

Review Article

Using the Process of Reactive Immunization to Induce Catalytic Antibodies with Complex Mechanisms: Aldolases*

Richard A. Lerner and Carlos F. Barbas III

Departments of Chemistry and Molecular Biology, The Scripps Research Institute, La Jolla, CA 92037, USA

Lerner, R. A. and Barbas, C. F. III, 1996. Using the Process of Reactive Immunization to Induce Catalytic Antibodies with Complex Mechanisms: Aldolases. – Acta Chem. Scand. 50: 672–678. © Acta Chemica Scandinavica 1996.

The process of reactive immunization has been used to induce efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes. Reactive immunogens are those that react chemically during induction of the immune response. This same reaction is used later in catalysis. In essence one immunizes with the equivalent of a mechanism-based inhibitor. The difference is that instead of inhibiting a mechanism, a mechanism is induced. This advance allows the experimenter to dictate the exact mechanism by which catalytic antibodies proceed. The hapten used in the present study is a 1,3-diketone that both traps the requisite lysine residue to initiate formation of the enamine and induces a binding pocket that overcomes the entropic barrier of this bimolecular reaction.

Over the past ten years antibodies that catalyze a wide variety of chemical transformations have been induced.¹ In most cases the antibodies have been prepared to an analogue of the rate-limiting transition state such that upon binding the antibody lowers the energy of the actual transition state and increases the rate of the reaction. These catalysts can be programmed to bind to geometrical and electrostatic features of the transition state so that the reaction route can be controlled by neutralizing unfavorable charges, overcoming entropic barriers, and dictating stereoelectronic features of the reaction. By this means even reactions that are otherwise highly disfavored have been catalyzed. However, for some reactions that require complex mechanisms (such as covalent catalysis) the exact nature of the functionalities in the protein that contribute to the binding energy are of critical importance and until recently there was no way to dictate the nature of the amino acid side chains that participate in the binding of the hapten. However, recently a new procedure called reactive

immunization has been developed which in some cases allows the experimenter to dictate the exact chemical mechanism by which the antibody catalysis occurs.^{2,3} Here we describe the concept of reactive immunization and illustrate its power by demonstrating how it has been used to induce highly efficient aldolase catalytic antibodies that use the enamine mechanism of natural enzymes.

Concept of reactive immunization.

Since its inception immunochemists have sought to use, as antigens, materials that have as little chemical reactivity as possible. In fact, when one has immunized with proteins it is often stated as a matter of pride that the immunogen is of highly native conformation. This is understandable since it is almost always the case that one wishes the ultimate antibody to interact with native structures. For instance, if one assumes that the poliovirus infectious particle consists of proteins of native conformation, antibodies that neutralize the virus must also interact with proteins of native conformation. In reactive immunization the concept is just the opposite. One immunizes with compounds that are highly reactive so that upon binding to the antibody molecule during

* Contribution presented at the Nobel Symposium on *Catalytic Asymmetric Synthesis*, September 3–7, 1995 at Tammsvik, Bro, Sweden.

the induction process a chemical reaction ensues. Later this same chemical reaction becomes part of the mechanism of the catalytic event. In a certain sense one is immunizing with a chemical reaction rather than a substance *per se*. Reactive immunogens can be considered as analogous to the mechanism-based inhibitors that enzymologists use except that they are used in the inverse way in that instead of inhibiting a mechanism they induce a mechanism.

The aldol addition

To illustrate the power of reactive immunization we consider here how the process has been used to induce aldolase catalytic antibodies that utilize the complex mechanism of the natural enzymes.³ The basic goal of these experiments is to mimic the reaction mechanism of type I aldolases⁴ that feature an active site lysine residue that participates in the activation of the aldol donor [Fig. 1(a)]. Upon binding to the ketone substrate the active site lysine forms a Schiff base or iminium ion that

lowers the activation energy for proton abstraction and collapse to the enamine. The enamine functionality is central to the mechanism in that it ultimately functions as the carbon nucleophile. After addition to the electrophilic aldol acceptor and formation of the new carbon-carbon bond the Schiff base is hydrolyzed to release the aldol product which is shown as the β -hydroxy ketone. In order to mimic this complex multi-step mechanism it is necessary both to overcome the entropic barrier of this bimolecular reaction and form the enamine functionality that is central to the mechanism. To accomplish this by reactive immunization we designed compound 1 [Fig. 1(b)] that can be expected to induce antibodies that both function to overcome the entropic barrier and trap the critical lysine residue. This is an example of reactive immunization in that the central mechanistic idea is that one inductive phase of antibody formation involves a chemical reaction in the binding pocket. Further, the reaction coordinate traversed during the induction with 1 is designed to overlap that traversed in the desired catalytic reaction. It was expected that

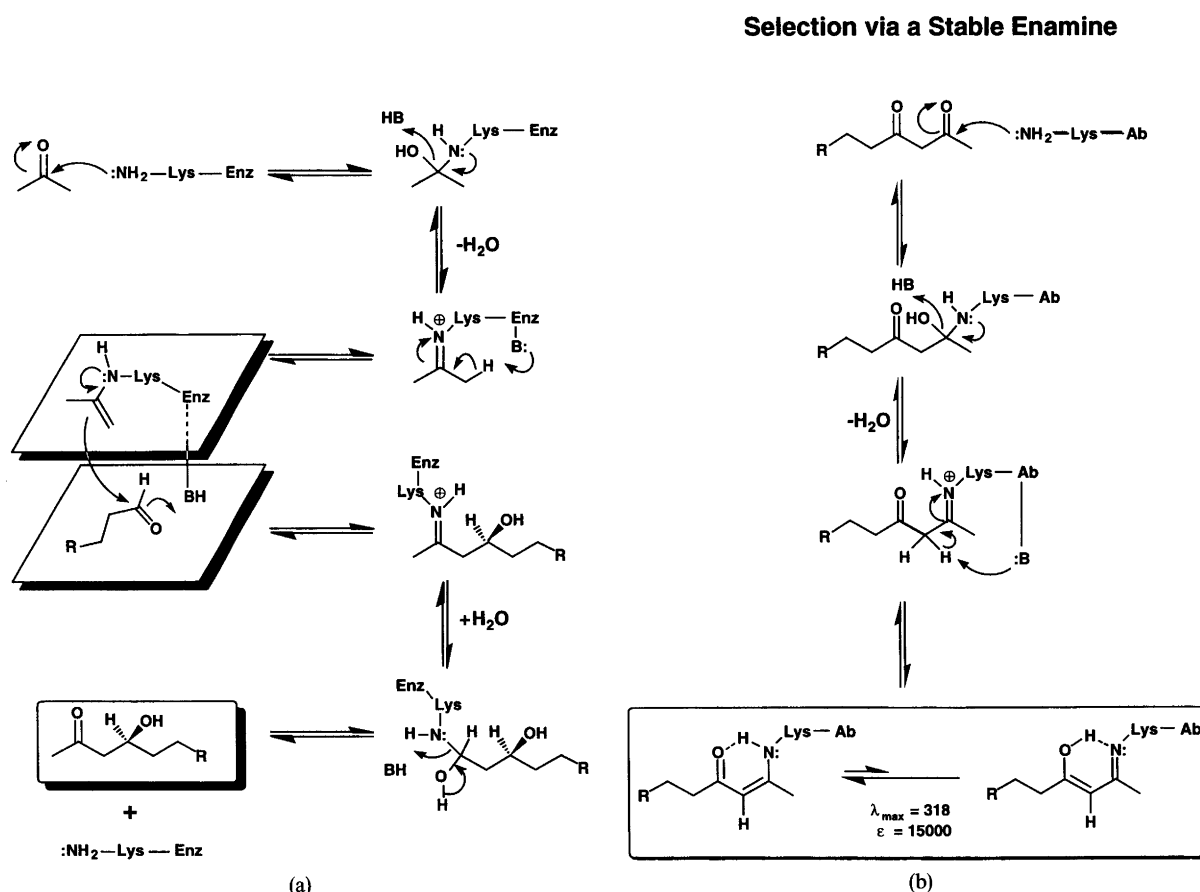


Fig. 1. (a) General mechanism of a class I aldolase catalyzed aldol addition reaction (4) (Enz, enzyme; B, base). (b) Mechanism of trapping the essential ϵ -amino group of a Lys residue in the antibody (Ab) binding pocket using the 1,3-diketone hapten 1. The formation of the stable covalent vinyllogous amide 2 can be detected at $\lambda = 316$ nm (ϵ is the extinction coefficient). $R = p\text{-[HOOC(CH}_2\text{)}_3\text{CONH]C}_6\text{H}_4^-$. (Reprinted with permission from *Science*. Copyright 1995 American Association for the Advancement of Science.)

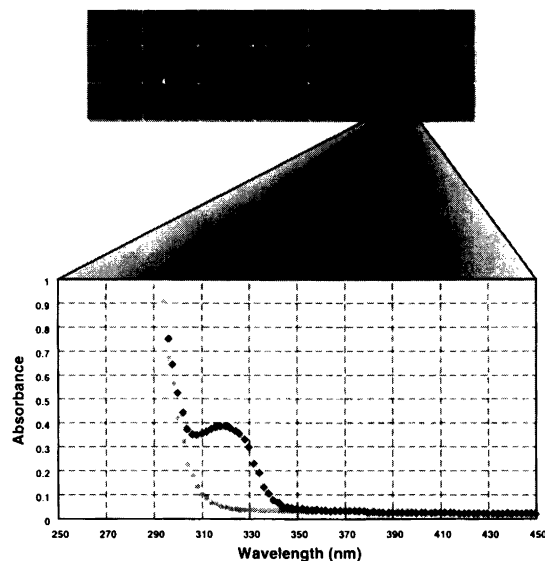


Fig. 2. Screening of antibodies for the formation of the vinylogous amide intermediate **2**. Hapten **1**, 5 equiv., was added to 20 mM solutions of each antibody in PBS buffer (pH=7.5) in a microtiter plate format. Antibodies with aldolase catalytic activity presented the typical absorption maximum of the vinylogous amide at 318 nm (example shown in black), whereas none of the inactive antibodies did (example shown in gray). Two antibodies out of 20 formed the intermediate **2** and were subsequently determined to be catalytic. This efficient method should allow rapid screening of a large number of antibodies in future studies. (Reprinted with permission from *Science*. Copyright 1995 American Association for the Advancement of Science.)

after attack of the 1,3-diketone by the lysine residue, the vinylogous amide **2** would be formed that, according to the Woodward rules for enones, should absorb in the ultraviolet region of the spectrum at about 318 nm.⁵

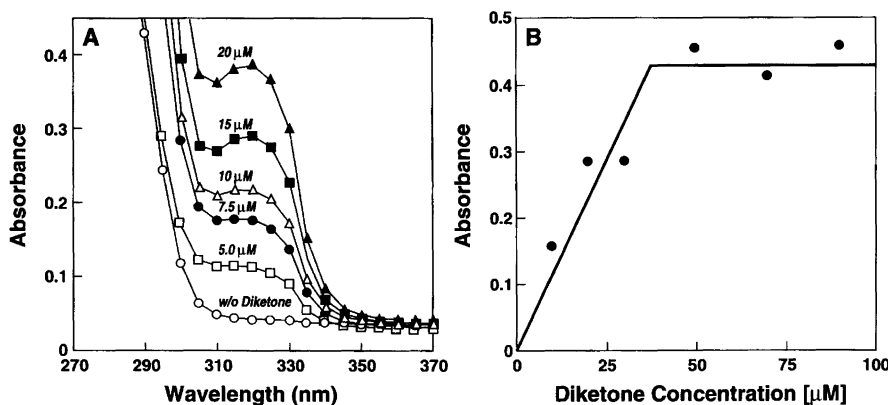


Fig. 3. (a) Determination of the extinction coefficient of **2**. A fixed concentration (100 mM) of hapten **1** was added to the indicated concentrations of antibody 33F12. The antibody enamine complex could easily be detected at an antibody concentration as low as 2 mM. (b) The active sites of antibody 38C2 were titrated with acetylacetone. The antibody concentration (20 mM) was fixed. Acetylacetone, (0–4.5 equiv.), was added and the absorption measured at 316 nm. The intersection of the two lines corresponds to a ratio of 1.9 of acetylacetone to antibody 38C2, indicating that both binding sites of the antibody form the enamine adduct **2**. (Reprinted with permission from *Science*. Copyright 1995 American Association for the Advancement of Science.)

Thus, instead of screening the antibody molecules for simple binding, one was now in a position to screen for formation of the critical intermediate in the catalysis, which in this case is expected to correspond to a known absorption maximum in the ultraviolet region of the spectrum.

Twenty monoclonal antibodies were obtained after the immunization. They were all screened for formation of the stable vinylogous amide **2** [Fig. 1(b)] by incubation with the diketone hapten **1** and two showed a strong absorption at 316 nm indicating that they had formed the predicted vinylogous amide **2** (Fig. 2). We determined the stoichiometry of the reaction by using the formation of the chromophore in the titration of the antibody with acetylacetone. The stoichiometry of the titration was 1.9 indicating that the two binding sites of the antibody molecule were involved in the reaction (Fig. 3). Both antibodies efficiently catalyzed the aldol addition and retroaldol reaction with a high degree of control over the stereochemistry. The k_{cat} value of the background reaction was determined to be $2.28 \times 10^{-7} \text{ M}^{-1} \text{ min}^{-1}$. This allows us to calculate the efficiency of the catalyzed reaction as $(k_{\text{cat}}/K_{\text{m}})/k_{\text{uncat}} \sim 10^9$. Thus, these remarkable antibody aldolases are nearly as efficient as the most studied natural aldolase, fructose 1,6-bisphosphate aldolase with its optimal substrates and more efficient than 2-deoxyribose 5-phosphate aldolase with the substrates shown in Fig. 4.

The stereochemical control of the reaction was also excellent. The antibodies catalyze the diastereoselective addition of ketones to the *Re*-face of **5** regardless of the stereochemistry at C-2 in both the Cram and anti-Cram mode of attack to yield the (4*S*,5*S*)-**6** and the (4*S*,5*R*)-**6** products, respectively, with some reactions giving greater than 95% de (Fig. 4).

Diastereofacial Selectivity

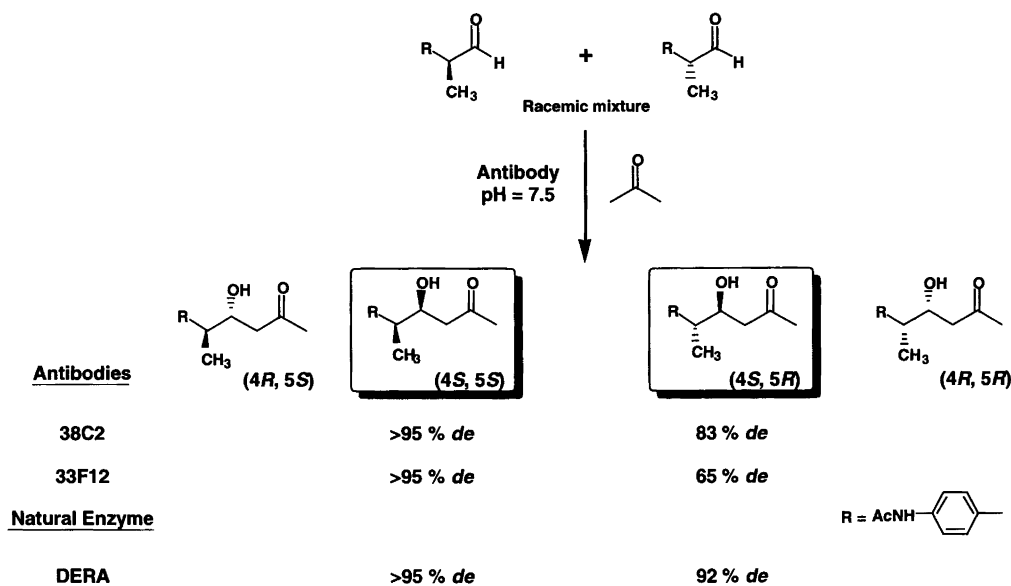


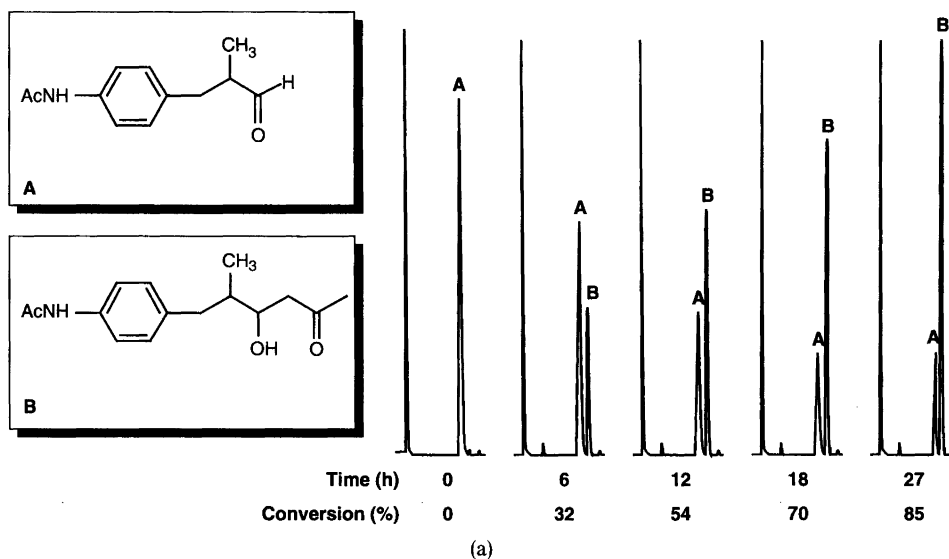
Fig. 4. The diastereoselectivity of the aldol addition between aldehyde 5 and acetone determined after 30% conversion. Both antibodies, 38C2 and 33F12, formed predominately the S configuration at C-4 of the aldol product. (Reprinted with permission from *Science*. Copyright 1995 American Association for the Advancement of Science.)

Table 1. Substrate specificity of antibody 38C2.

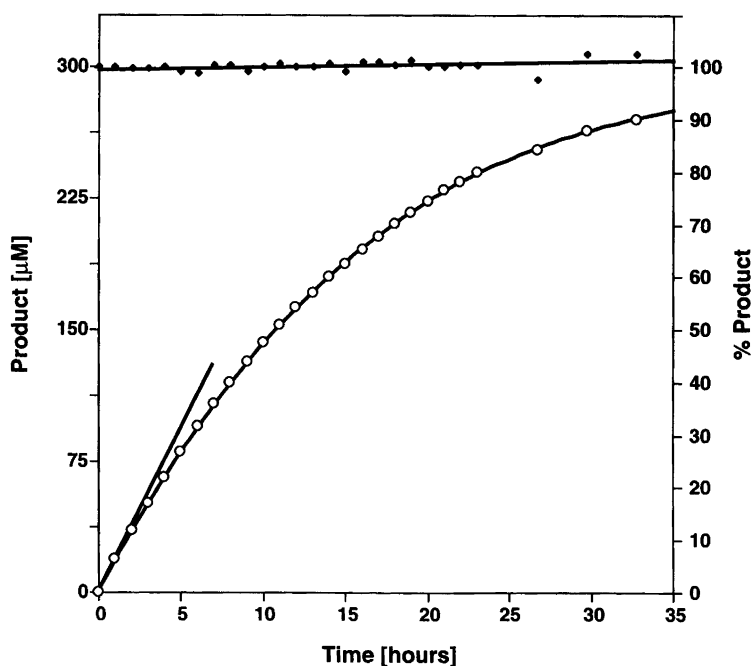
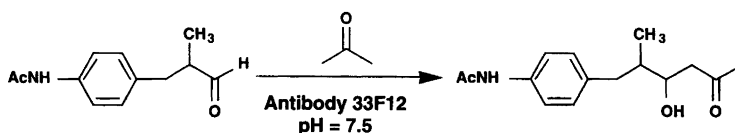
Reaction	$k_{\text{cat}}/\text{min}^{-1}$	$K_{\text{M}}/\mu\text{M}$
$\text{R-CH(CH}_3\text{)-CHO} \xrightarrow{\text{acetone}} \text{R-CH(CH}_3\text{)-CH(OH)-CH}_2\text{-C(=O)-CH}_3$	8.3×10^{-2a}	125 ^a
$\text{R-CH}_2\text{-CHO} \xrightarrow{\text{acetone}} \text{R-CH}_2\text{-CH(OH)-CH}_2\text{-C(=O)-CH}_3$	4.0×10^{-2}	48
$\text{R-CH}_2\text{-CHO} \xrightarrow{\text{acetone}} \left\{ \begin{array}{l} \text{R-CH}_2\text{-CH(OH)-CH}_2\text{-C(=O)-CH}_3 \quad 94 \\ \text{R-CH}_2\text{-CH(OH)-CH}_2\text{-C(=O)-CH}_2\text{-CH}_3 \quad 4 \end{array} \right.$	6.7×10^{-3}	17
$\text{R-CH}_2\text{-CHO} \xrightarrow{\text{acetone}} \text{R-CH}_2\text{-CH(OH)-CH}_2\text{-C(=O)-CH}_2\text{-CH}_3$	1.8×10^{-2}	52
$\text{R-CH}_2\text{-CHO} \xrightarrow{\text{acetone}} \left\{ \begin{array}{l} \text{R-CH}_2\text{-CH(OH)-CH}_2\text{-C(=O)-CH}_2\text{-CH}_2\text{-CH}_3 \quad 73 \\ \text{R-CH}_2\text{-CH(OH)-CH}_2\text{-C(=O)-CH}_2\text{-CH}_2\text{-CH}_2\text{-CH}_3 \quad 27 \end{array} \right.$	5.1×10^{-3}	115
$\text{R-CH}_2\text{-CHO} \xrightarrow{\text{formaldehyde}} \text{No reaction}$	—	—
$\text{R-CH}_2\text{-CHO} \xrightarrow{\text{acetone}} \text{No reaction}$	—	—

^a Reaction using antibody 33F12.

Progress of the Aldol Condensation Monitored by HPLC



(a)



(b)

Fig. 5. (a) The aldol addition reaction of aldehyde 5 and acetone was monitored over a 36 h period in the presence of 1.5% catalyst. The catalyst showed multiple turnovers (~ 2 turnovers hour^{-1}) and virtually no product inhibition. (b) A 90% conversion could be obtained in the presence of an excess of acetone (5% v/v) to minimize the retro-aldol reaction. The perfect mass balance (top line) indicates that no side reactions, such as elimination or polymerization, occurred over that period. Thus, the antibody-catalyzed aldol reaction is an exceptionally mild method of C-C bond formation. (Reprinted with permission from *Science*. Copyright 1995 American Association for the Advancement of Science.)

Influence of the Acetone Concentration on Catalysis

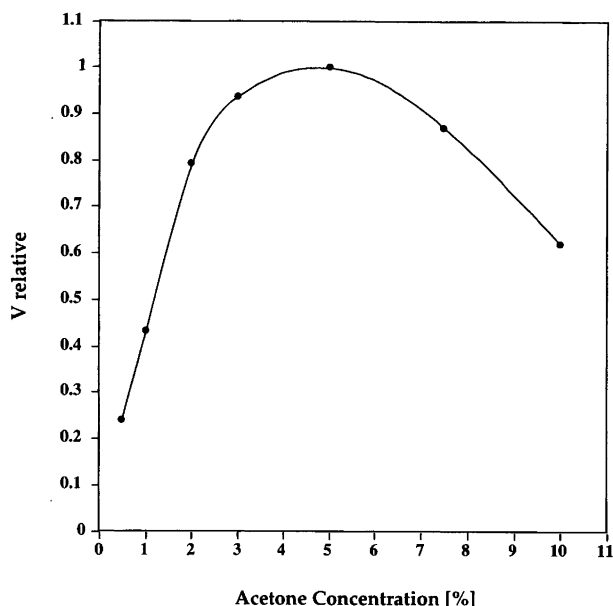


Fig. 6. The relative rate of the aldol addition reaction between aldehyde **5** and acetone was studied as a function of acetone concentration. Since the aldol is a readily reversible reaction the equilibrium favoring product formation for this reaction must be driven with an excess of the aldol donor, acetone in this case. As shown, synthesis of **6** is optimal in the presence of 5% v/v acetone for both antibodies.

The four diastereoisomers were separated on a DAICEL Chiralpak OJ column with an isocratic program, 7:1 hexane-ethanol, 1 ml min⁻¹, 254 nm. The retention times for the four isomers were: 19.74 (4*R*,5*R*), 23.32 (4*R*,5*S*), 25.15 (4*S*,5*R*) and 27.91 min (4*S*,5*S*). The relative configuration had been determined previously.³ The absolute configuration was deduced from an experiment wherein the catalyst was 2-deoxyribose 5-phosphate aldolase (DERA). DERA forms exclusively the aldol product possessing the *S* configuration at C-4. The aldol product generated by DERA consists of a 1:4.5 mixture of (4*S*,5*R*)-**6** (92% diastereomeric excess, de) and (4*S*,5*S*)-**6** (>95% de). The kinetic parameters of this particular transformation were $k_{\text{cat}} = 4.5 \times 10^{-2} \text{ min}^{-1}$ and $K_m = 3400 \text{ mM}$.

The broad specificity and synthetic efficiency of catalytic antibody aldolases

One of the main reasons to make catalytic antibody aldolases is that natural aldolases have a very limited substrate specificity. In principle since the substrate specificity is programmed by the experimenter, catalytic antibody aldolases could be induced to accept any aldol donor and acceptor. As indicated in Table 1 the antibodies studied here utilize a broad range of substrates, most of which are not utilized by natural aldolase. The anti-

bodies accept acetone, fluoroacetone, chloroacetone, 2-butanone, 3-pentanone, 2-pentanone and dihydroxyacetone as substrates. The use of dihydroxyacetone is particularly intriguing as it may provide an entry into the synthesis of sugars.

The efficiency of the reaction for synthesis was studied [Figs. 5(a) and (b)]. Less than 1 mol% catalyst is sufficient to effect complete conversion of the substrate in a short time at room temperature in water at pH 7.5. The reaction shows multiple turnovers and little product inhibition. The perfect mass balance shows that no side products were formed [Fig. 5(b)]. An analysis of the influence of the acetone concentration on catalysis shows a maximum rate at 5% acetone which is a concentration that allows one to minimize the retro-aldol reaction (Fig. 6).

Conclusions

The results presented here validate the concept of reactive immunization. We expect this process to be used widely in the antibody catalysis field to generate new catalysts in which at least some of the mechanistic details are programmed by the experimenter with a precision not heretofore possible. In addition the general availability of a wide variety of mechanism based inhibitors may allow one rapidly to induce catalytic antibodies without the need to synthesize new molecules. For example in the β -lactamase field one could couple immunization with the existing mechanism-based inhibitors with selection for penicillin resistance in phage-antibody systems. In addition there are mechanism-based inhibitors that are capable of probing multiple aspects of a mechanism. We expect the use of such inhibitors as immunogens will provide antibodies in which multiple components of a mechanism are induced thereby yielding catalysts with machinery so complex that it was until now the sole purview of Nature. The methodology reported here may offer a general solution to the aldolase problem in organic chemistry. A good test case will be the preparation of a hapten that is a 1,3-diketone in which C-2 is stereogenic such that one generates four different antibodies where each selectively catalyzes the formation of one of the four possible diastereoisomers. If this is successful, one can imagine the induction of multiple catalysts where each one catalyzes and controls the stereochemistry of one step in a synthetic scheme. Thus, one may envision a synthesis where many of the steps utilize catalysts that are customized to intermediates that are prepared as the synthesis proceeds. Such an approach may, for example, be applicable in the synthesis of the polypropionate natural products.

Acknowledgements. We thank Jurgen Wagner for his contributions to this work. Funding was provided by the US National Institutes of Health CA27489 and an Investigator Award to C.F.B. from the Cancer Research Institute.

References

1. Schultz, P. G. and Lerner, R. A. *Science* 269 (1995) 1835.
2. Wirsching, P., Ashley, J. A., Lo, C.-H. L., Janda, K. D. and Lerner, R. A. *Science* 270 (1995) 1775.
3. Wagner, J., Lerner, R. A. and Barbas III, C. F. *Science* 270 (1995) 1797.
4. Lai, C. Y., Nakai, N. and Chang, D. *Science* 183 (1974) 1204; Morris, A. J. and Tolan, D. R. *Biochemistry* 33 (1994) 12291; Rutter, W. J. *Fed. Proc. Am. Soc. Exp. Biol.* 23 (1964) 1248.
5. Pretsch, E., Seibl, J., Simon, W. and Clerc, T. *Springer-Verlag, Berlin, ed. 2*, (1989), p. U20.

Received October 17, 1995.