Spectral Hole Burning, Recovery, and Thermocycling in Chlorophyll-Protein Complexes: Distributions of Barriers on the Protein Energy Landscape

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Abstract: Chlorophyll-protein complexes are ideal model systems for protein energy landscape research. Here pigments, used in optical spectroscopy experiments as sensitive probes to local dynamics, are built into protein by Nature (in a large variety of local environments without extraneous chemical manipulations or genetic engineering). Distributions of the tunneling parameter, λ, and/or protein energy landscape barrier-heights, V, have been determined for (the lowest-energy state of) the CP43 core antenna complex of Photosystem II. We demonstrate that spectral hole burning (SHB) and hole recovery (HR) measurements are capable of delivering important information on protein energy landscape properties and spectral diffusion mechanism details. In particular, we show that tunneling rather than barrier-hopping is responsible for both persistent SHB and subsequent HR at 5-12 K, which allows us to estimate the md^2 parameter of the tunneling entities as $\sim 1.0 \cdot 10^{-46}$ kg·m^2. The sub-distributions of λ actually contributing to the non-saturated spectral holes (and affecting their recovery) differ from the respective full true distributions. In the case of the full λ–distribution being uniform (or the barrier height distribution $\sim 1/\sqrt{V}$, a model which has been widely employed in theories of amorphous solids at low temperatures and in HR analysis), the difference is qualitative, with λ sub-distributions probed in the HR experiments being highly asymmetrical, and barrier V sub-distributions deviating significantly from $\sim 1/\sqrt{V}$. Thus, the distribution of λ for the protein energy landscape tier directly probed by SHB is likely Gaussian and not uniform. Additionally, a Gaussian distribution of barriers, with parameters incompatible with those of the landscape tier directly probed by SHB, contributes to the thermocycling results.

ABBREVIATIONS:

CD – Circular Dichroism; FWHM – Full Width at Half Maximum; HB – Hole Burning;

HGK – Hole Growth Kinetics; HR – hole recovery; LIHF – Light-induced Hole Filling;

NPHB – Non-photochemical Hole Burning; OD – Optical Density; PS I – Photosystem I;

PS II – Photosystem II; SDF – Site Distribution Function; SHB – Spectral Hole Burning;

SPCS – Single Photosynthetic Complex Spectroscopy; TLS – Two-level System; ZPH –

zero-phonon hole; ZPL – Zero-Phonon Line.

1. INTRODUCTION

Proteins perform a variety of tasks in living organisms. Their ability to complete

these tasks depends critically on the final, tertiary structure of the protein, formed as a

result of the polypeptide chain folding. The resultant structure is stabilized by hydrogen

bonds and hydrophobic interactions, both between the peptides and with surrounding

water molecules. Due to the enormous number of degrees of freedom and relative

weakness of the above interactions, the final structure of the protein is not perfectly

determined, allowing for existence of multiple nearly- but not perfectly identical

conformational sub-states. These energy minima are separated by barriers and

collectively form the protein energy landscape, arranged in hierarchal tiers [1-3]. Transitions

between the sub-states are possible, with the rates depending on the barrier parameters.

Pigment molecules, embedded in the protein and in electrostatic interactions with it, can

"sense" subtle structural rearrangements of the protein and react to them by changing

their electronic transition energies. This phenomenon is referred to as "spectral

diffusion". Thus, transitions between different conformational sub-states of the protein

can be monitored by optical spectroscopic methods. These methods are most sensitive at very low temperatures. Therefore, they probe only a fraction of the full protein energy landscape, since the largest barriers cannot be crossed. However, this is an important fraction – the transitions between different sub-states around the global energy minimum are often relevant for proper protein function. And at low temperatures these transitions are sufficiently slow for convenient exploration of the energy landscape properties. Higher-barrier tiers of the protein energy landscape can be explored at higher temperatures, at the expense of the loss of small-barrier tier information. The hierarchal character of the protein energy landscapes has indeed been confirmed by a variety of methods, including optical ones, in particular Spectral Hole Burning (SHB) [4,5] and single molecule spectroscopy [3]. However, many important issues related to barrier distribution shapes and parameters, as well as to the exact atomic nature of protein structural rearrangements involved in this process, remain unresolved.

SHB involves selecting a sub-ensemble of molecules with narrow zero-phonon lines (ZPL) in resonance with the laser excitation frequency and inducing either photo-transformation of the molecules themselves, or a rearrangement of molecules' environment. In both cases, a resonant decrease of absorption (a "hole") appears at the illumination frequency, which is a mirror image of the ZPL. The environmental rearrangement case, which can be considered light-induced spectral diffusion [6-10], is encountered in amorphous solids, including proteins at low temperatures. This HB mechanism is known as Non-Photochemical Hole Burning (NPHB)[11,12]. A schematic representation of NPHB is shown in Figure 1. Part A shows the ground and excited electronic states of the pigment molecule in interaction with the amorphous solid (e.g.

protein). Only two minima of the energy landscape are depicted for the sake of simplicity; the actual number of minima on the protein energy landscape is certainly larger. If the barriers in the excited state are significantly lower than in the ground state, the system may, starting from well 1 with ΔE_1 corresponding to the laser frequency, experience transition from one well to another within the lifetime of the excited state, return to the ground state, and remain trapped in state 2 for a relatively long time, determined by the ground state barrier parameters. The barrier parameters are introduced in Figure 1B, which may represent either the ground or excited state. The transitions between the two wells may involve either tunneling or barrier hopping, with NPHB, according to[11,12], being due to tunneling in the excited state. Δ is the asymmetry, with the difference in ground and excited state asymmetries determining the frequency shift of the pigment upon NPHB. V is the barrier height and d is the change in generalized coordinate between wells 1 and 2. So far the model is no different from the two-level system (TLS) model [13,14] employed to explain SHB in glasses [11,12,15-19], but we further expand it to include the second, lower hierarchal tiers of the protein-energy landscape (Figure 1C). For the sake of simplicity the landscape is shown as one-dimensional, while in fact the number of relevant dimensions (independent generalized coordinates) may be much larger. A fraction of a 2D landscape is shown in the Graphical Abstract.

SHB has been widely applied to determine the details of spectral diffusion and energy landscapes in glasses[11,20-26] and proteins[4,27,28] at low temperatures. Chlorophyll-protein complexes in particular are ideal model systems for protein energy landscape research: here pigments / "probes" are built into protein by Nature (in a large variety of local environments) without chemical manipulations or genetic engineering. These are

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the pigment-protein interactions, which determine the transition energies of the pigments inside the pigment-protein complexes [29-32]. The site-energies, along with the inter-pigment interaction energies, determine the shapes of various optical spectra of the complexes [31-35], as well as the details of the energy-transfer processes, which are part of photosynthetic light-harvesting. The energy transfer processes are affected not only by the static structure of the complex, but also by the protein dynamics, including the slow processes. For example, anticorrelated behavior of the emission bands in single Photosystem I (PS I) complexes[36] indicates that energy transfer pathways can fluctuate following conformational changes in the protein. The light-induced shifts of the chlorophyll site energies (NPHB), which are determined by the properties of the protein energy landscapes, affect the positions and oscillator strengths of excitonic states [32-34]. These influence various optical spectra, including those of non-resonant NPHB[12,13] and δ-CD[35].

Several classes of SHB experiments probe different aspects of the protein energy landscapes. Experiments on hole evolution during the burning process, in particular hole growth kinetics (HGK) [15-19,37,38] measurements, probe the distribution of barriers in the excited electronic state of a pigment-protein system. It has been demonstrated that HGK results for a variety of glassy[15-19] and protein[37,38] systems are in good agreement with this model, employing Gaussian distributions of the tunneling parameter, λ [20,39]. The latter is related to barrier heights and other system parameters (see Figure 1B) via $\lambda = d\sqrt{2mV}/\hbar$, where d and V were defined above and m is the effective mass of the entity rearranging during conformational change. On the other hand, the distribution of barriers in the ground state has been explored by observing recovery of the previously

burned holes, either at a fixed, low temperature (same as the temperature at which the burning took place) or as a result of thermocycling [22,26,40]. It was usually assumed that the holes recover due to barrier hopping upon thermocycling, with the probability of crossing barriers of height, V, being temperature-dependent and proportional to $exp(-V/k_BT)$. The derivative of the dependence of the hole area on the cycling temperature yields the distribution of barrier heights [22,26]. It has been noticed long ago, that in the case of glasses the resulting ground–state barrier distribution is proportional to $1/\sqrt{V}$ [22,23,25] (corresponding to a uniform distribution of λ)[13,14]. A notable exception is the work of Love et al, who observed a Gaussian barrier distribution in the ground state of the $Tb^{3+}/Ba_{1-x-y}La_xTb_yF_{2+x+y}$ system[26]. In the case of proteins, a superposition of $1/\sqrt{V}$ and Gaussian components has been reported[4,40]. Distributions of d and m are usually assumed to be very narrow and are neglected[22].

A decrease in the area of a hole is usually accompanied by an increase in the hole width, described by a spectral diffusion kernel[4,24,41]. This broadening was also reported to conform to an $\sim 1/\sqrt{V}$ barrier distribution, although Jankowiak et al.[39,42] demonstrated that a Gaussian λ-distribution may yield similar hole-broadening results for certain parameters. One could argue that hole broadening is due to conformational changes on the fastest, smallest-barrier tier of the protein energy landscape[1,3] (Figure 1C), while a hole area decrease is due to relaxation on the next, higher-barrier tier. NPHB (and HR) with a higher-barrier tier supposedly involves relatively large shifts of the pigment's absorption frequency (large compared to the width of the hole and to the width of the high-resolution laser scan, ~ 1.5 cm^{-1} in this study). (NPHB on the lower-barrier tier can occur as well, but it contributes to the width of the initial hole.) Spectral shifts of different

magnitudes, corresponding to different tiers of the protein energy landscape, have been directly observed in Single Photosynthetic Complex Spectroscopy (SPCS) experiments[3,41,43]. A Gaussian spectral diffusion kernel has been observed in SPCS experiments on LH2 for the smallest line shift tier of the protein landscape (characteristic shift magnitude ~1 cm^{-1}) [41]. The shapes of the barrier and shift magnitude distributions on the tier of the energy landscape corresponding to ~10 cm^{-1} spectral line shifts, however, remains undetermined by SPCS so far.

SPCS is currently considered the technique of choice for spectral diffusion research as it is free from (sub-)ensemble averaging inherent in SHB. However, there are several areas where SHB, despite its seeming shortcomings, can provide information relevant for interpretation of SPCS experiments and beyond. For instance, one question is whether the phenomena observed in SPCS experiments are predominantly thermally-induced (i.e., are occurring anyway, whether one observes them in an optical experiment or not) or are measurement / light-induced (i.e., represent NPHB on a single molecule level; light-induced single molecule line jumps are well known in glassy systems[6-10]). In [37] we suggested that joint analysis of the excited state barrier distribution data (and resulting distributions of HB yields) obtained from HB experiments and of photon budgets of SPCS experiments allows one to distinguish between these possibilities (with the conclusion being that low-temperature SPCS observations are mostly light-induced). A related question is whether the observed line shifts are due to tunneling or barrier-hopping[44]. The latter question will be addressed in this manuscript. The SHB experiments also naturally deliver ensemble averages, which can be compared with the averages of the

SPCS data in order to tell if particular SPCS experiments probe relevant dynamics of the intact sample, not perturbed by preparation.

In summary, there is the need for improved qualitative and quantitative understanding of energy landscape barrier distributions. There is also a contradiction between the shapes of the barrier distributions for the tier of the protein energy landscape responsible for light-induced line shifts of around 10 cm^{-1}, with the HGK results being in agreement with a Gaussian barrier distribution in the excited state and thermocycling results suggesting a $1/\sqrt{V}$ barrier distribution in the ground state. The shape of this distribution is important in various theories concerning the low-temperature properties, not only of proteins but also of other amorphous solids (see [13,14,39,42] and references therein). We demonstrate that HB and hole recovery (HR) studies of the same system (and within the framework of one unified model) can resolve this contradiction. We test the ability of Gaussian and uniform λ-distributions to explain both the hole-burning and hole-recovery (including thermocycling) results in a unified way, and show that only Gaussian distributions properly describe all data discussed in this manuscript. We also present arguments showing that tunneling is the key mechanism responsible for NPHB in pigment-protein complexes (as well as for the line shifts in SPCS experiments).

2. MATERIALS AND METHODS

CP43 (proximal antenna complex of Photosystem II, PS II) from spinach was used as a model system since SHB processes of this complex have been thoroughly explored[33-35,38,45,46] and many parameters are known. Samples were isolated and purified as described in[33]. The absorption spectra, measured with a Varian Cary 5000

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spectrophotometer at a resolution of 0.5 nm, were nearly identical to those reported in[33,45]. The same can be said about emission spectra, measured with an Acton SP2356 spectrograph equipped with a Princeton Instruments Pixis CCD.

The high-resolution experiments have been performed in fluorescence excitation mode with the apparatus described in[38]. Here we point out that the Spectra-Physics / Sirah Matisse-DS dye laser employed in this work is capable of seamless high-resolution scans of ~45 GHz, and can be stabilized to less than 30 MHz for hours in HGK measurements. In thermocycling experiments, the temperature was controlled and stabilized using an UTREX temperature controller associated with a cryostat. The procedure for exploring HR and thermocycling was as follows: After burning a hole, the hole was first allowed to recover at a fixed (burn) temperature (5 K). The hole spectrum was scanned from time to time during this recovery phase. Alternatively, the signal at a fixed burn wavelength was monitored with low (read) intensity. Although the latter approach provided information only on the depth of the hole, it eliminated light-induced hole filling (LIHF)[47]. A couple of hours after burning, the thermocycling experiments were started. In these experiments, the temperature was raised to the desired level and then lowered back to the burn temperature of 5 K. The hole spectrum was always measured at 5 K. Then the cycles were repeated with increasing maximal temperatures.

3. RESULTS

3.1 Tunneling versus barrier hopping and the nature of the rearranging entities

Figure 2 depicts the HGK curves (noisy curves) obtained at a burn wavelength of 686.1±0.1 nm (The absorption at 686 nm is dominated by the so-called A-state of CP43

complex[33-35,38,45,46]. At this wavelength the respective pigment is the lowest-energy one and no downhill excitation energy transfer is expected, see insert) at different temperatures with burn intensities of ~2-12 μW/cm^2. The maximal temperature of this experiment was 13 K, as above 13 K the thermocycling-related recovery clearly becomes important (see below) and straightforward interpretation of the results becomes difficult. The burning process exhibits a slowdown with increasing temperature. The parameters of the Gaussian λ-distribution obtained from the 5 K curve (λ_0=10.2±0.1, σ_λ=1.1±0.1, with S=0.35±0.05) are somewhat in disagreement with the results of [38], where we reported a larger value of the tunneling parameter ($\lambda_0 = 11.0$). Careful analysis of the details of the experiments in this study and in[38] suggests that too large a reading intensity was employed for CP43 in[38] (but only for CP43, not for CP29 or LHCII, for which a somewhat different setup was used), which could result in (a) a fraction of molecules with the smallest λ being burnt even before the start of the HGK measurement and (b) in an increase of λ_0 and decrease of σ_λ obtained from the HGK curve. We have confirmed that burning of ~67%-deep zero-phonon holes (ZPH) is possible (i.e., the ZPH is almost at 100% of its theoretically possible depth, given $S(T=0) \sim 0.3$ and assuming downhill tunneling in the excited state[48]). Figure 2 also contains the results of HGK modeling (smooth curves), which was performed assuming that temperature influences the HGK only via the homogeneous line width[45] and a weak temperature dependence of the Huang-Rhys factor S ($S(T)\sim\coth(\hbar\omega/2k_BT)$[49]). The modeling was based on the SHB master equation, implying that SHB is due to tunneling (involving the higher-barrier tier, Figure 1B), with the absorption spectrum after burning at ω_B with photon flux, P, for time t being:

$$D(\Omega,t)=1.5\int d\omega L(\Omega-\omega)G(\omega)\int d\lambda f(\lambda)$$
$$\times \int d\alpha \sin\alpha \cos^2\alpha e^{-P\sigma\phi(\lambda,\tau_{fl})L(\omega_B-\omega)t\cos^2\alpha} \qquad (1)$$

Here σ and ϕ are the integral absorption cross section and the HB quantum yield, respectively. The latter depends on the tunneling parameter λ:

$$\phi(\lambda,\tau_{fl})=\frac{\Omega_0\exp(-2\lambda)}{\Omega_0\exp(-2\lambda)+\tau_{fl}^{-1}} . \qquad (2)$$

$f(\lambda)$ is the distribution of λ and τ_{fl} is the fluorescence lifetime. $L(\omega_B-\omega)$ is the single site absorption profile. $\Omega_0=7.6\cdot10^{12}$ s^{-1} can be interpreted as an attempt frequency; the value is borrowed from[15-18] for the sake of λ-distribution parameters for different systems being easily comparable. Alpha is the angle between the laser polarization and the transition dipole moment. $G(\omega)$ is the site distribution function (SDF), which is Gaussian before burning, and it describes the probability of finding a pigment with a zero-phonon line (ZPL) at a given frequency. Homogeneous line widths for several temperatures up to 13 K were measured in this study; lower-temperature widths were in good agreement with the data from [45]. The agreement between experimental and modeling results was fairly good (except for the latest stages of the burning process, where the equilibration between HR and HB led to an apparent slowdown of burning), indicating none or very weak temperature dependence of the SHB yield between 5 and 13 K. The dashed arrow indicates that at increased temperatures some small fraction of the systems exhibited somewhat accelerated burning.

The tunneling rate depends on λ as $exp(-2\lambda)$. The hopping rate depends on the barrier height, V, as $exp(-V/k_BT)$, and thus it depends on $\lambda=d\sqrt{2mV}/\hbar$ as

$exp(-\lambda^2\hbar^2/2md^2k_BT)$. The omitted prefactor in both rates is the same attempt frequency

Ω_0 on the order of 10^{12} Hz. From this rate representation one can obtain the upper limit of

md^2 for which the initial assumption of our HB model (tunneling in the excited state

being the dominant process responsible for NPHB), is still valid. Namely, this is true if

$$2\lambda << \lambda^2\hbar^2/2md^2k_BT \tag{3}$$

or $\lambda\hbar^2/md^2k_BT>>4$. At T=5 K, $md^2< \lambda\hbar^2/4k_BT=\lambda\cdot4.0\cdot10^{-47}$ kg·m^2. At T=13 K,

$md^2<\lambda\cdot1.5\cdot10^{-47}$ kg·m^2. Figure 3 depicts the dependence of the ratio of the tunneling rate

to the hopping rate on λ for $md^2 = 1.0\cdot10^{-46}$ kg·m^2 at 13 K (red solid curve, note the

logarithmic vertical scale). Tunneling strongly dominates at all λ, except for the smallest

ones, $\lambda \lesssim 6.5$. The increase of temperature or of md^2 would shift the curve towards larger

λ, meaning that barrier-hopping would be dominant for a larger range of λ. The dashed

curve in Figure 3 is a Gaussian λ-distribution resulting from the fit to the 5 K HGK data

in Figure 2. Figure 3 demonstrates that in order for NPHB to be dominated by tunneling

for any useful λ ($\lambda \geq 6$), Gaussian λ-distribution, and temperatures up to 13 K, the md^2

has to be less than $1.0 \cdot10^{-46}$ kg·m^2. (Somewhat larger md^2 can be in agreement with

tunneling in the case of uniform λ-distribution [dotted lines in Figure 3], but see the

discussion on distribution shapes below.) It is clear that for the situation depicted in

Figure 3 at (13 K), a small fraction of systems with excited state $\lambda \leq 6.5$ will experience

HB via barrier hopping, somewhat accelerating the initial stages of the HB process

(which is indeed observed, see dashed arrow in Figure 2) and somewhat decelerating the

HR (indeed observed, see the 13 K curve in Figure 4B). Thus, our data can be interpreted

as $md^2 = 1.0\cdot10^{-46}$ kg·m^2 being *the* true value of md^2, rather than its upper limit, although

the evidence might be somewhat weak to claim that with full certainty. If the displacement along the generalized coordinate, d, is ~1Å, the mass of the tunneling entity should be ~$1.0 \cdot 10^{-26}$ kg, which is significantly smaller than the mass of one carbon atom. Thus, for tunneling to be the dominant HB mechanism with conformational changes involving structural elements as large as protein side-groups, displacements along the generalized coordinate should be significantly smaller than 1Å. A plausible alternative would be proton tunneling. An extended discussion of the structural implications will be provided at the end of Section 4.1.

3.2. Hole recovery at burn temperature and partial λ- and barrier distributions.

Figure 4A depicts the 5 K recovery of the holes (relative area vs time) of different initial fractional depths burned at 686.1±0.1 nm. It is clear that the rate of recovery is dependent on the fractional depth of the hole originally burned. A larger fraction of the shallower holes is recovered within the same time interval than for the more saturated holes. The holes also experience weak broadening. Note that performing the laser scan takes some time and, therefore, the first hole in the series was measured approximately three minutes after the end of burning. Nevertheless, the fractional depths obtained from the first post-burn spectra and from the HGK curves were fairly consistent, within ~1%. Frame B contains hole-recovery data obtained by monitoring the fluorescence signal (i.e., hole depth rather than area) at fixed $\lambda_B \sim 686$ nm, and utilizing the same light intensity as was employed while measuring the hole spectra (~40 nW/cm^2) at several temperatures. The data for the ~20%-deep holes was collected immediately after measuring the respective HGK (a motorized filter flipper was employed). The holes experienced

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relatively slow recovery, with 1.4% (of the pre-burn absorption; 7% of the original 20% hole) recovering in the first 180 seconds (solid arrow, the approximate time between the end of the HGK measurement and the hole spectrum measured for the first time in other experiments). After each of the measurements depicted in Figure 4B, the sample was left in the dark for several minutes. Subsequent segments of the recovery curves (not shown) were located on the smooth line defined as further-extrapolated fit to initial recovery, which indicated that monitoring the recovery with the reading intensity has a small, if any, effect on the recovery rate. Interestingly, the recovery of the hole observed in this mode did not depend on the temperature up to 10 K, but did become slower at 13 K. The latter effect may be due to the increase of the efficiency of HB (competing with recovery). This increase in HB yield may be attributed to the onset of excited-state barrier-hopping (for the smallest-λ systems in the ensemble) in addition to tunneling. (See the previous section and Figure 3 for additional details.)

We also noticed that performing SHB experiments in the neighboring wavelength ranges resulted in somewhat faster recovery of a given hole. Qualitatively, this indicates that a fraction of NPHB photoproduct (anti-hole) was redistributed within several cm^{-1} from the original hole, and that a low-λ fraction of this anti-hole could be returned to the original wavelength via light-induced hole filling (LIHF). Although this is not immediately obvious from Figures 4A and 4B, as one of them depicts evolution of hole area while the other depicts evolution of hole depth, the data in frames A and B are in disagreement. Namely, when monitored at a fixed (burn) wavelength, the 20% hole recovered significantly slower than suggested by the data shown in Figure 4A (see blue diamonds). The most likely reason for this disagreement is, again, LIHF, caused by the

measurement (i.e., scanning of the HB spectra). In this scenario, the shallower (20%) hole would be more affected by LIHF, since the anti-hole would be dominated by lower values of λ (or barrier height). Figure 5 compares the hole-depth dependences on the recovery time for holes monitored at a fixed burn wavelength (black curve a, hole 55%-deep right after burning; blue curve b, 20%), and holes monitored via scanning (green (c), red (d); same holes as in Figure 4A). Note that in the fixed-wavelength experiments, the sample was not exposed to light continuously but just for short periods of time (except for the very beginnings of the curves). Thus, possible prevention of HR by the measuring light has been minimized. It is clear that the differences between the hole-depth data obtained in the two types of recovery experiments are dramatic, and one must conclude that the data in Figure 4A is significantly affected by LIHF. Therefore, the data obtained via direct hole depth monitoring, as shown in Figure 5 (black, blue), rather than data on hole areas (see Figure 4A), should be modeled to obtain distribution shapes and parameters, as discussed below.

As mentioned in the Introduction, the shape of the barrier height or λ distribution is a subject of debate. In order to resolve this issue, we will attempt to model our data within both Gaussian (characterized by mean λ_0 and STD σ_λ) and uniform (or, more precisely, rectangular; constant probability between λ_{min} and λ_{max}[13,14]) λ-distribution frameworks, assuming the existence of a correlation between the shapes of the protein energy landscapes (magnitudes of the relevant energy barriers) in the ground and excited states of the CP43 pigment-protein system. We will also make the assumption that the mass of the tunneling entity, m, and the displacement along the generalized coordinate, d, are the same in both the ground and excited states, and that the only difference between

the ground and excited states barriers is in the magnitude of the barrier heights. This is equivalent to requiring that, as depicted in Figure 1, the minima of the ground-state landscape are located below the minima of the excited-state landscape.

It is important to recognize that the HGK experiments, with the irradiation dose ($P \cdot t$ in Eq. 1) changing over several orders of magnitude, and with the holes eventually reaching saturation, yield the whole distribution of barriers (more precisely, the tunneling parameter, λ) in the excited state. Holes of various non-saturated depths, however, represent only a fraction of the original λ- (or barrier) distribution. Obviously, the subset of pigments experiencing the smallest barriers (smallest λ) undergoes NPHB first, while for shallow enough holes, the pigments with large λ remain mostly unaffected and do not contribute to the hole spectra.

The HB simulation software previously used in[37,38] as well as for modeling the HGK curves in Figure 2, has been modified to save and automatically analyze separate contributions to the spectral hole obtained for different values of λ. Contributions to the hole spectrum described in full by Eq. 1 were calculated separately for every λ over several fractional depths of the (total) hole, and respective contributions to the total resonant hole (ZPH) area and depth were determined. Figure 6 depicts partial excited-state λ-distributions, which actually contribute to the holes of the fractional depths of 20% and 55% for the Gaussian and uniform λ-distributions. The areas under the curves, which are proportional to the hole areas, were re-normalized to one for clarity. The modeling parameters correspond to burning at 686 nm into the (lowest-energy) A-state of CP43 (with the SDF peaking at 683 nm and an inhomogeneous width of 180 cm^{-1} [34,35,45]). The full λ–distribution parameters for both shapes of the distribution were obtained from

the best fit to the saturated 5 K HGK curve reported in Figure 2. For full, true Gaussian

λ-distribution, λ_0 is 10.2±0.1 and σ_λ is 1.1±0.1, with S=0.35±0.05. The latter value is in

agreement with the earlier SHB data [33-35,38,45,46], including the fitting of the whole SHB

spectra [35,46], not just the HGK. For a uniform λ distribution, a quite satisfactory fit to the

HGK curve can be obtained for λ_{min}=8.5±0.1 and λ_{max}=11.9±0.1.

In the case that we start from the Gaussian λ-distribution, the partial distributions

actually contributing to the holes resemble Gaussian curves (Frame A of Figure 6),

although the agreement is not perfect (low-λ tail somewhat extended; high-λ tail a bit

steeper than for a Gaussian). λ_0'=8.60, σ_λ'=0.99 were obtained for a 20% hole and

λ_0'=8.84, σ_λ'=1.08 were obtained for a 55% hole via Gaussian fits. However, the shape

of the partial λ–distribution in the case where the full λ-distribution is uniform (between

λ_{min} and λ_{max}) is highly asymmetrical (see Frame 6B). The partial distributions for holes

of 20% and 55% initial fractional depths are shown as solid black (a) and blue (b) curves,

respectively; full distributions are shown with dashed lines. Also presented in Figure 6

are the respective cumulative distributions (red and green curves c and d; i.e., integrals of

the partial distribution curves) and their differences (e, magenta curves). For both types

of distributions, the difference reaches a maximum value of about 12% by the time the

holes are roughly 70% recovered. This is in clear disagreement with the results in Figure

4A, where an 18% difference between the recovery curves for 20% and 55% holes was

achieved early in the process of recovery. The disagreement is attributed to LIHF.

The smallest-barrier fraction of the excited-state λ-distribution (see Figure 6)

corresponds to the smallest barrier fraction of the ground-state distribution. It is clear that

if tunneling is the dominant process behind NPHB at 5 K, it also would be an

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overwhelmingly dominant process behind HR in the ground state, where λ and V are larger (see Figure 3). The modified expression of Love et al.[26] for the fraction of systems not recovered after time τ, which includes both tunneling and barrier-hopping rates, can be written as

$$q(\lambda,\tau,T) = \exp(-\Omega_0\tau[(n(E)+1)\exp(-2\lambda) + \exp(-\lambda^2\hbar^2/2md^2k_BT)]) . \qquad (4)$$

Here $n(E)=(exp(E/k_BT)-1)^{-1}$ is the occupation number for phonons (bosons) necessary to satisfy the energy balance, and $E \cong \Delta \sim 10$ cm^{-1} is approximately equal to the ground-state TLS asymmetry (see Figure 1), which, in turn, is similar to a typical ZPL shift upon burning. One can easily calculate numerically the λ–(sub)-distributions, areas, and depths of the holes remaining after any time interval at any temperature by multiplying the post-burn fractional λ–distributions by the $q(\lambda,\tau,T)$ curves. As described above, we have reasons to believe that the area recovery data presented in Figure 4A is affected by LIHF. Thus, parameters of the ground-state λ sub-distributions determining the 5 K HR could be more reliably obtained from an analysis of the hole depths, rather than the hole areas. Note that our model does not take into account spectral-diffusion broadening[24,28]. Therefore, the actual decrease of the hole depth, not affected by LIHF, should be equal to or slightly faster than the depth decrease following from our model.

Figure 7 depicts results of hole-depth recovery modeling based on a Gaussian (Frame A) or a uniform (Frame B) original full λ-distribution. It is immediately clear that due to highly asymmetrical partial-distribution shapes (see Figure 6B), the uniform λ–distribution model predicts HR behavior, which is qualitatively different from that observed in the experiment. On the other hand, in the Gaussian λ–distribution model, one can successfully fit recovery of both shallow and deep holes, starting with the same set of

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parameters. The excited-state partial distributions depicted in Figure 6A have been stretched/rescaled by the same factor $k=\lambda_{ground}/\lambda_{excited}=2.35$ to yield the respective ground-state λ-distributions. This factor is in reasonable agreement with the results obtained by Reinot et al. for glasses[16,19].

3.3 Hole thermocycling

Figure 8 depicts results of a typical thermocycling experiment (circles). The hole, originally 37% deep, was allowed to recover for about 2.5 hours at 5 K before the thermocycling was started. This ensured that the smallest-barrier fraction of the hole had recovered, either spontaneously or via LIHF (see the previous section), and one could meaningfully follow the recovery upon thermocycling by monitoring the hole area rather than the depth. The latter would not be very informative, since spectral holes are known to broaden rapidly upon thermocycling[23,28,50]. That said, one can note that for the first three hours, recovery of the 37% hole lay between the 20% and 55%-depth data sets of Figure 4A. The hole broadened quickly upon thermocycling, which resulted in large uncertainties in the area of the hole for cycling temperatures over 40 K. The triangles in Figure 8A indicate the expected HR at 5 K, based on respective partial λ–distributions, for 37%-deep hole, similar to that in Figure 6A, and $md^2=1.0 \cdot 10^{-46}$ kg·m^2. It can be seen that up to about 13 K, the recovery of the hole was determined by the elapsed time, and not the cycling temperature. The other, open symbol, datasets in Figure 8A result from simulations and will be discussed below.

In order to predict the hole behavior upon thermocycling, one needs to calculate

numerically the λ-(sub-)distributions and areas, corresponding to the remaining holes

after each thermocycling step, according to

$$f(\lambda)_{next} = f(\lambda)_{previous} \prod_{T,\tau(T)} q(\lambda,T,\tau(T)), \tag{5}$$

where $\tau(T)$ is the time spent at temperature T during the thermocycle. Since the increase

and decrease of temperature is not instantaneous, one has to multiply many $q(\lambda)$ curves

with each other, according to the actual temperature-change profile.

Figure 8B depicts the post-burn partial ground-state λ-distribution (solid black

curve) obtained for 37%-deep hole in the Gaussian distribution model with the

parameters described above (including $k=\lambda_{ground}/\lambda_{exc}=2.35$). The dashed blue curve

represents the cutoff q-curve, Eq. 4, which results in an approximately 35% recovery of

the hole at 5 K, as in Figure 8A. The red solid curve represents the partial distribution

remaining after that initial recovery, i.e., present at the beginning of thermocycling. The

area under the red curve is 65% of the area under the black curve. The dotted magenta

curve is obtained for $\tau=60$ sec, $md^2=1.0 \cdot 10^{-46}$ kg·m^2, and T=40 K. It is still located at

significantly smaller λ values than the red curve. Thus, for $md^2=1.0 \cdot 10^{-46}$ kg·m^2, the

recovery upon thermocycling should be indistinguishable from the recovery at 5 K for

cycling temperatures up to about 40 K (see open circles in Figure 8A). On the other

hand, the dotted green and brown q-curves correspond to cycling to 45 and 50 K,

respectively. Since these curves intersect the red curve, significant recovery is expected

upon cycling to these temperatures. The calculated datapoints in Figure 8A were obtained

taking into account the entire actual temperature change profile. The difference between

experimental and modeling results indicates the presence of an additional recovery

mechanism, with respect to burnt molecules returning to their original frequencies. More

details will be provided in the Discussion section. Interestingly, the above analysis of the

thermocycling results provides the same upper limit of $md^2=1.0 \cdot 10^{-46}$ kg·m^2 as suggested

based on HB behavior. Consider instead the recovery expected upon thermocycling for

$md^2=1.4 \cdot 10^{-46}$ kg·m^2 (open squares). The situation when experimental data and the results

of simulations first diverge and then converge (at around 40 K) is unphysical. This

scenario would imply that the recovery due to the additional mechanism mentioned above

has been reversed. In other words, the derivative of the difference between experimental

results and those of simulations would not yield a meaningful barrier distribution.

The difference between the hole area as a result of thermocycling (closed circles in

Fig. 8A) and the hole area based on modeling for 5 K (triangles) is presented in Figure

8C. Although the derivative of the fit to the data (dashed red line) is not perfectly

Gaussian, it is clearly far from $1/\sqrt{V}$. The midpoint of the dependence of the hole area

on the cycling temperature is located at approximately 36 K, which with typical $ln(\Omega_0\tau_T)$

~ 30 [22,26] (where a τ_T of ~ 60 s is time spent at the highest temperature of the cycle),

corresponds to an average ground-state barrier height of $k_BT \cdot ln(\Omega_0\tau_T)\sim920$ cm^{-1}. With a

ground-state λ_0 of >20, this would result in a $md^2>1.5 \cdot 10^{-46}$ kg·m^2. This is larger than the

upper limit determined above, indicating likely involvement of another landscape tier.

4. DISCUSSION

4.1. HB mechanism: tunneling versus barrier-hopping

As stated in the Introduction, according to [11,12] the NPHB mechanism is based on tunneling in the excited state of the pigment/protein system (see Figure 1. In Section 3.1 we obtained the upper limit of $1.0 \cdot 10^{-46}$ kg·m^2 for md^2, assuming that weakly temperature dependent tunneling [16] is indeed the dominant HB mechanism up to 13 K. A significantly larger value of md^2 was reported in[50] for phycobiliprotein, which would require barrier-hopping to dominate at much lower temperature (see Figure 3). To confirm the dominance of tunneling as the HB mechanism in our experiments, we must consider if **excited-state** barrier hopping could be an alternative mechanism behind the HB process. If excited-state barrier hopping were the dominant NPHB mechanism, the HB yield in Eq. 2 would have to be modified:

$$\phi(V, \tau_{fl}) = \frac{\Omega_0 \exp(-V/k_B T)}{\Omega_0 \exp(-V/k_B T) + \tau_{fl}^{-1}} \ . \tag{6}$$

Obviously, in the case of a Gaussian barrier height V distribution, the resulting HGK curves and partial barrier distributions will qualitatively resemble those obtained for the Gaussian λ-distribution. The main problem with allowing barrier hopping to dominate in the excited state is that the HB yield would be much more strongly dependent on temperature than in the case of tunneling. With $\lambda \sim 10$ and $md^2 \sim 10^{-46}$ kg·m^2, the excited state barrier heights would have to be of the order of 100 cm^{-1}. However, changing the burn temperature, T, from 5.0 to 13.0 K, as in Figure 2, would result in a drastic (several orders of magnitude) increase of the HB yield, which is clearly not observed experimentally. Furthermore, the temperature-dependent HGK in Figure 2 exhibits deceleration of HB rather than acceleration with increasing temperature. These results can be quantitatively explained by just increasing the homogeneous line width [45] and

phonon Huang-Rhys factor, S, with temperature without any change in the HB yield, in agreement with the tunneling hypothesis, with the respective rate being only very weakly dependent on temperature[19]. In fact, our results indicate that either the burning in protein-chlorophyll complexes obeys the Shu-Small mechanism (outside-in migration of free volume, with TLS poised for downhill tunneling prior to burning)[48], or that the excited-state asymmetry, Δ (see Figure 1B), is large enough to render uphill tunneling improbable. Significant LIHF (with the anti-hole being located close to the hole, see the next section), on the other hand, indicates that the asymmetry is, at least for a significant fraction of complexes, relatively small. Thus, our results support the Shu-Small burning mechanism[48] in proteins.

One could still point out that the HR starts right after the start of burning (this could be the most likely reason for the small discrepancies between experimental and theoretical curves in Figure 2) and ask if, in the case of barrier hopping in the excited state, the HB and recovery rates could be changing with temperature in a concerted fashion, still yielding the results depicted in Figure 2. Given that the barriers in the ground state have to be significantly higher than in the excited state, and taking into account the exponential dependence of the hopping rate on barrier height, V, near-perfect mutual compensation of changes in the burning and recovery yields in the 5-13 K range is obviously impossible; the recovery rate, while smaller than the burn rate, will change with temperature orders of magnitude faster than the burning rate. A scenario in which barrier hopping is the dominant process in the excited state while tunneling is the dominant process in the ground state (md^2 only slightly larger than the upper limit derived in Section 3.1) is also not feasible. In this case the recovery rate would change

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too little with temperature, compared to the burning rate. Summarizing, barrier-hopping unlikely contributes to SHB up to 12 K in CP43.

We also note that the Bogner-Schwartz NPHB mechanism[51], involving **ground-state** barrier hopping utilizing energy locally dissipated in the electronic transition via phonons and vibrations, is quite unlikely since it would lead to independence of the NPHB yield on the excited state lifetime. This would contradict the observed dependence of the NPHB yield on wavelength within the B800 band in LH2 complex[37]. Additionally, positive correlation between the NPHB yield and electron phonon coupling, S (i.e., negative correlation between λ_0 and S), would be expected for the Bogner-Schwartz NPHB mechanism. However, this contradicts the results presented in[38], where the LHCII trimer exhibited both the highest S and highest λ_0 among the complexes studied. Additionally, the LHCII monomer exhibited the same λ_0 as the LHCII trimer, but a significantly lower S.

The nature of the tunneling entities could be suggested based on the md^2 value. Above we presented some evidence that $1.0 \cdot 10^{-46}\,kg \cdot m^2$ is the true value of the md^2 rather than just it's upper limit. In this case, if the tunneling involved a proton, the respective distance, d, would be 2.45Å, which is a typical hydrogen bond length. On the other hand, it is known that in the scenarios involving tunneling within a sufficiently long hydrogen bond yielding double-well potentials, the value of d is several times smaller than the hydrogen bond length [53,54]. Here it is worth mentioning that tunneling involving significant rearrangement of the C=O··H hydrogen bonds between protein and chlorophyll has been proposed as "photoconversion-HB" mechanism in CP43 [35,46] (but mainly for the B-state) to explain changes in the absorption spectrum occurring very far

away from the original hole. However, we recently demonstrated that this apparent large shift of anti-hole absorption can be explained with small site energy shifts, but it results in changes in the whole picture of excitonic interactions and redistribution of oscillator strengths [34]. Moreover, the presence of significant LIHF in our experiments, involving relatively narrow scanning range, speaks against too large a shift in pigment site energies upon HB. For a methyl group $md^2=1.0\cdot10^{-46}\,kg\cdot m^2$ would result in $d=0.37$Å. With respect to the latter possibility we need to stress that we did not observe any evidence of "population hole burning" related to rotational tunneling of the methyl groups [55-57]. There were no sharp anti-holes appearing or disappearing within the 45 GHz scan range centered on the original hole (or within ~120 GHz for broader, more saturated holes). Note that the sharp anti-hole effect has been observed for small chromophores, e.g., dimethyl-s-tetrazine and p-chlorotoluene, with the methyl group directly attached to the ring. The reduced coupling of the relevant methyl group to the chlorophyll's π-electrons expected in our system must result in poorly resolved hole and antiholes cancelling each other in the case where HB is limited to rotational tunneling. On the other hand, a 120° rotation of a C_3-symmetrical methyl group is not expected to cause large shifts in absorption frequency of nearby pigments by purely electrostatic effects. Thus, although rotational tunneling of methyl groups may occur in CP43, the HB mechanism must be different. Other interesting alternatives, especially in the case where the true md^2 is less than $1.0\cdot10^{-46}\,kg\cdot m^2$, include hindered rotation of a hydroxyl group (the mass of the hydroxyl group is close to that of the methyl group) and tunneling within a hydrogen bond. However, it is not clear which particular hydrogen bonds might be involved in the latter process. One cannot also exclude concerted motion of several groups of atoms.

4.2 Barrier and/or λ-distribution shapes

First we must point out that the existence of dependence of the hole recovery on the fractional depth of the original hole, as depicted in Figures 4A and 5, is a clear indication that CP43 manifests significant degree of spectral memory [16]. This means that holes recover mostly as a result of the previously burnt molecules returning to ω_B. This assumption was implicitly behind the idea that the recovery is governed by the hole-depth-dependent partial λ-distributions (Figure 6); here we provide justification. In the case of no spectral memory whatsoever, any molecule would be as likely to participate in the recovery of the hole as the molecules initially absorbing at ω_B and burnt away in the process of HB. This, however, would mean that a hole of any depth would recover according to the same full barrier height or λ-distribution, and there would be no difference in the recovery of shallow and nearly saturated holes. The high degree of spectral memory should not be understood as literally as in [16,19], where each pigment could assume only two spectral positions. Notably, modeling of HB and HR on a 20-well energy landscape (Najafi et al, in preparation) yields results similar to those in Figures 4 and 5. This can be understood if one notices that as long as the number of available conformational sub-states is limited, many individual pigment / protein systems will not possess a well which is resonant with ω_B. Thus, memory-less recovery (as proposed to explain part of the thermocycling results below) can contribute to only a fraction of the hole recovery. The limited number of available conformational sub-states in CP43 is in qualitative agreement with the SPCS results on LH2 complex [41,43].

As demonstrated in Figure 7, the uniform full λ-distribution assumption is in disagreement with the HR experimental results. The respective $\sim 1/\sqrt{V}$ distribution

originates from the theories explaining low-temperature properties of various amorphous solids [13,14], and has been applied to hole thermocycling experiments[22,25,40]. It is obvious that for a uniform full λ-distribution with highly asymmetrical partial λ-distributions (Figure 6B), the partial distributions of the ground state barrier heights, V, are expected to be even more asymmetrical, and deviate significantly (i.e., decrease much more steeply with increasing V) from $\sim 1/\sqrt{V}$. We note that in many papers devoted to hole-thermocycling studies of the barrier distributions in proteins, the fractional depth or the degree of saturation of the holes being thermocycled was not specified. Therefore it is hard to tell how far the partial distributions probed by the authors of these works were from true full barrier distributions. More importantly, the holes were often thermocycled right after burning, sometimes without proper correction for spontaneous recovery. (Love et al. in their work on $Ba_{1-x-y}La_xTb_yF_{2+x+y}$ recognized the importance of disentangling the recovery at a fixed-burn temperature resulting from thermocycling[26], but in protein studies, the issue was not always addressed sufficiently.) It may well be that thermocycling without correction yielded results, which were a superposition of the dependences presented in Figures 4A and 8B and that could be reasonably well fitted to $\sim 1/\sqrt{V}$ just by coincidence. A crucially important point here is that the line of reasoning yielding partial distribution shapes is not protein-specific, and it applies to NPHB in any kind of amorphous material, including glasses and polymers. Thus, fitting *any* HR or thermocycling results, including those obtained in glasses, with $\sim 1/\sqrt{V}$ does not appear to be sufficiently justified from a theoretical standpoint. As stated above, introduction of partial barrier- or λ–distributions is justified by the presence of spectral memory. In polymer [58,59] and *p*-terphenyl [8] glasses, single molecule spectral lines exhibited jumps

between a small number of wavelengths, consistent with the model where the pigment is interacting with a small number of TLS (see Figure 1A). In such cases a burnt molecule is likely to return to its original wavelength as the result of HR. Interestingly, in toluene glass additional slow drifts and irreproducible jumps were observed[59]. However, slow drifts would contribute mainly to hole broadening in HR experiments, and therefore for the purpose of our discussion centered on hole area or depth variations, toluene glass still would be a system with a fair degree of spectral memory. HB and HR experiments on glasses, analogous to those described here for CP43, need to be performed to further test these ideas.

One could also note that with $V \sim \lambda^2$, the Gaussian distribution of the tunneling parameter does not translate into a Gaussian distribution of barrier heights. Roughly, the Gaussian distribution of λ results in an asymmetrical, non-central, chi-square distribution for V. Conversely, the Gaussian distribution of V translates into an asymmetrical distribution of λ, which is the product of a Gaussian and a term linear in λ. (One can arrive at these results using the Leibnitz integral rule.) However, for the parameters reported here (i.e., for relatively small σ_λ), the difference between the latter distribution of λ and the Gaussian λ-distribution is small, and these two types of distributions cannot be distinguished, given a realistic amount of noise in the experimental data.

Finally, we comment on a disagreement between the parameters of the Gaussian distributions resulting from the analysis of the HB and fixed-temperature HR on one hand, and the recovery upon thermocycling on the other hand. We have no choice but to suggest that thermocycling probes certain features of the protein barrier distribution not probed by burning, and that the hole is filled not only by the molecules previously burnt

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out in the process of producing a hole, but by random molecules several cm^{-1} away from the hole as well. (This situation is different from spectral diffusion causing hole broadening, where single spectral shifts are much smaller than the hole width or a single-scan range; Figure 1C.) The distribution involved in this process will be the full ground-state barrier distribution for the respective protein landscape tier, not just the partial distribution corresponding to the hole. The excited-state barriers of that tier are characterized by higher λ than for the tier probed by burning, in agreement with the md^2 estimates above. Note that for this scenario to contribute to our observations, it is not necessary for the respective tier of the protein energy landscape to be out of thermodynamic equilibrium. As long as there is lack of molecules absorbing in the segment with the hole, there will be some net flux of molecules into this segment. (The tendency to increase the entropy may override the tendency to minimize the energy, and the hole represents "order".)

The recovery upon thermocycling depicted in Figure 8C is in reasonable agreement with the Gaussian barrier-height distribution for this additional spectral-diffusion tier. Therefore, there is no evidence for $\sim 1/\sqrt{V}$ barrier distributions in CP43. On the other hand, in light of the above arguments, the previously reported observations of $\sim 1/\sqrt{V}$ barrier distributions [22,23,25] just might be misinterpretations, even in glasses. Consequently, one of the two energy-landscape tiers observed in this work and characterized by Gaussian distributions may reflect the dynamics of the amorphous host surrounding the protein (i.e., the frozen buffer/glycerol glass), rather than of the protein itself. It has been argued that some features of the spectral diffusion observed in single LH2 complex experiments could be assigned to the dynamics of the amorphous solid

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outside of the protein [43] or to protein-host surface TLS [4,60]. More experiments, in particular with complexes where the lowest-energy state (the state being burnt and possessing narrow ZPL) is better screened by the protein from the surrounding buffer/glycerol glass, are necessary to clarify the origins of various tiers of the spectral diffusion dynamics in protein-chlorophyll complexes. This may not be an easy task, since in antenna complexes the lowest energy pigment is likely located on a periphery of the complex, at the side facing the next complex in the energy transfer chain (e.g., the reaction center). We note that hole broadening was extremely slow for the CP47-RC complex of PS II compared to isolated CP47 and PS II RC[61]. Another possible avenue of study involves exploring whether changes in the amorphous host (e.g., deuteration of buffer and/or glycerol) surrounding CP43 or other simple antenna complexes affect the parameters of HB and HR. A similar approach has been applied in[52] to single-complex PS I studies.

5. CONCLUSIONS

The dispersive character of NPHB results in the barrier height, V (or tunneling parameter λ-), distributions actually contributing to the holes that are significantly different with respect to true, full distributions. This becomes particularly important in the case of a uniform λ-distribution, corresponding to the $\sim 1/\sqrt{V}$ barrier-height distribution, widely employed in theories explaining low-temperature properties of amorphous solids. Partial λ-distributions in this case are highly asymmetrical and result in predictions concerning HR that are incompatible with experimental results. We demonstrated that tunneling is responsible for both NPHB and HR at 5-12 K in CP43

antenna protein complex. NPHB involves tunneling in the excited state (Figure 1), while HR at 5-12 K involves tunneling in the ground state of the system. A simple experiment involving the measurement of HGK curves (i.e., of HB yield) at different temperatures can be performed in a variety of amorphous systems, to further confirm that tunneling is the dominant NPHB mechanism in a given temperature range and to determine the upper limit of md^2 in these systems. In the case of CP43 protein complex, the upper limit of md^2 appears to be $\sim 1.0 \cdot 10^{-46}$ kg·m^2. Interestingly, the same upper limit can be obtained from thermocycling results (Figure 8A). However, the acceleration of the initial stage of burning (Figure 2) and slowdown of recovery observed at 13 K (possibly additional SHB induced by the measurement light; see Figure 4B) suggest that barrier hopping in the excited state may become important for the smallest λ at this temperature. If this is indeed the case, $1.0 \cdot 10^{-46}$ kg·m^2 is the true md^2 rather than its upper limit. Thus, the structural elements involved in tunneling in the CP43 protein are most likely the protein side chains (e.g., small amino acid ligands), although proton tunneling cannot be excluded at this point. It also appears that thermocycling in CP43 SHB experiments probes some barrier distribution features incompatible with the 5 K HB and HR data, (i.e., a distribution other than the sub-distributions directly created and probed by SHB). We suggest that HR at higher temperatures is partially due to spectral diffusion on the higher-barrier tier of the protein-energy landscape, with the respective generalized coordinate being different from that involved in the SHB process, as shown schematically in the Graphical Abstract.

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FIGURE CAPTIONS:

Figure 1: A: Schematics of the NPHB process. Both excited and ground states of the pigment in interaction with protein are shown. ω_B is the burn frequency; the pigment/TLS in well 1 is initially in resonance with the laser. After (the initially unspecified) transition between the TLS wells, occurring while the pigment is in the excited state, the system may get trapped in well 2. **B:** Detailed representation of the TLS (a fraction of the protein energy landscape) with the pigment in either the excited or ground states, introducing relevant parameters and possible transition processes. **C:** Second, lower-barrier hierarchal tier of the protein energy landscape. The larger-barrier tier is responsible for NPHB, while smaller barrier tier is responsible for hole broadening.

Figure 2: HGK curves for 5, 6, 7, 8, 10 and 13 K with fits produced assuming no temperature dependence of the HB yield, and temperature dependence of only the homogeneous line width and S. The dashed arrow indicates the discrepancy between the fit and the 13 K HGK curve at low burning doses. The insert depicts the absorption spectrum of CP43 (black) with the site distribution function of the A-state (red) and the B-state (blue). The down arrow indicates the burn wavelength. See text for additional details.

Figure 3: Dependence of the ratio of tunneling rate to hopping rate on tunneling parameter λ for $md^2 = 1.0 \cdot 10^{-46}$ kg·m^2 and T= 13 K, red solid curve. Horizontal dashed line corresponds to ratio=1; ratio is ~3 for λ=7.0. The full excited state λ-distributions (Gaussian: blue dashed line; uniform: black dotted line) are added for comparison.

Figure 4: Frame **A**: Recovery of the holes (λ_B=686 nm) at 5 K. Circles: originally a 55%-deep hole; diamonds: originally a 20%-deep hole. The data was obtained by measuring the hole spectra and determining the hole areas via a fit. The red arrow corresponds to an 18% difference in the recovery datasets, and this difference was achieved in about the first 3 hours, while the holes were still at 78% and 60% of their original areas, respectively. See Figure 6 for more details. Frame **B**: recovery of 20% holes at ~686.1 nm, monitored as the hole depth (fluorescence signal) versus time. Both burning and recovery for each hole were measured at the same temperature. These recovery curves belong to the respective holes found in Figure 2.

Figure 5: Recovery of the holes of different initial fractional depths measured either while keeping the laser at a fixed burn wavelength (using read intensity) or by scanning the hole spectra. Black dots (*a*): initially 55% hole, recovery monitored at fixed (burn) wavelength; blue dots (*b*): initially 20% hole, fixed wavelength; green dots (*c*): initially 55% hole, scanning; red dots (*d*): initially 20% hole, scanning.

Figure 6: Frame **A:** Calculated excited-state partial λ-distributions for Gaussian true full λ-distributions (black: 20%-deep hole, *a*; blue: 55%-deep hole, *b*). Areas under curves are normalized to 1. The red (*c*) and green (*d*) curves are integrals of the black and blue curves, respectively. The magenta curve (*e*) is the difference between these integrals. It reaches a maximum of ~12% when the holes are ~70% recovered. The dashed curve is the true full λ-distribution. Frame **B:** Same starting from the uniform λ-distribution.

Again, the maximal discrepancy between the expected 20% and 55% HR curves is 12%, which is reached gradually by the time holes are 70% recovered.

Figure 7: 5 K recovery of the holes of different initial depths measured while keeping the laser at a fixed burn wavelength (using read intensity). Black (curve b): 55% hole; blue: (curve a) 20% hole. **Frame A:** Recovery modeled starting from a Gaussian full λ-distribution and utilizing the partial excited state distributions depicted in Figure 6A. Long-dashed curves (*a* for a 20% hole and *b* for a 55% hole) correspond to $k=\lambda_{ground}/\lambda_{exc}$=2.35. Short-dashed curves correspond to k=2.25 and 2.30. **Frame B:** Recovery modeled starting from a uniform full λ-distribution and utilizing the partial distributions depicted in Figure 6B. Different dashed curves correspond to different values of *k* (2.20, 2.25 and 2.30).

Figure 8: Frame A: Recovery of the 37% hole at 5 K (first 2.5 hours) and then as a result of thermocycling (closed circles). The numbers next to the data points denote the maximal cycle temperature in K. Closed triangles represent the recovery expected at 5 K. Open circles represent recovery expected upon thermocycling for md^2=1.0$\cdot$10^{-46} kg$\cdot$m^2 and downhill tunneling only. Open squares represent recovery expected upon thermocycling for md^2=1.4$\cdot$10^{-46} kg$\cdot$m^2. **Frame B:** Black (curve *a*): partial ground-state λ-distribution corresponding to the hole of 37% initial fractional depth, obtained with the same parameters as the curves in Figs. 6A and 8A. Dashed blue (curve *b*): the q-curve obtained with Eq. 5 which corresponds to 35% recovery of the initial hole. Red curve (*c*) illustrates the partial distribution remaining after the hole is 35% recovered; it is a

product of the black and blue curves. Magenta (curve *d*): q-curve (Eq. 4) for thermocycling to any temperature up to 40 K, t = 60 sec, $md^2 = 1.0 \cdot 10^{-46}$ kg·m^2. Green (e): thermocycling up to 45 K. Brown (f): thermocycling up to 50 K. **Frame C:** Relative hole area versus cycle maximal temperature. The area of the hole after the first ~ 2.5 hours of recovery at 5 K is taken as 100%. The data has been corrected for recovery, which would occur anyway at 5 K (triangles in frame A). The dataset yields a Gaussian barrier distribution with a mean of 36 K and a FWHM of 35 K (dashed line).