Active-Site-Dependent Elimination of 4-Nitrophenol from 4-Nitrophenyl Alkylphosphonyl Serine Protease Adducts

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Abstract: Chymotrypsin and subtilisin BPN′ can be inhibited by bis(4-nitrophenyl) methylphosphonate (NMN) and bis(4-nitrophenyl) propylphosphonate (NPN) very efficiently with second-order rate constants, k_i/K_i, between 544 and 4300 M^{-1} s^{-1} at 25.0 ± 0.1 °C at the pH maxima. The second-order rate constants for the inhibition of trypsin are 26.3 ± 1.4 M^{-1} s^{-1} with NMN and 891 ± 14 M^{-1} s^{-1} with NPN at pH 8.3 and 25.0 ± 0.1 °C. A second stoichiometric equivalent 4-nitrophenol is also lost from 4-nitrophenyl alkylphosphonyl adducts of chymotrypsin but not from trypsin and subtilisin BPN′. Elimination of 4-nitrophenol from the propylphosphonyl adduct is at a rate only about twice the rate of hydrolysis of a comparable phosphonate diester, whereas 4-nitrophenol is eliminated 270 times faster from the methylphosphonyl adduct of chymotrypsin. The activation enthalpies, in kcal/mol, for 4-nitrophenol elimination from 4-nitrophenyl alkylphosphonylchymotrypsin are 15.0 ± 1.3 for the propyl derivative, 16.4 ± 0.5 for the methyl derivative in H_2O and 18.0 ± 0.5 in D_2O. The activation entropies, in cal mol^{-1} K^{-1}, are −29.7 ± 2.4 for the propyl derivative, −14.8 ± 0.5 for the methyl derivative in H_2O, and −10.3 ± 0.3 for the methyl derivative in D_2O. Partial solvent isotope effects for the elimination of 4-nitrophenol from 4-nitrophenyl methylphosphonylchymotrypsin give best fits to two-site proton models: These give primary isotope effects between 1.9 and 2.0 ($\phi_1^{\ddagger} = 0.52 \pm 0.14$ or 0.49 ± 0.07) for a proton in flight, possibly from the water attacking at phosphorus to the catalytic His, and an α-secondary effect of 1.3 ($\phi_2^{\ddagger} = 0.75 \pm 0.20$) or a term for solvent contribution of 1.25 ($\Phi = 0.80 \pm 0.10$). The secondary β-deuterium isotope effect on the elimination of the second 4-nitrophenol from the adduct of chymotrypsin with NMN-l_3 (l = *h* or *d*) is 0.94 ± 0.2 possibly of hyperconjugative origin. The occurrence and mechanisms of secondary reactions in phosphonylated serine protease enzymes are markedly different from those in phosphonylated cholinesterases.

The molecular origins of inhibition of serine hydrolase enzymes by phosphonate esters, as a question of fundamental science, has long been an interest in this laboratory.[1−7] A remarkable efficiency (60−70%)[6] of mobilization of the catalytic power of these enzymes was observed in the P−O bond formation at the active-site Ser in some cases. This catalytic assistance must, however, be diminished or absent in the dephosphorylation of the enzymes to account for their often irreversible inhibition and ensuing toxicity. Although acute toxicity of phosphonate esters is predicated on their action on acetylcholinesterase (AChE),[8] which regulates the concentration of the neurotransmitter, acetylcholine,[9] inhibition of other serine hydrolases[10−13] also causes physiological impairments that have not yet been studied in detail. A pertinent question in this context is the potential for secondary reactions of the covalently modified enzymes. A perhaps familiar term for a group of these reactions is *aging* having the connotation of total resistance to nucleophilic reactivation of the enzyme. The chemical transformations underlying the phenomenon follow different mechanisms.

Our current hypothesis is that some unique interactions exist in numerous organophosphate−serine hydrolase adducts that stabilize these intermediates and that their hydrolysis off the enzymes is hampered by some interference with the normal acid−base catalytic function of these enzymes.[6,14,15] To test this hypothesis, we have been studying the dynamics of phosphonylation and related enantioselectivity[15] and the nature of interactions leading to dephosphonylation versus aging in rationally selected phosphonylated adducts of serine hydrolase enzymes.[1−7] The architecture and electrostatic character of the active site governs the fate of a covalently attached phosphonyl

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fragment, as we have shown recently.[16] Strong negative electrostatic and hydrophobic forces in the cholinesterases preferentially promote C−O bond cleavage with occasional methyl migration[16] whereas this route of dealkylation is nearly absent in phosphonate esters of serine proteases. The active site of serine proteases is wide, near the surface, and void of the unique combination of carboxylic and aromatic residues present at the active site of cholinesterases.

The electronic properties of ligands attached to phosphorus is also decisive in whether C−O or P−O bond cleavage occurs in phosphonate diesters of serine hydrolases. Phenolate ions are good leaving groups and depart readily with P−O cleavage from chymotrypsin[17,18] and the cholinesterases.[6,13,19]

In the following we describe studies of bis(4-nitrophenyl) propylphosphonate (NPN), bis(4-nitrophenyl) methylphosphonate (NMN), and the β-deuterated isotopomer of NMN as inhibitors of chymotrypsin, trypsin, and subtilisin BPN′. This investigation has been focused on molecular features at the active site that promote P−O bond breaking and elimination of ligands from the central phosphorus of the covalent phosphonate adducts of serine proteases. In particular, the transition state properties of the chymotrypsin-catalyzed elimination of the second 4-nitrophenol have been characterized by the use of isotope effects and thermodynamic parameters.

Experimental Section

Materials. Inorganic salts and buffer components were reagent grade chemicals, which were used as purchased or dried, recrystallized, or distilled as necessary. Water was distilled from a copper-bottom still, passed through a Barnstead mixed-bed ion-exchange column, boiled for 20 min, and cooled suddenly. Heavy water 98% enriched was purchased from Aldrich and used directly, then stored under nitrogen. Porcine pancreatic trypsin (EC 3.4.21.4), bovine pancreatic α-chymotrypsin (EC 3.4.21.1), and subtilisin BPN′ (EC 3.4.21.62) were purchased from Sigma. 4-Methylumbelliferyl-4-guanidinobenzoate hydrochloride (MUGB) and 4-methylumbelliferyl-4-trimethylammonium cinnamate chloride (MUTMAC) were also from Sigma. The synthesis of NPN[1] and NMN and its CD_3 isotopomer was published earlier.[20]

Solutions. Buffer solutions were prepared fresh from the appropriate analytical grade buffer salts and double distilled water. Buffer solutions in light and heavy water were made with identical ratios of conjugate acid−base pairs of the buffer components. Enzyme solutions were made by dissolving the solid enzyme into buffer. Phosphate buffers at 0.05 M were used between pH 6.5 and 8.0 and Tris or borate buffers at 0.05−0.1 M were used between pH 7.5 and 9.5 for determination of the pH dependence of elimination of 4-nitrophenolate ion from the 4-nitrophenyl methylphosphonate adduct of chymotrypsin. Proton-inventory measurements were carried out at pH 7.7 in 0.05 M phosphate buffer and also at pH 8.1 in 0.05 M Tris buffer and their equivalent pL (L = H or D) in mixtures of isotopic waters. The molarity of active trypsin was determined by titration with MUGB and the molarity of active α-chymotrypsin and subtilisin BPN′ was determined with MUTMAC. NPN and NMN were used in $(1-3) \times 10^{-3}$ M stock solutions in dried acetonitrile. NMN solutions diluted with 0.0001 M HCl were used for stopped-flow kinetic runs. Diluted acetonitrile solutions of NMN were used for conventional spectrophotometric kinetic runs.

Inactivation of Serine Proteases. In stopped-flow kinetic runs, the enzyme concentration was in large (at least 40-fold) excess to either NMN or NPN. The reactions were conducted at pH 7.7 in 0.05 M phosphate buffer with chymotrypsin and at pH 8.2 and 8.3 in 0.1 M Tris and 0.05 M borate buffer with trypsin and subtilisin BPN′. Kinetic runs were followed by monitoring the absorbance of the released 4-nitrophenolate ion at 400 nm. Pseudo-first-order rate constants were obtained by fitting 200 data points.

In a typical conventional spectrophotometric kinetic run, 950 μL of an ∼1 mM enzyme solution was first incubated for about 10 min, then 20−50 μL of acetonitrile solution of NMN or NPN was injected to initiate the reaction. Automated acquisition of 200−1000 data points at 400 nm with a Perkin-Elmer Lambda-7 spectrophotometer interfaced to a PC was used. Pseudo-first-order rate constants were calculated for 4 half-lives by nonlinear least squares of absorbance/time coordinates. The fit was to a biexponential equation to separate any contribution from the release of the second 4-nitrophenol. The temperature was controlled with a Lauda K4/DR circulating water bath furnished with a thermistor probe attached to a digital read-out. Measurements of the pH of kinetic solutions before and after reaction were performed with a Radiometer RHM 84 pH meter.

Rate Measurements for Elimination of the Second 4-Nitrophenol from the Chymotrypsin Adducts. In these experiments, the appropriate volume of a buffered solution of chymotrypsin at 1 mM was equilibrated within 0.05 °C of the working temperature (as monitored with a thermistor probe) in a quartz cuvet in the cell compartment of the spectrophotometer. Ten microliters of the inhibitor solutions were introduced into a 1 mL total volume to initiate the reaction. The concentration of chymotrypsin was held at least 30% above the concentration of the inhibitor. Two hundred to a thousand absorbance/time data pairs were collected at 400 nm and were fit to the biexponential rate equation. The second exponential phase was completely isolable from the first one. All data reductions were performed by GraFit.[21]

Molecular Mechanics Minimization of Chymotrypsin Covalently Modified with 4-Nitrophenyl Methyl or Propylphosphonate at Ser195. Fully refined and solvated structures of the native enzyme generated in YETI (5.3)[22] were reported previously.[23] Optimized geometry and electrostatic potential (ESP)-derived partial atomic charges were generated for the tetracoordinate phosphonates in MNDO as implemented in MOPAC (6.0).[24] The united atom representation was used in YETI. The adducts were generated by incorporating the modified Ser fragment into solvated structures of the enzymes that had been energy-optimized. Acidic and basic residues were given unit electrostatic charges. All Tyr and Cys residues were neutral. The site of protonation of the His residues was based on the availability of H-bond donors or acceptors. The nitrogen on Nδ was protonated on the catalytic His. The enzymes thus modified with the tetracoordinate phosphonates were also optimized in YETI.

Results

The Inactivation of Serine Protease Enzymes by Bis(4-nitrophenyl) Alkylphosphonates. The inactivation of α-chymotrypsin by both NMN and NPN was measured by stopped-flow techniques at pH 7.7 in 0.05 M phosphate buffer at 25.0 ± 0.1 °C, under pseudo-first-order conditions with at least 40-fold excess of α-chymotrypsin over NMN or NPN. Single turnover eliminated interference from background hydrolysis of the compounds and provided the necessary condition for establishing the stoichiometry of 4-nitrophenol release.

The reactions of active trypsin with NMN and NPN were measured in Tris or borate buffer at pH 8.2 and 25.0 ± 0.1 °C either by stopped-flow or by conventional spectrophotometric techniques. Neither NMN nor NPN was as good an inhibitor

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Table 1. Second-Order Rate Constants[a] (M^{-1} s^{-1}) for the Inactivation of Serine Proteases by NMN and NPN at the Optimal pH for the Enzymes and at 25.0 ± 0.1 °C

enzyme	pH	NMN	NPN
chymotrypsin	7.7	4300 ± 120	2380 ± 30
trypsin	8.2	26.3 ± 1.4	890 ± 14
subtilison BPN′	8.3	544 ± 8	1660 ± 12

[a] Average of 3−4 measurements.

for trypsin as for α-chymotrypsin. In the case of the inactivation of trypsin by NMN, a typical absorbance−time trace recorded by spectrophotometry consisted of one fast phase, with 70% of the total 2 equiv of 4-nitrophenolate ion released, and another slow phase, with 12% of the total 4-nitrophenolate ion released in the span of 5 h. The remaining 18% 4-nitrophenol was released too slowly to be followed. Since more than 50% of the total 4-nitrophenol was released during the first phase of the reaction of trypsin with NMN, it was considered that a nonactive-site reaction between trypsin and NMN was involved. A similar reaction profile was observed for the bovine serum albumin (BSA)-catalyzed hydrolysis of NMN. This reaction was carried out, identically to those with the enzymes, with 25 mg/mL of BSA at pH 8.2 in 0.05 M borate buffer. Again, 70% of the total 4-nitrophenolate ion was released with a rate constant of $(1.06 \pm 0.01) \times 10^{-2}$ s^{-1} at 25.0 ± 0.1 °C.

To investigate a nonactive-site-catalyzed component of the observed reactions, active-site blocked trypsin and subtilisin BPN′ were prepared. The enzymes were fully inactivated by diisopropyl fluorophosphate (DFP),[13] which was confirmed by active-site titration with MUGB or MUTMAC. Then the reactions with NMN and NPN were studied with native and active-site-blocked enzymes under identical conditions by conventional spectrophotometric techniques. The rate constant for the nonactive-site-catalyzed hydrolysis approached 30% of the overall reaction rate constants for the inhibition of trypsin with NMN. They were less than 1−2% of the reactions of trypsin with NPN and that of subtilisin BPN′ with either NMN or NPN. The active-site specific rate constants were calculated by correcting the observed rate constants with the rate constants for the nonactive-site reaction and dividing by the active-site concentration of the enzyme. The resulting second-order rate constants are listed in Table 1. The results of inhibition of the enzymes with NPN agree well with those measured by using competition against a good substrate as published earlier.[1,2]

The Elimination of 4-Nitrophenol from Chymotrypsin-4-nitrophenyl Alkylphosphonate Adducts. Inactivation of chymotrypsin at pH 7.7 in 0.05 M phosphate buffer occurred instantaneously upon mixing with small volumes of inhibitor stock solutions in acetonitrile at 25.0 ± 0.1 °C.[1] The structure-dependent departure of the second 4-nitrophenolate ion in buffered H_2O and D_2O was then monitored at 400 nm. pH dependence of the reaction showed plateaus beginning at pH 7.7 (pD 8.2). The first-order rate constants for elimination of this second 4-nitrophenolate ion from the adducts of chymotrypsin formed with NPN and NMN in H_2O and in D_2O are listed in Table 2 as a function of temperature. The data were fit to the Eyring equation:[25]

$$k_2 = \frac{k_B T}{h} e^{-\Delta H^{\ddagger}/RT} e^{\Delta S^{\ddagger}/R} \quad (1)$$

Figure 1 shows the Eyring plot for NMN and Table 3 gives the activation parameters along with their errors obtained by nonlinear least squares.

Table 2. Temperature Dependence of the First-Order Rate Constants,[a] Solvent Isotope Effects, and β-Deuterium Secondary Isotope Effect for 4-Nitrophenol Release from Chymotrypsin−Phosphonate Ester Adducts at pH 7.7, the Maximum, in 0.05 M Phosphate Buffer

inhibitor	temp, °C (±0.1)	$10^5 k_{HOH}$, s^{-1}	$10^5 k_{DOD}$, s^{-1}	k_{HOH}/k_{DOD}
NMN	25.0	456.0 ± 5.7	182.0 ± 2.2	2.50 ± 0.04
NMN	30.0	752.0 ± 9.4	324.0 ± 4.3	2.40 ± 0.04
NMN	35.0	1205 ± 10	531.0 ± 8.0	2.30 ± 0.04
NMN	40.0	1760 ± 60	818.0 ± 8.0	2.20 ± 0.08
NPN	25.0	1.90 ± 0.20		
NPN	30.0	2.60 ± 0.20		
NPN	35.0	4.40 ± 0.36	1.90 ± 0.20	2.30 ± 0.30
NPN	40.0	6.60 ± 0.10	2.45 ± 0.07	2.67 ± 0.09
NMN (pH 7.6)	25.0	448.0 ± 6.0	479.0 ± 6.4[b]	0.94 ± 0.02[c]

[a] Average of 3−4 measurements. [b] NMN-d_3. [c] β-deuterium isotope effect.

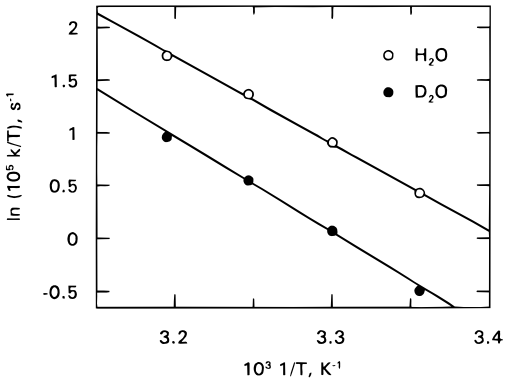

Figure 1. Eyring plots for 4-nitrophenol release from the 4-nitrophenyl methylphosphonate adduct of chymotrypsin.

Table 3. Activation Parameters for 4-Nitrophenol Release from Chymotrypsin Phosphonate Ester Adducts at pH 7.7 in 0.05 M Phosphate Buffer

system	$\Delta H^{\ddagger}$, kcal/mol	$\Delta S^{\ddagger}$, cal/(mol K)
NPN(H_2O)	15.0 ± 1.5	−29.7 ± 2.4
NMN(H_2O)	16.4 ± 0.5	−14.8 ± 0.5
NMN(D_2O)	18.0 ± 0.5	−10.3 ± 0.3

Isotope Effects. The solvent isotope effects are listed in the last column of Table 2. Solvent isotope effects for elimination of 4-nitrophenolate ion were between 2.2 and 2.6 from NMN-inhibited chymotrypsin and between 2.3 and 2.5 from NPN-inhibited chymotrypsin. The solvent isotope effects for the methyl adduct decreased with increasing temperature as expected on grounds of the theory of isotope effects.[26]

Figure 2 shows a plot of partial solvent isotope effects as a function of atom fraction deuterium (n) for the elimination of 4-nitrophenolate ion from the 4-nitrophenyl methylphosphonate adduct of chymotrypsin at pH 7.7 in 0.05 M phosphate buffer at 25.0 ± 0.1 °C. A three-point determination at pH 8.1 in 0.1 M Tris buffer according to Albery[27−29] and used recently by Kresge[30] has also been carried out with identical outcome to

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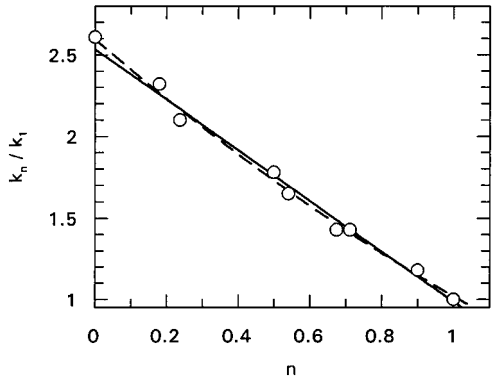

Figure 2. Partial solvent isotope effects (k_n/k_1) as a function of atom fraction deuterium (n) for 4-nitrophenol release from the 4-nitrophenyl methylphosphonate adduct of chymotrypsin. The symbols represent ratios, within one standard deviation, of the average of at least three rate constants, the heavy line is the best linear least-squares fit of the data to $k_n/k_1 = 2.54(1 - n + 0.39)$, and the dashed line is the nonlinear least-squares fit of the data to either $k_n/k_1 = 2.60(1 - n + 0.52n)(1 - n + 0.75n)$ or $k_n/k_1 = 2.60(1 - n + 0.49n)(0.8)^n$.

Figure 2. The β-deuterium isotope effect for the elimination of 4-nitrophenolate ion from the corresponding methylphosphonyl adduct of chymotrypsin at 25.0 ± 0.1 °C was calculated to be $k(CH_3)/k(CD_3) = 0.94 \pm 0.02$.

The Elimination of 4-Nitrophenol from Trypsin and Subtilisin BPN′-4-Nitrophenyl Alkylphosphonate Adducts. Only about 24% of the second equivalent of 4-nitrophenolate ion was released from NMN-inhibited trypsin and a negligible amount from the other adducts. The release of 4-nitrophenolate ion was exponential and associated with a solvent isotope effect of 1.60 ± 0.02. This reaction probably originated from nonspecific nucleophilic sites, which were shown to take up ~30% of NMN in trypsin. This pattern of 4-nitrophenolate ion release was also observed with BSA. The rest of the second equivalent of 4-nitrophenolate ion was released at a very slow rate.

Discussion

Inactivation of Serine Proteases by NMN and NPN. The bis(4-nitrophenyl) alkylphosphonyl esters of NMN and NPN are good inhibitors of serine proteases, particularly chymotrypsin and subtilisin BPN′. The second-order rate constant, k_i/K_i, for inactivation of chymotrypsin with NMN is nearly twice the second-order rate constant with NPN whereas the opposite is true for subtilisin BPN′. NPN also inhibits trypsin better than NMN; the difference is over 30-fold. Because phosphonylation of the active site of trypsin by NMN is relatively slow, other nucleophilic residues on the protein become phosphonylated under single-turnover conditions. The reaction between chymotrypsin and NPN takes place with the involvement of a single proton transferring at the transition state and a solvent isotope effect of 2.00.[1] This was attributed to His57 base-catalyzed attack of Ser195 at phosphorus.

Nucleophilic attack at phosphorus by the active-site Ser is most likely via an in-line displacement of one of the 4-nitrophenol groups,[6,15,16,23,31−33] resulting in a single diastereomer of the phosphonylated enzyme adduct. Minimization by molecular mechanics YETI (V 5.3)[22] of two possible diastereomers resulting from the inhibition of chymotrysin by NPN indicated a preference for the adduct (*S* configuration at P) in which the 4-nitrophenyl group binds in the specificity pocket of the enzyme instead of occupying the N-acyl binding site.[34] Inspection of the corresponding models of the adduct with trypsin permits the speculation that the electrostatic repulsion between Asp189 and the nitro group in the 4-position in phenol may disfavor this orientation and thus the aromatic residue may bind at the N-acyl binding site as in the diastereomer with *R* configuration at P. A sound model for the stereochemical outcome of inhibition in the three enzymes would have to wait for a competent simulation using molecular dynamics.

Chymotrypsin-Catalyzed Elimination of the Second 4-Nitrophenol. As results of this investigation demonstrate, the covalent adduct of 4-nitrophenyl methylphosphonylchymotrypsin undergoes removal of the second 4-nitrophenyl group quite efficiently and 270-fold faster than the propyl analogue. The rate constant for hydrolysis of the propyl analogue is only about twice the rate constant for hydrolysis of a close structural analogue, 4-nitrophenyl methoxyl propylphosphonate ester, $k_{obs} = 9.5 \times 10^{-6}$ s^{-1} at pH 7.7 in 0.05 M phosphate buffer at 25.0 ± 0.1 °C.[1a] Thus, the hydrolysis of the 4-nitrophenyl propylphosphonate adduct occurs essentially nonenzymatically. A 270-fold difference in the rate of 4-nitrophenol elimination between the methyl and propyl adducts cannot be due to simple electronic or steric effect on the rate of nonenzymic hydrolysis since these effects favor hydrolysis of the methylphosphonate diesters over the propyl analogue by probably 2-fold. Second-order rate constants for the hydrolysis of NMN catalyzed by phosphate dianion, the dominant buffer component, and imidazole base-catalyzed hydrolysis, a model of the enzymic general base, were found to be nearly identical in another study.[35] Consequently, the rapid elimination of 4-nitrophenolate ion from the methyl adduct is enzyme catalyzed.

Activation Parameters. The activation parameters calculated from the temperature dependence of the elimination of 4-nitrophenol from the adducts are given in Table 3. The activation enthalpies are almost the same within experimental error for all cases, whereas the activation entropies differ; the entropy of activation is twice as large negative for the propyl adduct as the values, in H_2O and D_2O, for the methyl adduct. Thus, the entire difference in reaction rates between the methyl and propyl adducts is entropic in origin, which is consistent with steric effects. It is not surprising that no difference in the internal energy of bond fission is observed.

Solvent Isotope Effects and Proton Inventories. The solvent isotope effects are between 2.2 and 2.6 for 4-nitrophenol elimination from both adducts of chymotrypsin. The solvent isotope effects for the methyl adduct decrease with increasing temperature as expected on grounds of the theory of isotope effects.[26] The origin of the solvent isotope effect is not entirely enthalpic ($\Delta H_H^{\ddagger} - \Delta H_D^{\ddagger} = -1.6$ kcal/mol), since there is a 4.5 cal mol^{-1} K^{-1} difference in the entropies ($\Delta S_H^{\ddagger} - \Delta S_D^{\ddagger}$) for the methyl adduct. This may reflect differences in conformation or the mode of solvation between reactant states and transition states. Kinetic data for the slow reactions of the propyl compound, below 35 °C, are not precise enough to warrant an analysis of the temperature dependence of the solvent isotope effect.

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The partial solvent isotope effects were also measured for the elimination of 4-nitrophenol from the 4-nitrophenyl methylphosphonyl adduct of chymotrypsin at pH 7.7 in 0.05 M phosphate buffer at 25.0 ± 0.1 °C and the proton inventory profile constructed from the data is shown in Figure 2. A solvent isotope effect of 2.61 ± 0.10 and a similar profile to Figure 2, with only three points, was also measured at pH 8.1. A comparison of the proton inventory profile of this work to that of a previous report[35] on the imidazole base-catalyzed hydrolysis of the parent compound, NMN, shows that the solvent isotope effects but not the proton inventory profiles are similar: a linear proton inventory with a solvent isotope effect of 2.7 ± 0.1 corresponding to a fractionation factor (inverse solvent isotope effect) of 0.37 ± 0.01 was found for NMN hydrolysis. Linear proton inventories were reported for imidazole-catalyzed hydrolysis of 1-acetylimidazole and ethyl trifluorothiolacetate.[36]

To account for the slightly curved dependence on n of the partial solvent isotope effects, k_n/k_1, in Figure 2, four forms of the Gross−Butler equation

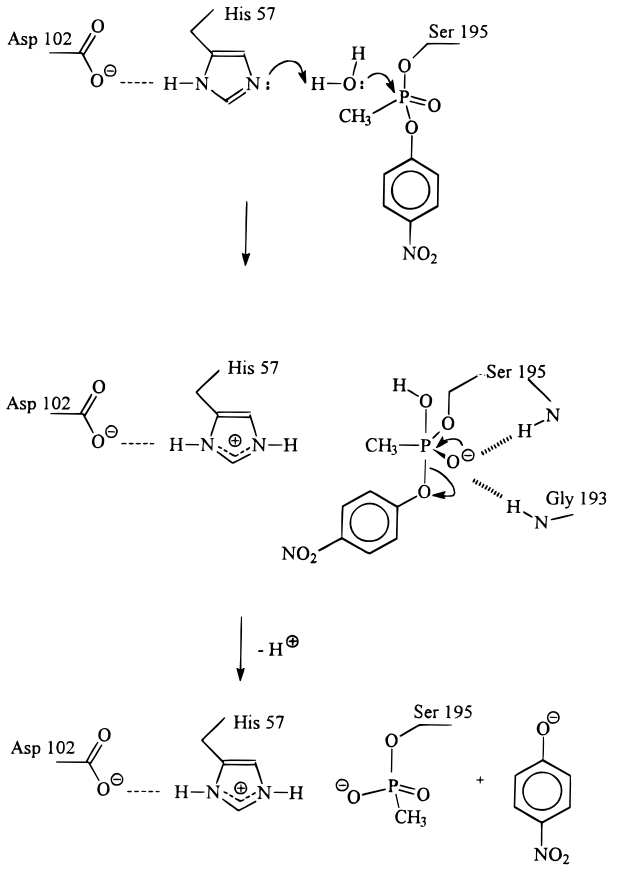
$$k_n/k_1 = (k_o/k_1)\prod_i(1 - n + \phi_i^{\ddagger}n)/\prod_j(1 - n + \phi_j^{R}n) \quad (2)$$

wherein $\phi^{\ddagger}$ is the transition state fractionation factor and ϕ^{R} is the reactant state fraction factor,[37,38] were considered for statistical fitting with the reactant state fractionation factor $\phi^{R} = 1.0$ for OH in water: (1) $k_n/k_1 = k_o/k_1(1 - n + \phi^{\ddagger}n)$ for a single fractionation factor, single-proton transfer; (2) $k_n/k_1 = k_o/k_1(1 - n + \phi^{\ddagger}n)^2$ for a two and equal fractionation factor, two-proton transfer; (3) $k_n/k_1 = k_o/k_1(1 - n + \phi_1^{\ddagger}n)(1 - n + \phi_2^{\ddagger}n)$ for a two and unequal fractionation factor, two-proton transfer; and (4) $k_n/k_1 = k_o/k_1(1 - n + \phi^{\ddagger}n)\Phi^n$ for a single fractionation factor, single-proton-transfer plus a term, Φ, for general solvation.[30]

The statistical F-test indicates modest improvements (only 73% probability of identity) for models 2−4 over model 1, but a consideration of higher order terms has no merit. Fitting to models 3 and 4 gives statistically indistinguishable results.

Although an unambiguous distinction among the three models in Figure 2 may seem debatable, the statistical relevance and mechanistic ramifications of model 3 are intriguing and merit consideration. Model 3 gives $k_n/k_1 = 2.60(1 - n + 0.52n)(1 - n + 0.75n)$, where $\phi_1^{\ddagger} = 0.52 \pm 0.14$ and $\phi_2^{\ddagger} = 0.75 \pm 0.20$ are the fractionation factors corresponding to solvent isotope effects of 1.9 and 1.3, respectively. A plausible and appealing rationale for model 3 may be that a proton is transferred from the attacking water to the active-site His of chymotrypsin in the rate-determining step with a primary isotope effect of 1.9. An α-secondary isotope effect of 1.3 may result from the other hydrogen on the nucleophilic water molecule (Scheme 1). The origin of this α-secondary isotope effect is that a vibrational motion of the α-H becomes freer (looser) at the transition state and thus the force constant becomes smaller. It is probably not a bending vibration[39] that undergoes these changes as in α-secondary sites on carbon reaction centers. The alignment

Scheme 1



of the electron pair of the catalytic His at a crowded active site may not be ideal for transferring one of the hydrogens in the attacking water molecule. Instead, it may be bifurcated and interact with both hydrogens to a different degree, which is discernible from the molecular model. It appears that the proton in transfer at the transition state for the hydrolysis of the adduct catalyzed by the imidazole of the catalytic His is less symmetric than that in the free imidazole base-catalyzed hydrolysis of NMN found earlier.[35] This can reflect the electronic and steric differences at the transition states between the phosphonate diesters: enzyme adduct and NMN. This effect, however, seems to be ameliorated by loosening of the other OH bond at the transition state of the enzymic reaction. The effect could be viewed as another manifestation of compression[28,37,40] at the active site.

Nevertheless, two alternative origins of the small component of the solvent isotope effect cannot be ruled out: (1) A contribution from the proton between Asp102 and His57, as some compression[28,37,40] at the transition state moves it in a shallow potential well,[28,37] may result in a small primary isotope effect. (2) Solvent reorganization at the transition state conforming to model 4 and advocated recently by Kresge[30] gives $\phi^{\ddagger} = 0.49 \pm 0.07$, a primary isotope effect of 2.0, and $\Phi = 0.8 \pm 0.10$, corresponding to a general solvation term of 1.25.

Secondary β-Deuterium Isotope Effect. The β-deuterium isotope effect is inverse, 0.94 ± 0.02 (per CD_3), for the elimination of 4-nitrophenol from the methyl derivative of the adduct. Inverse isotope effects of similar magnitude were observed for the inactivation of AChE by phosphonate esters and for imidazole-catalyzed reactions of phosphonate esters.[20]

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It is assumed that the effects are enthalpic in origin ($\Delta\Delta G^{\ddagger} \sim \Delta\Delta H^{\ddagger} = 37$ cal/mol). The interpretation of these secondary deuterium isotope effects is clouded by many unresolved issues; however, the loss of hyperconjugative overlap,[39,41] existing in the ground state between the σ orbitals of the C−H bond and the d orbitals at P, in the transition state was found to be the most correct explanation.[20] This reasoning may well be valid here as well since the inverse isotope effect is larger than that expected for inductive and steric reasons. Steric effects seem to be important in the enzymic reaction since the lack of enzymic catalysis for the elimination of 4-nitrophenol from the propyl adduct is best explained by a steric hindrance to assistance by the catalytic His at the active site of chymotrypsin. Therefore, a slight advantage of the less voluminous deuteria over the protia in the β-position may also be at play.

Secondary Reactions in Phosphonylated/Phosphorylated Serine Hydrolase Enzymes. Organophosphorus adducts of serine hydrolase enzymes as transition state analogues have been useful tools in mechanistic enzymology. Our long-standing interest in them is as active-site probes because of the presence of four ligands (five in transients) interacting with a large area of the active site. Secondary reactions investigated by our group are caused by the electrostatic and steric environment at the active site and conformational changes resulting from interactions between the phosphonate fragment and these components.

The results of these studies reveal that chymotrypsin provides base catalysis by His57 of the hydrolysis of the methylphosphonate diester of the enzyme with the better leaving group, 4-nitrophenolate ion, departing. The His57-catalyzed hydrolysis of the propyl analogue is not allowed for steric reasons, but it occurs with buffer catalysis (vide supra).[1a,35] Surprisingly, neither trypsin nor subtilisin BPN′ has the propensity for this catalytic assistance. A possible explanation is that His57 remains in the free base form under neutral pH to a greater extent, has lower p*K*, in covalently modified adducts of chymotrypsin than in trypsin and subtilisin BPN′. Inspection of models of covalently modified enzymes provides some ground for this speculation. The active sites are well solvated and suited for water attack. Chymotrypsin has a slightly narrower specificity pocket for binding one of the 4-nitrophenyl groups of the inhibitor than does trypsin. Trypsin also has a negatively charged residue, Asp189, in the specificity pocket that may repel the 4-nitrophenyl group with a high negative electron density and thus thwart departure of 4-nitrophenol from the diastereomer with P_S configuration. It is equally likely that adducts of trypsin and subtilisin BPN′, for electrostatic or steric reason, assume a conformation that does not lend support to water attack at phosphorus in the diesters. The latter idea may also be extended to explain a complete lack of an SN_1-type dealkylation of 2-(3,3-dimethylbutyl) methylphosphonofluoridate (soman)-inhibited trypsin.[16] The soman-inhibited trypsin adduct aged very slowly perhaps with P−O fission: The analogous adduct of chymotrypsin is also resistant to an SN_1-type reaction.[11,42] This circumstance stands in stark contrast to that observed with the cholinesterases. The narrow active site of AChE for instance can effectively sequester an inhibitor with the size of soman from water.[43] The negative electron density in the vicinity of the alkyl side chain together with the steric strain in cholinesterases enforces a methyl migration probably concerted with C−O bond cleavage.[16,44]

Conclusions

Inhibition of chymotrypsin and subtilisin BPN′ by bis(4-nitrophenyl) alkylphosphonates is very efficient and governed by electronic effects, whereas that of trypsin is more complex and probably governed by steric effects. Chymotrypsin is the most susceptible to inhibition by these phosphonate diesters among the three serine proteases studied. NMN with the small methyl group is better accommodated by chymotrypsin than NPN with the propyl group. The binding of the inhibitors at the active site of trypsin and subtilisin BPN′ must be different from chymotrypsin since the order of reactivity changes. Because the inactivation of trypsin is not efficient enough, nucleophilic sites other than the active-site residues can compete effectively for the electrophiles.

The elimination of 4-nitrophenolate ion from the 4-nitrophenyl methylphosphonyl adduct of chymotrypsin is 270 times faster than that from its propyl analogue due to enzyme catalysis of the former but not the latter. The catalyst is likely to be the active-site His: it removes a proton from the attacking water molecule with a solvent isotope effect of either 2.6 or 1.9 combined with a secondary isotope effect of 1.3 or solvation effect of 1.25. Steric restrictions imposed by the propyl group at the active site cause interference with the enzyme-assisted elimination of 4-nitrophenol from 4-nitrophenyl propylphosphonate ester of chymotrypsin. Steric effects and, even more importantly, loss of hyperconjugation at the transition state of the hydrolysis reaction can account for an inverse β-secondary deuterium (CD_3) isotope effect of 0.94 ± 0.2. Departure of a second equivalent of 4-nitrophenol from the active site of adducts of trypsin and subtilisin BPN′ is not observed.

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