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ORIGINAL PAPER

Stable misfolded states of human serum albumin revealed by high-pressure infrared spectroscopic studies

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Abstract Pressure unfolding–refolding and the subsequent aggregation of human serum albumin (HSA) was investigated by high-pressure Fourier transform infrared measurements. HSA is completely unfolded at 1 GPa pressure, but the unfolding is not cooperative. Hydrogen–deuterium exchange experiments suggest that a molten globule-like conformation is adopted above 0.4 GPa. An intermediate was formed after decompression, which differs from the native state only slightly in terms of the secondary structure, but this intermediate is more stable against the temperature-induced gel formation than the pressure-untreated native protein. This observation can be explained by assuming that the pressure unfolded–refolded protein is in a misfolded state, which is more stable than the native one.

Keywords High pressure · Human serum albumin · Misfolding · Aggregation · Conformational disease · Infrared · Spectroscopy

Introduction

Due to the progress in our understanding in protein folding and the recent interest in protein aggregation (Daggett and Fresht 2003; Dobson 2004), many protein folding studies have focussed on the population and characterization of non-native states that can act as precursors for protein aggregation. In particular, protein assemblies called amyloid fibrils, which are associated with debilitating diseases such as Alzheimer's disease and type II diabetes, have received a lot of interest. It was shown that the formation of such disease-associated aggregates requires at least a partial unfolding of the native structure (Dobson 2003). The population of these partially unfolded states can arise from a destabilization of the native state, for instance, due to a mutation, as in the case of familial amyloidosis, which is connected to mutations of lysozyme (Dumoulin et al. 2003). However, in some cases the interaction with misfolded aggregates is sufficient to destabilize the native proteins (Booth et al. 1997).

Studying folding requires performing refolding experiments. In such investigations, the native protein is first unfolded, generally by addition of a chemical denaturant, and the refolding is initiated by the sudden removal of this denaturing agent (by diluting the system) (Greubele 1999). However, complete removal of a chemical denaturant cannot be achieved. Instead, only its concentration can be reduced. Simultaneously, there is a drop in protein concentration, which may also influence the system. In the case of proteins, where the cold denaturation temperature does not fall below the freezing point, one can start from the cold denatured state and refold the protein with a temperature jump (Osvath et al. 2003). But this is possible only for a limited number of proteins.

We used an alternative approach, where the protein is unfolded by high hydrostatic pressure, which is known to unfold proteins (Mozhaev et al. 1996). The stability region of the native conformation can be described by an elliptic shape on the pressure–temperature plane (Smeller 2002). It is noteworthy that the pressure- and temperature-unfolded

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states differ in a very important respect, namely the pressure-unfolded protein is not aggregated, whereas the temperature-unfolded states often form aggregates (Meersman et al. 2002). This is mainly because the aggregated system occupies a larger volume, which is opposed by pressure (Randolph et al. 2002). This makes pressure a useful tool for the refolding studies.

In the present study we explore the effects of pressure unfolding–refolding and subsequent heating on human serum albumin (HSA) by Fourier transform infrared spectroscopy. HSA is a blood plasma protein, transporting fatty acids in the vascular system. Several studies have been performed on HSA since it is the most abundant protein in the plasma and it is relatively easy to purify. It consists of 585 amino acids forming a single chain and has a molar mass of 67 kDa. Its three-dimensional structure has been resolved by X-ray crystallography (He and Carter 1992), consisting of 34 helices which are connected by 28 β-turns and 3 γ-turns (Fig. 1). The helical content is around 70%, represented mainly by α-helices. Only 4 of the 34 helices are short 3_{10} helices. The tertiary structure consists of three domains, and it is stabilized by 17 disulphide bridges. Between the domains there are cavities to accommodate the substrate molecules.

Materials and methods

Human serum albumin was purchased from Sigma. HSA was dissolved in a pD = 7.0 Tris–DCl buffer.

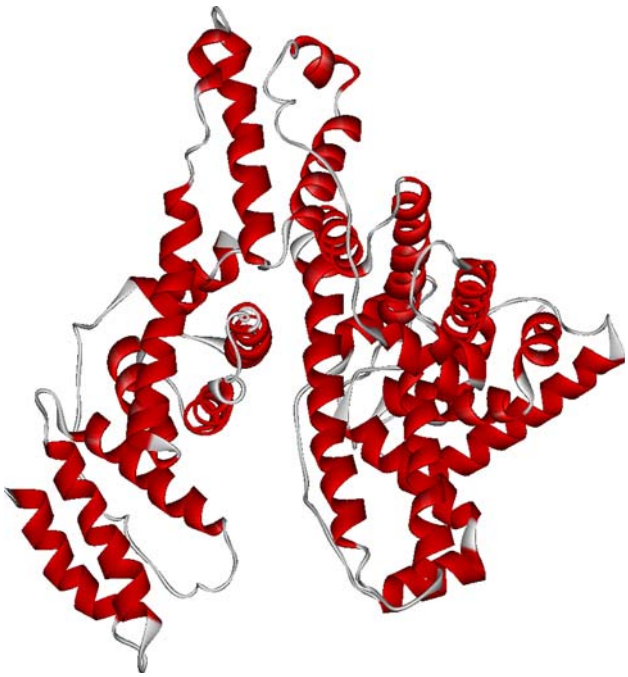

Fig. 1 Ribbon diagram representing the structure of human serum albumin (PDB code 1AO6). The figure was produced using Accerlys WieverLite

Pressure experiments were performed in a diamond anvil cell, as described previously (Meersman et al. 2002). High-pressure was achieved in a diamond anvil cell (Diacell, Leichester, UK). The pressure was monitored by the shift of the 983 cm^{-1} band of $BaSO_4$, which was used as internal calibrant (Wong and Moffat 1989).

Fourier transform infrared spectra were collected with a Bruker IFS66 spectrometer equipped with an MCT detector, using 2 cm^{-1} resolution. Two hundred and fifty six scans were added to reduce the signal-to-noise ratio. Fourier self-deconvolution was used to separate overlapping bands (Kauppinen at al. 1981; Smeller et al. 1995).

Results and discussion

Infrared spectrum

Figure 2 shows the conformation sensitive amide I region of the infrared spectra of HSA recorded under different conditions. The spectra are deconvoluted to enhance the spectral resolution. This way the fitting of the spectrum with Gaussian component curves was also possible. The spectrum of the native protein has a major peak at 1,652 cm^{-1}, with shoulders at lower and higher wavenumbers (1,636 and 1,663 cm^{-1}). The area percentages of the

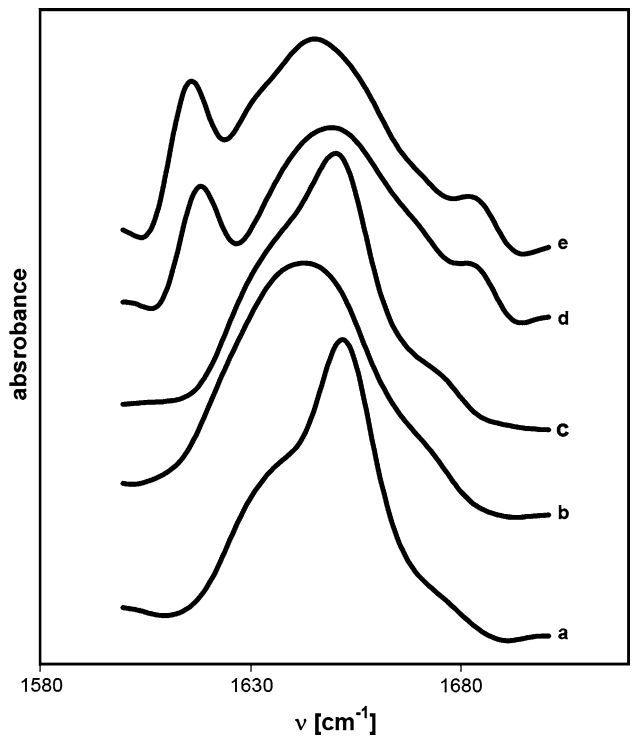

Fig. 2 Amide I region of the infrared spectrum of HSA during successive pressure–temperature cycling. Curves from bottom to top 25 °C, ambient pressure; 25 °C, 1.2 GPa; 25 °C, back to ambient pressure; 90 °C, ambient pressure; back to 25 °C, ambient pressure

fitted Gaussian curves are 41, 39 and 20% for the peaks at 1,652, 1,636 and 1,663 cm^{-1}, respectively. The position of the main peak is characteristic for the α-helical structure (Susi and Byler 1986). This is consistent with the crystallographic structure (Seugio et al. 1999), which contains 30 α-helices. The higher wavenumber spectral component at 1,663 cm^{-1} can be attributed unequivocally to the short turn structures connecting the helices. The interpretation of the shoulder band at 1,636 cm^{-1} is more complex. According to the classical assignment, these frequencies correspond to β-sheet structures (Susi and Byler 1986), but this protein does not have any β-sheet structure. Later it was shown that such a shoulder can be assigned to solvent exposed helices or to the residues at the end of the helices (Harris and Chapman 1995). According to the crystal structure, 400 of the 585 residues (70%) form α-helical structure. About 120 of these amino acids are at the end of the helix, which gives 21% of the total residues. Thus less than half of the residues are in the inner positions of the helices. This is close to the area of the central peak of the amide I band, which gives 41% of the total area. The residues at the end of the helices presumably contribute to the peak at 1,636 cm^{-1}. These assignments are also supported by the fact that similar shoulder at 1,632 cm^{-1} can be observed in the infrared spectrum of the myoglobin, which is also a completely helical protein (Meersman et al. 2002).

Pressure unfolding is not cooperative

The pressure stability of HSA was investigated by monitoring the maximum position of the amide I band as a function of pressure. Figure 3a shows that as the pressure increases the maximum gradually shifts from 1,652 to 1,642 cm^{-1}, a band maximum typical of a disordered structure. This is also apparent from the fact that at 1.0 GPa the amide I band is broad and featureless (Fig. 2b). The pressure unfolding of the protein seems to be non-cooperative, if one only observes the shift of the amide I peak. A similar non-cooperative unfolding behavior was observed by Krishnakumar and Panda (2002) using a chemical denaturant. Later the same group suggested sequential unfolding of the three domains, based on the fluorescence dyes attached to different domains (Kumar et al. 2005). Tanaka et al. (1997) also found that only the domain III is unfolded at 200 MPa, but the practically unchanged tryptophan fluorescence suggested that domain II is intact until 400 MPa. Likewise, equine serum albumin also shows a broad pressure transition (Okuno et al. 2007).

However, the maximum position of the band alone can be misleading, because the band is the sum of several components. Changes in the intensity of the component bands can result in the shift of the overall band maximum, but changes in the intensities of the minor components can be hidden by the larger components. Therefore it is worthwhile to look to other spectral parameters, such as the intensity of the amide I band at wavenumbers characteristic

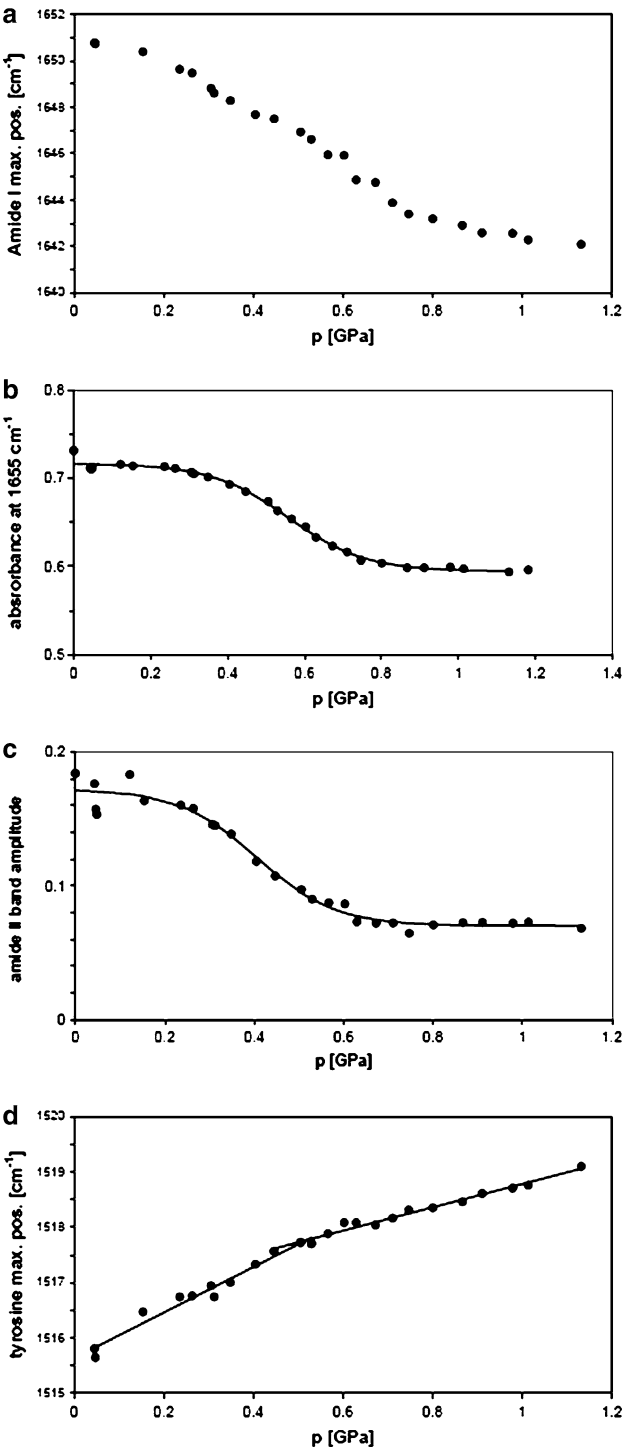

Fig. 3 **a** The maximum position of the amide I band of HSA versus pressure. **b** The absorbance at 1655 cm^{-1} versus pressure. **c** The intensity of the amide II band of HSA versus pressure. **d** The position of the tyrosine ring vibration as function of the pressure

for certain secondary structures. Figure 3b shows the absorbance at 1,655 cm^{-1} as a function of pressure. A broad transition spanning a pressure interval from 300 to 800 MPa can be observed. It can be fitted with a sigmoid shape with a mid-point of 560 MPa. Another important parameter in characterizing the protein structure and flexibility is the amide II band, which gives information about the hydrogen–deuterium (H–D) exchange. The normal mode of the amide II vibration contains N–H bending and C–N stretching. Since 60% of the energy of the vibration is located at the N–H bending, the frequency is very sensitive for the H–D exchange (Englander and Kallenbach. 1984). The hydrogenated protein backbone shows the amide II band at 1545 cm^{-1}, while band shifts to lower wavenumbers in the deuterated case (called amide II′ band). Plotting the intensity of the 1,545 cm^{-1} band shows the amount of the non-exchanged part of the protein. Since the protein solution had a few hours to exchange before the start of the experiments, all the solvent accessible protons are already exchanged at the beginning of the experiment. This means that hydrogen atoms in the solvent exposed surface and in the flexible regions of the protein are already exchanged at the beginning of the experiment. Figure 3c shows a clear sigmoidal transition in the intensity of the amide II peak, with a midpoint at ~410 MPa. This suggests that around this pressure the protein is losing its compactness, and even the most buried part of the polypeptide chain has become accessible for the deuterated water. The exchange of the inner core of the protein at the unfolding pressure was observed for many proteins, e.g. myoglobin horseradish peroxidase and lysozyme (Smeller et al. 1999, 2003), but in case of bovine pancreatic trypsin inhibitor (BPTI), which is a very pressure stable protein, the exchange was already completed before the onset of the conformational changes of the protein (Goosens et al. 1996). The similarity with BPTI lies in the presence of the structure stabilizing disulphide bonds. So it cannot be excluded that the disulfide bonds stabilize HSA above 410 MPa in a molten globule state, where the loose structure allows the H–D exchange, but the secondary structure elements are partially retained. The infrared spectrum at 1.0 GPa, however, clearly shows that at this pressure the protein does no longer contain a significant amount of secondary structure.

Further information on the pressure unfolding process can be obtained from the behavior of the tyrosine residues, which have a characteristic ring vibration at 1,515 cm^{-1} (Fig. 3d). Pressurizing leads to the increase of the band frequency, due to a simple physical effect of bond compression caused by the environment of the vibrating part of the molecule. It can be observed that the tyrosine peak shifts linearly with pressure, showing a reduction in the slope of the curve from 4.1 to 2.1 cm^{-1}/GPa at ~0.5 GPa, indicating that at this pressure the environment of the tyrosine rings has changed (Fig. 3d). A possible explanation is the swelling of the protein into a molten globule-like state by squeezing the water molecules inside the protein, which causes a change in the polarity of the tyrosine environment. Since HSA contains 18 tyrosine residues, located throughout the molecule (7 in domains I and II each and 4 of them in domain III), the tyrosine vibration reports on the whole protein.

The pressure effect on HSA was studied by Kunugi and co-workers (Tanaka 1997) using fluorescent labels attached to different domains and using the intrinsic fluorescence of the TRP214 residue located in domain II. While the dansylsarcosine and walfarin dyes dissociated from the protein at 200 MPa, the intrinsic fluorescence did not show any significant change up to 400 MPa, which was the limit of their experimental setup. The authors concluded that domain III is unfolded in the investigated pressure range.

After decompression, the amide I band maximum and the tyrosine peak return to their native positions, indicating a refolding of the secondary structure. The difference spectrum of the refolded and the original sample (not shown), however, suggests a decrease in the helical structure and an increase in the disordered structure. The loss of helical structure, is estimated from the difference infrared spectrum (not shown), is less than 5%.

The question can be asked whether the misfolded structure is a kind of molten globule with marked difference in tertiary structure. Usually tryptophan fluorescence or circular dichroism spectroscopy is used to address this question. Unfortunately circular dichroism spectroscopy cannot be applied at high-pressure. HSA has only one tryptophan residue, which would make this as a very local label. On the contrary, tyrosines are distributed along the whole polypeptide chain; therefore the reversibility of the tyrosine vibration band, which is also sensitive to the environment, suggests that large global changes in the tertiary structure can be excluded.

Pressure pre-treatment reduces the aggregation propensity

The temperature stability of the refolded HSA was investigated and compared to that of the non-pressure-treated protein. The amide I band of the heat denatured protein is dominated by the presence of two bands at 1618 and 1685 cm^{-1} (Fig. 2, curve d). These are typical of the formation of an intermolecular anti-parallel β-sheet (Ismail et al. 1992). The middle part of the amide I band is broad, indicating the remaining secondary structure is disordered. The above-described shape of the amide I band remains unchanged when the sample is returned to ambient temperature, indicating the irreversible character of the temperature unfolding. The spectra at high temperature (90 °C) and after cooling to 25 °C were identical, whether a previous pressure unfolding–refolding cycle was performed or not.

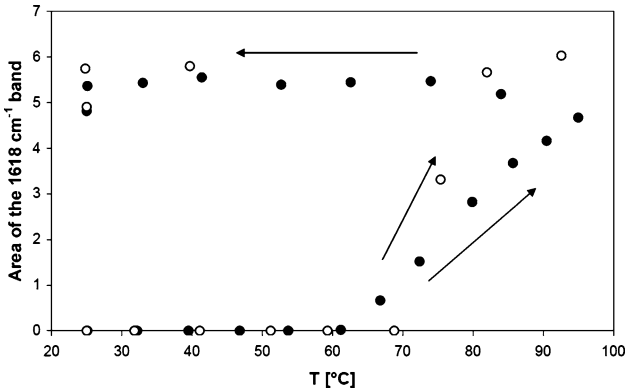

Fig. 4 Area of the aggregation sensitive infrared band (at 1618 cm^{-1}) of HSA during heating. *open circle* without previous pressure treatment, *filled circle* after pressure unfolding (1.2 GPa) and refolding

Figure 4 shows the fitted area of the 1618 cm^{-1} band as a function of the temperature for the pressure unfolded–refolded protein and for the control sample, which was not pressure treated before the temperature scan. It is clear that the midpoint of the aggregation is at higher temperature in case of the pressure treated protein. Fitting of a sigmoid curve gives the midpoint temperatures of 77.2 and 73.8 °C for the pressurized and the untreated proteins, respectively. Both the samples show the same maximal intensity (i.e. area) of the 1618 cm^{-1} band. This suggests that the refolded protein is more stable than the native one. In our previous experiments on a number of proteins, including myoglobin, lysozyme and horseradish peroxidase (Smeller et al. 1999, 2002, 2006), this effect was never observed. In all these cases folding intermediates obtained after pressure-refolding were more prone to aggregate when heated. Interestingly, in the case of a number of amyloid forming proteins, such as insulin, transthyretin and the prion protein (Foguel et al. 2003; Cordeiro et al. 2005, 2006; Grudzilanek et al. 2006; Torrent et al. 2004), pressure cycling resulted in the population of intermediate conformations that have an increased propensity to form fibrillar aggregates, even if the native state remains stable under the given experimental conditions. Conversely, the present data seem to suggest that the misfolded intermediates that appear after the pressure cycle do not significantly differ from the native state in their conformation, but their stability is slightly increased. It is not clear at present how such an effect could come about.

At closer inspection of the curves one can see that the pressure-treated protein shows a much broader transition. This can also indicate that the structure of the intermediate formed after the unfolding and refolding of HSA is more inhomogeneous. In the case of reversible transitions one can fit the transition using the van't Hoff equation to obtain the enthalpy change (ΔH). A broader transition in this theory corresponds to lower ΔH. In the present case, however, the unfolding process is not reversible and therefore an equilibrium thermodynamic analysis cannot be applied. In addition, it cannot be ruled out that in order to reach the intermediate state a high-energy barrier needs to be overcome. This would significantly affect the kinetics of the aggregation process, giving rise to a transition over a wider temperature range.

Since the pressure unfolding of the molecule is not a single cooperative process, presumably the refolding of the domains also happens both at different pressures and at different kinetic rates. The decompression in our experiment was considerably slower than in the classical (e.g. stopped flow) folding experiments, which means that the stable intermediate or misfolded state has a long lifetime and it is kinetically stable. The sequential folding of the domains can also cause a conformational drift similar to what was observed in case of oligomeric proteins (Ruan and Weber 1989) or mismatched docking that also can lead to stable misfolded structures.

Both the aggregates formed with and without pressure treatment, are stable upon cooling, which was also observed for many other proteins.

In summary, HSA is completely unfolded at 1 GPa pressure, but there is a molten globule formed at 0.4 GPa. Decompression results in the formation of an intermediate, which differs only slightly from the native state. This intermediate is more stable against the temperature-induced aggregation than the pressure untreated protein.

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