Utility of *N*-Peracetylation of Proteins for Their Structure Determination by Mass Spectrometry

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Acetylation of the amino groups (*N*-terminus and lysine) of proteins before enzymatic or chemical cleavage was explored as an approach to provide additional information in the course of the determination of amino acid sequences. The major advantage is the ability to differentiate glutamine from lysine, because only the latter is acetylated and thus increases in mass by 42 Da. Horse heart cytochrome *c* could be fully *N*-acetylated and even on prolonged digestion with chymotrypsin underwent very little tryptic cleavage, in contrast to the native protein where this side reaction is extensive. Sperm whale myoglobin is more difficult to acetylate, but even at 40%–50% average acetylation, all 19 lysines could be identified unambiguously. A proteolytic digest of acetylated protein is thus a useful component of strategies for the determination of the primary structure of proteins by tandem mass spectrometry. (*J Am Soc Mass Spectrom* 1993, 4, 87–96)

The potential of mass spectrometry for the determination of the primary structure of proteins is now well established [1–3]. The strategy involves the digestion of the protein with proteolytic enzymes, partial separation of the digest by high-performance liquid chromatography (HPLC), determination of the molecular weights of the proteolytic peptides, and, finally, sequencing of the latter by using collision-induced dissociation (CID) followed by tandem mass spectrometry (MS/MS). Fast-atom (or cesium ion) bombardment (FAB) is the most widely used ionization method, although electrospray (ES) and matrix-assisted laser desorption (MALD) have also been applied more recently. Because of its high specificity for cleavage at lysine and arginine, trypsin is generally the first protease chosen.

Only under exceptional circumstances would the data from a single digest contain sufficient information to deduce the complete sequence of a protein. It is therefore always necessary to analyze at least one digest obtained with a different enzyme, such as chymotrypsin, Endo Glu-C or Endo Asp-N. Trypsin (or Endo Lys-C) generates peptides with either arginine or lysine as the C-terminal amino acid (except for peptides representing the original C-terminus). This is useful in mass spectrometric peptide sequencing

because it has been demonstrated that on CID, the presence of a basic amino acid will strongly influence fragmentation and favor fragment ions that encompass the site of the basic residue [4]. The CID spectra of tryptic peptides will therefore be dominated by C-terminal fragment ions. For this reason, the resulting spectra are different from those generated by the other proteases, which produce peptides containing basic amino acids in the interior, at the *N*-terminus, or none at all.

The amino acid lysine has two characteristics that are important for peptide sequencing by MS/MS. The basic ϵ -amino group is easily protonated and thus directs the fragmentation of the $(M + H)^+$ ion of the peptide, and, more important, lysine has the same nominal residue mass (128 u) as glutamine from which it differs by only 0.036 u. A difference in 128 u between two fragment ions of the same ion type therefore indicates the presence of either Lys or Gln, which must then be differentiated by other means. One method is provided by the specificity of trypsin, which cleaves on the C-terminal side of Lys but not Gln. This information is reliable in positive cases (i.e., where the tryptic cleavage occurs) but not in the negative instances, because there are situations where the enzyme does not cleave, such as the Lys-Pro bond or where Lys becomes a C- or *N*-terminal amino acid (i.e., if it is preceded or followed by arginine in the original protein).

For all these reasons, it is necessary to be able to differentiate Lys from Gln independently, and this is

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generally achieved by acetylation of the peptide (or the HPLC fraction in which it occurs) [3, 5]. In addition to the *N*-terminal amino group, each lysine present adds 42 u to the molecular weight of the peptide, and the residue mass of lysine is shifted to 170 u, whereas that of glutamine remains unchanged.

Because the acetylation needs to be performed on a number of peptides, we have explored a variation of the strategy outlined earlier, namely, acetylation of all lysines in the intact protein, followed by digestion with an enzyme that does not depend on the basic character of the lysine side chain. The resulting digest would consist of peptides where the mass coincidence of Lys and Gln is already eliminated, and the CID fragmentation directing effect of the basic amino acids is reduced to those peptides containing arginine and to a lesser extent histidine. Furthermore, all of the peptides generated by digestion of a protein in which all of the lysines are acetylated would have only one primary or secondary amino group left, namely, the *N*-terminus (except for the original *N*-terminal peptide). This would make it easier to derivatize this group with a substituent that contains a quarternary ammonium [6] or phosphonium group [7] for the purpose of generating d_n or w_n ions that are necessary to differentiate leucine from isoleucine [4, 8] and hydroxyproline [9]. Finally, cleavages at lysine, which occur frequently in lengthy chymotryptic digests because of unwanted tryptic activity, are eliminated by acetylation of the lysines.

The goal of this work was to evaluate the utility of *N*-peracetylation of proteins as an aid in the determination of their linear structure by mass spectrometry. After mass spectral analysis of a tryptic digest of the protein, a second digest with a different enzyme would be performed on the acetylated protein. We believe that acetylation will aid significantly in the analysis of the CID data from the second digest and facilitate the location of sequence overlaps. With the recent development of MALD [10] and ES [11], it has become very easy to accurately measure the molecular weights of proteins. We have used the former method to follow the acetylation reaction and evaluate the utility of such measurements for determining the number of acetyl groups incorporated.

Experimental

Proteins, Enzymes, and Reagents

Cytochrome *c* from horse heart (lot C-7752), myoglobin from horse skeletal muscle (lot 75F7035), myoglobin from sperm whale skeletal muscle (lot 93F-0497), ubiquitin from bovine red blood cells (lot U-6253), glucagon (lot G-4250), glacial acetic acid, and acetic anhydride were purchased from Sigma Chemical Co. (St. Louis, MO). Trypsin (sequenal grade) and chymotrypsin were purchased from Boehringer-Mannheim (Indianapolis, IN).

Sample Preparation

***N*-Peracetylation of Proteins.** Cytochrome *c* was acetylated by dissolving 20 nmol of the protein in 50 μ L of glacial acetic acid and adding an equal volume of acetic anhydride. The solution was heated for 3 h at 38 °C. The reaction was stopped by adding 200 μ L of water and the reagents removed using a Speed Vac (Savant, Farmingdale, NY). The sample was not allowed to go to dryness. Additional water was added and the sample reevaporated until the pH was neutral, indicating that all of the reagents had been removed. The reagents can also be removed by HPLC with a C_{18} (300-Å pore size) guard column, using 60% aqueous acetonitrile (0.5% trifluoroacetic acid) as the elution solvent. The sample volume was then reduced in a Speed Vac.

Under these conditions, serine, tyrosine, and, to some extent, threonine will also be acetylated. Treatment of the acetylated protein with base for 2 h at room temperature will remove most of this unwanted *O*-acetylation. If the protein is to be digested with 0.1 M NH_4HCO_3 , 0.1 mM $CaCl_2$ buffer at pH 8.5, any *O*-acetylated tyrosine or serine will be hydrolyzed at this time.

Myoglobin was first converted to apomyoglobin by removal of the heme in a manner similar to the removal of the heme from hemoglobin [12]. Dry myoglobin (10–50 nmol) was dissolved in 50 μ L of distilled deionized water. The apomyoglobin was precipitated by addition of a 100-fold excess volume of ice cold acetone/0.3% HCl, left to settle for 15 min on ice and then pelleted with a laboratory centrifuge (3000 g). The supernatant was removed and the pellet washed once with ice cold acetone/0.3% HCl, which was then removed after centrifugation. The residual acetone was removed under a stream of argon. The apomyoglobin was then dissolved in 25–50 μ L of H_2O , and 100 μ L each of glacial acetic acid and acetic anhydride were added. The acetylation and workup were carried out as described previously.

Tryptic and Chymotryptic Digestion. A solution of 10–50 nmol of either the native or the acetylated proteins that had been concentrated into approximately 50 μ L of water was diluted with 100 μ L of 8 M urea and denatured for 20 min at 38 °C. The solution was then diluted with 400 μ L of 0.1 M NH_4HCO_3 , 0.1 mM $CaCl_2$, pH 8.5, and the protein digested with either trypsin or chymotrypsin (2% by weight) at 37 °C. Both myoglobin digests (trypsin and chymotrypsin) were stopped after 6 h by adding 200 μ L of 0.5% trifluoroacetic acid. The cytochrome *c* digests were terminated in a similar manner after 36 h. The resulting peptides were fractionated by reversed-phase HPLC. An aliquot of the crude digest corresponding to 2–5 nmol was injected onto a Vydac protein C_{18} column (4.6 $\times$ 25 cm) and eluted by using a gradient of 90% A (H_2O ,

0.05% trifluoroacetic acid) to 65% B (acetonitrile, 0.05% trifluoroacetic acid) over 50 min.

To prepare the samples for fast-atom bombardment mass spectrometry (FABMS) analysis, glycerol (1–2 μ L) was added to each fraction and the HPLC solvent removed with a Speed Vac. An aliquot from each fraction was analyzed by FABMS to determine the $(M + H)^+$ ions of the proteolytic peptides. Another aliquot from the same sample was later used to record the CID spectrum of the $(M + H)^+$ ions of the corresponding peptides.

Fast-Atom Bombardment Mass Spectrometry

The FABMS of peptides, dissolved in glycerol, was carried out in MS-I of a JEOL HX110/HX110 (JEOL Ltd., Akishima, Japan) high-resolution tandem mass spectrometer ($E_1B_1E_2B_2$ configuration) at an accelerating voltage of 10 kV and a mass resolution of 1:2600 (10% valley). The JEOL Cs^+ ion gun was operated at 26 kV. Single scans were acquired between m/z 200 and 2000 in 0.8 min. The instrument was calibrated by using $(CsI)_nCs^+$ cluster ions. Mass spectra were acquired with a JEOL DA5500 data system running on a DEC PDP11/73 computer. All FABMS data are reported as the monoisotopic mass, unless otherwise noted.

Tandem Mass Spectrometry

Tandem mass spectrometry was carried out by using all four sectors of the JEOL HX110/HX110. Collision-induced dissociation of protonated peptide molecules, selected by MS-I, took place in the field-free region after B_1 , thus operating both MS-I and MS-II as double-focusing instruments. The collision cell potential was held at 3.0 kV; therefore, the ion collision energies were 7.0 keV. Helium collision gas was introduced into the collision chamber at a pressure sufficient to reduce the precursor ion signal by 65%–70%. The CID mass spectra were recorded with 100-Hz filtering at a rate to scan from m/z 100 to 6000 in 2.5 min (e.g., a CID mass spectrum from m/z 50 to 1950 requires 1 min). The MS-I was operated at a resolution adjusted so that only the ^{12}C -species of the protonated peptide molecule to be analyzed was transmitted. The MS-II was operated at a resolution of 1:1000 and was calibrated with a mixture of CsI , NaI , KI , RbI , and $LiCl$ [13].

Matrix-Assisted UV Laser Desorption Time-of-Flight Mass Spectrometry

Spectra were acquired by using a modified Vestec VT2000 LD-TOF (VESTEC Corp., Houston, TX) operating at 30-kV accelerating voltage and equipped with a Laser Science N_2 laser (LSI, Inc., Cambridge, MA). This laser produces UV light at 337 nm with 8-ns-wide pulses and was operated at a repetition rate of 5 Hz.

Samples were mixed with a matrix of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid) and ionized by irradiation with the N_2 laser. Each spectrum is the sum of 50 laser shots.

Results and Discussion

The complete and lysine-specific acetylation of horse heart cytochrome *c* ($M_r = 12360.1$) [14] and sperm whale myoglobin ($M_r = 17199.9$) [15] with a mixture of acetic acid/acid anhydride has proved to be difficult, corroborating earlier findings [16]. Cytochrome *c* was chosen because it digests very slowly and therefore, when using commercially available chymotrypsin, leads to extensive cleavage at lysine. As a result, 13 of the 20 peptides identified from the digest (Table 1) are due to trypsinlike activity.

When cytochrome *c* containing 19 lysines and having an *N*-acetyl blocked *N*-terminus was acetylated, the MALD spectrum of the derivatized product (Figure 1) revealed an $(M + H)^+$ ion of m/z 13,211.0 measured at the apex of the peak. This average M_r of 13,210 ($\pm 13\%$ or $\pm 0.1\%$) corresponds to the addition of 20 acetyl groups [i.e., one more than predicted for the number of lysines alone ($M_r = 13,200$)]. The peak is slightly broader than that of the internal standard owing to the inhomogeneity of the acetylation product (the centroid of the top 25% of the peak falls at m/z 13,222.6).

After a 37-h digestion with chymotrypsin, a much more enzyme-specific peptide mixture (compare Table 2 with Table 1) was obtained, the components of which covered the complete sequence (see Table 2 and Figure 2). This is in marked contrast to the results from the chymotryptic digest of the native protein, where only 64% of the sequence could be accounted for. Because many of the lysines in cytochrome *c* are located near each other, the undesired cleavages at lysine produced many small peptides, a significant number of which escaped detection. Because the acetylation of the lysines renders them inert to trypsin, the chymotryptic digest of acetylated cytochrome *c* contains almost exclusively chymotryptic peptides, even after a 37-h digestion.

A detailed inspection of the FAB spectra of the HPLC fractions from the chymotryptic digest of acetylated cytochrome *c* (Figure 3) revealed that all of the $(M + H)^+$ ion signals corresponded to the mass-to-charge ratio values calculated for complete lysine acetylation, except for two. These two peptides ($M + H)^+ = m/z$ 850.6 and 1167.4, respectively, were 42 u heavier than the $(M + H)^+$ ions of the peptides (27–33) and (27–36), both of which contained the threonine at position 28 (see sequences with I in Table 2 and an asterisk in Figure 2). Thus, this amino acid must be the locus of "overacetylation." Incubation of the acetylated protein and proteolytic peptides in ammonium bicarbonate at pH 8.5 should remove any *O*-acetylation on serine and tyrosine. Threonine, however, although reacting more slowly than either serine

Table 1. Chymotryptic digest of cytochrome *c*

Position	(M + H) ⁺ measured (FAB) ^a	Sequence
8-10	407.3	(K)KIF
11-22	1991.3 ^b	(F)VQKC...TVEK
11-26	2370.0 ^b	(F)VQKC...GGKH
26-37	1220.9	(K)HKTGPNLHGLF
27-36	1083.8	(H)KTGPNLHGL
27-35	936.7	(H)KTGPNLHGLF
28-36	955.5	(K)TGPNLHGLF
28-35	808.6	(K)TGPNLHGL
40-46	677.4	(K)TGOAPGF
40-48	941.5	(K)TGOAPGFTY
55-59	604.4	(N)KGITW
56-59	476.4	(K)GITW
60-65	750.5	(W)KEETL
60-67	1042.7	(W)KEETLMEY
60-68	1155.3	(W)KEETLMEYL
80-82	410.3	(K)MIF
87-97	1365.8	(K)KKTE...LIAY
88-97	1237.8	(K)KTER...LIAY
89-97	1109.6	(K)TERE...LIAY
88-98	1351.1	(K)KTER...IAYL

^aFAB, fast-atom bombardment.^b(M + H)⁺ measured at the peak centroid.

or tyrosine, requires a pH above 10.5 to remove the *O*-acetyl group [16].

An example of the effect that acetyl lysine has on the CID spectrum of a chymotryptic peptide from an acetylated protein is shown in Figure 4a. First, its presence is revealed by the abundant ion at m/z 126 (labeled KAc), which is formed by the loss of ammonia from the immonium ion of acetylated lysine, m/z 143, and is thus the acetyl analog of the cyclic immonium ion of m/z 84, characteristic of lysine (m/z 84 is still formed by the loss of ketene from m/z 126). There is also an ion ($-KAc$) corresponding to the loss of 114 u (the entire acetylated side chain) from the (M + H)⁺ ion. Second, a mass difference of 170 u between the y_6 ion (m/z 638) and the (M + H)⁺ ion (m/z 808.6) also requires that the *N*-terminal amino acid be acetyllysine

and not glutamine. The fragment ion nomenclature used herein has been outlined elsewhere [1, 2].

The spectrum of the higher molecular weight component, (M + H)⁺ = m/z 850.5 (Figure 4b), from the same sequence (27-33) is similar, except that all of the peaks that are due to fragments containing the amino acid in position 28 are shifted by 42 u, which confirms that this threonine is acetylated. Of note, in this spectrum the w_{6a} ion is very abundant and the w_{6b} ion is absent, whereas in Figure 4a both are present but less abundant. This indicates that in the formation of w_n ions, a CH₃COO[•] radical is lost much more readily than an HO[•] radical.

The CID spectrum of the chymotryptic peptide (68-74) of (M + H)⁺ = m/z 975.6 is shown in Figure 5a. This spectrum consists mainly of b_n - and y_n -series ions and is characteristic of peptides that do not contain a basic amino acid. Although the b_2 ion at m/z 243 could be either X-E or N-Q (the possibility of K is eliminated, because it would be acetylated), the presence of abundant immonium ions at m/z 86 and 102 indicates that it is probably X-E, although the order cannot be established because of the absence of a y_6 ion. Because b_n - and y_n -series ions are not precursors for side-chain cleavages, these spectra do not therefore exhibit either d_n or w_n ions and as a result cannot differentiate leucine from isoleucine; however, the spectrum clearly demonstrates that the two amino acids in positions 72 and 73 are both lysine rather than glutamine. An overlapping tryptic peptide, (M + H)⁺ = m/z 1495.8 (Figure 5b), obtained from the

Figure 1. MALD spectrum of peracetylated cytochrome *c*. The peaks indicated by an asterisk are the (M + 2H)²⁺ and (M + H)⁺ ions, respectively, of the internal standard, sperm whale apomyoglobin (M_r = 17,199.9).

Table 2. Chymotryptic digest of acetylated cytochrome c

Position	(M + H) ⁺ measured (FAB) ^a	Sequence	HPLC ^b Fraction
1-10	1288.9	GDVEKGKKIF	13
11-26	2496.1 ^c	(F)VQKC...GGKH ^d	15
27-33	808.7	(H)KTGPNLH	2
27-33	850.6	(H)KTGPNLH ^e	3
27-36	1125.4	(H)KTGPNLHGLF	14
27-36	1167.4	(H)KTGPNLHGLF ^e	15
37-48	1324.7	(F)GRKT...GFTY	7
39-48	1111.6	(R)KTGQ...GFTY	7
49-59	1331.7	(Y)TDAN...GITW	10
60-67	1084.5	(W)KEETLMEY	9
60-68	1197.5	(W)KEETLMEYL	16
66-67	312.4	(M)EY	8
68-74	975.6	(Y)LENPKKY	5
75-80	688.4	(Y)IPGTKM	4
81-82	279.2	(M)IF	7
81-97	2121.2	(M)IFAG...LIAY	15
75-82	948.8	(Y)IPGTMIF	16
83-91	1156.7	(F)AGIKKKTER	3
83-97	1861.0	(F)AGIK...LIAY	11
83-98	1973.8	(F)AGIK...IAYL	16
95-97	479.3	(L)IAY	10
98-104	887.7	(Y)LKKATNE	1

^aFAB, Fast-atom bombardment.^bHPLC, high-performance liquid chromatography.^c(M + H)⁺ measured at the centroid.^dContains heme.^eI denotes O-acetylthreonine.

native protein underscores the complementarity of the CID mass spectra of the products of these two digests. This spectrum is dominated by C-terminal fragment ions owing to the presence of lysine at the C-terminus. It reveals both the order L-E (from the y_3 - y_5 series) and establishes that position 68 is leucine rather than isoleucine on the basis of the w_5 ion.

The complete acetylation of the 19 lysines and the N-terminal amino group of sperm whale apomyoglobin ($M_r = 17199.9$) is more difficult. After treatment of the acetylated protein with ammonium bicarbonate,

Figure 2. Amino acid sequence of horse heart cytochrome c. The peptides observed by FABMS on digestion of the acetylated protein with chymotrypsin are underlined by brackets; asterisks indicate that the peptide was found both with free and acetylated threonine (see Table 2 and text).

Figure 3. Separation by reversed-phase HPLC (214 nm) of a 28-h chymotryptic digest of peracetylated cytochrome c. The solvent gradient varied and was kept shallow in the middle of the chromatogram. 0-15 min: 10%-35% acetonitrile in water (containing 0.05% trifluoroacetic acid); 15-30 min: 35%-40%; 30-35 min: 40%-60%. Seventeen fractions were collected as indicated. Fraction 17 is the heme, (M + H)⁺ = m/z 617.7.

Figure 4. CID mass spectra of the chymotryptic peptides corresponding to the sequence 27-33 of cytochrome *c* and representing (a) the monoacetylated peptide, precursor ion of m/z 808.7, and (b) the diacetylated peptide, precursor ion of m/z 850.6. KAc refers to the cyclic immonium ion of acetylated lysine; -KAc indicates the loss of the acetyllysine side chain.

the product was analyzed by MALD (Figure 6). The broad peak (compared with the internal standard) indicates considerable inhomogeneity. The apex of the $(M + H)^+$ peak falls at m/z 17,531.4, whereas the centroid of the top 25% of the peak is found at m/z

Figure 5. CID mass spectra of (a) the chymotryptic peptide 68-74 from acetylated cytochrome *c*, and (b) the tryptic peptide 61-72 from the native protein. KAc and -KAc as in Figure 4.

Figure 6. MALD spectrum of acetylated sperm whale myoglobin. The peaks indicated by an asterisk are the $(M + 2H)^{2+}$ and $(M + H)^+$ ions, respectively, of the internal standard, horse heart apomyoglobin ($M_r = 16,951.5$) [15].

17,538.3 but at that point has a width of 200 u. These data suggest that although not all the lysines had been acetylated, a broad distribution of peracetylated species exists. Results from several of these experiments indicated that the average extent of acetylation was between 40% and 60%. The partial underacetylation of the lysines is indicated in Figure 7, which details the results of a chymotryptic digest of acetylated sperm whale apomyoglobin. Although the mass-to-charge ratio values of the $(M + H)^+$ ions do not as such differentiate between *N*-acetylated lysine and any *O*-acetylated amino acids, the CID spectra of these peptides clearly indicate that in this case, only the lysines have been derivatized.

The degree of acetylation within a particular lysine-containing chymotryptic peptide varies from 0%

Figure 7. Amino acid sequence of sperm whale myoglobin. The peptides observed by FAB/MS on digestion of the acetylated protein with chymotrypsin are underlined by brackets. The numbers below the brackets correspond to the mass-to-charge ratio values observed for the $(M + H)^+$ ion. Values in parentheses indicate the number of acetyl groups incorporated. For clarity of presentation, additional peptides detected but that do not add further information are not shown.

to 100% and can be random or specific. For example, reaction of the lysines at positions 140 and 145 gives rise to all four possible combinations for the sequence 139-146 (unreacted), two monoacetylated, and one diacetylated analog), $(M + H)^+ = m/z$ 964.7, 1006.6, and 1048.7, respectively. Because all of them have an *N*-terminal arginine, their CID spectra consist mainly of a_n and d_n ions. Figure 8a shows the CID spectra of the underivatized peptide, $(M + H)^+ = m/z$ 964.4, and although there is a complete series of a_n and d_n ions, the amino acids in position 140 and 145 can be either K or Q. Because these two amino acids have the same residue (128 u) and side-chain (58 u) mass, they give an identical set of a_n and d_n ions. The CID spectrum of the diacetyl derivative $(M + H)^+ = m/z$ 1048.6 shown in Figure 8c, however, allows unambiguous assignment of positions 140 and 145 as lysine. After acetylation, the mass-to-charge ratio values of the a_2 - a_6 and d_3 , d_4 ions have been shifted by +42 u and a_7 by +84 u, indicating that amino acids 140 and 145 must have

been lysine and not glutamine, information that could not have been deduced from Figure 8a alone.

Figure 8b is an interesting case. It represents the CID spectrum of a mixture of isomeric monoacetylated peptides $(M + H)^+ = m/z$ 1006.6 from the sequence 139-146, where the modification at position 145 is slightly more abundant. This conclusion can be easily reached from the fact that the ions a_2 - d_7 appear as doublets, spaced 42 u apart, because these fragments retain only one lysine, including its side chain. On the other hand, the d_2 and a_7 ions are singlets, as are all ions of mass lower than d_2 and higher than a_7 because they contain either none or both of the lysines present in the peptide.

In contrast to the previous example, the CID spectrum of the chymotryptic peptide 34-43 (Figure 9) shows that of the two lysines at positions 34 and 42, only the latter is acetylated. In this spectrum, which has an appearance typical of peptides with a basic amino acid at the *N*-terminus (mainly a_n and d_n ions), the mass-to-charge ratio values for all of the fragments are compatible with an unreacted *N*-terminal lysine. Furthermore, the mass difference between a_8 and $a_9 + 1$ (171 u) indicates that the amino acid at position 42 is acetyllysine. If the lysine at position 34 had been acetylated instead, the spectrum would be dominated by the corresponding C-terminal fragment ions (none of which is observed), and all of the *N*-terminal ions originating from below position 42 would be shifted by +42 u. Thus, in this case, both the appearance of the spectrum and the mass-to-charge ratio values of the fragment ions indicate that the amino acids 34 and 42 are Lys not Gln. This is also supported by the CID spectrum of the diacetylated peptide $(M + H)^+ = m/z$ 1299.9 (data not shown). Because the monoacetylated peptide 34-40 has been detected in addition to the unreacted analog (see Figure 4), some of the peptide 34-43 acetylated at Lys-34 should also have been observed. It must have been chromatographically separated from the other isomer.

Table 4 summarizes the extent of acetylation derived from these and other analogous data, whereas Figure 7 represents the assignments of the observed chymotryptic peptides to the amino acid sequence of sperm whale myoglobin. Although peptides 90-97 and 124-131 were not observed, they were also not found

Figure 8. CID mass spectra of the peptides corresponding to various degrees of acetylation of the region 139-146 of myoglobin: (a) unacetylated, (b) monoacetylated, and (c) diacetylated. Fragments labeled a'_n and d'_n in b refer to those due to the isomer acetylated at Lys-140.

Figure 9. CID mass spectrum of the chymotryptic peptide 34-43 from acetylated myoglobin $[(M + H)^+ = m/z$ 1258.7].

Table 3. Chymotryptic digest of sperm whale apomyoglobin

Position	(M + H) ⁺ measured (FAB) ^a	Sequence
1-7	819.4	VSLEGEW
8-11	472.4	(W)QLVL
12-14	441.3	(L)HVW
15-29	1523.1	(W)AKVE... QDIL
30-33	548.4	(L)IRLF
34-40	811.4	(F)KSHPETL
34-46	1633.9	(F)KSHP... FDRF
44-50	943.8	(F)DRFKHLK
48-55	1086.6	(K)HLKTEAEM
56-69	1525.1	(M)KASE... VTVL
64-69	625.5	(K)HGVTVL
70-76	658.6	(L)TALGAIL
77-89	1515.2	(L)KKKG... LKPL
98-103	761.5	(H)KIPIKY
98-104	874.7	(H)KIPIKYL
104-106	408.4	(Y)LEF
107-113	782.4	(F)ISEAIIH
107-115	994.8	(F)ISEAIIHVL
116-123	952.6	(L)HSRHPGDF
132-137	834.5	(M)NKALELF
134-140	876.4	(K)ALELFRK
139-146	964.7	(R)KDIAAKY
147-151	609.3	(Y)KELGY
147-153	794.5	(Y)KELGYQG

^aFAB, fast-atom bombardment.

in the digest of the native protein. In contrast to cytochrome *c*, sperm whale apomyoglobin can be fully digested in 3-6 h; thus, the unacetylated digest of this protein reveals little cleavage at tryptic sites. The extent of sequence coverage by the chymotryptic digests of native and acetylated apomyoglobin is therefore similar (compare Table 3 with the data in Figure 7); how-

ever, the results from the digest of the acetylated protein clearly define all of the lysines present and differentiate them from glutamine by a single experiment.

Although the molecular weight information provided by MALD indicated an average of only 40%-60% acetylation, the FABMS and CID data

Table 4. Distribution of the sites of acetylation in some chymotryptic myoglobin peptides containing more than one lysine

Position	Sequence	Mass-to-charge ratio observed (FAB) ^a	Site of acetylation
34-43	KSHPETLEKF	(0) ^b 1215.7 (1) ^c (1) 1258.7 (1) (2) 1299.9 (1)	Completely K42
47-55	KHLKTEAEM	(0) 1086.6 (1) (1) 1128.6 (4) (2) 1170.6 (2)	Predominately K47
98-103	KIPIKY	(0) 761.2 (2) (1) 803.5 (1) (2) 845.6 (3)	3:1 K98:K102
139-146	RKDIAAKY	(0) 964.7 (1) (1) 1006.6 (3) (2) 1048.7 (1)	Almost equimolar K140:K145

^aFAB, fast-atom bombardment.^bNumber of lysines acetylated.^cRelative abundance.

Figure 10. CID mass spectrum of the *N*-terminal chymotryptic peptide (1-7) from (a) acetylated and (b) native myoglobin.

showed that in all cases, fully acetylated lysine-containing peptides were detected. The reason for the difficulty in achieving complete acetylation is unclear but is in agreement with earlier observations that between 60% and 90% acetylation can be considered typical [16]. The problem of incomplete acetylation is likely to become more severe with much larger proteins. Because we have found that the previous acetylation protocol works well with smaller proteins, such as insulin and ubiquitin, an alternative strategy may be to first cleave the native protein into large ($M_r < 10,000$) polypeptides using CNBr. These fragments should be easily acetylated and could then be digested with an appropriate enzyme, such as chymotrypsin or Endo Glu-C. We have found that the C-terminal CNBr fragment (132-153) of sperm whale apomyoglobin, a region that was difficult to acetylate in the intact protein, readily adds five acetyl groups, indicating that all four lysines and the *N*-terminus have been acetylated.

Finally, the *N*-terminal peptide of the original protein can be easily identified (unless it is already blocked as in cytochrome *c*) in the digest of an acetylated product. The peptide incorporating the original *N*-terminal residue will be the only one with an acetylated *N*-terminus. Figure 10a represents the CID spectrum of one of the chymotryptic peptides $[(M + H)^+ = m/z 861.5]$ from acetylated sperm whale myoglobin. The abundant b_n and y_n ion series establish the sequence as BXSEGEW, where B is an amino acid of residue mass 141. This corresponds to *N*-acetyl valine. The assignment is further confirmed by the small but significant peak at $m/z 142$, which could be the b_1 ion,

a fragmentation that we have only observed in *N*-acetylated peptides [17]. This peptide must therefore represent the original *N*-terminus. Its unacetylated analog (Figure 10b) is observed in the chymotryptic digest of the native myoglobin. Because this peptide retains the basic *N*-terminal amino group, the corresponding CID spectrum is dominated by *N*-terminal ions, and the clearly observable d_2 ion defines the amino acid in position 2 as leucine rather than isoleucine.

Conclusions

Although it is difficult to completely and exclusively acetylate the α -amino groups in proteins (except smaller ones) under the conditions described, a proteolytic digest of the product provides very useful information complementary to that obtained from digests of the native protein. Most important, the CID spectra of the $(M + H)^+$ ions of the peptides from the acetylated protein permit direct differentiation of lysine from glutamine and eliminate the separate acetylation of various fractions containing peptides in which this ambiguity would otherwise remain. We have shown previously that acetylation of lysines, which occurs in the interior of the peptide sequence (such as are produced by chymotryptic and Endo Glu-C digests), can simplify the CID spectra by reducing the number of the fragmentation directing basic sites [18]. Furthermore, conversion of lysine to a nonbasic amino acid simplifies chymotryptic digests that have to be carried out over a relatively long time, which often leads to additional trypsinlike cleavage. Finally, the original *N*-terminal peptide will be acetylated at the first amino acid and thus will be readily recognized. The results presented here suggest that at least one digest of acetylated protein should be incorporated into the strategies for MS/MS sequencing of proteins.

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