Spectral Tuning in Biology I: Pigments

9

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9.1 Introduction

The justification for a book about photobiology rests partly on combination of various specialities for interdisciplinary comparisons. One topic suitable for comparisons is "spectral tuning." By this we mean the principles for how spectra of pigments, and factors that can modify their spectral responses, are adjusted to the needs of the organisms that produce them. A number of examples will be found in this chapter.

To pigments, substances that produce color by absorbing light of some wavelengths and reflecting or transmitting the rest, we must add a second class of color mechanism, *structural* colors. These are produced by the interaction of light with the detailed architecture of the material or structure on which it falls. We will begin with a discussion of biological pigments and in the next chapter move on to biological structural colors. We will also discuss some additional mechanisms by which organisms control their spectral presentation to the world.

Spectral tuning is relevant for vision (not only for color vision), photosynthesis, bioluminescence, flower colors, and adaptive coloration of animals, and especially for animals that move around among green plants, to increase contrast of edges. These processes are not independent. Flower colors are adapted to the vision of pollinators and as a contrast to photosynthetic pigments. Bioluminescence and vision have evolved together. Phytochrome has evolved to discriminate between direct daylight and light modified by chlorophyll absorption. The basis is the spectrum of the sun. To begin with, let us see what the relation is between the spectrum of the most important of all pigments, chlorophyll *a*, and the spectrum of the sun.

9.2 Why Are Plants Green?

Many people have discussed the spectrum of chlorophyll in relation to daylight. Some have come to the conclusion that they do not match well, as absorption "in the middle of the spectrum," i.e., the green band, is weak. A common idea is that an ideal pigment for photosynthetic energy conversion ought to be either black, absorbing all available radiation, or absorb most efficiently at the "peak" of daylight.

But what is the "peak" wavelength for daylight? The maximum of the daylight spectrum depends on how we plot it. For the present purpose, to simplify comparisons and calculations, we may represent the daylight spectrum by that of a 6,000 K blackbody radiator and thus apply Planck's radiation law (Chap. 1). We can then calculate that if we plot the spectrum as energy per uniform wavelength interval, the maximum is at 480 nm. But if we instead plot it as photons per uniform wavelength interval, the maximum is at 600 nm, and if we, following the habits of physicists, plot the spectrum as energy per uniform frequency interval, or photons per uniform frequency interval, the peak will be seen at frequencies corresponding to 800 nm and 1,200 nm, respectively.

Thus, the "maximum of the daylight spectrum" is an ambiguous concept, and we have to find another way of optimizing our pigment. As for the idea that an ideal pigment should absorb everything, we should remember that the better a substance absorbs, the better it emits, and the transformation of radiant energy into other energy forms is just the balance between absorption and reradiation. That total absorption is not an ideal is even more apparent in the case of color vision. Vertebrate cones, which are cells receiving light signals for color vision, are shorter than the rods involved in "noncolor night vision" (scotopic vision) and therefore absorb a smaller portion of the light and discriminate between wavelength bands better than they would if they were as long as rods.

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Photosynthesis depends on photochemistry, and photochemistry works particle to particle, photon to molecule. The useful energy storage can be regarded as the product of the number of reacting photons and the free energy that each converted photon contributes. Björn (1976) following this principle comes to the conclusion that the long-wave absorption band of a pigment giving maximum energy conversion in direct sunlight should be rather narrow and have a maximum at 707 nm. Furthermore, the pigment should be highly fluorescent (when not quenched by photochemistry). The maximum chemical potential difference that can be created by a one-step system is

$$\mu_o = kT \cdot \ln\left[\left(\Phi r^2 / 4R^2\right)\right] + h\nu_o\left(1 - T / T_S\right) - b^2\left[h^2/2\mathrm{k}\right]\left]T\left(1/T^2 - 1/T_s^2\right)\right.$$

where k = Boltzmann's constant, h Planck's constant, T ambient temperature, T_S the temperature of the radiating surface of the Sun, Φ the fluorescence yield, r the radius of the Sun, R the Earth-Sun distance, ν_o the frequency of the spectral peak of the absorption band, and b a parameter determining the width of the absorption band such that the half-band width is $2b\sqrt{(2\ln 2)} = 2.35b$. With numerical values inserted, this becomes $\mu_o = -0.342$ eV + (19/20) $h\nu_o$–$6.6.10^{-28}$b^{2}eV s^2 (eV stands for electron volts). The effective chemical potential difference under conditions of maximum energy conversion is 0.13 eV lower than that (just as the voltage of an electrical battery is lowered when power is drawn from it).

All this is for full sunlight. The optimum position of the absorption peak is lowered by 12 nm for every tenfold decrease of fluence rate, even if the spectrum of the daylight is not changed. Thus, it appears that the long-wavelength band of chlorophyll *a* in vivo is rather well matched to the conditions of our planet. The "blue" absorption band of chlorophyll (the Soret band) does not contribute to chemical potential, but leads to increased photon absorption and thus increased energy conversion, as do various accessory pigments.

Milo (2009) using partly different and refined arguments and seeking the optimum center wavelength of the reaction center for a system utilizing pigment antennae to feed the center with energy arrive at essentially the same result. The most refined analysis today of the reason that the spectra of photosynthetic systems have evolved to the properties that they have is that of Marosvölgyi and van Gorkom (2011). They have taken into account also the energetic cost of the construction of the pigments and can explain also the long-wavelength spectral band of chlorophyll *b* and the spectra of bacteriochlorophylls. By using real daylight spectra rather than a blackbody approximation of the solar spectrum, they also find that the optimum wavelengths are hardly dependent at all on photon fluence rate.

Mauzerall (1976) has speculated on how chlorophylls can have evolved from porphyrins along a path of increasing lipophilicity, making them suitable for incorporation into membranes. Björn et al. (2009) have concentrated their discussion specifically on the question of why chlorophyll *a* has been chosen as the key pigment for oxygenic photosynthesis. With the discovery that chlorophyll *d* can replace chlorophyll *a* not only in pigment antennae, but also in reaction centers, the latter has much of its uniqueness.

Various types of bacterial chlorophylls have absorption bands at longer wavelengths. They are not adapted to direct daylight, but to the light that penetrates down to the places (such as anoxic sediments below algae absorbing shorter-wavelength light) where these bacteria live. The record in long wavelengths is held by bacteriochlorophyll *b*. Its spectrum peaks at 1,020 nm, beyond two infrared absorption bands of water.

Kiang et al. (2007) and Stomp et al. (2007) have detailed how various photosynthetic pigments are adapted for different environments on Earth.

9.3 What Determines Spectra of Pigments?

Generally speaking, the absorption spectrum of a pigment is determined by (1) the structure of the chromophore(s) and (2) the environment of the chromophore.

The most important feature in chromophore structure is the arrangement of conjugated double bonds (alternating single and double bonds), i.e., the π electron clouds. The environment of the chromophore in many cases consists of, or is at least dominated by, a protein to which the chromophore is bound, but there are also important cases, such as vacuolar flower pigments, where the chromophore is not protein bound, and other factors are the main concern.

In general, the absorption peak with longest wavelength for a molecule with conjugated bonds increases with the length of the conjugated bond system. It depends, as we shall see, also very much on the shape of the conjugated system, whether it is straight or not and, in the case of macrocycles as in chlorophylls, on its symmetry. If conjugation is broken by single bonds and thus divided into two conjugated systems, the absorption spectrum is similar to the sum of the contributions from the two systems.

As a simple example of the effect of conjugated system length, let us consider a series of polyene hydrocarbons, $CH_2 = CH–(CH = CH)_{n-2}–CH = CH_2$, with n conjugated double bonds. Such a simple and regular system is fairly well understood. Energy levels for the ground state and the first excited state can be computed, and the wavelength of the long-wavelength absorption maximum corresponding to their difference determined. Two common approaches are the free electron (FE) theory and the linear combination of atomic orbitals (LCAO) method. In the former the Schrödinger

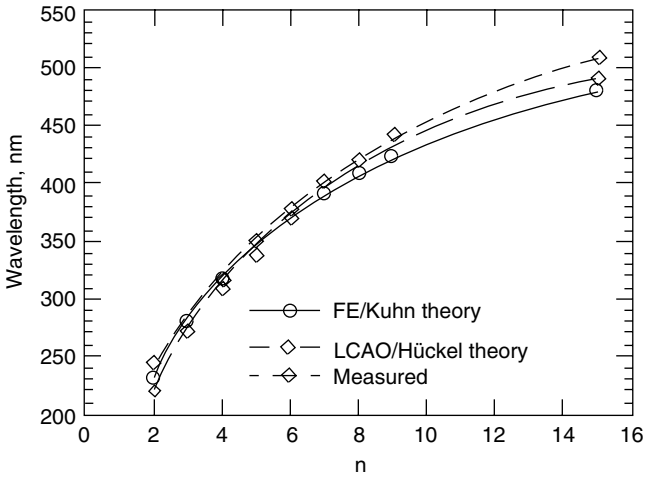

Fig. 9.1 The wavelength of the absorption maximum with longest wavelength for a series of polyenes with formula CH_2 = CH–(CH = CH)$_{n-2}$–CH = CH_2. The graph shows experimental values, as well as values calculated by two different methods, the Kuhn version of the free electron theory, and the Hückel version of the linear combination of atomic orbitals theory

equation is applied to a "gas" of π-electrons in a "box potential," i.e., a potential which is constant over an interval corresponding to the length of the conjugated system and zero outside of it. In the simplest version of this, the wavelength of the maximum would vary linearly with the length of the conjugated system, i.e., with n. However, one must consider that the length of the bonds between carbon atoms is not constant (every second bond is longer, and there are "edge effects"), and therefore, the potential is not constant in the "box"; and Kuhn has developed a method to allow for this, the result of which is shown in Fig. 9.1 (another complication, as we shall see later, is that the chain is not straight and may be folded in various ways). In the LCAO method, one starts by computing the orbitals of the individual atoms, and then sums up their wave functions. In a refined version due to Hückel, one allows for the variation in bond length. Both methods give quite good results for low values of n but underestimate the wavelength for large values of n. The reader interested in more information on these computational methods is referred to a textbook on electron spectra of organic molecules, such as that by Jensen and Bunker (2000) or the still very readable one by Murrell (1963).

The carotenoids, and the retinals of visual pigments, are biologically important molecules resembling polyenes. The principle of spectral tuning by variation of the conjugated bond system is beautifully exploited by plants in the regulation by the xanthophyll cycle of energy intake for photosynthesis (see Sect. 28.11). Also, the phycobiliproteins show a change in wavelength position related to the length of the conjugated system, but we shall see also that other factors are important for the tuning. A complication with simple polyenes and carotenoids is that the first excited state is "optically forbidden" or "dipole forbidden," i.e., cannot be reached from the ground state by light absorption (Schulten and Karplus 1972; see also Sect. 1.19). The absorption spectrum in the daylight region is due to transition to the second excited state (or, for carotenoids, the third excited state). The first excited state can still, for some carotenoids, participate in energy transfer to chlorophyll and make this very efficient (Thrash et al. 1979; Ritz et al. 2000), while for other carotenoids, it is too low (Polívka et al. 1999; but see Frank et al. 2000).

9.4 Relation Between the Absorption and Molecular Structure of Chlorophylls

Chlorophylls can be classified into three main groups, depending on whether the nucleus is that of porphin with 11 conjugated double bonds, dihydroporphin with 10, or tetrahydroporphin with 9 conjugated double bonds. In Fig. 9.2 it is shown which chlorophylls belong to each group, as well as the positions in organic solvent (in most cases ethyl ether) of their main absorption bands.

As is evident from Fig. 9.2, the long-wavelength transition, called Q_y, corresponds to a smaller energy change in the tetrahydroporphin type bacteriochlorophylls than in the dihydroporphin type pigments and to a smaller energy change in the dihydroporphin pigments than in the porphin pigments. Even in the porphin pigments, there is a certain asymmetry (not shown in Fig. 9.2) due to the side chains, so even in these one can distinguish between Q_x and Q_y transitions. Q_x and Q_y transitions can be distinguished through measurements of polarization of fluorescence excited by plane polarized light. Another way is to align the molecules in some way, for instance, in thin films, and study the absorption dichroism.

But the effects due to the chromophore environment are sometimes even larger than the effects of the differences in the conjugated double-bond system. Thus, the in vivo environment (mainly the bonding to protein) changes the Q_y band as shown in Table 9.1.

Those chlorophylls having the smallest energy gaps and the absorption bands at longest wavelengths are the reaction center special pairs (for exceptions, see Sect. 9.5). Their special absorption properties are due to formation of exciton complexes (see Chap. 1).

The reader who wishes to understand chlorophyll absorption spectra in more detail is referred to Linanto and Korppi-Tommola (2000). The theory of electronic spectra of organic molecules and the computational methods used to understand the spectra are treated in many books, e.g., Murrell (1963).

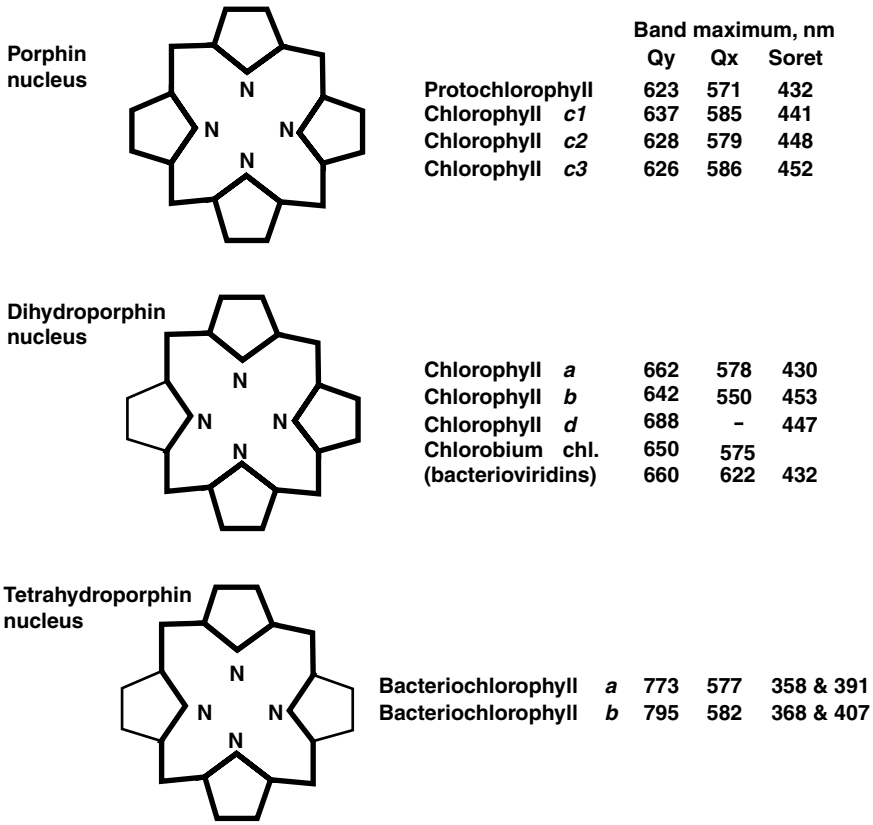

Fig. 9.2 The ring systems for the three main classes of chlorophylls. These ring systems are flat. The conjugated double-bond systems are indicated by heavy lines. While the conjugated system is rather isodiametric (approximating circular form) for the porphins, it is more elongated for the other two groups. The long axis of this elongated system is often referred to as the *y*-axis, and the transition moment for the so-called Q_y transition lies along this axis. The *x*-axis, and the direction of the Q_x transition, is almost perpendicular to this but also in the plane of the ring. The short-wavelength "Soret" band is complex with transitions along both axes. For instance, for bacteriochlorophyll *a* the 358 Soret component is in the *y*-direction and the 391 component in the *x*-direction

Table 9.1 Comparison of absorption spectra of chlorophyll pigments position of long-wavelength (Q_y) band (nm)

Pigment	In ethyl ether	In vivo
Protochlorophyll	624	630–650
Chlorophyll *a*	662	670–700
Chlorophyll *b*	644	650
Chlorophylls *c*	626–637	
Bacteriochlorophyll *a*	773	800–890
Bacteriochlorophyll *b*	795	1,020
Bacteriochlorophyll *c*		725–750

9.5 Tuning of Chlorophyll *a* and *b* Absorption Peaks by the Molecular Environment

Chlorophyll *a* is the key pigment in almost all organisms with oxygenic photosynthesis, i.e., cyanobacteria, algae, and plants. It functions in both reaction centers I and II and as antenna pigment in both photosystems. A great number of spectral forms can be distinguished in vivo and in thylakoid preparations.

In organic solvents the long-wavelength absorption peak of chlorophyll *a* lies at 662 nm (ethyl ether) to 663 nm (acetone), when the solution is dilute and the chlorophyll in a monomeric state. In vivo all chlorophyll a fractions are "red shifted," i.e., shifted to longer wavelengths compared to this. Some of the antenna chlorophylls in photosystem II (PSII) have the smallest shift, while a few molecules in photosystem I have the largest shift and, perhaps surprisingly, have peaks at even longer wavelength than photosystem I (PSI) reaction center chlorophyll (P700).

Cinque et al. (2000) were able to specify the spectra of two particular antenna chlorophyll chromophores in maize by comparing recombinant chlorophyll proteins lacking chromophores present in the wild type. They found that the long-wavelength (Q_y) peak of CP29 chlorophyll *a* is at 680 nm and that of LHC II chlorophyll *b* at 652 nm. Thus, the red shifts (bathochromic shifts) compared to solution in, e.g., ethyl ether are very different. However, the overall shapes of the two spectra are very similar in organic solution and protein environments.

Some chlorophyll species are extremely red shifted in vivo. Halldal (1968) discovered that the green alga *Ostreobium*, growing inside a coral on the Great Barrier Reef, contained unusually large amounts of such long-wavelength chlorophyll, "C_a720." It seemed to be an adaptation to the unusual environment of this organism. Other algae growing outside *Ostreobium* filtered away much of the daylight and left mostly far-red light for *Ostreobium* to use. Halldal asked his student Öquist to find out whether a more "ordinary" alga, *Chlorella*, would also be able to adapt to far-red light by forming more long-wavelength chlorophyll, and to some extent it could (Öquist 1969). But some

species of *Ostreobium* are exceptional and are still attracting the interest of scientists. Although most of the long-wavelength chlorophyll is associated to photosystem I, there seems also to be long-wavelength chlorophyll in photosystem II (Fuad et al. 1983; Zucchelli et al. 1990; Koehne et al. 1999). Investigators are puzzled by the fact that these pigament forms seem to be able to deliver energy to the reaction centers, which have absorption spectra peaking at shorter wavelengths and were thought to require higher energy quanta. The explanation may be, as in the case of dragonfish vision, that thermal quanta deliver the extra energy needed. This need not violate the second law of thermodynamics if the whole photosynthetic system is considered.

Some of the recent and detailed investigations have been done on cyanobacteria. It has been shown that also in this case the long-wavelength forms, at physiological temperatures (but not at very low temperature), can transfer absorbed energy to P700 (Pålsson et al. 1998). The main long-wavelength absorption peaks of these long-wavelength forms are variously given as 708 and 719 nm (Pålsson et al. 1998) and 705, 714, and 723 nm (Kochubey and Samokhval 2000), both for *Synechococcus elongatus*. For *Synechocystis* sp. PCC 6803, only a single long-wavelength form peaking at 710 nm was identified (Gill and Wittmershaus 1999), possibly due to methodological differences. The 719 nm form seems to arise from the 708 nm form when monomers of PSI are combined to the trimers present in vivo (Pålsson et al. 1998; Jordan et al. 2001). In a structural model (Jordan et al. 2001), for PSI the 710/719 nm chlorophyll is tentatively identified with the aC-A32/aC-B7 molecule pair, which seems to connect the monomers energetically. Thus, the long-wavelength band may arise by excimer splitting, just as in a long-wavelength spectral form of phycocyanobilin. However, Koehne et al. (1999) have obtained evidence that at least some long-wavelength forms in the eukaryotic alga *Oestrobium* have another origin.

To date the long-wavelength record for chlorophyll *a* tuning is held by the cyanobacterium *Spirulina platensis*, which has a form peaking at 738 nm (Shubin et al. 1991; Koehne and Trissl 1998; Karapetyan et al. 1997).

P700 of photosystem I as well as P680 of photosystem II are both dimers (within the photosystem monomer), with the tetrapyrrols closely stacked in a parallel manner and with tightly overlapping π orbitals, forming excimers. It is thus not difficult to understand that there is a large red shift from the absorption peak of chlorophyll *a* in dilute organic solution.

Carotenoids may be important for the spectral fine tuning of some other chlorophyll forms. Several of the carotenoids in PSI show extended overlap of their π orbitals with those of chlorophyll molecules (Jordan et al. 2001).

For cyanobacterial PSII a structural model has been published by Zouni et al. (2001). According to this model, $Chlz_{D1}$ and $Chlz_{D2}$ are identified with chlorophyll *a* bound to histidine in polypeptides D1 and D2. These chlorophylls have absorption peaks at 675 nm (Schelvis et al. 1994).

9.6 Phycobiliproteins and Phycobilisomes

In hardly any case is spectral tuning more important and critical than in photosynthetic antenna pigments. Light is absorbed by one chromophore and transferred over a series of other chromophores to photosynthetic reaction centers. The most common method for energy transfer is the Förster mechanism. For this to be efficient, the emission spectrum of the energy donor must in each step match the absorption spectrum of the receiver.

There are many kinds of antenna pigments in various organisms: chlorophylls, carotenoids, pteridines, and phycobiliproteins. We shall concentrate here on phycobiliproteins, which occur in cyanobacteria and several groups of algae, most important of which are the red algae. The description below will primarily reflect the conditions in cyanobacteria.

Cyanobacteria use several principles for spectral tuning, the first of which is variation of the structure of the chromophores (bilins). Different lengths of the conjugated bond system results in different transition energies: the greater the length, the lower the energy. The types of chromophores known are shown in Table 9.2 and their distribution among proteins in Table 9.3.

When the chromophores attach to proteins (which takes place via sulfur bridges at one or two points), two changes are immediately apparent: The absorption bands become sharper, and the intensity of the long-wavelength band increases in comparison to those at lower wavelengths. The sharpening of the bands takes place because the conformation of the chromophore, which in the free form is very

Table 9.2 Number of conjugated double bonds and absorption maxima for the phycobilins

Phycobilin	Number of conjugated double bonds	Absorption maximum in free form (nm)	Absorption maximum in phycobiliprotein
Phycourobilin (PUB)	5		495
Phycoerythrobilin (PEB)	6	530	545–565
Phycoviolobilin (PVB)	7		510–570
Phycocyanobilin (PCB)	8	600	610–671

Table 9.3 Occurrence of phycobilin chromophores among cyanobacterial phycobiliproteins and their *a* and *b* peptides

	On *a* peptide	On *b* peptide
Allophycocyanin	1 PCB	1 PCB
C-Phycocyanin	1 PCB	2 PCB
Phycoerythrocyanin	1 PVB	2 PCB
R-Phycocyanin II	1 PEB	2 PCB
Phycocyanin WH8501	1 PUB	2 PCB
C-Phycoerythrin	2 PEB	2 PEB
CU-Phycoerythrin (1)	3 PUB	1 PEB + 1 PUB
CU-Phycoerythrin (2)	1 PEB + 2 PUB	2 PEB
CU-Phycoerythrin (3)	3 PUB	2 PEB
CU-Phycoerythrin (4)	2 PEB + 1 PUB	2 PEB + 1 PUB

Source: After MacColl (1998), slightly simplified. For corresponding information for rhodophycean phycobiliproteins, see Table 1 of Holzwarth (1991), and for cryptophycean (cryptomonad) phycobiliproteins Glazer and Wedemayer (1995)

flexible and therefore distributed over a large number of conformational states, becomes restricted. The relative intensification of the long-wavelength band takes place because the chromophore becomes more straight, while in the free state, it is, on average, more circular, as one turn of a helix (Scheer and Kufer 1977; Knipp et al. 1998), with overlapping ends. The spectrum therefore is in this case more "porphyrin like," with a pronounced Soret-type band. Another effect of the binding to protein is that the position of the long-wavelength band is shifted, to very different extents in different cases which will be described below.

The chromophore is not fixed in the protein with complete rigidity. Of particular interest are cases in which the conformation of the chromophore can change under the influence of light, causing photochromicity of the chromoprotein, analogous to the behavior of phytochrome. The behavior was first noticed by Scheibe (1972) in an extract containing phycocyanin. The phenomenon was further examined in a number of papers by G.S. Björn, summarized by G.S. Björn (1980), L.O. Björn (1979), and Björn and Björn (1980). The experiments were mostly carried out on extracts, but in one case (G.S. Björn 1979), photoreversible photochromism was shown to occur also in vivo. In this case irradiation with light of 505 nm results in a decrease of absorption at this wavelength and an increase at 570 nm, while irradiation with 570 nm light reverses this effect, and the reaction can be repeated over and over again.

This particular case has been further explored by other researchers, in particular the group around H. Scheer in Germany (Zhao et al. 1995; Zhao and Scheer 1995; see also Ohad et al. 1979; Scharnagl and Fischer 1993). It has been found that the change in absorption spectrum is primarily due to rotation around a double bond between carbon atoms 15 and 16 in a phycoviolobilin chromophore in the a subunit of phycoerythrocyanin.

How rigidly the chromophore is held by the protein depends partly on the covalent (thioether) bonds between chromophore and protein. The bonds may go from either the A ring or the D ring, or both, and this affects the spectral properties. The extent to which the chromophore is stretched and kept rigid also affects another property of great importance for the function, namely, the excited state lifetime. A phycobilin chromophore which is not fixed in a protein has great flexibility, which gives greater possibilities for thermal relaxation, i.e., shorter lifetime and less efficient energy transfer and photochemical efficiency. This is in contrast to what is the case with chlorophylls, which are already in the free state rigid structures. The attachment to protein also favors a protonated state, which also lengthens the excited state life.

The ordered arrangement of chromophores in a protein matrix affects the chromophore spectrum by one more mechanism. It keeps certain chromophores in the close vicinity of one another, which results in the formation of exciplexes. As described in Sect. 1.17, this splits the energy levels of the isolated chromophores in a higher and a lower level, in turn splitting the absorption bands in a corresponding manner.

Thus, even though cyanobacterial phycocyanins and allophycocyanins all contain only a single type of chromophore, namely, phycocyanobilin, they exhibit a wide range of absorption bands, peaking from 620 nm in C-phycocyanin to 671 nm in allophycocyanin B and also in the "terminal linker polypeptide" in the center of the phycobilisomes, close to the chlorophyll in the thylakoid membranes.

Phytochromes constitute a quite different type of phycobiliproteins, which have a light-sensing function (Chaps. 11, 12, 13 and 19). Plant phytochromes, which are those studied in most detail and that contain phytochromobilin (closely related to phycocyanobilin) as chromophore, are interconvertible between two forms of which one has evolved to absorb maximally near the absorption maximum of chlorophyll, while the other one absorbs maximally just outside the chlorophyll absorption. Therefore, they are well suited for detecting the change in light spectrum caused by the presence of competing plants. Phytochromes of nonphotosynthetic bacteria, on the other hand (whose exact biological function is yet to be explored), contain a different type of phycobilin chromophore and absorb at longer wavelengths (Bhoo et al. 2001).

9.7 Spectral Tuning of Phycobilisomes: Chromatic Acclimation in Cyanobacteria

What we shall be dealing with in this chapter has traditionally been called chromatic adaptation, but in modern terminology, adaptation is hereditary and evolutionary adjustment to the environment. What we shall describe now is the nonhereditary adjustment to the spectrum of the surrounding light, and

this should for consistency be called chromatic acclimation. By this we mean, in a photosynthesis context, the ability of organisms to adjust their photosynthesis apparatus, and in particular the amounts of various light-harvesting pigments, to match the spectral composition of available light (Kehoe and Gutu 2006). (The term can also be used in vision science.) The phenomenon has been studied for a long time, but we shall focus here on recent developments.

Cyanobacteria have protein complexes called phycobilisomes attached to the outside of their photosynthetic membranes (thylakoid membranes). They are composed of phycobiloproteins serving to gather light energy that is efficiently channeled to the photosystems (mostly to photosystem II). There are several kinds of phycobiliproteins, but in the following, we shall focus on the blue (red-light-absorbing) phycocyanins and the red (green-light-absorbing) phycoerythrins of cyanobacteria. Light energy absorbed in phycoerythrin is transferred to phycocyanin and from there via another phycobiliprotein, allophycocyanin, to chlorophyll *a* in the thylakoid membrane.

Some other photosynthetic organisms are also capable of chromatic acclimation, but it is most dramatically obvious in some cyanobacteria. All cyanobacteria do not exhibit chromatic acclimation, but in all major phylogenetic groups of cyanobacteria, some members do. de Marsac (1977) divided cyanobacteria into three groups with respect to their ability in this respect:

Group I, not capable of chromatic acclimation

Group II, able to vary phycoerythrin content but not phycocyanin content

Group III, able to vary both phycoerythrin and phycocyanin contents

Gutu and Kehoe (2012) use the symbolism CA1, CA2, and CA3 for the corresponding variants of chromatic acclimation.

Of these, group III has, not surprisingly, attracted most interest from researchers (Fujita and Hattori 1962; Ohki and Fujita 1978), and during recent years, the mechanism has, to a large degree, been clarified for some cyanobacteria. However, it is also clear that the mechanism is not the same for all cyanobacteria. Cyanobacteria are well equipped with photoreceptors. In a single species there may be many different phytochrome-type proteins with GAF domains carrying bilin chromophores (Ma et al. 2012), but also rhodopsins can be involved in the regulation of photosynthetic pigment antennae (Irieda et al. 2012). We shall return to group III in the following, but first complete the picture with a couple of other kinds of chromatic acclimation discovered more recently.

Palenik (2001) found that at least some *Synechococcus* strains are able to increase their absorption of blue light when there is more of this available than light of longer wavelength. The effect was further studied by Shukla et al. (2012) and by them named CA4 (type 4 chromatic acclimation). These cells, when grown in blue light, increase their content of the chromophore phycourobilin at the expense of phycoerythrobilin, simply by isomerizing the latter to the former (Fig. 9.3) without changing the protein to which the chromophore is bound. This changes the absorption spectrum of the biliprotein. An isomerase, called MpeZ, catalyzing this reaction has also been found. The photoreceptor regulating the amount of isomerase is so far not characterized, but is likely to be a cyanobacteriochrome.

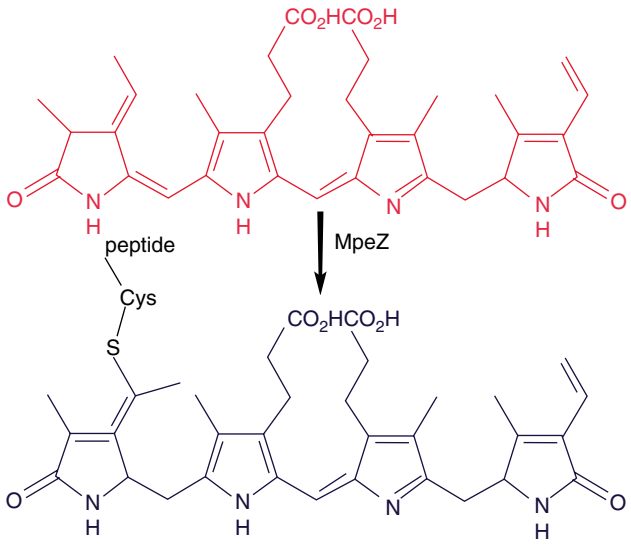

Fig. 9.3 The isomerization of phycoerythrobilin to phycourobilin. Note that the conjugated double-bond system is longer in phycoerythrobilin (*top*) (From Shukla et al. 2012)

Another type of chromatic acclimation that we can call type 5 (CA5) has been found by Duxbury et al. (2009). This is the ability of the strain of *Acaryochloris marina* (Chen et al. 2010; 2012; Chen and Blankenship 2013; Loughlin et al. 2013) to adjust the content of phycocyanobilin in relation to chlorophyll *d*, depending on the amount of orange or far-red light available.

We shall focus here on the best understood case, the type III acclimation of *Fremyella diplosiphon* (Fig. 9.6). Absorption spectra, visual appearances, and phycobilisome structures of this organism grown in red and green light are shown in Fig. 9.4. The photoreceptor that senses the light for this regulation is a cyanobacteriochrome called RcaE (Kehoe and Grossman 1996; Grossman and Baya 2001).

The photoreceptor for this regulation is a cyanobacteriochrome of the type that has only one cysteine link between the protein and the bilin (in this case phycocyanobilin). The switch between the two forms involves not only an isomerization around a double bond (15) from the 15Z configuration for the green-absorbing Pg form to the 15E configuration for the red-absorbing Pr form, but also a protonation when going from Pg to Pr. It is the protonation that causes most of the spectral change, not the isomerization. Pg is the active form,

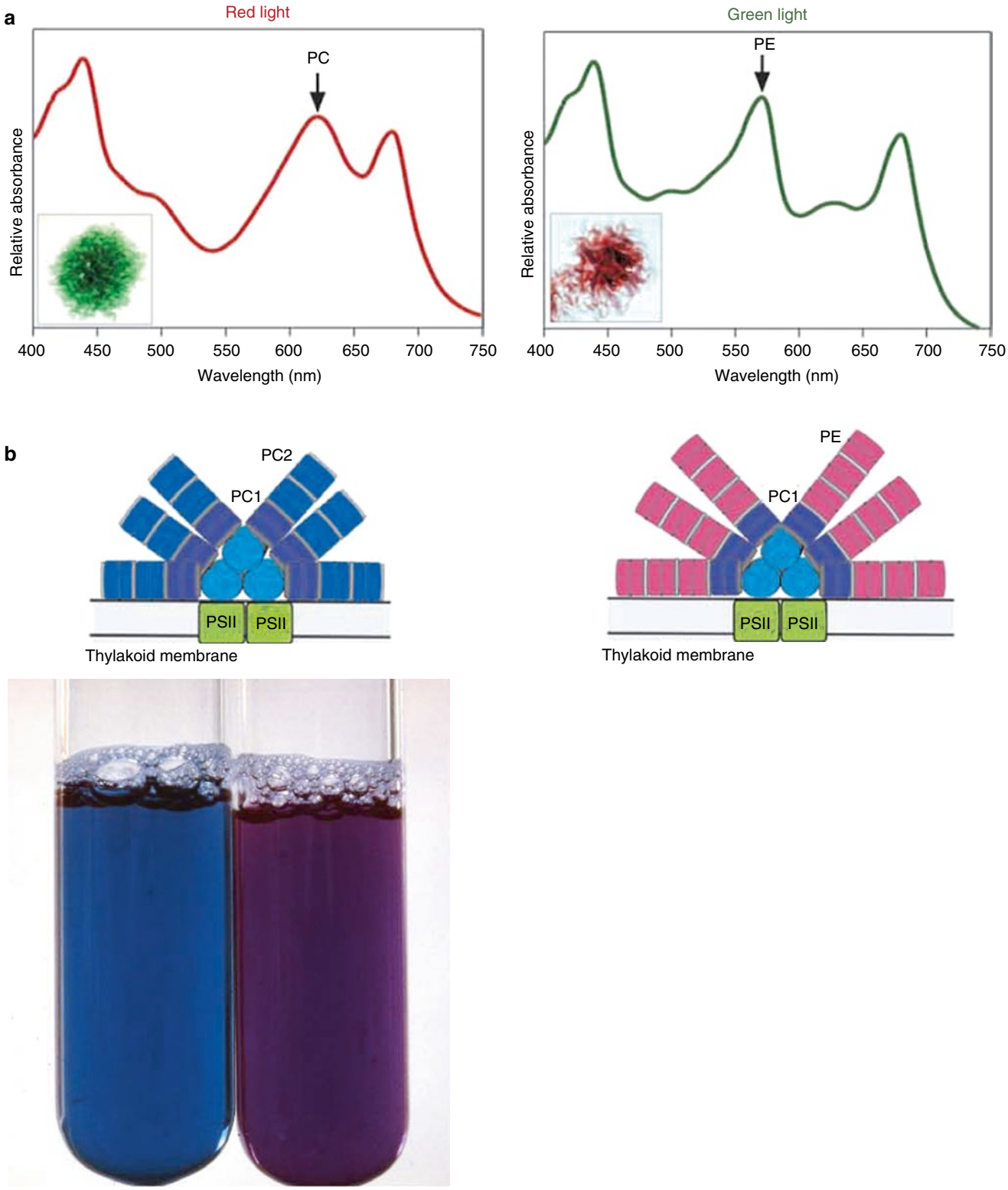

Fig. 9.4 (**a**) *Above*: Whole-cell absorption spectra of *Fremyella diplosiphon* grown in red (*left*) and green (*right*) light (*inset*, photos of the cultures). *Below*: Models of the corresponding phycobilisomes. PC1 and PC2, phycocyanins 1 and 2; PE, phycoerythrin; light blue, phycobilisome core with allophycocyanin; linker proteins not shown (From Gutu and Kehoe 2012). (**b**). Aqueous extracts of *Fremyella diplosiphon* grown in green (*left*) or red (*right*) light (Courtesy Gunvor Björn)

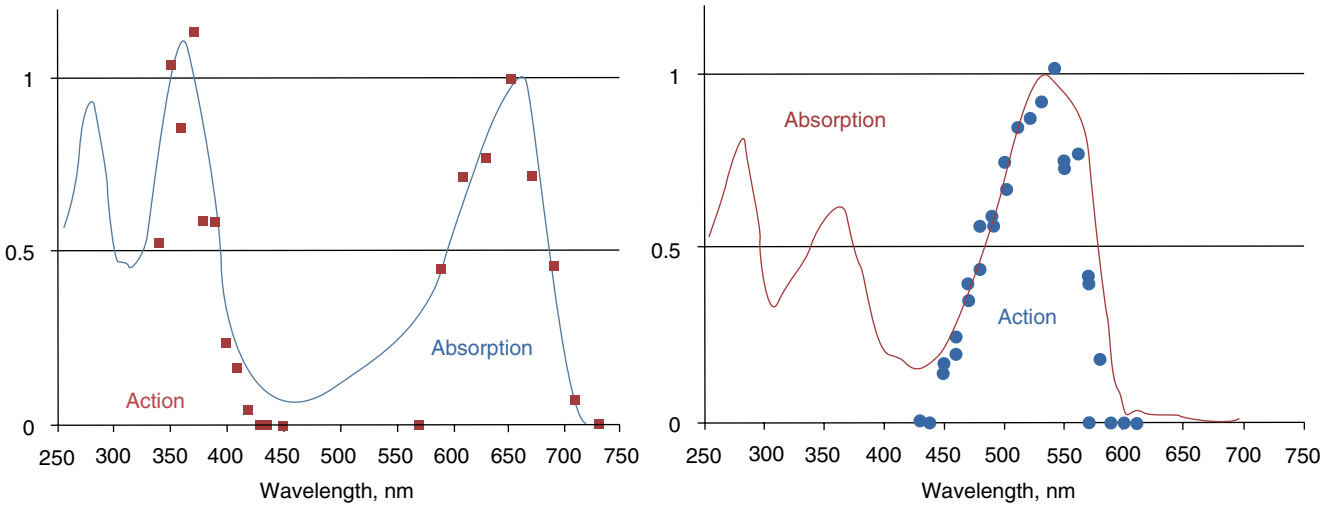

Fig. 9.5 The action spectrum for induction of phycoerythrin synthesis (*red squares*) and for its inhibition (*blue circles*) from Vogelmann and Scheibe (1978) compared to the Pr form (*left panel*, *blue line*) and the Pg form (*right panel*, *red line*) of RcaE (Hirose et al. 2013). All data are normalized to unity at the long-wavelength maximum

having histidine kinase activity (autophosphorylation in a first step, followed by transfer of a phosphate group to the protein RcaC via another one, RcaF). The phosphorylated form of RcaC inhibits transcription of phycoerythrin genes and increases transcription of phycocyanin genes (see detail in Gutu and Kehoe 2012). It is instructive to compare the action spectra for chromatic acclimation in *F. diplosiphon* (Vogelmann and Scheibe 1978) with the absorption spectrum for purified cyanobacteriochrome RcaE (Hirose et al. 2013) in Pr and Pg forms (Fig. 9.5).

We see from Fig. 9.5 that action and absorption spectra follow each other in the main bands around 350, 550, and 650 nm. However, between 420 and 570 nm, the action spectrum for induction of phycoerythrin synthesis drops to zero, while the corresponding absorption spectrum does not. This can be explained by the high rate of the reverse reaction in this spectral region. In the same way, one can understand that the action spectrum for inhibition of the induction drops to zero at short wavelengths, where the opposite reaction becomes important.

Let us try to express this a little more quantitatively. Since it is known now that it is the Pg form that is the active form, which inhibits phycoerythrin synthesis and promotes phycocyanin synthesis, let us try to model the effect of its formation.

Let us assume, based on Fig. 2 in Vogelmann and Scheibe (1978), that the span of PE/(PE + PC) after green-light treatment is from 0 to 0.6. Then halfway between is 0.3 and the corresponding log(fluence)/(J m^{-2}) ca 2.4 and thus the half-saturating 550 nm photon fluence ca 10^{-3} mol m^{-2}. This gives us the order of the action cross section as 1,000 m^2 mol^{-1}. The action cross section is the absorption cross-section times the quantum yield. The absorption cross section in turn is the absorption coefficient multiplied by ln(10), the latter factor coming from the fact that spectrophotometric absorption coefficients are based on decadic logarithms, while natural logarithms have to be used in kinetic computations.

From Fig. 3 in Vogelmann and Scheibe (1978), it appears that the reversal of PE induction saturates when there is still some PE synthesis remaining. The halfway saturation for 650 nm light is for a log(fluence) of about 1.8 (J m^{-2}), corresponding to a photon fluence of 3.4×10^{-4} mol m^{-2}. This gives us a rough value for the action cross section (σ) at 650 nm of 3,000 m^2 mol^{-1}. Thus, the action cross section of Pr at 650 nm appears to be about three times the size of the action cross section of Pg at 550 nm. Obviously estimated in a somewhat different way, Vogelmann and Scheibe (1978) arrive at a ratio of 7 instead of 3. Thus, these estimates are not very accurate, and in the calculations below, we have used the action cross sections 1,000 and 10,000 mol m^{-2} for the green-absorbing and the red-absorbing forms of the photoreceptor at their absorption maxima, i.e., a ratio of 10.

Let us now consider the reaction system

$$\mathrm{Pr} \leftrightarrow \mathrm{Pg}$$

where Pr and Pg are the red- and green-absorbing forms of photoreceptor pigment. With ε and ϕ standing for absorption cross sections and quantum yields, respectively, the rate of Pg formation is $\mathrm{d[Pg]/d}t = \mathrm{Frate}(\lambda)\{\varepsilon r(\lambda) \times \phi r \times [\mathrm{Pr}]$

$-\varepsilon g(\lambda)\times\phi g\times[\mathrm{Pg}]\}$, or with simpler notation $\mathrm{dPg/d}t=\mathrm{Frate}\times(\sigma r\times\mathrm{Pr}-\sigma g\times\mathrm{Pg})$, where t is time, Frate is photon fluence rate, σr and σg are wavelength-dependent action cross sections of the red- and green-absorbing forms of the photoreceptor, and Pr and Pg are the amounts of the two photoreceptor forms as fractions of the total. Considering that Pr = 1–Pg, and assuming that all photoreceptor is in the Pr form before irradiation with red light starts, this relationship can be integrated over time to yield

$\ln\{[\sigma r-(\sigma r+\sigma g)*\mathrm{Pg}]/\sigma r\}=-(\sigma r+\sigma g)\times\mathrm{Frate}\times t=-(\sigma r+\sigma g)\times F$, where $F=\mathrm{Frate}\times t$ is the fluence.

Thus, $\ln\{[\sigma r-(\sigma r+\sigma g)\times\mathrm{Pg}]\}/\sigma r]=-(\sigma r+\sigma g)\times F$ which can be rewritten as $1-(1+\sigma g/\sigma r)\times\mathrm{Pg}=\exp[-(\sigma r+\sigma g)\times F]$, i.e., $\mathrm{Pg}=\{1-\exp[-(\sigma r+\sigma g)\times F]\}/(1+\sigma g/\sigma r)$.

We have made the action cross sections for the green-absorbing and red-absorbing form 1,000 and 10,000 times the absorption coefficients with maxima normalized to 1, which we can call kr and kb, so we get

$$\mathrm{Pg}=\left\{1-\exp\left[-\left(10{,}000\times kr+1{,}000\times kg\right)\times F\right]\right\}/\left(1+0.1\times kg/kr\right)$$

We did not get a good fit with this. But by changing the factor 0.1 in the last parenthesis to 1, we obtained the violet curve marked "model" in Fig. 9.6, and by squaring it, we got a fit which is probably within experimental error.

Can we logically motivate the square relationship? We could get a square relationship if two parts of the phycoerythrin molecule (such as the protein part and the chromophore) were separately regulated by the photoreceptor.

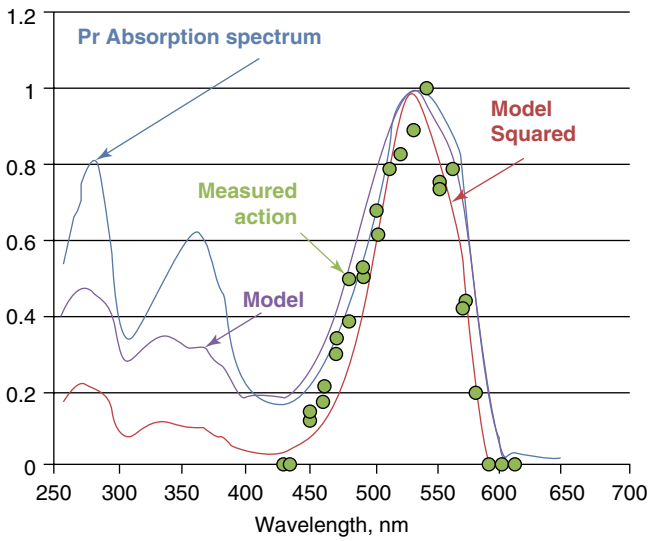

Fig 9.6 Measured action spectrum for phycoerythrin formation (Vogelmann and Scheibe 1975) compared to the Pr absorption spectrum (Hirose et al. 2013) and the modeled action spectrum as described in the text

9.8 Tuning of Phytochrome-Like Pigments

Since the publication of the second edition of this book, it has become obvious that there exist in many main groups of organisms pigments which are analogous to the plant photosensory pigments known as phytochromes (Rockwell and Lagarias 2010). Phytochrome-like pigments, or at least genes coding for such chromoproteins, have by now been found in various kinds of algae, fungi (Blumenstein et al. 2005; Rodriguez-Romero et al. 2010; Lamparter and Marwan 2001), Excavata (Bolige and Goto 2007), and in many major groups of bacteria: cyanobacteria (Rockwell et al. 2011), actinobacteria, α-proteobacteria, γ-proteobacteria, ∂-proteobacteria (White et al. 2008), Deinococcaceae, and Firmicutes. They have even been implicated in Archaea. The plant phytochromes have two main states with absorption peaks at about 660 and 730 nm, respectively. Their newly detected relatives in other organisms span over a very large spectral range, rivaling the spectral tuning of rhodopsin-type and chlorophyll-type pigments. This spectral variation is due to both chromophore modifications and protein interactions. The peaks of light sensitivity range from 382 nm (Song et al. 2011) for a *Synechocystis* pigment to 750 nm for a *Agrobacterium tumefaciens* pigment (Rottwinkel et al. 2010). In the latter case significant sensitivity extends above 800 nm.

The plant phytochromes and many of the related pigments in other groups have their open-chain tetrapyrrole (bilin-type) chromophore attached via a covalent bond to a cysteine in the apoprotein. The cyanobacteria exhibit a special richness and variability of phytochrome-like pigments. In a single cyanobacterial strain, *Nostoc* sp. PCC7120, seven different types have been found (Ma et al. 2012). In addition, this organism is equipped with a rhodopsin-type light sensor (Jung et al. 2003). More generally, there are two main types of phytochrome-like sensors in cyanobacteria, those which have their chromophore attached via a single cystein residue. These are appropriately called cyanobacterial phytochromes. An overview of this is given in the Introduction part of Anders et al. (2011). They exhibit red/far-red photoreversibility as plant phytochromes. The other types, called cyanobacteriochromes or cyanochromes, have two cysteine attachment points between chromophore and apoprotein. Their spectra are shifted to lower wavelengths, in an extreme case to 382 nm (Song et al. 2011).

Below we shall give some examples of the effect of chromophore and of protein environment on absorption (and sensitivity) spectra. It should be noted that in many cases described in the literature, chromoproteins have not been characterized after extraction from the original organism under investigation. Instead the corresponding gene, or even

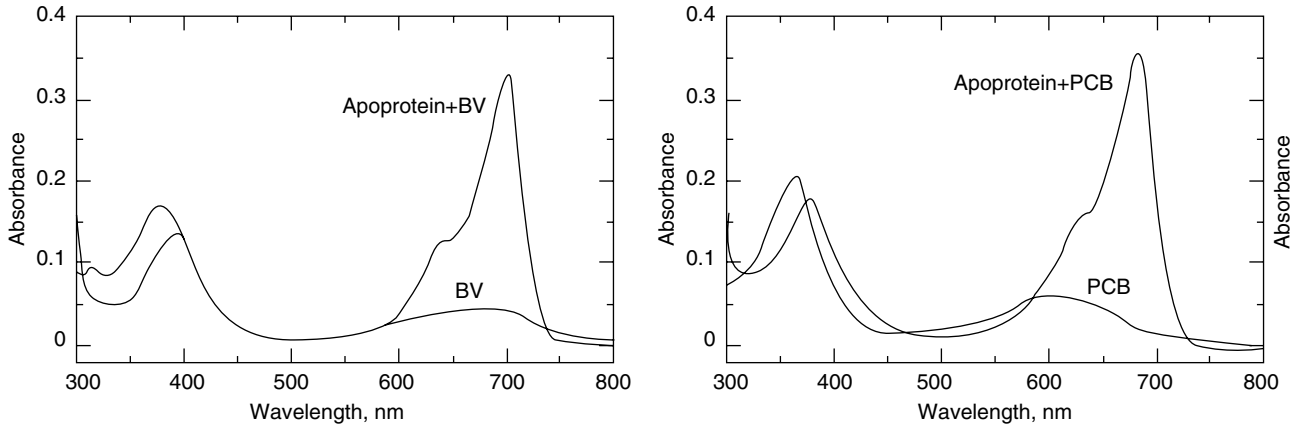

Fig. 9.7 Absorption spectra of biliverdin (*left*) and phycocyanobilin (*right*) in free form or combined with a phytochrome apoprotein (Agp1) from *Agrobacterium tumefaciens* (Lamparter et al. 2002). All pigments in unexcited dark state

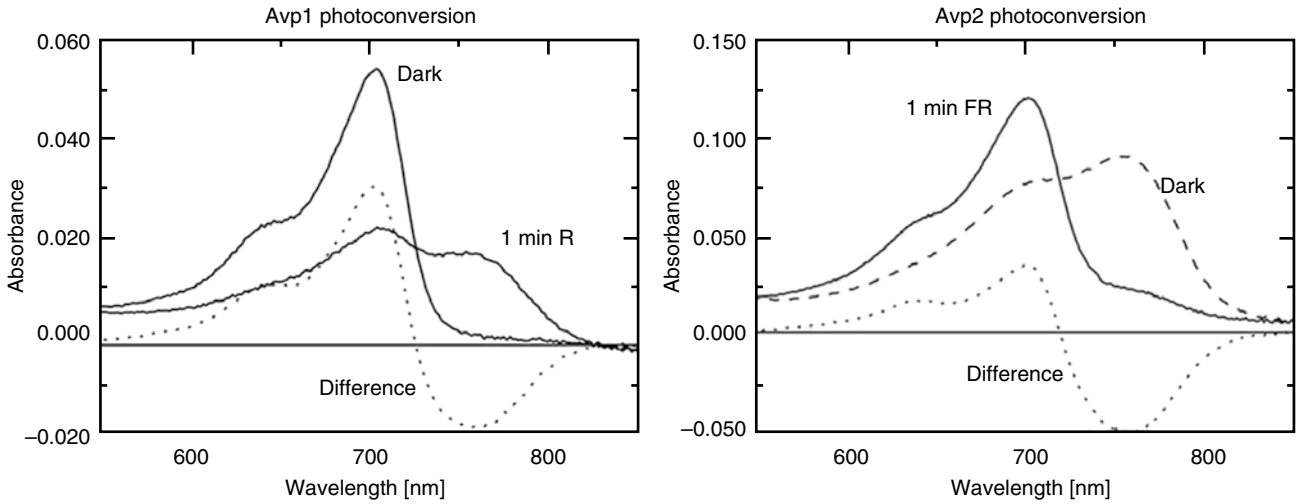

Fig. 9.8 Phototransformations of two phytochromes from *Agrobacterium vitis* according to Rottwinkel et al. (2010)

only part of it, has been cloned into another organism that has been able to produce the protein in sufficient amount for investigation.

From Fig. 9.7 we can learn several things: (1) The free forms of the bilins in solution have less sharp spectra because they are flexible, so the spectra are averaged over many conformations. The protein-bound forms, on the other hand, are fixed in a particular conformation. (2) The biliverdin chromoprotein has its absorption peak at longer wavelength (ca 703 nm) than the phycocyanobilin chromoprotein (ca 680 nm), because biliverdin has one double bond more.

Figure 9.8 shows the change of absorption spectra upon irradiation of two phytochromes prepared from genes of *Agrobacterium vitis*. Note that one of them changes towards longer wavelengths on irradiation with red light, in a way similar to the plant phytochrome. The other one, however, shifts to shorter wavelength upon irradiation. This type of phytochrome has been called bathyphytochrome (from Greek bathys, deep or low). As described in Fig. 12.14, these spectral changes are due to rotation of part of the bilin molecule around a double bond and changes in the protein that follow from this.

Figure 9.9 shows spectra of the other main type of photo sensory biliproteins, called cyanobacteriochromes (or cyanochromes), with two attachment points for the bilin via cysteine residues on the protein. One of these bonds is labile and may loosen in one of the spectral forms. The double attachment results in a very large shift to shorter wavelength (the chromophore in this case, as in others investigated, is phycocyanobilin). Irradiation with UV-A radiation results in the 534 nm form, while irradiation with green light produces the 382 nm form. By comparison with Fig. 9.7, we can see that the protein can modulate the phycocyanobilin absorption peak between 382 nm and 684 nm.

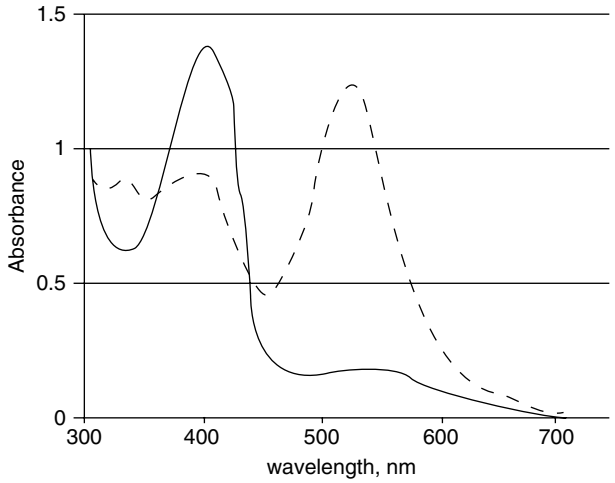

Fig. 9.9 *Green* irradiated (382 nm form), *solid line*, and UV-A irradiated (534 nm form), *dashed line*, of cyanobacteriochrome from *Synechocystis* sp. PCC 6803 (Redrawn from Song et al. 2011)

Further aspects of cyanobacterial chromatic acclimation are treated by Bussel and Kehoe (2014).

9.9 Visual Tuning

Visual pigments of animals span a spectral range of 300–700 nm (Marshall and Oberwinkler 1999), i.e., more than an octave of the electromagnetic spectrum. They are proteins with, in most cases, either 11-cis-retinal or 11-cis-3-dehydroretinal (Fig. 9.10: Makino et al. 1999) as chromophores. Proteins with 11-cis-retinal alone cover a range of absorption spectra with maxima from 360 to 635 nm (Kleinschmidt and Harosi 1992, Kochendoerfer et al. 1999). The term rhodopsin is somewhat ambiguous and sometimes covers all chromoproteins related to the human visual pigments, including light-sensitive proteins in algae, bacteria, and archaea, but sometimes visual pigments containing 11-cis-dehydroretinal (which are then termed porphyropsins) are excluded. The spectra of visual pigments to some extent depends on which chromophore they contain; dehydroretinal (also called *retinal*$_2$) with its longer conjugated double-bond system giving a red shift of 10–50 nm compared to 11-cis-retinal. Two other "primary chromophores" involved in animal vision are also known: 11-cis-4-hydroxyretinal has been found (as well as retinal and 3-dehydroretinal) in the eyes of the bioluminescent squid *Watasenia scintillans* (Matsui et al. 1988) and gives a blue shift compared to retinal. 3-Hydroxyretinal occurs in several insect orders (Vogt 1983; Vogt and Kirschfeld 1984; Tanimura et al. 1986; Seki and Vogt 1998), and different stereoisomers (3R and 3S enantiomers) of it occur, with different phylogenetic distributions (Seki and Vogt 1998). Many vertebrates, including humans, have a differentiation of light-sensitive cells in the retina between rods, specialized for "black-and-white vision" in weak light, and cones, specialized for color vision in stronger light.

In addition to these "primary chromophores," there are, in certain cases, "sensitizing chromophores" attached to the same proteins. These chromophores act in analogy to photosynthetic antenna pigments: they absorb light and transfer the energy to the primary chromophores. Only three such sensitizing chromophores have been detected so far: 11-cis-3-hydroxyretinol in Diptera (Vogt and Kirschfeld 1984) and defarnesylated *Chlorobium* pheophorbide methyl ester in bioluminescent dragonfish (Douglas et al. 1998, 1999) and chlorin e6 in salamander (Isayama et al. 2006). More about the dragonfish can be found in Chap. 26.

In addition to visual pigment structure, spectral filters in the form of colored oil drops contribute to spectral tuning of photoreceptor sensitivity in some animals, especially birds (Maier and Bowmaker 1993; Bowmaker et al. 1997; Vorobyev et al. 1998; Hart et al. 2000) and reptiles (Schneeweis and Green 1995). We also have filters in our own eyes, namely, in the yellow spot of the retina, macula lutea. This is the spot of highest visual acuity, devoid of blue-sensitive cones. Here the yellow pigment serves to prevent blue light to reach the green- and red-sensitive cones, for which it would degrade acuity by chromatic aberration.

However, most of the spectral tuning is achieved by variation of a few of the amino acids in the protein to which the chromophores are attached (Britt et al. 1993).

Humans use exclusively the 11-cis-retinal chromophore. In free form in methanol solution, 11-cis-retinal absorbs maximally at 380 nm (in protonated Schiff's base form, it absorbs maximally at 440 nm). Human rhodopsin, the protein-chromophore complex of rods, used in twilight vision, peaks at 493 nm (Wald and Brown 1958). The three human cone pigments, used in color vision, peak at 426, 530, and 552 nm (or 557 nm; all persons do not have exactly the same type). The human cone pigments are often referred to as SW (for short wavelength), MW, and LW pigments, respectively.

The effect of protein primary structure on spectral properties of visual pigments is studied (1) by comparing various naturally occurring pigments and (2) by site-directed mutagenesis experiments in which certain amino acids are changed.

An important determinant of the spectrum seems to be the negative charges on amino acids in proximity to the chromophore. We shall give two interesting examples of how this can work:

1. Ultraviolet vision has been demonstrated in many vertebrate (fish, amphibian, reptilian, avian, and mammal) species (Jacobs 1992). Yokoyama et al. (2000) have convincingly shown that ultraviolet-sensitive pigment in birds evolved from violet-sensitive pigments by a single

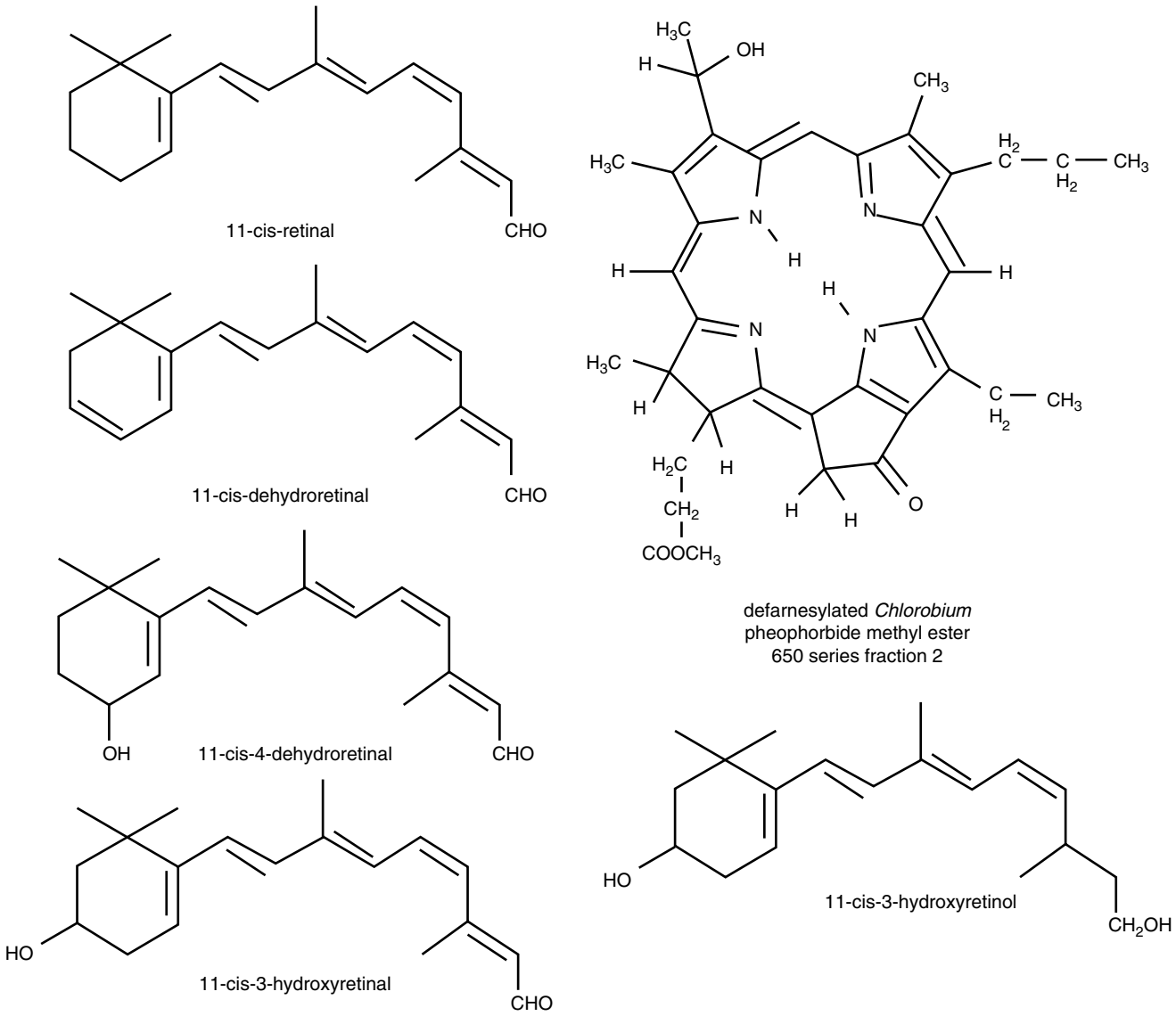

Fig. 9.10 Structures of five chromophores known from animal visual pigments. Several compounds related to the *Chlorobium* pheophorbide derivative also occur in dragonfish eyes. 11-cis-3-Hydroxyretinal occurs as two different enantiomers, with different phylogenetic distributions

amino acid substitution, namely, by a change of serine to cysteine at position 84. This shifts the absorption spectrum of the wild-type pigeon pigment with a maximum at 393 nm to one peaking at 358 nm. For the corresponding chicken pigment, the shift was from 415 to 369 nm. Conversely, the zebra finch UV pigment peaking at 359 nm could be shifted to 397 nm by a change at position 84 from cysteine to serine. It should be noted, however, that ultraviolet-absorbing pigments in other vertebrate groups have arisen independently and by other substitutions.

2. Human trichromatic color vision has arisen recently during evolution; most mammals have only dichromatic color vision and some, such as whales, do not have more than one type of cone pigment. Some New World monkeys also possess trichromatic color vision but that has arisen independently. The human type of trichromacy has arisen by gene duplication of a long-wavelength pigment and mutation of one of the gene copies to produce a middle-wavelength (MW) pigment. According to Neitz et al. (1991), the spectral differences between these pigments depend on three amino acid differences, and in addition there are several differences without spectral effect. Effects are detailed in Table 9.4 (values vary somewhat between investigators using different methods). In each case the change from a nonhydroxyl to a hydroxyl amino acid results in a "red shift," i.e., absorption at longer wavelengths.

Table 9.4 Spectral effects of amino acid (aa) substitutions in human cone pigments

Position	aa in MWP	aa in LWP	Δλ on change
180	Alanine	Serine	+6
277	Phenylalanine	Tyrosine	+9
285	Alanine	Threonine	+15

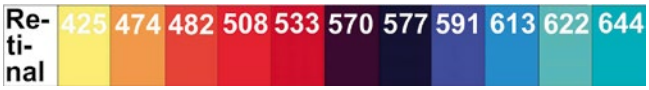

Fig. 9.11 Colors of the engineered rhodopsins produced by Wang et al. (2012). The numbers show the wavelengths of absorption maxima in nm. The free retinal (*far left*) is completely colorless

Those people who possess a 557 nm LW pigment have serine at position 180, while those with a 552 nm pigment have alanine. This position is also variable in the MW pigment but seems to produce a smaller spectral shift there, and investigations are not as thorough as for the LW pigment (see Sharpe et al. 1999 for details). The same person may, in fact, possess more than three different cone pigments (Neitz et al. 1993).

Fujimoto et al. (2005) have studied the spectral tuning of retinal proteins by quantum mechanical methods, while Nathans (1992) has discussed it in more general terms, easier to understand for the nonspecialist. Retinal undergoes a large decrease in dipole moment in going from the ground state to the photoexcited state: In the ground state, a positive charge is localized mainly to the Schiff's base nitrogen, and this charge is distributed more evenly throughout the π-electron system upon photoexcitation (Nathans 1990). A negative charge, such as from glutamate or aspartate, along the polyene chain of retinal would favor charge delocalization in the ground state and thus a smaller energy gap, i.e., a red shift. Polar groups along the polyene chain would favor or disfavor charge delocalization depending on orientation. Polarizable groups along the polyene chain would stabilize the excited state (produce a red shift) through compensatory charge movement. Twisting around single or double bonds would, respectively, decrease or increase charge delocalization. Moving the Schiff's base counterion further from the chromophore would decrease the effect of the ground state dipole moment and produce a red shift. A record shift for a single amino acid change (which has not been found in nature), from 500 to 380 nm, was produced experimentally by changing glutamic acid to glutamine at position 113 (references in Yokoyama 1997). Wang et al. (2012) performed systematic modifications of rhodopsin structure producing a whole range of rhodopsins with spectra peaking from 460 to 644 nm and having the colors in solution shown in Fig. 9.11.

It has been shown that in some cases the spectrum of a visual pigment can be modified even without change of amino acid in the protein and without change of chromophore, namely, by the concentration of chloride ions (Yamashita et al. 2013 and literature cited by them).

Returning to the human MW and LW pigments, one may wonder why their absorption spectra are not more different. Color vision would appear to be more efficient if they were. The difference between the LW (552 or 557 nm) absorption peak and that of the MW (530 nm) pigment is much smaller than between MW and SW (426 nm) pigment maxima. The difference between the human LW and MW pigments is much smaller than the difference between corresponding pigments in, e.g., the fruit fly *Drosophila* (in this animal the two pigments absorbing at longest wavelengths peak at 420 and 480 nm, respectively; in addition the fly has two pigments peaking in the ultraviolet). One explanation that has been proposed is that the image-forming optics of the human eye has a large chromatic aberration and the effect of this is minimized if the spectra are not too different. The perception of shapes and position depends mainly on the LW and MW cones, and the focusing of an image on the retina is adjusted for the average of their wavelengths. The blue color is mentally "painted" into the outlines formed by these receptors. In the part of the retina used for the sharpest vision, the luteum has very few SW receptors and contains a yellow pigment which absorbs blue light. The similarity of LW and MW spectra makes good focusing possible. On the other hand, the small difference in the human pigments may be just a consequence of the fact that the gene duplication has occurred so recently, and evolution has not had time to result in a bigger difference yet.

Insects have a completely different system for image generation (Chap. 15), without the chromatic aberration problems, but the visual acuity of the fruit fly eye is much lower than that of the human eye.

What are the evolutionary pressures causing visual pigment spectra to be tuned? Generally speaking, of course, color vision provides more information than monochromic vision. We prefer color television to black and white. According to Osorio and Vorobyev (1996) and Regan et al. (1998), the main importance of the differentiation into LW and MW pigments in primates has been to aid our forefathers in detecting fruits against a green background and judging the ripeness of fruits. This view has been questioned by Lucas et al. (1998) and Dominy and Lucas (2001), who provide evidence that trichromatic vision is important for the selection of leaves at an optimal developmental stage for consumption. As for ultraviolet vision in birds, one well-documented advantage for birds of prey is that UV vision allows them to see urine of rodents and thus to locate their whereabouts. Ultraviolet vision in birds is also important for recognition of plumage coloration of conspecifics and for detection and identification of edible berries (Siitari et al. 1999).

For insects depositing eggs on leaves, it is important to find the leaves and to judge their age and health status. It turns out that for this task *a red-light* receptor can be very important. Most insects do not have red-sensitive receptors,

but both sawflies (Peitsch et al. 1992) and moths (Kelber 1999) that oviposit (lay eggs) on leaves do. Excitation of green-sensitive photoreceptors gives an attractive signal, and the red-sensitive receptors provide a contrasting, repelling signal. In the case of moths, their ultraviolet-, violet-, and blue-sensitive receptors probably play a role in their orientation and choice of leaves (young leaves are preferred). On the other hand, in selecting green leaves, vision probably does not play an important role in the discrimination between plant species; for this, chemical cues are more important.

The daylight penetrating deepest into the ocean is in the blue-violet region, and consequently the vision of deepwater fish is tuned to this wavelength band (Lythgoe 1984), while surface-living fish and fish in shallow freshwater have a visual sensitivity peaking, like ours, in the green spectral region (although the span of pigments in fish is much wider than ours, with pigment absorption peaks spanning from the ultraviolet to the red). Although also several bioluminescent deepsea fishes have maximum sensitivity in the blue-violet region (Fernandez 1978), others, who use their bioluminescence for environmental illumination, show remarkable deviations from this rule (Douglas et al. 1998, 1999). Other aspects of the connection between bioluminescence and the vision of deepsea fishes have been treated by Warrant (2000).

Deep-diving whales have rod pigments peaking at 485 nm, while rod pigments of aquatic animals foraging closer to the surface (seals, manatees) peak near 500 nm. To the surprise of some investigators, none of six whale species and seven seal species possess SW cones (nor any LW cones), only MW cones (Peichl et al. 2001), with a pigment absorbing maximally around 524 nm (Fasick et al. 1998). It has been claimed that this means that they do not possess color vision (Peichl et al. 2001), but this may be jumping to a conclusion. Although color discrimination in whales has not been established, rods are saturated in strong light and cones useless in weak light; there may be intermediate depth and light levels where signals are obtained from both rods and cones and give whales and seals a dichromatic color vision (Fasick et al. 1998; Fasick and Robinson 2000). A corresponding phenomenon in humans was demonstrated many years ago through a very interesting experiment by John J. McCann and Jeanne L. Benton, described by Land (1964). They first illuminated a multicolored display with "monochromatic" (narrowband) light of 550 nm (500 nm would probably have worked as well), which was so weak that only the rods of a human observer were stimulated. Of course no colors could be discriminated under such circumstances. They then added a second narrowband beam of 656 nm wavelength. The irradiance of this second light was adjusted so that only the LW cones were stimulated. Thus, only the rods and the LW cones were operative. Nevertheless, the observer was able to give names to colors in the display almost as if it was illuminated by natural daylight and all three types of cones had been stimulated.

Color vision is not restricted to di-, tri-, and tetrachromatic versions. The mantis shrimp (Osorio et al. 1997; Marshall and Oberwinkler 1999; Cronin et al. 2001) may have up to 16 types of visual pigment, although this does not mean that it has a corresponding number of color channels. It also has the ability to further tune the sensitivity spectra of their receptors by color filters as required by the light environment they inhabit. These animals may live close to the water surface (in full daylight spectrum) or as deep as 30 m (in a restricted blue-light environment). All 16 types of light-sensitive pigments may not correspond to separate sensory input channels, but no doubt they provide polychromatic vision.

Rhodopsins are used not only for vision but also for energy collecting by microorganisms. Proteorhodopsins, a group of rhodopsins widely spread among many microorganisms, is particularly worth mentioning in this context. It is spectrally tuned to different habitats mainly by substitution at a single amino acid site (Man et al. 2003).

9.10 Tuning of Anthocyanins

Anthocyanins are the most common vacuolar pigments, giving color to many flowers, fruits, and autumn leaves. We may think of the cell sap of plant vacuoles as structureless and of interactions between anthocyanins and their environment as a dull subject, but if we do so, we are in error.

The great pioneer in the elucidation of chemical structures of plant compounds, Richard Willstätter, got a surprise when he compared the structures of the blue pigment of the cornflower (*Centaurea cyanus*) with that of the red pigment of a rose. He found that the pigments were chemically identical (he was not completely right, but that does not destroy the story). He thought that the difference in color came about from a difference in pH of the cell sap of the two plants. He was not right there either, but he got the main point—that anthocyanins can produce very different colors with practically identical chromophores.

The basic structure of an anthocyanin is shown in Fig. 9.12. The molecule consists of two fused six-membered rings connected to a third six-membered ring. A system of

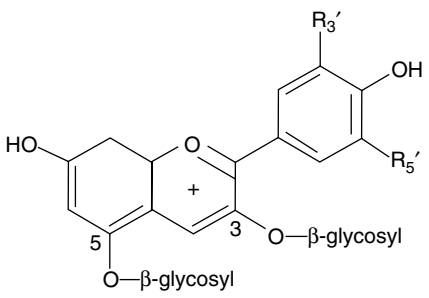

Fig. 9.12 The general structure of an anthocyanin in the flavylium cation form

conjugated double bonds extends over all rings. The fused rings carry three hydroxy groups, one or two of which form glycoside bonds with sugar molecules, often glucose. The sugar-free compound (the aglycon) is called anthocyanidin. The anthocyanidin of red rose flowers is cyanidin and that of cornflower succinyl-cyanidin (so they are indeed closely related, and the little difference does not explain the color difference). Pure cyanin (the glycosylated cyanidin) is red in acid solution, and if it is made alkaline, the color changes towards blue, so it is understandable that Willstätter ascribed the color difference between roses and cornflowers to a pH difference of the cell sap. However, the blue color acquired

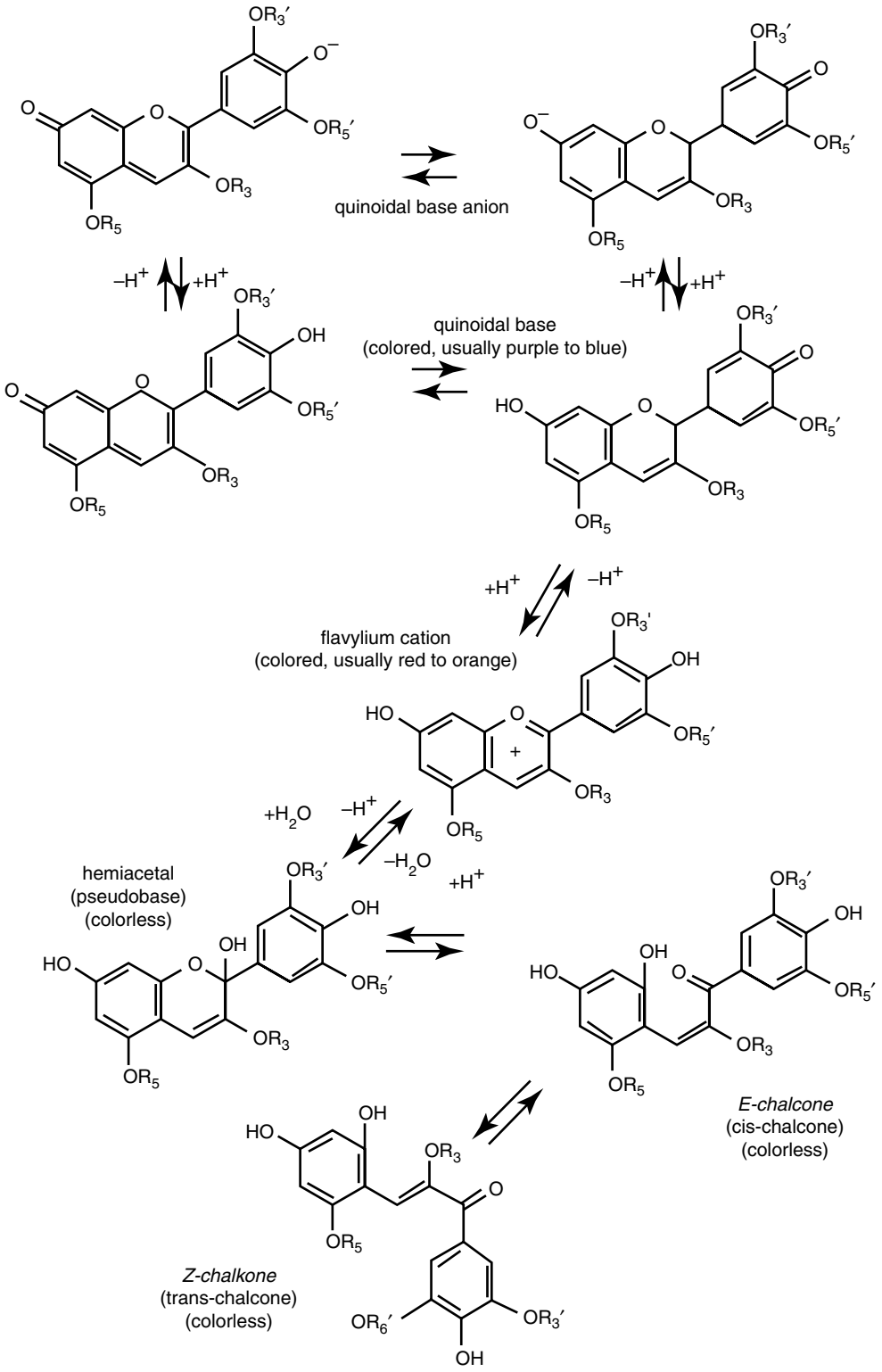

Fig. 9.13 An anthocyanin can exist in many different interconvertible molecular forms, as shown in this diagram: as different quinoidal bases anions or unionized quinonoidal bases, as flavylium cation, as hemiacetal (pseudo base), or as chalcone (E or Z form). Although hemiacetals and chalcones are colorless, they absorb ultraviolet radiation and can therefore appear colored to some animals. Their presence can also modify the hue of the colored forms

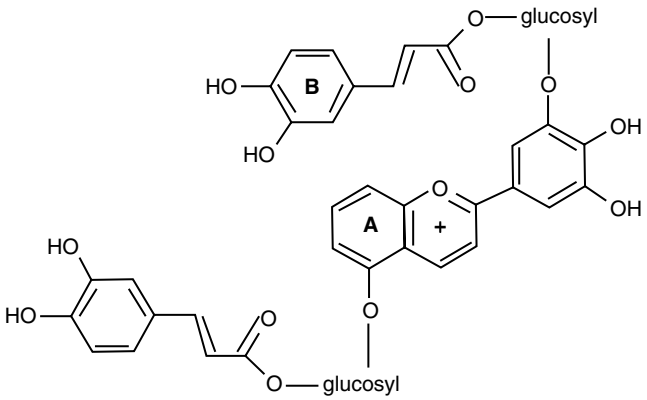

Fig. 9.14 The structure of gentiodelphin, schematic. In reality the molecule is more bent, so ring B closely overlaps the double ring A and the π orbitals of the ring systems fuse

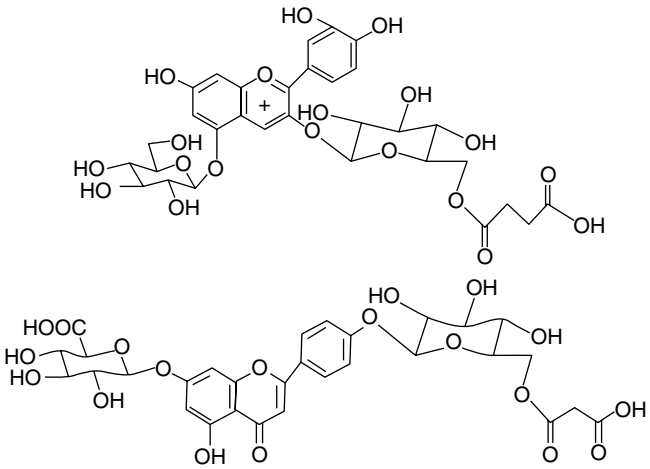

Fig. 9.15 According to Goto and Kondo (1991), the blue pigment of cornflower, protocyanin, is built up of six molecules each of the succinylcyanin cation (top) and the malonylflavone shown in the figure, plus one Fe and one Mg ion

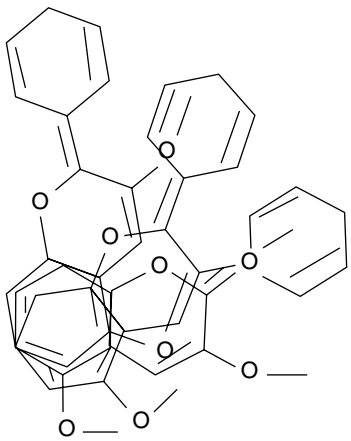

Fig. 9.16 An attempt to show the chiral stacking of delphin molecules. The sugar groups are omitted for clarity

upon alkalinization does not last long, the color fades away completely. This is because the molecule already, when the pH exceeds about 5, takes up water (Fig. 9.13). Furthermore, no plants are known with an alkaline cell sap, so the cornflower's blue color must be explained in another way.

The R-groups in Fig. 9.13 are numbered according to the conventional numbering of the carbon atoms to which they are bound. The groups can all be hydroxyl and can also be hydrogen; methoxyl; a chelated metal ion, such as Fe^{3+} or Al^{3+}; or a sugar or sugar derivative, while R_3 and R_5 can be a sugar or an acylated sugar. The equilibria between different forms depend on many things, such as the chelation with metal ions. In at least one case, it is also light dependent (Figueiredo et al. 1994).

The reason for the blue color of the cornflower rests in the phenomena of copigmentation and self-association. Copigmentation means that the color of the anthocyanin is influenced by other molecules in its environment. Self-association means that the anthocyanin molecule can associate with other anthocyanin molecules, and this also affects its color. Both self-association and associations between anthocyanins and uncolored phenolic compounds cause overlaps between the π-electron clouds of the individual molecules, and hence changes in the electron levels (Fig. 9.14). The concentration of anthocyanin in cell sap can exceed 20 m*M*, and this is more than sufficient for the molecules to associate to one another and sometimes form helical stacks through a combination of hydrophobic bonds between the rings and hydrophilic bonds between the sugar residues. These associations also prevent the formation of pseudo base, hence the bleaching of color that would otherwise take place at the pH prevalent in the cell sap.

Many different kinds of (uncolored) molecules and ions produce copigmentation effects with anthocyanins. The most important ones are some metal ions and colorless flavonoids (absorbing in the ultraviolet spectral region) and other phenolic compounds. In the case of the blue cornflower pigment (Fig. 9.15), Fe^{3+} and Mg^{2+} have been reported as copigmenting ions and a flavone as copigmenting phenol. Anthocyanins having two ortho-hydroxy groups at the B-ring (the leftmost ring in Fig. 9.15) form blue chelate complexes with trivalent metal ions such as iron(III) and aluminum(III), but not with magnesium. This latter ion is, however, very important in some other cases.

Goto and Kondo (1991) have written a very readable account of copigmentation, illustrated with color pictures. They describe structures of several more complex anthocyanins, with aromatic groups attached to the sugar residues, and a particularly interesting case of pigment complex, commelinin (rendering the blue color to *Commelina communis*). This consists of six molecules each of malonylawobanin (a complex anthocyanin) and flavocommelinin (a flavone glycoside) arranged around two magnesium ions. The intense blue color is partly due to exciton coupling between adjacent anthocyanin units. The molecular mass of this complex is nearly 1,000. Also chiral stacking (Fig. 9.16) can affect color.

The importance of the sugar groups in anthocyanins lies not in a direct effect on light absorption, but in their contribution to the folding and ordering of the chromophoric groups such that overlaps of π-electron clouds can take place.

The color of anthocyanins is affected not only by their chemical environment but also by physical factors: temperature and light. One summer day when one of the authors and his wife were having tea in their garden, she remarked that she had noticed how some flowers of a variety of *Phlox paniculata* had shifted color when they were reached by sunlight. He delivered a lecture on the psychology of color perception and the mistakes we can make, but she insisted. So we took the flowers to the lab and measured the reflection spectra after dark adaptation and after exposure to strong light for an hour (Björn et al. 1985). And indeed, the reflectance spectrum did change with light conditions. Color photos of the phenomenon have been published in Swedish journals (Björn 1985a, b). At the time we hypothesized that the color shift was caused by a light-activated proton pump in the tonoplast (the membrane surrounding the vacuole) which would change the pH of the cell sap. We attempted to show such a pH change using a white *Phlox* variety and artificial pH indicator dyes, but did not succeed. About a decade later, Figueiredo et al. (1994) demonstrated that the absorption spectrum of an artificial anthocyanin-like compound, 4′,7-dihydroxyflavylium chloride, could be reversibly affected, due to Z/E photoisomerization, by ultraviolet radiation (changing under irradiation from pale yellow to intense yellow), so it is possible that the color shift of our *Phlox* is due to an analogous phenomenon.

Thus, a number of factors can affect the color of anthocyanin-containing flower petals. Obviously the reason that plants have evolved colored flowers is that they attract pollinators. The pollinators also benefit from this, so a question naturally arises: To what extent have flowers adapted to pollinator vision, and to what extent has pollinator vision (and mental capacity, such as long-term and short-term memory) adapted to flower colors?

Chittka and coworkers (Chittka and Menzel 1992; Chittka 1996; Chittka et al. 1999; Chittka and Dornhous 1999) have studied this question. By comparison of color vision in various arthropod groups, they claim to be able to follow the evolution of color vision in insect pollinators. Photoreceptors among most Crustacea and Insecta fall into three rather distinct spectral classes: ultraviolet receptors with maxima around 350 nm, blue receptors with maxima at 400–460 nm, and green receptors with maxima from 470 to 550 nm. All these types seem to be very ancient. In addition, a few groups have red receptors with maxima around 600 nm. Chittka and coworkers see little evidence of insect adaptation to flower colors and believe that the main adjustment has been of flower colors to insect vision.

Only a minority of pollinators, such as hummingbirds and some butterflies, have red-sensitive photoreceptors. However, it must be borne in mind that pollinators without such receptors can see red flowers and distinguish them from other flowers and from green leaves.

The flowers of many plants vary in color with stage of development (Lunau 1996) and in this way signal to pollinators when a visit will be rewarded Lunau (2004). Of particular interest are flowers that change color during the year in synchronization with the availability of different pollinators.

Fig. 9.17 A flower of creeping tormentil, *Potentilla reptans* photographed in visible light (*left*) and in UV-A radiation (*right*) (Photo by L.O. Björn)

What has been described here for flower colors has, to some extent, a counterpart in the coloration of fruits of plants which depend on animals for seed dispersal.

The ultraviolet receptors of insects make it possible for them to see patterns invisible to us. Figure 9.17 shows an example of such a flower pattern due to ultraviolet-absorbing flavonoids.

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