Trehalose Effect on Low Temperature Protein Dynamics: Fluctuation and Relaxation Phenomena

J. Schlichter,* J. Friedrich,* L. Herenyi,† and J. Fidy†
*Lehrstuhl für Physik Weihenstephan, Technische Universität München, D-85350 Freising, Germany and †Institute of Biophysics, Semmelweis University, Budapest, Hungary

ABSTRACT We performed spectral diffusion experiments in trehalose-enriched glycerol/buffer-glass on horseradish peroxidase where the heme was replaced by metal-free mesoporphyrin IX, and compared them with the respective behavior in a pure glycerol/buffer-glass (Schlichter et al., *J. Chem. Phys.* 2000, 112:3045–3050). Trehalose has a significant influence: spectral diffusion broadening speeds up compared to the trehalose-free glass. This speeding up is attributed to a shortening of the correlation time of the frequency fluctuations most probably by preventing water molecules from leaving the protein interior. Superimposed to the frequency fluctuation dynamics is a relaxation dynamics that manifests itself as an aging process in the spectral diffusion broadening. Although the trehalose environment speeds up the fluctuations, it does not have any influence on the relaxation. Both relaxation and fluctuations are governed by power laws in time. The respective exponents do not seem to change with the protein environment. From the spectral dynamics, the mean square displacement in conformation space can be determined. It is governed by anomalous diffusion. The associated frequency correlation time is incredibly long, demonstrating that proteins at low temperatures are truly nonergodic systems.

INTRODUCTION

A central problem of spectral diffusion experiments on chromoproteins concerns the question of to what extent the chromophore, which serves as a probe for the conformational dynamics, is also sensitive to processes that occur close to the surface of the protein or in the host matrix (Fritsch et al., 1996, 1998; Thorn-Leeson et al., 1997). A straightforward way to solve this problem are comparative experiments between solutions of the same protein in different host glasses.

A glass-forming material with a very specific interaction with proteins is trehalose (Branca et al., 1999; Crowe et al., 1994, 1998; Green and Angell, 1989). Trehalose is a disaccharide that has a high H-bonding affinity, thus may replace the water molecules close to the protein surface. It has a rather high glass-transition temperature, much higher, for instance, than a glycerol/water glass (Green and Angell, 1989; Miller et al., 1999), which we use as a reference matrix. Hence, the naive expectation is that the spectral diffusion dynamics of a protein may slow down in a trehalose environment because the rigidity of the protein is increased, thus the number of conformational degrees of freedom is reduced.

To check this idea, we performed a series of low-temperature spectral diffusion experiments with a modified horseradish peroxidase protein in a trehalose environment. The native heme chromophore of the protein was replaced by mesoporphyrin IX, whose inner ring protons undergo a light-induced proton transfer reaction. This reaction is exploited for spectral hole burning. Horseradish peroxidase was selected because of its stability, known crystal structure (Gajhede et al., 1997) and central heme position. We have recently investigated this protein in a glycerol/buffer glass (Schlichter et al., 2000a). Thus, a comparison of its spectral diffusion behavior in glycerol/buffer and in the respective trehalose-enriched glass became possible.

The influence of trehalose on protein dynamics has been investigated by different groups (Gottfried et al., 1996; Hagen et al., 1995, 1996; Sastry and Agmon, 1997). Hagen et al. investigated CO-rebinding in myoglobin after flash photolysis. The kinetics in trehalose was found to be faster than in glycerol. This result was attributed to the high viscosity of trehalose, which prevents protein relaxation and thus keeps the barriers for rebinding low. Another interpretation was offered by Sastry and Agmon (1997), who argued that the sugar matrix preserves water in the heme pocket. Water molecules in the protein interior may retain a high internal flexibility and thus speed up the rebinding kinetics. Relaxation of the protein, in contrast, may not be hampered at all through the trehalose environment. As we will show, this latter view is also favored by our experiments.

EXPERIMENTAL

Mesoporphyrin IX substituted horseradish peroxidase was prepared and purified as described elsewhere (Paul and Stigbrand, 1970; Teale, 1959). The protein was dissolved in a saturated trehalose/buffer mixture at pH 8, which, in turn, was mixed with glycerol. Glycerol was necessary to ensure a sufficiently good glass quality. We investigated two samples to check a possible dependence of the results on the concentration of trehalose. In sample 1, the concentration of

Received for publication 24 October 2000 and in final form 4 January 2001.

Address reprint requests to J. Friedrich, Technische Universität München, Lehrstuhl für Physik Weihenstephan, D-85350 Freising, Germany. Tel.: 0049–8161-71–3294; Fax: 0049–8161-71–4517; E-mail: J.Friedrich@lrz.tu-muenchen.de.

0006-3495/01/04/2011/07 $2.00

saturated trehalose/glycerol/buffer in the respective mixture was 40/50/10% v/v. In sample 2, the respective concentrations were 60/33/7% v/v. In the comparative experiment without trehalose, a glycerol/buffer mixture of 50/50% v/v was used (Schlichter et al., 2000a). The samples were quickly frozen to 4.2 K by plunging them into liquid Helium.

Hole burning was performed with a dye ring laser pumped by a frequency-doubled Nd:vanadate laser. Typical burn intensities were of the order of a few μW. During hole reading, the laser power was reduced by a factor of ~5000. We measured the spectral diffusion broadening σ of burnt-in holes as a function of waiting time t_w and aging time t_a. We define t_a as the time elapsed after the sample has reached its final temperature but before it is labeled with a hole. The first waiting/aging time experiment (Fig. 1 *A*) ran for about two weeks. During this time period, seven holes were burnt in a very narrow frequency range at different aging times reaching from 40 min to about 264 h. The second waiting time experiment (sample 2, Fig. 1 *B*) ran for about four days. In addition, we measured the so-called quasi homogeneous line width γ at 4.2 K.

The quantity measured in a spectral diffusion waiting-time hole-burning experiment is the change σ of the hole width as a function of time (Friedrich and Haarer, 1986; Breinl et al., 1984; Schlichter et al., 1999). σ is extracted from a line shape which is a convolution of the initial hole with the so-called spectral diffusion kernel which is the time-dependent part. To extract σ from the measured holes, a model is needed. Our data evaluation is based on the assumption that the diffusion kernel is a Gaussian (Skinner et al., 1999) with a variance σ^2. This assumption is quite in contrast to the TLS-model used to describe spectral diffusion in glasses, which predicts a Lorentzian diffusion kernel (Reinecke, 1979; Pack et al., 1990). The data evaluation procedure is described elsewhere (Schlichter et al., 2000a).

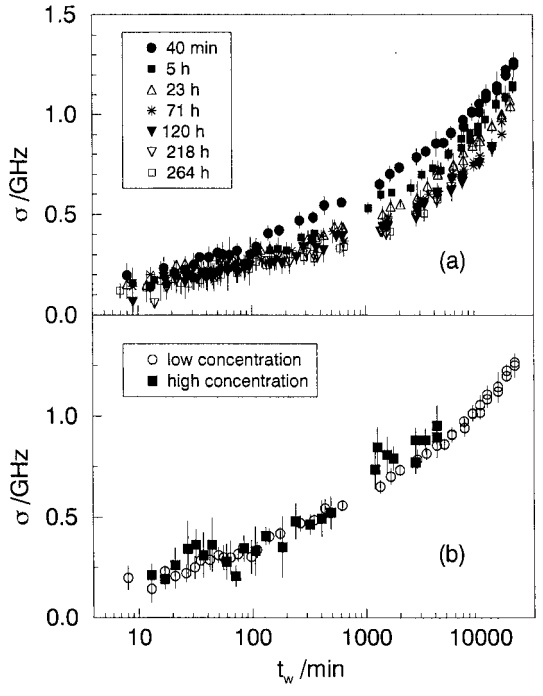

FIGURE 1. (*A*) Spectral diffusion width σ (square root of variance of the Gaussian diffusion kernel) as a function of waiting time t_w for various aging times (*insert*) in a semilogarithmic representation. Temperature 4.2 K. Sample: Mesoporphyrin IX-substituted horseradish peroxidase in a trehalose/glycerol/water glass. The overall width of the holes after burning ($t_w = 0$) was ~3 GHz. (*B*) A comparative experiment with a higher trehalose concentration.

RESULTS

Figure 1 *A* shows the width σ of the spectral diffusion kernel as a function of waiting time t_w for a series of aging times t_a at 4.2 K. Figure 1 *B* shows the comparative experiment where the trehalose concentration was changed from 40% to 60%. Three features should be stressed: First, in the representation of σ over log t_w, it is immediately obvious that $\sigma(t_w)$ does not increase proportional to log t_w. Otherwise, the data points should fall on straight lines. Second, spectral diffusion broadening is clearly subject to aging: as the aging times increase, σ becomes progressively smaller. However, the change of σ with aging time is much smaller than the respective change with waiting time. Third, an increase of the concentration of trehalose does not have a significant influence on spectral diffusion broadening: the data points for sample 1 and sample 2 (40% versus 60% trehalose concentration) measured at the same aging time fall on top of each other (Fig. 1 *B*).

Figure 2 shows the aging-time dependence of spectral diffusion broadening for the trehalose-enriched glass in comparison with the trehalose-free glass. In this experiment, the waiting time t_w was kept fixed at 10^4 min, and σ was

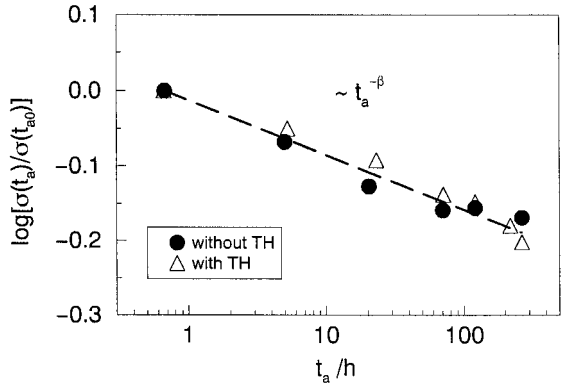

FIGURE 2 The decay of the spectral diffusion broadening with aging time t_a in a log-log representation measured at a waiting time $t_w = 10^4$ min. The data sets for the trehalose-enriched (*TH*) and for the trehalose-free glass follow the same power law. The respective coefficient $\beta = 0.07 \pm 0.01$. Note that the data points at the beginning of the aging experiments (t_{a0}) are arbitrarily normalized to 1.

measured as a function of t_a. The aging kinetics is the same for the two glasses within the error limits of the experiment. In both glasses, aging follows a power law governed by the same exponent $\beta = 0.07 \pm 0.01$.

Figure 3 *A* shows that all the spectral diffusion data of Fig. 1 fall on the same master plot if σ is scaled with the respective aging-time dependence as obtained from Fig. 2. Again, this scaling behavior holds for both glasses. The important message, which follows from this behavior, is that the two processes that contribute to spectral diffusion, namely fluctuation and relaxation (i.e., aging) are decoupled processes. The two data series in Fig. 3 *A* clearly demonstrate the trehalose effect on spectral diffusion. Figure 3 *B* shows the inhomogeneous width in the two glasses with and without trehalose. There is no significant difference

Figure 4 shows the hole widths as a function of hole area for the two glasses. The hole area is proportional to the absorbed radiation energy, hence, is a measure of spectral saturation. Extrapolation to area zero yields the so-called quasihomogenous hole width γ. For the trehalose-enriched glass, the respective value is 1.4 GHz, for the trehalose-free glass 0.9 GHz, i.e., γ differs by the same amount as σ, namely by $\sim(35 \pm 5)\%$. γ may be influenced by phonon scattering and by spectral diffusion processes. From the measured difference in the two glasses, we conclude that the latter processes dominate. Note that the results are independent of the power level of the laser (*different symbols*).

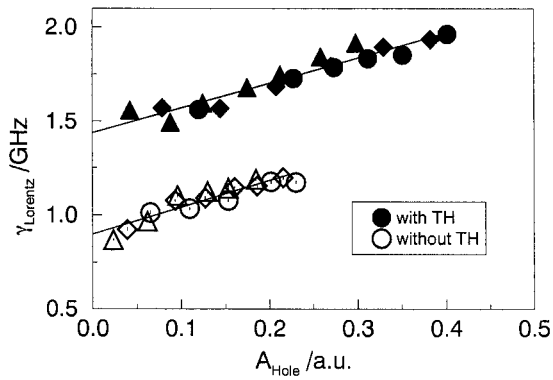

FIGURE 4 The hole width in the trehalose-enriched (*TH*) and the trehalose-free glass as a function of hole area. The extrapolation to area zero yields the so-called quasihomogenous line width. Different symbols indicate different burning intensities of the laser. Temperature: 4.2 K.

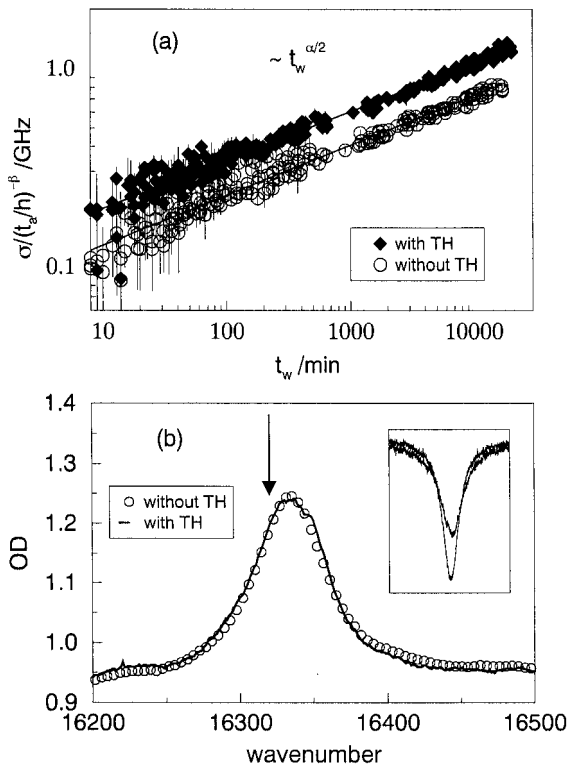

FIGURE 3 (*A*) Scaling behavior of the spectral diffusion data of horseradish peroxidase in trehalose (*TH*, see also Fig. 1) and trehalose-free glass in a log-log representation. The influence of aging time on spectral diffusion is scaled out by dividing σ by the respective aging time dependence $(t_a/h)^{-\beta}$. Both data sets follow the same power law. The exponent $\alpha/2 = 0.25 \pm 0.02$. (*B*) The inhomogeneous line width in the trehalose-enriched and the trehalose-free glass. The arrow indicates the frequency range where hole burning was performed. The insert shows a hole at the beginning and at the end of a spectral diffusion waiting-time experiment. The respective frequency scale covers 20 GHz. All data are taken at 4.2 K.

DISCUSSION

Conformational diffusion and spectral diffusion: The model

The data in Fig. 1 and the respective representation in Fig. 3 demonstrate that spectral diffusion is governed by a power law in time. Although the respective exponents are almost exactly the same for the two glasses, spectral-diffusion broadening is significantly larger in the presence of trehalose. What could be a possible reason for this behavior?

Either the increased spectral diffusion is an outside effect, i.e., structural dynamics in the trehalose environment is faster as compared to the pure glycerol/water environment, and this increased dynamics is reflected by an increased spectral diffusion of the chromophore via long range interactions, or the trehalose environment influences the internal parameters of the protein, which govern spectral diffusion. Let us, for the moment, assume that it is the internal parameters that are influenced by the trehalose environment. Then, the interesting question is which of these parameters could possibly be affected by trehalose. (Below we will present arguments that support the internal effect.)

The dynamics of a protein in conformation space is generally assumed to be governed by diffusive processes (Agmon et al., 1983). In the following, we base our arguments on the assumption that this is also true at 4 K. As a consequence, we describe the low-temperature spectral dy-

namics as a diffusive process in frequency space (Skinner et al., 1999).

Note that spectral diffusion in glasses is, at sufficiently low temperature, well described by the so-called Two Level System (TLS) model. In the TLS model, the dynamics is based on quantum jumps in rather localized independent double wells. Because such a dynamics leads to a logarithmic time pattern (Friedrich and Haarer, 1986), we do not consider it as appropriate for describing proteins.

It was shown by Stephens et al. (1997) that fluctuations in frequency space are directly related to fluctuations in conformation space via the frequency autocorrelation function $C(t)$. $C(t)$ determines the variance $\sigma^2(t)$ of the spectral diffusion kernel

$$\sigma^2(t) = \sigma_0^2[1 - C^2(t)]. \quad (1)$$

σ_0 is the spectral diffusion width for an infinitely long waiting time. To account for the observed power law, $C(t)$ has to be a stretched exponential,

$$C(t) = \exp[-(t/\tau)^\alpha]. \quad (2)$$

τ is the frequency correlation time. As documented by our experiments, spectral diffusion at 4 K is an extremely slow process. τ is many orders of magnitudes larger than the experimental time t. Hence, a first-order expansion of $C(t)$ is a very good approximation. From Eqs. 1 and 2, we get

$$\sigma(t_w) = 2^{1/2}\sigma_0(t_w/\tau)^{\alpha/2}. \quad (3)$$

Here, we have indexed the time t with a w to stress that the relevant time in Eq. 3 is the waiting time, i.e., the time span elapsed after burning the hole. Eq. 3 represents the desired power law for the spectral diffusion width σ.

The concept of a spectral diffusion experiment is that of an ideal diffusion experiment: an initially prepared δ-function-like hole diffuses apart. According to this view, Eq. 3 can be written in terms of a time-dependent spectral diffusion coefficient $D_\nu(t_w)$,

$$\sigma^2(t_w) = 2D_\nu(t_w)t_w, \quad (4)$$

with $D_\nu(t_w)$ given by

$$D_\nu(t_w) = [\sigma_0^2/\tau][t_w/\tau]^{\alpha-1}. \quad (5)$$

As is known from microscopic models, correlation functions of the stretched exponential type occur within some time window, only. However, compared to practical time scales, this time window may cover many orders of magnitude (Palmer et al., 1984). As a consequence, also Eq. 5 holds within a certain, yet huge, time window, only (Berlin, 1996; Berlin and Burin, 1996). As $t_w \to 0$, it breaks down. Note, however, that the zero time limit does not touch the results of our experiments because the shortest times (burning + first reading of a hole) are of the order of a few minutes. This is already well within the above-mentioned time window, considering the fact that spectral diffusion is also influenced by rather fast processes, which, in many cases, even dominate the quasihomogeneous width (Fig. 4 and respective discussion).

The trehalose effect in spectral diffusion

Eqs. 3–5 are characterized by three parameters, namely σ_0, τ, and α. Within an infinite time span the protein will explore the total conformational phase space, which is accessible at the beginning of the experiment. Hence, we identify σ_0 with the inhomogeneous width, which is generally assumed to scale with the area of the accessible conformational phase space. Because trehalose has a much higher glass transition temperature than glycerol/water, one would expect that, in the trehalose glass, a larger area of the conformation space gets frozen in, and, therefore, the inhomogeneous width would be larger. However, this is not the case. From this observation, it is obvious that conformations of the protein surface do not contribute to the inhomogeneous line broadening of a chromophore that is buried within the protein. The conclusion is that probing the protein with a chromophore concerns the nearby environment of the probe only.

From Fig. 3 *A*, it is obvious that, apart from the inhomogeneous width σ_0, also the slope parameter α is unaffected by the trehalose environment. Hence, the only parameter responsible for the influence of trehalose on spectral diffusion must be the frequency correlation time τ. The frequency correlation time, which characterizes spectral diffusion, also characterizes conformational diffusion (Stephens et al., 1997; Schlichter et al., 2000b). Then, the obvious influence of trehalose is to increase the conformational flexibility of the protein by decreasing τ. This finding may be counterintuitive, because one would expect that the higher glass transition temperature of trehalose would lower the flexibility of the protein. However, the same conclusion was also obtained by Sastry and Agmon (1997) when treating the influence of trehalose on the CO recombination in Mb. According to these authors, trehalose prevents the internal water molecules from leaving the heme pocket. Internal water obviously lowers the rigidity of the protein, thereby enhancing its conformational fluctuations. This seems to be one of the functional effects of internal water (Green et al., 1994). We stress that, also in horseradish peroxidase, there are several internal water molecules close to the heme pocket (Gajhede et al., 1997), so that the idea of fluctuation enhancement by reducing the rigidity should hold in this case, too.

From the fact that a trehalose environment affects the quasihomogeneous line width γ in the same way as the spectral diffusion broadening σ (Fig. 4), we conclude that γ, too, must be largely determined by spectral diffusion processes occurring, however, on time scales shorter than typically a minute. This observation yields additional support

to the idea that spectral diffusion is governed by one general parameter, namely the frequency correlation time τ.

The relation between spectral and conformational diffusion

Correlations between structural and spectral properties are quite common and have been addressed in the literature several times, also in context with the spectroscopy of chromoproteins (Agmon, 1988; Campbell et al., 1987; Srajer and Champion, 1991; Steinbach et al., 1991). In the following, we show that a simple relation exists between the mean square displacement in conformation space, $\langle x^2 \rangle$, and the spectral diffusion width σ. This relation allows us to relate the spectral dynamics in a straightforward way to the dynamics in conformation space (Schlichter et al., 2000b).

Consider, for example, a chromophore that interacts with an amino acid residue separated from it by a distance R_i. The interaction depends strongly on the distance, say like R_i^{-n}, and gives rise to a frequency shift,

$$\nu_i = cR_i^{-n}. \tag{6}$$

c is a difference coupling constant that is responsible for the solvent shift. The solvent shift originates, as a rule, from dispersion or higher-order electrostatic interactions, so a good number for n is 6. The total shift ν is obtained from summing Eq. 6 over all residues. However, because the protein is constantly diffusing around in its conformation space, R_i is uncertain within a bound x_i. This uncertainty gives rise to fluctuations $\delta\nu$ of the solvent shift of an individual protein molecule. In lowest order in x_i, we get

$$\delta\nu = -nc\Sigma_i x_i R_i^{-(n+1)}. \tag{7}$$

We take the square average of Eq. 7 over the whole ensemble. Assuming for the moment that the various residues move independently (Frauenfelder et al., 1979; Frauenfelder, 1989) and introducing for $\langle x_i^2 \rangle$ an average value $\langle\langle x^2 \rangle\rangle$ (averaged over the ensemble of protein molecules and over the residues), we arrive at

$$\langle \delta\nu^2 \rangle = \sigma^2 = \langle\langle x^2 \rangle\rangle n^2 c^2 \Sigma_i R_i^{-2(n+1)}. \tag{8}$$

Eq. 8 represents a transparent and practically important result: it tells us that the mean square displacement in frequency space, which is the quantity measured in a spectral diffusion experiment, is directly proportional to the average mean square displacement in conformation space.

There is another interesting aspect of Eq. 8. Because the distances R_i appear with a high negative power, it is only the first shell of residues around the chromophore that contributes significantly to the spectral diffusion fluctuations. Hence, the experiment is only sensitive to a few essential coordinates of the whole protein, which are the distances to the nearest neighbors around the chromophore. We call the respective part of the conformation space the "light" space to distinguish it from the rest of the conformation space, which we call the "dark" space.

Eq. 8 seems to be the key for understanding the origin of the stretched exponential dynamics: if the degrees of freedom in the light space are strongly coupled to the degrees of freedom in the dark space via constraining conditions, the dynamics will be governed by stretched exponential correlation functions (Berlin, 1996; Berlin and Burin, 1996; Palmer et al., 1984). In our case, the situation is even more complicated because, in addition to the spectral diffusion dynamics, we observe aging. Aging, too, seems to be governed by a stretched exponential (Fig. 2). In a similar way as relaxation in the dark space influences the dynamics in the light space, the opposite may occur as well. Hence, relaxation in the dark space slows down as time goes on. This slowing down manifests itself as a time dependence of the correlation time τ, which governs the dynamics in the light space. To get rid of the aging effect in our data (Fig. 1 *A*) we scaled σ with $t_a^{-\beta}$ (Fig. 2). This scaling results in the master plots shown in Fig. 3.

We measure a power law for $\langle \delta\nu^2 \rangle$, hence, according to Eq. 8, the mean square displacement in conformation space evolves with the same power law,

$$\langle\langle x^2 \rangle\rangle \propto t_w^{\alpha}. \tag{9}$$

Accordingly, conformational dynamics of proteins is governed by anomalous diffusion (Fritsch et al., 1998; Schlichter et al., 1999, 2000a; Skinner et al., 1999). The respective coefficient α, which is about 0.5 ± 0.04 for both glasses, is a measure of the inhomogeneity of conformation space. This inhomogeneity may arise from the respective inhomogeneity of the energy landscape (Frauenfelder, 1984, 1995; Ansari et al., 1985; Frauenfelder et al., 1988, 1991; Frauenfelder and Wolynes, 1994; Frauenfelder and Leeson, 1998). However, this inhomogeneity is not changed by the trehalose glass, despite its higher glass transition. α is the same for the two glasses.

Eq. 9 can be written in an analogous way to Eq. 5 by introducing a time-dependent conformational diffusion coefficient $D_x(t_w)$,

$$\langle\langle x^2 \rangle\rangle = 2D_x(t_w)t_w, \tag{10}$$

with $D_x(t_w)$ given by

$$D_x(t_w) = D_\nu(t_w)/[c^2 n^2 \Sigma_i R_i^{-2(n+1)}]. \tag{11}$$

From Eq. 3 we can determine τ, taking σ_0 and α from the experiment. From Eq. 8 we can determine the interaction factor $f^2 = n^2 c^2 \Sigma_i R_i^{-2(n+1)}$ by exploiting that, for $t_w \to \infty$, $\sigma^2(t_w) \to \sigma_0^2$. The associated $\langle\langle x^2 \rangle\rangle$ coincides with the values as obtained from x-ray experiments. We denote it by $\langle\langle x^2 \rangle\rangle_x$. An order of magnitude number is 0.05 Å^2 (Frauenfelder, 1989; Frauenfelder et al., 1979, 1988). We obtain $f^2 = \sigma_0^2/\langle\langle x^2 \rangle\rangle_x$. With this result, the conformational diffusion

coefficient can be written as

$$D_x(t_w) = [t_w/\tau]^{\alpha-1}\langle\langle x^2\rangle\rangle_x/\tau. \quad (12)$$

Considering absolute numbers: nonergodicity of low temperature proteins

We consider the factor f^2, first. Inserting for σ_0 a value of 25 cm^{-1}, and for $\langle\langle x^2\rangle\rangle_x$ a value of 0.05 $Å^2$, we obtain $|f| \cong$ 110 $cm^{-1}/Å$. This is a reasonable number. It tells us that an average displacement of about 1 Å of the nearest neighbor residues causes a spectral shift of the order of 100 wavenumbers.

Next we consider the absolute length scale probed by our experiment. The total spectral diffusion width probed by our experiment is about 1 GHz (Fig. 1). This width corresponds with a length scale $\langle\langle x^2\rangle\rangle^{1/2}$ that can be determined from the experimental results. From Eq. 8 we obtain

$$\sigma^2/\sigma_0^2 = \langle\langle x^2\rangle\rangle/\langle\langle x^2\rangle\rangle_x.$$

Inserting again the respective numbers for σ_0^2, σ^2 and $\langle\langle x^2\rangle\rangle_x$, we obtain, as an order of magnitude number, $\langle\langle x^2\rangle\rangle^{1/2} \cong 10^{-4}$Å. Note that the motion associated with this length scale is quasistatic.

We can also determine the correlation time τ. At 4 K it is incredibly long, of the order of 10^{17} s. We obtained similar numbers for cytochrome c and myoglobin-type proteins. This huge number just shows that proteins at 4 K are truly nonergodic systems. They never have a chance to sample their structural phase space in reasonable times. Nevertheless, as our experiments show, conformational dynamics can be probed, although the respective area of the phase space that is sampled is extremely small. The question immediately arises whether general conclusions on protein dynamics can be drawn from spectral diffusion experiments since the phase space sampled is so small. According to our view the answer is yes. One should keep in mind that the number of conformational states of a mid-sized protein is extremely large so that, even if the dynamically accessible conformational phase space is small, the associated number of states may still be large enough to comprise a statistically representative ensemble. The conclusion then is that the general dynamic laws, as reflected for instance in Eqs. 3, 10, and 12, may hold up to physiologically relevant temperatures, and that the dynamic changes that occur are solely due to the temperature dependence of the relevant parameters, i.e., of the correlation time τ or, equivalently of the diffusion coefficient D_x. As is well known, these parameters may change with temperature extremely rapidly (Zwanzig, 1988).

Summarizing conclusions

From the spectral diffusion experiments the time evolution of the mean square displacement in conformation space could be deduced. It is governed by anomalous diffusion.

Two independent processes determine spectral diffusion in proteins, namely relaxation and fluctuations. Relaxation shows up as an aging effect. Both processes follow a power law in time. The origin of these power laws is explained in terms of a "light" conformational phase space whose associated motions are directly seen in the experiment, and of a "dark" conformational phase space whose motions are observed only via their influence on the light motions. It is this mutual coupling that gives rise to the observed power laws. The influence of trehalose is traced back to the internal water molecules around the heme pocket, which cannot escape from the protein and which obviously speed up the motions in the light phase space by lowering the rigidity of the protein lattice. Because of this, the correlation time is shortened. As to our reasoning, this is the only influence of trehalose on protein dynamics. Because neither the aging dynamics nor the inhomogeneous width is changed, we infer that motions from the surface or from the host glass cannot significantly contribute to spectral diffusion. Obviously, it is largely determined by the nearby protein environment.

J. Friedrich acknowledges stimulating discussions with Y. Berlin, Northwestern University, Evanston, Ill.; S. F. Fischer, Technische Universität München; Y. Kagan, Kurchatov Institute, Moscow; and J. L. Skinner, University of Wisconsin, Madison. J. Fidy thanks S. Stavrov, Tel Aviv University, for suggesting experiments in trehalose glass.

Support from the Deutschen Forschungsgemeinschaft (SFB 533), the German–Hungarian collaboration program (UNG 005 97), from the Fonds der Chemischen Industrie and from the Hungarian grants OTKA T25545, ETT425/1996, and MKM FKFP 1191/1997 is gratefully acknowledged.

We thank Mrs. Rozsa Markacs for technical assistance.

REFERENCES

Agmon, N., and J. J. Hopfield. 1983. CO binding to heme proteins: a model for barrier height distributions and slow conformational changes. *J. Chem. Phys.* 79:2042–2053.

Agmon, N. 1988. Reactive lineshape narrowing in low-temperature inhomogeneous geminate recombination of CO to myoglobin. *Biochemistry.* 27:3507–3511.

Ansari, A., J. Berendzen, S. F. Bowne, H. Frauenfelder, I. E. T. Iben, T. B. Saucke, E. Shyamsunder, and R. D. Young. 1985. Protein states and proteinquakes. *Proc. Natl. Acad. Sci. USA.* 82:5000–5004.

Berlin, Y. A., and A. L. Burin. 1996. Hierarchically constrained dynamics of the configurational coordinate for rate processes in complex systems. *Chem. Phys. Lett.* 267:234–243.

Berlin, Y. A. 1996. Irreversible random transition theory as applied to rate processes in condensed media: transient effects of constrained configuration rearrangements in complex systems. *Chem. Phys.* 212:29–39.

Branca, C., S. Magazu, G. Maisano, and P. Migliardo. 1999. Anomalous cryoprotective effectiveness of trehalose: raman scattering evidences. *J. Chem. Phys.* 111:281–287.

Breinl, W., J. Friedrich, and D. Haarer. 1984. Spectral diffusion of a proton-transfer system in an amorphous organic host: quinizarin in alcohol glass. *J. Chem. Phys.* 81:3915–3921.

Campbell, B. F., M. R. Chance, and J. M. Friedman. 1987. Linkage of functional and structural heterogeneity in proteins: dynamic hole burning in carboxymyoglobin. *Science.* 238:373–376.

Crowe, J. H., J. F. Carpenter, and L. M. Crowe. 1998. The role of vitrification in anhydrobiosis. *Annu. Rev. Physiol.* 60:73–103.

Crowe, J. H., S. B. Leslie, and L. M. Crowe. 1994. Is vitrification sufficient to preserve liposomes during freeze-drying. *Cryobiology.* 31:355–366.

Frauenfelder, H. 1984. From atoms to biomolecules. *Helv. Phys. Acta.* 57:165–187.

Frauenfelder, H. 1989. The Debye–Waller-factor from villain to hero in protein crystallography. *Int. J. Quantum Chem.* 35:711–715.

Frauenfelder, H. 1995. Complexity in proteins. *Nature Struct. Biol.* 2:821–823.

Frauenfelder, H., F. Parak, and R. D. Young. 1988. Conformational substates in proteins. *Ann. Rev. Biophys. Chem.* 17:451–479.

Frauenfelder, H., G. A. Petsko, and D. Tsernoglou. 1979. Temperature-dependent x-ray diffraction as a probe of protein structural dynamics. *Nature.* 280:558–563.

Frauenfelder, H., S. G. Sligar, and P. G. Wolynes. 1991. The energy landscapes and motions of proteins. *Science.* 254:1598–1603.

Frauenfelder, H., and D. Thorn-Leeson. 1998. The energy landscape in non-biological and biological molecules. *Nature Struct. Biol.* 5:757–759.

Frauenfelder, H., and P. G. Wolynes. 1994. Where the physics of complexity and simplicity meet. *Physics Today.* 47:58–64.

Friedrich, J., and D. Haarer. 1986. Structural relaxation processes in polymers and glasses as studied by high resolution optical spectroscopy. *In* Optical Spectrocopy of Glasses. I. Zschokke, editor. Reidel, Dordrecht, The Netherlands.149–198.

Fritsch, K.-D., A. Eicker, J. Friedrich, B. M. Kharlamov, and J. M. Vanderkooi. 1998. Spectral diffusion in proteins. *Europhys. Lett.* 41: 339–344.

Fritsch, K.-D., J. Friedrich, F. Parak, and J. L. Skinner. 1996. Spectral diffusion and the energy landscape of a protein. *Proc. Natl. Acad. Sci. USA.* 93:15141–15145.

Gajhede, M., D. J. Schuller, A. Henriksen, A. T. Smith, and T. L. Poulos. 1997. Crystal structure of horseradish peroxidase C at 2.15 Å resolution. *Nature Struct. Biol.* 4:1032–1038.

Gottfried, D. S., E. S. Peterson, A. G. Sheikh, J. Wang, M. Yang, and J. M. Friedman. 1996. Evidence for damped hemoglobin dynamics in a room temperature trehalose glass. *J. Phys. Chem.* 100:12034–12042.

Green, J. L., and C. A. Angell. 1989. Phase relations and vitrification in saccharide–water solutions and the trehalose anomaly. *J. Phys. Chem.* 93:2880–2882.

Green, J. L., J. Fan, and C. A. Angell. 1994. The protein-glass analogy: some insights from homopeptide comparisons. *J. Phys. Chem.* 98: 13780–13791.

Hagen, S. J., J. Hofrichter, and W. A. Eaton. 1995. Protein reaction kinetics in a room-temperature glass. *Science.* 269:959–962.

Hagen, S. J., J. Hofrichter, and W. A. Eaton. 1996. Geminate rebinding and conformational dynamics of myoglobin embedded in a glass at room temperature. *J. Phys. Chem. B.* 100:12008–12021.

Miller, D. P., J. J. de Pablo, and H. R. Corti. 1999. Viscosity and glass transition temperature of aqueous mixtures of trehalose with borax and sodium chloride. *J. Phys. Chem. B.* 103:10243–10249.

Pack, D. W., L. R. Narasimhan, and M. D. Fayer. 1990. Solvation shell effects and spectral diffusion: photon echo and optical hole burning experiments on ionic dyes in ethanol glass. *J. Chem. Phys.* 92: 4125–4138.

Palmer, R. G., D. L. Stein, E. Abrams, and P. W. Anderson. 1984. Models of hierarchically constrained dynamics for glassy relaxation. *Phys. Rev. Lett.* 53:958–961.

Paul, K. G., and T. Stigbrand. 1970. Four isoperoxidases from horseradish roots. *Acta Chem. Scand.* 24:3607–3617.

Reinecke, T. L. 1979. Fluorescence linewidth in glasses. *Sol. State Commun.* 32:1103–1106.

Sastry, G. M., and N. Agmon. 1997. Trehalose prevents myoglobin collapse. *Biochemistry.* 36:7097–7108.

Schlichter, J., J. Friedrich, L. Herenyi, and J. Fidy. 2000a. Protein dynamics at low temperatures. *J. Chem. Phys.* 112:3045–3050.

Schlichter, J., K.-D. Fritsch, J. Friedrich, and J. M. Vanderkooi. 1999. Conformational dynamics of a low temperature protein: free base cytochrome *c*. *J. Chem. Phys*. 110:3229–3234.

Schlichter, J., K.-D. Fritsch, and J. Friedrich. 2000b. Dynamics of frozen proteins as investigated by optical hole burning experiments. *J. Mol. Liquids.* 86:127–136.

Skinner, J. L., J. Friedrich, and J. Schlichter. 1999. Spectral diffusion in proteins: a simple phenomenological model. *J. Phys. Chem. A.* 103: 2310–2311.

Srajer, V., and P. M. Champion. 1991. Investigation of optical line shapes and kinetic hole burning in myoglobin. *Biochemistry.* 30:7390–7402.

Steinbach, P. J., A. Ansari, J. Berendzen, D. Braunstein, K. Chu, B. R. Cowen, D. Ehrenstein, H. Frauenfelder, B. Johnson, D. C. Lamb, S. Luck, J. R. Mourant, G. U. Nienhaus, P. Ormos, R. Phillipp, A. Xie, and R. D. Young. 1991. Ligand binding to heme proteins: the connection between dynamics and function. *Biochemistry.* 30:3988–4001.

Stephens, M. D., J. G. Saven, and J. L. Skinner. 1997. Molecular theory of electronic spectroscopy in nonpolar fluids: ultrafast solvation dynamics and absorption and emission line shapes. *J. Chem. Phys*. 106: 2129–2144.

Teale, F. W. J. 1959. Cleavage of the hemoprotein link by acid methyl ethyl ketone. *Biochim. Biophys. Acta.* 35:543–550.

Thorn-Leeson, D., D. A. Wiersma, K.-D. Fritsch, and J. Friedrich. 1997. The energy landscape of myoglobin: an optical study. *J. Phys. Chem. B.* 101:6331–6340.

Zwanzig, R. 1988. Diffusion in a rough potential. *Proc. Natl. Acad. Sci. USA.* 85:2029–2030.