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Use of High-Speed Size-Exclusion Chromatography for the Study of Protein Folding and Stability†

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ABSTRACT: The urea denaturation of sperm whale myoglobin and the thermal denaturation of ribonuclease have been studied by following the associated volume changes by size-exclusion chromatography on a Toya Soda TSK 3000SW gel permeation column. The permeation properties of the gel have been shown to be invariant in the following solvent systems: 0.2 M NaCl; 8.0 M urea–0.2 M NaCl; and 6.0 M guanidinium chloride (GdmCl). A precise measurement of the volume changes associated with solvent-induced protein denaturation is thus practicable. The column was calibrated in the above solvent systems by using 12 well-characterized proteins as standards. In the case of the denaturation of myoglobin by urea, the rate of equilibration of folded and unfolded species is slow on the time scale of the chromatographic experiment, and the two forms are well separated on the column in the transition region. Both the folded and unfolded species are shown to undergo significant swelling in urea. This result suggests that the view of denaturation based solely on the preferential solvation of the unfolded protein is incorrect. The rate of interconversion between folded and unfolded ribonuclease is fast relative to the time scale of the chromatographic experiments performed in this study. This is reflected in the fact that only one peak is observed in the elution profiles of ribonuclease in the transition region. Thermally unfolded ribonuclease has a smaller volume than the unfolded state in urea or GdmCl, suggesting that it has residual structure. The van't Hoff ΔH for the thermal unfolding of ribonuclease calculated from the size-exclusion chromatographic experiments (36 ± 3 kcal/mol) is significantly lower than previously reported values. This suggests that there are contributions to the enthalpy of unfolding which have negligible volume changes.

The elucidation of the folding pathways of globular proteins remains one of the major objectives of contemporary protein chemistry (Anfinsen & Scheraga, 1975; Nemethy & Scheraga, 1977; Schultz, 1977; Jaenicke, 1980). Among the many theoretical and experimental approaches to this problem, denaturation studies continue to play a crucial role (Tanford, 1968, 1970; Privalov, 1979; Schellman & Hawkes, 1980). A thermodynamic analysis of thermal or solvent-induced unfolding processes provides the free energy of stabilization of the native state of globular proteins and a measure of the cooperativity (Privalov, 1979, 1982) of the unfolding process. Many properties can be used to monitor folding–unfolding equilibria, including the large volume changes associated with these processes. Although intrinsic viscosity remains the most theoretically straightforward method for determining such volume changes, gel filtration techniques offer an alternative approach (Ackers, 1970).

More than a decade ago, Tanford and co-workers showed that analytical gel filtration can be used to study proteins, denatured in a variety of solvents (Fish et al., 1970). However, the high resistance to flow, low mechanical strength, and poor chemical resistance of the soft gels based on dextran, polyacrylamide, and agarose, which were the only ones available at that time, made it difficult to apply this approach to denaturation studies requiring high concentrations of denaturants

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such as urea, guanidinium chloride, or organic solvents. The use of a rigid matrix such as controlled-pore glass resulted in higher flow rates but gave poor resolution (Ansari & Mage, 1976). Recently, advances in the technology of liquid chromatography and the development of a new class of column packing materials have led to a higher level of performance in analytical gel filtration. A reevaluation of the use of gel filtration techniques in protein denaturation studies is, therefore, worthwhile. In particular, the TSK SW series of gels developed by Hashimoto and co-workers (Kato et al., 1980) are representative of the new class of column packings and have been compared favorably with other commercial high-performance gels (Pfannkoch et al., 1980). These gels have been shown to give excellent calibration curves [partition coefficient (K_d) vs. log molecular weight] for proteins and other macromolecules in the presence (Ui, 1979; Imamura et al., 1981) or absence (Himmel & Squire, 1981) of denaturants. It is clear from the latter studies that size-exclusion chromatography (SEC)[1] should be able to resolve the large changes in volume associated with the unfolding of globular proteins and allow a systematic study of such processes. Furthermore, if partially unfolded species occur as stable intermediates on the folding pathway, it may be possible to quantitate their concentration and properties if they are stable enough kinetically to occur on the same time scale as the chromatographic experiment.

In this paper, we report the first systematic study of the application of SEC to the quantitation of the volume changes occurring upon protein denaturation and to the determination of equilibrium data for folding–unfolding processes. In particular, the results obtained for the urea denaturation of sperm whale myoglobin and the thermal denaturation of ribonuclease are presented.

Materials and Methods

The following proteins were used for column calibration or unfolding studies under a variety of conditions: (1) bovine liver catalase (Sigma); (2) rabbit muscle lactate dehydrogenase (Sigma type XI); (3) human transferrin (Sigma grade II); (4) bovine serum albumin (Sigma MW-SDS-70 kit); (5) ovalbumin (Sigma MW-SDS-70 kit); (6) bovine erythrocyte carbonic anhydrase (Serva Biochemicals); (7) bovine milk β-lactoglobin (Sigma MW-SDS-70 kit); (8) human hemoglobin (Sigma type VI); (9) whale skeletal muscle myoglobin (Sigma type II); (10) bovine pancreatic ribonuclease A (Sigma type III-A); (11) horse heart cytochrome *c* (Sigma type VI); (12) bovine pancreatic insulin (Sigma). Blue dextran (Sigma) and sodium azide (Fisher) were also used in the column calibration. Gdn·HCl and urea were both ultrapure reagents from Schwarz/Mann. Piperazine-*N*,*N*′-bis(2-ethanesulfonic acid) (Pipes), tris(hydroxymethyl)aminomethane (Tris), and (+)-10-camphorsulfonic acid (CSA) were obtained from Sigma.

The in-house distilled water available to our lab was further purified by passage through organic removal, high-capacity ion-exchange, and ultrapure ion-exchange hose–nipple cartridges (Barnstead Co.) connected in series.

All solutions prepared for HPLC were passed through a 0.2-μm nylon 66 filter (Rainin Instrument Co.). HPLC measurements were performed on a Varian 5040 liquid chromatograph equipped with a Varian UV 50 absorbance monitor (set at 220 nm, 16-nm bandwidth), a Valco loop injector (10- or 50-μL loop), and an 8055 autosampler attached to a Varian 401 data system. Analytical gel filtration experiments were performed by using a Toya Soda micropak TSK 3000SW column (30 cm × 7.5 mm i.d.). Column temperature was maintained (±0.5 °C) by housing the column in a glass jacket attached to a Lauda K-4/RD circulating water bath which also held the solvent reservoir and protein samples. For the column calibration and equilibrium thermal denaturation studies, the column solvent and sample were preincubated at the temperature of interest for 30 min before injection of the protein solution onto the column. It should be noted that the manufacturer recommends a maximum operating temperature of 45 °C and a pH range of 2–7.5. When the denaturant concentration was changed, at least 100 mL of solvent was passed through the column before a sample was injected. By monitoring the temperature of the effluent from the solvent mixing chamber, it was established that the on-line mixing of high concentrations of denaturant (e.g., 9.0 M urea or 6.0 M Gdn·HCl) with a denaturant-free buffer solution did not result in a measurable heat of mixing. Thus, all intermediate denaturant concentrations were obtained by specifying different proportions of solvent from two channels containing the highest denaturant concentration and buffer with no denaturant, respectively. The flow rate through the column was maintained at 0.5 mL/min; the pressure head varied from 8 to 22 atm depending on the temperature and concentration of urea or Gdn·HCl being pumped.

All CD measurements were performed on a Jasco J-500 A spectropolarimeter equipped with a DP-500 N data processor and calibrated with CSA by using the procedure of Chen & Yang (1977). Typically a protein solution (0.1–1.0 mg/mL) was placed in a jacketed cylindrical cell with a path length of 0.045 cm and was scanned 4 times from 250 to 200 nm (20 nm/min; time constant 4 s). The spectra obtained were then averaged and smoothed once by using the data processor.

All UV measurements were performed on a Cary 210 spectrophotometer at 23 °C. Reported protein concentrations are based on the weight of the protein powder except in the following cases: myoglobin [ϵ_{405} = 171 000 M^{-1} cm^{-1} (Puett, 1973)]; ribonuclease A [ϵ_{278} = 9800 M^{-1} cm^{-1} (Sela et al., 1957)].

Myoglobin was purified by passage down a K16-30 Pharmacia column containing Sephadex G-75 and equilibrated with buffer solutions containing 0.1 M NaCl, 0.01 M Tris, pH 7.0, and 1.0 mM EDTA. Ribonuclease was purified by dialysis (Spectropor type I membrane) against a solution containing 0.174 M KCl, pH 2.0, ionic strength 0.2 M. Other proteins for column calibration were used as supplied from the commercial source without further purification.

To produce randomly coiled proteins for column calibration, approximately 2.0 mg of each protein was incubated at 25 °C for 24 h in 2 mL of solution containing either 6.0 M Gdn·HCl or 8.0 M urea and 0.01 M Tris (pH 8.0), 1.0 mM EDTA, and 0.01 M dithiothreitol (DTT). Proteins unfolded, but with disulfide bonds intact, were incubated as described above at pH 7.0 with no DTT present. Proteins to be chromatographed under nativelike conditions were dissolved in buffer solutions containing 0.2 M NaCl, 0.01 M Tris (pH 7.0), and 1.0 mM EDTA.

Results and Discussion

Calibration of the TSK 3000SW Column under Various Solvent Conditions. In general, it was found that the proteins

[1] Abbreviations: SEC, size-exclusion chromatography; GdmCl, guanidinium chloride; Pipes, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; CSA, (+)-10-camphorsulfonic acid; HPLC, high-performance liquid chromatography; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; CD, circular dichroism; erf^{-1}, inverse error function; BSA, bovine serum albumin; LDH, lactate dehydrogenase; Gdn·HCl, guanidine hydrochloride.

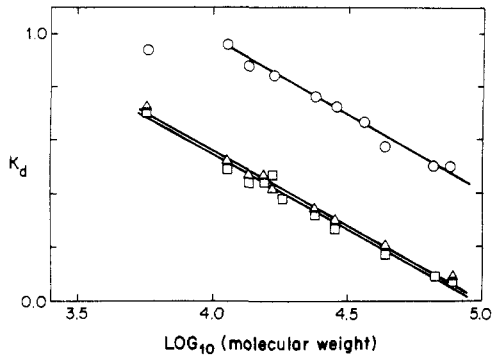

FIGURE 1: Protein molecular weight calibration curves for the TSK 3000SW column in 6.0 M Gdn·HCl (□), 8.0 M urea plus 0.2 M NaCl (△), and 0.2 M NaCl (○). All solutions contained 0.01 M Tris, pH 7.0, and 1.0 mM EDTA and were maintained at 25 °C. Typically, 10 μL of protein solution (1 mg/mL) was injected onto the column.

Table I: Comparison of Column Parameters for the TSK 3000SW Column in Various Solvents

solvent system[a]	V_0 (mL)[b]	V_t (mL)[c]
0.2 M NaCl	4.56 (4.10)[d]	10.41 (10.56)[d]
8.0 M urea + 0.2 M NaCl	4.11	10.24
6.0 M GdmCl	4.12	10.46

[a] All solvents contained 0.01 M Tris-1.0 mM EDTA, pH 7.0, and were maintained at 25.0 °C. [b] V_0, the void volume, is based on the elution volume of blue dextran. [c] V_t, the total solvent-accessible column volume, is based on the elution volume of sodium azide. [d] Values in parentheses were calculated from the data of Himmel & Squire (1981) by using the average of the elution volumes reported for glutamic dehydrogenase and thyroglobulin as the value for V_0 and the elution volume of sodium azide as the value for V_t and by taking the total volume of the column used by Himmel and Squire as exactly twice that of our column.

used for calibration gave constant retention times in the range from 0.2 to 1.0 M NaCl. However, several of the high molecular weight multisubunit proteins were found to have higher K_d values than those expected on the basis of the calibration curves obtained with native proteins (Figure 1, open). Thus, it would appear that 0.2 M NaCl is a high enough salt concentration to cause partial dissociation of the subunits of catalase, lactate dehydrogenase, and hemoglobin at the concentrations they reach during elution from the column. Consistent with the findings of Himmel & Squire (1981), we found that blue dextran is strongly retained and retarded on this column in the presence of low NaCl concentrations. Increasing the NaCl concentration to 1.0 M increased the amount of blue dextran eluting from the column, but the elution time for the peak was still anomalously long. In the presence of 6.0 M Gdn·HCl or 8.0 M urea and 0.2 M NaCl, the interaction of blue dextran with the column appears to be reduced. The V_0 calculated for our column is in good agreement with the value determined by Himmel and Squire when the difference in the volumes of our column and theirs is taken into account (Table I). The fact that very similar V_0 and V_t values are obtained for our column under three different solvent conditions suggests that the TSK gel does not undergo solvent swelling or contraction to any measurable extent. The fact that all three calibration curves in Figure 1 show identical slopes, within experimental error, further supports the conclusion that the permeation properties of this gel do not change significantly in going from 0.2 M NaCl to 8.0 M urea–0.2 M NaCl or to 6.0 M Gdn·HCl, respectively.

The data shown in Figure 1 have also been plotted in the form suggested by Ackers (1967) (Figure 2). Also included

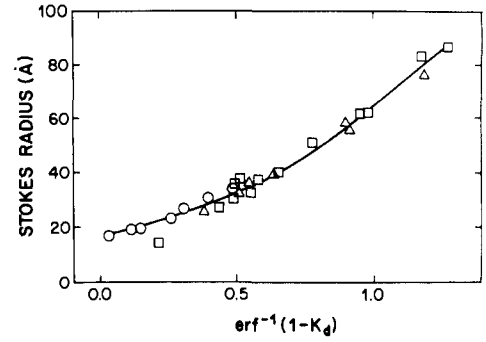

FIGURE 2: Relationship between the Stokes radius and $erf^{-1}(1 - K_d)$ of calibration proteins. The symbols correspond to the same solvents listed in Figure 1. In some cases, the proteins in urea or GdmCl had disulfide bonds intact. The radii were calculated from literature intrinsic viscosity data (Table II) by using the relationship $[\eta] = (2.5N/M_r)^4/_3\pi R_s^3)$, where N is Avogadro's number and M_r is the molecular weight.

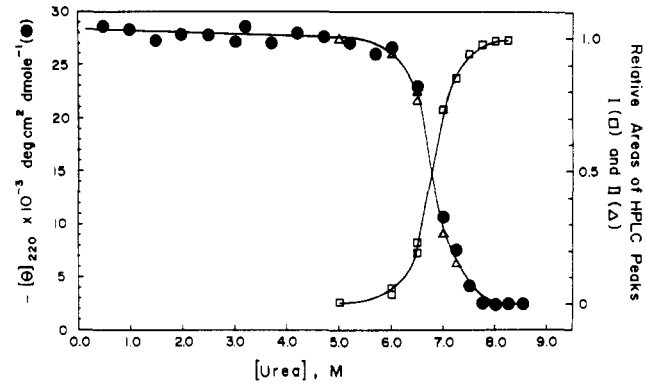

FIGURE 3: Comparison of the urea-induced unfolding of sperm whale myoglobin as measured by CD (●) and SEC–HPLC (□, △). All solutions contained 0.72 mg/mL myoglobin which had been incubated 6 h at the same urea concentration as that of the buffer used to equilibrate the column. All solutions contained 0.2 M NaCl, 0.01 M Tris, and 1.0 mM EDTA, pH 7.0, and were maintained at 25 °C. Peaks I and II refer to the peaks in Figure 4.

in this plot are data for proteins unfolded with disulfide bonds intact, and whose intrinsic viscosities have been reported in the literature. The data for the three different solvent conditions (Table II) all fall along the curve shown in Figure 2. Figure 2 clearly illustrates the large increases in the Stokes radius which occur upon protein unfolding and also sugests that size-exclusion chromatographic techniques may be used to determine the corresponding volume changes with some precision. In the remainder of this report, we illustrate how this may be done by using myoglobin and ribonuclease as examples.

Equilibrium Unfolding of Sperm Whale Myoglobin in Urea. The molar ellipticity of myoglobin at 220 nm remains constant up to 5.0 M urea (Figure 3), suggesting that little change in the secondary structure occurs in this range of denaturant concentration. At higher concentrations of urea, a marked decrease in molar ellipiticity occurs with a transition midpoint at 6.8 M urea. This denaturation curve is in very good agreement with the one reported by Puett (1973), who followed the denaturation of whale myoglobin by measuring the change in the extinction coefficient of the Soret band at 409 nm. The coincidence of the two curves is consistent with a highly cooperative, two-state unfolding of the molecule under these denaturing conditions.

When myoglobin is injected onto the SEC column, equilibrated with from 6.0 to 8.5 M urea, two peaks are observed to elute from the column (Figure 4). On the basis of their

Table II: Intrinsic Viscosity, Stokes Radius, and K_d Values Determined on the TSK 3000SW Column

protein	solvent system[a]	M_r ($\times 10^{-3}$)	K_d[b,d]	$[\eta]$[c] (mL/g)	R_s[c]	ref
catalase	0.2 M NaCl	220	0.508		52	LeMaire et al. (1980)
LDH	0.2 M NaCl	141	0.514	3.8	43.9	Castellino & Barker (1968)
	6.0 M GdmCl (R)	35		32.1	56.2	
transferrin	0.2 M NaCl	81	0.490		36	LeMaire et al. (1980)
	6.0 M GdmCl	81	0.071		86.8	Fish et al. (1970)
BSA	0.2 M NaCl	66.3	0.492	3.7	33.9	Tanford et al. (1967)
	8.0 M urea (0) + 0.2 M NaCl	66.3	0.196	16.6	55.9	Tanford et al. (1967)
	8.0 M urea (R) + 0.2 M NaCl	66.3	0.093	43.2	76.8	Lapanje (1969)
	6.0 M GdmCl (0)	66.3	0.166	22.9	62.2	Tanford et al. (1967)
	6.0 M GdmCl (R)	66.3	0.094	52.2	81.8	Tanford et al. (1967)
ovalbumin	0.2 M NaCl	43.5	0.576	4.4	31.2	Castellino & Barker (1968)
	8.0 M urea (R) + 0.2 M NaCl	43.5	0.205	29.5	58.8	Ahmad & Salahuddin (1974)
	6.0 M GdmCl (R)	43.5	0.175	34.6	62.0	Castellino & Barker (1968)
carbonic anhydrase	0.2 M NaCl	28.8	0.719	2.9	23.6	Wong & Tanford (1973)
	6.0 M GdmCl (R)	28.8	0.270	29.6	51.3	Wong & Tanford (1973)
β-lactoglobulin	0.2 M NaCl	36.8	0.666	3.4	27.1	Tanford et al. (1967)
	8.0 M urea (0) + 0.2 M NaCl	18.4	0.438	16.2	36.1	Tanford et al. (1967)
	8.0 M urea (R)	18.4	0.374	21.6	39.8	Lapanje (1969)
	6.0 M GdmCl (0)	18.4	0.415	19.1	38.2	Tanford et al. (1969)
	6.0 M GdmCl (R)	18.4	0.376	22.8	40.5	Tanford et al. (1967)
hemoglobin	0.2 M NaCl	64	0.757	3.6	33.2	Tanford et al. (1967)
	6.0 M GdmCl	16	0.441	18.9	57.6	
myoglobin	0.2 M NaCl	16.9	0.843	3.1	20.2	Tanford et al. (1967)
	6.0 M GdmCl	16.9	0.469	20.1	37.8	Ahmad & Salahuddin (1974)
ribonuclease	0.2 M NaCl	13.7	0.876	3.3	19.3	Tanford et al. (1967)
	8.0 M urea (0) + 0.2 M NaCl	13.7	0.590	7.6	25.5	Tanford et al. (1967)
	8.0 M urea (R) + 0.2 M NaCl	13.7	0.471	15.6	32.4	Lapanje (1969)
	6.0 M GdmCl (0)	13.7	0.536	9.4	27.3	Tanford et al. (1967)
	6.0 M GdmCl (R)	13.7	0.437	16.3	32.8	Tanford et al. (1967)
cytochrome *c*	0.2 M NaCl	11.7	0.969		17	Tanford (1974)
	6.0 M GdmCl (R)	11.7	0.487		30.4	Fish et al. (1970)
insulin	6.0 M GdmCl (0)	5.78	0.709	5.5	17.1	Tanford et al. (1967)
	6.0 M GdmCl (R)	2.97[e]	0.759	6.1	14.2	Tanford et al. (1967)

[a] See footnote *a* of Table I for a complete description of the solvent composition. (R) denotes that the protein was preincubated in 0.01 M DTT while (0) indicates that no reducing agent was present in the sample solution. [b] Values determined in this study. [c] The Stokes radii (R_s) of proteins for which intrinsic viscosity data ($[\eta]$) are available were calculated by using the relationship $[\eta] = (2.5N/M_r)(^4/_3\pi R_s^3)$ as suggested by Tanford et al. (1970), where N is Avogadro's number and M_r is the molecular weight. [d] In order to reduce the systematic error, the elution volume of DTT was used as an internal standard marker for V_t. For samples containing DTT, the values listed in Table I were used. [e] This value refers to the average molecular weight of insulin a and b chains.

$\text{erf}^{-1}(1 - K_d)$ values and the corresponding Stokes radii (Figure 2), we can conclude that peaks I and II (Figure 4) correspond to the folded and unfolded states of the myoglobin molecule, respectively. Clearly, the rate of interconversion of the two species is slow under the column conditions defined in the legend to Figure 4. As the concentration of urea is increased, the area of peak I (folded) decreases with a corresponding increase in the area of peak II (unfolded). When the areas of the two peaks are normalized by taking into account the differences in absorbance at 220 nm between folded and unfolded myoglobin, it is found that the transition curve for the urea-induced unfolding has the same midpoint as that obtained by following changes in molar ellipticity (Figure 3). In order to assess the reversibility of the transition, samples of a stock solution of myoglobin in 8 M urea were diluted to the various concentrations of urea indicated in Figure 4, incubated, and then injected onto the SEC column equilibrated with the same concentration of urea. The elution profiles obtained were identical with those shown in Figure 4, indicating that the transition is reversible. The coincidence of the midpoints of the SEC transition curves with those obtained by using other properties demonstrates that, although they are well separated as they elute from the column, the folded and unfolded states maintain their equilibrium concentrations and thus a true equilibrium transition curve is obtained.

As a result of the favorably slow kinetics prevailing under the conditions of these experiments, it is possible to study the effect of urea concentration on the size of the folded protein throughout the range of denaturant concentrations up to 8.5 M urea. The results obtained (Figure 5) clearly indicate that urea causes a significant swelling of the folded conformation of myoglobin up to and beyond the equilibrium transition point. The difference in volume between the native fold in the absence of urea and the swollen but still globular molecule in 8.0 M urea is 1.07×10^4 Å^3/molecule. This corresponds to a 29%

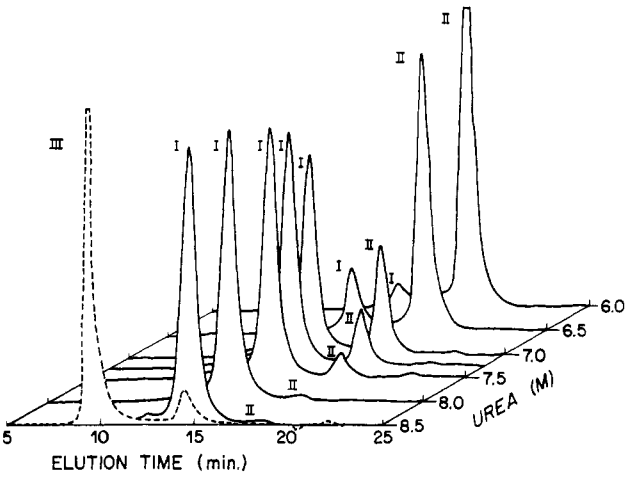

FIGURE 4: Urea-induced unfolding of sperm whale myoglobin as determined by SEC–HPLC. Illustrated are the elution profile obtained when 50 μL of myoglobin solution was injected onto the TSK 3000SW column at various urea concentrations. Buffer conditions are given in Figure 3. The labeled peaks refer to (I) unfolded myoglobin, (II) folded myoglobin, and (III) oligomeric myoglobin formed after 24-h incubation in 8.5 M urea.

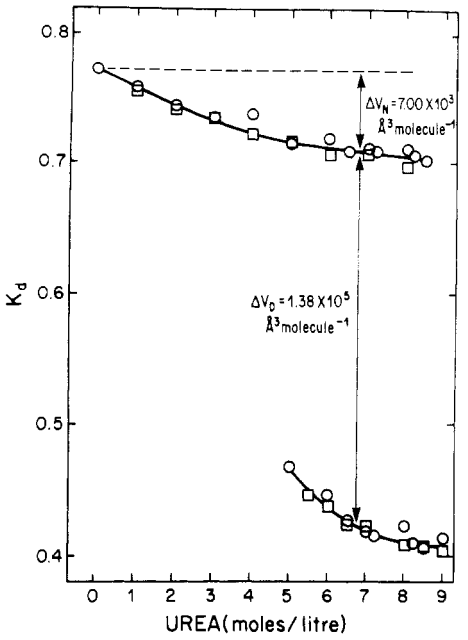

FIGURE 5: Effect of urea concentration on the K_d value of folded (upper curve) and unfolded (lower curve) myoglobin. (○) Unfolding experiments; (□) refolding from 8 M urea. Volume changes (ΔV) were calculated from R_s values obtained from the calibration curve of Figure 2 and $V = {}^4/_3\pi R_s^3$.

swelling of the folded molecule. The swelling of the unfolded molecule in the range from 6.7 to 8 M urea, 2.6×10^4 Å^3, is much greater and reflects the higher accessibility of the unfolded polypeptide coil domain to solvent.

The volume of the unfolded molecule in 8.0 M urea obtained in this study is 2.21×10^5 Å^3, which compares well with the volume, 2.08×10^5 Å^3, calculated from Tanford's expression for the intrinsic viscosity of random-coil proteins in 8 M urea: $[\eta] = 76n^{0.655}$ (Tanford, 1968). By the same token, the volume change for the transition from the native fold to the random coil in 8 M urea, 1.70×10^5 Å^3, compares favorably with the value of 1.73×10^5 Å^3 calculated from the intrinsic viscosities of the native structure [3.1 cm^3/g (Tanford, 1968)] and the random coil [18.24 cm^3/g; calculated from the expression given above (Tanford, 1968)]. These results serve to demonstrate the accuracy with which Stokes radii and hence equivalent hydrodynamic volumes may be determined by using the SEC–HPLC method.

The swelling results (Figure 5) provide useful insights into the mechanism of urea denaturation of globular proteins. For many years, the mechanism of solvent denaturation based on the concept of preferential solvation of the unfolded polypeptide coil by denaturant has been in vogue. However, because both the folded and unfolded conformations of myoglobin interact with and are swollen by urea, the unfolding cannot be thought of as being due solely to the preferential solvation of the unfolded form by urea. The preferential solvation mechanism has been critically reevaluated in general terms by Schellman & Hawkes (1980). They conclude that this model should now be treated with caution. The present results support this point of view and suggest that, in the case of myoglobin, the molecule unfolds gradually as a result of the urea-induced swelling of the native globule over the whole range of denaturant concentrations up to and beyond the equilibrium transition point. Evidence for the massive penetration of a globular protein by urea has been obtained in the case of crystalline α-chymotrypsin by X-ray crystallographic methods (Hibbard & Tulinsky, 1978). The latter authors also demonstrated that urea differes significantly from Gdn·HCl in its interactions with chymotrypsin, which suggests that attempts to develop a general molecular mechanism of solvent denaturation which does not take into account such differences between denaturants are unrealistic.

A major objective of denaturation studies is the evaluation of the free energy of stabilization, $\Delta G°_u$, of the native fold under physiological conditions. As Schellman & Hawkes (1980) have pointed out, this requires a rather long extrapolation of equilibrium data from the transition region which is usually far from physiological. Another point raised by the latter authors relates to the linearity of the extrapolation of $\Delta G°_u$ from the transition region to zero denaturant concentration, an extrapolation which is often assumed to be linear in the absence of substantiating experimental evidence. The data summarized in Figure 5 indicate that a linear extrapolation of ΔV to zero denaturant concentration would be invalid. However, the extrapolation of the free energy of swelling, $\Delta G_s = -RT \ln K_d$, of the folded form is linear and gives a value of 0.9 kcal/mol in the absence of urea. The $\Delta G°_u$ extrapolation is linear in the region from 5.0 to 8.5 M urea. The results of this study do not allow an evaluation of the linearity of the free-energy plot below 5 M urea. On the assumption that the free energy of unfolding is a linear function of urea concentration, we obtain a value of 14.1 kcal/mol for the free energy of stabilizaton of myoglobin. The latter value compares well with the value of 13.0 kcal/mol obtained by Puett (1973) under the same conditions as used in the present study.

It was found that myoglobin, incubated in 7–9 M urea for 24 h or longer, formed high molecular weight oligomers (peak III, Figure 4) with a maximum apparent molecular weight of 64 000 under the conditions of these experiments. Dilution of the oligomeric material to lower urea concentrations led to precipitation. The slow aggregation of proteins in the presence of high concentrations of denaturants has been observed in many studies. Goldberg & Zetina (1980) have studied this phenomenon in the case of the β2 subunit of *Escherichia coli* tryptophan synthetase and, after an exhaustive search, have established conditions under which the reversible transition of the subunit can be studied. We suggest that SEC techniques could be used effectively to explore protein aggregation phenomena systematically.

Thermal Unfolding of Bovine Ribonuclease A. Hermans & Scheraga (1961) have reported that, at pH 2.0 and ionic strength 0.16 M, ribonuclease has a transition temperature of 33 °C. Under similar solvent conditions, we have established that it is possible to obtain on the TSK 3000SW column a complete thermal unfolding curve for ribonuclease with disulfide bonds intact. As the plot in Figure 6 indicates, the retention time of ribonuclease decreases markedly as the column temperature is increased. The transition temperature is 32 °C under the conditions specified in the legend to Figure 6. The decrease in the K_d values, from 0.895 (5 °C) to 0.769 (50 °C), corresponds to an increase in the Stokes radius from 19.5 to 22.5 Å (increase in volume of 1.66×10^4 Å^3/molecule) over the temperature range studied. This is comparable to the change in radius (from 19.5 to 23.5 Å) calculated from the increase in intrinsic viscosity (from 3.4 to 5.95 mL/g) measured in the same temperature range at pH 2.8 and 0.15 M KCl by Holcomb & van Holde (1962). These results demonstrate that the permeability properties of the TSK SW3000 column are not significantly dependent on temperature in the range up to 50 °C.

It is also interesting to compare the retention times of thermally unfolded ribonuclease with ribonuclease unfolded with GdmCl or urea, in each case with the disulfide bonds

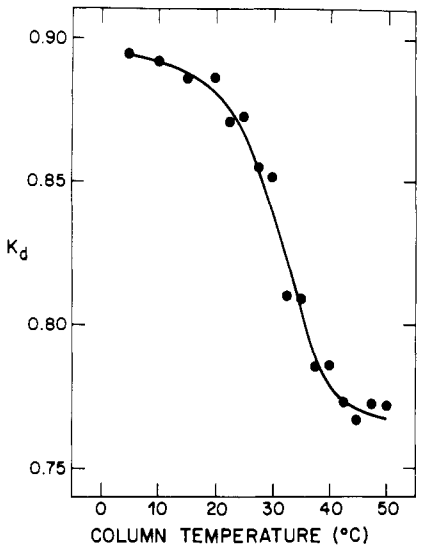

FIGURE 6: Thermal unfolding of ribonuclease as determined by SEC–HPLC. Ribonuclease (0.85 mg/mL) was incubated for 30 min in 0.17 M KCl/HCl, pH 7.0, at the temperature of interest. Fifty microliter samples of equilibrated protein solutions were injected onto the column which had been preequilibrated at the same temperature. The K_d values were calculated according to the equation $K_d = (V_s - V_0)/(V_t - V_0)$ by using the V_0 and V_t values reported in Table I.

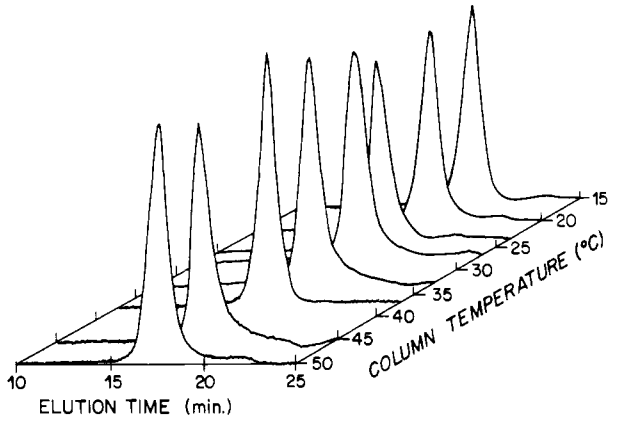

FIGURE 7: SEC–HPLC elution profiles for ribonuclease at various temperatures. Experimental conditions are given in the legend to Figure 6.

intact. It is apparent that ribonuclease at 45 °C (K_d = 0.767; R_s = 22.5 Å) does not have as expanded a structure as it has in 8.0 M urea (K_d = 0.590; R_s = 28.3 Å) or in 6.0 M GdmCl (K_d = 0.536; R_s = 30.5 Å) at 25 °C. Evidence that heat-denatured ribonuclease contains residual structure is provided by the observation that addition of Gdn·HCl to the thermally unfolded protein produces another transition (Aune et al., 1967). The present results provide further evidence for multiple denatured states in this protein (Ahmad & Bigelow, 1979).

The chromatograms for ribonuclease shown in Figure 7 have only a single peak. Although the difference in volume between folded and unfolded ribonuclease with disulfide bonds intact is not as large as that observed for the folded and unfolded forms of myoglobin, the two forms differ sufficiently in volume to be resolved on the TSK 3000SW column. The fact that, in contrast to the behavior of myoglobin, only a single peak is seen in the elution pattern for ribonuclease can be accounted for in terms of the kinetics of thermal folding–unfolding processes. If conformational equilibration is rapid relative to the time scale of the column elution process, then a single peak with an elution time which is the weighted average of the forms in equilibrium will be observed.

Although the transition temperature obtained, 32 °C, is in good agreement with the value of Hermans & Scheraga (1961), the van't Hoff ΔH for thermal unfolding, 36 ± 3 kcal/mol (derived from the transition curve shown in Figure 6), is significantly lower than most of the values reported in the literature [summarized by Privalov (1979); see his Table I]. As Privalov (1979) has pointed out, the data in the literature provide equivocal evidence for the two-state model in the case of ribonuclease. Westmoreland & Matthews (1973) have concluded on the basis of their NMR study of the thermal denaturation of ribonuclease that a multistate mechanism is operative at pH 1.3. Tsong et al. (1970) have performed a calorimetric study of the thermal unfolding of ribonuclease in which they find evidence for a multistate transition at pH 2 and higher. Temperature jump experiments performed at low pH also indicate that intermediate states are present on the unfolding pathway (Tsong & Baldwin, 1972; Tsong et al., 1971). The value for the van't Hoff enthalpy determined in the present study is a measure only of the enthalpy of the volume changes. Large contributions to the enthalpy from processes which have negligible volume changes will not be detected by the HPLC–SEC method.

Conclusions

It is clear from the results obtained in the present study that the TSK 3000SW gel column has considerable potential for the study of proteins under a wide variety of solvent conditions and temperatures in the range 5–50 °C. The technique of size-exclusion chromatography on HPLC columns is considerably faster than other transport techniques such as viscosity and sedimentation and other conventional gel chromatographic techniques. The use of the TSK 3000SW column is limited by the fact that it is not stable outside the pH range from 2.0 to 7.5 and for the extended periods above 45 °C. Also proteins with a high pI, such as lysozyme, have been shown to be retarded on this column, even at high ionic strengths (Imamura et al., 1981). The unique feature of SEC–HPLC demonstrated in the present study is the ability to separate and characterize slowly interchanging conformational states of a given protein. Although myoglobin has been shown to unfold and refold rapidly under some conditions (Litman & Schellman, 1968), the present study has demonstrated that, under conditions which allow for slow unfolding–refolding, it is possible to separate and study the folded and unfolded species without significantly disturbing the equilibrium. The implications for other systems are clear. One potential application of SEC–HPLC in protein folding studies is the systematic exploration of solvent conditions for the reversible folding of large multimeric systems. Another application is to follow the kinetics of unfolding processes (Corbett & Roche, 1983a,b).

The technique described in this paper is conceptually related to the urea gradient electrophoresis method for the study of protein denaturation developed by Creighton (1974, 1980). However, we believe that the SEC technique offers several advantages: (1) It is possible to follow relatively more rapid folding–unfolding transitions by using SEC. (2) In addition to following denaturation by urea, SEC may be used to follow thermal denaturation as well as denaturation by acids and ionic denaturants such as guanidinium chloride. (3) A variety of optical detection methods could potentially be used to study the properties of the eluted species. (4) Protein samples can be recovered at the end of the experiment.

Registry No. Catalase, 9001-05-2; lactic dehydrogenase, 9001-60-9; carbonic anhydrase, 9001-03-0; trypsinogen, 9002-08-8; ribonuclease,

9001-99-4; cytochrome *c*, 9007-43-6; insulin, 9004-10-8.

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