Reconstructing Aldolase Antibodies to Alter Their Substrate Specificity and Turnover

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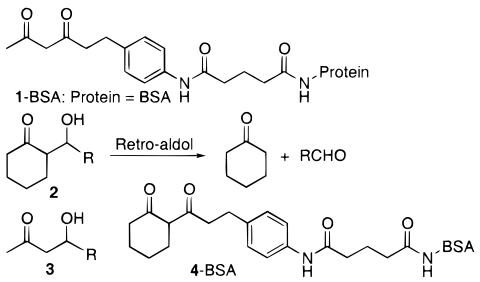
Many enzymes use common catalytic mechanisms in the catalysis of analogous chemical transformations, for example in the hydrolysis of esters and amides. Conservation of catalytic mechanism is observed not only among evolutionarily related and highly homologous enzymes but also between structurally different and evolutionarily unrelated enzymes.[1] 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 with different substrates where chemical mechanism is conserved. In the case of catalytic antibodies, immunization of mice with transition-state analogues designed for the preparation of hydrolytic antibodies has provided a variety of catalytic antibodies that demonstrate high levels of homology at both structural and mechanistic levels.[2] 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.[3] This fact acts to experimentally limit our opportunities to probe the structural repertoire available to antibodies more completely. To search for novel aldolase antibodies we sought a strategy that would not be limited by the need to reimmunize animals but one that would take advantage of insights gained by the study of existing catalysts.

Here we examine a new strategy to obtain improved catalytic antibodies by recombining the catalytic machinery of parental antibodies with a naive V gene repertoire. Antibody libraries of this type may provide new evolutionary opportunities for catalysis not accessible through the relatively small, five or six amino acid residues changes[4] in protein sequence that are typically probed to prepare modified or enhanced catalysts.

Aldolase antibodies 38C2 and 33F12 were previously generated by reactive immunization with 1,3-diketone **1**.[5] The antibodies are highly homologous with respect to sequence, structure, and catalytic mechansim.[5,6] Both antibodies possess a highly reactive lysine residue (LysH93)[7] in their active sites that is essential to their catalytic mechanism. The ϵ-amino group of the lysine residue is key in the formation of Schiff base and enamine intermediates that appear along the reaction coordinate of the aldol reaction.[5,6] Although these antibodies possess unusually promiscuous active sites capable of catalyzing a wide variety of aldol and retro-aldol reactions, the efficiency with which any given aldol is processed can vary significantly. For example, retro-aldol reactions of cyclohexanone-aldols **2** are relatively slow compared to those involving acetone-aldols **3**. The keto functionality appears to be key in determining the relative efficiency of processing by these catalysts since **3** with a wide variety of R groups are processed efficiently. Therefore, we focused on the alteration of substrate specificity using this strategy with the aim of preparing catalysts that would more efficiently process **2**.

To retain the catalytic function of the residue LysH93, we took advantage of information provided by the crystal structure of 33F12.[6a] The structure showed the reactive lysine at the bottom of a deep binding pocket where most of the residues within a 4 Å radius of the ϵ-amino group of LysH93 are hydrophobic and are thus likely key in tuning the pK_a of this amine group. These residues are SerH35, ValH37, TrpH47, TyrH95, TrpH103, and PheL98. TyrH95 and TrpH103 are found in HCDR3 (heavy chain complementarity determining region 3), and PheL98 is in LCDR3. Therefore, the sequences of the LysH93, HCDR3, and LCDR3 of the aldolase antibodies were retained in the library. 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.[8] 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.[9] The phage displayed libraries[8,10] were selected by three rounds of panning against 1,3-diketones **4**- and **1**-BSA (bovine serum albumin) in order to select antibodies that would accept both **2** and **3**. In a



subsequent diversification step, V_L libraries were combined with the selected V_H libraries, and three additional rounds of selection were performed.

Screening of the phage selected clones by ELISA for the production of soluble Fab capable of binding to both **4**- and

(1) (a) Branden, C.; Tooze, J. *Introduction to Protein Structure*; Garland Publishing: New York, 1991; p 236. (b) Russell, R. B. *J. Mol. Biol.* **1998**, *279*, 1211. (c) Rao, J. K.; Erickson, J. W.; Wlodawer, A. *Biochemistry* **1991**, *30*, 4663.

(2) (a) Angeles, T. S.; Smith, R. G.; Darsley, M. J.; Sugasawara, R.; Sanchez, R. I.; Kenten, J.; Schultz, P. G.; Martin, M. T. *Biochemistry* **1993**, *32*, 12128. (b) Miyashita, H.; Hara, T.; Tanimura, R.; Tanaka, F.; Kikuchi, M.; Fujii, I. *Proc. Natl. Acad. Sci. U.S.A.* **1994**, *91*, 6045. (c) Fujii, I.; Tanaka, F.; Miyashita, H.; Tanimura, R.; Kinoshita, K. *J. Am. Chem. Soc.* **1995**, *117*, 6199. (d) MacBeath, G.; Hilvert, D. *Chem. Biol.* **1996**, *3*, 433. (e) Buchbinder, J. L.; Stephenson, R. C.; Scanlan, T. S.; Fletterick, R. J. *J. Mol. Biol.* **1998**, *282*, 1033. (f) Charbonnier J.-B.; Golinelli-Pimpaneau, B.; Gigant, B.; Tawfik, D. S.; Chap, R.; Scindler, D. G.; Kim, S.-H.; Green, B. S.; Eshhar, Z.; Knossow, M. *Science* **1997**, *275*, 1140.

(3) Kaartinen, M.; Solin, M.-L.; Mäkelä, O. *Eur. J. Immunol.* **1991**, *21*, 2863.

(4) (a) Baca, M.; Scanlan, T. S.; Stephenson, R. C.; Wells, J. *Proc. Natl. Acad. Sci. U.S.A.* **1997**, *94*, 10063. (b) Fujii, I.; Fukuyama, S.; Iwabuchi, Y.; Tanimura, R. *Nat. Biotechnol.* **1998**, *16*, 463.

(5) Wagner, J.; Lerner, R. A.; Barbas, C. F., III. *Science* **1995**, *270*, 1797.

(6) (a) 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. (b) 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. (c) Zhong, G.; Hoffmann, T.; Lerner, R. A.; Danishefsky, S.; Barbas, C. F., III. *J. Am. Chem. Soc*. **1997**, *119*, 8131. (d) List, B.; Shabat, D.; Barbas, C. F., III; Lerner, R. A. *Chem. Eur. J.* **1998**, *4*, 881. (e) Zhong, G.; Shabat, D.; List, B.; Anderson, J.; Sinha, R. A.; Lerner, R. A.; Barbas, C. F., III. *Angew. Chem., Int. Ed.* **1998**, *110*, 2609. (f) List, B.; Shabat, D.; Zhong, G.; Turner, J. M.; Li, A.; Bui, T.; Anderson, J.; Lerner, R. A.; Barbas, C. F., III. *J. Am. Chem. Soc.* **1999**, *121*, 7283. (g) Shabat, D.; Rader, C.; List, B.; Lerner, R. A.; Barbas, C. F., III. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6925. (h) List, B.; Lerner, R. A.; Barbas, C. F., III. *Org. Lett.* **1999**, *1*, 59. (i) Shabat, D.; List, B.; Lerner, R. A.; Barbas, C. F., III. *Tetrahedron Lett.* **1999**. *40*, 1437. (j) Sinha, S.; Barbas, C. F., III; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 14603.

(7) The numbering is according to Kabat et al. Kabat, E. A.; Wu, T. T.; Perry, H. M.; Gottesman, K. S.; Foeller, C. *Sequences of Proteins of Immunological Interest*, 5th ed.; U.S. Public Health Service, National Institute of Health: Bethesda, MD, 1991.

(8) Barbas, C. F., III, Burton, D. R., Scott, J., Silverman, G., Eds. *Phage Display of Proteins and Peptides: A Laboratory Manual*; Cold Spring Harbor Laboratory Press: Cold Spring Harbor, New York, 2000.

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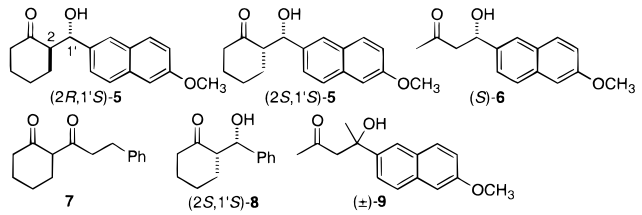
Table 1. Kinetic Parameters of the Retro-Aldol Reactions of 5[a]

	anti-**5**				*syn*-**5**			
		K_m, μM	k_{cat}, min^{-1}	k_{cat}/k_{uncat} [b]		K_m, μM	k_{cat}, min^{-1}	k_{cat}/k_{uncat} [b]
Fab 28	±	1.8×10^2	1.0×10^{-1}	2.3×10^4	±	1.1×10^2	2.9×10^{-1}	1.2×10^4
Fab 28[c]	2*R*,1′*S*[d]	1.0×10^2	9.5×10^{-2}	2.1×10^4	2*S*,1′*S*[d]	87	2.9×10^{-1}	1.2×10^4
38C2	±	36	3.4×10^{-2}	7.5×10^3	±	1.1×10^2	9.7×10^{-2}	4.0×10^3
33F12	±	97	1.1×10^{-2}	2.5×10^3	±	1.3×10^2	4.7×10^{-2}	1.9×10^3

[a] Reaction conditions: 5% DMSO/PBS (pH 7.4), 25 °C except as noted. [b] The first-order kinetic constant of the background reaction (k_{uncat}) was 4.5×10^{-6} min^{-1} for (±)-*anti*-**5** and 2.4×10^{-5} min^{-1} for (±)-*syn*-**5**. [c] Reaction was performed in 5% CH_3CN/PBS (pH 7.4). The k_{uncats} were the same as those observed in 5% DMSO/PBS (pH 7.4). [d] See ref 15.

1-BSA, identified 23 clones. The five clones that showed the strongest ELISA signal were chosen for further study, and the antibody Fab fragments were purified.[11] Screening for the ability to catalyze the retro-aldol reaction of fluorogenic substrate **5**[12] using 0.5 μM of Fab and a mixture of (±)-*anti*-**5** (125 μM)−(±)-*syn*-**5** (125 μM) in 5% DMSO/PBS (pH 7.4) at 25 °C, identified antibody Fab 28 as the best catalyst, and this antibody was studied in detail. Fab 28-catalyzed reactions displayed saturation kinetics described by the Michaelis−Menten equation in the steady-state of the retro-aldol reaction. The kinetic parameters are shown in Table 1. The k_{cat} values of Fab 28 were superior to those of the parental antibodies for these substrates by approximately 3 to 10-fold. In addition, Fab 28 catalyzed the retro-aldol reaction of (±)-**6**.[13] The k_{cat} value is similar to that of antibody 33F12.[13] Fab 28-catalyzed reactions were inhibited by the addition of diketone **7**. The K_i was determined by Dixon analysis to be 1.0 μM in 28-catalyzed retro-aldol reaction of (±)-*syn*-**5**. To determine the enantioselectivity of the catalyzed reaction, each of the enantiomers of these substrates were separately studied.[14] Fab 28 catalyzed the retro-aldol reactions of (2*R*,1′*S*)-**5**, (2*S*,1′*S*)-**5** (Table 1), and of (*S*)-**6**.[15] Catalysis of retro-aldolization of the opposite optical isomers was not detected. The stereochemistries of the preferred substrate enantiomers of Fab 28 are the same as those of the parental antibodies. Fab 28 also catalyzed the retro-aldol reaction of (2*S*,1′*S*)-**8**.[15−17] This reaction was also stereoselective, and the ratio of the initial velocities of 28-catalyzed reactions using each of the enantiomers of *syn*-**8**[15] (200 μM) was >99:1. This contrasts the results obtained for 38C2-catalyzed reactions of the enantiomers of *syn*-**8** (200 μM) where an insignificant velocity ratio of 2.4:1 was observed. Fab 28 also catalyzed the retro-aldol reactions of tertiary aldol (±)-**9**.[6f,18]



The key elements of the catalytic mechanism of the parental antibodies appear to be conserved in Fab 28-catalyzed reactions. When Fab 28 (2.6 μM) was mixed with 2,4-pentanedione (**10**) (500 μM) in 5% CH_3CN/PBS (pH 7.4), a new absorption at 318 nm was observed. This result is consistent with enaminone formation[5,6] between Fab 28 and **10**, and with the reaction mechanism of the parental antibodies. The amino acid sequences of V_L and V_H of Fab 28 and of the parental antibodies are shown in Figure 1 in the Supporting Information. As expected from the library construction, Fab 28 retained sequence elements of the parental antibodies as was the design of the library. The remaining protein sequence is of human origin and is not related in primary sequence to the parental antibodies. To further evaluate the role of LysH93 in Fab 28, it was mutated to alanine and methionine. The mutants did not catalyze any of the reactions studied above and displayed reduced binding affinities for diketones.[19] Further, enaminone formation between the mutants and diketones was not detected. These results are consistent with an essential role of LysH93 in the catalytic mechanism of Fab 28.

In this work, we have demonstrated that a V gene shuffling strategy can be used to reconstruct aldolase antibodies while retaining the catalytic residues and mechanism of the parental antibodies. This strategy that provides meaningful structural diversity is complementary to the existing lower-complexity randomization procedures[4,11] and is analogous to family-based DNA shuffling strategies.[20] Since the aldolase antibody selected here is now humanized with respect to its primary sequence, it has potential utility in the activation of designed prodrugs for cancer therapy.[6g] In addition to selection with novel diketones, the libraries in this report might also allow for the selection of catalysts not accessible though immunization, for example, novel catalysts by posttranslational modification at LysH93 within the library to prepare cofactor-dependent catalytic antibodies.[21]

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Supporting Information Available: Alignment of the amino acid sequences, synthesis of **5**, assays, enaminone formation, construction of the libraries, panning selection, protein production and purification, and preparation of mutants (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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(9) The antibody constant regions were taken from the human anti-tetanus toxoid Fab p313. The antibody light chain of this clone was also used as the recipient of the LCDR3s of 38C2 and 33F12. Barbas, C. F., III; Kang, A. S.; Lerner, R. A.; Benkovic, S. J. *Proc. Natl. Acad. Sci. U.S.A.* **1991**, *88*, 7978.

(10) Phage display approach for humanization of binding antibodies: Rader, C.; Cheresh, D.; Barbas, C. F., III. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 8910.

(11) Yang, W.-P.; Green, K.; Pinz-Sweeney, S.; Briones, A. T.; Burton, D. R.; Barbas, C. F., III. *J. Mol. Biol.* **1995**, *254*, 392.

(12) List, B.; Barbas, C. F., III; Lerner, R. A. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 15351.

(13) The kinetic parameters for (±)-**6**: Fab 28; K_m 6.5×10^2 μM, k_{cat} 9.5×10^{-2} min^{-1}, and k_{cat}/k_{uncat} 2.1×10^5. Antibody 33F12; K_m 43 μM, k_{cat} 1.1×10^{-1} min^{-1}, and k_{cat}/k_{uncat} 2.4×10^5 (per active site).

(14) Tanaka, F.; Kinoshita, K.; Tanimura, R.; Fujii, I. *J. Am. Chem. Soc.* **1996**, *118*, 2332 and references therein.

(15) The purity of the enantiomeric substrates that were used in the reactions is as follows: (2*R*,1′*S*)-**5**, 99.0% ee; (2*S*,1′*R*)-, (2*S*,1′*S*)-, and (2*R*,1′*R*)-**5**, >99.5% ee; (*S*)-**6**, 98% ee; (*R*)-**6**, 99.5% ee; (2*S*,1′*S*)- and (2*R*,1′*R*)-**8**, >99.5% ee. The enantiomers were purified using chiral phase HPLC, and the ee was determined by the HPLC analysis. The absolute stereochemistry of *syn*-**5** was determined by the analogy of the reactivity in the 28-catalyzed retro-aldol reactions of known enantiomers, (2*S*,1′*S*)- and (2*R*,1′*R*)-**8**.[16] For the determination of absolute stereochemistry of *anti*-**5**, an enantiomer of *anti*-**5** (>99.5% ee) was epimerized by treating with K_2CO_3 in MeOH to provide *syn*-**5**.[16]

(16) Denmark, S. E.; Stavenger, R. A.; Wong, K.-T.; Su, X. *J. Am. Chem. Soc.* **1999**, *121*, 4982.

(17) The kinetic parameters for (2*S*,1′*S*)-**8**: K_m 2.3×10^2 μM, k_{cat} 4.6×10^{-2} min^{-1}, and k_{cat}/k_{uncat} 1.5×10^4 in 10% CH_3CN/PBS (pH 7.4). The reaction was monitored by HPLC analysis.

(18) The kinetic parameters for (±)-**9**: K_m 2.0×10^2 μM, k_{cat} 2.9×10^{-1} min^{-1}, and k_{cat}/k_{uncat} 2.0×10^5 in 5% CH_3CN/PBS (pH 7.4).

(19) The K_d of Fab 28 for diketone **7** was determined to be 0.8 μM by competitive ELISA while the K_ds of the alanine and methionine mutants were determined to be 6×10^2 and 8×10^2 μM, respectively. The K_d was determined as the inhibitor concentration required to inhibit 50% of the maximal binding in the competitive ELISA using **1**-BSA coated well.

(20) Crameri, A.; Raillard, S. A.; Bermudez, E.; Stemmer, W. P. *Nature* **1998**, *391*, 288.

(21) Tanaka, F.; Lerner, R. A.; Barbas, C. F., III. *Chem. Commun.* **1999**, 1383.