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Manipulation of Temperature To Improve Solubility of Hydrophobic Proteins and Cocrystallization with Matrix for Analysis by MALDI-TOF Mass Spectrometry

Gregory H. Bird, Ajay R. Lajmi, and Jumi A. Shin*

Department of Chemistry, University of Pittsburgh, Pittsburgh, Pennsylvania 15260

Matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) requires cocrystallization of analyte with a large excess of matrix, which must be mutually soluble in a solvent that encourages crystal growth upon evaporation. MALDI-MS of hydrophobic proteins can be difficult, because they tend to aggregate in polar solutions. High concentrations of denaturants and salts are often employed to combat protein aggregation, but this can result in signal suppression. By using various organic cosolvent systems and matrixes at different protein:matrix ratios, we were able to use MALDI-TOFMS to detect four bacterially expressed hydrophobic proteins comprising alanine-rich mutants of the basic region/leucine zipper protein (bZIP) GCN4. By manipulating sample temperature, we were able to maintain protein solubility. Protein aggregation was suppressed when mixing the protein and matrix solutions at 4 °C prior to warming to 37 °C, following the temperature-leap technique described by Xie and Wetlaufer (*Protein Sci.* 1996, *5,* 517−523), who used this method to renature bovine carbonic anhydrase II. Manipulation of temperature encouraged our hydrophobic proteins to adopt conformations leading to the nonaggregating state, and solubility was maintained even when the concentration of denaturant was reduced from 4 M to 400 mM. The temperature-leap tactic was critical for maintaining protein solubility, preventing signal suppression normally seen with higher concentrations of salts, allowing for generation of superior spectra, and should prove applicable to other systems prone to aggregation.

With the era of proteomics upon us, characterization of biopolymers has become more important than ever.[1] Analysis of the entire collection of proteins for a particular organism or cell type requires intensive fractionation or separation, or a system that is adept at identifying components of mixtures.[2] Additionally, the ideal system would not discriminate among proteins based on their hydrophobicity or any other parameter. Molecular weights of proteins and protein fragments can be ascertained by mass spectrometry, in particular electrospray ionization (ESI) and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF); these techniques have risen in popularity as the demand for molecular characterization of biological systems has increased. The use of MALDI-TOF has become widespread and accessible, because it has evolved to become a valuable tool for the structural characterization of proteins because of the low amount of sample required for analysis (femtomoles to picomoles of analyte), its high mass accuracy (0.01−0.1%), and its tolerance to buffers and salts.[3]

Hydrophobic proteins, however, present a challenge, because they are not soluble in polar solvents. Moreover, characterization of hydrophobic and aggregating proteins, including those involved in amyloidogenesis, is of great interest to the research community. To facilitate matrix−sample cocrystal formation, organic solvents are often used. Mixtures of chloroform:methanol or 2-propanol: acetonitrile have been effective in solubilizing hydrophobic proteins and peptides, and generation of abundant molecular ions has been seen without degradation of sample.[4−6] Formic acid has been used in combination with acetonitrile, 2-propanol, or hexafluoro-2-propanol to dissolve membrane and nuclear proteins.[7−12] In all of the above cases, quality spectra were obtained by using organic solvents, but utilization of organic solvents can potentially destroy the substrate, and therefore, its use is not universally applicable.[8]

* Corresponding author. Phone: (412) 624-4927. Fax: (412) 624-4255. E-mail: jumi+@pitt.edu.

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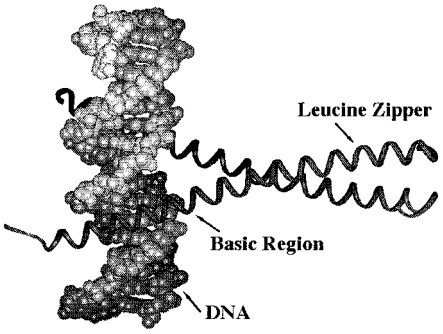

Figure 1. GCN4 bZIP in complex with the AP-1 DNA site, 5′-TGACTCA.[21] DNA is the vertical double helix at the left of the figure, and the bZIP is the horizontal α-helical dimer. The leucine zipper dimerizes into the coiled-coil structure shown at the right of the figure; the helical zipper then smoothly forks to either side of the DNA major groove.

Detergents are excellent at stabilizing and solubilizing hydrophobic proteins, because they prevent aggregation. Nonionic detergents, such as *n*-octyl-*β*-D-glucopyranoside, were used to solubilize bovine cytochrome *c* oxidase[13] and glycoproteins.[7] Sodium dodecyl sulfate (SDS) concentrations below 0.50% dissolved hydrophobic peptides yet did not suppress the mass signal.[14] Other comprehensive detergent studies have consistently shown that lower concentrations of nonionic detergents do not interfere with sample-matrix cocrystallization.[15,16] All of these techniques are useful, because they solubilize the protein and encourage cocrystallization with matrix. Once the cocrystal has been formed, superior spectra can be generated by removing salts; washing the sample plate with cold water exploits the water solubility of signal suppressing salts and denaturants without dissolving the matrix or protein.[17,18]

Our work focuses on alanine-scanning mutants of the α-helical basic region/leucine zipper motif (bZIP) of GCN4, which binds the major groove of DNA with high affinity and sequence specificity (Figure 1).[19−22] GCN4 is a dimeric transcriptional regulatory protein that governs histidine biosynthesis in yeast under conditions of amino acid starvation.[19] The full-length GCN4 monomer is 281 amino acids, and the bZIP monomer is composed of ∼60 residues. The leucine zipper provides a hydrophobic interface for protein dimerization, and the basic regions bind opposite sides of DNA.

Of the naturally occurring amino acids, alanine possesses the highest propensity for forming and stabilizing α-helical protein

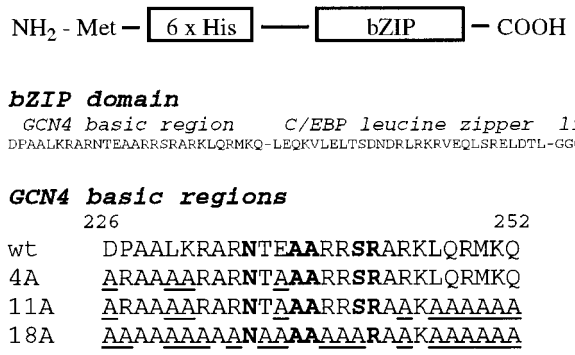

Figure 2. (Top) Schematic representation of expressed protein. Basic region/leucine zipper (bZIP) proteins were cloned into expression vector pTrcHis B (Invitrogen), which contains a six-histidine tag for protein purification and enterokinase cleavage site (DDDDK). The bZIP is at the carboxyl termini of the expressed proteins, which is the same positioning of the bZIP domain in native GCN4. The fully expressed bZIP comprises ∼35 residues from the pTrcHis expression vector, basic region mutants of GCN4 (residues 226−252), leucine zipper from C/EBP (residues 312−338), plus a linker (GGCGGYYYY) for covalent protein dimerization (cysteine) and chemical derivatization (tyrosines).[45] (Middle) Sequence of the bZIP domain. (Bottom) Sequences of the basic regions. The sequences for alanine mutants **4A**, **11A**, and **18A** are shown below wt; these proteins are the same as wt, except for the mutated basic regions. Alanine substitutions are underlined, and highly conserved residues are in boldface. We note that Pro[227] is arginine in both **4A** and **11A**; this is a cloning artifact, and this residue has no interaction with DNA.[21,22,46]

structures.[23,24] To explore the structural and functional aspects of DNA recognition by α-helical proteins, we substituted alanines into the basic regions of bacterially expressed GCN4 bZIP derivatives comprising the GCN4 basic region (residues 226−252) and C/EBP leucine zipper (residues 312−338).[25] The wild-type GCN4 derivative and alanine mutants are shown in Figure 2 with alanine replacements underlined; wt (wild-type) is the "native" variant comprising the GCN4 basic region and C/EBP leucine zipper. These Ala-based mutants are unusual proteins for expression in that they are short (∼100 amino acids) and hydrophobic (Ala-mutated basic regions, leucine-zipper dimerization domains). Circular dichroism demonstrates that these bZIP mutants assume the properly folded α-helical structure, and DNase I footprinting analysis shows that all of the mutants bind specifically to the AP-1 DNA site, the in vivo target site of GCN4 in yeast.[26]

Although our mutant bZIP proteins maintain native α-helical structure and sequence-specific DNA-binding function, they are poorly soluble in buffered solutions; tendency toward aggregation can likely be attributed to the Ala-rich basic regions and amphipathic leucine zipper. Protein hydrophobicity poses significant problems throughout the protein expression and purification stages, as well as during characterization. We, therefore, use high concentrations of denaturant (at least 4 M) throughout all of the steps of protein isolation,[25] and after purification, protein stock solutions are stored in 4 M urea or guanidine.

To maintain protein solubility at denaturant concentrations <4 M, we utilized a temperature leap tactic (T-leap) that aids in

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maintaining protein solubility when we drop the concentration of denaturant to 400 mM.[27] Xie and Wetlaufer studied the kinetics of renaturation of bovine carbonic anhydrase from 4 °C to 36 °C at a protein concentration of 136 μM. When denaturant (guanidine hydrochloride) concentration was reduced below 1 M, protein aggregation occurred. However, if refolding under lower concentrations of denaturant was conducted at 4 °C for 2 h, aggregation was significantly suppressed (37% enzyme activity). Moreover, if the enzyme was then rapidly warmed to 36 °C, the activity increased to 95%. Their explanation for these observations is that there are two sequential, slow-folding intermediates, the first of which is prone to aggregation, the second leading to native enzyme. At 4 °C, the aggregation-prone first intermediate is depleted after 120 min, and a rise in temperature allows the second intermediate to convert rapidly to the native, active form.[27]

By incorporating the T-leap tactic to aid in protein−matrix cocrystallization, we were able to detect strong mass spectrometric signals for all four mutants by MALDI-TOF. No spectra were generated in the absence of T-leap. When the T-leap was not utilized, no signal was seen, even when detergents and organic solvents were used. With the T-leap, protein aggregation was suppressed and protein−matrix cocrystallization occurred, allowing for accurate mass measurements of our four hydrophobic bZIP proteins (wt, **4A**, **11A**, and **18A)**, even in the presence of salts and denaturant.

EXPERIMENTAL SECTION

Materials. The matrixes 2,5 dihydroxybenzoic acid; *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid); 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid); 3-dihydroxycinnamic acid; *trans*-3-indoleacrylic acid; 5-chlorosalicylic acid; 6-aza-2-thiothymine; 2′,4′,6′-trihydroxyacetophenone; and α-cyano-4-hydroxycinnamic acid were purchased from Aldrich and used without further purification. The solvent systems used were 1:2 (by volume), acetonitrile:water, 0.1% trifluoroacetic acid (TFA); 2:1, acetonitrile:water, 0.1% TFA; 1:1:1, acetonitrile:2-propanol:water, 0.1% TFA; 1:2:3, formic acid:2-propanol:water; 1:2:3, formic acid:hexafluoro-2-propanol:water; 1:2:3, formic acid:tetrahydrofuran:water; and 2:5:2, chloroform:methanol:water, 0.1% TFA.[12] All solvents were of HPLC grade and were used without further purification. Water was purified through a Milli-Q filtration system (Millipore). Myoglobin (16 951 amu, ICN) and cytochrome *c* (12 359 amu, Acros) were dissolved in 1:2, acetonitrile:water, 0.1%TFA to concentrations of 193 μM and 100 μM, respectively, and stored at −20 °C.

Protein Preparation. Protocols for DNA oligonucleotide synthesis, gene construction and cloning, and protein overexpression and purification have been described in detail.[25] A brief summary of these procedures is as follows: genes for expression of bZIP proteins were constructed by mutually primed synthesis, cloned into protein expression vector pTrcHis B (Invitrogen), and transformed into *E. coli* strain BL21(DE3) (Stratagene) by electroporation (Bio-Rad). These 6xHis-tagged proteins were purified first on TALON cobalt-metal-ion-affinity resin (Clontech), followed by further purification by size-exclusion chromatography (Superdex 75 HR 10/30 column, Pharmacia) or by reversed-phase HPLC on a C4 column (Vydac) on a Beckman System Gold HPLC. Protein purification was monitored by SDS polyacrylamide gel electrophoresis (SDS-PAGE) and Western immunoblot assay. Purified stocks of wt bZIP, **4A**, **11A**, and **18A** were stored in SEC buffer (50mM phosphate, pH 6.8, 10% acetonitrile, 150 mM NaCl, 4 M urea) at final concentrations of 28.4, 24.6, 28.5, and 23.5 μM, respectively, and stored at −80 °C with protease inhibitors (1 mM PMSF and 1 μg/mL pepstatin).

MALDI Sample Preparation. Matrix solutions were prepared by saturating 1 mL of the appropriate solvent system with matrix at 4 °C overnight. This saturated solution was then vortexed ∼20 s and passed through a disposable filter to collect undissolved solid. The filter was dried in a 37 °C oven overnight and then weighed. By comparing the masses of the filters before and after collection of the insoluble portion, concentrations were ascertained. The protein solution, matrix solution, and a 0.6 mL Eppendorf tube were equilibrated at 4 °C for 30 min. For a standard matrix:protein ratio of 10 000:1 (mole ratio), 7.1 μL of saturated matrix solution was added to 1.0 μL of protein solution at 4 °C. The tubes were then spun down in a centrifuge for 1 min at 4 °C to ensure that all of the solution was collected at the bottom of the tube; any residual solid was scraped to the bottom of the tube with a pipet tip.

Only the amount of protein to be used for that day's experiments was renatured to active form following the T-leap tactic described by Xie and Wetlaufer.[27] The tubes containing protein and matrix solution were incubated at 4 °C overnight, although 2 h at 4 °C was found to be sufficient (as in the original protocol of Xie and Wetlaufer); tubes were then placed in a 37 °C bath for 1 h. After this time, the entire contents of each tube (1.9−23 μL) was taken up in a pipet and spotted onto a 100-well gold MALDI-TOFMS sample plate (Applied Biosystems) at room temperature (high matrix-to-protein ratios necessitated these unusually large volumes). The droplets were air-dried at room temperature for ∼10 min, followed by drying under vacuum for ∼10 min to remove residual solvent. Two procedures were used for addition of the calibrants. Typically, cytochrome *c* and myoglobin (0.5 μL and 1.0 μL of 30 μM protein solutions, respectively) were spotted on the partially dried droplet (air-dry at room temperature for ∼10 min; droplet is dried and crystallized along edges), followed by drying for ∼10 min under vacuum; this procedure was used in Figures 4-8. Alternatively, the same volumes of calibrants as in the first procedure were added to the solution of matrix and protein at 4 °C, and a T-leap was performed.

Mass Spectrometry. Mass spectra were obtained on a PerSeptive Voyager-DE STR MALDI-TOF (Applied Biosystems) at the Center for Molecular Analysis, Carnegie Mellon University. The 337-nm nitrogen laser had a pulse width of 3 ns. The accelerating voltage was 25 kV, and the sample plate grid voltage was set at 93% of the accelerating voltage. After a delay of 250 ns, the accelerating voltage was used to extract the ions. A guide wire voltage of 0.15% of the accelerating voltage was applied to refocus the desorbed ions along the 2.0-m flight tube. A low-mass gate of 4500 amu was used to prevent detector saturation. Positive ions were detected using a high-mass detector, which sent the signal to the digitizer at a rate of 500 MHz. Two hundred laser shots were accumulated and averaged for each sample. When acquiring spectra, we found that moving the laser around the periphery of

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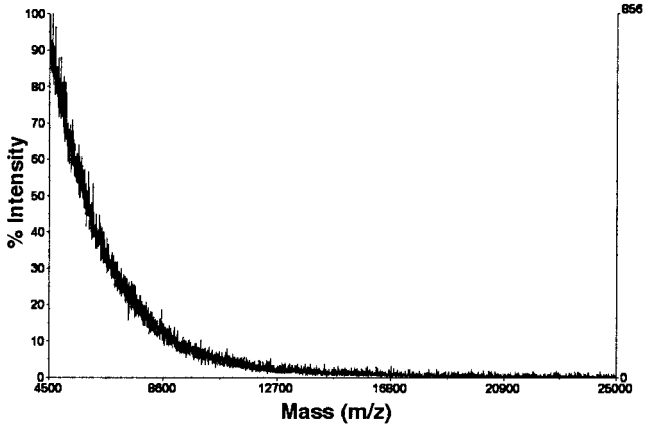

Figure 3. MALDI-TOF spectrum, without T-leap, of **4A** in sinapinic acid matrix at matrix:protein ratio of 10 000:1. Solvent system is 1:2, acetonitrile:water, 0.1% TFA. Similarly, the spectra of wt bZIP, **11A**, and **18A** show no peaks in the absence of the T-leap tactic.

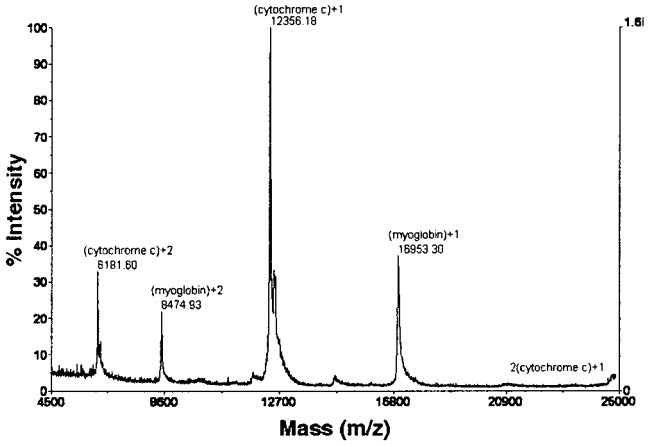

Figure 4. MALDI-TOF spectrum, without T-leap, of **4A** in sinapinic acid matrix at matrix:protein ratio of 10 000:1. Calibrants are myoglobin and cytochrome *c*. Solvent system is 1:2, acetonitrile:water, 0.1% TFA.

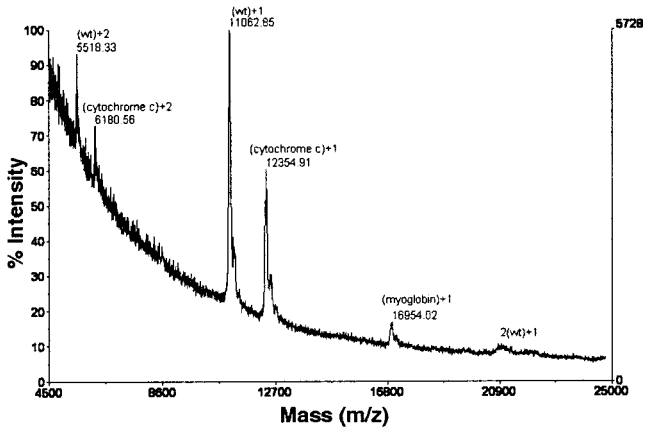

Figure 5. MALDI-TOF spectrum, with T-leap, of wt bZIP in ferulic acid matrix at matrix:protein ratio of 50 000:1. Solvent system is 1:2, acetonitrile:water, 0.1% TFA.

the dried spot and using 50−80% of the maximum laser intensity achieved the best results. Data Explorer software (version 3.4) from Applied Biosystems was used to work up data. Cytochrome *c* and myoglobin served as internal standards for calibration of the mass spectrometer.

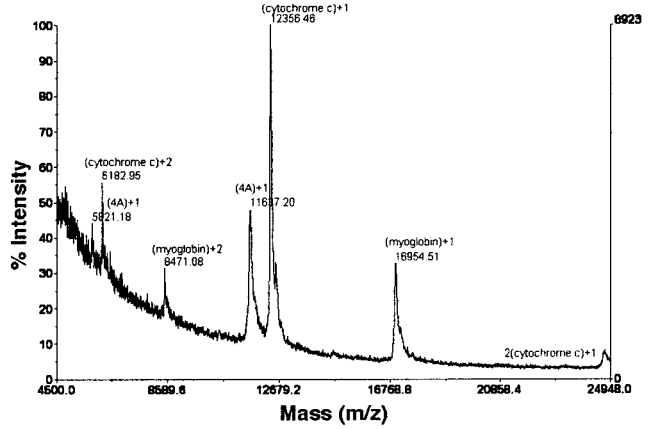

Figure 6. MALDI-TOF spectrum, with T-leap, of **4A** in sinapinic acid matrix at matrix:protein ratio of 100 000:1. Solvent system is 1:2, acetonitrile:water, 0.1% TFA.

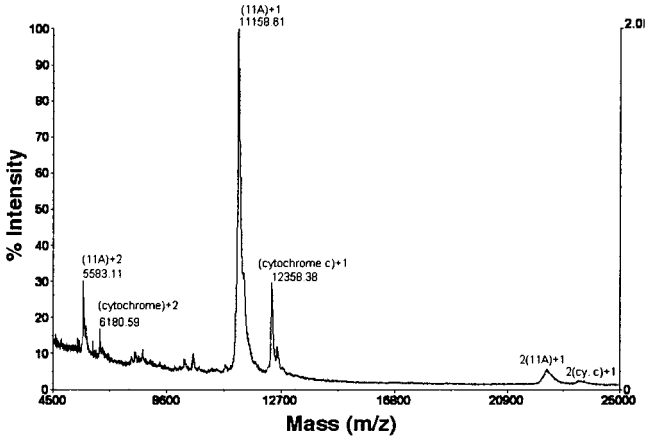

Figure 7. MALDI-TOF spectrum, with T-leap, of **11A** in ferulic acid matrix at matrix:protein ratio of 100 000:1. Solvent system is 1:2, acetonitrile:water, 0.1% TFA.

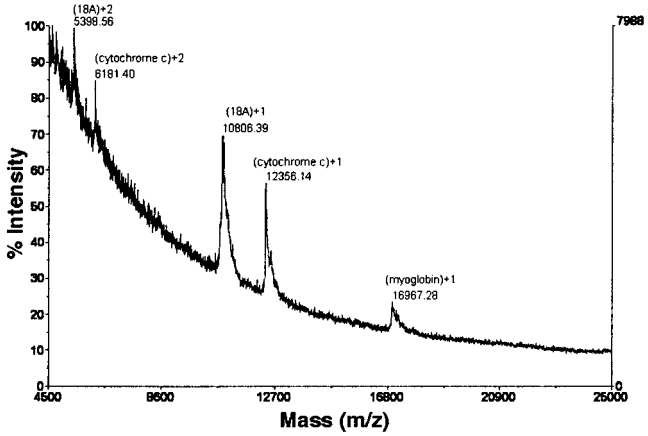

Figure 8. MALDI-TOF spectrum, with T-leap, of **18A** in ferulic acid matrix at matrix:protein ratio of 100 000:1. Solvent system is 1:2, acetonitrile:water, 0.1% TFA.

RESULTS AND DISCUSSION

To improve solubility of hydrophobic proteins, detergents and denaturants are often employed; unfortunately, these additives often degrade the quality of the spectra.[15,16] Our initial attempts to acquire mass spectra of our proteins were unsuccessful. We investigated nine matrixes, seven solvent systems, and various matrix-to-protein ratios in order to find the combinations that

yielded a signal at the expected mass-to-charge ratios. With the standard matrix-to-protein ratios of 1000:1, 5000:1, and 15 000:1 (mole ratio), all attempts resulted in failure; Figure 3 illustrates this failure with **4A**, and similar results were seen with the other proteins. Figure 4 shows the identical experiment, except that calibrant was added to the partially dried droplet; the presence of strong calibrant peaks but no signal from **4A** suggests that cocrystallization between our bZIP proteins and the matrix does not occur under these conditions. We attributed these failures to protein aggregation upon dilution of urea below 4 M when the matrix and protein were mixed and spotted on the plate or when matrix and protein were spotted sequentially and mixed by pipetting. Even when protein was applied to a dried matrix spot, no signal was observed. Detergents such as Triton X-100, Triton X-114, *n*-octyl-*β*-D-glucopyranoside, *n*-octyl-*β*-D-thioglucopyranoside, dimethyloctyl(3-sulfopropyl)ammonium hydroxide, dimethyltetradecyl(3-sulfopropyl)ammonium hydroxide, CHAPS (3-([3-cholamidopropyl]dimethylammonio)-1-propanesulfonate), CHAPSO (3-([3-cholamidopropyl]dimethylammonio)-2-hydroxy-1-propanesulfonate), and dimethyldodecylamine oxide were employed, similar to work by Rosinke et al.[16] Organic solvent systems described in the Experimental Section were also attempted.[12] Rinsing the dried sample with cold water to remove salts also had no effect on signal enhancement.[17] All of these efforts resulted in failure to generate spectra.

Previously, the T-leap tactic had been successfully utilized on enzymes such as carbonic anhydrase[27] and the P22 tailspike protein.[28,29] These enzyme catalysts, however, are of very different structure and function in comparison with our short, ~100-residue, α-helical DNA-binding bZIP mutants. After failing to obtain any mass spectra, as discussed above, we successfully utilized the T-leap to generate MALDI-MS for all four proteins in a variety of matrixes and solvent systems. Only after suppression of protein aggregation by using the full T-leap procedure, a 4 °C incubation of sample solution followed by a 37 °C incubation, were we able to see a signal. When we performed MALDI on samples that had been manipulated and incubated at 4 °C without the subsequent 37 °C incubation, no spectra were obtained; an example is shown in Figure 3 in which no T-leap was performed on **4A**, and no signal is detected whatsoever. Therefore, the T-leap tactic was indispensable to our gaining MALDI data.

Using the T-leap tactic, we again evaluated the nine matrixes with the standard matrix-to-protein ratios listed above. The best results were observed by using α-cyano-4-hydroxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid (sinapinic acid), and *trans*-4-hydroxy-3-methoxycinnamic acid (ferulic acid); in some instances, 2′,4′,6′-trihydroxyacetophenone, 2,5-dihydroxybenzoic acid, and *trans*-3-indoleacrylic acid yielded signals near the expected masses; however, 3,4-dihydroxycinnamic acid, 5-chlorosalicylic acid, and 6-aza-2-thiothymine failed to give any signals. The spectra of **11A** and **18A** showed peaks that were intense (4500 counts) with high signal-to-noise ratios (S/N) of 265:1. Spectra of wt bZIP were of average quality with intensities of 3500 counts and S/N ratios of 145:1. Spectra of **4A** were of the poorest quality with intensities of 3000 counts and S/N ratios of 47:1. For **4A**, more intense peaks (10 000 counts) with better S/N ratios (253:1) were produced from ferulic acid dissolved in 1:1:1, acetonitrile:2-propanol:water, 0.1% TFA, combined with **4A** at a ratio of 10 000:1; although the signal for **4A** was strong under these conditions, mass accuracy was unacceptable. Lower quality spectra for **4A** were seen from 2′,4′,6′-trihydroxyacetophenone dissolved in 2:5:2, chloroform:methanol:water, 0.1% TFA, and α-cyano-4-hydroxycinnamic acid dissolved in 1:1:1, acetonitrile:2-propanol:water, 0.1% TFA, both at ratios of 10 000 to 1. Therefore, with utilization of the T-leap, spectra of modest quality were obtained at the standard matrix-to-protein ratios of 1000:1, 5000:1, and 15 000:1; in contrast, no spectra were obtained in the absence of the T-leap tactic.

Table 1. Parameters for Highest-Quality Spectra

bZIP	matrix[a]	M:P[b]	solvent system[c]	intensity[d]	S/N[e]
wt	ferulic	50 000:1	MeCN/TFA	5709	179:1
4A	sinapinic	100 000:1	MeCN/TFA	4300	160:1
11A	ferulic	100 000:1	MeCN/TFA	20000	335:1
18A	ferulic	100 000:1	MeCN/TFA	4300	160:1

[a] Ferulic acid or sinapinic acid. [b] Matrix:protein ratio. [c] MeCN/TFA refers to 1:2, MeCN:water, 0.1% TFA. [d] Intensity measured in counts. [e] Signal-to-noise ratio.

By increasing the matrix-to-protein ratio to 50 000:1 and 100 000:1, spectra of the highest quality were generated. Studies by Wilkins and co-workers found that higher matrix:analyte ratios can allow quality MALDI spectra to be generated in the presence of buffers (phosphate, TRIS, etc.), which suppress signals at lower ratios.[30] This effect is largely due to a concurrent increase in the matrix:buffer ratio, allowing the sample and matrix to cocrystallize with less interference from buffer contaminants. Table 1 presents the details from the best spectra for each protein. Figures 5−8 illustrate the highest quality spectra for each protein, achieved at matrix-to-protein ratios of 50 000:1 or 100 000:1. The spectrum for **11A** shown in Figure 7 was the best that was seen for any of our proteins under any conditions. Therefore, these higher matrix-to-protein ratios and incorporation of the T-leap significantly improved the quality of the spectra, for these were the best spectra produced. Figures 5−8 show shoulders on the wt bZIP and cytochrome *c* peaks that are consistent with matrix adduct formation. Table 2 summarizes results from enzyme digestions, reductions, and other experiments (data not shown) that confirmed that postranslational modifications have occurred: the initiating methionine at the amino terminus was cleaved by proteolysis on all four proteins.[31] Carbamylation at the amino terminus from decomposition of urea (necessary for maintaining protein solubility in all solutions) occurred on three proteins,[32,33] and *β*-mercaptoethanol (used during protein purification) adduct formation at cysteine was found for two proteins.

In some instances, proteins and matrix were mixed together with the calibrant, and T-leap was performed (data not shown). The relative peak strengths and mass-to-charge ratios were similar to those acquired when the calibrant was applied to a dried matrix−analyte spot, although fewer picomoles of calibrant were

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Table 2. Mass Spectrometry Results

bZIP	expected mass[a] (amu)	deMet[b] (amu)	carbamylation (amu)	BME adduct[c] (amu)	calculated mass (amu)	observed mass (amu)	% difference
wt	11073.16	−132.08	+43.03	+76.11	11060.22	11062.85 ± 2.61	0.02
4A	11731.85	−132.08	+43.03	NA	11642.80	11637.20 ± 6.57	0.05
11A	11288.27	−132.08	NA	NA	11156.19	11158.61 ± 4.14	0.02
18A	10816.76	−132.08	+43.03	+76.11	10803.82	10806.39 ± 2.40	0.02

[a] For full intact protein with no posttranslational modifications. [b] Demethionylation (loss of initiating methionine at amino terminus). [c] BME is β-mercaptoethanol.

needed relative to analyte in order to get comparable peak intensities. As we have illustrated, the solubility of hydrophobic proteins in reduced concentrations of denaturants is aided by manipulation of temperature, but the solubility of hydrophilic proteins should not be affected by denaturant or temperature change. Breaux et al. utilized sodium dodecyl sulfate (SDS) when analyzing simultaneously short hydrophobic and hydrophilic peptides of <1000 amu in aqueous solutions; there were no interfering surfactant background ions, little or no loss of acid-labile protecting groups, and an abundant pseudomolecular ion of the analyte.[14]

Xie and Wetlaufer studied the kinetics of the refolding of bovine carbonic anhydrase at high micromolar concentrations; in contrast, solubility of our bZIP mutants becomes problematic at such concentrations, even when correctly folded.[27] The authors attributed aggregation of carbonic anhydrase to an early intermediate in the folding process that is prone to aggregation. The aggregation-prone intermediate can be thought of as a molten globule, a partially folded state that rapidly interconverts with the fully unfolded form but only slowly converts to the fully folded state; depending on the protein, the molten globule can be completely unstable or stable under certain conditions.[34] Association of such species by hydrophobic interactions would not be surprising.[27] Therefore, allowing protein to fold slowly and correctly at low temperature for a period of time is critical for maintaining the soluble, active species, whether for MALDI or any other experiment. During the T-leap tactic, when matrix and protein are incubated at 4 °C for 2 h or more, it is possible that the slow crystallization process is also occurring. In slow crystallization, matrix is allowed to crystallize, forming a suspension in solution; protein can then adhere to the face of the growing matrix crystal, thereby being concentrated in the crystal.[35] This process is time-consuming.[36] Working with recombinant mouse leptin (~16 000 amu), Cohen and Chait needed a day for slow crystallization to occur, and the matrix−protein solutions showed visible turbidity.[37] Although no turbidity is observed in our solutions and T-leap with a much shorter 2-h incubation leads to strong spectra, we cannot discount the possibility that slow crystallization occurs in our solutions, and this may aid in generation of superior spectra.

Both in vivo and in vitro, the partitioning between aggregation and productive folding can be very sensitive to temperature,[38] and aggregation-prone intermediates have been shown for several proteins.[39,40] For example, when folding of the β-coil tailspike trimer of phage P22, a bacterial endorhamnosidase, was initiated at low temperature and later warmed, intermediates were stable and resistant to aggregation.[28,29] Interestingly, folding of tailspike is sensitive to environment, and mutations can create temperature-sensitive derivatives in which folding is shifted from the productive to the competing aggregation-prone pathway. In our case, Ala replacements in the basic region generate proteins prone to aggregation. Therefore, the utility of the T-leap in improving solubility of hydrophobic proteins and subsequent cocrystallization with matrix for analysis by MALDI-TOFMS may be widespread.

Although extremely efficient for rapid generation of large amounts of protein, overexpression can lead to protein aggregation as inclusion bodies in the cell. Normally, organisms use foldases and chaperones to assist the correct folding process, but these systems often become overwhelmed during overexpression. Aggregation, presumed to occur between hydrophobic areas of proteins, is a major cause of decreased yields of active protein.[41] Additionally, protein aggregation in vivo appears to be an important process in amyloid diseases, wherein protein deposits may be involved in pathogenicity. Such aggregation-prone proteins, including membrane-bound proteins and ion channels and even misfolded proteins that have aggregated before folding properly, can be analyzed by MALDI-MS only after solubilization.

Other researchers have shown that rather than renaturing protein aggregates and inclusion bodies, expressed soluble proteins can be generated by use of *E. coli* fusion protein expression systems in which soluble protein segments are fused to desired proteins.[42] Inclusion body formation is the result of an unbalanced equilibrium between correct (soluble) and incorrect (insoluble) protein folding. Arresting protein synthesis in vivo can allow for disintegration of aggregates, followed by resolubilization and correct refolding,[43] but not all systems display reversible aggregation and folding. In luciferase folding, a kinetic trap is accessible from two inactive equilibrium intermediates that can lead to irreversible protein association, which can be avoided by molecular chaperones.[44] Our overpression protocols were designed to promote slower protein synthesis in vivo, thereby intending to reduce inclusion body formation.[25] Although more soluble protein was synthesized, inclusion bodies remained. With

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proteins such as ours that are prone to aggregation, the T-leap tactic is absolutely indispensable for maintaining protein solubility during experimental manipulations and subsequent MALDI-MS analyses.

CONCLUSIONS

Understanding diverse biological systems at the molecular level requires sensitive and efficient analytical techniques. MALDI is one such technique that has been developed to handle some of the needs of researchers in the proteomic era. Characterization of hydrophobic, aggregating proteins is difficult. There exist several methods for refolding aggregated and misfolded proteins, including slow addition of denatured protein to a buffer promoting refolding, dilution of denaturant concentrations to levels that are high enough to solubilize protein but low enough to encourage folding, and use of the T-leap tactic.[41] In our case, we found that protein would aggregate rather than fold correctly, regardless of buffer or denaturant, unless temperature was also manipulated. A major advantage of the T-leap is that it is very straightforward and simple to implement with MALDI-MS. Additionally, the T-leap tactic should be useful in diverse protein systems, because it has proven to be useful in solubilizing enzymes as well as our small DNA-binding bZIP mutants. Thus, temperature manipulation may be a general method for solubilizing a wide variety of unwieldy protein systems and even other biomolecules that are prone to low mass spectrometric signal strength due to aggregation.

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