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The 64 kDa polypeptide of spinach may not be the LHCII kinase, but a lumen-located polyphenol oxidase

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Abstract **Phosphorylation of chlorophyll *a/b*-binding proteins of the of photosystem II light-harvesting assembly controls the energy distribution between the two photosystems as well as the turnover of thylakoid membrane proteins. The LHCII kinase, suggested to be a 64 kDa protein, is light-regulated by a mechanism involving reduction of plastoquinone and the participation of the cytochrome b_6/f complex. A cDNA encoding that protein has been isolated from a lambda gt11-based library made from spinach polyadenylated RNA using a two-step strategy involving screening by polyclonal monospecific antisera and plaque hybridization. The protein of 73.1 kDa molecular mass represents a precursor which contains a bipartite transit peptide of 101 amino acid residues (11.0 kDa) that directs the protein into the thylakoid lumen. It can be phosphorylated in vitro, and exhibits significant homology to plant polyphenol oxidases, not to kinases. The gene was therefore designated *PpoA*. Reinvestigation of components in the molecular mass range of 50–70 kDa disclosed five additional proteins which can accompany kinase-active cytochrome b_6/f, photosystem II and AMS [1] preparations. Four of them can be phosphorylated in vitro; two with apparent molecular masses of 53 and 66 kDa are capable of phosphorylation, and represent new, yet unidentified proteins.**

Key words: Thylakoid kinase; LHCII phosphorylation; PPO; cDNA

1. Introduction

The thylakoid-bound protein kinase responsible for the phosphorylation of the chlorophyll *a/b*-binding proteins of the photosystem II light-harvesting complex (LHCII; [2]) as well as of the 43 kDa, D2 and D1 photosystem II core proteins and the 9 kDa protein identified as the product of he *psbH* gene [3] represents an unique signal transduction system characteristic of the photosynthetic membranes of oxygen-evolving organisms. The uniqueness of this regulatory protein kinase resides in its activation by light via a sensing mechanism based on changes in the redox state of the photosynthetic membrane [2,4–6]. Evidence as it stands suggests that the state of the plastoquinone pool reduction and the interaction of plastoquinol with the cytochrome b_6/f complex mediate the kinase activation/deactivation process [7–10].

Despite various efforts, the identities of the kinase and of the corresponding phosphatase [11] have remained enigmatic, and all attempts to isolate the constituent polypeptides of this redox-controlled kinase system have failed so far. Following several publications demonstrating that a protein isolated from spinach thylakoids migrating as a single polypeptide band of 64 kDa exhibits histone, LHCII as well as self phosphorylation activity it was inferred that this protein may be responsible for the phosphorylation of LHCII [12–14]. However, the isolated kinase did not exhibit redox control. Using a different approach fractions enriched in the cytochrome b_6/f complex could be obtained exhibiting kinase activity with histone as a substrate as well as self phosphorylation. Such preparations phosphorylated isolated LHCII with a substantial degree of redox control [1] and their polypeptide composition besides the components of the cytochrome complex included a minor polypeptide with an apparent molecular mass of 64 kDa. The N-terminal amino acid sequence of this protein from such a preparation was determined [15], and it appeared that it was identical with that of the putative 64 kDa protein kinase (G. Hind personal communication, also plenary lecture at the 1992 Harden Conference).

In this communication we describe the isolation and characterization of a full-size cDNA of the 64 kDa polypeptide from spinach. The results of this analysis cannot be reconciled with the idea of a kinase. This finding prompted us to pursue the search for the protein, i.e. to reinvestigate various subthylakoidal fractions enriched in protein kinase activity and to monitor for potential kinase components in various molecular mass ranges.

2. Materials and methods

2.1. Construction of phage-based expression libraries; library screening

Screening of the cDNA library, constructed from polyA$^+$-RNA isolated from etiolated spinach cotyledone after 16 h illumination, was done as previously described [16].

2.2. Thylakoid fractionation; phosphorylation and protein analyses

Photosystem II and cytochrome b_6/f complex were isolated according to [17,18], respectively, AMS fractions with high kinase activity according to [15]. Phosphorylation assays were performed as described in [7].

The kinase renaturation assay was carried out according to a modified protocol of [19]. Kinase-active fractions were separated on 10–17.5% SDS/polyacrylamide gradient gels [20], electroblotted onto a PVDF membrane (Immobilon P, Millipore, Bedford, USA) in 25 mM Tris, 192 mM glycine buffer, pH 8.2, containing 10% methanol. The membrane was then incubated in 7 M guanidinium hydrochloride, 50 mM Tris/HCl, pH 9.5, 50 mM DTT and 2 mM EDTA. After rinsing in 30 mM Tris/HCl, pH 7.5, proteins were renatured over night at 4°C in 10 mM Tris/HCl, pH 7.5, 140 mM NaCl, 2 mM DTT, 2 mM EDTA, 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1% BSA, 0.05% casein, and 0.1% Nonidet P-40. During this step the kinase substrate casein bound to the membrane. The membrane was then saturated with 1.2% casein, 5% BSA

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in 30 mM Tris/HCl, pH 7.5. Phosphorylation was performed for 1 h at room temperature in a buffer consisting of 30 mM Tris/HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM $MnCl_2$, 1 mM $CaCl_2$ in the presence of 100 μCi of [γ-^{32}P]ATP. The membrane was then washed twice with 30 mM Tris/HCl, pH 7.5, once in the same buffer in the presence of 0.05% Nonidet P-40, and again twice in the first buffer. Loosely bound [γ-^{32}P]ATP was removed by incubation of the membrane in 1 M KOH for 15 min, the membrane neutralized with 30 mM Tris/HCl, pH 7.5, air dried, and exposed to Kodak Biomax X-ray film.

Proteins were electrophoretically separated over the entire width of slab gels, individual bands were excised, equilibrated in buffer (0.125 Tris/HCl, pH 6.8, 1 mM EDTA and 0.1% SDS), and loaded on a 12.5% polyacrylamide concentrating gel. For N-terminal sequencing with an Applied Biosystems 475A Gas Phase Microsequencer, the proteins were electroblotted onto Glassybond GB10 membrane (Biometra, Göttingen) according to the manufacturer's recommendations.

3. Results and discussion

3.1. Protein and phosphorylation patterns

Among various subthylakoidal fractions checked, protein phosphorylation could be observed with purified cytochrome complex, AMS preparations [1], remarkably also with purified photosystem II (PSII), which may contain minor components in the molecular mass range of 60–65 kDa [17]. No activity was found with photosystem I assembly and ATP synthase. Closer inspection of the protein patterns of kinase-active AMS, cytochrome complex and PSII preparations on high resolution SDS/polyacrylamide gels revealed up to six minor bands in the range of 50–70 kDa, designated AMS-5 to -10 (Fig. 1A, lanes 1–3; cf. also [21]). Some of these comigrated with minor components detectable in purified PSII and cytochrome b_6/f complex. Determination of the N-terminal amino acid sequences for all these components uncovered that AMS-8, with an apparent molecular mass of 62 kDa estimated under conditions of high resolution, was identical with the previously published component [15]. AMS-7 respresents most likely an Hsp60 homolog; AMS-6 does not exhibit homology to any known protein or translated nucleic acid sequence, and AMS-5, -9 and -10 were N-terminally blocked. The details of this analysis will be presented elsewhere.

Assays of the kinase-active AMS, PSII and cytochrome b_6/f preparations in the presence of [γ-^{32}P]ATP [15], separation of the labelled products on high resolution SDS/polyacrylamide gels and fluorography showed that AMS-6, -8, -9 and -10, and to a significantly lesser extent bands in the PSII and cytochrome fractions comigrating with AMS-8 and -9 became phosphorylated (Fig. 1B, lanes 1–3). Provided the latter represent identical components, this result suggests that either less kinase is present in PSII and cytochrome assemblies as compared to AMS preparations, or the kinase becomes partially inactivated during purification of these membrane complexes.

3.2. Clone selection and characterization

AMS-8 identical with the 64 kDa component, which also yielded by far the strongest phosphorylation signal (Fig. 1B, lane 1), was chosen for further analysis. Six positive recombinant phage were serologically isolated after screening 10^6 *pfu* of a lambda gt11-based cDNA library with a mixture of two polyclonal antisera elicited against the purified protein and an N-terminal oligopeptide of the 64 kDa component, respectively [1]. Five of the cDNAs isolated were identical in sequence in their overlapping segments. A 5′ terminal 193 bp *Eco*RV (restriction site in the vector)/*Xmn*I fragment of the largest insert (1353 bp) was chosen for library rescreening [22], and yielded a single positive phage which contained the entire protein coding region.

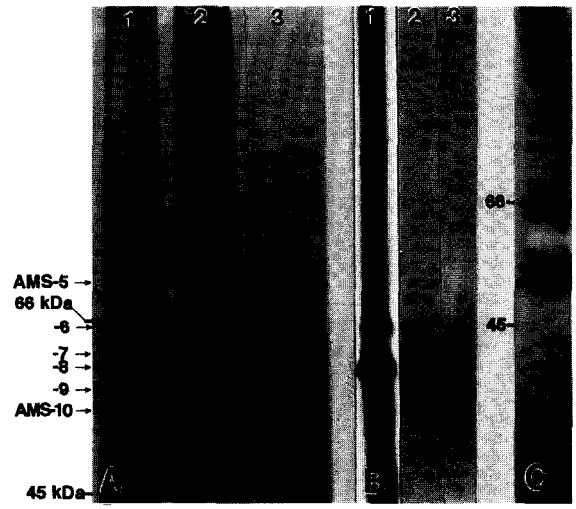

Fig. 1. High resolution electrophoresis of kinase-active subthylakoidal fractions. (A) Silver staining of an AMS fraction (lane 1), photosystem II (2), and cytochrome complex (3). (B) Autoradiograph of the phosphorylation patterns with the fractions shown in Panel A. Panels A and B illustrate the same 7.5% SDS/polyacrylamide gel, but with different slot widths. Note that the major components of the membrane complexes, such as cytochrome *f*, that have migrated out of the gel would form bands of 3–4 mm width under the chosen loading. (C) Autoradiograph illustrating protein kinase activity after protein renaturation on a blot. Primary separation of proteins was on a 12.5% SDS/polyacryamide gel (see text). Left: molecular weight standards or AMS-components.

The nucleotide sequence of the 2107 bp insert of that phage is deposited at the EMBL Databank, accesssion number x. Translation of this sequence uncovered an open reading frame of 638 amino acid residues with a deduced mass of 73.1 kDa, and 24 and 169 nucleotides of 5′ and 3′ untranslated regions, respectively (Fig. 2). The assumed initiation codon is surrounded by a nucleotide sequence that correlates well with the canonical sequence deduced for translation start in plant cytosolic mRNAs [23], and is preceded by an in-frame stop codon at position −19/−21. The determined N-terminal sequence of 15 residues [15] is found in the amino acid residues 102–116; the preceding segment displays features characteristic of bipartite transit peptides for nuclear-encoded plastid proteins. The presequence possesses an amino-terminal envelope transit signal which possesses a net positive charge and is rich in hydroxylated amino acid residues. It is followed by a thylakoid transfer signal which usually consists of a charged section, followed by a hydrophobic segment, helix-breaking and turn-inducing residues, and ends with a terminal processing site containing short-chain residues at positions −3 and −1 [24].

The search of the complete EMBL DNA Database uncovered unexpectedly that the deduced amino acid sequence exhibits similarity with plant polyphenol oxidases (Fig. 2, [25–28]). The Cu-containing metalloproteins from tomato, potato, pea and grapes share an overall 31%, 34%, 39% and 39% amino acid identity, respectively, including the potential heteroatom ligands. They are of nuclear origin, and that of tomato has

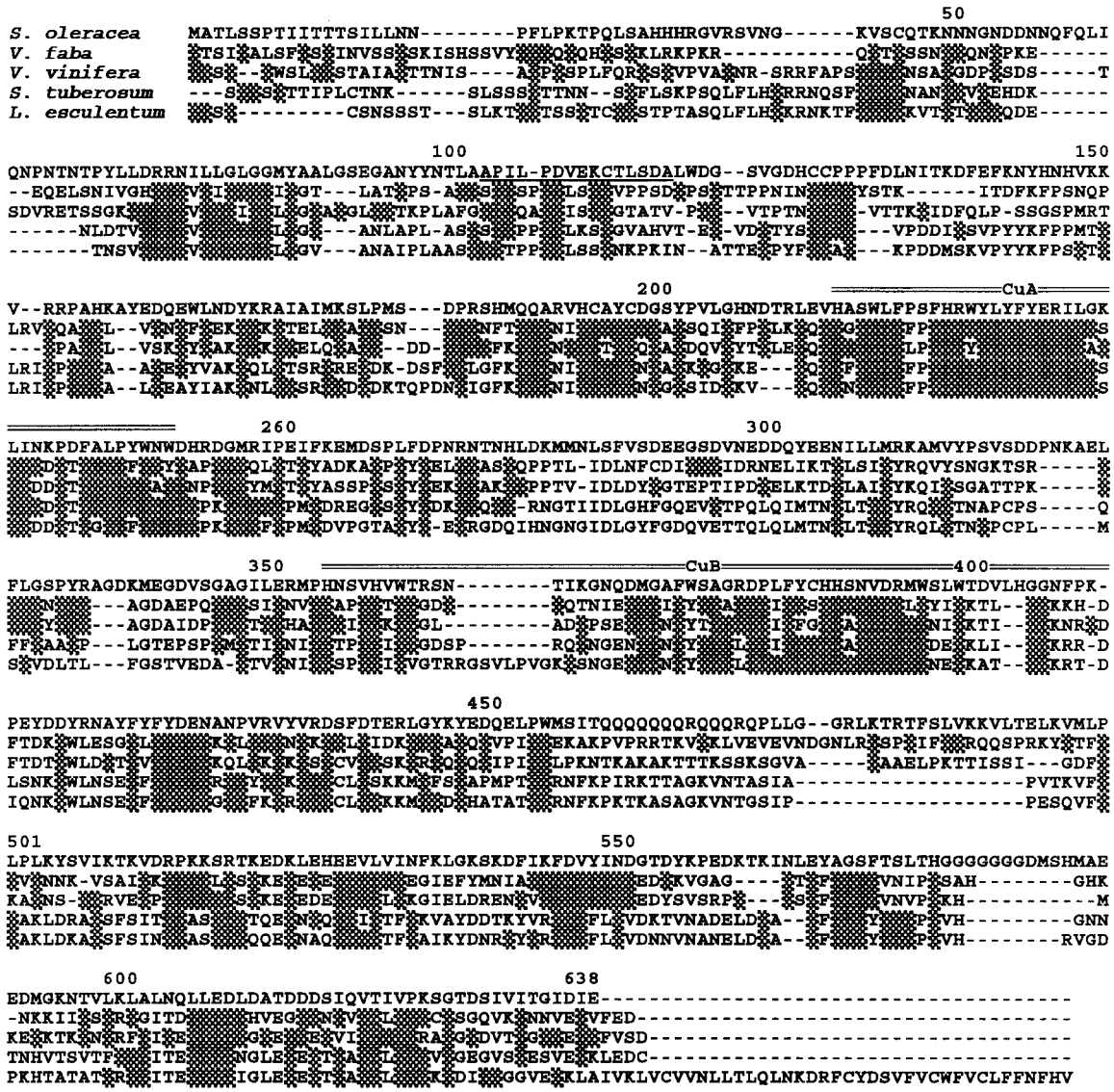

Fig. 2. Alignment of the deduced amino acid sequence of the putative polyphenol oxidase (PPO) from spinach with sequences from pea [25], grape [26], potato PPO B [27] and tomato PPO A [28]. Numbering refers to the spinach sequence. Amino acid residues identical with the spinach PPO are represented by shaded boxes; gaps are marked by dashes. The terminal processing site between the transit peptide and the mature protein is indicated by an arrowhead; the determined N-terminal sequence of the protein [15] is underlined. The highly conserved copper binding sites of the PPOs are marked with double lines (CuA and CuB).

recently been demonstrated to be located in the lumen of thylakoid membranes [29], consistent with transit peptide secondary structure predictions.

In order to verify the targeting of the cDNA-encoded protein to the chloroplast and its location in the thylakoid lumen, the precursor protein made in vitro from the cDNA was incubated with isolated chloroplasts from spinach [30]. The in vitro translation product (Fig. 3, lane 1) could, in fact, be imported into the organelle, and is processed in two steps (Fig. 3, lanes 2 and 3) to a protein comigrating with AMS-8 in different gel systems (Fig. 3, lane 3, and data not shown). Different from the gamma subunit of the thylakoid-located ATP synthase and similar to plastocyanin, chosen as controls, the imported product was protease resistant, commensurate with its expected intraorganelle location in the thylakoid lumen (Fig. 3, lane 4). Assays in the presence of appropriate antimetabolites (nigericin) suggest that the protein follows the delta pH route across the thylakoid membrane, as the 23 and 16 kDa polypeptides of the oxygen-evolving system associated with photosystem II [data not shown, 31, 32]. These results are in agreement with data obtained with polyphenol oxidase from tomato. We infer from the outlined findings that the recombinant DNA isolated does not represent the thylakoid protein kinase, but encodes a lumenal polyphenol oxidase, and hence tentatively designate the corresponding gene *PpoA*. After this manuscript was submitted, Hind et al. [33] published independently the deduced amino acid sequence of a 64 kDa protein also inferred to represent a polyphenol oxidase. The sequence is identical to ours except of an Asp to Glu exchange at position 55 and an additional Thr residue between positions 409 and 410. The lumenal location of the protein was not demonstrated.

The physiological role of polyphenol oxidases in plants has

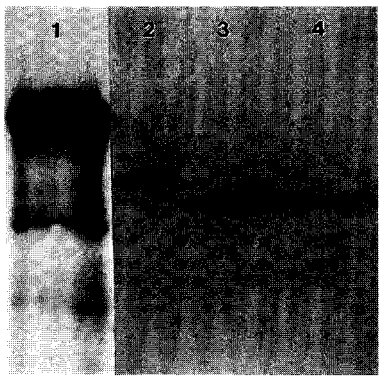

Fig. 3. In vitro transcription/translation of *PpoA* cDNA and import of the in vitro precursor product into isolated spinach chloroplasts. Fluorograph of a 12.5% denaturing SDS-containing polyacrylamide gel. (Lane 1) Translation of *PpoA* cDNA in a wheat germ extract; (lanes 2–4) products after import of the precursor and thermolysin treatment of the organelles, (2) stroma fraction, (3) thylakoid membranes, and (4) thylakoid membranes after treatment with thermolysin. For molecular weights see text.

not yet been unequivocally established [34], and various features of these proteins have remained enigmatic. The localization in the thylakoid lumen [cf. also 29] verified by the presence of a bipartite transit sequence, two-step processing, and protease protection suggests the possibility that the enzyme is sequestered and its substrates become available after mechanical damage [35]. However, polyphenol oxidases may be located in other subcellular compartments, including envelope membranes [36,37]. This is consistent with the finding that these enzymes are encoded by gene families [28], and that their expression is developmentally regulated [38]. Their amounts vary in different plant tissues; a transient, high concentration has, for instance, been observed in fruits prior to ripening [35]. Presently, there is no evidence that the enzyme is phosphorylated in situ in the lumenal space in agreement with the current view that ATP is not present in this compartment. All known thylakoid kinase(s) active sites are oriented toward substrates exposed to the matrix face of the membrane (LHCII and PSII polypeptides), and thus the lumenal enzyme may not be available to the kinase. However, it is conceivable that once liberated from the lumen into the chloroplast matrix, the protein may rapidly become phosphorylated as indicated by its phosphorylation in vitro in the AMS preparation. Furthermore, postmaturation processes such as controled proteolysis to different molecular sizes [39] may be involved in regulation. We have noted a distinct, non-phosphorylatable component of 39 kDa apparent mocleular mass with an identical N-terminal sequence implying that an 21 kDa C-terminal segment must have been removed (data not shown).

3.3. Search for kinase(s)

The outlined results rendered it highly unlikely that AMS-8, although strongly phosphorylated in vitro, respresents the proposed LHCII kinase. Since the phosphorylation assay is not free of pitfalls, for instance, a kinase may use various substrates present in addition to autophosporylation, a different strategy was employed to search for kinases [19]. This assay rests on the separation of kinase-active components in denaturing polyacrylamide gels, the Western transfer of the polypeptides to PVDF membranes, renaturation of the blotted proteins, and checking for their activity in the presence of casein and [γ-^{32}P]ATP. Alternatively, bands of appropriate molecular weight were excised after high resolution electrophoresis, and subjected to the same transfer, renaturation and assay procedures. Two kinase-active components with apparent molecular weights of 53 kDa (AMS-9) and 67 kDa (AMS-6) could be autoradiographically detected (Fig. 1C). None of them coincided with AMS-8. From our data it is obvious that three reasons caused the confusion as to the nature of the 64 (62) kDa polypeptide: that the protein is a highly efficient phosphorylation substrate (Fig. 1B, lane 1), the impurity of the subcellular fractions used [12–15], and the poor resolution of the chosen gel systems.

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