Conformational Relaxation of Single Bacterial Light-Harvesting Complexes†

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ABSTRACT: We have employed the technique of single-molecule fluorescence microspectroscopy to investigate the spontaneous conformational evolution of individual peripheral LH2 complexes from the purple bacterium Rhodopseudomonas acidophila. Fluorescence microscopy is a sensitive tool, which allows the spectral changes of single complexes to be monitored on a time scale from 0.1 s to many minutes. Here we have investigated “natural” (occurring in the absence of excitation) spectral diffusion after a spectral jump has occurred. In a quarter of all the observed spectral jumps recorded with the LH2 complexes, a further spontaneous evolution occurs, in the absence of illumination, that results in the formation of a different spectroscopic state. We suggest that this is due to a natural conformational development of the pigment–protein complex, which so far has not been observed for this type of complex at the single-molecule level. The functional significance of such structural rearrangements is not yet clear but may be associated with the necessity for the light-harvesting complexes to adjust their shape in the densely packed photosynthetic membrane.

Although textbooks frequently picture proteins as static structures, they are in reality dynamic structures (1, 2). Despite this awareness, the study of protein motions, their classification and characterization, and investigation of their functional significance are still at an embryonic stage, because of the bewildering variety of possible protein motions and the intrinsic complexity of even the simplest molecules. Even a relatively short polypeptide is characterized by a very large number of degrees of freedom. These are called conformations or conformational substates and are associated with variations of the potential energy of the system. In a sense, conformations are analogous to energy levels in much simpler systems such as atoms. In the protein world, the concept of a one-dimensional energy level scheme has been substituted with the much more imaginative idea of a conformational energy hypersurface or landscape, which is a function of all the degrees of freedom in the system. For simplicity, usually a one-dimensional cross section of this conformational landscape is presented to demonstrate the arrangement of the interchanging energy barriers and minima between them. Each conformation corresponds to a minimum, while a transition to a different conformation is associated with the crossing of an energy barrier (3).

Despite this developing conceptual framework, rather little is known about the detailed conformational landscape and dynamics of it for any single protein. Probably one of the best studied proteins is the relatively small protein myoglobin, the biological function of which is to reversibly bind molecular oxygen. Its properties have been investigated extensively during the past few decades using an array of different techniques, which have resulted in a model for its energy landscape and ensuing dynamics (4–6). In this model, all the relevant conformational states are classified into a hierarchy of tiers according to the size of the energy barriers separating different conformations and, consequently, also according to the extent of the structural rearrangement required to alter a specific conformation. The zero tier consists of a limited number of well-characterized so-called taxonomic states, each with a well-defined biological function. Furthermore, each of these states can be subdivided into a number of substates that are no longer characterized individually but treated as a statistical distribution. It has been suggested that this hierarchical structure can be further extended by structuring each minimum of a higher tier into a number of minima of the subsequent tier. While protein movements between conformations in the higher tiers are thought to be functionally significant, the function of the small conformational changes in the lower tiers is not yet clear.

The aim of this study in combination with the previous work (7, 8) is to visualize the natural protein motions of a membrane protein, peripheral light-harvesting complex 2 (LH2)† from the photosynthetic purple bacterium Rhodopseudomonas acidophila. The function of LH2 is to capture solar energy and transfer that energy to the reaction center where it is used to initiate the charge separation reaction that eventually leads to the synthesis of ATP (9).

The structure of LH2 has been determined to a resolution of 2 Å (10, 11); the complex is a highly symmetric ring of

† Abbreviations: FL, fluorescence; FLP, fluorescence peak; LH2, peripheral light-harvesting complex 2; BChl, bacteriochlorophyll.
nine pigment–protein subunits, each containing two helical transmembrane polypeptides: the α-polypeptide on the inner and the β-polypeptide on the outer side of the ring. Close to the C-terminus the protein binds a ring of 18 tightly coupled bacteriochlorophyll (BChl) a molecules, separated center to center by a distance of less than 1 nm. This ring is responsible for the intense absorption of LH2 around 850 nm (B850 ring). A second ring of nine weakly interacting BChl a molecules is located closer to the proteins’ N-terminus and is largely responsible for the absorption peak at 800 nm (B800 ring).

Thorugh investigations of its electronic and energy transfer properties have resulted in a rather consistent physical model of LH2 (12–18). The two basic features of this model are the excitonic coupling between pigments and the effect of static disorder of the pigments’ electronic transition energies, which arises from the intrinsic disorder of the protein.

The available crystal structure of LH2 is, of course, by definition an average of many different conformational substates, and little is known about the dynamics of these on a slow (milliseconds to minutes) time scale. Due to the strong protein–pigment interactions (19, 20), changes of the pigment site energies and consequently fluorescence (FL) spectral properties are expected to be coupled to these protein movements. Thus, the dynamics of the observable FL spectrum can be used to follow these protein movements.

In our previous work, we observed spontaneous and light-induced conformational changes; in this study, we set out to remove the complex from its initial state and then to monitor the result of the subsequent “natural” spectral evolution, which is similar to the flash photolysis measurements carried out previously on myoglobin (21). However, since our experiment is conducted on individual light-harvesting complexes (LHCs), we avoid ensemble averaging that complicates the analysis and interpretation of the relevant kinetic traces. In this way, we are able to obtain direct information about the spontaneous pigment–protein motions.

MATERIALS AND METHODS

Purified LH2 complexes of Rps. acidophila 10050 were prepared as described previously (22, 23). A stock solution of the sample in buffer [20 mM Tris-HCl (pH 8.0) and 0.1% lauryldimethylamine oxide (LDAO)] was divided into aliquots and kept at −80 °C until it was thawed and used only once, thus preventing damaging repetitive thawing and freezing. The complexes in the stock solution were diluted with the same buffer to a picomolar concentration. The buffer was deoxygenated and used to flush the immobilized complexes. Dissolved oxygen was removed by first ventilating the buffer with nitrogen gas while stirring with a magnetic spinner for at least 0.5 h; the remaining oxygen was chemically removed by adding a small amount of sodium dithionite without opening the deoxygenation chamber. In the oxygen-free environment, LH2 complexes were fluorescent for a number of minutes and the sample was fit for measurement for at least 1 day. Isolated LH2 complexes were immobilized on a standard microscope coverslip as described previously (8). The confocal setup utilized to acquire sequences of single-molecule FL spectra was based on a commercial biological microscope as described previously (8).

RESULTS

In our earlier work, we obtained sequences of FL spectra of continuously illuminated individual bacterial LHCs (8). For most complexes, the spectral trace starts with the FL peak wavelength (FLP) close to 870 nm. This wavelength is the peak wavelength of the bulk spectrum and is the most frequent value in the FLP distribution of the single complexes. Over time, the FL trace develops according to various scenarios. Transitions between different FLP values are associated with spontaneous and light-induced conformational changes of the pigment–protein complex. The light effect is due to the nonradiative relaxation of the excitation energy, which is transformed into vibrational modes and facilitates crossing of the potential energy barrier in the conformational landscape.

FLP deviations from 870 nm are accompanied by a spectral broadening and a change in the spectral asymmetry. To explain these spectral variations, we applied the disordered exciton model (12–18), in which both the excitonic coupling between the pigments and the static disorder of the pigment electronic transition energies are taken into account. In this model, the observable spectral occurrences are associated with different realizations of the static disorder which are linked to changes in protein conformations by means of microscopic parameters such as hydrogen bonds between specific amino acids and functional groups on the BChl molecules. Although phenomenological, this model satisfactorily accounts for the measured changes of the FLP and the FL band shape.

Furthermore, a significant fraction of the measured complexes exhibits FLP evolution through a number of quasi-stable states, i.e., levels of distinctly different and relatively constant magnitude in the time trace. We suggest that these surprisingly long-lived states (tens of seconds) are associated with conformations that are both difficult to access and difficult from which to escape. The applied high excitation intensity (100–1000 W/cm²) facilitates the transitions to and from these states. The duration of the quasi-stable states varies, probably due to differences in the heights of the conformational energy barriers and the probabilistic nature of the barrier crossing.

The intense excitation accelerates conformational transitions into states that are difficult to access and at the same time promotes their further evolution. The aim of this work is to observe the natural conformational relaxation. To this end, excitation was turned off after a spectral jump (meaning that a conformational change has occurred) to allow the spontaneous evolution of the complex. Thus, a typical measurement begins with the acquisition of a FL spectrum and its fitting to obtain a value of FLP, which is then compared with the average of FLP values of the previous spectra. A spectral jump is detected if the difference between the newly acquired point in the FLP time trace and the average of the values of the previous points is larger than a selected threshold; in this way, immediate and overall spectral changes are monitored. After a dark period, the laser light is switched on again and the whole cycle is repeated: FLP of each collected spectrum is compared with the average
of the preceding FLP values, starting from the end of the last “dark” period, and in case of a new spectral jump, the excitation is blocked again. An example of such a spectral time trace is presented in Figure 1. For clarity, periods of the FLP time trace in the absence of the excitation are filled with the value of the last spectrum acquired before the switch-off.

This experimental procedure allows the comparison of the spectral state before the laser light is switched off and the state immediately after the excitation is switched on again. Thus, it yields information about the result of a natural conformational evolution during a period without excitation. We observed, as is not surprising given the complexity of the conformational landscape, a multitude of scenarios of this evolution. Figure 1 contains a trace with periods without excitation followed by a spectrum deviating insignificantly from the state before the switch-off. This shows that during a dark period (15 s) this complex does not undergo a significant spontaneous development. Figure 2 shows some characteristic cases of changes among the conformational (spectral) substates. The complex in Figure 2A evolves through at least three states; while in the first destabilized state with FLP around 880 nm the complex does not change in the absence of the excitation, it is driven to a different state when the excitation is applied for some time. The other two states around 905 and 890 nm switch to the first state if left in the dark.

Figure 2B is a surprising example of a complex cycling between a state with an intermediate FLP value around 870 nm and states around 860 nm; transitions from 870 to 860 nm occur in the presence of the excitation light, whereas the transition from 860 to 870 nm occurs spontaneously (in the absence of the excitation light). The latter two are examples of spectral jumping to the red and blue, respectively. However, they are not sufficient to draw conclusions about the general character of spontaneous evolution from the red or blue states. Figure 2C demonstrates the possibility of a drastic spontaneous change: the second state with an FLP value around 910 nm spontaneously moves to a state around 850 nm and is driven back to the intermediate position by continuing excitation. Finally, Figure 2D shows an example of a state with an intermediate value of FLP around 870 nm (second state in the trace) that spontaneously moves to a very short-lived state around 900 nm that is then driven by light to a different spectral position. This demonstrates that even from the initial conformation with the FLP around 870 nm a complex can spontaneously move to a state with a different spectral peak wavelength.

The general trends of the natural spectral evolution of different conformations are envisaged from the statistics of the FLP values before and after the dark period for all such periods. Figure 3 presents an example of a scattered distribution of such pairs of FLP values demonstrating the extent of spontaneous spectral diffusion of conformations with various FLPs. The two diagonal lines confine conformations that change by less than the value of the spectral jump threshold. Dots outside this area represent conformations spontaneously evolving into significantly different spectral states. Points above the confined area denote states that are relatively blue and change to relatively red. Points below this area indicate the opposite relationship between the initial and final states.

The overall statistics of spectrally diffusive conformations for the measurements with different excitation intensities and durations of the applied dark period are summarized in Table 1. The conformation is considered to be evolving if corresponding FLP values differ by more than a set threshold of the spectral jump. In general, ~20–30% of the conformations evolve in the dark into significantly different spectral
is a wide spread of FLP values along the ordinate axis. Thus, the same value of FLP evolve rather differently since there the diffusive states. It also appears that conformations with significantly exhibit a distribution of FLP values similar to that of analysis of Figure 3: spectral states that diffuse insignifi-
evolution. This complexity is further demonstrated by the examples in panels A and C of Figure 2 where the red
substates. For example, with an excitation intensity of 3 \( \mu W \), a dark period duration of 15 s, and a jump threshold of 10 nm, 29% of the observed conformational substates move into conformations with an FLP value that differs from the FLP before the dark period by more than 10 nm. Therefore, spontaneous spectral diffusion is significant compared to the light-induced effects.

DISCUSSION

The results of spontaneous changes from the different spectral states described here allow us to visualize the character of the conformational landscape of LH2. For example, from Figure 2A it appears that the conformational energy barriers separating the 880 nm state from the 890 and 905 nm states are asymmetric since the transition from the former state to the latter two is more difficult than in the backward direction. At the same time, once in the 880 nm state the complex is in a conformational energy minimum from which the transition to the initial 870 nm state is more difficult than a further evolution to states that are more red. Figure 2B demonstrates another such example of energy barrier asymmetry where the transition from the intermediate state of 870 to 860 nm requires excitation energy, whereas the thermal energy of the protein lattice is sufficient for the transition from 860 to 870 nm. At the same time, spontaneous transitions from the 870 nm state are not prohibited as is apparent from Figure 2D.

From the detailed analysis of Figure 3, it appears that the total number of conformations that are relatively blue and change to relatively red is comparable in magnitude to the number with the opposite relationship. Probably due to the limited statistics, we do not observe a preferential FLP value of spectrally diffusive states: the points outside the confined area in Figure 3 do not exhibit any clustering. However, most of those states are to the blue of 870 nm. This means that they are rather blue and exhibit a possibility of further evolution even more to the blue or, on the contrary, to the red.

The multitude of different evolution scenarios of a certain conformation is apparent from the comparison of the examples in panels A and C of Figure 2 where the red spectral states with similar FLP values evolve rather differently, implying that the conformational energy landscape of the complex exhibits a multitude of routes for natural evolution. This complexity is further demonstrated by the analysis of Figure 3: spectral states that diffuse insignificantly exhibit a distribution of FLP values similar to that of the diffusive states. It also appears that conformations with the same value of FLP evolve rather differently since there is a wide spread of FLP values along the ordinate axis. Thus, the FLP value does not unambiguously determine the conformational evolution of a complex. This is probably related to the complexity of the potential energy hypersurface: from a particular conformation a complex can move to a number of other conformations, and a probabilistic character of the barrier crossing then results in different final states. Or it is possible that due to the complexity of the LH2 structure some of the spectroscopic states are degenerate; i.e., multiple structural conformations correspond to the same spectroscopic state. In that case, different initial conditions set diverse evolutionary pathways resulting in various final states.

It should be noted that some conformations with an FLP either to the blue or to the red of 870 nm evolve to a state with a FLP value close to 820 nm. With 800 nm excitation, a spectrum peaking at 820 nm is, in fact, just a wing of an even bluer spectrum strongly distorted by the FL filter. From the comparison of the data sets acquired with 800 and 594 nm excitation, it appears that the corresponding spectrum peaks around 810 nm and is associated with the temporary bleaching of the B850 ring, as was already discussed in an earlier work (D. Rutkauskas et al., submitted for publication). It is an astonishing observation that in a number of cases an intact light-harvesting complex spontaneously moves to a state with a bleached B850 ring.

Within the framework of the disordered exciton model, spectra blue-shifted relative to the intermediate position of 870 nm correspond to coherently delocalized excitation relatively unperturbed by the static disorder when pigment site energies are of similar magnitude (8, 24; V. Novoderezhkin et al., submitted for publication). A spectral blue shift also occurs when electronic transition energies are uniformly shifted to the blue. This can be due to microscopic factors such as breakage of hydrogen bonds to the acetyl carbonyl group of the BChls (25–27; D. Rutkauskas et al., submitted for publication). The red-shifted spectral states correspond to a localized excitation seriously perturbed by the energetic disorder. A red shift of just one pigment site energy can significantly shift the whole spectrum to the red. This might be caused by the strengthening of the hydrogen bonding. Therefore, transitions between the conformations of various FLPs must be associated with significant and in some cases possibly correlated structural rearrangement of the binding pocket of one or a number of chromophores. Apparently at room temperature, the protein lattice of a bacterial light-harvesting pigment–protein complex possesses sufficient vibrational energy to induce such changes.

In this work, we observed the spontaneous nonequilibrium relaxation of individual bacterial light-harvesting pigment–protein complexes from spectral states arbitrarily prepared by the excitation light. Application of a single-molecule technique is paramount since it allows the detection of spectral changes that otherwise would be masked by ensemble averaging. The natural spectral development is associated with the transitions of the pigment–protein complex in its conformational potential energy landscape. To the best of our knowledge, these spontaneous pigment–protein motions, unaffected by the probe of the excitation light, have not been observed previously on a single-molecule level for the light-harvesting complexes. Transitions among spectral forms differing by tens of nanometers occur in the absence of the illumination. We discovered that a multitude

<table>
<thead>
<tr>
<th>excitation (( \mu W ))</th>
<th>no. of measured molecules</th>
<th>duration of the dark period (s)</th>
<th>jump threshold (nm)</th>
<th>% jumpy states in the dark$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td>118</td>
<td>15</td>
<td>10</td>
<td>29</td>
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<tr>
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<td>1</td>
<td>147</td>
<td>15</td>
<td>10</td>
<td>27</td>
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$^a$ A fraction of dark periods after which the FLP value is different from that before blocking the excitation by more than the threshold value.
of different spectral evolutionary scenarios are possible, indicating the anticipated complexity of the conformational landscape. Such changes must be associated with significant structural rearrangements leading to an alteration of microscopic factors, such as the strength of hydrogen bonding to the side groups of the BChls in the complex. That these alterations occur naturally suggests an intriguing level of structural flexibility in the light-harvesting ring, which may have some functional significance. The implications of these changes in the spectral properties remain open to speculation.

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REFERENCES