Site-Selective Modifications of Arginine Residues in Human Hemoglobin Induced by Methylglyoxal

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Received July 13, 2006; Revised Manuscript Received October 31, 2006

ABSTRACT: Methylglyoxal (MG) is an important glycating agent produced under physiological conditions. MG could react with DNA and proteins to generate advanced glycation end products. Human hemoglobin, the most abundant protein in blood cells, has not been systematically investigated as the target protein for methylglyoxal modification. Here we examined carefully, by using HPLC coupled with tandem mass spectrometry (LC−MS/MS), the covalent modifications of human hemoglobin induced by methylglyoxal. Our results revealed that hemoglobin could be modified by methylglyoxal, and the major form of modification was found to be the hydroimidazolone derivative of arginine residues. In addition, Arg-92 and Arg-141 in the α chain as well as Arg-40 and Arg-104 in the *β* chain were modified, whereas two other arginine residues, that is, Arg-31 in the α chain and Arg-30 in the *β* chain, were not modified. Semiquantitative measurement for adduct formation, together with the analysis of the X-ray structure of hemoglobin, showed that the extents of arginine modification were highly correlated with the solvent accessibilities of these residues. The facile formation of hydroimidazolone derivatives of arginine residues in hemoglobin by methylglyoxal at physiologically relevant concentrations suggested that this type of modification might occur *in vivo*. The unambiguous determination of the sites and extents of methylglyoxal modifications of arginines in hemoglobin provided a basis for understanding the implications of these modifications and for employing this type of hemoglobin modification as molecular biomarkers for clinical applications.

Methylglyoxal (MG[1]) is a highly reactive α-oxoaldehyde that can be induced endogenously (*1*). The primary source of MG is from triose phosphate generated during glycolysis, dihydroxyacetone phosphate, and glyceraldehyde-3-phosphate (*2*). The level of MG can be elevated upon the increase of metabolites which stimulate the influx of triose phosphate, i.e., glucose, fructose, dihydroxyacetone, and hydroxyacetone (*3*−*5*). The rate of MG formation was calculated to be 120 *μ*M/day under normoglycemic conditions (*1*). Many factors could trigger the accumulation of MG *in vivo*, which include aging, hyperglycemia, inflammation, oxidative stress, and uremia (*6*). In this respect, the median concentration of MG is increased by 5−6-fold and 2−3-fold in blood samples of diabetic patients with insulin-dependent (type I) and non-insulin-dependent (type II) diabetes mellitus, respectively (*7*). In addition, the concentrations of MG in blood samples of patients with type I diabetes mellitus correlate positively with the duration of the disease and increase at a rate of approximately 10% of the control values per year (*7*).

As an important glycating agent, MG could react with proteins, DNA, and other substrates to generate advanced glycation end products (AGEs) (*8*−*12*), which could cause enzyme activity inhibition (*13*), transcriptional activation (*14*, *15*), apoptosis (*16*), and tissue injury (*17*). Although arginine (*8*, *10*), lysine (*10*, *18*), and cysteine (*11*) residues could be modified by MG, proteins are predominantly modified on arginine residues (*19*) and the dominant arginine adduct was reported to be N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine (MG-H1, Scheme 1) (*20*−*22*).

Previous studies revealed that MG could induce the modifications of several human proteins, which include human serum albumin (*12*), heat-shock protein 27 (*23*), and α-crystallin (*17*). Hemoglobin (Hb), the most abundant protein in human blood cells, has not been well studied as the target for MG modification, though HbA1c, a subfraction of hemoglobin in which the N-terminal valine residue of the *β* chain is nonenzymatically glycated, was identified more than 40 years ago and it has been employed as a biomarker for diabetes mellitus (*24*). In this context, there is only one very brief publication reporting that the incubation of human hemoglobin with MG could result in the formation of MG-H1 adducts. The sites of modification were reported to be arginine residues 31 in the α chain as well as arginine residues 30, 40, and 104 in the *β* chain, with the latter two being the most reactive sites (*25*).

Herein, we investigated systematically the sites of MG modifications in human hemoglobin and found that four out of six arginine residues in this protein could be modified by MG *in vitro*. In addition, we quantified the percent yields of MG-modified peptides by LC−MS/MS, and it turned out that the levels of arginine modification correlated well with the solvent accessibilities of these arginine residues.

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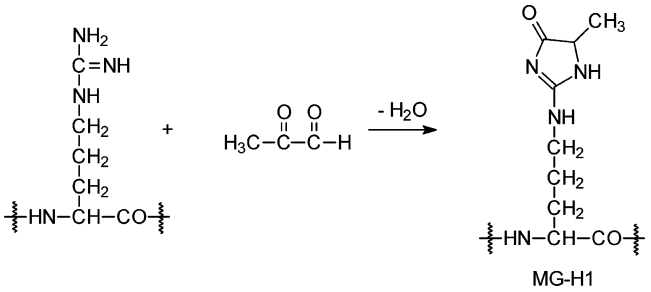
[1] Abbreviations: MG, methylglyoxal; MG-H1, N^{δ}-(5-hydro-5-methyl-4-imidazolon-2-yl)-ornithine; Hb, hemoglobin; AGEs, advanced glycation end products; MS/MS, tandem mass spectrometry.

10.1021/bi061410o CCC: $33.50
Published on Web 12/19/2006

Scheme 1: Reaction of Methylglyoxal with Arginine



MATERIALS AND METHODS

Materials. MG (40% aqueous solution) and human hemoglobin were purchased from Sigma (St. Louis, MO). Synthetic peptides were obtained from Quality Controlled Biochemicals, Inc. (Hopkinton, MA).

Preparation of Modified Hemoglobin. Human hemoglobin at a concentration of 100 μM was incubated with varying concentrations of MG (10 μM−200 mM) in PBS buffer (pH 7.4) at 37 °C for 24 h. Excess MG was removed by using Microcon YM-10 centrifugal filters (Millipore, Billerica, MA). The sample in the control experiment was treated in the same way except that no MG was added.

Preparation of MG-Modified Peptides. Synthetic peptides at a concentration of approximately 1 mM were treated with 4 mM MG at 37 °C for 24 h, and the resulting mixtures were separated by HPLC on a Surveyor system (ThermoFinnigan, San Jose, CA) with a photodiode array detector, which was set at 220 nm for monitoring the effluents. A 4.6 × 250 mm Vydac reversed-phase C18 column (5 μm in particle size and 300 Å in pore size, Grace Vydac, Hesperia, CA) was used. The gradient was 8 min of 0−18% acetonitrile in 0.1% TFA followed by 33 min of 18−40% acetonitrile in 0.1% TFA, and the flow rate was 1.0 mL/min. The HPLC fractions were then dried in a Speed-vac and stored in a −20 °C freezer.

Enzymatic Digestion, LC−MS/MS, and Database Search. The modified hemoglobin was subjected directly to digestion by either Lys-C or chymotrypsin (Roche Applied Science, Indianapolis, IN). The enzyme-to-substrate ratio was 1/10 (by weight), and the digestion was carried out in a 50 mM NH_4HCO_3 buffer (pH 8.0) at 37 °C for 18 h. MALDI-MS analysis of the resulting digestion samples showed no signals for the full-length protein, indicating that the digestion was close to completion.

LC−MS/MS experiments were performed with a Zorbax SB C18 column (0.5 × 150 mm, 5 μm in particle size, and 80 Å in pore size, Agilent Technologies, Palo Alto, CA) and an LTQ linear ion-trap mass spectrometer (Thermo Electron Co., San Jose, CA). A gradient of 65 min of 2−65% acetonitrile in 0.6% acetic acid was employed. The flow rate was 6.0 μL/min, which was delivered by an Agilent 1100 capillary pump (Agilent Technologies).

For the identification of peptides, MassAnalyzer 1.03 (*26*), which was kindly provided by Dr. Zhongqi Zhang at Amgen Inc., was employed to search against the sequence of human hemoglobin.

Semiquantification of MG Modification on Arginines. The quantification experiments were carried out by using LC−MS/MS in selective-ion monitoring (SIM) mode (the precursor ions chosen for quantification are listed in Table 1). The percent yield for the formation of MG-H1-bearing peptide was calculated based on the ratio of peak area for the ion corresponding to the modified peptide over the sum of peak areas corresponding to both the modified and unmodified forms of the same peptide; in this respect, the peak areas were obtained from the total ion chromatograms (TICs) for monitoring the fragmentation of the chosen precursor ions.

Heme Release Analysis. Hemoglobin (100 μM) was incubated with MG at a concentration of 10 μM, 200 μM, or 4 mM at 37 °C for 24 h, and the released heme from the resulting mixtures was isolated by using YM-10 Microcon centrifuge filters. The heme content was measured by LC−MS, and a 45 min gradient of 50−90% acetonitrile in 0.6% acetic acid was employed for the separation.

RESULTS

Sequence Coverage of Hemoglobin from LC−MS/MS Analysis. Human hemoglobin is a tetramer, which mainly consists of two identical α chains and two identical β chains. The sequence coverages for the α and β chains of hemoglobin from our LC−MS/MS experiments are 94% and 97%, respectively (Figure 1). The high sequence coverage makes it feasible to investigate thoroughly the MG modifications of the protein.

Hydroimidazolone Is the Major Form of Arginine Modification in Hemoglobin Induced by MG. In proteins, arginine residues are the dominant targets for MG modification (*19*). Although the predominant MG derivative of arginine was reported to be MG-H1 (Scheme 1) (*20*−*22*), other studies showed that argpyrimidine could be the major form (*8*, *27*). In our experiment, we first explored both possibilities by using LC−MS/MS to monitor the enzyme-digested hemoglobin samples that had been treated with MG at a concentration of 10 μM, 200 μM, or 4 mM. The arginine modification was found to increase the mass of the peptides by 54 Da, which is consistent with the formation of arginine-derived MG-H1, but not the argpyrimidine adduct. This result, therefore, supports that the major arginine adduct induced by MG is MG-H1.

To further substantiate this conclusion, we treated three synthetic peptides (Ac-MAKLRVDPVNFK-COOH, Ac-YKRLL-COOH, and Ac-MKYR-COOH) with MG. In this respect, each of the three peptides carries the sequence of one of reactive arginine-bearing hemoglobin peptides (*vide infra*). We then separated the reaction mixtures by HPLC and analyzed the HPLC fractions by ESI-MS and MS/MS (Figures S3−S8, Supporting Information). ESI-MS analysis showed that each peptide was modified by MG with a mass increase of 54 Da, and the MS/MS results demonstrated that the arginine residues in these peptides were converted to the MG-H1 derivatives (Figures S4−S8).

Arg-92 and Arg-141 in the α Chain as Well as Arg-40 and Arg-104 in the β Chain Could Be Modified by MG in Vitro. Both the α and β chains of human hemoglobin harbor three arginine residues, i.e., Arg-31, Arg-92, and Arg-141 in the α chain and Arg-30, Arg-40, and Arg-104 in the β chain (Figure 1). The high sequence coverage offered by LC−MS/MS analysis enabled us to monitor specifically the formation of the MG-H1 adduct at individual arginine residues. To this end, we subjected the enzymatic digestion mixtures of MG-treated hemoglobin to LC−MS/MS analysis,

Table 1: Arginine-Containing Peptides in Human Hemoglobin Identified by LC−MS/MS and Ions Used for the Semiquantification of the Levels of Arginine Modification

		unmodified peptides			MG-H1-bearing peptides		
arginine position	peptide	calcd *m/z*, $[M + H]^+$	charge state	measd *m/z*	calcd *m/z*, $[M + H]^+$	charge state	measd *m/z*
R31, α	VGAHAGEYGAEALERMFLSFPTTK[a]	2583.9	+3	862.2	2638.0	nd[b]	nd
R92, α	LRVDPVNFK[a]	1088.3	+2	544.8	1142.4	+2	571.8
R141, α	YR[a]	338.4	+1	338.4	392.4	+1	392.4
R30, *β*	GKVNVDEVGGEALGRLL[c]	1727.0	+2	864.0	1781.0	nd	nd
R40, *β*	TQRFFESFGDLSTPDAVM[c]	2049.3	+2	1025.0	2103.3	+2	1052.0
R104, *β*	RLL[c]	401.5	+1	401.5	455.6	+1	455.5

[a] From Lys-C digestion. [b] Not detectable. [c] From chymotrypsin digestion.

α Subunit (From Lys-C Digestion, % AA=94)

VLSPADKTNV KAAWGKVGAH AGEYGAEALE **R**MFLSFPTTK
TYFPHFDLSH GSAQVKGHGK KVADALTNAV AHVDDMPNAL
SALSDLHAHK L***R***VDPVNFKL LSHCLLVTLA AHLPAEFTPA
VHASLDKFLA SVSTVLTSKY ***R***

β Subunit (From Chymotrypsin Digestion, % AA=97)

VHLTPEEKSA VTALWGKVNV DEVGGEALG**R** LLVVYPWTQ***R***
FFESFGDLST PDAVMGNPKV KAHGKKVLGA FSDGLAHLDN
LKGTFATLSE LHCDKLHVDP ENF***R***LLGNVL VCVLAHHFGK
EFTPPVQAAY QKVVAGVANA LAHKYH

FIGURE 1: A summary of sequence coverage for human hemoglobin from the peptides identified by LC−MS/MS analysis of the peptide mixtures resulting from Lys-C (for the α chain, top panel) and chymotrypsin (for the *β* chain, bottom panel) digestion. The arginine residues are shown in bold letters, and MG-modified arginines are also shown in italics. The covered residues are underlined. The human hemoglobin sequence was obtained from SwissProt.

where we monitored specifically the fragmentations of unmodified arginine- and MG-H1-bearing peptides (Table 1). It turned out that, other than Arg-40 and Arg-104 in the *β* chain which were previously shown to be the major sites modified by MG *in vitro* (*25*), Arg-92 and Arg-141 in the α chain could also be modified by MG *in vitro* to give MG-H1 adducts (Figures 2 and 3 and Figures S1 and S2, Supporting Information). We also examined the possibility of the modification of Arg-31 in the α chain and Arg-40 in the *β* chain. It turned out that we failed to find the MG-H1-bearing peptides, though we observed the corresponding unmodified arginine-carrying peptides (MS/MS shown in Figures S9 and S10, Supporting Information). Moreover, none of six arginine residues were modified in the control sample while no MG was added (data not shown).

Here we discuss the tandem mass spectra supporting the assignments of the arginine modifications. In the MS/MS of the $[M + 2H]^{2+}$ ions of the unmodified and MG-H1-bearing peptide LRVDPVNFK (residues 91−99 in the α chain), the *m/z* difference for the b_2 ions observed in the top (for unmodified peptide) and bottom (for modified peptide) panels of Figure 2 is 54 Da, which is consistent with the modification of Arg-92. In addition, the measured *m/z* values for all y ions (i.e., y_2, y_3, y_5, y_6, and y_7 ions) found for the modified peptide are the same as those for the corresponding y ions observed for the unmodified peptide (Figure 2), again showing that Arg-92 is modified.

Comparison of the product-ion spectra for RLL (residues 104−106 in the *β* chain) and the same peptide with the arginine being replaced with a MG-H1 also confirmed the arginine modification (Figure 3). In this respect, both b_1 and b_2 ions found for the modified peptide exhibited a 54 Da upshift in mass, whereas the *m/z* value for y_2 ion is the same as that for the unmodified peptide. In addition, we observed an ion carrying the side chain of the MG-H1 component (*m/z* 166, Figure 3b), supporting the presence of MG-H1 in this tripeptide. Similar MS/MS analysis revealed that Arg-141 in YR (residues 140−141 in the α chain) and Arg-40 in TQRFFESFGDLSTPDAVM (residues 38−55 in the *β* chain) could be modified to give MG-H1 upon MG treatment (spectra shown in Figures S1 and S2).

Semiquantitative Measurement of Dose-Dependent Modification of Hemoglobin by MG. After having identified the arginine residues that are susceptible to MG modification, we carried out a dose-dependent experiment, where we treated hemoglobin (100 *μ*M) with MG at a concentration of 0, 10, 45, 200, 900 *μ*M, 4 mM, or 18 mM. We then estimated, based on the intensity ratios of ions corresponding to unmodified and MG-H1-bearing peptides, the yields for the formation of MG-H1 at the above four reactive sites. In this context, previous positive-ion ESI-MS measurements showed that the N-terminal peptides resulting from the Glu-C digestion of both unmodified hemoglobin *β* chain and HbA1c gave the doubly charged ions as the most abundant ionized species; however, the singly charged ions of these two peptides exhibited different relative abundances (*28*). As a result, calibration plots for the measurements of HbA1c based on singly or doubly charged ions of the N-terminal peptides gave different slopes (*28*). In positive-ion ESI-MS, peptides RLL and YR only exhibit as singly charged ions; thus, singly charged ions were employed for the quantification of arginine modification in these two peptides. Peptides LRVDPVNFK and TQRFFESFGDLSTPDAVM, on the other hand, can be both di- and triprotonated. We, however, did not observe significant difference in the charge state distributions for the modified and unmodified forms of the latter two arginine-bearing peptides. Along this line, we found that the quantification based on the triply charged ions of the latter two peptides (data not shown) gave results similar to those from the measurements based on doubly charged ions (Figure 4 and Figure S11, Supporting Information).

As depicted in Figure 4 and Figure S11, the percentage of arginine modification rose with the increase of MG concentration. Moreover, at low and medium concentrations of MG, the percent yield of modification of each of the four arginines was found to be proportional to MG concentration (Figure 4).

It is worth emphasizing that the above quantification is based on an assumption that the unmodified peptide and the

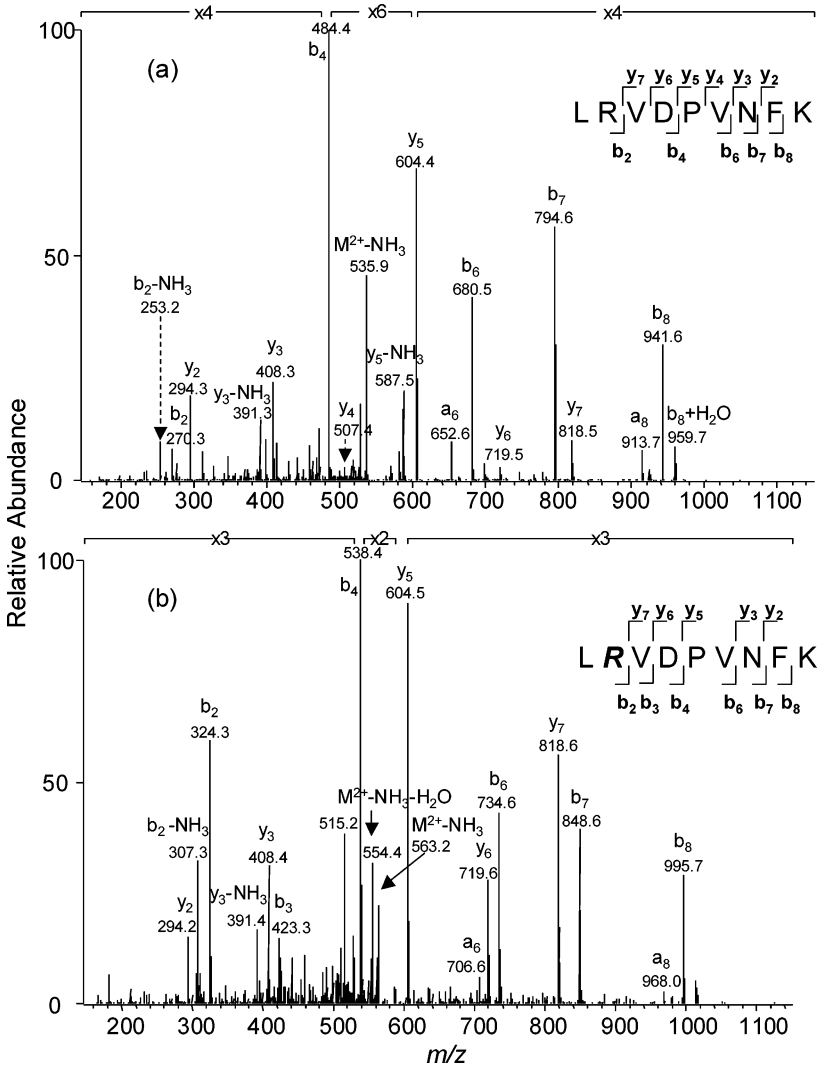


FIGURE 2: Product-ion spectra of the $[M + 2H]^{2+}$ ions of the unmodified (top panel) and MG-modified (bottom panel) peptide L91-K99 of the α chain of human hemoglobin. A summary of the observed b^+ and y^+ ions is shown above the spectrum. The "R" in boldface and italics indicates the MG-modified arginine residue.

MG-H1-bearing peptide share similar ionization efficiency under our experimental conditions. The conversion of arginine to MG-H1 may alter the ionization efficiency of peptides; the actual yield for the formation of MG-H1, therefore, may be somewhat different from what we estimated above.

The Extents of Arginine MG-Modifications Correlate with Their Surface Exposable Areas. The above data revealed that there is a remarkable selectivity for the arginine residues being modified (Table 2 and Figure 4). We next asked whether the selectivity in reactivity of arginines could be attributed to the differences in solvent accessibility of these residues. To this end, we employed a web-based program GETAREA (http://www.scsb.utmb.edu/cgi-bin/get_a_form.tcl) and measured the surface exposable area for each arginine residue in human hemoglobin based on the X-ray structure of the protein (Table 2).

It turned out that indeed the reactivity of arginine residues in hemoglobin toward MG is highly correlated with the solvent accessibilities of these residues. For instance, Arg-31 in the α chain and Arg-30 in the β chain are buried inside the protein and their surface exposable areas are 3.8 and 3.6 Å^2, respectively (Figure 5). This is consistent with the fact that we were not able to detect the peptides bearing a MG-H1 derived from these two arginines (Tables 1 and 2). Among the six arginines in human hemoglobin, Arg-104 in the β chain is the most accessible to solvent (103 Å^2 in surface exposable area), which is in keeping with the highest yield for the formation of MG-H1 at this arginine (Table 2 and Figure 4). In addition, Arg-92 and Arg-141 in the α chain as well as Arg-40 in the β chain exhibit surface exposable areas of 40, 69, and 61 Å^2, respectively, which is in agreement with our experimental observations that these three arginines are susceptible to MG modification, but the yields for the formation of adducts at these three sites are lower than that for Arg-104 in the β chain (Table 2).

Formation of MG-H1 Does Not Cause Obvious Heme Release from Hemoglobin. Hemoglobin in vertebrates transports oxygen from the lungs to the rest of the body. In this process, heme is responsible for the binding of oxygen. Therefore, it is important to examine whether MG treatment could induce the release of heme from this protein. In this respect, an earlier report showed that the treatment of hemoglobin with the anticancer drug cisplatin under physiological conditions could cause the relesase of heme from hemoglobin (*29*). We followed similar procedures, and it

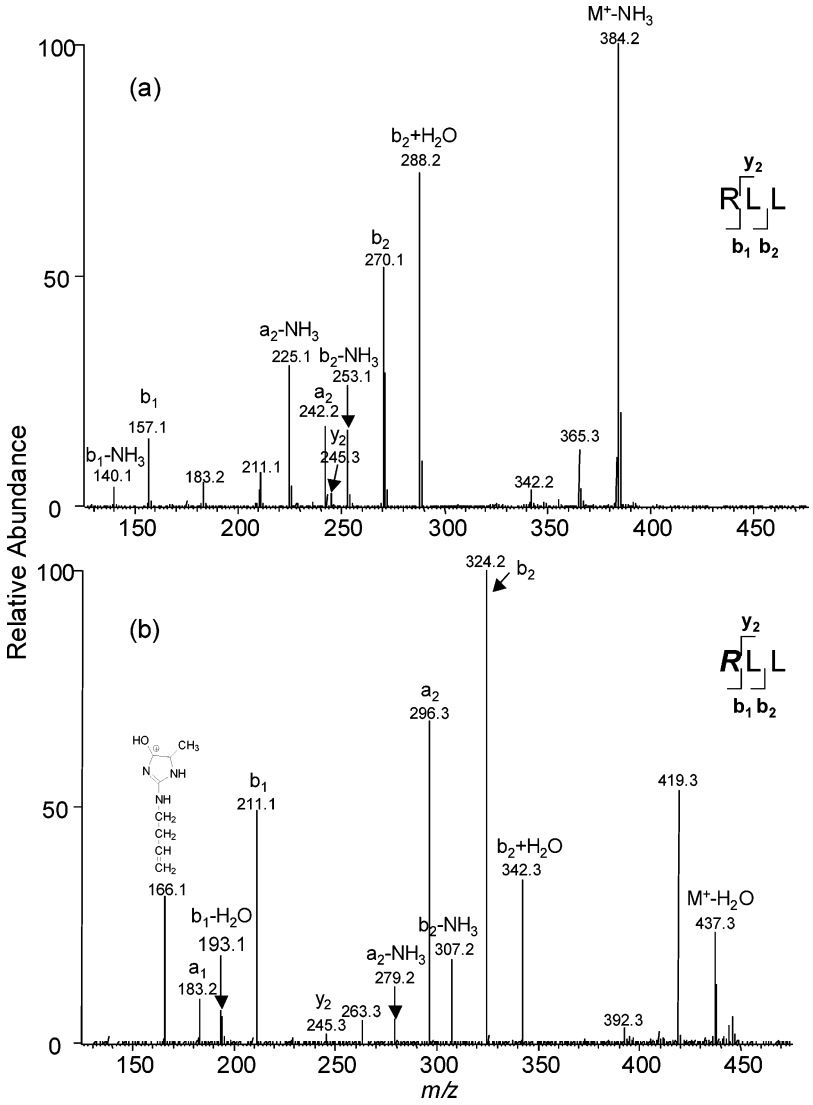


FIGURE 3: Product-ion spectra of the $[M + H]^+$ ions of the unmodified (top panel) and MG-H1-containing (bottom panel) peptide R104-L106 of the β chain of human hemoglobin. A summary of the observed b^+ and y^+ ions is shown above the spectrum. The "R" in boldface and italics represents the MG-modified arginine residue.

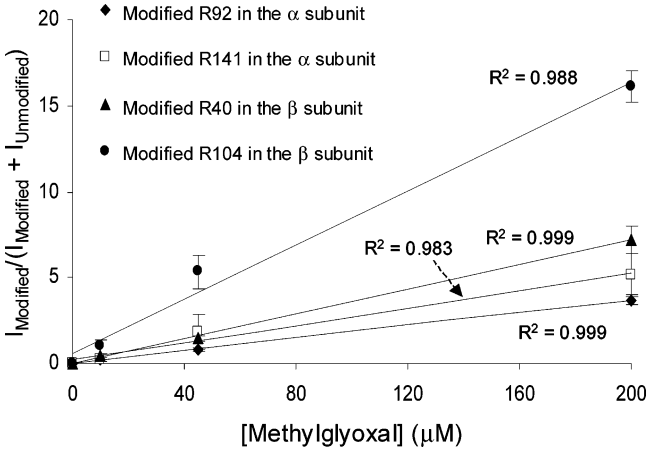


FIGURE 4: Percent yield of MG-modified arginine containing peptides upon MG treatment under low and medium MG concentrations. The data represent the means and standard deviations of results from three independent oxidation and LC−MS/MS measurements.

turned out that there is no obvious difference in the amount of heme released from hemoglobin with or without MG treatment. This is very likely due to the fact that the four reactive arginine residues are not directly involved in heme binding. In addition, the conversion of arginine to MG-H1 on the surface of hemoglobin may not induce significant change of the conformation of hemoglobin. These factors may explain why the MG-induced covalent modification does not result in heme release from this protein.

Table 2: The Extent of MG-H1 Formation Is Correlated with the Surface Exposure Areas of Arginine Residues in Human Hemoglobin[a]

arginine residue	surface exposure area[b] (Å^2)	% of modified arginine upon 200 μM MG treatment
R31, α chain	3.8	nd[c]
R92, α chain	40	3.7
R141, α chain	69	5.2
R30, β chain	3.6	nd
R40, β chain	61	7.2
β104, β chain	103	16.1

[a] Based on the X-ray structure of the Oxy T state of hemoglobin (PDB ID: 1GZX). [b] Solvent-accessible surface areas were calculated by using GETAREA 1.1 with water probe at a radius of 1.4 Å. [c] Not detectable.

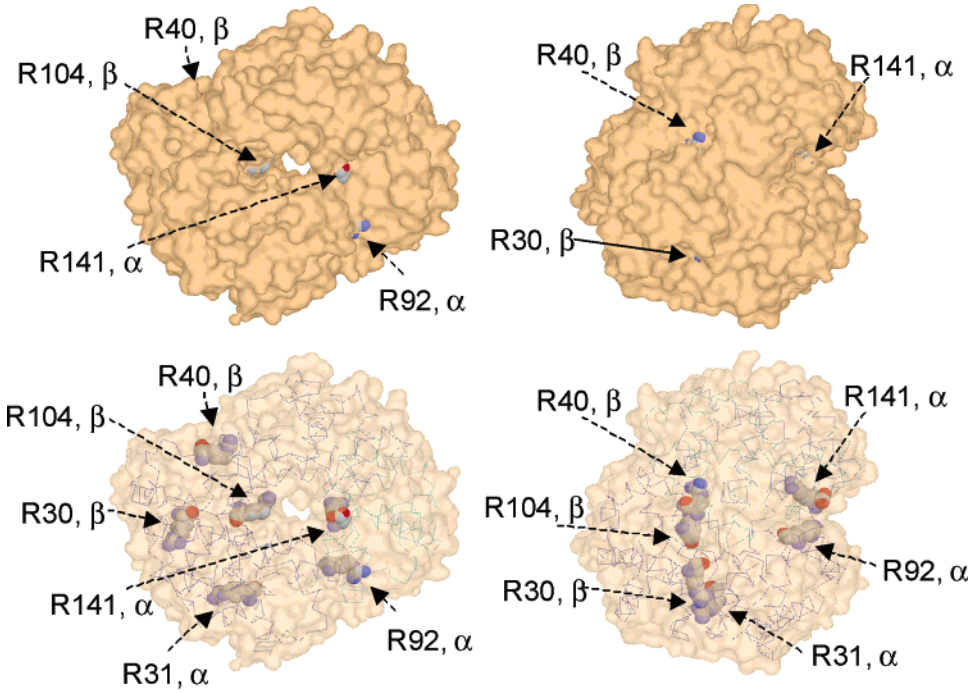


FIGURE 5: Structure of Oxy T state hemoglobin (PDB ID: 1GZX) shown in surface mode (top and bottom panels depict solid and transparent surfaces, respectively). The arginine residues are depicted in space-filling model. Graphs were generated by using Protein Explorer (http://molvis.sdsc.edu/protexpl/frntdoor.htm).

DISCUSSION

Our study established without ambiguity the nature of arginine modification in human hemoglobin induced by MG. We found that, upon treatment with physiologically relevant concentrations of MG, four out of six arginines in hemoglobin could be modified to give MG-H1 as the dominant adduct. The facile formation of the MG-H1 products in hemoglobin suggests that these modifications may occur under physiological conditions *in vivo*. We also demonstrated that MG modification occurred only on those arginine residues that are solvent-exposable and the percent yields for the MG-H1 modification of arginine residues were correlated with the solvent accessibilities of these residues.

The unambiguous identification of the modification sites and the quantitative information about the yields for the modifications of arginine residues may facilitate the potential application of the MG-H1 adducts of hemoglobin as biomarkers for clinical and pathological applications. In this context, the MG-H1 product of hemoglobin, similar as HbA1c, may serve as an indicator for long-term glycemic control in patients with diabetes mellitus. In addition, our results may provide a basis for understanding the biological implications of elevated concentrations of methylglyoxal, which are associated with pathological conditions including diabetes and age-related pathologies, such as atherosclerosis and Alzheimer's disease.

Phenylglyoxal and 1,2-cyclohexanedione have long been employed as arginine-specific reactive reagents for probing the surface arginine residues in proteins and active site arginine residues in enzymes (*30*, *31*). The combination of this chemical modification with mass spectrometry has also been used for mapping the surface arginine residues in proteins (*32*). Our data here suggest that MG might be employed for similar purposes.

SUPPORTING INFORMATION AVAILABLE

HPLC traces for the separation of the reaction mixtures of synthetic peptides with methylglyoxal; MS/MS data for unmodified arginine- and MG-H1-carrying peptides. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI061410O