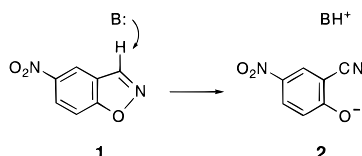
# Albumin-Catalyzed Proton Transfer

Kazuya Kikuchi, Simon N. Thorn, and Donald Hilvert*

*Departments of Chemistry and Molecular Biology*
*The Scripps Research Institute*
*10550 North Torrey Pines Road*
*La Jolla, California 92037*

*Received May 22, 1996*

The origins of the high efficiencies of enzymatic proton transfers are of considerable current interest.[1] Tailored protein catalysts, such as catalytic antibodies,[2] provide an opportunity to investigate the factors that influence efficiency using well-characterized model systems like the base-promoted rearrangement of benzisoxazoles to salicylonitriles.[3] The latter reaction is an exothermic E2 elimination that is highly sensitive to base strength and, in the case of carboxylate bases, solvent polarity.[4,5]




Recently, we reported that antibodies generated against a benzimidazolium hapten accelerate the decomposition of **1** with high rates and multiple turnovers.[6] Consistent with our experimental design, a carboxylate induced in response to the positive charge of the hapten functions as the catalytic base in the binding pocket. Although effective molarities (EM values) for intramolecular general base catalysis in model systems seldom exceed 10 M,[7] EMs greater than $10^4$ M were observed for this carboxylate relative to free acetate. EM values are usually interpreted as a measure of positional ordering of reactive groups at the active site, but other factors, including the increase in carboxylate basicity due to desolvation of the oxyanion and direct stabilization of the highly polarizable transition state by the local reaction microenvironment, are likely to contribute substantially to the catalytic efficiency of the antibodies. The potential importance of medium effects is underscored by the large rate accelerations ($>10^7$-fold) that result when the nonenzymatic reaction of **1** with acetate is transferred from protic to aprotic solvents.[5] In contrast, the deprotonation of benzisoxazoles by amine bases is notably insensitive to solvent change.[5] Protein catalysts containing an amine rather than a carboxylate base would consequently be valuable for examining the relative importance of base positioning versus environmental effects on proton transfer efficiency.

During the course of screening hybridoma supernatants for catalytic activity,[6] we noticed that albumins present as tissue culture additives significantly accelerated benzisoxazole decomposition. Serum albumins are abundant proteins that bind a wide range of compounds[8] and also promote a variety of chemical transformations.[9] The decomposition of a Meisenheimer complex by bovine serum albumin (BSA)[10] is notable in the present context, because evidence has been adduced for the participation of a lysine as the catalytic base.[11] These observations prompted us to investigate the reaction of benzisoxazoles with BSA in greater detail.

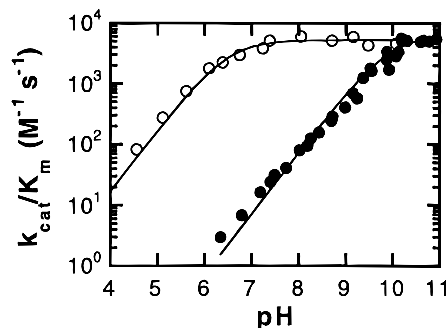



**Figure 1.** Plot of log ($k_{cat}/K_m$) versus pH for the conversion of **1** to **2** catalyzed by BSA (●) and antibody 34E4 (○). Crystallized BSA, treated to remove bound lipids, was purchased from Sigma and used without further purification; 34E4 was isolated and purified as previously described.[6] Kinetics were performed at 20 °C in the following buffers (40 mM, containing 100 mM NaCl): sodium acetate (pH < 6); sodium phosphate (6 < pH < 8); sodium borate (8 < pH < 10); and sodium carbonate (pH > 10). Initial rates were determined spectrophotometrically by measuring the absorbance increase at 380 nm as a function of time; stopped flow techniques were used for measurements at high pH (>8.5). The data were fit to the equation $k_{cat}/K_m = (k_{cat}/K_m)^{max}/(1 + 10^{pK_{EH}-pH})$. The kinetic constants for this and the corresponding $k_{cat}$ versus pH profile are summarized in Table 1.

Under the original assay conditions (40 mM phosphate, 100 mM NaCl, pH 7.4, and 20 °C), the BSA-catalyzed rearrangement of 5-nitrobenzisoxazole follows Michaelis−Menten kinetics with $k_{cat} = 0.017 \pm 0.001\ s^{-1}$ and $K_m = 720 \pm 68\ \mu M$. The corresponding values of $k_{cat}$ and $K_m$ for the catalytic antibody 34E4 are 0.66 $s^{-1}$ and 120 $\mu$M, respectively.[6] Thus, depending on substrate concentration, BSA must be present at 40−230-fold higher concentrations than the antibody to achieve comparable initial rates at pH 7.4, which explains how 34E4 could be detected in tissue culture supernatants containing large excesses of albumin. Furthermore, in contrast to 34E4,[6] BSA is strongly and competitively inhibited by product ($K_i = 51\ \mu M$), further accentuating the catalytic advantage of the antibody under multiturnover conditions. As shown in Figure 1, however, the rate of the BSA reaction increases substantially with pH. The data can be fit to an equation for the ionization of a single active site residue with a $pK_a \approx 10$ (Table 1). At their respective pH optima, BSA and 34E4 have similar values of $k_{cat}/K_m$. Moreover, BSA's $k_{cat}^{max}$ parameter is ≈9-fold larger than that of the antibody. Abstraction of the C-3 proton is rate limiting over the entire pH range as judged by deuterium kinetic isotope effects of 6.36 (pH 7.4) and 6.24 (pH 10.1) on $k_{cat}/K_m$.

(1) See, for instance: (a) Kuliopulos, A.; Talalay, P.; Mildvan, A. S. *Biochemistry* **1990**, *29*, 10271−10280. (b) Rose, I. A.; Warms, J. V. B.; Kuo, D. J. *Biochemistry* **1992**, *31*, 9993−9999. (c) Gerlt, J. A.; Gassman, P. G. *Biochemistry* **1993**, *32*, 11943−11952. (d) Guthrie, J. P.; Kluger, R. *J. Am. Chem. Soc.* **1993**, *115*, 11569−11572. (e) Glickman, M. H.; Wiseman, J. S.; Klinman, J. P. *J. Am. Chem. Soc.* **1994**, *116*, 793−794.
(2) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659−667.
(3) Casey, M. L.; Kemp, D. S.; Paul, K. G.; Cox, D. D. *J. Org. Chem.* **1973**, *38*, 2294−2301.
(4) Kemp, D. S.; Casey, M. L. *J. Am. Chem. Soc.* **1973**, *95*, 6670−6680.
(5) Kemp, D. S.; Cox, D. D.; Paul, K. G. *J. Am. Chem. Soc.* **1975**, *97*, 7312−7318.
(6) Thorn, S. N.; Daniels, R. G.; Auditor, M.-T. M.; Hilvert, D. *Nature* **1995**, *373*, 228−230.
(7) Kirby, A. J. *Adv. Phys. Org. Chem.* **1980**, *17*, 183−278.
(8) Peters, T., Jr. *Adv. Prot. Chem.* **1985**, *37*, 161−245.
(9) For representative examples, see: (a) Tilden, J. T.; Ogilvie, J. W. *J. Biol. Chem.* **1972**, *247*, 1265−1271. (b) Means, G. E.; Bender, M. L. *Biochemistry* **1975**, *14*, 4989−4994. (c) Baba, N.; Matsumura, Y.; Sugimoto, T. *Tetrahedron Lett.* **1978**, *44*, 4281−4284. (d) Sugimoto, T.; Matsumura, Y.; Tanimoto, S.; Okano, M. *J. Chem. Soc., Chem. Commun.* **1978**, 926−927. (e) Sugimoto, T.; Kokubo, T.; Miyazaki, J.; Tanimoto, S.; Okano, M. *J. Chem. Soc., Chem. Commun.* **1979**, 402−404. (f) Sugimoto, T.; Kokubo, T.; Miyazaki, J.; Tanimoto, S.; Okano, M. *J. Chem. Soc., Chem. Commun.* **1979**, 1052−1053. (g) Colonna, S.; Banfi, S.; Fontana, F.; Sommaruga, M. *J. Org. Chem.* **1985**, *50*, 769−771. (h) Colonna, S.; Banfi, S.; Annunziata, R.; Casella, L. *J. Org. Chem.* **1986**, *51*, 891−895. (i) Nango, M.; Kimura, Y.; Kanda, S.; Ihara, Y.; Koga, J.; Kuroki, N. *Chem. Lett.* **1986**, 229−232. (j) Pfeifer, U.; Fukumura, H.; Misawa, H.; Kitamura, N.; Masuhara, H. *J. Am. Chem. Soc.* **1992**, *114*, 4417−4418.
(10) (a) Taylor, R. P.; Vatz, J. B. *J. Am. Chem. Soc.* **1973**, *95*, 5819−5820. (b) Taylor, R. P.; Chau, V.; Bryner, C.; Berga, S. *J. Am. Chem. Soc.* **1975**, *97*, 1934−1943.
(11) Taylor, R. P. *J. Am. Chem. Soc.* **1976**, *98*, 2684−2686.

S0002-7863(96)01739-8 CCC: $12.00 © 1996 American Chemical Society

**Table 1.** Steady-State Kinetic Parameters for BSA and Antibody 34E4 at Their Respective pH Optima[a]

| catalyst | $(k_{cat}/K_m)^{max}$ ($M^{-1}$ $s^{-1}$) | $pK_{EH}$ | $k_{cat}^{max}$ ($s^{-1}$) | $pK_{EHS}$ |
|---|---|---|---|---|
| BSA | 6450 ± 432 | 10.0 ± 0.09 | 6.02 ± 0.24 | 10.3 ± 0.04 |
| 34E4 | 5180 ± 240 | 6.50 ± 0.12 | 0.66 ± 0.01 | 6.02 ± 0.06 |

[a] The data were obtained and analyzed as described in the legend to Figure 1. $pK_{EH}$ and $pK_{EHS}$ are the apparent ionization constants for the enzyme and enzyme–substrate complex, respectively.

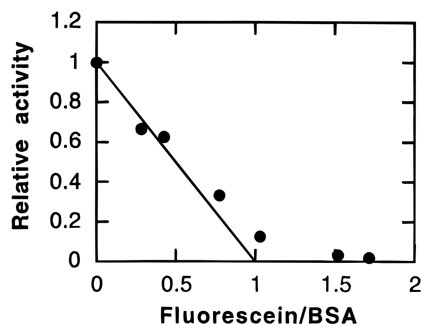



**Figure 2.** Correlation of the stoichiometry of BSA labeling by fluorescein isothiocyanate and the loss of catalytic activity. BSA was modifed as previously described.[11] Protein concentrations were determined by the method of Smith,[19] and an extinction coefficient of 15 900 $M^{-1}$ $cm^{-1}$ was assumed for fluorescein coupled to BSA.[11] Assays were performed with 55 $\mu$M 5-nitrobenzisoxazole in carbonate buffer (pH 10.2) as described in the legend to Figure 1.

The identity of the catalytic base in BSA was probed by chemical modification.[12] Cysteine alkylation with iodoacetamide has no effect on rates, and modification of tyrosines (and possibly lysines) with a 100-fold molar excess of acetylimidazole reduces the specific activity by only 54%. In contrast, modification of a single lysine with fluorescein isothiocyanate is apparently sufficient to abolish activity (Figure 2). This reagent has been previously shown[11,13] to react preferentially with Lys220 located in a binding pocket in subdomain IIA of the protein.[14] Although the normal caveats apply, the chemical modification results and the pH-rate data are most consistent with this lysine acting as the catalytic base. The non-enzymatic reaction of **1** with *n*-butylamine can consequently be used to assess the catalytic efficiency of BSA. The ratio of $(k_{cat}/K_m)^{max}$ and $k_{BuNH_2}$, the second order rate constant for the nonenzymatic reaction,[15] gives a rate enhancement of $2.0 \times 10^5$; the EM for this system ($k_{cat}^{max}/k_{BuNH_2}$) is 184 M. For comparison, antibody 34E4 with its carboxylate base catalyzes the decomposition of **1** with a rate acceleration of $3.4 \times 10^8$ relative to acetate and achieves an EM of 41 000 M.[6]

The EM for BSA, while smaller than that of the antibody, is more than an order of magnitude larger than values seen for most model systems[7] and can be compared with EM values in the range 60–80 M recently observed for the conversion of **1** to **2** catalyzed by a xylene-bridged host molecule containing a pyridine base.[16] Given the relative insensitivity of the amine-promoted deprotonation reaction to medium effects,[5] these results suggest that the BSA binding site is reasonably effective at aligning the catalytic lysine with the C-3 proton of **1**. However, other contributions to transition state stabilization cannot be excluded. Structural studies on the closely related human and equine serum albumins[14] show that the highly conserved IIa binding site contains, in addition to a reactive lysine, a cluster of positively charged arginines which could stabilize the incipient phenolate through charge complementarity. The importance of such interactions might also explain the strong inhibition observed for the product anion. The comparatively larger EM attained by 34E4 can be attributed in part to the enhanced basicity of its carboxylate relative to acetate ($\Delta pK_a = 1.25$)[6]—presumably a consequence of its location in an active site that is less polar than water. After correcting for differences in basicity,[17] the EM for 34E4 (5000 M) is still 13-times larger than the similarly adjusted EM for BSA (364 M), reflecting better positioning of the antibody base, specific interactions between the tailored binding pocket and the transition state, or both.

In summary, BSA is an effective catalyst for the deprotonation of benzisoxazoles. That this is not a general property of proteins with generic hydrophobic pockets containing reactive groups is shown by the many antibodies we have generated against benzimidazolium haptens[6,18] that are inactive or have substantially reduced activity relative to BSA and 34E4. Further work, especially investigations of structure-reactivity relationships in these two differently functionalized catalysts, may permit dissection of the specific factors that contribute to the efficiency of proton transfer. Insights gained in the course of these studies will be invaluable to efforts to optimize the activity of these and other first generation catalysts.

**Acknowledgment.** The authors are grateful to Professor A. J. Kirby for communicating the results of a similar study prior to publication. This work was supported in part by the National Institutes of Health (GM38273 to D.H.). K.K. is the recipient of a fellowship from the Naito Foundation (Tokyo, Japan).

JA9617395

(12) Lundblad, R. L. *Chemical Reagents for Protein Modification*, 2nd ed.; CRC Press, Inc.: Boca Raton, FL, 1991.
(13) Andersson, L.-O.; Rehnström, A.; Eaker, D. *Eur. J. Biochem.* **1971**, *20*, 371–380.
(14) (a) He, X. M.; Carter, D. C. *Nature* **1992**, *358*, 209–215. (b) Carter, D. C.; Ho, J. X. *Adv. Prot. Chem.* **1994**, *45*, 153–203.
(15) The rate constant $k_{BuNH_2}$ (0.0326 $M^{-1}$ $s^{-1}$) was determined in water ($\mu = 0.1$, pH 11.2, 20 °C) at 50 $\mu$M **1** using butylamine concentrations in the range 10–200 mM. The amine–amine hydrochloride ratio was 3:1. In contrast to the rate constant for the acetate-promoted deprotonation ($k_{AcO^-} = 1.62 \times 10^{-5}$ $M^{-1}$ $s^{-1}$, ref 6), $k_{BuNH_2}$ deviates negatively from the previously reported Brønsted equation correlating the rates of deprotonation of **1** by tertiary amines (ref 4).
(16) Kennan, A. J.; Whitlock, H. W. *J. Am. Chem. Soc.* **1996**, *118*, 3027–3028.
(17) A Brønsted coefficient $\beta = 0.73$ was assumed in all cases (refs 4 and 6).
(18) Kikuchi, K.; Jiang, N.; Kirby, A. J.; Hilvert, D. Unpublished results.
(19) Smith, P. K.; Krohn, R. I.; Hermanson, G. T.; Mallia, A. K.; Gartner, F. H.; Provenzano, M. D.; Fujimoto, E. K.; Goeke, N. M.; Olson, G. J.; Klenk, D. C. *Anal. Biochem.* **1985**, *150*, 76–85.