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Thermal Unfolding of Proteins Studied by Coupled Reversed-Phase HPLC−Electrospray Ionization Mass Spectrometry Techniques Based on Isotope Exchange Effects

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In this study, a deuterium exchange procedure has been employed to evaluate the thermal stability of globular proteins under conditions that replicate their interactive behavior in reversed-phase high performance chromatographic (RP-HPLC) systems. In particular, this investigation has permitted the conformational stability of two proteins, hen egg white lysozyme (HEWL) and horse heart myoglobin (HMYO) to be examined under different temperature and low-pH solvent regimes. The results confirm that this experimental approach provides an efficient strategy to explore fundamental conformational features of polypeptides or proteins in their folded and partial unfolded states under these interactive conditions. In particular, this analytical procedure permits insight to be readily gained into the processes that occur when polypeptides and globular proteins interact with lipophilic liquid/solid interfaces in the presence of water−organic solvent mixtures at different temperatures.

Over the past two decades, reversed-phase high performance liquid chromatography (RP-HPLC) with porous *n*-alkylsilicas has become the dominant technique for the analytical separation, as well as the micropreparative isolation, of polypeptides and proteins.[1,2] As an experimental procedure, RP-HPLC can, in addition, be employed to probe in detail the thermodynamics of the interaction of polypeptides and proteins with hydrophobic ligands, such as immobilized *n*-alkyl groups or their lipophilic analogues.[3−6] Although valuable physicochemical information can be retrieved from such studies, a fundamental consideration frequently arises with polypeptides and proteins in terms of their conformational status and structural stability in the presence of such hydrophobic environments. The heterogeneous nature of the surface of the sorbent and the influence of the low-pH water−organic solvent conditions that are traditionally used often combine to induce unfolding processes with these biomacromolecules, a process that can be particularly exacerbated when elevated temperatures are employed. Such behavior has previously been visualized in, for example, RP-HPLC, high performance hydrophobic interaction chromatography (HP-HIC), and other adsorption modes of HPLC as multiple peak zones or distorted peak shapes under various conditions. Formation of multiple peak zones with compositionally pure samples, loss of biological activity or the reduction in mass yields can all be attributed[7−9] to folding/unfolding phenomena arising from the interaction of polypeptides or proteins with the *n*-alkyl ligand or as a result of solvational effects during the separation process.

A variety of spectroscopic procedures have previously been employed to monitor such conformational changes with polypeptides and proteins in bulk solution or at solid/liquid interfaces, including diffuse reflectance infrared spectroscopy (DRIFT),[10,11] circular dichroism (CD),[3,12−15] second derivative and difference UV−vis spectroscopy,[16,17] and intrinsic fluorescence spectros-

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copy.[6,18] Thus, changes in the intensity of the amide I and amide II bands detected by one- and two-dimensional Fourier transform IR spectroscopy have been employed[19] to monitor structural changes in cytochrome c_{552} from *Thermus thermophilus* on adsorption to anionic and hydrophobic surfaces. Analogous procedures have been exploited to follow conformational changes of other proteins, such as serum albumins on adsorption to various clays,[20] porous ion exchangers,[21] and reversed-phase sorbents.[22] Such techniques provide useful global assessments of the conformational stability of a particular polypeptide (or protein) species under different solvational or solid/liquid interfacial conditions. This outcome is, however, usually achieved without regard to the changes in the secondary structure or the local architecture of the polypeptide or protein in its folded or partially folded state. The above spectroscopic techniques are, moreover, dependent on the presence of suitable reporter groups or regions within the polypeptide or protein, such as Tyr or Trp residues or α-helical or β-sheet secondary structures, to permit the changes in the conformational state(s) to be reflected as variations in electronic spectral characteristics as the microenvironments surrounding these residues/regions are manipulated. To obtain more detailed information at the level of the atomic microenvironment on the effect of changes in the microenvironment surrounding particular regions of a protein in a partly folded state, recourse to high-field two- and three-dimensional heteronuclear nuclear magnetic resonance (2-D or 3-D NMR) spectroscopic methods can be employed. Although important results[16,23−28] on the conformational flexibility of several small proteins, for example, lysozymes or cytochrome *c*'s, have been obtained by NMR techniques, including the demonstration that reversed-phase chromatographic surfaces can result in significant amide exchange of these proteins through increased solvent exposure and some loss of native structure, generally no simple correlation has been found between chromatographic retention and the degree of surface-induced unfolding. Variations in residual conformation may, thus, explain the complex retention behavior seen for proteins under RP-HPLC conditions, as compared to small peptides or organic molecules. Since routine application of sophisticated high field NMR methods is not feasible in the majority of laboratories, other alternative methods are needed.

When a protein adopts a "nativelike" folded structure in solution, a complex series of physicochemical effects come into play, mediated inter alia by the hydrogen bonding characteristics of different hydrogen bond donor and acceptor groups within the amino acid sequence, as well as those present in the solvent. For fully folded polypeptides and proteins, two classes of intra- and intermolecular hydrogen bond processes ("progressive" or "chaotic") can be identified, depending on whether the amino acid residues and the backbone amides are fully or partially accessible to the solvent or, alternatively, are buried within the interior of the folded structure. The progressive processes are associated mainly with backbone amide hydrogen bonds that are relatively inaccessible with folded polypeptides and globular proteins to the surrounding solvent. These intramolecular hydrogen bonds can be extremely stable, with half-lives for exchange ranging from seconds up to months.[29] Typically, these long-lived intramolecular hydrogen bonds are associated with stabilization of the secondary and tertiary structure of the folded protein and involve internalized regions of α helices, β sheets, type-II β-turn loops, $\beta\alpha\beta$ motifs, α_2- to α_4-helical coiled-coil bundles or other supramolecular structures derived, for example, from side-chain-to-side-chain interactions, such as salt bridges. Progressive processes, thus, involve exchange phenomena that are kinetically slow when the polypeptide or protein is in its folded, nativelike conformational state and multistage. Chaotic processes of hydrogen bond exchange with folded polypeptides and globular proteins, on the other hand, have much shorter half-lives and involve side chain moieties containing exchangeable/ionizable protons, or backbone amides that are highly accessible to the solvent. Consequently, when a folded polypeptide or protein is exposed, for example, to H_2O or D_2O or under low-pH conditions, the "solvent-accessible" protons of the polypeptide or protein will rapidly exchange with other protons or deuterons in the bulk solvent. Relatively mild conditions can be employed to achieve this chaotic process whereby a large polypeptide or protein does not necessarily enter a folding/unfolding trajectory given by the sequential mechanism N ↔ I ↔ II ↔ U, where N, I, II, and U correspond to the native state, the intermediate states I and II (the former also known as the "molten-globule-like" state and the latter, the "partially nucleated state" lacking significant secondary structure) and the unfolded state. Thus, conditions can be selected such that these amide hydrogen exchange effects can occur with activation energies falling well below the activation energy required for the polypeptide or protein to commence to lose its secondary structure or to unfold. With polypeptides that lack any well-developed secondary structure in solution, or alternatively, with unfolded (denatured) proteins in which the tertiary/secondary structure has become extensively disordered without becoming amyloid- or fibril-like, most of the amide hydrogens and all of the protons associated with the ionizable N-terminal amino group, the C-terminal carboxyl group, and the side chain groups involved in acid/base equilibrium or other chemical exchange events are accessible to the surrounding solvent. As a consequence, with disordered molecules, these amide hydrogens/ionizable protons have exchange rates as short as microseconds to seconds.[30] This difference of 1000-fold or more in hydrogen exchange rates

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between the progressive and chaotic processes of a polypeptide or protein in its folded versus its unfolded state offers an exquisitely sensitive probe to examine conformational fluctuations that these macromolecules can undergo in response to changes in the surrounding chemical or physical environment, such as variations in the composition of the solvent, the temperature, or the nature of an interactive solid/liquid surface to which the polypeptide or protein has docked.

Amide hydrogen exchange experiments involving proton replacement by deuterium (i.e., $^1H \rightarrow {}^2H$) have previously been studied with proteins in the presence of different substrates, inhibitors, or chemical surfaces by ^1H NMR[23,24,27,31] and neutron diffraction[32] procedures to establish the kinetics of enzyme catalysis. Similarly, $^1H \rightarrow {}^2H$ isotope effects have been employed to assess the extent of surface accessibility to the bulk solvent of specific amino acid residues after chemical modification of adjacent regions of a particular protein domain.[33] From the isotopic exchange rates of the amide hydrogens of a protein in bulk solution in the presence of proton and deuteron donor solvents, it is also possible to identify functionally different allosteric intermediates, protein−protein complexes, and different folded states.[34,35] Enzymatic fragmentation/mass spectrometric procedures can also be used to measure amide hydrogen exchange, allowing the stability of folded domains of proteins, such as aldolase,[36] in bulk solution to be characterized. Alternatively, different mechanisms of hydrogen exchange can be distinguished[27,37] using complementary mass spectrometry and nuclear magnetic resonance techniques with $^2H \rightarrow {}^1H$ back-exchange of perdeuterated protein samples in proton donor solvents, such as water. A decade ago, Chait and co-workers first demonstrated that electrospray ionization mass spectrometry (ESI-MS) can be an effective off-line method to probe the conformational changes of proteins, such as bovine ubiquitin, on exposure to acidic solutions of methanol[38] or as a function of temperature.[39] Similarly, the use of protein fragmentation methods coupled with microbore high performance liquid chromatography can be employed[40,41] to ascertain the exchange rates of peptide amide hydrogens within proteins undergoing isotopic exchange from the deuterium levels detected by mass spectrometry in the proteolytic fragments of the protein. With the advent of routine on-line LC−MS techniques, adaptation of these isotope exchange procedures represents an attractive opportunity to explore the effects of different chemically defined solid/liquid interfacial environments on the conformational status of intact polypeptides or proteins under, for example, various chromatographic conditions. In this case, a $^1H \rightarrow {}^2H$ exchange mass profile, unique for each polypeptide or protein, can be generated in response to perturbations of their conformations as the polypeptide or protein interacts with ligands present at the solvated surface of the sorbent. Changes in this mass profile will reflect conformational populations of the polypeptide or protein with distinctly different molecular masses as the biomolecule unfolds in the deuterated solvent environments. A corresponding mass change will not occur in the equivalent protonated solvent environment. Measurement of these characteristic $^1H \rightarrow {}^2H$ amide exchange mass profiles should potentially offer a rapid and sensitive means to study the stability of polypeptides and proteins in low-pH water−organic solvent environments in the presence/absence of hydrophobic *n*-alkylsilica sorbents, such as those employed in reversed-phase HPLC.

As an extension to our studies[3,9,42−45] into the conformational and thermodynamic behavior of polypeptides and proteins in different hydrophobic/lipophilic environments, we have validated this hypothesis in the present investigation. In particular, the mass spectrometric characteristics of hen egg white lysozyme (HEWL) and horse heart myoglobin (HMYO) have been investigated following incubation of these proteins in low-pH water−organic solvent mixtures in the presence/absence of an *n*-octadecylsilica sorbent over a wide range of temperature conditions. These proteins were selected because their three-dimensional structures in the crystalline and solution states have been determined[23−27,46,47] to very high resolution by X-ray, neutron diffraction, and multidimensional heteronuclear NMR methods (HEWL to 0.95 Å resolution and HMYO to 2.0 Å resolution), including definition of the extent of surface accessibility of individual amino acid residues where hydrogen bonding to structured water can occur. In the studies described below, changes in the hydrogen bond characteristics and, thus, variations in residual conformational status of these proteins were assessed by following the changes in their molecular masses in response to variations in the bulk solvent or chromatographic conditions as the operating temperature or residency time of the system was increased. The results document that conformational changes of these proteins can be directly monitored from the resultant mass ladder profiles as the progressively exchanging amide hydrogen bonds involved in the stabilization of the native tertiary structure are slowly disrupted by perturbing solvational, thermal or solid/liquid interfacial conditions.

MATERIALS AND METHODS

Chemicals and Reagents. Hen egg white lysozyme (HEWL) and horse heart myoglobin (HMYO), obtained from Sigma Chem. Co. (St Louis, MO), was repurified and characterized as described previously.[9] Highest-grade reserpine, trifluoroacetic acid (TFA), acetonitrile (ACN), methanol (MeOH), deuterated water (D_2O), and deuterated trifluoroacetic acid (DTFA, >99% D) were purchased from Nacalai Tesque (Kyoto, Japan) or Pierce Chemical Co. (Rockford, IL). The water (H_2O) used was double-distilled

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and purified through a Milli-Q system (Millipore, Medford, MA). The sheath liquid was 50% methanol in purified H_2O. To make the sample solutions, appropriate amounts of each protein were separately dissolved in (i) 0.1% TFA in Milli-Q-purified H_2O, (ii) 0.1% DTFA in D_2O, (iii) 0.1% TFA in 35% ACN/Milli-Q-purified H_2O, and (iv) 0.1% DTFA in 35% ACN/D_2O. This choice of acetonitrile percentage corresponded to the volume fraction at which these proteins eluted from the reversed-phase sorbent. The protein concentrations were ca. 500 μg/mL.

Apparatus and Instrumental Techniques. Reversed-phase HPLC procedures were based on methods described previously[3,42−45] using a Beckman System Gold programmable HPLC system with a Beckman (Fullerton, CA) Solvent Mixing Module 126, a Beckman model 126 UV detector, or an Applied Biosystems Integral system (Framingham, MA) coupled to a microbore (150 × 1.0 mm) column packed with porous *n*-octadecylsilica (3 μm mean diameter and ca. 120 Å mean pore diameter) (YMC, Shermbeck, FGR). The flow rate was controlled at 50 μL/min throughout these studies, and all injections of 5 μL were made via a Rheodyne (CotatiInc., CA) model 8125 injector. Peak profiles were monitored at 215 nm. Column and extra-column dead volumes were measured independently with the noninteractive solute, sodium nitrate. The temperatures investigated were from 298 K to 338 K. All data points were derived from at least triplicate experiments with the variation in the relative retention factor measurements between replicates typically less than 5%. Bulk solvents were filtered through 0.2-μm filters and then degassed by sparging with nitrogen. The pH measurements were recorded using an Beckman F34 pH meter (Fullerton, CA) or an Orion model SA520 pH meter (Cambridge, MA).

Electrospray ionization mass spectral data were collected on a Finnigan LCQ ion trap mass spectrometer (Finnigan MAT, San Jose, CA) and a PE-Sciex AP-111 mass spectrometer (Thornhill, Ontario, Canada) equipped with an electrospray source. A microsyringe pump (Harvard Apparatus, South Natick, MA) was used to deliver the sample solution via a 250-μL syringe (Ito Microsyringe Co., Tokyo, Japan) and a 50-μm-i.d. capillary (Polymicro Technologies Inc, Phoenix, AZ) into the electrospray source. The built-in microsyringe pump of the Finnigan LCQ ion trap mass spectrometer was used to deliver the sheath liquid via a 500-μL syringe (Unimetrics, Shorewood, IL) and polymer tubing. A laboratory-made water bath consisting of a Hot Stirrer S-300H (Iuchi, Osaka, Japan) and 500-mL Pyrex beaker, or alternatively, a Biorad module 486 recirculating water bath (Biorad, Richmond, CA), were used for the temperature incubations. The temperatures were monitored using a laboratory thermometer.

Methods. For the bulk solution studies, the protein samples were incubated for 2 min at different temperatures ranging from 293 K to 353 K. In the chromatographic experiments, the proteins in the appropriate buffers were eluted at a flow rate of 50 μL/min using a linear gradient of acetonitrile−H_2O mixtures, generated from buffer A (0.1% TFA in H_2O) to buffer B (0.09% TFA in acetonitrile:H_2O (65:35 v/v)). Alternatively, linear gradients of acetonitrile/D_2O at the same flow rate were generated using 0.1% DTFA in D_2O (buffer A) to 0.09% DTFA in acetonitrile:D_2O (65:35)(buffer B). An aliquot of the column effluent was directed to the nebulizing needle of the ion spray interface via a standard in-line 1:10 Valco (Houston, TX) Tee-stream splitter using a length of 50-μm-i.d. coated fused-silica tubing. A 5 μL/min flow of the sample solution and 1 μL/min flow of the sheath liquid was simultaneously injected into the electrospray source ,and the mass spectra were acquired at 3 microscans and 50 ms maximum injection time over a m/z range of 150−2000. A dwell time of ca. 0.5 ms/step with an orifice voltage of 40 V was used. MS full-scan mode was conducted in all experiments. The input method was first/last and the CID source was turned off. A capillary temperature of 473 K, along with a ionization spray voltage of 5 kV, and nitrogen as the sheath gas were employed. Data collection and analysis were performed on a PC using the Navigator 1.1 Software (Finnigan MAT, San Jose, CA) and Bioexplore Version 1.0 beta 8 for LCQ 1.1 (Finnigan MAT, San Jose, CA), respectively. A solution of reserpine (MW 608.7) in the sheath liquid was used for tuning of the mass spectrometer.

RESULTS AND DISCUSSION

In the experiments described below, $^1H \rightarrow {}^2H$ exchange was initiated by dissolving the fully ^1H form of the protein in low-pH, deuterated buffers, and then incubating the sample for different time intervals at different temperatures in the presence or absence of the *n*-octadecylsilica reversed-phase chromatographic sorbent. This strategy differs from previous hydrogen exchange approaches,[28,29,33−37] since it monitors the unfolding process directly from the $^1H \rightarrow {}^2H$ mass gain rather than from a $^2H \rightarrow {}^1H$ mass decrease of the intact protein in bulk solution. Moreover, protein fragmentation procedures are not employed as a prelude to determine the extent of the isotopic exchange. Thus, when hen egg white lysozyme (HEWL) was incubated in 0.1% TFA in H_2O, irrespective of the incubation temperature or time, the same m/z charge states and, thus, the same calculated molecular mass for the HEWL species were obtained (Figure 1A,B). This behavior can be contrasted to the corresponding results for HEWL incubated in 0.1% DTFA in D_2O for the same time duration (2 min) in which similar charge states were observed Figure 1C,D, but in this case, the values of the m/z ratios for HEWL were significantly higher, particularly when the protein was incubated at elevated temperatures.

Several differences are noteworthy from these results. First, an increase in the value of the m/z ratio was evident when D_2O was employed, that is, for the +8 charge state, the m/z value increased from 1789.2 in the proton exchange experiment to 1806.9 for the deuterium exchange experiment (when both samples were incubated at 293 K). It can be noted that the theoretical molecular mass calculated from the amino acid sequence of HEWL was 14313.13 Da, but the experimentally determined coefficient-weighted value deconvoluted from the +11 to +8 charge state m/z values with the 293 K H_2O experiment was 14310.0 Da. Second, the difference in the m/z ratios became progressively larger when the temperature was increased, that is, for the +8 charge state the m/z value increased from 1789.5 for the proton exchange experiment to 1820.8 for the deuterium exchange experiment (when both samples were incubated at 353 K). Third, no significant differences in the corresponding m/z charge states were evident when 35% (v/v) acetonitrile was present in either of the incubation solvents at 353 K; however, at lower temperatures, the m/z ratios for the incubation experiments using 0.1% DTFA in 35% (v/v) ACN−D_2O indicated that partial destabilization of the HEWL had occurred, since these m/z values were

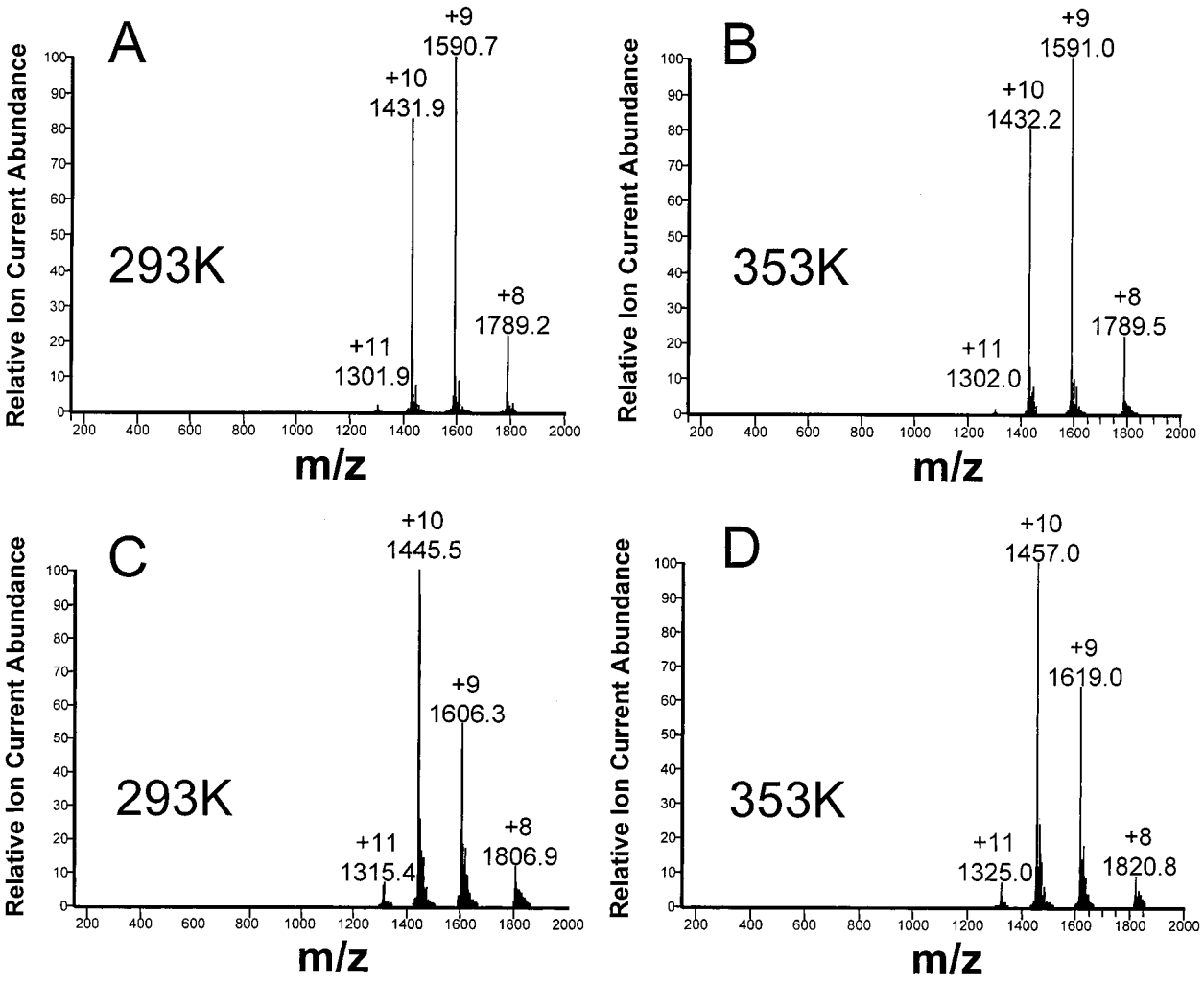

Figure 1. Representative mass spectral *m*/*z* data for hen egg white lysozyme (HEWL) following incubation in 0.1% TFA in purified H_2O (A and B) or 0.1% DTFA in D_2O (C and D) at temperatures of 293 and 353 K, respectively.

larger than obtained in the absence of this cosolvent. This finding confirms previous CD studies[48] with HEWL in which the presence of intermediate to high concentrations of acetonitrile has been shown to act as a denaturant. Thus, it can be concluded from these mass spectroscopic studies that as the temperature was increased, additional backbone amide hydrogens of HEWL underwent $^1H \rightarrow {}^2H$ exchange; that is, the protein progressively unfolds with loss of native structure. However, complete exchange of backbone amide hydrogens does not occur at low temperatures, irrespective of whether acetonitrile is present or not in the incubation mixture. Horse heart myoglobin (HMYO) exhibited analogous behavior (data not shown) with no change in the *m*/*z* ratio evident for the proton experiments at 293 or 353 K respectively (i.e., *m*/*z* for the +9 charge state remained at 1884.6), but an increase in the value of the *m*/*z* ratio for the + 9 charge state from *m*/*z* o1906.5 to *m*/*z* 1911.0, respectively (and corresponding *m*/*z* increases for the other charge states), was evident for the deuterium exchange experiments. The mass spectral data for the isotope exchange with HMYO indicated that the protein lost most of its native structure, even at relatively low temperatures. The situation with HMYO is, however, more complex that for HEWL because of the presence of the haem moiety in HMYO. The theoretical molecular mass calculated from the amino acid sequence for the *holo*-HMYO is 17466.59 and for the *apo*-HMYO is 16951.48 Da, and the experimentally determined coefficient-weighted, average molecular mass derived from the deconvoluted +22 to +9 charge state *m*/*z* value for the 20 °C H_2O experiment was 16955.0 Da. Thus, under the studied incubation conditions, the haem group (M_r = 515.1 Da) dissociated from the protein, resulting in a molecular mass characteristic of the *apo*-HMYO species. This behavior is consistent with other observations[9,49−51] on the thermal denaturation of myoglobins when this protein is chromatographed on *n*-butyl and *n*-octadecylsilicas using low-pH water−organic solvent mixtures. We have previously shown[42] that even at higher pH values, for example, pH 7.0, HMYO still loses the haem group under these reversed-phase conditions.

As noted above, various reports have documented the use of analytical RP-HPLC or HP-HIC procedures to probe polypeptide and protein conformation. For example, RP-HPLC and HP-HIC methods have been employed to assess ligand-induced stabilization of coiled-coil[52,53] and amphipathic helical peptides[13,42−45,54,55]

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as well as used to study the solvent- or ligand-induced denaturation of various polypeptides and proteins, including cytochrome *c*,[56] insulin-related polypeptides,[57] growth hormone,[58] α-lactalbumin,[59,60] α- and *β*-lactoglobin,[61] papain,[8] ribonuclease A,[6,62] soybean trypsin inhibitor,[63] and trypsin.[64] Although off-line CD or in situ intrinsic fluorescence spectroscopic measurements have been used in some of these studies, generally, the conformational status of the polypeptide or protein has not been characterized in these RP-HPLC or HP-HIC systems, but rather has been inferred from the change in the retention time(s) or shape of the eluted peak zone(s) obtained at a single temperature or, in a few cases, over a range of different temperatures. Thus, it has frequently been concluded that an unfolded polypeptide or proteins under these hydrophobic conditions will have a longer retention time and a more asymmetric peak shape than the same molecule in the folded states, although evidence validating this postulate is often lacking. Some investigators have recently asserted,[65,66] solely on the basis of retention time arguments, that α-helical or *β*-sheet secondary structures have been induced with relatively small synthetic peptides (i.e., <20 residues) by the lipophilic surfaces of nonpolar sorbents. Again, definitive, independent evidence substantiating these conclusions has not been forthcoming. Considerable ambiguities, thus, remain about the significance of these findings or their interpretation. The procedures described in this paper provide one avenue to ascertain whether such conformational behavior actually occurs when a polypeptide or protein interacts with the solvent or chemically modified sorbent under these conditions.

Anomalous multizoning or non-Gaussian peak shape behavior has been previously documented under certain RP-HPLC conditions[5,42−45,67,68] with polypeptides and globular proteins. In the present investigations, complex peak profiles were similarly observed for HEWL and HMYO when these proteins were chromatographed on *n*-octadecylsilica sorbents at elevated temperatures. Illustrative of these effects are the representative elution

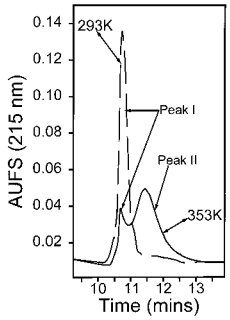

Figure 2. Representative RP-HPLC elution profile for HEWL chromatographed on a C_{18} *n*-octadecyl silica sorbent at 298 (profile I, - - -) and 338 K (profile II, —) using a linear 15-min gradient of acetonitrile−H_2O (or −D_2O) mixtures, generated from buffer A (0.1% TFA in H_2O (or 0.1% DTFA in D_2O)) to buffer B (0.09% TFA in acetonitrile−H_2O (or 0.9% DTFA in acetonitrile−D_2O) (65:35 v/v). The elution positions of peaks 1 and 2 discussed in the text are indicated by arrows. Other conditions are given in the Materials and Methods section.

profiles (Figure 2) for HEWL at low (profile I, 293 K) and high (profile II, 353 K) temperatures using a linear gradient of acetonitrile/H_2O mixtures, generated from buffer A (0.1% TFA in H_2O) to buffer B (0.09% TFA in acetonitrile:H_2O (65:35 v/v), with a 3-*μ*m *n*-octadecylsilica sorbent packed in a microbore (150 × 1.0 mm) column at a flow rate of 50 *μ*L/min. Similar non-Gaussian peaks have been observed in our earlier work[9] with HEWL chromatographed on other C_{18}- and C_4- sorbents with gradient times of 30−90 min and at temperatures of 278 K to 358 K, respectively. The absence of significant band broadening in the elution profile of HEWL at the lower temperature (peak I, 293 K experiment) indicated that the overall kinetics associated with the adsorption/desorption of HEWL were relatively rapid and that the protein may have adopted under these conditions an averaged conformation, which is similar to that occurring in the bulk solution. The more complex elution profile that arose at the higher temperatures, coupled with the increase in elution time for the broad peak II, was compatible with the presence of ensembles of conformational species due to the further unfolding of the protein and exposure of additional hydrophobic amino acid residues for interaction with the *n*-octadecyl ligands. The appearance of this more complex elution profile at higher temperatures, thus, could represent an extreme example of the multizoning "split-peak" effect.[5,7] This split-peak effect occurs when the differences in the interactive structures of slowly interconverting conformational ensembles of a protein, as it undergoes a transition from the fully folded state to a partially or fully unfolded state or vice versa, are sufficiently large to permit these transient molecular species to be (partially) resolved and, thus, to form a distorted or even a distinct peak envelope as part of the RP-HPLC elution profile. Since degradation of HEWL does not occur under these conditions, the appearance of two (or more) chromatographic peaks suggests the presence of distinct conformational states of HEWL in the RP-HPLC environment. Under the conditions employed in this study, these HEWL (and HYMO) species eluted at an acetonitrile content of ca. 35% (v/v). To assess the extent that HEWL and HMYO were denatured under these low-pH, thermal conditions in the presence of water/acetonitrile mixtures, the corresponding $^1H \rightarrow {}^2H$ exchange experiments were undertaken under both bulk solution incubation conditions and microbore RP-HPLC elution

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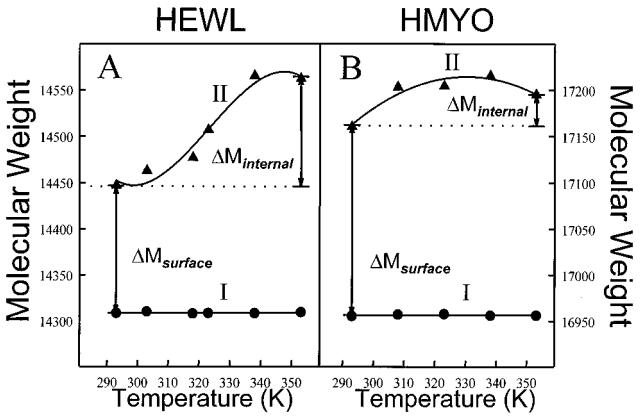

Figure 3. Plot of molecular mass versus temperature (reflecting the $^1H \rightarrow {}^2H$ replacement of the protein exchangeable hydrogens) for HEWL (A) and HMYO (B) following incubation in 0.1% TFA in H_2O (plot I, ●−●−●) or 0.1% DTFA in D_2O (plot II, ▲−▲−▲) as a function of temperature from 293 to 353 K. The molecular mass gain due to the chaotic and progressive processes as HEWL and HYMO unfold as the temperature is increased are shown as $\Delta M_{surface}$ and $\Delta M_{internal}$, respectively.

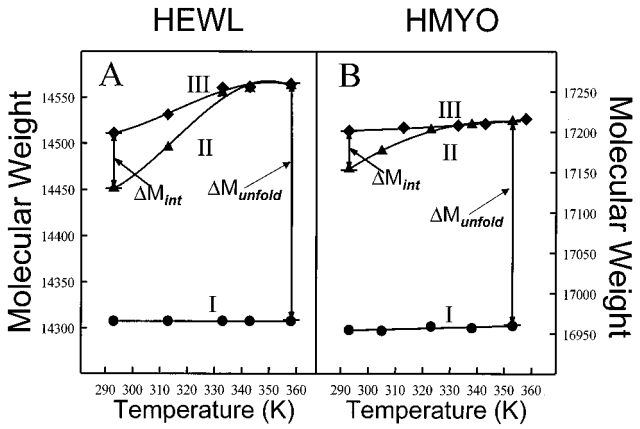

Figure 4. Plot of molecular mass versus temperature (reflecting the $^1H \rightarrow {}^2H$ replacement of the protein amide hydrogens) for HEWL (A) and HMYO (B) following incubation in 0.1% TFA−35% (v/v) acetonitrile−H_2O (plot I, ●−●−●) or 0.1% DTFA 35% (v/v) acetonitrile−D_2O (plot II, ▲−▲−▲), as a function of temperature from 293 to 353 K. Also shown in this figure are the data for the molecular mass changes as a function of temperature (reflecting the deuterium exchange, as compared to proton exchange) for HEWL and HMYO following interaction and elution from the RP-HPLC sorbent at temperatures from 298 to 353 K (plot III, ◆−◆−◆) using a linear 15-min gradient generated from buffer A (0.1% DTFA in D_2O) to buffer B (0.9% DTFA in acetonitrile−D_2O, 65:35 v/v), as described in the Materials and Methods section. The molecular mass gain due to the overall unfolding process and the surface-induced interaction of HEWL and HYMO as the temperature was increased is shown as ΔM_{unfold} and ΔM_{int}, respectively.

conditions. The results from these experiments are shown in Figures 3 and 4.

In these experiments, the molecular mass for HEWL or HMYO species in the deuterated solvent environments progressively increased as the temperature was increased and the protein adopted different ensembles of conformational states. Because of the experimental design used in these investigations in which the incubation time was at least 100× larger than the time for chaotic $^1H \rightarrow {}^2H$ exchange, it is possible that distinct populations of protein molecules exist with specific backbone amide or side chain hydrogens still protected from exchange. However, once steady state equilibrium for isotopic exchange at a particular site has been reached in the presence of a vast excess of deuterons, the protein conformational population will exist almost exclusively as the deuterated species at that site, rather than a transient mixture with some molecules containing a deuteron and others a proton at that site. Depending on whether the exchange rate, k_{ex}, is slow or fast, the observed molecular mass will, as a consequence, represent the atomic mass of the protein plus the number of deuterons arising from the $^1H \rightarrow {}^2H$ exchange at specific sites under those conditions, rather than the weighted sum average of a population distribution of the protein as a mixture of protonated and deuterated species at those sites.

In Figure 3 are illustrated the plots of molecular mass versus temperature from 293 to 353 K for HEWL (A) and HMYO (B) incubated in 0.1% TFA in H_2O (I) and 0.1% DTFA in D_2O (II). As is evident from these plots, no change in the molecular masses arose for these two proteins when these incubations were carried out as the proton experiments. This situation can be contrasted with the corresponding data for the deuterium exchange experiments, in which significant increases in molecular masses were apparent as the temperature was increased from 293 to 353 K. HEWL contains a total of 255 exchangeable protons, with 126 side chain protons, 126 protons from the backbone amides, and 3 protons from the termini, including 82 side chain hydrogens partially accessible to a 1.4-Å solvent molecule (i.e., a molecule of the dimensions of H_2O or D_2O) and 10 highly buried side chain hydrogens, as assessed by neutron diffraction and X-ray crystallographic experiments.[26,45] As a consequence, HEWL could potentially generate a molecular mass change of 255 amu if all of these protons simultaneously were to undergo chaotic deuterium exchange. However, many of these protons are either associated with salt-bridged structures or form part of the intramolecular amide hydrogen bond network that stabilizes the various α-helical or β-sheet structures that have only partial accessibility to the solvent under the low-pH conditions employed. The mass increase ($\Delta M_{surface}$) of 136 Da that occurred at 293 K thus represents the contribution from the chaotic process(es) involving the more readily exchangeable surface-accessible protons and indicates that ca. 53.3% (136/255) of these potential exchanges had occurred. After incubation for 2 h at 293 K at low pH in reversed-phase sorbents, other studies have shown[27] that although significant amide exchange has occurred, a significant amount of secondary structure was still preserved, with the most protection in the α helix domain. In pH 2.0 solutions, HEWL is known from NMR studies[24−27] to still contain an average of about 80 backbone amide and side chain protons that are protected from $^1H \rightarrow {}^2H$ exchange. The observed difference of 119 potentially exchangeable sites detected in the present study correlates reasonably well with these previous observations. In addition, the additional mass increase ($\Delta M_{internal}$) of 116 Da that subsequently occurred as the temperature was increased up to $T = 353$ K, corresponded to the progressive $^1H \rightarrow {}^2H$ amide hydrogen exchange process(es) of these less accessible 119 protons, which formerly were involved in intramolecular hydrogen bonds associated with the stabilizing secondary and tertiary structural characteristics of the "native" protein. Because HEWL contains 7 disulfide bonds, the formation of a completely extended, random coil structure typified by the

reduced species will not occur even at 353 K, although a species approximating an unfolded intermediate state, II, with the coincident loss of both the secondary and tertiary structures, appears to be generated under these low-pH conditions at elevated temperatures. The thermal melting of the secondary and tertiary structures of HEWL in low-pH water is known to occur with a T_m of 347.8 ± 0.4 K and 347.3 ± 0.7 K, respectively. The ESI-MS data obtained for the thermal melting of HEWL in 0.1%TFA (or DTFA) in the presence or absence of 35% acetonitrile−water (v/v) mixtures (see below) indicate that similar transitions occurred under these conditions near these temperatures, that is, near 374 K, leading to the loss of structure of this protein.

The shape of the molecular mass versus temperature plots with the deuterium exchange experiment was, moreover, significantly different for HEWL and HMYO, suggesting that the application of such $^1H \rightarrow {}^2H$ amide hydrogen exchange plots thus provide a useful approach to discriminate between the conformational characteristics and stability of these and other globular proteins in water−organic solvent mixtures under different conditions. In the case of HMYO, a mass increase of 192 Da was observed following the $^1H \rightarrow {}^2H$ exchange experiment at 293 K. From X-ray diffraction studies,[69] HMYO theoretically contains 80 side chains bearing exchangeable hydrogens that are surface-accessible and could potentially undergo this fast, chaotic deuterium exchange, but 12 are buried. HMYO contains 153 amide hydrogens. Significantly, under these conditions, the haem group had dissociated from the HMYO, generating the *apo*-protein. The mass increase ($\Delta M_{surface}$) of 192 Da that occurred for HMYO at 293 K, thus, represents the contribution from the chaotic process(es) and indicates that ca. 78% (192/245) of the theoretical number of exchangeable hydrogens have undergone this isotope exchange. In addition, the overall mass increase ($\Delta M_{internal}$) of ca. 35 Da (i.e., 227 of the total of 245 hydrogens had exchanged) that subsequently occurs due to the progressive $^1H \rightarrow {}^2H$ amide hydrogen exchange process(es) as the temperature was increased up to $T = 353$ K, predominantly corresponded to many of the remaining 53 exchangeable protons, which were formerly involved in intramolecular hydrogen bonds associated with the stabilizing secondary and tertiary structural characteristics of the "native" HMYO. These results indicate that the protein has unfolded by ca. 90% (227/245) under these low-pH conditions at elevated temperature conditions. At low temperatures, *apo*-HMYO can maintain in low-pH water-only solutions a considerable amount of its secondary structure (ca. 60% helix). From the results obtained in this study, it is apparent that the majority of the amide hydrogens and ionizable protons of the *apo*-HMYO species that were generated in this binary water−acetonitrile solution were readily exchanged, suggesting that little residual secondary structure or few salt bridges remain under these low-pH conditions, even at temperatures of 293 K. Similar observations have been made on the thermal stability of myoglobins by other spectroscopic techniques, such as CD, when an organic solvent is present as a cosolvent. Thus, depending on the protein under study, these $^1H \rightarrow {}^2H$ exchange experiments also permit the impact of organic solvent(s) to be assessed independently of the influence of temperature, pH, or buffer ion effects.

The fact that the molecular mass remained constant in the proton experiments with both HEWL and HYMO also confirms that amide bond fragmentation does not occur under these low-pH conditions. In this context, it is also interesting to note that the deuterium exchange results for HEWL followed a sinusoidal dependency on temperature, reminiscent of the thermal unfolding curve of this protein. Thus, it can be concluded from these proton/deuterium exchange data that at 293 K, HEWL still maintains some three-dimensional features of a "nativelike" structure (i.e., it exists in a compact state(s) with a significant amount of secondary structure that may approximate the molten-globule-like intermediate state I or after further unfolding the partially nucleated, near-random-coil intermediate state II) in the bulk solution of 0.1% TFA−H_2O or 0.1% DTFA−D_2O, a conclusion in accord with related CD and ^1H NMR studies.[15,70−74] In contrast, at 353 K, the hinged two-domain structure of HEWL has largely been destabilized, and the protein is approaching its random coil conformation. A different, but related, set of considerations applies to HMYO, in which the low-pH conditions clearly have induced the dissociation of the haem moiety, leading to the formation of the *apo*-HMYO species. As the temperature was increased, the deuterium exchange resulted in smaller increases in molecular mass, indicating that most of the exchangeable amide and side chain hydrogens of the *apo*-HMYO were much more accessible to the solvent than what was the case for HEWL. Again, these results are consistent with the known thermal denaturation of HMYO at low pH values.[12,75−78]

The molecular mass versus temperature dependencies of these HEWL and HMYO under conditions that replicated their behavior in reversed-phase HPLC systems are shown in Figure 4. The relative difference in stability for HEWL and HMYO in the free and bound states can be determined from the value of ΔM_{int} (equals mass change following adsorption minus mass change in bulk solution). At 353 K, considerable unfolding (given by the value of ΔM_{unfold}) was evident for both HEWL and HMYO at a rate similar to that observed under the bulk incubation conditions at elevated temperatures. Thus, in the presence of 0.1% TFA−35% acetonitrile−H_2O following elution from the nonpolar sorbent, the molecular mass of both proteins again remained essentially constant over the temperature range 293−353 K under the proton exchange conditions. With the deuterium exchange conditions of 0.1% DTFA−35% acetonitrile−D_2O, on the other hand, progressive increases in the molecular mass were apparent for both proteins. In the case of HEWL, the same final molecular mass value (14 560 Da) was achieved with the 0.1% DTFA−D_2O incubation and the 0.1% DTFA−35% acetonitrile−D_2O elution condition. When compared to the results obtained at 293 K for HEWL with the 0.1% DTFA−D_2O incubation condition, the molecular mass values were different (14 491 Da in the 0.1%

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DTFA−35% acetonitrile−D_2O incubation system, as compared to 14 447 Da in the 0.1% DTFA−D_2O incubation system). However, the later-eluting zone for HEWL (Figure 2, peak II) exhibited deuterated species ranging in molecular mass from 14 516 Da to 14 561 Da, suggesting that this complex elution profile contained ensembles of conformational species that were different from that found in bulk solution, ranging from a partially unfolded near-native state to states that were more substantially unfolded. In this context, it is interesting to note that Wu et al.[79,80] have observed a similar phenomenon by UV/vis difference spectroscopy for the thermal unfolding of HEWL, α-lactalbumin (α-LAC), and several other proteins by hydrophobic interaction chromatography. These investigators have similarly concluded that the later-eluting species corresponded to a more unfolded conformational state(s) of the protein. The corresponding data for HMYO suggested that the protein has lost the haem moiety during or prior to adsorption/desorption with the *n*-octadecylsilica sorbent but undergoes relatively little further conformational change induced by the solvated *n*-octadecyl ligands as the temperature is increased. Such behavior, reflected as a small ΔM_{int} value, would be consistent with this protein existing in a disordered *apo*-HMYO state under these conditions, a conclusion consistent with other spectroscopic and chromatographic observations[9,56,57] with this protein in water−organic solvent solutions of low pH and elevated temperatures.

The above findings are consistent with increased deuterium exchange and greater amide/side chain accessibility of HEWL following desorption from the reversed-phase sorbent with 0.1% TFA (or DTFA)−35% acetonitrile−H_2O (or −D_2O). This finding suggests that HEWL and HMYO unfold more readily in the presence of the *n*-octadecylsilica sorbent with this binary solvent mixture, despite the known propensity[3,12,39] of acetonitrile to stabilize the α-helical conformations of some polypeptides in bulk solution, as assessed from CD measurements, but also to destabilize β-sheet-rich proteins at intermediate to higher concentrations.

The present study, thus, has documented an approach to examine the conformational stability of proteins in water−organic solvent mixtures and at hydrophobic solid/liquid interfaces. These findings confirm the unique ability of ESI-MS procedures to distinguish between populations of different protein conformers during the unfolding process. These results are, moreover, reminiscent of the observations made on the transitions of HEWL from the fully unfolded state to the corresponding folded state.[81] Clearly, the procedures described for the assessment of RP-HPLC behavior of proteins could be adapted to study the conformational vagaries of polypeptides and globular proteins in the other modes of interactive HPLC, including high performance ion exchange (HP-IEX), hydrophobic interaction (HP-HIC), affinity (HP-AC) and metal ion affinity (HP-IMAC) chromatographic methods. For each of these different high-resolution separation procedures, the approach described in this paper should enable the solution component of the conformational cycle(s) to be assessed independently from the contributions of the surface interactive component. Such information would have important ramifications for the characteristics of recombinant protein variants in terms of their relationship to the wild-type protein, as well as in assessing the conformational integrity of proteins following their purification, handling, or storage. Additional studies exploring the utility of this approach to characterize the conformational behavior of proteins in binary or ternary solvent systems, as well as under circumstances in which isodesmic self-association of polypeptides or proteins can occur in various solid/liquid interfacial environments, will be reported in subsequent papers.

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