Anal. Chem. **2002,** *74,* 1884−1890

Optimization of Guanidination Procedures for MALDI Mass Mapping

Richard L. Beardsley and James P. Reilly*

Department of Chemistry, Indiana University, Bloomington, Indiana 47405

Improved procedures for guanidination of lysine-containing peptides, a derivatization that results in increased MALDI mass spectral signal intensities are presented. The complete conversion of lysines to homoarginines can be accomplished in as little as 5 min. The method is demonstrated on a model peptide and on tryptic digests of three proteins. To demonstrate the applicability to proteomics samples, it is successfully applied to the digest of 50 fmol of a protein. Approaches for concentrating and purifying low-quantity protein digests following guanidination are evaluated. Experiments with the model peptide GRGDSPK enable investigation of the specificity of the guanidination reaction.

Over the past decade, the desire to rapidly identify and sequence proteins has grown. Mass spectrometry has greatly facilitated these goals by offering fast and sensitive analyses that lead to the accurate characterization of proteins. Whether electrospray or matrix-assisted laser desorption/ionization (MALDI) is employed, the two general types of experiments that are commonly performed involve either sequencing a single protein fragment or enzymatically digesting a protein and mapping the proteolytic fragments.[1−3] The mass spectral data are then compared to theoretical masses derived from genomic databases. Since interpretation of mass spectra is based on this comparison, it is desirable to generate data that contain the maximum amount of information about the protein or protein fragment.

Despite the fact that trypsin is expected to cleave with equal propensity at lysine and arginine, Krause et. al. demonstrated that signals from arginine-containing peptides are generally stronger.[5] The ionization efficiency of arginine was postulated to be greater than that of lysine as a result of its more basic side chain. Based on this, a recent flurry of research has been directed toward increasing mass spectral signal intensities from lysine-containing peptides.[6−8] Researchers have modified lysine residues with *O*-methylisourea to form more basic homoarginine residues. This guanidination reaction is largely specific for the ϵ-amine of lysines, occurring only minimally at the α-amine of the peptide's N terminus.[9] This derivatization leads to a striking increase in the intensities of lysine-containing peptide masses, observation of increased numbers of lysine-containing tryptic peptides, and improved sequence coverages.[6−8] In related work, Keough et. al. combined guanidination of lysine residues with the addition of a sulfonic acid group to the N terminus to facilitate de novo sequencing of low-abundance, lysine-containing tryptic peptides by post-source-decay (PSD).[10] The sulfonic acid derivatization alone results in modification of both the N termini and the ϵ-amines of lysine-containing peptides. Peptides with multiple sulfonic acid additions are not useful for de novo sequencing because of poor sensitivity and fragmentation in the positive-ion mode. Therefore, de novo sequencing following this modification was previously limited to arginine-containing peptides.[11] Following guanidination of lysine ϵ-amines, introduction of sulfonic acid groups to tryptic peptides was possible solely at the N termini, thereby allowing de novo sequencing of lysine-containing tryptic peptides. In recent work by Brancia et. al., guanidination of lysines coupled with modification of N termini by phenylthiocarbamoyl (PTC) provided the basis for a PSD ion fragmentation method to increase the confidence limit of protein identifications via genomic database searches.[12] The PTC modification facilitated identification of the N-terminal residue by enhancing fragmentation of the first peptide bond, and guanidination provided some sequence-specific information (by differentiating between C-terminal lysine- and arginine-containing tryptic peptides) and increased peptide ion yields. Guanidination distinguishes between arginine and lysine, because the reaction results in a 42 Da mass shift for every modified lysine. Utilizing the sequence information derived from these two procedures, the number of false protein assignments was shown to be greatly reduced.

Despite the benefits that guanidination conveys, there have been some drawbacks to using the technique. One has been the relatively long reaction times employed (typically several hours to overnight).[6−10,12] Additionally, despite these long reaction times,

(1) Perkins, D. N.; Pappin, D. J. C.; Creasy, D. M.; Cottrell, J. S. *Electrophoresis* **1999**, *20*, 3551−3567.
(2) Quadroni, M.; James, P. *Electrophoresis* **1999**, *20*, 664−677.
(3) Burlingame, A. L.; Boyd, R. K.; Gaskell, S. J. *Anal. Chem.* **1998**, *70*, 647R−716R.
(4) Fersht, A. *Structure and Mechanism In Protein Science: A Guide to Enzyme Catalysis and Protein Folding*; W. H. Freeman: New York, 1999, pp 26−30.
(5) Krause, E.; Wenschuh, H.; Jungblut, P. R. *Anal. Chem.* **1999**, *71*, 4160−4165.
(6) Brancia, F. L.; Oliver, S. G.; Gaskell, S. J. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2070−2073.
(7) Beardsley, R. L.; Karty, J. A.; Reilly, J. P. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2147−2153.
(8) Hale, J. E.; Butler, J. P.; Knierman, M. D.; Becker, G. W. *Anal. Biochem.* **2000**, *287*, 110−117.
(9) Kimmel, J. R. *Methods Enzymol.* **1967**, *11*, 584−589.
(10) Keough, T.; Lacey, M. P.; Youngquist, R. S. *Rapid Commun. Mass Spectrom.* **2000**, *14*, 2348−2356.
(11) Keough, T.; Lacey, M. P.; Fieno, A. M.; Grant, R. A.; Sun, Y.; Bauer, M. D.; Begley, K. B. *Electrophoresis* **2000**, *21*, 2252−2265.
(12) Brancia, F. L.; Butt, A.; Beynon, R. J.; Hubbard, S. J.; Gaskell, S. J.; Oliver, S. G. *Electrophoresis* **2001**, *22*, 552−559.

10.1021/ac015613o CCC: $22.00
Published on Web 03/13/2002

incomplete conversion of lysines to homoarginines has been reported (80−85% yield).[7,10] This results in increased spectral complexity, because signals are observed from both the derivatized and underivatized forms of lysine-containing peptides. In addition, the maximum signal enhancement is not attained if the overall signal for a given peptide is distributed over more than one mass. Another drawback is that the reagents used for guanidination result in significant contamination of the protein sample, thus necessitating a purification step prior to MALDI analysis. Although the benefits of sample cleanup and enrichment with a reversed-phase microextraction column have been demonstrated,[13] analyte losses are often unavoidable when this is employed.[14] In particular, we have observed that high-mass peptide signals are not increased and are in some cases absent following guanidination.[7] Because of the inefficiency of sample handling, guanidination's utility with low-femtomole quantities of protein is therefore unproven.

The motivation for this study was to improve the guanidination protocol to a more routine level, thereby expanding the potential of this derivatization by rendering it easy to implement in the analysis of tryptic peptides. By investigating factors that control the success of this reaction, we were able to develop a procedure that offers several advantages over those previously used. The application of this new protocol to low-quantity protein samples is evaluated using a tryptic digest of cytochrome *c*. We previously observed guanidination of a peptide that did not contain lysine and suggested that the reaction had occurred at its N terminus.[7] To follow up on this observation, we have studied the specificity of guanidination using the model peptide GRGDSPK.

MATERIALS AND METHODS

Materials. TPCK-treated trypsin (bovine pancreas), myoglobin (horse), model peptide GRGDSPK, cytochrome *c* (horse), hemoglobin (human), octadecyl-derivatized silica gel (Zorbax LP 100/40 C_{18}), and α-cyano-4-hydroxycinnamic acid (αCHCA) were supplied by Sigma (St. Louis, MO). Sequencing grade modified trypsin (bovine) was provided by Promega Co. (Madison, WI). 2,5 Dihydroxybenzoic acid (DHB) was obtained from Aldrich (Milwaukee, WI). *O*-Methylisourea hemisulfate was purchased from Acros Organics (Janssens, Belgium). Ammonium bicarbonate was supplied by Fisher (Fair Lawn, NJ). Trifluoroacetic acid (TFA) and acetonitrile were purchased from EM Science (Gibbstown, NJ). Ammonium hydroxide was obtained from J. T. Baker (Phillipsburg, NJ). The water used in these experiments was obtained from an E-pure water purification system (Barnstead Thermolyne Co., Dubuque, IA).

Tryptic Digestion of Proteins. Myoglobin was digested by the method of Russell et al.[15] A 22-μg portion of myoglobin was incubated with 5 μg of trypsin for 5 min at 37 °C. The digestion mixture was composed of 80% acetonitrile (v/v), 25 mM NH_4HCO_3, 13 pmol/μL myoglobin, and 2 pmol/μL TPCK-treated trypsin in a total volume of 100 μL. The reaction was terminated by addition of 10 μL of 10% TFA (v/v). The acidified digestion mixture was completely dried in a speed-vac (Jouan, Winchester, VA), and the contents were reconstituted in 100 μL of water.

Peptides were generated from cytochrome *c* by digestion with sequencing grade modified trypsin in 10 mM ammonium bicarbonate at 37 °C. The reaction was in a total volume of 100 μL and was quenched by 5 μL of 10% TFA (v/v) after 13 h. The ratio of trypsin to cytochrome *c* was 1:11.5 (mol/mol), and the acidified mixture was 1 pmol/μL of cytochrome *c*. For this protein, a long digestion time and sequencing grade modified trypsin were employed to increase tryptic peptide recovery. The modified trypsin increases the recovery of tryptic peptides by eliminating the chymotryptic activity that can result from using unmodified trypsin.[16,17] Since the purpose of guanidination experiments with cytochrome *c* was to evaluate the application to low-level protein samples, it was important to attain a thorough digestion so that the tryptic peptide quantity would be more accurately estimated.

Guanidination and Purification/Concentration of Tryptic and Model Peptides. An *O*-methylisourea stock solution was prepared by dissolving 0.050 g in 51 μL of water. The guanidination reaction mixture was prepared by mixing a 5-μL aliquot of digested protein with 5.5 μL of 7 N NH_4OH and 1.5 μL of *O*-methylisourea stock solution. Freshly prepared *O*-methylisourea stock solution was used in every reaction. Use of older stock solution resulted in incomplete guanidination. A pH of 10.6 was measured with a glass micro-pH electrode (Orion Research Inc., Beverly, MA) for this mixture. After incubation for 5−10 min at 65 °C in an oven, the reaction was terminated by adding 15 μL of 10% TFA (v/v). The acidified reaction mixture was partially dried in a speed-vac to a final volume of ∼10 μL. Microextraction of guanidinated peptides was accomplished using a micropipet tip packed with octadecyl-derivatized silica gel. After equilibrating the extraction media with 50% acetonitrile (v/v) in water followed by 0.1% TFA (v/v), binding of the peptides was accomplished directly from the partially dried reaction mixture. The bound peptides were washed by flowing two 15-μL aliquots of 0.1% TFA (v/v) through the microextraction column and discarding each aliquot to waste. Next, elution of the peptides was accomplished using a solution of 50% acetonitrile (v/v) in 0.1% TFA (v/v). For the study involving low-quantity digests of cytochrome *c*, only 1 μL of elution mixture was employed, since small elution volumes can be helpful for enriching low-abundance peptides. With higher levels of protein or peptide, (myoglobin, hemoglobin, and GRGDSPK), 5 μL of this mixture was used instead.

Matrix-Assisted Laser Desorption/Ionization Time-of-Flight Mass Spectrometry. All spectra were acquired in positive ion mode using a Reflex III, reflectron time-of-flight mass spectrometer (Bruker, Bremen, Germany). MALDI spot preparations for low- and high-quantity proteins differed. The dried-droplet method was used for hemoglobin, myoglobin, and model peptide GRGDSPK. A 0.7-μL aliquot from the elution mixture containing ∼10 pmol of sample and 10 g/L αCHCA was applied to a stainless steel probe and allowed to air-dry. For high-sensitivity experiments with cytochrome *c*, an AnchorChip[18] (Bruker, Bremen, Germany)

(13) Bagshaw, R. D.; Callahan, J. W.; Mahuran, D. J. *Anal. Biochem.* **2000**, *284*, 432−435.

(14) Erdjument-Bromage, H.; Lui, M.; Lacomis, L.; Grewal, A.; Annan, R. S.; McNulty, D. E.; Carr, S. A.; Tempst, P. *J. Chromatogr. A* **1998**, *826*, 167−181.

(15) Russell, W. K.; Park, Z.-Y.; Russell, D. H. *Anal. Chem.* **2001**, *73*, 2682−2685.

(16) Keil-Dlouha, V.; Zylber, N.; Imhoff, J. M.; Tong, N. T.; Keil, B. *FEBS Lett.* **1971**, *16*, 291−295.

(17) Rice, R. H.; Means, G. E.; Brown, W. D. *Biochim. Biophys. Acta* **1977**, *492*, 316−321.

(18) Schuerenberg, M.; Luebbert, C.; Eickhoff, H.; Kalkum, M.; Lehrach, H.; Nordhoff, E. *Anal. Chem.* **2000**, *72*, 3436−3442.

with 400-μm diameter targets was used. MALDI spots were prepared by first applying 0.5 μL of 5 g/L DHB in water to an anchor target, allowing this to completely dry, and then adding the entire 1 μL of elution mixture from the microextraction step.

RESULTS AND DISCUSSION

General Considerations for Improved Guanidination Conditions. As first priority, improved reaction conditions that resulted in the complete conversion of lysines to homoarginines in a short time were sought. The most critical factors affecting the efficiency of this derivatization are pH, *O*-methylisourea concentration, and temperature. Since deprotonation of lysines is required for this reaction, a pH minimum of 10 has been used in past research.[6−10,12] Typically, the base chosen for reaching this high pH has been either NaOH[6,9,10,12] or Na_2CO_3;[8] however, we have found that there are some advantages to using NH_4OH to meet this requirement. These include its easy removal by evaporation and lowered contamination of MALDI spots with Na^+. In addition, Keough et. al. reported that peptide hydrolysis dominates when using enough NaOH in the reaction to obtain a pH of 13.6.[10] We have not observed any deleterious side effects, such as peptide hydrolysis, when using NH_4OH in our reactions.

Another factor that can affect the success of a guanidination reaction is the *O*-methylisourea salt that is employed. Most investigators have chosen *O*-methylisourea hydrogensulfate for this purpose,[6,7,10,12] whereas some have used *O*-methylisourea hemisulfate.[8] Unlike the hydrogensulfate salt, the crystals of which are composed of one HSO_4^- per *O*-methylisourea, the hemisulfate salt is composed of one SO_4^{2-} for every two *O*-methylisourea molecules. In this salt, each hydrogen from H_2SO_4 is associated with an *O*-methylisourea, whereas the hydrogensulfate crystal possesses a proton that completely dissociates in water. We measured the pHs of saturated solutions of *O*-methylisourea hemi- and hydrogensulfate to be 2.60 and 0.74, respectively. Since the hemisulfate salt is not as acidic as the hydrogensulfate, it can be used at a higher concentration without sacrificing the basic pH requirement or necessitating a titration that would further contaminate the sample. As expected, we found that increasing the *O*-methylisourea concentration is an effective way to decrease the time required for a complete reaction.

Yet another variable that is critical for attaining a fast and complete guanidination is the temperature that is used. In early studies involving the guanidination reaction, temperatures of 5 °C or lower were typically used with reaction times ranging from overnight to several days.[19,20] Since the emphasis of those studies was to probe the characteristics of intact proteins, denaturation was avoided by using low temperatures. In recent guanidination studies, temperatures as high as 37 °C were used as a means of shortening the reaction time to several hours.[7,8] In this work, we have utilized a temperature of 65 °C and found that a complete reaction could be attained much faster than was possible using the lower temperatures. Detrimental side effects, such as the rapid hydrolysis of *O*-methylisourea or the tryptic peptides, were not observed at this temperature. Either these hydrolysis reactions do not occur, or are they are much slower than guanidination at this temperature.

Guanidination of Model Proteins. We have used myoglobin as a model protein to demonstrate the application of our new guanidination protocol. Myoglobin was chosen for study because it contains 19 lysines and only 2 arginines, suggesting that MALDI analysis of its tryptic peptides would benefit from conversion of its lysine residues to homoarginines. As previously reported by Russell et al.,[15] efficient generation of tryptic peptides from myoglobin requires that the protein be denatured because of its tightly packed, globular structure. For this reason, we followed their method of digestion in which the proteolysis was done in a strongly organic environment. This method was also attractive to us, because it could be accomplished in as little as 5 min.

The results from a tryptic digest of myoglobin are shown in Table 1 and Figure 1. The spectrum in Figure 1A is of the digest without guanidination, and Figure 1B shows the guanidinated peptide spectrum on the same intensity scale. Consistent with previous reports,[6−8] the most striking result evident in Figure 1 is a dramatic increase in the signals of most lysine-containing peptides. There were several lysine-containing peptides whose peak intensities were improved by guanidination (**4**, **5**, **6**, **8**, and **15**). Most noteworthy of these are the peptides **4**, **6**, and **15**, which dominate the mass spectrum acquired from the guanidinated sample. Additionally, many of the peptides (**1**, **3**, **10**−**13**, **16**, **17**, and **20**) could be observed only following guanidination; 13 were identified without guanidination, and 22 peptides could be identified with guanidination. Some high-mass peptide signals (**18**, **19**, **21**, and **22**) increased very little or not at all by guanidinating their lysine residues. This trend is consistent with our previous work[7] and may be related to the poor solubilities of high-mass peptides.[22,23] Partial guanidination of the N termini of three peptides was observed. Peaks 13a, 14a, and 21a were shifted 42 Da from the lysine-guanidinated masses of peptides **13**, **14**, and **21**. These peptides were unique in that they all possessed an N-terminal glycine. For the other peptides observed, none of which have an N-terminal glycine, there is no evidence of guanidination at their N termini. In the data shown, an already impressive sequence coverage of 90% before guanidination improved to 100% afterward. In several trials with this digest, the sequence coverage observed from guanidinated samples always ranged from 95 to 100%, whereas the underivatized digest yielded 80−90% sequence coverage. The conversion of lysines to homoarginines is essentially complete, since no signals from unguanidinated, lysine-containing peptides were observed. This consideration is especially critical when analyzing much lower quantities of protein digests, for which the signals of some peptides are likely to be very weak. An incomplete reaction and the consequent dilution of signal intensity could result in the absence of some mass spectral peaks. To attain a complete conversion of lysines to homoarginines in the myoglobin digest, a reaction time of 10 min was employed. In several trials using a reaction time of 5 min ,we found that guanidination of high-mass peptides (>2.5 kDa) was incomplete. This effect was also observed in our work with tryptic digests of hemoglobin using the improved guanidination procedure. We have found that peptides smaller than 2.5 kDa, are completely derivatized in 5 min. With this in mind, we

(19) Hughes, W. L., Jr.; Saroff, H. A.; Carney, A. L. *J. Am. Chem. Soc.* **1949**, *71*, 2476−2480.
(20) Chervenka, C. H.; Wilcox, P. E. *J. Biol. Chem.* **1956**, *222*, 635−647.
(21) Prowl is found at http://prowl.rockefeller.edu.
(22) Cohen, S. L.; Chait, B. T. *Anal. Chem.* **1996**, *68*, 31−37.
(23) Gobom, J.; Schuerenberg, M.; Mueller, M.; Theiss, D.; Lehrach, H.; Nordhoff, E. *Anal. Chem.* **2001**, *73*, 434−438.

Table 1. Experimental Data for Myoglobin Tryptic Digest[a]

ID	theoretical unmodified mass	experimental mass (Figure 1A)	theoretical guanidinated mass	experimental mass (Figure 1B)	sequence range	no. arginines	no. lysines
1	684.36		768.41	768.38	(43−47)	0	2
2	748.43	748.19	748.43	748.38	(134−139)	1	0
3	790.42		874.47	874.47	(57−63)	0	2
4	941.47	941.40	983.49	983.51	(146−153)	0	1
5	1271.66	1271.62	1313.68	1313.77	(32−42)	0	1
6	1360.75	1360.83	1402.77	1402.90	(134−145)	1	1
7	1378.83	1378.90	1420.86	1420.96	(64−77)	0	1
8	1506.93	1506.93	1590.97	1591.03	(64−78)	0	2
9	1606.85	1606.91	1606.85	1606.99	(17−31)	1	0
10	1635.02		1761.09	1761.12	(63−78)	0	3
11	1651.91		1735.95	1736.05	(134−147)	1	2
12	1661.85		1745.89	1745.95	(32−45)	0	2
13	1815.89		1857.92	1857.99	(1−16)	0	1
14	1853.95	1854.04	1938.00	1938.07	(80−96)	0	2
15	1937.01	1937.09	2063.08	2063.13	(32−47)	0	3
16	1982.05		2108.11	2108.15	(79−96)	0	3
17	2150.25		2276.31	2276.32	(57−77)	0	3
18	2283.21	2283.27	2367.25	2367.25	(134−153)	1	2
19	2601.48	2601.47	2727.55	2727.52	(97−118)	0	3
20	3217.79		3427.90	3427.63	(48−77)	0	5
21	3403.73	3403.28	3445.75	3445.32	(1−31)	1	1
22	4084.15	4084.50	4252.24	4252.59	(97−133)	0	4

[a] Data shown are for Figure 1A,B. Experimental masses were internally calibrated. Theoretical masses were determined from Prowl.[21] All masses listed are protonated.

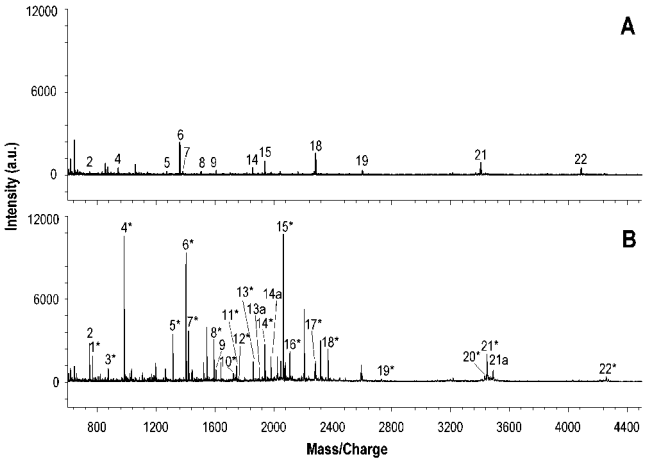

Figure 1. Mass spectra of myoglobin tryptic digest: (A) without guanidination, (B) with guanidination. All peaks are labeled per ID numbers from Table 1, with guanidinated peaks followed by an asterisk (*). Each MALDI spot contained no more than 9 pmol of myoglobin.

have been careful to use 10 min reaction times when high-mass tryptic peptides may be observed in the mass spectrum.

Contrary to the general tendency for arginine-containing peptides to dominate MALDI mass spectra, peaks 2 and 9 were relatively weak in both Figure 1 spectra. This observation, coupled with the lack of signal enhancement for certain guanidinated peptides, serves as a reminder that the dominance of arginine- or homoarginine-containing peptides is only a generalization and that the MALDI signal intensity of some peptides may depend heavily on factors other than the gas-phase basicity of their amino acids.[24−29]

The reproducibility of the signal enhancement is another issue that deserved attention. To investigate this, we studied 10 unguanidinated and 10 guanidinated samples derived from a single hemoglobin tryptic digest. To minimize any discrepancies, both samples were microextracted. Mass spectra are available in Supporting Information, and Figure 2 summarizes the results. The average relative intensities of unguanidinated and guanidinated peptides are portrayed as bars. Labels above the bars denote the number of lysines (K) or arginines (R) in the peptides and standard deviations are displayed as error bars. The masses of peptides before and after guanidination are displayed on the *x* axis. Relative intensities rather than absolute intensities were used for comparison, since the mass-integrated ion signals varied by up to a factor of 3 from spectrum to spectrum. (This is a familiar idiosyncrasy of MALDI that apparently results from localization of analyte into different regions of the crystalline matrix.) It is evident from the data that guanidinated tryptic peptides consistently yield greater signals than their unguanidinated counterparts, although the extent of signal enhancement varies for different peptides. In the most encouraging cases (818/860 and 933/975 Da pairs), only the guanidinated peptide was detectable. Most peptide signals at least doubled. Consistent with previous observations, the signals of the three heaviest peptides did not increase upon guanidination (original masses 2228, 2529, and 2996 Da).[7] Although the relative intensities of many arginine-containing

(24) Kussman, M.; Nordhoff, E.; Rahbek-Nielsen, H.; Haebel, S.; Rossel-Larsen, M.; Jakobsen, L.; Gobom, J.; Mirgorodskaya, E.; Kroll-Kristensen, A.; Palm, L.; Roepstorff, P. *J. Mass Spectrom.* **1997**, *32*, 593−601.

(25) Figueroa, I. D.; Torres, O.; Russell, D. H. *Anal. Chem.* **1998**, *70*, 4527−4533.

(26) Zhu, Y. F.; Lee, K. L.; Tang, K.; Allman, S. L.; Taranenko, N. I.; Chen, C. H. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 1315−1320.

(27) Olumee, Z.; Sadeghi, M.; Tang, X.; Vertes, A. *Rapid Commun. Mass Spectrom.* **1995**, *9*, 744−752.

(28) Wenschuh, H.; Halada, P.; Lamer, S.; Jungblut, P.; Krause, E. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 115−119.

(29) Kratzer, R.; Eckerskorn, C.; Karas, M.; Lottspeich, F. *Electrophoresis* **1998**, *19*, 1910−1919.

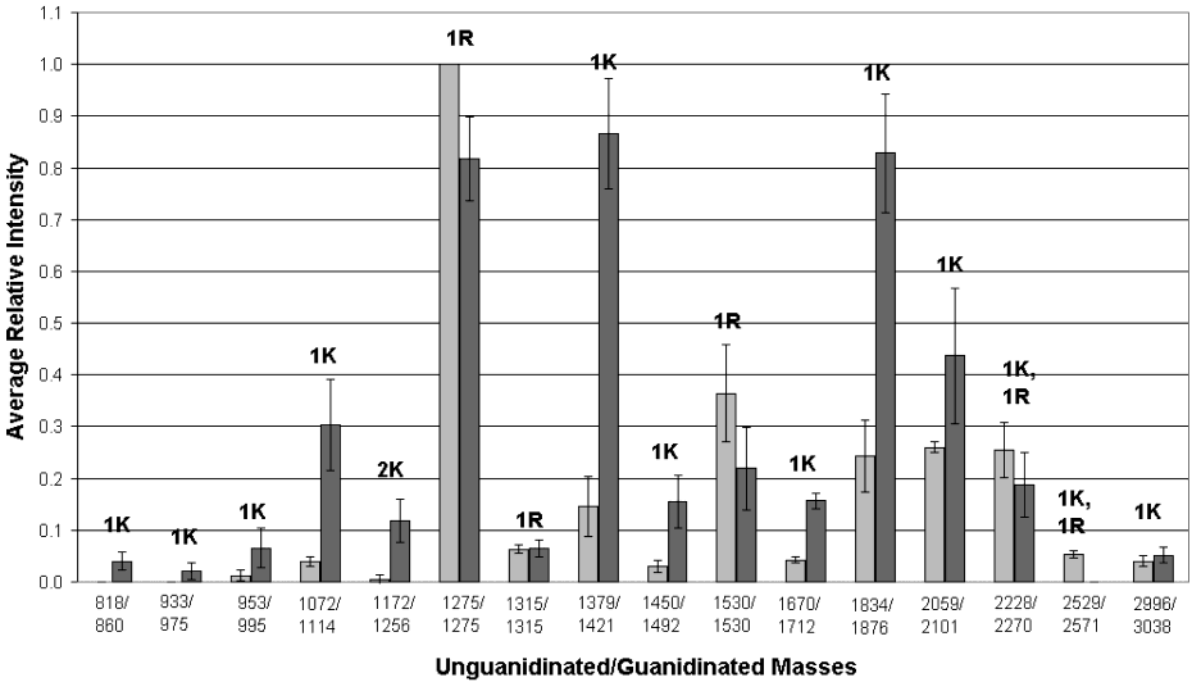

Figure 2. Comparison of relative intensities of hemoglobin tryptic peptides before and after guanidination. The average relative intensities of unguanidinated and guanidinated peptides are displayed as light and dark bars, respectively. The averages were derived from 10 spectra of each type of sample, and the error bars represent standard deviations. The number of lysines (K) and arginines (R) in the peptides are indicated. The *x* axis denotes the peptide's mass before and after guanidination.

peptides were lower in spectra of guanidinated samples, this may be somewhat misleading. The decrease primarily reflects the improvement in lysine-containing peptide signals rather than an absolute decrease of sensitivity for arginine-containing peptides.

Guanidination of Low-Abundance Proteins. In work published thus far, the ultimate sensitivity of guanidinated analyses has not been explored. Since it is common in proteome studies to encounter femtomole quantities of protein, we wished to evaluate guanidination of a tryptic digest on this scale. The focus of these experiments was to determine whether analyte losses incurred by the additional sample handling required following the guanidination reaction (e.g., peptide purification with reversed-phase microextraction columns) would cripple the analysis of low levels of protein. Cytochrome *c* was chosen as the model protein to perform this study with, because like myoglobin, the majority of tryptic peptides generated from it contain lysine rather than arginine. In previous guanidination studies on cytochrome *c*, the sensitivity limits of the technique were not considered.[8,10]

For all experiments with cytochrome *c*, aliquots of the tryptic digest were taken from a 1 pmol/μL stock solution and diluted to the desired concentration. Complete drying of the sample was avoided in all steps of the procedure, since in our experience, this can result in significant loss of some tryptic peptides. In addition, AnchorChip technology was employed in these experiments to improve detection sensitivity.[18]

Typical experimental data from 50 fmol of a cytochrome *c* tryptic digest are presented in Table 2, and Figure 3. All spectra in Figure 3 were taken using an AnchorChip with 400-μm diameter targets. The spectrum in Figure 3A is from a 50-fmol aliquot of unguanidinated cytochrome *c* digest that was applied to the MALDI spot without microextraction. The Figure 3B spectrum is also from 50 fmol of unguanidinated digest, but microextraction was employed. Figure 3C shows a spectrum of guanidinated cytochrome *c* that was also handled by microextraction. Comparison of parts A and B of Figure 3 reveals the extent of analyte loss caused by use of a microextraction column. The intensities of nearly all peptides were diminished when a microextraction column was used. Although this result was disappointing, a comparison of spectra acquired from microextracted samples (Figure 3B,C) reveals the benefit of guanidinating low-quantity protein digests. The signal intensities of all guanidinated peptides but one in spectrum 3C were increased relative to that of lysine-containing peptides in 3B. An improvement in sequence coverage from 46 to 63% was attained by guanidination, and the number of observed peptides increased from 7 to 14. Without guanidination, the lysine-containing peptides were barely detectable, but the only arginine-containing peptide (**10**) in the spectrum was easily observed. With guanidination of lysines, the arginine-containing peptide was still dominant; however, many lysine-containing peptides yielded much stronger signals than without guanidination. An important observation from these spectra is that the signal from peptide **10** was essentially unchanged for the two trials. We believe that this reflects the similarity of the MALDI spots, thereby verifying that the observed signal enhancements are not an artifact of differences in MALDI spot quality. In typical experiments with low-quantity proteins (i.e., proteins from 2-D gel electrophoresis experiments) preconcentration and purification are likely to be required prior to MALDI analysis. Therefore, the comparison of parts B and C of Figure 3 is more relevant to proteomics, since microextraction will often be employed even without contamination from guanidination reagents. In further support of the utility of guanidinating low-abundance protein samples, we are applying the improved guanidination procedure on a routine basis to study bacterial proteins obtained from 2-D gels.[30]

Table 2. Experimental Data for 50 fmol of Cytochrome *c* Digest[a]

ID	theoretical unmodified mass	experimental mass (Figure 3A)	experimental mass (Figure 3B)	theoretical guanidinated mass	experimental mass (Figure 3C)	sequence range	no. arginines	no. lysines
1	604.34	604.35		646.36	646.28	(56−60)	0	1
2	634.39	634.39		676.41	676.37	(9−13)	0	1
3	678.38	678.39		720.39		(74−79)	0	1
4	762.48	762.51		846.52		(8−13)	0	2
5	779.44	779.47	779.45	821.46	821.46	(80−86)	0	1
6	795.43	795.47	795.44	837.45	837.44	(80−86)*	0	1
7	806.47	806.52		890.51		(73−79)	0	2
8	907.54	907.59		991.58	991.57	(80−87)	0	2
9	964.53	964.60		1006.55	1006.50	(92−99)	0	1
10	1168.61	1168.63	1168.64	1168.61	1168.62	(28−38)	1	0
11	1350.72	1350.74		1392.74	1392.73	(89−99)	1	1
12	1433.77		1433.77	1475.79		(26−38)	1	1
13	1470.68	1470.68	1470.67	1512.70	1512.70	(40−53)	0	1
14	1478.81	1478.82		1562.85	1562.80	(89−100)	1	2
15	1495.69			1537.71	1537.69	(61−72)	0	1
16	1598.77	1598.73	1598.77	1682.81	1682.78	(39−53)	0	2
17	1606.91	1606.80		1732.97		(87−99)	1	3
18	1623.79	1623.75		1707.83	1707.82	(61−73)	0	2
19	1633.81	1633.54		1717.85		(9−22)	0	2
20	1639.78	1639.70	1639.64	1723.82	1723.81	(61−73)*	0	2

[a] Data shown are for Figure 3A−C. An asterisk * indicates oxidized methionine. Each spectrum was internally calibrated. Theoretical masses were determined from Prowl.[21] All masses listed are protonated.

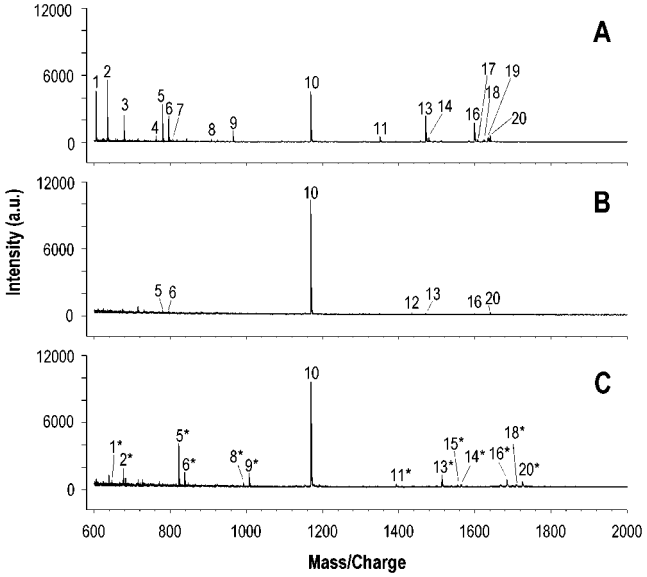

Figure 3. Mass spectra of diluted cytochrome *c* digest (A) without guanidination or microextraction, (B) without guanidination but with microextraction, and (C) with guanidination and microextraction. All peaks are labeled per ID numbers from Table 2, with guanidinated peaks followed by an asterisk (*). Each MALDI spot contains no more than 50 fmol of cytochrome *c*.

The choice of matrix was an especially important factor that we considered for these high-sensitivity experiments. We chose not to use αCHCA, because the quality of MALDI spot preparations on the AnchorChip targets was far more consistent using DHB. An additional point about DHB is that the signal enhancement from guanidinated peptides may not be as great as it is using αCHCA. Krause et al. reported that the extent to which arginine-containing peptides dominate in MALDI mass spectra is at least a factor of 2 lower if DHB, rather than αCHCA, is the matrix.[5] Since this factor did not impair our ability to evaluate the sample-handling efficiency of low quantity proteins, we found DHB to be an adequate matrix.

In addition to evaluating sample losses incurred by use of microextraction columns, we also analyzed a guanidinated sample of hemoglobin digest without microextraction. Rather than concentrating the guanidinated digest by microextraction, the sample was completely dried and reconstituted in a small volume of 10 g/L αCHCA matrix solution. The MALDI spots were prepared by applying the reconstituted peptides to our sample probe without purification. To our knowledge, MALDI has not been attempted in the presence of *O*-methylisourea, and the effect of this reagent on MALDI could not be predicted. If the MALDI phenomenon were not impaired, then this approach would be advantageous because of the reduction in sample handling steps. In addition, sample losses may be less significant than with microextraction. Much to our surprise, it is possible to acquire mass spectra from MALDI spots prepared in the presence of excess *O*-methylisourea (data not shown). Unfortunately, though, we found this approach to be impractical for routine use: MALDI spots dry very slowly (several hours), and signals are observed only with a great deal of searching on the spot.

Reaction at the N Terminus. The extent of guanidinating N-terminal amines under the fast and rather aggressive reaction conditions described above was next considered. In previous work, this phenomenon has been observed only when the N-terminal residue was a glycine.[7,31] In this work, three N-terminal glycines of myoglobin were guanidinated, as noted above. Mass spectra of the arbitrarily chosen model peptide GRGDSPK before (A) and after (B) guanidination (5-minute reaction time) are displayed in

(30) Karty, J. A.; Ireland, M. M. E.; Brun, Y. V.; Reilly, J. P. Submitted to *J. Proteome Res.*

(31) Cotter, R. J.; Ramirez, S. M.; Soloski, M. J. Proceedings of the 49th ASMS Conference on Mass Spectrometry and Allied Topics, Chicago, IL May 27−31, 2001.

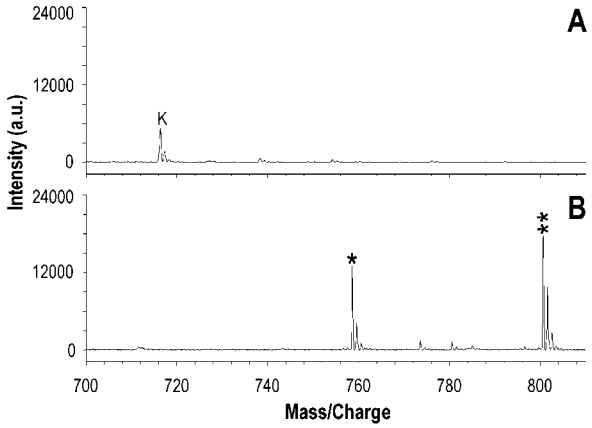

Figure 4. Mass spectra of GRGDSPK (A) without guanidination and (B) with guanidination. K indicates the underivatized peptide, and the asterisk (*) and double asterisk (**) denote the singly and doubly guanidinated peptides, respectively.

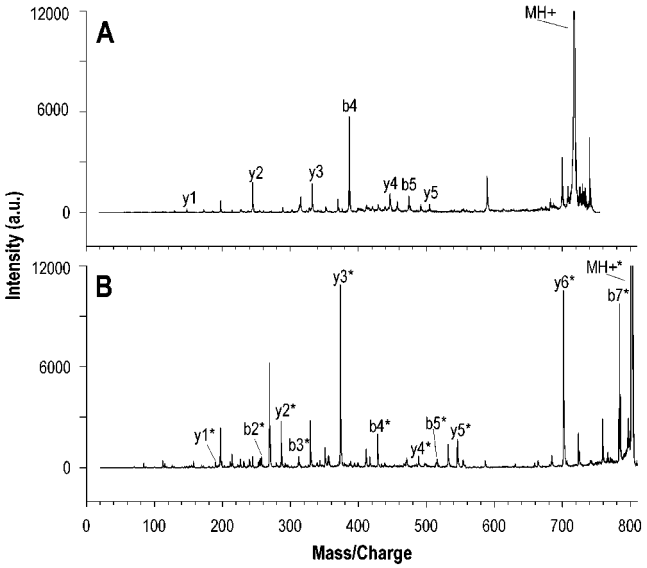

Figure 5. PSD mass spectra of GRGDSPK: (A) unguanidinated and (B) guanidinated. Only b and y ions are labeled. Labels that are followed by an asterisk (*) indicate a guanidinated fragment.

Figure 4. The two intense peaks in the guanidinated spectrum are shifted 42.0 and 84.0 Da from the unguanidinated mass of 716.4 Da. Since this peptide contains only one lysine, we hypothesized that the 84.0 Da mass shift must have resulted from guanidination at both the N-terminal amine and the ϵ-amine of the lysine. To confirm this we performed a PSD ion fragmentation experiment. PSD spectra of GRGDSPK before (A) and after (B) guanidination are shown in Figure 5. In the 5B spectrum, every predicted y ion from this peptide is observed, and all are shifted 42.0 Da from their unguanidinated masses. Additionally, the b ions that are observed also possess a 42.0 Da mass shift. Since every y ion is shifted by only 42.0 Da, the second guanidination must have occurred at the N terminus. In our data for Figures 1 and 3, the only evidence of N-terminal guanidination involved the amino acid glycine. The reaction times were 10 and 5 min with myoglobin and cytochrome *c*, respectively. However, when the reaction was extended to several hours, we observed partial guanidination at the N terminus of M, S, V, L, F, E, and A residues. This indicates that reaction with the N terminus is not limited to glycine, but it must be slower when the N-terminal residue is another amino acid. The increased reaction rate observed with glycine may be due to reduced steric hindrance. More significant to proteomics, the occurrence of N-terminal guanidination could adversely affect mass fingerprinting applications. The intensities of singly and doubly guanidinated peptides in Figure 4 are roughly equal, indicating that partial guanidination of the N terminus has resulted in some loss of sensitivity for detecting either. For low-abundance peptides that are observed only weakly, this loss of sensitivity could result in absent peaks. Conversely, the modification of N-terminal glycine residues could be a useful tool for deriving sequence specific information that may facilitate the identification of some proteins. In experiments in which such a partial modification is observed, it would be possible to remove many of the false-positive hits attained from genomic database searches by eliminating from consideration those peptides that do not contain an N-terminal glycine. As further support to our observations with GRGDSPK and myoglobin, we have obtained similar results using the improved guanidination procedure in proteomics applications.[30] In studies of bacterial proteins, we commonly observe guanidination of N-terminal glycines. N-terminal, glycine-specific guanidination is being used in these studies to facilitate protein identifications via genomic databases.

CONCLUSION

In this paper, we have presented dramatic improvements to the guanidination technique. The new procedure is rapid and simple to apply, and the results that follow are reproducible. Additionally, we have demonstrated that this procedure is applicable using two of the most common MALDI matrixes for peptides (2,5-DHB and αCHCA). With a reaction time as short as 5 min, the complete conversion of lysines to homoarginines is possible. However, guanidination of high-mass peptides is slower and may necessitate as long as 10 min for complete conversion. Through study of a model peptide and several tryptic peptides, we have presented additional evidence of partial guanidination at N-terminal glycines. In addition, we have demonstrated that this procedure can be easily applied to improve the MALDI mass spectral quality of even low- to midfemtomole quantities of protein. Through these studies, we have further characterized some of the benefits and complications that can be encountered with guanidination.

ACKNOWLEDGMENT

This research has been funded by NIH grant 1R01 GM6133601 and NSF grant CHE-0094579.

SUPPORTING INFORMATION AVAILABLE

Mass spectra of 10 unguanidinated and 10 guanidinated samples derived from a single hemoglobin tryptic digest. This material is available free of charge via the Internet at http://pubs.acs.org.

Received for review August 30, 2001. Accepted January 30, 2002.

AC015613O