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Hypothesis

Is the 9 kDa thylakoid membrane phosphoprotein functionally and structurally analogous to the 'H' subunit of bacterial reaction centres?

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Although the amino acid sequence of the 9 kDa (phospho)protein of chloroplasts has been determined, the function of this thylakoid membrane protein in photosynthetic electron transport and the reason for its physiological control remains unclear. In this paper, I briefly review the evidence which indicates that the phosphorylation of the 9 kDa protein results in a partial inhibition of photosynthetic oxygen evolution by increasing the stability of the semiquinone bound to Q_A the primary, plastoquinone-binding site of photosystem II (PS II). I propose that in its dephosphorylated state, the 9 kDa thylakoid membrane protein may serve PS II to ensure efficient photochemical charge separation by aiding the transfer of reducing equivalents out of the reaction centre to the attendant plastoquinone pool. This function is analogous to that proposed for the H-subunit of the reaction centre of photosynthetic eubacteria. Whether these two proteins have evolved from a common ancestral reaction centre protein is discussed in the light of a comparison of their amino acid sequences and predicted secondary structures.

Thylakoid membrane; Protein phosphorylation; 9 kDa phosphoprotein; Photosystem II; Reaction center; Plastoquinone; (Bacterium)

1. THYLAKOID MEMBRANE PROTEIN PHOSPHORYLATION REGULATES THE DISTRIBUTION OF EXCITATION ENERGY BETWEEN PS I AND PS II

It is well established that several thylakoid membrane proteins, notably including the 26 kDa and 24 kDa components of the light-harvesting chlorophyll *a/b* protein complex II (LHC II) and a 9 kDa species, can be phosphorylated when the plastoquinone pool is reduced [1]. The reversible phosphorylation of LHC II is thought to regulate the distribution of excitation energy between PS II and PS I, and thereby permit the chloroplast to maximise the overall efficiency of photosynthesis under any given light condition [2,3]. The molecular mechanism of this control process may involve the lateral migration of phospho-LHC II from the PS II-enriched appressed membranes of the granal stacks to the PS I-enriched non-appressed granal end-membranes and stromal lamellae (see [4]). Traditionally, the 9 kDa thylakoid membrane (phospho)protein has not been considered to be part of the regulation pro-

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Abbreviations: PS II, photosystem II; D1 and D2, the two reaction centre subunits of PS II; L, M and H, the light, medium and heavy subunits of the reaction centre from photosynthetic eubacteria; Q_A and Q_B, the primary and secondary plastoquinone-binding sites of PS II reaction centres

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cess, because the kinetics of its dephosphorylation are slower than those for LHC II [5] and for the increased absorption cross-section of PS II [6].

2. THE PRIMARY AND PREDICTED SECONDARY STRUCTURES OF THE 9 kDa (PHOSPHO)PROTEIN

The 9 kDa phosphoprotein has been isolated from thylakoid membranes and its N-terminal amino acid sequence determined [7]. Recent studies show that the threonine residue at position 2 in the mature protein is phosphorylated [8]. The N-terminus sequence identified the 9 kDa (phospho)protein as the product of the *psb*H gene of the chloroplast genome, and the complete primary structure of the protein has been deduced from the nucleotide sequence [9]. Secondary structure analysis of the amino acid sequence predicts that the 9 kDa (phospho)protein has a single membrane-spanning α-helix situated towards the C-terminus of the protein (see fig.1).

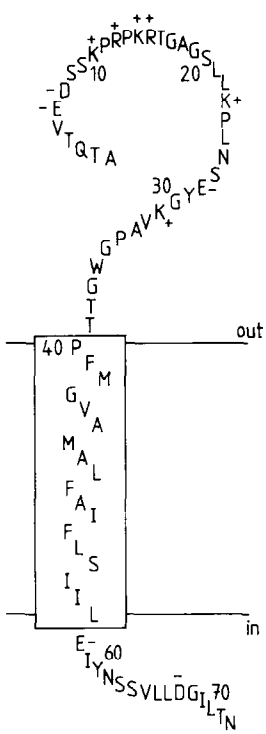

Fig.1. A possible folding pattern of the wheat *psb*H gene product based on a hydropathy plot analysis [9]. The single membrane-spanning α-helix is located towards the C-terminus.

The absence of histidines and cysteines in its primary structure suggests that the 9 kDa (phospho)protein may not be a light-harvesting pigment protein directly involved in light interception, although a weak homology has been recognised between the region of the 9 kDa (phospho)protein flanked by residues Asn-26 and Tyr-60 and the first 35 residues of the mature LHC II of wheat and lemna [9]. A comparison of the aligned amino acid sequences revealed that 5 of the 35 (14%) residues were identical in all three proteins. The 24 and 26 kDa proteins of the LHC II are, however, phosphorylated on threonine residues [1] located near the N-terminus of the mature protein [10]. Thus, although the 9 kDa (phospho)protein and LHC II are probably phosphorylated by different thylakoid membrane protein kinases (see below), it is possible that the weak identity between these two proteins may reflect a homology in the binding sites of the protein kinases (and phosphoprotein phosphatases).

3. PHOSPHORYLATION OF THE 9 kDa THYLAKOID MEMBRANE PROTEIN INHIBITS PS II PHOTOCHEMISTRY BY INHIBITING ELECTRON TRANSFER BETWEEN THE PRIMARY, Q_A, AND SECONDARY, Q_B, PLASTOQUINONE ACCEPTORS OF THE PS II REACTION CENTRE

Thylakoid membrane protein phosphorylation has been shown to introduce a partial (approx. 20%) inhibition of light-dependent oxygen evolution rate under fully-oxidising conditions [11,12]. This result is expected if LHC II phosphorylation causes a decreased light-harvesting capacity of PS II. The degree of inhibition was not, however, reversed as the actinic light intensity is increased to saturating levels, indicating that thylakoid membrane protein phosphorylation must also have a direct inhibitory effect on PS II photochemistry. Chlorophyll *a* fluorescence emission studies have suggested that thylakoid membrane protein phosphorylation affects the acceptor side reactions of PS II and results in an increased stability of the anionic semiquinone at the primary, Q_A, plastoquinone binding site [12,13].

PS II centres which retain their Q_A in the Q_A^- state following flash excitation are 'closed' to fur-

ther photochemistry until Q_A^- is re-oxidised either by Q_B (halftime of 100–200 μs) (see [14]) or by the S2 oxidation state of PS II (halftime of 1–5 s) [14,15]. The equilibrium constant for electron transfer between Q_A^- and Q_B has been measured to be about 20; equivalent to an 80 mV difference in redox potential between the Q_A/Q_A^- and Q_B/Q_B^- couples. Thus the vast majority (about 95%) of reaction centres will have their Q_A^- re-oxidised by Q_B. Since the re-oxidation of Q_B^- by S2 has a halftime of 25–40 s [14,16], the lifetime of the S2 oxidation state is a sensitive indicator of the acceptor-side reactions of PS II.

The effect of thylakoid membrane protein phosphorylation on the lifetime of the S2 oxidation state has been examined by flash-induced oxygen yield measurements [17]. It was found that with non-phosphorylated thylakoid membranes only about 5% of the S2 population is re-reduced by Q_A^-, which concurs with the predicted equilibrium constant for electron transfer between Q_A^- and Q_B. With phosphorylated thylakoid membranes, however, the extent of the fast phase in the biphasic kinetics of S2 re-reduction, monitoring the re-oxidation of Q_A^-, is increased to 20%. This observation supports the claim that thylakoid membrane protein phosphorylation increases the stability of Q_A^- in the light. From the increased fast re-reduction of S2, we have calculated that the equilibrium constant for electron transfer between Q_A^- and Q_B is decreased from 20 to 6 upon thylakoid membrane protein phosphorylation, suggesting that the redox potential difference between the Q_A/Q_A^- and Q_B/Q_B^- redox couples is decreased by about 30 mV.

The precise molecular mechanism by which thylakoid membrane phosphorylation affects the stability of Q_A^- in the light remains unclear. Is the effect due to an increased negative surface charge density on the stability of the anionic semiquinone in the Q_B pocket, or due to a conformational change in PS II leading to a decreased plastoquinone binding in the Q_B pocket? A study of the flash-induced electrochromic 515 nm absorption bandshift has indeed indicated that the redox midpoint potential of the Q_A/Q_A^- couple can be altered by -150 mV if the surface potential is made more negative in the cation-depleted medium [18]. Phosphorylation of thylakoid membrane proteins would be expected to increase the negative surface charge density of thylakoid membranes. Thus, if the altered surface potential-dependence was restricted to a change in redox midpoint potential of Q_B/Q_B^- and not Q_A/Q_A^-, then this could account for an increased stability of Q_A^- after flash excitation. Since the Q_A and Q_B sites are presumed to be close, it is unlikely that the effect of thylakoid membrane protein phosphorylation is directly related to a change in the negative surface charge density around the Q_B pocket. A more probable explanation is that thylakoid membrane phosphorylation alters the conformation of the Q_B pocket and thereby impedes plastoquinone binding. It has been reported that the efficacy of inhibitors, which can displace plastoquinone from the Q_B pocket [19], is increased upon thylakoid membrane protein phosphorylation [20,21]. An increased affinity for the herbicide may be associated with a corresponding decrease in plastoquinone binding in the phosphorylated PS II centres.

Is the inhibition of PS II the result of phosphorylation of LHC II as Horton and Lee [22] have proposed, or due to the phosphorylation of the 9 kDa protein? It is increasingly evident that the LHC II and 9 kDa thylakoid membrane proteins are phosphorylated by different protein kinases. This is based on studies which have shown that protein kinase inhibitors, such as *N*-ethylmaleimide (NEM) and fluorosulphonylbenzoic acid (FSBA), selectively inhibited the phosphorylation of LHC II with little effect on the phosphorylation of the 9 kDa (phospho)protein [23,24]. Using these inhibitors, a correlation in the inhibition of the oxygen evolution rate with the phosphorylation of the 9 kDa protein and not the LHC II can be demonstrated [25]. It might be argued that these results do not rule out the possibility that the inhibition of PS II results from the phosphorylation of other thylakoid membrane proteins which are also substrates for the 9 kDa protein kinase. However, the conditions employed in these studies did not result in a significant phosphorylation (i.e. incorporation of the phosphate label at amounts stoichiometric to the number of PS II centres) of any other thylakoid membrane protein. It was therefore deduced that the inhibition of electron transfer between Q_A and Q_B is due to the specific phosphorylation of the 9 kDa protein, and not to any other thylakoid membrane phosphoprotein.

4. A POSSIBLE FUNCTIONAL AND STRUCTURAL RELATIONSHIP BETWEEN THE 9 kDa THYLAKOID MEMBRANE PROTEIN AND THE H-SUBUNIT OF THE ANOXYGENIC PROKARYOTIC REACTION CENTRE

The 9 kDa protein is located in the appressed thylakoid membranes, and is therefore in close proximity to the (dephosphorylated) LHC II and PS II reaction centre [26]. However, unlike the phospho-LHC II, the 9 kDa phosphoprotein is not able to migrate from the appressed, PS II-enriched, thylakoid membranes to the unappressed, PS I-enriched, regions [27]. Thus, phosphorylation of the 9 kDa protein must exert its effect through a conformational change in the protein, rather than through a change in its lateral distribution within the thylakoid membrane. The 9 kDa (phospho)protein is not considered to be a component of the PS II reaction centre. The isolated reaction centre contains only three proteins; the D1 and D2 proteins and cytochrome *b*-559 [28]. This reaction centre preparation lacks plastoquinone and is unable to catalyse stabilised electron transfer unless supplemented with an artificial electron acceptor which can reoxidise the photoreduced pheophytin [29]; exogenous quinones are unable to support electron transport. Full photochemical activity can be obtained with less-pure 'core' PS II preparations which contain bound plastoquinone and the 9 kDa phosphoprotein [30].

That the D1 and D2 thylakoid membrane proteins comprise the PS II reaction centre was first suggested by the identification of homologies in their amino acid sequences to the L- and M-subunits, respectively, of the anoxygenic prokaryotic reaction centres of the *Rhodospirillaceae* (see [31,32]). Although the identity in the amino acid alignment of the eukaryotic and prokaryotic proteins is low (only 14% for D1 against the L-subunit and 12% for D2 against the M-subunit from *Rhodopseudomonas viridis* and *Rhodobacter capsulatus*: higher identities are found if one aligns the eukaryotic sequence against either bacterial sequence), hydropathy index plot analysis of the amino acid sequences suggests that all four proteins have similar secondary structures [31]. The secondary structures of the L- and M-subunits have been confirmed by the elucidation of the crystal structure of the *Rhodopseudomonas viridis* reaction centre by Michel, Deisenhofer and colleagues [32,33]. Moreover, a close functional similarity between the bacterial and eukaryotic reaction centres has long been assumed, based on spectrophotometric analyses of the electron acceptor reactions (see [34]) and on their similar sensitivities to triazine herbicides [35,36].

The bacterial reaction centre also contains a third subunit, termed H [37]. This subunit has no prosthetic group, and is not involved in the primary photochemical charge separation reactions [37,38]. The H-subunit of the *Rps. viridis* reaction centre has a single membrane-spanning α-helix at the N-terminus (between residues Ile-12 and Arg-37). The remainder of the molecule (with the exception of the sequence between residues Leu-48 and Tyr-56 which is disordered in the crystal) forms a globular domain on the cytoplasmic side of the photosynthetic membrane [32,33]. Studies with LM complexes, depleted of the H-subunit, have suggested that the H-subunit is needed for the full reduction of plastoquinone to plastohydroquinone at the Q_B binding site. The removal of the H-subunit was shown to cause a tenfold decrease in ubiquinone binding at the Q_B site, which would account for the increased stability of Q_A^- in the LM complexes [38].

I propose that the 9 kDa (phospho)protein of thylakoid membranes is the eukaryotic equivalent to the H-subunit of the anoxygenic bacterial reaction centre. The 9 kDa (phospho)protein functions to ensure a high quantum efficiency for the primary photochemical charge separation reactions in PS II by aiding the transfer of reducing equivalents out of the reaction centre to the attendant quinone pool. This is achieved by promoting the binding of quinone to the Q_B pocket. It is envisaged that phosphorylation of the 9 kDa thylakoid membrane protein may be expected to affect the conformation of the Q_B pocket and thereby increase Q_A^- stability by decreasing plastoquinone binding to the PS II reaction centre. This model predicts that full plastoquinone reductase activity in the isolated PS II reaction centre preparation [28] requires the reconstitution of the D1-D2 heterodimer with the 9 kDa (phospho)-protein.

Fig.2 shows a comparison of the amino acid sequence of the 9 kDa (phospho)protein to the

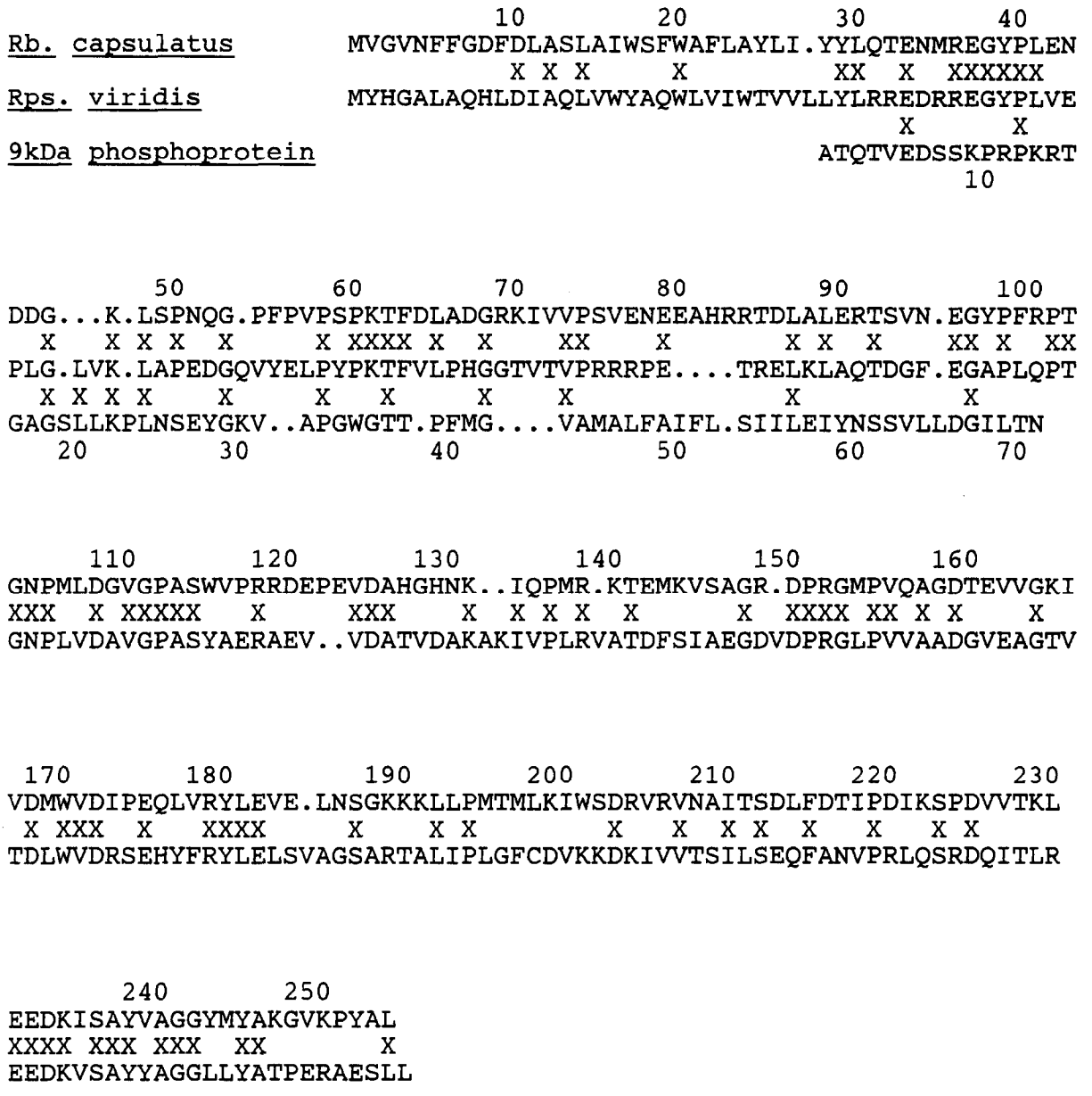

Fig.2. Comparison of the amino acid sequences of the reaction centre H-subunit from *Rps. viridis* [39] and *Rb. capsulatus* [40] and the thylakoid membrane 9 kDa (phospho)protein [9].

bacterial H-subunit from two photosynthetic eubacteria, *Rps. viridis* [39] and *Rb. capsulatus* [40]. The two bacterial sequences can be aligned to give 38% identity, indicating that the H-subunit is not strongly conserved. The amino acid sequence of the 9 kDa polypeptide can be aligned to the 'consensus' prokaryotic sequence to give 18% identity (table 1). A higher identity (22%) can be obtained if one aligns the 9 kDa (phospho)protein against the H-subunit of *Rps. viridis*. Moreover, a 23% identity (with the inclusion of 4 insertions/deletions) can be obtained if one aligns only the N-terminal amino acid sequence of the 9 kDa (phospho)protein (from Ala-1 to Thr-39) which is

not thought to be involved in the membrane-spanning α-helix. Thus, in the light of the low level of conservation in the primary structures of the H-subunits from the two bacterial species, it is tempting to speculate that the N-terminus of the 9 kDa (phospho)protein may be structurally similar to the H-subunit of the photosynthetic bacteria and that both have evolved from a common ancestral reaction centre protein. I have also compared the amino acid sequence of the 9 kDa (phospho)protein with the primary structure of the putative ubiquinone-binding protein (QP-C) of the cytochrome b-c_1 complex of the mitochondrial respiratory chain [40]. The low identity (less than 10%) indicates that there is no homology between these two proteins: a result which is not surprising if neither the 9 kDa (phospho)protein nor the bacterial H-subunit directly bind quinone.

5. A POSSIBLE PHYSIOLOGICAL ROLE OF THE 9 kDa (PHOSPHO)PROTEIN IN PROTECTING PS II CENTRES AGAINST PHOTOINHIBITION

The physiological role of the 9 kDa (phospho)protein remains unclear. Allen and Holmes [42] have proposed that the phosphorylation of this protein assists in the lateral migration of (phospho)LHC II from the PS II centres in the appressed thylakoid lamellae to the PS I centres located in the non-appressed regions. Other authors suggest the phosphorylation of the PS II-associated proteins might be involved in either the correct assembly of the PS II complex [26], or in ear-marking the proteins for degradation [43]. However, thylakoid membrane protein phosphorylation has been shown to partially protect PS II from photoinhibition [22]. Although this must, in part, be a consequence of the decreased light-harvesting capacity of PS II resulting from the displacement of (phospho)LHC II, Horton and Lee [22] have suggested that a cyclic electron transport around the PS II reaction centre could be an additional protective mechanism. Such a pathway around the PS II reaction centre would serve to remove the charged radicals possibly involved in the photoinhibitory effect. As outlined above, the phosphorylation of the 9 kDa protein inhibits the photochemical reduction of plastoquinone and promotes the re-oxidation of Q_A^- by S2 [17]. Thus, the 9 kDa (phospho)protein can be said to activate a cyclic electron transport around the PS II reaction centre. These results are therefore consistent with the proposal by Horton and Lee [22] and indicate a possible physiological role of the 9 kDa (phospho)protein in helping protect PS II complexes from photoinhibition by catalysing the sequestration of radicals generated within the reaction centre.

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