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Comparative Fourier Transform Infrared Spectroscopy Study of Cold-, Pressure-, and Heat-Induced Unfolding and Aggregation of Myoglobin

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ABSTRACT We studied the cold unfolding of myoglobin with Fourier transform infrared spectroscopy and compared it with pressure and heat unfolding. Because protein aggregation is a phenomenon with medical as well as biotechnological implications, we were interested in both the structural changes as well as the aggregation behavior of the respective unfolded states. The cold- and pressure-induced unfolding both yield a partially unfolded state characterized by a persistent amount of secondary structure, in which a stable core of G and H helices is preserved. In this respect the cold- and pressure-unfolded states show a resemblance with an early folding intermediate of myoglobin. In contrast, the heat unfolding results in the formation of the infrared bands typical of intermolecular antiparallel β-sheet aggregation. This implies a transformation of α-helix into intermolecular β-sheet. H/^{2}H-exchange data suggest that the helices are first unfolded and then form intermolecular β-sheets. The pressure and cold unfolded states do not give rise to the intermolecular aggregation bands that are typical for the infrared spectra of many heat-unfolded proteins. This suggests that the pathways of the cold and pressure unfolding are substantially different from that of the heat unfolding. After return to ambient conditions the cold- or pressure-treated proteins adopt a partially refolded conformation. This aggregates at a lower temperature (32°C) than the native state (74°C).

INTRODUCTION

Cold unfolding was predicted for a number of proteins on the basis of the temperature dependence of the unfolding enthalpy (Brandts, 1969). A thermodynamic analysis of the temperature and pressure dependence of the unfolding of chymotrypsinogen shows an elliptical phase diagram as presented by Hawley (1971) with a reintersection on the pressure axis at low temperatures, consistent with the predictions by Brandts (1969). Since then, cold, heat, and pressure unfolding of proteins have been extensively studied using a variety of methods such as nuclear magnetic resonance (Zhang et al., 1995; Jonas et al., 1998), ultraviolet spectroscopy (Mombelli et al., 1997), differential scanning calorimetry (Privalov et al., 1986), small angle X-ray scattering (Panick et al., 1998, 1999), and molecular dynamics (Wroblowski et al., 1996; Floriano et al., 1998). More recently, it was shown that high pressure and low temperature can be applied to inactivate viruses (Oliveira et al., 1999).

The cold unfolding is particularly interesting because a possible resemblance of the cold unfolded state of certain proteins, such as RNAse A and lysozyme, with an early folding intermediate of these proteins was suggested (Nash and Jonas, 1997). This conclusion is based on the fact that these intermediates are characterized by protection factors for hydrogen-deuterium exchange that are similar to the ones found for the cold unfolded state. Indirectly these protection factors can be linked to a remaining amount of secondary structure. Because of the presence of secondary structure in the pressure-unfolded state one might expect this state to resemble certain folding intermediates as well. However, most of the techniques used to study the cold and pressure unfolding so far give no direct information on the secondary structure.

The nature of the cold unfolding compared with heat unfolding is still a matter of controversy. According to Huang and Oas (1996) the heat and cold unfolded states are thermodynamically and conformationally equivalent. Privalov (1990) referred to the unfolded state as being universal. This was based on the finding that the thermodynamic parameters for the cold and heat or chemically unfolded states are much alike and only depend on the solution conditions. In contrast, others (Griko and Kutyshenko, 1994; Richardson et al., 2000) observed structural and thermodynamic differences between the cold and heat denaturation processes. Similarly this question arises for pressure versus temperature effects.

The role of water as the molecular basis for the cold unfolding of proteins has been emphasized (Huang and Oas, 1996). A similar model has been proposed for the pressure unfolding on the basis of high-pressure molecular dynamics calculations by Wroblowski et al. (1996). It is suggested that the water that is forced into the protein interior by the increasing pressure eventually breaks the structure apart (Hummer et al., 1998). Vidugiris and Royer (1998) determined the volume changes for pressure-induced transitions of apomyoglobin. They concluded that a hydrophobic model as such could not explain the pressure and cold unfolding.

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In this paper we study the cold unfolding of myoglobin and compare it with the pressure and heat unfolding as observed with Fourier transform infrared spectroscopy. This paper is organized as follows. First, the infrared vibrations of interest are described, and the native state is characterized. Then we discuss the cold, pressure, and heat unfolding and investigate the aggregation behavior of the different unfolded states. The aggregation phenomenon is of growing interest because of its role in certain molecular diseases (Booth et al., 1997; Carrell and Gooptu, 1998; Kim et al., 2000) and biotechnology (Wetzel, 1994; Carpenter et al., 1999). Finally, we discuss the effect of cold on the pressure-induced state and of heat on the cold-induced state.

MATERIALS AND METHODS

Sample preparation

Horse heart metmyoglobin was purchased from Sigma (Bornem, Belgium) and used without further purification. Metmyoglobin was dissolved at 75 mg/mL in 10 mM deuterated TRIS-HCl buffer (p^2H 8.2). The pH meter was calibrated by reference to standard buffers in H_2O, and the reading was corrected for the deuterium isotope effect according to p^2H = pH meter reading + 0.4. The sample was stored overnight to ensure complete $H/^2H$-exchange of all solvent accessible protons.

Cold unfolding

Cold unfolding studies were performed at a pressure of ~2 kbar, in which the phase diagram of water allows the possibility of lowering the temperature down to −20°C before the formation of ice occurs (Bridgman, 1935). We used a Graseby Specac (Orpington, UK) variable temperature cell in which the classical temperature cell is replaced with a diamond anvil cell (Diacell Products, Leicester, UK) that allows measurements at variable temperatures under pressure. The pressure was increased up to 2 kbar by means of a membrane that upon expansion by helium gas pushes the diamond anvils against one another (www.diacell.co.uk). After reaching 2 kbar at room temperature the sample was cooled using a solid carbondioxide-acetone mixture and an automatic temperature controller (Graseby Specac). Barium sulfate was used as an internal pressure standard (Wong and Moffat, 1989).

Heat unfolding

Heat unfolding was followed using a temperature cell with CaF_2 windows separated by a 50-μm teflon spacer, which was placed in a heating jacket controlled by a Graseby Specac Automatic Temperature Controller.

Pressure unfolding

Pressure unfolding was achieved using a diamond anvil cell, where the pressure was built up by means of a screw mechanism. Barium sulfate was used as an internal pressure standard (Wong and Moffat, 1989). The diamond anvil cell was thermostated at 25°C.

Fourier transform infrared spectroscopy

The infrared spectra were obtained with a Bruker IFS66 Fourier transform infrared (FTIR) spectrometer equipped with a liquid nitrogen cooled broad band mercury-cadmium-telluride solid-state detector. Two-hundred, fifty interferograms were co-added after registration at a resolution of 2 cm^{-1}.

Fourier self-deconvolution

The overlapping components of the amide I′ band were narrowed by the Fourier self-deconvolution developed by Kauppinen et al. (1981). The optimal parameters were determined from the observation of the power spectrum as described by Smeller et al. (1995a). A resolution enhancement factor (Kauppinen et al., 1981) of 1.5 was reached using the Lorentzian band shape of 20 cm^{-1} bandwidth. A triangular square apodization function was used (Griffiths and de Haseth, 1986).

Fitting

The secondary structure was determined by fitting the self-deconvoluted amide I′ band of the spectrum using Gaussian functions (Byler and Susi, 1986; Smeller et al., 1995b). The fitting of component peaks was performed by a program developed in our laboratory, using the Levenberg-Marquard algorithm (Press et al., 1986).

Transition midpoint determination

The amide I′ band maximum versus pressure or temperature was fitted by a sigmoid function to determine the transition midpoint. The sigmoid curve describes a transition between states where $\nu(T, p)$, the maximal position of the amide I′ band, depends linearly on temperature or pressure, according to $\nu(T, p) = a + bx$ far below the transition, and $\nu(T, p) = c + dx$ far above the transition. These fitted sigmoid functions are similar to the ones that could be calculated from the Planck and van't Hoff relations, but because in our case the reversibility of the transition cannot be guaranteed, the thermodynamic parameters of the transition cannot be determined. Only the transition midpoint of the sigmoid curve is used as a parameter to characterize the transition.

RESULTS

Structural analysis and assignments

The two protein infrared absorption bands of interest are the amide I and amide II bands. The amide I band is mainly due to the C═O stretching vibration of the backbone chain and is sensitive to the secondary conformation (Jackson and Mantsch, 1995). It is a rather broad band, but by Fourier self-deconvolution and fitting of the spectrum, the component peaks can be visualized (Fig. 1). These bands can be assigned to specific types of secondary structure (Byler and Susi, 1986; Goossens et al., 1996; Panick et al., 1998). The assignments used in this work are summarized in Table 1. Although single wave numbers are given, it should be kept in mind that these are mean band positions.

The amide II band at 1545 cm^{-1} is due to the amide N—H bending vibration (Wong, 1991) and shifts to 1457 cm^{-1}, the so-called amide II′ band, upon $H/^2H$-exchange. In our procedure all the solvent accessible protons will be exchanged, but the buried ones will remain unexchanged until the protein unfolds. This will allow further monitoring of the unfolding process using the amide II/II′ band.

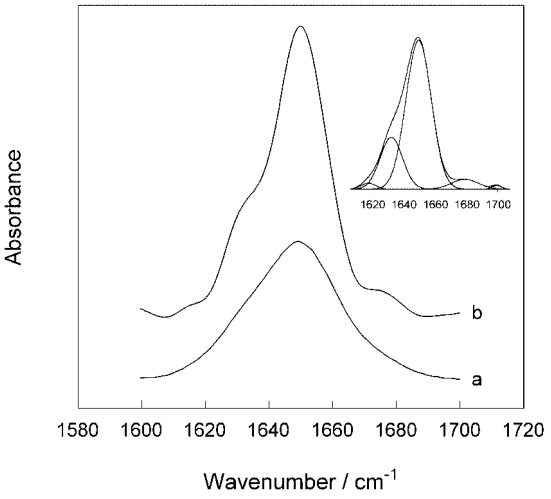

FIGURE 1 Original (*a*) and deconvoluted (*b*) infrared spectrum of native myoglobin (1 bar, 25°C) between 1600 and 1700 cm^{-1}. The inset shows the fit of the deconvoluted spectrum. Each Gaussian curve can be assigned to a type of secondary structure.

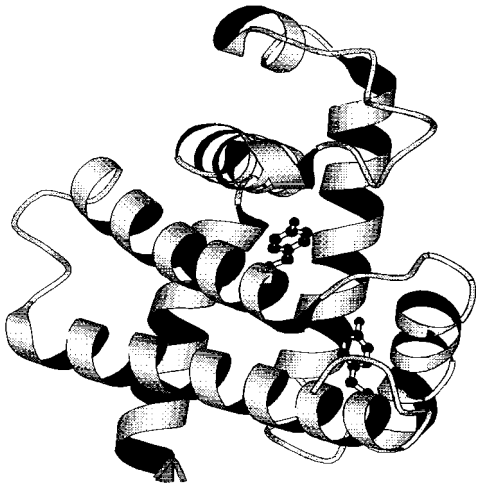

FIGURE 2 Three-dimensional structure of horse heart myoglobin. Two tyrosines at positions 103 and 146 are shown. Atomic coordinates are from the PDB database (Evans and Brayer, 1990; 1YMB). The structure was designed with MOLSCRIPT (Kraulis, 1991).

Finally, a few amino acid side chain vibrations can be observed outside the region of the amide bands. Myoglobin contains two tyrosine residues at positions 103 and 146 in helices G and H, respectively (Evans and Brayer, 1990). Tyrosine 103 is partially exposed to the solvent, whereas tyrosine 146 is solvent protected (Fig. 2). The frequency of the tyrosine ring vibration can be found at 1517 cm^{-1} (Rahmelow et al., 1998). The band at 1566 cm^{-1} was attributed by Ismail et al. (1992) to the antisymmetric COO^- stretching vibration of the side chain carboxylate groups of glutamate. Myoglobin contains 13 glutamate residues evenly distributed over the different helices (Evans and Brayer, 1990).

Native structure

Fig. 1 shows the amide I′ band of the native myoglobin. Its main component is the α-helix band at 1650 cm^{-1}. The α-helix makes up almost three-quarters of the total amount of secondary structure (Table 2). This corresponds with the x-ray analysis data of Evans and Brayer (1990), which show that ~74% of the secondary structure consists of α-helix.

TABLE 1 Assignments of the components of the amide I′ band (Byler and Susi, 1986)

Position (cm^{-1})	Secondary structure
1618	Intermolecular β-sheet
1630	Extended chain
1643	Unordered
1653	α-Helix
1668	Turn
1683	Intermolecular β-sheet

The wavenumbers given are mean positions.

Generally the bands at 1624 cm^{-1} and at 1632 cm^{-1} are assigned to β-sheet, whereas the band at 1668 cm^{-1} is assigned to turn structure. However, because the x-ray crystal structure of myoglobin indicates that there is no β-sheet present, Byler and Susi (1986) suggested that these bands must be associated with extended chains that connect the helical cylinders rather than with β-structure. On the other hand, Torii and Tasumi (1992) used model calculations to show that α-helices might absorb in the region below 1640 cm^{-1} as well. More recently, it was suggested that solvent exposed helices absorb between 1630 and 1645 cm^{-1} (Haris

TABLE 2 Fitted values (in %) for the secondary structures present in the native (ambient conditions), the heat-unfolded (90°C), the cold-unfolded (−21°C, 2 kbar), and the pressure-unfolded (10 kbar) state of myoglobin at pH 8.2, as well as for the heat, the cold, and the pressure unfolded state after return to ambient conditions, as determined with FTIR spectroscopy

State	α-Helix + random*	Extended chain†	Turn‡	Intermolecular β-sheet§
Native	73.5	15	11.5	
Heat unfolded	47.5	14	11.5	27
Heat unfolded¶	47	13	15.5	24.5
Cold unfolded	42	52	6	
Cold unfolded¶	37.5	49.5	13	
Pressure unfolded	32	61	7	
Pressure unfolded¶	45.5	45.5	9	

*Band at 1650 cm^{-1} for α-helix and at 1645 cm^{-1} for random structure.
†Band at 1632 and 1624 cm^{-1}
‡Band at 1668 cm^{-1}.
§Bands at 1618 and 1683 cm^{-1}.
¶After return to ambient conditions (25°C, 1 bar).
The fitting was performed as explained in the Materials and Methods section.

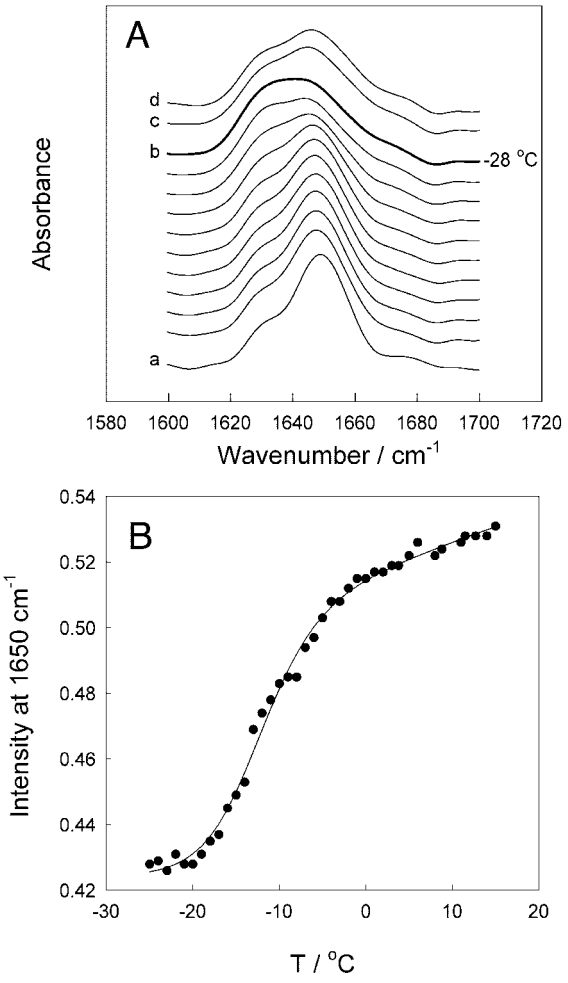

FIGURE 3 (*A*) Stacked plot of the deconvoluted 1600 to 1700 cm^{-1} region of myoglobin upon cold unfolding. The sequence of the spectra is from bottom to top. All spectra were taken at 2 kbar except for the bottom [*a*] and top [*d*] spectrum, which are taken at atmospheric pressure and 20°C before and after the cold unfolding, respectively. The temperatures at which each spectrum was taken are 20, 15, 11, 5, 0, −5, −10, −15, −20, −25, [*b*] −28, [*c*] 10, and [*d*] 20°C. Spectrum [*c*] is taken at 2 kbar and 10°C after the cold unfolding. (*B*) Intensity of the band at 1650 cm^{-1} versus decreasing temperature. Dots are the experimental data, and the full line is the fitted curve.

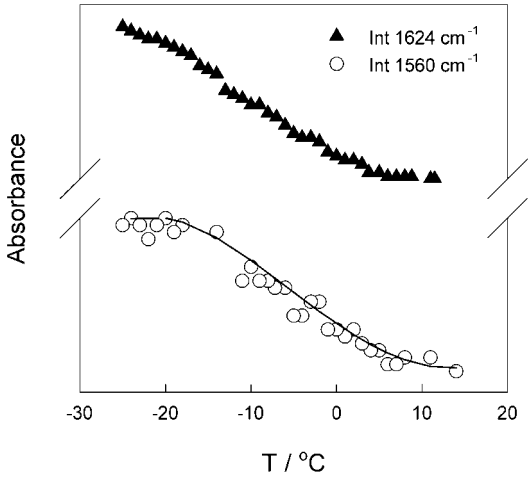

FIGURE 4 Intensity of the (○) 1566 cm^{-1} (COO^- stretching of the glutamate side chain carboxylate groups) and intensity the (▲) 1624 cm^{-1} (assigned to extended chain) band versus temperature, in the cold unfolding experiment. The solid line is the fit of the intensity of the 1566 cm^{-1} band.

and Chapman, 1995; Gilmanshin et al., 1997). Comparing our spectral fitting with the x-ray data, the assignment by Byler and Susi (1986) agrees well with the crystal structure.

Effect of cooling on the FTIR spectrum

Fig. 3 *A* shows the changes in the amide I′ band upon cooling. The accompanying Fig. 3 *B* shows that the intensity of the strong α-helix band at 1650 cm^{-1}, which is present in the native structure, gradually decreases during cooling. The transition midpoint can be found at -12.7 ± 0.4°C. Concomitant with the loss of α-helix the absorbance in the region of 1620 cm^{-1} to 1640 cm^{-1} increases (Fig. 3 *A*). This increase is due to the development of the broad-band characteristic of the unordered structure and a new component at 1624 cm^{-1}. The increase of the band at 1624 cm^{-1} can be correlated with the increase of the band at 1566 cm^{-1}, which is assigned to the antisymmetric COO^- stretching vibration of the carboxylate groups of the glutamate side chains (Fig. 4).

The amide II band does not disappear completely during the cooling. It can still be seen even on the spectra recorded at −25°C, which is well below the cold unfolding temperature. This suggests that even in the cold unfolded state not all the protons are accessible for exchange with D_2O and that some stable core might still remain.

The tyrosine band does not show any significant frequency shift during the cooling cycle (Fig. 5), which suggests that the tyrosine residues do not become solvent exposed. Similar observations have been made by Kaposi et al. (1999) for horseradish peroxidase down to −260°C.

The cold unfolding is not completely reversible (Fig. 3). We find that return to ambient conditions (1 bar and 20°C) results in a decrease of the 1632 cm^{-1} area (extended chain), whereas a distinct band ~1646 cm^{-1}, probably representing a more native-like structure, appears (Table 2). On the other hand the width of the amide I′ band is still the same as in case of the unfolded state. This suggests that we are dealing with a heterogeneous ensemble of refolded and unfolded species.

Pressure dependence of the FTIR spectrum

In Fig. 6 *A* the effect of increasing hydrostatic pressure on the infrared spectra of myoglobin is shown. It can be seen that at high pressure the amide I′ band converts into a broad band as the result of a significant loss of α-helix structure

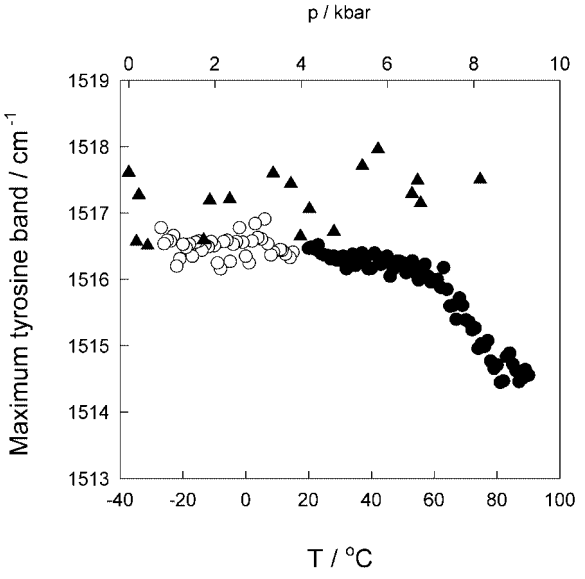

FIGURE 5 Temperature (○) and pressure (▲) dependency of the frequency of the tyrosine band at 1517 cm^{-1}. Black dots indicate the heat unfolding experiment, white dots the cold unfolding one. Temperature data (○) should be read from the bottom axis and pressure values (▲) from the top axis.

(1650 cm^{-1}) and an increase of random structure (1645 cm^{-1}) (Krimm and Bandekar, 1980; Byler and Susi, 1986). Furthermore an intensity increase ~1624 and 1632 cm^{-1} can be observed. Both are attributed to the formation of the extended chain structure, which originates from the partial unfolding of the α-helix structure. The increase of the 1624 cm^{-1} band is correlated to the changes of the 1566 cm^{-1} band, which also gains intensity and has a red shift upon pressure increase. A plot of the intensity of the band at 1650 cm^{-1} shows a curve with two transitions (Fig. 6 *B*), suggesting the existence of an intermediate. The respective transition midpoints ($p_{1/2}$) are found at 4.03 ± 0.04 and 5.9 ± 0.05 kbar.

The pressure dependence of the frequency of the tyrosine ring vibration at 1517 cm^{-1} shows a general trend with the pressure, which can be expected from the compression of the ring vibrations (Zakin and Hersbach, 1986) (Fig. 5). The lack of any changes in the band position near the transition midpoint suggests that the tyrosines do not become solvent exposed in the pressure unfolded state.

Pressure release causes a decrease of the intensity ~1632 cm^{-1} and the appearance of a distinct band ~1648 cm^{-1}, typical of the native protein (Fig. 6 *A*). Nevertheless a significant amount of extended chain remains present at 1 bar (Table 2). This indicates that the pressure unfolding is only partially reversible.

Temperature dependence of the FTIR spectrum

At ambient conditions the amide I′ band is dominated by the α-helix band at 1650 cm^{-1} as can be seen in Fig. 7 *A*.

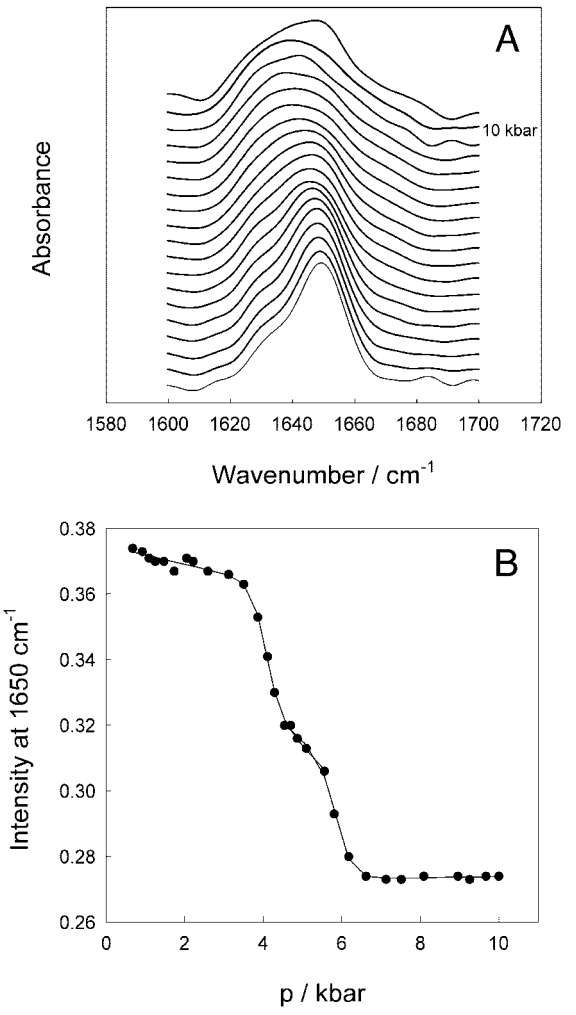

FIGURE 6 (*A*) Stacked plot of the changes with pressure of the deconvoluted 1600 to 1700 cm^{-1} region of the IR spectrum of myoglobin. The sequence of the spectra is from bottom to top. Spectra were taken at 0.7, 1.3, 1.8, 2.6, 3.2, 3.9, 4.1, 4.6, 4.9, 5.1, 5.6, 5.8, 6.2, 6.6, 7.2, 8.1, 9, and 10 kbar, respectively. The spectrum at the top is taken at 1 bar after decompression. (*B*) The intensity of the α-helix band at 1650 cm^{-1} versus pressure. The dots represent the experimental data, and the full line is the fitted curve.

Above the unfolding temperature, this component disappears and the formation of a broad band is observed in the region between 1620 and 1680 cm^{-1}. As can be seen in Table 2 this is a mixture of ordered and random structure. Plotting the intensity of the band at 1650 cm^{-1} versus temperature, a sigmoidal curve is found with a midpoint temperature, $T_{1/2}$, at 72.3 ± 0.04°C (Fig. 7 *B*). The broadening is accompanied by the appearance of two new bands at 1618 and 1683 cm^{-1}, characteristic of intermolecular antiparallel β-sheet aggregation (Ismail et al., 1992; Martinez et al., 1996; Dong et al., 2000; Damaschun et al., 2000). These bands are formed in the beginning of the transition region and gain intensity as the temperature further increases. Formation of these bands is correlated with

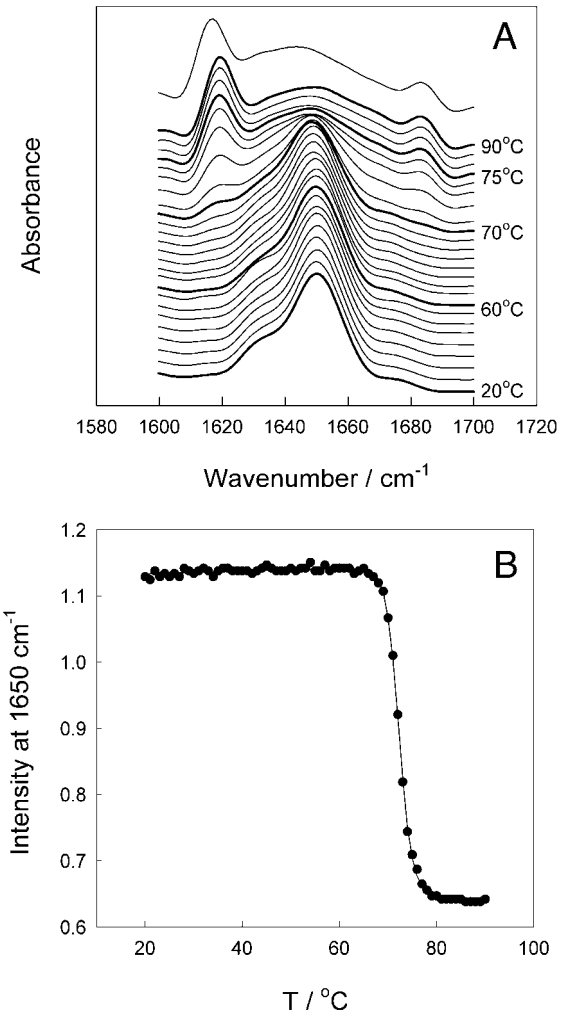

FIGURE 7 (*A*) Stacked plot of the deconvoluted IR spectra of myoglobin between 1600 and 1700 cm^{-1} with increasing temperature. The sequence of the spectra is from bottom to top with respective temperatures of 20, 25, 30, 35, 40, 45, 50, 55, 60, 62, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 80, 85, and 90°C. The top spectrum is taken at ambient conditions after cooling. (*B*) The intensity of the band at 1650 cm^{-1} versus temperature. The dots represent the experimental data, and the full line is the fitted curve.

the partial loss of the original secondary structure. However, we do not observe any change in secondary structure up to the temperature where the aggregation bands first appear (70°C, Fig. 7 *A*). The bands do not disappear after cooling the sample back to room temperature, but shift ~2 cm^{-1} toward lower wave numbers. The shift is due to the strengthening of the hydrogen bonds during the gelation.

Between 60°C and 67°C the amide II band (1545 cm^{-1}) disappears, whereas the intensity of the amide II′ band increases. Fig. 8 shows the ratio of the 1457/1545 cm^{-1} bands as a function of temperature, which indicates the completion of the H/^{2}H-exchange of the hydrogens that were not solvent accessible in the folded state. This also implies that the tertiary structure is already lost before the

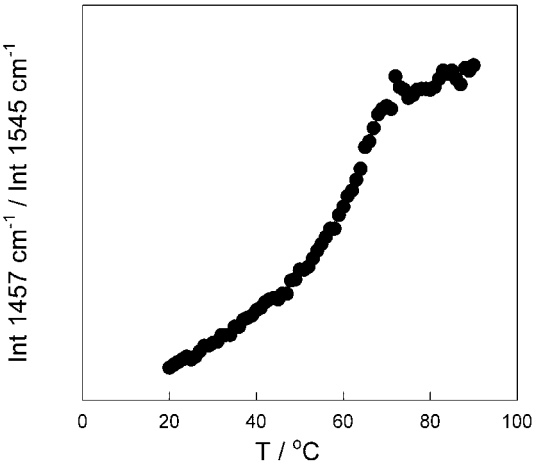

FIGURE 8 Ratio of the 1457/1545 cm^{-1} bands versus temperature indicating completion of the H/^{2}H exchange.

observed main transition. Correlated with the intensity ratio of amide II′/amide II bands, a small down shift of the amide I band position can also be observed in the temperature range of 60°C to 70°C, which can also be attributed to the completion of the exchange.

The tyrosine ring vibration at 1517 cm^{-1} shifts toward a lower wave number in a cooperative manner upon unfolding and concomitant aggregation (Fig. 5). The transition midpoint can be found at 72.2 ± 2.9°C.

Effect of cooling on the pressure unfolded protein

First the native protein is unfolded by pressure (7 kbar, one-half hour) and then, after releasing the pressure to 2 kbar, we cool the system to a temperature well below the transition temperature of the cold unfolding (−25°C). The conformation that was obtained after the pressure treatment remains unaffected by the cooling. Only a small reversible intensity change in the 1632 to 1624 cm^{-1} region of the amide I′ band is observed. When conditions return to ambient, a small shift of the amide I′ band maximum toward higher wave numbers is observed.

Effect of heating on the cold unfolded protein

Myoglobin is first subjected to a cold unfolding (2 kbar, −19°C). After return to 20°C and a pressure below 0.5 kbar a heat unfolding at this pressure is performed. The main observation here is the appearance of the band at 1618 cm^{-1} at 32°C and its further intensity increase upon increasing temperature (Fig. 9). At 78°C the midpoint $T_{1/2}$ of the main transition is observed, which is accompanied by a strong growth of the bands at 1618 and 1683 cm^{-1}. The band at 1683 cm^{-1} could not be observed before the main transition, probably due to its lower

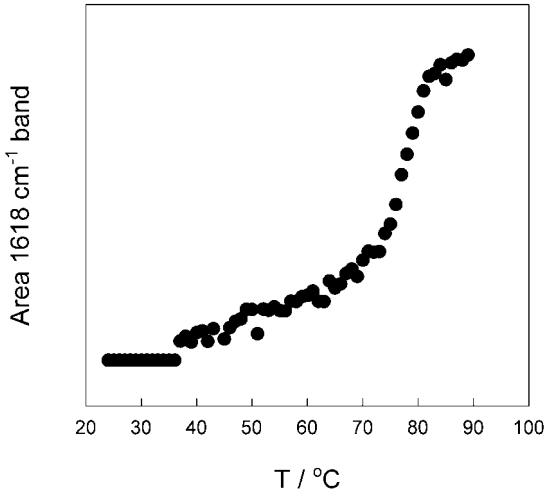

FIGURE 9 Area of the 1618 cm^{-1} band versus temperature when heating the previously cold unfolded (−19°C, 2 kbar for 15 min) myoglobin.

intensity compared with the 1618 cm^{-1} band (Ismail et al., 1992; Martinez et al., 1996; Dong et al., 2000; Damaschun et al., 2000). This observation suggests that part of the cold unfolded molecules already aggregates at moderate temperatures well below the temperature observed for the untreated protein (74°C). As the temperature increases the molecules further unfold and more aggregation takes place, giving rise to more intense bands.

DISCUSSION

Cold unfolding

Nash and Jonas (1997) suggested that for some proteins the cold unfolded state might resemble an early folding intermediate. Other studies have shown that the cold unfolded state retains a native-like core structure (Zhang et al., 1995). In agreement with these findings we show that the cold unfolded state of myoglobin has a persistent amount of secondary structure. Furthermore the cold unfolding does not cause a complete H/^{2}H-exchange of the buried hydrogens, nor a change of the tyrosine position (Fig. 5). These tyrosine residues are part of the H and G helices, respectively, which are the most stable helices and are probably part of an early folding intermediate as suggested by computer simulations (Floriano et al., 1998). In case of apomyoglobin the A/G/H helix intermediate has been shown to be a kinetic folding intermediate (Jennings and Wright, 1993). Thus, the tyrosine data suggest that a rigid core containing the helices H and G may remain intact. We conclude that the cold-induced unfolding results in the formation of a partially unfolded state in which some of the secondary contacts are still present.

Ismail et al. (1992) pointed out that the intensity increase of the coupled bands at 1566 and 1624 cm^{-1} is probably due to reorganization of the hydrogen bonding and the ionic charges within the protein. The reorganization is thereby attributed to an increase in the solvent exposed extended chain conformation and to additional hydrogen bonding between the amide groups and water molecules. The growth of these bands is not related to aggregation. This reorganization might as well explain why we do not observe any aggregation in the cold unfolding process. The water could have solvated the hydrophobic patches, thereby avoiding a preferential protein-protein interaction upon exposure of these groups to the bulk solvent as a consequence of the unfolding. This protein-protein interaction could otherwise cause demixing and protein cross-linking (San Biagio et al., 1996, 1999).

Our experiments show that the applied pressure plays an important role in the cold unfolding. Without the pressure-assistance no cold unfolded state can be obtained because of the ice formation. Tsuda et al. (1999) have recently assumed that pressure-assisted experiments rather reflect the effect of pressure than that of low temperature. We think this to be quite unlikely. First, from the phase diagram for the stability of proteins it can be expected that pressure shifts the cold unfolding transition to a higher temperature (Heremans and Smeller, 1998). In this respect pressure is acting like urea, bringing the cold transition within a reasonable experimental temperature range. The latter approach has been used with other spectroscopic techniques (Huang and Oas, 1996; Mombelli et al., 1997). Second, a thermodynamic analysis of the stability phase diagram shows that the high pressure- and the low temperature-induced unfolding are both driven by negative volume changes (Heremans and Smeller, 1998). This fact provides a theoretical basis for the observed similarities between the pressure and the cold unfolded states. The stability diagram also shows that the heat unfolding is driven by positive entropy changes. Thus, the thermodynamic parameters of the heat unfolding provide a basis for the differences between unfolding due to heat on the one hand and pressure and cold on the other hand.

Pressure unfolding

Pressure unfolding is found to proceed in a similar way as cold unfolding. Both processes result in a partially unfolded structure, which still consists of a significant amount of secondary structure (~50%) (Table 2). However, it should be emphasized that although they are similar, they are definitely not identical. As can be seen from Table 2 more extended chain is formed under pressure.

Molecular dynamics calculations by Floriano et al. (1998) predicted a pressure unfolding mechanism in which a molten globule state is formed at 7 kbar. This is the pressure at which we find the pressure unfolding to have come to an end (Fig. 6 *B*). The molten globule was charac-

terized by a percentage of secondary structure (α-helix) between 30 and 40% and with less intrachain hydrogen bonds than in the native state (Floriano et al., 1998). Given the fact that there have been changes in secondary structure it would be better to refer to this state as a partially unfolded state in which a rigid core made up of the H and G helices is still present. Similarly, pressure denaturation of apomyoglobin was recently shown to result in the formation of an intermediate state in which a core consisting of the A/G/H helices is conserved (Bondos et al., 2000). However, it was possible to further denature this intermediate at higher pressures ($\sim$5 kbar).

The spectral changes upon decompression indicate that decompression gives rise to a conformation that differs from that of the pressure unfolded state. Table 2 shows that there is an increase in the amount of unordered structure and α-helix during decompression. This result is in contradiction to the work of Zipp and Kauzmann (1973) who observed a completely reversible pressure unfolding. A possible explanation for this is the fact that the protein concentration in our FTIR experiments is two orders of magnitude higher than in the case of absorption spectroscopy experiments (Zipp and Kauzmann, 1973). One could speculate that due to these high concentrations aggregation might occur during refolding. However, we have no evidence for such aggregation.

Heat unfolding

According to the classical idea, the heat unfolding leads to the complete loss of secondary structure, but several studies (Zhang et al., 1995; Holzbaur et al., 1996) have shown that the heat unfolded state consists of a persistent amount of secondary structure. In our experiments we observe that the unfolding is accompanied by aggregation without the preceding population of an intermediate. The aggregation is reflected by the IR spectroscopy in the formation and growth of two new bands at 1618 and 1683 cm^{-1}, which are characteristic for intermolecular antiparallel β-sheet aggregation (Ismail et al., 1992; Martinez et al., 1996; Dong et al., 2000; Damaschun et al., 2000). It is the result of a kinetic competition between the unfolded state and an off-pathway aggregate. Due to the high protein concentrations often used in FTIR experiments the aggregation process is often favored. However, it is noteworthy that certain proteins do not exhibit these bands upon heat unfolding (Fabian and Mantsch, 1995; Panick et al., 1998; Dong et al., 2000; Panick and Winter, 2000). This indicates that the formation of an aggregate is still dependent on the dynamics of the investigated protein.

Although there are no structural clues for the existence of an intermediate from the amide I′ band, the $H/^2H$-exchange suggests some mechanism for the observed transformation of α-helix into intermolecular β-sheet. Completion of the exchange not only implies the loss of tertiary structure, but it also requires a local or global unfolding of the secondary structure (Englander et al., 1996; Englander, 2000). This suggests that the helices are unfolded into random coil structure, which then can aggregate. The absence of any structural changes in the amide I′ band before the appearance of the aggregation bands suggests the assumption that in the case of myoglobin a local unfolding takes place that triggers the aggregation in a cooperative manner. The behavior of the tyrosine vibration is in contrast to earlier FTIR observations where the tyrosine peak was found to shift to higher wave numbers upon unfolding (Fabian et al., 1993; Panick and Winter, 2000). Such a shift is explained by the fact that the tyrosine side chain experiences less strong hydrogen bonding when exposed to the solvent than when buried in the protein interior. Our finding suggests that the tyrosine residues are involved in the aggregation, thereby experiencing stronger hydrogen bonding with neighboring groups. This implies that, in contrast to cold and pressure unfolding, the helices G and H become disrupted during the aggregation.

Unfolding and aggregation

Recently, the effect of heating on the pressure unfolded state of myoglobin was investigated (Smeller et al., 1999). It was found that a pressure treatment of the protein at room temperature significantly lowered the aggregation temperature. This led to the conclusion that the formation of an aggregate stabilized by hydrogen bonds requires a partially unfolded protein and a certain temperature. Likewise, Ferrão-Gonzales et al. (2000) demonstrated that a pressure treatment of native transthyretin could convert the protein into its amyloidogenic state. They referred to the pressure unfolded state as the preaggregated state.

The effect of cooling on the pressure unfolded protein shows that the pressure unfolded state is stable even at low temperatures. This supports the idea that pressure and cold affect the protein in a similar manner. The small intensity change in the spectrum is probably due to the changing water structure upon cooling. In contrast, heating of the cold pretreated protein results in the early formation of the 1618 cm^{-1} band. Such an effect was also observed for the pressure pretreated protein by Smeller et al. (1999). This also leads to the assumption that pressure and cold are similar ways of unfolding, yielding partially unfolded states with a high amount of extended chain. It is consistent with the view that an intermediate conformation is formed during the cold or pressure unfolding that has a strong tendency to aggregate at moderate temperatures. These moderate temperatures are sufficient to induce a further conformational change leading to aggregation. Our results are in agreement with recent work by Dobson and co-workers (Fändrich et al., 2001), who showed that under conditions that destabilize the native fold, apomyoglobin is capable of aggregating into fibrils.

To summarize our comparison of the cold, heat, and pressure unfolding of myoglobin, we show that the cold and pressure unfolded states are only partially unfolded and that a rigid core of the G and H helices is maintained. In this respect these states resemble early folding intermediates. These states have a high content of extended chain (50–60%) and easily aggregate at moderate temperatures. In contrast, the heat unfolding readily leads to aggregation whereby almost 30% of helix is transformed into intermolecular β-sheet, whereas the amount of extended chain remains unchanged. Thus, we show for myoglobin that the cold, pressure, and heat unfolding do not follow the same pathway.

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