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ORIGINAL PAPER

An Insight into the Biophysical Characterization of Insoluble Collagen Aggregates: Implication for Arthritis

Samreen Amani · Anas Shamsi · Gulam Rabbani · Aabgeena Naim

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Abstract Misfolding and aggregation of proteins is involved in some of the most prevalent neurodegenerative disorders. The importance of collagen stems from the fact that it is one of the dominant component used for tissue engineering and drug delivery applications and is a major component of skin, tendon, bone and other connective tissues. A systematic investigation on the conformation of collagen at various concentrations of glyoxal is studied by various biophysical techniques such as Trp fluorescence, ANS binding, Circular dichroism (CD), ATR-FTIR, Congo red (CR) assay, Rayleigh light scattering and Turbidity measurements. At 60 % (*v*/*v*) glyoxal, collagen retains native-like secondary structure, altered Trp environment and high ANS fluorescence, characteristic of molten globule (MG) state. At 80 % (v/v) glyoxal, insoluble collagen aggregates are detected as confirmed by decrease in Trp and ANS fluorescence, increase in non-native β sheet structure as evident from far-UV CD and FTIR spectra, increase in Thioflavin T fluorescence, Rayleigh light scattering, Turbidity measurements, as well as red shift in CR absorbance.

Keywords Aggregation · Collagen · Glyoxal · Fluorescence spectroscopy · Molten globule

S. Amani · A. Shamsi · A. Naim (✉)
Department of Biochemistry, Faculty of Life Sciences, Aligarh Muslim University, Aligarh 202002, India
e-mail: aabgeenanaim@gmail.com

A. Naim
e-mail: anaeem.bc@amu.ac.in

G. Rabbani
Interdisciplinary Biotechnology Unit, Aligarh Muslim University, Aligarh 202002, India

Abbreviations

ANS	8-anilino-1-naphthalene-sulphonic acid
ATR-FTIR	Attenuated total reflection fourier transformed infra red spectroscopy
CD	Circular dichroism
CR	Congo red
Hyp	Hydroxyproline
MG	Molten globule
PPII-type	Polyproline II-type
ThT	Thioflavin T

Introduction

For a protein to be properly functional it must be in its properly folded form and when it fails to fold appropriately, it results in aggregation. The accumulation of misfolded or aggregated proteins can lead to cell death or dysfunction [1]. A protein can exist as intermediate state between its unfolded and native state depending upon its environment, such as pH, temperature, different denaturants, etc. Under certain conditions, proteins can also exist as a collapsed state with partial ordered structure known as the "molten globule" [2, 3]. A 'Molten Globule' (MG) has native-like secondary structure with radius only 10–20 % larger than that of the native structure but there is absence of specific tertiary packing interactions of the side chains [4]. Thus protein aggregation has become one of the debated topics of current science as aggregates formation can be accounted for various disorders currently under research.

Collagen is rich only in three amino acids and its major role is structural. It is the most abundant fibrous protein in vertebrates found in tendons, cartilage and cornea of eye.

It has unique structure in which three polypeptide strands, each possessing left-handed polyproline II-type (PPII-type) helix, twisted together into a right-handed coiled coil structure, called as triple helix or Tropocollagen [5, 6]. Though the chains are covalently cross linked by Lys-Lys and Lys-Hydroxylysine bonds but it has been well established that the major forces stabilizing the collagen triple helices are hydrogen bonding [7]. The presence of glycine at every third residue in a Xaa-Yaa-Gly (where Xaa and Yaa can be any amino acid) repeating motif makes it possible for the existence of a tightly packed triple helical structure in collagen. Hydroxyproline (Hyp) residues play a significant role, as it is suggested to form water mediated bridges with free carbonyl groups in the triple helix [8]. However, recently it has been demonstrated that Hyp residues stabilize the triple helix via a stereoelectronic effect that organizes appropriate backbone torsion angles for triple-helix formation [9]. Collagen-derived products as biomaterials have a tremendous impact in biomedical applications because the natural collagen triple helical structure act as a biological support for cells and scaffold for tissue repair and regeneration [10].

There are various compounds that can denature proteins and forms aggregates in solution. Native tertiary structure of proteins may get altered by organic solvents which disrupt hydrophobic interactions between the non-polar side chains of the amino acids. Relatively high concentrations of these solvents are required to unfold the ordered secondary structure of polypeptide chains [11, 12]. One such solvent is glyoxal which is usually supplied as 40 % aqueous solution and is found in beer, tea, wine and frequently detected in food and beverages. The main routes of occupational exposure to glyoxal during use as a disinfectant are via inhalation and dermal absorption. It rapidly reacts with amino groups of proteins, nucleotides and lipids and considered as an important intermediate in the formation of advance glycation end products (AGE's). It is formed in small quantity during carbohydrate metabolism under in vivo environment but the level may rise in hyperglycemic patients suffering from diabetes mellitus [13].

Our present study tries to analyze the aggregation pattern of collagen with varying glyoxal concentrations.

Materials and Methods

Materials

Human collagen was purchased from Sigma (St. Louis, MO, US). 8-Anilino-1-Naphthalene-Sulphonic acid (ANS), Thioflavin T (ThT) and Congo red (CR) were bought from Sigma (St. Louis, MO, USA). Monosodium phosphate and disodium phosphate were purchased from Qualigens fine chemicals (Mumbai, India) for making sodium phosphate buffer.

The stock solution of collagen (5 mg/ml) was prepared in 20 mM sodium phosphate buffer of pH 7.0 and it was then dialyzed in the same buffer to remove any impurity if present. The concentration of native protein in 20 mM sodium phosphate buffer, pH 7.0, was determined by Lowry method [14]. Spectral subtraction has been performed with the help of software available in Shimadzu RF-5301 spectrofluorophotometer. This software records the reading of blank sample of either solvent alone or dye and solvent in the absence of protein. Later signals of sample having dye, solvent and protein were recorded. Then both these signals were subtracted and final subtracted spectra is presented by the software.

Aliquot Preparation

Aliquots of collagen were prepared with varying concentration of glyoxal i.e. 0 % to 80 % (Unless otherwise indicated, all glyoxal concentrations are mentioned in % v/vv) at pH 7. Samples were incubated at room temperature for 4 h prior to performing spectroscopic measurements. Three replicates for each set were analyzed for the results.

Spectrofluorometric Measurements

Trp Fluorescence Measurements

The fluorescence spectra were recorded on a Shimadzu RF-1501 spectrofluorophotometer (Tokyo, Japan) in a 10 mm path length quartz cell. The excitation wavelength was 295 nm and the emission was recorded in the range of 300–400 nm [15]. Concentration of collagen in the aliquots was 1.1 μM.

ANS Fluorescence Measurements

ANS binding was measured by fluorescence emission with excitation wavelength at 380 nm and emission spectra were recorded from 400 to 600 nm. Protein (1.1 μM) was incubated with 100 M excess of ANS for 30 min [16].

Acrylamide Quenching Studies

In the acrylamide-quenching experiments, aliquots of 5 M acrylamide stock solution were added to a protein stock solution (15 μM) to achieve the desired acrylamide concentration. Aliquots were excited at 295 nm so as to excite only Trp fluorescence and the emission was recorded in the range of 300–400 nm. The decrease in

fluorescence intensity at λmax was analyzed according to the equation given by Stern–Volmer [17]:

$$F0/F = 1 + Ksv\,[Q]$$

where F_0 and F are the fluorescence intensities at an appropriate wavelength in the absence and presence of acrylamide, respectively, K_{SV} is the Stern–Volmer constant for the collisional quenching process, and [Q] is acrylamide concentration.

Conformational Analysis

Attenuated Total Reflection Fourier Transformed Infra Red (ATR-FTIR) Spectroscopy

ATR-FTIR spectra were recorded with an Interspec 2020 FTIR spectrometer in deuterated buffer of pH 7. The samples for FTIR studies were prepared by dissolving different states of collagen in deuterated buffer. Solutions are placed between two CaF_2 windows with a spacer. Since the D_2O bend vibration absorbs strongly below 1500 cm^{-1} the path length must be kept to a minimum, therefore sample concentrations must be relatively high. Protein concentration was 20 μM. The scanning wave number was from 1000 to 4000 cm^{-1} [18].

Circular Dichroism (CD) Measurements

CD was measured with a JASCO J-815 spectropolarimeter equipped with a Jasco Peltier-type temperature controller (PTC–424S/15) and calibrated with ammonium D-10-camphorsulfonate. All the CD measurements were carried out at 25 °C with scan speed of 100 nm/min and response time of 1 s. Cells of path lengths 0.1 cm were used for scanning between 190 and 250 nm. For good signal to noise ratio, each spectrum was the average of 4 scans. Base lining and analysis were done using Jasco J-720 software. The results were expressed as MRE (Mean Residue Ellipticity) in deg cm^2 $dmol^{-1}$ which is defined as:

$$MRE = \frac{\theta obs \times MRW}{c \times l \times 10}$$

where θ_{obs} is the CD in milli-degree, where, n the number of residues, Cp the molar fraction, and l the length of light path in cm [19].

Aggregation Studies

Precipitation Reaction

Interaction of collagen (1.1 μM) with varying concentration of glyoxal (0 % to 80 %) was studied in 20 mM sodium phosphate buffer (pH 7) by turbidity method at 350 nm on Shimadzu UV-1700 spectrometer with the help of 1 cm path length cuvette. For the analysis proper blank of native collagen in buffer was taken into account.

Rayleigh Scattering Measurements

Rayleigh scattering measurement was performed on Shimadzu RF-5301 spectrofluorophotometer (Tokyo, Japan) in a 1 cm path length quartz cell. The excitation wavelength was 350 nm and emission was recorded in the range of 300–400 nm. Both excitation and emission slit width were fixed at 5 nm. Fluorescence intensities at 350 nm were plotted. The final concentration of collagen incubated with varying concentration of glyoxal was 1.1 μM.

Thioflavin T assay

Fluorescence spectra were recorded with a Shimadzu RF-5301 spectrofluorophotometer in a 10 mm path length quartz cell. The parameters used for the analysis of ThT binding to aggregates were as follows: excitation wavelength was 440 nm and the emission range was 450–600 nm. Final concentration of collagen was 1.1 μM while that of ThT was 10 μM [20]. ThT was prepared in 20 mM sodium phosphate buffer, pH 7.

Congo Red Assay

Aggregation of collagen was further analyzed by red shift of CR upon binding with aggregates. Spectra was recorded in the range of 400–700 nm on Shimadzu UV-1700 spectrophotometer by using cuvette of path length 1 cm. Aliquots were prepared in presence of different concentration of glyoxal with collagen concentration of 2 mg/ml and incubated for 4 h. 60 μl of each aliquot was added to 440 μl of a solution containing 10 μM CR in 20 mM sodium phosphate buffer and thus maintaining a ratio of 1:6. After 2–3 min of equilibration, absorbance was recorded [21].

Results and Discussion

Spectrofluorometric Analysis

Trp Fluorescence Measurements

Proteins possess three intrinsic fluorophores Trp, tyrosine and phenylalanine, although the later has a very low quantum yield and its contribution to protein fluorescence emission is thus negligible. Of the remaining two residues, tyrosine has the lower quantum yield and its fluorescence is almost entirely quenched when it becomes ionized, or is located near an

amino or carboxyl group, or Trp residue. Excitation at 295 nm results in fluorescence spectra owing to Trp residues. There are 15 Trp residues in human collagen triple helix structure which contribute to the intrinsic fluorescence. Curves 1–9 in Fig. 1a represent Trp fluorescence intensity of collagen with increasing concentration of glyoxal (0–80 %). About 7 fold increase in fluorescence was observed at 60 % glyoxal (curve7) as compared to native collagen (curve 1) suggesting the exposure of buried Trp residues in the non-polar environment. This state can be considered as intermediate state or most probably 'MG' state. Interestingly further increase in concentration of glyoxal to 80 % results decrease in fluorescence intensity (curve 9). This decrease may be attributed to burial of earlier exposed Trp residues owing to aggregation due to protein-protein interaction.

ANS Fluorescence Measurements

ANS is believed to strongly bind cationic groups of proteins through ion pair formation. It is an extensively utilized fluorescent probe for the characterization of protein binding sites. It emits weak fluorescence in polar environment, however in non-polar environment e.g. when bound to hydrophobic patches on proteins, its fluorescence emission significantly increases and the spectrum shows a hypsochromic shift. ANS is thus a valuable tool for assessment of the degree of non-polarity [22]. The observed features of ANS, a blue shift of fluorescence emission maxima and the increase in fluorescence intensity, are generally attributed to the hydrophobicity of the binding site [23]. ANS fluorescence has been widely used as probe to monitor the conformational transitions in proteins due to its affinity for partially exposed hydrophobic regions of protein structure. A steady increase in fluorescence intensity on increasing glyoxal concentration reaching maximum at 70 % and 80 % (curve 8 and 9) was observed indicating the possibility of MG state (Fig. 1b). A 20-fold increase in fluorescence was observed at 60 % with a blue shift of 35 nm (curve 7) and 22 fold increase at 70 and 80 % with a blue shift of 35 and 30 nm respectively.

To clear the exact glyoxal concentration at which MG state occurs, collagen aliquots with increasing concentration of glyoxal having ANS were centrifuged. Prior to centrifugation, a continuous increase in fluorescence intensity as a function of increasing glyoxal concentration was observed (Fig. 1b (inset)). However, when aliquots were centrifuged and only the supernatant was evaluated, there was an abrupt decrease in ANS-fluorescence above 60 % glyoxal. This suggests the existence of MG state at 60 %. Increase in fluorescence beyond 60 % i.e. at 70 % and 80 % before centrifugation might be attributed to the existence of insoluble aggregates in which clefts are present. ANS binds to hydrophobic patches in the cleft of aggregates thereby

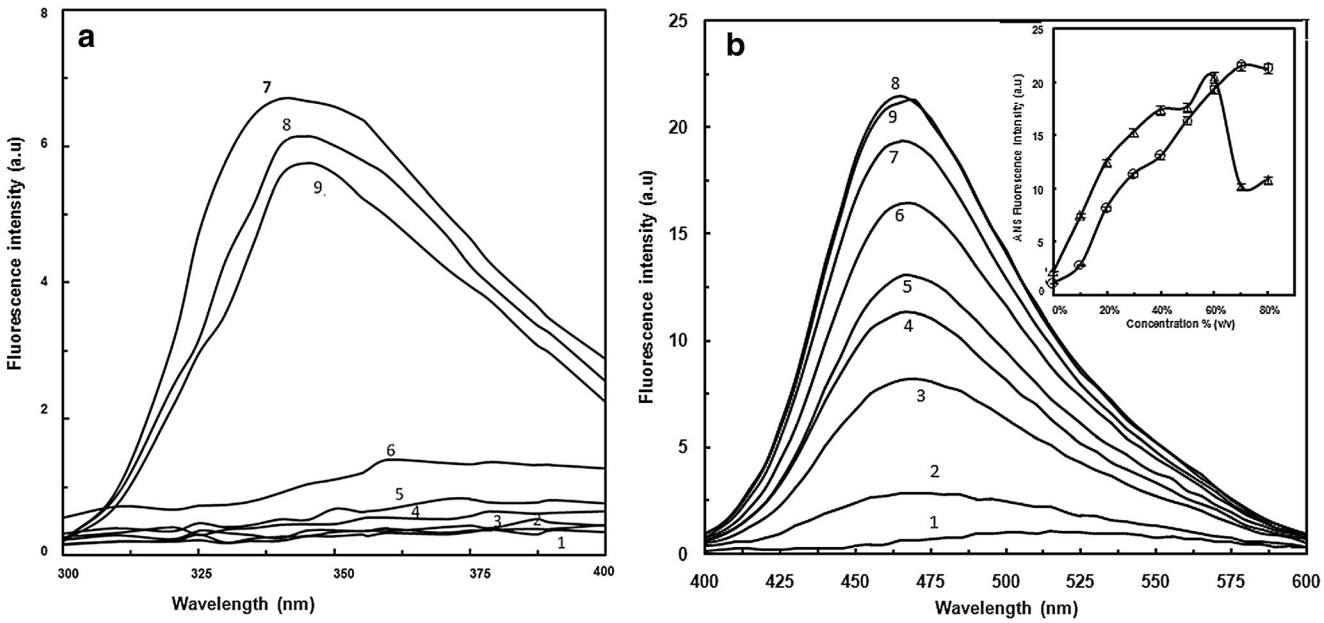

Fig. 1 Spectroflorometric analysis: Trp fluorescence (**a**) ANS fluorescence (**b**) emission spectra of native collagen in 20 mM sodium phosphate buffer, pH 7.0 (curve 1); curves 2–9 represent collagen at 10 %–80 % glyoxal respectively. Excitation wavelength for the Trp and ANS fluorescence was 295 and 380 nm whereas the emission range was 300–400 and 400–600 nm respectively. Inset Fig. 1b: Relative ANS fluorescence intensity of collagen as a function of varying concentration of glyoxal before (○) and after (Δ) centrifugation. Collagen concentration was 1.1 μM and the pathlength was 1 cm

leading to increase in the fluorescence. Presumably it is the pelleting of these insoluble aggregates by centrifugation that resulted in lowering of ANS fluorescence intensity [24].

Acrylamide Quenching Studies

To rule out the possibility that the change in intrinsic fluorescence intensity upon increasing glyoxal concentration is only due to fluorescence quenching of Trp, acrylamide quenching experiment was performed for collagen at 0 and 80 % glyoxal (Fig. 2). The quenching data analyzed from Stern-Volmer plots are shown in Table 1. Quenching by acrylamide resulted in linear Stern-Volmer plots in absence and presence of glyoxal. The Stern-Volmer constant (K_{SV}) value of collagen at 80 % glyoxal was lower as compared to native collagen. This indicates that Trp residues in aggregates possess lesser accessibility to the quencher. As acrylamide is water soluble quencher and does not penetrate into the hydrophobic core of aggregates, very less acrylamide quenching of collagen in the presence of glyoxal suggests the possibility of aggregation. Ksv for acrylamide quenching was found to decrease with aggregation of protein. This decrease may be due to decreased accessibility of Trp to solvent owing to aggregation. Results of Trp analogue NATA are also included as a standard for complete accessibility to quencher. Ksv value of NATA were significantly higher than those in the native and aggregated state signifying that even in the native state, Trp residues were not fully accessible.

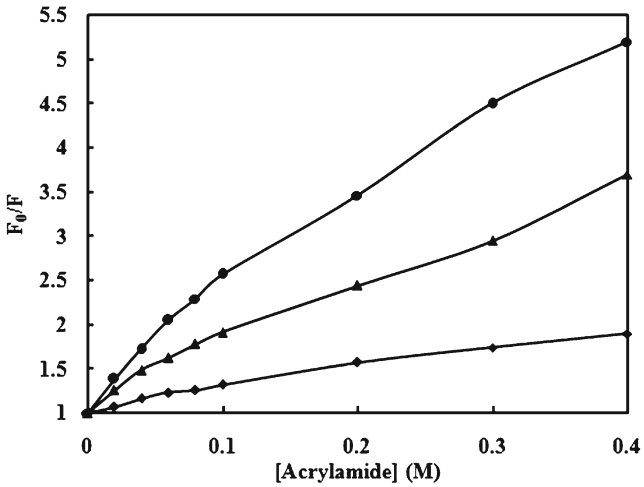

Fig. 2 Acrylamide quenching. Stern–Volmer plots for acrylamide quenching of tryptophan fluorescence of native collagen in 20 mM sodium phosphate buffer, pH 7 (▲); collagen at 80 % glyoxal (♦) and NATA alone (●). Values shown are the ratios of fluorescence in the absence of acrylamide (F0) to the fluorescence at that concentration of quencher (F). The excitation wavelength was 295 nm and emission wavelength was in the range 300–400 nm. The protein concentration was 1.1 μM and the path length was 1 cm

Conformational Analysis

ATR-FTIR Spectroscopy

ATR-FTIR spectroscopy is perhaps the most versatile spectroscopic technique for analyzing protein secondary structure in diverse physiochemical environments. FTIR spectroscopy has been applied to investigate protein structure in solution in aggregates and inclusion bodies [25]. Infrared spectroscopy predicts β-strands much better than α-helices [26]. It is often used as a complementary technique for CD for elucidation of secondary structure. Amide I is the most intense absorption band in proteins. It is primilary governed by the stretching vibrations of the C=O (70–85 %) and C-N groups (10–20 %). Its frequency is found in the range between 1600 and 1700 cm^{-1}. The exact band position is determined by the backbone conformation and the hydrogen bonding pattern. Fig. 3a exhibits the FTIR spectra of collagen in the presence and absence of glyoxal. As expected, native collagen (curve 1) shows an amide I band at 1654 cm^{-1} which are signatures of collagen PPII-type helical structure. At 60 % glyoxal (curve 2), amide band is observed at 1656 cm^{-1} suggestive of PPII-type helical structure but with lesser helical content. Thus, state observed at 60 % glyoxal possess secondary structure similar to native state and hence can be regarded as MG state. At 80 % glyoxal (curve 3), broad amide I band centered at 1635 cm^{-1} appears which is characteristic of β- sheet structure suggesting the transition of PPII-type helical structure to β-sheet conformation. As aggregates are known to possess non-native β-sheet conformation, this state can be regarded as aggregated state.

CD Measurements

CD spectroscopy is the most widespread technique used for estimating the secondary structures of proteins and polypeptides in solution. Secondary structure can be determined by CD spectroscopy in the far-UV region. At these wavelengths (190–250 nm), the chromophore is the peptide bond, and the signal arises when it is located in a regular, folded environment. The approximate fraction of each secondary structure type viz. α-helix, β-sheet, and random coil structures that are present in any protein can thus be determined by analyzing its far-UV CD spectrum as a sum of fractional multiples of such reference spectra for each structural type. Fig. 3b depicts the far-UV CD spectra of native collagen (curve 1) which shows a

Table 1 Acrylamide quenching parameters of collagen in different conditions

Conditions	K_{sv} (M^{-1})
Native collagen	6.244
Collagen +80 % glyoxal	2.217
NATA	10.25

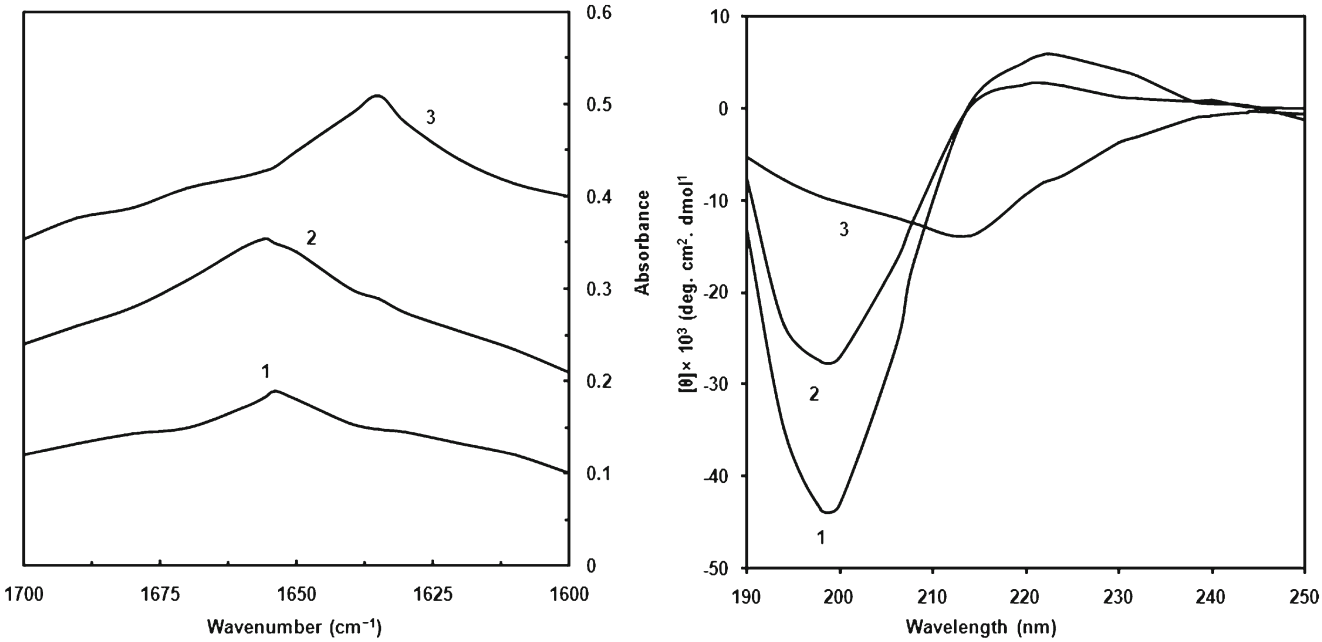

Fig. 3 Conformational analysis ATR-FTIR spectra (**a**) Far-UV CD spectra (**b**) of collagen with different glyoxal concentration. Curve 1 shows native state, curve 2 and 3 corresponds to collagen at 60 % and 80 % glyoxal respectively. Protein concentration for FTIR was 20 μM and absorbance was recorded in the amide I region in the range of 1700–1600 cm^{-1}. Concentration of collagen for CD analysis was 2 μM and the path length was 0.1 cm

positive band at 222 nm followed by a strong negative band at 198 nm, which is characteristic of left-handed PPII-type helix structure thus demonstrating that collagen exits in native conformation with triple helical structure. At 60 % glyoxal, the CD spectrum exhibits a decrease in the positive ellipticity at 222 nm and negative ellipticity at 198 nm, suggesting the presence of triple helix structure but with lower content as compared to native (curve 2). At 80 % glyoxal, the positive ellipticity of collagen at 222 nm disappeared with the appearance of a strong negative band at~214 nm, thus suggesting the transition from helical to β-sheet structure.

Precipitation Reaction and Rayleigh Light Scattering Measurements

Turbidity is the haziness or cloudiness of a fluid caused by individual particles. The precipitation reaction curve of collagen in the presence of varying concentration of glyoxal has been shown in Fig. 4a. Turbidity was monitored spectrophotometrically by change in absorbance at 350 nm, a wavelength where absorbance of most of the proteins is negligible. Negligible absorbance was observed in collagen up to 30 % glyoxal. On increasing the concentration of glyoxal from 40 % onwards there is a steady increase in absorbance approaching maximum at 80 % thereby confirming the presence of aggregates at this concentration. As determination of light scattering is a very sensitive probe for detection of protein aggregation, light scattering of collagen in varying glyoxal concentration (0–80 %) was monitored. As depicted by the Fig. 4b, a clear transition from native to aggregates in collagen is observed on increasing the glyoxal concentration. At low concentration of glyoxal light scattering is very low. A prominent increase in light scattering (~10 times of native) of collagen incubated at 80 % glyoxal was observed suggestive of aggregate formation [27]. Although turbidity and light scattering are not expected to be linearly proportional with aggregation, our results showed increase in turbidity and light scattering with formation of higher order aggregates.

ThT Fluorescence

To further analyze that the ordered β sheet structure and exposed hydrophobic surface of collagen at 80 % glyoxal as a result of aggregation, ThT assay was performed. ThT is the most commonly used dye to diagnose amyloid fibril formation, both in vitro and in vivo. The binding of glyoxal (0–80 %) to ThT dye was taken into account. Fig. 5a depicts ThT spectra of human collagen as a function of increasing glyoxal concentration (0–80 %) incubated for 24 h. There is a constant increase in fluorescence intensity with about 9 folds increase observed at 80 % as compared to native thus indicating the formation of aggregates at 80 %. Thus it can be concluded that at 80 % glyoxal, formation of extensive β-sheet aggregates with the exposure of side chain residues of collagen occur thereby leading to steric interaction between these residues and ThT dye and this ultimately results in increase in fluorescence.

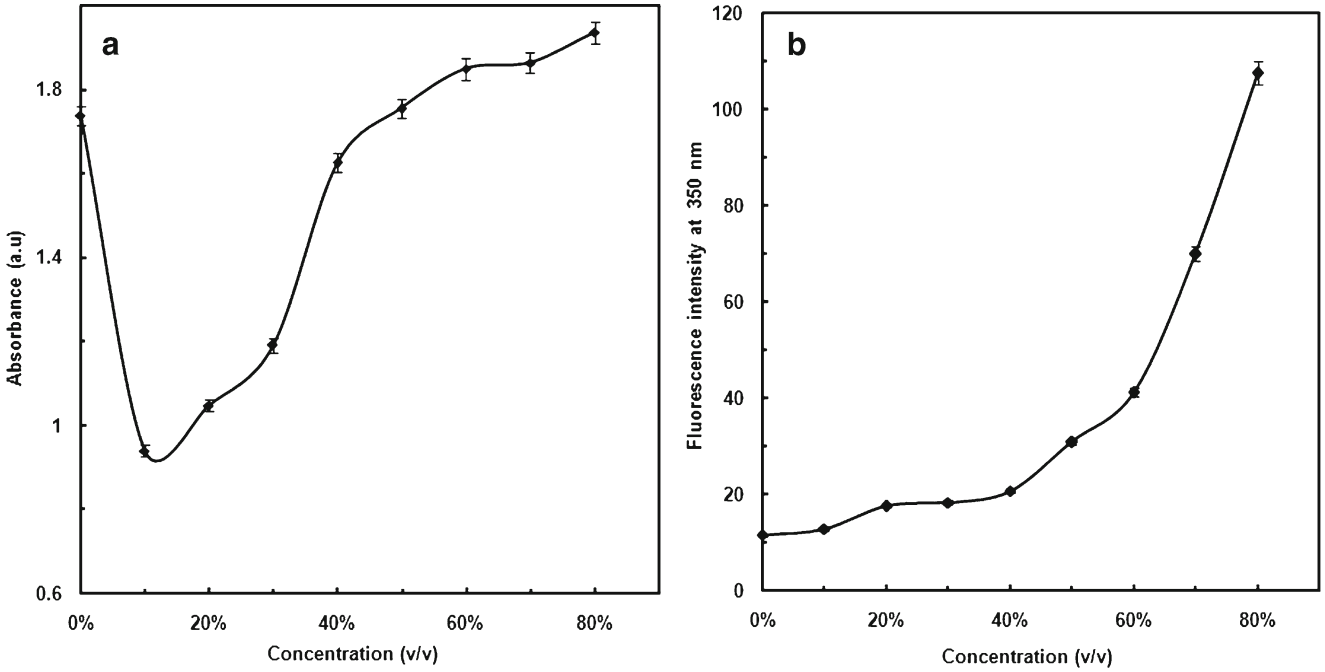

Fig. 4 Turbidity measurement (**a**) Rayleigh measurement (**b**) of collagen as a function of varying concentration of glyoxal (0–80 %). Collagen concentration was 2.5 μM and path length was 1 cm for turbidity measurements. For rayleigh light scattering measurements the excitation wavelength was 350 nm and emission was recorded in the wavelength range of 300–400 nm. Path length of cuvette was 1 cm and the protein concentration in the aliquots was 1.1 μM

CR Assay

The azo dye CR has a high affinity with the β-pleated structure of all forms of amyloid. The repeating β-sheet structure allows the hydrophobic dye like CR to interact with regularly spaced protein chains, which is commonly used to monitor amyloid formation in vitro. The interaction between CR and protein is due to the electrostatic interactions between sulphonic group of CR and positively charged amino acids of protein. As it can be made out from Fig. 5b, native collagen

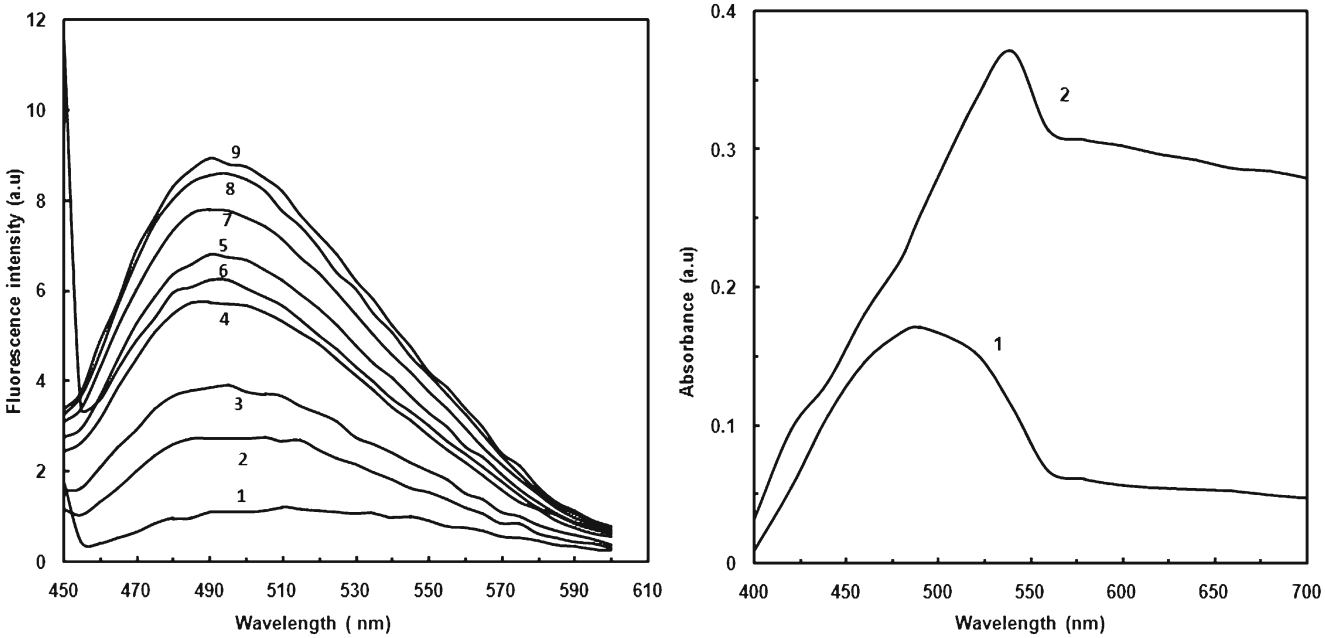

Fig. 5 Aggregation analysis: ThT spectra of collagen in the presence of glyoxal. Curve 1 shows native state while curves 2–9 represent varying glyoxal concentration (10–80 %). ThT fluorescence was monitored at an excitation wavelength of 440 nm. Concentration of collagen for the analysis was 1.1 μM and the path length was 1 cm. CR dye binding (**b**) of collagen in the presence of 80 % glyoxal (curve 2). Native protein is denoted by curve 1. Concentration of collagen was 1.1 μM and the path length was 1 cm. Absorbance was recorded in the range of 400 to 700 nm

(curve 1) showed peak at around 490 nm. At 80 % glyoxal (curve 2), a red shift of 50 nm with 2 folds increase in absorbance was observed. The absorbance peak was observed at 540 nm.

Elucidation of the molecular mechanism of protein folding from a disordered polypeptide chain to specific native state, that is the deciphering of second half of the genetic code, remains one of the major challenges in biochemistry. As aggregation process is considered as process acting in competition with the normal folding pathway, the native protein and the amyloid aggregates can, therefore, be seen as originating from a common population of partially unfolded, interconverting molecules. Aggregation of proteins is a common problem in bioengineering and is also intimately linked to the pathogenesis of most neurodegenerative diseases in humans. In this study, glyoxal is used as a solvent for the formation of collagen amyloid fibrils in vitro. When the results of ANS fluorescence were taken into account it revealed the existence of MG state at 60 % glyoxal as defined for other proteins. Our far-UV CD studies show that native collagen shows a positive band at 222 nm along with a negative band at 198 nm which are characteristics of left handed PPII-type helix structure thus demonstrating that collagen exits in native conformation with triple helical structure. Addition of 60 % glyoxal to collagen exhibits a decrease in the positive as well as negative ellipticity, suggesting that the formation of triple helix decrease with increasing glyoxal concentration. At 80 % the positive ellipticity of collagen at 222 nm disappeared with the appearance of a strong negative band at~214 nm, characteristic of β-sheet structure. In FTIR analysis band appearance at 1656 cm^{-1} in collagen with 60 % glyoxal confirms the presence of α helical structure and the appearance of band at 1635 cm^{-1} in collagen with 80 % glyoxal after an incubation of 4 h further confirm the presence of β sheet structure. This is consistent with CD results where collagen showed conformational transition from triple helix to non-native β-sheet with increasing concentration of glyoxal. Identification of the structural characteristics of these partially folded states is important for understanding the pathway of protein folding. Further incubation of collagen at high concentration of glyoxal (at physiological pH), i.e.80 % results in the formation of aggregates as confirmed by rayleigh scattering measurements and turbidity measurements. These aggregates were further confirmed by ThT and CR analysis. The misfolding and aggregation of proteins is a very common phenomenon now a days and is an area of growing interest as understanding the mechanistic aspect of the aggregation and misfolding of various proteins would surely help us combat various diseases viz. many neurodegenerative disorders which arise as a result of protein misfolding and aggregation. Our work will facilitate further understanding the mechanistic aspects of aggregated protein and conformational changes in the presence of organic solvent and can prove to be very useful for investigating the molecular basis of diseases. This study can be important as collagen is one of the most important component of connective tissue and we are living in what is called 'Era of Arthritis'.

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