ISOLATION, PURIFICATION AND CHARACTERIZATION OF THE ANTENNA COMPLEXES FROM *CHROMATIUM VINOSUM* STRAIN D

A Thesis Submitted For The Degree of

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by

MING LI

Department of Biochemistry/
Botany
University of Glasgow

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ABBREVIATIONS

ADH:	Alcohol dehydrogenase
Bchl(s):	Bacteriochlorophyll(s)
Bchl **a** (**b**):	Bacteriochlorophyll **a** (**b**)
$Bchl_2$:	Bchl dimer
Bpheo:	Bacteriopheophytin
BSA:	Bovine serum albumin
Chr30:	Cells of *Chr. vinosum* strain D grown at 30 °C
Chr40:	Cells of *Chr. vinosum* strain D grown at 40 °C
EDTA:	Ethylene diaminetetraacetic acid
FPLC:	Fast protein liquid chromatography
HFo:	Formic acid
ICM :	Intracytoplasmic membrane
LDAO:	Lauryldimethylamine-N-oxide
NIR	Near infra-red
PMSF:	Phenylmethylsulfonylfluoride
Q_A:	Primary quinone
Q_B:	Secondary quinone
RC:	Reaction centre
RP-FPLC:	Reverse phase FPLC
SDS:	Sodium dodecyl sulphate
SDS-PAGE:	Polyacrylamide gel electrophoresis in the presence of SDS.
TEMED:	NNN'N'-Tetramethylenediamine
Tris:	Tris (hydroxymethyl) methylamine

Summary

The B800-820 and B880 antenna complexes from 30 and 40 °C grown cells of *Chr. vinosum* strain D have been exhaustively purified and extensively characterized in this research.

The mild non-ionic detergent, Triton X-100, was found to be the most suitable for isolation of the B800-820 and the B880 antenna complexes from the intracytoplasmic membranes (ICMs) of the cells after preliminary experiments with the detergents SDS, LDAO, Brij-58 and cholic acid. The ICMs were first incubated in 30 % (v/v) Triton X-100 for 1 h at 0 °C and then were diluted to 4% (v/v) of Triton X-100. These solubilised membranes were finally subjected to sucrose density gradient centrifugation. The B800-820 and the B880 antenna complexes were initially obtained after 16 h of centrifugation at 197,000 xg. Each of these two complexes was further purified by DE-52 anion exchange chromatography, and eluted with a step-wise gradient in the range of 0 to 200 mM sodium chloride.

Preparation of the B800-850 antenna complex was attempted by using a different detergent, SDS, to solubilise the membranes. The solubilised extract was then precipitated with increasing concentrations of ammonium sulphate. However, we found that almost all the fractions collected from ammonium sulphate precipitation contained the B800-850 antenna complexes as judged by their NIR absorption spectra. Surprisingly, we failed

to detect the existence of the other two antenna complexes, i.e. the B800-820 and B880 antenna complexes (see Chapter 3 for details).

The protein compositions of the B800-820 and the B880 antenna complexes were visualized by SDS polyacrylamide gel electrophoresis. The B880 antenna complex contains two antenna apoproteins whereas the B800-820 antenna complex consists of at least three apoproteins. This result was confirmed by reverse phase liquid chromatography where a total of five individual apoproteins could be fractionated from these two complexes.

A highly reproducible method was developed for fractionation of antenna apoproteins from *Chr. vinosum* strain D by reverse phase liquid chromatography. The solvent system used was isopropanol, formic acid and water. This solvent system has the potential for general use in separating hydrophobic membrane proteins.

Amino acid sequencing of these five antenna apoproteins from *Chr. vinosum* strain D is still in progress. The amino acid compositions of three apoproteins have been determined so far. Two antenna apoproteins, the B880 α- and β-apoproteins, were estimated to contain at least 50 and 56 amino acid residues respectively; one of the three apoproteins of the B800-820 antenna complex contains approximately 60 amino acid residues. Similar to the antenna apoproteins sequenced from purple non-sulphur bacteria, these three apoproteins contain either one or two histidine residues, which are thought to be involved in binding to the Mg atom of Bchl, and a high content of aromatic amino acid residues.

The individual carotenoids from the B800-820 and the B880 antenna complexes were separated by silica thin layer

chromatography, and were identified by their R_f value and absorption spectra. The identification of each of the carotenoids was then positively confirmed by mass spectroscopy. The ratio of Bchl **a** to carotenoids was measured to be 2:1 for the B800-820 antenna complex and 1.5:1 for the B880 antenna complex in this research (see Chapter 5 for further explanations). Growth temperature appeared to have only a slight effect on the ratios of Bchl **a** to carotenoids for both the B800-820 and the B880 antenna complexes; however, it has considerable effects both on the carotenoid compositions of B800-820 antenna complexes and the ratio of Bchl **a** to protein of B880 antenna complexes.

The membrane topology of the antenna complexes of *Chr. vinosum* strain D was studied in a preliminary fashion by mild non-specific proteolysis with proteinase K as well as immunological means. Polyclonal antibodies against the purified B800-820 and B880 antenna apoproteins have been successfully raised. In the future, these antibodies could be employed to probe the lateral distribution of the individual antenna complexes on the intracytoplasmic membranes.

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Chapter 1

Introduction

§1-1 Overview of Photosynthesis

Photosynthesis is the most fundamental process on the earth; every plant and animal relies, directly or indirectly on it to survive. It is a complex process in which radiant solar energy is captured and converted into chemical free energy in the form of NAD(P)H and ATP (Clayton, 1971). NAD(P)H and ATP are needed to convert carbon dioxide into carbohydrate (van Niel, 1935; Gaffron, 1960).

The overall mechanism of photosynthesis, so far as it is understood is fundamentally similar in all organisms and can be expressed by the following simple universal equation:

$$2\,H_2A + CO_2 \xrightarrow{h\nu} (CH_2O) + 2A + H_2O$$

The reducing substrate H_2A is H_2O for oxygen-evolving photosynthesis, as found in higher plants or algae. For anoxygenic photosynthesis, however, H_2A can be a great variety of organic or inorganic substances such as H_2S , $S_2O_3^{2-}$, ethanol and organic acids.

From the evolutionary point of view anoxygenic photosynthetic bacteria are the precursors to chloroplasts as well as mitochondria and these bacteria have been used as useful experimental models for understanding the mechanism of

photosynthesis in general. They have proven themselves to be solid stepping-stones to probe and reveal the secret of the light reactions of photosynthesis.

§1-2 Classification of Purple Bacteria

§1-2-1. Taxonomic Classification

Purple bacteria belong to the order of *Rhodospirillales*. These are bacteria which contain Bchl **a** or Bchl **b** and perform anoxygenic photosynthesis (Fig1-1 illustrates the classification of the order of *Rhodospirillales)*.

Initially, purple bacteria were divided into two taxonomic groups: *Chromatiaceae* (previously *Thiorhodaceae*) or purple sulphur bacteria, and *Rhodospirillaceae* (previously *Athiorhodaceae*) or purple non-sulphur bacteria.

All members of *Chromatiaceae* can grow with sulphide and some of them can also use elemental sulphur, sulphite, or thiosulphate as electron donors. During cell growth, sulphide or thiosulphate is oxidized *via* elemental sulphur to sulphate, and the resultant sulphur is deposited inside the cells. More recently the *Chromatiaceae* were subdivided and a third family, *Ectothiorhodaceae*, was created based on the feature that these bacteria oxidize sulphide but deposit the resultant sulphur extracellularly (Imhoff, 1984; Imhoff et al., 1984). The bacteria of *Rhodospirillaceae* grow preferentially under photoheterotrophic conditions and many of them are aerotolerant and can grow as chemoheterotrophs in the dark.

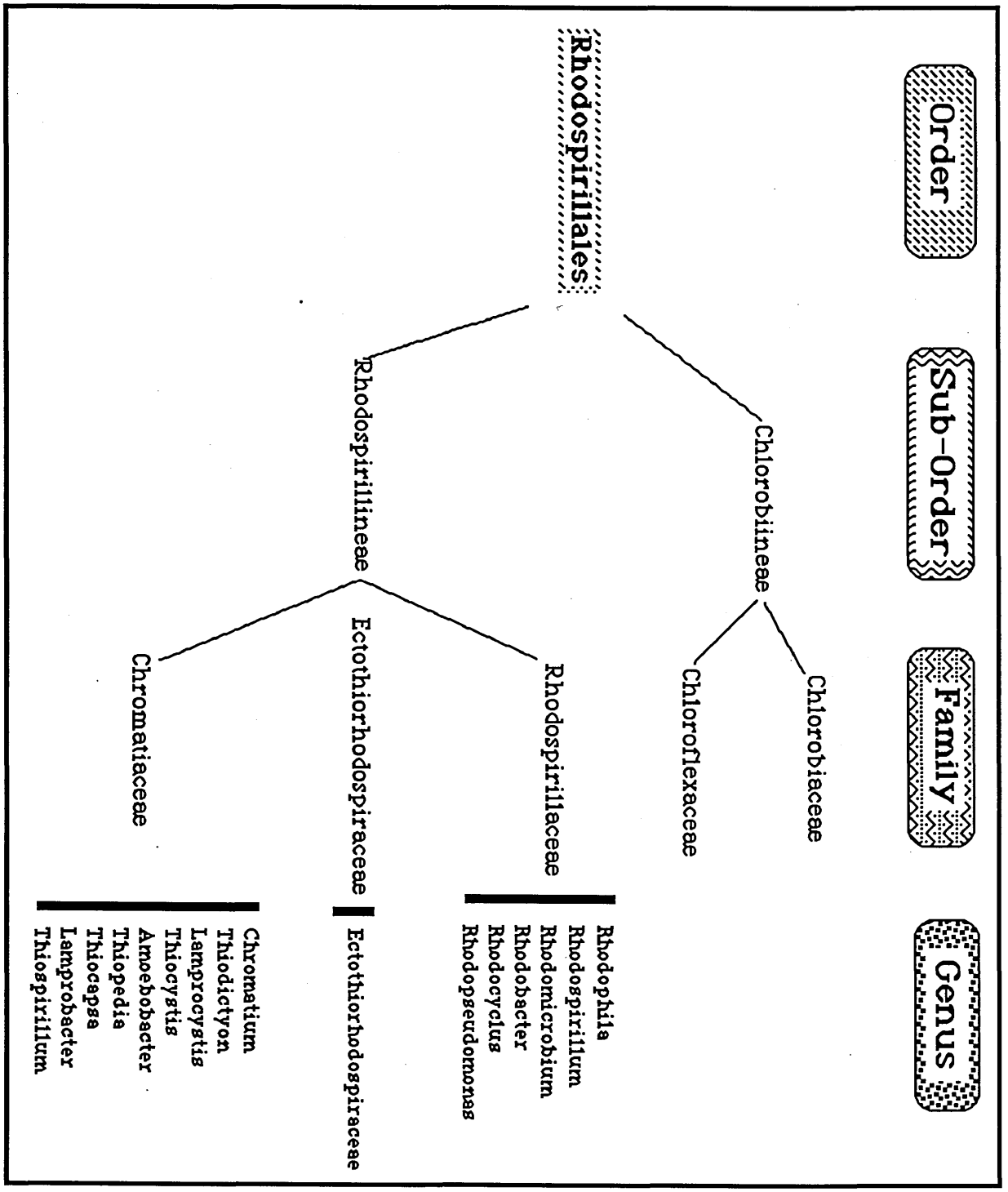


Fig. 1-1 Illustration of the classification of the order of *Rhodospirillales*.
(Adopted from Pfennig and Truper, 1984)

Although some species are able to use sulphide as an electron donor, only very few oxidize sulphide *via* elemental sulphur to sulphate (Hansen & Imhoff, 1985) and typical globules of elemental sulphur seen in *Chromatiaceae* never appear inside the cells of *Rhodospirillaceae*.

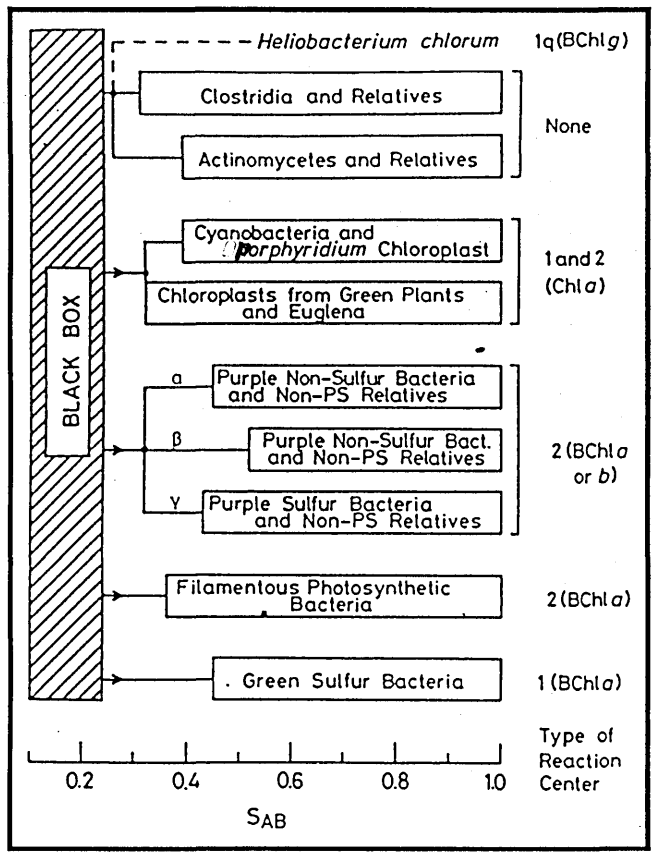


Fig. 1-2 Dendrogram of relationship between photosynthetic prokaryotes and their relatives. This dendrogram is taken from Pierson and Olson (1987). S_{AB}: association coefficient.

§1-2-2. Phylogenetic Classification

Purple bacteria are divided into three major subdivisions on the basis of oligonucleotide catalogues of 16S rRNA (Woese et al., 1984[a,b]). The alpha subdivision (see Fig. 1-2) includes most of

Rhodospirillaceae and several non-photosynthetic relatives (Woese et al., 1984[a]). The beta subdivision contains three other species of *Rhodospirillaceae* and several species of non-photosynthetic relatives (Woese et al., 1984[b]). All members of the *Chromatiaceae* and *Ectothiorhodaceae* are included in the gamma subdivision (Woese et al.,1985; Fowler et al.,1984)

This scheme of classification reveals the interesting phylogenetic relationship between these bacteria and their non-photosynthetic relatives.

§1-3 Photosynthetic Apparatus of Purple Bacteria

The primary reactions of photosynthesis are processes in which light energy is converted into chemical free energy. These processes take place in the photosynthetic apparatus which contains mainly antenna complexes and reaction centres.

In this process, light energy is absorbed predominantly by the antenna complexes. This creates excited states of electrons, called excitons, and these excitons are then funneled to and trapped in the reaction centre. In the reaction centres charge-separation occurs, which leads to the processes which eventually produce a membrane potential and a proton gradient across the membrane (Parson and Ke, 1982).

The photosynthetic apparatus consists of a large number of photosynthetic units. The term, photosynthetic unit, was originally defined as the number of Bchls per reaction centre; however, this unit can now be unambiguously referred to as the assemblies of antenna complexes and reaction centres. Several photosynthetic units are interconnected to form a functional domain for energy

transfer (Clayton, 1967). In each domain, reaction centres are embedded as if in a "lake" of antenna complexes which are ready to trap oncoming excitons from any direction within the "lake" (Monger and Parson, 1977; van Grondelle, 1985).

§1-4 Membrane Location of Photosynthetic Apparatus

Most of the photosynthetic components involved in the primary reactions of bacterial photosynthesis are located within intracytoplasmic membranes (ICMs or chromatophore membranes), including reaction centre complexes, antenna complexes, ATPase and $\mathbf{b/c_1}$ complex (Bachofen and Wiemken, 1986).

§1-4-1 Structure and Development of ICM of Purple Bacteria

There are three main types of ICMs observed in purple bacteria, namely vesicular, tubular and lamellar membranes. Fig. 1-4 shows in diagrammatic form these different types of ICMs. There are, however, a few exceptions to the morphologies described in Fig. 1-4. For instance, *Rhodospirillum tenue* contains no ICMs and the photosynthetic apparatus for this species is located in the cytoplasmic membrane.

These ICMs are physically connected to the CM which can be seen clearly under the electron microscope (Sprague and Varga, 1985). It is presumed that ICMs are developed from CM under certain conditions such as low oxygen tension in the cell environment and different light intensities (Drews, 1986). The

photosynthetic components are synthesized and assembled along with the formation of ICMs.

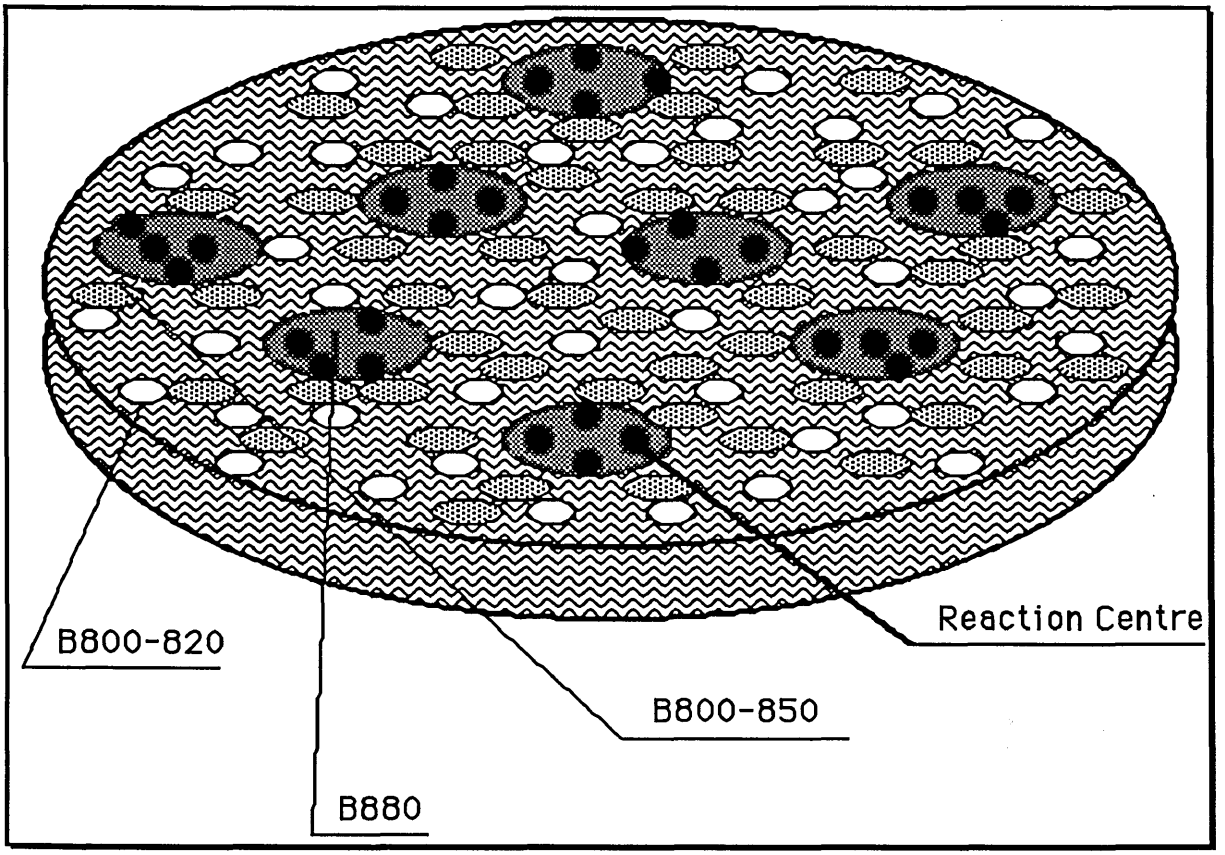


Fig. 1-3 Schematic Lake Model (adopted from van Grondelle, 1985)

Recent research on the development of new ICMs by Chory and his collegues(Chory et al., 1984) has provided new insights into the mechanism of ICM development. In this study, cells of *Rb. sphaeroides* were allowed to grow firstly under aerobic conditions. Neither ICMs nor pigment-protein complexes were found in the cells at this stage. However, they found that indentations were present on the CM; in addition, they also found low levels of the H subunit of RCs. When the aerobic growth condition was changed to strictly anaerobic conditions, ICMs, along with photosynthetic

apparatus, were progressively formed, and the cells adapted to the photosynthetic mode of growth. It was proposed from this experiment that the indentations and the presence of the H subunit may provide assembly sites for the formation of ICM as well as photosynthetic apparatus.

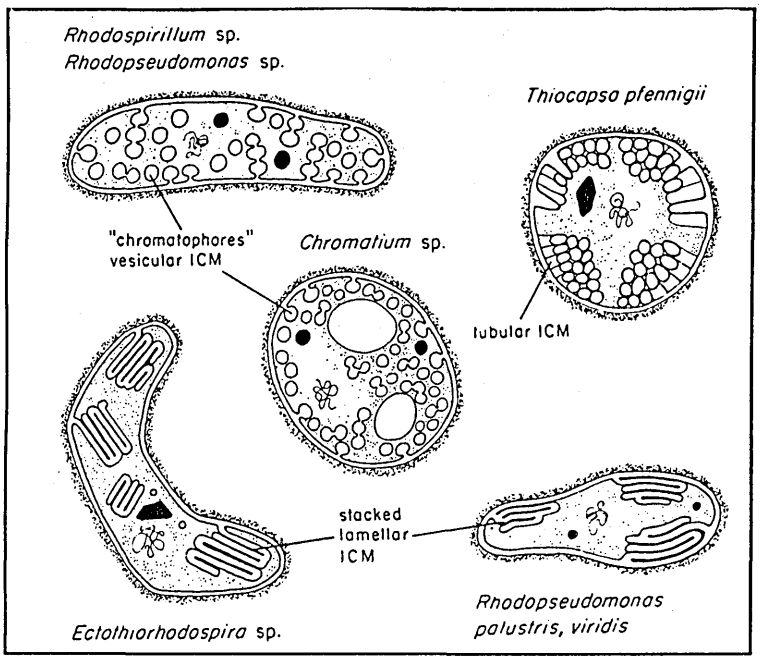


Fig. 1-4. Illustration in diagrammatic form of the different types of intracytoplasmic membranes of purple bacteria. Most purple bacteria elaborate an extensive system within the cytoplasm for the photosynthetic components, (taken from Sprague and Varga, 1985).

More recently, using chemical and transposon mutagenesis techniques, Hunter's group produced several mutants which have permitted further elucidation of the development of ICM (Hunter, 1988). From a wild-type strain of *Rb. sphaeroides*, they successfully isolated *Rb. sphaeroides* mutants NF57 ($B875^-$, RC^-), M21 ($B800^-$, 850^-) and M2192 ($B800^-$, 850^-, RC^-). Their studies suggest that (i) the B800-850 apoproteins, either α- or β-

apoprotein, is necessary for the formation of vesicular ICM; (ii) the maturation process can be completed without B875-RC conjugates and (iii) morphogenesis of the membrane is arrested at the tubular stage in the absence of B800-850 complexes.

The structure of ICMs can be influenced by several other factors: (i) B880 antenna complexes (in *Rps. palustris*) may act as a main membrane adhesion factor for stacking and appression of ICMs (Varga and Staehelin, 1985); (ii) irreversible destruction of the pigments leads to a loss of the typical ordered membrane structure (Ghosh and Bachofen, 1984); (iii) carotenoids may play an important role in ICM structure, judging by comparison of the ICM structure of the carotenoid-less strain R-26 of *Rb. sphaeroides* with its parent wild-type strain. (Lommen & Takemoto, 1978); (iv) the ratio of lipid to protein (Fraley et al., 1979; Chory et al., 1984) seems to be important for the formation of the ICM structure of *Rb. sphaeroides*, since the synthesis of lipids and antenna proteins is related to the formation of the ICM.

§1-4-2 Membrane Location of Photosynthetic Apparatus

The ICMs contain all of the components of the light reactions of bacterial photosynthesis. However, little is known about how these functional components are organized within the photosynthetic membranes to allow the whole system to function efficiently.

Three major biochemical methods have been used to investigate the membrane location of each component (Bachofen and Wiemken, 1986): (i) immunological approaches (Brunisholz et al., 1986); (ii) mild non-specific proteolysis (Tadros et al., 1986);

and (iii) hydrophobic / hydrophilic labelling (Meister et al., 1985). The current picture of the topology of membrane components was obtained basically from the information revealed by these three methods, in addition to some physical methods.

a. Membrane Location of Reaction Centre Complexes

Reaction centre complexes are the most thoroughly studied photosynthetic components. A typical RC complex consists of three subunits in 1:1:1 stoichiometry with apparent M_r values of 20 to 30 kD. The primary structures of the L and M subunits from *Rb. capsulatus* (Youvan et al., 1984), *Rb. sphaeroides* (Williams et al., 1983; 1984[a&b]) and *Rps. viridis* (Michel et al., 1986) have been determined. The amino acid sequences are highly conserved from species to species and the L and M sequences exhibit a high degree of homology. It has been suggested, based on the length of the helical regions and the hydrophobic nature of amino acid residues, that the L and M subunits could span the membrane five times in the form of alpha-helixes. The H subunit, however, could only span the membrane once (Youvan et al., 1984; Michel et al., 1985). This assumption was tentatively confirmed by CD and IR spectroscopy which indicated that the reaction centre contains considerable amounts of alpha-helical structure. By all three biochemical methods, evidence was also obtained to suggest that all three subunits of the reaction centre cross the membrane and are exposed on both membrane surfaces. Confirmation of these assumptions, however, was made after a high-resolution 3-D structure of reaction centre complexes became available (Deisenhofer et al., 1985[a&b]; Yeates, et al., 1987). Fig. 1-5 shows

the current structural model of the membrane location of the reaction centre complexes from *Rb. sphaeroides* strain R-26, which was based on the three dimensional structure of the RC from *Rb. sphaeroides* strain R-26, and the hydrophobic energy function of Eisenberg and McLachlan (1986).

b. Membrane Location of Antenna Complexes

Most of the antenna complexes from purple bacteria contain two or three low M_r hydrophobic polypeptides to which pigment molecules are bound and oriented in the plane of the membrane for efficient energy harvesting and transfer. The antenna polypeptides sequenced so far share a common feature: hydrophobic in the central region (approx. 20 amino acid residues) and hydrophilic at both C- and N-terminal regions. Far ultraviolet CD spectra of intact antenna complexes have confirmed the prediction of an alpha-helical structure in the hydrophobic stretch (Cogdell and Scheer, 1985), which is tilted at less than 40° to the plane of the ICM as proposed from earlier polarized IR studies (Nabedryk and Breton, 1981). It is assumed that these polypeptides cross the membrane once in the form of an alpha-helix on comparing the length of hydrophobic region, 30-35Å long, with the thickness of the membrane.

Immunological studies show that antenna polypeptides of B890 complexes from *R. rubrum* are located at both cytoplasmic and periplasmic membrane surfaces (Brunisholz et al., 1986). Mild proteolysis of antenna polypeptides from *Rb. sphaeroides, Rb. capsulatus* and *R. rubrum* with the non-specific protease, proteinase K, further reveals that the N-terminal regions of

antenna apoproteins are located at the cytoplasmic membrane surface and the C-termini are most probably exposed to the periplasmic surface (Brunisholz et al., 1986; Drews, 1986).

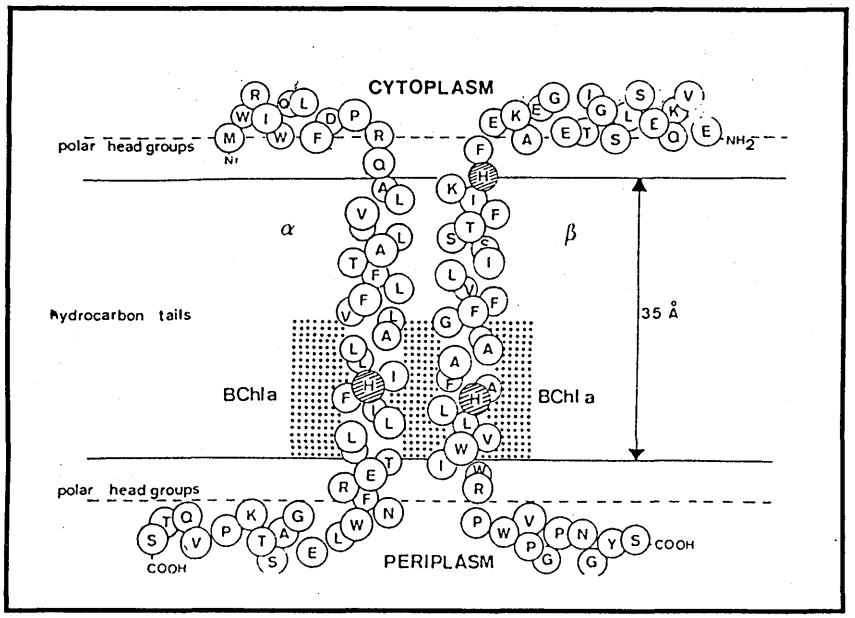


Fig. 1-6. Transmembrane arrangement of α- and β-apoproteins of the B880 antenna complex from *R. rubrum*. (taken from Brunisolz et al., 1986)

Similar results were also obtained by using chemical markers (Meister et al., 1985; Bachofen et al., 1986). All this evidence strongly supports the predicted model of membrane location of antenna complexes as proposed initially by Zuber and Bachofen and their colleagues. (Brunisholz et al., 1984). Fig 1-6 showes the proposed model for the organisation of the two antenna apoproteins of the B890-complex from *R. rubrum*. In this model, the two hydrophobic regions of the two apoproteins are embedded in the membrane in the form of alpha-helices which are perpendicular to the plane of the membrane. Two histidine residues, which are thought to be possible ligands to the Mg atom

of Bchl, are found within the alpha-helixes and are close to the periplasmic membrane surface (Zuber et al., 1987).

§1-4-3 Lateral Distribution of Photosynthetic Components within ICM

Most of our knowledge on the membrane location of individual photosynthetic components comes mainly from analysis of the topographical distribution of the constituent apoproteins. Once the primary structure of an apoprotein is known, it is theoretically possible to predict the location of its cofactors to a certain degree of accuracy within the complex. Practically, however, a detailed three-dimensional structure will certainly come from high resolution X-ray analysis of the crystallized complex.

Over the last decade, evidence has accumulated that most of the intracytoplasmic membrane proteins of purple bacteria investigated so far are exposed to both inside and outside membrane surfaces and seem to cross the membrane at least once. It is possible now to draw a picture of the lateral distribution of photosynthetic components within the ICM (Fig. 1-7). This picture will certainly be further developed from the coordinated efforts of several major groups in the near future.

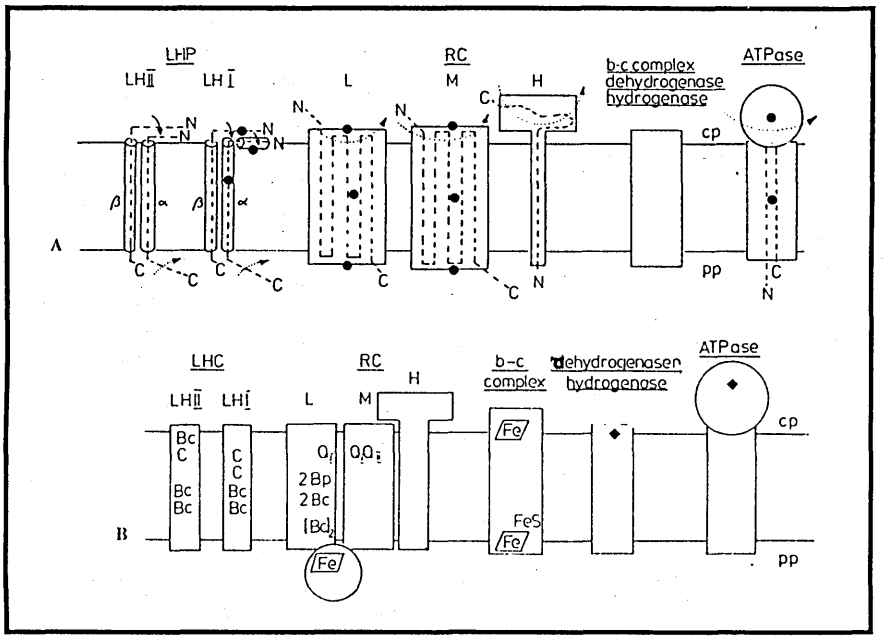


Fig. 1-7 Lateral distribution of membrane proteins within ICMs (taken from Bachofen et al., 1986).
Known structure (----); labelling site (.); cytoplasmic side (cp); periplasmic side (pp); LHP (light-harvesting polypeptides); LHC (light-harvesting complexes)

§1-5 The Structure and Function of the Bacterial Reaction Centre

Since the first reaction centre complexes were isolated from a purple bacterium in the late 1960s (Reed and Clayton, 1968), there have been very many studies on the structure and function of reaction centres from various species of purple bacteria. The dramatic recent breakthrough in this field has been the crystallization of reaction centre complexes from *Rps. viridis* (Michel, 1982). These crystals have been used to produce the first high-resolution structural model for a hydrophobic membrane protein complex. The current structural model of the reaction centre, based on X-ray diffraction data from *Rps. viridis* and *Rb.*

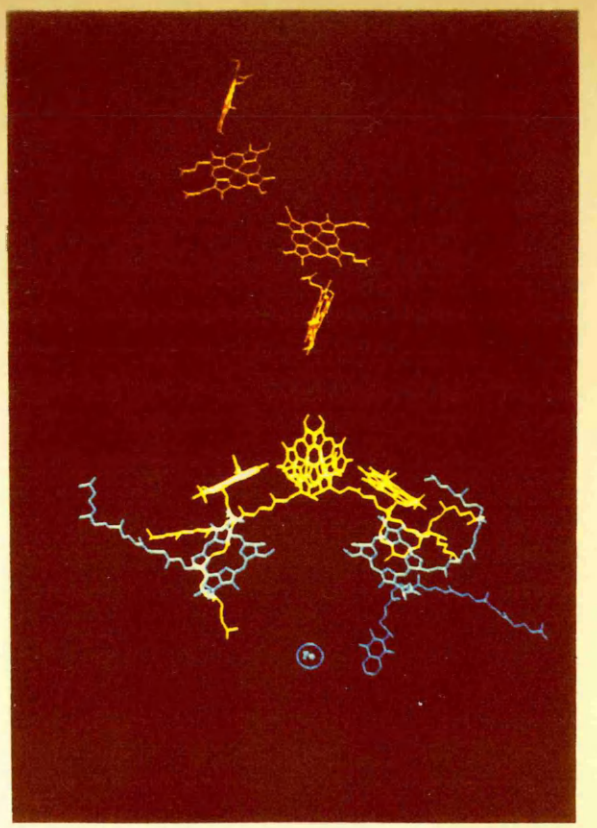
sphaeroides, strain R-26, has been obtained to a resolution of less than 3.0 Å and is still undergoing refinement (Michel and Diesenhofer, 1986; Allen et al., 1987a&b; 1988; Yeates et al., 1987). The structural information provides the means to understand the primary photochemical reactions of photosynthesis in three-dimensional terms.

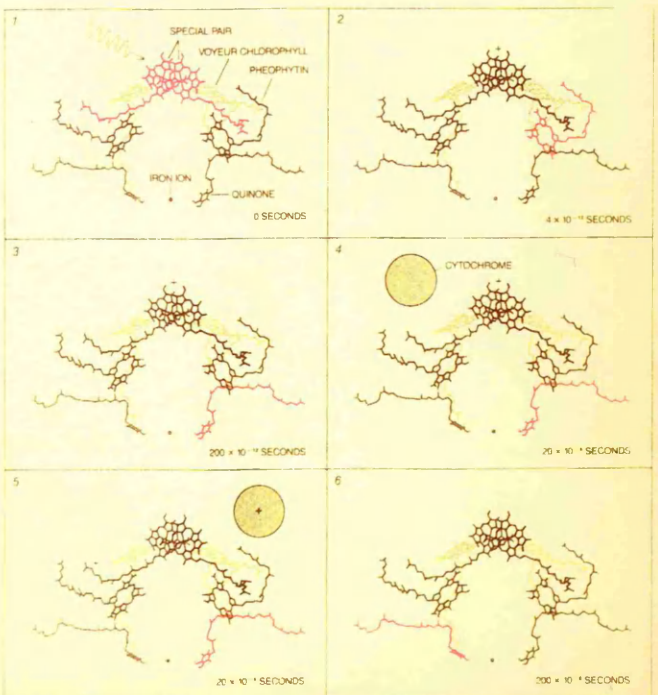
Fig. 1-5 shows the three-dimensional structure of the reaction centre from *Rb. sphaeroides* strain R-26, which was determined by X-ray diffraction at a resolution of 2.8 Å. This structure consists of three protein subunits (L, M, H polypeptides), four molecules of Bchl **b**, two molecules of Bpheo **b**, two molecules of quinone (Q_A and Q_B) and one atom of ferrous iron. A water soluble cytochrome c_2 was added into the structure by computer simulation (Allen et al., 1987a&b). The L, M and H polypeptides have been completely sequenced and contain 281, 307 and 260 amino acid residues respectively (Williams et al., 1983; 1984a&b). Each of the L and M subunits contains five transmembrane helices and are related to each other by a two-fold rotational symmetry axis. The H subunit has one transmembrane helix and a globular domain on the cytoplasmic side.

Fig 1-8 shows the model of the RC-cytochrome complex of *Rb. sphaeroides* strain R-26. The binding site of the cytochrome c_2 is on the periplasmic side of the membrane (Prince et al., 1975), which is shared by both the L and M subunits (Rosen et al., 1983). The binding domain on the RC contains negtively charged carboxylate groups that are believed to interact electrostatically with lysine residues surrounding the haem crevice of cytochrome c_2 (Margoliash et al., 1983; Hall et al., 1987).

Fig. 1-9 Cofactor structure of the RC from *R. viridis*. The top four molecules are haems. (see Fig 1-13 for more clear illustration of the other cofactors), taken from Cogdell (1988).

Fig. 1-10 Current knowledge about the mechanism of primary photochemical reactions within the RC, taken from Youvan et al.,(1987)





CHEMICAL REACTIONS that make up the early stages of photosynthesis transport an electron to one end of the reaction center while leaving behind a region of positive electric charge at the other end. In the first step of photosynthesis (*1*) a photon is absorbed by a special pair of chlorophyll molecules and transfers its energy to an electron in the special pair. (In each step the molecules bearing an electron in an excited state are shown in red.) The electron then moves (*2*) to a pheophytin molecule, passing a voyeur chlorophyll molecule and leaving a positive charge on the special pair. From there the electron travels (*3*) to the quinone molecule at the end of one spiraling chain of prosthetic molecules. At this stage a cytochrome molecule (a globular molecule) moving freely in solution approaches the special pair (*4*) and transfers an electron to it. The cytochrome molecule thereby acquires a positive charge (*5*) and the special pair is neutralized. Later the excited electron that had traveled to one quinone molecule passes to the second quinone molecule (*6*). The separation of the electron and the region of positive charge represents stored energy.

An approximate twofold axis of symmetry of pigments in the structure is maintained by the L and the M subunits. The function of the H subunit, which does not bind pigments, is not clear so far. Speculations have been made based on its position within the membrane and its molecular microenvironment. This subunit may be involved in the electron transfer from Q_A to Q_B; it may be needed to maintain an adequate conformation of antenna complexes for energy transfer and/or serve as the target site for the assembly of the photosynthetic apparatus (Chory et al., 1984).

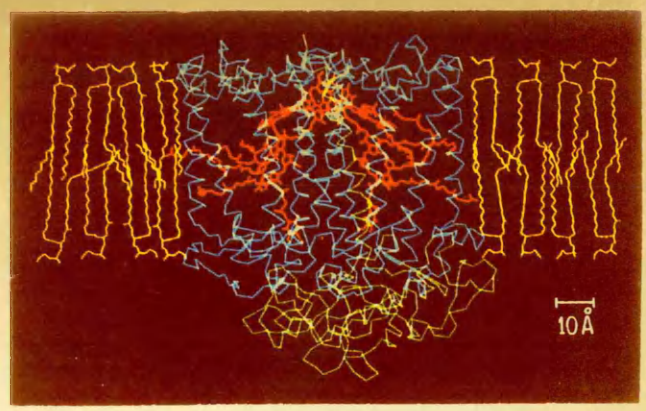
Fig 1-9 shows the spatial arrangement of the cofactors from *R. viridis*. The four haems lie on the top of the Bchl **b** dimer, and this dimer, which lies on this axis of symmetry is stacked with pyrrole rings I on top of each other. The other two Bchl **b** and two Bpheo **b** are allocated equally to form two arms; the right-hand side arm is called the L branch and the left-hand side arm is called the M branch, which are named after the reaction centre subunits with which they are mainly associated.

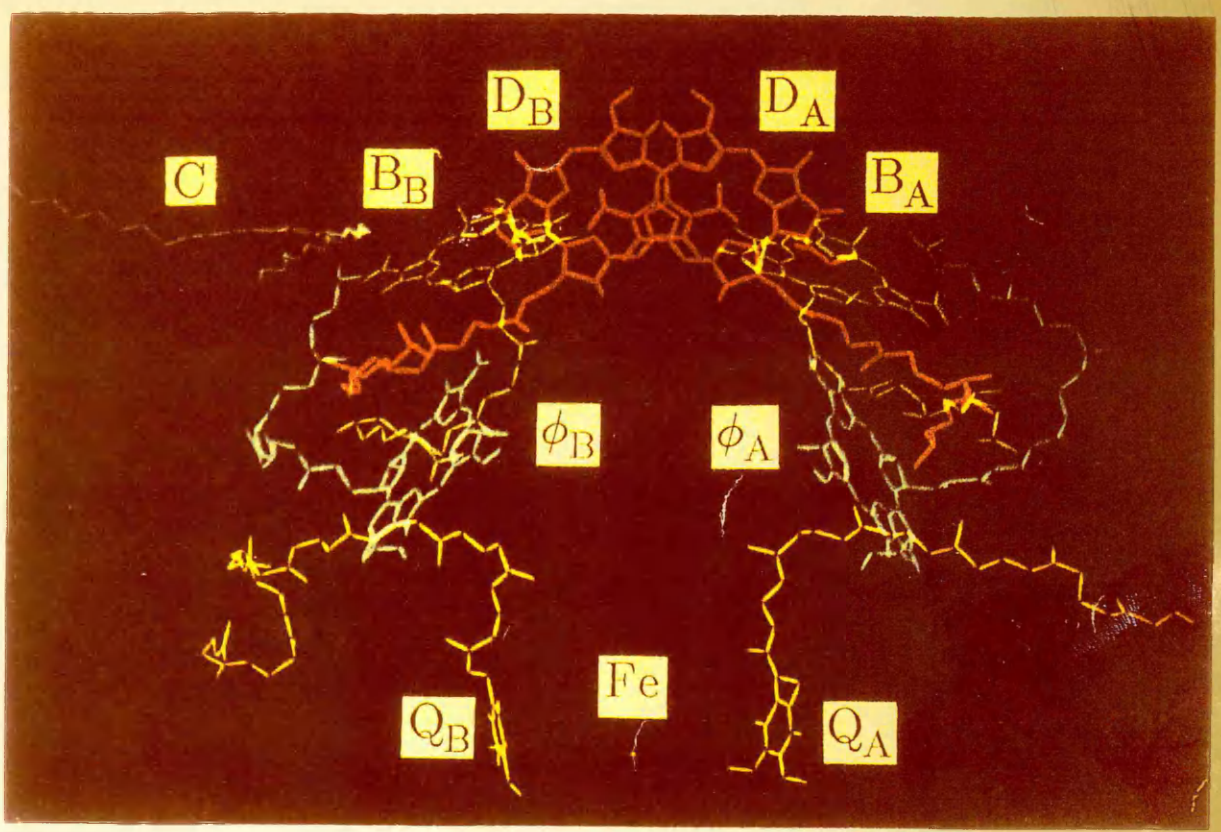
The radiant solar energy is harvested by antenna complexes, creating excited electrons (energy) and funnelling this energy to the reaction centres. In the reaction centre, the Bchl **b** dimer receives the energy which is then transferred to Bpheo **b** and further to Q_A, leaving a hole on the Bchl **b** dimer which is filled by the electrons come from the top, four haems. Spectroscopic evidence on the reaction centre shows that, either in solution or in the crystalline state, only the L branch is used for electron transfer (Zinth et al., 1983). The function of the M branch, however, is unknown so far. It has been suggested that, as L, M, (H) subunits are necessary for maintaining an optimal conformation for pigments, the Bchl **b** and Bpheo **b** in the M

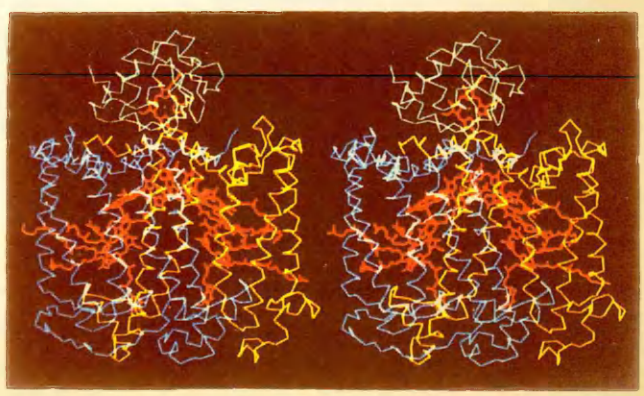
Fig. 1-5 Structural model of membrane location of the reaction centre complex from *Rb. sphaeroides* strain R-26, (LM complex, blue; H subunit, green; cofactors, red; lipid bilayer, yellow), (from Yeates et al., 1987).

Fig. 1-14 The structure of the chromatophores in RC based on the RC X-ray diffraction data from *Rb. sphaeroides* wild type strain 2.4.1. C, the carotenoid; D_A and D_B, the primary donor; B_A and B_B monomeric Bchl; ϕ_A and ϕ_B, monomeric Bpheo; Q_A and Q_B, quinones. takenfrom Allen et al.,(1988)

Fig. 1-8 A model of cytocrome c_2-RC complex of *Rb. sphaeroides* strain R-26, (L subunit, yellow; M subunit, blue; cofactors, red; cytochrome c_2 , green with its haem, red).The periplasmic side of the membrane is near top and cytoplasmic side is near the bottom., taken from Allen et al.,(1988)


10 Å


D_B
D_A
C
B_B
B_A
ϕ_B
ϕ_A
Fe
Q_B
Q_A



branch may be needed to guarantee the correct spatial structure of the L and M subunits.

Based on the structural model of the reaction centre from *Rps. viridis* and *Rb. sphaeroides*, the primary photochemical events taking place within bacterial membranes now can be expressed precisely as in Fig. 1-10.

§1-6 The Structure and Function of Antenna Complexes in Purple Bacteria

The photosynthetic apparatus also houses a large number of antenna complexes which are integrated with the reaction centres (van Grondelle, 1985).

The function of antenna complexes is to harvest radiant solar energy and to transfer that energy, in the form of excitons, to reaction centres with high efficiency and little energy dissipation (Thornber et al., 1983; Drews, 1985).

§1-6-1 Nomenclature of Antenna Complexes

Antenna complexes of most purple bacteria can be classified into two groups: light-harvesting complex, type I (LH I) and light-harvesting complex, type II (LH II). Owing to the lack of information in these titles, however, these trivial names are now tending to be replaced by a newly proposed system of nomenclature (Cogdell et al., 1985), with antenna complexes now often called B890, B800-850 complexes and so forth. B stands for "Bulk", whereas the numbers refer to the Q_y absorption maximum(a) of the appropriate Bchl. The following antenna

complexes are often encountered in purple bacteria: (i) B-875, B-880, B-890, and B-1020 complexes which are equivalent to LH I complexes or "core" complexes; (ii) B-800-820 and B-800-850 complexes which correspond to LH II or "peripheral" complexes. Purple bacteria are able to adapt themselves to changed external conditions in several ways and exhibit their adaptation in the form of alterations in the shape of their NIR absorption bands. To address this point, B800-850 complexes are subdivided into two types: (i) B800-850 complexes, type I with high 850 nm absorption and low 800 nm absorption, and (ii) B800-850 complexes, type II with low 850 nm absorption and high 800 nm absorption (Thornber et al., 1986).

§1-6-2 Structure and Function of Antenna Complexes

Currently there are two experimental strategies to study the structure and function of antenna complexes: (i) antenna complexes are obtained by solubilizing membranes followed by fractionation techniques such as ion exchange chromatography, sucrose density gradient centrifugation and electrophoresis. Most of our present knowledge of purified antenna complexes is based on this strategy; (ii) the second strategy has been developed only recently. This strategy relies on the use of chemical and transposon mutagenesis techniques to produce strains which contain either a single antenna complex or combinations of antenna complex with other antenna complexes or reaction centres (Drews, 1988; Hunter, 1988). This second strategy has several advantages over the first one. One advantage is that the structure and function of the antenna complexes can be

investigated *in vivo*. Therefore, possible artifacts caused by fractionation procedures, especially by detergents, can be eliminated.

There are two main types of antenna complexes found in purple bacteria: (a) the "core" antenna complexes, and (b) the peripheral or "variable" antenna complexes. The core antenna complexes closely surround and interconnect the reaction centres, forming reaction centre-antenna conjugates (Dawkins, 1988). They are usually found in a constant amount with respect to the concentration of reaction centres, and are more dedicated to that reaction centre (Aagaard and Sistrom, 1972). The peripheral antenna complexes are present in photosynthetic apparatus in variable amounts with respect to reaction centre concentration and are able to transfer their absorbed energy to several different reaction centres (Cogdell, 1988).

The organization of antenna complexes of purple bacteria has been studied intensively during the last decade. A minimal model for the B800-850 antenna complex of *Rb. sphaeroides* has been recently proposed based on the results of fluorescence polarization spectra (Kramer et al., 1984). In this model, the B800-850 complex of *Rb. sphaeroides* consists of two $Bchl_{800}$ molecules, four $Bchl_{850}$ molecules, and three carotenoids which are non-covalently bound to four apoproteins (see Fig. 1-11). The 850 nm absorption corresponds to a Bchl dimer, and a Bchl monomer is responsible for the 800 nm absorption. The controversial ratio of Bchl a to carotenoids has been recently redetermined by using HPLC and two other methods (Evans et al., 1988; Evans, 1989) and a ratio of 2:1, instead of 3:1, was obtained and is in agreement with this model.

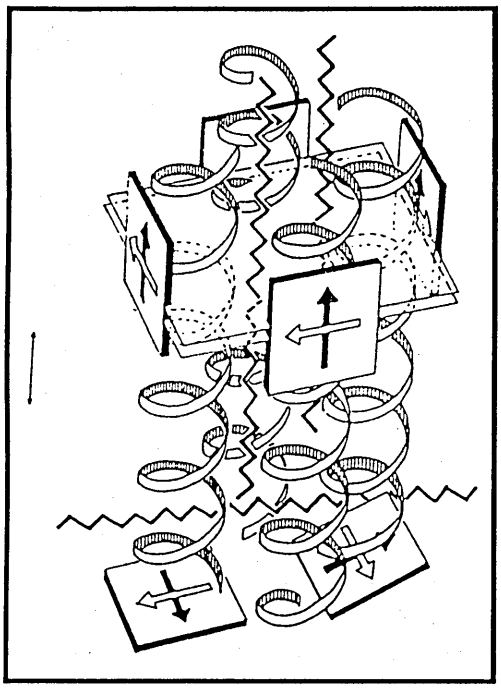


Fig. 1-11 A minimal model for the B800-850 antenna complex of *Rb. sphaeroides*. The basic unit consists of four Bchl 850 molecules (upper boxes), two Bchl 800 molecules (lower boxes), three carotenoids (zigzag lines) and two proteins. The open arrows represent Q_y dipoles and the block arrows represent Q_x dipoles (taken from Kramer et al., 1984).

a. Structure and Function of Antenna Polypeptides

All antenna complexes isolated from purple bacteria so far contain two or three low M_r (5.0-9.0 kD), strongly hydrophobic polypeptides which have been named α- and β- (or γ-) apoproteins. These proteins are found in a 1:1 (or:1) stoichiometry (Drews, 1985; Zuber et al., 1987).

During the last decade, more than 20 apoproteins have been successfully sequenced from purple bacteria. The primary structures of all these apoproteins share a common tripartite

feature: hydrophobic in the central domain (approx. 20-23 amino acid residues) and hydrophilic at either end.

The α- and β-polypeptides were found to exhibit only slight sequence homologies (7-13%); however, the α- or β-polypeptides in the corresponding individual complexes in various purple bacteria have a relatively high sequence homology (30-80%) (Zuber, 1986), indicating the phylogenetic relationship among those bacteria.

One conserved histidine residue has been found in the hydrophobic domain of both the α- and β-apoproteins, which is the most probable candidate for a ligand to the Mg-tetrapyrrole of the Bchl dimer. Another conserved histidine residue was also found in the N-terminal domain of the β-apoprotein, and this histidine is suggested as a possible binding site for monomeric Bchl (see Fig. 1-11).

Structural analysis of the apoproteins also shows that α- and β-apoproteins contain several aromatic amino acid residue clusters (Brunisholz, 1988). These clusters, apart from affecting spectral properties of antenna complexes, are probably playing vitally important roles in this highly efficient energy harvesting and trapping network due to their unique molecular structures.

b. The Functional Size of Antenna Complexes

The basic functional units of photosynthesis of several purple bacteria and their mutants have been determined recently by van Grondelle's group using singlet-singlet annihilation techniques.

The functional size of "core" antenna complexes, B875 complex in *Rb. sphaeroides* and B880 complexes in *R. rubrum,* contains

100-150 energetically connected Bchl **a** molecules at 4 °K and the "peripheral" antenna complexes of B800-850 complex in *Rb. sphaeroides* contain 50 Bchl **a** (Bakker et al., 1983; Vos et al., 1986; Vos, 1987). At room temperature, these basic functional units tend to form aggregates acting as domains for energy transfer. It is observed that at least 8 of the functional units in *Rb. sphaeroides* mutant M21 are aggregated to form a single domain and 15-25 units are connected in *R. rubrum*. Reaction centres seem to be necessary for the aggregation of those units. It is also assumed from these studies that antenna complexes are spectrally heterogeneous.

§1-7 The Function of Pigment Molecules in Purple Bacteria

Purple bacteria contain either Bchl **a** or Bchl **b** together with the carotenoids. These pigments are all located in the pigment-protein complexes, i.e. antenna complexes and reaction centres, which are associated with intracytoplasmic membranes (Thornber, 1978; Siefermann-Harms, 1985).

§1-7-1 Bacteriochlorophylls

The structures of Bchl **a** and Bchl **b** in purple bacteria are presented in Fig. 1-12. *In vivo* Bchls are associated and interact with membrane proteins to maintain adequate structures for these Bchls to function efficiently.

Biosynthesis of Chl (Bchl) involves a complex pathway which has been recently reviewed in great detail (Rudiger and Schoch, 1988).

In this pathway, 5-aminolaevulinic acid (ALA) is the first specific precursor for formation of (B)Chls. Condensation of two molecules of ALA with elimination of two molecules of water leads to the first pyrrole compound, porphobilinogen (PBG). Four molecules of PBG then combine to give rise to the first tetrapyrrole molecule, uroporphyrinogen III. (B)Chls are finally formed after a series of enzyme catalysed reactions and modifications.

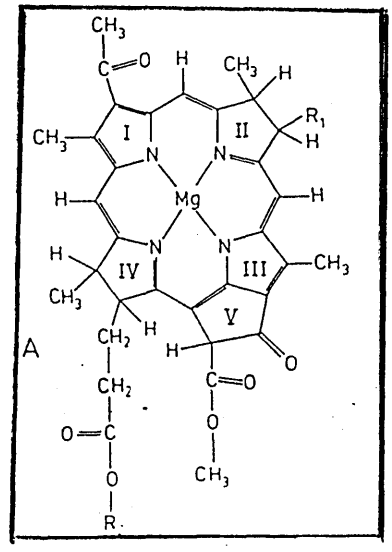


Fig. 1-12 Structures of Bchl a and b. For Bchl a, R_1=H, R_2=phytyl or geranyl-geranyl. For Bchl b, R_1=C-CH_3, R_2= phytyl (*R. viridis*)

The organization of Bchls located within antenna complexes is different from that of the Bchls located in reaction centres. This is why the function of the Bchls is different in these two types of complexes. The role of antenna Bchls is to harvest light energy and

funnel that energy to reaction centres, whereas the role of reaction centre Bchls is to transform the collected energy into biochemical energy needed for bacterial photosynthesis (see §1-3 for details).

§1-7-2 Carotenoids in the Photosynthetic Apparatus of Purple Bacteria

Carotenoids of purple bacteria are a class of C_{40} tetraterpenes and their oxgenated derivatives (Isler et al., 1971; Goodwin, 1980, 1988) which are mostly found associated with intracytoplasmic membranes where they are bound, non-covalently, to specific pigment-protein complexes (Cogdell, 1985[a], 1985[b]; Siefermann-Harms, 1985).

The major functions of carotenoids in photosynthesis are (i) photoprotection, which is essential for photosynthesis in the presence of oxygen, and (ii) light-harvesting, which allows the photosynthetic organism to utilize a wider range of light wavelengths, in the 450-570 nm region (Cogdell and Frank, 1987).

a. Photoprotection Role of Carotenoids

Without carotenoids there would be no photosynthesis in the presence of oxygen (Cogdell, 1988). This can be easily demonstrated by illuminating the carotenoidless mutant of *Rb. sphaeroides* R26 in the presence of oxygen. This results in the death of these cells (Griffith, 1955). The following mechanism is most probably responsible for the harmful process:

$$Bchl \xrightarrow{h\nu} Bchl^{*} \longrightarrow {}^{3}Bchl^{*} \xrightarrow{O_2} Bchl + {}^{1}\Delta_g O_2^{*}$$

Singlet oxygen is extremely toxic for living cells and will rapidly kill them by its powerful oxidizing nature, oxidizing BChl, lipids and nucleic acids as well (Halliwell and Gutteridge, 1985).

Thanks to the carotenoids which have the ability to prevent this photooxidative killing by quenching the singlet oxygen or the triplet excited ^{3}BChl* (Foote and Denny, 1968), the processes of photoprotection of carotenoids can be finally summarized as in Fig. 1-13.

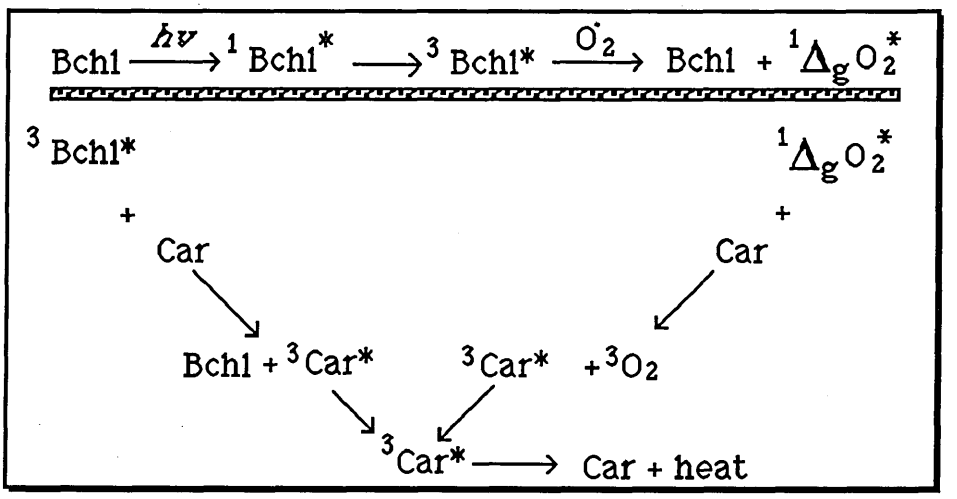


Fig. 1-13 The process of photoprotection of carotenoids in purple bacteria (adapted from Cogdell and Frank (1987)).

b. Structure and Location of Carotenoids in RC and Antenna Complexes

Apart from Bchl, carotenoids are the major pigments found in the photosynthetic apparatus of purple bacteria. The light-harvesting and photoprotection roles of carotenoids have been well established; however, there is no evidence so far for these attractive pigments to be involved directly in the primary electron-transfer reactions which occur in the reaction centres.

Isolated RCs from the carotenoidless mutant of *Rb. sphaeroides* R 26 show that carotenoids are not essential for the charge-separation process. Recently, the first carotenoidless B800-850 complex was reported from *Rb. capsulatus* mutant strain GK2, indicating that carotenoids were not essential either for the formation of the B800-850 complex (Dorge et al., 1987). However, all RCs and antenna complexes isolated from wild-type species contain a specific amount of carotenoid, one carotenoid per reaction centre for RC complexes and normally one carotenoid for every 2 or 3 Bchls in antenna complexes (Thornber, 1983; Cogdell, 1988; Evans et al., 1988).

Several approaches have been used to study the structure and function of carotenoid: (i) reconstitution of RC (Chadwick et al., 1986); (ii) spectroscopic techniques such as ESR (McGann et al., 1985), NMR and Raman spectroscopy (Lutz et al., 1987). Moreover, the precise location and structural information on carotenoids in RCs has now been obtained by X-ray diffraction analysis of crystallized RCs from *Rps. viridis* (Deisenhofer and Michel, 1988), *Rb. sphaeroides* strain Y1 (Ducruix and Reiss-Husson, 1987) and *Rb. sphaeroides* strain 2.4.1. (Frank et al., 1987; Allen et al., 1988).

Fig. 1-14 shows the state of art knowledge on carotenoid structure and location in RCs based on the RC X-ray diffraction data from the 2.4.1. strain (Allen et al., 1988). In this model, the carotenoid is situated between the B and C helices of the M subunit near the monomeric accessory Bchl (and approximately 11 Å from the primary donor for the RC of *Rps. viridis*). The carotenoid was found lying in the plane of the membrane with the two-fold symmetry axis of the RC perpendicular to this plane. The

position of the carotenoid in the RC of *Rps. viridis* is essentially the same as that in the *Rb. sphaeroides* 2.4.1. strain.

The structure and location of carotenoids in antenna complexes have not been revealed as yet. However, there has been some progress in this field, particularly in the context of crystallization of antenna complexes. High-quality crystals, diffracting to beyond 3.5Å have been produced from *R. acidophila* 10050 (Cogdell, R.J., unpublished results), and hopefully a high resolution structure for antenna complexes will be elucidated in the foreseeable future.

Like Bchl, the carotenoids are non-covalently bound to antenna polypeptides. The precise binding site(s) is (are) also unknown so far, although, CD spectra suggested that the binding site is different from that of RCs (Cogdell et al., 1978).

The conformation and the configuration of the antenna carotenoids have been studied by resonance Raman spectroscopy and all-*trans* isomers of the carotenoids were suggested (Iwata et al., 1985; Lutz et al., 1979; Hayashi et al., 1987), in contrast to the *cis*-isomer of RC carotenoids (Chadwick et al., 1986; Lutz et al., 1987).

§1-7-3 Energy Transfer between the Pigments

The efficiency of singlet-singlet energy transfer from carotenoid to Bchl has been measured from a range of antenna complexes. In most cases, efficiencies are higher than 60% (van Grondelle, 1985).

Singlet-singlet energy transfer can proceed *via* at least two known types of mechanism: (i) coulombic or dipole-dipole

resonance interaction, which is commonly called the Forster mechanism (Forster, 1959), and (ii) exchange resonance interaction, called the Dexter mechanism (Dexter, 1953).

§1-8 Energy Transduction in Purple Bacteria

The primary reaction of bacterial photosynthesis takes place in the chromatophore membrane, which contains a well-defined network of antenna complexes, reaction centres, electron carriers such as the $\mathbf{b/c_1}$ complex, and ATP synthetase etc.

When photons of radiant energy are absorbed by pigments within antenna complexes, excitons are created. Immediately these excitons migrate through the antenna network and are eventually trapped in the reaction centres provided that this reaction centre is "open" (Fig. 1-15).

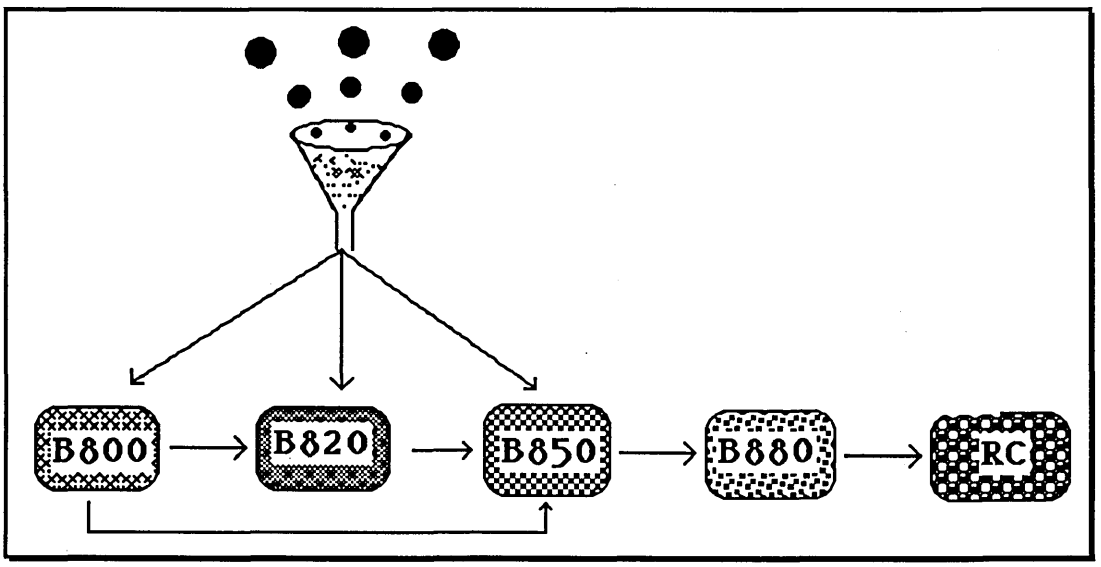


Fig. 1-15 A cartoon shows the energy transfer from the antenna complexes to a RC.

If this reaction centre is "closed", these excitons may migrate to other available reaction centres to give up their energy in order to return to their stable ground state. If the reaction centres within the microenvironment are all closed, the excited energy may have to dissipate by an alternative way: (i) radiative fluorescence emission or (ii) phosphorescence emission through intersystem crossing or (iii) non-radiative heat loss. The efficiency of energy transfer to reaction centres depends on the number of antenna complexes per reaction centre and the topography of the photosynthetic units, (or more precisely depends on the structure of the ICM), as well as the external growth conditions

Once excitons are trapped in the reaction centres, charge separation occurs, leading ultimately to the production of ATP and generation of NAD(P)H, both of which are necessary for the subsequent carbon metabolism to fix carbon dioxide in the synthesis of carbohydrate. The overall processes of photosynthesis are summarized simply in Fig. 1-16.

§1-9 Aims and Background of This Research

The photosynthetic apparatus of *Chr. vinosum* strain D has been studied since the 1950s. (Komen, 1956; Garcia, 1966; Thornber, 1970; Mechler and Oelze; 1978, Hayashi et al., 1980). Garcia (1966) reported initially that two Bchl-containing fractions could be isolated from Triton X-100-treated chromatophores of *Chr. vinosum* strain D. Thornber (1970) observed that this purple sulphur bacterium contained three spectrally different pigment-protein complexes by using the detergent SDS to solubilize the chromatophore membranes followed by chromatography on

hydroxylapatite and ammonium sulphate precipitation. These three complexes have now been designated as B800-820, B800-850 and B880 antenna complexes.

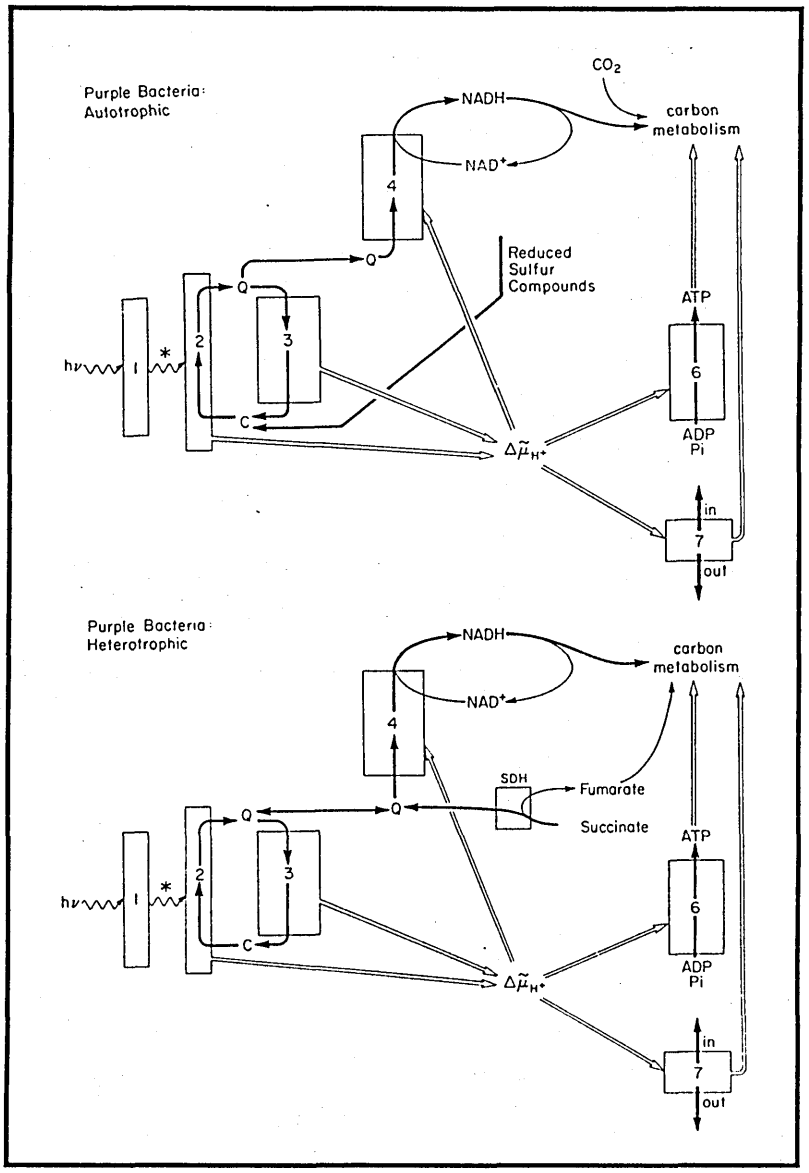


Fig. 1-16 Simplified process of photosynthesis of purple bacteria, taken from Dutton (1986). This figure is represented in the reverse direction as accepting $\Delta\mu_H{}^+$ and using it to oxidize quinol to quinone and reduce NAD^+ to NADH for use in the cell. Box 1: antenna complexes; box 2: reaction centre; box 3: cytochrome b/c_1; box 4: NADH-quinone oxidoreductase; box 6: ATP synthase complex, box 7: transmembrane metabolite transporters. C: cytochrome c_2; Q: membrane quinone pool; SDH: succinate dehydrogenase.

These antenna complexes are thought to account for the near infrared (i.e. Qy) absorption spectra of the cells of *Chr. vinosum* strain D. It has been observed that the near infrared spectrum of this bacterium can be influenced by temperature, light intensity and composition of the growth medium (Mechler and Oelze, 1978; Hayashi et al., 1980). This purple bacterium is, therefore, a good experimental model for investigating the mechanism of primary reactions of photosynthesis, especially for understanding how photosynthesis is regulated by changed external conditions. In the first stage, however, it is necessary to determine the correlation between the expression of the various types of antenna complexes and the different growth conditions.

To fulfil the above purpose, it is essential, first of all, to obtain pure antenna complexes. Secondly, it is necessary to characterize the purest possible individual antenna complexes. Once the methods for purification and characterization of antenna complexes are developed, it will be helpful to perform comparative studies on individual antenna complexes from the cells grown in different culture conditions to find out which parameter affects the synthesis of individual antenna complexes.

The aims of this research can be specifically divided into three parts. At the beginning, it was required to develop a practical method for isolation and purification of individual antenna complexes from *Chr. vinosum* strain D. Purity of the specific antenna complexes at this stage was the key to the success of subsequent research. Once the purified antenna complexes were obtained, characterization of the individual antenna complexes needed to be carried out. This included (i) fractionation of individual antenna apoproteins and determination of the primary

structure of these proteins; and (ii) determination of carotenoid compositions and the Bchl to carotenoid ratios in individual antenna complexes. At the same time comparative studies on different antenna complexes from the cells grown under different conditions should also be carried out. Finally, topology studies on the intramembrane organisation of individual antenna complexes should be performed by using immunological approaches and mild surface-specific proteolysis.

Chapter 2

Materials and Methods

§2-1 Cell Cultivation

§2-1-1 Cell Growth

Cells of *Chromatium vinosum* strain D were grown anaerobically under photoautotrophic conditions in Fuller's medium, using sodium hydrogen carbonate as the carbon source and thiosulphate as the electron donor (Bose, 1963).

The cells were grown in tightly-sealed, flat-sided 1 litre bottles which were kept at 30 °C in a growth room or at 40 °C in a thermostatically controlled water bath in the same room. The light intensity at which cells were grown was $42Wm^{-2}$, which was achieved by continuous illumination with 2 parallel racks of 150 W tungsten lamps.

§2-1-2 Measurement of Growth

A small amount of cells was transferred to a quartz spectrophotometer cuvette, which was sealed with a tightly-fitting stopper. The absorbance at 650 nm was recorded at intervals over several days against a blank of growth medium. Growth curves of absorbance at 650 nm (which is proportional to cell concentration) versus time were plotted.

§2-2 Cell Harvesting

Cells were harvested in the stationary phase of growth (usually after 7 days of growth) by centrifugation at 2800 xg for 60 min at 4 °C. For the preparation of spheroplasts, cells were harvested in the log phase (after approximately 20 h of culture). They were washed and resuspended in 20 mM Tris-HCl, pH8.0. The cells were then either used immediately or stored frozen at -20 °C until required.

§2-3 Preparation of Photosynthetic Vesicles

§2-3-1 Chromatophore (Inside-Out Vesicles) Preparation

Chromatophores of *Chr. vinosum* strain D were prepared by French pressure disruption at 10 ton/inch^2 in the presence of a little bovine pancreas DNAase I (Sigma) and magnesium chloride (Van der Rest et al., 1974). The disrupted membranes were then centrifuged at 12000 xg for 10 min and unbroken cells and large debris were removed. The chromatophores were pelleted by centrifugation at 197,000 xg for 1 h at 4 °C, homogenized, resuspended in a minimal volume of 10 mM Tris-HCl pH8.0 and stored frozen at -20 °C until required.

§2-3-2 Spheroplast (Right-Side Vesicles) Preparation

Cells were grown in 1 litre flat-sided bottles for 20 h in relatively high light (more than 40 Wm^{-2}). Cells were harvested by centrifugation at 2800 xg for 60 min at 4 °C, and then

resuspended in the lysozyme digestion buffer to give an absorbance of 40 cm^{-1} at 680 nm. The lysozyme digestion buffer contained 25% (w/v) sucrose as an osmotic buffer, 100 mM Tris-HCl pH 6.0 and 6mM EDTA. The cells were incubated with 2 mg/ml lysozyme (Sigma egg-white lysozyme grade 1, EC 3.2.1.17) at 37 °C for 1 h, in a shaking water bath. After the incubation the reaction was stopped by the addition of 80 mM magnesium chloride. The mixture was then subjected to a centrifugation at 5700 xg for 10 min at 4 °C, and the supernatant fraction was further centrifuged at 10,500 xg for 10 min at 4 °C. These two pellets were resuspended in a minimal volume of 25% (w/v) sucrose, 100 mM Tris-HCl, pH 6.0 and examined under the light microscope to see the yield of spheroplasts by comparing the pellets with untreated whole cells.

The degree of intactness was checked by monitoring the release of of the soluble cytoplasmic enzyme malate dehydrogenase (Takemoto et al., 1979).

§2-3-3 Electron Microscopy of Chromatophores and Spheroplasts

A small amount of a concentrated sample of either chromatophores or spheroplasts was clotted in fibrin and then cut into small pieces. This clotted sample was transferred into 3% (v/v) glutaraldehyde, 0.2 M sodium phosphate (pH 7.5) for 12-18 h for fixation , rinsed three times in sodium phosphate (10-15 min each) and post-fixed in 1% (w/v) osmium tetroxide, 0.2 M sodium phosphate (pH 7.5) for 1-3 h. The sample was then briefly rinsed in distilled water, block stained in 2% (w/v) aqueous uranyl

acetate, dehydrated in an acetone series and embedded in SPURR resin for 12 h at 60 °C. Thin sections were cut (approx. 60 nm thick) with an LKB III ultratome and picked up on naked 300 mesh copper grids. These were stained with saturated ethanolic uranyl acetate for 10 min and lead citrate for 2 min, prior to examination in a Philips EM 301 electron microscope.

For spheroplasts, 30% (w/v) sucrose was included in all steps as an osmotic support.

§2-4 Isolation and Purification of Light-Harvesting Complexes

§2-4-1 Solubilization of Photosynthetic Membranes.

Photosynthetic membranes of *Chr. vinosum* strain D were solubilized with Triton X-100, a mild detergent, to obtain B800-820 and B880 antenna complexes. To obtain B800-850 complexes SDS, a strong detergent, was used.

Chromatophores were resuspended in 10mM Tris-HCl, pH 8.0, to give an absorbance at 850 nm of 50 cm^{-1} for *Chr. vinosum* strain D grown at 30°C and at 802 nm of 70 cm^{-1} for *Chr. vinosum* strain D grown at 40°C. The solutions were then made 30% (v/v)* with Triton X-100, mixed rapidly and incubated in the dark in an ice bath for 1 h. After incubation, these solutions were diluted to a concentration of 4% (v/v) of the detergent and homogenized with a glass homogenizer. Without further centrifugation, this solubilized membrane preparation was subjected to either sucrose

* This concentration could actually be reduced.

gradient centrifugation or DE-52 ion-exchange chromatography to isolate B800-820 and B880 complexes.

§2-4-2 Isolation of B800-820 and B880 Antenna Complexes.

Sucrose Density Gradient Centrifugation

Sucrose gradients were prepared in 28 ml polycarbonate centrifuge tubes (Du Pont) , a linear gradient of 0.25 to 1.25 M sucrose was carefully prepared by a gradient maker connected to a peristatic pump at a constant delivery rate of 4 ml/min. The gradients were prepared in 10mM Tris-HCl, pH 8.0, in the presence of 0.4% (v/v) Triton X-100. The detergent-treated chromatophores were layered onto the linear sucrose gradients and then centrifuged at 195,000 xg for approximately 16 h. Centrifugation was stopped without using a brake to prevent disturbance of the gradients. Two purple bands were obtained and were removed individually by using a syringe with a flat-headed needle. Absorption spectra of the individual fractions were recorded. The bottom band contained the B880 antenna complexes and the upper band contained the B800-820 complexes as shown by the absorption spectra. Best results were obtained with 3 ml samples loaded onto each tube.

Further purification of the two complexes was carried out by ion-exchange chromatography on DE-52 cellulose columns.

§2-4-3 Preparation of Spectrally Pure B800-850 Antenna Complexes

Ammonium Sulphate Precipitation

Chromatophores were resuspended in 10mM Tris-HCl, pH8.0, to give an absorbance at 850 nm of 50 cm^{-1} for Chr30 and at 802 nm of 70 cm^{-1} for Chr40.

The solutions were then made 1% (w/v) with SDS and stirred for 10 min at room temperature. After solubilization, ammonium sulphate was added to a concentration of 5% (w/v), and samples were stirred for 5 min at room temperature. The precipitate was collected by centrifugation at 10,000 rpm for 10 min in an MSE 18 and then resuspended in a minimal volume of the Tris buffer. This procedure was repeated after addition of ammonium sulphate (5g/100ml) to the supernatant fluid until all the pigmented material had been collected. The quality of B800-850 antenna complex preparation was judged by its near-infra absorption as well as the ratio of Bchl a to protein.

§2-4-4 Purification of B800-820 and B880 Antenna Complexes

DE-52 Anion Exchange Chromatography

This method can be used (i) for concentrating antenna complexes; (ii) for separating one antenna complex from another, especially in removing small amounts of contaminating complex (iii) for isolating individual antenna complexes from detergent solubilized membranes (which is preferable on a large scale then the sucrose gradient method).

Pre-swollen Whatman DE-52 DEAE cellulose anion exchanger was prepared in 20mM Tris-HCl, pH 8.0, gently stirred and then slowly poured into a sintered glass column (3 X 8 cm) to a height of approx. 5 cm depending on the amount of sample. The column was equilibrated with 2-3 column volumes of 20 mM Tris-HCl, pH 8.0, buffer containing 0.4% (v/v) Triton X-100.

Samples of membranes were treated with 30% (v/v) Triton X-100 for 1 h at 0 °C, diluted to 4% (v/v) Triton X-100 and then incubated at 4 °C for at least 1 h. After dilution, the solubilized membranes were loaded onto a column of DE-52, equilibrated with 20 mM Tris-HCl, pH8.0 in the presence of 0.4% (v/v) Triton X-100, and then eluted by a stepwise NaCl gradient with 0, 75, 100, 125, 150 and 200 mM in Tris-HCl, pH8.0 with 0.4% (v/v) Triton X-100. Fractions were monitored by their near-infra red absorbance. Usually B800-820 complexes were eluted in 100 mM NaCl and completely washed off with 125 mM NaCl. After 2-3 column volumes of wash with 125 mM NaCl (depending on the volumes loaded) to ensure the complete removal of B800-820 complexes (judged either by eye or more sensitively by their near infra-red absorption), B880 complexes were eluted with 200 mM NaCl. These antenna complexes were dialysed against distilled water and stored at -20 °C .

§2-5 Fractionation and Purification of Antenna Apoproteins

§2-5-1 Isolation of Antenna Polypeptides

Gel Filtration Chromatography

Freeze-dried isolated complexes were extracted extensively with dichloromethane/methanol (1:1) containing 0.1 M ammonium acetate. The extract was centrifuged and supernatant fractions were combined.

After extraction, the supernatant fluid was applied to a Sephadex LH-60 column (3 X 150 cm) and eluted with the dichloromethane /methanol /ammonium acetate (1:1:0.1M) solution. The eluate was monitored by absorption at 280 nm on a LKB Uvicord UV monitor. The individual protein-containing fractions were collected and freeze-dried. (for more detail, see Brunisholz et al., 1981).

§2-5-2 Fractionation of Individual Antenna Polypeptides

Reverse Phase FPLC

Samples of freeze-dried antenna polypeptides collected from Sephadex LH-60 columns were dissolved in 10% (v/v) formic acid for B800-820 complexes and in 10% (v/v) formic acid in the presence of 30% (v/v) isopropanol for B880 complexes. These samples were centrifuged at 75,000 xg for 5 min to remove any insoluble materials.

Routinely 100-500µl samples were loaded onto a ProRPC^{TM} 15 µm10/10 column (Pharmacia)*, the amount varying according to the original concentration of the sample. A gradient elution was carried out, controlled by the FPLC microprocessor. Peaks were collected and pooled. Each peak was dried down by using a rotary vacuum evaporator, then reinjected into the FPLC system, and eluted with the same solvent, to check that it was a single polypeptide.

§2-6 SDS Polyacrylamide Gel Electrophoresis

§2-6-1 General Description

The overall protein composition of the samples used in this research was visualized by SDS-PAGE. Different types of gradient slab gels were used in order to produce good resolution of the proteins of interest. The protocol for preparation of the slab gels and the pretreatments of the samples are presented in Appendix I. The gels were normally stained with Coomassie brilliant blue. On some occasions, however, they were stained with silver stain.

§2-6-2 Analytical SDS-PAGE

A polyacrylamide gradient slab gel of 12.5-16.5% (w/v) acrylamide was used for monitoring the purity of the complexes and fractions from Sephadex LH-60 column. The assignment of polypeptides to specific elution peaks from FPLC was fulfilled by

* ProRPC^{TM} 15 µm10/10 column (Code No. 17-0578-01) Reverse phase silica, C_8 300Å bed size 10 X 10 mm

running a slab gel (12.5 to 20% (w/v) polyacrylamide gradient) essentially as described by the method of Laemmli (1970).

§2-6-3 Protein Staining

(i) Coomassie Brilliant Blue Stain

The gel was stained overnight in stain solution[1] and then washed several times at 30 min intervals with destainer[2] until a clear background was obtained.

(ii) Silver Stain (SwitzerIII et al., 1979; Oakley et al., 1980; Morrissey, 1981)

This silver stain method is 100 times more sensitive than Coomassie brilliant blue stain and therefore was used for visualizing individual proteins purified by reverse phase FPLC.

Since the silver stain can detect as little as 0.001 μg protein, all glassware used in the experiment must be extremely clean and the reagents must be pure.

Procedure

After electrophoresis, the gel was prefixed in about 100 ml of 50% (v/v) methanol 10% (v/v) acetic acid for 30 min, followed by 5% (v/v) methanol 7% (v/v) acetic acid for 30 min. The gel was then fixed in 10% glutaraldehyde (E.M. Science, biological grade) for 30 min and rinsed by either of the following two methods (i) soaking overnight in distilled water and washing for 30 min in fresh distilled water the next day; (ii) washing four or five times

[1]Stain solution: 5% (v/v) methanol, 7% (v/v) acetic acid and 0.1% (w/v) coomassie brilliant blue G

[2] Destainer: 10% (v/v) methanol, 10% (v/v) acetic acid in water.

over a 2 h period. This gel was incubated in 5 μg/ml DTT (dithiothreitol) for 30 min and then the solution was discarded. The gel was left in 0.1% (w/v) $AgNO_3$ solution for 30 min and this solution was discarded also. Then, the gel was rinsed twice briefly; firstly in distilled water and secondly in developer[3] . Staining of the gel was achieved by soaking it in developer until the desired level of staining was reached, and stopped by adding 12 ml of 2.3 M citric acid directly to the developer and agitating for 10 min. This solution was then discarded and the gel was washed several times in distilled water over a 30 min period.

(For storage it was best to soak the gel for 10 min in 0.03% (w/v) sodium carbonate to prevent bleaching.)

§2-6-4 Determination of Protein Concentration

Tannin Assay

Tannin assay was used to estimate the total protein content of antenna complexes as well as whole cells of *Chr. vinosum* strain D. The principle of this assay is that protein concentration is proportional to the relative turbidity of the protein-Tannin solution (Mejbaum-Katzenellenbogen and Drobryszycka, 1959).

Procedure

Samples were made up in 1 ml of distilled water, and a variety of concentrations of standard protein (0.0-100 μg/ml) were also prepared in a 1 ml volume. All the test tubes were preheated for 2 min at 30 °C in a water bath. 1ml of Tannin

[3] developer: 50 ml 37% (v/v) formaldehyde, 100 ml 3% (w/v) sodium carbonate.

reagent was added to start the reaction and samples were incubated at 30 °C for 10 min. After removal of the samples from water bath, 1ml of 0.2% (w/v) gum arabic was added to each tube to stabilize the turbidity and then samples were left at room temperature for 2 min. Absorbance of the samples and standards at 500 nm were recorded. A calibration curve of absorbance *vs* concentration of standard protein was drawn, and the concentrations of unknown proteins were estimated from this calibration curve.

It was found that a linear relationship between protein concentration and relative turbidity was obtained over the range of 0-100 μg protein/ ml.

§2-7 Determination of the Carotenoid Composition

Experiments in this section were done in the dark to prevent possible degradation.

§2-7-1 Extraction of Pigments

Initial samples for determination of carotenoid composition can be freeze-dried antenna complexes or concentrated antenna complex solution.

5 ml antenna complexes (OD_{800nm}=40 cm^{-1}) were precipitated by addition of an equal volume of methanol and centrifuged for 5 min at 6000 xg (for freeze-dried samples, this step is unnecessary). The pellet was extracted with methanol to remove Bchl, prior to extraction with a solvent mixture of acetone/methanol (7:2) until the pellet was colourless. The

extracts were centrifuged at 2000 xg for 5 min and pooled in a separation funnel. Diethylether (approx. 10 ml) or light petroleum (40-60 °C) was added to extract carotenoids from this water-containing organic solvent mixture. Two layers were formed on addition of increasing amounts of water. These two layers were carefully separated and the lower aqueous phase was further extracted in diethylether or petroleum ether (40-60°C) until colourless (3-4 times). The carotenoid-containing ether layer was combined and evaporated to dryness under O_2 free nitrogen gas prior to storage or further immediate use.

§2-7-2 Separation of Bchl from Carotenoids

The methanolic extract of the antenna complex pellet contains a large amount of Bchl **a** and a trace amount of carotenoids. For quantitative work in the determination of carotenoid composition, however, this small amount of carotenoid needs to be combined with the rest of carotenoids. This was achieved by phase separation with diethylether and 90% methanol/water (Davies, 1965).

The methanolic extract was made up to 90% methanol/water in a separation funnel and an equal volume of diethylether was added to extract the carotenoids. Two phases were formed, the upper ether phase was collected and combined with the rest of the carotenoid-containing ether layers for quantitative analysis.

§2-7-3 Thin-layer Chromatography

Two kinds of TLC plates were used in the experiment; (i) home-made silica gel plates (0.5-0.75 mm), which were prepared

using Kieselgel G (Merck) mixed in water in a ratio of 1 g : 2 ml in the presence of a little potassium hydroxide, and Whatman AL SIL G (0.25 mm) silica gel plates.

Total carotenoid extract from an antenna complex was evaporated to dryness under oxygen free nitrogen gas and dissolved in a small volume of dichloromethane. The carotenoid mixture was carefully loaded on the plates in a narrow band and developed in a solvent system containing petroleum ether (40-60 ^{o}C), dichloromethane and diethylether in a ratio of 7:1:2 under total darkness. Individual carotenoids were carefully scraped off the TLC plate and extracted in diethylether until no more colour was released. Each sample was carefully filtered through a pasteur pipette which was tightly plugged with cotton wool and finally dried under a constant stream of oxygen free nitrogen gas for storage or for further immediate use.

For identification of individual carotenoids collected from TLC, all the solvents used throughout the procedure were either HPLC grade or distilled Analar grade to eliminate any minor contaminations. For identification of carotenoids by mass spectroscopy, in addition to the solvents, the TLC plates were pre-developed in diethyl ether and the cotton wool was extracted extensively in ether. The collected individual bands were re-chromatographed on the same silica thin layer plates and developed in the same solvent system.

§2-7-4 Identification of Carotenoids

Preliminary identification of individual carotenoids was carried out by the R_f value and the visible absorption spectra. These assignments were then confirmed by mass spectral analysis.

§2-7-5 Determination of Carotenoid Composition

A small amount of antenna complexes, as either freeze-dried sample or concentrated solution, was extracted in methanol and acetone as described in §2-7-1 and §2-7-2. Individual carotenoids were then separated by thin-layer chromatography. Each carotenoid was carefully recovered from the TLC plate (see §2-7-3) and evaporated to dryness under nitrogen gas. The separated carotenoids were then dissolved in a known volume, 1.5 to 5.0 ml of petroleum ether (40-60 °C), depending on the relative amount of the sample. The absorbance of each carotenoid was scanned quantitatively from 550 to 350 nm. The relative concentration of each sample was then calculated from its absorption coefficient $\varepsilon^{1\%}_{1cm}$ and its absorbance at its wavelength of maximal absorption, O.D.max, then

$$S_i = \frac{O.D._{max}}{\varepsilon^{1\%}_{1cm} \cdot 100} \cdot vol$$

$$C_i = \frac{S_i}{\sum S_i} \cdot 100\%$$

Where: S_i: The amount of a carotenoid (mg)

C_i: Relative carotenoid concentration

Vol : Volume of petroleum ether used to dissolve the carotenoid

§2-8 Determination of the Bchl **a** to Carotenoid Ratios

§2-8-1 General Principles

Theoretically, the relative amounts of Bchl **a** and total carotenoids of an antenna complex can be calculated from their absorption spectra, provided that all the pigments are released by organic solvent treatment and extracted totally in this solvent.

The concentration of Bchl **a** in each antenna complex was calculated based on its absorbance at 772 nm in acetone/methanol (7/2, v/v) and the published absorption coefficient (ε_{772}=76 $mM^{-1}\cdot cm^{-1}$) (Clayton, 1966) because carotenoids do not absorb at this wavelength.

The calculation of the concentration of carotenoids, however, requires a number of procedures, which was the major theme of this part of the research.

Unlike Bchl **a**, carotenoids are a group of pigments which are present in different amounts in the various antenna complexes. Therefore, to calculate the amounts of total carotenoid in an antenna complex the composition of carotenoids in that antenna complex has to be determined.

Once the composition of carotenoids in an antenna complex is known, an average absorption coefficient for carotenoids at the wavelength of maximal absorption of total carotenoids in this antenna complex can be worked out, and then the concentration of total carotenoids can be calculated from this average absorption coefficient.

Unfortunately, Bchl **a** has some absorption at this wavelength; therefore, a correction must be made so that the data obtained are attributable only to the absorption of carotenoids. This correction was made by quantitatively recording a spectrum of pure Bchl **a** and then determining the absorption coefficient for Bchl **a** at the wavelength of

maximal absorption of total carotenoids of the antenna complex in the required solvent.

In summary, the concentration of Bchl **a** and total carotenoids in an antenna complex can be calculated from the following equations:

$$[\text{Bchl a}] = \frac{\text{O.D.}_{772}}{\varepsilon_{772}^{\text{Bchl a}}}$$

$$[\text{Car.}] = \frac{1}{\bar{\varepsilon}_{\text{max}}^{\text{car}}} \left(\text{O.D}_{\text{max}} - \frac{\text{O.D.}_{772}}{\varepsilon_{772}^{\text{Bchl a}}} \cdot \varepsilon_{\text{max}}^{\text{Bchl a}} \right)$$

$\bar{\varepsilon}_{\text{max}}^{\text{car}}$ represents the average absorption coefficient for the carotenoids of an antenna complex at the wavelength of maximal absorption of total carotenoids of the antenna complex, whereas $\varepsilon_{\text{max}}^{\text{Bchl a}}$ stands for the absorption coefficient for the Bchl **a** of the antenna complex at this carotenoid absorption maximum (The cuvette path length used is 1 cm).

§2-8-2 Determination of Average Absorption Coefficients $\bar{\varepsilon}_{\text{max}}^{\text{car}}$ for the Total Carotenoids of the Antenna Complexes

The individual carotenoid was firstly separated by TLC as described previously. Each carotenoid was then collected, extracted completely in diethyl ether and then evaporated to dryness under nitrogen.

Two groups of quantitative spectra of the individual carotenoids obtained from TLC were recorded from 350 to 550 nm. The samples of the first group were dissolved in a known volume of petroleum ether (40-60 °C). After quantitative spectra were recorded the sample were evaporated to dryness under N_2 and redissolved in another known volume of acetone/methanol (7/2). A second group of spectra were quantitatively recorded as previously, then,

$$\frac{O.D_{max}^{(i)}}{\varepsilon_{1cm}^{1\%}(i)} \cdot vol(i) = \frac{O.D.(x)}{\varepsilon_{1cm}^{1\%}(x)} \cdot vol_{(x)}$$

Rearrange the equation to yield:

$$\varepsilon_{mM}^{(x)} = \frac{O.D(x)}{O.D_{max}^{(i)}} \cdot \frac{vol(x)}{vol_{(i)}} \cdot \varepsilon_{1cm}^{1\%}(i) \div \frac{10}{Mt} \cdot 10^{-3}$$

where $\varepsilon_{mM}^{(x)}$ represents the millimolar absorption coefficient of a carotenoid in a known volume, $vol_{(x)}$, of acetone/methanol at the wavelength of maximal absorption (with the absorbance of $O.D_{(x)}$) of total carotenoid in an antenna complex. $O.D._{max}$ (i) is the maximal absorbance of carotenoid i in a certain volume, $vol_{(i)}$, of petroleum ether.

The average millimolar absorption coefficient can be calculated from the following equation:

$$\bar{\varepsilon}_{mM} = \sum_{i} C_i \cdot \varepsilon_{mM}^{(x)}(i)$$

where C_i represents the relative concentration of each carotenoid in an antenna complex.

§2-8-3 Determination of Absorption Coefficient for Bchl a at the Absorption Maximum of the Total Carotenoid of Antenna Complexes

Bchl **a** was extracted in acetone/methanol (7/2, v/v) from either of the two carotenoid-less mutants, *R. sphaeroides* R-26 or *R. rubrum* G 9^+ and quantitative spectra were recorded from 900 nm to 350 nm. ε_x^{Bchla} can then be calculated from this equation:

$$\varepsilon_x^{Bchla} = \varepsilon_{772} \cdot \frac{O.D_x}{O.D_{772}}$$

where x is the absorption maximal wavelength of the total carotenoid of an antenna complex, for B800-820 antenna complex x=470 nm whereas for B880 antenna complex x=493 nm.

§2-9 The Membrane Location of Pigment-Protein Complexes

§2-9-1 Immunological Approach

1. **Antibody production**

Production of conventional monospecific and polyclonal antibodies requires methods for the introduction of immunogen into animals, withdrawal of blood for testing the antibody titre,

and finally exsanguination for collection of large amounts of immune sera.

(i) Immunization

B800-820 and B880 antenna complexes from *Chr. vinosum* strain D (grown at 30 °C), which were used as immunogens in the experiments, were carefully prepared by a combination of sucrose gradient centrifugation and DE-52 ion-exchange chromatography. The purity of the sample was checked by SDS-PAGE and only highly purified samples were used to raise antibodies. These complexes were extensively dialysed against distilled water and lyophilized. The antenna apoproteins from the B800-820 and the B880 antenna complexes were then obtained by Sephadex LH60 gel filtration chromatography as described in §2-5-1. Antisera were then raised against the purified antenna apoproteins of the B800-820 and the B880 complexes respectively as described previously (De Marcucci et al., 1985).

(ii) Bleeding and isolation of serum from blood

A small amount (approx. 30ml) of blood, which was collected from the rabbit's ear vein, was left to clot at room temperature for about 1 h, then at 4 °C overnight. Antiserum was poured off into centrifugation tubes and then centrifuged at 2500 xg for 30 min at 4 °C. The sera were aliquoted and stored at -20 °C.

2.**Immunoprecipitation**

This method was used to see whether antenna polypeptide fragment(s) are located at the membrane surface. Two types of membrane vesicles were used in this experiment, chromatophores

(inside-out vesicles) with cytoplasmic membrane exposed to the external medium, and spheroplasts (right-side out vesicles) with periplasmic surface exposed to the external environment.

Membranes of either chromatophores or spheroplasts were made up in 1ml of mixture of buffer* (see below) and antiserum to give an absorbance of 0.5 cm^{-1}, at 800 nm for the chromatophores of Chr30 or 880 nm for those of Chr40. In this way a set of test tubes were set up each containing a final volume of 1 ml, but with increasing amounts of antiserum. A similar set of control tubes were made up using equal volumes of control serum. The tubes were incubated at 37 °C for 30 min and then for 4 h at 4 °C. Any agglutinated material was removed by low speed centrifugation at 1000 xg for 5 min and the concentration of the membranes remaining in solution was assayed by measuring the absorbance at their NIR absorption maximum.

3. **Immunoblotting** (Western blotting)

Apoproteins of the B800-820 and B880 antenna complexes were resolved on 12.5-16% (w/v) SDS polyacrylamide gradient slab gels. These proteins were then transferred onto nitrocellulose sheets (Towbin et al., 1979; Batteiger et al., 1982) by electrophoresis. After incubation with specific antisera, ^{125}I-labeled protein A, the antigen-antibody conjugates were detected by autoradiography (De Marcucci et al., 1985).

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*Buffer: This buffer contains 150 mM sodium chloride 20 mM sodium phosphate (pH 7.4) for chromatophores; additional 40% (w/v) sucrose was added to this buffer as an osmotic support for spheroplasts.

Following SDS-PAGE, a slab gel was cut into two pieces. One piece was stained with Coomassie brilliant blue and the other piece was used for transfer to nitrocellulose sheets.

Transfer of the antenna polypeptides was carried out for either 1-4 h at 0.40 A or 16-20 h at 40 mA in transfer buffer[a]. The nitrocellulose paper was pre-incubated with washing buffer[b] for 1 h at room temperature to reduce non-specific binding, and then incubated with antisera (diluted 1:50 or 1:100) for 60 min in washing buffer at room temperature. This nitrocellulose was washed five times with washing buffer over 30 min intervals to remove excess antisera and then incubated with ^{125}I-labeled protein A for 1 h in washing buffer. Excess unbound radiolabel was removed by following with another 5 times wash with washing buffer at 5-10 min intervals. Finally, the nitrocellulose paper was dried before exposure to Kodak X-Omat S film with an intensifying screen at -80 ^{o}C to enhance autoradiographic detection.

§2-9-2 Mild Proteolysis with Proteinase K

Chromatophores of the *Chr. vinosum* strain D were digested with proteinase K in a ratio of 1 mg of enzyme to 50 mg of chromatophore protein. The membranes were preincubated for 5

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[a] Transfer buffer : 25 mM Tris, 192 mM glycine, 0.02% SDS and 20% methanol

[b] Washing buffer : 20 mM Tris-HCl pH 7.2 containing 0.15 M NaCl, 0.5% (v/v) Tween 20

min at 37 °C in a shaking water bath prior to addition of proteinase K. Samples were withdrawn at 1, 10 and 60 min of digestion. The reaction was stopped by chilling the samples on ice and by adding the protease inhibitor PMSF (phenylmethyl-sulphonylfluoride) to a final concentration of 2.5 mM. The membranes were then pelleted by centrifugation at 220,000 xg for 1 h and resuspended in 10 mM Tris-HCl (pH 8.0).

Chapter 3

Isolation, Purification and Partial Characterization of Antenna Complexes from *Chr. vinosum* Strain D

§3-1 Introduction

Purple bacteria have been used as basic models for understanding the general mechanism of photosynthesis, because they are prokaryotes which contain only one photosystem. The concepts generated from studies on these photosynthetic bacteria have been very helpful to and widely adopted by researchers working on photosynthesis in higher plants as well as cyanobacteria.

In the last decade, dramatic advances have been made in understanding the molecular events of bacterial photosynthesis. This has culminated in the award of the Nobel prize in 1988 for research on elucidation of the structure and function of reaction centres (Diesenhofer et al., 1985[b]). In the case of antenna complexes, however, progress has not been so rapid. Researchers in this field are now engaged in trying to match the achievements made in the study of the reaction centres.

Most of our knowledge on the bacterial photosynthetic apparatus was acquired from purple non-sulphur bacteria, *Rhodospirillaceae*. However, so far information about the structure and function of the antenna system of purple sulphur bacteria, *Chromatiaceae*, has been minimal. Therefore, *Chr.*

vinosum strain D, a typical purple sulphur bacterium, was chosen for investigation in this research

Chr. vinosum strain D was reported to contain three antenna complexes: B800-820, B800-850 and B880 (Thornber, 1970). These three antenna complexes, existing *in vivo*, exhibit characteristic NIR absorption spectra with three absorption maxima at 800, 820 or 850, and 880 nm. It appeared that the profile of the absorption spectrum of this bacterium can be influenced by temperature, light intensity and growth medium (Mechler and Oelze, 1978; Hayashi and Morita, 1980), indicating that adaptation occurred to the changed conditions to meet the needs of optimal photosynthesis. This bacterium is, therefore, quite useful for understanding how photosynthesis is regulated and how the individual photosynthetic components respond to the external conditions. However, initial characterization of individual antenna complexes is a prerequisite for a more detailed study on the regulation of expression of these complexes.

In this research, a quick, preparative method for complete separation of B800-820 and B880 antenna complexes was developed, by combining sucrose gradient centrifugation with chromatography on DE-52 columns.

§3-2 Results and Discussion:

§3-2-1 Adaptation of Cells of *Chr. vinosum* Strain D to the Changed Growth Conditions

The near infrared spectrum of *Chr. vinosum* strain D can be influenced by temperature, light intensity and composition of the

growth medium. Two typical spectral forms were observed under the culture conditions in this experiment (Fig. 3-1 and 3-2). When cells were grown photoautotrophically at 30 °C, using $NaHCO_3$ as the carbon source and sodium thiosulphate as an electron donor, they exhibited a high 850 nm and low 800 nm absorption profile. To some extent, the absorption at 850 nm increases with the increase of light intensity at 30 °C. This feature of absorption *vs* light-intensity change was not observed when cells were grown at 40 °C and only low absorption at 850 nm was observed. In contrast, when grown heterotrophically, using malate as the carbon source, the cells exhibit high 850nm absorption at 40 °C and low at 30 °C (Mechler & Oelze, 1978). Furthermore, the two spectral forms can even be switched over from one type to another when the medium is supplemented with sodium sulphide (Hayashi & Morita, 1980).

Since the Qy state of Bchl is responsible for NIR absorption (see Fig. 3-3), any factors which change the Qy levels, or further change the Bchl **a** microenvironment will change the profile of NIR absorption.

Bchls are known to be non-covalently bound to specific antenna apoproteins. The interactions between Bchls and apoproteins are the key to the function of the antenna system. The different absorption maxima at 800, 820 (850) and 880nm are attributed to the different interactions between Bchl **a** and the various antenna apoproteins. The spectra of the whole cells suggest that there are at least 3 types of Bchl **a** and antenna protein interaction, and indeed 3 types of antenna complexes were reported to be isolated by using different detergents (Thornber, 1970; Mechler & Oelze, 1978; Hayashi & Morita, 1980).

However, the type of antenna complex and the assignment of different antenna complexes very much depends on the nature and concentration of detergents used because spectral alterations can be induced by the presence of detergents.

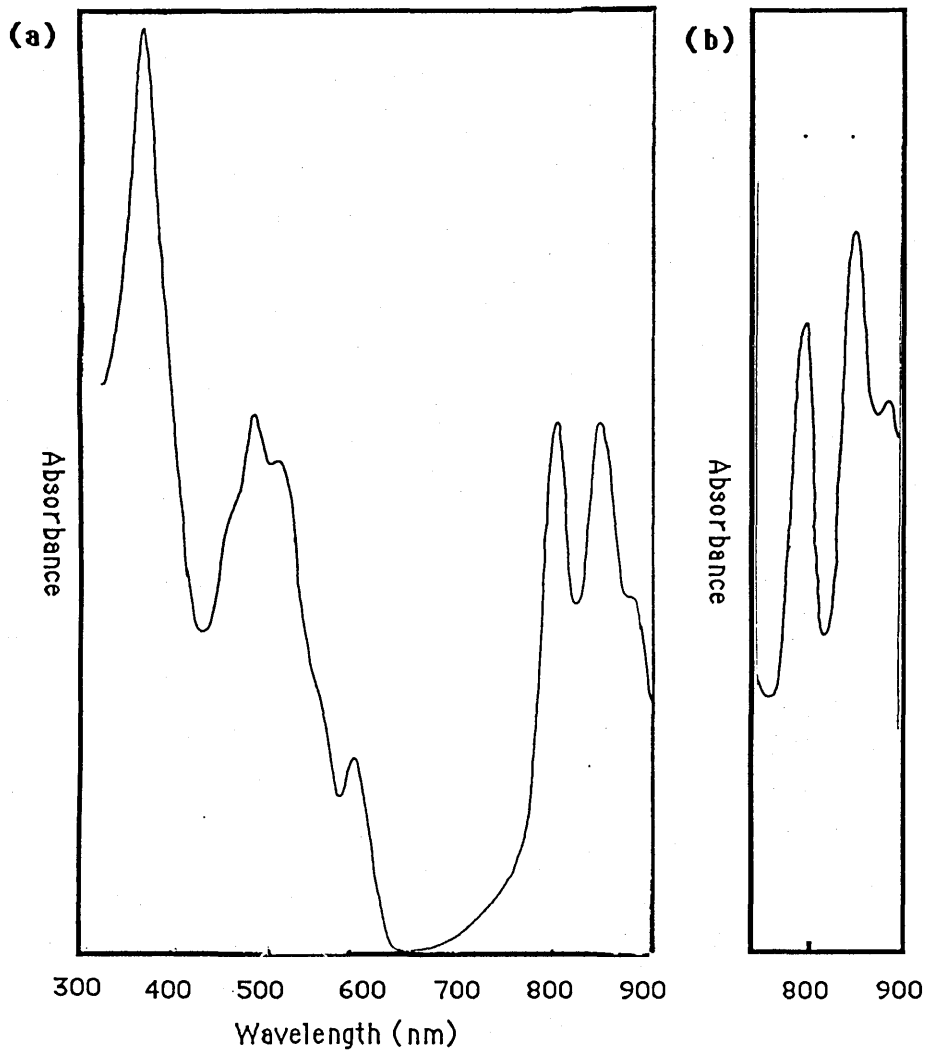


Fig. 3-1 Absorption Spectra of Chromatophores from *Chr.vinosum* Strain D
(a) Cells grown at 30 °C under low light intensity
(b) Cells grown at 30 °C under high light intensity (see Chapter 2 for details)

§3-2-2 Isolation and Purification of the B800-820 and the B880 Antenna Complex

In this research a range of detergents were tried out (SDS,

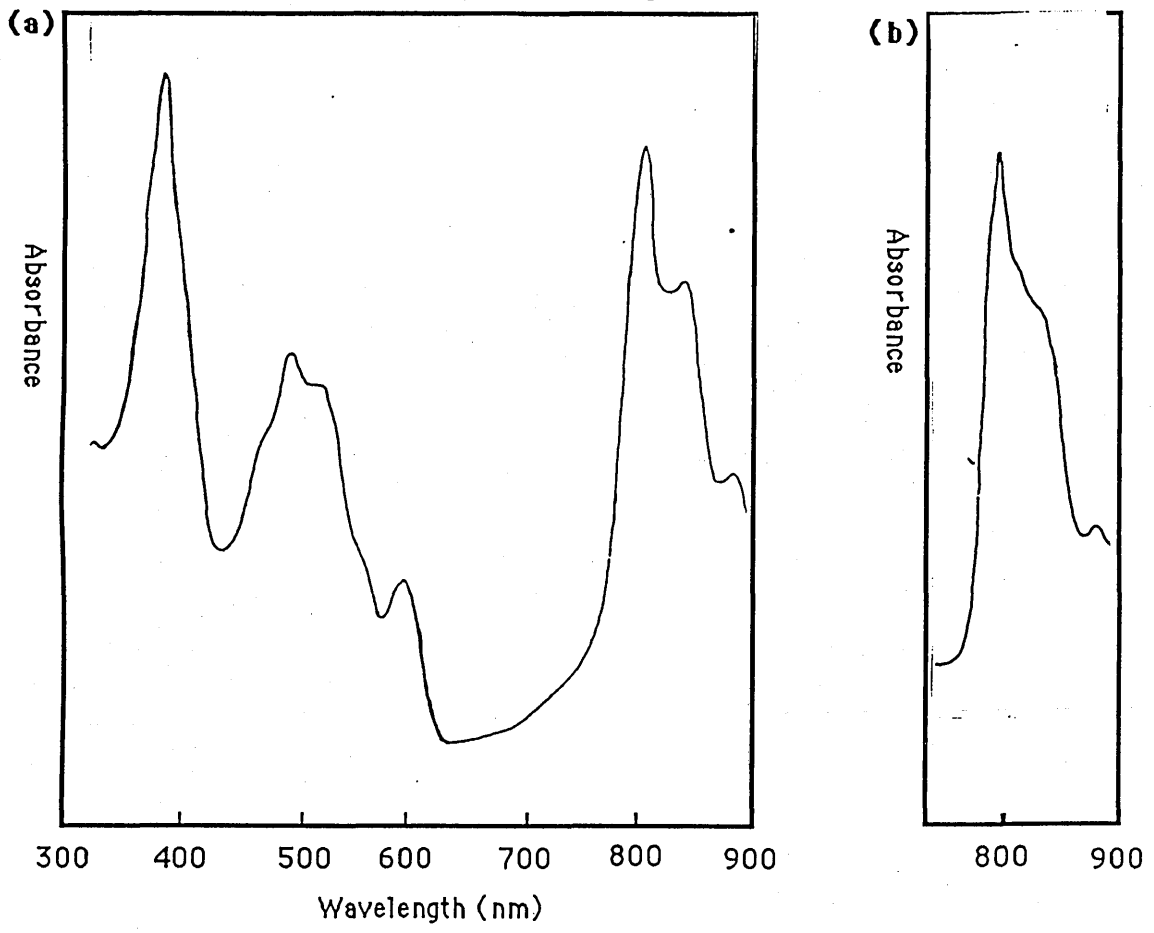


Fig. 3-2 Absorption Spectra of Chromatophores from *Chr. vinosum* Strain D (a) Cells grown at 40 ^{o}C under low light intensity (b) Cells grown at 40 ^{o}C under high light intensity

LDAO, cholic acid and Triton X-100) and their molecular structures are presented in Table 3-1. In the trial and error experiments, the ICMs were solubilised in low concentrations (usually1-5%) of these detergents respectively, and then were subjected to sucrose density gradient centrifugation to separate the individual antenna complexes. Absorption spectra for each fraction collected from

sucrose gradient centrifugation were recorded. The results are presented in Table 3-2. In most cases, each of the fractions contains only one type of antenna complex and a large amount of free pigments and proteins, indicating that extensive denaturation of the pigment-protein complexes had occurred under these conditions.

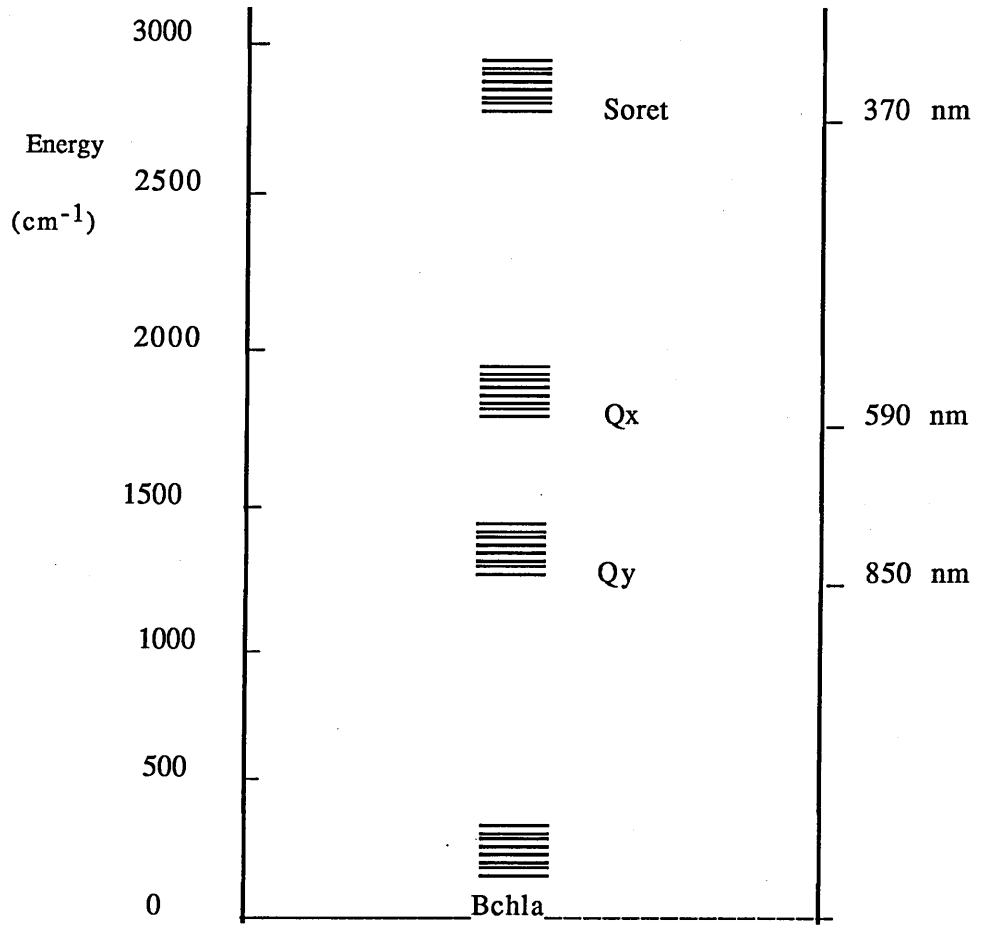


Fig. 3-3 Schematic illustration of energy map of Bchl a adapted from Siefermann-Harms, (1985)

High concentrations of the non-ionic detergent Triton X-100, however, were found to be effective in solubilising the intracytoplasmic membranes of *Chr. vinosum* strain D and also

maintaining the antenna complexes in a native state. When the solubilized membranes were subjected to sucrose gradient centrifugation a good separation of three coloured bands was achieved (Fig. 3-4). Spectra of individual bands showed that the top faint band contained free pigment from denatured antenna complexes with a major absorption maximum at 772 nm; the middle band contained the B800-820 complex and the bottom band contained the B880 complex (see Fig. 3-5).

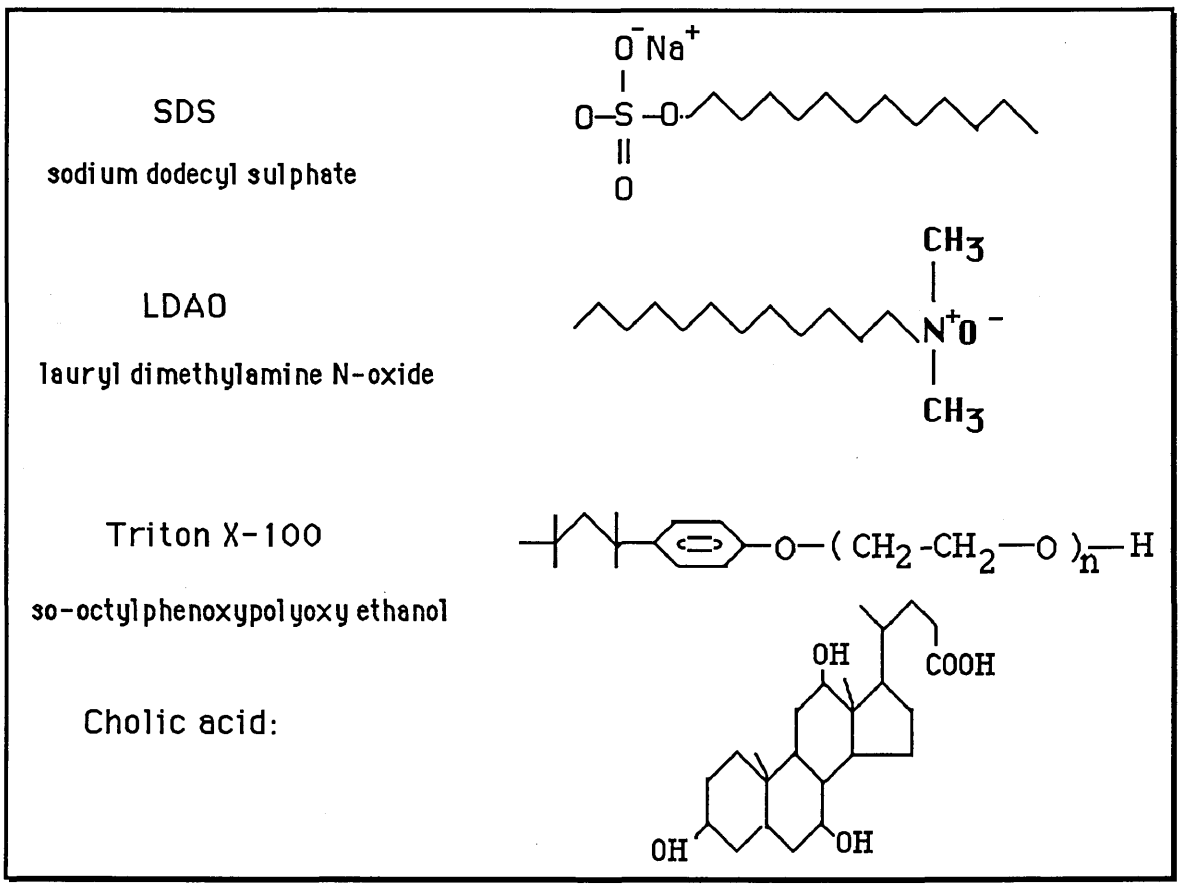


Table 3-1 Molecular structure of the detergents used in this research

Fig. 3-6 shows an SDS-PAGE analysis of the two antenna complexes from both Chr30 and Chr40. Four proteins appear on

the slab gel with M_r values less than 12 kD for samples of both B800-820 and B880 antenna complexes, indicating cross contamination between the two antenna complexes. Each antenna complex, therefore, needs to be purified before any further characterization of the complex.

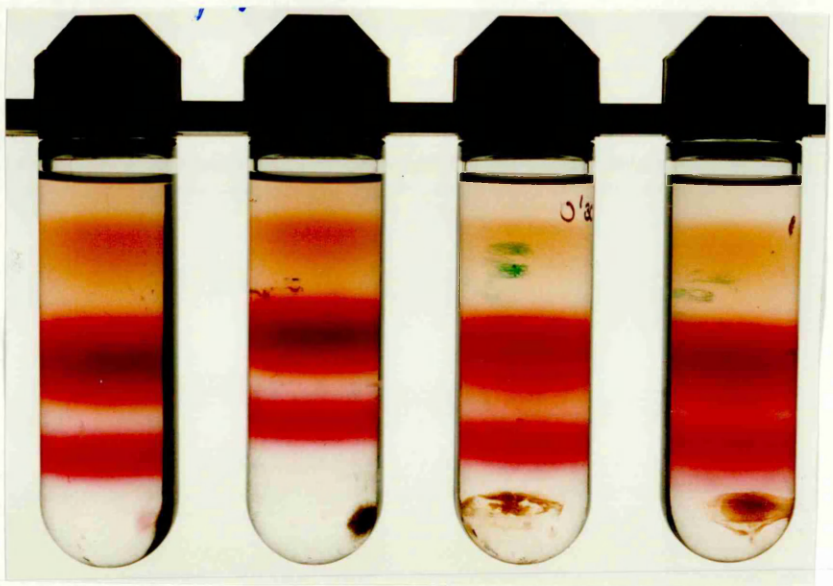


Fig. 3-4 Sucrose gradient centrifugation of Triton X-100 solubilized membranes after 18 h centrifugation at 197000 xg. The bottom band is B880 antenna complexes and the middle band is B800-820 complexes whereas the top band is free pigments from denatured antenna complexes.
The two tubes on the left show the samples from 40 ^{o}C grown cells and the two on the right are from 30 ^{o}C grown cells.

DE-52 anion exchange chromatography was used at this stage to purify the individual antenna complexes. A relatively small amount of sample, collected from sucrose gradient centrifugation, was loaded onto the column and then eluted with

a carefully chosen sodium chloride gradient (see §2-4-4 for details). Routinely, fractions were monitored by their NIR absorption spectra and the OD_{802} or OD_{880} to OD_{280} ratios (see Table 3-2 and 3-3). These ratios were used as indicators for the purity and intactness of the B800-820 and the B880 antenna complex. It was found that fractions collected at 100 and 125 mM sodium chloride contained the B800-820 complex, whereas fractions collected at 200 mM comprised the B880 complex.

Detergent	B800-820	B800-850	B880
SDS 0.7, 1.0% (w/v)	-	+	-
LDAO 0.2,0.5,1.0%(v/v)	+	-	-
Brij-58 0.5% (v/v)	+	-	-
Triton X-100 >4% (v/v)	+	-	+

Table3-2 Separation of antenna complexes using different detergents
"+" (or "-") means that fractions collected from sucrose gradient centrifugation contain (or do not contain) the corresponding antenna complex.

The success of this complete separation can be seen from the gel (Fig. 3-7) as well as from the spectra (Fig. 3-5). B880 antenna complexes resolved on SDS-PAGE into two bands, rather than four as seen in Fig. 3-6, in the M_r range below 12 kD. These two apoproteins seem to be the minimal requirement for this complex to maintain its function *in vitro,* or at least its absorption properties.

Stepwise gradient (mM NaCl)	Antenna complexes	(a) O.D802/O.D280 (b) O.D880/O.D280
0 (20 mM Tris-HCl, pH 8.0)	---	0.030
75	---	0.107
100	B800-820 (major fraction)	3.33
125	B800-820	2.63
150	B800-820 + B880	(a) 1.62 (b) 1.64
200	B880 (major fraction)	(a) 0.806 (b) 1.08

Table 3-3 Illustration of the isolation of Triton X-100 solubilised B800-820 and B880 antenna complexes by DE-52 chromatography

The B800-820 antenna complex, on the other hand, appeared to contain at least three apoproteins. In addition to the broad band (possibly more than one band), which has a similar mobility to that of the bands of the B880 complex, another two bands were resolved at the bottom of the slab gel. These two proteins can be completely removed from B880 antenna complex by passing the sample through the DE-52 column. However, the B800-820 antenna complex always contains these two proteins under all conditions tested.

In conclusion, B800-820 antenna complex contains at least three apoproteins whereas B880 complex contains two apoproteins. This conclusion is confirmed by the results of reverse phase FPLC where five apoproteins have been fractionated from these two antenna complexes (see chapter 4 for details).

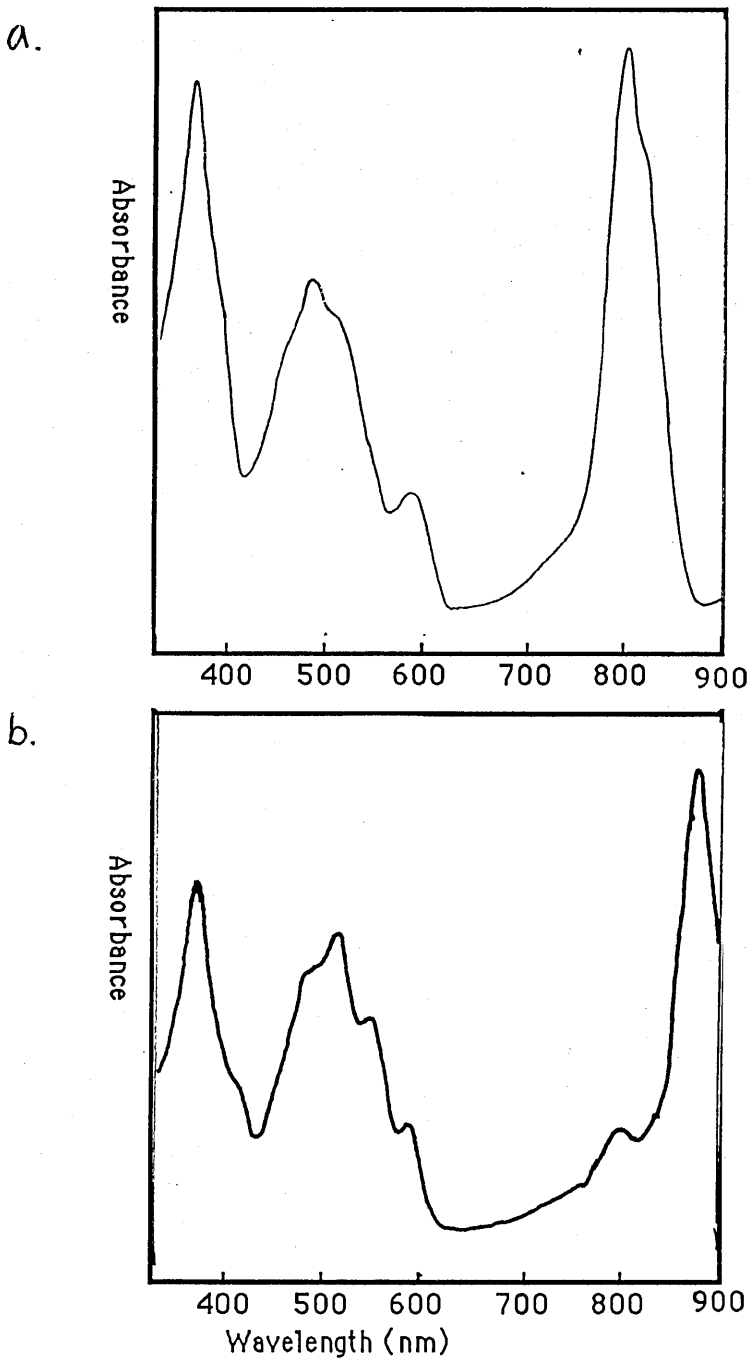


Fig. 3-5 Absorption spectra of purified (a) B800-820 antenna complexes (b) B880 antenna complexes

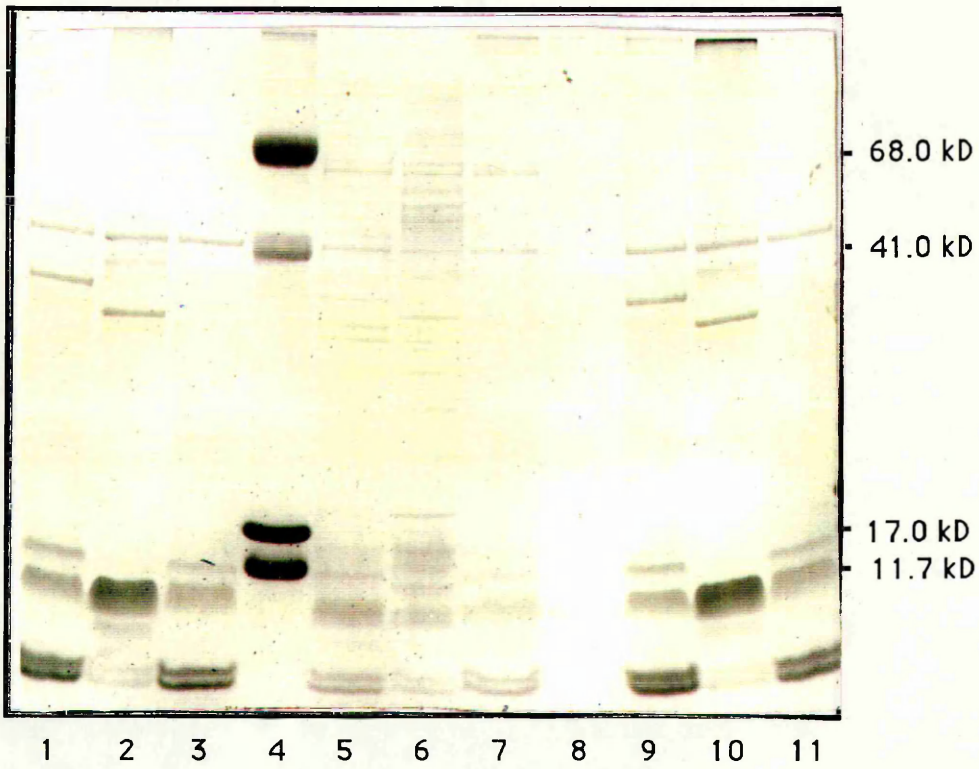


Fig. 3-6 SDS-PAGE of bottom and middle bands from sucrose gradient centrifugation.
Lane 1 and **9**: the B800-820 complex (collected from middle bands) of Chr40; **lane 2** and **10**: the B880 complex (collected from bottom bands) of Chr 30; **lane 3** and **11**: the B800-820 complex of Chr30; **lane 4**: M_r standards; **lane 5** and **7**: Chr30, and **lane 6**: Chr40. (see Chapter 2 for further details).

Sample		Bchl a (mg/ml)	Protein (mg/ml)	Bchl a / Protein
Chr30	B800-820	0.108	2.2	0.049
	B880	0.030	0.65	0.046
Chr40	B800-820	0.113	3.0	0.038
	B880	0.028	1.1	0.025

Table 3-4 Bchl **a** to Protein ratios of B800-820 and B880 antenna complexes from Chr30 and Chr40. Protein and Bchl **a** concentrations were determined as described in Chapter 2.

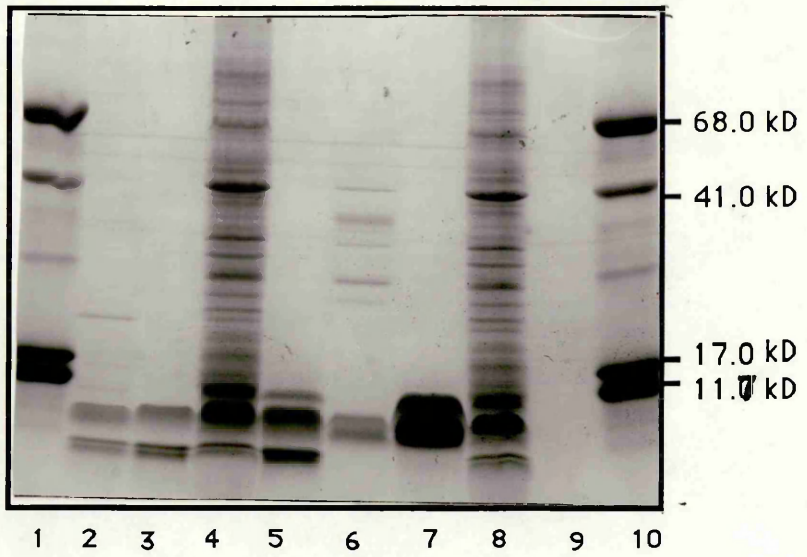


Fig. 3-7 SDS polyacrylamide slab gradient gel (10-16.5%) stained with Coomassie brilliant blue. **Lane 1**: M_r markers (cytochrome *c*, 11.7kD, myoglobin 17.0kD, ADH, 41.0kD, BSA, 68.0kD). **Lane 2**: B800-820 antenna complex. **Lane 3:** antenna apoproteins of B800-820 antenna complex collected from LH-60 column. **Lane 4**: Whole cells of *Chr. vinosum* strain D. **Lane 5:** total antenna apoproteins collected from LH-60 column. **Lane 6**: B880 antenna complex. **Lane 7:** antenna apoproteins of B880 complex collected from LH-60 column. **Lane 8**: the same as lane 4. **Lane 9**: blank. **Lane 10:** M_r markers.

Table 3-4 shows the ratios of Bchl **a** to protein in each antenna complex. It appears that these ratios are generally decreased for both B800-820 and B880 antenna complexes at the higher growth temperature. It was found that for B800-820 antenna complexes, the ratio of Bchl **a** to protein is only decreased slightly as the growth temperature increases from 30°C to 40°C under constant light intensity. In contrast, for B880 antenna complexes, the ratio of Bchl **a** to protein from Chr40 is nearly two times lower than for that from Chr30. The reasons for

these differences are far from being understood. In addition these ratios can be varied in different antenna complex preparations, reflecting differences in nativity, which is difficult to maintain, especially for B880 antenna complexes.

In this research, an alternative way to isolate B800-820 and B880 antenna complexes for *Chr. vinosum* strain D by DE-52 chramotography was also developed. This isolation is quite useful in a practical sense, because it enabled us to obtain individual antenna complexes on a relatively large scale, facilitating the subsequent characterization of each complex.

Table 3-3 gives the result of this method. This table is based on cells of Chr40; however, there is little or no difference in the elution behaviour on DE-52 columns between antenna complexes isolated from Chr30 and Chr40. This may suggest that the primary structure or at least the charge state of corresponding individual antenna apoproteins are quite similar or possibly the same. However, further conclusions can only be made when the sequence data of the apoproteins are available.

We are still uncertain about whether there are any changes in the primary structures of the antenna apoproteins since the protein sequences of the five apoproteins are not available so far. Similarities in the polypeptide profile of antenna complexes from both Chr30 and Chr40 suggest that there seem to be no changes in M_r of the corresponding apoproteins from these two cells. In contrast, dramatic differences did appear at higher M_r values above 17.0 kD (Fig. 3-8). Research on these polypeptides is worth pursuing in future for further understanding the adaptation of this bacterium to the external conditions

§3-2-3 Preparation of the B800-850 Antenna Complex

B800-850 antenna complexes were also isolated in this research by solubilizing the chromatophore membranes with a harsher detergent, SDS, followed by precipitation with increasing concentrations of ammonium sulphate.

The results of this isolation are presented in Table 3-5. It is interesting to notice that nearly all the pigmented fractions collected (five out of seven for Chr30 and seven out of seven for Chr40) contained B800-850 antenna complexes judging by the NIR absorption of each fraction. These results are quite confusing. Naturally, the results lead us to try to detect the presence of the other two antenna complexes, B800-820 and B880 antenna complex. One possibility is that these two complexes were denatured; however, we found that there were virtually no free pigments either in any of the pigmented fractions or in the final supernatant. Also SDS-PAGE of these B800-850 antenna complexes shows the presence of several low M_r polypeptides (less than 12 kD) exhibiting a similar pattern to that obtained with the whole chromatophore membranes (result not shown).

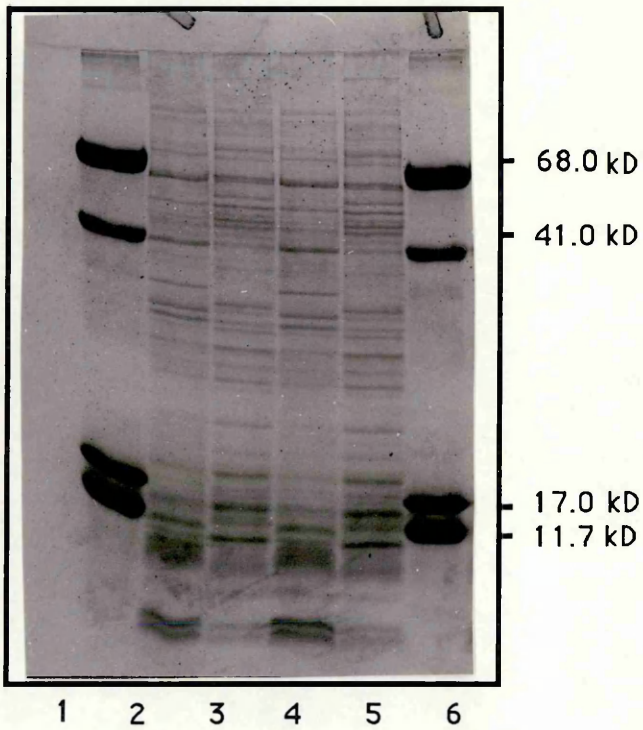


Fig. 3-8 SDS-PAGE analysis of protein compositions of whole chromatophore membranes grown at different temperatures.
Lane 1 and **6**: standards; **lane 2** and **4**: membranes of Chr30; **lane 3** and **5**: membranes of Chr40

(i) Chr30

Pellet No.	1	2	3	4	5	6	7
$(NH_4)_2SO_4$	5%	10%	15%	20%	25%	30%	40%
Protein (mg/ml)	5.2	8.0	5.2	2.8	4.0	3.4	10
Bchl**a** (mg/ml)	0.22	0.56	0.36	0.16	0.17	0.20	0.63
$\frac{\text{Bchl}a}{\text{Protein}}$	0.042	0.070	0.069	0.057	0.043	0.059	0.063
Type of antenna	B800-850 complexes			Mixture of B800-850 & B880		B800-850	

(ii) Chr40

Pellet No.	1	2	3	4	5	6	7
$(NH_4)_2SO_4$	5%	10%	15%	20%	25%	30%	35%
Protein (mg/ml)	5.9	6.1	3.6	6.7	2.2	9.5	10.8
Bchla (mg/ml)	0.52	0.40	0.29	0.33	0.13	0.71	0.87
$\frac{\text{Bchla}}{\text{Protein}}$	0.089	0.065	0.081	0.050	0.059	0.075	0.081
Type of antenna	← B800-850 complexes →						

Table 3-5 Isolation of B800-850 antenna complex of (i) Chr30 (ii) Chr40. Membranes were solubilized in 1% (w/v) SDS followed by ammonium sulphate precipitation. Pellets were collected by centrifugation at 10,000 rpm for 10 min in an MSE 18.

It seems, therefore, that these B800-850 complexes prepared in this experiment may be artifacts or at least do not represent the minimal basic building block of the native B800-850 antenna complexes. They may be solubilized membrane fractions with the detergent SDS causing spectral shifts such that the absorption profiles of the isolated fractions all resemble those of the native B800-850 antenna complexes.

Chapter 4

Fractionation of Individual Antenna Polypeptides from B800-820, B880 Light-Harvesting Complexes of *Chr. vinosum* Strain D

§4-1 Introduction:

Antenna apoproteins can probably be described as providing the appropriate framework for maintaining the adequate structure and conducting the functions of antenna complexes. These polypeptides optimize the distance, orientation and arrangements of the pigment molecules for efficient energy transfer and transduction (Drews, 1985; Zuber, 1987). They are therefore fundamental for understanding the structure and function of antenna complexes as well as the architecture of intracytoplasmic membranes in which the photosynthetic apparatus is organized.

Since the first antenna apoprotein was sequenced from a purple bacterium in 1981 (Brunisholz et al., 1981), the primary structures of more than 20 antenna apoproteins from purple non-sulphur bacteria have now become available. However, no antenna apoproteins have ever been isolated and purified from purple sulphur bacteria, i.e. *Chromatiaceae*. It is therefore quite important to investigate the antenna systems of *Chromatiaceae*, and to understand how the photosynthetic apparatus in these bacteria functions at a molecular level.

In this research, five antenna apoproteins from antenna complexes of *Chr. vinosum* strain D have been isolated and purified. Sequencing of the 5 apoproteins is still in progress. The partial preliminary results, i.e. the amino acid compositions of 3 antenna apoproteins, show that the overall structures of these apoproteins are quite similar to those from purple non-sulphur bacteria.

§4-2 Results and Discussion

Antenna apoproteins are membrane proteins which are unfortunately water insoluble. Therefore, there are only limited types of protein fractionation techniques available for these proteins.

It is theoretically possible to separate water-insoluble proteins in aqueous buffers in the presence of detergent; however, usually only poor resolution can be achieved because of the multiple interactions between the non-denaturing detergent and the protein complexes of interest, including the formation of protein-lipid micelles.

In this experiment, a combination of gel filtration and reverse phase liquid chromatography was used to fractionate the individual apoproteins. Initial separation of the apoproteins was carried out by gel filtration on Sephadex LH 60 (hydroxypropylated dextrans) which can be used with mixtures of organic solvents and acid.

Antenna apoproteins were then applied to reverse phase liquid chromatography for final resolution.

§4-2-1 The Protein Compositions of the B800-820 and the B880 Antenna Complexes

The protein compositions of the B800-820 and B880 antenna complexes were examined by SDS-PAGE. SDS polyacrylamide slab gel electrophoresis of the purified B800-820 and B880 antenna complexes is shown in Fig. 3-7, in comparison with the intracytoplasmic membranes. The B800-820 antenna complex appears to contain three polypeptides (lane 2), two clear narrow bands and one faint broad band, in the low M_r region. These three polypeptides can be further fractionated (in a denatured form) in an organic solvent mixture of methanol /dichloromethane (1:1) /0.1M ammonium acetate and were recovered completely after gel filtration chromatography on Sephadex LH-60 columns as seen in lane 3. The B880 antenna complex resolved into two bands of low M_r (lane 6) and several other bands at high M_r above 17 kD, which are most likely to be RC polypeptides (L, M, H) and cyt *c*. This specialized cyt *c* was reported to be bound to the reaction centre complex in *Chr.vinosum* strain D (Thornber and Olson, 1971; Coremans et al., 1985). The two low Mr polypeptides from this antenna complex are soluble in organic solvent and are also recovered in high yields from the LH-60 column as shown in lane 7.

To fractionate those low M_r hydrophobic polypeptides can be a difficult and tedious task, especially in the case of this research where the M_r and hydrophobicities (known from RP-FPLC experiment) of these antenna polypeptides are so close to

each other. Any impurities of the antenna complexes, especially cross-over contamination between the two antenna complexes may lead to a total failure of the fractionation. The antenna complexes of B800-820 and B880 for this experiment were applied twice to a DE-52 column, eluted with a carefully chosen NaCl gradient and detergent concentration to make certain that B800-820 complexes or other possible impurities were completely removed from B880 complexes and *vice versa*. Analytical SDS-PAGE was necessarily performed at this stage to monitor the purity of the complexes before any further fractionation. Only highly purified samples with minimal protein contamination while retaining the spectroscopic properties of the native complexes were used for the subsequent fractionation.

§4-2-2 Isolation of Antenna Apoproteins

Fig. 4-1 and Fig. 4-2 present the elution profiles of Sephadex LH-60 chromatography of organic solvent extracts from the antenna complexes and the whole cells grown at 30 °C and 40 °C. This size exclusion chromatography separated antenna polypeptides from high M_r polypeptides, i.e. L and M polypeptides of reaction centres or aggregates of antenna polypeptides, as well as from small molecules of free pigments and lipids.

Fig. 4-1 shows the elution profiles of (a) extracts of B800-820 antenna complex and (b) extracts of B880 antenna complex. Peak II in both figures contains antenna polypeptides, which was confirmed by SDS-PAGE as seen in lanes 3 and 7 in Fig. 3-7. Peak III contains small molecules e.g. free pigments and lipids etc. Peak I in Fig. 4-1 (a) contains unidentified protein(s) which are

probably aggregates of antenna polypeptides, and this peak is much smaller than peak II. Peak I in Fig. 4-2 (b) is about the same height as peak II. This peak contains mostly, if not all, reaction centre polypeptides, i.e. L and M polypeptides, since B880 antenna complex is always found closely packaged with the reaction centres, forming an antenna-RC conjugate, and the M_r of all of RC polypeptides isolated so far are in the range of 20-40 kD.

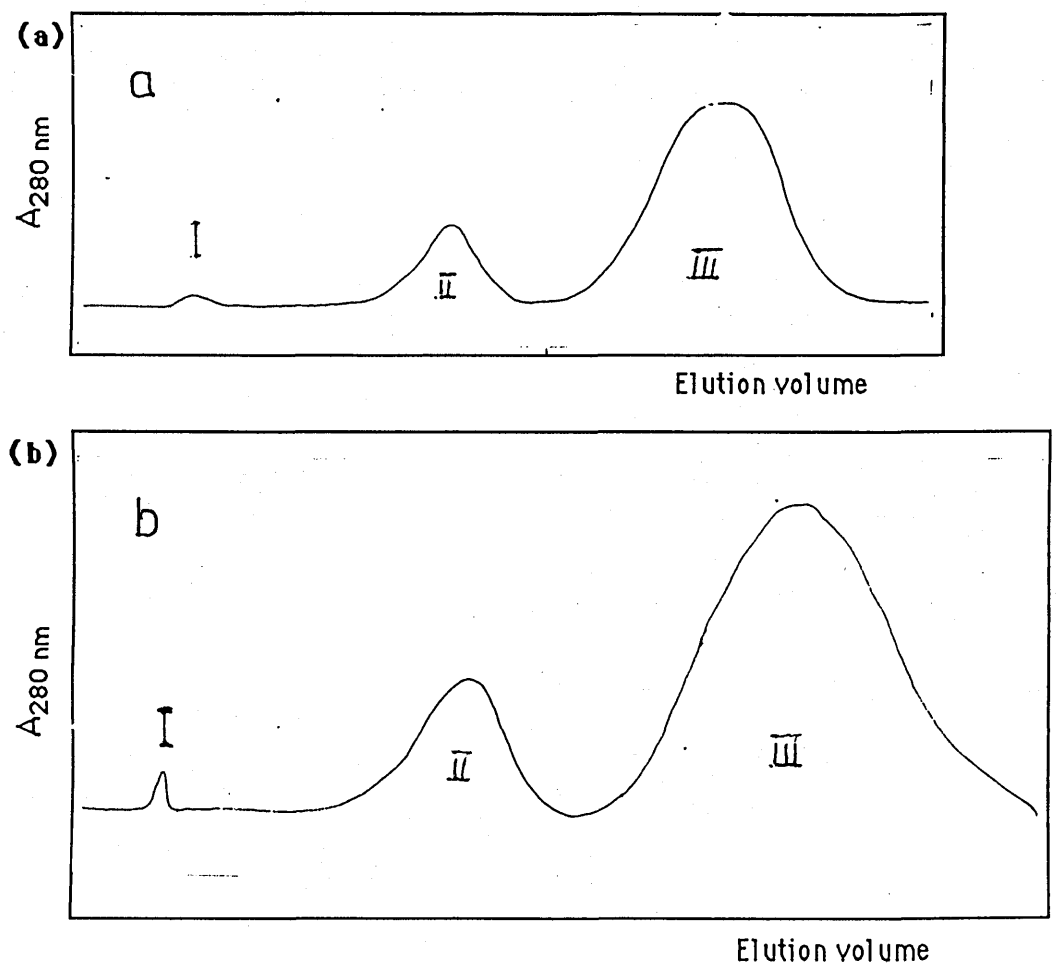


Fig. 4-1 (a) An elution profile of B800-820 antenna complex from LH-60 column
(b) An elution profile of B880 antenna complex from LH-60 column (see Chapter 2 for further details)

Fig. 4-2 shows the elution profiles of the organic solvent extracts of whole cells grown (a) at 30 °C and (b) at 40 °C. Characteristic traces of three peaks also appeared; however, instead of the smooth peak II found in Fig. 4-1 (**a**) and (**b**), peak II in Fig. 4-2 (**a**) or (**b**) is split. The small shoulder is possibly attributable to the top band in lane 5 (see Fig. 3-7) and this band was often visible on the gel if the antenna complexes were not carefully purified by DE-52 chromatography as shown in lanes 1, 3, 5, 6, 7, 9 and 11 (Fig. 3-6). This band was considered initially as a possible candidate for one of the antenna polypeptides since it is also a low M_r hydrophobic protein and can be extracted in the same solvent system, methanol /dichloromethane /0.1M ammonium acetate. However, later work showed that this polypeptide could be removed from antenna complexes without affecting the absorption spectra of the antenna complexes and their stability. Therefore, this polypeptide appeared not to be a necessary component of the antenna complexes.

§4-2-3 Fractionation of the Individual Antenna Apoproteins

The mixtures of antenna apoproteins collected from Sephadex LH-60 column (peak II in Fig. 4-1 and Fig. 4-2) were pooled separately and freeze-dried. These samples were then subjected to reverse phase liquid chromatography, using a pro RPC HR 10/10 column in conjunction with a Phamacia FPLC apparatus.

During the last few years reverse phase FPLC has appeared as a powerful tool for protein fractionation. It can be used to fractionate proteins with very small differences in their

composition. However this technique has been less successfully applied to hydrophobic membrane proteins due mainly to the difficulties of finding a suitable solvent/eluent system.

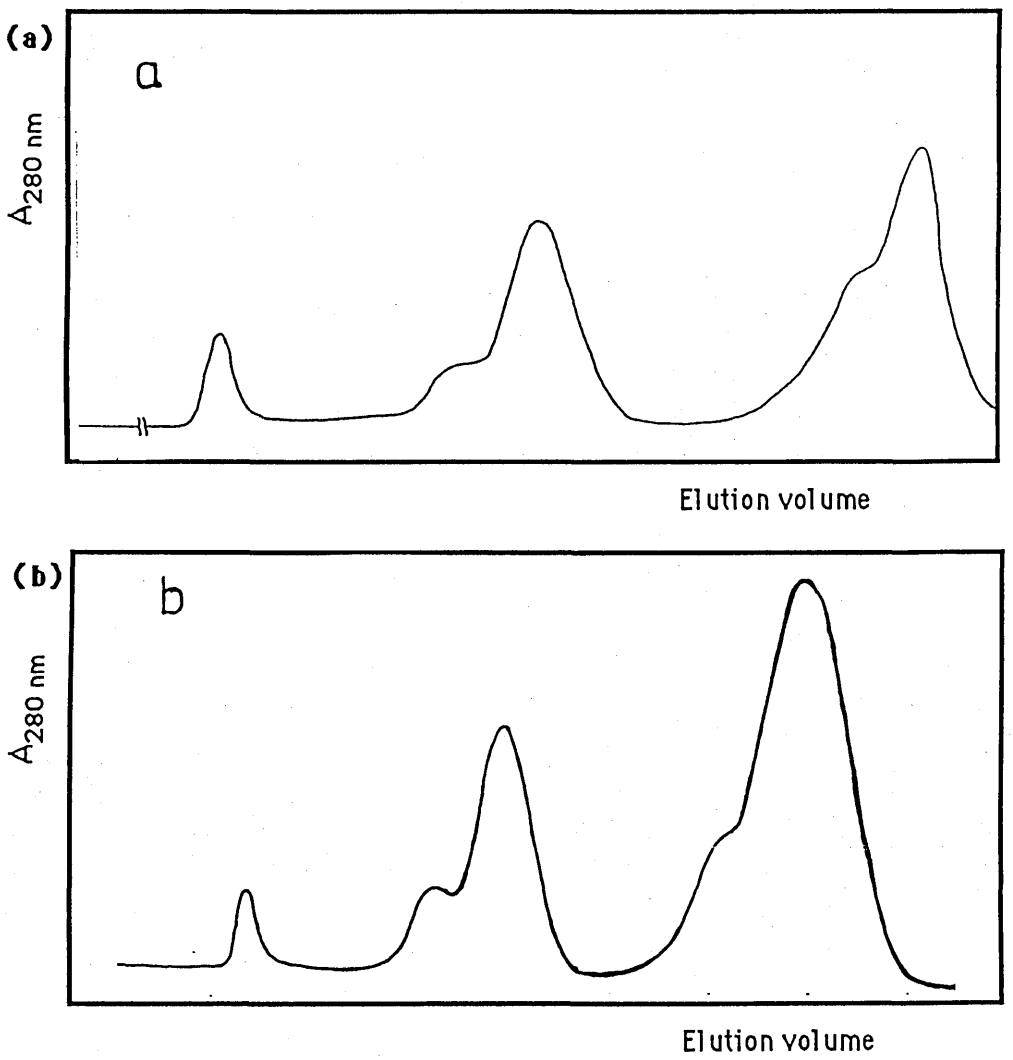


Fig. 4-2 The elution profiles of organic solvent extract of protein from whole cells of *Chr. vinosum* strain D on Sephadex LH60 columns.
(a) protein extracts of Chr30; (b) protein extracts of Chr40 (see Chapter 2 for details)

In principle, the resolution of liquid chromatographic separation of a mixture of proteins depends on the equilibrium distribution of each component between stationary and mobile phases. Once the stationary phase, i.e. column, and the temperature, usually at room temperature, are chosen, successful liquid chromatographic separation depends on selecting a suitable mobile phase, solvent, with the appropriate k' and α values. The capacity factor, k', is defined as

$$k'=(Ve-Vo)/Vo$$

where Ve is the elution volume of the eluted peak, and Vo is the void volume of the column used. The selectivity factor, α, is defined as

$$\alpha = \frac{k'_{e_2}}{k'_{e_1}} = \frac{V_{e_2} - V_o}{V_{e_1} - V_o}$$

This factor is actually the retention of one substance compared to the substance eluting just before it.

At the beginning of this research, the eluent system used was water and acetonitrile in the presence of 1 % (v/v) TFA, an ion-pairing agent. The sample was dissolved in methanol/dichloromethane because the sample was not soluble in water. Acetonitrile was used at the beginning of the research since it is a preferred solvent in reverse phase liquid chromatography owing to its low viscosity and specific solvating properties. It was observed, however, that this solvent system was not suitable for this fractionation of antenna apoproteins from *Chr.vinosum* strain D, because (i) the pattern of eluent traces were often irreproducible under the same conditions; (ii) reinjection of a previously collected single peak resulted in multiple peaks, and

(iii) different sample preparations, changing the ratio of methanol to dichloromethane to dissolve the sample, can change the eluent profiles dramatically. Furthermore, most importantly, it was observed later that the freeze-dried sample dissolved very poorly not only in eluent A, i.e. water, 1% (v/v) TFA, but in eluent B, i.e. acetonitrile, 1% (v/v) TFA, as well. Therefore, this eluent system was abandoned and trial and error experiments were initiated to find a suitable solvent system.

A range of organic solvents has been tried out and these solvents are methanol, dichoromethane, isopropanol as well as acetonitrile. As the eluent B, they were either used solely or in a combination of any two solvents. It was found that the eluent system of water and isopropanol in the presence of 10% (v/v) formic acid can give the best resolution of the sample, and the results were highly reproducible.

Formic acid in this eluent system is the key to the success of this fractionation. The polypeptide mixture was completely dissolved in 10% (v/v) formic acid in the mobile phase. Using formic acid, a low pH was also maintained so that the apoproteins exist predominantly in one charged state. In this state, possible coulombic forces were reduced dramatically and the hydrophobicity of each apoprotein was retained.

An ion pairing agent, e.g. TFA, is often included in the eluent system for reverse phase liquid chromatography (RPLC). However, in this research it was omitted deliberately after the pilot experiments. TFA was originally recommended by Dunlap III (Dunlap III et al., 1978) as a potential ion pairing agent for RPLC since it is volatile and can be easily removed by lyophilization. It was observed that this agent can decrease elution time without

loss of resolution, because "TFA acts by some mechanism to decrease interaction of the solutes with the stationary phase of the column" (Dunlap III et al., 1978). However, for the purpose of this research, resolution of the sample mixture is the priority. The differential interaction of each apoprotein with the stationary phase is the major factor determining the success of the resolution, which has proved to be the case from a practical standpoint in this research. From the research of Dunlap III et al., (1978) similar conclusions could also be drawn. Comparisons were made between using methanol-formic acid and methanol-TFA mixtures to separate a drug mixture, 5-benzomophan. In that paper, $\Delta k'_{HFo}$ is considerably larger than $\Delta k'_{TFA}$ although there is not much difference between the α values. When methanol-TFA is substituted for methanol-HFo, the capacity of the column is greatly reduced. Therefore, as a different interpretation of the results obtained in Dunlap III's research as well as a conclusion of the results achieved in our own research, formic acid rather than TFA is preferred for inclusion in the elution system, especially when the sample is strongly hydrophobic and the properties of each component are very close to each other.

Fig. 4-3 presents the results of fractionation of the individual antenna apoproteins using the isopropanol-formic acid eluent system. The eluent gradient was controlled by the microprocessor of the FPLC system.

Three peaks were resolved from the antenna apoproteins of B800-820 complex as seen in Fig. 4-3 (a), and this result is in agreement with the previous SDS-PAGE result (lane 3, Fig. 3-7). To confirm this result, these collected peaks were analysed by SDS-PAGE with an acrylamide gradient of 12.5-20%. This slab gel (see

Fig. 4-4) shows that each peak corresponds to a single band: lane 1 (peak I) has the lowest M_r value; lane 3 (peak III) has an M_r higher than that of lane 1; lane 2 (peak II) has the highest M_r

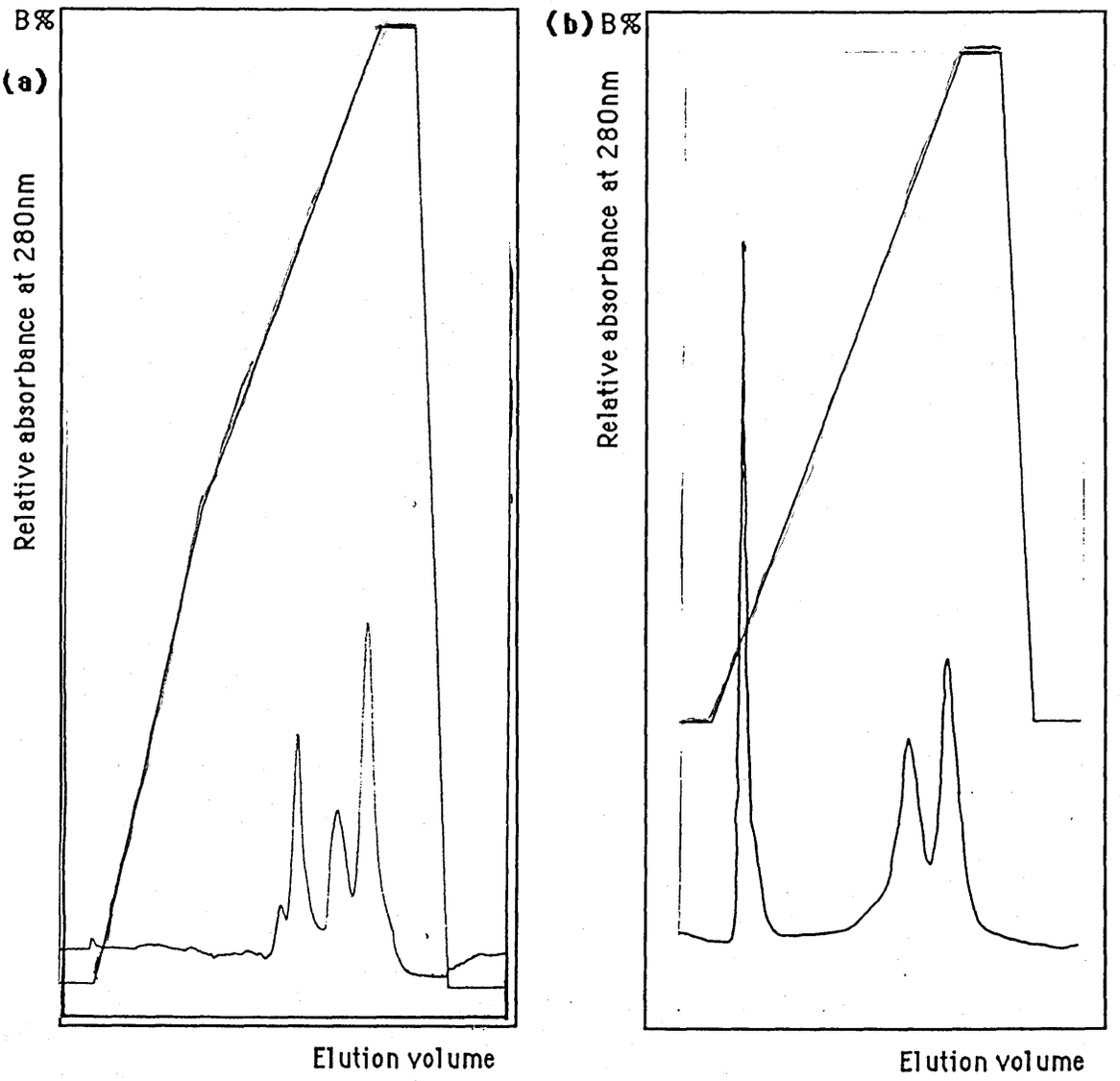


Fig. 4-3 A typical FPLC chromatogram of protein extracts from
(a) the B800-820 antenna complex.
(b) B880 antenna complex.
Eluent A: water/ formic acid (9:1);
Eluent B: isopropanol/ water/ formic acid (6:3:1 v:v:v)
The red lines in the Fig.s are the gradient lines
(see Chapter 2 for further details)

of the three apoproteins. However, a little contamination appeared in lane 2. Apart from the top dark band, two faint bands also appeared which are equivalent to peak I and III in migration. These contaminations can be removed by rechromatography under the same conditions as used to separate the apoproteins.

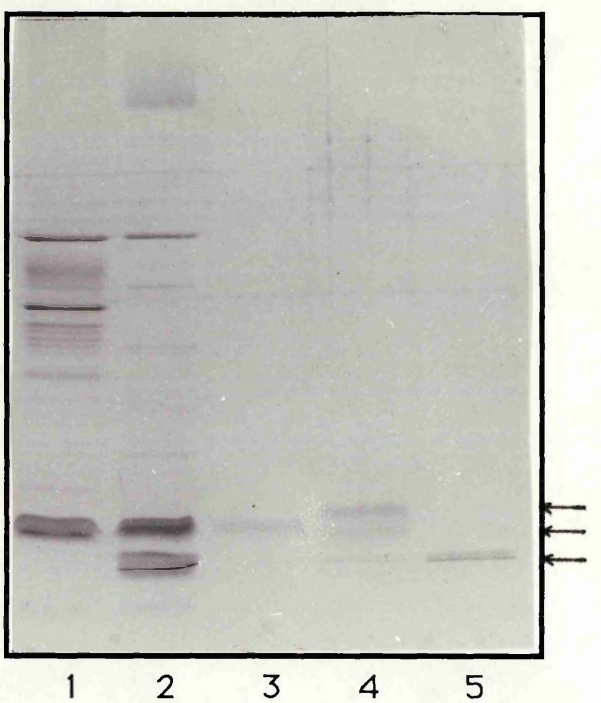


Fig. 4-4 SDS-PAGE (12.5-20% (w/v) acrylamide) analysis of the protein peaks collected from reverse phase FPLC. **Lane 1**: the B880 antenna complex; **lane 2**: the B800-820 antenna complex; **lane 3**: peak III (see Fig 4-3 (a)); **lane 4**: peak II; **lane 5**: peak I.

The antenna apoproteins of B880 complexes resolved into two peaks by reverse phase FPLC as shown in Fig. 4-3 (b). Results of SDS-PAGE show that these two peaks correspond to two polypeptides and these polypeptides can also be purified by rechromatography. Finally, these five purified polypeptides (three

from B800-820 and two from B880 complexes) were applied to a SDS-PAGE gel with a gradient of 12.5-20%. The result of this slab gel is shown in Fig. 4-5.

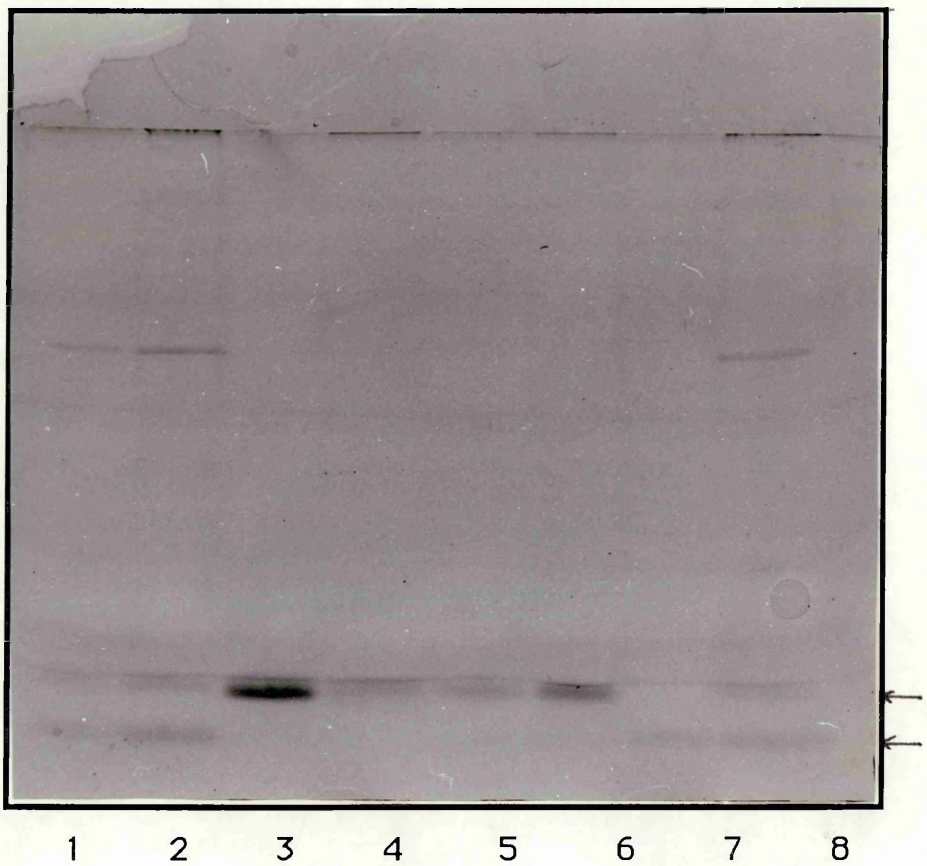


Fig. 4-5. SDS-PAGE (12.5-20% acrylamide) of the individual apoproteins of the B800-820 and the B880 antenna complex which were collected from FPLC. Lane 1, 2, and 8: the B800-820 complex; lane 3: peak II of B880 (see Fig. 4-3(b)); lane 4: peak I of B880; lane 5: peak III of B800-820 (see Fig. 4-3 (a)); lane 6: peak II of B800-820; lane 7: peak I of B800-820.

§4-2-4 Amino Acid Compositions of the Individual Apoproteins

Two purified apoproteins from B880 antenna complex were hydrolysed for either 24 or 72 h in 6 M HCl at 110 °C. The samples were then applied to an automatic amino acid analyser and the results were presented in Table 4-1 and Table 4-2.

Determination of the amino acid composition of the apoproteins of B800-820 complex is still in progress and so far only the data from one apoprotein are available. These are presented in Table 4-3.

Table 4-1 Amino acid composition of B880 β-apoprotein (previously assigned as peak II) of Chr30

A.A	mol% hydrolysis for 24 h	mol% hydrolysis for 72 h	estimated number of a.a.residues	number of a.a. residues after correction
Asp	3.39	2.72	3	3
Thr	1.34	1.20	1	1
Ser	3.88	2.63	3-4	4
Glu	2.05	1.71	2	2
Gly	2.80	2.73	3	3
Ala	3.52	3.32	3-4	4
Val	2.39	2.67	2-3	3
Met	1.47	1.30	1-2	2
Ile	4.54	4.15	4-5	5
Leu	9.26	9.10	9	9
Phe	5.00	5.00	5	5
Lys	0.95	0.94	1	1
His	1.47	1.50	1-2	2
Arg	2.70	2.08	2-3	3
Pro	2.62	2.44	2-3	3
Cys	0.00	0.00	0.00	0

Total number of amino acid residues : 50 + Trp

Amino acid composition analysis is the first step towards sequencing a protein. Before subjecting to amino acid analysis, a protein sample must be cleaved completely into individual amino acids. This was done by acid hydrolysis at high temperature (110 °C) for either 48 h or 72 h. Under such conditions, however, some of the amino acids could be completely or partially decomposed, i.e. approx. 100% trp, 15% serine, 6% threonine, 4% cysteine and

10% tyrosine are decomposed by acid hydrolysis for 72 h. On the other hand, some of the amino acids, i.e. valine and leucine, are hydrolysed more slowly than the others. These figures provide the basis for correction of the experimental data of amino acid composition to the nearest integral values for each amino acid.

Table 4-2 Amino acid composition of B880 α-apoprotein (previously assigned as peak I) of Chr30

A.A.	mol% hydrolysis for 24 h	mol% hydrolysis for 72 h	estimated number of a.a.residues	number of a.a. residues after correction
Asp	3.67	2.37	3-4	4
Thr	3.52	2.90	3-4	4
Ser	4.16	2.27	3-4	4
Glu	7.28	5.69	6-7	7
Gly	5.93	4.41	5-6	6
Ala	4.82	4.43	4-5	5
Val	2.23	2.07	2	2
Met	2.23	2.08	2	2
Ile	3.97	3.89	4	4
Leu	5.80	5.18	5-6	6
Tyr	0.73	0.40	1	1
Phe	4.10	4.00	4	4
Lys	1.24	1.20	1	1
His	1.22	1.14	1	1
Arg	2.04	1.49	2	2
Pro	2.59	1.94	2-3	3
Cys	0.00	0.00	0.00	0

Total number of amino acid residues : 56 + Trp

B880 α-apoprotein was estimated to contain a minimum of 56 amino acid residues, whereas B880 β-apoprotein was estimated to contain a minimum of 50 residues. It is found that, similar to antenna apoproteins from purple non-sulphur bacteria, each of the two apoproteins contains one histidine residue, which is most likely to be the ligand to the magnesium of the Bchl a

dimer. In contrast to the B880 α-apoprotein, B880 β-apoprotein contains another histidine residue. This histidine residue is suggested as a possible binding site for the Mg of monomeric Bchl a; however, there is no direct evidence to prove this assumption as yet. Relatively large amounts of aromatic amino acid residues were found in both α- and β- apoproteins of B880 antenna complexes. These aromatic amino acid residues are often located close to the conserved histidine residue on the primary structures of most other apoproteins from purple bacteria. Having unique structural properties (e.g. aromaticity), these residues are probably heavily involved, in a subtle way, in the energy processing network.

Table 4-3 Amino acid composition of a B800-820 antenna apoprotein (previously assigned as peak III) of Chr30

A.A.	mol% hydrolysis for 24 h	estimated number of a.a. residues
Asp	4.12	4
Thr	2.86	3
Ser	0.96	1
Glu	6.13	6
Gly	3.63	4
Ala	9.42	9
Val	5.19	5
Met	1.68	2
Ile	4.04	4
Leu	7.02	7
Tyr	1.89	2
Phe	3.89	4
Lys	1.87	2
His	1.67	2
Arg	0.43	1
Pro	4.03	4
Cys	0.00	0

Total number of amino acid residues : 60 + Trp

The amino acid composition of one of the three apoproteins from B800-820 antenna complex is presented in Table 4-3. This apoprotein consists of at least 60 amino acid residues. Similarly, two histidine residues and some aromatic amino acid residues are also represented in the composition.

The amino acid compositions of the two additional apoproteins of B800-820 complex are not available at the time of writing up this thesis. However, sequence work on these five apoproteins is in progress. Hopefully, the primary structures of the individual apoproteins of the antenna complexes from *Chr. vinosum* strain D will be available in the near future.

Chapter 5

Determination of the Carotenoid Compositions and Bchl a to Carotenoid Ratios of B800-820 and B880 Antenna Complexes from *Chr. vinosum* Strain D

§5-1 Introduction

The amino acid compositions of the antenna apoproteins of B800-820 and B880 antenna complexes from this purple sulphur bacterium, *Chr.vinosum* strain D, have been presented in Chapter 4. These polypeptides are fundamental to the organization of antenna complexes to allow these complexes to function efficiently. However, the key components of antenna complexes in the primary reactions are Bchls and carotenoids. It is within these pigments that energy collecting and transfer processes take place.

Energy transfer from carotenoids to Bchls and from short-wavelength absorption Bchl, e.g. $Bchl_{800}$, to long-wavelength absorption Bchls, e.g. $Bchl_{850}$, from a range of antenna complexes has been studied. It was observed that (in most cases) the energy transfer efficiency from carotenoids to Bchls was over 60% (Cogdell et al., 1981; Cogdell and Frank, 1987; van Grondelle, 1985, Kramer et al., 1984). Moreover, the efficiency of energy transfer from $Bchl_{800}$ to $Bchl_{850}$ is relatively high (van Grondelle et al., 1982). How excitation energy migrates between these pigments within antenna complexes and what kinds of roles these carotenoids play are still open to debate.

In this chapter, the compositions and the ratios of Bchl a to carotenoids of the B800-820 and B880 antenna complexes from *Chr vinosum* strain D are presented. These data are absolutely essential for subsequent research to reveal the mechanism of energy transfer within antenna complexes.

5-2. Results and Discussion

§5-2-1. Separation and Identification of Individual Carotenoids

The individual carotenoids from *Chr. vinosum* strain D were resolved by silica thin layer chromatography. A typical result was photographed and presented in Fig 5-1. All TLC plates in this research were developed in diethylether/ dichloromethane / petroleum ether (40-60°C) (in a ratio of 2/1/7, v/v/v). A complete separation of five bands from the sample of whole cells was achieved by using this solvent system as seen in Fig 5-1. Three strong bands and two faint bands appear on the TLC plate. The R_f value of each band is presented in Table 5-1.

Similar results were also obtained when the sample was replaced by B800-820 or B880 antenna complexes. Each band was carefully removed from the TLC plate and dissolved in petroleum ether (40-60°C) (HPLC grade). The absorption spectrum of each carotenoid was then recorded. These five bands were tentatively identified as (from the top) lycopene, anhydrorhodovibrin, spirilloxanthin, rhodopin and rhodovibrin, which were judged by comparing the R_f value and the absorption maximum of each band

with well documented R_f data as well as absorption maxima of individual carotenoids (Davies, 1965).

No. of band (from top)	Rf
1. Lycopene	0.95
2. Anhydrorhodovibrin	0.75
3. Spirilloxanthin	0.43
4. Rhodopin	0.22
5. Rhodovibrin	0.09

Table 5-1 Absorption affinity of the individual carotenoids on the TLC plate (see chapter 2 for experimental details)

Since R_f values and absorption maxima are not totally reliable in providing unambiguous identification of individual carotenoids, e.g. spectrum shifts can be caused by different purity of solvents, each carotenoid was then subjected to mass spectral analysis to confirm the previous results. Bands 2, 3 and 4 were positively confirmed as anhydrorhodovibrin, spirilloxanthin and rhodopin in this research as shown in Fig 5-3 (for interpretations of the spectra see Isler, 1971). Although identification of band 1 and band 5 by mass spectroscopy was not successful because of the experimental problems of acquiring enough highly purified samples, results obtained in this research, i.e. R_f and absorption maxima, and previous studies by Schmidt (Schmidt et al., 1965;

Schmidt, 1978), Evans (Evans, 1989) strongly suggest that they are lycopene and rhodovibrin.

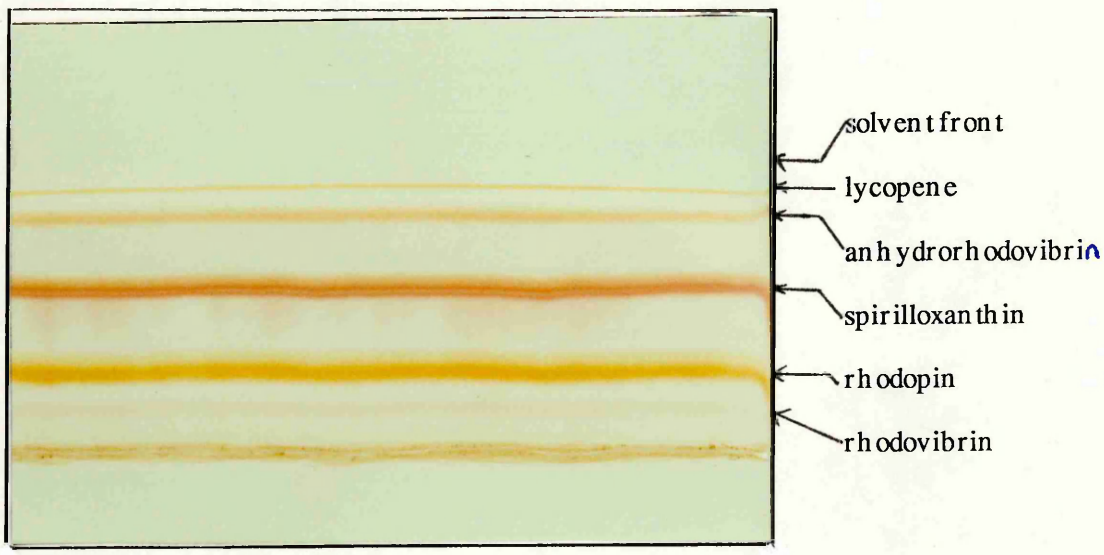


Fig 5-1 Silica TLC of total pigments extracted from the whole cells of Chr30. These five bands were identified as (from top) lycopene, anhydrorhodovibrin, spirilloxanthin, rhodopin, and rhodovibrin (the bottom band was origin line). A similar result was obtained when the Chr30 was replaced by Chr40, B800-820 or B880 complexes.

§5-2-2 Carotenoid Composition of B800-820 and B880 Antenna Complexes

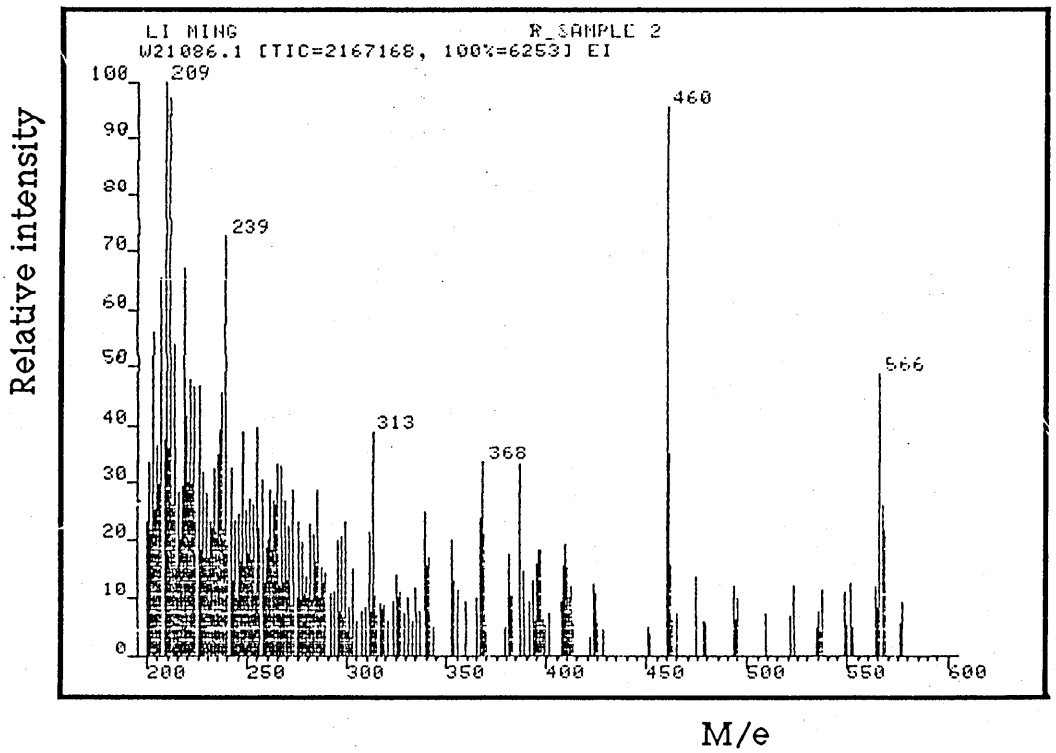
The carotenoid compositions from these two types of antenna complexes from 30 and 40 °C grown cells of *Chr. vinosum* strain D are presented in Table 5-2. The data show that the B880 antenna complex contains predominantly spirilloxanthin and a small amount of rhodopin. This result differs considerably from an earlier estimation (Cogdell & Thornber, 1979). Spirilloxanthin

seems to be an essential component of the "core" antenna complex, since the B880 antenna complex of Chr30 comprises 94% spirilloxanthin out of total carotenoids and similarly the B890 antenna complex of *R. rubrum* contains 90% of this carotenoid out of the total carotenoids (van der Rest et al., 1974; Evans et al., 1988). The other carotenoids may simply be intermediates in the synthesis of the end product, spirilloxanthin, which is required for the proper functioning of the antenna complexes (see Fig 5-4). These intermediate carotenoids, however, seem to be necessary for the formation and/or function of B800-820 complexes, judging by the substantial amounts within the complexes.

Growth temperature seems to have little effect on the B880 complex. In contrast, the carotenoid composition of the B800-820 complex changes markedly at increased growth temperature. This result suggests that the "peripheral" antenna complex is more dependent on the external conditions.

§5-2-3 The Ratios of Bchl a to Carotenoids of B800-820 and B880 Antenna Complexes

The ratios of Bchls to carotenoids of the B800-820 and the B880 antenna complexes from 30 and 40 °C grown cells of *Chr. vinosum* strain D are presented in Table 5-3.



Major predicted fragmentation pattern:

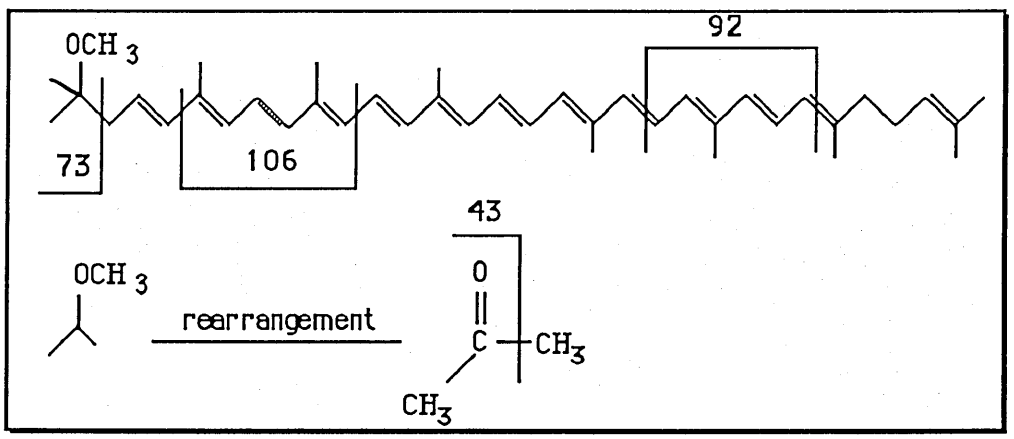


Fig 5-3 (a) Mass spectrum of anhydrorhodovibrin, M_r=566. Major diagnostic fragment ions (see fragmentation pattern above):

M/e=523:	M_r-43
M/e=493:	M_r-73
M/e=474:	M_r-92
M/e=460:	M_r-106
M/e=368:	M_r-106-92

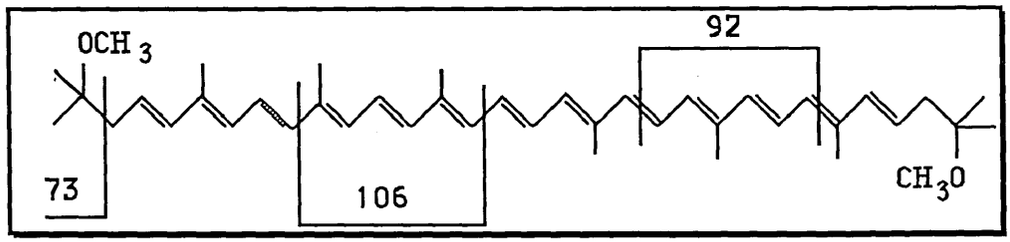
The Bchl a concentration of each antenna complex was calculated based on the published absorption coefficient (76 mM cm^{-1}) at 772 nm. The concentration of carotenoids of each antenna complex, however, was estimated based on the average coefficient at the absorption maximum of the total carotenoids of each antenna complex. These average absorption coefficients were calculated from the carotenoid compositions of the antenna complexes, and are presented in Table 5-4.

The quantitative absorption spectra of total pigment extracts of the antenna complexes are presented in Fig. 5-5. The concentrations of Bchl a and carotenoids of each antenna complex were calculated from the same quantitative spectrum of the extracts of that antenna complex.

	B800-820, Chr30			B880, Chr30		
	No. sample	Composition	$\overline{SD}$	No.sample	Composition	$\overline{SD}$
Lycopene	4	0.91%	0.16%	4	0	-
Anhydro-rhodovibrin	4	14%	0.56%	4	1.8%	0.06%
Spirilloxanthin	4	29%	1.4%	4	93.5%	0.16%
Rhodopin	4	55%	0.86%	4	3.9%	0.04%
Rhodovibrin	4	0.96%	0.21%	4	0.75%	0.11%

Table 5-2 Carotenoid composition of B800-820 antenna complexes from *Chr. vinosum* Strain D grown at 30 °C and 40 °C. SD: standard deviation

Major predicted fragmentation pattern of Fig5-3 (b):



Major diagnostic fragment ions (see fragmentation pattern as above):

M/e=596:	M_r
M/e=490:	M_r-106
M/e=417:	M_r-106-73
M/e=398:	M_r-106-92
M/e=384:	M_r-106-106
M/e=311:	M_r-106-106-73

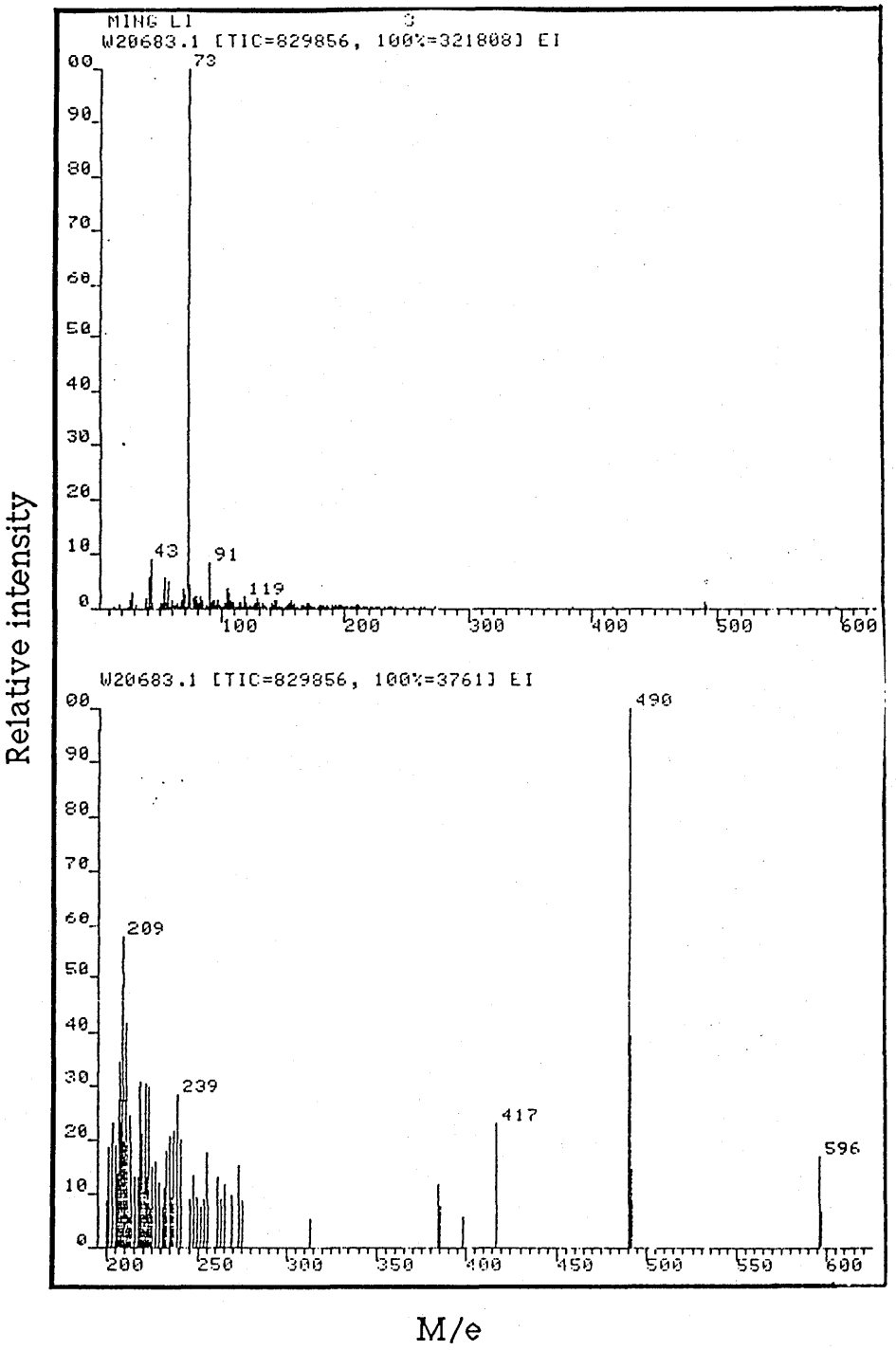
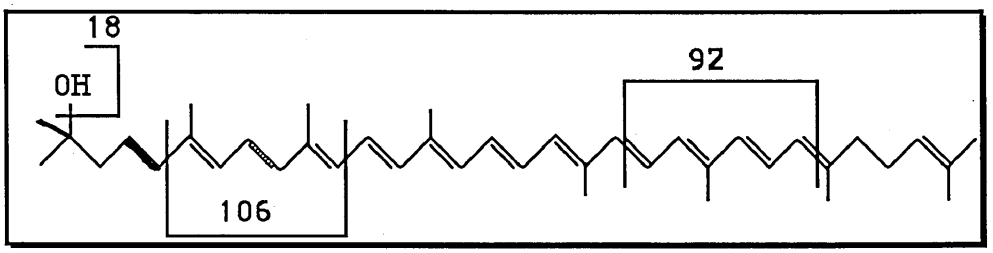


Fig 5-3 (b) Mass spectrum of spirilloxanthin (M_r=596).

Major predicted fragmentation pattern of Fig. 5-3 (c):



Major diagnostic fragment ions (see fragmentation pattern as above):

M/e=554:	M_r
M/e=536:	M_{r-18}
M/e=462:	M_{r-92}
M/e=448:	M_{r-106}
M/e=338:	$M_{r-106-92-18}$

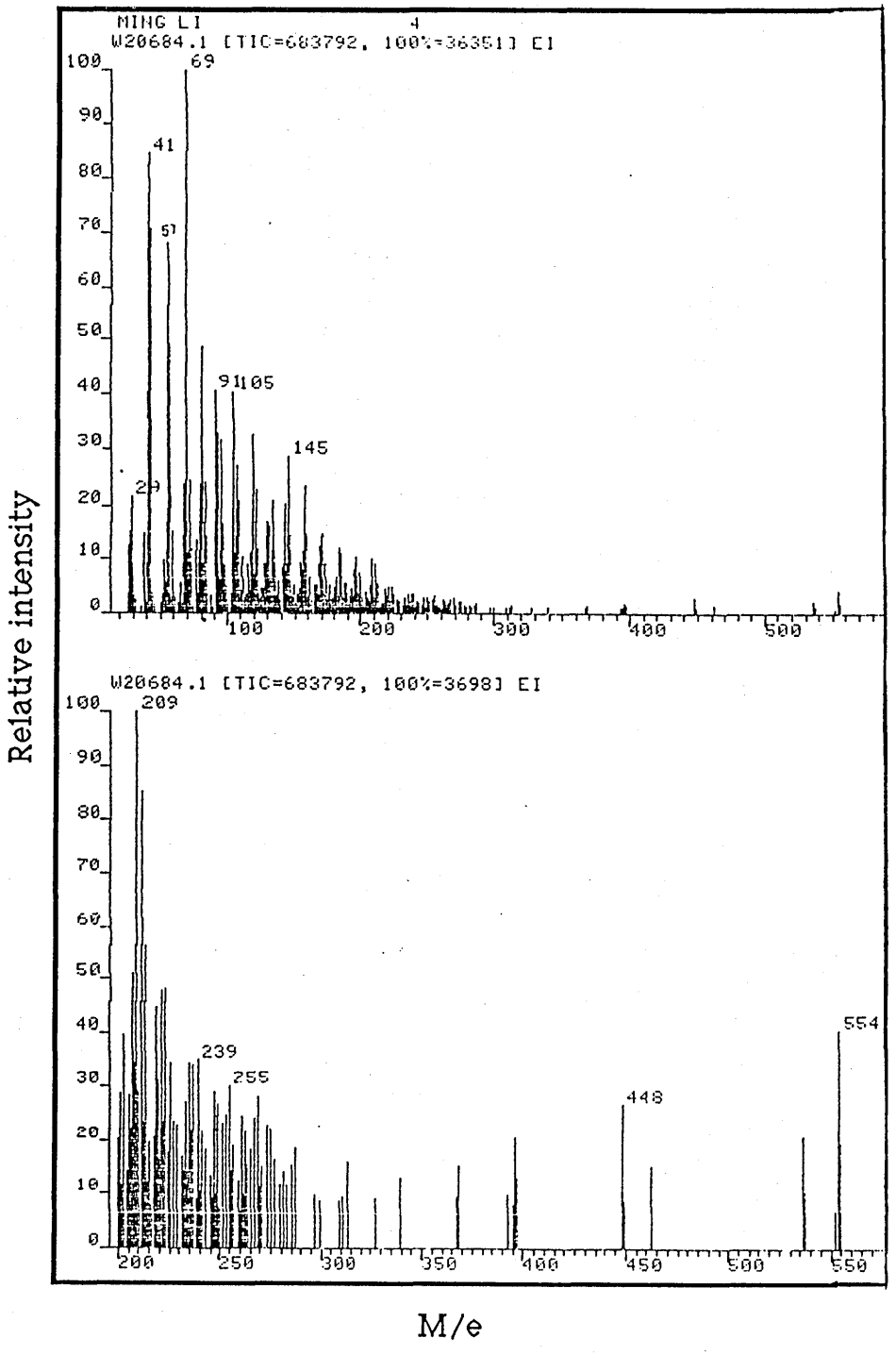


Fig. 5-3 (c) Mass spectrum of rhodopin, M_r=554

Composition	B800-820, Chr40			B880, Chr40		
	1st sample	2nd sample	Average value	1st sample	2nd sample	Average value
Lycopene	0.4%	0.41%	0.40%	-	-	-
Anhydro-rhodovibrin	17.6%	17.5%	17.5%	6.36%	3.22%	4.8%
Spirilloxanthin	47.6%	47.9%	47.8%	86.0%	90.5%	88.3%
Rhodopin	32.6%	33.4%	33.0%	6.37%	5.31%	5.8%
Rhodovibrin	1.45%	0.90%	1.18%	1.33%	0.94%	1.1%

Table 5-3 Carotenoid composition of B880 antenna complexes from *Chr. vinosum* Strain D grown at 30 °C and 40 °C.

The ratio of Bchl a to carotenoids of the B800-820 antenna complex was determined to be nearly 2:1, whereas the ratio of the B880 antenna complex was measured to be approximately 1.5:1. The ratio of 1.5:1 is not in agreement with the early estimation of 2:1 (Cogdell and Thornber, 1979). One could say that the ratio of 2:1 was only an estimation since no experimental data were presented and the carotenoid composition, or possibly further the purity, of the antenna complex is different from that in this research. On the other hand the ratio of 1.5:1 determined in this research is also tentative because of (i) the limited accuracy of the experimental techniques used for the measurements of carotenoid composition and $\overline{\varepsilon}^{Car}_{max}$ (see next page for abbreviation); (ii) that the nativity of the antenna complexes, especially B880 antenna

complexes, is difficult to maintain, resulting in the possible loss of Bchl a.

Growth temperature appears to have a little effect on the ratios of Bchl a to carotenoids of both the B800-820 and B880 antenna complexes. The ratios are slightly decreased if the antenna complexes were isolated from the cells grown at 40 °C. The reason for this has yet to be discovered.

	λ_{max}	$\overline{\varepsilon}^{Car}_{max}$	$\varepsilon^{Bchl\ a}_{max}$
B800-820 Chr30	470 nm	132	3.52
B800-820 Chr 40	470 nm	120	3.52
B880 Chr 30	493 nm	130	3.33
B880 Chr 40	493 nm	129	3.33

Table 5-4 The average absorption coefficients of total carotenoids of each antenna complex from *Chr. vinosum* strain D

λ_{max}: Maximal wavelength of absorption of the total carotenoids of each antenna complex

$\overline{\varepsilon}^{Car}_{max}$ Average millimolar absorption coefficient at wavelength of λ_{max} nm

$\varepsilon^{Bchl\ a}_{max}$ Millimolar absorption coefficient at wavelength of λ_{max} nm

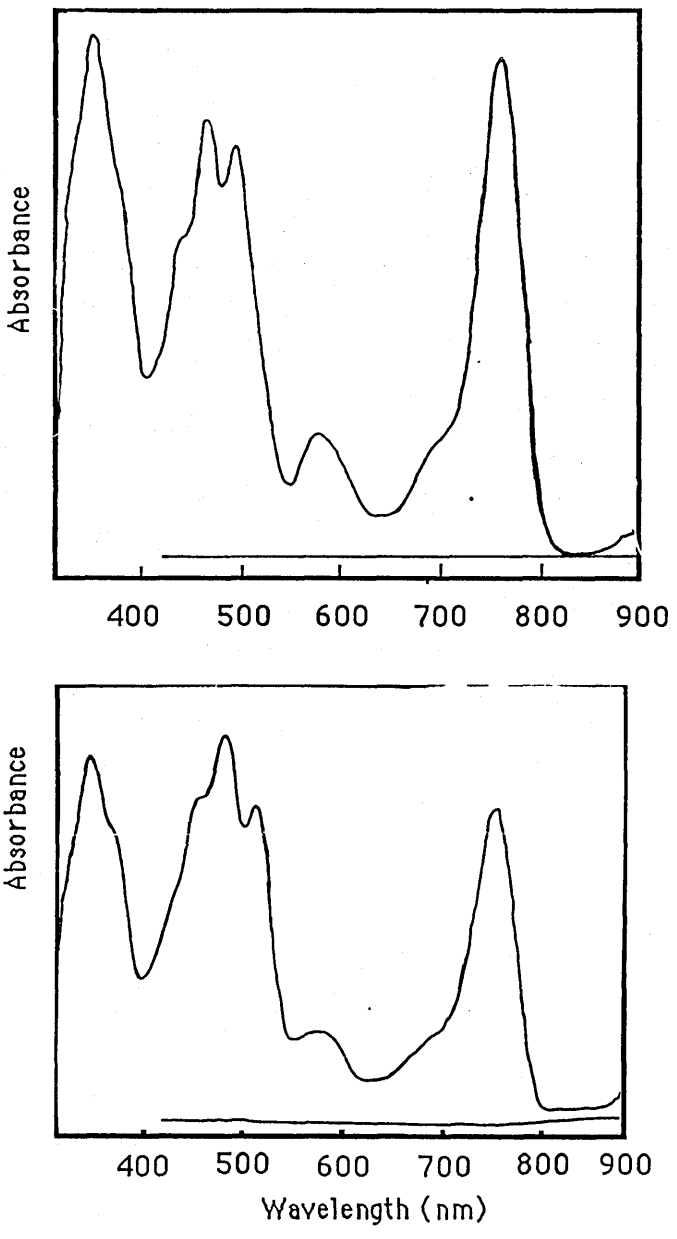


Fig 5-5 The quantitative absorption spectra of total pigment extracts of (a) B800-820 and (b) B880 antenna complexes. The bottom lines in the two spectra are the base lines

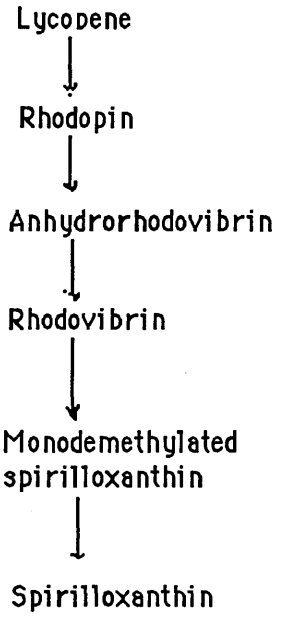


Fig 5-4 The biosynthetic pathway of carotenoids in Chr. vinosum strain D

Bchl a / Carotenoid / Sample	B800-820 Chr30		B800-820 Chr40		B880 Chr30		B880 Chr40	
	E. Data	SD	E. Data	SD	E.Data	SD	E.Data	SD
1	2.20	0.01	1.90	0.01	1.40	0.052	1.36	0.017
2	2.24	0.12	1.89	0.01	1.60	0.064	1.40	0.006
3	2.07	0.07	1.82	0.03	1.50	0.006	1.43	0.023
4	2.09	0.06	1.91	0.02	1.45	0.023	1.35	0.023
Average value	2.19	0.063	1.88	0.017	1.49	0.036	1.39	0.017

Table 5-3 The ratios of Bchl a to carotenoids of the B800-820 and the B880 antenna complexes from 30 and 40 °C grown cells of *Chr. vinosum* strain D. E. Data: experimental data; SD: standard deviation.

In summary *Chr. vinosum* strain D contains spirilloxanthin series carotenoids. All of the carotenoids are suggested to be synthesized through one biosynthetic pathway as shown in Fig. 5-4. The carotenoid composition of the B880 antenna complex comprises mainly spirilloxanthin, which is the end product of the biosynthetic pathway. The B800-820 antenna complex, however, contains relatively large amounts of rhodopin and anhydrorhodovibrin in addition to spirilloxanthin. The ratio of Bchl **a** to carotenoids for the B800-820 antenna complex is 2:1 and the tentative ratio of that for the B880 antenna complex could be 1.5:1.

Chapter 6

Preliminary Studies on Membrane Location of Antenna Complexes

§6-1 Introduction

In the last few years, topological investigations on the intramembrane organization of protein complexes have increasingly drawn the attention of scientists. The photosynthetic membranes of purple bacteria have provided an experimental system to investigate the three-dimensional structure of biological membranes.

It is found in purple bacteria that the ICMs contain only a few different phospholipids (Snozzi & Bachofen, 1982). For ICMs of *Rb. sphaeroides, R. rubrum* and *Chr. vinosum* strain D, the distribution of the major phospholipids (PE, PG) is asymmetric (Al-Bayatti & Takemoto, 1981, DaPra et al., 1982; Shimada & Murata, 1976). It is suggested that the lateral distribution of the membrane lipids is also rather inhomogeneous based on the results obtained from *R. rubrum* (Snozzi & Bachofen, 1979; Picorel et al., 1983). The ratio of proteins to lipids of ICMs of purple bacteria is about 2-3 to 1 by weight (Niedermann et al., 1978). Most importantly, the photosynthetic components, which consist mainly of proteins, are all organized within these membranes. Therefore understanding the mechanism of

photosynthesis has to be twinned with understanding the architecture of photosynthetic membranes (or ICMs).

The membrane locations of antenna complexes of purple bacteria have been probed by (i) immunological approaches, (ii) mild non-specific proteolysis and (iii) hydrophobic/ hydrophilic labelling. These methods have proven to be powerful tools for studying the topology of membrane protein components. A model of the localization of the membrane protein components was proposed on the evidence obtained from these methods and was presented in Fig 1-7 (Chapter 1).

The purple bacteria used for studies of membrane topology so far contain only one type of antenna complex. In Zuber's, Bachofen's and Cogdell's laboratories, *R. rubrum* was chosen (Brunisholz et al., 1986; Meister et al.,1985). The reasons for choosing this bacterium are not only that it contains only B890 antenna complex but this antenna complex has been well characterized. Furthermore, the primary structures of the two antenna apoproteins are known. For similar reasons, Drews's group selected a *Rb. capsulatus*, Y5 mutant, which contains only a B800-850 antenna complex (Tadros et al., 1986).

In this research, however, the species employed is *Chr. vinosum* strain D. This purple sulphur bacterium contains more than one type of antenna complex and at least five antenna apoproteins. This bacterium has the potential to provide much more information on membrane topology than *R. rubrum* or *Rb. capsulatus*, Y5. Evidence on the lateral distribution of different antenna complexes and stoichiometry of each antenna complex within the membranes can possibly be obtained from this bacterium.

§6-2 Results and Discussion

§6-2-1. Immunological Approach

In this research two types of antibodies were successfully raised. These are antibodies to B800-820 and B880 antenna apoproteins. Immunoblotting of the antibodies against their native antenna complexes and the whole cells, which were resolved by SDS-PAGE, shows that these polyclonal antisera are highly specific (Fig. 6-1), and can be used as probes for the future work on membrane location of the individual antenna complexes.

Fig. 6-1 (a) shows the immunoblotting analysis of the specificity of the reaction of the anti-B800-820 apoproteins of Chr30 with the whole cells of Chr30 (lane 1), B880-820 antenna complexes from Chr30 (lane 2) and B880 antenna complexes from Chr30. Although samples were not well resolved on the SDS-PAGE, the specificity of this polyclonal antibody was reasonably high. (The multiple bands in lane 1 are due to overloading of the sample, causing smearing artifact on the gel). The band in lane 3 suggests that one of the B880 apoproteins is partially homologous with one of the B800-820 apoproteins if there was no cross contamination at all between these two complexes. The homology of the apoproteins between these two complexes was also observed in Fig. 6-1 (b), one faint band appeared in lane 2 and 4. The specificity of the antiserum of anti-B880 apoproteins is also high (lanes 3 and 5). This antiserum reacts only with the apoproteins to which it was raised (compare the Coomassie blue stained gel in Fig. 3-7).

§6-2-2. Mild Proteolysis with Proteinase K

Chromatophores of cells of *Chr. vinosum* strain D were digested with proteinase K for 1, 10 and 60 min. After this digestion, dramatic differences were observed on SDS-PAGE analysis compared to the untreated membranes (Fig. 6-2). The gel shows that high M_r polypeptides are more susceptible to proteinase K treatment. The three dark bands in the middle of lanes 1 and 5 are thought to be RC polypeptides. The smallest one of the three polypeptides was totally digested. The digestion took place almost instantly, with the band disappearing within 1 min of enzyme treatment. This result suggests that this polypeptide is exposed on the chromatophore membrane surface. The antenna apoproteins can be subjected to mild proteolysis with proteinase K. However, no conclusion can be made from this gel. This is because most of the large proteins were cleaved into small fragments and the M_r of some of the fragments can be as large as those of the antenna apoproteins.

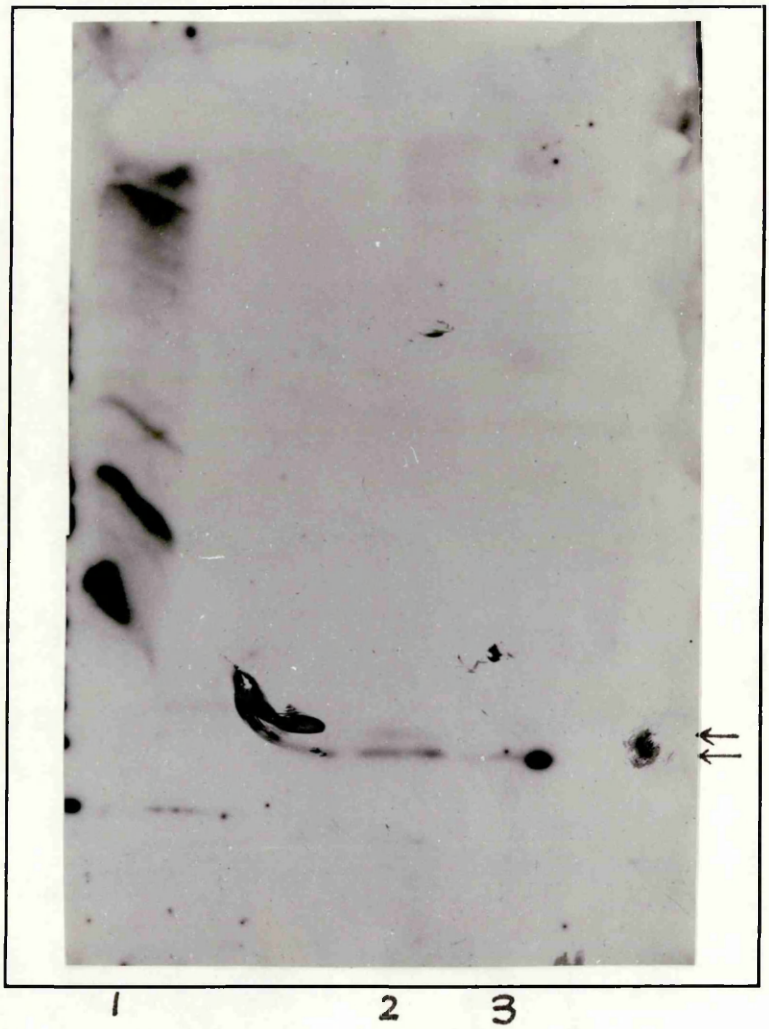


Fig. 6-1 (a) Immunoblotting of antiserum to anti-B800-820 against **lane 1**: Whole cells of Chr30.; **lane 2**: B800-820 complex of Chr30; **lane 3**:B880 antenna complex of Chr30. (for experimental details see §2-9-1, and see text for explanations).

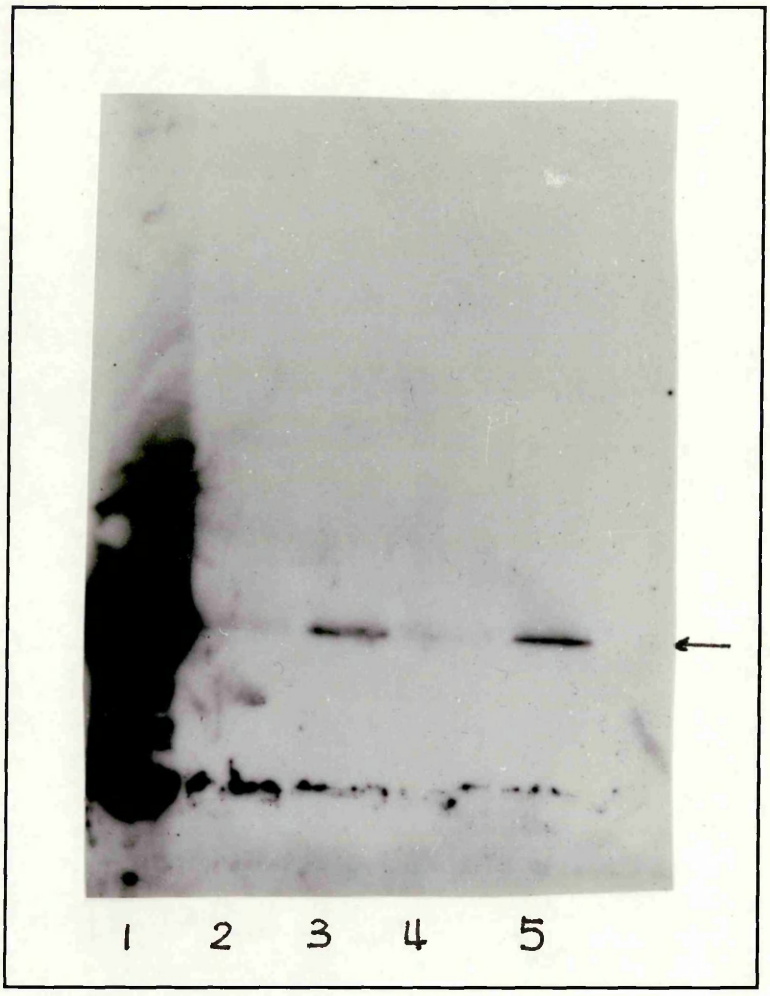


Fig. 6-1 (b) Immunoblotting of antiserum to anti-B880 against **lane 1**: Chr 30; **lane 2** and **4**: B800-820 complex; **lane 3** and **5**: B880 complex (see §2-9-1 for experimental details, and see text for explanations)

The digested membranes were treated with Triton X-100 and subjected to sucrose density gradient centrifugation as described in § 2-4-2. Two clearly separated bands were obtained after 16 h centrifugation at 197,000 xg. The absorption spectrum of each band appears virtually the same as that of each intact antenna complex, i.e. B800-820 or B880 antenna complex. This result indicates that the binding sites and their microenvironment were not severely influenced by the enzyme treatment.

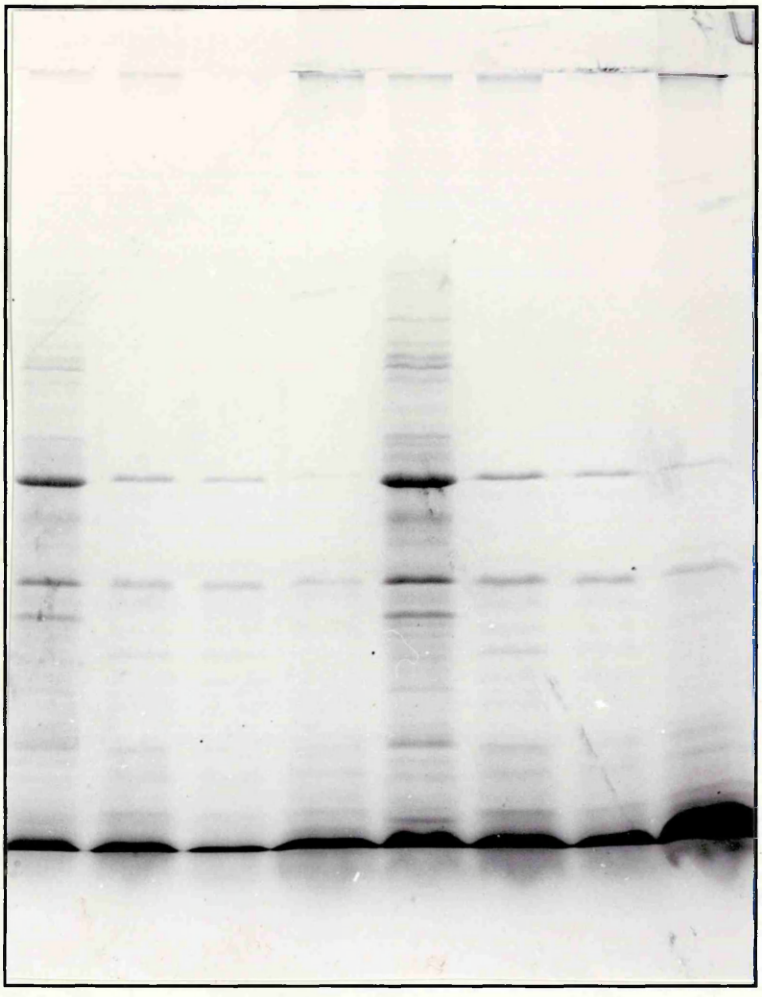


Fig. 6-2 SDS-PAGE showing protein compositions of chromatophores after treatment with proteinase K. **Lane 1** and **5**: untreated chromatophores; **lane 2** and **6** chromatophores were treated for 1 min; **lane 3** and **7**: chromatophores were treated for 10 min; **lane 4** and **8**: chromatophores were treated for 60 min.

Future work in this area could be easily performed if a protein sequencer is available. The individual apoproteins or derived fragments can be fractionated by using the method developed in this research as in §2-4 to §2-5 in Chapter 2. If each apoprotein can be sequenced successfully, the membrane

organisation of each apoprotein will become known by comparing the primary structures of intact apoproteins with that of proteinase K treated apoproteins.

Chapter 7

General Discussion

The light reactions of photosynthesis in purple bacteria are a set of processes in which the radiant solar energy is converted into an electrochemical potential gradient across the plasma membrane. This leads to the production of ATP and generation of reducing power (NAD(P)H), which is required to reduce CO_2 to carbohydrates.

The basic principles of the light reaction are the same no matter which photosynthetic organism is concerned. It is, therefore, rational to choose simple organisms, e.g. purple bacteria, initially to investigate the mechanism of photosynthesis in general.

In this research, the antenna system of *Chr. vinosum* strain D, a purple sulphur bacterium, was extensively investigated. In order to understand how the antenna system of this bacterium functions, it is desirable, first of all, to obtain substantial amounts of pure antenna complexes. It is then essential to characterize the different antenna complexes isolated from this bacterium before embarking on any further investigation.

The first part of this project, involving isolation, purification and partial characterization of the antenna complexes, was discussed in Chapter 3. This represents the first study in which the antenna complexes from a purple sulphur bacterium have been purified and extensively characterized. Although a number

of researchers have managed to isolate different antenna complexes from this bacterium (Garcia et al., 1966; Thornber, 1970; Mechler and Oelze, 1978; Hayashi and Morita, 1980), no antenna complexes were pure enough for subsequent studies such as structural and biochemical characterization and photochemical measurements.

Chr. vinosum has been widely accepted to contain 3 types of antenna complexes, one core antenna complex, i.e. the B880 complex, and two peripheral antenna complexes, i.e. the B800-820 and B800-850 complexes. The B800-820 and the B880 antenna complexes were successfully isolated and purified in this project by a combination of sucrose gradient centrifugation and DE-52 anion exchange chromatography. The B800-850 antenna complex was "obtained" by a separate method in this research. The chromatophore membranes were solubilized in the presence of 1% (w/v) SDS. The different fractions were then collected by ammonium sulphate precipitation. However, nearly all the pigmented fractions contained B800-850 type antenna complexes, as judged by their absorption spectra. There was no trace of any other antenna complexes. Furthermore, the protein compositions of these complexes within the antenna apoprotein region (M_r 5-10 kD) on a slab gel looked rather like those of the whole membranes. These B800-850 antenna complexes prepared in this research could, therefore, be spectrally altered from their *in vivo* forms.

After the purification of the B800-820 and the B880 antenna complexes, their protein compositions were determined by SDS-PAGE. The B880 antenna complex consists of two antenna apoproteins, whereas the B800-820 antenna complex contains three (or more) apoproteins. The protein compositions of the two

complexes are quite different and can be seen clearly on SDS-PAGE (see Fig 3-7). Any impurities or cross contamination between the two complexes, if any, can also be easily recognised on the gel.

Optimisation of procedures for fractionating the individual antenna apoproteins from *Chr. vinosum* was discussed in Chapter 4. A highly reproducible method of separating hydrophobic membrane proteins by reverse phase liquid chromatography (RPLC) was successfully developed in this research. There have been problems in using RPLC to fractionate hydrophobic proteins, mainly owing to difficulties in finding a suitable eluent system. These difficulties were overcome by including 10% (v/v) formic acid in the eluent system of water and isopropanol. Formic acid can improve the solubility of the hydrophobic proteins and provide a low pH value so that the proteins exist predominantly in one charge state. Under such conditions, possible Coulombic forces were reduced and the hydrophobicity of each protein was retained.

Five apoproteins were fractionated, for the first time, from a purple sulphur bacterium. Sequencing of these apoproteins is still in progress. The preliminary results, i.e. amino acid composition, show that these apoproteins are quite similar to those from purple non-sulphur bacteria. Three of the five apoproteins consist of about 50, 56 and 60 amino acid residues respectively, and each of the apoproteins contains either one or two histidine residues which are thought to be the possible ligand to the Mg of Bchl.

The carotenoid compositions and the ratios of Bchl a to carotenoids of the B800-820 and the B880 antenna complexes were determined and discussed in Chapter 5.

Although the cells of *Chr. vinosum* were previously determined to contain normal spirilloxanthin series carotenoids (Schmidt,1965), it could be argued that the methodology in use at that time was not reliable for any structural analyses. Besides, Thornber (Thornber, 1970) later reported that the antenna complexes isolated from this bacterium contained spirilloxanthin, lycoxanthin and lycophyll. To settle the controversy, the individual carotenoids were carefully identified and confirmed by mass spectroscopy. The cells were found to contain lycopene, anhydrorhodovibrin, spirilloxanthin, rhodopin, and rhodovibrin. This result is basically in agreement with Schmidt's observation (1965); however, neither lycoxanthin nor lycophyll was found either in whole cells or in individual antenna complexes.

There has been controversy on the ratios of Bchl to carotenoids in antenna complexes. It was widely accepted that the ratio of Bchl to carotenoids of the peripheral antenna complex, e.g. B800-820 or B800-850, was 3:1. This ratio was challenged by Kramer et al. (1984). They proposed a "minimal unit" model of the B800-850 antenna complex of *Rb. sphaeroides*. The Bchl **a** to carotenoid stoichiometry is 2:1 in this model. In an attempt to settle the controversy, Evans (1989) investigated extensively the ratios of Bchl to carotenoids from a range of antenna complexes, and he reported that the ratios were 2:1 for most of peripheral antenna complexes and 2:1 and 1:1 for the core antenna complexes. The ratios of Bchl a to carotenoids were previously estimated to be 3:1 for the B800-820 antenna complex and 2:1 for the B880 antenna complex from *Chr. vinosum* strain D (Cogdell and Thornber, 1979). Since no experimental description was given and the carotenoid compositions presented were incorrect, these

estimations needed to be reevaluated. After the antenna complexes were carefully purified, these ratios were then redetermined in this research. This ratio was measured to be 2:1 for the B800-820 and 1.5:1 (or 3:2) for the B880 antenna complexes (see Chapter 5 for further comments).

The membrane topology of the B800-820 and B880 antenna complexes from *Chr. vinosum* strain D was studied in a preliminary fashion by immunological and surface-specific proteolysis approaches. Polyclonal, monospecific antisera against the B800-820 and B880 antenna apoproteins have been successfully raised. In future, these antibodies can be used as powerful probes for elucidating the membrane location of the individual antenna complexes. A method for studies on chromatophore membrane locations of the protein-pigment complexes from *Chr. vinosum* strain D by mild non-specific proteolysis with proteinase K was also developed. The enzyme digested membranes were subjected to sucrose centrifugation as described in the preparation of the antenna complexes from untreated membranes. Two fractions were obtained after 16 h centrifugation at 197,000 xg. The absorption spectral profile of each fraction looks like that of an intact antenna complex; one fraction resembled the B800-820 complex and the other resembled the B880 complex. This suggests that the Bchl **a** binding sites to the antenna complexes are buried inside the chromatophore membranes.

To summarize, the results obtained in this research are presented in the following table (Table 7-1).

Table 7-1 Summary of part of the results obtained in this research.

		Chr 30		Chr 40	
Antenna complex:		B800-820	B880	B800-820	B880
No. of antenna apoproteins		3	2	3	2
Carotenoid composition	lycopene	0.91%	0	0.40%	0
	anhydro-rhodovibrin	14 %	1.8	17.5 %	4.8 %
	spirilloxanthin	29 %	93.5 %	47.9 %	88.3 %
	rhodopin	55 %	3.9 %	33 %	5.8 %
	rhodovibrin	0.96 %	0.8 %	1.2 %	1.1 %
$\overline{\varepsilon}^{Car}_{max}$		132	130	120	129
$\varepsilon^{Bchla}_{max}$		3.52	3.33	3.52	3.33
Ratios of Bchla to Carotenoids		2.19	1.49	1.88	1.39
Ratios of Bchl a to protein		0.049	0.046	0.039	0.025

(see Table 5-4 for the definition of $\overline{\varepsilon}^{Car}_{max}$ and $\varepsilon^{Bchla}_{max}$)

Appendix:

A .

Stock Solutions and the *Protocol* for SDS-Polyacrylamide Gradient Slab Gel Electrophoresis

1. Regents and stock solutions

(a). Acrylamide- bisacrylamide (30: 0.8)

It was prepared by dissolving 75 g acrylamide and 2 g bisacrylamide in 250 ml distilled water.The solution was then filtered and stored at 4 °C in a dark bottle.

(b). Ammonium persulphate (1.5% w/v)

This solution is unstable and should be made fresh before use.

(c). Running Gel Buffer

Tris: 18%. SDS: 0.4% (w/v)

(d). Stacking Gel Buffer

Tris: 0.5M. SDS: 0.4%

(f). Electrolyte (% w/v)

Tris: 0.3% SDS: 0.1% Glycine: 1.5%

(g). Boiling Solution

Tris:	50mM
SDS:	2% (w/v)
Glycerol:	20% (v/v)
Mercaptoethanol:	2% (v/v)
Bromophenol blue:	0.1% (w/v)

(h). M_r standards

Cytochrome *c*	11.7 kD	5mg/ml
Myoglobin	17.0 kD	5mg/ml

Alcohol Dehydrogenase	41.0kD	5mg/ml
B. S. A	68.0kD	5mg/ml

(i). TEMED
use as provided (see the following Table)

2. *Protocol* for preparation of a gradient slab gel

1. Clean the plates seqentially in water, detergent, water, and methanol. Allow the plates to be air-dried.
2. Attach the glass plates to each other, separated by lightly greased spacer(s).
3. Prepare two required solutions of different acrylamide concentration(A% and B%) according to the following table for the resolving gel.
4. Add a certain amount of TEMED to catalyze polymerization. Immediately fill up the set-up plates with a gradient of A-B% acrylamide using a gradient maker connected with a peristatic pump. Gently layer a few milliliters of methanol/water (1/1) on top to form a flat surface while acrylamide polymerizes.
5. After polymerization of the gel, pour the upper unpolymerized liquid out. Prepare a top stacking gel according to the following table. Pour enough of this stacking gel mixture to fill the upper 3 cm of the gel. Insert the sample well comb, and allow the gel to polymerize.
6. Attach gel to electrophoresis unit. Fill up both top and bottom chambers with electrode buffer and check for leaks.
7. Mix or dissolve the protein sample (1-100 μg) with 10-100 μl of boiling solution. Heat to 100 °C for 3 min and load samples on gel. Include molecular weight standards in one or two lanes.
8. Run gel until blue dye front runs 1 cm to the end of the gel. The typical setting is 40-45 mA constant current, which should run in about 4-5 hr.
9. Gel can be stained with silver stain but usually with Coomassie brilliant blue.

Table **Recipe for gradient gel preparation**

Stock solution	Stacking gel	Final acrylamide concentration in resolving gel					
		7.5%	10%	11.5%	15%	16.5%	20%
Stack gel buffer	5.0 ml						
Running gel buffer (ml)		5.0	5.0	5.0	5.0	5.0	5.0
Acrylamide-bisacrylamide (30: 0.8) (ml)	3.0	5.0	6.6	7.5	10.0	11.0	13.2
Distilled water	6.0						
15% , 70%(w/v) sucrose(ml)		10	8.4	7.5	5.0	4.0	1.8
15% Ammonium persulphate (µl)	60	100	100	100	100	100	100
TEMED (µl)	20	13	13	13	13	13	13

B.

Tannin assay solutions

Tannin regent:

1 M HCl: 196ml

Tannin acid: 20g

Phenol: 4 ml

To prepare this solution, heat to 80 °C, filter and store at 4 °C

Gum arabic:

acacia: 0.2 % (w/v)

C .

Fuller's medium:

Solution 1 1 litre pH 7-8

Sodium Chloride	60 g
Di-potassium hydrogen orthophosphate	1 g
Potassium dihydrogen orthophosphate	1 g
Ammonium chloride	2 g
$CaCl_2\ 2H_2O$	0.25 g
Magnesium chloride , $6H_2O$	1 g

Solution 2 1 litre pH 7.8-8.0

Sodium thiosulphate, $5H_2O$	6 g
Sodium hydrogen carbonate	8 g

Solution 3 1 litre

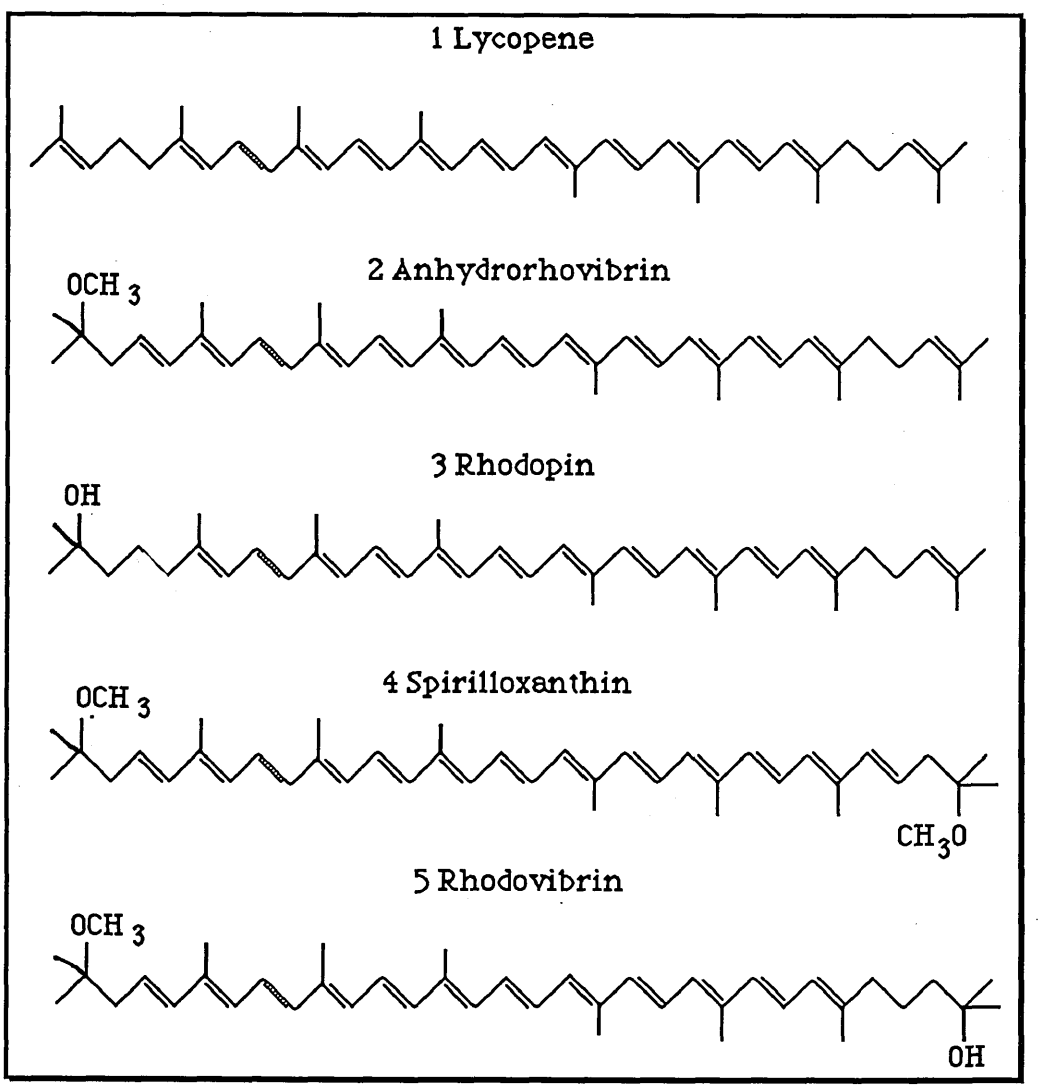
$FeSO_4$, $7H_2O$	1.6 g
EDTA	3 g

To make up Fuller's medium :

1 litre of solution 1 + 1 litre of solution 2 + 8 mililitre solution 3

D .

The Molecular Structures of Carotenoids



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