# Spectral Diffusion Experiment with a Denatured Protein

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Spectral diffusion broadening of cytochrome *c* carrying the free-base analogue of heme is investigated in its unfolded state and compared with the corresponding broadening in the native state. Spectral diffusion is much larger in the unfolded state, in comparison to the native state. Interestingly, the time law that governs spectral diffusion changes as the aging time increases, from a power-law behavior in the native state to an apparent logarithmic behavior in the unfolded state.

## 1. Introduction

Proteins do show features of randomness. On nanoscopic scales and below, there is a distribution of structures and, as a consequence, there is a distribution of energies, barriers, folding rates, folding trajectories, etc.[1,2] Accordingly, on the proper time scales, a single protein may be considered as an "individual" that is well-distinguished from another protein of the same species, e.g., by a different energy of the native state, a different thermal stability, a different folding rate, etc. This situation is described in a most transparent fashion within the concept of an energy landscape.[3,4] Within such a framework, it is clear that the native state, for example, consists of many structural substates in all of which the protein is, at least in principle, functioning. Apart from the ensemble of native states, a protein has a much larger ensemble of denatured states. Within this ensemble, there are characteristic sets of substates e.g., the so-called molten globule.[5] Although the molten globule belongs to the ensemble of denatured states, its substates are, in regard to their structural features, similar to those of the native state.[6,7] Another characteristic set of states within the ensemble of denatured states is the random coil. The associated substates are structurally very dissimilar from the native state and, accordingly, comprise a much larger region in structural phase space.[8]

An immediate consequence of this distribution of structures is structural dynamics. At sufficiently high temperature, this is most obvious: The protein can cross structural barriers by thermal activation,[9,10] including a transfer from the ensemble of native states to the ensemble of denatured states.[11,12] Even at extremely low temperatures, structural dynamics are not forbidden and do occur, for instance, through tunneling between closely related structures.[13] Activated processes also may occur, as long as $V/k_BT$, which is the ratio of the structural barrier $V$ to the thermal energy $k_BT$, is in an appropriate range.[14,15] At low temperatures, this motion in structural phase space can be precisely measured with spectral diffusion experiments.[16] In these experiments, the line width of an optical transition of a marker molecule is measured as a function of time. Whenever the protein undergoes a structural change, the frequency of the marker molecule changes. The frequency changes due to structural relaxation are small; therefore, the associated spectral jumps can only be observed if the monitoring lines are sufficiently sharp. In this respect, hole-burning techniques offer several advantages. On one hand, the lines are very narrow, with a width that is similar to the homogeneous line width. On the other hand, holes may live almost forever, which allows even the very slow dynamics, which occur on the scale of days or longer, to be investigated. As a consequence, structural jumps can be observed as long as they produce frequency jumps on the order of the homogeneous line width. Typically, at 4 K, the respective frequency scale is on the order of a gigahertz. In an ensemble experiment, these frequency jumps manifest themselves as a broadening of the burnt-in hole. This broadening is dependent on the temperature of the sample. The homogeneous line width is strongly temperature dependent;[17,18] therefore, the contribution to the hole from spectral diffusion can be measured most precisely at liquid helium temperatures.[19] Broadening of a spectral hole due to spectral diffusion processes reflects the dynamics of structural fluctuation and relaxation processes that occur in disordered systems such as glasses, polymers, and also proteins. These systems are characterized by a broad distribution of rates.[20] This distribution of rates reflects features of the energy landscape and the associated structural phase space, which is the objective of our present investigation.

Spectral diffusion was originally discovered in glasses.[21] Indeed, glasses are also characterized by an energy landscape, however, obviously by one that differs significantly from that of proteins. The protein landscape has the shape of a narrow

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funnel, where the average gradient toward the native state is steep, which is a necessary requirement for a high driving force toward the native state.[5] A narrow funnel structure of the free-energy landscape implies a limited region in structural phase space where the protein can move around. In contrast, a glass does not have a distinguished set of states that is similar to that of a protein and, accordingly, has no pronounced funnel structure. As a consequence, the area in structural phase space spanned by the accessible states is much larger, as is reflected in a significantly stronger spectral diffusion broadening as well as in a different time dependence.[22,34]

In this paper, it is our goal to (i) investigate the spectral diffusion dynamics of a protein, namely cytochrome *c* carrying the free base analogue of heme, in its unfolded state and (ii) compare the results with the corresponding behavior of the same protein in its native state. We have investigated the spectral diffusion dynamics in the native state previously under different experimental conditions.[28,33] In the unfolded state, the protein attains most probably a random coil structure. Consequently, the number of accessible structural states is larger than that in the native state. This structural heterogeneity should be reflected in spectral diffusion broadening. Whether this is true or not is one goal of the present investigation. A second goal is to determine whether the time law of the structural dynamics changes in the random coil structure, as compared to the native structure. In most cases, it has been found that structural dynamics in the native state are governed by a power law.[23] In contrast, the corresponding dynamics in glasses follow a logarithmic time law.[21] We consider the time laws that govern spectral diffusion to be interesting features, because they may shed light on the dynamics at higher temperatures,[9] where the biologically important processes occur.

## 2. Experimental Section

**Materials.** Horse heart cytochrome *c* (Fe−Cc) was from Sigma, and cytochrome *c* carrying the free base analogue of heme ($H_2$−Cc) was prepared as described previously by the extraction of iron from the chromophore.[24] Guanidinium hydrochloride (Gua, from Sigma) and urea (Schwarz-Mann) were of ultrapure grade; all other chemicals and solvents were reagent grade.

**Sample Preparation and Stock Solutions.** For the unfolding experiments, Fe−Cc was dissolved in potassium phosphate buffer (50 mM, pH 7.3) and $H_2$−Cc was dissolved in *tris*-HCl buffer (10 mM, pH 8.3) at an optical density of 0.6 in a 1 cm cuvette at 280 nm. These solutions were prepared fresh every day. Gua was dissolved in a 2:1 (v/v) mixture of glycerol and potassium phosphate buffer (50 mM, pH 7.3, for Fe−Cc) or a 1:1 (v/v) mixture of glycerol and *tris*-HCl (10 mM, pH 8.3, for $H_2$−Cc). For the spectral diffusion experiments, 6 M Gua was dissolved in a 2:1 (v/v) mixture of glycerol and potassium phosphate buffer. $H_2$−Cc was added to this solution, resulting in a final concentration of ∼10 mg/mL. To avoid protonation, the pH was adjusted to ∼7 via the addition of KOH (1 M). This mixture was sealed in a homemade cuvette with a path length of 6 mm. The optical density in the spectral range where the hole-burning experiments were performed (615 nm) was 0.3 at liquid helium temperature.

**Unfolding Experiments.** Unfolding of $H_2$−Cc was induced by treatment with increasing concentrations of Gua.[25,26] Just before the measurements, appropriate aliquots of the respective protein stock solution—the Gua solution and a Gua−free glycerol/buffer solution—were mixed. The circular dichroism (CD) spectra (using Jobin-Yvon circular dichrograph V) were recorded immediately after mixing in a range of 208−250 nm using a 1-mm cuvette. From each spectrum, the CD signal ($\Delta A_{248} - \Delta A_{224}$) was extracted.[27] In Figure 1, this quantity is plotted on a relative scale against the Gua concentration. A similar series of experiments was conducted with urea as the denaturant, using a maximum concentration of 10 M.

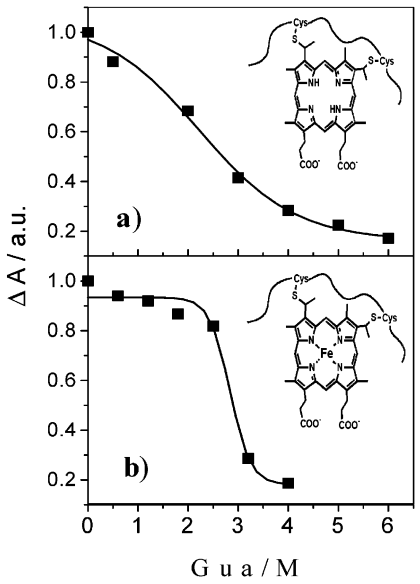


**Figure 1.** Circular dichroism (CD) signal $\Delta A$ at 224 nm (in arbitrary units), as a function of concentration of the denaturing agent, guanidinium hydrochloride (Gua). Data are normalized to the CD signal at zero concentration of Gua. The respective data for $H_2$−Cc (Figure 1a) are compared with those for Fe−Cc (Figure 1b). The respective structures of the two chromophores are shown in the insets. The full lines are guides to the eye.

**Spectral Diffusion Experiment.** The spectral diffusion experiments were performed at a temperature of 4.2 K. The sealed sample was cooled to 4.2 K within 20 min. Hole burning was performed with a dye ring laser (Coherent, model CW 899-21) pumped at 532 nm by a frequency-doubled neodymium: vanadate laser (Verdi). The width of the laser line was <1 MHz. Power levels for burning were 30−60 $\mu$W/cm$^2$. To avoid reburning, the reading signal was reduced by more than 3 orders of magnitude. The holes were monitored in the transmission mode and processed by a computer. In our experiment, we vary two time parameters: the aging time ($t_a$) and the waiting time ($t_w$). The aging time is defined as the width of the time window between cooling and hole burning, and the waiting time is defined as the width of the time window between hole burning and hole reading. Several holes were burned at different aging times varying from $t_a = 1$ to $t_a = 192$ h. The hole broadening was measured over a period of approximately two weeks. The time resolution of the experiment, as defined by the time needed to burn and read a hole, was a couple of minutes.

**Data Evaluation.** The quantitative evaluation of a spectral diffusion experiment relies on a certain assumption about the shape of the spectral diffusion kernel, that a portion of the line shape that causes the time evolution of the hole within the experimental time window is set by the waiting time $t_w$. We have been analyzing spectral diffusion in proteins within the framework of a conformational diffusion model, which implies a Gaussian line shape of the respective kernel. To evaluate all the experiments with the same procedure, we fitted Voigtians to the measured hole shapes and extracted the associated Gaussian kernel. The Lorentzian component of this Voigtian can be considered to be an empirical fit to the hole when it is measured for the first time at the beginning of the each waiting

time experiment. In all cases, the hole immediately after burning was well fit by a Lorentzian. Note that spectral diffusion processes may occur on time scales that range from nanoseconds to practically infinity. Our experimental time window covers just a small range, extending from minutes to several days. Hence, the initial shape of the hole may already contain a spectral diffusion kernel, which, however, does not influence our evaluation procedure, because it is incorporated into the initial hole shape that is deconvoluted from the experimentally measured dependence on $t_w$. For clarity, we mention that, for all the holes in a given $t_w$ experiment, the initial empirical Lorentzian line shape was kept constant.

We call the standard deviation $\sigma$ of the Gaussian kernel the spectral diffusion width. This is the quantity in which we are interested. We stress that the Gaussian line shape assumption might not reflect the proper physics for the unfolded protein. In the unfolded state, the chromophore may become exposed to the solvent; hence, glasslike excitations (two-level systems, TLS), might contribute to spectral diffusion and change the features of the respective line shape. However, we did not change the evaluation procedure, so data are treated in the same fashion. This is mandatory for comparative experiments. Note that, within the restrictions of our evaluation procedure, the fits to the data are unique within the error margins. For $t_w$ values larger than $\sim 10^3$ min, the error bars barely exceed the size of the data point symbol in the graphs. At shorter times, they are larger, because the time-dependent component that we extract from the line shape becomes smaller.

In regard to the inhomogeneously broadened spectrum of the unfolded protein, we decomposed it into a minimum number of Gaussians. It is clear that such a procedure carries some arbitrariness; however, it should be good enough for an estimation of the inhomogeneous bandwidths involved. The line shape of the native protein, too, is a superposition of several components that we associate with the tautomeric states of the inner-ring protons. However, the features of the central component are sufficiently well-separated from the others, so that its Gaussian shape could be independently fitted.[33]

## 3. Results

Cytochrome *c* is largely α-helical; hence, the far-UV CD signal is expected to decay as the protein unfolds. The experiments show that a concentration of the denaturing agent as high as 4 M is needed for complete unfolding of Fe−Cc and even 6 M for $H_2$−Cc. Both $H_2$−Cc and the unmodified protein (Fe−Cc) show a sigmoidal decrease of the CD signal as the concentration increases, reaching the same final plateau near zero CD at Gua concentrations of >5 M (see Figure 1). However, for $H_2$−Cc, a 50% decrease in the CD signal is already attained at a concentration of 2.4 M, as compared to 2.8 M for Fe−Cc. The cooperativity of the denaturing transition, as reflected in the slope of the CD signal in the transition region, is much lower for $H_2$−Cc than for Fe−Cc. The major difference between the two samples is the presence and absence, respectively, of the central Fe atom (see structure insets in Figure 1); therefore, it seems that coordination of the latter with the protein increases stability and cooperativity, most likely by decreasing sample heterogeneity. A similar behavior has been found for myoglobin, where a variety of chromophores can be inserted. These results will be published elsewhere. Apart from the unfolding experiments with Gua, we also performed control experiments with urea. Unfolding with this reagent was never complete, not even at the highest accessible concentrations (~10 M). Therefore, the spectral diffusion experiment was done with

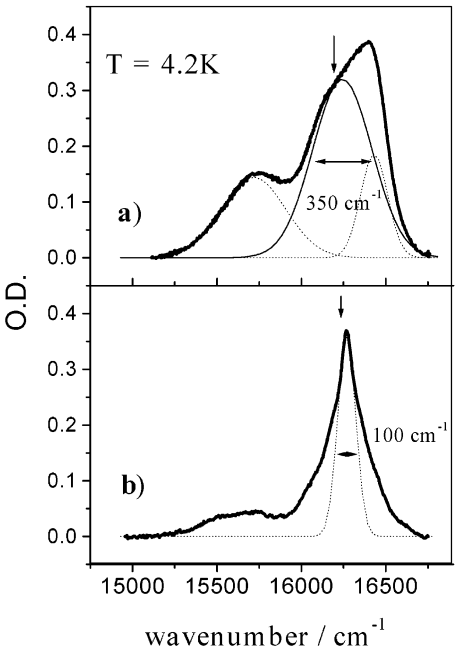


**Figure 2.** Inhomogeneous long-wavelength absorption for $H_2$−Cc (a) in the chemically unfolded state (6 M Gua) and (b) in the native state. The spectrum of the denatured protein clearly is composed of several components that belong to different substates, such as, for example, conformers. The arrow indicates the spectral region where hole burning was performed.

Gua. Also, the low-temperature spectra in the unfolded state were somewhat different: The peak at 16 400 $cm^{-1}$ (Figure 2a) was absent in the urea-denatured sample. From a comparison of the spectra in the two different denaturing agents, it seemed that the dominant transition in the denatured state is around 16 250 $cm^{-1}$. Accordingly, we conducted our spectral diffusion experiments in a narrow range around this frequency.

Figure 2 shows the inhomogeneous spectra of $H_2$−Cc in the unfolded state (Figure 2a) and in the native state (Figure 2b).[28] We observe a series of interesting features. Compared to the native state, the spectrum becomes more complex. First, a band at around 15 700 $cm^{-1}$ grows; this band is only weakly indicated in the native state. We observed that the intensity of this band increases as time increases at room temperature and that it grows much faster in the unfolded state than in the folded state. On the basis of this observation and on the red shift of the band, we assign it to the formation of an aggregated species. Second, the band at ~16 250 $cm^{-1}$ strongly changes shape and becomes much broader. Already in the native state, this band is composed of at least three subbands, which may originate from the tautomeric states of the inner-ring protons of the free base porphyrin. Obviously, these states shift and broaden as the protein becomes unfolded. The spectral diffusion experiments were performed in a narrow frequency range of ~50 $cm^{-1}$ around the positions marked in Figure 2 with an arrow.

Figure 3 shows the spectral diffusion behavior of the unfolded protein, which is compared to the respective behavior of the protein in the native state in Figure 4.[28] Two distinct features catch the eye. First, broadening in the unfolded protein (6 M Gua) is significantly larger (approximately twice as large as that in the native state). Second, in both states, spectral diffusion broadening is subject to aging. Aging is manifested by the slowing of spectral diffusion. In the native state, aging does not change the time law of this broadening process in a significant fashion. At all aging times (up to 120 h) at which we performed experiments, spectral diffusion is governed by a

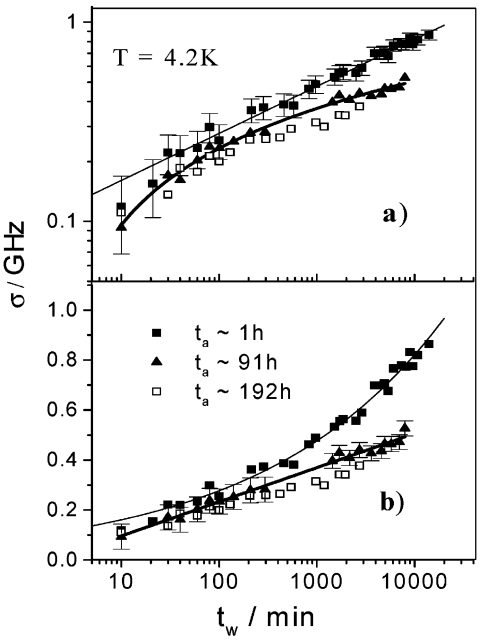


**Figure 3.** Time evolution of the line width of spectral holes burned at various aging times ($t_a$). The behavior of the unfolded free-base chromoprotein ($H_2$−Cc, 6 M Gua) is shown: Figure 3a shows a log−log plot, whereas Figure 3b shows the same data in a linear−logarithmic representation. This representation clearly demonstrates the striking change in the time evolution of the spectral holes as $t_a$ increases.

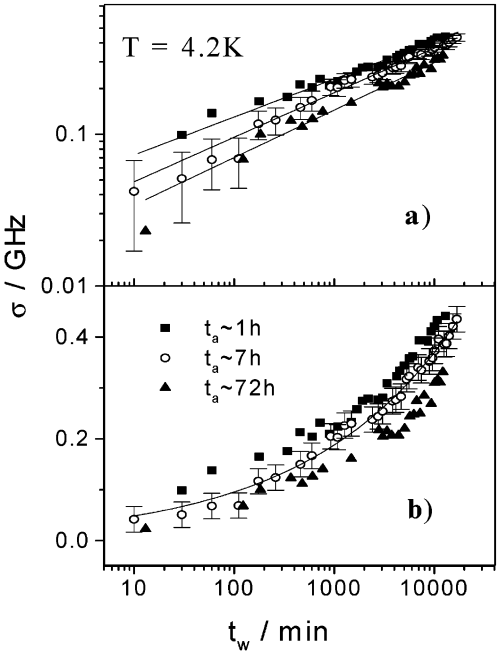


**Figure 4.** Comparative data of the spectral diffusion width, as a function of the waiting time $t_w$ of the native free base chromoprotein ($H_2$−Cc)[28] (a) in a log−log representation and (b) in a linear−logarithmic representation. Note that (i) the spectral diffusion width ($\sigma$) is significantly smaller than that of the unfolded protein (see Figure 3), and (ii) the power-law behavior of the time evolution does not change as the aging times $t_a$ increase. The power-law exponent is $\alpha = 0.29 \pm 0.03$.

power law.[28] The respective exponent is 0.29 ± 0.03. In the unfolded state, however, the spectral diffusion behavior changes significantly as the aging time $t_a$ progresses. At short aging times, the time law is still well-described by a power law, similar to that of the native state. The corresponding exponent is 0.26 ± 0.01. As the aging time increases, spectral diffusion broadening slows dramatically for long waiting times. We compare aging times of 91 h for the unfolded protein with 72 and 120 h for the folded protein.[28] The experiments with the unfolded protein at $t_a = 192$ h demonstrate that an additional increase in the aging time does not further change the peculiar deviation from the power law. The 192 h data almost fall on top of the 91 h data, as is very clearly seen in the linear−logarithmic representation in Figure 3b. Within the 3 orders of magnitude of our experimental time window, the time evolution can be well fit by assuming log $t_w$ behavior. Because we cannot offer an interpretation of this behavior, we consider the logarithmic fit curve to be a guide for the eye, rather than a definitive statement. However, the main result—that the time evolution changes with aging time in the unfolded protein—is definitive.

## 4. Discussion

**Aspects of Spectral Diffusion Physics.** Spectral diffusion dynamics are quite intimately associated with structural disorder. Structural disorder, on the other hand, results in an energy landscape that is characterized by structural traps with a broad distribution of barriers separating these traps. The dynamics of a system in a rough energy landscape may be characterized by two types of processes, namely, fluctuations that are associated with a local equilibrium and aging processes. The latter are typical nonequilibrium processes and are a characteristic feature of deeply frozen structurally disordered materials. Aging processes are indicative of the fact that the system under investigation has become trapped for some time in a local energy minimum during cooling from which it may eventually get expelled, just to become trapped again in another local minimum.[29] They are a consequence of the fact that the system moves toward its global energy minimum. At sufficiently low temperatures, these processes become extremely slow. If the residence time in a local structural trap is long enough, a local thermodynamic equilibrium may be established between structural states accessible at the experimental bath temperature. Accordingly, the relaxation processes connected with sample aging are accompanied by structural fluctuation processes. In a series of spectral diffusion experiments with various deeply frozen proteins, we have investigated the related structural dynamics.[28,30] The main outcome of these experiments can be summarized: Structural dynamics at low temperatures are so slow that one can safely characterize proteins as nonergodic systems. The spectral diffusion width is governed by a power law in time whose exponent varies in a narrow range of ~0.25. This behavior is contrary to that of glasses, where the spectral diffusion width is governed by logarithmic time laws. In all cases where proteins could be compared with glasses, the spectral diffusion width was significantly smaller for proteins.[22]

It is a major goal of any model of spectral diffusion to relate the spectral diffusion width to the characteristic features of the energy landscape. In glasses, for example, this landscape is represented by almost-degenerate, localized two-level systems (the TLSs) that represent the two lowest states in a double well potential. The spectral diffusion width is proportional to the number of TLSs that have changed their state during a certain waiting time $t_w$. This is directly related to the change of conformational entropy ($\Delta S$), which the system experiences during $t_w$. Along these lines of reasoning, one can say that the measured width is related to the area in structural phase space that the system has explored during $t_w$.

In proteins, spectral diffusion dynamics have been directly connected with conformational diffusion in phase space, which

is a very different approach, in comparison to the glass case.[30] Hence, in this case, the relation between the explored area in structural phase space and the line width is even more obvious because the width scales directly with the mean square displacement $\langle x^2 \rangle$ in conformation space. If one assumes that conformational dynamics are governed by a random walk along a one-dimensional (1D) trajectory in conformation space, the spectral diffusion width scales with the end-to-end distance, which itself scales with $t_w^{1/4}$, in agreement with the experiments.[31] The structure of a protein is well-defined but not as well as the structure of a crystal. Low temperature $\langle x^2 \rangle$ values, for instance, are much larger than those in crystals.[32] Hence, on length scales at or below ~0.1 Å, the average structure is much less defined. Consequently, structural fluctuation and relaxation processes do occur and can be monitored via spectral diffusion experiments. However, because of the narrow funnel structure of the energy landscape of the native protein, the processes must be much more restricted, in comparison to systems without such a funnel structure.

**Spectral Diffusion and Protein Unfolding.** The results in Figure 3 definitely show that the protein in the unfolded state is subject to significantly enhanced spectral diffusion dynamics. We stress that, in cytochrome *c*, the chromophore is covalently bound to the polypeptide chain, unlike, for instance, that in myoglobin. Therefore, unfolding does not mean that the chromophore just diffuses into the solvent environment. An enhancement of the spectral diffusion dynamics may have two reasons: (i) enhanced structural dynamics, because the accessible phase space has been increased (as previously discussed), and/or (ii) an enhanced coupling of the chromophore to the solvent environment. After unfolding, the concentration of the denaturing agent is high, and, hence, we cannot definitively exclude the possibility that the chromophore solvent coupling may be influenced. However, for the following reasons, we do not consider such a possibility as the main factor for the tremendous increase in spectral diffusion broadening. First, because the chromophore is still covalently bound to the polypeptide chain, its nearby solvent environment is still significantly determined by the amino acid residues. Second, a doubling of the chromophore solvent interaction due to the presence of Gua seems to be, by far, too large, considering the fact that the solvent shift does not change at all in the unfolded state (see Figure 2). On the other hand, the inhomogeneous line width changes strongly, which is consistent with the assumption of a larger phase space. Along these lines of reasoning, we attribute at least a major portion of the enhanced spectral diffusion broadening to the breaking up of the native structure and the concomitant transition from a folded protein to a random coil. In the random coil state, structural dynamics are much less restricted; the accessible structural phase space is larger, and this is obviously reflected in the enhanced spectral diffusion broadening.

**Time Evolution of the Hole Width.** An interesting observation is the fact that the dependence of the hole width on waiting time $t_w$ changes in a qualitative fashion as the aging time $t_a$ increases. As stressed previously, the time evolution of the hole width in native proteins is governed by a power law in time, according to various experiments that have been performed to date. The origin of this power law can be seen in an anomalous structural diffusion process in conformation space. Quite in contrast to the protein case, spectral diffusion in random solvents, similar to glasses or polymers, is governed by logarithmic time laws. The origin of the logarithmic behavior may be, as in glasses, the occurrence of TLS whose relaxation rates are distributed over many orders of magnitude, because of the random structure of the material.

What we observe here is really very surprising. The first data set of our spectral diffusion experiments at a short aging time $t_a$ is well-described by a power law in $t_w$. The exponent is not very different from that which is observed in the native state (see Figure 3a), irrespective of the fact that the magnitude of the broadening is tremendously increased. Although the power law is retained in the native state data as the sample ages, it changes gradually to a different behavior in the unfolded state. We stress that we can only speculate on the underlying reasons for this behavior. It seems that irregular behavior is observed, in comparison to that of the native state, which is responsible for this observation. This irregular behavior could lead to a time dependence of the power law exponent $\alpha$, in the sense that it slows as a function of time so that the slope becomes smaller as time increases. In regard to the physics behind such behavior, it would mean that the dispersion of fluctuation rates involved would grow with time. Another possibility could originate in the TLS of the host glass. Because protein unfolding exposes the chromophore to the solvent, these TLSs might become important as the relaxation of the polypeptide chains slows as the aging time $t_a$ increases.

Currently, we cannot really solve the puzzle as to what governs the breakdown of the power-law behavior. More experiments are required to reach more-definitive conclusions.

## 5. Conclusions

We investigated spectral diffusion in a protein in the chemically unfolded state and compared it with results from earlier experiments on the same protein in the native state. Unfolding increases the inhomogeneous broadening tremendously, and it also increases spectral diffusion. We related this observation to an increase in the accessible structural phase space in the unfolded state. A most interesting observation is the fact that the qualitative behavior of the time evolution of the hole width changes in the unfolded state from a power law at short aging times to a significantly slower time dependence. We have discussed possible reasons; however, for an unambiguous interpretation, more experiments are needed.

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