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Sequence analysis reveals new membrane anchor of reaction centre-bound cytochromes possibly related to PufX

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Abstract **Most of the bacterial photosynthetic reaction centres known to date contain a cytochrome subunit with four covalently bound haem groups. In the case of *Blastochloris viridis*, this reaction centre subunit is anchored in the membrane by a lipid molecule covalently attached to the cysteine which forms the N-terminus of the mature protein after processing by a signal peptidase. We show that posttranslational N-terminal cleavage of the cytochrome subunit does not occur in the aerobic photosynthetic bacterium *Roseobacter denitrificans*. From sequence analysis of the resulting elongated N-terminus it follows that a transmembrane helix is anchoring the reaction centre-bound cytochrome in the membrane. Comparative sequence analysis strongly suggests that all cytochrome subunits lacking the lipid coupling cysteine share this structural feature. Comparison of the N-terminal segment of the cytochrome subunit of *Roseobacter denitrificans* with the sequences of the PufX proteins from *Rhodobacter sphaeroides* and *Rhodobacter capsulatus* suggests a phylogenetic relation.**
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1. Introduction

Bacterial reaction centres (RCs) are membrane-spanning pigment protein complexes performing the primary processes of photosynthesis in photosynthetic bacteria. They contain three protein subunits, called H, L and M. The L and M subunits form five transmembrane helices each. The largest part of the H subunit, which contributes an additional transmembrane helix, is found in the cytoplasm. Four bacteriochlorophylls, two bacteriopheophytins, two quinones, one non-haem iron atom and a carotenoid molecule are bound to the helix bundle of the L and M subunits forming the core of the RC. These cofactors perform the directed light-induced electron transfer across the photosynthetic membrane resulting in the transformation of light into chemical energy (reviewed e.g. in [1,2]).

The majority of the known RCs have an additional subunit, which is located in the periplasm and usually contains four covalently bound haem groups, therefore called cytochrome or tetrahaem subunit [3]. For *Blastochloris viridis* (formerly *Rhodopseudomonas viridis*) the RC-bound cytochrome was shown to be anchored in the cytoplasm membrane by a diacylglycerol moiety covalently attached to the thiol group of the N-terminal cysteine residue. This cysteine becomes the N-terminal residue of the tetrahaem subunit after cleavage of an N-terminal segment of 20 amino acids by a signal peptidase II [4]. Examples of photosynthetic bacteria with RCs lacking the cytochrome subunit are *Rhodobacter sphaeroides*, *Rhodobacter capsulatus* and *Rhodospirillum rubrum*.

The bacterial RC is part of a supramolecular complex with the light-harvesting complex I (LHI) (designated 'core complex') which surrounds the RC as a cylinder as shown for *Rsp. rubrum* [5]. As recently observed by electron microscopy, dimeric core complexes are present in the photosynthetic membrane of *Rh. sphaeroides*. They were assumed to contain two RCs and possibly one cytochrome bc_1 complex as well as two PufX proteins (see below) [6].

In photosynthetic bacteria, the *puf* operon encodes the L, M, and C subunits of the RC as well as the α and β subunits of LHI. Moreover, in the phylogenetically closely related species *Rh. sphaeroides* and *Rh. capsulatus* the structural gene for the PufX protein, *pufX*, is also found on the *puf* operon. Barz et al. [7] have shown that PufX is required for the ubiquinol/ubiquinone exchange between the bacterial RC and the cytochrome bc_1 complex and the structural organisation of the photosynthetic membrane [8,9]. The mature PufX proteins from *Rh. capsulatus* and *Rh. sphaeroides* contain 78 and 80 amino acids, respectively [10]. How this small protein affects these functional and structural properties is under debate. The ability of isolated PufX to inhibit the in vitro reassembly of LHI from its components has led to the proposal that PufX might prohibit the formation of a closed LHI circle around the RC, thus enabling efficient quinone exchange between the RC and the quinone pool [11]. From the primary sequence [12,13] and circular dichroism data [10] it was deduced that the core region of PufX forms a transmembrane helix. Core segments of PufX showed the characteristic inhibition of in vitro LHI reconstitution thought to be associated with the in vivo function of PufX. However, neither N- nor C-terminal

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Abbreviations: A., Acidophilum; Achr., Allochromatium; B., Blastochloris; Chl., Chloroflexus; E., Ectothiorhodospira; R., Roseobacter; Rh., Rhodobacter; Rsp., Rhodospirillum; Rv., Rubrivivax; Rvu., Rhodovolum; T., Thermochromatium; RC, reaction centre; LHI, light-harvesting complex I

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fragments alone exhibited a similar function [10]. More recently, Francia et al. have shown that the core region of PufX is sufficient to allow fast quinone exchange between the RC and the cytochrome bc_1 complex [14].

In this work we report the N-terminal sequence of the mature cytochrome subunit of the RC from *Roseobacter denitrificans*, providing insights into the membrane-anchoring mechanism of RC-bound cytochromes. Further, we present indications of a phylogenetic relation between this cytochrome N-terminus and the PufX protein. This hypothesis is discussed with respect to the function of PufX and the RC evolution.

2. Materials and methods

2.1. N-terminal sequencing of the RC-bound cytochrome subunit

Cells of the aerobic photosynthetic bacterium *R. denitrificans* (ATCC number: 33942) were grown semi-aerobically in the dark according to [15,16]. Reaction centres were isolated and purified following known procedures [17,18]. SDS–PAGE was performed as described earlier [19]. The RC preparation was heated in SDS probing buffer for 30 min at 60°C.

The proteins were semi-dry blotted onto a polyvinylidene difluoride membrane in 200 mM glycine, 25 mM Tris, 0.01% SDS, 20% methanol and stained with Ponceau S. The excised band of the cytochrome subunit was subjected to automated Edman degradation using an Applied Biosystems protein sequencer (model 477A/120A) and following the manufacturer's instructions.

2.2. Computational sequence analysis

Sequences of RC-bound cytochromes were obtained with the sequence retrieval system (SRS6 [20,21]) at the European Bioinformatics Institute (http://srs.ebi.ac.uk/) by searching the SWALL databank. The multiple alignment of these sequences was prepared with the program CLUSTALW [22] (http://www.ebi.ac.uk/clustalw) using default parameters.

The homology between the N-termini of the cytochrome subunits of the RCs from *R. denitrificans* and *Rhodovulum sulfidophilum* with the PufX proteins was examined with both the PSI-BLAST service of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/BLAST/) and the multiple sequence alignment program MATCHBOX [23] (http://www.fundp.ac.be/sciences/biologie/bms/matchbox_submit.shtml).

The BLOSUM80 scoring matrix was used for PSI-BLAST searches for proteins which are homologous to the first 78 amino acid residues of the cytochrome subunit or to the PufX proteins to account for the short length of the sequences. Putative transmembrane helices formed by the N-termini of RC-bound cytochromes were predicted with the program TMHMM [24] (http://www.cbs.dtu.dk/services/TMHMM-2.0/). In a recent test case [25] the ability of 13 different methods to predict 883 membrane-spanning segments of biochemically characterised proteins was examined. TMHMM correctly predicted 415 of 469 (i.e. 88%) membrane-spanning regions not used in the training set of the program, 27 sequence segments were wrongly assigned as transmembrane segments. Thus, TMHMM was the best performing of the 13 tested methods. Programs for the prediction of membrane-spanning segments tend to assign N-terminal signal sequences as transmembrane helices [25]. In the test described above seven of 34 N-terminal signal sequences were erroneously assigned by TMHMM. In contrast no false assignment was obtained with the program ALOM2 [26]. We therefore checked the TMHMM predictions with ALOM2, which is available via the PSORT server at http://psort.nibb.ac.jp/.

In the case of the cytochrome subunit of *R. denitrificans*, two additional methods were applied (DAS [27], http://www.sbc.su.se/~miklos/DAS/, and TopPred [28,29], http://bioweb.pasteur.fr/seqanal/interfaces/toppred.html).

3. Results and discussion

The complete sequence of the cytochrome subunit of the RC from the aerobic photosynthetic bacterium *R. denitrificans* was published recently [30]. A multiple alignment with the sequences of nine RC-bound cytochromes showed that the amino acids involved in the attachment of four *c*-type haem groups to the protein, i.e. the cysteine residues for covalent linkage to the porphyrin system and the axial ligands of the haem iron atoms, are conserved in all species except from *Rvu. sulfidophilum* and *Chloroflexus aurantiacus*. The RC of *Rvu. sulfidophilum* contains only three instead of four haems [31]. The sequence of the cytochrome subunit from the green non-sulphur bacterium *Chl. aurantiacus* shows overall only a low similarity with the sequences from the purple bacteria. It also lacks one haem binding motif.

The *R. denitrificans* sequence differs from that of the RC-bound cytochrome of *B. viridis*. The cysteine which carries the lipid molecule anchoring the tetrahaem subunit in the membrane is replaced by an isoleucine (Fig. 1). Since no cysteine is found within the first 126 amino acids of the sequence it could be excluded that this is due to an alignment error. Therefore, the membrane-anchoring mechanism of the tetrahaem subunit of the *R. denitrificans* RC must be different from that of *B. viridis*. The question arose if the cleavage of the N-terminal segment of the tetrahaem subunit by a signal peptidase occurs in *R. denitrificans*, as found for *B. viridis*. N-terminal sequence determination was performed to clarify this point. The sequence obtained was MFPKWFD which matches the N-terminus encoded in the *pufC* gene, i.e. posttranslational cleavage does not take place. The analysis of the sequence with the program TMHMM showed that the N-terminus most probably forms a transmembrane helix. This prediction was confirmed with two different methods (DAS and TopPred) providing a consensus sequence for the transmembrane region spanning residues 21–40. The TMHMM analysis was repeated

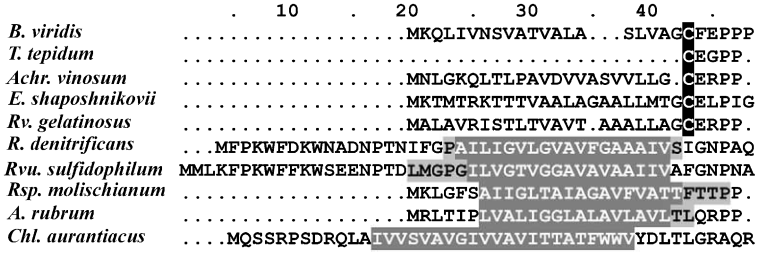


Fig. 1. Multiple sequence alignment of the N-termini of RC-bound cytochromes. The upper five sequences correspond to cytochromes which are probably anchored in the photosynthetic membrane by a lipid molecule. The lipid is covalently attached to an N-terminal cysteine residue as highlighted in black. The lower five N-termini lack the cysteine. They probably form transmembrane helices as membrane anchors of the cytochrome. Sequence regions with transmembrane helix probabilities $>50\%$ and $>90\%$ are shown with light grey and dark grey background, respectively. Transmembrane helix probabilities were determined with the program TMHMM (see Section 2).

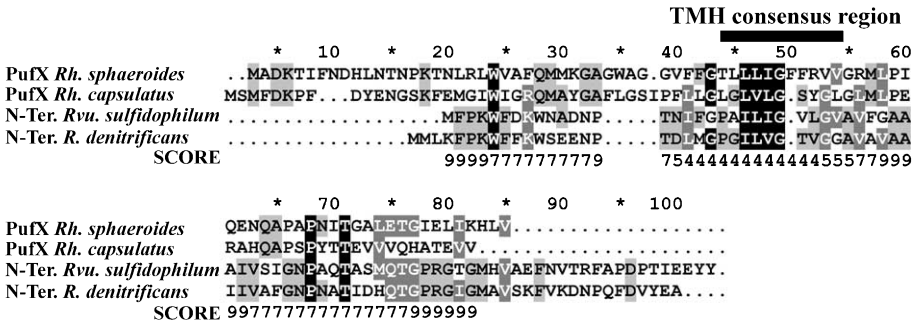


Fig. 2. Alignment of the N-termini of the cytochrome subunits of the RCs from *R. denitrificans* and *Rvu. sulfidophilum* with the PufX sequences of *Rh. capsulatus* and *Rh. sphaeroides*. The original MATCHBOX alignment was slightly modified by moving a gap from position 62 to position 50 to enhance the sequence identity for the two PufX proteins from 16 to 24%. This had no significant effect on the other identity levels. Shown in the last line is the MATCHBOX score for the corresponding alignment position. The lower the score the more reliable the alignment at this position. Nine of 12 residues with the lowest score, corresponding to a probability of $>74\%$ for a correct alignment, were found within the consensus region of predicted transmembrane helices.

with all available sequences of RC-associated cytochromes (Fig. 1). It turned out that for all sequences lacking the cysteine corresponding to the lipid-coupling cysteine of the *B. viridis* sequence continuous segments of 18–23 residues with probabilities for the formation of a transmembrane helix greater than 0.5 were found in the N-terminal regions. Within these sequence sections, for 16–18 amino acids transmembrane helix probabilities above 90% were obtained. This property was also shared by the cytochrome subunit from *Chl. aurantiacus*, which is more distantly related to the purple bacteria. Checking the TMHMM assignments with ALOM2 (see Section 2) confirmed the formation of transmembrane helices by the N-termini of the RCs from *R. denitrificans*, *Rvu. sulfidophilum*, *Rhodospirillum molischianum* and *Chl. aurantiacus*. Since this program is much less sensitive with respect to the detection of transmembrane helices than TMHMM [25], these findings strongly support the assignments of TMHMM for these four organisms. For *Acidophilum rubrum* ALOM2 did not predict a membrane-spanning segment.

On the other hand, for none of the sequences showing the characteristic cysteine residue a transmembrane helix was predicted. On the basis of these results we conclude that at least two groups of RC-bound cytochromes can be distinguished: those having an N-terminal cysteine are anchored in the membrane by a covalently bound diacylglycerol molecule. The second group, lacking this cysteine residue, uses an N-terminal transmembrane helix for this purpose.

From the structure of the *puf* operon of *R. denitrificans* [30] it is obvious that in organisms with cytochrome subunit the corresponding gene appears immediately downstream of the structural gene for the M subunit. In *Rh. capsulatus* [32] and *Rh. sphaeroides* [33,34] the *pufX* gene encoding the PufX protein is found at the same position of the operon. As outlined above, the N-terminus of the tetrahaem subunit from *R. denitrificans* forms a transmembrane helix. From a sequence analysis it was concluded that PufX also forms a transmembrane helix [12,13]. These data suggest that PufX is related to the N-terminus of those cytochrome subunits forming transmembrane helices.

To test this hypothesis, the PSI-BLAST program [35] was used to search for sequences that are homologous to the first 78 amino acids of the RC-bound cytochrome from *R. denitrificans* as the length of the shorter of the two known *pufX* genes from *Rh. capsulatus* corresponds to 78 amino acids. The PufX proteins were not detected. Instead, the cytochrome subunit of the RC from *Rvu. sulfidophilum* was found. As is also obvious from Fig. 1, the N-termini of the RC-bound cytochromes from *R. denitrificans* and *Rvu. sulfidophilum* are highly homologous. The sequence identity amounts to 50% within the first 78 residues of the *R. denitrificans* cytochrome. The PufX sequences were also used as PSI-BLAST input. PufX from *Rh. sphaeroides* was identified as being homologous to PufX from *Rh. capsulatus*. On the other hand, searching the database with the PufX sequence of *Rh. sphaeroides* did not lead to the detection of the *Rh. capsulatus* sequence.

Since the PSI-BLAST method was unable to detect PufX from *Rh. capsulatus* as a sequence related to PufX from *Rh. sphaeroides* although these sequences are without any doubt related, we used the program MATCHBOX [23]. MATCHBOX is known to deliver alignments with a high level of confidence compared to other methods, especially at low levels of sequence identity [36]. Furthermore, the program quantifies the reliability of the different regions in the alignment simplifying its interpretation. To analyse a possible relation between PufX and the N-termini of the tetrahaem subunits we prepared a multiple sequence alignment of the first 78 residues of the RC-bound cytochromes of *R. denitrificans* and *Rvu. sulfidophilum* with the PufX sequences of *Rh. sphaeroides* and *Rh. capsulatus* (Fig. 2). The highest level of identity between a PufX sequence and the sequence of a cytochrome N-terminus is obtained for the pair *Rh. sphaeroides*/*R. denitrificans*. The sequence identity amounts to only 12% (17% when similar amino acids were considered). The alignment scores indicated a low reliability of most regions in the alignment. However, the core region of 12 residues exhibited an alignment score of 4, corresponding to a probability of $>74\%$ for the correctness of this part of the alignment indicating a relationship between the sequences of PufX and the cytochrome subunits. It should be noted that nine of the 12 residues in the region with the highest reliability of the alignment are found within the consensus region of the predictions of transmembrane helices.

Bott et al. [37] discovered a membrane-anchored homologue of mitochondrial cytochrome *c* which may serve as an electron carrier between the cytochrome bc_1 complex and the terminal oxidase cytochrome aa_3. More recently, similar cytochromes were found in several other organisms [38–42]. The cytochromes c_y of *Rh. sphaeroides* [40,41] and *Rh. capsulatus*

[38,39] also belong to this group. In the MATCHBOX alignment (not shown) of the N-termini of these two proteins with the N-terminal segments of the tetrahaem subunits of the RCs from *R. denitrificans* and *Rvu. sulfidophilum* a score of 5 was assigned to the most reliable region corresponding to a probability of $>49\%$. This region does not include the transmembrane helices as determined with TMHMM. These findings provide no evidence for a relation of the membrane-anchored cytochromes c_y with the membrane-anchored tetrahaem subunits.

Several properties of the PufX protein are in agreement with our idea of a relation to the cytochrome N-termini: Pugh et al. [43] have shown that the N-terminus of PufX in *Rh. sphaeroides* is exposed to the cytoplasmic side of the photosynthetic membrane. The N-terminus of RC-bound cytochromes with one N-terminal transmembrane helix must also be located on the cytoplasmic side of the membrane because the major part of the cytochrome is located in the periplasm.

Tests of the ability of several truncated forms of PufX have shown that a core segment of the protein, encompassing the transmembrane helix, is sufficient for the inhibition of LHI reconstitution in vitro. In the case of *Rh. sphaeroides* even a core segment of only 25 amino acids, i.e. not considerably longer than the transmembrane helix, was found to be active [10]. The in vivo effects of truncations of the N- and the C-termini of PufX in *Rh. sphaeroides* have also been examined [14]. It turned out that the presence of the central hydrophobic portion of PufX is sufficient for a fast ubiquinone exchange between the RC and the cytochrome bc_1 complex. In summary, it can be concluded that the transmembrane part is the key component with respect to the functional properties of PufX. Therefore this region of the sequence is expected to show the highest level of conservation. This is in agreement with our finding that in the multiple sequence alignment of the PufX sequences with the N-terminal segments of the RC cytochrome subunits indications of the homology are found in the consensus region of the predicted transmembrane helices.

PufX is part of the core complexes in photosynthetic membranes from *Rh. sphaeroides* as well as in isolated core complexes and is present in a 1:1 molar ratio to the RCs [8]. It strongly interacts with the α subunit of LHI [11]. Obviously, the molar ratio of the N-terminal helix of the cytochrome subunit to the RC is also 1:1. From the homology model of the structure of the RC of *R. denitrificans* [44] it follows that the cytochrome transmembrane helix is located on the surface of the RC enabling the interaction with the LHI transmembrane helices surrounding the RC in the core complex.

Matsuura and Shimada analysed the phylogenetic relations between 14 different organisms of the α subgroup of purple bacteria considering the presence or absence of the RC-bound cytochrome [45]. They concluded that the ancient form of the bacterial RC contained such a subunit. Accordingly, in all four lines of this subgroup (α_1–α_4) deletions took place independently, leading to the disappearance of the cytochrome subunits in some species. *R. denitrificans*, *Rvu. sulfidophilum*, *Rh. sphaeroides* and *Rh. capsulatus* are members of the α_3 subgroup of purple bacteria [46]. Therefore our hypothesis, that the N-terminal transmembrane helices are related to PufX, might be rationalised as follows: the RCs of *R. denitrificans* and *Rvu. sulfidophilum* correspond to the ancient form of this protein. In a common ancestor of *Rh. sphaeroides* and *Rh. capsulatus* incomplete deletion of the *pufC* gene encoding the cytochrome subunit caused the disappearance of the major part of this subunit leaving behind little more than the N-terminal transmembrane part, which has an important function for the structural organisation of the photosynthetic membrane in this group of photosynthetic bacteria.

The possibility of a relationship between PufX and the N-termini of the RC-bound cytochromes has important implications: if the structure of an RC with an N-terminal transmembrane helix was known, the interaction of this helix with other parts of the RC would provide insights into the way in which PufX interacts with the RC. This should significantly contribute to the understanding of the mechanisms enabling PufX to exert such drastic effects on the structural organisation of the photosynthetic membrane and the efficiency of the quinone exchange between the RC and the cytochrome bc_1 complex. Such a structure of the RC could also be used to deduce the position of PufX within the RC core complexes from *Rh. sphaeroides* which have recently been characterised by electron microscopy.

An experimental approach for the verification of our hypothesis can be deduced from the observation that the transmembrane part of PufX was found to be sufficient for its function. In analogy to the experiments described by Parkes-Loach et al. [10] the transmembrane part of the N-terminus of the cytochrome subunit of the RC from *R. denitrificans* could be synthesised. If our hypothesis is correct, we would expect that this synthetic protein, like synthetic PufX core segments, inhibits the in vitro reconstitution of LHI.

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