Spontaneous Hydrolysis of Ionized Phosphate Monoesters and Diesters and the Proficiencies of Phosphatases and Phosphodiesterases as Catalysts

Richard Wolfenden,* Caroline Ridgway, and Gregory Young

Department of Biochemistry and Biophysics
University of North Carolina
Chapel Hill, North Carolina 27599

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The quest for efficient man-made catalysts has led to increasing appreciation of the attainments of biological catalysts, but the actual rate enhancements produced by enzymes have been established in only a few cases.[1] Information about the rate of an uncatalyzed reaction can be used to estimate an enzyme's minimal affinity for an inhibitor that perfectly resembles the altered substrate in the transition state[2] and to analyze the effects of mutation on enzyme action, by comparing the transition state affinity of an enzyme with the transition state affinities of its structural elements.[3] For comparisons involving phosphatases and phosphodiesterases, it seemed desirable to determine the uncatalyzed rates of hydrolysis of unactivated monoesters and diesters of phosphoric acid, in the fully ionized forms that appear to serve as substrates for the corresponding enzymes. In the experiments described here, esters (0.05 M) were typically adjusted to the appropriate pH and then incubated in sealed quartz tubes at elevated temperatures for various time intervals in the absence or presence of potassium phosphate or carbonate buffers (0.1−0.3 M) whose pH had been established at room temperature.[4] After the tubes had cooled to room temperature, the contents were diluted 100-fold with D_2O containing added pyrazine as an integration standard. Analysis of product mixtures by proton NMR showed that these reactions follow simple first-order kinetics to completion, without formation of detectable byproducts. The resulting Arrhenius plots indicate that these reactions proceed very slowly at room temperature and that phosphomonoesterases and phosphodiesterases produce rate enhancements far larger than those that have been recorded for other hydrolases.

Methyl phosphate, previously studied at lower pH values,[6] undergoes hydrolysis at elevated temperatures (110−200 °C) at rates that decrease as the pH increases from 6 to 9, approaching a value that remains constant above pH 10, consistent with reaction of the fully ionized compound ($CH_3OPO_3^{2-}$) (Figure 1a). GC-MS analysis of the methanol produced by hydrolysis at pH 10 and 11, in the presence of $H_2{}^{18}O$ (70 atom % excess), shows that this reaction occurs >99% by phosphorus−oxygen cleavage (no detectable exchange of ^{18}O from water to methanol was found to occur under these conditions). An Arrhenius plot (Figure 2) indicates that the heat of activation for cleavage of $CH_3OPO_3^{2-}$ is 37.2 kcal/mol, and the entropy of activation is small and positive (+3.7 cal deg^{-1} mol^{-1}), consistent with the possibility that this reaction, like that of the monoanion,[7] may proceed through a metaphosphate-like transition state. At 100 °C, $CH_3OPO_3^{2-}$ is cleaved 800-fold less rapidly than the analogous phosphorus−oxygen cleavage of $CH_3OP(OH)O_2^-$ at 100 °C,[6] providing a measure of the possible role played by the hydroxyl group (directly or through the solvent) in the elimination of metaphosphate from $CH_3OP(OH)O_2^-$, as proposed by Butcher and Westheimer.[7] Methyl phosphate is cleaved considerably more rapidly

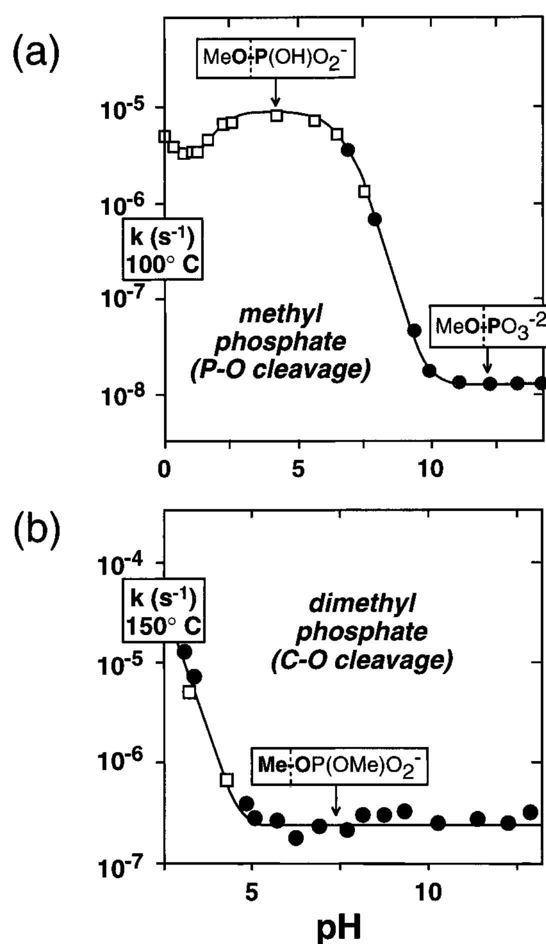

Figure 1. Apparent first-order rate constants for hydrolysis, obtained by extrapolation to zero buffer concentration, for (a) monomethyl phosphate at 100 °C (●), including values (□) reported by Bunton et al. (ref 6). (b) Rates of hydrolysis of dimethyl phosphate at 150 °C (●), including values (□) reported by Bunton et al. (ref 13).

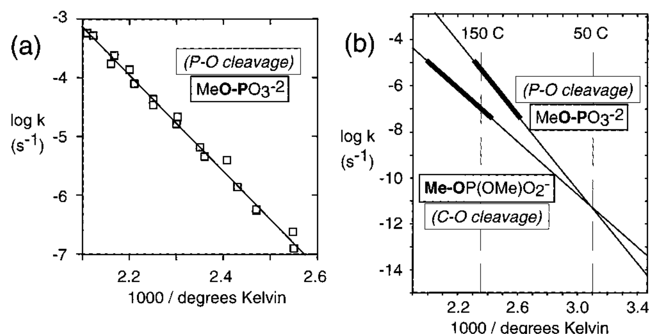

Figure 2. (a) Apparent first-order rate constants for hydrolysis of methyl phosphate at pH 10, plotted as a function of reciprocal temperature (Kelvin). (b) Arrhenius plots for the hydrolysis of the dimethyl phosphate anion at pH 6.8 (based on data given in ref 1) and of the methyl phosphate dianion at pH 10 (based on data in panel a). The range of experimental rate measurements is indicated by bold parts of the lines.

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(3) Bearne, S. L.; Wolfenden, R. *Biochemistry* **1997**, *36*, 1646−1656.
(4) The acidities of substrates, buffers, and solvent water vary with temperature (ref 5). These complications are not expected to interfere with determination of the rates of the uncatalyzed reactions, which are pH-independent.
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than expected on the basis of a Brønsted plot based on the behavior of several activated phosphodiesters examined earlier by Kirby and Varvoglis.[8] Extrapolation to room temperature (Figure 2) yields a first-order rate constant of $4.6(\pm 2.0) \times 10^{-14}$ s^{-1} at 25 °C. Comparison of this value with the kinetic constants reported for *Escherichia coli* alkaline phosphatase acting on methyl phosphate[9] indicates that this enzyme enhances the rate of reaction by a factor of at least 10^{15}-fold and that, in the transition state, the formal dissociation constant of the enzyme−substrate complex is less than 3×10^{-20} M. When the rate of an enzyme reaction is governed by a physical process such as substrate binding or product release, which has no counterpart in the uncatalyzed reaction, comparison of enzymatic and nonenzymatic reaction rates gives only a minimal impression of the enzyme's ability to stabilize the transition state for substrate transformation according to the mechanism by which the reaction proceeds at the active site.[2] The rate of the alkaline phosphatase reaction has been shown to be limited by a physical step, rather than by the making and breaking of chemical bonds in substrates.[10−12] Accordingly, this dissociation constant may represent an upper limit.

Similar experiments, conducted on dimethyl phosphate at 140−230 °C, show that this compound undergoes hydrolysis with rate constants that do not vary significantly between pH 5 and 13, after extrapolation to zero buffer concentration at any single temperature, consistent with decomposition of $(CH_3O)_2PO_2^-$ (Figure 1b).[14] An Arrhenius plot indicates (Figure 2) that the heat of activation for hydrolysis of $(CH_3O)_2PO_2^-$ is 25.9 kcal/mol, and the entropy of activation is −34 cal deg^{-1} mol^{-1}. $(CH_3O)_2PO_2^-$ is hydrolyzed >99.5% by cleavage between carbon and oxygen, as indicated by GC-MS analysis of the methanol produced; this reaction was found to be catalyzed by nucleophilic anions. Extrapolation to room temperature yields a first-order rate constant of $1.6(\pm 0.8) \times 10^{-13}$ s^{-1} at 25 °C for the uncatalyzed carbon−oxygen cleavage of $(CH_3O)_2PO_2^-$ by water.[16] This rate constant, as low as it is, is inappropriate for comparison with reactions catalyzed by phosphomonoesterases, which proceed with P−O cleavage. Instead, this value, combined with the finding that less that 0.5% of the nonenzymatic reaction occurs by phosphorus−oxygen cleavage, places an upper limit of 10^{-15} s^{-1} on the first-order rate constant for the phosphorus−oxygen cleavage of $(CH_3O)_2PO_2^-$ at 25 °C, substantially lower than suggested in a preliminary report in which the site of bond cleavage had not been established.[1] Comparison with kinetic constants reported for staphylococcal nuclease[17] indicate that this enzyme enhances the rate of phosphorus−oxygen cleavage by a factor of at least 10^{17}-fold and that the formal dissociation constant of this enzyme's complex with the activated substrate is less than 10^{-22} M in the transition state.

The present rate comparisons suggest that the transition state affinities of phosphoric ester hydrolases approach the extremely low value (5×10^{-24} M) recorded earlier for orotidine 5′-phosphate decarboxylase[1] and identify these hydrolases as proficient catalysts that should furnish sensitive targets for inhibitor design. It is also evident that phosphodiesters, with a half-life of 130 000 years at 25 °C, are well-suited to the storage of genetic information. Even at this rate, a human DNA molecule, with $\sim 3 \times 10^9$ base pairs, would be expected to experience one bond cleavage in 24 min in the absence of repair, if the phosphodiester bonds of DNA are assumed to be equivalent to dimethyl phosphate in their rates of hydrolysis.

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(16) Because of major differences in energy of activation, dimethyl phosphate anion is more rapidly hydrolyzed than methyl phosphate dianion at temperatures below 50 °C. These rates of reaction are too low to be of practical value in the preparation of monoesters, but see footnote 14 above.

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