A Structural Basis of Light Energy and Electron Transfer in Biology (Nobel Lecture)**

By Robert Huber*

Dedicated to Christa

Aspects of intramolecular light energy and electron transfer are discussed for three protein cofactor complexes whose three-dimensional structures have been elucidated by X-ray crystallography: the light harvesting phycobilisomes of cyanobacteria, the reaction center of purple bacteria, and the blue multi-copper oxidases. A wealth of functional data is available for these systems which allows specific correlations to be made between structure and function and general conclusions to be drawn about light energy and electron transfer in biological materials.

Introduction***

All life on earth depends ultimately on the sun, whose radiant energy is captured by plants and other organisms capable of growing by photosynthesis. They use sunlight to synthesize organic substances which serve as building materials or stores of energy. This was clearly formulated by *L. Boltzmann*, who stated that "there exists between the sun and the earth a colossal difference in temperature ... The equalization of temperature between these two bodies, a process which must occur because it is based on the law of probability will, because of the enormous distance and magnitude involved, last millions of years. The energy of the sun may, before reaching the temperature of the earth, assume improbable transition forms. It thus becomes possible to utilize the temperature drop between the sun and the earth to perform work as is the case with the temperature drop between steam and water ... To make the most use of this transition, green plants spread the enormous surface of their leaves, and, in a still unknown way, force the energy of the sun to carry out chemical syntheses before it cools down to the temperature level of the earth's surface. These chemical syntheses are to us in our laboratories complete mysteries..."[1]

Today many of these 'mysteries' have been resolved by biochemical research and the protein components and their basic catalytic functions have been defined.[2]

I will focus in my lecture on *Boltzmann*'s 'improbable transition forms', namely excited electronic states and charge transfer states in modern terminology, the structures of biological materials involved and the interplay of cofactors (pigments and metals) and proteins. I will discuss some aspects of the photosynthetic reaction center of *Rhodopseudomonas viridis* (see the original publications cited later and short reviews[3-5]) and of functionally related systems, whose structures have been studied in my laboratory: light harvesting phycobilisomes of cyanobacteria and blue oxidases. A wealth of structural and functional data is available for these three systems, which makes them uniquely appropriate examples to derive general principles of light energy and electron transfer in biological materials. Indeed, there are very few systems known in sufficient detail for such purposes.*

We strive to understand the underlying physical principles of light and electron conduction in biological materials with considerable hope for success as these processes appear to be more tractable than other biological reactions, which involve diffusive motions of substrates and products and intramolecular motions. Large-scale motions have been identified in many proteins and shown to be essential for many functions.[12,13] Theoretical treatments of these reactions have to

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[***] Abbreviations:
PBS phycobilisomes, light harvesting organelles peripheral to the thylakoid membrane in cyanobacteria, which carry out oxygenic photosynthesis and have photosystems I and II.
PE, PEC, PC, APC phycoerythrin, phycoerythrocyanin, phycocyanin, allophycocyanin, biliprotein components in PBS with covalently attached tetrapyrrole (bilin) pigments.
PS I, II photosynthetic reaction centers in chloroplasts and cyanobacteria.
RBP retinol binding protein.
BBP bilin (biliverdin IXγ) binding protein in *Pieris brassicae*.
Rps. viridis Rhodopseudomonas viridis, bacteriochlorophyll-b containing purple bacterium carrying out anoxygenic photosynthesis.
RC reaction center.
C, H, L, M the four subunits of the reaction center from *Rps. viridis*: the cytochrome c subunit (C), with four hemes displaying two redox potentials (c_{553}, c_{558}) is located on the periplasmic side of the membrane; the L- and M-subunits are integrated in the membrane and their polypeptide chains span the membrane with 5 α-helices each, labeled A, B, C, D, E; they bind the bacteriochlorophyll-b (BChl-b or BC), bacteriopheophytin-b (BPh-b or BP), menaquinone-9 (Q_A), ubiquinone-9 (UQ, Q_B) and Fe^{2+} cofactors; the subscripts P, A, M, L indicate pair, accessory, M-, L-subunit association, respectively; the H-subunit is located on the cytoplasmic side and its *N*-terminal α-helical segment (H) spans the membrane.
P_{680}, P_{960} primary electron donors in PS II and the RC of *Rps. viridis*, respectively, indicating the long wavelength absorption maxima.
P*, D* electronically excited states of P, D (donor), A (acceptor)
LHC light harvesting complexes.
$LH_{a,b}$ light harvesting protein pigment complexes in BChl-a, b containing bacteria.
Car carotenoids.
Sor Soret bands of chlorophyll and bacteriochlorophyll.
PCY plastocyanin, electron carrier in the photosynthetic apparatus of plants.
LAC laccase, oxidase in plants and fungi.
AO ascorbate oxidase, oxidase in plants.
CP ceruloplasmin, oxidase in mammalian plasma.

[*] The structure of the *Rb. sphaeroides* RC is closely related to the *Rps. viridis* RC [6, 7, 151]. A green bacterial bacteriochlorophyll-a containing light harvesting protein is well defined in structure [9] but not in function. In the multiheme cytochromes [10, 11] the existence/or significance of intramolecular electron transfer is unclear.

take flexibility and solvent into account and become theoretically tractable only by applying the rather severe approximations of molecular dynamics[14, 15] or by limiting the system to a few active site residues, which can then be treated by quantum mechanical methods.

Light and electron transfer processes seem to be amenable to a more quantitative theoretical treatment. The substrates are immaterial or very small and the transfer processes on which I focus are intramolecular and far removed from solvent. Molecular motions seem to be unimportant, as shown by generally small temperature dependences. The components active in energy and electron transport are cofactors, which, in a first approximation, suffice for a theoretical analysis, simplifying calculations considerably.

1. Models for Energy and Electron Transfer

To test theories developed for energy and electron transfer appropriate model compounds are essential. Although it would be desirable, these models need not be mimics of the biological structures.

Förster's theory of inductive resonance[16] treats the cases of strong and very weak coupling in energy transfer. Strong interactions lead to optical spectra which are very different from the component spectra. Examples include concentrated solutions of some dyes, crystalline arrays, and the bacteriochlorophyll pair (BC_P) discussed in Section 3.1. The electronic excitation is in this case delocalized over a molecular assembly. A very weak coupling produces little or no alteration of the absorption spectra, but the luminescence properties may be quite different. Structurally defined models for this case are scarce. The controlled deposited dye layers of *Kuhn* and *Frommherz* et al.[17, 18] may serve this purpose and have demonstrated the general validity of *Förster*'s theory, but with deviations.

Synthetic models with electron transfer are abundant and have recently been supplemented by appropriately chemically modified proteins.[19–21] They are well covered in reviews (see, e.g., Refs. [22–29]). Figure 1 shows the essential elements of such models: Donor D (of electrons) and acceptor A may be connected by a bridging ligand (B) with a pendant group (P) embedded in a matrix M.

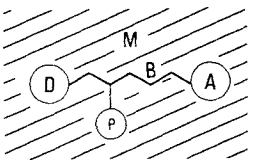

Fig. 1. Determinants of electron transfer models. D: Donor, A: Acceptor, B: Bridge, P: Pendant group, M: Matrix

Models with porphyrins as donors and quinones as acceptors are mimics of the reaction center (RC).[30, 31] Models with peptide bridging ligands[32] merit interest especially in relation to the blue oxidases. The effect of pendant groups (P), which are not in the direct line of electron transfer[22] is noteworthy in relation to the unused electron transfer branches in the RC and the blue oxidases. It is clear, however, that the biological systems are substantially more complex than synthetic models. The protein matrix is inhomogeneous and unique in each case. Despite these shortcomings, theory and models provide the framework within which the factors controlling the transfer of excitation energy and electrons and competing processes are to be evaluated.

1.1. Determinants of Energy and Electron Transfer

The important factors are summarized in Table 1. They may be derived from *Förster*'s theory and variants of *Marcus*' theory[26] for excitation and electron transfer, respectively. These theoretical treatments may in turn be derived from classical considerations or from *Fermi*'s Golden Rule with suitable approximations (see, e.g., Ref. [33]). Excitation and electron transfer depend on the geometric relation between donors and acceptors. Excitation energy transfer may occur over wide distances when the transition dipole moments are favorably aligned. Fast electron transfer requires sufficient electronic orbital overlap. Fast electron transfer over wide distances must therefore involve a series of closely spaced intermediate carriers with low lying unoccupied molecular orbitals or suitable ligands bridging donor and acceptor. Bridging ligands may actively participate in the transfer process and form ligand radical intermediates (chemical mechanism) or the electron may at no time be in a bound state of the ligands (resonance mechanism).[34] The spectral overlap and the 'driving force', for energy and electron transfer, respectively, have obvious effects on the transfer rates and are largely determined by the chemical nature and geometry of donors and acceptors. Nuclear reorganization of donor, acceptor, and the surrounding medium accompanying electron transfer is an important factor but difficult to evaluate, even qualitatively, in a complex protein system; we observe that the protein typically binds donors and acceptors firmly and rigidly, keeping reactant reorganization effects small. Surrounding polar groups may, as a result of their reorientation, slow down rapid electron transfer. However, a polar environment also contributes to the energetics by stabilizing ion pairs ($D^+ A^-$) or lowering activation and tunneling barriers and may thus increase 'driving force' and rate. The energy transfer also depends on the medium and is disfavored in media with high refractive index.

Table 1. Factors controlling the rate of transfer of excitation energy and electrons.

Excitation energy transfer $D^* + A \rightarrow D + A^*$ (very weak coupling)	Distance and orientation (coupling of excited states); Spectral overlap of emission and absorption of D and A; Refractive index of medium
Electron transfer from excited state $D^* + A \rightarrow D^+ + A^-$	Distance and orientation (electronic coupling, orbital overlap);
and from ground state $D^- + A \rightarrow D + A^-$	Free energy change ('driving force'); Reorganization in D and A; Orientation polarization of medium

Processes competing with productive energy and electron transfer from excited states 'lurk' everywhere (Table 2). Quite generally they are minimized by high transfer rates and a conformational rigidity of the cofactors in the protein ensemble.

I will discuss these factors in relation to the biological structures later on.

Table 2. Processes competing with the transfer of excitation energy and electrons.

Excitation energy transfer $D^* + A \rightarrow D + A^*$ (very weak coupling)	Non-radiative relaxation of D^* by photoisomerization and other conformational changes; Excited state proton transfer; Intersystem crossing; Chemical reactions of D^*, A^*, D^+, A^- with the matrix; Fluorescence radiation of D^*
Electron transfer from excited state $D^* + A \rightarrow D^+ + A^-$	Energy transfer; as above; Back reaction to ground state D,A
from ground state $D^- + A \rightarrow D + A^-$	–

2. The Role of Cofactors

The naturally occurring amino acids are transparent to visible light and seem also, with the exception of tyrosine, to be unsuitable as single electron carriers. Tyrosyl radicals have been identified in PS II as excited intermediates Z* and donors D*, which are involved in electron transfer from the water-splitting manganese protein complex to the photooxidized P^+_{680} (for reviews, see Refs. [35, 36]). Their identification has been assisted by the observation that Tyr L162 lies in the electron transfer path from the cytochrome to the bacteriochlorophyll pair (BC_{LP}) in the bacterial RC[37] (see Section 3.2.2.2 and Fig. 10c). A tyrosyl radical is not generated in the bacterial system, because the redox potential of P^+_{960} does not suffice.

Therefore cofactors, i.e. pigments and metal ions, generally serve as light energy acceptors and redox active elements in biological materials.

Figure 2 is a gallery of the pigments and metal clusters which will be discussed further on, namely the bile pigments phycocyanobilin and biliverdin IXγ in the light harvesting

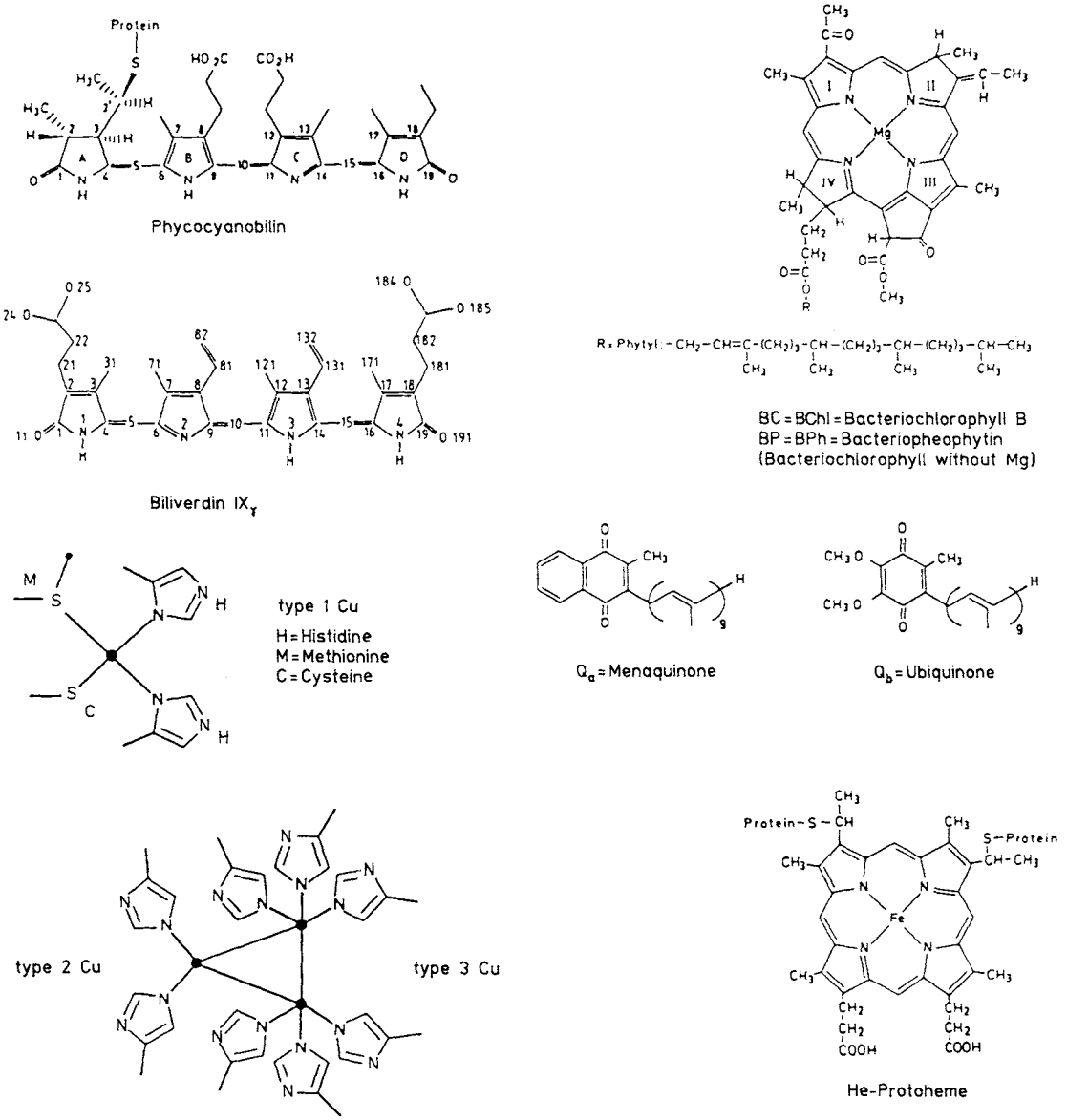

Fig. 2. Cofactors in phycocyanin (PC), bilin binding protein (BBP), ascorbate oxidase (AO) and the reaction center (RC) of the purple bacteria. Phycocyanobilins are covalently bound by thioether linkages to the protein. Biliverdin IXγ is non-covalently bound to the protein BBP. Type-1, type-2, type-3 copper ions are linked to the enzyme AO by coordination to the amino acid residues indicated. Four BChl-b molecules and two 2 BPh-b molecules are bound to the reaction center (RC) of the purple bacteria. A pair of BChl-b serves as the primary electron donor, a menaquinone-9 (Q_A) is the primary electron acceptor and an ubiquinone-9 (Q_B) the secondary acceptor. The four heme groups are bound by thioether linkages to the cytochrome c.

complexes, the BChl-b, BPh-b and quinones in the purple bacterial RC, and the copper centers in the blue oxidases.

The physicochemical properties of these cofactors determine the general features of the protein pigment complexes, whereas the protein part has a decisive influence on the spectral and redox properties.

3. The Role of the Protein

The role of the protein follows a hierarchy in determining the properties of the functional protein cofactor complexes shown in Table 3. The interactions listed in the table are different for the various systems and shall be described separately, except point 1, as there are common features in the action of proteins as polydentate ligands which can be ascribed to a 'rack mechanism'.

Table 3. Hierarchy of protein cofactor interactions.

1. Influence on configuration and conformation of the cofactors by the nature and geometry of ligands (the protein as a *polydentate ligand*)
2. Determination of the spatial arrangements of arrays of cofactors (the protein as a *scaffold*)
3. The protein as the *medium*
4. Mediation of the interaction with other components in the supramolecular biological system

3.1. The Protein as Polydentate Ligand

The 'rack mechanism' was introduced by *Lumry* and *Eyring*[38] and *Gray* and *Malmström*[39] to explain unusual reactivities and spectral and redox properties of amino acids and cofactors by the distortion enforced by the protein.

A comparison of isolated and protein bound bile pigments clearly demonstrates this effect. Isolated bile pigments in solution and in the crystalline state prefer a macrocyclic helical geometry with configuration *ZZZ* and conformation *syn,syn,syn* and show a weak absorption in the visible range and a low fluorescence quantum yield.[40-42] When bound as cofactors to light harvesting phycocyanins, however, they absorb and fluoresce very strongly in the visible range (Fig. 3). The auxochromic shift, essential for the light harvesting functions, is due to a strained conformation of the chromophore, which has configuration *ZZZ* and conformation *anti,syn,anti* stabilized by tight polar interactions with the protein[44-46] (Fig. 4). Particularly noteworthy is an aspartate residue (A87 here) bound to the central pyrrole nitrogen atoms, and conserved in all pigment binding sites. It influences the protonation, charge, and spectral properties of the tetrapyrrole systems. Tight binding is also effective against deexcitation by conformational changes. The structure shown in Figure 3 as representative for the free pigment is in fact observed in a bilin binding protein from insects.[41,42] This protein serves a different function and prefers the low energy conformer. The open chain tetrapyrrole bilins are conformationally adaptable, a property which makes them appropriate cofactors for different purposes.

The cyclic bacteriochlorophyll in the reaction center of purple bacteria is conformationally restrained but responds

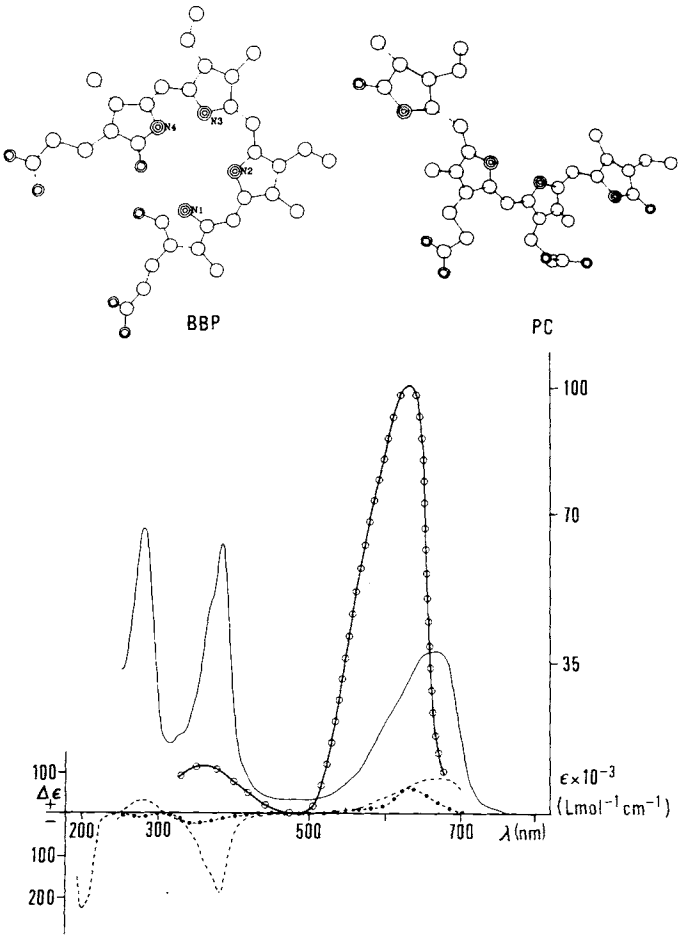

Fig. 3. Tetrapyrrole structures in PC and BBP and the associated optical and circular dichroism spectra [42, 44]. Absorption (———) and CD spectrum (– – –) of BBP; absorption (⊖) and CD spectrum (– · –) of PC.

to the environment by twisting and bending of the macrocycle. This may be one of the reasons for the different electron transfer properties of the two pigment branches in the RC (cf. Section 3.2.2.2). A more profound influence of the protein on the RC pigment system is seen in the absorption spectra, which differ from the composite spectra of the individual components (Fig. 5). The protein binds a pair of BChl-b (BC_P) in such a way that the two BChl-b molecules interact strongly via the pyrrole rings I, the acetyl substituents, and the central magnesium ions.[47] Alignment of the transition dipole moments and proximity of the BChl-b molecules lead to excitonic coupling, which partly explains the long wavelength absorption band P_{960}.[49]

The change in the optical spectra of blue copper proteins compared with those of cupric ions in normal tetragonal coordination is even greater (Fig. 6). The redox potential is also raised, to about 300–500 mV vs. 150 mV for Cu^{2+} (aq).[51] These effects are caused by the distorted tetrahedral coordination of the type-1 copper (a strained conformation stabilizing the cuprous state) and a charge transfer transition from a ligand cysteine $S^- \rightarrow Cu^{2+}$.[39,52]

The examples presented demonstrate the influence of the protein on the cofactors by various mechanisms: the stabilization of unstable conformers and strained ligand geometries and the generation of contacts between pigments leading to strong electronic interaction.

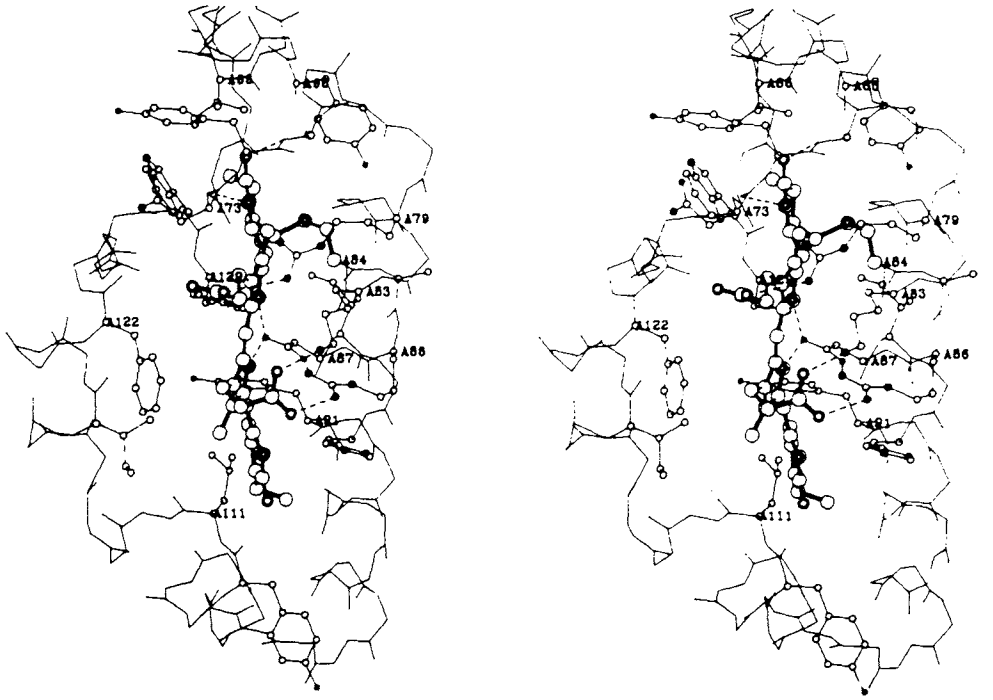

Fig. 4. Stereodrawing of phycocyanobilin A84 (thick bonds) and its protein environment (thin bonds). All polar groups of the bilin except those of the terminal D pyrrole ring are bound by hydrogen bonds and salt links to protein groups [44].

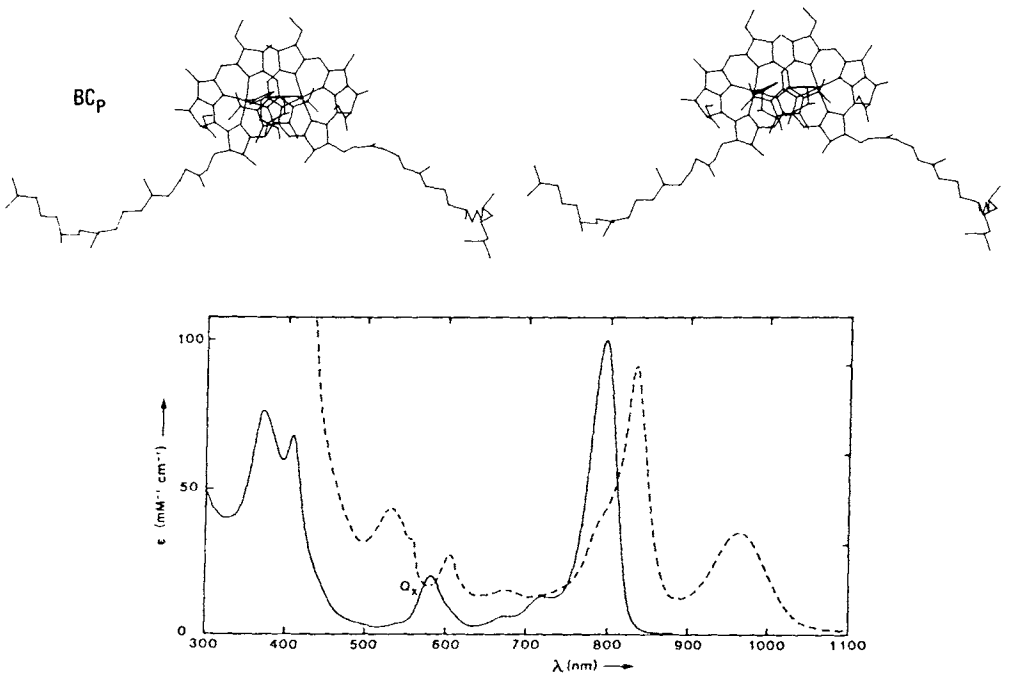

Fig. 5. Stereodrawing of the special pair BC_P in the reaction center (RC) [47] mainly responsible for the spectral alterations and the long wavelength absorption of the RC of *Rps. viridis* (– – –) compared with the spectra of BChl-*b* in ether solution (———) (spectra from Ref. [48]).

The fixation of the relative arrangements of systems of cofactors forms the basis of the energy and charge transfer properties in each system.

3.2. Protein as a Scaffold

3.2.1. Light Harvesting by Phycobilisomes

The limited number of pigment molecules associated with the RC would absorb only a small portion of incident sunlight. The RCs are therefore associated with LHC, which may be located within the photosynthetic membrane or form layers or antenna-like organelles in association with the photosynthetic membrane. Cyanobacteria contain particularly intricate light harvesting systems, the phycobilisomes (PBS), as organelles peripheral to the thylakoid membrane. Compared to PS I and PS II they absorb light of shorter wavelengths and thus use a wider spectral range of sunlight (Fig. 7). The PBS are assembled from components with fine-

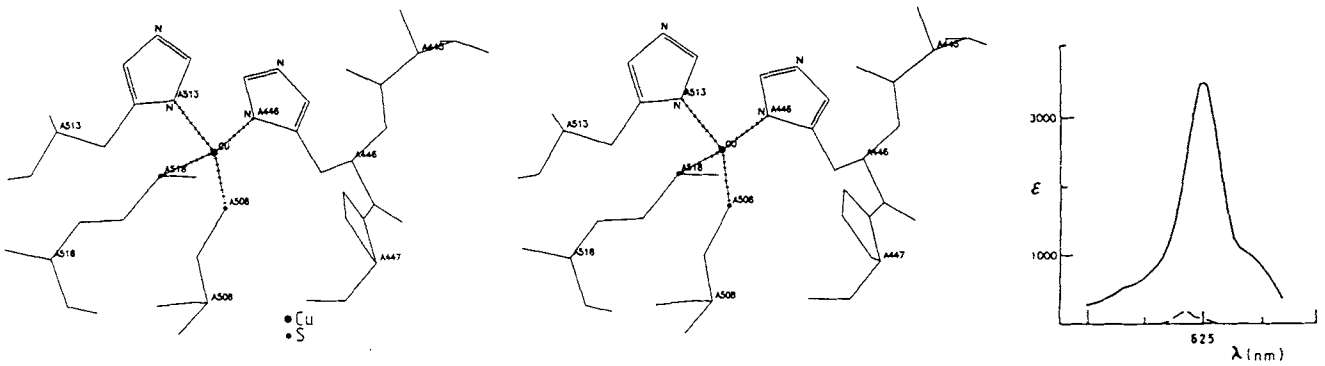

Fig. 6. Stereodiagram of the type-1 copper and its ligands in the ascorbate oxidase (AO). The coordination of the copper is to His A446, His A513, Met A518, Cys A508 [50]. The optical absorption spectra of 'blue' copper in copper proteins (———) are compared with the spectrum of normal tetragonal copper (– – –) (spectra from Ref. [51]).

ly tuned spectral properties such that the light energy is channeled along an energy gradient to PS II.

3.2.1.1. Morphology of the Phycobilisomes

The phycobilisomes consist of biliproteins and linker polypeptides. Biochemical and electron microscopy studies[54–57] led to the model representative of a hemidiscoidal phycobilisome (PBS) in Figure 7. Accordingly, PBS rods are assembled in a polar way from phycoerythrin (PE) or phycoerythrocyanin (PEC) and phycocyanin (PC), which is attached to a central core of allophycocyanine (APC). APC is next to the photosynthetic membrane and close to PS II (for a review, see Ref. [53]). The PC component consists of α- and β-protein subunits, which are arranged as $(\alpha\beta)_6$ disc-like aggregates with the dimensions 120 Å × 60 Å (for reviews, see Refs. 58–63]).

From crystallographic analyses, a detailed picture of the PC and PEC components has emerged.[44–46, 64, 65] The homology in the amino acid sequences suggests that all components have similar structures.

3.2.1.2. Structure of Phycocyanin

The α- and β-subunits of the phycocyanin (PC) (in *Mastigocladus laminosus*) have 162 and 172 amino acid residues, respectively. The phycocyanobilin chromophores (cf. Fig. 2) are linked via thioether bonds to cysteine residues at position 84 of both chains (A84, B84) and at position 155 of the β-subunit (B155).[66] Both subunits have similar structures and are folded into eight α-helices (X, Y, A, B, E, F, G, H: see Fig. 13). A84 and B84 are attached to helix E, B155 to the G-H loop. α-helices X and Y form a protruding antiparallel pair essential for formation of the (αβ) unit.

The isolated protein forms $(\alpha\beta)_3$-trimers with C_3 symmetry and hexamers $(\alpha\beta)_6$ as head-to-head associated trimers with D_3 symmetry (Fig. 8). The inter-trimer contact is exclusively mediated by the α-subunits, which are linked by an intricate network of polar bonds. The inter-hexamer contacts within the crystal (and in the native PBS rods) are made by the β-subunits.[44]

3.2.1.3. Oligomeric Aggregates: Spectral Properties and Energy Transfer

The spectral properties, absorption strength and fluorescence quantum yield of biliproteins depend on the state of aggregation. The absorption spectrum of the (αβ) unit resembles the sum of the spectra of the constituent subunits, but the fluorescence quantum yield is somewhat higher than

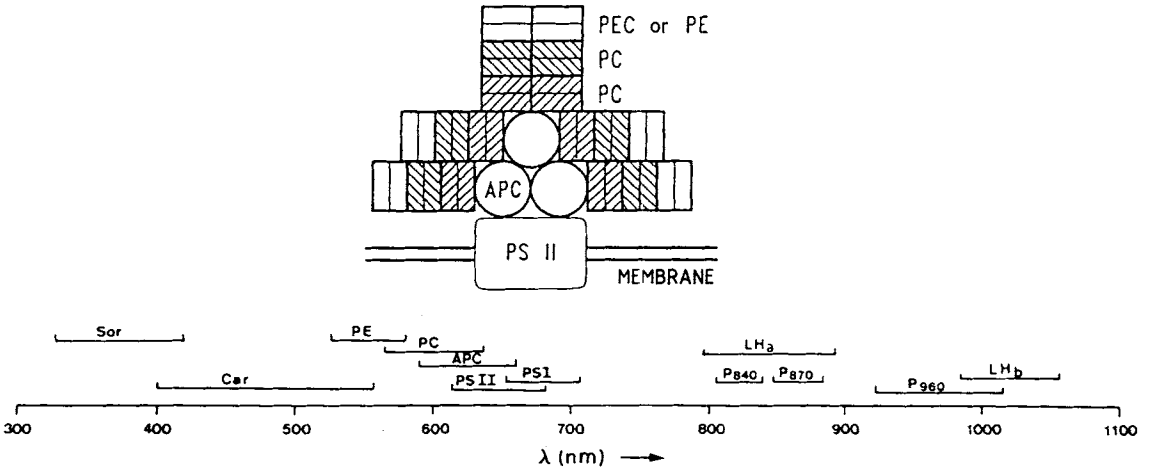

Fig. 7. Scheme of a typical phycobilisome (PBS) with the arrangements of the components and the putative spatial relationship to the thylakoid and photosystem II (PS II; for reviews see Refs. [53, 54]). The component labeled PS II is thought to represent PS II and the phycobilisome attachment sites. The main absorption bands of photosynthetic protein cofactor complexes in photosynthetic organisms are also shown. The PBS components absorb differently to cover a wide spectral range and permit energy flow from phycoerythrocyanin (PEC)/phycoerythrin (PE) via phycocyanin (PC) and allophycocyanin (APC) to PS II (for abbreviations see footnote [***] to the Introduction).

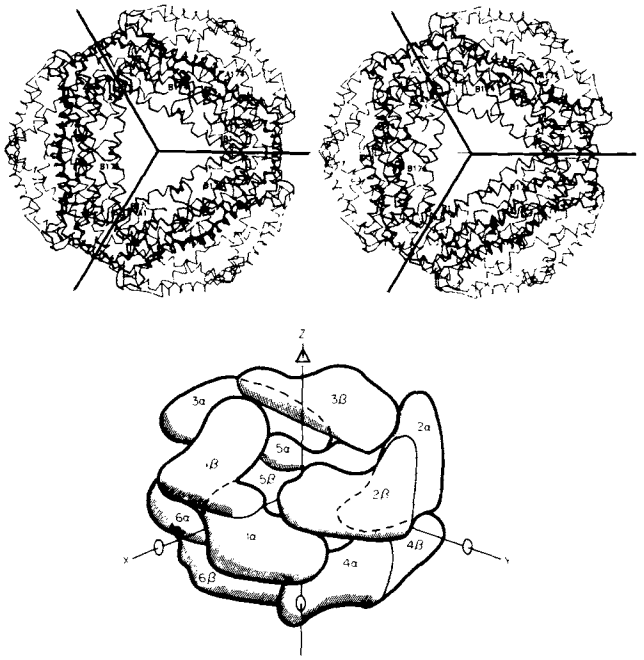

Fig. 8. Stereodrawing of the polypeptide chain fold of an $(\alpha\beta)_6$ hexamer of phycocyanin (PC) seen along the disk axis (top). The scheme (bottom) indicates the packing of subunits in the hexamer seen from the side.

the sum of the fluorescence yields of the constituent subunits. Upon trimer formation, the absorption is red-shifted and its strength and the quantum yield of fluorescence are increased[67, 68] (for a review, see Ref. [60]). In the $(\alpha\beta)_6$-linker complexes, the fluorescence is further increased and the absorption spectrum further altered.[69]

These observations can be rationalized by the structure of the aggregates. Formation of $(\alpha\beta)$ units causes little change in the environment of the chromophores. They remain quite widely separated at distances of > 36 Å (Fig. 9a). Upon trimer formation, the environment of chromophore A84 changes profoundly due to approach of chromophore B84 of the related symmetrically bound unit (Fig. 9a, top). In the hexamer (Fig. 9a, middle) the A84 and B155 chromophores strongly interact pairwise across the trimer interface. Furthermore, the molecular structures become more rigid with increasing size of the aggregates, as seen in the crystals of the trimeric and hexameric aggregates.[44, 46] Rigidity hinders excitation relaxation by isomerization and thus increases the fluorescence quantum yield.

The chromophores can be divided into subsets of s (sensitizing) and f (fluorescing) chromophores.[70, 71] The s-chromophores absorb at the blue edge of the absorption band and rapidly transfer excitation energy to the f-chromophores. This transfer is accompanied by depolarization.[72] Excitation at the red absorption edge (f-chromophores), however, results in little depolarization, suggesting that the energy is transferred along stacks of similarly oriented f-chromophores (Fig. 9a, bottom).[73] The assignment of the chromophores to s and f was accomplished by steady-state spectroscopy on various aggregates,[68] by chemical modification guided by the spatial structure,[74] and conclusively by measurement of the linear dichroism and polarized fluorescence on single crystals.[75] Accordingly, B155 is the s-, B84 the f-, and A84 the intermediate chromophore.

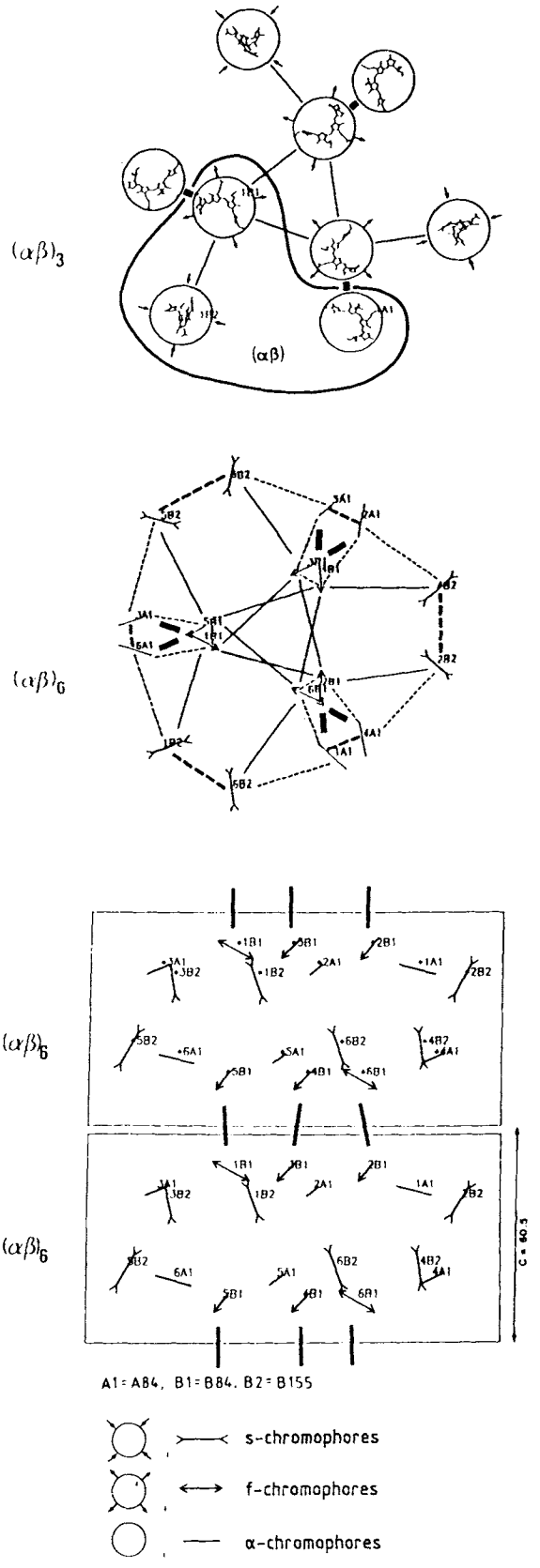

Fig. 9a. Arrangement of chromophores and preferred energy transfer pathways in $(\alpha\beta)_3$ trimers, $(\alpha\beta)_6$ hexamers and stacked hexamers (based on Table 10 from Ref. [44]). For the trimer the detailed structures of the chromophores are drawn, otherwise their approximate transition dipole directions are indicated. For the trimer and hexamer the view is along the disk axis; for the stacked hexamers it is perpendicular. In the stacked hexamers only the inter-hexamer transfers are indicated. The strength of coupling is indicated by the thickness of the connecting lines. Transfer paths within and between the trimers are represented by full and broken lines, respectively.

Light energy is transferred rapidly within 50 to 100 ps from the tips to the core of the PBS (for reviews, see Refs. [60, 73, 76–80]). The transfer times from the periphery to the base are several orders of magnitude faster than the intrinsic fluorescence life-times of the isolated components.[72, 76] The distances between the chromophores within and between the hexamers are too large for strong (excitonic) coupling, but efficient energy transfer by inductive resonance occurs. A Förster radius of about 50 Å has been suggested by *Grabowski* and *Gantt*.[81] The relative orientations and distances of the chromophores as obtained by *Schirmer* et al.[44] were used as basis for the calculation of the energy transfer rates in Figure 9a, which shows the preferred energy transfer pathways in $(\alpha\beta)$ units, $(\alpha\beta)_3$ trimers, $(\alpha\beta)_6$ hexamers and stacked disks as models for native antenna rods. There is very weak coupling of the chromophores in the $(\alpha\beta)$ units. However, some energy transfer takes place, probably between B155 and B84, as indicated by steady-state polarization measurements.[68, 82] Trimer formation leads to strong coupling between A84 and B84, but B155 is integrated only weakly. In the hexamer many additional transfer pathways are opened and B155 is efficiently coupled. Hexamers are obviously the functional units, as the energy can be distributed and concentrated on the central f-chromophores, which couple the stacks of hexamers. Kinetic studies[60, 68, 73, 83] have confirmed the picture of energy transfer along the rods as a random walk (trap or diffusion limited) along a one-dimensional array of f-chromophores. *Sauer* et al.[84] have successfully simulated the observed energy transfer kinetics in PC aggregates on the basis of the structures using *Förster*'s mechanism. The PEC component at the tips of PBS rods is extremely similar to PC.[64, 65] Its short wavelength absorbing chromophore A84 is located at the periphery (cf. Fig. 10a), as are the additional chromophores in PE, which is also a tip component (Fig. 9b).

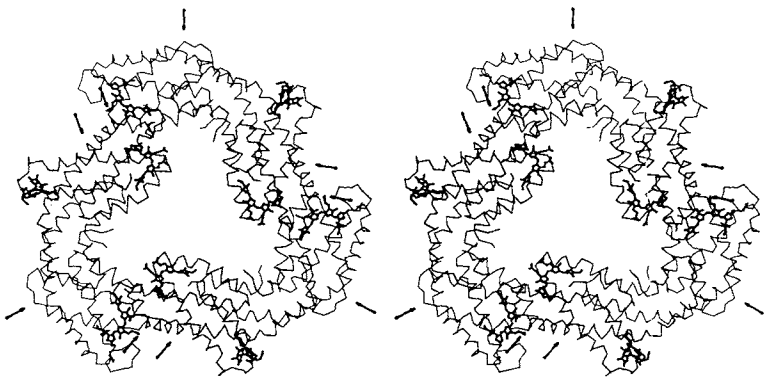

Fig. 9b. Model of the PE $(\alpha\beta)_3$ trimer of phycoerythrin (PE) on the basis of the structure of phycocyanin (PC). The locations of the additional phycoerythrobilins are indicated by arrows.

The phycobilisome rods act as light collectors and energy concentrators from the peripheral to the central chromophores, that is, as excitation energy funnels from the periphery to the center and from the tip to the bottom.

The linker polypeptides determine the functional properties of the aggregates. Some of them are believed to be located in the central channel of the hexamers, where they may interact with B84.

3.2.2. Electron Transfer in the Reaction Center[*]

3.2.2.1. The Composition of the Reaction Center[**]

The reaction center of *Rps. viridis* is a complex of the four protein subunits C, L, M, H and cofactors arranged as in Figure 10a. The proteins consist of 336, 273, 323, and 258 amino acid residues, respectively.[43, 87, 89] The c-type cytochrome contains four heme groups covalently bound via thioether linkages. The cofactors are four BChl-b (BC_{MP}, BC_{LP}, BC_{LA}, BC_{MA}), two BPh-b (BP_M, BP_L), one menaquinone-9 (Q_A), and a ferrous ion involved in electron transfer. A second quinone (ubiquinone-9 UQ, Q_B), which is a component of the functional complex, is partially lost during preparation and crystallization of the reaction center.

3.2.2.2. Chromophore Arrangement and Electron Transfer

The chromophores are arranged in L- and M-branches, which meet at BC_P and are related by an axis of approximate two-fold symmetry.[47] This axis is normal to the plane of the membrane.

While many of the optical properties of the pigment system are rather well understood on the basis of the spatial structure,[49] electron transfer is less well understood. The excited BC_P is quenched by electron transfer to BP_L in 3 ps and then further to the primary acceptor Q_A in about 200 ps, driven by the redox potential gradient between P^*/P^+ (about -760 mV) and Q_A/Q_A^- (about -110 mV). The redox potential of BP/BP^- is intermediate with a value of about -400 mV.[90–99] These functional data are summarized in Figure 10a. General factors controlling the transfer rates have been summarized in Table 1 and are detailed here specifically for the reaction center:

Fast electron transfer requires effective overlap of the molecular orbitals. The orbital interaction decreases exponentially with the edge-to-edge distance of donor and acceptor and is insignificant at distances larger than about 10 Å.[29, 100] In the reaction center the distance between BC_P

[*] A historical background of the development of concepts and key features of the purple bacterial reaction center is given by *Parson* [85].

[**] The arrangement of the reaction center in the thylakoid membranes of *Rps. viridis* as obtained by electron microscopy is described by *Stark* et al. [86].

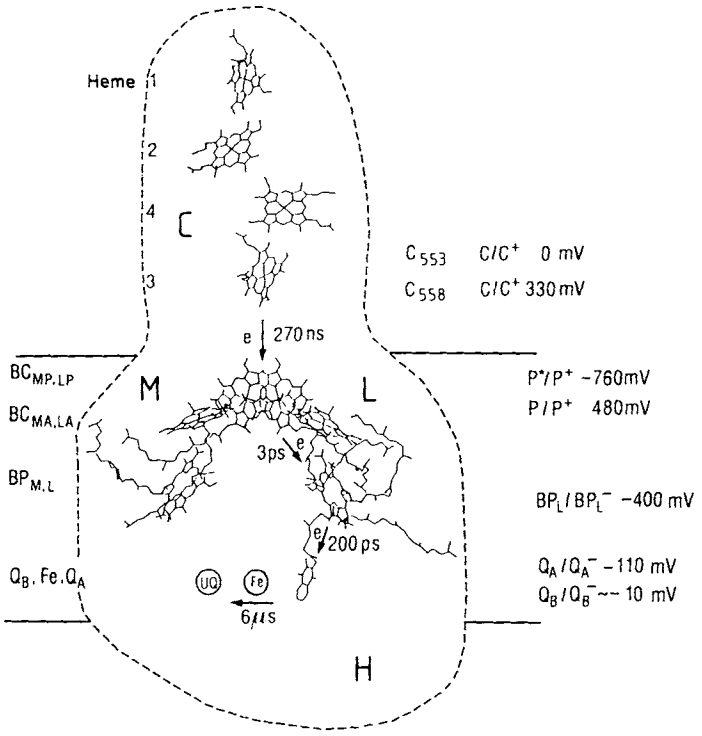

Fig. 10a. Scheme of the structure of the reaction center of *Rps. viridis* showing the cofactor system, the outline of the protein subunits (C, M, L, H), the electron transfer half-times, and the redox potentials of defined intermediates (for references see Section 3.2.2.2; for abbreviations see footnote [***] to the Introduction).

and Q_A is far too large to allow fast direct electron transfer; instead the electron migrates via BP_L. BP_L^- is a spectroscopically and kinetically well defined intermediate. Although located between BC_P and BP_L, BC_{LA}^- is not an intermediate but probably involved in electron transfer by a 'superexchange' mechanism mediating a strong quantum mechanical coupling[101] (for a review, see Ref. [102]). The distance between BP_L and Q_A seems large for a fast transfer. Indeed, in the L branch of the pigment system, this gap is bridged by the aromatic side chain of Trp M250 (Fig. 10b),[37,88] which might mediate coupling via appropriate orbitals. In addition, the isoprenoid side chain of Q_A is close to BP_L. Electron transfer via long connecting chains by through-bond coupling of donor and acceptor orbitals has been observed[100,103,104] but, in the reaction center, there are only Van der Waals contacts.

A second important factor for electron transfer is the free energy change (ΔG), which is governed by the chemical nature of the components, by geometrical factors, and by the environment (solvent polarity). It depends on the ionization potential of the donor in its excited state, on the electron affinity of the acceptor, and on the coulombic interaction of the radical ion pair, which is probably small, as donor and acceptor (BC_P and Q_A in the RC) are far apart. The influence of the environment may be substantial in the stabilization of the radical ion pair by ionic interactions and hydrogen bonds. ΔG is *one* important factor of the activation energy of electron transfer. Also important is the nuclear rearrangement of the reactants and the environment. As the charge on donor and acceptor develops, the nuclear configurations change. These changes are likely to be small in the RC, as the BChl-b macrocycles are relatively rigid and are tightly packed in the protein, and the charge is distributed over the extended aromatic electron system. Reorientable dipolar groups (peptide groups and side chains) may contribute considerably to the energy barrier of the electron transfer. On the other hand, a matrix with high electronic polarizability stabilizes the developing charge in the transition state of the reaction and reduces the activation energy. An alternative picture is that the potential energy barrier to electron tunneling is decreased. Aromatic compounds which are concentrated in the vicinity of the electron carriers in the RC have these characteristics (see Trp M250).

The electron transfer from P* to Q_A occurs with very low activation energy[91,94,96,105-108] and proceeds readily, even at 1 K. Thermally activated processes, nuclear motions, and collisions are therefore not important for the first very fast charge separation steps. There is even a slight increase in rate with decrease in temperature, due either to a closer approach of the pigments at low temperature or to changes of the vibrational levels, which may lead to a more favorable Franck-Condon factor.

The electron transfer between primary and secondary quinone acceptors, Q_A and Q_B, is rather different from the previous processes, because it is much slower (about 6 μs at pH 7 according to *Carithers* and *Parson*[91]) and has a sub-

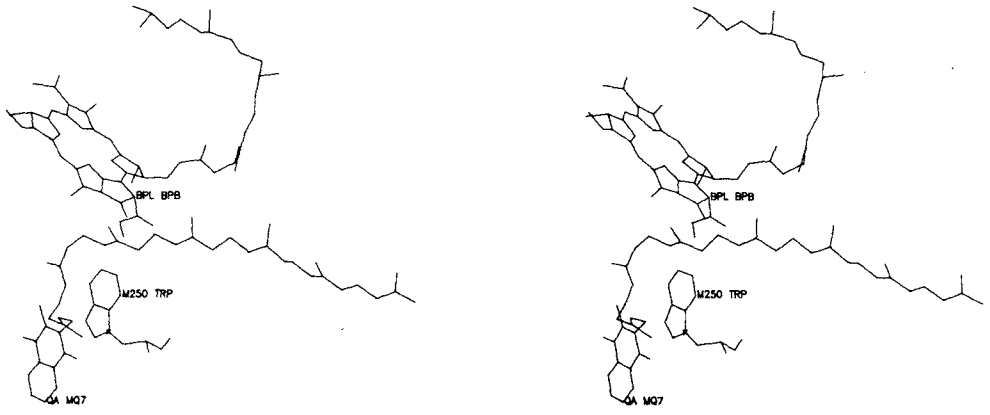

Fig. 10b. Stereodrawing of the arrangement of BP_L, Trp M250 and Q_A in the L-branch of the pigment system of the reaction center.

stantial activation energy of about 8 kcal mol^{-1}. In *Rps. viridis*, Q_A is a menaquinone-9 and Q_B a ubiquinone-9, which differ in their redox potentials in solution by about 100 mV. In other purple bacteria, both Q_A and Q_B are ubiquinones. The redox potential difference required for efficient electron transfer in these cases is generated by the asymmetric protein matrix. The protein matrix is also responsible for the quite different functional properties of Q_A and Q_B. Q_A accepts only one electron (leading to a semiquinone anion), which is transferred to Q_B before the next electron transfer can occur. Q_B, however, accepts two electrons and is protonated to form a hydroquinone, which diffuses from the reaction center (two-electron gate[109]). Q_B is close to Glu L212, which opens a path to the H-subunit and may protonate Q_B. The electron transfer between Q_A and Q_B takes place in an environment which differs considerably from the environment of the primary electron transfer components. The line connecting Q_A and Q_B (the Q_B binding site has been inferred from the binding mode of competitive inhibitors and ubiquinone-1 in *Rps. viridis* crystals[37]) is occupied by the iron and its five coordinating ligands – four histidine residues (M217, M264, L190, L230) and a glutamic acid (M232) residue. His M217 forms a hydrogen bond to Q_A. His L190 is close to Q_B. Q_A and Q_B are separated by an edge-to-edge distance of about 15 Å, which might explain the slow transfer. If electron transfer and protonation are coupled, the observed pH dependence of the electron transfer rate from Q_A to Q_B[110] could be explained, and conformational changes required for proton transfer may generate the observed activation energy barrier. The role of the charged Fe-His_4-Glu complex in the Q_A to Q_B electron transfer is poorly understood at present, as it occurs also in the absence of the iron;[111] its role seems to be predominantly structural.

The cycle of electron transfer is closed by the rereduction of BC_P^* by cytochrome c (subunit C of the reaction center), whereby a distance of about 11 Å between the pyrrole ring I of heme 3 and the pyrrole ring III of BC_{LP} has to be bridged. The transfer time is 270 ns[95] (cf. Ref. [147]); the transfer is considerably slower than the first electron transfer steps. Tyr L162, which is located midway (Fig. 10c), may facilitate electron transfer by mediating electronic coupling between the widely spaced donor and acceptor. The biphasic temperature dependence indicates a complex mechanism in which nuclear motions play a role at high temperatures (for reviews, see Refs. [112, 113]).

The favorable rate controlling factors that have been discussed are a necessary but not sufficient condition for the electron transfer, which competes with other quenching processes summarized in Table 2 and now described in detail for the reaction center:

The transfer of energy from P* back to the light harvesting complex or to other pigments may be favored by orientation and proximity but is disfavored energetically. The special pair BC_P usually absorbs (but not in *Rps. viridis* where the absorption maxima of the RC and the LHC are at 960 and 1020 nm respectively, see Fig. 7) at longer wavelengths than other pigments of the photosynthetic apparatus and thus represents the light energy sink. The natural radiative lifetime of the excited singlet state P* is around 20 ns[107, 114] and may serve as an estimate of the times involved in the other wasteful quenching processes. Clearly, electron transfer is much faster. Non-radiative relaxation of BC_P^* by isomerizations and conformational changes is unlikely, since the cyclic pigment systems are tightly packed in the protein matrix.

The back reaction $P^+Q_A^-$ to PQ_A has a favorable driving force (Fig. 10a) and is temperature independent, but is slow and insignificant under physiological conditions (for a review, see Ref. [94]). The physical basis for this has yet to be explained. It may be related to a gating function of BP_L by its negative redox potential compared to Q_A, to electronic properties of P^+ disfavoring charge transfer, and to conformational changes induced by electron transfer.

The profound influence of the protein matrix on electron transfer in the reaction center manifests itself in the observed asymmetry of electron transfer in the bacteriochlorophyll-b (BC) and bacteriopheophytin (BP) pigments, depending upon whether they belong to the L or the M branch. Only the

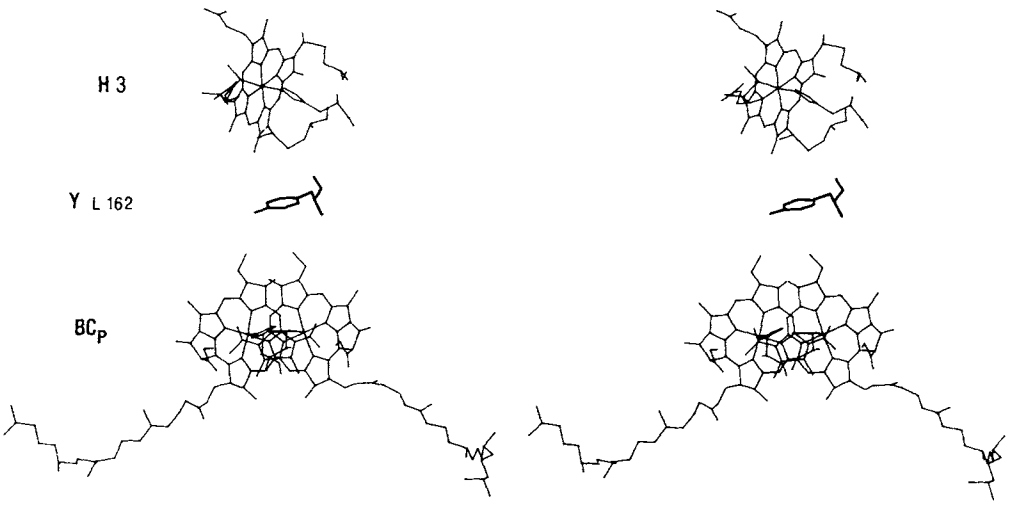

Fig. 10c. Stereodrawing of heme 3 (H3) of the cytochrome c, the special pair BC_P and the intercalating Tyr L162 (Y L162) of the L-subunit [37]. The His and Met ligands to the iron of H3 and the His ligands to the magnesium ions of the BC_P are also shown.

branch more closely associated with the L-subunit is active. An explanation is offered perhaps by the finding that the protein environment of the two branches, although formed by homologous proteins (L and M), is quite different, in particular by virtue of the Trp M250 located between BP_L and Q_A and the numerous differences in the Q_A and Q_B binding sites.[37, 88] Asymmetry is observed in the BC_P due to different distortions and hydrogen bonding of the macrocycles and in the slightly different spatial arrangements of the BC_A and BP. It has been suggested that electron release into the L-branch is thereby facilitated.[115] The M-branch may, however, have influence as a pendant group.

The protein matrix also serves to dissipate the excess energy of about 650 mV[92] of the excited special pair ($P^* Q_A$) compared to the radical ion pair $P^+ Q_A^-$. These processes are probably very fast.

In summary, the very fast electron transfer from BC_P^* to Q_A occurs between closely spaced aromatic macrocycles with matched redox potentials. The protein matrix in which the pigments are tightly held is lined predominantly with apolar amino acid side chains with a high proportion of aromatic residues. The path taken by the electrons is far removed from the water phase.

3.2.3. *The Blue Oxidases*

Oxidases catalyze the reduction of dioxygen by single electron transfers from substrates. Dioxygen requires four electrons and four protons to be reduced to two water molecules. Oxidases must provide recognition sites for the two substrates, a storage site for electrons and/or the means to stabilize reactive partially reduced oxygen intermediates.[116–118]

The 'blue' oxidases are classified according to the distinct spectroscopic properties of the three types of copper which they contain: Type-1 Cu^{2+} is responsible for the deep blue color of these proteins; type-2 or normal Cu^{2+} has no recognizable optical absorption; type-1 and type-2 cupric ions are paramagnetic; type-3 copper has a strong absorption around 330 nm and is antiferromagnetic, indicating coupling of the spin of a pair of cupric ions. The characteristic optical and electron paramagnetic resonance spectra disappear upon reduction.

Studies of the catalytic and redox properties of the 'blue' oxidases have been well documented in several recent reviews (e.g. for laccase,[119] for ascorbate oxidase,[120] for ceruloplasmin[121]). Basically, type-1 Cu^{2+} is reduced by electron transfer from the substrate. The electron is transferred further to the type-3 and type-2 copper ions. The second substrate, dioxygen, is associated with the type-3 and/or type-2 copper ions.

3.2.3.1. *Ascorbate Oxidase, Composition and Copper Arrangement*

Ascorbate oxidase is a polypeptide of 553 amino acid residues folded into three tightly associated domains.[50] It is a dimer in solution; the functional unit, however, is the monomer. Ascorbate oxidase belongs, together with laccase and ceruloplasmin, to the group of 'blue' oxidases.[122]

Structures of copper proteins containing only one of the different types of copper are known: Plastocyanin has a 'blue' type-1 copper, which is coordinated to two histidine residues and the sulfur atoms of cysteine and methionine in a distorted tetrahedral geometry.[123] Cu–Zn-superoxide dismutase contains a type-2 copper, which has four histidine ligands with slightly distorted square coordination.[124] Hemocyanin of *Panulirus interruptus* contains as type-3 copper, a pair of copper ions 3.4 Å apart with six histidine ligands.[125]

In domain 3 of ascorbate oxidase (see Section 4.4) a copper ion is found in a strongly distorted tetrahedral (approaching trigonal pyramidal geometry) coordination with the ligands His, Cys, His, Met as has already been shown in Figure 6. It resembles the blue type-1 copper in plastocyanin. A trinuclear copper site is enclosed between domains 1 and 3 of ascorbate oxidase (Fig. 11a). Four (–His–X–His–) amino acid sequences provide the eight histidine ligands. The trinuclear copper site contains a pair of coppers (Cu 31, Cu 32), each with three histidyl (A108, A451, A507; A64, A106, A509) ligands, forming a trigonal prism. It is the type-3 copper pair; a comparable arrangement is observed in

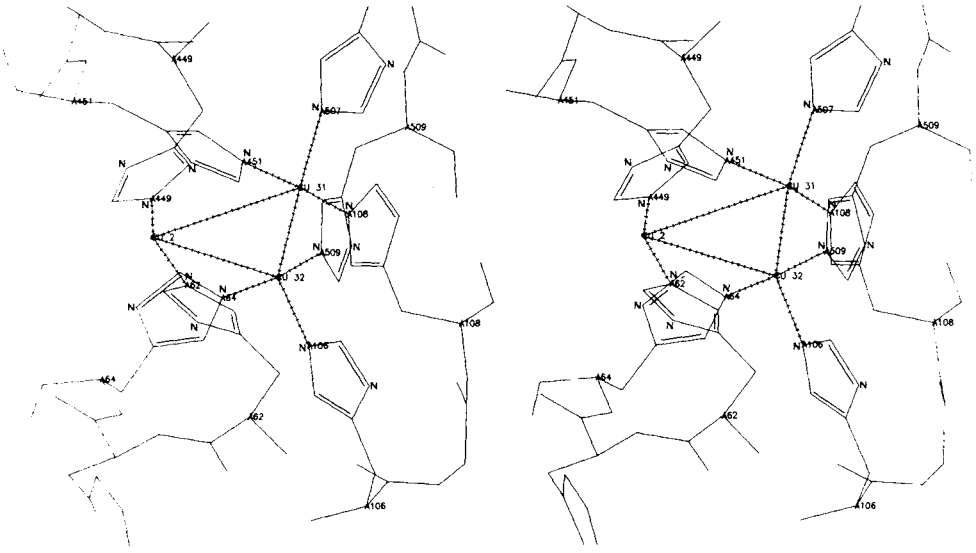

Fig. 11a. Stereodrawing of the trinuclear copper site in ascorbate oxidase. Coordination bonds between the copper ions and the protein residues are marked (dotted lines) [50].

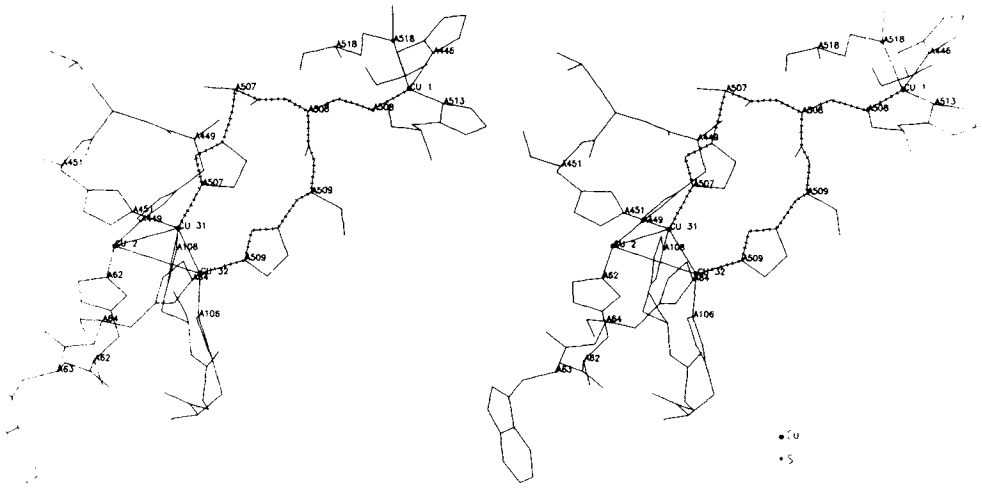

Fig. 11 b. Stereodrawing of the tridentate peptide ligand (–His 507–Cys 508–His 509–) in ascorbate oxidase bridging type-1 copper (Cu 1) and the trinuclear cluster (Cu 31, Cu 32, Cu 2) [50].

hemocyanin. The remaining copper (Cu 2) has two histidyl ligands (A62, A449) and is type-2 copper. The trinuclear copper cluster is the site where dioxygen binds; details of the structure including the presence of exogenic ligands still require clarification. The close spatial association of the three copper ions in the cluster suggests facile electron exchange. The cluster serves as electron storage site and may function as cooperative three-electron donor for the oxygen molecule, to irreversibly cleave the O–O bond.

3.2.3.2. Intramolecular Electron Transfer in Ascorbate Oxidase

Electrons are transferred from the type-1 copper to the trinuclear site. The shortest pathway is via Cys-A508 and His-A507 or His-A509. The (His–X–His–) segment links electron donor and acceptor and thus bridges a distance of 12 Å (Fig. 11 b). The cysteine sulfur and the imidazole components of the bridging ligand have low lying unoccupied molecular orbitals and may favor a chemical mechanism of electron transfer, but the intervening aliphatic and peptide chains are unlikely to form transient radicals and may well participate by resonance. The optical absorption of the 'blue' copper assigned to a cysteine $S^- \rightarrow Cu^{2+}$ charge transfer transition supports the suggested electron pathway.

The putative electron path branches at the C^α atom of Cys A508. Model compounds have shown inequivalence and faster transfer in the N–C direction of amide linking groups.[131] This may apply also to the blue oxidases and cause preferred transfer to A507.

The difference in redox potential between the type-1 copper and the type-3 copper is –40 mV in ascorbate oxidase. Unfortunately, there are no direct measurements of the intramolecular electron transfer rates available. The turnover number serves as a lower limit and is $7.5 \times 10^3\ s^{-1}$ in ascorbate oxidase.[126, 127] indicating a fairly rapid transfer despite the long distance and small driving force. The electron pathway is intramolecular and far removed from bulk water.

The characteristic distribution of the redox centers as mono- and trinuclear sites in the blue oxidases may also be found in the most complex oxidase, cytochrome oxidase (see the hypothetical model of *Holm* et al.[128]) and in the water-splitting manganese protein complex of the photosystem II, which carries out the reverse reaction of the oxidases. For its $(Mn)_4$ cofactor, either two binuclear or a tetranuclear metal center is favored,[129] but mono- and trinuclear arrangements cannot be excluded.

3.3. The Protein as Medium

The boundary between protein as ligand and as medium is fluid. The extreme microscopic complexity of structure, polarity and polarizability of protein media may influence the energy and electron transfer. There are no obvious common structural features in the protein systems discussed except a high proportion of aromatic residues (particularly tryptophans) bordering the electron transfer paths in the reaction center of purple bacteria and in ascorbate oxidase and their wide separation from bulk water by internal location within the protein and the hydrocarbon bilayer (in the reaction center). These effects have been mentioned in Sections 1.3 and 3.2.2.2.

4. Structural Relationships and Internal Repeats

All four protein systems mentioned in the following show internal repetition of structural motifs or similarities to other proteins of known folding patterns. This is a quite common phenomenon and not confined to energy and electron transfer proteins. It is also not uncommon that these relationships often remained undetected on account of the amino acid sequences; in effect this reflects our ignorance of the sequence structure relationships. An analysis of the structural relationships will shed light on the evolution and function of protein systems and is thus appropriate here.

4.1. Retinol and Bilin Binding Proteins

The simplest case is shown in Figure 12, where bilin-binding protein (BBP)[142] is compared with retinol-binding

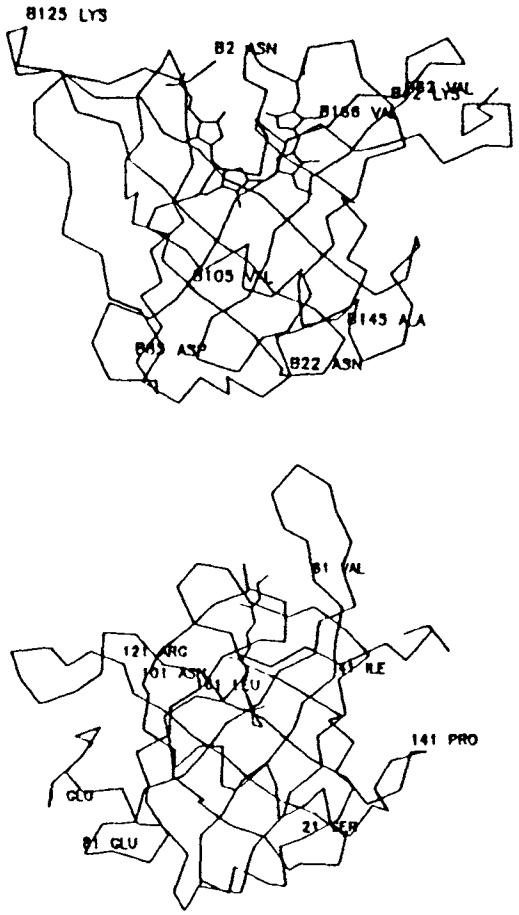

Fig. 12. Comparison of the polypeptide chain folds of bilin-binding protein (BBP) and retinol-binding protein (RBP) with bound cofactors.

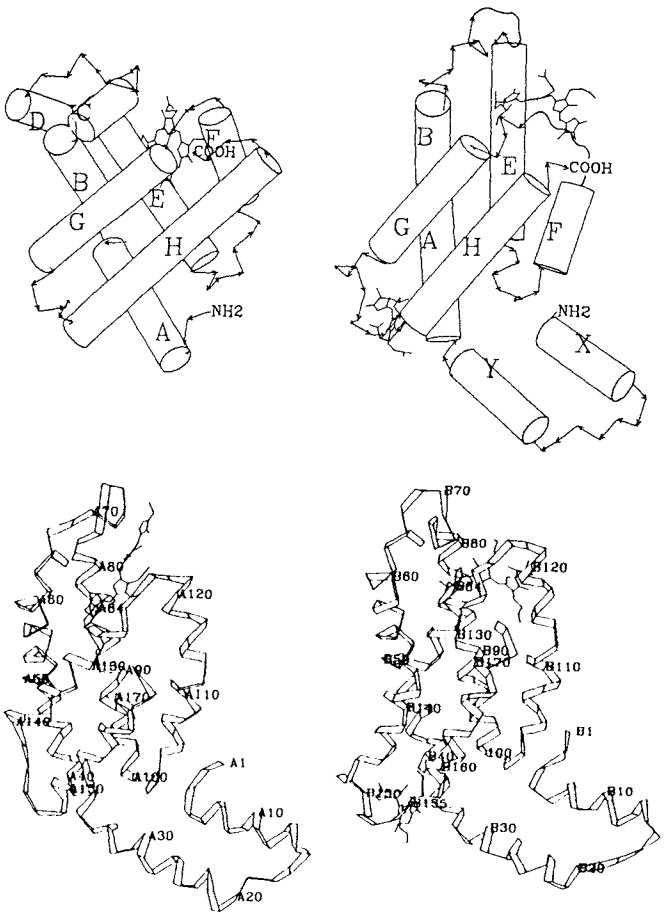

Fig. 13. Polypeptide chain folds of the α- and β-subunits of phycocyanin [44] (lower part, left and right) and comparison of the arrangements of α-helices in myoglobin and phycocyanin (upper part, left and right).

protein (RBP).[130] For the bottom of the β-barrel structure, the structural similarity is obvious, while the upper part, which is involved in the binding of the pigments biliverdin and retinol, differs considerably. The molecule is apparently subdivided into framework and hypervariable segments which determine binding specificity in analogy to the immunoglobulins.[131] This relationship suggests carrier functions for BBP as for RBP, although RBP also serves for pigmentation in butterflies.

4.2. Phycocyanin

In the protein part, phycocyanin (PC) consists of two polypeptide chains α and β which are clearly related in structure (Fig. 13, bottom), and probably originate from a common precursor.

The α-subunit is shorter in the G–H turn and lacks the s-chromophore B155 (see Section 3.2.1.3.). The loss or acquisition of chromophores during evolution may be less important than differentiation of the α and β subunits, which occupy non-equivalent positions in the $(\alpha\beta)_3$ trimer, so that the homologous chromophores A84 and B84 are non-equivalent with B84 lying on the inner wall of the disk. In addition the α and β subunits play very different roles in the formation of the $(\alpha\beta)_6$ hexamer, as has already been shown in Figure 8. Symmetrical precursor hexamers might have existed and could have formed stacks, but would lack the differentiation of the chromophores, in particular the inequivalence and close interaction of A84 and B84 in the trimer. Functional improvement has probably promoted divergent evolution of the α- and β-subunits.

A most surprising similarity was discovered between the PC subunits and the globins shown in Figure 13 (top). The globular helical assemblies A to H show similar topology. The U-shaped extension of phycocyanin formed from the *N*-terminal X,Y α-helices is essential for the formation of the αβ substructure. Comparison of the amino acid sequence after structural superposition reveals some homologies, suggesting divergent evolution of phycobiliproteins and globins;[44] however, what function a precursor of light harvesting and oxygen binding proteins might have had remains a mystery.

4.3. Reaction Center

The reaction center of *Rps. viridis* lacks symmetry across the membrane plane. This is not surprising for a complex which catalyzes a vectorial process across the membrane. However, there is quasi-symmetry relating the L- and M subunits and the pigment system. The structural similarity and the homology of the amino acid sequences of the L- and M-subunits suggest a common evolutionary origin. This relationship is extended to the photosystem II components D1 and D2 on the basis of sequence homology and conservation of residues involved in cofactor binding (for reviews, see

Refs. [132, 133]). The putative precursor was a symmetrical dimer with identical electron transfer pathways. The interaction with the H subunit introduces asymmetry, particularly noteworthy at the *N*-terminal transmembrane α-helix of the H-subunit (H), which is close to the E transmembrane α-helix of the M-subunit, the L-branch of the pigment system, and Q_A (Fig. 14). The improvement of the interaction with the H-subunit, which appears to play a role in the electron transfer from Q_A to Q_B and in protonation of Q_B might have advanced divergent evolution of the L- and M subunits at the expense of the inactivation of the M pigment branch. However, the electron transfer from BC_P to Q_A is extremely fast and not rate-limiting for the overall reaction. The evolutionary conservation of the M branch of pigments may be of functional significance as a pendant group in light harvesting and electron transfer. There are also structural reasons, for its deletion would leave a void.

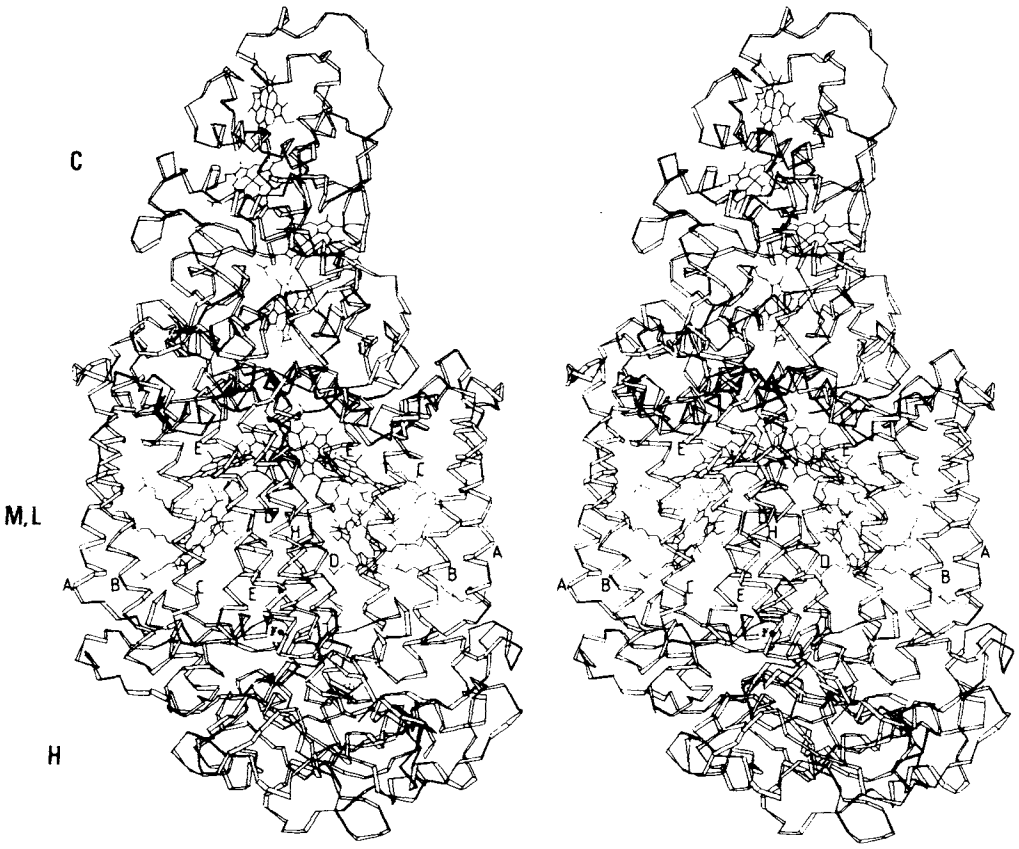

Fig. 14. Stereodrawing of the reaction center of *Rps. viridis* (complete complex of subunits and cofactors). The membrane-spanning α-helices of the L- and M-subunits (A, B, C, D, E in sequential and A, B. C, E, D in spatial order) and the H-subunit (H) are labeled [37] (cf. Fig. 10 a).

The cytochrome subunit (C) contributes to the asymmetry of the L–M complex and shows, in itself, an internal duplication.[37] All four heme groups are associated with a helix-turn-helix motif, but the turns are short for heme groups 1 and 3 and long for heme groups 2 and 4.

4.4. Blue Oxidases

Gene multiplication and divergent evolution is most evident in the blue oxidase, ascorbate oxidase. Figure 15 shows the polypeptide chain of 553 amino acid residues folded into three closely associated domains of similar topology.[50] Although nearly twice as large, they resemble the simple, small copper protein plastocyanin (Fig. 16).[123] In the blue oxidase the domains I and III enclose the trinuclear copper cluster in a quasi-symmetrical fashion, but only domain III contains the type 1 copper, the electron donor to the trinuclear site. A potential electron transfer pathway in domain I is not realized, reminiscent of the M-branch of pigments in the reaction center (cf. Section 3.2.2.2). Similarly to the H subunit in the reaction center, the linking domain II introduces asymmetry in the ascorbate oxidase, which might have promoted evolutionary divergence of domains I and III.

The proteins plastocyanin, ascorbate oxidase, laccase, and ceruloplasmin are members of a family of copper proteins, as indicated by structural relations and sequence homology.[50, 135–137] They provide a record from which an evolutionary tree may be mapped out (Fig. 17). The simplest molecule is plastocyanin containing only a type-1 copper. A dimer of plastocyanin-like molecules could provide the two times four histidyl ligands for the trinuclear copper cluster, representing a symmetrical oxidase. From this hypothetical precursor the modern blue oxidases and ceruloplasmin might have evolved following different paths of gene (domain) insertion and loss or acquisition of copper. In both, the arrangement of the *N*- and *C*-terminal domains, which

Fig. 15. Stereodrawing of the polypeptide chain folds of ascorbate oxidase and exploded view of its three domains from top to bottom [50]. β-strands are indicated as arrows and α-helices as cylinders (produced by the plot program of *Lesk* and *Hardman* [134]).

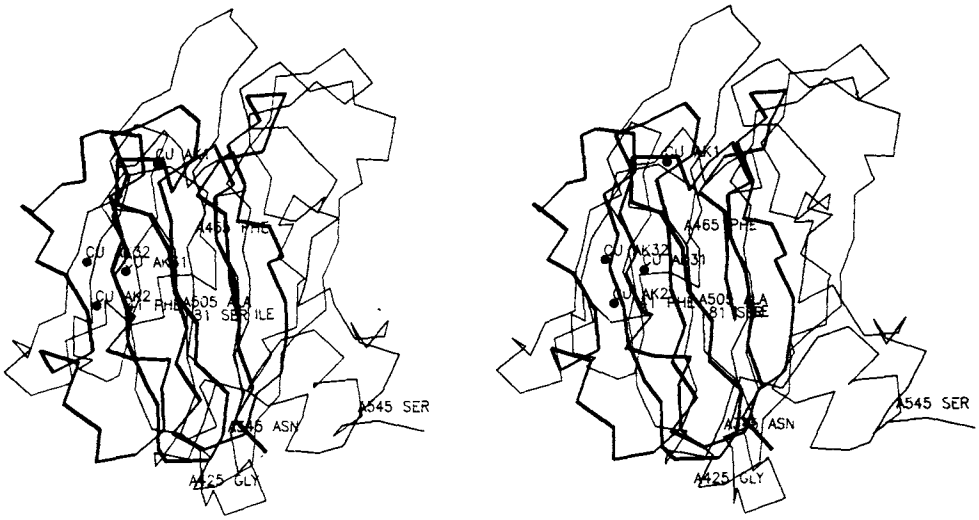

Fig. 16. Stereodrawing and superposition of domain III of ascorbate oxidase (thin lines) and plastocyanin (thick lines). The trinuclear copper site in ascorbate oxidase is buried between domain I (not shown) and domain III.

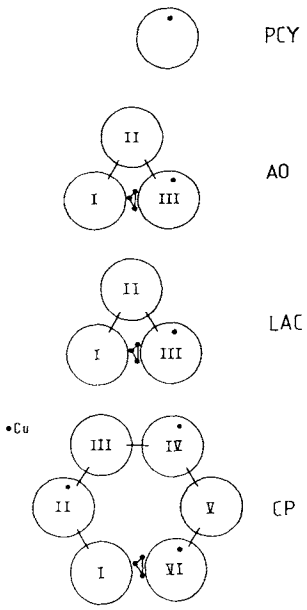

Fig. 17. Homologous domains in plastocyanin (PCY), ascorbate oxidase (AO), laccase (LAC) and ceruloplasmin (CP). The mono- and trinuclear copper sites are indicated.

contain the functional copper cluster, has been preserved. Recombinant DNA technology provides the possibility of reconstructing the hypothetical precursor oxidase. This is under investigation.

5. Implications from the Structure of the Reaction Center for Membrane Proteins in General

The structures of water soluble proteins show a seemingly unlimited diversity, despite their being built from only a few well defined secondary structural elements such as helices, β-sheets and turns and despite their construction from domains and recurring structural motifs. The proteins under discussion provide ample evidence of this. That there seems to be a limited set of basic folds may be related to the evolution of proteins from a basis set of structures and/or to constraints by protein stability and rates of folding. These basic folding motifs do not represent rigid building blocks, however, but adapt to sequence changes and respond to the environment and to association with other structural elements. Adaptability and plasticity (which is not to be confused with flexibility) is related to the fact that the entire protein and solvent system, and not its individual components, must attain the global energy minimum. Water is a good hydrogen bond donor and acceptor and is thus able to saturate polar peptide groups exposed on the surface almost as well as intraprotein hydrogen bonds do (entropic effects excluded).

Membrane proteins face the inert hydrocarbon part of the phospholipid bilayer and must saturate their hydrogen bonds intramolecularly. Only two secondary structures fulfill this condition for the polypeptide main chains, namely the α-helix and the β-barrel. For the assembly of α-helices packing rules have been derived which predict certain preferred angles between the helix axes albeit with a broad scatter. Similarly, the arrangement of strands in β-sheets and β-barrels follows defined rules.[138]

5.1. Structure of the Membrane Associated Parts of the Reaction Center

The reaction center was the first membrane protein whose structure could be determined at atomic resolution. Only the low resolution structure of bacteriorhodopsin was known previously. Both structures have some common features.[139] The structure of the reaction center may permit some conclusions to be drawn about membrane proteins in general. The reaction center contains 11 transmembrane α-helices, which, with 26 residues (H-subunit) or 24–30 residues (L- and M-subunits), have appropriate lengths to span the membrane. The amino acid sequences of these segments are devoid of charged residues (Fig. 18). Few charged residues occur close to the ends of the α-helices. Glycine residues initiate and terminate almost all α-helical segments, both the transmembrane and the connecting α-helices. It is well known from soluble proteins that glycine residues are abundant in turns and often associated with flexible regions of proteins.[12] They may be important for the insertion into the membrane by enabling structural rearrangements. The angles between the axes of the contacting α-helices of the L- and M-complex are 20° to 30°, a packing angle range also preferred by α-he-

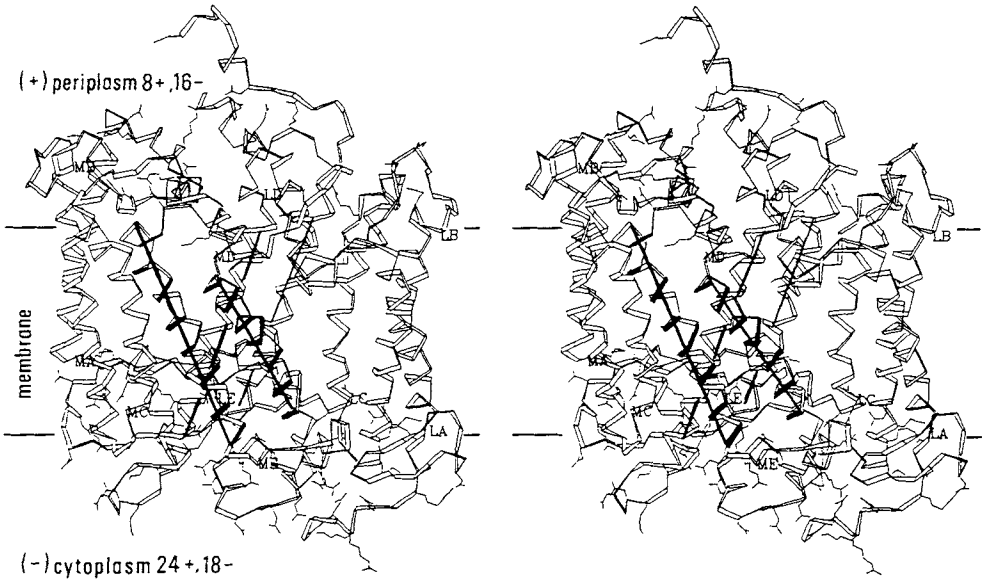

Fig. 18. Stereodrawing of the polypeptide chains of the L- and M-subunits of the reaction center in ribbon representation. The *N*-terminal residues of the membrane-spanning α-helices are labeled (including the prefix M and L) and the tetrahelical motif of the D and E α-helices is marked by shading and lines. The side chains of charged residues are drawn. Asp, Glu, and carboxy-termini are counted as negatively charged, and Lys, Arg and amino-termini are counted as positively charged and added for the cytoplasmic and periplasmic sides [5, 37].

lices in soluble proteins. They have features in common with buried α-helices in large globular proteins, which are also characterized by the absence of charged residues and the preferred location of glycines and prolines at the termini.[140, 141] Furthermore, the D- and E-α-helices of the L- and M-subunits (Fig. 18) find counterparts in soluble proteins. They are associated around the local diad axis and form the center of the L–M module, which binds the iron and the bacteriochlorophyll pair BC_P.

The four D- and E-α-helices of the L- and M-subunits are arranged as a bundle, held together by the iron ion and splayed out towards the cytoplasmic side to accommodate the large special pair. This motif is quite common in water-soluble electron transfer proteins.[142] I will resume this discussion later and suggest appropriate substructures of soluble proteins as models for pore forming membrane proteins.

5.2. Membrane Insertion

The structure of the reaction center is also important regarding our views of the mechanism of integration of membrane proteins into the phospholipid bilayer. The reaction center is composed of components which are quite differently arranged with respect to the membrane: The C-subunit is located on the periplasmic side. The H-subunit is folded into two parts: a globular part located on the cytoplasmic side and a transmembrane α-helix. The L- and M-subunits are incorporated into the phospholipid bilayer. Consequently, cytochrome (the C-subunit) has to be completely translocated across the membrane from its intracellular site of synthesis. In the H-, L-, and M-subunits the transmembrane α-helices are embedded in the bilayer. Only the *N*-terminal segment of H and the *C*-termini and connecting segments of the α-helices located at the periplasmic side of L and M (A–B, C–D) require transfer.

It is interesting to note that only the cytochrome gene possesses a prokaryotic signal sequence, as indicated by the sequence of the gene.[89] Transfer of the large hydrophilic C-subunit may require a complex translocation system and a signal sequence, while H, L, and M may spontaneously insert into the bilayer due to the affinity of the contiguous hydrophobic segments for the phospholipids (for a review of this and related problems see Ref. [143]). A "simple" dissolution still requires transfer across the membrane of those charged residues which are located at the periplasmic side.[37, 88] The increasingly favorable protein lipid interaction which develops with insertion may assist in this process. M and L have considerably more charged residues at the cytoplasmic side (41) than at the periplasmic side (24), providing a lower activation energy barrier for correct insertion. The net charge distribution of the L–M complex is asymmetric, with six positive charges at the cytoplasmic side and eight negative charges at the periplasmic side. As the intracellular membrane potential is negative, the observed orientation of the LM complex is energetically favored (Fig. 18).

The H-subunit has a very polar amino acid sequence at the *C*-terminus of the transmembrane α-helix with a stretch of seven consecutive charged residues (H33–H39)[37, 87] which may efficiently stop membrane insertion. Similarly, there are three to eleven charged residues in each of the connecting segments of the α-helices at the cytoplasmic side of the L- and M-subunits which might stop the transfer of α-helices or α-helical pairs.[144] As an alternative to sequential insertion the L- and M-subunits may be inserted into the membrane as assembled protein pigment complexes, because they cohere tightly by protein–protein and protein–cofactor interactions.

5.3. Models of Pore Forming Proteins

It is not absolutely clear whether the structural principles observed in the reaction center apply also to 'pore' or 'channel' forming α-helical proteins. These could, in principle, elaborate quite complex structures within the aqueous chan-

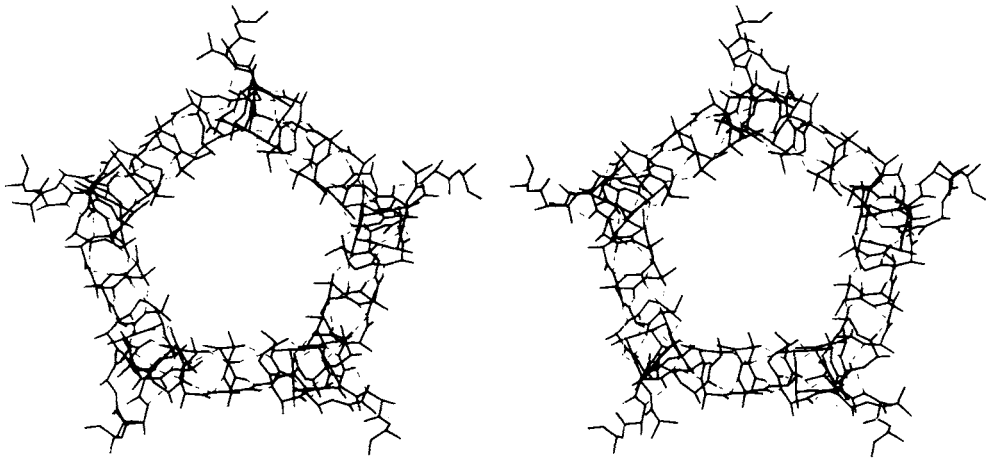

Fig. 19. Stereodrawing of the pentahelical pore in heavy riboflavin synthase [148].

nel,[145] but available evidence at low resolution for gap junction proteins[146] indicates in this specific case a simple hexameric arrangement of membrane-spanning amphiphilic α-helices, whose polar sides face the aqueous channel.

Guided by the observation that rules for structure and packing of α-helices derived for soluble proteins apply also to the reaction center, we may derive models for membrane pore forming proteins from appropriate soluble protein substructures. The penta-helical pore seen at high resolution in the icosahedral multi-enzyme complex riboflavin synthase seems to be a suitable model[148] (Fig. 19). Five amphiphilic α-helices of 23 residues each are nearly perpendicular to the capsid surface. The coiled coil of α-helices has a right-handed twist and forms a pore for the putative import of substrates and export of products. They pack with their apolar sides against the central four-stranded β-sheet of the protein, which mimics the hydrocarbon part of a phospholipid bilayer, and project charged residues into the aqueous channel.

Similar modeling of membrane protein structures may be extended to another class of membrane proteins which have β-structures spanning the outer membrane, the bacterial porins.[149] The β-barrels observed in soluble proteins have four to eight or more strands. The lower limit is determined by the distortion of the regular hydrogen bonds. An upper limit may be given by the possible sizes of stable protein domains. A four-stranded β-barrel with 4 parallel strands duplicated head-to-head with symmetry D_4 is observed in the ovomucoid octamer.[150] The β-strands lean against the hydrophobic core of the molecule and project their (short) polar residues into the channel (which is extremely narrow here).

6. Some Thoughts on the Future of Protein Crystallography

Thirty years after the elucidation of the first protein crystal structures by *Perutz* and *Kendrew* and after steady development, protein crystallography is undergoing a revolution. Recent technical and methodical developments enable us to analyze large functional protein complexes like the reaction center,[37,151] large virus structures (to mention only Refs. [152–154]), protein DNA complexes (to mention only Ref. [155]), and multienzyme complexes like riboflavin synthase.[148]

The significance of these studies for understanding biological functions is obvious and has excited the interest of the scientific community in general.

In addition, it was recognized that detailed structural information is a prerequisite for the rational design of drugs and proteins. As an illustration I chose human leucocyte elastase, which is an important pathogenic agent. On the basis of its three-dimensional structure (Fig. 20)[156] and the criteria of optimal stereochemical fit, potent inhibitors are

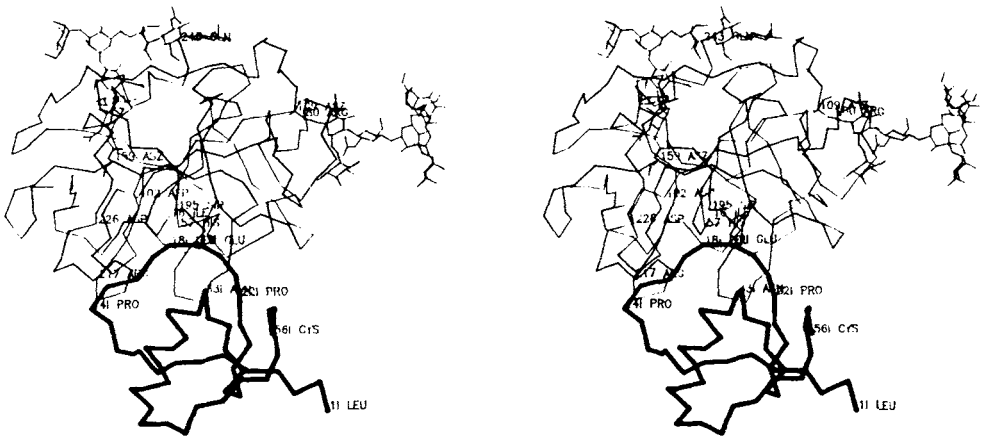

Fig. 20. Stereodrawing of the complex between human leucocyte elastase (thin lines) and turkey ovomucoid inhibitor (thick lines) [156].

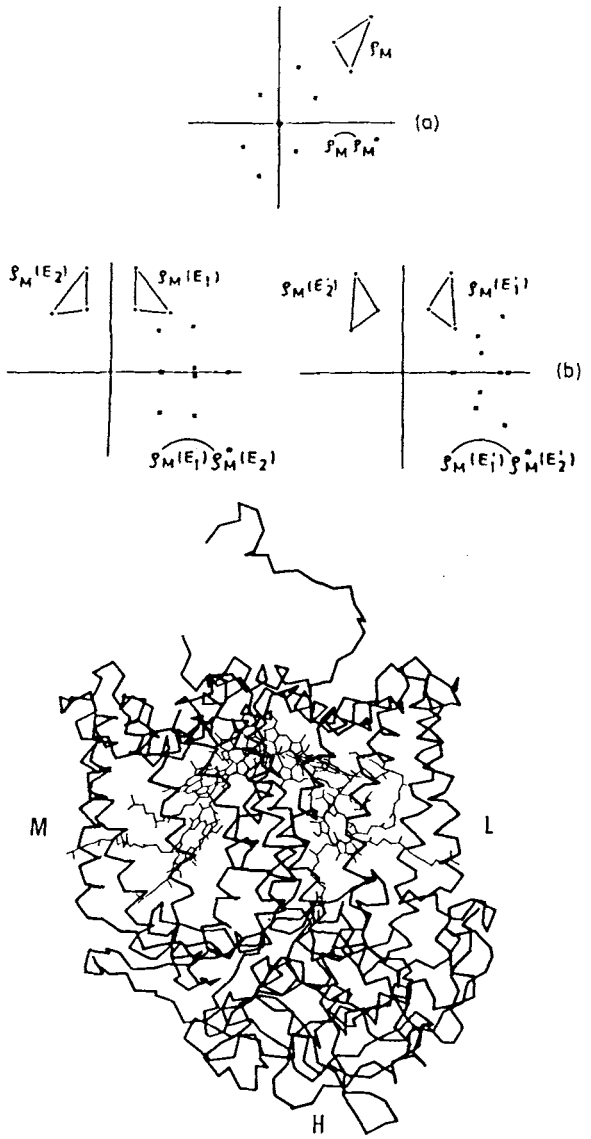

Fig. 21. Faltmolekül construction. Upper part: $\varrho_M \varrho_M^*$ (a) and $\varrho_M(E1)\varrho_M^*(E2)$ (b) are the intra- and intermolecular vector sets of a triangular structure ϱ_M, respectively. Their sum represents the Patterson function. The intramolecular vector set can be constructed from the molecular structure, is located at the origin, and permits the determination of the orientation. From the intermolecular vector set the translation component relative to the mirror plane can be derived. In (b) the intermolecular vector sets corresponding to two different orientations of ϱ_M are shown [164]. Lower part: Drawing of the main chain of the M-, L-, H-subunits and the cofactors which served as search model to solve the phase problem for the crystal structure of the reaction center of *Rb. sphaeroides*. For the calculation all homologous main chain and side chain atoms were included [6].

now being synthesized or natural inhibitors modified by use of recombinant DNA technology in many scientific and commercial institutions. Other, equally important proteins are also being studied. This field especially benefits from the readily available molecular modeling software (e.g. FRODO[157]) and a standard depository of structural data, the Protein Data Bank.[158]

Success *and* the new technical and methodical developments spur protein crystallography's progress. These new developments are indeed remarkable: Area detectors for automatic recording of diffraction intensities have been designed. Brilliant X-ray sources (synchrotrons) are available for very fast measurements and now permit use of very small crystals or radiation-sensitive materials. Their polychromatic radiation is used to obtain diffraction data sets within milliseconds by Laue techniques,[159] and their tunability allows the optimal use of anomalous dispersion effects.[160, 161]

Refinement methods including crystallographic and conformational energy terms provide improved protein models. Methods which allow the analysis of large protein complexes with internal symmetry averaging procedures have been developed[162] leading from blurred to remarkably clear pictures. A priori information of a relationship to known proteins can be used to great advantage as it is possible to solve an unknown crystal structure using a known model of a variant structure by a method discovered and named the 'Faltmolekül' method by my teacher, *W. Hoppe*. It has become a very powerful tool in protein crystallography.

With this last paragraph I wish to pay tribute to *W. Hoppe* who in 1957 laid the foundation to Patterson search methods by discovering that the Patterson function (the Fourier transform of the diffraction intensities) of molecular crystals can be split into sums of intra- and intermolecular vector sets[163] from which orientation and translation of the molecules can be derived when their approximate structure is known (Fig. 21). *Hoppe*'s method has been elaborated, computerized, and reformulated.[8, 165, 166] It provided a shortcut to the crystal structure of the reaction center of *Rb. sphaeroides* which was solved on the basis of the molecular structure of the reaction center of *Rps. viridis* and subsequently refined.[6, 151] The molecular architectures are very similar, although the *Rb. sphaeroides* reaction center lacks the permanently bound cytochrome. The structure solution was independently confirmed using similar methods.[7] With the Faltmolekül method the orientation and location of a molecule in a crystal cell can be determined. The detailed molecular structure and its deviations from the parent model have to be worked out by crystallographic refinement, to which *W. Steigemann* and *J. Deisenhofer* (in his thesis work) laid a foundation in my laboratory.[167, 168]

Recently, NMR techniques have demonstrated their efficiency in the determination of three-dimensional structures of small proteins in solution. In one case a detailed comparison of crystal and solution structures has shown very good correspondence,[169, 170] but further development is called for to extend the power of the method to larger protein structures.

Protein crystallography is the only tool available to unravel in detail the architecture of the large protein complexes described here, and it will continue in the foreseeable future to be the only experimental method that provides atomic resolution data on atom – atom and molecule – molecule interactions. It is such a method which *Emil Fischer* had in mind in 1907 in his 9th Faraday Lecture when pointing out that an understanding of "the precise nature of the assimilation process ... will only be accomplished when biological research, aided by improved analytical methods, has succeeded in following the changes which take place in the actual chlorophyll granules."[171] Yet an ultimate goal for which we all struggle is the solution of the folding problem. The growing number of known protein structures and the design of single residue variants by recombinant DNA technology and their analysis by protein crystallography has brought us nearer to this goal. We are able to study contributions of

individual residues to rates of folding, structure, stability, and function. Also theoretical analysis of protein structures has progressed (to mention only *Levitt* and *Sharon*[172]), but a clue to the code relating sequence and structure is not in sight.[173] Like *Carl von Linné* who 250 years ago created a system of plant classification on the basis of morphology (Genera plantarum, Leiden 1737), we classify proteins according to their shapes and structures. Whether this may lead to a solution of the folding problem is unclear, but it is certain that the end of protein crystallography will only come through it itself.

Acknowledgement

J. Deisenhofer's and my interest in structural studies on the photosynthetic reaction center of *Rps. viridis* was awakened by the establishment of *D. Oesterhelt*'s department in Martinsried in 1980; he brought with him *H. Michel*, with whom a fruitful collaboration on the analysis of the crystal structure of this large protein complex began. Later, other members of my group, *O. Epp* and *K. Miki*, became involved. We had been studying enzymes, proteases and their natural inhibitors, immunoglobulins, and had developed methods to improve data collection, electron density map interpretation, and crystallographic refinement. The tools were available to solve the structure of the reaction center, which was and still is the largest asymmetric protein whose structure has been determined with atomic resolution.

The "eureka" moment of protein crystallography is at the very end when one sees for the first time a new macromolecule with the eyes of a discoverer of unknown territories. To reach this moment much, sometimes tedious work has to be done with the ever present possibility of failure. I am deeply grateful to my collaborators, present and past, for their dedicated and patient work over many years. I mention by name those involved in the studies of the light harvesting cyanobacterial proteins and the blue oxidases: W. Bode, M. Duerring, R. Ladenstein, A. Messerschmidt, T. Schirmer. These projects were collaborative undertakings with biochemists in Switzerland (H. Zuber, W. Sidler), USA (M. L. Hackert) and Italy (M. Bolognesi, A. Marchesini, A. Finazzi-Agro).

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