Photochemical hole burning: A means to observe high resolution optical structures in phycoerythrin

J. Friedrich,[a),c)] H. Scheer,[b)] B. Zickendraht-Wendelstadt,[c)] and D. Haarer

IBM Research Laboratory, San Jose, California 95193
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We have investigated the lowest electronic transitions of phycoerythrobilin using laser-induced low temperature photochemistry. Narrow band irradiation into the absorption bands of the native chromophore leads to photochemical holes of the width of about 1–2 cm^{-1}. We attribute the hole burning mechanism to reversible proton rearrangement processes. The high resolution of the experiments allows the resolution of well defined substructures in the broad absorption bands which we interpret as being due to discrete vibrational states or due to a nonresonant energy transfer mechanism within the highly ordered chromophore–protein configuration.

I. INTRODUCTION

Phycocyanins and phycoerythrins are light harvesting pigments which are found in blue–green and red algae.[1,2] In the photosynthetic assembly the pigments are covalently bonded to large protein units which stabilize certain configurations of the chromophores which, as free molecules, can attain either cyclic, prophyrin type configurations or more elongated, polyene type configurations.[3,4] From spectroscopic studies, it was concluded that in the native chromophore–protein assemblies the chromophore is stabilized in an elongated configuration which leads to a very strong optical absorption in the visible range between 5000 and 6700 Å and to a correspondingly weaker UV absorption.[5–7]

Recent spectroscopic studies on light harvesting pigments have led to interesting conclusions concerning the structure of the native chromophore–protein assembly and related questions of light absorption and energy transfer.[7–9] Nevertheless, the above investigations were limited by the low resolution which is inherent in straightforward optical spectroscopy of large biological molecules.

In this article we want to report narrow band photochemical hole burning (PHB) experiments on native C–phycoerythrin (C–PE). These experiments, which yield a 100-fold increase in resolution (1.5 cm^{-1}), will, for the first time, show a series of narrow optical structures in the region of the two lowest optical absorption bands of the pigment. We propose two possible explanations for the experimental data:

(a) The narrow structures correspond to vibrational satellites of the lowest electronic pigment transitions. Such vibronic side bands however, have thus far not been observed in light harvesting pigments.

(b) The structures are due to a nonresonant energy transfer mechanism. In this case the existence of sharp "sideholes" would require that the protein–chromophore assembly exhibits a highly ordered structure.

If the latter interpretation is correct, then PHB should be an excellent optical tool to label various chromophore configurations in complex biological structures.

Before presenting detailed spectroscopic data, however, we want to briefly discuss the phenomenon of photochemical hole burning in biological molecules. This frequency selective, low temperature photochemistry has been observed for a related biological system[10] and for smaller molecules.[11–13] We will argue that the reported photochemistry is due to reversible, light induced proton rearrangement processes. We think that, in analogy to smaller model systems, the existence of hydrogen bonds and the state of protonation of the molecule play an important role in the photochemical scheme. Since hydrogen bonding is a common feature in biological systems, it is to be expected that similar PHB experiments will be feasible in other biological systems.

II. EXPERIMENTAL

A. Sample preparation

C–PE from *Phormidium persicinum* was prepared according to previously published procedures.[8] The method involves breakage of the cells in a glass ball mill, high-speed centrifugation to remove membrane bound chlorophyll, and ammonium sulfate fractionation of the crude extract. Due to the facile proteolysis of C–PE, rapid workup and addition of PMSF to all buffers used is necessary. The freeze-dried samples were dissolved in a 1:3 mixture (v/v) of potassium phosphate buffer (0.01 M, pH 7.5) and glycerol at concentrations corresponding to an optical density of 0.7 at 5650 A and an optical pathlength of 1 cm (2.8×10^{-6} M). At this concentration an aqueous solution of C–PE is known to be monomeric.

B. Optical experiments

The observed narrow band photochemistry was performed with a pulsed dye laser with an optical bandwidth of about 0.1 Å. The laser pulses were about 10 ns long and had typical peak intensities of several tens to a hundred kW at repetition frequencies of about 20 Hz. Typical hole burning times were on the order of minutes of unfocused laser irradiation. The irradiated sample area was several square millimeters. The low resolution absorption experiments were performed with

[a)] IBM Postdoctoral Fellow.
[b)] Permanent address: Institut fuer Botanik, Universitaet Muenchen, West Germany.
[c)] Permanent address: Institut fuer Physikalische und Theoretische Chemie, TU, Muenchen, West Germany.

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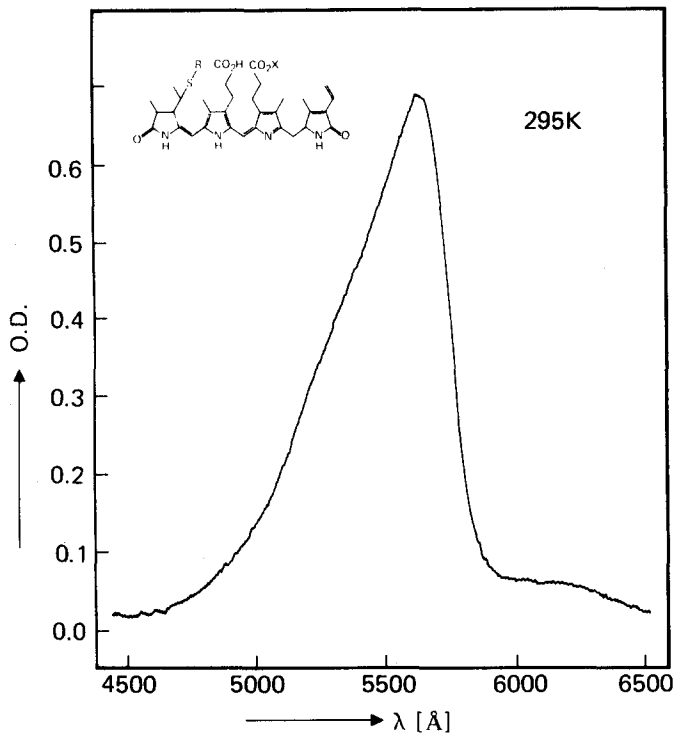

FIG. 1. Visible absorption spectrum of C–phycoerthrin at room temperature. Solvent: glycerol/buffer 3:1.

a commercial spectrophotometer with a resolution on the order of 1.5 Å and with a built-in low temperature unit which could be operated between 4 and 400 K.

The high resolution experiments were performed with a 1 m spectrometer in second order with a linear dispersion of about 4 Å/mm. Typical slit widths were 50–100 μm. The sample was immersed in superfluid helium at temperatures between 1.7 and 2 K. A dual beam feature of the spectrometer allowed the direct experimental observation of I/I_0. As light source we used a 75 W xenon lamp which was monochromatized before irradiating the sample. The latter feature is important in order to prevent photochemistry due to broadband light irradiation.

III. RESULTS AND DISCUSSION

A. Low temperature absorption spectra and photochemical holes

Photochemical hole burning has been observed recently in phycocyanin (C–PE).[10] C–PE differs from C–PC mainly by having one reduced bond in its tetrapyrrolic chromophores, thus providing a shorter conjugated π-electron system. Therefore, its visible optical absorption is at shorter wavelength than the corresponding spectrum of C–PC. Figures 1 and 2(a) show absorption spectra of the native C–PE at room temperature and at 6 K. At low temperatures the absorption splits into two bands at about 5650 and 5500 Å. Their width, however, is still large and there is very little structure, even at 6 K. In principle this large width can have various different origins:

(a) It can be due to phonon effects, i.e., due to a strong coupling of the electronic transition to its protein environment through which part of the photon energy is transmitted to its environment via phonons. Phonon coupling mechanisms of this kind lead to the dissipation of a fraction of the photon energy into lattice motion and to a large Stokes shift between the absorption and the emission spectrum.

(b) It can be due to large inhomogeneous broadening mechanism, i.e., to statistical variations in the local structure of the chromophore and its immediate environment which are reflected by a large spread in the electronic origins of the corresponding species.

(c) It can be due to the presence of many, well defined transitions of different electronic energy which cannot be resolved in a straightforward optical experiment because of their inhomogeneous width. The multiplicity of these transitions could either be due to vibronic structures and/or due to the existence of various well defined, yet different chromophore conformations.

With the aid of PHB experiments, we will be able to rule out the possibilities (a) and (b) as major line broadening mechanisms and will discuss the mechanism (c) at length. In this context we will investigate the relation between photochemical hole burning and energy transfer, and we will also discuss the origin of inhomogeneous broadening in proteins. Before going into details, however, we want to describe the observed PHB phenomena.

Figures (2b) and 2(c) show low resolution absorption spectra of a sample which has been irradiated with a narrow-band laser at frequencies ν_1 and ν_2. At both frequencies, one observes a small dip in the absorption spectrum. These narrow-band areas of reduced absorption, so-called photochemical holes, show up clearly in the higher resolution scans of Figs. 3(b) (ν_1)

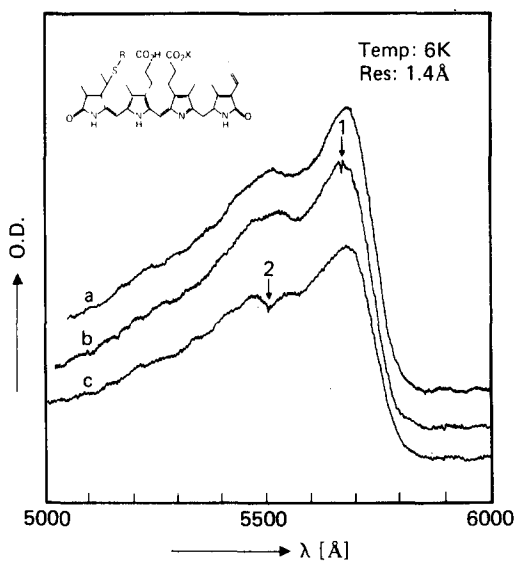

FIG. 2. Visible absorption spectrum of phycoerythrin at 6 K. Solvent: glycerol/buffer 3:1. Trace (a): unburnt spectrum. Trace (b): hole 1 is burnt by irradiation with a narrow bandwidth laser at 5682 Å (30 min). Trace (c): hole 2 is burnt at 5499 Å, while hole 1 is simultaneously filled. The low depth of the holes is an experimental artifact (see the text).

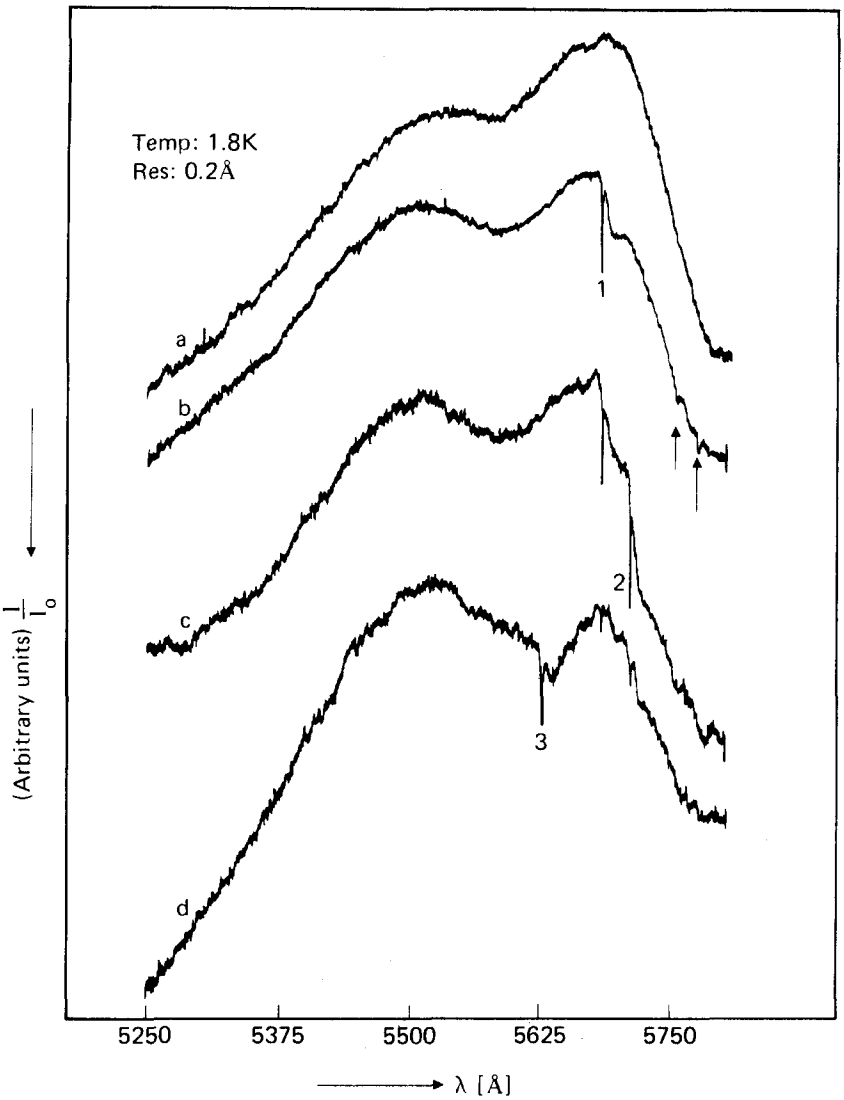

FIG. 3. Multiple hole burning in the lower energy peak of the the visible band of phycoerythrin. Solvent: glycerol/buffer 3:1. High resolution scan. Trace (a): unburnt spectrum. Trace (b): after 30 min laser irradiation at 5683 Å (hole 1). The arrows mark two nonresonant PHB satellites. Trace (c): hole 2 is burnt (30 min); hole 1 is not affected. Trace (d): hole 3 is burnt. The lower energy holes (1 and 2) are filled by reverse photochemistry.

and 4(c) (ν_1). In these scans, the optical density at the laser frequency is reduced by about 30%. At low temperatures the narrow holes in the above absorption spectra are time independent features; they are due to a photochemical conversion of centers which originally absorbed at the laser frequency ν_L and which are photochemically converted to centers absorbing at a different frequency ν_p, the absorption of the photoproduct. This photoproduct can either be due to a photochemical reaction,[14-15] or it can be due to photophysical solvent molecule rearrangement processes.[16,17] In the present PHB experiments with C-PE we propose a reversible proton rearrangement as the main mechanism of the observed hole burning process. We propose this scheme on the grounds of its thermal reversibility and its dependence on protonation, which were observed for a related pigment, the octaethyldihydrobiliverdin.[10] We also believe that the presence of the proton donating N-H groups and the proton accepting carbonyl and -N= groups allows one to draw analogies to the observed PHB photochemistry of smaller, hydrogen bonded systems.[13,18] In the case of the smaller, hydrogen bonded hydroxyquinones, it is suggested that the breakage of an intramolecular hydrogen bond and the subsequent formation of an external hydrogen bond is responsible for the photochemical reaction which occurs with a large quantum yield. The higher energy absorption of the photoproduct and the matrix dependence of the photochemical reaction strongly support such a proton-rearrangement scheme.[13] Quite in analogy to smaller molecular systems, the PHB process is optically reversible upon irradiation into the higher energy photoproduct. Figure 2(c) shows that the creation of a higher energy hole at frequency ν_2 eliminates the original hole at the lower frequency ν_1. PHB at lower energies, however, does *not* lead to reverse photochemistry as shown in Fig. 3. Here the photochemical hole at ν_2 does not affect the original hole at ν_1.

The above experiments demonstrate that, as in C-PC,[10] the hole burning photochemistry does not directly involve the population of the lowest excited singlet state of the product because its energy is above the reactant state (in analogy to Ref. 13). Therefore, we conclude that the first step in the reaction scheme is a monomolecular relaxation process. This observation links the time scale of the photochemical reaction to that of the involved intramolecular relaxation processes. This

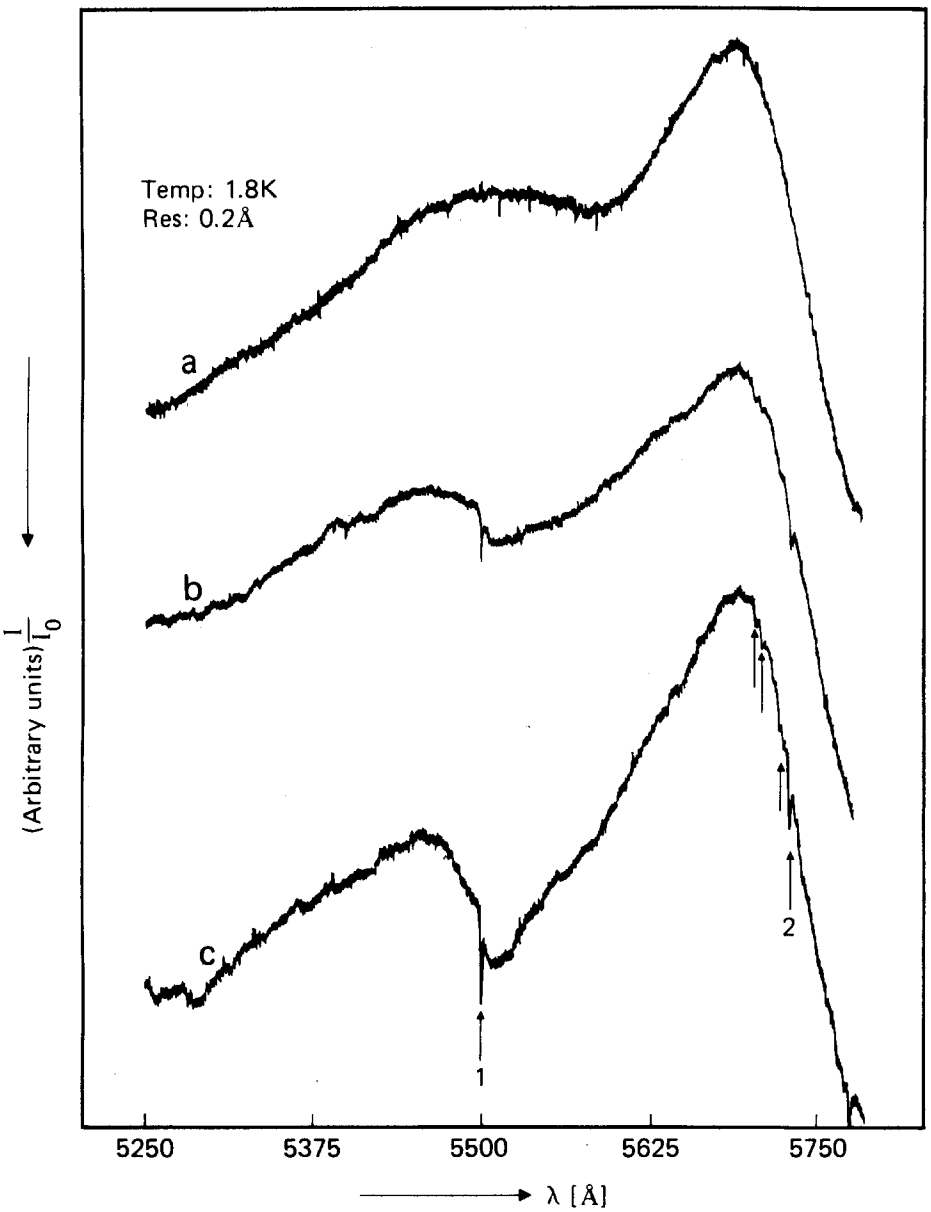

FIG. 4. Hole burning in the higher energy peak of the visible band of phycoerythrin. Solvent: glycerol/buffer 3:1. Trace (a): unburnt spectrum. Trace (b): after 10 min laser irradiation at 5499 Å. Trace (c): after 30 min laser irradiation at 5499 Å. In (b) and (c) the broad phonon side hole is clearly discernible. The arrows mark the nonresonant PHB satellites, the most prominent of which is at the frequency position 2.

view is well corroborated by the intense low temperature fluorescence.[8]

In this first contribution, we cannot deal with the microscopic aspects of the observed photochemistry of C-PE in an exhaustive fashion. We would at this point like to suggest a possible photochemical scheme, namely, proton rearrangement, and in the following section we use photochemical holes as spectroscopic markers to gain high optical resolution spectra of the large biological molecule.

B. PHB line shapes and chromophore-protein coupling

The most surprising fact of the above PHB experiments is the appearance of very narrow, purely electronic transitions [see Fig. 3 (ν_1, ν_2, and ν_3) and Fig. 4(ν_1)]. These zero-phonon transitions, which are a condition for narrow-band PHB,[19] reflect the fact that the chromophore is in a fairly rigid environment. This rigid local environment is spectroscopically reflected in the small intensity of the phonon sideband which is the broad shoulder on the low energy side of the narrow holes at frequencies ν_1 and ν_3 (Figs. 3 and 4). The low intensity of the sidebands reflects a small electron-phonon coupling with little excited state distortion of the chromophore environment.[20]

The above spectroscopic observations show that only a very small fraction of the photon energy is transferred to the chromophore environment as useless heat. Most of the energy is available for electronic energy transfer to the photochemical reaction center. Figure 5 shows the photochemical hole of Fig. 3 (ν_1) in an expanded scan. The line shape is close to Lorentzian and is about 1.4-1.7 cm^{-1}.[21] If one extrapolates to zero burning time (see Fig. 6), one gets linewidths of 1.4 and 2.3 cm^{-1}, respectively, for the low (5683 Å) and high (5499 Å) energy bands of native C-PE. Assuming that both linewidths are Lorentzian and reflect a true dynamical feature of the excited state, these widths re-

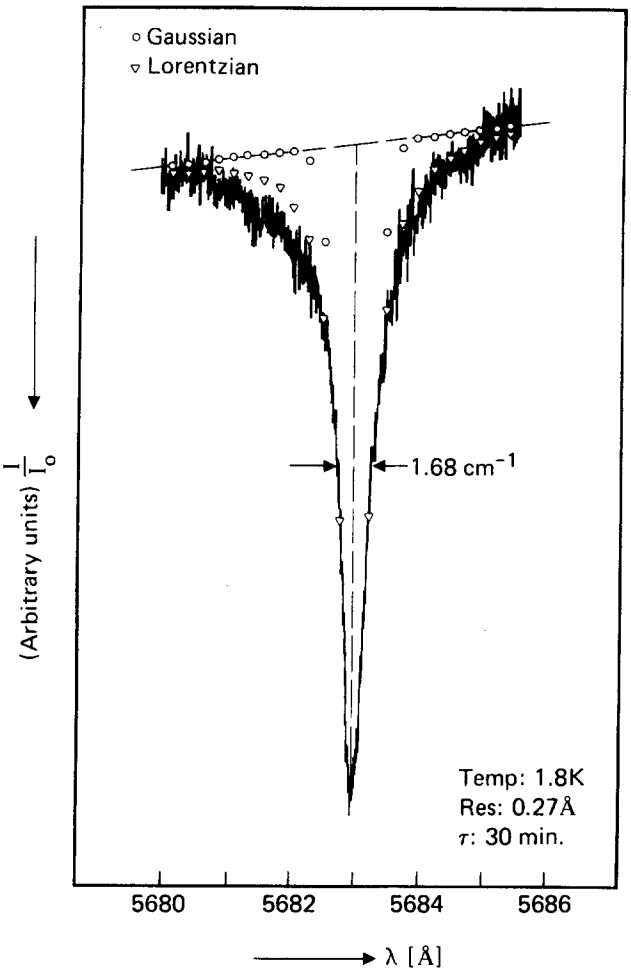

FIG. 5. Expanded high resolution scan of hole 1 of Fig. 3. Burning time 30 min. The triangles and circles represent fits to a Lorentzian and a Gaussian lineshape, respectively.[21]

flect excited state scattering processes (T_2) on the order of picoseconds. These fast scattering processes, however, are energy conserving and are typical of hydrogen bonded systems in alcoholic glasses.[13] Speculations about the microscopic origin of the homogeneous linewidths cannot be made considering the present, limited knowledge about the microscopic protein–chromophore structure.

From the above line shape analysis one can conclude that the large width of the measured low energy bands of C–PE is not due to phonon coupling and hence in agreement with experiments which report a small Stokes shift between absorption and emission spectra.[8]

C. PHB satellites

Thus far we have discussed the spectroscopic features of photochemical holes in C–PE near the PHB frequency ν_L. A closer look at the experimental data, however, reveals narrow, photochemical satellites at frequencies which are several hundred wave numbers away from the laser frequency. In Fig. 4 for instance, PHB at the frequency ν_1 yields at least four clearly discernible photochemical satellites at spacings between 644 and 727 cm^{-1} on the low energy side of the original photochemical hole. Figure 7 shows the original hole at ν_1 (5499 Å) for two different burning times [Figs. 7(a), (b)] and the most pronounced satellite at 5729 Å [Fig. 7(c)]. Whereas the holes at the laser frequency are quite narrow and Lorentzian in shape,[21] the satellites are relatively broad and have Gaussian line shape. A similar phenomenon occurs if one irradiates the low energy band. Figure 3(b) shows that a hole of frequency ν_1 creates at least two satellites with a spacing of 263 and 330 cm^{-1} [see arrows, Fig. 3(b)].

There are two ways to interpret the observed PHB satellites:

(a) population of several discrete sites via overlapping vibrational bands ("vibrational hole burning");

(b) nonresonant energy transfer within a highly ordered protein–chromophore assembly.

We will discuss our results within the framework of both possible explanations. It is important to note, however, that our present understanding of PHB in very complex molecular systems does not allow us to rule out one of the two possibilities.

1. *Vibrational hole burning*

If the protein–chromophore assembly exhibits several (in our case at least four) well defined vibronic transitions which are associated with the lowest electronic transition and which exhibit a large enough inhomogeneous linewidth as to overlap with the laser frequency, then the laser induced photochemistry occurs in as many discrete sites as there are absorbing vibrational states.[22] After PHB in the vibrationally excited states, a relaxation to the electronic origin will give rise to a multitude of "nonresonant" holes which accompany the

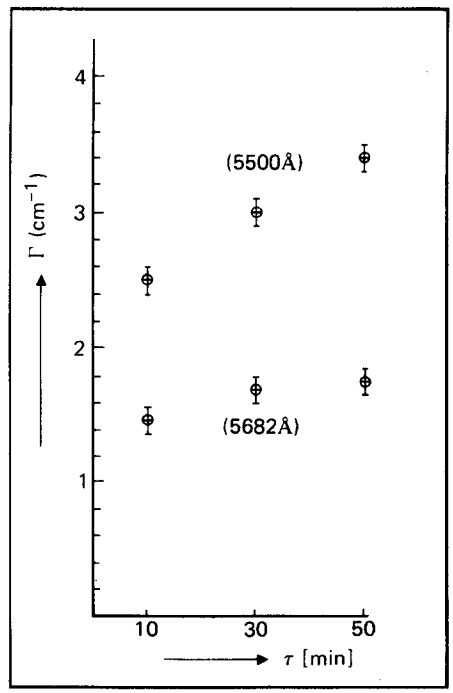

FIG. 6. Hole widths Γ as a function of burning time τ. The wavelength of the laser light is given in parentheses.

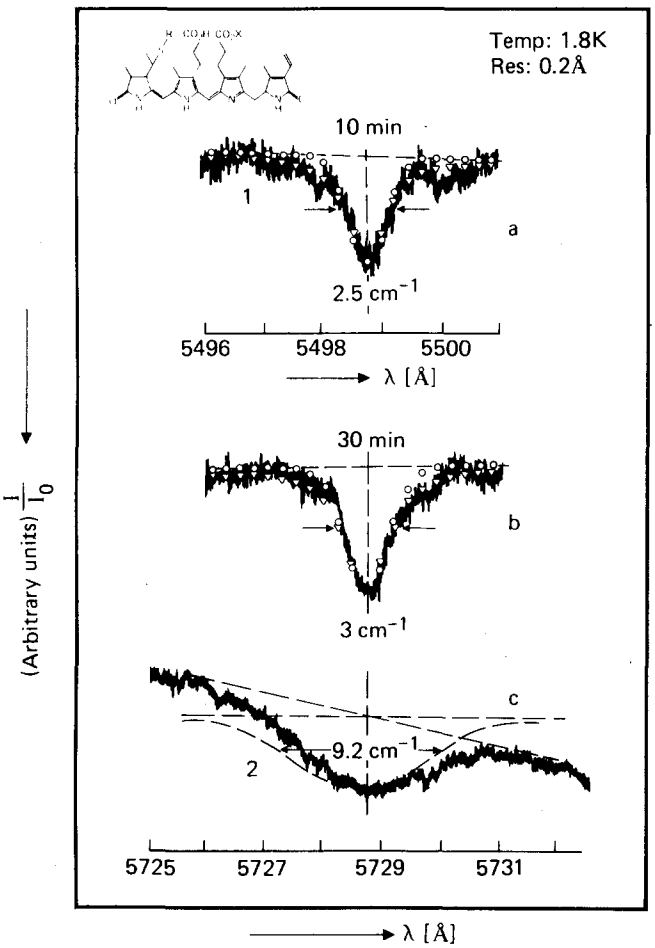

FIG. 7. High resolution scan of the hole at 5499 Å [in Fig. 4(b)] for two burning times (10 and 30 min). The triangles and circles represent the fit to a Lorentzian and a Gaussian line shape, respectively. The lower trace is a nonresonant satellite hole (hole 2 in Fig. 4).[21]

"resonant" hole at the laser frequency. The energy spacing between the laser frequency and the satellite holes is given by the quanta of the excited vibrations.

If the photochemical doorway state is the electronic origin S_1, as is the case in presently known proton transfer PHB systems, then the width of the resonant hole (2.4 cm^{-1}) is determined by vibrational relaxation processes.[23,24] In our case the measured linewidth would correspond to a T_2 of about 4 ps. The width of the "relaxed holes" is much larger (9.2 cm^{-1}) and cannot be related to a dynamical parameter of the system. Its broad Gaussian line shape can be understood in terms of large vibrational inhomogeneities which are typical of hydrogen bonded systems.[23]

2. *Energy transfer*

It is well known that the visible bands of light harvesting pigments are made up by the absorption of various different chromophores (in our case, six[8,25]). This overlap of optical bands leads to a broadening of the observed spectra, which cannot be removed by going to low temperature spectroscopy (see Figs. 1 and 2), because the inhomogeneous widths associated with the different chromophores are larger than their energy splittings.

Chromophores in antenna pigments are commonly subdivided into two empirical categories[25] sensitizing (s) and fluorescing (f) chromophores. This classification is based on the fact that higher energy chromophores usually transfer their electronic energy to lower energy chromophores, which, in the absence of further acceptors (like photosynthetic centers), release the excitation energy by fluorescence emission. It is this energy transfer process that can modify straightforward PHB experiments. Assuming that the laser induces a photochemical reaction in a higher energy (s-type) chromophore, we can distinguish three cases:

(1) Slow transfer limit: The rate of energy transfer K_{ET} is slow compared to the rate of the photochemical reaction K_{PR}:

$$K_{ET} \ll K_{PR} .$$

In this case we expect a hole resonant with the laser frequency.

(2) Fast transfer limit: The rate of energy transfer is fast compared to the photochemical reaction

$$K_{ET} \gg K_{PR} .$$

We expect a nonresonant (broadened) hole shifted to lower energies.

(3) Intermediate transfer limit: Both rate constants are of the same order of magnitude

$$K_{ET} \approx K_{PR} .$$

In this case a fraction of the initially excited molecules undergoes photochemical reaction while the remaining molecules transfer their excitation energy and hence the photoreaction may occur in the acceptor. Therefore, we expect a resonant and a nonresonant hole (or holes).

The PHB data (Figs. 3 and 4) show that our experiments would fall into the category of intermediate transfer because both the resonant hole and the satellites are clearly observable. If we interpret our experiment in terms of energy transfer, then the spacings between the resonant hole and the satellites would correspond to the energy differences between the various chromophores. A comparison between Figs. 3 and 4 shows that these spacings depend on the excitation energy, i.e., are different in the two broad low energy bands.

Another, quite important conclusion pertains to the dynamical aspects of the energy transfer process. We know that in both systems C-PE and C-PC,[10] the time scale of the photochemical reaction is given by an intramolecular relaxation process which occurs within nanoseconds.[25] Therefore, we would have to conclude that, at low temperatures, the excitation transfer is a comparatively slow process since all chromophores show up in our satellite spectra. These conclusions corroborate earlier experiments showing that, at 4 K, almost all chromophores in phycoerythrin are not only sensitizing but also fluorescing. Picosecond data of Kobayashi *et al.*[26] were taken at 300 K where transfer

times may be much faster than in the low temperature regime.[27]

A very remarkable feature of the observed PHB satellites is their narrow width. This narrow width would imply, in the framework of the previous section (Sec. III.C.1), a rather rigid vibrational spacing in these very large chromophore–protein assemblies. The implications within the second model (Sec. III.C.2) are as surprising: One has to assume that the chromophore–protein assembly has a very well defined geometry and hence the spacing of the satellites is defined by the local geometry of the protein rather than by the random glass environment. The remaining "disorder," which gives rise to the increased satellite linewidth of about 10 cm^{-1}, could reflect both random protein or environment fluctuations.

IV. CONCLUSIONS

We have reported photochemical hole burning experiments in native C–phycoerythrin, and we have tentatively attributed the observed low temperature photochemistry to light induced proton transfer processes. This photochemical scheme was adopted due to obvious similarities with the photochemsitry of small hydrogen bonded molecules.

From the existence of narrow holes with small phonon sidebands we have concluded that the chromophore–protein structure is comparatively rigid and that most of the photon energy is absorbed as purely electronic energy. In this scheme only a small amount of the light energy is dissipated as heat by phonon relaxation and hence lost for utilization in the photosynthetic process.

We have reported the first observation of photochemical satellites in large biomolecules which accompany the original photochemical holes and are separated from the laser frequency by several hundred wave numbers. The observed photochemical satellites are the key for an understanding of the large spectroscopic width of the lowest C–phycoerythrin transitions. We could clearly show that the large linewidth is not due to a large random inhomogeneous broadening mechanism, but is instead due to either the existence of well defined vibrational frequencies or due to the existence of various well defined chromophores within the same protein unit. Both these interpretations would explain the large observed optical width. At the present state of our understanding of the reported PHB experiments, we have a slight preference for the interpretation which is based on the existence of several chromophores (in this case *b*) which are coupled via energy transfer processes.

Whichever model we take for the interpretation of our data, we have to assume the existence of a very well defined chromophores–protein unit which has either quite well defined vibrational frequencies or very well defined sites of the dye-like molecules which act as antennas for the incident photons. We believe that both interpretations lead to far reaching consequences and ask for further high resolution laser experiments on complicated biological structures such as the investigated bile proteins.

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