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Enzymic Hydrolysis of Phosphonate Esters. Reaction Mechanism of Intestinal 5′-Nucleotide Phosphodiesterase†

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ABSTRACT: The mechanism of bovine intestinal 5′-nucleotide phosphodiesterase was investigated by determining kinetic constants of systematically varied substrates, with emphasis on esters of phosphonic acids (which have much higher V_{max} values than conventional phosphodiester substrates), and by pre-steady-state kinetics using bis(4-nitrophenyl) phosphate as substrate. The results suggest a ping-pong type mechanism, with participation of a covalent enzyme intermediate.

We have previously demonstrated that enzymes which hydrolyze phosphonate esters[1] are widely distributed in nature (Kelly and Butler, 1975) and that this activity is due to and characteristic of 5′-nucleotide phosphodiesterase enzymes (Kelly et al., 1975). These substrates thus provide a convenient and economical means of assaying 5′-nucleotide phosphodiesterases and of distinguishing them from phosphodiesterases which produce 3′-nucleoside monophosphates and do not hydrolyze phosphonate monoesters (Kelly et al., 1975). Several other names have been applied to enzymes having properties similar to the enzyme which we refer to as 5′-nucleotide phosphodiesterase (Kelly and Butler, 1977).

We have investigated the mechanism of bovine intestinal 5′-nucleotide phosphodiesterase by several techniques. This paper describes steady-state kinetic parameters obtained using a group of substrates with systematically varied structures, and pre-steady-state kinetics using one of these substrates. The results suggest a "ping-pong" type mechanism (Cleland, 1967) involving an intermediate consisting of a phosphonyl (phos-

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[1] As used here, the term "phosphonate esters" refers only to *monoesters* of phosphonic acids.

phonate ester substrates) or monoesterified phosphoryl (phosphodiester substrates) moiety bound to the enzyme.

Materials and Methods

Materials. The synthesis of 4-nitrophenyl and 2-naphthyl monoesters of phenyl-, methyl-, and chloromethylphosphonic acids has been described by Purdum et al. (1976). Bis(4-nitrophenyl) phosphate (Aldrich Chemical Co.) was purified as previously described (Kelly and Butler, 1975). The source of all other chemicals was as previously described (Kelly and Butler, 1975) or the best grade commercially available. The bovine intestinal 5′-nucleotide phosphodiesterase was the homogeneous preparation (specific activity, 47.7 units mg^{-1}) described by Kelly et al. (1975).

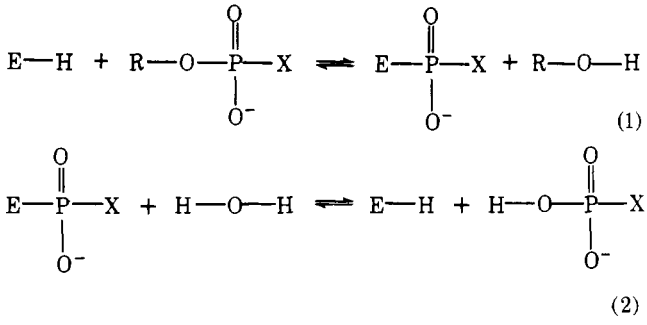
Assays. Hydrolysis of 2-naphthyl esters of phosphonic acids was quantitated spectrophotometrically at 330 nm: under the conditions of the standard assay (0.1 M Tris,[2] pH 8.0, 30 °C), the extinction coefficient of 2-naphthol is 1500 M^{-1} cm^{-1}, while the extinction coefficient of the 2-naphthyl esters is approximately 23 M^{-1} cm^{-1}. Hydrolysis of 4-nitrophenyl esters was quantitated spectrophotometrically at 400 nm as previously described (Kelly and Butler, 1975).

Stopped-flow experiments were performed with a Durrum-Gibson stopped-flow spectrophotometer with a 2-cm light path and equipped with an air drive system. The temperature was maintained at 30 °C with a Precision Scientific Lo-Temptrol constant-temperature unit. Changes in transmittance of light by the reaction mixture were monitored using a Tektronix Type 564 storage oscilloscope with a Type 2A63 differential amplifier and Type 2B67 time base. The traces were recorded permanently with a Polaroid camera and kinetic parameters evaluated from the photographs.

Results and Discussion

Scheme I represents a simple reaction sequence for the hydrolysis of phosphonate esters and phosphodiesters by bovine intestinal 5′-nucleotide phosphodiesterase which is in accord with the present observations.

Scheme I



(1)

(2)

In this formulation, X represents an alkyl or aryl (phosphonate ester substrates) or alkoxy or aryloxy (phosphodiester substrates) substituent; R can be alkyl,[3] aryl, or a phosphate. Hydrolysis via a covalent enzyme intermediate has previously been suggested for spleen 3′-nucleotide phosphodiesterase (Razzell and Khorana, 1961); analogous mechanisms (where X represents -OH) have been established for several phosphomonoesterases (Reid and Wilson, 1971; Hickey and Van Etten, 1972; Fernley and Walker, 1970).

TABLE I: Kinetic Parameters.[a,b]

Substrate	V_{max} (μmol min^{-1} mg^{-1})	K_m (mM)
1. 4-Nitrophenyl methylphosphonate	850	66
2. 2-Naphthyl methylphosphonate	830	120
3. 4-Nitrophenyl phenylphosphonate	740	11
4. 2-Naphthyl phenylphosphonate	780	18
5. 4-Nitrophenyl chloromethylphosphonate	500	41
6. 2-Naphthyl chloromethylphosphonate	530	83
7. 4-Nitrophenyl 5′-TMP[c]	270	0.043
8. Bis(4-nitrophenyl) phosphate[c]	25	0.85
9. NAD[c]	10.6	0.03
10. 3′,5′-cAMP[c]	7.0	0.1
11. ADP[c]	6.4	
12. ATP[c]	4.6	0.2

[a] Calculated by least-squares linear regression analysis of $1/v$ vs. $1/S$ plots. [b] Conditions were 0.1 M Tris, pH 8.0, 30 °C. [c] From Kelly et al. (1975).

Effect of Substrate Substituents on Kinetic Parameters. Kinetic parameters for the hydrolysis of 4-nitrophenyl and 2-naphthyl monoesters of phenyl-, chloromethyl-, and methylphosphonic acids are presented in Table I, along with those previously reported for nucleotides and other substrates (Kelly et al., 1975). The data indicate that the 4-nitrophenyl and 2-naphthyl esters of the same phosphonic acid (different R, same X in Scheme I) have V_{max} values which are very similar. In contrast, potato nucleotide pyrophosphatase, which resembles intestinal 5′-nucleotide phosphodiesterase in several respects, shows V_{max} values for 4-nitrophenyl esters which are 25–200 times faster than for the corresponding 1-naphthyl esters (Kole et al., 1976). Similar V_{max} values are also observed (Table I) for four different nucleotide derivatives which this enzyme hydrolyzes to 5′-adenosine monophosphate (Kelly et al., 1975); in this series, X represents the common 5′-adenosyl moiety and R varies. The apparent independence of V_{max} on R indicates that the rate-limiting step occurs after cleavage of the ester linkage of the substrate (reaction 1 in the above formulation).

The data in Table I demonstrate that V_{max} is strongly dependent on the nature of the X substituent (Scheme I) and is consistent with the occurrence of a covalent intermediate containing X whose hydrolysis is the rate-limiting step (reaction 2). The larger values of V_{max} for phosphonates than for phosphates might not be expected for water attack on the phosphorus atom, which should have greater electron density in the phosphonates than in the corresponding phosphates because the carbon atom is less electronegative than oxygen. However, the larger values of V_{max} for phosphonates are consistent with the observation of up to 100-fold greater second-order rate constants for solvolysis of phosphonates than for the corresponding phosphates (Cox and Ramsay, 1964). Such comparisons are quite complex (Hudson, 1965); even within the phosphonates there is no obvious relationship between our observed V_{max} values and the relative elecron-withdrawing power of the phosphonyl substituent, as determined by the ^{31}P chemical shift (Van Wazer and Letcher, 1967).

[2] Abbreviation used: Tris, tris(hydroxymethyl)aminomethane.

[3] Preliminary observations indicate that simple alkyl esters (e.g., methyl) are hydrolyzed at very slow rates, if at all; however, the oligonucleotides are alkyl esters which are hydrolyzed by the enzyme (Kelly et al., 1975).

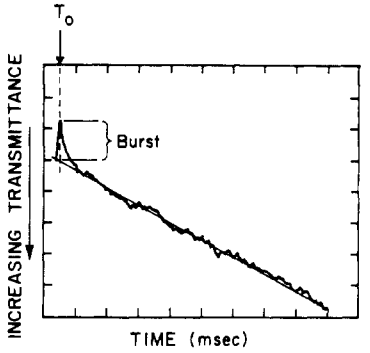


FIGURE 1: Pre-steady-state hydrolysis of bis(4-nitrophenyl) phosphate by bovine 5′-nucleotide phosphodiesterase. The release of 4-nitrophenol was followed at 400 nm in 0.05 M imidazole, pH 7.0, in the stopped-flow spectrophotometer described in Materials and Methods. Concentrations after mixing were 0.645 μM enzyme and 0.5 mM substrate. Instrument settings were: wavelength, 400 nm; monochromator slit width, 0.30 mm; noise filter time constant, 0.5 ms, 5 mV per vertical division (100% T, 1000 mV), and 10 ms per horizontal division.

The nucleotide substrates in which X represents a 5′-O-nucleoside have K_m values at least two orders of magnitude lower than do the nonphysiological phosphonates. The 13-fold difference in K_m values for similar phosphonate and phosphate compounds which have the same 4-nitrophenolate leaving group (lines 3 and 8 in Table I) suggests a role in substrate binding for the bridge oxygen in X. Within the phosphonate series, the relative K_m values suggest size of substituents as a factor recognized by the enzyme; the largest X substituents and the smallest R substituents have the lowest values for K_m. The hydroxyl group is not an acceptable X substituent (neither 4-nitrophenyl phosphate nor PP_i are substrates). Further work with more widely varied X substituents should permit more accurate description of this binding site with respect to size, charge, and hydrophobicity of the X moiety.

Pre-Steady-State Kinetics. Mechanisms in which the rate-limiting step occurs after release of the first product can result in a transient pre-steady-state "burst" of product on initial mixing (Hartley and Kilby, 1954). When 4-nitrophenyl esters of phosphonates were used as substrates, the reaction was so rapid that no burst of 4-nitrophenol could be resolved by the stopped-flow spectrophotometer. Using as substrate bis(4-nitrophenyl) phosphate, which is hydrolyzed much more slowly than phosphonate esters (Table I), a rapid burst of 4-nitrophenol production was readily observed, as predicted by Scheme I with reaction 2 rate limiting. In the experiment shown in Figure 1, the burst corresponds to 0.34 mol of 4-nitrophenol released per mol of enzyme. At 0.5 and 2.5 mM bis(4-nitrophenyl) phosphate, pH 7.0, the magnitude of the burst was 0.27 ± 0.06 and 0.58 ± 0.10 mol of 4-nitrophenol released per mol of enzyme. Under these conditions (0.05 M imidazole acetate, pH 7.0, 30 °C), a K_m value of 0.37 mM was observed for this substrate; thus the burst experiments were done at concentrations of substrate which do not saturate the enzyme. At pH 8, a less well-defined burst was observed, possibly due to a more similar rate of the two partial reactions.

The magnitude of the burst, when corrected to saturating substrate concentration (this correction assumes that reaction 2 is strictly rate limiting) (Faller and Sturtevant, 1966), was approximately 0.8 mol per mol of enzyme under the conditions described in Figure 1. The enzyme consists of identical dimers (Kelly et al., 1975); a burst of close to 1 mol per mol of dimer suggests that this enzyme may exhibit half-site reactivity (Seydoux et al., 1974). Similar observations of a burst corresponding to only one site of a dimeric enzyme have been made with bovine intestinal alkaline phosphatase (Chappelet-Tordo et al., 1974) which intestinal phosphodiesterase resembles in many respects (Kelly et al., 1975), and which operates by an analogous mechanism involving a covalent phosphoryl enzyme intermediate (Engstrom, 1961). Similar V_{max} values at pH 8 are observed with intestinal alkaline phosphatase (Chappelet-Tordo et al., 1974) for a wide variety of substrates, suggesting a common rate-limiting step which is probably a conformational change (Fernley, 1971). In contrast, the dependence of V_{max} values for the intestinal phosphodiesterase on the nature of the moiety of substrate rules out, as a rate-limiting step, a common reaction independent of substrate, such as a conformational change. The much lower V_{max} values for nucleotide substrates than for phosphonates and the strong competitive inhibition by 5′-AMP (K_i = 1.2 μM) (Kelly et al., 1975) suggest that, with nucleotide substrates, dissociation of the second product may be rate limiting.

These observations provide strong evidence for the mechanism presented in Scheme I. Future investigations will involve direct attempts to isolate and characterize the postulated covalent intermediate.

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