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Site-specific modification of positively-charged surfaces on human serum albumin by malondialdehyde

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ABSTRACT

Malondialdehyde (MDA), a lipid peroxidation product, reacts with lysine residues in proteins. Human serum albumin (HSA) is a major target of MDA-modification of serum proteins. To identify, the modification sites of HSA by MDA *in vitro*, MDA-treated HSA was digested with a protease and the resulting peptides were subjected to liquid chromatography-tandem mass spectrometry. We identified six peptides, which contained a *N*-propenal adduct at Lys136, Lys174, Lys240, Lys281, Lys525, and Lys541, and revealed that Lys525 is the most reactive residue for MDA modification. Analysis of electrostatic surface potential of a 3-D model structure of HSA indicates that Lys525 is located at the center of positively charged grooves. The results of this study indicate that the modification of proteins by lipid-derived aldehydes may be influenced by the electrostatic potential of the protein surface.

Malondialdehyde (MDA) is often the most abundant aldehyde resulting from lipid peroxidation, and occurs in biological materials in various covalently bound forms. MDA primarily forms adducts with lysine residues of proteins, with amine headgroups of phospholipids, and with DNA bases [1–3]. The major reaction of MDA is its addition to lysine, generating N^{ε}-(2-propenal) lysine (*N*-propenal-lysine) [4,5]. This adduct is the major form in which endogenous MDA is excreted in urine in rats and humans [5]. MDA also forms fluorescent products such as dihydropyridine (DHP)-lysine, a model of fluorescent components in lipofuscin [6,7].

Human serum albumin (HSA) is a 66 kDa protein that plays an important role in the physiological transport of many compounds such as free fatty acids, steroids, and some metals and drug metabolites. HSA is an attractive target for biomarker studies of oxidative stress because it is highly abundant, constituting over half the total serum protein [8,9]. Albumin contains numerous nucleophilic residues that react with electrophiles such as MDA. In addition, the concentration of MDA in plasma is increased in most (if not all) acute and chronic diseases [10], exposing the protein to high concentrations of MDA. MDA-modified HSA, and antibodies against MDA-modified HSA, are often increased in patients with diseases related to oxidative stress [11–14]. However, the mechanism by which MDA modifies HSA has not been characterized in detail.

The purpose of this study was to identify the MDA-modification sites on HSA using mass spectrometric techniques. Thus, HSA was exposed to MDA, and the modified proteins were digested with V8 protease and analyzed by liquid chromatography–tandem mass spectrometry (LC–MS/MS) in order to characterize the HSA target sites of MDA modification.

Materials and methods

Materials. The sodium salt of MDA (3-hydroxy-2-propenal, sodium salt) was prepared by hydrolysis of malondialdehyde bis (diethyl acetal) [15]. HSA (fatty acid-free) was obtained from Sigma. V8 protease, dithiothreitol (DTT) and iodoacetic acid were purchased from Wako Pure Chemical Industries, Ltd.

Reaction of HSA with MDA. HSA (6.6 mg/mL) was incubated with 0.1–10 mM MDA (HSA: MDA = 1: 1–100, mol/mol) in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C for 24 h. The reaction was terminated by centrifugal filtration (Microcon-50, molecular weight cut-off of 50,000; Millipore, Belford, MA, USA) to remove the low molecular weight reactants. Control experiments were performed under the same conditions without addition of MDA. Fluorescence spectra (excitation, 387 nm; emission, 455 nm) were recorded with a F-2000 spectrofluorometer (Hitachi, Ibaraki, Japan).

Matrix-assisted laser desorption and ionization time-of-flight mass spectrometry (MALDI-TOF MS). Native or MDA-treated HSA were mixed with a 25% solution of sinapinic acid containing 80% acetonitrile and 0.1% trifluoroacetic acid. The mixture was spotted and dried on stainless steel targets at room temperature. The analyses were performed using an UltraFLEX MALDI-TOF MS (Bruker Daltonics, Ltd., Bremen, Germany). All analyses were carried out in the positive ion mode, and the instrument was calibrated immediately prior to each series of studies.

Liquid chromatography–mass spectrometry/mass spectrometry (LC–MS/MS). Native and MDA-modified HSA (1 mg/mL) were subjected to reduction and alkylation. Briefly, samples were reduced with 10 mM DTT in 50 mM ammonium

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bicarbonate buffer (pH 8.0) for 30 min at 56 °C, then carboxymethylated with 50 mM iodoacetic acid in the dark for 30 min at room temperature. DTT and iodoacetamide were removed by centrifugal filtration using a Microcon-50. The alkylated samples were digested with V8 protease in 50 mM sodium phosphate buffer (pH 7.2) at 37 °C for 24 h using an enzyme: substrate ratio of 1:100 (w/w). Products were separated on a nanospace SI-1 HPLC system (Shiseido, Tokyo, Japan) using a Capcell Pak C18 UG120 column (2.0 × 250 mm i.d., Shiseido). Samples were eluted with a linear gradient of 0.1% formic acid in water (solvent A) and 0.1% formic acid/acetonitrile (solvent B) (time = 0 min, 5% B; 0–10 min, 5% B; 10–90 min, 40% B, 90–95 min 85% B). The flow rate was 0.2 mL/min and the column temperature was controlled at 40 °C. The elution profiles were monitored by absorbance at 215 nm. MS (MS/MS) analyses were performed on an LCQ ion trap mass system (Thermo Fisher Scientific, San Jose, CA, USA) equipped with an electrospray ion source using a spray voltage of 5 kV and a capillary temperature of 260 °C. Spectra were acquired in the positive ion mode, with a scan range from *m/z* 200 to 2000. Collision induced dissociation experiments in the positive ion mode were performed by setting the relative collision energy at 25% using helium as the collision gas.

Electrostatic surface potential. A large number of three dimensional structures of HSA have been reported, but there is no high resolution structure due to HSA's conformational flexibility. The atomic structure of HSA complexed with myristic acids and heme (PDB ID: 1N5 U) provides the most reliable structure showing the surface residues [16], some of which are often invisible in the low-resolution electron density maps. Surface electrostatic potentials of HSA were calculated by the Poisson–Bolzmann equation as implemented in eF-surf server [17], and all figures were prepared using MIfit [18] and jV viewer [17].

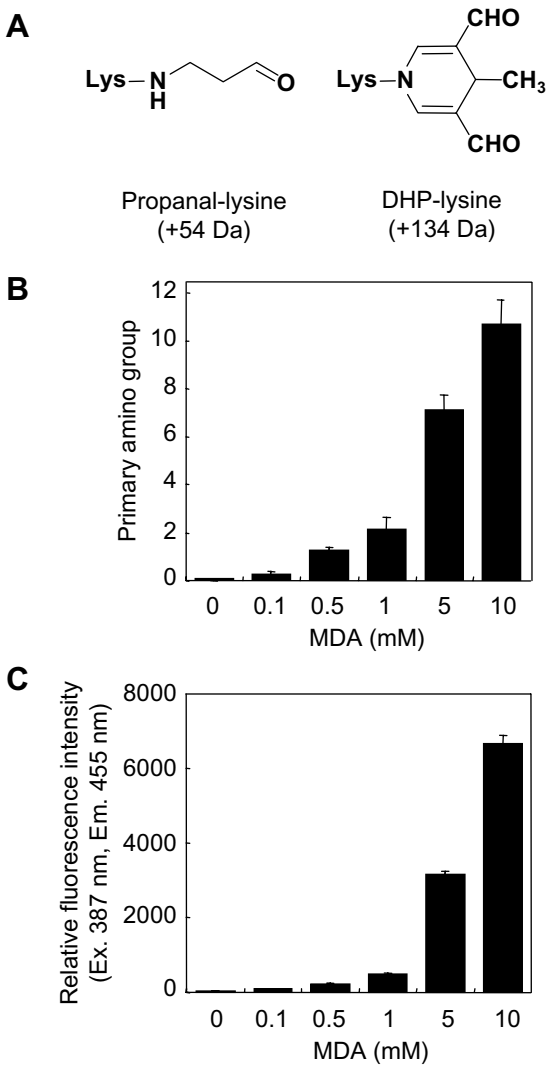

Fig. 1. MDA modification of HSA. HSA (6.6 mg/mL) was incubated with 0–10 mM MDA in 50 mM sodium phosphate buffer (pH 7.4) at 37 °C. (A) Chemical structures of *N*-propenal- and DHP-lysine MDA adducts. The numbers represent the increments of molecular weight as adducts are formed. (B) Loss of primary amino group. (B) Increase in fluorescence intensity (excitation, 387 nm; emission, 455 nm).

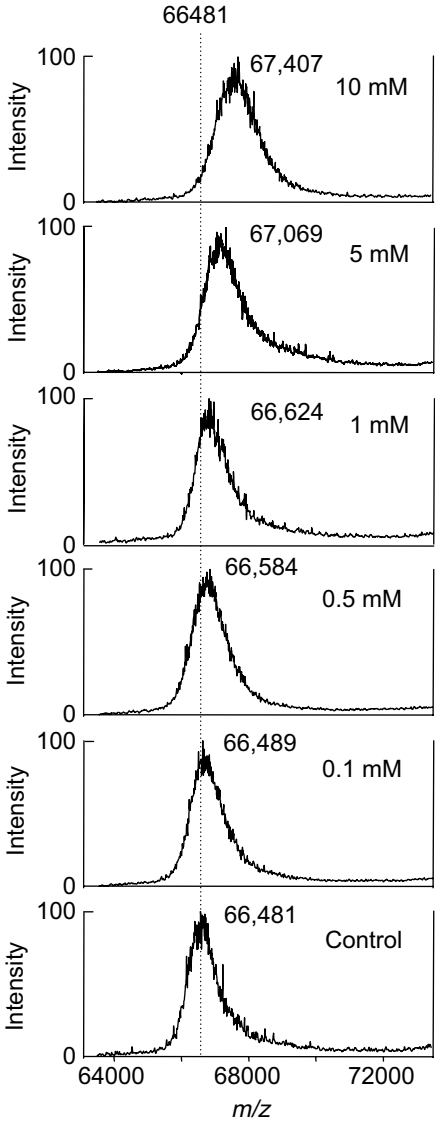

Fig. 2. MALDI-TOF MS spectra of native and MDA-modified HSA. Molecular mass and the number of MDA-modifications of each peak are shown in Table 1.

Table 1
Summary of MDA-modified HSA

MDA concentration (mM)	*m/z*	Δ*m* (Da)	Δ*m* (Da)/MDA (74 Da)
0	66,481	0	0
0.1	66,489	8	0.11
0.5	66,584	103	1.39
1	66,624	143	1.93
5	67,069	588	7.95
10	67,407	926	12.5

* Δ*m* (Da) is the increase in weight against native HSA.

Results and discussion

Modification of HSA by MDA

The reaction of MDA with proteins produces two major types of adducts, *N*-propenal- and DHP-lysine, resulting in mass increases of 54 and 134 Da, respectively (Fig. 1A) [19]. We first examined the modification of HSA during 24 h incubation with MDA. As shown in Fig. 1B, incubation of HSA with MDA resulted in a

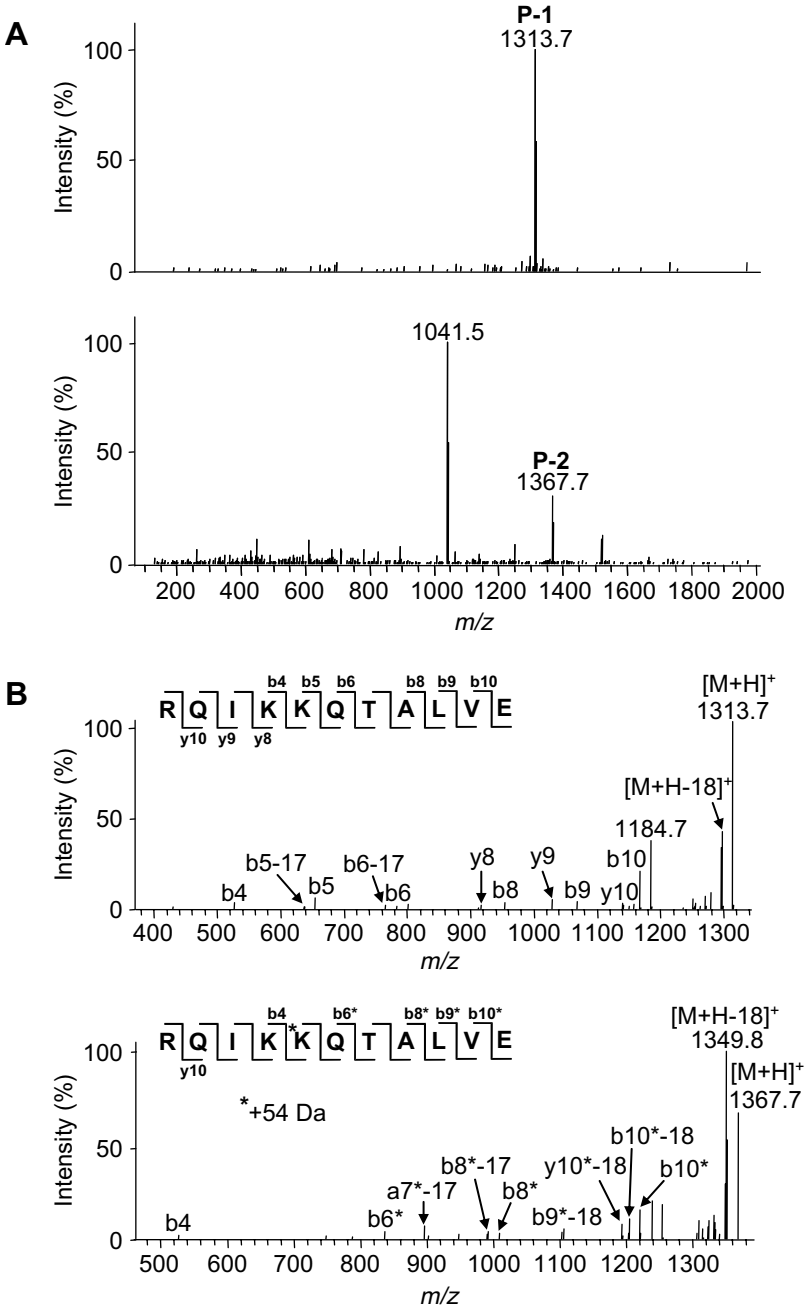

Fig. 3. LC–MS (MS/MS) spectra of native and MDA-modified HSA digested with V8 protease. (A) MS spectra of the $[M+H]^+$ ion at *m/z* 1313.7 (namely, P-1: *upper*) and 1367.7 (namely, P-2: *bottom*). (B) MS/MS spectra of P-1 (*upper*) and P-2 (*bottom*). Label* denotes modification by MDA (corresponding to the formation of *N*-propenal-lysine adduct).

dose-dependent loss of primary amino groups, suggesting that MDA reacts with lysine residues in HSA. The loss of amino groups was accompanied by an increase in protein fluorescence (Fig. 1C), suggesting that DHP-lysine adducts were generated in the MDA-modified HSA. Subsequently, aliquots of native and MDA-modified HSA were analyzed by MALDI-TOF-MS. As shown in Fig. 2, MS analysis of native HSA revealed a peak of *m/z* 66,481. When HSA was incubated with 0.1–10 mM MDA in 50 mM sodium phosphate buffer (pH 7.2) for 24 h, dose-dependent increase of the molecular mass was observed (Fig. 2 and Table 1). This increase in molecular weight indicates that MDA reacts with lysine residues in HSA dose-dependently. Incubation of HSA with 1 mM MDA produced a peak with molecular mass *m/z* = 66,624, which corresponds to a 143 Da increase in the mass of native HSA (Fig. 2). This is indicative of the addition of approximately two molecules of MDA. In addition, the increase in mass was accompanied by the loss of two lysine residues (Fig. 1B). Furthermore, the modified proteins were only

Table 2
Summary of the target sites of MDA-modification in HSA

Position of Lys in HSA	*m/z*	Peptide sequence	0.1 mM MDA	0.5 mM MDA
136	$(629.5)^{2+}$	TFLK*KYLYE	–	○
174	$(630.9)^{3+}$	CCQAADK*AACLLPKLDE	–	○
240	882.3	LTK*VHTE	–	○
281	$(552.5)^{2+}$	CCEK*PLLE	–	○
525	1367.7	RQIKK*QTALVE	○	○
541	$(444.7)^{3+}$	LVKHKPKATK*E	–	○

Label* denotes modification by MDA (corresponding to the formation of an *N*-propenal-lysine adduct).
○: detected, –: not detected.

weakly fluorescent at lower MDA concentrations (~1 mM). These results suggest that, at lower concentrations of MDA, the main reaction products are *N*-propenal-lysine adducts.

Identification of MDA modification sites in HSA by MS/MS analysis

Slatter et al. [20] reported two concurrent mechanisms for the formation of DHP-lysine adducts from MDA and propylamine. Formation of a propenal-lysine adduct is initially faster than that of a DHP-lysine adduct because direct formation of DHP-lysine via the proposed transient intermediates requires chemical cleavage of MDA into acetaldehyde and formic acid. Previously, we characterized the formation of DHP-lysine adducts in oxidized LDL and in atherosclerotic lesions of human aorta [21]. However, the formation of DHP-lysine adducts in HSA would be less likely under physiological conditions because (i) MDA in plasma is present in very low concentrations [22], (ii) plasma in patients would be competing with many other oxidized fatty acid or sugar products for reactive lysines in serum proteins [22], and (iii) the blood clearance rate of even lightly oxidized albumin is considerably faster than that of underivatized albumin [23]. In this study, there was a marked tendency for the *N*-propenal-lysine adduct to be preferentially formed compared to the DHP-lysine adduct at low MDA concentrations. Therefore, physiologically important MDA-lysine adducts in HSA are most likely to involve just one MDA molecule. To identify the major sites of MDA modification in HSA, MDA (0.1 and 0.5 mM)-treated HSA was digested with V8 protease, and the resulting peptides were subjected to LC–MS/MS. Peptide mass mapping by LC–MS analysis of native HSA digests identified peptides accounting for 83% of the protein sequence (data not shown). MDA-modified HSA digested with the protease was also analyzed by LC–MS/MS. After modification by MDA (0.1 mM), a peak (P-1, *m/z* 1313.7) decreased significantly in intensity (data not shown) and a new peak (P-2, *m/z* 1367.7) appeared (Fig. 3A). MS/MS analysis of P-1 confirmed the sequence of the peptide (residues 521–531 of HSA, RQIKKQTALVE) and their lack of modification (Fig. 3B, *top*). The MS/MS spectrum of P-2 revealed several singly charged product ions (b4, y10-H_2O) (Fig. 3B, bottom). Furthermore, the N-terminal fragment ions (b6, a7-H_2O, b8-H_2O, b8, b9-H_2O, b10, and b10-H_2O) and C-terminal ion (y10-H_2O) showed a 54-Da increase, suggesting that the *N*-propenal-lysine adduct is on Lys525. Table 2 summarizes the results of MS/MS analysis of V8 protease-digested MDA-modified HSA. From this MS analysis, we identified six target sites each Lys136, Lys174, Lys240, Lys281, Lys525, or Lys541, and revealed that Lys525 is the most reactive target for MDA modification.

Site-specific modification of positively charged grooves on HSA by MDA

HSA is the major transport protein for unesterified fatty acids, and also binds to a wide variety of metabolites, drugs and organic compounds. HSA is a 66 kDa monomer containing three homologous helical domains (I–III), each of which is divided into A and B subdomains (Fig. 4A). HSA contains 58 lysine residues (approximately 10% of the protein sequence), most of which are equally distributed throughout the surface of the protein, making them available to react with other molecules in the system. However, MDA treatment of HSA resulted in obvious site-specific MDA modification of nucleophilic lysine residues (Table 1), indicating that the local environment around the N^{ε}-atoms of lysine residues alters their specificity.

Electrostatic interactions and geometric complementarity (due to van der Waals interactions) play a significant role in substrate binding and catalysis [24,25], and provide clues regarding catalytic mechanisms and predictive ligand binding sites [26]. The model-based electrostatic surface of HSA (PDB ID 1N5 U, 1.9 Å resolution [16]) is overall positively charged, and fatty acids and one heme

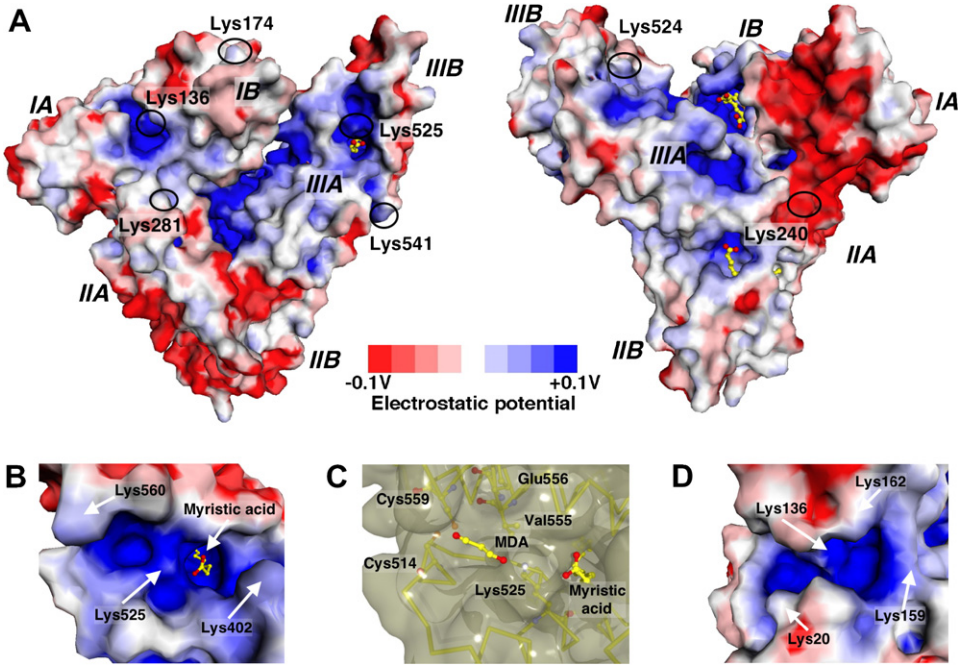

Fig. 4. Electrostatic surface potential of HSA complexed with heme and myristic acid. (A) HSA is composed of three homologous domains (I–III), each of which is divided into A and B subdomains. One heme, and two of the five molecules of myristic acid found to bind to HSA, are visible in these figures. The black circles indicate the positions of the N^{ε}-atoms of lysine, which are susceptible to modification by MDA. The red and blue colors correspond, respectively, to negative and positive potentials. (B) Close-up views of MDA-susceptible sites (Lys525). All lysine residues, including those resistant to MDA treatment, are also illustrated for comparison. (C) Hypothetical interactions between MDA and HSA. One MDA molecule is presumed to flank the main-chain atoms of Glu556 and Val555, and interact with the disulfide bond (between Cys514 and Cys559) and N^{ε}-atom of Lys525. Myristic acid and MDA are depicted in a ball-and-stick model colored according to atom type (nitrogen, blue; carbon, yellow; oxygen, red). (D) Close-up views of MDA susceptible sites (Lys136). All lysine residues, including those resistant to MDA modification, are also illustrated for comparison. (For interpretation of color mentioned in this figure the reader is referred to the web version of the article.)

bind to internal electropositive and hydrophobic sites (Fig. 4A) [27]. Five of the 6 MDA-susceptible lysine residues are located near positively charged grooves, indicating that electrostatic and topographic complementarity accelerates the reaction of MDA with lysine [28]. In particular, the N^{ε}-atom of Lys525, which is the most-specific MDA-modification site in HSA, is located at the center of strongly positively charged grooves in subdomain IIIB (Fig. 4B). It is interesting that the groove in subdomain IIIB containing Lys525 is located at the entrance of the myristic acid binding site (Fig. 4B) [27,29]. Fig. 4C illustrates several hypothetical MDA–HSA interactions: a disulfide bond (for which a preference for interaction with carbonyl oxygen has been reported) [30] and Val555/Glu556 residues help orient the binding of MDA towards the N^{ε}-atom of Lys525. Lys136 is also located at the center of a positively charged groove, subdomain IB (Fig. 4D). This groove contains Lys136 at the rear entrance of drug binding site I in HSA [29,31]. Both grooves have potential binding capacity and sufficient space to react with an MDA molecule, consistent with our MS analysis. These results and observations suggest that the modification of HSA by MDA may be influenced by positively charged grooves on the protein surface.

Protein targeting by electrophilic aldehydes is a complex process. Site selectivity for protein modification derives from the rate constants for multiple competing modification reactions. Each of these rate constants in turn is dependent on the basicity and nucleophilicity of individual nucleophilic residues, as well as their steric accessibility. Previously, we demonstrated that 4-hydroxy-2-nonenal (HNE) treatment of glyceraldehyde-3-phosphate dehydrogenase (GAPDH) results in the modification of amino acid residues primarily located on the surface of the GAPDH molecule [32]. Ahamed et al. [33] proposed that surface exposure and neighboring group effects on the basicity of arginine residues may account for the selectivity of glycation by methylglyoxal in HSA. Szapacs et al. [34] reported that H242 is the most reactive target on HSA for HNE, and that noncovalent complexation of the electrophile can drive reactivity by favorably orienting the electrophile towards the nucleophile target. In the present study, we demonstrate that protein modification by MDA may be influenced by electrostatic potential on the protein surface. The formation of lipid-aldehyde adducts in proteins in vivo may yield biomarkers of oxidative stress-related diseases. Adducts of HSA are of particular interest because HSA is an abundant blood protein that binds many endogenous and exogenous substances. Covalent modification of lipid-derived aldehydes to HSA may offer useful biomarkers of oxidative stress. The present findings may further our understanding of the damaging effects of lipid-derived aldehydes in various pathological states.

References

[1] M.E. Haberland, A.M. Fogelman, P.A. Edwards, Specificity of receptor-mediated recognition of malondialdehyde-modified low density lipoproteins, Proc. Natl. Acad. Sci. USA 79 (1982) 1712–1716.

[2] K. Uchida, Role of reactive aldehyde in cardiovascular diseases, Free Radic. Biol. Med. 28 (2000) 1685–1696.

[3] K. Stone, A. Uzieblo, L.J. Marnett, Studies of the reaction of malondialdehyde with cytosine nucleosides, Chem. Res. Toxicol. 3 (1990) 467–472.

[4] K. Uchida, K. Sakai, K. Itakura, T. Osawa, S. Toyokuni, Protein modification by lipid peroxidation products: formation of malondialdehyde- derived N^{ε}-(2-propenol) lysine in proteins, Arch. Biochem. Biophys. 346 (1997) 45–52.

[5] L.G. McGirr, M. Hadley, H.H. Draper, Identification of N^{α}-acetyl-N^{ε}-(2-propenal)lysine as a urinary metabolite of malondialdehyde, J. Biol. Chem. 260 (1985) 15427–15431.

[6] K. Kikugawa, Y. Ido, Studies on peroxidized lipids. V. Formation and characterization of 1,4-dihydropyridine-3,5-dicarbaldehydes as model of fluorescent components in lipofuscin, Rapid Commun. Lipids 19 (1984) 600–608.

[7] K. Uchida, Lipofuscin-like fluorophores originated from malondialdehyde, Free Radic. Res. 40 (2006) 1335–1338.

[8] A. Schmitt, J. Gasic-Milenkovic, J. Schmitt, Characterization of advanced glycation end products: mass changes in correlation to side chain modifications, Anal. Biochem. 346 (2005) 101–106.

[9] G. Aldini, M. Orioli, M. Carini, α,β -Unsaturated aldehydes adducts to actin and albumin as potential biomarkers of carbonylation damage, Redox Rep. 12 (2007) 20–25.

[10] D. Del Rio, A.J. Stewart, N. Pellegrini, A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress, Nutr. Metab. Cardiovasc. Dis. 15 (2005) 316–328.

[11] D. Vay, M. Parodi, R. Rolla, E. Mottaran, M. Vidali, G. Bellomo, E. Albano, Circulating antibodies recognizing malondialdehyde-modified proteins in healthy subjects, Free Radic. Biol. Med. 30 (2001) 277–286.

[12] E. Mottaran, S.F. Stewart, R. Rolla, D. Vay, V. Cipriani, M. Moretti, M. Vidali, M. Sartori, C. Rigamonti, C.P. Day, E. Albano, Lipid peroxidation contributes to immune reactions associated with alcoholic liver disease, Free Radic. Biol. Med. 32 (2002) 38–45.

[13] J.R. Palacio, A. Iborra, Z. Ulcova-Gallova, R. Badia, P. Martínez, The presence of antibodies to oxidative modified proteins in serum from polycystic ovary syndrome patients, Clin. Exp. Immunol. 144 (2006) 217–222.

[14] D.A. Slatter, N.C. Avery, A.J. Bailey, Identification of a new cross-link and unique histidine adduct from bovine serum albumin incubated with malondialdehyde, J. Biol. Chem. 279 (2004) 61–69.

[15] L.J. Marnett, M.A. Tuttle, Comparison of the mutagenicities of malondialdehyde and the side products formed during its chemical synthesis, Cancer Res. 40 (1980) 276–282.

[16] M. Wardell, Z. Wang, J.X. Ho, J. Robert, F. Ruker, J. Ruble, D.C. Carter, The atomic structure of human methemalbumin at 1.9 Å, Biochem. Biophys. Res. Commun. 291 (2002) 813–819.

[17] K. Kinoshita, H. Nakamura, eF-site and PDBjViewer: database and viewer for protein functional sites, Bioinformatics 20 (2004) 1329–1330.

[18] D.E. McRee, Differential evolution for protein crystallographic optimizations, Acta. Crystallogr. D Biol. Crystallogr. 60 (2004) 2276–2279.

[19] T. Ishii, S. Kumazawa, T. Sakurai, T. Nakayama, K. Uchida, Mass spectroscopic characterization of protein modification by malondialdehyde, Chem. Res. Toxicol. 19 (2006) 122–129.

[20] D.A. Slatter, M. Murray, A.J. Bailiey, Formation of a dihydropyridine derivative as a potential cross-link derived from malondialdehyde in physiological systems, FEBS Lett. 421 (1998) 180–184.

[21] S. Yamada, S. Kumazawa, T. Ishii, T. Nakayama, K. Itakura, N. Shibata, M. Kobayashi, K. Sakai, T. Osawa, K. Uchida, Immunochemical detection of a lipofuscin-like fluorophore derived from malondialdehyde and lysine, J. Lipid Res. 42 (2001) 1187–1196.

[22] J.W. Baynes, S.R. Thorpe, Glycoxidation and lipoxidation in atherogenesis, Free Radic. Biol. Med. 28 (2000) 1708–1716.

[23] R. Nagai, K. Matsumoto, X. Ling, H. Suzuki, T. Araki, S. Horiuchi, Glycolaldehyde, a reactive intermediate for advanced glycation end products, plays an important role in the generation of an active ligand for the macrophage scavenger receptor, Diabetes 49 (2000) 1714–1723.

[24] K.M. Shokat, C.J. Leumann, R. Sugasawara, P.G. Schultz, A new strategy for the generation of catalytic antibodies, Nature 338 (1989) 269–271.

[25] B. Honig, A. Nicholls, Classical electrostatics in biology and chemistry, Science 268 (1995) 1144–1149.

[26] K. Kinoshita, J. Furui, H. Nakamura, Identification of protein functions from a molecular surface database, eF-site, J. Struct. Funct. Genomics 2 (2001) 9–22.

[27] X.M. He, D.C. Carter, Atomic structure and chemistry of human serum albumin, Nature 358 (1992) 209. J. Struct. Funct. Genomics 215..

[28] A. Warshel, Electrostatic origin of the catalytic power of enzymes and the role of preorganized active sites, J. Biol. Chem. 273 (1998) 27035–27038.

[29] J. Ghuman, P.A. Zunszain, I. Petitpas, A.A. Bhattacharya, M. Otagiri, S. Curry, Structural basis of the drug-binding specificity of human serum albumin, J. Mol. Biol. 353 (2005) 38–52.

[30] R. Bhattacharyya, D. Pal, P. Chakrabarti, Disulfide bonds, their stereospecific environment and conservation in protein structures, Protein Eng. Des. Sel. 17 (2004) 795–808.

[31] G. Sudlow, D.J. Birkett, D.N. Wade, The characterization of two specific drug binding sites on human serum albumin, Mol. Pharmacol. 11 (1975) 824–832.

[32] T. Ishii, E. Tatsuda, S. Kumazawa, T. Nakayama, K. Uchida, Molecular basis of enzyme inactivation by an endogenous electrophile 4-hydroxy-2-nonenal: identification of modification sites in glyceraldehyde-3-phosphate dehydrogenase, Biochemistry 42 (2003) 3474–3480.

[33] N. Ahmed, D. Dobler, M. Dean, P.J. Thornalley, Peptide mapping identifies hotspot site of modification in human serum albumin by methylglyoxal involved in ligand binding and esterase activity, J. Biol. Chem. 280 (2005) 5724–5732.

[34] M.E. Szapacs, J.N. Riggins, L.J. Zimmerman, D.C. Liebler, Covalent adduction of human serum albumin by 4-hydroxy-2-nonenal: kinetic analysis of competing alkylation reactions, Biochemistry 45 (2006) 10521–10528.