An aspartate and a water molecule mediate efficient acid-base catalysis in a tailored antibody pocket

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Design of catalysts featuring multiple functional groups is a desirable, yet formidable goal. Antibody 13G5, which accelerates the cleavage of unactivated benzisoxazoles, is one of few artificial enzymes that harness an acid and a base to achieve efficient proton transfer. X-ray structures of the Fab-hapten complexes of wild-type 13G5 and active-site variants now afford detailed insights into its mechanism. The parent antibody preorganizes Asp^{H35} and Glu^{L34} to abstract a proton from substrate and to orient a water molecule for leaving group stabilization, respectively. Remodeling the environment of the hydrogen bond donor with a compensatory network of ordered waters, as seen in the Glu^{L34} to alanine mutant, leads to an impressive 10⁹-fold rate acceleration over the nonenzymatic reaction with acetate, illustrating the utility of buried water molecules in bifunctional catalysis. Generalization of these design principles may aid in creation of catalysts for other important chemical transformations.

catalytic antibody | crystal structure | enzyme design | enzyme mechanism | proton transfer

Proton transfer from carbon is an elementary step in numerous chemical transformations. To accelerate this kinetically and thermodynamically difficult reaction (1, 2), enzymes typically rely on multiple catalytic residues acting in concert. Incorporation of analogous arrays of acids and bases or electrophiles and nucleophiles into engineered catalysts represents a major design challenge (3). Lacking such, most enzyme mimics are many orders of magnitude less active than their natural counterparts.

The base-promoted Kemp elimination of benzisoxazoles to produce salicylonitriles (1 → 3, Fig. 1) (4–6) is an abiological reaction that is widely used as a model system to study proton transfer. Antibody 34E4, generated against hapten 4 (7), is among the most active catalysts for this transformation because it effectively exploits a combination of hydrogen bonding, π stacking, and van der Waals interactions to align the substrate with Glu^{H50}, the carboxylate base that was induced in response to haptenic charge (8). Although 34E4 achieves large rate accelerations (7, 9), it is substantially less efficient than catalysts like triose phosphate isomerase (TIM) and ketosteroid isomerase (KSI), which promote proton transfers near the diffusion limit (10, 11). Conformational isomerism of the free antibody (12) and reliance on a single catalytic residue (8, 9) apparently limit its efficacy.

Encouraged by a theoretical study predicting that the Kemp elimination would benefit from bifunctional catalysis (13), hapten 5 was subsequently designed to elicit an antibody combining site that would contain two functional groups: a base to initiate proton transfer and an acid to stabilize the developing negative charge at the phenoxide leaving group. Consistent with this design, antibody 13G5, which binds 5 with low nanomolar affinity, promotes selective cleavage of 6-glutaramidebenzisoxazole—an unactivated substrate—with multiple turnovers and rate accelerations >10⁵ over background (14). In contrast to the sigmoidal pH dependence of other antibodies that promote proton transfers, 13G5 exhibits a bell-shaped pH-rate profile

Fig. 1. Base-catalyzed Kemp elimination of 6-glutaramidebenzisoxazole [1, X = NHC(O)(CH₂)₃CO₂[−]]. Hapten 4 was used to generate antibody 34E4, whereas hapten 5 was used to elicit antibody 13G5.

that would be expected for bifunctional acid-base catalysis. Moreover, structural studies of the unliganded Fab fragment (15) identified two carboxylic acids, Asp^{H35} and Glu^{L34}, induced by the cationic hapten and positioned at the bottom of an otherwise hydrophobic pocket, as potential catalytic residues. Mutagenesis studies (15) showed that Asp^{H35} is absolutely required for catalysis and most likely acts, in its deprotonated form, as the catalytic base. In contrast, substitution of glutamine for Glu^{L34} had little impact on specific activity, but dramatically broadened the pH optimum of the reaction (15), suggesting that a protonated Glu^{L34} might stabilize the negative charge in the transition state by donating a hydrogen bond to the leaving group. Nevertheless, a polar residue at position L34 is not required for catalysis, as shown by the >10-fold increase in activity achieved when Glu^{L34} is replaced with alanine (15).

To resolve these mechanistic ambiguities and elucidate the origins of 13G5's catalytic efficiency, the Fab fragment of the parent antibody, as well as the Glu^{L34}Gln and Glu^{L34}Ala variants were crystallized with hapten 5. In addition to confirming the

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org [PDB ID codes 3FO0 (hapten complex of the 13G5 wild-type), 3FO1 (hapten complex of the 113G5-Glu^{L34}Ala variant), and 3FO2 (hapten complex of the 13G5-Glu^{L34}Gln variant)].

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Table 1. Data collection and refinement statistics

	13G5-5	Glu ^{L34} Gln 13G5-5	Glu ^{L34} Ala 13G5-5
Data collection			
Space group	<i>P</i> 2 ₁ 2 ₁ 2	<i>P</i> 2 ₁ 2 ₁ 2 ₁	<i>P</i> 2 ₁
Cell dimensions			
<i>a</i> , Å	84.1	83.1	59.6
<i>b</i> , Å	87.0	86.2	87.0
<i>c</i> , Å	59.4	113.6	84.4
α , °	90.0	90.0	90.0
β , °	90.0	90.0	91.3
γ , °	90.0	90.0	90.0
Resolution, Å	50.0–2.5 (2.56–2.50)	50.0–2.2 (2.25–2.20)	50.0–2.2 (2.25–2.20)
<i>R</i> _{sym}	0.076 (0.55)	0.093 (0.61)	0.072 (0.51)
$\langle I \rangle / \langle \sigma \rangle$	36.3 (3.8)	15.9 (2.2)	22.3 (2.4)
Completeness, %	99.7 (99.2)	92.4 (89.1)	99.8 (99.8)
Redundancy	6.6 (6.2)	3.2 (3.2)	3.5 (3.4)
Refinement			
Resolution, Å	50.0–2.5	50.0–2.2	50.0–2.2
No. reflections	15,696	39,309	43,563
<i>R</i> _{work} / <i>R</i> _{free}	0.22/0.27	0.20/0.25	0.23 / 0.28
No. atoms			
Protein	3,269	6,538	6,540
Ligand/ion	19	38	38
Water	49	162	165
<i>B</i> -values			
Protein	49.6	53.1	33.4
Ligand/ion	45.4	55.8	37.8
Water	50.1	38.1	42.2
rmsd			
Bond lengths, Å	0.011	0.014	0.012
Bond angles, °	1.4	1.5	1.4

Values in parentheses are for highest-resolution shell.

role of Asp^{H35} as the catalytic base, the structures of the complexes reveal that a buried water molecule, oriented by Glu^{L34} in the parent antibody, is the crucial hydrogen bond donor to the phenoxide leaving group. Optimization of the position and electrostatic environment of this water molecule, as seen in the Glu^{L34}Ala mutant, leads to very large catalytic effects. These three crystal structures highlight the catalytic potential of ordered waters, and provide firm evidence for bifunctional catalysis in an antibody combining site.

Results

Structure of the 13G5 Fab-Hapten Complex. The X-ray structure of 13G5, complexed with hapten **5**, was determined at 2.5 Å resolution (Table 1). Because the apo Fab possesses an open binding pocket large enough to house the 2-aminobenzimidazolium group of **5** (15), significant ligand-induced structural rearrangements were not anticipated. In fact, the variable domains of the 13G5 complex structure superimpose closely onto their apo counterparts (15) in two different space groups (Fig. 2), with root-mean-square deviations of 0.4 Å (apo structure in *P*2₁) and 0.6 Å (apo structure in *C*2), based on the C α atoms of the framework regions. Moreover, the variation in V_L-V_H rotation angles are small (1.7 and 1.2°, respectively) and fall in the range of values typically observed for molecules related by noncrystallographic symmetry. Minor adjustments to the side chains of Leu^{L94}, Ile^{H100A}, and His^{H27D} are observed at the mouth of the binding pocket (Fig. 2), but the complementarity determining region (CDR) loops remain essentially unchanged upon ligand binding. The modest rearrangement in CDR H3 reflects a modified backbone conformation remote (*ca.* 5 Å) from the hapten. As the electron density at the tip of H3 was

fairly weak in the apo structures, this hypervariable loop is apparently stabilized upon hapten binding. Unlike antibody 34E4 (12), however, 13G5 appears to exist in a binding-

Fig. 2. Superposition of antibody 13G5 in its unliganded state (gray) and in complex with hapten **5** (blue). Only minor adjustments take place at the mouth of the binding pocket, showing that the antibody does not undergo major remodeling upon ligand binding.

Fig. 3. Electrostatic and shape complementarity of the hapten to the 13G5 antibody combining site. A slice through the center of the binding site is shown. The electrostatic potential was calculated in APBS (43) and mapped onto the surface with the color code ranging from -30 kT/e (bright red) to +30 kT/e (dark blue). The base of the pocket has strongly negative electrostatic potential due to the two carboxylic acids, Asp^{H35} and Glu^{L34}. The architecture of the binding pocket reveals the structural basis for the observed substrate specificity.

competent state as it requires no major active-site remodeling to accommodate ligands.

Hapten Recognition. Ligand contacts are localized at the center of the 13G5 combining site. CDR L3 provides the majority of interactions (45.1% of the buried protein surface in contact with ligand) (Fig. 2); CDR L1 (5.8%), H1 (5.5%), H2 (16.0%), and H3 (22.3%) each contribute to a smaller extent. CDR L2 does not interact with hapten, as commonly observed in other antibody-hapten complexes (16). Although aromatic residues constitute 22.5% of the buried protein surface area, π stacking is not used to bind **5**. Instead, the hapten is clamped between two opposing prolines in CDRs L3 and H3: Pro^{L96} and Pro^{H100B} (Fig. 2). Additional van der Waals contacts are made by Phe^{L89}, Gly^{L91}, Leu^{L94}, Trp^{H47}, Val^{H50}, Trp^{H52}, His^{H95}, Ile^{H100A}, and Met^{H100E}.

As anticipated by the immunization strategy used to generate 13G5 (14), the polar guanidinium group of **5** is deeply buried in the binding pocket. Active-site residues Asp^{H35} and Glu^{L34} compensate for its positive charge, rendering the electrostatic surface at the base of the pocket strongly negative (Fig. 3). The Asp^{H35} carboxylate makes bidentate hydrogen bonds with the hapten N3 and N10 nitrogens (*ca.* 2.7 Å), while two well-defined water molecules, coordinated to Glu^{L34}, hydrogen bond with the N1 and N10 nitrogens on the opposite edge of the hapten (Fig. 4*A* and *D*). The orientation of the ligand within the pocket is further constrained by a hydrogen bond between the backbone carbonyl of Gly^{L91} and the amide group of the glutaramide linker.

Asp^{H35} Appears to Be Optimally Positioned for Proton Abstraction. The similarity in size and shape of the substrate and hapten dictates a binding geometry for 6-glutaramide benzisoxazole that

accurately orients its C3 hydrogen proximal to Asp^{H35}, the catalytic base. The linker is crucial in this regard, setting the proper binding register and, hence, controlling 13G5 specificity. For example, steric clashes preclude a productive binding mode for the 5-glutaramide derivative, which is not a substrate (14), whereas benzisoxazoles lacking the linker are presumably less optimally aligned than **1**. Although the absence of the hapten's exocyclic amine may result in small adjustments in the substrate binding mode, the slot-like nature of the binding site greatly restricts possible orientations. Furthermore, hydrogen bonding interactions with His^{H95} and Trp^{H47} fix the Asp^{H35} carboxylate and direct its more basic syn lone pairs (17) toward the ligand. The high degree of positional ordering of the catalytic base relative to bound substrate in this system is manifest, at least in part, in an unusually large effective molarity (EM). The EM corresponds to the concentration of base that would be needed in the absence of antibody to achieve the same rate (i.e., $k_{\text{cat}}/k_{\text{AcO}_-}$) and is $>10^5$ M for 13G5 (14). In stark contrast, EMs rarely exceed 10 M for general base catalysis in simple model systems (1, 18).

The enhanced basicity of the Asp^{H35} carboxylate also contributes to catalytic efficiency. The program PROPKA (19) predicts a pK_a of 5.9 for this residue, in good agreement with the observed value of 6.0 ± 0.1 for the acidic limb of the bell-shaped pH-rate profile (15). The calculation assumes that His^{H95} is not protonated at the active site. Consistent with this assumption, replacement with a neutral asparagine has only modest effects on the pH-dependence and steady state parameters of the Kemp elimination (15). The greater basicity of the active site carboxylate compared to free acetate ($\Delta pK \approx 1.2$) is presumably a consequence of its close proximity to the apolar side chains of Val^{H50} and Met^{H100E} (Fig. 4*D*).

An Ordered Water Molecule Stabilizes the Phenoxide Leaving Group.

The chemical transformation of benzisoxazoles at the 13G5 active site takes place in a hydrophobic pocket, largely shielded from bulk solvent. Thus, a mechanism for stabilizing the developing negative charge on the phenolate leaving group is required. Although the apo structure had suggested Glu^{L34} as a candidate for the obligate general acid, mutagenesis experiments showed that this residue is not essential (15). The crystal structure for the 13G5-hapten complex confirms that Glu^{L34} does not directly interact with bound ligand. Instead, a well-defined water molecule (W12) is positioned between the Glu^{L34} carboxylic acid and the hapten N1 nitrogen. This water molecule would be well suited to donate a hydrogen bond to the leaving group during catalysis (Fig. 4*A* and *D*).

Although Glu^{L34} cannot participate directly in the elimination reaction, its ionization state nevertheless influences antibody activity. PROPKA (19) predicts a pK_a of 7.5 ± 0.8 for this residue, consistent with the apparent ionization constant of 8.1 ± 0.1 for the basic limb of the pH-rate profile (15). At the pH optimum for catalysis (≈ 7), Glu^{L34} will be largely protonated, and, in this form, would serve as a proton donor to W12. As a consequence, this water could provide a hydrogen bond to the leaving group. At high pH, deprotonation of Glu^{L34} would be expected to disfavor substrate binding and inhibit formation of the incipient phenoxide electrostatically. In addition, when Glu^{L34} is deprotonated, it would be a better hydrogen bond acceptor than the nascent phenoxide. Since W12 is also engaged as a donor with the carbonyl group of Pro^{H100B}, neither of its protons would be available for transition state stabilization, explaining the observed drop in activity at high pH.

Support for this interpretation comes from biochemical and structural data on the Glu^{L34}Gln mutant. Replacement of the carboxylate with a neutral carboxamide does not significantly affect k_{cat} , but does greatly broaden the pH optimum for the

Fig. 4. Antibody combining site of 13G5 wild-type (A), Glu^{L34}Gln mutant (B), and Glu^{L34}Ala mutant (C) bound to its hapten. The light and heavy chains are colored in green and blue, respectively. The σ_a -weighted difference $F_o - F_c$ electron density maps (pink mesh) were calculated by omitting the ligand and active site water molecules and are contoured around the ligand at 3.0σ . The σ_a -weighted $2F_o - F_c$ electron density map (blue mesh) is contoured at 1.4σ . Hydrogen-bonding interactions of 13G5 wild-type (D), Glu^{L34}Gln mutant (E), and Glu^{L34}Ala mutant (F) with hapten 5. The proton-abstracting Asp^{H35} makes a bidentate hydrogen-bond interaction with the guanidinium group of the hapten. While only slight variations are observed in the Glu^{L34}Gln mutant to the wild-type, the immediate environment around the proton-donating water molecule W5 in the Glu^{L34}Ala mutant shows a marked difference in the solvent structure.

elimination reaction, increasing the apparent ionization constant for the basic limb of the pH-rate profile by more than two pK units (15). The crystal structure of the Glu^{L34}Gln mutant in complex with hapten 5 (2.2 Å resolution) indicates that the active-site architecture, including the network of buried water molecules, is unchanged by this substitution (Fig. 4 B and E). Since the glutamine side chain does not ionize under physiological conditions, it can serve as a hydrogen bond donor to water W15 (equivalent to W12 in the parent antibody) over the entire pH range. As a result, W15 may donate a hydrogen bond to the transition state even at elevated pH.

Optimizing Acid Catalysis. Although the buried water in 13G5 facilitates substrate cleavage, it is not an optimal general acid for the Kemp elimination. As already noted, simply mutating Glu^{L34} to alanine increases catalytic efficiency by more than an order of magnitude (15). To understand this finding, the crystal structure of the Glu^{L34}Ala mutant in complex with hapten 5 was determined at 2.2 Å resolution (Fig. 4 C and F).

Comparison with the parent antibody shows that the Glu^{L34}Ala mutation does not alter the position or orientation of the other active-site residues (Fig. 5). However, the solvent structure around the “catalytic water” (numbered here as W5) is substantially modified. W5 is hydrogen bonded to a new water

molecule (W37) that occupies the approximate position of the Glu^{L34} Oε2 atom in wild-type 13G5. In addition, water W17 is displaced from the position of the corresponding water molecule (W45) in the parent antibody, so that it now occupies approximately the same location as Oε1 of Glu^{L34} in the parent antibody. As a result of this solvent remodeling, W5 is involved in an extensive hydrogen-bonding network of four water molecules and nine hydrogen bonds that compensate for the loss of the carboxylate side chain. Since the buried solvent network is the only significant difference between the wild-type and mutant 13G5 structures, the properties of the Glu^{L34}Ala variant likely originate from this newly acquired feature. Although not observed crystallographically, subtle changes in protein structure and dynamics may also favorably modulate interactions of W5 with the transition state. As for the Glu^{L34}Gln variant, the broader pH optimum of Glu^{L34}Ala 13G5 (15) can be rationalized by the absence of an ionizing group in the immediate vicinity of W5.

Because natural enzymes often use lysines (20), arginines (21), and tyrosines (22) instead of ordered water molecules to stabilize developing negative charge on transition states, we replaced Glu^{L34} in 13G5 with these amino acids. Visual inspection of the active site had suggested that these residues might be able to replace the catalytic water, but the variants were 10 (Glu^{L34}Arg

Fig. 5. Overlay of wild-type (blue) and Glu^{L34}Ala (orange) 13G5 highlights the distinctive environments surrounding the respective catalytic waters, W12 and W5.

and Glu^{L34}Lys) to 100 times (Glu^{L34}Tyr) less active catalysts for the Kemp elimination of 6-glutaramidebenzoxazole than wild-type 13G5.

Discussion

Enzymes typically rely on multiple functional groups to accelerate difficult chemical reactions. The extraordinary efficiency of TIM (23) and KSI (24) ($k_{\text{cat}}/K_m \approx 10^8 \text{ M}^{-1} \text{ s}^{-1}$), for instance, arises from synergistic action of a carboxylate base, which initiates proton abstraction from carbon, and a general acid, either a histidine (25) or a tyrosine (26), that stabilizes the resulting anionic intermediate via hydrogen bonding.

The properties of antibody 13G5 demonstrate that artificial enzymes can similarly profit from concerted acid-base catalysis. The 13G5 active site provides a highly structured pocket of appropriate dimensions for sequestering 6-glutaramidebenzoxazole from solvent and aligning it with the catalytic functionality necessary for effecting the conversion to the corresponding salicylonitrile. The carboxylate base Asp^{H35} appears to be optimally positioned to abstract a proton from the substrate, and an ordered water molecule, fixed in place by Glu^{L34}, helps to shuttle negative charge from the base to the phenoxide leaving group as the reaction proceeds. This mechanism, effectively programmed by the 2-aminobenzimidazolium hapten, enables cleavage of unactivated benzoxazoles, which are poor substrates for 34E4 and other catalysts of the Kemp elimination.

Utilization of water as a catalytic group is not restricted to antibodies, of course. Natural aldolases, such as the 2-keto-3-deoxy-6-phosphogluconate aldolase (27) and the D-2-deoxyribose-5-phosphate aldolase (28), exploit ordered water molecules to mediate critical proton transfers. Explicit water molecules have also been successfully invoked for the computational design of artificial aldolases (29). Indeed, many side-chain functions in modern enzymes may well have been carried out by water molecules early in evolution. Beyond their versa-

tility as hydrogen bond donors and acceptors, water molecules have the potential advantage over networks of precisely positioned amino acid side chains in being able to respond flexibly to the changing catalytic requirements of a complex, multistep reaction manifold. Nevertheless, as with other catalytic groups, their position and electrostatic environment presumably have to be optimized for high efficiency.

In the case of 13G5, remodeling the network of buried waters through the Glu^{L34}Ala mutation serendipitously improved interactions of the “catalytic water” with the transition state. In fact, Glu^{L34}Ala 13G5 is among the most efficient catalytic antibodies yet described. Its EM is $>10^6 \text{ M}$ and its k_{cat}/K_m value is $>10^9$ times greater than the second-order rate constant for the acetate-promoted decomposition of 6-glutaramidebenzoxazole (15). These are exceptionally large effects for an artificial enzyme (30). By comparison, BSA, which accelerates the Kemp elimination of activated benzoxazoles at high pH, cleaves 10^3 times less efficiently than 13G5 (15), despite employing an intrinsically more reactive amine as the catalytic base. Antibody 34E4 exhibits a 10^2 -fold lower EM value for cleavage of the substantially more reactive 5-nitrobenzoxazole (9). The same is true for the best computationally designed enzymes for this reaction, even after optimization by directed evolution (31).

Although the magnitude of the individual mechanistic contributions to catalytic efficiency are difficult to tease apart, antibody 13G5 appears to capture many of the attributes and features that we associate with true enzymes. Nevertheless, it is still a modest catalyst when compared with its most active natural counterparts. This is not surprising, given that 13G5 was optimized immunologically to bind hapten 5 rather than to catalyze the Kemp elimination. Future efforts to augment its activity might, therefore, profitably focus on improving interactions with the developing charge in the transition state, for example by replacing the catalytic water with an accurately positioned protein side chain. Although our initial attempts to do so failed, the diffusion-controlled rates achieved by TIM and KS suggest that such groups may ultimately be required for maximal efficiency. Engineering such interactions represents an enormous challenge, but if 13G5 can be brought closer to catalytic perfection, perhaps through a combination of computational design and directed evolution (32), valuable clues regarding the origins of enzymatic efficacy should emerge. These, in turn, are likely to fertilize the fruitful field of enzyme design.

Materials and Methods

Materials. Chemicals were purchased from Sigma, Fluka, or Aldrich, unless otherwise stated. The substrates and hapten 5 were synthesized as previously described (14). Plasmid p4xH-M13 (33) was a gift from P. G. Schultz. All primers were purchased from Microsynth. *E. coli* XL1-blue and TOPP2 cells were obtained from Stratagene. Restriction enzymes and T4 DNA ligase for cloning were purchased from New England Biolabs. HotStarTaq DNA polymerase for PCR was purchased from Sigma. DNA sequencing was performed on an ABI PRISM 3100-Avant Genetic Analyzer from PE-Applied Biosystems.

Site-Directed Mutagenesis for Chimeric 13G5 Fab Mutants. Plasmid p4xH-13G5, which directs the production of 13G5 as a chimeric murine-human Fab fragment in *E. coli* (33), was used as a template for site-directed mutagenesis. Mutations were introduced as previously described (15) with the following primers: Glu^{L34}Lys sense (5'-GGAAACACCTATTTAAATGGTAC-CTGCAGAAA, the mutated codon in bold), Glu^{L34}Arg sense (5'-GGAAACACCTATTACGTTGGTACCTGCAGAAA), Glu^{L34}Tyr sense (5'-GGAAACACCTATTATCTGGTACCTGCAGAAA), and the flanking primers: V_H sense (5'-GGGAGAGTGTTAAGCTGGGATCC, BamH I restriction site shown in italics), V_H antisense (5'-GATACCGGTGACCGTGGTCCCTTG, BstE II), V_L sense (5'-TCTTGCAGATCTAGTCAGAGCATTGTA, BglII), V_L antisense (5'-GATCTCAAGCTTGGTGCCACC, Hind III). Coding regions of the cloned mutants were confirmed by DNA sequencing.

Production and Purification of Chimeric 13G5 Fab Proteins. CaCl₂-competent TOPP2 *E. coli* cells were transformed with the p4xH-13G5 plasmid containing the relevant chimeric Fab variant and fermented at high-density on a 2-L scale

with a BIOFLO 3000 bioreactor (New Brunswick Scientific). The Fab proteins were purified from the crude periplasmic lysate by Protein G affinity chromatography, followed by cation exchange chromatography (Mono S HR 10/10, Amersham Biosciences), as previously described (15).

Kinetic Assays. Kinetic experiments were performed at 20.0 ± 0.2 °C in 40 mM phosphate buffer containing 100 mM NaCl (pH 7.4). Reactions were initiated by addition of 6-glutamylbenzoxazole, and product formation was monitored spectroscopically, as previously described ($\Delta\epsilon = 6,820 \text{ M}^{-1}\text{cm}^{-1}$ at 329 nm) (15). Initial rates were corrected for the hydroxide- and buffer-catalyzed background reaction measured under the same conditions and fitted to the Michaelis-Menten equation $v_0/[E] = k_{\text{cat}}/[K_m + [S]]$, where v_0 is the initial rate, $[E]$ is the Fab concentration, and $[S]$ is the substrate concentration.

Crystallization and Data Collection. Crystallization trials of the wild-type and mutant 13G5-hapten complexes were performed by the sitting drop vapor diffusion method at 22.5 °C. The three proteins, concentrated to 15 mg/mL, crystallized in the presence of a 2-fold molar excess of hapten 5 under the same condition: 25% PEG 3350, 0.1 M Tris, pH 8.5. Crystal growth was significantly accelerated by streak seeding (34). For data collection, the crystals were flash-cooled to 100 K using 25% glycerol as cryoprotectant. Data were processed and scaled with HKL2000 (35) (Table 1). Although all three Fabs arrange in a similar fashion in their respective crystal lattices, they crystallized in different, but related space groups. Curiously, each Fab did not crystallize exclusively in the space group that is listed in Table 1, but could also adopt the other two space groups. While the crystal form in space group $P2_12_12_1$ contains only one Fab in the asymmetric unit, the $P2_1$ and $P2_12_12_1$ space groups harbor two Fabs.

Structure Determination and Refinement. The 13G5 complex structures were determined by molecular replacement using the program Phaser (36) and the coordinates of uncomplexed Fab (PDB ID code 2GJZ). The model was refined by alternating cycles of model building with the program O (37) and refinement with Refmac5 (38). During refinement, noncrystallographic symmetry restraints were applied to all main-chain atoms of the two Fab molecules in the asymmetric unit of $P2_1$ and $P2_12_12_1$, except for some loop regions. An all-atom contact analysis of hydrogen-bonding and steric clashes was performed in the program MolProbity (39) to assign the orientation of the Gln^{L34} side chain and the protonation state of His^{H95}. The rotamer in Fig. 4B accepts a hydrogen bond from Tyr^{L36}, which in turn acts as a hydrogen bond acceptor for the amide proton of Met^{H100E}. Furthermore, this rotamer has few van der Waals overlaps. By contrast, the flipped rotamer would undergo unfavorable interactions and cannot form an equivalently strong hydrogen bond with Tyr^{L36}. The final statistics of the three structures are shown in Table 1. The quality of the structures was analyzed using the programs MolProbity (39), WHATCHECK (40), and PROCHECK (41). Val^{L51} (CDR L2) of all 13G5-hapten complex structures is the only residue assigned by PROCHECK to a disallowed region, but with well-defined electron density. Val^{L51} is in a γ turn, as commonly observed in other antibody structures (42).

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- Kirby AJ (1997) Efficiency of proton transfer catalysis in models and enzymes. *Acc Chem Res* 30:290–296.
- Richard JP, Amyes TL (2001) Proton transfer at carbon. *Curr Opin Chem Biol* 5:626–633.
- Breslow R ed (2005) *Artificial Enzymes* (Wiley-VCH, Weinheim).
- Casey ML, Kemp DS, Paul KG, Cox DD (1973) Physical organic chemistry of benzisoxazoles. I. Mechanism of the base-catalyzed decomposition of benzisoxazoles. *J Org Chem* 38:2294–2301.
- Kemp DS, Casey ML (1973) Physical organic chemistry of benzisoxazoles. II. Linearity of the Bronsted free energy relation for the base-catalyzed decomposition of benzisoxazoles. *J Am Chem Soc* 95:6670–6680.
- Kemp DS, Cox DD, Paul KG (1975) Physical organic chemistry of benzisoxazoles. IV. Origins and catalytic nature of the solvent rate acceleration for the decarboxylation of 3-carboxybenzisoxazoles. *J Am Chem Soc* 97:7312–7318.
- Thorn SN, Daniels RG, Auditor MT, Hilvert D (1995) Large rate accelerations in antibody catalysis by strategic use of haptenic charge. *Nature* 373:228–230.
- Debler EW, et al. (2005) Structural origins of efficient proton abstraction from carbon by a catalytic antibody. *Proc Natl Acad Sci USA* 102:4984–4989.
- Seebeck FP, Hilvert D (2005) Positional ordering of reacting groups contributes significantly to the efficiency of proton transfer at an antibody active site. *J Am Chem Soc* 127:1307–1312.
- Knowles JR, Albery WJ (1977) Perfection in enzyme catalysis: The energetics of triosephosphate isomerase. *Acc Chem Res* 10:105–111.
- Hawkinson DC, Eames TCM, Pollack RM (1991) Energetics of 3-oxo- Δ^5 -steroid isomerase: Source of the catalytic power of the enzyme. *Biochemistry* 30:10849–10858.
- Debler EW, Müller R, Hilvert D, Wilson IA (2008) Conformational isomerism can limit antibody catalysis. *J Biol Chem* 283:16554–16560.
- Na J, Houk KN, Hilvert D (1996) Transition state of the base-promoted ring-opening of isoxazoles. Theoretical prediction of catalytic functionalities and design of haptens for antibody production. *J Am Chem Soc* 118:6462–6471.
- Kikuchi K, Hannak RB, Guo MJ, Kirby AJ, Hilvert D (2006) Toward bifunctional antibody catalysis. *Bioorg Med Chem* 14:6189–6196.
- Müller R, et al. (2007) Bifunctional catalysis of proton transfer at an antibody active site. *J Am Chem Soc* 129:460–461.
- Wilson IA, Stanfield RL (1993) Antibody-antigen interactions. *Curr Opin Struct Biol* 3:113–118.
- Gandour RD (1981) On the importance of orientation in general base catalysis by carboxylate. *Bioorg Chem* 10:169–176.
- Kirby AJ (1980) Effective molarities for intramolecular reactions. *Adv Phys Org Chem* 17:183–278.
- Li H, Robertson AD, Jensen JH (2005) Very fast empirical prediction and rationalization of protein pKa values. *Proteins* 61:704–721.
- Gerlt JA, Babbitt PC, Rayment I (2005) Divergent evolution in the enolase superfamily: The interplay of mechanism and specificity. *Arch Biochem Biophys* 433:59–70.
- Makinen MW, Wells GB, Kang SO (1984) Structure and mechanism of carboxypeptidase A. *Adv Inorg Biochem* 6:1–69.
- Kim SW, et al. (1997) High-resolution crystal structures of Δ^5 -3-ketosteroid isomerase with and without a reaction intermediate analogue. *Biochemistry* 36:14030–14036.
- Knowles JR (1991) Enzyme catalysis: Not different, just better. *Nature* 350:121–124.
- Pollack RM (2004) Enzymatic mechanisms for catalysis of enolization: Ketosteroid isomerase. *Bioorg Chem* 32:341–353.
- Lodi PJ, Knowles JR (1991) Neutral imidazole is the electrophile in the reaction catalyzed by triosephosphate isomerase: Structural origins and catalytic implications. *Biochemistry* 30:6948–6956.
- Kuliopulos A, Mildvan AS, Shortle D, Talalay P (1989) Kinetic and ultraviolet spectroscopic studies of active-site mutants of Δ^5 -3-ketosteroid isomerase. *Biochemistry* 28:149–159.
- Fullerton SW, et al. (2006) Mechanism of the Class I KDPG aldolase. *Bioorg Med Chem* 14:3002–3010.
- Heine A, et al. (2001) Observation of covalent intermediates in an enzyme mechanism at atomic resolution. *Science* 294:369–374.
- Jiang L, et al. (2008) De novo computational design of retro-aldol enzymes. *Science* 319:1387–1391.
- Hilvert D (2000) Critical analysis of antibody catalysis. *Annu Rev Biochem* 69:751–793.
- Röthlisberger D, et al. (2008) Kemp elimination catalysts by computational enzyme design. *Nature* 453:190–195.
- Jäckel C, Kast P, Hilvert D (2008) Protein design by directed evolution. *Annu Rev Biophys* 37:153–173.
- Ulrich HD, Patten PA, Yang PL, Romesberg FE, Schultz PG (1995) Expression studies of catalytic antibodies. *Proc Natl Acad Sci USA* 92:11907–11911.
- Stura EA, Wilson IA (1991) Applications of the streak seeding technique in protein crystallization. *J Cryst Growth* 110:270–282.
- Otwinowski Z, Minor W (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol* 276:307–326.
- Storoni LC, McCoy AJ, Read RJ (2004) Likelihood-enhanced fast rotation functions. *Acta Crystallogr D* 60:432–438.
- Jones TA, Zou JY, Cowan SW, Kjeldgaard M (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. *Acta Crystallogr A* 47:110–119.
- Murshudov GN, Vagin AA, Dodson EJ (1997) Refinement of macromolecular structures by the maximum-likelihood method. *Acta Crystallogr D* 53:240–255.
- Lovell SC, et al. (2003) Structure validation by α geometry: ϕ , ψ and χ deviation. *Proteins* 50:437–450.
- Vriend G (1990) WHAT IF: A molecular modeling and drug design program. *J Mol Graphics* 8:52–56.
- Laskowski RA, MacArthur MW, Moss DS, Thornton JM (1993) PROCHECK: A program to check the stereochemical quality of protein structures. *J Appl Crystallogr* 26:283–291.
- Al-Lazikani B, Lesk AM, Chothia C (1997) Standard conformations for the canonical structures of immunoglobulins. *J Mol Biol* 273:927–948.
- Baker NA, Sept D, Joseph S, Holst MJ, McCammon JA (2001) Electrostatics of nanosystems: Application to microtubules and the ribosome. *Proc Natl Acad Sci USA* 98:10037–10041.