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Short sequence-paper

A pyrophosphate synthase gene: molecular cloning and sequencing of the cDNA encoding the inorganic pyrophosphate synthase from *Rhodospirillum rubrum* [1]

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Abstract

The integrally membrane-bound, proton-pumping inorganic pyrophosphate (PPi) synthase in phototrophic bacteria is hitherto the only described alternative to the ATP synthase in biological electron transport phosphorylation. We have identified and sequenced the first gene coding for a pyrophosphate synthase. The deduced protein contains 660 amino acid residues and 15 putative membrane-spanning segments. It is homologous to the vacuolar pyrophosphatases from plants.

Keywords: Pyrophosphate synthase gene; Photophosphorylation; Proton pump; *Rhodospirillum rubrum*

The membrane-bound, proton-pumping inorganic pyrophosphate synthase (H^+-PPi synthase) was first described [1,2] in chromatophores from the phototrophic bacterium *Rhodospirillum rubrum*. Under physiological conditions this enzyme can both synthesize and hydrolyze PPi. It was also demonstrated [3–5] that PPi could drive a number of energy requiring reactions in chromatophores from *R. rubrum*, including ATP synthesis in the dark [6]. The enzyme can function in bacterial photosynthesis as a coupling factor, the first and only known alternative to the well-known ATP synthase in biological electron transport phosphorylation.

The solubilized enzyme is very hydrophobic, has an apparent molecular weight of only 56 000, as determined by SDS-PAGE [7] and consists of one polypeptide containing both the catalytic site and the proton channel, possibly as a homo-oligomer. In order to retain activity, phospholipid or detergent has to be present. Optimal activity is obtained in the presence of cardiolipin. The enzyme has the capacity to create a proton gradient, both in chromatophores and after solubilization, when reconstituted in artificial liposomes. As we have shown, this gradient suffices for a low rate of ATP synthesis [8] in liposomes evidently containing both the PPi synthase and the ATP synthase.

Higher plant vacuoles have been found to contain H^+-pumping membrane-bound pyrophosphatases (PPases). A number of these V-PPases have been

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isolated and purified, and in recent years the genes for several of them have been identified and sequenced [9–14]. A high degree of homology between these PPases is found, normally around 80% identity. One of the first V-PPases to be purified was the one from *Vigna radiata* [15] and we found that antibodies against this V-PPase cross-reacted with the purified *R. rubrum* PPi synthase [16]. This indicated some similarity between the plant V-PPases and the bacterial enzyme. Since the extreme hydrophobicity of the latter up to now has made it technically impossible to obtain an amino acid sequence, it was decided to make a molecular analysis of its gene.

Membrane-bound PPi synthase was isolated from *R. rubrum* (strain S1) [7] and purified further by electroelution. Rabbit polyclonal antibody was raised against the electroeluted *R. rubrum* PPi synthase and affinity-purified according to the method described by Sambrook et al. [17]. Rat polyclonal antibodies against the vacuolar PPase from *V. radiata* were a kind gift from Prof. M. Maeshima.

A *R. rubrum* cDNA library constructed in λZAP II was purchased from Clontech. Restriction enzymes and other DNA modification enzymes were purchased from Pharmacia, [α^{35}S]dATP from Amersham and all other commonly used chemicals from Sigma.

Antibodies raised against the PPi synthase were used to screen a *R. rubrum* cDNA expression library in λZAP II as described by Sambrook et al. [17]. Immunoscreening was also performed with antibodies against *V. radiata* vacuolar PPase as these antibodies have been shown to cross-react with our PPi synthase [16].

Positive phage plaques were detected by an enhanced chemiluminescence system (Amersham) using horseradish peroxidase-labelled secondary antibody and an amplified alkaline phosphatase immunoblot assay kit (Bio-Rad) with a streptavidin–biotinylated enzyme complex.

Insert DNA from positive λZAP II clones was excised in vivo by using helper phage R408 into a pBluescript SK(−) vector and subcloned according to the manufacturer's instructions (Clontech). The vector was transformed into *Escherichia coli* XL-1 Blue.

Manual DNA sequencing was performed by the dideoxynucleotide chain-termination method [18] using Deaza G/A^{T7} sequencing kits (Pharmacia) and M13 universal and reverse primers. For further sequencing oligonucleotide primers were then custom-synthesized (Kebo and Pharmacia). Twenty-one percent DMSO was added in order to facilitate the annealing of the G-C rich primer.

Automated DNA sequencing was done using an Applied Biosystems 373A sequencer and a Dye Terminator Cycle Sequencing Ready Reaction kit (Perkin-Elmer).

Three positive λZAP II clones (PP4, PP5 and PP6) were identified when screening the expression library with the two antibodies. The insert DNA from these three clones was excised and subcloned. Based on restriction digestion of the insert DNA from the three clones, the three appeared to be identical and approximately 4.5 kb in length.

The 4.5 kb insert DNA from one of the clones (PP4) was digested with *Eco*R1 and *Hin*dIII and the resulting three fragments (3 kb, 1.05 kb and 0.45 kb) were subcloned into pUC18. The nucleotide sequences of these three fragments were determined in both the 5′ and 3′ directions by manual DNA sequencing. The 4.5 kb insert DNA from all three positive clones (PP4, PP5 and PP6) were sequenced in both directions by automated DNA sequencing. All three clones were found to have identical sequences. A putative open reading frame of 1980 bp was found, starting from GTG (underlined in Fig. 1). Although GTG is used less frequently than ATG as a translation initiation codon, it has been shown to be an initiation site in certain proteins in *R. rubrum*, e.g., the δ subunit of the ATP synthase [19].

A Shine–Dalgarno sequence, AGGAGA, lies 17 bp upstream of the initiation codon. Downstream to the translational termination codon, TAA, is a potential σ-independent transcriptional terminator consisting of a G + C rich inverted repeat region (indicated by arrows in Fig. 1) followed by an A + T rich sequence.

The 1980 bp open reading frame encodes a protein of 660 amino acids with a calculated molecular mass (M_r) of 67 453 (Fig. 1). The calculated M_r is greater than the apparent M_r (56 000) of *R. rubrum* PPi synthase determined by SDS-PAGE. Since the membrane-bound *R. rubrum* PPi synthase is known to be a very hydrophobic protein [20], it is possible that it moves anomalously on gels giving rise to an underestimated M_r. This is not uncommon with membrane

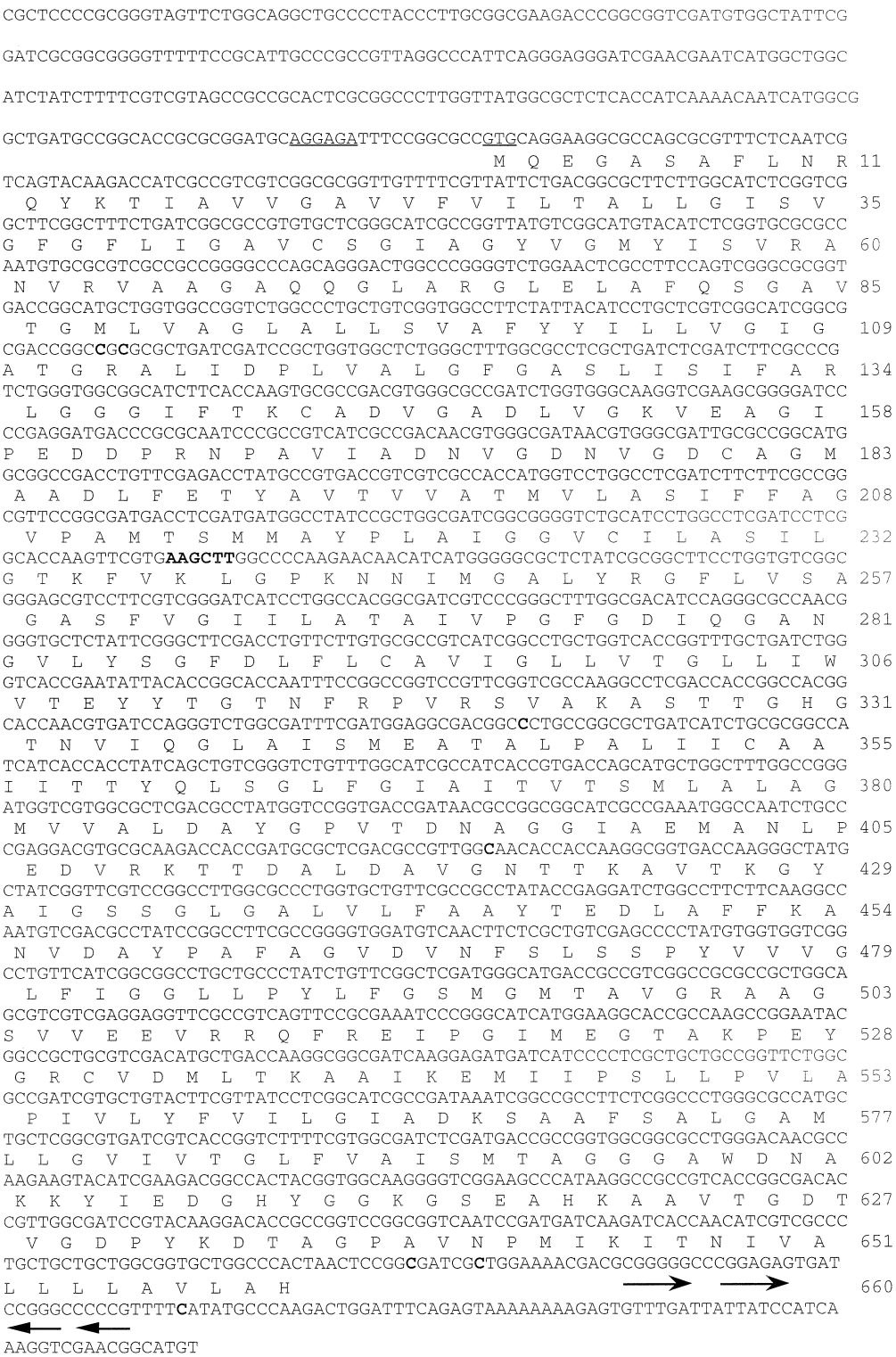

Fig. 1. The nucleotide sequence of parts of the 3.0 kb and the 1.05 kb subclones from PP4 and the deduced amino acid sequence. The nucleotides in bold script mark the *Hin*dIII restriction site. The Shine–Dalgarno sequence and the start codon are underlined. The arrows indicate the inverted repeat.

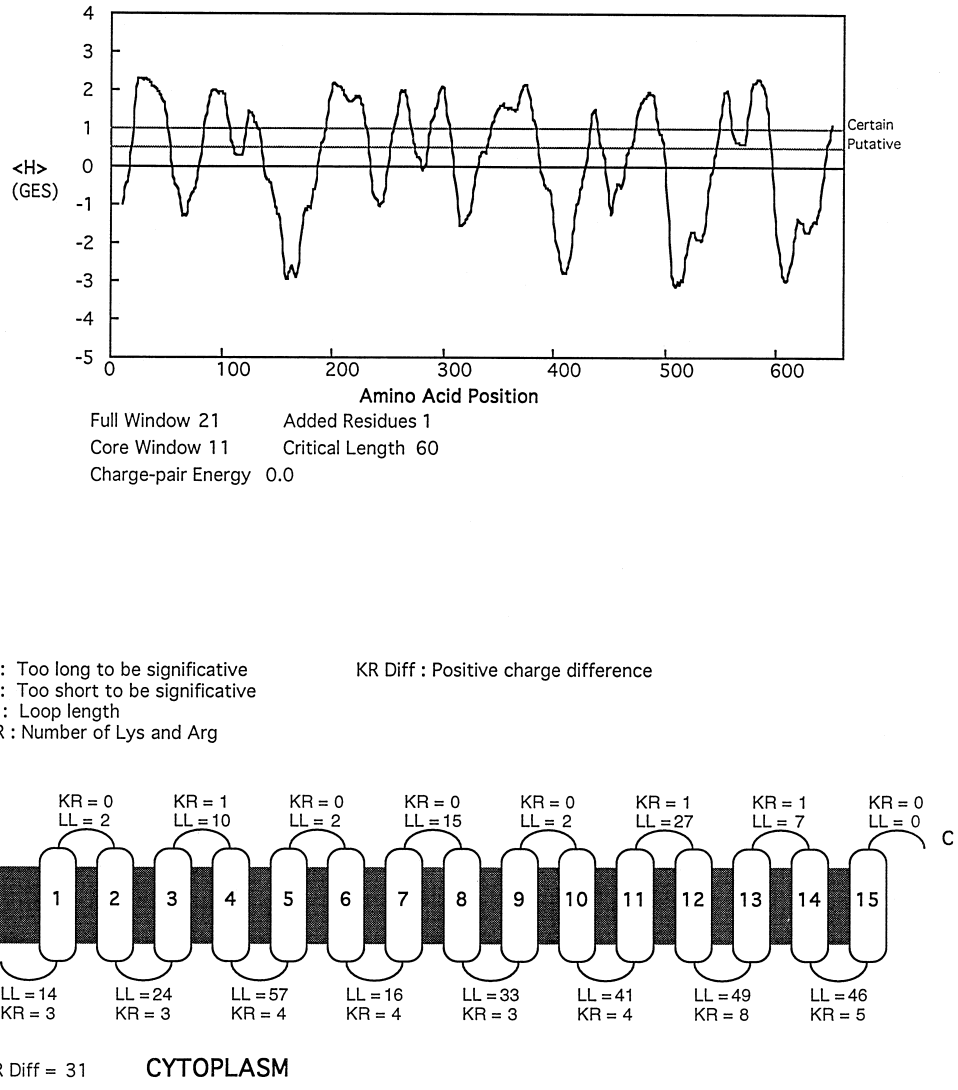

Fig. 2. Hydropathy plot and membrane topology of the deduced amino acid sequence of the PPi synthase as generated with the TopPredII [2] program.

proteins and is ascribed to the binding of nonsaturating amounts of SDS to the protein [21].

The extreme hydrophobicity of the protein is also reflected in the hydropathy plot and suggested topography, as analysed with the TopPred program [22] (Fig. 2). As many as 15 transmembrane segments are

R. rubrum	135	LGGGIFTKCADVGAD	LVGKVEAGIPEDDPR	NPAVIADNVGDNVGD	CAGMAADLFETY 191
V. radiata	243	VGGGIYTKAADVGAD	LVGKVERNIPEDDPR	NPAVIADNVGDNVGD	IAGMGSDLFGSY 299
A. thaliana	247	VGGGIYTKAADVGAD	LVGKIERNIPEDDPR	NPAVIADNVGDNVGD	IAGMGSDLFGSY 303
O. sativa	248	VGGGIYTKAADVGAD	LVGKVERNIPEDDPR	NPAVIADNVGDNVGD	IAGMGSDLFGSY 304
T. maritima	192	VGGGVYTKAADMAAD	LVGKTELNLPEDDPR	NPATIADNVGDNVGD	VAGLGADLLESF 248

Fig. 3. Alignment between two bacterial and three vacuolar H^+ PPases of the segment containing the motif proposed to be important for catalysis. The *R. rubrum* sequence represents the loop between transmembrane segments 4 and 5 in Fig. 2. Underlined amino acids are those differing from the consensus ones. The sequence from *Thermotoga maritima* was obtained from the Institute for Genomic Research (personal communication).

predicted, and account for 48% of the amino acids in the protein.

The deduced amino acid sequence of our protein (Fig. 1) shows homology with the plant vacuolar pyrophosphatases from *Arabidopsis thaliana*, *Beta vulgaris*, *V. radiata*, *Oryza sativa*, *Nicotiana tabacum* and *Hordeum vulgare* [9–14]. Comparing the parts that are nearly identical, in relation to the predicted topology of the enzymes, it is evident that great identity is confined to some of the hydrophilic loops connecting the transmembrane segments. It is as high as 82% for a loop of 57 amino acids, whereas the overall identity with the vacuolar PPases is 36–39%. This large loop, situated between transmembrane segments 4 and 5 in our enzyme and consisting of amino acids 135–191, contains a DX7KXE motif found conserved in all sequenced vacuolar PPases (DVGADLVGKVE, Fig. 3). It is similar to a EX7KXE motif found in most soluble PPases, which, has been proposed to participate directly in substrate and Mg^{2+} binding [23,24], based on the three-dimensional structure of the *E. coli* enzyme. Antibody to this sequence in *V. radiata* inhibits both the PPase activity and the proton pumping [25], a result which further substantiates the importance of this sequence.

A PPi synthase sequence, which may turn out to be very significant, is EYYT, which is found right after transmembrane segment 8 (AA 308–311) and which is identical to a corresponding sequence in *Arabidopsis* and other V-PPases. In a recent publication Zhen et al. [26] report an elaborate mutational analysis of the gene for the vacuolar PPase from *Arabidopsis*, where the mutation E427Q, of its EYYT sequence, uncouples the H^+ transport from the PPase activity.

The similarities in the loops between the transmembrane segments make it reasonable to assume that the *R. rubrum* PPi synthase is homologous with the V-PPase family. One has to bear in mind, though, that the *R. rubrum* enzyme, in contrast to the other members of the family has the unique ability also to physiologically synthesize PPi in addition to hydrolyzing this compound. The deduced sequence presented in this communication represents a polypeptide with a catalytic site for both the synthesis and the hydrolysis of pyrophosphate as well as a proton channel, which physiologically functions in both directions. By mutational analysis we hope to be able to shed more light on this (primitive?) coupling mechanism between biological electron transport and phosphorylation.

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