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Evolution of Aldolase Antibodies *in Vitro*: Correlation of Catalytic Activity and Reaction-based Selection

Fujie Tanaka*, Roberta Fuller, Hyunbo Shim, Richard A. Lerner and Carlos F. Barbas III*

The Skaggs Institute for Chemical Biology and Departments of Molecular Biology and Chemistry, The Scripps Research Institute 10550 North Torrey Pines Road BCC-550, La Jolla, CA 92037,USA

*Corresponding authors

Aldolase antibodies that operate *via* an enamine mechanism were developed by *in vitro* selection. Antibody Fab phage display libraries were created where the catalytic active site residues of aldolase antibodies 38C2 and 33F12 were combined with a naive human antibody V gene repertoire. Selection from these libraries with 1,3-diketones covalently trapped the amino groups of reactive lysine residues by formation of stable enaminones. The selected aldolase antibodies retained the essential catalytic lysine residue and its function in altered and humanized primary antibody structures. The substrate specificity of the aldolase antibodies was directly related to the structure of the diketone used for selection. The k_{cat} values of the antibody-catalyzed retro-aldol reactions were correlated with the K_d values, i.e. the reactivities of the selected aldolase antibodies for the corresponding diketones. Antibodies that bound to the diketone with a lower K_d value displayed a higher k_{cat} value in the retro-aldol reaction, and a linear relationship was observed in the plots of log k_{cat} *versus* log K_d. These results indicate that selections with diketones directed the evolution of aldolase antibodies *in vitro* that operate *via* an enamine mechanism. This strategy provides a route to tailor-made aldol catalysts with different substrate specificities.

Keywords: aldolase antibody; catalytic antibody; enamine; phage display; *in vitro* evolution

Introduction

Catalytic antibodies for diverse types of chemical transformations have been generated by immunization of animals with designed haptens.[1–5] These results demonstrate that the diverse repertoire of the immune system can be tapped to provide catalysts for a wide range of reactions. However, while the immune repertoire provides a highly diverse array of antibody genes from which to select catalysts, immune responses to haptens are often highly restricted to a few favored V genes.[6,7] Immunization of mice with transition state analogs designed for hydrolytic reactions has provided a variety of catalytic antibodies that demonstrate high levels of similarity at both structural and mechanistic levels.[8–15] This fact acts to experimentally limit our opportunities to probe the structural repertoire available to antibodies more completely. To search for novel catalytic antibodies, we sought a strategy that would not be limited by the need to re-immunize animals and one that could take advantage of insight gained by the study of existing catalytic antibodies. Our ability to tap the immune repertoire in a way that is not limited by *in vivo* immune responses would expand available opportunities to search for catalysts in a directed way.

Recent advances in phage displayed antibody Fab and scFv libraries permit the selection of monoclonal antibodies *in vitro*.[16] Since antibody phage libraries can be made from both immune and non-immune sources, *in vitro* selection with designed haptens using antibody phage libraries provides for access to catalytic antibodies in a way

Abbreviations used: HCDR, heavy chain complementarity determining region; LCDR, light chain complementarity determining region; BSA, bovine serum albumin; ELISA, enzyme-linked immunosorbent assay; DMSO, dimethyl sulfoxide; PBS, phosphate-buffered saline; ee, enantiomeric excess.

E-mail addresses of the corresponding authors: carlos@scripps.edu; ftanaka@scripps.edu

not limited by animal sources or by immune responses. However, the maximum number of library members that can be typically examined in an antibody phage library 10^8–10^{11} is a small portion of the potential diversity available to the immune repertoire.[16] Selections from such a small portion of the total repertoire does not allow for a comprehensive survey of possible catalysts. To overcome problems associated with the selection of efficient catalysts while exploiting the structural diversity available to antibodies more fully, we have taken lessons from Nature's methods of enzyme evolution. Many natural enzymes use common catalytic mechanisms in the catalysis of analogous chemical transformations or essentially the same chemical mechanism operant on different substrates, for example in the hydrolysis of esters and amides. Conservation of catalytic mechanism is observed not only among evolutionarily related and highly identical enzymes but also between structurally different and evolutionarily unrelated enzymes.[17–19] Such observations provide evidence for evolutionary convergence at the level of chemical mechanism. It is anticipated that structural heterogeneity may provide for distinct opportunities for the optimization of catalytic efficiency in different active site structures where chemical mechanism is conserved. In accord with Nature's strategy of convergent evolution, we sought an *in vitro* strategy for accessing catalytic antibodies that conserves catalytic mechanism while maximizing structural diversity. We prepared antibody libraries by the combination of the active site residues of existing catalytic antibodies with a human antibody V gene repertoire. This library strategy should provide greater opportunities to access catalytic antibodies while remaining rich in structural diversity as compared to natural immunization where only a fraction of a naive antibody repertoire is typically accessed. Selection with designed compounds that trap catalysts from these types of libraries should provide access to catalysts possessing distinct active site structures and that operate *via* an analogous chemical mechanism. This strategy would eliminate the need to re-immunize animals when new catalysts are desired and also addresses the limitations of such a strategy. This strategy is complementary to other *in vitro* methods for that aim to improve catalytic antibody function, *vida infra*.

We have demonstrated the potential of this strategy by preparing a family of novel aldolase antibodies (catalytic antibodies possessing aldolase activity). To accomplish this we took advantage of insight gained by the study of existing aldolase antibodies to obtain substrate specificity-altered and improved catalytic antibodies. We prepared antibody libraries programmed with the catalytic active site residues of aldolase antibodies 38C2 and 33F12, and selected these libraries with 1,3-diketone derivatives.[20] Aldolase antibodies 38C2 and 33F12 were previously generated by reactive immunization with 1,3-diketone **1**.[21] These antibodies are highly similar with respect to sequence, structure, and catalytic mechanism.[21–37] Both antibodies possess a highly reactive lysine residue (LysH93) (the numbering is according to Kabat *et al*.[38]) in their active sites and use the ε-amino group of the lysine to catalyze aldol, retro-aldol, and related reactions *via* an enamine mechanism.[21–37] The 1,3-diketone hapten acts as a mechanism-based trap of the requisite lysine residue in the antibody active site through formation of an enaminone. Exploiting the similarities of the reaction coordinate of enaminone formation with the reaction coordinate of an enamine-based aldol reaction allows for enamine-based aldolase activity to be readily programmed.

We have focused on the preparation of aldolase antibodies that process cyclohexanone–aldols efficiently, since reactions of substrates of this type are not efficiently catalyzed by the parental antibodies 38C2 and 33F12. We prepared phage displayed antibody Fab libraries and selected catalysts in covalent selections with 1,3-diketones designed for both acetone and cyclohexanone aldol reactions. These selections provided antibody Fab 28 that catalyzes retro-aldol reactions of cyclohexanone–aldol substrates efficiently as compared to the parental antibodies.[20] Fab 28 retained the same catalytic lysine residue and the enamine mechanism of the parental antibodies to catalyze the targeted aldol reactions. Here we report the family of *in vitro* selected aldolase antibodies obtained with Fab 28, and evaluate the library strategy and efficacy of 1,3-diketone binding selections for the catalysts possessing aldolase activity by the comparison of these aldolase antibodies.

Results

Libraries and selection

To retain the catalytic function of residue LysH93 that is found near the heavy chain complementarity determining region 3 (HCDR3) in the primary structure of aldolase antibodies 38C2 and 33F12, the sequences of the LysH93, HCDR3, and light chain complementarity determining region 3 (LCDR3) of the aldolase antibodies were retained in the library.[20] A naive antibody heavy chain variable domain (V_H) library was generated using human bone marrow cDNA and fused to the parental heavy chain sequences at H93. The LCDR3 sequences of the parental antibodies were placed in the context of an unrelated human light chain that possessed a radically different amino acid sequence as compared to the parental aldolase antibodies. To place aldolase antibody sequences into the libraries, chemically synthesized DNA oligomers were used. The phage-displayed libraries[16] were selected by panning against 1,3-diketone **1**-BSA[21] (bovine serum albumin) and **2**-BSA[20] in order to select antibodies that would

accept both **3** and **4** as substrates for retro-aldol reaction. In a subsequent diversification step, light chain variable domain (V_L) libraries were combined with the selected V_H libraries and the binding selections were repeated using the same 1,3-diketones. In addition to these libraries, libraries in which the residue at L87 was mutated to phenylalanine were also used (see below). Antibodies that bound to **1**-BSA and **2**-BSA were identified by enzyme-linked immunosorbent assay (ELISA) from the selected libraries. In total 13 Fabs that bound to the diketones were purified and assayed for retro-aldol activity using fluorogenic substrates **3**[39] and **4**.[20] The reactions were performed using 0.5 μM of Fab and (±)-**3** (200 μM) or a mixture of (±)-*anti*-**4** (125 μM)-(±)-*syn*-**4** (125 μM) in 5% DMSO/PBS (pH 7.4) at 25 °C. In addition to Fab 28, Fabs 3 and 9 also catalyzed the reactions. The remaining Fabs showed no catalytic activity or a velocity 40-fold lower than the highest velocity observed for each substrate. The relatively low catalytic activity of Fab 10 was detected using higher concentrations of Fab in the assay. Antibodies that showed lower catalytic activities under the typical conditions were not analyzed except Fab 10. Since the protein production level of Fab 10 was higher than that of other Fabs, we could obtain a large amount of Fab 10 protein from the same volume of culture and could assay the activity using higher concentrations of antibody. Fab 22 was also determined to be a non-active catalyst. The amino acid sequence of Fab 22 was identical with that of catalytic Fab 28 with the exception of two amino acid residues, residues L87 and L94. Fab 22 possesses TyrL87 and ValL94, while Fab 28 possesses PheL87 and LeuL94. The amino acid residues at L94 originated from the parental antibodies: Fabs 28 and 22 retained the amino acid at L94 of the parental antibodies 38C2 and 33F12, respectively. The phenylalanine mutant of Fab 22 at L87 (Fab 22 L87Phe) was prepared and examined for catalytic activity using the same substrates. This mutant catalyzed the reactions in the conditions described above. Therefore, antibody Fabs 28, 3, 9, 10 and 22 L87Phe were analyzed in detail.

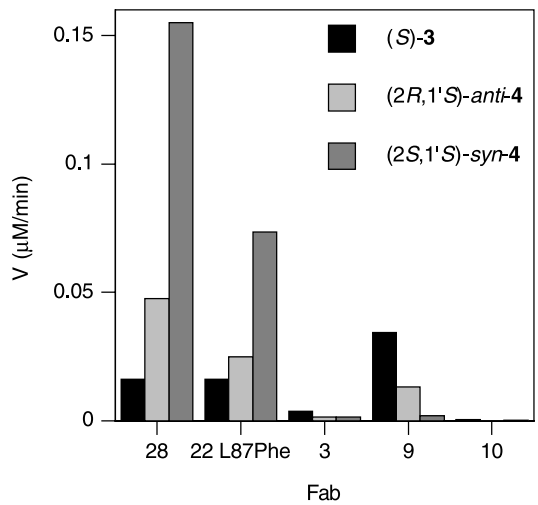
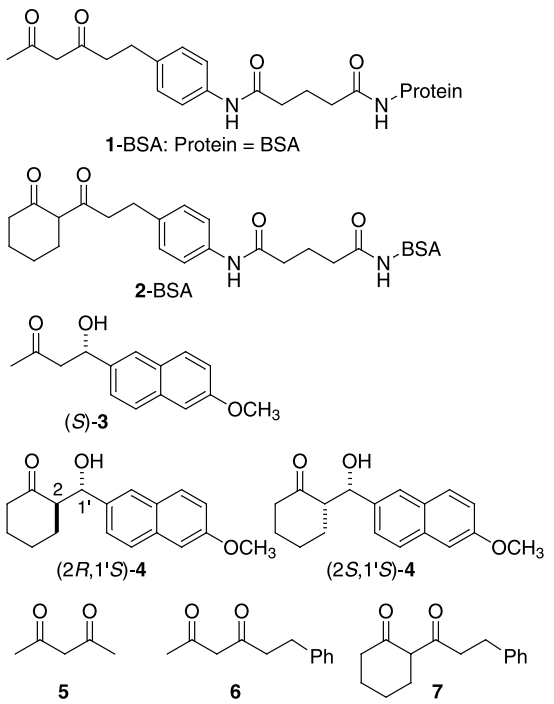


Figure 1. Comparison of the velocities of Fab-catalyzed retro-aldol reactions. Conditions: [Substrate] 100 μM, [Fab] 1 μM, 5% CH_3CN/PBS (pH 7.4). Substrate enantiomeric purity: (*S*)-**3**, 98% ee; (2*R*,1′*S*)-*anti*-**4**, 99% ee; (2*S*,1′*S*)-*syn*-**4**, >99.5% ee.



Catalytic activity and kinetics

To analyze the substrate specificity, the Fab-catalyzed retro-aldol reactions were performed using each enantiomer of substrates **3**, *anti*-**4**, and *syn*-**4** (100 μM), separately. All of the Fab-catalyzed retro-aldol reactions using these substrates were highly enantioselective and the preferred substrates were (*S*)-**3**, (2*R*,1′*S*)-**4**, and (2*S*,1′*S*)-**4**. The stereochemistries of the preferred substrate enantiomers of these Fabs are the same as those of the parental antibodies 38C2 and 33F12. The *S*-configuration at the carbon center bearing the hydroxy group was essential for catalysis by these antibodies. Catalysis of the aldol reaction with the opposite enantiomers was not detected, except in the case of Fab 3 that catalyzed the retro-aldol reaction of *syn*-**4**. The ratio of the initial velocities of Fab 3-catalyzed reactions of (2*S*,1′*S*)-**4** and (2*R*,1′*R*)-**4** was determined to be 96:4. The velocities of the Fab-catalyzed retro-aldol reactions using their preferred enantiomeric substrates (100 μM) are shown in Figure 1. Fabs 28 and 22 L87Phe catalyzed the retro-aldol reaction of cyclohexanone–aldol **4** efficiently. Although these *in vitro* selected aldolase antibodies had different substrate specificities and exhibited a wide range of catalytic activity, they retained the preferred

Table 1. Kinetic parameters of the Fab-catalyzed retro-aldol reactions of **3**

		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}[a]
Fab 28[b]	±	6.5×10^2 (± 10)	9.5×10^{-2} ($\pm 7.9 \times 10^{-3}$)	2.1×10^5
Fab 22L87Phe	±	4.4×10^2 (± 61)	2.8×10^{-2} ($\pm 1.8 \times 10^{-3}$)	6.3×10^4
Fab 3	±	1.1×10^3 ($\pm 2.4 \times 10^2$)	1.0×10^{-2} ($\pm 1.4 \times 10^{-3}$)	2.2×10^4
Fab 9	±	4.3×10^2 (± 55)	9.9×10^{-2} ($\pm 5.8 \times 10^{-3}$)	2.2×10^5
Fab 9[c]	*S*[d]	1.9×10^2 (± 20)	1.0×10^{-1} ($\pm 4.6 \times 10^{-3}$)	2.2×10^5
Fab 10	±	75 (± 27)	5.3×10^{-4} ($\pm 6.4 \times 10^{-5}$)	1.2×10^3
Ab 38C2[e,f]	±	14	1.0	2.2×10^6
Ab 33F12[b,f]	±	43 (± 2.6)	1.1×10^{-1} ($\pm 2.2 \times 10^{-3}$)	2.4×10^5

Reaction conditions: 5% DMSO/PBS (pH 7.4), 25 °C except where noted. Standard deviations for k_{cat} and K_m values are indicated in parentheses.
[a] The first-order kinetic constant of the background reaction (k_{uncat}) was 4.5×10^{-7} min^{-1} for (±)-**3**.
[b] The data taken from Tanaka *et al.*[20]
[c] Reaction was performed in 5% CH_3CN/PBS (pH 7.4). The k_{uncat} value was the same as that observed in 5% DMSO/PBS (pH 7.4).
[d] (*S*)-**3** (98% ee).
[e] The data taken from List *et al.*[39]
[f] Mouse IgG antibody.[21] The parameters are reported per active site.

stereochemistries of the substrates processed by the parental antibodies.

To survey the catalytic activity of the Fabs, the kinetic parameters of the Fab-catalyzed retro-aldol reactions of **3** and **4** were determined (Tables 1 and 2). The K_m values of these Fab-catalyzed reactions were in the range of 63 μM–1.1 mM. The k_{cat} values were up to 0.3 min^{-1} and the k_{cat}/k_{uncat} values were up to 2.2×10^5. The k_{cat} values of Fab 28-catalyzed reactions of *anti*- and *syn*-**4** were superior to those of the parental antibodies by approximately three- to tenfold. In the case of Fab 22 L87Phe-catalyzed reactions of *anti*- and *syn*-**4**, the k_{cat} values were superior to those of the parental antibodies by up to sixfold. Fab 28 exhibited a threefold higher k_{cat} value for *syn*-**4** than for **3**, and k_{cat} value for *anti*-**4** similar to that for **3**. This is in stark contrast to the catalytic profile of the parental antibodies. In the 38C2-catalyzed reactions, the k_{cat} value for *syn*-**4** is approximately 10% that of **3** and the k_{cat} value for *anti*-**4** is only 3% that of **3**. In the 33F12-catalyzed reactions, the k_{cat} value for *syn*-**4** is approximately half that of **3** and the k_{cat} value for *anti*-**4** is 10% that of **3**. Fab 28 and Fab 9 had k_{cat} values similar to those of parental antibody 33F12 in the retro-aldol reaction of **3**.

Enaminone formation with 2,4-pentanedione and binding affinity to 1,3-diketones

It was expected that the enamine mechanism of the parental antibodies would be favored, since

Table 2. Kinetic parameters of the Fab-catalyzed retro-aldol reaction of **4**

	anti-**4**				*syn*-**4**			
		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}[a]		K_m (μM)	k_{cat} (min^{-1})	k_{cat}/k_{uncat}[a]
Fab 28[b]	±	1.8×10^2 (± 31)	1.0×10^{-1} ($\pm 1.0 \times 10^{-2}$)	2.3×10^4	±	1.1×10^2 (± 11)	2.9×10^{-1} ($\pm 1.4 \times 10^{-2}$)	1.2×10^4
Fab 28[b,c]	2*R*,1′*S*[d]	1.0×10^2 (± 16)	9.5×10^{-2} ($\pm 6.8 \times 10^{-3}$)	2.1×10^4	2*S*,1′*S*[d]	87 (± 14)	2.9×10^{-1} ($\pm 2.2 \times 10^{-2}$)	1.2×10^4
Fab 22L87Phe[c]	2*R*,1′*S*[d]	1.6×10^2 (± 23)	6.5×10^{-2} ($\pm 5.1 \times 10^{-3}$)	1.4×10^4	2*S*,1′*S*[d]	63 (± 16)	1.2×10^{-1} ($\pm 1.1 \times 10^{-2}$)	4.8×10^3
Fab 3	±	8.7×10^2 ($\pm 3.0 \times 10^2$)	1.5×10^{-2} ($\pm 3.7 \times 10^{-3}$)	3.4×10^3		nd[e]		
Fab 9[c]	2*R*,1′*S*[d]	2.0×10^2 (± 46)	4.0×10^{-2} ($\pm 4.6 \times 10^{-3}$)	8.8×10^3		nd[e]		
Ab 38C2[b,f]	±	36 (± 2.0)	3.4×10^{-2} ($\pm 5.9 \times 10^{-4}$)	7.5×10^3	±	1.1×10^2 (± 5.2)	9.7×10^{-2} ($\pm 2.2 \times 10^{-3}$)	4.0×10^3
Ab 33F12[b,f]	±	97 (± 24)	1.1×10^{-2} ($\pm 1.3 \times 10^{-3}$)	2.5×10^3	±	1.3×10^2 (± 73)	4.7×10^{-2} ($\pm 1.1 \times 10^{-2}$)	1.9×10^3

Reaction conditions: 5% DMSO/PBS (pH 7.4), 25 °C except where noted. Standard deviations for k_{cat} and K_m values are indicated in parentheses.
[a] The first-order kinetic constant of the background reaction (k_{uncat}) was 4.5×10^{-6} min^{-1} for (±)-*anti*-**4** and 2.4×10^{-5} min^{-1} for (±)-*syn*-**4**.
[b] The data taken from Tanaka *et al.*[20]
[c] Reaction was performed in 5% CH_3CN/PBS (pH 7.4). The k_{uncat} values were the same as those observed in 5% DMSO/PBS (pH 7.4).
[d] (2*R*,1′*S*)-**4**, 99% ee; (2*S*,1′*S*)-**4**, >99.5% ee.
[e] Not determined.
[f] Mouse IgG antibody.[21] The parameters are reported per active site.

the catalytic lysine residue LysH93 of these antibodies is retained and phage selections were performed using 1,3-diketones. To support this mechanism, enaminone formation of the catalytic Fab with 2,4-pentanedione (**5**), the smallest compound containing 1,3-diketone structure, was studied. When **5** (500 μM) was mixed with Fab in 5% CH_3CN/PBS (pH 7.4), a new absorption at 318 nm was observed. All catalytic Fabs gave the same absorption in this experiment. This result is consistent with enaminone formation[21–37] between **5** and Fab, and with the reaction mechanism of the parental antibodies. Although all these catalytic Fabs formed an enaminone with **5**, the velocities of the enaminone formation varied. The relative initial velocities of the enaminone formation with **5** were 1:2.6:0.2 for Fab 28/Fab 9/Fab 10 when Fab (1 μM active site) was used in the conditions described above. The time required for 50% maximal enaminone formation was approximately 30 minutes for Fab 28, 15 minutes for Fab 9, and four hours for Fab 10. Fab 22 and non-catalytic mutants of Fab 28, Fab 28 H93Ala and Fab 28 H93Met (alanine and methionine mutant at LysH93, respectively), did not form the UV-observable enaminone with **5**.[20]

The catalytic Fabs bound both **1**-BSA and **4**-BSA. To analyze the reactivity of the antibodies with 1,3-diketones, apparent dissociation constant K_d of the catalytic Fabs to 1,3-diketones **6** and **7** was determined by inhibition ELISA.[40] This procedure is useful for ranking the reactivity of aldolase antibodies to the diketones by providing apparent dissociation constants. We used this procedure to estimate the antibody reactivity to the diketones. Limiting inhibition time (incubation time for mixing antibody and the diketone) in the inhibition ELISA provides apparent K_d values respecting the binding step (on rate). In the inhibition ELISA experiments, antibody reactivity to the diketone can be compared by using the diketone compounds that do not include linker moieties. In addition, inhibition ELISA experiments require only a small amount of antibody compared to enaminone formation by UV assays. The results are shown in Table 3. The K_d values of parental antibodies (human-mouse chimeric Fabs) 38C2 and 33F12 were also determined. To avoid avidity of the bivalent IgG, human-mouse chimeric Fabs 38C2 and 33F12 were used for the determination of the K_d values. The K_d values of catalytic Fabs selected from the libraries were in the range of 0.8–90 μM. Parental antibodies 38C2 and 33F12 had a lower K_d value for acetone-diketone **6** than that for cyclohexanone–diketone **7**. The binding profile of the *in vitro* selected aldolase antibodies was reversed. They had a lower K_d value for **7** than for **6**. Since Fabs 28 H93Ala and 28 H93Met displayed greatly reduced K_d values (600 and 800 μM for **7**, respectively),[20] the catalytic residue LysH93 contributes both to the catalysis and to binding of the diketones. In addition, the results indicate that enaminone formation is an important mechanism for binding to both **6** and **7**.

Four poorly active or non-catalytic binding Fabs were also characterized and the K_d values of diketones **6** and **7** were determined. The K_d range determined for these Fabs was 2–100 μM. Catalysts were found among the binding Fabs with a binding mechanism associated with enaminone formation, i.e. covalent bond formation. Simple non-covalent binding, however, did not correlate with catalytic function.

Table 3. Apparent dissociation constant to diketones

	K_d (μM)	
	6	**7**
Fab 28	3	0.8 (K_i = 1.0 μM)[a]
Fab 22 L87Phe	10	2
Fab 3	20	5
Fab 9	7	3
Fab 10	90	40
Fab 38C2M/H[b]	0.2	6
Fab 33F12M/H[b]	2	70

The K_d value was determined as diketone concentration required for 50% inhibition of maximal binding in the inhibition ELISA using **1**-BSA coated plates.

[a] The K_i value was determined by Dixon analysis in Fab 28-catalyzed retro-aldol reaction of (±)-*syn*-**4**.[20]

[b] Human-mouse chimeric Fab.

Relationship between catalytic activity and binding affinity to 1,3-diketones

To evaluate the binding selection strategy, the relationship between the k_{cat} value of the Fab-catalyzed retro-aldol reaction and the K_d value of the corresponding diketone, i.e. reactivity with the diketone, of the selected catalysts was analyzed. The k_{cat} values of **3** and of *anti*-**4** were compared to the K_d values of diketone **6** and of **7**, respectively. The plots of the k_{cat} *versus* K_d are shown in Figures 2 and 3. The Figures also include data of

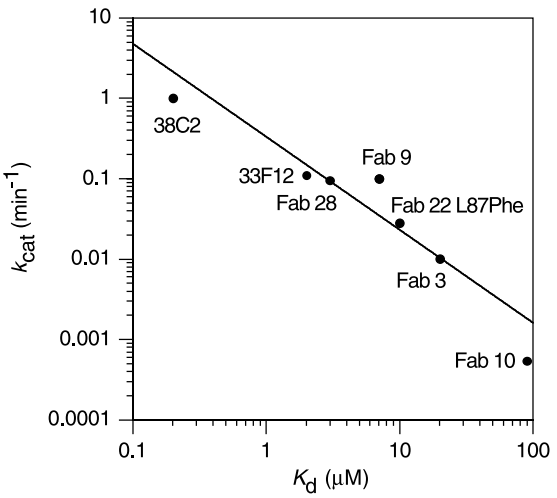


Figure 2. Plot of k_{cat} of the retro-aldol reaction of **3** *versus* K_d of diketone **6**. Slope = −1.16, r^2 = 0.87.

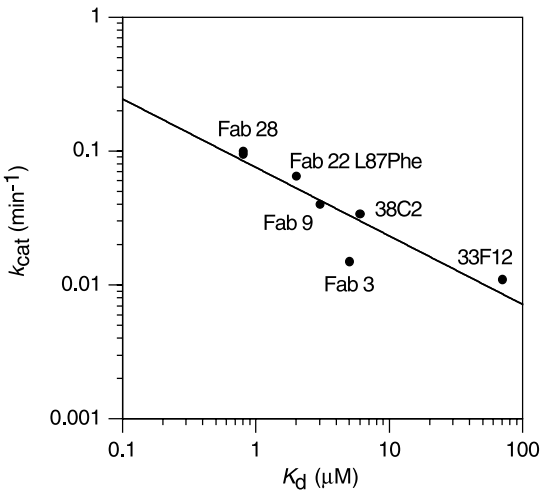


Figure 3. Plot of k_{cat} of the retro-aldol reaction of *anti*-**4** *versus* K_d of diketone **7**. Slope = −0.51, $r^2 = 0.82$.

the parental antibodies. In both cases, a stronger binding (lower K_d value) to the diketone, i.e. a higher reactivity with the diketone, correlated with a higher k_{cat} value. A rough linear relationship was observed when the log axes were used in the graphs (slope −1.16, $r^2 = 0.87$ in Figure 2 and slope −0.51, $r^2 = 0.82$ in Figure 3). The parental antibodies bound to acetone-diketone **6** more strongly (with lower K_d) than the *in vitro* selected Fabs and catalyzed the retro-aldol reaction of acetone–aldol **3** more efficiently (with higher k_{cat}) than the *in vitro* selected Fabs. On the other hand, Fab 28, Fab 22 L87Phe, and Fab 9 bound to cyclohexanone–diketone **7** more strongly than the parental antibodies and catalyzed the retro-aldol reaction of cyclohexanone–aldol *anti*-**4** more efficiently than the parental antibodies. No relationship between the K_d and K_m values was observed. Improvement in binding or reactivity to cyclohexanone–diketone **7** directly correlated with improvement in k_{cat} of the retro-aldol reaction of cyclohexanone–aldol *anti*-**4** and did not correlate with the improvement in substrate binding. Although Fabs that bind to the diketones in the ELISA include both catalysts and non-catalysts, the apparent dissociation constants determined by inhibition ELISA correlated with the catalytic activities within the family of catalytic antibodies. Selection using a structure-altered diketone provided catalytic antibodies that have altered substrate specificity as directed by the structure of the selecting diketone.

Sequences

Amino acid sequences of the V_L and V_H regions of the catalytic Fabs and of the parental antibodies 38C2 and 33F12 are shown in Figure 4. As expected based on the libraries' design, the Fabs retained sequence elements of the parental antibodies. In the V_L sequence, Fabs 28, 3, and 10 retained the 38C2 sequence segment and Fabs 22 L87Phe and 9 retained the 33F12 sequence segment. In the V_H sequence, Fab 10 retained the 38C2 sequence segment and the others retained the 33F12 sequence segment. The remaining protein sequence is of human origin and is not related in primary sequence to the parental antibodies. The key residue LysH93 retained its function in the context of a dramatically altered protein sequence. Dramatic differences in the lengths of the LCDR1, HCDR1, and HCDR2 regions utilized by the Fabs are seen as compared to those used by the parental aldolase antibodies. The identities of the sequences between the parental antibodies and Fabs 28, 22 L87Phe, 3, 9, and 10 are 55–75% in the light chain frame regions 1–3 (FR 1-3) and 44–65% in the heavy chain FR1-3. These identity values are low compared to those of a panel of catalytic antibodies obtained from a single hapten immunization and in the range observed for any randomly compared human antibody sequences.[9] In addition, the comparison of the family of *in vitro* selected catalytic Fabs among themselves also indicated significant structural diversity. The identities of Fabs 28, 3, 9, and 10 are 60–100% in the light chain FR1-3 and 48–87% in the heavy chain FR1-3. Thus, the *in vitro* selection protocol proved to be an efficient method of trapping the potential structural diversity of the immune system.

In the crystal structure of 33F12, residues SerH35, ValH37, TrpH47, TyrH95, TrpH103 and PheL98 are within 5 Å of the ε-amino group of LysH93.[22,41] In the *in vitro* selected aldolase antibodies, TyrH95, TrpH103, and PheL98 were retained by the library design and TrpH47 was also retained in the heavy chain FR2. SerH35 and ValH37 were varied depending on the Fabs. The *in vitro* selected aldolase antibodies also retained TyrL36 that may act as a general acid/base catalyst.[23]

Discussion

Phage display antibody Fab libraries were prepared wherein the catalytic active site residues of parental aldolase antibodies were combined with a naive V gene repertoire. Selections of these libraries were performed using 1,3-diketone compounds designed to covalently trap catalysts that operate *via* a predefined enamine mechanism for aldolase activity. The *in vitro* selected aldolase antibodies retained the enamine mechanism for catalysis of retro-aldol reactions. Significantly, the functional characteristics of the essential ε-amino group of LysH93 were retained within the context of antibodies of dramatically different primary amino acid sequences. These results indicate that antibody libraries of this type provide new evolutionary opportunities for catalysis and that selections using compounds designed for trapping catalytic function provide catalytic antibodies that have conserved catalytic reaction mechanisms programmed by the selecting compounds.

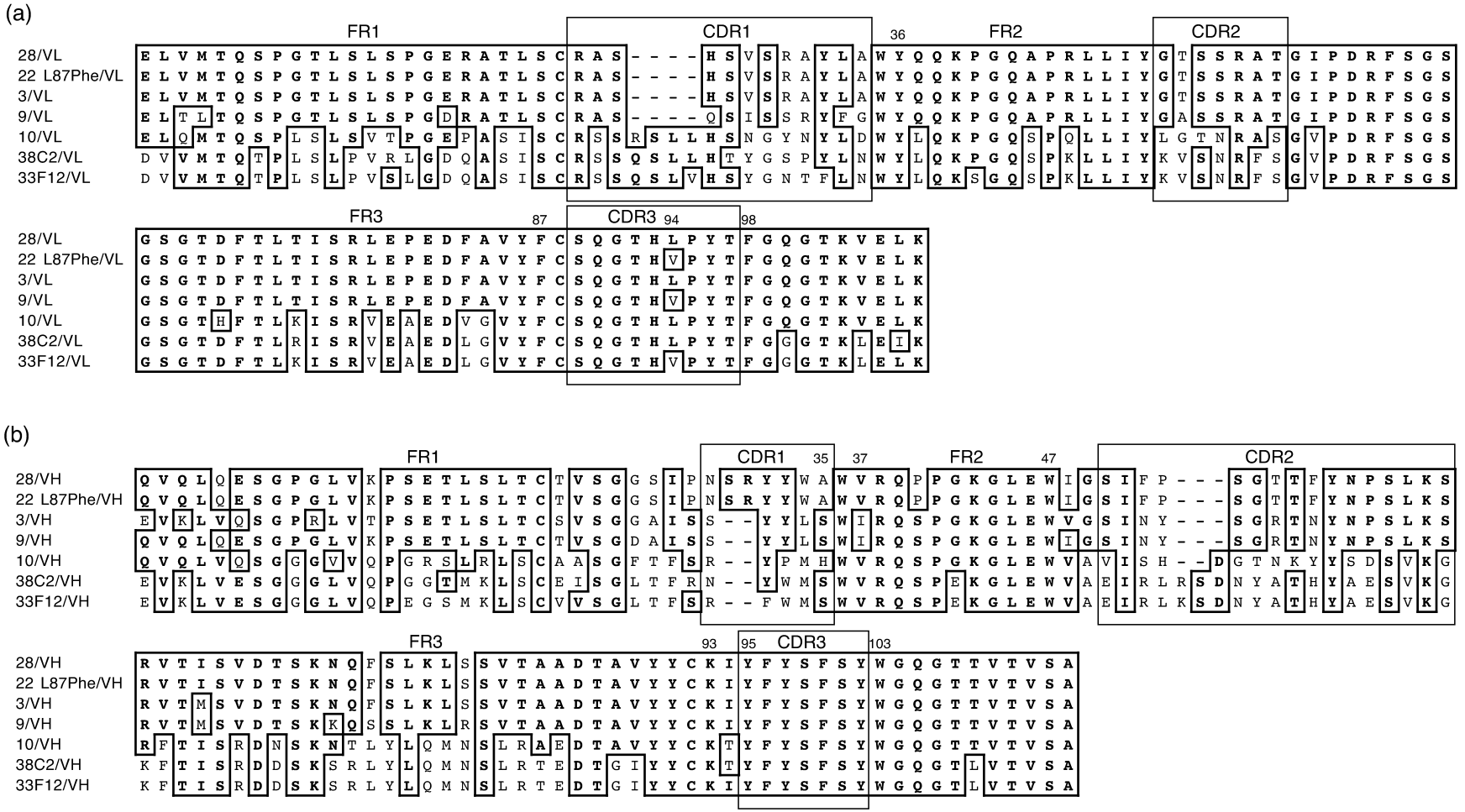
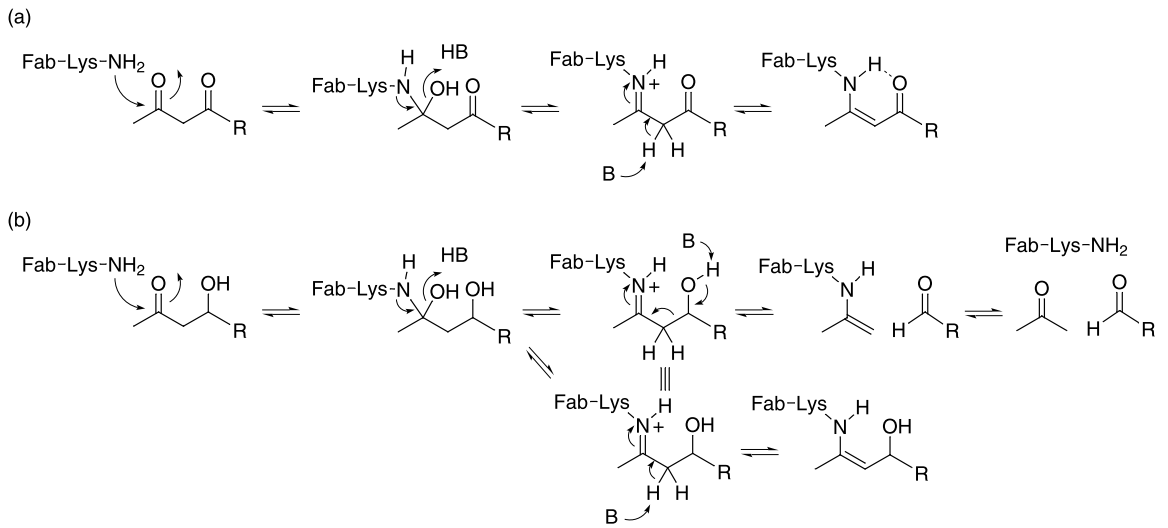


Figure 4. Alignment of the amino acid sequences of aldolase antibodies. (a) V_L segments; (b) V_H segments. CDRs are indicated.

A positive relationship was observed between the catalytic activity of selected antibodies and their binding affinity to particular 1,3-diketones, i.e. their reactivity with the diketones. Antibodies that bound to the acetone-type 1,3-diketone **6** with a lower K_d value displayed a higher k_{cat} value for the retro-aldol reaction of acetone–aldol **3** among the catalysts, while antibodies that bound to the cyclohexanone-type 1,3-diketone **7** with a lower K_d value displayed a higher k_{cat} value for the retro-aldol reaction of cyclohexanone–aldol *anti*-**4**. A rough linear relationship was observed in the plots of $\log k_{cat}$ *versus* $\log K_d$. Selections using cyclohexanone–diketone provided improved catalysts for cyclohexanone–aldol substrates. These results indicate that covalent binding selections with 1,3-diketones can be directly related to enamine-based aldol catalysis.

Catalysis of enaminone formation between a lysine group and the diketone is accelerated by general acid catalysis for dehydration of the carbinolamine intermediate and/or general base catalysis for abstraction of the α-proton of the carbonyl group (Scheme 1(a)).[21] The general acid catalyst of enaminone formation should also accelerate Schiff base formation with the aldol substrate (Scheme 1(b)).[42,43] The general base catalyst for abstraction of the α-proton of the carbonyl group in the enaminone formation pathway should contribute to increasing the concentration of the iminium ion intermediate for the retro-aldol reaction by tautomerization to the enamine.[42,43] The slope −1.16 is close to −1 in Figure 2, indicating that the difference in binding energies for diketone **6** between the catalysts is approximately equal to the energy difference corresponding to the k_{cat} value of the retro-aldol reaction of **3**. (The difference in binding energies to the diketone between Fab A and Fab B is described as $-RT \ln[(K_d \text{ of Fab A})/(K_d \text{ of Fab B})]$. The difference in energies calculated from k_{cat} between Fab A and Fab B is described as $-RT \ln[(k_{cat} \text{ of Fab A})/(k_{cat} \text{ of Fab B})]$.) Since the α-proton abstraction step is not directly related to the C–C bond cleavage, the slope in Figure 2 suggests that carbinolamine formation and/or dehydration from the carbinolamine intermediate are substantially rate-limiting in the antibody-catalyzed retro-aldol reaction of **3**. In the case of natural aldolase enzymes, the rate-limiting step can vary although the enzymes share a common enamine mechanism.[43] If the rate-limiting step of the catalyzed retro-reaction varied among Fabs, the linear relationship found in Figure 2 should not have been observed. For example, if abstraction of the alcoholic proton is the rate-limiting step in a Fab-catalyzed reaction, a deviation from the linear relationship observed in Figure 1 should occur, since this step is not existent in the enaminone formation reaction pathway involving the diketone. The linear relationship suggests that Schiff base formation is key both in the catalytic process and in diketone binding, and that the catalytic mechanism is conserved among the catalysts. Insight into the importance of Schiff base formation with the aldol substrate for the retro-aldol reaction suggests that the amino acid residue that acts as an acid catalyst for enamine formation may act as a base for deprotonation of the alcohol hydroxy group of the intermediate. In the case of the relationship observed between substrate *anti*-**4** and diketone **7** in Figure 3, the slope −0.51 indicates that the difference in binding energies for **7** between the catalysts is approximately half the energy difference corresponding to the k_{cat} value of the retro-aldol reaction of *anti*-**4**. Although a detailed mechanism including the rate-limiting step is not determined for the Fab-catalyzed retro-aldol reactions, the relationship between the k_{cat} and the K_d among the Fabs indicates a similarity in catalytic mechanism within the family of antibody aldolases inclusive of the rate-limiting step.



Scheme 1. (a) Enaminone formation with 1,3-diketone; (b) retro-aldol reaction.

Non-catalytic antibodies that bound to diketones were also obtained along with the catalysts in our selections. Similar results have been reported in hydrolytic esterase antibodies generated using phosphonate transition state analogs.[9,44–47] In these studies, a higher binding affinity to the phosphonate analog did not correlate with higher catalytic activity, since binding provides interactions not only with features of the transition state analog key to the transition state of the reaction but also with other parts of the hapten molecule present in the ground state of the molecule. The binding interaction between non-catalytic antibodies and diketones may involve exclusively non-covalent interactions, since non-covalent antibody–antigen interactions constitute the prototypical mode of binding for antibodies. The ε-amino group of LysH93 in non-catalytic antibodies could form hydrogen bonds with the diketone without forming a Schiff base. Simple binding assays based on ELISA did not provide sufficient information to discriminate amongst these binding mechanisms. Once the catalysts were compared, the binding affinity to the diketones, determined by an inhibition ELISA, correlated with catalytic activity. Selections with 1,3-diketones therefore directed the evolution of the chemical mechanism of the aldolase catalysts.

Libraries that retain the catalytic residues of the parental antibodies may increase opportunities to select varied catalysts. Although random antibody libraries that do not bias the library towards any particular catalytic reaction can be prepared, experimentally it is unfeasible to completely survey all possible protein variants using phage display.[16] Biased libraries that retain the parental catalytic residues described here effectively provided catalysts through the selection. The *in vitro* selected catalysts retain the function of the essential catalytic active site residue and the catalytic mechanism, while allowing the substrate specificity of the catalysts to be altered. Primary amino acid sequences unrelated to the parental antibodies in the library regions may contribute to substrate specificity alteration. This library strategy therefore provides a meaningful source of diversity. The library strategy we have used here provides for tremendous diversification of the antibody in a way that is not accessible by focused mutagenesis of five or six amino acid residues, a strategy that has been typically used to prepare modified or enhanced catalysts.[44–47] The library strategy described here may also be useful for the development of other catalytic activities when the appropriate biases are set in the library design. When human antibody gene segments are used for gene recombination with the catalytic machinery of an extant antibody as described here, humanized catalytic antibodies result. Humanized catalytic antibodies are desirable for therapeutic applications such as the activation of designed pro-drugs for cancer therapy or pro-proteins for diabetes.[28,34,36,48]

Most typically, catalytic antibodies have been prepared by animal immunization. Antibodies that bind to the designed hapten used for the immunization are amplified *in vivo* and catalytic antibodies are screened from among the binding antibodies. Selection of catalytic antibodies by phage display systems *in vitro* have also been performed using designed haptens.[44–47,49–52] In these experiments, antibody libraries were prepared from immunized animals that had already generated affinity mature antibodies, or antibody libraries were prepared by randomizing a small part of the parental antibodies providing for the sampling of a small amount of structural diversity. Significantly, a fully synthetic antibody library (functional size 2×10^9) was recently used for the phage selection with a reactive compound and hydrolytic antibodies were obtained.[53] Our results described here indicate that designed reactive compounds are also useful for the selection of catalytic antibodies *in vitro* from the libraries with considerably more structural diversity providing for the selection of structurally varied catalysts. 1,3-Diketones have also been used for phage selections of small peptides that catalyze aldol and retro-aldol reactions *via* an enamine mechanism[54] and have been employed to trap nucleophilic ε-amino groups of lysine residues of peptides[55] and enzymes[56–60] *via* enaminone formation. Selection with 1,3-diketones appears to be a general and effective route to catalysts that operate *via* an enamine mechanism.

According to insights from studies of evolutionary convergence within Nature's enzymes, we proposed a strategy to access improved catalytic antibodies *in vitro*. We demonstrated the efficiency of this strategy with the evolution of aldolase antibodies utilizing enamine-based reaction mechanism. Retention of chemical mechanism was found within the family of selected aldolase antibodies where substrate specificity could be purposefully modified by structural modification of the selecting 1,3-diketone. This library strategy and selection strategy with compounds designed for catalysis should also be useful for the development of other catalytic antibodies including humanized catalytic antibodies.

Materials and Methods

Catalytic assay and kinetics

Reactions were initiated by adding 5 μl of a stock solution of substrate in DMSO or CH_3CN to 95 μl of Fab solution in PBS (pH 7.4) at 25 °C for a 96 well plate, or 1 μl of a stock solution of substrate to 19 μl of Fab solution for a 384 well plate. The increase in the fluorescence (λ_{ex} 330 nm, λ_{em} 452 nm) was monitored using a Spectra Max Gemini (Molecular Devices). The observed rate was corrected for the uncatalyzed rate in the absence of Fab. The kinetic parameters, k_{cat} and K_m, were determined by curve-fitting of S–V plots as described by the Michaelis–Menten equation using MacCurveFit (Kevin

Raner). The enantiomeric purity of the substrates used in the reactions were as follows: (*S*)-**3**, 98% ee; (*R*)-**3**, 99.5% ee; (2*R*,1′*S*)-**4**, 99.0% ee; (2*S*,1′*R*)-**4**, >99.5% ee; (2*S*,1′*S*)-**4**, >99.5% ee; (2*R*,1′*R*)-**4**, >99.5% ee.

Inhibition ELISA

Prior to carrying out inhibition ELISA, the optimum Fab concentration was determined by ELISA. Microtiter plates (Costar 3690) were coated with **1**-BSA (0.5 μg/25 μl of PBS/well), at 37 °C for one hour, washed twice with water, and blocked with 3% BSA/PBS (50 μl/well) at room temperature for one hour. Blocking solution was removed, the ELISA sample (25 μl/well) was added, and the plate was incubated at room temperature for one hour. The well was washed ten times with water and the bound Fab was detected using alkaline phosphatase conjugated goat anti-human IgG $F(ab')_2$ (PIERCE) and the phosphatase substrate, *p*-nitrophenyl phosphate. The resulting yellow color was measured at 405 nm. The Fab concentration was defined as the concentration at which $0.3 < OD < 0.7$.

To 60 μl of Fab solution in PBS was added 0.6 μl of diketone in CH_3CN at room temperature. After 20 minutes, 25 μl of the Fab-inhibitor-mixture was added to the **1**-BSA (bovine serum albumin) coated well described above and ELISA was performed. The apparent dissociation constant, K_d was determined as the inhibitor concentration required to inhibit 50% of the maximal binding in a sigmoidal plot of the log of the inhibitor concentration *versus* inhibition percentage to the maximal binding.

Construction of the libraries and phage panning

Library construction and panning were described in the previous report.[20] In addition to these libraries, the libraries in which the L87 was mutated to the phenylalanine were used. The plasmid DNA, pComb3X,[16] possessing Fab genes from a naive V_L library and of the selected V_H library using **1**- and **2**-BSA, in which the sequences of the LysH93, HCDR3, and LCDR3 of the aldolase antibodies 38C2 and 33F12 were retained,[20] was used as a template for PCR. The light chain FR1-FR3 domain was amplified by PCR using the 5′-primer, RSC-F (5′-GAGGAGGAGGAGGAGGAGGCGGGGCCC AGGCGGCCGAGCTC-3′) and 3′-antisense primers that have the codon of Phe mutation at L87, 5′-CCTTGA GAGCAGAAATACACTGCAAAATC-3′ and 5′-CTTTG AGAGCAGAAATAAACCCCAACATC-3′. The remaining part of the Fab gene was amplified by PCR using 5′-primers, 5′-GATTTTGCAGTGTATTTCTGCTCTCAA GG-3′ and 5′-GATGTTGGGGTTTATTTCTGCTCTCA AGG-3′, and 3′-primer, RSC-B (5′-GAGGAGGAGGAGG AGGAGCCTGGCCGGCCTGGCCACTAGTG-3′). The PCR products were fused by PCR using the primers RSC-F and RSC-B. To recombine the light chain domain and the heavy chain domain, the light chain domain was amplified by PCR using 5′-primer RSC-F and 3′-primer Kpel (5′-CGGCTGCCGTAGGCAATAGGT-3′), and the heavy chain domain was amplified by PCR using 5′-primer pelseq (5′-ACCTATTGCCTACGGCA GCCG-3′) and 3′-primer RSC-B, and then the PCR products were fused using primers, RSC-F and RSC-B. The un-recombined and recombined fusion PCR products were digested with *Sfi* I. The *Sfi* I-digested fragments encoding the Fab were ligated to a *Sfi* I-digested pComb3X. The panning of the libraries containing L87Phe was performed by using the same procedure as described.[20]

Screening for soluble functional Fab and protein production

After the panning, the final round plasmid DNA was digested with *Sfi* I and the *Sfi* I-digested fragments encoding the Fab were ligated to a *Sfi* I-digested pAra vector.[20] Individual colonies were picked from the plates and grown in SB (5 ml) containing chloramphenicol (30 μg/ml) at 37 °C for seven hours. Expression of the Fab genes was induced by addition of arabinose (0.4%) and the cultures were incubated over night. The culture was centrifuged and the supernatant was used for assessing the binding activity by ELISA using **1**- and **2**-BSA. The Fab proteins were produced and purified as described.[20]

38C2M/H and 33F12M/H

Human-mouse chimeric Fab was prepared by the reported procedure.[16] The constant regions of these chimeric Fabs are the same as those of the *in vitro* selected aldolase Fabs. The antibody constant regions were taken from the human anti-tetanus toxoid Fab p313.[61] The antibody light chain of this clone was also used as the recipient of the LCDR3 regions of 38C2 and 33F12.

GenBank accession numbers

Fab 28 VL, AY260104; Fab 28 VH, AY260105; Fab 3 VL, AY263785; Fab 3 VH, AY263786; Fab 9 VL, AY263787; Fab 9 VH, AY263788; Fab 10 VL, AY263789; Fab 10 VH, AY263790; Fab 22L87PheVL, AY263791; Fab 22L87Phe VH, AY263792.

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References

1. Schultz, P. G. & Lerner, R. A. (1995). From molecular diversity to catalysis: lessons from the immune system. *Science*, **269**, 1835–1842.
2. Hilvert, D. (2000). Critical analysis of antibody catalysis. *Annu. Rev. Biochem.* **69**, 751–793.
3. Blackburn, G. M. & Garcon, A. (2000). Catalytic Antibodies. In *Biotechnology Biotransformations II*, Wiley-VCH, Weinheim pp. 404–490.
4. Schultz, P. G., Yin, J. & Lerner, R. A. (2002). The chemistry of the antibody molecule. *Angew. Chem., Int. Ed.* **41**, 4427–4437.
5. Tanaka, F. (2002). Catalytic antibodies as designer proteases and esterases. *Chem. Rev.* **102**, 4885–4906.
6. Kaartinen, M., Solin, M.-L. & Makela, O. (1991). V genes of oxazolone antibodies in 10 strains of mice. *Eur. J. Immunol.* **21**, 2863–2869.
7. Solin, M. L., Kaartinen, M. & Makela, O. (1992). The

same few V genes account for a majority of oxazolone antibodies in most mouse strains. *Mol. Immunol.* **29**, 1357–1362.
8. Angeles, T. S., Smith, R. G., Darsley, M. J., Sugasawara, R., Sanchez, R. I., Kenten, J. *et al.* (1993). Isoabzymes: structurally and mechanistically similar catalytic antibodies from the same immunization. *Biochemistry*, **32**, 12128–12135.
9. Miyashita, H., Hara, T., Tanimura, R., Tanaka, F., Kikuchi, M. & Fujii, I. (1994). A common ancestry for multiple catalytic antibodies generated against a single transition-state analog. *Proc. Natl Acad. Sci. USA*, **91**, 6045–6049 and see also 10757.
10. Fujii, I., Tanaka, F., Miyashita, H., Tanimura, R. & Kinoshita, K. (1995). Correlation between antigen-combining-site structures and functions within a panel of catalytic antibodies generated against a single transition state analog. *J. Am. Chem. Soc.* **117**, 6199–6209.
11. MacBeath, G. & Hilvert, D. (1996). Hydrolytic antibodies: variations on a theme. *Chem. Biol.* **3**, 433–445.
12. Buchbinder, J. L., Stephenson, R. C., Scanlan, T. S. & Fletterick, R. J. (1998). A comparison of the crystallographic structures of two catalytic antibodies with esterase activity. *J. Mol. Biol.* **282**, 1033–1041.
13. Charbonnier, J. B., Carpenter, E., Gigant, B., Golinelli-Pimpaneau, B., Eshhar, Z., Green, B. S. & Knossow, M. (1995). Crystal structure of the complex of a catalytic antibody Fab fragment with a transition state analog: structural similarities in esterase-like catalytic antibodies. *Proc. Natl Acad. Sci. USA*, **92**, 11721–11725.
14. Charbonnier, J.-B., Golinelli-Pimpaneau, B., Gigant, B., Tawfik, D. S., Chap, R., Scindler, D. G. *et al.* (1997). Structural convergence in the active sites of a family of catalytic antibodies. *Science*, **275**, 1140–1142.
15. Tantillo, D. J. & Houk, K. N. (2001). Canonical binding arrays as molecular recognition elements in the immune system: tetrahedral anions and the ester hydrolysis transition state. *Chem. Biol.* **8**, 535–545.
16. Barbas, C. F. III; Burton, D. R.; Scott, J. K.; Silverman, G. J. (eds) (2001). *Phage Display of Proteins and Peptides: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
17. Branden, C. & Tooze, J. (1991). *Introduction to Protein Structure*, Garland Publishing, New York p. 236.
18. Russell, R. B. (1998). Detection of protein three-dimensional side-chain patterns: new examples of convergent evolution. *J. Mol. Biol.* **279**, 1211–1227.
19. Rao, J. K., Erickson, J. W. & Wlodawer, A. (1991). Structural and evolutionary relationships between retroviral and eukaryotic aspartic proteinases. *Biochemistry*, **30**, 4663–4671.
20. Tanaka, F., Lerner, R. A. & Barbas, C. F., III (2000). Reconstructing aldolase antibodies to alter their substrate specificity and turnover. *J. Am. Chem. Soc.* **122**, 4835–4836.
21. Wagner, J., Lerner, R. A. & Barbas, C. F., III (1995). Efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. *Science*, **270**, 1797–1800.
22. Barbas, C. F., III, Heine, A., Zhong, G., Hoffmann, T., Gramatikova, S., Bjornestedt, R. *et al.* (1997). Immune *versus* natural selection: antibody aldolases with enzymic rates but broader scope. *Science*, **278**, 2085–2092.
23. Hoffmann, T., Zhong, G., List, B., Shabat, D., Anderson, J., Gramatikova, S. *et al.* (1998). Aldolase antibodies of remarkable scope. *J. Am. Chem. Soc.* **120**, 2768–2779.
24. Zhong, G., Hoffmann, T., Lerner, R. A., Danishefsky, S. & Barbas, C. F., III (1997). Antibody-catalyzed enantioselective Robinson annulation. *J. Am. Chem. Soc.* **119**, 8131–8132.
25. List, B., Shabat, D., Barbas, C. F., III & Lerner, R. A. (1998). Enantioselective total synthesis of some Brevicomins using aldolase antibody 38C2. *Chem. Eur. J.* **4**, 881–885.
26. Zhong, G., Shabat, D., List, B., Anderson, J., Sinha, R. A. & Barbas, C. F., III (1998). Catalytic enantioselective retro-aldol reactions: kinetic resolution of β-hydroxyketones with aldolase antibodies. *Angew. Chem., Int. Ed.* **37**, 2481–2484.
27. List, B., Shabat, D., Zhong, G., Turner, J. M., Li, A., Bui, T. *et al.* (1999). A catalytic enantioselective route to hydroxy-substituted quaternary carbon centers: resolution of tertiary aldols with a catalytic antibody. *J. Am. Chem. Soc.* **121**, 7283–7291.
28. Shabat, D., Rader, C., List, B., Lerner, R. A. & Barbas, C. F., III (1999). Multiple event activation of a generic prodrug trigger by antibody catalysis. *Proc. Natl Acad. Sci. USA*, **96**, 6925–6930.
29. List, B., Lerner, R. A. & Barbas, C. F., III (1999). Enantioselective aldol cyclodehydration catalyzed by antibody 38C2. *Org. Letters*, **1**, 59–61.
30. Shabat, D., List, B., Lerner, R. A. & Barbas, C. F., III (1999). A short enantioselective synthesis of 1-deoxy-L-xylulose by antibody catalysis. *Tetrahedron Letters*, **40**, 1437–1440.
31. Sinha, S., Barbas, C. F., III & Lerner, R. A. (1998). The antibody catalysis route to the total synthesis of epothilones. *Proc. Natl Acad. Sci. USA*, **95**, 14603–14608.
32. Tanaka, F., Lerner, R. A. & Barbas, C. F., III (1999). Thiazolium-dependent catalytic antibodies produced using a covalent modification strategy. *Chem. Commun.* 1383–1384.
33. Turner, J. M., Bui, T., Lerner, R. A., Barbas, C. F., III & List, B. (2000). An efficient benchtop system for multigram-scale kinetic resolutions using aldolase antibodies. *Chem. Eur. J.* **6**, 2772–2774.
34. Shabat, D., Lode, H. N., Pertl, U., Reisfeld, R. A., Rader, C., Lerner, R. A. & Barbas, C. F., III (2001). *In vitro* activity in a catalytic antibody-prodrug system: antibody catalyzed etoposide prodrug activation for selective chemotherapy. *Proc. Natl Acad. Sci. USA*, **98**, 7528–7533.
35. Tanaka, F., Kerwin, L., Kubitz, D., Lerner, R. A. & Barbas, C. F., III (2001). Visualizing antibody-catalyzed retro-aldol–retro-Michael reactions. *Bioorg. Med. Chem. Letters*, **11**, 2983–2986.
36. Worrall, D. S., McDunn, J. E., List, B., Reichart, D., Hevener, A., Gustafson, T. *et al.* (2001). Synthesis of an organoinsulin molecule that can be activated by antibody catalysis. *Proc. Natl Acad. Sci. USA*, **98**, 13514–13518.
37. Tanaka, F. & Barbas, C. F. (2002). III Reactive immunization: a unique approach to catalytic antibodies. *J. Immunol. Methods*, **269**, 67–79.
38. Kabat, E. A., Wu, T. T., Perry, H. M., Gottesman, K. S. & Foeller, C. (1991). *Sequences of Proteins of Immunological Interest*, 5th edit., US Public Health Service, National Institute of Health, Bethesda, MD.
39. List, B., Barbas, C. F., III & Lerner, R. A. (1998). Aldol sensors for the rapid generation of tunable fluorescence by antibody catalysis. *Proc. Natl Acad. Sci. USA*, **95**, 15351–15355.

40. Friguet, B., Chaffotte, A. F., Djavadi-Ohaniance, L. & Goldberg, M. E. (1985). Measurements of the true affinity constant in solution of antigen–antibody complexes by enzyme-linked immunosorbent assay. *J. Immunol. Methods*, **77**, 305–319.
41. Karlstrom, A., Zong, G., Rader, C., Larsen, N. A., Heine, A., Fuller, R. *et al.* (2000). Using antibody catalysis to study the outcome of multiple evolutionary trials of a chemical task. *Proc. Natl Acad. Sci. USA*, **97**, 3878–3883.
42. Littlechild, J. A. & Watson, H. C. (1993). A data-based reaction mechanism for type I fructose bisphosphate aldolase. *Trends Biochem. Sci.* **18**, 36–39.
43. Morris, A. J., Davenport, R. C. & Tolan, D. R. (1996). A lysine to arginine substitution at position 146 of rabbit aldolase A changes the rate-determining step to Schiff base formation. *Protein Eng.* **9**, 61–67 and references therein.
44. Baca, M., Scanlan, T. S., Stephenson, R. C. & Wells, J. (1997). Phage display of catalytic antibody to optimize affinity for transition-state analog binding. *Proc. Natl Acad. Sci. USA*, **94**, 10063–10068.
45. Fujii, I., Fukuyama, S., Iwabuchi, Y. & Tanimura, R. (1998). Evolving catalytic antibodies in a phage-displayed combinatorial library. *Nature Biotechnol.* **16**, 463–467.
46. Arkin, M. R. & Wells, J. A. (1998). Probing the importance of second sphere residues in an esterolytic antibody by phage display. *J. Mol. Biol.* **284**, 1083–1094.
47. Takahashi, N., Kakinuma, H., Liu, L., Nishi, Y. & Fujii, I. (2001). *In vitro* abzyme evolution to optimize antibody recognition for catalysis. *Nature Biotechnol.* **19**, 563–567.
48. Bagshawe, K. D. (1989). Toward generating cytotoxic agents at cancer sites. *Br. J. Cancer*, **60**, 275–281.
49. Janda, K. D., Lo, C.-H. L., Li, T., Barbas, C. F., III, Wirsching, P. & Lerner, R. A. (1994). Direct selection for a catalytic mechanism from combinatorial antibody libraries. *Proc. Natl Acad. Sci. USA*, **91**, 2532–2536.
50. Janda, K. D., Lo, L.-C., Lo, C.-H. L., Sim, M.-M., Wang, R., Wong, C.-H. & Lerner, R. A. (1997). Chemical selection for catalysis in combinatorial antibody libraries. *Science*, **275**, 945–948.
51. Gao, C., Lavey, B. J., Lo, C.-H. L., Datta, A., Wentworth, P. & Janda, K. D. (1998). Direct selection for catalysis from combinatorial antibody libraries using boronic acid probe: primary amide bond hyrolysis. *J. Am. Chem. Soc.* **120**, 2211–2217.
52. Tanaka, F., Almer, H., Lerner, R. A. & Barbas, C. F., III (1999). Catalytic single-chain antibodies possessing β-lactamase activity selected from a phage displayed combinatorial library using a mechanism-based inhibitor. *Tetrahedron Letters*, **40**, 8063–8066.
53. Cesaro-Tadic, S., Lagos, D., Honegger, A., Rickard, J. H., Partridge, L. J., Blackburn, G. M. & Pluckthun, A. (2003). Turnover-based *in vitro* selection and evolution of biocatalysts from a fully synthetic antibody library. *Nature Biotechnol.* **21**, 679–685.
54. Tanaka, F. & Barbas, C. F., III (2001). Phage display selection of peptides possessing aldolase activity. *Chem. Commun.* 769–770.
55. Tanaka, F. & Barbas, C. F., III (2002). A modular assembly strategy for improving the substrate specificity of small catalytic peptides. *J. Am. Chem. Soc.* **124**, 3510–3511.
56. Tagaki, W., Guthrie, J. P. & Westheimer, F. H. (1968). Acetoacetate decarboxylase. Reaction with acetopyruvate. *Biochemistry*, **7**, 905–913.
57. Gilbert, H. F., III & O'Leary, M. H. (1975). Modification of arginine and lysine in proteins. *Biochemistry*, **14**, 5194–5199.
58. Gilbert, H. F., III & O'Leary, M. H. (1977). Reversible modification of amino groups in aspartate aminotransferase. *Biochim. Biophys. Acta*, **483**, 79–89.
59. Otwell, H. B., Cipollo, K. L. & Dunlap, R. B. (1979). Modification of lysyl residues of dihydrofolate reductase with 2,4-pentanedione. *Biochim. Biophys. Acta*, **568**, 297–306.
60. Guidinger, P. F. & Nowak, T. (1991). An active-site lysine in liver phosphoenolpyruvate carboxykinase. *Biochemistry*, **30**, 8851–8861.
61. Barbas, C. F., III, Kang, A. S., Lerner, R. A. & Benkovic, S. J. (1991). Assembly of combinatorial antibody libraries on phage surfaces: the gene III site. *Proc. Natl Acad. Sci. USA*, **88**, 7978–7982.

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