5′-Nucleotide Phosphodiesterase: Features of the Substrate Binding Site As Deduced from Specificity and Kinetics of Some Novel Substrates†

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ABSTRACT: Phosphonate monoesters and phosphate diesters with systematically varied substituents and leaving groups were synthesized and tested as substrates for homogeneous 5′-nucleotide phosphodiesterase from bovine intestine. The enzyme was shown to hydrolyze phosphorothioate and phosphonoamidate compounds but at significantly lower rates than comparable oxy compounds. The effects of bulk and structure of the ester or phosphonate substituents were also investigated. Dibenzyl phosphate, an ester of an aliphatic alcohol, was a poor substrate. The enzyme did not hydrolyze aliphatic monoesters of phosphonates, regardless of bulk. Kinetic parameters of several nitrophenyl phosphonomonoesters and phosphodiesters are presented. The results suggest that synthetic nonnucleotide substrates can bind in two different modes, only one of which is productive. Incidence of nonproductive binding, with consequent kinetic effects, is increased by increasing the symmetry of the substrates.

5′-Nucleotide phosphodiesterase (EC 3.1.4.1) catalyzes the hydrolysis of a wide range of synthetic phosphoesters in addition to naturally occurring nucleotide substrates. Nucleic acids are degraded at the 3′ end to liberate 5′-nucleotides; NAD, ATP, ADP, and cyclic 3′,5′-AMP are hydrolyzed to 5′-AMP (Kelly et al., 1975), and phosphate diesters such as bis(4-nitrophenyl) phosphate are hydrolyzed to a phosphate monoester. Also substrates are phosphonate monoesters such as 4-nitrophenyl phenylphosphonate, which provides a convenient, inexpensive, and specific assay for this enzymatic activity (Kelly & Butler, 1975).

Snake venom (Laskowski, 1971) and mammalian small intestine (Kelly et al., 1975) are rich sources of 5′-nucleotide phosphodiesterase. Details of the specificity of the snake venom enzyme have been examined by using nucleotides modified by substituting various phosphate oxygens with nitrogen (Chambers & Moffat, 1958; Tsou & Yip, 1973; Letsinger et al., 1976; Yarbrough, 1978), sulfur (Eckstein & Goody, 1976; Dudman & Benkovic, 1977), fluorine (Nichol et al., 1967; Alvarez et al., 1974), and carbon (Engel, 1977), by esterification of phosphate oxygens (Smith et al., 1961; Miller et al., 1971), and by variant nucleotides (Gray, 1976; Uesugi et al., 1977). Stereochemical specificity (Ogilvie & Hruska, 1976; Bryant & Benkovic, 1979) and susceptibility to inhibition by Cibacron Blue (Cory et al., 1978) have also been determined for the snake venom enzyme.

A method of preparing large amounts of homogeneous enzyme from bovine intestine has been developed (Landt & Butler, 1978); detailed characterization of this enzyme is now possible. This report describes the synthesis of several new phosphodiesters and phosphonate monoesters and their use, along with previously employed compounds, to elucidate several features of the substrate specificity of the bovine intestinal 5′-nucleotide phosphodiesterase.

Materials and Methods

Materials. 4-Nitrophenyl phenylphosphonate was purchased from Regis Chemical Co., Morton Grove, IL. Dicyclohexylcarbodiimide, tris(hydroxymethyl)aminomethane (Tris), and bis(4-nitrophenyl) phosphate were products of Sigma Chemical Co. Diethyl (4-nitrobenzyl)phosphonate was obtained from the Aldrich Chemical Co., A. E. Bader collection. Dibenzyl phosphate and diphenyl phosphate were obtained from Aldrich Chemical Co. Dimethyl benzylphosphonate was a product of Chemical Procurement Laboratories. CM 23 carboxymethylcellulose was obtained from Whatman Co.

Enzyme. 5′-Nucleotide phosphodiesterase was purified from fresh bovine intestine by the method of Landt & Butler (1978). For the several preparations which were employed, specific activities ranged from 38 to 46 μmol/mg under standard conditions (see Assays).

Assays. Enzyme activity was determined at 30 °C by using 1.0 mM 4-nitrophenyl phenylphosphonate as the substrate in 100 mM Tris-HCl, pH 8.0. The release of 4-nitrophenol was measured spectrophotometrically at 400 nm with an extinction coefficient of 18 340 cm^{-1} mol^{-1} (Kezdy & Bender, 1963). One unit of activity produced 1 μmol of 4-nitrophenol per min under standard conditions. Protein concentration was determined from the absorbance at 280 nm with the mass extinction coefficient of 0.98 OD mg^{-1} mL^{-1} (Kelly et al., 1975).

Synthesis of Monoesters and Amide of (4-Nitrobenzyl)-phosphonic and Benzylphosphonic Acid. The free acids were obtained from the dimethyl or diethyl esters by methods previously described (Landt et al., 1978). The desired monoesters were prepared by dissolving 5 mmol of the phosphonic acid and 20 mmol of an alcohol in 50 mL of dry pyridine. Dicyclohexylcarbodiimide, 20 mmol, was added with stirring, and the cloudy suspension was stirred at room temperature overnight. Water, 20 mL, was added to destroy the remaining carbodiimide, and the mixture was stirred for 20 min and then filtered to remove precipitated dicyclohexylurea. After three extractions with ether (discarded), the aqueous layer was reduced to a syrup by rotary evaporation. The ester was precipitated by addition of 1.0 M cyclohexylammonium chloride, until no further precipitation occurred. The precipitate was recrystallized from hot water and collected by filtration.

Ethyl (4-nitrobenzyl)phosphonate cyclohexylammonium salt: yield, 13%; mp 133–136 °C. Anal. Calcd for $C_{15}H_{25}O_5N_2P$: C, 52.32; H, 7.27; P, 9.01. Found: C, 51.12; H, 7.15; P, 9.10.

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Butyl (4-nitrobenzyl)phosphonate cyclohexylammonium salt: yield, 35%. Anal. Calcd for $C_{17}H_{29}O_5N_2P$: C, 54.84; H, 7.80; P, 8.33. Found: C, 54.54; H, 7.95; P, 8.44.

Cyclohexyl (4-nitrobenzyl)phosphonate cyclohexylammonium salt: yield, 32%. Anal. Calcd for $C_{19}H_{31}O_5N_2P$: C, 57.28; H, 7.79; P, 7.79. Found: C, 57.43; H, 8.07; P, 7.45.

Phenyl (4-nitrobenzyl)phosphonate cyclohexylammonium salt: yield, 47%. Anal. Calcd for $C_{19}H_{25}O_5N_2P$: C, 58.16; H, 6.38; P, 7.91. Found: C, 57.90; H, 6.57; P, 7.77.

Benzyl (4-nitrobenzyl)phosphonate cyclohexylammonium salt: yield, 59%. Anal. Calcd for $C_{20}H_{27}O_5N_2P$: C, 59.11; H, 6.65; P, 7.63. Found: C, 58.88; H, 6.94; P, 7.42.

4-Nitroanilino(4-nitrobenzyl)phosphonate pyridinium salt. Crystals of product formed upon concentration following extraction with ether: yield, 13%; mp 100–104 °C. Anal. Calcd for $C_{18}H_{17}O_4N_4P{\cdot}H_2O$: C, 49.76; H, 4.38; P, 7.14. Found: C, 49.62; H, 4.56; P, 6.97.

4-Nitrophenyl (4-nitrobenzyl)phosphonate ammonium salt. The ammonium salt was formed by adding 1 mL of concentrated NH_4OH in place of cyclohexylammonium chloride, taking the solution to dryness, and crystallizing the residue from ethanol with addition of ether: mp 144–146 °C. Anal. Calcd for $C_{13}H_{14}O_7N_3P$: C, 43.94; H, 3.95; P, 8.73. Found: C, 43.74; H, 4.24; P, 8.58.

4-Nitrophenyl benzylphosphonate sodium salt. The sodium salt was formed by passing the solution, following extraction with ether, through a column containing 10 g of Whatman CM 23 carboxymethylcellulose (sodium form) equilibrated with water. The UV-absorbing fractions were concentrated to dryness, and the compound was precipitated from 10 mL of ethanol by addition of 30 mL of ether: yield, 38%; mp 255–258 °C. Anal. Calcd for $C_{13}H_{11}O_5N_1P_1Na$: C, 49.52; H, 3.49; P, 9.84. Found: C, 49.31; H, 3.72; P, 9.62.

Synthesis and Purification of Other Derivatives. 4-Nitrophenyl phenylphosphonothioate cyclohexylammonium salt was synthesized by the method of Dudman & Benkovic (1977): yield, 53%; mp 155–157 °C. Anal. Calcd for $C_{18}H_{23}O_4N_2S_1P$: C, 54.81; H, 5.84; P, 7.86; S, 8.12. Found: C, 54.82; H, 6.02; P, 7.59; S, 8.41.

Bis(4-nitrophenyl) phosphorothioate pyridinium salt and 4-nitrophenyl phenyl phosphate cyclohexylammonium salt were synthesized with the method described by Eckstein & Frischauf (1974) for the synthesis of 4-nitrophenyl phenyl phosphorothioate. The 4-nitrophenyl thiophosphonic dichloride required as a synthetic intermediate for the first of these compounds was prepared by the method of Tolkmith (1958). Bis(4-nitrophenyl) phosphorothioate was purified by the method of Eckstein & Frischauf (1974) and crystallized as the pyridinium salt upon concentration in H_2O: yield, 50%; mp 128–133 °C. Anal. Calcd for $C_{17}H_{14}O_7N_3S_1P$: C, 46.90; H, 3.22; S, 7.36; P, 7.13. Found: C, 46.76; H, 3.41; S, 7.70; P, 7.58. 4-Nitrophenyl phenyl phosphate was purified by extracting the reaction mixture (stopped with 25 mL of H_2O) with ether 3 times, acidifying the H_2O layer to 1 M HCl, and extracting the compound into benzene. The benzene was removed by rotary evaporation and the residue was dissolved in a minimum of water. Addition of 1 M cyclohexylammonium chloride precipitated the desired compound: yield, 14%; mp 164–168 °C. Anal. Calcd for $C_{18}H_{23}O_6N_2P$: C, 54.82; H, 5.84; P, 7.87. Found: C, 54.56; H, 5.92; P, 7.69.

Tests to Determine Hydrolysis of Synthetic Substrates. Substrate analogues were tested at 10 mM concentration with phosphodiesterase in 100 mM Tris-HCl, pH 8.0. The reactions were run at 30 °C for up to 115 h. Tubes containing 1.0 mL (final volume) contained 0, 17, or 179 μg of phosphodiesterase having a specific activity of 30 units mg^{-1}. At selected times 10-μL aliquots of each incubation mixture were spotted on Whatman MM1 paper and chromatographed with a solution of 2-propanol–H_2O–NH_4OH, 80:20:0.2 (v/v). Enzyme activity was still present when the incubations were terminated. The compounds were visualized by their absorption of ultraviolet light [compounds containing (4-nitrobenzyl)phosphonate absorb intensely] or by a phosphate spray (Hanes & Isherwood, 1949). All of the compounds originally produced a single spot, with $R_f > 0.50$, in this system, and were stable in the absence of enzyme over the time of the experiments. Utilization of a given compound as a substrate was indicated by the appearance of a new spot due to production of phosphonic acid or phosphate monoester, which have much lower R_f values in this system.

Kinetics. The rate of hydrolysis of 4-nitrophenyl esters was determined at 30 °C in 100 mM Tris-HCl, pH 8.0. Continuous assays in 3-mL cuvettes were performed in a Beckman DB-G spectrophotometer connected to a recorder. Production of 4-nitrophenol was measured as described for the standard assay. Data were plotted as the least-squares fit on double-reciprocal graphs and values of K_m and V_{max} were determined from intercepts and slopes.

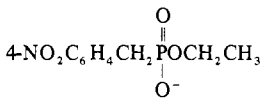
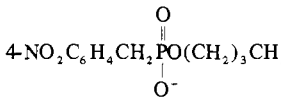
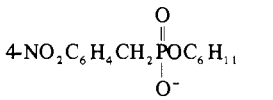
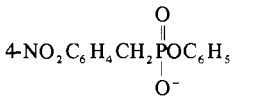
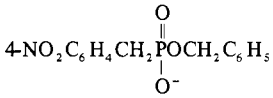
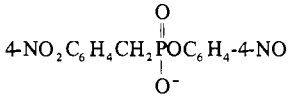
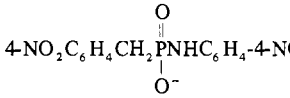
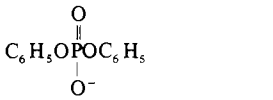
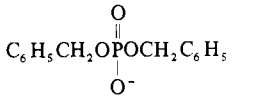
Results

Testing of Compounds as Substrates. A series of monoesters of (4-nitrobenzyl)phosphonic acid, as well as its 4-nitroanilide, were synthesized and incubated with two concentrations of 5′-nucleotide phosphodiesterase from bovine intestine. Also tested as substrates were the phosphodiesters dibenzyl phosphate and diphenyl phosphate. The results are presented in Table I, along with the structures of the tested compounds. Phenolic esters of (4-nitrobenzyl)phosphonic acid such as phenyl (4-nitrobenzyl)phosphonate are substrates, but aliphatic esters of phosphonates were not hydrolyzed, even if the alcohol contains an aromatic ring (benzyl ester). The aliphatic esters, if hydrolyzed at all, were cleaved at least 10 000-fold more slowly than 4-nitrophenyl (4-nitrobenzyl)-phosphonate, which was completely hydrolyzed in 1 h at the lower enzyme concentration. The phosphoramidate bond of 4-nitroanilino(4-nitrobenzyl)phosphonate was hydrolyzed, but at a 1000-fold slower rate than the analogous 4-nitrophenyl ester. Diphenyl phosphate was hydrolyzed rapidly and completely, but dibenzyl phosphate was hydrolyzed about 100-fold more slowly, presumably due to the aliphatic ester linkage. Phosphonate and phosphate esters of 4-nitrophenol, including compounds in which a nonbridge oxygen bonded to phosphorus is replaced with sulfur, are substrates of phosphodiesterase.

Compounds which are not hydrolyzed by phosphodiesterase (Table I) were tested as inhibitors of phosphodiesterase-catalyzed hydrolysis of 4-nitrophenyl phenylphosphonate. The ethyl, *n*-butyl, and cyclohexyl esters of (4-nitrobenzyl)-phosphonate did not show significant inhibition in concentrations up to 15 mM. The benzyl ester showed weak inhibition but was far less inhibitory than nonesterified (4-nitrobenzyl)phosphonate, which is also a strong inhibitor of intestinal alkaline phosphatase (Landt et al., 1978). The inhibition of phosphodiesterase by these compounds showed certain anomalous features which will be described in a subsequent report.

Kinetic constants were determined for several esters having the same 4-nitrophenyl leaving group. The results are shown in Table II. For 4-nitrophenyl phenyl phosphate (line 5), which presumably can be hydrolyzed by the enzyme to either 4-nitrophenol and phenyl phosphate or phenol and 4-nitrophenyl phosphate, only the former reaction was measured. For

Table I: Phosphonate Monoesters and Phosphodiesters Tested as Substrates

name	structure	result[a]
ethyl (4-nitrobenzyl)phosphonate		–[b]
n-butyl (4-nitrobenzyl)phosphonate		–[b]
cyclohexyl (4-nitrobenzyl)phosphonate		–[b]
phenyl (4-nitrobenzyl)phosphonate		++[b]
benzyl (4-nitrobenzyl)phosphonate		–[b]
4-nitrophenyl (4-nitrobenzyl)phosphonate		+++[b]
4-nitroanilino(4-nitrobenzyl)phosphonate		+[b]
diphenyl phosphate		+++[c]
dibenzyl phosphate		+[c]

[a] A plus sign indicates detectable hydrolysis of the compound by phosphodiesterase, three plus signs indicate essentially complete hydrolysis, and two plus signs indicate an intermediate amount of hydrolysis. A minus sign indicates no detectable hydrolysis. [b] Result of 115-h incubation. [c] Result of 72-h incubation.

simplification of the presentation, data for V_{max} will be considered separately from data for K_m.

Despite the common leaving group, the values for V_{max} vary over 5000-fold. Substitution of sulfur for a nonbridge oxygen resulted in a decrease of V_{max} up to 100-fold (line 1 vs. line 2 and line 6 vs. line 7). Substitution of a methylene group for a bridge oxygen (phosphate diester compared to the isosteric phosphonate monoester) decreased V_{max} approximately 3.3-fold (line 5 vs. line 3 and line 6 vs. line 4). Insertion of a methylene group between the phenyl group and the phosphorus atom decreased V_{max} by over 50-fold (line 1 vs. line 3 and line 1 vs. line 5). Substitution of a 4-nitro group on the non-leaving-group aromatic ring decreased V_{max} about 2.4-fold (line 3 vs. line 4 and line 5 vs. line 6). These last comparisons are notable for a consistent decrease in V_{max} as the internal molecular symmetry increased with methylene group insertion or nitro group substitution.

The corresponding effects on K_m are less regular than those on V_{max}. The only consistent pattern was for substitution of sulfur for a nonbridge oxygen, which increased K_m twofold (line 2 vs. line 1 and line 7 vs. line 6).

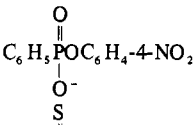
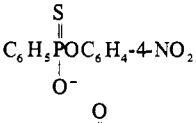
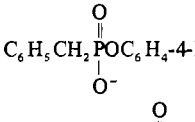
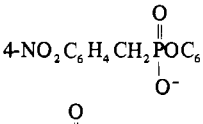
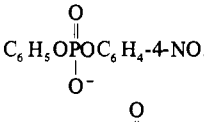
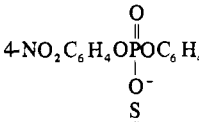
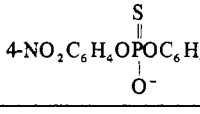
4-Nitrophenyl phenylphosphonothioate possesses a chiral phosphorus atom and is synthesized as a racemic mixture (Dudman & Benkovic, 1977). When a 3.0 mM solution of this compound in 100 mM Tris-HCl, pH 8.0, was treated with 15 μg of enzyme, 50% of the compound was hydrolyzed within 2 h and no further hydrolysis was detected. Addition of concentrated HCl with heating hydrolyzed the remainder of the compound as determined after neutralization by measurements of free 4-nitrophenol.

Discussion

Previous work has demonstrated that bovine intestinal 5′-nucleotide phosphodiesterase catalyzes hydrolysis of phosphonate monoesters and phosphate diesters with a ping-pong mechanism involving formation of a covalent intermediate (Kelly & Butler, 1977; Landt & Butler, 1978). Catalysis involves formation of a covalent bond between a specific residue of the enzyme and the phosphorus atom of the phosphate/phosphonate concomitant with cleavage and release of an esterified leaving group. A subsequent step involves hydrolysis of the intermediate–enzyme bond with release of phosphonic acid/phosphomonoester.

Kelly & Butler (1977) have shown that the enzyme hydrolyzes naphthyl and 4-nitrophenyl esters of several phosphonates in which the substituents bonded to phosphorus by a C–P bond range from a methyl group to a phenyl group. These esters are much more rapidly hydrolyzed under saturating conditions than are nucleotide substrates, but K_m values are much higher than for nucleotides. Typical is 4-nitrophenyl methylphosphonate which has a V_{max} of 850 μmol min^{-1} mg^{-1} (or more than 100-fold greater than the V_{max} for ADP) but a K_m of 66 mM (more than 300-fold higher than for ADP). The rate-limiting step in hydrolysis of these phosphonate esters appears to be hydrolysis of the covalent intermediate (Kelly & Butler, 1977). The hydrolysis rate for nucleotide substrates

Table II: Kinetic Constants for Several Phosphonate Monoesters and Phosphodiesters

name	structure	K_m[a] (mM)	V_{max}[a] (μmol min^{-1} mg^{-1})
(1) 4-nitrophenyl phenylphosphonate		11	418
(2) 4-nitrophenyl phenylphosphonothioate		21	180
(3) 4-nitrophenyl benzylphosphonate		3.5	8.0
(4) 4-nitrophenyl (4-nitrobenzyl)phosphonate		6.7	3.4
(5) 4-nitrophenyl phenyl phosphate		21	26.3
(6) bis(4-nitrophenyl) phosphate		1.0	11
(7) bis(4-nitrophenyl) phosphorothioate		2.2	0.08

[a] These constants were determined at 30 °C in 100 mM Tris-HCl, pH 8.0.

appears to be limited by the slow rate of dissociation of nucleotide product from the enzyme (Landt & Butler, 1978).

The 50-fold greater value of V_{max} for 4-nitrophenyl phenylphosphonate than for the corresponding benzylphosphonate (lines 1 and 3 in Table II) is a much larger difference than previously observed with a series of phosphonate monoesters, in which the 4-nitrophenyl esters of phenyl-, methyl-, and (chloromethyl)phosphonate gave V_{max} values which differed less than twofold (Kelly & Butler, 1977). The much larger effect observed by changing the distance of the phenyl group from the phosphorus atom than by exchanging it for a methyl or chloromethyl group suggests that the geometry of arrangement of the aromatic phenyl ring, rather than inductive effects, determines the rate of the slowest step in the reaction sequence, which for these substrates is probably hydrolysis of the covalent phosphonyl enzyme intermediate (Kelly & Butler, 1977). Even phosphodiester substrates [e.g., bis(4-nitrophenyl) phosphate] are hydrolyzed far more slowly than the phenylphosphonate (lines 5 and 6 vs. line 1, Table II) although slightly faster than their isosteric benzylphosphonate analogues (lines 3 and 4).

Part of the impetus for the present study arose out of our repeated observation that 5′-nucleotide phosphodiesterase failed to hydrolyze simple alkyl monoesters of phosphonic acids. Table I illustrates these observations and shows that even an ester of a secondary alcohol such as cyclohexanol is not a substrate, although the enzyme readily cleaves the alkyl secondary alcohol ester linkage to the 3′-hydroxyl group of ribose in its natural substrates, the oligonucleotides. The failure of the enzyme to cleave phosphonate alkyl esters is thus apparently not due to inability to cleave this linkage but to failure to recognize and bind these phosphonates to the active site, as confirmed by their failure to inhibit the hydrolysis of other esters. The benzyl ester did show weak inhibition, suggesting the importance of aromatic groups in promoting binding to the active site.

Aryl esters provide good leaving groups for displacement reactions such as those catalyzed by phosphodiesterase. On the other hand, the enzyme hydrolyzes, at a relatively slow rate, dibenzyl phosphate, showing it is capable of cleaving alkyl phosphodiesters, which more resemble natural substrates than do the corresponding alkyl monoesters of phosphonates, which are not hydrolyzed. Reports of enzymes known to cleave alkyl phosphodiesters are quite limited (Gerlt & Whitman, 1975), although snake venom 5′-nucleotide phosphodiesterase apparently cleaves methyl esters of 5′-nucleotides (Chambers & Moffat, 1958).

The effects of various substituents on the values of the kinetic constants for different substrates are probably at least partially due to internal symmetry considerations. Most of the tested compounds possess approximate twofold symmetry with an axis through the phosphorus atom, permitting binding to the active site in two different modes. In the instance of a phosphonate substrate, one mode places the P–C bond in the active site, while the other places the P–O bond in the active site. Only the latter mode is productive (e.g., leads to hydrolysis) and the other must be inhibitory. The observed kinetic constants are probably composites which include factors for correct binding which results in hydrolysis and "wrong-way" binding which is inhibitory (Osterman & Walz, 1978). A straightforward example is provided by comparison of the symmetrical bis(4-nitrophenyl) phosphate with the unsymmetrical but isosteric 4-nitrophenyl (4-nitrobenzyl)phosphonate (lines 6 and 4, Table II). For the latter compound only one 4-nitrophenyl group is subject to hydrolysis; the occurrence of wrong-way, nonproductive binding is consistent with the 3-fold slower rate and 6.7-fold greater K_m than for the symmetrical analogue which can be hydrolyzed equally well regardless of binding orientation. As would be expected for substrate inhibition of a completely competitive nature (Webb, 1963), the double-reciprocal plots were linear.

The enzyme active site can be considered to include two

Table III: Relative Effects of Changes in Symmetry or Chemical Structure in Compounds from Table II

less symmetrical to more symmetrical[a]	type of change	effect on V_{max}	effect on K_m
1 → 3	$-CH_2-$ group inserted	↓↓	↓
1 → 5	-O- atom inserted	↓↓	↑
3 → 4	$-NO_2$ group substitution	↓	↑
5 → 6	$-NO_2$ group substitution	↓	↓↓
chemical changes	**type of change**	**effect on V_{max}**	**effect on K_m**
3 → 5	$-CH_2-$ → -O-	↑	↑
4 → 6	$-CH_2-$ → -O-	↑	↓
2 → 1	S → O	↑	↓
7 → 6	S → O	↑↑	↓

[a] For identity of compounds, see Table II.

separate binding domains. In terms of oligonucleotide substrates, one of these domains contains a binding site (A) for the nucleotide at the 3′ terminus (this nucleoside 5′-phosphate is hydrolytically removed in the reaction). The other domain contains a binding site (B) for the penultimate nucleotide and possibly for others along the oligomer. The amino acid residue to which the terminal nucleotide becomes transiently esterified through its 5′-phosphate in the course of the catalytic reaction (Landt & Butler, 1978) is interposed between these two domains, with P–O cleavage occurring on the B side. The symmetry effects reported here can be rationalized by postulating that the B site has a much greater affinity for aromatic groups than does the A site. By use of the highly aromatic nitrophenyl ester substrates, V_{max} would be expected to be greatest when the phosphonate substituent (in the A site) was least aromatic (e.g., nitrophenyl *methyl*phosphonate) because wrong-way nonproductive binding should be minimal. Conversely, phosphonate substrates which are more symmetrical due to the presence of aromatic groups on both sides of the P atom [e.g., nitrophenyl (nitrobenzyl)phosphonate] should have a greater incidence of wrong-way binding, resulting in the observed lower V_{max} values. This postulate also explains the enzyme's failure to hydrolyze aliphatic esters of phosphonates, which would have little affinity for the B site.

It is thus proposed that increasing the symmetry of an unsymmetrical substrate leads to an increase in the incidence of nonproductive binding (actually the ratio of nonproductive binding to productive binding) with consequent effects upon the value of apparent (composite) kinetic constants. In particular, when the other effects of introducing a substituent are minimal, increasing the incidence of nonproductive binding lowers the value for V_{max}. Examples of patterns of symmetry effects deducible from Table II are presented in Table III. All the substrates which are compared contain the same 4-nitrophenolate leaving group; the various substituents introduced are elsewhere in the molecule. It can be seen that in certain cases (e.g., insertion of a methylene group or addition of a 4-nitro group) the predicted decrease in V_{max} is observed. In other cases the change in the substrate involves changing a phosphorus-bound sulfur atom to the natural oxygen form, producing an increase in V_{max} due to chemical considerations which outweigh symmetry effects. Subtle changes, such as interposition of a methylene group between the phosphorus atom and the phenyl ring of a phosphonate substrate, may lead to relatively large changes in V_{max} which appear to be due largely to effects of nonproductive binding.

The symmetry effects on K_m are less predictable because the change which increases the substrate symmetry may either decrease or increase its affinity for the enzyme in either the productive or nonproductive modes. Table III illustrates both types of effect.

Symmetry considerations involving exchange of sulfur for nonbridge oxygen on the phosphorus atom constitute a special class because of the chirality of the phosphorus atom which may result. Only one diastereomer of 4-nitrophenyl phenylphosphonothioate (line 2, Table II) is cleaved at an appreciable rate, in agreement with the observation of Dudman & Benkovic (1977). Kinetic constants for this compound, which have been determined for the first time with this enzyme and are presented in Table II, differ from the values for the corresponding oxygen analogue by only a factor of about 2, suggesting that not only can the enzyme hydrolyze phosphonothioate esters at a rapid rate but also the diastereomer which is a poor substrate has relatively little affinity for the active site and that nonproductive binding of either form is of minor importance. If this enzyme has the same stereospecificity as the analogous snake venom enzyme, the *S* enantiomer is the one which is a good substrate (Bryant & Benkovic, 1979).

In contrast, the V_{max} for bis(4-nitrophenyl) phosphorothioate is over 100-fold lower than that for the corresponding oxygen phosphodiester (Table II). Thus, the enzyme may catalyze phosphorothioate hydrolysis much less efficiently than phosphonothioate or phosphodiester hydrolysis. Eckstein & Frischauf (1974) reported that snake venom phosphodiesterase was unable to hydrolyze dithymidine 3′,5′-phosphorothioate and 4-nitrophenyl phenyl phosphorothioate. Alkaline phosphatase, a closely related enzyme mechanistically, has been shown to have similar reactivity, with an approximately 100-fold lower rate of hydrolysis of 4-nitrophenyl phosphorothioate (Breslow & Katz, 1968), compared to 4-nitrophenyl phosphate.

Stereochemical considerations suggest an alternate explanation for the large rate difference. Although the phosphorothioate diester is symmetrical, it has two nonequivalent binding modes which differ with respect to the positions of the nonbridge oxygen and sulfur atoms. The present observations could be due to a reduced rate of hydrolysis on binding in one of the modes.

In summary, 5′-nucleotide phosphodiesterase from calf intestine is capable of hydrolyzing a wide range of compounds which contain phosphonothioate, phosphorothioate, or phosphonoamidate bonds. Alkyl phosphonate monoesters are resistant whereas dibenzyl phosphate, an alkyl diester, is a substrate. Aryl monoesters of phosphonates are hydrolyzed by this enzyme much more rapidly than "natural" nucleotide substrates (Kelly & Butler, 1977). Some of the kinetic effects with near-symmetrical artificial substrates are due to nonproductive modes of substrate binding.

References

Alvarez, R., Moore, T. C., & Vanderpent, J. (1974) *Plant Physiol. 53*, 144.

Breslow, R., & Katz, I. (1968) *J. Am. Chem. Soc. 90*, 7376.

Bryant, F. R., & Benkovic, S. J. (1979) *Biochemistry 18*, 2825.

Chambers, R. W., & Moffat, J. G. (1958) *J. Am. Chem. Soc. 80*, 3752.

Cory, J. G., Fleisher, A. E., & Munro, J. B. (1978) *J. Biol. Chem. 253*, 2898.

Dudman, N. P., & Benkovic, S. J. (1977) *J. Am. Chem. Soc. 99*, 6113.

Eckstein, F., & Frischauf, A. M. (1974) *Methods Enzymol. 34*, 605.

Eckstein, F., & Goody, R. S. (1976) *Biochemistry 15*, 1685.

Engle, R. (1977) *Chem. Rev. 77*, 349.
Gerlt, J. A., & Whitman, G. J. R. (1975) *J. Biol. Chem. 250*, 5053.
Gray, M. W. (1976) *Can. J. Biochem. 54*, 413.
Hanes, L. S., & Isherwood, F. A. (1949) *Nature (London) 164*, 1107.
Kelly, S. J., & Butler, L. G. (1975) *Biochem. Biophys. Res. Commun. 66*, 316.
Kelly, S. J., & Butler, L. G. (1977) *Biochemistry 16*, 1102.
Kelly, S. J., Dardinger, D. E., & Butler, L. G. (1975) *Biochemistry 14*, 4983.
Kezdy, F. J., & Bender, M. L. (1962) *Biochemistry 1*, 1097.
Landt, M., & Butler, L. G. (1978) *Biochemistry 17*, 4130.
Landt, M., Boltz, S. C., & Butler, L. G. (1978) *Biochemistry 17*, 915.
Laskowski, M., Sr. (1971) *Enzymes, 3rd Ed. 4*, 313.
Letsinger, R. L., Wilkes, J. S., & Dumas, L. B. (1976) *Biochemistry 15*, 2810.
Miller, P. S., Fang, K. N., Kondo, N. S., & T'so, P. O. P. (1971) *J. Am. Chem. Soc. 93*, 6657.
Nichol, A. W., Nomura, A., & Hampton, A. (1967) *Biochemistry 6*, 1008.
Ogilvie, K. K., & Hruska, F. H. (1976) *Biochem. Biophys. Res. Commun. 68*, 375.
Osterman, H. L., & Walz, F. G., Jr. (1978) *Biochemistry 17*, 4124.
Smith, M., Brummond, G. I., & Khorana, H. G. (1961) *J. Am. Chem. Soc. 83*, 698.
Tolkmith, M. C. (1958) *J. Org. Chem. 23*, 1685.
Tsou, K. C., & Yip, K. F. (1973) *Life Sci. 13*, 1505.
Uesugi, S., Yano, J., Yano, E., & Idehara, M. (1977) *J. Am. Chem. Soc. 99*, 2313.
Webb, J. L. (1963) *Enzyme and Metabolic Inhibitors*, Vol. 1, Academic Press, New York.
Yarbrough, L. E. (1978) *Biochem. Biophys. Res. Commun. 81*, 35.

Adenosine Cyclic 3′,5′-Monophosphate Dependent Protein Kinase: Active Site Directed Inhibition by Cibacron Blue F3GA†

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ABSTRACT: Cibacron Blue F3GA, the polycyclic blue chromophore of Blue Dextran 2000, inhibits the catalytic subunit of bovine brain protein kinase. The rate of inactivation exhibits a hyperbolic dependence on the dye concentration. This suggests that an enzyme–dye complex forms prior to inactivation. Protein and peptide substrates or MgATP protects the enzyme against dye inactivation. Kinetic measurements show that the dissociation constant is ~100 μM and the maximal rate of inactivation is 0.13 min^{-1} at 22 °C. Inactivation is temperature and time dependent. Exhaustive dialysis, gel filtration, or the addition of substrate fails to reactivate inhibited enzyme. The failure to reverse the inhibition suggests that the dye forms a covalent complex with the enzyme. Denaturation by sodium dodecyl sulfate also fails to dissociate the dye from enzyme. The hyperbolic kinetics, moreover, suggest that the dye functions as an active site directed reagent. The holoenzyme is resistant to Cibacron Blue inactivation. Addition of cAMP converts the enzyme to a form susceptible to inhibition. In agreement with our previous studies, these results also suggest that the regulatory subunit shields, either physically or functionally, the active site of the catalytic subunit.

Adenosine cyclic 3′,5′-monophosphate (cAMP)[1] dependent protein kinase consists of two dissimilar subunits (Gill & Garen, 1970; Tao et al., 1970; Kumon et al., 1970; Reimann et al., 1971). The activation of this enzyme by cAMP is summarized by

$$R_2C_2 + 2cAMP \rightleftharpoons (R\text{-}cAMP)_2 + 2C$$

in which R_2C_2 is the holoenzyme and R and C are the regulatory and catalytic subunits, respectively (Rosen & Erlichman, 1975; Hofmann et al., 1975). The free C subunit is active and cAMP independent. Combining C subunit and the $(R\text{-}cAMP)_2$ complex results in the release of cAMP and the regeneration of the cAMP-dependent holoenzyme.

We reported that the free C subunit binds to Blue Dextran–Sepharose affinity resin. The blue chromophore of Blue Dextran, Cibacron Blue F3GA, is a nucleotide analogue (Thompson et al., 1975). These investigators proposed that this nucleotide analogue binds to those proteins which contain the super secondary structure termed the dinucleotide fold. The binding of free C subunit to Blue Dextran–Sepharose and the failure of the holoenzyme (R_2C_2) to bind support the notion that the active site is inaccessible in the holoenzyme form.

We present experiments which show that the free chromophore of Blue Dextran, which contains a triazine ring chloride, inhibits the free C subunit in an apparently irreversible manner and exhibits the characteristics of an active site directed reagent. Furthermore, RI, RII, and the protein kinase inhibitor protein (PKI) also protect against blue dye inactivation. These results support the idea that each of these proteins physically or functionally shields the active site of the

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[1] Abbreviations used: C, catalytic subunit; RI and RII, regulatory subunits I and II, respectively; cAMP, adenosine cyclic 3′,5′-monophosphate; PKI, protein kinase inhibitor protein; Mops, 3-(*N*-morpholino)propanesulfonic acid.

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