Canaani, D., Revel, M., & Groner, Y. (1976) *FEBS Lett. 64*, 326.
Chu, L.-Y., & Rhoads, R. E. (1978) *Biochemistry 17*, 2450.
Chu, L.-Y., Lockard, R. E., RajBhandary, U. L., & Rhoads, R. E. (1978) *J. Biol. Chem. 253*, 5228.
Desrosiers, R., Friderici, K., & Rottman, F. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 3971.
Hickey, E. D., Weber, L. A., & Baglioni, C. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 19.
Hunt, R. T., Hunter, A. R., & Munro, A. J. (1968) *Nature (London) 220*, 481.
Kemper, B., & Stolarsky, L. (1977) *Biochemistry 16*, 5676.
Lodish, H. F. (1974) *Nature (London) 251*, 385.
Lodish, H. F. (1976) *Annu. Rev. Biochem. 45*, 39.
Lodish, H. F., & Jacobsen, M. (1972) *J. Biol. Chem. 247*, 3622.
Lodish, H. F., & Rose, J. K. (1977) *J. Biol. Chem. 252*, 1181.
Malek, L. T., Breter, H.-J., Hellmann, G. M., Friderici, K. H., Rottman, F. M., & Rhoads, R. E. (1979) *J. Biol. Chem. 254*, 10415.
MacDonald, C. T., Gibbs, J. H., & Pipkin, A. C. (1968) *Biopolymers 6*, 1.
Palmiter, R. D. (1973) *J. Biol. Chem. 248*, 2095.
Pelham, H. R. B., & Jackson, R. J. (1976) *Eur. J. Biochem. 67*, 247.
Rhoads, R. E. (1975) *J. Biol. Chem. 250*, 8088.
Rhoads, R. E., McKnight, G. S., & Schimke, R. T. (1971) *J. Biol. Chem. 246*, 7407.
Rhoads, R. E., McKnight, G. S., & Schimke, R. T. (1973) *J. Biol. Chem. 248*, 2031.
Roman, R., Brooker, J. D., Seal, S. N., & Marcus, A. (1976) *Nature (London) 260*, 359.
Schreier, M. H., & Staehelin, T. (1973) *J. Mol. Biol. 73*, 329.
Shafritz, D. A., Weinstein, J. A., Safer, B., Merrick, W. C., Weber, L. A., Hickey, E. D., & Baglioni, C. (1976) *Nature (London) 261*, 291.
Sharma, O. K., Hruby, D. E., & Beezley, D. N. (1976) *Biochem. Biophys. Res. Commun. 72*, 1392.
Sonenberg, N., Morgan, M. A., Merrick, W. C., & Shatkin, A. J. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 4843.
Suzuki, H. (1976) *FEBS Lett. 72*, 309.
Suzuki, H. (1978) *J. Biochem. (Tokyo) 84*, 1125.
Weber, L. A., Hickey, E. D., Nuss, D. L., & Baglioni, C. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 3254.
Weber, L. A., Hickey, E. D., & Baglioni, C. (1978) *J. Biol. Chem. 253*, 178.
Willems, M., Wieringa, B., Mulder, J., Ab, G., & Gruber, M. (1979) *Eur. J. Biochem. 93*, 469.
Wu, J. M., Cheung, C. P., & Suhadolnik, R. J. (1977) *Biochem. Biophys. Res. Commun. 78*, 1079.

Magnetic Osmometry: Association of Two Peptic Fragments from Bovine Serum Albumin at Micromolar Concentrations†

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ABSTRACT: The magnetic osmometer was employed to study at micromolar concentrations the known association of the two nearly equal sized fragments of bovine serum albumin (BSA) formed by peptic cleavage between amino acid residues 306 and 307. An association constant of $\sim 1.8\ \mu M^{-1}$ was determined at pH 8.6 (0.1 M KCl, 20 °C); similar association constants were obtained by equilibrium sedimentation and from the catalytic decomposition of a Meisenheimer complex (MC) by mixtures of the fragments where the substrate, MC, served as a reporter group (intact BSA exhibits a similar activity). A volume change for the association (~−80 mL/mol) was determined by density experiments, suggesting that nonpolar rather than ionic interactions predominate in the formation of the association complex. This was supported by titration experiments which showed that the net charge on the parent BSA was conserved in the isolated peptic fragments and in the association complex. Intrinsic viscosity values, however, indicated that the complex had a substantially larger hydrodynamic volume than the parent BSA (viz., 5.6 vs. 4.2 mL/g); the C-terminal peptic fragment from BSA exhibited a similarly large value, whereas that for the N-terminal fragment was more BSA-like. Dissociation of the complex was observed in high centrifugal fields and also by osmotic pressure and electrophoresis when the fragments had been exposed to decane; the N-terminal peptic fragment when so exposed exhibited a changed behavior in electrophoretic and centrifugal fields and it was catalytically incompetent, whereas these tests with the other fragment and with BSA, after similar exposure, revealed little or no change. These results and those reported by others illustrate that this BSA system provides a useful model for comparing struction–function relationships in the quasi-independent domains of two compositionally alike macromolecular entities (BSA and the complex).

The osmotic pressure is a direct measure of the change in chemical potential when adding macromolecules to a system. The osmotic method, however, has not been generally useful at macromolecule concentrations of much less than 10^{-4} M, thus excluding many of the association reactions of interest to protein chemists. The magnetic osmometer introduced recently by Beams et al. (1973) can be used in the micromolar range of concentrations with useful precision. A modified version of this osmometer was employed here to elucidate the putative association of the two nearly equal sized fragments of bovine serum albumin (BSA)[1] as obtained by limited pepsin

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[1] Abbreviations used: BSA, bovine serum albumin; MC, 1,1-dihydro-2,4,6-trinitrocyclohexadienate (a Meisenheimer complex); BTP, 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (also called Bistris-propane).

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digestion (King, 1973). This relatively new protein system is interesting because the binding of certain ligands to BSA [cf. Reed et al. (1976)] and an enzyme-like activity of BSA (Taylor & Silver, 1976) are substantially restored upon mixing the isolated fragments, which alone have either a much reduced or negligible activity. [Certain other binding functions of BSA, however, are not disturbed appreciably by this cleavage, indicating that BSA is comprised of several, somewhat independent, structural domains (Reed et al., 1975).] The above studies on BSA have suggested that a 1:1 molar complex of these two fragments, BSA-P-(1–306) and BSA-P-(307–581),[2] forms by inducing some of the structural integrity of the parent BSA into domains which have been disturbed by the peptic cleavage. Stable associations of the two kinds of fragments in a 1:1 molar ratio (called BSA-P′)[2] have been indicated by cellulose acetate electrophoresis, and approximate values for the association constants (e.g., $\sim 16\ \mu M^{-1}$ at pH 8.6) have been calculated (Reed et al., 1976). Clearly, an equilibrium method is desirable for determining the association behavior unequivocally. When the magnetic osmometer was employed, a stronger association than that indicated by the foregoing transport method was observed; thus, measurements in the micromolar range were required. Ancillary to the osmotic study on the association of the BSA fragments, we report results also on further characterizations of the system. These include density increments, the volume change for the association, pH–titration profiles, specific absorbancies, and sedimentation, viscosity, and electrophoresis experiments.

For a functional integrity indicator, we have utilized the enzyme-like activity of BSA discovered by Taylor & Vatz (1973), who noted that BSA decomposes the Meisenheimer complex, 1,1-dihydro-2,4,6-trinitrocyclohexadienate (MC), at neutral pH $\sim 10^4$ times faster than that by conventional alkaline catalysis. [For a review of this activity of BSA, which activity satisfies the usual criteria for that of an enzyme, cf. Taylor (1977).] This activity is of particular interest because the active site has been associated with the lysine residue (Taylor, 1976) in position 220 of the sequence of BSA proposed by Brown (1975). The fragment, BSA-P-(1–306), containing this residue, however, possesses little or no activity unless the other fragment, BSA-P-(307–581), is present. This observation suggests that the latter fragment acts as if it is a kind of allosteric effector or contains a region which cooperates as part of the active site when the fragments interact. This BSA system, therefore, is a model for comparing structure–function relationships between two compositionally alike polypeptide structures (BSA and BSA-P′) in which a single peptide bond is missing in one of them.

Experimental Section

Materials and Preparations. The procedures employed for the peptic cleavage of BSA and for the isolation of the BSA fragments, BSA-P-(1–306) and BSA-P(307–581), were as outlined by Feldhoff & Peters (1975). BSA was obtained from Reheis Chemical Co. (Stock No. 2266101); octanoic acid, Tris, and 1,3-bis[[tris(hydroxymethyl)methyl]amino]propane (BTP) were from Sigma Chemical Co.; acetonitrile, HCl, and KCl were the most pure grades of Fisher Scientific Co. The free sulfhydryl group of BSA was blocked with half-cystine [cf. Peters et al. (1973)]. Specifically, 21.7 mg of cystine (Sigma Chemical Co.) per g of BSA (2% in 0.05 M sodium phosphate, pH 8.0) was stirred for 5 h at room temperature; the solution was then concentrated to 5% BSA by pressure filtration. The SH-blocked monomer of BSA was isolated by passing 20–40 mL of the concentrated solution through a Sephadex G-150 column (4 × 100 cm) equilibrated with 0.1 M Tris-HCl–0.1 M NaCl, pH 8.0. The monomer fraction was then pooled, concentrated, and defatted by the method of Chen (1967). The peptic fragments, BSA-P-(1–306) and BSA-P-(307–581), were prepared from the SH-blocked monomer in the presence of octanoic acid (Feldhoff & Peters, 1975). All protein solutions were exhaustively dialyzed against deionized water at 4 °C and were then deionized by passage through columns of Amberlite MB-1 (Mallinkrodt Chemical Co.). The final products were concentrated, filtered through 0.2-μm Nucleopore filters, and stored at −15 °C until used.

All studies on the isolated SH-blocked BSA and the isolated fragments therefrom were related to masses determined by dry-weight analysis after deionizing, dialyzing against deionized water, concentrating, and passing through 0.2-μm Nucleopore filters. The operational definition used for the assignment of a dry mass was as described by Kupke & Dorrier (1978), utilizing a procedure similar to one developed by Hunter (1966). The unsmoothed, extreme deviations in weight fraction among samples in quadruplicate on each of the protein materials in water ranged from 1 part in 350 to <1 part in 10^3. Extinction coefficients in the ultraviolet region based on these dry weights were compared in two spectrophotometers, a Cary Model 14 and a Beckman Acta M-VI. The wavelength of maximum absorbance at 20–21 °C for each material (SH-blocked BSA and the two fragments) was the same in both instruments, although the optical densities per unit of dry mass were consistently about 3 parts per 10^3 higher in the latter instrument. The coefficients in terms of milliliters per milligram dry material (1-cm path) in the solvent 0.1 M KCl–0.01 M BTP, pH 8.6, by the Cary instrument were 0.6868 (277.7 nm) for SH-blocked BSA, 0.7940 (278.0 nm) for BSA-P-(1–306), and 0.5516 (277.5 nm) for BSA-P-(307–581).

Catalytic Activity Method. The tetramethylammonium salt of the Meisenheimer complex (MC), which was used as the substrate in the assays for the catalytic activity of BSA, was freshly prepared by Dr. R. P. Taylor according to the method described by Taylor et al. (1975a). This substrate was stored in vacuo at −15 °C in the dark until used. Acetonitrile solutions of the MC were used in the assay procedure with BSA or its fragments under the conditions outlined by Taylor and co-workers; our assay results were in good agreement with those reported by them. For our assays, ~ 4–$7\ \mu$L of a solution of the tetramethylammonium salt of the MC (~ 4.5 mg/mL) in acetonitrile was added to a final volume of 1 mL containing the appropriate protein material (SH-blocked BSA, the isolated fragments, and mixtures of the latter) in an aqueous solvent consisting of 0.1 M KCl–0.01 M BTP buffer, pH 8.6. The pH chosen is approximately at the pK of an abnormal lysine residue (at position 220 in the BSA sequence) which is believed to be functional in the active site when unprotonated (Taylor, 1976); base decomposition of the substrate becomes significant at higher pH. The change in absorbance relative to that of the control solution was monitored at 478 nm in a

[2] These two peptic fragments of BSA, known formerly as "A" and "B" with various prefixes, have been redesignated in Reed et al. (1976) by an adaptation of the rules of the Commission on Biochemical Nomenclature (IUPAC–IUB Commission on Biochemical Nomenclature, 1967). The fragment formerly designated by the letter "B" was assigned to BSA-P-(1–306), which denotes the source, BSA, of the peptide arising from digestion with pepsin, P; the numerals refer to the amino- and carboxy-terminal amino acid residues, respectively, of the peptide in terms of the sequence numbers established for the parent protein. Thus, the peptide formerly referred to with an "A" was assigned as BSA-P-(307–581). The complex formed from two isolated fragments was designated BSA-P′. We follow the nomenclature proposed by Reed et al. (1976) in this paper.

Cary Model 14 spectrophotometer (20–21 °C).

The assays were used also for estimating an association constant upon mixing the two isolated fragments. It was found that by saturating one fragment, at any given concentration, with the other fragment (50- to 100-fold excess), the rate of MC decomposition was directly proportional to the putative concentration of BSA-P′, the association complex formed by one molecule of each fragment; i.e., the activity was proportional to the concentration of the fragment being saturated. Hence, when the concentration of one fragment at a sufficiently low, though fixed, concentration of the other fragment was varied, an association constant, independent of the osmotic method, could be calculated. In these experiments it was not feasible to completely saturate the protein with the MC substrate. The same association constant was obtained, however, at MC concentrations which were only 10% of the normal assay conditions. Thus, the association appeared to be independent of the presence of MC within the compositional range and temperature of the present studies.

Time-course studies were also carried out concurrently with different concentrations of the BSA in parallel to those for the putative amounts of BSA-P′. The data from both sets fit the expression

$$-\frac{d[MC]}{[E]dt} = \frac{k_{cat}[MC]}{K_m + [MC]} \quad (1)$$

where E refers to BSA or BSA-P′, k_{cat} is the maximum velocity for the decomposition of MC per unit of E, and K_m is the Michaelis–Menten constant. The values of the derivatives, $d[MC]/dt$, when fitted into eq 1 exhibited little or no indication of product inhibition arising from the MC decomposition in the presence of either BSA or BSA-P′.

Osmometry. The magnetic osmometer of Beams et al. (1973) was modified to be somewhat more convenient and so that the magnetically controlled Cartesian diver containing an air space could be placed on the solvent side of the membrane. The latter change was made to eliminate possible buoyancy effects on the diver from surface tension differences when varying the protein concentration. [Details for this particular model are available (Crouch, 1977).] Otherwise, the principles and method of measurement were as described by Kupke & Crouch (1978). Membranes cut from size 18 cellulose casing (Union Carbide Corp.) were used after being boiled in deionized water for 5 min and then equilibrated 12 h with solvent.

Since this osmotic pressure technique is relatively new, a few additional remarks are included. The measurement of interest is the amount of electric current delivered to a solenoid system when the diver is stably suspended within the solution at a preset height. The diver (~6 × 11 mm hollow cylinder with a sealed top to contain an air space of ~60 μL) consists of an annealed (hysteresis free) thin-walled ferromagnetic open cylinder (Molypermalloy, Allegheny-Ludlum Steel Co., or HyMu-80, Carpenter Steel Co.) suitably jacketed inside and outside with fitted caps of Kel-F or polypropylene. As the pressure on the liquid changes, the volume of the gas phase in the diver adjusts in accordance with Boyle's law, producing a change in the density of the diver. At osmotic equilibrium, the current which is required to maintain the diver at the preset height is related to the osmotic pressure by calibrations. The calibration consists of forcing amounts of solvent-equilibrated air, via a gas-tight syringe, into a constant-temperature air tank of known volume (~4 L) which is connected to the osmometer; this tank and a larger one serve as a constant atmosphere during the experiment because atmospheric pressure fluctuations are too severe. The volume of added air as a function of current to the supporting solenoid was linear to ~10 cm of H_2O. Since many of the osmotic pressures in this study were <1 cm of H_2O, a calibration was made immediately after each measurement (~1 min) so that the temperature changed less than 0.0003 °C (Tronac controller, Model CTB-405, Tronac Inc., Orem, UT). [A pressure change of 10^{-3} cm of H_2O (~10^{-6} atm) changes the density of our divers (~2–3) × 10^{-7} g/mL. Since a change of 0.001 °C affects the density of aqueous solutions ~2 × 10^{-7} g/mL (at 20 °C), a temperature change of <0.001 °C is desired for the interval between measurement of the current and the calibration so that sensitivities on the order of 10^{-3} cm of H_2O can be achieved (air density changes are about 2 orders of magnitude less than that for liquid water per unit of temperature change).] Since nondiffusible solutes at a concentration of 1 μM produce an osmotic pressure of ~0.025 cm of H_2O, the magnetic method was found suitable for studying this associating system. Five to thirty minutes were required for equilibrium to be achieved, but usually the experiment was allowed more time. Several measurements with calibrations were often made on the same sample after achieving equilibrium each time; the precisions noted in this exercise were better than 2 × 10^{-3} cm of H_2O.

The experiments with the magnetic osmometer were carried out at 20 °C and 1 atm of pressure in a solvent containing 0.1 M KCl–0.01 M BTP buffer, pH 8.6. This pH was chosen to correspond with the most favorable conditions for assaying the catalytic activity and also because at this pH the association of the fragments appears to be over 80% complete by zone electrophoresis (Reed et al., 1976).

With the present magnetic instrument, it was not convenient to measure pressure differences of more than 10 cm of H_2O. In an attempt to evaluate the effects of nonideal behavior on the association at higher protein concentrations (>10^{-4} M), a capillary-type, metal-free osmometer was employed (Kupke, 1961). During the course of these measurements, we observed that the osmotic pressures of solutions containing a mixture of the two fragments (0.16–0.5 mM each) were higher than expected, being nearly that calculated for the unassociated species. These results, while reproducible, were traced to an effect on the BSA-P-(1–306) fragment by the decane used as a capillary liquid overlaying the protein solution. Concurrent zone electrophoresis experiments (cellulose acetate–mylar strips, Millipore Biomedica) demonstrated that this peptide after deliberate exposure to decane (>1 day) or after an osmotic experiment migrated identically with BSA and/or BSA-P′ at pH 8.6. Thus, mixtures of the two fragments when exposed to decane presented a pronounced but spurious BSA-P′ band and an undiminished BSA-P-(307–581) band with no apparent BSA-P-(1–306) material between them. Without exposure to decane, such mixtures showed trace amounts of both fragments trailing the major BSA-P′ band, quite similar to the pictures published by Reed et al. (1976). [Normally, the BSA-P-(1–306) band migrates to the anode head of the BSA-P-(307–581) band and behind the BSA and/or BSA-P′ band at this pH.] Almost no MC decomposition activity was observed for mixtures by the assay procedure (as above) when BSA-P-(1–306) had been in contact with decane; the other fragment appeared to be unaffected in control studies. Intact BSA exposed to decane was also unaffected electrophoretically, and it did exhibit the enzyme-like activity approaching that of the control. Presumably, the BSA-P-(1–306) peptide interacts with the hydrocarbon to change the net charge and peptide-binding properties. The sedimentation rate by velocity ultracentrifugation of this decane-exposed fragment was about

10% lower than that of the unexposed fragment at comparable concentrations. Except for noting this interesting interaction with decane, further osmotic experiments at higher protein concentrations, while necessary, were postponed pending appropriate modifications to the magnetic osmometer.

The osmotic pressure data on the individual fragments and on the SH-blocked BSA (nonassociating systems) were treated by fitting them to the relationship

$$\Pi/(RTC) = 1/\mathrm{MW_n} + \beta C \qquad (2)$$

where Π is in centimeters of water, RT = 24870 L·cm of H_2O·mol^{-1} at 20 °C, C is the protein concentration in grams per liter, $\mathrm{MW_n}$ is the number-average molecular weight, and β is the coefficient reflecting nonideal behavior. The latter parameter was observed to be constant with concentration over the range studied for these nonassociating systems in terms of present experimental error. The data from the fragment mixtures were analyzed by assuming that the nonideal term in eq 2 was both constant and equivalent to that observed for BSA over the region of study. These assumptions were based upon the apparent values of β for the individual fragments and for the complex (BSA-P′). That is, the median of the values for the fragments was not grossly dissimilar from that for BSA, and the β value for BSA-P′, estimated from the concentration region where association was maximal, was similar to the β for BSA; in addition, the net charge was conserved upon peptic scission of the BSA and upon association of the fragments (see Results). For this association case may may write

$$M = \Pi/(RT) - \beta C^2 \qquad (3)$$

where $M = M_A + M_B + M_{AB}$, the molarities of the two fragments, A and B, and of the complex, AB, at osmotic equilibrium. The molarities of each species in the reaction A + B $\rightleftarrows$ AB are obtained in terms of the known molar quantities of the two fragments which were added independently to the system. The total molarity, M_T, of the added components, therefore, is

$$M_T = M_{AT} + M_{BT} \qquad (4)$$

where AT and BT refer to the total added amounts of A and B, respectively. By definition, $M_A = M_{AT} - M_{AB}$, and $M_B = M_{BT} - M_{AB}$; hence, $M = M_T - M_{AB}$. Accordingly, the value of K_a for the system A + B $\rightleftarrows$ AB, becomes (eq 3 and 4)

$$K_a = [M_T - \Pi/(RT) + \beta C^2]/\{[M_{AT} + \Pi/(RT) - \beta C^2 - M_T][M_{BT} + \Pi/(RT) - \beta C^2 - M_T]\} \qquad (5)$$

which is similar to a treatment by Adams et al. (1978).

Other Methods. The volume change, ΔV, for the association of the two isolated fragments from BSA was determined by a density method (Kupke & Beams, 1972) using the magnetic densimeter of Senter (1969) as modified by Kupke & Crouch (1978). Densities were determined at 20 °C in triplicate (0.25 mL per measurement) on solutions of each isolated fragment ($\sim 5 \times 10^{-4}$ M) in the identical solvent (0.1 M KCl–0.01 M BTP buffer, pH 8.6). About 0.75 mL of each solution of known density was added sequentially on the analytical balance, and each addition was weighed to ±5 μg, with application of a protocol to minimize evaporation (Kupke & Crouch, 1978). The density of this mixture was then determined from measurements on triplicate samples. The procedure was repeated with another set of solutions of the two peptides. The precision of the measurements for the density as determined on replicate samples of a given solution was 2×10^{-6} g/mL. Accordingly, the method is capable of yielding values for ΔV_{mix} on volumes of ~1 mL of each of the premixed solutions to a precision of ±5 nL, since a microgram is virtually equivalent to a nanoliter of aqueous solution. Because the solvent medium was identical in each solution of peptide prior to mixing, the ΔV of mixing gives immediately the volume change for the peptide–peptide interaction (Crouch & Kupke, 1977). Thus, if two sets of molecules, denoted as A and B, associate completely upon mixing in a common solvent medium, the volume change for the association is given by

$$\Delta V = m_{(AB)}/\rho_{(AB)} - [m_{(A)}/\rho_{(A)} + m_{(B)}/\rho_{(B)}] \qquad (6)$$

where m is grams of a solution, ρ is the density, (A) and (B) refer to the two initial solutions containing molecules A and B, respectively, and (AB) refers to the solution after mixing (A) and (B).

pH titration experiments were carried out on the SH-blocked BSA, on the BSA fragments, and on 1:1 molar mixtures of the fragments in order to estimate the Donnan effect and to determine whether a change in net charge accompanies the peptic cleavage of BSA and the complexing of these fragments when mixed. The experiments were performed with a Radiometer TTT-1C automatic titrator and chart recorder at 21 °C. The titrations were made on the isoionic peptides in the presence of KCl and by using HCl and KOH containing KCl so that the ionic strength remained at 0.1. The acid and base solutions were standardized against Tris base and potassium acid phthalate, respectively, and then against each other as a cross-check. The initial concentrations of isolated fragments were 10^{-4} M, while those of the BSA and the 1:1 molar mixture of the fragments were 0.5×10^{-4} M. The peptides were first titrated with base, then with acid, and again with base between pH 3 and 11; no significant hysteresis was observed.

Variation of the density with concentration, c (in grams per milliliter), under isopotential conditions was determined at 20 °C as described by Kupke & Beams (1972) on SH-blocked BSA, on the BSA fragments, and on 1:1 molar mixtures of the fragments as equilibrated in the KCl–BTP solvent, pH 8.6. Triplicate determinations via the magnetic densimeter were made on each sample at a given concentration of each peptide; six to eight concentrations sufficed to determine the slope, $(\partial\rho/\partial c)_\mu^0$, at the zero concentration intercept. These plots were linear within experimental error (SE < $\pm 10^{-5}$ g/mL). The values of these isopotential slopes were utilized directly as the buoyancy term in the sedimentation experiments (Reisler & Eisenberg, 1969). These values were also utilized in combination with the corresponding isomolal ones, $(\partial\rho/\partial c)_m^0$, in order to estimate the preferential interaction parameter, ξ, where ξ is the excess grams of salt or of water per gram of protein relative to dialyzate composition [Güntelberg & Linderstrøm-Lang, 1949; Casassa & Eisenberg, 1961; for details with the density method, cf. Kupke (1973)]. The purpose of estimating ξ was to evaluate its depressant effect on the second virial coefficient in the osmotic experiments [Scatchard, 1946; cf. Kelly & Kupke (1973) for additional discussion).

Equilibrium sedimentation was carried out at 20 °C on a 1:1 molar mixture of the purified BSA fragments at 24000 rpm in a Spinco Model E ultracentrifuge equipped with scanning ultraviolet optics. The meniscus depletion method of Yphantis (1964) was employed with the scanner set at a wavelength of 277.7 nm. The weight-average molecular weight, $\mathrm{MW_n}$, for evaluating the association constant, K_a, was related to the variation of the absorbance, A, with the distance, r, from the axis of rotation by

$$\mathrm{MW_w} = \left(\frac{2RT}{\omega^2(\partial\rho/\partial c)_\mu^0}\right)\left(\frac{\mathrm{d}\ln A}{\mathrm{d}r^2}\right) \qquad (7)$$

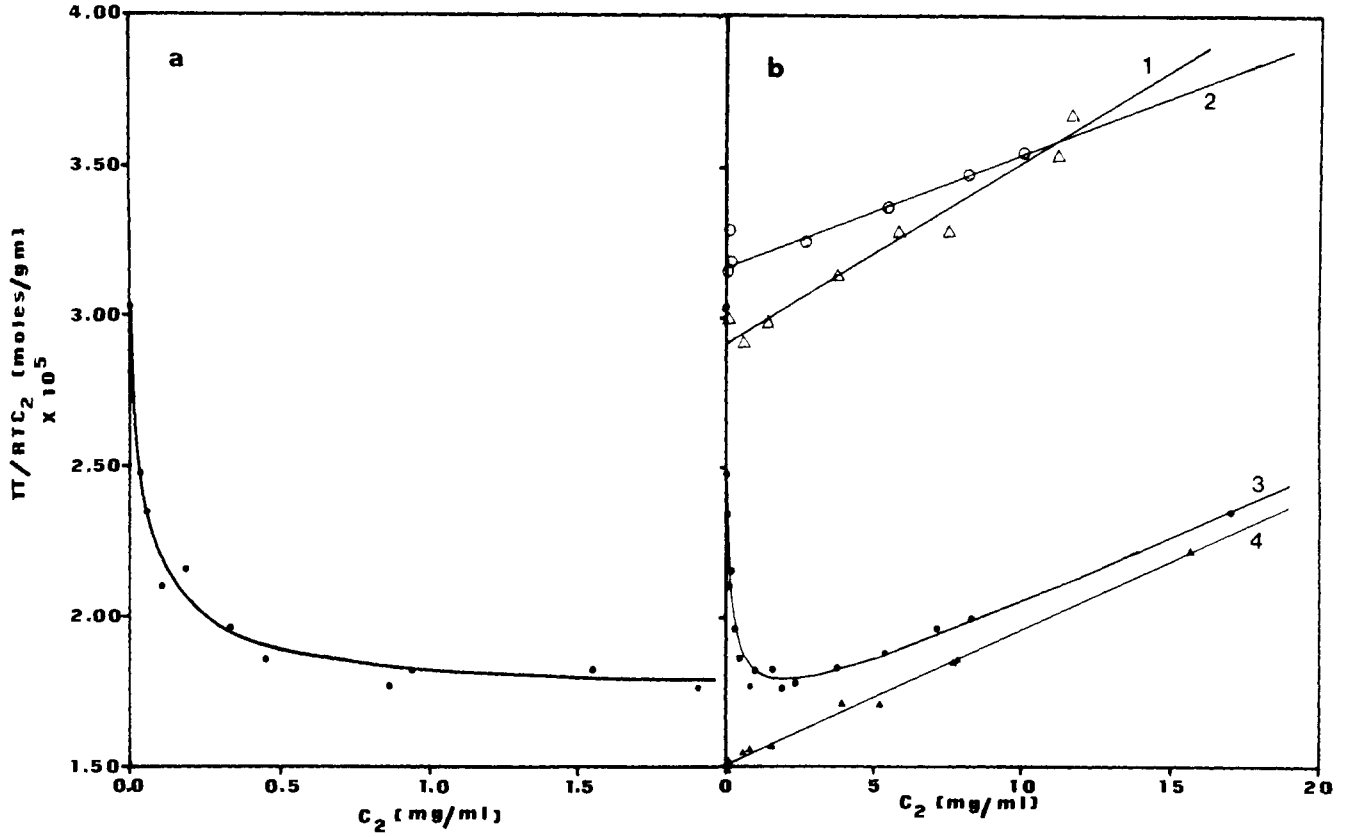

FIGURE 1: (a) Reduced osmotic pressure as a function of total protein concentration for an equimolar mixture of fragments BSA-P-(1–306) and BSA-P-(307–581). Conditions: 20 °C; 0.1 M KCl–0.01 M BTP, pH 8.6. The solid line is a simulation with $K_a = 1.8\ \mu M^{-1}$. (This is an expanded scale of part of curve 3 of Figure 1b.) (b) Reduced osmotic pressure as a function of protein concentration. Curve 1, BSA-P-(1–306); curve 2, BSA-P-(307–581); curve 3, equimolar mixture of BSA-P-(1–306) and BSA-P-(307–581); curve 4, BSA. The solid lines of curves 1, 2, and 4 are linear regressions by the method of least squares (see text); the solid line of curve 3 is a simulation with $K_a = 1.8\ \mu M^{-1}$.

where ω is the angular velocity. This study was carried out at pH 8.6 in the KCl–BTP solvent at an initial concentration of 2 μM per fragment as a check on the value of K_a determined by osmometry.

Sedimentation velocity experiments in the pH 8.6 solvent were performed on the SH-blocked BSA, on the isolated BSA fragments, and on 1:1 molar mixtures thereof at 48 000–60 000 rpm at 20 °C in a Spinco Model E ultracentrifuge using standard Schlieren optics. Values of the observed sedimentation coefficient, s_{20}, at finite concentrations were extrapolated linearly by the method of least squares to zero concentration in order to evaluate s^0_{20}. In the case of mixtures, the lowest concentration used (~2.5 mg/mL) was sufficient to ensure maximal association under equilibrium conditions. Hence, these experiments at different speeds served to determine whether dissociation could be observed by this transport method as well as to characterize the sedimentation rates of the purified BSA fragments.

Specific viscosities, η_{sp}, were determined on the SH-blocked BSA, on the isolated BSA fragments, and on 1:1 molar mixtures of the latter with the magnetic viscometer of Hodgins & Beams (1971). Evaluation of the intrinsic viscosity, $[\eta]$, for each of the systems was carried out by using 0.2 mL of solution per measurement as described by Kupke et al. (1972). All measurements were made at 20 °C with an applied shear stress of ~0.002 dyn/cm^2 in the KCl–BTP solvent, pH 8.6. The concentration range in which the fragments in a mixture series dissociate under equilibrium conditions (<10 μM) was too low to test for curvature in plots of the reduced viscosity, η_{sp}/c, vs. concentration, c (grams per milliliter). All values of $[\eta]$ were obtained by extrapolating to zero concentration a linear least-squares fit of the reduced viscosities.

Results

Figure 1a shows the reduced osmotic pressure $[\Pi/(RTC)]$ as a function of concentration for a 1:1 molar mixture of the purified fragments, BSA-P-(1–306) and BSA-P-(307–581), between 0.03 and 2 mg/mL peptide material (~5×10^{-7} to 3×10^{-5} M in terms of BSA-P′). The solid line is drawn on the basis of an association constant, $K_a = 1.8\ \mu M^{-1}$, for a single association reaction of the type A + B $\rightleftarrows$ AB, where A and B refer to the isolated fragments and AB represents the complex, BSA-P′. The calculation for K_a was based upon the total data to 2×10^{-4} M as shown in curve 3 of Figure 1b. For these calculations, it was assumed that the total nonideality for any equilibrium mixture of the three species was like that for the SH-blocked BSA. The average of the second virial coefficients for the fragments (curves 1 and 2, Figure 1b) and the apparent coefficient for the complex (curve 3) above ~5 mg/mL seem not to be seriously different from that for the parent BSA (curve 4). Moreover, the conservation of charge upon association of the fragments (presently) suggests that no major difference between the average nonideality of the fragments and that of the complex need be expected if all species remain essentially globular (as indicated by viscosity, Table I). This procedure neglects the effect of cross terms arising when mixtures of nondiffusible components are present (Scatchard, 1946); with globular proteins, however, such effects are small if the net charge does not change when the species interact. [Adams et al. (1978) have treated this kind of a nonideal associating system in detail. According to their guidelines, a plot of the difference in the quantity $C_T/(MW_{n,app})$ vs. the product of the concentrations of the fragments should exhibit

Table I: Intrinsic Viscosity, $[\eta]$,[a] of BSA and BSA Peptic Fragments[b]

peptide	$[\eta]$ (mL/g)[c]	σ (mL/g)[d]
BSA	4.23	±0.011
BSA-P′	5.63	±0.005
BSA-P-(1–306)	4.42	±0.022
BSA-P-(307–581)	5.48	±0.041

[a] Conditions: 0.1 M KCl–0.01 M BPT, pH 8.6, at 20 °C. [b] Fragment designations are as defined in footnote 2; BSA was SH-blocked and defatted. [c] The linear, least-squares slopes (mL^2/g^2) for the reduced viscosity (η_{sp}/c) vs. concentration on the basis of four different concentrations (in triplicate) are BSA, 26.9; BSA-P′, 4.90; BSA-P-(1–306), 30.8; and BSA-P-(307–581), 45.7. [d] These values represent the standard deviation of the data points from the line given by the linear, least-squares analysis.

a downward curvature; this was observed in our case ($MW_{n,app}$ is the apparent number-average molecular weight at finite concentrations). An upward curvature would have indicated additional nondiffusible species.] Owing to current, but correctable, limitations with the magnetic osmometer, experiments were not carried out at sufficiently high concentrations in order to determine whether a fixed proportion of the fragments remain uncomplexed (a small fraction of the fragments, <6%, may have remained as monomers at the highest concentrations employed; see Discussion). Confirming osmotic experiments were carried out at molar ratios for [BSA-P-(1–306)]/[BSA-P-(307–581)] of 1:2 and 2:1 (not shown). The lines calculated on the basis of K_a = 1.8 μM^{-1} fit these data as well as in the case of 1:1 molar mixtures. Furthermore, the value obtained for K_a by equilibrium sedimentation on the 1:1 molar mixture of the fragments was 1.74 ± 0.2 μM^{-1}, assuming again that the only macromolecular species present were the two fragments and BSA-P′ at any position r along the axis of rotation.

A value for K_a was also calculated via the catalytic activity exhibited by mixtures of the BSA fragments in decomposing the Meisenheimer complex (MC). For this purpose, the concentration of one fragment was held constant while that of the other fragment was varied. The total change in absorbance per unit of time at 478 nm was found to be constant after a 10- to 20-fold excess of one of the fragments had been added. This absorbance change was arbitrarily assigned to a molar concentration of the active complex equal to that of the fragment not being varied. Hence, lesser changes in absorbance per unit of time as a function of the amount of fragment being varied yield the concentrations for the calculation of K_a. This assumes that the amount of active complex is approximately equal to that of a stable molecular complex (BSA-P′). The data points in Figure 2 are for three different preparations of the fragments, where BSA-P-(1–306), containing the active-site lysyl residue, was held constant at 2 μM while fragment BSA-P-(307–581) was varied. Because different preparations of the unstable substrate, MC, were used, somewhat different rates of decomposition were observed. The average value of K_a under these circumstances was found to be 1.33 ± 0.55 μM^{-1}. The solid line of Figure 2 represents the calculated values of active BSA-P′ concentration assuming an equilibrium constant of 1.33 μM^{-1}. Although 1:1 molar mixtures seldomly exhibited more than ~35% of the activity given by equivalent concentrations of the SH-blocked BSA [cf. also Taylor & Silver (1976)] at these low assay levels, saturation of one fragment by the other yielded 61% or more of this control activity (i.e., where moles of BSA = moles of fragment held constant).

The number-average molecular weights, MW_n, for the isolated fragments and for the SH-blocked BSA monomer

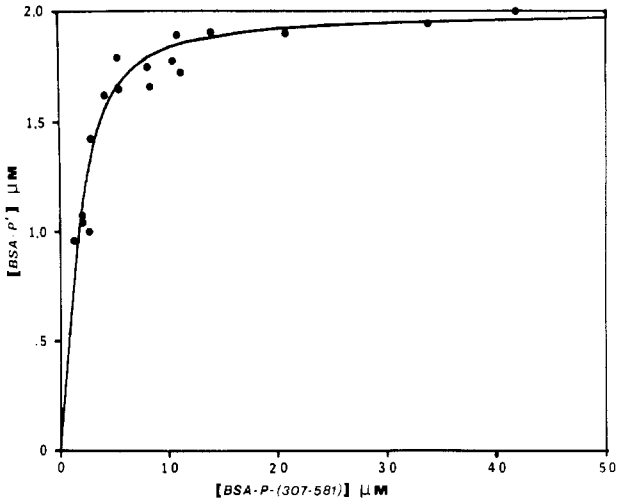

FIGURE 2: Catalytic activity determination of K_a (20 °C). Molar concentration of the complex (BSA-P′) as a function of concentration of fragment BSA-P-(307–581) in the presence of a constant concentration (2 μM) of fragment BSA-P-(1–306). The solid line represents a simulation with K_a = 1.33 μM^{-1}.

were estimated from the osmotic pressure experiments at C = 0 (where $\Pi/(RTC) = MW_n^{-1}$) by linear, least-squares analysis (solid lines of curves 1, 2, and 4 of Figure 1b). The values in daltons were 34 500 for BSA-P-(1–306), 31 700 for BSA-P-(307–581), and 66 100 for the SH-blocked BSA. The corresponding values calculated from the amino acid composition as proposed by Brown (1975) are 34 953, 31 275, and 66 228 daltons, respectively. The equations for curves 1, 2, and 4 were expressed in the manner employed by Scatchard (1946) for dilute protein solutions whereby $\Pi = AC + ABC^2$, in which $A = RT/MW_n$ and B (liters per gram) is the coefficient representing the departure from the van't Hoff proportionality ($\Pi \propto C$). For experiments at 20 °C, RT = 24 870 L·cm of H_2O·mol^{-1}. [By utilization of the measured densities, the foregoing relationship between Π and C is convertible to terms of the molal scale, as was actually employed by Scatchard, since $C_i = 1000\rho W_i$, where W is the weight fraction and the density, ρ, is in grams per milliliter.] For curve 1 of Figure 1b, $\Pi = 0.7203C + 0.01531C^2$, for curve 2, $\Pi = 0.7839C + 0.009242C^2$, and for curve 4, $\Pi = 0.3763C + 0.01095C^2$. The major factors contributing to the value of B in dilute protein solutions are the Donnan effect, protein–protein interactions (which are comparatively small for unassociating globular proteins), and the redistribution of diffusible components when more than one such component is present (Scatchard, 1946; Güntelberg & Linderstrøm-Lang, 1949). Except for the mixtures (curve 3, Figure 1b), the Donnan term was the major contribution to the slopes of curves 1, 2, and 4 since self-association in these cases was negligible and since the negative effect on B from the redistribution of the KCl and water (an undistributing process) was found to be very small. [The density results for this redistribution or preferential interaction effect indicated a slight increase in the molality of the KCl on the protein side of the membrane in all the systems ($\xi \sim +0.02$ g of salt per gram of protein), but values could not be assigned owing to an estimated overall error of ±0.02 g/g.] In curve 3, the negative contribution to B from the associative interaction of the 1:1 molar mixtures in the very low concentration range clearly overrides even the Donnan effect until the degree of association was maximal.

The Donnan contribution to the value of B was evaluated from titration experiments which are shown in Figure 3. From

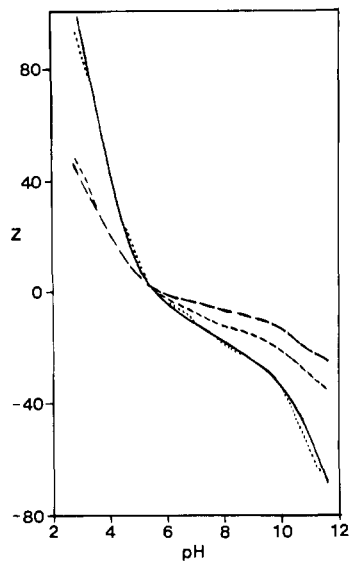

FIGURE 3: Net charge, Z, as a function of pH at constant ionic strength (0.1) with KOH and HCl containing KCl, 21 °C. (—) BSA (initially at 0.5×10^{-4} M); (···) BSA-P′ (initially at 0.5×10^{-4} M); (- - -) BSA-P-(1–306) (initially at 1×10^{-4} M); (— — —) BSA-P-(307–581) (initially at 1×10^{-4} M). The BSA-P′ (···) refers to an equimolar mixture of the latter two fragments.

these data, a net negative charge of 22 ± 1 was estimated at pH 8.6 for both the 1:1 molar mixture of the fragments and for the SH-blocked BSA, 13–14 for BSA-P-(1–306) and 8–9 for BSA-P-(307–581). The sum of the charges for the two fragments, within experimental error, is equal to that found for either the BSA or the 1:1 molar mixture of the fragments (predominantly BSA-P′). This result suggests that charge is conserved upon scission of the parent albumin and upon association of the two fragments into BSA-P′. The Donnan contribution to B [cf. Scatchard et al. (1946)] was estimated as ~85% of the total value of B for both BSA and BSA-P′ (the latter determined at >5 mg/mL).

The difference in volume before and after mixing the fragments (0.75-mL samples, each containing 0.34 μmol of isolated fragment) ranged from −25 to −28 nL as calculated by eq 6. This result corresponds to about −80 mL/mol (66 000 g) of the complex. The fact that this small value was clearly negative suggests that the net charge of the fragments was not altered during the association but rather that caged water on nonpolar sites was released from the contact areas (Richards, 1977).

The values for the intrinsic viscosity, $[\eta]$, for each of the fragments, for a 1:1 molar mixture of them, and for SH-blocked BSA are shown in Table I. These values reflect the magnitude of the excluded volume effect when inserting macromolecules into a solvent (Tanford, 1961) but are not directly equatable with the protein–protein interactions contributing to the thermodynamic value of B. The concentrations employed (3–20 mg/mL) were sufficiently large to ensure that the fragments in the 1:1 molar mixtures were maximally complexed (the rate of shearing was only about 0.1 s^{-1} which probably did not give rise to any appreciable net transport of the solutes). The significantly higher value of $[\eta]$ for BSA-P′ over that for BSA apparently reflects the contribution from a comparatively high value of $[\eta]$ for fragment BSA-P-(307–581). If so, the conformation of this fragment does not adopt a more compact or native-like structure upon association with the seemingly less deformed fragment BSA-P-(1–306). The effect of this larger hydrodynamic volume of BSA-P′ on the osmotic nonideality, however, is small. Possibly, a slightly larger positive slope beyond ~1% BSA-P′ compared to that for BSA might have been established if the osmometry at relatively high concentrations had been successful; a small difference in charge between BSA-P′ and BSA, however, would have camouflaged such an effect at this ionic strength. The value of $[\eta]$ for BSA is in good agreement with that (4.17 mL/g) reported by Tanford & Buzzell (1956) at this pH and ionic strength; our material, however, was defatted and SH-blocked.

The linear equation relating the sedimentation coefficients with concentration of the SH-blocked BSA (60 000 rpm) in the KCl–BTP solvent, pH 8.6, was $s_{20} = 0.06616C + 4.55$; the last term is the value for s^0_{20}, which is in good agreement with accepted values for the monomer [cf. Peters (1975)]. The equations for the individual fragments are as follows: for BSA-P-(1–306), $s_{20} = -0.0394C + 3.27$; for BSA-P-(307–581), $s_{20} = -0.0089C + 3.07$. These values of s^0_{20} for the fragments relative to that for BSA are consistent with the rule of thumb that doubling the molecular weight of globular proteins increases s^0_{20} by ~50%. In the case of the 1:1 molar mixtures of the fragments, the Schlieren patterns and the sedimentation coefficients clearly indicated dissociation of the complex (BSA-P′) during transport. At 60 000 rpm, the regression pattern of s_{20} vs. C was not linear; values of s_{20} below ~7–8 mg/mL usually remained at ~3.0 Svedberg units (S) or a little higher. Above this concentration range, the values extrapolated to ~3.6 S, or considerably smaller than that for BSA. At the reduced rotor speed (48 000 rpm) the regression pattern was linear, giving the relation $s_{20} = 0.0597C + 3.56$. The Schlieren patterns with the mixtures exhibited substantially more spreading with time than for BSA. Apparently, the larger hydrodynamic volume of the fragment BSA-P-(307–581) results in its lagging further behind the faster and more compact fragment BSA-P-(1–306) on the trailing side of the main BSA-P′ boundary. Hence, at the higher speed, more dissociation would be expected, leading to nearly complete loss of the complex as the concentration was reduced. An apparent slowdown of the peak position was also observed during a given run, although these positions could not be assessed with confidence. In the low concentration range (2.5–7.5 mg/mL), where s_{20} values were nearly constant with concentration at the highest speed, maximal complexing was observed in the osmotic pressure experiments (Figure 1b).

For the calculation of the above s_{20} values, the densities were determined as a function of the concentration under isopotential conditions. The linear slopes, $(\partial\rho/\partial c)_\mu^0$ (evaluated by least-squares analysis), were applied in place of the conventional buoyancy term. The values of $(\partial\rho/\partial c)_\mu^0$ were 0.279 for BSA, 0.291 for BSA-P-(1–306), 0.292 for BSA-P-(307–581), and 0.293 for the 1:1 molar mixtures of the latter two fragments (or BSA-P′). The latter value was utilized also in the equilibrium sedimentation study.

Discussion

The results with the magnetic osmometer demonstrate that the colloid osmotic pressure can be utilized effectively in the micromolar range of macromolecule concentration. The association constant obtained with this technique for the peptic fragments of BSA is in good agreement with those from the other methods used in this study and also with that (1 μM^{-1}) of Pedersen & Foster (1969) via equilibrium sedimentation on a highly similar BSA system derived from subtilisin digestion. An approximate value of K_a for the peptic fragments at pH 8.6 from electrophoretic experiments, however, is an order of magnitude larger (Reed et al., 1976). The osmotic pressure, being unencumbered by density and pressure gra-

dients and by hydrodynamic and net transport effects and also being less complicated with multicomponent systems, is a potentially powerful method for the study of associating macromolecules. As noted previously, the magnetic osmometer can be made more convenient and versatile than was practiced here. Unfortunately, it was not feasible for these experiments to adapt the instrument for concentrations sufficiently high to evaluate with confidence the second virial coefficient on mixtures after the association to BSA-P′ was maximal. However, the interesting effect of decane, which was observed when attempting higher concentrations by the traditional osmotic method, may be useful in designing other experiments with fragments of BSA. Decane appears to act on fragment BSA-P-(1–306), inhibiting the association with BSA-P-(307–581) and abolishing the catalytic activity exhibited by mixtures of these fragments. This observation is consistent with the volume change and titration experiments which suggest that the association is largely via nonpolar interactions between the fragments.

It is clear from the catalytic activity experiments that the complex, BSA-P′, is less competent in decomposing the MC substrate than is intact BSA. The larger value of K_m (2×10^{-4} M) of the substrate for BSA-P′ indicates a lower affinity than for BSA ($K_m = 1.74 \times 10^{-5}$ M); correspondingly, the rate of the MC decomposition (k_{cat}) in the presence of BSA-P′ reflects ~60% efficiency relative to comparable concentrations of BSA (0.723 s^{-1} vs. 0.436 s^{-1}, respectively). The osmotic pressure results, however, indicate substantially more complexing of the fragments than 60%. We estimate that at most 6% of the fragments in a 1:1 molar mixture may not have complexed at total protein concentrations above 5 mg/mL. [This calculation assumes that the osmotic nonideality of such mixtures at concentrations >0.5% protein is about the same as that of the SH-blocked BSA and that the only association product is BSA-P′ (as was suggested by the sedimentation velocity and electrophoretic experiments). The value of MW_n of the 1:1 molar mixtures is 62 200 by extrapolating to zero concentration the solid line from values above 0.5% protein in curve 3, Figure 1b.]

The results, overall, are consistent with the evaluation of Taylor et al. (1975b) that some domains in BSA, such as the one containing the MC catalytic site, have greater structural stability than other domains. In our experiments, however, the domains are potentially destabilized by the peptic cleavage rather than by the unfolding and disulfide cleavage procedure of Taylor and co-workers. Despite the gross conformational differences between the association complex (BSA-P′) and the parent BSA as noted in this study, the return of catalytic activity, approaching that observed upon renaturation of the intact molecule by Taylor and co-workers (~73%), suggests at this point that the cooperating regions in the two fragments which produce the active site do not require a native configuration of the other domains. Nonetheless, the degree of specificity of the fragment BSA-P-(307–581) in combining with the fragment BSA-P-(1–306), containing the active site lysine, remains open. The unexpectedly large value of $[\eta]$ for the former fragment prompts the suggestion that other fragments, such as the C-terminal half of human serum albumin, should be tested for this and other activities when mixed with BSA-P-(1–306).

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References

Adams, E. T., Wan, P. J., & Crawford, E. F. (1978) *Methods Enzymol. 48*, 69.

Beams, J. W., Hodgins, M. G., & Kupke, D. W. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 2785.

Brown, J. R. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 591.

Casassa, E. F., & Eisenberg, H. (1961) *J. Phys. Chem. 65*, 427.

Chen, R. F. (1967) *J. Biol. Chem. 242*, 173.

Crouch, T. H. (1977) Dissertation, University of Virginia.

Crouch, T. H., & Kupke, D. W. (1977) *Biochemistry 16*, 2586.

Feldhoff, R. C., & Peters, T., Jr. (1975) *Biochemistry 14*, 4508.

Güntelberg, A. V., & Linderstrøm-Lang, K. (1949) *C. R. Trav. Lab. Carlsberg 27*, 1.

Hodgins, M. G., & Beams, J. W. (1971) *Rev. Sci. Instrum. 42*, 1455.

Hunter, M. J. (1966) *J. Phys. Chem. 70*, 3285.

IUPAC–IUB Commission on Biochemical Nomenclature (1967) *J. Biol. Chem. 242*, 555–557.

Kelly, M. J., & Kupke, D. W. (1973) in *Physical Principles and Techniques in Protein Chemistry* (Leach, S. J., Ed.) Part C, p 77, Academic Press, New York.

King, T. P. (1973) *Arch. Biochem. Biophys. 156*, 509.

Kupke, D. W. (1961) *C. R. Trav. Lab. Carlsberg 32*, 107.

Kupke, D. W. (1973) in *Physical Principles and Techniques in Protein Chemistry* (Leach, S. J., Ed.) Part C, p 1, Academic Press, New York.

Kupke, D. W., & Beams, J. W. (1972) *Methods Enzymol. 26*, 74.

Kupke, D. W., & Crouch, T. H. (1978) *Methods Enzymol. 48*, 29.

Kupke, D. W., & Dorrier, T. E. (1978) *Methods Enzymol. 48*, 155.

Kupke, D. W., Hodgins, M. G., & Beams, J. W. (1972) *Proc. Natl. Acad. Sci. U.S.A. 69*, 2258.

Pedersen, D. M., & Foster, J. F. (1969) *Biochemistry 8*, 2357.

Peters, T., Jr. (1975) in *The Plasma Proteins* (Putnam, F. W., Ed.) Vol. I, 2nd ed., p 133, Academic Press, New York.

Peters, T., Jr., Taniuchi, H., & Anfinsen, C. B., Jr. (1973) *J. Biol. Chem. 248*, 2447.

Reed, R. G., Feldhoff, R. C., & Peters, T., Jr. (1975) *Biochemistry 14*, 4578.

Reed, R. G., Feldhoff, R. C., & Peters, T., Jr. (1976) *Biochemistry 15*, 5394.

Reisler, E., & Eisenberg, H. (1969) *Biochemistry 8*, 4572.

Richards, F. M. (1977) *Annu. Rev. Biophys. Bioeng. 6*, 151.

Scatchard, G. (1946) *J. Am. Chem. Soc. 68*, 2315.

Scatchard, G., Batchelder, A. C., & Brown, A. (1946) *J. Am. Chem. Soc. 68*, 2320.

Senter, J. P. (1969) *Rev. Sci. Instrum. 40*, 334.

Tanford, C. (1961) *Physical Chemistry of Macromolecules*, Wiley, New York.

Tanford, C., & Buzzell, J. G. (1956) *J. Phys. Chem. 60*, 225.

Taylor, R. P. (1976) *J. Am. Chem. Soc. 98*, 2684.
Taylor, R. P. (1977) in *Albumin Structure, Function & Uses* (Rosemoer, V. M., Oratz, M., & Rothschild, M. A., Eds.) p 183, Pergamon Press, New York.
Taylor, R. P., & Vatz, J. B. (1973) *J. Am. Chem. Soc. 95*, 5819.
Taylor, R. P., & Silver, A. (1976) *J. Am. Chem. Soc. 98*, 4650.
Taylor, R. P., Chau, V., Bryner, C., & Berga, S. (1975a) *J. Am. Chem. Soc. 97*, 1934.
Taylor, R. P., Berga, S., Chau, V., & Bryner, C. (1975b) *J. Am. Chem. Soc. 97*, 1943.
Yphantis, D. A. (1964) *Biochemistry 3*, 297.

Urea Denaturation of Horse Heart Ferricytochrome *c*. Equilibrium Studies and Characterization of Intermediate Forms†

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ABSTRACT: Equilibrium studies of the urea denaturation of horse heart ferricytochrome *c*, pH 7.0, through alteration of the absolute extinction of the 695-nm band and of the fluorescence efficiency of the tryptophan side chain, have been reported. The denaturation profiles using the two probes have been analyzed in terms of similarities and differences as well as to determine the nature of the intermediate forms. The intermediate forms in the 4–4.5 M urea concentration range have been further characterized by circular dichroism spectroscopy in the Soret and the intrinsic absorption regions, stability to temperature and pH, and reactivity of the methionine and histidine side chains to bromoacetic acid. The scheme $N \rightleftharpoons X_1 \rightleftharpoons X_2 \rightleftharpoons D$, in which N and D are the 0 and 9 M urea forms and X_1 and X_2 are the two intermediate forms, with midconcentrations of urea of 2–2.5, 6.2, and 6.9 M, respectively, for the three transitions, is proposed as an explanation of the observed denaturation profiles of the protein. The N to X_1 transition is seen in the enhanced absorptivity of the 695-nm band and the further quenching of tryptophan fluorescence. Form X_1 exhibits lowered temperature and pH stability, a nativelike intrinsic CD spectrum, and reactivity of both His-18 and Met-80 to bromoacetic acid. The X_1 to X_2 transition is apparent only in the 695-nm absorptivity–urea profile, while the X_2 to D transition is discerned from both the enhancement of tryptophan fluorescence and the final quenching of the 695-nm absorptivity. The extent of alteration of $|\epsilon|_{695}$ during the X_1 to X_2 transition is about two-thirds the total extinction. The two high molar urea transitions yield reference free-energy changes of 25.1 and 9.1 kcal/mol, and the corresponding parameter determining the extent of solvent exposure, the parameter *m*, amounts to about 4000 and 1300 cal/M^2, respectively. The course of urea denaturation of horse heart ferricytochrome *c*, in terms of the alteration of protein structures, is thus described as follows. Step 1, N to X_1, reflects in the main the loosening of the frontal section of the heme crevice, without alteration of either the coordination configuration of the native protein or its polypeptide structures. However, definite destabilization of the molecular conformation, as well as of the heme coordination configuration, occurs, which is detailed with reference to the reciprocity of the heme crevice to molecular stability. Step 2, the X_1 to X_2 transition, is characterized simply as a reflection of the solvent exposure of the protein backbone, again without alteration of either the coordination configuration of heme iron or the tryptophan–heme domain of the molecule. The reduction of the 695-nm extinction during this step is considered to be linked to the molecular conformation. Step 3, the X_2 to D transition, is regarded as a composite of both the disruption of the Met-80–S–iron linkage and the loosening of the crevice in the tryptophan–heme domain of the molecule; it may reflect the reorganization of the polypeptide chain.

The structural, conformational, and possibly functional aspects of enzymes can be better understood from a detailing of the folding and unfolding processes. In the case of the constituents of the electron-transport chain, cytochrome *c* is the only component susceptible to such investigation, primarily because of its availability in a pure, well-defined form and the wealth of information regarding its structure, conformation, and function (Margoliash & Schejter, 1966; Harbury & Marks, 1973; Dickerson & Timkovich, 1975; Myer, 1978; Myer & Pande, 1978; Ferguson-Miller et al., 1979). The availability of the three-dimensional structures of a number of preparations, on the one hand, and details of structural–conformational–functional relationships, on the other, provide the necessary bases for the interpretation of not only the course of the folding and unfolding processes but also of the state of the intermediate forms.

Horse heart ferricytochrome *c* has been denatured by a number of agents and studied by both kinetic and equilibrium approaches, yet there is no consistent view regarding either the mechanism for denaturation and folding of the protein or an understanding of the processes in terms of various structural aspects of the molecule. From earlier studies with urea using circular dichroism and absorption spectroscopy, it was proposed that the course of urea denaturation can be described by a two-step process, i.e., a three-state model (Myer, 1968a; Stellwagen, 1968). Similar inferences were made when the denaturation was induced with a variety of alcohols (Kaminsky et al., 1973). Using fluorescence spectroscopy as the probe,

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