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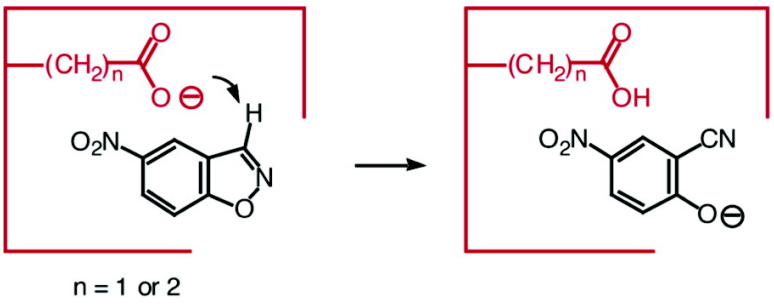

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Positional Ordering of Reacting Groups Contributes Significantly to the Efficiency of Proton Transfer at an Antibody Active Site

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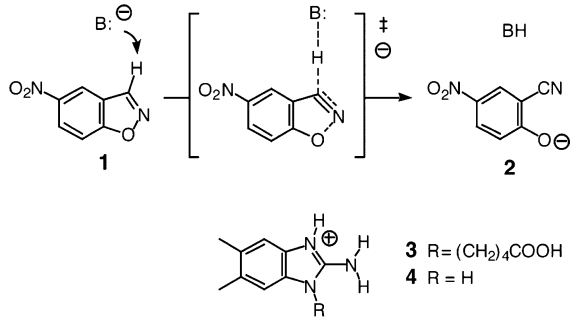
Abstract: Catalytic antibody 34E4 accelerates the conversion of benzisoxazoles to salicylonitriles with surprising efficiency, exploiting a carboxylate base with an elevated pK_a for proton abstraction. Mutagenesis of this antibody, produced as a chimeric Fab, confirms the prediction of a homology model that Glu^{H50} is the essential catalytic residue. Replacement of this residue by glutamine, alanine, or glycine reduces catalytic activity by more than 2.6×10^4-fold. By comparing the chemical proficiencies of the parent antibody with the chemical proficiencies of acetate and the mutants, the effective concentration of the catalytic side chain was estimated to be >51 000 M. The 2.1 kcal/mol destabilization of the transition state observed when Glu^{H50} is replaced by aspartate suggests that positional ordering imposed by the antibody active site contributes significantly to the efficiency of proton transfer. The observation that the Glu^{H50}Ala and Glu^{H50}Gly variants could not be chemically rescued by exogenous addition of high concentrations of formate or acetate further underscores the advantage the antibody derives from covalently fixing its base at the active site. Although medium effects also play an important role in 34E4, for example in enhancing the reactivity of the carboxylate side chain through desolvation, comparison of 34E4 with less proficient antibodies shows that positioning a carboxylate in a hydrophobic binding pocket alone is insufficient for efficient general base catalysis. Our results demonstrate that structural complementarity between the antibody and its substrate in the transition state is an important and necessary component of 34E4's high activity. By harnessing an additional catalytic group that could serve as a general acid to stabilize developing negative charge in the leaving group, overall efficiencies rivaling those of highly evolved enzymes should be accessible.

Introduction

The cleavage of C−H bonds is a fundamental, yet chemically demanding reaction. Enzymes, which accelerate these inherently slow proton transfers by enormous factors, must often overcome both kinetic and thermodynamic barriers.[1,2] Insights into the origins of their remarkable efficiencies can be gained through studies of well-defined model systems such as antibody 34E4,[3] which efficiently catalyzes the base-promoted E2 elimination of substituted benzisoxazoles (Scheme 1),[4,5] also known as the Kemp elimination. This antibody was generated against a cationic 2-aminobenzimidazolium hapten **3**, and combined kinetic and chemical modification studies have implicated a complementarily charged active-site carboxylate as a likely general base. This catalyst is $>10^8$ times more efficient in promoting the decomposition of benzisoxazoles than acetate [(k_{cat}/K_m)/k_{AcO^-}], and the rate acceleration over the spontaneous background reaction in water is $>10^6$ at pH 6. Moreover, the effective molarity of the active-site carboxylate is exceptionally high (EM = $k_{cat}/k_{AcO^-} > 10^4$ M). By way of contrast, EMs rarely exceed 10 M for intramolecular general base catalysis in model systems[2] or for general acid/base catalysis in other antibodies.[6,7] As judged by these properties, 34E4 is superior to a variety of catalysts reported for the Kemp elimination, including other

Scheme 1



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proteins,[8−10] synzymes,[11,12] organic host molecules,[13] imprinted polymers,[14] cationic vesicles,[15] and natural coals.[16]

Because of its unusual efficiency and the relative simplicity of the reaction it catalyzes, antibody 34E4 presents a fine case to evaluate the factors that influence successful general base catalysis at protein active sites. As pointed out previously,[3] at least three effects are likely to contribute to its observed activity: (1) an apolar active site that increases the reactivity of the antibody carboxylate through desolvation, (2) a microenvironment that favors delocalization of the carboxylate's negative charge to the nascent phenolate anion and stabilization of the polarizable transition state, and (3) orientation effects associated with correctly aligning the catalytic base with the 3-hydrogen of the benzisoxazole substrate. Although the importance of these individual aspects is not in contention, their relative magnitude is a matter of considerable debate.

Although almost certainly important in natural enzymes,[17,18] the role of precise positioning of catalytic and substrate groups has been questioned in the case of 34E4. The observation that serum albumins[8−10] and alkylated polyethylamines[11,12] significantly accelerate the elimination of **1**, together with the known sensitivity of the acetate-promoted reaction to solvent polarity,[5] has been used to argue[8,11,12] that reactant preorganization plays a subordinate role to nonspecific medium effects in the antibody-catalyzed reaction. However, in contrast to 34E4, these other catalysts exploit amine bases, which are relatively insensitive to medium effects and intrinsically several orders of magnitude more reactive than carboxylates in the decomposition of benzisoxazoles.[5] As a consequence, mechanistic conclusions about 34E4 based on the properties of these chemically distinct systems must be viewed with caution. Comparisons of 34E4 with elimination catalysts in which the orientation of the carboxylate base relative to substrate is varied in an otherwise similar (ideally unchanged) active-site microenvironment would be more revealing about the importance of base positioning.

Here, we identify Glu^{H50} as the catalytic base in 34E4, confirming predictions based on a homology model,[19] and examine the consequences of repositioning this essential residue by (a) mutating glutamate to aspartate and (b) replacing the carboxylate side chain altogether with noncovalently bound formate or acetate. Our results suggest that effective positioning of the catalytic carboxylate in 34E4 relative to bound substrate contributes significantly to the efficiency of proton transfer and that this residue is comparable to analogous groups in enzymes that catalyze proton transfers in its effectiveness as a base. However, the absence of a general acid that could stabilize developing negative charge at the leaving group probably prevents this antibody from achieving the overall efficiency of highly evolved natural enzymes.

Table 1. Kinetic Parameters for Antibody 34E4 Fab and Mutants[a]

	k_{cat} [s^{-1}]	K_m [μM]	k_{cat}/K_m [$M^{-1}s^{-1}$]	K_d [nM]	pK_{EH}
34E4 Fab	0.45 ± 0.03	90 ± 11	5.0×10^3	1.5 ± 0.3[c]	6.4 ± 0.1
$E^{H50}D$	0.015 ± 0.001	110 ± 8	1.4×10^2	233 ± 9[c]	6.6 ± 0.1
$E^{H50}Q$	n.d.[b]	n.d.	≤0.19	114 ± 10[d]	n.d.
$E^{H50}A/G$	n.d.	n.d.	≤0.19	n.d.	n.d.
$Y^{L32}K$	0.03 ± 0.005	10^3 ± 100	30	3.2 ± 0.4[d]	n.d.
$Y^{L32}R$	n.d.	n.d.	≤0.19	n.d.	n.d.

[a] Kinetics were measured with 5-nitro-benzisoxazole (**1**) at 20 ± 1 °C in 40 mM phosphate buffer (pH 7.4) containing 100 mM NaCl and 2% acetonitrile. The k_{cat} and K_m parameters were calculated from initial rates using the Michaelis−Menten equation. The uncatalyzed reaction measured under the same conditions afforded $k_{non} = 3.1 \times 10^{-5}$ s^{-1}; for comparison, $k_{AcO^-} = 1.6 \times 10^{-5}$ M^{-1} s^{-1}.[3] [b] n.d. = not determined. [c] Determined by surface plasmon resonance from k_{on} and k_{off} at three different Fab concentrations (1.0, 0.6, and 0.4 μM) at pH 7.0. [d] Fluorescence titration with (**4**) at 20 ± 1 °C in 40 mM Tris-HCl, 100 mM NaCl (pH 9.0). Determination of the K_d for wild-type 34E4 by fluorescence titration gave the same value within experimental error as the surface plasmon resonance measurements.

Results

Recombinant Antibody Production. To enable mutagenesis, 34E4 was engineered as a chimeric murine-human Fab fragment[20] and expressed in *Escherichia coli*. The recombinant protein binds hapten **4** with a K_d of 1.5 nM and catalyzes the conversion of **1** to **2** with a k_{cat} of 0.45 s^{-1} and a K_m of 90 μM (Table 1). The K_d for hapten binding is consistent with initial estimates (ca. 1 nM), while catalytic efficiency is only decreased somewhat (<2-fold) compared with the original murine IgG.[3] The slight deviations in the values reported here from those published earlier are likely due to the chimeric nature of the 34E4 Fab fragment.

Identification of the General Base in 34E4. On the basis of a homology model, we predicted residue Glu^{H50} to be the catalytic base in 34E4.[19] Consistent with this proposal, mutation of Glu^{H50} to glutamine reduces activity by more than 2.6×10^4-fold. The Glu^{H50}Gln variant also binds hapten analogue **4** >10^4-fold less well than the parent antibody as determined by surface plasmon resonance measurements and fluorescence titration at pH 7.0. Under these conditions, 2-aminobenzimidazoles are largely protonated (pK_a = 7.8), and the loss of affinity accompanying the Glu^{H50}Gln mutation can be attributed to removal of a complementary negative charge from the antibody binding pocket. At pH 9.0, however, where compound **4** is deprotonated, significant binding (K_d = 114 nM)—but no catalysis—is observed.

The side-chain amide of Gln^{H50} could conceivably provide either monodentate or bidentate interactions with the neutral hapten analogous to the interactions possible between glutamate and the protonated benzimidazole (Chart 1). The 100-fold difference in affinity observed for the wild-type and mutant antibodies correlates with the difference in binding energy expected for an ion pair versus a hydrogen bond between a neutral donor and a neutral acceptor.[21]

Repositioning the General Base. To alter the position of the carboxylate base in the 34E4 active site, Glu^{H50} was replaced with aspartate, which has a shorter side chain by one methylene

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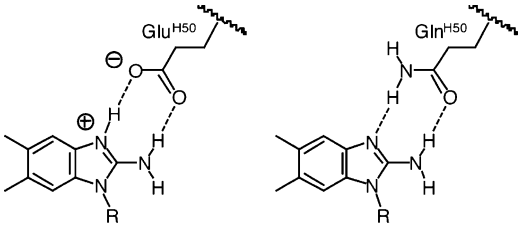
Chart 1



group. This mutation affords a catalyst with a 30-fold decreased k_{cat}. Because the Michaelis constant K_m remains essentially unchanged, transition-state stabilization is reduced by 2.1 kcal/mol at 20 °C. Hapten affinity also decreases 150-fold as determined by surface plasmon resonance. The pH dependence of the reaction was examined to detect possible changes in the solvation of the catalytic base. As shown in Figure 1, the pK_a determined for the mutant (~6.6) is essentially the same as that for the parent catalyst, indicating that the two carboxylates are perturbed to similar extents by their respective microenvironments. This result suggests that the decrease in rate observed for the Glu^{H50}Asp variant can be largely ascribed to less optimal positioning of its carboxylate base rather than to a medium effect.

Chemical Rescue. The effects of an even less ordered base on the efficiency of 34E4 were assessed by replacing Glu^{H50} with alanine or glycine and adding acetate and formate exogenously to compensate for the missing side chain. However, no activity over background was observed for the Glu^{H50}Ala and Glu^{H50}Gly variants at acetate or formate concentrations up to 2 M. The inability of these small carboxylic acids to replace Glu^{H50} in the mutants reflects both the low probability of forming a ternary complex between the protein, substrate, and external base, as well as the increased mobility or inaccurate positioning of the carboxylate once it is bound at the active site.

General Acid Catalysis. Gas-phase calculations on the Kemp elimination suggest that provision of a general acid in the vicinity of the benzisoxazole 1-oxygen would stabilize accumulating negative charge in the transition state.[22] It was estimated that such a residue might enhance overall catalytic efficiency by several orders of magnitude. On the basis of the homology model of 34E4,[19] we identified Tyr^{L32} as a possible (although not ideal) residue for replacement by a cationic lysine or arginine, which might serve as both general acid and counterion to the developing charge in the transition state. However, the Tyr^{L32}Lys variant is 170-fold less active than wild-type antibody, and the Tyr^{L32}Arg mutant is completely inactive (Table 1). Conceivably, formation of an intramolecular salt bridge between Glu^{H50} and the Arg^{L32} or Lys^{L32} side chains across the binding pocket might inhibit productive binding of the substrate.

Discussion

Antibodies raised against rationally designed transition-state analogues catalyze many reactions.[23−26] Nevertheless, activities

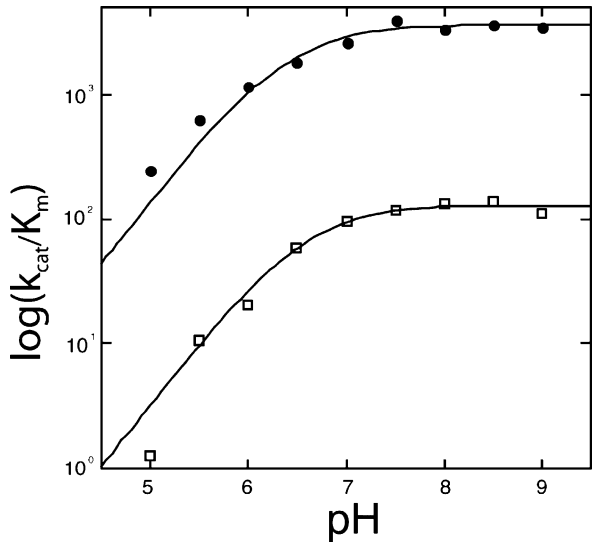


Figure 1. Plot of log(k_{cat}/K_m) in M^{-1} s^{-1} versus pH for the elimination of **1** catalyzed by wild-type Fab 34E4 (●) and the Glu^{H50}Asp variant (□).

in such systems are usually low compared to real enzymes, reflecting the rather simplistic mechanistic strategies generally utilized by antibody combining sites. The low probability of eliciting functional groups using haptens designed solely to mimic transition-state shape has been identified as one reason for this inefficiency, and a variety of strategies have been explored to induce catalytic residues directly. Reactive immunization protocols[27,28] and exploitation of charge complementarity between hapten and protein[3,7,29,30] have been the most fruitful approaches in this regard. Antibody 34E4 is a particularly successful example of the strategic use of haptenic charge to elicit a catalytic base.

Efficiency of General Base Catalysis in 34E4. As anticipated in the design of cationic hapten **3**,[3] the antibody contains a complementary and essential carboxylate base, Glu^{H50}, at its active site. As described by Bearne and Wolfenden,[31] the effective concentration of this functional group in the transition state of the antibody-catalyzed reaction can be estimated from the connection free energy (ΔG^S) that describes the change in probability of binding that results when the catalytic group is connected to a truncated variant to give the wild-type antibody. This parameter is related to the transition-state stabilization provided by the wild-type antibody (ΔG_{tx}^{wt}), the intrinsic binding energy provided by the antibody variant from which the active-site functional group has been removed ($\Delta G_{tx}^{i(variant)}$), and the intrinsic binding energy provided by the functional moiety alone ($\Delta G_{tx}^{i(piece)}$), in this case represented by acetate, as shown in eq 1:

$$\Delta G^S = \Delta G_{tx}^{wt} - \Delta G_{tx}^{i(variant)} - \Delta G_{tx}^{i(piece)} \quad (1)$$

Rewriting this expression in terms of rate constants affords eq 2:

$$\Delta G^S = RT \ln[(k_{cat}/K_m)^{wt}/(k_{cat}/K_m)^{variant}](k_{non}/k_{piece})] \quad (2)$$

From the kinetic data summarized in Table 1, we calculate ΔG^S

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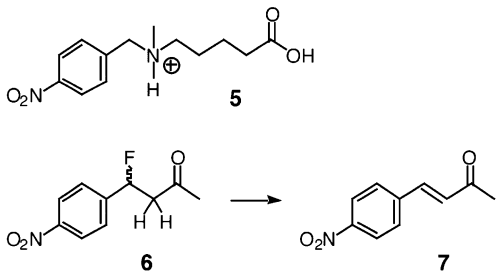
to be ≥6.3 kcal/mol for Glu^{H50} in 34E4, which corresponds to an effective concentration ≥51 000 M for this residue in the transition state. This EM compares favorably to the originally calculated value[3] of k_{cat}/k_{AcO^-} and to EM values obtained for the individual catalytic residues at the active site of the enzyme mandelate racemase,[31] which catalyzes the interconversion of (*R*)- and (*S*)-mandelic acid by a two-base mechanism. In mandelate racemase, the effective concentrations of the catalytic side chains of Lys^{166}, His^{297}, and Glu^{317} were calculated to be ≥622, ≥3 × 10^3, and ≥3 × 10^5 M, respectively.[31] By this criterion, general base catalysis in 34E4, mediated by Glu^{H50}, appears to be at least as efficient as in this evolutionarily optimized natural enzyme.

Contributions to Catalytic Efficiency. Although EMs are often taken as a measure of the entropic advantage an enzyme gains by having a catalytic group covalently bound at its active site, free energy changes arising from relief of strain, solvation effects, or changes in protein conformation can contribute significantly to the absolute magnitude of this parameter.[32] For instance, in the case of 34E4, medium effects are certainly important. The elevated pK_a of the active-site carboxylate indicates that this residue is activated by desolvation due to its apolar surroundings. At least a factor of 10 toward the catalytic efficiency of the antibody can be attributed directly to the difference in basicity between Glu^{H50} and acetate ($\Delta pK_a \approx 1.25$).[3] Depending on the nature of the residues lining the binding pocket, the transition state of the reaction might be additionally stabilized via a specific medium effect achieved through hydrogen bonding or dispersive interactions.[3,5,12]

Although precise positioning of the carboxylate base in 34E4 has been argued to contribute only modestly to the observed EM,[8,11,12] the significant decrease in activity observed upon replacing the catalytic base Glu^{H50} with the shorter side chain of aspartate and the inability of exogenously added formate or acetate to rescue truncated variants lacking a carboxylate side chain demonstrate that juxtaposition of substrate and reactant groups is important in this system.

The consequences of mutating the catalytic base are manifest predominantly as a destabilized transition state for proton abstraction. This destabilization cannot be explained as a medium effect, since the wild-type and Glu^{H50}Asp mutant antibodies have similar pH-rate profiles and affinity for substrate. Instead, removal of a methylene group from the side chain of residue H50 likely moves the base away from the substrate by something less than 1 Å;[33] it may also alter the geometry of proton abstraction.[34] Both factors have been invoked to explain the ca. 1000-fold reduction in the rate constant for proton abstraction seen when the catalytic glutamate in triose phosphate isomerase (TIM) was replaced by aspartate.[17,33] A definitive interpretation of the mutagenesis results obtained with 34E4 must await high-resolution structures of the wild-type and mutant antibodies, but a smaller displacement of the catalytic base in the antibody would explain the reduced sensitivity to substitution compared with TIM. Indeed, the magnitude of the effect of the Glu^{H50}Asp mutation is well within the range reported for a variety of other enzymes that exploit carboxyl groups to promote proton transfers and show 3−400-fold reductions in specific activity upon replacement of glutamate by aspartate or aspartate by glutamate.[35−40] The observed variation in these natural systems probably reflects the specific geometric constraints of their respective active sites. Functional groups that interact synergistically with other catalytic groups may also be more sensitive to mutation than those that act in isolation, as in 34E4.

Scheme 2



Comparison with Other Antibodies that Promote Proton Transfers. Like 34E4, antibodies 4B2[29] and 43D4-3D12[7] were generated in response to cationic haptens and exploit carboxylate bases for proton transfer from carbon. However, they are considerably less efficient than 34E4, suggesting that simply positioning a glutamate within a hydrophobic pocket is insufficient for high catalytic efficacy.

Antibody 43D4-3D12 was raised against the trialkylammonium cation **5** and accelerates the elimination of HF from *β*-fluoroketone **6** to give the unsaturated ketone **7** (Scheme 2).[7] Mechanistic studies suggest either an E1cb or an E2 elimination mechanism, in which Glu^{H50}, the same functional group utilized by 34E4, serves as the general base.[41,42] As in 34E4, this residue has an elevated pK_a (∼6.2), but it is much less successful as a base, judging by its low effective concentration in the transition state. Using eq 2 and published kinetic data for the uncatalyzed ($k_{non} = 2.2 \times 10^{-6}$ s^{-1}), acetate-catalyzed ($k_{AcO^-} = 1.9 \times 10^{-4}$ M^{-1} s^{-1}), and antibody-catalyzed ($k_{cat}/K_m = 16$ M^{-1} s^{-1}) reactions at 37 °C,[7] and assuming that the Glu^{H50}Gln mutant has a $(k_{cat}/K_m)^{variant} \leq 0.004$ M^{-1} s^{-1}, we obtained a ΔG^S value of 2.4 kcal/mol, which corresponds to an EM of 46 M. Correction for the difference in basicity between Glu^{H50} and acetate will reduce this value by a further factor of 10.

The 1000-fold lower efficiency of 43D4-3D12 compared to that of 34E4 can be largely attributed to the ability of its substrate to rotate relatively freely within the binding pocket. Experiments with selectively deuterated substrates show that 43D4-3D12 favors an anti over a syn elimination but exhibits little stereofacial selectivity, abstracting either the pro*R* or pro*S* proton from C-3 of **6**.[41] In contrast to planar hapten **3**, which correctly mimics the transition-state geometry of all reacting

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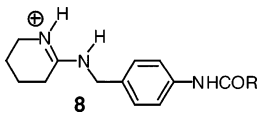
Chart 2



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bonds in the Kemp elimination, hapten **5** is a relatively flexible molecule with multiple rotatable bonds. It is consequently not terribly surprising that the complementary antibody binding pocket does not force substrate **6** to adopt an optimal conformation for proton abstraction by Glu^{H50}.

The second antibody, 4B2, tightly binds the cyclic amidinium salt **8** (Chart 2) and was found, serendipitously, to accelerate the Kemp elimination of **1** with a k_{cat}/K_m of 29 M^{-1} s^{-1} at 30 °C.[29] Chemical modification studies and the pH dependence of k_{cat}/K_m indicate that the catalytic base is a carboxylate with a pK_a of 5.8. The crystal structure of this antibody confirms the presence of a glutamate at position L34 within an otherwise hydrophobic binding pocket;[43] its carboxylate side chain is directed into the interior of the combining site where it forms a salt bridge with the amidinium group of the bound hapten. Although Glu^{L34} is "precisely positioned" through a hydrogen-bonding network with His^{L36} and Trp^{H103}, it is a substantially poorer base than Glu^{H50} in 34E4. Neither the Glu^{L34}Gln nor Glu^{L34}Ala mutations has been described, but if we assume that these variants would have no activity over background, i.e., $(k_{cat}/K_m)^{variant} \leq 0.19$ M^{-1} s^{-1} (see Table 1), then the effective concentration of the catalytic carboxylate can be calculated according to eq 2 to be ca. 5 M (the rate constants k_{non} and k_{AcO^-} reported in ref 29 are 1.9×10^{-6} s^{-1} and 5.3×10^5 M^{-1} s^{-1}, respectively). In other words, despite its hydrophobic surroundings and similar pK_a, Glu^{L34} in 4B2 appears to be 10 000 times less proficient than Glu^{H50} in 34E4 in the transition state for proton abstraction.

While most residues that line a protein binding pocket are likely to be more or less precisely positioned, such positioning is not useful for catalysis if a functional group is not aligned with the substrate in the transition state. Benzisoxazoles bear little resemblance to hapten **8** and can presumably bind at the relatively spacious 4B2 binding pocket[43] in a variety of orientations that are suboptimal for proton abstraction by the active-site carboxylate. Indeed, the high K_m value reported for **1** of 1.2 mM implies relatively weak and nonspecific interactions with the binding pocket.[29] We conclude that positioning a carboxylate in a hydrophobic binding pocket is insufficient for efficient catalysis of the Kemp elimination. While medium effects undoubtedly contribute to the observed activities of these catalysts, a high degree of positional ordering imposed by the 34E4 binding site would appear to distinguish it from a more primitive catalyst such as 4B2.

General Acid Catalysis. Given the high effective concentration of its catalytic base, why is the overall catalytic efficiency of 34E4 more than 4 orders of magnitude lower than that for natural enzymes such as triose phosphate isomerase[44] or 3-oxo-Δ^5-steroid isomerase,[45] which also utilize carboxylate bases for proton abstraction? In a sense, 34E4 is only half an enzyme. In contrast to its highly evolved natural counterparts, which use multiple catalytic groups in synergistic fashion to stabilize the high energy transition states of the reactions they catalyze, 34E4 appears to rely on only a single functional group, namely Glu^{H50}.

A Brønsted analysis has shown that the antibody-catalyzed reaction of benzisoxazoles is more sensitive to leaving group effects than the uncatalyzed reaction, catalysis being most efficient with substrates containing strong electron-withdrawing groups.[19] Comparison of the β-values obtained for the elimination under various conditions shows that the developing negative charge is relatively destabilized by the antibody binding pocket and suggests that the leaving group phenol binds in a region that is less polar than acetonitrile. Unfortunately, our initial attempts to provide an antibody residue able to stabilize the oxy-anion through hydrogen bonding or electrostatic interactions have not met with measurable success.

Conceivably, detailed crystallographic data for 34E4 will provide a better basis for rational optimization of this catalyst than the available homology model. However, the failure to improve 34E4 by mutating Tyr^{L32} to lysine or arginine may be a consequence of the predicted location of this residue near the mouth of the binding pocket where potentially beneficial electrostatic effects of the cationic group with the leaving group oxygen would be attenuated through solvation by the bulk medium. To achieve efficient bifunctional catalysis, burial of the general acid and general base within the active site may prove necessary.[22] Appropriately configured antibodies might be obtained by linking the benzimidazolium hapten through its benzene ring rather than through the imidazolium moiety, making both N1 and N3 available for induction of two embedded functional groups preorganized for cleavage of benzisoxazole substrates. Our results suggest that such interactions will be required if antibody catalysts are to achieve the overall efficiencies of enzymes that promote proton transfers.

Experimental Procedures

Materials. All primers were purchased from Microsynth (Baglach, Switzerland); all other chemicals were purchased from Sigma or Fluka unless otherwise stated. The synthesis of **1** and preparation of the BSA-hapten conjugate have been described elsewhere.[3] The expression plasmid p4xH-M13 was a gift from Prof. P. G. Schultz.[20] *E. coli* XL1-blue and TOPP2 strains were obtained from Stratagene (La Jolla, CA). Restriction enzymes and T4 ligase were purchased from New England Biolabs (Beverly, MA) and Fermentas (Vilnius, Lithuania), respectively. Thermostable HotStarTaq DNA polymerase was purchased from Quiagen (Basel, Switzerland). All nucleic acid manipulations were according to standard procedures.[46] DNA sequencing was performed on an ABI PRISM 310 Genetic Analyzer from PE-Applied Biosystems (Foster City, CA).

Site-Directed Mutagenesis for Chimeric Fab 34E4 Mutants. Plasmid p4xH-34E4, which was previously constructed and described for optimized production of the chimeric 34E4 Fab fragment in *E. coli*,[19] served as a template for site-directed mutagenesis. Mutations were introduced using the QuickChange site-directed mutagenesis kit (Stratagene) using the following primers: Glu^{H50}Ala sense (5′-AATGGATTGGC**GCT**ATTAATC-3′, the mutated codon in bold), Glu^{H50}Ala antisense (5′-CTGGATTAAT**AGC**GCCAATC-3′), Glu^{H50}Gly sense (5′-AATGGATTGGC**GGT**ATTAATC-3′), Glu^{H50}Gly anti-sense (5′-CTG-

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GATTAAT**ACC**GCCAATC-3′), Glu^{H50}Gln sense (5′-AATGGATTG-GC**CAG**ATTAATC-3′), Glu^{H50}Gln anti-sense (5′-CTGGATTAAT**CTG**-GCCAATC-3′), Glu^{H50}Asp sense (5′-AATGGATTGGC**GAC**ATTAATC-3′), Glu^{H50}Asp anti-sense (5′-CTGGATTAAT**GTC**GCCAATC-3′), Tyr^{L32}Lys sense (5′-TAGTAAT**AAA**GCCACCTGGGTCCAA-3′), Tyr^{L32}Lys anti-sense (5′-CCCAGGTGGC**TTT**ATTACTAGTTG-3′), Tyr^{L32}Arg sense (5′-TAGTAAT**CGA**GCCACCTGGGTCCAA-3′), Tyr^{L32}Arg anti-sense (5′-CCCAGGTGGC**TCG**ATTACTAGTTG-3′). All coding portions of the constructed plasmids were confirmed by DNA sequencing.

Expression and Purification of the Fab 34E4 Protein. Calcium-competent TOPP2 *E. coli* cells were transformed with the p4xH-34E4 plasmid and fermented at high density on a 2-L scale with BIOFLO 3000 (New Brunswick Scientific, Edison, NJ) as previously described.[20] The chimeric Fab fragment was purified from crude periplasmic lysates by protein G affinity chromatography, followed by MonoS cation exchange chromatography. Sample purity was assessed by SDS-PAGE. The protein was dialyzed into a storage buffer (20 mM Tris HCl, 100 mM NaCl, 0.5 mM EDTA, 1 mM methionine), concentrated to 5 mg/mL, and sterile filtered. The final yield of Fab protein ranged from 5 to 15 mg/L for the various mutants. Protein concentration was determined spectroscopically at 280 nm, using molar absorption coefficients calculated as described.[47]

Kinetic Assays. All reactions were carried out at 20 ± 1 °C in aqueous buffer (40 mM phosphate, 100 mM NaCl, pH 7.4). Kinetics at various pH's were performed in the following buffers (40 mM, containing 100 mM NaCl): sodium acetate (pH < 6), sodium phosphate (6 < pH < 8), and sodium borate (8 < pH < 10). Acetonitrile at a final concentration of 2% was used as cosolvent to ensure substrate solubility. Initial rates for the consumption of **1** were determined spectrophotometrically, measuring the increase in absorbance at 380 nm ($\Delta\epsilon$ = 15 800 M^{-1} cm^{-1}). The data were corrected for the hydroxide- and buffer-catalyzed background reaction measured under the same conditions. Steady-state kinetic parameters k_{cat} and K_m were derived by fitting the experimental data to the Michaelis−Menten equation: $v_0/[E] = k_{cat}[S]/(K_m + [S])$, where v_0 is the initial rate, [E] is the antibody concentration, and [S] is the substrate concentration. Active-site titration with **4** was used periodically to confirm active-site concentrations determined spectroscopically. Reduced affinity of weakly active variants precluded a similar analysis. To assess trace activity in inactive 34E4 variants, protein concentrations as high as 33 μM were used. The apparent ionization constant for the enzyme (pK_{EH}) was determined by fitting the pH dependence of the apparent second-order rate constant k_{cat}/K_m using the equation:

$$k_{cat}/K_m = \frac{(k_{cat}/K_m)_{max}}{1 + 10^{(pK_{EH} - pH)}}$$

BIAcore Measurements. Binding kinetics were determined by surface plasmon resonance using a BIAcore 3000 instrument (Pharmacia).[48] The surface of a dextran chip was activated with 1-[3-dimethylamino)propyl]-3-ethylcarbodiimide hydrochloride and *N*-hydroxysuccinimide in 10 mM acetate (pH 5.0) and covalently linked to the BSA-hapten conjugate according to the manufacturer's instructions. The association rate constant (k_{on}) was determined by measuring the rate of binding to the hapten at three different Fab concentrations (1, 0.6, and 0.4 μM). The dissociation phase (k_{off}) was monitored after the association process was complete. All measurements were conducted in HEPES-buffered saline (pH 7) with a flow rate of 5 μL/min. After each measurement, the chip surface was regenerated with 10 mM HCl. Rate constants were calculated assuming a single-site binding model ($A + B \rightleftharpoons AB$). The apparent dissociation constant ($K_d = k_{off}/k_{on}$) was calculated from the individual association and dissociation rate constants.

Fluorescence Titrations. Thermodynamic dissociation constants were determined as previously described.[49] Titration curves were recorded by stepwise addition of **4** to a diluted solution of the chimeric Fab fragment (25 nM) and subsequent measurement of the fluorescence. The excitation and emission wavelengths were 290 and 340 nm, respectively, and the corresponding band-pass was 8 and 16 nm. The high voltage of the detector was set to 900 V. These measurements were carried out in 20 mM Tris-HCl (pH 9.0) and 100 mM NaCl at 20 °C.

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