

Hydrolysis of Phosphate Diesters as Nucleic Acid Model

Sung, Nack-Do

Department of Agricultural Chemistry,
Chungnam National Univeristy, 305-764, Taejon

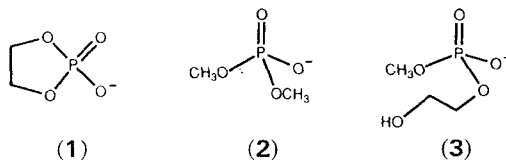
Abstract : Rate of hydrolysis ethylene phosphate, dimethylphosphate and hydroxyethylmethylphosphate in neutral water have been measured. Hydrolysis of ethylene phosphate proceeds with P-O bond cleavage ($k_{\text{obs}}=3\times 10^{-7}\text{s}^{-1}$ at 100°C , $\Delta H^{\ddagger}=24$ kcal, $\Delta S^{\ddagger}=25.5$ eu). In contrast, hydrolysis of dimethylphosphate proceeds with C-O bond cleavage ($k_{\text{obs}}=3\times 10^{-7}\text{s}^{-1}$ at 150°C). The rate constant for P-O bond cleavage of dimethylphosphate is estimated at $1\times 10^{-11}\text{s}^{-1}$ at 150°C ($\Delta H^{\ddagger}=36$ kcal, $\Delta S^{\ddagger}=25.5$ eu). A phosphodiesterase catalyzed hydrolysis of dimethylphosphate is 10^{17} times faster than the simple water rate. The observed rate of hydrolysis of hydroxyethylmethylphosphate is comparable to that of dimethylphosphate indicating C-O bond cleavage ($k_{\text{obs}}=6\times 10^{-7}\text{s}^{-1}$ at 150°C). (Received October 3, 1994; accepted November 18, 1994)

Introduction

The phosphate diester bond plays an enormously important role in nature.¹⁾ It forms the backbone of the genetic material DNA and it also occurs in phosphatidyl choline, one of the major components of cell membranes. One reason for nature's choice for the phosphate diester linkage for DNA and phosphatidyl choline appears to be its tremendous resistance to hydrolysis.²⁾ There are many enzymes that are highly efficient in catalyzing the hydrolysis of unactivated phosphate diesters (eg. dimethylphosphate and RNA and DNA) in neutral water. However, unactivated phosphate diesters are so stable that until the present study, their simple rates of hydrolysis in neutral water have not been measured.

In this paper, the author report the kinetics and mechanisms of hydrolysis in neutral water of ethylene phosphate (1), dimethylphosphate (2) and hydroxyethylmethylphosphate (3). Dimethylphosphate and hydroxyethylmethylphosphate are models for the phosphodiester backbones of DNA and RNA

respectively.³⁾ Ethylene phosphate is a model for the cyclic phosphate intermediate in RNA hydrolysis.⁴⁾ The kinetic data obtained in this study will be useful for evaluating the efficiencies of phosphodiesterases and synthetic catalysts⁵⁾ that hydrolyze phosphate diesters.



Materials and Methods

Materials

Dimethylphosphate,⁶⁾ sodium ethylene phosphate⁷⁾ and hydroxyethylmethyl phosphate⁸⁾ were prepared according to known procedures.

Kinetics

Hydrolysis of the phosphate diesters (dimethylphosphate, ethylene phosphate and hydroxyethylmethylphosphate) were monitored by using an

Key words : Hydrolysis, Phosphodiester, Nucleic acid model.

Corresponding author : N.-D. Sung

NMR method. The phosphate diester (0.1 M) solutions in D₂O buffered with 0.2 M phosphate (pD 5.5 to 8.5) were sealed in an NMR tube. Effect of buffer catalysis and solvent isotope effect for hydrolysis of phosphate diesters are small.⁹⁾ The pD values of the reaction solutions were measured at 25°C following the procedure of Fife and Bruce.¹⁰⁾ The reactions were conducted at 150°C for dimethylphosphate and hydroxyethylmethylphosphate and at 100°C for ethylene phosphate. The reactions were followed to only about one half-life since even at elevated temperatures the reactions were extremely slow.

The rate constants were obtained by monitoring the following ¹H NMR (200 MHz) signals: methyl groups due to dimethylphosphate (δ 3.6, d, J_{P-H} = 10 Hz, internal reference TMS),¹¹⁾ hydroxyethylmethylphosphate (δ 3.6, d, J_{P-H} = 10 Hz) and methanol (δ 3.3, s); and methylene groups due to ethylene phosphate (δ 4.2, d, J_{P-H} = 10 Hz) and ethylene glycol (δ 3.6, s).

Results and Discussion

Hydrolysis of ethylene phosphate (1)

The mechanism of hydrolysis of ethylene phosphate in neutral water involves nucleophilic attack of a water molecule on the phosphorus resulting in P-O bond cleavage.¹²⁾ The products of the hydrolysis reaction are ethylene glycol and inorganic phosphate. The phosphate monoester intermediate, hydroxyethylphosphate, does not accumulate an observable extent during the hydrolysis reaction in neutral water.¹³⁾ Hydrolysis of phosphate monoesters proceed rapidly by the metaphosphate mechanism at neutral pH.

The rate of hydrolysis of ethylene phosphate under neutral pH conditions is quite slow even at 100°C (Table 1). Kirby *et al.*⁹⁾ showed that the activation entropy for the solvent catalyzed hydrolysis of bis-2,4-dinitrophenyl phosphate is 25.5 eu. Assuming that the activation entropy for the hydrolysis of ethylene phosphate is the same as that for the hydrolysis of bis-2,4-dinitrophenylphosphate, the

rate constant for hydrolysis of ethylene phosphate in neutral water at 25°C and 150°C should be $8.2 \times 10^{-11} \text{s}^{-1}$ and $1.6 \times 10^{-5} \text{s}^{-1}$ respectively ($\Delta H^\ddagger = 24$ Kcal). The author's experimentally determined rate constant for the hydrolysis of ethylene phosphate at 150°C ($1.5 \times 10^{-5} \text{s}^{-1}$) is in good agreement with the above estimation.

Hydrolysis of dimethylphosphate (2)

Hydrolysis of dimethylphosphate in neutral water gave two equivalents of methanol and inorganic phosphate without any observable build up of the monophosphate intermediate during the reaction.¹¹⁾ Reactivities of methyl phosphate, dimethylphosphate and trimethylphosphate had been neatly summarized in the form of complete pH-rate profiles by Guthrie.¹³⁾ However, due to the lack of experimental data, it had not been clear whether the hydrolysis of dimethylphosphate in neutral water proceeds with P-O bond cleavage or C-O bond cleavage. The author's experimentally determined rate constant for the hydrolysis reaction at 150°C is $3 \times 10^{-7} \text{s}^{-1}$ (Table 1). This rate should represent C-O bond cleavage since, as shown below, it is 10^4 times too fast to represent P-O bond cleavage. It is well known that at 25°C, the rate of P-O bond cleavage of ethylene phosphate is 10^8 times faster than that of dimethyl phosphate when hydroxide is the nucleophile.¹²⁾ If it is assumed that this ratio of reactivity holds when water is the nucleophile, the rate constant for P-O bond cleavage of dimethylphosphate at 25°C should be $8.2 \times 10^{-19} \text{s}^{-1}$. From the reactivity and selectivity principle,¹⁴⁾ it is expected that water, being the weaker nucleophile, would be more selective than hydroxide. Therefore, the above rate constant ($8.2 \times 10^{-19} \text{s}^{-1}$) should be an upper limit. Assuming that the activation entropy of the hydrolysis reaction is 25.5 eu⁹⁾ the activation enthalpy of the hydrolysis reaction calculated from the observed rate constant is 35 Kcal. The rate constants for P-O bond cleavage of dimethylphosphate, with water as the nucleophile, at 100°C and 150°C should be $1.3 \times 10^{-13} \text{s}^{-1}$ and $3.5 \times 10^{-11} \text{s}^{-1}$ respectively.

A second method of estimating the water rate

for the P-O bond cleavage of dimethylphosphate is shown below. Kirby *et al.*⁹⁾ studied the rates of hydrolysis in neutral water of diarylphosphates. The mechanism of the hydrolysis reactions involves nucleophilic attack of water on the phosphoryl group of diarylphosphate anion followed by the P-O bond cleavage. There is a linear relationship (eq. 1) between the logarithm of the rate constant for the hydrolysis reaction (at 100°C) and pKa of the conjugate acid of the leaving group.⁹⁾

$$\log k = 1.57 - 0.97 \text{ pKa} \quad (1)$$

The rate constant calculated from eq. 1, for cleavage of the P-O bond of dimethyl phosphate is $3.4 \times 10^{-14} \text{ s}^{-1}$ (pKa of methanol=15.5). Assuming that the activation entropy of the hydrolysis reaction is 25.5 eu, the rate constants for P-O bond cleavage of dimethylphosphate, with water molecule as the nucleophile, at 25°C and 150°C should be $1.5 \times 10^{-19} \text{ s}^{-1}$ and $1.1 \times 10^{-11} \text{ s}^{-1}$ respectively. The calculated activation enthalpy of the hydrolysis reaction is $36 \text{ kcal} \cdot \text{mol}^{-1}$. The above two estimates for the activation enthalpy, obtained by two distinct methods, are in very good agreement.

Hydrolysis of hydroxyethylmethylphosphate (3)

Under neutral conditions the products of hydrolysis of hydroxyethylmethylphosphate are methanol, inorganic phosphate and ethylene glycol. It is well known that in 1 N NaOH solution the rate of hydrolysis of hydroxyethylmethylphosphate is 10^6 times greater than the rate of hydrolysis of dimethylphosphate.⁷⁾ The enhanced reactivity of hydroxyethyl-

methylphosphate is due to the intramolecular alkoxy group which can attack the phosphoryl group. In neutral pH however, the reactivity of hydroxyethylmethylphosphate (Table 1) is comparable to that of dimethylphosphate. It appears that in neutral water the hydrolysis of hydroxyethylmethylphosphate involves C-O bond cleavage as in the case of dimethylphosphate hydrolysis.

Efficiency of phosphodiesterases

There are many enzymes that catalyze the hydrolysis of phosphate diesters. The specificity of phosphodiesterases vary over a wide range. For example, restriction enzymes catalyze the hydrolysis (P-O bond) of double-stranded DNA sequence specifically whereas an enzyme isolated from *Enterobacter aerogenes* catalyzes the hydrolysis of simple phosphate diesters such as dimethylphosphate, diethylphosphate and ethylene phosphate.¹⁵⁾ Restriction endonucleases typically cleave DNA with k_{cat} values of about 10^{-2} s^{-1} to 10^{-1} s^{-1} .¹⁵⁾ The k_{cat} values for the *Enterobacter aerogenes* enzyme catalyzed hydrolysis of diethylphosphate and ethylene phosphate at pH 7.2, 30°C are 10^{-2} s^{-1} and 10^0 s^{-1} respectively.¹⁵⁾ By comparison, the water rates for P-O bond cleavage of dimethylphosphate and ethylene phosphate are $1.5 \times 10^{-19} \text{ s}^{-1}$ and $8.2 \times 10^{-11} \text{ s}^{-1}$ at 25°C.

In conclusion, phosphate diesters are highly stable in neutral water. The half-life for P-O bond cleavage of dimethylphosphate at 25°C through solvent catalyzed hydrolysis is over a hundred billion years. It is not surprising that nature has selected such a strong linkage for the backbone of DNA since it is important that the genetic material be preserved for the survival of any living organism. A human genome contains approximately six billion phosphate diester bonds. Uncontrolled cleavage of even one of those phosphate diester bonds may have serious consequences in a cell. However, controlled cleavage of phosphate diesters is necessary for normal functioning of a cell. Phosphodiesterases can catalyze the hydrolysis of phosphate diesters within minutes. In the case of a phosphodiesterase isolated from *Enterobacter aerogenes*, the enzyme ca-

Table 1. Observed rate constants (k_{obs} in s^{-1})^a for hydrolysis of ethylene phosphate(1), dimethylphosphate(2) and hydroxyethylmethylphosphate(3) in D_2O , 0.2 M phosphate buffer

pD	1 ^b	2 ^c	3 ^c
6.5	2.6	3.5	6.3
7.0	3.0	3.0	6.1
7.5	3.0	3.0	5.8
8.5	2.6	2.9	6.3

^a $k_{\text{obs}} \times 10^{-7} \text{ s}^{-1}$, ^bAt 100°C., ^cAt 150°C.

talyzed hydrolysis of diethylphosphate is 10^{17} times faster than the simple water rate.

Acknowledgements

This paper was supported in part by the grant (901-1502-033-2) from KOSEF, 1992. The author thank to prof. J. Chin, department of chemistry, McGill University, for several helpful discussions.

References

1. Stryer, L. (1981) Biochemistry, Second Ed., 599, Freeman and Co., New York
2. Westheimer, F. H. (1987) Why nature chose phosphates, *Science*, **235**, 1173-8
3. Dugas, H. and C. Penny (1980) Bioorganic Chemistry, 113, Springer Verlag, New York
4. Walsh, C. (1977) Enzymatic Reaction Mechanisms, 150, Freeman and Co., New York
5. Chin, J. and X. Zou (1988) Cobalt complex promoted hydrolysis of phosphate diester, *J. Am. Chem. Soc.*, **110**, 223-5
6. Kosolapoff, G. M. and C. Maier (1950) Organic Phosphorus Compounds, 214, Wiley, New York
7. Kluger, R. and P. Wasserstein (1974) Inhibition of acetoacetate decarboxylase by ketophosphates. Structure and dynamic probes of the active site. *Tet. Lett.*, **38**, 3451-5
8. Kluger, R., F. Covitz, E. Dennis, D. L. Williams and F. H. Westheimer (1969) pH-product and pH-rate profiles for the hydrolysis of methylethylphosphate. *J. Am. Chem. Soc.*, **91**, 6066
9. Kirby, A. J. and M. Younas (1970) The organic chemistry of phosphorus (Reaction mechanisms in organic chemistry, Monograph 5). *J. Chem. Soc. (B)*, 510-6
10. Fife, T. H. and T. C. Bruice (1961) Temperature dependence of the ΔpD correction for the use of the glass electrode in D_2O . *J. Phys. Chem.*, **65**, 1079-84
11. Kim, J. H. and J. Chin (1992) Dimethylphosphate hydrolysis at neutral pH., *J. Am. Chem. Soc.*, **114**, 9792-5
12. Jr. Cox, J. R. and B. Ramsay (1964) Mechanism nucleophilic substitution in phosphate esters., *Chem. Rev.*, **64**, 317-352
13. Guthrie, J. P. (1977) Hydration and dehydration of phosphoric acid derivatives. *J. Am. Chem. Soc.*, **99**, 3991-4001
14. Leffler, J. E. and J. Grunwald (1963) Rates and Equilibria of Organic Reactions, Wiley, New York, 162~168
15. Gerlt, J. A. and F. H. Westheimer (1973) A phosphodiesterase from *Enterobacter aerogenes*., *J. Am. Chem. Soc.*, **95**, 8166-8
16. Wells, R. D., R. D. Klein and C. K. Singleton (1981) *Enzymes*, **14**, 157-61

핵산 모델로서 Phosphate Diester들의 가수분해 반응

성낙도(충남대학교 농화학과)

초록 : RNA의 가수분해시에 생성되는 cyclic phosphate 중간체의 모델 화합물인 ethylene phosphate의 P-O결합 분해속도 상수는 100°C 에서 $k=3 \times 10^{-7} \text{s}^{-1}$ ($\Delta H^\ddagger=24 \text{ kcal}$, $\Delta S^\ddagger=25.5 \text{ eu}$)이었으며 DNA모델 화합물인 dimethylphosphate는 150°C 에서 $1 \times 10^{-11} \text{s}^{-1}$ ($\Delta H^\ddagger=36 \text{ kcal}$, $\Delta S^\ddagger=25.5 \text{ eu}$)이었다. RNA모델 화합물인 hydroxyethylmethylphosphate의 가수분해는 dimethylphosphate의 C-O결합이 가수분해되는 반응속도와 비교될 만 한 정도의 반응속도가 관측되었다.