(CO); uv λ_{max} (EtOH) 245 nm (log ϵ 3.74); mass *m/e* (calcd for $C_{20}H_{32}O_4$, 336.230) found, 336.229 ($M^{+\cdot}$).

Dihydrokromycin (5b). A solution of **2b** (1.0 g) in ethanol (50 ml) with hydrogenated over 5% Pd/C (0.25 g). The filtrate was evaporated under a reduced pressure to yield crude **4b** as a white powder (0.95 g), and this was used for the next reaction without further purification. **4b** was dissolved in 1 *N* hydrochloric acid (20 ml), which was adjusted to pH 6.8 with 0.1 *N* sodium hydroxide. After warming the solution at 60° for 120 hr, the separated crystals were recrystallized from petroleum ether–ether to **5b** (0.12 g) as colorless needles: mp 134–136°; ir (KBr) 1720 (lactone), 1701, 1665, 1632 cm^{-1} (CO); uv λ_{max} (EtOH) 233 nm (log ϵ 4.13), 297 (1.90).

Anal. Calcd for $C_{20}H_{32}O_5$: C, 68.15; H, 9.15. Found: C, 68.12; H, 9.20.

Tetrahydrokromycin (7). To a solution of **5b** (100 mg) in methanol (3 ml), a solution of sodium borohydride (100 mg) in methanol (10 ml) and water (1 ml) was added. After standing for 12 hr at room temperature, the reaction mixture was treated with acetone to decompose the excess reagent. The residue after evaporation of the ether extract was crystallized from petroleum ether–ether to yield 80 mg of **6** as white needles: mp 112–113°; mass *m/e* 356 ($M^{+\cdot}$). This was used for the next reaction without further purification.

A solution of **6** (70 mg) in acetone (30 ml) was stirred for 24 hr at room temperature with active manganese oxide (1 g). After evaporation of the filtrate, the residual white powder was crystallized from petroleum ether–ether to yield **7** (28 mg) as white needles: mp 137–138°; $[\alpha]^{20}D$ −15.4 (*c* 1.0, MeOH); ir (KBr) 3480 (OH), 1726 (lactone), 1670, 1635 cm^{-1} (CO); uv λ_{max} (EtOH) 245 nm (log ϵ 3.65); mass *m/e* (calcd for $C_{20}H_{34}O_5$, 354.241) found, 354.240 ($M^{+\cdot}$).

Anal. Calcd for $C_{20}H_{34}O_5$: C, 67.76; H, 9.67. Found: C, 67.55; H, 9.46.

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Bovine Serum Albumin as a Catalyst. II. Characterization of the Kinetics

Ronald P. Taylor,* Vincent Chau, Claudia Bryner, and Sarah Berga

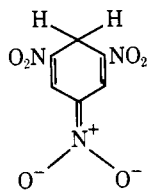
Contribution from the Department of Biochemistry, School of Medicine, University of Virginia, Charlottesville, Virginia 22901. *Received September 23, 1974*

Abstract: The accelerated decomposition of the Meisenheimer complex, 1,1-dihydro-2,4,6-trinitrocyclohexadienate, by bovine serum albumin (BSA) is reported in detail. In the pH range of 7–9 the BSA-catalyzed reaction is accelerated by a factor of about 10^4 relative to the rate of decomposition of the substrate alone. This catalysis requires an unprotonated base on the protein (pK_a = 8.4) and can be stoichiometrically inhibited by binding of 1 equiv of pyridoxal phosphate (PP) to the protein. Chemical modification with acetic anhydride and fluorodinitrobenzene along with previous studies on the PP–BSA complex indicates that the active site of the molecule is rich in lysine and is probably coincident with a unique PP binding site in the protein. This unusual catalytic function has also been used as a sensitive probe to generate new information on some of the ligand-binding properties of the protein.

Numerous examples of catalytic activity of apparently nonbiological significance have been reported for a variety of proteins,[1,2] synthetic macromolecules,[3,4] and micellar complexes.[5,6] Recent examples include the bovine serum albumin (BSA) catalyzed decomposition of *p*-nitrophenyl acetate[1] and the catalyzed hydrolysis of 4-nitrocatechol sulfate by a synthetic polymer.[3] Often there are distinct differences between these activities and those associated with enzymes. Generally speaking, manifestation of specificity for both substrates and inhibitors is considerably more restricted in enzymes. In addition, but for one outstanding exception,[3] the relative rate enhancement due to the "nonbiological" catalyst is generally significantly less than the rate enhancements observed in typical enzyme systems.

The presence of unique specific active sites within enzymes probably constitutes the most significant distinguishing property of true enzyme systems.[7,8] These active sites are generated by the proper juxtaposition of catalytic residues which in turn depend upon the proper three-dimensional folding of the protein.[9,10] It is not surprising, therefore, that those parameters (temperature, pH, solvent, ionic strength) which perturb the delicate balance of forces stabilizing native protein configuration[10-12] often have profound effects on enzyme activities. For example, whereas in principle inorganic catalysts such as hydroxide ion and oxonium ion can function in 8 *M* urea, enzymes are inactive in this medium. As a second example, it is well known that the interaction of sodium dodecyl sulfate (SDS) with most proteins causes severe conformational refolding[13] which generally results in the loss of biological activity.[14] However, SDS micelles themselves can act as moderately efficient catalysts over wide ranges of pH and temperature.[5,6]

We recently reported an unusual catalytic activity exhibited by BSA, namely, the accelerated decomposition of the Meisenheimer complex, 1,1-dihydro-2,4,6-trinitrocyclohexadienate (**1**).[15] This catalysis should certainly qualify as a nonbiological activity, as the substrate is clearly unlikely to be found in bovine serum. We report here a more complete characterization of this catalysis.



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In this and future papers we will show that the catalytic activity we study is a sensitive probe of the conformational integrity and known physical properties of the molecule. In addition, we will demonstrate that this catalysis occurs within a unique specific active site within the molecule. An immediate aim of these investigations will be to prove that this unusual "nonbiological" catalytic property of BSA does, in fact, meet the requirements we have specified for biological activity in true enzyme systems.

Experimental Section

Bovine Serum Albumin. BSA was obtained from a variety of sources in different states of purity (see Table II). Unless otherwise noted, all experiments were performed on crystalline BSA obtained from Armour (Lot No. L72410 and L72510) which had been purified by the following procedures. The free sulfhydryl group was blocked by reacting the protein with about a tenfold excess of iodoacetamide (IA) for 24 hr in 0.05 *M* phosphate, pH 7.0, at 4°. After dialysis against water in the cold, the protein was defatted following the methods of Foster.[16] The monomeric form of the protein was obtained by passing about 1 g of this defatted, IA blocked protein down a 1 m long (4 cm diameter) Sephadex G-150 column equilibrated with 0.1 *M* NaCl and 0.1 *M* Tris–HCl at pH 8.0 and 4°[17] at a flow rate of about 30 ml/hr. Subsequently the protein was dialyzed exhaustively in the cold against distilled and deionized water. Small aliquots which were to be used for direct kinetic assays were diluted with water to a concentration of about 4 mg/ml ($E_{279}^{1\%}$ 6.67)[16] and stored frozen at −15°. The remainder of the solution was concentrated to about 20 mg/ml and was also stored frozen. Samples of Armour crystalline BSA purified by these procedures (i.e., defatted IA blocked monomers) will be designated as IA–BSA. The molecular weight of BSA was taken as 66,000.[18]

Substrate. It was found that the method of handling substrate (exposure to light, air, and water) significantly affected the observed kinetics. The following protocol was found to be sufficient for achieving reproducible kinetics. Tetramethylammonium 1,1-dihydro-2,4,6-trinitrocyclohexadienate was synthesized by previously published methods[19] except great care was taken to exclude light from the product while in solution. In addition, it was not necessary to perform the reaction under nitrogen. The product was stored in vacuo in desiccators filled with Drierite at −15°. Acetonitrile solutions used for assay procedures were prepared in volumetric flasks and wrapped in aluminum foil to exclude light and also stored at −15° when not in use. Under these conditions, stock solutions in acetonitrile could be stored 2–3 days at −15° with no harmful effects on kinetics. Storage of the substrate as a solid in vacuo at −15° for periods of up to 3 weeks was possible. By this time (sometimes less, depending upon the number of times the desiccator was opened) the substrate would start to "go bad" as evidenced by lower reaction velocities in our "standard assay" (see below) and fresh substrate would be prepared.

Reagents, pH, and Buffers. Spectroquality grade acetonitrile (Mallinckrodt) was used without further purification. pH was measured on a Corning Model 110 pH meter equipped with either Corning or Thomas combination electrodes. Buffers from Mallinckrodt were used for standardization. Sodium phosphate buffers prepared from monobasic sodium phosphate and either sodium hydroxide or dibasic sodium phosphate were used. Tris–HCl [tris(hydroxymethyl)aminomethane hydrochloride] was from Sigma and was adjusted to pH with sodium hydroxide. All water used in the experiments was distilled deionized water. Pyridoxal phosphate (PP) was obtained from Nutrition Biochemicals Corp. and used according to the published procedures of Dempsey and Christensen.[20] Fluorodinitrobenzene (FDNB) was obtained from Eastman and dissolved in ethanol (stock solutions were approximately 6×10^{-2} *M*) before reacting it with aqueous solutions of the protein. Sodium dodecyl sulfate (SDS), ultrapure, was obtained from Schwarz/Mann. Palmitic acid was from Sigma. Iodoacetamide (IA) was from Pierce and was recrystallized from water before it was used. Penicillin G (benzylpenicillin, sodium salt) was from Sigma. The pH of all solutions used in assays was determined on preparation and immediately after each experiment. All reported pH values are precise to ±0.02 pH units. Salts were obtained from either Sigma or Mallinckrodt and used without further purification.

Assays. We followed the general spectrophotometric procedures developed by Bender and coworkers.[21] In our "standard assay", approximately 13 μl of a solution of the tetramethylammonium salt of the substrate **1**, dissolved in acetonitrile (approximately 5 mg/ml), was added to 3 ml of buffer solution (0.05 *M* phosphate, pH 8.0) previously equilibrated at 25.0 ± 0.2° in a cuvette in a Coleman 124 spectrophotometer. This should give an OD of approximately 1.6 at 478 nm. Our control experiments indicate that the small amounts of acetonitrile used in the assay are not inhibitory. A neutral density filter (OD of 1) was used in the reference beam of the spectrophotometer to allow us to select a recorder output of 0–1 OD full scale. After a suitable base line was obtained 50 μl of a BSA stock solution in water (4 mg/ml) was added and the rate of decrease of absorbance at 478 nm was determined. The "initial rate" of decrease of absorbance upon addition of BSA was used to calculate reaction velocities from a knowledge of the difference in molar extinction coefficients between reactants and products. These values were determined by completely decomposing a known amount of substrate with BSA and determining the total absorbance change at 478 nm. Corrections for BSA absorbance at 478 were small. The differences in molar absorptivities between reactants and products were pH dependent and are reported in Table I. Under the above conditions (i.e., substrate concentration of about 75 μ*M*) the initial rate of decomposition of substrate by BSA was approximately independent of substrate concentration, as saturation of "enzyme" (i.e., BSA) by substrate was almost complete (see below). Unless otherwise stated all experiments were performed at this level of substrate concentration.

Calculations. Apparent catalytic rate constants, k_c, were determined assuming the "active site" concentration in BSA to be identical with the protein concentration. That is

$$k_c = \frac{[dA/dt]_0/\Delta\epsilon}{[P]}$$

where $[dA/dt]_0$ is the "initial" rate of decrease of absorbance of substrate at 478 nm on adding BSA, $\Delta\epsilon$ is the difference in molar

Table I. Molar Absorptivities of 1 and Its Decomposition Products[a,b] at 478 nm, 25.0°

pH	Buffer	Molar absorptivity of 1 ($\times 10^{-4}$)	Molar absorptivity of decomposition products ($\times 10^{-4}$)	$\Delta\epsilon$ ($\times 10^{-4}$)
7.00	Phosphate	2.16	0.03	2.13
7.50	Phosphate	2.18	0.05	2.13
8.00	Phosphate	2.16	0.04	2.12
8.00	Tris–HCl	2.18	0.04	2.14
8.50	Phosphate	2.15	0.11	2.04
8.90	Tris–HCl	2.16	0.13	2.03
9.20	Tris–HCl	2.14	0.25	1.89
9.42	NH_4Cl	2.16	0.35	1.81
9.66	Tris–HCl	2.09	0.40	1.69
9.98	NH_4Cl	2.04	0.44	1.60
10.11	NH_4Cl	2.05	0.45	1.60
10.42	NH_4Cl	1.88	0.42	1.46

[a] All buffers are 0.05 *M*. [b] Uncertainties are ~3% or less in $\Delta\epsilon$, based on at least two or three independent determinations for each point.

absorptivity between reactants and products, and [P] is the concentration of BSA in the cuvette in which the reaction is monitored.

The apparent catalytic rate constant, k_c, determined for IA–BSA under the "standard assay" conditions was 0.55 ± 0.03 sec^{-1} over a period of 6 months using two different initial lots of Armour BSA (L72410 and L72510). Standard assays which preceded all other experiments had to be within the aforementioned limits before an experiment was done. Individual experimental points for assays (e.g., at a given pH) were generally repeated two or three times for each experiment on a given day and had to be within 3% agreement in order to be acceptable. Most experiments were repeated independently weeks or months apart. We estimate the uncertainty in individual apparent catalytic rate constants as 6% or better based on the observed reproducibility of these experiments over long periods of time. At higher values of pH or lower substrate or protein concentrations, the experimental uncertainties were somewhat higher (approximately 10% at most) and are so noted in the figures.

Chemical Modification Experiments. IA–BSA was acetylated with acetic anhydride by adding about 50 μl of acetic anhydride in a total of three portions to 2 ml of a half-saturated solution of sodium acetate containing 20 mg/ml of BSA at 4°. After 2 hr the solution was dialyzed exhaustively against distilled water, and the modified protein was assayed for activity.

IA–BSA was labeled with FDNB by adding small aliquots of alcoholic solutions of FDNB (about 6×10^{-2} *M*) to solutions of the protein in 0.1 *M* $NaHCO_3$. The final protein concentration was usually about 1.9 mg/ml and the final FDNB concentration was about 3×10^{-4} *M*. After about 30 min the reaction was quenched by adding 0.1 *M* HCl to a pH of about 5.5. Subsequently the labeled protein was dialyzed in the cold against 0.05 *M* acetate buffer, pH 5, and then against water.

Preparation of solutions of IA–BSA complexed with PP was achieved by incubating both protein (about 10 mg/ml) and PP together in the dark, at pH 8, for 24 hr in water which had been previously deoxygenated by bubbling nitrogen through it for 0.5 hr at 4°. If the samples were to be reacted with FDNB the solutions were then made 0.1 *M* in $NaHCO_3$, the protein concentration was adjusted to 1.9 mg/ml, and appropriate aliquots of FDNB were added as described above. After a period of incubation (about 30 min) the solutions were quenched (see above) and dialyzed in the cold against 0.05 *M* acetate buffer, pH 5, to remove unreacted FDNB. Subsequently, the samples were dialyzed for at least 2 days against pH 4.0, 0.05 *M* acetate to remove any remaining PP. Finally the samples were dialyzed exhaustively against distilled water.

Difference Burst Titrations. In a typical experiment 3 ml of a solution of IA–BSA (1.9 mg/ml) which had been previously incubated with a given amount of PP and then made 0.1 *M* in $NaHCO_3$ was placed in the reference cuvette of the spectrophotometer. An identical quantity of IA–BSA in 0.1 *M* $NaHCO_3$ (but lacking PP) was placed in the sample compartment. At zero time identical aliquots (about 13 μl) of alcoholic solutions of FDNB (about 6×10^{-2} *M*) were added to the sample and reference cuvettes and the increase in absorbance at 360 nm was monitored as a function of time. The molar extinction coefficient for the formation of 1 mol of dinitrophenyllysine was taken as 17,400.

Table II. Apparent Catalytic Rate Constants for Decomposition of 1 by Various Samples of BSA[a,b]

Co.	State of protein	Batch no.	k_c, sec^{-1}
Armour	Crystalline	L72510	0.27
Armour	Crystalline, defatted	L72510	0.53
Nutritional Biochemicals	Crystalline	3701	0.27
Pentex	Crystalline	25	0.41
Schwarz/Mann	Crystalline	X3894	0.25
Sigma	Fraction V	33C-2940	0.42
Sigma	"Fatty acid free" from fraction V	42C-7660	0.43
Armour	IA–BSA	L72410 L72510	0.57
Armour	Iodoacetamide blocked only	L72510	0.32
Armour	Dimer from preparation of IA–BSA	L72410	0.415
Armour	Mercaptoalbumin	*c*	0.43

[a] All samples were examined with the "standard assay" procedure, 25.0°. [b] Each sample was examined at least twice. Results were reproducible to about 5% or better. [c] We thank Professor Foster for providing this sample. It was prepared according to Hagenmaier and Foster, *Biochemistry*, 10, 637 (1971), except SP Sephadex was used instead of SE Sephadex.

Spectroscopic Investigations. Absorption spectra were taken on a Cary 15 spectrophotometer. Difference spectra were taken on an Aminco-DW-2 spectrophotometer. Studies of the time-dependent changes in the absorption spectrum of BSA–PP complexes and HSA–PP complexes were performed following the basic protocol of Dempsey and Christensen[20] on a Beckman Acta VI spectrophotometer.

Results

Figure 1 is a typical recorder output which illustrates the rapid decomposition of **1** caused by the addition of small quantities of BSA. It should be noted that the general shape of the curve is typical for what would be expected for decomposition of a substrate by an enzyme. It is also clear that the reaction goes to completion. A variety of samples of BSA in varying states of purity all give rise to the observed activity (Table II). It is likely that those samples of lower activity have a higher concentration of dimer, fatty acid, or extraneous protein. We note that purification of crystalline, Armour BSA significantly increases the activity of this species. The fact that the dimer is less active suggests that dimerization may block the "active site" in some of the molecules. Blocking the free sulfhydryl group of BSA has little effect on activity (Table II). This procedure was followed to generate a conformationally more stable species (see following paper).

There is a slight dependence of activity upon protein concentration (Figure 2). One possibility is that at low concentrations the protein is adsorbed to the walls of the cuvette. It is also possible that more than one protein molecule is needed to generate an "active site". This, however, is unlikely (see below). Another possibility is that very low concentrations of impurities within the substrate may act to poison the reaction by acting as inhibitors. All experiments were performed along the plateau level of the curve (Figure 2) to minimize effects due to changes in protein concentration.

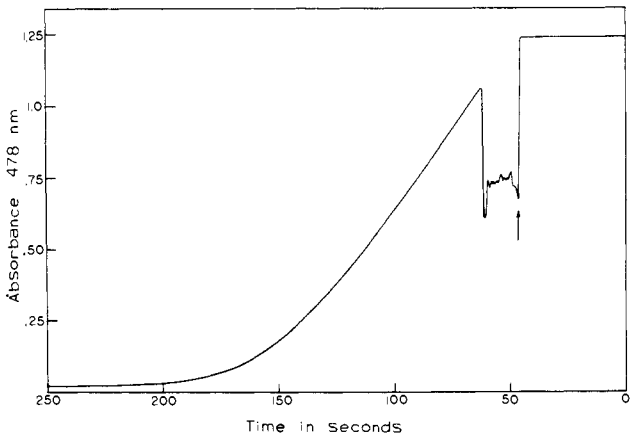


Figure 1. Recorder output illustrating the effect on the absorbance at 478 nm of adding 50 μl of BSA solution (4 mg/ml) to an aqueous solution of **1** (0.05 *M* phosphate, pH 8.0) in a 3-ml cuvette at 25°. The arrow indicates the time of addition of protein.

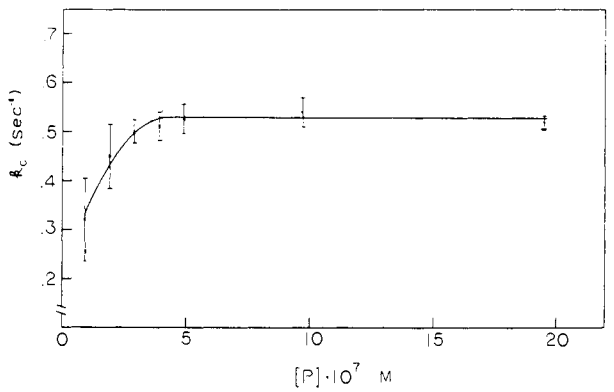


Figure 2. Effect of protein concentration on the catalytic activity of 1A-BSA. Experiments were performed under the conditions of the "standard assay", except the number of μl of BSA added was varied. The error bars represent the result of between four and seven independent experiments for each point.

Effects of Substrate Concentration. Besides exhibiting turnover, the reaction is characterized by saturation kinetics (Figure 3). The apparent K_m for the reaction is rather low (about 7 μM) and therefore it has been difficult to obtain highly precise data for analysis in terms of Michaelis-Menten kinetics. For this reason the data are plotted directly as apparent catalytic rate constant vs. substrate concentration to emphasize the apparent saturation behavior we obtain. With more conventional linear plots such as the Lineweaver-Burke type most of the data congregate close to zero values of the abscissa (that is, $1/S$ is very large) and little information is conveyed. Under the conditions of our experiments it was difficult to obtain highly precise data at lower substrate concentrations because lower protein concentrations would then also be required and we know that under these conditions k_c can change (see Figure 2). Similar data were obtained in Tris-HCl buffer, pH 8.0. At pH 9 and 10, the observed velocity is also approximately independent of substrate concentration for substrate concentrations of about 75 μM or higher. The data displayed in Figure 1 were also analyzed by using the integrated form of the Michaelis-Menten equation, following exactly the procedures described by Kumar and Hein.[22] In this way data from the whole course of the reaction are used to calculate K_m and k_c max. Using this analysis we obtained a K_m of 9 μM and a maximum k_c of 0.66 sec^{-1} which are in fair agreement with the values obtained by initial rate methods (7 μM and 0.57 sec^{-1}, for K_m and k_c max, respectively), considering

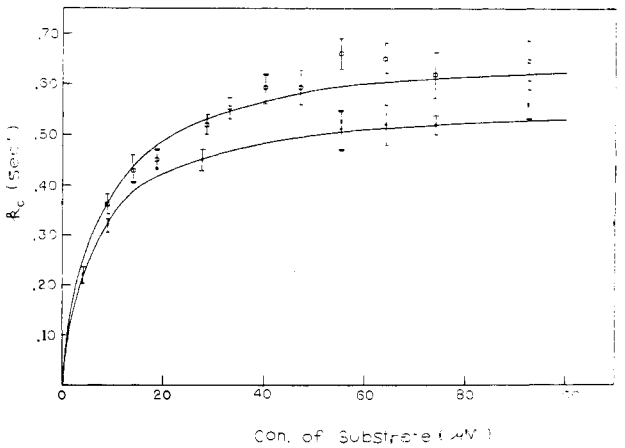


Figure 3. Effect of substrate concentration on the rate of decomposition of **1** by 10^{-6} *M* 1A-BSA in phosphate buffer, 25.0°. The lower set of points represents data obtained at 0.05 *M* buffer; the solid curve was calculated assuming the system obeys Michaelis-Menten kinetics with a K_m of 7 μM and maximum k_c of 0.57 sec^{-1}. The upper set of data was obtained in 0.02 *M* phosphate buffer and the solid line is based on a K_m of 7.5 μM and maximum k_c of 0.67 sec^{-1}. Within experimental error identical results were obtained using 4×10^{-7} *M* 1A-BSA. Similar curves were obtained in Tris-HCl buffer.

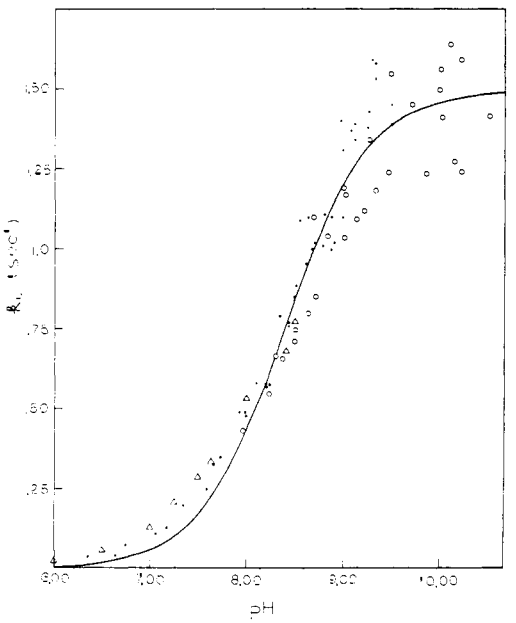


Figure 4. pH dependence for decomposition of **1** by 1A-BSA at 1 μM protein and 74 μM substrate, 25.0°. Key: (●) Tris-HCl; (△) phosphate; (○) NH_4Cl; all buffers are 0.05 *M*. The solid line represents the least-squares best fit to the data assuming a single group must be unprotonated for activity. The maximum value of the k_c was calculated to be 1.50 ± 0.01 sec^{-1}, and the pK_a of the group was 8.38 ± 0.02. Phosphate results were the average of between two and four independent determinations and were reproducible to about 5% or better. Error bars were omitted for clarity. Results for Tris-HCl and NH_4Cl were obtained over a period of 3 months and were not averaged in order to display the true experimental reproducibilities.

that our reproducibility in obtaining initial velocities from individual curves was ca. ±5%.

Effects of pH, Buffers, and Anions. The reaction is accelerated at higher pH (Figure 4). However, the base-catalyzed decomposition of **1** occurs at a significant rate at higher pH (see below) and therefore corrections were made to obtain the catalysis attributable to the protein itself. The results suggest a group of pK_a of ca. 8.4[23] (perhaps a lysine of abnormal pK_a, see below) must be unprotonated for activity. The fit of experimental data to the calculated curve is

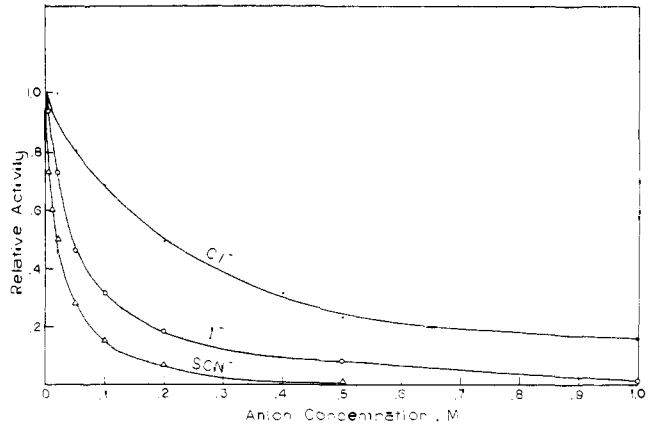


Figure 5. Inhibition of IA-BSA by anions. Experiments were performed under the conditions of the "standard assay". Relative activities were reproducible to 3% or better. Error bars were omitted for clarity. Substrate concentration was 74 μM, except for iodide, where concentrations of both 74 μM and approximately 18 μM were examined, and the results were superimposable at these two different substrate concentrations.

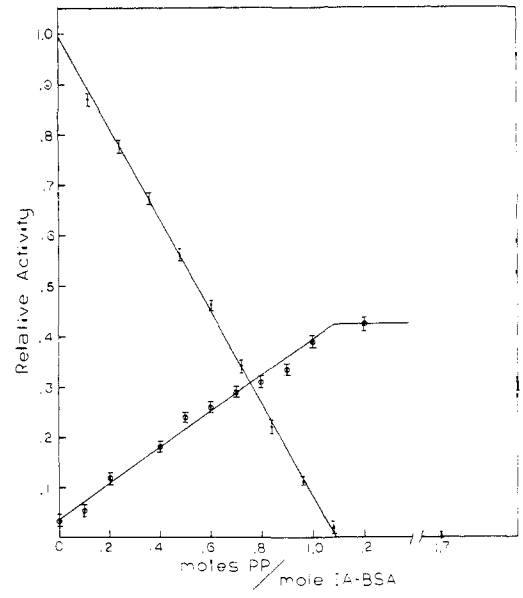


Figure 6. Descending lines: inactivation of IA-BSA by titration with pyridoxal 5-phosphate. Aliquots of IA-BSA (approximately 10 mg/ml) were incubated with various concentrations of PP in the dark at pH 8.0. After 24 hr 100-μl aliquots were assayed for activity. Results of a 1-hr incubation were the same within experimental error. However, activities were considerably higher if assays were performed immediately after incubation started. The time to reach 90% inhibition for the 1.1:1 sample was ca. 15 min. Incubations with other samples of the protein (Sigma defatted or Armour crystalline) gave similar results. Ascending line: results of reacting samples of IA-BSA (incubated with different molar ratios of PP) with FDNB. Both excess FDNB and PP were removed before activity was measured (see Experimental Section) in this part of the experiment.

less satisfactory at low pH. Microheterogeneity in the albumin sample could be a possible source of the deviations. In addition, as anions do cause inhibition (see below) it is possible that the inhibition by anions is pH dependent, and this could also distort the results. There is not a pH-independent component to the activity, however. As the pH is lowered k_c continues to decrease. For example, in 0.02 M acetate buffer, k_c at pH 5.5 is about $\frac{1}{9}$ its value at pH 6.5. The buffers do not actively engage in the reaction, as in fact use of lower buffer concentrations generally resulted in higher velocities (Figures 4 and 5), which is due to a decrease in inhibition of the anion of the buffer salt. In addition, similar results were obtained for different buffers (Figure 4). The binding of anions to BSA has been well documented[24] and at sufficiently high anion concentration there is significant inhibition of activity. The results for iodide indicate that inhibition is *independent* of substrate concentrations (Figure 5). This suggests the iodide inhibition must be *noncompetitive*. It is well known[24] that albumin has a variety of anion binding sites of differing affinities. It is clear that under the conditions of our experiment many of the moderate and even weak binding sites must be occupied. Whether the inhibition is caused by nonspecific effects of multiple anion binding (such as subtle conformational rearrangements) or the effect of the binding of an anion to a specific functional site cannot be determined at this time. However, if the results for iodide can be generalized, it is clear that the inhibition is *not* caused by the binding of anions to the *strongest* anion binding site on the protein. For example, the highest affinity iodide binding site on albumin has an association constant of about 10^4 M^{-1}.[24a] (As most anions still bind avidly to BSA at pH 8, we assume the results obtained at lower pH are valid at pH 8). If occupation of this site by iodide was sufficient to cause inhibition, then we would expect less than 1% activity at an iodide concentration of 10^{-2} M, where, in fact, only slight inhibition is observed.

Identification of an Active Site. Dempsey and Christensen[20] reported the specific binding of pyridoxal 5′-phosphate (PP) to BSA. They noted time-dependent changes in the absorption spectra of freshly prepared BSA-PP solutions in the region of 300–430 nm. They interpreted their experiments in terms of two high affinity sites on the molecule. One site has an association constant of about 10^5 M^{-1} at pH 7.5 and is favored kinetically. The other site is the stronger site and has an association constant of greater than 10^6 M^{-1} at pH 7.5, but it requires more time (somewhere between 2 and 24 hr) for final equilibration. Unfortunately, they did not report any experiments on the binding of PP to human serum albumin (HSA). We have repeated and confirmed the basic observations of Dempsey and Christensen[20] on both unpurified BSA and IA-BSA. However, we could not detect similar time-dependent changes in the absorption spectrum (in the region of 300–430 nm) of freshly prepared PP-HSA solutions, even for defatted HSA. In terms of the reported analysis for BSA,[20] our results for HSA suggest that site II (the *weaker* site which is favored kinetically) is occupied by PP, but we have no spectrophotometric evidence for the existence of the *stronger site* in HSA, which we believe is the catalytic site in BSA (see below).

We have been able to "titrate" BSA with PP (Figure 6). The results indicate that about 1 mol of PP binds to the strongest binding site on BSA and thus destroys activity. This provides very strong evidence for the existence of a unique site within the molecule which cannot be occupied by ligand (in this case PP) if catalysis is to occur. As it is believed that PP is coordinated to one or more lysines in BSA[20] this also suggests lysine is present at the catalytic active site. The inhibition caused by PP is reversible, however. Solutions of IA-BSA complexed with PP which had negligible activity recovered full catalytic activity when the PP was removed by prolonged dialysis at pH 4.0. Whether inhibition is reversible *after* the PP-IA-BSA complex comes in contact with substrate is under investigation. In addition, chemical modification of the amino groups of BSA with acetic anhydride (see above) destroyed all activity.

Green[25] has reported that FDNB labels two highly reactive amino groups (presumably lysine) in BSA approximately 500 times more rapidly ($t_{1/2}$ ~3 min) than the re-

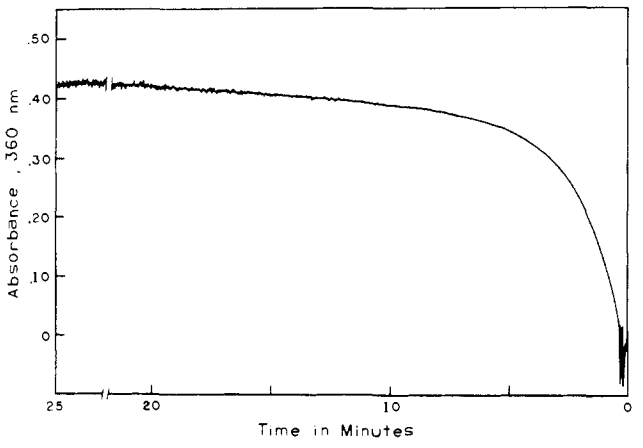


Figure 7. "Difference burst" titration of IA-BSA (see Experimental Section) with FDNB, 25.0°. The protein concentration in the sample and refence was 2.47×10^{-5} *M*. The reference cuvette also contained about 2.6×10^{-5} *M* PP. The theoretical limit for formation of 1 mol of dinitrophenyl lysine in the sample would be 0.42. When the reaction leveled off, about 4 equiv of dinitrophenylamino acid/mol of IA-BSA had been formed in the reference cuvette.

maining lysines at pH 8.3. He has suggested that the enhanced reactivity of these groups may indicate that they have abnormally low pK_a's and, in fact, are unprotonated at this pH. It is therefore possible that at least one of these groups is one of the lysines at the catalytic active site of the protein and the following experiments support this proposal.

Difference burst titrations (see Experimental Section and Figure 7) demonstrate that complexation of PP with IA-BSA results in the protection of up to one rapidly reacting lysine from FDNB. In these experiments we are examining the *difference* in reactivity toward FDNB of two solutions of IA-BSA. In one solution the IA-BSA is complexed with and partially protected from attack by FDNB by a known amount of PP. In the control solution, the reaction of FDNB with the protein is not inhibited and proceeds more rapidly. The fact that the extent of protection is stoichiometric (1:1) in moles of PP per mole of IA-BSA (Figure 8) localizes the action of PP to the protection of one specific residue.

This protective capacity is also manifested when one examines the *activities* of various samples of IA-BSA which were previously complexed with different ratios of PP before subsequent reaction with FDNB (Figure 6). In these studies, *after* the "difference burst titration" was completed the unreacted FDNB and PP were removed prior to the assay by prolonged dialysis at pH 4.0. Although the results do not point to an "all or none" effect, it is clear that PP helps to protect IA-BSA from activity losses which are due to reaction with FDNB. In addition, we have found that the *remaining* active site concentrations (as judged by the number of moles of PP per mole of protein necessary to reduce residual activity by a factor of 20) approximately correlated with the number of moles of PP which originally *protected* the active site of IA-BSA from modification by FDNB. For example, a sample "protected" with 0.5 mol of PP/mol of IA-BSA gave an "active site" titration of 50% after modification with FDNB followed by dialysis. Protection at a 1.2:1 ratio led to an "active site" titration of about 90%, although the level of activity was about 45%. These results indicate that, in fact, the catalytic site and the PP binding site must be coincident and this site must include one of the active lysines identified via FDNB modification. It is possible that the second unprotected residue is not lysine but, in fact, the N-terminal aspartate, which should be ionized and therefore highly reactive at pH 8.3. We also cannot exclude histidine as one of the other groups either.

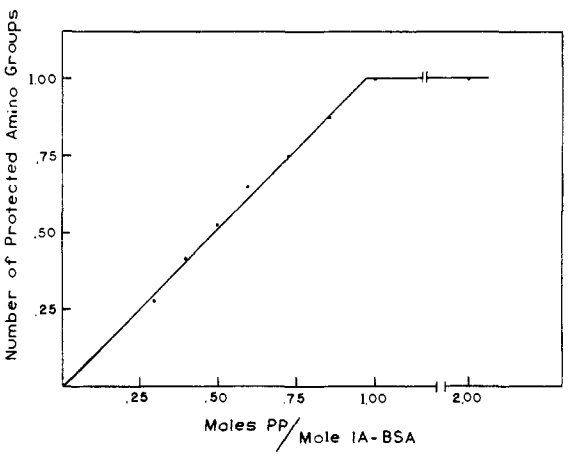


Figure 8. Results of difference burst titrations indicating the number of moles of amino groups protected/mol of IA-BSA for samples of IA-BSA incubated with various ratios of PP.

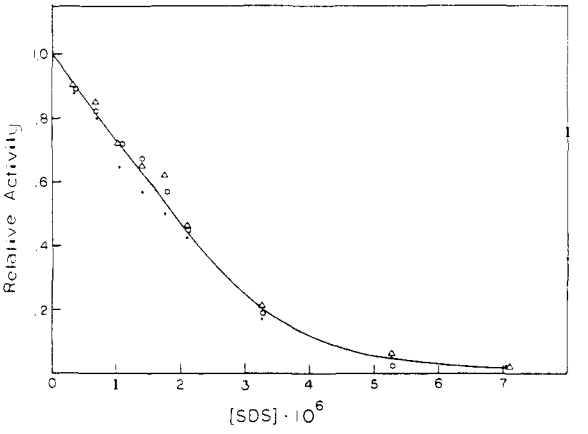


Figure 9. Inhibition of IA-BSA by sodium dodecyl sulfate, 25.0°. Key: (Δ) substrate = 74 μ*M*; (●) substrate = 28 μ*M*; (○) substrate = 9 μ*M*. IA-BSA concentration was 1 μ*M*. Similar results were obtained with palmitic acid as an inhibitor. 1×10^{-6} *M* palmitate caused approximately 33% inhibition, and 2×10^{-6} *M* palmitate gave approximately 65% inhibition. In these later experiments the palmitic acid was added as an ethanolic solution to the cuvette. Corrections for ethanol inhibition were small and were made. Within experimental error (±5%) the extent of inhibition was again independent of substrate concentration in the range of 74–9 μ*M*. Similar results were obtained if these inhibitors were first incubated with the protein for up to 24 hr and then the protein-amphiphile complexes were added to the cuvette.

Finally, we note that both the PP titration and the "difference burst titration" with FDNB provide independent means for measuring the concentration of active sites in BSA. These techniques can in principle be applied to other chemical modification reactions in order to differentiate nonspecific reduction of activity in BSA from those which specifically modify the active site of the protein.

Binding of Amphiphiles. It is generally believed that fatty acid binding is one of the physiologically important functions of albumin.[26] Recent evidence indicates that there may be one extremely strong fatty acid binding site and a number of secondary sites of moderate affinity.[27,28] An important question in this research is whether the strongest fatty acid binding site on the protein is coincident with the catalytic site. We find that both palmitic acid and sodium dodecyl sulfate inhibit the catalytic properties we have described (Figure 9). The inhibition is noncompetitive as it is independent of substrate concentration (Figure 9).

Our velocity-substrate concentration profiles do not contain sufficient information for analysis of the nature of the inhibition in the usual ways. However, we can show by cal-

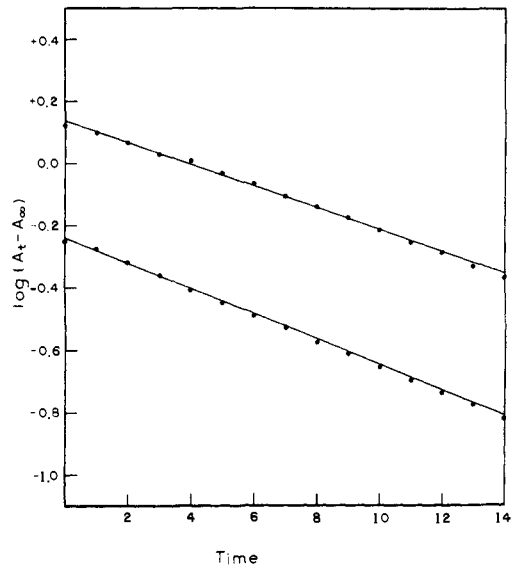


Figure 10. Typical first-order plots for decomposition of **1** in the *absence* of BSA, 25.0°. The upper curve was obtained in 0.05 *M* Tris-HCl, pH 8; each unit of time represents 20 min. The lower curve was obtained in 0.05 *M* NH_4Cl, pH 10.5; each unit of time represents 1 min.

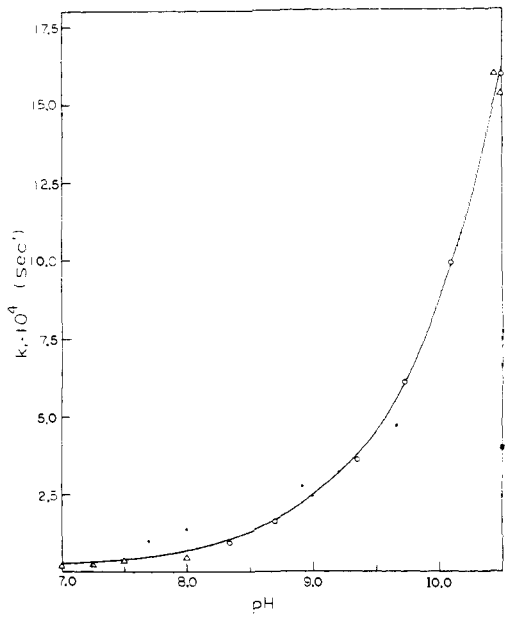


Figure 11. First-order rate constants for decomposition of **1** as a function of pH, 25.0°. All buffers are 0.05 *M*; the key is the same as in Figure 4. The darkened square represents the observed rate at pH 10.5 (0.05 *M* NH_4Cl) in the presence of 0.02 *M* trimethylhexadecylammonium bromide.

culation that amphiphile inhibition must be noncompetitive. At a substrate concentration of 74 and 1 μM IA-BSA, 1 μM SDS gives 28% inhibition. If the inhibition were competitive, this would correspond to a K_I of about 0.16 μM (taking K_m as 7 μM). Therefore, at a substrate concentration of 9 μM and identical concentrations of SDS and BSA, we would expect 54% inhibition. As we observe the same inhibition at both low and high substrate concentrations (and points in between) we are forced to conclude that the inhibition is noncompetitive. At higher ratios of amphiphile to substrate, multiple binding occurs.[24b,28] However, the results still argue in favor of a noncompetitive form of inhibition, in that the level of inhibition is independent of substrate concentration even at a 3:1 ratio of amphiphile to BSA. In addition, it should be noted that the inhibition is considerably weaker than that induced by PP (Figure 6). This result is not due to differences in incubation conditions for these amphiphiles vs. those used for PP (Figure 9). Based on the reported binding constants of fatty acids and SDS for BSA,[24b,27,28] under the conditions of these experiments all the fatty acid (and probably all the SDS) is bound. There is significant residual activity even at a ratio of amphiphile to protein of 2:1 and this indicates that the strongest fatty acid binding site cannot be coincident with the catalytic site.

Other Inhibitors. It has long been recognized[24,29-31] that albumin binds a wide variety of ligands of varying shapes and sizes. We have found that a number of these ligands have only slight to moderate effects on the activity of IA-BSA, even at concentrations at which they definitely should be bound to the protein. The ligands examined, concentrations used, and the observed levels of inhibition are as follows: *N*-acetyl-L-tryptophan, 2×10^{-4} *M*, no inhibition; tryptophan, 2×10^{-4} *M*, no inhibition; octanoic acid, 3×10^{-5} *M*, 13% inhibition; penicillin, 5×10^{-5} *M*, less than 10% inhibition. All of the inhibitors were examined under conditions of our "standard assay". We noted a slight increase (a factor of 1.3) in rates in the presence of 10^{-4} *M* copper in both Tris and phosphate. We believe the catalysis does *not* require metal ions, however, as the reaction in the presence of 10^{-3} EDTA was only slightly inhibited (about 10%) in both Tris and phosphate buffers.[32]

Model Studies. The decomposition of **1** in the absence of BSA follows first-order kinetics (Figure 10). The pH dependence for decomposition of **1** in the absence of BSA suggests that a specific base-catalyzed reaction is involved (Figure 11). We note in passing that Fendler and associates[33] have found that the decomposition of Meisenheimer complexes which have alkoxy moieties as leaving groups is *acid catalyzed*. Hydride anion is a very poor leaving group and this would explain why the decomposition of **1** must follow a significantly different mechanism (see Discussion). A comparison of the ratios of the velocities in Figure 4 and the first-order rate constants in Figure 11 indicates that the rate accelerations for decomposition of **1** by BSA average ca. 10^4 in the pH range 7-9.

The catalytic reaction requires more than a hydrophobic binding site. The absorption spectrum of **1** is very sensitive to solvent (Figure 12). The difference spectrum obtained when it is dissolved in a solution of trimethylhexadecylammonium bromide suggests that **1** is placed into a nonaqueous environment, presumably in the hydrophobic core of the cationic micelles of this compound (Figure 13). The base-catalyzed decomposition of **1** is *decreased* by a factor of about 4 when it is incorporated into the cationic micelle (Figure 11). Therefore, hydroxide anion must be at least partially excluded from those micelles which bind **1**. Alternatively, hydroxide anion may penetrate the micelles, but the activation free energy for attack of hydroxide on **1** is raised, because attack of the hydroxide anion on this substrate anion is unfavorable in the low dielectric medium of the micelle interior.

Bunton et al.[34] reported that the attack of hydroxide anion on 2,4- and 2,6-dinitrophenyl phosphate is not accelerated when these substrates are incorporated into cationic micelles of trimethylhexadecylammonium bromide. Their experiments indicated that incorporation of one anion into a micelle inhibits the approach of a second anion. Our results are consistent with these studies. Fendler[35] has shown that incorporation of a variety of alkoxy Meisenheimer complexes into cationic micelles has little effect on their activity coefficients. He concluded that the decomposition of these

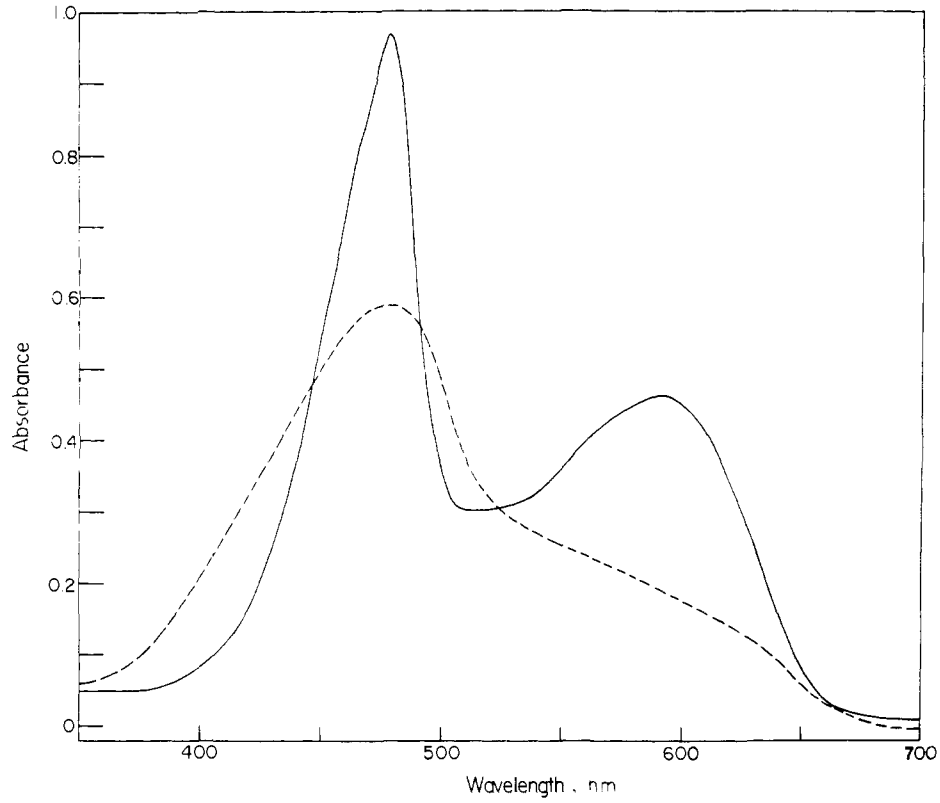


Figure 12. Visible absorption spectrum of **1** in acetonitrile (solid line) and water (0.05 *M* phosphate, pH 6.0) (dashes), 23°. Concentration is about 3×10^{-5} *M*.

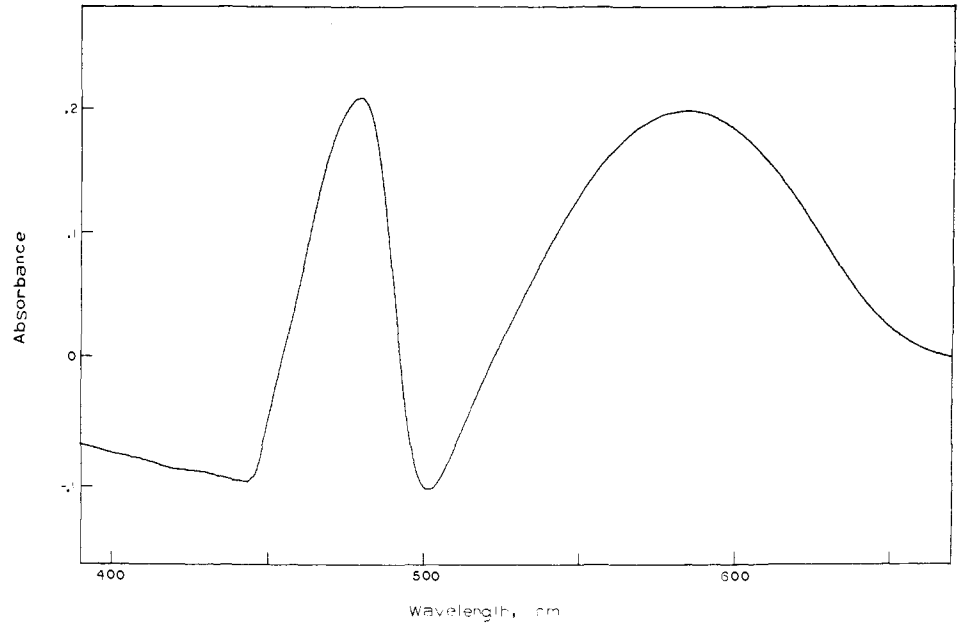


Figure 13. Difference spectrum for **1** illustrating its incorporation into cationic micelles, 23°. Reference cuvette: 0.05 *M* phosphate, pH 6.0, concentration of **1** is approximately 5.5×10^{-5} *M*. Sample cuvette: same as reference and in addition solution is 0.02 *M* in trimethylhexadecylammonium bromide.

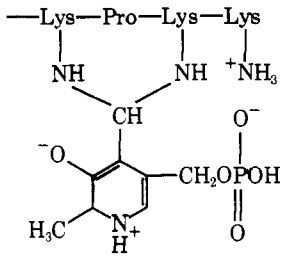
species was inhibited in cationic micelles due to a destabilization of the transition state for reaction. Considering the similarity of **1** to the species studied by Fendler, it is unlikely that its activity coefficient would be significantly affected in cationic micelles either. It is most likely that the transition state for the reactions we study (which we have noted must involve attack of hydroxide on **1**) is also destabilized in the micelle interior.

Our model studies with fairly concentrated (2–8 m*M*) solutions of either lysine or histidine indicated these molecules do not significantly (more than a factor of 2) accelerate decomposition of **1** at either pH 9 or 11, even in the presence of trimethylhexadecylammonium bromide. In addition, at pH 9 and 11, polylysine is also not capable of accelerating the decomposition of **1**. Finally, we also have found that micelles of sodium dodecyl sulfate have no appreciable effects on decomposition of **1**. It is therefore obvious that a hydrophobic binding site alone cannot cause catalysis.

Discussion

The BSA-catalyzed decomposition of **1** manifests those characteristics which are typical for biologically significant

enzyme-catalyzed reactions. Turnover, saturation, inhibition, and significant rate accelerations are observed. Our pH profile data indicate that a deprotonated base (probably lysine) is required at the catalytic site. This is further substantiated by active site studies with PP and FDNB. Anderson et al.[36] have determined the sequence of residues in the PP binding site of BSA and have suggested a possible structure for the PP-BSA complex, **2**. It is not hard to extrapo-



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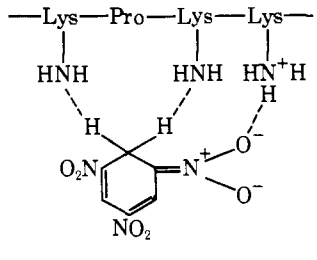

3

late from their work to arrive at a possible structure of the catalytic configuration of **1** and BSA, **3**. Presumably the rate-determining step in the reaction would be removal of a proton from substrate (presumably transferred to one of the unprotonated lysines) followed by protonation of oxygen (perhaps by the protonated lysine) on the trinitrobenzene dianion and loss of hydroxide to generate 3,5-dinitronitrosobenzene. While other pathways are possible and in fact we as yet lack the details for any firm discussion of mechanism, it seems likely that the crucial step in the decomposition of substrate is the initial removal of a proton from **1**,[15] either by hydroxide anion in aqueous solution or by either a basic residue (probably lysine) or hydroxide within the BSA active site. The state of protonation of the lysines in the catalytic configuration is clearly open to question. We have noted that the pH profile for activity indicates only *one* basic group needs to be unprotonated in order for catalysis to occur. The configuration *in* **3** would require *two* unprotonated lysines, and this would presumably be reflected in a sharper pH-activity profile than we observed. One possibility is that one of the lysines in **3** has a very low pK_a (ca. 7) and does not significantly affect the profile. This might also explain the apparent deviation at low pH of the theoretical pH profile for activity from the experimental data. Alternatively, it could be that the binding of ligands (or substrate) *shifts* the pK_a of one of the lysines to much lower values such that its contribution to the pH dependence of catalysis goes unnoticed. Halfman and Nishida[37] have shown the binding of amphiphiles to BSA does apparently decrease the pK_a's of some of its ϵ-amino groups. Alternatively, the true catalytic configuration could require that only one lysine be unprotonated in **3**.

It is possible that the unusual catalytic activity we have described is in fact related to a biological function, though we have no evidence on this point yet. One possibility is the pyridoxal phosphate binding site is physiologically important, and it coincidentally has the proper characteristics for catalyzing the decomposition of **1**. Perhaps there is some as yet unknown biological activity which can be attributed to the PP-BSA complexes.

In terms of enzyme catalysis in general it could be that the unusual array of amino acids within the PP binding site is particularly suited for proton-transfer reactions through general-base catalysis. If this is the case, then it is conceivable that other enzymes which either bind PP or catalyze reactions through general-base catalysis might have similar sequences at their active sites. It will be interesting to see if any of those enzymes[36] which have PP binding sites containing adjacent lysines also decompose **1**.

Surprisingly, while a variety of animal serum albumins manifests at least some activity, human serum albumin (HSA) has very low activity. As the general physical properties of these proteins are so similar, this result is quite unexpected. Whether this is due to some inhibitor present in the HSA (crystalline HSA, obtained from Schwarz/Mann, defatted and exhaustively dialyzed at pH 4.2 had less than 0.05% of the activity of BSA) remains to be seen. The answer may be that there are differences in amino acid sequence between HSA and BSA at the "active site." (Alternatively, there may be some nondialyzable inhibitor in HSA.) We have found (see above) that defatted HSA does *not* bind PP in a manner analogous to the time-dependent binding reaction reported for BSA and PP. This strongly supports our view that a unique active site of high specificity must exist in the BSA molecule.

One of the most unusual observations in this work has been the lack of *competitive* inhibition by any ligands which are known to bind to BSA.[38] Therefore inhibition must result from either steric blocking of catalytic sites or some form of "allosteric" regulation of the active site configuration. The catalytic site (which is presumably the pyridoxal phosphate binding site) apparently does not bind directly to amphiphiles or anions but shows high specificity for both substrate and pyridoxal phosphate. The binding of ligands to serum albumin has generally been characterized as a *nonspecific* binding phenomenon. The results of this study in fact suggest there must be a number of *independent* specific sites which have varying degrees of interaction. For example, the binding of tryptophan has no effect on activity. We suggest the tryptophan binding site is in a different globular region of the protein from the catalytic site.

The binding of long-chain fatty acids causes partial inhibition through steric blocking of the active site (or possibly through an allosteric effect). Inhibition by shorter chain fatty acids is significantly less efficient. If they do bind at the same sites as the long-chain fatty acids, then it is conceivable that they are not long enough to protrude into the active site and cause steric inhibition. Alternatively, there could be specific sites which require long-chain fatty acids but not short ones. A systematic investigation of the effects of low concentrations of fatty acids of different lengths on binding of pyridoxal phosphate to albumin should provide one means of clarifying these questions. A second independent approach would be to determine if shorter chain fatty acids can effectively *compete* for the strongest fatty acid binding sites in BSA. Further tests of the specificity and *independence* of the ligand binding sites of BSA could include an examination of the ligand-binding properties of the protein after reaction with moderate amounts of FDNB.

In summary, we have examined a catalytic property of BSA which meets the requirements generally specified for "true" enzyme catalysis. In the following paper we will show that this catalytic activity is in fact intimately related to the comformational characteristics of the protein. While we do not know as yet if this activity has a physiological analog, it is clear that it serves as a unique probe of the physical chemistry of this protein.

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Bovine Serum Albumin as a Catalyst. III. Conformational Studies

Ronald P. Taylor,* Sarah Berga, Vincent Chau, and Claudia Bryner

Contribution from the Department of Biochemistry, University of Virginia School of Medicine, Charlottesville, Virginia 22901. *Received September 23, 1974*

Abstract: We have used the recently discovered catalytic activity of bovine serum albumin (BSA) as a sensitive probe of the conformational properties of this protein in solution. When the protein is unfolded in 8 *M* urea and the disulfide bonds are broken with mercaptoethanol, as much as 73% of full activity can be regained if the protein is diluted and allowed to slowly air reoxidize and the monomer is isolated. Our evidence indicates that irreversible denaturation of the protein in 8 *M* urea alone is caused by an intermolecular reaction which must involve a disulfide exchange reaction which is facilitated by the free unprotonated sulfhydryl group of the protein. Irreversible thermal denaturation also is strongly aided by the free sulfhydryl group. The results of these and other experiments are in general agreement with recent theories about domain structure in proteins. The data suggest that certain domains in BSA have greater conformational stability than other domains. Our evidence indicates that the catalytic active site of the molecule is part of one domain of relatively high conformational stability.

The plasma albumins have occupied center stage in a number of protein chemistry laboratories throughout the world.[1-6] Plasma albumin is inexpensive and can be obtained in a relatively pure form. It binds a variety of ligands[7] and can be found in a number of different conformational states[1] depending upon such conditions as solvent, temperature, pH, ionic strength, etc. For these reasons there have been a number of studies aimed at characterizing the physical properties of this protein in an effort to obtain information on the physical chemistry of proteins in general.

These physical studies have generated substantial information on the factors which influence the structure of albumin in solution. Unfortunately, many of them have suffered from a lack of an operational definition of the true "native state". In this paper we show that the catalytic activity we have described for bovine serum albumin (BSA)[8] serves as a uniquely sensitive probe of the conformational integrity of the molecule. In fact, our results suggest that this activity is indicative of the degree of "native" conformation of the protein in the vicinity of its active site.

Experimental Section

Bovine Serum Albumin. Three types of protein preparations were used in our studies: IA–BSA (see preceding paper);[8] Sigma Type F defatted BSA (Lot No. 63C 7290), S–BSA; and Armour crystalline BSA (Lot No. L72410 and L72510), which was defatted by the method of Chen[9] as modified by Foster,[10] AR–BSA. In this paper, the initials BSA signify the protein in general rather than one of the specific preparations.

Reagents and Assays. Urea was of ultrapure grade and was obtained from Schwarz/Mann. Mercaptoethanol (ME) was from Pierce Chemical Co. Ammonium sulfate used in the thermal denaturation experiments was ultrapure grade from Schwarz/Mann. Other reagents, activity assays, active site titrations, and pH measurements and data analyses were described in the preceding paper.[8]

Reduction and Reoxidation Experiments. Andersson's[5] procedures for the reduction of BSA in 8 *M* urea and 0.3 *M* ME followed by dilution and air reoxidation were followed. Incubation with ME was extended over a 3-hr period to ensure reduction of all disulfide bonds. After the specified time for air reoxidation, the sample was concentrated and dialyzed in the cold against 0.05 *M* KCl for up to 5 days. If the sample had been reoxidized in the