FTIR Spectroscopic Study of the Dynamics of Conformational Substates in Hydrated Carbonyl-Myoglobin Films via Temperature Dependence of the CO Stretching Band Parameters

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ABSTRACT Two hydrated carbonyl myoglobin (MbCO) films, one containing (0.30 g water)/(g MbCO) from MbCO solution in water at pH 5.5 and the other (0.32 g water)/(g MbCO) from 0.1 M potassium phosphate buffer solution at pH 6.8, were studied by FTIR spectroscopy from 293 K to 78 K at selected temperatures on cooling and reheating. Above ≈180 K the general trend in temperature dependence of half-bandwidths, peak maxima, and band area ratios of the A_1 and A_3 conformer bands is similar to those reported by Ansari et al. (1987. *Biophys. J.* 26:337) for MbCO in 75% glycerol/water solution, but abrupt changes in slopes at ≈180–200 K and freezing-in of conformer populations, which could be taken as indicator for glass transition of the solvent or the protein, are absent for the hydrated MbCO films. This is interpreted in terms of an exceptionally broad distribution of relaxation times, and is in accord with conclusions from recent calorimetric annealing studies of hydrated protein powders (Sartor et al. 1994. *Biophys. J.* 66:249). Exchange between the three A conformers does not stop at ≈180–200 K but occurs over the whole temperature region studied. These results are then discussed with respect to MbCO's behavior in the glass→liquid transition region of glass-forming solvents, and it is concluded that, in analogy to the behavior of low-molecular-weight compounds with a distribution of rapidly interconverting conformers, freezing-in of MbCO's A conformer populations by the solvent should not be mistaken for a glass transition of MbCO.

INTRODUCTION

In solutions of carbonyl-myoglobin (MbCO) at ambient temperature, the presence of four bands in the CO stretching band region indicates four discrete conformers, or conformational substates, which interconvert rapidly on the ^{13}C-NMR time scale (Caughey et al., 1981). FTIR spectroscopic studies of the CO stretching band region have shown that on cooling of MbCO dissolved in glass-forming solvents, marked decrease in the rate of interconversion of the conformers close to the onset temperature of the glass→liquid transition (or T_g) of the solvents occurs which is for 75% (v/v) glycerol/water at ≈180 K. These are seen in plots of log(conformer band area ratio) vs. 1/T in form of abrupt changes of slope close to the T_g of the solvent (Ansari et al., 1987; Hong et al., 1990). This dependence of conformer transition on solvent characteristics has been explained as "slaved glass transition" (Ansari et al., 1987; Iben et al., 1989; Young et al., 1991), but has recently been attributed to the enormous viscosity of the solvent at T_g (Ansari et al., 1992).

For hydrated films or aqueous solutions, however, a similar FTIR spectroscopic study extended to cryogenic temperatures has to my knowledge not been reported. (The report by Ansari et al. (1987) of a glass transition at ≈260 K in freeze-concentrated aqueous solution of MbCO was subsequently shown to be caused simply by lowering of pH due to the sodium phosphate buffer artifact (Astl and Mayer, 1991)). Studies of protein crystals and of hydrated powders or films as a function of temperature by various techniques have further led to the concept of a so-called glass transition of a protein at ≈180–200 K (Morozov and Gevorkian, 1985; Frauenfelder and Gratton, 1986; Parak, 1986; Doster et al., 1986, 1989, 1990, 1991, 1993; Goldanskii and Krupyanskii, 1989; Rupley and Careri, 1991; Srajer et al., 1991; Champion, 1992; Pethig, 1992; Pissis et al., 1992) whose temperature range is similar to that observed for MbCO in glass-forming solvents. So, at first sight it appears that hydrated powders and glass-forming solutions of proteins behave in a similar way with respect to their dynamics.

However, influence of the solvent's viscosity on the dynamics of the protein has been reported (Beece et al., 1980; Steinbach et al., 1991; Ansari et al., 1992; Settles et al., 1992), and recently Doster et al. (1993) have compared MbCO's ligand kinetics in hydrated films and concentrated glycerol/water solution and attributed differences in ligand escape rates to strong correlation between ligand transfer at the protein-solvent interface and structural relaxation of the solvent.

In this FTIR spectroscopic study of the CO stretching band region of two hydrated MbCO films, with 0.30 and 0.32 g water/g MbCO, I report half-bandwidths (HBWs), peak maxima, and relative band areas of the three conformer bands at ≈1966 cm^{-1} (conformer A_0), ≈1945 cm^{-1} (A_1), and ≈1934 cm^{-1} (A_3) for the temperature range 293 K to 78 K. Their temperature dependencies are then compared with those reported for MbCO in 75% glycerol/water solution (Ansari et al., 1987). It is shown that, where comparison is possible, above ≈180 K the general trend with temperature is similar but that in hydrated MbCO films abrupt changes in slope at

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≈180–200 K are absent and that the conformer populations are not frozen-in. This is interpreted in terms of a much broader distribution of relaxation times in hydrated MbCO films than in glass-forming solution.

This FTIR spectroscopic study is in accord with the recent study of Sartor et al. (1994a) by differential scanning calorimetry (DSC) where annealing effects have been observed in hydrated myoglobin, hemoglobin and lysozyme powders from ≈150 K up to the temperature of denaturation. These effects have been attributed to an exceptionally broad distribution of local relaxation times in its different segmental regions. Alternatively stated, the glass transition in the hydrated proteins does not occur at a single temperature or in a narrow range of temperature, such as for example in 75% glycerol/water solution, but it occurs over a broad temperature range that extends from ≈150 K up to the temperature of denaturation.

However, by using in this study the CO stretching band region as a spectral probe, it is possible to go beyond the conclusions of the previous calorimetric study (Sartor et al., 1994a), and to follow the dynamics of the three A conformer substates as a function of temperature. I then discuss the results obtained for hydrated MbCO films with respect to the behavior of MbCO dissolved in glass-forming solutions such as in 75% glycerol/water, and I conclude that, in analogy to the behavior of low-molecular-weight compounds with a distribution of rapidly interconverting conformers, freezing-in of MbCO's A conformer populations near the glass→liquid transition region of the solvent should not be mistaken for a glass transition of MbCO.

MATERIALS AND METHODS

Myoglobin (Mb) from horse skeletal muscle was obtained either from Sigma Chemical Co. (St. Louis, MO, No. M0630) or from Fluka Chemical Co. (Buchs, Switzerland, No. 70025) and used as received. Both were characterized in aqueous solution (phosphate buffered at pH 6.8) by their ultraviolet (UV)-visible spectra to be MetMb (Rothgeb and Gurd, 1978). Solutions of MbCO were prepared by dissolving in one case 0.10 g Mb in 2.2 g water, adding excess dithionite and by saturating the solution repeatedly with CO (99.97%). The final pH of the MbCO solution was 5.5. In the second case, 0.20 g Mb was dissolved in 2.2 g of 0.1 M potassium phosphate buffer and converted into MbCO as above, but with a final pH of the solution of 6.8. MbCO formation was controlled by its UV-visible spectrum.

Films of hydrated MbCO were obtained by keeping the MbCO solutions on CaF_2 discs over saturated ammonium nitrate solution under an atmosphere of CO for about one week (Poole and Finney, 1986). For the phosphate-buffered MbCO film, the weight of the buffer components is about 1/6th of that of MbCO. The hydrated MbCO films were thereafter covered with a second CaF_2 disc, taped, and transferred to the cryostat.

The FTIR spectra were recorded in transmission on BioRad's FTS 45 at 2 cm^{-1} resolution (UDR1), by coadding 250 scans. Temperature was regulated with the Paar controller model TTK-HC and was constant to ± 0.1 K. The rate of heating or cooling was between 2 and 5 K min^{-1}, and at each selected temperature the sample was kept for 5 min before recording its spectrum.

Water vapor was subtracted from the spectra, and the sloping background originating mainly from water's combination band was subtracted by using the multipoint spline function routine provided by BioRad. The spectra were thereafter transferred to a PC and curve-fitted by using SpectraCalc's software. The band parameters obtained from the curve fits did not depend on the starting conditions of the curve-fitting procedure.

RESULTS

Two hydrated MbCO films were studied, one where MbCO dissolved in pure water was used as stock solution and the pH was 5.5, and the other where MbCO was dissolved in 0.1 M potassium phosphate buffer solution and the pH was 6.8. The solutions were dehydrated on CaF_2 plates over saturated ammonium nitrate solution to an extent that on slow cooling to 78 K no crystalline ice was formed. The water content was determined thereafter from percent relative area of the band at ≈1966 cm^{-1} (conformer A_0) at 293 K according to Fig. 4 of Brown et al. (1983) where the relative area of this conformer is given as a function of water content of the protein. This evaluation gave a value of (0.30 g water)/(g MbCO) for the buffer-free sample and (0.32 g water)/(g MbCO) for the buffer-containing sample, which is within the range expected for unfreezable water (Rupley and Careri, 1991). According to the error bar in Fig. 4 of Brown et al. (1983), such an evaluation contains an appreciable uncertainty. However, the absence of formation of ice on cooling to 78 K in both samples further confirms that the water content must be below ≈(0.4 g water)/(g MbCO).

In the following I show first the results for the buffer-free hydrated MbCO film and then the results for the buffer-containing film. The data points of the first scatter considerably more than those of the second, but the general features of the various parameters as a function of temperature are similar for the two samples.

Fig. 1 shows at several selected temperatures on cooling from 293 K to 78 K the original curves from 2020 to 1880 cm^{-1}, but after subtraction of water vapor. The sloping background at high frequency is caused by the combination band of water. The changes with temperature are slight: the peak maximum of the most intense composite band which is at 1945.6 cm^{-1} at 293 K, decreases first on cooling gradually to 1945.3 cm^{-1} and increases thereafter again to 1945.6 cm^{-1} at 78 K, the second peak maximum decreases from 1966.3 cm^{-1} at 293 K to 1967.3 cm^{-1} at 78 K and the relative intensity of the composite band at low frequency decreases on cooling.

The composite bands were curve-fitted for the three component bands at ≈1966 cm^{-1} (conformer A_0), ≈1945 cm^{-1} (A_1), and ≈1934 cm^{-1} (A_3). The very weak component band at ≈1941 cm^{-1} (A_2) was neglected, in line with Ansari et al. (1987). For this analysis of the spectra, the sloping background was subtracted with BioRad's multipoint spline function routine. This is the critical step in the procedure because the shape of the composite band can be altered by the setting of the breakpoints. This is expected to influence mainly the two bands at high and low frequency of the most intense band centered at ≈1945 cm^{-1}, and is particularly troublesome at high frequency where a sloping background from water's combination band has to be subtracted. Consequently the error is largest for the A_0 band component centered at ≈1966 cm^{-1}. The influence of this error was investigated systematically for all spectra, by varying during background subtraction the upper breakpoint limit between ≥1990 and ≥2010 cm^{-1}. This had a strong influence on the HBW of the

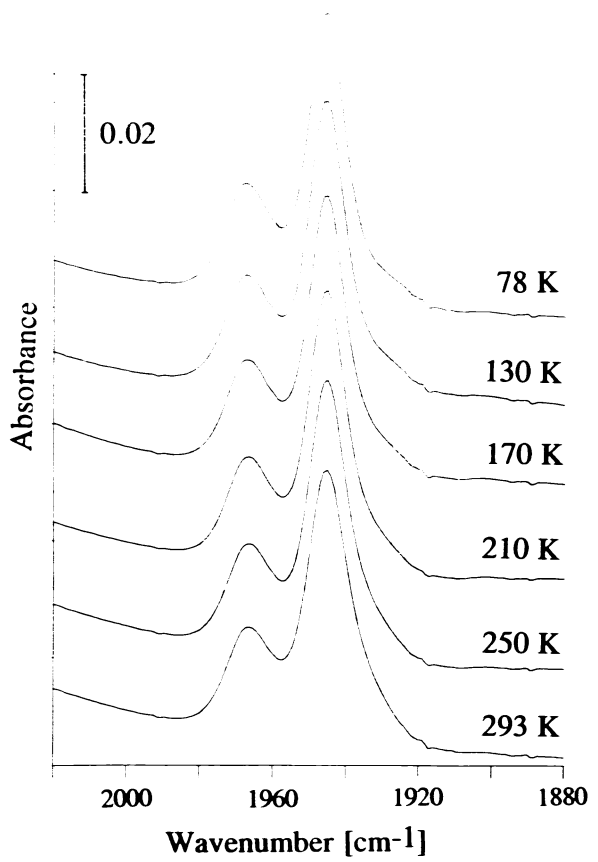

FIGURE 1 FTIR spectra of hydrated unbuffered MbCO film containing (0.30 g water)/(g MbCO) from 2020 to 1880 cm^{-1}, recorded on cooling from 293 K to 78 K at selected temperatures. Water vapor has been subtracted. The ordinate scale is for the spectrum recorded at 293 K. The other spectra are shown at similar size and are shifted vertically for clarity.

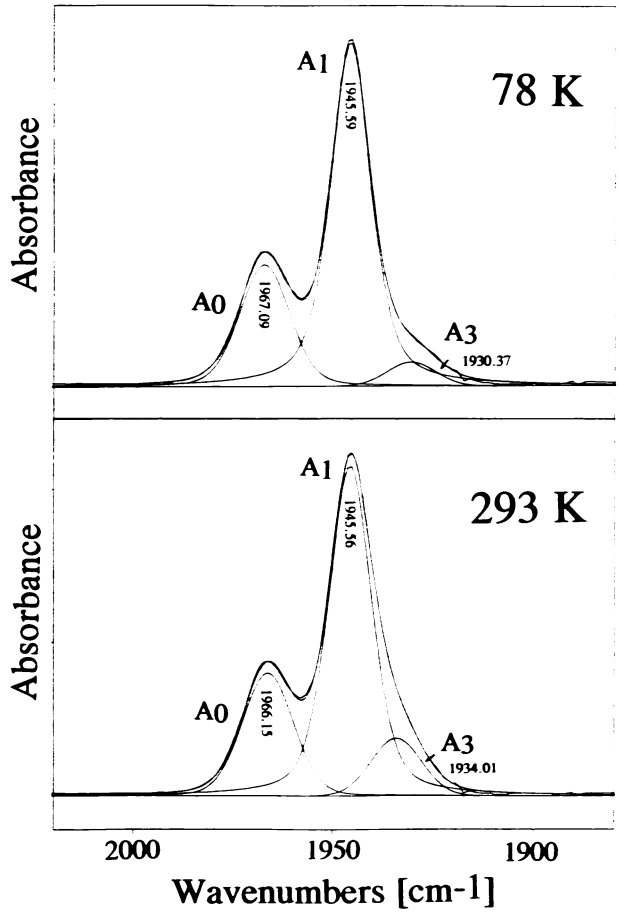

FIGURE 2 Background-corrected spectra of the hydrated unbuffered MbCO film with (0.30 g water)/(g MbCO) recorded at 293 K and at 78 K, and the curve-fitted component bands due to conformer A_0, A_1, and A_3.

band at ≈1966 cm^{-1} and to a lesser extent on its relative intensity, but very little influence on its peak maximum and on the parameters of the other two band components. In particular, the general features of the latter parameters as a function of temperature were unaffected and are therefore considered to be real. An upper breakpoint limit of ≥2000 cm^{-1} and a lower one of ≤1910 cm^{-1} was finally used as a compromise for the following reasons. For the various limits tested, a mixed Gaussian/Lorentzian band shape, or Voigt sum, was obtained in curve fits for the A_0 and A_1 conformer bands, which is typical for FTIR spectra in condensed phase (Reilly et al., 1992). For all composite band shapes studied, the values were between 70 and 90% Gaussian for the A_0 conformer band shape and between 50 and 60% Gaussian for the A_1 band. The A_3 band had consistently 100% Gaussian band shape. For an average HBW of ≈15 cm^{-1} of the A_0 conformer band, an upper limit of 2000 cm^{-1} is shifted from the A_0 band center by about 2.3 times of its HBW. This is sufficient to retain most of its intensity. Similar argument holds for the A_3 conformer band at low frequency.

Fig. 2 shows curve fits of two background corrected spectra which had been recorded at 293 K and 78 K. The most obvious change is the strongly decreasing intensity of the band at ≈1930 cm^{-1} (A_3) from 11.2% relative area at 293 K to 4.7% at 78 K. The relative areas of the bands due to the A_1 and A_0 conformers are 63.4 and 25.5% at 293 K and 68.5 and 26.8% at 78 K.

Fig. 3 shows the peak maxima of the three component bands due to conformers A_1, A_0, and A_3 as a function of temperature. In this and the following figures, triangles are for data points obtained on cooling which are connected by solid lines, whereas inverted triangles are for data points obtained on subsequent heating which are connected by dashed lines. Changes of slope both on cooling and heating are pronounced for the peak maxima values of the A_1 band, whereas those of the A_0 and A_3 band are less distinct. For plots with changes of slope, curves are approximated to the data points by third- or fourth-order polynomials in this and the following figures.

Fig. 4 shows the HBWs of the three bands due to conformers A_1, A_0, and A_3 as a function of temperature. The HBW of conformer A_1 clearly shows a gradual change in slope both on cooling and reheating. The data points of conformer A_0 scatter much more, probably for the reasons outlined above. Despite these uncertainties, a change in slope also seems to occur. The HBWs for conformer A_3 are scattered even more, probably partly because of its low intensity

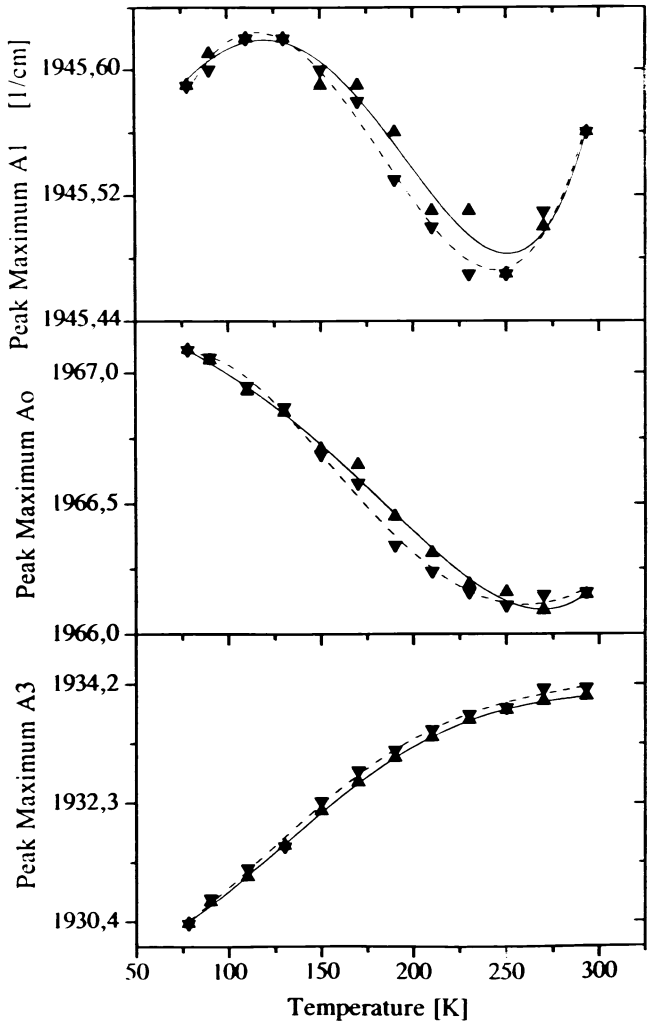

FIGURE 3 Temperature dependence of the peak maxima of the CO stretching bands of conformers A_0, A_1, and A_3 in hydrated unbuffered MbCO film with (0.30 g water)/(g MbCO). In this and the following figures, ▲ are for data points obtained on cooling which are connected by solid lines, whereas ▼ are for data points obtained on subsequent heating which are connected by dashed lines.

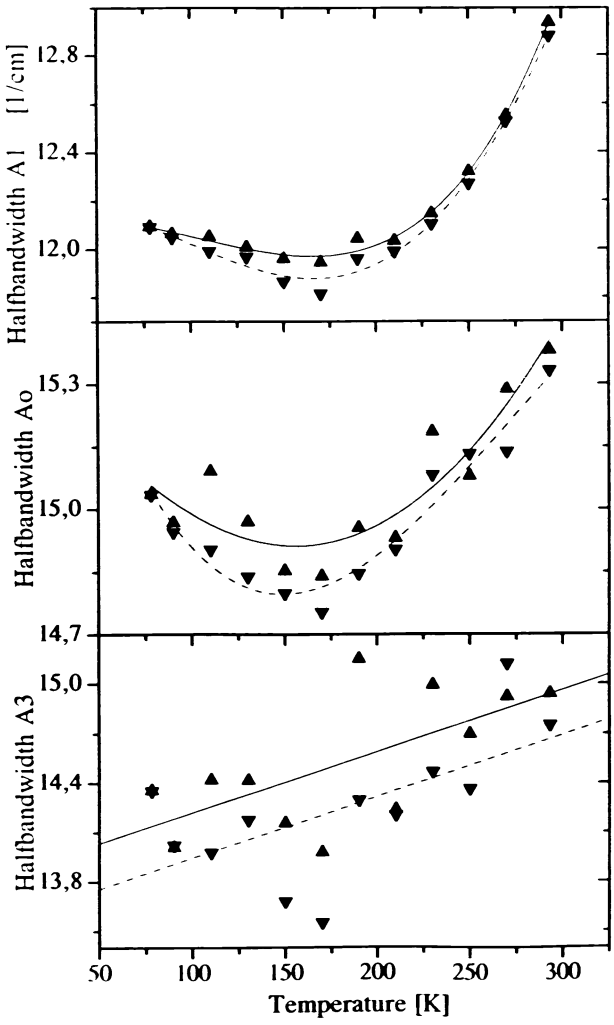

FIGURE 4 Temperature dependence of the half-bandwidths of the CO stretching bands of conformers A_0, A_1, and A_3 in hydrated unbuffered MbCO film with (0.30 g water)/(g MbCO). See Fig. 3 for details.

and also because of errors in background subtraction, and they are shown for completeness only. Nevertheless, a decrease in HBW with decreasing temperature is indicated by linear regression.

Fig. 5 shows percent relative areas of the component bands due to conformers A_1, A_3, and A_0 as a function of temperature. For conformers A_1 and A_0, curves are approximated to the data points by linear regression, but for conformer A_3 a weak change of slope seems to occur which is more pronounced on heating. In Fig. 6 the log(area ratios) of A_3/A_1, A_3/A_0, and A_0/A_1 are plotted versus $10^3/T$. For the first two, gradual changes of slope are apparent both on cooling and reheating, but for the last one strong scattering of the data points is seen, probably for reasons discussed above. Nevertheless, the $\log(A_0/A_1)$ area ratio is also shown for completeness and the curves are approximated to the data points by linear regression.

The results of the curve-fitting analysis of the buffer-containing MbCO sample are shown in Figs. 7-9. Fig. 7 shows the peak maxima of the three band components of conformers A_1, A_0, and A_3 as a function of temperature. Fig. 8 contains the temperature dependence of the HBWs and of % relative area of conformers A_1 and A_3. The data for conformer A_0 are not shown because the data points scatter strongly, similar to those shown in Fig. 5 for the buffer-free MbCO sample, but a small increase in % relative area of the A_0 conformer band from 23.1% at 293 K to 24.8% at 78 K is observed. In Fig. 9 the log(area ratios) of A_3/A_1 and A_3/A_0 are plotted versus $10^3/T$.

For both hydrated MbCO films, the total integrated area, i.e., the sum of the three band areas, seems to be constant from 293 K to 78 K. However, the data points scatter strongly and small changes in total band area would not be noticed.

The time dependence of the band parameters of the buffer-free hydrated MbCO film was also investigated. Isothermal annealing at 190 and 170 K for 260 and 220 min, respectively, after cooling from 293 K at an average rate of 4 K

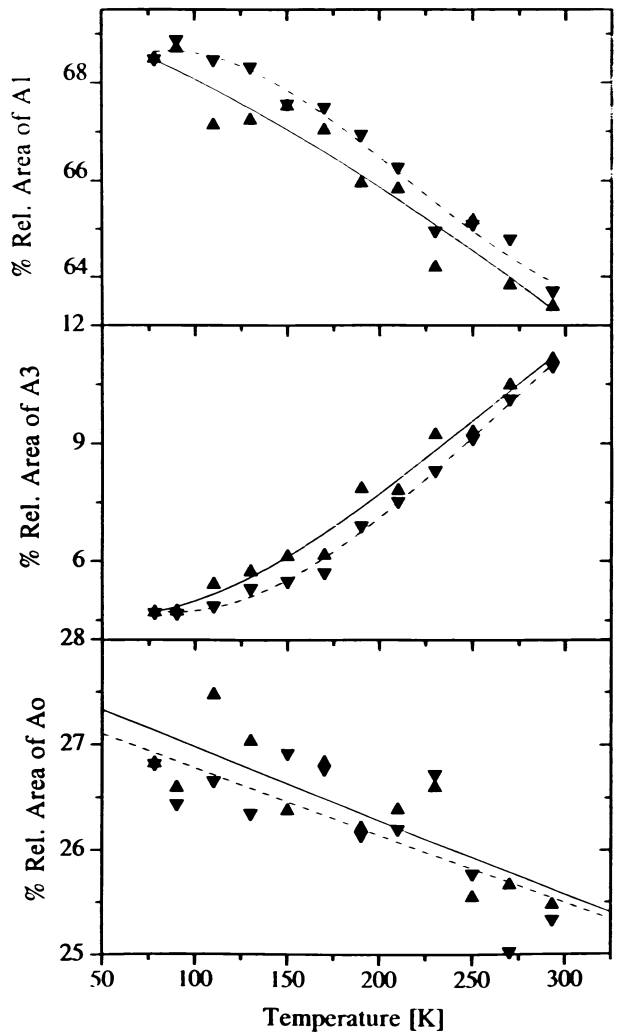

FIGURE 5 Temperature dependence of percent relative areas of the CO stretching bands of conformers A_0, A_1, and A_3 in hydrated, unbuffered MbCO film with (0.30 g water)/(g MbCO). See Fig. 3 for details.

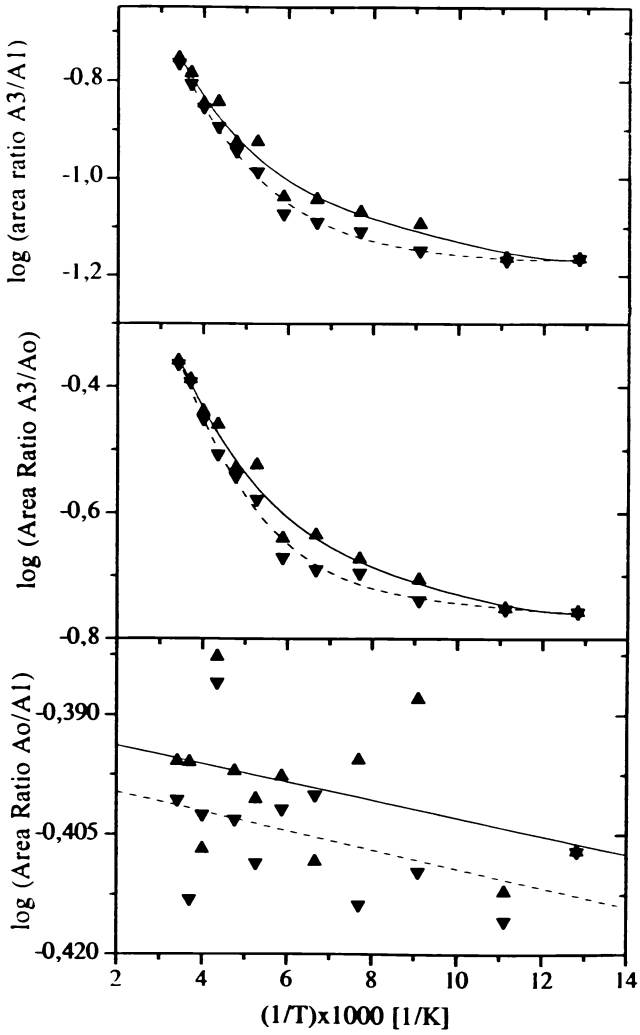

FIGURE 6 Plots of log(area ratios) for conformer band ratios A_3/A_1, A_3/A_0, and A_0/A_1 versus $10^3/T$ in hydrated, unbuffered MbCO film with (0.30 g water)/(g MbCO). See Fig. 3 for details.

min^{-1}, did not produce a systematic change in any of the band parameters of the three bands. For isothermal annealing at 150 K for 102 min, after cooling from 170 K at a rate of ≈10 K min^{-1}, a small shift in the peak maximum of the band due to conformer A_1 was observed from 1945.59 cm^{-1} to 1945.60 cm^{-1} after 30 min and to 1945.61 cm^{-1} after 70 min, and an increase in percent relative area of conformer A_1 by 0.6% was compensated by a decrease of those of conformers A_0 and A_3 by 0.3% each.

DISCUSSION

Vandeginste and De Galan (1975) have proposed objective criteria for the resolvability of overlapping of infrared absorption bands which are based on separation between peak maxima of the bands (reviewed by Maddams, 1980). For the case of this study where the number of bands is known and their HBWs are similar, curve fitting gives good results when the separation of peak maxima of the bands is larger than their HBWs, even when the mathematical model is an approximation (see Table V in Vandeginste and DeGalan, 1975). According to Gans and Gill (1980) this criterion of peak maxima separation further depends on the signal-to-noise (S/N) ratio of the composite band, increasing S/N ratios allowing curve resolution despite decreasing separation of peak maxima. According to both criteria, curve fitting of MbCO's overlapping CO stretching bands gives reliable results because separation of peak maxima is larger than the HBWs of the component bands and/or the S/N ratio is high.

The similar temperature dependencies for the band parameters of the two hydrated films, one obtained from unbuffered stock solution at pH 5.5 and the other from a potassium phosphate-buffered solution at pH 6.8, clearly establish that the general features are not influenced by the presence of potassium phosphate buffer or by small changes in pH of the stock solutions used for preparing the films. Small differences in the two sets of data are apparent, and in particular for the buffer-containing film, data points obtained on cooling and on heating overlap more closely than those of the buffer-free film. These small differences might be due

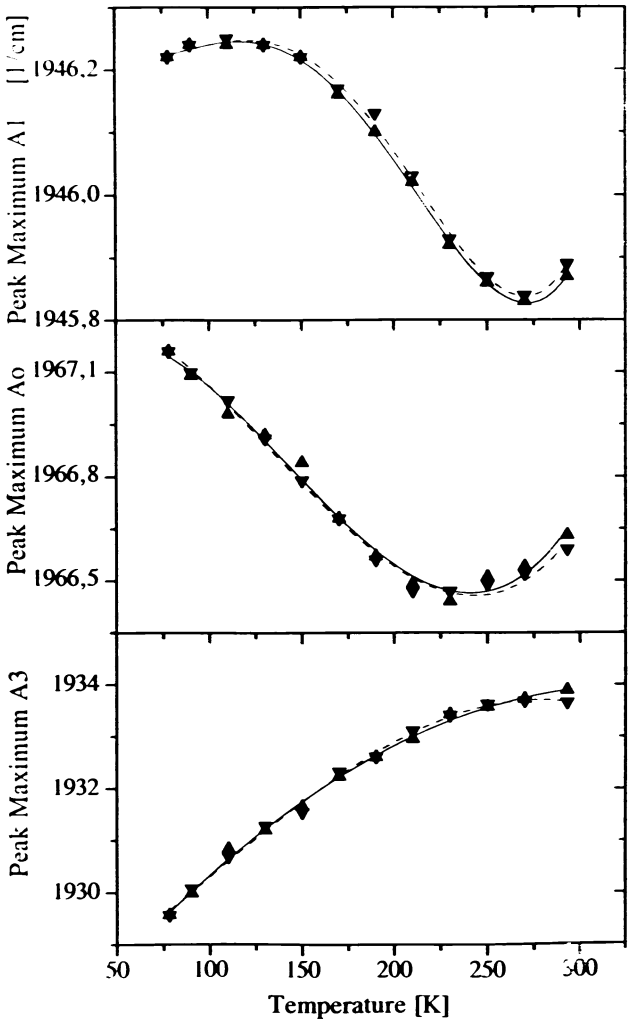

FIGURE 7 Temperature dependence of the peak maxima of the CO stretching bands of conformers A_0, A_1, and A_3 in hydrated, buffer-containing MbCO film with (0.32 g water)/(g MbCO). See Fig. 3 for details.

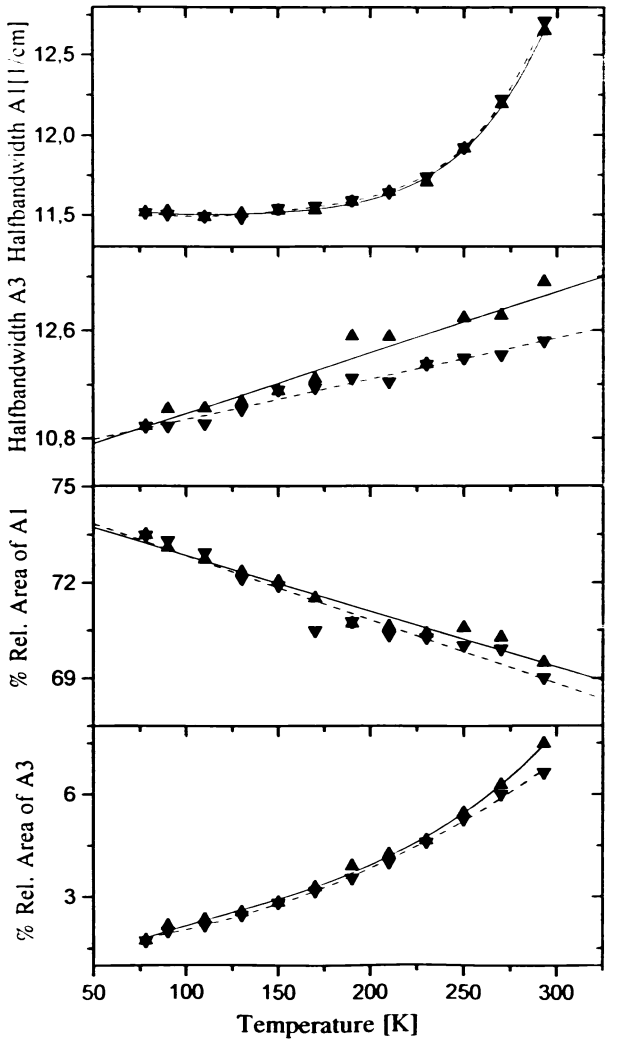

FIGURE 8 Temperature dependence of the half-bandwidths and percent relative areas of the CO stretching bands of conformers A_1 and A_3 in hydrated, buffer-containing MbCO film with (0.32 g water)/(g MbCO). See Fig. 3 for details.

to the presence of buffer in one sample or to changes in pH on cooling of the buffer-free film, but it should be noted that the meaning of pH at low temperatures, and in particular in a hydrated film, is ambiguous (Taylor, 1981). Because of these complexities the differences between the two sets of data are not discussed here further, but in paragraph 2 below an attempt is made to rationalize the differences in the unbuffered MbCO film between plots obtained on cooling and reheating.

In the following the new data for hydrated MbCO films are compared with those of Ansari et al. (1987) obtained for MbCO in 75% (v/v) glycerol/water solution. For the latter study Mb from sperm whale was used, whereas for this study horse Mb was taken. Small differences in ligand rebinding kinetics between the two types of Mb are known, and Post et al. (1993) have compared the two in their Fig. 10. Minor spectral differences between the two types of Mb in the UV-visible region were also reported by Hanania et al. (1966). However, since above ~180K the general trend in temperature dependence of the various band parameters is similar, a comparison seems to be justified.

The at first sight most pronounced differences between the sets of curves for the two hydrated MbCO films shown here to those of Ansari et al. (1987) is that the band area ratio A_0/A_1 changes very little and cannot be used as an indicator. This is not due to differences between horse and sperm whale Mb, but is so because for hydrated MbCO containing only (0.30 g water)/(g MbCO), the relative band area at 293 K is already 25.5% (Brown et al., 1983), and it increases on cooling from 293 K to 78 K only to 26.8% (Fig. 5). Furthermore, uncertainties in band parameters are most pronounced for the A_0 conformer band as outlined above. Therefore, the temperature dependence of other parameters has to be used.

In Fig. 10 the plots of the log(area ratio A_3/A_1) vs. $10^3/T$ of the unbuffered (*b*) and buffered (*c*) hydrated MbCO films are compared with that of MbCO in 75% glycerol/water solution (*a*) taken from Fig. 6 of Ansari et al. (1987). The dashed vertical line is at 180 K. The most pronounced difference between the two sets of plots is that for (*a*) the change

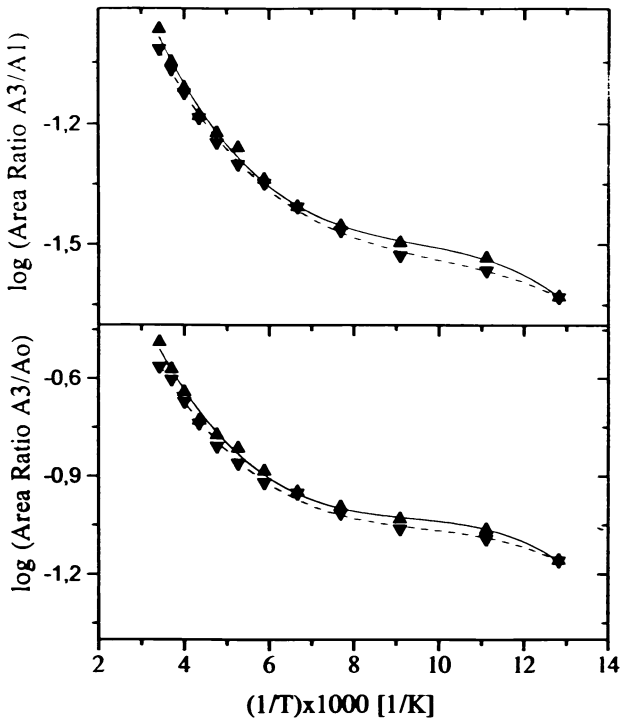

FIGURE 9 Plots of log(area ratio A_3/A_1) and log(area ratio A_3/A_0) versus $10^3/T$ in hydrated, buffer-containing MbCO film with (0.32 g water)/(g MbCO). See Fig. 3 for details.

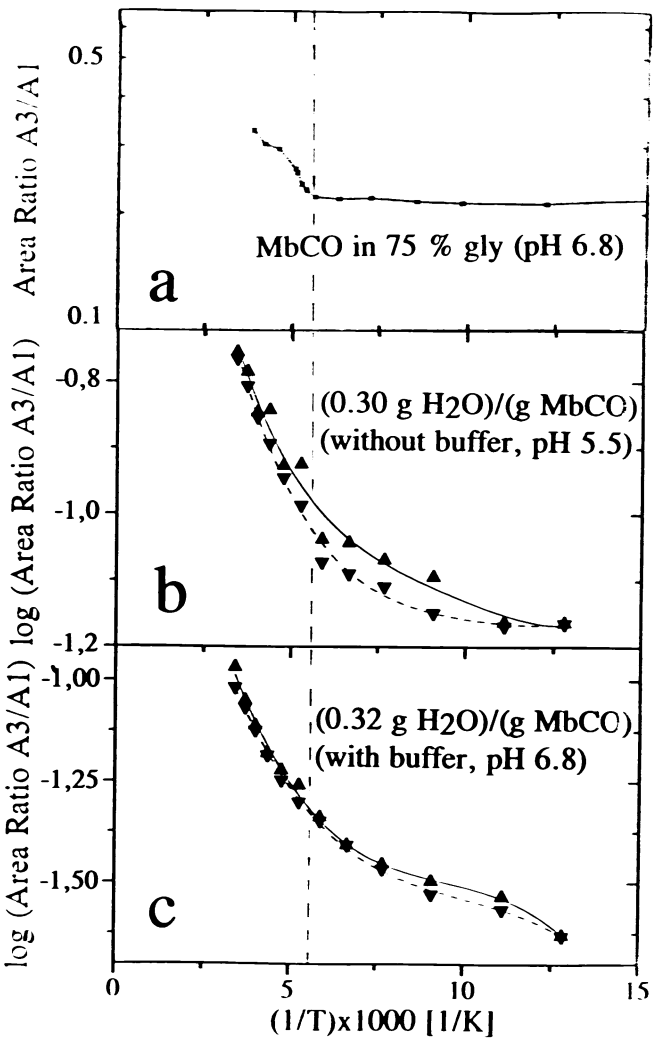

FIGURE 10 Comparison of log(area ratio A_3/A_1) plots versus $10^3/T$ in hydrated MbCO film containing (0.30 g water)/(g MbCO) in (*b*) and (0.32 g water)/(g MbCO) in (*c*), with that of MbCO in 75% glycerol/water solution in (*a*) which is taken from Ansari et al. (1987, Fig. 6). The vertical dashed line is at 180 K. See Fig. 3 for details.

in slope is abrupt at ≈180 K and that below that temperature the curve is parallel to the abscissa, but for (*b*) and (*c*) the slope changes only gradually over a wide temperature range. In Fig. 11 the HBWs of the band due to conformer A_1 in buffered (*b*) and unbuffered (*c*) hydrated MbCO films are shown as a function of temperature, and compared with those of MbCO in 75% glycerol/water solution (*a*) taken from Fig. 9 of Ansari et al. (1987). The dashed vertical line at 180 K again emphasizes that the change in HBWs is abrupt for (*a*) but gradual for (*b*) and (*c*). This difference in behavior must be due to differences in the solvents and their interaction with the protein and is interpreted as follows.

Ansari et al. (1987) have attributed the abrupt change at ≈180 K in plots of log(area ratio) of A_0/A_1 and A_3/A_1 vs. $1/T$ to a "slaved glass transition": below 180 K interconversion of conformers is frozen in, above ≈180 K the conformers interconvert freely within the time scale of the experiment. The dependence of conformer transitions on solvent characteristics is also seen in Fig. 4 of Hong et al. (1990) where the temperature dependence of MbCO dissolved either in 75% glycerol/water or in 60% ethylene glycol/water is compared in form of plots of log(area ratio A_0/A_1) vs. $1/T$. For MbCO in 60% ethylene glycol solution, the change in slope is shifted ≈20° to lower temperature. This scales nicely with differences in the glass transition temperatures of the two solvents (Luyet and Rasmussen, 1968). The glass→liquid transitions of the pure solvents have a width of a few degrees which indicates a comparatively narrow distribution of relaxation times.

However, for hydrated myoglobin, hemoglobin and lysozyme powders with water contents ≤(0.30 g water)/(g protein), Sartor et al. (1994a) have recently reported calorimetric annealing effects at all temperatures between ≈150 K and the denaturation temperature of the hydrated proteins and have attributed these to an exceptionally broad distribution of relaxation times. Alternatively stated, the glass transition in these hydrated proteins does not occur at a single temperature or in a narrow range of temperature, as it has been suggested for MbCO in glycerol/water solution (Ansari et al., 1987), but it occurs over a broad temperature range that extends from ≈150 K up to the denaturation temperature. Therefore, no single glass transition can be assigned to the three hydrated proteins. The gradual changes in slope seen in Figs. 10 and 11 for hydrated MbCO films is expected for such a broad distribution of relaxation times in the hydrated proteins because the width of a glass transition increases with increasing distribution of relaxation times (Moynihan et al.,

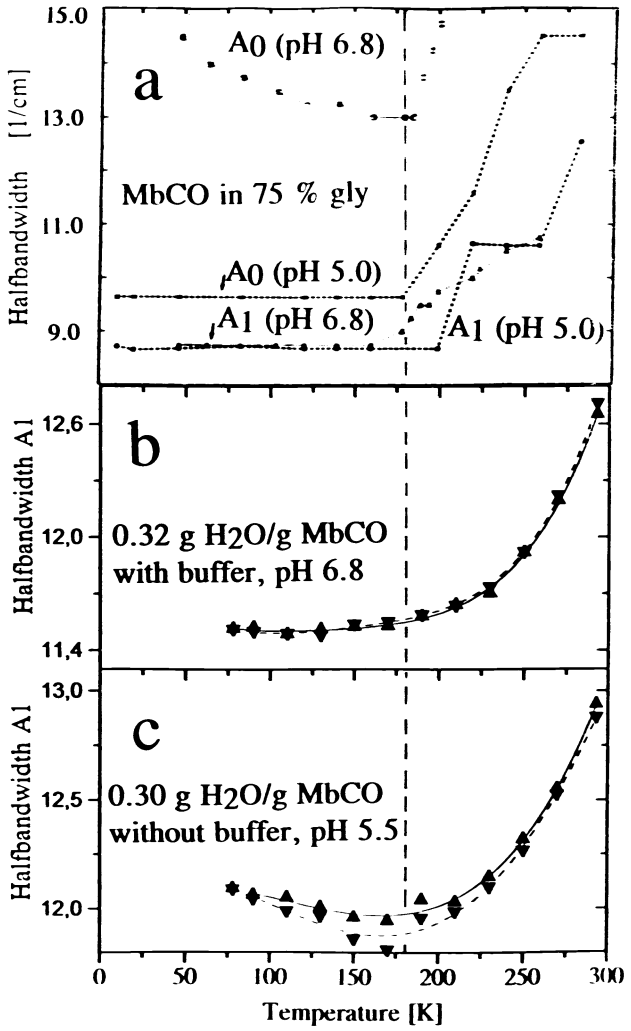

FIGURE 11 Comparison of temperature dependence of half-bandwidths of the A_1 conformer band in hydrated MbCO film containing (0.32 g water)/(g MbCO) in (*b*) and (0.30 g water)/(g MbCO in (*c*), with those of the A_1 and A_0 conformer bands of MbCO in 75% glycerol/water solution in (*a*) which is taken from Ansari et al. (1987, Fig. 9). The vertical dashed line is at 180 K. See Fig. 3 for details.

1976; Hodge and Berens, 1982). A striking example for exceptionally broad distribution of relaxation times in a synthetic polymer recently has been reported by Sartor et al. (1994b). As a consequence of the large breadth of relaxation time distribution, the glass transition endotherm in a DSC measurement becomes spread out over a very broad temperature range.

The gradual change in slope of the HBWs of the A_1 and A_0 conformer bands of hydrated HbCO films shown in Figs. 4 and 8 is also not that expected for a system with a glass→liquid transition of a typical width of a few degrees. For example, Clarke and Miller (1972) have reported that in the glass forming $Ca(NO_3)_2$-3/2KNO_3 solution the HBW of nitrate's symmetric stretching vibration decreases on cooling until T_g is reached, and then remains constant on further cooling in the glassy state. The sharp break in slope at T_g was attributed to decreasing reorientational mobility of the nitrate ion with decreasing temperature and its absence in the glassy state. Generally speaking, change in slope of the HBW of a vibrational band parameter at T_g is expected when this particular parameter depends on the volume because on supercooling of a solution the volume decreases and has a break at T_g; on further cooling in the glassy state the rate of decrease is much less. A sharp break in HBWs close to the temperature of the solvent's T_g is seen clearly for the A_1 and A_0 conformer bands of MbCO in 75% glycerol/water solution (Fig. 11a, taken from Ansari et al., 1987). It absence in hydrated MbCO films implies a *gradual* decrease in volume with decreasing temperature. For metMb crystal, decrease in volume on cooling from ≈300 K to 80 K by ≈3% has been reported ((Frauenfelder et al., 1987), but it is not known whether or not it decreases at a constant rate.

In Fig. 12 the temperature dependence of peak maxima of the A_1 (*b*) and A_0 (*c*) conformer bands of hydrated MbCO film (from Fig. 7) is compared with those of MbCO dissolved

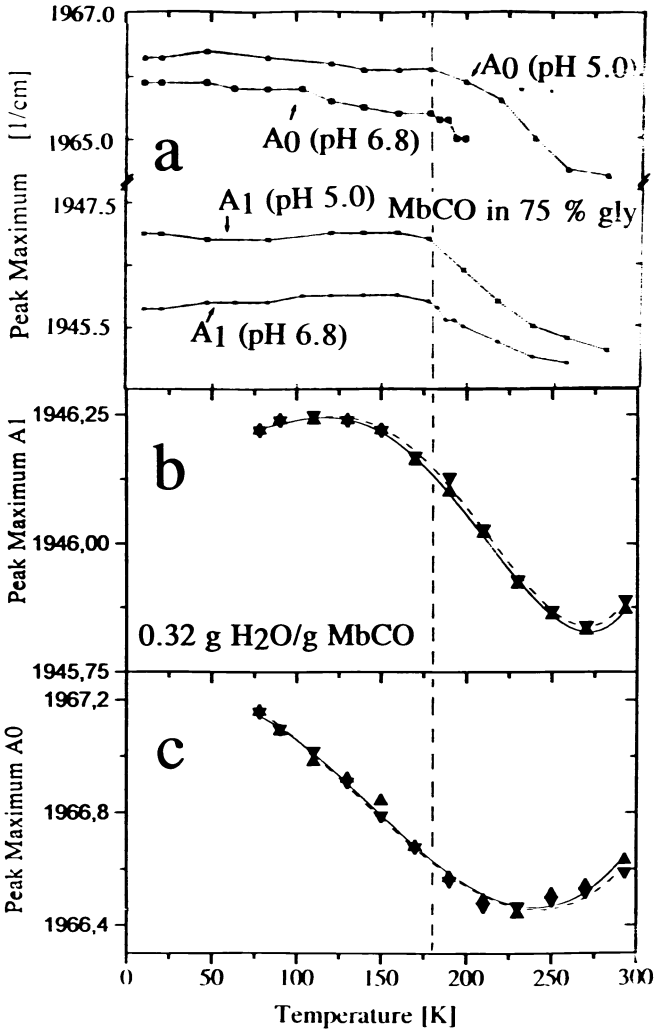

FIGURE 12 Comparison of temperature dependence of peak maxima of the A_1 and A_0 conformer bands in hydrated MbCO film containing (0.32 g water)/(g MbCO) in (*b*) and (*c*), with those of the A_1 and A_0 conformer bands of MbCO in 75% glycerol/water solution in (*a*) which is taken from Ansari et al. (1987, Fig. 8). The vertical dashed line is at 180 K. See Fig. 3 for details.

in 75% glycerol/water solution (*a*) taken from Fig. 8 of Ansari et al. (1987). The curves from Fig. 7 are for buffer-containing hydrated MbCO, but its features are very similar to those of unbuffered hydrated MbCO shown in Fig. 3. The dashed vertical line is at 180 K. For the A_1 conformer band, the peak frequencies increase with decreasing temperatures in both systems at T<250 K, reach a maximum value and a plateau region and thereafter decrease slightly again. For the hydrated MbCO film the change in slope where the plateau region is reached, is shifted to lower temperature. For the A_0 conformer band, similar increase in peak frequencies with decreasing temperature at $T < 220$ K is noted for both systems, but the maximum and the plateau region, seen for MbCO in 75% glycerol/water solution (*a*) at ≈180 K, is absent in hydrated MbCO (*c*). However, in curves (*c*) and also in those of conformer band A_0 in unbuffered hydrated MbCO shown in Fig. 3, the increase in peak maximum values with decreasing temperature seems to slow down at the lowest temperatures measured in these experiments. A further feature in the curves of the hydrated MbCO films is puzzling: for conformers A_1 (*b*) and A_0 (*c*), a distinct minimum is seen in the temperature range 240–270 K. This minimum is absent in the curves of MbCO in 75% glycerol/water solution (*a*). These changes in slope indicate a further process in hydrated MbCO which is absent in MbCO in 75% glycerol/water solution.

An important test for freezing-in of conformer equilibria is the time dependence of a band parameter at a given temperature, after cooling from a temperature where the conformers are still in equilibrium, and its approach toward the equilibrium value. This has been demonstrated by Ansari et al. (1987) in their Fig. 10, and on cooling from 195 K to 190 K approach to equilibrium of the A_0 conformer band area was observed on a time scale of several hours. Isothermal studies of the buffer-free hydrated MbCO film at 190 K or 170 K over several hours did not reveal systematic change of any of the band parameters. However, isothermal annealing at 150 K, after cooling from 170 K at a rate of ≈10 K min^{-1}, did show a systematic change in the value of the peak maximum of the A_1 conformer band from 1945.59 cm^{-1} to 1945.61 cm^{-1}, and small changes in % relative areas of the tree conformers. While the magnitude of these changes is hardly convincing, it is in the direction expected for an approach to equilibrium (see Figs. 3 and 4).

This study is in accord with the previous calorimetric study of hydrated protein powders, where physical aging effects at ≈≥150 K up to the denaturation temperature were attributed to an exceptionally broad distribution of relaxation times (Sartor et al., 1994a), but it goes beyond it for the following reasons.

1. For hydrated MbCO films exchange of the conformers A_1, A_2 and A_3 does not stop at ≈180 K, as in 75% glycerol/water solution, but it occurs over the whole temperature range investigated which is down to 78 K. This is a basic difference and it is clearly seen in Fig. 10 where for MbCO in 75% glycerol/water solution the log(area A_3/A_1 ratio) is parallel to the temperature axis below ≈180 K, but for hydrated MbCO it gradually changes over the whole temperature range down to 78 K. It is also seen in the other log(conformer area ratio) vs. $1/T$ plots (Figs. 6 and 9) and in Figs. 5 and 8 where the temperature dependence of percent relative area of the three A conformers is shown. These changes in conformer populations slowly decrease with decreasing temperatures, as can be seen by gradual changes in slope, but the curves do not become parallel to the temperature axis even for the lowest temperatures investigated here. The influence of the solvent and of its T_g has been interpreted in terms of a "slaved glass transition" (Ansari et al., 1987; Iben et al., 1989; Young et al., 1991). Recently Ansari et al. (1992) argue that the marked decrease in the rate of conformer interconversion near the solvent's T_g results from the enormous viscosity of the solvent, and that the conformational substates may not be "frozen" so much than "stuck." Whatever the correct interpretation, the freezing-in of conformer exchange in glassy glycerol/water solution, and its absence for hydrated MbCO films emphasizes the importance of the rigid medium. It is important to note that the temperature where in hydrated MbCO exchange of the A conformers is still observable, is also much lower than that of T_g of glassy bulk water made by "hyperquenching" which is at 136 ± 1 K for heating at a rate of 30 K min^{-1} (Johari et al., 1987; Hallbrucker et al., 1989), or of that of the vitreous, but freezable water fraction in hydrated methemoglobin observed at 169 ± 2 K for the same rate of heating (Sartor et al., 1992). In analogy to the behavior of MbCO in glycerol/water solution, I expect that in glassy dilute aqueous solution of MbCO which could be made by hyperquenching into the glassy state, exchange between the A conformers is also slowed down to a very low rate of interconversion or stopped below the solvent's T_g, e.g., below ≈130 K.

2. For the buffered MbCO film, plots of the log(area ratio) versus $1/T$ obtained on cooling and reheating are nearly superimposable (see Fig. 9). This suggests that for the time scale of the experiment the three A conformers are in, or close to, equilibrium over the whole temperature range investigated both on cooling and reheating. For the unbuffered MbCO film, however, the log(area ratio) versus $1/T$ plots obtained on reheating deviate at low temperatures from those obtained on cooling, and only on heating above ≈200 K do the two curves merge (see Fig. 6). This type of behavior and the direction of the deviation at low temperatures is consistent with the notion that at low temperatures conformer exchange is slowed down, but not completely frozen-in, and that it requires heating to ≈200 K for attaining the equilibrium distribution. This is in line with the isothermal annealing experiment at 150 K mentioned above. The difference in behavior of the two hydrated MbCO films is seen most clearly in Fig. 10, where in (*b*) the log(area A_3/A_1 ratio) versus $1/T$ plot of the unbuffered MbCO film is compared with that of the buffered film (*c*). For the same time protocol of the two experiments, deviation on heating is much more pronounced in (*b*) than in (*c*). I conclude that the presence of the buffer components accelerates exchange between the A conformers.

3. This study raises the question as to the values of the activation energy barriers for exchange between the three A conformer substates. Here it may be useful to compare the behavior of MbCO in glass-forming solution with those of other conformationally nonhomogeneous and rapidly interconverting compounds. Fishman et al. (1986, 1993) have first investigated by infrared spectroscopy for several low-molecular-weight solutes the influence of a solvent's T_g on the dynamics of conformer exchange. For example, for pure 2-chlorobutane freezing-in of its conformer populations was observed at 97 K, but for 2-chlorobutane dissolved in nujol exchange between its conformers stops already at 205 K. For pure 2-chlorobutane, the temperature region where exchange between the conformers stops, nicely correlates with its calorimetric T_g of ≈97 K, and this demonstrates that changes in conformational mobility coincide with those of translational and rotational mobility at its T_g. However, for 2-chlorobutane dissolved in nujol, freezing-in of its conformer populations correlates with the calorimetric T_g of the solvent of ≈210 K. In other words, if the activation energy for conformer exchange in 2-chlorobutane would have been based on its immobilization at ≈205 K in nujol, the value would have been much higher than the 19 kJ mol^{-1} reported in the literature. The above example is only one of many cited by Fishman et al. (1986), and it seems that this type of behavior is general. The authors clearly distinguish between the two cases and stress the point that freezing-in of conformer exchange at the pure liquid's T_g should not be confused with freezing-in at a solvent's T_g, and that in the latter case the solute is simply immobilized because of the extreme viscosity of the solvent. A solute even can be used as a conformational probe for determining a solvent's T_g, and Fishman et al. (1986) give several examples for this approach. Similar argument is expected to hold for MbCO in glass-forming solution such as in 75% glycerol/water, and it suggests that immobilization of the A conformer populations near the solvent's T_g is not due to the activation energy barriers for exchange between the A conformational substates, but is caused by the rigid medium and its extreme viscosity. Therefore, immobilization of MbCO's conformer population at the solvent's T_g should not be considered to be a glass transition of the protein. The exchange between the A conformational substates in hydrated MbCO films reported in this study for temperatures down to 78 K is further support for this view. I note that Ansari et al. (1992) also have attributed the marked decrease in the rate of interconversion of conformational substates near the solvent's T_g to the enormous viscosity of the solvent, and not to potential energy barriers for exchange between the conformers.

4. The exchange between the A conformers in hydrated MbCO films, with water contents of 0.30 and 0.32 (g water)-/(g MbCO), to temperatures as low as 78 K demonstrates water's influence on the dynamics of this process. This has to be compared with reports by Brown et al. (1983), where for MbCO samples with ≈≤0.2 (g water)/(g MbCO) uncoupling of CO conformer interconversion and freezing-in of their populations was reported even at 293 K (see Figs. 2 and 9 in Brown et al., 1983). Since the time scale of their infrared measurements is apparently similar to that used in this study, it follows that the enormous increase of the rate of interconversion between the A conformers seen in this study must be attributed to the higher water content. Extension of this study to even higher water content was not attempted because formation of ice on slow cooling to subzero temperatures interferes.

5. The results of this work are next compared with the many other studies of the dynamics of hydrated proteins and of protein crystals as a function of temperature and water content by various techniques (Morozov and Gevorkian, 1985; Frauenfelder and Gratton, 1986; Parak, 1986; Doster et al., 1986, 1989, 1990, 1991, 1993; Nienhaus et al., 1989; Goldanskii and Krupyanskii, 1989; Smith et al., 1990; Rupley and Careri, 1991; Srajer et al., 1991; Champion, 1992; Pethig, 1992; Pissis et al., 1992; Sartor et al., 1994). These studies have shown that at a given temperature internal mobility of the protein increases with increasing hydration. This is also called the "plasticizing effect" of water on the protein, thus increasing its mobility (Parak, 1986), and the increase in the exchange rate of the A conformers at 293 K with increasing hydration which is discussed above in paragraph 4 is consistent with these reports. Studies of the mobility of hydrated proteins or proteins crystals as a function of temperature indicate for a given water content increasing mobility of the protein at ≈180–200 K (Morozov and Gevorkian, 1985; Frauenfelder and Gratton, 1986; Parak, 1986; Doster et al., 1986, 1989, 1990, 1991, 1993; Nienhaus et al., 1989; Goldanskii and Krupyanskii, 1989; Smith et al., 1990; reviewed by Rupley and Careri, 1991; Srajer et al., 1991; Champion, 1992; Pethig, 1992; Pissis et al., 1992), or at ≈150–170 K on heating at a rate of 30 K min^{-1} (Sartor et al., 1994a). These temperatures are much higher than those reported in this study where exchange between the A conformer substates is still observable. This is not necessarily a contradiction because in most of these studies the whole protein is used as a probe whereas in this study a particular site of the protein is investigated. Nevertheless, it appears that increase in protein's mobility at ≈180–200 K clearly seen by other techniques also contributes to the shape of the plots reported in this study: first, in the two plots of the log(area ratio) of A_3/A_0 and A_3/A_1 vs. $1/T$ shown in Fig. 6, the approach of the heating curve to the cooling curve in this temperature region which has been interpreted above as attainment of the conformers' equilibrium distributions, could be due to increasing mobility of the protein. And second, in these plots and in those of Fig. 9, the change in slope is most pronounced between ≈150 and 200 K.

6. The temperature where for MbCO dissolved in, for example, 75% glycerol/water its A conformer populations are frozen-in on cooling, is similar to the temperature range where in protein crystals, and in hydrated protein films and powders internal mobility of the protein strongly decreases upon cooling (~180 K vs. ~180–200 K). This suggests a common origin. I propose that this correspondence of the two temperature regions is accidental, and that it is simply caused

by the value of the solvent's calorimetric T_g. Hong et al. (1990) already have noted the influence of the solvent on freezing-in of MbCO's conformer population: for MbCO dissolved in 60% ethylene glycol/water solution freezing-in occurs at ~160 K, which is ~20 K below the value in 75% glycerol/water solution (read from Fig. 4 of Hong et al., 1990). These values nicely scale with the T_g's of the two solvents (Luyet and Rasmussen, 1968). I expect that freezing-in of MbCO's A conformer populations also can be shifted to higher temperatures by choosing a suitable solvent with, e.g., a $T_g > 200$ K.

7. The ligand rebinding kinetics of photo-dissociated MbCO depends on the population of the A, or bound, conformer substates. For studies in glass-forming solution it is common practice to use below the solvent's T_g a temperature-independent distribution of conformer population for analyzing the data because exchange between the A conformers is immobilized by the solvent (Ansari et al., 1987; Hong et al., 1990). For studies of hydrated MbCO films, however, this work suggests that for optimal analysis of the data a temperature-dependent distribution of conformer populations should be used even for temperatures down to 78 K, and possibly below 78 K.

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