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Thermal aggregation of α-chymotrypsin: Role of hydrophobic and electrostatic interactions

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Abstract

We have recently reported that electrostatic interactions may play a critical role in alcohol-induced aggregation of α-chymotrypsin (CT). In the present study, we have investigated the heat-induced aggregation of this protein. Thermal aggregation of CT obeyed a characteristic pattern, with a clear lag phase followed by a sharp rise in turbidity. Intrinsic and ANS fluorescence studies, together with fluorescence quenching by acrylamide, suggested that the hydrophobic patches are more exposed in the denatured conformation. Typical chaperone-like proteins, including α- and β-caseins and α-crystalline could inhibit thermal aggregation of CT, and their inhibitory effect was nearly pH-independent (within the pH range of 7–9). This was partially counteracted by α-, β- and especially γ-cyclodextrins, suggesting that hydrophobic interactions may play a major role. Loss of thermal aggregation at extreme acidic and basic conditions, combined with changes in net charge/pH profile of aggregation upon chemical modification of lysine residues are taken to support concomitant involvement of electrostatic interactions.

Keywords: α-Chymotrypsin; Thermal aggregation; Hydrophobic interactions; Chaperone-like proteins; Kosmotropic and chaotropic ions

1. Introduction

Protein aggregation, the self-association of non-native polypeptide chains to form amorphous or ordered multimeric structures occurs in a wide variety of conditions. It may appear in the form of inclusion bodies during protein expression in host cells or as a kinetically competing reaction during the recovery of active proteins, thereby diminishing the yield of productive refolding in biotechnological processes [1,2]. Additionally, protein misfolding followed by aggregation into amyloid fibrils may lead to a group of pathologic states known as amyloid diseases, like Alzheimer's, Parkinson's and Huntington's diseases, transmissible spongiform encephalopathies and systemic amyloidoses [3–5]. Furthermore, protein products which are now increasingly introduced to the pharmaceutical market may undergo non-native aggregation during purification, sterilization, shipping and storage processes [6]. Due to the clinical and industrial importance of protein aggregation, there is now a strong motivation to investigate more closely the issue of protein aggregation, i.e. characterize the initial aggregation-prone state of the polypeptide chains and the final morphology of the aggregated state, elucidate the mechanism of protein aggregation and the interactions involved, and examine the effect of various conditions on the kinetics and thermodynamics of protein aggregation.

Although there are some reports suggesting that protein aggregation may proceed through a transiently expanded conformational species within the native state ensemble [7–9], the first event in the aggregation process is often the unfolding of the native state conformation [10]. It is well established that the protein native structures are marginally stable, with free energies about 5–20 kcal mol^{-1} lower than the unfolded "state", so that a relatively small perturbation in the environmental conditions may result in destabilization of the native state [11]. Various physical measures, including mechanical stress, extremes of temperature and pressure, acidic or basic pH, and presence of ligands or

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cosolutes which could variously interact with the native and unfolded states have been employed to destabilize the native structure of proteins causing complete or partial unfolding [12,13].

In addition to the structural changes that occur during protein aggregation, protein molecules should also be assembled to form higher order aggregates. The assembly process occurs as a result of the protein–protein pair potential of mean force, which include hard-sphere, electrostatic, van der Waals', desolvation, hydrophobic and all other short-range interactions and are quantitatively reflected in the osmotic second virial coefficient (B_{22}) [14,15]. It is widely believed that the partially-unfolded molten globule-like protein structures are especially susceptible to aggregation [10,12,16,17]. The colloidal stability of the unfolded protein against aggregation is strongly dependent on medium conditions, including temperature, pH, salt type and concentration [14,18]. Some small molecule additives such as arginine, putrescine, spermidine and spermine have proven useful as inhibitors of the aggregation process [19,20]. Several studies have also revealed the anti-aggregation effects of some macromolecules, e.g. heparin, dextran sulfate and proteins like caseins and α-crystallin [21–24]. α-Crystallin, a prominent member of small heat shock protein family (sHSPs) and a major structural protein of the mammalian eye lens, has been shown to act physiologically as the inhibitor of protein aggregation in the lens [25] and also play a role as a molecular chaperone [26]. It has been proposed and generally accepted that α-crystallin suppresses the aggregation of other proteins mainly through the interaction between hydrophobic patches on its surface and the exposed hydrophobic sites of partially-unfolded substrate proteins [27,28] despite some observations to the contrary [29].

The kinetic mechanism of protein aggregation has been extensively studied [30–32]. The basic models of aggregation include the *sequential particle–cluster aggregation* where the monomeric units add individually to a growing aggregate, *nucleation-dependent aggregation* characterized by slow formation of nucleus followed by rapid growth of the aggregate and *multimeric cluster–cluster aggregation* in which multimers of any size associate as a diffusion- or reaction-limited process and no sequential addition of monomers to a growing aggregate occurs [23,33,34].

α-Chymotrypsin is a well-known serine protease with three chains connected by five inter- and intra-chain disulfide bonds. This protein is folded into two anti-parallel β-barrel domains consisting of a Greek key motif followed by an anti-parallel hairpin motif [35]. It has been recently demonstrated that α-chymotrypsin may be driven toward amyloid aggregation by addition of 2,2,2,trifluoroethanol (TFE) at intermediate concentrations [36,37]. The critical role of electrostatic interactions in the TFE-induced structural changes and aggregation of α-chymotrypsin has been demonstrated [37,38]. In the present study, thermal unfolding and aggregation of α-chymotrypsin have been studied in some detail and the effect of various salts, natural and artificial chaperone-like compounds, pH, and chemical modification of lysine residues on these processes examined. It is suggested that both hydrophobic interactions and the net charge of protein play critical roles in the heat-induced aggregation of this protein.

2. Experimental

2.1. Materials

α-Chymotrypsin, α- and β-caseins, α-crystalline, *N*-benzoyl-L-tyrosyl-ethyl ester (BTEE), phenyl methane sulphonyl fluoride (PMSF), tosyl phenylalanyl chloromethyl ketone (TPCK), putrescine dihydrochloride, spermidine trihydrochloride, lysine, arginine, citraconic anhydride, fluorescamine and sodium sulfate were purchased from Sigma (St. Louis, MO). 1-Sulfonato-8-anillinonaphthalene (ANS), di-potassium hydrogen phosphate, sodium thiocyanate and sodium chloride were obtained from Merck (Darmstadt, Germany). Unless otherwise stated, all solutions were made in 50 mM potassium phosphate buffer (pH 7.0). Protein concentration was determined by measuring 280-nm absorbance using an absorbance of 20.4 for 1 g/100 ml protein solution [39]. The reported results are averages of 2–4 separate experiments whenever the coefficients of variation were less than 5%.

2.2. Turbidity measurements

Turbidity measurements were made at specified wavelengths on a Cary-100 Bio VARIAN spectrophotometer. Temperatures were controlled to within ±0.1 °C by a Cary temperature controller. The path length of the sample cell used was 10 mm. To determine the wavelength (λ) dependence of turbidity (τ), the apparent absorbance of protein samples were obtained by scanning between 350 and 750 nm in 1 nm intervals, then $\ln\tau$ was plotted against $\ln\lambda$ and the slope of the best fitted line was calculated as the turbidity-wavelength exponent.

2.3. SDS-PAGE

Possible occurrence of fragmentation or autolysis of α-chymotrypsin in various conditions was examined by SDS-PAGE on 12% polyacrylamide gels according to the procedure described first by Lamelli [40].

2.4. Fluorescence spectroscopy

Fluorescence experiments were performed on a Cary Eclipse VARIAN fluorescence spectrophotometer, with temperature controlled within ±0.1 °C by a Cary temperature controller. The intrinsic emission spectra were obtained at a protein concentration of 0.01 mg/ml. Temperature-dependent changes of the fluorescence emission intensities were measured between 20 and 75 °C with a scan rate of 1 °C min^{-1} and protein concentration of 0.01 mg/ml. ANS, a polarity-sensitive probe, was used to investigate the nature of conformational states promoted by high temperatures. Fluorescence emission spectra of ANS were taken by using the probe at 20 μM concentration and a 0.05 mg/ml protein solution. The excitation wavelength was 365 nm and excitation and emission slit widths were 5 and 10 nm, respectively.

2.5. Quenching experiment by acrylamide

Fluorescence quenching was analyzed according to the Stern–Volmer relationship ($F_0/F = 1 + K_{SV}[Q]$) [41], where F_0 is the fluorescence in the absence of quencher, F is the fluorescence at molar quencher (acrylamide) concentration [Q], and K_{SV} is the Stern–Volmer constant obtained from the slope of a plot of F_0/F versus [Q]. The excitation wavelength was 280 nm and the acrylamide concentration ranged from 0 to 0.22 M.

2.6. Circular dichroism

Circular dichroism (CD) measurements in the far-UV region (190–260 nm) were performed by an AVIV 215 spectropolarimeter using 1 mm-path cell and protein concentration of 4 μM (0.1 mg/ml). Temperature-dependent variation of 230-nm CD signal was measured between 20 and 75 °C with a scan rate of 1 °C min^{-1} and protein concentration of 4 μM (0.1 mg/ml). The temperature was controlled within ±0.1 °C by a PolyScience recirculating chiller.

2.7. Thermal stability determination

Thermal denaturation of α-chymotrypsin was first studied by differential scanning calorimetry (DSC) using a Scal 1 differential scanning microcalorimeter equipped with 0.355 ml capillary glass cells, under 2 atmosphere pressure over cells. The temperature range of 10–80 °C was scanned with various scan rates of 0.25–1 °C/min. Protein concentration was 1.5 mg/ml. The calorimetric data were analyzed by Scan Scal C program and the excess C_p (in kJ K^{-1} mol^{-1}) was finally plotted as a function of temperature. To further investigate thermal denaturation of α-chymotrypsin, temperature-dependent changes of UV-absorbance (at 280 nm), far-UV CD (at 230 nm) and fluorescence emission (at 335 nm upon excitation at 280 nm) were also measured. The wavelengths were selected as those where maximal changes occur upon unfolding. The temperature-dependent fraction of native species (f_N) was estimated through the following formula,

$$f_N = (I_{obs} - I_U)/(I_N - I_U) \quad (1)$$

where I_{obs} is the observed intensity at a specific temperature and I_N and I_U are the values obtained by extrapolation of the native and unfolded lines, respectively, to that temperature. $T_{1/2}$ of denaturation process was calculated as the temperature with $f_N = 1/2$. Using the two-state approximation, the apparent van't Hoff enthalpic change of denaturation process was obtained from the slope of $\ln[(1-f_N)/f_N]$ versus $1/T$ (reciprocal of the absolute temperature) plot. Thermal denaturation process was considered irreversible if the calorimetric peak or sigmoidal change of the spectroscopic probe was absent during the reheating phase.

2.8. Estimation of pH-dependent net charge of the protein

After estimating pK_a of titratable α-chymotrypsin groups through PCE (Protein Continuum Electrostatics)–pK_a calculations by D. Bashford's MEAD program [42] (accessible at http://bioserv.rpbs.jussieu.fr/cgi-bin/PCE-pKa), the net charge of protein was obtained as a function of pH. For pKa calculations, the PDB file of 1ACB was used. The protein internal dielectric constant was set at 4 and the corresponding value for buffer was 80. The ionic strength was assumed to be 0.1 and aspartate, glutamate, histidine, lysine and arginine residues in addition to N- and C-terminals were considered as titratable groups.

2.9. Modification of lysine residues

Citraconic anhydride was used to modify lysine residues in α-chymotrypsin, according to the procedure described in [43]. Protein samples were then dialyzed extensively against 50 mM phosphate buffer, pH 7.0. To determine the number of modified lysine residues, the number of free amino groups in the protein was measured using the fluorescamine method described in [44].

2.10. Enzymatic activity assay

Residual enzymatic activity was determined according to Hummel [39] by measuring an increase in absorbance at 256 nm resulting from the hydrolysis of BTEE.

3. Results and discussion

3.1. Thermal stability of α-chymotrypsin

Thermal stability of α-chymotrypsin was studied by a variety of techniques, including DSC and UV-absorbance, far-UV CD and fluorescence emission measurements. α-Chymotrypsin underwent a structural transition with an apparent T_m (and $T_{1/2}$) of 49.5±0.3 °C (and 53.6±0.2 °C), according to DSC (and spectroscopic measurements). The heat-induced structural transition was characterized by an endothermic peak in DSC, a slight decrease in 280-nm absorbance, an obvious rise in the intensity of 230-nm CD combined with a marked decrease in intrinsic fluorescence. Fig. 1 presents data on excess C_p and calculated fractions of native species during thermal transition. The apparent calorimetric and van't Hoff enthalpy changes related to thermal

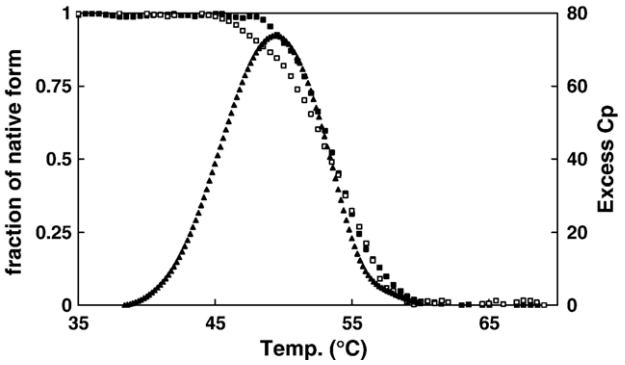


Fig. 1. Thermal unfolding of α-chymotrypsin, as followed by changes occurring in the excess C_p (▲) or in the fraction of native α-chymotrypsin, calculated on the basis of temperature-dependent variation in 230-nm CD (□) or 335-nm emission intensity (■) following excitation at 280 nm. The excess C_p unit was kJ K^{-1} mol^{-1}.

denaturation of α-chymotrypsin were 673±19 and 536±13 kJ mol^{-1}, respectively. Thermal unfolding of the protein was found to be irreversible, since no apparent peak was observed upon reheating. Since T_m was increased when the scan rate was raised between 0.25 and 1 K min^{-1} (Fig. 2), protein unfolding seemed to be under the kinetic control of an irreversible step. Aggregation does not appear to be the cause of irreversibility of thermal unfolding as no apparent turbidity developed between 350 and 650 nm upon incubation at high temperatures (45–65 °C) for one hour. Instead, it is quite likely to have occurred as a result of autolytic digestion, which causes extensive fragmentation of the protein molecule [45,46].

3.2. Induction of thermal aggregation after inhibition of α-chymotrypsin activity

Chymotrypsinogen, the inactive precursor of α-chymotrypsin, was found to aggregate extensively at 65 °C (data not shown), suggesting that the proteolytic activity of α-chymotrypsin was probably responsible for the observed lack of aggregation. To check this possibility, we employed PMSF for irreversibly inhibiting the enzymatic activity of α-chymotrypsin, and then monitored turbidity at 65 °C. Using 1 mM PMSF, the enzymatic activity of α-chymotrypsin was completely lost at room temperature. It is well known that PMSF modifies a reactive serine (serine 195) located in the active site of α-chymotrypsin, and the secondary and tertiary structure of the protein remain unchanged. Moreover, the observed structural changes are quite limited to some fine perturbations of side chains in the active site (e.g. change in conformation of His 57 from *gauche* to *trans*, as reported in Ref. [47]). However, as depicted in Fig. 3a, $T_{1/2}$ of α-chymotrypsin inhibited by PMSF was found to be higher than that of the non-inhibited enzyme (58.9±0.2 vs. 53.6±0.2 °C). The apparent van't Hoff enthalpy change of α-chymotrypsin during its thermal denaturation was also increased after PMSF-mediated inhibition (791±19 vs. 536±13 kJ mol^{-1}). Since the unfolded species of α-chymotrypsin is expected to be prone to the proteolytic activity of the active enzyme [45,46], the lower T_m of the non-inhibited enzyme may be attributed to the occurrence of proteolysis concurrent with protein unfolding. Similarly, the greater enthalpy change of the inhibited enzyme may be explained

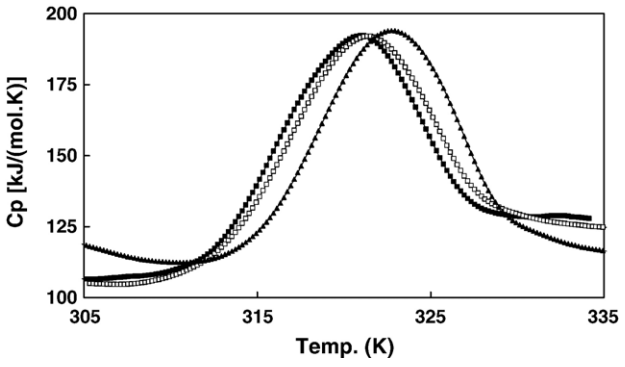


Fig. 2. Temperature-dependent changes in C_p, as measured by DSC with scan rates of 0.25 (■), 0.5 (□) and 1 (▲) K min^{-1}. The C_p unit was kJ K^{-1} mol^{-1} and α-chymotrypsin concentration was 1.5 mg/ml.

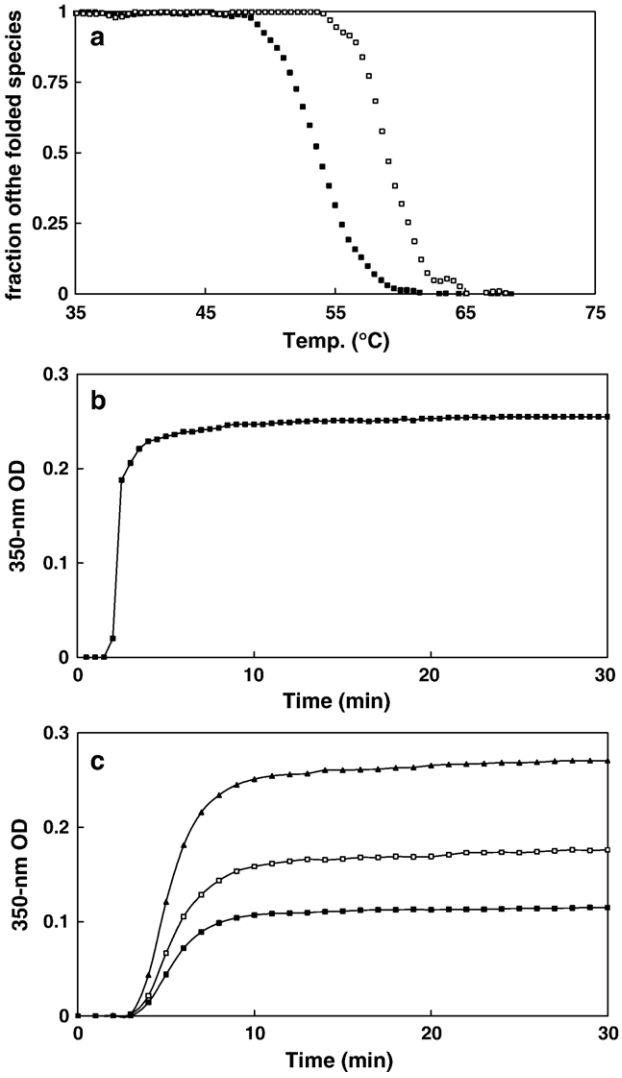


Fig. 3. a. Thermal unfolding of the native (■) and PMSF-inhibited α-chymotrypsin (□), as followed by changes occurring in the fraction of the folded protein, calculated on the basis of temperature-dependent variation in 335-nm emission intensity following excitation at 280 nm. b. Temporal development of 350-nm turbidity during aggregation of 0.05 mg/ml PMSF-inhibited α-chymotrypsin solution at 65 °C. c. Concentration-dependent effect of TPCK on inducing thermal aggregation of α-chymotrypsin at 65 °C. TPCK concentration was 100 (■), 200 (□) and 300 (▲) μM and α-chymotrypsin concentration was 0.05 mg/ml.

by the absence of exothermic autolysis reaction. Also, as indicated in Fig. 3b, the inhibited α-chymotrypsin was aggregated at 65 °C in a characteristic manner, showing a well-defined lag period followed by a sharp increase in turbidity.

TPCK, another irreversible inhibitor, which specifically modifies histidine 57 at the active site of α-chymotrypsin was examined. Thermal aggregation of the TPCK-treated product was also observed in a concentration-dependent manner (Fig. 3c), suggesting that inhibition of proteolysis, irrespective of the nature of the inhibitor, is an essential requirement for aggregation to occur. However, no thermal aggregation was evident at acidic and alkaline pH values (3 and 11) at which the enzyme is known to be inactive [48]. This observation can be taken to suggest that loss of proteolytic activity is a necessary

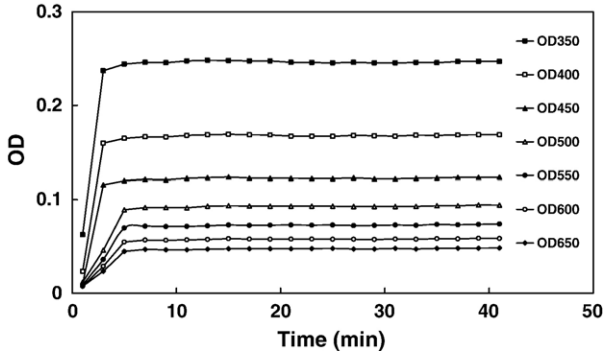


Fig. 4. Wavelength-dependence of the temporal development of turbidity in 0.05 mg/ml PMSF-inhibited α-chymotrypsin solution at 65 °C.

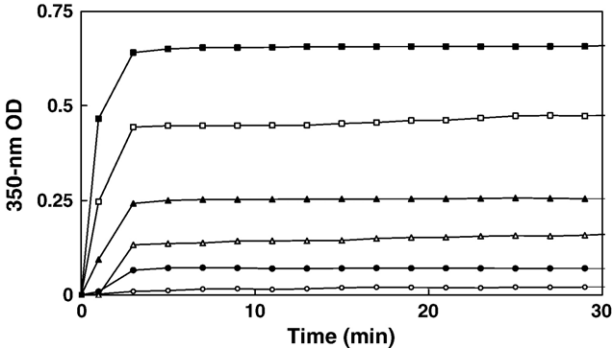


Fig. 6. Effect of protein concentration on the kinetics of PMSF-inhibited α-chymotrypsin aggregation at 65 °C, as followed by measuring the relative OD at 350 nm. Protein concentration was 10 (○), 20 (●), 35 (△), 50 (▲), 75 (□) and 100 (■) μg/ml.

but not sufficient requirement for thermal aggregation of α-chymotrypsin to occur.

Fig. 4 illustrates the temporal development of PMSF-treated α-chymotrypsin at 65 °C as determined at several wavelengths (350, 400, 450, 500, 550, 600 and 650 nm). Because of the intensity and rapidity of changes appearing at 350 nm and the observation that the final turbidity at 350 nm showed an excellent linear relationship with protein concentration (data not shown), 350 nm was selected as the most appropriate wavelength for monitoring thermal aggregation of α-chymotrypsin. The calculated turbidity-wavelength exponent, shown in Fig. 5, was found to gradually increase, indicating changes in distribution of protein population due to formation of larger species [49].

Fig. 6 demonstrates a gradual development of turbidity at 350 nm with increase in α-chymotrypsin concentration. In the range of 0.035–0.1 mg/ml (1.4–4 μM), the initial rate of aggregation obeyed an obvious linear relationship with protein concentration, i.e. the kinetic order was close to 1 (see Fig. 7).

3.3. Thermally-induced hydrophobic exposure over the surface of α-chymotrypsin

Fig. 8 and its inset illustrate emission spectra of α-chymotrypsin (inhibited by PMSF) following excitation at 280 and 265 nm, respectively. The emission spectrum of the native protein upon excitation at 280 nm showed a peak at 338 nm (with spectral width of 60 nm), indicating that the related fluorophores of α-chymotrypsin were considerably shielded from the solvent in the native conformation. A diminished intensity and a prominent red shift observed at 65 °C suggested that the related fluorophores (tyrosine and tryptophane for 280 and tyrosine for 265 nm) were more exposed to the solvent in the thermally-unfolded aggregation-competent species of the protein. The larger spectral widths of emission spectra also indicated the higher heterogeneity of fluorophore environments in the unfolded structures. Similar results were observed after specific excitation of tryptophanes at 295 nm. The fluorescence emission spectrum of ANS, a widely used polarity-sensitive probe, was taken in the presence of α-chymotrypsin at room and high temperatures. As depicted in Fig. 9, this was peaked at 525 nm with significant enhancement and blue shift (about 40 nm) following addition of the protein at 65 °C, with little change observed at room temperature.

Fluorescence quenching by acrylamide was conducted and the Stern–Volmer constant (K_{SV}) calculated for the inhibited α-chymotrypsin at room and high temperatures. As manifested by Fig. 10, K_{SV} was increased about three fold at 65 °C, suggesting that protein fluorophores had become more solvent accessible

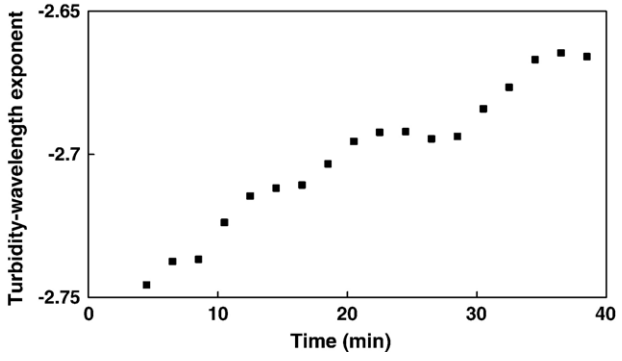


Fig. 5. Time-dependent evolution of turbidity-wavelength exponent during aggregation of 0.05 mg/ml PMSF-inhibited α-chymotrypsin solution at 65 °C. (see Methods for calculation of turbidity-wavelength exponent). For each point, the calculated standard error was less than 0.005.

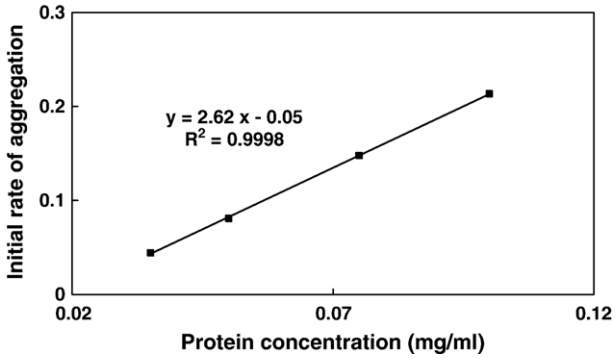


Fig. 7. The initial rate of thermal aggregation (the change in 350-nm OD per minute) obeyed a clear linear relationship with PMSF-inhibited α-chymotrypsin concentration between 0.035 and 0.1 mg/ml.

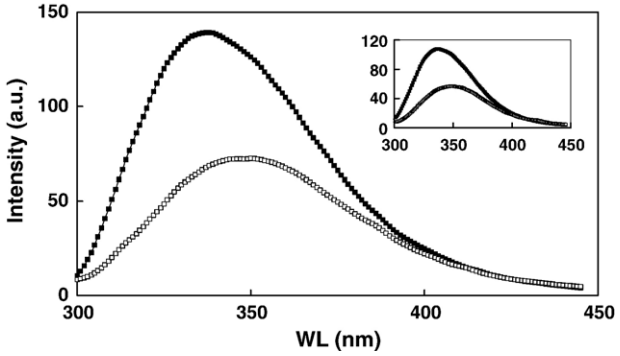


Fig. 8. The intrinsic fluorescence emission spectrum of PMSF-inhibited α-chymotrypsin at 25 °C (■) and 65 °C (□). The excitation wavelength was 280 and 265 nm in the main figure and the inset. Similar results were observed after excitation at 295 nm.

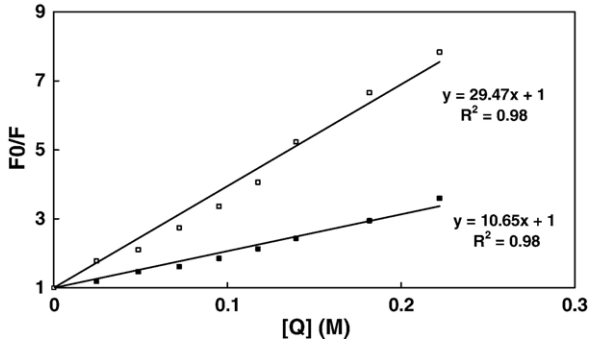


Fig. 10. The quenching effect of acrylamide on the intrinsic fluorescence of PMSF-inhibited α-chymotrypsin at 25 °C (■) and 65 °C (□) as a function of quencher concentration. The excitation wavelength was 280 nm.

and/or mobile at this high temperature. Taking the results of intrinsic, ANS fluorescence, and acrylamide quenching experiments into account, it appears reasonable to conclude that the hydrophobic residues (including the aromatic fluorophores) are more solvent exposed and mobile in the thermally-unfolded aggregation-competent species of α-chymotrypsin and the inter-molecular hydrophobic interactions may play a determining role in the heat-induced aggregation of this protein.

3.4. Anti-aggregation effect of chaperone-like proteins: caseins and α-crystallin

Possible anti-aggregation effects of a variety of natural and artificial chaperones were explored. No prominent result was evident with artificial chaperones α-, β- and γ-cyclodextrins or the mono-, poly- amines lysine, arginine, putrescine and spermidine. The natural chaperones, α-casein, β-casein and α-crystallin, were then separately incubated alone at 65 °C, where in accord with the earlier reports [e.g. 50], no considerable turbidity appeared. However, when these proteins which are widely believed to interfere with inter-molecular hydrophobic interactions [28,29] were added to α-chymotrypsin solution, they afforded significant protection against thermal aggregation in a concentration-dependent manner (Fig. 11 a–c). Amongst these, the inhibitory effect of α-crystalline was quite

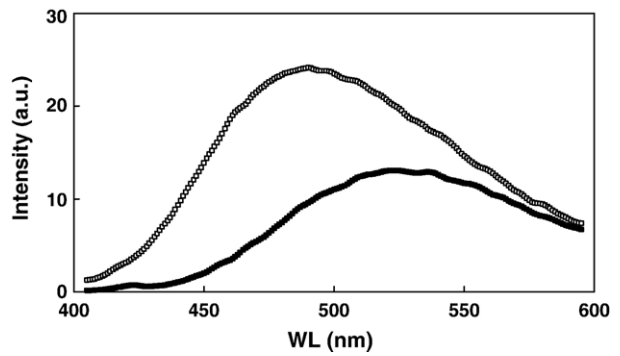


Fig. 9. The fluorescence emission spectrum of ANS at 65 °C, before (■) and 2-minutes after addition of PMSF-inhibited α-chymotrypsin (□). The excitation wavelength was 365 nm.

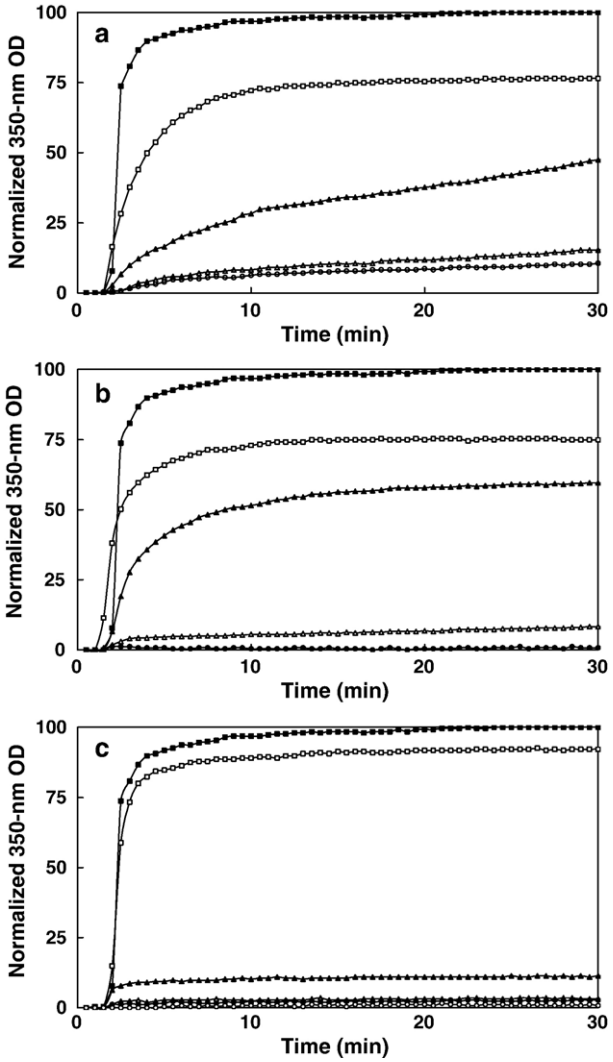


Fig. 11. The anti-aggregation effect of α- and β-caseins and α-crystallin on PMSF-inhibited α-chymotrypsin aggregation at 65 °C. Protein concentration was 0.05 mg/ml. a. with α-casein concentrations of 0 (■), 0.5 (□), 1 (▲), 1.5 (△) and 2 (●) mg/ml. b. with β-casein concentrations of 0 (■), 0.05 (□), 0.1 (▲), 0.25 (△) and 0.5 (●) mg/ml. c. with α-crystallin concentrations of 0 (■), 0.02 (□), 0.05 (▲), 0.125 (△), 0.25 (●) and 0.5 (○) mg/ml.

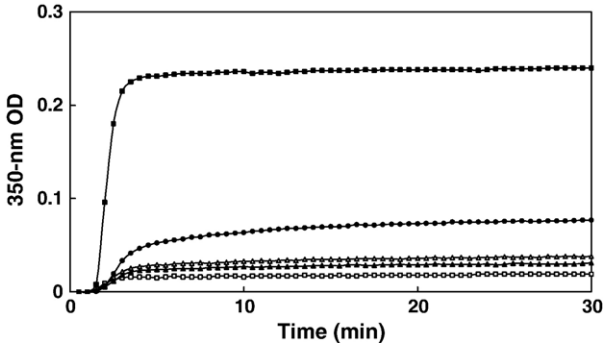


Fig. 12. α-, β- and γ-cyclodextrins remove partially the anti-aggregation effect of α-crystallin. The PMSF-inhibited α-chymotrypsin concentration was 0.05 mg/ml and the temperature was 65 °C. The α-crystallin concentration was also 0.05 mg/ml except in (■) where its concentration was zero. In (□), no cyclodextrin was present, but in (▲), (△) and (●), respectively, α-, β- and γ-cyclodextrins were added at 0.5 mM concentration.

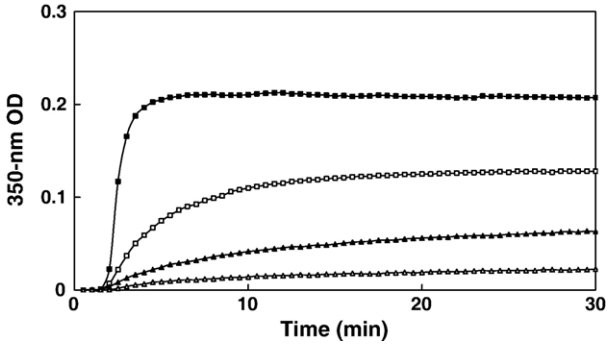


Fig. 13. Effect of 0 (■), 300 (□), 600 (▲) and 900 mM (△) SCN^{-1} on thermal aggregation of 0.05 mg/ml PMSF-inhibited α-chymotrypsin at 65 °C.

dramatic. α-Crystallin is known to undergo a heat-induced structural transition with a T_m of around 62 °C [51] which significantly enhances its anti-aggregation effect [28,50]. Inhibition of thermal aggregation by the chaperone-like proteins and lack of any significant effect of amino acids or polyamines were in contrast with those observed in a recent study on alcohol-induced aggregation of this protein [37,38]. During the process of monitoring the temperature-induced changes of fluorescence emission, it was observed that α- and β-caseins and α-crystallin diminished the apparent $T_{1/2}$ of α-chymotrypsin denaturation (data not shown). In agreement with previous reports [27–29], the destabilizing effect of these chaperone-like proteins may indicate that they preferentially interact with the thermally-unfolded species of α-chymotrypsin which exposes larger hydrophobic patches over its surface (see the above section).

The anti-aggregation effect of α-crystallin was examined in a pH range of 7–9 and the presence of various cyclodextrins. Little variations in the effectiveness of α-crystallin were observed within the above-mentioned pH range where the net charges of both α-crystallin and α-chymotrypsin were altered considerably (data not shown). However, the presence of various cyclodextrins, especially the γ variant with the largest and most flexible hydrophobic cavity among the cyclodextrins [52], could partially remove the inhibitory effect of α-crystallin (Fig. 12). This observation suggested that the availability of hydrophobic patches over α-crystallin surface is required for its interaction with the unfolded α-chymotrypsin. Through such interferences, α-crystallin may enhance the colloidal stability of the unfolded α-chymotrypsin molecules, thereby preventing them from interacting with each other toward formation of aggregates. According to an alternative mechanism proposed recently, [23], the anti-aggregation effect of these natural chaperones may be due to their subsequent incorporation into the "start aggregates" formed during the apparent lag phase and their retardation effects on the rate of cluster–cluster sticking. This would lead to changes in the kinetic regime from diffusion-limited cluster aggregation (DLCA) to reaction-limited cluster aggregation (RLCA) [34].

3.5. The opposite effects of kosmotropic and chaotropic anions on thermal aggregation of α-chymotrypsin

Thermal aggregation of α-chymotrypsin was also studied in the presence of some anions (SO_4^{-2}, Cl^{-1} and SCN^{-1}) which differ with respect to their impacts on water structure [15]. Firstly, the effects of these anions on $T_{1/2}$ of α-chymotrypsin denaturation were examined. As expected from the position of anions within the Hoffmeister series [15], the highest and lowest $T_{1/2}$ were observed in the presence of SO_4^{-2} and SCN^{-1}, respectively ($T_{1/2}$ was 61.0, 60.5 and 52.5 °C in the presence of 100 mM SO_4^{-2}, 300 mM Cl^{-1} and 300 mM SCN^{-1}, respectively). However, at the studied temperature of 65 °C, the unfolding process was complete in each case. It was found that, at identical ionic strengths, Cl^{-1} and especially SO_4^{-2} enhanced the heat-induced aggregation of α-chymotrypsin but the chaotropic anion SCN^{-1} considerably diminished it. This is in contrast with the results of the TFE-induced aggregation of α-chymotrypsin [37], where all the salts tested, irrespective of their kosmotropic or chaotropic behavior, diminished the extent and rate of aggregation. The anti-aggregation effect of SCN^{-1} revealed a clear dependence on its concentration (Fig. 13). Interestingly, addition of SO_4^{-2}, a kosmotropic anion, could gradually release inhibition of aggregation provided by 600 mM SCN^{-1} (Fig. 14). Taking together, these observations provide further support for the critical involvement of hydrophobic

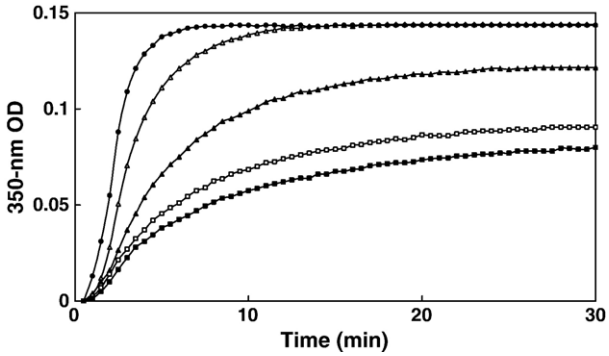


Fig. 14. Partial removal of the anti-aggregation effect of 600 mM SCN^{-1} by 0 (■), 60 (□), 120 (▲), 240 (△) and 360 (●) mM SO_4^{-2}. The PMSF-inhibited α-chymotrypsin concentration was 0.05 mg/ml and the temperature was 65 °C.

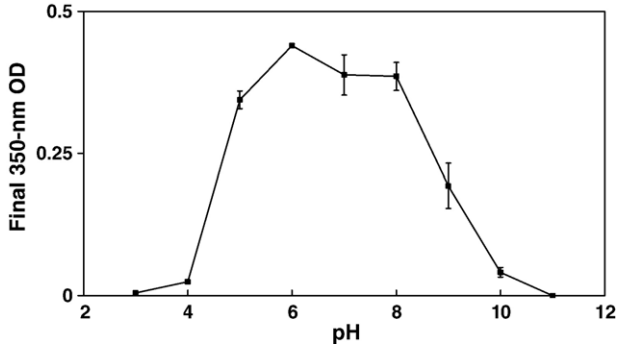


Fig. 15. Effect of pH on the extent (in terms of final turbidity) of PMSF-inhibited α-chymotrypsin thermal aggregation; Protein concentration was 0.075 mg/ml and temperature was 65 °C.

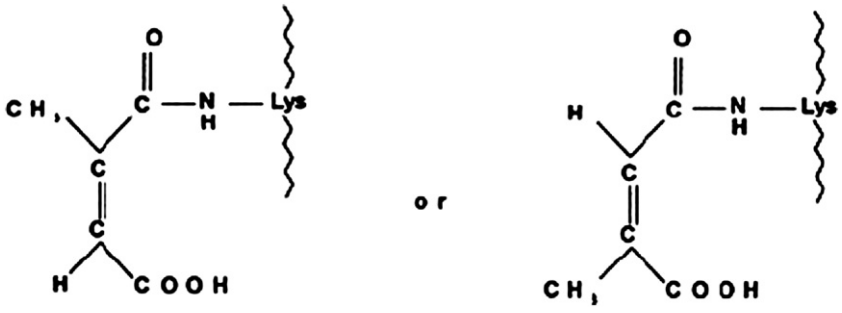
interactions in thermal aggregation of α-chymotrypsin. Accordingly, water structure breakage by a chaotropic anion such as SCN^{-1}, which leads to the weakening of hydrophobic interactions, may diminish the rate and extent of aggregation.

3.6. Effect of variation of pH and modification of lysine residues on thermal aggregation of α-chymotrypsin

The extent of thermal aggregation of the protein was studied in a wide range of pH, from 3 to 11. It has been reported that in alkaline conditions (pH 10–11), the α-amino group of Ile 16 becomes deprotonated and the salt bridge between Ile 16 and Asp 194, critical for the enzyme active conformation, is broken [48]. Therefore, the enzyme adopts a conformation similar to chymotrypsinogen, its inactive precursor [48]. On the other hand, in extreme acidic conditions, the side chain of His 57, which is involved in the general acid–base mechanism of catalysis, becomes protonated causing loss of activity of the enzyme. However, α-chymotrypsin is also likely to undergo some conformational changes at these acidic pHs. With regard to thermal stability, it has been already reported that thermal unfolding of α-chymotrypsin is completed at 65 °C at all pH values studied [53]. Moreover, the observed enthalpy change during thermal unfolding of the protein [54] is comparable to the theoretical one calculated on the basis of complete unfolding. It is therefore reasonable to assume that α-chymotrypsin may adopt a completely unfolded conformation at such high temperatures.

As manifested in Fig. 15, the extent of heat-induced aggregation was significant at pH values 5, 6, 7 and 8, with an apparent maximum at pH 6. At extreme basic and acidic conditions, α-chymotrypsin is minimally aggregated. As a possible mechanism for the loss of thermal aggregation at acidic pH, it has been proposed that the PMSF-inhibited enzyme might be reactivated, thereby regaining its (auto) proteolytic activity [55], which could in turn result in loss of heat-induced aggregation. However, the SDS-PAGE of the inhibited enzyme, after heat treatment at 65 °C, showed that the enzyme remained intact at both pH extremes (data not shown), ruling out the proposed mechanism. As an alternative mechanism related to our own observations, it was conjectured that thermal unfolding of α-chymotrypsin at acidic pH may not sufficiently expose the required hydrophobic patches on its surface. To test this possibility, ANS fluorescence was studied in the presence of α-chymotrypsin in acidic conditions. ANS emission spectrum was found to be significantly enhanced and shifted to the blue side (data not shown) providing evidence for exposure of large hydrophobic patches on the protein surface and consequently making the above-mentioned mechanism quite unlikely. As another alternative, it was proposed that extensive repulsive electrostatic interactions occurring as a result of a large net charge of the protein molecule at pH 3–4 may be responsible for lack of thermal aggregation. Accordingly, chemical modification of lysine residues was carried out to change protein net charge, estimated to be about +14 and +5, at pH 3 and 7, respectively. Using citraconic anhydride as the modifier, the net charge of the protein molecule was diminished through replacing the positive charge of some lysine ε-amino groups by the potentially negative charge of carboxylic groups (Scheme 1). By following the procedure outlined in Materials and methods, modification of 5 lysine residues of native α-chymotrypsin was achieved, without any significant change in its catalytic activity or far-UV CD spectroscopic properties (data not shown). Then, after inhibiting the modified enzyme by PMSF, the extent of thermal aggregation at pH 3 was examined. Interestingly, the modified protein was intensely aggregated at 65 °C.

Based on this finding, the above-mentioned proposition that large patches of positive charges in the protein structure may be responsible for the lack of thermal aggregation at pH 3–4 was confirmed. A similar mechanism may be involved in the absence of thermal aggregation at pH 10–11, although more elaborate experiments would be needed to confirm it.



Scheme 1.

4. Conclusions

In conclusion, thermal aggregation of α-chymotrypsin was induced and studied under various conditions, following inhibition of its enzymatic activity. The observed effects of artificial and natural chaperone-like compounds as well as the influence of kosmotropic and chaotropic salts on conformational and colloidal stability of the protein indicated a critical role for hydrophobic interactions. Additionally, the importance of protein net charge combined with the significance of electrostatic interactions was demonstrated. It is suggested that a comparison of the present results with those reported earlier on TFE-induced aggregation of the protein [36–38] may be helpful toward gaining a clear insight into the diversity of the aggregation-prone states and the mechanisms involved. This is especially true when different conditions are employed to induce aggregation.

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