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Research paper

Glycation of Ribonuclease A affects its enzymatic activity and DNA binding ability

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ABSTRACT

Prolonged non-enzymatic glycation of proteins results in the formation of advanced glycation end products (AGEs) that cause several diseases. The glycation of Ribonuclease A (RNase A) at pH 7.4 and 37 °C with ribose, glucose and fructose has been monitored by UV–vis, fluorescence, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) and matrix assisted laser desorption ionization spectroscopy-time of flight (MALDI-TOF) methods. The enzymatic activity and DNA binding ability of glycated RNase A was also investigated by an agarose gel-based assay. A precipitation assay examined the ribonucleolytic activity of the glycated enzyme. An increase in incubation time resulted in the formation of high molecular weight AGEs with a decrease in ribonucleolytic activity. Ribose exhibits the highest potency as a glycating agent and showed the greatest reduction in the ribonucleolytic activity of the enzyme. Interestingly, glycated RNase A was unable to bind with the ribonuclease inhibitor (RI) and DNA. The glycated form of the protein was also found to be ineffective in DNA melting unlike native RNase A.

1. Introduction

Modification of proteins via a non-enzymatic reaction between reducing sugars and side chains of proteins constitutes the glycation of proteins, commonly known as the Maillard reaction. The non-enzymatic glycation pathway involves a cascade of steps. In the initial step, the N- terminal and side chain amino groups of proteins interact with the carbonyl group of sugars to form a labile Schiff base. This Schiff base undergoes Amadori rearrangement and thus a relatively stable ketoamine adduct is generated [1,2]. The Amadori product, then undergoes a series of complicated reactions and is transformed into a variety of chromophores, fluorophores and other products, collectively known as advanced glycation end products (AGEs) [3] such as N^{ϵ}- (carboxymethyl) lysine (CML). Involvement of an irreversible process, during the formation of AGEs results in structural and functional changes in the protein. Due to glycation, proteins may lose their biological activity, become toxic and cause cell death [4,5]. This non-enzymatic glycation of proteins leading to the generation of AGEs has been implicated in several chronic diseases like diabetes [6], inflammation [7], cancer [8], atherosclerosis [9], rheumatoid arthritis, osteoarthritis [10–12], renal disease [13], Alzheimer's disease [14,15] in addition to normal aging [15,16].

We have chosen Ribonuclease A (RNase A) as a model enzyme because of its well defined three-dimensional structure, thermal stability and susceptibility towards glycation [17–22]. RNase A is a stable enzyme of 124 amino acids with a molecular mass of ~13.7 kDa and a p*I* of 9.3. The protein catalyzes the hydrolysis of RNA in which the first step involves the cleavage of RNA to produce a 2′,3′-cyclic phosphodiester intermediate that in the next step forms a 3′-phosphomonoester. Residues responsible for the catalytic action are His12, His119 and Lys41 which are located in the P1 subsite of the active site [23]. The role of Lys41 is to stabilize the excess negative charge on the nonbridging phosphoryl oxygens in the transition state during RNA cleavage [24]. Along with Lys41, Lys7 and Lys66 are also present at the RNA binding site. In addition to the ribonucleolytic activity, RNase A also possesses DNA melting properties. The DNA melting property of RNase A is attributed to its preferential binding to single strands of DNA over the double

Abbreviations: AGEs, advanced glycation end products; RNase A, Ribonuclease A; CML, N^{ϵ}- (carboxymethyl) lysine; SDS-PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; MALDI-TOF, matrix assisted laser desorption ionization spectroscopy-time of flight; RI, ribonuclease inhibitor; ct-DNA, calf thymus DNA.

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stranded DNA [25,26]. RNase A contains one alpha amino group at the N-terminal and 10 epsilon (ε-) amino groups from 10 Lys residues [27]. In RNase A, the alpha amino group of Lys1 is the primary site of Schiff base formation where the extent of glycation is 15% [18]. Ketoamine adduct formation occurs to an extent of ~ 29% and ~38% at the epsilon amino groups of Lys7 and Lys41 respectively [18]. The other reactive sites that take part to a smaller extent in the glycation of RNase A are N^{ε}-Lys1 (9%), N^{ε}-Lys37 (9%), N^{ε}-Lys66, N^{ε}-Lys98 and N^{ε}-Lys104 [18,21]. Apart from the Lys residues two Arg residues (Arg39 and Arg85) also participate in glycation [21]. The higher reactivity of the alpha amino group of Lys1 is due its relatively low pK_a value (pK_a ~7.8) [18]. Glycation occurs with high potency at Lys41 also due to the low pK_a ~8.8 value of the amino group and its presence in the active site (phosphate binding site) which is a relatively basic region of RNase A. The reactivity of Lys7 is also due to its presence in the relatively basic region of the macromolecule. The presence of an acidic amino acid adjacent to Lys1 and Lys37 makes the epsilon amino group of these residues reactive towards glycation [18,28]. Due to modification of Lys residues during glycation, located at or near the active site, the ribonucleolytic activity of RNase A decreases [18,22]. A comparative study of the glycation of RNase A, at physiological pH (7.4) and temperature (37 °C) over a time period of 30 days by glucose, fructose and ribose and its effect on the enzymatic activity of RNase A has been investigated. In addition, the effect of glycation on the ribonuclease inhibitor (RI) binding, DNA binding, and DNA melting properties of RNase A have been looked into.

Glycation of proteins can occur in the presence of various molecules, such as reducing sugars and ascorbic acid [29,30]. Among the reducing sugars, glucose is frequently used in various studies due to its abundance in cells of nearly all living organism and its association with diabetic complications [4,31,32]. Apart from glucose, other sugars such as fructose and ribose are also used for glycation due to several reasons [Fig. 1] [33,34].

In the present study, RNase A has been incubated separately with three common reducing sugars (D-ribose, D-glucose and D-fructose) at physiological pH (7.4) and 37 °C over a time period of 30 days. Formation of AGEs and progression of glycation of RNase A with increasing time of incubation was monitored by UV–vis studies, fluorescence spectroscopic techniques and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. The number of sugar moieties attached to each RNase A molecule on glycation by the three sugars was determined using matrix assisted laser desorption ionization spectroscopy-time of flight: (MALDI-TOF) spectroscopy. We have compared the ribonucleolytic activity of RNase A due to glycation by these sugars by an agarose gel-based assay and a precipitation assay. Ribonuclease inhibitor (RI) binding assays and native-PAGE and were also employed to check the binding ability of glycated RNase A with RI. It is known that the native protein is able to melt DNA and preferentially binds to single stranded DNA [26]. The DNA binding ability and melting property of glycated RNase A was also investigated.

2. Materials and methods

2.1. Materials

RNase A (Type XII-A; purity ≥90%) and yeast RNA (impurities: Ca ≤ 0.03%; H_2O ≤ 10%) were obtained from Sigma–Aldrich (USA) and used as received. Supercoiled *pBR322* DNA (CsCl purified) and RI (purity>90%) were purchased from Bangalore (Genei), India. D-glucose (purity ≥99.73%; extrapure for biochemistry), D-fructose (purity ≥99.38%; extrapure for biochemistry), D-ribose (purity ≥99.89%; extrapure for biochemistry), calf thymus DNA (ct-DNA; extrapure for biochemistry) and other analytical grade reagents were from SRL, India. Spectral measurements were performed using Shimadzu-1800 UV–vis spectrophotometer and a Horiba Jobin Yvon Fluoromax-4 spectrofluorimeter. The concentration of RNase A and ct-DNA were measured spectrophotometrically using a molar extinction coefficient value of $\varepsilon_{278.5}$ = 9800 M^{-1} cm^{-1} and ε_{260} = 6600 M^{-1} cm^{-1} (per nucleotide) respectively [35,36].

2.2. Preparation of glycated RNase A

RNase A (10 mg/ml) was incubated with 0.5 M D-ribose, D-glucose and D-fructose separately in sodium phosphate buffer (0.4 M, pH 7.4) under sterile conditions at 37 °C [19]. The buffer solution was prepared using autoclaved water containing 0.02% sodium azide to prevent bacterial growth during incubation. Under similar conditions, one control set was incubated using protein

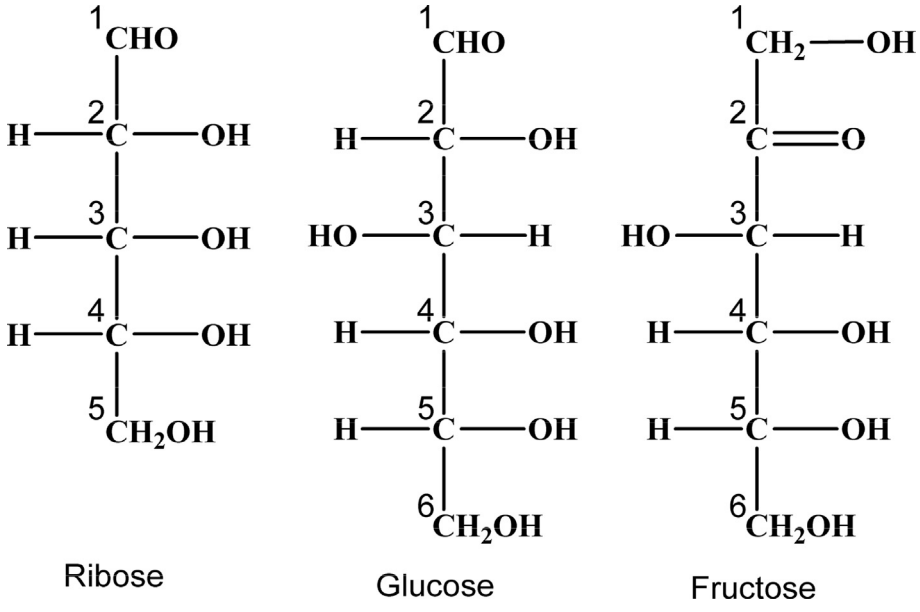

Fig. 1. Open chain structure of reducing sugars (D-ribose, D-glucose and D-fructose).