Carotenoid-Induced Cooperative Formation of Bacterial Photosynthetic LH1 Complex†

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ABSTRACT: A simple reconstitution technique has been developed and then applied to prepare a series of light-harvesting antenna 1 (LH1) complexes with a programmed carotenoid composition, not available from native photosynthetic membranes. The complexes were reconstituted with different C_{40} carotenoids, having two structural parameters variable: the functional side groups and the number of conjugated C−C double bonds, systematically increasing from 9 to 13. The complexes, differing only in the type of carotenoid, bound to an otherwise identical bacteriochlorophyll−polypeptide matrix, can serve as a unique model system in which the relationship between the carotenoid character and the functioning of pigment−protein complexes can be investigated. The reconstituted LH1 complexes resemble the native antenna, isolated from wild-type *Rhodospirillum rubrum*, but their coloration is entirely determined by carotenoid. Along with the increase in its conjugation size, the carotenoid absorption transitions gradually shift to the red. Thus, the extension of the conjugation size of the antenna carotenoids provides a mechanism for the spectral tuning of light harvesting in the visible part of the spectrum. The carotenoids in the reconstitution system promote the LH1 formation and seem to bind and transfer the excitation energy specifically only to a species with characteristically red-shifted absorption and emission maxima, apparently, due to a cooperative effect. Monitoring the LH1 formation by steady-state absorption and fluorescence spectroscopies reveals that in the presence of carotenoids it proceeds without spectrally resolved intermediates, leading directly to B880. The effect of the carotenoid is enhanced when the pigment contains the hydroxy or methoxy side groups, implying that, in parallel to hydrophobic interactions and $\pi-\pi$ stacking, other interactions are also involved in the formation and stabilization of LH1.

In purple photosynthetic bacteria, the conversion of light energy into chemical energy takes place in a specialized photochemical nanodevice, which constitutes the central part of their photosynthetic apparatus. The device performs very specific functions; first, the photon capturing (= light harvesting) and energy transfer by the antennae, and consequently the energy conversion in the reaction centers (RCs).[1] The basic functional entity, which effectively carries out these primary steps of photosynthesis in bacteria, is the core complex (RC−LH1), which consists of the light-harvesting antenna 1 (LH1) and reaction center (RC), tightly arranged with 1:1 stoichiometry, as found, for example, in *Rhodospirillum rubrum* (*1*−*3*) and in *Rhodopseudomonas palustris* (*4*). To increase the efficiency of light absorption, many other species of purple photosynthetic bacteria develop another auxiliary light-harvesting antenna 2 (LH2), located peripherally with respect to the core complex (*5*).

Structural modeling (*6*) and low-resolution crystallographic data (*2*, *7*) showed that the RC fits into the inner space of the LH1 cylinder, but a possibility remains that the LH1 complex is not fully closed and another small-sized polypeptide might be involved in the functional assembly (*8*, *9*). Indeed, the recent relatively high-resolution crystal structure of the RC−LH1 complex reveals more structural features of the assembly, in which the RC is surrounded by an oval nonsymmetrical LH1 antenna. The symmetry of the complex is broken by an additional transmembrane helix, creating a gap in the LH1 cylinder near the ubiquinone binding site of the RC (*4*). Yet, the resolution of the structure (4.8 Å) does not allow for the accurate location of every pigment molecule in the complex. Therefore, the interactions between its components as well the mechanism and control of its formation in vivo remain unclear (*10*−*12*). From the in vitro reconstitution studies (*13*, *14*) it seems that the LH1 antenna and the entire core complex represent a self-organizing system, but knowledge about the factors that govern the system is quite limited.

The photosynthetic pigments, (bacterio)chlorophylls and carotenoids (Crts), are required for the correct assembly of naturally occurring photosystems, where they function both as photoactive and as structural components. Their proper

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[1] Abbreviations: Anv, anhydrorhodovibrin; BChl, bacteriochlorophyll; β-OG, *n*-octyl β-glucopyranoside; CD, circular dichroism; cmc, critical micelle concentration; Crt, carotenoid; LDAO, *N*,*N*-dimethyldodecylamine *N*-oxide; LH, light harvesting; Lyc, lycopene; Neu, neurosporene; RC, reaction center; Rhd, rhodopin; *Rb.*, *Rhodobacter*; *Rsp.*, *Rhodospirillum*; Sph, spheroidene; Spx, spirilloxanthin; WT, wild type.

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functioning requires the involvement of proteins, which noncovalently bind the chromophores and actively participate in adjusting chromophores' properties, relevant in mediating the energy and electron transfer. Early studies on Crt biosynthesis inhibition provided hints as to the importance of Crts for both oxygenic (plant) and anoxygenic (bacterial) types of photosynthesis (*15*). Among the most important functions of Crts is that of photoprotection against excess radiative energy (*16*) and of providing structural stabilization (*17*, *18*). In the antennae, these pigments also contribute to the light harvesting in spectral regions where the absorption of light by tetrapyrroles is minimal (*19*). The efficiency of the energy transfer from the Crt to BChl molecules varies from 30% to nearly 90%, clearly depending on the number of conjugated double bonds in Crt. However, despite intensive structural and spectroscopic investigations, the questions as to the molecular mechanisms involved in the Crt-to-BChl energy transfer remain open (*19*−*21*) and there is a need for spectroscopic studies on LH complexes where the factors determining the rates of energy transfer can be systematically varied.

The reconstitution from individual components and pigment replacement proved highly successful approaches to understanding the structure−function relationships in multicomponent biological systems, such as photosynthetic complexes (*22*−*24*). There are many reports on preparation of reaction centers with selectively modified pigment composition (*23*, *25*) and several examples of pigment replacement in the LH2 antenna (*25*−*27*). The techniques of LH1 complex reconstitution with native as well as modified bacteriochlorophylls are particularly well established (*14*, *28*, *29*).

In most cases, the LH1 reconstitution relies on the phenomenon of reversible dissociation of this modular complex in micellar media (*30*, *31*). The reversibility appears to be restricted, however, to the carotenoidless and carotenoid-depleted variants of LH1, and therefore only a few examples exist of a complete reconstitution of the complex, which would include Crts as well. For instance, the effect of the number of conjugated C═C bonds in Crt on the energy transfer from the incorporated Crt to bacteriochlorophyll (BChl) in LH1 complexes from several strains of purple bacteria has been studied by Noguchi et al. (*32*). In another study, Davis et al. (*18*) have shown efficient energy transfer from incorporated Crts to BChls in reconstituted LH1 complexes of *Rb. sphaeroides* and *Rsp. rubrum* and strong effects of Crts on the LH1 subunit oligomerization. In a recent study on the LH1 from *Rb. sphaeroides*, involving a replacement of native BChl with its Ni-substituted analogue, the measurements of the efficiency of energy transfer from the reconstituted native Crt to BChls (B875) were used to determine the exciton delocalization size (*14*) and to investigate the dynamics of energy relaxation in this complex (*33*).

Here we present a convenient way of constructing bacterial LH1 antenna with modified pigment composition. By use of the reconstitution approach, a series of model LH1 complexes has been prepared, differing only in the incorporated C_{40} Crts, which have various side groups and systematically varying conjugation sizes ranging from 9 to 13. Access to such model complexes provides an opportunity to reveal the structural features of Crts, relevant to their engagement in the photosynthetic apparatus.

Another aim was also to gain access to model pigment−protein complexes, in which the Crt structure would be the only variable parameter, and in which the fates of the Crt and BChl excited states could be investigated as a function of that parameter. The preliminary results of the time-resolved spectroscopic studies on these complexes were published elsewhere (*34*), while their biochemical properties and the effects of carotenoid substitution are discussed in the present paper. A detailed mechanism of the in vitro formation of LH1 antenna in the presence of Crts and its implications for the in vivo system are also given.

EXPERIMENTAL PROCEDURES

Purification of Native LH1 Antenna. Native LH1 antenna was prepared from *Rsp. rubrum* (S1 strain) by a modification of a previously published method (*35*). Freshly prepared chromatophores (final $A_{880} \sim 38$) were suspended in 20 mM Tris-HCl buffer (pH 7.8) containing sodium ascorbate and 0.1% LDAO (v/v). After 10−20 min of stirring at 4 °C, the absorbance of the suspension was measured again and readjusted to 38. The detergent concentration was then increased to 0.45%, and vigorous stirring at 4 °C was continued for 1 h in the dark. After 4-fold dilution, the solubilized chromatophores were centrifuged first at low speed (8000*g*, 10 min, 4 °C) and then pelleted at high speed (105000*g*, 60 min, 4 °C). The RC-depleted pellet was collected and extracted twice with 0.3% LDAO; the combined LH1 extracts were extensively dialyzed against 20 mM Tris-HCl buffer (pH 7.8) containing sodium ascorbate. This crude LH1 antenna preparation, obtained after dialysis as a precipitate, was practically free of RC as judged by its absorption spectrum and the analysis by SDS−PAGE (not shown). It was stored under nitrogen at 4 °C in the dark until further use.

For the final purification by ion-exchange chromatography, a portion of the pellet (equivalent to ∼1.3 mg of BChl) was suspended in 4 mL of 40 mM Tris-HCl buffer (pH 7.8), containing sodium ascorbate and 2.35% β-OG (w/v). The suspension was stirred for 60 min on ice and afterward centrifuged (8000*g*, 20 min, 4 °C). The supernatant, containing solubilized LH1, was loaded onto a small column (0.5 cm × 3.5 cm) of DEAE-cellulose (DE52 Whatman), equilibrated in 0.8% β-OG in 20 mM Tris-HCl buffer (pH 7.8)/10 mM NaCl. The contaminations were eluted with 6−8 mL of the same buffer containing 50 mM NaCl and then with 4 mL of 60 mM NaCl. The fraction of pure LH1 antenna was eluted with 180 mM NaCl as a dark-red band.

Isolation of Carotenoids. The carotenoids (see their structures and absorption spectra in Figure 3 in the Results section) were isolated from purple photosynthetic bacteria following the methods previously described. Neurosporene (Neu) was extracted from the cells of *Rb. sphaeroides* strain G1C (*36*) and purified by two-step column chromatography, on alumina and on silica gel, respectively. The final purification was done by recrystallization from *n*-hexane. Spheroidene (Sph) was isolated from the wet cells of wild-type *Rb. sphaeroides* 2.4.1 and purified by a similar method (*36*). Lycopene (Lyc) was extracted from tomato concentrate and purified by column chromatography on alumina, fol-

lowed by repeated crystallization from a mixture of tetrahydrofuran and *n*-hexane (*37*). Rhodopin (Rhd) and anhydrorhodovibrin (Anv) were extracted from the cells of *Allochromatium vinosum* (D). Both pigments were purified by column chromatography on alumina and then recrystallized, Rhd from *n*-hexane and Anv from a mixture of tetrahydrofuran and *n*-hexane (*38*). Spirilloxanthin (Spx) was extracted from the wet cells of the *Rhodospirillum rubrum* S1 strain and purified by low-pressure column chromatography on $Ca(OH)_2$, with an acetone/benzene mixture for elution, and repurified by recrystallization in *n*-hexane (*39*).

Reconstitution of LH1 Complex. Freeze-dried chromatophores from wild-type *Rsp. rubrum* were treated with benzene until a complete extraction of Crts was achieved, as confirmed by the absorption spectrum of the extract. After a thorough removal of the residual benzene under vacuum, the powder of carotenoid-depleted chromatophores was stored at −20 °C under nitrogen atmosphere until further use. A 100 mg portion of the carotenoid-depleted chromatophores was suspended in 8 mL of 40 mM Tris-HCl buffer (pH 7.8) containing sodium ascorbate and 0.3% LDAO (v/v). After 45 min of vigorous stirring at 4 °C in the dark, the suspension was centrifuged at 7000*g* (40 min, 4 °C). The supernatant, containing solubilized carotenoid-depleted LH1 antenna, was collected and stored at −30 °C.

A 0.5 mL aliquot of the carotenoid-depleted antenna, containing ∼0.1 μmol of BChl, was diluted with 15 mL of 40 mM Tris-HCl buffer (pH 7.8), and then 300 μL of a 5% LDAO solution was added, followed by dilution with an additional 35 mL of the buffer. The mixture, while stirred on ice, was slowly titrated with 0.5 mL portions of acetone solution of a desired Crt (absorbance 1−1.4 at maximum absorption, concentration ∼ 9 μM). The volume of the reconstitution mixture was kept constant around 60 mL by removing excess acetone under a stream of nitrogen, if necessary. The progress of reconstitution was monitored by absorption and emission spectroscopy. The titration with the Crt solution was carried out until a shift of the BChl Q_y band to 880 nm was observed (Figure 6). Usually, a 12−24 h period of stabilization at 4 °C in the dark was required.

After complex stabilization, the reconstitution mixture was charged on a DEAE-cellulose (DE52, Whatman) column (1.5 cm × 3 cm), preequilibrated at 4 °C in 20 mM Tris-HCl buffer (pH 7.8), containing 0.025% LDAO. The excess Crt was removed by elution with 40 mL of the same buffer containing 0.045% detergent and 50 mM NaCl. By increasing the salt concentration to 175 mM, the fraction of purified reconstituted complex was eluted. For purification of less stable complexes, i.e., those reconstituted with Lyc and Neu, the elutions were done in 0.030−0.035% LDAO. In the case of complexes reconstituted with Spx and Rhd, Triton X-100 had to be used instead of LDAO for the final elution. If necessary, the purification by ion-exchange chromatography was repeated. All chromatographic steps were done at 4 °C in dim light. The purified complexes were stored at −30 °C. Depending on the stability of the complex, yields of the purified complexes were between 40% and 75%, with respect to the carotenoid-depleted complex.

Analysis of Pigment Composition. The pigments were extracted by shaking for 1 min a 150 μL aliquot of a purified complex with 2 mL of a cold acetone/methanol mixture (7:2 v/v). The mixture was centrifuged (10000*g*, 5 min, 4 °C),

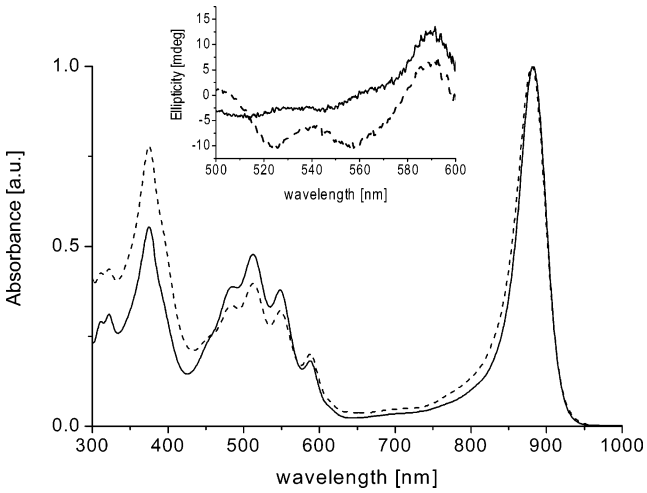

FIGURE 1: Absorption and circular dichroic (inset) spectra of LH1 antenna isolated from the chromatophores of *Rsp. rubrum* S1 and purified by ion-exchange chromatography in the presence of 0.8% β-OG (solid line) and of LH1 complex reconstituted with Spx (dashed line).

the supernatant was collected, and the pellet was treated with 0.3 mL of tetrahydrofuran. After centrifugation, the supernatants were combined and the absorption spectrum of the total extract was measured. The amounts of the pigments were estimated by use of the value of $\epsilon_{770} = 7.15 \times 10^4$ (M^{-1} cm^{-1}) for BChla (*40*), and for the Crts, the values of their extinction coefficients as determined by Fujii et al. (*38*) and by Zhang et al. (*41*).

SDS−PAGE Analysis. The polypeptide composition analyses of the isolated and reconstituted complexes were done by the tricine−SDS−PAGE technique, modified for membrane proteins (gel conditions 10% T and 3% C), according to Schägger and von Jagow (*42*). The samples of the complexes were incubated for 60 min at 40 °C with 4% SDS before loading. After the electrophoretic separation, the gels were stained with Coomassie blue.

Absorption, Emission, and CD Measurements. The absorption spectra were measured on Hitachi U-2000 and Cary 50 (Varian) spectrophotometers. The steady-state emission spectra were recorded at room temperature in 1 cm quartz cells, on a Spex fluorolog 1680 spectrofluorometer, equipped with 0.22 m double monochromators. Circular dichroic spectra were recorded at 15 °C in 2 mm quartz cells on a J-710 (JASCO) spectropolarimeter.

RESULTS

Isolation of Native LH1. It was difficult to obtain satisfactorily pure LH1 by use of LDAO alone or even in combination with Triton X-100, as recommended in the original protocol. The purification was less problematic when a milder detergent, β-OG, for the final solubilization and chromatography was applied, already used for the preparation of LH1 antenna from *Rsp. rubrum* G-9 (*2*) and from *Rb. sphaeroides* (*7*, *14*). The absorption spectrum of the purified LH1 antenna, obtained by ion-exchange chromatography on DEAE-cellulose in the presence of 0.8% β-OG, is shown in Figure 1. This single-step purification procedure yields the LH1 complex with the near-IR absorption band located at 883 nm and the value of A_{883}/A_{275} ratio as high as 3. The electrophoretic analysis, done by tricine−SDS−PAGE,

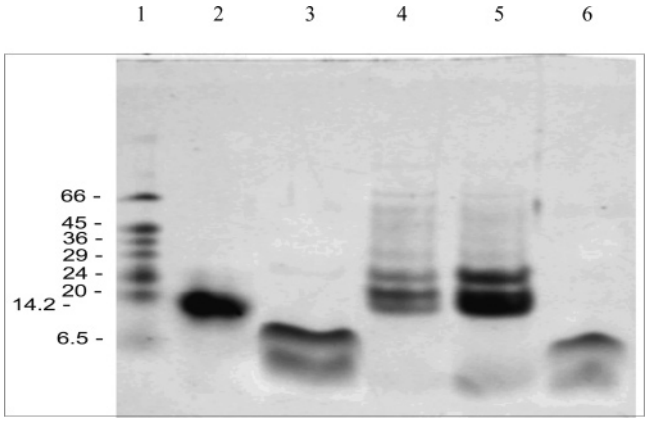

FIGURE 2: Tricine−SDS−PAGE analysis of the polypeptide composition of the reconstituted and native LH1 complexes, followed by Coomassie staining, according to Schägger and von Jagow (*42*): lanes 1 and 2, protein molecular mass standards (molecular masses indicated in kilodaltons); lane 3, LH1 complex reconstituted with Spx; lanes 4 and 5, RCs purified from *Rsp. rubrum*; lane 6, native LH1 complex, isolated from *Rsp. rubrum*.

revealed the presence of only the LH1 polypeptides in the preparation (Figure 2). Significantly, in the absorption spectrum there are no bands seen near 770 and 820 nm, which indicates no contamination by free BChl and the B820 subunit, respectively. These results confirm the high purity of the LH1 antenna isolated by use of β-OG.

Interestingly, the same mild detergent was found to be rather ineffective and destructive to the core antenna when applied directly for solubilization of the photosynthetic membranes of *Rsp. rubrum*. Even a short treatment of the chromatophores with 2−3% β-OG at 4 °C resulted in the appearance of an absorption at 820 nm and a blue shift of the LH1 Q_y transition (data not shown), indicating a loss of Crts from the complex.

LH1 Reconstitution with Spirilloxanthin. The method of LH1 complex reconstitution in LDAO, described under Experimental Procedures, was first applied to the insertion of Spx. In our study, three different detergents, β-OG, Triton X-100, and LDAO, were tested as a medium for reconstitution and all gave positive results (not shown), but the best results were obtained for the last one. A stable B880 complex was readily formed upon the addition of Spx into the carotenoid-depleted reconstitution mixture, at the ratio of 0.095 μmol of Crt/0.1 μmol of BChla, provided very pure preparations of the Crt were used. The red shift of the Q_y transition to 880 nm could be achieved only in the presence of Crt in the reconstitution system. Similar results were obtained for the LH1 complex isolated from the carotenoidless G-9 strain of *Rsp. rubrum* (*34*). A control experiment, done by titrating the carotenoidless reconstitution mixture with neat acetone, showed that initially acetone indeed induces a partial reassociation of the subunits to some aggregated forms with the Q_y transition at 860−865 nm (not shown). However, further additions of the solvent lead to a disruption of the fine interactions between the antenna components and a complete breakdown of the aggregates (not shown), still below the level of acetone content usually reached at the final stage of reconstitution with Crt.

Although the yields of the complex formation with Spx were high in LDAO, almost quantitative, as judged from the absorption spectra of the mixture (see below), the chromatographic separation on DEAE-cellulose in the presence of that detergent was poor and resulted in the dissociation of the product. A more efficient purification of the reconstituted complex was achieved by replacing LDAO with Triton X-100 during the chromatography. The electronic absorption spectra of the purified reconstituted and native complexes (*Rsp. rubrum* S1) are shown in Figure 1. The spectrum of the reconstituted complex closely matches the spectrum of the native one, in particular in terms of reproducing the red shifts of BChl Q_y transition and the positions of Crt absorption bands (Table 1).

Table 1: Pigment Stoichiometry (BChla:Crt molar ratio) and Energies of BChl Q_y and Carotenoid Transitions in LH1 Complexes Containing Different Carotenoids

incorporated carotenoid	BChl-to-Crt ratio	LH1 Q_y (nm)	transition $0 \rightarrow 0^a$		
			acetone	LH1	ΔE
Neu	2:1.13	880	468	483	15
			21 368	20 704	664
Sph	2:1.27	883	486	502	16
			20 576	19 920	656
Lyc	2:1.21	880	504	519	15
			19 841	19 268	573
Rhd	2:0.96	882	500	516	16
			19 984	19 380	604
Anv	2:1.24	883	516	536	20
			19 380	18 657	723
Spx	2:0.93	883	526	549	23
			19 011	18 215	796
native	2:1	883	525	547	22
			19 048	18 282	766

[a] The first value for each transition is given in nanometers, and the second is given in reciprocal centimeters.

The incorporation of Spx into the LH1 antenna also has a noticeable effect on the optical activity of that pigment; in the CD spectrum, shown in the inset of Figure 1, a clear nonconservative (negative) signal appears in the region of Crt absorption. The CD signal attributed to Crts (mainly Spx) in the native LH1 complex is slightly less pronounced (Figure 1 inset).

LH1 Reconstitution with Other Carotenoids. The reconstitution method, very effective in reconstructing the LH1 complex with Spx ($n = 13$), was successfully applied to the incorporation of other Crts into the same carotenoid-depleted LH1 system. The following Crts, having different functional groups and conjugation lengths varying from 9 to 12—Neu ($n = 9$), Sph ($n = 10$), Lyc ($n = 11$), Rhd ($n = 11$), and Anv ($n = 12$)—were used in the reconstitution studies and all gave high yields of the reconstituted complexes. As the conjugation size in the Crts systematically increases from 9 to 13 and the energetic gap between the S_0 state and the $1Bu^+$ state (S_3 in the new notation) narrows (*43*), a progressive red shift of the absorption transitions can be observed in the absorption spectra of the isolated pigments (Figure 3).

In each case, the Crt added to the reconstitution system strongly affects the equilibrium state between the antenna subunits, inducing their aggregation to LH1 complex with the Q_y band red-shifted to 880 nm. However, the Crts show differences in the amounts required to achieve the complete red shift. The B880 complexes were readily formed when around 0.1 μmol of Sph, Rhd, or Anv per 0.1 μmol of BChl was applied, comparably to Spx. The ease of B880 formation with Neu and Lyc was somewhat reduced, as reflected by

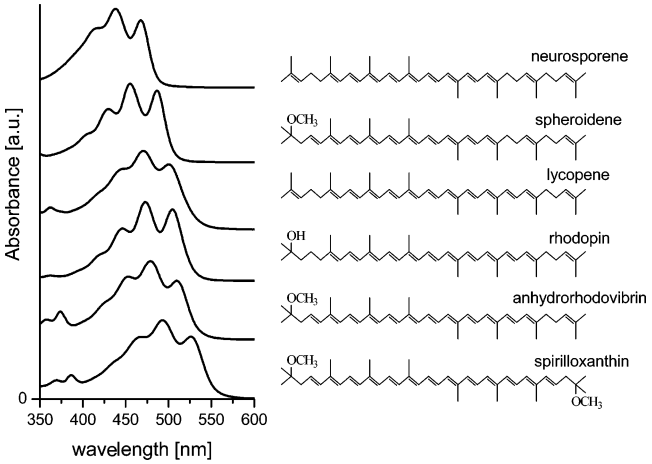

FIGURE 3: Absorption spectra and chemical structures of the carotenoids used in the present study for reconstitution into the LH1 complex from wild-type *Rsp. rubrum*. The spectra, recorded in acetone at room temperature, were normalized to match the absorbance at maximum absorption.

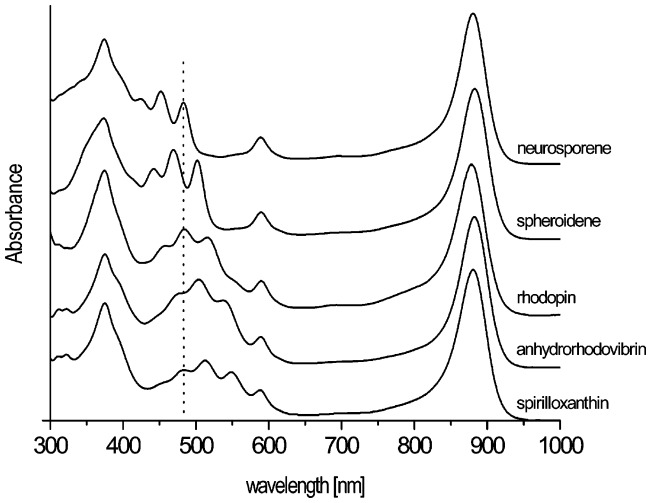

FIGURE 4: Absorption spectra of model LH1 complexes, reconstituted with different carotenoids with the conjugation size increasing systematically from 9 to 13.

the necessity of applying their larger quantities: 0.16 and 0.15 μmol, respectively, and by the need of longer periods of stabilization of the reconstituted complexes at 4 °C.

The reconstituted complexes were purified by generally the same method, i.e., ion-exchange chromatography on DEAE-cellulose, in the presence of detergent LDAO or Triton X-100; the exact conditions somewhat differed, depending on the stability of the complexes. Thus, 0.045% LDAO was used for the isolation of complexes with Sph and Anv. The best results with LH1 reconstituted with Spx and Rhd were obtained by replacing LDAO with 0.04% Triton X-100. The purification of complexes with Neu and Lyc was more problematic, due to their lability, and could be done efficiently in LDAO only at concentrations near the cmc value (0.03%).

The absorption spectra of all reconstituted complexes (Figure 4) are very similar in the Q_x and Q_y regions of BChl absorption; the positions of the relevant absorption bands are listed in Table 1. In this respect, they all resemble the spectrum of the native LH1 antenna, while there are some distortions in the intensity of the Soret band (Figure 1). In all cases a characteristic red shift of the Q_y transition is

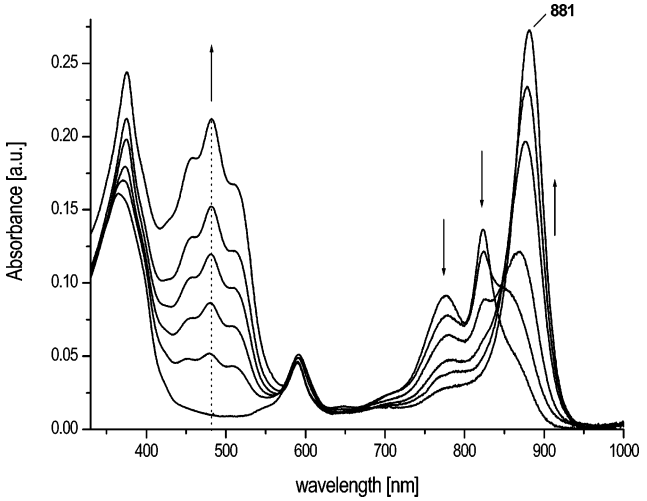

FIGURE 5: Changes in the absorption spectra of the reconstitution mixture, recorded at different stages of LH1 complex formation with rhodopin (see Experimental Procedures for details). The spectra were taken against acetone/Tris-buffer mixture at the ratio appropriate for each stage and are not normalized.

reproduced but its exact position of varies (by 3−4 nm), depending on the Crt. The red-most Q_y positions (883 nm) show the complexes reconstituted with Spx, Anv, Rhd, and Sph. Repeatedly, for the complexes with Lyc and Neu, the Q_y maximum never shifts above 879−880 nm.

Considerably larger differences are seen in the spectra of the model LH1 complexes in the region between 400 and 550 nm, which is characteristic for the light absorption by Crts (Figure 3). In the complexes reconstituted with Neu and Sph (conjugation sizes 9 and 10, respectively), the Crt absorption bands appear the most blue-shifted and therefore the BChl Q_x transition can be seen as a separate band (Figure 4). In the LH1 complexes with other Crts, as the conjugation length increases, the carotenoid absorption bands gradually shift to the red; with Spx, the spectral gap between the BChl Soret and Q_x transitions is filled up by the Crt absorption.

The light absorption by the reconstituted complexes in the visible range is solely determined by the type of Crt bound. In fact, their colors closely resemble the coloration of the strains of photosynthetic bacteria, which accumulate the corresponding pigments as their major Crts, namely, the G1C mutant of *Rb. sphaeroides*, wild type *Rb. sphaeroides* 2.4.1, *Rsp. molischianum*, *Rd. marinum*, and *Rsp. rubrum* S1, respectively.

Pigment Stoichiometry in Reconstituted LH1 Complexes. The quantification of pigment stoichiometries has been done by a total extraction to organic solvent, applying two different solvents to ensure a complete pigment recovery. The results, listed in Table 1, show that molar ratio of BChl to Crt in the complexes reconstituted with Rhd and Spx is slightly lower than in the native complex (as seen also in the absorption spectrum, Figure 1). The Crt content in the complexes reconstituted with the other four Crts is by 13−27% higher with respect to the BChla level.

Spectroscopic Monitoring of LH1 Formation. The progress of the LH1 complex formation and the binding of Crts into the complexes was followed by electronic absorption and emission spectroscopies, directly in the preparative reconstitution system. The absorption spectra of the initial reconstitution mixture (Crts absent) and the subsequent stages

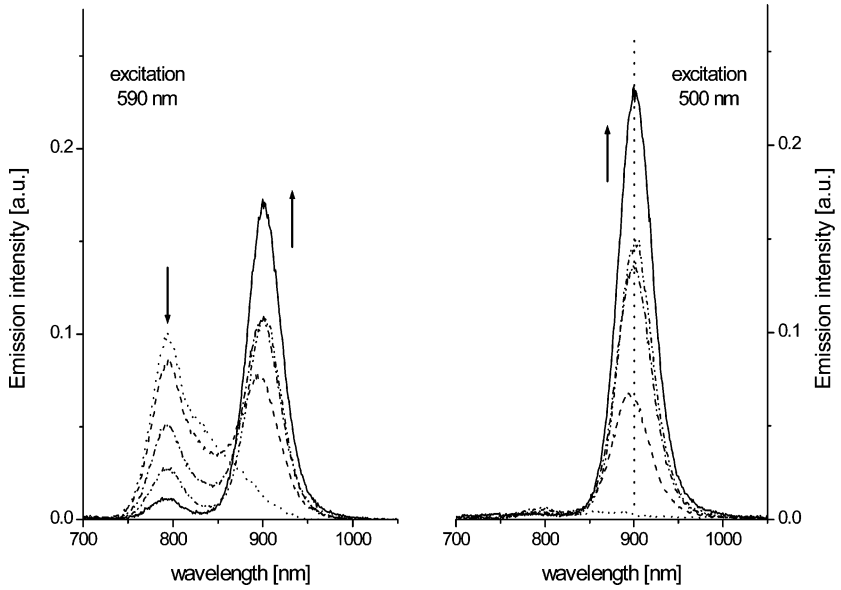

FIGURE 6: Progress of B880 complex formation in the presence of Crt (Sph), monitored by fluorescence spectroscopy, applying the excitation to BChl at 590 nm (left panel) and to the Crt at 500 nm (right panel). The spectra were recorded at consecutive stages of reconstitution (see text for details) and were not normalized.

of LH1 formation in the presence of increasing amounts of Rhd are shown in Figure 5. Initially, a partial dissociation of the antenna is induced by the detergent (0.033% LDAO) and therefore all forms of the carotenoid-depleted antenna, B780, B820, and B870, are present in equilibrium. Upon introducing the Crt into the reconstitution system, the equilibrium between the subforms shifts and the carotenoid-binding B880 complex is formed, apparently through a series of intermediate stages. A gradual disappearance of the subunit forms B780 and B820 occurs with a concomitant increase in the Crt absorption between 420 and 550 nm (Figure 5). Interestingly, from the onset of the complex formation, the Crt absorption transitions are already maximally red-shifted. In parallel, a new BChl band appears in the near-IR, gradually reaching 880−882 nm at the point of saturation with Crt. Judging from the complete disappearance of the absorption bands corresponding to the free LH1 subunits, the formation of the carotenoid-binding complex is almost quantitative.

The fluorescence spectra, recorded during the monitoring of the LH1 reconstitution in the presence of Sph by emission spectroscopy, are presented in Figure 6. The position of the Crt $0 \rightarrow 0$ transition in the complex (502 nm; Table 1, Figure 4), conveniently coinciding with the minimum of BChl absorption, allowed for a very selective excitation to the absorption band of the complex-bound Crt.

The shapes as well as the trends in the emission spectra strongly depend on the excitation wavelength (Figure 6). Initially, in the absence of Crts, when the excitation is applied at 590 nm (BChl Q_x transition), the emission spectrum of the reconstitution mixture is dominated by a strong band at 800 nm with shoulders near 830 and 870 nm (Figure 6, left panel). The presence of these three emission bands corresponds well to the detergent-determined equilibrium between the antenna subunits, seen also in the absorption spectra (Figure 5). Further, with increasing Crt content in the reconstitution system, the emission signals due to the antenna subforms gradually disappear. In parallel, a signal near 900 nm builds up, which reflects the changes in position of the equilibrium and the appearance of a new fluorescent species.

When the same reconstitution mixture is excited at 486 or 500 nm (the Crt absorption), a much different emission pattern is observed (Figure 6, right panel). Before addition of the Crt into the system (initial stage), the reconstitution mixture obviously shows no emission. However, as soon as the Crt is introduced to the mixture, only a single emission band appears, with the maximum near 900 nm, whose intensity increases with the amounts of Crt added, until saturation.

DISCUSSION

LH1 Complexes with Designed Carotenoid Content. The reconstitution technique was applied first to incorporate pure Spx into the carotenoid-depleted LH1 complex from *Rsp. rubrum*. The resulting LH1 complex is stable and can be isolated by ion-exchange chromatography in the presence of Triton X-100. The protein analysis of this complex by SDS−PAGE confirmed the presence of only two polypeptides of low molecular masses, (electrophoretically) identical to the ones found in the isolated antenna. The spectral properties of the model LH1 compare also very well with those of the native LH1 complex, confirming that the pigment−pigment as well as pigment−polypeptide interactions within the reconstituted complex are well reproduced. In the absorption spectrum, both the BChl and Crt transitions are characteristically red-shifted (*18*, *32*). Minor differences in the peak positions (Table 1) very likely reflect the difference in the Crt composition (*44*), while the increase of the Soret band intensity in the reconstituted antenna can be accounted for by a Rayleigh type of light scattering due to the protein association, induced by a nonspecific binding of Crts to the complexes. The formation of such nonspecific associates with Crts could be indeed confirmed by finding excess Crts in the reconstituted complexes, not reflected, however, in the intensities of the Crt absorption in the visible.

The specific binding of Spx in the complex is further confirmed by circular dichroic spectroscopy. A broad nega-

tive band, seen in the region of Spx absorption (Figure 4), similar to that reported previously (*18*), indicates the occurrence of strong interactions of the Crt with the chiral pigment−protein environment of the complex. The native antenna shows somewhat weaker CD features in that region (Figure 1), perhaps due to the presence of lipid molecules, remaining bound to the antenna and thus reducing the contact of Crts with the antenna polypeptides (*45*).

The other five Crts used in the present study also form stable LH1 complexes, in which they apparently are involved in strong interactions, causing characteristic red shifts of the absorption transitions of both Crts and BChl. The correct functional assembly of the model complexes is evidenced in particular by their emission properties, i.e., showing the restored Crt-to-BChl energy transfer (see below). These positive results of Crt insertion into LH1 are not quite unexpected, as the whole series, except Sph, belongs to the normal biosynthetic pathway of Spx and is found in the LH1 antenna isolated from young cells of wild-type *Rsp. rubrum* (*44*). The reconstitution of the LH1 antenna from this bacterium with Sph has already been described (*18*).

The presence of Crts affects the properties of LH1 complexes on several levels. The complex formation itself, triggered by Crt added to the mixture of the antenna subunits, is the earliest indication of the Crt effect. The assembly, discussed in more detail below, occurs under conditions that otherwise favor a complete dissociation of the complex into the subunits, as observed previously in a similar LH1 reconstitution system (*18*). Another such indication is the enhanced resistance of the Crt-binding LH1 to organic solvents, to as much as 20% aqueous acetone at the final stage of reconstitution. As indicated by the BChl to Crt molar ratios in the resulting complexes, during the reconstitution there is some degree of a nonspecific Crt binding. Nevertheless, the comparison of the relative amounts of Crt required for the formation of stable B880 and also varying stabilities of the complexes reconstituted with different Crts (reflected, for example, in the chromatographic conditions) allows for the conclusion that the Crt stabilizing effect is not the same in each case. Consequently, the six Crts, used in the present study, can be divided into two groups; Lyc and Neu (plain hydrocarbons) give less stable complexes and thus higher concentrations are required to form B880, while Spx, Anv, Rhd, and Sph form products of higher stability. The latter four pigments show higher affinity to the BChl-$\alpha\beta$ subunits, perhaps due to the presence of the hydroxy (Rhd) and methoxy side groups (Anv, Sph, Spx). The strong binding and larger stabilizing effect in the case of Rhd and Spx is also evidenced by their ability to form, at slightly substoichiometric content (Table 1), stable LH1 complexes with far red-shifted Q_y transitions. These observations indicate that, in addition to hydrophobic interactions (*46*) and more specific $\pi-\pi$ stacking interactions (*47*), interactions of other types also play a role in binding the Crt in the LH1 and the stabilization of the complex (see below).

The present reconstitution approach offers a general, simple, and efficient way of obtaining LH1 complexes with programmed Crt composition. The insertion of the exogenous Crt is practically quantitative, and significant amounts of purified complexes can be prepared. Thus, it allows for the obtaining of model LH systems of designed and well-defined Crt content, in which the relevant parameters of Crt can be varied in a systematic way, while the polypeptide−tetrapyrrole counterpart remains invariant. The preliminary ultrafast spectroscopic investigations on the dynamics of the Crt-to-BChl energy transfer in the model LH1 complexes have already shown the usefulness of the reconstitution approach (*34*). The model LH1 complexes provide perhaps a simpler alternative to widely studied and structurally better characterized LH2 complexes, in which the presence of two forms of BChl (B800 and B850) and two different Crt-binding sites, at least in some LH2 antennae (*48*), generates somewhat troublesome complexity of the intracomplex energy transfer pathways (*41*, *49*, *50*).

LH1 Reconstitution System. In the absence of Crts, the antenna subunits exist in a detergent-defined equilibrium, according to the following equation, in which d denotes the involvement of the detergent:

$$B870_d \leftrightarrow B820_d \leftrightarrow B780_d$$

At a constant temperature, the actual position of this equilibrium seems to be determined mainly by the detergent concentration; that is, it depends on the number of detergent micelles per number of individual copies of LH1, in a close analogy to a model system of BChl dimerization in the micelles of Triton X-100 (*51*).

Several parameters have been found to be critical in the present reconstitution system. In particular, a partial dissociation of the complex into free subunits is required; the Crt insertion technique, applied to intact carotenoidless LH1 (B870), produces no positive results (not shown). Therefore, the detergent concentration has to be high enough both to induce the dissociation of the complex (the initial stage of reconstitution, Figure 5), and to solubilize the externally added Crt molecules, to facilitate their interactions with the subunits. When the detergent concentration is too high, these interactions are disrupted and binding of Crt does not occur; and further, the subunits irreversibly dissociate into the separate components, free BChl and the polypeptides. The LDAO concentration of 0.033% (v/v) in the initial reconstitution mixture, slightly above its cmc value (0.03%), was found to be optimal for the binding of Crt and for the B880 complex formation. During titration with Crt in acetone, the volume of the reconstitution mixture increases by ~20%, and at saturation point, the detergent concentration is likely to drop below the cmc. One would also expect an increase in the cmc value, due to the enhanced solubility of the detergent in aqueous acetone. These conditions seem to favor the interactions of Crts with the LH1 subunits and lead to the complex's assembly. To reach these conditions, the choice of organic solvent as the Crt carrier is of importance, too; several solvents tested for Crt incorporation, such as benzene, *n*-hexane, tetrahydrofuran, and diethyl ether, produced no positive results (not shown). Perhaps here the unlimited miscibility of acetone with water is of relevance, as strong hydration of acetone molecules in an aqueous solution may prevent the solvent from interfering with the complex formation. Finally, the presence of the Crt is crucial for the formation of nativelike LH1 antenna with the NIR transition shifted to 880 nm (B880).

Cooperative Assembly of LH1 Complex. The presence of Crts in the reconstitution system provides a convenient intrinsic probe for spectroscopic monitoring of the assembly

process. Initially, even if only a small amount of Crt is present and all subforms (B780, B820, and B870) of the antenna are clearly observed, the Crt absorption bands appear already maximally red-shifted, to almost the same positions as in the final reconstitution stage (Figure 5) and in the purified product (Figure 1). If one considers this red shift as a measure of the Crt interactions with its environment ($\alpha\beta$-BChl units), it can be seen that, throughout the complex formation, these interactions do not change and are similarly strong in the reconstitution mixture (solution) and in the isolated complex. The monitoring of the process by emission spectroscopy further supports this conclusion. From the early stages of the Crt-induced complex formation, energy transfer from Crt to the long-wavelength forms of BChl can be observed, being a good verification for the correct assembly. Surprisingly, the energy transfer occurs exclusively to the B880, as indicated by the position of the emission maximum near 900 nm. This lack of other fluorescent forms implies the appearance of only a single type of Crt-binding species under the conditions of the reconstitution. As soon as the Crt is added to the LH1 subunit mixture, the pigment molecules strongly interact with the subunits and cause their rapid aggregation to the B880 form. Consequently, the B780−B820−B870 bands seen in the absorption spectra (Figure 5) and the fluorescence maxima, appearing upon excitation at 590 nm (Figure 6), can be ascribed to the carotenoidless forms. The presence of Crts markedly shifts the equilibrium between the LH1 subunits toward the B880 formation, as noted previously by Davis et al. (*18*), implying that Crts have a high affinity to their binding sites and are involved in strong interactions from the very moment of being introduced into the reconstitution system. However, they do not distribute evenly among the available binding sites on the carotenoid-depleted $\alpha\beta$-BChla heterodimers but rapidly induce the B880 formation. The Crt-to-$\alpha\beta$-BChla heterodimer binding constant as well as the aggregation constant of the initially formed Crt−$\alpha\beta$-BChla unit must be relatively high, as the far-red-emitting B880 complex is formed even when the ratio of the Crt to the carotenoidless $\alpha\beta$-BChla subunits is still very low. This points to the cooperative mechanism of LH1 formation in the presence of Crt. However, it should be noted here that B880 species do not necessarily represent the complete rings (32 BChls) of the LH1 antenna. B880 may correspond to Crt−$\alpha\beta$-BChla complexes with a distribution of molecular sizes but of similar spectral properties, as the ones shown by Westerhuis et al. (*52*).

The observations made on the present LH1 reconstitution system are also suggestive as to a possible mechanism of the cooperative effect of Crt on the LH1 formation. Probably, the interactions of Crt molecules with the $\alpha\beta$-BChla units cause structural rearrangements of the latter and an exposure of some hydrophobic amino acid residues in the transmembrane domains of the α and β polypeptides. The cooperative (recognition) effect originates then from the increased hydrophobicity of this (short-lived) Crt-binding intermediate species. This is supported by the fact that the LH1 complex can be formed with pure Neu and Lyc (hydrocarbons with no functional groups). Although various types of interactions play roles in the assembly of the antenna, the interactions of a hydrophobic character (and perhaps the $\pi-\pi$ stacking) would be the major interactions responsible for the cooperative effect and are sufficiently strong for the formation of a stable LH1 complex. The cooperative effects are enhanced and an additional stabilization of the complex occurs, for example, when the side functionalities of the Crt molecule are able to form hydrogen bonds to the amino acids residues of α and β polypeptides.

Tuning Effects of Crts in LH1 Complex. The series of model LH1 complexes, in which various Crts are inserted into identical pigment−polypeptide, provides a unique opportunity to check for the correlations between the Crt structure and LH1 spectral features (Figure 4, Table 1). The comparison of the absorption spectra of model LH1 complexes reveals that the extent of complementing the BChl light harvesting by Crts depends mostly on the conjugation size: the greater the conjugation size, the better the coverage of the green part of the spectrum. Furthermore, upon binding in the complex, the Crt absorption transitions experience a significant red shift, which ranges from 570 to 800 cm^{-1} (15−23 nm, Table 1) and places these intense transitions closer to the weaker BChl Q_x transition. For the conjugation length $n = 9-11$, this shift in LH1 does not exceed 670 cm^{-1}, whereas for $n = 12$ and 13, the shifts are noticeably larger, above 720 cm^{-1}. Two effects may in parallel underlie this apparent structural dependence. The Crts in the series, along with increasing conjugation size, possess more functional side groups, which are able to participate in a larger number of (stronger) interactions in the complex. In addition, the greater conjugation size renders the Crt molecule more polarizable and thus more sensitive to the environmental effects (*53*, *54*).

As yet another tuning effect in LH1, the Crt-induced 10 nm red shift of the BChla lowest energy transition may be considered. The exact origin of this shift remains unclear and at least two effects are usually taken into account: either enhanced BChl−BChl interactions due to the bound Crt and/or Crt−BChl−polypeptide interactions. In the model LH1 complexes, the BChl Q_y transition shifts to 880−883 nm and appears not to depend much on the Crt structure (Table 1), therefore indicating that the 10 nm red shift is not likely to originate mainly from the direct Crt−BChl interactions. However, as observed by Picorel et al. (*35*) and noted as well in our experiments, the position of the Q_y transition in LH1 is detergent-sensitive and the values of the shift must be interpreted with caution.

Relevance to the Assembly and Functioning of LH1 Antenna in Vivo. The Crts are not as essential for the formation of the LH1 antennae as for the assembly of the LH2 complexes, since both in vivo and in vitro the carotenoidless LH1 complexes seem correctly assembled and remain functional, and mainly their photo- and to some extent thermodynamic stability is affected by the absence of Crts. The Crts, however, promote cooperative aggregation of the antenna subunits and strongly enhance the antenna formation. Apparently, these pigments may also contribute an important driving force for the assembly of the photosynthetic apparatus in vivo. This would explain why, in the native photosynthetic membranes, at various stages of their development, the "free" intermediates (subforms) of LH1 complexes are practically never observed (*10*, *11*).

Upon the binding of Crt into the complex, as discussed above, the tuning effects of the Crt and BChl spectral properties are observed, which may result in the optimization

of the LH functions. The complex-bound Crt transition bands are red-shifted (15−23 nm) and thus their overlap with the BChl Soret band is decreased, resulting in a more effective light absorption by Crts in the green-yellow region of the spectrum. Considering the light conditions in most niches inhabited by photosynthetic bacteria, this might be an important factor. The antenna BChls also undergo some tuning; the bound Crt induces the 10 nm red shift of the antenna Q_y transition, which renders this transition a better excitation sink, shallower by 130 cm^{-1}.

It seems tempting to regard the accumulation of Crts differing in conjugation length in photosynthetic membranes of the photosynthetic bacteria as one of the evolutionary mechanisms of spectral tuning of their antenna absorption to the visible light conditions, in analogy to the chromatic adaptation known in cyanobacteria. In addition, the structural modifications of Crts seem to enhance their ability to stabilize the photosynthetic pigment−protein complexes. However, it was shown that the LH1 complexes binding Crts with short conjugation size, such as Sph in *Rb. sphaeroides*, are characterized by high yields of energy transfer, up to 90%, whereas quite low efficiencies, on the order of only 20−30%, were found for the antennae containing, for example, Spx (*Rsp. rubrum*) (*18*, *32*). It seems that the overall enhancement of light harvesting achieved by the bacterium due to the spectral tuning to the green region of the solar spectrum, via the enlargement of the conjugation size in the light-harvesting Crt, counterbalances a considerable drop in the efficiency of energy transfer from Crt to BChl.

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