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Chemically Reactive Immunogens Lead to Functional Convergence of the Immune Response

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Abstract: An aldolase antibody, 24H6, which was obtained from immunization with the large hapten **2**, is shown to possess an active-site lysine residue with a perturbed pK_a of 7.0. This antibody catalyzes both the aldol addition and the retrograde aldol fragmentation with a broad range of substrates that are structurally different from the hapten. This observation suggests that in reactive immunization with 1,3-diketones, the hapten structure governs the chemistry but not the overall organization of the active site. Hammett correlation studies of the 38C2- and 24H6-catalyzed aldol and retroaldol reactions revealed that although the two antibodies exhibit broad substrate specificities, they utilize slightly different mechanisms. While antibody 38C2 adopts a mechanism that is reminiscent of an acid-catalyzed aldol reaction, antibody 24H6 follows a mechanism that is similar to the base-catalyzed reaction.

Introduction

The recently introduced technology of reactive immunization[1] employs a chemically reactive group in the hapten to elicit an appropriate functionality for a desired reaction mechanism. This approach is very different from the traditional immunization process, in which a chemically inert hapten induces only structural complementarity in the antibody. Consequently, reactive immunization offers unprecedented strategies to learn about the evolution and the optimization processes of the immune response. Furthermore, this technology presents new opportunities in chemical immunology in general, and in the field of catalytic antibodies in particular.[2]

For example, the 1,3-diketone functionality was designed as a chemical trigger to evoke covalent bonding between the hapten and an active-site lysine residue via formation of a vinylogous amide.[1a] Thus, hapten **1** was used to elicit the aldolase antibodies, 38C2 and 33F12.[1a] One of the most remarkable features of these antibodies is their ability to accept a broad range of substrates.[3] It has been postulated that this feature reflects termination of the immunization process before the

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(1) (a) Wagner, J.; Lerner, R. A.; Barbas, C. F., III. *Science* **1995**, *270*, 1797. (b) Wirsching, P.; Ashley, J. A.; Lo, C. H. L.; Janda, K. D.; Lerner, L. A. *Science* **1995**, *270*, 11775.

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(3) (a) Hoffmann, T.; Zhong, G.; List, B.; Shabat, D.; Anderson, J.; Gramatikova, S.; Lerner, R. A.; Barbas, C. F., III. *J. Am. Chem. Soc.* **1998**, *120*, 2768. (b) Zhong, G.; Shabat, D.; List, B.; Anderson, J.; Sinha, S. C.; Lerner, R. A.; Barbas, C. F., III. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 2481.

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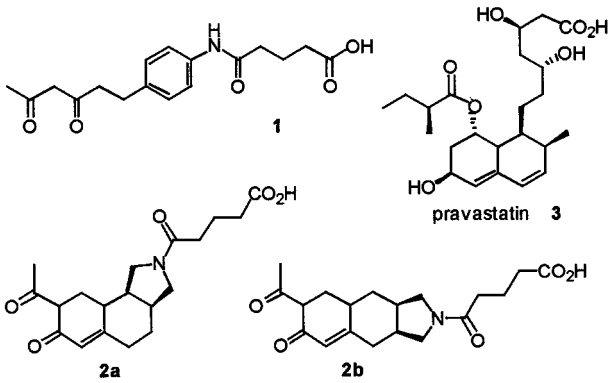

antibody shape complementarity has been fully optimized via somatic mutations.[4]

This hypothesis raises two interesting questions concerning the evolution of a binding site against a reactive immunogen. The first question refers to the relative importance of the hapten general shape vs the reactivity of its chemical trigger. The second question is even more intriguing: Can one intercept the immune response at a desired stage of the optimization process by fine-tuning of the hapten reactivity?

Here we address the first question, while studies related to the second one are underway. We report that an aldolase antibody, 24H6, generated against the large hapten **2** (mixture of isomers), prefers substrates that are structurally different from the hapten. Furthermore, we show, on the basis of Hammett correlations, that the catalytic mechanisms of antibodies 24H6 and 38C2 are different. These Hammett correlations, together with the substrate specificities of both antibodies, suggest that the hapten structure has little bearing on an immune response that is dominated by the covalent bond formation.

Results and Discussion

One way to address the first question mentioned above is to carry out a comparative experiment in which reactive immunization is carried out with haptens that have similar chemical triggers but different molecular shapes. Accordingly, we designed a group of isomeric haptens, **2**, which contain the 1,3-diketone trigger. The tricyclic ring system of **2** is substantially different in terms of molecular shape and electron distribution from the flexible aliphatic chain with an aromatic ring that characterizes hapten **1**. Anti-**2** aldolase antibodies could be useful catalysts for the synthesis of the bicyclic portion of various mevinic acids, such as pravastatin, **3**, which are used effectively to treat hypercholesterolemia.

Hapten Synthesis. The hapten molecules **2a** and **2b** were prepared from tetrahydrophthalimide **4** (Scheme 1). Lithium aluminum hydride (LAH) reduction produced the corresponding secondary amine, which was protected with di-*tert*-butyl dicarbonate (BOC anhydride) to afford compound **5**. Oxidative hydroboration of the double bond using borane−dimethyl sulfide and basic hydrogen peroxide produced alcohol **6**, which was oxidized with PCC to give the corresponding ketone **7**. The enamine formed from **7** and morpholine underwent Robinson annulation with methylvinyl ketone to produce a racemic mixture of two isomeric tricyclic enones, **8a** and **8b**. Kinetic enolization of **8** was carried out by selective deprotonation with lithium diisopropylamide. The resultant mixture of isomeric enolates was treated with acetyl chloride to produce the endione derivatives **9a** and **9b**.[5] Finally, deprotection of the amine and reaction with glutaric anhydride produced the racemic hapten as a mixture of two structural isomers, **2a** and **2b**.

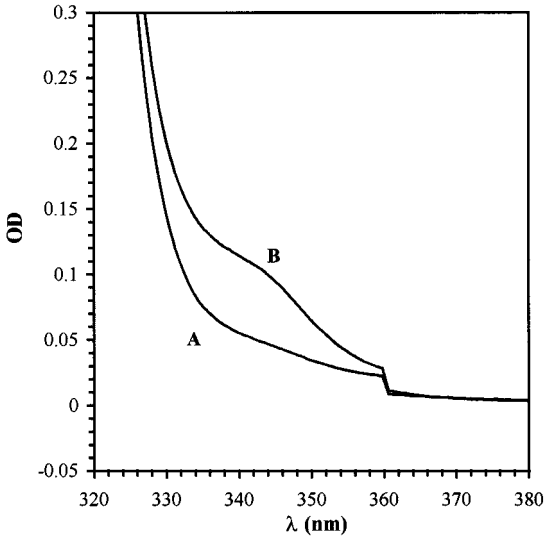

Figure 1. Irreversible inhibition of antibody 24H6 by reductive alkylation.

Immunization and Screening. The hapten was conjugated with carrier proteins, keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA), using known procedures.[6] 129G^{IX+} mice were immunized with the KLH conjugate, and monoclonal antibodies were produced using common protocols.[7] Twenty-four antibodies that exhibited the highest affinities toward the BSA conjugate were produced from ascites fluid.[8] Antibodies from each cell line were purified by ammonium sulfate precipitation followed by protein G affinity chromatography.

All antibodies were screened for their ability to produce the typical UV absorption of the proposed vinylogous amide with 1,3 diketones[1a] [λ_{max} = 316 nm (ϵ = 15 000) for acetylacetone, 335 nm (ϵ = 9100) for 3-methylpentane-2,4-dione,[9] and 340 nm (ϵ = 14 400) for 2-acetylcyclohexanone]. One antibody, 24H6, was chosen for further studies on the basis of its specific interaction with 2-acetylcyclohexanone. This specific interaction suggests the presence of an active-site lysine residue. Antibody 24H6 was further purified by ion-exchange chromatography on a Mono-Q column.

To confirm the presence of a lysine residue within the antibody binding site, we attempted irreversible reductive alkylation of that primary amine group.[10] Thus, antibody 24H6 was first incubated with acetone and then treated with Na(CN)BH_3. Indeed, this treatment followed by extensive dialysis against buffer solution produced a chemically modified antibody that was unable to exhibit the above-mentioned 340-nm absorption upon incubation with 2-acetylcyclohexanone (Figure 1A). In a

(4) Baltimore, D. *Cell* **1981**, *26*, 295.

(5) Under the same conditions, a simpler model compound, cyclohex-2-en-1-one, was acetylated to produce 6-acetylcyclohex-2-en-1-one as the sole product. ^{1}H NMR (enol form): 6.7 (dt, J = 10.0, 5.5 Hz, 1H), 6.0 (dt, J = 10.0, 1.8 Hz, 1H), 2.5 (t, J = 7.0 Hz, 2H), 2.1 (s, 3H).

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(9) Barbas, C. F., III; Heine, A.; Zhong, G. Hoffmann, T.; Gramatikova, S.; Björnestedt, R.; List, B.; Anderson, J.; Stura, E. A.; Wilson, I. A.; Lerner, R. A. *Science* **1997**, *278*, 2085.

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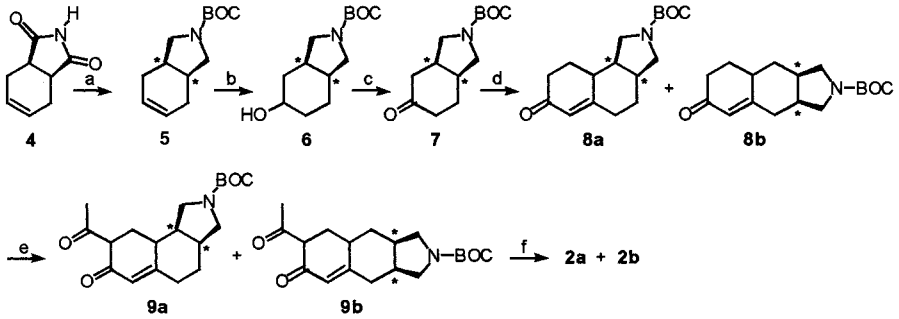
Scheme 1. Synthesis of Hapten **2**[a]



[a] Key: (a) (i) LAH, THF, ether; (ii) BOC anhydride, Et_3N, THF. (b) (i) BH_3−DMS; (ii) MeOH; (iii) NaOH, H_2O_2. (c) PCC, CH_2Cl_2, Celite. (d) (i) morpholine, toluene, azeotropic distillation; (ii) methylvinyl ketone, benzene, reflux; (iii) MeOH/H_2O (1:1), reflux. (e) LDA, HMPA, acetyl chloride, THF. (f) (i) TFA, CH_2Cl_2; (ii) glutaric anhydride, pyridine. The * symbol represents relative stereochemistry, not absolute configuration.

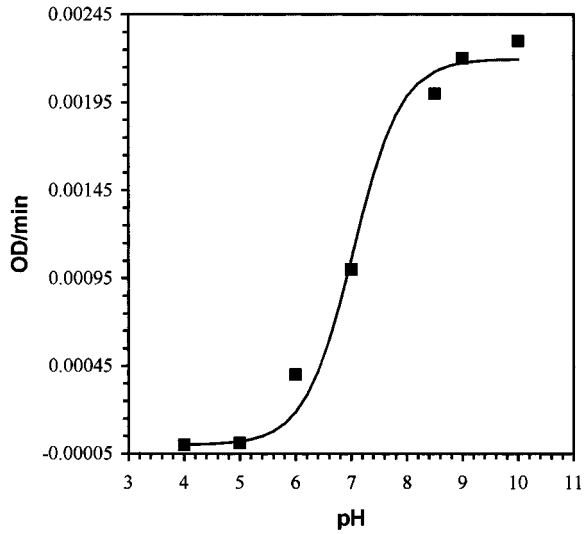


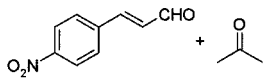
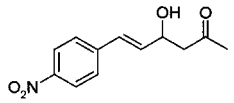
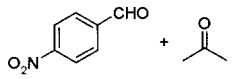
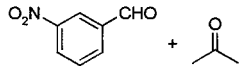
Figure 2. Determination of the pK_a of the active-site lysine in 24H6. The line represents the equation $y = y_{max}/(1 + 10^{(pKa - pH)})$. A p$K_a$ value of 7.03 was derived from this equation. The standard error was 0.0001235.

control experiment, antibody 24H6 was treated with Na(CN)BH_3 in the absence of acetone. This treatment did not impair the antibody's ability to generate the characteristic UV absorption with 2-acetylcyclohexanone (Figure 1B).

Determination of the pK_a of the Active-Site Lysine. Evaluation of the pK_a of the active-site lysine was based on the rate of enaminone formation with 2-acetylcyclohexanone (Figure 2).[9] The incubation mixtures contained 10 μM antibody and 833 μM diketone in 0.05 M phosphate−citrate buffer in a pH range between 4 and 10. The pH dependence of this reaction showed that the active-site lysine has a perturbed pK_a of approximately 7. This value is higher than the pK_a values of 5.5 and 6 previously observed for the aldolases 38C2 and 33F12. This observation could reflect the relative positioning of the lysine residues within the active sites. In antibodies 38C2 and 33F12, the lysine residue is located in the framework region before the complementarity-determining region (CDR), H3 (Lys H93). According to X-ray crystallographic data, the residue is buried deep in a hydrophobic pocket. The observed higher pK_a of the active-site lysine residue in 24H6, as compared with 38C2, probably implies that this lysine is located in a more solvent exposed, less hydrophobic environment (vide infra).

Catalysis of Aldol and Retroaldol Reactions. It has been shown that the lysine antibodies, 38C2 and 33F12, can catalyze both the aldol addition and the retroaldol fragmentation. We found that our antibody, 24H6, also catalyzes these reactions. In all cases, catalysis followed Michaelis−Menten kinetics and was completely inhibited by acetylcyclohexanone. The kinetic parameters (apparent K_M and apparent k_{cat}) for representative substrates are listed in Table 1.

Table 1. Kinetic Data for the Antibody 24H6-Catalyzed Aldol and Retrograde Aldol Reactions[a]

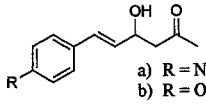
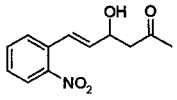
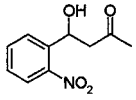
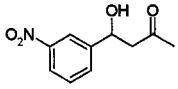
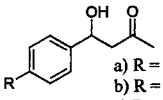
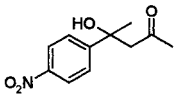
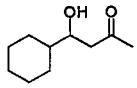
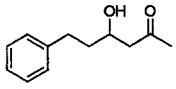
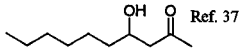
Entry	Substrates	K_M (mM)	k_{cat} (sec^{-1})
1		4.7 ± 1.8	$2.5\cdot10^{-4} \pm 6\cdot10^{-6}$
2		9.2 ± 4.8	$1.2\cdot10^{-4} \pm 6\cdot10^{-5}$
3		1.7 ± 0.16	$5.0\cdot10^{-4} \pm 5\cdot10^{-5}$
4		2.4 ± 0.36	$9.3\cdot10^{-4} \pm 1\cdot10^{-5}$

[a] All reactions were carried out in phosphate-buffered saline (50 mM phosphate, 100 mM NaCl, pH 7.4) containing 10% organic solvent (a 1:1 mixture of acetonitrile and acetone in the aldol reactions and pure acetonitrile in the retrograde aldol reactions). Active site concentrations ranged from 10 to 100 μM, and substrate (aldehyde) concentrations ranged from 0.5 to 5.0 mM. The progress of the reaction was monitored by HPLC. The K_M values refer to the aldehyde/aldol substrates.

The relative rates of the aldol and retroaldol reactions catalyzed by 24H6, in addition to the corresponding data reported for antibodies 38C2 and 33F12,[3] provide an interesting opportunity to learn about the relevance of the hapten size and shape to the actual size and shape of the resultant catalytic antibody. Although both haptens used to elicit 24H6 and 38C2 have the same chemical trigger, the one used for 24H6 is significantly larger than the one used to raise 38C2. If the structure of the hapten had a significant influence on the dimensions of the resultant active site, then antibody 24H6 should exhibit a high preference for larger aliphatic substrates than those preferred by 38C2. Conversely, if both antibodies exhibit similar substrate selectivities, then one could conclude that the hapten structure has only a small influence in shaping the active site.

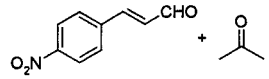
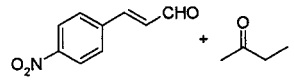
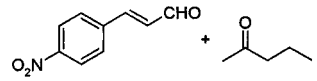
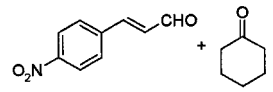
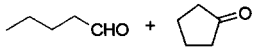
We first studied the aldol addition and the retroaldol fragmentation reactions with acetone and a variety of aliphatic as well as aromatic aldehydes (Table 2). In another set of experiments, we studied the aldol additions of a variety of ketones to *p*-nitrocinnamaldehyde and the corresponding retroaldol fragmentation reactions (Table 3).

Table 2. Substrate Specificities for the 24H6-Catalyzed Aldol Addition and Retrograde Aldol Fragmentation Reactions with Acetone and Various Carbonyl Acceptors[a]

Entry	Substrates	Retro $10^3 \cdot k_{cat}/K_M$ ($sec^{-1}M^{-1}$)	Aldol $10^3 \cdot k_{cat}/K_M$ ($sec^{-1}M^{-1}$)
1	 a) R = NMe_2	6.7	*b*
	b) R = OMe	2.6	0.35
	c) R = H	0.7	0.78
	d) R = Cl	2.9	20
	e) R = CF_3	1.1	23
	f) R = NO_2	12.5	53
2		1.8	14.5
3		0	62.5
4		1.4	389
5	 a) R = NO_2	4.5	294
	b) R = Br	0.3	190
	c) R = NMe_2	2.8	*b*
6		3.4	0
7	 Ref. 36	0	0
8		0	0
9	 Ref. 37	0	0

[a] The kinetic parameters, K_M and k_{cat}, were elucidated from Lineweaver−Burk plots of the appropriate kinetic data, as described in Table 1. The error limit is approximately 15%. [b] Not determined.

Table 3. Substrate Specificities for the 24H6-Catalyzed Aldol Addition and Retrograde Aldol Fragmentation Reactions with Various Carbonyl Donors[a]

Entry	Substrates	Retro $10^3 \cdot k_{cat}/K_M$ ($sec^{-1}M^{-1}$)	Aldol $10^3 \cdot k_{cat}/K_M$ ($sec^{-1}M^{-1}$)
1		12.5	53
2		1.4[c]	17[b]
3		2.4[c]	0
4		2.5	10.8
5	 Ref. 3a	0	0

[a] The kinetic parameters, K_M and k_{cat}, were elucidated from Lineweaver−Burk plots of the appropriate kinetic data, as described in Table 1. The error limit is approximately 15%. [b] Total value for all three possible isomeric products. [c] This value is for the pure kinetic aldol substrate.

The data in Tables 2 and 3 point at interesting specificity trends that characterize antibody 24H6. This catalyst exhibits a remarkable preference for unsaturated and aromatic aldehydes over saturated aldehydes, which is observed in both the aldol and the retroaldol reactions. Furthermore, both reactions are strongly influenced by steric effects in the substrate. For example, the ortho-substituted aromatic substrates react more slowly than their para-substituted counterparts (Table 2, entries 1f and 2; 3 and 5a). It is clear that both the aldol and retroaldol reactions using acetone as the donor are preferred over those with the sterically larger ketones, such as butanone, pentanone, and cyclohexanone (Table 3, entries 1−4).

Interestingly, antibody 24H6 shows high selectivity for aromatic substrates that are different from its aliphatic hapten. Likewise, antibody 38C2 is also known to accept substrates that are structurally different from its relatively small hapten. Again, these observations show that, as was initially proposed for reactive immunization, when the covalent event appears early in the evolution process, further refinement of the binding pocket ceases.[9] Apparently, the structure of the hapten does not determine precisely the shape of the antibody binding site.

Linear Free Energy Relationships. The aldol condensation is a nontrivial multistep reaction that is catalyzed either by bases or by acids as well as by metals.[11] Linear free energy relationship studies of biocatalyzed aldol and retroaldol reactions are useful because they can teach us about charge development in the transition state.[12] The three major events in the antibody-catalyzed retroaldol fragmentation are formation of a Schiff base intermediate, deprotonation of the alcohol group by a general base, and cleavage of the C−C bond. Since all processes concerning formation or fragmentation of the Schiff base intermediate do not occur in conjugation with the aromatic moiety of the substrate, they are not expected to affect the Hammett correlations for the antibody-catalyzed reactions. Additionally, it has already been shown that the reversible formation of Schiff base intermediates with antibody 38C2 may be considered as a rapid preequilibrium.[13] Since protonation and deprotonation of heteroatoms are known to be fast steps, the

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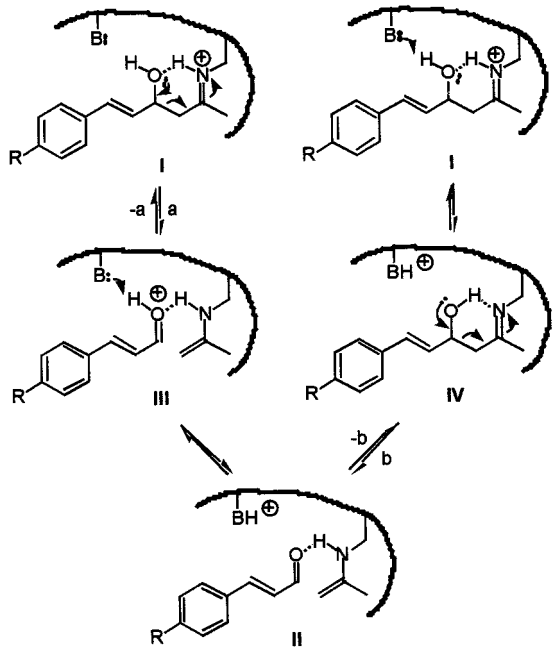
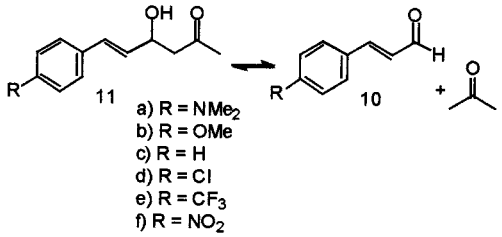
Scheme 2. Mechanistic Pathways of the Aldol and Retrograde Aldol Reactions



Scheme 3



rate-determining step in the retrograde aldol reaction sequence is the cleavage of the carbon−carbon bond (**I** to **II**, Scheme 2).[14] Thus, one of the key issues concerns the timing of the proton abstraction by a general base.

The relative timing of the proton abstraction and the C−C bond cleavage could define two mechanistic pathways for the antibody-catalyzed retrograde aldol reaction, leading from the protonated Schiff base **I** to the substituted cinnamaldehyde **10** and acetone (Schemes 2 and 3): (a) initial C−C bond cleavage to produce intermediate **III**, followed by deprotonation, and (b) deprotonation of **I**, leading to intermediate **IV**, followed by C−C bond cleavage to form **II**. If pathway (a) dominates, then accumulation of positive charge in the transition state is expected. By contrast, if the reaction follows pathway (b), then this transformation will involve the neutral intermediate **IV**. Mechanistic studies via linear free energy relationships could allow for useful comparison of the catalytic machinery in aldolase antibodies and enzymes and teach us about the structural parameters of their active sites.

All antibody-catalyzed reactions with substrates **11a**−**f** (Scheme 3) were carried out in phosphate-buffered saline (PBS), pH 7.4, 26 °C, and monitored by HPLC. The kinetic parameters (apparent k_{cat} and apparent K_M, Tables 4 and 5) were extracted from Lineweaver−Burk analyses of the kinetic data.

We first compared the catalytic behavior of antibodies 24H6 and 38C2 in the retroaldol fragmentation reaction, as reflected by the correlation between log(k_{cat}) and the Hammett substituent constant, σ.[15] As can be seen from Tables 4 and 5, the Hammett

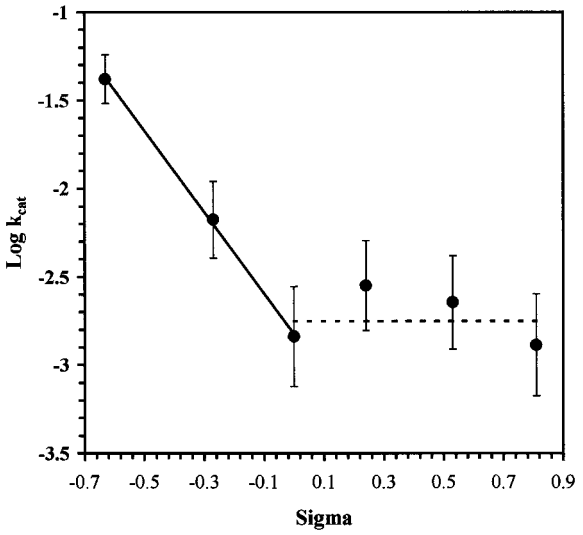


Figure 3. Hammett correlation for the 38C2-catalyzed retroaldol fragmentation.The linear correlation for the electron-rich substrates is represented by $Y = -2.31X - 1.05$ ($R^2 = 0.99$).

correlations between log(k_{cat}) and σ for the two antibodies are different. For antibody 38C2 (Figure 3) the correlation is not linear.[16] In general, as we proceed from electron-donating toward electron-withdrawing substituents, reaction rates decrease. With electron-rich systems, the reaction rate is very sensitive to the nature of the substituent ($\rho = -2.3$). However, with electron-deficient systems, reaction rates are essentially independent of the substituent ($\rho \approx 0$). The break in the Hammett line points at a change in mechanism when going from one group of substituents to the other.[17] The negative Hammett correlation coefficient exhibited by the electron-donating substrates, **11a**−**c**, points at a positively charged transition state. Such a transition state is expected for the transformation of **I** into **III**, supporting the intermediacy of **III** for these substrates. In contrast, with electron-deficient substrates, **11d**−**f**, k_{cat} values for this antibody are essentially independent of the substituent R. This observation stands in agreement with mechanistic pathway (b) (Scheme 2), where changes in the charge density in the transition state are not affected by the aromatic group for lack of conjugation. Under such circumstances, ρ is expected to be essentially zero. The reason for the change in mechanism may stem from the fact that the positively charged transition state leading to intermediate **III** is no longer stabilized by the electron-withdrawing substituents on these substrates.

The above-described interpretation of the experimental results for substrates **11a**−**c** with antibody 38C2 is supported by literature precedents on the acid-catalyzed retrograde aldol reaction. For example, the fragmentation rate of 4-phenyl-4-hydroxy-3-methylbutan-2-one to benzaldehyde and butanone was qualitatively reported to be highly sensitive to the *p*-substituent on the aromatic ring.[18] This high sensitivity was explained by formation of a protonated aldehyde and an enol as the initial products. Conversely, the base-catalyzed retrograde aldol reaction is expected to be essentially independent of the nature of the substituent because changes in charge density (neutralization of the alkoxide anion) occur on an atom that is

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Table 4. Kinetic Parameters for 24H6-Catalyzed Retroaldol and Aldol Reactions[a]

	retroaldol		aldol	
R	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
NMe_2	1.9 ± 0.1	$1.3 \times 10^{-5} \pm 6.6 \times 10^{-7}$		
OMe	4.1 ± 0.5	$1.0 \times 10^{-5} \pm 1.3 \times 10^{-6}$	3.9 ± 0.4	$1.4 \times 10^{-6} \pm 1.4 \times 10^{-7}$
H	3.7 ± 1.1	$2.6 \times 10^{-6} \pm 8.1 \times 10^{-7}$	8.5 ± 2.5	$6.7 \times 10^{-6} \pm 2.0 \times 10^{-6}$
Cl	3.0 ± 0.2	$8.5 \times 10^{-6} \pm 7.3 \times 10^{-7}$	1.2 ± 0.2	$2.5 \times 10^{-5} \pm 2.5 \times 10^{-6}$
CF_3	1.7 ± 0.1	$2.0 \times 10^{-6} \pm 9.1 \times 10^{-7}$	2.5 ± 0.2	$5.7 \times 10^{-5} \pm 5.0 \times 10^{-6}$
NO_2	9.2 ± 4.7	$1.1 \times 10^{-4} \pm 6.0 \times 10^{-5}$	4.7 ± 1.8	$2.5 \times 10^{-4} \pm 6.7 \times 10^{-6}$

[a] The K_M values refer to the aldehyde/aldol substrates.

Table 5. Kinetic Parameters for 38C2-Catalyzed Retroaldol and Aldol Reactions[a]

	retroaldol (ref 16)		aldol	
R	K_M (mM)	k_{cat} (s^{-1})	K_M (mM)	k_{cat} (s^{-1})
NMe_2	1.25 ± 0.3	0.041 ± 0.01		
OMe	0.52 ± 0.06	$6.7 \times 10^{-3} \pm 6.7 \times 10^{-4}$	0.31 ± 0.03	$3.2 \times 10^{-4} \pm 3.3 \times 10^{-5}$
H	0.42 ± 0.06	$1.5 \times 10^{-3} \pm 1.6 \times 10^{-4}$	0.057 ± 0.004	$1.6 \times 10^{-4} \pm 8.3 \times 10^{-6}$
Cl	0.32 ± 0.07	$2.8 \times 10^{-3} \pm 2.5 \times 10^{-4}$	0.054 ± 0.005	$6.8 \times 10^{-4} \pm 2.6 \times 10^{-5}$
CF_3	0.24 ± 0.06	$2.3 \times 10^{-3} \pm 2.3 \times 10^{-4}$	0.041 ± 0.003	$1.7 \times 10^{-3} \pm 2.5 \times 10^{-4}$
NO_2	0.14 ± 0.01	$1.3 \times 10^{-3} \pm 1.0 \times 10^{-4}$	0.009 ± 0.002	$3.0 \times 10^{-4} \pm 1.6 \times 10^{-5}$

[a] The K_M values refer to the aldehyde/aldol substrates.

not conjugated with the substituted aromatic ring. Indeed, we measured the base-catalyzed rates of the retrograde aldol reaction with all substrates **11a**−**f** and found no significant differences.[19]

In contrast to the results obtained with antibody 38C2,[16] the reaction rates for the antibody 24H6-catalyzed retroaldol fragmentation reaction were found to be essentially independent of the substituent R (with one exception, substrate **11f**). We conclude that antibody 24H6 operates with all substrates via the above-described mechanism proposed for 38C2 with the electron-deficient substrates. This mechanism does not involve accumulation of charge on the acceptor carbonyl carbon in the transition state (Scheme 2, pathway b). The preference of antibody 24H6 to promote pathway (b) could stem from specific structural parameters of the active site. For example, one could envision electrostatic destabilization of a positively charged transition state by a positively charged residue, which would discourage pathway (a). Additionally, close proximity of a general base residue to the hydroxyl group in **I** could further encourage pathway (b). These hypotheses will probably be verified by future crystallographic studies with 24H6.

Next, we studied the substituent effect in the antibody-catalyzed aldol addition reactions with both antibodies. We found that with 24H6, the correlation between $\log(k_{cat})$ and σ is linear with a positive slope ($\rho = 2.0$) (Figure 4). This observation suggests that a negative charge is being developed in the transition state of this reaction. A similar slope has been reported for the nucleophilic addition of HCN to benzaldehyde[20] ($\rho = 2.3$), which also indicated the development of a partially negatively charged transition state. Conversely, the rate of the 38C2-catalyzed aldol addition reaction was found to be essentially independent of the substituent R.

It is known that while the rate of the acid-catalyzed aldol addition of ketones to substituted benzaldehydes is nearly independent of the nature of the substituent, the base-catalyzed reaction is faster with electron-deficient aldehydes.[21] We

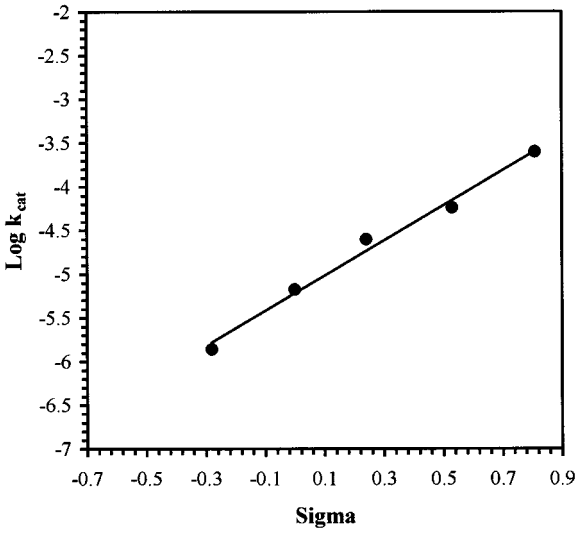

Figure 4. Hammett correlation for the 24H6-catalyzed aldol addition. The linear correlation is represented by $Y = -2.0X - 3.4$ ($R^2 = 0.99$).

therefore conclude that the 24H6-catalyzed process is reminiscent of the base-catalyzed aldol addition in which formation of the new C−C bond (step −b in Scheme 2) precedes protonation. However, in the 38C2-catalyzed reaction, protonation of the aldehyde precedes the rate-limiting step of C−C bond formation (step −a in Scheme 2), resulting in a negligible ρ value.

We observed that in both the aldol and retroaldol reactions catalyzed by antibody 38C2, $\log(K_M)$ is inversely proportional to σ ($\rho = -1.2$, -0.6, respectively, Figures 5 and 6). We therefore conclude that the interactions between the substrates' aromatic portion and antibody 38C2 contribute significantly to the binding phenomenon, probably via π-stacking and hydrophobic interactions with electron-rich amino acid side chains such as tyrosine and tryptophan. In fact, the recently reported crystal structure of a closely related aldolase antibody, 33F12, and the amino acid sequences of both 38C2 and 33F12 have confirmed that the binding pockets of these antibodies are, indeed, very rich in aromatic residues, and in tyrosines and tryptophanes in particular.[9] Moreover, considering the fact that the active site does not have positively charged residues other than the lysine group, the hypothesis of π-stacking and

(19) All reactions were carried out in carbonate buffer (25 mM, pH 10.05) containing 10% acetonitrile. The progress of the reactions was monitored by HPLC. Substrate, relative rate: **1a**, 1.6; **1b**, 1.3; **1c**, 1.5; **1d**, 1.1; **1e**, 1.4; **1f**, 1.0.

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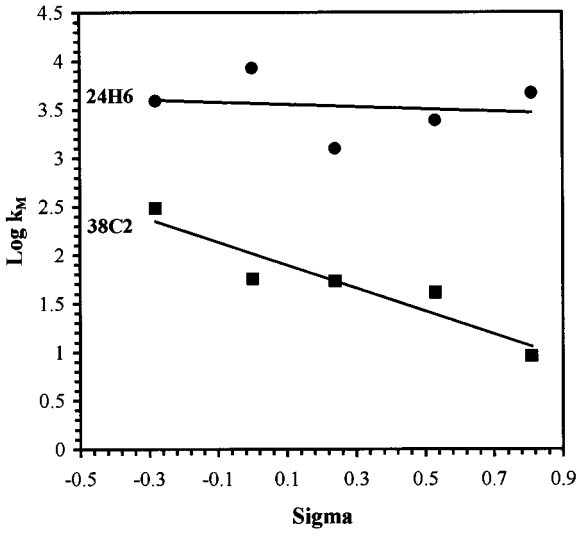

Figure 5. Log K_M vs σ for the aldol addition reaction with antibodies 24H6 and 38C2. The linear correlations are represented by the following: for 38C2, $Y = -1.19X + 2.02$ ($R^2 = 0.87$); for 24H6, $Y = -0.12X + 3.57$ ($R^2 = 0.03$).

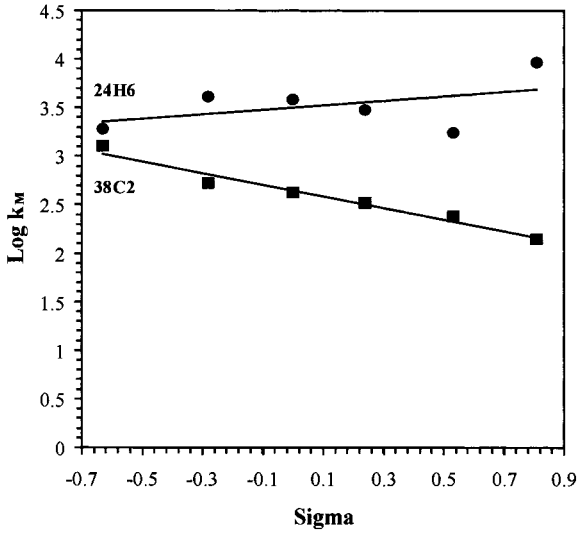

Figure 6. Log K_M vs σ for the retroaldol fragmentation reactions with antibodies 24H6 and 38C2. The linear correlations are represented by the following: for 38C2, $Y = -0.60X + 2.65$ ($R^2 = 0.97$); for 24H6, $Y = -0.23X + 3.50$ ($R^2 = 0.2$).

hydrophobic interactions rather than elctrostatic interaction between the protein and the substrate is further reinforced. Positive charge stabilization by electron-rich aromatic residues has been widely observed in antibodies and enzymes.[22] A recent example of this phenomenon is the active site of a polyene cyclase catalytic antibody which was designed to reduce the positive charges that develop along the reaction coordinate.[23]

Interestingly, the log(K_M) values of the same reactions catalyzed by antibody 24H6 are independent of σ ($\rho \approx 0$). Considering the fact that the K_M values in the 38C2-catalyzed reactions are approximately 1−2 orders of magnitude smaller than the values observed in the reactions catalyzed by 24H6, it is not surprising that the substituent effect is negligible for the loosely bound substrates in 24H6. The larger K_M values for most substrates with 24H6 as compared with 38C2 may, again, reflect the relative positioning of the catalytic lysine residue within the active site.

Cloning, Sequence Analysis, and Production of Antibody 24H6 Fab. In general, the antibody combining site is predominantly comprised of six CDRs, with the antibody light and heavy chains each contributing three CDRs. While the light chain is the product of two fused genes, V_L (variable) and J_L (joining), the heavy chain is coded for by three genes, V_H, D (diversity), and J_H. The heavy chain also typically displays randomly inserted nucleotides (termed P and N nucleotides) at the junction of each gene segment. The amino acid sequences of the variable heavy (V_H) and variable light (V_L) chains of antibody 24H6 are shown in Figure 7. On the basis of the homology between 24H6 and published sequences, it is possible to identify likely germline gene candidates.[24] Antibody 24H6 employs a λ light chain composed of a variable region, which is highly homologous with the $V_{\lambda 1}$ gene of Balb/c[25] fused to the $J_{\lambda 1}$ gene. The mature antibody gene possesses six amino acid differences relative to Balb/c $V_{\lambda 1}$: Y34F, P42S, T47S, S54Y, A57P, and F89L. It is interesting to note that three of the differences occur in the hypervariable loops, Y34F in CDR1 as well as S54Y and A57P in CDR2. At this point, it is not possible to determine whether these differences originate from somatic mutations or from strain differences.

Inspection of the V_H region of 24H6 reveals that this catalytic antibody uses a member of the V_H subgroup 2 family.[26] Comparing the V_H chain of 24H6 to all of the reported V_HII germline genes reveals that the V_H nucleotide sequence is 91% identical to the Balb/c germline gene H10.[27] Two other Balb/c germline genes were found from the same family, which also had high homology to the 24H6 V_H: H4a-3 is 88% identical, while H2b-3 is 87% identical.[27] While a definitive germline assignment cannot be made at this time, it seems likely that the 24H6 V_H gene originated from a $129G^{IX+}$ H10-like germline gene. The 24H6 V_H gene was rearranged onto a D minigene with high homology to the Balb/c gene DFL16.1, followed by the J_H2 gene.

Another antibody, 17.2.25, uses V_L and V_H chains with high homology to 24H6.[28] This antibody was raised in Balb/c mice against the unrelated hapten (4-hydroxy-3-nitrophenyl)acetyl (NP). The 24H6 light chain is 95% identical and 96% similar to the V_λ chain used by 17.2.25.[29] The 17.2.25 heavy chain is 82% identical and 90% similar to that of 24H6. Remarkably,

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(24) An unambiguous assignment of the germline gene progenitor of 24H6 would require sequencing of the 5′ untranslated regions of candidate germline genes from $129G^{IX+}$ (the inbred strain of mouse from which the antibody was isolated) and comparison with the 5′ untranslated region of the rearranged hybridoma DNA.

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(28) Loh, D. Y.; Bothwell, A. L. M.; White-Scharf, M. E.; Imanishi-Kari, T.; Baltimore, D. *Cell* **33**, *85*, 1983.

(29) As no published sequence for the mature light chain of 17.2.25 is available, we used the germline sequence (ref 25).

a. Heavy Chains

```
                                          CDR1                    CDR2
H10(Balb/c) EVQLQQSGAELVKPGASVKLSCTASGFNIK DTYMH WVKQRPEQGLEWIG RIDP**ANGNTKY
VH 17.2.25  ------------R-------------P--- ----- -------------- ----**-------
VH 24H6           ---D----------A--------- ---IQ ------G---D--- G---**-T-KF-C
VH 38C2         LE--GG--Q--GTM----EI--LTFR NYW-S --R-S--K----VA E-RLRSD-YA-H-
VH 33F12        LE--GG--Q--GSM----VV--LTFS RFW-S --R-S--K----VA E-RLKSD-WA-HT

                                                  CDR3
DPKFQG KATITADTSSNTAYLQLSSLTSEDTAVYYCAR
------ -----V---T---------------------- YYRYPYYAMDY WGQGTSVTVSS
G----- ---L----------V----------------- --KF--FS--- -----------
AESVK- -F--SR-D-KSRL---MN--RT---GI---KT -FYSFS-**** -----L-----
AESVK- -F--SR-D-KSRL---MN--RT---GI---KI -FYSFS-**** -----L-----
```

b. Light Chains

```
                                    CDR1                      CDR2
V_1(Balb/c) ISQAVVTQESALTTSPGETVTLTC RSSTGAV**TTSNYAN WVQEKPDHLFTGLIG GTNNRAP
VL 17.2.25  ------------------------ -------**------- --------------- -------
VL 24H6       ---------------------- -------**----F-- -----S----S---- --Y--P-
VL 38C2      ELVMTQTPLS-PVRL-DQASIS- ---QSLLHTYG-P-L- -YLQ--GQSPKL--Y KVS--FS
VL 33F12     ELVMTQTPLS-PV-L-DQASIS- ---QSL-HSYGNTFL- -YLQ-SGQSPKL--Y KVS--FS
                                          CDR3
GVPARFSGSLIGDKAALTITGAQTEDEAIYFC ALWYSNH
-------------------------------- ------- WVFGGGTKLEIKRTV
-------------------------------L- ------- ---------------
---D-----GS-TDFT-R-SRVEA--LGV--- SQGTHLP YT-------
---D-----GS-TDFT-K-SRVEA--LGV--- SQGTHVP YT-------
```

Figure 7. Translated sequences from heavy- and light-chain genes. H10 and V$_\lambda$1 correspond to germline genes from Balb/c.[27] The light-chain sequence of 17.2.25 is inferred from reported gene use.[28] The amino-terminal sequences of both 24H6 chains are not reported because they are obscured by 5′ primer design (see Experimental Section). Deletions are represented by *. Residue numbering and alignment are based on Kabat.[26]

this high homology extends through the V_H CDR3 loop, which is 11 amino acids in length for each antibody. Seven of these are identical, while three represent conservative substitutions (R101K, Y102F, and Y105F). The DNA codings for the V_H CDR3 loop of 24H6 and 17.2.25 are identical at 28 of their 33 nucleotides. Not only do the two antibodies rearrange the same combination of germline genes, but they also do so to yield very similar junctions and/or P and N nucleotides. Moreover, the conservation of antibody-combining sites is likely to have been even more pronounced at the germline level prior to hypermutation, since this process introduces somatic mutations that will contribute to the sequence differences of 24H6 and 17.2.25.

This particular gene combination may code for an antibody combining site capable of multiple specificities,[30] at least for the haptens **2** and NP. The demonstrated polyspecificity of the 24H6 catalytic antibody, as well as the other aldolase antibodies which were elicited by reactive immunization, may derive in part from an inherent polyspecificity of the antibody genes.[30] Interestingly, although the same mouse strain (129G^{IX+}) was used to elicit both 24H6 and 38C2, these aldolase antibodies were evolved from two different germline genes.

Conclusion

The aldolase antibody, 24H6, which was obtained from immunization with the large hapten **2**, was shown to possess an active-site lysine residue with a perturbed pK_a of 7.0. This antibody catalyzes both the aldol addition and the retrograde aldol fragmentation with a broad range of substrates that are structurally different from the hapten. This observation suggests that in reactive immunization with 1,3-diketones the hapten structure governs the chemistry but not the overall organization of the active site. Hammett correlation studies of the 38C2- and 24H6-catalyzed aldol and retroaldol reactions revealed that although the two antibodies exhibit broad substrate specificities, they utilize slightly different mechanisms. While antibody 38C2 adopts a mechanism that is reminiscent of an acid-catalyzed aldol reaction, antibody 24H6 uses a mechanism that is similar to the base-catalyzed reaction.

The immune response proceeds by a binding energy competition between individual antibodies.[31] Usually, the competition is based upon selection of those clones that can develop and optimize a concert of binding energy parameters, including shape and charge complementarity. However, when the ability to form

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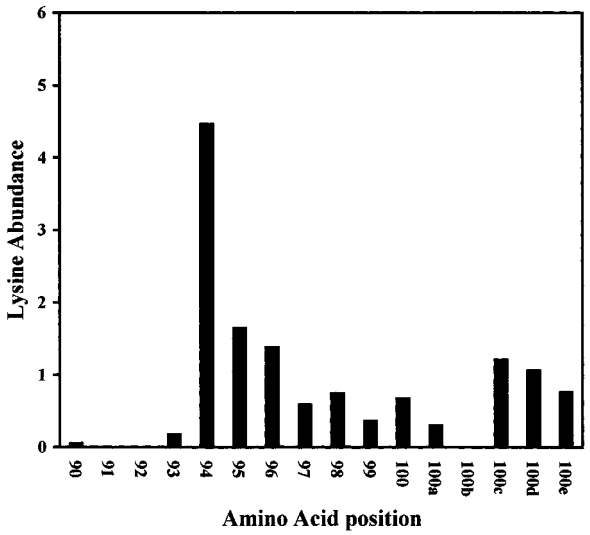

Figure 8. Statistical probability of a lysine residue in the CDR H3 in published antibody sequences. Lys, Met, and Cys are the three least common amino acids in CDR H3.[26]

a covalent bond is added to this set of parameters, the entire nature of the competition changes radically. It is remarkable that although the same mouse strain ($129G^{IX+}$) was used to elicit both 24H6 and 38C2, these aldolase antibodies were evolved from two different germline genes, and yet they utilize similar catalytic machinery. The fact that, when offered, the covalent chemical opportunity is taken, means that the system truly drives to optimal binding energy.

The early interception of the somatic optimization of the immune response certainly reflects the high reactivity of the 1,3-diketone group functioning as a chemical trigger. Yet another, and probably more important, determining factor of this early interception is the degree of difficulty requested by the experimenter. Although lysine residues are rarely found in the antibody CDRs (Figure 8), our effort to solicit an active-site lysine does not seem to be very difficult. We expect that more complex catalytic machinery, such as a complete catalytic triad[32] or other polyfunctional catalytic devices, would take longer to appear via somatic mutations. Such catalytic antibodies are expected to exhibit higher substrate specificity and to carry out even more complex chemistry than the aldolase antibodies.

Experimental Section

General Methods. ^{1}H NMR spectra were recorded on a Bruker AM200 spectrometer, operating at 200 MHz, using $CDCl_3$ as a solvent (unless otherwise specified). Positive ion mass spectra, using the fast atom bombardment (FAB) technique, were obtained on a VG ZAB-VSE double-focusing, high-resolution mass spectrometer equipped with either a cesium or a sodium ion gun. The instrument used for EI MS was a Finnigan MAT-711; that for CI-MS was a Finnigan TSQ-70. UV−vis spectra were recorded on a Shimadzu UV-1601 spectrometer. TLC was performed on glass sheets precoated with silica gel (Merck, Kieselgel 60, F254, Art. 5715). Column chromatographic separations were performed on silica gel (Merck, Kieselgel 60, 230−400 mesh, Art. 9385) under pressure. Dry THF (Sure-seal) was purchased from Aldrich. HPLC analyses were carried out with a Merck-Hitachi Lachrom system equipped with an L-7100 pump, an L-7400 UV−vis detector, and a D-7000 system manager using a Supelco RP LC-18 analytical column. All starting materials and reagents, including *cis*-1,2,3,6-tetrahydrophthalimide, **4**, were purchased from Aldrich. The preparation of the non-commercially available cinnamaldehyde derivatives was reported earlier.[16] Keyhole limpet haemocyanine (KLH) and bovine serum albumin (BSA) were purchased from Sigma.

***N*-BOC-2,3,3a,4,7,7a-Hexahydro-1*H*-isoindole, 5.** Lithium aluminum hydride (2.25 g, 80 mmol) was dissolved in ether (200 mL) and cooled to 0 °C. A solution of 1,2,3,6-tetrahydrophthalimide, **4** (3 g, 19.8 mmol), in THF (100 mL) was added dropwise, and the mixture was warmed slowly to room temperature, heated to 40 °C, refluxed for 3 h, and then cooled to 0 °C. Water (20 mL) was slowly added, followed by sodium sulfate, and the mixture was filtered. The solids were washed with dichloromethane which was added to the ether solution. Solvents were removed under reduced pressure, and the crude product (2 g) was used in the next step without further purification. ^{1}H NMR (300 MHz): 5.65 (br s, 2H), 3.00 (dd, J = 10.2, 5.93 Hz, 2H), 2.65 (dd, J = 10.2, 5.93 Hz, 2H), 2.2 (m, 5H), 1.85 (m, 2H).

The crude pyrrolidine product (0.933 g, 7.6 mmol) was dissolved in THF (40 mL), and the solution was cooled to 0 °C under argon. Triethylamine (1.05 g, 11.4 mmol) and di-*tert*-butyl dicarbonate (1.83 g, 8.4 mmol) were added, the mixture was stirred at room temperature for 7 h, water and dichloromethane were added, and the aqueous phase was extracted with dichloromethane. The organic extract was washed with saturated aqueous sodium bicarbonate and dried over $MgSO_4$, and the solvent was removed under reduced pressure. Column chromatography (silica gel, 10% ethyl acetate in hexane) afforded the BOC derivative **5** (50%) in the form of a colorless oil. ^{1}H NMR (300 MHz): 5.61 (br s, 2H), 3.36 (m, 2H), 3.12 (dd, J = 10.3, 4.7 Hz, 1H), 3.05 (dd, J = 10.3, 6.3 Hz, 1H), 2.2 (m, 4H), 1.89 (m, 2H), 1.42 (s, 9H). MS (EI): 223 (M^+).

***N*-BOC-Octahydro-1*H*-isoindol-5-ol, 6.** Compound **5** (3.16 g, 14.2 mmol) was dissolved in dry THF (35 mL) and cooled to 0 °C under argon. BH_3−DMS (7.1 mL, 2 M in THF, 14.2 mmol) was added dropwise, and the mixture was warmed slowly to room temperature and stirred overnight. The mixture was cooled again to 0 °C, and then methanol (8 mL) was added dropwise, followed by a mixture of aqueous NaOH (5 mL, 3 N) and H_2O_2 (5 mL, 30%). The mixture was stirred at 60 °C for 1.5 h, cooled to room temperature, and worked up with ether and water to produce crude **6** (2.6 g) in the form of a white solid, which was used in the next step without further purification. ^{1}H NMR (300 MHz): 3.86 (m, 1H), 3.3 (m, 2H), 3.1 (m, 2H), 2.5 (m, 1H), 2.1 (m, 1H), 1.8 (m, 5H), 1.5 (m 2H), 1.42 (s, 9H). MS (CI): 242 (MH^+).

***N*-BOC-Octahydro-1*H*-isoindol-5-one, 7.** The crude alcohol **6** (1.6 g, 6.64 mmol) was dissolved in dichloromethane (70 mL) under argon. PCC (2.26 g, 10.5 mmol) and Celite (2.3 g) were added, and the mixture was stirred at room temperature for 1 h and then filtered through silica gel. The crude product was purified by column chromatography (silica gel, ethyl acetate−hexane, 1:1) to give **7** (70%) in the form of a colorless oil. ^{1}H NMR (300 MHz): 3.5 (dd, J = 10.9, 6.8 Hz, 2H), 3.3 (m, 1H), 3.0 (m, 1H), 2.65 (m, 1H), 2.4 (m, 5H), 2.05 (m, 1H), 1.85 (m, 1H), 1.42 (s, 9H). MS (EI): 239 (M^+).

Tricyclic Ketones 8a and 8b. Compound **7** (0.5 g, 2.1 mmol) was dissolved in dry benzene (30 mL). Molecular sieves (3 Å) and morpholine (0.5 mL) were added, and the mixture was refluxed under argon overnight using a Dean Stark condenser. The solvents were removed under reduced pressure, dry benzene was added followed by methylvinyl ketone (0.52 mL, 6.3 mmol), and the mixture was refluxed for 3 h. The solvent was removed again under reduced pressure, and the crude product was dissolved in methanol−water (1:1 v/v). The mixture was refluxed overnight and then worked up with ether and water, and the residue was purified by flash chromatography (silica gel, ethyl acetate−hexane, from 3:7 to 1:1) to give a mixture of racemic isomers, **8a** and **8b** (90 mg, 15%), in the form of a yellowish oil. ^{1}H NMR (300 MHz, partial): 5.89 (br d, 1H), 3.3 (m, 5H), 2.95 (br q, 1H), 2.7 (m, 1H), 1.4 (s, 9H). ^{13}C NMR (100.56 MHz): 199.2, 163.2, 162.9, 126.6, 126.4, 79.3, 79.1, 52.5, 52.2, 51.9, 51.7, 47.6, 46.8, 45.3, 45.1, 43.5, 41.5, 38.2, 38.0, 37.1, 37.0, 36.9, 36.8, 36.5, 36.0, 35.9, 34.2, 34.0, 33.7, 29.6, 28.5, 26.6, 26.5, 26.4 ppm. MS (FAB): 292 (MH^+), 314 (MNa^+).

Diketones 9a and 9b. The above-described isomeric mixture of **8a** and **8b** (160 mg, 0.55 mmol) was added dropwise to a cold (−78 °C) solution of LDA (0.66 mmol, prepared from diisopropylamine and BuLi in dry THF), and the mixture was stirred at the same temperature for 2 h. Dry HMPA (0.12 mL, 6.6 mmol) was added, followed by acetyl

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chloride (0.037 mL, 0.55 mmol). The mixture was warmed slowly to room temperature over 3.5 h and then worked up with aqueous NH_4Cl and ether followed by column chromatography (silica gel, ethyl acetate−hexane, from 3:7 to 1:1) to give a mixture of racemic isomers, **9a** and **9b** (11%), in the form of a yellowish oil. ^{1}H NMR (300 MHz, partial): 5.91−5.85 (br d, 1H), 3.45 (m, 4H), 1.4 (s, 9H). MS (FAB): 356 (MNa^+).

Haptens 2a and 2b. The above-described isomeric mixture of **9a** and **9b** (1.5 g, 4.5 mmol) was dissolved in dichloromethane (20 mL) under argon. Excess trifluoroacetic acid was added, and the reaction was stirred at 40 °C for 24 h. The solvent was removed under reduced pressure to produce the crude unprotected amine (0.685 g). ^{1}H NMR (300 MHz, partial): 5.8−6.0 (m, 1H), 3.5−2.9 (m, 5H), 2.05 (s, 0.7H) 2.04 (s, 1H). MS (FAB): 234 (MH^+), 256 (MNa^+).

The crude amine (0.685 g) was dissolved in pyridine (15 mL), glutaric anhydride (0.67 g, 6.7 mmol) was added, and the mixture was stirred at room temperature for 1 h and then worked up with dichloromethane and water followed by purification using preperative TLC (silica gel, hexanes−ethyl acetate, 7:3) to give a mixture of racemic isomers, **2a** and **2b** (50%), in the form of a yellow oil. ^{1}H NMR (300 MHz): 5.89 (m, 1H), 3.6−1.9 (m, 23H). MS (FAB): 348 (MH^+), 370 (MNa^+).

Protein Conjugates of Haptens 2a and 2b. The hapten mixture was conjugated to the carrier proteins, KLH and BSA, by activation of its carboxylic groups with EDC and sulfo-NHS.[33] Two stock solutions were prepared as follows: (A) EDC (29.5 mg, 0.154 mmol) in water (0.051 mL), and (B) SulfoNHS (31.7 mg, 0.146 mmol) in DMF−water (1:1, 0.097 mL). The above-described isomeric mixture of **2a** and **2b** (4 mg, 0.0115 mmol) was dissolved in DMF (0.195 mL). Solution A (0.005 mL) and solution B (0.013 mL) were added, and the mixture was stirred at room temperature overnight. This mixture (0.095 mL) was added to a cold (4 °C) protein solution that was prepared from KLH (5 mg) and PBS (pH 7.4, 0.9 mL), and the mixture was gently shaken at 4 °C overnight. The same procedure was repeated with BSA.

Immunization and Production of Antibodies. $129G^{IX+}$ mice were immunized with the KLH conjugate in complete Freund's adjuvant. Mice with a high serum titer were used to generate hybridomas by fusion of their spleen cells with myeloma cells.[34] Twenty-four hybridoma cells producing anti-**2** antibodies were selected on the basis of ELISA tests for further studies.[35] Antibodies from each cell line were produced in larger amounts from ascites fluid and then purified by ammonium sulfate precipitation and protein-G affinity chromatography.

Antibody Screening with 1,3-Diketones. Each of the 24 anti-**2** antibodies [7 μM in phosphate-buffered saline (PBS, 50 mM phosphate, 100 mM NaCl, pH 7.4) containing 10% acetonitrile] was mixed with acetylcyclohexanone (513 μM). The mixture was incubated at 25 °C for 24 h, and the formation of a vinylogous amide was monitored by observing the growth of the characteristic absorption at 340 nm using a UV spectrometer. Antibody 24H6 was selected for further studies because it exhibited the most significant absorption at 340 nm.

Irreversible Inhibition of 24H6 by Reductive Amination. Antibody 24H6 (12 μM in PBS, pH 7.4, containing 3% v/v acetone) was incubated at 25 °C for 48 h. An aqueous solution of $Na(CN)BH_3$ (38 μL, 0.83 M) was then added, and the mixture was kept at room temperature for 2 h before undergoing extensive dialysis (the 100-μL antibody solution was dialyzed repeatedly 10 times against 250 mL each of the same buffer solution). The modified antibody was assayed as described above, and the typical absorption at 340 nm was not observed. Similar results were obtained with cyclohexanone instead of acetone. In a control experiment we used acetonitrile instead of acetone and found no change in the 340-nm absorption.

Evaluation of the pK_a of the Active-Site Lysine. Antibody 24H6 (10 μM in 50 mM phosphate−citrate buffer) was incubated with acetylcyclohexanone (833 μM) within a pH range between 4 and 10. The initial rate of formation of the typical UV absorption at 340 nm was measured.

Antibody-Catalyzed Reactions. All antibody-catalyzed reactions were carried out in PBS (50 mM phosphate, 100 mM NaCl, pH 7.4) containing 10% organic cosolvent. In the aldol reactions, the organic cosolvent was CH_3CN (5%) and acetone (5%), while in the retroaldol reactions only CH_3CN (10%) was used. The progress of the reactions was monitored by HPLC using an RP Supelcosil LC18 column, with the initial rates being calculated by regressional analysis. Antibody active sites' concentrations ranged between 4 and 12 μM for 38C2, and between 30 and 120 μM for 24H6. The rate of the uncatalyzed reactions was subtracted when significant.

Preparation of Aldol Substrates. General Procedure. The appropriate ketone (1 mmol) was added to a freshly prepared, cold (−78 °C) solution of LDA (1.05 mmol) in dry THF (2 mL), and the mixture was stirred at the same temperature for 30 min. A solution of the appropriate aldehyde (1 mmol in 2 mL of THF) was added dropwise, and the progress of the reaction was monitored by TLC. Upon completion, the reaction was quenched with saturated aqueous NH_4Cl. The mixture was allowed to warm to room temperature and then extracted with ethyl acetate and dried over $MgSO_4$. The solvent was removed under reduced pressure, and the residue was purified by flash chromatography.

Physical Data of Representative Aldol Products. 6-Phenyl-4-hydroxyhex-5-en-2-one (II-1c).[36] ^{1}H NMR: 7.3 (m, 5H), 6.6 (d, J = 16 Hz, 1H), 6.2 (dd, J = 16, 6 Hz, 1H), 4.7 (m, 1H), 3.1 (d, J = 3.3 Hz, 1H), 2.75 (d, J = 6 Hz, 2H), 2.2 (s, 3H). MS (EI): 190 (M^+).

6-(4′-Chlorophenyl)-4-hydroxyhex-5-en-2-one (II-1d). ^{1}H NMR: 7.25 (br s, 4H), 6.5 (d, J = 15.6 Hz, 1H), 6.1 (dd, J = 15.6, 6.1 Hz, 1H), 4.7 (m, 1H), 2.7 (m, 2H), 2.2 (s, 3H). MS (EI): 258 (M^+).

6-(4′-Methoxyphenyl)-4-hydroxyhex-5-en-2-one (II-1b). ^{1}H NMR: 7.3 (d, J = 8.6 Hz, 2H), 6.8 (d, J = 8.6 Hz, 2H), 6.5 (d, J = 15.9 Hz, 1H), 6.1 (dd, J = 15.9, 6.5 Hz, 1H), 4.7 (m, 1H), 3.78 (s, 3H), 2.97 (d, J = 3.7 Hz, 1H, OH), 2.74 (d, J = 6 Hz, 2H), 2.2 (s, 3H). MS (EI): 220 (M^+).

6-(4′-Trifluoromethylphenyl)-4-hydroxyhex-5-en-2-one (II-1e). ^{1}H NMR: 7.5 (m, 4H), 6.7 (d, J = 16.0 Hz, 1H), 6.3 (dd, J = 16.0, 5.7 Hz, 1H), 4.75 (m, 1H), 3.18 (d, J = 3.0 Hz, 1H), 2.7 (m, 2H), 2.2 (s, 3H). MS (CI): 241 (MH^+-H_2O).

6-(4′-*N*-Dimethylaminophenyl)-4-hydroxyhex-5-en-2-one (II-1a).[3b] ^{1}H NMR: 7.24 (d, J = 8.6 Hz, 2H), 6.6 (d, J = 8.6 Hz, 2H), 6.5 (d, J = 15.9 Hz, 1H), 6.0 (dd, J = 15.9, 6.6 Hz, 1H), 4.7 (m, 1H), 2.9 (s, 6H), 2.7 (d, J = 6.0 Hz, 2H), 2.2 (s, 3H). MS (EI): 233 (M^+).

6-(4′-Nitrophenyl)-4-hydroxyhex-5-en-2-one (II-1f).[3a] ^{1}H NMR: 8.05 (d, J = 8.6 Hz, 2H), 7.3 (d, J = 8.6 Hz, 2H), 6.6 (d, J = 15.9 Hz, 1H), 6.3 (dd, J = 15.9, 5.2 Hz, 1H), 4.7 (m, 1H), 3.25 (d, J = 3.7 Hz, 1H), 2.65 (m, 2H), 2.1 (s, 3H). MS (EI): 235 (M^+).

6-(2′-Nitrophenyl)-4-hydroxyhex-5-en-2-one (II-2). ^{1}H NMR: 7.95 (d, J = 8.3 Hz, 1H), 7.5 (m, 3H), 7.1 (d, J = 15.2 Hz, 1H), 6.2 (dd, J = 15.2, 6.9 Hz, 1H), 4.8 (m, 1H), 3.3 (br, 1H), 2.8 (d, J = 6.9 Hz, 2H), 2.2 (s, 3H). MS (−DCI): 235 (M^-). MS (+DCI): 218 ($MH^+ - H_2O$).

4-(4′-Nitrophenyl)-4-hydroxybutan-2-one (II-5a).[37] ^{1}H NMR: 8.13 (d, J = 8.5 Hz, 2H), 7.5 (d, J = 8.5 Hz, 2H), 5.2 (t, J = 7.2 Hz, 1H), 3.6 (br s, 1H), 2.8 (d, J = 6.25 Hz, 2H), 2.2 (s, 3H). MS (EI): 209 (M^+). MS (CI): 210 (MH^+).

4-(2′-Nitrophenyl)-4-hydroxybutan-2-one (II-3).[37b] ^{1}H NMR: 7.93 (d, J = 8.3 Hz, 1H), 7.88 (d, J = 8.0 Hz, 1H), 7.65 (t, J = 8.0 Hz, 1H), 7.4 (t, J = 8.3 Hz, 1H), 5.68 (br d, J = 9.3 Hz, 1H), 3.7 (d, J = 3.0 Hz, 1H), 3.1 (dd, J = 17.9, 2.2 Hz, 1H), 2.7 (dd, J = 17.9, 9.3 Hz, 1H), 2.2 (s, 3H). MS (CI): 210 (MH^+).

4-(3′-Nitrophenyl)-4-hydroxybutan-2-one (II-4).[37b,38] ^{1}H NMR: 8.18 (s, 1H), 8.1 (d, J = 8.0 Hz, 1H), 7.6 (d, J = 8.0 Hz 1H), 7.43 (t,

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J = 8.0 Hz, 1H), 5.2 (t, J = 6.1 Hz, 1H), 3.75 (br s, 1H), 2.83 (d, J = 6.2 Hz, 2H), 2.2 (s, 3H). MS (CI): 210 (MH^+).

4-(4′-Bromophenyl)-4-hydroxybutan-2-one (II-5b).[39] ^{1}H NMR: 7.46 (d, J = 8.4 Hz, 2H), 2.2 (d, J = 8.4 Hz, 2H), 5.1 (m, 1H), 3.4 (d, J = 3.0 Hz, 1H), 2.81 (d, J = 3 Hz, 1H), 2.77, (s, 1H), 2.2 (s, 3H). MS (−DCI): 242, 244 (M^-).

4-(4′-Dimethylaminophenyl)-4-hydroxybutan-2-one (II-5c).[39] ^{1}H NMR: 7.2 (d, J = 8.6 Hz, 2H), 6.67 (d, J = 8.6 Hz, 2H), 5.0 (m, 1H), 2.9 (s, 6H), 2.8 (m, 2H), 2.2 (s, 3H). MS (CI): 208 (MH^+).

4-(4′-Nitrophenyl)-4-hydroxypentan-2-one (II-6). ^{1}H NMR: 8.2 (d, J = 10.0 Hz, 2H), 7.6 (d, J = 10.0 Hz, 2H), 4.6 (s, 1H, OH), 3.2 (d, J = 16.7 Hz, 1H), 2.9 (d, J = 16.7 Hz, 1H), 2.1 (s, 3H), 1.5 (s, 3H).

6-Phenyl-4-hydroxyhexan-2-one (II-8). ^{1}H NMR: 7.2 (m, 5H), 4.0 (m, 1H), 3.1 (d, J = 5.3 Hz, 1H), 2.7 (m, 4H), 2.1 (s, 3H), 1.7 (m, 2H). MS-CI: 193 ($M + H^+$).[40]

7-(4′-Nitrophenyl)-5-hydroxyhept-6-en-3-one (III-2). ^{1}H NMR: 8.16 (d, J = 8.8 Hz, 2H), 7.5 (d, J = 8.8 Hz, 2H), 6.7 (d, J = 16.0 Hz, 1H), 6.3 (dd, J = 16.0, 5.2 Hz, 1H), 4.8 (m, 1H), 3.3 (d, J = 3.65 Hz, 1H, OH), 2.7 (m, 2H), 2.46 (q, J = 7.3 Hz, 2H), 1.1 (t, J = 7.3 Hz, 3H). MS (CI): 250 (MH^+).

6-(4′-Nitrophenyl)-4-hydroxy-3-methylhex-5-en-2-one (Mixture of Anti and Syn Isomers of III-2). ^{1}H NMR: 8.13 (d, J = 8.6 Hz, 2H), 7.5 (d, J = 8.6 Hz, 2H), 6.73 (two doublets, J = 15.4 Hz each, together 1H), 6.3 (dd, J = 15.4, 5.8 Hz, and dd, J = 15.4, 5.2 Hz, together 1H), 4.7 (m, 0.5 H), 4.4 (m, 0.5 H), 3.0 (d, J = 2.95 Hz, 0.5H, OH), 2.9 (d, J = 5.2 Hz, 0.5 H, OH), 2.8 (m, 1H), 2.2 (s, 3H), 1.2, (m, 3H). MS (CI): 250 (MH^+).

8-(4′-Nitrophenyl)-6-hydroxyoct-7-en-4-one (III-3). ^{1}H NMR: 8.13 (d, J = 8.6 Hz, 2H), 7.5 (d, J = 8.6 Hz, 2H), 6.7 (d, J = 15.9 Hz, 1H), 6.3 (dd, J = 15.9, 5.2 Hz, 1H), 4.8 (m, 1H), 3.3 (d, J = 3.3 Hz, 1H), 2.7 (m, 2H), 2.43 (t, J = 7.3 Hz, 2H), 1.6 (m, 2H), 0.9 (t, J = 7.3 Hz, 3H). MS (CI): 264 (MH^+).

3-(4′-Nitrophenyl)-1-(2″-oxocyclohexyl)prop-2-en-1-ol (III-4). ^{1}H NMR: 8.1 (d, J = 8.7 Hz, 2H), 7.4 (d, J = 8.7 Hz, 2H), 6.6 (d, J = 15.8 Hz, 1H), 6.3 (dd, J = 15.8, 5.0 Hz, 1H), 4.8 (br, 1H), 3.0 (br, 1H, OH), 2.4 (m, 3H), 1.5 (m, 6H). MS (−DCI): 275 (M^-). MS (+DCI): 276 (MH^+).

Antibody Cloning. Total RNA was isolated from the hybridoma cell line by the method of Chomczynski and Sacchi.[41] Total RNA was enriched for antibody encoding messenger RNA by affinity chromatography with oligo (dT) cellulose (Pharmacia). A cocktail of 3′ primers corresponding to each J_λ and J_H region, modified to contain unique restriction sites, was used to reverse transcribe cDNA. PCR amplification with the same 3′ primers and the 5′ primers described by Huse et al.[42] yielded sufficient DNA for cloning. The V_λ and V_H genes were cloned into plasmid p4X.[43] This construct fuses the murine V_λ and V_H genes to human $C_\kappa 1$ and $C_H 1$ genes, respectively. Sequence determination was performed at the core facility at TSRI (TSRI sequencing facility). The Fab fragment was isolated from *Escherichia coli* 25F2 transformed with the p4X plasmid, according to published protocols.[42] The Fab fragment was further purified by protein G affinity chromatography (Sepharose CL-6B, Pierce). The Fab fragment was concentrated and stored in phosphate-buffered saline (Ca^{2+}, Mg^{2+} free, deionized water). The authenticity of the Fab fragment was verified by ELISA with immobilized hapten.

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