Studies on a bacterial photosensor

Kort, R.

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Chapter 1
General introduction


1.1 Purple bacteria

Life in the microbial world. Life on earth shows great diversity. In order to come to a better understanding of life and its evolution over a period of about 4 billion years, taxonomists have developed a system for the division of living organisms in separate groups. In contrast to our teacher in history, who taught us that we have to know the past to understand the present, systematic biologists try to understand the past by knowing the present. One of the first man-made divisions of life on earth is that between plants and animals. Single-celled life forms however, do not fit in either category, as observed by Haeckel (1866), who added this group as protists to the tree of life. New branches of this tree split out over the years by the addition of the kingdoms Monera (bacteria) and Fungi, leading to a five-kingdom scheme: Animalia, Plantae, Fungi, Protista and Monera. A completely different concept was proposed by Chatton (1938), who separated life in eukaryotes and prokaryotes. This dichotomy of life, which is still used today, defines prokaryotes by differences with respect to the organization of their cellular machinery. These differences can be found in the organization of the nuclear region and the machinery for respiration and photosynthesis, present in eukaryotic cells as membrane enclosed organelles, like the nucleus, mitochondria and chloroplasts. In contrast, in prokaryotes no structural unit smaller than the entire cell is recognizable as the site of respiration or photosynthesis (Stanier & Van Niel, 1962). The division of life in two primary kingdoms came to an end, as well as the prokaryotic kingdom as a phylogenetically valid taxon, by the use of biomolecular sequences as a new phylogenetic marker. The concept of biomolecules as documents of evolutionary history was first introduced by Zuckerlandl & Pauling (1965).

A comparison of ribosomal ribonucleic acid (rRNA) sequences led to the proposal for a division of life in three domains: the Bacteria, the Archaea and the Eukarya, each containing several kingdoms (Woese, Kandler & Wheelis, 1990). The new domain of Archaea consists of three kingdoms, the Euryarchaeota, comprising the methanogens and the halophiles, the Crenarchaeota, including the extremely thermophilic archaeabacteria, and the Korarchaeota (figure 1). The latter kingdom was added recently as a result from the phylogenetic analysis of rRNA sequences obtained from uncultivated organisms of a hot spring in Yellowstone National Park (Barns et al., 1996). Although archaeabacteria can still be considered as prokaryotes by their cytological characteristics, their biomolecules generally resemble their eukaryotic homologs more than their eubacterial ones. This is also demonstrated by the phylogenetic tree based on 16S rRNA sequences, where the root separates the eubacteria from the other two primary groups. The use of rrm gene sequences (encoding 16S or 23S rRNA) and highly conserved protein sequences as phylogenetic markers in the construction of the tree of life has proven to be more successful than previous methods based on morphological and physiological characteristics. In addition, amplification of the marker genes by the polymerase chain reaction (PCR) overcomes the problem of isolating species from their natural environment. However, genes within one organism may evolve fast or slowly, depending on the importance of their function and the specific environment of the cell. Also exchange of genes among organisms, lateral gene transfer, may create a problem. Indeed, the comparison of several gene sequences, recently released by the completion of microbial genome sequences (the sequences of at least 20 prokaryotic genomes and 1 eukaryotic genome have been elucidated now) reveals unexpected connections between prokaryotes thought to have diverged hundreds of millions of years ago (Pennisi, 1998).

Anoxygenic phototrophic prokaryotes. The anoxygenic phototrophic bacteria carry out photosynthesis without oxygen evolution on the basis of a bacteriochlorophyll-mediated process. The transformation of light into chemical energy can be achieved by several types of bacteriochlorophyll and a variety of carotenoids as pigments. Photosynthesis in
anoxygenic phototrophic bacteria mostly depends on oxygen-deficient conditions, because synthesis of the photosynthetic pigments is usually repressed by oxygen. Unlike cyanobacteria, algae and plants, anoxygenic bacteria are unable to use water as an electron donor, as donors of a lower redox potential are required. Sulfide, reduced sulfur compounds, hydrogen and small organic molecules are used as electron donors instead. In spite of their common theme of photosynthesis, anoxygenic phototrophic bacteria are extremely diverse on the basis of morphological, physiological and molecular characteristics. They include green sulfur bacteria, green non-sulfur bacteria, heliobacteria, purple

![Universal phylogenetic tree based on 16S rRNA sequences. Numbers indicate the percentage of bootstrap resampling that support the indicated branches in the maximum likelihood (before slash) or the maximum parsimony method (after slash). Analyses of duplicated protein genes placed the root on the branch at the base of the bacteria. The sequence amplified from the Pacific indicates a low-temperature member of the Crenarchaeota. Figure taken from Barns et al. (1996) with permission from the Proceedings of National Academy of Sciences USA.](image-url)

Figure 1. Universal phylogenetic tree based on 16S rRNA sequences. Numbers indicate the percentage of bootstrap resampling that support the indicated branches in the maximum likelihood (before slash) or the maximum parsimony method (after slash). Analyses of duplicated protein genes placed the root on the branch at the base of the bacteria. The sequence amplified from the Pacific indicates a low-temperature member of the Crenarchaeota. Figure taken from Barns et al. (1996) with permission from the Proceedings of National Academy of Sciences USA.
sulfur bacteria and purple non-sulfur bacteria. A high metabolic versatility is particularly found in the group of purple bacteria. Photoautotrophic growth is typical for purple and green sulfur bacteria, while photoheterotrophic growth is typical for purple and green non-sulfur bacteria. Chemoheterotrophic growth in the presence of oxygen is common among purple non-sulfur bacteria as well. Under anaerobic conditions in the dark, some species are able to grow by respiratory electron transport in the presence of nitrate, nitrite, nitrous oxide, dimethylsulfoxide (DMSO) or trimethylamine-N-oxide (TMAO). These electron acceptors may also serve as auxiliary oxidants to provide a sink for electrons during photoheterotrophic growth on highly reduced carbon substrates (Ferguson, Jackson & McEwan, 1987). In addition to the diverse pathways for energy generation, there is a considerable variation in the utilization of carbon, nitrogen, and sulfur compounds for assimilation and dissimilation among the phototrophic bacteria (Imhoff, 1995).

In all purple bacteria the photosynthetic apparatus is located within intracytoplasmic membranes. Initially, two types of purple bacteria were distinguished on the basis of their ability to form globules of elemental sulfur inside the cell: Thiorhodacaea and Athiorhodaceae. Later, these two groups were renamed to Chromatiaceae and Rhodospirillaceae, respectively. When it became known that some purple bacteria were able to accumulate elemental sulfur outside the cell, they were considered as a separate family, the Ectothiorhodospiraceae. Surprisingly, analysis of 16S rRNA sequences revealed deep branches among different groups of phototrophic purple bacteria as well as close relationships of phototrophic purple bacteria with some non-phototrophic chemotrophic bacteria. This led to the proposal of a new group, containing all purple bacteria and their chemotrophic relatives, called the proteobacteria. This group is divided into five subgroups called α, β, γ, δ, and ε, among which only the first three include phototrophic bacteria. The Chromatiaceae and Ectothiorhodospiraceae form separated groups within the γ-subgroup of the proteobacteria, whereas the purple non-sulfur bacteria encompass a more heterogeneous group of bacteria, belonging to the α- and β-subgroups of the proteobacteria (Imhoff, 1995).

In many cases 16S rRNA sequences of purple non-sulfur bacteria are more similar to those of chemotrophic bacteria than to those of other members of the group of purple non-sulfur bacteria (Stackebrandt, Rainey & Ward-Rainey, 1996). A striking example is the high similarity between the chemotrophic Paracoccus group and the phototrophic Rhodobacter group. Another relevant taxonomic insight, evolving from the analysis of fatty acid composition and 16S rRNA sequences, is the reevaluation of phylogenetic relationship among the Ectothiorhodospiraceae (Imhoff & Suling, 1996). This led to the removal of extremely halophilic species from the genus Ectothiorhodospira and their transfer to the new genus Halorhodospira. So far, the renaming of Ectothiorhodospira halophila has been mostly neglected in the literature. Also in this thesis Halorhodospira halophila is consistently referred to as Ectothiorhodospira halophila, since the renaming took place during the period of research described here. Three species of photosynthetic bacteria were cultured for and used in a wide variety of studies reported in this thesis: Ectothiorhodospira halophila, Rhodospirillum salexigens and Rhodobacter sphaeroides. Each of these species will be described in the paragraphs below.

Ectothiorhodospira halophila strains have been isolated from salt lakes like Summer Lake, Oregon, USA and the Wadi Natrun in Egypt. The type strain SL-1 has been isolated from Summer Lake (Raymond & Sistrom, 1967; Raymond & Sistrom, 1969) and 4 other strains from the Wadi Natrun (Imhoff, Hashwa & Truper, 1978). As can be concluded from its name, Ectothiorhodospira halophila is a red-colored spirillum that deposits sulfur outside the cell and is dependent on high salt concentrations. E. halophila is a motile, Gram-negative bacterium; it swims by use of two single flagella present at the poles of the cell. The width of E. halophila cells is 0.8 μm and their length 5 μm; it should
be noted that the size of the cells is strongly dependent on the growth conditions. The DNA base composition is 68.4% guanine plus cytosine. *E. halophila* is able to use sulfide, sulfur, thiosulfate, succinate and acetate as photosynthetic electron donors and requires at least one reduced sulfur source for growth. Photoautotrophic growth occurs only under anaerobic conditions with a minimum doubling time of about 6.5 hours. The temperature optimum for growth is 47°C and the maximum temperature is 50°C. The pH optima were different for the type strain and the strains isolated from alkaline brines in the Wadi Natrun with pH values up to 11; the pH optima ranged from 7.4-7.8 to 8.5-9.0, respectively. Growth occurs in sodium chloride concentrations from 8% to 30%, but is optimal between 11% and 22%. Below 3% sodium chloride cells start to lyse and form faint spiral ghosts. *E. halophila* belongs to the γ-subgroup of the proteobacteria and is the most halophilic bacterium isolated so far.

*Rhodospirillum salinum* WS 68 has been isolated by Sistrom from partially evaporated pools of seawater with decaying plants on the Oregon coast, USA (Drews, 1981). Cells are curved in a spiral of one or two complete turns, 0.8 μm wide and 3.5 μm long. The bacterium has a Gram-negative cell envelope and is motile by means of bipolar polytrichous flagella. The guanine plus cytosine content of its DNA is 64 ± 2%. *Rs.* salinum grows under photoheterotrophic conditions with acetate as a carbon source, optimally at 30°C with a doubling time of 7 hours. At temperatures higher than 45°C cells grow slowly and are transformed into spheroplasts. *Rs.* salinum is also able to grow under chemoheterotrophic conditions (aerobically in the dark) with similar doubling times as phototrophic cultures. Photoautotrophic growth in the presence of carbon dioxide and reduced sulfur compounds in mineral medium was not observed. In addition, the cells do not show globules of intracellular sulfur. *Rs.* salinum grows in sodium chloride concentrations from 5% to 20%, but growth is optimal between 6% and 8%, with a pH optimum of 7.0 ± 0.4. *Rs.* salinum belongs to the γ-subgroup of the proteobacteria and is the first described species of the family of the Rhodospirillaceae that is salt-dependent.

*Rhodobacter sphaeroides* (previously known as *Rhodopseudomonas sphaeroides*) has been isolated from mud and stagnant bodies of water exposed to light (Van Niel, 1944). The cells are spherical from 0.7 μm to 4 μm in diameter. The bacterium is Gram-negative, non-halophilic and motile by a single flagellum. Its guanine plus cytosine content is 68.4-69.9%. *Rh.* sphaeroides is photoheterotrophic, facultatively aerobic, growing either anaerobically in the light (greenish brown cultures) or aerobically in the dark (red cultures). Its photosynthetic pigments consist of bacteriochlorophyll a and carotenoids including spheroidene and hydroxyspheroidene, which are converted into the corresponding ketocarotenoids under aerobic conditions, causing the color change. Growth occurs in mineral media of simple organic substrates and bicarbonate, supplemented with thiamine, biotin and nicotinic acid. Molecular hydrogen can serve as an electron donor for growth. The pH range for growth is 6.0-8.5 with an optimum at pH 7.0; the optimal growth temperature is 30°C. *Rh.* sphaeroides belongs to the α-subgroup of the proteobacteria.

**Molecular genetics in *Rh. sphaeroides***. Genetic techniques have become widely used in the study of photosynthetic purple bacteria. The majority of these have been applied to the two closely related species of purple non-sulfur bacteria *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, establishing them as model systems for studies on many aspects of important biological processes. Under aerobic growth conditions *Rhodobacter* has a physiology similar to the colorless, non-photosynthetic members of the proteobacteria, but a reduction of oxygen tension induces differentiation of the intracellular membrane, resulting in the formation of a membrane system which contains pigment-protein complexes. These protein complexes constitute the photosynthetic machinery, consisting of the reaction center and the light harvesting complexes LHI and LHII. Light energy is absorbed by the light harvesting complexes and directed to the reaction center, where
excitation energy drives a cyclic flow of electrons, generating a proton motive force. Radiation damage is avoided by among others the carotenoid pigments, which dissipate excessive light energy. Besides regulation of photosynthesis (Bauer & Bird, 1996; Zeilstra-Ryalls et al., 1998), fields of Rhodobacter research include nitrogen fixation (Krnan & Cullen, 1995), carbon dioxide fixation (Tabita, 1995), photo- and chemotaxis (Armitage, 1997), transport (Forward et al., 1997) and quorum sensing (Puskas et al., 1997).

A common method for transferring DNA into purple bacteria is conjugation with use of Escherichia coli as a donor strain. Matings are performed by mixing donor and recipient strain and plating on a solid surface, like an aged agar plate or a membrane filter. Selection for the recipient strain Rhodobacter can be achieved by an auxotrophic marker in the E. coli donor strain. A plasmid often used for conjugal transfer to Rhodobacter is the pBR325 derivative pSUP202 (Simon, Priefer & Puhler, 1983). This plasmid is mobilizable by an inserted Mob site from the broad host range plasmid RP4. This Mob site includes the origin of transfer (oriT) and acts a recognition site for RP4 transfer functions. These transfer functions are provided in trans either from a helper plasmid in a triparental mating, or from a donor strain such as E. coli S17-1, in which the transfer genes of RP4 have been integrated in the chromosome. The RP4 kanamycin resistance marker has been inactivated in E. coli S17-1 by a Tn7 insertion in order to maintain this marker for positive selection of the recipient (Simon et al., 1983).

An alternative method for DNA transfer is transduction by use of bacteriophages. Although a number of endogenous phages have been isolated from purple bacteria, they have not shown to be useful for transduction. A related system for gene transfer though, has been found in Rhodobacter capsulatus, called the gene transfer agent (GTA), which consists of phage-like particles that package approximately 4.5 kb linear DNA fragments (Marrs, 1974). GTA particles are not capable of transferring the ability to produce GTA particles to recipients, so they can be considered as pre-phage particles that confer the advantage of genetic exchange or as a defective phage population. The ability of GTA to transfer short linear DNA fragments has been used extensively in mapping genes and gene replacements (Williams & Taguchi, 1995). In addition, protocols for alternative ways of DNA transfer to Rhodobacter like transformation of CaCl2-treated cells and electroporation, have been developed. The efficiency of DNA transfer using the latter two methods, expressed as the number of transformants per microgram of DNA, is a factor of about 10⁶ lower as compared to the efficiency of similar methods applied to E. coli (Donohue & Kaplan, 1991).

Broad host range plasmids can be used to shuttle DNA fragments between E. coli and Rhodobacter in order to complement genetic defects or to overexpress genes. Broad host range vectors have been reduced in size and engineered such that they contain antibiotic resistance markers and unique restriction sites for cloning. A set of widely used vectors includes derivatives of RK2 (Pansegrau et al., 1994), such as pARO180 (Parke, 1990), pRK415 (Keen et al., 1988) and pRK290 (Ditt et al., 1980). These incompatibility group Pa (IncPa) plasmids are lost from up to 50% of the cells when Rhodobacter is grown for six to eight generations without antibiotic pressure. Not only IncPa plasmids are suitable for DNA shuttling, but also plasmids derived from RSF1010 (Scholz et al., 1989), which belong to the IncQ incompatibility group, like pKT210 (Bagdasarian et al., 1981). In addition, cosmid derivatives of pRK290 were constructed, like pLA2917 (Allan & Hanson, 1985), allowing the selection of large DNA insertions of up to 30 kb by in vitro packaging into bacteriophage lambda, suitable for the construction of genomic libraries. Rhodobacter is sensitive to most common antibiotics used for selection, like ampicillin, chloramphenicol, kanamycin, streptomycin and tetracyclin; it should be noted that the resistance genes encoding the resistance towards the former two antibiotics are expressed insufficiently for use in Rhodobacter. To overcome the problem of low (heterologous) expression of genes in photosynthetic bacteria from their own
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promoter, overexpression vectors have been constructed, like pCHB500, in which the promoter region upstream of the *Rb. capsulatus* cycA gene is inserted into pRK415 (Benning & Somerville, 1992).

Random mutagenesis in photosynthetic bacteria can be performed by UV irradiation or by exposure to chemical agentia, like nitrosoguanidine (NTG) or ethylmethane sulfonate (EMS). To overcome the problem of small mutation frequencies due to *in vivo* DNA repair mechanisms, one could subject cloned fragments to mutagens *in vitro* and transfer them back into the host for screening. Random insertions in chromosomal or plasmid DNA can also be obtained by transposon mutagenesis. Transposons are generally transferred by a mobilizable suicide vector, a strategy where survival of the recipient depends on the insertion of the transposon with a selection marker in the genome before the plasmid is lost. The most widely used system of this type is the suicide plasmid pSUP2021 (Simon *et al.*, 1983), which contains the Tn5 transposon (Reznikoff, 1993). After a phenotypic screen, the insertion site of the transposon can be identified by cloning a restriction fragment containing the antibiotic resistance marker or by complementation with a genomic library (Williams & Taguchi, 1995).

Alternatively, specific genes can be inactivated with a site-directed chromosomal insertion by homologous recombination through a double crossing-over event. To carry out this strategy, referred to as interposon mutagenesis (Prentki & Krisch, 1984), an antibiotic cassette is inserted into a restriction site of a clone from the target strain followed by insertion of the interrupted gene into a suicide vector and transformation of the target strain. The *ColEl* plasmid pSUP202 (Simon *et al.*, 1983) is often used for this purpose, since it is unable to replicate outside the enteric bacteria. In addition, an in frame deletion of a gene can be created by a method, consisting of two steps: (i) chromosomal integration through a single crossing-over event of a non-replicating plasmid containing only the adjacent flanking regions of the relevant gene, followed by (ii) excision of the integrated plasmid from the chromosome. One can select for the first event by resistance against an antibiotic, encoded by the integrated plasmid and for the second event by sensitivity towards this antibiotic, after growing the cells for several generations. The advantages of this method over interposon mutagenesis include the absence of a marker in the chromosome of the mutant and the minimization of polar effects. The disadvantages are the absence of a marker for selection of mutants in step two (excision of the plasmid can result in wild-type as well) and the low frequency of the excision event in step two. To overcome the latter problem, a strategy for positive selection in step two was tested in *Rb. sphaeroides* and proven successful (Hamblin *et al.*, 1997b). This method includes the use of the vector pK18mobsacB, constructed by Schafer *et al.* (1994). This vector contains the *sacB* gene, encoding an extracellular enzyme that hydrolyzes sucrose, a reaction with a toxic by-product. This allows positive selection for the second recombination event, which leads to the loss of the *sacB*-containing vector, resulting in sucrose resistance.

A macrorestriction map, representing the complete physical map of *Rb. sphaeroides* type strain 2.4.1, has been constructed with the use of restriction enzymes that rarely cut GC-rich DNA (*e.g.* *AseI*, which cuts the DNA sequence 5'-ATTAAT-3'). The large DNA fragments generated by these enzymes were analyzed through separation by transverse alternating field electrophoresis (TAFE). This method, reviewed by Dawkins (1989), is also useful for the generation of DNA fingerprints in order to differentiate between species. The TAFE in combination with Southern hybridization analysis resulted in the estimation of the size of the entire genome of 4.5 Mb, comprising two different circular chromosomes: chromosome I of 3 Mb and chromosome II of 0.9 Mb (Suwanto & Kaplan, 1989). In addition, *Rb. sphaeroides* 2.4.1 harbors 5 endogenous plasmids of approximately 42, 95, 97, 105 and 110 kb (Fornari, Watkins & Kaplan, 1984). A number of genes have been shown to exist in duplicate copies in chromosome I and chromosome II. The iso-enzymes
encoded by these duplicate genes are structurally similar, but in most cases differentially expressed. The recent publication of the sequence of 291 kb of chromosome II shows that major metabolic functions are represented on this chromosome (Choudhary et al., 1997). In addition, a 189 kb segment of the closely related *Rb. capsulatus* has been sequenced, i.e. 5% of the single 3.8 Mb chromosome in this bacterium (Vlcek et al., 1997).

1.2 How bacteria respond to their ambient environment

Sensing and signaling in bacteria: two-component systems. Research on the mechanism and function of signal transduction systems in bacteria has evolved from being non-existent in the sixties into a mature field of science in the nineties. Bacteria were initially considered to be too small and too simple to possess or need signal transduction systems, as discussed by for example Hellingwerf (1988), but it is now known that most of these organisms do contain a multitude of modules dedicated to this task. Below, the limited number of basic types that predominate among them will be discussed.

To define the process of signal transfer in bacteria precisely is a difficult task, but there is consensus that it encompasses the response of microorganisms to chemical or physical signals from their environment. These environmental stimuli elicit a change in gene expression (genomic response), or modulate the migration pattern of the cell (locomotor response). In this introduction, the definition of signal transfer is limited to those processes in which an extracellular signal leads to an intracellular response in terms of one of the processes summed up above. Considering signal transfer in bacteria in these terms, it appears that two basic modes predominate. In the first, true transmembrane signal transfer takes place, based on the conversion of the presence of an extracellular signaling molecule into an intracellular response of an entirely different chemical nature, by a transmembrane signal transfer protein. In the alternative process, the signaling molecule can enter the cell, either through passive diffusion or via one of the intrinsic permeases of the cell. The molecule is then reacted upon by the first cytoplasmic receptor protein in the chain that can respond to the presence of that particular signaling compound. The responses to physical stimuli as temperature, light or even sound (Matsuhashi et al., 1996), to which neither the cytoplasmic membrane, nor the entire cell envelope is a barrier, would also fall into the latter category, unless membrane incorporated receptors are also involved in the perception of such signals.

In the early eighties, with the first releases of sequence analysis software, a surprising relationship was uncovered between proteins that had never been thought to be related (Kofoid & Parkinson, 1988; Nixon, Ronson & Ausubel, 1986). These proteins play a role in the regulation of nitrogen metabolism in enterobacteria, in chemotaxis in *E. coli* and in sporulation in *Bacillus subtilis*. They were classified into two protein families: the sensors and the regulators (see figure 2), each corresponding pair forming the basic unit of a two-component regulatory system. Most sensors

![Figure 2. The two-component system. A sensor protein, which in most systems is present as an intrinsic membrane protein, recognizes and binds a periplasmic signaling molecule (1); this leads to an increase in the kinase activity of its cytoplasmic transmitter domain (2). Phosphoryl groups are subsequently transferred to the receiver domain of a cognate cytoplasmic regulator (3). The effector domain of this latter protein mediates the response (4).](image-url)
are intrinsic membrane proteins with two or more N-terminal transmembrane α-helices. The C-terminus forms an independently folded domain that extends into the cytoplasm, binds ATP and displays autokinase activity. This C-terminal domain, which is called a transmitter domain, is approximately 250 amino acid residues in size and displays significant similarity among all members of the sensor-family (figure 3A). Within this domain various signature sequences for nucleotide binding can be detected, as well as a conserved histidine.
residue, which is the target for the autokinase activity. This kinase activity is modulated by the presence of the cognate signal molecule, which is often sensed by binding to the periplasmic domain of the sensor. Most regulators are cytosolic proteins, composed of two independently folded domains (figure 3B). Of these, the N-terminal domain (approximately 125 amino acid residues in size) is the recurring element among the regulators, the C-terminal domain displays significant homology only amongst certain subclasses of regulators as for instance, those that function in combination with a specific minor Sigma-factor. The N-terminal domain of a regulator, called the receiver domain, can be phosphorylated by phosphoryl transfer from the autokinase domain of the cognate sensor. The enzyme activity required for this phosphoryl transfer and for determining its specificity is located in the regulator rather than in the sensor (Lukat et al., 1992). This phosphorylation activates the C-terminal domain of the regulator, resulting in the activation of the corresponding response system. In many systems, the sensors also have phosphatase activity towards the phosphorylated regulators, particularly when their signaling molecules are absent (Ninfa et al., 1993).

Current thinking about the structure of sensors and transmembrane signaling is guided by what is known about the methyl-accepting chemotaxis proteins from *E. coli*, though these proteins lack the typical histidine protein kinase domain of the usual two-component sensors, as described above. The spatial structure of the periplasmic domain of Tar, the MCP that functions in the detection of aspartate, has been determined by X-ray crystallography (Jancarik et al., 1991). A hypothetical structure of the transmembrane and periplasmic part of this sensor has been proposed on the basis of molecular modeling (Milburn et al., 1991). The conformational change during transmembrane signaling has been investigated by use of the X-ray crystallographic data and cysteine scanning mutagenesis (Careaga & Falke, 1992; Scott & Stoddard, 1994). Differences were revealed in the formation of disulfide bridges between engineered cysteines in the presence and absence of the signaling ligand as well as in the rate of formation of these bridges. The model resulting from these studies describes the conformational change as a piston movement of one of the 4 transmembrane α-helices of a dimer of MCPs (Chervitz & Falke, 1996). A second study based on crystallographic data of apo- and aspartate-bound forms of the Tar sensing domain did not reveal any conformational changes in the relative positions of α-helices within a receptor monomer, but detected an intersubunit rotation between the two monomers (5-8°) as the presumed transmembrane signal upon ligand binding (Chi, Yokota and Kim, 1997). Recent results indicate that Tar is present in a cluster of dimers in a signaling array, which may suggest a role for lateral interactions in transmembrane signaling (Levit, Liu & Stock, 1998). Lateral signal propagation may also play a role as a mechanism to control sensitivity by increasing the gain for a response, by switching neighbouring receptors to a signaling state (Bray, Levin & Morton-Firth, 1998).

Information available on the chemotaxis regulator protein CheY has provided insight into the structure of the regulator domains. The structure of the non-phosphorylated form of this protein was resolved through X-ray crystallography (Stock et al., 1989a). CheY essentially corresponds with the N-terminal domain of the average regulator and has an α/β-barrel structure with 5 α-helices surrounding a 5-stranded parallel β-sheet. In the regulators, which are folded into two separate domains, phosphorylation of this N-terminal domain must modulate the activity of the independently folded C-terminal domain. Recently, the structure of CheY and CheY-P were compared using 1H-NMR spectroscopy. These studies revealed that upon phosphorylation nearly half of all the resonances in the spectrum of CheY were shifted to a different position (Lowry et al., 1994), indicating that a major conformational transition is induced by the phosphoryl transfer. It was already predicted on the basis of sequence comparisons that the so-called γ-turn loop of the regulator domains may form a hinge for the
conformational transition that moves regulators into the signaling state (Volz, 1993).

Little is known about the actual signal molecule that is perceived by the sensor for most two-component regulatory systems. This is because most of these systems were identified in sequence analysis projects (figure 3C). There are exceptions like UhpB, which detects the periplasmic concentration of glucose 6-phosphate (Weston & Kadner, 1988), FixL which detects molecular oxygen (Gilles-Gonzalez, Ditta & Helinski, 1991) and NarX and NarQ which sense nitrate and nitrite (Stewart & Rabin, 1995; Williams & Stewart, 1997).

Variations on the basic theme as outlined above do occur. The sensor may be a soluble cytoplasmic protein as in the Ntr system (Miranda-Rios et al., 1987), and even when the sensor is an intrinsic membrane protein, the signal sensing domain may be located on the cytoplasmic rather than on the periplasmic side of the membrane as in PhoB/R (Scholten & Tommassen, 1993). Also, the regulator may essentially be a single-domain protein, like CheY and SpoOF (Stock et al., 1989a; Trach et al., 1985), and the sensor and regulator domain may be combined into a single protein, like in FrzE (McCleary & Zusman, 1990).

Recently, it has been demonstrated that signaling components with homology to bacterial sensors and regulators also occur in eukaryotic cells, such as yeast (Ota & Varshavsky, 1993) and Arabidopsis (Chang et al., 1993). These eukaryotic systems form a specific subset of the two-component systems together with for instance Arc from *E. coli* (Iuchi & Lin, 1992), Kin/Spo from *B. subtilis* (Burbulya, Trach & Hoch, 1991) and Bvg from Bordetella pertussis (Uhl & Miller, 1996). Members of this subclass contain the so-called hybrid sensory kinases (figure 3A), in which three amino acid side chains (His, Asp and His) subsequently carry the phosphoryl from ATP to the regulator. The two histidines are part of a histidine protein kinase domain and a histidylphosphate transfer domain, respectively, while the aspartate is part of a regulator domain. These systems with more than two consecutive phosphoryl-carrying amino acid side chains are referred to as multi-step phosphorelay systems (Appleby, Parkinson & Bourret, 1996). It remains to be determined whether a general rule for the direction of the flow of phosphoryl-groups in the multi-step phosphorelay systems can be formulated. It has been reported for the yeast Sln/Ypd/Ssk system that phosphoryl flow is by necessity unidirectional from the histidine protein kinase domain to the second regulator (Posas et al., 1996). However, in the Arc system it has been reported that phosphoryl groups can flow in both directions, so also from the histidylphosphate transfer domain to the first regulator domain (Tsuzuki, Ishige & Mizuno, 1995). The observations made in the former systems create a conceptual problem. The free energy of hydrolysis of a histidylphosphate is much larger than the free energy of ATP-hydrolysis, while the free energy of an aspartyl-phosphate is less than that of ATP-hydrolysis (Stock, Ninfa & Stock, 1989b). Thus, it is difficult to understand how a regulator can phosphorylate a histidylphosphate transfer domain. A way to solve this dilemma is to assume that the protein environment in the sensor or regulator in these multi-step phosphorelay systems significantly influences the thermodynamics of the (de)phosphorylation reaction of the histidyl- and aspartyl-phosphate.

In many two-component systems the situation is more complex than in the basic two-component module. Some phosphoryl-transfer pathways diverge, as more than one regulator is phosphorylated by a single kinase, like the Spo system in *B. subtilis* which is involved in the regulation of sporulation as well as competence development (Burbulya et al., 1991). Others converge because more than one kinase phosphorylates a single response regulator, as for instance PhoB (Wanner & Wilmes-Riesenberg, 1992), NarL (Schröder et al., 1994), RegA (Mosley, Suzuki & Bauer, 1994), and LuxN (Bassler, Wright & Silverman, 1994). The phenomenon of divergence and convergence of phosphoryl transfer pathways and the striking sequence homology between sensors and regulators brings up the question of the degree of specificity in phosphoryl transfer activity among the sensors and regulators of the
various pathways that operate in parallel in a single cell. Assuming that neither the sensors nor the regulators have absolute specificity for a single partner, it follows that also phosphoryl transfer between pathways will occur. This phenomenon is referred to as cross-talk. Ample evidence is available that cross-talk occurs in vitro; the amount of evidence for the occurrence of this phenomenon in vivo is more restricted (McCleary, Stock & Ninfa, 1993; Wanner & Wilmes-Riesenberg, 1992; Yackel et al., 1997). Detailed understanding of the different responses of bacteria to signals from the fluctuating environment will be impossible without a quantitative description of the degree of cross-talk between various systems in a single cell. It remains to be determined whether the involved components form one large interconnected network and whether the strength of these connections varies to such an extent that it is relevant to discriminate, more or less isolated, subdomains within a presumed 'phospho-neural network' of individual bacterial cells (Hellingwerf et al., 1995). Extensive mutagenesis screens in both B. subtilis and E. coli have revealed another basic component that functions in signaling via the two-component system based phosphoryl-transfer pathways. These are protein phosphatases, which function in the Spo system of B. subtilis (Perego & Hoch, 1996) and in signaling for the presence of denatured proteins in the periplasm of E. coli through CpxA and CpxR (Missiakas & Raina, 1997). These proteins display homology to serine/threonine and tyrosine phosphatases and are also active towards phosphorylated histidines and aspartates, as has been demonstrated in vivo (Perego, 1997) and in vitro (Missiakas & Raina, 1997). These protein phosphatases provide yet another level at which interactions between phosphoryl transfer pathways may occur.

The histidine-kinase activity of several sensors occurs through phosphoryl transfer between the two halves of a dimer of sensor molecules (Ninfa et al., 1993; Pan et al., 1993; Yang & Inouye, 1991). For the sensing part of the kinases that function in signal transfer in chemotaxis (the MCPs), the importance of dimer formation has been explicitly demonstrated in a study of in vitro ligand-binding to isolated MCPs (Biemann & Koshland, 1994). It was observed that ligand binding to MCPs shows strong negative cooperativity. Recent results indicate that transmembrane signaling occurs within receptor clusters rather than through isolated dimers (Levit et al., 1998). This functional clustering of receptors may be related to the non-uniform lateral distribution of the MCPs in the cell-surface, as indicated by the results of immunogold labeling studies on thin sections of E. coli. In these studies it was reported that the MCPs cluster in the pole of the cell, opposite to the side where the flagella bundle (Maddock & Shapiro, 1993, Shapiro & Losick, 1997). It is not yet known whether such a non-random distribution, or even clustering of receptors, is relevant for other sensors nor even whether negative cooperativity plays a role in signal transfer through regular sensors of a two-component system. Interestingly, deviations from Michaelis-Menten type of kinetics were observed in an analysis of the intensity of signal transfer through the Uhp system in response to variations in the concentration of glucose 6-phosphate (Verhamme & Hellingwerf, unpublished observations). This deviation from Michaelis-Menten kinetics may be explained by lateral interactions between adjacent sensor molecules.

The great majority of the known transmembrane signal transfer systems belong to the sensor-class of the two-component systems. Nevertheless, there are some exceptions, for example, the sensors involved in the detection of ϒ-lactam antibiotics, BlaR (Hardt et al., 1997) and for ferric citrate in the periplasm of enteric bacteria, FecR (Braun, 1997) are of an entirely different type. However, these two signal transduction proteins may also be structurally similar, based upon the speculation that both are members of an emerging class of sensors that function through the activation of a cytoplasmic protease domain by their respective signaling molecules. Even light may activate such a pathway, like in the light-activated carotenoid synthesis in Myxococcus mediated by
CarQ/R/S (Gorham *et al.*, 1996). The viscosity induced, flagella-mediated, signal transfer system in *Vibrio* is an example of yet another completely unrelated system (Kawagishi *et al.*, 1996).

**Chemotaxis: the enteric paradigm versus *Rb. sphaeroides***. Chemotaxis in the enteric bacteria *E. coli* and *Salmonella typhimurium* can be considered as well-understood systems of signal transduction in biology. These cells swim by rotation of six to eight flagella, which are inserted at random in their cell envelope. They move in a three-dimensional random walk, by changing intermittently the direction of rotation of the flagellar motors. Rotation in the counterclockwise direction causes coalescence of the flagella into a bundle, propelling the cells forward (smooth swimming). Clockwise rotation disperses the bundle, resulting in a chaotic motion that randomly reorients the cell (tumbling). In homogeneous environments, cells tumble about once a second, each time randomizing the next swimming direction. In case cells swim towards increasing concentration of attractants (*e.g.* sugars and amino acids) or a decreasing concentration of repellents (*e.g.* fatty acids and alcohols), tumbles will be suppressed, leading to an extension of the runs into the direction of the beneficial environment. The small size and rapid movements of bacteria exclude sensing of gradients based on spatial comparisons. Instead, spatial gradients are sensed by a temporal mechanism. Cells determine the temporal change in concentration of chemoeffectors by comparison of the occupancy of their chemoreceptors with that of a few seconds ago. The mechanism for this temporal comparison includes sensory adaptation by reversible methylation of the chemoreceptors, which cancels the receptor output in a homogenous environment, independent of the ambient chemoeffector concentration. Typically, attractants and repellents are sensed in the periplasm by direct interaction with specific chemoreceptors, and not by their physiological effects during or after transport into the cell. Exceptions to this rule are the carbohydrate phosphotransferase system for sensing of sugars and presumably, the sensing mechanism for aerotaxis, as described in more detail below.

The proteins essential for chemotaxis can be divided into three classes: transmembrane chemoreceptors, cytoplasmic signaling components and enzymes involved in the adaptation mechanism. The chemoreceptors that mediate transmembrane signaling are also known as transducers or methyl-accepting chemotaxis proteins (MCPs), due to the presence of four or five glutamate residues, which can be reversibly methylated. *E. coli* contains four MCPs that primarily sense serine (*Tsr*), aspartate and maltose (*Tar*), ribose, galactose and glucose (*Trg*), and dipeptides (*Tap*). *S. typhimurium* lacks Tap but possesses a citrate sensor (*Tcp*) instead. Serine, aspartate and citrate bind directly to the receptors, whereas maltose, ribose, galactose and glucose bind to periplasmic binding proteins, which then interact with the respective MCPs. MCPs are present as homodimers, each unit consisting of a transmembrane helix (TM1), a periplasmic sensing domain, a second transmembrane helix (TM2), and a cytoplasmic signaling domain. The cytoplasmic signaling components, which mediate communication between the receptors and the flagellar switch protein, include the histidine protein kinase CheA, the response regulator CheY, the receptor-linker protein CheW and CheZ, which enhances dephosphorylation of CheY. CheA and CheY constitute a two-component system, be it with several deviations from the standard system: CheA contains a C-terminal input domain, the transmitter in the center and a separate, small N-terminal domain with the phospho-accepting histidine (figure 3A). CheY contains only a receiver domain, it does not have an effector domain for DNA-binding as present in many other response regulators (figure 3B). The activity of CheA is controlled by the MCP-signaling domain in a ternary complex. This complex consists of an MCP dimer, or rather a cluster of MCP dimers (Levit *et al.*, 1998), which is linked to a CheA dimer by two CheW monomers. When an attractant binds to the receptor, it inhibits autophosphorylation activity of CheA, decreasing phosphotransfer from
CheA-P to the conserved aspartate residue in CheY. The latter protein binds in its phosphorylated form to the flagellar motor-switch complex, causing clockwise rotation of the flagella and tumbling of the cell. Thus, the receptors trigger behavioral responses by decreasing the level of phosphorylated CheY, leading to a suppression of tumbling, thus an increase of the run lengths, when the concentration of attractant increases. The accumulation of CheY-P is prevented by CheZ, which accelerates the conversion of CheY-P into its non-phosphorylated form. Binding of a repellent to the receptor will result in an increase of the level of CheY-P and an induction of tumbling, leading to smaller run lengths.

The ability of a receptor to stimulate CheA is not only determined by the binding of stimulatory ligands, but also by its level of glutamate methylation. The process of adaptive receptor methylation is mediated by the methyltransferase CheR and the methylesterase CheB. The methyltransferase is constitutively active and transfers methyl groups from S-adenosylmethione to glutamate residues in the cytoplasmic domain of the MCPs. In contrast, the activity of CheB is inducible: it only removes these methyl groups from the MCPs when its N-terminal domain is phosphorylated by CheA. In the steady state, the activity of CheR and CheB results in an average receptor methylation level of 0.5 to 1 methyl group per subunit, maintaining a certain tumble frequency, hence a random walk.

Figure 4 shows that binding of an attractant to the receptor inhibits CheA activity, resulting in a decrease of the level of CheY-P, as well as that of CheB-P (slower than that of CheY-P, because CheB-P is not a substrate for CheZ). The latter effect of CheA activity provides a negative feedback loop, since it results in a decrease of the rate of receptor demethylation, causing an increase in the methylation level of the receptor, which tends to have a stimulating effect on CheA activity. Thus, the process of adaptive receptor methylation results in the return of the run lengths of the cell to prestimulus level, even in the presence of the attractant. For a more detailed description of signaling in chemotaxis of enteric bacteria, see reviews by Hazelbauer (1988), Levit et al. (1998), Manson et al.,

![Diagram of the chemotactic signaling pathway](image-url)

**Figure 4.** Diagram of the chemotactic signaling pathway (see text for details). Abbreviations: OM, outer membrane; IM, inner membrane; A, CheA; W, CheW; Y, CheY; Z, CheZ; R, CheR; B, CheB; G, FliG; M, FliM; N, FliN; p, phosphate; CH₃, methyl group (shown as lollipop-like objects on the cytoplasmic domains of the receptors). Figure taken from Manson et al. (1998) with permission from Journal of Bacteriology.
(1998), Parkinson (1993) and Stock and Surette (1996). An alternative sensing mechanism is known for some sugar molecules that function as attractants (e.g., mannitol, mannose and glucitol). These substrates are transported into the cell by the phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (PTS). Uptake of PTS carbohydrates requires phosphorylation through a histidine kinase (EI), a phosphohistidine carrier protein (Hpr) and sugar-specific phosphoryl-transfer proteins. Addition of one or more of these sugars to the cell will lead to a lowering of the average phosphorylation level of EI. The dephosphorylated EI molecules inhibit auto-phosphorylation activity of the autokinase CheA, thus connecting this pathway to the MCP-dependent chemotactic signaling pathway (Lux et al., 1995). Quite some amount of progress in the understanding of another alternative sensing mechanism resulted from E. coli genome sequencing. Analysis of open reading frames led to the identification of a fifth transducer protein, designated to Aer, because of its role in aerotaxis and energy responses (Bibikov et al., 1997; Rebbapragada et al., 1997). The Aer protein contains a hydrophobic region that could anchor the protein to the membrane and a putative cytoplasmic signaling domain for the modulation of CheA autophosphorylation, but deviates in many other ways from the classical MCPs. It contains an N-terminal FAD-binding domain, but lacks a periplasmic sensing domain. Its cytoplasmic C-terminal domain lacks the consensus sequence for reversible methylation, which is A/S-X-X-E*-X-A/S/T-A-A/S/T (Hazelbauer, 1988). A disruption of the aer gene in E. coli diminishes, but does not abolish aerotaxis. This and other observations suggest a role for the serine receptor as a second transducer protein in aerotaxis (Rebbapragada et al., 1997). Indeed, an aer tsr double mutant shows no aerotaxis, while this mutant still responds to aspartate and mannitol, as mediated by Tar and the PTS, respectively. In addition, Aer and Tsr were found to mediate responses to other compounds (e.g., quinone analogs), that like oxygen, affect the electron transport chain. Aer and Tsr sense the overall energy-state of the cell, rather than the concentration of specific compounds. Redox sensing by the FAD-binding domain in Aer may include a widespread mechanism, as the N-terminal part of the protein contains a PAS domain, which is shared by many other proteins involved in oxygen or light sensing (Zhulin, Taylor & Dixon, 1997). The membrane topology of Aer points into the direction of a new mechanism for tactic signal transfer in E. coli, i.e., between two cytoplasmic domains, instead of the transmembrane signaling between a periplasmic and a cytoplasmic domain in case of the classical MCPs. The mechanism of adaptation in aerotaxis in E. coli is not clear. Aerotaxis can occur in absence of protein methylation and clear consensus recognition sites for the methyltransferase CheR are absent (Rebbapragada et al., 1997). Unlike Aer, the Tsr chemosensor is hard to picture as a redox sensor, because it does not contain a redox-sensitive cofactor. Alternatively, Tsr has been proposed to sense the proton motive force directly, as an indicator for the energy-state of the cell (Rebbapragada et al., 1997; Stock, 1997).

Motility in Rb. sphaeroides is different from that in the enteric bacteria; it swims by unidirectional, clockwise rotation of a single flagellum at speeds of up to 80 µm/s with an average of 35 µm/s (for comparison E. coli swims with an average speed of 20 µm/s). About every 10 seconds swimming by Rb. sphaeroides is interrupted by a stop with a duration of about 1 s (Armitage & Macnab, 1987). While stopping, the flagellum relaxes from the distal end into a coiled conformation (large-amplitude, short-wavelength). During the stop, the flagellum slowly rotates, contributing to reorientation of the cell (Armitage & Schmitt, 1997). During the stop, the cell displays short stops that resemble the tumbles of E. coli. Chemotactic responses in Rb. sphaeroides to chemoeffectors (small organic acids and sugars) are most pronounced after removal of attractants, leading to an increase in the stop frequency and shorter runs (Packer, Gauden & Armitage & Schmitt, 1997).
In addition to chemotactic responses, *Rhizobium sphaeroides* also shows chemokinesis: a sustained increase in the rate of flagellar rotation after addition of an attractant (Packer & Armitage, 1994). Several studies showed that transport and metabolism of attractants is required for chemotaxis in *Rhizobium sphaeroides*, but transport is not the source of responses, targeting has been studied extensively over the past years (Armitage, 1997; Armitage & Schmitt, 1997). One of the genetic tools, which has been used to identify the components involved in the chemosensory system of *Rhizobium sphaeroides* is Tn5 transposon mutagenesis. Screening of Tn5 mutagenized cells never led to the isolation of a mutant defective in chemotaxis (Armitage, personal communication). The breakthrough eventually came with the isolation of chemotactic Tn5 mutants and subsequent identification of a chemotaxis operon in the soil bacterium *Sinorhizobium meliloti* (previously known as *Rhizobium meliloti*) (Greck et al., 1995). The *S. meliloti* chemotaxis genes are excellent probes for Southern blots with *Rhizobium sphaeroides* DNA, because of their close phylogenetic relationship and the similarity in guanine plus cytosine content of both organisms. Probing *Rhizobium sphaeroides* DNA with the 3' terminal part of the *S. meliloti* cheA led to the identification of a large chemotaxis operon in *Rhizobium sphaeroides* (Ward et al., 1995a). The operon structure is similar to that in *S. meliloti*: 5' cheY1-cheA-cheW-cheR-cheY2 3'. Interestingly, both contain two homologues of the cheY genes, but lack a cheZ homologue. A CheB homologue is present in the *S. meliloti* operon in between cheR and cheY2, but is absent in the *Rhizobium sphaeroides* chemotaxis operon. The most striking difference is the absence of a clear phenotype when cheA is knocked out in *Rhizobium sphaeroides*, while this obviously is not the case in *S. meliloti*, where cheA was identified as one of the Tn5 insertion sites in the screen for chemotaxis deficient mutants. This strongly suggests that a second pathway for chemosensory signaling in *Rhizobium sphaeroides* is present, although Southern blots with *Rhizobium sphaeroides* chromosomal DNA did not reveal a second copy of cheA (Ward et al., 1995a). Although the function of the *Rhizobium sphaeroides* chemotaxis operon is unclear, at least one of the chemotaxis genes encodes a functional protein: chemotaxis of an *E. coli* cheW mutant can be restored by the introduction of the *Rhizobium sphaeroides* cheW gene (Ward et al., 1995a).

The finding of the chemotaxis operon in *Rhizobium sphaeroides* suggests an MCP-dependent adaptation pathway. This is in contrast with results of previous studies, in which *in vitro* and *in vivo* methylation, methanol production assays and the use of antibodies raised against Tar in Western analyses, all indicated the absence of such a pathway (Sokett, Armitage & Evans, 1987). The presence of methylation-dependent chemotaxis in *Rhizobium sphaeroides* became more likely with the identification of two genes encoding MCP homologues upstream of the chemotaxis operon (Armitage & Schmitt, 1997; Ward et al., 1995b). These proteins, designated to TlpA and TlpB (transducer like proteins), contain the highly conserved signaling domain, but no transmembrane regions nor a periplasmic domain. Both proteins are probably located in the cytoplasm. Analysis of a tlpA mutant showed that this transducer mediates chemotaxis towards a wide range of chemoeffectors, but only under aerobic growth conditions, while tlpB is primarily expressed under anaerobic conditions. In addition, a gene encoding an MCP homologue (mcpA) was discovered by analysis of the sequence of chromosome II. This gene is not present in a chemotaxis operon, but adjacent to the dadA gene, which encodes a D-amino acid dehydrogenase (Choudhary et al., 1997). A more detailed study on CheW revealed that overproduction of this coupling factor completely inhibited motility in a flagellated *Rhizobium sphaeroides* strain (this mutant is the equivalent of an *E. coli* tumbling mutant), while there was no clear effect of a cheW deletion (Hamblin, Bourne & Armitage, 1997a). This is different in *E. coli*, where both overexpression and deletion of cheW lead to a smooth swimming phenotype, as if cells are constantly responding to an attractant (Sanders, Mendez & Koshland, 1996).
The induction of a smooth swimming signal in *E. coli* by overproduced CheW is generated by CheW-mediated inhibition of kinase activity of CheA (Ninfa *et al.*, 1991). The phenotype of the cheW deletion in *Rh. sphaeroides* suggests an alternative pathway for chemosensing, but no evidence for genes homologous to cheW was found by Southern analyses (Hamblin *et al.*, 1997a).

The approach to identify alternative pathway(s) started with the construction of a *Rh. sphaeroides* mutant with a deletion of the entire chemotaxis operon. This mutant strain was then subjected to Tn5 mutagenesis, followed by two separate screenings for mutants lacking chemotaxis and phototaxis (Hamblin *et al.*, 1997b). Only a single chemotactic mutant was isolated, which appeared to contain Tn5 insertion in a metabolic gene, encoding glucose-6-phosphate-dehydrogenase, in agreement with the finding that metabolism of attractants is required for chemotaxis (Jeziore-Sassoon *et al.*, 1998). The isolation of phototactic mutants was carried out by screening photo-trophically cultured cells for their disability to sense a light-dark boundary (Hamblin *et al.*, 1997b). One out of 12 selected mutants was analyzed and showed a Tn5 insertion into a second chemotaxis operon, with the following organization: 5' cheY3-cheA2-cheW2-cheW3-cheR2-cheB-tlpC 3' (see figure 5 for the role of the components encoded by these genes). A deletion of CheA2 was constructed in the

**Figure 5** Multiple sensory pathways for chemotaxis in the α-subgroup of proteobacteria. The pathway indicated by the single-headed broken arrows indicates an additional signaling pathway in *Rh. sphaeroides*. In *Rhodospirillum centenum*, CheA and CheY2 are fused into a single gene product. Some MCPs require the presence of CheD for CheR to methylate them, so far a unique feature of chemotaxis in the Gram-positive bacterium *Bacillus subtilis*. Abbreviations: A, CheA; A, CheA2; B, CheB; MCP, methyl-accepting chemotaxis protein; P, phosphate; R, CheR; Tlp, transducer like protein; W, CheW; Y, CheY1; Y, CheY2; Y, CheY3. Figure taken from Manson *et al.* (1998) with permission from *Journal of Bacteriology.*
wild-type strain and in a background, where the first-identified chemotaxis operon was deleted. Both mutants showed inverted responses towards the strong attractant acetate and photosynthetic light. This means that these mutants decrease the stop frequency when acetate is added and increase the stop frequency after removal of acetate. Both mutants showed no response to a decrease of photosynthetic light, but increased the stop frequency when the light intensity is increased.

Besides the complexity of *Rb. sphaeroides* chemotaxis, as reflected by the presence of at least two operons and multiple copies of the Che components, other interesting differences to the enteric paradigm can be observed in this organism (figure 5). First, while there are 2 copies of cheY in the first identified chemotaxis operon, there is no gene in *Rb. sphaeroides*, encoding the CheZ phosphatase. This suggests an alternative mechanism for CheY-P dephosphorylation. Indeed, in *S. meliloti* it has been shown that the active state of the main response regulator CheY2-P is controlled by CheY1 (Sourjik & Schmitt, 1996). In vitro phosphotransfer studies indicate that CheY1 acts as a phosphoryl sink for CheY2-P, when unphosphorylated CheA is present (Sourjik & Schmitt, 1998). Second, the loss of chemotaxis and phototaxis in *Rb. sphaeroides* deletion mutants is not reflected by swimming behavior of non-stimulated cells, unlike the tumbling and smooth swimming phenotypes in *E. coli* che mutants. Apparently, part of the motor control in *Rb. sphaeroides* is independent of the chemosensory pathways identified so far (Armitage & Schmitt, 1997).

**Phototaxis in purple bacteria.** The description of light-induced tactic migration of photosynthetic bacteria has a long history. In 1883 Engelmann reported that motile, photosynthetic purple bacteria (referred to as *Bacterium photometricum*, which were most likely *Chromatium* cells) accumulated at specific wavelengths in a dispersed spectrum, including the infrared region. These bacteriospectrograms show that the cells were predominantly present at spectral regions where the photosynthetic pigments absorb maximally. Two different types of tactic light responses of *Chromatium* cells were reported by Engelmann. The first response is a change in the velocity of movement, as a result of the change of light intensity, the velocity of swimming cells increases when the intensity of photosynthetic light increases. The second response is a reversal of swimming direction when cells enter a region of reduced light intensity (Engelmann, 1883). The latter response could explain the accumulation patterns in the observed bacteriospectrograms. When swimming cells enter a ‘dark’ region, the reversal frequency will increase (compare tumbling in *E. coli*), resulting in a net migration towards spectral regions, where the photosynthetic pigments absorb maximally. The tactic response towards a decrease of light intensity has been referred to by Engelmann as the ‘Schreckbewegung’ and became known as the photophobic response, or more correctly, the scotophobic response, meaning fear of darkness (Ragatz et al., 1994). The term scotophobic becomes especially useful, if one takes into account that the ‘Schreckbewegung’ of swimming cells could also result from a sudden exposure to high light intensities. This latter response has been observed in the phototrophic consortium *Chloronium mirabile* (Buder, 1914) and the free-swimming halophilic purple sulfur bacterium *E. halophila*, as described in more detail below (Sprenger et al., 1993) and can be considered as a true photophobic response, a fear of light. This fear of light is also displayed by swimming *Rb. sphaeroides* cells and the discovery of the latter response is described in this thesis. Interestingly, this response does not match the absorbance spectra of the photosynthetic pigments, so this would imply that bacteriospectrograms of cells, showing the scotophobic as well as the true photophobic response, would change as a function of light intensity.

The observations made by Engelmann suggest that the light-induced tactic response in *Chromatium* cells occured as a result of changes in photosynthetic electron transport, rather than excitation of specific photoreceptors. The linkage between photosensory responses and the photosynthetic pigments in purple bacteria was confirmed by the work of
Clayton, who showed that the scotophobic response of *Rhodospirillum rubrum* only occurred with light that is photosynthetically active (Clayton, 1953). In addition, no light-induced tactic responses could be identified in photosynthetic reaction center mutants of *Rhodopseudomonas sphaeroides*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum*, even though these mutants showed normal motility and chemotaxis (Armitage & Evans, 1981). Such mutants still absorb light via their photosynthetic antenna pigments, but cannot use the associated free energy for electron transport.

Since bacteria use free energy of the electrochemical proton gradient to drive flagellar rotation (Glagolev & Skulachev, 1978), photokinesis is probably a direct result of a change in the rate of electron transfer, whereas the scotophobic response occurs as a result of sensory signaling, through alteration of the flagellar switching frequency. An intriguing problem, which still has to be solved, is the sensing mechanism that mediates the scotophobic response.

Light can be detected by its intensity, color and direction. The latter aspect particularly, has led to the introduction of a rather complicated terminology regarding the characterization of tactic responses of microorganisms. Purple bacteria are only capable of sensing light-intensity and color. Recently however, it was reported that colonies of *Rhodospirillum centenum* are capable of sensing the direction of light; this might provide the first report of true phototaxis (migration towards or away from light by sensing its direction) in prokaryotes (Gest, 1995; Ragatz et al., 1994; Ragatz et al., 1995). So far bacteria were only known to sense spatial differences in light intensity by a temporal mechanism, as also described above for chemotaxis. To substantiate this possibility of true phototaxis in prokaryotes, motion analysis of single cells is required to exclude that colony migration towards light is a result of sensing differences in light intensity caused by shading within a colony, rather than sensing of the direction of illumination by individual cells. Recent studies with single cells exposed to a light beam emitted from an optical fiber, however, have indicated that *Rs. centenum* accumulated uniformly in all parts of the beam, whereas the eukaryote *Chlamydomonas reinhardtii* was capable of swimming towards the light source, showing true phototaxis (Sackett et al., 1997). In this thesis the term phototaxis will be used quite loosely, to refer to processes in which individual bacteria show a net migration in response to changes in their ambient light climate. Both a positive and a negative phototactic response were observed in *Rs. centenum* colonies, depending on the light intensity used (Ragatz et al., 1995). At low-light intensity the positive phototactic response was observed, with a wavelength-dependence that suggests that this response is mediated through the photosynthesis pigments. The tactic response of *Rs. centenum* at high light intensities, which caused the colonies to migrate away from the light source, was elicited mainly by light in the wavelength region between 550 and 600 nm. Also in *Rs. centenum* a gene cluster has been identified that encodes the Che signal transduction components that mediate phototaxis as well as chemotaxis (Jiang, Gest & Bauer, 1997).

In 1993 a light-induced repellent response in the halophilic purple-sulfur bacterium *E. halophila* was reported (Sprenger et al., 1993). The initial observation that led to these studies was that in a light spot of red or infrared-light (in other words light that can be absorbed by the photosynthesis machinery) cells of this species accumulate, whereas a different response is observed in a blue-light spot. With light of the latter color cells accumulate at the edge of the spot, indicating that besides an attractant response, selectively elicited by red-light, these cells additionally display a repellent response towards blue-light. Subsequent motion analyses of *E. halophila* cells showed an increase in reversal frequency of swimming direction, upon a step-up in the intensity of blue-light, in the physiological range of light intensities. This response to blue-light showed adaptation, with kinetics similar to the kinetics of adaptation in chemotaxis of enterobacteria. Because it was known at that time that a low-abundant, highly absorbing photoactive protein was present in *E. halophila*, which is called photoactive yellow protein (PYP), the
wave length dependence of this response was subsequently investigated. Light of wavelengths longer than 500 nm did not elicit any increase in the probability of directional switching of the cells, whereas light of 450 nm elicited a maximal effect. Thus, this new repellent response is probably not mediated by the photosynthesis machinery as the primary photoreceptor, like the scotophobic response in this and other purple bacteria (Hustede, Liebergesell & Schlegel, 1989). In contrast, its wavelength dependence matches the absorption spectrum of PYP, which makes this latter protein the designated candidate for the photoreceptor of this newly discovered repellent response (figure 6).

1.3 Photoactive yellow protein: a bacterial photosensor

Discovery and(2,11),(992,980)(3,7),(996,992) diversity. Photoactive yellow protein (PYP) was discovered as part of a study on eight colored proteins isolated from the water soluble fraction of extracts from the halophilic purple sulfur bacterium E. halophila (Meyer, 1985). In addition to cytochromes and ferredoxins, a purple and a small yellow-colored protein were purified and partly characterized. The yellow protein showed an absorbance peak at 446 nm (figure 7), which disappeared at low pH, concomitant to the formation of a peak at about 350 nm (Meyer, 1985). Two years later the photoactivity of PYP was reported (Meyer et al., 1987). After absorption of a blue photon, PYP enters a photocycle with striking resemblance to the photocycle of the retinal-containing membrane-spanning sensory rhodopsins, as identified in the archaeon Halobacterium salinarum (Bogomolni & Spudich, 1982; Hoff, Jung & Spudich, 1997a; Marwan & Oesterhelt, 1987). In both photocycles, light-induced formation of a short-lived, red-shifted intermediate is followed by the dark conversion to a relatively long-lived, blue-shifted intermediate (as in more detail described below). Chromophore extractions on PYP carried out according to methods described
for rhodopsins did not result in the isolation of retinal, the chromophoric group in the latter proteins. Circular dichroism spectra indicated 19% α-helix and 30% β-sheet secondary structure, while rhodopsins contain relatively more α-helices, in accordance with their membrane-spanning nature. These findings and the photochemical characteristics of PYP raised a paradox: how is it possible that two photoactive proteins with a very different structure show such a similar photocycle?

Figure 7. Absorption spectrum of photoactive yellow protein (PYP).

In 1986 the crystallization of PYP in space group P63 was reported, including a very important observation: a PYP crystal can be reversibly bleached by visible light, while it is not bleached by exposure to X-rays (McRee et al., 1986). This allows crystallographic studies to light-induced conformational changes on PYP crystals. In disagreement with the spectroscopic studies described above, the crystallographic structure of PYP at 2.4 Å resolution was initially reported to consist entirely of β-strands, forming two perpendicular β-sheets (a β-clam fold), that enclose a retinal chromophore, linked to Lys111 by a Schiff base (McRee et al., 1989). Subsequent amino acid sequencing and mass spectrometric analysis of proteolytic digests however, revealed a protein of 125 residues, containing a chromophore attached to Cys69 with a molecular weight of ~147, thus excluding a retinal chromophore linked to Lys111 (Van Beeumen et al., 1993).

In 1994 the chromophore in PYP was reported to be 4-hydroxy cinnamic acid, which is covalently bound to Cys69 via a thiol ester bond, as presented in figure 8 (Baca et al., 1994; Hoff et al., 1994a). The evidence for the nature of the chromophore was obtained via a wide variety of techniques. Hoff et al. isolated a fraction absorbing at 307 nm from a proteolytic digest of PYP by high performance liquid chromatography (HPLC). 1H-NMR spectroscopy of this fraction, containing exclusively the chromophore of PYP, led to the identification of its chemical structure. Alkaline hydrolysis confirmed the thiol ester linkage of the chromophore to the protein and capillary electrophoresis confirmed that a compound from PYP was released at high pH with the same electrophoretic mobility as 4-hydroxy cinnamic acid (Hoff et al., 1994a). Baca et al. isolated the chromophore from the protein by aminolysis, resulting in the amide derivative of the chromophore. Mass spectrometric fragmentation suggested the presence of a primary amide and a phenolic structure. A base titration of free chromophore showed a shift in the UV spectrum from ~300 nm to 340 nm with a pKa of ~9.0, in agreement with the deprotonation of phenolic hydroxyl group. Refining the PYP protein with the attached 4-hydroxy-cinnamyl chromophore against a new 1.4 Å resolution diffraction data revealed the chromophore structure (Baca et al., 1994).

Interestingly, both groups initially proposed a different reversible chemical reaction in the chromophore as the photochemical basis for the photocycle of PYP: trans to cis photoisomerization of the vinyl double bond (Hoff et al., 1994a) versus protonation of the deprotonated phenolic hydroxyl group (Baca et al., 1994). Both proposals do not exclude

Figure 8. Chemical structure of the 4-hydroxy-cinnamic acid chromophore of photoactive yellow protein; the chromophore is covalently linked to a cysteine residue in the protein via a thiol ester bond.
each other, since these reversible chemical reactions can subsequently take place in time while PYP progresses through its photocycle. The photo-isomerization was hypothesized on the basis of the analogy with the sensory rhodopsins. The first experimental evidence that was found for photo-isomerization during the photocycle of PYP is provided in this thesis (chapter 4.1). The proposal for protonation of the chromophore was supported by three pieces of experimental evidence. First, uptake and release of a proton associated with the photocycle was observed by use of a bromocresol pH indicator (Meyer, Cusanovich & Tollin, 1993). Second, UV spectroscopic data showed that the free chromophore is blue-shifted when the 4-hydroxy group becomes protonated, like the formation of the PYP photocycle intermediate (Baca et al., 1994). In support of the presence of a deprotonated 4-hydroxyl group of the chromophore in the ground state of PYP, which could act as the proton acceptor during the PYP photocycle, the new 1.4 Å resolution diffraction data set indicated a relatively short C-O bond that connects the phenolic ring to the oxygen, indicative of a phenolate anion (Baca et al., 1994).

As mentioned above, PYP was first discovered in the halophilic purple sulfur bacterium *E. halophila*. Five years later a second PYP was purified from the moderately halophilic purple non-sulfur bacterium *Rs. salexigens* (Meyer et al., 1990). This protein is very similar to *E. halophila* PYP regarding its absorbance spectrum and photoactivity. A third PYP with similar characteristics was isolated from the halophilic purple bacterium *Chromatium salexigens*. The amino acid sequences of all three PYPs were determined by proteolytic digestion, followed by Edman degradation, revealing that all three proteins contain 125 amino acids with high mutual identities of 70-76% (Koh et al., 1996; Van Beeumen et al., 1993). The *E. halophila* PYP gene was amplified by use of the polymerase chain reaction (PCR) with degenerated oligonucleotides derived from the amino acid sequence and using chromosomal template DNA, as described in this thesis and by Baca et al., (1994). The PCR-product was used to probe *E. halophila* chromosomal DNA for cloning the pyp gene. The translation of the pyp gene sequence matched the PYP amino acid sequence, except for Glu56, which appeared to be Gln56 (Baca et al., 1994). An extensive description of the cloning, sequencing and analysis of the pyp genes and their flanking regions from *E. halophila* and *Rs. salexigens* is presented in chapter 2.1.

A study with a highly specific polyclonal antiserum against *E. halophila* PYP indicated the presence of a single, cross-reacting protein of similar size as PYP in a large number of tested eubacteria, including non-phototrophic species, like for example *E. coli* (Hoff et al., 1994b). Among the tested organisms, a cross-reacting protein was only present in representatives of the domain of Bacteria. The archaeon *Halobacterium salinarum* for example, does not contain a cross-reacting protein. The key question here is whether all the cross-reacting proteins are PYP homologues. In support of this, *Rs. salexigens* and *C. salexigens* cell-free extracts showed a single cross-reacting protein, while the PYPs purified from these organisms cross-reacted with purified antiserum against *E. halophila* PYP (Hoff et al., 1994b). In 1995 an even more specific polyclonal antiserum was raised against *E. halophila* PYP (Thiemann & Imhoff, 1995). This antiserum only cross-reacted with a single protein in *Rs. salexigens* and *C. salexigens* cell-free extracts and confirmed the cytoplasmic localization of PYP. This result does not exclude however, the presence of less homologous proteins in other bacteria. In this thesis the identification of a pyp gene in *Rh. sphaeroides* is described, extending the group of bacteria known to contain PYP with a genetically well-characterized non-halophilic purple non-sulfur bacterium (chapter 2). Until now, no data have been obtained that indicate the presence of pyp genes in other organisms than purple bacteria.

Purification of recombinant PYP, obtained from an *E. coli* strain that overproduces the protein, indicated that the protein was produced in its colorless apoform (chapter 2.1). The protein can be reconstituted *in vitro* with activated forms of the 4-hydroxy cinnamic acid chromophore: the 4-hydroxy...
cinnamyl thiophenyl ester or the 4-hydroxy cinnamic anhydride (Imamoto et al., 1995). In addition, apoPYP can be reconstituted with activated chromophore analogues, containing substituents at the aromatic ring, including 3,4-dihydroxy cinnamic acid (caffeic acid), 3-methoxy-4-hydroxy cinnamic acid (ferulic acid) and 3,5-dimethoxy cinnamic acid (sinapinic acid), resulting in hybrids with red-shifted absorbance maxima of 457 nm, 460 nm and 488 nm, respectively (Kroon et al., 1996). Acid titrations show that the pKa for the formation of the bleached species (pBdark) is increased in these hybrids, indicating a decreased stability of these PYP hybrids.

The difference between the absorption maximum of the free trans 4-hydroxy cinnamic acid chromophore (284 nm) and that of PYP (446 nm) raises the question of the molecular basis of this red-shift. Three factors contributing to the tuning of the absorption of the chromophore have been identified and quantified: (i) the deprotonation of the para hydroxyl group in the free chromophore shifts the absorption maximum from 284 nm to 340 nm, (ii) the presence of a thiol ester bond in a deprotonated model compound indicates a shift from 340 nm to 410 nm, and (iii) the protein environment around the chromophore binding pocket red-shifts the absorption maximum another 36 nm to 446 nm (Hoff, 1995; Kroon et al., 1996).

The role of amino acid residues in the chromophore-binding pocket was investigated by construction of the following site-directed mutants of PYP: Y42F, E46Q, T50V, R52A, R52Q and C69S (Genick et al., 1997b; Mihara et al., 1997). The lack of the unique cysteine residue in an E. halophile PYP mutant confirmed that this residue is essential for chromophore binding, because no yellow-colored protein was formed in reconstitution experiments with this mutant (Mihara et al., 1997). All the other constructed PYP mutants showed a red-shift in their absorption spectra and, as far as measured, an increase in the pKa values for pBdark formation. The effect of amino acid substitutions on photocycle kinetics is further discussed below.

Structure and photocycle. The photocycle of PYP and the associated structural changes have been thoroughly investigated, since the discovery of the photoactivity of PYP (Meyer et al., 1987). After absorption of a blue photon, the ground state of PYP (pG446) enters the photocycle with a quantum yield of ~0.35 (Van Brederode et al., 1995). Recent studies with picosecond transient absorption spectroscopy revealed two intermediates on the sub-nanosecond time scale. The first intermediate (I0) is formed within ~3 picoseconds and exhibits a maximal absorption at 510 nm. The second intermediate (I0') shows a similar wavelength maximum, but a lower extinction coefficient, and is formed in ~0.2 nanosecond (Ujj et al., 1998). The latter intermediate decays with a ~3 nanoseconds time constant to form pR455. This intermediate is converted into a blue-shifted intermediate (pB355) on a sub-millisecond time scale, followed by the recovery of the ground state (pG446) on a sub-second time scale (Hoff et al., 1994c), as presented in figure 9. Low temperature spectroscopy shows that a red-shifted intermediate (λmax ~490 nm) can be trapped at temperatures below -180°C (Hoff et al., 1992; Imamoto, Kataoka & Tokunaga, 1996). Whether this red-shifted intermediate reflects one of those identified at room temperature on the nanoseconds time scale is not yet established. The formation of pB355, which is associated with major protein conformational changes, is blocked at

![Figure 9](https://via.placeholder.com/150)
temperatures below -50°C (Imamoto et al., 1996). At room temperature, a light-dependent branching reaction was identified from pB\textsubscript{355} to pG\textsubscript{446} with a high quantum yield of ~0.5, which may suggest a biological function for this reaction (Miller et al., 1993).

The effects of temperature, pH and hydrophobicity of the solvent on the photocycle kinetics have been extensively investigated (Genick et al., 1997b; Hoff et al., 1997b; Meyer et al., 1989; Van Brederode et al., 1996). The temperature dependence of the photocycle kinetics deviates from normal Arrhenius behavior. The changes in kinetics can be explained by the assumption that heat capacity changes are associated with the PYP photocycle (Van Brederode et al., 1996). These heat capacity changes may be caused by the exposure of hydrophobic residues in the pB\textsubscript{355} intermediate of the photocycle to water. This exposure of hydrophobic residues in pB\textsubscript{355} is in agreement with previous experiments, showing that an increase of the solvent hydrophobicity increases the rate constant for pB\textsubscript{355} formation, but decreases the rate constant for pG\textsubscript{446} recovery (Meyer et al., 1989). At low pH in the dark PYP can be reversibly converted into a stable blue-shifted state (pB\textsubscript{dark}), which maximally absorbs at 350 nm; the pKa for this transition is -2.8 (Hoff et al., 1997b; Meyer, 1985). The pB\textsubscript{355} to pG\textsubscript{446} transition in the photocycle of PYP is strongly decelerated as a result of acidification (Hoff et al., 1997b). A rationale for this can be provided by a model in which the deprotonated chromophore takes up a proton from the solvent and releases it when pG\textsubscript{446} is reformed, as supported by laser spectroscopy with a pH indicator (Meyer et al., 1993). Recently, it was discovered that the pB\textsubscript{355} to pG\textsubscript{446} transition is also decelerated at high pH values. Thus, when the rate constant for this transition is plotted as a function of pH, one observes a bell-shaped curve (Genick et al., 1997b). The latter observation makes an explanation for the pH-dependence of the photocycle kinetics less straightforward.

The re-determined crystal structure of PYP at 1.4 Å resolution shows an unusual α/β-fold, indicated in figure 10, that is similar to that of the eukaryotic signal transduction proteins profilin and the SH2 domain (Borgstahl, Williams & Getzoff, 1995). The 4-hydroxy cinnamic acid chromophore is buried within the major hydrophobic core of the protein and covalently linked to the sulfur atom of Cys69 by a thiol ester bond. In this active site, the chromophore is present in a deprotonated state, as also shown by resonance Raman spectroscopy (Kim et al., 1995). The chromophore is stabilized by a hydrogen-bonding network. The phenolic oxygen of the chromophore hydrogen bonds with the side-chain oxygen atoms of Tyr42 and Glu46. The acidic amino acid Glu46 is present in the protein in the protonated state and shares this proton with the phenolic oxygen of the chromophore. In addition, the chromophore carbonyl oxygen atom hydrogen bonds with the main-chain amide group of Cys69. The side-chain oxygen atom of Thr50 hydrogen bonds to that of Tyr42, while its main-chain carbonyl oxygen hydrogen bonds with Arg52. Arg52 shields the chromophore from the solvent and is kept in position by hydrogen bonds with the carbonyl oxygen atoms of Tyr42 and Tyr98. The positively charged guanidinium group of Arg52 might play a role in stabilizing the buried negative charge on the chromophore (Borgstahl et al., 1995).

What happens to the PYP structure when
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the protein absorbs a blue photon and progresses through the photocycle? The very first structural event in the photocycle is probably trans to cis photo-isomerization of the vinyl double bond in the chromophore. Fourier transform infrared spectroscopic studies led to the proposal of a structural model for this isomerization reaction. The $\text{pR}_{\text{lowT}}$-$\text{pG}_{\text{lowT}}$ difference absorbance spectra at 80 K indicated the structural perturbation of a protonated carboxyl group, which was assigned to Glu46 by use of the three-dimensional structure of PYP; this structure indicated that the only buried carboxyl group in the protein was belonging to Glu46 (Xie et al., 1996). The correctness of this assignment was confirmed by FTIR studies on a E46Q mutant of PYP (Imamoto et al., 1997). This observation is important, because it implies that in the $\text{pR}_{\text{lowT}}$ intermediate the hydrogen bonding between Glu46 and the phenolic oxygen of the chromophore is still intact. This suggests that the chromophore isomerizes to the 7-cis 9-S-trans conformation, resulting from co-isomerization of both the $\text{C}_7$-$\text{C}_8$ and the $\text{C}_9$-$\text{C}_{10}$ bond (figure 11). In addition, $\text{pB}_{355}$-$\text{pG}_{446}$ FTIR difference spectra indicated a deprotonation of Glu46 in $\text{pB}_{355}$, suggesting that Glu46 donates a proton to the chromophore during the photocycle (Xie et al., 1996). Surprisingly, E46Q still displays a photocycle; this would suggest that the chromophore in this mutant takes up a proton from an alternative proton donor, for example Tyr42, or from the solvent (Imamoto et al., 1997).

The two intermediates identified on the sub-nanosecond time scale most likely contain the cis isomer of the chromophore. This change however, does not explain the strong red-shift of the $\lambda_{\text{max}}$ of these intermediates. The free trans 4-hydroxy cinnamic acid chromophore absorbs maximally at 284 nm, while the cis isomer absorbs maximally at 265 nm. More importantly, the trans chromophore has a more stretched conformation than the cis chromophore, meaning that the distance between the phenolic oxygen and its hydrogen-bonding partner Glu46 increases going from the trans to the cis isomer. This could add to the red-shift by an increase of negative charge on the chromophore. The importance of the distance between the phenolic oxygen and Glu46 for the spectral tuning of PYP can be tested by the construction of a E46D mutant. The decrease of the extinction coefficient in $I_\lambda$ may be caused by protein relaxation, including twisting of the chromophore and reorientation of protein side-chains. The formation of $\text{pR}_{465}$ may result from even further relaxation of the protein, which may include the formation of new hydrogen bond interactions (Ujj et al., 1998).

Most of the conformational changes associated with the photocycle appear to occur during the formation and decay of $\text{pB}_{355}$.
General introduction

(amount of data regarding its structure and photocycle. However, there is still no conclusive evidence for the function of PYP in the living cell. The question of the physiological role of PYP is one of the major subjects in this thesis (chapter 3) and has been speculated upon before by others. On the basis of the photoactivity of PYP and its cytoplasmic nature it was suggested that PYP could be a sensory protein that binds to a membrane receptor or modulates enzyme activity or gene expression to control a metabolic system (Meyer et al., 1987). On the basis of the high quantum efficiency of the photocycle of PYP, of the kinetic similarity to the sensory rhodopsin I photocycle and of positive phototaxis in terms of accumulation of E. halophila cells in a capillary, it was postulated that the biological role for PYP was to mediate a phototactic response by binding a specific receptor (McRee et al., 1989). Sprenger et al. (1993) presented the first experimental evidence for the function of PYP by the demonstration that E. halophila is negatively phototactic with a wavelength dependence that matches the absorbance spectrum of PYP. In a saturating background of photosynthetic light, E. halophila cells increased the number of reversals after a step-up in blue-light, whereas they suppressed these reversals after a decrease in the blue-light intensity (Sprenger et al., 1993).

Another clue about the in vivo role of PYP or more correctly, about the nature of the downstream signaling partner of PYP came from sequence comparisons. One of the highly conserved regions present in PAS domains, the S1 box, has been identified in PYP (Lagaris, Wu & Lagarias, 1995; Pellequer et al., 1998; Zhulin et al., 1997). PAS domains were initially found in three eukaryotic regulators, the Drosophila Period clock protein, vertebrate Aryl hydrocarbon receptor nuclear translocator and the Drosophila Single-minded. Typically, PAS domains are involved in protein-protein interactions. Recently, the two highly conserved regions S1 and S2 present in PAS domains have been found in many archaeal and bacterial sensory proteins, like oxygen and redox sensors (Zhulin et al., 1997). Some of the latter proteins have been

The function. Research on photoactive yellow protein so far has generated a large
shown to contain a prosthetic group. In FixL, for example, a heme group is coordinated by His194, while Aer and NifL bind FAD. The chromophore in PYP is located at the C-terminal boundary of the S₀ box. In response to input signals, a conformational change in the S₀ fold may activate another protein domain. Reduction or, in the case of PYP, isomerization of the prosthetic group, may switch a transient domain interacting with the S box to an active form. A proposed PAS/PYP three-dimensional fold is displayed in figure 10 and the related multiple sequence alignment in figure 12. A dimerization interface with the PYP-downstream partner has been proposed to include (i) the central region, residues 51-68 (ii) the loop 95-103 and (iii) two residues from the α3 in the PAS core domain (Pellequer et al. 1998). Interestingly, two PAS domains have been found in a Rb. sphaeroides protein that is homologous to methyl-accepting chemotaxis proteins (Ponting & Aravind, 1997), which puts this protein high on the list of possible candidates for heterodimer formation with PYP.

1.4 Outline of this thesis. Before providing the contours of this PhD-study in terms of the techniques and strategies involved, I would like to outline the general importance of this study. If one considers our current knowledge about the subject of this thesis, the molecular basis for ‘vision’ in prokaryotes, it is clear that the photosensory system in the archaeon Halobacterium salinarum is by far the best understood (for a recent review see Hoff, Jung & Spudich, 1997). The beauty of this system is demonstrated by the fact that the effect of changes in the components of this photosensory system, obtained for example through site-directed mutagenesis or chromophore substitutions, can be studied in vitro as well as in vivo. Thus, for example, the effect of a substitution of a single amino acid residue in one of the two photosensors of this system can be determined on the level of photocycle kinetics of the photosensor, but also on the level of tactic behavioral responses of the bacterium to photostimuli. Studies on the archael photosensory system already led to a very detailed insight in the signaling mechanisms between the two membrane-spanning proteins (Ponting & Aravind, 1997) with permission from the Proceedings of National Academy of Sciences of the USA.
photosensors and their transducers.

So, why do we want to study photoactive yellow protein? First of all, in eubacteria a photosensory system with retinal-containing chromoproteins, like that of archaeabacteria and eukaryotes, has not been characterized. Instead, a completely different, 4-hydroxy cinnamic acid-containing chromoprotein has been identified: photoactive yellow protein. This protein shows a photocycle with a remarkable similarity to that of the sensory rhodopsins, but in contrast to the latter, is water-soluble. Thus, photoactive yellow protein does not only provide a model system to study a new way of light-sensing in another domain of life, the Bacteria, but also to study a new signaling mechanism, starting with a water-soluble photosensor. Because of its water-soluble nature, it has been relatively easy to obtain crystals and the structure of PYP has been resolved down to a very high resolution (0.82 Å). Taking this together with its intrinsic photoactivity, photoactive yellow protein has the potential to become the candidate for resolving the signal transfer of a protein to its downstream partner at extremely high structural and temporal resolution. In order to achieve this, the biological function of this protein needs to be proven and its downstream partner needs to be identified. Thus, one of the aims in this thesis is to add biological significance to the wealth of biophysical data about photoactive yellow protein, obtained so far. After reading this thesis, it will be clear that, although we may be close, this mission has not been completed yet.

The study described here is aimed at the elucidation of the function of photoactive yellow protein in the living cell and the characterization of structural changes associated with its photocycle. Cloning of pyp genes and subsequent analysis of flanking regions is carried out to reveal functionally related genes (chapter 2.1). Screening for the presence of pyp genes in genetically well-characterized bacteria by use of the polymerase chain reaction is described in chapter 2.1. Such a bacterium would be an excellent candidate for the genetic approach to resolve the function of PYP, provided that the organism shows physiologically relevant (tactic) blue-light responses, which may be mediated by PYP.

Studies on the characterization of these blue-light responses in a pyp-containing bacterium, carried out in the research group of Prof. Spudich at the University of Texas, Houston, USA, are described in chapter 3. The techniques involved in the characterization of these responses include computer-assisted motion analysis and methanol release assays. The pyp gene has been inactivated by gene replacements, followed by phenotypic analysis of the mutants obtained, leading to a preliminary model for light-induced signal transduction in phototaxis in Rb. sphaeroides (chapter 3).

Characterization of the structural changes associated with the PYP photocycle has been carried out with a wide range of techniques. The chomophore of PYP, in its ground state (pG446) and its blue-shifted photocycle intermediate (pB355), has been extracted and subsequently characterized by high performance capillary zone electrophoresis (chapter 4.1). The indispensability of isomerization of the vinyl double bond in the chromophore has been investigated by characterization of reconstituted photoactive yellow proteins with chromophore analogues (chapter 4.2). In addition, structural events in the photocycle of PYP on the nano- and milliseconds time scale are discussed, partly based on time-resolved Laue diffraction data, resulting from a collaborative project with the research group of Prof. Moffat at the University of Chicago, USA (chapter 4.3).

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