

Glycerol-Induced Aggregation of the Oligomeric L-Asparaginase II from *E. coli* Monitored with ATR-FTIR

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Abstract: In this paper attenuated total reflectance Fourier transform infrared spectroscopy has been employed for the study of the structural composition of aggregates of the oligomeric L-asparaginase II from *E.coli* formed in the presence of glycerol after the induction of refolding of the protein. Apart from the perfect coincidence of the secondary structure composition of EcA2 as determined by FTIR and crystallography [1], it has also been shown that secondary structure of protein in asparaginase deposits is similar to that of the native conformation: 20.7% extended, 22.3% disordered, 31.4% helix and 25.6% turn/bend/ β sheet. Certain structural similarities in the range of experimental error was observed for all three protein deposits presented in this paper, indicating a common structural basis for the composition of this types of aggregates. It is concluded that in the constitution of such precipitates, a partially folded (molten globule like) state(s) is involved, and its stabilisation is a key factor leading to the aggregation. The results presented in this paper might serve to be a good explanation and an excellent basis for the fundamental theory of protein (oligomers) precipitation by osmotic substances.

Keywords: attenuated total reflectance Fourier transform infrared spectroscopy, the oligomeric L-asparaginase II, secondary structure

Abbreviations: EcA2[WT] - *E.coli* L-asparaginase II wild type. ATR-FTIR - attenuated total reflectance Fourier transform infrared spectroscopy. MW - molecular weight. PEG – poly(ethylene glycol). IRE - internal reflection element.

1. Introduction

It has previously been shown [2] that high molecular weight linear polymers are able to precipitate almost all plasma proteins (mainly oligomers) without denaturation occurring. Although this suggests a

new and very useful method for protein fractionation, no acceptable theory has been offered, and the mechanism underlying this process still remains undiscovered. L-asparaginase II from *E.coli*. has first, been thought to undergo simple cooperative transition during unfolding [3-6], second, to show strange behaviour for the denaturation of oligomeric proteins [unpublished data] and third, to have a double-exponential kinetic profile of refolding, as monitored by stopped flow CD (Figure 6, *vide infra*). Extensive studies of a number of different types of protein deposits, including those involved in several human diseases [7-12] have been conducted with the intention of understanding the general forces driving precipitate formation and finding ways to avoid it. It has been found that in protein aggregates a substantial amount of native-like secondary structures are present [13] leading to the suggestion that improper protein association/misfolding involves a number of ordered structures which could be listed as native like molten globule, molten globule, pre-molten globule states [14]. If this is the case, it should be expected that the observed precipitation will be caused by a molten globule like equilibrium state, highly populated under physiological conditions [15]. Keeping this in mind, we have found precipitation induced by glycerol to be a useful tool to enable the capture of the intermediate equilibrium state of L-asparaginase II from *E.coli*. ATR-FTIR used in this study seems to be one of the few experimental techniques allowing the analysis of protein deposits; the deformation of the protein structure due to an interaction with an incorporated IRE is negligible for this method. It is anticipated, with the further development of this type of research, that the description of structure(s) in differing protein deposits will aid a better understanding of the natural events occurring during very early stages of the *in vivo* protein folding pathway. We would also expect to have a powerful test for the elucidation of two-state model transitions, a better understanding of the general features of the polypeptide chain, means for the deflecting of various diseases caused by aggregations, and new tools for the engineering of very important oligomeric enzymes in cancer therapy [16].

2. Materials and Methods

Reagents. Ultra pure quality guanidinium hydrochloride and 87% glycerol were purchased from Sigma. The concentration of denaturant in all solutions were determined by refractive index measurements [17].

Production of EcA2[WT]. The protein purification and over-expression has been performed using a combination of the fractional ammonium sulfate precipitation, chromatofocusing and rechromatofocusing as described elsewhere [18].

Preparation of protein precipitates for ATR-FTIR. For the study of protein aggregates by FTIR, ammonium sulfate precipitates of asparaginase II from *E.coli* was centrifuged, dissolved in 50 mM Tris/HCl buffer (pH = 8.0) and dialysed against the same buffer. The estimated concentration of protein in the final stock solution was 2.5 mg ml⁻¹ as measured by UV absorption at a wavelength of 280 nm and assuming that $A(1\%)_{280} = 7.7$. This solution was directly applied to the surface of a germanium crystal for FTIR measurements of the native protein. For the precipitation experiments, individual 1 ml samples of the protein stock solutions were mixed with 6 M GuHCl solution in a ratio resulting in either a 1 M, 2 M or 3 M GuHCl concentration in the final solution, and stored for at least three hours to allow it to equilibrate. Following this, glycerol was added to make a final concentration of 25%. The resulting mixture was dialyzed overnight against 2 l of Tris/HCl buffer containing 25% of glycerol. The protein was then aggregated and collected by centrifugation. To remove the glycerol, the

pellets were dissolved in a minimum volume of Tris/HCl. The loss of the protein during this step was minimal. The resultant solution was applied to the surface of IRE for FTIR measurements.

ATR-FTIR spectroscopy. Experiments were recorded on a Nicolet 800SX FTIR spectrometer equipped with an MCT detector. An internal reflectance element - 45° trapezoidal germanium crystal with a high-out-turn of 72×10×6 mm was used. The solution was spread using a spatula during evaporation under dry nitrogen to ensure an equal distribution of protein on the crystal surface. For each experiment, 1024 scans were set. First, we recorded the spectrum of the clean crystal, followed by the spectra of the buffer and the asparaginase refolded from the 1 M, 2 M and 3 M GuHCl solutions. After loading each sample, the crystal was cleaned with soap and water and a spectrum of the clean crystal recorded. Finally, water vapour data was also collected.

FTIR spectral analysis. The spectra analysis and secondary structure calculation were performed with the Lab Calc and GRAMS/32 (Galactic Industries) programs. For the Fourier transformation of interferograms, the Mertz method was used [19]. Spectra were converted to absorbance by ratioing against the spectrum of the clean IRE. Water vapour and buffer spectra were subtracted and five independent spectra from different measurements were obtained. For all spectra, resolution enhancement was performed by using Fourier Self Deconvolution (FSD); second derivatives of the spectra were obtained using the Savitsky-Golay method. Peak positions were identified by both transformations and used in the curve-fitting routine. The program *curvefit.ab* from Galactic Industries was used for the curve-fitting procedure. This included possibilities for the automatic determination of initial positions of peaks and the manual changing of certain parameters. The peak positions as defined from FSD and their second derivative were used for the initial fitting of raw spectra. These bands were then fixed and the peak width was increased by up to 10 cm⁻¹. Following this, peak height, width and % Lorentzian were allowed to vary until the solution converged to a minimum. Finally, all parameters including the band centers were freed and 300 iterations under these conditions were performed. The final value of goodness-of-fit (reduced chi-squared) was in the range 1-4. Peak assignment and the calculation of the individual contributions was made based on data published elsewhere [20-22]. The sum of the curve areas obtained from the curve-fit procedure was fully covered by the experimental peak areas (Figure 3).

3. Results

Crystal structure of native wild type L-asparaginase II from E-coli. The crystal structure of wild type L-asparaginase II has been resolved [1] at 2.3Å resolution using single heavy atom derivatives and molecular replacement. L-asparaginase II is a 222 symmetry homo-tetramer and a member of the α/β protein family. The subunits contain two domains with unique topological features, being composed of 62 β conformations and 37 α-helix and various numbers of 310 helices, hydrogen bonded turns, bends and isolated β bridges. As expected, the major component of the FTIR spectra of native asparaginase is a band at 1657 cm⁻¹ corresponding to a helical conformation. The band at 1653 cm⁻¹ is comparable in intensity, and corresponds to a disordered/irregular part of the molecule. Turns and β sheets have been assigned to bands at the 1694 cm⁻¹, 1681 cm⁻¹ and 1672 cm⁻¹ positions and in part at 1614 cm⁻¹. The peak at 1637 cm⁻¹ belongs to extended β strands. The estimated proportions of different secondary structural elements are as follows: 18% extended, 25% disordered, 31% helix, turn/bend/β 26% (table 1) and coincides with the corresponding results from X-ray analysis. Therefore, we conclude that these data are a good approximation and can be used for the structural analysis of L-

asparaginase II, although it was also expected that absorptive interaction of proteins with the germanium crystal could be a reason for the structural distortion of the protein [20].

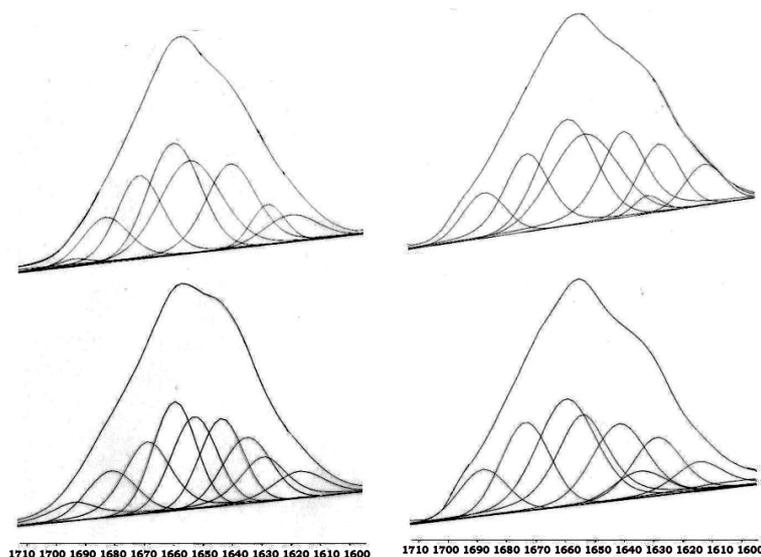


Figure 3

Figure 3. Presentation of curve-fit spectra of amide I region of the ATR-FTIR spectrum after FSD resolution enhancement. A: Native state , b: Refolded and aggregated from 1M GuHCl, c and d:from 2M GuHCl and from 3M GuHCl, respectively. Clear similarities in aggregated forms can also be seen. Notice that the peak positions are slightly shifted and a number of fitted peaks have also changed.

Table 1. Comparison of secondary structure content of wild type L-asparaginase II ^a

	Refolded from 3 M GuHCl	Refolded from 2 M GuHCl	Refolded from 1 M GuHCl	Native	X-ray Native
Extended	21.2±0.2	21.1±1.3	21.0±1.1	18.0±0.4	20.7
Disordered	26.7±0.3	26.5±1.0	26.8±1.4	24.5±0.2	22.3
Helix	26.3±0.6	26.4±1.5	25.3±1.6	30.9±0.9	31.4
Turn/bend/β	26.8±1.2	26.1±2.0	26.9±2.4	26.6±1.1	25.6

^a The percentage of secondary structure elements was calculated from curve fit analysis of the amide I region.

Structure of the L-asparaginase precipitates refolded from 1 M GuHCl. As mentioned earlier, ATR-FTIR is one of the few techniques which is able to estimate the protein conformation in aggregates. The secondary structural composition of L-asparaginase aggregates refolded after dilution in 1 M GuHCl in the presence of 25% of glycerol is given in Table 1. As can be seen, the percentage parts of the different structural units are comparable to the native conformation within the range of experimen-

tal error. We have also concluded that, based on the changes in peak position of the second derivative of amide I, that the tertiary and quaternary structure of the protein in aggregated conformation is significantly different from the native one. The positions of the peaks are split and the number in, and shape of, the whole raw amide I region is altered. Nevertheless, based on previous studies [20-22] we were able to assign peaks to appropriate structural units and obtain the following composition for the secondary structure of protein: 21% extended, 27% disordered, 25% helix and 27% turn/bend/ β , which slightly differs from the same parameters for the native protein obtained by ATR-FTIR measurements (see Table 1). The shift of peaks along the x-axis and their splitting could be due to the aggregation of, and the tight interaction between, different parts of the molecule. This could also be the cause of the appearance of shoulders in the amide I region of the raw and FSD spectra which grow upon increasing the initial concentration of GuHCl. This suggests that a population of uniquely conformed proteins, which will be discussed below, is responsible for the precipitation being accumulated in a greater amount, although at 1 M GuHCl concentration it is less populated. This experiment has provided us with clear evidence that the conformation of proteins in glycerol precipitates differs from that of native protein, but has certain similarities in the amounts of secondary structural elements. Once more, this validates the ability and accuracy of the ATR-FTIR measurements for the study of protein deposits.

Structure of L-asparaginase precipitates refolded from 2 M and 3 M GuHCl. From the amide I and the second derivative spectra analysis, as well as from the quantitative estimation of secondary structure elements and band positions from the curve-fitting routine, we have concluded that these aggregates contain almost identical conformations. For each of these we obtain ratios of 21% extended, 27% disordered, 26 % helix and 27% turn/bend/ β (see table 1). The band positions in both cases are similar, with no additional peaks or peaks splittings being observed. Such results can be readily interpreted as being obvious, since at the given concentrations of GuHCl the protein is fully denatured and its consequent refolding should yield equal populations of aggregated conformations. The increasing of amount of aggregated units can also be seen from the amide I region of the corresponding spectra and its second derivative (Figures 1 and 2).

Amide II and amide III of spectra. The amide II and amide III spectral regions from 1200 cm^{-1} to 1600 cm^{-1} contain information mainly about the out-of-phase combinations of NH, the in-plane-bending of NH, and the in-phase combinations of CN with some CC and C=O [31]. These spectral regions (Figures 4 and 5) also display quite similar shaped band positions, although the three aggregate spectra differ from the native one. This is due to the tertiary structural difference between them, given that spectral regions are also sensitive to changes in the global fold of a molecule. The analysis of FSD and the second derivative of the amide II and III regions confirms once more that structures of these three aggregates are very similar, but that they differ substantially from the tertiary structure of the native protein.

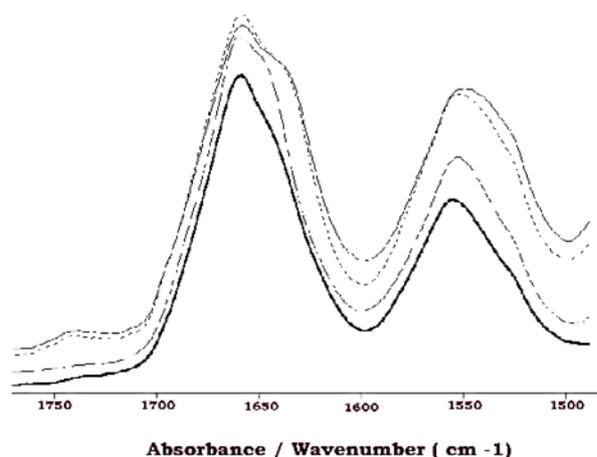


Figure 1

Figure 1. Comparison of the amide I and II regions of the ATR-FTIR spectrum of L-asparaginase II from *E.coli* in its native state (wide connected) , refolded and aggregated from 1M GuHCl (dashed), from 2M GuHCl (connected) and from 3M GuHCl (dotted). FSD resolution enhancement of raw amide I spectra has not been performed.

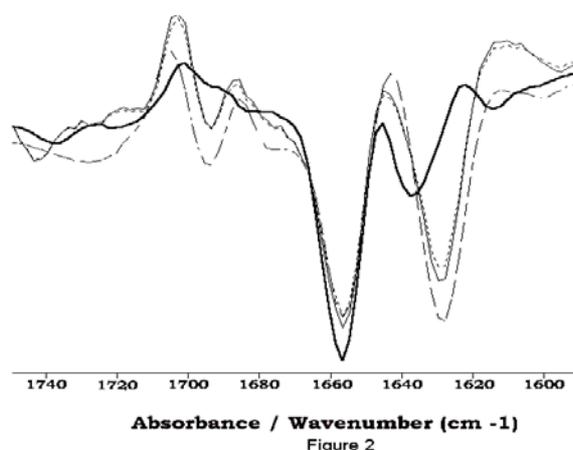


Figure 2

Figure 2. The second derivative spectra of the amide I region of the ATR-FTIR spectrum of L-asparaginase II from *E.coli* in its native state (wide connected) , refolded and aggregated from 1M GuHCl (dashed) ,from 2M GuHCl (connected) and from 3M GuHCl (dotted). FSD resolution enhancement of raw amide I spectra has been performed. The best coincidence between all aggregated forms is evident.

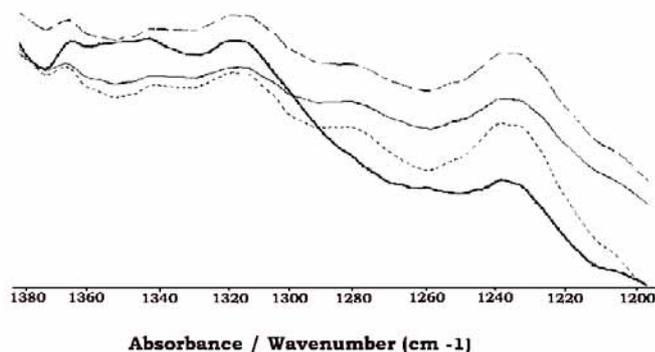


Figure 4

Figure 4. The spectra of the amide III region from the ATR-FTIR spectrum of L-asparaginase II in the native state (wide connected) , refolded and aggregated from 1M GuHCl (dashed) ,from 2M GuHCl (connected) and from 3M GuHCl (dotted). All three deposits of protein exhibit a nearly identical shape, suggesting a similar structural composition.

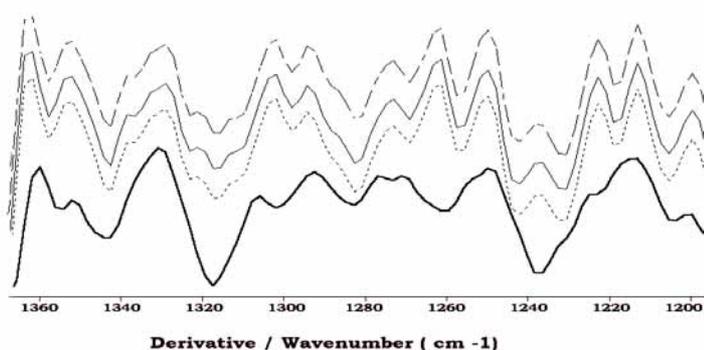


Figure 5

Figure 5. The second derivative of amide III region of FTIR spectra of L-asparaginase II in its native state (wide connected) , refolded and aggregated from 1M GuHCl (dashed) ,from 2M GuHCl (connected) and from 3M GuHCl (dotted). A substantial difference from the native spectra of all protein aggregates can easily be noticed.

4. Discussion

Effect of glycerol on protein conformation. Polson and co-workers [2] performed a detailed study of the serum proteins fractionation by linear polymers. Using poly(ethylene glycol), they were able to selectively precipitate almost all the proteins from human plasma. Using PEG of different molecular weights, the authors came to the conclusion that for the precipitation of, for instance, γ -globulin, a low concentration of high molecular weight polymer is required. The same effect can be seen with a high concentration of low molecular weight polymers. This observation excluded the possible explanation of PEG action acting as a precipitant by dehydration, since the osmotic pressure induced by higher

MW polymers will dehydrate the protein to a higher degree. No satisfactory theory for this phenomenon has been offered to date. Gekko and Timasheff [23] performed an extensive study in order to understand the mechanism of protein conformational changes caused by glycerol. Using different ratios of glycerol/water mixtures, they concluded that the protein is preferentially hydrated, and therefore has increased chemical potential. Sinanoglu and Abdunur [24] have also shown that glycerol is able to repel nonpolar substances, as it interacts favourably with water [25] by the disruption and strengthening of the water structure [26] and increasing protein hydrophobicity. It should therefore be expected that the hydrophobic groups will move from the protein surface to the interior, and consequently, that the volume of the molecule will be decreased, and the protein will contract. This serves as the best explanation for the recently observed phenomenon when using glycerol as a "chaperone" for the correct folding of monomeric rhodanese [27], which does not aggregate unless refolding is assisted. Rhodanese is one of the few proteins that clearly exhibits the existence of a stable folding intermediate when studied using any of the available biophysical techniques, with two independent steps of the first phase order transitions [[28,29]. It has been suggested for the refinement of the structure affected by glycerol during refolding, that the specific aggregation prone intermediate is stabilised by glycerol, yielding an increasing amount of active rhodanese upon the increase of glycerol concentration. The ability of glycerol to modify the stability of folding intermediates has been also proved in the work of Schumann and co-workers [30]. The act of stabilising a protein's structure and increasing the activity of an enzyme in general has been noticed in a number of previous studies [31-40]. The stabilizing effect of glycerol has been shown even when there is temperature related unfolding of the protein [41]. Glycerol also works as a cryoprotectant [42], and this type of interaction has been fully described by Fink [43]. The effect of glycerol on dimer stability has also been studied [44]. It was shown that the DNA-binding Arc repressor dimer cannot be denatured under pressure, if water is not present. The authors followed pressure-denaturation of the Arc repressor by the measurements of fluorescence in the presence of glycerol, and observed the dissociation of native dimer into denatured, molten-globule monomers. For these dimers, two-dimensional NMR also demonstrated the accumulation of partially folded states [45] and found that the entropy-driven step in the dimerization of protein occurs within the temperature range between -15 to 20°C [46]. The free energy coupling between transition molten globule \leftrightarrow native dimer and DNA binding has also been visualized [47]. The stabilising effect of glycerol on the leucine zipper-coiled coil dimer's early stage molten-globule-like intermediate, in the previously mentioned temperature range, was also found [own unpublished data]. The unfolding of bovine pancreatic ribonuclease by polyethylene glycol and glycerol has been tracked using fluorescence, CD and optical rotation [48]. It was found that the protein was not fully denatured in 97% glycerol, since the transition has been observed with all three methods. These data suggest that glycerol predominantly affects the medium or solvation layer of the protein, when it is increasing the stability of the native molecule.

Folding/unfolding of E.coli L-asparaginase II. The assay of stability of the system studied in this work has rather complicated history. From the fluorescence measurements of the chemical denaturation of wild type EcA2 and several of its mutants, single transition profile characterised one step highly cooperative unfolding has been observed [3-6]. However, it is worthwhile to notice that for some other, particularly oligomeric proteins that have been previously studied [49], intrinsic fluorescence has proven to be a rather insensitive method for the exploration of changes accompanying the transi-

tion from the native state to the closely related molten-globule state. Furthermore, *E.coli* L-asparaginase II has absolutely no dependence of position of the denaturation curves when it is measured using circular dichroism and fluorescence, on initial concentration of protein in solution [unpublished data], thus, indirectly suggesting a non two-state model transition [50]. Calorimetric measurements have clearly demonstrated that there are significant discrepancies between van't Hoff and calorimetric enthalpies, this being the best indication for a non two-state model transition [51-53], as well as for aggregation at the end of temperature denaturation [unpublished data] - one more piece of evidence for the existence of residual structure at the final stage of denaturation [54]. Although the mechanisms of chemical and temperature denaturation differ, this observation serves as a further evidence for a non two-state model transition, assuming that unfolding from the native tetramer with the both ways of denaturation results in monomers. We have also conducted stopped-flow CD measurement of L-asparaginase II refolding following unfolding by diluting a sample in 3 M GuHCl (Figure 6). The results are consistent with a non two-state model of protein folding.

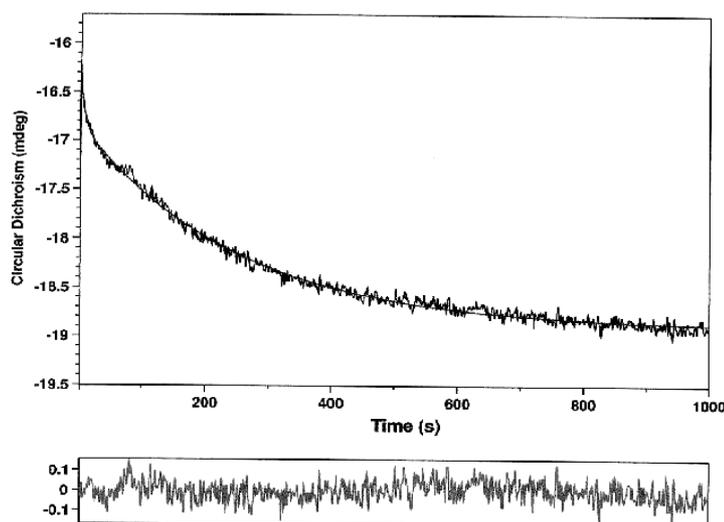


Figure 6

Figure 6. Kinetics of L-asparaginase refolding monitored with circular dichroism at 225 nm and 22°C in Tris/HCl buffer pH=8.0. The protein concentration was 2 mg/ml. The enzyme was unfolded in 3M GuHCl solution and refolding was initiated by diluting into refolding buffer. The upper bar indicates kinetic traces measured on stopped-flow device. The lower bar theoretical double-exponential curve fit the stopped flow data. The kinetic parameters are as follow: $A_1 = -0.67 \text{ deg cm}^2 \text{ dmol}^{-1}$, $A_2 = -2.09 \text{ deg cm}^2 \text{ dmol}^{-1}$, $A_{\text{infinity}} = -1.89 \text{ deg cm}^2 \text{ dmol}^{-1}$, $k_1 = 0.15$, $k_2 = 0.0041$.

Interpretation of ATR-FTIR data for E.coli L-asparaginase II. Diversity of equilibrium intermediates of globular proteins have been described in detail by Uversky [14] and Fink and co-workers [55], suggesting the existence of a pre-molten globule type intermediate with native protein like topology [56] and a practical absence of tertiary contacts. From the excellent works on mechanisms of aggregation [9,57,58] it can be concluded that the precursor of all types of aggregations are the partially folded intermediates with native protein like topology. Since changes of standard free energy caused by the stabilising effect of glycerol on protein conformation is too small ($\sim 0.1 \text{ kcal mol}^{-1}$ of protein and added

glycerol [59,60]) for simple kinetic intermediates to overcome the free energy barrier between two states [13,61,62], while the molten globule like states are energetically closely related to the native state and in some cases are more stable than the native one [63], the glycerol should be considered as a compound able to selectively affect and to accumulate equilibrium molten globule like intermediates. For the asparaginase, we observed nearly identical profiles of amide I, II, and III in the aggregated states, no shift along the x-axis was observed between the three precipitates of the protein, and since the FTIR spectra are very sensitive to the changes of dihedral angles φ and ψ [64], based on the curve-fit data (table 1) and the above mentioned studies, we can conclude that there is only one structure, partially folded molten globule like on the pathway of L-asparaginase folding, stabilised by glycerol and followed by aggregation upon refolding. For the account of aggregation we can only propose, based on the studies by Broglia *et al.* [65], Eliezer *et al.* [66] and Istrail *et al.* [67], that the spread of secondary structural elements along the polypeptide chain are slightly changed in the compact equilibrium intermediate state, and that this is followed by stabilisation and speeding up of the association of monomers to the native oligomer by glycerol and subsequent collisions in search of energetically most favourable conformation, resulting in aggregation/misassociation, rather than correct folding to the native, functional state. If we assume that the proteins in the work of Polson *et al.*, [2] are natively unfolded under physiological conditions and probably unstructured [68,69], too, then the effect of the linear polymers on the serum proteins can be explained by the stabilisation of partially folded molten globule like equilibrium states resulting in the misfolding of the proteins. In order to verify this concept, it is necessary to perform high resolution NMR experiments similar to those by Eliezer *et al.* [66] using a transverse relaxation optimised NMR technique, which provides the best resolution and signal to noise ratio, even in integral membrane protein/detergent systems [70].

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