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Modification of chickpea cystatin by reactive dicarbonyl species: Glycation, oxidation and aggregation

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ABSTRACT

Reactive dicarbonyl species such as methylglyoxal (MGO) and glyoxal (GO) have recently received extensive attention due to their high reactivity and ability to modify biological substances such as proteins, phospholipids, and DNA. In case of proteins these reactive species mainly react with lysine and arginine residues to form AGEs, oxidative products, and aggregates. Chickpea cystatin (CPC) was incubated with varying concentrations of glyoxal and methylglyoxal which caused, along with altered secondary and tertiary structures, glycation, functional inactivation, altered redox state, cross-linking and high-molecular-mass aggregation. All these processes were examined and characterized by UV-Vis, fluorescence, and CD spectroscopies. Further characterization of CPC modified by reactive dicarbonyls was done by polyacrylamide gel electrophoresis which also showed alterations in the CPC molecules. Thus, in addition to describing the effects of GO and MGO on structure, conformation and function of CPC, this study also shows the relatively superior modifying effect of methylglyoxal for CPC in terms of glycation, oxidation and aggregation. This model system could shed some more light on the role of the reactive dicarbonyls in the specific alterations of proteins with different biological consequences having implications to ageing and disease such as diabetes.

1. Introduction

There is overwhelming evidence for involvement of reactive oxygen species (ROS) in a number of pathophysiological conditions such as diabetes, cancer, and aging but the studies linking reactive dicarbonyl species (RDS) to these conditions are limited [1,2]. RDS, such as MGO and GO are produced by degradation of monosaccharides, degradation of lipid peroxidation products, early protein glycation adducts, and as a byproduct of glycolysis. The reported estimates of the concentrations of methylglyoxal and glyoxal in human blood plasma are in the range 100–120 nM [3,4] and cellular concentrations of methylglyoxal and glyoxal are reported to be in the range of 1–5 μM, and 0.1–1 μM, respectively [5]. RDS, sometimes also being referred to as reactive acyclic α-oxoaldehyde metabolites or simply α-oxoaldehydes, irreversibly modify proteins under physiological conditions in which the reactions proceed even at their physiological concentrations [6] and form fluorescent products, characteristics of which resemble those with occurring in proteins in aging and diabetes [7]. These RDS also are reported to modify histones which results in cross-linking of proteins and induces ROS-dependent cleavage of plasmid DNA [8]. The proteasome degradation of RDS products is not complete and remnants may accumulate and cause epigenetic changes as well as further DNA and protein damage [9].

GO and MGO are potent glycating agents and interact with the side chains of many amino acids especially lysine, arginine and even cysteine too. Glycation of proteins is a complex series of parallel and sequential reactions collectively called the Maillard reaction. It occurs in all tissues and body fluids. Early stage reactions in glycation of protein by glucose lead to the formation of reversible fructosyl-lysine (FL) and *N*-terminal amino acid residue-derived fructosamines, sometimes also called as Amadori products. Later stage reactions form irreversible end-stage adducts called advanced glycation endproducts (AGEs) [10]. FL degrades slowly to form AGEs - and also glyoxal and methylglyoxal [11]. In contrast, GO and MGO react with proteins to form AGE residues directly and relatively rapidly. These chemically stable AGEs are formed on extracellular long-lived proteins such as skin collagen as well

Abbreviations: AGEs, Advanced glycated end products; ANS, 8-Anilino-1-Naphthalene-Sulphonic acid; CPC, Chickpea cystatin; DNPH, 2, 4-dinitrophenylhydrazine; GO, Glyoxal; MGO, Methylglyoxal; RDS, Reactive dicarbonyl species; SDS-PAGE, Sodium dodecyl sulphate-polyacrylamide gel electrophoresis; TNBS, Trinitrobenzene sulphonate; Th T, Thioflavin T

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as cellular and short-lived extracellular proteins. Protein glycation is an important physiological phenomenon and is under intensive investigation. Particularly damaging effects are produced by covalent crosslinking of proteins which confers resistance to proteolysis [12]. This crosslinking of proteins may further lead to amyloid type aggregation and thus giving rise to more disease complications. Protein modification is also damaging when amino acid residues such as arginine, lysine, cysteine etc., are located in sites of protein-protein interaction, enzyme-substrate interaction and protein-DNA interaction (for transcription factors). A bioinformatics analysis of receptor binding domains indicated that arginine residues have the highest probability (19.6%) of being located at such sites [13]. The major modification of proteins by GO and MGO is on arginine residues. Formation of hydroimidazolones causes structural distortion, loss of side chain charge and functional impairment [14]. Furthermore, it is also reported that glycation and oxidation are strictly interconnected [15], in fact a synergism has been postulated and a new term, glycoxidation, has already been coined for the same [16].

In continuation of the study of protein modification by these RDS, the present communication describes the detailed and comparative account of CPC modification by MGO and GO with respect to glycation, oxidation and aggregation besides structure, conformation and function, to correlate it with ageing and diseased conditions. The study may fill the lacuna that exists currently with regard to implicating RDS led protein alterations in pathogenic complications. Cystatins are a superfamily of cysteine proteinase inhibitors that constitute a powerful regulatory system for endogenous cysteine proteinases, which may otherwise cause uncontrolled proteolysis and tissue damage. A cysteine proteinase inhibitor (CPC) was isolated from chickpea seeds by the method described earlier [17], and used as a model protein to study the comparative effects of two RDS namely MGO and GO on various parameters of the purified inhibitor. It was envisaged that this model system through a systematic study will be useful to understand the role of these reactive species mediated protein modifications in ageing and disease. We have for the first time studied a cystatin for its modification in the presence of two highly reactive physiological metabolites with respect not only to the formation of AGEs but also shift in the redox potential, and aggregation. Though a drop in bucket, it gives some in depth knowledge about the role of glyoxal and methylglyoxal mediated protein modifications in pathological conditions of the protein.

2. Materials and methods

2.1. Materials

Methylglyoxal, glyoxal, ethylene diamine tetra acetate (EDTA), L-cysteine, sephacryl S-100 HR, and papain were obtained from Sigma (ST. Louis, MO). All other chemicals and reagents used in the study were of the highest analytical grade, and available commercially.

2.2. Methods

2.2.1. Preparation of RDS-Modified CPC

Unless otherwise indicated all incubations were carried out in 0.05 M sodium phosphate buffer, pH 7.5, containing 0.02% NaN_3 to prevent bacterial growth. The incubations were set-up containing final concentrations of 0.5 mg ml^{-1} of CPC and 1 mM, 5 mM, and 10 mM each of GO and MGO in separate sets in a total volume of 2 ml. Control incubation was set up containing the isolated CPC in buffer alone. Control incubations were also set up that contained 5 mM MGO, and 5 mM GO in buffer, separately. All solutions were filtered through 0.22 μm syringe filters (sterilized, max. pressure 4.5 bars) under aseptic conditions into sterile eppendorfs and incubated at 37 °C for 7 days. Appropriate aliquots of treated CPC samples were removed at regular time intervals (0, 1, 3, 5, and 7 days), dialyzed to remove unbound dicarbonyls, and subjected to studies. Also, the samples were kept undisturbed for some time to allow the insoluble aggregates and other debris settle down, and then carefully aliquots were withdraw from the upper part for spectroscopic analyses. This was done largely to reduce distortion and light scattering effects in the samples during our studies.

2.2.2. Antiproteolytic activity assay of CPC

Antiproteinase activity of unmodified and methylglyoxal/glyoxal modified CPC was probed by measuring the ability of this inhibitor to inhibit the caseinolytic activity of papain, as described by Kunitz [18]. This cysteine proteinase inhibitory activity of all the CPC samples was measured at different time intervals, and the antiproteolytic activity of untreated CPC was taken as 100% for reference.

2.2.3. UV-Vis spectrophotometry

The UV-Vis absorption characteristics of control and treated CPC were recorded on UV-visible spectrophotometer (UV-Vis 1700 Shimadzu, Japan) between 200 and 400 nm using UV quartz cuvettes of 1 cm path length. The final concentration of protein taken for the study was 0.3 mg/ml.

2.2.4. Intrinsic fluorescence measurements

The fluorescence data were recorded with a RF-1501 spectrofluorophotometer (Shimadzu Co. Japan) as described earlier [19]. The fluorescence was recorded at regular intervals as mentioned above, for control as well as α-diacarbonyl treated samples. Each spectrum was the average of three scans and concentration of CPC for the scans was 0.5 mg/ml.

2.2.5. AGEs/glycation specific fluorescence

Glycation specific fluorescence measurements were made by exciting the samples at 325 nm and 485 nm, and emissions recorded at wavelengths of 395 nm (emission range 350–550 nm) and 530 nm (emission range 500–600 nm) respectively. Total AGE fluorescence was also measured by exciting the samples at 370 nm and recording the emission spectrum in the range of 390–550 nm [20]. Rest of the experimental conditions were the same as that of intrinsic fluorescence measurements.

2.2.6. Functional group estimation (free amino and thiol group determination)

The free amino groups present in native and RDS-modified CPC samples were determined by 2, 4, 6-trinitrobenzene sulphonate (TNBS) method, as described earlier [21].

Free sulfhydryl groups were measured by Ellman's reagent in 50 mM Tris-EDTA buffer, pH 8.0; absorbance was read at 412 nm [22].

2.2.7. Determination of carbonyl content as protein oxidation index using 2, 4-DNPH

Carbonyl groups resulting from glyco-oxidative damage in RDS-incubated CPC samples were evaluated by a 2, 4-dinitrophenylhydrazine (DNPH) assay as described elsewhere [23].

2.2.8. Surface hydrophobicity of the RDS modified CPC by ANS fluorescence assay

Surface hydrophobicity as an additional measure of protein modification upon incubation with MGO or GO was examined by fluorescence of ANS in the presence of native and methylglyoxal- and glyoxal-modified CPC [24].

2.2.9. Polyacrylamide gel electrophoresis (SDS-PAGE)

Samples of native and treated CPC samples were examined for RDS induced alterations in CPC by SDS-PAGE performed in 12.5% gels as described by Laemmli using tris-glycine buffer pH 8.3 [25]. About 60 μl (30 μg) of protein samples were loaded in each of the wells and electrophoresed. After running, staining by coomassie brilliant blue dye of the gels was performed for the visualization of the protein bands.

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