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Glycation of Amino Groups in Protein

STUDIES ON THE SPECIFICITY OF MODIFICATION OF RNase BY GLUCOSE*

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Ribonuclease A has been used as a model protein for studying the specificity of glycation of amino groups in protein under physiological conditions (phosphate buffer, pH 7.4, 37 °C). Incubation of RNase with glucose led to an enhanced rate of inactivation of the enzyme relative to the rate of modification of lysine residues, suggesting preferential modification of active site lysine residues. Sites of glycation of RNase were identified by amino acid analysis of tryptic peptides isolated by reverse-phase high pressure liquid chromatography and phenylboronate affinity chromatography. Schiff base adducts were trapped with $NaBH_3CN$ and the α-amino group of Lys-1 was identified as the primary site (80–90%) of initial Schiff base formation on RNase. In contrast, Lys-41 and Lys-7 in the active site accounted for about 38 and 29%, respectively, of ketoamine adducts formed via the Amadori rearrangement. Other sites reactive in ketoamine formation included N^{α}-Lys-1 (15%), N^{ϵ}-Lys-1 (9%), and Lys-37 (9%) which are adjacent to acidic amino acids. The remaining six lysine residues in RNase, which are located on the surface of the protein, were relatively inactive in forming either the Schiff base or Amadori adduct. Both the equilibrium Schiff base concentration and the rate of the Amadori rearrangement at each site were found to be important in determining the specificity of glycation of RNase.

Glycation or nonenzymatic glycosylation of protein occurs as the result of a reaction between reducing sugars and the primary amino groups on protein. The initial product is a labile Schiff base derivative of the protein which slowly isomerizes to the more stable ketoamine adduct via the Amadori rearrangement (Fig. 1) (1–3). Although formation of Schiff bases is known to depend on the pK_a (nucleophilicity) of amino groups, much less is known about factors which promote the formation of the Amadori product at specific amino groups in protein. Glycation takes place at ε-amino groups of lysine or hydroxylysine residues as well as at α-amino groups of amino-terminal residues (3). The β-chain terminal valine residue has been identified as a highly reactive site in hemoglobin (3, 4) and specific lysine residues in hemoglobin (5) and human serum albumin (6) have also been identified as preferential sites of glycation of these proteins *in vivo*. In hemoglobin, the most reactive lysine residues appeared to be located adjacent to carboxylate groups in the primary or three-dimensional structure of the protein (5), while in albumin the reactive lysine is adjacent to another lysine residue in the primary sequence (6). With these exceptions, however, there is little information regarding specificity in glycation of protein.

In previous work, we had obtained evidence for a range in rates of glycation of lysine residues in bovine pancreatic RNase[1] (7) and we expected that further study of the reactivity of lysine residues in this protein would help in understanding the specificity in modification of other proteins by glucose. RNase (Fig. 2) (8, 9) contains 10 lysine residues including the amino-terminal residue, for a total of 11 primary amino groups. Two amino groups have low pK_a values, N^{α}-Lys-1 with $pK_a \sim 7.8$ and N^{ϵ}-Lys-41 with $pK_a \sim 8.8$ (8), making them potentially more reactive in formation of the Schiff base, while the remainder of the lysine amino groups with $pK_a \sim 10.5$ should be less reactive. Three lysines (1, 7, and 41) are in or near the active site and modification of any one of these three has been shown to decrease the specific enzymatic activity of RNase (8–10). In this paper, we compare the sites of formation of Schiff base and Amadori adducts to RNase and describe factors which affect the specificity of glycation of amino groups in this protein.

EXPERIMENTAL PROCEDURES

Materials—Bovine pancreatic RNase A (Type XII-B), tosylphenylalanyl chloromethyl ketone-treated trypsin (Type XIII), yeast RNA, sodium borohydride, and sodium cyanoborohydride were obtained from Sigma. Affi-Gel 601 (phenylboronate affinity resin) was purchased from Bio-Rad and β-D-glucose from P-L Biochemicals. $NaB[^3H]_4$ and D-[6-^{3}H]glucose were purchased from New England Nuclear. Radioactive glucose was purified (11) by column chromatography using Dowex 50W-X8 in the Ca^{2+} form (12). The glucose was eluted with deionized water, concentrated to dryness by rotary evaporation, dissolved in incubation buffer, and normally used within 24 h of preparation.

Reaction of RNase with Glucose—RNase (25 mg/ml) was incubated with 0.4 M glucose in 0.1 M sodium phosphate buffer, pH 7.4 at 37 °C. The concentration of native RNase in solution was determined by measuring absorbance at 280 nm ($E_{1\%} = 7.1$) (8). RNase enzymatic activity was measured using yeast RNA as substrate (13). The actual glucose concentration in the incubation was measured by the glucose oxidase-peroxidase assay (Amresco). Reaction mixtures were filtered through 0.2 filters (Gelman) into sterile microfuge tubes to prevent bacterial contamination. Aliquots were removed under sterile conditions at desired times and frozen at −20 °C until assayed.

Reduction of Ketoamine Adducts—Prior to reduction of glycated protein, samples (typically 5 mg RNase) were diluted to 1 ml with 0.2 M sodium acetate, pH 5, and incubated at 37 °C for 2 h to discharge glycosylamine adducts (14). The pH 5-treated protein was then di-

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[1] The abbreviations used are: RNase, bovine pancreatic ribonuclease A; RP-HPLC, reverse-phase high pressure liquid chromatography.

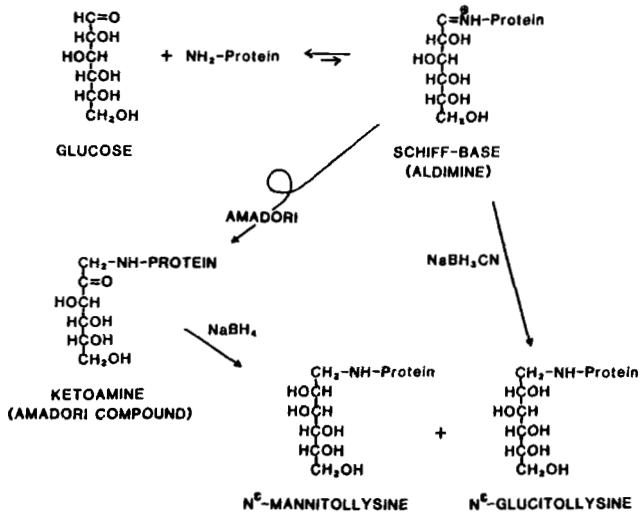

FIG. 1. **Pathway for glycation of protein.** The Schiff base intermediate and the ketoamine (Amadori compound) exist primarily in cyclic furanose and pyranose conformations (2). The Schiff base adduct can be trapped by reduction with cyanoborohydride to N^{ϵ}-glucitollysine, while the ketoamine is reduced by borohydride to a mixture of N^{ϵ}-glucitol- and N^{ϵ}-mannitollysine.

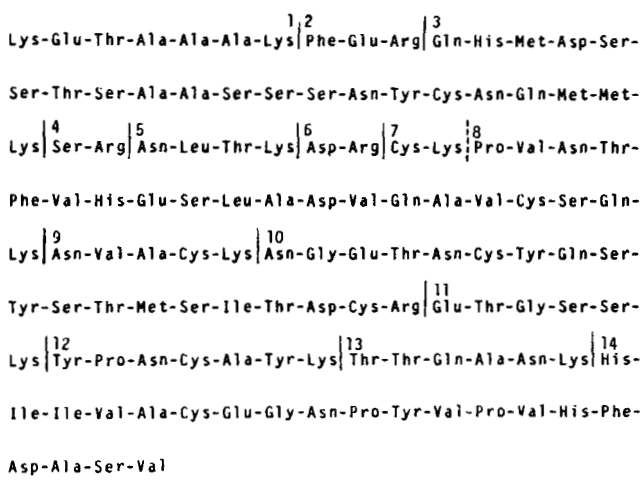

FIG. 2. **Sequence of amino acids (8) and sites of trypsin cleavage (9) of RNase.**

alyzed overnight against 4 liters of 0.1 M sodium phosphate, pH 7.4, at 4 °C to remove glucose. The dialyzed protein was reduced by adding a 50-fold molar excess of NaB[^{3}H]$_4$ (70 μCi/μmol) in 0.1 M NaOH. The pH of the solution was estimated using narrow range pH paper and adjusted as required to pH 8–9 with 1 N NaOH. After 4 h at room temperature, the reaction was terminated by the slow addition of 1 N HCl to destroy excess $NaBH_4$. The samples were then dialyzed overnight at 4 °C against 4 liters of distilled water.

Determination of the Extent of Modification of RNase—An aliquot of reduced RNase was diluted with an equal volume of 12 N HCl and hydrolyzed at 110 °C for 24 h. The sample was then analyzed on a Beckman Model 119C amino acid analyzer. The extent of lysine modification was calculated by comparing the lysine:alanine ratio in glycated *versus* native RNase, using the alanine in RNase as an internal standard.

Quantitation of the Ratio of N^{α}- to N^{ϵ}-Hexitollysine—Aliquots of NaB[^{3}H]$_4$-reduced glycated protein or peptides were hydrolyzed in 6 N HCl at 95 °C for 18 h. These hydrolysis conditions do not yield quantitative hydrolysis of all peptide bonds in RNase, but do yield quantitative recovery of lysine with minimal degradation of hexitollysines (15). The radioactive hydrolysates were chromatographed on a cation exchange column using a Waters HPLC amino acid analyzer. To optimize separation of the lysine derivatives, a complex gradient was generated from two buffers (A: 0.2 N Na^+ citrate, pH 3.5; B: 0.8 N NaCl in 0.2 N Na^+ citrate, pH 7.4; initial conditions: flow rate 0.5 ml/min, 100% A; 50 min, 30% A, linear gradient (Waters Curve 6); 60 min, 0% A, convex gradient (Waters curve 8); 80 min, 0% A. Fractions (3–5 drops) were collected directly into 5-ml scintillation vials and counted in Budget-Solv scintillation fluid (Research Products International). The radioactive peaks were identified by comparison to N^{α}- and N^{ϵ}-glucitollysine and N^{α}- and N^{ϵ}-mannitol lysine standards synthesized as described previously (7).

Peptide Mapping of RNase—Reduced RNase was treated with performic acid (16), evaporated to dryness, and then resuspended in 0.1 M sodium phosphate, pH 8.0. Trypsin (0.1% w/w) was added and, after a 24-h digestion at 37 °C, was inactivated by adjusting to pH 2 with 1 N HCl. The samples were frozen at −20 °C until analyzed. Peptides were separated on a Beckman ODS ultrasphere (reverse-phase) column using a Varian 5020 HPLC. A water-acetonitrile gradient was developed, based on that previously reported by Blackburn *et al.* (17, 18). Buffer A consisted of 0.1% trifluoroacetic acid (Sequanal grade, Pierce) in distilled water and Buffer B was 0.1% trifluoroacetic acid in 50% acetonitrile (HPLC grade, Burdick and Jackson). The column was equilibrated with 4% buffer B at 1 ml/min and the peptides eluted with a combination of isocratic and linear gradient steps. The program was as follows: 0 min, 4% buffer B; 5 min, 4% B; 25 min, 20% B; 45 min, 20% B; 75 min, 40% B; 95 min, 65% B; 100 min, 65% B; 115 min, 70% B. Peptides were detected by their absorbance at 214 nm and the various peak fractions were pooled, dried under nitrogen, and characterized by amino acid analysis. The order of elution of peptides (shown in Fig. 6*A*) was identical with that previously reported by Blackburn *et al.* (17, 18). Glycated peptides, labeled either with radioactive glucose or by reduction with NaB[^{3}H]$_4$, were located by assaying aliquots of column fractions for radioactivity.

Characterization of Glycated Peptides—Separation of glycated from unmodified peptides was achieved by affinity chromatography using phenylboronic acid resin which selectively binds vicinal diols of the reduced glucose residue in glycated peptides. The radioactive fractions eluted from the reverse-phase column were pooled, evaporated under nitrogen, and redissolved in 0.5 ml of 0.025 M sodium phosphate, pH 8.5. The sample was applied to a 2-ml affinity column, equilibrated with 20 ml of 0.025 M sodium phosphate, pH 8.5. The column was washed with 5 ml of starting buffer and the bound glycated peptides eluted with 7 ml of 50 mM acetic acid. Aliquots (0.1 ml) of each fraction (1 ml) were counted, radioactive peaks were pooled, and peptides were identified by amino acid composition.

RESULTS

Kinetics of Modification of RNase by Glucose—RNase was incubated with glucose in order to study both its rate of reaction with glucose and the effect of glycation on its enzymatic activity. In earlier studies (7), we had observed that the rate of glycation of RNase *in vitro* was first order in glucose concentration up to at least 1 M glucose. The present studies were done at 0.4 M glucose for convenience, in order to yield protein with about 1 mol of Glc/mol of RNase within 3 days at physiological temperature and pH. The kinetics of glycation and loss of enzymatic activity are shown in Fig. 3. The initial rate of glycation was approximately 12% lysine modification · $mol^{-1}_{RNase} \cdot M^{-1}_{Glc} \cdot day^{-1}$; however, the rate of modification was nonlinear on the semilog plot, indicating that not all lysines were reacting at the same rate. In addition, the initial rate of loss of enzymatic activity was 42% inactivation · $M^{-1}_{Glc} \cdot day^{-1}$, *i.e.* about 3.5 times the rate of modification of lysine residues. The enhanced rate of inactivation of the enzyme compared to the rate of lysine modification suggested that glycation, *i.e.* formation of the Amadori adduct, was occurring preferentially at active site lysine residues.

When the glycation reaction is carried out in the presence of $NaBH_3CN$, this reagent traps the Schiff base intermediate by reducing it directly to glucitollysine (Fig. 1). Proteins are modified more rapidly in this case because the rate of formation and reduction of the Schiff base is more rapid than that of the Amadori rearrangement (1, 3). As shown in Fig. 4, the initial rate of modification of RNase by glucose in the presence

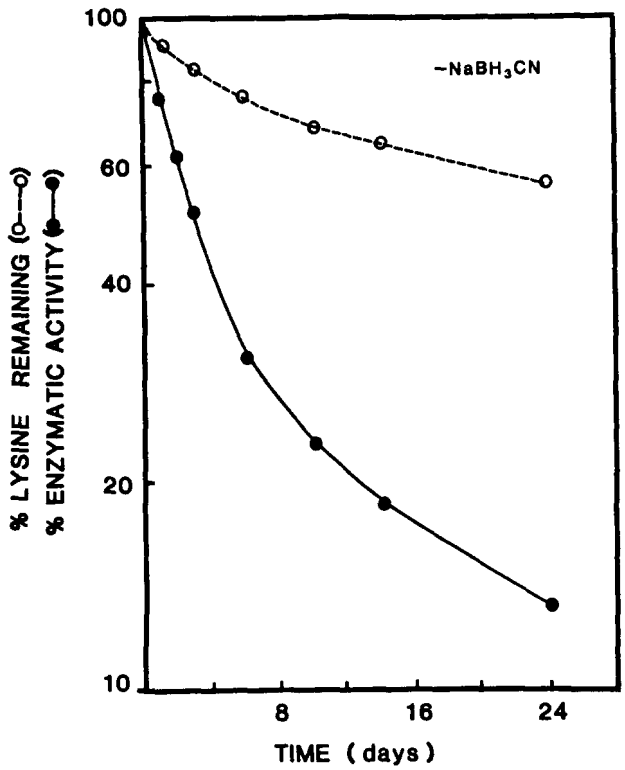

FIG. 3. **Kinetics of glycation and inactivation of RNase by glucose.** RNase (25 mg/ml) was incubated in 0.4 M glucose in 0.1 M sodium phosphate, pH 7.4, at 37 °C. The extent of glycation was determined from the loss of lysine residues as determined by amino acid analysis. The loss of enzymatic activity in control incubations in buffer without glucose was less than 10% at 25 days.

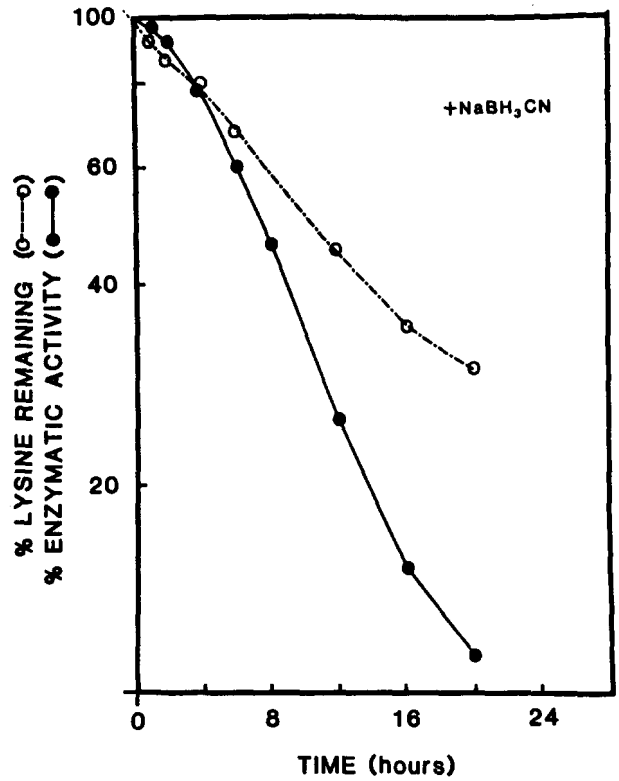

FIG. 4. **Kinetics of glycation and inactivation of RNase by glucose in the presence of $NaBH_3CN$.** RNase was reacted with glucose as described in Fig. 3 except that the incubation also contained 0.4 M $NaBH_3CN$. No loss of enzymatic activity was observed in control incubations of RNase with $NaBH_3CN$ (without glucose) after 24 h.

of a large excess of $NaBH_3CN$ is about 12% lysine modification $\cdot mol^{-1}_{RNase} \cdot M^{-1}_{Glc} \cdot h^{-1}$, *i.e.* more than 20 times faster than the rate of the Amadori rearrangement (Fig. 3). The rate of loss of enzyme activity was 14% inactivation $\cdot M^{-1}_{Glc} \cdot h^{-1}$. Thus, during the early stages of the reaction in the presence of $NaBH_3CN$, before extensive modification of the protein had occurred, the rates of modification of lysine residues and loss of enzymatic activity were similar. In Fig. 5, the loss of lysine residues is compared to the loss of enzymatic activity for protein modified by glucose in the presence and absence of $NaBH_3CN$. In the presence of $NaBH_3CN$, 15% of the enzymatic activity is lost when 20% of the lysine residues are modified; while in the incubation without the reducing reagent, 65% of the activity is lost at the same degree of glucose modification. These data suggest not only that there are differences in the sites of modification of RNase in the presence and absence of $NaBH_3CN$, but also that, in comparison to Schiff base formation, glycation of RNase occurs preferentially at active site lysine residues.

Initial Sites of Formation of Amadori Adducts to RNase—The sites of modification of RNase by glucose were examined by tryptic peptide analysis of RNase modified with 1 mol of Glc/mol of protein, *i.e.* after 3 days incubation with glucose (Fig. 3). In the initial experiments, the peptides were labeled by reaction of RNase with 6-[^{3}H]glucose, followed by reduction with $NaBH_4$. An alternate and less expensive method of labeling the glycated peptide was also evaluated, *i.e.* $NaB[^3H]_4$ reduction of RNase modified with unlabeled glucose. Despite potential problems from nonspecific reduction of cystine and peptide bonds by $NaB[^3H]_4$ (19), the two labeling methods yielded identical radioactive peptide profiles on RP-HPLC (Fig. 6), so long as the reduction reaction was

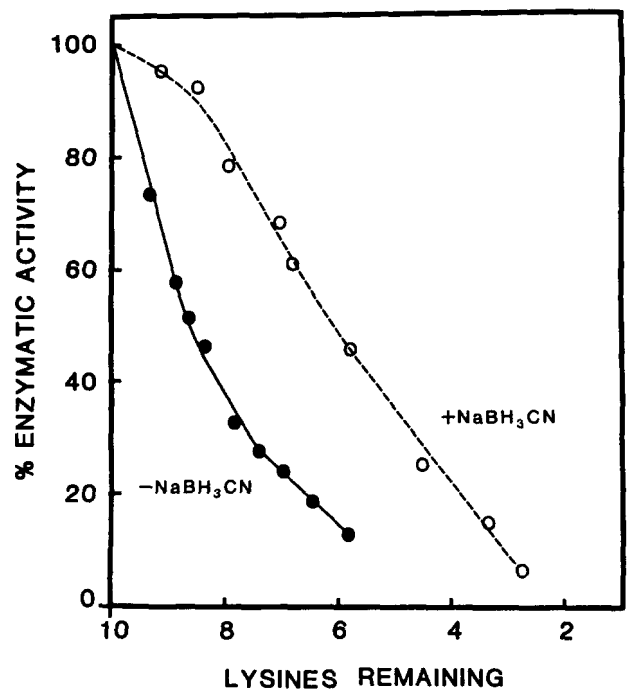

FIG. 5. **Comparative loss of RNase enzymatic activity *versus* lysine modification by glucose in the presence and absence of $NaBH_3CN$.**

maintained at pH 8–9. Higher pH during the $NaB[^3H]_4$ reduction yielded increased incorporation of radioactivity into the protein and a more complex peptide map, resulting in part from incomplete digestion of the protein. Since the

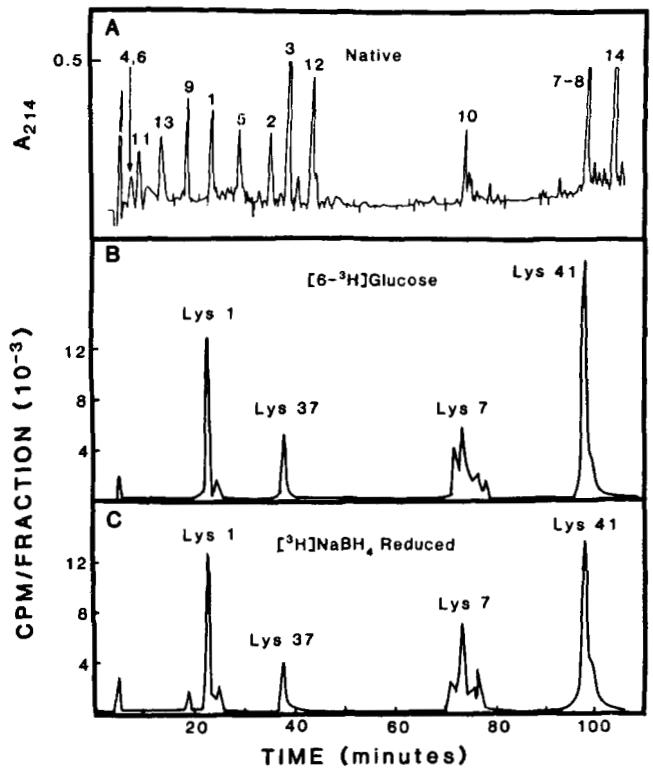

FIG. 6. **Comparison of tryptic peptide maps obtained by RP-HPLC.** *A*, peptide map of native RNase. Peptides are numbered as shown in Fig. 2. *B*, radioactive peptide map of RNase glycated with 6-[^{3}H]glucose, followed by reduction with $NaBH_4$. The modified lysine residue is identified in each peak. *C*, radioactive peptide map of RNase glycated with nonradioactive glucose, then reduced with $NaB[^3H]_4$.

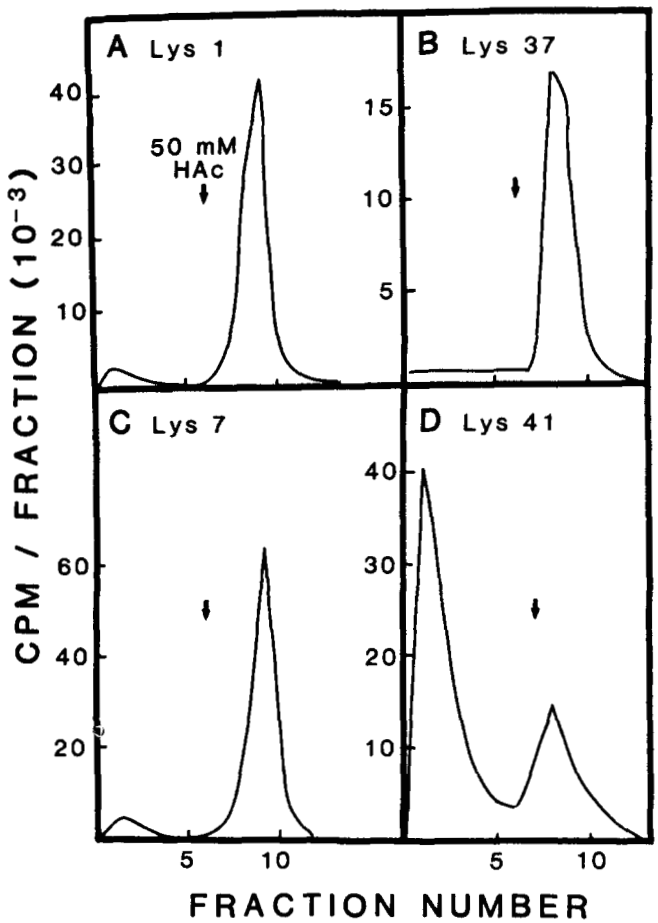

FIG. 7. **Isolation of glycated peptides by affinity chromatography on phenylboronate resin (Table I).** *A*, peptide T_1 containing glycated Lys-1. *B*, peptide T_{5-6} containing glycated Lys-37. *C*, peptide T_{1-2} containing glycated Lys-7. *D*, peptide T_{7-8} containing glycated Lys-41.

results of the two procedures were identical, further characterization of glycated peptides was done using $NaB[^3H]_4$-reduced protein. To identify the peptides, radioactive peaks were pooled from the reverse-phase column and the glycated peptides were isolated by affinity chromatography on phenylboronic acid resin (Fig. 7) and identified by amino acid analysis (Table I).

The first peak, which eluted from the RP-HPLC column at 21 min and contained approximately 24% of the total peptide radioactivity, was identified as glycated T_1, containing lysine residues 1 and 7 (Table II). This peptide was not resolved from the unmodified peptide in our chromatographic system and, since glycation of Lys-7 would have blocked tryptic cleavage at that site (5), Lys-1 must be the site of modification. To determine whether the α- or ϵ-amino group was modified, aliquots of both total protein and glycated peptide T_1 were hydrolyzed and the N^{α}- and N^{ϵ}-hexitollysine derivatives were separated by ion exchange HPLC (Fig. 8). The radioactivity profile from the total protein (Fig. 8*A*) indicated a distribution of 17α N^{α}- and 83% N^{ϵ}-modification of the protein, compared to a distribution of 61% N^{α}- and 39% N^{ϵ}-modification of glycated peptide T_1 (Fig. 8*B*). A trailing peak at 22–23 min was consistently observed in the chromatograms (see Fig. 9 and discussion below) and was identical with T_1 by amino acid composition and the ratio of α- to ϵ-amino group modification. This peak was included in the total of 24% glycated T_1 (Table II). The estimate of α-amino group glycation of the total protein (17%, Fig. 8*A*) was in good agreement with the estimate of 15% α-modification based on 61% α-modification of peptide T_1 (Fig. 8*B*) which accounted for 24% of glycated peptides.

The second peak of radioactivity, eluting from the RP-HPLC column at 36 min, contained 9% of the total radioactivity (Table II). This peptide was identified as T_{5-6}, residues 34–39 (Table I), containing Lys-37. The glycated peptide eluted approximately 9 min later than the native peptide which contains Lys-37 (T_5, residues 34–37, at 27 min).

The third peak at 73 min contained 29% of total radioactivity and was identified as T_{1-2}, containing residues 1–10. This peptide contains 2 lysine residues (1 and 7), with Lys-7 being modified. Amino acid analysis of aliquots taken from across the peak were identical indicating that even though the peak appeared heterogeneous by RP-HPLC, it was essentially homogeneous in amino acid composition. As indicated above, a satellite peptide was also observed following peptide T_1 and was indistinguishable from T_1 by amino acid analysis. Some heterogeneity was also observed for many of the peptides in the native protein (Fig. 6*A*), suggesting that these peaks were probably artifacts or ghost peaks generated by nonuniform sample loading or transport on the HPLC column. The fourth peak at 97 min, with 38% of total radioactivity, contained the most reactive site of glycation of RNase. This peptide was unusual because typically only about 35% of the applied radioactivity was retained on the phenylboronic acid affinity column (Fig. 7*D*). This behavior was reproducible and a fraction of the unbound peptide would bind to the phenylboronic acid column on reapplication. Similar results were obtained with this peptide prepared from incubations of

TABLE I

Identification of glycated peptides by amino acid analysis

Amino acid[b]	Peptide identification							
	T_1 (21)[a]		T_{5-6} (36)		T_{1-2} (73)		T_{7-8} (97)	
	Observed	Theoretical	Observed	Theoretical	Observed	Theoretical	Observed	Theoretical
Asx			2.1	2	0.5		2.0	2
Thr	1.2	1	1.1	1	1.5	1	0.8	1
Ser							1.9	2
Glx	1.1	1	0.5		2.1	2	2.1	3
Gly							0.7	
Ala	3.0*	3	0.5		3.0*	3	2.0*	2
Val								
Ile								
Leu			1.0*	1			0.8	1
Tyr								
Phe					1.0	1	0.8	1
His							0.8	1
Lys[c]	1.0	2	0.3	1	1.1	2	1.0	2
Arg			1.0	1	0.9	1	0.2	

[a] Numbers in parentheses represent retention time in minutes.

[b] Amino acid composition is based on asterisked residue, alanine or leucine.

[c] Hexitollysine was not quantitated. Observed lysine content should be one less than theoretical for run-on peptides.

TABLE II

Distribution of glycated peptides in RNase

Retention time (min)	Peptide number	Lysine-modified	% radioactivity[a]		
			Glucose + $[^3H]NaBH_4$[b]	6-[^{3}H] Glucose + $NaBH_3CN$[c]	
				A	B
21	T_1	1	24[d]	82[e]	66
36	T_{5-6}	37	9	3	7
73	T_{1-2}	7	29	6	14
97	T_{7-8}	41	38	9	14

[a] Per cent of total radioactivity in peptides. The fraction of radioactivity at the void volume (3 min) was variable (5–10%). Analysis of this fraction from several different column runs indicated that it did not contain hexitollysines.

[b] RNase glycated with unlabeled glucose (1 mol/mol), then reduced with $NaB[^3H]_4$.

[c] RNase reacted with [6-^{3}H]glucose in the presence of $NaHB_3CN$. Columns A and B represent protein labeled with 0.6 and 1.5 mol of Glc/mol of RNase, respectively.

[d] N^{α}:N^{ϵ}-hexitollysine = 61:39 (Fig. 8).

[e] N^{α}:N^{ϵ}-hexitollysine = 86:14 (Fig. 10*A*).

RNase with 6-[^{3}H]glucose and $NaBH_3CN$ (see below). Both the bound and unbound peptides could be identified as tryptic peptide T_{7-8} containing residues 40–61. This peptide has two lysine residues (41 and 61) and, because it co-elutes with native T_{7-8}, Lys-41 must be the site of glycation. This is a large, relatively hydrophobic peptide and its binding to the phenylboronic acid column may be inhibited by tertiary structure in the peptide.

The overall results of the peptide mapping indicate that lysines 1, 7, 37, and 41 are the most reactive sites for modification of RNase by glucose. The high reactivity of lysines 7 and 41 which are in the active site and of lysines 1 and 37 which neighbor the active site cleft is consistent with the earlier suggestion that the rate of inactivation of RNase by glucose indicated preferential modification of active site residues.

Sites of Formation of Schiff Base Adducts to RNase—To determine if the same lysine residues in RNase were equally reactive in formation of both Schiff base and Amadori adducts, the Schiff base adducts were trapped by glycation of RNase with 6-[^{3}H]glucose in the presence of $NaBH_3CN$. Two separate incubations were conducted for 15 and 30 min, respectively. The reactions were quenched by adjusting to pH 4 with acetic acid, then quickly chromatographed on Sephadex G-25 at 4 °C. This yielded preparations of RNase containing 0.6 and 1.5 mol of Glc/mol of protein. Peptide analysis by RP-HPLC indicated that the most reactive amino acid was Lys-1 accounting for about 82 and 66% of all lysine modification in the protein at 0.6 and 1.5 mol/mol substitution, respectively (Fig. 9 and Table II). Comparison of the peptide maps in Figs. 6 and 9 indicates that the remaining lysine residues modified in the presence of $NaBH_3CN$ were the same residues which were glycated via the Amadori rearrangement.

To determine whether the Schiff base was formed at the α- or ϵ-amino group of Lys-1, aliquots of $NaBH_3CN$-reduced glycated protein and peptide T_1 were hydrolyzed and their N^{α}- and N^{ϵ}-glucitollysine content was analyzed by ion exchange HPLC. As shown in Fig. 10*A*, the α-amino group of Lys-1 was the primary site (86%) of glycation of this peptide isolated from protein at 0.6 mol/mol substitution. These data indicate that N^{α}-Lys-1 is about 6 times as reactive as N^{ϵ}-Lys-1 and more than 10 times as reactive as the average ϵ-amino group of RNase in forming the Schiff base adduct. The presence of 66% of the radioactivity in peptide T_1 after 1.5 mol/mol substitution suggests that the α-amino group of the protein had been quantitatively modified by glucose at this point. However, analysis of the total protein at 1.5 mol/mol substitution, shown in Fig. 10*B*, yielded equal amounts of N^{α}- and N^{ϵ}-glucitollysine. The discrepancy may be explained in part by the unidentified peak at 31 min which may represent a dihexitollysine adduct to Lys-1.

Overall, the peptide maps obtained in the presence and absence of $NaBH_3CN$ were qualitatively similar but quantitatively distinct. The preferred site of glycation of RNase in the presence of $NaBH_3CN$ was at N^{α}-Lys-1, consistent with the low pK_a of the α-amino groups. In contrast, the preferred site of modification of the protein via the Amadori rearrangement was at Lys-41 in the active site. In addition to N^{α}-Lys-1 and N^{ϵ}-Lys-41, the N^{ϵ}-amino groups of lysines 1, 7, and 37 were also more reactive than other amino groups in either the presence or absence of $NaBH_3CN$.

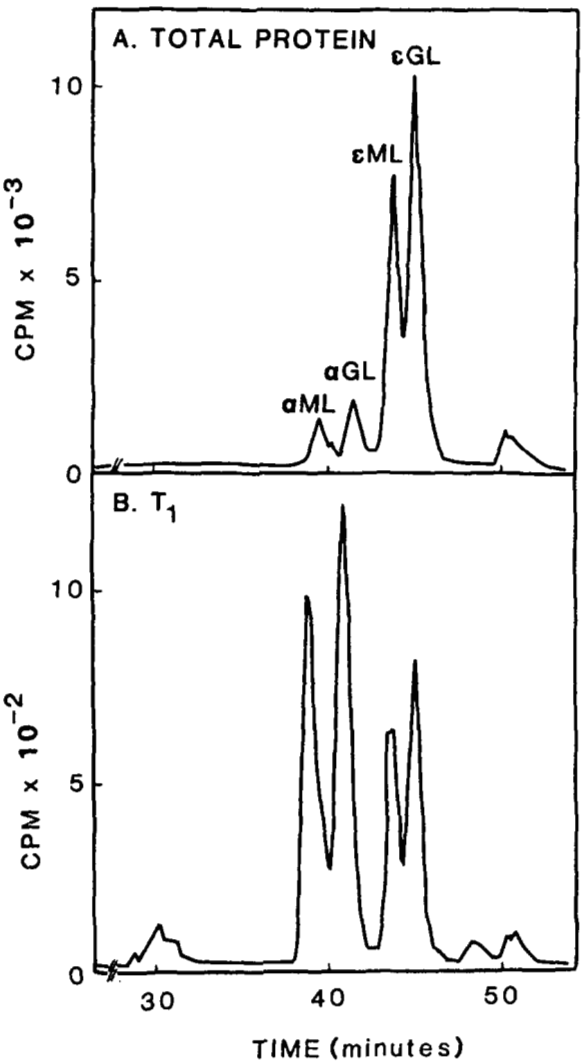

FIG. 8. **Determination of N^{α}- and N^{ϵ}-hexitollysines in total glycated RNase and in peptide T_1.** *A*, chromatogram obtained from hydrolysate of NaB[^{3}H]$_4$-reduced, glycated RNase. Distribution of radioactivity is 17% N^{α}- and 83% N^{ϵ}-hexitollysine in total protein. *B*, chromatogram from isolated peptide T_1. Distribution is 61% N^{α}- and 39% N^{ϵ}-hexitollysine in peptide T_1. αML = N^{α}-mannitollysine; αGL = N^{α}-glucitollysine; ϵML = N^{ϵ}-mannitollysine; ϵGL = N^{ϵ}-glucitollysine.

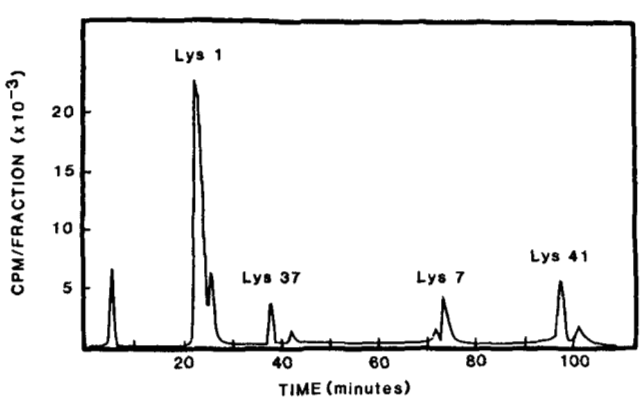

FIG. 9. **Radioactive tryptic peptide map of RNase reacted with 6-[^{3}H]glucose in the presence of $NaBH_3CN$.** This preparation contained 1.5 mol of Glc/mol of protein. The preparation containing 0.6 mol of Glc/mol of RNase was essentially identical, except for the increased relative intensity of glycated peptide T_1.

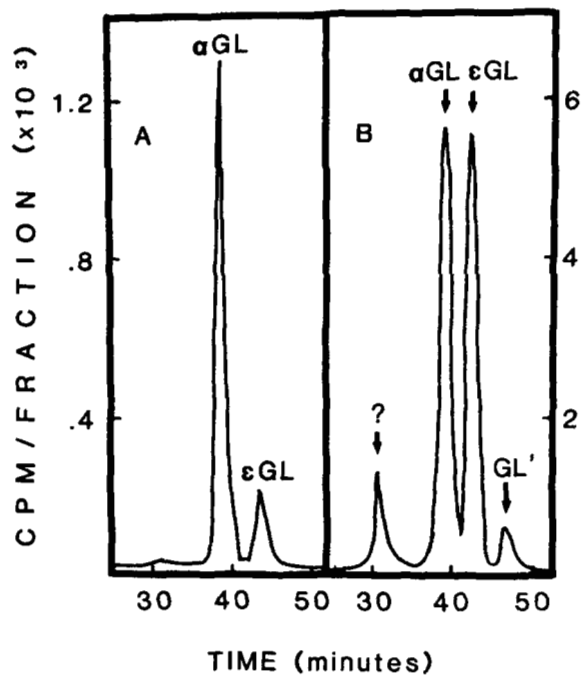

FIG. 10. **Determination of N^{α}- and N^{ϵ}-glucitollysine in RNase glycated in the presence of $NaBH_3CN$.** *A*, radioactivity in glycated peptide T_1 isolated from RNase containing 0.6 mol of Glc/mol of protein. Distribution is 86% N^{α}- and 14% N^{ϵ}-glucitollysine in peptide T_1. *B*, radioactivity in total glycated protein containing 1.5 mol of Glc/mol of RNase. Distribution is 45% N^{α}- and 45% N^{ϵ}-glucitollysine. Abbreviations as described in legend to Fig. 8, except *GL'* which is a degradation product formed on hydrolysis of N^{ϵ}-glucitollysine (7).

DISCUSSION

Glycation is a common post-translational modification of proteins in the body. Individual proteins may be more or less reactive with glucose, but their rates and extents of glycation are dependent on both the ambient glucose concentration and the biological half-life of the protein. Increased glycation and subsequent Maillard reactions of glycated proteins have been implicated as factors contributing to pathology in the lens, kidney, connective tissue, and vasculature in diabetes (3, 4, 20, 21). This reaction may also be involved in the normal aging of tissues because the extent of protein glycation increases with age in man (20), possibly in response to decreased protein turnover and the development of glucose intolerance. Knowledge of the factors affecting the specificity of protein glycation may be important in understanding the role of this chemical modification in the structural and functional changes in protein which occur both in diabetes and during normal aging.

The work described here began with the observation that the inactivation of RNase by glucose proceeded more rapidly than the overall rate of glycation of its lysine residues. A number of earlier studies had described the inactivation of RNase by chemical modification of lysine residues (8–10) and several reagents are known to react preferentially in the active site and to inactivate the enzyme at a rate much faster than the overall rate of lysine modification. This occurs, for example, on dinitrophenylation of RNase (22) or on modification of the enzyme via Schiff base formation with pyridoxal phosphate (23). The goal of the present research was to characterize the sites of modification of RNase by glucose and to make use of this model protein system to identify factors

which might affect the specificity of glycation of other proteins.

Of the 10 lysine residues in RNase, a set of 4 (lysines 1, 7, 37, and 41) has been identified as preferential sites of reaction with glucose and Lys-1 was observed to react at both its α- and ϵ-amino group. The enhanced reactivity of these four lysine residues was apparent in the formation of both Schiff base and Amadori adducts to RNase, although the different sites varied in their reactivity at the two steps in the glycation reaction. Adducts of N^{α}- and N^{ϵ}-Lys-1 should be equally accessible to reduction by $NaBH_3CN$, but, consistent with its lower pK_a, substantially more Schiff base is formed at the α-amino group. As shown in Table II, the initial distribution of Schiff base adducts to RNase (Fig. 9) differs remarkably from the eventual distribution of Amadori adducts (Fig. 6). Thus, not all Schiff bases are equally efficient in undergoing the Amadori rearrangement. Comparison of the initial distribution of Schiff base adducts to Lys-1 (α:ϵ = 86:14, Fig. 10*A*) with the final distribution of Amadori adducts (α:ϵ = 61:39, Fig. 8*B*) indicates that the Amadori rearrangement occurs about 4 times faster at the ϵ-amino group. Similarly, from data in Table II, Lys-41 has about 13% (9/(0.86 × 82) = 0.13) as much Schiff base modification as N^{α}-Lys-1, but accounts for more than twice as much of the Amadori adduct to RNase (38/(0.61 × 24) = 2.6). This indicates that the Schiff base at Lys-41 is about 20 times more efficient than that at N^{α}-Lys-1 in undergoing the Amadori rearrangement. It is significant that the final distribution of N^{ϵ}-Lys-41 to N^{α}-Lys-1 modification of RNase is not 20:1, but closer to 3:1. Thus, both the initial distribution of Schiff base adducts and the rate of the Amadori rearrangement at each site play a role in determining the final distribution of Amadori adducts to the protein. Similar calculations based on the data in Table II show that Schiff base adducts to Lys-7, which is in the active site near Lys-41, are about as reactive as those at Lys-41 in undergoing the Amadori rearrangement. Those at Lys-37 appear to be intermediate in reactivity between N^{α}-Lys-1 and lysines 7 or 41. The remainder of the amino groups in RNase were not particularly reactive in forming either the Schiff base or Amadori adduct. The unreactive sites include lysines 31, 61, 66, 91, 98, and 104. The only feature common to these amino acids is their location on the surface of the protein, directly exposed to solvent. It should be noted that all of these amino groups may eventually react with glucose, but that after modification of the first five amino groups on RNase the rate constant for the glycation reaction declined to about 20% of its original value (7).

There are several features which distinguish the reactive from the unreactive amino groups in RNase. Thus, lysines 1 and 37 are adjacent to acidic amino acids, Glu-2 and Asp-38, which may be involved in local catalysis of the Amadori rearrangement. This is consistent with the observations of Shapiro *et al.* (5) who noted that a feature common to several lysine residues glycated in hemoglobin was their nearness to carboxylic acid residues in the primary or three-dimensional structure of the protein. Similarly, both lysines 7 and 41 are located in the active site cleft of RNase. There is evidence that either or both of these ϵ-amino groups can participate in the catalytic process (8–10), although modification of Lys-41 by bulky reagents generally results in near complete loss of enzymatic activity (8, 9, 24, 25). In our experiments, the 40% decrease in activity of RNase containing 1 mol of Glc/mol of protein (Fig. 3) is consistent with the 38% glycation of Lys-41 (Table II). The comparison between glycation of RNase and hemoglobin may be extended by noting that the N^{α}-amino termini are reactive sites for glycation of both proteins, although in hemoglobin the β-chain terminal valine is much more reactive than the α-chain valine and also more reactive than any lysine residue in the protein (5). It is worth noting that the amino groups most reactive to glycation in RNase and hemoglobin, Lys-41 in RNase and β-Val-1 in hemoglobin, share similar environments. Thus, they are both located in high affinity binding sites for phosphate ion or organic phosphates, *i.e.* the binding site for the substrate, RNA, in RNase and the binding site for the allosteric effector, 2,3-diphosphoglycerate, in hemoglobin. In both proteins these are relatively basic regions of the molecule, containing arginine and histidine residues in addition to the reactive amino groups. Local catalysis of the Amadori rearrangement of lysines 7 and 41 in RNase and β-Val-1 in hemoglobin could be effected by either the phosphate ions or histidine residues in these binding sites. This hypothesis would explain the much higher reactivity of the β-chain *versus* the α-chain valine residues in hemoglobin; despite essentially identical pK_a values (24), only the reactive β-terminal valine residue is located in the phosphate binding site. In support of this interpretation, we have observed that the enhanced reactivity of Lys-41 in RNase, as well as the rate of inactivation of the enzyme by glucose, is clearly dependent on the concentration of phosphate ion in the incubation buffer.[2]

Based on the specificity of glycation of RNase and hemoglobin, it seems possible that phosphate binding allosteric or active sites of other proteins may also be preferred sites of reaction with glucose. It is of interest that, for proteins studied in detail thus far, such as hemoglobin (5, 25, 26), albumin (6, 27), low density lipoprotein (28–30), and RNase, there appears to be a high degree of specificity in the reaction of amino groups with glucose. Thus, glycation of relatively few amino groups in these proteins seems to have pronounced effects on their allosteric sensitivity (25), ligand binding (26, 27), receptor recognition (28–30), and enzymatic activity (this work), respectively. Although glycation of protein is a slow reaction, it is tempting to remark that glucose has some of the characteristics of a site-specific reagent for enzymes and proteins. The increased glycation of protein in diabetes, coupled with the specificity of this reaction, suggests that further study of protein glycation in diabetes will be important for understanding the metabolic and structural consequences of hyperglycemia.

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