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# Chemical Deglycosylation Can Induce Methylation, Succinimide Formation, and Isomerization

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Interpretation of deglycosylation studies relies heavily on the absence of modifications to the polypeptide chain. We have found that by using a common chemical deglycosylation technique, one can effect at least three changes in a peptide's structure: methylation, isomerization, and ring formation. It was determined that the conditions of chemical deglycosylation introduce a +14 Da shift in the masses of our model peptides, RKDVY, RKEVY, and horseradish peroxidase. This shift is localized to acidic functional groups and is interpreted as methylation of the free carboxylates in our models. An additional shift in mass of −18 Da is found in the model peptide RKDVY consistent with the loss of water associated with succinimide ring formation in this peptide. Chemical treatment induced isomerization of aspartyl residues to isoaspartyl residues in another model peptide, tetragastrin. These results indicate that one should use caution when interpreting the results of chemical deglycosylation experiments.

**KEY WORDS:** Isoaspartate; chemical deglycosylation; methylation; succinimide formation.

## 1. INTRODUCTION

Deglycosylation of glycoproteins is a widely practiced technique used to elucidate the function of carbohydrate moieties on polypeptide chains. The carbohydrate chain may play a role in the resistance of a glycoprotein to proteolytic degradation (Sareneva *et al.*, 1995), transduction of information between cells (Sacchettini *et al.*, 2001), and intracellular adhesion through specific receptor interactions (Ono *et al.*, 2000). When using deglycosylation to attribute the above functions solely to the carbohydrate, one assumes that the deglycosylation process splits the protein from the carbohydrate component without impairing the physicochemical integrity of the polypeptide chain.

Deglycosylation can occur via enzymatic or chemical methods. Enzymatic deglycosylation involves the incubation of the glycoprotein with exo- and/or endoglycosidases (Thotakura and Bahl, 1987). One of the disadvantages of this method is the extensive incubation periods with numerous enzymes to obtain near complete deglycosylation. Chemical deglycosylation is an attractive alternative to enzymatic deglycosylation because the reaction occurs relatively quickly and, in most cases, results in the complete removal of carbohydrates (Sojar and Bahl, 1987). Chemical deglycosylation is typically carried out in the presence of either trifluoromethanesulfonic acid (TFMS)[4] (Tams and Welinder, 1995) or hydrofluoric acid (HF) (Axelsson *et al.*, 1998). Both reagents were initially used to remove the protecting groups from synthetic peptides (Thompson *et al.*, 1995; Lebl *et al.*, 1999). TFMS has been shown to be superior because it is more potent and does not require any special handling.

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[4] Abbreviations: CID, collision induced dissociation; DTT, dithiothreitol; HF, hydrofluoric acid; HFBA, heptafluorobutyric acid; HRP, horseradish peroxidase; MS, mass spectrometry; PAGE, polyacrylamide gel electrophoresis; SAM S-adenosyl-L-methionine; SDS, sodium dodecyl sulfate; TFMS, trifluoromethane sulfonic acid; Tris, tris[hydroxymethyl]aminomethane.



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Our data suggest that chemical deglycosylation using established protocols (Sojar and Bahl, 1987) or commercial kits (Glyko Inc., 2001) may introduce numerous unanticipated modifications in the polypeptide chain via acid catalyzed methylation, succinimide formation, and the conversion of aspartyl residues to isoaspartyl residues.

## 2. MATERIALS AND METHODS

### 2.1. Chemicals and Reagents

Tris[hydroxymethyl]aminomethane (Tris), dithiothreitol (DTT), ammonium bicarbonate, and model peptides were obtained from Sigma (St. Louis, MO). Acetonitrile was from Mallinckrodt Laboratory Chemicals (Phillipsburg, NJ). All other reagents were of the highest quality commercially available.

### 2.2. Deglycosylation of Peptides and Protein

Arg-Lys-Asp-Val-Tyr (RKDVY), Arg-Lys-Glu-Val-Tyr (RKEVY), tetragastrin, and horseradish peroxidase (HRP) (Sigma, St. Louis, MO) were deglycosylated using the GlycoFree® chemical deglycosylation kit (Glyko Inc, Novato, CA). Stock solutions of peptide and protein at 1 mg/mL were lyophilized to complete dryness. Reactions were carried out in duplicate with 50 μL of TFMS added to one of each sample and water added to the other as a negative control. Samples were placed on dry ice for 20 s and moved to −20°C for 4 h, after which time the reaction was stopped by adding 150 μL of methanol. Subsequent to incubation on dry and wet ice, respectively, 400 μL of 0.5% ammonium bicarbonate were added. All samples were then stored at −80°C until further use.

### 2.3. Electrophoresis

HRP was run on SDS-PAGE (Laemmli, 1970) 4%–20% linear gradient minigels (Bio-rad, Hercules, CA). SDS-PAGE gels were silver stained according to the method of Blum *et al.,* (1987) or stained with Coomassie Blue according to established methods.

### 2.4. Tryptic Digests

Untreated and deglycosylated HRPs were digested in solution with trypsin (Promega, Madison, WI) (Covey, 1998.). Briefly, both samples were denatured and reduced in 8 M urea, 10 mM DTT for 30 min at 37°C. Iodoacetamide and Tris, pH = 8.0, were added to the samples to a final concentration of 25 mM and 10 mM, respectively, and incubated in the dark at 37°C for 1 h. Samples were then dialyzed into 100 mM ammonium bicarbonate, pH = 7.5, using Slide-A-Lyzer® mini dialysis units with a 3500 molecular weight cut off (Pierce, Rockford, IL). TPCK-trypsin was added at 1:50 w/w. The samples were left at room temperature for 8 h, at which time another aliquot of trypsin was added and the reaction was continued overnight.

### 2.5. Chromatography

Peptide reaction products were run on a model 140C microgradient HPLC (Applied Biosystems, Forster City, CA). A linear 0 to 100% gradient from water to acetonitrile with 0.02% heptafluorobutyric acid (HFBA) and 0.2% acetic acid as the ion pairing agents was used to separate the reaction products on a HIASIL 300 C18 5 micron analytical column (Higgins Analytical Inc., Mountain View, CA). Peaks were collected and lyophilized to dryness on a Savant speed-vac (Savant Instruments Inc., Holbrook, NY) and reconstituted in 50% methanol, 5% acetic acid for electrospray mass spectrometry.

### 2.6. Mass Spectrometry (MS)

Peptides were analyzed on an ion trap LCQ mass spectrometer (Finnigan, San Jose, CA). Peptides were infused at 3 μL/min via the electrospray interface. Mass spectra were acquired over a mass range of 150 to 2000 D in MS mode. Peptides were selected to undergo collision-induced dissociation (CID) with collision energies from 30% to 60%. The solution digested tryptic peptides were analyzed by fractionation on a capillary LC column connected online to the LCQ through a microelectrospray interface (Gygi *et al.,* 2000). Peptides were concentrated on a micro C18 column (100 μm internal diameter, 12 cm long). Separation was accomplished by applying a gradient from 5% to 65% (A: 0.2% acetic acid and 0.005% HFBA, B: 80% acetonitrile in A) over 20 min. The gradient was delivered by a 140C microgradient HPLC system. Ion signal above a predetermined threshold automatically triggered the instrument to switch from MS to MS/MS mode, generating CID spectra. To determine the locale of modifications (methylation and ring formation), tandem mass spectra were searched against the protein database by using the Sequest search program (Eng *et al.,* 1994).

### 2.7. Detection of Isoaspartyl Residues

Radioactive detection of L-isoaspartyl residues was performed using the Isoquant® isoaspartate detection kit (Promega, Madison, WI). [Methyl-$^3$H]-S-adenosyl-L-methionine (SAM) stock solution was prepared with unlabeled SAM at a final concentration of 0.1 mM and [$^3$H] SAM at a final activity of 0.1 microCi. A master mix was prepared by combining equal amounts of water, 5× reaction buffer, isoaspartyl methyltransferase and [$^3$H] SAM stock solution. Isoaspartyl delta sleep-inducing peptide provided with the kit was used as the reference standard. 40 μL of master mix were added to 10 μL of each of the reactants to be tested and incubated at 30°C for 30 min. The reaction was quenched by adding 50 μL of stop solution. Fifty μL of reaction product was added to a sponge insert placed into the cap of a scintillation vial half filled with ReadySafe® liquid scintillation cocktail for aqueous samples (Beckman-Coulter, Palo Alto, CA). Scintillation vials were incubated in a 37°C oven for 1 h, the caps were removed from the vials, and scintillation counts taken with a Multi-Purpose Scintillation Counter LS 6500 (Beckman-Coulter, Palo Alto, CA). Base radioactivity was obtained using the values from the negative controls.

## 3. RESULTS

### 3.1. Detection of Methylation

Mass spectrometric analyses of deglycosylated, C18 fractionated RKDVY, RKEVY, tetragastrin, and HRP were used to test for methylation. As a model for chemical deglycosylation of a protein, HRP was treated with the GlykoFree chemical deglycosylation kit and run out on a gel to verify successful removal of the carbohydrate moeties (Fig. 1). Mass shifts of 14 atomic mass units (loss of –H with simultaneous gain of -$CH_3$) above those predicted for native peptides confirmed methylation may be occurring in both the deglycosylated RKDVY and RKEVY (Fig. 2, Panel A). Fragmentation of the modified ions indicated that the alteration was occurring at either the aspartyl or the glutamyl sidechains or at the carboxy terminus of the peptide (Fig. 2, Panel B) consistent with the idea that the free carboxylates are being modified. 28 Da molecular weight shifts were also seen in the peptide models (Fig. 2, Panel C) and upon fragmentation were determined to be doubly methylated species. No changes were seen in the deglycosylated tetragastrin by mass spectrometry. Modifications to HRP were detected by different methods than those used on the peptides due to the inherent difficulties in directly analyzing large polypeptides by mass spectrometry. Using this methodology, three instances of methylation were found in the model protein: two modified glutamyl residues as well as methylation at the carboxy terminus of the protein (data not shown).

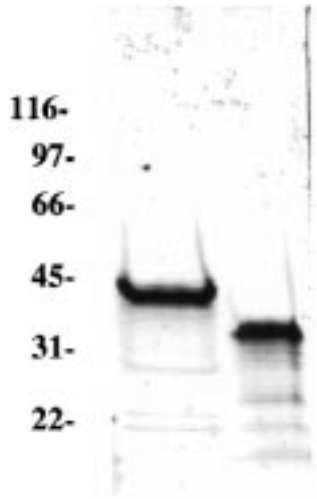


**Fig. 1.** Chemical deglycosylation of model protein. 1 μg of HRP and 1 μg of chemically deglycosylated HRP were run out on a Biorad 4%–20% gel and silver stained. Chemically deglycosylated HRP (right lane) shows an approximate 10,000 Da loss in mass compared with untreated HRP (left lane). HRP, horseradish peroxidase.

### 3.2. Detection of Succinimide Ring Formation

Mass spectrometric analysis of the deglycosylated peptides was also used to check for the formation of succinimide rings. Succinimide ring formation proceeds via the loss of a water with an expected apparent loss of 18 Da. This shift was observed only in the deglycosylated RKDVY (Fig. 2, Panel A) at the aspartyl residue consistent with the expectation that glutamyl-residues cannot form a stable ring in the peptide chain. Fragmentation of this ion confirmed that the 18 Da loss was on the aspartyl residue. No evidence for succinimide ring formation was found in deglycosylated RKEVY or tetragastrin. Deglycosylated HRP was analyzed for the presence of succinimide ring formation by using the amino acid sequence for HRP and looking for 18 Da mass shifts of predicted tryptic fragments. No evidence for succinimide ring formation was found in deglycosylated HRP.

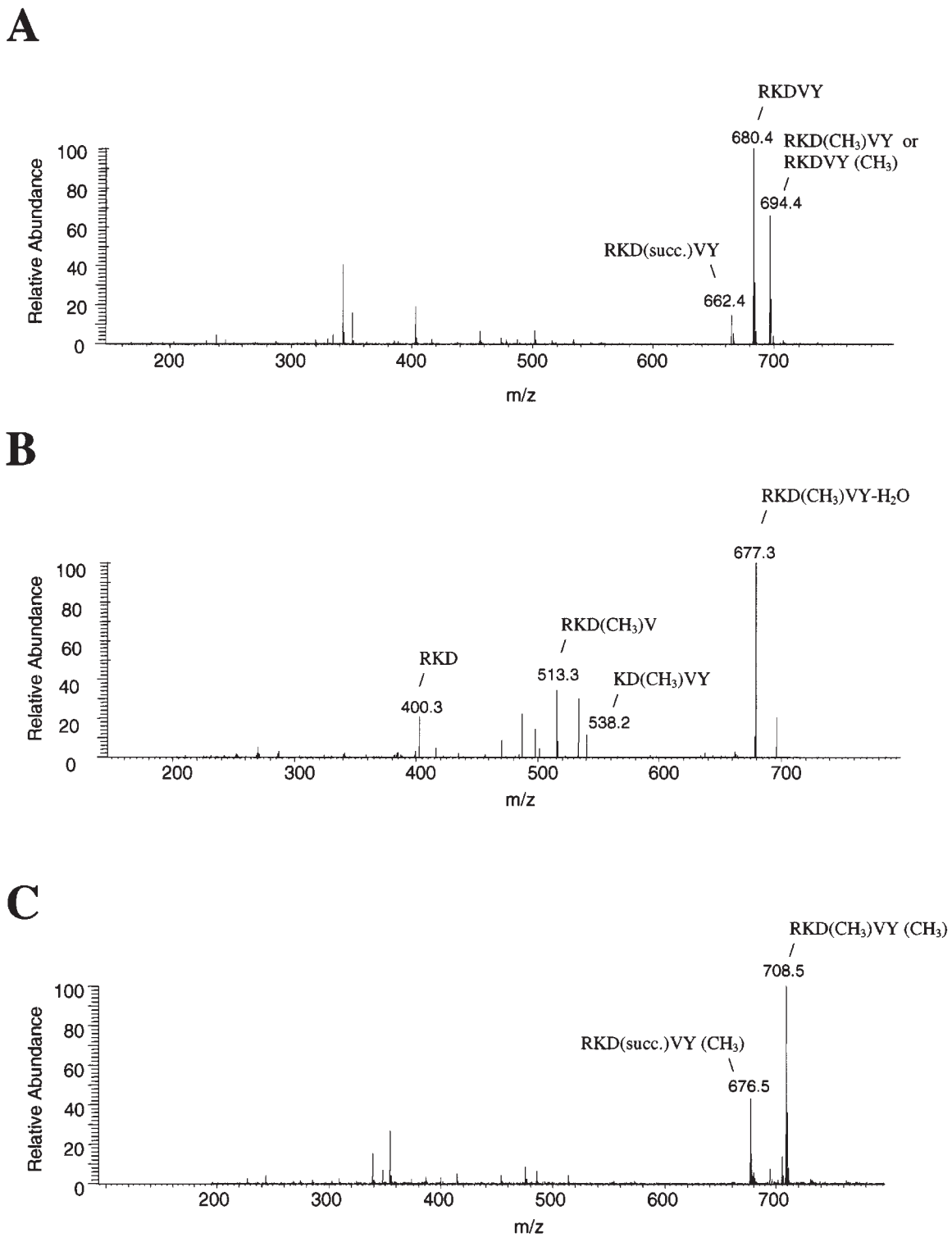


**Fig. 2.** Methylation and succinimide formation in model peptides. **A:** Full MS spectra from peak 1 of C18 separated, deglycosylated RKDVY: ion of m/z 680.4 corresponds to unmodified form of RKDVY. Ion at m/z of 662.4 corresponds to the succinimide form of RKDVY and the ion at m/z of 694.4 corresponds to either aspartic acid methylated or carboxy terminal methylated RKDVY. **B:** MS/MS spectra of 694.4 ion: ion of m/z of 538.2 corresponds to the $y_4$ ion of aspartic acid methylated RKDVY. Ion at m/z of 513.3 corresponds to the $b_4$ ion of aspartic acid methylated RKDVY and ion at m/z of 400.3 corresponds to the $b_3$ ion of carboxy terminal methylated RKDVY. **C:** Full MS spectra from peak 2 of C18 separated, deglycosylated RKDVY: Ion at m/z of 708.5 corresponds to the doubly methylated form of RKDVY and ion at m/z of 676.5 corresponds to the carboxy terminal methylated succinimide ring form of RKDVY.

### 3.3. Detection of Isoaspartyl Residues

Isoaspartyl formation was determined using the Isoquant® isoaspartate detection kit as described in Materials and Methods. Model peptides were treated with TFMS or mock-treated. The positive control peptide included in the kit gave consistent counts of 11,000 CPM above background. Deglycosylated RKDVY, RKEVY, and HRP were found to have $^{3}H$ methanol release levels indistinguishable from the background. Deglycosylated tetragastrin had highly elevated counts, indicating that a large portion of aspartyls in this peptide had been converted to isoaspartyls by the chemical treatment (Fig. 3).

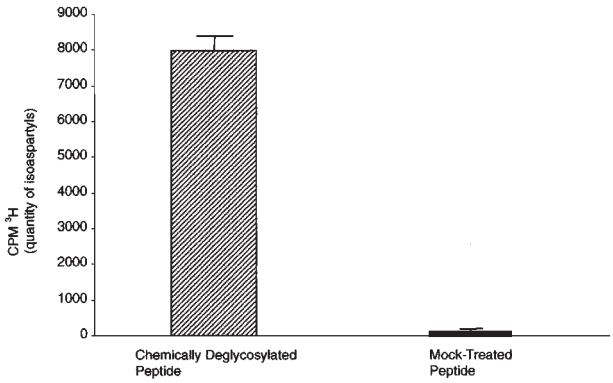


**Fig. 3.** Chemical deglycosylation treatment induces isoaspartyl formation. Isoaspartyl content of chemically deglycosylated tetragastrin was monitored using the Isoquant isoaspartyl determination kit. The counts obtained for chemically deglycosylated tetragastrin were highly elevated with respect to the mock-treated tetragastrin.

## 4. DISCUSSION

Using our model peptides and proteins, we have established that the use of chemical deglycosylation may cause significant levels of methylation, succinimide ring formation, and aspartyl isomerization in any polypeptide. The levels of each modification may be dependent on the local environment of the modified free carboxylate. Thus, there were no detectable amounts of isoaspartyl residues in the deglycosylated RKDVY, RKEVY, and HRP. The converse was seen in the model peptide tetragastrin. This may be explained by the proposed reaction mechanism (McFadden and Clarke, 1986) (Fig. 4). Methylation and succinimide ring formation found in deglycosylated RKDVY, RKEVY, and HRP are intermediates in the reaction process, which may be stable under the conditions and amino acid environments found in these model peptides. If these are stabilized, one would expect to see the

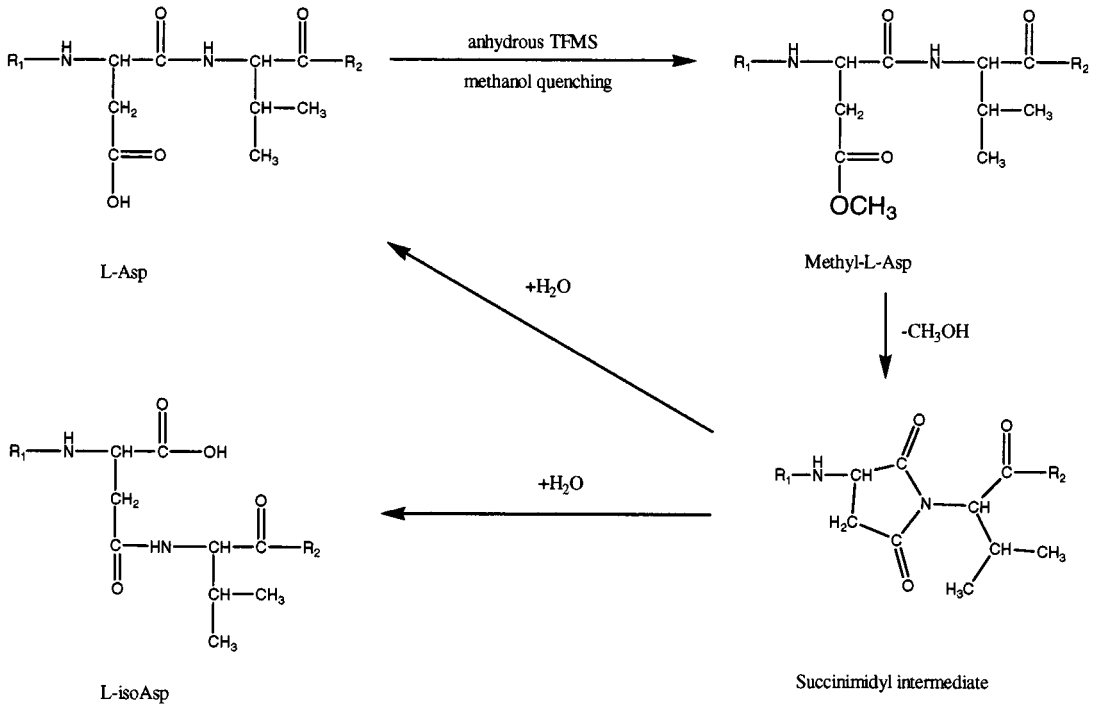


**Fig. 4.** Proposed reaction mechanism for the formation of methyl-, succinimidyl, and isoaspartyl residues. The formation of methylated intermediates, succinimide, and isoaspartyl is induced via an acid catalyzed esterification at the free carboxylates of aspartate.

accumulation of intermediates with little isoaspartyl end product. Likewise, the physicochemical properties of tetragastrin may destabilize the intermediates and favor the conversion of aspartic acid to isoaspartic acid upon chemical treatment. This is consistent with undetectable levels of methyl- and succinimidyl-tetragastrin accompanied by high levels of isoaspartyl tetragastrin. All peptides we have studied have shown some form of modification—methylation, succinimidyl formation, or aspartyl isomerization—indicating that it is likely that, under the conditions commonly used for chemical deglycosylation, the polypeptide portion of any glycoprotein will be modified in an undesirable manner.

It is important to bear in mind that changes in polypeptide structure subsequent to chemical deglycosylation can lead to changes in a protein's properties. Alteration of catalytic sites by loss of charges may lead to changes in both binding and catalytic constants of an enzyme. Chemical modifications which occur within an immunological epitope, or induce conformational changes which affect such an epitope, may bring about changes in the properties of an antigen. Due to these modifications, chemically deglycosylated proteins may not reflect native scenarios and the experimental conclusions drawn from such studies may be based on side-reactions occurring during the deglycosylation process. This means that the loss or gain of function observed with chemical deglycosylation may be ascribed to the loss of the carbohydrate moiety, when in fact it reflects a modification of the polypeptide chain.

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