Peptide Mapping Identifies Hotspot Site of Modification in Human Serum Albumin by Methylglyoxal Involved in Ligand Binding and Esterase Activity*

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Methylglyoxal is a potent glycating agent under physiological conditions. Human serum albumin is modified by methylglyoxal in vivo. The glycation adducts formed and structural and functional changes induced by methylglyoxal modification have not been fully disclosed. Methylglyoxal reacted with human serum albumin under physiological conditions to form mainly the hydroimidazolone \( \text{N}^\text{H}-\text{5-hydro-5-methyl-4-imidazolazon-2-yl-ornithine} \) (MG-H1). There are two other related hydroimidazolone structural isomers, but MG-H1 dominates in proteins glycated under physiological conditions (2, 3, 5, 6). Other minor methylglyoxal-derived AGEs are a tetrahydropyrimidine \( \left( \text{N}^\text{H}-\text{5-[2,3,4-trihydroxybutyl]-5-hydro-4-imidazolazon-2-ylornithine} \right) \), argpyrimidine, \( \text{N}^\text{H}-\text{carboxethyllysine, and methylglyoxal-derived lysine dimer (1,3-dimethylimidazolium salt)} \) (reviewed in Ref. 6).

Glycation by methylglyoxal, unlike glycation by glucose, is mainly arginine-directed and produces a loss of positive charge by hydroimidazolone formation (7). An important target of glycation in vivo is human serum albumin (HSA), the major protein of human blood plasma. The sites and functional effects of formation of the major glucose-derived glycation adduct fructosyl-lysine have been determined (8). In contrast, the sites of glycation of HSA by methylglyoxal have not, and neither have structural and functional changes induced by methylglyoxal modification been characterized. Related to this, however, glyoxal derivatives have been used to interrogate active sites and functional domains of proteins for critical arginine residues, exploiting the mainly arginine-directed modification by these agents (9).

In this report, we describe the sites of modification of HSA by methylglyoxal under physiological conditions and show that similar glycation occurs in vivo. The associated functional effects, inhibition of ligand binding and esterase activity, were characterized, and structural changes were predicted to explain the consequences of methylglyoxal modification for albumin functionality.

EXPERIMENTAL PROCEDURES

Materials—HSA (fatty acid-free) was purchased from Sigma. Methylglyoxal, \( \text{N}^\text{H}-\text{(1-deoxy-o-fructos-1-yllisine) (referred to as fructosyl-lysine)} \) and AGE authentic standards, including stable isotope-substituted analogues, were prepared as described (6). High purity methylglyoxal was prepared and purified as described (10).

Preparation of Human Serum Albumin Glycated by Methylglyoxal—HSA glycated minimally by methylglyoxal-derived glycation adducts \( \text{(MG}_{\text{min}}-\text{HSA)} \) was prepared by incubation of methylglyoxal (500 \( \mu \text{M} \)) with HSA (6.6 mg/ml) in sodium phosphate buffer (100 mM, pH 7.4) at 37 °C for 24 h. A similar preparation was made of control protein of HSA incubated without methylglyoxal. Further preparations were made with and without methylglyoxal but preincubated for 30 min and throughout the glycation period with 120 \( \mu \text{M} \) ketoprofen (to block drug-matography with triple quadrupole mass spectrometric detection; \( \text{MG}_{\text{min}}-\text{HSA} \), human serum albumin minimally modified by methylglyoxal; MG-H1, \( \text{N}^\text{H}-\text{5-hydro-5-methyl-4-imidazolazon-2-yl-ornithine} \).
binding site II). The glycated and control HSA preparations were dialyzed against 30 mM ammonium bicarbonate at 4°C, lyophilized to dryness, and stored at -20°C. Validation studies showed that lyophilization did not change the AGE content of these proteins significantly.

**Protein Glycation Adduct Determination by LC-MS/MS and Tryptic Digestion and Peptide Mapping**—Protein glycation adduct residues were determined in exhaustive enzymatic digests (50 μg of protein equivalent) by LC-MS/MS and stable isotope-substituted standard internal standardization, as described (3). MG<sub>min</sub>-HSA and HSA control (100 μg) were diluted to 20 μl with water. Aliquots of 40 mM HCl (25 μl), peptin solution (2 mg/ml in 20 mM HCl; 5 μl), and thymol solution (2 mg/ml in 20 mM HCl; 5 μl) were added, and the sample was incubated at 37°C for 24 h. The sample was then neutralized and buffered at pH 7.4 by the addition of 25 μl of 0.5 M potassium phosphate buffer, pH 7.4, and 5 μl of 260 mM KOH. Pronase E solution (2 mg/ml in 10 mM potassium phosphate buffer, pH 7.4, 5 μl) was added, and the sample was incubated at 37°C for 48 h under nitrogen (6). This gave the final enzymatic hydrolysate (100 μl) for the AGE assay. AGE and amino acid recoveries were 70–100%; fructosyl-lysine recovery was 71% (3). Analytes released by self-digestion of glycation adduct samples were assayed for glycation adducts by LC-MS/MS and stable isotope-substituted standard internal standardization.

The samples were assayed for glycation adducts by LC-MS/MS and stable isotope-substituted standard internal standardization. The samples were assayed for glycation adducts by LC-MS/MS and stable isotope-substituted standard internal standardization. The spectra were solvent base line-corrected, and the concentration of the control HSA and MG<sub>min</sub>-HSA was determined assuming ε<sub>730</sub> = 0.531 mg/ml (12). The spectra were deconvoluted using a principle component regression analysis method to deduce the proportion of α-helix, β-sheet, and random coil in the secondary structure of the proteins (13, 14).

**Drug Binding Studies and Esterase Activity Studies**—The binding of ketoprofen to binding site II, domain 3A to HSA, and MG<sub>min</sub>-HSA was studied. Ketoprofen (0.1–2 μM) and albumin derivative (10 μM) were incubated in 67 mM sodium phosphate buffer, pH 7.4, and 37°C for 30 min. The unbound ligand fractions were separated by ultrafiltration centrifugation (20,000 × g at 4°C for 5 min) using a 12-kDa cut-off membrane. Adsorption of ketoprofen onto the filtration membrane and apparent reversibility were negligible. The concentration of unbound ligand was determined by HPLC. The HPLC system consisted of Waters 717 plus autosampler (with samples maintained at 4°C), Waters 600 quaternary pump, and Waters 481 Lambda Max absorbance detector. Columns for reversed phase HPLC were a 3.9 × 150-mm NOVAPAK<sup>TM</sup> ODS (4 μM) fitted with a 3.9 × 20-mm NOVAPAK<sup>TM</sup> ODS Sentry guard column. The mobile phase was 10 mM sodium phosphate buffer with 50% (v/v) methanol, and the sample was detected at 230 nm. Esterase activities of MG<sub>min</sub>-HSA and HSA were determined with the synthetic substrate p-nitrophenylacetate following the hydrolysis to p-nitrophenol and acetate. The reaction was followed by monitoring the appearance of p-nitrophenol, detected spectrophotometrically at 400 nm. The reaction mixtures contained 50 μM p-nitrophenylacetate and 20 μM protein in 50 mM sodium phosphate buffer, pH 7.4, and 37°C. The reaction velocity was monitored over the pH range 6.4–9.4 using phosphate buffer in the pH range 6.4–7.8 and pyrophosphate buffer in the pH range 8.4–9.4. Extinction coefficients for p-nitrophenol were determined at each pH value studied, and esterase activity was deduced from the initial rates of absorbance (da<sub>400</sub>/dt).

**Molecular Dynamics, Arginine Residue Surface Exposure, and Surface Charge Density**—The AMBER 7.0 force field (15) was used to model the effect of modified MG<sub>min-HSA</sub> on the native protein glycation HSA (Protein Data Bank identification number 1ao6) at 2.5 Å (16) obtained from the Research Collaboratory for Structural Bioinformatics data base. Arg-410 was replaced with the hydroimidazolone MG-H1 residue. The native and modified protein structures were equilibrated using the Generalized Born solvent model (17) and then subjected to 100 ps of molecular dynamics using the AMBER force field until the total energy of each system reached equilibrium. Final energy minimization of each protein was carried out within AMBER. Energy minimizations of modifications and molecular graphics were performed using DS Viewer Pro 5.0 (Accelrys, San Diego, CA). The electrostatic potential was calculated using the Poisson-Boltzmann (18) method as implemented in the University of Houston Brownian Dynamics (19) software (21) by rolling a probe of a defined radius (water molecule) over the surface of the protein using Gopenmol (20). The surface exposure of each of the Arg residues within the structure of HSA was calculated using the NACCESS software (22) by rolling a probe of a defined radius (water molecule) over the surface of the protein to determine residue accessibility.

**RESULTS**

**Modification and Glycation Adduct Quantitation by LC-MS/MS**—We and other investigators have previously prepared and studied the molecular characteristics of human and bovine serum albumins modified to minimal and high extents by methylglyoxal (22–24). We examined the presence of glycation adducts by quantifying the loss of lysine and arginine residues by chromophoric and fluorimetric lysine- and arginine-specific reagents, and amino acid analysis with and without dicyclohexylcarbodiimide (22). This showed convincingly that arginine residues were lost preferentially by glycation with methylglyoxal. With recent advances in the application of LC-MS/MS to the detection of protein glycation adducts (3), we are now able to quantify methylglyoxal-derived glycation adducts in such modified proteins. Studies of the concentrations of methylglyoxal-derived glycation adducts in human plasma proteins and molecular mass measurements of human serum albumin in vivo indicated that HSA is also modified minimally by methylglyoxal-derived glycation adducts in vivo (3, 25, 26). Therefore, to quantify the
glycation adducts formed and identify sites in HSA reactive toward methylglyoxal under physiological conditions in a physiologically relevant model glycated protein, we prepared HSA modified minimally by methylglyoxal (MGmin-HSA). The type and amount of glycation adducts in MGmin-HSA were assessed by quantitative glycation adduct screening with LC-MS/MS (Table I). Estimates of other glycation adducts, oxidation, and nitration adducts in HSA that were not changed significantly by glycation with methylglyoxal were (mmol/mol protein; mean ± S.D., n = 4): N'-fructosyl-lysine, 10.0 ± 0.9; N'-carboxymethyl-lysine, 5.7 ± 0.8; glyoxylic-derived hydroimidazolone N5-(hydro-4-4-imidazol-2-yl)ornithine, <0.5; 3-deoxyglucose-derived hydroimidazolone N5-(hydro-5-(2,3,4-trihydroxybutyl)-4-imidazol-2-yl)ornithine and related structural isomers, 7.8 ± 2.8; pentosidine, 0.073 ± 0.013; methionine sulfoxide, 124 ± 11; and 3-nitrotyrosine, 0.22 ± 0.05. The main methylglyoxal-derived glycation adduct in MGmin-HSA was the hydroimidazolone MG-H1, found at −2.5 molar equivalents and representing 91% of total methylglyoxal-derived glycation adducts. The LC-MS/MS detection of MG-H1 showed the expected partial resolution of two epimers of the hydroimidazolone (Table I). Estimates of other glycation adducts, oxidation, and nitration adducts in HSA that were not changed significantly by glycation with methylglyoxal were (mmol/mol protein; mean ± S.D., n = 4): N'-fructosyl-lysine, 10.0 ± 0.9; N'-carboxymethyl-lysine, 5.7 ± 0.8; glyoxylic-derived hydroimidazolone N5-(hydro-4-4-imidazol-2-yl)ornithine, <0.5; 3-deoxyglucose-derived hydroimidazolone N5-(hydro-5-(2,3,4-trihydroxybutyl)-4-imidazol-2-yl)ornithine and related structural isomers, 7.8 ± 2.8; pentosidine, 0.073 ± 0.013; methionine sulfoxide, 124 ± 11; and 3-nitrotyrosine, 0.22 ± 0.05. The main methylglyoxal-derived glycation adduct in MGmin-HSA was the hydroimidazolone MG-H1, found at −2.5 molar equivalents and representing 91% of total methylglyoxal-derived glycation adducts. The LC-MS/MS detection of MG-H1 showed the expected partial resolution of two epimers of the hydroimidazolone (Fig. 1, a and b). Minor methylglyoxal-derived adducts were: argpyrimidine, 7%; N'-carboxyethyl-lysine, 1%; and methylglyoxal-derived lysine dimer, <1% of total methylglyoxal glycation adducts. The total methylglyoxal-derived adducts in MGmin-HSA, deduced by preparation of MGmin-HSA with radiolabeled [2-14C]methylglyoxal (27), was 2.45 ± 0.30 mol/mol protein, indicating that all of the methylglyoxal-derived adducts were detected. High resolution isoelectric focusing and SDS-PAGE of MGmin-HSA showed a small decrease in pI with respect to HSA (5.62 versus 5.76) and no evidence of significant intermolecular cross-linking (Fig. 2), consistent with loss of arginine residue positive charge by formation of MG-H1 residues.

**Location of the Hydroimidazolone MG-H1 by Peptide Mapping, Molecular Dynamics and Modeling, Surface Arginine Exposure, and Charge and Secondary Structure**—The locations of MG-H1 residues in MGmin-HSA were identified by tryptic peptide mapping by cationic electrospray LC-MS. Tryptic peptide responses were normalized to the response of the C-terminal peptide. Loss of peptide (by methylglyoxal modification) was deduced by comparison of normalized peptide responses in MGmin-HSA to that of control HSA in triplicate. The normalized peptide ion response gave reproducible detection when singly charged peptide responses were quantified; doubly and triply charged peptide ions suffered variable ion quenching and were not used in deducing peptide detection responses. The summation of all peptide responses showing statistically significant decrease in MGmin-HSA, with respect to HSA, gave a total molar equivalent peptide loss of 2.06. This loss is assumed to be due to modification of peptides by MG-H1. The total modification by methylglyoxal in MGmin-HSA was −2.5 molar equivalents; the residual −0.5 molar equivalents of modified peptide difference between the total and that located in tryptic peptides is accounted for by hydrolysis of MG-H1 during the tryptic digestion (expect loss of 26% of the total modification or 0.6 molar equivalents) (6). The individual peptides with decreased detection in MGmin-HSA are given in Table II. They represent modification of arginine residues (mol % modified) at Arg-114 (36%), Arg-186 (25%), Arg-218 (31%), Arg-410 (89%), and Arg-428 (25%). For the detection of T31213–218, there was significant interference from a dipeptide T67–68. Pepsin T31 contained a chromophoric tryptophan residue, the only tryptophan residue in HSA, whereas T67–68 did not. Hence, absorbance detection at 286 nm was used to quantify the loss of T31. The peptide containing the Arg-410 residue, T52, showed the most decreased response in MGmin-HSA and hence had the highest modification. Formation of MG-H1 residues in MGmin-HSA prevented trypsin cleavage at that site such that corresponding uncleaved tryptic dipeptides were detected. MG-H1-containing dipeptides in tryptic digests of MGmin-HSA were detected for Arg-186, Arg-218, Arg-410, and Arg-428 but not for Arg-114. Hence for the hotspot modification of Arg-410, the modified dipeptide T52–53 (FQNLLVRMG-H1-YTK) was detected in digests of MGmin-HSA but not of HSA (Fig. 1, c and d). Tryptic digests of plasma protein glycated in vivo also contained the MG-H1-modified peptide detected by matrix-assisted laser desorption ionization time-of-flight mass spectrometry (predicted FQNLLVRMG-H1-YTK 1406.9; found peptide mass 1406.8 Da; Fig. 2c). This suggests that Arg-410 is a hotspot for albumin modification by methylglyoxal experimentally and may also be so physiologically too (Fig. 1, e and f). MG-H1 residues have been detected and quantified by LC-MS/MS in plasma protein in vivo where increases were found in diabetes and uremia (3, 28).

Crystallographic and mutational studies indicated that Arg-410 is in drug-binding site II of HSA, located in helix 3Ah2 of subdomain 3A (29, 30). In the native state, the guanidino nitrogen of Arg-410 is hydrogen-bonded to the side chain carbonyl group of Asn-391 (bond length, 1.9 Å), and there is a weak electrostatic interaction with the phenolic OH group of Tyr-411 (bond length, 3.9 Å) when the phenolic group is considered to be unionized. Molecular simulations with the phenoxo anion of Tyr-411 indicated an electrostatic interaction of the Arg-410 guanidinium and Tyr-411 phenoxide groups: with energy minimization, the Arg-410 guanidinium ω-NH to Tyr-411 phenoxide −O− bond length is predicted to be 2.0 Å. Conversion of Arg-410 to a MG-H1 residue abolishes the hydrogen bonding to Asn-391. Helix 3Ah2 is distorted such that Arg-410 and Tyr-411 swing away from Asn-391, increasing the spatial separation of Asn-391 and Arg-410 (Fig. 3, a and b). This changes functional activity at this site.

The susceptibility of arginine residue in HSA in modification by methylglyoxal was examined by deducing the percentage surface exposure of arginine residues (Fig. 2d). This indicated that although Arg-114 and Arg-186 modified by methylglyoxal had high surface exposure (80–100%), the remaining arginine residues modified by methylglyoxal, Arg-218, Arg-410, and Arg-428, had low surface exposure (25–40%). There were also some arginine residues with high surface exposure that were not modified significantly by methylglyoxal: Arg-81 and Arg-209. Calculations of the surface charge of HSA with MG-H1 at residues 114, 186, 218, 410, and 428 showed no marked decrease in surface positive charge, except in the surface region close to residue 410. The CD spectrum of HSA and MGmin-HSA were identical in the far and near UV (185–360 nm) and were similar to those reported previously (30). There was 72% α-helix, 0% β-sheet, and 28% other conformation (data not shown).

**Functional Effects: Ligand Binding and Esterase Activity**—The nonsteroidal anti-inflammatory drug ketoprofen binds to drug-binding site II in HSA (29, 30). We determined the disso-



cation constant $K_D$ values of ketoprofen binding to HSA and MG$_{\text{min}}$-HSA. The $K_D$ values were: HSA, 5.31 ± 1.73 μM; and MG$_{\text{min}}$-HSA, 20.4 ± 4.1 μM ($p < 0.001$; $n = 7$) (Fig. 4, a and b). This indicated that drug-binding site II had markedly decreased affinity for ketoprofen in MG$_{\text{min}}$-HSA. When HSA was glycated by methylglyoxal with site II saturated with ketoprofen, LC-MS/MS quantitation of MG-H1 residues gave only 1724 ± 231 mmol/mol protein, a decrease of 769 mmol/mol protein and consistent with blocking of modification of Arg-410 by methylglyoxal. This corroborates and confirms the modification of Arg-410 by methylglyoxal indicated by the peptide mapping studies. Molecular graphics studies of the docking of ketoprofen into drug-binding site II of HSA predicts that ketoprofen binding is stabilized by hydrogen bonding of the propi-onyl carboxylic acid to the guanidino hydrogen of Arg-410 and phenolic hydrogen of Tyr-411 (bond length, 2.2 Å; Fig. 3c). This bonding is not available in MG$_{\text{min}}$-HSA, consistent with the decreased binding affinity of ketoprofen and hotspot modification of Arg-410.

Esterase activity of HSA is also associated with subdomain 3A of HSA. Tyr-411 has been proposed as the catalytic base because the mutational replacement Y411A led to a complete
loss of esterase activity. Arg-410 is thought to stabilize negatively charged intermediates in the catalytic mechanism because mutational replacement R410A decreased esterase activity by 88% with respect to wild-type HSA (30). We studied the pH dependence of esterase activity of HSA. This showed a pH dependence indicative of an amino acid residue involved in the catalytic mechanism with a $pK_a = 7.9 \pm 0.1$ (Fig. 4, c). This is assigned to the ionization of the Tyr-411 phenolic group, lowered by the positive electrostatic interaction with Arg-410. Modification of Arg-410 in MG$_{\text{min}}$-HSA inhibited esterase ac-

### Table II

| Arg position | Peptide            | HSA $R_t$ | M (Da) | (M+H)$^a$ |
|--------------|--------------------|-----------|--------|-----------|
| 114          | DDNPNIPEVR         | 25.3      | 939.44 | 940.45    |
| 168          | LDELR (T23–24162–163) | 25.5      | 664.4  | 665.4     |
| 218          | AWAVVAR (T31–23–213) | 28.8      | 672.4  | 673.4 (A286–ve) |
| 410          | FQNALLVR           | 35.9      | 959.6  | 960.6     |
| 428          | VPQVSTPTLVEVR      | 37.0      | 1510.8 | 1511.8    |

$^a$ ND, not determined.

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**Fig. 2.** Two-dimensional gel electrophoresis of human serum albumin modified minimally by methylglyoxal. a, HSA (pI = 5.76) and b, MG$_{\text{min}}$-HSA (pI = 5.02). c, section of a matrix-assisted laser desorption ionization time-of-flight mass spectrum of a tryptic digest in protein of human plasma glycated in vivo. The arrow indicates the peak assigned to peptide FQNALLVR$_{\text{MG-H1}}$YTK (predicted mass, 1406.9 Da). d, percentage surface exposure of arginine residues in HSA (see “Experimental Procedures”).
activity by 63% (Fig. 4e; cf. a decrease of 88% by R410A mutation; Ref. 30). The pH dependence of the esterase activity of MGmin-HSA showed a shift of \( pK_a \) to 8.8, consistent with the loss of the catalytic base stabilization. When the pH was increased to 9.4, however, the esterase activity of MGmin-HSA was higher than that of HSA (Fig. 4d). Inhibition of esterase activity was prevented by glycation of HSA with methylglyoxal when site II was saturated with ketoprofen (Fig. 4e), consistent with the involvement of Arg-410 in the catalytic mechanism of esterase activity and its modification to MG-H1 in esterase activity inhibition.

To model the transition state structure of the esterase reaction, we positioned the carbonyl carbon of the synthetic substrate, p-nitrophenylacetate, close to the phenoxide catalytic base of Tyr-411 (bond length, 1.44 Å, assumed from known bond lengths of similar structures). Energy minimized molecular dynamics studies predicted hydrogen bonding between the guanidino hydrogen of Arg-410 and carbonyl oxygen in the substrate (bond length, 2.3 Å) ligand. Electrostatic interaction also stabilizes the increasing negative charge on the substrate carbonyl oxygen during nucleophilic attack of Tyr-411 (Fig. 2d).

In the putative mechanistic intermediate, site II with O-acetylated Tyr-411, there is also hydrogen bonding of Arg-410 to the acetyl carbonyl oxygen (bond length, 2.3 Å; structure not shown). Throughout the catalytic reaction, Arg-410 remains hydrogen-bonded to Asn-391. This supports the proposal of a catalytic triad of amino acid residues sustaining esterase activity of HSA: Tyr-411, Arg-410, and Asn-391 (Fig. 3e).

DISCUSSION

Takahashi (9) and others pioneered the use of glyoxal derivatives for probing the active sites of enzymes and other proteins for functionally active arginine residues. The use of these agents was based on their specificity for modification of arginine residues, but the modified structure formed was not identified. The significance of arginine residue modification in pro-
tein glycation in vitro and in vivo is a relatively recent development. We characterized recently the products formed by the reaction of arginine residues with an asymmetric glyoxal derivative, methylglyoxal. The product formed is a hydroimidazolone; the three possible structural isomers may all be formed, but one isomer, MG-H1, predominates in protein residues (6, 7). MG-H1 residues were found in relatively high amounts in cellular and extracellular proteins, 1–5 mmol/mol Arg (3). Under such conditions, from human proteome statistics (with an average arginine residue content/protein of 26), it is predicted there will be one MG-derived AGE on 3–13% of all proteins. This is expected to have significant effects on protein structure and function, contributing to protein misfolding (31), with cellular proteolysis liberating free MG-H1, which was indeed found (3). How hydroimidazolone affects the structure and function of the active site and binding domain of a protein has not been revealed at the molecular level.

Modification of Human Serum Albumin by Methylglyoxal to Minimal Extent Revealed Hotspots of Arginine Residue Glycation—Minimal modification of albumin by methylglyoxal produced significant formation of MG-H1 adducts at residues 114, 186, 218, 410, and 428 only. To understand the basis for this selectivity, we investigated surface exposure of arginine residues. This showed that Arg-114 and Arg-186 had high surface exposure; whereas Arg-218, Arg-410, and Arg-428 had low surface exposures and were nevertheless modified. Arg-81 and Arg-209 had high surface exposure but were unmodified. Arg-81 has weak electrostatic interaction with Asp-89; the
guanidinium $\omega$-NH to carboxylate $-\text{O^}\text{-}$ distance of closest approach was 5.84 Å. This may stabilize the guanidinium group and decrease its reactivity. Similarly, Arg-209 may be stabilized by interaction with Glu-354: the guanidinium $\omega$-NH to carboxylate $-\text{O^}\text{-}$ distance of closest approach was 3.56 Å. Arg-218 may be activated toward modification by methylglyoxal by being within the pocket of drug-binding site 1 and with a weak electrostatic interaction with the guanidinium group of Arg-222; the inter-guanidinium $\omega$-NH distance of closest approach was 4.73 Å. Arg-410 may be activated toward modification by methylglyoxal being within the pocket of drug-binding site 2. Analogous to the proposed catalytic mechanism of the esterase activity, methylglyoxal may form an initial hemiacetal linkage to Tyr-411, facilitating attachment to the neighboring Arg-410. Arg-428 may be activated toward modification of methylglyoxal by decreased $pK_a$ imposed by the close proximity of its guanidinium group to the alkyl side chain of Ile-523 and the ammonium cation group of Lys-432; the guanidinium $\omega$-NH distances of closest approach to the side chains of Ile-523 and Lys-432 were 4.90 and 5.21 Å, respectively. Surface exposure and neighboring group effects on the basicity of arginine residues may therefore account for the selectivity of glycation by methylglyoxal in HSA.

Although the formation of MG-H1 residues decreased the $pI$ value of HSA, very little change in surface charge was noticed, however, except for the region of MG-H1–410. This region may therefore be a key site involved in the binding of methylglyoxal-modified albumin to cell surface receptors (32), particularly because there was little change in secondary structure in MG$_{min}$-HSA, as indicated by the CD spectra.

**Methylglyoxal Modification Changes Ligand Binding and Enzymatic Activity of Domain 3A of Human Serum Albumin—** The crystal structure of HSA and discrete expression of conserved domains revealed that domains 2 and 3 have pockets formed by hydrophobic and positively charged residues that bind a wide variety of drugs and other ligands (16, 33–35). Fatty acids bind in these and other sites distributed symmetrically throughout the molecule (36). The accessibility and reactivity of arginine residues in HSA are probable determinants of reactivity with methylglyoxal. The accessibility of Arg-218 and Arg-410 to drugs and fatty acids indicates that these sites within HSA are accessible to small molecules such as methylglyoxal. Petersen et al. (37) considered these residues as “gatekeepers” influential on ligand interactions in drug-binding sites 1 and 2, respectively. Arg-114, Arg-186, and Arg-428 are on the surface of HSA (Fig. 1e). It was surprising that only Arg-410 of the gatekeeper arginine residues was modified highly by methylglyoxal in MG$_{min}$-HSA. The proximity of the activated Tyr-411 to Arg-410 may lead to the reversible binding of methylglyoxal to the Tyr-411 phenoxy anion initially with subsequent transfer to the neighboring guanidino group of Arg-410. Therefore, Tyr-411 may facilitate glycation at neighboring Arg-410. Drug site II (in subdomain 3A) of HSA is the active site for esterase activity as well as binding of ketoprofen, diazepam, and fatty acids. Single residue mutation studies have indicated that Arg-410 and Tyr-411 are necessary for these activities (30). Studies of the $pH$ dependence of esterase activity suggested that the $pK_a$ of the Tyr-411 is 7.9. Proximate groups imposing a strong positive electrostatic field decreases the $pK_a$ of the phenolic OH group of tyrosine residues, $cf.$ the lowering of the $pK_a$ of the catalytic tyrosine residue in UDP-galactose 4-epimerase to 6.0 (38). At high $pH$ ($pH 9.4$), the esterase activity of MG$_{min}$-HSA was higher than that of native HSA. The explanation for this may be that although Arg-410 stabilizes the phenoxy anion of Tyr-411, it also partially screens the negative charge. In MG$_{min}$-HSA, when the $pH$ is increased so that Tyr-411 is deprotonated, the lack of the proximate positive charge leaves the phenoxy anion unscreened and realizes maximum catalytic basicity.

We also found that Arg-410 is hydrogen-bonded to Asn-391. The side chains of these two residues project into the binding site and remain in close proximity to each other. In the native protein, the phenolic oxygen of Tyr-411 was found to be 3.9 Å from the guanidino group of Arg-410 in the crystal structure (39) but predicted to be 2.0Å apart if the ionization of Tyr-411 was taken into account. The predicted bond length implies a strong electrostatic interaction between Arg-410 and Tyr-411. NMR studies have shown that the fatty acid binding in this site is predominantly maintained by the binding of the carboxylate head group of the fatty acid in this site (40). Crystal structures of albumin complexed with fatty acids indicate that fatty acid binding within this site also involves interaction of the Arg-410 guanidinium and Tyr-411 phenolic groups with and the carboxylate head group of the fatty acid (41, 42). The guanidinium moiety of Arg-410 also interacts electrostatically with the carboxyl group of ketoprofen, and the phenolic oxygen of Tyr-411 forms a hydrogen bond with the keto group of the ketoprofen. In the catalytic reaction of esterase activity, the putative transition state involves the esterase substrate also interacting with these groups. Molecular modeling of the consequences of hydroimidazolone modification of Arg-410 indicated that these interactions critical for high affinity binding of ketoprofen and catalytic hydrolysis of $p$-nitrophenyl acetate are disrupted.

Glyoxal derivative modification of arginine residues forms hydroimidazolones with consequent loss of arginine side chain positive charge and change in structure. These features are critical for ligand binding and substrate orientation and stabilization. They explain why glyoxal reagents have proven utility. Bioinformatics analysis indicates that arginine residues have a high probability (19.6%) of being located in ligand and substrate-binding sites of proteins (43). Glycation of arginine residues by methylglyoxal and other $\alpha$-oxoaldehydes is therefore expected to have a significant impact on the functional activity of proteins. Methylglyoxal-derived hydroimidazolone is a major glycation adduct of albumin and other extracellular and cellular proteins in vivo, increasing in diabetes, uremia, and aging, although the extent of modification is normally 2% and does not normally exceed 4% in diabetes and uremia (3). The consequent protein misfolding may also target proteins for proteasomal degradation (31), as evidenced by the relatively high concentrations of hydroimidazolone free adducts in plasma and excretion in urine in vivo (3).

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