

Overalkylation of a Protein Digest with Iodoacetamide

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Cystine linkages in proteins are often opened with reducing agents, sometimes to improve their digestion, often to eliminate disulfide linkages from complicating analysis of the digest. After reduction, the sulfhydryls are usually reacted with iodoacetamide (IAM), iodoacetic acid (IAA), or another electrophile to prevent reformation of disulfide linkages in a random manner. When the amount of protein may be reliably estimated, side reactions from excess IAM or IAA can be avoided. When this is not so, removal of excess iodoalkane can be accomplished by HPLC, by dialysis, or simply by allowing a reducing thiol to consume any excess. In mass spectrometric analysis of proteins isolated by 1D or 2D gels, removal of the excess iodoalkane is often accomplished simply by washing the gel prior to proteolytic digestion. During a recent study of the glutathionylation site mapping of actin, IAM was used to block any residual sulfhydryl groups remaining on the protein so that they would not displace glutathione from its initial site. In addition, to avoid losses due to actin polymerization during dialysis, the IAM was allowed to remain during the digestion. This further ensured that any sulfhydryl groups liberated during the digestion would be similarly blocked by the IAM. Under these conditions, we observed the peptides to undergo N- as well as S-carbamidomethylation. In examining a series of other peptides alkylated with IAM in this way, we have found N-alkylation to be the rule rather than the exception and even O-alkylation was detected. The main sites to which the carbamidomethyl group attaches to the peptides have been located with LC–MS² using an ion trap mass spectrometer and found to be the N-terminal amino group. A simple expedient to prevent such reactions when an excess of reducing agent must be avoided is to run the alkylation in the presence of a thioether such as 2,2'-thiodiethanol rather than a thiol.

Reduction and carbamidomethylation with iodoacetamide (IAM) or carboxymethylation with iodoacetic acid (IAA) of the cystine disulfide groups in proteins is a common procedure used today in protein modification^{1,2} and peptide mapping.³ Its use may also help overcome the resistance of many native proteins to

enzymatic degradation, although the reverse has been observed, e.g., with bovine albumin.^{4a} Where applicable, maximal digestion ensures the production of a full range of proteolytic fragments and this is especially important when mass spectrometry is used for identification.³ Interestingly, long before it was applied analytically, iodoacetate was identified as an inhibitor of muscle contraction.⁵ Arguments about its mechanism of action were finally settled when Dickens showed that alkylation of the sulfhydryl groups of the glutathione coenzyme of glyoxalase (the glutathione-dependent glyoxalase I–II system) was responsible.⁶ Later, Michaelis and Schubert pointed out⁷ that IAA also reacts with amines such as those found in amino acids, so that reaction with the cysteines in proteins could not be automatically assumed. Although reaction with SH was generally found to be more rapid than with amines, it did depend strongly on the exact structure of the thiol, so that, “If the (biological) effect of IAA occurs only at pH >7–8 the point of attack may be just as well an amino group” (ref 7, p 334). Smythe further examined the reaction of both IAA and IAM⁸ with a range of thiols and aminothiols at pH 6 and 7, confirming the range of their reactivities but failing to find evidence for reaction with amino groups under these conditions. He also found reactions with IAM to be about 2.7–5.4 times as fast as IAA, and in agreement with Hellerman et al.,⁹ they find that the amide is a better inhibitor of urease than the acid but that the opposite is true in the case of yeast fermentation. More recently, the reaction rate of IAM with thioredoxin was found to be ~20-fold greater according to Kallis and Holmgren¹⁰ and 60 times greater in the case of soybean amylase.

Our interest in iodoalkylation arose from our desire to identify the glutathionylation site on actin that had been reacted with this tripeptide under oxidizing conditions (full details to be reported elsewhere). Here the alkylation step with IAM was performed in order to block any glutathionylation-unreactive cysteines located elsewhere on the protein from displacing glutathione from its original location. Since additional cysteines might be exposed during the digestion, and in order to prevent actin polymerization during attempts to remove the IAM, it was allowed to remain in the solution during the entire digestion period. Electrospray mass

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spectrometry of the digest then revealed that several of the tryptic peaks showed an increase in mass by multiples of 57 Da as a result of IAM reacting with amino groups in addition to sulfhydryls. To explore this phenomenon in more detail, we have also alkylated a series of peptides with and without lysine and glutamic or aspartic acid in the side chains.

EXPERIMENTAL SECTION

Materials. Bovine muscle actin, oxidized form of glutathione (GSSG), IAM, ammonium bicarbonate, 2,2'-thiodiethanol (thiodiglycol, 2-hydroxyethyl sulfide), and synthetic peptides VGDE and angiotensin I were purchased from Sigma Co. (St. Louis, MO). Peptide PEQECEQQK was a gift from Dr. P. Steinert¹¹ (NIAMSS, NIH). Acetic anhydride was obtained from Fluka Chemika. Sequencing grade trypsin (Promega, Madison, WI) was used for digestions in a water bath at 37 °C.

LC-ESI-MS Analysis. Mass spectrometry was performed using a HP1100 binary pump system (Hewlett-Packard, Palo Alto, CA) coupled on-line to a quadrupole ion trap LCQ mass spectrometer (ThermoFinnigan, San Jose, CA) through its electrospray interface. Microflow was generated by a precolumn AC-400-VAR variable-microflow processor (LC Packings, San Francisco, CA) that was connected to the gradient system and a calibrator for a 0.3-mm-i.d. column. A peptide CapTrap loop (0.5 × 2 mm, Michrom BioResources, Inc., Auburn, CA) was placed directly on the injection valve to serve as an on-line sample cleanup device. MS data were acquired in positive ion mode using centroid format. Protein digest and peptide samples were separated on a 100 mm × 0.3 mm i.d. capillary LC column (Keystone Scientific, Inc., Bellefonte, PA) packed with 5- μ m C₁₈ base-deactivated stationary phase (particle size 150 Å) at a flow rate of 5 μ L/min. Mobile phase A consisted of 0.1% formic acid in CH₃CN–H₂O (2:98, v/v) and mobile phase B of 0.1% formic acid in H₂O–CH₃CN (5:95, v/v). Reaction products (100 pmol) were analyzed using a gradient of 0–100% B in 15 min. Tandem MS² and zoom scans were performed in the data-dependent mode where an ion was fragmented after a threshold of at least 5 × 10⁵ total ion counts was exceeded in a preliminary survey scan. The mass isolation width was set at 2 Da, the scan range during parent ion scans was from *m/z* 100 to 2000, and the arbitrary relative collision energy was set at 40%. The heated capillary was maintained at 200 °C and the spray voltage at 3.5 kV.

Tryptic Digest of Bovine Actin. Bovine muscle actin (50 μ M) in 50 mM Tris-HCl, pH 7.5 was incubated with GSSG (1 mM) at 37 °C for 1 h. Iodoacetamide was added to the reaction mixture to a final concentration of 2 mM to block residual cysteine residues. This reaction was allowed to proceed in the dark at room temperature for 1 h. Promega trypsin (0.5 μ g) was then added to an enzyme-to-substrate ratio of 1:85 (w/w), and digestion in the same buffer continued for 18 h at 37 °C in the presence of IAM. The alkylation and trypsinization reactions were quenched by adding 5 μ L of 5% acetic acid prior to mass spectrometric analysis.

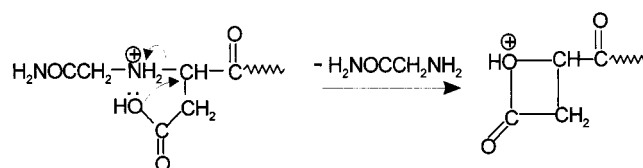
Model Peptide Studies with IAM. Synthetic peptides VGDE, PEQECEQQK, and angiotensin I (DRVYIHPFHL) were dissolved in water to a final concentration of 50 μ M before IAM (0.5 M stock solution in 50 mM NH₄HCO₃) was added to a molar ratio

of 10:1 (IAM/peptide). The pH of the final solution was ~7.0 as measured by test paper (Micro Essential Laboratory Inc., Brooklyn, NY). Alkylation was allowed to proceed at room temperature for 1 h prior to incubation at 37 °C for an additional 18 h to simulate the condition of the tryptic digestion of actin. A second experiment was run on PEQECEQQK dissolved in 50 mM NH₄HCO₃ in an effort to have the ammonia compete with the amino functions on the peptide.

The short peptide VGDE was also N-acetylated to block the N-alkylation with IAM. Two drops of 100% acetic anhydride were added to a stock solution of 5 mM peptide on ice. N-Acetylation proceeded at room temperature for 2 h, and an aliquot of 5 μ L was withdrawn for LC-MS analysis to ensure the completion of this reaction. Without further purification, IAM was added to the N-acetylated peptide to a molar ratio of 10:1 and incubated in the same fashion.

RESULTS AND DISCUSSION

After alkylating bovine actin with IAM at pH 7.5 for 1 h in the absence of any thioethers or reducing thiols, the sample was trypsinized at pH 7.5 for 18 h. As discussed above, we omitted steps to remove excess IAM such as HPLC, dialysis, etc., because of concerns regarding migration of glutathione to sulfhydryls exposed during digestion and actin polymerization. The digest provided the LC-MS shown in Figure 1a. Several peaks are seen to be accompanied by one or more satellites 57 Da higher in mass due to the addition of carbamidomethyl groups during and after trypsinization. Three were identified as arising from bovine actin while two others, X with its single and double addition of 57 and Y singly adducted, are from an unidentified source. The most abundant peak set represents DSYVGDEAQS⁺ of *m/z* 1198.6, 1255.6, and 1313.5. The MS² of the first, even in its singly charged form, provides an excellent accounting for b₄–b₁₀ and y₃–y₁₀ peaks (Figure 1b). Its monoalkylated homologue (Figure 1c) exhibits ions for b₄–b₁₀, all of which also contain the 57 mass units, reducing its position to the DSYV portion while the abundances and masses of y₄–y₁₀ are very similar to the unalkylated form. These force the alkyl group to be located on the N-terminal aspartate, either on the nitrogen or, less likely, on the carboxylate group. The MH⁺ ions of both alkylated and unalkylated forms lose water, but the alkylated ion also loses 74 Da to give the ion at *m/z* 1181.4. We suggest that this involves loss of the carbamidomethyl group attached to the N-terminal aspartate nitrogen, perhaps facilitated by the side-chain carboxyl:



Alkylation likely occurs at the less basic N-terminus (pK_a 7.4) of DSYVGDEAQS⁺ instead of the lysine nitrogen at the C-terminus because at pH 7.5 the lysine (pK_a 10.5) is still largely protonated.

The MH⁺ ion at *m/z* 976.5 (AGFAGDDAPR) in a second peptide from actin also shows a nearly complete set of b and y ions on MS² (Figure 1d). Once again, its 57-Da satellite at *m/z*

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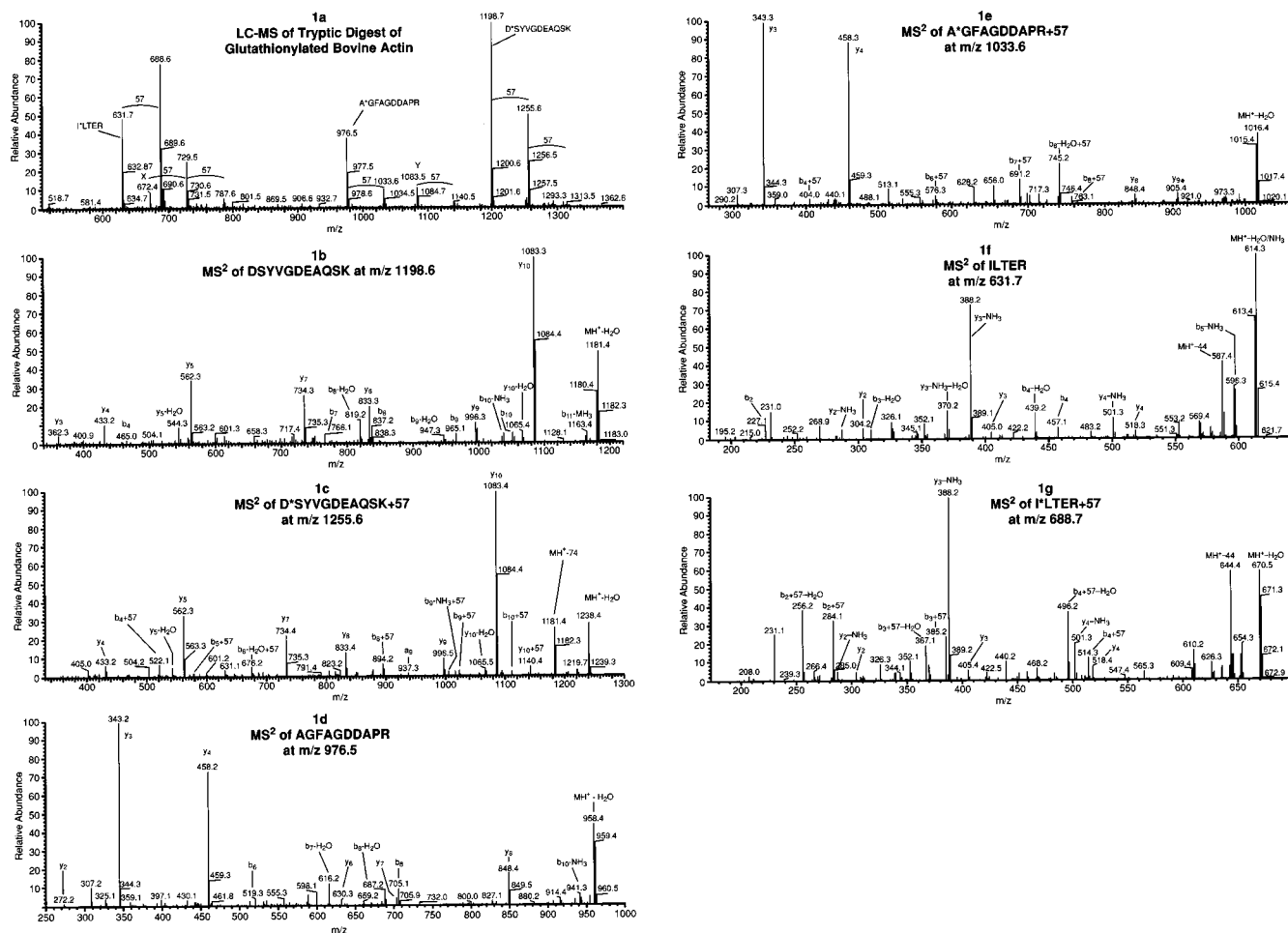


Figure 1. (a) LC–MS spectrum of a glutathionylated actin digested with trypsin at pH 7.5. (b) MS² of DSYVGDEAQS* at *m/z* 1198.6. (c) MS² of monoalkylated DSYVGDEAQS* at *m/z* 1255.6. (d) MS² of AGFAGDDAPR* at *m/z* 976.5. (e) MS² of monoalkylated AGFAGDDAPR* at *m/z* 1033.6. (f) MS² of ILTER* at *m/z* 631.7. (g) MS² of monoalkylated ILTER* at *m/z* 688.7. An asterisk labeled after an amino acid indicates the site of alkylation with IAM.

1033.6 presents a similar spectrum (Figure 1e) with *y*₃–*y*₉ ions at the same masses as in the unalkylated product while *b*₄–*b*₈ all contain the 57 Da of the carbamidomethyl group. Thus, the nitrogen of the N-terminal alanine is again its location since GFA cannot support alkylation nor is arginine known to be alkylated by the haloacids.^{4b}

Finally, the third, short peptide from actin at *m/z* 631.7 (ILTER) is likewise alkylated at the N-terminal isoleucine as clearly shown by the unalkylated *y*₂–*y*₄ ions along with alkylated *b*₂–*b*₄ ions (Figure 1, g vs f).

In their recent review of sample preparation for peptide mapping,³ Lundell and Schreitmüller stressed that IAM and IAA are known to react with other amino acids such as methionine, histidine, and lysine in proteins, but specific examples are not provided in the context of mass spectral analyses. We felt it would be helpful, therefore, to examine the reaction with several other peptides. To this end, we first alkylated the short peptide VGDE with IAM at pH ~7.0 and panels a and b of Figure 2 compare the MS² spectra of its unalkylated (MH⁺ 419.1) and monoalkylated forms (MH⁺ 476.3), respectively. That alkylation is again on the N-terminal valine is shown by the unalkylated *y*₁–*y*₃ ions along with *b*₂ + 57 and *b*₃ + 57 ions. We then removed the ability of this peptide to N-alkylate in this fashion by simply acetylating its

terminal nitrogen with acetic anhydride. Under these conditions, the main product was still monoalkylated (MH⁺ 518.1), but now the carbamidomethyl group has been attached to the GDE end of the molecule as shown by the *y*₂ + 57 and the abundant *y*₃ + 57 ions (Figure 2, d vs c). The ions at *m/z* 205.1 (*y*₁ + 57) and *m/z* 313.9 (*b*₃) suggest that the glutamic acid is O-alkylated, but *b*₃ + 57 at *m/z* 370.9 implies the alkylation occurred on the aspartic acid residue. Presumably, both are in fact present. Of course, the glutamate alkylation may be at either the C-terminus or on the side-chain carboxyl and there appears to be no way to distinguish among them. A trace of the dialkylated species was also seen at *m/z* 575.1.

A more complex case is afforded by a peptide prepared during a study of involucrin,¹¹ PEQECEQQK (MH⁺ 1246.6), where a range of possible O-, N-, and S-alkylation sites is available. Here alkylation under the same conditions proceeded to give mostly a dialkylated product (MH⁺ 1360.6) with minor amounts of mono-, tri-, and tetraalkylated products also detected at *m/z* 1303.6, 1417.7, and 1474.8, respectively. The monoalkylated product, MH⁺ 1303.5, was surprisingly found to be alkylated at the N-terminal proline rather than the more nucleophilic cysteine (Figure 3, b vs a). Thus, all of the ions from *b*₃–*b*₉ contain the carbamidomethyl group while *y*₄–*y*₉ are unalkylated. Clearly, one must adjust the pH to

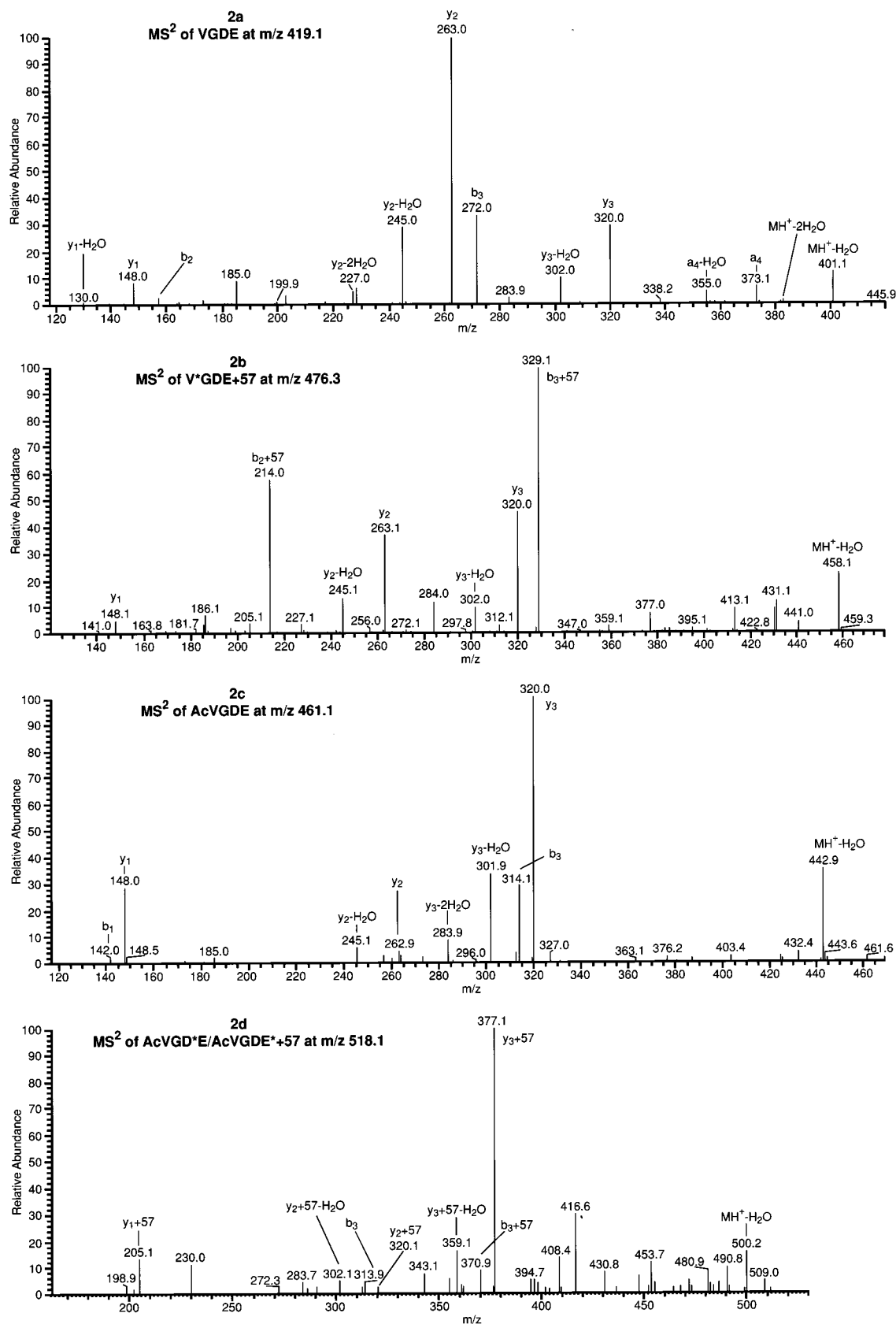


Figure 2. (a) MS² spectrum of peptide VGDE at *m/z* 419.1. (b) MS² of monoalkylated VGDE at *m/z* 476.3. (c) MS² of acetylated VGDE at *m/z* 461.1. (d) MS² of monoalkylated acetylated VGDE at *m/z* 518.1.

values lower than 7.0 to completely prevent N-alkylation of terminal amino groups, but it may be that higher pH for shorter

periods of time would provide preferential S-alkylation due to formation of the very nucleophilic RS[−] anion.

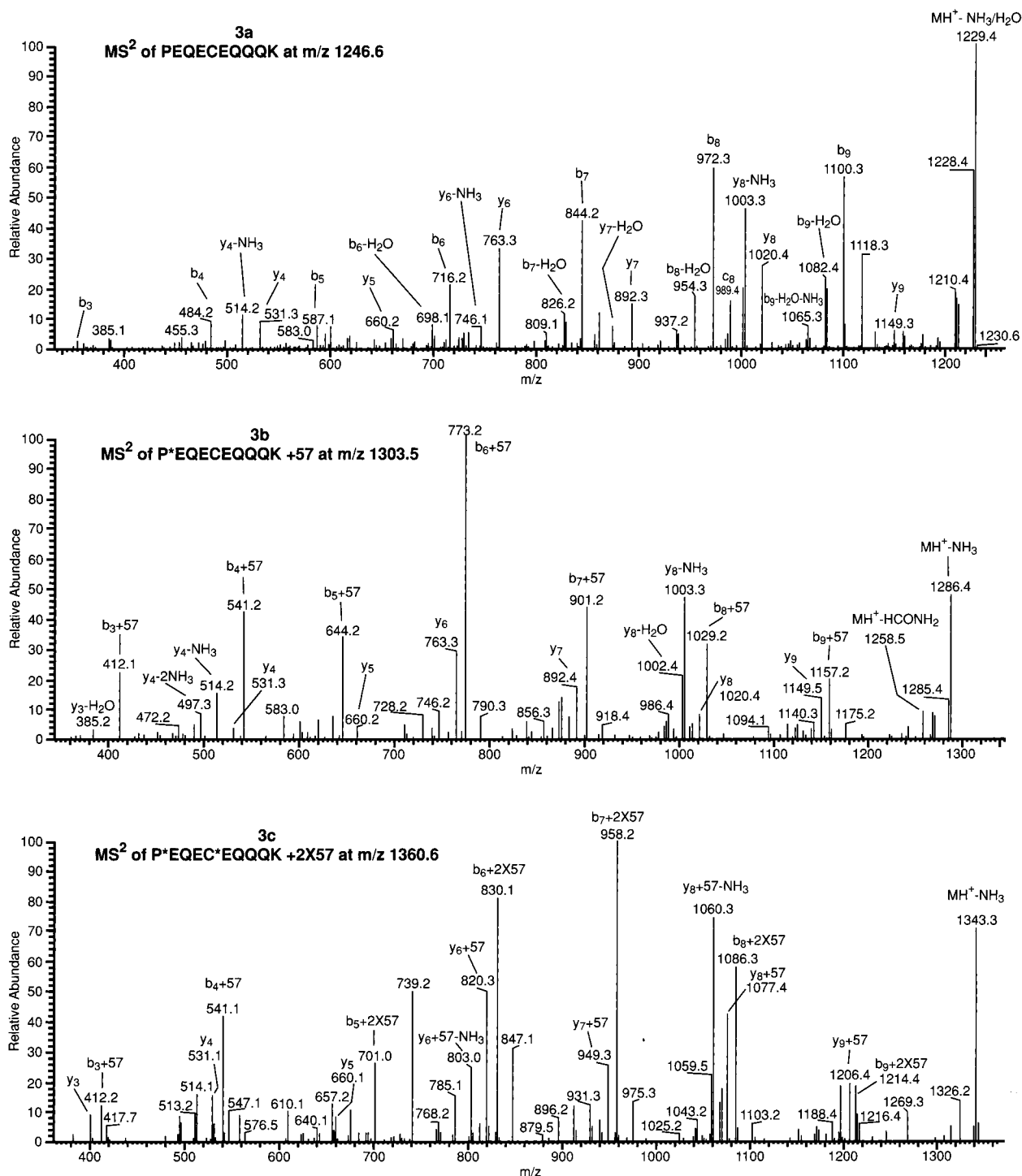


Figure 3. (a) MS² spectrum of peptide PEQECEQQQK at *m/z* 1246.6. (b) MS² of monoalkylated PEQECEQQQK at *m/z* 1303.5. (c) MS² of dialkylated PEQECEQQQK at *m/z* 1360.6.

Comparing the MS² spectra of the dialkylated with the monoalkylated species (Figure 3, c vs b) reveals that alkylation has taken place on the former at both the N-terminal proline and, as expected, the cysteine. Thus, *b*₃ + 57 and *b*₄ + 57 at *m/z* 412.2 and 541.1, respectively, locate one carbamidomethyl group to either the N-terminal proline or the glutamate residue at position 2 while the ions from *b*₅ to *b*₉ are doubly alkylated due to the cysteine. Confirming this, *y*₃–*y*₅ are at their normal unalkylated positions at *m/z* 400.1, 531.3, and 660.1 while *y*₆–*y*₉ are all monoalkylated via the cysteine. The *y*₉

+ 57 ion confirms that the second alkyl group is attached to the N-terminal proline rather than the first glutamate. Without MS² data, this result would not be entirely straightforward since it is well known that any primary amino group in a peptide can be dialkylated^{4b} as well. A second experiment on this peptide was carried out by allowing its alkylation to proceed in the presence of 50 mM NH₄HCO₃ in hopes of suppressing N-alkylation through competition by free ammonia. However, the LC–MS obtained was identical with that described above.

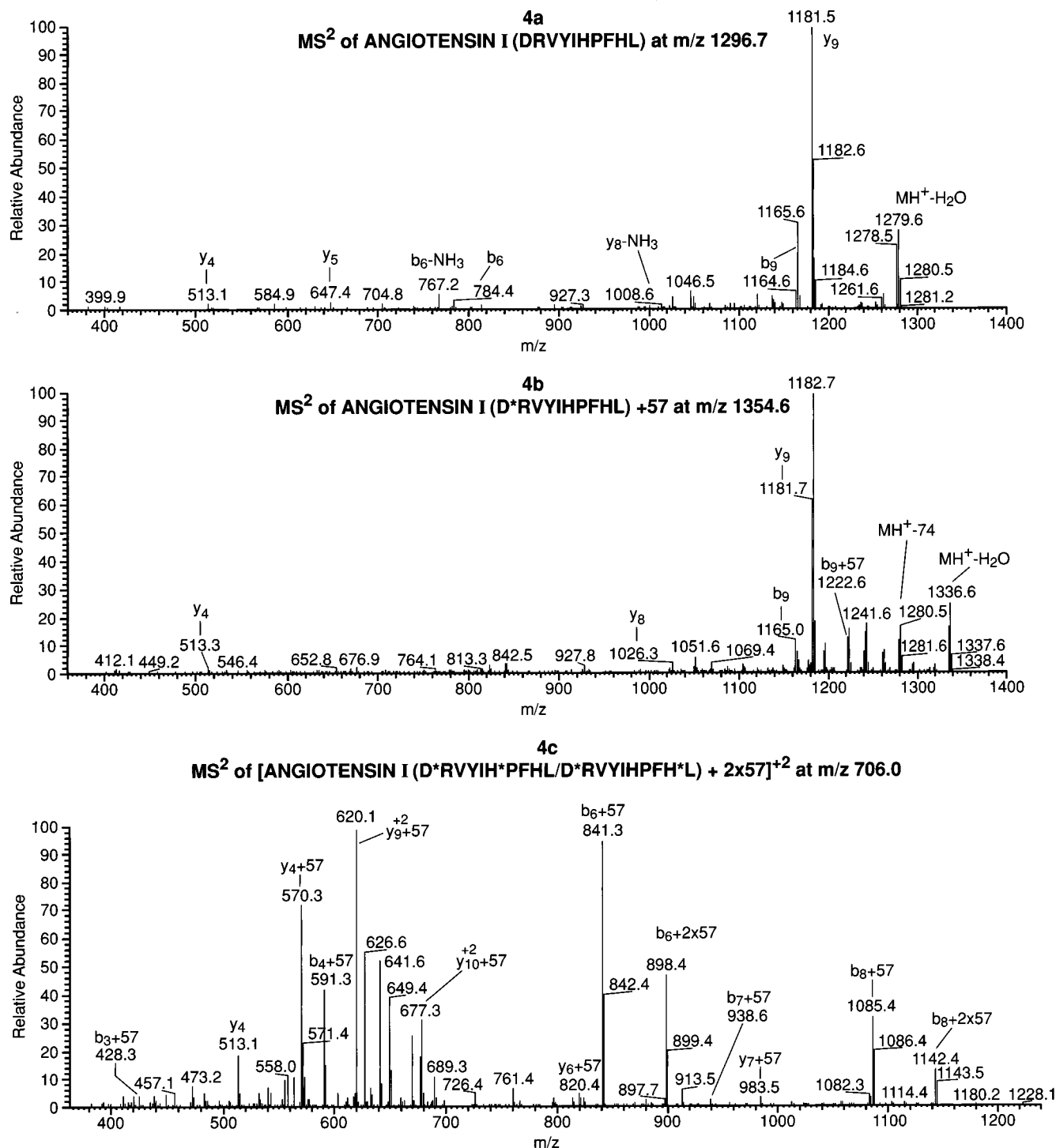


Figure 4. (a) MS² of angiotensin I (DRVYIHPFHL) at m/z 1296.7. (b) MS² of monoalkylated angiotensin I at m/z 1354.6. (c) MS² of doubly charged dialkylated angiotensin I at m/z 706.0.

In the case of the more readily available angiotensin I, DRVYIHPFHL, LC-MS revealed the presence of mono-, di-, and trialkylated derivatives in decreasing order of abundance (chromatogram not shown). Panels b and a of Figure 4 compare the MS² of the monoalkyl derivative with that of the underivatized peptide, respectively, neither of which yields many sequence ions. However, they do suffice to demonstrate that the major portion is alkylated at the N-terminal aspartate, either on the nitrogen or on the carboxylate groups since the most abundant y_9 ion and

$y_9 - \text{NH}_3$ are at the same mass as the unalkylated species. The MH^+ ion also shows the same loss of 74 Da as seen above in an N-alkylated peptide from actin. Here again, the N-terminus is aspartic acid whose side-chain carboxyl may facilitate the reaction. To the extent that this is true, it may be diagnostic for N-terminal aspartic and perhaps glutamic acid as well.

The MS² of the singly charged dialkylated ion at m/z 1410.7 refused to fragment to any degree, but the spectrum of the doubly charged ion at m/z 706.0 (Figure 4c) suggests the presence of

Table 1. Summary of Peptides Analyzed by LC–MS^{2a}

peptides	MH ⁺		
	unalkyl	monoalkyl	dialkyl
D*SYVGDEAQS	1198.6	1255.6	
A*GFAGPDAPR	976.5	1033.6	
I*LTER	631.7	688.7	
V*GDE	419.1	476.3	
Ac–VGD* ^a E* ^b	461.1	518.1	
P*EQEC*EQQQK	1246.6	1303.5	1360.6
D*RVYIH* ^c PFH* ^d L	1296.7	1354.6	(1410.7) ²⁺ /706.0

^a All amino acid residues found to be alkylated with IAM are labeled with asterisks (*) at their top right corner. O-Monoalkylation in the N-acetylated peptide VGDE occurred at either Asp-3*^a or Glu-4*^b. Dialkylation in peptide DRVYIHPFHL occurred at either Asp-1 and His-6*^c or Asp-1 and His-9*^d.

two species at this mass. The ions at y_4 , $b_3 + 57$, $b_4 + 57$, $b_6 + 2 \times 57$, $b_7 + 57$, and $b_8 + 2 \times 57$ at m/z 513.1, 428.3, 591.3, 898.4, 938.6, and 1142.4, respectively, show that the N-terminal aspartate nitrogen is monoalkylated together with the histidine at position 6. Alternatively, $y_4 + 57$ at m/z 570.3 shows the presence of a second species where monoalkylation of the histidine at position 9 occurred. The singly charged $y_7 + 57$ and the doubly charged $y_9 + 57$ and $y_{10} + 57$ ions at m/z 983.5, 620.1, and 677.3, respectively, show that an additional species with both histidine residues involved is not present. Here again, MS² is essential when histidine is involved since even that residue is known to be able to doubly alkylate forming a quaternary salt.^{4b} All of the above overalkylations are summarized in Table 1 where the alkylated site is indicated by an asterisk.

Since overalkylation can occur easily at near-neutral pH under these conditions, we sought a remedy that did not involve thiol reduction of protein-bound glutathione in our actin experiment. The commercially available 2,2'-thiodiethanol, recommended by Moore and Stein for the protection of methionine from oxidation,¹² was an obvious choice since the excess IAM would be expected to react with this thioether forming a sulfonium salt instead of attacking the nucleophiles on actin and its peptides. Indeed, the LC–MS of an actin alkylation preparation to which 2,2'-thiodiethanol had been added showed the disappearance of all 57-Da satellites; only the unalkylated peptides were detected (data not shown).

CONCLUSIONS

In the above examples, it is seen that overalkylation can occur when the reaction is run at pH 7.0–7.5 for longer periods of time. Reaction occurs at sites of relatively low basicity such as terminal amino groups and histidines in preference to lysine side-chain

amino groups, and under certain conditions, this reaction may even take place in preference to cysteine. Nor is it clear that merely lowering the pH will prevent this N-alkylation, although the more basic lysine side chains do seem to be protected at the usual pH of 7–8.5. Since raising the pH to higher values will greatly accelerate the alkylation of cysteine through formation of the very nucleophilic RS[−], one might expect their alkylation still to exceed that of the lysine nitrogens, particularly if the contact time is more limited and this is usually the case. Generally, when these alkylation experiments are run on macroquantities of intact proteins, one can carefully adjust the amount of IAM and reductant to avoid overalkylation. However, when it is a matter of running such reactions on a protein band isolated from a PAGE gel, such material balance is difficult to achieve and no recipes to avoid this problem seem suited for all purposes. As mentioned above, in one experiment, we ran the alkylation in NH₄HCO₃ buffer in an attempt to prevent N-alkylation through competition by the NH₃, but this was clearly not successful, possibly due to the higher pK_a of ammonia (9.2). Another, more direct approach, would be to use a buffer of glycylglycine or another amine whose pK_a is around 7.0 such as imidazole. None of the examples shown above contained methionine but that residue is also easily alkylated.^{13,14} If this occurs, it may be easy to detect since, as with dialkylated histidine, a charge is introduced with the reaction changing the parity of the ion and perhaps helping in its location. Fortunately, peaks at M + 57 from extraneous sources do not appear to occur very often during electrospray mass spectrometry¹⁵ so the excess alkylation should be relatively easy to detect. Of course, in peptide and protein work, one must always be aware that glycine also has this residue mass. We have shown that in cases where reducing agents such as thiols must be avoided, 2,2'-thiodiethanol (and presumably, other thioethers) can serve to consume excesses of IAM and help to prevent this confusion. Considering the importance of glutathionylation in response to oxidative stress in the cellular environment,^{16,17} this approach may find future application.

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