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**Unfolding and aggregation of lysozyme:
a thermodynamic and kinetic study by FTIR spectroscopy**

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Abstract

The unfolding of hen egg-white lysozyme dissolved both in D₂O and CH₃CH₂OD/D₂O was studied by Fourier Transform Infrared (FTIR) absorption spectroscopy at different protein concentrations. A detailed description of the local and global rearrangement of the secondary structure upon a temperature increase, in the range 295 to 365 K, was obtained through the analysis of the amide I band. Thermodynamic parameters for the melting, and the effect of the co-solvent in determining a change in thermal stability of the protein were evaluated. The protein-protein interactions were also followed as a function of temperature: a strong dependence of the cluster stability and aggregation yield on the solvent composition was observed. Finally, FTIR spectra taken at successive time steps of the aggregation enabled intermolecular contacts to be monitored as a function of time, and kinetic information to be obtained showing that both unfolded and folded states of lysozyme act as reactants for the clustering event.

Keywords: lysozyme, unfolding, aggregation, FTIR, kinetics

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Introduction

Self-assembling of polypeptide chains to form ordered structures is a general property of protein systems. It is promoted by denaturing conditions such as acidity, heating, and addition of alcohols or reducing agents[1,2]. The strong attractive interaction between protein molecules can lead to irreversible aggregation, with the formation of a stable solution of protein clusters. Association among protein side-chains can develop through a complex mechanism, from the formation of small oligomers to the creation of long fibers having a few nanometer diameter and a hundred nanometer length[3]. The study of aggregation phenomena is of great importance, owing to their well recognized implication into a number of systemic and neurodegenerative diseases[4,5]; it offers the possibility of identifying pathways able to reduce the risk of a pathological event. Also, the shelf-life of foods and drugs can be sensitively extended if one of the dominant degradation routes, i.e. aggregation of proteins therein, is depleted[6,7].

The formation of ordered ensembles seems to be connected with the nature of intermolecular interactions instead of the structural properties of native monomers or their primary structure. In fact, it is well known that proteins having a different native structure may show the same tendency to produce filaments or fibrils[1,2]. Intermolecular interactions are probably induced or enhanced by the tendency to minimize the contacts with the solvent (hydrophobic effect); from this point of view an irreversible aggregation is competitive with the refolding of the single molecule.

Owing to its relatively simple structure and reduced size, hen egg white lysozyme is one of the best suited models to study protein unfolding and aggregation[8-14]. The thermal denaturation of lysozyme has been the subject of a very large number of studies, however, both aggregation and unfolding mechanisms still leaves unanswered questions; whether the solvent is able to stabilize intermediates of unfolding, and the aggregation to evolve from these species[14-16]. Lysozyme is known to form fibrils upon a rather long incubation time, at temperatures above 65°C and low pH values. These experimental conditions favor the production of multimers with a high content of β -

sheet structures whose stability is enhanced upon a temperature decrease (down to room temperature) [17-21].

The environment of proteins in solution may strongly influence structural motifs and, consequently, the biological function[20,21]. Intermediates of the unfolding process can lead to different pathways of aggregation, which is generally initiated by partially unfolded species [3,7,22]. Thus, specific solvent conditions have to be explored in order to follow the effects on the melting of single molecule on one side, and then on the intermolecular interactions on the other. In particular, adding a co-solvent to a protein solution is useful since hydrophilic and/or hydrophobic interactions between molecules can be suitably tuned[8-14,23]. Besides, specific intermediates within the mechanism of unfolding can be stabilized: a recent study on the melting process of lysozyme dissolved in water/glycerol solution, has revealed[24] the formation of a molten globule intermediate, and this has not been detected for lysozyme in water.

When using alcohol as a co-solvent, the conformational stability of the protein is a complicated function of the alcohol mole fraction[9,10,12,13]. At ambient temperature, the addition of small amounts of ethanol to aqueous lysozyme solution can increase the hydrophobic effect and hence lead to a compact structure (α -helix) of the protein [10,11]; reversely, high concentrations of alcohol favor the unfolding and aggregation of the macromolecule[11,12].

At pH=3.0, the folded state of lysozyme is maintained at ambient temperature if the ethanol mole fraction is less than 0.25[8,9]; as a consequence, one can follow the unfolding process upon heating the sample [14], and a deeper comprehension of reversible and irreversible denaturation processes can be achieved modulating both temperature and ethanol concentration. A detailed description of both these effects is lacking in the literature.

In the present study, we used FTIR spectroscopy to investigate unfolding and aggregation properties of lysozyme in two different media, that are D₂O and the D₂O/CH₃CH₂OD mixture. Infrared absorption spectra were measured at different temperatures, in the 295-365K range where both unfolding and aggregation are expected to occur. Deuterated solvents were employed to avoid

the strong absorption of water in the mid-IR region. Relevant information about conformational changes in the structure of the protein was obtained through the analysis of amide spectral bands, without interfering signals from the solvent[25]. Unfolding and aggregation of lysozyme were revealed, and the use of two different protein concentrations enabled us to distinguish between effects of self-assembling and melting of the secondary structure. Aggregation of the protein was followed at successive time steps of the process, and kinetic parameters were determined; this allowed evidencing the conditions favorable to protein association, according to temperature and solvent composition. Moreover, the kinetic law of the nucleation event in polymer formation was obtained: this allowed evidencing the conditions favorable to protein association.

Experimental Section

Materials

Hen egg-white lysozyme (purity $\geq 90\%$), D₂O (99.990 atom % D) and CH₃CH₂OD (99.5 atom % D) were purchased from Sigma-Aldrich and used without further purification.

Solutions

A solution was prepared by dissolving the lysozyme in D₂O and then left to equilibrate at room temperature (generally two hours was sufficient). Full deuteration of the amide groups of the protein was achieved heating the solution up to 343K and leaving it rest for 15 minutes. Cooling down the mixture, lysozyme in its native form was recovered, as evidenced by the FTIR spectrum. The solution was dissolved into additional D₂O and in CH₃CH₂OD - ethanol mole fraction (x_{Et}) of 0.15 and 0.18 - to obtain both compositions of 30 mg/ml (*diluted* samples) and 120 mg/ml (*concentrated* samples). A few drops of chloride acid were added to the solutions to obtain a pD of 3.0 ± 0.1 ; taking account of the deuterated solvent, measured values were corrected by adding 0.4 units. pD conditions were also checked during the heating process and a few percent variation was observed in the 295-365K range.

FTIR spectra

Infrared spectra were recorded using a Bruker IFS28 spectrometer equipped with a Deuterium Triglycine Sulphate (DTGS) detector. Each spectrum was the average of fifty scans, measured with a resolution of 2 cm^{-1} . Transmission spectra were collected at various temperatures, in the 295-365 K range, using a specially designed cell with CaF_2 windows[26]. Temperature of the experiment was controlled through a circulating water bath (Haake F6 ultra-thermostat), with a precision of 0.1K. Before each measurement, a 15-minute wait was allowed to well thermalize the sample at a given selected temperature. The whole temperature scan was replicated three times on a fresh solution in order to test the reproducibility of results.

FTIR spectra were normalized to the intensity (peak area) of the amide I band in the spectrum of the D_2O solution at 298 K.

Aggregation of lysozyme at different temperatures was assessed through the evolution of the signal at 1618 cm^{-1} over time (nearly one hour from the beginning of the experiment), in the $x_{\text{Et}}=0.18$ sample: in this environment the formation of aggregates was particularly favored and the spectrum easily revealed their concentration variation. Each experiment was performed on the fresh solution thermalized at a temperature comprised in the 313-343 K interval. A spectrum was obtained by averaging 20 scans, each taken every 40s within the first ten minutes, and then every 120s at longer times.

Data analysis

The position of the amide I profile in the spectrum of lysozyme, accounts for a distribution of spectral components from distinct secondary structures of the protein[27,28]. Owing to the asymmetry of such distribution, the position of the band was evaluated through the frequency first moment (P_T) expressed as :

$$P_T = \frac{\int_{\text{band}} \bar{\nu} I(\bar{\nu}) d\bar{\nu}}{\int_{\text{band}} I(\bar{\nu}) d\bar{\nu}} \quad (1)$$

Since P_T is a mean vibrational frequency of oscillators involved in various secondary structures, one can refer to it as a measure of the conformational distribution of lysozyme[29].

It is known from the literature that the unfolding of lysozyme is properly described by a two-state model[23,30,31]. Within this model the observed spectroscopic parameter P_T can be expressed as the value of the folded state times its population, plus the signal of the unfolded state times its population[32]:

$$P_T = \frac{P_F + m_F \cdot T + (P_U + m_U \cdot T) K_T}{1 + K_T} \quad (2)$$

where K_T is the equilibrium constant at T temperature between folded and unfolded state, P_F and P_U denote the values of P_T at T=0 K in the folded and unfolded state respectively, and m_F and m_U their temperature dependence. In particular, m_F and m_U parameters describe the linear T-dependence of the band position in the pre- and post-melting region, and this trend suggests a continuous variation for the arrangement of both folded and unfolded structures.

The equilibrium constant K_T can be expressed as a function of the unfolding enthalpy variation ΔH_{U-F} and of melting temperature T_m :

$$\ln(K_T) = \frac{\Delta H_{U-F}}{R} \left(\frac{1}{T_m} - \frac{1}{T} \right) \quad (3)$$

We applied the non-linear least squares method of OriginPro 8 software, by OriginLab Corporation, to fit experimental values; hence, the evolution of P_T as a function of temperature allowed thermodynamic parameters of melting of the secondary structure to be evaluated for different environments.

Results and Discussion

Reversible Denaturation

Figure 1 shows the evolution of the amide I band in the IR spectrum of a 30 mg/ml solution of lysozyme in D₂O as a function of temperature, in the range 291-363K. An up-shift of the peak position along with a decrease of intensity of the amide I band is apparent through this evolution, particularly above 333 K (Figure 1b) due to a decrease of ordered conformations of lysozyme in the solution (from helices and sheets to turns and bends) [28].

Figure 2 shows plots of the first moment of the amide I band as a function of temperature for a diluted solution of lysozyme at different ethanol mole fractions: an S-shaped curve is found in all cases and reproduced according to eq.(3) (see Experimental Section). Thermodynamic parameters of the thermal unfolding were estimated and listed in Table 1. Values of ΔH_{U-F} , T_m and ΔS_{U-F} appear to decrease upon ethanol addition to the solution. Note that enthalpy changes and melting temperatures are in close agreement with previously reported values [8]. Our results indicate that ethanol reduces the melting temperature of the polypeptide, with a stronger effect at higher concentration. This behavior is known from the literature[8-10] but our experimental results also show the sensitivity of IR measurements to reveal the weak restructuration of conformational arrangements within the folded and unfolded states, through the evaluation of m_F and m_U parameters [33-35]. In fact, within the main macroscopic states of folded and unfolded forms of the molecule, some local motions can take place depending on temperature, and a progressive change of conformations can be observed upon heating. This is not in contrast with the two-state description of the unfolding process, since local rearrangements may only slightly alter the distribution of secondary structures of the protein without compromising the activity[35-37]. On the other hand, enzymes are able to perform their functions owing to some flexibility of the polypeptide chain, and this is particularly true at physiological conditions.

A comparison of m_F and m_U values in the three solutions (Table 1) indicates an enhanced flexibility of the polypeptide chain both in the folded and unfolded states due to the presence of ethanol, since a continuous and substantial rearrangement of the local structure is achieved for the $x_{Et}=0.15$ and $x_{Et}=0.18$ samples upon a temperature increase. For the aqueous solution, a change of slope is observed within the pre-melting region, at $T_c=315$ K (Figure 2a), with a higher value below T_c . This latter represents a kind of critical value for the aqueous solution since, at this temperature, a maximum in the free energy and hydrodynamic radius has previously been observed[38-41]. These findings seem to suggest that at T_c , a local conformational transition occurs in the aqueous environment and not in the mixed solvent. In the ethanol-free sample, this conformational transition

could be related to the temperature dependence of hydrophobic interactions; these interactions are characteristic of water, hence they are expected to be less effective in a different environment. This pre-melting transition, which has not been observed for lysozyme dissolved in other mixed solvents[24], is characteristic of lysozyme in aqueous solution. To the best of our knowledge, this is the first time that such a local effect has been evidenced by FTIR spectroscopy.

Aggregation and dissociation of protein clusters

At higher protein content, the macromolecular system may undergo a self-assembling process upon heating. In relation to the aggregation process, the presence of intermolecular β -sheet conformation can be evidenced by characteristic FTIR absorptions at 1618 and 1690 cm^{-1} [16,25].

FTIR spectra of the concentrated aqueous solution here investigated, lack the signature of protein clusters; in fact, the amide I band profile perfectly overlaps with that of the 30 mg/ml D_2O -solution (data not shown). On the contrary, FTIR spectra of concentrated $\text{D}_2\text{O}/\text{CH}_3\text{CH}_2\text{OD}$ solutions of lysozyme, show the presence of 1618 and 1690 cm^{-1} absorptions. Figure 3 displays the temperature dependence of the amide I band of a 120 mg/ml lysozyme in mixed solvent with $x_{\text{Et}}=0.15$. Differently from the spectra obtained at low protein concentration, one can see that the amide I profile shows the superposition of intra- and inter-molecular vibrations having different temperature evolution.

Specifically, the band at 1650 cm^{-1} shows a double behavior: its intensity decreases with increasing temperature in the range 310-337 K (Figure 3a), while it increases above 337 K (Figure 3b). This band essentially arises from α -helix arrangements of the native structure of the protein, with minor contributions from turns (at 1670 cm^{-1}). As a consequence, the decrease of intensity up to 337 K (indicated by a red arrow in Figure 3a) is associated with the unfolding of helix structures; on the other hand, the rise at higher temperatures (red arrow in Figure 3b) is probably due to an increase of different conformations. In fact, along with an increased intensity, the band shows a blue shift of its position, indicating a growing contribution of turn arrangements (see below). The temperature dependence of 1650 cm^{-1} intensity is shown in Figure 4 for both diluted and

concentrated solutions (blue circles in Figure 4(a) and 4(b), respectively); a loss of absorbance is observed in the 310-337 K range for both solutions. Hence, in analogy with the aqueous environment, the unfolding process of helix structures is likely to take place regardless of the protein concentration, at least within the range of 30-120 mg/ml. However, a different conformational state is observed in concentrated solution due to the loss of helix arrangements: in fact, up to 337 K the position of amide I main peak red-shifts (see Figure 3a) and this is in contrast with the trend of Figure 2b.

The temperature dependence of 1618 cm^{-1} intensity is also shown in Figure 4(b) for the 120 mg/ml sample. The behavior is opposite to that of the band at 1650 cm^{-1} : an increase up to 337K is related to the formation of ordered aggregates, whilst the decrease further on suggests the rupture of these aggregates.

It is worth noting that the aggregate formation develops together with the loss of native structure of the protein (Figure 4(b)); this is also observed at higher ethanol mole fraction (see Figure 5). Concomitantly with the decrease of α -helices, the concentration of intermolecular β -sheets increases. This indicates that the clustering process is triggered by unfolded species and develops with the fraction of those. In Figure 6, the comparison between intensity variations of 1618 cm^{-1} signal (v_{AGG}) for solutions at different ethanol content, evidences that intermolecular contacts are stable only in a limited T-interval: 333-337 K at $x_{\text{Et}}=0.15$ and 328-337 K at $x_{\text{Et}}=0.18$.

Figure 6 also shows that the dissociation of clusters occurs at $T>337\text{ K}$ for both ethanol/water solutions. Aggregate dissociation has been also observed in lysozyme under reducing conditions, in a similar temperature range[16]; in that case authors have suggested that the driving force for the dissociation of ordered assemblies is a further conformational change rather than the reversal of the aggregation process; this change coincides with the complete unfolding of lysozyme chains. Actually, we observed that the T-range of aggregate dissociation coincides with the unfolding range of lysozyme in D_2O : this is also proven by the increase of absorbance at 1650 cm^{-1} (Figure 4(b) and 5). Moreover, at 363K we obtained the same unfolded state for the three

concentrated samples, regardless the aggregation event being observed only in ternary solutions. From analysis of Figure 7 one can note that both room-temperature and high-temperature conformational states show the same secondary structure, regardless of the presence of ethanol; thermodynamic (and kinetic) effects induced by the co-solvent are observed in the temperature variations between these two situations since, in ethanol solution, a stable folded-non native state is visited by the single molecule on passing from active to open conformation.

As stated above, Figure 6 shows that the stability range of supra-molecular species increases with x_{Et} ; also, the aggregation yield observed in the two ethanol solutions is different. This is probably the result of two opposite effects: the temperature-induced aggregation of monomers and the temperature-induced dissociation of aggregates. As we stated above, only the former process is solvent-dependent, and consequently a different superposition of effects is observed on heating the two ternary systems. In particular, the aggregation temperature (T_{AGG} in Figure 6) of the $x_{Et}=0.18$ sample is lower than that of the $x_{Et}=0.15$ solution; this suggests a wider stability range for lysozyme clusters and hence the possibility of having a higher aggregation yield before the dissociation prevails.

Aggregation kinetics

Our data support the idea that heat-induced aggregation is triggered by the unfolding event and non-native monomers are involved as reactive precursors for the formation of oligomers. According to the Lumry-Eyring model[42], an irreversible and cooperative rearrangement of unfolded clusters may give rise to ordered aggregates (with a high content of β -sheet structure). This reaction constitutes the nucleation step for the polymer formation. At this stage of the mechanism, inter-chain contacts are established, as indicated by the band at 1618 cm^{-1} in the FTIR spectrum. We cannot exclude that this band is also sensitive to the growth of oligomers; nevertheless, it has been observed that the IR technique is almost blind to the formation of larger species (pre-fibrillar aggregates) and the spectral evolution of signals is only in the first stages of assembling[43].

Within the framework of this description, both unfolding and aggregation processes occur in the mechanism, with hardly separable effects. However, the intense IR absorption assigned specifically to intermolecular bonds (band ν_{AGG} at 1618 cm^{-1}) allows us to recognize and to probe the clustering event as a function of both temperature and time. Therefore, we monitored the evolution of the ν_{AGG} intensity as a function of the time (within an hour from the beginning of the experiment) at different temperatures. In particular, we followed the growth of intermolecular β -sheets structures in the $x_{\text{Et}}=0.18$ solution: in this environment, the formation of aggregates is particularly favored and the spectrum can easily reveal their concentration variation.

Figure 8 displays the time evolution of ν_{AGG} intensity at different temperatures in the 313-343 K range. These data exhibit a general trend: an initial increase, within the first ten minutes of the measurement, followed by a much slower augment.

The temperature domain here explored corresponds to the melting range: for the $x_{\text{Et}}=0.18$ solution $T_{\text{m}}=323 \text{ K}$. At $T < 313 \text{ K}$, the band at 1618 cm^{-1} was not revealed: this suggests that at least a small fraction of unfolded species is required for the formation of aggregates. On the other hand, at temperatures above 343 K, when the intra- and inter-molecular interactions are disfavored, the complete unfolding of protein sample was achieved and no evidence of aggregate formation was observed. At $T=343 \text{ K}$ a sensitive decrease in the ν_{AGG} intensity and in its variation was measured.

In Figure 8b, the initial aggregate formation rate v_0 can be easily recognized in the slope of the different curves. It is noteworthy that this initial rate has a maximum close to the melting temperature, as shown in Figure 9. According to this finding we cannot assume that the aggregation rate is only dependent on the unfolded fraction: in this case we should observe a maximum v_0 value at $T=338 \text{ K}$, when the total amount of protein molecules has an unfolded structure. At this temperature lysozyme experiences a maximum aggregation yield but not a maximum aggregation rate; the latter was rather obtained at $T \approx T_{\text{m}}$. This could suggest that self-assembling involves both folded and unfolded species [42], and the initial rate has the following expression:

$$v_0 = k_{agg} [F]^n \cdot [U]^m \quad (4)$$

where [F] and [U] are the concentrations of the folded and unfolded states, respectively, n and m their reaction orders, and k_{agg} is the aggregation rate constant. If we refer to the total protein concentration [P] and the mole fraction x_F of the folded state, then we obtain:

$$[F] = x_F \cdot [P] \quad (5a)$$

$$[U] = x_U \cdot [P] = (1 - x_F) \cdot [P] \quad (5b)$$

$$v_0 = k_{agg} \cdot [P]^{n+m} \cdot x_F^n \cdot (1 - x_F)^m \quad (5c)$$

In case that $n=m=1$, one should observe:

$$v_0 = k_{agg} \cdot [P]^2 \cdot x_F \cdot (1 - x_F) \quad (6)$$

According to equation (6) v_0 has a maximum when $x_F=0.5$, and this is verified at $T=T_m$. In our data this maximum was observed at x_F slightly lower than 0.5, thus m is probably higher than n ; nevertheless, a more accurate study is requested to define the kinetic law precisely.

The participation of native molecules to protein self-assemblies was also proposed for lysozyme under reducing condition[44], and authors suggested a possible inclusion of folded molecules within the structure or at the surface of the clusters during the growing phase of polymerization. Our results demonstrate that together with unfolded chains, also native molecules act as reactants of the nucleation event, as expressed by the dependence of v_0 on x_F and x_U .

The gelation process

One of the most striking results of our kinetic measurements is that, within the reaction time (1h), we obtained different aggregate fractions at different temperatures, thus resembling the trend of Fig.6 where a single sample was heated in the whole T range and measurements were taken after 15-minute equilibration. The presence of such limiting concentrations is not obvious since one can imagine the irreversible self-assembling to consume the protein completely, even though at different rates. This is not the case and we obtained different samples (e.g. different cluster/monomer fractions) at different temperatures.

We believe this to be connected with the marked increase of viscosity of the sample due to an increase in cluster concentration. In fact, we observed the formation of transparent gels with a higher strength at higher temperatures (data not shown). Heat-set gelation of globular proteins is a well known phenomenon[17-19,45], and the formation of a transparent gel is usually connected with the growth of ordered clusters[45], as revealed by our data. The formation of a gel seems to hinder the conformational rearrangements of the protein, and this may substantially reduce the rate of any further assembling.

Conclusions

In the present study, conformational changes induced by temperature on lysozyme dissolved in different environments were analyzed in relation to protein unfolding and self-assembling.

A detailed analysis of pre-melting effects was performed for different solutions; in particular, a local rearrangement of the secondary structure was evinced only for the binary system. An enhanced sensitivity of protein conformations to the temperature variation was revealed with increasing the fraction of ethanol as a co-solvent, and a reduced thermal stability of the folded state of lysozyme was evidenced from the behavior of the amide I band profile. The reduced thermal stability and the continuous rearrangement of the secondary structure suggested the presence of weakened hydrophobic interactions in the solution with ethanol. On the other hand, an enhancement of intermolecular interactions was observed upon increase of alcohol concentration.

From the comparison of data at low and high protein concentration, a clear comprehension of effects due to the unfolding on one side, and the aggregation on the other was obtained for the protein in water/ethanol solutions. The evidence of an irreversible association initiated by the unfolded state of the macromolecule was verified; nonetheless, the participation of the native state of the protein was also revealed from the temperature dependence of the initial rate of aggregate formation.

The complete unfolding of lysozyme chains was observed as the result of aggregate dissociation. For both water/ethanol solutions this dissociation was detected at the temperatures of lysozyme melting in water ($T > 337$ K). On the contrary, a lower aggregation temperature was observed on increasing the ethanol content, thus: a different range of cluster stability and a different aggregation yield was evidenced in the two ternary systems.

Monitoring the aggregation process as a function of time we also observed the formation of transparent gels: we believe that the increase of viscosity connected to gelation is responsible for the decrease of aggregation rate observed at each temperature and long reaction times. Due to the “freezing” of conformational motions, we obtained different transparent gels (e.g. different cluster/monomer fractions) at different temperatures; the characterization of the systems obtained by different thermal treatments, will be the subject of our future studies.

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Table 1. Parameter evaluated for diluted lysozyme solutions

x_{Et}	ΔH_{U-F} /kcal mol ⁻¹	T_m /K	ΔS_{U-F} /cal K ⁻¹ mol ⁻¹	m_F /K ⁻¹ cm ⁻¹	m_U /K ⁻¹ cm ⁻¹
0.00	116.7±6.0*	348.0±0.5	335.3±19.0	0.019**±0.002	0.021±0.002
0.15	95.0±9.0	328.4±0.7	289.3±29.0	0.065±0.005	0.060±0.004
0.18	73.0±8.0	323.6±0.9	225.6±28.0	0.067±0.005	0.060±0.003

* Uncertainties are derived from the applied fitting procedure

** Value obtained at temperatures higher than $T_c=315$ K.

Figure captions

Figure 1: FTIR spectrum in the region of the amide I band of a 30 mg/ml solution of hen egg-white lysozyme in D₂O at different temperatures, in the 291 to 333K (a), and 333 to 363K (b) ranges.

Figure 2: Evolution of the first moment P_T of the amide I band for a 30 mg/ml solution as a function of temperature. The frequency first moment was evaluated from FTIR spectra of $x_{Et}=0.00$ solution (a), $x_{Et}=0.15$ solution (b), and $x_{Et}=0.18$ solution (c); black, pink and green squares refer to the three determinations performed to test the reproducibility of results on each sample. Red curves are the result of fitting procedure described by equation (2) (see text), and performed on the complete data set; blue line is only a guide for the eyes.

Figure 3: FTIR spectra of lysozyme 120 mg/ml solution in D₂O/CH₃CH₂OD $x_{Et}=0.15$, in the region of the amide I absorption band.

Figure 4: IR intensity of the 1650 cm⁻¹ band (scale on right axis) and 1618 cm⁻¹ band (scale on left axis) measured on spectra of diluted (a) and concentrated (b) lysozyme solutions in D₂O/CH₃CH₂OD -solution, $x_{Et}=0.15$.

Figure 5: IR intensity of the 1650 cm⁻¹ band (scale on right axis) and 1618 cm⁻¹ band (scale on left axis) measured on spectra of lysozyme 120 mg/ml D₂O/CH₃CH₂OD -solution, $x_{Et}=0.18$.

Figure 6: IR intensity of the 1618 cm⁻¹ band (ν_{AGG}) measured on spectra of a 120 mg/ml D₂O/CH₃CH₂OD -solutions: red circles $x_{Et}=0.15$; black squares $x_{Et}=0.18$. The aggregation temperature T_{AGG} is referred to the midpoint of the intensity increase.

Figure 7: Amide I profile for lysozyme 120 mg/ml D₂O-solution (black curves), D₂O/CH₃CH₂OD-solution $x_{Et}=0.15$ (blue curves), D₂O/ CH₃CH₂OD-solution $x_{Et}=0.18$ (red curves). The superposition of curves suggests the presence of the same folded state at 298 K, and the same unfolded state at 363 K, for lysozyme in the three solvents.

Figure 8: Increase of IR intensity at 1618 cm⁻¹ (v_{AGG}) measured on spectra of a 120 mg/ml D₂O/CH₃CH₂OD -solution $x_{Et}=0.18$, at different temperatures. Data shown in graph (b) refer to the first minutes of reaction and are the same of graph (a). Dashed lines are the results of a linear curve-fitting applied to the initial intensity increase.

Figure 9: Initial aggregate formation rate v_0 as evaluated from the slope of lines reported in Fig.8b

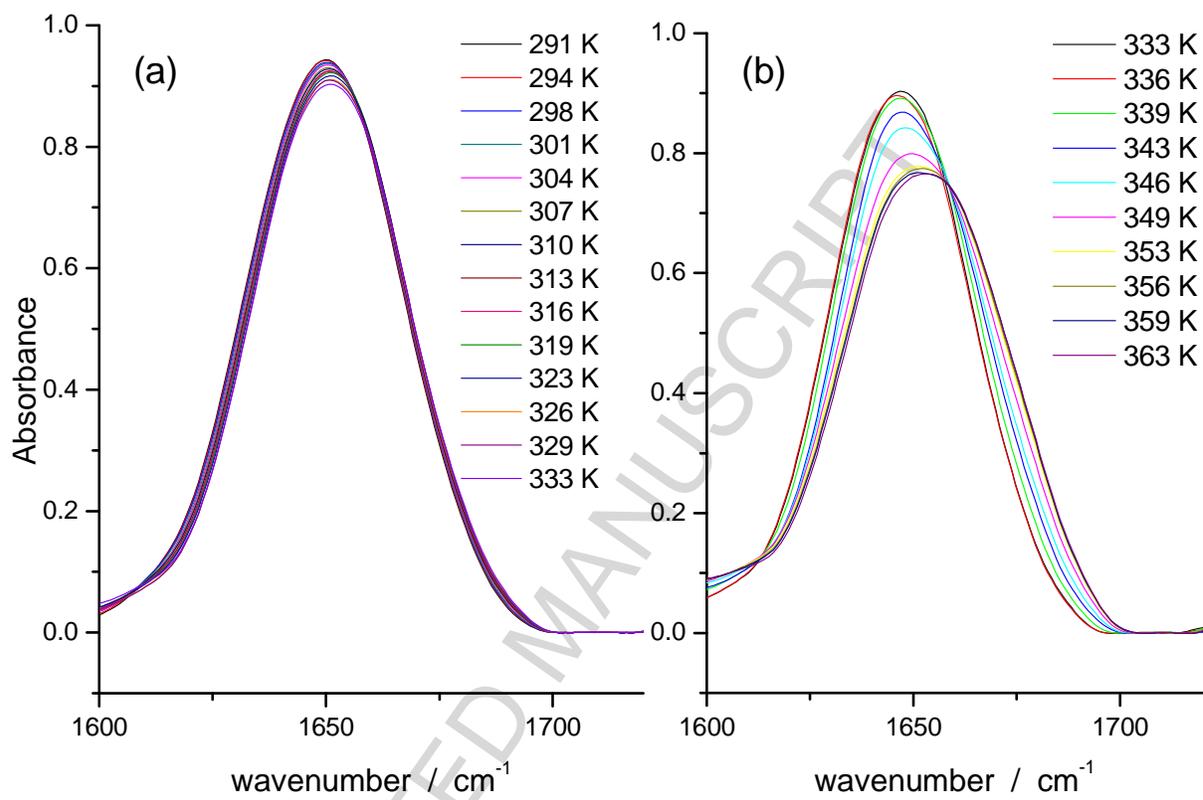


Figure 1

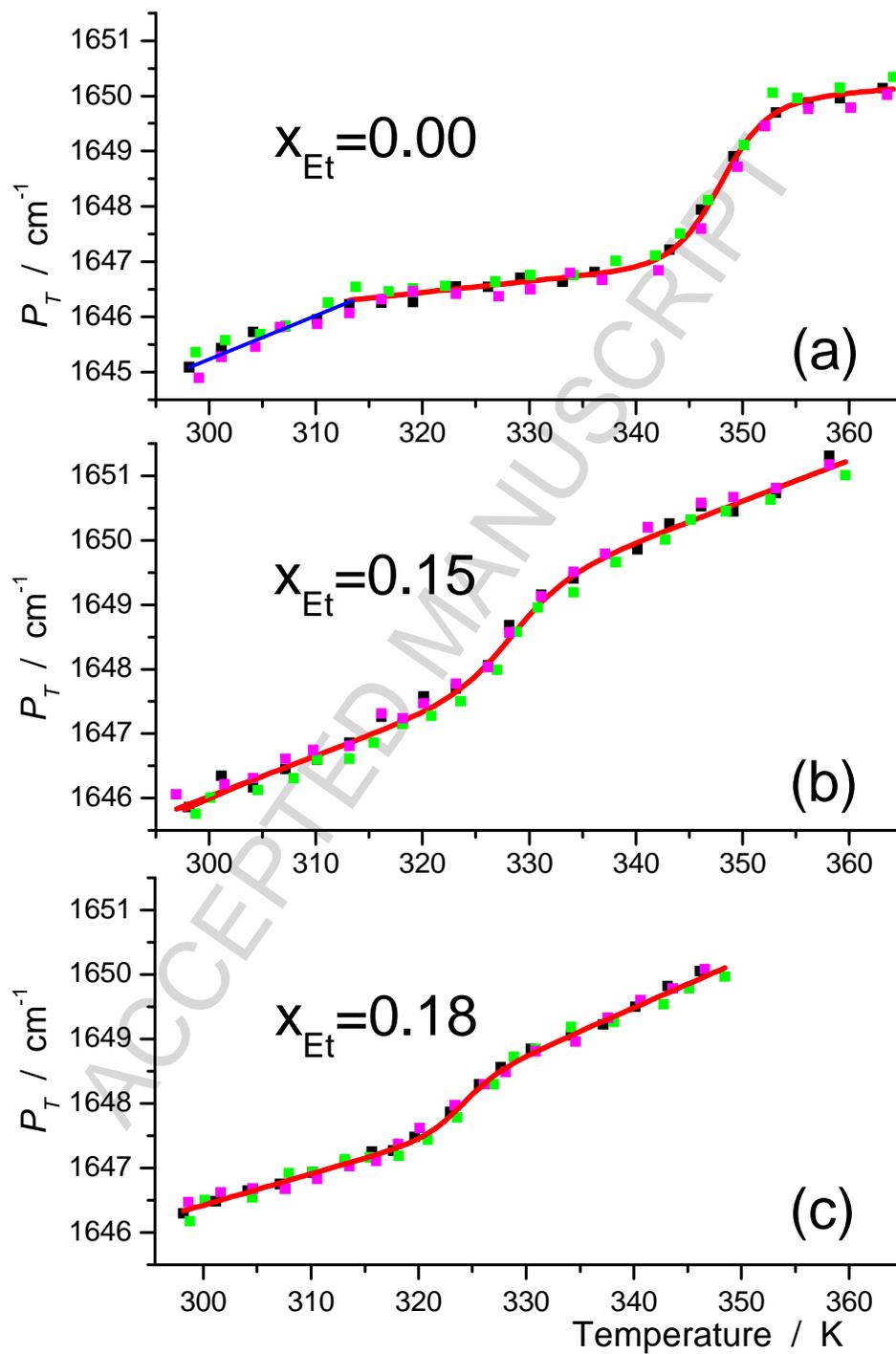


Figure 2

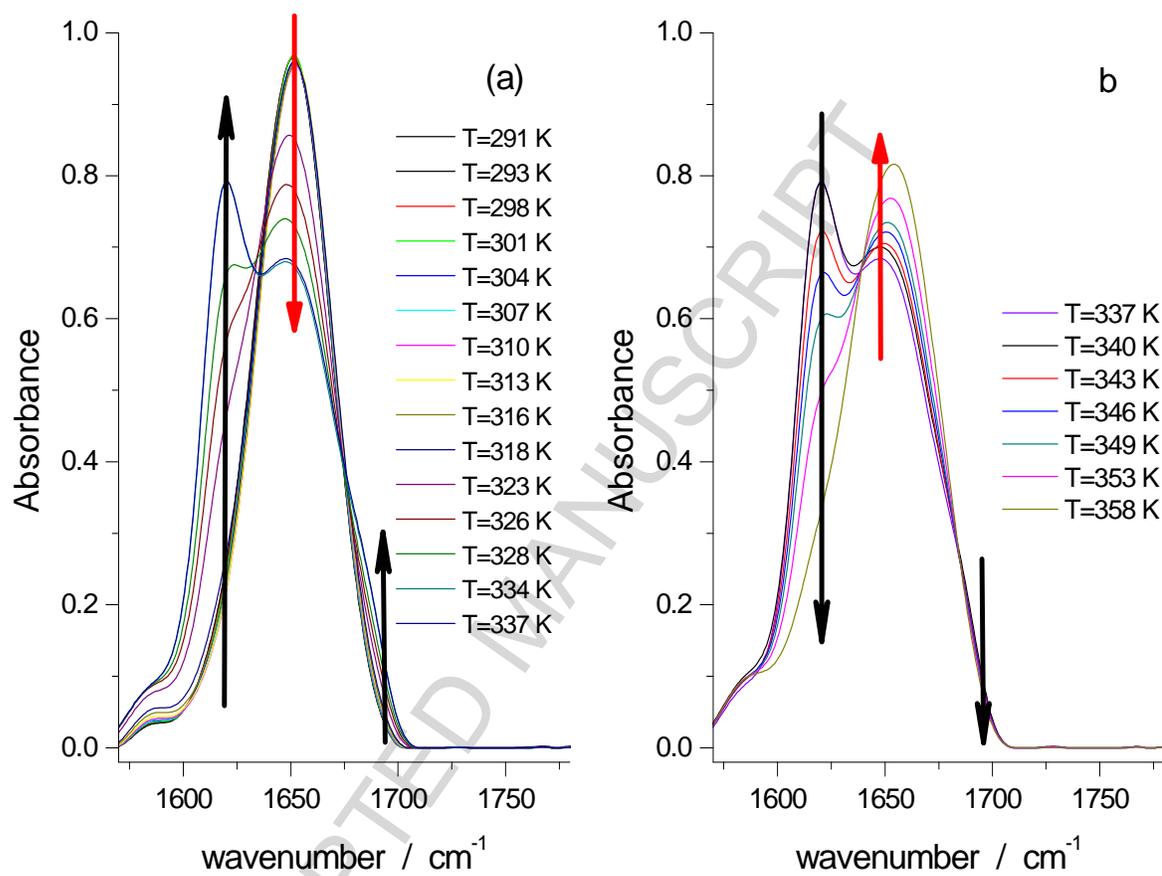


Figure 3

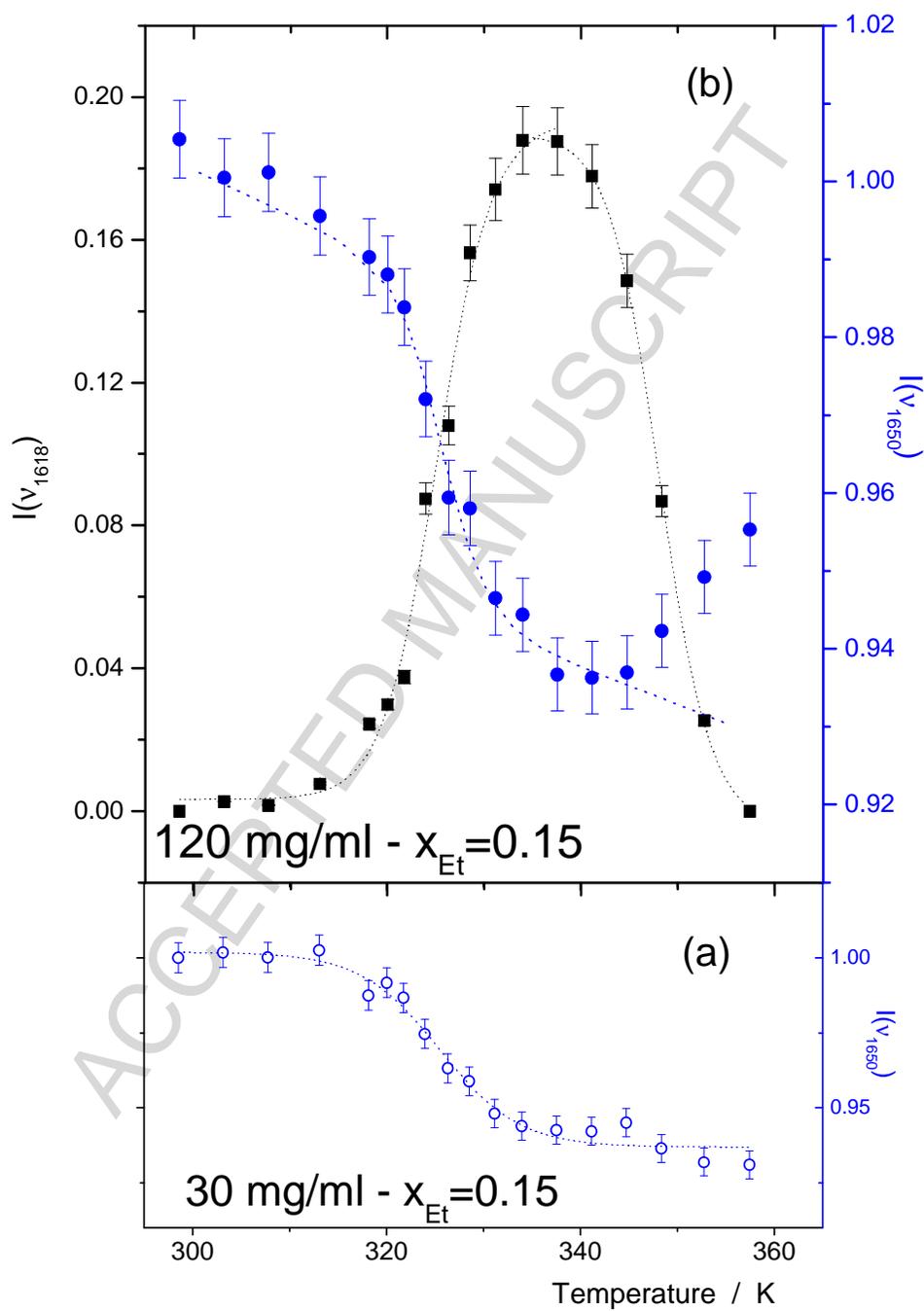


Figure 4

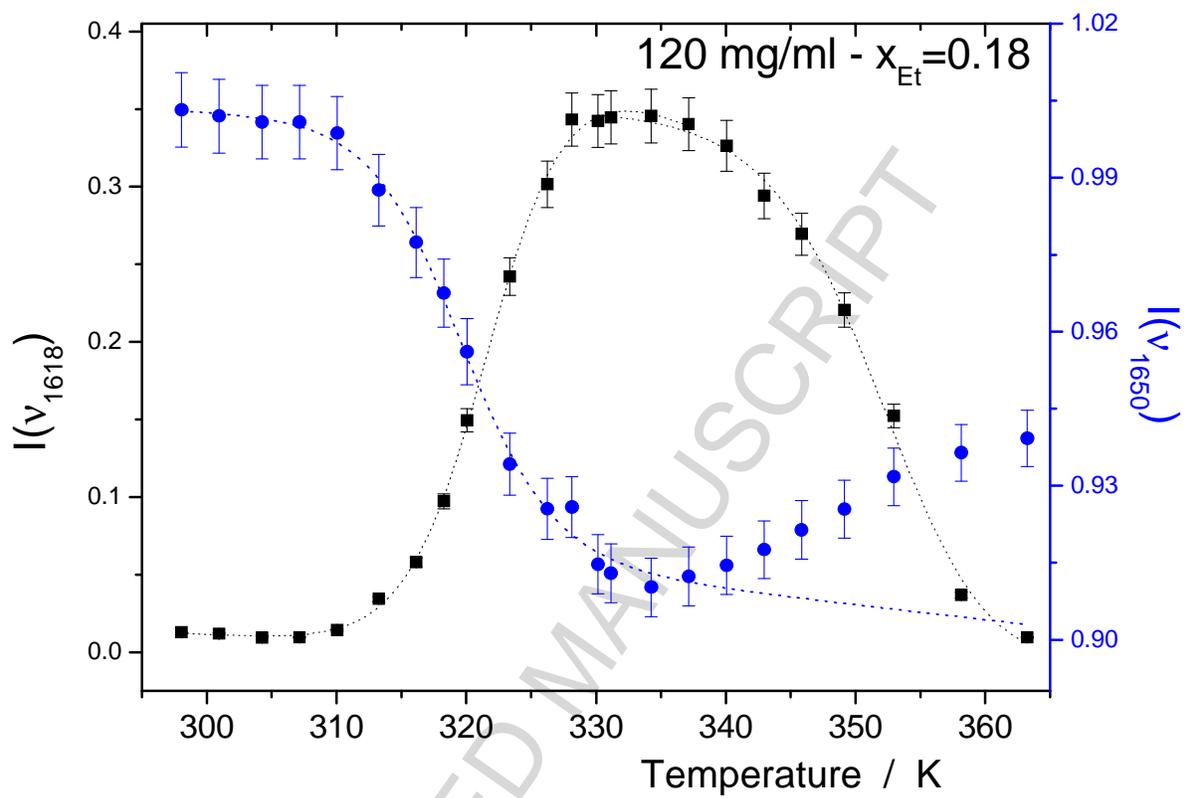


Figure 5

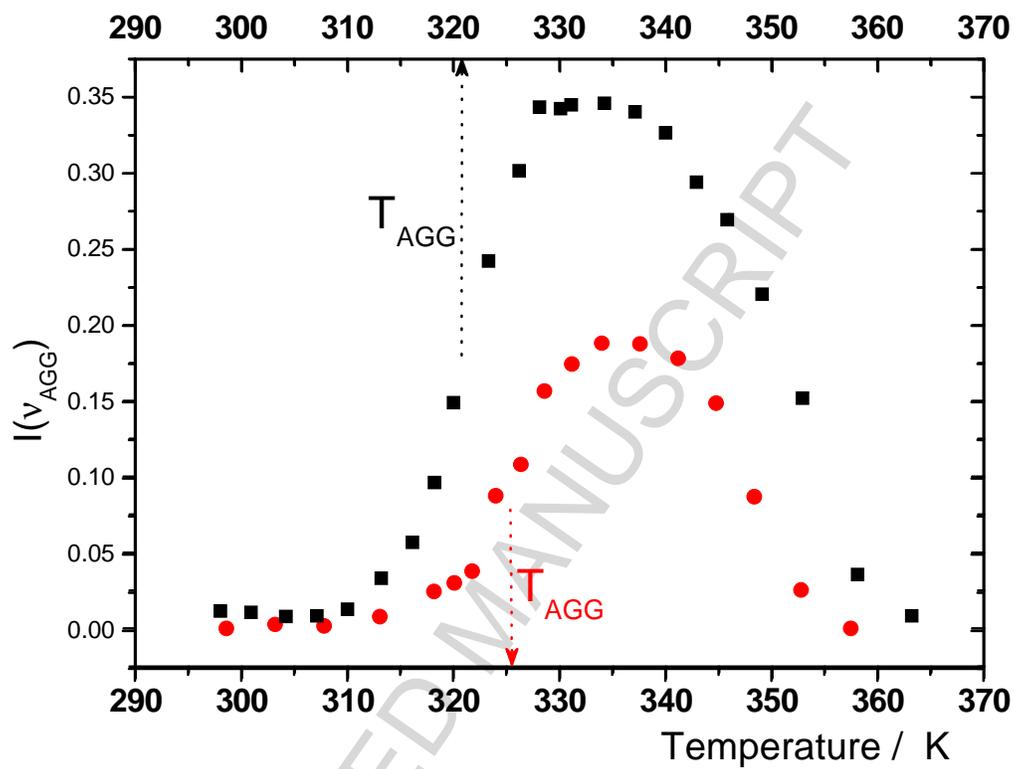


Figure 6

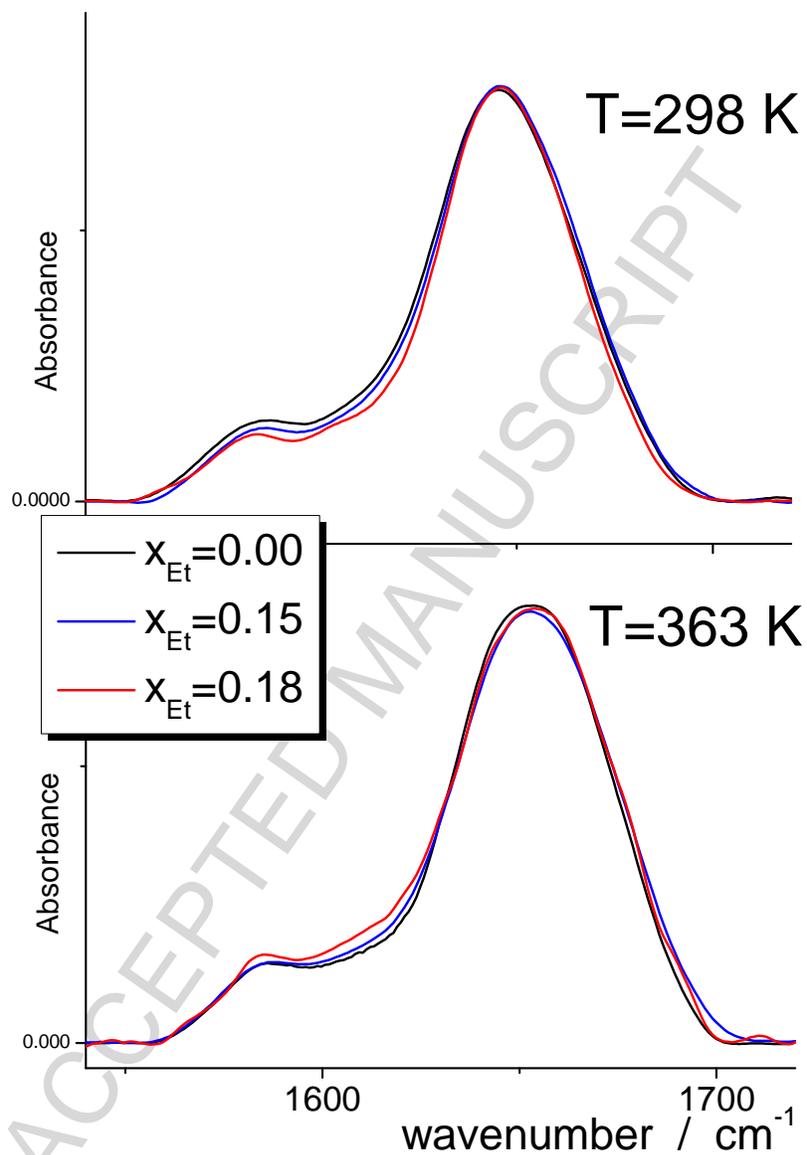


Figure 7

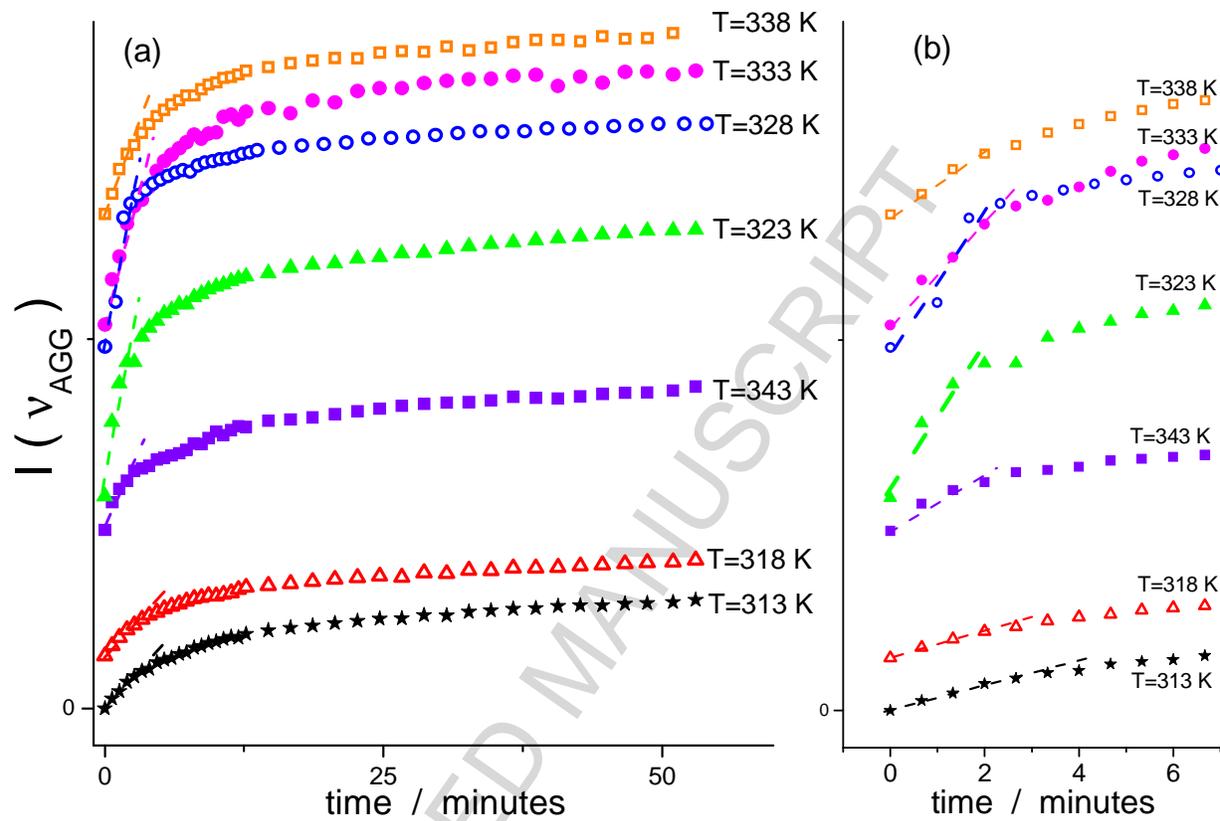


Figure 8

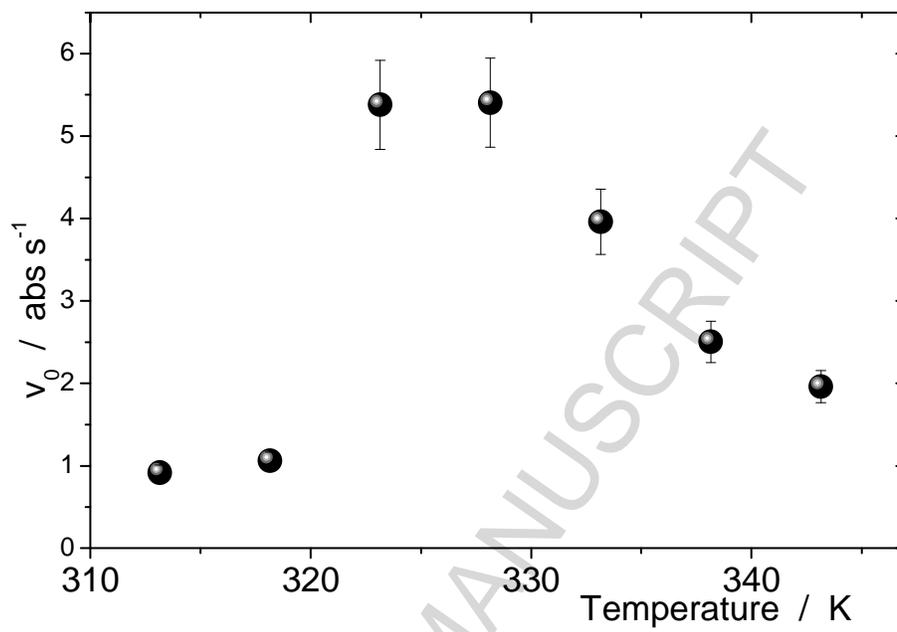
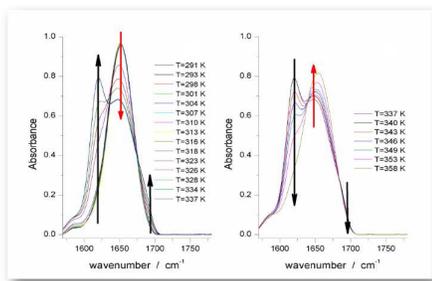
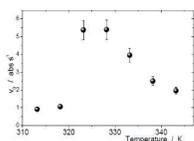


Figure 9

Folded + Unfolded = 

Graphical Abstract

ACCEPTED MANUSCRIPT

Research Highlights –

> We followed the effects of ethanol addition to lysozyme/water solutions. > A reduced stability of the folded state was revealed on increasing ethanol fraction. > An enhancement of intermolecular interactions between protein chains was observed upon increase of alcohol concentration. > We evidenced an irreversible association initiated by the unfolded and the folded states of the macromolecule. > We obtained different hydrogels under different thermal treatment of a single protein solution.