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ORIGINAL PAPER

Existence of Different Structural Intermediates and Aggregates on the Folding Pathway of Ovalbumin

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Abstract Structural modifications of ovalbumin in presence of different concentration of guanidine hydrochloride (Gdn HCl) and glucose were investigated by using intrinsic fluorescence, Fourier transform infra-red spectroscopy, circular dichroism and 8-anilino-1-naphthalene-sulphonic acid, to confirm that partially folded intermediates of ovalbumin lead to aggregation. The two partially folded intermediates of ovalbumin were observed one at 1 M Gdn HCl and another in the presence of 20 mM glucose at 3 M Gdn HCl. Both intermediates exist as compact states with altered intrinsic fluorescence, prominent β-sheet secondary structure and enhanced ANS binding. Ovalbumin in the presence of glucose required more concentration of Gdn HCl (3 M) to exist as an intermediate state than control (1 M). Such alpha-helix/beta-sheet transition of proteins is a crucial step in amyloidogenic diseases and represents an internal rearrangement of local contacts in an already folded protein. Further, incubation for 24 h resulted in the formation of aggregates as detected by thioflavin T-assay. On further increasing the concentration of glucose to 50 mM and incubation time for various days resulted in the formation of molten globule state of ovalbumin at 6th day. Later on, at 10th day advanced glycated end products were observed.

Keywords Aggregation · Advanced glycated end products · Glucose · Ovalbumin · Partially folded state

Introduction

Proteins are biologically active macromolecules that require the correct three dimensional structures of proteins [1, 2]. Several studies have shown that the amount of secondary structure and the compactness of the intermediate state formed in the folding pathway of protein are not necessarily close to those of the native state, but vary greatly depending on the protein species [3]. This suggests the presence of various intermediate states, from one close to the fully unfolded state to one close to the native state, depending upon the experimental conditions [4]. These intermediates are partially stable collapsed folded states of protein (equilibrium states) found in mildly denaturing conditions such as high concentration of solutes, low pH, mild denaturant or high temperature [5, 6]. Guanidine HCl (Gdn HCl), a strong chaotropic agent can solubilise insoluble or denatured proteins such as inclusion bodies [7]. Guanidine hydrochloride solutions are used to denature native globular proteins by disturbing hydrogen bonds, which hold the protein in its unique structure. However, there is also evidence suggesting that guanidine HCl may also disrupt hydrophobic interactions by promoting the solubility of hydrophobic residues in aqueous solutions [8]. Due to disruption of internal stabilizing forces, unfolding results in the expansion of a molecule [9]. When a protein folds into the compact native globular structure, a large number of hydrophobic residues get buried inside the protein core. These hydrophobic residues impart proteins the tendency of aggregation when they are unfolded or partially folded [10]. Partially folded and misfolded

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proteins that escape the cellular quality control mechanism have the high tendency to form inter-molecular hydrogen bonding between the same protein molecules resulting in aggregation [11]. These conformational states of a polypeptide chain form the precursor aggregates, eventually leading to amyloid formation. A number of severe human pathologies such as type II diabetes, Parkinson's diseases, Alzheimer's diseases and transmissible spongiform encephalopathy's (TSEs; prion diseases such as bovine spongiform encephalopathy, BSE, or Mad Cow disease and Creutzfeldt–Jacob disease, CJD, in humans) are based on extracellular deposition of insoluble protein aggregates known as amyloid fibrils [12, 13]. The likelihood of aggregation is further triggered by post-translational modifications, occurring as chemical modifications at amino acid residues, including glycation, glycosylation, phosphorylation, sumoylation etc. During glycation, amino group (NH_2-group) of proteins comes in contact with carbonyl (CO) group of sugars, Amadori Rearrangement Product or Schiff base is formed first, which later on is transformed into Advanced Glycated end products (AGEs) [14]. AGEs have the property of exhibiting fluorescence as well as strong tendency to aggregate. Glycation has several pathological effects on human body. It leads to several diseases such as renal failure, diabetic nephropathy, retinopathy etc. as well as damages non-structural proteins such as enzymes and serum proteins. Various proteins such as collagen, myelin basic protein, lens crystallin, LDL as well as hemoglobin become glycated in diabetes [15–18]. The covalent binding of proteins with sugars such as glucose causes conformational changes in these glycated proteins which in turn modify their functional properties [19–21].

Chicken egg ovalbumin, the major secretary product of oviduct cells is a 45-kd protein [22–24]. The glycoprotein consists of a single polypeptide chain of 385 amino acids residues that fold into a globular conformation with a high secondary structure content (30.6% α-helix and 31.4% β-strand) [25]. The folding of ovalbumin is of interest because to initiate the folding process, N-terminal signal sequence is required but ovalbumin lacks this initiating signal so it accomplishes its folding process with the help of its internal hydrophobic signal sequence [26]. Ovalbumin is also an important model system to study helix/sheet transitions, because it has almost equal amount of α-helix and β-sheet, i.e., 30.6% α-helix and 31.4% β-strand. Besides, it also has bioinformatics applications such as discovering new proteins of serpin super family by using BLAST and CLUSTALW tools. As these distinct structures are highly compact, the transition of α-helix to β-sheet structural changes can be a promising approach to discover new therapies as this transition leads to severe maladies.

Here in this study ovalbumin in the presence of different concentration of glucose on different incubation time varying from hours to days exists in different intermediates forms on folding and aggregation pathways. In our studies, in the presence of glucose, a higher denaturant concentration (3 M Gdn HCl) was required to obtain an intermediate state that was previously observed at lower concentration of Gdn HCl (1 M Gdn HCl) in absence of glucose. Glucose at low concentration serves as a food for energy and also provides defense against physiological stress by preventing cellular proteins from being damaged. Here at low concentration, it serves as a protective additive against Gdn HCl. High concentration of glucose destabilizes the glycoprotein resulting in formation of AGEs. The present study mimics the in vivo situation; in providing rapid results that investigates the role of hyperglycemia in the development of pathological diseases.

Materials and Methods

Ovalbumin from *Gallus gallus* was purchased commercially from genei (Bangalore, India); guanidine HCl was from Sigma Chemical Co. (St. Louis, MO, USA). Glucose, acrylamide, EDTA, Tris base, glycine, NAH_2PO_4 and NA_2HPO_4 were from Sisco Res. Lab., India. All other chemicals were of analytical grade. Protein concentration of ovalbumin stock solutions was typically 5 mg/ml for folding and 10 mg/ml for glycation studies.

Protein Estimation

Protein concentration was determined spectrophotometrically using molar extinction coefficient on Hitachi single beam spectrophotometer using 1 cm path length [27].

Effect of Gdn HCl and Glucose on Ovalbumin

Ovalbumin solutions of varying concentration of Gdn HCl (0 to 5 M) in absence and presence of 20 mM glucose were prepared in 20 mM sodium phosphate buffer (pH 7.2) and incubated at 37 °C for 4 h prior to carrying out spectroscopic measurements.

Preparation of Glycated Samples

For kinetics experiments, ovalbumin was incubated in 50 mM glucose and 50 mM fructose separately at 37 °C for various days. The final concentration of ovalbumin in the incubation mixture at the start of the reaction was 2 mg/ml. All solutions were prepared under sterile condition by adding sodium azide (0.002%) to avoid contamination (bacterial or fungal). Aliquots were withdrawn on alternate day from incubated samples for further studies.

Intrinsic Tryptophan Fluorescence

The fluorescence spectra were recorded on a Shimadzu RF-1501 spectrofluorophotometer (Tokyo, Japan) in a 1 cm path length quartz cell in the wavelength range of 300–400 nm using protein concentration of 2.2 μM. The excitation wavelength was 280 nm and the emission was recorded from 300 to 400 nm [28].

Thioflavin T (Th T) Assay

Fluorescence spectra were recorded with a Shimadzu RF-1501 spectrofluorophotometer in a 1 cm path length quartz cell. The excitation wavelength was 440 nm and the emission was recorded from 450 to 600 nm [29]. Final concentration of glycoprotein was made 2.2 μM in 15 μM Th T dye and 20 mM sodium phosphate buffer. Samples were incubated for 24 h at room temperature before taking fluorescence.

Fourier Transform Infra Red Spectroscopy (FTIR)

FTIR spectra were recorded with an Interspec-2020 Fourier transform spectrometer in D_2O. Each spectrum was the average of 6 scans. Protein concentration of the samples was typically 2.2 μM. The scanning wave number was from 1000 to 4000 cm^{-1}.

Circular Dichroism (CD) Spectrum Measurements

CD was measured with a JASCO J-815 spectropolarimeter calibrated with ammonium D-10-camphorsulfonate. A cell of path lengths 0.1 cm was used for scanning between 250 and 190 nm. Protein concentration of the samples was typically 2.2 μM in 20 mM phosphate buffer of pH 7.2 for the far-UV studies. Each spectrum was the average of four scans. The results were expressed as mean residue ellipticity (MRE) in degree cm^2/dmol, which is defined as $MRE=\theta_{Obs}$ (mdeg)/10×n×Cp×l; where θ is the observed ellipticity in degrees, Cp is the molar fraction, and l is the length of light-path in cm [30].

8-Anilino-1-Naphthalene-Sulphonic acid (ANS) Fluorescence Measurements

ANS binding was measured by fluorescence emission spectra with excitation at 380 nm and emission was recorded from 400 to 600 nm, respectively [31]. Typically, ANS concentration was 100 molar excess of protein concentration and protein concentration was in the vicinity of 2.2 μM in 20 mM phosphate buffer, pH 7.2. It should be noted that Gdn HCl itself binds to ANS, therefore to avoid anomaly, proper blank was made.

SDS- PAGE (Sodium Dodecyl Sulphate Poly Acrylamide Gel Electrophoresis)

12% SDS PAGE was prepared by incubating ovalbumin with 50 mM glucose/fructose for 10 days at 37 °C to run the glucated/fructated ovalbumin [32]. The samples were withdrawn on alternate day from the respective samples and were loaded. The purity of the protein was also confirmed by this SDS-PAGE, a single monomeric band appearing in case of ovalbumin whereas band multiplicity was observed for glucated and fructated samples.

Results and Discussion

Intrinsic Fluorescence

Intrinsic fluorescence is a sensitive index of alteration in protein conformation. There are three tryptophan residues: Trp148 in helix F, Trp184 as the nearest neighbor residue of the C-terminus of strand 3A and Trp 267 in helix H as well as 8 tyrosine residues in a single polypeptide chain of ovalbumin glycoprotein [33]. The conformational states of ovalbumin were analyzed by monitoring the polarity of three tryptophan and eight tyrosine residues when ovalbumin was incubated with varying concentration of Gdn HCl (0–5 M) in absence and presence of 20 mM glucose. Figure 1(a) showed the relative fluorescence of ovalbumin as a function of increasing concentration of Gdn HCl in absence and presence of 20 mM glucose. On increasing concentration of Gdn HCl, there was an increase in fluorescence intensity up to 1 M, indicating the presence of a partially folded intermediate state at 1 M Gdn HCl. In the presence of glucose; this partially folded state was shifted to 3 M Gdn HCl concentration. On further increasing denaturant concentration, beyond 1 M (with no glucose) and 3 M (with 20 mM glucose), there was a regular decrease in relative fluorescence intensity in both the curves, suggesting that the aromatic amino acid residues are getting denatured on increasing the Gdn HCl concentration. Figure 1(b) depicted the intrinsic emission spectrum of ovalbumin in presence of varying concentration of Gdn HCl. Native ovalbumin has emission maxima at 335 nm (curve 1). On increasing Gdn HCl concentration from 0.5 M (curve 2) to 1 M (curve 3), there was of roughly ~73% increase in fluorescence intensity (curve 2 to curve 3) and λ_{max} underwent a blue shift of 5 nm i.e. at 330 nm. We speculate that the tryptophan residues at 1 M Gdn HCl induced state are somewhat exposed to the environment in contrast to the highly buried tryptophan residues in the native protein. These partially exposed tryptophan residues face more and more non-polar environment explaining the blue shift in λ_{max} and enhancement in intrinsic fluores-

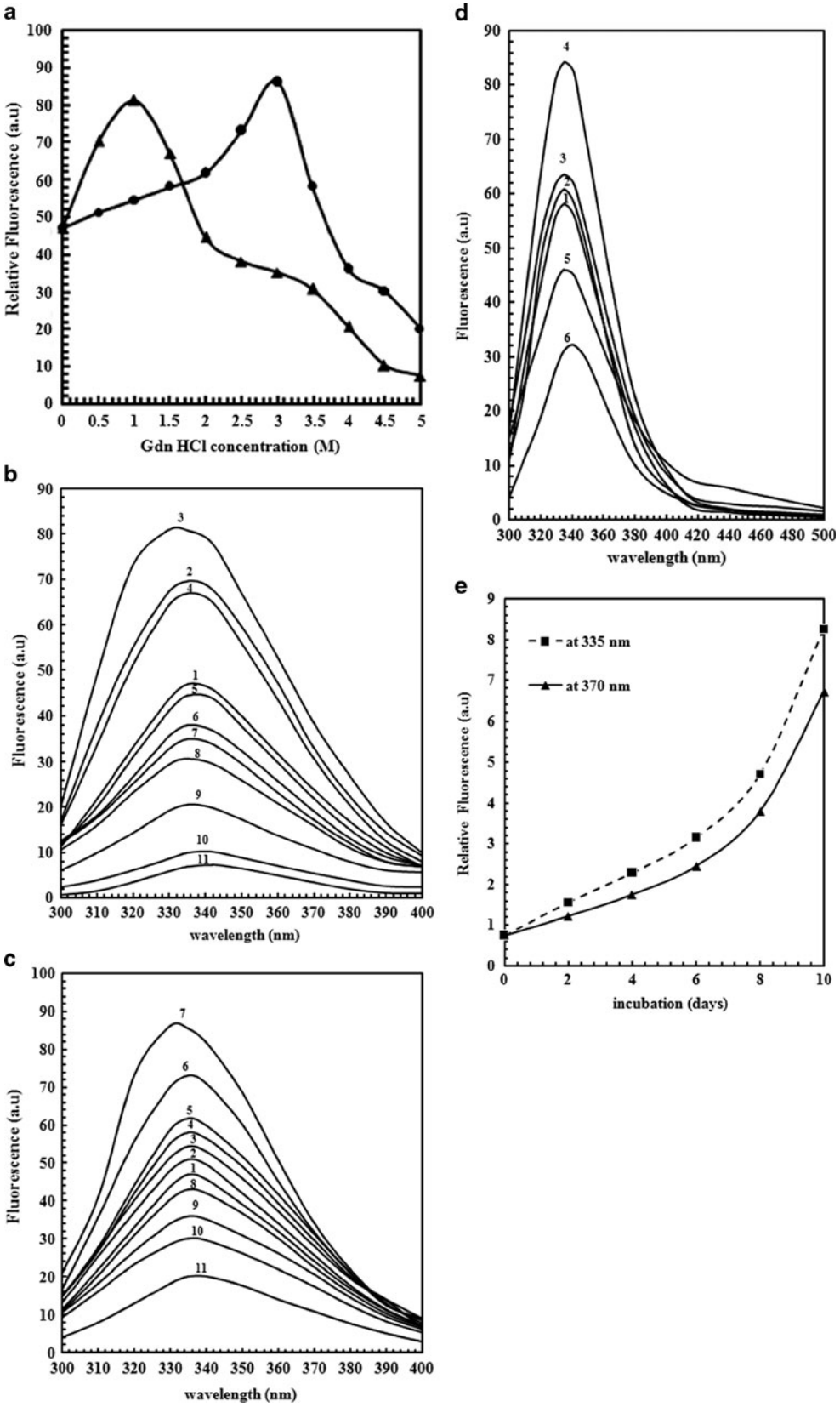

Fig. 1 **a** Relative intrinsic fluorescence intensity of ovalbumin as a function of increasing concentration of Gdn HCl without glucose [▲] and in presence of 20 mM glucose [●]. The protein concentration was 2.2 μM and the path length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 280 nm. **b** Intrinsic fluorescence emission spectra of native ovalbumin in 20 mM sodium phosphate buffer pH 7.2 (curve 1) and ovalbumin at 0.5 M–5 M Gdn HCl is shown in curve 2–11). The protein concentration was 2.2 μM and the path length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 280 nm and emission was recorded in the range of 300–400 nm. **c** Intrinsic fluorescence emission spectra of native ovalbumin in 20 mM sodium phosphate buffer pH 7.2 (curve 1) and ovalbumin at 0.5 M–5 M Gdn HCl in presence of 20 mM glucose is depicted in curves 2–11. The protein concentration was 2.2 μM and the path length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 280 nm and emission was recorded in the range of 300–400 nm. **d** Intrinsic Fluorescence emission spectra of native ovalbumin in 20 mM sodium phosphate buffer pH 7.2 (curve 1) and ovalbumin in presence of 50 mM glucose incubated for 2, 4, 6, 8 and 10 days at 37 °C is shown in curves 2–6 respectively. The protein concentration was 2.2 μM and the path length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 280 nm and emission was recorded in the range of 300–500 nm. **e** Time course of two AGEs products (pentosidine (■), excitation 335 nm and malondialdehyde [▲], excitation 370 nm) of ovalbumin in presence of 50 mM glucose at 37 °C

cence. On further increasing Gdn HCl concentration, fluorescence intensity was found to decrease regularly and a red shift of 5 nm in the λ_{max} to 335 nm was observed in 1.5 M (curve 4), 2 M (curve 5), 2.5 M (curve 6), 3 M (curve

7), 3.5 M (curve 8) and 4 M Gdn HCl (curve 9) as compared to ovalbumin in 1 M Gdn HCl (curve 3). The addition of 4.5 M (curve 10) and 5 M Gdn HCl (curve 11) caused further decrease in fluorescence intensity and a further red shift of 7 nm in the λ_{max} to 342 nm. Maximum decrease was found at 5 M Gdn HCl suggesting that protein is almost completely denatured at this stage due to increased polarity of environment. Similarly, intrinsic emission spectra of ovalbumin in varying concentration of Gdn HCl and 20 mM glucose are shown in Figure 1(c). The maximum increase in fluorescence intensity was observed at 3 M (curve 7) with ~84% increase in fluorescence intensity with respect to native and a blue shift of 3 nm in λ_{max} i.e. at 332 nm suggesting the conformational changes in the vicinity of surface-exposed aromatic amino acid residues, presumably due to internalization in a more hydrophobic environment. Fluorescence intensity of tryptophan beyond 3 M was found to decrease regularly and a red shift in λ_{max} to 335 nm was observed up to 4.5 M (curve 8 to curve 10) as compared to ovalbumin in 3 M Gdn HCl (curve 7). Maximum decrease was found at 5 M Gdn HCl and a further red shift in λ_{max} to 340 nm was observed (curve 11). At 5 M Gdn HCl, when the protein is in polar environment, the tryptophan residues which were buried in the native environment get exposed due to Gdn HCl unfolding; this is evident by low fluorescence intensity and a red shift in λ_{max} of 5 nm as compared to the native spectrum. Similar blue shifted and red shifted fluorescence has been reported earlier by Naeem et.al on ovalbumin and papain [33, 34]. These results indicated that glucose shifted the partially folded intermediate state at 3 M Gdn HCl and hence protected the glycoprotein from being exposed to the outer environment, consequently higher concentration of Gdn HCl was required to achieve the same partially folded intermediate state as same as found in absence of glucose. A parallel experiment carried out in ovalbumin at 3 M Gdn HCl on increasing concentration of glucose up to 100 mM to prove the protective effect of glucose was provided in the supporting information (Fig. S1). Ovalbumin in 3 M Gdn HCl and 20 mM sodium phosphate buffer has emission maxima at 335 nm (curve 1). There was concomitant increase in fluorescence intensity with increasing glucose concentration from 10 mM to 100 mM (curves 2–7). Maximum fluorescence intensity was observed at 100 mM suggesting the presence of an intermediate state. We previously mentioned that ovalbumin (in presence of 20 mM glucose) possesses maximum fluorescence intensity at 3 M Gdn HCl. Thus we can suggest that glucose in the presence of 3 M Gdn HCl has a significant role in stabilizing the protein structure.

The fluorescence spectra of ovalbumin during glucation reaction shown in Fig. 1(d) indicated changes in protein structure due to reaction with glucose (50 mM at 37 °C, incubation temperature raised to achieve faster glycation kinetics). Control ovalbumin was found to have no alteration in intrinsic fluorescence intensity compared with native ovalbumin. In control ovalbumin (curve 1), a peak at 335 nm was observed which got steadily increased on incubating ovalbumin from 2nd day to 6th day (from curve 2 to curve 4). The increasing trend of fluorescence reversed from the 8th day (curve 5) showing the decrease of fluorescence intensity on 10th day of glucation. Maximum decrease was observed on 10th day of glucation with 7 nm red shift in λ_{max} (curve 6). These results indicated that glucated ovalbumin's amino acid residues are gradually exposed to the outer environment on incubating for 6 days, consequently showing maximum fluorescence on 6th day hence suggesting a molten globule state at this day. Molten globule state has been reported in human serum albumin [35]. The isoemissive point at 400 nm was observed in the spectra, indicating a conversion of early glycation adduct to late stage glycation product. Similar isoemissive point has been reported earlier in hemoglobin [36]. Our findings suggest that, in the initial phase with advancement of Maillard reaction active participation of the free -NH2 group (amino group) and glucose takes place to form a Schiff's base linkage, leading to increase in protein fluorescence with peak at 335 nm up to 6 days of incubation, beyond 6 days reduction in protein fluorescence was observed accompanied by 7 nm red shift after 10 days of incubation.

AGE Accumulation

The intrinsic molecular nature of ovalbumin-AGEs can prove to be useful for long-term monitoring of conformational diseases i.e. diabetes mellitus. In vitro studies showed that two different fluorophore adducts developed after a prolonged period of incubation of ovalbumin with glucose. These fluorophore adducts having the excitation wavelengths of 335 nm and 370 nm seem to be characteristic of ovalbumin-AGEs and are specific for the fluorescent AGEs formed during glucation of ovalbumin. The fluorescence emission intensity at 335 nm and 370 nm also steadily increased on incubating from 0 to 10 days (data not shown). Figure 1(e) depicted the temporal accumulation of ovalbumin-AGEs using excitation wavelengths of 335 nm and 370 nm. Fluorescence for the known fluorescent adducts like pentosidine (335 nm) and malondialdehyde (370 nm) was found to be high on 10th day.

Thioflavin T-assay

The β-sheet structure and high hydrophobic residues of ovalbumin observed in the presence of 3 M Gdn HCl and 20 mM glucose were further analyzed for aggregation. This

was assessed by using thioflavin T, a benzothiazole dye that specifically binds to ordered β-sheet aggregates [37]. Figure 2 depicted the thioflavin T fluorescence spectra of ovalbumin at 3 M Gdn HCl in absence and presence of 20 mM glucose incubated for 24 h. Samples of control ovalbumin (curve 1) and in 3 M Gdn HCl (curve 2) showed less enhancement than that observed for ovalbumin in 3 M Gdn HCl, 20 mM glucose and phosphate buffer at pH 7.2 (140 times in curve 3). This implied that 20 mM glucose in 3 M concentration of Gdn HCl induces an environment which enhances the formation of aggregates in native ovalbumin as confirmed by thioflavin T-assay. This can be interpreted as when aggregation takes place Th T comes in proximity of side chains of β-sheet and it results in steric interaction between them. These interactions could ensure that dye molecule remains in a flat conformation even when excited by the absorbance of photon of light [38]. Thus with increase in aggregation, when progressively β-sheets are formed in solution, large amount of Th T comes in contact with side chains leading to enhanced fluorescence as shown in ovalbumin at 3 M Gdn HCl and 20 mM glucose incubated for 24 h.

FTIR Analysis

FTIR has been used to probe protein structure, and the amide I band has been used to estimate spectroscopy protein secondary structure content [39]. Infrared spectroscopy predicts β-strands much better than α-helices [40]. Figure 3 depicted wavenumber analyses of ovalbumin between 1550 and 1750 cm^{-1} revealing a peak corresponding to the region of amide band protein (NH-CO). The conformational changes in ovalbumin by adding

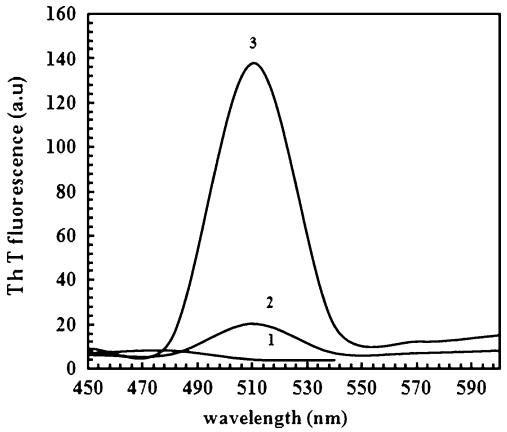

Fig. 2 Thioflavin T spectra of native ovalbumin in 20 mM sodium phosphate buffer (curve 1); curve 2 represents ovalbumin at 3 M Gdn HCl and curve 3 at 3 M Gdn HCl in presence of 20 mM glucose. The protein concentration was 2.2 μM and the path length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 440 nm and emission was recorded in the range of 450–600 nm

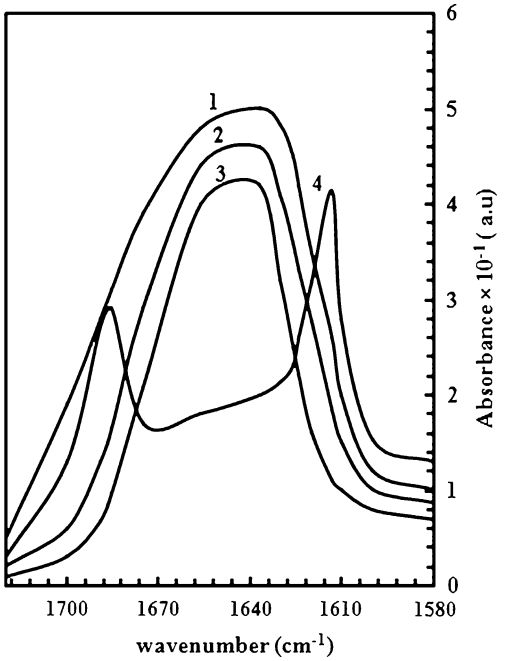

Fig. 3 FTIR spectra of ovalbumin in the amide I region. Curve 1 shows spectra of native ovalbumin in 20 mM sodium phosphate buffer, pH 7.2; curve 2 shows the spectra of molten globule state at 1 M Gdn HCl; curve 3 and curve 4 depict spectra of ovalbumin at 3 M Gdn HCl in presence of 20 mM glucose incubated for 4 h and 24 h respectively

1 M Gdn HCl and 3 M Gdn HCl (in presence of 20 mM glucose) were monitored in the amide I band region of FTIR spectra. Native ovalbumin showed a broad peak around 1,636 to 1,656 cm^{-1} indicating presence of α-helix and β-sheet structure (curve 1). On addition of 1 M Gdn HCl, peak was observed at around 1637 cm^{-1} indicating the presence of β-sheet structure (curve 2). This indicates that this state corresponds to the β-sheet organization similar to one present in native state. At 3 M Gdn HCl and 20 mM glucose (curve 3), retention of this peak was observed at 1637 cm^{-1} but with lower absorbance suggesting that this state of ovalbumin is closer to native as compared to 1 M Gdn HCl state. These states can be characterized as partially folded intermediates. Moreover, on increasing the incubation time for 24 h of the ovalbumin intermediate state at 3 M Gdn HCl (in the presence of 20 mM glucose), concomitant appearance of the two bands at 1685 cm^{-1} and 1613 cm^{-1} in the amide I region of the FTIR spectrum suggests that such β- structures result from hydrogen bonding with in protein aggregates (curve 4). A peak at 1613 cm^{-1} in the amide I region implies that aggregated species possess extensive β- sheet structure. Significantly, this type of infrared spectrum has been shown to be characteristic of amyloid fibrils [41, 42]. Kauffmann et al. reported transition from α-helix to β-sheet in β-lactoglobulin using a diffusive IR mixture [43]. B-Lactoglobulin is an important model system for their study, because it

represents a protein that would be predicted by modeling to be α-helix rather than β-sheet. Ovalbumin is selected as a model to study helix/sheet transitions, because it represents a protein that possesses equal% of α-helix and β-sheet, i.e., 30.6% α-helix and 31.4% β-strand. An erroneous transition from α-helix to β-sheet structure has fatal consequences in prior and other amylogenic diseases. As these distinct conformations are both highly compact, an important study can be the interconversion of α-helix to β-sheet structural changes.

Circular Dichroism Measurements Far-UV CD

The changes in secondary structure of ovalbumin as a function of Gdn HCl (0–5 M) in absence and presence of 20 mM glucose were followed by far-UV CD by measuring ellipticity at 222 nm (Fig. 4a). As can be seen from the figure, transition was occurred in the vicinity of 1 M Gdn HCl and 3 M Gdn HCl in presence of 20 mM glucose. Both the curves are sigmoid shape indicating that this transition approximates to a two step process. Figure 4(b) showed the far-UV CD spectra of native ovalbumin (curve 1), in 1 M Gdn HCl (curve 3), in 3 M Gdn HCl with 20 mM glucose (curve 2) and in 5 M Gdn HCl concentration (curve 4). Native ovalbumin showed the minima at 208 nm and 222 nm indicating the presence of α- helical structure (curve 1). Addition of 1 M Gdn HCl to ovalbumin resulted in the appearance of peak at 219 nm, suggesting the transition from α- helix to β- sheet structure (curve 2). In the presence of 20 mM glucose, peak at 219 nm was retained in 3 M Gdn HCl with more negative MRE value (curve 3) suggesting the increase in β- sheet content. On further increasing Gdn HCl concentration, its denaturing effect increases resulting in the complete disruption of secondary structure at 5 M (curve 4). CD measurements were also performed to investigate glucation on ovalbumin samples withdrawn on alternate day up to ten days. Figure 4(c) showed the far-UV CD spectra of ovalbumin with 50 mM glucose in the 190–250 nm range on 6th, 8th and 10th day. Peak at 208 nm and 222 nm in native ovalbumin (curve 1) was observed showing the presence of α-helical structure. There were no significant changes observed on 2nd and 4th day (data not shown). Ovalbumin on incubating with 50 mM glucose for 6 days turned into β-sheet structure with the appearance of peak around 219 nm indicating the presence of a compact intermediate state on 6th day (curve 2). In contrary to this, CD signal in

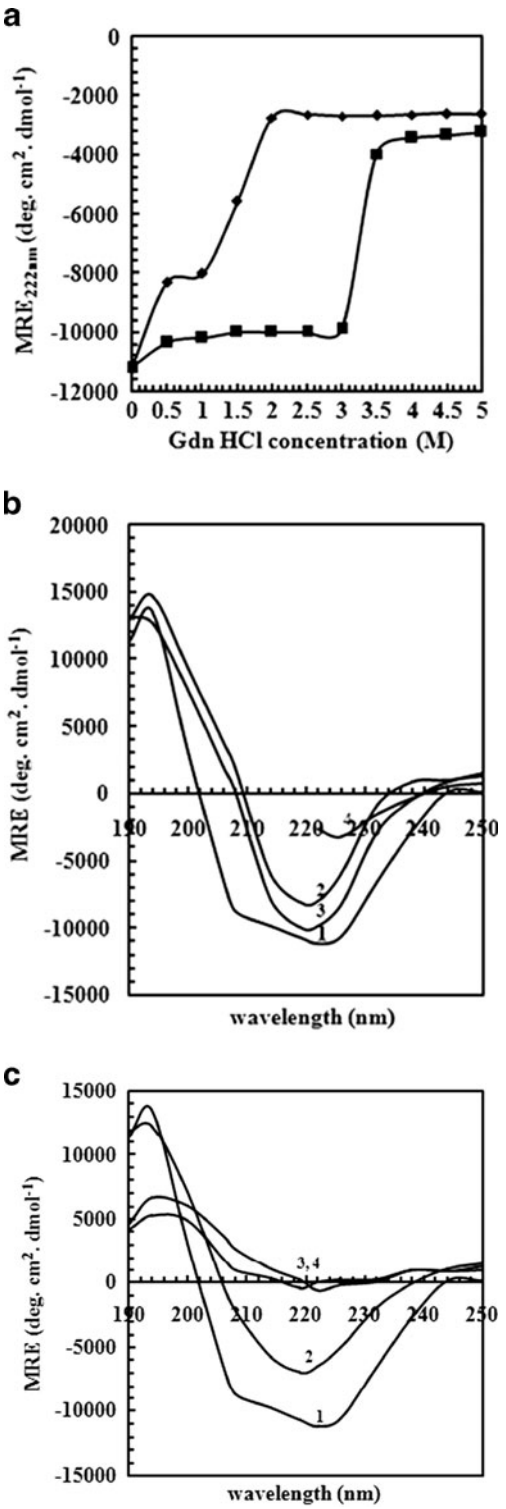

Fig. 4 **a** Effect of increasing concentration of Gdn HCl (0 M–5 M) on ovalbumin with no glucose [♦] and in presence of 20 mM glucose [■] as followed by MRE measurements at 222 nm. **b** Far-UV CD spectra of ovalbumin in the presence of Gdn HCl in 20 mM sodium phosphate buffer, pH 7.2. Curve 1 shows spectra of native ovalbumin; curve 2 corresponds to the ovalbumin spectra at 1 M Gdn HCl, curve 3 depicts spectra at 3 M Gdn HCl in the presence of 20 mM glucose and curve 4 shows spectra at 5 M Gdn HCl respectively. The protein concentration was 2.2 μM and the path length was 0.1 cm. **c** Far-UV CD spectra of ovalbumin at different days of incubation. Curve 1 shows the spectra of native ovalbumin; curves 2–4 show spectra of ovalbumin in presence of 50 mM glucose incubated for 6, 8 and 10 days respectively. The protein concentration was 2.2 μM and the path length was 0.1 cm

this range disappeared on further incubating ovalbumin for 8 days (curve 3). On 10th day, no CD signal was observed (curve 4), probably due to considerable scattering of light as soluble glycoprotein converted to insoluble cross-linked Amadori products. After 6 days of incubation, the secondary structure of glucated ovalbumin was retained, and thus this incubation time was related to the molten globule state of Maillard reaction.

Incubation with glucose induced various conformational changes in ovalbumin in a concentration and time dependent manner. These changes were due to extensive hydrogen bonding, which usually occurs between glucose and ovalbumin, and directly enhances or reduces the extent of H-bonding [44].

ANS Fluorescence Measurements

Since ANS is known to bind the hydrophobic patches on proteins, binding of ANS to hydrophobic regions of protein has been widely used to study the folding intermediates formed during protein folding [45]. The intermediate state induced by Gdn HCl and glucose was further confirmed by extrinsic fluorescence studies. Figure 5a depicted the relative ANS fluorescence intensity measurements of ovalbumin (at 480 nm) as a function of varying concentration of Gdn HCl (0 M to 5 M) in absence and presence of glucose (20 mM). As can be seen from this figure on increasing Gdn HCl concentration in absence of glucose, ANS showed maximum binding at 1 M and in presence of glucose, maximum binding was observed at 3 M Gdn HCl. This observation could be attributed to the fact that when protein was allowed to denature in absence of glucose, some hydrophobic pockets which were earlier not accessible in native ovalbumin, became now accessible so that ANS molecules bind in these pockets, generate fluorescence and hence produce signal at 1 M. While in presence of glucose, higher concentration of Gdn HCl (3 M) is required for the hydrophobic packets to be exposed for ANS binding. The further decrease in ANS binding on increasing Gdn HCl concentration in presence of glucose is due to disruptions of hydrophobic patches. Glycoprotein's hydrophobic compact state appeared at 1 M Gdn HCl with no glucose shifted to 3 M in presence of glucose. This is because glucose acted as a protector by forming hydrogen bonds with the charges induced by Gdn HCl on protein and hence high concentration of denaturant is required to achieve this partially folded state in presence of glucose. The protective effect of glucose was also confirmed by ANS fluorescence. The relative intensity measurements of ovalbumin in 3 M Gdn HCl as a function of increasing concentration of glucose up to 100 mM was provided in supporting information (Fig. S2). Maximum ANS binding was observed at 100 mM showing the presence of a compact state at 100 mM glucose. These results also suggest the protective behavior of glucose in ovalbumin at 3 M Gdn HCl.

Figure 5b showed the relative ANS fluorescence intensity measurements (at 480 nm) of ovalbumin in presence of 50 mM glucose as a function of days. The affinity of ANS for ovalbumin increased after 6 days incubation time with 50 mM glucose. Collectively, these data suggest that the formation of molten globule state of ovalbumin occurs during the progression of Maillard reaction in the presence of 50 mM glucose after 6 days of incubation. The observed maximum ANS fluorescence intensity on 10th day resulted from dramatic conformational state alterations in the ovalbumin structure to enhance hydrophobicity of the protein due to developing of Amadori products [35].

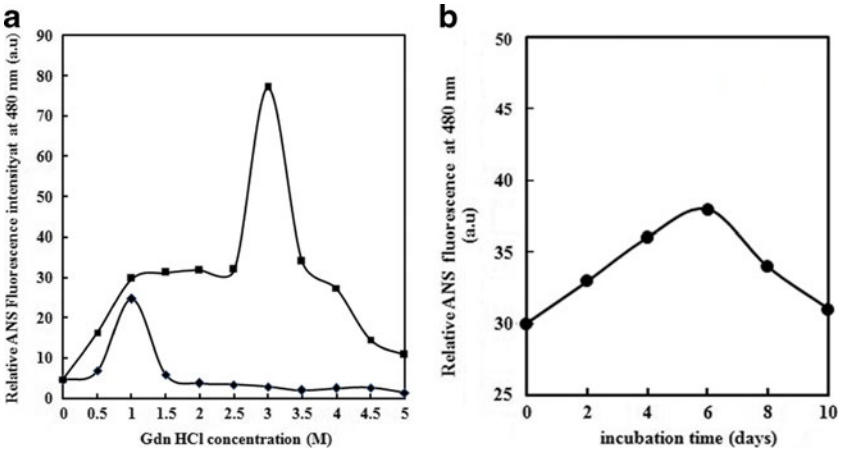

Fig. 5 **a** Relative ANS fluorescence intensity at 480 nm of ovalbumin as a function of increasing concentration of Gdn HCl without glucose concentration [♦] and in presence of 20 mM glucose [■]. The protein concentration was 2.2 μM and the path length was 1 cm. The fluorescence intensity measurement was carried out at an excitation wavelength of 380 nm. **b** The effect of glucation time on ANS binding fluorescence of ovalbumin: plots were constructed at maximum emission wavelength at 480 nm against glucation time (●)

SDS-PAGE Analysis

To investigate further whether the tertiary structure is disrupted, we incubated ovalbumin with 50 mM glucose and fructose independently up to 10 days and then aliquots were withdrawn and analyzed on alternate day by SDS-PAGE. Figure 6 showed SDS gel migration profiles of glucated and fructated ovalbumin. Retardation of protein bands were observed during the glycation of ovalbumin with glucose and fructose on 10th day. It is also proved that damage induced by fructose is greater than glucose by observing cross linked dimeric band earlier in the presence of fructose. The first lane from extreme left corner showed the control while lanes 2, 3 and 4 showed the formation of aggregated cross-linked species due to glucation and fructation reactions. The cross-linked dimeric band started to appear in glucose on 10th day of incubation (lane 2) while dimeric as well as aggregated species started to appear in case of fructose from 8th day onwards (lane 3) which faded with time and a smeared zone emerged at 10th day (lane 4). No significant band retardation was exhibited in control ovalbumin (data not shown). These results demonstrated that incubation of ovalbumin with glucose and fructose led to AGEs formation. The induced protein damage could be traced back to protein cross linking induced by glycation at specific sites (say, lysines). The evidence for such cross linking was shown by presence of an additional SDS resistant dimeric band in SDS-PAGE of glucated ovalbumin. Such covalent cross linking in turn might induce stress in the backbone finally triggering the overall structural changes.

To summarize: from the far-UV CD spectra, FTIR spectra, fluorescence, ANS binding and Th T assay, we showed that the two partially folded states of ovalbumin exist as compact intermediates with altered secondary structure, intrinsic fluorescence and high ANS binding. Both states have similar characteristics with difference only in amount of structure and microenvironment. In the presence of glucose, a high concentration of Gdn HCl is required to achieve the intermediate state. This may be due to the hydroxyl groups of glucose moiety that protected the glycoprotein by forming intermolecular hydrogen bonds between glycoprotein and glucose. These states on increasing the incubation time as well as concentration of glucose resulted in formation of molten globule, aggregates and AGEs. The molten globule state was also specified after 6 days incubation. Thus, exposure of ovalbumin to high glucose concentration for a long period of time may have a great impact on its structural properties.

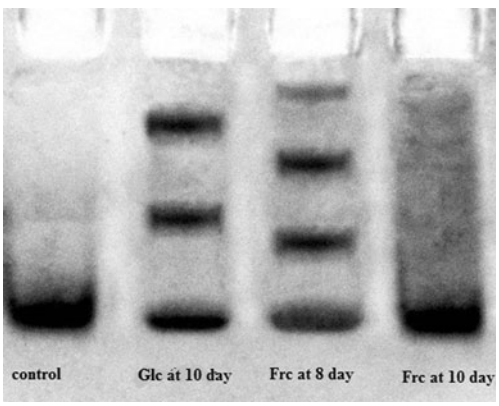

Fig. 6 SDS-PAGE pattern of ovalbumin showing changes in mobility as a result of modification by sugars. The lanes from left represents control ovalbumin, 2nd lane shows ovalbumin in the presence of 50 mM glucose after 10 days of incubation. 3 rd and 4th lanes show ovalbumin in the presence of 50 mM fructose after 8 and 10 days of incubation respectively. Temperature of incubation was 37 °C

Conclusions

Mimicking the in vivo conditions are valuable approaches for studying the conformational changes occurring in proteins due to physiological stress which in turn will be helpful in understanding the pathological conditions responsible for disease. Glucose assists the folding of protein under physiological stress conditions. Since glucose has five hydroxyl groups (−OH) and one aldehyde group (−CHO). Glucose on incubation for short period of time showed stabilizing effect as hydroxyl groups being more in number formed hydrogen bonding with ovalbumin. On increasing incubation time, glucose generated aggregation-prone partially folded state of ovalbumin in 3 M Gdn HCl. Exposure of macromolecules in vitro to high glucose concentration is considered as a relevant model for studying the structural alterations occurring in glycation. This is because on incubating for longer time period aldehyde group form Schiff's base between protein and sugar moiety subsequently disrupting the native conformation of protein. Characterization of such partially folded states has important implications for protein folding and aggregation studies. The problem of stimulating glycation in vivo is that in the cell the glucose concentration is of the order of 5 mM, which is ten times lower than the concentration used in vitro (50 mM) to study glycation. However the ovalbumin concentration used in this study is 0.1 mg/ml which is very much lesser than present in oviduct cells. According to law of mass action, rate of reaction depends on all of the reactants present in a reaction. Reaction rate will be equivalent if concentration of first reactant is low and concentration of second reactant is high or vice versa. Therefore, given the rates are similar, in vivo, the damage will be higher if the protein concentration is high, as the cross linking may have a cascade effect. By using concentration of glucose higher than physiological level in

our study, a chemically equivalent condition was being prevailed. Our studies conclude that changes in the microenvironment of ovalbumin destabilize the native structure forming the aggregates and AGEs. We suggest that under appropriate conditions, it is the intrinsic property of natural polypeptide to form protein aggregates and AGEs. Both protein aggregates and AGEs are found in pathological conditions such as Diabetes Mellitus (DM), Alzheimer's disease, Parkinson's disease, nephropathy, retinopathy. A step ahead to the present study will encourage a more proactive approach to early diagnosis as well as therapy of conformational diseases and nutritional counseling.

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