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USE OF HIGH-PERFORMANCE SIZE-EXCLUSION, ION-EXCHANGE, AND HYDROPHOBIC INTERACTION CHROMATOGRAPHY FOR THE MEASUREMENT OF PROTEIN CONFORMATIONAL CHANGE AND STABILITY

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SUMMARY

Size-exclusion, ion-exchange, and hydrophobic interaction chromatographic techniques able to detect conformational changes induced by urea were developed for three globular proteins: bovine serum albumin, lysozyme, and trypsin. Alterations in tertiary structure were manifest chromatographically by highly reproducible changes in peak height, retention, and appearance of multiple peaks. Denaturation equilibria and kinetics obtained by classical physical methods, such as fluorescence intensity measurements for bovine serum albumin and enzyme activity for trypsin, could be correlated to particular changes in chromatographic behavior. The chromatographic methods utilized were selective toward specific structural changes and monitored independent denaturation steps via multiphasic kinetics. The correlation of chromatographic behavior to both independent measures of conformational changes and to assays which measure loss in biological activity, in the case of select proteins indicates that non-denaturing high-performance liquid chromatography can be a useful tool to detect and quantitate perturbations of protein three dimensional structure which result in a loss in biological activity.

INTRODUCTION

A major current challenge to the pharmaceutical industry is the development of proteins as drugs. Macromolecular drug substances have unique and complex analytical and stability problems[1]. While biological activity will reside in part in the primary structure of a protein, in the majority of cases the functional (native) state is due to a specific three dimensional configuration assumed by the polypeptide chain. Changes in protein primary structure (and perhaps activity changes which are due to primary structure alteration) due to chemical reactions can generally be quantitatively measured by chromatographic techniques such as reversed-phase high-per-

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formance liquid chromatography (HPLC)[2], which are widely used in the pharmaceutical industry. The number of analytical methods readily applicable to the detection and quantitation of changes in tertiary structure —that is, conformation changes and denaturation, which can lead to loss of biological activity— are, however, quite limited. A number of examples exist for correlations between instrumental measurements of protein physico-chemical properties and the bioactivity of such substances. Correlations between circular dichroism measurements and biological activity have been found for pyruvate kinase[3], soybean amylase[4], and fibronectin[5]. Fluorescence measurements provide good correlation with activity for antithrombin III[6], but poor correlation with activity loss in creatine kinase[7]. Even changes in solubility have been found to correlate to "catalytic activity" of bovine serum albumin (BSA)[8]. While no correlations between protein chromatographic behavior and biological activity have been published, activities have been measured for antithrombin III[9] and alpha amylase inhibitor[10] by measuring decreases of native protein peaks by size-exclusion chromatography. Due to the familiarity with and heavy reliance upon HPLC methods of analysis in the pharmaceutical industry, analytically valid chromatographic procedures which can detect and quantitate protein conformational alterations are in development. The eventual goal is to employ HPLC as one of several simple, rapid, rugged, inexpensive, and reproducible analytical methods which can measure biological activity loss via physico-chemical measurements.

Numerous studies have shown that high-performance size-exclusion chromatography (SEC) can perform high-resolution separations of proteins in a non-denaturing manner[9–17]. High-performance ion-exchange chromatography can also effect separations of similar proteins under non-denaturing conditions[11,18–26]. Due to the potential for very high resolution, hydrophobic interaction chromatography (HIC) is being actively investigated as a non-denaturing protein separation technique[27–37].

The ability of chromatography to detect conformational differences between proteins is a new area of investigation. With the recent advent of high-performance analytical columns and non-denaturing chromatographic methods, various preliminary studies indicate potential for protein HPLC as an analytical tool to resolve and measure differences in proteins, based on tertiary structure[38]. Conformational changes in several proteins have been detected by size-exclusion chromatography, through changes in retention time[15,17,39–42] where distribution coefficients correlate with Stokes radii of protein conformers[15,16,42], and via separation of new peaks[9,10,12,15,41,43,44]. Ion-exchange chromatography has detected conformational changes induced in lysozyme[45], chymotrypsinogen[24], and in proteins denatured by reversed-phase HPLC[46]. Hydrophobic interaction chromatography has detected calcium-induced conformational changes in calmodulin[35], pH-induced conformational transitions in alpha fetoprotein[36], a time dependent conformational change in ovalbumin[47], and denaturation induced by HIC[48].

Development of stability indicating HPLC assays analogous to the above, for protein drugs, can potentially complement imprecise, costly, and time consuming biological assays (especially for the large number of characterization and stability assays required during the pharmaceutical development of a protein drug substance). Realization of such new methods will aid protein drug formulation by allowing rapid and accurate evaluation of stability (activity) of drug dosage forms based on their

tertiary structure, and determination of changes in protein chemical and physical properties upon long term storage. Familiarity with analytical chromatographic methods for physico-chemical analysis of proteins in the pharmaceutical industry might also allow the ready determination of properties relevant to activity and delivery of polypeptide substances.

Bovine serum albumin (BSA), trypsin, and lysozyme serve as models for protein drugs in this study. These proteins were chosen because of their well characterized structures and denaturation processes, and because each is readily amenable to chromatographic analysis.

BSA has a molecular weight of 66000, an isoelectric point (p*I*) of 4.9 and one free cysteinyl residue[49]. The protein's tertiary structure consists of three spatially distinct domains and several subdomains[49,50] and possesses substantial microheterogeneity[51]. Furthermore, several isomers of BSA exist at various pH's, the most important to this study being the neutral to basic transition which occurs at pH 8[50]. This protein should therefore be susceptible to several different conformational changes and to free sulfhydryl catalyzed disulfide isomerization and aggregation.

Serum albumin has previously been analyzed by several HPLC techniques. Concentrations of monomeric, dimeric, and polymeric serum albumin species in commercial preparations have been determined by SEC[52]. Size-exclusion chromatography has also been employed to measure the rate of serum albumin aggregation upon thermal denaturation[53]. Changes in BSA tertiary structure brought about by reversed-phase HPLC analysis of the protein have been detected by ion-exchange chromatography[46]. BSA has been analyzed by hydrophobic interaction chromatography to assess the resolving power of the method[31]. Hydrogen ion concentration-dependent changes in bovine serum albumin conformation have also been measured by HIC, employing both retention time and peak area changes[36].

High resolution separation of trypsin from other basic proteins has previously been performed using high-performance cation-exchange chromatography[54]. Trypsin has been analyzed in terms of resolution and enzyme activity during high-performance hydrophobic interaction chromatography[31]. HIC of trypsin has also been examined as a function of pH, ionic strength, and bonded phase properties[27,28].

Denaturation of lysozyme by urea and guanidine hydrochloride (which acts in a manner similar to urea[55]) is a well characterized process which has been examined by several methods[45,56–59]. High-performance chromatographic separation of lysozyme have been performed by cation-exchange[18,23,54] and hydrophobic interaction methods[31]. Size-exclusion chromatography has not been possible on currently available columns under non-denaturing conditions due to the pronounced adsorption of lysozyme onto the silica based gel packing[16]. Under denaturing conditions (guanidine hydrochloride present in the mobile phase), changes in lysozyme tertiary structure induced by denaturant can be measured by SEC. Disappearance of native peak, formation of a peak due to denatured protein, and changes in retention times of both peaks all correlate to the extent of conformational change produced by increasing guanidine concentration[41]. Extensive thermal denaturation of lysozyme in urea solutions, and the effect of urea on the cation exchange process have also been measured chromatographically[45].

None of the above studies have, however, attempted to detect multiple conformational changes, to compare different high-performance techniques in terms of

the reproducibility of chromatographic changes due to change in conformation, to correlate biological activity with chromatographic properties, or to study mechanisms of denaturation using chromatographically derived kinetic data. This study is the first to address the above issues, analyzing the above mentioned proteins from a pharmaceutical point of view of "can we reproducibly measure the structural changes in proteins associated with biological activity loss?" Chromatographic methods employed for BSA included size-exclusion, anion-exchange, and hydrophobic interaction chromatography. Denaturation of lysozyme and trypsin was studied by cation-exchange chromatography. These techniques were compared in terms of sensitivity and reproducibility, in terms of the multiple structural changes detected, in terms of ability to measure rates of conformational and/or primary structure change, and for trypsin, in terms of bioactivity change.

Urea was chosen as the denaturing species due to its well documented action[55–57]. Realistically, however, the major causes of destabilization for a pharmaceutical protein product will result from natural (or necessary) environmental conditions such as light and heat, formulation excipients, surface effects, and manufacturing processes. Urea-catalyzed denaturations were examined as models for pharmaceutically relevant conformational changes, primarily because of the magnitude of the urea-induced conformational changes and the ease with which they can be effected. In addition, data obtained from chromatographic methods could be compared to those obtained by other methods which successfully measure urea-induced conformational changes. Denaturation extent and kinetics obtained by classical physical methods (in systems identical to those analyzed chromatographically) such as fluorescence (which represents assays based on energy transfer phenomena) and enzyme activity (which represents assays based on specific quantitative consumption of chemical species) were examined to determine the degree of correlation with specific changes in protein chromatographic behavior and chromatographically measured rates of native protein loss.

EXPERIMENTAL

Chemicals

Ammonium sulfate was purchased from Bio-Rad (Richmond, CA, U.S.A.). Bovine serum albumin (BSA), trypsin, lysozyme, benzoyl-DL-arginine *p*-nitroanilide (BAPNA) and urea were obtained from Sigma (St. Louis, MO, U.S.A.). The albumin employed was fatty acid free. Phosphoric acid, sodium hydroxide, sodium chloride, and sodium azide were purchased from Fisher (Fair Lawn, NJ, U.S.A.). Urea solutions were further treated to remove ionic impurities by stirring with activated charcoal for approximately four hours. Removal of impurities was monitored by ion-exchange chromatography. All other chemicals were used without further purification.

Apparatus

Synchropak S300 column (250 × 4.1 mm I.D.) and Synchropak Propyl columns (250 × 4.1 mm I.D.) were obtained from Synchrom (Linden, ID, U.S.A.). Protein Pak 300 SW column (300 × 7.5 mm I.D.) and Protein Pak DEAE-5PW columns (75 × 7.5 mm I.D.) were purchased from Waters (Milford, MA, U.S.A.). A Zorbax GE-250 column was obtained as a gift from Du Pont.

The chromatographic system used for analyses consisted of a Waters M-490 programmable multiwavelength detector or a Schoeffel (Ramsey, NJ, U.S.A.) GM 770 multiwavelength detector, a Waters Model 510 pump or an Altex (Berkeley, CA, U.S.A.) 110a pump, and a Waters intelligent sample processor. A Hewlett Packard (Avondale, PA, U.S.A.) 3390A integrator or a Linear (Irvine, CA, U.S.A.) 1200 strip chart recorder was used for quantitation. Fluorescence measurements were performed with a Perkin Elmer (Norwalk, CT, U.S.A.) 650-40 spectrofluorophotometer. Colorimetric enzyme assays were performed using a Hewlett Packard 8450A spectrophotometer. Circular dichroism spectra were obtained with a Jasco J-500A spectropolarimeter connected to a DP-500 N signal averager.

Analytical methods —BSA

Chromatography. BSA solutions were prepared at a concentration of 1 mg/ml in 0.02 *M* phosphate buffer pH 7.0, 0.1 *M* sodium chloride and 0–6 *M* urea, and sampled at various times after preparation. An isocratic system for the size-exclusion separation of BSA employed a mobile phase containing 0.02 *M* phosphate buffer pH 7.0, 0.1 *M* sodium chloride, and urea at a concentration equivalent to that in the injected sample. Analyses were performed with a Protein Pak 300 SW size-exclusion column and a flow-rate of 0.75 ml/min. Size-exclusion was the only continuously denaturing chromatographic system employed in the analysis of BSA (that is, containing urea in the mobile phase, equivalent to the sample concentration).

Anion-exchange chromatography was performed using a 20-min linear gradient from 0.0 *M* to 0.14 *M* sodium chloride in 0.075 *M* phosphate buffer (pH 6.5). A Protein Pak DEAE-5PW (weak anion exchanger) column and a flow-rate of 0.9 ml/min were employed. Separation by hydrophobic interaction chromatography was performed using a 20-min linear gradient ranging from 1.6 *M* to 0.2 *M* ammonium sulfate in 0.075 *M* phosphate buffer (pH 7.0). A Synchropak Propyl column and a flow-rate of 0.9 ml/min were used.

Chromatographic peaks were measured at 280 nm (absorbance due to aromatic amino acids) for all proteins and peptides examined. All kinetic studies and chromatographic analyses were performed at controlled room temperature (25–27°C). Efficiency of resolution by a given chromatographic method assumed that urea-catalyzed denaturation is a two state transition with either height (area) or retention changing with increasing urea concentration. For structural changes which were manifest as a decrease in native peak size, attempts were made to detect the denatured protein and to maximize separation of such a peak from that of native proteins by changes in stationary and mobile phases and experimental conditions. Key chromatographic experiments, such as those assessing resolution and quantitation of a given method for a specific protein system, were performed in triplicate to assess reproducibility.

Fluorescence determinations. BSA solutions were prepared at a concentration of 1 mg/ml in 0.02 *M* phosphate buffer (pH 7.0), 0.1 *M* sodium chloride, and 1.0–6.5 *M* urea, and sampled at various times after preparation. Fluorescence intensity measurements of BSA in urea solutions were obtained at an excitation wavelength of 278 nm and an emission wavelength at the λ_{max}. The λ_{max} shifted (between 342 and 349 nm) in a reproducible manner as a result of protein conformational changes and/or urea concentration.

Circular dichroism spectra. Circular dichroism spectra were obtained for BSA concentrations of 1 mg/ml in 0.02 *M* phosphate buffer (pH 7.0), 0.1 *M* sodium chloride, and 0, 3, and 6 *M* urea. An individual spectrum resulted from the average of eight measurements of the spectrum determined with the aid of a DP-500 N signal averager.

Analytical methods —trypsin

Chromatography. A cation-exchange chromatographic method was developed to detect alterations in the native structure of trypsin induced by urea solutions and to separate autolysis degradation products from intact enzyme. Separation was achieved in an isocratic system with a mobile phase consisting of 0.02 *M* phosphate buffer (pH 7.0), 0.1 *M* sodium chloride, and urea equivalent to the sample concentration. Chromatography was also performed without urea in the mobile phase for comparative purposes. A strong cation-exchange column, Synchropak S300, and a flow-rate of 0.9 ml/min were employed. Trypsin solutions were prepared at concentrations of 0.4 mg/ml in 0.02 *M* phosphate buffer (pH 7.0), 0.1 *M* sodium chloride, and 0–6 *M* urea.

Enzyme activity. Enzyme activity of trypsin in urea solutions was determined by measurement of the rate of trypsin-catalyzed hydrolysis of benzoyl-DL-arginine *p*-nitroanilide (BAPNA). Trypsin was dissolved in 0.075 *M* phosphate buffer (pH 7.0) containing 0–6 *M* urea. Enzymatic activity measurements were initiated by adding an equal volume of 0.6 m*M* BAPNA dissolved in the same solvent as the trypsin solution. Absorbance of *p*-nitroaniline (hydrolysis product of BAPNA) was recorded at 390 nm as a function of time to obtain zero order rates of hydrolysis, the magnitude of which were a measure of enzyme activity.

Analytical methods —lysozyme

Chromatography. A cation-exchange chromatographic method was developed for lysozyme which detected changes in retention of lysozyme as a function of urea concentration. Separation was achieved in an isocratic system with a mobile phase containing 0.02 *M* phosphate buffer, 0.26 *M* sodium chloride and urea equivalent to sample concentration, at pH 7.0. A strong cation-exchange column, Synchropak S300, and a flow-rate of 1.0 ml/min were employed. Lysozyme solutions were prepared at a concentration of 0.6 mg/ml.

Enzyme activity. Enzyme activity of lysozyme in solutions containing urea was determined by the rate of lysis of *Micrococcus Lysodeikticus* cells in suspension. Lysozyme solution (0.6 mg/ml) was prepared in 0.02 *M* phosphate buffer (pH 7.0) containing 0.26 *M* sodium chloride and 0–5 *M* urea. Cell suspension of *M. Lysodeikticus* was prepared by suspending 5 mg of cells in 5 ml of the solvent used in the preparation of lysozyme, and triturated gently. An additional 15 ml of solvent was added to the suspension to give a final concentration of 0.25 mg/ml. A 200 μl volume of lysozyme solution was added to 2 ml of cell suspension in a cuvette. Absorbance of the mixture was recorded at 450 nm as a function of time to obtain zero order rates of lysis, which are a measure of enzyme activity.

RESULTS

Bovine serum albumin

Initial rapid changes in BSA conformation produced by urea can be detected by size-exclusion chromatography. At initial time, immediately after mixing BSA with urea, a decrease in the relative peak height of native (N) protein occurs with a corresponding increase in peak height of an intermediate (X), the change in both peak heights (or areas) being dependent on urea concentration. These chromatographic changes observed for N and X in the presence of urea are shown in Fig. 1. The peak identified as D in the chromatograms of both native and denatured proteins in Fig. 1 is consistent with a dimeric form of BSA, based upon calibrated elution volume in the size exclusion separation. Elution volume was correlated with protein molecular weight employing a series of both native and denatured reference proteins. Dimer D was present in all samples of BSA raw material examined and has been previously observed in other chromatographic studies[52].

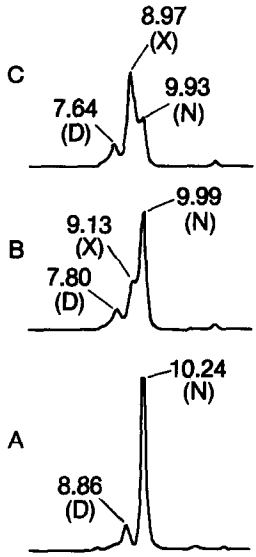

Fig. 1. Changes in high-performance size-exclusion chromatograms of BSA as a function of urea concentration. (A) No urea; (B) 4.4 *M* urea; (C) 4.8 *M* urea. N represents the native protein, D a BSA dimer, and X a conformationally altered monomeric species. Run time for all chromatograms was 16 min.

A sigmoidal transition curve was observed for the loss of relative peak height of N and increase in peak height of intermediate X as a function of urea concentration (Fig. 2). Each data point represents the average peak height value for equal injections of three identical solutions, with the standard deviation indicated by error bars. Chromatographic measurements of peak heights and areas were reproducible, with the coefficient of variation (at different urea concentrations) of the analyses for three identical samples ranging from 0.43 to 14.1% (n = 3 at each urea concentration). The conformational equilibrium between N and X was achieved immediately upon

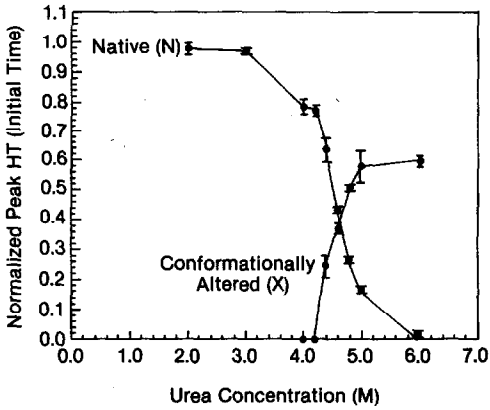

Fig. 2. Changes in initial peak heights of native (N) and conformationally altered forms (X) of BSA as a function of urea concentration, measured by SEC (n = 3 at each urea concentration).

mixing BSA with urea, since peak areas associated with these species remained constant at all early measurement times. The transition curves obtained chromatographically indicate a two-step denaturation process, which is observed to be reversible upon removal of urea from the protein samples (either by dialysis, or SEC with urea absent from the mobile phase).

Loss in peak height of N was compared with loss in fluorescence intensity of BSA at initial time (Fig. 3). Sigmoidal transition curves obtained by both chromatographic and fluorescence measurements were equivalent, showing identical midpoints in terms of urea dependence, and comparable relative changes in signal based on amount of native protein present. Minor additional changes in intensity were observed upon several days storage in urea. The time scale for this secondary fluorescence change was comparable to that observed for urea catalyzed BSA dimerization (see below).

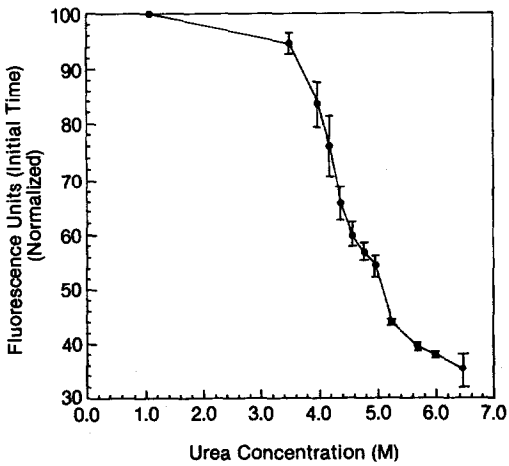

Fig. 3. Changes in relative fluorescence intensity of BSA (n = 3) as a function of urea concentration.

Decreases in size-exclusion elution volumes of all BSA species N, X, and D were found to occur with an increase in urea concentration (Fig. 4). The observed retention time changes were invarient with time and highly reproducible for all BSA species, with the coefficient of variation (at different urea concentrations) ranging from 0.0 (identical results) to 1.47% (n = 3 at each urea concentration). Retention time shifts for N, X, and D detected by SEC indicate possible additional conformational equilibria $N \rightleftarrows N_1$, $X \rightleftarrows X_1$, and $D \rightleftarrows D_1$ leading to apparent increase in molecular volume for the various structures. While changes in retention time of N and X may be due in part to changes in peak heights of overlapping peaks, a pronounced change in retention time is also observed for the BSA dimer D peak, which was completely resolved from other species. The presumed equilibria are rapid relative to the chromatographic permeation–elution time scale, thereby manifesting a shift in retention time of species such as N, X, and D. The N and X conformers being coupled by an equilibrium which is slow relative to the chromatographic time scale, were on the other hand resolved as discrete peaks (Figs. 1 and 2) at the various urea concentrations[15].

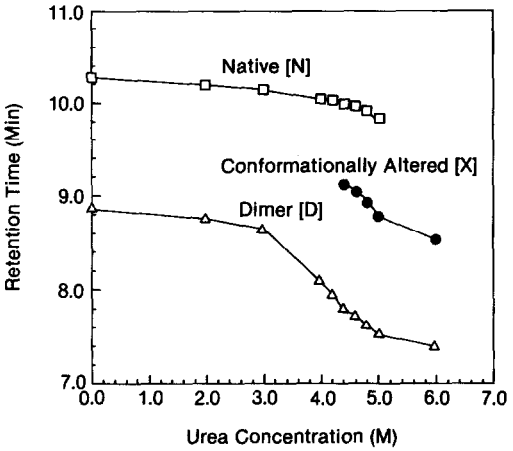

Fig. 4. Changes in size-exclusion retention times of native (N), conformationally altered (X), and dimeric (D) forms of BSA as a function of urea concentration (n = 3 at each urea concentration).

The pore structure of the stationary phase employed in protein SEC has been shown by others to be invarient with concentration of urea present in sample and mobile phase[15,16,39,41,42]. Further investigations of the observed decrease in retention time of BSA species with increasing urea concentrations were performed to assure detection of conformational changes, as opposed to chromatographic changes due to solute effects of urea in the system, or changes in the pore structure of the stationary phase.

Various molecular weight dextrans (9000–150000 MW), a random coil uncharged polymer, whose structure theoretically should be unaffected by urea, were chromatographed in a non-denaturing size-exclusion system without urea, and in a denaturing system containing 5.0 M urea. In Fig. 5 the plot of log MW of dextrans

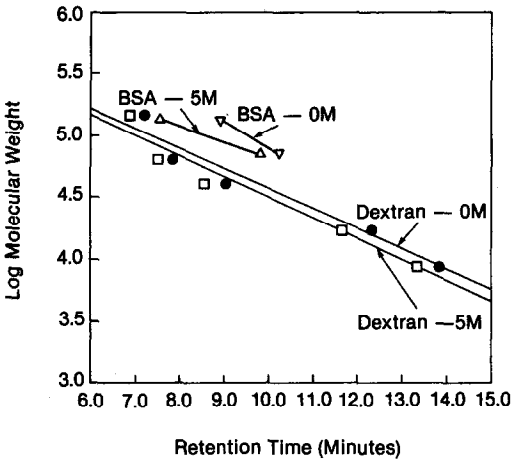

Fig. 5. Changes in size-exclusion retention times for random coil dextrans and globular albumins as a function of species molecular weight and urea concentration.

versus retention time (min) results in a linear relationship in both solvent systems, as expected. Equivalent slopes were obtained for both systems indicating solvent effects rather than changes to the pore structure of the stationary phase. A similar experiment was performed for BSA by plotting the log MW of the monomer and dimer *versus* retention time (min) at various urea concentrations. Although only two points were available for slope determination, the different relative slope values obtained show that a much larger change in elution volume resulted when the apparent molecular weight of serum albumin was doubled (monomer *vs.* dimer) in the presence of 5 *M* urea. This suggests urea-dependent changes in molecular volume of BSA species, as well as possible solvent effects. The possibility that the observed apparent changes in molecular volume are not due to changes in protein conformation but rather to extensive urea binding, is unlikely since no difference in retention times were observed between SEC of BSA in the absence of urea *versus* chromatography in the presence of 2.0 *M* urea. Since a large number of urea molecules would be expected to bind to BSA at 2 *M* concentration[60], one would expect to observe a change in retention time if urea binding *per se*, increased effective molecular volume.

Other evidence that rapid conformational changes resulting in retention time shifts occurred concurrently with observed changes in conformer concentrations (for example, the N to X equilibrium), includes the sigmoidal transition curve obtained by plotting retention time *versus* urea concentration for the dimer species D, as shown in Fig. 4. The urea concentration corresponding to the midpoint of this transition curve is very similar to that observed in the N to X transition curve (changes in peak heights of N or X at various urea concentrations, also at initial time). Behavior similar to the sigmoidal change in retention time for the BSA dimer, seen as a function of urea concentration, was observed for retention times of N and X. While neither species displayed a full sigmoidal profile (perhaps due to detection limits for N and X at high and low respective urea concentrations), superposition of the two retention time curves as a function of urea concentration produced a profile indicative of a

two-state transition. The fact that apparent shifts in retention time can occur with two peaks which are not completely resolved in the course of relative peak height changes, complicates the assessment of retention time changes for N and X. Changes in retention times of these peaks with increase in urea concentration, comparable to the difference in retention time between N and X at a given urea concentration, however, suggested that effects independent of measurement artifacts were occurring.

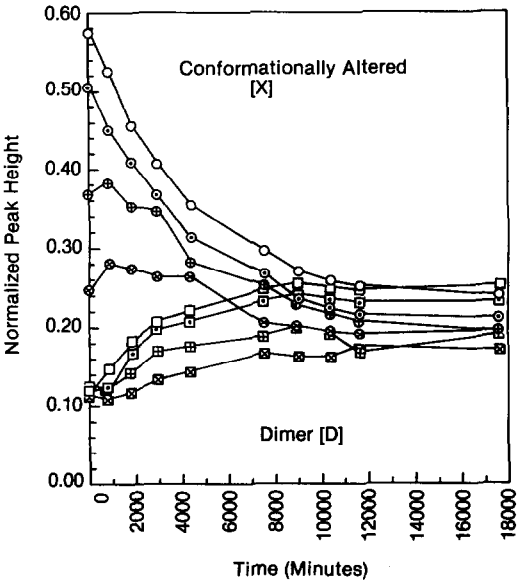

Fig. 6. Changes in peak height of conformationally altered (X) and dimer (D) forms of BSA as a function of time, at various urea concentrations. ○, □ = 5.0 *M*; ⊙, ⊡ = 4.8 *M*; ⊕, ⊞ = 4.6 *M*; ⊗, ⊠ = 4.4 *M*.

Storage of equilibrium mixtures of N and X at specific urea concentrations for several days resulted in additional changes in composition. The effect of urea on BSA species over time, as determined by SEC, indicated a slow (relative to initial conformational changes) dimerization reaction manifested by decrease in peak heights of X with corresponding increase in peak height of D, leading to equilibration (Fig. 6). This chromatographic result indicated that an additional kinetic process $2X \rightleftarrows D$, was occurring. In Fig. 7 by plotting the reciprocal of [X] *versus* time, second-order rate constants were obtained for the dimerization reaction, showing no dependence on urea concentration. The lack of deviation from a simple second order analysis at time points in Fig. 7 near completion of the observed conversion, suggested that the rate for dissociation of D to 2 X is slow relative to the dimerization rate. During the time period of increasing dimer concentration, the peak height of the native species (N), as measured by SEC, remains unchanged (Fig. 8). This suggests that additional equilibria involving the native species (N) are occurring. One hypothesis is that the native species may also be in a urea-dependent equilibrium with the dimer species, in ad-

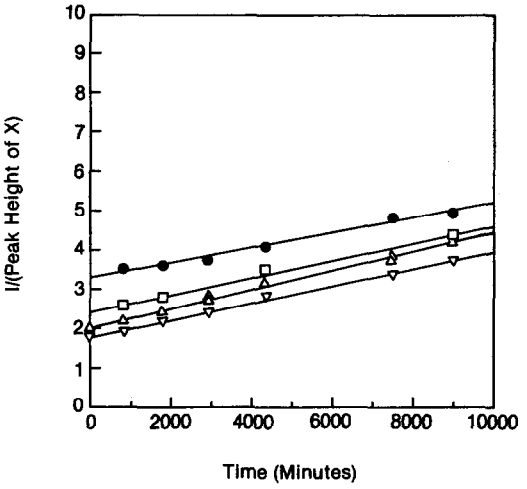

Fig. 7. Second-order rate plots for the disappearance of conformationally altered (X) BSA as a function of urea concentration. ● = 4.4 *M*; □ = 4.6 *M*; △ = 4.8 *M*; ▽ = 5.0 *M*.

dition to the N to D equilibrium which exists in non-denatured samples.

The identity of putative species, detected by SEC which are claimed as being conformationally altered forms of BSA, has been examined by circular dichroism (CD) spectroscopy. Examination of the aromatic circular dichroic spectra (which are a measure of the optical activity intrinsic to tertiary structure)[61] of BSA which has been altered by urea, showed that a difference in chiral structure exists between the native albumin and the species X which predominates in 6 *M* urea (Fig. 9). A time

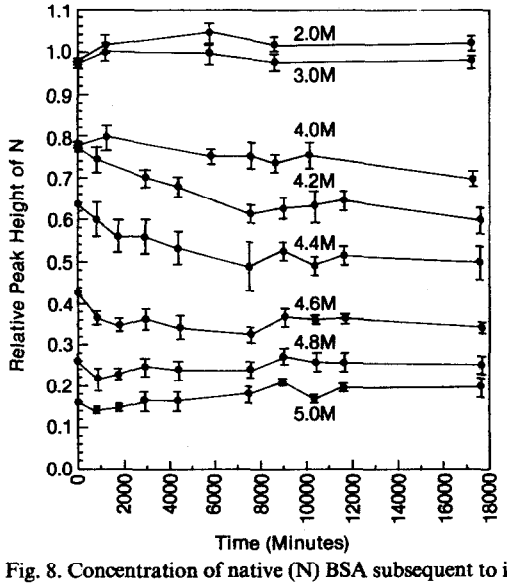

Fig. 8. Concentration of native (N) BSA subsequent to initial concentration changes as a function of time, at various urea concentrations (n = 3 at each time point).

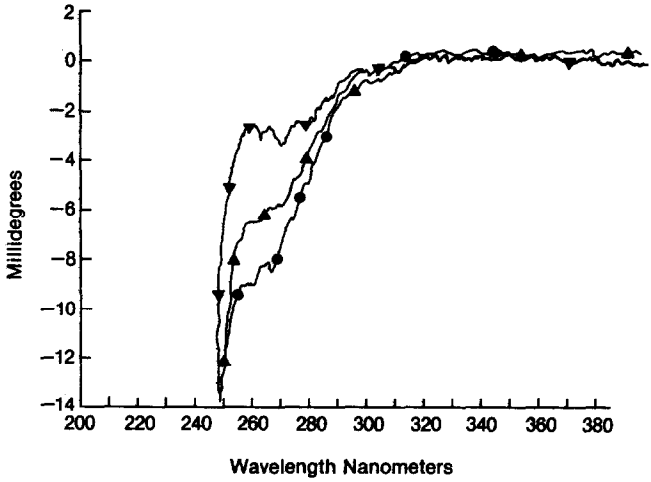

Fig. 9. Changes in aromatic circular dichroic spectra of BSA as a function of urea concentration. (●) 0 *M*; (▲) 3 *M*; (▼) 6 *M*.

dependent change in the CD spectrum of X, but not N, was also observed, suggesting that the chromatographically detected 2X ⇄ D process involves additional change in BSA tertiary structure (Fig. 10). (The spectra shown in Fig. 10 were obtained upon completion of the 2X ⇄ D equilibration, approximately one week.)

The analysis of urea-induced conformationally altered states of BSA, by high-performance anion-exchange chromatography, cannot be as readily performed as with SEC. This is due to significant alterations in the ion-exchange process produced by the presence of urea in the mobile phase. An absence of urea in the mobile phase

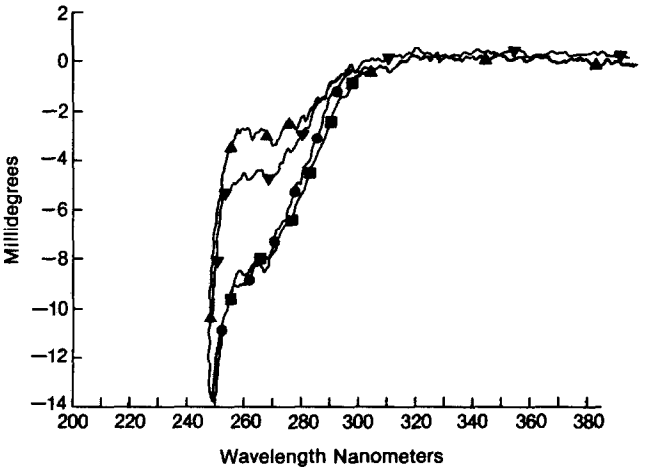

Fig. 10. Changes in CD spectra of native and conformationally altered forms of BSA after one week's storage. (A) Native BSA in buffer. (●) t = 0, (■) t = 1 week. (B) Conformationally altered species in 6 *M* urea. (▲) t = 0, (▼) t = 1 week.

in turn allows renaturation of several of the conformationally altered BSA species which have been shown to be maintained during SEC with a mobile phase containing urea. (SEC in the absence of urea showed complete reconversion to native for BSA samples which had been in contact with urea for but a few minutes, that is for chromatography performed at initial times.)

Storage of BSA in urea solutions for prolonged periods of time resulted in additional changes (of conformational and/or chemically modified nature) which are not readily reversible upon urea dilution in the course of ion-exchange chromatography. Denaturation (or degradation) kinetics of BSA due to prolonged exposure to urea were determined using anion-exchange chromatography, by monitoring the loss of the major (native) peak with time. Sample chromatograms of the native and denatured protein solutions are shown in Fig. 11. In anion-exchange chromatography, much smaller losses of native BSA were observed at initial time (freshly prepared samples at a given urea concentration) relative to losses of N detected by the size exclusion system where in the latter technique a given conformational state or mixture of states was maintained during chromatography. This is indicative of the above mentioned partial renaturation to native during the anion-exchange separation due to absence of urea in the mobile phase. Denaturation (or degradation) of BSA with urea over time detected by anion-exchange chromatography can be analyzed as a simple first order process (Fig. 12). The observed apparent first order rate constants (Fig. 13) for various urea concentrations, in addition to being urea concentration dependent, are significantly faster than the rates of dimerization observed by SEC (completion of the conversion in 500–2000 min, rather than 10000 min as seen for dimerization, at identical BSA starting concentrations). The observed loss of N is also very much slower than the conversion of N to X measured by SEC.

Other results which show a clear differentiation between the loss of native BSA measured by anion-exchange and the equilibria observed by SEC include (a) insignificant loss of N plus X was observed by SEC over the time interval for observed

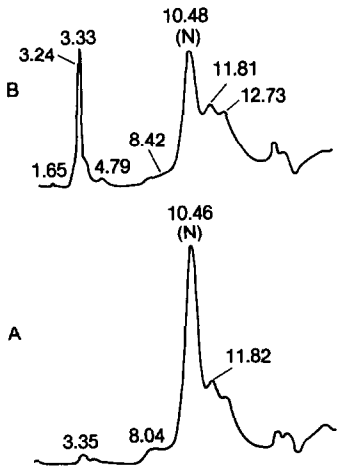

Fig. 11. Changes in high-performance anion-exchange chromatograms of BSA upon prolonged exposure to urea. (A) No urea; (B) sample stored in 4.8 *M* urea for 120 min. N represents native protein. Run time for all samples was 22 min.

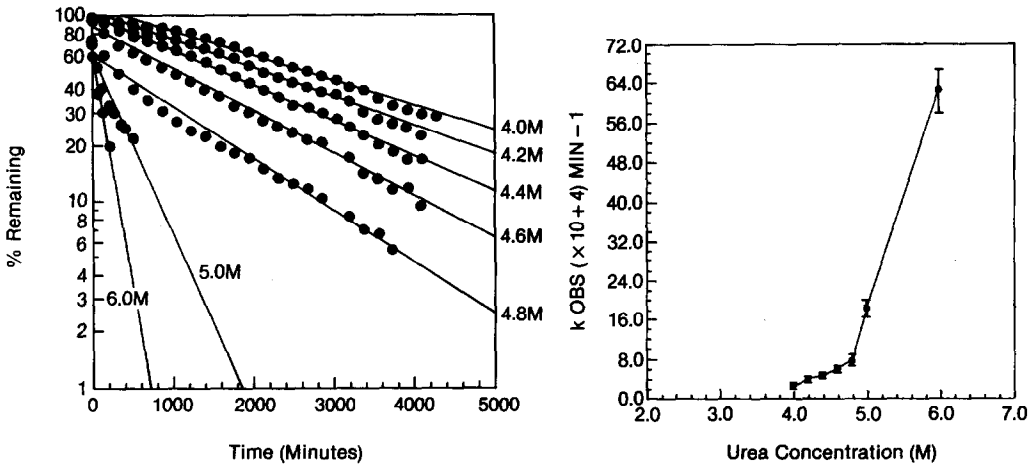

Fig. 12. First-order decrease in native BSA concentration measured by anion-exchange chromatography as a function of time, at various urea concentrations.

Fig. 13. First-order rate constants (n = 3) for irreversible disappearance for native BSA as a function of urea concentration.

substantial loss of native protein measured by anion exchange, and (b) the kinetic process observed by ion-exchange chromatography shows a different urea concentration dependence than the rapid native (N) to intermediate (X) equilibrium observed by SEC. These results indicate formation of a second species (Y), which less readily renatures to native BSA than does X under the non-denaturing environment which results during anion-exchange chromatography. (The point should again be made that upon removal of urea from a protein sample by performing chromatography with urea absent from the mobile phase, intermediates such as X immediately and quantitatively reconvert to native albumin.)

Formation of species Y was confirmed by performing SEC on BSA samples which has been stored in urea solutions for several hundred minutes, with urea absent from the mobile phase. This allowed the same potential extent of renaturation to occur as during anion-exchange chromatography. First order loss of major peak occurred for samples stored in 5 *M* urea, without the detection of any intermediate. Rate constants for disappearance of N from 5 *M* urea solution, when determined by SEC were equivalent to those obtained by anion-exchange chromatography, indicating the detection of identical kinetic processes. The extent of reconversion of Y to N upon urea removal (effected during either SEC or anion-exchange chromatography) was small, suggesting a urea-catalyzed conformational change (or degradation) which was essentially irreversible, which was detectable by SEC only in the absence of urea, and which did not result in any apparent changes in concentrations of N and X as monitored by SEC in the presence of urea. That is, the total peak areas of N, X, and D (after either 500 min or 10000 min) were equivalent to the initial peak area of native BSA, showing that no net loss of protein occurred when urea was present in the mobile phase. Species Y may, therefore, be a confomer or

chemical modification of BSA which does not produce a perceptible change on the molecular volumes of N or X, and which in the absence of urea adsorbs strongly to surfaces such as chromatographic stationary phases. (In the presence of urea, species Y must therefore have a retention time identical to N during SEC.)

Extent and kinetics of denaturation of BSA by urea were also determined by hydrophobic interaction chromatography. In this method denaturation (or degradation) of BSA was detected by loss of major (native) peak. Chromatography was performed under non-denaturing conditions (no urea in mobile phase) because of the marked perturbation of HIC by urea. Sample chromatograms of native and denatured protein are shown in Fig. 14. When examined by HIC, native BSA concentration was found to decrease with time, as a function of urea concentration. On a conversion time scale equivalent to that employed in kinetic measurements by anion-exchange and size-exclusion chromatography, results obtained by HIC (Fig. 15) showed the disappearance of N at rates slower than those measured by the other methods. This difference was more apparent at low urea concentrations. At high urea levels the observed kinetics appeared to become independent of urea concentration (unlike rates measured by anion exchange which increased markedly at high urea concentration).

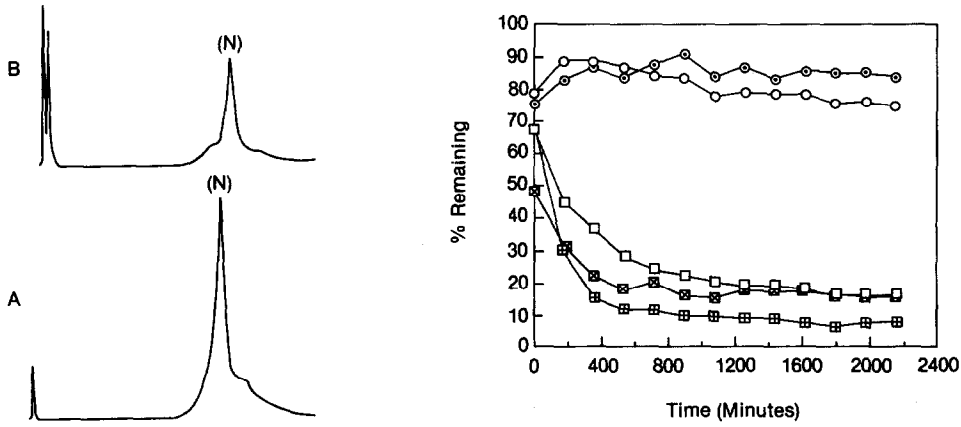

Fig. 14. Changes in high-performance hydrophobic interaction chromatograms of BSA upon prolonged exposure to urea. (A) No urea; (B) sample stored in 5 *M* urea for 180 min, (N) represents native protein. Run time for all samples was 22 min.

Fig. 15. Decrease in native BSA concentration measured by HIC as a function of time at various urea concentrations. ⊙ = 3 *M*; ○ = 4 *M*; □ = 5 *M*; ⊞ = 5.5 *M*; ⊠ = 6 *M*.

Comparisons of residual concentrations of native BSA remaining after 2000 min storage in urea (as assessed by HIC), as a function of urea concentration, showed that a possible sigmoidal relationship exists between the amount of native BSA recoverable (after diluting out urea) and the urea concentration in which a protein sample had been stored (for prolonged periods). These data, as shown in Fig. 16, suggest that the chromatographic behavior changes observed with HIC indirectly monitor the rapid N ⇄ X equilibrium observed by continuously denaturing SEC. Total correlation was, however, not observed between the two measurements, in that the

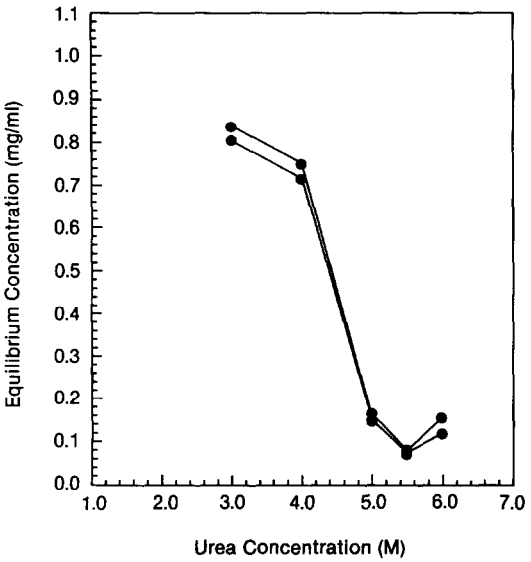

Fig. 16. Concentration of native BSA measured by HIC in samples incubated in urea solution for 2000 min, as a function of urea concentration.

HIC results showed minor difference between the concentrations of N in 3 and 4 *M* urea, while SEC consistently showed a significant difference (Figs. 2 and 8). It is possible that conversion of native BSA to species Y occurs through intermediate X, the concentration of which is dependent upon urea concentration.

Assessment and extrapolation of protein stability (especially for polypeptide drug substances) requires reproducible kinetic data. In the case of the model protein BSA, rate constants for the irreversible conversion of N to Y obtained by anion-exchange chromatography, were highly reproducible as shown by the one standard deviation ($n = 3$) error bars in Fig. 13. A similar high reproducibility was obtained with raw kinetic data, for example the one standard deviation ($n = 3$) error bars in Fig. 8 indicated that conformational changes in BSA can be measured chromatographically, with acceptable precision.

Trypsin and lysozyme

High-resolution separation of trypsin (pH 7.0) based upon the molecular volume of the native protein (using size-exclusion chromatography) could not be achieved. A cation-exchange mechanism appeared to occur on size exclusion stationary phases, which possess a net negative charge[16]. Trypsin was found to elute much more slowly than expected based on its molecular weight (24000) during SEC on several different columns. This anomalously strong retention has been observed with other basic proteins (p*I* of trypsin is 9.1), such as lysozyme[16]. Size-exclusion columns investigated included Du Pont's Zorbax Bio-Series GE-250 and Water's Protein Pak 300 SW.

Conformational changes in trypsin induced by urea were detected by cation-exchange chromatography, by both changes in retention times and loss of the major

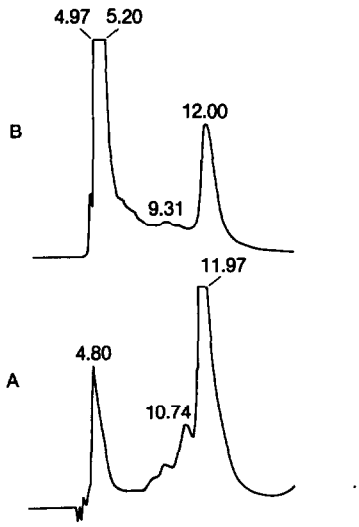

Fig. 17. Changes in high-performance cation-exchange chromatography of trypsin upon prolonged exposure to urea. (A) No urea; (B) sample stored in 5.5 *M* urea for 260 min. Run time for all samples was 18 min (employing a mobile phase without urea).

peak. The chromatography was performed both in the absence and presence of urea in the mobile phase for comparative purposes. Sample chromatograms for native and denatured protein are shown in Fig. 17. Very rapid conformational changes of trypsin occurred in urea solutions in that changes in enzyme activity and chromatographic behavior were observed immediately after adding urea to protein solutions. A decrease in enzyme activity, determined by the trypsin-catalyzed rate of BAPNA hydrolysis, occurred with an increase in urea concentration (Fig. 18). In terms of

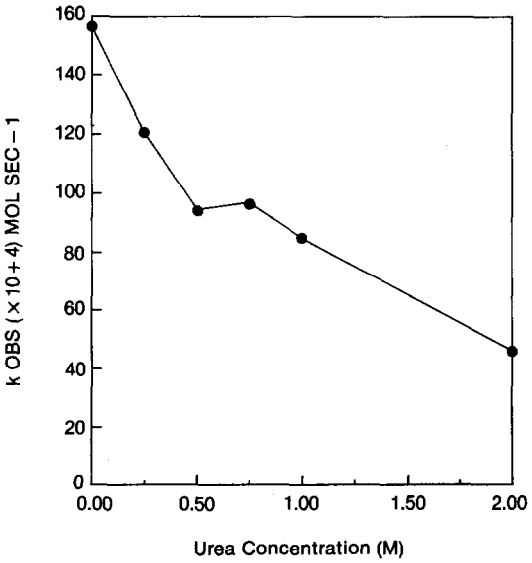

Fig. 18. Initial decrease in trypsin activity as a function of urea concentration in sample.

chromatographic behavior, retention times of trypsin generally decreased as a function of increasing urea concentration in the sample and mobile phase. An exceptional increase in retention time occurred at low urea concentrations (Fig. 19). No other changes in chromatographic behavior were observed at initial time. Since urea affects the retention of charged species in ion-exchange chromatography[45], this behavior was further investigated.

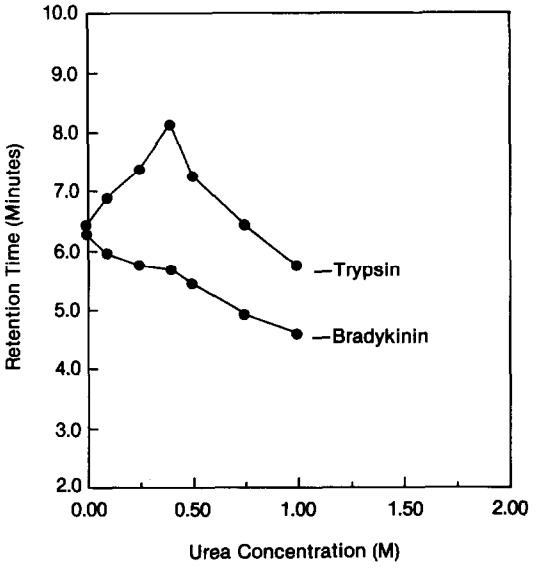

Fig. 19. Changes in cation-exchange retention times for trypsin and bradykinin at low urea concentration as a function of urea concentration in sample *and* mobile phase.

Retention behavior of bradykinin, a random coil cationic peptide, whose structure should remain unaffected by urea, was examined at varying urea concentrations by the cation-exchange chromatography method developed for trypsin. At all urea concentrations employed, a linear decrease in the retention of bradykinin occurred. This effect was opposite that observed with trypsin at low urea concentrations (Fig. 19). The observed increase in retention time at low urea concentrations appeared to be due to effects which occur concurrently with the anticipated urea-dependent solvent perturbations which can change the dielectric constant of the medium separating charged groups on the stationary phase from charged groups on the eluting species (resulting in changes in stationary phase pK_a values and binding constants, and/or protein p*I*).

The very rapid partial loss in enzyme activity which occured at a given urea concentration (and associated chromatographic retention behavior changes) suggest the formation of a non-active (or low activity) conformationally altered intermediate (X) in rapid equilibrium with native trypsin. This conversion was observed to be reversible upon removal or dilution of urea. On longer time scales, for example upon storage of trypsin in urea solutions for several hours, losses in the residual enzymatic activity remaining were observed (Fig. 20A). The rate constants for loss of residual

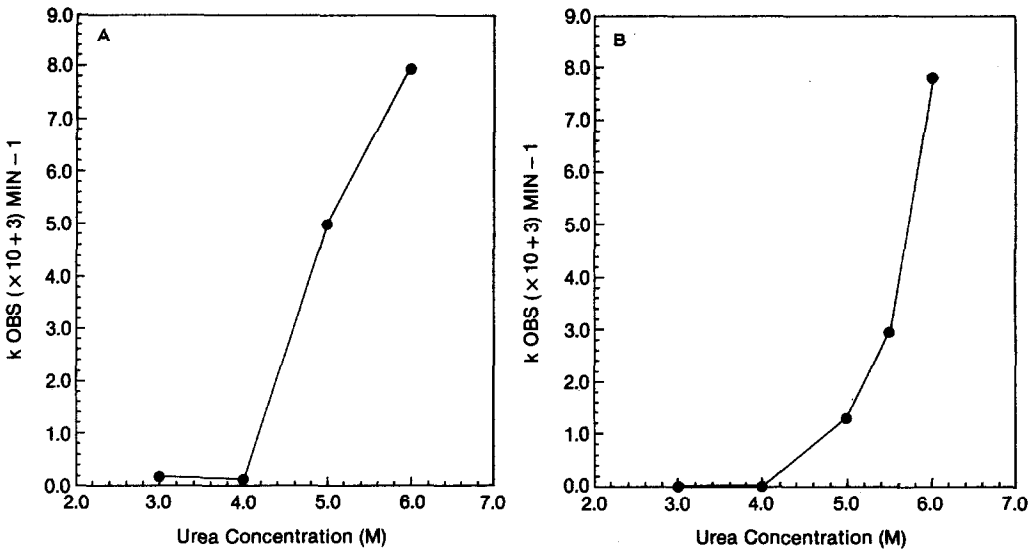

Fig. 20. (A) First-order rate constants for residual trypsin activity loss as a function of urea concentration during sample storage. (B) First-order rate constants for disappearance of residual trypsin peak measured by cation-exchange chromatography as a function of urea concentration during sample storage.

enzyme activity (determined by measuring rates of enzyme catalyzed BAPNA hydrolysis using samples of trypsin incubated in urea for various time periods) were comparable to first-order rate constants for loss of native enzyme measured by cation-exchange chromatography at identical urea concentrations (Fig. 20B). This indicated that two conformationally altered species of trypsin form upon denaturation, an intermediate (X) which either possesses partial activity or is in equilibrium with native trypsin (N) and a more completely denatured (or degraded) species (D), which is completely inactive and forms irreversibly.

$$[\mathrm{N} \overset{K_1}{\rightleftharpoons} \mathrm{X}] \overset{k_1}{\rightarrow} \mathrm{D}$$

First-order rates of conversion of native trypsin to D, obtained chromatographically by measurement of loss of major peak were similar regardless of whether urea is present in the mobile phase. These results further substantiate the fact that N and X are in rapid equilibrium in that identical total losses are observed regardless of whether the concentration of N or X is being measured.

Cation-exchange chromatographic retention behavior was also determined for another highly basic protein, lysozyme (pI = 11.0), as a function of urea concentration (Fig. 21). Similarly to trypsin, this protein's enzyme activity decreased with increasing urea concentration (Fig. 22A). Unlike trypsin, a decrease in retention time of lysozyme occurred at all urea concentrations examined (Fig. 22B). Potential chromatographic changes (retention times) due to any changes in protein conformation which occur at low denaturant concentrations may therefore be obscured in lysozyme by other effects of urea which may affect retention time in the opposite direction to a greater proportionate degree.

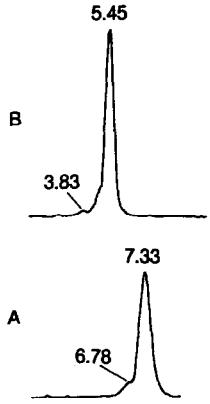

Fig. 21. Changes in high-performance cation-exchange chromatography of lysozyme upon exposure to urea. (A) No urea; (B) sample in 0.5 *M* urea (equivalent urea concentration in mobile phase).

DISCUSSION

Change in conformation of a proteins tertiary structure can be expected to result in several changes in physico-chemical properties[56,57]. Partial denaturation would be expected to expose portions of the polypeptide chain to solvent, which are buried in the native protein. Since the majority of buried amino acid residues are hydrophobic in nature, a conformational change should increase the effective hydrophobicity of a protein. The net ionization state of a protein can also be expected to

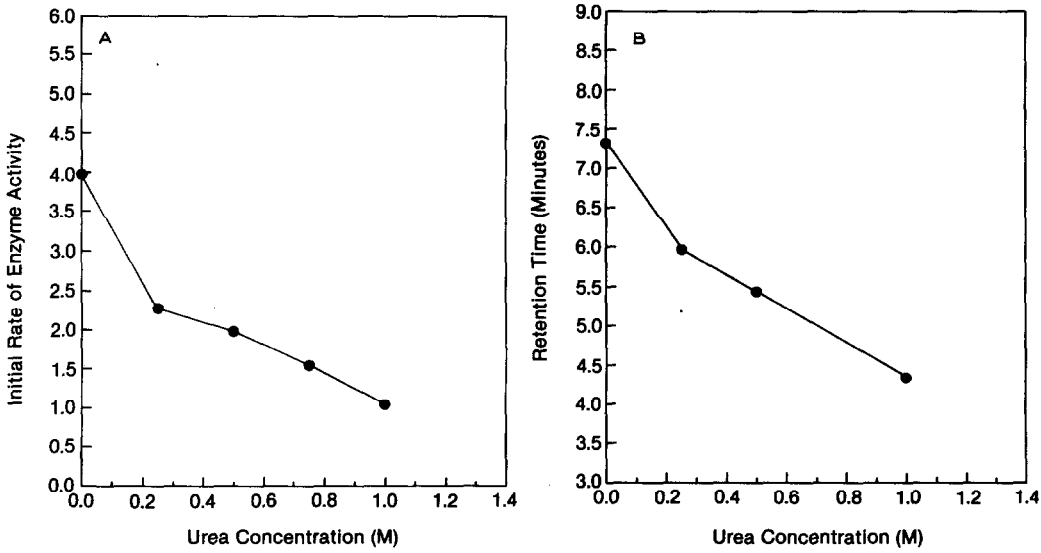

Fig. 22. (A) Initial decrease in lysozyme enzymatic activity as a function of urea concentration in sample. (B) Decrease in cation-exchange retention time of lysozyme as a function of urea concentration in sample and mobile phase.

change, either by altering the distance between two charged residues which influence each others pK_a values, or removal of a potentially ionizable group from a hydrophobic environment where it is uncharged to an aqueous environment where it can exist in an ionized form. Finally, the majority of protein denaturations and conformational changes result in an expansion of the molecule. It could, therefore, be expected that a perturbation of tertiary structure would lead to net increase in effective molecular radius[15,16,39,41].

The hypotheses being tested are:

(a) conformational changes which result in changes in molecular radius can be measured by size-exclusion chromatography;

(b) conformational changes altering net charge, charge density, or charge accessibility can be measured by ion-exchange techniques;

(c) conformational changes affecting hydrophobic surface area can be measured by hydrophobic interaction chromatography.

Conformational changes in BSA correlating to changes in chromatographic behavior during size-exclusion separation include appearance of multiple peaks, changes in peak size, and changes in retention of the various protein species detected. Size exclusion can detect native BSA (N), an intermediate (X) in slow (relative to the permeation–elution time scale, but rapid relative to the rates of formation of other species measured through changes in peak heights) equilibrium with the native, possible additional intermediates N_1 and X_1 which are in rapid (relative to the chromatographic process) equilibrium with N and X, dimeric BSA (D), and a conformationally altered form of the dimer D_1 which is in rapid equilibrium with the native dimer present in the absence of urea. Changes in chromatographic behavior of BSA in urea at initial time, such as the transition which occurs at approximately 4.5 *M* urea concentration (detected by size exclusion) correlate well with changes in fluorescent intensity and other physico-chemical changes induced by urea. Change in tertiary structure has previously been measured as a function of urea concentration by techniques such as ultraviolet difference spectroscopy[58,62,63], viscometry[64–67], optical rotation[66,68–70], dielectric dispersion[67,71], dilatometry[72], hydrogen ion titration[73], isoelectric focusing[74–76], gel electrophoresis[77], and "catalytic activity"[8]. These studies also show that a major conformational change occurs in approximately 4.5 *M* urea. Kinetic studies of urea-catalyzed BSA denaturation have shown that the major detected conformational change occurs rapidly[64,66,77]; that is, in less than several minutes. The initial changes in peak areas observed by SEC were also found to occur immediately, in agreement with literature results. High-performance size-exclusion chromatography methods, therefore, have the ability to detect changes in protein tertiary structure (and provide physico-chemical measurements based on conformational changes), which in the past have been obtained primarily by classical spectroscopic and hydrodynamic techniques.

Existence of a subtle conformational isomerization known as the "neutral" to "basic" transition[50] which occurs in BSA at pH 7–8[50,78], may have bearing on urea-catalyzed conformational changes observed by chromatography. If the presence of urea perturbs the "neutral" $\rightleftarrows$ "basic" equilibrium, then the relative concentrations of the two forms present at pH 7.0 will change. Since the "basic" and "neutral" forms are protonated to different degrees at a given pH[79] and exhibit different degrees of hydrophobicity[80], one might expect that the two isomers are also different in size. It

is, therefore conceivable that this equilibrium is being detected during SEC (either due to size differences or a mixed retention mechanism also involving charge and hydrophobicity).

The interconversion between the "neutral" and "basic" isomers of BSA occurs over approximately 20 ms[81]. If this rate is rapid relative to the time scale of size-exclusion elution process, then it is possible that this conformational transition, manifest at the proposed $N \rightleftarrows N_1$ equilibrium, was detected by retention time changes in SEC. If neutral and basic forms of X and D also exist, a similar argument can be made for the proposed species X_1 and D_1.

The slow dimerization observed chromatographically subsequent to the above-mentioned phenomena has also been measured by other methods[8,64,66]. Second order rates for denaturation events subsequent to the initial change in conformation were found by others to be independent of urea concentration[64] when measured viscometrically, and increase with pH when measured via loss of "activity"[8]. ("Activity" in the context of BSA is defined as the rate constant for a specific chemical reaction catalyzed by native BSA, which decreases upon protein denaturation.) While the dimerization reaction measured by SEC is independent of urea concentration, the reaction time scale (time for reaction to go to greater than 90% completion) is about a factor of ten greater than for the "activity" loss reaction performed at a comparable pH. Viscometric rates, which are also independent of urea concentration, cannot be directly compared to the SEC results due to a 3 pH unit difference in solution acidities.

Ion-exchange and hydrophobic interaction methods can quantitatively detect only the native form of BSA. Differences between data obtained by size-exclusion, anion-exchange, and hydrophobic interaction chromatography for the irreversible loss of native BSA during extended storage in urea solutions, indicated that processes in addition to the equilibria observed by continuously denaturing SEC, are occurring. These differences may reflect non-identical rates and extents of renaturation in the presence of different stationary phases, during the chromatographic removal of urea from injected protein samples. Alternatively, they may be an indication of multiple conformational changes or degradations which occurred during the overall denaturation process, which were preferentially detected by the different modes of chromatography employed.

In the case of serum albumin, the measurement of kinetic processes by anion-exchange and hydrophobic interaction chromatography with rates different from the conformational and dimerization processes observed by SEC, shows that at least one additional conformationally (or chemically) altered species (Y) exists in the overall denaturation pathway. This postulated species may be the result of disulfide isomerization, which can occur upon protein denaturation[82]. Regardless of the nature of the actual modification of N to yield Y, it appears that Y adsorbs readily to surfaces. The observed loss of native BSA upon storage in urea and subsequent chromatography in the absence of urea may be due to conversion of N to Y, with Y adsorbing to various stationary phases upon chromatography. Such losses were not observed during the majority of SEC analyses perhaps due to the presence of protein solubilizing urea in the mobile phase. The rates of disappearance of native BSA ($N \rightarrow Y$) measured by anion-exchange and HIC were more rapid than other previously observed denaturation processes such as the rate of above mentioned

"activity" loss under comparable conditions (a 90% conversion time of ≈300 min measured in 6 *M* urea, pH 7.0, by anion exchange versus ≈500 min measured in 8 *M* urea, pH 7.4 via "activity" assay[8]). These results suggest the chromatographic detection and rate measurement of a conformational and/or chemical denaturation process which has heretofore not been detected by other analytical methods. Further evidence for the unique nature of this species is the lack of observed change in BSA fluorescent intensity or emission wavelength over the course of the kinetic process leading to Y.

Changes in retention times and peak heights of the various species resolved by high-performance protein chromatography methods can therefore be sensitive measures of protein conformational changes. In the case of BSA, evidence of sigmoidal changes in retention time of N, X, and D with increasing urea concentration indicate the possible presence of very rapid conformational equilibria in addition to a major transition which correlates with fluorescence spectroscopy and other measurements described above. While the latter conformational transition (which occurs with a midpoint at approximately 4.5 *M* urea) and subsequent dimerization, have been detected by other techniques, the fast equilibria $N \rightleftarrows N_1$, $X \rightleftarrows X_1$, and $D \rightleftarrows D_1$ have not been previously reported. The pathway converting native BSA to species Y also has no literature precedent. Protein chromatography can therefore provide evidence for conformational and/or chemical perturbations which are not readily detected by classical techniques of protein analysis.

Transformation rates of various conformational states occurring during urea-catalyzed BSA denaturation can be determined, via the denaturation kinetics measured by the various chromatographic methods. Conformational states for which conversion rates could be measured by at least one of the chromatographic methods employed in the study of urea-catalyzed BSA denaturation include the native N, intermediate X, and dimeric D species. Chromatography can, therefore, also be a tool for the elucidation of protein denaturation mechanisms via kinetic techniques.

The possibility of measuring activity loss due to conformational changes, by chromatographic methods, was examined with trypsin and lysozyme. Retention time changes, observed during urea-catalyzed denaturation of lysozyme show the same trend as urea concentration dependent losses in enzyme activity. Possible reasons for changes in retention times of polypeptides upon apparent change in tertiary structure in an ion-exchange chromatographic experiment in the presence of urea, include (1) detection of conformationally altered species in rapid equilibrium with native protein, (2) urea-induced change in protein/stationary phase affinity (due to changes in dielectric or ionization constants), and (3) urea-induced size-exclusion mechanism occurring on an ion-exchange column (changes in effective pore size). Retention time changes measured for lysozyme at low urea concentration, while decreasing to a slightly greater extent (with increasing urea concentration) than non-denaturable cationic peptides such as bradykinin, cannot as unambiguously be attributed to a change in conformation as results observed at high urea levels[4,5]. That is, a combination of the above retention time modifying mechanisms may combine to yield the observed net decrease in retention time with increasing urea concentration.

Changes in retention time as a function of urea concentration for trypsin are fundamentally different in character. As previously mentioned, retention times for positively charged species, such as lysozyme and bradykinin, decreases with increas-

ing urea concentration in cation-exchange chromatography. While both trypsin and lysozyme possess a net positive charge under the chromatographic conditions employed (pH 7.0, trypsin pI = 9.1, lysozyme pI = 11.0), trypsin exhibits an increase in retention time with increasing urea at low urea concentrations. This strongly suggests effects on the chromatography independent of any modifications of the stationary phase by urea. While it is possible that the presence of urea can raise the pI of trypsin by perturbing ionization constants of individual amino acids, the fact that a similar retention effect is not observed with lysozyme (which also possesses a large positive charge excess) at equivalent urea concentrations, suggests that the altered retention is due to a conformational change. The low urea concentration required (0.1 to 0.5 M) suggests that the conformational change is not extensive since most proteins require exposure to high (>3 M) urea concentrations to effect the major conformational changes which are typically measured by spectroscopic, hydrodynamic, and calorimetric techniques[45,56,57]. Trypsin itself shows evidence of a major conformational change which occurs at urea concentrations greater than 4 M (measured by both rate of loss of residual enzyme activity and rate of disappearance of chromatographically renaturable enzyme). Ion-exchange chromatography can, therefore, detect and quantitate relatively subtle changes in a protein's tertiary structure.

Since a correlation was also obtained for trypsin between retention time changes and initial activity losses (at given urea concentrations), cation-exchange chromatography can potentially be a method for detection of conformational changes which result in enzyme activity loss*. The potential therefore, exists for the use of analytical chromatographic methods to not only assess purity and molar concentration of proteins but also to assay biological activity in a convenient and highly reproducible fashion. However, the degree of correlation between specific chromatographic behavior resulting from conformational changes in biologically active proteins, and any actual change in activity, must be determined on a case-by-case basis for each enzyme or bioactive protein under study.

Reproducibility of data indicative of conformational changes was found to be dependent on specific mobile phase composition, and stationary phase properties of an individual column. For example, chromatographic resolution of all proteins which were eluted in the presence of urea (BSA, trypsin, lysozyme) was affected by the presence of small amounts of ionic impurities present in reagent grade urea. Depending on the protein and chromatographic conditions, retention times of both native and conformationally altered species could change in consistent manner upon extended use (week to week and even day to day) of an individual column. This latter phenomenon may be due to a slight but continuous irreversible binding of proteins, especially in their conformationally altered or denatured states. Observed coefficients of variation ranging as large as fourteen percent may be due to the above factors. While large, relative to typical small-molecule HPLC assay variability (2–3%), the variability obtained for conformationally altered proteins is much smaller than the scatter of data observed for the bioassay of protein drugs, where coefficients of vari-

* *Note added in proof.* The observed decrease in enzymatic activities in the presence of urea appears to be due to conformational changes rather than urea specific inhibition of catalytic active sites, since a similar effect was observed for both lysozyme and trypsin, enzymes with markedly different catalytic mechanisms.

ation of 35% and greater are accepted[83]. Therefore, if the same degree of correlation between chromatography and biological (enzymatic) activity can be shown for polypeptide drug substances, as for trypsin, a marked improvement in pharmaceutical analysis in terms of reproducibility and convenience will occur.

CONCLUSIONS

For the proteins examined, HPLC methods, which are intrinsically mild and non-denaturing, can be simple and reproducible tools to detect two-state protein conformational changes. The conformational alterations detected chromatographically correlate directly with changes detected by classical, physical, and biochemical methods such as fluorescence and enzyme activity. Several different conformational states and associated transformation rates can be detected and quantitated by various chromatographic methods. Denaturation kinetics obtained through chromatography complements data obtained by other methods, allowing more thorough elucidation of denaturation mechanisms. Moreover, chromatographic sensitivity appears to be sufficient to allow detection of either small changes in tertiary structure, or alternatively, denaturation in only a small fraction of the total protein present, with a degree of reproducibility acceptable in pharmaceutical analysis.

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