

Antibody-Catalyzed Retro-Aldol Reaction

Mark E. Flanagan, John R. Jacobsen, Elizabeth Sweet, and Peter G. Schultz*

Howard Hughes Medical Institute and
Department of Chemistry, University of California
Berkeley, California 94720

Received December 18, 1995

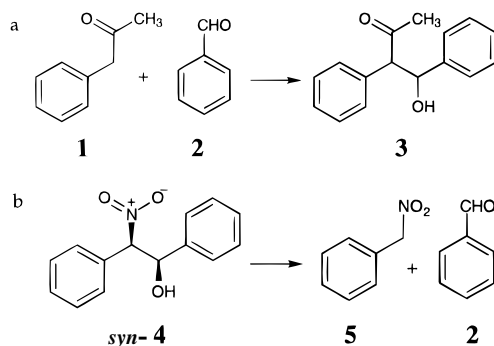
The aldol reaction plays a central role in cellular metabolism,¹ as well as in synthetic chemistry,² and is therefore an obvious choice for exploiting the diversity of the immune system^{3,4} to find new and selective catalysts. A number of recent examples of antibody-catalyzed aldol reactions have appeared 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}

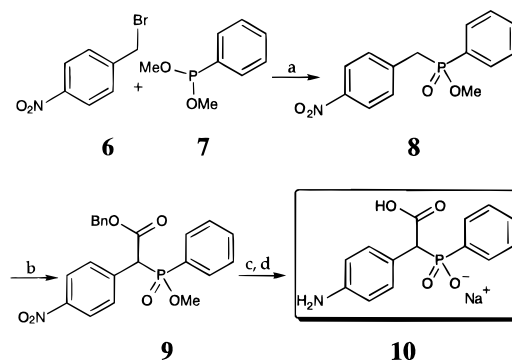
Hapten **10** was converted to the diazonium derivative by reaction with NaNO₂ and coupled to the carrier proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) in water (pH 11–12).⁸ Twenty clonal cell lines exhibiting binding specificity for BSA-**10** were prepared by standard hybridoma 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

Scheme 1



Scheme 2^a



^a Synthesis of hapten **10**. Key: (a) neat, 80 °C, 38%; (b) lithium bis(trimethylsilyl)amide, benzyl cyanofornate, HMPA/THF, 40%; (c) NaI, acetone (reflux); (d) Pd(OH)₂/C, H₂ (1 atm), MeOH/H₂O, 34% from **9**.

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

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

(1) Marsh, J. J.; Leberer, H. G. *Trends Biochem. Sci.* **1992**, *17*, 110.

(2) (a) Heathcock, C. H. *Aldrichimica Acta* **1990**, *23*, 99. (b) Evans, D. A. *Science* **1988**, *240*, 420.

(3) (a) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566. (b) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, *234*, 1570.

(4) (a) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391. (b) Schultz, P. G.; Lerner, R. A. *Science* **1995**, *269*, 1835.

(5) (a) Reymond, J.-L.; Chen, Y. *Tetrahedron Lett.* **1995**, *36*, 2575. (b) Koch, T.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1995**, *117*, 9383. (c) Wagner, J.; Lerner, R. A.; Barbas, C. F., III *Science* **1995**, *270*, 1797.

(6) Henry, L. C. *R. Acad. Sci. Paris* **1895**, *120*, 1265.

(7) (a) Jencks, W. P. *Catalysis in Chemistry and Enzymology*; Dover: Mineola, NY, 1987; p 11501. (b) Fersht, A. *Enzyme Structure and Mechanism*; W. H. Freeman: New York, NY, 1985.

(8) Epitope densities of KLH and BSA conjugates were measured by UV absorbance at 370 nm using ε₃₇₀ = 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.

(10) Fredriksson, U.-B.; Fagerstam, L. G.; Cole, A. W. G.; Lundgren, T. *Protein A-Sepharose Cl-4B Affinity Purification of IgG Monoclonal Antibodies from Mouse Ascites*; Pharmacia AB: Uppsala, Sweden, 1986.

(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.

(13) (a) Wittig, G.; Reiff, H. *Angew. Chem., Int. Ed. Engl.* **1968**, *7*, 7. (b) Wittig, G.; Suchanek, P. *Tetrahedron Suppl.* **1967**, *8*, 347.

(14) Alberty, W. J. *Adv. Phys. Org. Chem.* **1993**, *28*, 139.

(15) Compound **5** was prepared by treatment of benzylbromide with AgNO₂ (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).

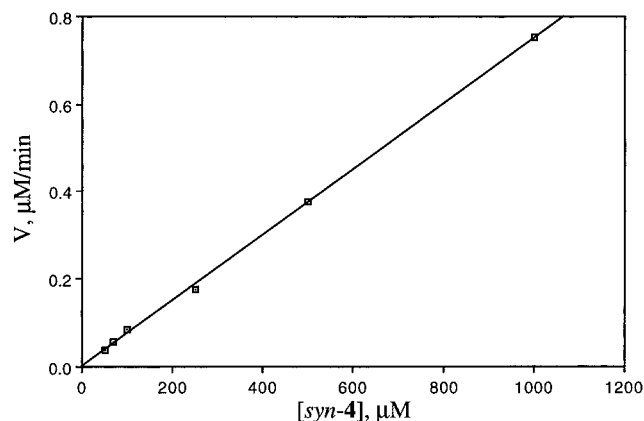


Figure 1. Velocity (V) vs substrate concentration [syn-4] plot for antibody-catalyzed retro-aldol reaction (Scheme 1b). Initial velocities were measured by monitoring the rate of formation of **2** by HPLC (UV, 260 nm).

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 ($k_{\text{cat}}/K_{\text{M}}$) of 125 $\text{M}^{-1} \text{min}^{-1}$ (the limited solubility of *syn-4* under the reaction conditions precluded determination of k_{cat} and K_{M}). The inhibition constant $K_{\text{i,app}}$ 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: $V_i/V_o = 1/2 E_t \{ (E_t -$

(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.

$I - K_i') + [(E_t + I + K_i')^2 - 4E_t I]^{1/2}$, 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 modification²⁰ 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 ($k_{\text{imid}} = 2.5 \times 10^{-4} \text{M}^{-1} \text{min}^{-1}$). This affords a ratio of second-order rate constants ($k_{\text{cat}}/K_{\text{M}}/k_{\text{imid}}$) 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 isoctane 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.

Acknowledgment. We are grateful for financial support for this work from the National Institutes of Health (Grant No. R01 AI24695). P.G.S. is a Howard Hughes Medical Institute Investigator. M.E.F. acknowledges a fellowship from the National Institutes of Health (Grant No. 1F32AI09093-01A1), and J.R.J. acknowledges an NIH Molecular Biophysics Training Grant.

JA954221U

(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 $^\circ\text{C}$.

(20) Holbrook, J. J.; Ingram, V. A. *Biochem.* **1973**, *131*, 729.

(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 ($k_{\text{uncat}} = 7.4 \times 10^{-4} \text{min}^{-1}$ 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.