Characterization of Proton-Transfer Catalysis by Serum Albumins

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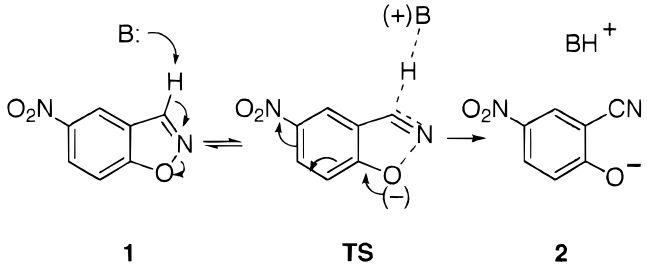
Abstract: Two independent investigations of catalysis by BSA and other serum albumins are combined to provide detailed insight into the mechanism of a classical proton-transfer reaction taking place on the surface of a protein. The Kemp elimination involves the general base-catalyzed removal of a proton from carbon and is known to be highly sensitive to medium effects. The serum albumins bind and catalyze the eliminative fragmentation of 5-nitrobenzisoxazole with remarkable efficiency and "accidental specificity." A binding site and a likely catalytic lysine are identified, and factors contributing to the efficiency of catalysis analyzed. A key factor appears to be a differentiated microenvironment, which allows delocalized negative charge to develop in a stabilizing hydrophobic pocket next to a polar region where the developing ammonium cation is not disfavored. Catalytic efficiency is discussed in terms of effective molarities for the reaction catalyzed by serum albumins and by catalytic antibodies and compared with a selection of published EMs for enzyme-catalyzed reactions.

Proton transfers are involved in almost every enzyme reaction. Although normally fast, they can become rate determining—and thus need efficient catalysis. This is especially true when the proton is transferred to or from carbon or when the reaction is concerted with the formation or cleavage of a bond between heavy (i.e., non-hydrogen) atoms. How enzymes stabilize proton-transfer transition states is the subject of vigorous ongoing debate.[1−10] Induced intramolecularity is an obvious candidate, but effective molarities (EM; the nominal concentration of a catalytic group in solution needed to match the rate of the intramolecular reaction under identical conditions[11]) for general acid/base catalysis in model systems are notoriously low—typically below 10 M. For nucleophilic catalysis, by contrast, values of up to 10^9 M (up to 10^{13} when ground-state strain is relieved during the reaction) can be achieved with relative ease when catalytic and substrate group are brought together on the same molecule. It has been suggested that this low efficiency of intramolecular general acid/base catalysis may reflect the rather loose transition-state binding of the in-flight proton—or simply our deficient understanding of the optimal positional requirements for a proton-transfer reaction. The recent calculation of an EM of the order of 10^5 M for the proton-transfer catalyzed by mandelate racemase,[12] shows how enzymes can take advantage of proximity effects to catalyze efficient proton transfer. Results with intramolecular model systems designed to show a large increase in hydrogen bond strength during the course of proton transfer show that high EMs (up to 10^6 M) can be attained, given rather precise control of geometry.[13,14] Control of geometry is of course a basic property of enzyme active sites, and results with Glu→Asp mutants show how the minimal change in the position of a general acid[15] or general base[16] reduces catalytic efficiency.

Of particular interest in this context is an estimated EM of up to 40 000 M for a reaction catalyzed by man-made antibody 34E4, generated by the bait-and-switch approach, using a cationic hapten to elicit an active-site carboxylate general base.[17] The model for much more demanding enzyme-catalyzed proton transfers was the Kemp elimination (**1** → **2**), a base-catalyzed proton transfer from carbon, in which the removal of the proton is concerted with N−O-cleavage.[18]



1 **TS** **2**

Catalytic antibodies are the best available enzyme mimics and have many interesting and relevant properties, but high

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efficiency is not usually one of them. So an EM as high as 40 000 M raises important fundamental questions. As noted by Thorn et al., the analysis in terms of catalytic efficiency is complicated by the high sensitivity of the reaction to medium effects,[19] so that the catalytic package even in this simple one-step model enzyme reaction is difficult to unravel. The Cambridge group thought to gain insight into what constitutes the main source of catalysis in the antibody 34E4—a medium effect or a "perfectly poised" base—by looking for other protein scaffolds with an appropriate combination of hydrophobic binding sites with potential general bases in close proximity. The family of serum albumins from different species shows the desired property and catalyses the Kemp elimination with k_{cat} values of the same order of magnitude. The Scripps/ETH group observed catalysis by bovine serum albumin (BSA) independently, in the course of their work with catalytic antibodies.[17]

This paper is based on our two independent preliminary reports of catalysis of the Kemp elimination by serum albumins.[20,21] We present detailed information about these systems, a discussion of the likely catalytically active residues and the global environment of the proton-transfer active site, and comparison with structural data. A comparison of serum albumins with other available catalysts for this reaction allows some insight into the sources of catalysis and points to a significant contribution of medium effects in bringing about rate acceleration.

Experimental Procedures

Materials. Bovine serum albumins were used as supplied by Sigma (St. Louis, MO) (A-7511, A-3294, A-7030) and Boehringer Mannheim (fraction V). (Preparation A-7030 ("essentially fatty acid free") was used for the majority of the experiments reported below, except where otherwise indicated.) Other proteins assayed for catalytic activity toward **1** were barnase, lysozyme, trypsin, chymotrypsinogen and chymotrypsin, and calmodulin (from Sigma; conditions: 0.2 mg/mL, 30 mM HEPES, pH 7.4). Many of these are known to have hydrophobic binding sites, but none catalyzed the Kemp elimination significantly.

Substrate Synthesis. The published preparation of **1**[22] was improved as follows. 1,2-Benzisoxazole (Aldrich, Milwaukee, WI, 4.6 g, 38.6 mM) was dissolved at 0 °C in concentrated H_2SO_4 (19.7 cm^3) and a mixture of concentrated nitric acid (2.6 cm^3) and concentrated H_2SO_4 (1 cm^3) was added slowly. The solution was stirred for 30 min and poured onto an ice/water mixture (1;1, 100 cm^3). The crude product was collected as a precipitate and recrystallized from anhydrous ethanol to yield the nitrobenzisoxazole **1** (3.1 g, 50%) as colorless needles, mp 126−7 °C;[22] ν_{max} ($CDCl_3$)/cm^{-1} 3100 (CH), 1620 (arom); 1530, 1350 (NO_2), δ_H (400 MHz; $CDCl_3$) 8.90 (1 H, d, J 0.8, H-3), 8.72 (1 H, d, J 2, H-4), 8.50 (1 H, dd, J 2 and 9, H-6), 7.75 (1 H, d, J 9, H-7); δ_C (400 MHz; $CDCl_3$) 164.4− (arom C−O), 147.1+ (arom CH═N), 144.8 (arom C−NO_2), 125.6+, 121.9−, 119.3+, 110.5+ (arom); *m*/*z* 164 (100%, M^+), 134 (23%, $M^+ - NO$), 118 (28%, $M^+ - NO_2$) (Found: $[M]^+$ 164.0222 $C_7H_4N_2O_3$ requires M^+, 164.0222). (Found: C, 51.1; H, 2.6; N, 17.1. $C_{12}H_{14}N_2O$ requires C, 51.2; H, 2.5; N, 17.1).

Rate Measurements. In Cambridge initial rates were determined spectrophotometrically by monitoring the release of product **2** at 405 nm in a Thermomax microtiter plate reader (Molecular Devices) and analyzed using the programs SOFTmax 2.32 and Kaleidagraph 3.0.5. Initial velocities were determined at 25 ± 1 °C with **1** (0.15−0.8 mM) and serum albumin (15 μM) and are corrected for the rate of the background reaction under the same conditions. Data points are means from three measurements. A methanolic stock solution of substrate **1** was diluted with water (1:10) and added to a solution of SA in buffer (30 mM containing 150 mM NaCl). The final methanol concentration did not exceed 2% of the final volume of 200 μL. The rate of the SA-catalyzed reaction was approximately halved in the presence of 150 mM NaCl compared to that in salt-free buffer solution. The use of buffers bearing hydrophobic groups (e.g., cyclohexyl in 2-(*N*-cyclohexylamino)ethanesulfonic acid (CHES) or 3-(cyclohexylamino)-1-propanesulfonic acid (CAPS) was avoided since they inhibited the reaction (by a factor of about 2 in 30 mM buffer, pH 9.5). Kinetic parameters were derived by Lineweaver−Burk and Eadie−Hofstee methods and by fitting directly to the Michaelis−Menten equation. Calculated errors are based on standard deviations. The low solubility of **1** in water prevented measurements at substrate concentrations above K_M; hence, errors greater than the indicated limits cannot be rigorously excluded. The rate constant k_{uncat} for the uncatalyzed reaction was obtained by extrapolation to zero buffer concentration (30−210 mM bis-Tris buffer, [S] = 10−37.5 mM]). Repetitive scans showed a clean isosbestic point at 304 nm, as for the uncatalyzed reaction, ruling out the possibility that a binding step was being monitored.

The Scripps/ETH group monitored reactions of **1** spectrophotometrically at 380 nm as a function of time. The reactions were carried out at 20 ± 0.2 °C in the following buffers (40 mM, containing 100 mM NaCl): sodium acetate (pH < 6), sodium phosphate (6 < pH < 8), and sodium carbonate (pH > 8). In all cases, addition of substrate dissolved in 2% acetonitrile was used to initiate reaction. Stopped-flow techniques were used for measurements at high pH (>8.5). Equal volumes of substrate and BSA solutions were rapidly mixed, and the time-dependent increase in absorption at 380 nm was recorded. Initial velocities, calculated from the first 5−10% of the reaction, were measured in triplicate and corrected for the background reaction of **1** in the absence of BSA. For saturation kinetics, the concentration of substrate was varied from approximately 50 μM to 1 mM with BSA concentrations around 10 μM. The catalyst concentration was increased up to ∼50 μM for particularly slow reactions to obtain reliable rates over background. Kinetic constants were derived by direct computer fit of the data to the Michaelis−Menten equation: $v_0 = k_{cat}[BSA][\mathbf{1}]/(K_m + [\mathbf{1}])$. Apparent second-order rate order constants (k_{cat}/K_m) were also obtained directly and independently from initial velocities obtained at low substrate concentration (50 μM ≪ K_m). The pH-rate data were analyzed as described in the text. Measurements by the two groups were generally in good agreement, but diverged above pH 9.3, where the Scripps stopped-flow results gave significantly higher initial rates. At these high rates (> 0.2 O. D./s) strong product inhibition had set in before the manual measurements commenced. The stopped-flow data for BSA gave a plateau rate of 360 min^{-1} and the apparent pK_a of 10.3 which we use to calculate the EM.

Inhibition Studies. Noncovalent inhibition. Noncovalent inhibition was measured for HSA and is expressed as the concentration of inhibitor for which the activity was halved (IC_{50}). Strong inhibition by product **2** was observed in all experiments ($K_i \approx 50$ μM). Up to 30 turnovers were observed before the background rate caught up with the catalyzed rate. Dialysis of product-inhibited HSA against phosphate buffer (pH 7.4) restored activity in full.

Covalent inhibition. SAs (150 μM) were incubated with pyridoxal phosphate (0−0.9 mM) at pH 8.1 (50 mM bicine) for 2 h in the dark. Following preincubation the solution was diluted into reaction buffer, and initial rates were measured as described above. SAs were also modified with acetylsalicylic acid following the procedure of Walker.[23] For modification with 4-nitrophenyl anthranilate,[24] SAs (4 mg/mL) were incubated with 4-nitrophenyl anthranilate (0−90 μM) for 24 h (100 mM phosphate, pH 7.4, 50 mM NaCl), and the release of nitrophenoxide was followed. The sample was then dialyzed against phosphate buffer (pH 7.4, 50 mM) and assayed as above. There was a good correlation between the release of nitrophenoxide and the residual activity for the

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Table 1. Sequence Alignment of Primary Structure for Serum Albumins from Different Species[a]

	IIA Site																	
	199	211	214	215	**218**	219	**222**	223	234	238	242	**257**	260	261	264	290	291	292
human	**K**	F	W	A	**R**	L	**R**	F	L	L	H	**R**	L	A	I	I	A	E
bovine	**R**	L	W	S	**R**	L	**K**	F	L	L	H	**R**	L	A	I	I	A	E
equine	**K**	V	W	S	**R**	L	**K**	F	I	L	H	**R**	L	A	I	I	A	E
rat	**K**	F	W	A	**R**	M	**R**	F	L	V	N	**R**	L	A	M	L	A	E
chicken	**F**	F	R	Q	**Y**	L	**K**	Y	F	S	H	**M**	M	M	L	I	M	E
sheep	**R**	L	W	S	**R**	L	**K**	F	I	L	H	**R**	M	L	I	I	A	E
porcine	**K**	F	W	S	**R**	L	**R**	F	I	L	H	**R**	M	L	I	I	A	E
murine	**K**	F	W	A	**R**	L	**T**	F	L	L	N	**R**	M	L	M	L	S	E
rabbit	**R**	Y	W	A	**R**	L	**R**	F	I	L	H	**R**	L	A	M	I	Y	G

	IIIA Site															
	384	**387**	388	391	392	**395**	410	**411**	430	433	438	449	**450**	453	485	489
human	P	**L**	I	N	C	**F**	R	**Y**	L	V	C	A	**E**	L	R	S
bovine	H	**L**	I	N	C	**F**	R	**Y**	L	V	C	T	**E**	L	R	S
equine	P	**L**	V	N	C	**F**	R	**Y**	L	V	C	S	**E**	L	R	S
rat	P	**L**	V	N	C	**Y**	R	**Y**	L	V	C	V	**E**	L	R	S
chicken	T	**V**	V	N	C	**L**	R	**Y**	M	I	C	S	**E**	L	R	T
sheep	P	**L**	I	N	C	**F**	R	**Y**	L	L	C	T	**E**	L	R	S
porcine	P	**L**	I	N	C	**F**	R	**Y**	L	L	C	A	**E**	L	R	S
murine	P	**L**	V	N	C	**Y**	R	**Y**	L	L	C	V	**E**	L	R	S
rabbit	P	**L**	V	N	C	**Y**	R	**Y**	L	V	C	V	**E**	L	R	S

[a] Residues are grouped according to interactions with ligands in two small-molecule binding sites as described by Carter.[26,35] The numbering is based on the assignment residue 214 as the only Trp in HSA. The IIA and IIIA sites are identical with Sudlow's sites I and II[56] and Ozeki's sites U and R,[57] respectively. Possible bases (or their substitutes) are printed in bold, other residues constituting the two binding sites are predominantly hydrophobic.

Kemp elimination, consistent with one equivalent of *p*-nitrophenyl anthranilate reacting. At complete modification of HSA (almost 90% reaction, extrapolated to 60 μM *p*-NPOH), 70% of the Kemp elimination activity remained. Lysine residues in BSA were modified with fluorescein isothiocyanate (FITC) as previously described.[25] Unreacted FITC was removed by gel filtration (Sephadex G-25). The concentration of the modified protein was determined by the method of Smith, and an extinction coefficient at 491 nm of 15,900 M^{-1} cm^{-1} was assumed for fluorescein coupled to BSA.[25] Residual activity was measured with 55 μM **1** in carbonate buffer (pH 10).

Sequence Alignment and Comparison. The unique residue Trp-214 of human serum albumin is taken as the anchoring point for numbering in Table 1. Sequence data were obtained from the SwissProt database.

pK_a Measurements and Reactions of 1 in Solvent Mixtures. The pK_a's of acetic acid and *n*-butylamine were measured under the reaction conditions as the pH of a 50% free base solution using a Russell CMAWL 757 electrode connected to a Radiometer PHM 83 pH-meter. [T = 25 °C, [CH_3COONa] = 50 mM, [I] = 25 mM.] Data are displayed in Figure 3. Acetate-catalyzed reactions were monitored by following the appearance of the product chromophore at 405 nm in a Cary 3 spectrophotometer. (T = 25 °C; [CH_3COONa] = 50−5 mM; [I] = 25 mM; [S] = 0.1−0.14 mM.) Pseudo-first-order rate constants were calculated from exponential fits of the increase in absorbance with time and, for the reaction in 100% water, by the method of initial rates. Pseudo-first-order rate constants were plotted against acetate concentration to yield second-order rate constants k_{OAc^-} (Figure 1).

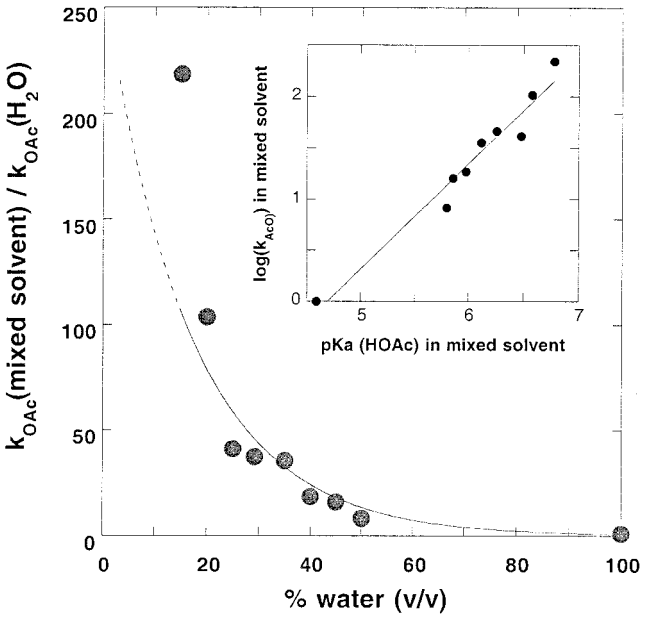

Figure 1. Second-order rate constants for the acetate-catalyzed reaction **1**→**2** (relative to the rate constants in water) plotted against the solvent composition in acetonitrile/water systems. Inset: Relative rates plotted logarithmically against the measured pK_a values of acetic acid in water/acetonitrile mixtures (data from Figure 3).

Results and Discussion

Magnitude of Rate Accelerations. All of the SAs tested accelerate the Kemp elimination. Catalytic activity was similar (±5%) under the same conditions for different commercial preparations, with the exception of the globulin-free cold alcohol precipitated albumin A-7638 (80% of typical activity at pH 7.4 and 65% at pH 8.5). For example, the activities of different preparations of BSA differed by no more than ±20%. The observed catalysis is thus independent of origin or method of purification and constitutes a genuine catalytic effect. This conclusion is further strengthened by the observation that albumins from other species, with known high sequence homology, share the catalytic power of BSA (Table 1). While most proteins can be denatured thermally, BSA is known to be particularly heat-resistant.[26] Attempted heat inactivation (94 °C, 10 min) resulted in a loss of no more than 40% of activity, thus ruling out labile proteins as catalytic contaminants.

Reactions were analyzed according to the Michaelis−Menten model (Figure 2). For BSA derived k_{cat} values (Cambridge, at 25 °C) ranged from 0.71 min^{-1} at pH 6.8 to 14.6 min^{-1} at pH 9.0; stopped-flow measurements by the Scripps/ETH group show the rate finally leveling out near pH 11 (k_{cat}^{max} = 360 min^{-1}, measurements at 20 °C). K_M values fall in the mM range, 2 mM at pH 8 and 0.7 mM at pH 9, although these data should be taken with a degree of caution since limited substrate solubility precluded measurements above K_M (Figure 2). The Scripps/ETH data yield K_M values between 0.4 and 0.7 mM for BSA above pH 7; at lower pH values K_M appears to increase making accurate determinations difficult. The corresponding k_{cat} for HSA falls between 3.2 min^{-1} at pH 8 and 28.8 min^{-1} at pH 9.0 (K_M 1.55 mM at pH 8 and 3.2 mM at pH 9, k_{cat}^{max} 48 min^{-1}). The k_{cat} values for both proteins correspond to rate accelerations

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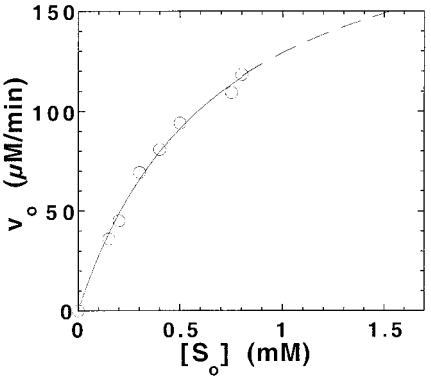


Figure 2. Plots of initial rates vs initial substrate concentration [S_o] for catalysis by BSA at pH 8.97. [Conditions: $T = 25$ °C, [BSA] = 15 μM in 30 mM buffer, 150 mM NaCl].

Table 2. Relative Activities toward **1** of Serum Albumins from Different Species

species[a]	relative activity[b] at pH 6.8	at pH 7.4	at pH 8.4	at pH 9.04
human (A-1653)	22	51	51	69
bovine (A-3249)	100	100	100	100
dog (A-3184)	40	48	35	34
horse (A-9888)	45	11	24	18
chicken (A-3014)	222	170	90	92
pig (A-2764)	106	95	95	95
rat (A-6272)	105	70	49	49
rabbit (A-0639)	169	95	34	31
mouse (A-3139)	78	62	44	41

[a] Sigma ordering number in brackets. [b] Measured as initial velocities. [Conditions: 25 °C, 30 mM buffer (BTBS, phosphate, bicine, AMPSO), 150 mM NaCl, [SA] = 2 mg /ml, [S_o] = 0.5 mM]. The relative activity of BSA at the respective pHs was set to 100.

over background of 10^3-10^4 (accurate only to an order of magnitude).[27]

Rate ratios for the different SAs vary with pH (Table 2), indicating that the catalytic base has a slightly different pK_a in each case: evidently the catalytic groups occupy different environments. In the pH-independent regions the plateau k_{cat} values are similar, differing by factors of no more than 5. These remarkable rate accelerations are by no means a general property of proteins, even those that are good substrate binders. The great majority of antibodies screened in previous studies exhibited no significant activity in the Kemp elimination, [17,28] and we found the same to be true of calmodulin, and such enzymes as barnase, lysozyme, trypsin, chymotrypsinogen, and chymotrypsin.

Identification of Active-Site Residues and Binding Site Mapping. The pH dependence of k_{cat} and k_{cat}/K_M suggests the involvement of catalytic bases of pK_a in the region of 9−10 for both BSA and HSA in the low pH region.[20] The stopped-flow measurements at Scripps/ETH reveal a further rate increase for the BSA-catalyzed reaction at higher pHs,[21] with a corresponding increase in the apparent pK_a to 10.3. It is not unexpected for the microenvironment of the catalytic group to change as other ionizing groups are deprotonated. (The activity at higher pH could also be evidence for a second catalytic site.) A suitable candidate in each case is a lysine (p$K_a \approx 11$ in small peptides) in a somewhat hydrophobic environment which destabilizes the ionic form to some extent. We have mimicked the proposed pK_a depression by measuring the pK_a of *n*-butylamine in acetonitrile/water mixtures, a simple homogeneous model for

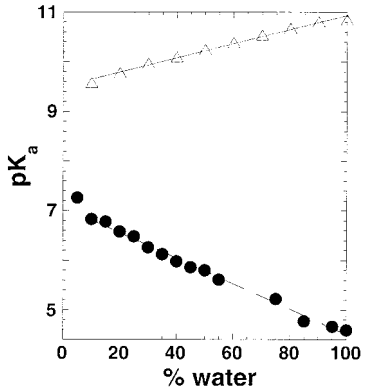
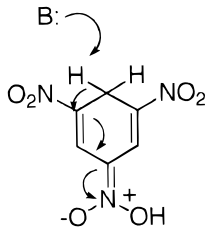


Figure 3. Measured pK_a values for acetic acid (●) and *n*-butylamine (△) vs percentage of water in acetonitrile/water mixtures. The equations for the lines drawn are $pK_a(HOOCCH_3) = 7.1-0.026\%$ water and $pK_a(BuNH_3^+) = 9.5 + 0.014\%$ water (correlation coefficients are >0.99).

a more or less hydrophobic active site. As the hydrophobicity of the mixed solvent medium increases with added acetonitrile, the pK_a falls to about 9.2 (Figure 3, upper dataset).

The dangers of overinterpreting pH dependencies of steady-state parameters are well-known,[29] and our evidence for the catalytic role of a lysine, although substantial, is necessarily indirect. We therefore tested directly for an active-site lysine by looking for inhibition by covalent modification. Pyridoxal-5-phosphate (PyrP) is known to bind covalently to two sites on BSA, the aldehyde group forming a Schiff-base (or a *gem*-diamine) with one (or two) lysine ϵ-amino groups.[30] Thus covalent modification with *1* equiv of PyrP completely inhibits the observed catalysis (by a lysine in its basic form with an apparent pK_a of 8.4) of the decomposition of Meisenheimer complex **3**.[31] We conclude below that the same lysine and the same binding site are involved in catalysis of the Kemp elimination.



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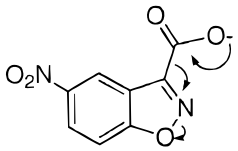

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Under the same conditions the BSA-catalyzed reaction of **1** is inhibited by *2* equiv of PyrP, but only to an extent of 80%. The pyridoxal treatment applied to HSA gave only 40% inhibition. This suggests the possibility that both PyrP binding sites described by Dempsey[30] may be catalytically active to some extent. On the other hand, 1 equiv of fluorescein isothiocyanate (FITC), known to react preferentially with Lys-222 of BSA,[25,26,32] inhibits some 90% of the catalytic activity.[21] This residue is located in a binding pocket in subdomain IIA of the protein.[33] The extent of modification by FITC is directly quantifiable, and the loss of activity was found to correlate with derivatization of a single lysine residue,[21] providing a strong indication that the decomposition of **1** occurs predominantly at a single site

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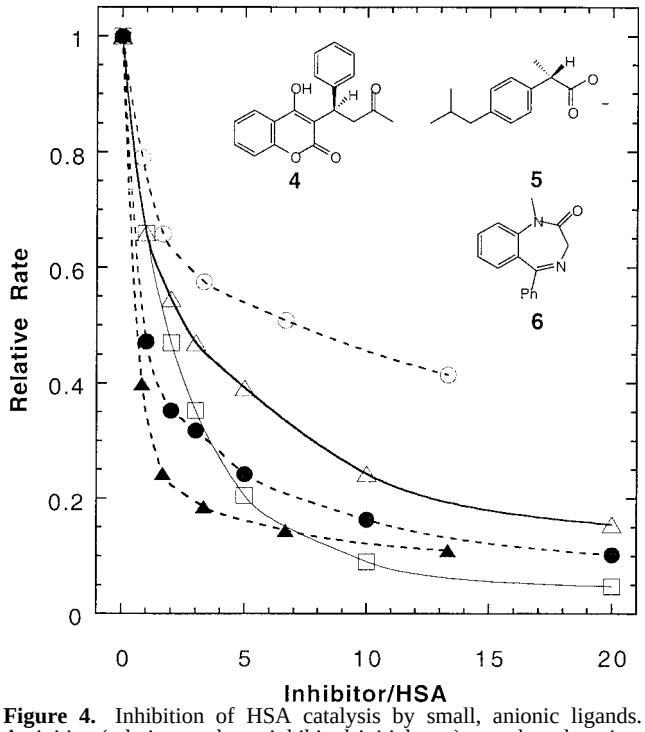

Figure 4. Inhibition of HSA catalysis by small, anionic ligands. Activities (relative to the uninhibited initial rate) are plotted against the ratio [inhibitor]/[HSA]. Inhibitors used: (●) warfarin (**4**), (□) ibuprofen (**5**), (○) diazepam (**6**), (Δ) octanoate ($CH_3(CH_2)_6COO^-$), (▲) and triiodobenzoate. [Conditions: 25 °C, [HSA] = 3.75−60 μM, [S_o] = 0.1 mM, in 30 mM bicine, pH 8.7].

on BSA. In contrast, alkylation of cysteines with iodoacetamide had no effect on the BSA-catalyzed reaction, and modification of tyrosines (and possibly lysines) with a 100-fold molar excess of acetylimidazole reduced the specific activity by only 54%. With HSA 4-nitrophenyl anthranilate (believed to react with Tyr-411[24]) reduced the activity by about 30%, suggesting that either Tyr-411 or a residue nearby in the IIIA site may contribute to the observed catalysis.

Several noncovalent binding sites have been identified on HSA for small, pharmacologically relevant molecules.[26] Figure 4 shows how some of the compounds involved in the binding studies (**4**−**6**) can inhibit catalysis more or less completely. The detailed analysis is complicated by the fact that the guests have individual preferences for one of the two sites (e.g., warfarin **4** for IIA, ibuprofen **5** for IIIA), with relative affinities differing by factors less than 100.[26] Further conformational change may be induced by a binding event at one of the two sites or possibly by binding of fatty acids (e.g. octanoic acid) at a third site.[26] Thus diazepam, which was located in the crystal structure in the IIIA site, inhibited the reaction by up to 30%. Since only a single association constant was measured[34] this may be the only inhibitor that is specific for a single site (K_d = 2 μM), although it is possible that a second association constant was missed. This is possible also for warfarin: up to 3 molar equiv gave 54% of inhibition, but at higher molar excesses further inhibition could be observed.

Taken together, the inhibition data suggest that catalysis by BSA occurs predominantly at the IIA binding site where an amine base, presumably a lysine, serves as the catalytically active residue. The well-defined IIIA binding pocket, implicated in various hydrolytic reactions, appears to be less important: if it contributes to the observed catalysis, it is to a much lesser extent. On the other hand the IIIA site in HSA appears to make a more significant contribution of up to some 30% of overall activity, as indicated by the results with diazepam and 4-nitroanthranilate.

The amino acid sequences of the two sites implicated in small ligand binding by He and Carter[35] for different SAs (Table 1) show predominantly hydrophobic side chains, together with potential general bases and some permanently cationic residues (e.g., Arg 257, 410, and 485). The positively charged residues could be involved in binding negatively charged portions of the ligands **4**−**6** or product **2** (and perhaps the transition state leading to it); with the hydrophobic residues binding the aromatic rings of these compounds. This is consistent with the binding properties of **2** and **4**−**6** and some pK_a-lowering effect of a hydrophobic environment on an amine base. In HSA the IIA site, presumably with Lys-199 acting as the general base, would be responsible for most of the activity. For BSA there is no lysine in position 199, but a different lysine (222) that is in turn absent in HSA is close by and available for catalysis.

Potential Sources of Rate Acceleration. We consider first the mechanisms available for efficient catalysis of the Kemp elimination.

(a) A catalytic base needs to be available in the free base form at the pH of the reaction, properly positioned with respect to the bound substrate in the catalyst active site. Its effectiveness will depend on its positioning, and will contribute to its observed effective molarity (EM, see above). (Assistance to leaving group departure by protonation or by interaction with a cationic group is also a possibility. However, Kemp observed no general acid catalysis even for the reaction of unsubstituted benzisoxazole ($pK_a^{\text{leaving group}} \approx 6.9$), so for substrate **1** ($pK_a^{\text{leaving group}} \approx 4.1$) such catalysis is even less likely, at least in a protic environment.)

(b) The work of Kemp shows that there is a large, accelerating medium effect of aprotic solvents on the reactions of anions with **1**, a consequence of the absence of hydrogen-bond solvation. Acetate is effectively destabilized and thus activated, compared to the hydrogen-bonded form in water. Studies in aprotic solvents indicate that this effect makes a major contribution to the over 10^7-fold rate accelerations observed for the decarboxylation of **7** and for catalysis of the elimination reaction **1** by acetate. Homogeneous aprotic solvents in Kemp's studies[19] achieved rate accelerations of at least this magnitude, regardless of their polarity. In the case of protein catalysts the thermodynamic cost of any desolvation of an active-site carboxylate has been paid at the stage of protein synthesis, raising its ground-state energy and thus increasing its intrinsic reactivity. However, the relatively modest perturbation of the observed (kinetic) pK_a under both saturating and nonsaturating conditions argues against substantial desolvation of the carboxylate base in the active site of 34E4.[17]

For catalysis by amines this effect will be in the opposite direction. The pK_a of an ammonium ion is depressed in less polar media (Figure 3) because the cation is less effectively solvated, and the same will be true of (this part of) the transition state in reactions where an amine acts as a general base. The modest rate increases observed by Kemp for the amine-catalyzed elimination reaction of **1** must therefore result from a different effect or combination of effects.

(c) In addition to medium effects involving hydrogen bonding we can identify *specific* medium effects on the free-energy of activation. These include fine-tuning of the effective dielectric constant of the active site, through-space electrostatic interactions, and dispersion interactions, in particular those involving

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the delocalized π-system of the benzisoxazole ring in the transition state. Large, polarizable anions are better solvated in aprotic solvents,[19,36] although data which allow the effect to be isolated and quantified are sparse.[37] It is possible to make limiting, order-of-magnitude estimates of the importance of these effects.

(i) By comparing the effects of different aprotic solvents tested by Kemp. In the absence of H-bonding solvation rates vary by up to 100-fold, consistent with a specific medium effect of at least this magnitude.

(ii) By measuring transfer parameters for the species of interest. In a rare example, the enthalpy of transfer of the Meisenheimer adduct of 2,4,6-trinitroanisole and methoxide from methanol to DMSO has been measured as at least 6 kcal mol^{-1}.[38] This would correspond in isolation to a rate effect of greater than 10^4. Evidently large effects on the solvation of delocalized nitroaromatic anions are possible.

Calculation of Effective Molarities. The EM is the ratio of the first-order rate constant for an intramolecular reaction and the second-order rate constant for the corresponding bimolecular reaction, measured under the same conditions.[11] The EM is interpreted in model systems in terms of the (fairly standard) entropic benefit of proximity plus any enthalpic advantage derived from the specific positioning of substrate and catalytic groups with respect to each other. For an enzyme an EM can be obtained by dividing k_{cat} by the second order rate constant for the intermolecular reaction. This ratio includes not only the effect of induced intramolecularity, but any effect (medium effects, desolvation, etc.) involved in stabilizing the transition state in question and destabilizing the corresponding ground-state Michaelis complex. Thus, an EM^{enz} is an *overall* measure of efficiency, providing only an upper limit for the effect due to positioning.

For any EM it is important to compare second- and first-order rate constants as far as possible under identical conditions. For BSA and HSA the Cambridge group measured second-order rate constants in both water and acetonitrile for catalysis of the Kemp elimination of 5-nitrobenzisoxazole **1** by 2-methoxyethylamine. The rate constant in water, 1.02 M^{-1} min^{-1}, corrected, using Kemp's Bronsted β of 0.72[39] to the pK_a of the catalytic base gives an EM of 150 M for the lysine NH_2 group of BSA when compared with the plateau rate of 360 min^{-1} (apparent pK_a = 10.3) reached near pH 11. Using the second-order rate constant for catalysis by *n*-butylamine[21] gives a corrected EM of 360 M.

These are relatively high EMs for proton-transfer reactions and clearly refer in the case of BSA to the same catalytic lysine, presumably in somewhat different microenvironments. (The active site of HSA appears to be closely similar.) In intramolecular reactions in water, general acid/base catalysis is almost always very inefficient and correspondingly insensitive to geometry. On the other hand, the small number of highly efficient intramolecular systems now known are typically rigid and highly sensitive to geometry.[40] It seems unlikely that such geometrical precision would be attained in several different protein binding sites with proximate lysine side chains.

EM^{enz} for the same reaction catalyzed by the carboxylate group in the active site of antibody 34E4,[17] corrected for the difference in basicity between acetate and the active-site carboxylate, is 5000 M. An EM of this magnitude for a proton-transfer process is even more remarkable, and raises in more acute form the question of how far the figure results from precise positioning of the active-site carboxylate, and how far it reflects the special microenvironment of the active site. It is not possible to dissect the observed catalysis in these systems neatly in terms of specific and nonspecific medium effects vs positioning of the general base. However, the relatively small shifts of the pK_a's from normal solution values suggest that hydrogen-bonding desolvation of the general base in the active site is not the major factor and is worth no more than perhaps an order of magnitude in rate for 34E4. For the serum albumins this factor must be is smaller still, since it is in the opposite direction, and unlikely to be worth more than 5.[19] The most convincing candidate for the major factor, certainly in the case of BSA and HSA and probably in the case of 34E4, is specific solvation of the transition state (source (c) above). This still leaves scope—but does not formally require—a contribution to the EM of the order of 10 M from positioning, which falls in the range we might expect for general base catalysis in such a system.

Implications and Conclusions

The rationale behind using SAs was simple: to "partition" the substrate from solution into a hydrophobic binding site of a protein known to bind a number of small, hydrophobic molecules.[26] To achieve catalysis in such an environment a catalytic base must be part of the binding site. Given the relative simplicity of this working hypothesis for the search of a suitable protein, it is remarkable that the search was successful: especially in view of the statistically low success rate for designed catalytic antibody catalysts. This success encouraged us to try to explore the factors involved in catalysis in this simple system, perhaps to shed light on more efficient—and more complex catalytic systems. One trivial reason for our success in observing catalysis is undoubtedly that the single transition-state reaction **1** → **2** has modest energetic requirements for catalysis,[41] compared with more demanding proton transfers in nature.[42] Another is likely to be the convenient geometry of the process.[43] Our analysis suggests a linear transition state with at one end a delocalized anion developing in a hydrophobic, stabilizing environment. This is generated by the removal of a proton by an adjacent catalytic general base at the other end which remains largely solvated and thus can take advantage of hydrogen-bonding solvation as the ammonium cation develops.

Efficiency of Antibody Catalysis. Antibodies can catalyze a wide variety of reactions and are indisputably the best enzyme mimics currently available. They can match natural enzymes in selectivity and stereospecificity,[44] but rate accelerations k_{cat}/k_{uncat} greater than 10^6 are rare exceptions (Table 3). Thus, calculations of EMs for reactions catalyzed by catalytic antibodies (Table 3) show that these are generally low, with very few exceptions. The highest EMs (as high as 10^4—10^5M[45,46]) typically come at the price of severe product inhibition.[46] A large hapten gives the antibody extensive recognition possibilities which allow it to bind the substrate well, but since most recognition interactions do not change during the course of the reaction, turnover is prevented.

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Table 3: EM^{enz} Values for Antibody-Catalyzed Reactions

system	reaction type	no. of substrates	EM^{enz} (M)[a]	ref
34E4	proton transfer	1	4.1×10^4 $(5000)^{b,c}$	17
35F10	proton transfer	1	2.2×10^4 $(6100)^{b,c}$	17
43D4-3D3	proton transfer	1	40.5 (3.3)	58
MOPC 315[d]	ester hydrolysis	1	0.05	59, 60
PCP21H3	transesterification	2	4×10^5	45
PCP21H3	transesterification	2	4.4×10^5	45
18R.136.1	transesterification	2	5.9×10^4	46
18R.136.1	transesterification	2	2.5×10^3	46
9B5.1	acyl azide aminolysis	2	0.158	61
17G8	ester aminolysis	2	10.3[e]	62
24B11	ester aminolysis	2	7.86	63
163G	ester aminolysis	2	10^3	64
163G	ester aminolysis	2	194	64
17C5-11C2	imine formation	2	0.21	65
7D4	Diels−Alder	2	4.75	66
22C8	Diels−Alder	2	18.3	66
1E9	Diels−Alder	2	1000	67, 68

[a] EM^{enz} are derived from k_{cat}/k_2, and thus include both the effects of positioning *and* other catalytic effects. [b] Corrected for the difference in pK_a values of using $k_2^{corr} = k_2/10^{-pKa}$. [c] Using k_2 in water. [d] Modified antibody. [e] The k_2 measured at pH 8 for butylamine was corrected for the fraction of deprotonated amine (pK_a 11) at this pH.

How do these data compare with EMs for natural enzymes? Page and Jencks estimated an efficiency gain simply from intramolecularity of the order of 10^8-fold,[47,48] and cyclization reactions of model systems in which the reacting groups are integrated into the same molecule show rate accelerations of up to this magnitude.[11] Intramolecular general acid−base catalysis is typically much less efficient, but EMs as high as 10^5 M can be attained with tight control of geometry,[40] and comparable values have been derived for enzymes. Thus Wolfenden estimated EMs for general species catalysis by mandelate racemase by comparing (extrapolated) uncatalyzed rates with those for the native and mutant enzymes.[12] For general acid catalysis by Glu-137 and general base catalysis by Lys-166 EMs were 3×10^5 and for 622 M, respectively. Similarly the mutation Glu→Asp, a change primarily in positioning, reduces efficiency by 2−3 orders of magnitude but does not eliminate catalysis entirely in such enzymes as triose phosphate isomerase[16] and certain glycosidases,[15] and catalytic groups built-in to the substrate can compete with[49] or replace[50,51] active-site general acids and bases in suitable mutants, substituting simple intramolecular for specific active-site positioning relatively effectively when the active site is otherwise intact. Thus, for general acid−base catalysis EMs due to positioning in enzyme active sites appear to fall within the extended range established by recent measurements with model systems.[40]

Interestingly, EMs for nucleophilic catalysis as low as 100 M have recently been obtained, by comparing wild type k_{cat} with reactions of mutants where a deleted active-site catalytic group is replaced by a corresponding exogenous nucleophile, but the active site is otherwise intact. Examples include the use of imidazole to replace His-122 of NDP kinase,[52] and formate for Glu-358 of an *Agrobacterium* β-glucosidase (acting on an activated substrate)[53]

Such figures do not look very different from those for catalytic antibodies (Table 3). However, calculation of the EM in the same way as for the antibodies (i.e., by comparison with the corresponding intermolecular reaction in solution, $EM = k_{cat}/k_2$) reveals the true extent of catalysis by enzymes. For NDP kinase the comparison with the second-order reaction of imidazole with ATP gives a figure of the order of 10^{15} M,[52] and a figure of the same order of magnitude is likely for imidazole catalysis of the racemisation of mandelate anion.[12] "Overall" EMs for enzyme reactions include contributions from a range of catalytic effects and can be substantially higher than those for the positioning of any particular group. Although the number of specific examples is limited, we can conclude that high EMs are commonplace in enzymes but a rare exception in reactions catalyzed by catalytic antibodies.

The failure of antibodies to attain the levels of efficiency of natural enzymes also stems from a combination of factors: (a) transition-state analogues are at best poor mimics of true transition states; (b) many reactions involve more than one transition state that requires stabilization; (c) the majority of catalytic antibody reactions do not actually involve a functional group acting as a catalyst, but simply stabilize the transition state for the reaction; (d) even if functional group(s) are involved in catalysis the probability of generating arrays capable of interacting synergistically, even given "good TSAs", is infinitesimally small. Even *bait and switch* haptens (exemplified by the entry for 43D4-3D3), although designed to tap the catalytic potential of conveniently located functional groups, appear to give EMs no higher than do TSA haptens. More generally, (e) only a tiny fraction of the immune response is typically sampled; and (f) hapten binding rather than catalysis drives selection in most cases (removing the possibility of optimizing catalysis by Darwinian evolution in the immune system). It is also conceivable that the immunoglobulin fold places intrinsic limitations on what can be accomplished catalytically.[54] As a result, reactions of unactivated substrates that require a complex assembly of catalytic groups acting at different loci along the time axis are not promising targets at this time.

More practical objectives are reactions with modest thermodynamic requirements, perhaps accelerated by medium effects, although recent experiments that exploit reactive mechanism-based inhibitors such as haptens suggest that some more complex reaction manifolds can be successfully addressed. The generation of highly versatile aldolase antibodies that exploit the enamine-mechanism of natural aldolase enzymes is notable

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in this regard.[55] The impressive and reliable chiral selectivity routinely available in their reactions makes further development of these tailor-made catalysts interesting for synthetic as well as potential medical applications.

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