Journal of Chromatography, 314 (1984) 337–347
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 17,135

EFFECTS OF UREA-THERMAL DENATURATION ON THE HIGH-PERFORMANCE CATION-EXCHANGE CHROMATOGRAPHY OF α-CHYMOTRYPSINOGEN-A

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(Received August 8th, 1984)

SUMMARY

Retention parameters of α-chymotrypsinogen-A were determined by isocratic elution for a series of concentrations of calcium acetate and sodium acetate both in the presence and absence of urea. Under non-denaturing conditions of temperature and urea concentration, urea facilitated elution. Under reversible denaturing conditions a sharp drop in chromatographic retention was observed over a narrow temperature range which could be correlated with equilibrium measurements of the protein fluorescence.

Retention of both native and denatured protein could be fit to a non-mechanistic retention model by plotting the log k' against log salt concentration. Conventional interpretation of these findings indicates that, while the number of ions displaced during binding is greater for the denatured protein, the affinity per ion decreases since the retention of denatured protein is much less than native.

Elution profiles obtained under partially denaturing conditions showed a strong flow-rate dependence. We attribute these observations to a rate of equilibration between native and denatured protein that is on the timescale of the chromatographic rate processes.

INTRODUCTION

Since the development of cellulose-based ion exchangers by Peterson and Sober[1], ion-exchange chromatography has been an important tool for the separation of proteins. The recent development of new ion-exchange column materials[2–4] has given rise to high-performance ion-exchange chromatography (HPIEC). Like the cellulose based materials, the new column packings are macroporous and have a hydrophilic surface chemistry. Unlike the cellulose based materials, they are rigid microparticles that can be used at elevated flow-rates and pressures. These new materials afford the advantages of both speed and efficiency compared to the more traditional carbohydrate-based materials[5] (hence, the designation high-performance).

In its simplest formulation, the ion-exchange retention of a protein may be

0021-9673/84/$03.00

viewed as being the result of the interaction of a polyelectrolyte with a charged surface[6,7]. Based on electrostatic considerations several authors have proposed mathematical models for the retention of proteins by ion exchange[8,9]. Recently, Kopaciewicz *et al.*[10] and Rounds and Regnier[11] have derived a model for HPIEC, based on an earlier treatment of Boardman and Partridge[12] which has been shown to be useful for a number of proteins. These models are non-mechanistic insofar as neither the nature of the interaction of the protein with the surface nor the implications of the protein structure in determining retention are fully known. The problem of predicting protein retention is exacerbated by the complex and diverse chemical and physical properties of proteins as a class of compounds. A discussion of some of the problems involved in predicting protein retention and the assumptions and caveats of current theoretical models has been given by Scopes[13].

In light of the complex structure and chemical nature of proteins, it is not surprising that there have been various reports of anomalous retention behavior (*i.e.* multiple peak formation, band shape problems, sharp drops in retention, etc.) using high-performance size-exclusion chromatography (HPSEC)[14], reversed-phase[15] and ion-exchange[16] chromatography. In general these observations have been attributed to changes in the three dimensional structure of the protein which are caused by denaturation.

In a previous investigation[16], we reported that the seemingly anomalous HPIEC retention behavior of hen egg white lysozyme in the presence of urea could be attributed to its urea-thermal denaturation. The present investigation is a continuation of our initial study of the effects of protein denaturation on HPIEC retention parameters. For the purposes of this study, we have selected a globular protein, α-chymotrypsinogen-A, that is somewhat less stable to urea-thermal denaturation than lysozyme and is reported to be reversibly denatured under some conditions[17]. The retention characteristics of both the native and denatured forms of this protein are examined within the framework of the current theoretical models. We believe that this study also provides some practical insights related to the diagnosis of denaturation-related chromatographic artifacts.

EXPERIMENTAL

Materials

Salt-free, six-times crystallized, type II α-chymotrypsinogen-A (bovine pancreas No. C4879) was purchased from Sigma (St. Louis, MO, U.S.A.) and used without further purification. Ultrapure urea was obtained from Schwarz/Mann (Spring Valley, NY, U.S.A.). All other reagents were ACS certified analytical reagent grade. Vydac 101TPB7.7 spherical silica (lot No. 180031) was used in the column packing preparation and was a gracious gift of The Separations Group (Hesperia, CA, U.S.A.). High-purity, HPLC grade water was used throughout and was prepared in-house using a purification system from Mar Cor Medical Services (Harleysville, PA, U.S.A.).

Equipment

The chromatographic system employed has been previously described[16] and consists of a Waters M6000A pump, a Rheodyne Model 7125 injection valve

equipped with a 20-μl sample loop, a Waters Lambda Max Model 480 spectrophotometer and a Hewlett-Packard 3390A reporting integrator. The column was thermostated in a water jacket using a circulating water bath to maintain the temperature to ±0.2°C.

The chromatographic column packing was a weak cation exchanger that was prepared according to the procedure of Alpert[3]. This material has a hydrophilic, poly(aspartic acid) coating bonded on a silica base which has a nominal pore size of 300 Å and an average particle size of 7 μm. A 250 × 4.6 mm I.D. column was packed to a constant pressure of 5000 p.s.i. using a Micromeretics Model 705 stirred-slurry column packer (Norcross, GA, U.S.A.). The column was operated at pressures well below the packing pressure, typically 500–1000 p.s.i., to prevent voiding.

The retention behavior of a test solute, hen egg white lysozyme, was very similar to that previously obtained using a commercially-available column of this type, The PolyCAT A WCX column (Custom LC, Houston, TX, U.S.A.). Symmetrical peaks were observed for both lysozyme and α-chymotrypsinogen-A. Our column had an efficiency of *ca.* 500 theoretical plates for α-chymotrypsinogen-A (1 ml/min, 24°C, sodium acetate–acetic acid buffer at pH = 4.80 and $k' \approx 4$). The capacity factor, k', for a standard sample of α-chymotrypsinogen-A was found to gradually increase *ca.* 10% during the first 50 h of use before stabilizing. It has been suggested that this break-in period may be due to residual ongoing hydrolysis, or some time dependent change in the polymeric phase[3]. Intrinsic fluorescence measurements were made as previously described[16].

Buffers

Mobile phase buffers containing calcium were made by the dilution of a concentrated stock solution prepared from calcium carbonate and glacial acetic acid. The pH of each buffer was adjusted to 4.80 with glacial acetic acid at room temperature (21–24°C) using a glass electrode. The final calcium ion concentration was determined by titration with EDTA after dilution and pH adjustment.

Buffers containing sodium were similarly prepared using a standardized stock solution of carbonate free sodium hydroxide and glacial acetic acid.

All mobile phase buffers were filtered through a Millipore type HA (0.45 μm) filter and degassed by sparging with helium before use. Solutions containing urea were prepared fresh daily.

Chromatography

All chromatograms were run using isocratic conditions at a nominal flow-rate of 1 ml/min unless otherwise specified. A 20-μl sample size was injected manually using the filled loop technique. The capacity factor, k', was determined using the observed time of the solvent perturbation as the retention time of an unretained component*. Measurements of k' made by replicate injections were found to be reproducible to better than ±1% in the k' range of 2–12. The day-to-day k' reproducibility was generally better than ±2%. This was accessed by using standard samples under standard conditions to insure column stability during the course of the study.

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* The capacity factor, k', was calculated using the equation $k' = (t_R - t_0)/t_0$ where t_R = component retention time and t_0 = retention time for unretained component.

Sample preparation

Protein samples were prepared to contain *ca.* 1 mg/ml by dissolving the protein in the mobile phase. In some cases a small amount of water was added to the sample to facilitate measurement of the unretained peak from the solvent perturbation. This was found not to affect the retention time of the protein component.

RESULTS

When the retention of α-chymotrypsinogen-A was measured as a function of temperature in a urea-containing calcium acetate–acetic acid buffer, a sharp drop in the capacity factor, k', was observed to occur over a narrow temperature range as shown in Fig. 1. The midpoint of this transition, $T_{1/2}$, was found to shift to a lower temperature when the urea concentration was increased from 5.0 to 5.5 *M*. Shown in the inset to Fig. 1 are the results of a set of measurements of the intrinsic fluorescence spectrum of the protein in the same buffers used in the chromatographic retention studies. Qualitatively, when the protein is heated, the wavelength of the emission maximum, λ_{max}, shifts to a longer wavelength with a concomitant decrease in the overall fluorescence intensity. These changes are attributed to the changing microenvironment of tryptophan residues in the protein concomitant with conformational changes in protein structure due to urea-thermal denaturation. As indicated by the inset, λ_{max} sharply increases over a narrow temperature range and $T_{1/2}$ of the observed transition also shifts to a lower temperature as the urea concentration is increased. Similar results were obtained using urea-containing sodium acetate–acetic acid buffers although in these cases the $T_{1/2}$ for the transitions were generally shifted to lower temperatures (Fig. 2). The higher $T_{1/2}$ observed with calcium can be rationalized, since the binding of calcium by this protein is known to enhance its stability[18]. The midpoint temperature of each transition in both the chromatographic studies

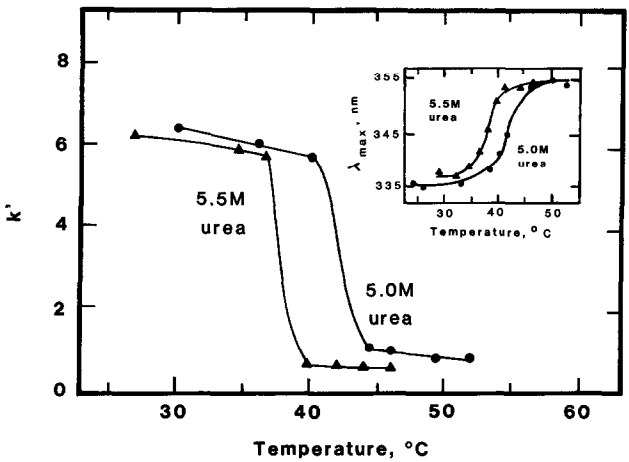

Fig. 1. Effect of urea-thermal denaturation on the HPIEC retention of α-chymotrypsinogen-A using a calcium acetate buffer (pH 4.80, $[Ca^{2+}]$ = 0.0294 *M*) containing (●) 5.0 *M* and (▲) 5.5 *M* urea. Inset: wavelength of maximum intensity derived from equilibrium measurements of the intrinsic fluorescence spectrum in the same buffers used in the chromatography.

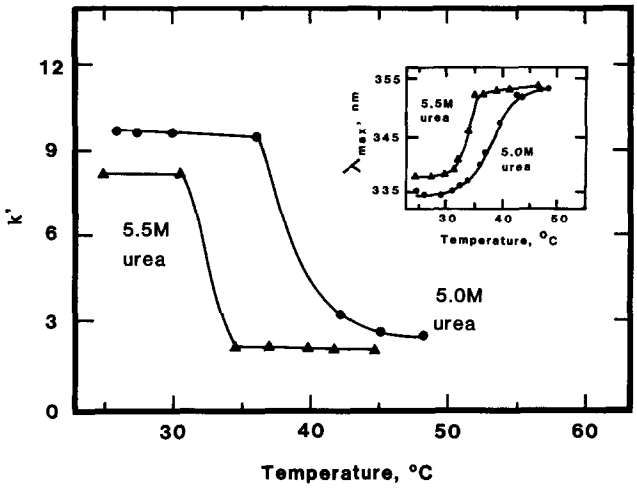

Fig. 2. Effect of urea-thermal denaturation on the HPIEC retention of α-chymotrypsinogen-A using a sodium acetate buffer (pH 4.80, $[Na^+]$ = 0.1589 *M*) containing 5.0 *M* (●) and 5.5 *M* (▲) urea. Inset: wavelength of maximum intensity derived from equilibrium measurements of the intrinsic fluorescence spectrum in the same buffers used in the chromatography.

and the fluorescence studies was determined graphically and are compared in Table I. In all cases, the $T_{1/2}$ from chromatographic retention data and fluorescence are well correlated.

Typical chromatograms obtained through the transition range are shown in Fig. 3. Once again, gross changes in the chromatograms are observed to occur over a narrow temperature range. It is seen that the peak collapses and becomes ill-defined in the temperature range of the denaturation transition. This presents a problem in obtaining retention data in the transition region.

The capacity factor, k', was measured as a function of calcium ion concentration both in the presence and absence of urea and at temperatures well above and below the midpoint transition temperature previously determined. As shown in Fig. 4A, generally, k' had a strong dependence on calcium ion concentration and in-

TABLE I

COMPARISON OF THE MIDPOINT TEMPERATURES OF THE UREA-THERMAL TRANSITION, $T_{1/2}$, DETERMINED USING CHROMATOGRAPHIC RETENTION DATA AND INTRINSIC FLUORESCENCE

*Buffer composition**	$T_{1/2}$** (°C)	$T_{1/2}$*** (°C)
0.0294 *M* Ca^{2+} plus 5.0 *M* urea	42 ± 1	41 ± 1
0.0294 *M* Ca^{2+} plus 5.5 *M* urea	38 ± 1	38 ± 1
0.1589 *M* Na^+ plus 5.0 *M* urea	39 ± 1	38 ± 1
0.1589 *M* Na^+ plus 5.5 *M* urea	32 ± 1	33 ± 1

* Metal acetate–acetic acid buffers at pH 4.80.
** Determined graphically from chromatographic retention data.
*** Determined graphically from intrinsic fluorescence data.

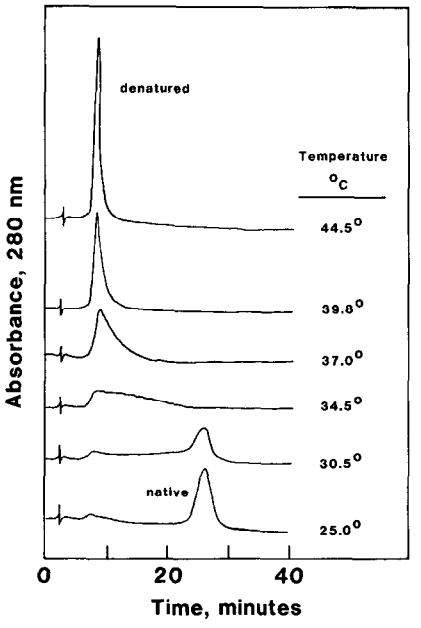

Fig. 3. Typical chromatograms obtained at selected temperatures through the urea-thermal transition region in a sodium acetate buffer containing 5.5 *M* urea (pH 4.80, $[Na^+]$ = 0.01589 *M*).

creased rapidly as the concentration was decreased. Increasing the temperature was found to decrease retention both in the absence and presence of urea. The addition of urea to the buffer was found to decrease overall retention. It is interesting to note that under denaturing conditions (47°C and 5.0 *M* urea) the retention characteristics of the denatured protein were qualitatively similar to those observed under non-denaturing conditions.

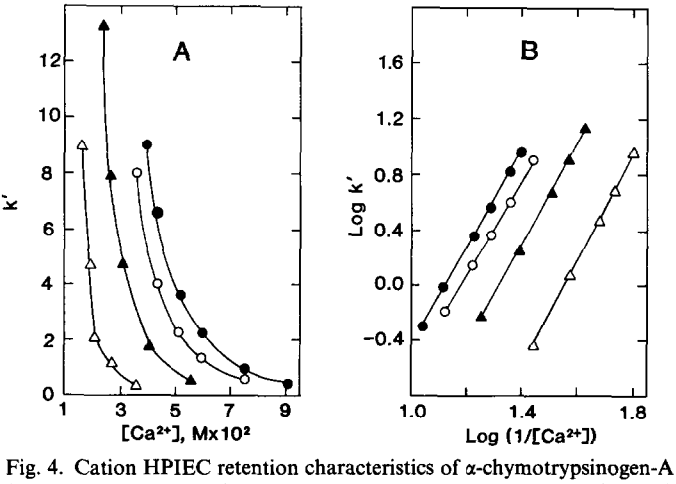

Fig. 4. Cation HPIEC retention characteristics of α-chymotrypsinogen-A in various calcium acetate buffers (pH 4.80) at selected temperatures. (A) Retention under non-denaturing conditions at 26°C (●), 47°C (○) and 25.6°C (▲) plus 5.0 *M* urea and denaturing conditions (△) 47°C plus 5.0 *M* urea. (B) Retention data from A replotted using the retention model given by eqn. 1.

Using the protein retention model proposed by Kopaciewicz *et al.*[10], the data from Fig. 4A were replotted using the equation:

$$\log k' = {}^3/_2 Z \log\left(\frac{1}{[Ca^{2+}]}\right) + \log c \quad (1)$$

where k' = capacity factor, Z = number of charged groups involved in the adsorption–desorption process, c = collection of constants.

As shown in Fig. 4B, linear relationships were obtained using this formalism for both denaturing and non-denaturing conditions. Similar results were obtained when sodium was substituted for calcium as shown in Fig. 5. In this case, however, the equation:

$$\log k' = 2 Z \log\left(\frac{1}{[Na^{+}]}\right) + \log d \quad (2)$$

(where d = collection of constants) was used as the retention model to reflect the use of a monovalent ion (*i.e.*, Na^+).

Using linear regression, the slopes and intercepts of data from Fig. 4B and 5B (plus some additional data not shown) were calculated and are summarized in Table II. The quantity Z, which is conventionally interpreted as being related to the number of charged groups involved in the adsorption/desorption process[10], was calculated from the line slope, using

$$Z = {}^3/_2 \text{ (slope)} \quad (3)$$

for calcium, and

$$Z = {}^1/_2 \text{ (slope)} \quad (4)$$

for sodium.

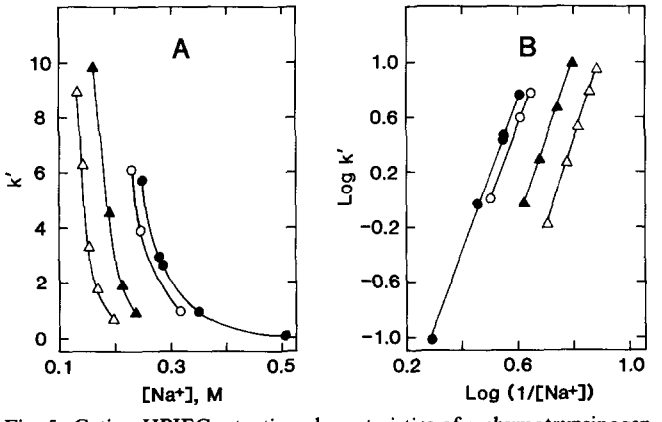

Fig. 5. Cation HPIEC retention characteristics of α-chymotrypsinogen-A in various sodium acetate buffers (pH = 4.80) at selected temperatures. (A) Retention under non-denaturing conditions at 26°C (●), 47°C (○) and 26°C (▲) plus 5.0 *M* urea and denaturing conditions 47.2°C (△) plus 5.0 *M* urea. (B) Retention data from A replotted using the retention model given by eqn. 2.

TABLE II

LINEAR PARAMETERS CHARACTERIZING THE HPIEC RETENTION OF α-CHYMOTRYPSINOGEN-A

Arrow indicates denaturing conditions.

*Elution buffer**	*Temperature (°C)*	*Slope***	*Intercept***	*Z****
Ca^{2+} (no urea)	26.0	3.50 ± 0.04	−3.94 ± 0.09	2.33 ± 0.03
	35.5	3.40 ± 0.03	−3.91 ± 0.04	2.27 ± 0.02
	39.0	3.41 ± 0.04	−4.06 ± 0.05	2.27 ± 0.03
	47.0	3.39 ± 0.04	−4.00 ± 0.05	2.26 ± 0.03
Ca^{2+} plus 5.0 *M* urea	25.6	3.52 ± 0.01	−4.64 ± 0.01	2.35 ± 0.01
	32.0	3.49 ± 0.03	−4.63 ± 0.05	2.33 ± 0.02
	→47.0	3.89 ± 0.16	−6.06 ± 0.26	2.59 ± 0.11
Na^{+} (no urea)	26.0	5.83 ± 0.20	−2.75 ± 0.09	2.91 ± 0.13
	47.0	5.53 ± 0.22	−2.75 ± 0.13	2.77 ± 0.15
Na^{+} plus 5.0 *M* urea	26.0	5.75 ± 0.06	−3.60 ± 0.04	2.88 ± 0.04
	→47.2	6.52 ± 0.02	−4.80 ± 0.02	3.26 ± 0.01

* Metal acetate–acetic acid bufffers at pH 4.80.
** Error estimates for 90% confidence limit.
*** Calculated from the slope using eqn. 3 for calcium and eqn. 4 for sodium buffers (see text).

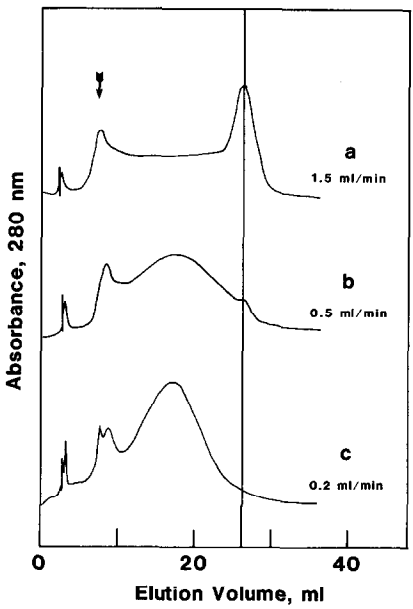

Fig. 6. Effect of flow-rate on the chromatographic profile near the midpoint of the urea-thermal transition (sodium acetate buffer, $[Na^{+}]$ = 0.1589 *M* plus 5.5 *M* urea, 32.5°C). The peak corresponding to native protein is indicated by the fiducial line at *ca.* 26 ml. Early eluting peaks (indicated by the arrow) are due to some irreversible decomposition of the protein.

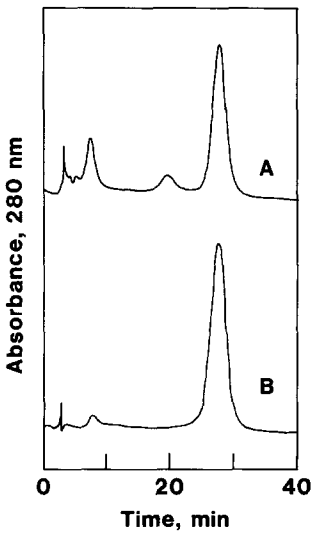

Fig. 7. Chromatographic evidence for the apparent reversibility of the urea-thermal denaturation. (A) A sample of α-chymotrypsinogen-A was heated in the same buffer described in Fig. 6 for 2.5 h at 39°C (denaturing conditions), cooled and chromatographed at 24°C. A control sample which had not been heated is shown in B. Note the appearance of minor degradation products at shorter retention times.

As shown in Table II, Z was found to be relatively independent of temperature for buffers containing calcium without urea. In the presence of calcium and urea under non-denaturing conditions the Z values were essentially the same as those determined in the absence of urea. Under denaturing conditions, however, there is a small but measurable increase in the value of Z. Although the data for sodium are not as complete as for calcium, the result is similar (*i.e.*, the Z value increases with denaturation).

A preliminary investigation of some of the kinetic aspects involved in the chromatography of proteins subjected to denaturing conditions was undertaken. When α-chymotrypsinogen-A was chromatographed under conditions near its $T_{1/2}$ value, the chromatograms obtained were found to be a function of the column flow-rate. For the purpose for comparison, elution volumes rather than retention times were used (Fig. 6). As the flow-rate is decreased, the peak corresponding to the native protein (as indicated by the fiducial line) diminishes and a broad peak with a retention volume intermediate between native and denatured is observed. Similar experiments outside the denaturation transition do not show flow-rate dependence.

In a control experiment, the reversibility of the urea-thermal transition under these conditions was studied. In Fig. 7A a chromatogram is shown which was obtained after the protein was heated in urea-containing buffer under denaturing conditions for an extended period, cooled and then chromatographed under non-denaturing conditions. Although some minor peaks were observed at shorter retention times compared to a sample that had never been heated (Fig. 7B), the peak corresponding to native protein was by and large intact. These minor peaks are believed to be due to either carbamylation or degradation of α-chymotrypsinogen-A by chymotrypsin contamination in the sample preparation. In a separate experiment, it was

found that samples of protein which had been heated and cooled as for Fig. 7A gave a fluorescence spectrum indistinguishable from that of native protein which had never been heated.

DISCUSSION

Based on the close correlation between the $T_{1/2}$ measured by two independent techniques (*i.e.*, chromatographic retention and intrinsic fluorescence), we believe that the sharp drop in retention can be attributed to conformational changes in the protein which occur during urea-thermal denaturation. It has been reported that under certain conditions this protein undergoes a reversible transition during its denaturation which can be approximated by a two-state model (*i.e.* native denatured)[17,19,20]. Although we make no claim as to the validity of the two-state approximation under these conditions, the results of our control experiments indicate that the conformational changes involved can be reversed.

The drop in HPIEC retention upon denaturation can be contrasted with reported increases in the retention of denatured proteins for reversed-phase chromatographic separation[15]. In the case of reversed-phase chromatography, the increase in retention has been rationalized on the basis that during denaturation additional hydrophobic residues from the protein interior which are exposed when the protein unfolds participate to produce stronger binding of the denatured protein[21]. We do not find a parallel in the present HPIEC studies. In terms of current theoretical models of retention behavior, it would seem that even though the net number of ions displaced was greater for the denatured protein compared to the non-denatured form (*i.e.* $Z_{\text{denatured}} > Z_{\text{native}}$), the net binding strength of the denatured protein was decreased, as evidenced by the sharp drop in HPIEC retention concomitant with denaturation. It should be pointed out, however, that even though the interpretation of the Z number is based on theoretical considerations, its meaning is still somewhat uncertain since the retention model itself is non-mechanistic.

It is interesting to note that the retention behavior of the denatured protein is consistent with expectations based on the proposed HPIEC retention model and is qualitatively similar to that observed for the undenatured form. One possible explanation for the similarity in retention behavior between the native and denatured states outside the transition region is that on the chromatographic timescale the observed retention reflects macroscopic properties of each particular state. We emphasize that denatured and native states cannot be distinguished by their fit to model behavior. Linear plots were obtained (Figs. 4 and 5) describing the elution of both native and denatured protein, with only small differences in slope. Additional criteria are required to determine whether the protein is native or denatured.

Even in our limited kinetic experiments, the value of varying the column flow-rate in diagnosing denaturation artifacts is evident. Changes in the chromatographic pattern with flow-rate would be expected in a system equilibrating slowly during transport[22,23]. We believe that changes in peak shape and retention time with flow-rate that occur in the transition region are due to slow equilibration kinetics[24]. Assuming for the sake of simplicity a reversible two-state denaturation model, one can propose the following scheme for the binding of native and denatured protein to the ion-exchange stationary phase:

$$\begin{array}{ccc} \text{Native}_{\text{Free}} & \rightleftharpoons & \text{Denatured}_{\text{Free}} \\ \downarrow \uparrow & & \downarrow \uparrow \\ \text{Native}_{\text{Bound}} & \rightleftharpoons & \text{Denatured}_{\text{Bound}} \end{array}$$

In a case where the interconversion between native and denatured states is slow compared to the chromatographic timescale, two peaks could be expected to be observed, corresponding to native and denatured forms. For a fast equilibration rate, one peak corresponding to the average retention time would be predicted. In an intermediate kinetic region, asymmetrical bands may result. Thus, by changing the chromatographic timescale, in some cases secondary equilibrium effects resulting from on-column denaturation may be detected. Without any loss of generality, the scheme above can be extended to multi-state denaturation models so long as the denaturation processes involved are relevant on the chromatographic timescale.

Although forcing conditions were used in this study to produce denatured protein, we believe that our results may prove useful in understanding the retention behavior of less stable proteins.

ACKNOWLEDGEMENT

This work was supported in part by a Biomedical Research Grant from the NIH Division of Research Resources. The authors thank Dr. Joseph Glajch for reviewing their manuscript.

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