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Inhibition and Labeling of Enzymes and Abzymes by Phosphonate Diesters

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Abstract

Reactive phosphonate diesters were designed and prepared as inhibitors of serine proteases and esterases. Inactivation of trypsin, chymotrypsin, and butyrylcholinesterase was determined by residual enzymatic activity as well as by the release of a chromogenic or fluorogenic product of the inhibition reaction. Second-order rate constants were determined from rates of nitrophenol formation. Application of the reaction for active-site titration of enzyme preparations is demonstrated. A basic functional group present in the nitrophenyl tropane phosphonate diester was shown to confer selectivity for inactivation of trypsin and chymotrypsin. Biotinylated derivatives of the phosphonate diesters were prepared to permit analysis of proteins modified in the inhibition reaction. Labeled polypeptides were resolved by SDS-PAGE, electroblotted, and detected by streptavidin-peroxidase staining. A detection limit of less than 4 ng, corresponding to 20 n*M* of trypsin, was demonstrated. Pretreatment of enzymes with DFP or nonbiotinylated phosphonates specifically blocks the labeling. This technique permits identification of serine proteases in complex mixtures with good sensitivity and specificity.

Introduction

Serine hydrolases participate in numerous biological processes ranging from digestion to immune responses. Substrate specificities of these enzymes can vary widely, and may define their functional niche in nature. Trypsin-like proteases cleave polypeptides at basic residues and with little discrimination for sequence or context. Other, more specialized, proteases have restricted hydrolytic activity toward a peptide bond within a specific

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peptide or protein sequence. Despite their wide range of functions, these enzymes utilize a common mechanism characterized by covalent chemical catalysis in peptide- or ester-bond cleavage. Recent evidence suggests that both naturally occurring and artificially derived catalytic antibodies with peptidase or esterase activities may also employ a mechanism analogous to that of the serine hydrolases *(1–3)*.

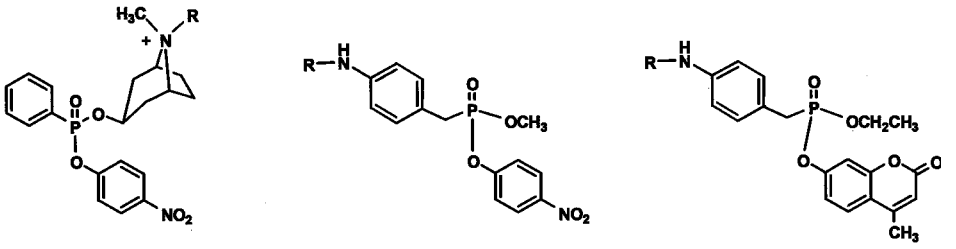
The ability to discriminate among numerous peptide bonds within a polypeptide substrate suggests targeted reactivity of the enzyme's catalytic groups. In effect, the protein structure can exert significant control over the chemical potential of a nucleophilic serine residue at the enzyme-active site. It is generally assumed that extended binding interactions between enzyme and substrate are responsible for channeling specificity and reactivity of highly specific serine proteases. Recognition of conformational determinants or tertiary structure of a protein substrate by the enzyme could further restrict proteolytic activity.

The enhanced reactivity of the catalytic serine hydroxyl group at the enzyme-active site is commonly manifested in reactions with a variety of small unnatural substrates and inhibitors. Such reagents include amides or esters, as well as phosphoryl and sulfonyl halides. Unlike the reaction with acyl substrates, phosphoryl and sulfonyl transfer to this site is generally irreversible, and leads to permanent inactivation of the enzyme. The prototypical reagent DFP contains a highly reactive fluorophosphate moiety, which reacts indiscriminately with a broad range of serine proteases and esterases. Selectivity can be conferred through chemical modifications that effectively modulate reactivity *(4–6)*. Recently, a renewed effort has been made to fine-tune the inhibitory activity of phosphonate esters through structural designs that exploit specific binding interactions approximating those between the enzyme and its natural substrates *(7–10)*. Furthermore, attenuating reactivity of the phosphorus center by the choice of ester substituent can confer stability against nonspecific phosphonylation of protein residues and against spontaneous hydrolysis of the reagent. The combination of these two strategies has been useful in the design of selective and potent inhibitors that target defined serine hydrolases *(10–12)*. The formation of a covalent adduct with the enzyme has additional advantageous features. An irreversible modification allows the introduction of molecular tags useful for localization or immobilization of the modified proteins *(12,13)*. Here we further develop this technique and demonstrate its usefulness for study of conventional enzymes as well as for identification of new catalytic antibody species that may arise from the immunological repertoire.

Results and Discussion

Design of Reactive Phosphonate Diesters

Phosphonate esters containing a *p*-nitrophenyl group were selected as a class of reagent with relatively high reactivity toward the hydroxyl



1; R = H
4; R = $CH_2CONH(CH_2)_6NH$-Biotin

2; R = $COCF_3$
5; R = Biotin

3; R = $COCF_3$

Fig. 1. Structures of phosphonate ester inhibitors and their biotinylated derivatives. Compounds were obtained by chemical synthesis, and had analytical characteristics in accord with their assigned structure. Synthetic methods are described elsewhere *(14)*.

group of an enzyme's catalytic serine residue *(4,14)*. Nevertheless, these esters provide considerably greater stability than the highly reactive fluorophosphates, represented by the common serine-protease inhibitor, diisopropylfluorophosphate (DFP). DFP breaks down by hydrolysis with a half-life of less than 10 min in aqueous buffers near neutral pH, whereas *p*-nitrophenyl phosphonates are stable for 6–18 h under similar conditions. The asymmetrical phosphonate diesters **1–3** (*see* Fig. 1) were designed to examine the influence of diverse structural and electrostatic properties of the phosphonyl moiety on the relative inhibitory activity toward typical serine hydrolases.

Inactivation of Trypsin, Subtilisin, and Butyrylcholinesterase

The nitrophenolate product released in the reaction of phosphonates **1–3** with nucleophiles provides a convenient signal for continuous determination of either enzyme inactivation or background hydrolysis reactions. Reaction of the phosphonate with enzyme at high concentrations could be observed directly by absorbance changes (*see* Fig. 2). Reagents were diluted into a freshly prepared solution of trypsin, chymotrypsin, or subtilisin, and the progress of the reaction was plotted over time. Inactivation rates determined by this method were in agreement with the values derived by conventional measurement of residual activity in discontinuous assays. Inactivation of butyrylcholinesterase was determined only by the discontinuous assay method, as the concentration of enzyme was too low to be observable by direct titration of *p*-nitrophenol. The second-order rate constants of inactivation are shown in Table 1. The two compounds had very different inhibitory activity in the reaction with subtilisin, whereas the rates of inactivation of trypsin and chymotrypsin by the neutral and positively charged phosphonate esters were comparable. These findings are consistent with the expected selectivity of these enzymes. The tropane functional group of compound **2** provides a basic tertiary amine in prox-

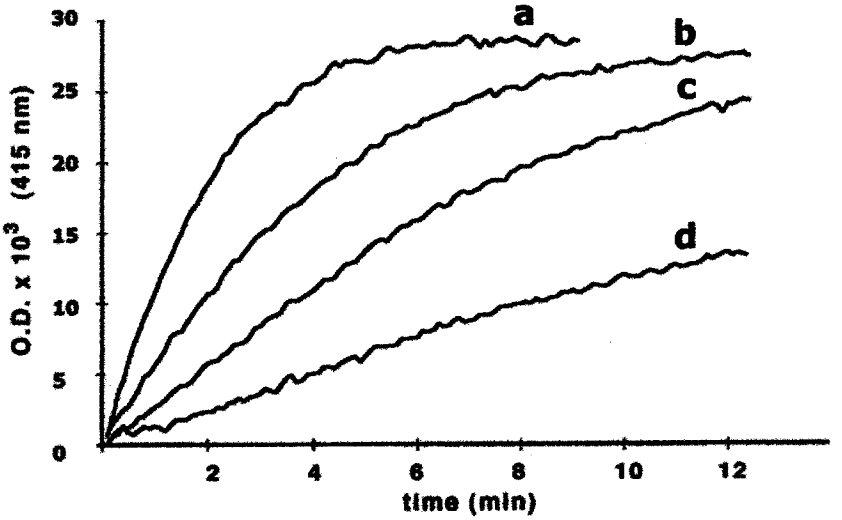


Fig. 2. Inactivation of α-chymotrypsin by phosphonate **1**. An aliquot of enzyme in 10 m*M* sodium acetate, pH 4.0, was diluted to a final concentration of 2 μ*M* in sodium phosphate 0.1 *M*, pH7.8, containing **(a)** 200 μ*M*, **(b)** 100 μ*M*, **(c)** 50 μ*M*, **(d)** 20 μ*M* of **1**. Absorbance changes were recorded continuously at 412 nm, on a Perkin Elmer lambda 6 spectrophotometer.

imity to the phosphonate ester moiety. It was anticipated that this electrostatic group could approximate the chemical character at lysine or arginine residues that are preferred sites for cleavage by trypsin-like enzymes. The neutral phosphonate ester **1** was a better inhibitor of subtilisin.

Direct observation of nitrophenol released in the inactivation reaction also serves to titrate the enzyme-active sites, although sensitivity is limited by the molar absorptivity of nitrophenol. Low micromolar concentrations of enzyme active site are readily detectable (*see* Fig. 2). The sensitivity could be further enhanced by using phosphonate esters containing a fluorogenic leaving group. The coumarin phosphonate ester **3** was used for active-site titration of a crude commercial preparation of butyrylcholinesterase (*see* Fig. 3). Fluorescence changes were linear over a range of 40–200 n*M* of enzyme, corresponding to 10–50 U/mL of enzyme. Inactivation rates were consistent with those estimated for inhibitor, **2,** determined by discontinuous assay of residual activity.

Inhibition of a Proteolytic Antibody Light Chain

The recombinant immunoglobulin kappa light chain derived from a murine monoclonal antibody (MAb) C23.5 has been shown to cleave its antigenic peptide VIP at a rate similar to the parent antibody *(1,2)*. Kinetic constants indicate saturation of the reaction at 10^{-8} to 10^{-7} *M*. Cleavage of ^{125}I-labeled VIP as assessed by the reported procedure demonstrated degradation of more than 80% of the available peptide. Previous reports showed

Table 1
Second-Order Rate Constants for Enzyme Inhibition by *p*-Nitrophenyl Phosphonates **1** and **2**[a]

	Rate (M^{-1}, min^{-1})	
Inhibitor	**1**	**2**
Subtilisin	N/R[b]	350
Butyrylcholinesterase[c]	1800	6×10^4
Trypsin	4100	
α-Chymotrypsin	2600	

[a]Enzymes (α-Chymotrypsin; Sigma, bovine, type II, EC 3.4.21.1. Trypsin; Boehringer Mannheim, EC 3.4.21.4. Subtilisin; Boehringer Mannheim, EC 3.4.21.62) were diluted to a final concentration of 2–10 μ*M* in 0.1 *M* phosphate buffer, pH 7.8, in a quartz cuvet. Phosphonate ester at concentrations ranging from 100–800 m*M* was added, and rates determined by absorbance changes at 412 nm due to nitrophenol release. Rates were calculated from a replot of initial rates vs concentration. Assay of residual activity was determined using chromogenic substrates for subtilisin (ZGGL-pNA) or trypsin (BAPA) and α-chymotrypsin (sucF-pNA).

[b]Inhibition was not apparent.

[c]Butyrylcholinesterase (Sigma, from horse serum, 300 U/mg, EC 3.1.1.8) was dissolved in 0.1 *M* phosphate buffer, pH 7.8, to a final concentration of 1 U/mL. Inhibitor was added to obtain concentrations from 0, 25, 50, and 100 μ*M*. Rates from assay of residual activity in hydrolysis of benzoylthiocholine, using Ellman's reagent for colorimetric product, were used to derive rate constants as described *(15)*

that the activity was most efficiently inhibited by DFP. Mutagenesis and modeling studies further suggest that the mechanism of the antibody is likely related to that of serine proteases *(2)*. Susceptibility to inhibition by reactive phosphonate esters was examined, which would provide additional evidence to implicate the covalent mechanism of catalysis. Incubation of the antibody in the presence of 100 μ*M* phenyl phosphonate ester **1**, or 1 m*M* DFP, was followed by assay for peptidase activity using the standard ^{125}I-labeled VIP assay. The phosphonate **4** was an effective inhibitor of the peptidase activity at a 10-fold lower concentration than VIP (*see* Fig. 4). The antigenic peptide is both a specific substrate and a high-affinity ligand for the antibody. Incubation of the antibody in the presence of VIP specifically protected the catalyst against inactivation by either DFP or the nitrophenyl phosphonate esters (not shown). These data support the suggested involvement of a nucleophilic mechanism in the catalysis of VIP cleavage by this antibody. The identity of the catalytic residue and its configuration in the putative active site must still be determined. The phosphonate modification could provide a useful label to assist in such characterization.

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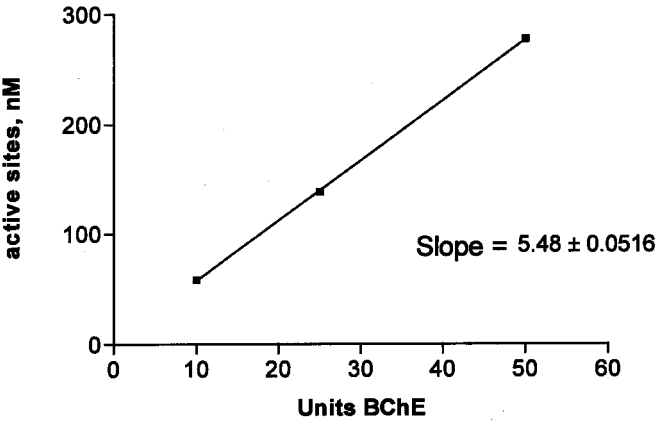

Fig. 3. Active-site titration of butyryl cholinesterase. Fluorescence changes due to release of 7-hydroxy-4-methylcoumarin from phosphonate **3** (20 μ*M*) were recorded in 0.1 *M* sodium phosphate, pH 7.8, in a 3-mL cuvet on a Perkin Elmer LS 50 fluorometer (Ex. 345 nm, Em. 457 nm). Three enzyme concentrations were employed, corresponding to 10, 25, and 50 U. Final fluorescence values were extrapolated from a curve obtained by continuous recording of changes over a 30-min reaction interval. Active-site concentrations were calculated from the fluorescence of authentic 7-hydroxy-4-methylcoumarin under these conditions (1.4 U/n*M*).

Specific Labeling of Enzymes with Biotinylated Phosphonate Esters

The active site-directed irreversible phosphonylation of a catalytic polypeptide allows the introduction of a label for detection or immobilization of the modified enzyme. This strategy can be implemented by conjugation of the phosphonate inhibitors to an affinity tag. The biotin-containing phosphonate ester derivatives **4** and **5** were prepared for this purpose by chemical transformation of available esters **1** and **2**. Modification at the amine function of the tropane phosphonate **1** by alkylation with iodoacetyl-LC-biotin *(16)* provides cationic molecule as the quarternary ammonium salt. This positive charge was expected to reinforce the selective reactivity of the phosphonylating reagent for trypsin-like enzymes that prefentially cleave at basic residues. On the other hand, presence of the long-chain biotin tag could hinder the interaction at the active site of some enzymes. Therefore, the inhibitory properties of the original phosphonate esters **1** and **2** and the biotinylated derivatives would not necessarily correspond.

Modification of crude mixtures or tissue samples containing the enzyme by biotinylated phosphonates **4** or **5** would incorporate a biotin tag. The specifically tagged catalyst could then be detected in the immobilized or fixed species with an avidin-peroxidase detection system *(12)*. This application was demonstrated by electrophoretic resolution and electroblotting of chymotrypsin and trypsin after treatment with the biotin-phosphonate esters. The specificity of labeling and limits of detection were

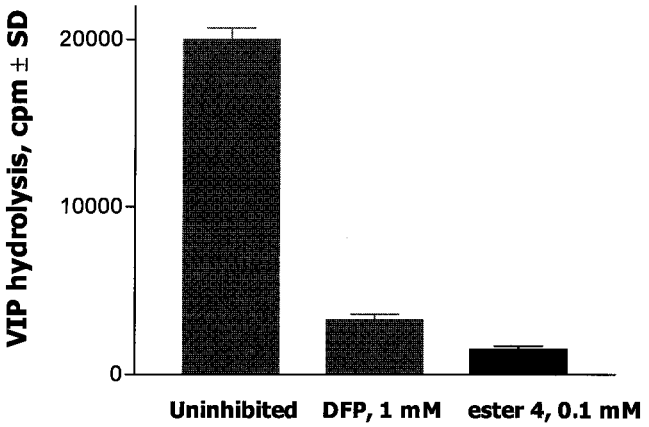

Fig. 4. Inhibition of cloned light chain of MAb C23.5 by phosphonate **4** (0.1 m*M*) or by DFP (1 m*M*). Light chain and inhibitor were incubated in 0.1 *M* Tris-glycine, pH 7.8, for 30 min at 37°C. Residual activity was determined by incubation of an aliquot with ^{125}I-VIP for 3 h at 37°C and determination of peptide cleavage by TCA precipitation of uncleaved peptide *(1,2)*.

investigated by reaction of phosphonate ester **5** with chymotrypsin in the presence of excess BSA. The reaction mixture was analyzed by SDS-PAGE, electroblotting, and staining with streptavidin-HRP, using chemiluminescent substrates (*see* Fig. 5). These data demonstrate detectable labeling of the serine protease at concentrations of less than 80 n*M*. Albumin was stained to a much lesser extent, even though its concentration is 10–100 times greater than that of the enzyme. The phosphonylated polypeptides survive the denaturing and reducing conditions of the electrophoretic separation. The charged phosphonate **4** was also useful for detection of chymotrypsin. Although the rates of inhibition by the neutral and charged phosphonate esters may be substantially different, there was no observable difference in chymotrypsin staining. However, nonspecific staining of BSA was significantly greater with this reagent (data not shown). Labeling of chymotrypsin was blocked by preincubating the enzymes with either DFP 250 μ*M* or nonbiotinylated phosphonate **1** at 100 μ*M*.

Similarly trypsin was analyzed by SDS-PAGE and streptavidin-HRP blot after treatment with the same reagents. In this case, the positively charged reagent **4** was superior for specific staining, permitting detection of trypsin at a lower limit of about 20 n*M*, whereas the neutral reagent **5** stained a 100-n*M* solution of enzyme only weakly. The latter reaction could not be blocked by pretreatment of trypsin with DFP or with nonbiotinylated reagent **2**. However, either 250 μ*M* DFP or 100 μ*M* **1** effectively blocked labeling by **4** (*see* Fig. 6). It is likely that these reactive phosphonate esters can attack at other sites on protein surfaces, as evidenced by the increased staining of BSA seen in blots. The positively charged ester **4** is particularly

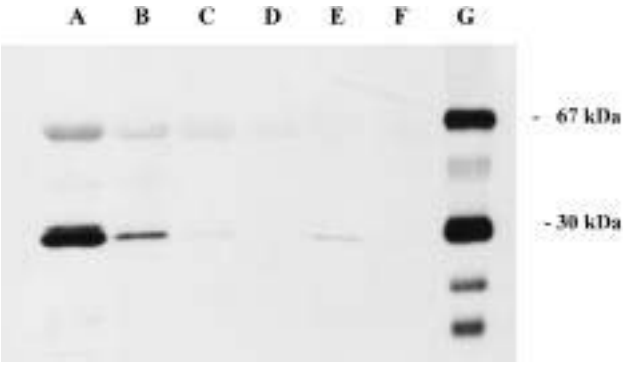

Fig. 5. SDS-PAGE, avidin-HRP blot analysis of α-chymotrypsin treated with biotinylated inhibitor **5** (MW 25 kDa). Phosphonate **5** was diluted to 10 μ*M* in 0.1 *M* sodium phosphate, pH 7.8, containing 0.25 mg/mL BSA containing active enyzme **(A)** 86, **(B)** 17, **(C)** 3, **(D)** 0.6 μg/mL or enzyme (86 μg/mL) pretreated with 0.1 m*M* phosphonate **2 (E)** or 0.25 m*M* DFP **(F)**. MW standards shown in lane G. Mixtures were incubated for 60 min at 23°C, then separated on SDS gel and electroblotted on PVDF. The membrane was blocked with 3% BSA and stained with streptavidin-HRP, using ECL detection (Amersham).

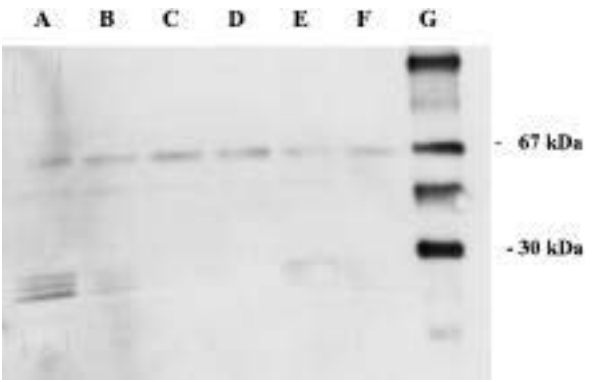

Fig. 6. SDS-PAGE, avidin-HRP blot analysis of trypsin treated with biotinylated inhibitor **4** (MW 23 kDa). Inhibitor was diluted to 10 μ*M* in 0.1 *M* sodium phosphate, pH 7.8 containing 0.25 mg/mL human serum albumin containing active enyzme **(A)** 100, **(B)** 20, **(C)** 4, **(D)** 0.8 μg/mL or enzyme (100 μg/mL) pretreated with 0.1 m*M* phosphonate **1 (E)** or 0.25 m*M* DFP **(F)**. MW standards shown in lane G. Analysis was carried out as per the legend to Fig. 5.

reactive toward bovine albumin. Human serum albumin is a less nonspecific competitor protein, as demonstrated in Fig. 6. On the other hand, DFP can protect the proteases against labeling by the more specific biotinylated reagents, suggesting that in these cases modification occurs at the same sites. Since DFP is known to specifically modify serine proteases at the active-site serine residue, it can be deduced that the selective phosphonate esters also attack this site.

Conclusion

The classical covalent mechanism of serine proteases predisposes these enzymes to irreversible inactivation by electrophilic phosphorus compounds. The chemical and structural properties of a phosphonate ester determine its reactivity toward a specific serine protease. Substrate-like features have been shown to contribute to specificity of the inhibitor. Several studies have suggested the incorporation of peptidic structures into a phosphonate-ester inhibitor to optimize selectivity toward specialized peptidases *(7–13)*. Simpler structural attributes, such as electrostatic charge and hydrophobicity, appear to play a role in the inhibitory activities reported here. Small phosphonate esters containing a basic functionality could react selectively toward a group of enzymes that cleave polypeptides at lysine or arginine residues *(17,18)*. The tropane derivative **1** is an example of such a molecule, which may be useful as a generic inactivator of cholinesterases as well as peptidases. It demonstrates selectivity for trypsin and chymotrypsin, as well as for a peptidase antibody light chain that cleaves at basic residues. The electrophilic reactivity at phosphorus provides another determinant of inhibitory activity. The nitrophenyl-leaving group enhances reactivity, and also introduces chirality at the phosphonyl center. The chiral environment of an enzyme-active site is likely to favor phosphonylation in one of the two possible configurations of the phosphonate ester *(5)*. Racemic mixtures of the phosphonates were used in this study, to allow for either possibility.

Covalent labeling by phosphonate esters constitutes a useful modification for molecular or histochemical analysis of biological samples containing the active enzymes *(12,13)*. Our study demonstrates this method for detection and quantitation of serine hydrolases. Additional applications of the labeling procedure include the structural analysis of the enzyme-active site by X-ray crystallography *(19,20)* and in chemical selection of biocatalysts from phage-display libraries. The implications for catalytic antibody studies are of particular interest. Further studies in these areas are currently under way.

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Discussion

Schowen: Is it conceivable that in the immunological selection induced by phosphonate haptens, you actually have a serine that interacts with the electrophilic phosphorous and is acidified in the presences of a base next to it—and then that interaction conditions the presence of a further base, selecting for the catalytic triad? Or is that pure fantasy?

Tramontano: Obviously, it is not clear how nucleophilic catalysis evolved in enymes, let alone in antibodies, but it is perfectly conceivable that these proteases arise either stochastically or by the type of mechanism you stated. Originally, we thought that nucleophilic catalysis is precluded because of the noncovalent nature of the antibody–antigen complex. It would be interesting to arrive at a model or reason for this kind of chemistry.

Schowen: The mechanism I was trying to imagine was not really for the catalytic activity, but for the immunological selection. If one takes a tetrahedral phosphonate, one should think it will then generate a series of groups around it in the antibody that interact favorably with the tetrahedral phosphonate. Ordinarily one would not expect to get covalently reactive groups. If the phosphorous was sufficiently electrophilic, a serine might crop up next to it, because it might be acidified to some degree—which might in turn lead to selection for a basic group next to it.

Tramontano: Yes. Whatever the process is in affinity maturation, nucleophilic catalysis has been observed in antiphosphonate esterolytic antibodies. The best example is from the UCSF lab where they suggest that the nucleophilic serine preserves structure in the CDR region by hydrogen bonding to some other residue away from the ligand. If that side chain is rotated by 180°, the serine then sits over the scissile bond.

Green: The suggestion of a nucleophilic serine in the example you cited has really been ruled out from Hilvert's analysis and our own analysis of a very similar antibody. Our antibody without serine has the same effect. I think mutation data also argue against a role for the serine. On the other hand, your idea of reactive immunization might generate chemically reactive functional groups in the antibody.

Tramontano: Sure. Sudhir and I are working on that proposal.

Paul: The work you cited was a presumed diad in the heavy chain generated by somatic hypermutation. In the natural antibody protease, we are dealing with a catalytic triad in the conserved sequence in the light chain. I just wanted to make sure that we don't mix apples and oranges.