Two-Level Systems in Myoglobin Probed by Non-Lorentzian Hole Broadening in a Temperature-Cycling Experiment

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We have measured the broadening of persistent spectral holes in H_2-protoporphyrin-substituted myoglobin caused by temperature cycling between 4 and 70 K. Non-Lorentzian hole broadening has been observed for excursion temperatures above 40 K. This effect has been explained by assuming that the hole broadening is due to the transition-frequency shift of the chromophore by the random flips of the two-level systems, and that the these systems in the interior and in the exterior of the myoglobin molecule make different contributions to the broadening. From the analysis of the hole spectra, the number and the coupling strength of the TLS's in myoglobin have been determined.

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For an understanding of the protein state of matter and protein functions, it is important to obtain a clear picture of the low-energy excitations and their relaxations in a protein. At low temperatures, many properties of proteins are described well by treating proteins as glasses [1]. Proteins and glasses show structural relaxation even at very low temperatures, and the relaxation is modeled by transitions between two metastable states in localized double-well potential systems, i.e., two-level systems (TLS's) [2,3]. Thermally activated transitions between the metastable states can be detected by hole-burning spectroscopy of dye molecules doped in glassy materials combined with a temperature-cycling technique. In this method, thermally activated transitions below a certain temperature T_c are probed by the irreversible broadening of a spectral hole burned at a low temperature T_b after the temperature is once raised to T_c and then lowered to the original temperature T_b. The irreversible broadening of the hole is considered to result from a shift of the transition frequency of the dye molecule in response to a change in the local environment of the dye, which corresponds to the transitions in the TLS's. The inhomogeneous distribution of absorption lines that originally have a degenerate transition frequency and spread in this way is called the spectral diffusion kernel (SDK) of a hole spectrum. The SDK usually shows a Lorentzian shape, and its width broadens with T_c. So far, a steplike broadening of holes as a function of temperature has been observed in proteins, which was attributed to a small number of degrees of freedom in a protein [4].

In this Letter, we report a temperature-cycling hole-burning experiment made on H_2-protoporphyrin-substituted myoglobin (H_2PP-Mb). A hole was burned at 4 K in the absorption band of H_2PP near 620 nm, and the excursion temperature T_c was varied up to about 70 K. For excursion temperatures above 40 K, the SDK was found to deviate from a Lorentzian shape. We infer that this comes from the finite size of the protein. We analyzed the hole profiles by assuming different number densities and chromophore-TLS coupling strengths for the TLS's in the interior and in the exterior of the myoglobin molecule. Hole shapes with a host matrix consisting of two spatial domains have already been analyzed by Pack, Narasimham, and Fayer [5] and Kador [6]. Pack, Narasimham, and Fayer calculated the hole shapes in a case where dynamic properties of the host glass varied between the solvation shell and bulk regions. Kador derived a formulation for temperature-cycled hole shapes or SDK's assuming a dead volume for the TLS, around the dye molecule. Both analyses demonstrated that hole shapes deviate from Lorentzian under certain conditions, and Kador showed that it is possible to estimate, from the non-Lorentzian SDK, the number density and the coupling strength of the TLS's with the dye. However, both of them failed to observe such a deviation in dye-doped polymers or organic glasses. In our system, the space around the chromophore is divided into two parts more distinctly by the protein surface. We extended Kador's formulation and fitted the non-Lorentzian SDK. By the fitting, both the number and the coupling constant of TLS's in the protein were determined for the first time.

The preparation of H_2PP-Mb has already been reported in a previous paper [7]. The protein was dissolved in a water-glycerol mixture (volume ratio 1:3) with a concentration of about 1 mM, and then contained in a glass cell with a path length of 1 mm. The optical density of the sample was about unity at 620 nm. The sample temperature was controlled with an accuracy of 0.1 K in a He-gas flow-type cryostat. A linearly polarized multimode dye laser with a bandwidth of about 0.3 cm^{-1} was employed to burn a hole (burning power 3.5 mW, exposed area 0.06 cm^2). The hole spectra were measured at 4 K before and after temperature cycles by passing unpolarized light from a halogen lamp through a 1 m single monochromator with a resolution of about 0.3 cm^{-1}, and then the sample. The time to measure a spectrum and the time to change the temperature were

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on the order of 10 min. In order to detect even subtle changes in the hole shape, hole spectra were measured with a rather long signal averaging time. In particular, we ascertained that there was no drift in the baseline of the spectrum during the temperature-cycling experiment. No further changes were observed in the hole spectra when the holding time at T_c was prolonged to about 1 h or the cycling to the same temperature was repeated. Accordingly, it is certain that the change in the hole spectra is not dependent on the time after the hole burning but fixed by the cycling temperature.

Figure 1(a) shows the hole spectrum of H_2PP-Mb just after hole burning at 4 K and after temperature cycling to $T_c = 69$ K. The initial hole shape is irrelevant to our discussion although the experimental sensitivity of the change is dependent on it. We notice that hole broadening occurs mainly in the tail of the hole, while the full-width at half maximum (FWHM) of the hole is hardly increased by temperature cycling. The closed circles in Fig. 1(a) represent a Lorentzian curve convoluted with the original hole shape. The width of the Lorentzian was chosen so as to reproduce the FWHM of the experimental data. The calculated spectrum deviates from the observed one in the tail part, and it is clear that the SDK of the hole spectrum of H_2PP-Mb is not a Lorentzian for T_c of ~70 K. This type of non-Lorentzian hole broadening was observed for excursion temperatures above 40 K, while the broadening was almost Lorentzian for T_c below 30 K. We also carried out a control experiment in which the hole profiles of H_2PP doped into an organic glass DDG [DMF:DMSO:glycerol, volume ratio 1:1:3 (where DMF and DMSO denote *N*, *N*-dimethylformamide and dimethyl sulfoxide, respectively)] with a concentration of 1 m*M* were measured. As shown in Fig. 2, the hole profiles of H_2PP are fitted well by convoluting Lorentzians with the hole spectrum before the cycling. Thus it is indicated that the non-Lorentzian line shape of the SDK observed in H_2PP-Mb is related to the particular structure and relaxation of the chromoprotein system, and that the dead volume around H_2PP that was assumed by Kador has no relation to the non-Lorentzian SDK of H_2PP-Mb.

Hole shapes in the temperature-cycling experiment can be calculated if we know the SDK, which is expressed for a dye-glass system as [6]

$$I(\nu) = \frac{1}{2\pi} \int_{-\infty}^{+\infty} dx\, e^{i\nu x} i(x), \tag{1}$$

with

$$i(x) = \left(\int_V d\mathbf{R}\, G(\mathbf{R}) e^{-i\tilde{v}(\mathbf{R})x} \right)^N, \tag{2}$$

where ν is the light frequency, V is the sample volume, N is the total number of flipping TLS's in the sample, $G(\mathbf{R})$ is their spatial distribution with respect to the dye located at $\mathbf{R} = 0$ [$\int d\mathbf{R}\, G(\mathbf{R}) = 1$], and $\tilde{v}(\mathbf{R})$ is the interaction potential between a TLS and a dye. In the derivation of these expressions, we have employed a stochastic model in which each TLS is assumed to have no correlation with any other and to flip randomly.

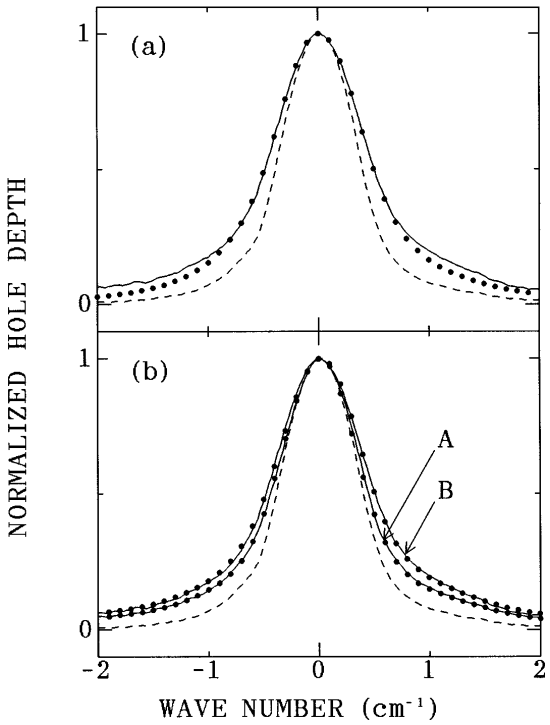

FIG. 1. (a) Hole spectrum of H_2PP-Mb burned at 4 K (dashed line) and that after temperature cycling to $T_c = 69$ K (solid line). The hole depth is normalized to unity. The closed circles denote the convolution of a Lorentzian with the initial hole shape. (b) The dashed line and curve *B* are identical with the data in (a). Curve *A* is the hole spectrum of H_2PP-Mb after temperature cycling to $T_c = 56$ K. The closed circles denote the fitting curves obtained from Eq. (4) with the parameters listed in Table I.

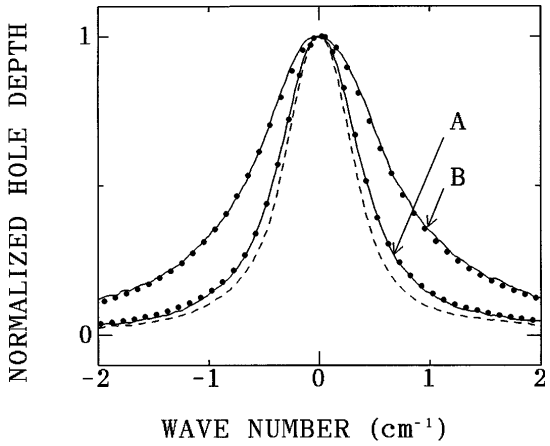

FIG. 2. Hole spectrum of H_2PP in DDG burned at 4 K (dashed line), and those after the temperature cyclings of $T_c = 37$ K (curve *A*) and $T_c = 62$ K (curve *B*). The closed circles denote the convolution of Lorentzians with the initial hole shape.

As for the dye-TLS interaction potential, we adopt the dipole-dipole type interaction [6]. Then $\tilde{\nu}(\mathbf{R})$ is approximately given as

$$\tilde{\nu}(\mathbf{R}) = -\frac{2\Delta\boldsymbol{\mu}\cdot\boldsymbol{\mu}_M}{4\pi\varepsilon_0 hc}\frac{1}{R^3} \equiv -\beta\frac{\cos\theta}{R^3}, \qquad (3)$$

where ε_0 is the permittivity of vacuum, h is Planck's constant, c is the light velocity in vacuum, $\Delta\boldsymbol{\mu}$ is the difference between the static dipole moments of a dye molecule in the excited and ground states, $\boldsymbol{\mu}_M$ is the change in the dipole moment of a TLS when it flips, and θ is the angle between $\Delta\boldsymbol{\mu}$ and $\boldsymbol{\mu}_M$. We assume a random distribution for θ. With this interaction potential and a uniform spatial distribution of TLS's, Eq. (1) gives Lorentzian hole broadening [6]. Thus the results observed for H_2PP in DDG glass can be explained with this model by neglecting the dead volume around H_2PP.

Now, we extend the above expressions to describe spectral diffusion in myoglobin. A myoglobin molecule is approximated by a sphere with radius $R_0 = 1.7$ nm, which contains a dye molecule at its center. In reality, the dye molecule, or the heme group, is not at the center of the myoglobin molecule. This will give angular dependence to the spatial distribution of TLS's. However, its effect on the calculated hole spectrum is considered to be small. When the properties of TLS's inside and outside the protein are different, $i(x)$ is given as

$$i(x) = \left[\sum_{N_{\rm in}} P(N_{\rm in}) i_{\rm in}(x)\right] i_{\rm out}(x), \qquad (2')$$

with

$$i_j(x) = \left(\int_{V_j} d\mathbf{R}\, G_j(\mathbf{R}) e^{-i\tilde{\nu}_j(\mathbf{R})x}\right)^{N_j}, \qquad (2'')$$

where j stands for "in" or "out." Since $N_{\rm in}$ may be different for each protein molecule, we have introduced the distribution function $P(N_{\rm in})$. For simplicity, we employ a Poisson distribution for $P(N_{\rm in})$. This corresponds to an assumption that the total number of TLS's has no upper limit, and they are activated independently of each other. As to the outside region, we use the relation $\lim_{N\to\infty}(1 - A/N)^N = \exp(-A)$, because $V_{\rm out}$ is large, and accordingly $N_{\rm out}$ is much larger than unity. The spatial distribution $G_j(\mathbf{R})$ is assumed to be uniform in each region. For the interaction between the dye and the outside TLS's, the interaction potential is considered to have the same R dependence as Eq. (3) for $R \gg R_0$ even if the protein shields the dye from the solvent. Then, substituting Eq. (2′) into Eq. (1), we have

$$I(\nu) = \frac{1}{2\pi}\int_{-\infty}^{+\infty} dx \exp[i\nu x - J_{\rm in}(x) - J_{\rm out}(x)], \qquad (4)$$

with

$$J_{\rm in}(x) = \overline{N}_{\rm in}\int_{V_{\rm in}} d\mathbf{R}\, G_{\rm in}(\mathbf{R})\left[1 - e^{-i\tilde{\nu}_{\rm in}(\mathbf{R})x}\right] = \overline{N}_{\rm in} u_{\rm in}\int_{u_{\rm in}}^{\infty}\left(1 - \frac{\sin u}{u}\right)\frac{du}{u^2}, \qquad (5)$$

$$J_{\rm out}(x) = N_{\rm out}\int_{V_{\rm out}} d\mathbf{R}\, G_{\rm out}(\mathbf{R})\left[1 - e^{-i\tilde{\nu}_{\rm out}(\mathbf{R})x}\right] = \overline{N}_{\rm out} u_{\rm out}\int_0^{u_{\rm out}}\left(1 - \frac{\sin u}{u}\right)\frac{du}{u^2}, \qquad (6)$$

where $u_j = \beta_j|x|/R_0^3$, $\overline{N}_{\rm in}$ is the average value of $N_{\rm in}$, and $\overline{N}_{\rm out} = N_{\rm out}V_{\rm in}/V_{\rm out}$. Equation (4) implies that the SDK is given by the convolution of inverse Fourier-transformed spectra of $\exp(-J_{\rm in})$ and $\exp(-J_{\rm out})$. In each spectrum, β_j determines the lateral scale of the spectrum, while $\overline{N}_j$ determines the spectral shape. The contributions of $J_{\rm in}$ and $J_{\rm out}$ approach Lorentzian when $\overline{N}_{\rm in} \gg 1$ and $\overline{N}_{\rm out} \ll 1$, respectively. On the other hand, the contribution of $J_{\rm out}$ approaches Gaussian when $\overline{N}_{\rm out} \gg 1$ [6]. Thus, only $J_{\rm in}$ with $\overline{N}_{\rm in} \lesssim 1$ can yield the SDK that explains hole broadening observed in H_2PP-Mb in which the tail region is raised compared with the Lorentzian. The deviation of Eq. (4) from the Lorentzian is prominent around $\nu = \beta_{\rm in}R_0^{-3}$.

Under temperature-cycling condition, $I(\nu)$ is a function of T_c. Let T_n be the excursion temperature of the nth cycle. Then we write SDK in the temperature range between T_n and T_{n+1} as $I(\nu, T_n \longrightarrow T_{n+1})$, which is defined by $I(\nu)$ with $J_j(x)$ replaced by $J_j(x, T_n \longrightarrow T_{n+1})$. Here $J_j(x, T_n \longrightarrow T_{n+1})$ is given by Eqs. (5) and (6), where $\overline{N}_j$ and β_j are values for TLS's that do not flip below T_n, but between T_n and T_{n+1}.

We made a least-mean-square fit to the data to estimate the parameter values. The fitting was made on Fourier transformed data, i.e., in the x space. This method has several advantages in that we can dispense with both the Fourier transform of Eq. (4) and the convolution of SDK with the initial hole spectrum, and the antisymmetric component of the hole spectrum that is due to drift, etc., is averaged by neglecting the imaginary part of the Fourier transformed spectrum. The parameter values thus estimated are given in Table I. In Fig. 1(b), the closed circles denote the convolution of the calculated $I(\nu, 43\ \text{K} \longrightarrow 56\ \text{K})$ and $I(\nu, 56\ \text{K} \longrightarrow 69\ \text{K})$ with the hole spectra of $T_c = 43$ K and $T_c = 56$ K, respectively. We see that the agreement between the calculated and the measured spectra is satisfactory in both the central and wing regions. We further confirmed that the experimental data in which the depth or the profile of the initially burnt hole is different from that in Fig. 1 can also be reproduced well with the above parameter values.

In Table I, we notice that only about 0.24 (= 0.16 + 0.08) TLS's flip, on an average, in each Mb molecule in

TABLE I. Parameters for fits to the hole-burning data of Fig. 1.

Temperature	$\beta_{\rm in}$ ($\text{cm}^{-1}\,\text{A}^3$)	$\beta_{\rm out}$ ($\text{cm}^{-1}\,\text{A}^3$)	$\overline{N}_{\rm in}$	$\overline{N}_{\rm out}$
43 K → 56 K	4.5×10^3	8.0×10^2	0.16	1.2
56 K → 69 K	3.8×10^3	6.2×10^2	0.08	3.8

the temperature interval from 43 to 69 K. (The relative uncertainty in the estimation of β_{in} and $\overline{N}_{\text{in}}$ is about 10%.) It should be noted that N_{in} does not include the number of the protein TLS's which flip an even number of times during the temperature cycling. Thus the number of protein TLS's which can flip in this temperature range is estimated to be about 0.5 ($\doteqdot 0.24 \times 2$). In a previous paper, from a measurement of hole filling in a temperature-cycling experiment, we speculated that H_2PP-Mb has a few TLS's that cause the nonphotochemical hole-burning effect [8]. It was also suggested that the number of active TLS's in Mb is about 3 in the temperature interval between 0 and 100 K based on a specific heat measurement at low temperatures [9]. These results are consistent with the present estimation. Hence, it is tempting to consider that the broadening in the wing of the hole in the 43–69 K temperature range is induced by only one kind of TLS in Mb.

In the above analysis, we assumed that TLS's in the protein interact with the dye via electrostatic interaction, and each TLS has the same coupling constant β_{in}. We also assumed that the dispersion of the frequency shift of the dye molecule comes from the spatial distribution of TLS's and the random direction of $\Delta\boldsymbol{\mu}$ with respect to $\boldsymbol{\mu}_M$. However, if the ordered structure of a protein is taken into account, it seems more natural to assume that the TLS is located in a well defined region of the protein, or that the transition in the TLS corresponds to a global conformation change and is delocalized within the protein. Moreover it is possible that the dominant mechanism of the dye-TLS interaction is not electrostatic but elastic. Even in these cases, the dispersion of the frequency shift exists because each protein molecule occupies a different substate in a hierarchically arranged phase space, and will give similar hole spectra. One TLS with a fixed frequency shift gives an SDK as

$$I_{\text{in}}(\nu) = \frac{n}{2}\,\delta(\nu + \Delta\nu) + (1 - n)\delta(\nu) + \frac{n}{2}\,\delta(\nu - \Delta\nu), \tag{7}$$

where n is the average number of flipping TLS's, $\Delta\nu$ is the frequency shift by the TLS flipping, and $\delta(\nu)$ is the delta function. This SDK with $n \doteqdot \overline{N}_{\text{in}}$, $\Delta\nu \doteqdot \beta_{\text{in}} R_0^{-3}$, and additional broadening by J_{out} roughly reproduces the experimental data. However, to get agreement as fine as with the SDK of Eq. (4), it is necessary to give a finite width to the first and the third delta functions in Eq. (7), and accordingly three parameters (n, $\Delta\nu$, and the width) are needed for the inside TLS's. Since the structure of TLS's and the dye-TLS interaction in a protein are not known at present, we have adopted the model of Eq. (5) with two parameters ($\overline{N}_{\text{in}}$ and β_{in}) as one of the most simple models. In any case, a definite result derived from our experimental data is that the frequency shift of the dye by the flipping of a TLS in the protein is on the order of $\beta_{\text{in}} R_0^{-3}$. If we assume the dipole-dipole interaction and adopt 0.214 D as the value of $\Delta\boldsymbol{\mu}$, which was obtained for chlorin in poly(vinylbutyral) [10], $\boldsymbol{\mu}_M$ is estimated to be ~1.9 D. This value is similar to the dipole moment of typical polar molecules such as H_2O (~2 D) and is several times larger than that of a TLS activated around 5 K (~0.6 D), which was estimated from dielectric constant data [9]. The values of β_{out} and $\overline{N}_{\text{out}}$ in Table I may be less accurate than those of β_{in} and $\overline{N}_{\text{in}}$. However, it is certain that, for TLS's in a protein, the coupling constant β is larger, and the number density is smaller compared with TLS's in the solvent of the protein.

As described above, we detected deviations from a Lorentzian hole shape by the use of a multimode laser and a monochromator. Here, we stress that a multimode laser is much more advantageous than a single-mode laser for our purpose. That is, even if we make the same experiment using a single-mode laser of a wide tunable range, it will be difficult to detect the deviation of the SDK from a Lorentzian. With a single-mode laser, the hole area is very small even if a deep hole is burned because the hole is narrow. If the initial hole area is small, the change in the tail region of the temperature-cycled hole spectrum is also small and hardly discernible because the structure in the tail region of the SDK is not sharp. On the other hand, a multimode laser can burn a large hole. Thus, laser light that is not single frequency but sufficiently narrow to resolve the hole broadening in the tail region is optimum for the detection of the present type of hole broadening.

[1] H. Frauenfelder, S. G. Sligar, and P. G. Wolynes, Science **254**, 1598 (1991).
[2] P. W. Anderson, B. I. Halperin, and C. M. Varma, Philos. Mag. **25**, 1 (1972).
[3] W. A. Phillips, J. Low Temp. Phys. **7**, 351 (1972).
[4] J. Zollfrank, J. Friedrich, J. M. Vanderkooi, and J. Fidy, Biophys. J. **59**, 305 (1991).
[5] D. E. Pack, L. R. Narasimhan, and M. D. Fayer, J. Chem. Phys. **92**, 4125 (1990).
[6] L. Kador, J. Lumin. **56**, 165 (1993).
[7] A. Kurita, Y. Kanematsu, and T. Kushida, J. Lumin. **45**, 317 (1990).
[8] A. Kurita, R. Ohmukai, and T. Kushida, J. Lumin. **53**, 255 (1992).
[9] G. P. Singh, H. J. Schink, H. V. Lohneysen, F. Parak, and S. Hunklinger, Z. Phys. B **55**, 23 (1984).
[10] L. Kador, D. Haarer, and R. Personov, J. Chem. Phys. **86**, 5300 (1987).