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Characterization of glycated hemoglobin in diabetic patients: usefulness of electrospray mass spectrometry in monitoring the extent and distribution of glycation

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Abstract

A combination of chromatographic and mass spectrometric techniques was used to evaluate the extent and distribution of glycation within the glycated hemoglobin (GHb) molecule. Studies on quantification of hemoglobin (Hb) glycation by electrospray ionization mass spectrometry (ES-MS) of intact globins employed specimens from 10 diabetic individuals and five normal controls. Detailed structural analysis of the phenylboronate affinity chromatography/ion-exchange (IE) HPLC-separated sub-populations of GHb was performed on a specimen carrying 13.7% GHb. An efficient protocol for mapping glycation sites within α and β globins was developed, e.g., Glu-C/Asp-N proteolytic digestion followed by LC–ES-MS. Relative site occupancy within discrete components of GHb was evaluated. A correlation between the degree of glycation measured at Hb level (by affinity chromatography) and at globin level (measured by ES-MS) was carried out. The above studies led us to conclude that during the process of phenylboronate chromatography GHb dimers, rather than tetramers, are bound to the affinity resin so a fraction of glycated dimers rather than tetramers is measured. This finding implies that a process of glycation affects a much higher number of native Hb tetramers than was previously contemplated. No glycation sites appear to be missed by phenylboronate affinity chromatography. We have found no evidence of the presence of multiple glycations within a single globin chain. While glycation of both globins within a dimer cannot be excluded, it is unlikely to be a significant phenomenon. According to ES-MS data, an equivalent of about one globin per αβ dimer of the affinity chromatography-isolated GHb carried glycation. © 2001 Elsevier Science B.V. All rights reserved.

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1. Introduction

Protein glycation is one of the most common post-translational modifications within the class of

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enzyme catalysis-independent processes. It is triggered by a Schiff base modification of active amino group(s) by reducing sugars to be followed by a slower step of product stabilization through a process of tautomerization (Amadori rearrangement) and results in ketoamine formation: reaction with glucose generates an amino-1-deoxyfructos-1-yl derivative [1]. Protein glycation is highly dependent upon substrate concentrations and, given the relatively slow kinetics of the overall process, it is best observed for long-lived proteins. In diabetes, in which the hallmark feature is an elevated glucose level in the peripheral circulation, the extent of red cell hemoglobin (Hb) glycation has been extensively used as a marker of glycemic control [2]. Either total glycated hemoglobin (GHb) or Hb fraction modified at the N-terminus of β chain (Hb A1c) typically is reported. In addition to glucose level, Hb glycation is controlled by a plethora of cellular conditions. Thus, while glycated Hb is an important indicator of ongoing modification of more crucial proteins, it should be used with caution if it is to serve as a sole marker of glycemic status [3]. Glycated Hb per se is an unlikely factor in the pathophysiology of diabetic complications [4].

Constitution of Hb A1c and of its non-beta N-terminally modified glycated counterpart (GHb A0) as well as distribution of glycation within the α and β globins were extensively studied in the past [5–9]. Shapiro et al. [9] elegantly demonstrated that major target sites of Hb glycation in vivo and in vitro were distinct (βVal-1>βLys-66>αLys-61>βLys-17> αVal-1 and βVal-1>αLys-16>βLys-66>βLys-17> αVal-1>αLys-7>βLys-120, respectively). Subtle differences in experimental conditions (Hb concentration and heme-ligand status) were postulated to exert changes in protein conformation which, in turn, effected unique glycation patterns. An impact of protein conformation on the outcome of glycation was further demonstrated in studies on reactivities of two target sites of α globin (αVal-1 and αLys-16) upon placing them within different structural context: in Hb tetramer, in isolated α chain and in the segment α1-36 [10]. Recent studies on Hb glycation have employed modern mass spectrometric techniques: electrospray ionization mass spectrometry (ES-MS) and matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF-MS). ES-MS was introduced to characterize glycated Hb species isolated by boronate affinity and/or cation-exchange chromatography [11] and to establish the purity of standard preparations of Hb A1c [12,13]. MALDI-TOF-MS was used for qualitative and quantitative evaluation of products of glycation in total Hb as well as in Hb A1c fraction [14].

The phenomenon of multiple glycations within either the same globin chain or, more likely, within the Hb multimer has not been systematically studied. A review of the literature points to some ambiguity regarding the number of glycation sites within a species classified as GHb. Shapiro et al. [9] estimated that a minimum of 0.8 residue of lysino-1-deoxysorbitol per αβ dimer of GHb was bound. Walton and McPherson measured 0.96 mol and 0.54 mol of *N*-(1-deoxyfructos-1-yl) derivative per mol of total glycated Hb and glycated Hb A0 dimer, respectively [15]. While the above data argue against multiple glycation within native Hb molecule, the results of rod isoelectric focusing of globin chains point to the contrary [16]. In the latter case, globin glycation values were found to be much higher than expected on the basis of GHb level measured by affinity chromatography (18%/28% and 8%/12% for β and α globin in normal/diabetic subjects, respectively). The authors attributed this discrepancy to an inefficient binding between sterically hindered sites within glycated Hb and affinity resin. Assays aimed at direct detection of the protein bound-sugar derivatives were also developed, such as colorimetry of the thiobarbituric acid-released 5-hydroxymethylfurfural [17,18], and radioactive, UV and/or fluorometric detection of hexitolamino acid derivatives generated via reduction of ketoamine function [8,15,19]. However, they suffer from limitations of differential reactivity of N-terminal valine- vs. internal lysine-bound derivatives and, for the latter subpopulation, of the linkage site-dependence of the reaction kinetics [8]. Recent papers on quantification of Hb glycation by ES-MS [10,20] and MALDI-TOF-MS [21] showed good correlation between mass spectrometric and conventional methods (ion-exchange high-performance liquid chromatography (IE-HPLC) and phenylboronate affinity column [22,23]). However, a direct comparison of absolute glycation levels measured for native Hb and denatured globin molecules was not performed.

Reports suggesting an impact of antioxidants on Hb glycation raised the possibility of changes in glycation pattern following treatment [24–27]. This prompted our examination of the potential of ES-MS in monitoring the extent of total glycation, its allotment to discrete GHb components as well as its distribution between various sites within α and β globins. To this end, we have examined the utility of ES-MS of intact globins in measuring absolute levels of Hb glycation, have developed a protocol to map a distribution of glycation within α and β globins, and have examined in detail a composition of discrete fractions of glycated Hb.

2. Experimental

Blood from diabetic donors and normal controls was collected in EDTA tubes. Plasma was separated by centrifugation. The red cells were washed three times with saline and stored at −70°C before further analysis.

2.1. Isolation of GHb with phenylboronate affinity chromatography

To isolate the total GHb, hemolysate was first subjected to phenylboronate affinity column chromatography (IsoLab, Akron, OH, USA). Non-GHb was eluted with 50 m*M* phosphate buffer (pH 9.05–9.25) and the bound GHb fraction was eluted with 100 m*M* sorbitol in the above buffer (pH 9.16) [23]. GHb fractions were re-equilibrated to the initial buffer conditions of the subsequent IE-HPLC (buffer A–buffer B, 84:16, see below) and concentrated utilizing a Centricon YM-10 microconcentrator (Millipore, Bedford, MA, USA).

2.2. Separation of Hb A1c and GHb A0 using cation-exchange HPLC

The concentrated GHb fractions were further separated on a cation-exchange column (200×4.6 mm I.D., 5 μm, 1000 Å; PolyCAT, Columbia, MD, USA) with a flow-rate of 0.8 ml/min and detected at 450 nm, following the method described by Ou and Rognerud [28]. A binary buffer system was used: buffers A and B consisted of 20 m*M* Bis-Tris–1 m*M* KCN (pH 6.7) and 20 m*M* Bis-Tris–1 m*M* KCN–0.2 *M* NaCl (pH 6.5), respectively. Buffers were titrated to a target pH with HCl. The column was equilibrated with 16% B, was maintained at 16% B for the first 1 min of elution and then two steps of a linear gradient were employed: from 16% B to 40% B in 40 min and then to 100% B in 12 min. Fractions were manually collected.

2.3. Enzymatic digestion

Hb species separated by IE-HPLC were transferred to water solution and concentrated utilizing a Centricon YM-10 microconcentrator (Millipore). Globins were de-hemed in acidified acetone, carboxyamidomethylated in 0.4 *M* ammonium carbonate (pH 7.8) containing 6 *M* guanidine hydrochloride and diluted 10× with water [29]. Incubation with endoproteinase Glu-C (Boehringer Mannheim, Germany), enzyme-to-substrate ratio 1:20 (w/w), was then carried out at room temperature for 17 h. Subsequently, endoproteinase Asp-N (Boehringer Mannheim), enzyme-to-substrate ratio 1:100 (w/w), was added and incubation at 37°C was continued for additional 6 h. Alternatively, endoproteinase Glu-C proteolytic fragments were isolated by narrow bore HPLC, lyophilized, dissolved in 50 m*M* sodium phosphate (pH 8.0) and further digested with endoproteinase Asp-N using an enzyme/substrate ratio of 1:100 (w/w) at 37°C for 6 h. To fully cleave the N-terminal part of the beta chain between two glutamic acid residues at positions 6 and 7, purified Hb A1c was incubated with endoproteinase Glu-C according to the published procedure [12,13]. All the digests were fractionated and mass analyzed by HPLC–ES-MS.

2.4. Mass spectrometry

Electrospray mass spectra were acquired on a BioQ triple quadrupole instrument (Micromass, Altringham, UK). The capillary potential was approximately 3900 V. The resolution was set to afford the 0.7–0.8 Da width at half height of the m/z 998 myoglobin peak. Profile data were acquired. For intact globin analysis, the cone voltage was ramped from 35 V to 50 V within the m/z range of 920–1420, 10 to 15 5-s scans were accumulated. Spectra

were internally calibrated utilizing human α globin as a standard. For peptide analysis, a cone voltage was alternated between 25 V and a cone ramp of 45–60 V allowing for acquisition of intact peptide molecular ions and products of in-source fragmentations, respectively. The m/z range of 400–1600 was scanned at a rate of 3 s/scan. The instrument was controlled and data were processed utilizing the MassLynx software, including MaxEnt algorithm (Micromass).

A narrow bore HPLC system (Michrom BioResources, Auburn, CA, USA) interfaced to BioQ mass spectrometer was operated at a flow-rate of 50 μl/min. The flow was split after the flow cell at a ratio of 1:7 to ultimately deliver 7 μl/min to the electrospray source. Protein and peptide elution was monitored at 214 nm.

Hemolysates were diluted 300 times with 0.2% formic acid in 50% acetonitrile and 10 μl aliquots were directly injected into a stream of 50% acetonitrile pumped at a flow-rate of 10 μl/min (2248 HPLC pump; Pharmacia, Alameda, CA, USA). Hb fractions isolated by affinity chromatography/IE-HPLC were on-line desalted and subsequently separated utilizing protein trap cartridge and PLRP-S column (50×1.0 mm I.D., 5 μm, 1000 Å), respectively, both from Michrom BioResources. Globins were eluted with a linear gradient of acetonitrile in 0.1% TFA (20% B to 80% B in 20 min, where A and B were 20% and 60% acetonitrile in 0.1% TFA, respectively). Proteolytic digests were on-line desalted using a peptide trap cartridge (Michrom BioResources) prior to separation on a Reliasil C_{18} column (150×1.0 mm I.D., 5 μm, 300 Å; Michrom BioResources). Peptide elution was afforded with a gradient of 5% B to 65% B in 20 min, where A and B were 2% and 90% acetonitrile in 0.1% TFA, respectively. Fractions were manually collected.

MALDI-TOF and MALDI-TOF post-source decay (PSD) experiments were carried out on a Voyager DE STR mass spectrometer (Applied Biosystems, Framingham, MA, USA). α-Cyano-4-hydroxycinnamic acid (Agilent Technologies) was used as a matrix. Two-point external calibration was applied for the mass measurement of the peptides, while ACTH (18–39) clip peptide was used for the PSD calibration. The reflector voltage was lowered to 75% in each subsequent PSD step, and data were acquired in 10–12 steps, depending on the peptide size. The PSD data segments were combined in a single spectrum and smoothed to yield average masses of product ions.

2.5. Quantification of glycation of intact globins by ES-MS

The most intense molecular ions derived from human globins are typically observed within a 600–900 m/z range of ES-MS spectra. However, the ions corresponding to 22- and 18-protonated glycated β globin happen to fall within one mass unit of the much more intense ions of glycated and non-glycated α globin carrying 21 and 17 positive charges. When a quadrupole detector of unit resolution is employed, those ions are not fully resolved and thus, the above mass range is not appropriate for quantification purposes. Therefore, the data acquisition was performed within the mass range of 920–1420 m/z where all the ions of interest are well resolved, i.e., under the conditions used by Roberts et al. [20] and Nakanishi et al. [30]. The intensity of mass spectrometric signal was very susceptible to changes in cone voltage values. In our hands, the best reproducibility and precision of quantification results was achieved when a cone voltage ramp of 35–50 V was employed. Due to a lack of standard data on absolute responses of glycated and non-glycated globin counterparts in ES-MS, the following studies relied upon an assumption that the close structural similarity of glycated and non-glycated analytes will result in close resemblance of their ES-MS signals [20].

Glycated and non-glycated α and β globin counterparts were manually identified as components in electrospray mass spectra. The ES mass spectra were deconvoluted using a ''transformation algorithm'' of MassLynx software. Relative abundance of species was estimated on the basis of heights of their corresponding smoothed and centroided peaks in the deconvoluted spectra: %glycα and %glycβ were defined as 100×glycα/(α+glycα) and 100×glycβ/(β+glycβ), respectively. Total globin glycation (%ES-MS) was defined as (%glycα+%glycβ).

2.6. ES-MS quantification of glycation of proteolytic fragments

Estimation of amounts of glycated and non-glycated peptide counterparts was based on the peak

Table 1
distribution of glycation within alpha and beta globin

Proteolytic fragment	Known	Found*	Helical position	Contact site	Relative distribution of glycation (%)		
					''Hb A1c''	''Hb A1d''	''CHb A0''
Alpha globin							
α1–5	V1	**V1**	**NA1**	External	0	47	0
α6–30	–	*K16*/K7	*A14*/*A5*	External	2	0	1
α31–46	K40	**K40**	**C5**	α1β2	6	6	5
α47–63	K61	**K61**	**E10**	External	76	39	72
α126–141	–	*K139*/K127	*HC1*/H10	External	16	8	22
Beta globin							
β1–6	V1	**V1**	**NA1**	2,3-DPG binding	90	68	0
β8–22	K8; 17	*K17*/K8	*A14*/A5	External	0	4	8
β52–72	K66	*K66*/K61/K65	*E10*/E5/E9	Heme contact	7	14	54
β102–121	K120	–	GH3	External	0	0	0
β122–146	K144	*K144*/K132	*HC1*/H10	External	3	14	38

*Unequivocal and least likely assignments are shown in bold and plain font, respectively. Educated guesses based upon an assumption that alpha and beta globins are glycated at the same helical positions are marked in italics.

areas of extracted ion chromatograms generated for those molecular ion charge states that were observed in both the glycated and non-glycated forms of each peptide. A ratio between the amount of glycated peptide and a sum of glycated and non-glycated counterparts was then calculated to derive an absolute site occupancy level. The likelihood of the presence of 1-deoxyfructos-1-yl group at a specific site of α and β within ''Hb A1c'', ''Hb A1d'' and ''GHb A0'' was defined as a ratio of the absolute occupancy level at this site to a sum of all absolute site occupancy levels for this globin (Table 1).

3. Results and discussion

3.1. Quantification of hemoglobin glycation utilizing ES-MS analysis of hemolysates

A hypothesis that some of the glycation sites might not ''be seen'' by affinity column was addressed [16]. To examine a possibility that affinity chromatography might provide incomplete description of the Hb glycation status, a comparison of glycation levels of native Hb (measured by phenylboronate affinity chromatography, %GHb) and denatured globins (measured by ES-MS and defined as %glycα+%glycα=%ES-MS) was performed. While the problem appears trivial at the first glance, it actually presents the following serious technical challenges: (a) difficulty in generating standards of glycated Hb and glycated α and β globins [12,13], and (b) ambiguity regarding multimeric state of Hb under the conditions of IE-HPLC [13] and phenylboronate affinity chromatography.

Native Hb is the $\alpha_2\beta_2$ tetramer in a rapid equilibrium with the αβ dimer. The rate of a tetramer–dimer exchange is strongly affected by a heme-ligand status (fast and slow for oxy- and deoxy Hb, respectively). The position of equilibrium is highly dependent upon pH and Hb concentration and it is influenced both by the tetramer–dimer interface and by the regions distant from this interface (Kd for Hb A equals 0.68 μM) [31]. Assuming that only one 1-deoxyfructos-1-yl group per Hb tetramer is required for its binding to the affinity resin, an equivalent of one glycated and three non-glycated globins would be released upon tetramer dissociation to monomers (e.g., under conditions of ES-MS experiment). Thus a population of Hb tetramers carrying %GHb glycation would produce (0.5× %GHb) glycated monomers ''seen'' by ES-MS (%ES-MS). On the other hand, should %GHb glycation be measured on a dimeric population, the fraction of ES-MS-detected monomers (%ES-MS) would be equal to %GHb. Hence, the %ES-MS/%GHb ratios of 0.5 and 1.0 are expected for the phenylboronate column-bound tetramer and dimer populations, respectively.

As illustrated in Fig. 1a, the ratio between the

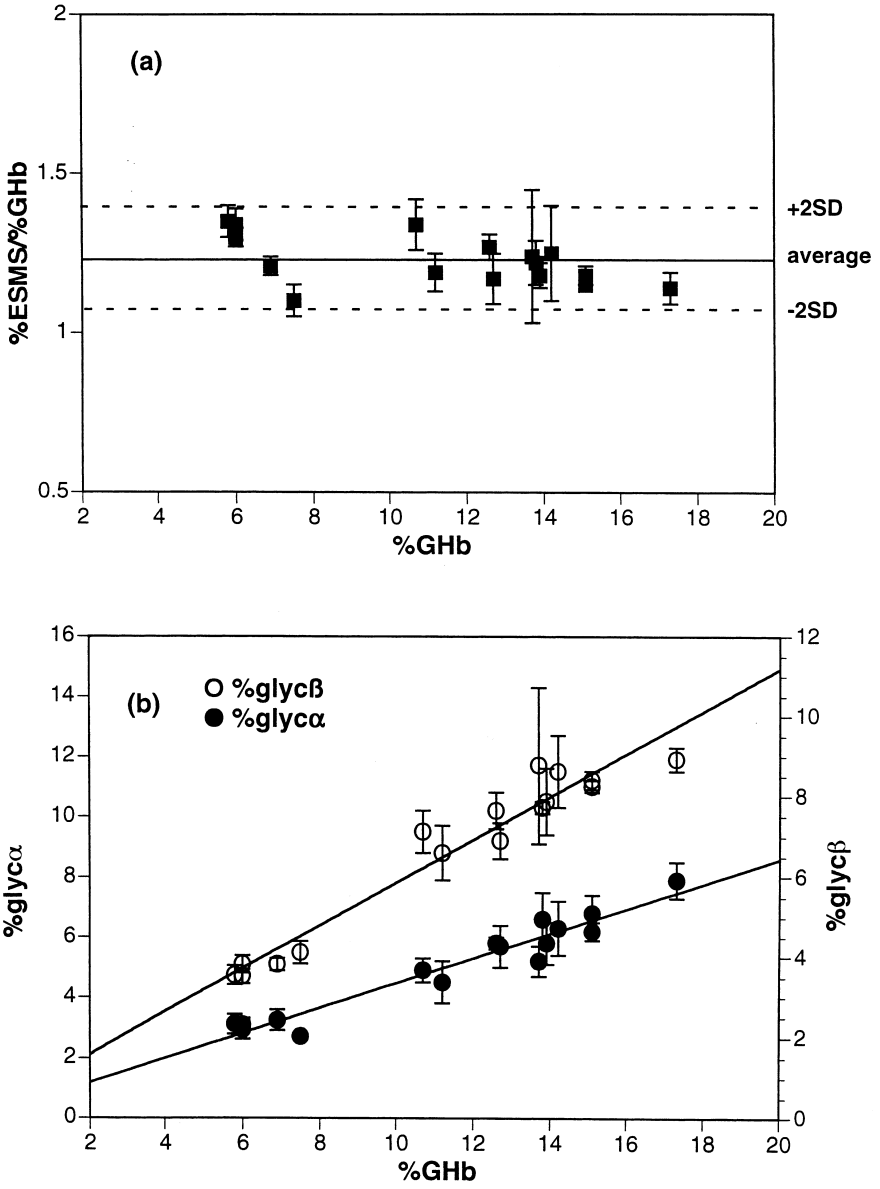


Fig. 1. (a) Correlation between the extent of globin glycation measured by ES-MS (%ES-MS) and defined as a sum of α and β globin glycation (%glycα+%glycβ) and the level of Hb glycation measured by phenylboronate affinity chromatography (%GHb). %ES-MS/%GHb=1.23±0.08, n=15, RSD=6.5%. All values for normal and diabetic subjects fall within two standard deviations of the mean, thus substantiating their independence from the total glycated Hb level. (b) The extent of α (%glycα) and β (%glycβ) glycation as a function of %ES-MS showed the following linear relationships: glycated α: (%glycα)=0.36 (%ES-MS)+0.022 (R^2=0.94); glycated β: (%glycβ)=0.64 (%ES-MS)+0.044 (R^2=0.98). The %glycα/%glycβ ratio is independent of total glycation level.

percentage of glycated globin determined by ES-MS (%ES-MS) and the percentage of glycated Hb determined by phenylboronate chromatography (%GHb) was 1.23±0.08 (measured in triplicate for five normal and 10 diabetic specimens, RSD=6.5%). No difference between the diabetic (1.21±0.06) and non-diabetic (1.26±0.11) subjects was observed. The ratio between the α and β globin glycation was

0.58±0.06 (RSD=10.4%) and was independent of the degree of glycation, in agreement with the previously reported values measured either utilizing radioactive tracers [8] or ES-MS [20], albeit contrary to the observation reported by Peterson et al. [11]. The slope of linear relationships between the α (%glycα) and β (%glycβ) globin glycation and total glycation (%ES-MS) measured by ES-MS (Fig. 1b) closely followed the published values [20].

The %ES-MS/%GHb ratio of 1.23±0.08 points to Hb dimers rather than tetramers as the major species undergoing separation on phenylboronate affinity column. This result is surprising given that the concentration of Hb applied to the affinity column is 5–10 μ*M*, i.e., at least one order of magnitude higher than the Kd of a Hb A tetramer–dimer equilibrium. If affinity separation of tetrameric molecules were to be postulated, the above result would imply that more than 50% of all glycation sites were missed by affinity chromatography. Should it be the case, the plausible expectation would be to see some of the ''missed'' glycation products within a fraction not bound to affinity resin. According to our own (not shown) as well as literature data [11,13], no glycated species were detected by ES-MS in the fraction that did not bind to phenylboronate affinity column. We thus postulate that glycated Hb species binds to a phenylboronate affinity column in a dimeric rather than tetrameric form.

3.2. Separation and mass analysis of discrete components of GHb

A combination of phenylboronate affinity chromatography and IE-HPLC (PolyCAT resin) followed by ES-MS was employed to address the question of a putative presence of multiple glycations within native Hb molecules as well as to re-examine an issue of a multimeric form of the phenylboronate affinity resin-bound GHb.

GHb was isolated and quantified by a phenylboronate affinity column [6,9,22,23], separated into distinct fractions utilizing IE-HPLC [28] (PolyCAT column) and analyzed by ES-MS. While employing the commercial IsoLab affinity columns for the first chromatographic step, we have substituted the manufacturer's buffer with our own preparation devoid of detergents whose presence appeared detrimental to the subsequent steps of analysis. Of note, we observed that the extent of non-specific Hb binding was very sensitive to minor changes in experimental conditions and thus, optimization of the pH of our buffers for each batch of the resin was required. The results of affinity chromatography performed under the laboratory-customized and standard procedure using a Primus CLC 330 analyzer (Primus, Kansas City, MO, USA) showed satisfactory correlation ($y=0.95x+0.90$, $R^2=0.96$, $P<0.05$, $n=14$, range of total GHb=10–17%).

Fig. 2 shows PolyCAT A chromatograms of (a) the whole hemolysate from a diabetic individual and

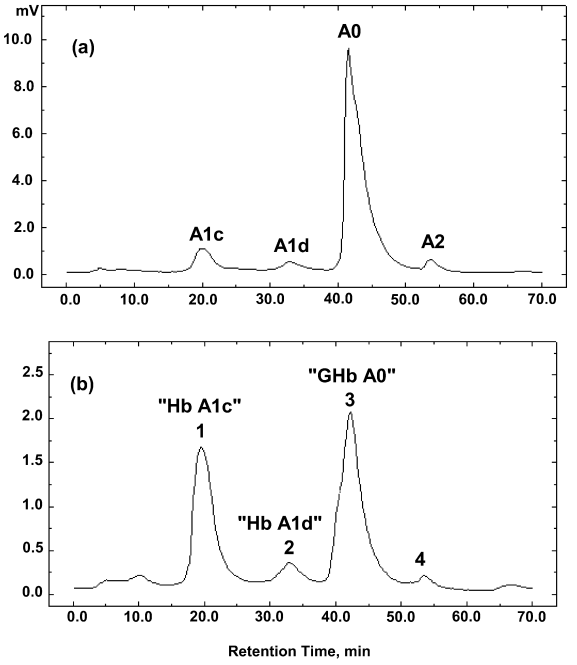


Fig. 2. Ion-exchange HPLC (PolyCAT A column) separation of hemoglobins of a diabetic individual: panels (a) and (b) show Hb species present in whole hemolysate and in the phenylboronate chromatography-isolated glycated Hb (IsoLab column, modified buffer elution system), respectively. Peak annotation in the top figure follows the customary nomenclature used for normal major (Hb A0) and minor (Hb A1c, Hb A1d and Hb A2) Hb fractions. Hb names in the bottom chromatogram depict the types of glycated Hb species eluting in the positions characteristic for Hb types described above. As we have indicated in the text, constitution of the affinity chromatography-captured/ion-exchange-separated glycated Hbs observed in this experiment is not necessarily identical to that of their in vivo counterparts demonstrating the same IE-HPLC mobilities.

(b) the GHb after isolation by phenylboronate affinity chromatography. Typically ~88% protein eluted in two major fractions: fraction 1 ''Hb A1c'' (39±2%) and fraction 3 ''GHb A0'' (48±3%), consistent with the results of Garlick et al. [8]. Few minor fractions were also observed: fractions 2 and 4 eluting in the positions of Hb A1d and Hb A2 were always clearly discerned at the levels of 5.2±0.5% and 2.3±0.2%, respectively.

Fractions 1, 2 and 3 were subjected to ES-MS analysis before and after carboxyamidomethylation (MaxEnt deconvoluted mass spectra of the *S*-alkylated species are shown in Fig. 3). Fraction 4, containing glycated and non-glycated α, β and δ globins, was analyzed by ES-MS without *S*-alkylation (data not shown). All IE-HPLC fractions contained mixtures of glycated and non-glycated forms of α and β globins. This observation strongly suggests that IE-HPLC-separated species contain more than one copy of the same type of globin, i.e., they do not migrate under conditions of IE-HPLC as αβ dimers. The leading, top and tailing portions of the ''Hb A1c'' peak were separately examined by ES-MS. The leading edge of ''Hb A1c'' peak contained much higher level of glycated α than the rest of the peak, thus pointing to a co-elution of another glycated species with Hb A1c. At the same time however, there was no difference between the top and tail portions of the fraction suggesting that ''pure'' Hb A1c might carry a discernable level of α globin glycation. The latter result is consistent with the data of Bookchin and Gallop [5] and Garlick et al. [8] who both detected low levels of α globin labeling following reductive tritiation of their Hb A1c preparations; this was recently confirmed by Finke et al. [13].

The exact position of alpha glycation within the Hb A1c fraction has not been established before. While the α N-terminal valine was postulated [2,13], our peptide mapping (reported in the next paragraph) does not support this hypothesis. The glycation levels of α and β globins (measured by ES-MS before alkylation) were glycα=15% and glycβ=89% in fraction 1, glycα=48% and glycβ=24% in fraction 2, glycα=57% and glycβ=22% in fraction 3, and glycα=49% and glycβ=25% in fraction 4. A slight decrease in α and β globin glycation levels following a process of reduction and alkylation was

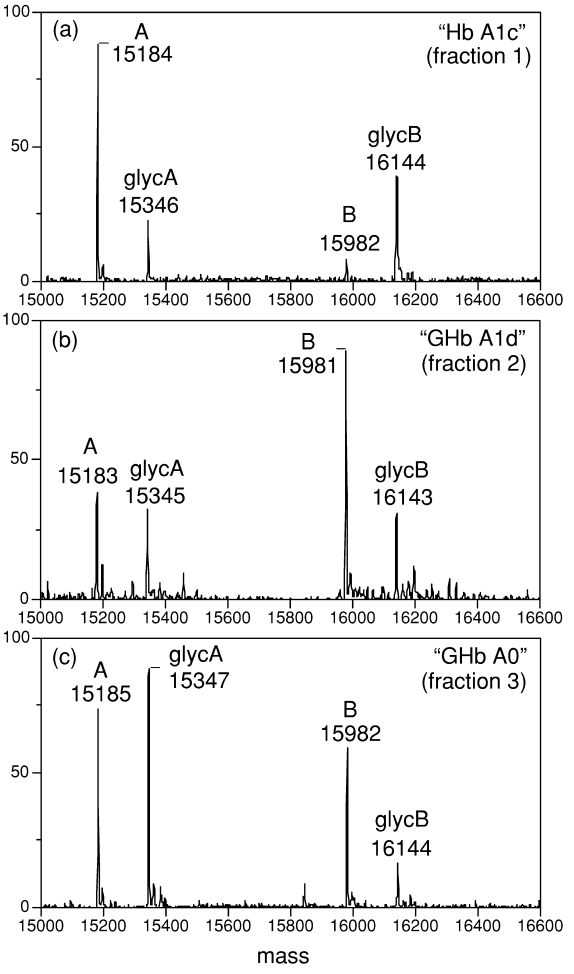


Fig. 3. Maximum entropy (MaxEnt)-deconvoluted electrospray mass spectra of two major and one minor fractions of the phenylboronate chromatography-isolated glycated Hb eluting in the positions of Hb A1c (fraction 1), Hb A1d (fraction 2) and Hb A0 (fraction 3) are shown in panels (a), (b) and (c), respectively. A and B represent α and β globins carrying one and two *S*-carboxyamidomethyl groups, respectively.

observed (some instability of 1-deoxyfructos-1yl lysine upon exposure to nucleophilic environment was reported before [32]).

On the basis of the known relative abundance of IE-HPLC fractions and ES-MS-derived levels of α and β globin glycation in all these fractions, the overall extent of α and β glycation in phenylboronate affinity-bound Hb (fractions 1–3) was found to be glycα=35% and glycβ=52%, respectively. The theoretically expected extent of total globin glycation

within the phenylboronate affinity-bound Hb is 25% and 50% for the tetrameric and dimeric bound forms, respectively. Assuming the α-to-β globin glycation ratio of 0.6, a distribution of glycation between α and β globins would be glycα=20%:glycβ=30% and glycα=40%:glycβ=60% for the tetrameric and dimeric bound forms, respectively. Experimental results are closer to the latter prediction, corroborating our earlier postulate that Hb dimers rather than tetramers bind to phenylboronate affinity column. Taken together the above results imply that the majority of αβ dimers carry only single glycation (either within α or β globin) and that no significant level of glycation is ''missed'' under the conditions of affinity separation.

An important consequence of the above argument is that a constitution of Hb assessed following its isolation by affinity chromatography reflects an artificial phenomenon of a re-assembly of glycated dimers in the absence of their non-glycated counterparts. Hence, it is not necessarily representative of the in vivo distribution of glycation between native Hb tetramers. Indeed, a significant difference in glycated globin status was observed between an authentic Hb A1d fraction (isolated from hemolysate by IE-HPLC, data not shown) and its affinity chromatography/IE-HPLC-isolated counterpart (fraction 2 in Fig. 3a): glycated β globin was present only in the latter case. While the existing data do not allow us to rule out a possibility of a preferential assembly of glycated/glycated versus glycated/non-glycated dimers, there is no evidence to support a notion of non-random distribution of glycated dimers within Hb tetramers. To assess an authentic distribution of glycation within native Hb tetramers, a reversed order of chromatographic steps (i.e., ion-exchange chromatography first, affinity chromatography second) should be employed. While Finke et al. [13] published ES data on Hb A1c purified following this very order of chromatographic steps, dimeric Hb was a major species under their IE-HPLC conditions (pH 6.2) and thus, no information about a native Hb A1c tetramer was obtained.

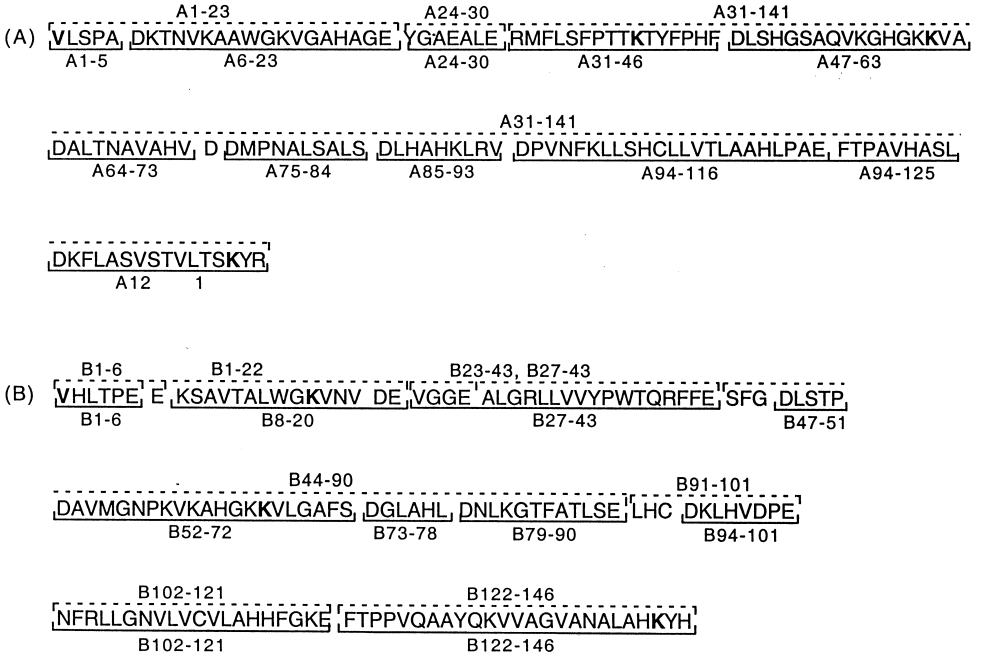
Our conclusions presented above in regard to absolute level of Hb glycation measured by ES-MS are based upon an assumption of identical responses of glycated and non-glycated species in the process of electrospray ionization. If, for the sake of argument, we assume that glycated globins are detected with twice the sensitivity of their non-glycated counterparts, our results would then lead to a contrary conclusion of a tetrameric rather than dimeric nature of the affinity chromatography-bound Hb. In support of our supposition of close similarity between the ES-MS responses of glycated and non-glycated globins we emphasize that molecular ion envelopes generated in the process of electrospray from glycated and non-glycated forms of α and β globins are the same.

3.3. Peptide mapping of glycated hemoglobin

Our goal was to develop a method for monitoring distribution and relative ratios of glycation at the known α and β globin sites within different types of glycated Hb species. To this end, LC–ES-MS analysis of proteolytic fragments generated via a combination of digestion with endoproteinase Glu-C and Asp-N was employed.

Theoretically, Glu-C endoproteinase digestion should segregate all known glycation sites on the β chain into distinct peptides (N-terminal Val-1, Lys-66, Lys-17, Lys-8, Lys144 and Lys-120) [8]. All the expected proteolytic fragments have indeed been recovered (Scheme 1). However, efficiency of proteolysis at the positions βGlu-6 and βGlu-7 under the conditions of Glu-C digestion at pH 7.8 was very low. When Glu-C digestion was performed at pH 4.0, as described by Finke et al. [13], a recovery of the peptide β1–6 was much better. At the same time though, neither efficiency nor specificity of this digestion protocol were adequate for unequivocal mapping of modifications in the C-terminal portion of β globin.

Mapping glycation sites within α globin (N-terminal Val-1, Lys-61 and Lys-40 [13]) utilizing a single digestion scheme is difficult. The number of active Glu-C sites within α globin is limited, and the only useful separation occurs between the N-terminal and C-terminal portions of the molecule. When denatured α globin is treated with Glu-C at pH 7.8, the major proteolytic cleavage occurs at αGlu-30, with the minor cut at αGlu-23. No information regarding putative cleavage at αGlu-27 was obtained. No cleavage at αGlu-116 was observed, in accord with the observation of Seetharam et al. [33].



Scheme 1. Amino acid sequences of human Hb: (a) α and (b) β globin. The dashed (---) and solid (—) lines indicate the peptides generated by Glu-C digestion at pH 7.8 and a combination of Glu-C and Asp-N digestion, respectively. Glycation sites observed in this study are shown in bold.

An alternative Glu-C digestion procedure employing the conditions of pH 4.0 [13] seemed to have slightly diminished efficiency of cleavage at αGlu-23 so that α1–27 peptide could be observed. Still no cleavage at the αGlu-116 site was achieved. Some of the expected cleavages at C-termini of Asp were observed, albeit at low yield. At the same time, a number of non-specific cleavages within the internal and C-terminal portion of α globin sequence was generated (main cleavage at the C-terminus of αLeu-105). Due to these non-specific cleavages, all known α globin glycation sites were separated into individual peptides. However, with an exception of two peptides α31–105 and α106–141, other species were present at low levels thus limiting the general applicability of the GluC digestion at pH 4.0 to detect and quantify minor glycation sites in α globin.

To resolve internal glycation sites within α globin, the fragments α1–30 and α31–141 were digested with endoproteinase Asp-N. All expected proteolytic peptides were observed (Scheme 1) and thus, the secondary digestion step allowed us to map all potential glycation sites within α globin. To facilitate analysis, a combination of Glu-C and Asp-N enzymatic digestion was subsequently examined and it provided excellent coverage of sequences of α and β globins (Fig. 4): with the exception of αLys-7/Lys-11 and βLys-8/Lys-17, all other known glycation sites were separated into distinct proteolytic peptides.

In summary, a comprehensive mapping of all α and β globin glycation sites requires a combination of proteolytic strategies. We have proposed an efficient protocol for analysis of Hb glycation based upon LC–ES-MS of Glu-C (pH 7.8)/Asp-N generated proteolytic fragments.

3.4. Distribution of glycation within α and β globins

Table 1 summarizes the data on distribution of glycation within α and β globins in Hb fractions 1, 2 and 3. LC–ES-MS analysis confirmed great majority of known glycation sites in α and β globin: αLys 61(E10) and βLys 66(E10) constituted the major internal glycation sites, modification of βLys 20(GH3) was not detected. Two new minor sites

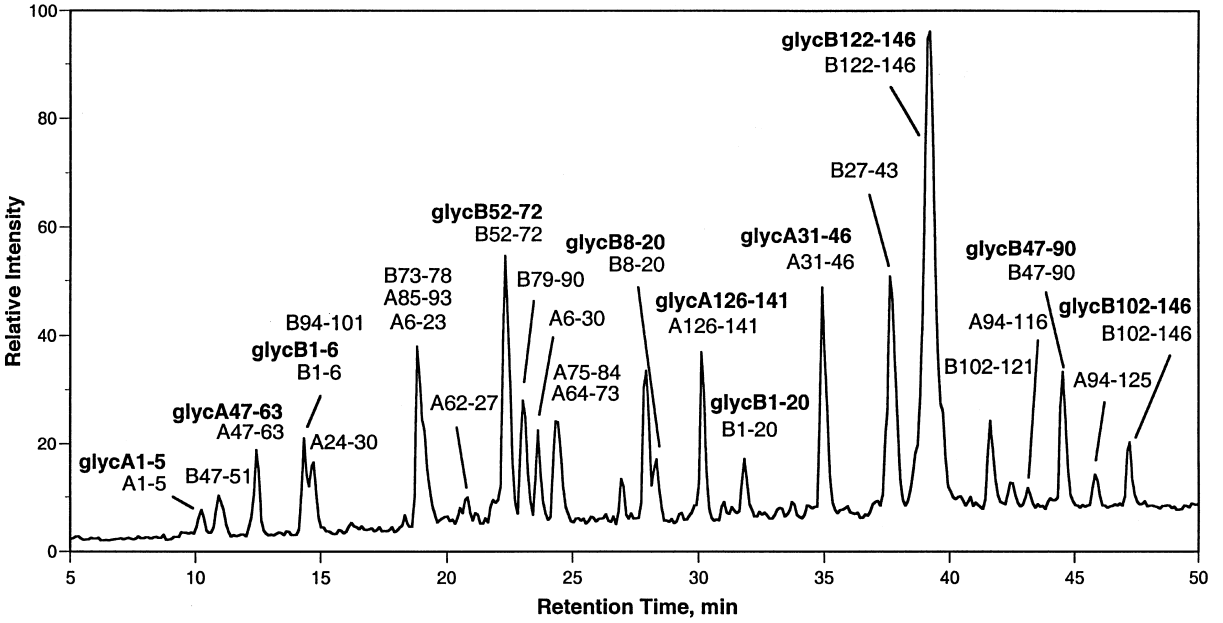


Fig. 4. Total ion current chromatogram (TIC) of proteolytic fragments generated from minor glycated Hb (fraction 2) upon a sequential digestion with endoproteinases Glu-C and Asp-N. With the exception of αLys7/Lys11 and βLys8/Lys17, all other known glycation sites are segregated into different proteolytic peptides. Two new minor sites were detected in α globin: (i) within the peptide α126–141, either at αLys 127(H10) or αLys 139(HC1), and (ii) within the peptide α6–30, either at αLys 16(A14) or αLys 7(A5).

were detected in α globin: (i) within the peptide α126–141, either at αLys 127(H10) or αLys 139(HC1), and (ii) within the peptide α6–30, either at αLys 16(A14) or αLys 7(A5). Regarding the latter site, we postulate that αLys 16(A14), which constitutes the major glycation site in vitro, is also utilized in vivo, albeit to much lower extent.

The major sites of α and β globin glycation are located at the same helical position E10: while in α globin this position is external, in β globin it resides within a heme contact site. The majority of glycation sites are located on the external surface of intact Hb tetramer: the exception is αLys 40(C5); β globin carries glutamine in this position. Given that a rate of Amadori rearrangement is ternary-structure dependent [10], it is plausible to expect that the same helical positions within highly homologous α and β globin will be targeted. Compilation of the literature and own data strongly suggest that this may be the case (Table 1).

The values of relative distribution of glycation (expressed as a percentage) depict a likelihood of the presence of glycation at the specific sites of α and β globins. They are based upon semi-quantification of electrospray signals generated by glycated and non-glycated peptide counterparts in the course of LC–ES-MS. Hb fractions eluting in positions of Hb A1c and Hb A0 demonstrated very similar relative distribution of glycation within α globin. Thus, α globin glycation within these fractions is unlikely to contribute to their distinct mobilities in IE-HPLC. The presence of glycation at the N-terminal Val of α globin was demonstrated in fraction 2 (position of Hb A1d), as postulated before [5]. However, this modification represents less than 50% of total α globin glycation in this fraction. The rest is distributed among the same internal glycation sites as observed before, albeit the relative occupancy of the major αLys 61 site is two times lower. As we have argued above, none of internal α glycation sites appear to affect Hb mobility in IE-HPLC. Thus, the presence of a substantial amount of the non-N-terminally glycated α within fraction 2 cannot be explained.

As expected, no N-terminally glycated β species was observed in fraction 3 (Hb A0) position. At the same time, relative distribution of internal β glyca-

tion in Hb A1c and GHb A0 fractions were very similar, thus arguing against a significant contribution of the internal β glycation to hemoglobin mobility in IE-HPLC. What we found surprising was the presence of high level of N-terminally glycated β peptide in fraction 2 (A1d position) and a reversed order of occupancy of two main internal sites β66 Lys and β144 Lys, as compared to Hb A1c and GHb A0 fractions. However, as we have pointed out before, fraction 2 is unlikely to represent the authentic Hb A1d.

Semi-quantification of glycation occupancy at various sites was based upon an assumption that glycated and non-glycated peptides produced the same responses in ES-MS. No systematic studies were performed to address this issue. In many cases only one charge state of glycated peptide was seen and then it corresponded to the major charge state of its non-glycated counterpart. However, a significant difference in relative intensities of the singly- and doubly-charged ions derived from the non-glycated and glycated forms of β1–6 hexapeptide was observed [12].

In summary we have confirmed the majority of known sites of glycation within α and β globins and have found two new minor glycation sites in α globin. The mobility of glycated Hbs in IE-HPLC is unlikely to be affected by modifications of internal sites and thus, the presence of the α non-N-terminally glycated material in fraction 2 remains unexplained.

3.5. Gas phase fragmentation of glycated peptides

The LC–ES-MS spectra of glycated peptides were complicated by extensive fragmentation of sugar residues. Fig. 5 shows the ES mass spectrum obtained for a mixture of non-glycated and glycated β1–22. In addition to the triply-/doubly-protonated molecular ions derived from non-glycated and glycated β1–22 (m/z 808.2/1212.2 and 862.6/1293.2, respectively), strong ions at m/z 844.5, 834.6, 850.7 and 856.6 were also detected. The ions

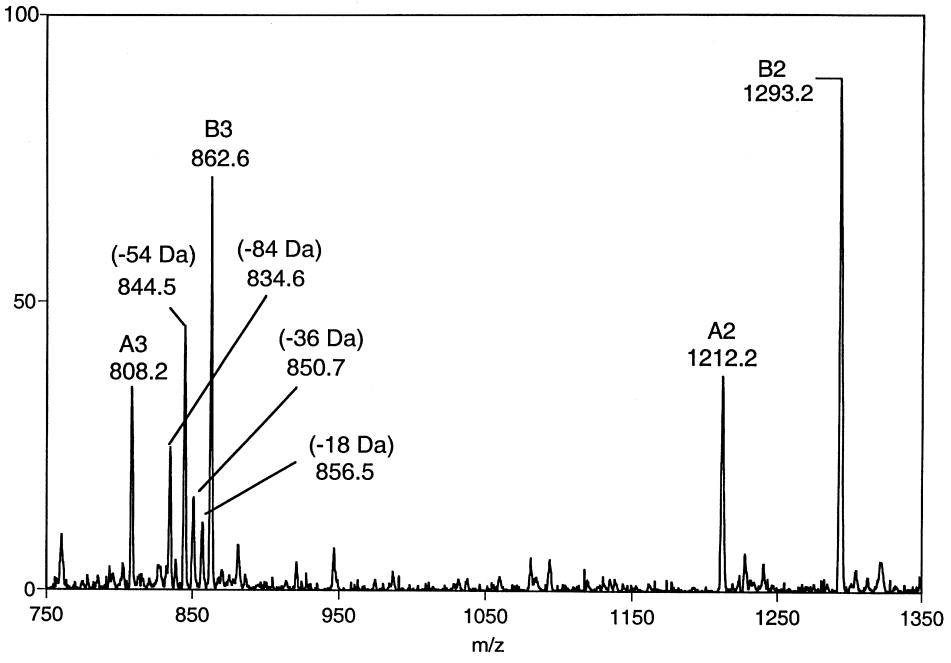


Fig. 5. ES spectrum of a mixture of glycated (B) and non-glycated (A) peptide β1–22 generated from Hb A1c (fraction 1) after its digestion with endoproteinase GluC. Doubly and triply charged states of molecular ions are annotated as B2, A2 and B3, A3, respectively. Peaks marked with −36 Da and −18 Da correspond to the triply-charged products of sugar moiety dehydration and those marked with −54 Da and −84 Da denote ions corresponding to furylium ion and a ''CHOH'' neutral loss from the furylium ion, respectively.

at m/z 844.5 and 834.6 are consistent with the formation of furylium ion and a ''CHOH'' loss from the furylium ion, respectively, as described in detail by Mollé et al. [34] who reported the fragmentation pathway of lactolated peptides. The one and two water losses from sugar moiety result in the dehydrated ions ($MH_3^{3+}-6$) and ($MH_3^{3+}-12$) at m/z 850.7 and 856.6, respectively. Under the LC–ES-MS conditions employed in this study, peptides of M_r around 2000 demonstrated high level of sugar moiety dehydration for the triply- (but not doubly-) charged molecular ions: neutral losses of 54 Da (formation of furylium ion) and 84 Da (CHOH loss from the furylium ion) were responsible for the major product ions (Fig. 5).

The MALDI-TOF-PSD spectrum of the glycated peptide α47–63 is shown in Fig. 6. Extensive b-ion series allowed us to establish the position of glycation at αLys 61(E10); a series of internal fragment ions was also seen. Significantly, with the exception of an immonium ion derived from modified lysine, no product ions carrying intact 1-deoxyfructosyl group were observed. Thus, no y-series ions were generated from the glycated peptide α47–63 while an efficient production of these ions was seen for its non-glycated counterpart (data not shown).

4. Conclusions

Comparative quantification of Hb glycation was performed utilizing a combination of chromatographic and mass spectrometric steps: phenylboronate chromatography, ES-MS of hemolysates and Hb sub-

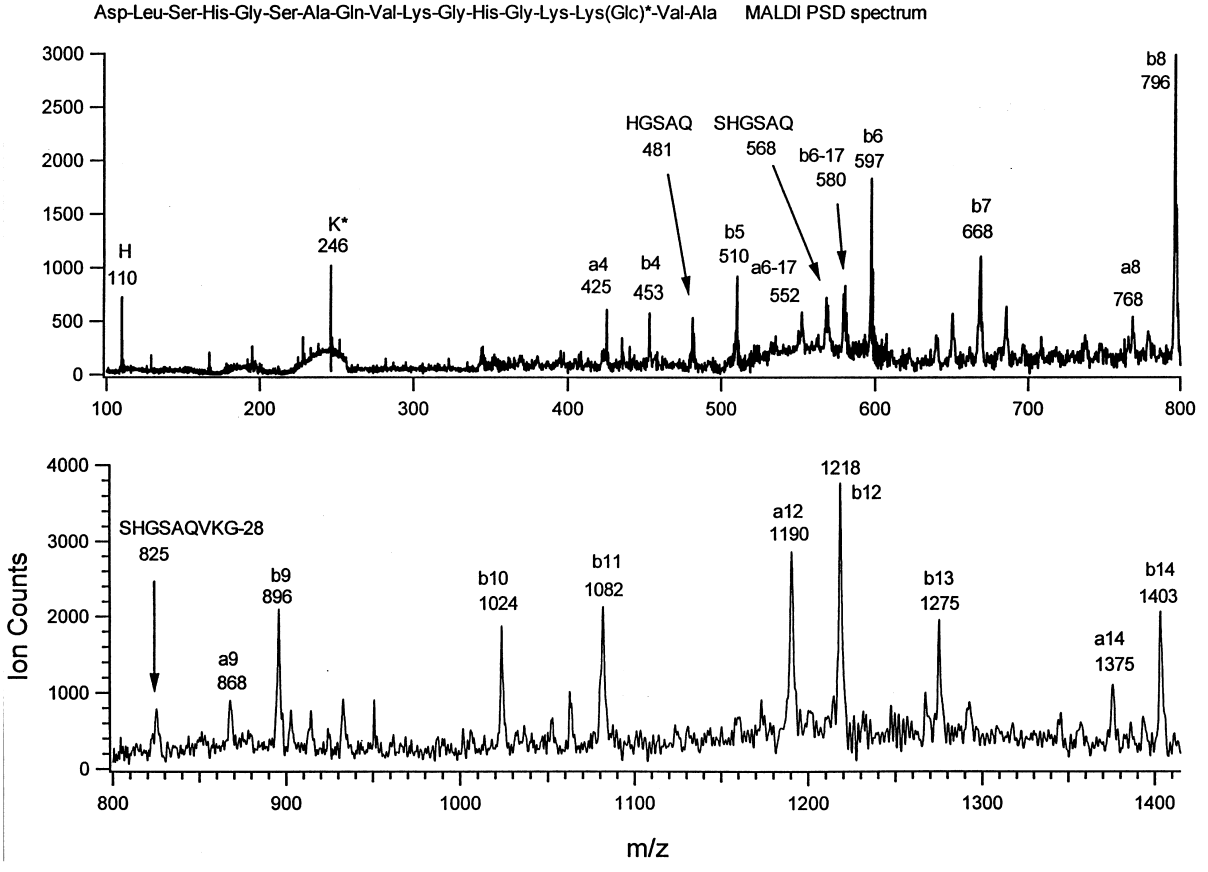


Fig. 6. MALDI-TOF-PSD spectrum of the peptide α47–63 glycated at Lys-61; the position of glycation is annotated with an asterisk.

populations isolated by a combination of phenylboronate affinity chromatography and IE-HPLC. The results of the above experiments allow us to draw the following conclusions. (1) Glycated Hb is preferentially bound to phenylboronate resin in a dimeric rather than a tetrameric form. Hence, previous studies based on affinity chromatography underestimated by twofold the fraction of native tetramers carrying glycation. (2) Phenylboronate chromatography demonstrates good sensitivity (all glycated dimers are detected and no glycation sites are missed) and good specificity (negligible amount of non-glycated Hb dimers are co-purified). (3) The allocation of glycation between α and β globins is independent of the total glycation level, i.e., it is the same for normal and diabetic subjects. (4) Multiple derivatization within single globin chain is not discernable even at the highest level of Hb glycation studied (17.5%). While the presence of αβ dimers glycated at both globins cannot be excluded, these products of ''over-glycation'' at best represent the minor species.

Two-step protein digestion with endoproteinases Glu-C and Asp-N, followed by LC–ES-MS of proteolytic fragments offers an efficient method of mapping all known glycation sites in human Hb. Given the low level of glycation at many sites, use of this method for accurate quantification of glycation site occupancy will require rigorous validation with appropriate standards to account for putative differences in ES-MS responses of glycated and non-glycated Hb peptides.

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