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Rhodopsin(s) in eubacteria

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Abstract

While the biochemical basis of photosynthesis by bacteriochlorophyll-based reaction centres in purple phototrophic Eubacteria and retinal-based bacteriorhodopsin in the Archaebacterium *Halobacterium salinarium* has been elucidated in great detail, much less is known about photosensory signal transduction; this is especially the case for Eubacteria. Recent findings on two different photosensory proteins in two different Eubacteria, which both show clear resemblances to the rhodopsins, will be presented.

The photoactive yellow protein (PYP) from the purple phototrophic organism *Ectothiorhodospira halophila* probably functions as the photoreceptor for a new type of negative phototaxis response and has been studied in some detail with respect to its structural and photochemical characteristics. On basis of crystallographic an photochemical data it has been proposed that PYP contains retinal as a chromophore. However, we have unambiguously demonstrated that the PYP chromophore is different from retinal, in spite of the fact that PYP's photochemical properties show striking similarities with the rhodopsins.

The cyanobacterium *Calothrix* sp. displays complementary chromatic adaptation, a process in which the pigment composition of the phycobilisomes is adjusted to the spectral characteristics of the incident light. In orange light the blueish chromophore phycocyanin is present, in green light the reddish phycoerythrin is synthesized. On the basis of the action spectrum of this adaptation process, we hypothesized that a rhodopsin is the photosensor in this process. In line with this, we found that nicotine, an inhibiter of the biosynthesis of β-carotene (which is the precursor of retinal), abolishes chromatic adaptation. Direct proof of the involvement of a photosensory rhodopsin was obtained in experiments in which the chromatic adaptation response was restored by the addition of retinal to the cultures.

The two photosensory proteins mentioned above represent the first examples of eubacterial photoreceptors that can be studied at a molecular level. Our current knowledge on these two proteins and their status as retinal proteins will be reviewed.

Keywords: Rhodopsin; Eubacteria

1. Eubacterial photoreceptors

Light is of primary importance as an energy source for phototrophic organisms. In addition, light plays an important role in the regulation of a large number of processes; many organisms can use external light stimuli as a source of information to respond to changes in the environment. A large body of knowledge has been obtained for photoreceptors from eukaryotic organisms (phytochromes in plants and rhodopsins in animals and unicellular organisms) and for archaebacterial photoreceptor proteins (the

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sensory rhodopsins in *Halobacterium*). However, detailed knowledge of eubacterial photoreceptors was lacking until recently. Here we will review data on two proteins which function as receptors for light stimuli: (1) the photoactive yellow protein (PYP) from the purple sulfur phototrophic Eubacterium *Ectothiorhodospira halophila* which probably is involved in negative phototaxis and (2) a rhodopsin from the cyanobacterium *Calothrix* sp. that regulates complementary chromatic adaptation in response to changes in light climate. These two proteins are the first candidates for being *eubacterial* retinal proteins, while rhodopsins in both Eukaryotes and Archaebacteria have been studied extensively.

2. Photoactive yellow protein, a new family of photoreceptor proteins

A photoactive yellow protein (PYP) has been isolated from *Ectothiorhodospira halophila*, *Rhodospirillum salexigens* and *Chromatium salexigens* [1–3]. PYP is a 14 kDa water soluble protein that is very stable towards denaturation. The visible absorption spectrum of PYP consists of a strong and broad band at 446 nm [1]. At pH values below 3, PYP is reversibly converted from the ground state to a state absorbing at approximately 345 nm [4]. After absorption of a blue photon the protein and chromophore undergo a number of dark transformations in which PYP changes its absorption spectrum and then returns to the initial state. This photocycle and other photophysical/chemical characteristics of PYP strongly resemble those observed in the (archaebacterial) rhodopsins [4–9].

The three-dimensional structure of the protein part of PYP is completely different from that of the rhodopsins. PYP consists of two perpendicular antiparallel β-sheets, forming a so-called β-clam structure [10]. In contrast, rhodopsins are all-helical transmembrane proteins (e.g. [11]). The complete amino acid sequence of PYP has been determined and shows no obvious sequence similarity to any other protein, including those with a β-clam structure [12].

The results of the characterization of the PYP photocycle are summarized in Fig. 1. The photocycle of PYP involves two intermediates in the time domain between 2 ns and 2 s: a red-shifted intermediate pR is formed (its formation has not been time-resolved) which is converted into a blue-shifted intermediate pB that returns to the ground form pG (Fig. 1A; [4,6,13,14]). The absorbance spectra of these intermediates have been estimated using a global analysis approach (Fig. 1B; [14]). At 77 K a batho-intermediate of PYP (which is similar to pR) is trapped, indicating that the photocycle is blocked

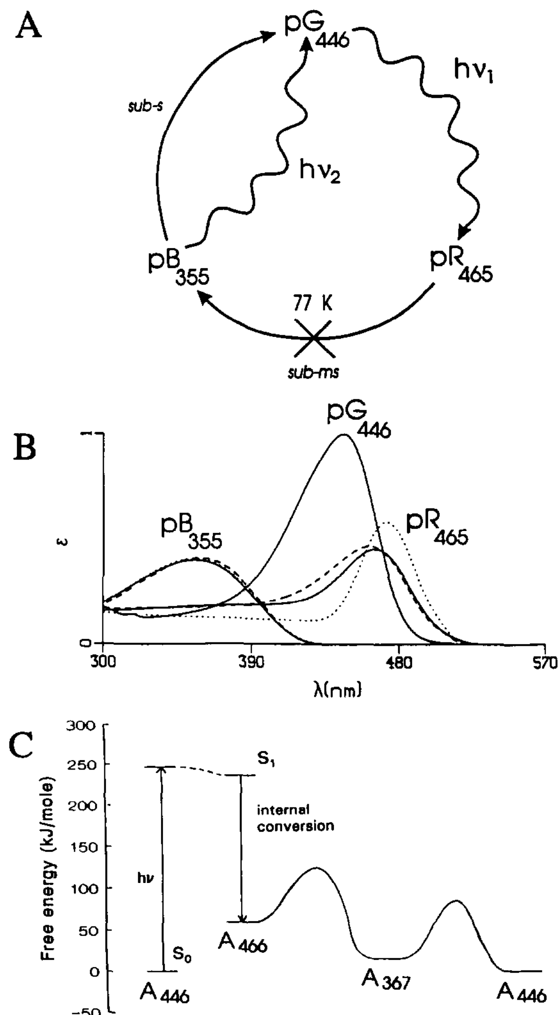

Fig. 1. The PYP photocycle. In (A) a schematic representation of the PYP photocycle is shown. Wavy lines indicate photochemical processes, subscripts indicate absorbance maxima of the photocycle intermediates. The absorbance spectra of the two intermediates in the PYP photocycle are shown in (B), together with and scaled to the absorbance spectrum of the ground state of PYP. The free energy profile of the PYP photocycle is depicted in (C).

after its first step at this temperature. In addition, a strongly fluorescent intermediate is formed in parallel with this red-shifted intermediate. This situation closely resembles that in bacteriorhodopsin at 77 K. In PYP a light-induced branching reaction from pB to pG has been described ([8]). In this process pB, which thermally decays *slowly* to the ground state, is converted to pG at a strongly increased rate (in comparison with the thermal reaction) after the absorption of a photon. Both the initial and the branching photoreaction in PYP have a high quantum yield [8,13]. A similar situation exists in the bacterial rhodopsins ([15]).

Concerning the biological function of PYP, evidence has been obtained that the protein is a photoreceptor for a negative phototactic response of *Ectothiorhodospira halophila* (Fig. 2A; [16]). This is the first example of negative phototaxis in a free-swim-

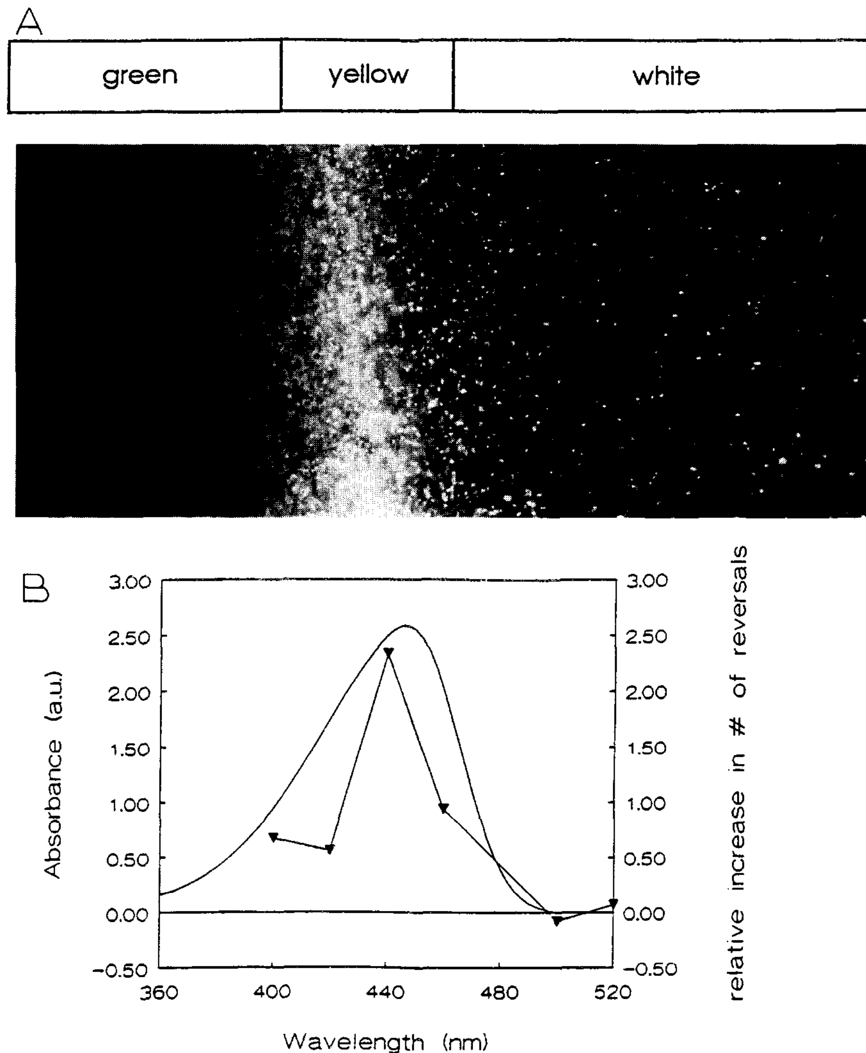

Fig. 2. Negative phototaxis in *E. halophila*. An example of the negative phototaxis response in *E. halophila* is depicted in (A), which shows the cell distribution pattern induced by the illumination of a flat capillary with a half-green, half-white light spot (200 ×). The blue component in the white light causes the cells to accumulate in the green area. The picture was kindly provided by W.W. Sprenger. The wavelength dependence of this negative phototaxis response is shown in (B) (see [16]).

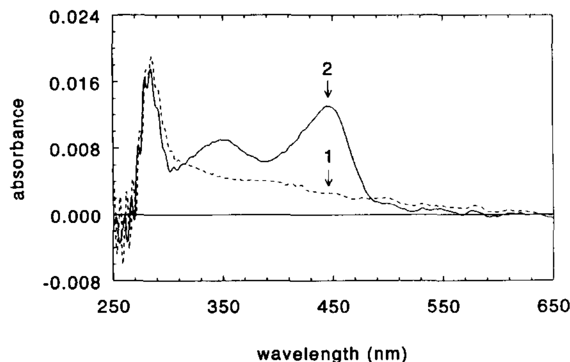

Fig. 3. Absorbance spectrum of PYP from *E. halophila* after incubation in sample buffer with or without DTT, followed by SDS-PAGE. PYP from *E. halophila* was incubated in sample buffer with (trace 1, broken line) or without (trace 2, solid line) 0.1 M DTT for 1 hour at 37° C and was then subjected to SDS-PAGE. Subsequently the gel was placed in a spectrophotometer to determine the UV/VIS absorbance spectrum of the PYP samples in the gel. The spectra shown have not been scaled and were smoothed to reduce scatter. The smoothing had no effect on the position or height of the peaks.

ming Eubacterium. The wavelength dependence of the response fits the absorbance spectrum of PYP (Fig. 2B). This is a strong indication that PYP is the photoreceptor for this response (see also [16]).

3. The chromophore of the photoactive yellow protein

On the basis of the crystallographic and photochemical data discussed above, it was proposed that the PYP chromophore is retinal. However, attempts to extract retinal from isolated PYP were never successful (Hoff et al., unpublished). Therefore, we followed a strategy in which we proteolytically degraded native PYP and isolated small peptides containing the chromophore. Amino acid sequencing of these peptides showed that the chromophore is attached to Cys-69, the unique cystein residue in PYP [12]. This was confirmed by experiments showing that DTT removes the chromophore from the protein (Fig. 3). The molecular weight of the chromophore has been determined to be approximately 147 Da by electrospray ionization mass spectrometric measurements on PYP before and after treatment with DTT [12]. From these results, it must be concluded that the PYP chromophore differs strongly from retinal. However, its chemical structure has not yet been reported. Since apparently the structure of both the protein (a water-soluble β-clam instead of a transmembrane 7 α-helix bundle) and chromophore part of PYP are very different from those found in the rhodopsins, this leads to a paradoxical situation: how can two structurally very different proteins display the same photochemical activities?

4. Protein conformational changes in the PYP photocycle: the signalling state

From the effects of solvent hydrophobicity and viscosity on the PYP photocycle kinetics it has been concluded that during progression through the photocycle a conformational change exposing hydrophobic sites takes place in the protein [13]. This suggested that changes in heat capacity may play a role in the photocycle (see [17]). With a thermodynamic model, developed for the description of protein (un)folding processes and incorporating changes in heat capacity, we were able to quantitatively explain (M.E. Van Brederode, W.D. Hoff and K.J. Hellingwerf, unpublished results) the unusual temperature dependence of the photocycle that had been reported previously [13]. The activation changes in heat capacity that we found to be associated with the PYP photocycle are comparable to those found for the (un)folding of small water-soluble proteins. Therefore, the pB photocycle intermediate is thermodynamically equiva-

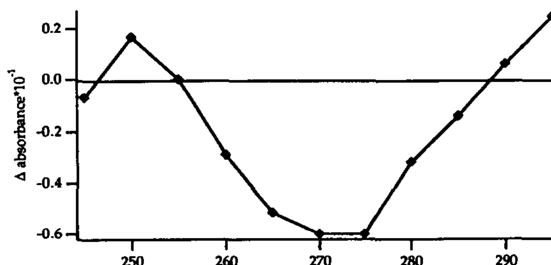

Fig. 4. Light-induced UV absorbance difference spectrum of PYP from *E. halophila*. PYP was illuminated for five seconds with white light to induce full bleaching. During the recovery of the ground state after turning off the light, the absorbance changes at different wavelengths were followed in time. The data points shown are those for $t = 0$, immediately after turning off the light.

lent to a (partially) unfolded protein state. These results establish an unexpected link between protein (un)folding events and the processes occurring in the light-triggered catalytic cycle of this photoreceptor protein. With these data and results from optoacoustic calorimetric experiments and steady state thermal

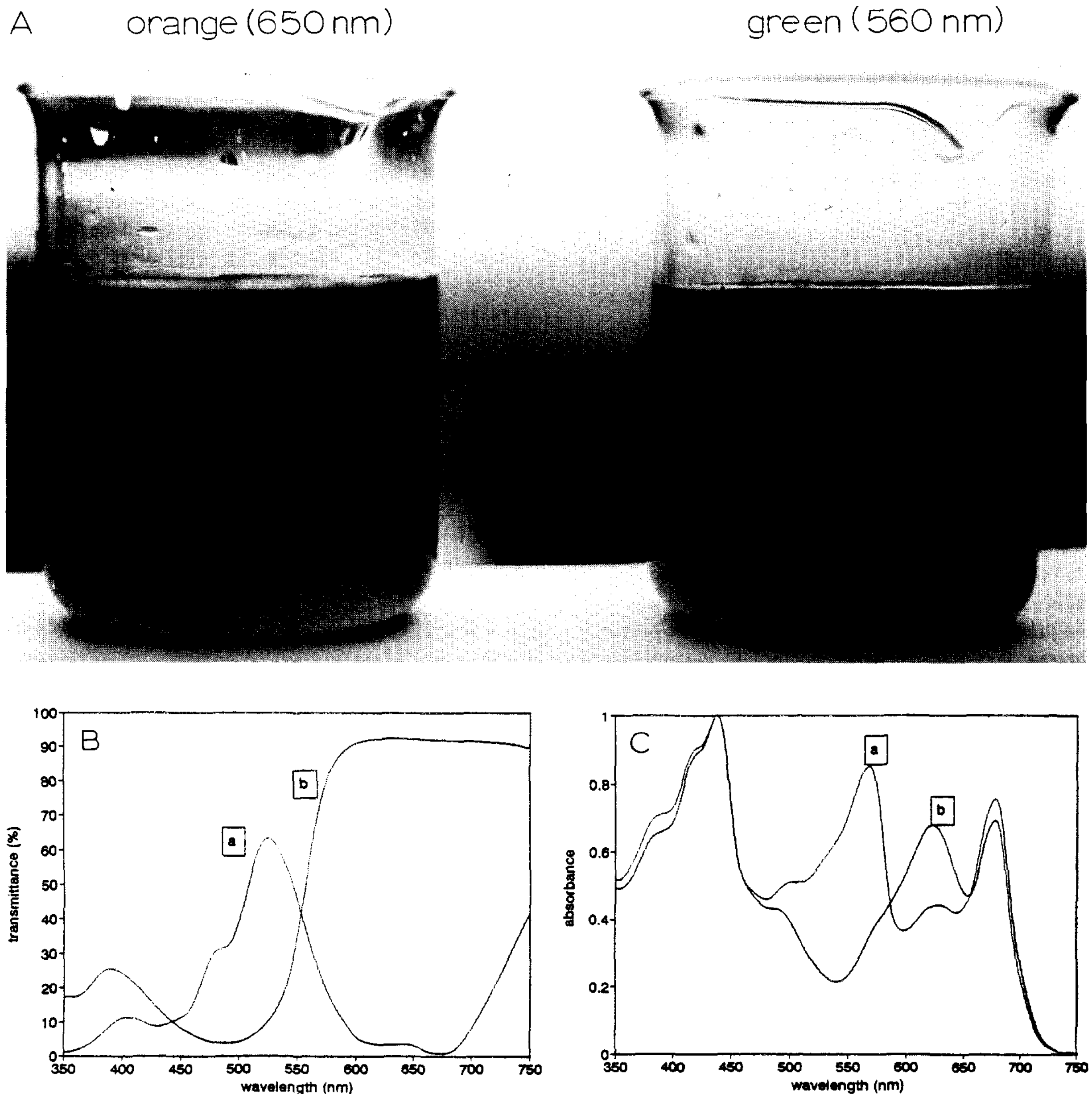

Fig. 5. Complementary chromatic adaptation in *Calothrix*. Cultures of *Calothrix* sp., which were grown in green or orange light, are shown in (A). In (B) the transmittance spectra of the green (a) and orange (b) filters used are shown. The in vivo absorbance spectra of the cultures grown in green (a) and orange (b) light are depicted in (C).

denaturation experiments we were able to construct a preliminary but complete thermodynamic description of the PYP photocycle (Fig. 1C).

We have obtained other, independent evidence for a large conformational change in the PYP photocycle by measuring the changes in absorbance in the aromatic region (near 280 nm) associated with the formation of pB. Fig. 4 shows a preliminary light-induced difference spectrum of PYP in the near UV region. The amplitude of the bleaching at 275 nm is approximately 15% of the absorbance band at 276 nm, indicating that the environment of (a number of) aromatic amino acids changes drastically during the photocycle. We are currently performing CD and NMR measurements on the protein in the dark *and* during steady state illumination with laser light, to study these conformational changes with atomic resolution to gain insight in the molecular basis of signal transduction in this photoreceptor protein.

5. Complementary chromatic adaptation in cyanobacteria

In so-called complementary chromatic adaptation (CCA), some strains of cyanobacteria, such as *Calothrix* sp., change the pigment composition of their antennae (phycobilisomes) to optimally adapt to the prevalent light conditions. If green light prevails, the red coloured C-phycoerythrin (PE) is synthesized and in orange or red light the blue coloured C-phycocyanin (PC; [18]; Fig. 5). The genes involved in this adaptive response have been mapped and the information available on gene activation occurring in CCA [19], including phosphorylation-dependent binding of an activator protein in the promoter region of genes encoding antenna polypeptides [20], suggests the involvement of a two-component signal transduction system, for which a sensor protein has been identified via complementation analysis of CCA-deficient mutants [21]. The protein identified in this complementation, RcaC, shows similarities with other sensor proteins of the two-component family [22]. The identity of the actual light-sensing component in this adaptation response, however, remains to be elucidated. The involvement of phytochrome, a major photosensing pigment in plants, in CCA is highly unlikely in view of the wavelengths involved [23] and phytochromes have only been detected in higher plants until now. Rhodopsins on the other hand cover the relevant spectral region [23].

6. A rhodopsin as the photosensor in complementary chromatic adaptation in *Calothrix* sp.

Recent work in our department has provided evidence for the involvement of a retinal protein (i.e. a rhodopsin) in light sensing in CCA (H. Schubert, H.C.P. Matthijs, L.R. Mur and K.J. Hellingwerf, unpublished results). First, the presence of retinal in the cyanobacterium *Calothrix* sp. could be demonstrated (not shown). Second, inhibition of retinal synthesis with nicotine (an inhibitor of β-carotene synthesis, the direct precursor of retinal) caused full inhibition of PE synthesis in *Calothrix*, upon transfer of the cells from orange to green light. Third, the inhibition of chromatic adaptation by nicotine could be reversed by the addition of exogenous retinal (Fig. 6). These results provide the first evidence that rhodopsins may also be functioning in the 'Kingdom of the Bacteria'. The biochemical properties of this cyanobacterial photosensory rhodopsin are currently being investigated.

Fig. 6. Reconstitution of complementary chromatic adaptation by the addition of retinal to nicotine-treated cultures of *Calothrix* sp. Cultures were switched from orange to green light and 60 h later in vivo absorbance spectra were recorded. Spectrum (a) +150 μM nicotine + 100 μM retinal + 1 mM DTT; spectrum (b) control, no additions; spectrum (c) +150 μM nicotine.

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