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Structural fluctuations and aging processes in deeply frozen proteins

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Frozen proteins are nonergodic systems and are subject to two types of structural motions, namely relaxation and fluctuation. Relaxation manifests itself in aging processes which slow the fluctuations. Within certain approximations we are able to experimentally separate the aging dynamics from the fluctuation dynamics by introducing two time parameters, namely an aging time t_a and a waiting time t_w. Both processes follow power laws in time. The fluctuation dynamics shows features of universality characterized by a rather uniform exponent of $1/4$. This universality features were shown to be possible due to a random walk on a 1D random trajectory in conformational phase space. A very interesting aspect of protein dynamics concerns the influence of the host solvent on structural motions of the protein cores. We present results for sugar solvents and discuss possible mechanisms.

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Introduction

Investigating structural dynamics of low temperature proteins, it is a fair question to ask, whether specific features as compared, for instance, to ordinary polymers or glasses are to be expected. Proteins are the nanomachines which keep life running. It is obvious, that this cannot be accomplished by just random motions which characterize the dynamics of dead polymers. Proteins have very specific structural features which are obviously linked to specific dynamical features, even in the frozen state. They are heteropolymers built from a stock of 20 well-defined aminoacids. Typical molecular weights are between 10^4 and 10^5, but there are also much larger assemblies. A characteristic feature is the rich variety of molecular interactions, most important among them the hydrophilic and the hydrophobic interactions. These two types of interactions are mainly responsible that proteins attain well defined structures. But, as is clear from the nature of these interactions, this is only possible if the solvent contains a sufficient amount of water. The strong interaction of proteins with water makes them sensitive to changes in their environment, which then may show up as changes in their structure and their respective dynamics.

Because of their well defined structure, Schrödinger called proteins «aperiodic crystals» to stress the fact that they are structurally well defined but lack translational periodicity. However, organization and order is just one aspect of proteins. A quite specific feature is that order in proteins is accompanied by disorder. It is this in-between nature which makes them very different from dead polymers and which points to possible specific features in their dynamics.

In the following we present an overview of our spectral diffusion experiments on a series of modified heme-proteins. We will show that low temperature proteins are truely nonergodic systems which never reach equilibrium. In addition we will show that their dynamics at low temperature differs in a characteristic fashion from the respective one of glasses. It shows certain aspects of universal features which we believe to be due to random walks on 1D stochastic conformational trajectories.

Experimental

In our experiments we investigate the dynamical behavior of low temperature proteins by measuring the absorption frequency of a dye molecule, a so-called chromophore, in the interior of the protein. Since this chromophore couples to the aminoacids in its environment, dynamical processes of the protein lead to fluctuations in its absorption frequency. Hence, if the linewidth of the chromophore is small enough, one has

a very sensitive tool for the measurement of protein dynamics.

All our measurements so far were done with heme proteins. The heme group, however, which is a natural constituent of this type of proteins, is not suitable as chromophore for our experiments, since the natural widths of its optical transitions are rather broad. To circumvent this problem, we used slightly modified proteins, where the natural heme group was substituted by very similar dye molecules, which show linewidths below 1 GHz (at a temperature of 4.2 K). In this case, even dynamical processes of the proteins which are associated with small changes in the transition energy of the chromophore are — in principle — measurable.

However, another problem has to be solved, to do this. Low temperature proteins do not have a well defined groundstate, but can exist in a great number of almost degenerate «conformational substates» [1,2], which are characterized by small differences in the structural arrangement of their aminoacids. Hence, in an ensemble of proteins every chromophore interacts with a slightly different environment. Accordingly, the absorption profile of an ensemble is inhomogeneously broadened. Typical band widths of such inhomogeneous protein spectra can be of the order of hundred wavenumbers. As a consequence, the small frequency fluctuations associated with conformational changes are completely hidden below this inhomogeneous line. To monitore them, one needs a more sophisticated experimental technique than simple absorption spectroscopy. In our measurements, spectral hole burning was used [3]. For this kind of experiment, a narrow-line laser illuminates the protein ensemble at a certain position in the inhomogeneous band. Proteins with suitable absorption frequencies are excited and photochemically bleached. Hence, the number of absorbing molecules at the laser frequency is decreased, and a gap (a so-called spectral hole) occurs in the absorption spectrum of the protein ensemble. The width of such a hole is comparable to the linewidth of a single chromophore. In addition it can be shown, that a spectral hole behaves exactly like an ensemble of proteins with identical absorption frequencies at the time of hole burning. The problem of inhomogeneous broadening can therefore be circumvented.

In our experiments the so-called spectral diffusion, i.e., the broadening of spectral holes (due to structural fluctuations) is monitored as a function of time. Two experimental parameters are important. After cooling the sample very rapidly from room temperature to 4.2 K, we wait for a certain time, the so-called *aging time* t_a, before burning a hole. Then, after a second time period, called the *waiting time* t_w, the shape of the (broadened) hole is measured. We will show later, that the broadening of spectral holes as a function of t_a can be connected with relaxational processes in the protein, whereas the dependence on t_w monitores (stationary) fluctuations of the molecule.

Mathematically the form of a spectral hole at later times is given by a convolution of the initial holeshape with a so-called spectral diffusion kernel $p(\nu_1, t_1|\nu_0, t_0)$. The width $\sigma(t_a, t_w)$ of this kernel is extracted from our experiments. Of course, since the hole-broadening is caused by structural fluctuations, the time dependence of $\sigma(t_a, t_w)$ monitores the structural dynamics of the proteins.

Results

Typical experimental observations

We did a series of spectral diffusion experiments with different heme-proteins (horseradish peroxidase, myoglobin, cytochrome c) containing various types of chromophores. In addition the effect of different solvents as well as the influence of deuteration on the low temperature dynamics of these proteins was investigated. In all these experiments we found a very similar behavior of our samples, which seems to be of a rather universal nature. As an example, measurements on horseradish peroxidase (HRP) in a water/glycerol matrix will be presented in this chapter [4]. (Free-base mesoporphyrin IX served as a chromophore in this protein.)

In Fig. 1 the results of this experiment are given. The broadening of six spectral holes (burned after aging times between 40 min and about eleven days) is shown as a function of the waiting time t_w. Two of the main results of our measurements can be recognized immediately from this figure: obviously the broadening of the holes is governed by a power law in t_w — the data follow straight lines in a double-logarithmic representation. In addition there are clear aging effects, the broadening of the holes which were burned at longer aging times t_a is smaller. This can be seen more clearly in the insert of the figure, which shows the dependence of the hole broadening on t_a at a fixed waiting time t_w as obtained from a «cut» through the data of Fig. 1 along the dashed line. Since the representation is double-logarithmic again, obviously this time dependence is governed by a power law, too. So what we find is:

$$\sigma(t_a, t_w) \propto \left(\frac{t_a}{T}\right)^{-\beta} \left(\frac{t_w}{\tau_0}\right)^{\alpha/2}. \qquad (1)$$

For the exponents $\alpha/2$ and β all our experiments yielded very similar values, namely about 0.25 and

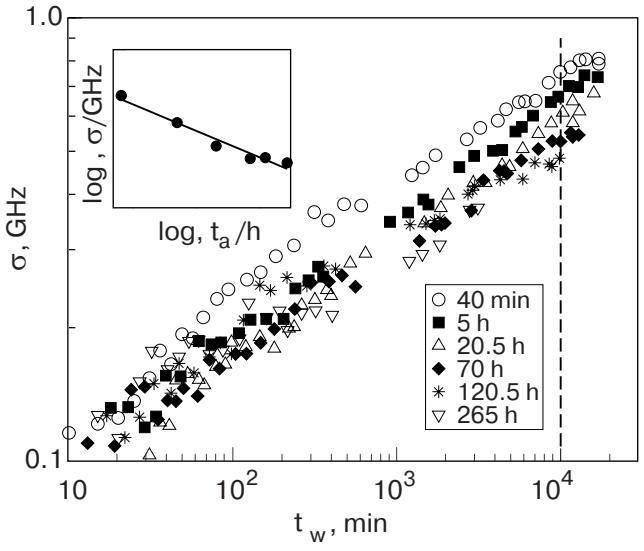

Fig. 1. Results of a spectral diffusion experiment on horseradish peroxidase at $T = 4.2$ K. The broadening of six spectral holes burned after different aging times t_a (see legend) is shown as a function of the waiting time t_w. Obviously the time dependence on t_w is given by a power law: $\sigma \propto t_w^{\alpha/2}$. The insert shows the hole-broadening at a fixed waiting time as a function of t_a. Again a power law is found: $\sigma \propto t_a^{-\beta}$.

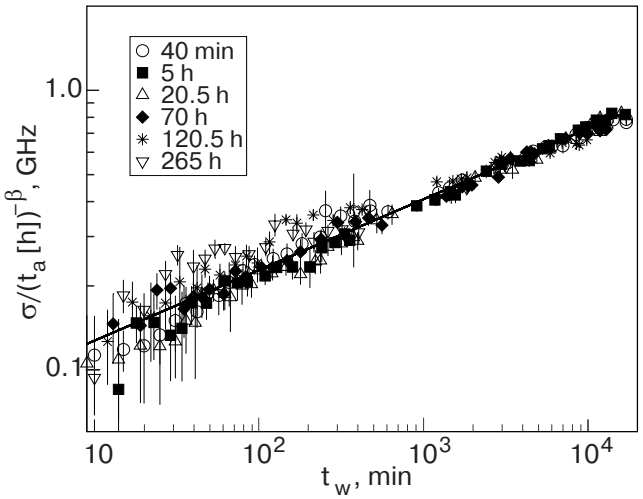

Fig. 2. Scaling the data of Fig. 1 with their aging time dependence yields a so-called «masterplot». It contains only the stationary contributions to the spectral diffusion. Again, the power-law behavior in t_w can clearly be seen.

0.07, respectively. A possible explanation for this apparently universal behavior will be given below.

As can be seen from Eq. (1), it is possible to separate the influence of aging time and waiting time in the experimental results, i.e., we can scale our data with their t_a-dependence to get a plot that is independent of t_a (see Fig. 2). In this so-called masterplot only the *stationary fluctuations* of the proteins show up; in contrast the t_a-dependence monitors nonequilibrium processes in the proteins, i.e., their relaxation to an equilibrium structure.

It should be emphasized that the numerical values of the above mentioned exponents depend on the microscopic picture which is used for the interpretation of the data (and which determines the mathematical form of the diffusion kernel); however, the power-law behavior itself is an independent experimental fact. In our earlier works we tried to analyze our spectral diffusion measurements in the framework of a TLS-theory, which is well known from the theory of low temperature glasses [5]. However, when we started to pay more attention to the aging effects which can be observed in protein dynamics, it turned out that a satisfactory explanation of our data with a TLS-theory was impossible [6]. This is seen very well in Fig. 3. Here a fit of the data of Fig. 3 by a (nonequilibrium) TLS-theory is attempted; obviously the agreement with the experiment is very poor.

However, this can be understood quite easy. In the TLS-theory spectral diffusion is explained by coupling the chromophore to an ensemble of two-level-systems with a broad range of relaxation times. At a time t_a after cooling down the sample, only TLS with time constants smaller than t_a have reached thermal equilibrium. Their influence on the broadening of a spectral hole will saturate after a waiting time $t_w > t_a$, since then any subensemble of these TLS, which has been «marked» by hole burning, has reached its equilibrium distribution again. If there are TLS with time constants larger than t_a, there will be spectral diffusion even for longer waiting times, but in addition

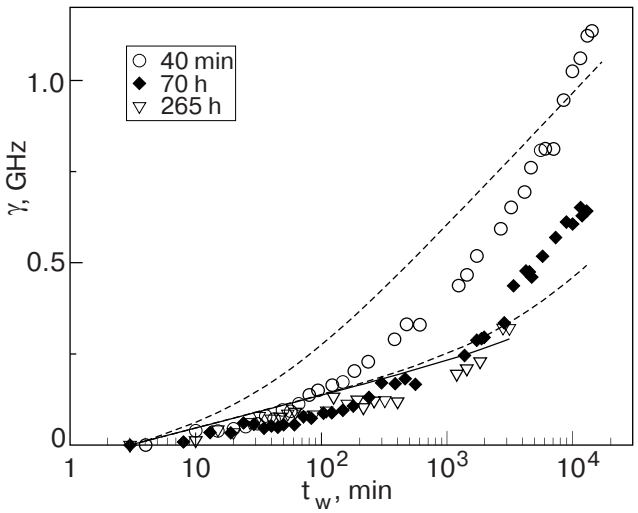

Fig. 3. An interpretation of the data of Fig. 1 according to a nonequilibrium TLS-theory (dotted lines) fails completely. (Here the broadening of the holes is labeled by γ instead of σ to clarify that the data have been analysed in a different way than in the upper figures.)

there will be aging effects, since these TLS still relax to their equilibrium distribution. A behavior as found in our protein experiments (i.e., almost no aging effects after an aging time of about a few days but still nonsaturating spectral diffusion for much longer waiting times) can thus not be explained by a TLS-theory. To solve this problem, we invented a new statistical ansatz to interpret our measurements, which will be discussed below. In this new model spectral diffusion is explained by diffusion-like motions of the aminoacids in the proteins; the diffusion kernel then takes the form of a Gaussian, and an analysis of our data based on this kernel leads to the exponents $\alpha/2$ and β given above.

Solvent effects on spectral diffusion

Horseradish peroxidase in a sugar matrix

In an important part of our experiments we investigated the role of solvent effects on protein dynamics. To learn something about this interesting question, we dissolved HRP in a trehalose-enriched water/glycerol solution [7]. (In addition, as will be presented in the next chapter, the behavior of cytochrome c in a dry trehalose-film was investigated.) Trehalose is a disaccharide with a high affinity for the formation of hydrogen-bonds [8] (and is therefore believed to replace water molecules at the surface of proteins) and with remarkably high glass-transition temperatures of its solutions [9]. It is well known that trehalose is of great importance for biological systems, helping them to survive phases of strong dehydration or low temperatures. For this reason, the influence of trehalose on protein dynamics was investigated before. For example, Hagen et al. [10,11] measured the kinetics of CO-rebinding in myoglobin in a trehalose-environment; this reaction is dominated by the relaxational behavior of the myoglobin. Similar experiments on hemoglobin have been done by Gottfried et al. [12].

In Fig. 4 the results of our own HRP/trehalose experiments are given. We compared the spectral diffusion behavior of a sample, where the proteins were dissolved in a water-glycerol mixture with the respective one in a trehalose-water-glycerol solution. (For experimental details, see [7].) Shown are masterplots, i.e., the data have already been scaled with their aging time dependence as explained above. As can clearly be seen in the insert of this figure, the aging behavior of the two samples is identical, hence the exponent β does not depend on the trehalose content of the solvent. In addition the exponent $\alpha/2$ (which is given by the slope in a double-logarithmic representation) is not affected by the solvent, too. So the only effect of the trehalose is a *decrease* of the parameter τ_0, which

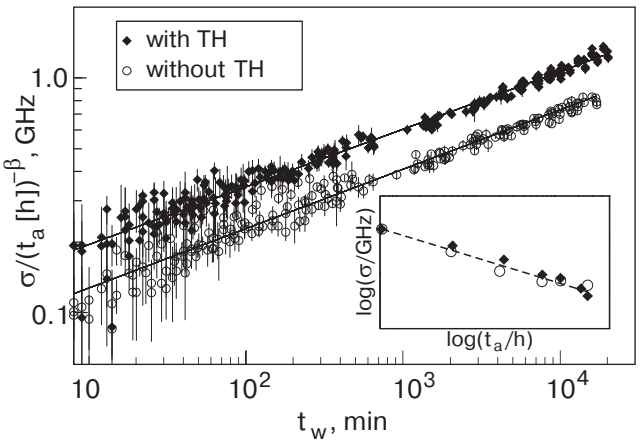

Fig. 4. Masterplots of spectral diffusion experiments on HRP dissolved in a water/glycerol matrix and a trehalose (TH)-enriched water/glycerol matrix. Only the intercept in a double-logarithmic plot is affected by the trehalose. The insert shows the aging behavior of the two samples (normalized to their values at $t_a = 40$ min), which is independent of the composition of the solvent.

is a correlation time for the *stationary* fluctuations of the proteins. In a trehalose-environment, these fluctuations are *faster* compared to a trehalose-free one by a factor of four, i.e.:

$$\tau_0(\text{trehalose}) \approx \frac{1}{4}\tau_0(\text{no trehalose}). \qquad (2)$$

In the literature, there are controversial opinions about the influence of trehalose on protein dynamics: The authors of [10,11] concluded from their CO-rebinding experiments, that trehalose prevents relaxation processes in proteins; in contrast Sastry et al. [13] believe that trehalose leads to faster *internal* fluctuations of the proteins; this is, of course, in complete agreement with the results of our own experiments, which show clear indications for faster stationary fluctuations as well as for the existence of relaxational processes in the presence of trehalose. As a possible microscopic reason for this faster dynamics, Sastry et al. propose that internal water molecules are hindered by the trehalose to leave the protein; these water molecules bind via hydrogen-bonds to aminoacid-residues of the protein and thus prevent the formation of further protein-internal bonds, i.e., the protein is kept in a more flexible state, which manifests itself in faster spectral diffusion.

Cytochrome c in a dry trehalose film

As mentioned above, we investigated the influence of trehalose on protein dynamics in a second series of experiments, this time with cytochrome c-type proteins, which were embedded in a dry trehalose film. (For details, see [14].) The motivation for these

experiments came from an interesting neutron-scattering experiment, which was performed by Cordone et al. [15] on myoglobin in pure trehalose. On the short time scales that are probed in such an experiment, the myoglobin showed at all temperatures only harmonic motions as they are found in a crystal. As a rule, in crystals no spectral diffusion is observed (except in some special cases [16]). Hence, it was interesting to test if this was true on the much longer timescales of a spectral diffusion experiment.

Our measurements were performed in complete analogy to the ones described in the previous chapter, i.e., the spectral diffusion (as a function of aging and waiting time) in the cytochrome/trehalose sample was compared with the respective one in a water/glycerol matrix. (In this case, the chromophore was the free-base analogue of the native heme group.) The main result of the experiments are given in Fig. 5, where again masterplots of the data are shown. Obviously in both cases spectral diffusion can be observed (so we do not find the crystal-like behavior of the neutron-scattering experiments). Again, power-law time dependencies are found; however, marginal changes in the exponents $\alpha/2$ and β for the different solvents can be observed. Nevertheless, the numerical values of the exponents are close to the ones found in HRP. Most important of all, the spectral diffusion in the trehalose sample is greatly enhanced, even to a much bigger extent than in our HRP experiments. We stress that the inhomogeneous band in the trehalose sample is wider by a factor of about two than in the water/glycerol sample. The reason for this is the rather high glass-transition temperature of the trehalose matrix (about 330 K) compared to about 200 K for the water/glycerol mixture. The inhomogeneous linewidth monitores the static disorder of the chromophore-environments, i.e., the proteins. During cooling of the sample, this disorder is frozen in, when the solvent reaches its glass transition temperature T_G, since below T_G large amplitude motions of the aminoacids are suppressed by the almost infinitely high viscosity of the solvent. Thus, in the trehalose sample the proteins are frozen in a much more «disordered» state, which manifests itself in the bigger inhomogeneous linewidth.

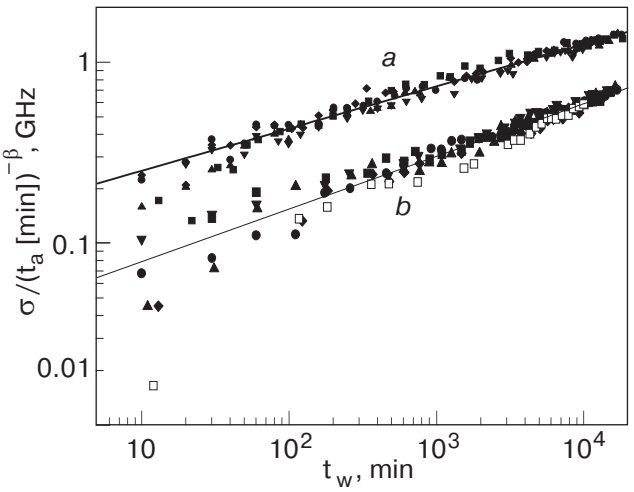

Fig. 5. Results of spectral diffusion experiments with (free-base mesoporphyrin) cytochrome c, solved in a dry trehalose film (*a*), and in a water/glycerol matrix (*b*). Again, a power law behavior is found. In the trehalose environment, the spectral diffusion is greatly enhanced.

Although being of static nature the inhomogeneous linewidth σ_0 is important for an interpretation of dynamical properties as well, because spectral diffusion scales linearly with σ_0, as will be shown in detail in the next chapter. However, the observed differences in the spectral diffusion cannot be explained only by the differences in σ_0. In addition, as an exact numerical analysis of the data shows, the correlation time for the stationary fluctuations in the proteins, τ_0, must again be shorter by a factor four in the trehalose sample. So this experiment is in complete agreement with our measurements on HRP: in a trehalose environment, the internal fluctuations of a protein are *faster*.

Theoretical frame: the diffusion model

Inhomogeneous line broadening

It is the purpose of spectral diffusion experiments to gain insight in the structural processes, which take place in a protein. To do so, one needs to know how structural rearrangements are connected with the frequency fluctuations of the chromophore. This can be achieved by a simple model [7]: we consider a chromophore inside a protein, which is separated from the various aminoacid-residues by distances R_i. The interaction with them gives rise to a frequency shift, which is simply the sum over the contributions of all residues:

$$\nu = \sum_i c R_i^{-n}. \tag{3}$$

(For a van der Waals interaction, for example, $n = 6$.) However, in an ensemble not all proteins are in the same conformational substate, the positions of the aminoacids are therefore undetermined by small values x_i. Because of this, the absorption line of each protein in the ensemble is shifted by a small amount $\delta\nu$, which is given by:

$$\nu + \delta\nu = \sum_i c(R_i + x_i)^{-n} \approx \sum_i c R_i^{-n} - nc \sum_i x_i R_i^{-(n+1)}. \tag{4}$$

The (static) distribution of the $\delta\nu$ in an ensemble of proteins is the reason for the inhomogeneous broadening, which is observed in experiments. The width σ_0 of the inhomogeneous band is therefore given by:

$$\sigma_0^2 = \langle \delta\nu^2 \rangle = \left\langle \overline{x^2} \right\rangle \left[n^2 c^2 \sum_i R_i^{-2(n+1)} \right]. \qquad (5)$$

Here $\langle . \rangle$ indicates an average over the protein ensemble, whereas averaging over the different aminoacids of one protein is denoted by a bar. Obviously, the width of the inhomogeneous line and the mean square displacement of the residues in the protein are proportional to each other. Interestingly, the proportionality factor (the bracket in the upper formula) can be determined experimentally. While σ_0 is easy to measure spectroscopically, the mean square displacement $\left\langle \overline{x^2} \right\rangle$ can be obtained from a x-ray diffraction experiment, which, of course, is done with an inhomogeneous ensemble of proteins, too. A typical value for the proportionality factor is about 100 cm $^{-1}$/Å, i.e., the absorption frequency of the chromophore is shifted by about 100 cm^{-1}, if all interacting aminoacid-residues in the protein are moved altogether over a distance of 1 Å, on average.

Anomalous diffusion in conformation space

As stated above, in our model spectral diffusion is treated as a real diffusion-like process. Though it is known from single-molecule experiments, that the frequency changes of a protein are discontinuous, the diffusion-picture is still a very good approximation, because the length of the frequency-jumps is very small compared to the inhomogeneous linewidth. The situation is therefore in complete analogy to a classical random walk with an (almost) infinite small step-length. It is well known, that this problem can be treated very good by a diffusion equation, too. As a second important assumption in our modeling, the spectral diffusion kernel is approximated by a Gaussian function. Considering that the absorption frequency of a protein is modulated by the (more or less) independent movements of several aminoacids, this Gaussian shape of the spectral diffusion kernel results from the central limit theorem and is a well-justified approximation, too.

With this two assumptions it is now possible to derive an analytic expression for the spectral diffusion kernel. It is a Gaussian with the time-dependent width

$$\sigma^2(t_w) = \sigma_0^2 (1 - C^2(t_w)). \qquad (6)$$

$\sigma(t_w)$ is the hole-broadening, that is measured in the experiments. Its time-dependence is given by the frequency correlation function $C(t_w)$. A reasonable ansatz for this correlation function is a stretched exponential:

$$C(t_w) = \exp\left[-\left(\frac{t_w}{\tau} \right)^{\alpha} \right]. \qquad (7)$$

Correlation functions of this type are usually found in systems with a hierarchy of degrees of freedom [17,18] and have been observed in various experiments with proteins. Since the correlation time τ is huge compared to experimental time scales (at least at a temperature of 4.2 K), it is possible to expand Eq. (7) to the lowest order in t_w/τ. From Eq. (6) we then get

$$\sigma(t_w) = \sqrt{2}\sigma_0 \left(\frac{t_w}{\tau} \right)^{\frac{\alpha}{2}}, \qquad (8)$$

which is just the power-law behavior that is found in our measurements.

With exactly the same argumentation like in the previous chapter it is now possible to connect $\sigma(t_w)$ with $\left\langle \overline{x^2} \right\rangle (t_w)$, i.e., with the structural movements of the protein. Again we find a proportionality between these variables with the same proportionality factor like in Eq. (5). This means, that *the conformational dynamics of low temperature proteins is governed by anomalous diffusion*. The value of the exponent $\alpha/2$ in this diffusion law (about 1/4) seems to be of a rather universal nature.

Burin et al. [19] proposed a simple model to explain this value of $\alpha/2$. According to them the structural changes, which are responsible for the spectral diffusion, can be seen as a random walk along an one-dimensional *statistical* trajectory in the complicated conformation space of the proteins. The mean separation between the initial and final point of such an trajectory after N steps scales like $N^{1/2}$. Hence, if every step shifts the transition energy of the chromophore by a certain amount, but with a *random sign*, the number of noncorrelated frequency shifts scales as $N^{1/2}$, too. The total frequency change scales then like $N^{1/4}$, and since the number of steps is proportional to t_w, one has a simple explanation for the time dependence that is found in our experiments.

Until now, we did not include aging effects in our model. However, this can be done in a rather straightforward fashion. If a system is nonstationary, its correlation functions do not only depend on the time interval t_w between hole burning and hole reading, but on the time t_a as well: $C = C(t_a, t_w)$. Empirically, such a time dependence can be included in Eq. (8) by

looking at the experimental results once more. Obviously in our measurements the exponent $\alpha/2$ does not depend on t_a. We could never observe a time-dependence of the inhomogeneous linewidth σ_0, too. So the only parameter of our model which could be influenced by t_a is the correlation time τ. To be in agreement with our measurements (see, for example, the insert in Fig. 4), τ must have an aging time dependence of the form

$$\tau = \tau_0 \left(\frac{t_a}{T} \right)^{\frac{2\beta}{\alpha}} . \qquad (9)$$

Here T is a typical time constant for those processes, which lead to an aging time dependence of the spectral diffusion. As mentioned before, these processes are the relaxational movements of the proteins towards their equilibrium state. Equation (9) can be interpreted as a short-time expansion of a stretched exponential, again. Doing this, one finds that τ_0 is the correlation time of the system, when all relaxational processes have come to an end, so it describes the *equilibrium fluctuations* of the protein. Inserting Eq. (9) in Eq. (8) one finds finally

$$\sigma(t_a, t_w) = \sqrt{2}\sigma_0 \left(\frac{t_a}{T} \right)^{-\beta} \left(\frac{t_w}{\tau_0} \right)^{\alpha/2} , \qquad (10)$$

which is exactly the result that has been derived from the experimental data (see Eq. (1)).

It is now possible to extract some numerical values from our experiments. One variable of interest is the correlation time τ for the structural fluctuations of the proteins. Neglecting all aging effects, τ is included in the intercept of a double-logarithmic plot of our data (see Eq. (8)). As a typical order of magnitude one finds for τ about 10^{17}s, which is a time span comparable to the age of the universe. This clearly demonstrates the nonergodicity of low temperature proteins: in «reasonable times» they can never explore their complete configuration space or reach thermal equilibrium.

One can estimate the length-scales of the structural motions, which are responsible for the observed spectral diffusion, too. A typical hole-broadening, that is observed after a waiting time of about two weeks, is in the range of 1 GHz. Since the proportionality factor between spectral and structural disorder is known (see. Eq. (5)), the respective length-scale of the structural motions can be estimated to be of the order of 10^{-4} Å. Only the high resolution of the hole-burning spectroscopy allows for the observation of such small movements.

Summary

We demonstrated how hole-burning can be used to investigate protein dynamics. The main result of these experiments is a power-law behavior for spectral diffusion, which differs completely from the time-dependencies found in low temperature glasses. In addition, the exponent of this power law (which has a value of about $\alpha/2 = 1/4$) seems to be of a rather universal nature.

A theoretical treatment of our results is possible in the framework of the so-called diffusion model, which holds diffusive movements of the aminoacid-residues in the protein responsible for the observed spectral diffusion. In [19] it was suggested, that this diffusion takes place on one-dimensional random trajectories in the conformation space of the proteins. In this case a time dependence like the one found in our experiments is expected.

By analysing our data according to the diffusion model it is possible to estimate a correlation time for the structural fluctuations of the proteins. This time is incredibly long, showing clearly the nonergodicity of low temperature proteins. In addition, typical length-scales for the movements of low temperature proteins can be determined.

Besides this global behavior the influence of different solvents on protein dynamics was tested experimentally. We compared the spectral diffusion of proteins, which were embedded in a trehalose matrix with the respective one in a water/glycerol environment. Spectral diffusion, i.e., the internal motion of the proteins, was greatly enhanced by the trehalose. As a possible explanation for this behavior the presence of internal water molecules in the trehalose-solved proteins was suggested.

Though not shown here in detail, it should be mentioned, that the influence of deuteration on protein dynamics was investigated by our group, too [20,21]. The results of these experiments fit perfectly in our diffusion model, as it was presented in the preceding chapters.

Acknowledgments

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