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Lanthanide Catalyzed Cyclization of Uridine 3′-*p*-Nitrophenyl Phosphate

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Steady state kinetics and ^{15}N isotope effects have been used to study the cyclization reaction of uridine 3′-*p*-nitrophenyl phosphate. The cyclization reaction is catalyzed by transition metal ions and lanthanides, as are substitution reactions of many phosphate esters. Kinetic analysis reveals that the erbium-catalyzed cyclization reaction involves the concerted deprotonation of the 2′-OH group and departure of the leaving group. The transition state is very late, with a very large degree of bond cleavage to the leaving group, which could be due to a large degree of polarization of the P–O bonds by erbium.

Nucleophilic substitution reactions of phosphate esters have been the subject of much research (*1*). Of special interest is the hydrolysis of phosphate diesters, for this class of compounds includes DNA and RNA, and hydrolysis of these compounds is the function of many enzymes. Metal ions have been found to be important in both enzymatic and nonenzymatic reactions of phosphate esters (*2*,*3*). Metal ions can catalyze substitution reactions of phosphates in several ways and understanding the role of metal ions in these reactions is critical to understanding the entire mechanism.

Metal ions in the lanthanide series have received some attention as exceptional catalysts of phosphate ester substitution reactions in several systems (*4*,*5*). Lanthanide complexes have been used as catalysts of phosphate ester substitutions with the eventual goal of preparing complexes that can cleave specific nucleic acids sequences (*6–11*). Under certain conditions, a lanthanide ion dimer has been observed with incredible catalytic capability toward phosphate ester substitution reactions of a dinucleotide (*12*). In this work, we have studied the cyclization reaction of uridine-3′-*p*-nitrophenyl phosphate, a model for RNA with an activated leaving group, by lanthanide ion catalysts. We report the results of steady state kinetics and ^{15}N isotope effects using several lanthanides as well as Zn^{+2} and imidazole as catalysts for comparison. The results provide insight into the transition state of this lanthanide ion catalyzed phosphate ester substitution reaction.

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EXPERIMENTAL PROCEDURES

Materials. Uridine and dibutyl tin oxide from Aldrich were used without further purification. Benzoyl chloride, triethylamine, dioxane, pyridine, methanol, and dihydropyran from Aldrich were distilled prior to use. *p*-Toluenesulfonic acid from Aldrich was recrystallized prior to use. Zinc sulfate was from MCB. Lanthanides were from Alfa except for europium which was from Aldrich. All lanthanides were nitrates except lutetium which was the chloride. *p*-Nitrophenyl phosphorodichloridate was prepared as previously described (*13*). Triethylammonium bicarbonate was prepared by bubbling CO_2 through a solution of 200 ml of triethylamine in 1080 ml of water until the pH of the solution was 8.0.

3′-O-Benzoyluridine. Uridine (4.86 g) was dissolved in 600 ml of distilled methanol in a dried flask and the solution was heated to reflux under nitrogen. Dibutyl tin oxide (4.92 g) was added and the solution was refluxed for about 1 h, after which the cloudy solution had clarified. The solution was cooled to room temperature and triethylamine (8.4 ml) was added followed by benzoyl chloride (7.2 ml). After 10 min, the reaction was evaporated *in vacuo* to a viscous oil. The oil was partitioned between water and ether, and the water was evaporated *in vacuo* until a precipitate was observed. The water fraction was refrigerated overnight and the crude precipitate was collected by filtration. The white powder was recrystallized from ethanol.

2′,5′-Di-tetrahydropyranyluridine. A solution of 3′-*O*-benzoyluridine (3.2 g), dihydropyran (17.3 ml), and *p*-toluenesulfonic acid (0.45 g) in 60 ml of dry dioxane was stirred at room temperature. After 90 min, saturated NH_4OH (0.16 ml) was added and the solution was evaporated *in vacuo* to a viscous oil. The oil was taken up in methanol (50 ml) and the solution was cooled to 4°C. Ammonia was bubbled through the solution until the solution was saturated, and the solution was stirred for 64 h. Silica gel was added to the solution, which was evaporated to dryness *in vacuo*. The silica was dry loaded onto a silica column (4 × 30 cm) and the column was eluted with 19:1 chloroform: methanol. Fractions were analyzed by TLC in the chromatography solvent. Peaks exhibiting UV absorbance were pooled, evaporated to dryness, and analyzed by ^{1}H NMR. The light orange glassy product was dried in a vacuum desiccator over P_2O_5.

2′,5′-Di-tetrahydropyranyluridine 3′-p-nitrophenyl phosphate. p-Nitrophenyl phosphorodichloridate (2.98 g) was dissolved in 10 ml of dry dioxane with pyridine (1.8 ml). A solution of 2′,5′-di-tetrahydropyranyluridine (2.3 g) in 10 ml of dry dioxane was added to the first solution by syringe over 20 min. After stirring for 2 h, a solution of pyridine (1.8 ml) in water (8 ml) was added rapidly and the solution was stirred for 5 min. The solution was evaporated *in vacuo* and the crude oil was taken up in 25 ml of water. The aqueous solution was extracted with chloroform (3 × 50 ml) and the organic layers were dried over $MgSO_4$, filtered, and evaporated to a green foam. The foam was taken up in 2 ml of chloroform and this solution was loaded onto a silica column (5 × 15 cm). The column was eluted with 5:1 ethyl acetate: methanol. Fractions were analyzed by TLC in the chromatography solvent. Peaks exhibiting UV absorbance were pooled, evaporated to dryness, and analyzed by ^{1}H NMR.

The crude product was taken up in 250 ml of water, titrated to pH 7, and loaded

onto a Sephadex A25 anion exchange column (2.5 × 10 cm), which had been equilibrated with 0.1 M triethylammonium bicarbonate, pH 8.0, and washed with deionized water until the eluate was free of buffer salt. The column was eluted with a linear gradient from 0 to 0.25 M TEAB and the eluate was monitored at 280 nm. Fractions containing product were evaporated to dryness, and the crude oil was repeatedly taken up in methanol and evaporated to dryness. The TEAB-free oil was taken up in 200 ml of water and passed through a Sephadex C25 cation exchange column (2.5 × 10 cm), which had been equilibrated with 1 M sodium acetate, pH 5.5, and washed with deionized water until the eluate was free of buffer salts. The column was washed with water and the eluate was monitored at 280 nm. Fractions containing product were titrated to pH 6.0 and lyophilized.

Uridine 3′-p-nitrophenylphosphate. The amount of 2′,5′-di-tetrahydropyranyluridine 3′-*p*-nitrophenyl phosphate required for a particular experiment was taken up in water sufficient to make a 10 mM solution and 6 N HCl was added to make the solution 0.1 N in HCl. After 40 min the solution was placed on ice and used as needed.

Determination of product. A sample of 2′,5′-*di*-tetrahydropyranyluridine 3′-*p*-nitrophenyl phosphate (6 mg) was taken up in 0.5 ml of D_2O and deblocked by addition of 25 μl of 1 N HCl. This sample was scanned by ^{31}P NMR at 202.34 MHz. The sample was titrated to pH 5.5 by addition of 50 mM MES at pH 6.0, and 0.09 ml of 10 mM Eu^{+3} was added. The sample was again scanned by ^{31}P NMR after 1 h.

Determination of cyclization rates. The rates of cyclization of uridine 3′-*p*-nitrophenyl phosphate were determined by spectrophotometric assay at 330 nm. The extinction coefficient was determined by complete reaction of a known amount of substrate with ribonuclease. Reactions with lanthanides were performed in 3 ml quartz cuvettes at 37°C in 5 mM MES, pH 5.5. The pH of reactions was found to remain constant to any extent of reaction.

pH Variation of cyclization rates. The rates of cyclization of uridine 3′-*p*-nitrophenyl phosphate were determined with erbium as above except that 5 mM MES was replaced by 25 mM Homopipes (pH 4–5.5) or 25 mM MES (pH 5.5–6), and that reactions were performed in 1 ml quartz cuvettes. The pH of reactions was found to remain constant to any extent of reaction. As ionization of *p*-nitrophenol becomes significant at the higher pH values, the extinction coefficients were determined at 330 nm at each individual pH.

Nitrogen isotope effects with lanthanides and zinc. All of the procedures described below were performed in triplicate for a given experiment. 2′, 5′-*Di*-tetrahydropyranyluridine 3′-*p*-nitrophenyl phosphate (60 mg) was deblocked in 10 ml of dilute HCl and the solution was mixed with an equal volume of 0.5 M MES, pH 6.0. To this solution was added 1 ml of 50 mM erbium nitrate or zinc sulfate, or 0.5 ml of 50 mM europium nitrate. After 15 min, the erbium reactions were quenched by titration with 5 N sulfuric acid to give a pH of 3–4. The europium reactions were likewise quenched after 8 min, while the zinc reactions were quenched after 60 min. The acidified reaction was extracted three times with an equal volume of ether. The aqueous layer was made basic with 1 ml of 5 N NaOH. The ether layers were combined and evaporated to dryness and the extracted *p*-nitrophenol was taken up in water and made basic with 1 ml of 5 N NaOH. The concentration of *p*-nitrophenol in both samples was determined by spectrophotometric assay at 400 nm. Both samples

were extracted three times with equal volumes of ether and the aqueous layers were acidified with 5 N sulfuric acid until the solutions were colorless. The solutions were extracted three times with equal volumes of ether. The ether layers were combined, dried over $MgSO_4$, and filtered. The ether was removed by evaporation *in vacuo* and the remaining *p*-nitrophenol was purified by vacuum sublimation. The *p*-nitrophenol was washed into quartz tubes (24 cm × 9 mm o.d., 7 mm i.d.) with ether. The ether was removed by aspiration and copper oxide (10 g), copper (0.2 g), and silver (0.2 g) were placed in the tube. The quartz tube was evacuated on a vacuum line, sealed with a torch, and heated to 850°C for 2 h. The quartz tubes were cracked on a high vacuum line and the nitrogen gas was distilled through dry ice-2-propanol and liquid nitrogen traps and collected on molecular sieves chilled with liquid nitrogen. The purified nitrogen gas was analyzed on a Finnegan MAT Delta isotope ratio mass spectrometer.

2′,5′-Di-tetrahydropyranyluridine 3′-*p*-nitrophenylphosphate (30 mg) was deblocked in 5 ml dilute HCl. This solution was made basic with 0.2 ml of 5 N NaOH and the reaction was monitored at 400 nm until no further increase was observed. The solution was acidified and extracted three times with equal volumes of ether. The ether layers were combined, dried over $MgSO_4$, and further worked up as described above. The isotopic abundance determined by this procedure is that of the nitro group in the starting material.

Nitrogen isotope effects with imidazole. 2′,5′-Di-tetrahydropyranyluridine 3′-*p*-nitrophenylphosphate (60 mg) was deblocked in 10 ml of dilute HCl and this solution was added to 40 ml of imidazole buffer (0.625 N, pH 6.75). After 20 min, the reaction was quenched by addition of 5 N sulfuric acid to pH 3–4. All of the procedures above were followed for the imidazole reactions except that the final ether extraction of the basic aqueous solutions was replaced by an extraction with dichloromethane. The pH of the aqueous layer was checked to ensure that it was greater than 12 before the basic ether extraction.

Data analysis. The abundances of ^{15}N in unreacted starting material (R_o), in remaining substrate (R_s), and product (R_p) were determined as described, as was the fraction of reaction (f). For comparisons between R_o and R_s, the isotope effects were calculated using Eq. [1].

$$\text{Isotope effect} = \log(1 - f)/\log((1 - f)(R_s/R_o)) \quad [1]$$

For comparisons between R_o and R_p, the isotope effects were calculated using Eq. [2].

$$\text{Isotope effect} = \log(1 - f)/\log(1 - f)(R_p/R_o)) \quad [2]$$

RESULTS

Determination of product. The reaction of uridine 3′-*p*-nitrophenyl phosphate was scanned before and after addition of metal ion catalyst by ^{31}P NMR. The peak observed after addition of metal ion was at 17.8 ppm, which is in the region where cyclic phosphates are observed, and no other peaks were observed. The only detectable product of the metal catalyzed cyclization was uridine 2′,3′-cyclic phosphate.

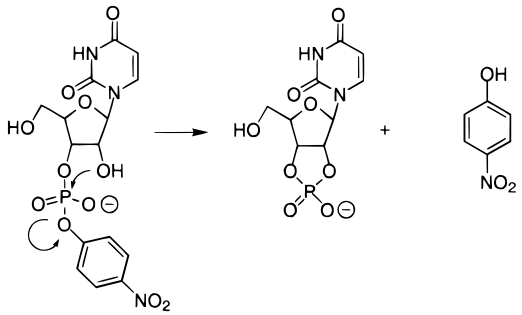

FIG. 1. Cyclization reaction of uridine 3′-*p*-nitrophenyl phosphate.

Spectrophotometric rate determinations. At pH 5.5, the product of the cyclization reaction (Fig. 1) is protonated *p*-nitrophenol. The difference in the UV-vis spectrum between this product and the substrate is greatest at 330 nm, so this wavelength was selected for rate determinations. As the change in extinction coefficient varies somewhat with pH, ribonuclease was used to completely cyclize a known amount of **1** and the change in extinction coefficient was measured. The rate of cyclization of **1** was determined at two different concentrations of metal ion and substrate for every lanthanide and compared to the uncatalyzed rate (Table 1).

TABLE 1

Rate Enhancement of Cyclization by Lanthanides

Background cyclization[a]	2.49×10^{-5} s^{-1}	
Metal ion	0.1 mM[b] (k_{cat}/k_{uncat})	0.5 mM[c] (k_{cat}/k_{uncat})
La^{+3}	26.6	51.6
Ce^{+3}	37.0	81.0
Pr^{+3}	41.2	81.0
Nd^{+3}	34.5	76.6
Sm^{+3}	50.7	107.6
Eu^{+3}	57.4	106.1
Gd^{+3}	46.0	78.1
Tb^{+3}	53.0	81.0
Dy^{+3}	54.5	79.5
Ho^{+3}	43.0	56.0
Er^{+3}	48.6	61.9
Tm^{+3}	42.8	72.2
Yb^{+3}	46.5	113.5
Lu^{+3}	43.0	70.7

[a] pH 5.5, 9 mM MES, 37°C. [Uridine 3′-*p*-nitrophenyl phosphate] = 0.291 mM.
[b] pH 5.5, 9 mM MES, 37°C. [Uridine 3′-*p*-nitrophenyl phosphate] = 0.291 mM.
[c] pH 5.5, 5 mM MES, 37°C. [Uridine 3′-*p*-nitrophenyl phosphate] = 0.454 mM.