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Bifunctional Catalysis of Proton Transfer at an Antibody Active Site

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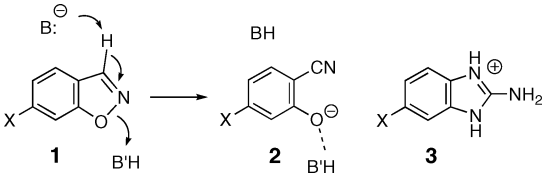
Highly evolved enzymes often depend on constellations of catalytic residues to promote otherwise difficult reactions. While functional arrays of acids, bases, and nucleophiles should similarly extend the properties of artificial enzymes, their incorporation into protein active sites represents a considerable challenge.

One relatively simple strategy to create functionalized binding pockets exploits charge complementarity between antibody and antigen.[1,2] This approach is exemplified by efforts to create bifunctional catalysts for the Kemp elimination of benzisoxazoles (**1**) to give salicylonitriles (**2**) using the cationic benzimidazolium derivative **3** as a hapten (Scheme 1).[3] The guanidinium group of **3** was expected to induce a base and an acid, preorganized to initiate proton transfer and stabilize developing negative charge at the phenoxide leaving group, respectively. Consistent with this design, antibody 13G5, which binds **3** with low nanomolar affinity, promotes the selective cleavage of 6-glutaramidebenzisoxazole—an unactivated substrate—with multiple turnovers and rate accelerations $>10^5$ over background. In contrast to the sigmoidal pH dependence seen for other antibodies that promote proton transfers,[1,2] 13G5 exhibits a bell-shaped pH-rate profile,[3] raising the possibility that bifunctional acid−base catalysis may be operative. In the current study, we have utilized X-ray crystallography and site-directed mutagenesis to gain additional insight into the origins of the catalytic effects in this system.

The crystal structure of the 13G5 Fab fragment, prepared by conventional proteolysis of the murine IgG2b/κ, was determined in its unliganded form to 2.65 Å resolution. The overall structure resembles other antibodies in its general features. The binding pocket is a deep cavity located between the variable light and heavy chains. Mirroring the design features of the immunizing hapten, it contains three polar groups in an otherwise hydrophobic environment: an aspartic acid−histidine dyad at the base of the active site (Asp^{H35}−His^{H95}) and a glutamic acid at position L34 (Figure 1). For comparison, 34E4, an antibody that was raised against an *N*-alkyl benzimidazolium derivative,[2] utilizes a single carboxylate residue (Glu^{H50}) that is located near the mouth of an apolar pocket to deprotonate activated benzisoxazoles.[4,5]

For mutagenesis studies, 13G5 was engineered as a chimeric human−murine Fab.[6] The recombinant protein was produced in *E. coli* by high-density fermentation on a 2 L scale and purified from the periplasmic lysate by Protein G and cation exchange chromatography. The chimeric Fab binds hapten **3** with a dissociation constant (K_d) of 2.0 nM and catalyzes the ring opening reaction of 6-glutaramidebenzisoxazole with $k_{cat} = 0.023\ s^{-1}$ and $K_m = 1.6$ mM (Table 1), in good agreement with the values reported for the original IgG2b/κ antibody.[3]

Scheme 1. The Base-Catalyzed Kemp Elimination[7] of 6-Glutaramidebenzisoxazole (**1**, X = -NHC(O)$(CH_2)_3CO_2H$) and Hapten **3** Which Was Used To Generate Antibody 13G5[3]



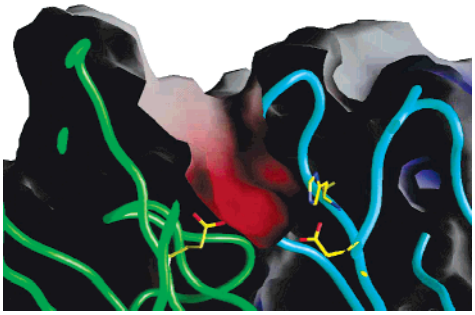


Figure 1. The active site of antibody 13G5 in the absence of ligand (PDB code 2GJZ). A slice through the center of the binding pocket is shown. The heavy and light chains are displayed as cyan and green coils, respectively. The side chains of the three polar residues, Glu^{L34}, Asp^{H35}, and His^{H95}, which are highlighted, project into an otherwise hydrophobic cavity. The electrostatic potential is mapped onto the surface with the color code ranging from −40 kT/e (bright red) to +40 kT/e (dark blue).

Table 1. Kinetic Parameters for the Antibody-Catalyzed Decomposition of 6-Glutaramidebenzisoxazole[a]

variant	k_{cat} [s^{-1}]	k_{cat}/K_m [$M^{-1}s^{-1}$]	pK_{a1}[b]	pK_{a2}[b]	K_d[c] [nM]
13G5 Fab	0.023	14	6.0	8.1	2.0
$D^{H35}N$		≤0.01	n.d.	n.d.	n.d.
$H^{H95}N$	0.007	2.0	5.8	8.4	n.d.
$E^{L34}Q$	0.017	9.1	6.3	10.2	87
$E^{L34}A$	0.18	220	6.6	10.8	140

[a] Kinetics were determined at 20.0 ± 0.2 °C in 40 mM phosphate buffer (pH 7.4) containing 100 mM NaCl. The standard error on the steady-state parameters is ≤11%. n.d. = not determined. [b] Deduced from the bell-shaped pH-dependence of the catalyzed reaction. [c] Fluorescence titration with **3** at 15.0 ± 0.2 °C in 20 mM Tris-HCl (pH 7.3) containing 100 mM NaCl; the standard error on the calculated dissociation constants is ≤25%.

The roles of the three polar residues at the active site were probed by site-directed mutagenesis (Table 1). Conservative mutation of Asp^{H35} to asparagine abolishes catalytic activity. Given the sensitivity of benzisoxazoles to base,[7] it is quite likely that this amino acid, in its ionized form, initiates proton transfer. In contrast, replacement of His^{H95} with asparagine reduces catalytic efficiency only about 7-fold. Since the latter mutation does not significantly alter the pH-

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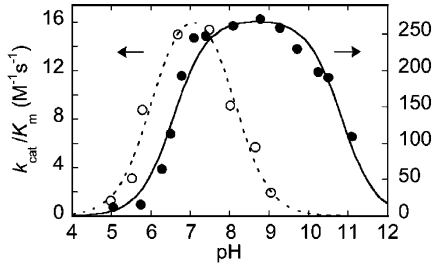

Figure 2. pH-dependence of the 13G5-catalyzed Kemp elimination of 6-glutaramidebenzisoxazole: wild-type Fab (○); $Glu^{L34}Ala$ variant (●).

dependence of the reaction (Table 1), the imidazole side chain probably does not act as either a general base or acid. Instead, given its proximity to Asp^{H35}, His^{H95} may simply enhance the reactivity of the catalytic base by helping to position it optimally for proton abstraction.

Because of the way in which the antigen was presented to the immune system,[3] the His^{H95}-Asp^{H35} dyad resides at the bottom of a hydrophobic cavity, and the chemical transformation promoted by 13G5 must take place in an environment largely shielded from bulk solvent. As a consequence, cleavage of the N−O bond of the unactivated benzisoxazole substrate would benefit from assistance from a hydrogen-bond donor or general acid.[8] An obvious candidate for this task is Glu^{L34}, which is separated from Asp^{H35} by about 7 Å. At acidic pH, it could stabilize the developing negative charge in the transition state by donating a proton to the leaving group. At basic pH, this residue will be ionized, disfavoring substrate binding and potentially destabilizing formation of the incipient phenoxide electrostatically. However, while replacement of Glu^{L34} by glutamine or by alanine reduces the affinity of the antibody for the positively charged hapten **3** by 40- to 70-fold, catalytic efficiency is not impaired by mutation (Table 1). In fact, the $Glu^{L34}Ala$ substitution leads to a substantial boost in both k_{cat} and k_{cat}/K_m. Both mutations also cause a dramatic broadening of the k_{cat}/K_m versus pH profile, primarily because of an increase in the apparent ionization constant for the basic limb, pK_{a2}, by more than two p*K* units (Table 1 and Figure 2). Although the data do not allow unambiguous assignment of the observed inflections to specific ionizing groups, the loss of activity at high pH appears to be a substrate binding effect, since both k_{cat} and K_m increase sharply above pH 8.[3,9] Unfavorable interactions between the negatively charged glutamate and the neutral substrate are presumably relieved when this residue is replaced by glutamine or alanine. Although these results do not rule out a role for Glu^{L34} in stabilizing the developing phenoxide anion at acidic pH, they show that it is not essential for catalysis and can be effectively replaced as proton donor by a buried water molecule or another protein group.

The efficiency of proton abstraction by Asp^{H35} at the 13G5 active site is highlighted by comparison with the acetate-promoted decomposition of 6-glutaramidebenzisoxazole: the $(k_{cat}/K_m)_{max}$ value for the $Glu^{L34}Ala$ variant (270 M^{-1} s^{-1}) is more than 10^9-times larger than the second-order rate constant for the acetate reaction (1.5×10^{-7} M^{-1} s^{-1}). The effectiveness of general base catalysis in the modified antibody active site is further underscored by an apparent effective molarity (EM = k_{cat}/k_{AcO^-}) of $>10^6$ M. These are extraordinarily large effects. For comparison, the carboxylate base in antibody 34E4, the best previously characterized protein catalyst for the Kemp elimination,[5] achieves an EM of ca. 50 000 M for the cleavage of the substantially more reactive 5-nitrobenzisoxazole.[4] Bovine serum albumin (BSA), which also promotes the cleavage of activated benzisoxazoles,[10] is a poor catalyst for the Kemp elimination of 6-glutaramidebenzisoxazole. Although it was not possible to determine an EM value for BSA's amine base because substrate saturation could not be achieved, the apparent second-order rate constant, k_{cat}/K_m, is only 0.09 $M^{-1}s^{-1}$ at pH 10, the pH optimum for this catalyst.

Our results show how an appropriately designed hapten can successfully elicit multiple functional groups within an antibody active site. As judged by its unusually large EM value, Asp^{H35} in 13G5 appears to be as effective at proton abstraction as analogous groups in highly evolved natural enzymes.[11] Moreover, efficient cleavage of an unactivated substrate, as opposed to the highly activated 5-nitrobenzisoxazole used in many model studies,[2,10,12] attests to this antibody's ability to coordinate proton abstraction and stabilization of the developing negative charge at the phenoxide leaving group. Structures of the antibody and its mutants complexed to the hapten should enhance our understanding of this process and aid future efforts to optimize this catalyst.

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Supporting Information Available: Experimental details for the production and characterization of the chimeric Fab fragments (CIF and PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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