Pressure Effect on the Temperature-Induced Unfolding and Tendency To Aggregate of Myoglobin†

L. Smeller,*,‡ P. Rubens,§ and K. Heremans§

Institute of Biophysics, Semmelweis University of Medicine, Budapest, Puskin u. 9 PF 263, H-1444 Hungary, and Department of Chemistry, Katholieke Universiteit Leuven, Celestijnenlaan 200D, B-3001 Leuven, Belgium

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ABSTRACT: This work demonstrates that pressure-induced partially unfolded states play a very important role in the aggregation of proteins. The high-pressure unfolding of horse heart metmyoglobin results in an intermediate form that shows a strong tendency to aggregate after pressure release. These aggregates are similar to those that are usually observed upon temperature denaturation. Infrared spectra in the amide I region indicate the formation of an intermolecular antiparallel β-sheet stabilized by hydrogen bonding. The formation of the aggregates is temperature-dependent. Below 30 °C, no aggregation is taking place as seen from the infrared spectra. At 45 and 60 °C, two types of aggregates are formed: one that can be dissociated by moderate pressures and one that is pressure-insensitive. When precompressed at 5 °C, temperature-induced aggregation takes place at lower temperature (38 °C) than without pressure pretreatment (74 °C).

Protein folding is a very active field of research. In many in vitro studies the formation of aggregates during the folding process is usually considered as a undesirable side effect which obscures the folding process as such. However, because of its important role in a number of diseases, the mechanism of the formation of aggregates, and the possible role of folding intermediates, deserves closer attention (*1*, *2*). Recent studies on lysozyme mutants have indicated that studies on model systems may help in obtaining a better insight into the molecular mechanisms underlying the formation of aggregates (*3*). The general picture that is emerging from a number of studies is that under partial denaturation conditions proteins may acquire conformational intermediates that have a strong tendency to aggregate (*4*).

The effect of pressure on protein−protein interactions is well documented (*5*). At low pressures, multimeric proteins dissociate reversibly into subunits. It is assumed that the free volumes at the interface of the subunits disappear at high pressure and are filled by the solvent. Under these conditions, pressure is used as a tool to study the assembly of viruses and macromolecular assemblages (*6*).

It has been known for a long time that the native structure of a protein can be unfolded not only by temperature but also by high pressure (*7*, *8*). The pressures needed for the unfolding are usually higher than those needed for the dissociation of protein−protein interactions. At high concentrations, the unfolding may lead to nonspecific interactions which gives rise to the formation of gels (*9*).

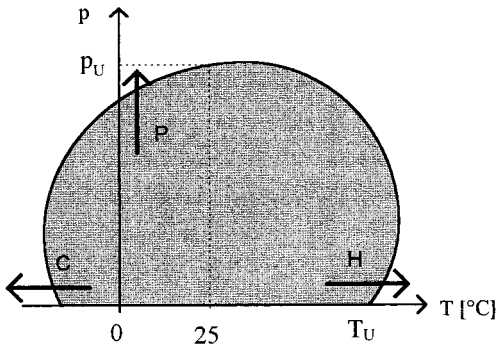

FIGURE 1: Schematic representation of the elliptic phase diagram for the stability of proteins. The protein is in the folded state in the shaded area. The arrows indicate the heat (H), pressure (P), and cold (C) unfolding. For horse heart metmyoglobin, the unfolding temperature (T_U) at ambient pressure is 74 °C. The pressure of unfolding (p_U) is 6 kbar at room temperature.

The structural differences between the pressure and the temperature unfolded proteins have not been clarified yet. The question of the protein unfolding became even more fascinating with the recently discovered low-temperature-induced unfolding (*10*, *11*). The reason the three effects can lead to unfolding of proteins can be explained by the elliptic phase diagram, which gives a description of the stability conditions in solution (*12*−*15*). In Figure 1 the protein is more stable in its native form inside the elliptic boundary (shaded area), while elsewhere the unfolded form is thermodynamically more stable. The phase diagram, however, does not tell anything about the mechanism of the unfolding and the aggregation of the unfolded state. Nor does it give information on the possible role of intermediates in the unfolding which have been observed in a number of temperature (*16*) and high-pressure studies (*17*, *18*). Such a phase diagram, based on a two-state model, suggests that

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* To whom correspondence should be addressed. Phone: +36-1-2662755/4033. Fax: +36-1-2666656. E-mail: smeller@puskin.sote.hu.

‡ Semmelweis University of Medicine.

§ Katholieke Universiteit Leuven.

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the heat, pressure, and cold unfolded states of the protein do not differ from each other qualitatively. This, however, is not supported by the recent NMR[1] data for ribonuclease A, which indicate different structural changes for heat, cold, and pressure unfolding (*19*).

The effect of pressure on protein−protein interactions has been studied with light scattering or turbidity methods (*20*, *21*). These methods give information on the degree of association and aggregation. Information on conformational changes or on the nature of the intermolecular interactions may be expected from spectroscopic studies. Differences between the structural changes induced by temperature and pressure can be most conveniently studied with Fourier transform infrared spectroscopy (FTIR). The infrared spectra of most heat unfolded proteins show two specific bands for the aggregated protein in the amide *I′* band range at 1615 and 1685 cm^{-1}. Notable exceptions to this rule are lysozyme (*3*) and ribonuclease T1 (*22*). These aggregation specific bands are not observed in the case of the pressure unfolded protein (*23*−*25*). These bands have been assigned to an intermolecular antiparallel β-sheet structure stabilized by hydrogen bonding (*26*−*28*). Such a structure should not be confused with the classical antiparallel intramolecular β-sheet secondary structure for which bands are expected in the region between 1630 and 1640 cm^{-1} (*29*). In the present case, the hydrogen bonds stabilize the intermolecular network of the heat unfolded protein. The formation of the network after heat unfolding is an irreversible process, because the specific bands remain visible even after the cooling of the protein to room temperature. The FTIR study of many proteins leads to the conclusion that pressure unfolding can be reversible, while temperature unfolding is almost always irreversible (*30*).

The pressure-induced denaturation of myoglobin has been studied with absorption spectroscopy (*14*) and with electrophoresis (*31*). The protein was found to form aggregates at neutral pH. In this paper we report on the effect of pressure on the unfolding of metmyoglobin at various temperatures. The aim is to investigate the possible role of pressure effects on the tendency of aggregation of the protein and to investigate in more detail the mechanism of aggregation.

EXPERIMENTAL PROCEDURES

Materials. Myoglobin from horse heart was purchased from Sigma and was dissolved in 10 mM Tris at pD 7.6 in the solvent D_2O using a protein concentration of 75 mg/mL. Experiments performed at lower (40 mg/mL) and higher (150 mg/mL) protein concentrations gave essentially the same results. The solution was left at room temperature overnight to allow the H−D exchange of the amide group protons.

Infrared Spectroscopy. Infrared spectra were recorded by a Bruker IFS66 FTIR spectrometer. Spectral resolution enhancement was applied to emphasize the spectral features of the overlapping amide *I* band. The resolution enhancement factor reached was approximately 1.5. The details of this procedure are given elsewhere (*32*). High pressure was generated in a diamond anvil cell (DAC) (Diacell Products, Leicester, U.K.), and the temperature was measured by a thermocouple. The heat unfolding at atmospheric pressure was measured in a CaF_2 cell, using a Graseby-Specac automatic temperature controller.

[1] Abbreviations: NMR, nuclear magnetic resonance; FTIR, Fourier transform infrared spectroscopy.

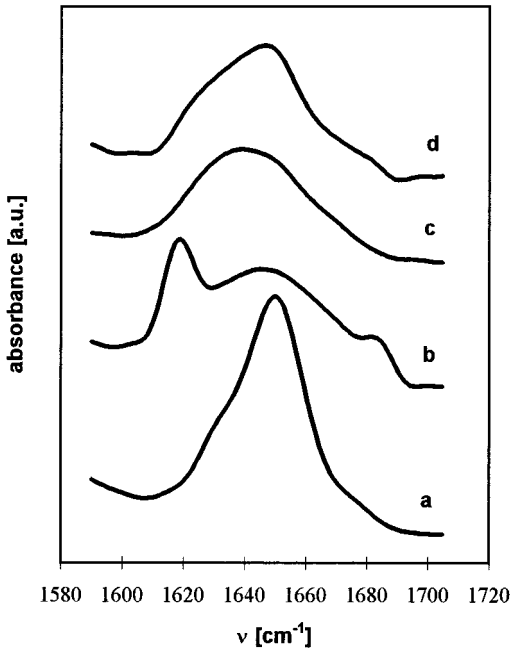


FIGURE 2: Amide I region of the infrared spectra of horse heart myoglobin. (a) Native (30 °C, 1 bar); (b) heat unfolded (90 °C, 1 bar); (c) pressure unfolded (27 °C, 10 kbar); (d) spectrum at 27 °C and 1 bar after a pressure treatment of 10 kbar.

RESULTS

Pressure and Temperature Unfolding. Figure 2 shows the amide *I* region of the FTIR spectra of native, pressure unfolded, and heat unfolded myoglobin. The dominant peak at 1650 cm^{-1} of the native protein is characteristic for the helical secondary structure (*33*), which is known to be the major structural component of myoglobin (*34*). The pressure and the heat unfolded proteins show a broad featureless spectrum, which is characteristic for the unordered, random structure. The bands specific for intermolecular hydrogen bonded structure can clearly be seen at 1616 and 1685 cm^{-1} in the case of the heat unfolded protein. When the protein is unfolded by high pressure at 27 °C, such bands are not observed.

Pressure Effect on the Aggregation. To investigate the effect of temperature on the pressure-induced unfolding, experiments were performed at 45 and 60 °C. At these conditions, the protein does not unfold at atmospheric pressure. The unfolding temperature at 1 bar is 71.2 °C, and the transition region is ca. 15 °C wide as obtained from the frequency shift of the maximum of the amide *I* band (data not shown). Figure 3 shows the most characteristic infrared spectra recorded at 45 °C as a function of pressure. The first pressurization up to 12 kbar denatures the protein; the spectrum is typical for the random structure. Unexpected results were obtained after releasing the pressure. The appearance of bands at 1616 and 1685 cm^{-1} indicates the formation of protein aggregates stabilized by intermolecular hydrogen bonding. As has been emphasized before, the simultaneous occurrence of these bands has been repeatedly assigned to an *inter*molecular antiparallel β-sheet structure

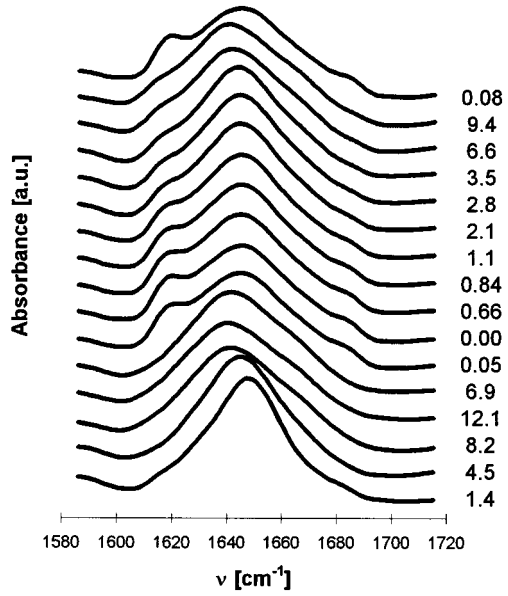


FIGURE 3: Infrared spectra of myoglobin from horse heart at 45 °C. Pressures are given in kbar. Spectra were recorded in chronological order from bottom to top.

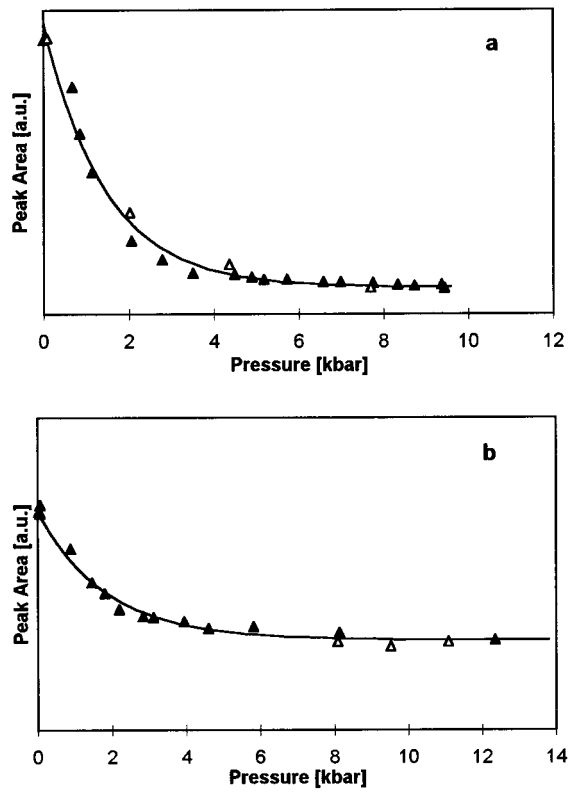


FIGURE 4: Area of the 1616 cm^{-1} band versus pressure during the second pressurizing cycle. (a) At 45 °C; (b) at 60 °C. Filled symbols indicate pressure increase, open symbols pressure decrease.

(*26*−*29*). It is remarkable that these aggregates do not form *at high pressure*, but only *after* the release of the pressure. These effects were not observed at lower temperatures (5 and 27 °C). The second pressurizing cycle again shows interesting results. Application of pressure to the slightly aggregated protein destabilizes the intermolecular hydrogen bonds, which is reflected in the weakening of the specific bands in the amide *I* region. As can be seen in Figure 4, the pressure for the dissociation of the aggregate is considerably less (2−3 kbar) than for the unfolding (6−7 kbar). A similar

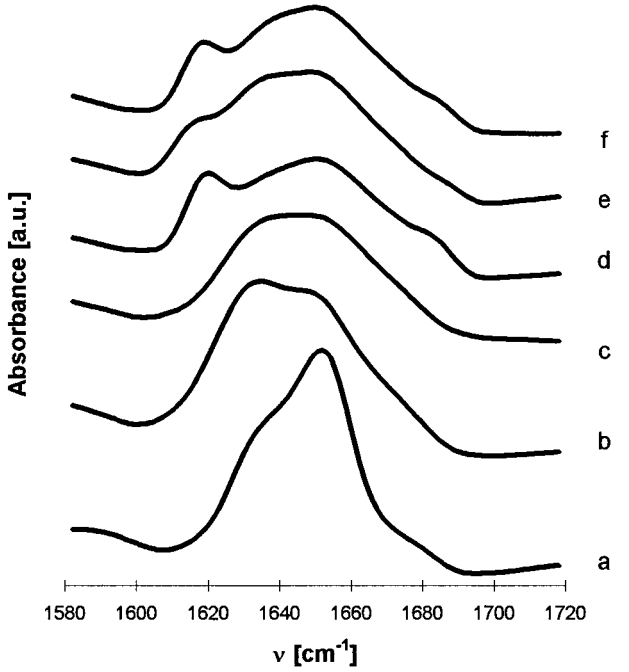


FIGURE 5: High-pressure, high-temperature treatment of horse heart metmyoglobin starting from ambient pressure at 30 °C (a). (b) Spectrum at 10 kbar and 30 °C; (c) 12 kbar and 90 °C; (d) 90 °C and ambient pressure; (e) second pressure cycle at 10 kbar and 90 °C; (f) depressurization at 90 °C.

pressure cycle was performed at 60 °C. As shown in Figure 4, the results are qualitatively the same as those observed at 45 °C.

Temperature−*Pressure Cycle.* At higher temperatures, it was not possible to use the same experimental procedure as used at 45 and 60 °C because the protein is already strongly aggregated at 90 °C at ambient pressure. This can be seen from the presence of bands due to strong intermolecular hydrogen bonding in Figure 2b. Therefore, the following pressure−temperature cycle was performed starting from ambient conditions as shown in Figure 5a. The protein was first pressure-unfolded by 10 kbar at 30 °C, showing an increase in β-structure as seen in Figure 5b. The pressure unfolded protein was then heated to 90 °C under pressure. At 12 kbar and 90 °C, no specific bands due to possible aggregation were observed as is seen in Figure 5c. The release of the pressure to near the atmospheric value resulted in the appearance of the specific bands (Figure 5d). The bands disappear by a second pressure increase at 90 °C (Figure 5e). After depressurization, the specific bands became stronger again (Figure 5f), and the spectrum after cooling to room temperature (not shown) is similar to the spectrum in Figure 5f.

Temperature Effect on the Intermediate. To test the possible role of pressure in the formation of intermediates, the following experiment was performed. The sample was pressurized at 5 °C and 10 kbar. After releasing the pressure, the protein was heated at low pressure (<0.5 kbar). Figure 6 shows the normalized intensity of the 1616 cm^{-1} band as a function of the temperature for the pressure unfolded and native protein, respectively. The spectrum of the protein pretreated by pressure is very similar to the spectrum of the intermediate as given in Figure 2d. In the case of pressure unfolded protein, the specific sidebands characteristic for the aggregation appear already at 38 °C which is lower than the

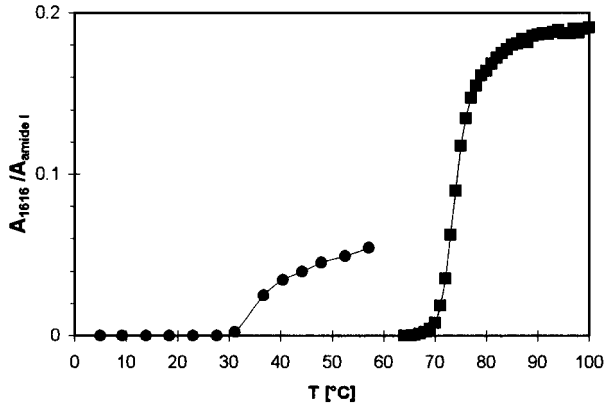
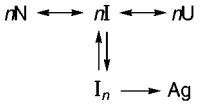


FIGURE 6: Ratio of the area of the 1616 cm^{-1} band, normalized for the area of the amide I band, as a function of temperature. (●) After pressure unfolding of the protein at 10 kbar and 5 °C; (■) without pressure treatment.

Scheme 1



thermal unfolding temperature of the native protein. A further increase of the temperature results in full development of the intensity of the 1616 cm^{-1} band at 74 °C (not shown). Without pressure treatment, the specific aggregation bands appear only at 74 °C as shown in Figure 6. The fact that only a fraction (ca. 30%) of the pressure unfolded protein starts to develop the specific sidebands below the normal transition can be explained by the assumption that the pressure effect at 5 °C is partially reversible.

DISCUSSION

The present study shows that pressure induces a conformation in horse heart metmyoglobin that gives rise to the formation of aggregates after the pressure is released. These aggregates show infrared bands specific for intermolecular antiparallel β-structure that differs clearly from intramolecular β-structures. The results can be interpreted by Scheme 1.

According to this scheme, high pressure unfolds the native protein (N) into a state (I) which may lead to the unfolded state at higher pressures (U). The intermediate (I) has a strong tendency to form aggregates (I_n) that can be dissociated by pressure. However, the aggregates convert slowly into a network which is not pressure-sensitive (Ag). This can be inferred from the observation that the concentration of the pressure-sensitive form (I_n) becomes smaller in time. Also the amount of pressure-stable aggregates is higher at 60 °C than at 45 °C as may be seen from the change in amplitude of the pressure-sensitive aggregates in Figure 4. At 90 °C, the development of the aggregates is too fast to be studied at ambient pressure so that a different experimental approach was used (see Figure 5). At this temperature, it is still possible to dissociate the aggregates with pressure.

The difference in conformation between (N), (I), and (U) can be seen from the amide I region of the spectra shown in Figure 2. The spectrum of the native state (Figure 2a) shows the predominance of the helix content. Figure 2c gives the spectrum of the unfolded state (U) at 12 kbar. This is a spectrum typical for an unordered state. Figure 2d shows the spectrum after decompression to 1 bar at 27 °C. It can be seen that the band is less broad (42 cm^{-1}) than in the pressure unfolded state (U) (46 cm^{-1}). The frequency of the maximum of the band is close to that of the native state, suggesting a conformation intermediate between the native and the completely unfolded state. The symbol I is used for this state.

When the protein aggregates from (*n*I) to (I_n) there are no major changes in the amide I band except for the appearance of the sidebands. This suggests that it is the intermediate (I) and not the unfolded state (U) that gives rise to the aggregates. This is also supported by the observations of the effect of temperature on the protein unfolded by pressure at low temperature. The results presented in Figure 6 show that the intermediate starts to unfold at a lower temperature than the native protein. The spectral differences between (I_n) and (Ag) are also minor as can be seen from the spectra of Figure 3 from 0.05 to 3.5 kbar. In all cases, the infrared spectra suggest a conformation for (I) that is between the native state and the completely unfolded state. In the absence of major spectral differences between (I_n) and (Ag), we suggest the following possible differences between these two states: The interaction between the units may be stronger due to an increased number of intermolecular hydrogen bonds. This stronger interaction may need higher pressure for dissociation. Small differences in conformation may exist that are not detectable with the infrared technique.

The present observations on the partial reversibility of the association should be contrasted with the reversible pressure effects that are observed in most cases of protein association (5, *20*, *21*). In these cases it is assumed that no changes in conformation take place so that the association is considered as a specific interaction between proteins. For the present case, nonspecific interactions are assumed which make the packing at the interface less efficient so that larger volume changes are expected (*20*). It has been shown, however, that the formation of inclusion bodies may result from the specific aggregation of partially folded proteins (*35*, *36*). The infrared spectra of inclusion bodies (*36*) show similarities with the spectra of the aggregates observed in the present work. Specificity was also observed in the assembly of immunoglobulin light chain deposition disease and amyloidosis (*37*).

The volume changes for the pressure dissociation of the aggregates can be calculated from the changes of the intensities of infrared bands with pressure from a simple two-state model provided the number of interacting protein units is known. Since the latter quantity is unknown for the present case, only qualitative statements can be made. The area of the 1616 cm^{-1} band falls off with pressure at a rate independent of the protein concentration between 40 and 150 mg/mL. This suggests that the system shows similarities with large protein aggregates such as erythrocruorin (*38*) and the chaperonin GroEL (*39*) where the pressure-induced dissociation is entirely concentration-independent. This has been interpreted as a heterogeneity of the free energy of association of the subunits in the assembled particle (*40*). For the present system, this implies that the interprotein contacts are not identical but show differences that are related to small changes in conformation of the individual units. These differences cannot be characterized from the infrared spectra that show an average of the protein population.

Previous studies on the formation of protein aggregates support the hypothesis that partially folded intermediates are key precursors in the process of aggregation (1−4, *20*). The infrared spectra support this hypothesis. It remains to be seen whether the present experiments on horse heart myoglobin, which is mainly composed of helices, can be extended to proteins with other composition in the secondary structure.

CONCLUSION

The pressure-induced unfolding of myoglobin forms two different states of aggregation: one that can be dissociated by high pressure, and another that is insensitive to pressure. The infrared spectra suggest that for the formation of the aggregates a partial unfolding is necessary either by pressure or by temperature. The aggregates are stabilized by intermolecular antiparallel β-structures stabilized by hydrogen bonds. Our observations suggest that mild pressures could be used to prevent protein aggregation under in vivo folding conditions.

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