Catalytic Recruitment in the Inactivation of Serine Proteases by Phosphonate Esters. Recruitment of Acid-Base Catalysis[1]

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Abstract: Two new inhibitors, bis(4-nitrophenyl) propylphosphonate (NPN) and methyl 4-nitrophenyl propylphosphonate (MPN), react irreversibly with the serine proteases bovine pancreatic α-chymotrypsin (EC 3.4.21.1), porcine pancreatic elastase (EC 3.4.21.11), and subtilisin (EC 3.4.21.3) (NPN also reacts with bovine pancreatic trypsin (EC 3.4.21.4)) to liberate 1 mol of 4-nitrophenol with production of an inactive enzyme. As with many other organophosphorus inhibitors, the inactivation reactions (presumed to involve phosphonylation of the active-site serine) are strongly accelerated by the enzyme. The catalytic acceleration factors of up to 10^8–10^9 are similar to the acceleration factors for acylation of the enzyme by natural substrates, showing that the inhibitors recruit essentially the entire catalytic power of the enzyme. The inhibitors react with the same pH/rate dependence as natural substrates and with solvent isotope effects k_{HOH}/k_{DOD} of 1.7–2.1, suggesting that the recruited catalytic power includes action of the acid–base catalytic entity of the active site. In all cases the dependence of the second-order rate constant for inactivation upon atom fraction of deuterium in the solvent (proton inventory) is linear, showing that a single protonic site generates the solvent isotope effect. Thus the recruited catalytic power does not include any potential of the enzyme for multisite protonic catalysis.

Organophosphorus compounds with good leaving groups such as fluoride or 4-nitrophenolate are powerful inhibitors of serine proteases, the good leaving group being displaced by the active-site serine hydroxyl group.[2] The resulting phosphorylated enzyme fails to undergo hydrolysis, and being itself catalytically inactive, the result is irreversible enzyme inhibition. Such inhibitors are used in research and in agriculture, for example, as pesticides, and are in some cases exceedingly toxic to human beings; the "nerve gases" fall into the latter category.[3]

These inhibitors are not simply reactive phosphorylating agents; they are instead quite specific for the active sites of serine hydrolases. This implies that the enzyme may itself be involved in stimulating its own inactivation, and information showing this to be true has long existed. The second-order rate constants k_i/K_i (M^{-1} s^{-1}) for reaction with acetylcholinesterase of a series of reagents $PO(OEt)_2X$, with X = F, $OPO(OEt)_2$, and OAr, were found by Aldridge and Davison[4] in 1952 to exhibit a linear free energy relationship (eq 1) with the (estimated) second-order rate

$$\log (k_i/K_i) = 14.5 + 1.5 \log k_w \quad (1)$$

$$\log [(k_i/K_i)/k_w] = 14.5 + 0.5 \log k_w \quad (2)$$

constants (M^{-1} s^{-1}) for neutral hydrolysis. This is readily recast as eq 2, where the left-hand side gives the catalytic acceleration factor. This equation emphasizes that very reactive phosphate reagents, such as tetraethyl pyrophosphate with $k_w \sim 10^{-6}$ M^{-1} s^{-1}, could have catalytic acceleration factors for phosphorylation of acetylcholinesterase greater than 10^{10}. This is essentially equal to the factor ($10^{10.2}$) by which acetylcholinesterase accelerates its own acetylation by the natural substrate acetylcholine.[5] These inhibitors may thus accurately be said to *recruit the catalytic power* of acetylcholinesterase in the phosphorylation reaction. The catalytic power is not similarly recrcuited in the dephosphorylation of the phosphoryl enzyme. The dephosphorylation is therefore slow, so that the enzyme is irreversibly inactivated. This combination of circumstances accounts for the inhibitory character of organophosphorus reagents in vitro and their toxicity in vivo.

The phenomenon of catalytic recruitment in serine hydrolases by organophosphorus reagents presents a biochemical puzzle. The tetrahedral reactant structure of the inhibitors does not resemble the trigonal reactant structure of the natural ester and amide substrates of the enzymes, nor does the expected quasi-trigonal-bipyramidal activated complex for displacement at phosphorus[6] obviously resemble the quasi-tetrahedral activated complex for carbonyl displacement in the substrates. It is therefore far from clear how the enzyme, evolved to stabilize the activated complex for acylation vs. the corresponding reactant state, can be activated by the inhibitor into stabilizing the phosphorylation activated complex vs. its reactant state.

In this paper, we report solvent isotope effect studies of the interaction of two phosphonate inhibitors with several serine proteases. The results bear on the question of how fully the inhibitors are able to recruit one particular aspect of the capacity of these enzymes, namely, their acid–base catalytic power. The overall solvent isotope effect (ratio of rates in H_2O and D_2O) provides a measure of the extent to which acid–base catalysis is important in determining the rate. Values of around 2–4 are commonly seen for hydrolytic enzymes, including those under study here, all of which have kinetically significant components of acid–base catalysis. In addition to the overall solvent isotope effects, proton inventories[7] are reported. These are measurements of the dependence of the rate constant on the atom fraction of deuterium in binary mixtures of H_2O and D_2O. The shape of the dependence gives information on the number of exchangeable protonic sites which are present in the transition state or reactant state and which contribute to the isotope effect. In the simplest case, when one site in the transition state produces the entire observed effect (as in simple acid–base catalysis in nonenzymic systems[8]), this dependence is linear (eq 3, where ϕ is the isotopic

$$k_n = k_0[(1 - n) + n\phi] \quad (3)$$

fractionation factor for the single site and n is the atom fraction

(1) This research was supported by the U.S. Army Medical Research and Development Command under contract no. DAMD-17-83-3199.

(2) Aldridge, W. N.; Reiner, E. *Enzyme Inhibitors as Substrates. Interactions of Esterases with Esters of Organophosphorus and Carbamic Acids*; American Elsevier: New York, 1972.

(3) (a) O'Brien, R. D. *Toxic Phosphorus Esters. Chemistry, Metabolism, and Biological Effects*; Academic; New York, 1960. (b) Heath, D. F. *Organophosphorus Poisons. Anticholinesterases and Related Compounds*; Pergamon: Oxford, 1961. (c) "Cholinesterase and Anticholinesterase Agents", *Handbuch der Experimentellen Pharmakologie*; Koelle, G. B., Ed.; Springer-Verlag: Berlin, 1963; Ergänzungswerk XV.

(4) Aldridge, W. N.; Davison, A. N. *Biochem. J.* **1952**, *52*, 663.

(5) Schowen, R. L. In *Transition States of Biochemical Processes*; Gandour, R. D., Schowen, R. L., Eds.; Plenum: New York, 1978; p 86.

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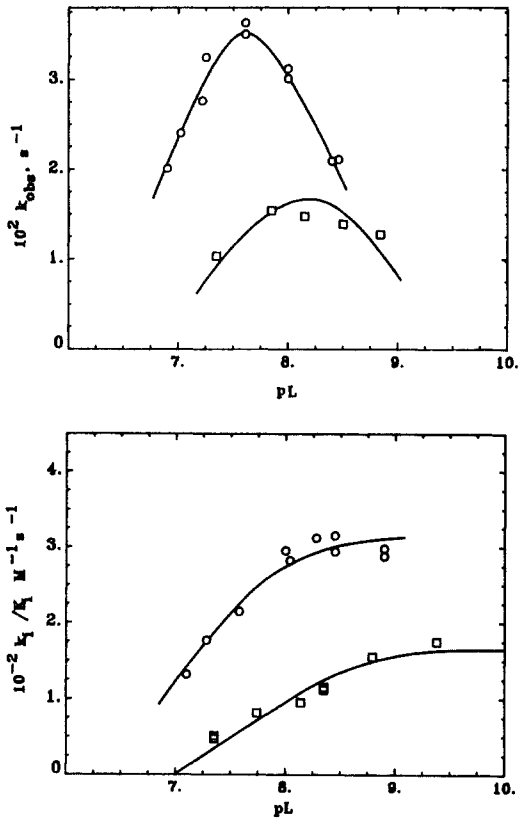

Figure 1. (a, top) pH(D) dependence of the inactivation rate constant for reaction of NPN with chymotrypsin. The upper curve is for H_2O, the lower for D_2O. The lines are calculated from the parameters of Table I. (b, bottom) Similar data for reaction of NPN with trypsin. Parameters are also in Table I.

of deuterium in the solvent). As more sites contribute, the dependence becomes nonlinear, with the rate constant being a polynomial in the atom fraction of deuterium. Roughly speaking, the order of the polynomial measures the number of sites; thus a quadratic dependence (eq 4) is characteristic of two sites.

$$k_n = k_0[(1 - n) + n\phi_1][(1 - n) + n\phi_2] \qquad (4)$$

Results

Measurements were carried out with two compounds, bis(4-nitrophenyl) propylphosphonate (NPN) and methyl 4-nitrophenyl propylphosphonate (MPN). The abbreviations adopted for the two compounds are meant to be general for compounds of the formula X—R(P=O)—Y, where Y is a leaving group, R a substituent attached to phosphorus, and X a second substituent, possibly also a leaving group. The abbreviations are of the form XRY, so that N = 4-nitrophenoxy, P = propyl, and M = methoxy.

Stoichiometry. Both reagents react at 25 °C and pH 7–8 with chymotrypsin, elastase, and subtilisin to generate 1 mol of 4-nitrophenol per mole of enzyme active sites. NPN also reacts similarly with trypsin; MPN shows no reaction with trypsin within 30 min.

The reactions of NPN, when either NPN or enzyme is in substantial excess, are strictly first order. MPN, which is a racemic mixture, shows biphasic kinetics with the first 0.5 equiv of 4-nitrophenol appearing several times more rapidly than the second 0.5 equiv. This is consistent with the expected enantioselectivity. A later report will give details. In the solvent isotope effect and proton-inventory studies for MPN, reported herein, initial rates were employed. The results, therefore, refer to the more reactive enantiomer.

pH and pD Dependence. Table I and Figure 1 show the dependence on pH in protium oxide and pD in deuterium oxide of the second-order rate constants for inactivation of chymotrypsin and trypsin by NPN. These data tend to confirm previous observations (by Douglas and Williams[9] for reaction of a phosphinate

Table I. pH(D) Dependences of the Second-Order Rate Constants for Inactivation of Serine Proteases by NPN at 25 °C

system	$(k_i/K_i)_{lim}$, $M^{-1}\ s^{-1}$ (SD)	10^8K_1 (SD)	10^9K_2 (SD)
chymotrypsin, 0.05 M phosphate buffers, HOH	4900 (400)	7.9 (1.4)	6.1 (1.4)
chymotrypsin, 0.05 M phosphate buffers, DOD	2300 (400)	(2.5)[a]	(1.9)[a]
trypsin, 0.05 M phosphate buffers, HOH	318 (8)	6.4 (0.9)	
trypsin, 0.05 M phosphate buffers, DOD	169 (10)	1.3 (0.3)	

[a] These values, corresponding to $pK_{HOH} - pK_{DOD} = 0.5$, were assumed.

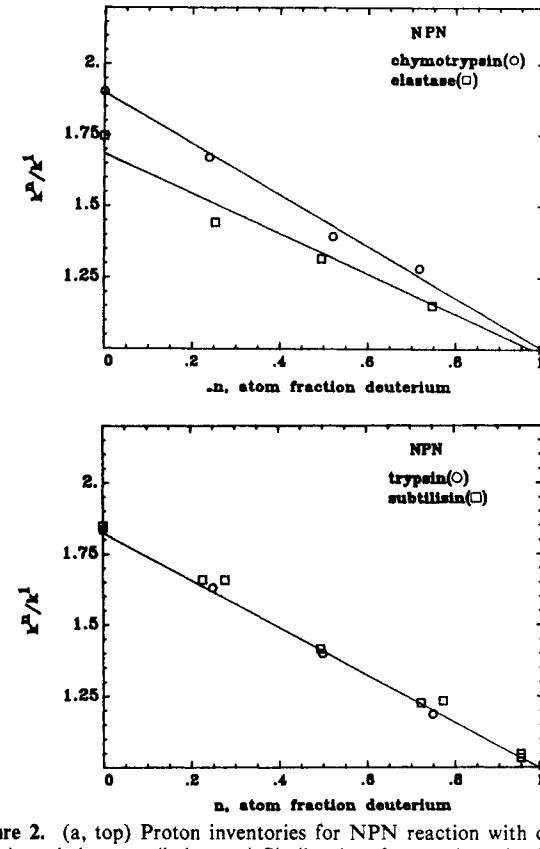

Figure 2. (a, top) Proton inventories for NPN reaction with chymotrypsin and elastase. (b, bottom) Similar data for trypsin and subtilisin. All curves are calculated from parameters given in Table II.

inactivator with chymotrypsin and by Demuth[10] for reaction of diisopropyl phosphonofluoridate with chymotrypsin, elastase, and subtilisin) that such inactivation rates exhibit the same pH (pD) dependence as does k_{cat}/K_m for specific substrates.[11,12] The reaction with chymotrypsin gives the characteristic bell-shaped curve[11a] and the reaction of trypsin a simple titration curve.[12] The pK values for the chymotrypsin reaction are 7.1 ± 0.1 and 8.2 ± 0.1 in H_2O. The values in D_2O are not well-defined, but the data are consistent with values greater by 0.5 unit. The pK values for trypsin are 7.2 ± 0.1 in H_2O and 7.9 ± 0.1 in D_2O. The values in D_2O are thus shifted to the basic side from the pK in H_2O as is expected for "normal" behavior of the enzyme.[7] The magnitude of the shift, ΔpK, is 0.7 ± 0.2 for trypsin, which is slightly large

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(10) Demuth, H.-U., unpublished results.
(11) (a) Chymotrypsin: Bender, M. L.; Clement, G. E.; Kezdy, F. J.; Heck, H. D'A *J. Am. Chem. Soc.* **1964**, *86*, 3680. (b) Elastase: Stein, R. L. *J. Am. Chem. Soc.* **1983**, *105*, 5111. (c) Subtilisin: Philipp, M.; Bender, M. L. *Mol. Cell. Biochem.* **1983**, *51*, 5.
(12) Spomer, W. E.; Wootton, J. F. *Biochim. Biophys. Acta* **1971**, *235*, 164.

Table II. Second-Order Rate Constants and Solvent Isotope Effect Information for Inactivation of Serine Proteases by NPN at 25 °C and Appropriate[a] pL

enzyme (pH), k_{HOH}/k_{DOD}	$(k_i/K_i)_{HOH}$, M^{-1} s^{-1} (SD)	$(k_i/K_i)_{DOD}$, M^{-1} s^{-1} (SD)	ϕ_1 (SD)	ϕ_2 (SD)
chymotrypsin (7.45), 1.92 ± 0.06	2260 (59)	1178 (17)	0.57 (0.06)	0.93 (0.09)
elastase (8.45), 1.65 ± 0.05	1840 (50)	1120 (20)	0.77 (1.03)	0.66 (1.15)
trypsin (8.48), 1.83 ± 0.06	264 (8)	145 (3)	0.58 (0.04)	0.93 (0.05)
subtilisin (8.40), 1.85 ± 0.07	1330 (49)	721 (20)	0.58 (0.02)	0.94 (0.02)

[a] For chymotrypsin, a value approximating the pH of maximum rate (actually 7.65 ± 0.10) was used. For other enzymes, a pH in the pH-independent region was chosen. Corresponding pL in mixtures of HOH and DOD was achieved by employment of constant buffer ratios.

Table III. Second-Order Rate Constants and Solvent Isotope Effect Information for Inactivation of Serine Proteases by MPN at 25 °C and Appropriate[a] pL

enzyme (pH), k_{HOH}/k_{DOD}	$(k_i/K_i)_{HOH}$, M^{-1} s^{-1} (SD)	$(k_i/K_i)_{DOD}$, M^{-1} s^{-1} (SD)	ϕ_1 (SD)	ϕ_2 (SD)
chymotrypsin (6.90), 2.12 ± 0.05	0.870 (0.016)	0.410 (0.005)	0.40 (0.02)	1.17 (0.05)
elastase (8.43), 2.01 ± 0.15	3.19 (0.20)	1.59 (0.08)	0.51 (0.07)	0.97 (0.10)
trypsin (8.50)	*b*	*b*	*b*	*b*
subtilisin (8.40), 2.06 ± 0.05	2.57 (0.01)	1.24 (0.02)	0.50 (0.08)	0.96 (0.13)

[a] For chymotrypsin, a value approximating the pH of maximum rate was used. For other enzymes, a pH in the pH-independent region was chosen. Corresponding pL in mixtures of HOH and DOD was achieved by employment of constant buffer ratios. [b] No reaction observed ($k_i/K_i < 0.1$ M^{-1} s^{-1}).

but near the normal value[7] of 0.5.

Proton Inventories for NPN. In Table II and Figure 2 the solvent isotope effects and proton inventories for the reactions of NPN with chymotrypsin, elastase, trypsin, and subtilisin are presented. The solvent isotope effects are 1.7–1.9 for all four enzymes. As Figure 2 shows, the data in mixtures of H_2O and D_2O are quantitatively consistent with a linear dependence on n in each case. The lines shown are best-fit, least-squares straight lines. The values of ϕ_1 and ϕ_2 given in Table II are obtained by fitting the data to eq 4 above, which assumes that two contributions (rather than one as implied by the linear dependences) determine the solvent isotope effect. For chymotrypsin, trypsin, and subtilisin, the one-contribution hypothesis is confirmed: the value of the second contribution (ϕ_2) is estimated as 0.93, 0.93, and 0.94, respectively, or essentially unity. By thus demonstrating that even when a two-site model is used the entire isotope effect appears at one site and ϕ for the second site is calculated as unity or nearly unity, we confirm the one-site character of the isotope effect. For elastase, the two-contribution fit does not yield $\phi_2 \sim 1$. Instead, both fractionation factors are calculated to have large errors. In this case, then, we cannot be sure whether the entire effect arises from one site. The data lead to no definite conclusion for elastase on the number of protonic sites which contribute to the isotope effect.

Proton Inventories for MPN. Table III and Figure 3 give the solvent isotope effects and proton inventories for the reactions of chymotrypsin, elastase, and subtilisin with MPN; no reaction of this compound with trypsin was observed. The solvent isotope effects are 2.0–2.1. The proton-inventory plots exhibit a linear form and are well accounted for by the best-fit, least-squares straight lines drawn in Figure 3. If, as with NPN, we allow for the possibility of a two-contribution dependence by fitting the data to eq 4, one of the fractionation factors always emerges as very close to unity (1.17, 0.97, and 0.96). The linear character of the proton inventory and thus the one-site origin of the isotope effect for chymotrypsin and subtilisin are thus confirmed. The larger errors in the data for elastase preclude a definite conclusion for it.

Nonenzymic Hydrolysis. MPN undergoes hydrolysis at 25 °C in 0.05 M phosphate buffer (0.043 M KH_2PO_4, 0.007 M K_2HPO_4; pH 7.70) with a first-order rate constant of 9.5 ± 0.1 × 10^{-6} s^{-1}. NPN liberates the first mole of 4-nitrophenol under the same conditions with a first-order rate constant of 9.1 ± 0.1 × 10^{-5} s^{-1}.

Discussion

Kinetics and Stoichiometry. In our kinetic studies of the inactivation of these four serine proteases by NPN and MPN, no departure from second-order kinetics was observed. Up to the highest inactivator concentrations employed by us (around 10 μM), there was therefore no sign of saturation through the formation of enzyme–reagent complexes. Although these complexes probably form, their dissociation constants apparently exceed about 20–100 μM.

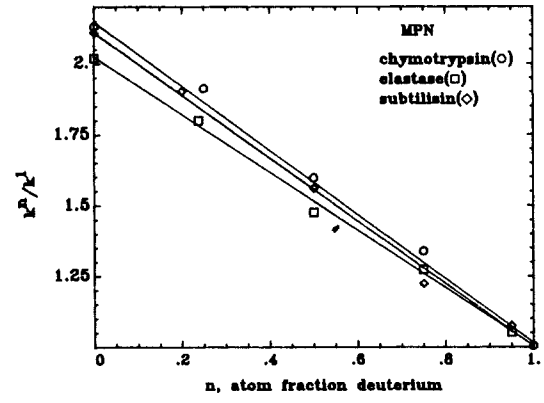

Figure 3. Proton inventories for MPN reaction with chymotrypsin, elastase, and subtilisin. Parameters are in Table III.

Both inhibitors liberated 1 mol of 4-nitrophenol per mole of enzyme active sites upon inactivation of the enzymes. This is consistent with the view that phosphonylation of only the active-site serine (and not other functional groups on the enzyme) is occurring with both reagents.

For NPN, liberation of a second mole of 4-nitrophenol, after the initial phosphonylation, occurs relatively slowly, if at all. Liberation of a second leaving group, when one is present in the derivatized enzyme, is known for other reagents[13,14] and is called "aging". Aging thus appears to be slow for 4-nitrophenyl propylphosphonyl enzymes ("NP-enzymes").

The biphasic kinetics observed for MPN indicate, as already noted, the expected enantioselectivity. Current work is expected to clarify which isomer is reacting more rapidly and the relative rates for the two isomers, but we do not yet have this information. The isotope effects discussed below, as already mentioned, were determined from initial rates. They refer, therefore, to the more reactive enantiomer.

Specificity. Some specificity is observed in these reactions: each reagent discriminates among the four enzymes, and each enzyme reacts differentially with the two reagents. Because the structures of these enzymes and the interactions responsible for their substrate specificity are so well understood, some conclusions should be

(13) Ooms, A. J. J.; van Dijk, C. *Biochem. Pharmacol.* **1966**, *15*, 1361.
(14) Bender, M. L.; Wedler, F. C. *J. Am. Chem. Soc.* **1972**, *94*, 2101.

possible about the origins of the rate differences.

For example, the presence of the second aryloxy group in NPN (as opposed to the methoxy group in MPN) is obviously advantageous: NPN reacts with every enzyme at least 400-fold more rapidly than MPN. This is not a simple electronic effect. In nonenzymic hydrolysis, NPN shows a rate constant only 10-fold larger than for MPN.

For chymotrypsin, trypsin, and subtilisin, a logical hypothesis for the greater reactivity of NPN would have been that the second aromatic group inserted itself into the "pocket" that accommodates a relatively large C_α side chain of the natural substrate for each of these enzymes.[15] However, this hypothesis is excluded by the large rate constant for elastase: only a small pocket is present in the active site of elastase,[15] not capable of accepting a 4-nitrophenyl group. Thus, binding into the C_α "specificity pocket" cannot explain the preference for NPN over MPN, if it is assumed that all four enzymes employ a common binding mode for the reagents.

An alternative hypothesis we favor is that the second aryloxy group binds to one of the *N*-acyl binding sites, which normally accommodate the amide linkages of residues along the "polypeptide tail" of the natural substrates.[15] The rate factor of 400 or more between NPN and MPN would then arise from the loss of the corresponding transition-state binding energy of 3.6 kcal/mol or more when the 4-nitrophenyl group of NPN is replaced by the methyl group of MPN. It may further be suggested that it is the propyl group that occupies the C_α side-chain pocket. This is clearly sterically possible with chymotrypsin, trypsin, and subtilisin, which have large pockets. It may be possible with elastase to orient the propyl group so that it lies in the depression which customarily accommodates the C_α methyl group of an alanine residue. This could be done by placing either the α or the β CH_2 unit in the depression and orienting the "leftover" ethyl or methyl group away from the enzyme.

If this hypothesis is correct, then NPN could bind to the enzymes in either of two orientations. In both orientations, the propyl group would occupy the C_α side-chain pocket in the "southeast" of the active site.[16] An aryloxy group would occupy an *N*-acyl site along the "southwest" edge of the active site. The other two phosphorus ligands (the oxygen atom and the second aryloxy group) would then have two potential orientations: (a) the oxygen toward the enzyme, near the oxy anion hole, and the aryloxy away from the enzyme, extending into the solution, and (b) the reverse orientation, with the oxygen directed toward the solution and the aryloxy toward the enzyme.

Obviously, orientation a can readily lead to backside displacement of aryl oxide by the active-site serine, possibly with stabilization of $P{=}O^{\delta-}$ in the oxy anion hole and general-base catalysis of serine attack by the active-site histidine. Orientation b should be nonproductive, and its formation should also be sterically retarded by interference between active-site groups and the inwardly directed aryloxy. The tentative conclusion is then that NPN will tend to bind with orientation a and undergo backside, in-line displacement[6] to generate the NP-enzyme. An adjacent mechanism would appear more difficult to accommodate but is by no means rigorously excluded.

In the case of MPN, the propyl group and the aryloxy group might be expected to take up the same preferred positions, in the C_α pocket and the *N*-acyl site, respectively, as with NPN. The oxygen atom and the methoxy group would then have two potential orientations, but *neither* should be a productive orientation. Displacement of methoxy by serine should be quite difficult; the enzyme can assist nucleophilic attack by general-base catalysis, but with the leaving group directed into the solution, there is no way of assisting departure of the strongly basic methoxide leaving group. With NPN, the much better aryloxide leaving group can depart without assistance. What is a productive complex for NPN becomes, therefore, an unproductive complex for MPN.

For MPN to react, it must interchange the aryloxy and methoxy ligands. The methoxy ligand will now occupy the *N*-acyl binding site, apparently with less affinity than aryloxy. The lower affinity, which corresponds to smaller transition-state stabilization, would then account for part or all of the rate differences between MPN and NPN.

This hypothesis about the structure of the inactivation transition state is certainly speculative, but it leads to a prediction and an explanation. It predicts the direction of enantioselectivity in inactivation by MPN, and it explains the failure of the NP-enzyme to undergo aging.

The reactive binding mode postulated for MPN, which projects the aryloxide leaving group away from the enzyme surface, dictates that the molecule with R_P configuration should react more rapidly. We, therefore, make this prediction, which we are currently testing.

The reluctance of the NP-enzyme to age is explained if the second 4-nitrophenyl group remains bound to the *N*-acyl binding site in the NP enzyme following displacement of the first 4-nitrophenolate by the serine hydroxyl. If the attack of water on this structure were to occur in an in-line mechanism,[6] general-base catalysis by the active-site histidine would be impossible. The water would attack from the fact opposite the 4-nitrophenolate leaving group; the histidine is near the face opposite the propyl group. Aging should, therefore, occur only at a rate corresponding to uncatalyzed hydrolysis, and this is expected to be very low. A fluxional interchange of the phosphorus ligands among the binding sites will not affect this argument as long as the major population is that postulated. However, whether any aspect of the proposed model is valid remains to be seen.

Recruitment of Acid–Base Catalytic Machinery. The solvent isotope effect and proton-inventory results for all systems studied are essentially the same: the overall solvent isotope effects are in the range 1.7–2.1, and the proton inventories are linear (except for elastase, where they are not adequately defined), indicating that the solvent isotope effect arises from a single transition-state site.[7] This is exactly the result which is obtaned with serine proteases and substrates which do not adequately simulate the natural polypeptide substrates: an overall solvent isotope effect around 2 in magnitude, produced by a single site in the transition state.[7]

With both truncated peptide-analogue substrates, which give one-site catalysis,[7] and the inactivators NPN and MPN, the most straightforward assumption is that the single site involved in acid–base catalysis is the hydrogen bridge between the active-site serine hydroxyl and the imidazole nitrogen of the active-site histidine. This component of the catalytic power of the enzyme has, therefore, been recruited by the organophosphorus reagents. The likelihood of this interpretation is further increased by the dependence of the rate on what is apparently the histidinium ionization, as seen in the pH/rate profiles.

On the other hand, the inhibitors have not recruited the multiproton catalytic capability of the enzyme, seen with good simulators of the natural substrates.[7] Oligopeptides of sufficient length usually "couple" the acid–base catalytic entity of the serine proteases to produce multiproton catalysis and therewith nonlinear ("bowl-shaped") proton inventories. Since the inhibitors, even in the cases with the greatest rates, give linear proton inventories, the multiproton component of the catalytic apparatus remains unrecruited.

The interaction of the P=O function in the transition state with the oxy anion hole is a readily conceived form of catalytic recruitment. If this is a feature of recruitment by NPN and MPN, the simplest view is that it generates no solvent isotope effects. The recruitment by these inhibitors is extensive, their inhibition rate constants being 10^{8-9}-fold larger than their spontaneous-hydrolysis rate constants. This is a factor similar to the catalytic acceleration of serine proteases with their natural substrates.[5]

Experimental Section

Materials. Inorganic salts, buffer components, and preparative reagents were reagent grade chemicals, which were used as purchased or dried, recrystallized, or distilled as necessary. Protium oxide was distilled, deionized, and degassed with nitrogen. Deuterium oxide (Norell Inc.,

(15) Fersht, A. *Enzyme Structure and Mechanism*, 2nd ed.; Freeman; San Francisco, 1985; Chapter 1.

(16) See the portrayal of the chymotrypsin active site in ref 5, p 375.

Table IV. Stoichiometry of *p*-Nitrophenol (PNP) Release during Inactivation of Serine Proteases by MPN

enzyme	10^5(active sites), M[a]	10^5NPN, M	10^5PNP, M[b] (%)	10^5(active sites remaining), M[c]
chymotrypsin	129 ± 5	7.22 ± 0.01	6.71 ± 0.3 (93%)	123 ± 5
elastase	5.00 ± 0.10	10.2 ± 0.1	5.0 ± 0.5 (100%)	0.77 ± 0.07
subtilisin	4.00 ± 0.20	10.0 ± 0.01	4.08 ± 0.2 (102%)	0.01 ± 0.01

[a] Titration with 7×10^{-4} M 4-nitrophenyl acetate, average of results from burst and rate assays. [b] Release in 15–23 h (PNP concentration as percent of the limiting reactant). [c] From active-site titration at the end of the inactivation experiment.

Table V. Stoichiometry of *p*-Nitrophenol (PNP) Release during Inactivation of Serine Proteases by NPN

enzyme	10^6(active sites), M[a]	10^6NPN, M	10^6PNP, M (%)[b]	10^6(active sites remaining), M[d]
chymotrypsin	4.4 ± 0.1	6.12 ± 0.01	4.3 ± 0.5 (98)	
	8.8 ± 0.2	61.25 ± 0.5	8.2 ± 0.2 (93)	
	8.8 ± 0.2	12.25 ± 0.2	8.64 ± 0.4 (98)	0.1 ± 0.1
	7.36 ± 0.2	12.25 ± 0.2	7.14 ± 0.2 (97)	0.1 ± 0.1
trypsin[c]	5.75 ± 0.2	12.25 ± 0.2	6.15 ± 0.1 (107)	0.1 ± 0.1
	5.75 ± 0.2	12.25 ± 0.2	6.90 ± 0.1 (120)	0.1 ± 0.1
elastase	5.00 ± 0.10	12.25 ± 0.2	5.6 ± 0.5 (112)	0.1 ± 0.1
	5.00 ± 0.10	12.25 ± 0.2	5.5 ± 0.5 (110)	0.1 ± 0.1
subtilisin	40.0 ± 2.0	61.25 ± 0.5	41.0 ± 0.4 (102)	0.1 ± 0.1
	4.0 ± 0.2	12.25 ± 0.2	4.6 ± 0.6 (115)	0.1 ± 0.1

[a] Titration with 7×10^{-4} M 4-nitrophenyl acetate, average of results from burst and steady-state assays in duplicate or triplicate. [b] Release in <20 min (PNP concentration as percent of the limiting reactant). [c] Titration with 1.9×10^{-4} M 4-nitrophenyl 4′-guanidinobenzoate hydrochloride. [d] Result of an attempted active-site titration of the inhibited enzyme.

99.9% deuterium) was distilled (100–102 °C fraction) and stored under nitrogen. Acetonitrile for stock solutions of kinetic measurements was purified by distillation from drying agents.[17]

Substrates. *N*-Succinyl-Ala-Ala-Ala-*p*-nitroanilide was purchased from Sigma Chemical Co. Glu-Leu-Phe-*p*-nitroanilide was a gift from Prof. A. Barth and Dr. H-U. Demuth.

Methyl 4-Nitrophenyl Propylphosphonate (MPN). The known compounds potassium 4-nitrophenolate and methyl propylphosphonochloridate were prepared and purified as previously reported.[18] Coupling of the two reagents was effected by dropwise addition of a solution of 1.0 mmol of methyl propylphosphonochloridate in 3 mL of dry benzene (0.4 mmol of propylphosphonodichloridate was present as an impurity) to a suspension of 2 mmol of potassium 4-nitrophenolate in 5 mL of benzene under constant stirring at room temperature. The reaction progress was monitored on TLC. After a few days the supernatant solution was washed with benzene, the KCl was filtered out, and the benzene was removed at reduced pressure. The products were taken up in 15 mL of dry methanol, and the insoluble portion was filtered to give a white powder, mp 132–140 °C (MPN).

The methanol was removed from the filtrate, and a yellow oil was obtained, which separated into the desired product and contaminants on TLC. The oil was placed on preparative TLC and double developed until complete isolation of MPN from other contaminants was achieved. [TLC: (1) ethyl acetate:cyclohexane = 3:2, (2) ethyl acetate:pentane = 3:2. R_f: 4-nitrophenol 0.74, NPN 0.64, MPN 0.42.] The MPN product (ca. 70% of theoretical) was scraped off the plate and eluted with ethyl acetate. Hydrolysis in 1 M NaOH produced 1.05 ± 0.02 equiv of 4-nitrophenolate as monitored spectroscopically at 400 nm: NMR (CCl_4, 2% Me_4Si) δ 0.8 (t, 3 H, CH_3), 1.1–2.0 (m, 4 H, CH_2CH_2), 3.6 (d, 3 H, OCH_3, J(P–H) = 11 Hz), 7.15 (d, 2 H, Ar(1,6), $J_{2,3}$ = 5 Hz), 8.0 (d, 2 H, Ar(3,5), $J_{5,6}$ = 5 Hz); MS (70 eV), *m/e* (relative intensity) 259 (14.4), 244 (13.9), 139 (56.3), 79 (100e, 43 (96.1). Anal. Calcd for $C_{10}H_{14}NO_5P$: C, 46.33; H, 5.41; N, 5.41. Found: C, 46.70; H, 5.56; N, 5.50.

Bis(4-nitrophenyl) Propylphosphonate (NPN). Potassium 4-nitrophenolate (see above) and propylphosphonodichloridate (Aldrich) were coupled in stoichiometric amounts in benzene as described above. The crude product was filtered out of methanol (mp 132–140 °C) and recrystallized from ethyl acetate–pentane (nearly quantitative yield). Basic hydrolysis of an aliquot yielded 2 equiv of 4-nitrophenolate ion (400 nm) in 102 ± 1% yields: mp 132–133 °C; NMR (CCl_4, 2% Me_4Si) δ 0.8 (t, 3 H, CH_3), 1.1–2.0 (m, 4 H, CH_2CH_2, J(P–H) = 11 Hz), 7.15 (d, 2 H, Ar(1,6), $J_{2,3}$ = 5 Hz), 8.0 (d, 2 H, Ar(3,5), $J_{5,6}$ = 5 Hz); MS (70 eV), *m/e* (relative intensity) 366 (15.1), 228 (4.9), 186 (17.6), 139 (27.1), 43 (100). Anal. Calcd for $C_{15}H_{15}N_2O_7P$: C, 49.18; H, 4.10; N, 7.65. Found: C, 49.38; H, 4.48; N, 7.52.

Enzymes. Bovine pancreatic α-chymotrypsin (EC 3.4.21.1) was obtained from Sigma Chemical Co. as a salt-free, lyophilized, 3-times recrystallized powder with an activity of 52 BTEE units/mg. Bovine pancreatic trypsin (EC 3.4.21.4) from Sigma Chemical Co., a dialyzed, recrystallized, salt-free powder with an activity of 7500 BAEE units/mg, was treated by storing buffered aqueous solutions at room temperature for at least 2 h to obtain autolytically stable solutions. Elastase (Pancreatopeptidase E, EC 3.4.21.11, from hog pancreas, a chromatographically purified, lyophilized, water-soluble powder with an activity of 90 elastin units/mg) and subtilisin (Protease Nagarse, EC 3.4.21.3, from *Bacillus amyloliquefaciens*, crystallized and lyophilized with an activity of 10 tyrosin units/mg) were both obtained from Sigma Chemical Co.

Kinetic Procedures. All reaction rates were monitored spectrophotometrically either by the direct release of 4-nitrophenol at 400 nm or, for fast inhibition, with a good, competitive, chromogenic substrate. Substrate solutions were prepared in 10^{-3} M stock solutions with 10% acetone or acetonitrile cosolvent at pH <7.0 (NaOH). Inhibitor solutions were freshly prepared in methanol for each experiment. An aliquot was hydrolyzed to 1 equiv of 4-nitrophenol for MPN and to 2 equiv of 4-nitrophenol for NPN to determine the actual concentration spectroscopically at 400 nm.

Inhibition by MPN. Direct measurements of the inhibition of chymotrypsin, elastase, and subtilisin by MPN were conducted under zero-order conditions, and the data were fit to a linear dependence on time by linear least squares. Second-order rate constants were calculated from $k_i/K_i = (\Delta A/\Delta t)/[E_0][I]\Delta\epsilon$, where $[E_0]$ = initial concentration of the enzyme, $[I_0]$ = initial concentration of the inhibitor, and $\Delta\epsilon$ (400 nm) = $6.33 \times 10/^3$ M^{-1} cm^{-1} at pH 6.90 and 17.40×10^3 M^{-1} cm^{-1} at pH 8.40.

Inhibition by NPN. Inhibition by NPN was studied in the presence of 5×10^{-4}–5×10^{-5} M Glu-Leu-Phe-*p*-nitroanilide with chymotrypsin and subtilisin proteinase and in the presence of *N*-succinyl-Ala-Ala-Ala-*p*-nitroanilide with elastase, all at 390 nm. An aliquot of the substrate solutions was also hydrolyzed to 4-nitroaniline and the concentration determined at 390 nm (ε 11 520). The inhibitor was held in 30–1000-fold excess over the enzyme concentration to obtain a sufficiently large change in absorbance at less than 10% decomposition of the substrate. In a typical run, buffer and substrate were thermally equilibrated in a volume of ~900 μL in a cell, and the background absorbance was recorded. Then the inhibitor was introduced in a volume of <50 μL, the absorbance was checked, and finally, the reaction was initiated by the introduction of 20–50-μL stock solutions of the enzyme. The total concentration of cosolvents was kept constant at <5% (mostly MeOH), any decomposition in the substrate or inhibitor was monitored, and the results were discarded or corrected as necessary.

First-order rate constants were obtained from a nonlinear least-squares fit of 500–1000 absorbance/time coordinates. Substrate-independent, second-order inhibition constants were calculated, as in previous re-

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ports,[19,20] from $1/k_{obsd} = 1/k_i + \{(K_i/k_i)/[I]\}\{1 + [S]/K_m\}$. Extrapolation of $1/k_{obsd}$ to [S] = 0 by linear least squares gives $(K_i/k_i)/[I]$ since $1/k_i$ is negligible in our experiments.

In experiments with trypsin, an excess of >20-fold in enzyme over NPN was used. Liberation of 4-nitrophenol was first order. Division of k_{obsd} by the enzyme concentration yielded k_i/K_i.

Stoichiometry. These measurements were conducted at the pH optimum for each enzyme in the buffer systems used for kinetic studies and are summarized in Tables IV and V for MPN and NPN, respectively. Aliquots of the methanolic stock solutions of the inhibitors were injected into preequilibrated buffer solutions followed by the injection of the enzymes from stock solutions. Release of 4-nitrophenol, monitored at 400 nm, leveled off within 20 min with NPN and within 15-23 h with MPN. Remaining enzyme activities were measured after these times by the addition of the appropriate reagents to the reaction mixture.

Enzyme-active-site molarities of stock enzyme solutions and inhibited enzyme solutions were determined according to Bender et al.[21] for chymotrypsin, elastase, and subtilisin. To 940 μL of a buffer solution was added 10 μL of a stock solution of 4-nitrophenyl acetate (stock solution, 70 mM; final concentration after enzyme addition, 0.7 mM). The absorbance was recorded for several minutes to determine the nonenzymic rate. A 50-μL aliquot enzyme stock solution was then added and the absorbance monitored through the burst phase and into the steady-state reaction. Active-site molarities were calculated both from the burst amplitude and the enzyme-dependent part of the steady-state rate, the average values being taken. Experiments were done in duplicate or triplicate.

The active-site molarity for trypsin was determined[22] from the burst phase of the hydrolysis of 4-nitrophenyl 4'-guanidinobenzoate hydrochloride (Sigma Chemical Co.).

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Oxygen Transfer Involving Non-Heme Iron. The Reaction of (EDTA)Fe^{III} with *m*-Chloroperbenzoic Acid

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Abstract: Oxygen transfer from percarboxylic acids to (ethylenediaminetetraacetato)iron(III) ((EDTA)Fe^{III}) has been investigated (solvent CH_3OH; *T* 30 °C), using 2,4,6-tri-*tert*-butylphenol (TBPH) as a trapping reagent for the reactive iron-oxo intermediate formed upon oxygen transfer. In the presence of TBPH the percarboxylic acid peroxo bond undergoes 100% heterolytic cleavage during oxygen transfer to (EDTA)Fe^{III}, as demonstrated with phenylperacetic acid. This requires the reaction to involve the transfer of an oxene or oxygen atom to the iron(III) moiety of the (EDTA)Fe^{III} catalyst to form what may be formally written as (EDTA)$Fe^{V}O$. Since the so-constituted oxidant oxidized $PhCH_2COH$ by $1e^-$ transfer to provide $(PhCH_2)_2$ at a rate greater than the oxidation of carboxylate ligand, the electron deficiency of the oxidant must reside at the oxygen to a greater extent than on iron. The structure of the oxidant is, therefore, best represented as something between (EDTA)$Fe^{V}O$ and one in which the iron(III) species stabilizes the oxygen atom by d-orbital overlap ((EDTA)$Fe^{III}O$) species. The kinetics of the reaction of a percarboxylic acid with (EDTA)Fe^{III} were studied by using *m*-chloroperbenzoic acid (MCPBA). The reaction was monitored by following the appearance of 2,4,6-tri-*tert*-butylphenoxyl radical (TBP$^•$). Oxygen transfer was shown to be rate limiting from the first-order dependence upon [MCPBA] and zero-order dependence upon [TBPH]. At constant [MCPBA] and [TBPH], the pseudo-first-order rate constants, k_{obsd}, for the appearance of TBP$^•$ increases linearly with increase in [(EDTA)Fe^{III}] and reaches a limiting value at high (EDTA)Fe^{III} concentrations. Oxidation of TBPH to TBP$^•$ is shown to compete with methanol solvent oxidation to formaldehyde and the oxidative coupling of MCPBA with TBPH to provide a peroxidic component, OXO. The percentage yields of TBP$^•$, CH_2O, and OXO are independent of the concentration of (EDTA)Fe^{III}, provided the concentrations of MCPBA and TBPH are kept constant. However, increasing the initial concentration of MCPBA increases the concentration of OXO, and, correspondingly, the yield of the other two products decreases. Similarly, the yield of phenoxyl radical increases with increase in [TBPH]. The initial addition of formaldehyde retards the rate of the oxidation reactions. A kinetically competent series of reactions which allow the calculation of experimental values of k_{obsd} and the yields of products have been arrived at by computer simulation (Scheme III). Comparison of the second-order rate constants (in methanol) for oxygen transfer from MCPBA to (tetraphenylporphyrinato)iron(III) chloride ((TPP)$Fe^{III}Cl$) and to (EDTA)Fe^{III} establishes the former to be but 45-fold a better acceptor.

Iron complexes, both heme and non-heme, play important roles as catalysts in biochemical oxidation reactions at the level of ligand-Fe(III) + 2e- + O_2. Among the (protoporphyrin IX)-Fe(III) enzymes are the peroxidases,[1] which carry out 1e- oxidations when charged by hydroperoxide, and cytochrome P-450 enzymes,[2] which perform substrate oxygen chemistry when charged by ordered combination with 1e-, O_2, and 1e-. Non-heme iron plays an important but, as yet, poorly understood role in the mechanism of the pterin-requiring mammalian phenylalanine hydroxylase[3] and in a class of α-ketoglutarate-requiring enzymes (prolyl, lysyl, etc. and hydroxylases[4]) which perform oxygen insertions into C-H bonds (much as in the case of some cytochrome P-450 reactions).

Studies from this laboratory have recently been directed to the oxygen-transfer chemistry of (*meso*-tetraphenylporphinato)-metal(III) chlorides ((TPP)$M^{III}Cl$).[5] The second-order rate

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