Isolation of the PufX Protein from *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*: Evidence for Its Interaction with the α-Polypeptide of the Core Light-Harvesting Complex†

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ABSTRACT: Using mutant strains of *Rhodobacter capsulatus* and *Rhodobacter sphaeroides* in which the *pufX* gene had been deleted, it was possible to identify by HPLC membrane protein components present in *pufX*$^+$ cells but absent in *pufX*$^-$ cells. In parallel preparations, membrane proteins soluble in chloroform/methanol containing ammonium acetate were first extracted from lyophilized membrane fractions of the *pufX*$^+$ cells and separated from pigments and larger protein material by gel-filtration chromatography. Protein-containing fractions were examined by HPLC, and several peaks were collected from *pufX*$^+$ material that were not present in *pufX*$^-$ material. From N-terminal amino acid sequencing, the PufX protein of *Rb. capsulatus* was identified, and from positive interaction with a PufX protein antibody, the *Rb. sphaeroides* PufX protein was identified. Although overall yields were very small, sufficient quantities of these proteins were isolated to evaluate their effect on the reconstitution of the core light-havesting antenna (LH1) and its subunit complex. From the behavior of the PufX protein and the α-polypeptide of LH1 on HPLC, qualitative evidence was obtained that the two proteins have a high affinity for each other. In reconstitution assays with bacteriochlorophyll (Bchl) and the LH1 α- and β-polypeptides of *Rb. capsulatus*, the PufX protein of *Rb. capsulatus* was inhibitory to LH1 formation at low concentration. A similar inhibition was exhibited by *Rb. sphaeroides* PufX protein for reconstitution of LH1 with Bchl and the LH1 α- and β-polypeptides of *Rb. sphaeroides*. In both cases, the ratios of concentrations of the PufX protein to the α-polypeptide causing 50% inhibition were approximately 0.5. Formation of the heterologous (αβ) subunit-type complex formed with Bchl and the α- and β-polypeptides of LH1 of *Rb. capsulatus* was also inhibited by low concentrations of the *Rb. capsulatus* PufX protein (approximately 50% inhibition at PufX:α-polypeptide ratios = 0.5). However, neither PufX protein inhibited formation of a homologous (ββ) subunit-type complex, which indicates that the PufX proteins do not interact with the β-polypeptides.

The photosynthetic apparatus of purple bacteria consists of two different types of pigment−protein complexes: the reaction center (RC) and the light-harvesting (LH) complexes. The main function of the LH complex is to absorb light and to transfer the excited-state energy to the RC where the primary photochemical events occur (*1*−*4*). In the RC, a charge separation across the membrane occurs, and an oxidant (ferric cytochrome *c*) and a reductant (ubihydroquinone) are produced that are substrates for secondary electron and proton transport in the bc_1 complex. These two supramolecular complexes, the RC and the bc_1 complex, working in tandem produce an electrochemical gradient of protons that drives ATP synthesis (*3, 4*).

All photosynthetic bacteria contain a core light-harvesting complex (LH1), and many also contain accessory LH complexes (e.g., LH2). The core light-harvesting complex (LH1) is composed of multiple bacteriochlorophyll *a* molecules (Bchl) that are held rigidly in place by two polypeptides, α and β, each about 6 kilodaltons. Analyses of amino acid sequences and topographical studies indicate the LH1 polypeptides span the membrane once with the C-terminus outside and the N-terminus inside (*2*). On the basis of recent crystal structural information on the closely related LH2 complexes (*5, 6*) and electron cryomicroscopic studies of the isolated LH1 complex of *Rhodospirillum rubrum* (*7*), it has been proposed that LH1 forms a ring around the RC (*7*). Such a ring would block exchange of ubihyroquinone reduced in the RC with free ubiquinone of the membrane

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(Q-pool) and, consequently, stop light-driven electron transport (*3, 4*). In *Rb. capsulatus* and *Rb. sphaeroides*, another protein, encoded for by the *pufX* gene, seems to be important in facilitating this exchange between ubihydroquinone of the RC and ubiquinone of the Q-pool (*8*−*13*).

The *puf* operon of purple bacteria contains the structural genes for the α- and β-polypeptides of LH1, the structural genes for the L- and M-polypeptides of the RC, the *pufQ* gene involved in pigment biosynthesis, and in *Rhodobacter capsulatus* and *Rhodobacter sphaeroides*, the *pufX* gene (*14*−*16*) whose protein product has not previously been isolated or characterized. In *Rb. capsulatus*, the levels of transcript encoding the PufX protein are about one-tenth the amount of that encoding the α- and β-polypeptides of LH1 (*17*). The *pufX* gene sequences in *Rb. capsulatus* and *Rb. sphaeroides* indicated that these polypeptides have 78 and 82 amino acids and are 8.5 and 9.0 kilodaltons, respectively (*14*−*17*). Upon comparison of the amino acid sequences (based on the nucleotide sequences of the genes), the proteins would have only a 20% identity in amino acid sequence. Inspection of the assumed amino acid sequences suggests there may be one membrane span located in the middle of each sequence, similar to those of the α- and β-polypeptides of LH1, but it is still unknown whether the *pufX* gene product actually spans the membrane (*18, 19*).

The importance of PufX protein in bacterial photosynthesis became evident when it was found that deletion of *pufX* caused changes in the LH1 content (*20*) and also resulted in photosynthetic incompetence (*21*). Moreover, adding *pufX* back to *pufX*$^-$ strains resulted in a recovery of photosynthetic competence (*22*). Furthermore, supressor mutants of *Rb. capsulatus* and *Rb. sphaeroides* lacking the *pufX* gene were shown to have mutations in the α- and β-polypeptides of LH1, apparently compensating for loss of PufX (*23*−*25*). In that work, it was suggested that point mutations in LH1 somehow compensate for the loss of PufX by structurally rearranging the photosynthetic unit and, thus, allowing ubiquinone/ubihydroquinone exchange between the reaction center and the bc_1 complex. These results suggest that PufX plays an essential role in bacterial photosynthesis. Moreover, "RC only" mutants (lacking LH) were found to be photosynthetically competent even when the *pufX* gene was deleted, suggesting the LH to be somehow connected to the function of PufX (*11*). Spectroscopic studies have shown that while PufX is essential for light-induced ATP synthesis, it is not required for a single-turnover photochemistry under anaerobic conditions. It is only under prolonged illumination that PufX is apparently required for optimal electron and proton transport (*13*). These results have led to the suggestion that PufX is involved in preventing LH1 from blocking the shuttling of ubiquinone/ubihydroquinone between the Q-pool and the RC (*13, 25*).

This paper describes the isolation of PufX protein from membranes of *Rb. capsulatus* and *Rb. sphaeroides*, which is the first report of the isolation of these important proteins. Reconstitution methodology (*26*−*28*), which has provided a powerful tool for probing structure−function relationships in LH1 polypeptides (*29*−*33*), was used to examine the effect of the PufX protein on LH1 formation. Initial characterization of these PufX proteins indicates they have a very high affinity for the α-polypeptides of these organisms, and it is shown that, as a result of this interaction, formation of the LH1 complexes was markedly inhibited by the PufX protein.

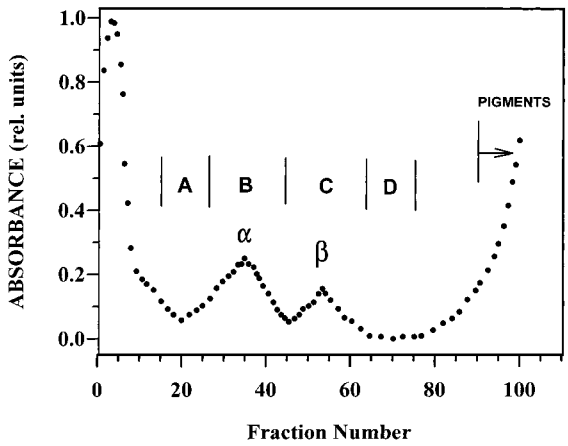

FIGURE 1: Sample of lyophilized membranes from *Rb. capsulatus* U43 mutant (PTB999) was extracted with $CHCl_3/CH_3OH$ (1:1) containing 0.1M NH_4OAc and separated on a Sephadex LH60 column using the same solvent. Fractions were collected beginning when material came off at the void volume (fractions 1−7) and continued through the beginning of pigment elution (fractions 80−100). Fractions where the α- and β-polypeptides eluted are indicated. The fractions in the regions labeled A, B, C, and D were pooled and subjected to HPLC.

MATERIALS AND METHODS

n-Octyl β-D-glucopyranoside (OG) was obtained from Sigma Chemical Co. All solvents were obtained from Burdick and Jackson and were HPLC grade. Hexafluoroacetone trihydrate (HFA) was purchased from Aldrich Chemical Co. Bchl was isolated from the R26 mutant of *Rb. sphaeroides* or the G-9 mutant of *Rs. rubrum* as previously described (*34, 35*) and modified as by Meadows et al. (*30*).

The *Rb. capsulatus* U43 mutants containing *pufX* (PTB999) and lacking *pufX* (pTL2) (*9, 25*) were used in the isolation of the *Rb. capsulatus* PufX protein. *Rb. sphaeroides* mutants RCO1X(+) and RCO1(X−) (*36*) were used in the isolation of the *Rb. sphaeroides* PufX protein. The *Rb. capsulatus* cells were grown on RCV medium (*37*), and the *Rb. sphaeroides* cells were grown in the dark on M22+ medium (*36*). Chromatophores were prepared, washed, and lyophilized to dryness in preparation for extraction with $CHCl_3/CH_3OH$ (1:1) containing 0.1 M ammonium acetate. Material dissolved in the extracting solvent was applied to an LH60 column at 4 °C, and fractions were eluted with the same solvent system (Figure 1). Several corresponding column fractions of material from each mutant were dialyzed and lyophilized to dryness. Polypeptides in these fractions were dissolved in HFA and analyzed by HPLC using a reverse-phase C18 column and a gradient as previously described (*30*). The peaks present in the material from *X*$^+$ cells, that were not present in corresponding fractions of material from *X*$^-$ cells, were collected and analyzed for their N-terminal amino acid sequence (*Rb. capsulatus*) or tested with an antibody to PufX protein from *Rb. sphaeroides* (*38*).

N-terminal amino acid sequence determinations were performed by the Department of Biochemistry and Microbiology, University of Victoria, British Columbia, and by the Structural Facility at Michigan State University. The identity of the *Rb. sphaeroides* PufX protein was determined

by ELISA assay using an antibody to PufX (*38*). Reconstitution assays and calculations of association constants for subunit formation were carried out as previously described (*26*, *27*, *30*). The concentration of the PufX protein was determined by measuring its UV absorption spectrum in HFA and calculating its concentration assuming an extinction coefficient at 287 nm = 3400 M^{-1} cm^{-1} per Trp residue (*27*).

HPLC was performed using a Waters system consisting of a Model 501 Solvent Delivery System, a system interface module, a 486 tunable absorbance detector, a U6K injector, and a Waters Baseline 810 chromatography software package. Absorption spectra were recorded with a Shimadzu UV-160 spectrophotometer interfaced to a microcomputer. To reduce the effects of scattered light, opal glass was placed between the sample and the detector. CD spectra were recorded at room temperature (usually 23 °C) using a Jasco J500C spectropolarimeter interfaced to a microcomputer. SDS−PAGE was performed with the PhastGel System. Following separation technique file number 120 in the owners manual, 2 μg of polypeptides in 1 μL of sample buffer was loaded onto a prepoured PhastGel gradient 8-25 (6−300 kDa) gel and run on an optimized voltage gradient program. After separation, the gels were stained either by Coomassie blue or by silver treatment and destained overnight.

RESULTS

Because the nucleotide sequence of the *pufX* genes indicated that the PufX proteins possess polar N- and C-terminal regions with nonpolar central regions, similar to the α- and β-polypeptides of the LH complexes, it was anticipated that the PufX protein would similarly dissolve in organic solvent. Initial extractions of lyophilized membranes from *pufX*$^+$ cells and *pufX*$^-$ cells seemed to confirm these expectations. Therefore, the first few steps commonly employed for isolation of LH polypeptides (as described under Materials and Methods) were used in each PufX protein isolation procedure.

Isolation of the PufX Protein of Rb. capsulatus. The PufX protein from *Rb. capsulatus* was identified by comparing the HPLC profile of *Rb. capsulatus pufX*$^+$ material taken from the LH60 column compared with the *pufX*$^-$ material taken from a similar LH60 column fraction (Figure 2A). Protein in material from *Rb. capsulatus pufX*$^+$ cells that was not present in the *pufX*$^-$ cells seemed to primarily be found in the LH60 column fractions that also contained the α-polypeptide. The major HPLC peaks found in *pufX*$^+$ material but not found in *pufX*$^-$ material in the 10−20 min region were collected and subjected to N-terminal amino acid sequencing. An expansion of the 5−20 min region (with a somewhat different gradient) shows the location of the PufX protein (see Figure 2B). The UV spectrum of the material in this 12.9 min peak was typical of protein, and its N-terminal amino acid sequence was the same as that predicted from the *pufX* gene sequence. Two different preparations of this protein were submitted for sequencing over a 3 year period. The first results obtained with a very small amount of sample showed that the sequence began at the second residue, indicating the original N-terminal methionine was absent. The N-terminal sequence determined was

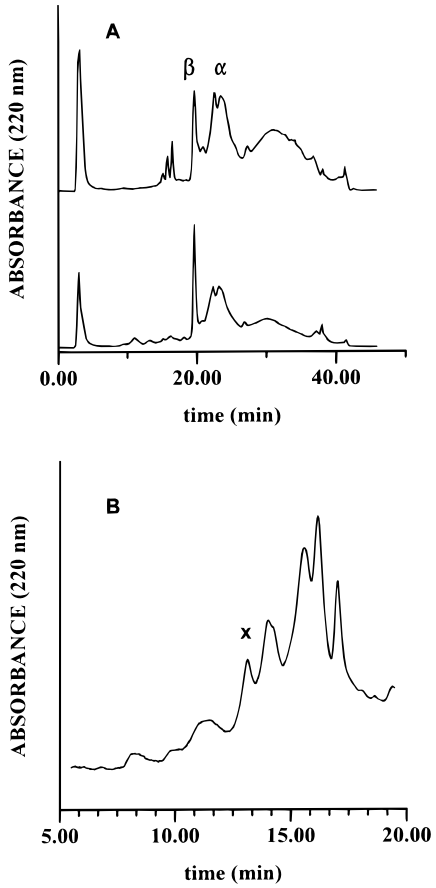

FIGURE 2: (A) HPLC profile of fraction B material from the LH60 column shown in Figure 1. The top trace is for material isolated from *pufX*$^+$-containing cells while the bottom trace is for corresponding material from *pufX*$^-$ cells. (B) Expansion of the 5−20 min region of the top trace of (A) showing the location of the PufX protein.

"SMFD-PFDYENGS...", which matched the known *pufX* gene product sequence (*15*) except that the fifth residue, Lys, was not determined. The later results obtained with a substantially larger sample gave the sequence SMFDK..., which agreed exactly with the first five amino acids (beginning after the N-terminal Met residue encoded by the *pufX* gene). In SDS−PAGE, this material ran as a single band slightly slower than the *Rb. capsulatus* α-polypeptide (data not shown).

Several factors contribute to the small quantities of material obtained. The ratio of the PufX protein peak to the α-polypeptide peak in HPLC runs is similar to the mRNA stoichiometry in which the concentration of mRNA for *Rb. capsulatus pufX* was only about 10% that of the *Rb. capsulatus* LH1 α- or β-polypeptide (*17*). Furthermore, the yields for HPLC isolation, based on the expected content of the starting material, ranged from 5 to 15% recovery for the α-polypeptide, but only 0.25−5% for the PufX protein. This very poor yield was not overcome by scaling-up HPLC runs because the PufX protein seemed to aggregate at higher concentrations as well as associate with the α-polypeptide. A typical yield was 0.05 mg of PufX protein per liter of cell culture. Isolating as much as 0.5 mg of PufX protein, which is enough for about 4−6 reconstitution experiments, is therefore quite labor-intensive. Attempts to separate α-polypeptide and the PufX protein-containing material on

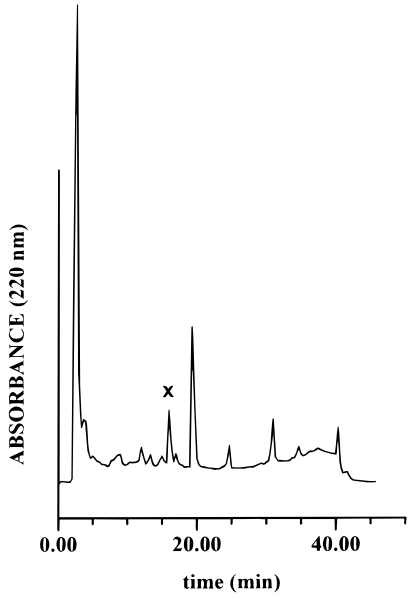

FIGURE 3: HPLC profile of LH60 column fractions pooled between the void volume and the pigment bands for material extracted from membranes of *Rb. sphaeroides* RCO1X$^+$ cells containing the *pufX* gene. The location of the PufX protein is indicated.

a reverse phase C-4 column resulted in higher yields, but did not resolve the PufX protein adequately.

Isolation of the PufX Gene Product of Rb. sphaeroides. An approach similar to that used to isolate the PufX protein from *Rb. capsulatus* was applied to *Rb. sphaeroides*. In this case, however, the two mutants used were LH1(−)LH2(−), which aided in the isolation process. HPLC analysis of the peaks found in LH60 column fractions from *pufX*(+) material is shown in Figure 3. The fraction marked X was found to strongly interact with the PufX antibody and was collected from multiple HPLC runs until enough sample was obtained for testing in reconstitution assays.

Effect of Rb. capsulatus PufX Protein on Reconstitution of LH1. In comparison to reconstitution of *Rs. rubrum* LH1, the reconstitution of LH1 of *Rb. capsulatus* was not as quantitative (*39*). Instead of equivalent concentrations of the α- and β-polypeptides, it typically required 2−4 times the amount of *Rb. capsulatus* α-polypeptide relative to β-polypeptide in order to approach complete formation of LH1. As is the case for the β-polypeptides of *Rs. rubrum* and *Rb. sphaeroides*, the *Rb. capsulatus* β-polypeptide alone readily forms a homologous (ββ) subunit-type complex (Figure 4A) so that the β-polypeptide seems to function well. It was also observed that in heterologous reconstitution experiments in which the β-polypeptide of *Rb. capsulatus* was paired with the α-polypeptide of *Rs. rubrum*, quite good formation of an LH1-type complex was obtained (*27*). Because the PufX protein migrated with the *Rb. capsulatus* LH1 α-polypeptide on the LH60 column and because it was necessary to inject very low concentrations of this LH60 material on HPLC in order to separate the PufX protein from the α-polypeptide, it seemed possible that the PufX protein was present as a contaminant in the α-polypeptide fraction and might be interfering with reconstitution of LH1. It was found that when the *Rb. capsulatus* α-polypeptide was further purified by HPLC using a very low concentration of the LH60 α-polypeptide fraction, and then utilized in the reconstitution system, not only was a subunit complex more

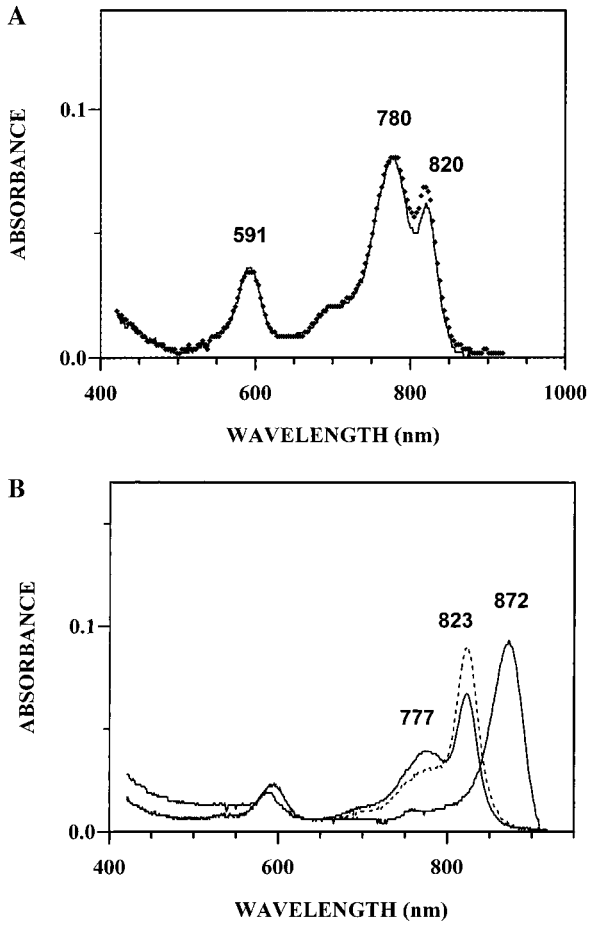

FIGURE 4: (A) Reconstitution of a homodimeric (ββ) subunit-type complex in 0.90% OG with Bchl and the β-polypeptide of *Rb. capsulatus* in the presence (◆) and absence (solid curve) of PufX protein isolated from *Rb. capsulatus*. Concentrations of Bchl, β-polypeptide, and PufX protein were 1.5 μM, 2.9 μM, and 2.6 μM, respectively. (B) Reconstitution of the subunit complex and LH1 with Bchl and the α- and β-polypeptides of *Rb. capsulatus* formed at 0.90% OG (solid curve with λ_{max} values = 823 and 777 nm), 0.75% OG (dashed curve), and the last sample diluted to 0.66% OG and chilled overnight at 5 °C (solid curve with λ_{max} = 872 nm). The concentrations of Bchl and the α- and β-polypeptides in 0.90% OG were 1.4 μM, 2.6 μM, and 2.9 μM, respectively.

readily formed, but so also was an LH1-type complex (Figure 4B).

When such an HPLC-purified α-polypeptide was used for reconstitution along with the *Rb. capsulatus* β-polypeptide, the addition of HPLC-purified PufX protein had a substantial effect on the formation of both subunit- and LH1-type complexes. Addition of increasing amounts of the PufX protein resulted in decreasing levels of formation of a subunit-type complex, especially at 0.90% OG (Figure 5A). The concentration ratio of the PufX protein to the α-polypeptide at which 50% inhibition of subunit formation was observed was 0.5. The inhibitory effect could be decreased by lowering the OG concentration, apparently favoring subunit formation (Figure 5B,C). Formation of LH1 from the subunit complex was also greatly inhibited, even when the subunit-type complex had been well-formed (Figure 6A,B). The concentration ratio of PufX protein to the α-polypeptide at which 50% inhibition was observed was again approximately 0.5 for LH1 formation after chilling the sample for 1 h (Figure 6A).

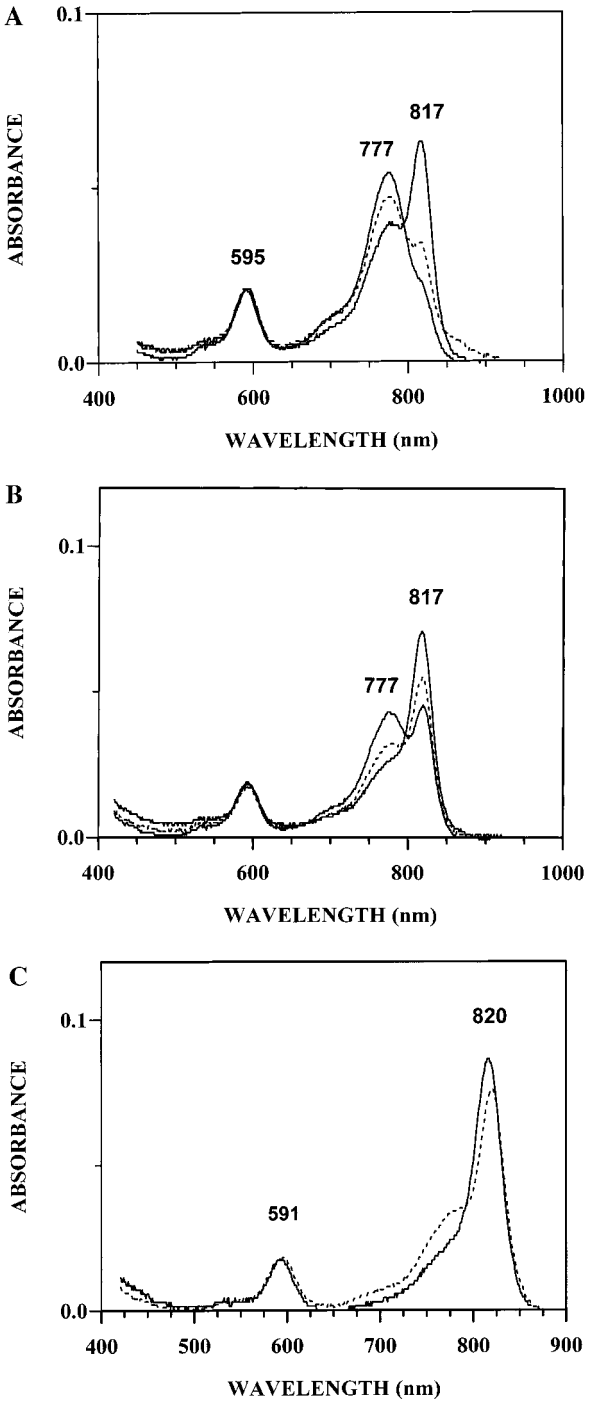

FIGURE 5: (A) Reconstitution of the subunit complex at 0.90% OG with Bchl and the α- and *β*-polypeptides of *Rb. capsulatus* in the presence of 0 μM (solid curve with λ_{max} = 817 nm), 1.2 μM (dashed curve), and 2.4 μM (solid curve with λ_{max} = 777 nm) PufX protein. The concentrations of Bchl and the α- and *β*-polypeptides were 1.4 μM, 2.7 μM, and 2.8 μM, respectively. (B) Absorption spectra of the same samples as for (A) except that the OG concentration was 0.75%. (C) Absorption spectra of the same samples as for (A) except that the OG concentration was 0.66%. Results at 0 and 1.2 μM PufX protein (solid curve) were identical; 2.4 μM, dashed curve.

It was important to establish that PufX protein interacts specifically with the reconstitution system and not just as a nonspecific, membrane protein which might have interfered in some way with subunit and LH1 formation. Accordingly, we used the *Rhodopseudomonas viridis* LH1 α-polypeptide as a control; we have previously shown that the *Rps. viridis*

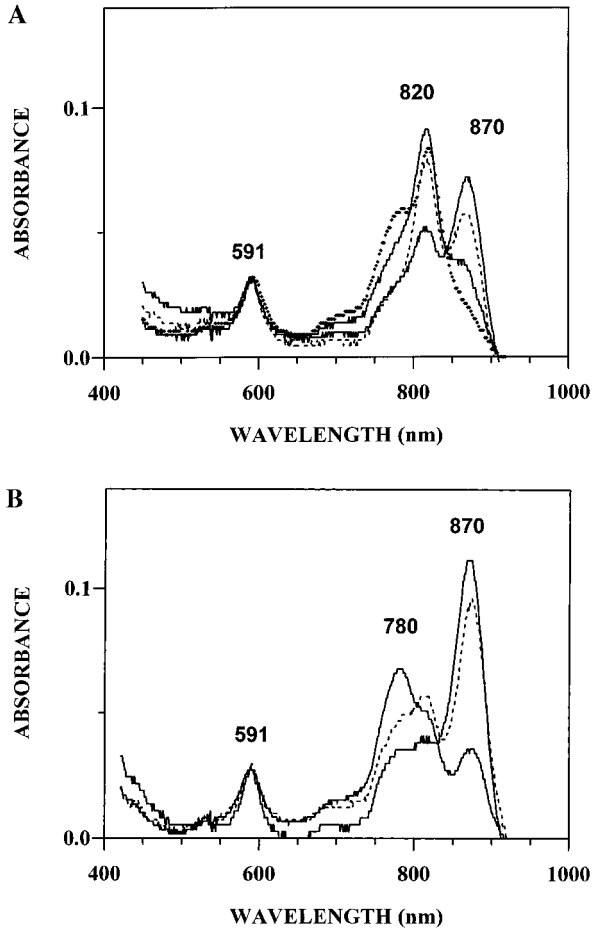

FIGURE 6: (A) Reconstitution of an LH1-type complex with Bchl and the α- and *β*-polypeptides of *Rb. capsulatus* in the presence of PufX protein of *Rb. capsulatus* at 0.66% OG after chilling the samples on ice for 1 h. The concentrations of Bchl and the α- and *β*-polypeptides were 1.0 μM, 2.0 μM, and 2.1 μM, respectively. The samples contained 0 μM (solid curve with λ_{max} values = 870 and 820 nm), 0.5 μM (dashed curve), 1.2 μM (solid curve with λ_{max} = 820 nm), and 2.4 μM (◆) PufX protein. (B) Absorption spectra of the same samples as for (A) chilled overnight. 0 μM (solid curve with λ_{max} = 870 nm), 1.2 μM (dashed curve), and 2.4 μM (solid curve with λ_{max} values = 870 and 780 nm) PufX protein.

α-polypeptide, which is an integral membrane protein, will not interact with the LH1 *β*-polypeptide of *Rb. sphaeroides*, *Rb. capsulatus*, or *Rs. rubrum* to form LH1 and will not form red-shifted complexes by itself (*27*). Reconstitution of Bchl with *Rb. capsulatus* LH1 α- and *β*-polypeptides in the presence of the *Rp. viridis* LH1 α-polypeptide showed no inhibition of LH1-type complex formation (data not shown). Other experimental controls of this type utilized the α-polypeptide of *Rb. sphaeroides* LH2, which was not active in heterologous reconstitution experiments with Bchl and the *β*-polypeptide of *Rb. sphaeroides* LH1, and was also without effect on the formation of the subunit and LH1-type complexes in the system containing Bchl and the α- and *β*-polypeptides of *Rb. sphaeroides* LH1. Also, we tested water-soluble proteins, such as cytochrome *c*, and found that they had no effect on the reconstitution of subunit and LH1-type complexes with the α- and *β*-polypeptides of LH1 (data not shown).

Effect of Rb. sphaeroides PufX Protein on the Reconstitution of LH1. In reconstitution experiments using only the *Rb. sphaeroides β*-polypeptide to form a homologous (*ββ*)

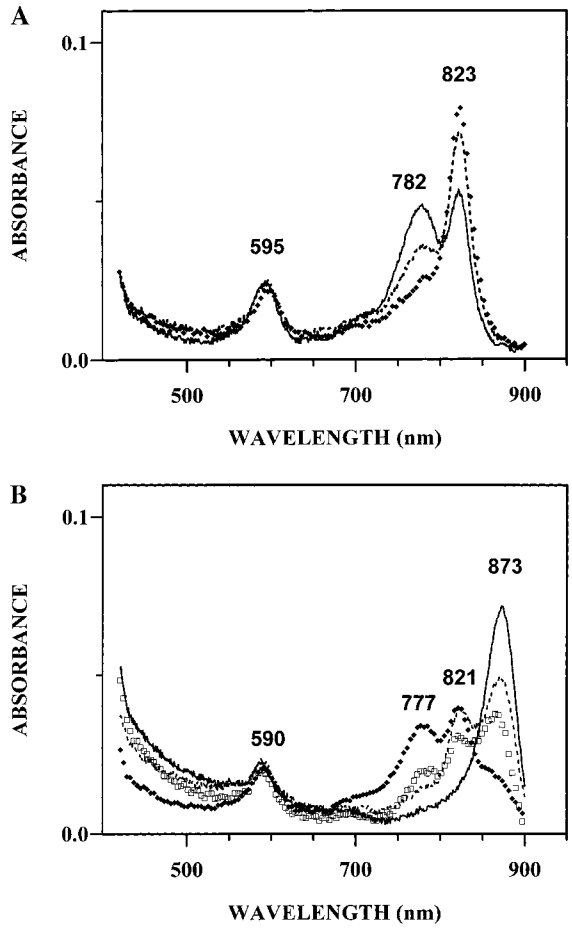

FIGURE 7: (A) Reconstitution of the subunit complex with Bchl and the α- and *β*-polypeptides of *Rb. sphaeroides* in the presence of the PufX protein of *Rb. sphaeroides* at 0.90% (solid curve), 0.75% (dashed curve), and 0.66% OG (◆). The concentrations at 0.90% OG were 1.5 *μ*M Bchl, 2.3 *μ*M α-polypeptide, 2.7 *μ*M *β*-polypeptide, and 2.6 *μ*M PufX protein. (B) Reconstitution of an LH1-type complex with Bchl and the α- and *β*-polypeptides of *Rb. sphaeroides* in the presence of the PufX protein of *Rb. sphaeroides* at 0.66% OG after chilling overnight at 4 °C. Concentrations of Bchl and the α- and *β*-polypeptides were the same as for the experiment shown in (A) with 0 *μ*M (solid curve), 0.5 *μ*M (dashed curve), 1.2 *μ*M (□), and 2.4 *μ*M (◆) PufX protein.

subunit-type complex, addition of the *Rb. sphaeroides* PufX protein was without effect (data not shown). This result is similar to that observed in reconstitution experiments with only the *β*-polypeptide of *Rb. capsulatus* (Figure 4A). Unlike the results with the α- and *β*-polypeptides of *Rb. capsulatus*, the *Rb. sphaeroides* PufX protein did not inhibit reconstitution of a subunit-type complex in the reconstitution system containing the *Rb. sphaeroides* α- and *β*-polypeptides (Figure 7A). However, it should be noted that because the *β*-polypeptide of *Rb. sphaeroides* readily forms a homodimer (*ββ*) subunit-type complex with a larger association constant than the analogous complex formed with the *β*-polypeptide of *Rb. capsulatus* (*40*), the lack of effect of the PufX protein on subunit formation in *Rb. sphaeroides* (Figure 7A) was likely due to the preferential formation of homodimer (*ββ*) subunit-type complex.

In the reconstitution system with Bchl and the *Rb. sphaeroides* α- and *β*-polypeptides, the formation of an LH1-type complex was markedly inhibited by the PufX protein (Figure 7B). The concentration ratio of the PufX protein to α-polypeptide causing 50% inhibition was approximately 0.5. Thus, this result is analogous to that obtained for the effect of the *Rb. capsulatus* PufX protein on LH1 formation with the *Rb. capsulatus* α- and *β*-polypeptides.

It should be noted that this experiment was conducted with HPLC-purified *Rb. sphaeroides* α-polypeptide to minimize the possible effect of PufX protein copurifying with the α-polypeptide through the LH60 column step in the isolation procedure. Because a mutant of *Rb. sphaeroides* was available which was LH1(+)X(−), an important control experiment could be conducted. The LH1 α-polypeptide was isolated from this mutant and tested in reconstitution experiments with Bchl and the native *β*-polypeptide. The results were essentially identical to the reconstitution experiments using the HPLC-purified α-polypeptide from puc705BA (*27*) which was used for the experiment whose results are shown in Figure 7. Thus, *Rb. sphaeroides* PufX protein seems to be satisfactorily removed from the LH1 α-polypeptide during HPLC purification at very low protein concentrations.

Assays were also conducted to examine whether the PufX protein of *Rb. sphaeroides* would affect the reconstitution of the subunit- and LH1-type complexes of *Rs. rubrum*. In reconstitution experiments with Bchl and the α- and *β*-polypeptides of *Rs. rubrum*, no significant effect was observed upon adding the PufX protein of *Rb. sphaeroides* (data not shown).

Also of interest is whether the PufX protein from *Rb. capsulatus* would cause inhibition of the formation of the subunit- and LH1-type complexes of *Rb. sphaeroides*, and vice versa. Only a relatively small effect (approximately 15% that observed with native systems) was observed by PufX protein in this kind of cross-reconstitution assay (data not shown). This is perhaps consistent with the low amino acid sequence identity (approximately 20%) between the PufX proteins of these two bacteria. Because of limited availability of PufX proteins, as well as poor solubility of the PufX proteins, these assays could not be conducted at higher concentrations of PufX.

Circular Dichroism Measurements. Although the α- and *β*-polypeptides of LH1 have been reported to have a high α-helical content (*26*, *41*−*43*) and the crystal structures of LH2 (*5*, *6*) and the RC show very high α-helical contents, the amino acid sequence of the PufX gene product indicates that the PufX protein would probably not contain extensive α-helical structure because of its high content of glycine and proline (Figure 8). Measurement of the CD spectra of the *Rb. capsulatus* and *Rb. sphaeroides* PufX proteins indicated that they do indeed have a much smaller fraction of α-helix per mole than the α- or *β*-polypeptides of LH1 (data not shown). Using an approximate calculation for the percent α-helix (*44*), the PufX proteins contain only 19% (*Rb. capsulatus* PufX) or 20% (*Rb. sphaeroides* PufX) α-helix compared with 56% for the α-polypeptide of LH1. Addition of the α- and *β*- polypeptides together with the PufX protein resulted in a simple addition of the spectra without evidence of change in α-helical content (data not shown). Thus, any interaction between polypeptides does not significantly affect the α-helical content of the polypeptides.

DISCUSSION

The PufX protein has been isolated from membrane fractions of two photosynthetic bacteria, *Rb. capsulatus* and

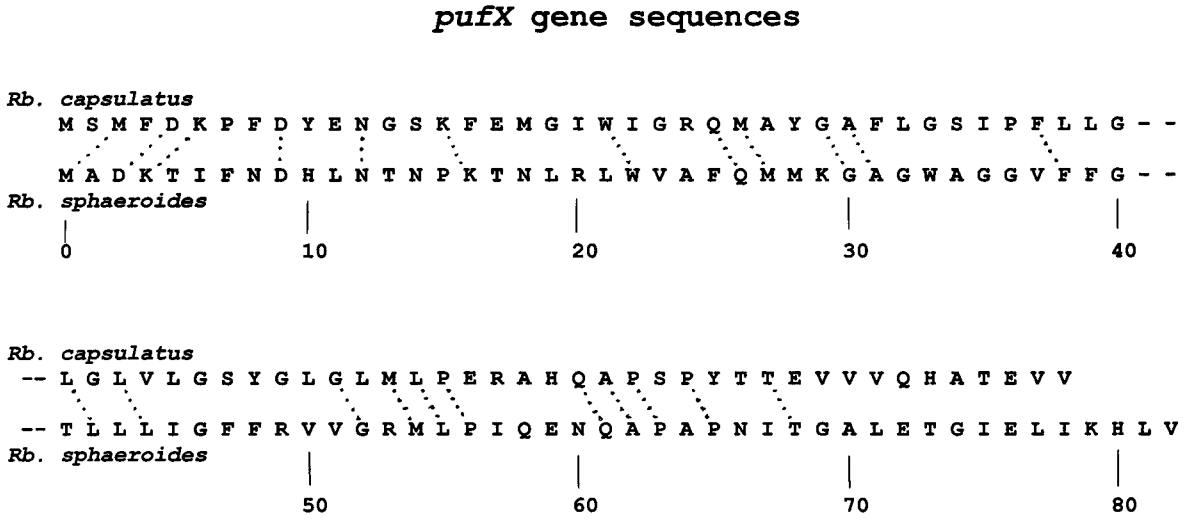

FIGURE 8: Amino acid sequence of the PufX proteins of *Rb. capsulatus* and *Rb sphaeroides* according to the nucleotide sequence of the corresponding *pufX* genes (*14*, *16*). Dotted lines indicate possible identities in amino acid sequence if the polypeptides were correspondingly aligned.

Rb. sphaeroides. From HPLC profiles of *Rb. capsulatus* membrane fractions, PufX protein appears to be present in approximately 1 copy per 10 copies of the LH1 polypeptides, which is about the same ratio as RC polypeptides/LH1 polypeptides and is consistent with the relative amounts of mRNA transcribed from the encoding genes. The PufX protein from both sources exhibited very low solubility in most solvents, and HPLC chromatographic yields were quite low. Thus, obtaining sufficient quantities for characterization was tedious. Eventual expression of the gene in *E. coli* or some other host to obtain larger quantities could be very helpful in this regard, although obtaining larger quantities of the purified protein may still be problematical. It is also possible that the PufX protein can be chemically synthesized, although achieving this for a 78 or 82 amino acid polypeptide which is not soluble in water may be a challenge. Even if larger quantities of PufX proteins become available by such means, it is still of considerable importance to isolate the protein from the organism in its native state so that its unique properties and relationships to the other components (e.g., LH1, LH2, RC, bc_1) may be ascertained.

Two results obtained in these studies indicate that PufX protein specifically associates with the LH1 α-polypeptide. First, the two proteins consistently comigrate during isolation procedures, especially at higher protein concentrations. Second, reconstitution of LH1, a process requiring the α-polypeptide in both the *Rb. capsulatus* and *Rb. sphaeroides* systems, was markedly inhibited by the PufX protein in both cases (Figures 6A,B and 7B), whereas the formation of the homologous ($\beta\beta$) subunit-type complex in both systems was unaffected by the presence of the PufX protein.

Because the reconstitution assay is, by its nature, conducted in the presence of detergent and utilizes conditions allowing progressive association of components, great care must be taken to guard against the effects of nonspecific association. In this regard, several experimental controls were conducted, the results of which may be summarized as follows. The presence of membrane-spanning polypeptides that should have no specific interactions with the α- and β-polypeptides of LH1 had no effect on the reconstitution of LH1. Second, addition of the *Rb. sphaeroides* PufX protein to Bchl and the α- and β-polypeptides of *Rs. rubrum* showed no significant effect on LH1 formation (a comparable *pufX* gene has not yet been observed in this bacterium). Globular proteins that are water-soluble, such as cytochrome *c*, did not affect the reconstitution system. Finally, and very importantly, reconstitution of homodimer ($\beta\beta$) subunit-type complexes is not inhibited by PufX protein, indicating that the β-polypeptide and the PufX protein do not interact.

The CD spectrum of the PufX protein is of interest in terms of structural predictions based on the nucleotide sequence of the PufX genes (*14*, *16*). If there were a membrane-spanning α-helical segment in the middle of the PufX protein, as has been suggested (*18*, *19*), according to our CD data there is only 19% α-helical structure in the *Rb. capsulatus* PufX protein compared with 56% for the LH1 α-polypeptide. Thus, the amount of α-helix indicated for the PufX protein would involve only about 16 amino acids. This is a little short for a membrane-spanning α-helix where one would expect about 20−23 amino acids. Moreover, there are seven Gly residues in this middle section of the protein (between amino acid residues 29 and 52; see Figure 8), suggesting a rather unusual structure, perhaps involving many turns or a U-shaped membrane anchor that extends only partway into the membrane.

CONCLUSIONS

For both the *Rb. capsulatus* and *Rb. sphaeroides* systems, evidence is provided that a specific association occurs between the PufX proteins and the α-polypeptides preventing formation of the heterodimeric ($\alpha\beta$) subunit or LH1 complexes. From measurements in which the PufX protein concentrations were varied, formation of the subunit complex of *Rb. capsulatus* and the LH1 complexes of both *Rb. capsulatus* and *Rb. sphaeroides* was inhibited by 50% at a ratio of approximately 0.5 PufX protein/α-polypeptide. This very strong and specific interaction is consistent with the expectation that at the levels of PufX protein predicted in vivo (approximately 10% of the α-polypeptide concentration as indicated by our isolation procedures), one PufX protein might be complexed with one or two α-polypeptides per LH1 complex. These results are congruent with the idea that the PufX protein modifies the proposed ring of LH1 around the

RC, thus enhancing ubiquinone/ubiquinol communication between the RC and the bc_1 complex (*13*, *25*).

Two aspects of our results are of great potential importance relative to the in vivo system. The very high affinity apparent between PufX and the LH1 α-polypeptide (indicating a 1:1 complex formation at nanomolar concentrations) implies these proteins have evolved to strongly interact. Second, the inability of the presumed PufX−LH1α-polypeptide complex to form LH1 suggests that the PufX role depends on this property. Several detailed mechanisms by which PufX could accomplish its task could be suggested. In one scenario as to how this might occur, a nearly completed LH1 ring could be "capped" by the introduction of one or two PufX polypeptides, thus leaving a "gap" in the ring near the Q_B binding site of the RC. Thus, LH1 might form a horseshoe that encompasses the RC with its opening at the Q_B binding site. A variant of this idea would involve incorporation of one or two PufX polypeptides into the α-polypeptide ring of LH1 near the Q_B binding site, leaving incomplete β-polypeptide, Bchl, and carotenoid rings. PufX might be especially effective in this role if it does not actually span the membrane, but only partially extends into it (recall that the middle segment has seven Gly). This would have the result of forming a "portal" (perhaps containing one or two lipid molecules) near the Q_B site of the RC. Note that for either of these possible mechanisms, a specific interaction between PufX protein and the RC would be required to make such a "gap" or "portal" in an appropriate location effective in allowing Q_B/Q_BH_2 exchange with the Q-pool and/or the bc_1 complex. Whatever the role, experiments investigating possible high-affinity interaction between the PufX protein, and the L-, M-, or H-polypeptide of the RC, should be of great interest to pursue. The importance of the results reported here is that biochemical experiments can now be designed to further evaluate these and other possibilities.

NOTE ADDED IN PROOF

Sufficient quantities of the peak labeled X in Figure 3 were collected subsequent to the submission of this paper to enable determination of the N-terminal amino acid sequence. This analysis further confirms that this material is the PufX protein as the first 10 amino acids were A_KTIFNDHL... which match the predicted sequence beginning with the second amino acid (Figure 8). The second amino acid in this N-terminal sequence could not be positively identified due to a high level of an unknown component (thought to be HFA) appearing at nearly the same peak location as Asp.

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