Using antibody catalysis to study the outcome of multiple evolutionary trials of a chemical task

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Catalytic aldolase antibodies generated by immunization with two different, but structurally related, β-diketone haptenes were cloned and sequenced to study similarities and differences between independently evolved catalysts. Kinetic and sequence analysis coupled with mutagenesis, structural, and modeling studies reveal that the defining event in the evolution of these catalysts was a somatic mutation that placed a lysine residue in a deep, yet otherwise unrefined, hydrophobic pocket. We suggest that covalent chemistries may be as readily selected from the immune repertoire as the traditional noncovalent interactions that have formed the basis of immunochemistry until this time. Further, we believe that these experiments recapitulate the defining events in the evolution of nature’s enzymes, particularly as they relate to chemical mechanism, catalytic promiscuity, and gene duplication.

One of the most fascinating questions in biology concerns what would happen if evolution started over again. For any system within an organism, the answer must, in part, depend on how many ways there are to accomplish a given task. For example, in the case of protein enzymes, the problem reduces to the purely chemical issue of the potential of the amino acids acting in concert to catalyze a given transformation. If there are many possible routes, multiple solutions to the problem may occur, such as for hydrolysis of the amide bond. However, even where there are multiple solutions, reoccurring motifs are found such as the catalytic triad of the serine proteases. The study of these questions is experimentally difficult because, other than “gedanken” experiments, it is generally not possible to restart the evolution of a system. However, the advent of the process of reactive immunization to generate antibody catalysts allows examination of the outcome of multiple evolutionary trials to accomplish a complicated chemical task by different members of a protein family.

Reactive immunization differs from the more usual immunization procedure in that reactive chemicals are used as immunogens. Thus, the selection parameters of the immune system are switched from simple binding to more complicated chemistry, such as the formation of a covalent bond between the antibody and the antigen. When the selectable chemistry is part of a catalytic mechanism, the result is often evolution of an efficient enzyme in real time (1–5).

Recently, we have used the reactive immunization procedure to generate catalytic antibody aldolases that are remarkably similar in efficiency and mechanism to the natural aldolases (1–3). One essential feature of the antibody aldolases is an active site containing a deeply buried lysine residue with a highly perturbed pKₐ was selected. This reactive lysine interacts with aldol donors to form an enamine that is the nascent carbon nucleophile that adds into the aldol acceptor to form the new carbon-carbon bond yielding a β-hydroxy ketone product with precise control of the stereochemistry at the carbinal carbon. Thus, reactive immunization allowed selection for a complex chemical process that requires, among other parameters: (i) selection of a binding pocket of suitable configuration to interact with two substrates in a bimolecular process; (ii) an active site with an accessible lysine residue; (iii) perturbation of the lysine pKₐ; (iv) the presence of a base; and (v) orchestration of the interaction with water allowing for the formation and hydrolysis of imine intermediates.

In the present work, we challenged the immune system with the same chemical task to study the outcome of multiple evolutionary attempts to create an enzyme. Here, we present our studies of antibody aldolases that exhibit the same enantioselectivity, the same apparent chemical mechanism, but were derived by independent immunization of mice with diketone haptenes 1 and 2 (Fig. 1).

Materials and Methods

Cloning of Antibody Genes. The antibody genes from hybridoma cell lines expressing the catalytic antibodies 38C2, 33F12, 40F12, and 42F1 were cloned as described (6, 7).

Site-Directed Mutagenesis. Lysine 93 in the heavy chain of 33F12 and 40F12 was mutated to an alanine residue by PCR using two overlapping oligonucleotides carrying the desired substitution. PCR was carried out by using the proof-reading Expand High Fidelity PCR System Pow/Taq DNA polymerase mix (Roche Molecular Biochemicals) to minimize PCR errors. The correct sequences of the mutants were confirmed by DNA sequencing.

Subcloning into the pIGG Expression Vector. The pIGG expression vector used here is the expression of whole human IgG in mammalian cells (C.R. and C.F.B., unpublished work). The heavy chain variable domains of the antibodies were PCR-amplified by using Expand High Fidelity PCR System Pow/Taq DNA polymerase mix and antibody-specific primers incorporating the restriction sites required for pIGG cloning. The PCR products were digested with SacI and ApaI and were ligated to SacI/ApaI-digested pIGG vector by using T4 DNA ligase (Life Technologies, Gaithersburg, MD). The ligation reactions were transformed into Escherichia coli strain XL-1 Blue (Stratagene) by electroporation. The light chain variable domains of the antibodies were PCR-amplified and were fused to a human kappa chain by overlap extension PCR using Expand High Fidelity PCR System Pow/Taq DNA polymerase mix and antibody-specific primers incorporating the restriction sites required for pIGG cloning. The PCR products were digested with SpeI and XbaI and were ligated to the vector with the appropriate heavy chain variable domain, prepared by digestion with SpeI and XbaI and 5′-desphosphorylation with alkaline phosphatase from calf intestine (Roche Molecular Biochemicals). The ligation reactions were transformed into XL-1 Blue by electroporation. The correct sequences of the pIGG antibody clones were confirmed by DNA sequencing.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. AF242212–AF242219).

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Expression and Purification of Recombinant Antibodies. The recombinant hybrid mouse/human IgG antibodies were expressed in transiently transfected human embryonic kidney 293 T cells. Near-confluent cells were transfected with pLGG-antibody plasmid DNA by LipofectAMINE PLUS (Life Technologies) according to the procedure recommended by the manufacturer. After transfection, the cells were cultured for 3 days in DMEM (Life Technologies) supplemented with 10% FBS (Life Technologies). Cell culture supernatant was collected and replaced with fresh medium every day. IgG were purified from concentrated cell culture supernatants by affinity chromatography using a 1-ml Protein A HiTrap column (Pharmacia) attached to an FPLC system (Pharmacia). PBS was used as equilibration and washing buffer and 0.5 M acetic acid for elution. Eluted fractions were neutralized immediately by using 0.5 volumes of 1 M Tris-HCl (pH 9.0) and were pooled, concentrated, and exchanged into PBS.

Activity Assays. Aldolase activity of the recombinant antibodies was measured by using the fluorigenic retro-aldol substrate method (8). The reactions were carried out in PBS (pH 7.4), with the substrate diluted from a 5 mM stock solution in acetonitrile to a final concentration of 200 μM. The assays were performed at room temperature in microtiter plates in a reaction volume of 100 μL. An antibody concentration of 1 μM was used. Formation of the aldehyde product was followed with a fluorescence plate reader by monitoring at λex = 330 nm and λem = 452 nm.

Computer-Based Homology Model. The template for the homology model of catalytic antibody 40F12 was selected by sequence comparison to our database of 120 structurally determined Fab's. Specifically, we used the Vλ region of Fab MOPC21 (9) and the VH region of Fab 33F12 (2), which had the greatest sequence identity to the Vλ and VH, respectively, of 40F12. The chosen VL and VH templates also had matching CDR lengths, with the exception of the heavy chain CDR3 loop (H3). Inspection of the database showed that Fab 730.1.4 (10) had an H3 loop with the same length. A least squares fitting of the Cα traces of 33F12 and 730.1.4, 10 residues before and 10 residues after the H3 loop, showed residues H92 and H104 were the points from which the CDR3 structures diverged. Hence, the H3 loop of 730.1.4 was spliced into 33F12 between residues H92 and H104. Where necessary, residues in this preliminary model were mutated to conform to the 40F12 sequence, and the conformations of conflicting sidechains were altered based on the standard rotamer library (11). Finally, the model was refined in two rounds of energy minimization with the program DISCOVER (Biosym Technologies, San Diego). In the first round, the mutated sidechains were minimized with the rest of the model constrained, and in the second round all sidechains were minimized with the backbone constrained.

Results and Discussion

A Second Evolutionary Trial. Our second evolutionary trial began with the synthesis of hapten 2 and the generation of monoclonal antibodies (5). Of 11 antibodies that were shown to catalyze the aldol reaction, antibodies 40F12 and 42F1 catalyzed aldol addition reactions and retro-aldol reactions with enantioselectivities similar to that previously observed for antibodies 33F12 and 38C2 (1–3). The remaining nine catalytic antibodies exhibited a reversal in enantioselectivity, preferentially forming aldols antipodal to those formed with 33F12 or 38C2. These antipodal catalysts are the subject of another report (5). We have restricted this analysis of aldolase antibodies to those catalysts that exhibit the same overall enantioselectivity in their catalysis of aldol reactions.

Antibodies 40F12 and 42F1 were first identified by their ability to react covalently with 2,4-pentanedione to form a stable enamine (UV at λmax, 318 nm) (1). Like antibodies 33F12 and 38C2, 40F12 and 42F1 showed the characteristic enamine absorption maximum at 318 nm after incubation with 2,4-pentanediene (Fig. 2A). The antibodies were then studied for their ability to catalyze a variety of aldol and retro-aldol reactions (Table 1). All antibody catalyzed aldol and retro-aldol reactions followed Michaelis-Menten kinetics and were inhibited by addition of a stoichiometric amount of 2,4-pentanediene. A summary of the kinetic parameters for antibody-catalyzed aldol and retro-aldol reactions is provided (Table 1). These results are consistent with the programming of a reactive amine in the catalytic mechanism (1–3). Further, these antibodies recapitulate the broad catalytic scope that was first observed with aldolase antibodies 33F12 and 38C2.

Sequence Analysis. To study the aldolase antibodies at the level of gene and protein sequence, their cDNAs were cloned, and expressed proteins were characterized. Alignment of aldolase antibody protein sequences shows that the two antibodies 38C2 and 33F12, generated by immunization with hapten 1, are highly homologous, contain the same VH CDR3 sequence and, therefore, originate from the same germline antibody. They differ from one another by 10 amino acid substitutions in VH and 9 in VL as a consequence of somatic mutation (Fig. 3) (2). Similarly, the amino acid sequences of the two antibodies 40F12 and 42F1, generated by immunization with hapten 2, are highly homologous and differ from each other only in 3 positions in VL and in 2 positions in VH. They too are somatic variants of one another. When the sequences of these two sets of antibodies are compared, it is evident that all of the antibodies have high homology in their VH gene segments. Searches of the available databases, including the IMGT database (13), suggest that the heavy chains of both sets of antibodies could be derived from the same germline gene because their nucleic acid sequences were found to be most homologous to the VH22.1 segment (Fig. 3) (2). Similarly, the amino acid sequences of the two antibodies 40F12 and 42F1, generated by immunization with hapten 2, are highly homologous and differ from each other only in 3 positions in VH and in 2 positions in VH. They too are somatic variants of one another.

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A Conserved and Chemically Reactive Lysine. Previous studies of antibodies 33F12 and 38C2 showed that these antibodies use a catalytic mechanism analogous to natural class I aldolase enzymes (1–3). In this mechanism, the $\epsilon$-amino group of a lysine residue located in the active site acts as a nucleophile to form an enamine with a donor ketone substrate, and subsequent reaction with an acceptor aldehyde substrate provides, after hydrolysis of the imine, the aldol product. The crystal structure of 33F12 (2) revealed that Lys 93 of the heavy chain [numbering according to Kabat (13)] contributes the best candidate catalytic $\epsilon$-amino group to the antibody binding pocket. Strikingly, lysine H93 is conserved in all four antibody sequences, although this residue is most typically conserved as alanine. Lysine at position H93 is very unusual, occurring in just 11 known antibody sequences (14). Comparison with the putative germline residue at this position indicates that the occurrence of lysine is the result of a somatic mutation, as no known germline $\text{V}_H$ segment encodes lysine at this position.

For the $\epsilon$-amino group of Lys to be nucleophilic, it must be uncharged. However, the $pK_a$ of the $\epsilon$-amino group in the free amino acid lysine in aqueous solution is 10.5 (15). Because catalytic activity depends on a nonprotonated lysine as a nucleophile and aldolases operate with maximal activity at neutral pH where the $\epsilon$-amino group of lysine normally would be protonated, the $pK_a$ of this group must be perturbed. To approximate the $pK_a$ of the essential lysine, we have used a kinetic assay based on the ability of the antibodies to form enaminones with $\beta$-diketones. We studied the reaction of 2,4-pentanedione with antibody 40F12 and spectrophotometrically monitored the absorption of the antibody-enamine complex at 318 nm. The pH dependence of this reaction is shown and is described by a simple titration curve with a $pK_a$ of 6.0 for 40F12 (Fig. 2B). Previous studies of antibodies 33F12 and 38C2 revealed $pK_a$s of 5.5 and 6.0, respectively, for their reactive lysines (2). The dependence of $k_{cat}/K_M$ as a function of pH for the retro-aldol reaction also showed an acidic limb $pK_a = 6.3–6.9$ for the family of aldol antibodies. This result approximates the pH dependence of catalysis of FDP aldolase (16). Thus, the aldol antibodies exhibit a chemically reactive amine group with a highly perturbed $pK_a$ in their active sites.

Mutational Studies. To confirm the identity of the chemically reactive lysine at position H93, site-directed mutagenesis studies of antibodies 33F12 and 40F12 were performed. Two mutants were constructed in which Lys$^{1093}$ was substituted with Ala. For efficient expression of the antibodies, the variable domains were cloned into a mammalian expression vector in which the heavy and the light chains were expressed from two CMV promoters to give an IgG1 hybrid antibody with a human kappa constant region and human $\gamma_1$ constant region (C.R. and C.F.B., unpublished data). The antibodies were expressed in a human cell line and were secreted into the cell culture medium, from which they were purified by protein A affinity chromatography. Subsequent analysis of the catalytic activity of the mutants and the wild-type hybrid antibodies was carried out by using the aldol sensor method, which, on catalytic conversion, provides a fluorescent product. This retro-aldol reaction was used because its fluorescent readout is the most sensitive indicator of aldolase catalytic activity (8). Analysis of recombinant 33F12 and 40F12 indicated that they maintained the catalytic activity of the hybridoma expressed antibody. However, the corresponding Lys$^{1093}$→Ala mutants of these antibodies showed a complete loss of catalytic activity. These results demonstrate the functional significance of this residue in both antibody families. Although structural alterations are difficult to exclude, the substitution of lysine with alanine is most likely tolerated at this position because alanine is the amino acid most typically found at this position. ELISA binding studies of the Lys$^{1093}$→Ala mutants to haptens 1 and 2 demonstrated that their specific binding to either of these diketones was abrogated. All of the hybridoma-expressed and recombinant aldolase antibodies bind both haptens with high affinity. Thus, these results imply that the loss of activity in the Lys$^{1093}$→Ala mutants is most likely caused by the substitution of a residue essential for the engagement of diketones in a covalent enamine linkage, consistent with the notion that this residue is the key catalytic lysine.

Homology in the Active Sites of Aldolase Antibodies and a Natural Aldolase Enzyme. In previous studies, we have reported the crystal structure of antibody 33F12 and an analysis of its active site. To gain insight into the active site of the 40F12 antibody, we constructed a model of the antibody by sequence comparison of 40F12 to our database of known Fab structures. The crystal structure of 33F12 and the homology model of 40F12 both show the reactive lysine at the bottom of a deep, hydrophobic binding pocket (Fig. 4 A and B) (2). In 33F12 and 40F12, most of the residues within a 5-Å radius of the $\epsilon$-amino group of lysine H93 are hydrophobic (Table 2). Further, the vast majority of these residues are encoded within the germline gene segment $\text{V}_H$ 22.1, with only His$^{135}$ in antibodies 40F12 and 42F1 resulting from somatic hypermutation. The dominant role of the $\text{V}_H$ segment in
shaping the binding sites of these antibodies around the critical lysine accounts for the ability of these two families of antibodies to accomplish the same catalytic task while using very different light chains. The similar architecture of the antibody binding sites in the two families of antibodies suggests that these aldolases function through analogous catalytic mechanisms using LysH93 as the nucleophile. The pKₐ of the ε-amino group of this lysine residue is, therefore, most likely perturbed by the hydrophobic environment of the binding sites present in both antibody families, as we originally proposed for antibody 33F12 (2). This hydrophobic microenvironment disfavors protonation and charge development on the ε-amino group of Lys H93, thereby reducing its pKₐ. To study the relationship of our antibody aldolases with their natural enzyme counterparts, we surveyed the crystal structures of natural aldolase enzymes. Analysis of the x-ray structure of N-acetylneuraminate lyase revealed a deep pocket, which is predicted to be the active site of the enzyme (17). In this enzyme, Lys165 lies at the bottom of the active site pocket in a hydrophobic environment (Fig. 4C) and has been predicted to be the key lysine residue in the catalytic mechanism

**Table 1. Kinetic parameters for antibody-catalyzed aldol and retro-aldol reactions**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Antibody</th>
<th>kcat, min⁻¹⁺</th>
<th>Kₐ, μM⁺</th>
<th>kcat/Km⁻¹⁺</th>
<th>(kcat/Km)/Km⁻²⁺</th>
</tr>
</thead>
<tbody>
<tr>
<td>40F12</td>
<td>33F12</td>
<td>0.0054</td>
<td>33</td>
<td>2.4 × 10⁴</td>
<td>7.2 × 10⁸</td>
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<tr>
<td>40F12</td>
<td>38C2</td>
<td>0.0026</td>
<td>41</td>
<td>1.1 × 10⁴</td>
<td>2.8 × 10⁸</td>
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<tr>
<td>38C2</td>
<td>42F1</td>
<td>0.0067</td>
<td>17</td>
<td>2.9 × 10⁴</td>
<td>1.7 × 10⁹</td>
</tr>
<tr>
<td>40F12</td>
<td>42F1</td>
<td>0.071</td>
<td>203</td>
<td>6.5 × 10⁴</td>
<td>3.2 × 10⁹</td>
</tr>
<tr>
<td>40F12</td>
<td>38C2</td>
<td>0.048</td>
<td>271</td>
<td>4.3 × 10⁴</td>
<td>1.6 × 10⁹</td>
</tr>
<tr>
<td>38C2</td>
<td>42F1</td>
<td>0.21</td>
<td>123</td>
<td>1.9 × 10⁴</td>
<td>1.5 × 10¹₀</td>
</tr>
<tr>
<td>40F12</td>
<td>33F12</td>
<td>0.11</td>
<td>130</td>
<td>6.1 × 10⁴</td>
<td>4.7 × 10⁹</td>
</tr>
<tr>
<td>38C2</td>
<td>42F1</td>
<td>0.21</td>
<td>184</td>
<td>4.6 × 10⁴</td>
<td>2.5 × 10¹⁰</td>
</tr>
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<td>38C2</td>
<td>33F12</td>
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<td>43</td>
<td>1.1 × 10⁴</td>
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<tr>
<td>38C2</td>
<td>33F12</td>
<td>0.11</td>
<td>14</td>
<td>1.0 × 10⁴</td>
<td>7.1 × 10¹⁰</td>
</tr>
</tbody>
</table>

*The kinetic data of kcat and Km (per antibody active site) were obtained in PBS at pH 7.4 by fitting experimental data to nonlinear regression analysis using GRAPIT software.

†Aldol reactions with a unit (M⁻¹).

‡Retro-aldol reactions with a unit (M⁻²).

§Ref. 1 and 2.

¶Ref. 8.

Fig. 3. Amino acid sequence alignment of aldolase antibodies. The framework regions (FR) and complementarity determining regions (CDR) are indicated. Dashes indicate identical amino acids. In contrast to the Vh sequences, the Vl sequences of the four aldolase antibodies are highly related. Databank screening revealed Vh 22.1 as the closest related germline gene.
of this aldolase. As with our aldolase antibodies, a hydrophobic microenvironment may be responsible for tuning the pK_a of the ε-amino group of this lysine residue. The majority of natural aldolase enzymes appear, however, to use an electrostatic mechanism for the perturbation of the pK_a of the ε-amino group of the lysine. This mechanism was first proposed by Westheimer in his studies of another amine-dependent enzyme, acetooacetate decarboxylase (ref. 18 and references therein). Multiple lysine residues are observed in the active sites of a variety of class I fructose 1,6-bisphosphate aldolases, suggesting that electrostatics might account for increased reactivity of the key lysine residue in this common glycolytic enzyme. In rabbit muscle aldolase (19), the distance between the ε-amino groups of Lys229 and Lys146 is 4.9 Å. (Fig. 4D), comparable to that observed for mandelate racemase (20) in which electrostatic effects are also believed to be critical in the perturbation of the pK_a of a key ε-amino group. Comparison of the side chains present within a 5-Å radius of the active site amino group shows the radical differences in the types of side chains found at the active sites of enzymes that use either a hydrophobic or electrostatic mechanism for lysine pK_a perturbation.

**Conclusions**

The formation of antibodies is an evolutionary process that selects on binding energy. If one compares the way that new functions are generated in biology in general to that of the immune system, many similarities are observed. In each case, genotypic parameters that generate new information are coupled to a selection system. The main differences are in the selection parameters and the time line. General evolution uses natural selection to select for function over a long time whereas the immune system uses clonal selection to select for improvement in the binding energy of antibodies in a matter of weeks. Reactive immunization allows one to switch the selection parameters of the immune system to those more like natural selection while preserving the favorable time line of antibody induction. One interesting feature of these experiments is that, because of threshold considerations, there was no assurance that, even if the system were offered the possibility to accomplish complex chemistry, it would be a selectable parameter. For example, in the experiments reported here, the system was set up

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**Table 2. Residues within 5 Å of the Nε group of the catalytic lysine (shown in bold)**

<table>
<thead>
<tr>
<th></th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>LysH93</td>
<td>LysH93</td>
<td>Lys165</td>
<td>Lys229</td>
<td></td>
</tr>
<tr>
<td>SerH35</td>
<td>HisH35</td>
<td>Tyr43</td>
<td>Ala31</td>
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</tr>
<tr>
<td>ValH37</td>
<td>ValH37</td>
<td>Tyr137</td>
<td>Ile77</td>
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</tr>
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<td>TrpH47</td>
<td>TrpH47</td>
<td>Thr167</td>
<td>Lys146</td>
<td></td>
</tr>
<tr>
<td>TyrH95</td>
<td>TyrH91</td>
<td>Gly189</td>
<td>Glu187</td>
<td></td>
</tr>
<tr>
<td>TrpH103</td>
<td>Thr H100d</td>
<td>Ile206</td>
<td>Leu270</td>
<td></td>
</tr>
<tr>
<td>Phel98</td>
<td>TrpH103</td>
<td></td>
<td>Ser300</td>
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</tr>
</tbody>
</table>

A, aldolase catalytic antibody Fab’ 33F12 [PDB ID code 1AXT (2)]; B, homology model of aldolase antibody 40F12; C, molecule 4 of N-acetylneuraminic lyase [PDB ID code 1NAL (17)]; D, molecule D of fructose 1, 6-bisphosphate aldolase from rabbit muscle [PDB ID code 1ADO (19)].
but a single somatic mutation at LysH93 provides a covalent diketone hapten that we have studied here with high affinity, pocket. The pocket in itself is not predisposed to bind the ment. This germline appears to encode a deep hydrophobic pocket. Thus, we find a similar result when covalent chemistry is involved reactions or for rapid evolution to high affinity hapten binding.

Our studies provide a picture of the evolutionary events that lead to the catalytic mechanism and substrate promiscuity observed for this family of catalysts. In both evolutionary trials, a lysine residue appeared early during somatic refinement in germline antibodies using the putative V<sub>H</sub> 22.1 germline segment. This germline appears to encode a deep hydrophobic pocket. The pocket in itself is not predisposed to bind the diketone haptons that we have studied here with high affinity, but a single somatic mutation at Lys<sup>493</sup> provides a covalent chemical solution for high affinity binding. The chemical reactivity that Lys<sup>493</sup> is endowed with in this type of binding site is readily selectable. Once this covalent chemistry appeared, further refinement of the binding pocket did not occur because it afforded no selective advantage to the system. This result complements studies of the ontogeny of other antibody catalysts and of other hapten binding antibodies in which noncovalent mechanisms are used (21–24). These studies have revealed that particular germline genes are poised for catalysis of particular reactions or for rapid evolution to high affinity hapten binding. Thus, we find a similar result when covalent chemistry is involved and suggest that covalent chemistries may be as readily selected from the immune repertoire as traditional noncovalent interactions that have shaped our thoughts regarding immunochimistry. Further, as we have discussed previously (2), we believe that these experiments recapitulate the defining events in the evolution of nature’s enzymes, particularly as they relate to chemical mechanism, catalytic promiscuity, and gene duplication (25, 26).

Thus, one of the key results from this study is that some parameters for aldol catalysts are critical whereas others are more degenerate. Although the four aldolase antibodies described here share the same V<sub>H</sub> germline segment, they exhibit very different V<sub>H</sub> CDR3 and light chain sequences. Although the structural correlates for covalent mechanism within this class of antibodies are now defined, the structural correlates for catalytic efficiency have yet to be uncovered. Nevertheless, we already know that the catalytic efficiencies of antibody aldolases can exceed 10<sup>13</sup> in some cases (5).

By necessity, our study depends on the chemical solutions allowed within an antibody framework. It is possible that alternative solutions might be favored in other protein frameworks. For example, we did not find a major solution to the lysine pK<sub>a</sub> problem that nature most commonly uses. In most natural aldolases, a second lysine appears in the vicinity of the lysine nucleophile and perturbs its pK<sub>a</sub> through electrostatic interactions. It might simply be that this second lysine so destabilizes the antibody fold that it is never seen or that the germline repertoire is poised structurally favoring the hydrophobic mechanism, as it arises from the common use of the same germline V<sub>H</sub> 22.1 segment by all four antibodies. Alternatively, the appearance of a second lysine may be a rarer event, and our sample size may have been too small to observe it.

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