ORIGINAL PAPER

Dynamics of apomyoglobin in the α-to-β transition and of partially unfolded aggregated protein

E. Fabiani · A. M. Stadler · D. Madern · M. M. Koza · M. Tehei · M. Hirai · G. Zaccai

Abstract Changes of molecular dynamics in the α-to-β transition associated with amyloid fibril formation were explored on apomyoglobin (ApoMb) as a model system. Circular dichroism, neutron and X-ray scattering experiments were performed as a function of temperature on the protein, at different solvent conditions. A significant change in molecular dynamics was observed at the α-to-β transition at about 55°C, indicating a more *resilient* high temperature β structure phase. A similar effect at approximately the same temperature was observed in holomyoglobin, associated with partial unfolding and protein aggregation. A study in a wide temperature range between 20 and 360 K revealed that a dynamical transition at about 200 K for motions in the 50 ps time scale exists also for a hydrated powder of heat-denatured aggregated ApoMb.

Keywords Amyloid former · Protein dynamics · Neutron · Circular dichroism

E. Fabiani · A. M. Stadler · M. M. Koza · M. Tehei · G. Zaccai (✉)
Institut Laue-Langevin, BP156, Grenoble, France
e-mail: zaccai@ill.fr

E. Fabiani · D. Madern
Institut de Biologie Structurale, Grenoble, France

E. Fabiani
Université Joseph Fourier, Grenoble, France

A. M. Stadler
Research Centre Juelich, Juelich, Germany

M. Hirai
Gunma University, Maebashi, Japan

Present Address:
E. Fabiani (✉)
CEA LETI Minatec Département des Technologies pour la Biologie et la Santé, Grenoble Cedex 9, France
e-mail: elisa.fabiani@cea.fr

Present Address:
M. Tehei
School of Chemistry, University of Wollongong, Wollongong, NSW 2522, Australia

M. Tehei
Australian Institute of Nuclear Science and Engineering (AINSE), Menai, NSW, Australia

Introduction

Amyloidosis is an emerging category of diseases characterised by the extracellular accumulation of protein aggregates in body organs or tissues, including brain, liver, spinal cord and intestine. Even if the first cases of amyloidosis were described over 300 years ago, it is only within the past 20 years that the specific chemical composition and structure of amyloid protein formations have been understood. More than 20 different kinds of amyloidosis are known currently. They include Alzheimer's disease, Parkinson's disease, Huntington's disease and the "prion diseases" (Scrapie, Kuru, CJD, BSE, etc.). Therapeutic approaches have focussed on reducing the production of the protein, with different treatments for the different cases of amyloidosis.

Amyloid aggregation is closely related to protein folding issues. "Amyloid fibrils" consist of polymerised cross-β-sheet structures in which the beta-strands are arranged perpendicular to the long axis of the fibre. There are various causes for protein misfolding that could lead to amyloid formation. For example, in the absence of chaperones, certain proteins will fail to achieve their native state

and may associate with other unfolded polypeptide chains to form large amyloid fibrils. Misfolding can also occur when a protein is subjected to particular conditions, such as extremes of heat or pH. Understanding the physical bases of misfolding in these cases is of fundamental scientific and biotechnological importance. In the context it is important to characterise the forces that stabilise protein structure, and therefore its dynamics under relevant solvent conditions.

Apomyoglobin (ApoMb) was chosen for neutron, X-ray scattering and circular dichroism (CD) measurements because previous studies on the protein have shown that its fibril formation appears under particular conditions of temperature and pH (Fändrich et al. 2003). Before amyloid formation, ApoMb can adopt two well-defined structural conformations at pH 9: below 55°C, the *helix-rich native-like structure* α, and above 55°C, *the cross-β structure* characteristic of proteins that can generate amyloid deposits in humans. The transition arises because the helical structures in ApoMb are destabilised partially so that neighbouring strands will interact to form the cross-β structure between them. Amyloid structures differ from globular protein structures, which are always encoded in the amino acid sequence, because they do not depend on the presence of distinctive sequence patterns or specific intramolecular side-chain interactions. The structural aspects of the transition have been characterised by small and wide angle X-ray scattering (WAXS; Onai et al. 2007). The total structure factor observed showed two peaks at 0.58 and 1.34 Å^{-1}, which are strongly correlated with amyloid transformation (Onai et al. 2007). The peaks appear at pH 9 at 55°C, and become more pronounced as the temperature is raised. Further investigations of structure (Booth et al. 1997; Chamberlain et al. 2001) and folding dynamics (Takano et al. 2001; Canet et al. 1999, 2002) have led to a better understanding of the process involved in the conversion of globular proteins into amyloid fibrils.

The work reported concerns the measurement and comparison of the structure and dynamics, in a wide temperature range, of ApoMb under different solvent conditions (at pH 9 and pH 7, in H_2O and D_2O), by using CD to characterise secondary structures, and energy resolved elastic neutron scattering. As it has been demonstrated for other biological systems, energy resolved neutron scattering provides quantitative information on the forces involved in the stabilisation of protein structures (Zaccai 2000). The scattering cross-section of ^1H dominates that of all other atoms in biological material, and of its isotope, deuterium ^2H (D). Thus, heavy water (D_2O) is often used to reduce the contribution of hydration water to the scattering signal. CD experiments were performed in H_2O and D_2O, therefore, to characterise whether or not the α-to-β transition is affected by the solvent isotope.

A significant change in molecular dynamics towards a more resilient structure was observed for ApoMb at pH 9, at the α-to-β transition at about 55°C. A similar observation for holo-myoglobin (HoloMb) was attributed to partial unfolding and protein aggregation, indicating that denatured and aggregated protein display similar dynamics as β-amyloid. In a further examination of the dynamics of irreversibly heat-denatured and aggregated protein, a hydrated powder sample of ApoMb was shown to undergo a dynamical transition at about 200 K as has been observed for native HoloMb (Doster et al. 1989), purple membranes (Ferrand et al. 1993; Lehnert et al. 1998).

Materials and methods

ApoMb preparation

Apomyoglobin was obtained by removing the heme from HoloMb. Several protocols yield the apo-protein with similar properties. We successfully adapted the method of Rothgeb (Rothgeb and Gurd 1978) to obtain the apo-protein form in gram quantities for the neutron scattering experiments. The process was promoted by acid conditions in suitable solvent. An appropriate amount of lyophilised horse heart HoloMb purchased from SIGMA-ALDRICH was dissolved in water. The pH of the HoloMb solution was then lowered to 1.5 with concentrated HCl at 4°C. The acidified solution was extracted as quickly as possible with 4 volumes of 2-butanone. The upper organic layer was decanted, and the extraction was repeated at least twice more in order to obtain a hazy, colourless aqueous layer which was dialysed exhaustively against a dilute bicarbonate solution followed by pure water. After salt was completely removed by dialysis, the protein was lyophilised. For the neutron experiments, the powder was hydrated by pipetting uniformly buffer solutions to the sample to a level of 0.73 g H_2O/g protein or corresponding 0.8 g D_2O/g protein. This corresponds to approximately two hydration layers per protein and allows for pH effects. CD experiments were performed on the protein solution before lyophilisation.

Solvent conditions

For neutron experiments, the lyophilised ApoMb was rehydrated under different solvent conditions in H_2O and D_2O potassium phosphate buffer (20 mM KH_2PO_4, pH/pD 7 and pH/pD 9). Potassium phosphate was purchased from SIGMA.

All solutions were obtained by dissolving the buffer in distilled water (H_2O) or heavy water (D_2O). The pH (pD) was adjusted to the desired value by adding acid or base.

The pD was calculated by adding 0.4 to the value measured on a normally calibrated pH metre (Lide 1999). The same buffer solution was used to adjust the pH (pD) of the protein solution for the CD experiments.

Circular dichroism experiments

Circular dichroism measurements were used to check the α-to-β transition, the pH (pD) and temperature dependence of secondary structure. CD spectroscopy measures differences in the absorption of left-handed polarised light vs. right-handed polarised light, which arise due to structural asymmetry. The absence of regular structure results in 0 CD intensity, while an ordered structure results in a spectrum, which can contain both positive and negative signals. CD spectroscopy is particularly good for determining whether or not a protein is folded, and if so characterising its secondary structure. CD measurements were performed at the Institut de Biologie Structurale (IBS) and EMBL in Grenoble (France) by using a CD spectrometer equipped with a temperature-controlled cuvette holder. The far-UV CD spectral region (190–250 nm) was explored for different temperatures, pH and buffer conditions. The protein concentration was about 20 μM for all samples. The experiments were performed in the temperature range between 25 and 95°C. The data were analysed by subtracting the background CD spectrum and converting the spectra from millidegrees to molar ellipticity (θ) in degrees·cm^2 per decimole residues using the measured protein concentration. Secondary structure analysis of the final CD data was carried out using the K2D web program (Andrade et al. 1993; Merelo et al. 1994).

Neutron scattering experiments

Neutron scattering has proven to be a versatile tool for the study of the molecular dynamics of condensed matter in general (Bée 2000), and biological macromolecules and solvent interactions, in particular (Gabel et al. 2002; Paciaroni et al. 2002; Lehnert et al. 1998; Gabel and Bellissent-Funel 2007; Andreani et al. 1995; Pieper et al. 2004). The complexity of protein dynamics is reflected in a vast range of types of motion and corresponding associated time and length scales. Neutrons are sensitive in different ways to the motions of hydrogen (H) and its heavy isotope deuterium (D). Isotope labelling as well as the use of spectrometers with different energy resolution (corresponding to different time scales) considerably enriches the interpretation of scattering spectra. Elastic incoherent neutron scattering is a specific technique for the study of dynamical processes occurring on a time scale defined by the spectrometer resolution in energy. It provides atomic mean square displacement values (dominated by H atoms because of their large scattering cross-section compared to all the other atoms in biological samples) as a function of temperature, from which effective force constants can be obtained (Zaccai 2000).

The mean square displacement $<u^2>$ is derived from the analysis of the Elastic Area vs. Q^2 in a Gaussian approximation, where the Elastic Area is the area of the elastically scattered intensity and Q is the modulus of the scattering vector, $(4\pi \sin \theta/2)/\lambda$ (Gabel et al. 2002) where θ is the scattering angle and λ is the neutron wavelength.

$$<u^2> = -6\left(\frac{d \ln(\text{Elastic Area})}{d(Q^2)}\right) \qquad (1)$$

The approximation is valid for values of $<u^2> Q^2$ smaller than or close to about 2, and the Q ranges for the fit were chosen appropriately.

In cases for which the data quality is not sufficient to derive precise mean square displacement values, an analysis as a function of temperature of the total intensity sum in the same Q range, can give a good indication of a transition in dynamical behaviour (Reat et al. 2000).

At the Institut Laue-Langevin reactor neutron source

Energy resolved elastic neutron scattering experiments on ApoMb and HoloMb were carried out at the Institut Laue-Langevin in Grenoble (France) on the spectrometer IN13 (http://www.ill.eu/YellowBook/IN13/). IN13 is a back-scattering spectrometer characterised by a high-energy resolution (of about 8 μeV, corresponding to 0.1 ns time scale) associated to a wide range of momentum transfer (Q_{max} $\sim$4.7 Å^{-1}, corresponding to a length scale $\sim$Ångstrom unit). Free and hydration water motions are too fast to be observed in this time-length window for $q > 1$ Å^{-1} and contributes to the measured signal only as a negligibly low background. Measured data was analysed in the q^2-range from 1.5 to 5 Å^{-2}. In this high scattering vector range experiments in natural abundance water solvent are feasible with negligible H_2O contribution (Tehei et al. 2004; Jasnin et al. 2008). An amount of 100 and 120 mg of re-hydrated ApoMb and HoloMb powder, respectively (0.73 g H_2O/g protein) at pH 9 was inserted in a flat aluminium cell with internal spacing of 0.3 mm, which was put in the IN13 sample environment at an angle of 135° with respect to the incident beam. The experiments were performed as a function of temperature (heating between 280 and 350 K). An displex device specific to IN13 was used for temperature control. The data were analysed as outlined above.

At the ISIS spallation neutron source

Neutron scattering experiments on a heat-denatured and aggregated ApoMb hydrated powder sample were carried out on the IRIS spectrometer at the ISIS spallation neutron

source, Chilton, UK. The denatured sample was obtained by heating the hydrated powder in a closed sample holder at 360 K and allowing incubation for 4 h. By exploiting the Pyrolitic Graphite PG(002) analyser configuration and the Beryllium filter, the covered momentum transfer range was between 0.3 and 1.8 Å^{-1} with an energy resolution of 17 μeV (corresponding to a 50-ps time scale). The experiment was performed with D_2O solvent, since diffusion of H_2O would contribute strongly to the measured signal at the small scattering vector range covered by the instrument (Jasnin et al. 2008. An amount of 130 mg hydrated powder (0.8 g D_2O/g protein) at pH 9 in D_2O was held in a flat aluminium cell with internal spacing of 0.3 mm, placed at an angle of 135° with respect to the incident beam. We estimated that D_2O solvent contributes only with 2% to the total incoherent scattering cross-section. Therefore, it is justified to neglect the solvent contribution of D_2O. Each temperature point was collected for 4 h. The temperature scan was performed in a temperature range between 20 and 360 K in order to examine the mean square atomic displacements and effective force constants (Zaccai 2000). It was reported in literature that water in the secondary hydration shell of proteins can be vitrified during flash cooling to 100 K and crystallises during reheating at 200–210 K to cubic ice (Sartor et al. 1995). Even if there was a secondary hydration shell present in our sample, we did not observe ice formation during the experiment at low temperature as evidenced by the absence of ice Bragg peaks. We can only speculate about the reason, but the presence of buffer solution and not pure water might be an explanation. The temperature was controlled by heating the sample in a standard IRIS cryofurnace. First the sample was inserted at 280 K. Then it was heated up to 360 K to allow for heat denaturation. Afterwards the sample was cooled down to 20 K and the scattering at low temperatures was measured as a reference. In the following, the temperature scan was measured for different temperature points from 20 to 360 K. Heating and cooling rates were approximately in the order of 1–3 K min^{-1}. Mean square displacements were calculated from the analysis of the integrated intensity over a frequency window corresponding to instrumental resolution around the elastic peak, according to Eq. 1.

Wide angle X-ray scattering experiments

To check an effect of change of solvent (H_2O or D_2O) on α-to-β transition and amyloid transition of ApoMb, we have carried out WAXS measurement under the same solvent condition used before (Onai et al. 2007), where the D_2O solvent was 50 mM Tris–DCl (2-amino-2-hydroxy-methyl-1,3-propanediol hydrochloride) buffer at pD 9. The ApoMb concentration was 1% w/v. The WAXS measurement was performed by using the BL-40B2 spectrometer

installed at the synchrotron radiation source (SPring-8) at the Japan Synchrotron Radiation Research Institute (JAS-RI), Japan. The X-ray wavelength, the sample-to-detector distance and the exposure time were 0.82 Å, 41 cm and 30 s, respectively. The details of the scattering data analysis were as reported previously (Hirai et al. 2004).

Results and discussion

Circular dichroism

The far-UV CD spectra of ApoMb are presented in Fig. 1 for all temperatures and solvent conditions explored. At room temperature, all spectra show two negative peaks at 207 and 222 nm and a positive one at 193 nm, characteristic of the alpha-helix structure (black curves). ApoMb shows the predominance of the alpha-helix structure until 55°C. Above 55°C and only at pH and pD 9, the beta-sheet contribution appears and replaces the alpha-helix secondary structure. This is evident in Fig. 1b, d by the shift of the minimum from 207 nm to at around 200 nm during heating (Fändrich et al. 2003) and by the behaviour of the beta-sheet content from the K2D analysis of the spectra shown in Fig. 2. Note that the K2D data show similar behaviour in H_2O and D_2O at corresponding pH or pD values, respectively. Synchrotron radiation experiments also showed similar behaviour at the large angle scattering features (indicative of secondary structure) irrespective of whether the sample was in H_2O or D_2O as shown below.

In Fig. 1a, b, at pD (pH) 7 in D_2O and H_2O, the amplitudes of the two peaks are lowered with increasing temperature, consistent with the expected partial unfolding of the protein. Analysis of the spectra with the programme K2D (Fig. 2) yielded helix fractions of 70–80% and 40% at 20 and 70°C, respectively, while the beta fraction, which was negligible at room temperature increased to about 15% at high temperatures. A random fraction increased from 20–30% to 40–60% between 20 and 70°C. However, in Fig. 2 at pH (pD) 9 in H_2O and D_2O, the helix fraction decreases rapidly to 15–25% between 20 and 70°C and the beta fraction increases up to 20–30% at 70°C, which is in agreement with an expected higher beta-sheet content at basic solvent conditions and high temperatures.

Previous CD experiments on HoloMb had shown partial unfolding of the protein at 65°C, total unfolding at 84.5 ± 1.0°C (Fändrich et al. 2003), with no indication of an α-to-β transition at 55°C at pH 9.

X-ray scattering

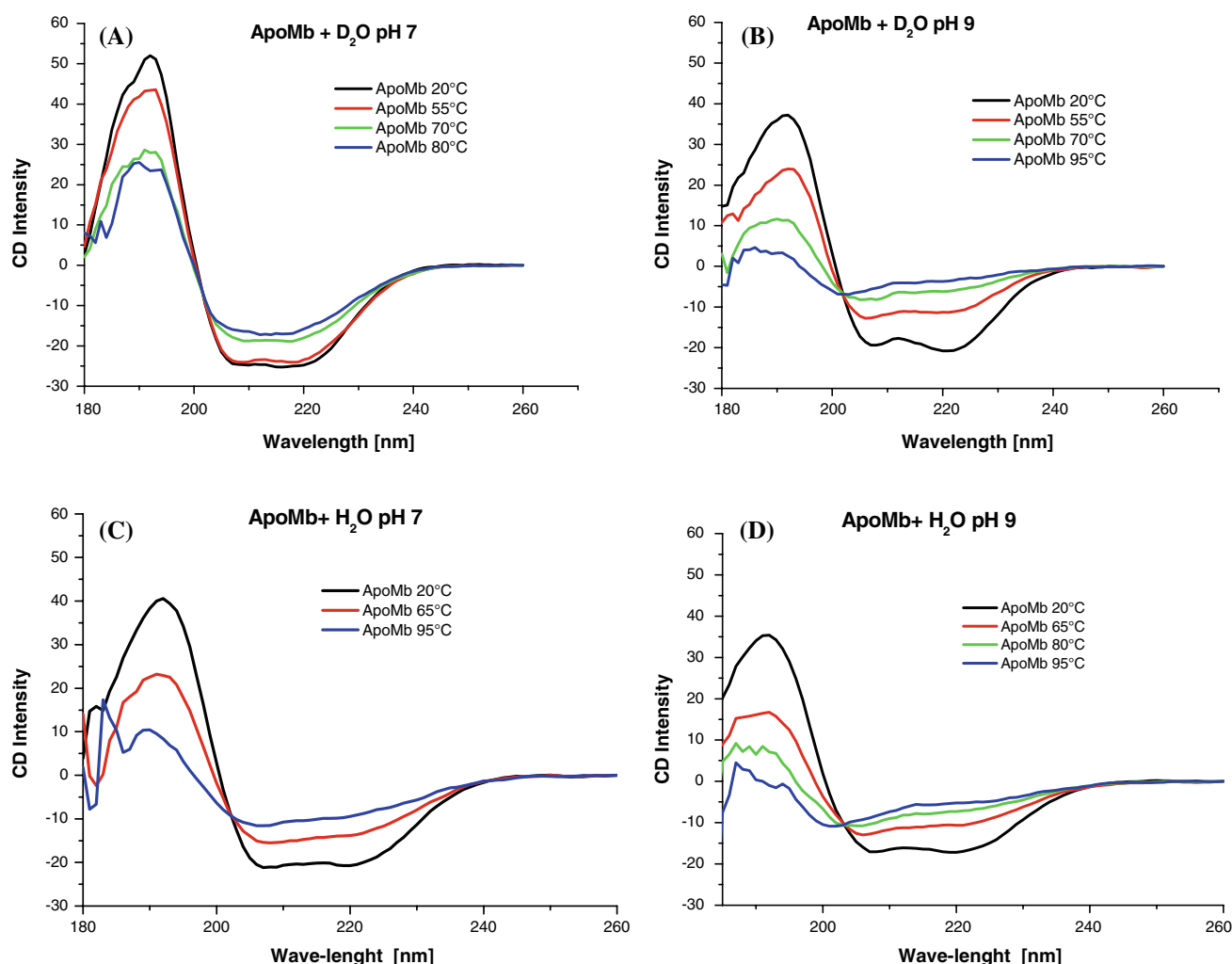Figure 3 shows the WAXS curve of ApoMb in D_2O buffer at pH 9 depending on temperature from 25 to 65°C. As

Fig. 1 Circular dichroism data for apomyoglobin **a** in D_2O at pH 7, **b** in D_2O at pH 9, **c** in H_2O at pH 7 and **d** in H_2O at pH 9 in the temperature range between 20 and 95°C

reported previously (Hirai et al. 2004), we can discuss about the details of the structural transition of ApoMb in its different hierarchical levels that are the quaternary and tertiary structures ($q < \sim 0.2 \text{ Å}^{-1}$), the inter-domain correlation and the intra-domain structure ($q = \sim 0.25$–0.8 Å^{-1}), and the secondary structure and the closely packed side chains in the hydrophobic cores ($q = \sim 1.1$–1.9 Å^{-1}), respectively. The arrows plotted in Fig. 3 indicate the typical features of the scattering curve of an amyloid formation. Namely, the peak at $q = \sim 1.35 \text{ Å}^{-1}$, the broad peak at $q = \sim 0.60 \text{ Å}^{-1}$ and the shoulder at $q = \sim 0.09 \text{ Å}^{-1}$ correspond to the α-to-β transition (cross-β structure), the pleated sheet stacking, and the oligomerisation of denatured ApoMb, respectively. These features appear simultaneously and are evidently seen above ~ 55°C. It should be mentioned that the positions of the peaks at $q = \sim 1.35$ and $\sim 0.60 \text{ Å}^{-1}$ are slightly larger than those observed in H_2O buffer (Onai et al. 2007). This suggests that in D_2O solvent the formed stacking of the

cross-β is more compact as compared with that in H_2O solvent.

Neutron scattering

The total intensity analysis of the IN13 data at pH 9 is shown in Fig. 4. The curves for ApoMb and HoloMb both show a "transition" at about 55°C indicative of a more resilient (smaller rate of decrease with temperature) structure at the higher temperature. The CD data showed that in the case of ApoMb, the effect is correlated with the α-to-β transition. In HoloMb, the more resilient state seems to be associated with partial unfolding of the protein and aggregation in accordance with Fändrich et al. (2003). We present only summed elastic intensities as these are more precise to identify dynamical transitions (Reat et al. 2000). Calculation of mean square displacements gave errors bars that were to big to make any conclusion about a dynamical transition around 55°C.
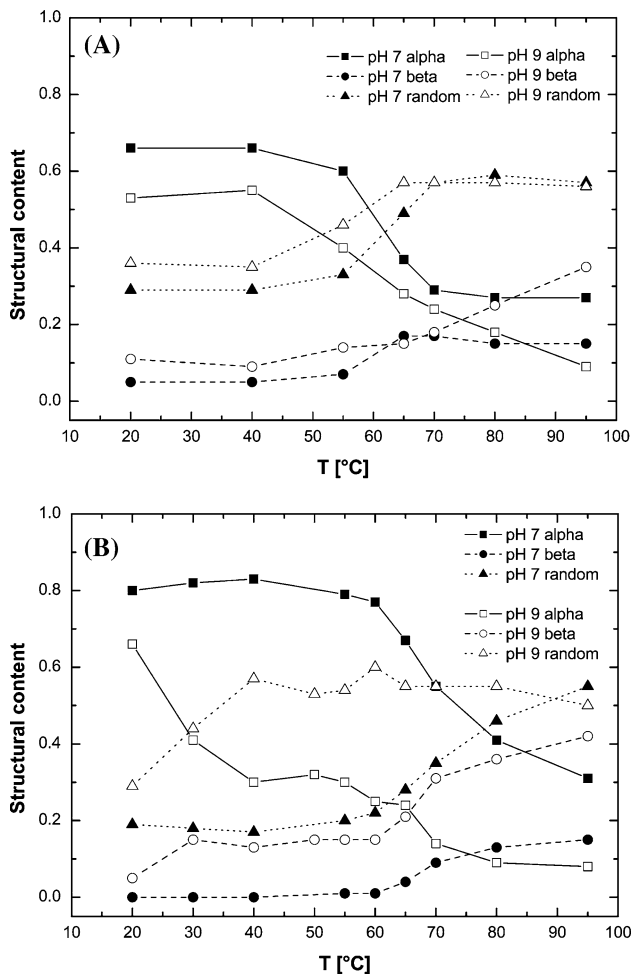
Fig. 2 Analysis of the structural content of ApoMb at pH 7 and pH 9 in **a** H$_2$O and **b** D$_2$O

Fig. 3 Wide angle X-ray scattering curve of apomyoglobin in D$_2$O solvent at pH 9 in the temperature range from 25 to 65°C. The *arrows* (the peaks at $q = \sim 1.35$ and ~ 0.60 Å^{-1}, the shoulder at $q = \sim 0.09$ Å^{-1}) indicate typical features of amyloid transition. This transition occurred above ~ 55°C

As the temperature is increased and before total unfolding at higher temperatures, both ApoMb and HoloMb display a transition to a more resilient aggregated dynamic structure at about 55°C. In the case of ApoMb at pH 9, the structure is the β-structure associated with amyloid fibre formation. While in the case of HoloMb, the structure is likely due to partial unfolding and an undefined aggregate state (Fändrich et al. 2003). The transition to a more resilient high temperature dynamics structure at ~ 55°C was also observed in IN13 experiments on ApoMb at pH 7 (data not shown), suggesting that like for HoloMb, ApoMb at pH 7 partially unfolds and aggregates in this pre-transition to total unfolding. In order to explore further the dynamic behaviour of aggregated protein, a temperature scan of the elastic intensity was performed on heat-denatured ApoMb on the IRIS spectrometer at ISIS (Fig. 5). The 17 μeV energy resolution condition chosen on the instrument corresponds to a time scale of 50 ps. Interestingly, the data showed a dynamical transition for the sample at about 200 K, similar to that observed in hydrated
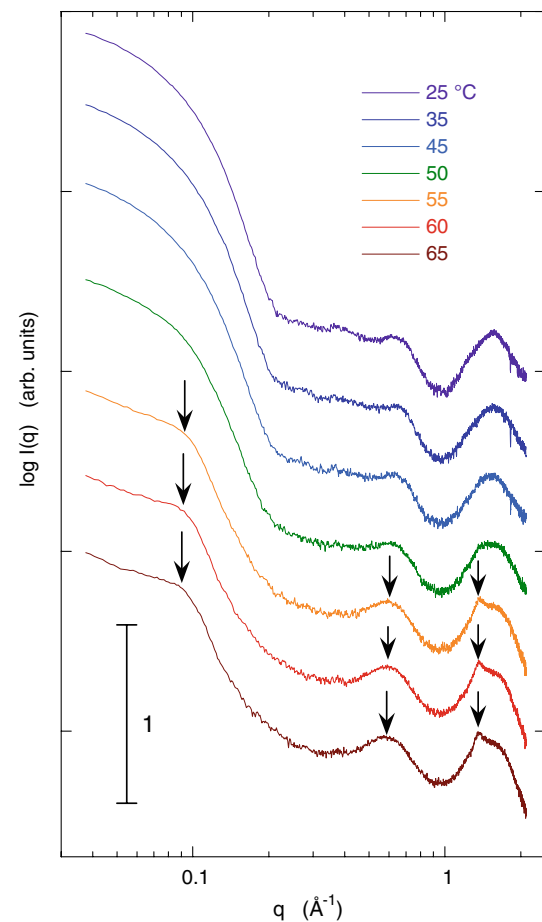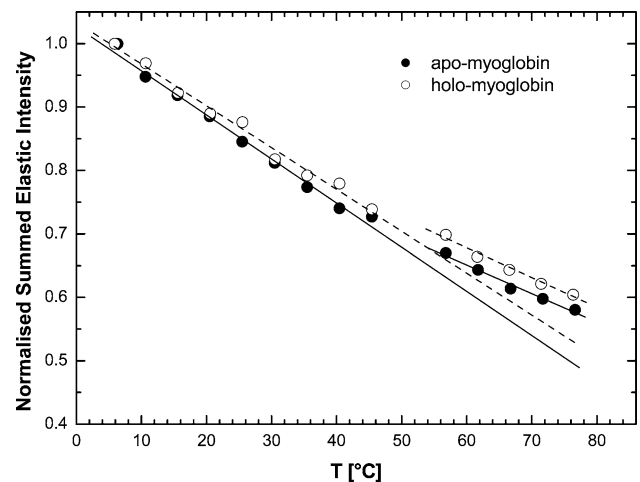

Fig. 4 IN13 sum of intensities vs. temperature for apomyoglobin and holo-myoglobin. The *broken line* is a linear fit for holo-myoglobin and the straight line is a linear fit to apomyoglobin. Due to limitations of the temperature control, no experimental points could be measured between around 45 and 55°C
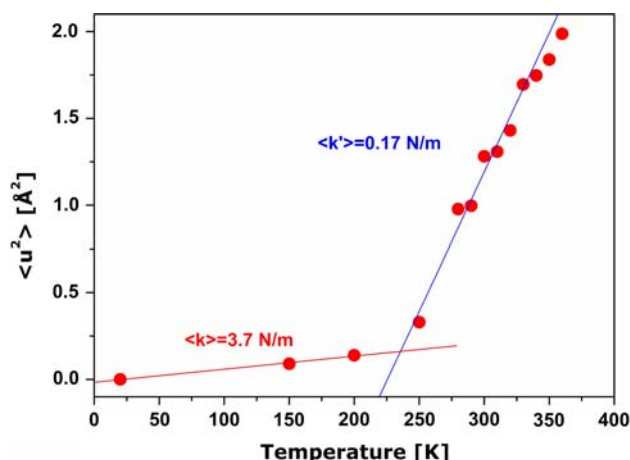
Fig. 5 Mean square displacement $<u^2>$ vs. temperature from IRIS elastic scan measurements on heat-denatured and aggregated ApoMb. *Error bars* are within the *symbols*

native HoloMb (0.38 g D_2O/g protein) by Doster et al. (1989), indicating that the transition is also a property of heat-denatured and aggregated protein. The force constant $<k>$ and the effective force constant $<k'>$ were determined for the low temperature range below 200 K with $<k> = 3.7 \pm 0.3$ N m^{-1} and above 200 K with $<k'> = 0.17 \pm 0.01$ N m^{-1} (Zaccai 2000). In the 100 ps time range values of $<k> = 3$ N m^{-1} and $<k'> = 0.3$ N m^{-1} were obtained for hydrated HoloMb powder (Zaccai 2000). At low temperatures the force constants $<k>$ between ApoMb and HoloMb are similar. Above the dynamical transition temperature, the effective force constant $<k'>$ of ApoMb is significantly smaller than that of HoloMb. The higher degree of hydration of the ApoMb sample might facilitate protein motion and be the cause of this discrepancy.

Conclusion

In this paper, ApoMb was used as a model biochemical system in order to explore the molecular changes of dynamics in the α-to-β transition associated with amyloid fibril formation. CD and neutron scattering experiments were performed on the protein, at pH 9 in H_2O and D_2O, in a wide temperature range. Similar experiments were carried out on HoloMb, on the same solvent condition. WAXS was used to confirm the occurrence of the α-to-β transition of ApoMb in D_2O solution at pD 9 by the appearance of characteristic Bragg peaks. CD experiments emphasised that the transition is present above 55°C and only at pH and pD 9 in dilute solution. ApoMb results show a significant change in molecular dynamics at the α-to-β transition at about 55°C, indicating a more *resilient* high temperature β structure phase. The behaviour of the holo-protein as function of temperature is associated with partial unfolding and protein aggregation.

References

Andrade MA, Chacón P, Merelo JJ, Morán F (1993) Evaluation of secondary structure of proteins from UV circular dichroism using an unsupervised learning neural network. Protein Eng 6:383–390

Andreani C, Filabozzi A, Menzinger F, Desideri A, Deriu A, Dicola D (1995) Dynamics of hydrogen atoms in superoxide dismutase by quasielastic neutron scattering. Biophys J 68:2519–2523

Bée M (2000) La diffusion quasiélastique des neutrons; introduction et principes généraux. Diffusion Quasiélastique des Neutrons. J Phys IV France 10:Pr1-1–Pr1-14

Booth DR, Sunde M, Bellotti V, Robinson CV, Hutchinson WL, Fraser PE, Hawkins PN, Dobson CM, Radford SE, Blake CCF, Pepys MB (1997) Instability, unfolding and aggregation of human lysozyme variants underlying amyloid fibrillogenesis. Nature 385:787–793

Canet D, Sunde M, Last AM, Miranker A, Spencer A, Robinson CV, Dobson CM (1999) Mechanistic studies of the folding of human lysozyme and the origin of amyloidogenic behaviour in its disease related variants. Biochemistry 38:6419–6427

Canet D, Last AM, Tito P, Sunde M, Spencer A, Archer DB, Redfield C, Robinson CV, Dobson CM (2002) Local cooperativity in the unfolding of an amyloidogenic variant of human lysozyme. Nat Struct Biol 9:308–315

Chamberlain AK, Receveur V, Spencer A, Redfield C, Dobson CM (2001) Characterization of the structure and dynamics of amyloidogenic variants of human lysozyme by NMR spectroscopy. Protein Sci 10:2525–2530

Doster W, Cusack S, Petry W (1989) Dynamical transition of myoglobin revealed by inelastic neutron scattering. Nature 337:754–756

Fändrich M, Forge V, Buder K, Kittler M, ChM Dobson, Diekmann S (2003) Myoglobin forms amyloid fibrils by association of unfolded polypeptide segments. Proc Natl Acad Sci USA 100:15463–15468

Ferrand M, Dianoux AJ, Petry W, Zaccai G (1993) Thermal motions and function of bacteriorhodopsin in purple membranes: effects of temperature and hydration studied by neutron scattering. Proc Natl Acad Sci USA 90(20):9668–9672

Gabel F, Bellissent-Funel MC (2007) C-phycocyanin hydration water dynamics in the presence of trehalose: an incoherent elastic neutron scattering study at different energy resolutions. Biophys J 92:4054–4063

Gabel F, Bicout D, Lehnert U, Tehei M, Weik M, Zaccai G (2002) Protein dynamics studied by neutron scattering. Q Rev Biophys 35:327–367

Hirai M, Koizumi M, Hayakawa T, Takahashi H, Abe S, Hirai H, Miura K, Inoue K (2004) Hierarchical map of protein unfolding

and refolding at thermal equilibrium revealed by wide-angle X-ray scattering. Biochemistry 43:9036–9049

Jasnin M, Moulin M, Haertlein M, Zaccai M, Tehei M (2008) Down to atomic-scale intracellular water dynamics. EMBO Rep 9:543–547

Lehnert U, Reat V, Weik M, Zaccai G, Pfister C (1998) Thermal motions in bacteriorhodopsin at different hydration levels studied by neutron scattering: correlation with kinetics and light-induced conformational changes. Biophys J 75:1945–1952

Lide DR (1999) CRC Handbook of chemistry and physics, 80th edn edn. CRC, Boca Raton

Merelo JJ, Andrade MA, Prieto A, Morán F (1994) Proteinotopic feature maps. Neurocomputing 6:443–454

Onai T, Koizumi M, Lu H, Inoue K, Hirai M (2007) Initial process of amyloid formation of apomyoglobin and effect of glycosphingolipid G_{M1}. J Appl Cryst 40:s184–s189

Paciaroni A, Cinelli S, Onori G (2002) Effect of the environment on the protein dynamical transition: a neutron scattering study. Biophys J 83:1157–1164

Pieper J, Irrgang KD, Renger G, Lechner RE (2004) Density of vibrational states of the light-harvesting complex II of green plants studied by inelastic neutron scattering. J Phys Chem B 108:10556–10565

Reat V, Dunn R, Ferrand M, Finney JL, Daniel RM, Smith JC (2000) Solvent dependence of dynamic transitions in protein solutions. Proc Natl Acad Sci USA 97:9961–9966

Rothgeb TM, Gurd FR (1978) Physical methods for the study of myoglobin. Methods Enzymol 52:73–86

Sartor G, Hallbrucker A, Mayer E (1995) Characterizing the secondary hydration shell on hydrated myoglobin, hemoglobin, and lysozyme powders by its vitrification behavior on cooling and its calorimetric glass → liquid transition and crystallization behavior on reheating. Biophys J 69:2679–2694

Takano K, Funahashi J, Yutani K (2001) The stability and folding process of amyloidogenic mutant human lysozymes. Eur J Biochem 268(1):155–159

Tehei M, Franzetti B, Madern D, Ginzburg M, Ginzburg BZ, Giudici-Orticoni MT, Bruschi M, Zaccai G (2004) Adaptation to extreme environments: macromolecular dynamics in bacteria compared *in vivo* by neutron scattering. EMBO Rep 5:66–70

Zaccai G (2000) How soft is a protein? A protein dynamics force constant measured by neutron scattering. Science 288:1604–1607