Nonenzymatic Glycosylation of Albumin in Vivo

IDENTIFICATION OF MULTIPLE GLYCOSYLATED SITES*

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Nonenzymatic glycosylation of albumin in vivo occurs at multiple sites. Glucose gets attached to Lys-199, Lys-281, Lys-439, and Lys-525 as well as to some other lysine residues. The principal glycosylated site is Lys-525. Approximately 33% of the overall glycosylation occurs at this site. This site specificity is remarkable and is postulated to be a consequence of local catalysis of the nonenzymatic glycosylation reaction. It appears that positively charged amino groups in the protein catalyze the Amadori rearrangement at specific sites. The principal glycosylated site, Lys-525, lies in a Lys-Lys sequence; other glycosylated sites lie in a Lys-Lys, Lys-His, and Lys-His-Lys sequence or are near disulfide bridges, which are likely to place amino groups of more remote parts of the protein closer to these sites. The occurrence of nonenzymatic glycosylation at most of the identified sites in albumin from diabetic patients is explained by the concept of local acid-base catalysis of the Amadori rearrangement.

Enhanced nonenzymatic glycosylation of proteins has been claimed to be of relevance in the development of some complications in diabetes mellitus (1-3). Because the glycosylation reaction is nonenzymatic in nature, it has been expected to occur generally with all kinds of proteins, but preferentially at primary amino groups with a low pK value. Although the occurrence of this reaction with a variety of proteins in vivo has been demonstrated, its apparent specificity has remained unexplained. The prediction that the reaction should occur at amino groups with a low pK value is supported by one example only: the glycosylation of the NH₂ terminus of the β-chain in hemoglobin which yields HbA1c (4). In both albumin (5-7) and hemoglobin (4), glycosylation generally occurs at sites irrespective of their pK value.

In RNase A, which was incubated with glucose, glycosylation was also found to occur at lysine residues irrespective of their pK value (8). It was realized that glycosylation occurs predominantly at lysine residues which are close to another amino group, and we hypothesized that acid-base catalysis of the Amadori rearrangement (9) by these close amino groups causes site specificity.

In this report we describe the results of our attempt to validate this concept for the in vivo glycosylation of proteins by elucidating the glycosylated sites in albumin. In addition to the principal glycosylated site in albumin identified by Garlick and Mazer (7) we identified several minor glycosylated sites. All these sites are likely to be close to charged amino groups that can act catalytically.

EXPERIMENTAL PROCEDURES

Materials—The affinity support Blue Trisacryl was a product of LKB (Bromma, Sweden), and the boronate affinity support Affi-Gel 601 was purchased from Bio-Rad. The gel filtration resins Sephadex G-15, Sephacryl S-200, and Trisacryl GF-05 were obtained from Pharmacia (Uppsala, Sweden) and LKB (Bromma, Sweden), respectively. [3H] Sodium borohydride was purchased from Amersham (Amersham, Buckinghamshire, UK).

Isolation of Albumin—Blood was obtained from a diabetic patient, in poor glycemic control, and anticoagulated with EDTA. The plasma was collected and stored at −70°C until used. Before application to the affinity column, 3-ml sample aliquots were filtered and passed over a Trisacryl GF-05 column, equilibrated with the application buffer of the affinity column. The plasma proteins were then applied to a column (diameter 1.6 cm) packed with 45 ml of Blue Trisacryl (10, 11), equilibrated with application buffer (0.05 M Tris/HC1, 0.5 M NaCl, pH 8.0), and the column was washed with 75 ml of application buffer at a flow rate of 30 ml/h. The adsorbed albumin was eluted with 2.5 M NaCl in 0.05 M Tris/HC1. Albumin from three runs was combined and concentrated to a volume of 16 ml in an Amicon filtration system. This yielded 330 mg (5 μmol) of albumin.

Labeling of the Glycosylated Sites—In order to label the glycosylated sites, the ketoamine adduct in albumin was reduced with [3H] labeled NaBH₄ (12). A 100-fold excess of radioactive NaBH₄ (20 mCi/mmol) was slowly added and the reaction allowed to proceed for 1 h on ice at pH 8.0. The reaction was stopped by the addition of acetic acid to a pH of 3.0.

Denaturation, Disulfide Cleavage, and Alkylation—Guanidinium/HCl and Tris/HCl were added to the above solution to yield a volume of 50 ml and a concentration of 6 M guanidinium/HCl and 0.5 M Tris/HCl. The pH was adjusted to 6.2. After 90 min at 50°C, 100 mg of dithiothreitol (5 mg/10 mg albumin) were added and disulfide cleavage allowed to proceed for 1 h (13). The solution was cooled to room temperature, 750 mg of iodoacetate (25 mg/10 mg albumin) were added (14), and after 1 h in the dark dialyzed extensively against 0.5% ammonium acetate. The solution was concentrated to 12 ml by Amicon ultrafiltration. 240 mg of alkylated and labeled albumin were obtained.

In order to isolate monomeric albumin, approximately 120 mg of the above material were passed over a column (diameter 2.2 cm) packed with 300 ml of Sephacryl S-200.

Tryptic Digestion—Tryptic digestion was performed with 90 mg of albumin. To destroy potential peptide activity, the albumin solution was heated for 5 min to 100°C. 1.6 mg of trypsin, purified by passage over Glycocel E, was added. Digestion was allowed to proceed for 8 h at 37°C and stopped by brief heating to 100°C. Experience showed that during digestion turbidity developed. This insolubility does not occur if digestion is performed in 2 M guanidinium/HCl in 0.5% ammonium acetate, pH 8.0 (15). The solution of tryptic peptides showed no turbidity nor was there any precipitate.

Isolation of the Tryptic Peptides—The glycosylated and labeled tryptic peptides were isolated by boronate affinity chromatography (16). The tryptic digest was applied to a column (diameter 2.2 cm) packed with 7 ml of Affi-Gel 601, equilibrated with application buffer...
(0.1 M ethylnorpholine, 2 M guanidinium/HCl, pH 9.0). For application, the 26 ml of digest were circulated over the column for 4 h at a flow rate of 50 ml/h. The column was washed with 100 ml of application buffer, and the adsorbed glycosylated peptides were eluted with 0.2 M sorbitol in the application buffer.

It is noteworthy that about 50% of the radioactivity did not bind to the affinity support. This radioactivity represents nonspecific incorporation, because the unbound material did not bind when reapplied. The nature of such nonspecific side reactions has been investigated. It originates from superoxides and radicals which are formed by decomposition of radioactive NaBH₄. These stabilized radicals lead to the reddish appearance of the commercial product.²

Separation of the Glycosylated Peptides—The glycosylated peptides were separated by HPLC,² using the reverse-phase column Aquapore RP-300 (Brownlee) and a detection system for peptides with fluorescamine (17). The column was operated at a flow rate of 50 ml/h. A gradient with buffer A (5% methanol in 0.025 M trifluoroacetic acid) and buffer B (75% methanol in 0.025 M trifluoroacetic acid) was used. For the first 15 min the column was run isocratically in buffer A, followed by 60 min linear gradient elution up to 35% buffer B, 15 min isocratic elution at 35% buffer B, and finally by 30 min up to 100% buffer B.

Lyophilized peptides dissolved in 2 ml of buffer A, corresponding to 2.5 mg of albumin, were applied and fractions of 1 min collected. Radioactivity was measured by liquid scintillation counting (Fig. 1). The pooled fractions with the glycosylated peptides were lyophilized. The estimated amount of the peptide corresponding to the principal glycosylated site (peak H) was 4–10 nmol.

Hydrolysis and Amino Acid Analysis—The composition of the isolated glycosylated peptides was determined by amino acid analysis after hydrolysis in 6 M HCl under N₂ at 110 °C for 24 h. Hydrolysates were lyophilized, dissolved in 0.1% acetic acid, and lyophilized a second time. Amino acid analysis was performed on the Pico Tag System from Waters (18). An estimated 100 pmol of amino acids of peak H, and about 10 pmol of the other peaks, were analysed.

RESULTS

Sequence assignment, based on the compositional data, was attempted by the following criteria. 1) It was assumed that

² R. Kissner, personal communication.
² The abbreviation used is: HPLC, high performance liquid chromatography.

tryptic digestion was complete, i.e. that all the radioactive peaks represented two tryptic peptides not split at the glycosylated lysine residue. 2) Mismatching “dipeptides” were excluded based on the reliably determined amino acids: Glu, Ala, Leu, Phe, Lys, and occasionally also Asp and Pro. 3) Positive sequence assignment required that every reliably determined amino acid in the peptide was detected in approximately the expected amount. “Noise” up to 0.5 residues was tolerated. By these criteria unequivocal assignment was possible for the compositional data of four peaks (Table I).

Due to the small amount of peptides of the minor glycosylated sites, there was considerable noise, and assignment and quantitation was not equally reliable for all peaks. Six other peptides could be assigned if more variability was accepted (Table II). Some radioactive peaks could not be assigned. These nonassigned radioactive peaks correspond to poorly
resolved peaks in the fluorescence monitoring. Interestingly, peaks B and K correspond to the same glycosylated site, a finding that remains unexplained.

**DISCUSSION**

The nonenzymatically glycosylated tryptic peptides of albumin were isolated by boronate affinity chromatography (16) and resolved by HPLC (Fig. 1). This separation showed that at least 10 different lysine residues out of a total of 59 get nonenzymatically glycosylated to some extent. Four glycosylated sites were clearly established: Lys-349, Lys-199, Lys-281, and Lys-525. Evidence for the assignment of five other sites is less certain but consistent with glycosylation at Lys-233, Lys-317, and Lys-439. Glycosylation at Lys-199 accounts for approximately one-third of the overall glycosylation, confirming an earlier report (16) that glycosylation at Lys-199 accounts for only approximately 5% of total glycosylation.

Nonenzymatic Glycosylation

| Peak Sequence | Glycosylated site |
|---------------|-------------------|
| B             | Lys-226-233       |
| C             | Lys-314-323       |
| E             | Lys-349-359       |
| F             | Lys-11-20         |
| G             | Lys-526-536       |
| H             | Lys-226-240       |
| I             | Lys-233           |
| J             | Lys-233           |

**TABLE II**

Amino acid composition of the other glycosylated tryptic peptides

| Amino acid | Composition (theoretical and experimental data) |
|------------|-----------------------------------------------|
| Asp        | 1.6                                           |
| Glu        | 2.1                                           |
| Ser        | 1.0                                           |
| His        | 0.6                                           |
| Thr        | 2.0                                           |
| Ala        | 2.1                                           |
| Tyr        | 1.3                                           |
| Val        | 1.3                                           |
| Cys*       | 1.0                                           |
| Leu        | 2.0                                           |
| Phe        | 0.8                                           |
| Lys        | 1.0                                           |
| Glc-Lys*   | 1.0                                           |

* see Table I.

It has been found that glycosylation of hemoglobin in vivo and in vitro affects different sites (4). This observation has not been explained but may be a consequence of differing reaction conditions or partial denaturation of the protein. In albumin, partial denaturation may explain why the indirect evidence for glycosylation of Lys-199 from in vitro glycosylation did not reveal the principal site glycosylated in vivo.

In summary, the concept of local acid-base catalysis of the nonenzymatic glycosylation reaction explains the site specificity of this reaction in all known instances: albumin, hemoglobin, and RNase A.

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Nonenzymatic Glycosylation of Albumin

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