Antibody-Catalyzed Retro-Aldol Reaction

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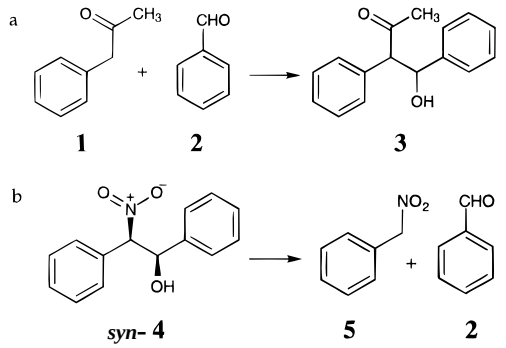
The aldol reaction plays a central role in cellular metabolism,[1] as well as in synthetic chemistry,[2] and is therefore an obvious choice for exploiting the diversity of the immune system[3,4] to find new and selective catalysts. A number of recent examples of antibody-catalyzed aldol reactions have appeared in which imine intermediates are involved.[5] In our efforts to develop antibodies to catalyze the bimolecular aldol condensation between phenylacetone (**1**) and benzaldehyde (**2**) (Scheme 1a), we discovered an antibody that catalyzes the retro-aldol reaction *Henry type*[6] illustrated in Scheme 1b, which we now report.

Monoclonal antibodies were elicited against the phosphinate transition state analog **10**. This hapten was designed to resemble the expected transition state for the addition of a phenylacetone-derived enolate to the carbonyl of benzaldehyde. The negatively-charged carboxylate group of hapten **10** was intended to approximate the negatively-charged enolate while the tetrahedral phosphinate group reflects the developing tetrahedral geometry and charge at the carbonyl group of the benzaldehyde. Consequently, antibodies generated against **10** were expected to catalyze the aldol condensation of **1** and **2** by a combination of both proximity and electrostatic effects.[4,7]

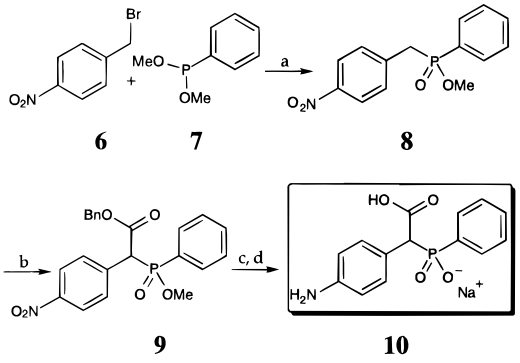
Hapten **10** was converted to the diazonium derivative by reaction with $NaNO_2$ and coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) in water (pH 11−12).[8] Twenty clonal cell lines exhibiting binding specificity for BSA-**10** were prepared by standard hybridoma technology.[9] Antibodies were purified by chromatography on protein A-coupled Sepharose 4B and determined to be greater than 95% homogeneous by SDS-polyacrylamide gel electrophoresis.[10]

Initially, antibodies were screened for catalysis of the condensation reaction of phenylacetone (**1**) and benzaldehyde (**2**) (Scheme 1a) under a variety of reaction conditions.[11] Product formation, over background, was observed for several antibodies. However, in no case was the observed product formation inhibited by the addition of up to 167 μM hapten **10**.[12] This lack of hapten inhibition suggested that the observed catalysis was not active site associated, but rather could be explained by a catalytic mechanism involving nonspecific imine formation with surface lysine residues.[13]

Scheme 1



Scheme 2[a]



[a] Synthesis of hapten **10**. Key: (a) neat, 80 °C, 38%; (b) lithium bis (trimethylsilyl)amide, benzyl cyanoformate, HMPA/THF, 40%; (c) NaI, acetone (reflux); (d) $Pd(OH)_2/C$, H_2 (1 atm), $MeOH/H_2O$, 34% from **9**.

We then examined phenylnitromethane (**5**) as a substrate for the antibodies since the electronic structure of the corresponding nitronate anion more closely matches that of the carboxylate group in hapten **10**. Antibodies generated against **10** might be expected to stabilize and thereby increase the effective concentration of the anion in the active site. However, the lower pK_a of **5** also significantly shifts the equilibrium of the addition reaction away from the condensation product. Consequently, based on the principle of microscopic reversibility,[14] the retro-aldol reaction was studied.

The retro-aldol reaction of **4**[15] (Scheme 1b) proceeded with a measurable background rate to give **2** and **5** in 50 mM NaOAc, 50 mM NaCl, 5% [v:v] acetonitrile (pH 5.0) at 4 °C. Antibodies were screened by monitoring production of benzaldehyde by reverse phase high-pressure liquid chromatography (HPLC) (C18, 50% acetonitrile in 50 mM NaOAc, pH 5.0, 260 nm detection). A single antibody (29C5.1) was identified that catalyzed this reaction. The *syn* diastereomer of **4** (illustrated in Scheme 1b) was found to be the better substrate for antibody

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(8) Epitope densities of KLH and BSA conjugates were measured by UV absorbance at 370 nm using $\epsilon_{370} = 22\,900$ for the azo linkage and determined to be 14 for KLH and 8 for BSA.
(9) (a) Harlow, E.; Lane, D. *Antibodies: A Laboratory Manual*; Cold Spring Harbor Laboratory: USA, 1988. (b) Shokat, K. M. Ph.D. Thesis, University of California, Berkeley, CA, 1991.
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(11) Antibodies were assayed for catalysis of the aldol condensation using reaction mixtures of benzaldehyde and phenylacetone at pH 8.0 and 9.0. 4-Fluorophenylacetone as well as the ketone containing an *ortho* azo linkage to *p*-cresol was assayed in pH 9.0 buffer.
(12) A derivative of hapten **10** was generated by diazotization and coupling to *p*-cresol. This analog was expected to have greater structural homology to the hapten−protein conjugate used for immunization and screening. No inhibition of catalysis was observed at concentrations up to 500 μM.
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(15) Compound **5** was prepared by treatment of benzylbromide with $AgNO_2$ (ether, room temperature, 24 h, 47%). Compound **4** was prepared from **2** and **5** by treatment with alumina, basic Brockman activity 1 (neat, 12 h, 42%). The *syn* diastereomer of **4** was the faster moving on silica (5:1 hexanes/ethylacetate, $R_f = 0.25$).

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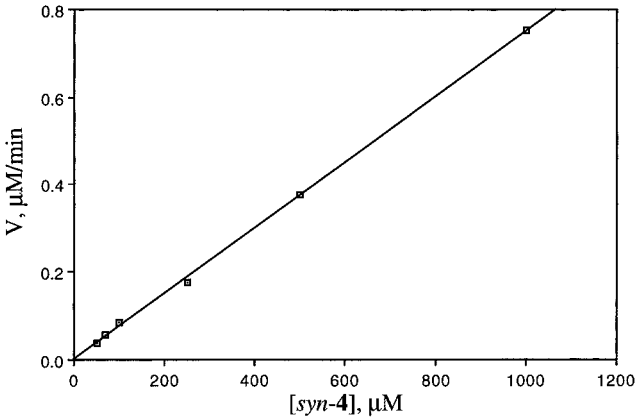

Figure 1. Velocity (*V*) vs substrate concentration [*syn*-**4**] plot for antibody-catalyzed retro-aldol reaction (Scheme 1b). Initial velocities were measured by monitoring the rate of formation of **2** by HPLC (UV, 260 nm).

catalysis by 2:1 over the *anti* diastereomer (250 μM substrate and 7 μM 29C5.1).[16,17]

Initial reaction rates were determined for the *syn* diastereomer of **4** at concentrations ranging from 50 μM to 1.0 mM in the presence of 3.0 μM IgG (6.0 μM combining site).[18] Analysis of the reaction rate (*V*) for antibody catalysis over background as a function of substrate concentration (Figure 1) afforded an apparent second-order rate constant (k_{cat}/K_M) of 125 M^{-1} min^{-1} (the limited solubility of *syn*-**4** under the reaction conditions precluded determination of k_{cat} and K_M). The inhibition constant $K_{i,app}$ for hapten **10** was determined to be 2.6 μM by curve-fitting data for V_i/V_o as a function of inhibitor (**10**) concentration to the equation for competitive inhibition: $V_i/V_o = {}^1/_2E_t\{(E_t - I - K_i') + [(E_t + I + K_i')^2 - 4E_tI]^{1/2}\}$, where V_i is the inhibited rate, V_o is the uninhibited rate, E_t is the total enzyme concentration, and I is the total inhibitor concentration.[18,19]

Chemical modification[20] of 29C5.1 with diethyl pyrocarbonate resulted in an ~4-fold reduction in catalytic activity (no inactivation was observed when the modification was carried out in the presence of 1 mM **10**). These results are consistent with the presence of an active site histidine. The rate of the antibody-catalyzed reaction can be compared with the rate of the reaction catalyzed by imidazole ($k_{imid} = 2.5 \times 10^{-4}$ M^{-1} min^{-1}). This affords a ratio of second-order rate constants ($k_{cat}/K_M/k_{imid}$) of 5.0×10^5 indicating the antibody to be an effective catalyst.[21]

An effort was made to alter the equilibrium of the reaction to favor formation of the condensation product (**4**) by reacting **2** and **5** in isooctane in the presence of purified Fab (29C5.1).[22] Unfortunately, these conditions also failed to produce a measurable rate of formation of **4** for both the background and antibody (Fab)-catalyzed reactions. In conclusion, these results demonstrate that antibodies can stabilize the aldol transition state but point to the need for improved strategies for enolate formation under aqueous conditions.

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(16) The relative stereochemical configuration of *syn*-**4** was determined by single-crystal X-ray analysis.

(17) No evidence of enantioselectivity was observed for catalysis by monitoring the relative consumption of the enantiomers of *syn*-**4** by chiral HPLC (Chiralcel OD-R column, 30−70% acetonitrile in 50 mM NaOAc, pH 5.0).

(18) (a) Cha, S. *Biochem. Pharmacol.* **1975**, *24*, 2177. (b) Angeles, T. S. *et al. Biochemistry* **1993**, *32*, 12128.

(19) Reactions were carried out in 50 mM NaOAc, 50 mM NaCl, 5% acetonitrile, pH 5.0 with 50 μM *syn*-**4** and 3 μM 29C5.1, 4 °C.

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(21) A study of pH effects on antibody catalysis revealed that the rates of the background and antibody-catalyzed reactions increased linearly as a function of hydroxide ion concentration from pH 4.5 to 6.5. Above pH 6.5 the fast background rate of the retro-aldol reaction complicated analysis ($k_{uncat} = 7.4 \times 10^{-4}$ min^{-1} at pH 5.0).

(22) Enzyme digests for Fab preparation used immobilized Papain (Pierce). Isooctane reactions were conducted in the presence of 1.0% Aerosol OT (Sigma) as described by Paradkar, V. M.; Dordick, J. S. *J. Am. Chem. Soc.* **1994**, *116*, 5009.