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Determination of dideoxyosone precursors of AGEs in human lens proteins

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ABSTRACT

Dideoxyosones (DDOs) are intermediates in the synthesis of advanced glycation endproducts (AGEs), such as pentosidine and glucosepane. Although the formation of pentosidine and glucosepane in the human lens has been firmly established, the formation of DDOs has not been demonstrated. The aim of this study was to develop a reliable method to detect DDOs in lens proteins. A specific DDO trapping agent, biotinyl-diaminobenzene (3,4-diamino-*N*-(3-{[5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl]aminopropyl)benzamide) (BDAB) was added during in vitro protein glycation or during protein extraction from human lenses. In vitro glycated human lens protein showed strong reaction in monomeric and polymeric crosslinked proteins by Western blot and ELISA. Glycation of BSA in the presence of BDAB resulted in covalent binding of BDAB to the protein and inhibited pentosidine formation. Mass spectrometric analysis of lysozyme glycated in the presence of BDAB showed the presence of quinoxalines at lysine residues at positions K1, K33, K96, and K116. The ELISA results indicated that cataractous lens proteins contain significantly higher levels of DDO than non-cataractous lenses (101.9 ± 67.8 vs. 31.7 ± 19.5 AU/mg protein, $p < 0.0001$). This study provides first direct evidence of DDO presence in human tissue proteins and establishes that AGE crosslink synthesis in the human lens occurs via DDO intermediates.

Introduction

The human lens proteins have negligible turnover rates and therefore accumulate post-translational modifications throughout life [1]. It is thought that these post-translational modifications cause the formation of high molecular weight protein aggregates, which diffract light in aged and cataractous lenses [1–4]. The major molecular mechanisms by which lens proteins are chemically modified include oxidation [5–7], glycation [8–10], carbamylation [11,12], truncation [13–15], and deamidation [16–18].

Glycation occurs between amino groups on lysine and arginine residues in proteins with carbonyl compounds [19,20]. The carbonyl compounds in the lens include glucose, fructose, methylglyoxal, glyoxal and ascorbate oxidation products [9,10,20]. A variety of stable protein adducts are generated by glycation and are collectively known as advanced glycation endproducts or AGEs. In the human lens, many AGEs have been reported, including pentosidine [9], glucosepane [10], K2P [21], *N*-carboxymethyl lysine [8], MOLD [22,23], and argpyrimidine [24]. AGEs accumulate progressively in lens proteins during aging and are generally present at higher levels in cataractous lenses than in non-cataractous lenses. AGE levels are positively correlated with yellow/brown pigmentation, non-tryptophan fluorescence and crosslinked aggregates in aged and cataractous lenses, suggesting that AGEs play a central role in these changes [25,26].

The glycation of lysine residues proceeds through the formation of an unstable aminoketose (Schiff's base), which rearranges to form a more stable structure known as the Amadori product. The Amadori product further reacts with neighboring lysine or arginine residues in proteins to form protein crosslinking AGEs. Although the role of the Amadori product is clear, the molecular mechanisms by which Amadori product generates AGEs are less clear. Several years ago, a novel pathway for Amadori-mediated AGE formation was discovered, which involved the transformation of the Amadori product into dideoxyosone (DDO)[1] structures [10,27]. DDOs are dicarbonyl compounds produced from the long-range shift of the carbonyl groups (through enolization and dehydration) in the Amadori product [27]. These intermediates exhibit high reactivity to-

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[1] *Abbreviations used:* AGEs, advanced glycation endproducts; DDOs, dideoxyosones; BDAB, 3,4-diamino-*N*-(3-[5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanoyl]aminopropyl)benzamide; BDNB, *N*-(3-(3,4-dinitrobenzylamino)propyl)-5-(2-oxo-hexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide; HLP, human lens proteins; MGO, methylglyoxal; TFA, trifluoroacetic acid, DTT, dithiothreitol.

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wards guanidino groups of arginine residues in proteins, and these reactions produce lysine–arginine protein crosslinking AGEs. Prominent examples of crosslinking AGEs are glucosepane and pentosidine, which are produced from hexose- and pentose-derived DDOs [10,28].

The observation that glucosepane is the dominant lysine–arginine crosslinking AGE in human skin collagen indicates that DDOs are major precursors of AGEs in vivo [29]. DDO-mediated AGE synthesis in tissue proteins becomes even more apparent when the potential of other sugars, such as ribose and fructose, are taken into consideration [28]. These sugars have much higher glycation capacity than glucose and therefore are expected to produce significantly more DDO than glucose. In the human lens, in addition to sugars, ascorbate is a potential source of DDO. The ascorbate concentration is relatively high in the human lens (~2 mM), and it is readily oxidized during cataract formation to form highly reactive sugars, such as erythrulose and threose [30]. These sugars rapidly react with lysine residues on proteins and generate significant quantities of DDOs. Taken together, it is conceivable that many carbohydrates in the lens can produce AGEs through DDO intermediates.

Despite the recognition that DDOs could be major precursors of AGEs, neither the chemical pathways by which DDOs generate AGEs nor their levels in tissues have been elucidated. Thus far, only two AGEs, glucosepane and pentosidine are known to be synthesized through DDO. These two AGEs probably account for a small fraction of AGEs that could be synthesized from DDOs, as Lederer's group estimated that glucosepane accounted for only ~8% of the DDOs in glycated proteins [10]. Thus, to understand the broader role of DDO in AGE synthesis in vivo, it is necessary to first understand the quantitative importance of DDOs for AGE synthesis. We previously provided immunological evidence for DDO in glycated proteins [31]. A specific monoclonal antibody reacted with quinoxaline (a product of the reaction between protein–DDO and diaminobenzene), but an ELISA using this antibody was not sufficiently sensitive to detect DDOs in tissue proteins.

This study was conducted (1) to identify DDO-bearing lysine residues in glycated proteins, (2) to develop a reliable and reproducible method for the detection and quantification of DDO in tissue proteins and (3) to understand the importance of DDO-mediated AGE formation in aging lenses and cataract formation.

Materials and methods

Chemicals and reagents

D-ribose (⩾99% pure), D-glucose (>99.9% pure), pentafluorophenol (⩾99%), *N*-hydroxysuccinimide (98%), 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (⩾97.0%), biotin (⩾99%), methylglyoxal (40% aqueous solution), trifluoroacetic acid (TFA; protein sequencing grade; ⩾99.5%), bovine serum albumin, lysozyme, formic acid (LC–MS grade) and ammonium bicarbonate were purchased from Sigma Chemical (St. Louis, MO). Sequencing grade trypsin was from Roche (Mannheim, Germany). Dithiothreitol (DTT) was obtained from Roth (Karlsruhe, Germany). *tert*-Butyl-*N*-(3-aminopropyl)carbamate and di-*tert*-butyl dicarbonate were purchased from AK Scientific (Mountain View, CA). N^{α}-t-Boc lysine was obtained from Bachem Americas, Inc. (Torrance, CA). 3,4-Diaminobenzoic acid (99.9%) and 3,4-dinitrobenzoic acid (99.9%) were from Alfa Aesar (Ward Hill, MA). Acetonitrile (HPLC grade), *N*,*N*-dimethylformamide (99.9%, anhydrous), and formic acid (analytical grade, 88%) were from Fisher Scientific (Pittsburg, PA). De-ionized water (18 MΩ or greater) was used throughout this project. All buffers used in this study were treated with Chelex-100 resin (10.0 g/l, 200–400 mesh, Bio-Rad Laboratories, Richmond, CA).

Synthesis of ribated lysine

Ribated lysine was prepared by incubating N^{α}-t-Boc lysine with D-ribose and was purified on Dowex 50X4 in the pyridinium form as previously described [32].

Synthesis of N-(3-((3,4-diaminobenzyl)amino)propyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (BDAB)

Biotin pentafluorophenyl ester was synthesized following a previously published procedure [33]. 3,4-bis((*tert*-Butoxycarbonyl)amino)benzoic acid (**1**) was synthesized as described by Tilley et al. [34], except that 2.2 M excess of di-*tert*-butyl dicarbonate over 3,4-diaminobenzoic acid was added to the reaction mixture, which was incubated for 16 h at room temperature. After another addition of di-*tert*-butyl dicarbonate (0.5:1 ratio of di-*tert*-butyl dicarbonate:3,4-diaminobenzoic acid), the mixture was incubated for another 120 h, yielding 90.5% 3,4-bis((*tert*-butoxycarbonyl)amino)benzoic acid. The succinimide ester of 3,4-bis((*tert*-butoxycarbonyl)amino)benzoic acid (**2**) was prepared following the procedure of Uchida et al. [35]. The biotinylated handle, *N*-(3-aminopropyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl) pentanamide formate salt, (**3**) was obtained using the two-step procedure described by Boudi et al. [36], except that biotin pentafluorophenyl ester was used instead of the biotin/*i*Bu-*O*-COCl/triethylamine mixture, which yielded 83.5% of the product.

Products (**2**) (1.7 mmol, 0.77 g) and (**3**) (1.9 mmol, 0.65 g) were suspended in 7 ml of a mixture of water:*N*,*N*-dimethylformamide (5:2) in the presence of 1.9 mmol of triethylamine and stirred under argon for 16 h at ambient temperature. The mixture was then evaporated to dryness under a vacuum. The target compound, di-*tert*-butyl (4-(((3-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl)amino)methyl)-1,2-phenylene)dicarbamate (**4**), was re-crystallized from this preparation with an ether:ethyl acetate mixture to yield 0.93 g (86.2% yield). This product (100 mg, 0.16 mmol) was further de-protected with neat trifluoroacetic acid (1.0 ml at room temperature for 1 h with stirring) and evaporated to yield 68 mg (85%) of crude *N*-(3-((3,4-diaminobenzyl)amino)propyl)-5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamide (**5**). This preparation was dissolved in water, lyophilized and re-dissolved in a minimal volume of 50 mM sodium phosphate buffer (pH 7.0); the compound was then purified by a semi-preparative RP-HPLC (R_t = 19.5–21.5 min) to yield impurity-free BDAB (25 mg) as a white amorphous powder (99% pure by HPLC analysis). The following conditions were used for BDAB purification: Prodigy C_{18} HPLC semi-preparative column (10 × 250 mm; Phenomenex, Los Angeles, CA); solvent A, 0.5% formic acid in water; solvent B, 0.5% formic acid in acetonitrile, linear gradient of 1–99% B, 0–35 min; 99% B at a flow rate 2.5 ml/min, 35–45 min; and the effluent absorbance was monitored at 270 nm.

The MS analysis (ESI+) of this compound showed $[M+H]^+$ m/z = 435.33 Th, which agreed well with the proposed structure. The ^{1}H NMR spectrum showed the following characteristics: 7.20 (d, J = 2 Hz, 1H), 7.14 (dd, J_1 = 8 Hz, J_2 = 2 Hz, 1H), 6.68 (d, J = 8 Hz, 1H), 4.46 (ddd, J_1 = 8 Hz, J_2 = 4.8 Hz, J_3 = 1 Hz, 1H), 4.27 (dd, J_1 = 7.6 Hz, J_2 = 4.4 Hz, 1H), 3.37 (t, J = 6.8 Hz, 2H), 3.26 (t, J = 6.8 Hz, 2H), 3.18 (m, 1H), 2.90 (dd, J_1 = 12.8 Hz, J_2 = 5.2 Hz, 1H), 2.68 (d, J = 12.4 Hz, 1H), 2.22 (t, J = 7.2, 2H), and 1.78–1.39 (8H).

Synthesis of 3,4-dinitro-N-(3-(5-(2-oxohexahydro-1H-thieno[3,4-d]imidazol-4-yl)pentanamido)propyl)benzamide (BDNB)

To synthesize BDNB, we first synthesized 3-(3,4-dinitrobenzamido)propan-1-aminium chloride (**6**) as follows: 3,4-dinitrobenzoic acid (1.06 g, 5 mmol) was dissolved in a mixture of anhydrous