Nucleophilic Proteolytic Antibodies

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Abstract

Proteolytic antibodies appear to utilize catalytic mechanisms akin to nonantibody serine proteases, assessed from mutagenesis and protease-inhibitor studies. The catalytic efficiency derives substantially from the ability to recognize the ground state with high affinity. Because the proteolytic activity is germline-encoded, catalysts with specificity for virtually any target polypeptide could potentially be developed by applying appropriate immunogens and selection strategies. Analysis of transition-state stabilizing interactions suggests that chemical reactivity of active-site serine residues is an important contributor to catalysis. A prototype antigen analog capable of reacting covalently with nucleophilic serine residues permitted enrichment of the catalysts from a phage-displayed lupus light-chain library. Further mechanistic developments in understanding proteolytic antibodies may lead to the isolation of catalysts suitable for passive immunotherapy of major diseases, and elicitation of catalytic immunity as a component of prophylactic vaccination.

Index Entries: Catalytic antibodies; phage display; serine proteases.

Introduction

Recurrent findings of catalytic antibodies (Abs) in autoimmune disease and multiple myeloma have suggested that a subset of Abs can develop the proteolytic function by entirely natural means. The *natural* origin of these Abs distinguishes them from designer esterolytic and amidase catalysts elicited by immunization with transition-state analogs. The latter type of Abs generally catalyze simple reactions with comparatively undemanding energetic needs—no success has been reported in generating proteolytic Abs by immunization with TSAs. Presumably, the TSA analog design does not adequately mimic the structure of the transition state.

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Protease inhibitor and mutagenesis studies *(1–3)* suggest that natural proteolytic Abs catalyze the cleavage of polypeptide antigens through a serine protease-like mechanism. Here, we assess the kinetic characteristics of available proteolytic Abs, describe evidence suggesting that their nucleophilic reactivity is an important factor enabling proteolysis, and evaluate the roles of inherited and adaptive genetic factors in the mounting of a proteolytic Abs response.

Serine Protease Reactivity

Early studies on polyclonal proteolytic antibodies consistently showed that diisopropyl fluorophosphate, a serine protease inhibitor, inhibited the catalytic activity, whereas inhibitors of metalloproteases, acid proteases, and cysteine proteases were with minimal effect or showed inhibition potentially attributable to global conformational transitions, as opposed to active site-directed effects *(1)*. Extending the polyclonal data, the light-chain (L chain) subunit of a MAb raised by immunization with vasoactive intestinal polypeptide (VIP) was described to bind *(4)* and cleave *(5)* this neuropeptide. A subtilisin-like arrangement of Ser27a, His93, and Asp1 residues was identified in the VIPase L chain by molecular modeling *(2)*. The hydrolysis of VIP was reduced by >90% by substitution of Ala residues for Ser27a, His93, or Asp1 by site-directed mutagenesis *(3)*. The turnover number of the Ser27a and His93 mutants was reduced by 100-fold, and of the Asp1 mutant, by 10-fold compared to the wild-type protein. The results were explained as arising from diminished transition-state binding and unaltered ground-state binding by the mutants. Thus, the Ser27a, His93, and Asp1 residues are the likely catalytic residues. Recent studies by Uda and colleagues also suggest the presence of a serine protease-like catalytic triad in an antibody L chain raised by immunization with the HIV coat-protein gp41 *(6,7)*. Matsuura and Sinohara described the use of a serine protease titrant to determine the active-site concentration in a catalytic L chain from a multiple myeloma patient *(8)*. We favor, therefore, the likelihood that antibodies frequently utilize their serine protease reactivity to effect antigen-specific proteolysis.

Conventional Abs bind only the long-lived ground states of the antigens. Proteolytic Abs must also bind and stabilize the transition state (*see* Fig. 1), but they are no different from conventional Abs in their high affinity for the antigen ground state, reflected by their low K_m values (*see* Table 1). The impressive catalytic efficiency $[(k_{cat}/K_m)/k_{uncat}]$ of previously identified proteolytic Ab light chains and Fv fragments, thus, is largely dependent on their ground-state binding affinity (ΔG_{GS}). The overall energy of transition-state stabilization (ΔG_{TS}) by the Ab fragments is comparable to that by non-Ab serine proteases, but the Ab fragments stabilize the ground state substantially better than the non-Ab proteases, presumably because the former molecules offer a greater contact area over which multiple electrostatic and hydrophobic packing interactions can occur. The
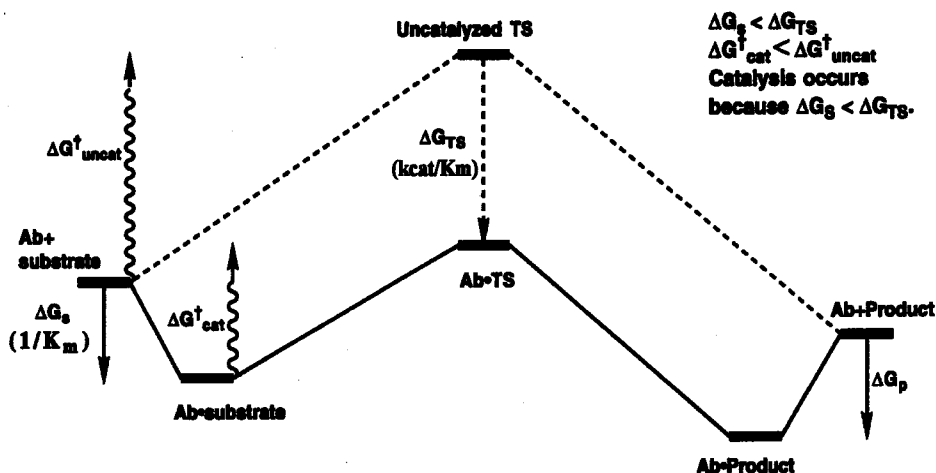
Fig. 1. Free-energy diagram for antibody catalysis involving stabilization of the substrate ground state (ΔG_S) and transition state (ΔG_{TS}). $\Delta G^\dagger_{uncat}$ and $\Delta G^\dagger_{cat}$ correspond to activation energies for the uncatalyzed and catalyzed reactions, respectively. K_m is a function of the extent of ground-state stabilization (ΔG_S). k_{cat}/K_m is a function of extent of transition-state stabilization relative to the catalyst-substrate ground-state complex.

turnover of the Abs, on the other hand, is comparatively slow (reflected by the magnitude of the ΔG_{uncat}–ΔG_{cat} term in Table 1). From the enzymological perspective, therefore, the serine-protease sites of the antibodies are by no means perfectly evolved. As in non-Ab serine proteases, hydrogen bonding with the His-Asp structure apparently confers enhanced nucleophilicity to the Ser hydroxyl group in the VIPase light chain clone c23.5. Mutations at the active site Asp of the light chain are less harmful than at the Ser/His residues, as also described for non-Ab serine proteases *(10)*. Perhaps the N-terminal location of Asp1 in the VIPase light chain is a factor limiting the turnover, in that protein termini tend to be mobile, which could interfere with precise positioning of the Asp residue necessary for hydrogen bonding with His93. Non-Ab serine proteases utilize an oxyanion hole to stabilize the transition state *(11)*. An absence of—or a defect in—the oxyanion hole in the VIPase light chain may also help explain its low turnover.

Germline Origin and Adaptive Diversification

Two independent explanations for the existence of proteolytic Abs can be conceived:

1. The V domains contain an inherited, germline-encoded catalytic site, which is recruited early in B-cell differentiation and then is improved (increased catalytic efficiency) or deteriorated (decreased catalytic efficiency) during somatic diversification.

Table 1
Peptidolysis by Ab Fragments and nonAb Serine Proteases:
Apparent Catalytic Proficiencies and Energy Requirements

Catalyst[1]	K_m (M)	$(k_{cat}/K_m)/k_{uncat}$ $(M)^6$	k_{cat}/k_{uncat}[6]	ΔG_{GS}[7]	ΔG_{TS}[8]	$\Delta G_{uncat} - \Delta G_{cat}$
Subtilisin	$1.8 \cdot 10^{-4}$	$2.3 \cdot 10^{13}$	$4.0 \cdot 10^9$	5.10	18.9	13.1
Trypsin[2]	$3.8 \cdot 10^{-4}$	$2.4 \cdot 10^{13}$	$9.2 \cdot 10^9$	4.8	19.0	14.1
c23.5 L chain[2]	$2.0 \cdot 10^{-7}$	$4.6 \cdot 10^{11}$	$9.1 \cdot 10^4$	9.5	16.5	7.0
c23.5 Fv[3]	$5.5 \cdot 10^{-9}$	$5.1 \cdot 10^{12}$	$2.8 \cdot 10^4$	11.7	18.0	6.3
hk14 L chain[4]	$2.0 \cdot 10^{-7}$	$9.1 \cdot 10^{13}$	$1.8 \cdot 10^7$	9.5	19.8	10.3
U24 L chain[5]	$2.6 \cdot 10^{-4}$	$1.6 \cdot 10^{10}$	$4.2 \cdot 10^6$	5.1	14.5	9.4

[1]Subtilisin values are for cleavage of a peptide-nitroanilide substrate *(23)*. Remaining data are for cleavage of VIP.

[2]Ref. *2*, L chain isolated following immunization with VIP.

[3]Ref. *13*, isolated following immunization with VIP.

[4]Ref. *12*, isolated from an asthma patient.

[5]Isolated from an unselected L-chain library from a mouse immunized with VIP.

[6]k_{uncat} of peptide-nitroanilide cleavage ($1 \cdot 1 \times 10^{-8}$ s^{-1}) is from ref. *23*. k_{uncat} for VIP cleavage (2×10^{-9} s^{-1}) is computed from model peptide-bond cleavage *(25)*. It is corrected for reaction temperature (37°C), and it is the sum of cleavage rates at four peptide bonds.

[7]$\Delta G_{GS} = -RT \ln K_m$ (represents stabilization of substrate ground state bound to catalyst compared to unbound substrate in kcal/mol).

[8]$\Delta G_{TS} = RT \ln (k_{cat}/k_m)/k_{uncat}$ (represents stabilization of transition state bound to catalyst compared to unbound transition state in kcal/mol). $\Delta G_{uncat} - \Delta G_{cat} = RT \ln (k_{cat}/k_{uncat})$ (represents transition-state binding energy utilized to accelerate the reaction under conditions of substrate excess in kcal/mol). ΔG_{uncat} of VIP and peptide-NA are, respectively, 30.5 and 28.3 kcal/mol.

2. The catalytic site is formed de novo during somatic sequence diversification.

We favor the former explanation based on the following findings: 1) the catalytic residues in a somatically matured VIPase light chain (clone c23.5) are also found in its germline VL gene homolog, and 2) the germline light chain obtained by reversion mutagenesis at four residues distinguishing the mature and germline sequences still expresses substantial catalytic activity *(10)*.

No direct evidence is presently available to evaluate improvements or deteriorations of the catalytic activity during B-cell clonal maturation and selection. However, highly mutated VIPase light chains isolated from asthma patients by phage display displayed significant proteolytic efficiencies (*see* Table 2), suggesting that somatic sequence diversification is not necessarily incompatible with maintenance and even improvement in the catalytic activity *(12)*. Another somatic diversification mechanism—i.e., combinatorial pairing of heavy and light chains—has previously been suggested to improve the catalytic efficiency *(13)*, based on findings that pairing of a VIPase VL domain with natural VH partner improved the catalysis, whereas pairing with an irrelevant VH resulted in reduced catal-

Table 2
Catalysis by Hypermutated L Chains[a]

Clone i.d.	k_{cat}/K_m (min^{-1}, M^{-1})	germline V_L homolog	Replacement mutations, number	
			CDR	FR
hk 14	1.1×10^7	02/012	12	3
hk 13	2.0×10^6	08/018	11	6

[a]hk14 and hk13 L chains were isolated by phage display. See reference *12* for methods.

ysis. Note further that nanomolar K_d and K_m values have been described for VIPase, thyroglobulinase, DNase, and factor-VIII-cleaving Abs *(14–17)*. Because high-affinity antigen recognition is a hallmark of V-domain affinity maturation, there is little doubt that expression of catalytic activity must be compatible with clonal selection under the specific immunological circumstances in which the proteolytic Abs have been identified.

No data are available to confirm whether the proteolytic activity can arise de novo during Ab maturation. Many enzymologists have reasoned that accidental formation of efficient catalytic sites occurs very rarely, and furthermore, unless discrete selection pressures select for catalysis, improvements in catalytic efficiency are highly improbable. It would appear intuitively valid to conclude, therefore, that the available examples of proteolytic antibodies result from recruitment of germline-encoded catalytic sites, as opposed to de novo formation of the catalytic sites.

At first glance, it might appear that rapid antigen cleavage and dissociation of antigen fragments from the cell surface must short-circuit the transmembrane signals needed to induce B-cell proliferation, thus aborting clonal selection. Under conditions of limiting antigen (i.e., [Ag] <K_m), the rate of catalysis can be computed as k_{cat}/K_m ratio. According to transition-state theory *(18)*, if improved binding of the antigen ground state is the only change occurring over the course of clonal selection, the catalytic competence of a germline-encoded Ab will remain constant and the turnover (k_{cat}) will decrease exactly in the same proportion as the improvement in antigen ground-state binding ($1/K_m$). This is apparent from Fig. 1, because increased ground-state binding will increase the activation energy (and thus decrease the k_{cat}). In this scenario, there will be no selection for or against catalysis. Viewed in terms of clonal selection theory, this conclusion remains tenable if the residence time of antigen bound to the catalytic Ab expressed on the B-cell surface is sufficient to trigger transmembrane signaling and cell proliferation. As long as the rate of transmembrane signaling is superior to the rate of Ag breakdown and dissociation, clonal expansion of protease-producing B cells will not be impeded.

There may be circumstances that would permit an improved catalytic activity and thus confer a survival advantage to B cells (*see* Fig. 2). If the
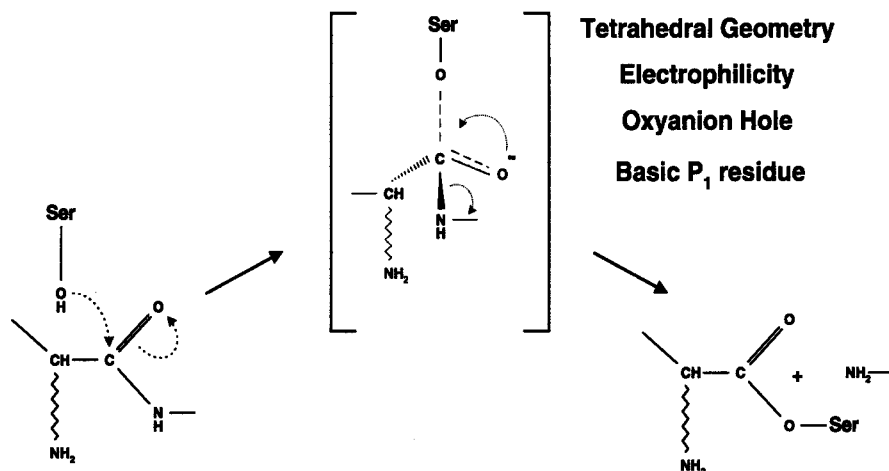
Fig. 2. The putative transition state of acyl-enzyme during peptide-bond cleavage by serine proteases. The acyl–enzyme complex (right structure) is deacylated by an attacking water molecule (hydrolysis step). Recognition of the basic side chain of the flanking residue may be a component of antibody binding to the extended transition state, although the flanking residues are not directly involved in bond making and breaking processes.

antigen is in excess (e.g., autoantigen excess encountered in autoimmune disease) or the antigen-binding affinity of the surface-expressed Ab is excessive (as may occur at the terminal stages of clonal selection), B cells can be silenced (and perhaps deleted) via receptor desensitization and down-regulation effects *(19,20)*. Antigen decomposition and dissociation from surface-expressed Abs can be theorized to reduce these effects. Thus, an improvement of the catalytic-rate constant brought about by somatic diversification could permit continued proliferation of the cells, resulting in rescue from clonal anergy or deletion.

Harnessing Proteolytic Immunity

Regardless of the genetic origins and immunological mechanisms of Ab proteases, the specificity and turnover capabilities of these molecules offer broad therapeutic possibilities, e.g., in removal of noxious microbial and cancer-associated antigens. Appropriately designed chemical probes that react with the Abs could be applied to isolate high-turnover proteolytic Abs of the desired specificity for passive immunotherapy and to elicit the Abs as mediators of prophylactic catalytic immunity. We have considered mechanistic information about serine proteases as an aid to designing the probes that bind the catalytic site, while discriminating Abs with differing specificity for the ground-state of various antigens. The latter property is necessary to distinguish antigen-specific catalysts, as opposed to Abs that cleave various polypeptides nonspecifically.
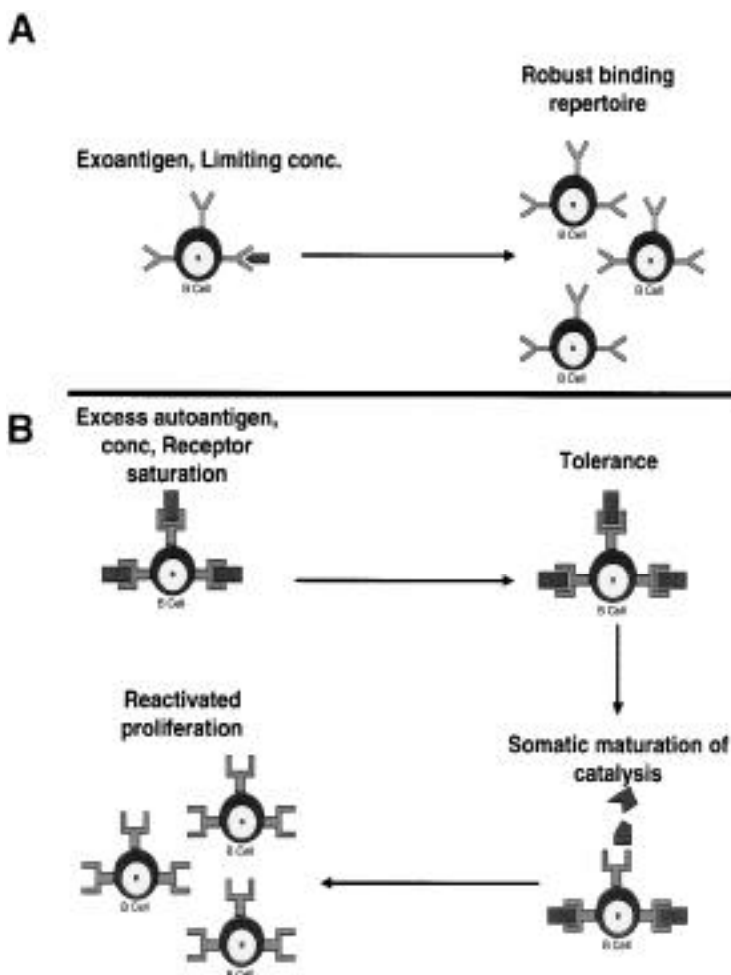
Fig. 3. Antigen concentration effects on catalyst development. **(A)** At limiting antigen concentration, affinity-maturation processes permit the development of antibodies capable of high-affinity antigen binding. Development of catalytic activity in antibody expressed on the surface of B cells (the B-cell receptor) under these conditions may be disfavored because increased catalytic rates will shorten the residence time of the antigen bound to the BCR, thus impeding completion of transmembrane signals required to stimulate clonal proliferation. **(B)** On the other hand, under conditions of excess antigen, B cells are rendered tolerant to the antigen. If the catalytic activity of antibodies improves somatically, the occupancy of the BCR by the antigen will be reduced, which may help the cells escape downregulation effects and permit selective clonal proliferation of the catalyst-synthesizing B cells.

Serine proteases utilize several distinct types of interactions to stabilize the transition-state of peptide-bond cleavage. Key structural features of the reaction include (*see* Fig. 3): 1) Stabilization of the tetrahedral geometry of the carbon atom formed in the transition state of the scissile peptide bond; 2) the chemical bonding of the carbon atom with a nucleophilic

serine residues in the catalyst; 3) stabilization of the oxyanionic character at the carbonyl oxygen by ion pairing with residues like Asn, Gln, or Arg in the catalyst (the so-called oxyanion hole); and 4) binding to the side chains of residues immediately flanking the scissile peptide bond and the sequence of the 5–10 residues flanking the bond. Note that the flanking residues, although not directly involved in bond making and breaking processes during catalysis, may occupy a different spatial position in the transition state and the ground state, permitting a variety of catalyst interactions at these structures in the two states of the antigen. This is possible because the partial double-bond character of the scissile peptide bond is lost upon formation of the transition state, permitting rotation around this bond, and consequent changes in the positions of remote groups. The feasibility of such remote spatial changes in the transition state has been deduced by computational modeling of a peptide substrate in the *sp2* (ground state) and *sp3* (transition state) configurations at the scissile bonds *(21)*.

Previous mutagenesis studies on non-Ab serine proteases and the VIPase light chain are helpful in dissecting the contributions of the nucleophilic serine reactivity vs the oxyanion hole. Recognition of the tetrahedral geometry of the transition state is estimated to contribute about 1 kcal/mol toward transition-state stabilization, a relatively minor effect *(22)*. On the other hand, covalent catalysis nucleophilic serine residues provides a significant catalytic contribution. Replacement of the nucleophilic serine with alanine residues in subtilisin *(23)* and the VIPase light chain reduces the rate of catalysis by a factor corresponding to 8.3 and 3.0 kcal/mol, respectively (ΔG^{app}_{nucl}; designated "nucleophilic energy"). These values represent 63% and 42% of the overall energy available for accelerating the reaction, respectively ($\Delta G_{uncat}-\Delta G_{cat}$; computed from the observed reaction rate at excess substrate concentration). Note that the term "nucleophilic energy" actually derives from all of the interactions that are lost by the serine mutation, possibly including the remote interactions at flanking substrate residues cited in the preceding paragraph ($\Delta G^{app}_{nucl} = \Sigma \Delta G_{nucl} + \Delta G_1 + \Delta G_2 + \cdots \Delta G_n$, where 1, 2, . . ., *n* are the remaining interactions destroyed by the mutation; note that the chemical reactivity of the serine nucleophile and other transition-state stabilizing interactions may be interdependent because they take place nearly contemporaneously and might be mutually reinforcing). In comparison, mutations at the Asn155 in subtilisin *(11)*, the apparent oxyanion hole in the enzyme, reduces the energy available for rate acceleration by 3.7 kcal/mol ($\Delta G^{app}_{oxyanion}$), or 28% of the overall available energy. As for ΔG^{app}_{nucl}, the term $\Delta G^{app}_{oxyanion}$ is the sum of the interactions interrupted by the mutation (at Asn155 in this case). Thus, it appears that the nucleophilic reactivity of the active-site serine in subtilisin provides a more important contribution than the oxyanion hole in stabilizing the transition state. This conclusion is valid notwithstanding the approximations inherent in computing the ΔG^{app}_{nucl} and $\Delta G^{app}_{oxyanion}$ terms, because the interactions outside the nucleophilic serine and the oxyanion are attendant to the existence
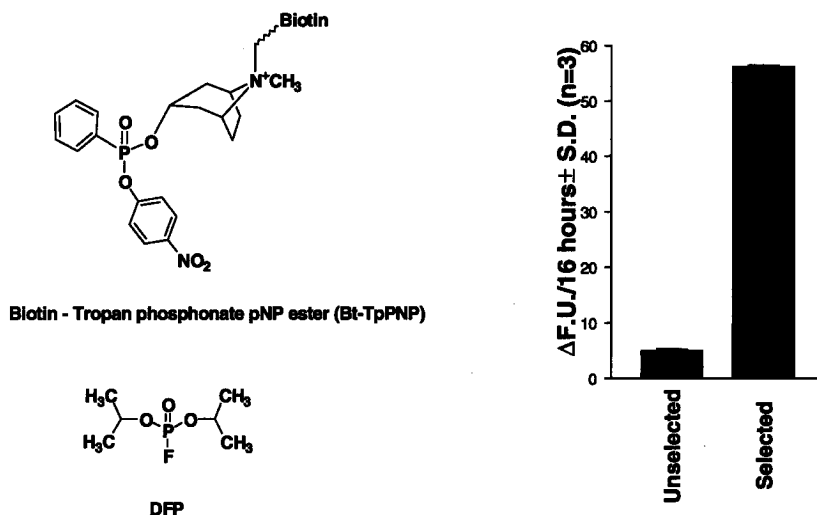
Fig. 4. Selection for catalytic antibody light chains by binding to a covalently reactive analog, biotinylated tropane p-nitrophenyl phenylphosphonate diester (Bt-TpPNP). Shown are values of cleavage of Pro-Phe-Arg-methylcoumarinamide (in fluorescence units, substrate conc 400 μM, 15 h, 37°C) by electrophoretically pure L chains (2 μM) prepared with and without selection of phage particles (10^{13} CFU) by binding to the Bt-TpPNP (0.1 mM, 30 min, 37°C). Unbound Bt-TpPNP was removed by precipitating phages with PEG (2%), and the phages were allowed to bind avidin-coated wells in a 96-well plate (overnight, 4°C). Following washing with buffers containing albumin and high salt, the specifically-bound phages were eluted with pyridine-2-aldoximemethlyiodide (10 mM). The selected phages were grown as plasmids in HB2151 cells and the recombinant L chains were purified to electrophoretic homogeneity from the periplasmic extracts by metal-affinity chromatography. See refs. *2* and *12* for cloning and purification methods. Synthesis of Bt-TpPNP is described by Tramontano et al. (this volume).

of these structures in the transition state and are included in the energy calculations from the mutation data.

An active-site serine residue is rendered nucleophilic because of intramolecular hydrogen bonding with histidine and aspartate residues, and because of medium effects that can perturb pK_a values of the hydroxyl group. Previous studies have suggested that phosphonate diester compounds, like DFP, can form stable covalent complexes with the active-site serine residues (*24*). One of us (S.P.) has suggested the utility of peptidyl phosphonate diester compounds as covalently reactive antigen analogs (CRAAs) in isolating proteolytic Abs and eliciting their synthesis on demand, as is required in prophylactic vaccination. As the first test of this proposal, a phage-displayed antibody light-chain library from lupus patients was subjected to selection using a prototype CRAA shown in Fig. 4. A biotin handle was placed in the CRAA to permit immobilization of the phage–CRAA complexes on immobilized streptavidin, and elution of the bound phages was done using pyridine-2-aldoximemethlyiodide, a

Table 3
Apparent Energetic Contributions of Serine Nucleophilicity
and Oxyanion Hole in Serine Protease Catalysis[a]

Catalyst	Substrate	k_{cat}/K_{uncut}	$\Delta G^{app}_{oxyanion}$ (kcal/mol)	ΔG^{app}_{nucl} (kcal/mol)	$\Delta G_{oxyanion} + \Delta G_{nuc}$ (kcal/mol)	$\Delta G_{uncat} - \Delta G_{cat}$ (kcal/mol)
Subtilisin	Peptide-pNA	4.0×10^9	3.7	8.3	12.0	13.1
Trypsin	VIP	1.2×10^{10}	n.d.	n.d.	n.a.	14.3
c23.5 light chain	VIP	1.2×10^5	n.d.	3.0	n.a.	7.2

[a]$\Delta G^{app}_{oxyanion} = -RT \ln(k_{cat}$ mutant$/k_{cat}$ wild-type); $\Delta G^{app}_{nuc} = -RT \ln(k_{cat}$ mutant$/k_{cat}$ wild-type); $\Delta G_{uncat} - \Delta G_{cat} = -RT \ln(k_{uncat}/k_{cat})$; k_{uncat} (peptide-pNA) = 1.1×10^{-8} s^{-1} (25°C) *(23)*; k_{uncat} (VIP) = 1.56×10^{-9} s^{-1} (37°C) *(25)*. Mutation data for the oxyanion hole are from ref. *11*, and for the active site serine, from ref. *23* (subtilisin) and ref. *3* (VIPase light chain).

reagent described to cleave the covalent bond formed between DFP and serine proteases. The model substrate Pro-Phe-Arg-methylcoumarinamide was cleaved by affinity-purified, CRAA-selected light chains >10-fold more rapidly than by unselected light chains (*see* Fig. 4), providing proof-of-principle that appropriately designed CRAAs can be utilized to isolate high-activity catalysts. The electronegativity of the oxygen atom permits presentation of a partial negative charge by the CRAA, but mimicry of the oxyanionic feature of the transition state is probably better achieved by negatively charged phosphonates, which have been explored extensively in catalytic antibody research without breakthroughs. Based on the energetic arguments, it is reasonable to hypothesize that covalently reactive tetrahedral compounds might prove effective in capturing high-activity proteolytic Abs.

Acknowledgments

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Discussion

Vijaylakshmi: We have studied different serine proteases, but not in the catalytic antibodies. It looks like very subtle differences in the flexibility of the histidine in the active site are important in determining the inhibition and K_m values. Have you tried TLCK inhibition of your catalytic antibody?

Gololobov: No.

Schowen: In the experiment in which you measured the contribution of the triad in the antibody catalysis—was that a triple mutant for the triad?

Gololobov: No, it was a single mutant. The rate becomes close to immeasurable, even in the single mutant.

Schowen: You mentioned that in some large fraction of the germline light chains there is a sign of a catalytic triad. Are they adjacent or nearly adjacent residues in sequence?

Gololobov: No, one of the residues is in CDR1, and the other in CDR3, and the aspartic acid is actually the N-terminal residue that approaches the histidine in CDR3 in the model.

Marchalonis: There are a lot of sequences of Bence Jones proteins available. Have you taken a *V*κ where the triad is not there, maybe you are out by one residue, and inserted the missing residue to see if you can then get the catalytic activity?

Gololobov: That is an exciting experiment, but we have not done it yet.

Gabibov: I wanted to comment that we have expressed your light chain in yeast, and observed catalytic activity.