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A reduction of protein specific motions in co-ligated myoglobin embedded in a trehalose glass

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Trehalose, a disaccharide composed of two (1→1)-linked α, α units of glucopyranose, is a non reducing sugar widespread in nature and found in large quantities in the so called resurrection plants. These plants grow in the desert and are subjected to quite high temperatures (>50 °C) and extremely dry conditions without suffering damage. Moreover, they can be kept dry and apparently dead for more than 50 years in the total absence of metabolic processes. Upon rehydration, however, the vegetative cycle restarts. This process can be repeated several times with no apparent damage for the organisms. The stabilising role against the effects of high temperatures and dehydration is believed to be played by trehalose (Madin and Crowe 1975; Crowe et al. 1984; Bianchi et al. 1991; Panek 1995; Crowe et al. 1996).

Recently, Hagen et al. (1995, 1996) and Gottfried et al. (1996) studied CO recombination after flash photolysis in carbon monoxy myoglobin (MbCO) and hemoglobin (HbCO) embedded in a trehalose glass. They found that some part of protein dynamics was strongly inhibited. The CO molecule was no longer able to leave the heme pocket even at room temperature. In this letter we investigate the influence of embedding MbCO in a trehalose glass by other techniques, namely Mössbauer spectroscopy and optical absorption spectroscopy. Such investigations are stimulated by another fact. Protein denaturation by unfolding is certainly a consequence of fluctuations with very large amplitudes. Protein dynamics and protein unfolding are, therefore, related. Trehalose coating of a protein, which prevents denaturation, should also influence protein dynamics. Its study could help to understand some features of denaturation on a molecular level. The final aim of this research is an understanding of the mechanisms through which, at the molecular level, trehalose protects proteins against denaturation by high temperature and dehydration.

Mössbauer (Parak et al. 1982) and optical absorption spectroscopy (Di Pace et al. 1992; Leone et al. 1994; Cupane et al. 1995) show that protein dynamics are characteristically different at low and high temperatures. While harmonic modes dominate at low temperatures (Melchers et al. 1996), additional non-harmonic motions occur above about 180 K. These non-harmonic motions are often called protein specific motions since they are typical for proteins and are a prerequisite for protein function. Physically, protein specific motions can be understood as thermal fluctuations of a molecule between conformational substates (Frauenfelder et al. 1988).

Optical absorption and Mössbauer spectroscopy are complementary in terms of different time windows and the observation of different types of motions coupled to the heme iron in MbCO. The optical absorption spectra of a chromophore embedded in a matrix depend on the temperature, owing to the coupling of the optic electron with low frequency motions (soft motions) of the environment. A narrowing of the band and a peak displacement are observed when the temperature is lowered. The interaction of the optic electron with nuclear vibrations can be described within the framework of the Franck-Condon approximation. In particular, the coupling with soft modes where the population varies with temperature, is expected to cause a temperature dependent Gaussian broadening of the absorption band. This Gaussian broadening can be expressed as (Di Pace et al. 1992; Cupane et al. 1994):

$$\sigma^2(T) = A\langle \nu^2 \rangle \coth\left[h\langle \nu \rangle/(2\,k_B\,T)\right] + \sigma_{in}^2 \qquad (1)$$

with $A = N\,S_1\,R_1^2$

where k_B is Boltzmann's constant, N, $\langle \nu \rangle$, S_1 and R_1 the total number, the effective frequency of the bath of low fre-

Dedicated to Prof. Massimo Ugo Palma on the occasion of his 70th birthday.

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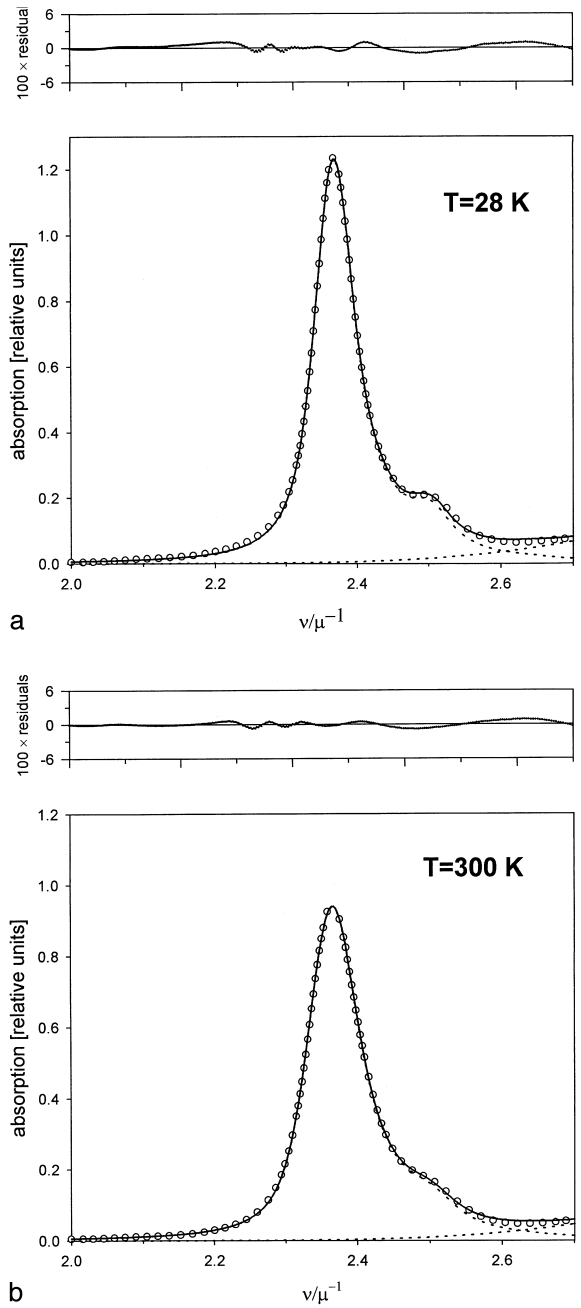

Fig. 1 a, b Spectra of "trehalose coated" horse MbCO at 28 K (**a**) and at 300 K (**b**). Circles represent the experimental points and the continuous line the fitting performed according to Eq. (3) of Di Pace et al. (1992). Dotted lines represent the fittings of the Soret band and the contribution of the N-band separately. For the sake of clarity not all the experimental points are reported. The residuals (i. e. the differences between the experimental and the reconstructed spectrum) are reported in the upper panel

quency modes and the effective linear and quadratic coupling constants, respectively. The term σ_{in} reflects an eventual broadening due to conformational heterogeneity. The value of the parameter A is obtained from the fitting procedure. The $\sigma^2(T)$ expression given by Eq. (1) has been obtained within the framework of the Franck-Condon harmonic approximation. Therefore, its temperature dependence is expected to obey the hyperbolic cotangent law only in the range where the soft nuclear motions to which the transition is coupled can be described within an harmonic approach. Non-harmonic contributions reveal themselves by a deviation. In the case of Soret band broadening of a heme protein, the vibrations of the iron with respect to the heme plane influence the optical absorption (Leone et al. 1994). Fluctuations of proteins among conformational substates can cause the appearance of non-harmonic contributions.

The area of the Mössbauer absorption spectrum of a ^{57}Fe nucleus is determined by the Lamb Mössbauer factor f:

$$f = \exp(-k^2 \langle x^2 \rangle) \quad (2)$$

where k is the wave vector of the ^{57}Fe Mössbauer radiation and $\langle x^2 \rangle$ is the mean square displacement of the iron with respect to the laboratory system. Note that all motions contribute which occur on a time scale faster than 100 ns. In contrast to optical absorption spectroscopy, which measures the vibrations of the iron with respect to the heme plane, Mössbauer spectroscopy is also sensitive to motions of the heme with respect to the protein molecule or of the molecules within the sample. The f-factor can easily be obtained from the absorption area of the Mössbauer spectrum (Parak and Reinisch, 1986). Besides the mean square displacement the shape of the absorption line allows one to draw conclusions about the type of motions. In a sufficiently thin absorber, harmonic motions yield narrow Lorentzian lines. Quasi diffusive motions occurring in deoxy myoglobin crystals above 200 K, which are protein specific, give rise to additional broad lines (Parak et al. 1982).

For our samples horse myoglobin (Ho-Mb) was purchased from Sigma (St. Louis, MO, USA) and used without further purification. For the sample used for optical absorption spectroscopy 2×10^{-5} mol/l lyophilized ferric protein was used in 10^{-1} mol/l phosphate buffer (pH = 7) and 10^{-2} mol/l trehalose. This solution was centrifuged, equilibrated with CO and reduced by anaerobic addition of about 3×10^{-2} mol/l sodium dithionite. Finally 1 ml of this solution was deposited on a suitable glass surface, which was then put in a desicator under CO atmosphere. For the sample used for Mössbauer spectroscopy 10^{-3} mol/l ^{57}Fe enriched myoglobin was used in 5×10^{-2} mol/l phosphate buffer (pH = 7.6) and 1 mol/l trehalose. The solution was equilibrated with CO and reduced by the addition of 10^{-2} mol/l sodium dithionite. The solution was dried under anaerobic conditions in a desicator under CO atmosphere. The sample was then put in a oven at 80 °C for about 10 hours.

Figure 1 shows the 28 K and the 300 K Soret band profile of trehalose coated MbCO and the fitting performed on the basis of the analysis proposed by Di Pace et al. (1992) and Cupane et al. (1995). In Fig. 2 a we compare the $\sigma^2(T)$ behaviour for MbCO in 65% v/v glycerol water solution and MbCO embedded in a trehalose glass. The solid lines show least squares fits of Eq. (1) to the σ^2-values obtained experimentally. The fitting parameters are given in Table 1. In water-glycerol solutions the harmonic

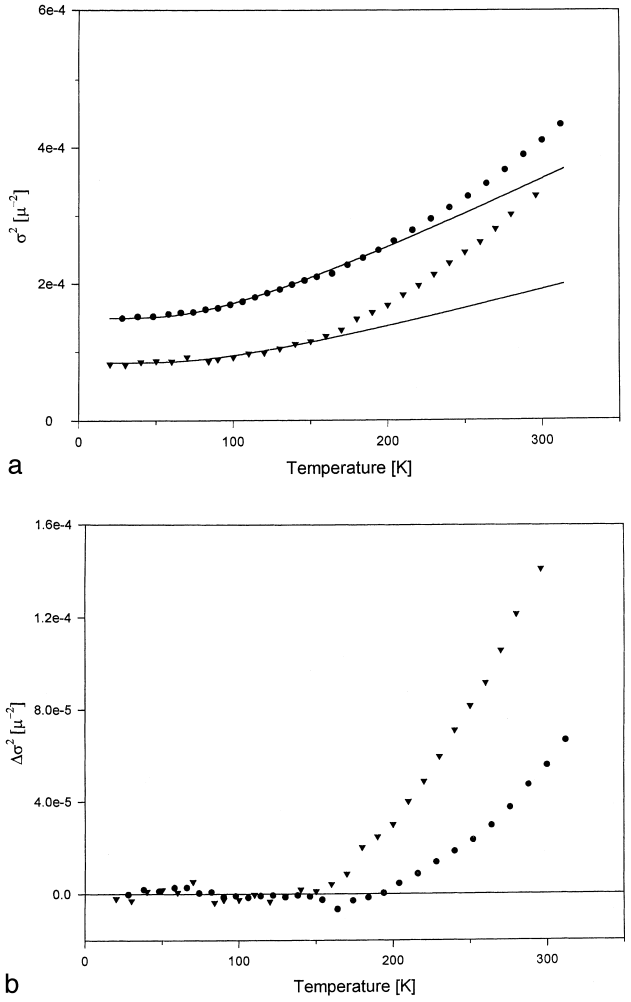

Fig. 2 **a** The width of the Gaussian, σ^2, as a function of temperature; circles: MbCO coated by trehalose, triangles: MbCO in glycerol water solution. **b** $\Delta\sigma^2$: the difference between data points and the continuous line in Fig. 2 a; circles: MbCO "trehalose coated", triangles: MbCO in 65% water glycerol. $\Delta\sigma^2$ reflects the amplitude of non-harmonic motions of the iron with respect to the heme plane

model reflected in Eq. (1) is obeyed only up to about 170 K, while it holds up to about 200 K in trehalose coated MbCO. The deviation from the harmonic part is more clearly seen in Fig. 2 b where the difference between the experimental σ^2 values and the solid line of Fig. 2 a is shown. Interestingly, the non-harmonic contributions to the motion of the iron with respect to the heme plane are also present when the protein is embedded in a trehalose glass i. e. under conditions where the constraints imposed by the solid external matrix are not released. However, these non-harmonic contributions have a much smaller amplitude in trehalose coated MbCO than in MbCO solution.

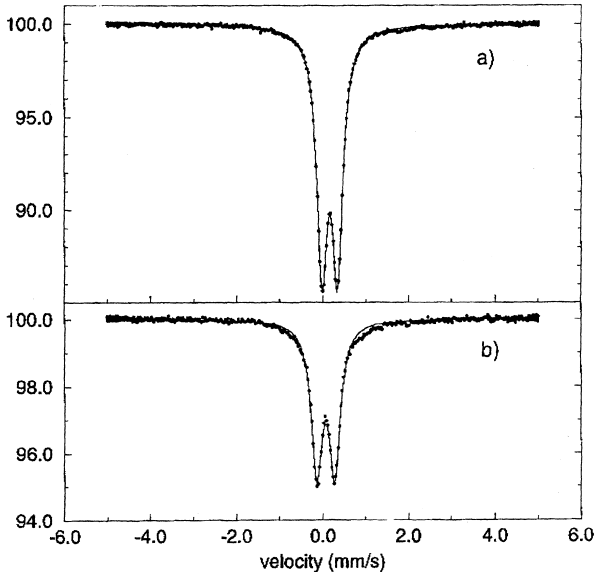

Fig. 3 Mössbauer spectra of trehalose coated MbCO **a**) at 80 K, **b**) at 320 K. The solid line gives at least squares fit of a quadrupole doublet of Lorentzians. Note the discrepancies with the experimental data at 320 K, indicating the presence of quasi diffusive motions

Figure 3 shows the Mössbauer spectrum of trehalose coated MbCO at 80 K (a) and at 320 K (b). The solid line is a least squares fit of a quadrupole doublet of Lorentzians with the well established parameters of MbCO (quadrupole splitting EQ = 0.36 mm/s, isomere shift IS = 0.29 mm/s with respect to metallic iron) and an experimental line width of 0.3 mm/s at 80 K. Note that the Lorentzians do not fit the 320 K spectrum very well. This discrepancy between the Lorentzian and the experimental data is a clear hint for additional broad lines indicating some diffusive motions in a limited space, as found in deoxy myoglobin crystals (Parak et al. 1982). Figure 4 shows the temperature dependence of the mean square displacement, $\langle x^2 \rangle$, of the trehalose coated MbCO, compared to deoxy myoglobin crystals. The dramatic reduction of the protein specific motions, much larger than the reduction detected by optical absorption spectroscopy, becomes obvious. While the onset of protein specific motions seen by Mössbauer spectroscopy is shifted to about 230 K by trehalose coating, the onset of non-harmonic Fe vibrations is shifted only to 200 K. In order to understand these results we have to emphasize once more that the mean square displacements determined by Mössbauer spectroscopy are sensitive to all motions within the protein while optical spectroscopy selects the vibrations of the iron with respect to the heme. Therefore, mean square displacements determined by Mössbauer spectroscopy are much larger than those deter-

Table 1 Values of parameters that characterize the coupling of the Soret band with low frequency motions

	$N S_1 R_1 \langle \nu \rangle^2 10^4/\mu^{-2}$	$\langle \nu \rangle$/cm^{-1}	σ_{in}/cm^{-1}
MbCO in 65% v/v glycerol/water	0.84±0.05	204±10	0
MbCO in trehalose, 14 hours in the oven (60 °C)	1.8 ±0.1	210±10	44±5

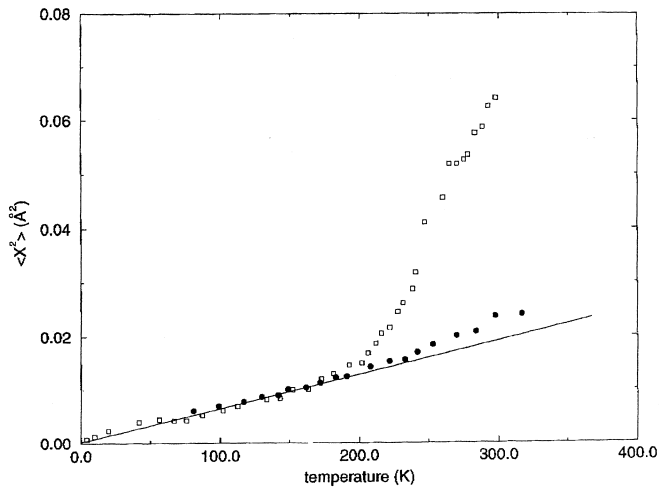

Fig. 4 Mean square displacement, $\langle x^2 \rangle$, of the heme iron as a function of temperature determined by Mössbauer spectroscopy. Values of the MbCO trehalose coated sample (circles) are compared with those of deoxy Mb crystals (squares). Protein specific motions reveal themselves as deviations from the straight line above about 180 K

mined by optical spectroscopy (Melchers et al. 1996). One also has to keep in mind that the CO molecule does not escape from the protein matrix if MbCO is coated by trehalose, even after prolonged illumination at room temperature. Obviously, the trehalose coating strongly hinders large scale diffusive fluctuations which open channels for the release of the CO into the solvent. Small scale motions localized within the molecule are less hindered. The reduction of the Mössbauer $\langle x^2 \rangle$-values in the high temperature region by trehalose coating is similar to that obtained by freeze drying (-Parak et al. 1987). The fluctuation of a molecule between different conformational substates is strongly reduced. Proteins are more rigid without a hydration shell. A comparison of the present results and the results on freeze dried myoglobin suggests that trehalose coating is extremely efficient in removing the water from the protein surface.

Our results are in full agreement with the findings reported by Hagen et al. (1995) and Gottfried et al. (1996) that trehalose coating suppresses any change in the average protein conformation after flash photolysis while not hindering the initial fast relaxation of the iron histidine linkage. Our findings also agree with the experimental facts used by Sastry and Agmon (1997). However, our experiments give no hint for an important role played by the water molecule within the heme pocket. The reported results enable us to conclude that trehalose coating prevents thermal denaturation by significantly reducing large scale fluctuations of protein specific motions that could bring about protein unfolding.

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