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Photoreception and photomovements of microorganisms †

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Many freely motile microorganisms can perceive and transduce external photic stimuli to the motor apparatus, eventually moving, by means of various behavioural strategies, into environments in which the illumination conditions are the most favourable for their life. In different microorganisms, a wide range of chromophores operate as light detectors, each of them set in a special molecular pocket that, in its turn, can be linked to another component of the transduction chain. The diverse photosensors are organized in special (and in many cases dedicated) photoreceptor units or subcellular organelles. The main molecular mechanisms connecting the early event of photon absorption to the formation of the signalling state down to the dark steps of the transduction chain are discussed in a selected number of case examples. The possible importance of an intensive multidisciplinary approach to these problems in an evolutionary perspective is finally briefly outlined.

Introduction

"Some people may think that study of the light-induced movement responses of insignificant microorganisms" (such as bacteria, algae, ciliates) "is an arcane pursuit with little significance to problems of the real world. However",[1]. . .. and there are

† This paper is dedicated to our querida Professor Silvia Braslavsky on the occasion of her 60th birthday.

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several howevers. For instance, about 30 years ago photosensory and photomotile responses in microorganisms were considered valuable "models" for a better and deeper understanding of the molecular basis of the visual process in higher organisms with neural networks. In microorganisms, in fact, the final behavioural reaction is derived directly from the primary event of the absorption of a photon through a chain of molecular reactions, without any neural integration/elaboration. Over the years, the idea of utilising microorganisms as a model system of vision was abandoned, but the research field of photomovements has progressively become more and more important because it allows clarification of the intimate nature of basic photosensory processes in living systems that are evolutionary closer to primeval organisms as well as components of an essential portion of existing natural ecosystems. Moreover, whereas in neural organisms rhodopsin represents the sole photosensory system, in unicellular life forms evolution has designed a large number of photoreceptors, each member of which suits the special requirements of a particular microorganism. In other words, natural selection has continued assaying chromophores other than retinal, bound to molecules other than transmembrane seven helices proteins, operating on mechanisms other than chromophore photoisomerisation.[2,3] Finally, the aforementioned wide variety of photoreceptors and transduction chains found in microorganisms might yield impressive advancements in natural pigment-based photonic devices for application in holography, neural network optical computing and optical memories.[4–6]

Why's and how's of photomovements

In a world in which light is by far the most important source of energy (photosynthesis) as well as a possible cause of damage (UltraViolet radiation, photosensitization processes), it is no wonder that microorganisms have been compelled by evolution to develop systems capable to perceive and "interpret" the different characteristics of light (direction, intensity, wavelength, polarization) in order to search for optimal illumination conditions and to escape noxious ones.

Photomotile responses allow photosynthetic bacteria (*e.g.*, *Rhodobacter sphaeroides*) and microalgae (*e.g.*, *Euglena gracilis*) to gather in environmental niches in which light is bright enough to efficiently drive the photosynthetic process and, at the same time, not too intense to lead to photoinhibition or photobleaching. In diatoms, for example, their different spectral sensitivities allow various species to occupy distinct spatial and temporal domains and in phytoplankton the integration of light and gravity perception allow vertical migration along the water column.

However, even for microorganisms that do not harvest and convert light energy directly for their metabolism, light can be an environmental cue to accumulate into habitats which can be favourable for reproduction (*e.g.*, *Dictyostelium discoideum*) or propitious for their prey (*e.g.*, *Fabrea salina*) and, in general, for food (*e.g.*, *Ophryoglena flava*). In microorganisms containing endogenous photosensitiser(s), used as defensive pigment(s) against predators (*e.g.*, *Blepharisma japonicum* and *Stentor coeruleus*), even relatively dim light can cause severe damage and their capability of escaping lighted spots is directly linked to their survival.[2] Finally, some microorganisms have been shown to be able to perceive and transduce UltraViolet radiation, again to run off from noxious environments.[7,8]

To achieve these goals, photomotile microorganisms adopt behavioural strategies based on directional (phototaxis) as well as non-directional (photophobic responses, photokinesis) movements.

Phototaxis results from the detection of the direction of light and is defined as positive or negative according to whether the oriented movement is toward or away from the source. Such a response implies the existence of a quite sophisticated photoreceptor apparatus. To perceive the position of the light source, in fact, an asymmetry in the photoreceptor apparatus, which allows the cell to sense the vectorial characteristics of the light signal, is required. This can be accomplished, for instance, if the photosensing unit is coupled to a screening/reflecting device that modulates the light signal on the photoreceptor, so that light direction is perceived by comparing the light absorbed by the photoreceptive unit at two instants in time.

In photophobic responses the sensory stimulus is a sudden change in light intensity regardless of its propagation direction. Step-up (step-down) photophobic responses are caused by a step-wise increase (decrease) in photon flux. This abrupt variation in photon flux elicits a transient modification in the microorganisms movement pattern that in turn depends on the morphology and on the type of locomotor apparatus (bacterial or algal flagella, cilia, pseudopodes) of the cell. In any case photophobic responses usually consist in a brief cessation of forward movement (stop response) followed by a random change of the direction of movement. A photophobic response typically lasts for a few seconds, after which the microorganism can become adapted to the new illumination conditions. If the sudden change in light intensity is experienced because the cell crosses a light-dark border, step-up (step-down) photophobic responses bring the cells to escape from lighted (shaded) regions and to accumulate in shaded (lighted) areas by means of a trial-and-error process. The final outcome of the step-up and step-down photophobic responses is cell population photodispersal and photoaccumulation, respectively.

In photokinesis light intensity affects the absolute value of the microorganism velocity. If the velocity increases (decreases) when the organism is exposed to light, photokinesis is positive (negative) and causes photodispersal (photoaccumulation).

It is worth noting that the same organism may be able to exhibit more than one photoresponse, which in some cases concur in causing the same result (*e.g.*, photodispersal induced by negative phototaxis and step-up photophobic responses in *S. coeruleus*) and, in other cases, depend on the environmental illumination condition (*e.g.*, positive or negative phototaxis elicited by low or high light intensities in *E. gracilis*).

To quantitatively measure light-elicited motile reactions in microorganisms, population methods as well as single cell track (usually computer-assisted[9]) analysis are currently used. Both of them have advantages and drawbacks, and here we only want to underline that these measurements require an accurate choice of meaningful parameters to reliably discriminate among different reactions. As mentioned above, photodispersal (photoaccumulation) may result not only from true negative (positive) phototaxis, but also from a series of step-up (step-down) photophobic reactions, as well as from positive (negative) photokinesis.

The photosensory transduction chain

In Fig. 1 the block diagram of the photosensory transduction chain is shown. Following absorption of a photon, the photosensing chromophore undergoes molecular modifications leading to the formation of the signalling state which triggers the transduction chain eventually acting on the motor apparatus.

We will go through the different blocks, briefly discussing the main recent advancements and the still open questions in a few case-examples.

Photoreceptor apparatus

In several microorganisms, not only the structure of the photosensing chromophore(s), but also the primary molecular events have been identified. In other ones, even the chemical nature of the molecule acting as light detector is still doubtful and the

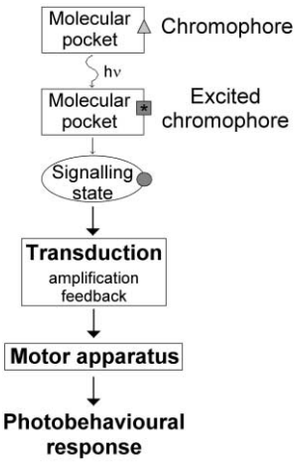

Fig. 1 Schematic block diagram of the photosensory transduction chain in microorganisms.

mechanism by which the sensory photoreceptors transmit the perceived informational light signals downstream to the transduction pathway is undetermined.

Chromophores

Photomotile microorganisms are present in almost every phyletic group that includes unicellular organisms. It is therefore not surprising that a multiplicity of chromophores with completely different molecular structures and spectroscopic features has been demonstrated to act as prosthetic group of the photoreceptor.

In prokaryotes, pigments used mainly for cell metabolism (*e.g.* photosynthetic pigments) can also play the role of photosensors, whereas in eukaryotes dedicated pigments (*i.e.*, those which capture light only for photosensory perception and transduction) are the rule.

In photoreceptor pigments, the presently known chromophores include 4-hydroxycinnamic acid (*e.g.*, in *Ectothiorhodospira halophila*), carotenoids (*e.g.*, retinal in *Halobacterium salinarum* and *Chlamydomonas reinhardtii*), pterins, flavins (*e.g.*, in *Loxodes striatus*) and dianthronic molecules (*e.g.*, stentorin in *S. coeruleus* and blepharismins in *B. japonicum*).

To whatever class they belong, in order to be able to detect light over a large range of the solar spectrum, biological chromophores usually have broad absorption bands with high molar extinction coefficients. In some cases the spectral sensitivity can be widened if different chromophores, absorbing in contiguous spectral ranges, are spatially assembled in molecular frameworks to maximize the collective interaction (as suggested in the case of *E. gracilis*, see below).

Because of their intrinsic multidisciplinary character, the problems of identification of the chromophore responsible for the photoresponse and of the subsequent steps of the photosensory transduction chain in microorganisms can be best faced with different experimental techniques and methodologies.

Action spectroscopy is an unparalleled powerful non-destructive technique for ascertaining *in vivo* the absorption properties and, in some cases, investigating the primary reactions of the chromophore. Most importantly, as they relate photoresponsiveness to the wavelength of the stimulating light, action spectra directly link the functional absorption of photons by the presumed photoreceptor pigment to the observed physiological reaction. The structure of an action spectrum should be proportional to that of the chromophore absorption spectrum. However, it may be affected by several artefacts (screening and/or reflecting organelles, energy transfer processes, multiple sensing chromophores, . . .) and could be made non-reliable by using the wrong choice of behavioural parameters describing photoresponsiveness. Even though it has some limitations and can be painstakingly time-consuming, action spectroscopy can, nonetheless, provide direct information on the photoreceptor pigment, it is non-invasive, it usually requires simple and inexpensive instrumentation, and it is one of the most, and in many cases the only, used technique for studying photoresponses in microorganisms.[10]

In vivo absorption and emission microspectroscopy allows not only the measurement of the spectroscopic characteristics of the candidate sensing chromophores in their physiological molecular environment, but also allows the localisation within the cell and, in some cases, the determination of spatial and spectral maps.

Whenever it is possible to extract the chromophore, or better to isolate the integral photoreceptor unit, many chemical, biochemical and physico-chemical assays can be used to carefully characterize the chromophore's structural and functional properties.

Mutants lacking one or more photopigment can be used in the determination of the pigment responsible for the photobehavioural reaction. In mutants whose photosensing unit is deprived of the light-detecting chromophore (blind mutants), exogenous addition of chromophores restoring the photoresponse can allow identification of the nature of the molecule whose role is perception and transduction of light stimuli.

When reliable hypotheses are available on the chemical nature of the sensing chromophore(s), specific inhibitors of its (their) biosynthesis can advantageously be employed.

The determination of action spectra of the photobehaviour of *C. reinhardtii* blind mutants incorporating different retinal analogues confirmed that in this flagellated green alga the photodetecting chromophore is retinal.[11]

Chromatographic analysis of whole cell extracts, the lack of photobehaviour in cells treated with retinal synthesis inhibitors and *in vivo* absorption microspectroscopy suggested that also in *E. gracilis* the photosensing chromophore is retinal.[12] In this same cell, however, a pterin–flavin system (Fig. 2) has been suggested as the light sensor from the results of action spectroscopy, also in cells incubated with riboflavin analogues absorbing in different spectral ranges (roseoflavin, in particular), and from spectroscopic studies of isolated photoreceptor structures.[13]

The chemical nature and the structural formula of the chromophores triggering photoavoidance responses of *E. halophyla* (4-hydroxycinnamic acid, Fig. 2) and step-up photophobic reactions of *S. coeruleus* and *B. japonicum* (stentorin, blepharismin and oxyblepharismin, Fig. 2) have been determined in detail by means of NMR spectroscopy and mass spectrometry measurements on biochemically isolated and purified chromophores.[14–18] In the case of *S. coeruleus* and *B. japonicum*, the assignment of stentorin and blepharismin as the photosensing chromophores has been clearly confirmed by the results of action spectroscopy. In *B. japonicum*, it has also been shown that not only native blepharismin but also oxyblepharismin elicits step-up photophobic responses, the latter being the product of an intracellular photooxidation of the native chromophore (see Fig. 3).[19,20]

Chromophore molecular pocket and primary reactions

In all photosensing chromophores the absorption of a photon (*e.g.*, step-up photophobic reactions) or the drop-down in the number of absorbed photons (*e.g.*, step-down photophobic reactions) induce molecular modifications that are the primary events of the complex sensory perception and transduction process. In this review we will discuss only the light triggered transduction pathways, mainly because of the almost complete lack of data on dark initiated chains.

Chemically different chromophores can undergo quite different early light-induced molecular transformations: for example, photoinduced charge transfer (flavins, stentorin

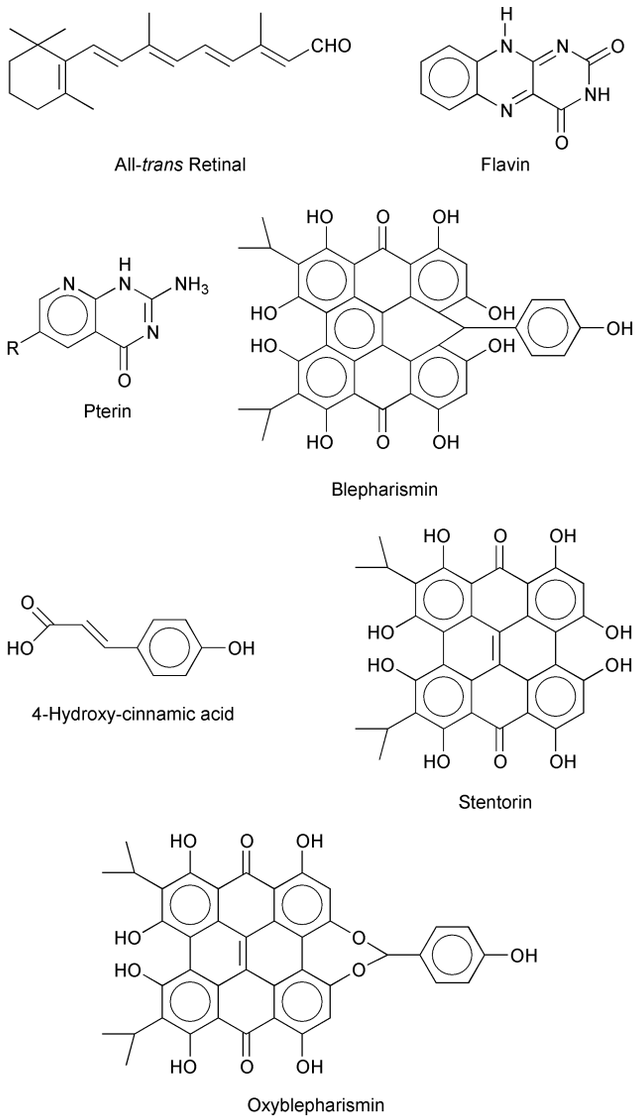

Fig. 2 Structural formulas of some photosensing chromophores.

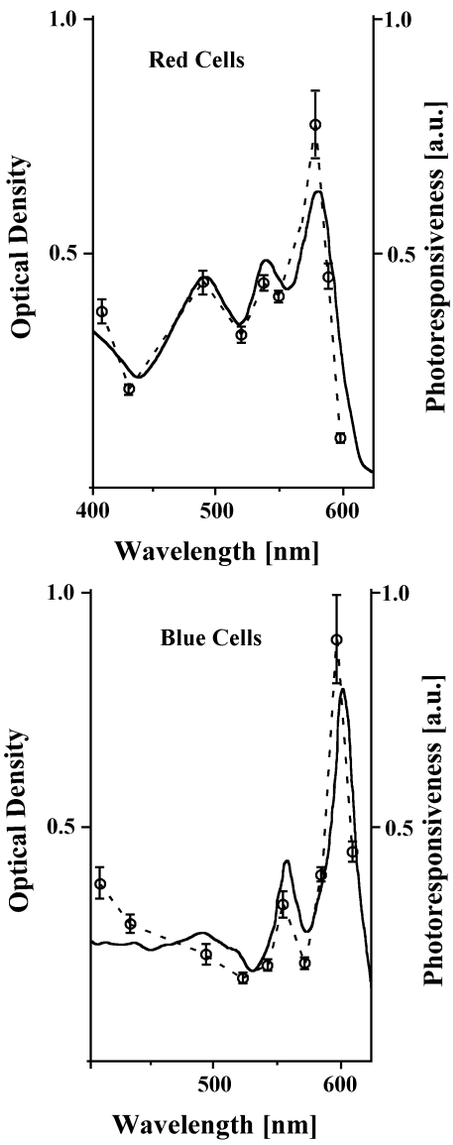

Fig. 3 Action spectra (dotted line) for the step-up photophobic response of *B. japonicum* red and blue cells compared to the optical absorption spectra (continuous line) of blepharismin (red cells) and oxyblepharismin (blue cells).

and blepharismins), photoisomerisation (retinals, 4-hydroxy-cinnamic acid) and energy transfer (pterins-flavins).

However, even the most comprehensive knowledge of the chemical properties of an isolated sensing chromophore is not enough for formulating reliable hypotheses on the primary molecular reactions occurring in the photoreceptive structure upon light absorption by the chromophore itself. The molecular interactions taking place between any chromophore and its surrounding microenvironment (its "molecular pocket") can, in fact, severely affect its photophysical and photochemical properties, the probability of radiationless and radiative transitions from the first excited singlet and triplet states, the yields of energy and charge transfer processes from excited and metastable states. The chromophore can then communicate its light-induced modifications to the molecular framework within which it is embedded and thus originate the signalling state. In other words, the very capability of a chromophore to act as a photosensing-phototransducing biological device depends on its molecular pocket and is linked to the structure which the pocket interacts with.[21]

Halobacterial retinal, for example, is bound to two different seven helices transmembrane opsins to make up Sensory Rhodopsin I (SRI) and Sensory Rhodopsin II (SRII). Even if the chromophore is the same, in dark kept samples the absorption maximum of the chromophore-protein complexes are centred at different wavelengths (587 nm for SRI and 487 nm for SRII) and the absorption of a photon by the retinal molecules gives rise to markedly different photocycles and motile responses. SRI, in fact, is responsible for both attractant and repellent responses following orange and near UV-blue light stimulation, respectively, whereas SRII elicits repellent responses upon absorption of blue light.[22]

The case of *H. salinarum* also exemplifies that the chromophore and its molecular pocket by themselves may not work as a photosensing/phototransducing device. In this bacterium, in fact, two more rhodopsins exist: bacteriorhodopsin (BR) and halorhodopsin (HR), both of which work as light-driven ionic pumps, for protons and chloride respectively. In SRI, retinal is bound to an apoprotein that is highly homologous to that of BR, in particular as far as the active site is concerned. As a matter of fact, *in vitro* also SRI works as a light-driven proton pump as BR does, whereas *in vivo* it can transfer the incoming photic signal across the plasma membrane just by means of a receptor-specific transducer protein (HTR I).[22]

As previously mentioned, in the photosynthetic flagellate *E. gracilis* retinal[12] and, alternatively, pterin-flavin complexes[13] are suggested to be the photodetecting chromophores, inserted in a three-dimensional crystal (the ParaFlagellar Body, PFB, see below). Retinal would be bound to a 27-kDa protein, with a photocycle similar to that of SR's, but with at least one stable intermediate. This rhodopsin-like photoreceptor, showing

optical bistability without thermal deactivation, may have quite interesting properties as an optical sensor for biomolecular devices.[12]

Much less is, at present, hypothesized about the molecular pocket of the pterin-flavin sensing chromophores in the same alga. Pterins are suggested to be located on the surface of the PFB and act as donors in an energy transfer process to flavins, which, in their turn, would be situated in the inner structure of PFB, but no clear evidence is available on the possible nature of the pterin-flavin-binding protein.[13]

On the basis of high resolution X-ray crystallographic and 1H-NMR data, it has been shown that in *E. halophila* 4-hydroxycinnamic acid is covalently bound to a 14 kDa water-soluble, cytoplasmatic protein to form a photosensor named photoactive yellow protein (PYP), with absorption maximum at 446 nm.[23] Time-resolved X-ray diffraction, NMR and FTIR techniques have been crucial to elucidate the PYP photocycle and characterize its intermediates.[24] As is the case of sensory rhodopsins, PYP undergoes a photocycle initiated by the photoisomerisation *trans–cis* of its chromophore. Following this primary photoreaction, global structural changes occur in the protein, mainly due to an intramolecular proton transfer to the anionic *cis* form of the chromophore from a glutamate residue, located in a highly hydrophobic pocket. Consequently, the negative buried charge of the glutamate residue gives rise to a partial protein unfolding. The PYP intermediate in which the chromophore is protonated, shows a main absorption blue-shifted band (λ_{max}=355 nm) and represents the signalling state, which can unlock the downstream transduction pathway.[25] Interestingly, also from an evolutionary point of view, the structure of PYP, with central five-stranded β-sheet and helical segments on either side, is typical of the PAS domain[26] (acronym from the three members of the Period clock, which regulates circadian rhythms; Aryl hydrocarbon receptor nuclear translocator, which activates the xenobiotic response; and Single-minded, which determines cell fate), a key element found in many eukaryotic and prokaryotic signal-transduction chains.

Stentorin and blepharismins, the photosensing chromophores of the ciliates *S. coeruleus* and *B. japonicum*, respectively, are located in molecular pockets whose nature is not yet unambiguously identified.

Two distinct forms of stentorin chromoproteins have been chromatographically isolated, stentorin I and II. The weakly fluorescent stentorin II contains two subunits, stentorin II-A and II-B, in which the chromophore is covalently bound to a 50 kDa protein.[27] This latter complex is suggested to be the photosensor of *S. coeruleus*, but, up to now, only short amino acids sequences are available which are too short to be able to perform reliable homology analyses with other gene products. Consequently, the protein structure is still unknown.

Even more uncertain are the molecular properties of the blepharismin-binding protein(s) and the nature of the binding itself. Different research groups have proposed different interpretations of their biochemical data. According to the resulting puzzling picture, the chromophore might be non-covalently bound either to an apoprotein with a 38 kDa molecular mass[28] or to a 200 kDa single polypeptide chain.[29] Finally, immunological and biochemical evidence suggests that a specific carrier protein for blepharismin chromophore might not exist at all.[30] Recent findings, obtained comparing spectroscopic properties and singlet oxygen production rate of the free pigment and of the putative pigment-protein complex, seem to support the 200 kDa hypothesis and suggest that at least oxyblepharismin is buried into the protein in a site poorly accessible to oxygen.[31]

Of course, because of the molecular structure of these hypericin-like chromophores, photophysical primary events other than photoisomerisation have to occur.

As these dianthronic molecules are efficient photosensitisers, able to yield singlet oxygen or other reactive oxygen species from their first excited triplet state, a reasonable hypothesis could be that, inside the cell, this reactive oxygen species is detected as a signal and transduced by a chemoreceptor (photochemosensing), similarly to what was observed in the gliding cyanobacterium *Anabaena variabilis*.[32] However, this hypothesis was ruled out by the lack of any significant effect on photomotile responses of crocetin, a singlet oxygen quencher, and DMPO (5,5-dimethyl-1-pyrroline *N*-oxide), a hydroxyl radical scavenger.[33,34]

In the past, a light-driven proton release from the first excited singlet state of the chromophores was suggested to be the very primary step of the transduction chain. In fact, photobehavioural studies carried out varying extracellular pH and in the presence of suitable protonophores or ammonium chloride, pointed to a photoinduced intracellular pH decrease as a key step for inducing the photomotile responses.[35,36] Time resolved fluorescence measurements on isolated stentorin and blepharismin, however, seriously challenged this direct proton release hypothesis from the first excited singlet state. It should be noted, however, that those experiments were not designed to investigate the occurrence of a proton release from the first excited triplet state, a hypothesis that could deserve deeper examination.

Recent EPR and fluorescence quenching experiments seem to indicate an electron transfer from the excited singlet state of the chromophore to a suitable physiological acceptor molecule (*e.g.* apoprotein) as the primary photoprocess in the photosensory transduction chain.[37,38] A possible reaction scheme is reported below:

$$\mathrm{HO–BP–OH} + h\nu \longrightarrow \mathrm{HO–BP–OH^*} \qquad (1)$$

$$\mathrm{HO–BP–OH^*} + \mathrm{R–S–S–R} \longrightarrow \mathrm{HO–BP–OH^+} + [\mathrm{R–S–S–R}]^- \qquad (2)$$

where R–S–S–R is the electron acceptor.

Proton transfer could be efficiently coupled to this electron transfer:

$$\mathrm{HO–BP–OH^+} + [\mathrm{R–S–S–R}]^- \longrightarrow \mathrm{O{=}BP{=}O} + 2\mathrm{H^+} + 2\mathrm{R–S^-} \qquad (3)$$

In fact upon electron transfer from stentorin and blepharismin, radical cations could be formed and the pK_a of their aromatic hydroxy protons could decrease generating a transient pH change as an important cellular signal.

Even though this scheme is only a hypothetical picture and further studies are necessary, a first check of its reliability can be provided by a rough assessment of the value of the photoinduced intracellular pH drop.

In blue cells, the measured granule OD at 590 nm is 0.8. As the molecular extinction coefficient of oxyblepharismin at 590 nm has been calculated to be of the order of 3×10^4, the chromophore concentration in the granule comes out to be about 10^{-2} M. Assuming the granule volume is of the order of 0.5×10^{-15} L, the number of oxyblepharismin molecules per granule is of the order of 3×10^6. Using a fluence rate of 10^{17} photons $s^{-1}m^{-2}$ (*i.e.* 10^5 photons $s^{-1}\mu m^{-2}$) for excitation at 590 nm, under the hypothesis that 50% of photons are absorbed by oxyblepharismin molecules, the number of excited chromophores per granule is of the order of 5×10^4. Envisaging that the quantum yields for photoinduced electron transfer and for the consequent H^+ release are both of the order of 0.1, about 500 H^+ per granule are released.

Taking into account the granule volume, the number of granules/cell and the cell volume, the number of oxyblepharismin molecules/L and the fact that the intracellular pH value of dark-kept cells is about 6.8, upon stimulation with 10^{17} photons $s^{-1}m^{-2}$ at 590 nm, the photoinduced electron transfer process and the following H^+ release would lower the

intracellular pH value from 6.8 to about 6.7 (assuming no intracellular pH buffer is operating).

Even though very approximate, these assumptions provide a result which is in reasonable agreement with Matsuoka's findings that in an aqueous solution of blepharismin a pH decrease of 0.04 units is observed following two minutes irradiation with white light 1000 W m^{-2}.[39]

A detailed comparative study of free and protein-bound blepharismin chromophores by means of transient absorption spectroscopy, with a femtosecond time resolution, could allow the detection of an electron transfer process as an intermediate step between the photoexcitation and the proton release.

Photoreceptor organelles

For the sake of efficiency in harvesting solar radiation and transducing the information coupled to the absorption of a photon, in most microorganisms the photosensors are usually organized in ordered structures, whose complexity spectacularly varies from prokaryotes to eukaryotes and within eukaryotes themselves.

In bacteria, for example, the photoreceptor molecules can be embedded into the cell membrane (as is the case of sensory rhodopsins in *H. salinarum*) or even simply located in the cytoplasm (as is the case of PYP in *E. halophila*).

In eukaryotes the wide variety of photoreceptor apparatuses ranges from the apparently simple pigment granules of *B. japonicum* and *S. coeruleus* to the elaborate eye-like structure of the ocellus of the Warnowiaceae Dinophyceae.

An exhaustive discussion of the various properties of these sophisticated subcellular structures is out of the scope of this review. It is, however, worthwhile mentioning that the combination of the structural, morphological and optical properties of the photoreceptor and of the different types of screening/reflecting organelles can be decisive for the cell to accomplish behavioural strategies and can have marked effects on the measured spectral responsiveness of the microorganism.[40]

We will focus our attention on the photoreceptor subcellular organelles of *B. japonicum* and *S. coeruleus*, the pigment granules, and of *E. gracilis*, the PFB, with which the stigma is associated.

Similarly to stentorin, blepharismin is localised in pigment granules distinct in nature from mitochondria, about 500 nm in diameter, membrane-limited and arranged in subcortical strings parallel to the ciliary rows, spread all over the cellular body.

The freeze-fracture electron micrograph of Fig. 4 (kindly provided by T. Matsuoka, Kochi University, Japan) shows the inside morphology of a *B. japonicum* pigment granule. The honeycomb-like structure (see inset) has been suggested to be made up of a folded membrane in order to harvest as much light as possible. The particles on the inner membrane have tentatively been identified as proteins of several hundreds kDa molecular weight,[41] thus supporting the hypothesis that the blepharismin binding protein could be that with a molecular weight of 200 kDa.[29,31]

Considerably more sophisticated is the photoreceptor system of *E. gracilis*, shown in Fig. 5 (kindly provided by P. Gualtieri, CNR, Pisa, Italy). The photoreceptor, the PFB, is a three-dimensional crystalline structure, about 1.0 × 0.7 × 0.7 μm, located at the base of the flagellum inside a subapical invagination. In front of the PFB, in the cytoplasm, lies the stigma, composed of red–orange lipid droplets. Whereas it has been known for a long time that the stigma droplets contain carotenoids, the nature of the pigment in the PFB is still under discussion and the contest rhodopsin *vs.* pterin-flavin is still open (see above). Here we only want to mention that, thanks to the fact that swimming cells rotate around their long axis, the stigma plays the key role of periodically shading the PFB. In this way the photic stimulus on the PFB is modulated and the light

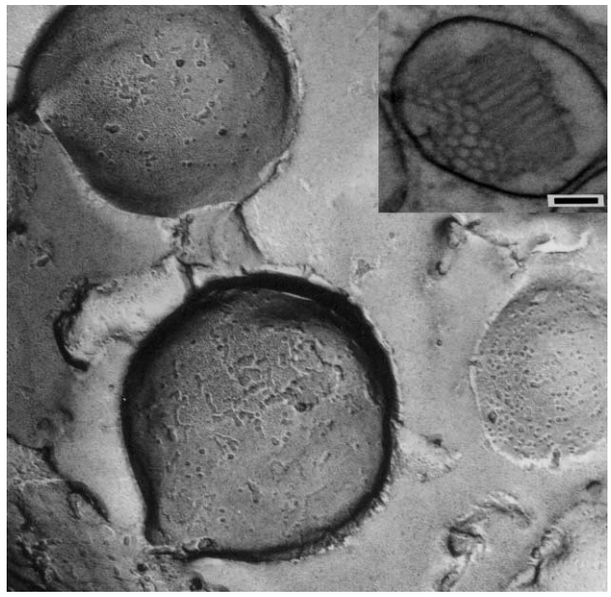

Fig. 4 Freeze-fracture electron micrograph of *B. japonicum* pigment granules; inset: TEM electron micrograph of a depigmented granule (kindly provided by T. Matsuoka, Kochi University, Japan).

Fig. 5 Scanning electron micrograph of *E. gracilis* PFB (kindly provided by P. Gualtieri, CNR, Pisa, Italy).

direction is perceived and tracked by comparing the light absorbed by the PFB at two instants in time. The ordered array of chromophores inside the PFB could finally provide the cell with the fine-tuning mechanism of perceiving the polarization plane of incident light.

The dark steps of the transduction chain

As shown in the block diagram of Fig. 1, the output signal from the photoreceptor must travel up to the motor apparatus in order to be finally converted into a cellular movement. The sequences of the transduction phases not directly dependent on the photic stimulus are called "dark steps".

In general, the events of this part of the transduction chain serve to amplify the signal without addition of noise in the system and activate feedback mechanisms, in order to extend dynamic ranges, improve the accuracy, prevent overloads as well as signal reduction and uncontrolled fluctuations.[42]

Of course, the molecular pathways of the light information transduction in prokaryotes are quite different with respect to those in eukaryotes and, in many cases, much better known, also thanks to the great amount of knowledge provided by intensive studies of chemoperception and chemotransduction in bacteria.

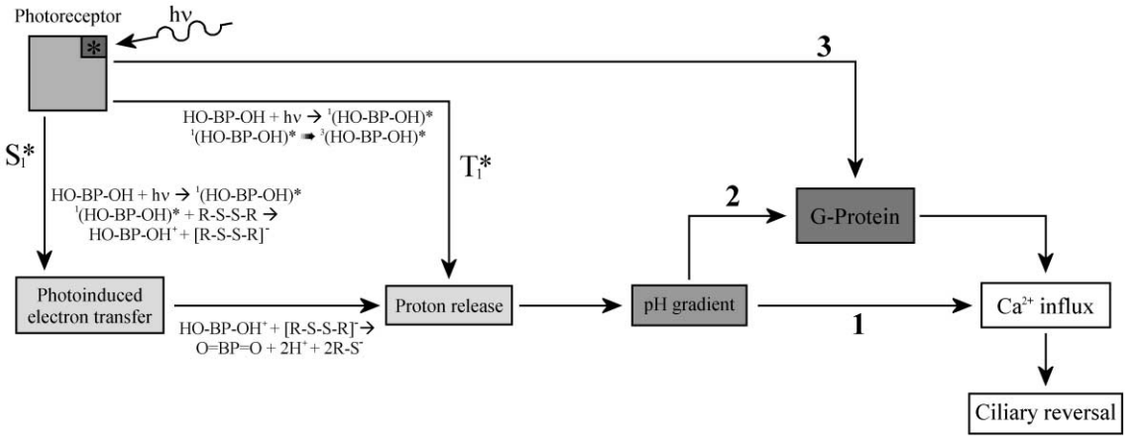

Fig. 6 Simplified scheme describing the possible dark steps in the sensory transduction chain of some ciliates (see text).

In the case of *H. salinarum*, in particular, it has been ascertained that the photosensor, the transducer and the motor switch are all inserted into the cellular membrane and the functional links among them are largely understood.[22]

For flagellated algae not very much is known. Spectroscopic evidence suggests that a cytochrome in the PFB of *E. gracilis* might be involved in light signal transduction.[13] The crystalline structure of the PFB and the lattice-like morphology of the paraflagellar rod, which runs along the emerging flagellum, led to the hypothesis of the photoelectric model. Following the light-generated photoelectric signal in the PFB, a long-range coherent displacement photocurrent would propagate along the paraflagellar rod finally causing flagellar stiffening and, consequently, a change in the swimming pattern of the cell.[43] Biochemical and immunological studies on isolated photoreceptors of *C. reinhardtii* point, instead, to the presence of GTP-binding proteins (G-proteins) as possible transducers.[44]

The case of ciliates

Ciliates are non-specialized eukaryotic cells, provided with sophisticated molecular machineries allowing them to exploit a large variety of biochemical pathways. The identification and the characterization of the molecular mechanisms underlying the sensory transduction chain are not trivial. In particular, in the case of *B. japonicum* and *S. coeruleus*, the dark steps are still largely "black boxes", notwithstanding the problem has been intensively studied.

As already discussed, many pieces of experimental evidence point to the occurrence in these protozoa of a light-induced transient intracellular pH decrease.[35,36] Furthermore, electrophysiological studies and experiments on the effect of calcium channel blockers and calcium ionophores on photomotile responses clearly indicate that calcium influx across the cellular membrane is responsible for ciliary stop and beating reversal (stop response and backward swimming).[45]

On the basis of these data a very first hypothesis was that, following light stimulation, an intracellular increase of proton concentration could lead to the opening of calcium channels, indirectly, by depolarising the membrane, or, directly, by altering the conductance of specific calcium channels (Fig. 6, path 1).

More recent results enrich and complicate this simple model, suggesting the involvement of amplification and transduction steps similar to those operating in the visual process of metazoa. The fact that in *B. japonicum* and *S. coeruleus* the light-induced ciliary beating stop occurs with a delay up to 1 second (pretty long in comparison with the milliseconds lag-time of mechanoresponses in the same organisms) has been considered indicative of specific time-limiting biochemical processes operating in the phototransduction chain.[46]

Modulators of G-Proteins have been tested on the photobehaviour of *B. japonicum* and *S. coeruleus*,[46] confirming their possible involvement in the photic signal processing of these ciliates (Fig. 6, path 2 and 3).

Other experiments carried out with molecular biology and biochemistry techniques seem to support this suggestion, indicating a partial homology between the G-proteins found in the cortex of *B. japonicum* and *S. coeruleus* and the α-subunit of transducin from photoreceptor cells of the retina.[46]

In the case of *S. coeruleus*, in particular, it has been shown that membrane permeable cGMP analogues as well as phosphodiesterase (PDE) antagonists influence specifically the photophobic response, while membrane permeable cAMP analogues have no effect.[47] In addition, patch-clamp experiments seem to indicate the presence of cGMP-dependent ionic channels in the cell membrane[48] and radioimmunoassays reveal a fast decrease in intracellular cGMP levels following light stimulation.[47] The schematic sequence presently available for *S. coeruleus* is similar to that of the visual system of vertebrates: photoexcitation of stentorin ⟶ G-protein activation ⟶ PDE activation ⟶ decrease in intracellular cGMP concentration ⟶ cell membrane depolarization (through inactivation of cGMP-dependent ionic channels).[47]

In the case of *B. japonicum*, the level of intracellular cGMP was found not to be significantly altered by light whereas the analysis of the intracellular level of the secondary messenger, inositol 1,4,5-trisphosphate ($InsP_3$), showed that light causes its temporary increase, thus suggesting it is the "real" secondary messenger. In analogy with the visual process of some invertebrates, the following scheme is presently suggested: photoexcitation of blepharismin ⟶ G-protein activation ⟶ Phospholipase C (PLC) activation ⟶ increase in intracellular $InsP_3$ concentration ⟶ cell membrane depolarization (mediated by an $InsP_3$-receptor).[49]

Among the most important questions to clarify in this framework is the mechanism of G-proteins activation in both *B. japonicum* and *S. coeruleus*. The presently available experimental results indicate that the G-protein could be activated by the intracellular pH variation following the release of protons from the photoreceptor (Fig. 6, path 2), but a direct interaction between the excited photoreceptor and the G-protein (Fig. 6, path 3) cannot be discarded, even though its nature is currently definitely unknown.

Another point that should be clarified is where these G-proteins are precisely located into the cells, as the cortex comprises also the pigment granules and the plasmatic membrane.

Conclusions

From this review the richness of the chromophore molecular structures and pockets and of the "dark steps" involved in

microorganisms photosensory perception and transduction should be apparent. An example is provided by the evolutionary close ciliates *B. japonicum* and *S. coeruleus*, in which perceptible differences are found in all the components of the transduction chain (from the chromophores to the secondary messengers).

Such a variety of structures and mechanisms make it, at present, impossible to provide any unitary description of the realm of these phenomena. The question is then authorized if the cause of such a multiplicity of interpretations is a consequence of a low level of knowledge and understanding or if it is an intrinsic feature of these natural phenomena.

It should also be taken into account that light is not the only environmental signal affecting the behaviour of microorganisms, and their sensory responses result from the integration of "internal needs" (such as, for instance, metabolism and cell cycle) with a diversity of external stimuli (chemical, mechanical, gravitational, photic. . .). Integrated investigations of their motile responses to different environmental signals (chemical and photic, for example) might, therefore, offer clues for a deeper understanding of sensory processes in microorganisms.

In conclusion, we think an intensive and multidisciplinary study of as many photosensing microorganisms as possible is desirable, planned and conducted with the aim of finally being able to frame photosensory transducing systems in an evolutionary perspective, which could facilitate revealing these studies as something more than "an arcane pursuit with little significance to problems of the real world".

Note added in proof

Iseki *et al.*[50] have recently discovered and biochemically characterized a new type of blue-light receptor flavoprotein, photoactivated adenylyl cyclase, which mediates the step-up photophobic response of *Euglena gracilis*.

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