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NOBEL LECTURE

A structural basis of light energy and electron transfer in biology*

Robert Huber

Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG

*Dedicated to Christa.

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

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....' (Boltzmann, 1886).

Today many of these mysteries have been resolved by biochemical research and the protein components and their basic catalytic functions have been defined (Calvin and Bassham, 1962). 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 centre of *Rhodopseudomonas viridis* [see the original publications cited later and short reviews (Deisenhofer *et al.*, 1985b, 1986, 1989)] and of functionally related systems, whose structures have been studied in my laboratory: light-harvesting cyanobacterial phycobilisomes and blue oxidases. A wealth of structural and functional data is available for these three systems, which make them uniquely appropriate examples from which to derive general principles of light energy and electron transfer in biological materials. Indeed, there are few systems known in sufficient detail for such purposes. [The strucure of the *Rhodobacter sphaeroides* reaction centre (RC) is closely related to the *R.viridis* RC (Allen *et al.*, 1986, 1987; Chang *et al.*, 1986). A green bacterial bacteriochlorophyll-*a*-containing light-harvesting protein is well defined in structure (Tronrud *et al.*, 1986) but not in function. In the multihaem cytochromes (Pierrot *et al.*, 1982; Higuchi *et al.*, 1984) the existence/or significance of intramolecular electron transfer is unclear.]

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 (Bennett and Huber, 1984; Huber, 1988). Theoretical treatments of these reactions have to take flexibility and solvent into account and become theoretically tractable only by applying the rather severe approximations of molecular dynamics (Karplus and McCammon, 1981; Burkert and Allinger, 1982) 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 seems to be unimportant, as shown by generally small temperature dependencies. 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 (Förster, 1948, 1967) 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 pair of bacteriochlorophyll *b* (BC_P) discussed in Section 3.1. The electronic excitation is in this case delocalized over a molecular assembly. Very weak coupling produces little or no alteration of the adsorption spectra but the luminescence properties may be

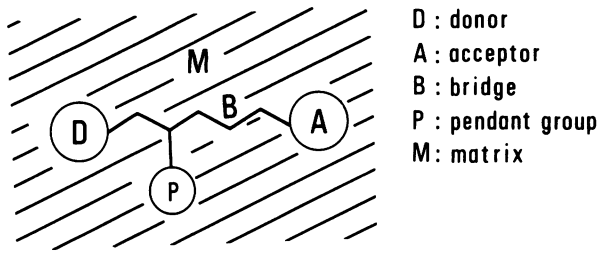


Fig. 1. Determinants of electron transfer models: D, donor; A, acceptor; B, bridge; P, pendant group; M, matrix.

Table I. Determinants of energy and electron transfer

	Factors controlling rates
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 $D^* + A \rightarrow D^+ + A^-$ and from ground state $D^- + A \rightarrow D + A^-$	Distance and orientation (electronic coupling, orbital overlap); free energy change ('driving force'); reorganization in D and A; orientation polarization of medium

Table II. Processes competing with productive transfer

	Competing processes
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^+ and 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 $D^- + A \rightarrow D + A^-$	–

quite different. Structurally defined models for this case are scarce. The controlled deposited dye layers of Kuhn and Frommherz (Kuhn, 1970; Frommherz and Reinbold, 1988) 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 (e.g. Gray, 1986; Mayo *et al.*, 1986; McGoutry *et al.*, 1987). They are covered in reviews (see, for example, Taube and Gould, 1969; Hopfield, 1974; Cramer and Crofts, 1982; Eberson, 1982; Marcus and Sutin, 1985; Mikkelsen and Ratner, 1987; Kebarle and Chowdhury, 1987; McLendon, 1988). Figure 1 shows 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.

Models with porphyrins as donors and quinones as acceptors are mimics of the RC (Gust *et al.*, 1987; Schmidt *et al.*, 1988). Models with peptide-bridging ligands (Isied *et al.*, 1985) 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 (Taube and Gould, 1969) 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 excitation energy and electron transfer and competing processes are to be evaluated.

1.1 *Determinants of energy and electron transfer*

The important factors are summarized in Table I. They may be derived from Förster's theory and forms of Marcus' theory (Marcus and Sutin, 1985) 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, for example, Barltrop and Coyle, 1978). 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 favourably 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 to the ligands (resonance mechanism) (Hains, 1975). The spectral overlap and the 'driving force', for energy and electron transfer respectively, are largely determined by the chemical nature and geometry of donors and acceptors with obvious effects on the transfer rates. Nuclear reorganization of donor, acceptor and the surrounding medium accompanying electron transfer is an important factor, but difficult to evaluate in a complex protein system, even qualitatively; we observe that the protein typically binds donors and acceptors firmly and rigidly, keeping reactant reorganization effects small. Surrounding polar groups may slow rapid electron transfer due to their reorientation. However, a polar environment also contributes to the energetics by stabilizing ion pairs ($D^+ A^-$) or lowering activation and tunnelling barriers and may increase 'driving force' and rate. Energy transfer depends also on the medium and is disfavoured in media with high refractive index.

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

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

2. The role of cofactors

The naturally occurring amino acids are transparent to visible light and seem also to be unsuitable as single electron carriers, with the exception of tyrosine. Tyrosyl radicals have

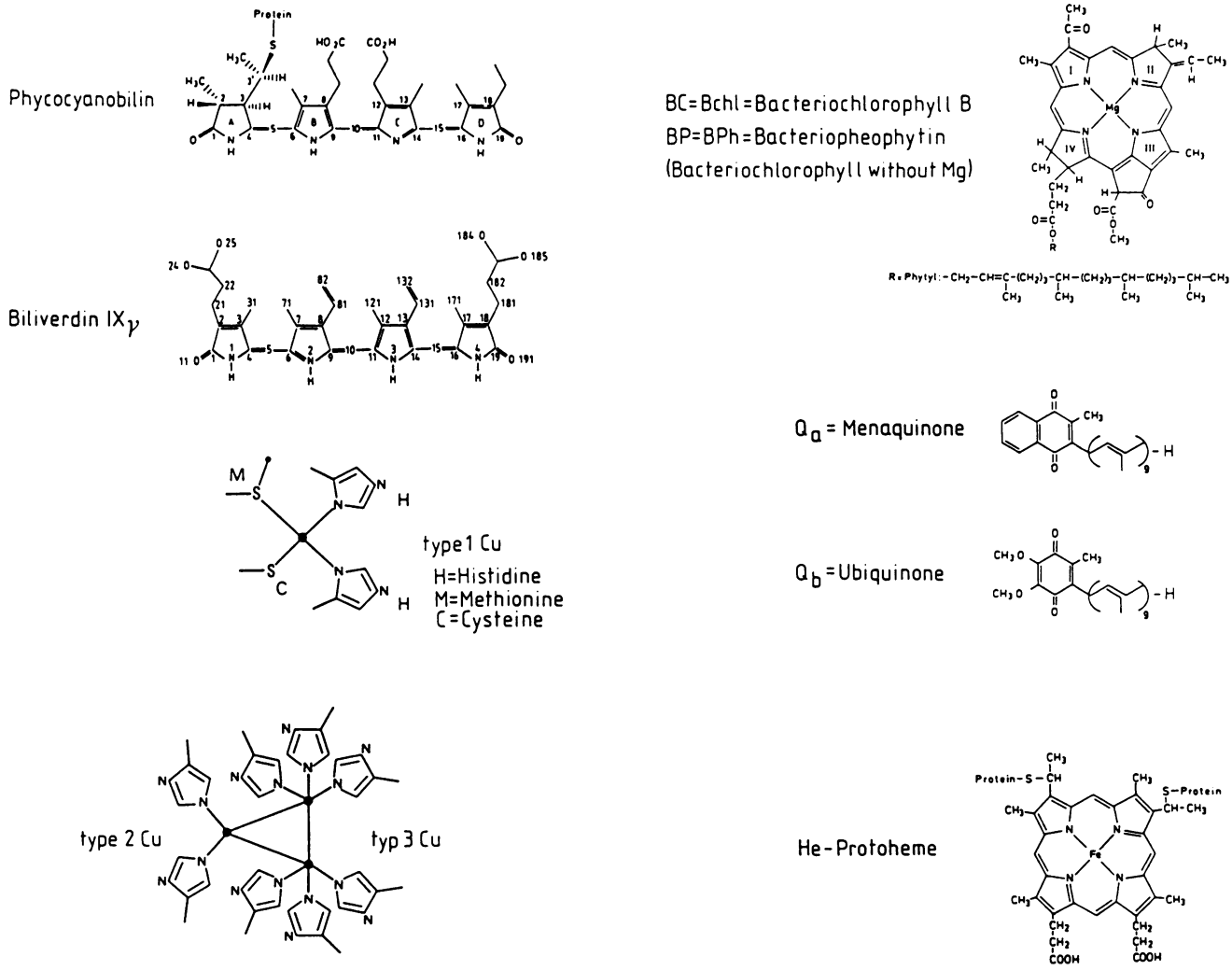


Fig. 2. Cofactors in PC, BBP, AO and RC. Phycocyanobilins are covalently bound by thioether linkages to the protein. Biliverdin IXγ is non-covalently bound to BBP. Type-1, type-2 and type-3 copper ions are linked to AO by coordination to the amino acid residues indicated. Four BChl-b and two BPL-b are bound to the RC. A pair of BCHl-b serves as the primary electron donor, a menaquinone-9 is the primary electron acceptor (Q_A) and an ubiquinone-9 the secondary acceptor (Q_B). The four haem groups are bound by thioether linkages to the cytochrome *c*.

been identified in photosynthetic reaction centre II (PS II) as Z* and D* intermediates, which are involved in electron transfer from the water-splitting manganese–protein complex to the photooxidized $P680^+$ [for review see Barber (1987) and Prince (1988)]. Their identification has been assisted by the observation that Tyr L162 lies in the electron transfer path from the cytochrome to BC_{LP} in the bacterial RC (Deisenhofer *et al.*, 1985a) (see Section 3.2.2.2.2 and Figure 10c). A tyrosyl radical is not generated in the bacterial system, because the redox potential of $P960^+$ is insufficient.

Generally therefore cofactors, pigments and metal ions serve as light energy acceptors and redox-active elements in biological materials.

Figure 2 is a gallery of the pigment and metal clusters which will be discussed further on, namely the bile pigments, phycocyanobilin and biliverdin IXγ in the light-harvesting complexes, the bacteriochlorophyll *b* (BChl-b or BC), bacteriopheophytin *b* (BPh-*b* or BP) and quinones in the purple bacterial RC and the copper centres in the blue oxidases.

The physical chemical properties of these cofactors determine the coarse features of the protein–pigment complexes, but the protein part exerts a decisive influence on the spectral and redox properties.

Table III. The role of the protein in determining protein–cofactor interactions

Hierarchy of protein–cofactor interactions
1. Influence of 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. 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 III. These interactions are different for the various systems and will be described separately, except point 1, as there are common features in the action of the protein as a polydentate ligand ascribed to a 'rack mechanism'.

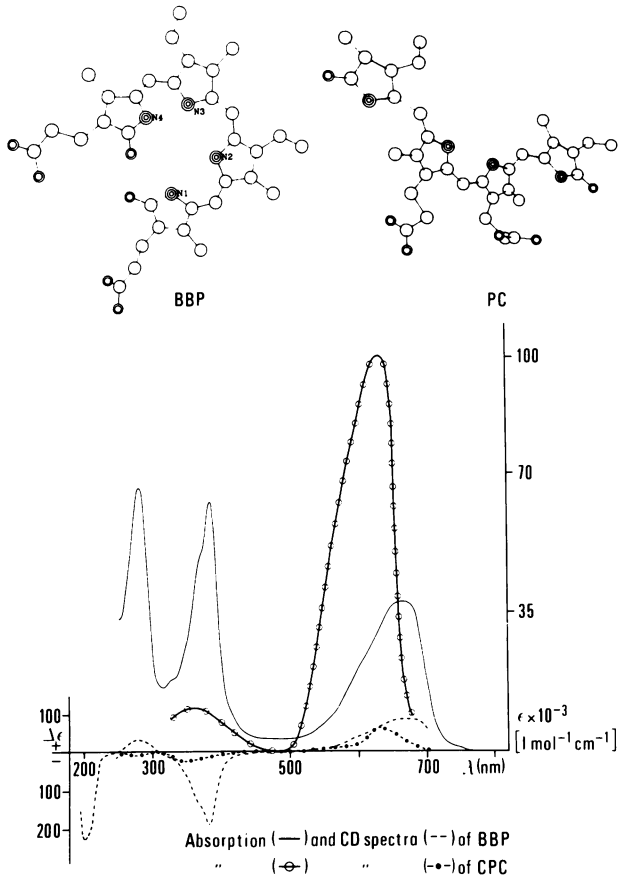


Fig. 3. Tetrapyrrole structures in PC and BBP and the associated optical and circular dichroism spectra (Huber, *et al.*, 1987a; Schirmer *et al.*, 1987).

3.1 *The protein as a polydentate ligand*

The 'rack mechanism' was introduced by Lumry and Eyring (1954) and Gray and Malmström (1983) to explain unusual reactivities, 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 gives a clear demonstration of 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 weak absorption in the visible range and low fluorescence quantum yield (Scharnagl *et al.*, 1983; Huber *et al.*, 1987b,c). When bound as cofactors to light-harvesting phycocyanins they have strong absorption in the visible range and high fluorescence yield (Figure 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 (Schirmer *et al.*, 1985, 1986, 1987) (Figure 4). Particularly noteworthy is an aspartate residue (A87 here) bound to the central pyrrole nitrogens and conserved in all pigment sites. It influences 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 (Huber *et al.*, 1987b,c). This protein serves a different function and prefers the low-energy conformation. The open chain tetrapyrrole bilins are conformationally adaptable, a property, which makes them appropriate cofactors for different purposes.

The cyclic BChl in the RC is conformationally restrained but responds to the environment by twisting and bending of the macrocycle. This may be one cause for the different

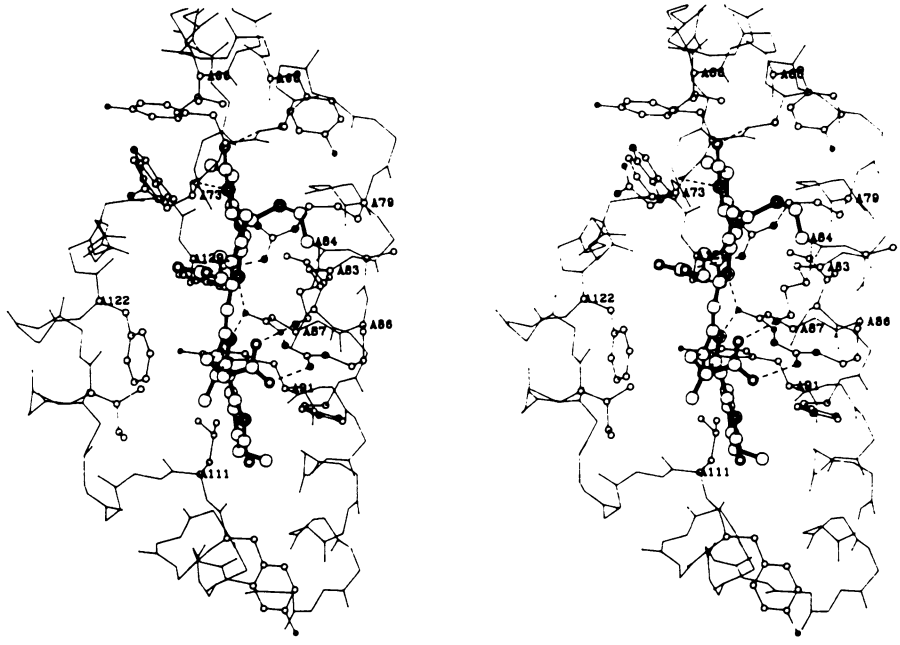


Fig. 4. Stereo drawing of phycocyanobilin 84 (thick bonds) and its protein environment (thin bonds). All polar groups of the bilin except those of the terminal D pyrrole ring are bound by hydrogen bonds and salt links to protein groups (Schirmer *et al.*, 1987).

electron-transfer properties of the two pigment branches in the RC as will be discussed later. 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 (Figure 5). The protein binds a pair of BChl-*b* (BC_P) so that the two BChl-*b* interact strongly between their pyrrole rings I, including the acetyl substituents and the central magnesium ions (Deisenhofer *et al.*, 1984). Alignment of the transition dipole moments and close approach cause excitonic coupling which partially

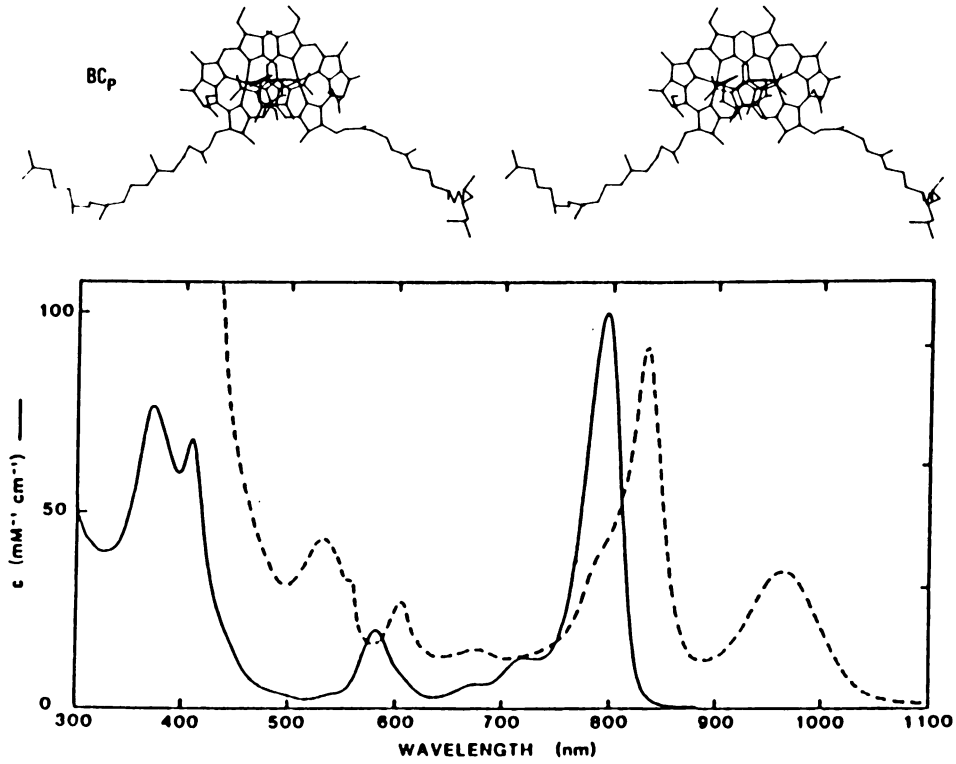


Fig. 5. Stereodrawings of the special pair BP_P in the RC (Deisenhofer *et al.*, 1984) mainly responsible for the spectral alterations and the long-wavelength absorption of the RC of *R. viridis* (- - -) compared with the spectra of BChl-b in ether solution (——) (spectra from Parson *et al.*, 1985).

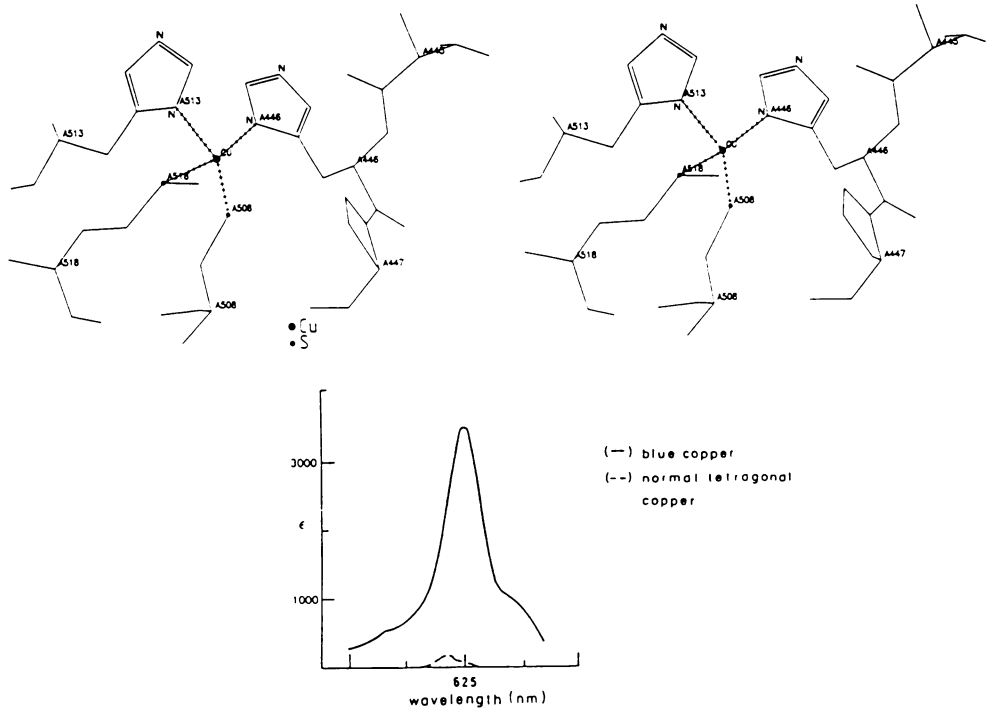


Fig. 6. The type-1 copper and its ligands in AO in stereo. The coordination of the copper is to His A446, His A513, Met A518, Cys A508 (Messerschmidt *et al.*, 1988). The optical absorption spectra of 'blue' copper in copper proteins (——) are compared with normal tetragonal copper (- - -) (spectrum from Gray and Solomon, 1981).

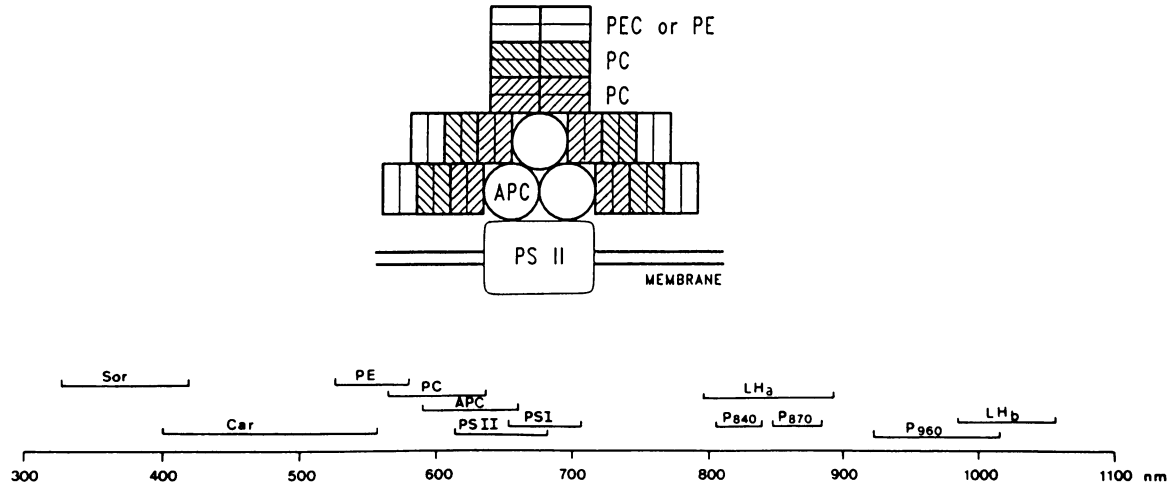


Fig. 7. Scheme of a typical PBS with the arrangements of the components and the putative spatial relationship to the thylakoid and PS II [for reviews see MacColl and Guard-Friar (1987) and Nies and Wehrmeyer (1981)]. The component labelled PS II is thought to represent PS II and the phycobilisome attachment 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 PEC/PE via PC and APC to PS II.

explains the long-wave-length absorption band P960 (primary electron donors in the RC of *R. viridis*) (Knapp *et al.*, 1985).

The optical spectra are even more perturbed in blue copper proteins compared with cupric ions in normal tetragonal coordination (Figure 6). The redox potential is also raised to $\sim 300-500$ mV versus 150 mV for Cu^{2+} (aq) (Gray and Solomon, 1981). 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 $S^{-1} \rightarrow Cu^{2+}$ (Blair *et al.*, 1985; Gray and Malmström, 1983).

The examples presented demonstrate the influence of the protein on the cofactors by various mechanisms, stabilization of unstable conformers and strained ligand geometries and the generation of contacts between pigments leading to strong electronic interaction. The fixation of the relative arrangements of systems of cofactors is 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 RC are therefore associated with light-harvesting complexes (LHC), which may be located within the photosynthetic membrane, or form layers or antenna-like organelles in association with the photosynthetic membrane. Cyanobacteria have particularly intricate light-harvesting systems, the phycobilisomes (PBS) organelles peripheral to the thylakoid membrane. They absorb light of shorter wavelengths than do PS I and II, so that a wide spectral range of sunlight is used (Figure 7). The PBS are assembled from components with finely tuned spectral properties such that the light energy is channelled along an energy gradient to PS II.

3.2.1.1 *Morphology.* PBS consist of biliproteins and linker polypeptides. Biochemical and electron microscopy studies (Gantt *et al.*, 1976; Mörschel *et al.*, 1977; Bryant *et al.*, 1979; Nies and Wehrmeyer, 1981) lead to the model representative of a hemidiscoidal PBS in Figure 7. Accordingly PBS rods are assembled in a polar way from phycerythrin (PE) or phycoerythrocyanin (PEC) and phycocyanin (PC), which is attached to a central core of allophycocyanin (APC). APC is next to the photosynthetic membrane and close to PS II (for a review see MacColl and Guard-Friar, 1987). The PC component consists of α- and β-protein subunits, which are arrranged as $(\alpha\beta)_6$ disc-like aggregates with dimensions 120 × 60 Å [For reviews see Scheer (1982), Cohen-Bazire and Briant (1982), Glazer (1985), Zilinskas and Greenwald (1986) and Zuber (1985, 1986)].

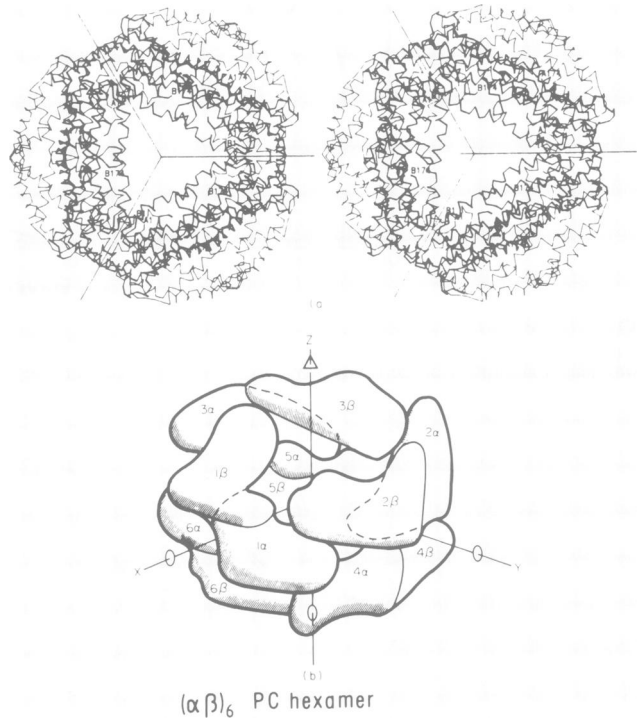


Fig. 8. Stereodrawing of the polypeptide chain fold of a $(\alpha\beta)_6$ hexamer of PC seen along the disc axis (upper panel). The scheme (lower panel) indicates the packing of subunits in the hexamer seen from the side.

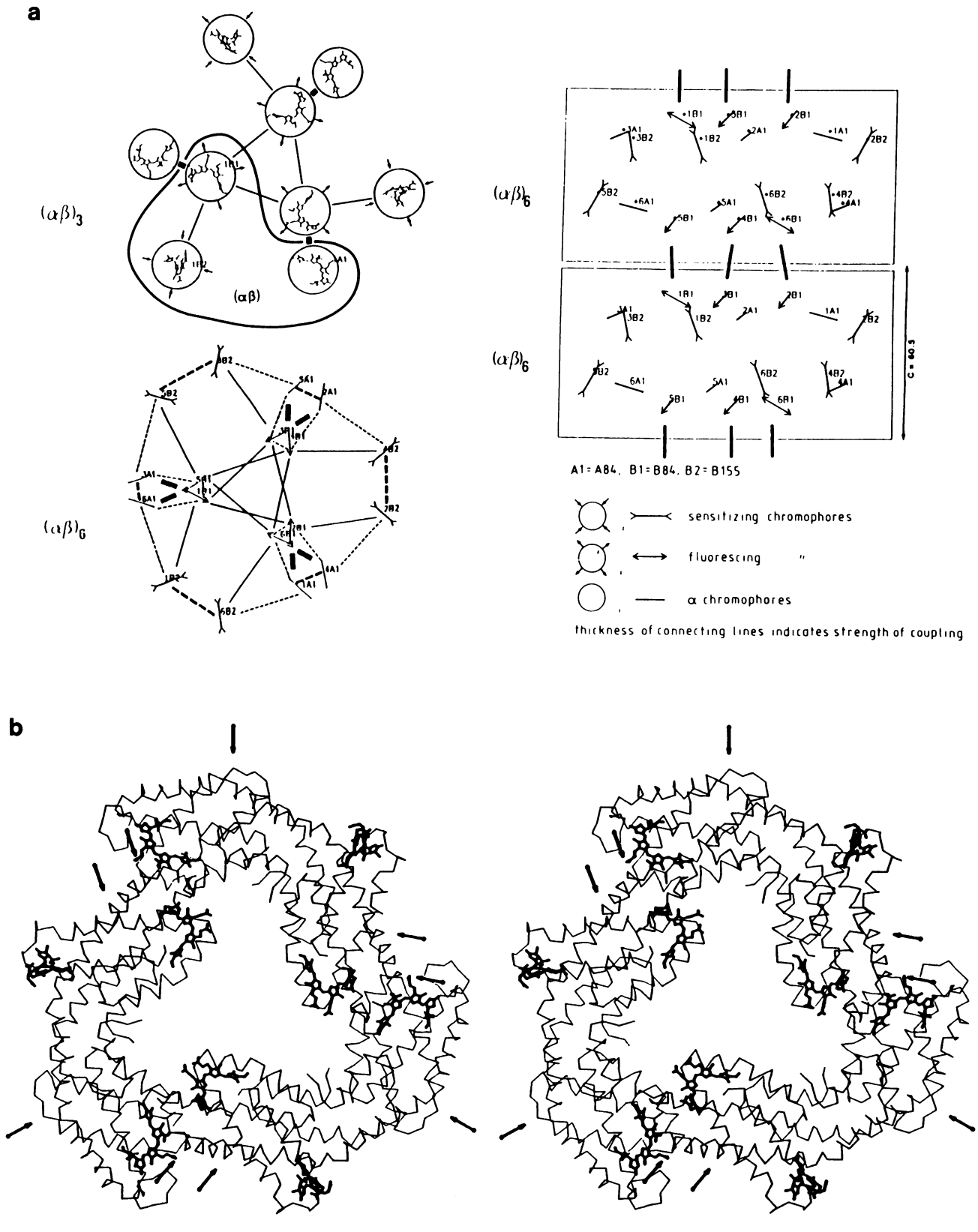


Fig. 9. (**a**) Arrangement of chromophores and preferred energy transfer pathways in $(\alpha\beta)_3$ trimers, $(\alpha\beta)_6$ hexamers and stacked hexamers based on Table 10 in Schirmer *et al.* (1987). For the trimer the detailed structures of the chromophores are drawn, otherwise their approximate transition dipole directions are indicated. For the trimer and hexamer the view is along the disc 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. (**b**) Model of PE $(\alpha\beta)_3$ on the basis of PC with the locations of the additional phycoerythrobilins indicated by arrows.

From crystallographic analyses, a detailed picture of PC and PEC components has emerged (Schirmer *et al.*, 1985, 1986, 1987; Duerring, 1988; Duerring *et al.*, 1989). Amino acid sequence homology suggests that other components have similar structures.

3.2.1.2 *Structure of phycocyanin.* The PC α-subunit and β-subunits have 162 and 172 amino acid residues respectively (in *Mastigocladus laminosus*). Phycocyanobilin chromophores are linked via thioether bonds to cysteine residues at position 84 of both chains (A84 and B84) and at position 155 of the β-subunit (B155) (Frank *et al.*, 1978). Both subunits have similar structures and are folded into eight α-helices (X, Y, A, B, E, F, G and H; see Figure 13). A84 and B84 are attached to helix E, B155 to the G−H loop. α-Helices X and Y form a protruding *anti*-parallel pair essential for formation of the (αβ)-unit.

The isolated protein forms $(\alpha\beta)_3$-trimers with C3 symmetry and hexamers $(\alpha\beta)_6$ as head-to-head associated trimers with D3 symmetry (Figure 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 (Schirmer *et al.*, 1987).

3.2.1.3 *Oligomeric aggregates: spectral properties and energy transfer.* The spectral properties, absorption strength and quantum yield of fluorescence 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. Upon trimer formation, the absorption is red-shifted and its strength and the quantum yield of fluorescence increased (Glazer *et al.*, 1973; Mimuro *et al.*, 1986; for a review see Glazer, 1985). In the $(\alpha\beta)_6$-linker complexes, the fluorescence is further increased and the absorption spectrum further altered (Lundell *et al.*, 1981).

These observations can be rationalized by the structure of the aggregates. Formation of (αβ)-units causes little change in the environment of the chromophores. They remain quite separated with distances >36 Å (Figure 9a). Upon trimer formation, the environment of chromophore A84 changes profoundly by approach of chromophore B84 of a related unit (Figure 9a, upper panel). In the hexamer (Figure 9a, middle panel) the A84 and B155 chromophores interact pairwise strongly across the trimer interface. Also the molecular structures become more rigid with increasing size of the aggregates as seen in the crystals of the trimeric and hexameric aggregates (Schirmer *et al.*, 1986, 1987). 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 (Teale and Dale, 1970; Zickendraht-Wendelstadt *et al.*, 1980). The s-chromophores absorb at the blue edge of the absorption band and transfer the excitation energy rapidly to the f-chromophores. This transfer is accompanied by depolarization (Hefferle *et al.*, 1983). 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 (Gillbro *et al.*, 1985). The assignment of the chromophores to s and f was made by steady-state spectroscopy on different aggregates (Mimuro *et al.*, 1986), by chemical modification guided by the spatial structure (Siebzehnrübl *et al.*, 1987) and conclusively by measurement of linear dichroism and polarized fluorescence in single crystals (Schirmer and Vincent, 1987). Accordingly B155 is the s-, B84 the f- and A84 the intermediate chromophore.

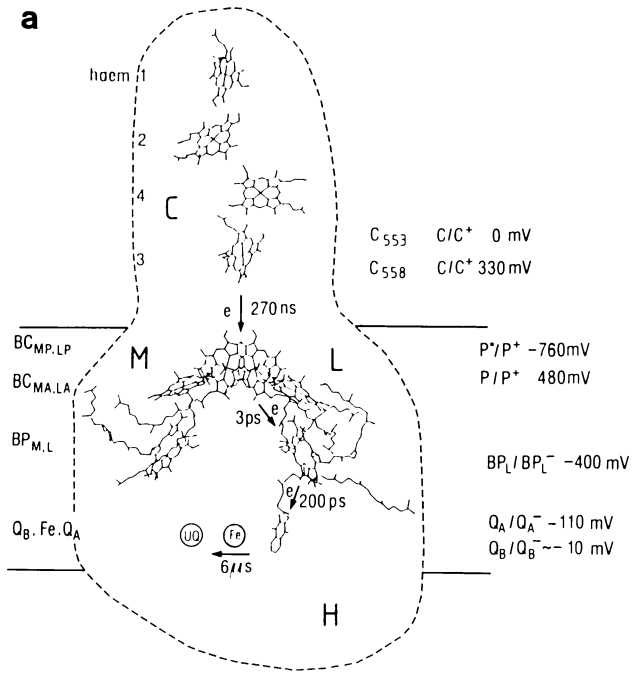
Light energy is transferred rapidly within 50−100 psec from the tips of the PBS to the core (for a review see, for example, Porter *et al.*, 1978; Searle *et al.*, 1978; Wendler *et al.*, 1984; Yamazaki *et al.*, 1984; Gillbro *et al.*, 1985; Glazer, 1985; Holzwarth, 1986). The transfer times from the periphery to the base are several orders of magnitude faster than the intrinsic fluorescence life-times of the isolated components (Porter *et al.*, 1978; Hefferle *et al.*, 1983). 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 ~50 Å has been suggested by Grabowski and Gantt (1978). The relative orientations and distances of the chromophores as obtained by Schirmer *et al.* (1987) were the basis for the calculation of the energy transfer rates in Figure 9a. It shows the preferred energy transfer pathways in (αβ)-units, $(\alpha\beta)_3$-trimers, $(\alpha\beta)_6$-hexamers and stacked discs as models for native antenna rods. There is very weak coupling of the chromophores in the (αβ)-units. Some energy transfer takes place, however, as indicated by steady-state polarization measurements (Switalski and Sauer, 1984; Mimuro *et al.*, 1986) probably between B115 and B84. Trimer formation generates 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 (Holzwarth, 1985; Gillbro *et al.*, 1985; Glazer *et al.*, 1985; Mimuro *et al.*, 1986) 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.* (1987) 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 (Duerring, 1988; Duerring *et al.*, 1989). Its short-wavelength-absorbing chromophore is A84 located at the periphery (Figure 10a), as are the additional chromophores in PE, which is also a tip component (Figure 9b).

The phycobilisome rods act as light collectors and energy concentrators from the peripheral onto the central chromophores, i.e. as excitation energy funnels from the periphery to centre and from the tip to the bottom.

We may expect functional modulations by the linker polypeptides. 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 RC.* A historical background of the development of concepts and key features of the purple bacterial reaction centre is given by Parson (1978).

3.2.2.1 *RC composition.* The RC of *R. viridis* is a complex of four protein subunits [the cytochrome *c* subunit (C), with four haems displaying two redox potentials (c_{553} and c_{558}),



RC, subunit and cofactor arrangement t 1/2 electron transfer times and redox potentials

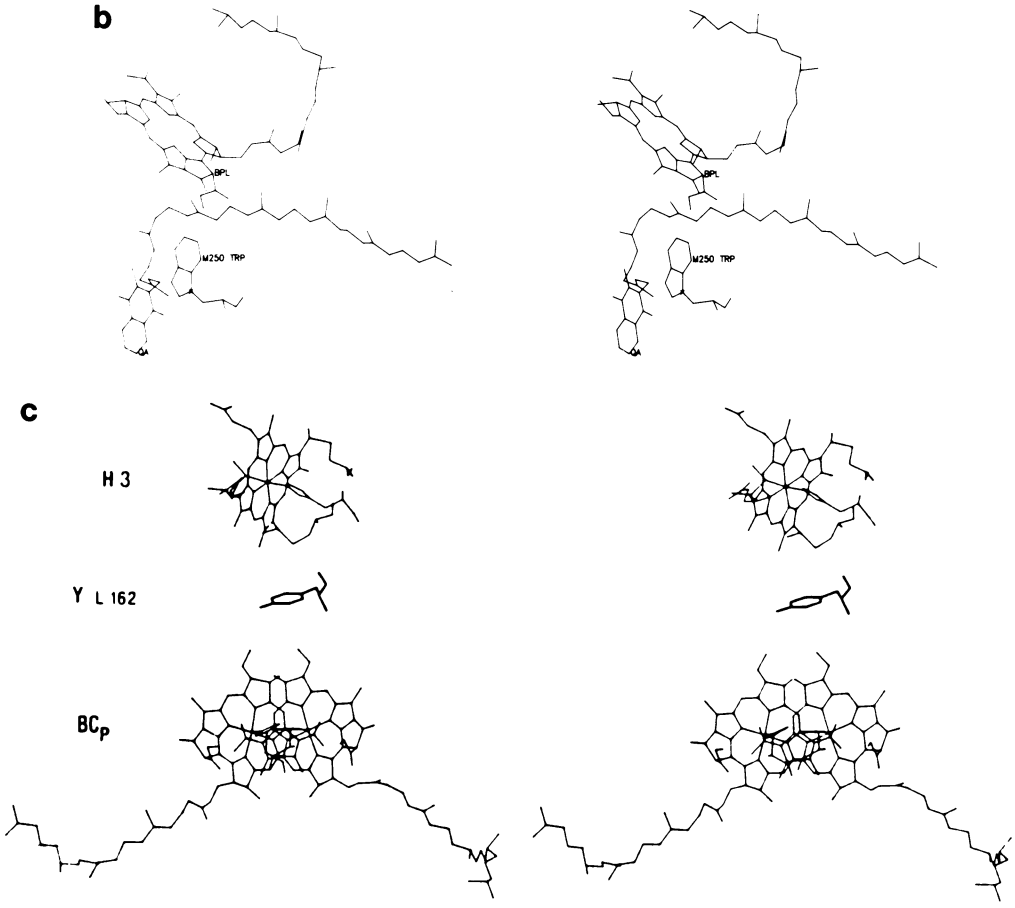


Fig. 10. (**a**) Scheme of the structure of RC cofactor system, the outline of the protein subunits (C, M, L and H), the electron transfer t1/2 times, and the redox potentials of defined intermediates (for references see Section 3.2.2.2). (**b**) Stereo drawing of the arrangement of BP_L, Trp M250 and O_A in the L-branch of the RC pigment system. (**c**) Stereo drawing of haem 3 (H3) of the cytochrome *c*, the special pair BCp and the intercalating Tyr L162 (Y L162) of the L-subunit (Deisenhofer *et al.*, 1985a). 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.

is located on the periplasmic side of the membrane; the L (light)- and M (medium)-subunits are integrated in the membrane and their polypeptide chains span the membrane with five α-helices each, labelled A–E; the H (heavy)-subunit is located on the cytoplasmic side and its N-terminal α-helical segment (H) spans the membrane] and the cofactors, which are bound by the L- and M-subunits, arranged as in Figure 10a. As shown by the amino acid sequence C, L, M and H consist of 336, 273, 323 and 258 residues respectively (Michel *et al.*, 1985, 1986a; Weyer *et al.*, 1987). The four haem groups in C are covalently bound via thioether linkages. The cofactors are four BChl-b (BC_{MP}, BC_{LP}, BC_{LA} and BC_{MA}), two BPh-b (BP_M and BP_L), one menaquinone-9 (Q_A) and a ferrous ion involved in electron transfer. A second quinone (ubiquinone-9) (Q_B), which is a component of the functional complex, is partially lost during preparation and crystallization of the RC. The subscripts P, A, M and L indicate pair, accessory M- and L-subunit association respectively.

3.2.2.2 *Chromophore arrangement and electron transfer.* The chromophores are arranged in L- and M-branches related by an axis of ~2-fold symmetry which meet at BC_P (Deisenhofer *et al.*, 1984). 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 (Breton *et al.*, 1985; Knapp *et al.*, 1985), electron transfer is less well understood. The excited BC_P is quenched by electron transfer to BP_L in 3 ps and further on to the primary acceptor Q_A in ~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 about −400 mV (Cogdell and Crofts, 1972; Carithers and Parson, 1975; Prince *et al.*, 1976; Netzel *et al.*, 1977; Bolton, 1978; Holten *et al.*, 1978; Woodbury *et al.*, 1985; Breton *et al.*, 1986). These functional data are summarized in Figure 10a. General factors controlling the transfer rates have been summarized in Table I and are detailed for the RC here.

Fast electron transfer requires effective overlap of the molecular orbitals by close approach. 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 (Kavarnos and Turro, 1986; McLendon, 1988). In the RC the distance between BC_P 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 (Fleming *et al.*, 1988; for a review see Barber, 1988). The distance between BP_L and Q_A seems large for a fast transfer. Indeed the gap is bridged by the aromatic side chain of Trp M250 in the L branch of the pigment system (Figure 10b) (Deisenhofer *et al.*, 1985a; Michel *et al.*, 1986b), 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 (Pasman *et al.*, 1982; Moore *et al.*, 1984; Kavarnos and Turro, 1986), but there is only Van der Waals contact here.

A second important factor for electron transfer is the free energy change (ΔG), which is governed by the chemical nature of the components, by geometric factors and by the environment (solvent polarity). It depends on the ionization potential of the donor in its excited state, the electron affinity of the acceptor, and on the coulombic interaction of the radial ion pair, which is probably small, as donor and acceptor (BC_P and Q_A in the RC) are far apart. The effect of the environment may be substantial by stabilizing the radical ion pair by ionic interactions and hydrogen bonds. ΔG is a determinant of the activation energy of electron transfer. Another determinant is nuclear rearrrangements 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 tightly packed in the protein and the charge is distributed over the extended aromatic electron systems. Reorientable dipolar groups (peptide groups and side chains) may contribute strongly to the energy barrier of electron transfer. A matrix with high electronic polarizability, on the other hand, 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 tunnelling 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 (Arnold and Clayton, 1960; Parson, 1974; Carithers and Parson, 1975; Parson and Cogdell, 1975; Bolton, 1978; Kirmaier *et al.*, 1985a,b; Woodbury *et al.*, 1985) and proceeds readily at 1°K. Thermally activated processes, nuclear motions and collisions are therefore not important for the initial very fast charge separation steps. There is even a slight increase in rate with temperature decrease either due to a closer approach of the pigments at low temperature, or to changes of the vibrational levels which may lead to a more favourable 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 (~6 μs at pH 7, derived from Carithers and Parson, 1975) and has a substantial activation energy of ~8 kcal/mol. In *R. viridis* Q_A is a menaquinone-9 and Q_B a ubiquinone-9, which differ in their redox potentials in solution by ~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 tranferred 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 RC [two-electron gate (Wraight, 1982)]. A Q_B is close to Glu L212, which opens a path to the H-subunit and may protonate Q_B. The path between Q_A and Q_B is very different from the environment of the primary electron transfer components. The line connnecting Q_A and Q_B [the Q_B binding site has been inferred from the binding mode of competitive inhibitors and ubiquinone-1 in *R. viridis* crystals (Deisenhofer *et al.*, 1985a,b)] is occupied by the iron and its five coordinating ligands, four histidine (M217,

M264, L190 and 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 have an edge-to-edge distance of ~15 Å which might explain the slow transfer. If electron transfer and protonation are coupled, the observed pH dependence of the electron transfer rate of Q_A and Q_B (Kleinfeld *et al.*, 1985) could be explained and nuclear motions 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 and Q_B electron transfer is poorly understood at present, as it occurs also in the absence of the iron (Debus *et al.*, 1986). Its role seems to be predominantly structural.

The cycle of electron transfer is closed by rereduction of BC_P* from the cytochrome bridging a distance of ~11 Å between pyrrole ring I of haem 3 and pyrrole ring II of BC_{LP}. The transfer time is 270 ns (Holten *et al.*, 1978), considerably slower than the initial processes. Tyr L162, which is located midway (Figure 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 at high temperatures nuclear motions play a role [for reviews see Dutton and Prince (1978) and De Vault and Chance (1966)].

The favourable rate-controlling factors discussed are a necessary but not sufficient condition for electron transfer, which competes with other quenching processes summarized in Table II and detailed for the RC.

Energy transfer from P* back to the LHC or to other pigments may be favourable from orientation and proximity considerations but is disfavoured out of energetic reasons. The special pair absorbs usually (but not in *R. viridis* where the maximal absorption of the RC and the LHC are at 960 and 1020 nm respectively; see Figure 7) at longer wavelengths than other pigments of the photosynthetic apparatus and represents the light energy sink. The natural radiative lifetime of the excited singlet state P* is ~20 ns (Slooten, 1972; Parson and Cogdell, 1975) 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 for the cyclic pigment systems tightly packed in the protein matrix.

The back reaction $P^+ Q_A^-$ to $P Q_A$ has a favourable driving force (Figure 10a) and proceeds independent of temperature, but is slow and insignificant under physiological conditions (for a review see Bolton, 1978). 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 with Q_A, to electronic properties of P^+ disfavouring charge transfer and to conformational changes induced by electron transfer.

The profound influence of the protein matrix on electron transfer in the RC is obvious in the observed asymmetry of electron transfer in the two branches of the BChl-b and BPh-b pigments. Only the branch more closely associated with the L-subunit is active. An explanation is offered by the fact that the protein environment of both branches, although provided by homologous proteins (L and M), is rather different in particular by the Trp M250 located between BP_L and Q_A and the numerous differences in the Q_A and Q_B binding sites (Deisenhofer *et al.*, 1985a; Michel *et al.*, 1986b). 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 it facilitates electron release into the L-branch (Michel-Beyerle *et al.*, 1988). The M-branch may have influence as a pendant group though.

The protein matrix also serves to dissipate the excess energy of ~650 mV (Prince *et al.*, 1976) of the excited special pair (P^* Q_a) over 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 a polar amino acid side chains with a high proportion of aromatic residues. The electron path is removed from bulk water.

3.2.3 *The blue oxidases*. Oxidases catalyse the reduction of dioxygen in 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 means to stabilize reactive partially reduced oxygen intermediates (Malmström, 1978, 1982; Farver and Pecht, 1984).

The 'blue' oxidases are classified corresponding to distinct spectroscopic properties of three types of copper which they contain: type-1 Cu^{2+} is responsible for the deep blue colour of these proteins; type-2 or normal Cu^{2+} has undetectable optical absorption (type-1 and type-2 cupric ions are paramagnetic); and type-3 copper has a strong absorption around 330 nm and is antiferromagnetic, indicating coupling 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 are well documented in several recent reviews [e.g. for laccase (LAC) (Reinhammar, 1984), for ascorbate oxidase (AO) (Mondovi and Avigliano, 1984) and for ceruloplasmin (CP) (Rydén, 1984)]. Basically type-1 Cu^{2+} is reduced by electron transfer from the substrate. The electron is transferred on 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 *AO, composition and copper arrangement*. AO an oxidase in plants, is a polypeptide of 553 amino acid residues folded into three tightly associated domains (Messerschmidt *et al.*, 1988). It is a dimer in solution, but the functional unit is the monomer. It belongs to the group of 'blue' oxidases, together with LAC, an oxidase in plants and fungi and CP an oxidase in mammalian plasma (Malkin and Malmström, 1970).

Structures of copper proteins containing only one of the different copper types are known. Plastocyanin an electron carrier in the photosynthetic apparatus of plants (PCY) has a 'blue' type-1 copper, which is coordinated to two histidine residues and the sulphur atoms of cysteine and methionine as a distorted tetrahedron (Guss and Freeman, 1983). $Cu-Zn-$superoxide dismutase contains a type-2 copper, which has four histidine ligands with slightly distorted quadratic coordination (Richardson *et al.*, 1975). Haemocyanin of *Panulirus interruptus* has type-3 copper, a pair of copper ions 3.4 Å apart with six histidine ligands (Gaykema *et al.*, 1984).

In domain 3 of AO (see Section 4.4), a copper ion is found

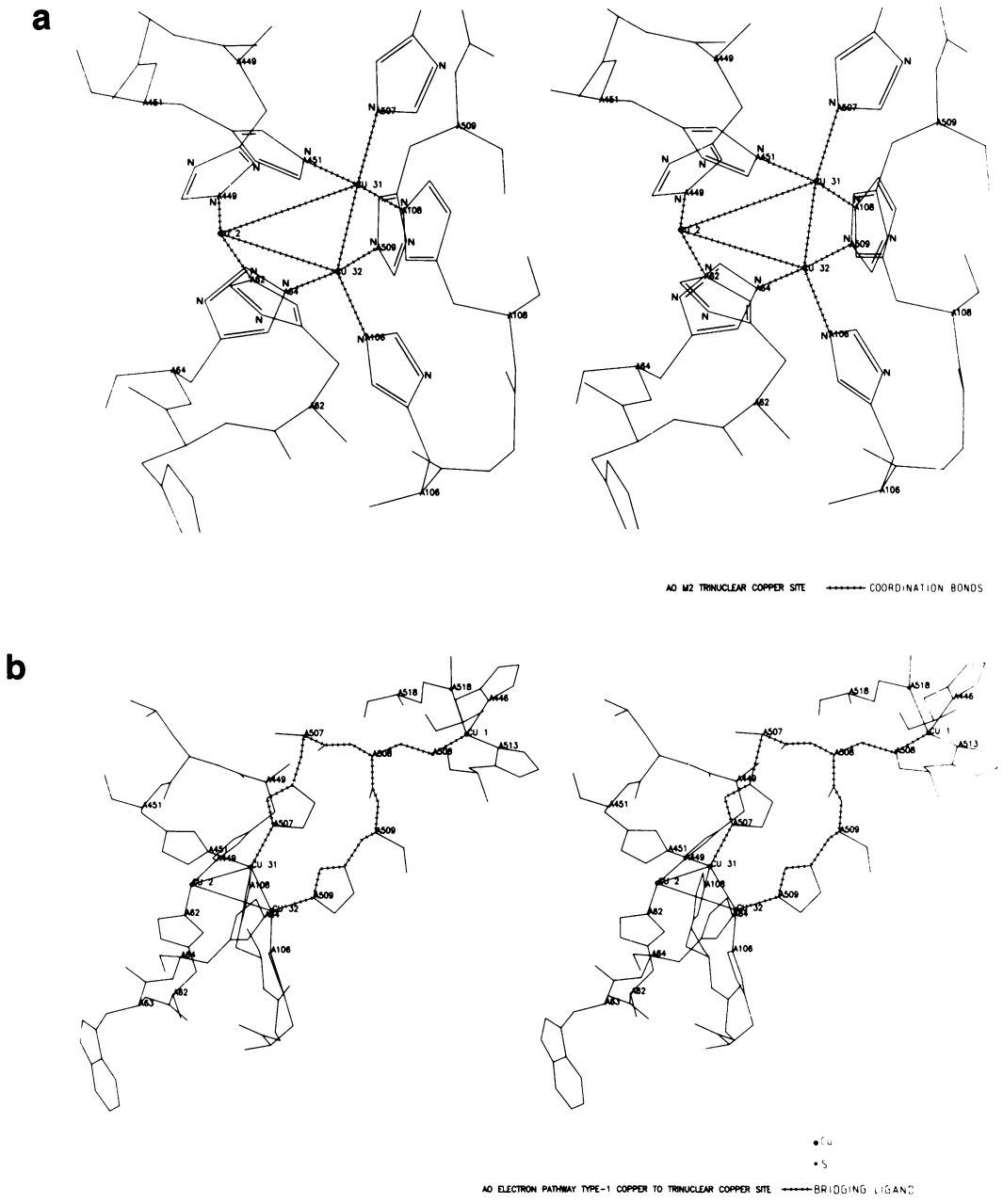


Fig. 11. (**a**) Stereodrawing of the trinuclear copper site in AO. Coordination bonds between the copper ions and the protein residues are marked (- - -) (Messerschmidt *et al.*, 1988). (**b**) Stereodrawing of the tridentate peptide ligand (-His 507-Cys 508-His 509-) bridging type-1 copper (Cu 1) and the trinuclear cluster (Cu 31, Cu 32 and Cu 2) (Messerschmidt *et al.*, 1988).

in a strongly distorted tetrahedral (approaching trigonal pyramidal geometry) coordination by the ligands His, Cys, His and Met as has been shown in Figure 6. It resembles the blue type-1 copper in plastocyanin. Between domain 1 and domain 3, a trinuclear copper site is enclosed and shown in Figure 11a. Four (His-X-His-) amino acid sequences provide the eight histidine ligands. The trinuclear copper site is subdivided in a pair of coppers (Cu31 and Cu32) with 2 × 3 histidyl (A108, A451 and A507; A64, A106 and A509) ligands forming a trigonal prism. It represents the type-3 copper pair, as a comparable arrangement is observed in haemocyanin. The remaining copper (Cu 2) has two histidyl ligands (A62 and A449). It is type-2 copper. The trinuclear copper cluster is the site where dioxygen binds, but the structural details including the presence of additional non-protein ligands require clarification. The close spatial association of the three copper ions in the cluster suggests facile electron exchange. It may function as an electron storage site and cooperative three-electron donor to dioxygen, to irreversibly break the O−O bond.

3.2.3.2 *Intramolecular electron transfer in AO.* 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, bridging a distance of 12 Å (Figure 11b). The cysteine sulphur and the imidazole components of the bridging ligand have low-lying unoccupied molecular orbitals and may favour a chemical mechanism of electron transfer, but the intervening aliphatic and peptide chains are unlikely

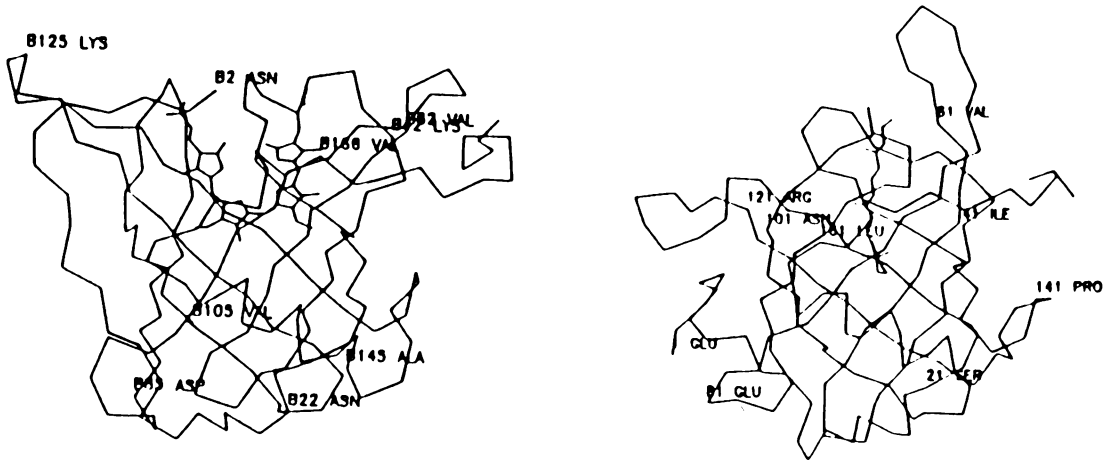


Fig. 12. Comparison of the polypeptide chain folds of BBP and RBP with bound cofactors.

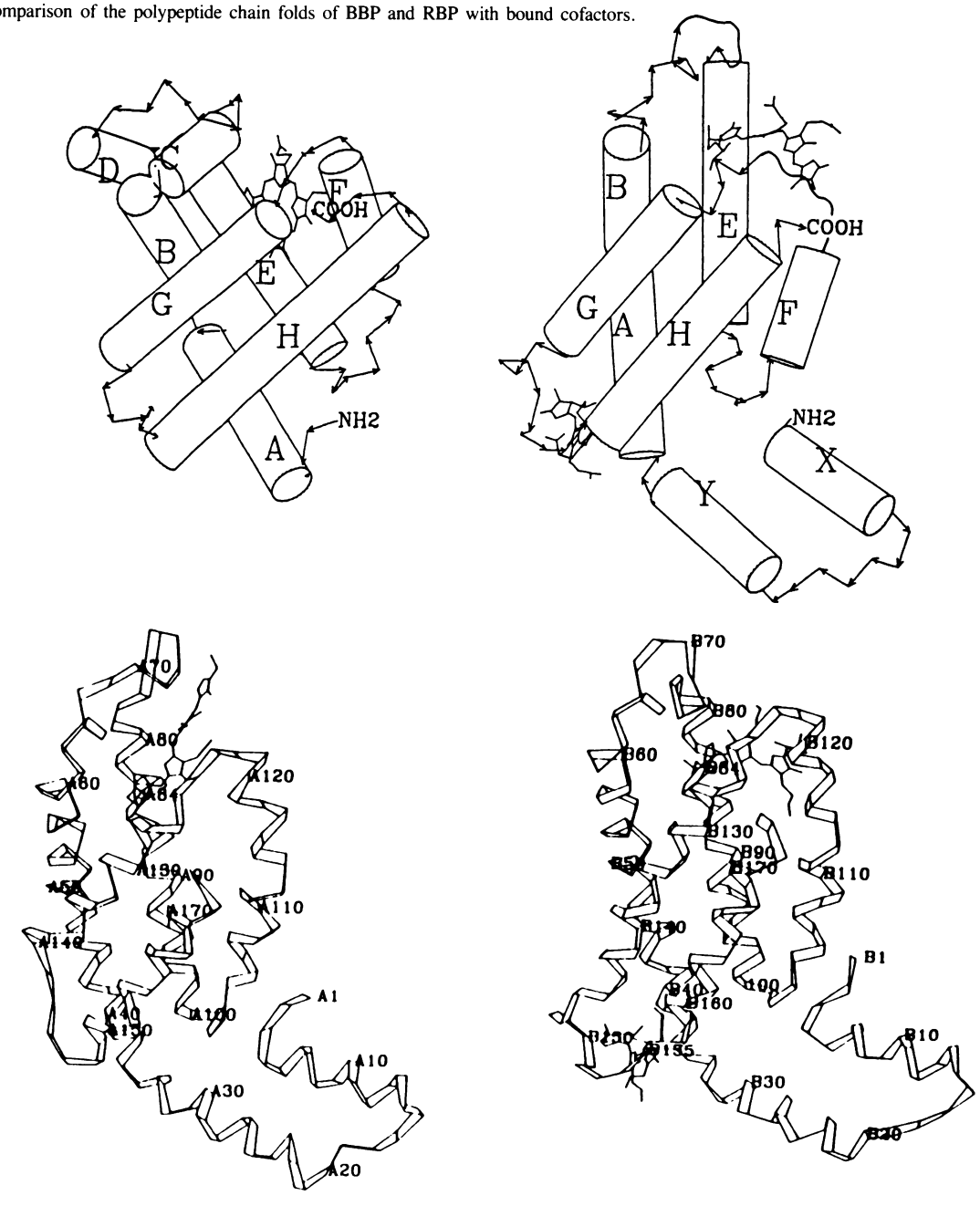


Fig. 13. Polypeptide chain folds of the α- and β-subunits of phycocyanin (Schirmer *et al.*, 1987) (**lower part**, left and right) and comparison of the arrangements of α-helices in myoglobin and phycocyanin (**upper part**, left and right).

to form transient radicals and may 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 (Schmidt *et al.*, 1988). This may apply also to the blue oxidases and cause preferred transfer to A507.

The redox potential differences between the type-1 copper and the type-3 copper are −40 mV in AO. Unfortunately there are no direct measurements of the intramolecular electron transfer rates available. The turnover number serves as a lower limit and is 7.5×10^3/s in AO (Dawson, 1966; Gerwin *et al.*, 1974) indicating a quite rapid transfer despite the long distance and small driving force. The electron pathway is intramolecular and removed from bulk water.

The characteristic distribution of redox centres as mono- and trinuclear sites in the blue oxidases may be found also in the most complex oxidase, cytochrome oxidase [see the hypothetical model of Holm *et al.* (1987)] and in the water-splitting manganese−protein complex of PS II, which carries out the reverse reaction of the oxidases. For its $(Mn)_4$ cofactor either two binuclear or a tetranuclear metal centre is favoured (Babcock, 1987), but mono- and trinuclear arrangements cannot be excluded.

3.3 *The protein as medium*

The boundary between the action of the protein as ligand and as medium is fluid. The protein medium is microscopically extremely complex in structure, polarity and polarizability, which may influence energy and electron transfer. There is no obvious common structural scheme in the protein systems discussed, except a high proportion of aromatic residues (particularly tryptophans) bordering the electron transfer paths in RC and AO and their wide separation from bulk water by internal location within the protein and the hydrocarbon bilayer (in RC). 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 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 the basis of the amino acid sequences; ultimately a reflection of our ignorance about the sequence−structure relationships. An analysis of structural relationships will shed light on evolution and function of the protein systems and is thus appropriate here.

4.1 *Retinol- and bilin-binding proteins*

The simplest case is shown in Figure 12, where a bilin (biliverdin IXγ)-binding protein found in *Pieris brassicae* and *R. viridis*, a BChl-b-containing purple bacterium carrying out anoxygenic photosynthesis (BBP) (Huber *et al.*, 1987a) is compared with a retinol-binding protein (RBP) (Newcomer *et al.*, 1984). The structural similarity is obvious for the bottom of the β-barrel structure, while the upper part which is involved in binding of the pigments, biliverdin and retinol, differs greatly. The molecule is apparently divided into framework and hypervariable segments which determine binding specificity in analogy to the immunoglobulins (Huber, 1984). The relationship suggests carrier functions for BBP as for RBP, although it serves also for pigmentation in butterflies.

4.2 *Phycocyanin*

The PC consist of two polypeptide chains α and β which are clearly related in structure (Figure 13) and probably originate from a common precursor.

The α-subunit is shorter in the globular helical (GH) turn and lacks the s-chromophore B155. 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 disc. In addition the α- and β-subunits play very different roles in the formation of the $(\alpha\beta)_6$-hexamer as has 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 driven 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. The GH assemblies A−H show similar topology. The N-terminal X,Y α-helices forming a U-shaped extension in PC is essential for formation for the $\alpha\beta$ substructure. The amino acid sequence comparison after structural superposition reveals some homology, suggesting divergent evolution of phycobiliproteins and globins (Schirmer *et al.*, 1987). However, what function a precursor of light-harvesting and oxygen-binding proteins might have had remains mysterious.

4.3 *Reaction centre*

The RC lacks symmetry across the membrane plane, not surprising for a complex, which catalyses a vectorial process across the membrane. However, there is quasi-symmetry perpendicular to it relating the L- and M-subunits and the pigment system. Structural similarity and amino acid sequence homology between the L- and M-subunits suggest a common evolutionary origin. This relationship is extended to the PS II components D1 and D2 on the basis of sequence homology and conservation of residues involved in cofactor binding [for reviews see Trebst (1986) and Michel and Deisenhofer (1988)]. 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 and the L-branch of the pigment system and Q_A (Figure 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 driven 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 in light harvesting and electron transfer as a pendant group.

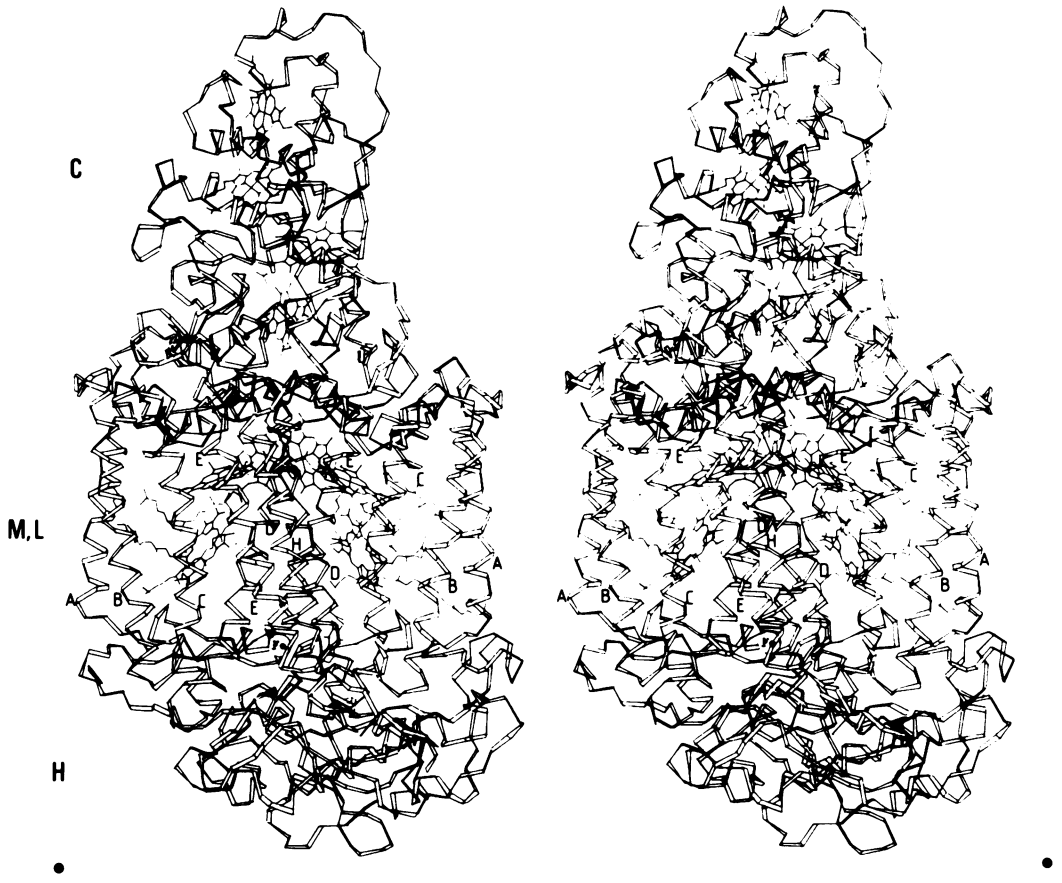


Fig. 14. Stereodrawing of the polypeptide fold of the RC subunits and the cofactor system. The membrane-spanning α-helices of the L- and M-subunits (A,B,C,D,E in sequential and A,B,C,E,D in spatial order) and the H-subunit (H) are labelled (Deisenhofer *et al.*, 1985a).

There are also structural reasons, as its deletion would have generated void space.

The cytochrome subunit adds to the asymmetry of the L−M complex and shows itself an internal duplication (Deisenhofer *et al.*, 1985a). All four haem groups are associated with a helix-turn-helix motif but the turns are short for haem groups 1 and 3 and long for 2 and 4.

4.4 *Blue oxidases*

Gene multiplication and divergent evolution is most evident in the blue oxidase, AO. Figure 15 shows the polypeptide chain of 553 amino acid residues folded into three closely associated domains of similar topology (Messerschmidt *et al.*, 1988). Although nearly twice as large, they resemble the simple, small copper protein, PCY (Guss and Freeman, 1983) (Figure 16). In the blue oxidase 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 RC. Similar to the H-subunit in the RC, the linking domain II introduces asymmetry in AO, which might have driven evolutionary divergence of domains I and III.

The proteins PCY, AO, LAC and CP are members of a family of copper proteins as indicated by structural relations and sequence homology (Takahashi *et al.*, 1984; Germann *et al.*, 1989; Messerschmidt *et al.*, 1989; Ohkawa *et al.*, 1988). They provide a record from which an evolutionary tree may be proposed (Figure 17). The simplest molecule is PCY, containing only a type-1 copper. A dimer of PCY-like molecules could provide the 2*4 histidyl ligands for the trinuclear copper cluster, representing a symmetrical oxidase. From this hypothetical precursor the modern blue oxidases and CP might have evolved following different paths of gene (domain) insertion and loss or acquisition of coppers. In both the arrangement of the N- and C-terminal domains, which contain the functional copper cluster, has been preserved. Recombinant DNA technology has the tools to reconstruct the hypothetical precursor oxidase. This is under investigation.

5. Implications from the structure of the RC for membrane proteins in general

The structures of water-soluble proteins show a seemingly unlimited diversity, although they are built from only a few defined secondary structural elements as helices, β-sheets and turns and, despite their construction, from domains and recurring structural motifs. The proteins discussed provide ample evidence that there seems to be a limited set of basic folds which may be related to the evolution of proteins from a basic 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 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 must attain the global energy minimum, not its individual

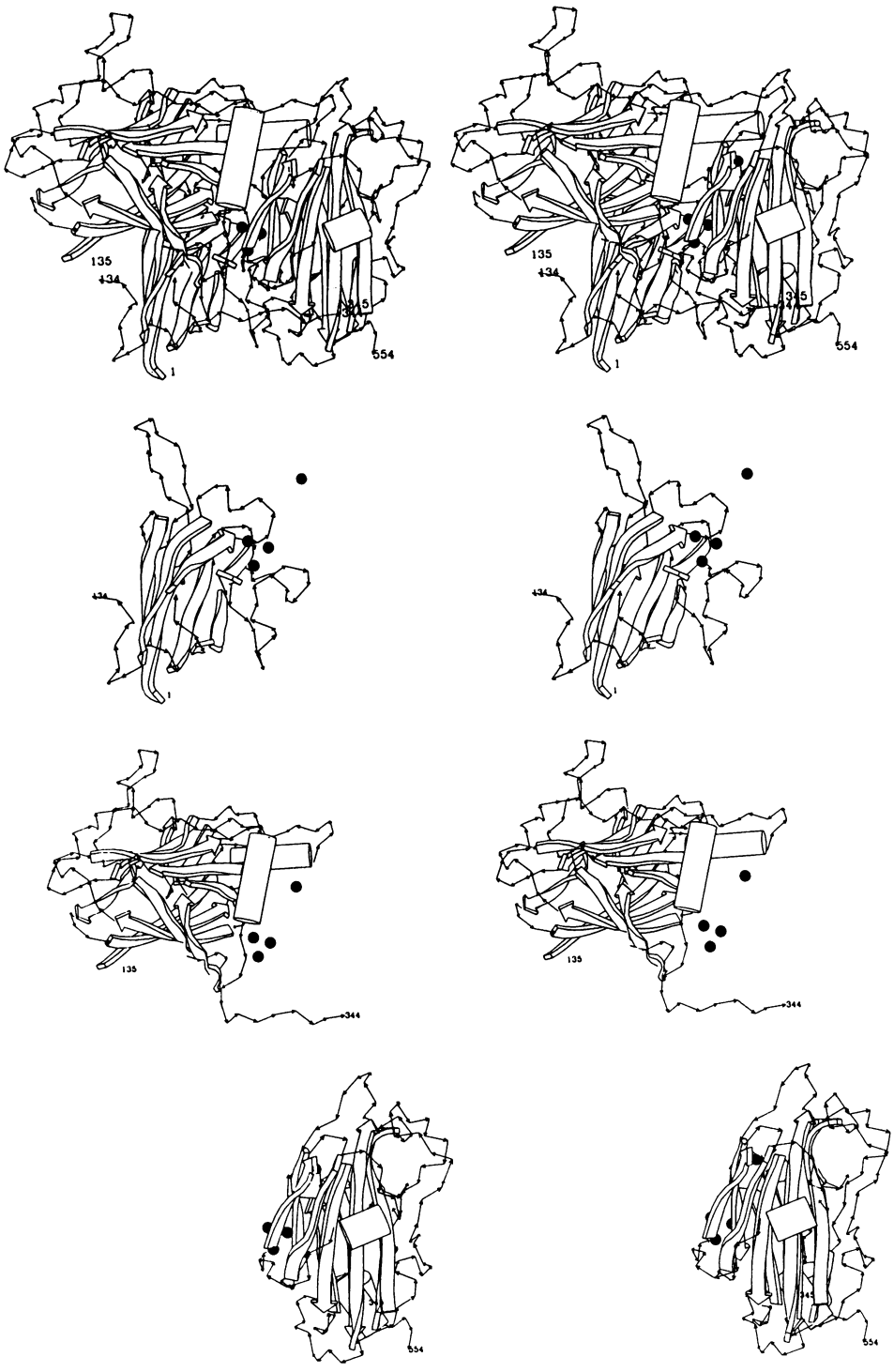


Fig. 15. Stereodrawing of the polypeptide chain folds of AO and explosion view of its three domains from top to bottom (Messerschmidt *et al.*, 1988). β-Strands are indicated as arrows and α-helices as cylinders (produced by the plot program of Lesk and Hardman, 1982).

components. Water is a good hydrogen-bond donor and acceptor and is thus able to saturate polar-surface-exposed peptide groups nearly as well as intraprotein hydrogen bonds do (except for entropic effects).

Membrane proteins face the inert hydrocarbon part of the phospholipid bilayer and must satisfy their hydrogen bonds intramolecularly. Only two secondary structures form closed hydrogen-bonding arrangements of their main chains, which satisfy this condition, namely the helix and the β-barrel. For assemblies of α-helices packing rules have been derived which predict certain preferred angles between the helix axes, although with a broad distribution. Similarly, the arrangement of strands in β-sheets and β-barrels follows defined rules (Chothia, 1984).

5.1 *Structure of the membrane-associated parts of the RC*

The structure of the RC may support some conclusions about membrane proteins in general, of which the RC structure was the first to be determined at atomic resolution after the

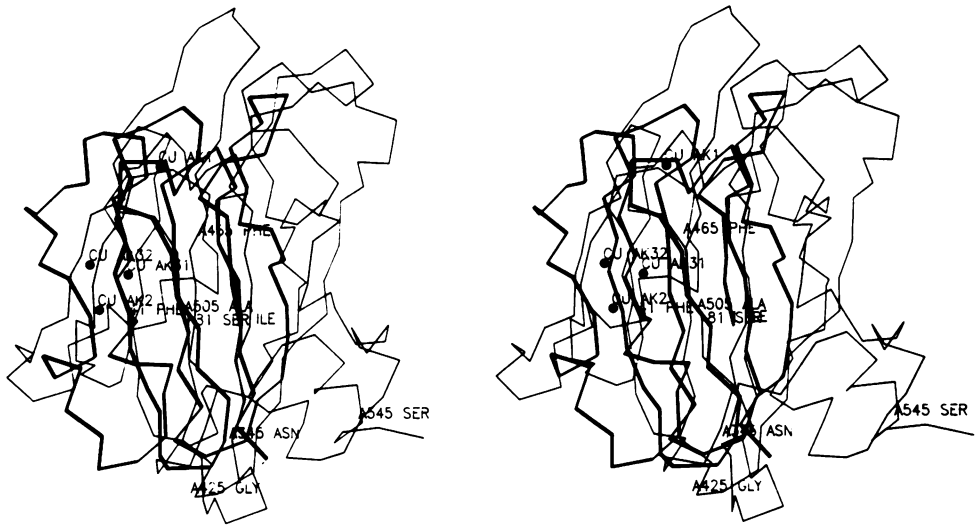


Fig. 16. Stereodrawing and superposition of domain III of AO (thin lines) and PCY (thick lines). The trinuclear copper site in AO is buried between domain I (not shown) and domain III.

low resolution structure of bacteriorhodopsin, which has some common features (Henderson and Unwin, 1975). The RC has 11 transmembrane α-helices, which consist of 26 residues (H-subunit) or 24−30 residues (L- and M-subunits), appropriate lengths to span the membrane. The amino acid sequences of these segments are devoid of charged residues (Figure 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 (Bennett and Huber, 1984). They may be important for the insertion into the membrane by allowing rearrangements. The angles between the axes of the contacting α-helices of the L- and M-complex are inclined by 20−30°, a preferred angular range for the packing of the α-helices 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 preference of glycines and prolines at the termini (Remington *et al.*, 1982; Loebermann *et al.*, 1984). In addition the D and E α-helices of the L- and M-subunits (Figure 18) find counterparts in soluble proteins. They are associated around the local diad axis and form the centre of the L−M module, which binds the iron and the BC_P. The four D and E α-helices of the L- and M-subunits are arranged as a bundle tied together by the iron ion and splay out towards the cytoplasmic side to accommodate the large special pair. This motif is quite common in soluble electron-transfer proteins (Weber and Salemme, 1980). I will resume this discussion later and suggest appropriate substructures of soluble proteins as models for pore-forming membrane proteins.

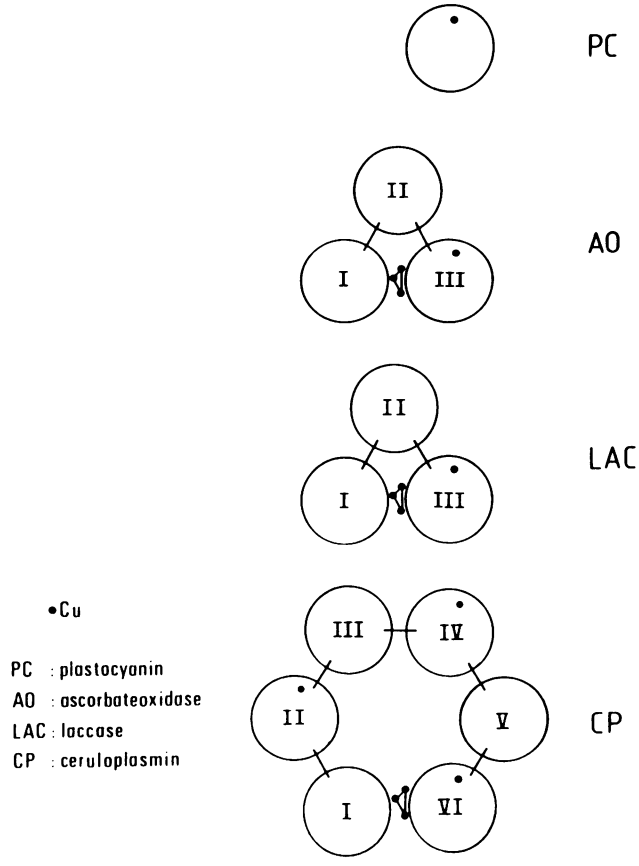


Fig. 17. Homologous domains in PCY, AO, LAC and CP. The mono- and trinuclear copper sites are indicated.

5.2 *Membrane insertion*

The structure of the RC is similarly important for our views of the mechanism of integration into the phospholipid bilayer. The RC 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, C 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 and C−D) require transfer.

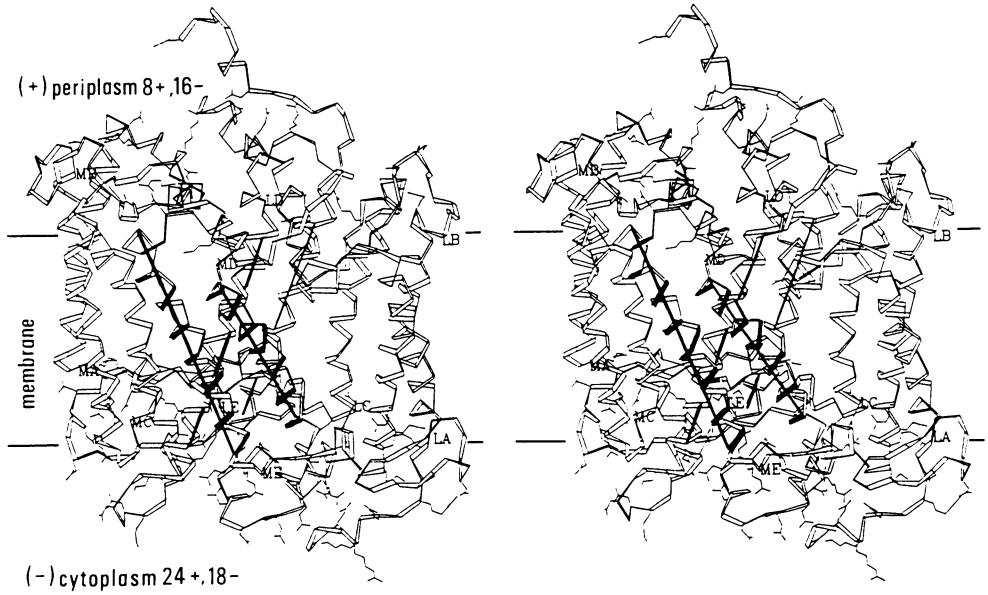


Fig. 18. Stereodrawing of the polypeptide chains of the L- and M-subunits of the RC in ribbon representation. The N-terminal residues of the membrane-spanning α-helices are labelled (including the prefix M and L) and the tetra-helical motif of the D and E α-helices is marked by shading and lines. The side chains of charged residues are drawn. Asp, Glu and C-termini are negatively charged, and Lys, Arg and N-termini are counted as positively charged and added for the cytoplasmic and periplasmic sides (Deisenhofer *et al.*, 1985a, 1989).

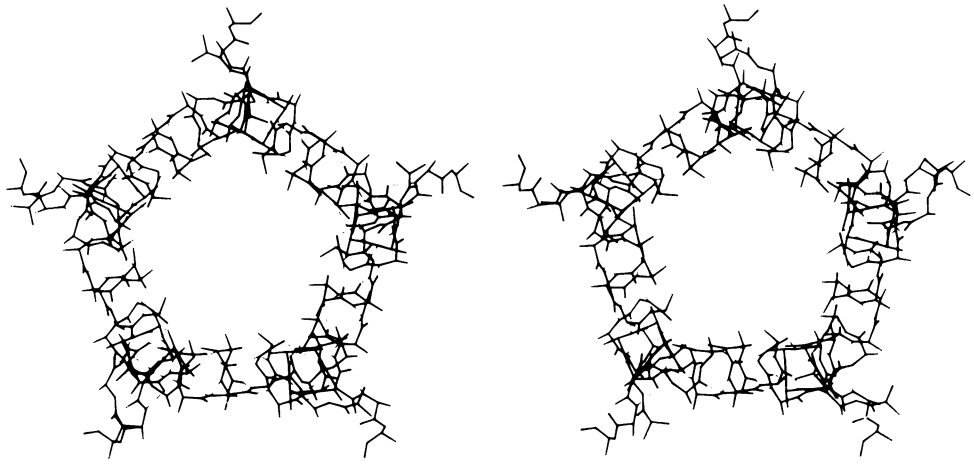


Fig. 19. Penta-helical pore in heavy riboflavin synthase in stereo (Ladenstein *et al.*, 1988).

It is interesting to note that only the cytochrome gene possesses a prokaryotic signal sequence, as indicated by the sequence of the gene (Weyer *et al.*, 1987). Transfer of the large hydrophilic C-subunit may require a complex translocation system affording a signal sequence, while H, L and M may spontaneously insert into the bilayer due to the affinity of the contiguous hydrophobic segments with the phospholipids (for a review of this and related problems see Rapoport, 1986). A 'simple' dissolution still requires transfer across the membrane of those charged residues which are located at the periplasmic side (Deisenhofer *et al.*, 1985a; Michel *et al.*, 1986a,b). The increasingly favourable 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 L–M complex is energetically favoured (Figure 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) (Deisenhofer *et al.*, 1985a; Michel *et al.*, 1985) which may efficiently stop membrane insertion. Similarly there are 3–11 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 (Engelman *et al.*, 1986). 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 obvious whether the structural principles observed in the RC apply also to 'pore'- or 'channel'-forming α-helical proteins. This could, in principle, elaborate quite complex

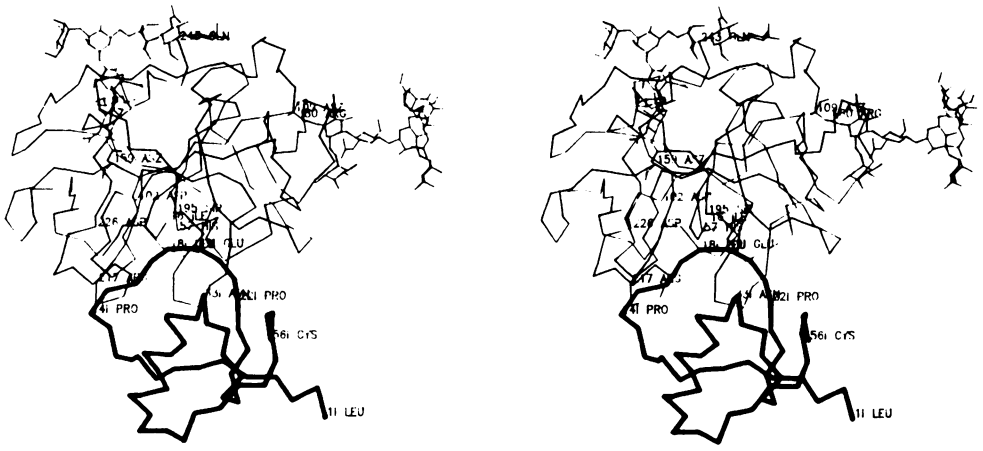


Fig. 20. Stereodrawing of the complex between human leucocyte elastase (thin lines) and turkey ovomucoid inhibitor (thick lines) (Bode *et al.*, 1986).

structures within the aqueous channel (Lodish, 1988), but available evidence at low resolution for gap junction proteins (Milks *et al.*, 1988) 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 RC, we may derive models for membrane pore-forming proteins from appropriate soluble protein sub-structures. The penta-helical pore seen at high resolution in the icosahedral multi-enzyme complex riboflavin synthase seems to be a suitable model (Ladenstein *et al.*, 1988) (Figure 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 modelling of membrane protein structures may be extended to another class of membrane proteins which have β-structures spanning the outer membrane, the bacterial porins (Kleffel *et al.*, 1985). In soluble proteins β-barrels observed have four to eight or more strands. The lower limit is determined by the distortion of regular hydrogen bonds. An upper limit may be given by the possible sizes of stable protein domains. A four-stranded β-barrel with four parallel strands duplicated head to head with symmetry D4 is seen in the ovomucoid octamer (Weber *et al.*, 1981). 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

Twenty-eight years after the elucidation of the first protein crystal structures by Perutz and Kendrew and after steady development, protein crystallography is undergoing a transformation. Recent technical and methodical developments enable us to analyse large functional protein complexes like the RC (Deisenhofer *et al.*, 1985a; Allen *et al.*, 1987), large virus structures (e.g. Harrison *et al.*, 1978; Rossmann *et al.*, 1985; Hogel *et al.*, 1985), protein–DNA complexes (e.g. Ollis *et al.*, 1985) and multienzyme complexes like riboflavin synthase (Ladenstein *et al.*, 1988).

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 rational design of drugs and proteins. For an illustration I chose human leucocyte elastase which is an important pathogenic agent. On the basis of its three-dimensional structure (Figure 20) (Bode *et al.*, 1986) and the criteria of optimal stereochemical fit, potent inhibitors are now being synthesized or natural inhibitors modified by use of recombinant DNA technology in many scientific and commercial institutions. Other, equally important proteins are similarly studied. This field especially benefits from the facile molecular modelling software (e.g. FRODO; Jones, 1978) and a standard and depository of structural data, the Protein Data Bank (Bernstein *et al.*, 1977).

Success and the new technical and methodological 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 (Hajdu *et al.*, 1988) and their tunability allows the optimal use of anomalous dispersion effects (Guss *et al.*, 1988; Hendrickson *et al.*, 1988).

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 were developed (Bricogne, 1976), 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.

At this point I wish to pay tribute to W.Hoppe who in 1957 laid the foundation to Patterson search techniques and thereby close the circle by returning to crystallographic work

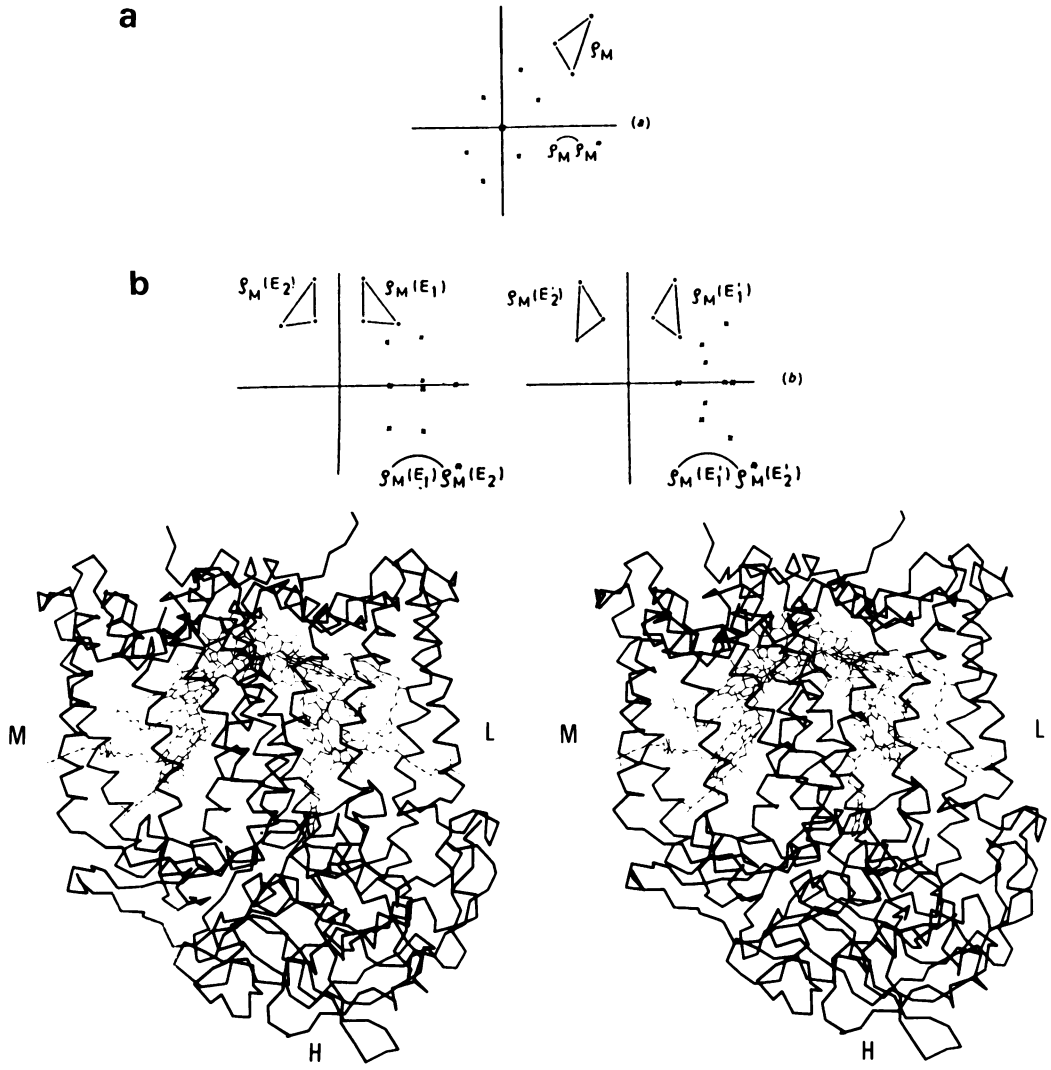


Fig. 21. Faltmolekül construct (**upper part**). $\varrho_M\varrho_M^*$ (**a**) and $\varrho_M(E1)\varrho_M^*(E2)$ (**b**) are the intra- and intermolecular vector sets of a triangular structure ϱ_M, respectively. Their sum represents the Patterson function. The intramolecular vector set can be constructed 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 line can be derived. In (**b**) the intermolecular vector sets corresponding to two different orientations of $_{rho}M$ are shown (Huber, 1985). **Lower part**. Drawing of the main chain of the M-, L- and H-subunits and the cofactors which served as search model to solve the phase problem for the *R.sphaeroides* RC crystal structure. For the calculation, all homologous main chain and side chain atoms were included (Allen *et al.*, 1986).

on the RC using this method. W.Hoppe discovered that the Patterson function (the Fourier transform of the diffraction intensities) of molecular crystals can be decomposed into sums of intra- and intermolecular vectors sets (Hoppe, 1957), from which orientation and translation of the molecules can be derived when their approximate structure is known (Figure 21). Hoppe's method was profoundly elaborated, computerized and reformulated (Rossmann and Blow, 1962; Huber, 1965; Crother and Blow, 1967). It provided a short-cut to the crystal structure of the RC of *R.sphaeroides* which was solved on the basis of the molecular structure of the RC of *R.viridis* and subsequently refined (Allen *et al.*, 1986, 1987). The molecular architectures are very similar although the *R.sphaeroides* RC lacks the permanently bound cytochrome. This was independently confirmed using similar methods by Chang *et al.* (1986). 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 (Huber *et al.*, 1974; Deisenhofer and Steigemann, 1975).

Recently nuclear magnetic resonance (NMR) techniques have demonstrated their capability to determine three-dimensional structure of small proteins in solution. In one case a detailed comparison between crystal and solution structure has shown very good correspondance (Kline *et al.*, 1986; Pflugrath *et al.*, 1986), but future developments will be needed to extend the power of the method to larger protein structures.

Protein crystallography is the only tool to unravel in detail the architecture of the large protein complexes described here and 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 the successful analytic method E.Fischer addressed by pointing out that 'the precise nature of the assimilation

process will only be accomplished when biological research, aided by much improved analytic methods, has succeeded in following the changes which take place in the actual chlorophyll granules' (Fischer, 1907). 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 (e.g. Levitt and Sharon, 1988) but a clue to the code-relating sequence and structure is not in sight (Jaenicke, 1988). Like Carl von Linne, who, 250 years ago, created a system of plants on the basis of morphology (*Genera Plantarum*, 1738), we classify proteins on 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 protein crystallography.

Acknowledgements

J.Deisenhofer's and my interest in structural studies of the photosynthetic reaction centre of *R.viridis* was raised 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 attack a problem which was and still is the largest asymmetric protein analysed at atomic resolution today.

The 'heureka' 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, those who are with me and those who had left, 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 and T.Shirmer. These projects were collaborative undertakings with biochemists in Switzerland (H.Zuber and W.Sidler), USA (M.L.Hackert) and Italy (M.Bolognesi, A.Marchesini and A.Finazzi-Agro).

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