Structural Studies on an Integral Membrane Light-Harvesting Complex

by

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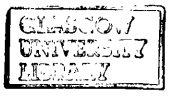

I declare this thesis is less than 100,000 words in length and that all of the work contained within was performed by myself unless otherwise stated.

Gerry McDermott

Glasgow, Jan 1997

This thesis is dedicated to my mother and father.

Abstract

The crystallographic structure of the B800-850 peripheral light harvesting complex, an integral membrane pigment-protein complex, from the non-sulphur purple bacteria *Rhodopseudomonas acidophila,* strain 10050, has been determined to a resolution of 2.5 Å (McDermott *et al.*, 1995).

This thesis outlines the changes made to the purification and crystallisation protocols which produced crystals which diffracted reproducibly to high resolution. In addition, microspectroscopy was utilised to determine the integrity of the complex in the crystal and to determine the effect of 'heavy atom soaking' procedures on the crystal order. The latter allowed the development of a soaking solution which maintained crystal order during heavy atom derivatisation trials.

The LH structure is described and some initial interpretations of the implications that the structure has for the study of bacterial photosynthesis.

Acknowledgements

I wish to acknowledge an enormous debt of gratitude to Professors Neil Isaacs and Richard Cogdell for giving me the opportunity to work on such a stimulating and exciting project and for providing the financial and material support necessary to carry out this work. More importantly, despite obvious pressure from their peers and funding bodies to produce results as rapidly as possible, I was given the luxury of being allowed the freedom to make, and hopefully learn from, my own mistakes. Once again, thanks for everything.

To Anna Hawthornthwaite-Lawless, Miroslav Papiz and Steve Prince I am also deeply grateful. All three taught me much more than they assume, and moreover did so with enormous patience and humour. Although Steve has a profound dislike of glib comments in acknowledgements, I have to say that their great expertise in their respective fields coupled with a profound sense of modesty and scientific integrity was and is inspirational.

Over the years that I spent in Glasgow, many people from various departments aided, abetted and generally helped me (oh, and put up with me and my horrible ways). I would like to thank (in no particular order, so don't go in the huff if you are near the end) the following people.....

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Finally, you are now adding weight to my theory (and that of the many people whose opinions I canvassed) that this is possibly the only page in the whole thing that gets read extensively. I hope if you were expecting to see your name that you found it. If not please write it in the space below and claim the drink that I now owe you !

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Abbreviations and symbols used in the text

Å	Ångstrom 10^{-10} m
B	Bulk (with reference to bacteriochlorophyll pigments)
BChl	Bacteriochlorophyll
β-OG	β-Octyl-glucopyranoside
Car	Carotenoid
CCP4	Collaborative Computational Project number 4
CD	Circular Dichroism
Chl	Chlorophyll
CMC	Critical Micelle Concentration
Da	Daltons
DDAO	Dimethyldecylamine *N*-oxide
DEAE	Diethylaminoethyl Cellulose
Fc	Calculated Structure Factor
Fo	Observed Structure Factor
F_P	Protein Structure Factor
F_H	Heavy Atom Structure Factor
F_{PH}	Protein + Heavy Atom Structure Factor
kDa	Kilo Daltons
LH	Light-Harvesting
LH I	'Core' Light-Harvesting Complex
LH II	Peripheral Light-Harvesting Complex
LHC-II	Plant Light-Harvesting Chlorophyll *a/b* Complex
LD	Linear Dichroism
LDAO	Lauryl-dimethylamine *N*-oxide
mMW	Micelle Molecular Weight
ms	Milli second (10^{-3} s)

OD	Optical Density
PAGE	Polyacrylimide Gel Electrophoresis
PEG	Polyethylene glycol
ps	Pico second (10^{-12} s)
PS I	Photosystem I
PS II	Photosystem II
Rps.	*Rhodopseudomonas*
Rb.	*Rhodobacter*
RC	Reaction Center
SDS	Sodium Dodecyl Sulphate
TGB 10	Triton TBG-10
Tris	Tris-hydroxymethyl-aminomethane

Chapter 1

Introduction

1.1 Introduction

Proteins are ubiquitous molecules, present in almost all biological systems and vital for their function. In the path towards fully understanding the mechanistic role a protein molecule plays in a particular process, the step of producing a high resolution structural model is now considered fundamental. The study of photosynthesis, where the existence of a large body of experimental data is concurrent with a paucity of structural information, can be taken as a case in point. In recent years, many points of controversy have arisen over specific aspects of the mechanisms involved. In most instances, these disputes have been directly attributed to a lack of structural information about the protein component of photosynthetic systems (Ford, 1992).

The work contained in this thesis was carried out with the ultimate aim of elucidating the X-ray crystallographic structure of a protein-pigment complex - the B800-850 light-harvesting complex (LH II) from *Rhodopseudomonas* (*Rps.*) *acidophila* strain 10050 - which is responsible for the initial photochemical event in bacterial photosynthesis, the capture of solar radiation. It was anticipated that elucidation of this structure, in conjunction with other published data, would provide definitive answers to long-standing questions regarding the molecular assembly of light-harvesting complexes and the effect that the structural design has on the photochemistry which occurs.

Furthermore, bacterial light-harvesting complexes are proteins of the integral membrane class. Prior to this work, the X-ray crystallographic structures of representatives from only two families of these functionally diverse and

fundamentally important proteins had been reported: the bacterial porins (reviewed in Cowan, 1993) and the bacterial photosynthetic reaction centers (Deisenhofer *et al.*, 1985; Komiya *et al.*, 1988; Chang *et al.*, 1991; Ermler *et al.*, 1994). Consequently, the prospect of elucidating the tertiary structure of a member from another family of integral membrane proteins was viewed as adding significantly to the structural information available on this class of protein (Kühlbrandt, 1992) and can be regarded as an aim of the work in this thesis in its own right.

1.2 Photosynthesis

Photosynthesis is the biochemical/biophysical process carried out by eukaryotic and prokaryotic phototrophic organisms, whereby solar radiation is captured and converted to chemical free energy. The scale on which photosynthesis is carried out is enormous. For example atmospheric carbon dioxide is recycled every 300 years, and each year $2x10^{11}$ tonnes of carbon are fixed (Barber & Anderson, 1994). Green plants, algae and cyanobacteria carry out oxygenic photosynthesis, with water as a substrate and gaseous oxygen as a by-product. Green and purple photosynthetic bacteria use alternative substrates, such as succinate, as the primary electron donor and carry out anoxogenic photosynthesis (Sistrom, 1978).

Ultimately all life on earth depends on photosynthesis: both as a source of oxygen and for the production of the chemical 'building blocks' required for the synthesis of all organic compounds found in biological systems. In addition, all organisms require energy to fuel essential cellular processes, heterotrophs can only

derive this energy by the controlled oxidation of carbohydrates and other similar molecules produced by phototrophs (Sistrom, 1978).

1.2.1 Photosynthesis: An historical perspective

It has been estimated that photosynthesis has been carried out on earth for at least 3.5 x 10^9 years (Nisbet *et al.*, 1995). Very strong similarities between photosynthetic apparatus isolated from evolutionary diverse sources have been identified (Ford, 1992). Coupled with the overall complexity of the process, these observations lead to difficulty in envisaging how photosynthesis could have arisen, by the process of natural selection, more than once (Ford, 1992). Based on the hypothesis that prior to photosynthesis life was closely associated with hydrothermal systems, a possible evolutionary pathway has been proposed (Nisbet *et al.*, 1995).

In hydrothermal systems the local environment encountered by an organism can vary enormously, both in terms of temperature and chemical composition. Therefore, an organism's chances of survival can be greatly increased if it develops the ability to move to an optimal position for life. If a system of phototaxis[1] evolved, based on the detection of infra-red radiation by bacteriochlorophyll like molecules, it is conceivable that from such a system photosynthesis may have arisen (Nisbet *et al.*, 1995).

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[1] The ability to move towards a light source.

1.2.2 Some landmarks in the study of photosynthesis

The study of photosynthesis can be traced back to 1771 when Joseph Priestley observed that the gas which bubbled to the surface of brewery fermentation vats had a detrimental effect on small animals. Priestley had discovered carbon dioxide, and more importantly the fact that plants can convert carbon dioxide to oxygen. The requirements of light and the chlorphyll containing leaves of the plant for the process were discovered in 1779 by Jan Ingen-Housz.

The observation that purple bacteria displayed photosynthetic activity was made by Engelmann in 1883. Initially he observed that purple bacteria exhibit phototaxis and move towards a source of light, and proved the requirement of light for their development. Further work led him to the conclusion that the wavelength of the light required for growth correlates to the absorption bands of the photosynthetic pigment bacteriochlorophyll.

C.B. van Niel characterised bacterial photosynthesis in the early 1940's (van Niel, 1941) He compared the chemistry involved when green plants undergo oxygenic photosynthesis with anoxogenic photosynthesis carried out by photosynthetic bacteria. He established that CO_2 reduction by photosynthetic bacteria is concomitant with oxidation of a substrate in the growth media. He also observed that in bacterial photosynthesis CO_2 assimilation is not accompanied by O_2 evolution. This resulted in his van Niel's general formula for photosynthesis:

$$2\,H_2A + CO_2 \rightarrow 2A + (CH_2O) + H_2O \quad \text{(van Niel, 1941)}$$

Where H_2A is an oxidisable substrate and CH_2O is representative of a stored organic compound. If H_2A is an organic compound it may serve as both a source

of carbon (in place of CO_2) and as a reducing substance. In the case of plants and algae, water is the oxidisable substrate. By using such an ubiquitous and readily available material, plants and algae developed a substantial advantage over photosynthetic bacteria.

1.3 Oxygenic photosynthetic systems

Photosynthetic membranes from oxygenic phototrophs contain a substantial number of pigment binding proteins organised into two cooperative Photosystems: Photosystems I and II (PS I and PS II, respectively). With each Photosystem being responsible for performing individual photoacts. The overall photochemistry of oxygenic photosynthesis was described as the "Z scheme of electron transport" by Hill and Bendal in 1960 (Hill & Bendal, 1960). To date their model is still central to the modern interpretation of oxygenic photosynthesis (Prince, 1996).

In recent years our knowledge of the structures and mechanisms involved in oxygenic photosynthesis has increased by virtue of the development of efficient isolation procedures and improved spectroscopic instrumentation and techniques. However, to complete the picture there is now a pressing requirement for the production of precise, high resolution structural models of the contents of the photosynthetic membrane (Ford, 1992). A full account of the oxygenic photosynthetic membrane will be best achieved by a combination of X-ray crystallography and electron microscopy based techniques (Tsiotis *et al.*, 1996). Even if the molecular structures of all subunits of the Photosystems are individually elucidated by X-ray crystallography, determining their relative locations and assembly *in* vivo will require the use of techniques such as electron microscopic

analysis of immunogold labeled membranes. Presently, the only available X-ray crystallographic structure is a low resolution model of Photosystem I (Krauss *et al.*, 1993; Fromme *et al.*, 1996). Electron microscopy techniques have proven slightly more successful in the study of proteins from oxygenic membranes (see Tsiotis *et al.*, 1996 for a review of the application of electron microscopy to the structure elucidation of Photosystem II). There are two complementary approaches to obtaining structural information on membrane proteins by electron microscopy based techniques: low resolution studies on intact membranes or detergent solubilised complexes, and high resolution electron crystallographic studies on 2-D crystals. To date, numerous membrane proteins have been 'reconstituted' in the presence of phospholipids into ordered crystalline sheets, vesicles and tubes. It has frequently been suggested that the reconstitution of 2-D protein-lipid crystals can be considered a routine procedure, with a significant likelihood of success (for reviews see Engel *et al.*, 1992; Jap *et al.*, 1992 and Kühlbrandt, 1992). Reconstitution experiments, and thereafter EM analysis, on Bacteriorhodopsin (Henderson *et al.*, 1990) and the plant light-harvesting chlorophyll *a/b* complex II (LHC-II) (Kühlbrandt *et al.*, 1994) have resulted in the establishment of three-dimensional models. The latter work is highly relevant in the context of this thesis, since it allows a direct comparison between two protein-pigment complexes which essentially perform a similar function, but originate from two evolutionary diverse sources.

1.3.1 Plant light-harvesting chlorophyll a / b complex

In vivo, the plant light-harvesting chlorophyll *a/b* complex (LHC-II) is associated with Photosystem II in the chloroplast and accounts for more than 50%

of the pigments involved in plant photosynthesis (Kühlbrandt *et al.* 1994). The complex is composed of a single 232 residue polypeptide, which binds a minimum of 12 chlorophyll (Chl) and 2 carotenoid (lutin) molecules (Cashmore, 1984; Michel *et al.*, 1991).

1.3.2 LHC-II: Electron crystallographic structural model

Electron crystallography data collection methodology imposes constraints on the collection of data at tilt angles above 60° (Kühlbrandt, 1994). The resultant incompleteness of data is manifest in a cone of reciprocal space where amplitudes and phases cannot be measured. Model calculations imply that the obtainable resolution in the direction perpendicular to the plane of the membrane is lower by a factor of 1.3 (Glaeser *et al.*, 1989). In the case of LHC-II, where the maximum observed resolution of data in the plane of the membrane is 3.4Å the theoretical maximum resolution obtainable perpendicular to this is 4.42Å, but in practice the best achieved is around 4.9 Å (Kühlbrandt, 1994). Despite this anisotropy of resolution, around 80% of the peptide content, and most of the associated pigment moieties have been fitted to the resulting calculated electron density. The published model has been refined to a free R-factor of 37.9% and a crystallographic R-factor of 33.0%.

The protein chain traverses the membrane three times, by way of three α helices, termed B, C, and A. In the model there is a further short extra-membranous α helix, D. Helices A and B are in close proximity, and have lengths of 43 Å and 51 Å respectively. Each helix begins with a proline and is tilted at an angle of 32° with respect to the membrane plane. Conversely, helix C begins with

a serine and extends for 31 Å with a 9° tilt with respect to the membrane plane. The first 24 residues of helices A and B are related by a local two fold axis. Two carotenoid molecules, lutein, have been assigned. The polyene chains of the lutein molecules are inclined at 50° to the membrane normal and are slightly curved, suggesting the all-trans conformation. The carotenoid molecules also display local two fold symmetry.

Twelve chlorophyll molecules, in six clusters, have been assigned to the calculated density. The presence of a thirteenth chlorophyll molecule is possible in a region of unassigned density near the N termini. In all cases, the plane of the chlorin rings is tilted with respect to the membrane normal, as can be seen in figure 1.1.

1.3.3 LHC-II: Structure function relationship

Kühlbrant suggests the location of the lutein molecules, across the A and B helices, may be necessary to promote structural rigidity in a structure with little helix-helix interactions. Their function in terms of photosynthetic mechanism is both as light harvesting molecules and as quenchers of triplet chlorphyll (Cogdell & Frank, 1987); the presence of which could result in the formation of highly destructive singlet oxygen (see Chapter 5 for discussion). The probability of this occurring is high, due to the relatively long lifetime of triplet chlorphyll (~1 ms).

Spectroscopic analysis has estimated that energy transfer from Chl *b* to Chl *a* occurs in less than 1 ps, significantly less than the time required for triplet formation, therefore only the Chl *a* species requires photoprotection (Kühlbrandt *et al.* 1994). At present, the resolution of the electron density map is insufficient to

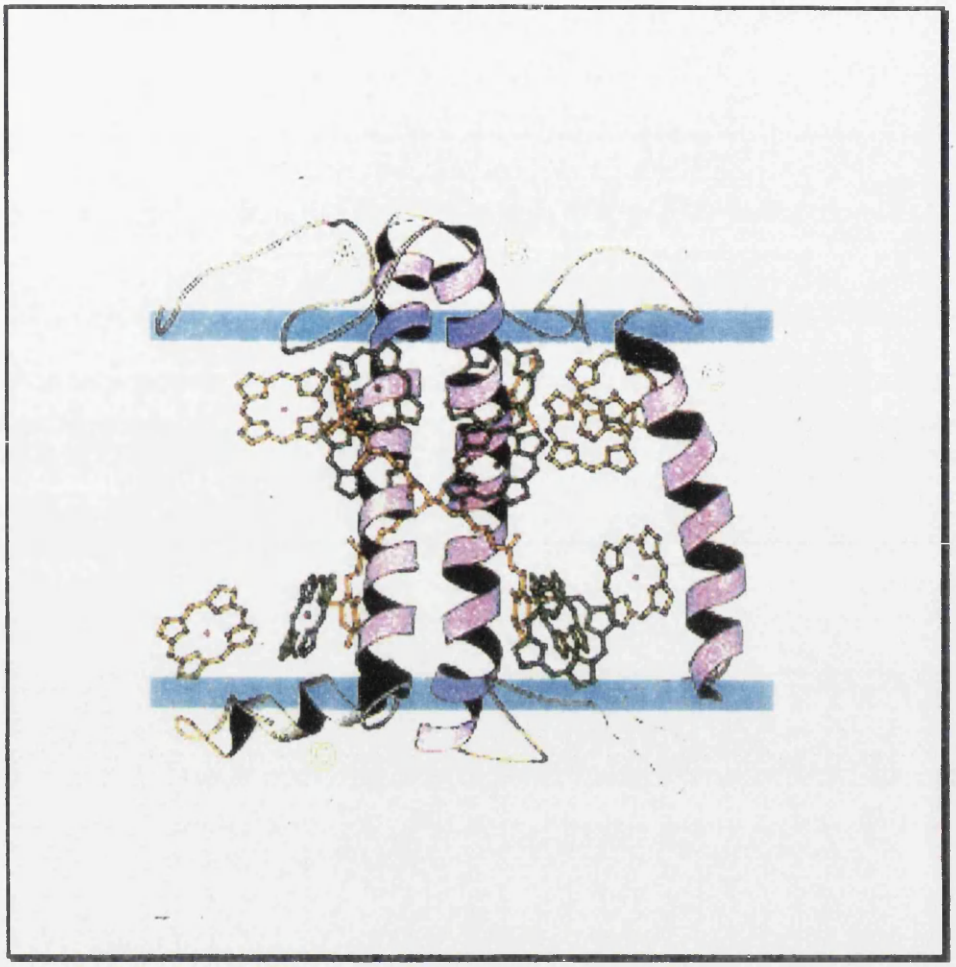

Figure 1.1. The plant light-harvesting chlorophyll *a/b* complex (Kühlbrandt *et al.*, 1994). Blue bands indicate the membrane surfaces. The presumed population of Chl *a* species are coloured green, and the Chl *b* yellow.

make the distinction between Chl *a* and Chl *b* (Kühlbrandt *et al.* 1994). Consequently, on the basis of photoprotection requirements, the seven chorophylls closest to the carotenoids have been assigned as Chl *a*, an assumption that has been verified spectroscopically. Six of the Chl *a* molecules are also related by local two fold symmetry. Kühlbrandt asserts that the presence of the local dyad probably has some underlying functional reason. This is not yet known, but Kühlbrandt suggests that the dyad arises from chlorophyll and carotenoids molecules locating in optimal positions for excitonic coupling, and Förster energy transfer[1] to other light-harvesting complexes, or to Photosystem II (Kühlbrandt *et al.*, 1994).

1.4 Photosynthetic purple bacteria

Non-sulphur photosynthetic purple bacteria are simple gram negative bacteria (Cogdell & Thornber, 1980). When grown under aerobic conditions, the cells have smooth inner membranes and respire aerobically. If the environmental oxygen tension decreases the cells synthesise, *de novo*, heavily pigmented, invaginated intracytoplasmic membranes termed "chromatophores" (Clayton & Haselkorn, 1972), as shown in figure 1.2. If exposed to light of an appropriate wavelength, the cells become capable of photosynthesis, and the photoassimilation of inorganic compounds (Hawthornthwaite & Cogdell, 1993).

The intracytoplasmic membrane is analogous to the thylakoid membrane of higher plants, and houses a range of photoactive protein-pigment complexes, with

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[1] See Chapter 5 for a discussion on the modes of energy transfer which may occur in photosynthetic systems.

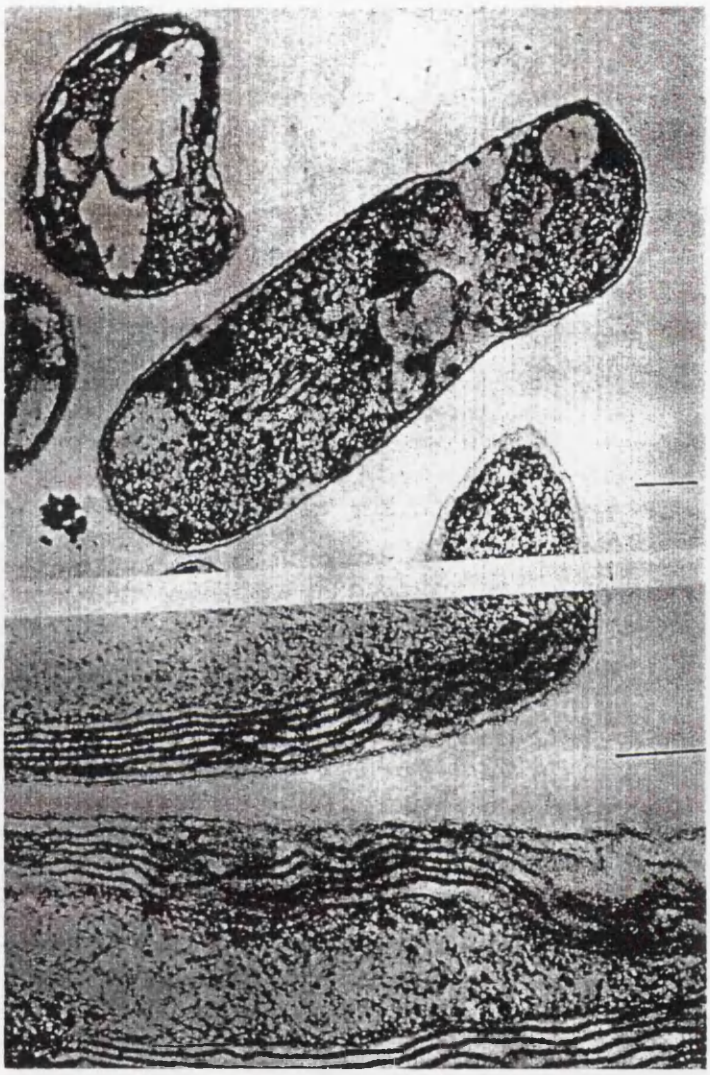

Figure 1.2. Electron micrographs of whole cells of *Rps. acidophila*.
Top: Cells grown aerobically.
Bottom: Cells grown photosynthetically.
Scale Bar: 200 nm.

the exception that in the case of anoxygenic bacteria the protein-pigment complexes only form a single Photosystem (Hawthornthwaite & Cogdell, 1993). The intracytoplasmic membrane can adopt several distinct topologies - either vesicles, lamellae or stacks (Imhoff, *et al.*, 1984). This variation in membrane morphology is the basis of the taxonomic differentiation between genera.

1.4.1 Taxonomy of photosynthetic bacteria

Photosynthetic bacteria are classified as the order *Rhodospiriallales* (Trüper & Pfennig, 1978). The defining characteristics of the order being the presence of bacteriochlorophyll and the ability to carry out anoxogenic photosynthesis. The *Rhodospiriallales* has two sub-orders: *Rhodospirillinea* (purple bacteria) and *Chlorobineae* (Green Bacteria). *Rhodospirillinea* contains three families: *Rhodospirillaceae, Chromatiaceae* and *Ectothiorhodospiraceae.* Of these the purple non-sulphur bacteria, *Rhodospirillaceae*, have the most diverse genera and have been the subject of extensive work. The work in this thesis was carried out on a genus of this family, the species *Rhodospeudomonas acidophila* (*Rps. acidophila)*, and in particular strain 10050. A schematic of the classification is given in figure 1.3.

1.4.2 The intracytoplasmic membrane

Two types of functionally distinct types of integral membrane protein-pigment complex are vectorially located into the intracytoplasmic membrane (Hawthornethwaite & Cogdell, 1993);

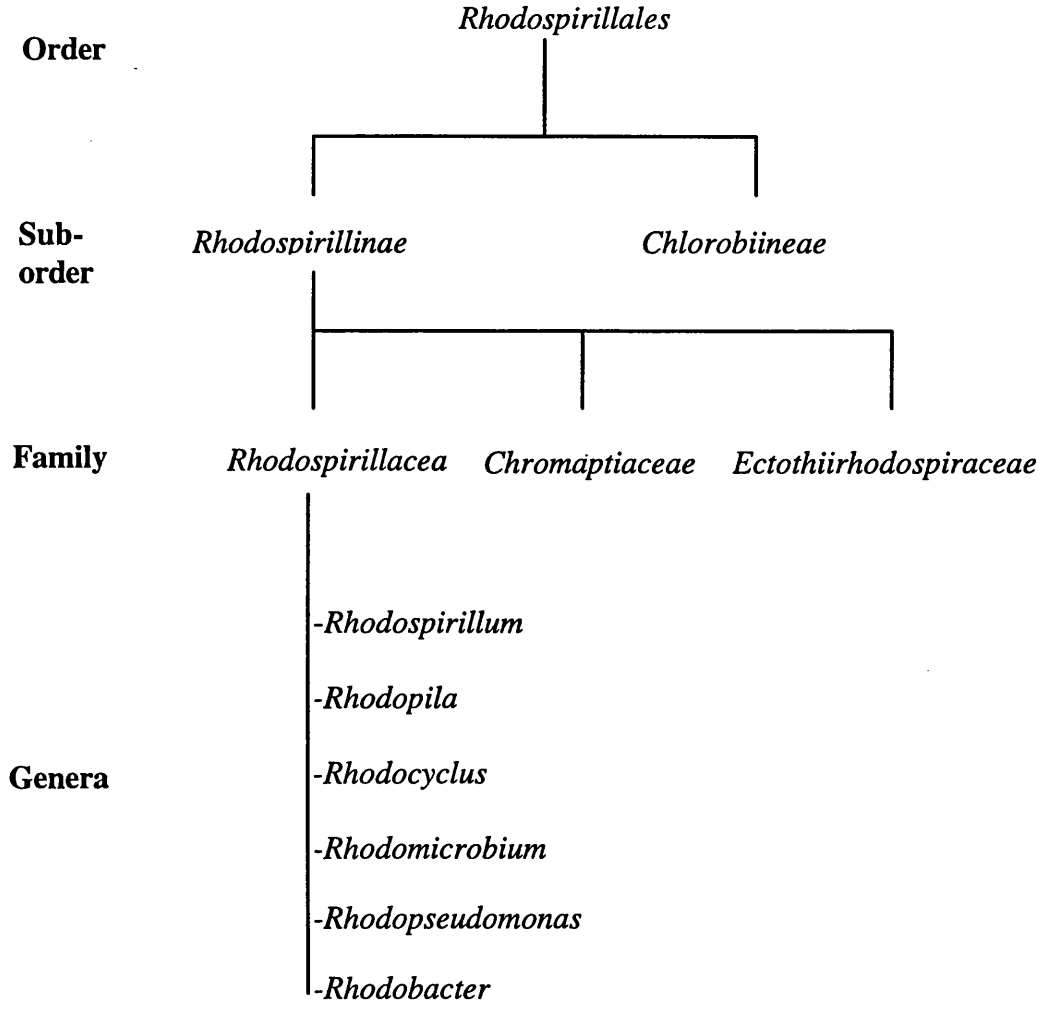

Figure 1.3. Taxonomy of purple bacteria (Imhoff *et al.*, 1984).

- **The Reaction Center** (RC): where primary charge separation across the photosynthetic membrane occurs.

- **Light-Harvesting Complexes** (LH): Protein-pigment complexes whose function is to capture solar radiation, and subsequently transfer the energy to the reaction center.

1.4.3 The bacterial reaction center

The existence of the bacterial reaction center was first postulated by Emerson and Arnold (Emerson & Arnold, 1932). The complex was first isolated from photosynthetic membranes by Reed and Clayton (Reed & Clayton, 1968). Bacterial reaction centers are composed of three protein chains: Light (L), Heavy (H) and Medium (M) (so called because of their apparent weight when determined by gel electrophoresis) (Clayton & Haselkorn, 1972). In *Rhodopseudomonas (Rps.) viridis* these consist of 258, 323 and 273 residues, respectively. The apoproteins bind: one carotenoid molecule; four bacteriochlorophyll; two bacteriopheopytins; one non-heme iron and two quinone[1] molecules. In this species the reaction center also has a tightly bound molecule of cytochrome *c* (Weyer *et. al.*, 1987). The reaction center from *Rps. viridis* was the first X-ray crystallographic structure of an integral membrane protein to be determined (Deisenhofer *et al.*, 1985). This was a landmark event in the field of protein crystallography, and resulted in Deisenhofer, Michel and Huber being awarded the Nobel prize for chemistry in 1988.

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[1] Q_A is menaquinone in *Rps. viridis* and ubiquinone in *Rhodobacter (Rb.) sphaeroides*. Q_B is ubiquinone in both species (Lancaster *et al.*, 1995).

The X-ray crystallographic structures of the reaction center from other strains and species of bacteria are now available. To date the structure of the reaction center from *Rps. acidophila* has not been elucidated, but several groups are now conducting experiments to rectify this situation. Comparison between reaction centers from *Rb. sphaeroides* and *Rps. viridis* revealed a very high level of structural homology (Elkabbani *et al.*, 1991). The domain of major functional importance is the hydrophobic transmembrane core. Constructed from α helices - five from both the L and the M subunit - this region is the location of the bound pigment co-factors. The transmembrane α helices follow approximate two fold symmetry, shown in figure 1.4. When viewed perpendicular to the plane of the membrane, the transmembrane domain is seen as an ellipse of dimensions 30Å by 70Å. In the direction perpendicular to the membrane plane, the reaction center - including the bound cytochrome - has a total length of 130Å.

The pigments are arranged with approximate two-fold symmetry into two branches, labeled A and B (see figure 1.5) and are coordinated to the L and M apoproteins (Elkabbani et al., 1991). The energy pathway is summarised in Deisenhofer & Michel, 1989. In brief, energy from the light harvesting system1 is transferred to a pair of exciton coupled BChl a molecules, termed the 'special pair'. This induces transition of the special pair to the lowest excited singlet state, quickly followed by the donation of an electron to the primary acceptor, bacteriopheophytin. The electron is transferred to the quinone Q_A. The bacteriochlorophyll dimer is re-reduced by the bound cytochrome subunit, whereupon the electron leaves Q_A and reduces Q_B. The process is completed when

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[1] See next section.

another quantum of absorbed energy results in the reduction of Q_{B^-} to hydroquinone. This leaves the reaction center and is replaced by a new Q_A molecule. The entire process only involves one side of the reaction center (the L-subunit). The reason for the continued presence of the functionally redundant pigments is unclear (Deisenhofer & Michel, 1989).

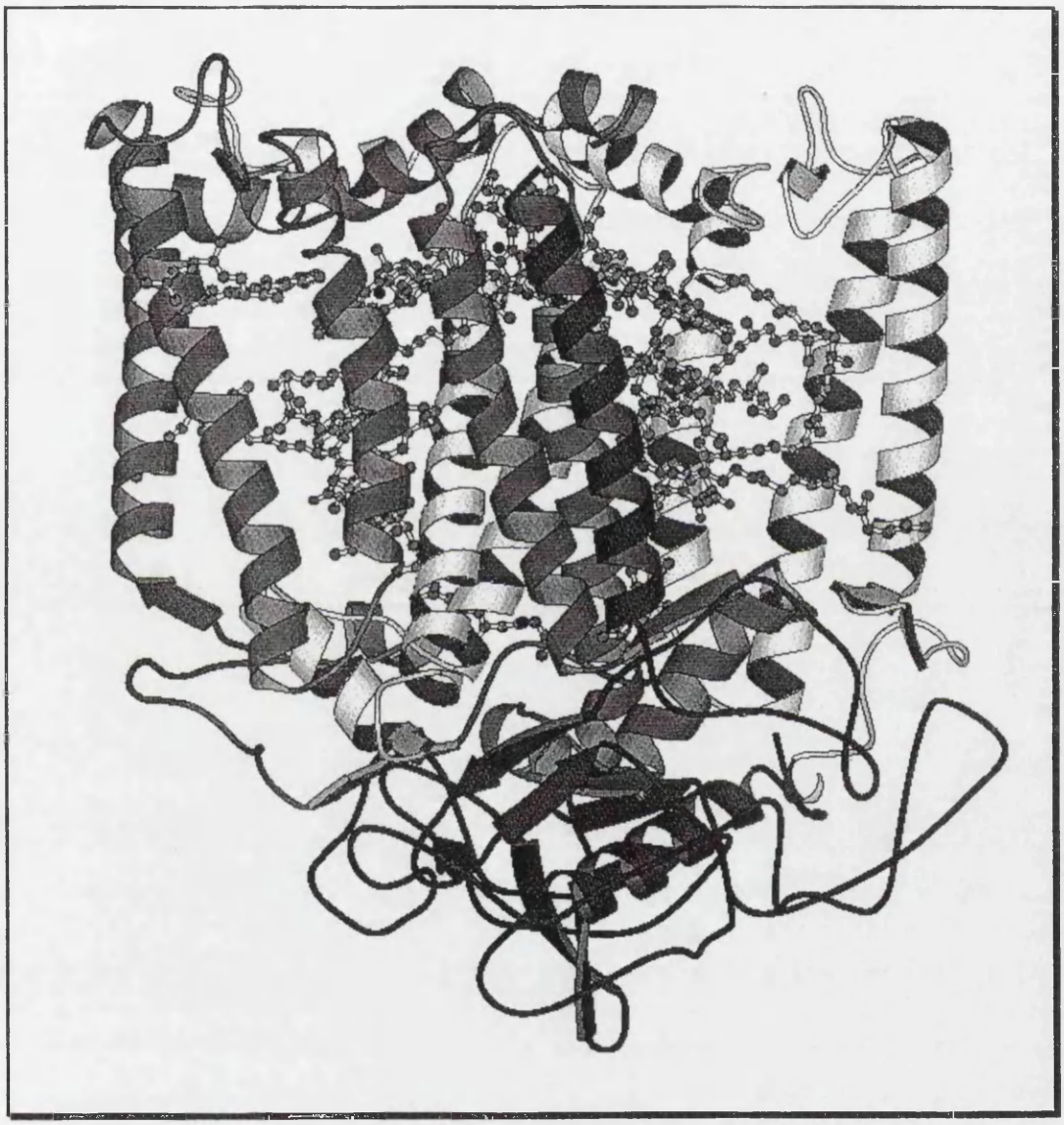

Figure 1.4. The apoproteins and pigments from *Rps. sphaeroides* reaction center.

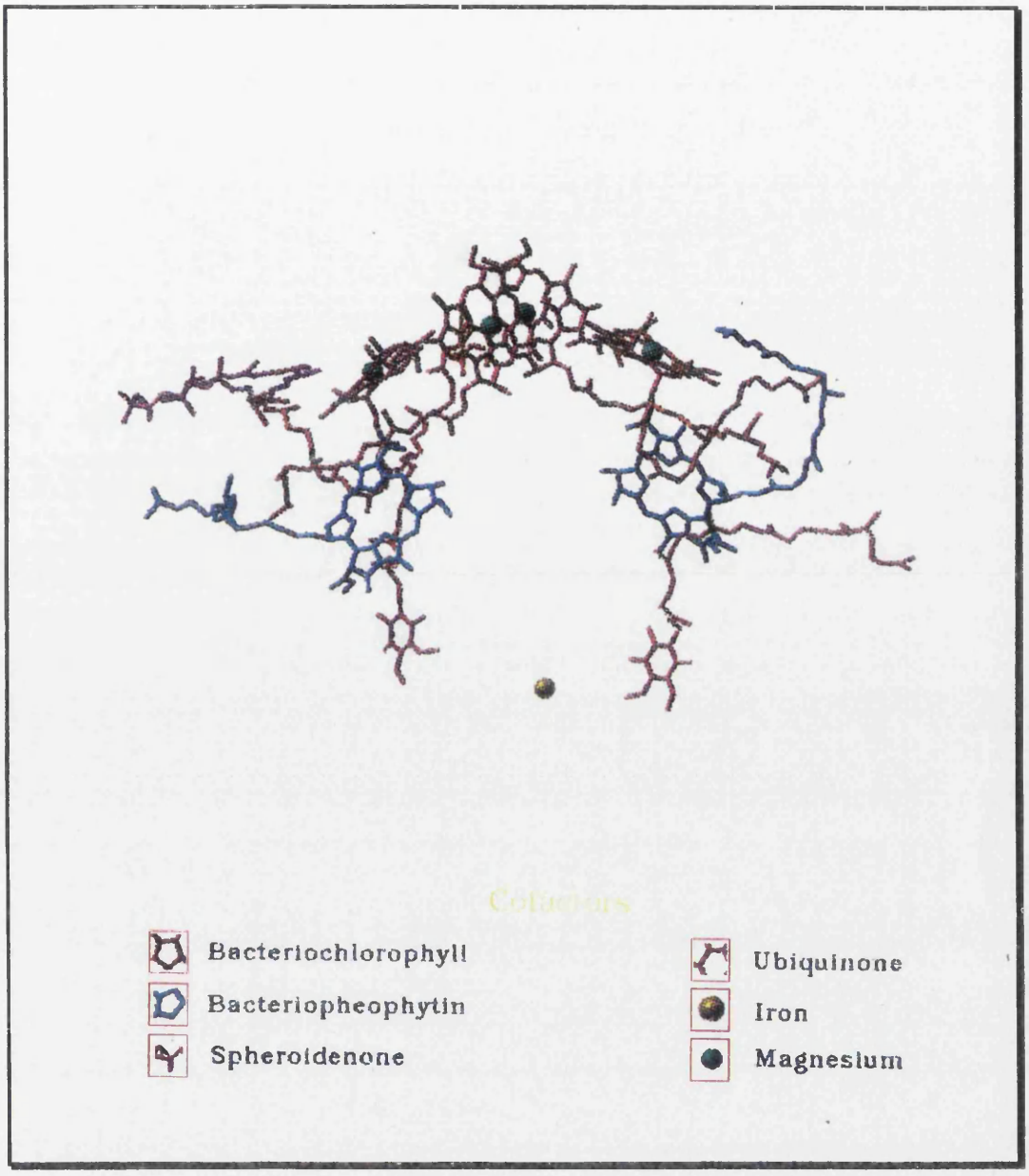

Figure 1.5. Organisation of the reaction center pigments.

1.5 Bacterial light-harvesting complexes

Most species and strains of purple bacteria synthesise two spectrally and functionally distinct populations of light-harvesting complex: LH I and LH II (Zuber, 1986). Categorisation of the complexes is based on distinctive absorbance features in the near-infra red region, and their presumed proximity to the reaction center (Zuber, 1986). The general distinctions between the two types of LH complex are summarised in Hawthornthwaite & Cogdell, 1993 and Zuber & Brunisholtz, 1993.

1.5.1 LH I

LH I is located in intimate association with the reaction center, and present in a fixed stoichiometric quantity with it. Isolated LH I complexes display a characteristic single absorption peak in the near infra-red, with maximum absorption occurring between 870 and 890nm. Generally this species of LH complex are referred to as 'core' light-harvesting complexes.

1.5.2 LH II

LH II complexes are found more distant than LH I from the reaction center, and present in the membrane in variable amounts. LH II complexes show two discrete near infra-red absorption peaks - typically at approximately 800 and 850nm - the exact wavelength dependent on species of origin and the environmental conditions encountered during isolation and purification of the complex. Due to their location relative to the reaction center, these LH complexes are usually referred to as 'peripheral' light-harvesting complexes. LH II functions as the main light-harvesting complex in most strains of bacteria and is therefore

generally present in greater numbers in the membrane than LH I (Cogdell & Thornber, 1980).

The nomenclature of LH complexes and the individual populations of bacteriochlorophylls, is based primarily on their absorbance characteristics. The prefix B (for bulk bacteriochlorophyll) is followed by the wavelength(s) of the absorption maxima in the region 800 to 900 nm (Cogdell *et al.,* 1985). Hence, LH II are termed B800-850 light-harvesting complexes. In some species and strains of bacteria, environmental conditions encountered during cell growth can result in the synthesis of a further type of peripheral LH complex. These complexes display absorption maxima at approximately 800 and 820 nm, hence are termed B800-820 LH II (or occasionally as LH III) (Gardiner *et al.*, 1993).

1.5.3 LH complexes: Common structural features

Biochemical composition and primary structure analyses of LH I and LH II complexes isolated from numerous strains and species of purple bacteria, resulted in the recognition of several common structural features (Zuber, 1990). All types of light-harvesting complex were found to be oligomers of two apoproteins (α and β) with relatively low molecular weights - both in the region of 5 to 7 kDa (Zuber, 1986). The apoproteins non-covalently, and stoichiometrically bind the pigment moieties bacteriochlorophyll *a* (BChl *a*) and carotenoid (Zuber, 1986). Hydropathy analysis of the apoprotein primary structures revealed a region of between 20 and 23 hydrophobic residues in both α and β (Brunisholz *et al.*, 1987). Based on circular dichroism and labeling analysis (Jay *et al.*, 1983) it was

postulated that this hydrophobic domain is α helical and constitutes the membrane spanning region (Brunisholz *et al.*, 1987).

Resonance Raman spectroscopy studies on LH complexes from *Rps. sphaeroides* strains 2.4.1 and R26, *Rps. capsulata*, *Rps. palustris* and *Rps. rubrum*, strains S1 and G9, indicated that conserved Histidine residues, located in the presumed membrane spanning region, were likely candidates as ligation sites for the B850 BChl *a* in LH II and B880 BChl *a* in LH I (Robert & Lutz, 1985). Conversely, the same study concluded that B800 BChl *a* ligation was not via a conserved Histidine residue, but via a different, unidentified residue.

1.5.4 Spectroscopic properties of LH II

The presence of stoichiometric quantities of specifically bound pigment moieties result in LH II displaying a highly characteristic absorption profile in the visible and near infra-red region (Angerhofer *et al.* 1986). A typical absorption spectrum, from *Rps. acidophila, 10050* is shown in figure 1.6. The BChl *a* absorption maximum occurs in the near infra-red, with the maximal transition Q_y being polarised along the Y axis, i.e. running through ring I and ring III nitrogen atoms of the bactriochlorin conjugated ring system. BChl *a* also shows a weaker absorption attributable to Q_x, the transition which runs perpendicular to Q_y (Angerhofer *et al.* 1986). A third band, the Soret band observed in the blue region, arises from the overlap of several bands which correspond to electronic transitions to higher energy states (Angerhofer *et al.* 1986).

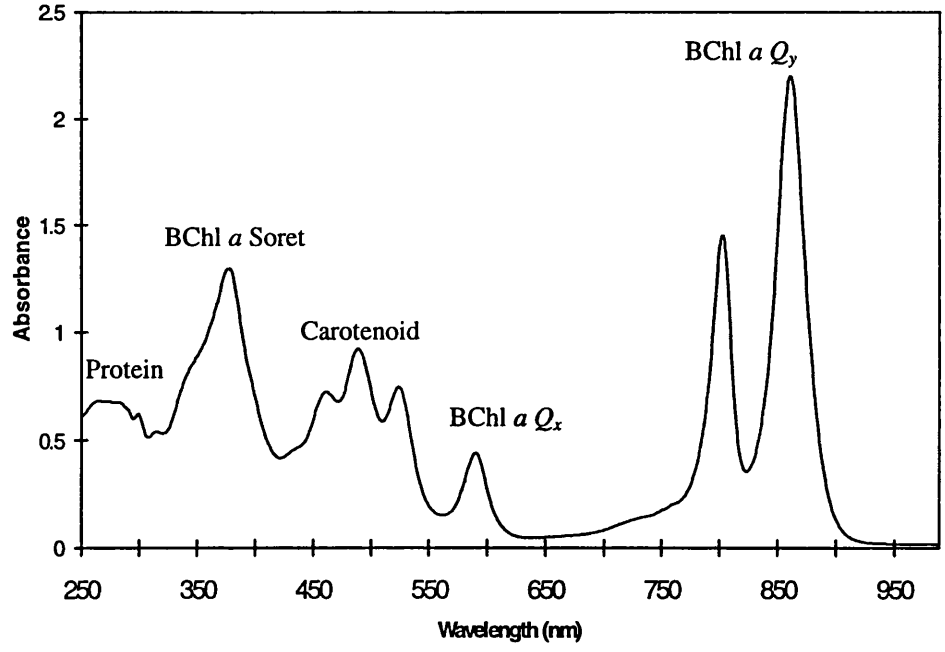

Figure 1.6. Absorbance spectra, LH II from *Rps. acidophila* strain 10050 in 20 mM Tris, pH 8.0, 0.1% LDAO.

The structural mechanisms involved in modulating the absorbance of BChl *a* was probed by site directed mutagenesis (Fowler *et al.*, 1992). The correlation between the presence of two aromatic residues in LH II from *Rhodobacter sphaeroides*, αTyr 44 and αTyr 45, and the absorbance spectra was examined. The mutations performed: αTyr 44, αTyr 45 → Phe Tyr and αTyr 44, αTyr 45 → Phe Leu, created an LH II complex whose sequence resembled the B800-820 from *Rps. acidophila* 7050. The single point mutation exhibited an 11 nm blueshift in the maximum absorbance of the 850nm peak, whereas in the double mutant the peak was blueshifted by 24 nm. The LH II circular dichroism (CD) signal in the 800 to 850 nm range is sensitive to structural change, and is thought to arise from pigment-pigment and protein-pigment interactions (Fowler *et al.*, 1992). CD analysis was carried out on native LH II and the above mutants. Although

blueshifted in this region, both mutants had a similar line shape to the native complex, suggesting that the mutants retained all of the important pigment interactions, and relative orientations (Fowler *et al.*, 1992). Consequently, it was concluded that the redshift of B850 BChl *a* arose in part from the close approach of a tyrosine residue with BChl *a* (Fowler *et al.*, 1992).

This effect had been predicted previously by Zuber and Brunisholz (Zuber & Brunisholz, 1988). They suggested that the redshift of BChl *a* pigments *in vivo* arose from interaction of the π-π^* orbitals of aromatic residues with the aromatic rings of the bacteriochlorin, but this factor alone does not account for the total redshift observed when BChl *a* molecules are bound to apoproteins in LH complexes. For example the B850 BChl *a* shows a shift of 80 nm compared to free aqueous pigment.[1] The residual redshift is attributed to excitonic interactions between pigments (Cogdell & Hawthornthwaite, 1993).

1.5.5 The macromolecular assembly

The overall macromolecular assembly of the apoproteins and pigments could not be resolved unambiguously, although several models were produced. These were recently reviewed by Olsen and Hunter (Olsen & Hunter, 1994) and will be discussed in Chapter 5. Briefly, of the models produced, those of Zuber were generally regarded as being the benchmark against which all other models were judged. Zuber postulated an oligomeric assembly consisting of six $\alpha\beta$ heterodimers, packed in three bundles with each bundle consisting of two $\alpha\beta$

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[1] Aqueous BChl *a* has an absorption maxima at 770nm (Weigl, 1953).

heterodimers. The overall packing of the apoproteins in each bundle resulted in the BChl *a* molecules being in close proximity and hence, forming an excitonic coupled array. In the model of the tetrameric bundles, the transmembrane helices are crossed at a point below the level of the bound B850 BChl molecules. It was presumed that the apoproteins were in tight association, and made extensive contact in the transmembrane region with the BChl molecules facing outwards into the lipid bilayer.

1.5.6 LH II from Rps. acidophila, strain 10050

The primary structure of the LH II apoproteins from *Rps. acidophila* strain 10050 were determined by Zuber and Brunisholz (Zuber & Brunisholz, 1993). The α polypeptide consists of 53 residues and has a molecular weight of 5.8 kDa. The β polypeptide is slightly smaller, consisting of 41 residues it has a molecular weight of 4.6 kDa. The sequence of both are given below, with the proposed (Zuber & Brunisholtz, 1993) membrane spanning regions shaded, and the conserved Histidine residues, postulated as B850 BChl *a* binding sites, highlighted:

α MNQGKIWTV PAIGIPALLGSVTVIAILVHLAIL SHTTWFPAYWQGGVKK

β AEQSEELHKYVIDGTRVFLGLALVAHFLAFSATPWLH

The pigment component of the complex was determined to be BChl *a* and the carotenoid rhodopin glucoside (Angerhofer *et al.* 1986; Evans, 1989). These were estimated to be present in the complex with a 2:1 ratio of BChl *a* to carotenoid (Evans, 1989).

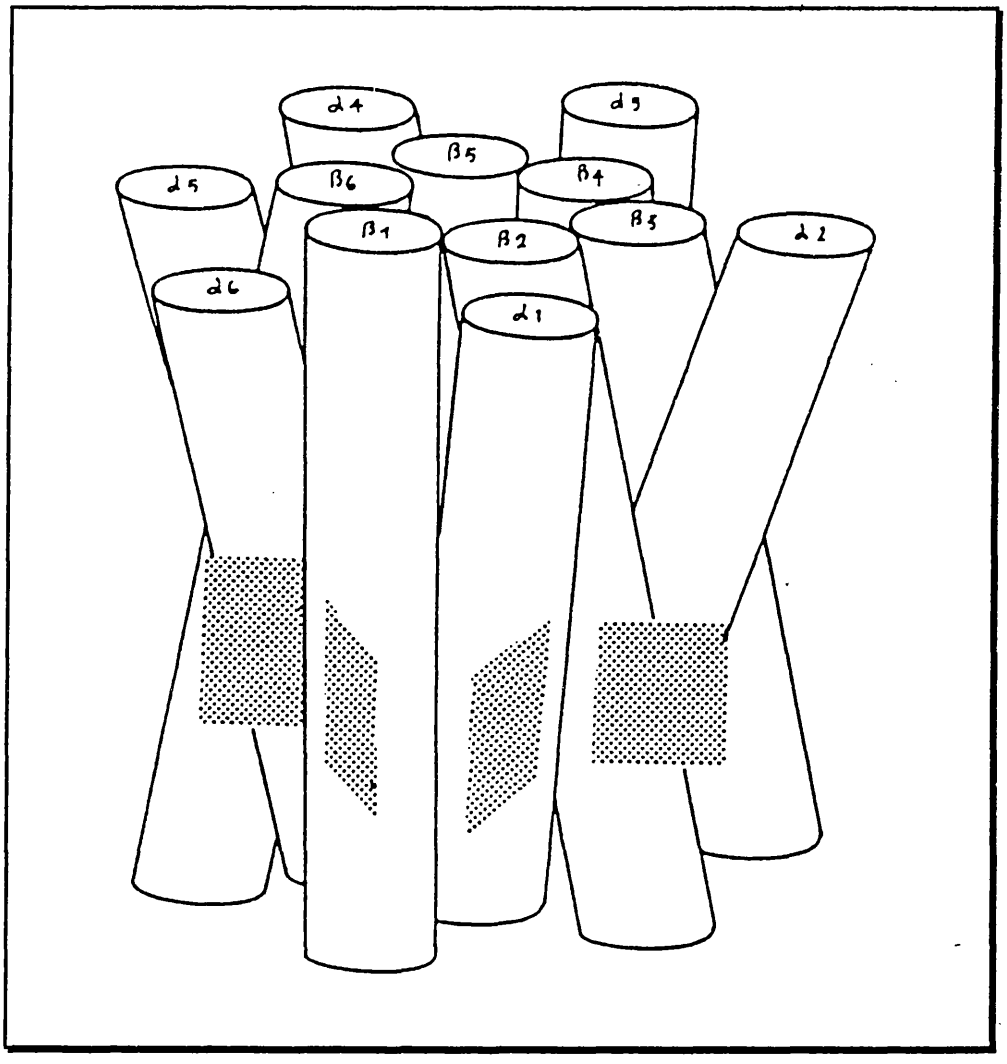

Figure 1.7. The postulated assembly of B800-850 LH II apoproteins and B850 BChl *a* (Zuber & Brunisholz, 1993). β apoproteins are located in the center of the complex. Shaded regions represent B850 BChl *a*

The overall assembly of LH II was proposed as a complex containing twelve apoproteins ($\alpha_6\beta_6$), binding 18 BChl *a* and 9 rhodopin glucoside molecules (Papiz *et* al., 1989; Cogdell & Hawthornthwaite, 1993) and arranged in the manner proposed by Zuber and Brunisholz, shown in figure 1.7 (Zuber & Brunisholz, 1993). A typical absorption spectra is shown in figure 1.6, the BChl Q_y maxima for this strain occurs at 801 and 863 nm.

1.6 X-ray crystallography

The study of molecular structure by X-ray crystallography can be traced back to 1913 when W.L. Bragg determined the molecular structure of several small inorganic salts. In the intervening years, X-ray crystallography has become highly developed, both in terms of comprehension of the underlying principles and improvements in the instruments and methods used for data collection and analysis. X-ray crystallography has been successfully applied to the study of a diverse range of molecules: from small organic/inorganic molecules to large macromolecules with molecular weights in the region of several hundred thousand Daltons. The theoretical basis of X-ray crystallography has been the subject of several lucid and in-depth textbooks, for example Stout & Jensen, 1968 and Ladd & Palmer, 1977. For a text specifically covering the application of X-ray crystallography to the structure solution of proteins, the reader should refer to the highly regarded work of Blundell & Johnson, 1976. Although comprehensive in coverage, this text is now dated in its coverage of the practical aspects of the X-ray crystallography, and should therefore be used in conjunction with more up to date texts such as

(Drenth, 1994) or the annual proceedings of the CCP4 workshops.[1] With the ready availability of textbooks on the subject, it would be superfluous to simply repeat the theory of X-ray crystallography of this thesis. Accordingly, only the basic crystallographic theory necessary for understanding the work in this thesis will be given below.

1.6.1 Some basic X-ray crystallography theory

When an incident X-ray beam impinges on a system of electrons the electrons oscillate with the same frequency as the incident beam. Energy is absorbed and emitted at the same frequency as the incident beam, i.e. elastic scattering occurs. If atoms are in the highly ordered form of a crystal, scattered waves from the electrons of each unique lattice element combine. The resultant wave vector is proportional to the sum of the wave vectors from each individual scattering element. Constructive addition of the wave vectors occurs when the Bragg relationship, equation 3.1, is fulfilled.

$$\mathbf{n\lambda = 2d \sin \theta} \qquad \textit{Eq.}\ 3.1$$

Where **d** is the inter atomic distance between crystal lattice planes, θ the angle subtended by the incident beam and the lattice planes, **n** an integer and λ the wavelength of the incident beam.

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[1] An excellent source of up to date information on practical and theoretical aspects of protein crystallography (Proceedings of CCP4 Daresbury study weekends, SERC Daresbury Laboratory, Warrington, England).

The overall scattering by the electrons in a crystal is the summation of an enormous number of waves - in the case of a protein crystal perhaps more than 10,000 electrons are present in each unit cell. The mode of X-ray scattering by a crystal is analogous to the diffraction of light. Except, unlike light, no optics have been found which refract X-rays. Such a material would make possible - in a manner analogous to an optical diffractometer - direct reconstruction of the image of the scattering material. The diffraction pattern seen when a crystal diffracts a collimated beam of X-rays is the Fourier transform of the electron component of the atomic structure in the asymmetric unit of the crystal. Therefore the inverse is possible: the mathematical reconstruction of an image of the scattering material by calculating an inverse Fourier transform from the diffraction data, i.e. the calculation of an interpretable electron density map. With the advent of fast and extremely powerful computers and algorithms this can be done readily. The electron density ρ at position (xyz) in a unit cell of volume V is related to the structure factor **F** by equation 3.2.

$$\rho(xyz) = \frac{1}{V} \sum_{h} \sum_{k} \sum_{l} \mathbf{F}(hkl) \exp[-2\pi i(hx + ky + lz)] \qquad Eq.\ 3.2$$

Since the structure factor $\mathbf{F} = |F| \exp[i\alpha]$, equation 3.2 can be re-written as equation 3.3.

$$\rho(xyz) = \frac{1}{V} \sum_{h} \sum_{k} \sum_{l} |F(hkl)| \exp[-2\pi i(hx + ky + lz) + i\alpha(hkl)] \qquad Eq.\ 3.3$$

$|F(hkl)|$ can be derived directly from I (hkl), the experimentally measured diffraction intensities,[1] but the phase angle α (hkl) can only be obtained indirectly. Four principle methods have been developed for obtaining phase information (Drenth, 1994):

- **Direct Methods**: A method for deriving the relative phases of diffracted beams by considering the relationships between indices and structure factor amplitudes of strong reflections. Since the electron density must be positive or zero, only certain phase values can be valid, hence certain permutations of phase and indices are not allowed. From this starting point it is possible to determine the phase of all reflections in an unknown structure.

- **Molecular Replacement**: A known homologous structure is placed, by way of rotation and translation functions, into the unit cell of a crystal of the unknown structure. If the positioning is optimal, phase information can be used to build an initial model of the unknown structure. This model can then be refined by comparison of the structure factors calculated from the model with experimentally observed structure factors.

- **Multiple Isomorphous Replacement**: More exactly the technique, in the context of its application to protein structure solution, is multiple isomorphous *addition*. An atom of considerably higher atomic number, and hence of significantly stronger scattering power, is added to the crystal lattice to produce

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[1] The measured intensity $I(hkl) = \mathbf{F}(hkl) \bullet \mathbf{F}^*(hkl)$ where $\mathbf{F}^*$ is the complex conjugate of the structure factor $\mathbf{F}$. $I(hkl)$ is proportional to $|F(hkl)|^2$, after allowing for geometric factors, absorption corrections and other minor effects. NB. $\mathbf{F}(hkl)$ is a vector, whereas $|F(hkl)|$ a scalar quantity.

a 'heavy atom derivative'. Provided the 'derivatised' form of the crystal remains crystallographically isomorphous with the native crystal, phase information can be obtained from the differences between native and derivative diffraction patterns.

- **Anomalous Dispersion:** Atoms absorb radiation of specific wavelengths, resulting in the breakdown of Friedel's Law i.e. reflections 180° apart do not have identical intensities: $I(h\,k\,l) \neq I(\bar{h}\,\bar{k}\,\bar{l})$. By collecting data, at wavelengths appropriate to the absorption characteristics of a particular atom present in the crystal lattice, phase information can be obtained.

1.6.2 Protein crystallography

A successful protein structure determination by X-ray crystallography is the culmination of the series of clearly defined experimental stages outlined in figure 1.8. Chronological progression towards the calculation of an interpretable electron density map rarely occurs in a linear fashion. As will be described below crystallogenesis and the multiple isomorphous replacement method for obtaining phase information remain, for the most part, heavily reliant on empirical trial and error methods. As such, these techniques are outwith the direct control of the crystallographer, and often prove to be obstacles which significantly hinder progress. This was found to be the case during the structure determination of LH II from *Rps. acidophila*. To date the application of direct methods techniques to the *ab initio* phasing of proteins has not been successful: the technique requires very high resolution data, and is not readily applicable to molecules containing more than a few hundred atoms (Drenth, 1994). Therefore in the case of LH II, where no homologous structure had been established, the only possible techniques

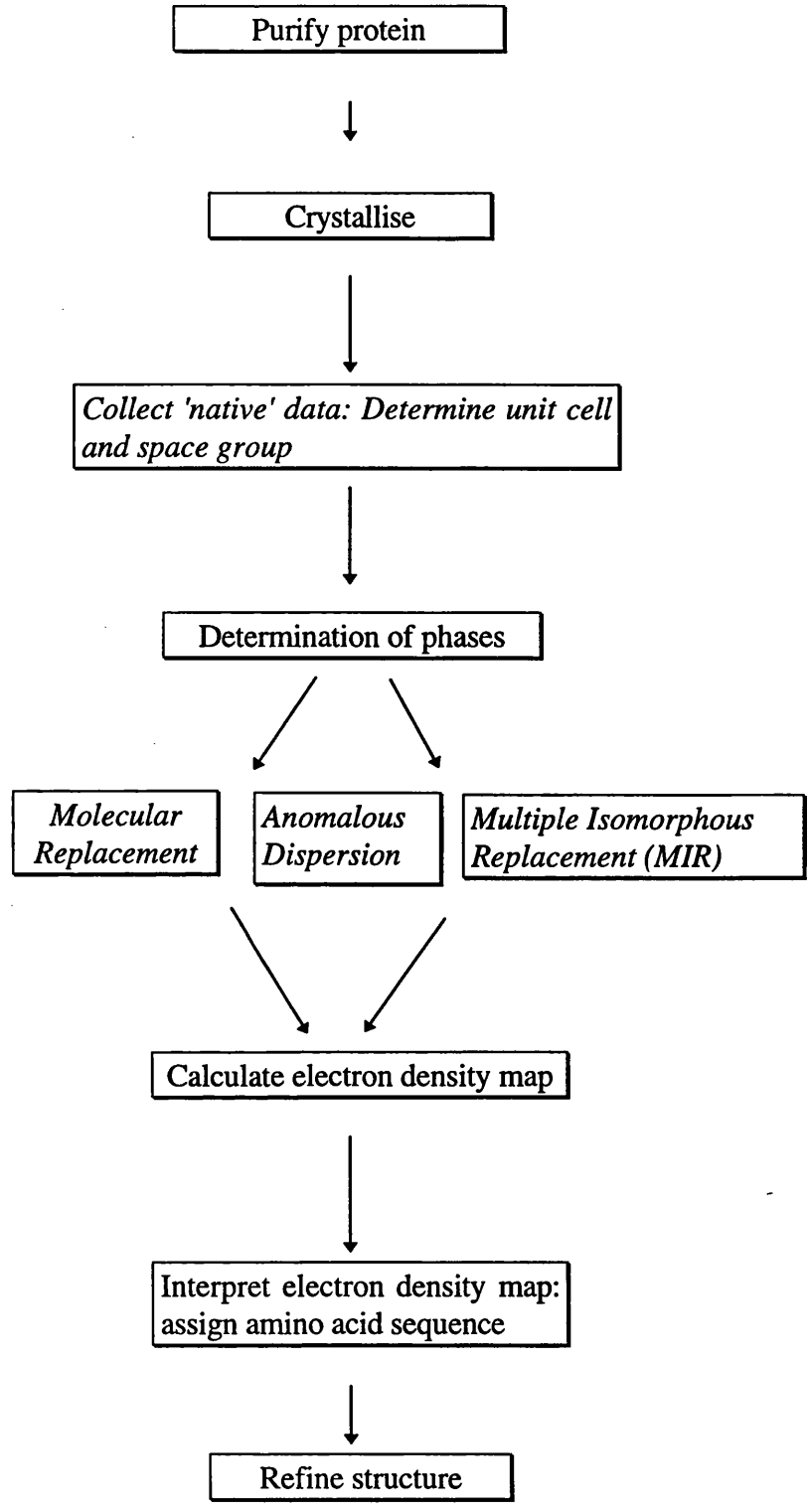

Figure. 1.8. The basic stages in elucidation of protein structure by X-ray crystallography.

available for obtaining phases were the MIR method and/or phasing by anomalous dispersion. The application of these techniques to the structure solution of LH II will be discussed in Chapter 3.

In total, more than ten years elapsed between results of initial crystallisation trials being reported (Cogdell *et al.*, 1985) and publication of the refined structural model of LH II (McDermott *et al.*, 1995). In the first instance it took approximately four years to produce crystals suitable for preliminary X-ray measurements, with a further five years of experimentation being required to fully optimise the observed diffraction characteristics. Overall, heavy atom derivatisation took five years of work. For almost the entire duration of the analysis, the structure solution of LH II was a highly iterative process. Poor quality diffraction displayed by crystals of the complex had the effect that heavy atom derivatisation trials were inconclusive. Therefore, experiments were conducted concurrently to improve the quality of observed diffraction and search for heavy atom derivatives: each time improvements were observed in the diffraction characteristics of the crystals putative 'heavy atom derivatives' were reassessed.

At this point, some consideration should be made of the particular biochemical and biophysical properties of membrane proteins, since they are directly responsible for making membrane proteins 'difficult' candidates for X-ray analysis.

1.7 Biological membranes

General analysis of biological membranes reveals they are composed from phospholipids and protein molecules; more detailed investigation exposes a prodigious diversity of composition, structure and function (Singer, 1971; Small, 1992). To explain much of the available data on biological membranes Singer and Nicholson developed the "Fluid Mosaic Model of Cell Membranes" (Singer & Nicholson, 1972). The model, which is now widely accepted as being an accurate blueprint depiction of cellular membranes, contrives to explain both the thermodynamics of membrane systems and the observed properties of lipids and proteins in functional membranes.[1]

Fundamental to the construction of the Singer and Nicholson model is the assumption that thermodynamic considerations predominate in determining the gross supramolecular structure of the membrane. The authors concede that noncovalent forces, such as hydrogen bonding and electrostatic interactions, do play some role, but these were considered to be of a secondary nature, and of only minor localised structural consequence. Thermodynamic factors orient membrane components in a manner that sequestrates hydrophobic, nonpolar regions away from aqueous environments, and conversely, orient polar hydrophilic components into solvent accessible positions. Consequently, the membrane lipids arrange systematically to form a bilayer,[2] with the polar head groups aligned to form two

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[1] For a recent review and a mathematical description of membrane conformation see Lipowsky, 1991.

[2] The only reported exception to this mode of organisation is the plasma membrane of the thermo-acidiphillic archaebacterium *Thermoplasma*. The membrane of this primitive organism is apparently constructed from a lipid monolayer formed by the lipid $C_{40}H_{82}$ poly-isoprenoid (Langworthy, 1977).

continuous hydrophilic surfaces in contact with the aqueous environment (Singer & Nicholson, 1972). The nonpolar fatty acid groups locate in the central region, between the two layers of polar head groups, in effect creating a nonpolar filling in a hydrophilic sandwich. The membrane lipid bilayer is not a rigid construction, rather it should be thought of as an oriented two dimensional viscous solution (Singer, 1971; Kornberg *et al.*, 1971).

1.7.1 Membrane proteins

Protein molecules affiliated with membranes are classified into in two distinct categories: peripheral and integral membrane proteins (Singer & Nicholson, 1972). Peripheral membrane proteins are weakly associated with the membrane, and are localised in the hydrophilic domain of the bilayer. Since their primary mode of adherence to the membrane is electrostatic they can be dissociated easily by a moderate increase in ionic strength, or by the addition of chelating agents. On release from the membrane, the protein is soluble in neutral aqueous solution and has no accompanying lipid. Conversely, integral membrane proteins are tightly associated with the bilayer and, in general, traverse the membrane at least once. Dissociation from the membrane can only be achieved by adopting relatively aggressive methods such as treatment with membrane disruptive agents (Singer & Nicholson, 1972). The requirement of such treatments to invoke dissociation is the determining factor in classification, not the presence of a transmembrane domain. Accordingly, prostaglandin-H_2-synthase is classified as an integral membrane protein, although the published structure reveals the mode of association with the membrane is via a short helical 'anchor' which only interacts with a single leaflet of the bilayer (Picot *et al.*, 1994).

According to Singer and Nicholson's study, integral membrane proteins account for more than 70% of the total protein associated with a typical membrane (Singer & Nicholson, 1972) and are of the greater functional importance. In general, it is this class of protein, and in particular those with at least one membrane spanning domain, which is implied by generic references to membrane proteins.

1.7.2 Intercalation of globular proteins into membranes

The thermodynamic principles utilised in building the membrane *per se* also govern the intercalation of globular proteins into the membrane. The outer surfaces of the transmembrane domain will be exposed to a non-polar environment of lipid fatty acid groups, and therefore should primarily be constructed from hydrophobic residues. Charged residues can be located in the transmembrane region provided the net thermodynamic gain is favourable. In terms of overall topology, integral membrane proteins generally have a tripartite construction of three spatially distinct macro-domains: two hydrophilic and one hydrophobic.

Once inserted into membrane the fluid nature of the membrane matrix allows translational freedom of movement in the plane of the membrane. However, the amphipathic nature, of both protein and membrane, prevent alterations to either the relative orientation or the degree of intercalation of the protein into the membrane (Singer & Nicholson, 1972).

1.7.3 Solubilisation of integral membrane proteins

On removal from the membrane, the amphipathic[1] nature of membrane proteins renders them insoluble in both polar and non-polar media (Michel & Oesterhelt, 1980). The working definition of solubilisation is "the preparation of a thermodynamically stable isotropic solution of a substance normally insoluble in a given solvent by the introduction of an additional amphiphilic component or components" (Lichtenberg *et al*, 1983). In the context of solubilising membrane proteins into aqueous media, suitable amphipathic molecules are detergents[2]. When added to an aqueous solution of biological membranes, detergents initiate solubilisation by partitioning into the membrane, causing the membrane to fragment. The degree of fragmentation, and the resultant species of membrane fragment formed, is a function of the detergent to protein ratio - outlined in Figure 1.9.

At relatively high detergent concentrations - several times the concentration at which detergent monomers spontaneously aggregate to form micelles[3] - complete membrane solubilisation is effected, resulting in detergent-protein, detergent-lipid and pure detergent micelles (Reynolds, 1982; Zulauf, 1990; Hjelmeland, 1990). When fully solubilised the hydrophobic domain of the protein is totally encapsulated within a detergent micelle, thus the protein molecule presents an entirely hydrophilic molecular surface to the bulk solvent (Michel,

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[1] Describes the hydrophobic and hydrophilic character (Greek: *amphi* = on both sides, of both kinds, dual; *pathos* = feeling)

[2] For a review of detergents and their use in this context see Zulauf, 1990.

[3] The critical micelle concentration (CMC).

1980; Zulauf, 1990; Hjelmeland, 1990). The generally accepted criterion indicative of complete solubilisation is retention of the protein in the supernatant after ultra-centrifugation at 105,000 *g* for 1 hour (Hjelmeland & Chrambach, 1984).

Once solubilisation has been effected, membrane proteins can generally be purified by conventional means, provided the protein environment is maintained at a detergent concentration sufficient to render the protein soluble (Findlay & Evans, 1987). Frequently, it has been found that the detergent which effectively dissociates the protein from the membrane is either unsuitable for maintaining protein solubility in the long term, or interferes with purification procedures (Michel, 1990; Hjelmeland, 1990). Consequently, it has become common practice to exchange the initial solubilisation detergent for another with more suitable physical characteristics on one or more occasions during purification (Michel, 1990; Hjelmeland, 1990; Cogdell & Hawthornthwaite,1993). As will be described in the next Chapter, detergent exchange is especially commonplace in the final purification stages when the protein is to be used in crystallisation trials.

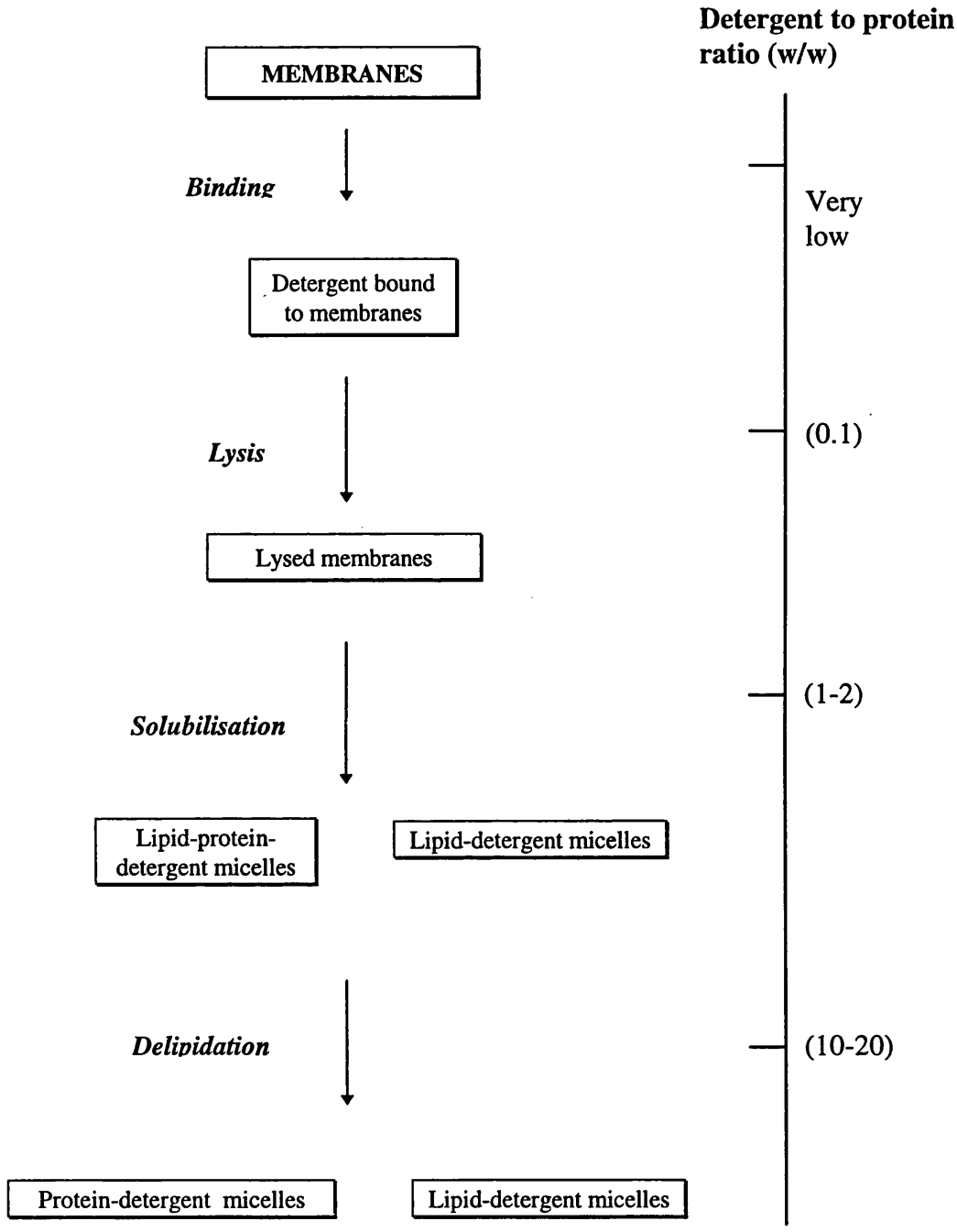

Figure 1.9. The effect of the detergent to protein ratio on membrane fragmentation during solubilisation (Hjelmeland, 1990)

Chapter 2

Purification and Crystallisation

2.1 Introduction

Protein crystals produced for X-ray analysis must adhere to certain criteria of size and internal order. Using a laboratory rotating anode X-ray source, crystals of at least 0.3 mm in all three dimensions are required if a full X-ray analysis is to be performed (Blundell & Johnson, 1972). A similar analysis using a synchrotron X-ray source - in particular one of the latest third generation instruments - can be carried out using crystals of half this size, or even smaller. It should be noted that these dimensions are only a rough indication of the crystal size required. The intensity of the observed diffraction pattern is approximately proportional to the volume of the crystal, and inversely proportional to the unit cell volume (Blundell & Johnson, 1972), therefore in general the larger the weight of the protein the larger the crystal which is required.

The resolution limit of the observed diffraction pattern is heavily dependent on the degree of order inherent in the crystal lattice. In this context, resolution is understood to mean the minimum interplanar spacing in the crystal lattice which gives rise to observable diffraction (Drenth, 1994).[1] For protein crystals, the current rule of thumb definition of 'high resolution' is diffraction data measurable to an interplanar spacing of 2.0Å, or better.[2] Although, it should be noted that the

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[1] The minimum interplanar spacing d_{min} is given by the equation:

$$d_{min} = \lambda/2\sin\theta_{max}$$

where λ is the wavelength of the incident X-ray beam and θ the Bragg angle.

[2] The inverse relationship between real space and the reciprocal space of the crystal lattice has the effect that, in terms of resolution, the lower the numerical value the greater the structural detail which can be delineated.

observed limit of diffraction is not automatically the resolution to which detail will be observed in a calculated electron density map. This is dependent on many additional factors, such as the degree of isomorphism between 'heavy atom derivative' and 'native' crystals, the precision of data measurements, the reliability and quality of calculated phase information and the presence of non-crystallographic symmetry (Drenth, 1994).

Historically, protein crystal growth (or crystallogenesis) has always been regarded as *'more of an art than a science'* (Garavito & Picot, 1990). A statement borne out of the fact that *a priori* it is not possible to determine the experimental parameters which induce, and subsequently sustain crystal growth (Garavito & Picot, 1990). Although work has been done towards the production of some rational theory, crystallisation of proteins remains essentially an empirical trial and error process (D'Arcy, 1994; McPherson *et al.*, 1995).

Details of the procedures and techniques described in this Chapter are given in Appendix C.

2.2 Protein crystallogenesis

Protein crystallogenesis proceeds on similar principles to those classically employed in the production of 'small molecule' crystals. By raising a protein solution to a supersaturated state, provided the equilibrium conditions are favourable, stable nuclei form which can support the inclusion of further molecules onto their surfaces, and hence form crystals (McPherson, 1990).

In a classical treatise on crystallisation the region of supersaturation is divided into two distinct regions: the metastable and the labile (Petrov *et al.*, 1969). On movement from an unsaturated state to a supersaturated one the molecule first enters the metastable region. It is commonly agreed that in the metastable region crystal growth can occur, but not the spontaneous formation of stable nuclei i.e. aggregates of sufficient size and physical coherence that support the inclusion of protein molecules onto growing surfaces faster than they are lost to solution (McPherson, 1990). To initiate nucleation the solution must be moved to a higher level of supersaturation, the labile region. In this region the formation of stable nuclei and crystal growth can occur. Despite the latter, for optimal crystal growth there should be minimal transgression into the labile region, otherwise the production of multiple nucleation sites would lead to showers of small, unusable crystals (McPherson, 1990). Therefore the optimal crystallisation experiment is one that moves the system from the labile region - after only a few nuclei have formed - to the metastable region.

In practice, the methodology used for the crystallisation of proteins differs from those used in traditional crystallisation, to take account of the relatively fragile nature of most proteins. A thorough description of the principles and practices of protein crystal growth can be found in many texts (Blundell & Johnston, 1976; McPherson, 1982; McPherson, 1985; Carter, 1990; McPherson, 1990, Ducruix & Giege, 1992) and will only be briefly outlined here.

In general four factors influence the solubility of a protein in solution: the ionic strength of co-solutes, temperature, the organic content of the solution and the pH (McPherson,1982). All of which can, and have been, successfully used to invoke protein supersaturation, and hence to induce crystal growth. The method

which is optimal for a particular protein crystallisation problem is very much a matter of trial and error. In practice, the method which has been most successfully and widely used is the judicious alteration of the ionic strength of a co-solute (McPherson, 1995). This technique relies on the long recognised fundamental property of almost all proteins to 'salt in' or 'salt out' i.e. to become more or less soluble as a function of salt concentration (McPherson, 1982). In the case of 'salting out', the protein is exposed to an increasing concentration of a co-solute molecule, known in the context of protein crystallogenesis as the 'precipitant'. Typically this is a small inorganic salt such as ammonium sulphate, or a relatively large organic molecule like polyethylene glycol. Although these are by no means unique in this regard, a very wide range of compounds have proven useful in protein crystallisation experiments (Ducruix & Giege, 1992). Competition between the protein and precipitant for the water of solvation eventually renders the protein insoluble (McPherson, 1982). Intuitively, formation of an amorphous precipitate would seem to be the lowest energy state of an equilibrium system such as this, and therefore the rational outcome of a 'salting out' experiment, but this is a false potential energy minima. The most energetically favorable outcome occurs when the optimal bonding pattern between molecules is repeated periodically in three dimensions, in other words by the formation of a crystal lattice (McPherson, 1990).

The converse technique of 'salting in' relies on the fact that the presence of a small amounts of ions in a protein solution has the effect of increasing the potential for favourable interactions with solvent water molecules, thus making the protein more soluble. Conversely, removal of these ions cause small changes in the

ionisation of amino acid side chains, resulting in protein aggregation and precipitation from solution (Blundell & Johnston, 1972).

The ability of a particular inorganic salt to induce salting out, or to salt in a protein, is a function of its ionic strength.[1] In general, the higher the ionic strength, the greater the efficacy of a particular salt for dehydrating solvated protein molecules (McPherson, 1990). The relative effect of ionic salts on proteins can be gauged by their position in the Hoffmeister series (McPherson, 1982).

Numerous different practical techniques have been developed for the controlled modification of the ionic strength of a protein in solution, most of these can be found Ducruix & Giege, 1992. The most widely used are methods which rely on 'vapour diffusion'. Details of the theory and practice of the technique are given in McPherson, 1972 and Ducruix & Giege, 1992 and in any textbook on protein crystallography (Blundell & Johnson, 1972; Drenth, 1994). In principle, the vapour diffusion technique depends on the equilibration of the vapour pressures of two spatial disparate solutions of different ionic strengths. A 'drop' containing protein and precipitant is allowed to equilibrate, by vapour diffusion, against a 'reservoir' solution at either higher or lower ionic strength, usually the former, to increase the ionic strength of the drop and thus instigate salting out of the protein.

The vapor diffusion technique is a very adaptable. All of the important physical parameters of a crystallisation protocol - the speed of equilibration, the initial and final precipitant concentrations, pH, protein concentration and

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[1] Ionic Strength = $\frac{1}{2}\sum [a]Z_a^2$: where [a] is concentration of ion a, and Z its charge.

temperature - can be tightly controlled and manipulated. This is essential in any protein crystallisation technique, since these parameters have a direct, and great bearing on the 'quality' of the crystals which result (McPherson, 1990).

Prior to conducting crystallisation experiments the physical characteristics of the subject protein should be considered (Ducruix & Giege, 1992). Optimistically, such analysis may illuminate a possible route to successful crystallisation, e.g. if it is known that a particular ion causes aggregation prudent use of this knowledge can be used in determining initial crystallisation conditions. More realistically, such information is principally useful in avoiding wasteful crystallisation protocols which induce denaturation. In the case of membrane proteins, their particular biochemical properties make such deliberations especially apposite (Michel, 1990).

2.2.1 Crystallisation of Membrane Proteins

For many years integral membrane proteins were thought to be unsuitable for crystallisation experiments. It was presumed that the necessary presence of highly disordered detergent molecules would preclude ordered crystal formation. This reasoning was unassailably challenged with the reported crystallisation of two integral membrane proteins: matrix porin from *E. coli* (Garavito & Rosenbusch, 1980) and Bacteriorhodopsin (Michel & Oesterhelt, 1980). In both cases the protein was present, at the outset of the crystallisation experiment, as a monodisperse solution of the protein solubilised into protein-detergent mixed

micelles of the non-ionic detergent β–Octyl-glucopyranoside.[1] In the years since, there have been several reports of the crytallisation of membrane proteins where the observed diffraction limit extends to 3Å, or better (Michel, 1982; Garavito *et al.*, 1983; Allen & Feher, 1984; Chang *et al.*, 1985; Michel, 1990; Kreusch *et al.*, 1991; Picot & Garavito, 1990; Buchanan *et al.*, 1993). All of these reports have been precursors to the publication of high resolution structures, although it should be stressed that these are only representative of two families of membrane protein (see section 1.1). In all of the reported crystallisation protocols, the protein was present as a protein-detergent mixed micelle and the crystallisation experiment carried out in a manner that mirrored that commonly used for soluble globular proteins.

To rationalise the processes involved, Michel proposed two possible models of how membrane proteins might crystallise (Michel, 1983):

- **Type I**: Essentially stacks of two dimensional crystals ordered in the third dimension. Hydrophobic interactions in the domains formerly located in the membrane, and polar interactions in the hydrophilic domains hold the crystal lattice together. This type of crystal is unlikely to result in good quality X-ray diffraction due to the non specific nature of the hydrophobic interactions.

- **Type II**: Lattice contacts are only made by residues in the hydrophilic domains. The hydrophobic domain is totally surrounded by a detergent micelle and plays no part in lattice formation.

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[1] Commonly known as β-Octyl-glucoside or β-OG.

In all of the reported membrane protein structures, the crystal lattice construction has been of the type II variety (Michel, 1990).

2.2.2 Detergents in membrane protein crystallisation

To avoid confusion, it should be stressed that the role of the detergent is not the paradoxical one of maintaining protein solubility during crystallisation. Rather, the function of the detergent should be thought of as essentially the conversion of hydrophobic domains to hydrophilic. Although the detergent micelle does not seem to actively take part in lattice formation *per se*, the choice of detergent can often have great influence on the outcome of crystallisation trials (Michel, 1990). As with most other crystallisation parameters, it is not possible to determine *a priori* which particular detergent will be optimal for the purpose. Therefore it is common to conduct parallel crystallisation trials on protein solubilised into several different species of detergent micelle.

Three techniques are commonly used to effect detergent exchange prior to crystallisation: chromatography; dialysis or precipitation of the protein followed by resuspension with a buffered solution of the replacement detergent (Hjelmeland, 1990b). The particular method used is dependent on the physical properties of both the protein and the two detergents which are to be exchanged, and will generally require some trial and error to determine which method is optimal (Hjelmeland, 1990b).

- **Chromatographic methods:** Two chromatographic techniques are applicable to exchanging detergents. If the protein is bound to a suitable adsorbent material, for example ion exchange matrix, and 'washed' with at

least 20 column volumes of buffer containing the new detergent, complete replacement of detergent takes place. Alternatively, if a gel permeation column is equilibrated with the replacement detergent, differences in the Stokes radius of pure detergent micelles and detergent protein micelles allows detergent exchange whilst a protein - loaded onto the column in the initial detergent - is being eluted.

- **Dialysis:** There are two limiting factors in the use of dialysis for detergent exchange: the stability of the protein during dialysis, and the critical micelle concentration and micelle molecular weight (mMW) of the two detergents involved. The first factor is, of course, specific to individual proteins and as such is difficult to comment on in general terms. The probability that two detergents will exchange by dialysis is heavily dependent on the mMW. In some instances the micelle weight is considerable with respect to the protein - for example, Triton X 100 has a mMW of 90 kDa - and may not pass through the dialysis membrane. In addition, detergents with low critcal micelle concentrations[1] tend to be more tightly bound to the protein than detergents with high CMC values, and consequently are difficult to remove by dialysis. Dialysis techniques where the driving force is osmosis, gas pressure or centrifugal force are all applicable to detergent exchange.

- **Precipitation:** Non-ionic detergents such as β–Octyl-glucopyranoside can be exchanged by precipitating the protein with polyethylene glycol (PEG) and then resuspending the pellet in buffered solution of the replacement detergent.

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[1] Critical micelle concentration (CMC) the concentration above which detergent monomers spontaneously form micelles (Zulauf, 1990)

The technique takes advantage of the fact that non-ionic detergents are often more soluble in PEG than membrane proteins, and therefore remain in the supernatant after precipitation.

2.2.3 The role of small amphiphilic additives

Whilst outlining possible models of membrane protein crystals Michel also proposed the 'small amphiphile concept' (Michel, 1983). He asserted that, in addition to detergent, small amphiphilic molecules[1] such as heptane-1-2-3-triol were essential for the crystallisation of membrane proteins. Michel suggested small amphiphiles performed several vital functions: the prevention of detergent phase separation, reducing the size of the detergent micelle and other unknown, but specific interactions with the protein molecule which enhanced lattice formation.

Some of the intrinsic precepts of Michel's arguments have now been given experimentally derived backing. Addition of 3% of the small amphiphile heptane-1-2-3-triol to a solution of the detergent β–Octyl-glucopyranoside was shown to reduce the micellular weight by 50% (Zulauf, 1990). Neutron diffraction studies on protein-detergent micelles have shown that the micellular radius is increased in the presence of small amphiphiles (Timmins *et al.*, 1991). The latter observation is particularly important. Although the detergent micelle should totally span the hydrophobic domain, it should protrude as little as possible outwards from the protein molecule. Otherwise the torus of detergent could hinder the close approach of protein molecules, and hence prevent inter-molecular protein-protein contacts (Michel, 1983). Therefore the observation that small amphiphiles increase

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[1] Differentiated from detergents by their inability to form micelles.

the micellular radius - thus decreasing the protrusion of the detergent outwards from the protein molecule - gives some credence to the use of small amphiphiles as additives.

To date, the reaction center from *Rhodopseudomonas viridis* (Michel, 1983) has been the only reported case where crystals could not be grown in the absence of small amphiphillic additives, although in many cases crystal quality has been greatly improved by their presence in the crystallisation protocol (Michel, 1990).

2.3 LH II crystallisation: Background and previous work

At the outset of the work contained in this thesis, a purification and crystallisation protocol for LH II from *Rps acidophila* strain 10050 had been established (Papiz *et al.* 1989). In the next Chapter the results of the X-ray analysis of crystals will be presented. In brief, it should be pointed out that in the initial stages of the analysis the quality of diffraction observed from crystals was very poor. Much of the work carried out during the structure determination was done with the purpose of increasing both the reproducibility and the resolution limit of the observed diffraction. As previously stated (see section 1.6.2), the time taken to finally elucidate the structure of LH II was still substantially longer than that normally taken to carry out a crystallographic analysis of a globular protein. The fact that LH II is an integral membrane protein undoubtedly contributed substantially to the length of time taken to perform the analysis.

In terms of crystallisation, detergent solubilised membrane proteins can be treated in a similar manner to globular proteins. However, the required presence

of two additional components - detergent and 'small amphiphile' - in the multiparametric crystallisation protocol lead to two main difficulties. Firstly, there is a large increase in the number of interdependent parameters which have to be considered and permuted when developing a crystallisation protocol (Michel,1990). Secondly, very few X-ray crystallographic membrane protein structures have been established (see section 1.1). Hence, at the time the LH II structural analysis was being carried out there was little precedent upon which to base a strategy for optimising the crystallisation protocol in the case of a membrane protein. An approach to solving the problem of why crystals exhibited such mediocre diffraction characteristics had to be developed virtually *a priori.* This was done, in the first instance, by considering and rationalising the possible causes of poor diffraction.

2.3.1 Some initial considerations on LH II crystallisation

Based on the assumption that empirical trial and error methods are inherent in the process of protein crystallisation it is logical to argue that the greater the number of experiments and trials which can be carried out, the greater the probability of achieving success. Furthermore, when crystallisation trials are conducted the goal is generally one of producing crystals with minimum dimensions of 0.3 mm. Typically, such crystals weigh approximately 15μg (Drenth,1994). Hence, only about 65 crystallisation trials can be conducted per mg of protein (Drenth,1994). These factors lead to the conclusion that the availability of protein - in terms of both quantity and regularity of supply - is a potentially significant factor in determining the overall viability of performing an X-ray crystallographic study on a protein, and therefore a first consideration.

LH II from *Rps. acidophila* strain 10050 is synthesised at high levels in the intercytoplasmic membranes. Hence, it is possible to obtain substantial amounts of the complex, in a semi-pure form, from a single cycle of cell growth[1] (Cogdell & Hawthornthwaite, 1993).

Of course, protein availability in terms of quantity alone is a basic consideration and requires further qualification. Despite some notable examples in the literature, such as the crystallisation of lysozyme directly from egg whites by merely adding seed crystals (Alderton & Feyvold, 1946), it has been generally found to be the case that crystallisation experiments are best conducted on highly purified, monodisperse preparations of protein (Giegé & Ducruix, 1992). In this context, 'highly purified' is considered to be a protein preparation which is not only free of contaminants, but is also free from structural heterogeneities, such those caused by the presence of active proteases or microheterogeneities in the form of naturally occurring isoforms. In effect this means a preparation of protein where the criteria of purity is much more rigorous than that required in any other branch of the biological sciences (Giegé & Ducruix, 1992).

The ready availability of LH II allowed extensive experiments to be conducted on the purification and crystallisation protocols to determine the optimum conditions. It would be of little value - and add enormously to the physical size of this thesis - to simply list all of these trials and experiments, and their subsequent outcomes. For the sake of clarity and brevity, the work done will

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[1] Typically LH II accounts for as much as 80% of protein content of the photosynthetic membrane (Cogdell & Hawthornthwaite, 1993). In practice, this means a 10 litre batch of *Rps. acidophila* strain 10050, yields approximately 50 mg of crude LH II.

be described in terms of the initial aims and objectives, and the philosophy of approach adopted to achieve them. To describe the work done in this manner can be considered legitimate in view of the successful elucidation of LH II to high resolution.

2.4 Rationalising poor diffraction from LH II crystals

Despite their poor diffraction properties, when viewed under the microscope crystals prepared by the protocol of Papiz and co-workers (Papiz *et al.*, 1989) gave indications of being highly ordered: the crystals routinely grew with dimensions of between 0.3 mm and 0.8 mm, there were no signs of splitting, cracking or twinning and all faces and edges appeared regular and smooth. This observation led to initial thoughts on possible causes of poor diffraction being centered around the purity of LH II used in crystallisation trails and not the crystallisation protocol itself.

Immediately prior to conducting crystallisation experiments, protein prepared as in (Papiz *et al.*, 1989) was seen as a single band when examined by non-denaturing polyacrylamide gel electrophoresis (PAGE) and as two bands by SDS-PAGE (Cogdell & Hawthornthwaite, 1993). Hence, contaminants in the protein preparation which may have been adversely affecting the diffraction characteristics of the crystals, were neither the reaction center polypeptides, nor unidentified globular cellular proteins. The possibility of contamination by other species of LH II, or the presence of more than one variant of each apoprotein was

considered. These would probably not be resolvable or identifiable by SDS PAGE gel electrophoresis.[1]

In some species and strains of purple bacteria the LH II β apoprotein component is not unique (Zuber & Brunisholz, 1993). For example, in the case of B800-820 LH II from *Rps. acidophila* strain 7750, at least two distinct β apoproteins have been identified and sequenced (β' and β"). These differ significantly in their N termini primary structure: the β" polypeptide is two residues longer, and comparison of sequences reveals few conserved residue in this region[2] (Zuber & Brunisholz, 1993). This has the effect that two species of αβ hetrodimer - αβ' and αβ" – are possible. Unless the assembled LH II oligomer is comprised of a fixed stoichiometric quantity of β' and β" containing hetrodimers, these complexes will have a great variance in apoprotein composition, and be unlikely to form highly ordered crystals.

Only single α and β LH II polypeptides have been isolated from *Rps. acidophila* strain 10050 (Zuber & Brunisholz, 1993). Furthermore, irrespective of

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[1] Isoelectric focusing proved ineffectual, the complex simply precipitated in a very broad band at approximately pH 6 (Dr. A.M. Hawthornthwaite-Lawless, personal communication).

[2] N terminal sequence of *Rps. acidophila* strain 7750 LH II B800-820. The polypeptides are aligned according to conserved residues, in particular the conserved B850 Bchl *a* binding histidines (not shown)(Zuber & Brunisholz, 1993). The symbol ▒ represents the start of the postulated transmembrane domain.

β' A V L S P E Q S E E L H ▒
β" A D K P L T A D Q A E E L H ▒

NB. Terminology has been altered from that used in the original paper i.e. β_2 to β" etc. to avoid misinterpretation of the subscript as being indicative of the presence of multiple copies of the apoprotein. See Appendix B for amino acid codes.

the environmental conditions encountered during cell growth, this strain of bacteria have not been observed to synthesize B800-820 type LH II complexes (Gardiner *et al.*,1993) thus also eliminating the possibility that B800-820 LH II apoproteins were present as contaminants.[1] The absence of B800-820 apoproteins was verified by electrospray mass spectroscopy - only two apoproteins were observed (Dr. N. Guthrie, personal communication).

Based on the assumption that poor diffraction was not attributable to the presence of protein contaminants, or microheterogeneitiy due to the presence of several variants of the apoproteins or other species of LH II, the following parameters were postulated as the putative causes of poor diffraction:

- Some unidentified element, or component of the crystallisation protocol hindered formation of ordered lattice contacts. This could either be a parameter of the crystallisation protocol *per se* such as the detergent system used, or an artifact carried through from the purification protocol.

- The complex underwent denaturation during crystallisation. Either denatured, or partially denatured complex was integrated into the lattice in the first instance, or alternatively the complex became denatured *in situ* in the crystal.

- Mechanical or thermal damage to the crystal lattice occurred whilst mounting crystals for data collection.

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[1] The potential presence of B800-820 polypeptides as impurities in B800-850 LH II preparations from species such as *Rps acidophila* strain 7750 makes this stain a poor source of LH II for future structural work. Both species of LH II are of a similar biochemical composition, and display similar characteristics during purification and crystallisation (Guthrie *et al.*, 1992). Consequently, B800-820 LH II would constitute a contaminant which is extremely difficult to purify out of a B800-850 preparation.

It has been suggested that rapidly occurring radiation damage - during collection of the first data image - is, in some instances, the root cause of apparent lack of diffraction from crystals (Dr. Z. Dauter, personal communication). In the case of LH II this was ruled out in the early stages of the analysis. Using a laboratory rotating anode source, it was generally possible to collect a complete data set from a single crystal on the occasions that good diffraction was observed.

2.4.1 The approach adopted

To comprehensively address the above postulates required experimental work to be carried out in several distinct areas. Experiments were carried with the following objectives:

- To determine spectroscopically the stability of the complex in solutions similar to those encountered during crystallisation, to ensure that the integrity of the complex was maintained throughout the period of crystal growth.

- By retaining the same crystallisation protocol, and modifying the purification protocol in a stepwise fashion, factors in the purification protocol which had a direct bearing on the diffraction characteristics of the crystals could be identified.

- Determination of the effect that individual crystallisation parameters, such as the detergent system used, had on the quality of the observed diffraction.

- The effect of alternative crystal mounting methods.

In related work, a further possible source of heterogeneity - the lipid content of different LH II preparations - was addressed in a series of experiments carried out by Ms. G. Dahler and Dr. P.J. Dominy of Glasgow University. They

determined the lipid composition of the complex in membrane fragments, during purification and immediately before crystallisation. Analyses were also carried out to compare the lipid content of crystals which diffracted to high resolution with that of crystals which displayed poor, or no diffraction. These experiments were carried out to ascertain if the degree of delipidation, or the presence of a particular lipid species, was an important factor in determining the level of diffraction observed. Dahler and Dominy concluded that the composition of lipid, in terms of relative ratios of the different constituent lipids, was constant - as far as they could determine with confidence - in all samples analysed (Ms. G. Dahler, personal communication).

2.5 Stability of LH II

The stability of the complex was determined primarily by spectroscopic methods. As discussed in section 1.5.4, the presence of fixed stoichiometric amounts of bound pigments results in characteristic spectral features that are assumed to be reliable indicators of structural integrity (Cogdell & Hawthornthwaite, 1993). In addition the absorbance (or optical density, OD) at 850 nm was used throughout to quantify the concentration of the complex, rather than an absolute measurement of the protein concentration. Experiments were conducted to ascertain the effect on LH II stability of the following parameters;

- Components and parameters of the crystallisation protocol.
- Time.

2.5.1 LH II stability: Temperature

A fundamental consideration when crystallising a membrane protein is to establish the relative stability of the protein in different detergent systems. This is perhaps somewhat obvious, but often ignored. If a particular detergent system only maintains protein integrity for a few hours at 4° C, there would seem to be little point in setting up crystallisation trails which take several weeks, especially if they are to be conducted at 15° C !

Differential calorimetry experiments carried out on LH II from *Rps. acidophila* solubilised into the detergent systems used for crystallisation (Ms. G. Dahler & Dr. P.J. Dominy, personal communication of unpublished work) showed that the first thermal transition of LH II occurred at around 56° C. It became apparent throughout the structural analysis that thermal degradation did not occur in the detergent systems used for crystallisation trials. The complex was stable at room temperatures for extended periods[1]. Therefore, the temperature chosen for crystallisation trials was done on the basis of modifying the rate of vapour diffusion, rather than on considerations of the stability of the complex.

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[1] As determined by spectroscopic measurements on samples which had been negligently left on the bench overnight. And more scientifically during later work attempting to apply the thermal methods developed by Sheer's group for pigment exchange (see section 2.8)

2.5.2 *LH II Stability: Other crystallisation components*

Stability trials were set up where the protein - prepared by the original protocol used in Papiz *et al.*, 1989 - was present in solutions similar to those encountered during crystallisation[1] namely:

- A protein concentration which gave an OD_{850nm} of 80
- 1% β-Octyl-glucoside
- 2.5% Benzamidine hydrochloride
- 350 mM NaCl
- 20 mM Tris
- Solution pH adjusted to 9.0 with solid ammonium hydroxide

Aliquots of this solution were made 0.5, 1.0 and 1.5 M di-potassium hydrogen orthophosphate (K_2HPO_4). The absorbance spectra from 400 to 900 nm was recorded for each of these solutions. The solutions were then stored at 12° C for 14 weeks, and the spectra again recorded. No discernible difference in the spectra was observed, shown in figure 2.1, either between those of solutions at different concentrations of K_2HPO_4, or as a function of time. The conclusion was therefore drawn that the complex was stable over long periods of time in conditions close to those which induced crystallisation. Hence, stability of the complex during crystallisation was eliminated as a possible cause of poor diffraction.

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[1] See section 2.6.2 and Appendix C for the protocol used to produce crystals for X-ray analysis.

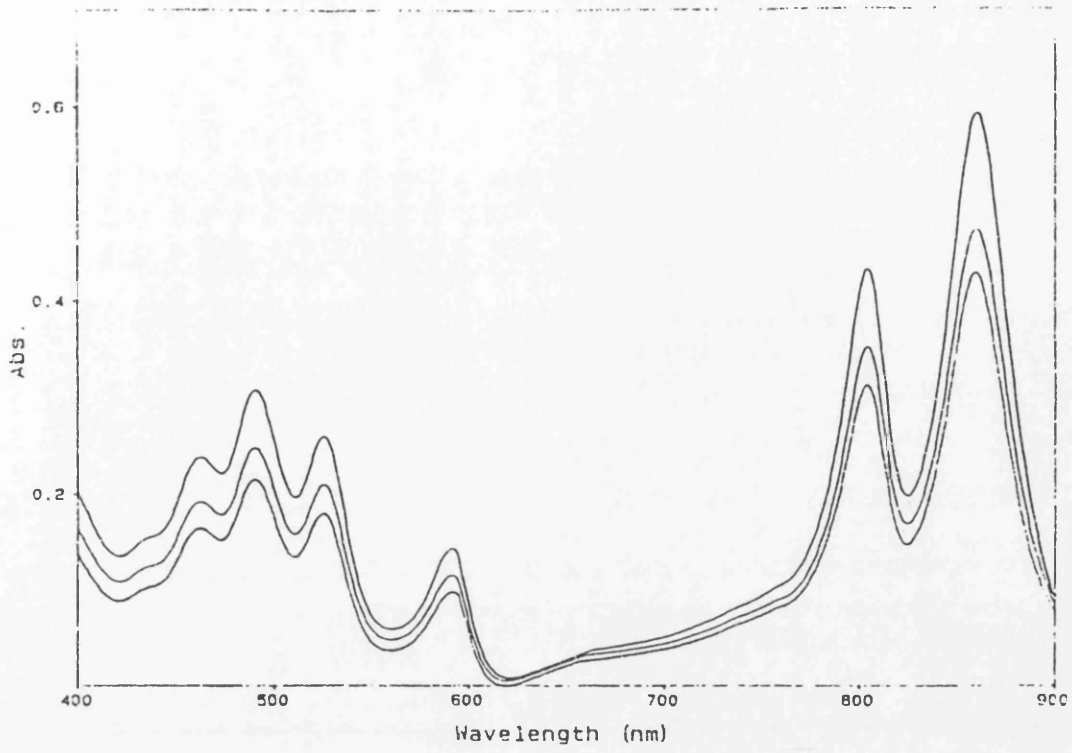

Figure 2.1. Absorption spectra from LH II in solutions outlined in section 2.5.2 after storage for 14 weeks. Concentration of K_2HPO_4: Top trace 0.5 M; middle 1.0 M and bottom 1.5M

To test this hypothesis further, stability trials were set up where the concentration of each of the crystallisation components was increased to a level much higher than that encountered during crystallisation. This was done initially for individual components, and then for a combination of components as a precaution against the possibility that there may be an effect which arose from interdependence of two components. To prevent precipitation of LH II the

protein was present in more dilute solution than would be used for crystallisation trials.[1] Protein was used at a concentration which gave OD_{850} of 1.0: this equates to 10 µl of protein at 'crystallisation concentration' diluted to 1ml. Consequently, 30 µl of protein with OD_{850} of 100 in a solution of 1% Octyl-glucoside, 350mM NaCl, 20 mM Tris pH 8.0 was transferred to 3 mls of the solutions given in Table 2.1. A representative absorption spectra is shown in figure 2.2.

Solution	Effect on spectra (200 - 900 nm)
3M Kpi, pH 8.97	No effect - after 16 hours
4M Kpi, pH 8.97	No effect - after 18 hours
3M Kpi, pH 8.97, 2.5 % Benzamidine HCl	No effect - after 16 hours
3M Kpi, pH 8.97, 2.5% Benzamidine HCl, 300 mM NaCl	No effect - after 18 hours

Table 2.1. Effect on LH II absorption spectra of components present during crystallisation. [Kpi is K_2HPO_4]

[1] This was seen as a valid experiment. Based on the experience of others (Dr. W.Welte & Dr. T. Wacker, personal communications) it has been observed that reducing the concentration of a membrane protein in solution tends to have a detrimental effect, rather than a positive, on the integrity of the protein. It should be noted that in these experiments the relative ratio of protein to detergent is also considerably higher than that encountered during purification or crystallisation, again a factor likely to be detrimental to protein integrity.

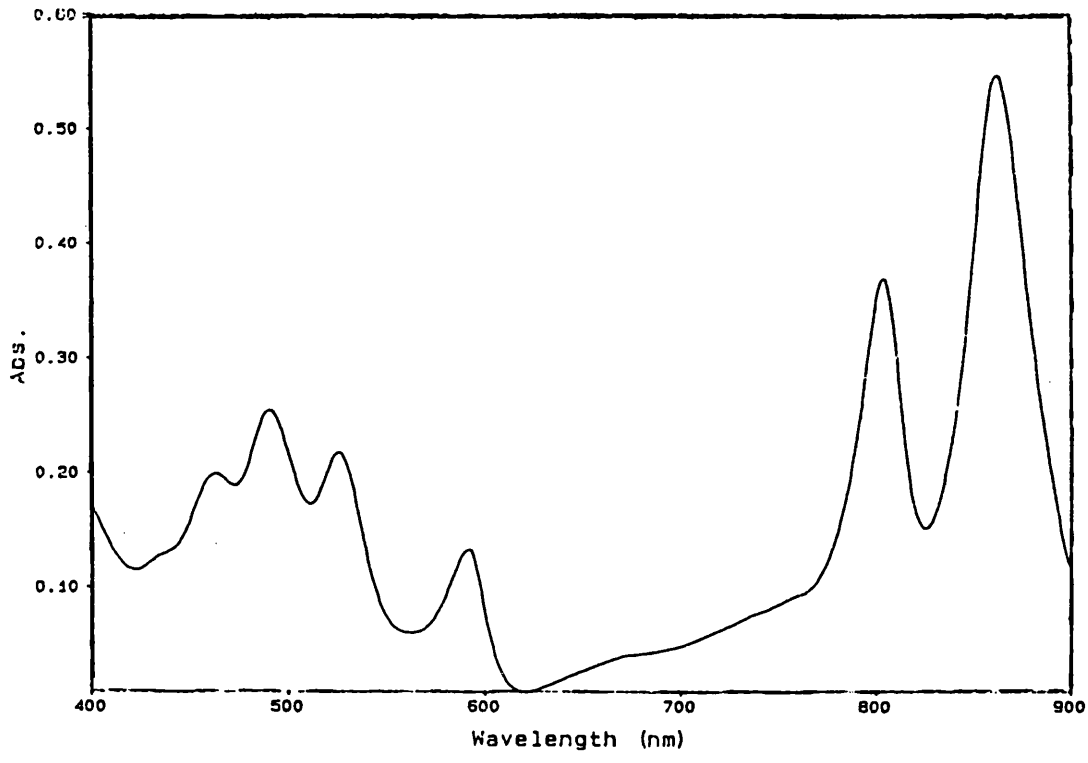

Figure 2.2. Absorption spectra of LH II in 3M K_2HPO_4, pH 8.97, 2.5 % Benzamidine HCl, pH 8.97.

2.6 Crystallisation of LH II

For the structural studies which were carried out on LH II - X-ray crystallography and microspectroscopy analysis - crystals fulfilling two distinct criteria of size and morphology were required. Full details of the procedures used are given in Appendix C.

2.6.1 Crystallisation for X-ray analysis

In the original crystallisation protocol developed by Papiz and others (Papiz *et al.*, 1989), two morphologically distinct forms of LH II crystal were produced. Of these the better diffraction was observed when the protein was exchanged into the detergent β-Octyl-glucoside prior to conducting crystallisation experiments. The protocol used was as follows: Protein in 20 mM Tris, pH 8.0, 250 -500 mM NaCl, 1% β-Octyl-glucoside was at a concentration which an OD_{850} of between 150 and 200. The solution was made 2.5 % benzamidine hydrochloride (w/v) and 0.9 M di-potassium orthophosphate (using a stock solution of 4M K_2HPO_4).

The pH of the resultant solution was adjusted to between 9.5 and 10.5 using small quantities of solid NaOH. Undissolved material was removed by centrifugation at 13,000 RPM for 10 minutes, in a small eppendorf centrifuge.

'Sitting drop' crystallisation trials were set up in home built containers (pictured in Cogdell & Hawthornthwaite, 1993). A drop of between 35 and 50 µl was equilibrated against a reservoir of 8ml of ammonium sulphate solution, adjusted to the same pH as the crystallisation drop with solid NaOH.

A typical LH II crystal is shown in figure 2.3.

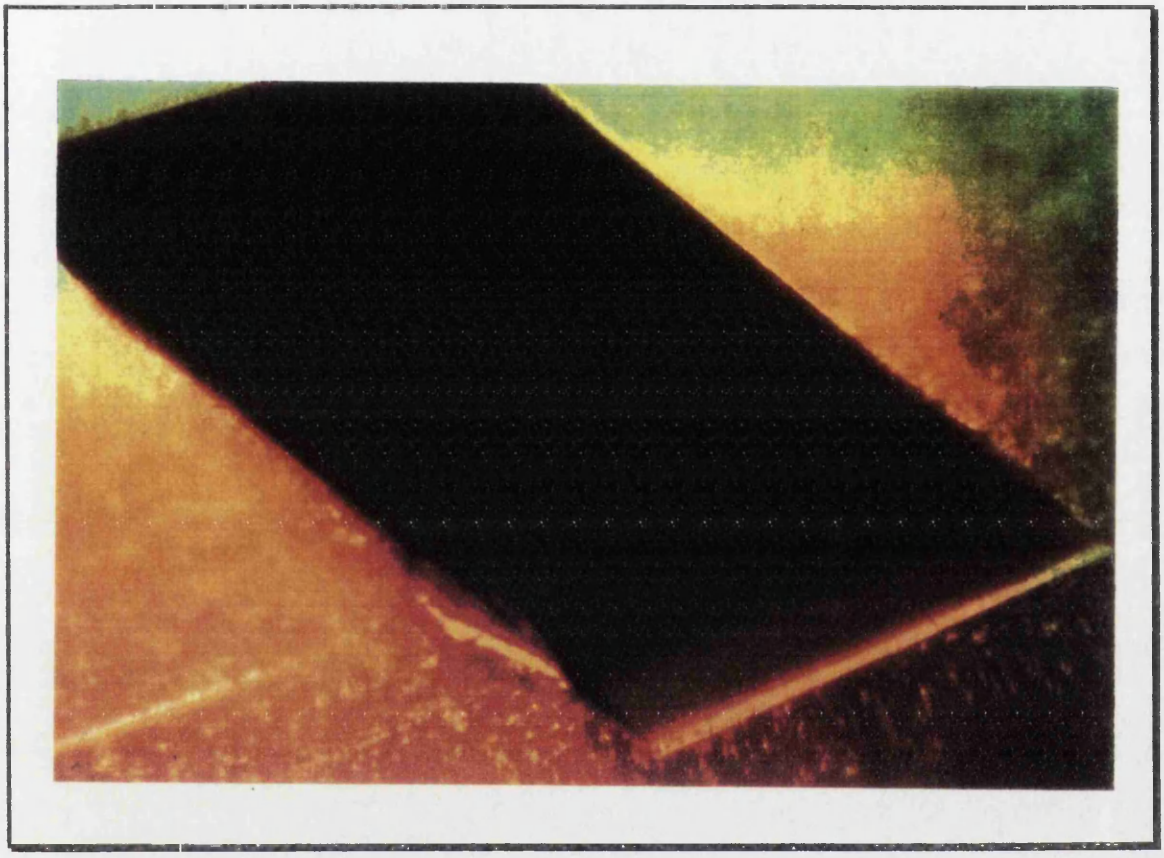

Figure 2.3. Typical crystal of LH II from *Rps. acidophila* strain 10050. Dimensions (in mm): 0.7 x 0.4 x 0.4

2.6.2 *Optimised crystallisation protocol*

The protocol which was found optimal, in terms of the quality and reproducibility of diffraction, was based closely on that originally used. Protein prepared as in section 2.7.2 was present at a concentration which gave an OD_{850} of 100 in 20 mM Tris, 1% β-Octyl-glucoside, 350 mM NaCl. The solution was made 2.5% w/v benzamidine hydrochloride and 1.0 M di-potassium orthophosphate (using a 4M stock solution). Without altering the pH, the solution was centrifuged as before to remove any debris which may act as nucleation sites. 'Sitting drop' crystallisation trials were set up in Cryschem trays (Charles Supper Company, Natrick, MA, USA). These consist of 24 'wells' with a central, elevated depression which can hold up to 25 µl of solution. Typically 15 µl drops containing protein were equilibrated against 1 ml of ammonium sulphate solution adjusted to pH 9.3 with solid NaOH.

2.6.3 *Crystallisation for microspectroscopy: background*

The predominant consideration when growing crystals of LH II for microspectroscopy measurements is one of crystal size and morphology. Ideally the crystal morphology should be tetragonal plates, as large as possible along the x and y edges[1] to permit accurate alignment of the crystal on the optical bench. But, due to the very high absorbance of the LH II, the crystal should be less than 6 µm in the z direction to prevent detector overload (Wacker, 1988).

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[1] See Appendix C for the laboratory convention on crystal morphology. The instrument used required crystals of minimum dimensions 10 µm by 30 µm in these axes.

Crystals of LH II from *Rps. acidophila* strain 10050 - together with LH II complexes from other strains of bacteria - had previously been crystallised specifically for microspectroscopy analysis (K.Stek *et al.*, 1990). The conditions used for crystallisation of LH II from *Rps. acidophila* were very markedly different from those used for producing crystals for X-ray analysis. In this study Steck and coworkers crystallised LH II using PEG 1000 as a precipitant, whereas all of the LH II crystals used for X-ray analysis were grow using inorganic salts as the precipitant. Therefore there was a driving force towards growing crystals suitable for microspectroscopy analysis by using crystallisation conditions similar to those used for producing X-ray sized crystals. This would allow comparison of the spectra in the conformationally sensitive visible/near infra-red region, from the complex present in membranes, throughout the purification protocol and in the crystal form used for tertiary structure determination. Furthermore, by using a polarised light source it was possible to measure the degree of linear dichroism exhibited by crystals, and hence estimate the level of crystalline order in crystals grown by various protocols (see next Chapter for details).

2.6.4 Crystallisation for microspectroscopy

Crystallisation for microspectroscopy was accomplished using the same solutions as had been developed for crystallising LH II for X-ray analysis but by altering the physical parameters of the experiment the crystals were 'forced' into adopting an alternative morphology. For microspectroscopy measurements the crystals are mounted in a cell, built from two glass coverslips and separating spacer. Therefore crystallisation trials were set up directly onto a 13mm circular glass coverslip. This was achieved by spreading 5 µl of the optimised

Figure 2.4. Microcrystals of LH II. Typical size (in μm) 40 x 30 x 5.

crystallisation solution over the surface of the coverslip. This was then rested on the wells in a microtitre plate, above 200µl of the reservoir solution. The tray was sealed and placed in an incubator at 17° C. Generally crystals suitable for analysis formed after two to five days, and on some occasions overnight.

Using this technique it was possible to assay the spectral effects when crystallisation trials were carried out using a variety of detergents, and precipitant/reservoir combinations. Microcrystals used are shown in figure 2.4.

2.7 Purification of LH II

The principle technique used throughout for assaying the purity of LH II preparations was a spectroscopic "purity index" (See Appendix C for details and explanation). In principle, the purification protocol used is primarily aimed at removing reaction center contamination. This is essential due to the inherent ease with which most reaction centers crystallise. Even if only present at very low concentration levels, the reaction center readily crystallises during LH II crystallisation trials (Dr. G. Fritzch, personal communication), and of course by virtue of its pigmentation could easily be mistaken for small LH II crystals.[1] Changes to the purification protocol will be discussed later in Chapter 5 in terms of their effect on the observed diffraction parameters of crystals. Full details of the purification protocols are given in Appendix C, the principal features of both protocols are outlined below.

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[1] To the authors knowledge this mistake has been made several times, in several different laboratories.

2.7.1 Original LH II purification protocol

As used in the original crystallisation protocol of Papiz *et al.*, 1989;

- Bacterial cells 'harvested' by centrifugation.
- Cells French pressed to release the photosynthetic membranes.
- Membranes solubilised by incubation - at a concentration which gives an OD_{850} of 50 cm^{-1} in 1% LDAO for 5 minutes at room temperature.
- Ion exchange chromatography using Watman DE52 matrix to separate LH II from reaction center.
- Second DE 52 column - under similar conditions - to remove any residual reaction center.
- Gel filtration; using Sephacryl S200 matrix.
- Detergent exchanged to β-Octyl-glucoside by ion exchange chromatography using a small Watman DE 52 column poured in a Pasteur pipette.

2.7.2 Optimised purification protocol

As used to prepare the crystals used in McDermott *et al*, 1995;

- Harvest cells by centrifugation.
- French press cells.
- Solubilise membranes by incubation, at a concentration which gives an OD_{850} of 25, with 2% LDAO for 2 hours at 4° C

- Sucrose gradient density centrifugation to remove reaction center (and all smaller contaminants such as unbound pigments and apoproteins).
- Gel filtration using FPLC quality Superdex 200 matrix.
- Detergent exchange to β-OG by centrifugation dialysis.

2.8 Pigment removal and reconstitution

A methodology for selectively removing, and subsequently reconstituting pigment moieties in bacterial reaction centers was developed by Scheer and co-workers - an overview of the work is to be found in (Sheer & Struck, 1993; Note: quotation marks used below are as used by the authors in this paper). The "secret" of their method being to heat a detergent solubilised solution of reaction centers to close to its "melting point" of around 40° C. This was assumed to allow the protein chains to "breathe", thus permitting the interchange of pigment molecules.

Adapting this method of pigment exchange to LH II was viewed as a rational method for the production of heavy atom derivatives. Experiments were initiated to develop a method of removing a specific subpopulation of Bchl *a* molecules and subsequently reconstituting the complex with a bacteriochlorin analogue containing a suitable heavy atom. As a means of obtaining heavy atom derivatives this method was envisaged as having several inherent advantages over the classical technique of 'soaking'. In the first instance it would be possible to quantify the level of heavy atom substitution. Secondly, the technique was seen as having the potential to minimise crystallographic non-isomorphism arising from either the binding of the metal to the crystal lattice, or from the soaking process itself.

2.8.1 B800 BChl a removal and reconstitution

Initial experiments to adapt the thermal technique used for pigment exchange in reaction center proved ineffective. At the elevated temperatures necessary to release pigment molecules the complex rapidly, and suddenly denatured. It was not possible to find a temperature which selectively allowed pigment exchange.

It had been previously reported that it was possible to remove the B800 BChl *a* population by treatment with reagents such as $NaBH_4$, $LiClO_4$ or SDS (Kramer *et al.* 1984), or by dilution of membranes with excess lipid (Pennoyer *et al.*, 1985). These results were taken to indicate that the B800 moieties are less firmly bound to the complex than the B850 BChl *a*. The resultant complex formed, the so called B850 LH II, was not stable and tended to denature after a short period of time. To be an effective route to producing 'heavy atom' derivatives, it was visualised that a method should be sought which produced a stable B850 LH II complex. This would allow the 'free' BChl *a* to be purified out of the solution, before reconstituting the complex with a heavy atom containing BChl *a* analogue.

In an attempt to selectively decouple the B800 BChls experiments were carried out on reducing the pH of the complex. This was attempted in the detergent systems used for purification and crystallisation. In all cases reducing the pH below pH 6.0 resulted in rapid denaturation of the complex. But, if the same experiments were conducted on LH II which had been exchanged into the glyco-

detergent[1] Triton TGB10 it was possible to reduce the pH, using acetate buffer, without causing denaturation. On reduction of the pH below 6.0, loss of absorption at 800 nm was apparent. If the pH was further titrated to around pH 5.3 the loss of 800 nm absorption was more pronounced (shown in figure 2.5) and continued over a period of approximately 40 minutes until a B850 LH II species was formed, figure 2.6. At this pH the B850 LH II complex was stable for some hours at room temperature or overnight at 4°C.

If the pH was then raised to above 6.0, the re-ligation of the B800 BChl was apparent from the reappearance of a peak at 800 nm in the absorption spectra. If a quantity of the detergent LDAO was also added this was seen to destabilise the B850 species and rapidly resulted in total reformation of a B800-850 complex, figure 2.7. From absorbance measurements before and after treatment it was estimated that almost 100% re-ligation of the B800 BChl *a* occurred.

2.8.2 Purification of B850 LH II

To allow reconstitution with a BChl *a* analogue containing a more electrodense central ion than magnesium it was necessary to remove the 'free' BChl *a* after titrating the pH to below 6.0. In some initial experiments, conducted on B850 complex derived from *Rb. sphaeroides* LH II, this was done with relative

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[1] The exact composition of the detergent was unknown. The detergent used was originally in an extremely crude form. It had a deep brown appearance and required thorough purification using ion exchange media immediately prior to use. Once purified the detergent was almost colourless, but still considered quite inhomogeous (Dr. M. Bandilla, personal communication; based on the observation that the detergent displayed multiple CMC values which varied with each batch purified). It was found to be possible to replace LDAO by Triton TGB 10 for solubilisation and purification of LH II.

ease using ion exchange chromatography [Work done in the University of Munich in collaboration with Prof. H. Scheer and Dr. M. Bandilla published in (Bandilla *et al.*, 1996)] Unfortunately, this could not be reproduced with B850 complexes from *Rps. acidophila.* Both the 'free' BChl *a* and the B850 complex eluted under the similar conditions. Rather than explore other possible ion exchange matrix, it was decided to take advantage of the obvious difference in molecular weight between 'free' BChl *a* and intact B850 complexes. If B850 complex was loaded onto an S200 sephacryl gel filtration column and eluted with a solution of Triton TGB 10 at pH 5.2, it was found that approximately 80% of the 'free' BChl *a* was removed. This was assayed by attempting to reconstitute the complex in the absence of exogenous bacteriochlorins. From the absorption spectra, it was seen that the 800 nm peak only partially regenerated.

Using material purified in this manner experiments were conducted adding exogenous bacteriochlorins. If the 'native' bacteriochlorin BChl *a* was used the resultant spectra was almost indistinguishable from untreated B800-850 complex, figure 2.8. Using BChl *a* analogues which contained Pd in place of Mg only about 50% to 60% of the 800 nm peak was reconstituted. This was taken as indicating the resultant complex had approximately 30% binding of the Pd analogue.

This partially Pd substituted B800-850 complex was then exchanged into β-Octyl-glucoside for crystallisation trials. Crystals of suitable dimensions were obtained using similar crystallisation conditions to that used for 'native' LH II. This work was being optimised when suitable derivatives were obtained by traditional soaking methods.

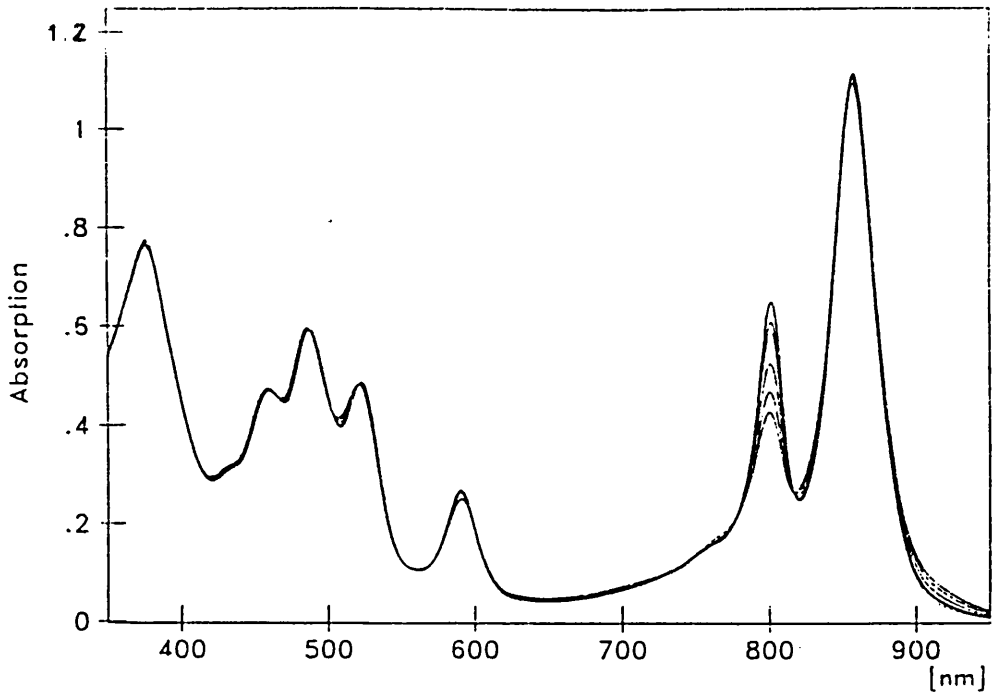

Figure 2.5. Removal of B800 BChl *a*. After titration to pH 5.3. Spectra recorded every 5 minutes.

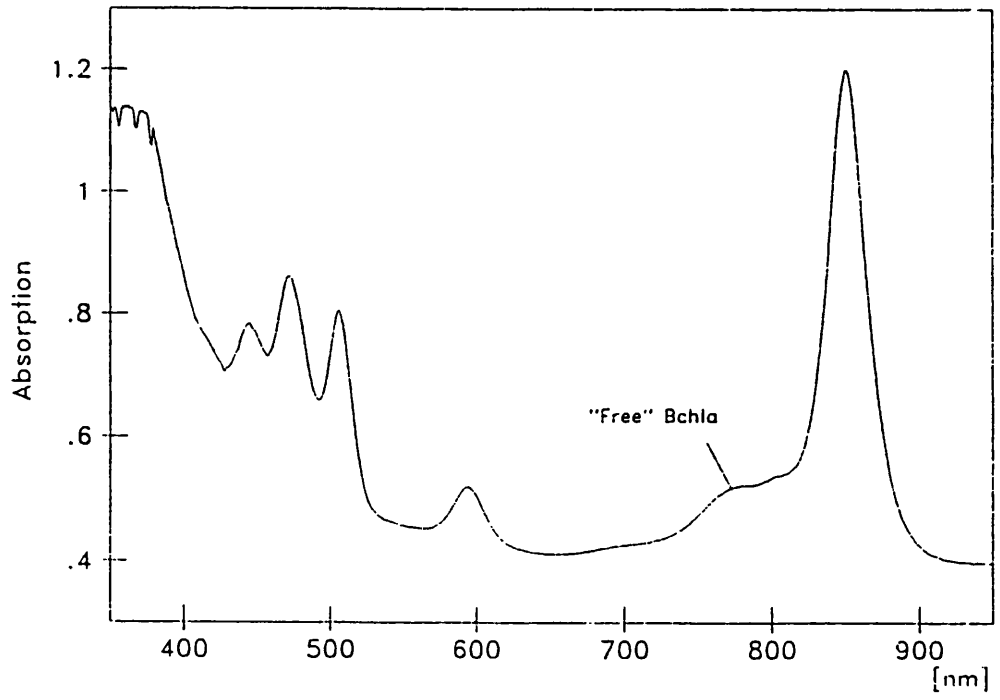

Figure 2.6. 50 minutes after titration to 5.3.

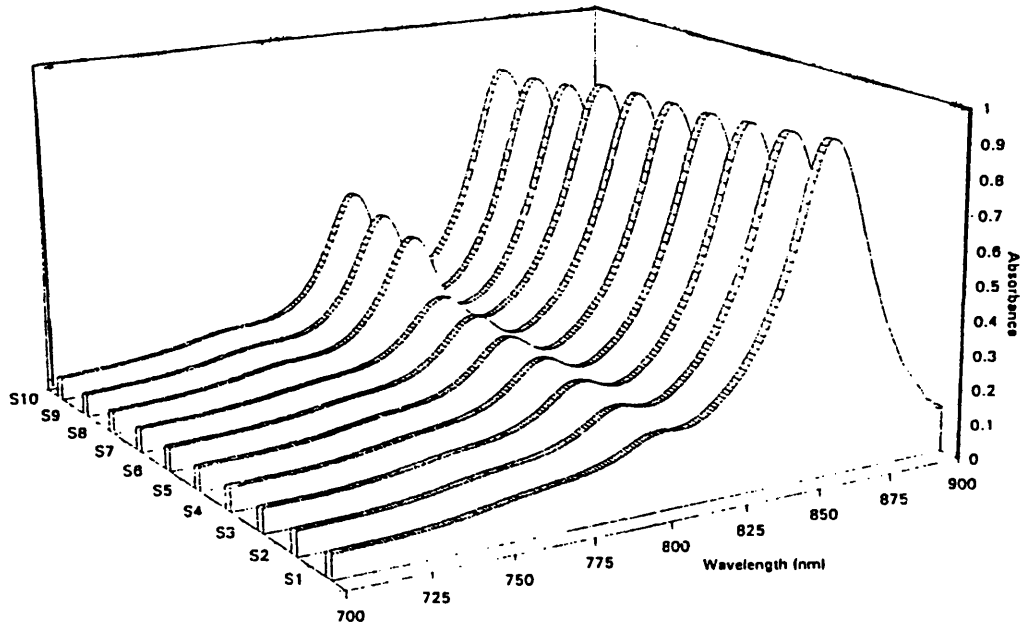

Figure 2.7. After titration of unpurified B850 LH II to pH 8.0. Spectra recorded every 12 minutes.

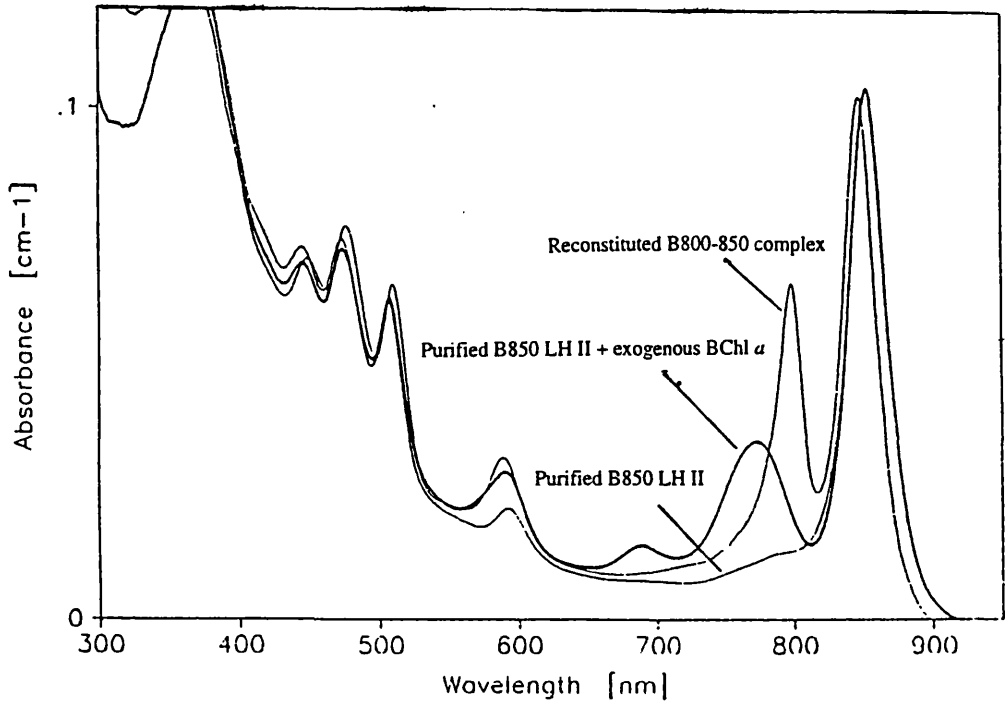

Figure 2.8. Absorption spectra for purified B850 LH II, B850 LH II + exogenous BChl *a* at pH 5.3 and after titration to pH 8.0.

2.9 Summary

The work described in this chapter can be regarded as groundwork for the structural analyses described in Chapter 3. LH II crystallisation and purification protocols were optimised by correlating modifications made to both with the observed levels of diffraction. This work ultimately allowed the production of crystals which diffracted reproducibly to high resolution.

Techniques were developed for the production of microcrystals of the complex. These could then be used for microspectoscopy and Linear Dichroism analysis. The information from which proved invaluable, and allowed a more rational approach when optimising the methods used for 'heavy atom' soaking experiments.

A novel method was developed for the selective removal of the 800nm absorbing Bchl *a*. The resultant B850 complex could be reconstituted with either the original bacteriochlorin or with an analogue containing Palladium as the central metal atom. Both of which retained full functionality and could be crystallised using similar conditions to the native complex.

Chapter 3

Spectroscopic and X-ray Crystallographic analyses

3.1 Introduction

Prior to the work contained in this thesis, an extensive range of crystallisation trials had been carried out on LH II from *Rps. acidophila* strain 10050. This resulted in the establishment of protocols for the production of two crystals forms of LH II suitable for initial X-ray characterisation. (Papiz *et al.*, 1989). The particular crystal form obtained depended solely on the detergent used during crystallisation. If the complex was present in the detergent dimethyldecylamine *N*-oxide (DDAO) a tetragonal crystal form was obtained. The space group was determined to be P4 and unit cell parameters a=b=75.8Å c=97.5Å. Unfortunately, the observed diffraction only extended to a maximum resolution of 12Å (Papiz *et al.*, 1989). Therefore, this crystal form was deemed unsuitable for further structural analyses.

If the complex was exchanged into the detergent β-Octyl-glucoside prior to crystallisation, a rhombohedral crystal form was obtained. Preliminary X-ray analysis determined the space group to be R32 with unit cell parameters a=b=121.1Å c=296.7Å α=90° γ =120°. For this crystal form the limit of diffraction was observed to extend to a maximum resolution of 3.5Å (Papiz *et al.*, 1989).

The work of this thesis began at this point in time. Unfortunately, the initial optimism fuelled by the observation of diffraction to 3.5Å was short lived. Rhombohedral crystals obtained by reproducing the procedures of Papiz and co-workers displayed very poor diffraction characteristics. On average only 1 crystal in 24 showed any diffraction whatsoever, and approximately only 1 crystal in 40

showed diffraction extending beyond 8 Å (McDermott, 1993). The quality of the observed diffraction was also highly unpredictable. To the extent that crystals taken from the same crystallisation drop were seen to exhibit both extremes[1] of diffraction character. The changes made to the purification and crystallisation protocols have been outlined in the previous Chapter and are discussed in terms of their effect on diffraction in Chapter 5.

3.2 Heavy atom derivitisation

Phase determination by the method of isomorphous replacement method typically involves the following experimental steps;

- Measure intensity data for native crystal(s).
- Crystals are transferred to a solution which resembles the crystal growth solution ('artificial mother liquor') containing a 'heavy atom' compound - typically at a concentration of between 0.1 and 5 mM.
- Allow the crystals to equilibrate, i.e. to 'soak'
- Measure intensities for putative heavy atom derivative crystals.
- Reduce, correct and scale data.
- In the first instance, the presence, or otherwise of heavy atoms in the crystal lattice is determined - normally by calculation of difference Patterson syntheses -

[1] i.e. either very low resolution diffraction (~15Å) or relatively higher resolution (~4Å) using a rotating anode source.

and if yes, their position in the unit cell is determined using programs adapted from 'small molecule' crystallography.

- If successful, calculate phases.

There are several inherent disadvantages of the MIR method. Firstly, *a prori* there is no indication which heavy atom compound will bind to the protein to produce a derivative. Even if likely binding sites are engineered into the protein by molecular biology techniques these can be involved in protein-protein contacts and therefore unavailable for heavy atom binding. Secondly, the soaking method requires the transfer of a mature crystal from the crystallisation drop into a solution containing the heavy atom solute. This solution must be sufficiently competent at mimicing the final environment of the crystallisation drop that it does not cause a loss of crystal order or induce non-isomorphism.

To obtain high quality derivatives the difference in signal between the native and derivative data sets should be maximised (Drenth,1994). Several aspects of the experiment are important in this respect: all data should be recorded as accurately as possible; the degree of isomorphism between native and derivative data should be as high as possible and the heavy atom bound should be large enough to induce significant changes in intensity.

Crick and Magdoff (1956) estimated that the intensity changes on binding a heavy atom were much greater than had been previously expected. The difference in intensity is given by equation 3.1 (Leslie, 1991).

$$\frac{\langle \Delta I \rangle}{I} = \gamma \frac{f_H}{f_P} \sqrt{\frac{N_H}{N_P}} \qquad \textit{Eq.}\ 3.1$$

where $\langle \Delta I \rangle$ is the r.m.s change in intensity, γ=2 for centric or $\sqrt{2}$ for acentric data, f_H is the heavy atom scattering factor, f_P is the protein atomic scattering factor, N_H the number of heavy atoms per protein molecule and N_P the number of protein atoms. Using this formula its can be shown that if a single mercury atom (with 80 electrons) is bound with 100% occupancy to a protein of molecular weight 24,000 Da the average change in intensity is approximately 40%. The relationship between occupancy and the average change in intensity is striking. A single mercury atom bound to a protein of molecular weight 360,000 Da with 100% occupancy can yield a 10% change in average intensity. If the occupancy is only 50%, the same change in intensity will only be seen if the protein has a molecular weight of 90,000 Da. In most modern texts on protein crystallography a 10% change in intensity is regarded as the minimum that can be measured with accuracy (Drenth, 1994).

Unfortunately, the problem is more complex than simply binding heavy atoms of suitable weight onto a minimum number of protein molecules in the crystal. The structure factor $\mathbf{F}_p$ for the native protein can be drawn as a vector diagram (known as the Harker construction), figure 3.1. $\mathbf{F}_P$ is a vector quantity composed of F_P the structure factor amplitude and phase α_P. Since the phase is unknown in a simple Harker construction the possible values of $\mathbf{F}_P$ can be drawn as a circle, figure 3.1.

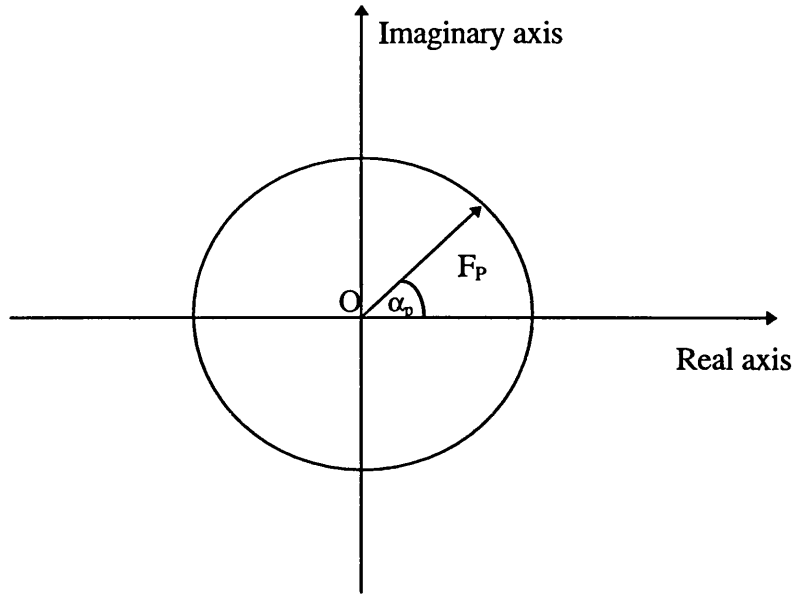

Figure 3.1. Harker construction for F_P.

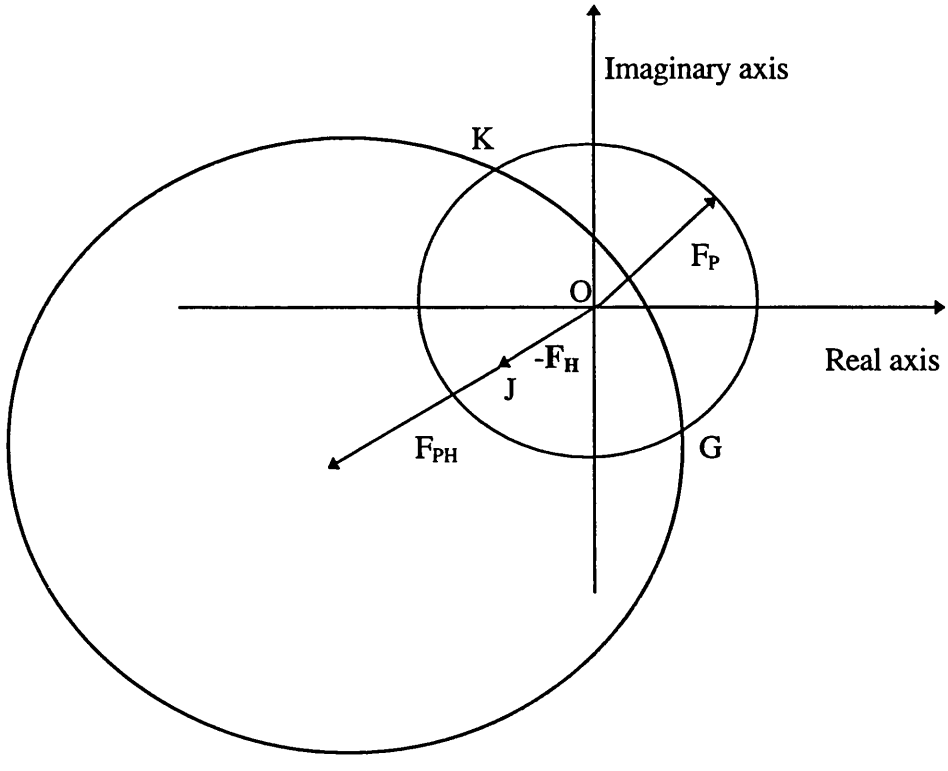

Figure 3.2. Harker construction for a single isomorphous replacement.

The structure factor of a heavy atom derivative $\mathbf{F}_{PH}$ is the vector addition of $\mathbf{F}_P$ and $\mathbf{F}_H$ the structure factor of the heavy atom alone. Since it is possible to measure F_{PH} and F_P, and calculate the position(s) of the heavy atom in the unit cell it is possible to calculate $\mathbf{F}_H$. In terms of the graphical Harker construction shown in figure 3.2 the value of $\mathbf{F}_P$ is the point where the circle of origin J intersects with the circle of origin O. As can be seen from the figure 3.2, this can have two possible solutions, the vectors OK and OG. The exception being when the vectors $\mathbf{F}_P$ and $\mathbf{F}_H$ are co-linear, when only one solution is possible. Otherwise, the vector solution for $\mathbf{F}_P$ can only be resolved unambiguously by obtaining from a further derivative with a different value of $\mathbf{F}_H$. In terms of the graphical Harker construction, this would give rise to a third circle of with a different origin. Theoretically the point where all three circles intersect is a unique value of $\mathbf{F}_P$. In practice, due to errors in data measurement it is unusual for all three circles to intersect with any degree of accuracy, therefore it is not uncommon that least one or more further derivatives are required before $\mathbf{F}_P$ can be calculated unambiguously. As an alternative to having to produce multiple derivatives anomalous scattering data can be used to resolve, either partially or totally, ambiguities in calculation of $\mathbf{F}_P$.

Even after considerable improvements were made to the quality of the diffraction obtained, conducting a search for heavy atom derivatives of LH II remained problematic. The process of soaking led to a serious reduction in the quality of the observed diffraction. For the most part this occurred irrespective of the heavy atom compound used, although some compounds exacerbated the effect. It was found that the maximum soak time which could be tolerated by crystals was

in the region of 20 to 30 minutes. Longer soaks resulted in a rapid and profound decrease in the resolution limit (McDermott, 1993).

In general, restricting soaking experiments to relatively short durations may be viewed as significantly decreasing the probability of obtaining heavy atom derivatives. Despite the observations that the half time for diffusion of phosphate into a crystal of phosphorylase *b* is about 1 minute (Hajdu *et al.*, 1987) and that intensity changes are complete if a lysozyme crystal is soaked in mercury acetate for only 30 minutes (Leslie, 1991), required soak times normally range from hours to several days (Leslie, 1991), or even longer (Drenth, 1994).

In the case of LH II it was assumed that a combination of factors made it unlikely that all intensity changes would be complete after soaks of 20 to 30 minutes. Firstly, in Michel's model of type II membrane protein crystals the detergent micelle covers the central region of the protein molecule and the hydrophilic regions are involved in inter-molecular contacts (Michel, 1983). Unlike the crystal lattice of the bacterial reaction center, the comparatively small hydrophilic domains of LH II have the effect that there are few solvent accessible regions. Secondly, it was considered a possibility that the diffusion of the heavy atom solute would be hindered by the presence of detergent 'congestion' in the solvent channels. Thirdly, the presence of ammonia in heavy atom soaking solution could interfere with the chemistry involved in the process of heavy atom binding. It is common that in the presence of ammonia, ligand exchange takes place. For example if there is an excess of free ammonia the following reaction takes place (Blundell & Johnson,1972);

$$PtCl_4^{2-} \rightarrow \text{cis } PtCl_2(NH_3)_2 \rightarrow Pt(NH_3)_4^{2+}$$

This reaction takes place slowly and it may be the case that only an intermediate species in the reaction binds to the protein (Drenth, 1994).

It was concluded that the optimal heavy atom derivatisation experiment would be one where soaking was carried out for a relatively long period of time, to ensure maximum diffusion and heavy atom attachment. To permit such experiments it was necessary to determine the reasons why the soaking procedure *per se* had such a detrimental effect on the diffraction. This was done primarily by measuring spectra from microcrystals grown using a protocol similar to that used to produce macrocrystals for X-ray analysis.

3.3 Microspectroscopy

The production of structures by X-ray crystallography frequently provokes the question: how closely does the structural model represent the *in vivo* molecule? This question is especially apposite when protein molecules experience vastly different environments *in vivo* and in the crystal form, as is clearly the case in structural studies on integral membrane proteins. By comparing the conformationally sensitive absorption spectra of LH II *in vivo*, during purification and crystallisation and in the crystal form itself, it is possible to address the above question. In terms of the practical side of X-ray analysis, making such measurements on a solution of the complex and on subsequent crystals will reveal if the crystallisation process has a detrimental effect on the integrity of the complex.

The fact that it was possible to make spectral measurements on crystals of LH II had also implications for the procedures used in heavy atom derivatisationb

trials. The presence of highly ordered pigment molecules in the crystal lattice leads to the crystals being dichroic (Cogdell & Hawthornethwaite, 1993). The level of dichroism observed is highly correlated with the degree of crystalline order. Therefore, by measuring the level of dichroism it was possible to examine the effect that heavy atom soaking procedures had on the crystal lattice.

3.3.1 Linear dichroism

A transition dipole absorbs radiation by oscillating in sympathy with the applied radiation. This oscillation has a certain direction associated with it, thus only components of the electromagnetic radiation that are aligned in the same direction as the transition dipole will cause transitions (Wacker *et al.*, 1988). In a solution all possible orientations of the molecules, and hence their transition dipole moments, are present. However, in a system of ordered molecules, such as a crystal, only a discrete number of transition dipole orientations will be found. Therefore, the absorption of plane polarised radiation will be a function of the crystal orientation. If the absorbance is recorded with the polariser in two positions 90° apart, the resulting difference in absorption, as a function of frequency, is termed Linear Dichroism (LD).

Microcrystals of LH II from *Rps. acidophila* strain 10050, and other LH complexes had previously been subject to LD analysis in two independent studies in an attempt to determine the relative orientations of the pigments (Stek *et al.*, 1990; Frank *et al.*, 1990). In both studies the crystallisation protocols used for the production of LH II crystals from *Rps. acidophila* strain 10050 were markedly different - in terms of detergent, additives, precipitant and reservoir solution - to

that used to produce macrocrystals for X-ray analysis. Therefore, there was a two-fold driving force towards producing microcrystals from conditions similar to those used for producing X-ray sized crystals: analysis of the spectra would yield information on any changes in the integrity of the complex which arose due to the environment encountered during crystallisation,[1] and by monitoring the spectra of crystals in solutions similar to those used during heavy atom soaks it would be possible to identify elements of the process which were detrimental to the crystal order.

3.4 Results: Microspectroscopy

A protocol was developed for the production of *Rps. acidophila* LH II microcrystals[2] which is very similar - in terms of drop and reservoir composition - to that used to produce macrocrystals for X-ray analysis. The only significant differences being the time taken for crystal growth and the final dimensions to which the crystals grow. A representative spectra obtained from a microcrystal is given in figure 3.3. From this it apparent that there is little difference between crystal and solution spectra. In particular, in the crystal spectra there is no indication of denaturation (i.e. no increase in the absorbance at 250 nm or in the appearance of a peak at 770 nm indicating the presence of 'free' BChl *a*).

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[1] This approach was preferable to simply dissolving crystals and measuring the spectra. It had been established that some structural effects - such as the removal of the B800 BChl *a* - were reversible. Therefore, the situation could have arisen where this particular BChl species became detached, or partially detached during crystallisation and caused dislocations in the crystal lattice, but if the conditions used to dissolve the crystal caused re-ligation no apparent difference would be observed in spectra recorded before and after crystallisation.

[2] Outlined in section 2.6.3 and Appendix C.

Therefore, it was concluded that the crystallisation protocol did not have an adverse effect on the integrity of the complex.

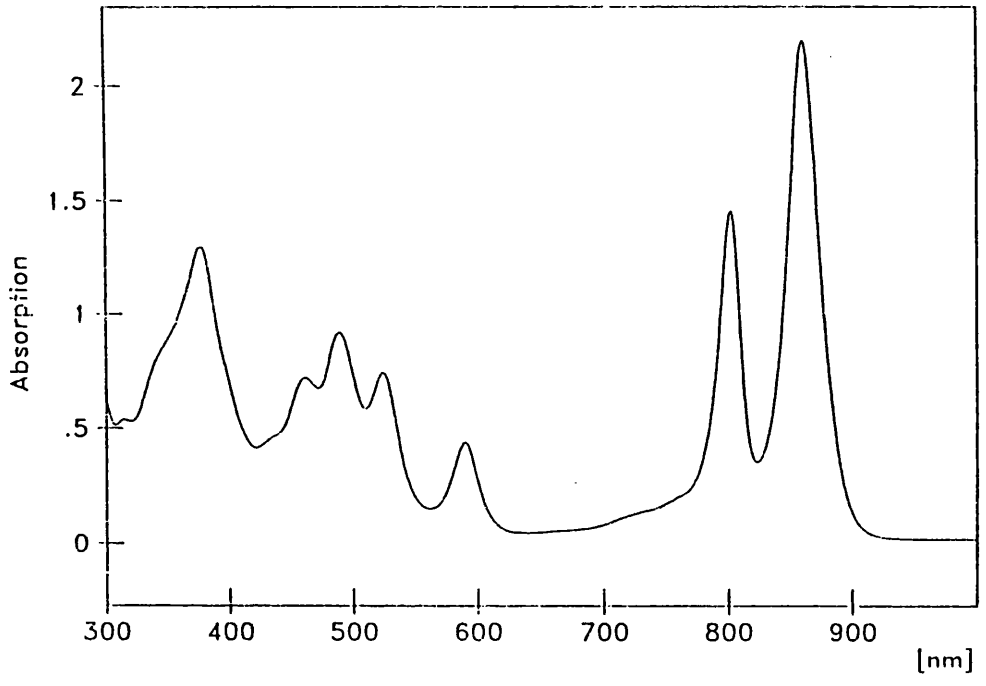

Figure 3.3. Absorbance spectra from microcrystals of LH II.
Crystallisation conditions: 5µl of protein at OD_{850} in 20 mM Tris, 1% β-Octyl-glucoside, 1.0 M K_2HPO_4, 300 mM NaCl, pH 8.0 equilibrated against 200 µl of 2.6 M ammonium sulphate, pH 9.6.

3.5 Results: Linear dichroism

Linear dichroism measurements were made on microcrystals grown by the method outlined in section 2.6.4 using a preparation of LH II described in section 2.7.2 with the exception that the final detergent exchange from LDAO to β-Octyl-glucoside was done chromatographically as in section 2.7.1. Measurement of LD from the resulting microcrystals revealed very high levels of dichroism, almost 100% for the BChl Q_y absorption, shown in figure3.4. This was significantly higher than that reported in the earlier studies (Steck *et al.*, 1990; Frank *et al.*1990), leading to the conclusion that the crystals were highly ordered. At this point in time, macrocrystals grown using similar conditions showed reproducible diffraction to a resolution of typically 3.0Å using a synchrotron source and 3.5Å using a laboratory rotating anode source.

3.5.1 The effect of heavy atom soaking on the LD value

The solution which had been developed for use in soaking experiments was tested for its effect on crystal order. Microcrystals were transferred into the original formulation of artificial growth liquor produced by conducting a crystallisation trial in the absence of protein (see Appendix C). The LD was measured immediately, and then at 10 minute intervals. A decrease in the LD was apparent after approximately 20 to 30 minutes, indicating a loss of crystalline order, shown in figure 3.5. This timescale correlated closely with the loss of diffraction observed when macrocrystals were treated similarly.

By formulating different compositions of soaking solution it was quickly established that the detergent concentration was the critical factor in maintaining

crystal order during soaking. Since the concentration of detergent in the drop increases during crystallisation it had been expected that the artificial mother liquor would require a higher concentration of detergent than that present at the start of crystallisation. In fact the converse was true. To the extent that microcrystals were marginally more stable in solutions of K_2HPO_4 alone than in the original formulation of artificial mother liquor.

Once the importance of detergent concentration had been identified, the optimal artificial mother liquor was developed by setting up a range of simulated crystallisation trials (in the absence of protein) over a range of initial detergent concentrations - 0.1% to 0.9% β-Octyl-glucoside. If these solutions were allowed to equilibrate for 5 to7 days, crystals could be soaked in the resulting solution for several days with no loss of resolution provided the initial detergent concentration had been 0.5% (± ~ 0.1%).

Over a period of time the composition of the artificial mother liquor was fine tuned. Omission of the small amphiphile benzamidine had no effect on the resolution or quality of diffraction, consequently as a precaution against its presence being detrimental to heavy atom binding it was excluded. The detergent concentration was modified further. By setting up simulated crystallisation trials at higher initial concentrations of K_2HPO_4 - and all other parameters remaining similar - the final equilibration point was reached faster, but with a lower final detergent concentration. Typically after equilibration for two days crystals could be maintained in this solution for a period of several weeks with no effect on the quality of diffraction.

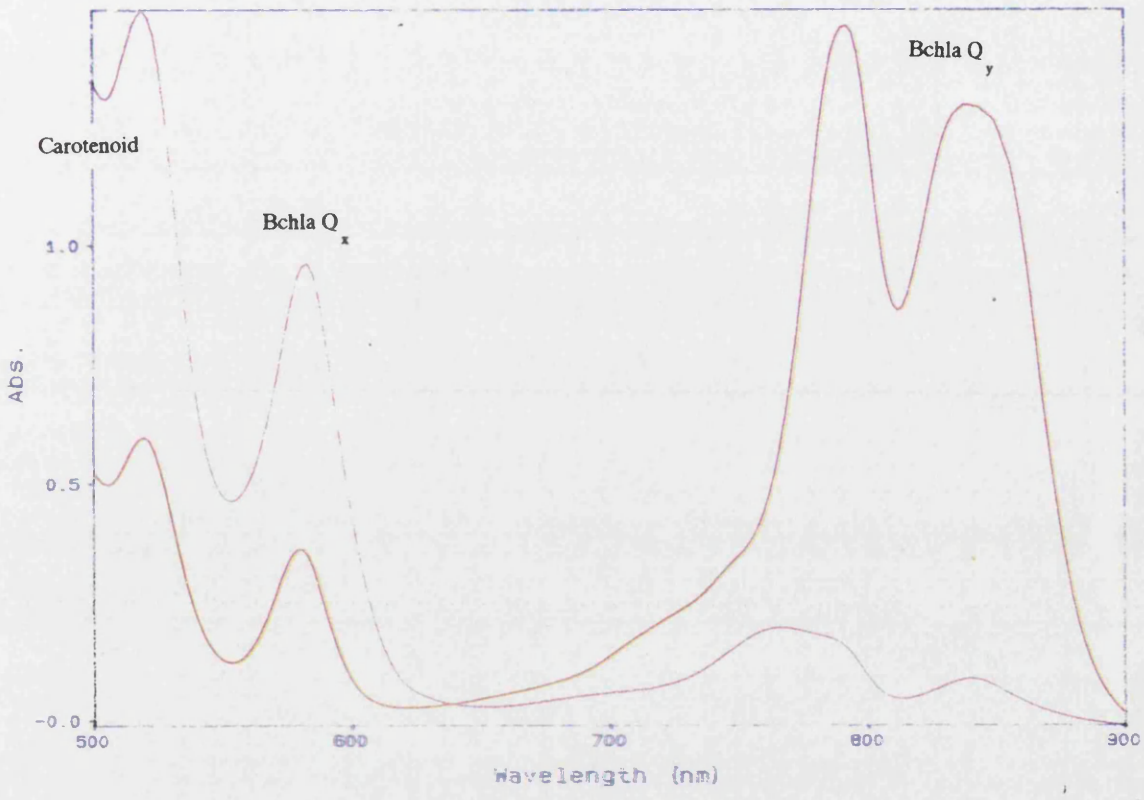

Figure 3.4. Linear dichroism spectra of LH II microcrystals. Red line: polariser set to 0°. Purple line: polariser set to 90°

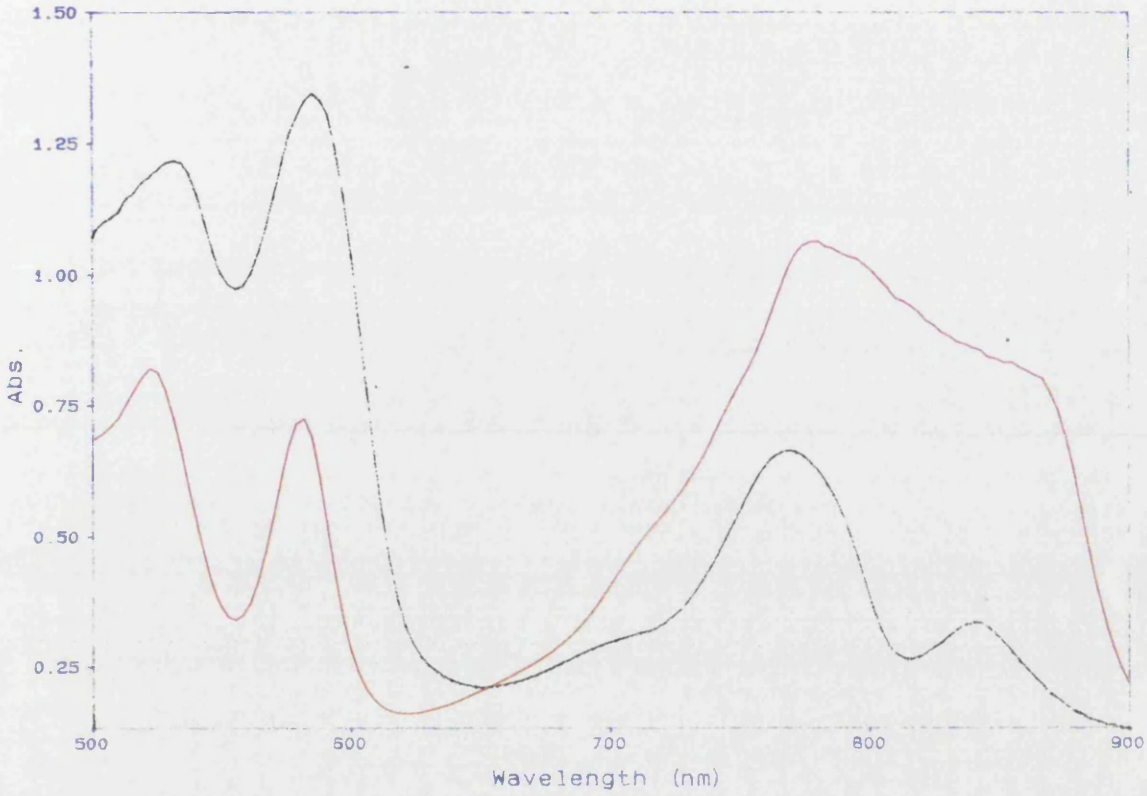

Figure 3.5. Linear dichroism spectra from crystals soaked in the original formulation of artificial mother liquor. Red line polariser set to 0°. Black line 90°

Equilibration of the artificial mother liquor against ammonium sulphate at elevated pH (i.e. above pH 9.0) was found to be essential, even though there was little alteration of the pH of the drop during the process. Even when using the optimised protocol for producing artificial mother liquor an equilibration time of at least 36 hours was required. If crystals were transferred into this solution after 12 hours equilibration, the limit of resolution was decreased by at least 2Å.

3.6 X-ray crystallographic analysis: Previous work

Gel electrophoresis and ultracentrifugation experiments had estimated the molecular weight of intact LH II complexes to be 84 kDa (Cogdell *et al.*, 1983). This weight was assumed to be an $\alpha_6\beta_6$ apoprotein assembly plus associated pigments (Zuber, 1986). The crystal density of the rhombohedral crystal form was found to be 1.17 g/cm^3. This was taken to suggest an asymmetric unit of molecular weight 146 kDa (assuming Z=18 in the hexagonal cell). Therefore, the conclusion was drawn, purely on mass terms, that the asymmetric unit consisted of a single 84 kDa LH II (Papiz *et al.*, 1989).

In the case of the tetragonal crystal form, the assumption that *Z*=4 together with crystal density measurements of 1.12 g/cm^3 led to the asymmetric unit being assigned a mass of 95 kDa (Papiz *et al.*, 1989). As with the rhombohedral crystal form, this was also assumed to consist of a single $\alpha_6\beta_6$ assembly. Although both asymmetric units were proposed to have similar contents, no explanation was given by Papiz and co-workers for the 51 kDa discrepancy in the estimated weight of the two asymmetric units.

Further analysis of the data obtained from the rhombohedral crystal form led to a model of the arrangement of the apoproteins in the asymmetric unit (Cogdell & Hawthornthwaite, 1993). Calculation of a section of Patterson space in the *a/b* plane revealed 18 distinct peaks (figure 3.6). These were seen as rings of density progressing outwards from the origin at distances of 10.5Å, 14.2Å and 24Å. These were proposed as being the distances between two helices, the diagonal between helices of a tetramer and the distance between tetramer bundles, figure 3.7. This model corresponded exactly with the 84 kDa molecular weight found by experimental methods, and with the hypothesis that LH II was an $\alpha_6\beta_6$ assembly, arranged into three tetrameric 'minimal units' (Cogdell & Hawthornthwaite, 1993).

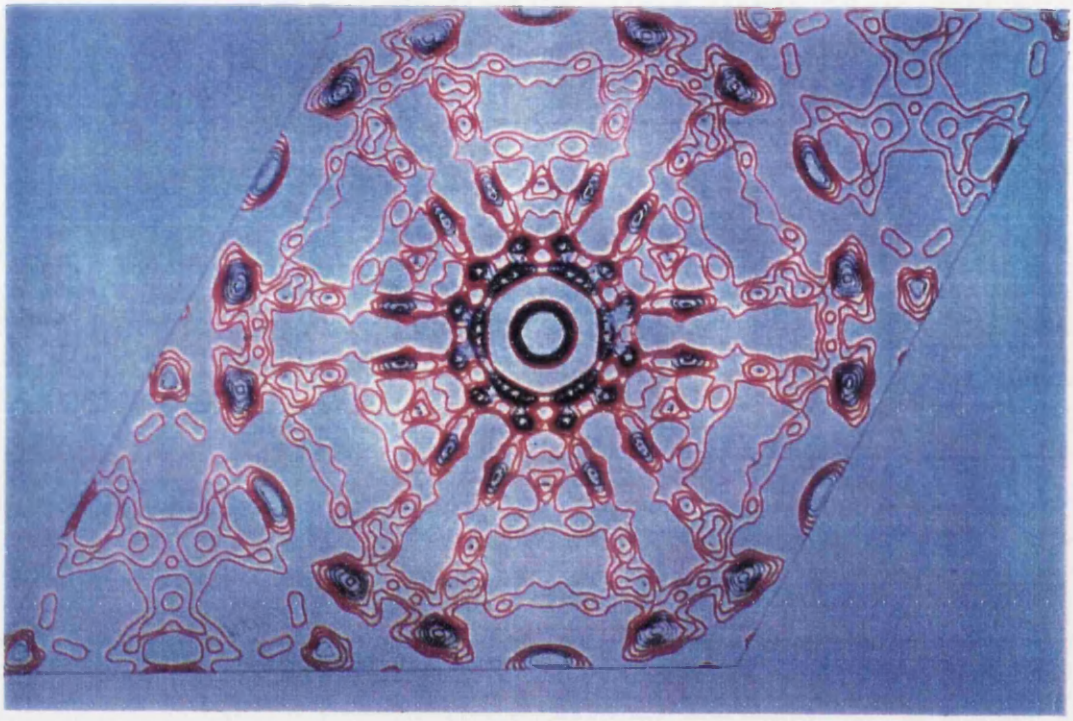

Figure 3.6. Harker section of a native Patterson syntheses in the *a/b* plane (Cogdell & Hawthornthwaite, 1993).

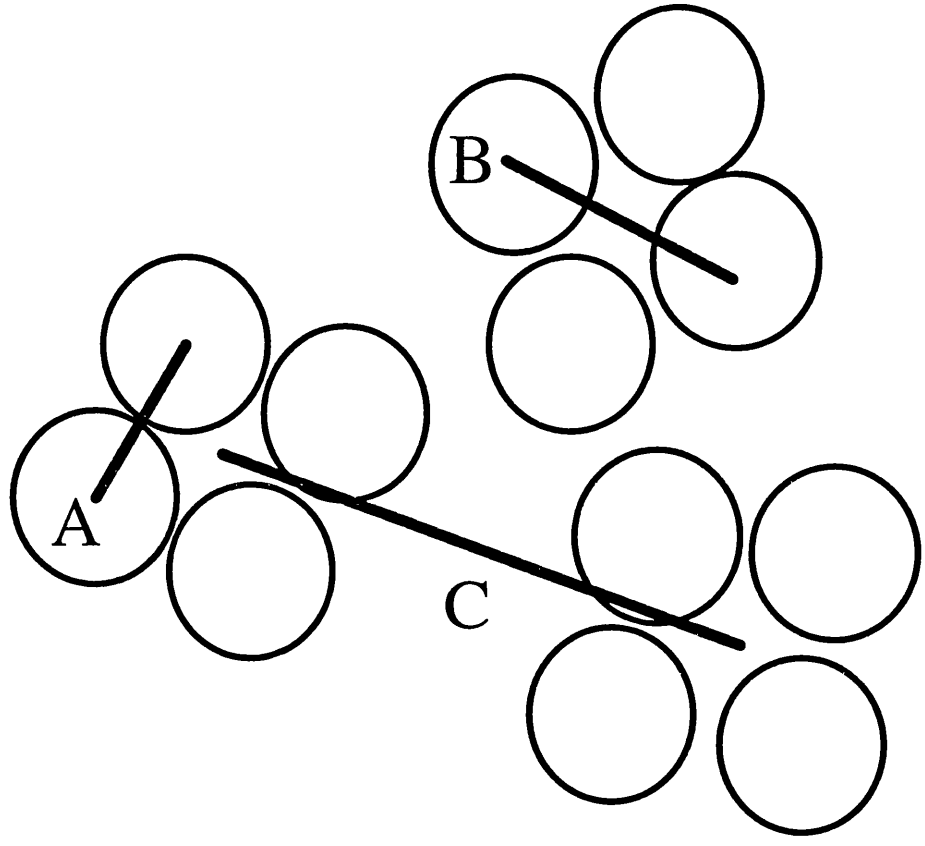

Figure 3.7. Interpretation of the native Patterson (Hawthornthwaite & Cogdell, 1993). circles represent transmembrane helices.

Scale bars: **A** =10.5Å **B** = 14.2Å **C** = 24.0Å

3.6.1 X-ray data collection and processing

The dynamic nature of the process by which the quality of diffraction was improved, and the consequential ambiguities which this factor had on the search for heavy atom derivatives, meant that data were routinely and extensively collected. Characterisation of diffraction was done primarily using an 'in-house' Siemens multiwire area detector on a laboratory Cu K_α rotating anode X-ray source. Data were processed and indexed using the XDS software suite (Kabsch, 1988) to produce intensities for all symmetry allowed indices *hkl*.

Data were also routinely collected on stations 9.5 and 9.6 at the Daresbury laboratory synchrotron source using Mar image plate detectors and processed using either DENZO (Otwinowski, 1993) or MOSFLM (Leslie, 1992).

On three occasions putative derivative and native data were collected at the DESY laboratory, Hamburg, at station X-11 using a Mar image plate detector, and processed using DENZO or MOSFLM. Several data sets for putative derivatives were collected at the EMBL, Heidelberg using a Siemens multiwire detector on a Cu K_α rotating anode X-ray source. As above, the latter data were processed and indexed using the XDS software suite (Kabsch, 1988).

3.6.2 Data reduction

Data were subsequently reduced using the following `programs` from the CCP4 software suite (Collaborative Computational Project Number 4, 1994) in sequence;

- `abscale` Applies geometric corrections.
- `rotaprep` Finds the symmetry equivalent measured indices.
- `sortmtz` Sorts data such that all equivalent indices are adjacent.
- `rotavata` Calculates scale factors.
- `agrovata` Merges equivalent reflections and applies scale factors.
- `agrovata` Identifies 'outliers'.
- `agrovata` Re-estimates standard deviations.
- `truncate` Checks that the final data is crystallographically reasonable.

3.7 Analysis of the Native Data

For reference purposes, a typical data image from a crystal regarded as exhibiting poor quality diffraction is reproduced in figure 3.8. Conversely, an image of diffraction from a crystal produced by the optimised purification and crystallisation protocols is given in figure 3.9.

Throughout the process of optimising the quality of the diffraction obtained, the unit cell remained close to that of that reported in Papiz *et al.*, 1989. Despite this, data from individual native crystals revealed significant levels of crystallographic non-isomorphous. Even when using the optimised purification and crystallisation protocols, calculation of difference Patterson syntheses between native crystals displayed significant levels of signal.

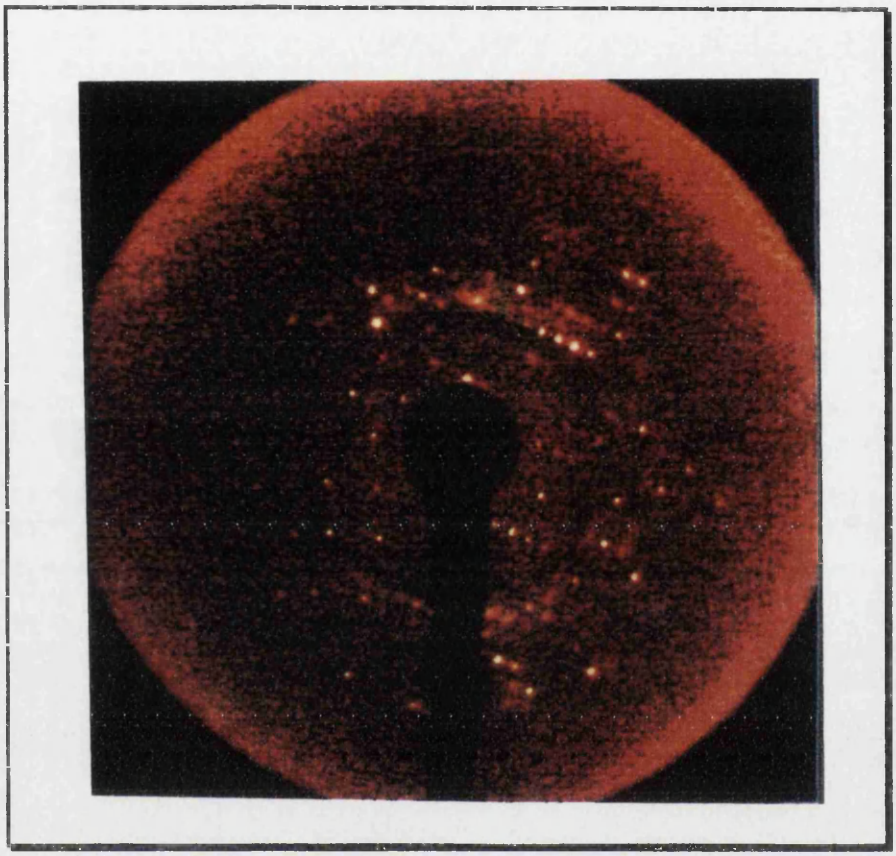

Figure 3.8. Representative of poor diffraction from native crystals. Siemens multiwire detector on a laboratory Cu k_α source. 0.25°rotation. Detector to crystal distance 150 mm.

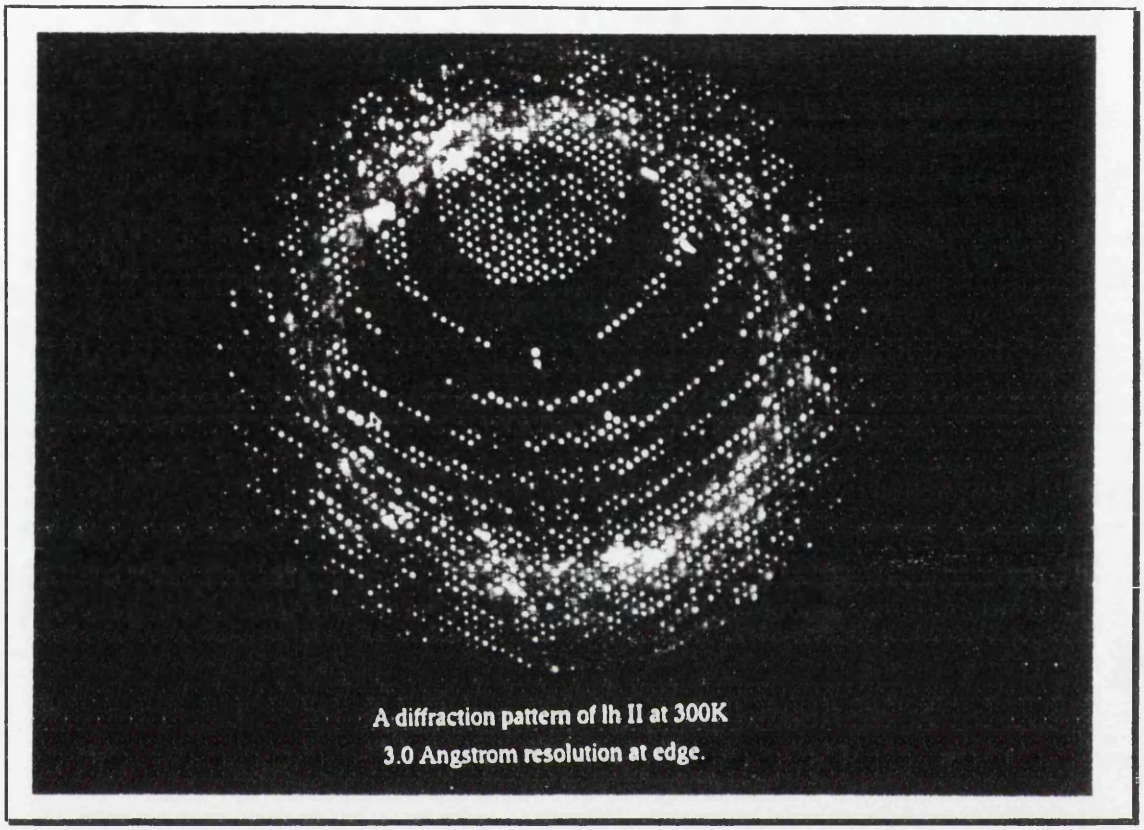

Figure 3.9. Diffraction image from 30 cm Mar image plate, Daresbury laboratory synchrotron source. 2° oscillation, detector crystal distance 500 mm. Resolution 3.0 Å at the edge of the detector plate.

3.7.1 Heavy atom derivitisation of LH II

An extensive selection of heavy atom compounds were used in heavy atom derivatisation trials. In total more than 60 compounds were assessed. Many of the compounds were re-assessed when improvements were evident in either the native diffraction or artificial mother liquor. In all cases there were no significant peaks in difference Patterson maps.[1] The difference Patterson maps looked strikingly similar for most of the heavy atom compounds assayed, and indeed similar to the maps obtained by difference syntheses between two native data sets. Therefore, the conclusion was drawn that either non-isomorphism was significant and masking the heavy atom signal, or that minimal heavy atom binding occurred.

3.7.2 Heavy atom derivatisation: The final solution

In an attempt to improve the level of crystallographic isomorphism between native and derivative crystals the following 'back-soaking' procedure was adopted;

- Crystals to be used for native data were transferred into the optimised artificial mother liquor to soak for 20 hours, and then mounted for data collection.

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[1] Difference Patterson maps are calculated directly from the observed intensities as coefficients;

$$\left|\Delta F\right|^2 = \left(|F|_P - |F|_{PH}\right)^2$$

Harker sections are essentially a vector maps of the heavy atoms alone.

- Two crystals were transferred from the same crystallisation drop to a solution containing heavy atom compound solubilised in the optimised artificial mother liquor.

- After allowing equilibration to take place for 16 hours, the crystals were removed from this solution and one crystal was mounted and used for data collection, the other was transferred back into a solution of artificial mother liquor only and allowed to soak for 4 hours.

- The back-soaked crystal was mounted and data measured.

This practice allowed three comparisons to be made per heavy atom compound assayed: native vs derivative, native vs back-soaked derivative and derivative vs back-soaked derivative. Crystals grown using the optimised purification and crystallisation protocols were transferred to the following soak solutions (see Appendix C for all solutions);

- **Pt combined** - $K_2Pt(CN)_4$ + $K_2Pt(NO_3)_4$ + $K_2Pt\ Cl_4$ (3 mM of each)

- **K_2HgI_4** - 1 mM K_2HgI_4 + 10 mM KI

Data were collected at the Daresbury laboratory on station 9.6 at a wavelength of 0.87Å using a Mar Research 30 cm image plate. The crystal to detector distance in all cases was 500 mm, and data were collected in 2° oscillations.

After processing and data reduction, scaling between native data and derivative data sets resulted in the statistics shown in table 3.1. Two difference Patterson syntheses were calculated: for the native vs K_2HgI_4 and K_2HgI_4 vs 'back-

soaked' K_2HgI_4 data, the Harker section is shown in figure 3.10. The latter analysis revealed a single mercury site. Further analysis finally resulted in establishing the positions of six mercury sites. In the first instance, analysis of the Pt combined data, again by calculating a soaked vs back-soaked difference Patterson, reveled 3 sites, which after cross phasing could be extended to include a further 3 sites.

Data set	No. of crystals	Resolution Å	Completeness %	R_{merge}	R_{iso}	No. of sites
Native	1	3.0	99	3.0		
K_2HgI_4	1	3.0	93.4	3.6	11.7	6
Pt combined.	1	3.0	90.6	3.7	12.8	6

Table 3.1. Merging and scaling statistics for the three data sets from which initial heavy atom positions were obtained.

$$R_{merge} = \frac{\sum_h \sum_j |I(h) - I(h)_j|}{\sum_h \sum_j I(h)_j}$$

where $I(h)$ is the mean intensity for reflection h.

$$R_{iso} = \frac{\sum_h \| F_{PH}| - |F_P \|}{\sum_h |F_P|}$$

where $|F_P|$ and $|F_{PH}|$ are the native and derivative structure factor amplitudes, respectively.

Further data were subsequently measured by Drs. S.M. Prince, M.Z. Papiz and A.M. Hawthornthwaite on crystals which had been soaked in two of the individual compounds of the Pt combined soak (both at a concentration of 3 mM) together with higher resolution native data from nine native crystals merged to give native(2), shown in table 3.2.

After many years of assuming that the lack of derivative data was due to too little binding of heavy atom, unexpectedly it was found that the converse was true. The absence of obvious indications of derivatisation were actually due to the exceptionally high levels of substitution which had been occurring during soaking experiments. The technique of back-soaking the crystals simple removed the less tightly bound sites - see Chapter 5 for a discussion

Data set	No. of crystals	Resolution Å	Completeness %	R_{merge}	R_{iso}	No. of sites	Phasing Power Acentric (centric)	R_{cullis}
Native (1)	1	3.0	99	3.0				
Native (2)	9	2.5	98.9	5.0				
K_2HgI_4	1	3.0	93.4	3.6	11.7	6	0.9 (0.8)	0.83
Pt comb.	1	3.0	90.6	3.7	12.8	6	1.4 (1.0)	0.71
$K_2Pt(CN)_4$	1	3.0	96.8	3.3	13.0	3	1.0 (1.0)	0.67
K_2PtCl_4	1	3.0	95.8	3.8	11.1	3	1.5 (1.2)	0.57

Table 3.2. Summary of the merging and scaling statistics of data used to calculate the refined heavy atom positions and phases.

$$R_{merge} = \frac{\sum_h \sum_j |I(h) - I(h)_j|}{\sum_h \sum_j I(h)_j}$$

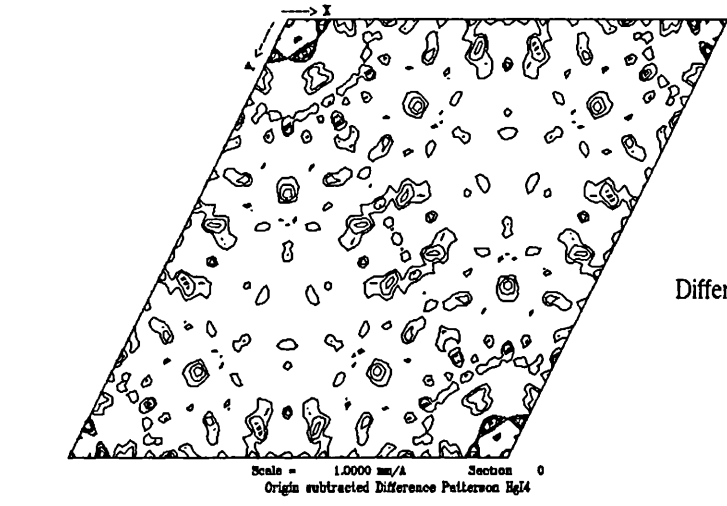
where $I(h)$ is the mean intensity for reflection h.

$$R_{iso} = \frac{\sum_h \| F_{PH}| - |F_P \|}{\sum_h |F_P|}$$

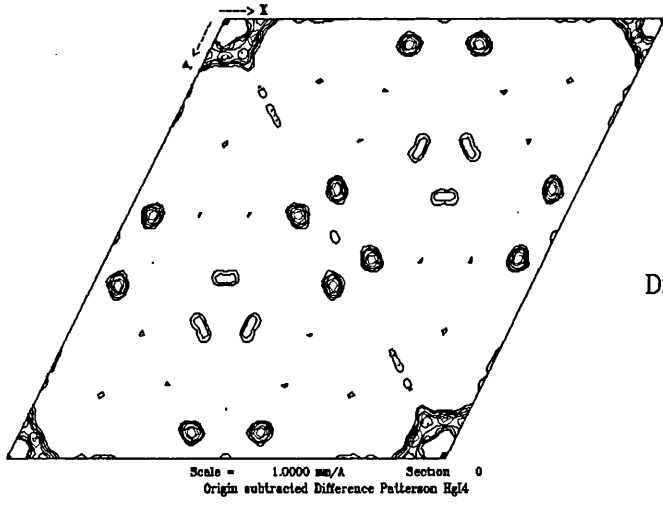
where $|F_P|$ and $|F_{PH}|$ are the native and derivative structure factor amplitudes, respectively.

$$R_{cullis} = \frac{\sum_h \| F_{PH} - F_P| - |F_{H\,calc}|}{\sum_h \| F_{PH}| - |F_P \|}$$

where $F_{H\,calc}$ is the calculated structure factor amplitude for the heavy atom.



Difference Patterson Native/Soak
Harker Section



Difference Patterson Soak/Back-soak
Harker Section

Figure 3.10. Difference Patterson syntheses calculated using data from:
Top - K_2HgI_4 soaked and Native (1)
Bottom - K_2HgI_4 soaked and back-soaked K_2HgI_4

3.8 Building the structural model

Locating the heavy atom positions, calculation of phases and resultant electron density maps, the application of non-crystallographic symmetry averaging to improve phasing and the calculated electron density, model building and its subsequent refinement were performed principally by Dr. Stephen M. Prince of Glasgow University, in collaboration with Dr. Miroslav Papiz of Daresbury SRS Laboratory. Valuable input - both practical and intellectual - to this stage of the structure determination was provided by Prof. Neil W. Isaacs and Dr. Andy A. Freer, of Glasgow University.

In the context of this thesis this work will not be discussed. Except to comment that even a cursory inspection of the initial calculated electron density map revealed that the overall molecular assembly of LH II was radically different from that previously predicted. In particular, it was strikingly apparent that the asymmetric unit did not consist of an entire complex as previously proposed (Papiz *et al.*, 1989), but instead contained one third of an LH II complex. In addition, the presence of nine-fold non-crystallographic symmetry, co-axial with the crystallographic three-fold axis was also strikingly apparent.

To allow non-specialists to gauge the accuracy of the structural model, representative examples of electron density into which the structural model was built are show in figures 3.11, 3.12, 3.13 and 3.14 - all figures were generated using the program O (Jones *et al.*, 1991).

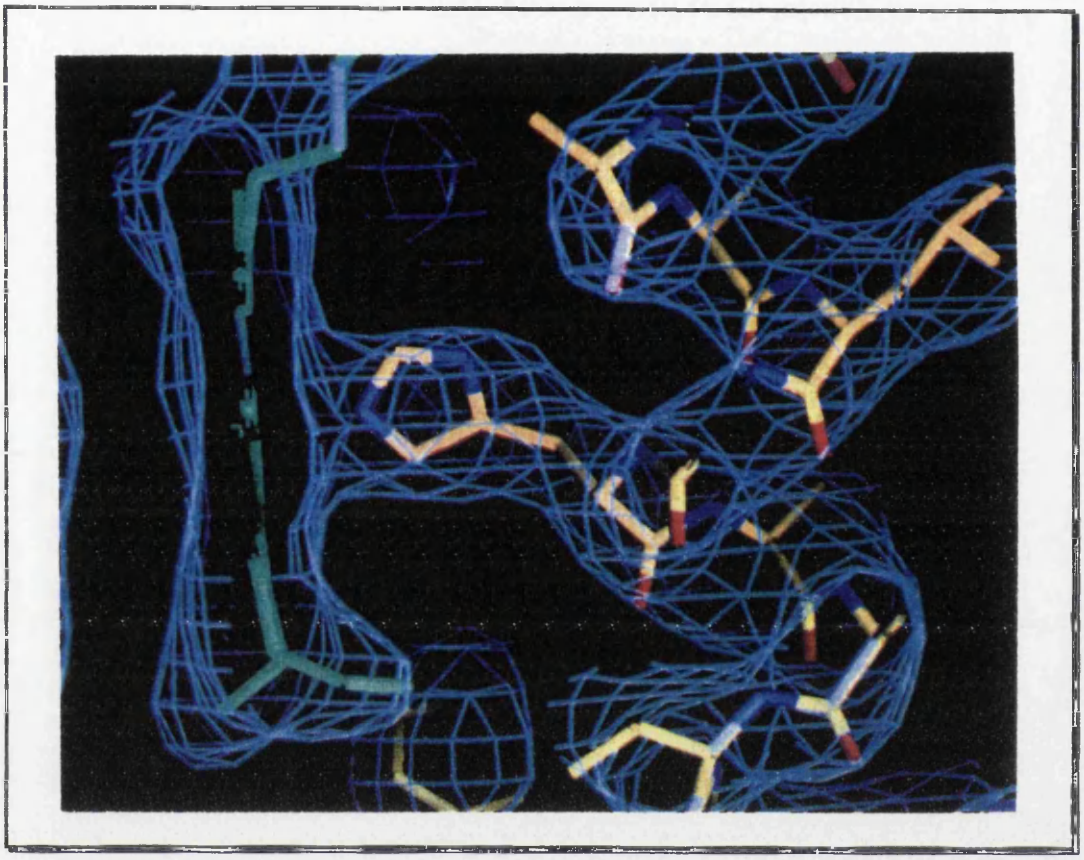


Figure 3.11. Section of apoprotein and pigment electron density

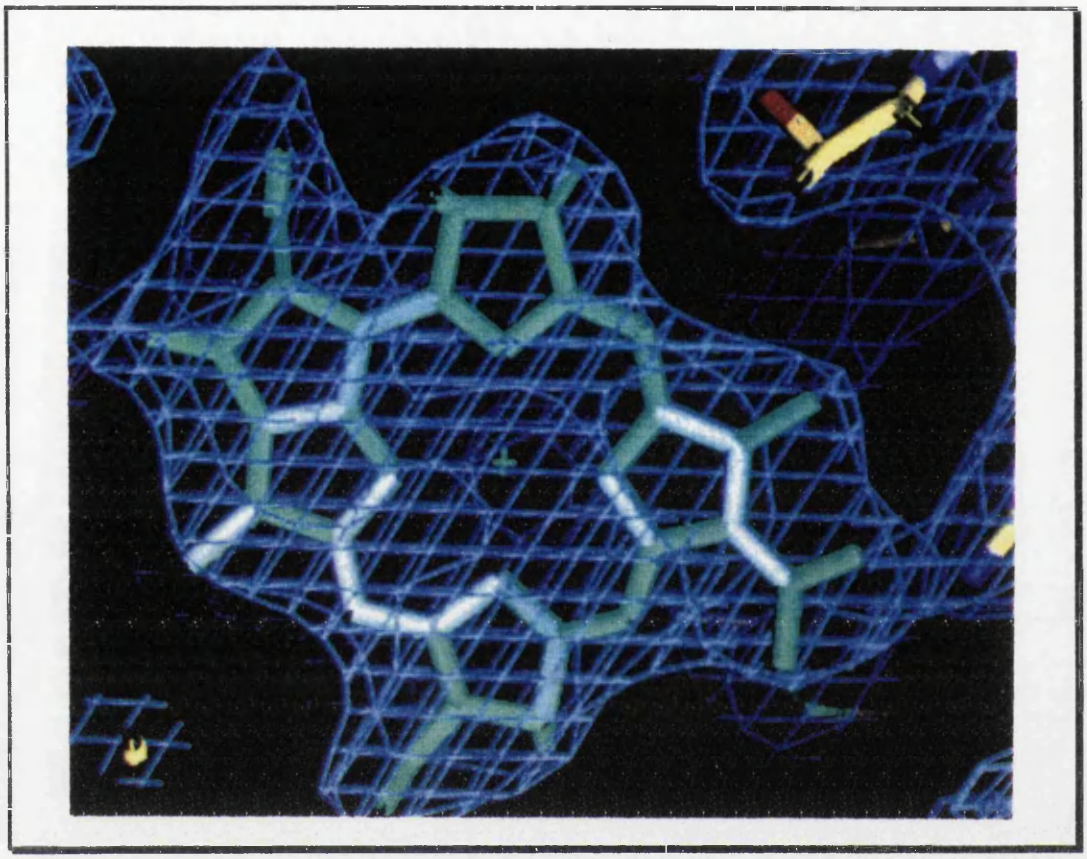


Figure 3.12. Electron density fitted with a Bacteriochlorophyll *a* molecule

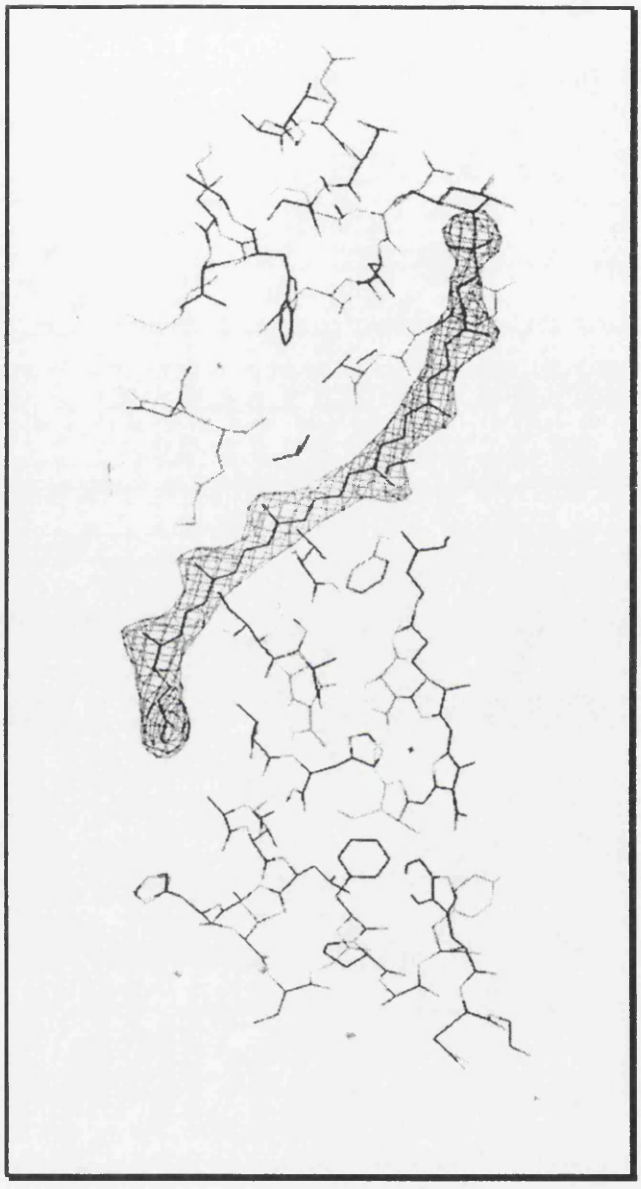


Fig 3.13 Electron density assigned to the carotenoid molecule rhodopin glucoside.

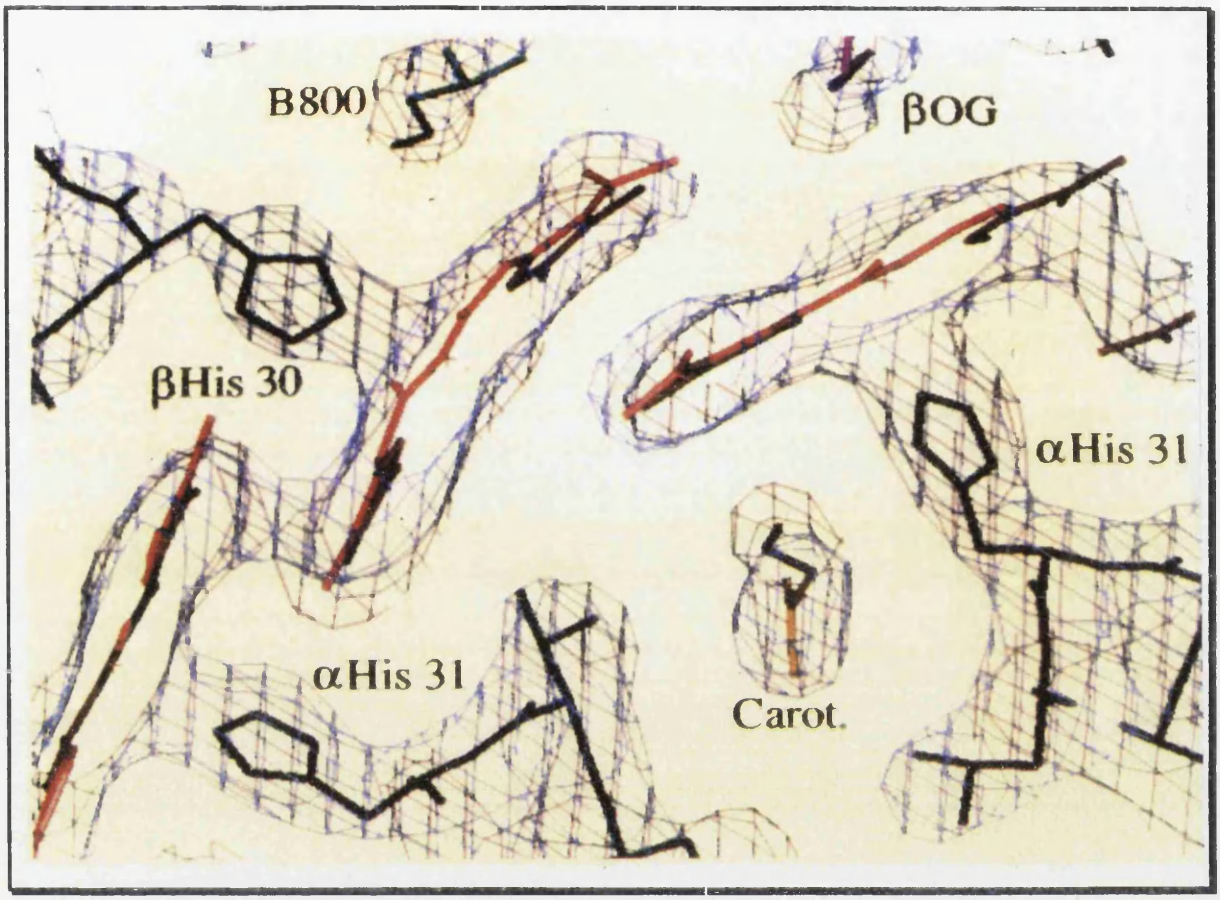


Figure 3.14 Fitted electron density in the region of the B850 BChl *a* molecules. Protein shown as black, B850 Bchl as red, carotenoid as yellow, a portion of a B800 phytl chain green and a portion of a detergent molecule (β–Octyl-glucoside) as magenta.

3.9 Summary

Microspectroscopy measurements made on crystals of LH II confirmed that the integrity of the complex was maintained during the crystallisation process. Linear dichroism analysis of crystals identified the criticality of the detergent concentration in the artificial mother liquor used for heavy atom derivatisation experiments. Once this parameter had been identified it was possible to conduct significantly longer heavy atom soaks. This eventually led to the development of a protocol which produced derivatives of sufficient quality to permit the calculation of a high resolution and easily interpretable electron density map.

Chapter 4

LH II Structural Model

4.1 Introduction

The structural model of LH II is described in McDermott *et al.*, 1995. This report is included as supplementary material, and will be central to the description of the structural model.[1] For the sake of improved clarity, the figures in this report will be reproduced in a larger format in this Chapter. The pigment-pigment interactions have been described in some detail in Freer *et al.* 1996. This can also be found in the supplementary materials section. The coordinates of the model have been deposited in the Brookhaven Protein Data Bank with the designated identity code 1KZU.

The presence of non-crystallographic symmetry allows description of the molecular structure of LH II at two levels. Firstly, as a structural account of the contents of the protomer unit, that is to say the unique portion of the molecule upon which the application of nine-fold non-crystallographic symmetry operates to construct a complete molecule, and secondly as a description of the assembled nonameric complex.

4.2 The protomer

The choice of protomer is arbitrary and is not correlated in any sense with photosynthetic function. The protomer unit bears no relation to the so called

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[1] NB. The spelling of BChl phytol chains has been changed to 'phytl chains' in this thesis. It had been pointed out that this is more correct, since phytol implies the chain contains an alcohol group.

photosynthetic 'minimal unit' commonly described in the literature by, for example, van Grondelle (van Grondelle, 1985) or Zuber & Cogdell (Zuber & Cogdell, 1996). The protomer chosen is composed of a radially associated α and β apoprotein pair, a rhodopin glucoside molecule and three bound BChl *a* molecules.

4.2.1 The protomer apoproteins

From the calculated electron density map it was possible to assign all of the β apoprotein sequence and residues 1 to 49 of the α apoprotein. The principal structural motif in both apoproteins is a long α helical domain, which is presumed to be membrane spanning (Zuber, 1986). These and the other helical components of both apoproteins are summarised in table 4.1.

In both α and β polypeptides the N terminus is located in the cytoplasmic side of the membrane (Zuber & Brunisholz, 1993). In the α apoprotein the N terminus is buried 9 Å into the presumed membrane surface, and ligands the central magnesium atom of the B800 BChl molecule (see below). Both apoproteins are approximately parallel to the three-fold crystallographic axis: α to within 2° and β with an inclination of 15° relative to the axis. The B850 binding Histidine residues (α His31 and βHis30) are located 10 Å into the membrane, from the presumed periplasmic side (figures 4.1, 4.2 and 4.3). Between apoproteins, there are no protein-protein contacts in the trans-membrane region, all contacts occur in the hydrophilic domains.

Helix No.	Sequence
1	GKIWT
2	PAIGIPALLGSVTVIAILVHLAILS
3	WFPAYWQ
4	AEQSEELHKYVIDGTRVFLGLALVAHFLAFSA

Helix No.	Residues	Helix type	Length (Å)	Residues per turn	Pitch
1	α4 - α8	3_{10}	10.04	3.23	6.60
2	α12 - α36	α	37.21	3.64	5.40
3	α40 - α46	α	10.38	3.60	5.09
4	β5 - β36	α	47.02	3.80	5.60

Table 4.1. LH II helices and their geometry. Generated by the CCP4 program PROCHECK. The β apoprotein helix is shaded.

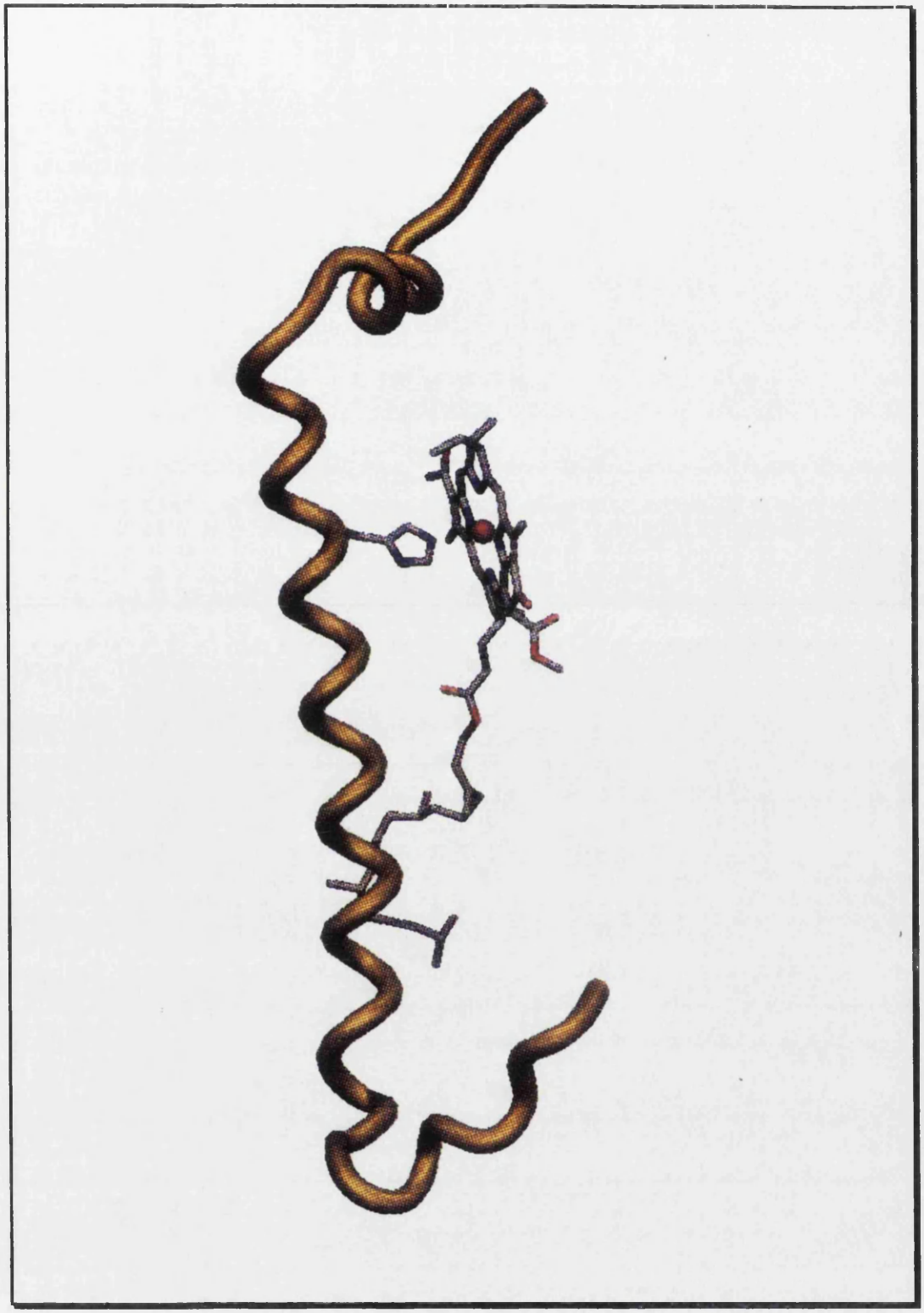

Figure 4.1. The α apoprotein with α His31 highlighted.

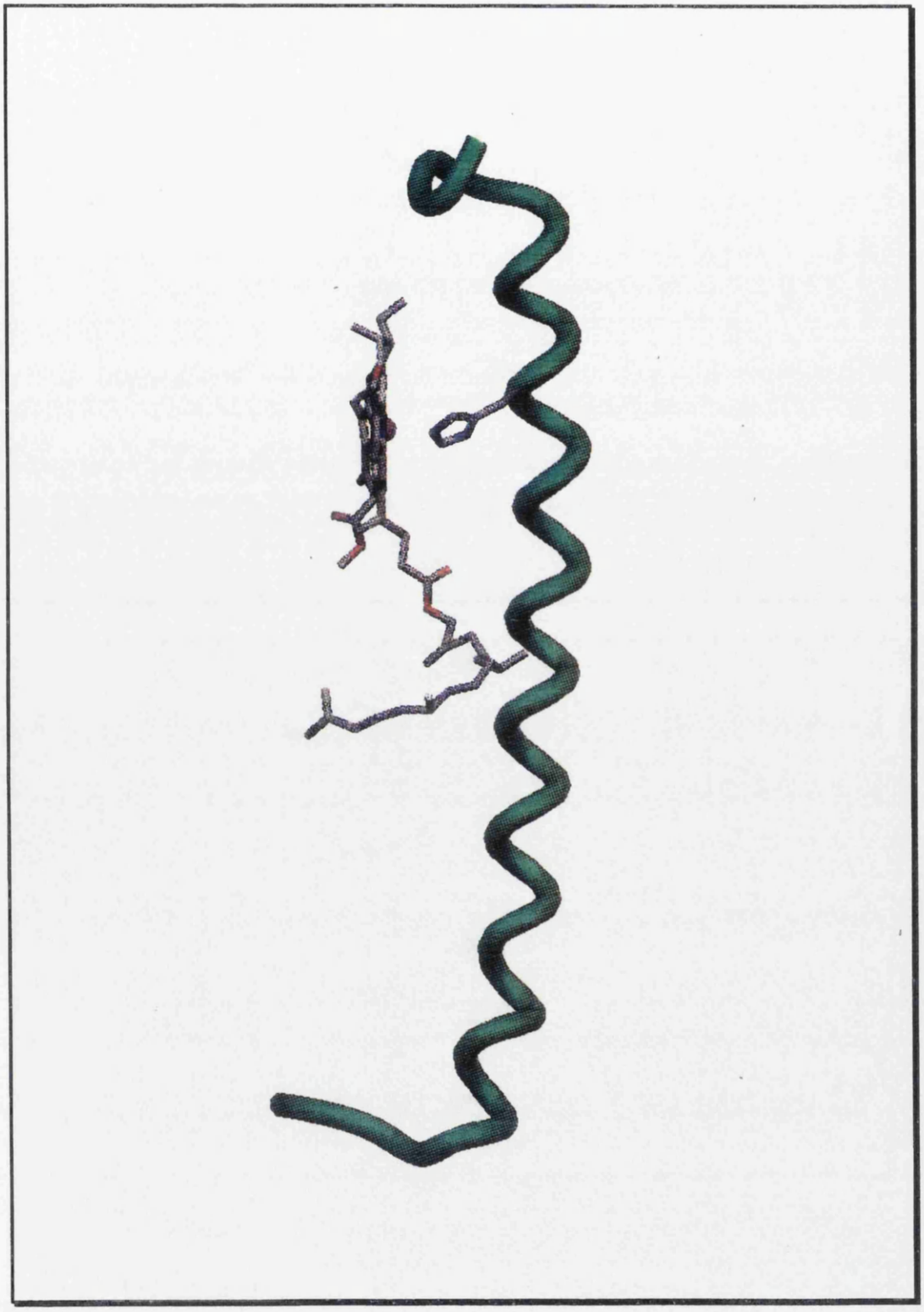

Figure 4.2. The β apoprotein with βHis30 highlighted

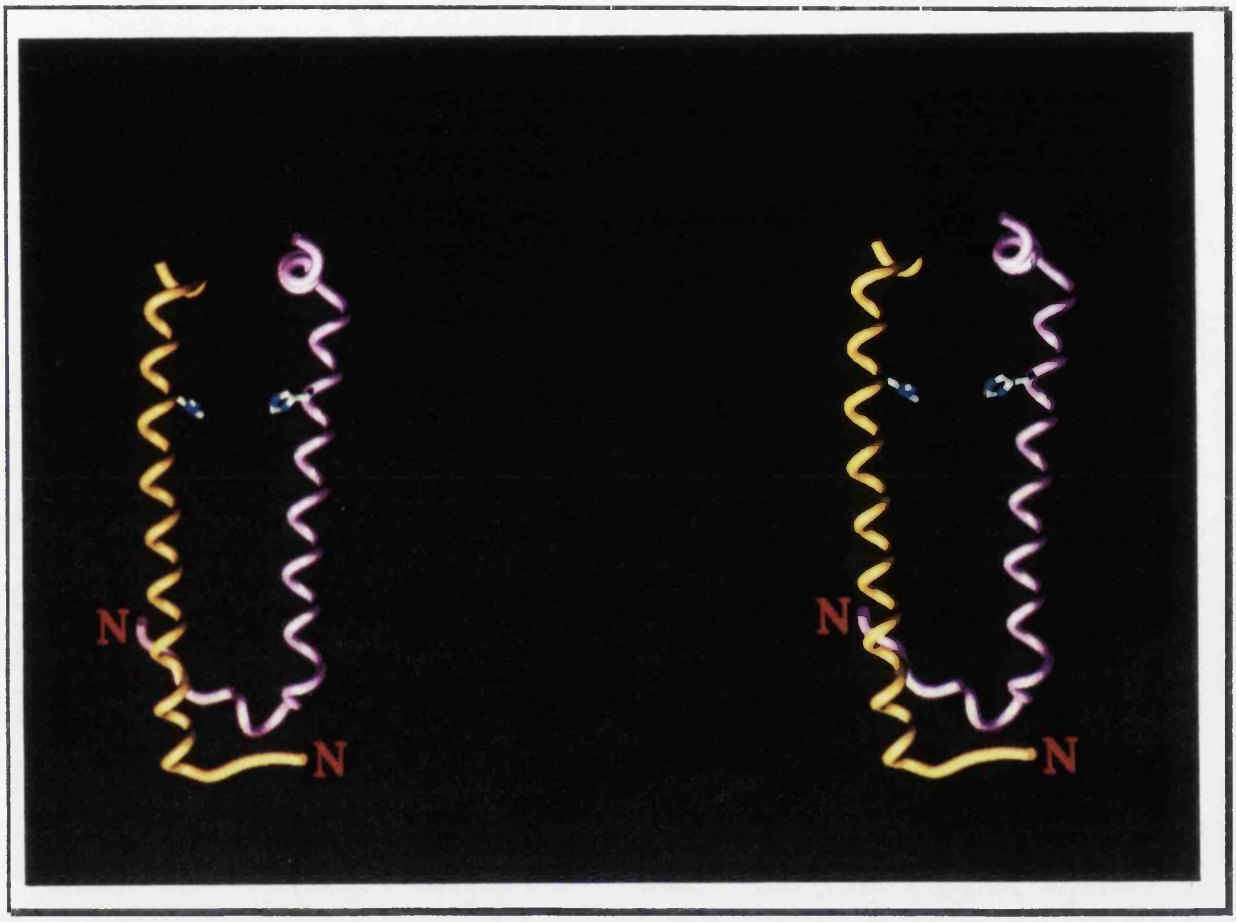

Figure 4.3. Stereo view of the apoproteins. α as magenta and β as yellow. The position of the His residues which ligand the B850 BChl *a* molecules are shown.

4.2.2 The protomer bacteriochlorophylls

With reference to presumed membrane surfaces, the BChl *a* molecules are located in two spatially disparate environments. Conserved Histidine residues at positions αHis 31 and βHis 30 are the ligation sites for the central Mg atom of two of the BChl *a* molecules, with the effect that the BChl *a* chlorin rings are perpendicular to the supposed membrane surface. Several large aromatic residues - α Trp40, α Trp45, α Tyr44 and βTrp34 - are involved in stabilising the binding of the bacteriochlorin ring, and form a binding pocket by way of hydrogen bonding and hydrophobic interactions, shown in figure 4.4.

The central Mg atoms of the B800 BChl ligands to the formyl oxygen of fMet1 on the N-terminal of the α apoprotein, shown in figure 4.5. The presence of fMet at the N terminal of α was unexpected, as the published amino acid sequence proposes this residue as being Methionine (Zuber & Bruinisholtz, 1993). The position of the fMet sulphur atom was located accurately by the production of a seleno-methionine derivative[2]. Consequently, ligation of the B800 BChl via the Methionine sulphur atom could be eliminated.

Spatially, the B800 BChl *a* molecules are located perpendicular to the 3-fold crystallographic axis and are approximately parallel with the presumed membrane surface.

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[2] Work was performed by Drs A.M. Hawthornthwaite-Lawless and M.Z. Papiz, Daresbury Laboratory, England.

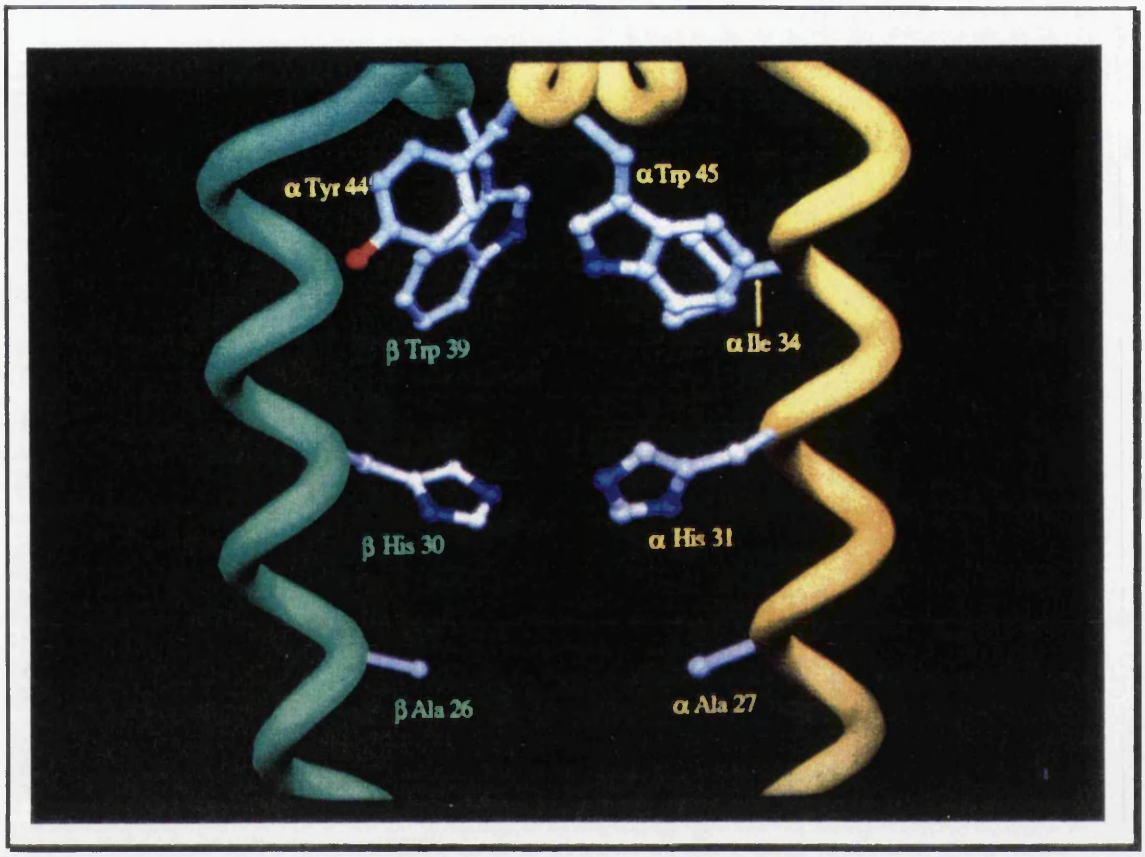

Figure 4.4. The residues which form the B850 BChl *a* binding pocket.

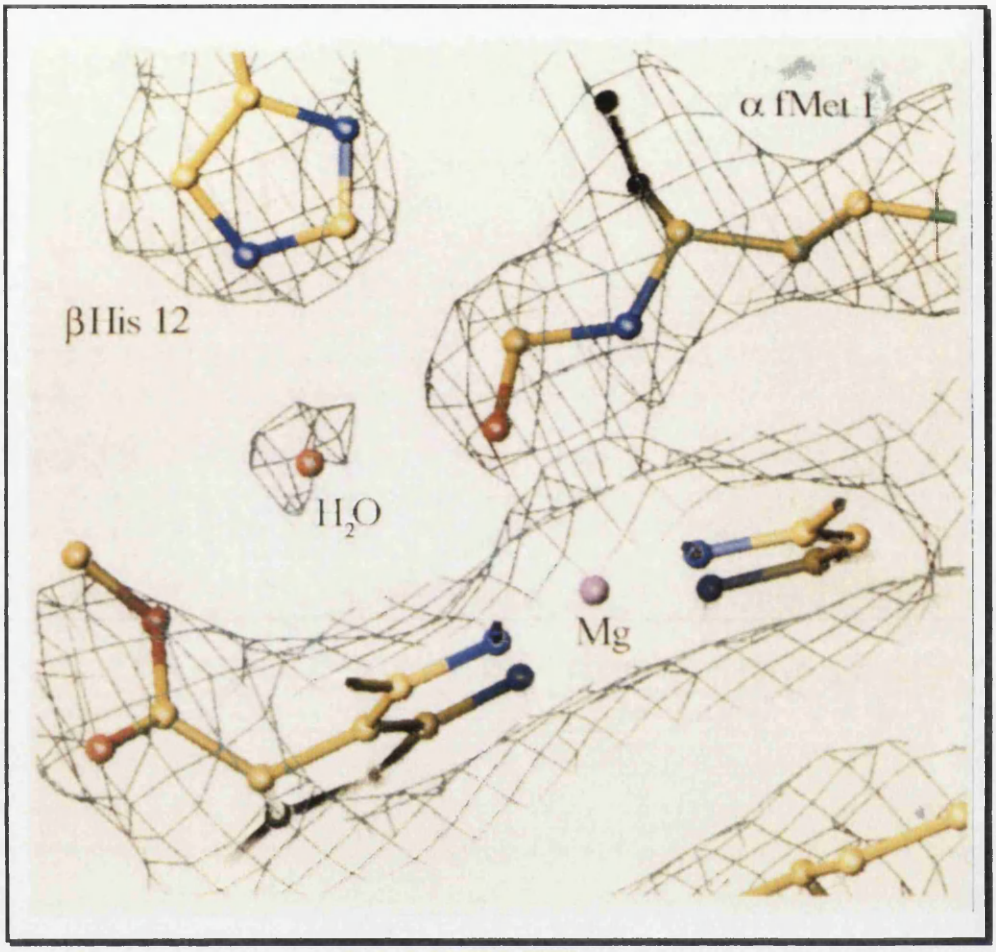

Figure 4.5. Fitted electron density (MIR phased, solvent flattened and symmetry averaged) at the location of the B800 ligation to α fMet 1. A fitted formyl group and adjacent buried water molecule are shown, together with fitted density from the β apoprotein. Distance from the formyl oxygen to BChl *a* Mg is approximately 2.46Å. The atoms are coloured red for oxygen, blue for nitrogen, yellow for carbon, magenta for magnesium and green for sulphur.

4.2.3 The protomer carotenoid

In the published model only one carotenoid molecule was fitted per protomer. A region of electron density was postulated as being a β Octyl-glucoside molecule which had replaced a carotenoid during purification. This would seem to be incorrect, since similar density has recently been observed with data from crystals grown in LDAO (Dr. S.M. Prince, personal communication). Therefore the presence of a second carotenoid molecule in the protomer is still open to question (see section 4.3.2). The carotenoid molecule (rhodopin glucoside) fitted to the model is not directly associated with any single apoprotein. The molecule spans the proposed distance of the membrane parallel to the crystallographic 3-fold axis. The sugar group is located in the extramembranous region in van der Wals contact with αLys5 and αThr8. The molecule has a twisted conformation, suggesting the all trans conformation. The carotenoid makes a number of van der Wals contacts with residues in both the α and β apoproteins and terminates with the terminal dimethyl groups being in close contact with the B850 BChl ligating residue α His 31.

4.3 The nonameric assembly.

The apoproteins and the pigment moieties of the asymmetric unit i.e. from three protomers, were individually fitted to the calculated electron density. The r.m.s of agreement between these is approximately of 0.25 Å, and hence indicative of the presence of near exact non-crystallographic symmetry.

The surface potential of the assembled nonamer was calculated using the program Grasp (Nichols, 1993) figure 4.6.

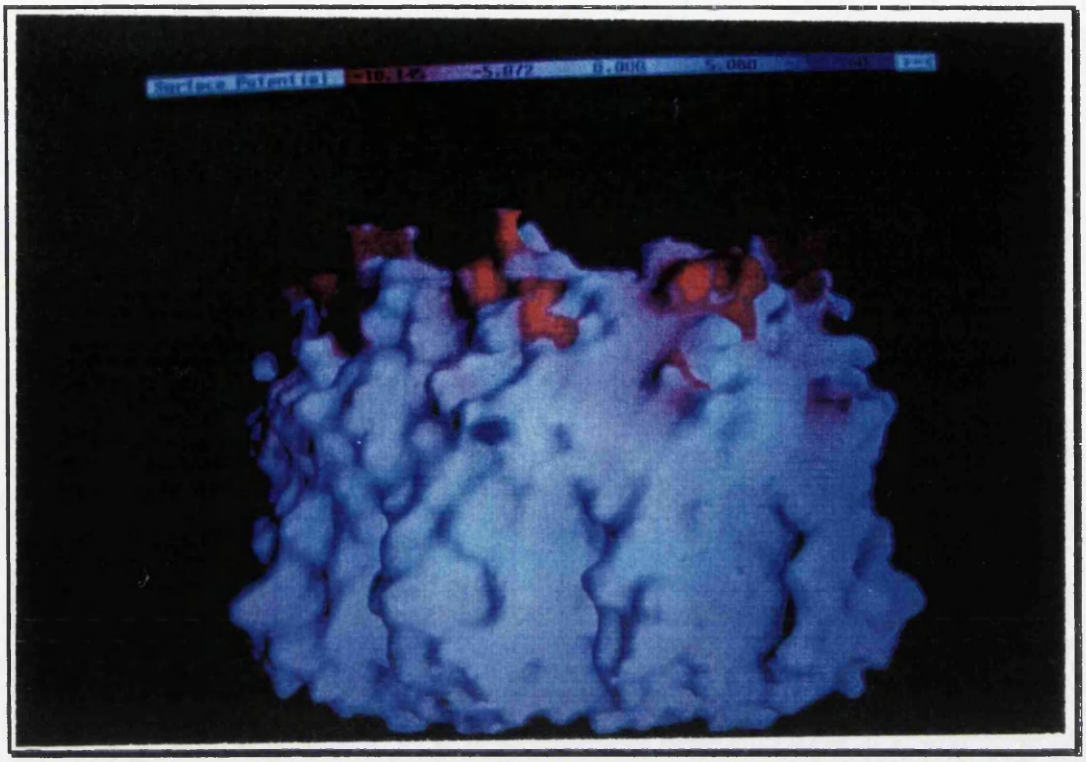

Figure 4.6. The surface potential of the nonameric assembly perpendicular to the proposed membrane surface.

In the proposed trans-membrane region, a lack of charged residues is apparent on both the 'inner' and 'outer' surfaces of the nonamer. The only exception being βArg20 and βAsp17, which form an ion pair on the outer sleeve of the nonamer.

The apparent lack of charged residues in the central region suggests that *in vivo* this is packed with lipid molecules. HPLC analysis to determine the lipid content of crystals (G. Dahler & P.J. Dominy, personal communication) revealed that crystals contained similar phospholipids to those found in the photosynthetic membrane *per se*. Despite this observation, no density was observed in a calculated Fo-Fc electron density map which could be attributed to either ordered lipid or detergent molecules.

4.3.1 The apoproteins

The apoproteins form two concentric cylinders; α apoproteins forming the inner sleeve of radius 14Å and β apoproteins an outer sleeve of radius 34Å. All of the pigments are located in the internal cavity formed by the two sets of apoproteins, shown in figures 4.7 and 4.8. Interaction between apoproteins only occur at the C and N termini. There are no inter molecular apoprotein contacts in the transmembrane domain. At the C terminus there are interactions between adjacent and radial α and β apoproteins, with the aromatic residues αTrp40, αTrp45, αTyr44 and βTrp36 binding the apoproteins together through a series of hydrogen bonds and hydrophobic interactions.

Figure 4.6. The assembled nonamer protein and pigments. Viewed parallel to the cytoplasmic membrane surface. Apoproteins are shown in white, B800 BChls shown in green, B850 BChls in red and the carotenoid in yellow.

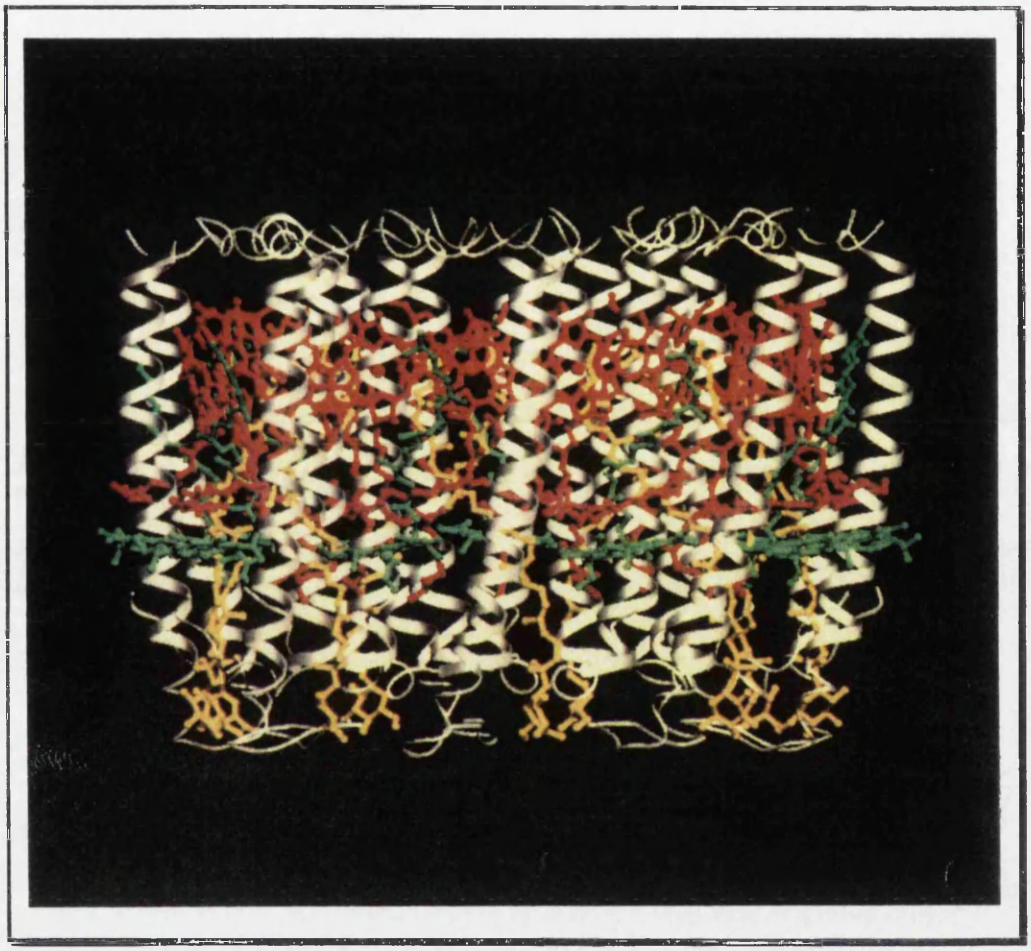

Figure 4.7. The assembled nonamer protein and pigments. Viewed perpendicular to the symmetry axis. Apoproteins are shown in white with broad ribbon representing the transmembrane helices, B800 BChls shown in green, B850 BChls in red and the carotenoid in yellow.

4.3.2 Carotenoid

Previous biochemical analysis suggested that the ratio of BChl *a*: carotenoid was 2:1 (Evans, 1988; Evans 1989; Zuber & Bruinisholtz, 1993; Hawthornthwaite & Cogdell, 1993), that is to say 13.5 carotenoids per nonameric assembly. It is unlikely that this number is close to being correct, especially in the light of the large error in the reported estimation of the molecular weight of the complex (Cogdell *et al.*, 1983). In addition, the environmental equivalence, which arises from the nine-fold symmetrical arrangement would imply that the only possible number of carotenoids which the molecule could house is nine, or higher multiples of nine. An arrangement where a carotenoid molecule is 'shared' between two protomers is not a viable proposition due to stoichiometric constraints imposed by a nonameric assembly. Although, no electron density which could account for a second carotenoid molecule was observed, a region of density, apparent in an Fo-Fc map, allowed the fitting of a β-Octyl glucoside detergent molecule. Clarification as to whether or not a carotenoid molecule has been replaced by a detergent molecule requires data obtained from LH II crystals grown in the presence of another detergent[1] which is of a markedly different molecular structure to rhodopin glucoside.

The position of the carotenoids relative to the two populations of BChl *a* molecules is shown in figure 4.8.

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[1] In particular LDAO, which does not contain a sugar head group

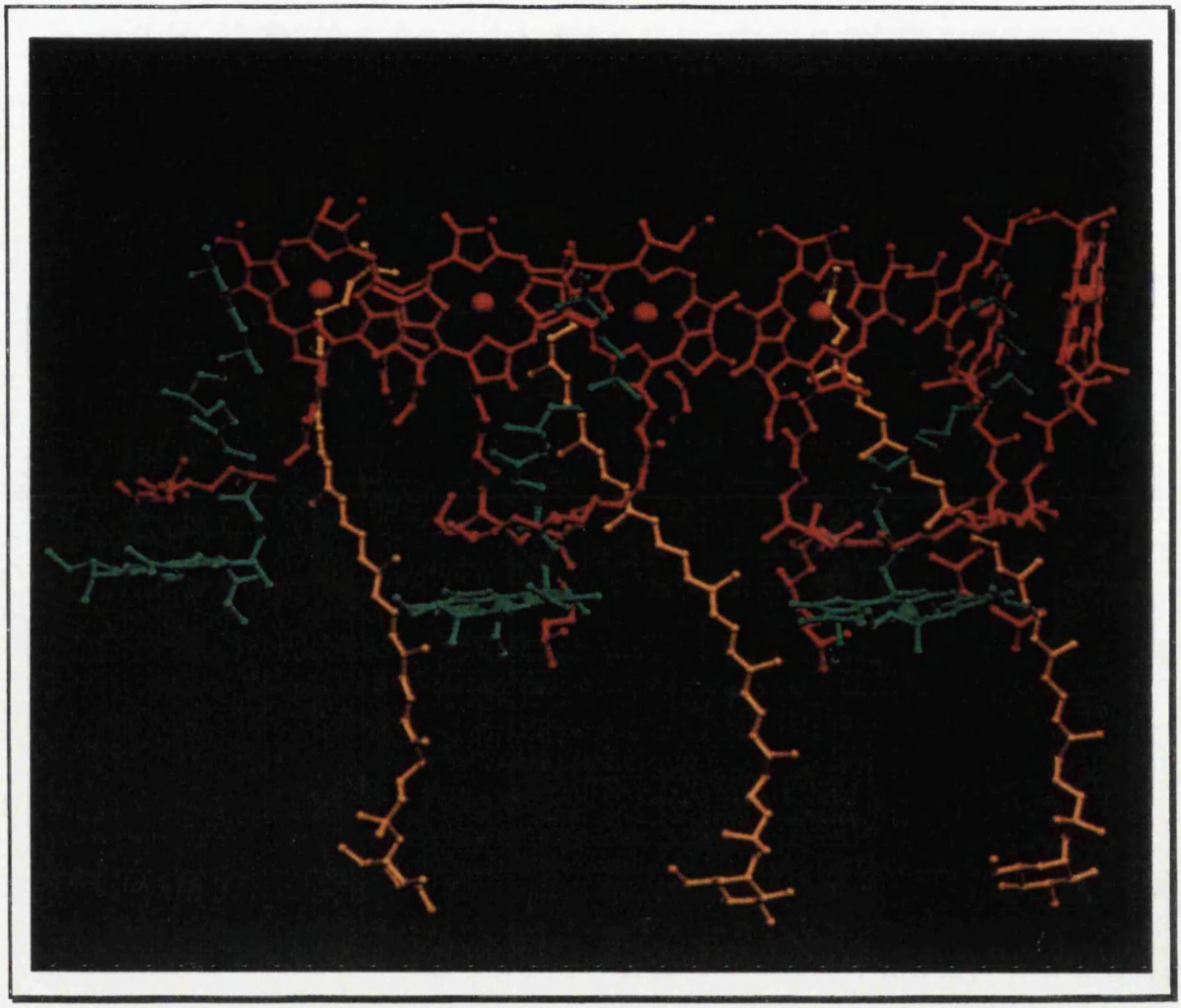

Figure 4.8. The pigments of the asymmetric unit. The carotenoid is coloured yellow, the B800 Bchls green, the B850 BChl bound to the α apoprotein orange and the B850 BChl bound to the β apoprotein red.

4.3.3 B800 BChls

The B800 BChls are located in the space between two β apoproteins, shown in figure 4.9. The distance from central Mg to central Mg of adjacent B800 BChls is 21 Å. Their location is such that they are not excitonically coupled, and can be regarded as being 'monomeric'.

4.3.4 B850 BChls

In the nonamer the location of the B850 BChls leads to the formation of an overlapping ring of 18 BChl molecules, shown in figure 4.10 There is a slight discrepancy in the distances between two BChl molecules in the same protomer and that between protomers. In the report of McDermott *et al.* 1995 this was quoted as 8.7Å between molecules in the same protomer and 9.7Å between molecules in adjacent protomers. After further refinement of the structure this discrepancy was reduced to 9.05Å and 9.53Å, respectively (shown in figure 4.11). The distances between the bacteriochlorin rings, at their extremities, allows for van der Waals overlap.

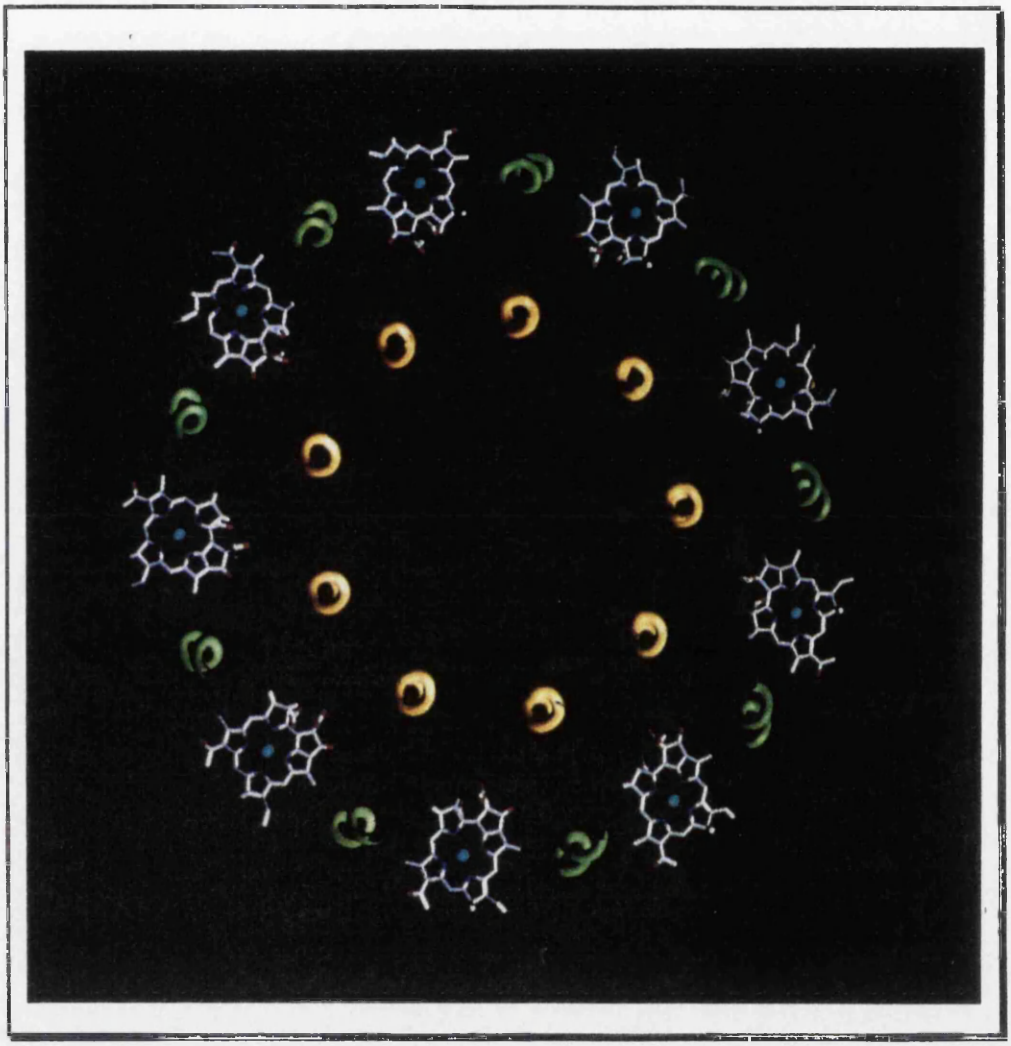

Figure 4.9. The position of the B800 BChl molecules relative to the apoproteins. The α apoprotein is coloured yellow and the β apoproteins green.

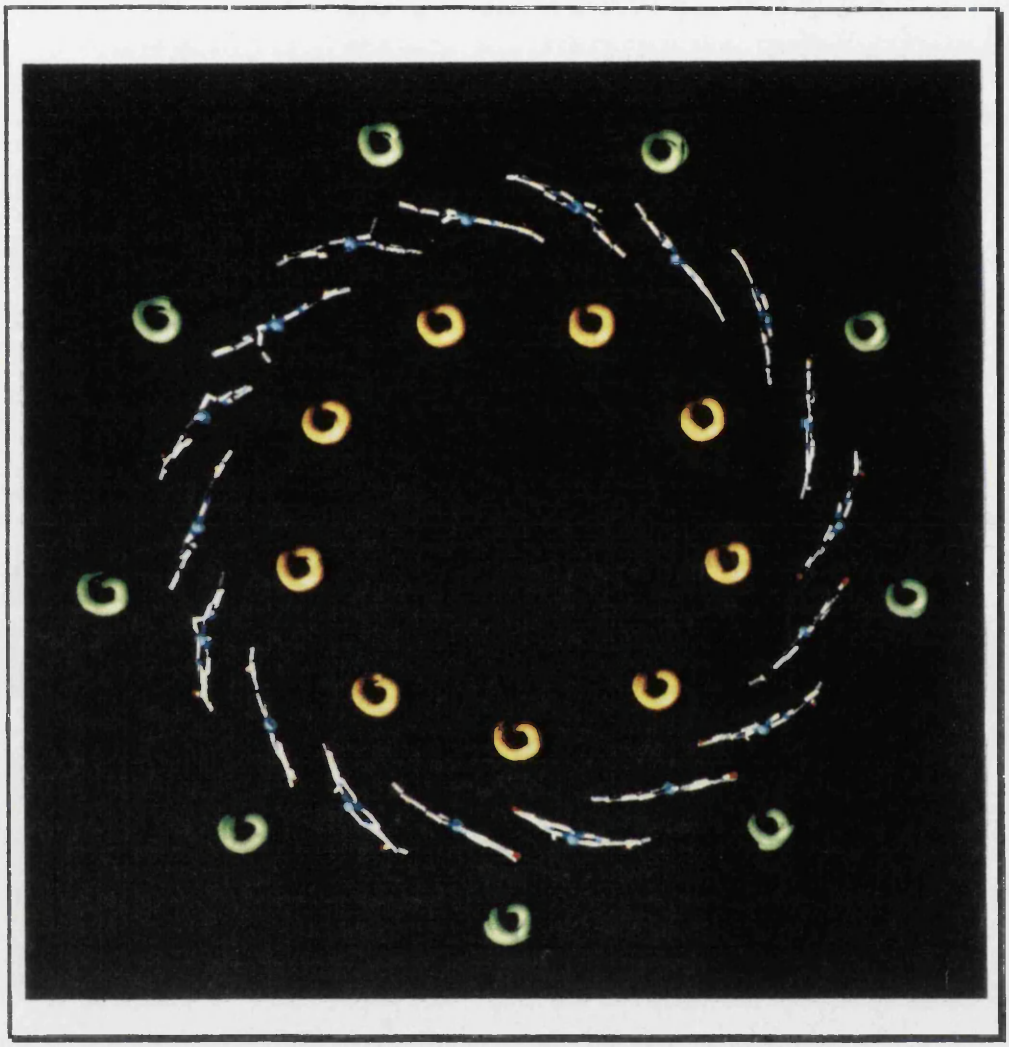

Figure 4.10. The 'ring' of 18 B850 Bchls. The α apoprotein is coloured yellow and the β apoproteins green.

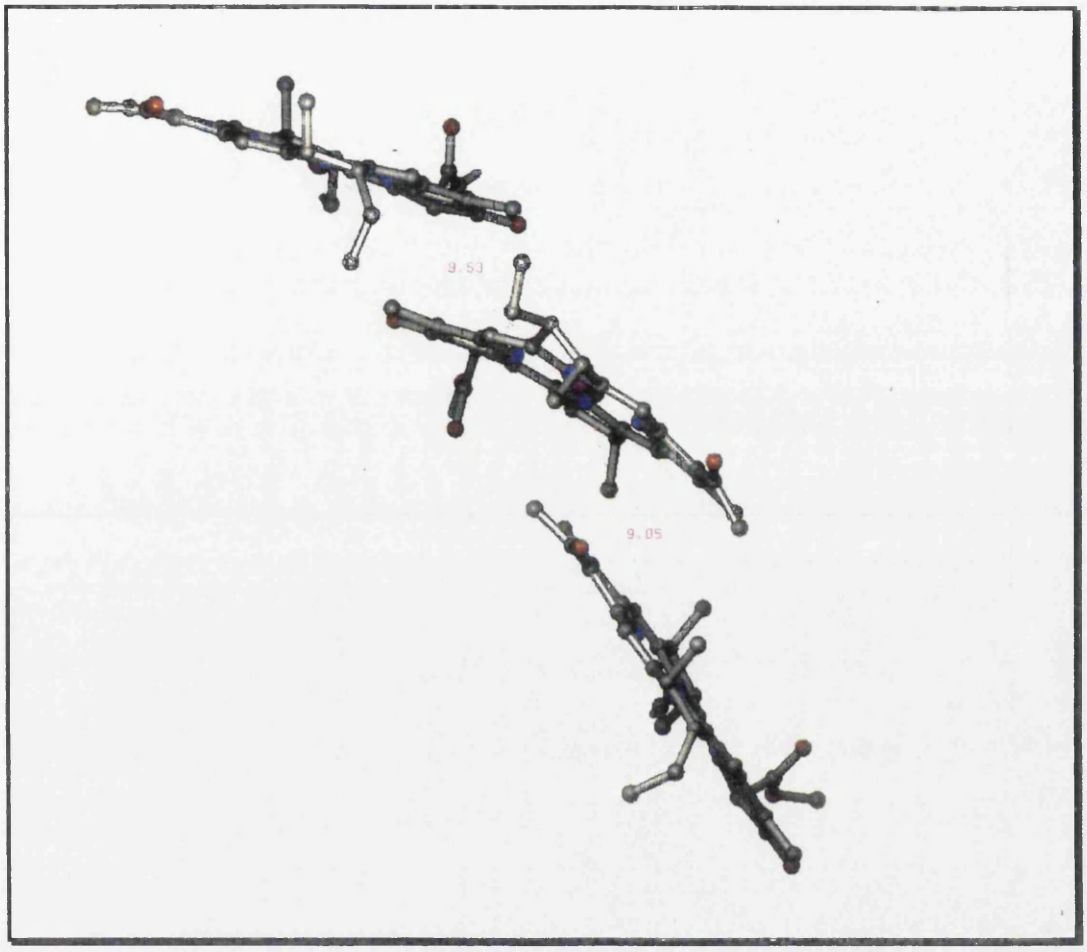

Figure 4.11. The distances between B850 BChls.

4.3.5 The phytl chains

The phytl chains of both the B800 and B850 BChl species make a number of close appproaches (within van der Waals distance). For example the phytl chain of the α B850 BChl terminates close to the B800 bacteriochlorin ring, shown in figure 4.12. The conformation of the phytl chains of each population of BChl is distinct. All three are shown in figure 4.13

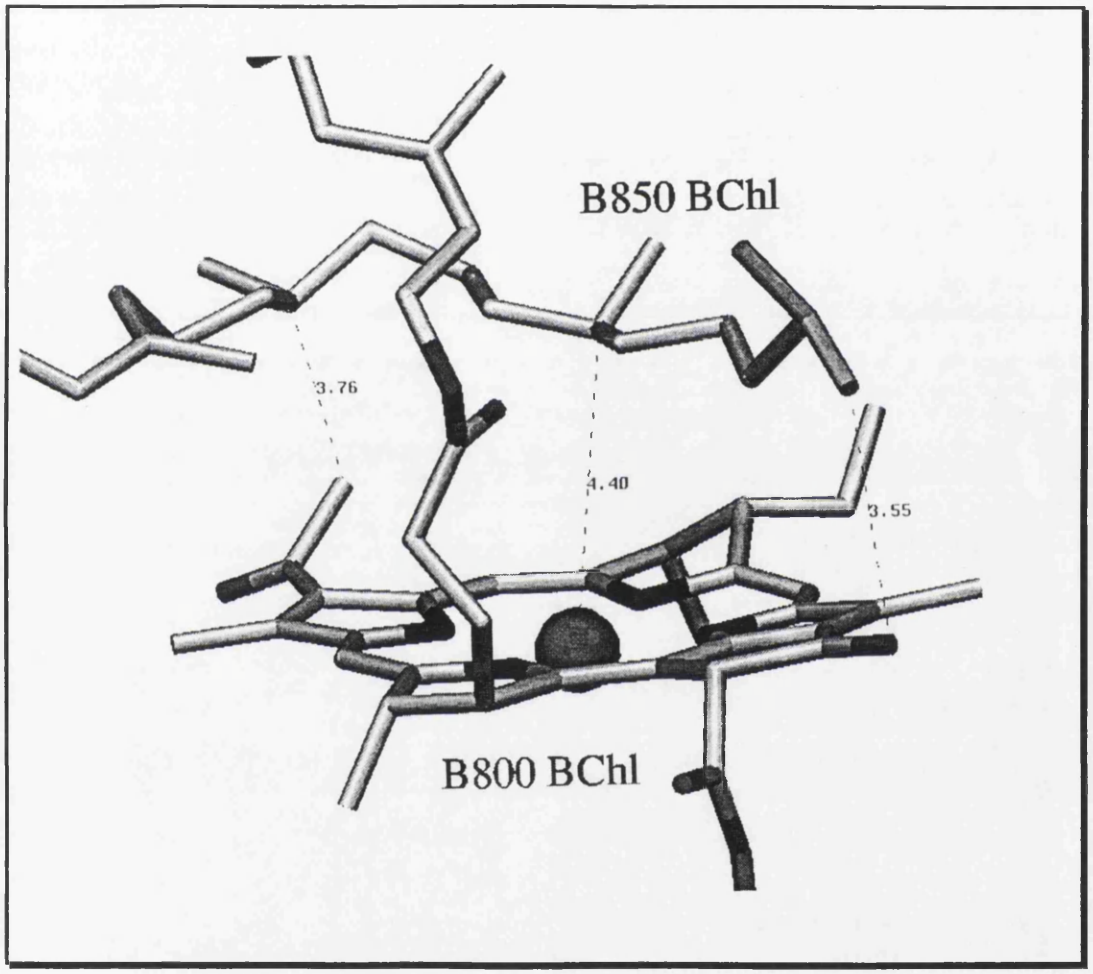

Figure 4.12. The close approach of the B850 phytl chain to the face of the B800 BChl.

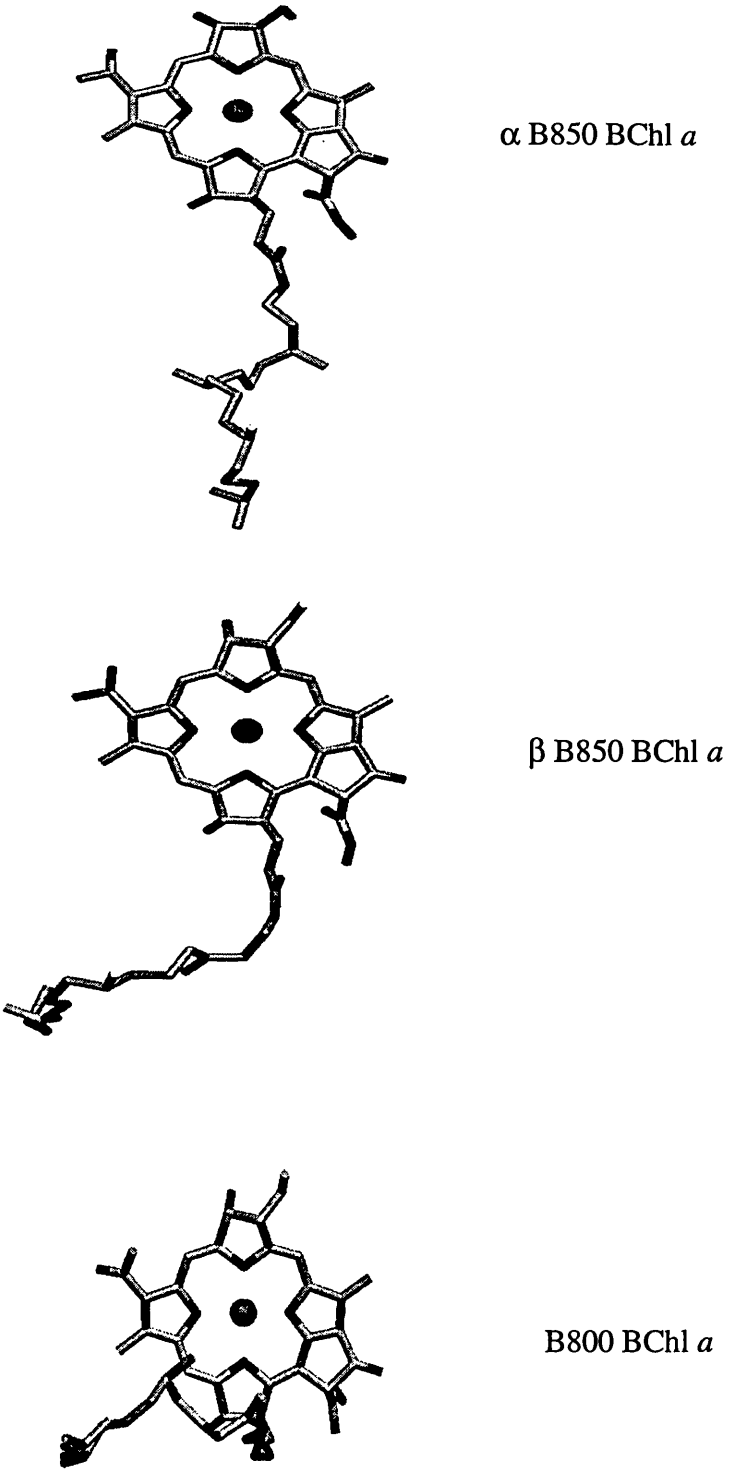

Figure 4.13. Illustration of the variations in conformation of the BChl *a* phytl chains.

Chapter 5

Discussion

5.1 Introduction

The structural model of LH II from *Rps. acidophila*, and the techniques employed to effect its elucidation, require discussion from within various contexts. In terms of this thesis, a complete and in-depth analysis of the knowledge which arises directly from elucidation of the LH II structure is impractical. For example, a full interpretation of the effect that the LH II structural design has on the photochemical properties of the pigments, or a rigorous account of the energetics of bacterial light-harvesting are significant undertakings, and will be the subject of further work and future theses and publications by other workers.

Prior to the elucidation of the LH II structure, the study of bacterial light-harvesting had reached an impasse. To advance the understanding of these systems clearly required an experimentally derived structural model (Olsen & Hunter, 1994; Cogdell & Zuber, 1995). Therefore, the most notable aspect of the work presented in this thesis is its ability to provide definitive answers to many long-standing questions. Undoubtedly, of greatest significance is the clear resolution of the uncertainty over the mode of assembly and the overall structural topology of bacterial light-harvesting complexes (Kühlbrandt, 1995). Furthermore, the resolution limit to which the observed diffraction extended, together with the enhancement of phase information produced by non-crystallographic symmetry averaging, results in a structural model which provides accurate, detailed information on the local molecular environment experienced by the pigment moieties (Freer *et al.*, 1996). In conjunction with available spectral data, such information permits unambiguous interpretation of the effect that protein-pigment and pigment-pigment interactions have on the photochemical properties of individual pigment species: Knowledge which can be used to correlate the

structural design of light-harvesting complexes with the mode of energy capture and transfer carried out (Freer *et al.* 1996).

In terms of bacterial photosynthesis in general, information on the assembly of light-harvesting complexes derived from the LH II structural model, together with data from low resolution electron microscopy studies on LH I (Karrasch *et* al., 1995), permits construction of a structural model of LH I. Consequently, the availability of models of both species of light-harvesting complex, together with the well established X-ray crystallographic models of the reaction center (Deisenhofer *et al.*, 1985; Komiya *et al.*, 1988; Chang *et al.*,1991; Ermler *et al.*, 1994), allows an initial model of the photosynthetic membrane to be built (Papiz *et al.*, 1996).

The crystallographic structure of LH II also has implications in the wider arena of photosynthesis *per se*. Together with the electron crystallographic structure of a plant light-harvesting complex (Kühlbrandt *et al.*, 1994) the structure of LH II allows a comparison to be made between two protein-pigment complexes which perform a similar photochemical function, but originate from evolutionary diverse organisms. Such comparisons will eventually be of great significance in understanding the tremendous evolutionary leap which occurred when anoxogenic photosynthetic systems became capable of oxygenic photosynthesis.

In the context of protein X-ray crystallography, the process of optimising the 'quality' of diffraction exhibited by crystals can be both critical and elusive. In the case of integral membrane proteins, it has become commonly accepted that the current paucity of X-ray crystallographic structures arises from the difficulties encountered in producing crystals which diffract X-rays to high resolution (Michel, 1990). Therefore, the general approach taken for optimising the observed diffraction from LH II crystals has the potential to be applicable in the structural

studies on other integral membrane proteins, especially other light-harvesting complexes. The protocol changes which enhanced the diffraction properties of LH II crystals will be discussed below. Together with the work done on 'heavy atom derivitisation' this work will be rationalised, and as far as possible, reduced to form some generally applicable practical guidelines for use in structural studies on other membrane proteins.

In the absence of a tertiary structure of LH II, biochemical and biophysical observations were interpreted in terms of notional, generic structural models LH II complexes (Olsen & Hunter, 1994; Cogdell & Zuber, 1995). For many years these models were central to the interpretation of experimental data, therefore they now merit some retrospective reassessment.

5.2 How accurate were the predictions of LH II structure ?

Unfortunately, very little of the published work on the prediction of the structure of light-harvesting complexes was performed specifically on LH II from *Rps. acidophila* - most work was carried out on LH II from *Rb. sphaeroides.* Consequently, a detailed comparison between predicted and actual structure is not entirely legitimate. But, if the assumption is made that strong similarities in function, photochemistry and biochemical composition indicates high structural homology existing between all LH II complexes, then structural predictions made about LH II, irrespective of species of origin, can be considered applicable to LH II from *Rps. acidophila* - with the obvious exception of any structural or functional effects which may arise due to oligomerisation states other than nonameric.

5.2.1 Early models of LH II: The gross topology and pigment location

The earliest predictions of the overall topology of LH II complexes were derived from neutron scattering studies on aqueous solutions of intact membranes (Sadler & Worcester, 1982). From this work, it was concluded that the complexes were mostly located in a transmembranous environment and only extended between 5 to 10 Å beyond the membrane (Sadler & Worcester, 1982). Although information on the location of the lipid bilayer is not directly obtainable from the X-ray structure (McDermott, *et al.*, 1995) the total length of LH II in the direction perpendicular to the presumed membrane surface confirms that the above conclusion was fairly accurate.

Basing their proposals on spectroscopic and linear dichroism data obtained from pressed gels containing isolated *Rb. sphaeroides* LH II complexes, Kramer and co-workers correctly identified two environmentally distinct populations of BChl *a* molecules and located their positions relative to the membrane with some degree of accuracy (Kramer *et al.*, 1984). In this study the authors also postulated two spectrally distinct populations of carotenoid molecule: one population located perpendicular to the membrane surface and associated with the B850 Bchl and the other population located parallel to the membrane and associated with the B800 BChl. Although there are some indications that there may be a second carotenoid molecule in LH II (Freer *et al*, 1996) this would also be in a position perpendicular to the membrane plane and not parallel with it.

Robert & Lutz used resonance Raman spectroscopic data to correctly assign the mode of BChl *a* binding: in particular to correctly assert that both B850 BChls are bound to conserved Histidine residues (Robert & Lutz, 1985; see section 1.5.3 for details of the LH II species used in this work). In addition, they

also correctly determined that conserved Histidine residues were not implicated in binding the B800 BChl (Robert & Lutz, 1985).

5.2.2 Later models: The apoprotein assembly and the 'minimal unit'

Prediction of the overall assembly of the complex, in terms of the structural assembly of the apoproteins, proved to be less successful. In all cases the models produced relied on two main assumptions. Firstly, that extensive close packing of the apoproteins occurred, especially in the α helical membrane spanning domains, and secondly that these interactions between α helices were dominant in determining the overall architecture of the complex (Donnelly & Cogdell, 1993; Zuber & Cogdell, 1995; Olsen & Hunter, 1994). The LH II structure clearly shows that both assumptions are completely incorrect: little protein-protein contact is made between polypeptides either within a protomer, or between the polypetides of adjacent protomers. The principal factor governing the construction and stabilisation of the complex is the highly structured, close interactions which occur between the pigments.

Understandably, in the light of the unexpected structural role played by the pigments, the most striking topological feature of LH II - the circular assembly of the apoproteins around a central 'hole'- had not been previously predicted. Furthermore, the existence of an LH II 'minimal functional unit' - postulated to be the basic constructional unit of all LH II complexes, an $\alpha_2\beta_2$ tetramer (reviewed in Olsen & Hunter, 1994) - which had become widely accepted in the literature (Kramer *et al.*, 1984; van Grondelle, 1985; van Grondelle *et al.*, 1994; Donelly & Cogdell, 1993; Zuber & Cogdell, 1996) clearly does not exist. The LH II crystallographic structure clearly shows that, in terms of structural construction

and photosynthetic function, there is no such 'minimal unit': the environment of all protomers is similar, and LH II only functions as an intact nonamer.

5.3 The basis of light-harvesting function

The basic function of the light-harvesting system is to collect solar radiation and funnel the energy to the reaction center (van Grondelle *et al.*, 1994; Zuber & Cogdell, 1995). To ensure maximum efficiency and minimal energy loss, the structural design of the photosynthetic components should be such that a unidirectional energy gradient is formed between the light-harvesting system and the reaction center (van Grondelle *et al.*, 1994). Of course, if more than one species of LH complex is present, then an energy gradient must also exist between the individual LH complexes (van Grondelle *et al.*, 1994). A theoretical description of the possible modes of energy transfer, both within and between LH complexes, is described in two comprehensive and authoritative reviews by van Grondelle and others (van Grondelle, 1985; van Grondelle *et al.*, 1994). The reader is directed to these for full exposition of the topic.

5.3.1 Possible modes of energy transfer between photosynthetic pigments

In light-harvesting systems, there are three possible mechanisms for energy transfer from a photoexcited pigment to other pigment molecules: nonradiative Coulombic coupling, Förster dipole-dipole transfer and the Dexter mechanism (van Grondelle, 1985). The mechanism effected during a particular energy transfer event is a function of the spatial distance between the energetically excited donor pigment and the acceptor molecule, and the relative strengths of their dipoles. During a particular energy transfer event, overlap in the criteria which

differentiates between the possible mechanisms has the effect that it may not be clear which mechanism is actually occurring, and in many circumstances it may be the case that more than one mechanism is simultaneously in effect (van Grondelle, 1994; Freer *et al.*, 1996).

5.3.2 Coulombic coupling

Coulombic coupling is a nonradative energy transfer arising from interaction between dipoles of an originally excited molecule and a nearby acceptor molecule. This mode of transfer can only occur when both are in close proximity (less than 10 Å). The rate of energy transfer is given by equation 5.1 (Freer *et al.*, 1996).

$$E_{ab} = \frac{C\mu_a\mu_b k}{R^3} \qquad \textit{Eq. } 5.1$$

Where R is the inter-molecular separation, C a constant, μ the transition dipole (of two molecules a and b) and k a scaling factor. E_{ab} is measured in volts per meter.

5.3.3 The Förster mechanism

The Förster mechanism describes the radiationless transmission of energy between two pigments 20 to 80Å apart. The process is primarily dependent on the overlap of the fluorescence spectrum of the donor with the adsorption spectrum of the acceptor molecule. During the transfer the donor returns to the ground state from an excited state by simultaneous exciting an acceptor molecule to its excited state. The difference in energies of the two transitions must be equal, or 'in resonance'. Förster derived an expression for the rate of energy transfer, based on the assumption that the interaction energy is small, i.e. where the coupling is small

(Förster, 1948). The transfer rate k and the efficiency of the energy transfer are given in equations 5.2 and 5.3, respectively.

$$k = \left(\frac{R_0}{R}\right)^6 . \frac{1}{\gamma} \qquad Eq.\ 5.2$$

$$\phi = \left(\frac{R_0}{R}\right)^6 \qquad Eq.\ 5.3$$

where

$$R_0^6 = Ck^2\eta^{-4}\int f(\nu)\varepsilon(\nu)\nu^{-4}d\nu \qquad Eq.\ 5.4$$

In equation 5.4, C is a constant, R the distance between pigments, R_0 the constant of proportionality, γ the intrinsic lifetime of the excited state of the donor, η the refractive index of the medium, k a geometric factor determined by the relative orientation of the transition dipoles, $f(\nu)$ the fluorescence of the donor and $\varepsilon(\nu)$ the absorption of the acceptor.

5.3.4 The Dexter mechanism

In contrast to the previous two methods, energy transfer by a Dexter type mechanism take place via electron exchange, hence there is a requirement that the donor and acceptor to be within van der Waals distance ($\approx$ 4 Å).

5.4 Energy transfer in LH II

LH II contains three photochemically distinct populations of pigment moieties: B800 Bchl *a,* B850 Bchl *a* and the carotenoid rhodopin glucoside.

Consequently, several modes of possible energy transfer must be considered: between carotenoid and Bchl *a* and within the Bchl *a* molecules themselves.

5.4.1 Energy Transfer in LH II: Carotenoid-BChl a

Energy transfer from carotenoid to BChl *a* takes place by single-singlet energy transfer (Frank and Cogdell, 1993). The electronic states of carotenoid and BChl *a* are shown in figure 5.1. The carotenoid is excited from the (S_0) 1^1Ag ground state to the optically allowed (S_2) 1^1Bu state by the absorption of light. This state rapidly relaxes to the optically forbidden (S_1) 2^1Ag excited electronic state by rapid internal conversion (Noguchi *et al.*, 1990). Since the lifetime of the 1^1Bu state is less than 1ps (Gilbro & Cogdell, 1989) and the energy level of the 2^1Ag state is close to BChl *a* Q_x it has been proposed that energy is transferred from the carotenoid 2^1Ag state to BChl *a* (Gilbro & Cogdell, 1989).

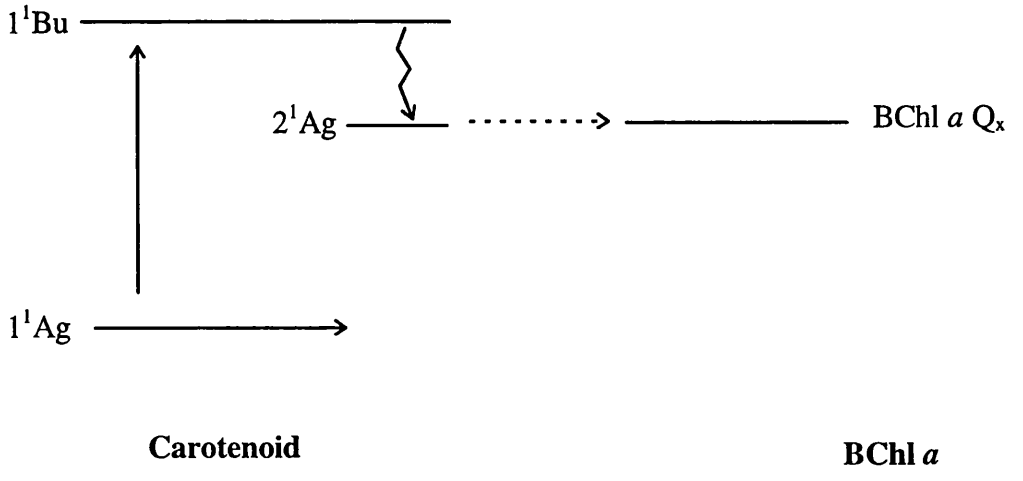

Figure 5.1. The relative energy levels of the ground and excited states of a carotenoid molecule relative to BChl *a*.

The LH II structure shows how the arrangement of the pigments allows energy transfer from carotenoid to BChl *a* Q_x via either the 2^1Ag or 1^1Bu states (Koyama, *et al.*, 1996) - the carotenoid and BChl *a* molecules are in close enough proximity to allow electron transfer, and the relative dipoles are sufficiently aligned to permit both Coulombic and/or Förster energy transfer (Freer *et al.*, 1996). The detailed mechanism which occurs is not clear and will require further experimentation and interpretation using the LH II structure as a reference point (Koyama *et al.*, 1996).

In general, energy transfer from carotenoids to Bchl in LH II occurs with between 30 and 100% efficiency (van Grondelle, 1984), depending on the species of origin and hence the species of carotenoid present (Frank & Cogdell, 1993). The efficiency of this transfer in LH II from *Rps acidophila* has been determined, by BChl *a* steady state carotenoid excitation induced fluorescence, to be 70 to 75% efficient (Angerhofer *et al.*, 1986).

In all photosynthetic systems carotenoids are multi-functional (Cogdell & Frank, 1987). In addition to their light-harvesting role, carotenoids photoprotect the system by quenching (bacterio)chlorophyll triplet states (BChlT) according to equation 5.5, where car^T is representative of triplet carotenoid (Krinsky, 1971; Seifermann-Harms, 1985; van Grondelle, 1985; Cogdell & Frank, 1987; Frank & Cogdell, 1993):

$$\mathbf{(B)Chl^T + car \rightarrow (B)\ Chl\ + car^T} \qquad \textit{Eq.}5.5$$

This transfer of excitation energy is necessary since intersystem crossing can occur between $BChl^T$ and environmental oxygen, leading to the formation of singlet oxygen: an extremely powerful oxidant whose presence results in rapid cell death (Cogdell & Frank, 1987). This energy transfer occurs via the Dexter

mechanism. The overriding criteria required for this type of energy transfer being that the acceptor and donor molecule are in close proximity, that is to say within van der Waals distance. In the LH II structure it can been seen that the carotenoid molecule is in very close contact with both populations of Bchl *a* by way of an extensive series of contacts (Freer *et al.*, 1996).

5.4.2 Energy Transfer: Bchl a to Bchl a

Within an LH II molecule there are two populations of BChl *a*, each with dissimilar photochemical properties. Hence two distinct possible energy transfer events must be considered: B800-B850 and B850-B850. Only very limited B800↔B800 transfer is thought to occur (van Grondelle, 1994). This assertion is based on the fact that the spectral overlap of B800 with B850 makes B800↔B800 transfer unfavourable, and the observation that B800↔B800 depolarization occurs on the 5-10 ps timescale at 77K whereas with the B800→B850 energy transfer occurs in 0.7 ps (Sundström & van Grondelle, 1995).

The transfer of energy from the B800 BChl *a* to B850 BChl *a* has been the subject of extensive spectroscopic examination (Sundström & van Grondelle, 1995). At room temperature the emitting B800 excited state is fully equilibrated with the B850 excited state in both *Rhodopseudomonas sphaeroides* and *Rhodopseudomonas capsulata* (van Grondelle, 1982). In the former species of LH II the room temperature transfer time for B800→B850 has been estimated to be 0.7ps ± 0.1ps. This photochemical event is followed by the ultra-fast depolarisation of the excited state over the excitonically coupled B850 species in 200 to 300 fs (van Grondelle *et al.*, 1994). It is apparent from the structure that the arrangement of B850 Bchls is optimal for the rapid depolarisation of energy

states: there is strong Coulombic coupling of the dipoles and the intermolecular distances are such that π-orbital overlap may occur.

Circular Dichroism measurements on the B800 absorbance indicated that this population of BChl is 'monomeric', in that there is no excitonic coupling between molecules (Cogdell & Scheer, 1985). From the LH II structure the monomeric character of this group of BChl *a* molecules is apparent. In the plane of the membrane the distance between the central magnesium atoms of adjacent B800 Bchl *a* molecules is 21Å, with no possibility of overlap of electron clouds in the bacteriochlorin rings.

5.5 The intracytoplasmic membrane

The structure of LH II, together with the available X-ray crystallographic structures of bacterial reaction centers and a low resolution electron microscopy projection of LH I (Karrasch *et al.* 1995), permit initial modeling of the arrangement of the pigmented photosynthetic components of a functional intracytoplasmic membrane.

Although of relatively low resolution (8.5Å), the projection of LH I by Karrasch and co-workers (Karrasch *et al.* 1995) shows that very strong similarities in structural design exists between both species of antenna complex. LH I is constructed in a modular fashion similar to LH II, only in this instance 16 protomers - each containing an αβ heterodimer - are arranged to form a torus of outer diameter 116Å and inner diameter 68Å. Such dimensions would accommodate a single reaction center molecule (Boonstra *et al.* 1994). Conserved Histidine residues in LH I α and β apoproteins are postulated to bind BChl *a* molecules in a similar spatial position, with respect to the membrane normal, as those in LH II and BChl 'special pair' of the reaction center (Zuber, 1986). A model of LH I was constructed by taking the coordinates of an αβ protomer from the LH II structure and moving them from the origin, radially in the plane of the membrane, by a factor of 16/9. The protomer was repeated 16 times, with a rotation of 22.5° about the membrane normal running through the origin (Papiz *et al.*, 1996).

The reaction center from *Rb. Sphaeroides* was placed in the center of the LH I ring, such that the 'special pair' was located at the same level, in a putative 'membrane', as the ring of LH I BChl molecules. Significantly, in this arrangement

the reaction center special pair is equidistant from all LH I BChl *a* molecules (Papiz *et al.*, 1996), shown in figures 5.2 and 5.3.

Biochemical analysis of purple bacterial photosynthetic membranes indicate a maximum of 300 BChl molecules are present per reaction center (Zuber & Cogdell, 1995). This would account for a stoichiometry of approximately eight LH II molecules per RC-LH I 'core' conjugate. This number of LH II complexes can be positioned closely around the entire circumference of the RC-LH I 'core' conjugate. Such an organisation is optimal for energy transfer from LH II to LH I. The fact that all LH complexes are exposed to identical environments, coupled with the fact that the both species of LH complex are circular removes any limitations of directionality on energy transfer. At this point in time this model is only putative, although some measure of confidence in this model can be derived from examining the location of the charged residues β Arg20 and β Asp 17. When these residues are highlighted in the model (figure 5.4) it can be seen that the ion pairing which arises 'locks' the LH II rings tightly around the RC-LH I 'core'.

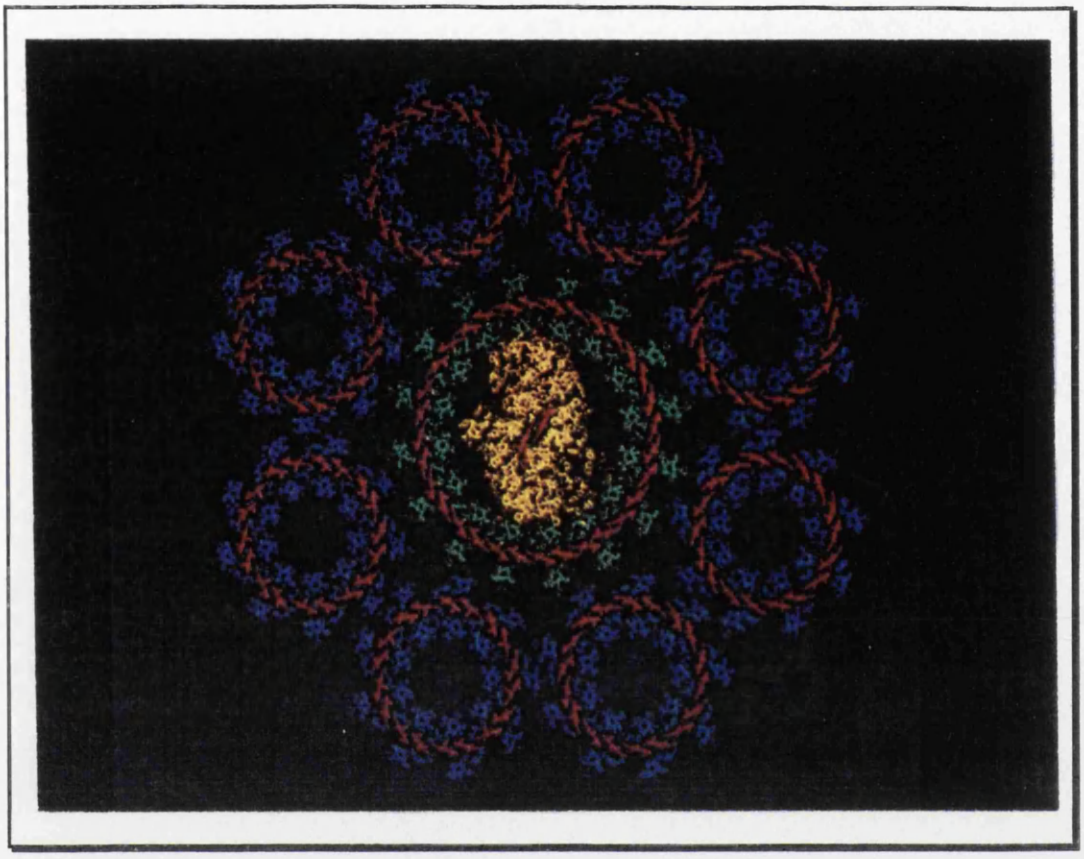

Figure 5.2. A putative model of the photosynthetic components, viewed parallel to the membrane surface. The larger green ring, surrounding the reaction center (shown in yellow), is LH I. LH I is are shown in blue. The B850 BChl in LH II, the B875 in LH I and the 'special pair' in the reaction center are shown in red.

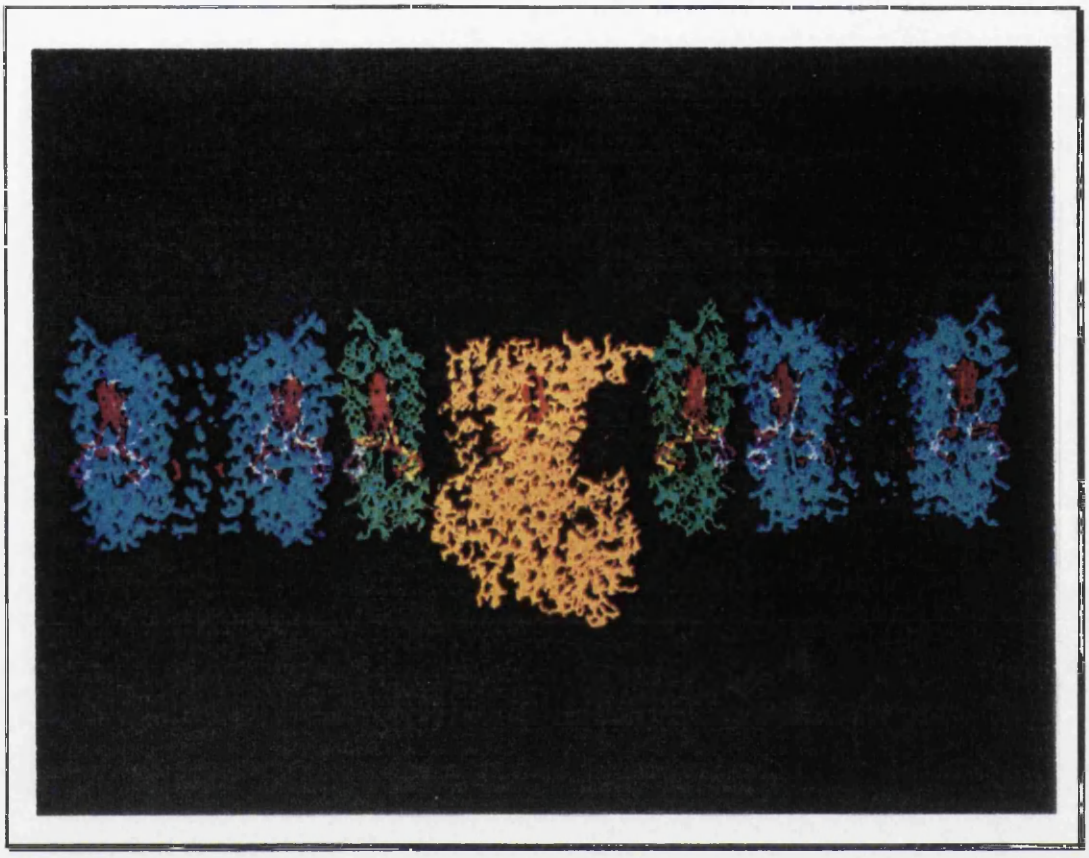

Figure 5.3. A putative model of the photosynthetic components, viewed perpendicular to the membrane surface. The larger green ring, surrounding the reaction center (shown in yellow), is LH I. LH I is shown in blue. The B850 BChl in LH II, the B875 in LH I and the 'special pair' in the reaction center are shown in red.

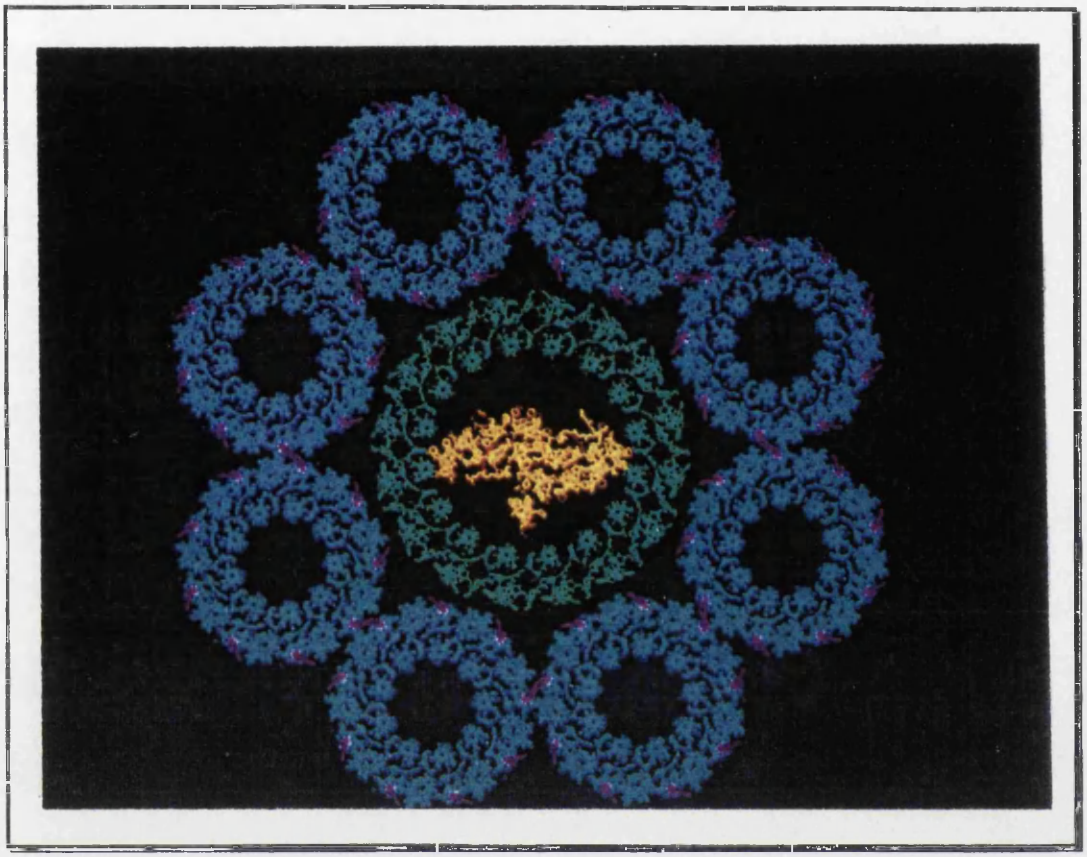

Figure 5.4. The arrangement of eight LH II complexes around the RC-LH I core. The ion pair βArg20-βAsp17 are shown in red (from Papiz *et al.*,1996).

5.6 Comparison of LH II with the plant light-harvesting complex

A comparison of the overall molecular topology of LH II with the electron crystallographic model of plant LHC-II (Kühlbrandt *et al.*, 1994) reveals that few structural similarities exist between the two types of light-harvesting complex. Plant LHC-II is composed of a single polypeptide chain which spans the membrane three times, compared with the 18 polypeptide/transmembrane spans of the bacterial version. This difference in polypeptide composition gives rise to the most striking structural difference between the two complexes: the absence of the circular mode of assembly in plant LHC-II. The reasons why the circular assembly was forsaken when plants developed the capacity to perform oxygenic photosynthesis are not immediately apparent. To date no structural information is available on Photosystem II (the plant analogue of the bacterial reaction center) or its complement of inner antenna light-harvesting complexes. Although both plant and bacterial reaction centers perform a similar basic function and are of homologous construction[1] (Trebst & Depka, 1985; Michel & Deisenhofer, 1989) the plant version contains many more inner antenna light-harvesting complexes, in addition to the subunits which form the oxygen evolving apparatus (Debus, 1992; Ghanotakis & Yocum, 1990). Only when the structure of Photosystem II and its accompanying inner antenna complexes are known will it be possible to speculate on why the circular assembly was abandoned.

On a more detailed molecular level, a comparison between the two types of light-harvesting complex also reveals little constructional similarities existing between the two types of complex. This is perhaps not unexpected in the absence

[1] This only holds true for the 'reducing side' of the two reaction centers. The absence of oxygen evolving apparatus in the bacterial version renders comparisons in the 'oxidizing side' somewhat uncertain.

of the circular mode of construction, and the significant differences in polypeptide and pigment composition. Possibly the only identifiable structural and functional similarity is the use of carotenoids to promote structural rigidity and in preventing the formation of highly destructive singlet oxygen (Kühlbrandt *et al.*, 1994). In the case of plant LHC-II, the resolution limit and resultant quality of the calculated electron density map did not allow the phytl chains of the majority of the chlorophyll molecules to be located (Kühlbrandt *et al.*, 1994). Hence a comparison with the highly structured interactions seen in the phytl chains of LH II is not possible.

5.7 The practical work carried out: Some conclusions

The purification and crystallisation protocols used during the structure determination of LH II had a great bearing on the level and reproducibility of the observed diffraction. As stated above, no biochemical or biophysical differences could be detected between complexes which had been prepared by the various purification protocols. The modifications made to the purification and crystallisation protocols and their consequential effect on the observed diffraction will be discussed below.

5.7.1 Solubilisation

The changes made to the protocol considerably increased the detergent to protein ration during solubilisation. Theoretically, this will result in more complete solubilisation of the photosynthetic membrane (Hjelmeland, 1990). In practice there was no noticeable change in either the behavior, or stability of the complex during subsequent purification and crystallisation steps. Increasing the level of

solubilisation resulted in crystallisation becoming significantly more reproducible. Using the original solubilisation conditions it was often the case that crystallisation trails resulted in showers of unusable micro crystals. These were usually seen to be embedded in a viscous semi-solid gel at the bottom of the crystallisation drop. Analysis of this material proved difficult, but indications were that in all likelihood this was phospholipid (Ms. G. Dahler, personal communication). Experiments using alternative detergents for the initial solubilisation were not carried out extensively. It was assumed that LDAO was a good choice of detergent for initial solubilisation, since it fulfilled the criteria of being cheap, produced adequate membrane fragmentation, maintained the integrity of the complex and was easily exchanged for other detergents.

5.7.2 Isolation of LH II.

The decision to use sucrose density centrifugation, in place of ion exchange chromatography, was based on the observation that the technique totally removed all traces of contaminating reaction center[1]. This was essential since the reaction center will form crystals, under numerous crystallisation conditions, if it is present as a contaminant in what is ostensibly an LH II crystallisation trial (Dr. G. Fritzch, personal communication; and the general experience of other workers in the laboratory in which the work for this thesis was carried out). When exploring alternative crystallisation conditions for LH II the formation of small RC crystals would have been misleading, and could have resulted in spending a considerable time attempting to develop a crystallisation protocol for an impurity.

[1] As determined by SDS-PAGE.

The use of an alternative matrix for carrying out molecular sieve chromatography had no identifiable direct consequence on the quality of crystals, or their diffraction properties. But, the 'purity index' used, suggested that the use of Sephacryl S200 resulted in a slightly 'purer' preparation, i.e. there was an increase in the ratio of the absorbance 850 nm relative to the absorbance at 254 nm. This could have been due to the removal of small amounts of a contaminating protein, but this was not detectable by coomasie stained SDS PAGE analysis. The contaminant could have been perhaps some 'free' apoproteins or an organic contaminant which absorbed at 254 nm.

5.7.3 Detergent exchange

The method used for exchanging the detergent was found to have significant effects on the degree of mosaicity[1] exhibited by crystals of the complex. If detergent exchange was carried out chromatographically, using a small ion exchange column poured in a Pasteur pipette, the mosaic spread was typically 0.6°. Whereas if centrifugation dialysis using 'Centricon' type concentrators was used to exchange the detergent, this was reduced to 0.3°. It is difficult to understand why this should be the case. Theoretically, the micellular weight of LDAO and β–OG are small enough to allow them to pass through the membrane during centrifugation in a 50 kDa 'Centricon' tube (Thomas & McNamee, 1990). Therefore, the technique used would seem unlikely to have resulted in a final protein preparation that is enriched in both detergents or at a significantly higher detergent concentration than expected. This aspect of the purification protocol needs further work, by using radiolabelled detergents to quantify the exchange.

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[1] Protein crystals are imperfect, and can be considered as being a mosaic of 'small blocks'. Each reflection has a certain width due to the angular spread of the mosaic blocks. Generally this is in the range 0.2 to 0.5°.

5.7.4 Crystallisation

All of the parameters of the crystallisation protocol were tested for their effect on the observed diffraction. Altering the individual components of the crystallisation drop resulted in the following conclusions being drawn.

Inorganic salts proved to be the most effective precipitants. The use of polyethylene glycols, even in the presence of 'small amphiphilic' additives, tended to induce serious phase separation, and usually resulted in denaturation of the complex. Other commonly used organic precipitants, such 2-methyl-2,4-pentanediol (MPD) usually caused rapid denaturation of the complex, even when used at low concentrations.

In terms of the optimised crystallisation protocol, the choice of detergent would appear not to be critical. Using the same crystallisation conditions - in terms of precipitant, pH etc. - it was possible to obtain crystals in a variety of detergents: for example LDAO, heptyl-glucoside and Triton TGB-10.

The 'small amphiphile' benzamidine hydrochloride would appear necessary for optimal crystal growth. Crystals generally do not occur if benzamidine hydrochloride is present at a concentration of less than 1% at the outset of the crystallisation experiment. The use of concentrations of 3%, or higher results in the formation of benzamidine crystals. Therefore, in the optimised crystallisation protocol benzamidine will be in a state of saturation, or near supersaturation after equilibrium.

In the case of LH II from *Rps. acidophila* alterations to the concentration of the small amphiphile had no effect on the resolution limit, or the reproducibility of the diffraction. Whereas, the crystallisation of LH II from *Rb. molischianum* required absolutely that the 'small amphiphile' heptane 1-2-3 triol (HPT) is

supersaturated at the end of the experiment (Michel, 1990). Any procedure which moved HPT out of supersaturation resulted in very rapid dissolution of the crystals (Prof. H. Michel, personal communication).

The use of ammonium sulphate in the reservoir at high pH has the effect that free ammonia will evolve and equilibrate into the crystallisation drop (Mikol *et al.*, 1989). From the trials conducted this seemed necessary for optimal crystallisation. If, for example potassium sulphate at the same ionic strength was used as the reservoir solution, poor quality crystals were produced. Typically, small clusters of micro-crystals which were of very poor external morphology were obtained.

Using 'Chryschem' crystallisation trays, in place of the original home built equipment, the surface area of the reservoir was very markedly reduced. By doing so the rate of equilibration is changed due to differences in the relative surface areas (Mikol *et al.*, 1989). Little effect on the crystallisation was observed in terms of resolution limit. The use of Chryschem trays had a very beneficial affect on the reproducibility of obtaining crystals of good external morphology. In retrospect this is understandable. In the home built apparatus, 8mls of reservoir solution was poured into a container of diameter 8 cm. Since, this volume of solution is insufficient to totally cover the bottom of the container there will be various surface areas possible - for example, the solution can form a thin band around the entire circumference of the dish, or alternatively sit as a large drop in the center of the container. In Chryschem trays the volume of solution used is sufficient to cover the entire surface of the reservoir, hence a vapour diffusion is carried out using a uniform surface area and hence uniform equillibration times.

5.7.5 The role of detergent

In terms of the role of detergent in crystallisation an examination of the crystal packing reveals that the distance, perpendicular to the crystallographic three fold axis, between adjacent nonamers is approximately 40Å, as can be seen in the crystal packing shown in figure 5.5. Therefore, large micellular aggregates can clearly be accommodated in the space between adjacent molecules. At this point in time the location of the detergent micelle is not known[1] but it would appear that to allow crystal packing of LH II, in space group R32, there are few dimensional constraints on the size of micelle, and hence on the length of detergent 'tail' or the choice of 'head' group. This assumption can be verified by the observation that crystallisation was possible in several detergents, all of which differed in chain length and head group.

The use of detergents in crystallisation experiments on soluble, globular proteins has been reported to lead to improvements in crystal size and observed diffraction limits (Cudney *et al.*, 1994). Consequently, it is not unreasonable to assume that there are potentially several roles played by these molecules in the overall process of crystallisation, and hence the particular properties of β-Octyl-glucoside which result in the optimal crystals of LH II may be more complex than the simple hypothesis that it form micelles of 'optimal size'.

[1] Obtaining this information requires further experiments: neutron diffraction data on crystals soaked in deuterated detergents.

5.7.6 Role of the 'small amphiphile' Benzamidine hydrochloride

The role of the 'small amphiphile' during crystallisation of LH II is one which requires further investigation to determine why the benzamidine hydrochloride was necessary, and what function it performed during crystallogenesis. It is unlikely that benzamidine was acting in its usual role as an inhibitor of serine protease, although it is certainly the case that the increase in pH of the crystallisation drop which occurs during equilibration would be sufficient to activate chymotrypsin type proteases which act on Histidine residues at high pH (Hjelmeland, 1990). No experimental work was performed to assess the importance, or otherwise, of benzamidine protease inhibition to obtaining crystals.

Since the intermolecular space available for detergent micelles is considerable, there is little need for benzamidine to function as an agent which 'shrinks' the micellular torus. In addition, even in the absence of small amphiphillic additives the detergent/precipitation combination used for LH II crystallisation seldom underwent phase separation. Hence, the required presence of benzamidine is difficult to rationalise.

5.7.7 Heavy atom derivatisation

To produce heavy atom derivatives it is necessary to transfer mature crystals into a solution which, although it contains appropriate quantities of the required 'heavy atom', maintains the crystallographic integrity of the crystal. In the case of LH II this proved problematic for a considerable time. It was apparent that after transfer to such solutions the 'quality' of diffraction exhibited by the crystals was markedly decreased. Data collected on crystals simply transferred to such solutions showed that it was inadequacies in the stabilising solution, rather than the heavy atom solute, which had caused this effect. Data from linear

dichroism experiments on crystals present in various stabilising solutions showed that the critical factor in maintaining crystallinity - and hence diffraction properties - was the amount of detergent present in the stabilising solution.

Varying other parameters or constituents of the supporting solution, for example solute concentration or pH, had minimal effects on the integrity of the crystal, provided the detergent was present at an appropriate concentration. In all cases it was found that the required amount of detergent was much less than one would have intuitively supposed. This could be attributed to the local detergent concentration inside the crystal, at the termination of crystal growth, being relatively high - consequently the detergent remaining in the drop is at a much lower concentration than would be produced by simply setting up a crystallisation in the absence of protein.

Finally, fears that the presence of high concentrations of detergent in the solvent channels would hinder diffusion of heavy atoms into the crystal lattice, or that there would be limited availability of suitable binding sites proved to be unfounded. In fact the opposite was found to be the case. The heavy atom positions from the selino-methionine derivative and the derviatives used to calculate the electron density maps are shown in figure 5.5. Once all of the heavy atom positions had been determined it was clear that earlier fears that there were insufficient binding sites available and that the diffusion of the solute into the crystal lattice was hindered were unfounded.

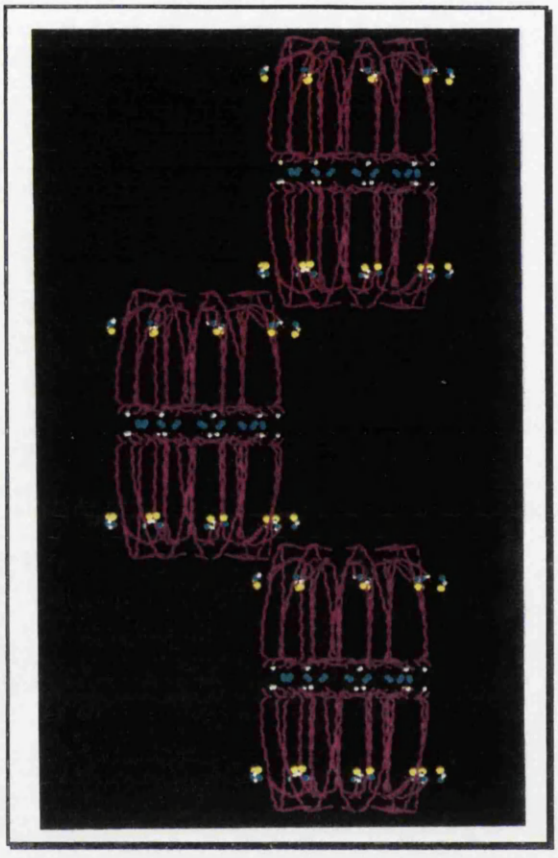


Figure 5.5. The heavy atom positions (shown in a portion of the unit cell). Selenium shown in yellow, platinum in white, mercury in blue.

5.7.8 Conclusion

In the final analysis, the chemical composition of the crystallisation drop is very complex at the outset, and with equilibration may become more so. It is generally accepted that the nucleation and subsequent crystal growth of proteins is not well understood, even the comparatively simple and most characterised case of lysozyme (McPherson, 1995). A system such as that employed to produce crystals of LH II is many times more complex: the presence of detergent, small amphiphilic molecules and inorganic salts in a media constantly increasing in ammonium ions, results in great difficulty in understanding the complex interactions which occur during crystallisation. Therefore, assuming an empirical approach is taken to conducting crystallisation trials, on the basis of the work performed on LH II the following conclusions can be drawn for structural studies on other membrane proteins, and in particular other LH II complexes.

It would appear, from the experiences of the work done on LH II, that in the first instance the production of initial crystallisation protocols would be best achieved using the well established screening methodologies (D'Arcy, 1994; Cudney *et al*, 1994) - avoiding conditions which are liable to induce detergent phase separation, for example PEG with molecular weights greater than 2000. Trials should be conducted, if possible, in several different detergents - although the choice of detergent wasn't significant in the case of LH II, in other cases it has proven critical (Michel, 1990). In the first instance crystallisation trials should be conducted in the absence of 'small amphiphilic' additives - these should be thought of as 'enhancers' of crystal quality, not necessarily a prerequisite for their production. In the absence of crystals, or in situations where detergent phase separation is a problem, conduct crystallisation trials in the presence of 2 to 5 % of

one -or preferably more - of the commonly used amphiphiles (see Michel 1990 for a list)

When initial crystallisation conditions are found - most often resulting in poor quality crystals unsuitable for initial X-ray analysis - a process of optimisation of the crystallisation protocol, similar to that used in the case of soluble globular proteins should be carried out. At this point the effect of additives, if not already used, should be explored.

Once crystals suitable for X-ray analysis are procured, the experience of this work, suggests the process of optimising diffraction should be done by retaining the crystallisation protocol whilst systematically modifying the purification protocol. Techniques based on molecular 'size' - such as sucrose density centrifugation or gel filtration seem particularly applicable, and if possible should be added to the purification protocol used.

And above all, no assumptions should be made. The work done on LH II has shown that relatively trivial modifications to protocols - for example, changing the procedure for detergent exchange - can have a significant effect on the diffraction properties of crystals.

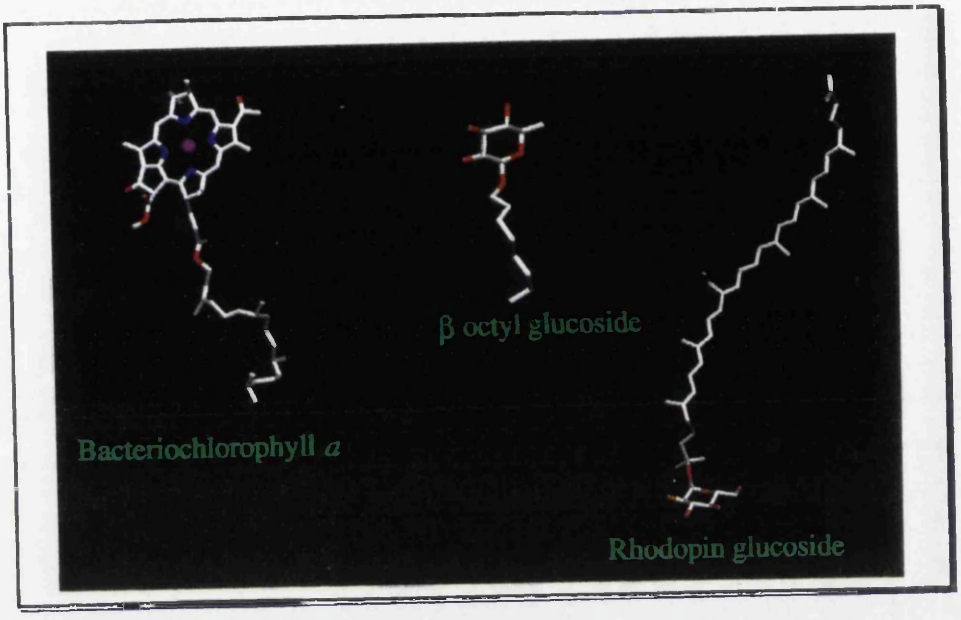
Epilogue

The work contained in this thesis has significance in the fields of photosynthesis and energy transfer, and in structural studies on integral membrane proteins. Perhaps most importantly, and of immediate impact, is the answering of long standing, controversial questions regarding the overall molecular assembly of bacterial light-harvesting complexes and the provision of information on the local molecular environment encountered by the individual populations of pigments present in LH II complexes.

Of course, a single crystallographic structure cannot provide all of the answers; some of the finer details of the structure and function of light-harvesting in bacteria still need to be clarified. Perhaps most enigmatic is the significance, if any, of the nonameric arrangement compared with other possible oligomerisation states.

Using the *Rps. acidophila* LH II structure as a search model, the structure of the B800-850 LH II complex from *Rhodospirillum molischianum* has been determined by molecular replacement (Koepke *et al.*, 1996). Intriguingly, this LH II complex is not a nonameric assembly but octameric. Future comparisons between these structures, and others, will perhaps reveal if these differences in oligomerisation number are functionally significant.

Appendix A: Structure of the non-protein component of LH II



Appendix B: Amino acid symbols

(IUPAC-IUB Joint commission on Biochemical nomenclature, 1984).

Non-polar

ALA	Alanine	A
VAL	Valine	V
LEU	Leucine	L
ILE	Isoleucine	I
PHE	Phenylalanine	F
TRP	Tryptophan	W
MET	Methionine	M
PRO	Proline	P

Polar uncharged

LYS	Lysine	K
ARG	Arginine	R
HIS	Histidine	H

Acidic

GLY	Glycine	G
SER	Serine	S
THR	Threonine	T
CYS	Cystine	C
TYR	Tyrosine	Y
ASN	Asparagine	N
GLN	Glutamine	Q

Basic

ASP	Aspartate	D
GLU	Glutamate	E

Appendix C: Materials and Methods

Cell Storage

Cell cultures were stored as agar stabs at room temperature.

Cell Culture

All cultures of *Rps. acidophila*, strain 10050, were grown anaerobically at 30° C in Pfennigs media (Pfenning, 1969) and illuminated with incandescent light of intensity 10 Wcm^{-2}.

Initially, starter cultures were prepared by the addition of 10 ml of Pfennigs media to cells stored in agar. After 2-3 days growth the cells were used to inoculate 500 ml flat sided bottles of the same media. After 2 days, cells grow to a sufficient density to allow inoculation of a 10 litre glass flask of media using a single 500 ml culture. Inoculations were carried out in aseptic conditions in a laminar air-flow cabinet.

Isolation of Chromatophores

Cells were harvested in the stationary phase by centrifugation at 2400 rpm for 15 minutes (Fison MSE Coolspin centrifuge). Pelleted cells were resuspended in the minimum amount of 20 mM Tris-HCl, pH 8.0, following which the cells were mechanically disrupted using a French Press at a pressure of 7-10 tons/$inch^{-2}$.

Solubilisation of Chromatophores

***Original method** - as used in the crystallisation protocol of Papiz et al., 1989*

Chromatophores were adjusted to a concentration which gave an absorbance of 50 cm^{-1} at a wavelength of 850 nm (i.e. OD_{850}) using 20mM Tris-HCl, pH 8.0.

This solution was made 1% LDAO (supplied by Millchem UK Ltd.) and incubated at room temperature for 5 minutes.

Optimised protocol - as used in McDermott et al., 1995

Chromatophores were adjusted to a concentration which gave an OD_{850} of 25 cm^{-1} using 20mM Tris-HCl, pH 8.0. This solution was made 2% (v/v) LDAO (Purum grade, supplied by Fluka Chemie AG) and incubated at 4° C for 2 hours.

Triton TBG 10 solubilisation

The chromatophore concentration was adjusted as to give an OD_{850} of 25 cm^{-1}. This solution was made 3% (v/v) Triton TBG 10 (gift by Union Carbide Ltd. to Prof. H. Scheer, University of Munich) and incubated for 2 hours at 4° C. Immediately prior to use, Triton TGB 10 was purified by passing a 25% (v/v) solution of the detergent in distilled water over Watman DE52 ion exchange matrix until the solution was colourless.

Isolation of solubilised chromophores

Immediately after solubilisation, the solution was centrifuged at 12,000 x g for 20 minutes. The pellet consisting of unsolubilised membranes and other cellular material was discarded.

Isolation of LH II

Sucrose Gradient Centrifugation

Discontinuous Sucrose-Density gradients were prepared by slowly layering 6 mls solutions of 0.8, 0.6, 0.4, and 0.2 M sucrose in 0.1% LDAO, 20 mM Tris-HCl, pH 8.0 into a 27 ml polycarbonate centrifuge tube. 3 ml of solubilised membranes were layered on top of the sucrose, and the tubes were centrifuged at 45,000 rpm for 16 hours in a Sorvall Ultracentrifuge. The solubilised membranes resolved into three distinct bands (shown in figure C.1). The bands were removed by pipette.

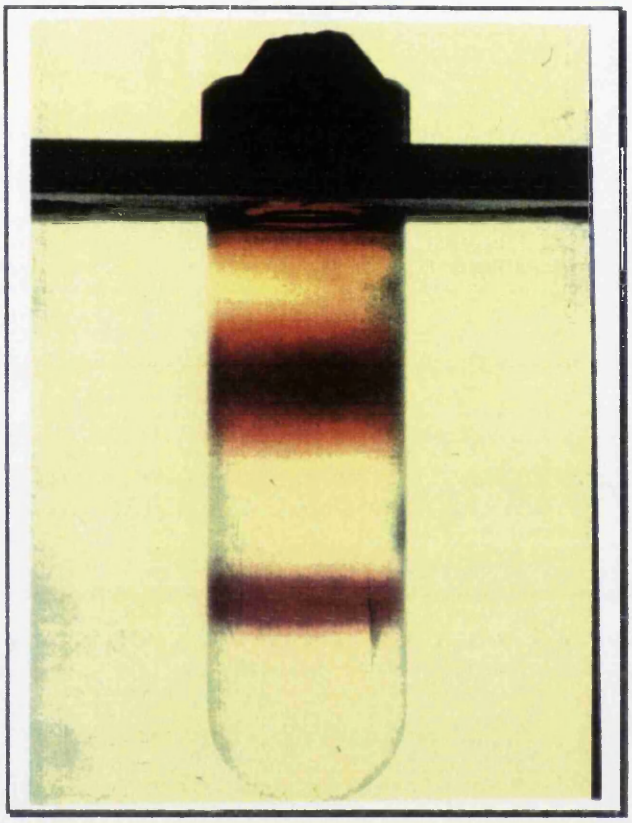


Figure C.1. After sucrose gradient separation of LDAO solubilised *Rps. acidophila* strain 10050 chromophores. The lower band consists of RC/LH I 'core'complex; the central band isolated LH II complex, and the uppermost band denatured apoprotein, lipids and "free" pigments.

Ion Exchange Chromatography

A slurry of Watman DE52 diethylethyl-aminoethylcellulose (DEAE) anion exchange matrix was prepared in 20 mM Tris-HCl, pH 8.0 as per manufacturers instructions. This was poured to a bed height of 8 to 10 cm in a 5 cm diameter gravity fed glass chromatography column,. The column was washed with 200 ml of 20 mM Tris-HCl, pH 8.0. To allow efficient binding the solubilised membranes were diluted with the same buffer to give an OD_{850} of 3 cm^{-1}. Elution was effected

by a stepwise gradient, in 50 mM steps, of 50 to 250 mM NaCl in 20 mM Tris-HCl, pH 8.0, 0.1% LDAO. With each step being allowed to flow until the eluent was colourless. At low salt concentration (~50 mM) 'free' pigments were eluted. The reaction centre 'core' complex eluted at a salt concentration of 100-150 mM. LH II eluted at approximately 200-250 mM. The exact salt concentration at which the various components eluted varied with batches of cells. All fractions were assayed spectrophotometrically (see next section). Suitable fractions were pooled, diluted with detergent free buffer to allow re-binding and applied to a second column. Elution was performed in a similar manner.

Spectrophotometric estimation of purity

Since bacteriochlorophyll *a* is stoichiometrically bound to the apoproteins, the ratio of absorbance at 850nm to 280nm is a good indication of both the integrity of the complex and the purity of the preparation (see figure 1.6 for representative absorption spectra). Any reduction of this ratio indicates an increase in concentration of either unbound apoproteins from denatured complexes or extraneous protein contaminants. During isolation by ion exchange chromatography, a ratio of 2.5:1 was used as the criterion for pooling fractions from the first DE52 column, and a ratio of 2.8:1 for pooling fractions from the second column. Alternatively, when sucrose gradient centrifugation was used to isolate LH II, a ratio of 3.0:1 was used.

Gel filtration chromatography

Isolated complex was applied straight from either an ion exchange column or sucrose gradients without further treatment other than filtered through a 22 μm filter.

Sephacryl S200

3 ml of complex was applied to a 1 metre long Sephacryl S200 Hi-Resolution gel permeation column (Pharmacia) previously equilibrated with 0.1% LDAO in 20

mM Tris-HCl, pH 8.0. The column was eluted with the same buffer, gravity fed at 4 °C. 2 ml fractions were collected and assayed spectrophotometrically. Those with a 850:280 ratio greater than 2.8 were pooled.

Superdex 200

A Superdex 200 16/60 FPLC column was equilibrated with two column volumes of 0.1% LDAO in 20 mM Tris-HCl, pH 8.0. A 5 ml aliquot of complex, from either ion exchange columns or sucrose gradients, was applied to the column and eluted with the equilibration buffer at a flow rate of 1ml/min. 1 ml fractions were collected and those with 850:280 ratios greater than 3.0:1 pooled.

Detergent exchange

Ion exchange chromatography

Watman DE52 was prepared as above. Using a small Pasteur pipette as a column, the slurry was poured i to produce a bed height of 3 cm. The column was equilibrated with 5 column volumes of 20 mM Tris-HCl, pH 8.0. Purified complex, in 0.1% LDAO, 20 mM Tris-HCl, pH 8.0 was applied to the column. When loading was complete the complex was washed with 3 column volumes of detergent free buffer, and then with two volumes of the new detergent in 20 mM Tris, pH 8.0. Elution was carried out by the addition of this buffered detergent solution containing in addition 350 mM NaCl.

Centrifugation Dialysis.

2 ml of purified protein were transferred to a Amicon 50 K 'Centricon' (Amicon, Inc., Beverly, MA 01915. USA) tube and was centrifuged at 5,000 rpm to minimum volume (approx. 300 µl). The complex was diluted with 2 ml of detergent free buffer and again centrifuged till minimum volume. This step was repeated twice to ensure removal of the original detergent. After the final wash the complex was diluted with buffer containing the new detergent, and any other additives (most usually 350 mM NaCl if the complex was intended for

crystallisation trials) and centrifuged to the required concentration. Again this step was repeated twice.

Linear Dichroism.

Crystallisation for Linear Dichroism Analysis

Crystals were grown by spreading 5ml of complex at an OD_{850} of 120 cm^{-1}, 350 mM NaCl, 1% β–Octyl-glucoside, 2.5% (w/v) benzamidine hydrochloride and 1 M K2HPO4, pH 9.5 into a well created by placing a 6 μm thick PTFE washer on a 13 mm glass coverslip. This was then placed on plastic supports in a microtiter plate (pictured in Cogdell & Hawthornthwaite, 1993) containing 200μl of 2.6 M ammonium sulphate, pH 9.5. The dish was sealed with tape and allowed to equilibrate at 17° C.

Data collection

Linear dichroism data were collected in the laboratory of Prof. W. Mantele, University of Freiberg, Germany, using a home built spectrophotometer (described in Wacker *et al.,* 1988). A cell was constructed by placing a second 13 mm coverslip over the 'well' in which the crystals grew. This was mounted in a home built cell holder, and clamped tightly. The laboratory convention is that the crystal is oriented in the instrument, with reference to the polariser, with the y-edge 'vertical' - consequently, the y-edge is aligned with the polariser in the 0° position figure C.2.

A screen was placed behind the crystal holder and the light beam slits adjusted till no extraneous light passed. The crystal was translated out of the light beam and, with the polariser set to 0°, a background spectra from 350 to 900 nm recorded. The crystal was moved back into the light beam and the spectra recorded over the same range. This procedure was repeated with the polariser rotated by 90°. After subtraction of the respective background measurements, both spectra were plotted and the degree of linear dichroism calculated.

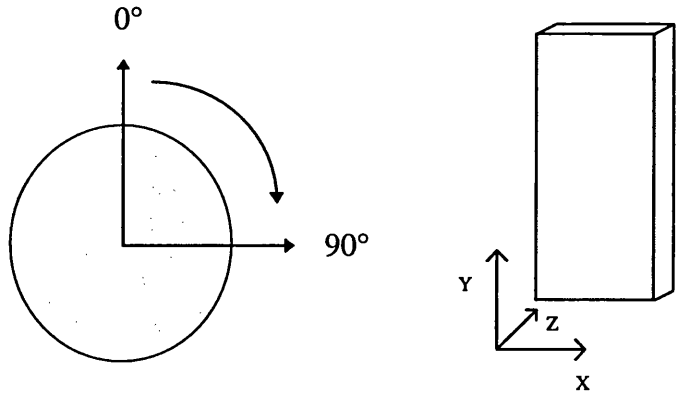


Figure C.2. Laboratory convention used to define crystal axes and polariser angle for linear dichroism experiments.

Crystallisation for X-ray analysis

***Original method** - as used in the crystallisation protocol of Papiz et al., 1989*

Crystallisation trials were conducted in 'large-scale vapour diffusion' dishes of diameter 10 cm (pictured in Cogdell & Hawthornthwaite, 1993). The dishes contained a perspex 'bridge' with a central depression which can hold crystallisation drops with a maximum volume of 50µl.

LH II at a concentration which gave an OD_{850} of 150-200 cm^{-1}, in 1% β-Octyl-glucoside, 350 mM NaCl, 20 mM Tris, pH 8.0 was made 2.5 % (w/v) benzamidine hydrochloride and 0.9 M K_2HPO_4. Prior to crystallisation the pH of the drops was increased to 9.5 using small quantities of solid NaOH. 30 to 50µl drops of this solution were equilibrated against 8 mls of reservoir solution consisting of 2.4 M ammonium sulphate, pH 9.5.

Optimised protocol - as used in McDermott et al., 1995

Protein at a concentration which gave an OD_{850} of 100 cm^{-1} in 1% b-Octyl-glucoside, 350 mM NaCl, 20 mM Tris, pH 8.0 was made 2.5 % (w/v) benzamidine hydrochloride and 1.0 M K_2HPO_4 (from a 4M stock solution in distilled water). The solution was centrifuged at 13,000 rpm in a Eppendorf mini-centrifuge for 10 minutes to pellet any solid material. Sitting drop crystallisation trials were set up in 'Cryschem' trays (Charles Supper Company, Natick, MA, USA). 15 µl drops were equilibrated against 1ml solutions of 2.2 M ammonium sulphate, pH 9.3.

Artificial mother liquor for heavy atom soaks

Original formulation

Using 'large-scale vapour diffusion' dishes, 35µl drops containing 1% β-Octyl-glucoside, 350 mM NaCl, 2.5% benzamidine hydrochloride and 1.0 M K_2HPO_4 were equilibrated against 8 mls of 2.2 M ammonium sulphate, pH 9.3 in for at least one week.

Optimised artificial mother liquor

10 cm diameter 'large-scale vapour diffusion' dishes (pictured in Cogdell & Hawthornthwaite, 1993) were altered to accomodate larger drops. Three 300µl drops containing 0.5% β-Octyl-glucoside, 350 mM NaCl and 1.5 M K_2HPO_4 were equilibrated against 8 mls of 2.3 M ammonium sulphate, pH 9.3 for between 36 and 48 hours.

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