL of 0.1 M NaOH was added at 0 °C. The resulting solution was extracted with ethyl acetate, and the water phase was evaporated. The crude product was applied to a Q-Sepharose (vol 60 mL) column and chromatographed at 5 °C using a linear gradient (500 mL) of 0–0.6 M triethylammonium bicarbonate buffer (TEAB), pH 8.5. The fractions from the main peak were pooled and evaporated. The residue was dissolved in water and lyophilized to remove excessive bicarbonate buffer. The yield obtained was 22%. The UV spectrum has an absorption max at 262 nm. The ^1H NMR was consistent with the proposed structure (Fig. 1). ^{31}P NMR (D_2O): δ 1.68 (1P, s, α-P), −0.08 (1P, s, β-P); negative ion FAB gave the expected ion $[M-H]^- = 386$.

General method for preparation of 5′-imidotriphosphate analogues of 2′-deoxyuridine and 2′-deoxythymidine

For the synthesis of dUPNPP and dTPNPP, the triethylammonium salt of dUPNP and dTPNP were used. The reaction was performed in 4 mL 0.1 M Tris–HCl, pH 8.5, containing 25 mM Mg^{2+}, 1 mM dithioerythritol, 60 mM phosphoenolpyruvate, 16 mM of the 5′-imidodiphosphate analogue and 0.1 M KCl. It was initiated by addition of 3 mg pyruvate kinase and monitored chromatographically by separating sampled reaction products on a MonoQ HR column using a linear gradient of 0–0.8 M TEAB, pH 8.5. The reaction was completed in 6 h for dUPNPP and 24 h for dTPNPP at 33 °C. The reaction mixture was diluted to 50 mM ionic strength in water and applied to a 60 mL Q-Sepharose column, equilibrated with water. The chromatogram was developed using a linear gradient (500 mL) of 0–0.8 M TEAB, pH 8.5. The fractions of the main peak were pooled and evaporated. The residue was dissolved in water and lyophilized to remove excessive bicarbonate buffer. The yields obtained were 92 and 90% for dUPNPP and dTPNPP, respectively. The retention time for dUPNPP and dTPNPP on MonoQ HR were comparable to dUTP and the structure was confirmed by ^{31}P NMR and mass analysis. For dUPNPP: ^{31}P NMR (D_2O): δ 0.92 (1P, d, $J = 6.5$ Hz), −5.94 (1P, d, $J = 20$ Hz), −11.20 (1P, d, $J = 20$ Hz); negative-ion FAB gave the expected ion $[M-H]^- = 466$. For dTPNPP, ^{31}P NMR (D_2O): δ 0.42 (1P, s), −7.99 (1P, s), −11.42 (1P, s); negative-ion FAB gave the expected ion $[M-H]^- = 480$.

Biochemistry

Enzyme preparation. dUTPase was isolated from *E. coli* strain pHW1, pRK248/MC1000 according to a method described by Hoffmann et al.[22] The enzyme was stored at −20 °C in 25 mM Bis–Tris, 100 mM NaCl, pH 7. Before the kinetic measurement, the enzyme solution was desalted on a PD-10 column (Pharmacia) equilibrated with 0.9 mM acetic acid, pH 5.8, in 0.1 M KCl.

Test of hydrolysis rate of dUPNPP by dUTPase. The hydrolytic activity of dUTPase towards dUPNPP was studied at 25 °C by incubating the nucleotide (0.9 mM, pH 7) with and without dUTPase (5 μM active site).

Samples from the incubation mixture were taken at regular time intervals, up to 30 h. The samples were analysed for hydrolysis products by ion-exchange chromatography using a MonoQ HR column on a FPLC (Pharmacia) (Larsson et al., personal communication). Buffer system was 10 mM MOPS, pH 7, with a 10 mL gradient of 0–0.6 M KCl. The samples were quenched by 10 mM EDTA, prior to analysis.

Kinetic measurements. Kinetic measurements were performed at 25 °C by a continuous assay using a stopped-flow apparatus as described by Larsson et al. (personal communication). Protons liberated by the hydrolysis of dUTP at pH 7.9 were buffered by 25 μM Cresol Red and 250 μM bicine in the presence of 100 mM KCl, 5 mM $MgCl_2$, and monitored spectrophotometrically at 573 nm. The enzyme activity was studied at two different enzyme and substrate concentrations (0.1 μM active sites and 2 μM dUTP, and 0.55 μM active sites and 10 μM dUTP, respectively) and the inhibition was monitored by including the dUPNPP at various concentrations (0–50 μM). Kinetic parameters were evaluated by application of the integrated Michaelis–Menten equation[23] as described (Larsson et al., personal communication).

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