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Phosphorylation of cytochrome *b6* by the LHC II kinase associated with the cytochrome complex

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The cytochrome *b6* polypeptide present in cytochrome *b6/f* preparations from spinach thylakoids is phosphorylated concomitantly with the autophosphorylation of the 64 kDa polypeptide identified as the redox-controlled LHCII kinase. The N-terminal sequence of the 64 kDa kinase and sequence analysis of cytochrome *b6* indicate the existence of putative phosphorylation sites in both proteins.

Cytochrome *b6*; LHCII kinase; N-Terminal sequence; Thylakoid membrane

1. INTRODUCTION

The redox-controlled, thylakoid-associated LHCII kinase is considered to be responsible for the regulation of energy distribution between photosystem II and I [1]. We have previously demonstrated that a fraction obtained from spinach thylakoids enriched in the cytochrome *b6/f* complex contains a protein kinase activity using both histone and isolated LHCII as substrates [2]. Based on the previous identification of a thylakoid-bound kinase from spinach as a 64 kDa polypeptide and its autophosphorylation properties [3], we have tentatively identified the 64 kDa polypeptide present in preparations of the purified cytochrome complex as being responsible for this phosphorylation activity. While the spinach LHCII kinase purified to homogeneity did not exhibit redox-controlled activation [4], the preparations containing both the cytochrome *b6/f* complex and the 64 kDa component, displayed an enhanced LHCII phosphorylation when reduced plastoquinone-1 was added. This activity was expectedly inhibited by DBMIB [5]. Collectively, these findings suggested that the 64 kDa polypeptide found in cytochrome *b6/f* preparations represents the redox-controlled LHCII kinase. Here we report studies on the enzyme in the *b6/f*-kinase complex including the determination of its N-terminal amino acid sequence to gain further information on the nature of the compound and with the purpose to isolate the corresponding gene(s).

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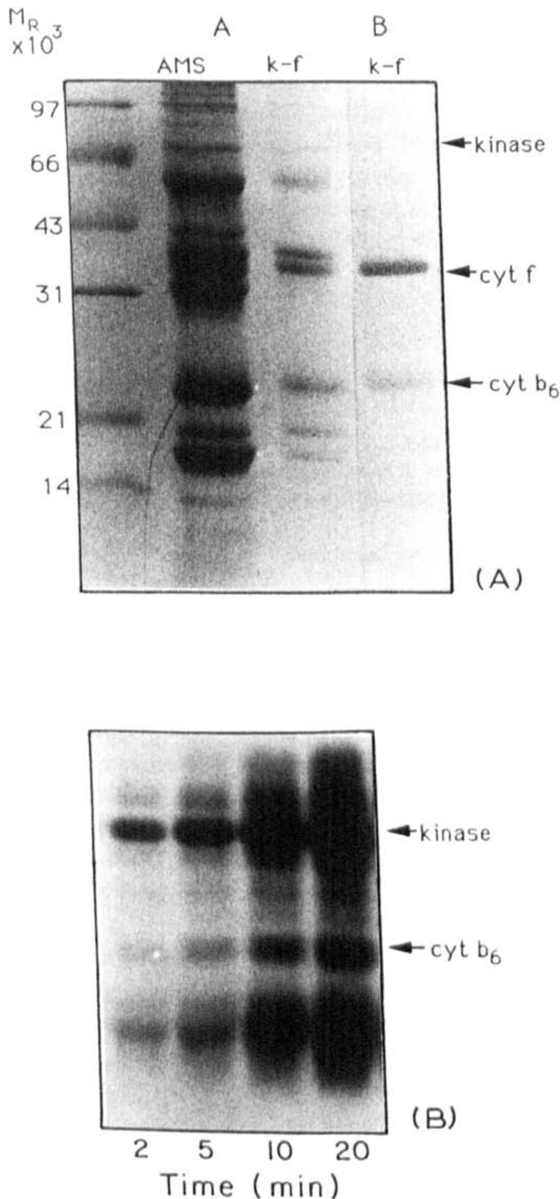
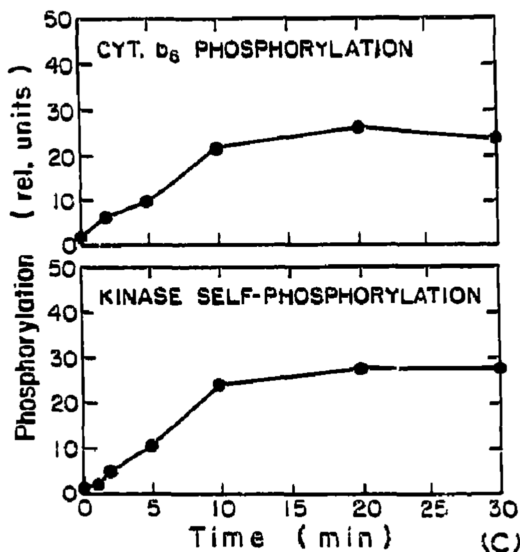
2. MATERIALS AND METHODS

The cytochrome kinase-active fractions used (K-F; cf. ref. 5) were prepared according to the following purification steps: (i) solubilization of spinach membranes by octyl-glucoside; (ii) ammonium sulfate fractionation of the solubilized membrane material; and (iii) chromatography of the dissolved compounds from the resulting pellet (AMS) on histone-Sepharose columns as detailed previously [5]. The affinity-purified fractions were incubated with [γ-^{32}P]ATP and the time course of the phosphorylation was monitored.

3. RESULTS AND DISCUSSION

Predominantly three components were heavily phosphorylated under the chosen experimental conditions: the 64 kDa polypeptide (LHCII kinase), an as yet unidentified 14 kDa polypeptide which is barley detectable by Coomassie blue staining and could be identical with the 14 kDa polypeptide phosphorylated in a redox-dependent fashion in isolated intact chloroplasts [6], and, relevant for this study, cytochrome *b6* (identified by heme staining; Fig. 1B). Although the total amount of radioactive phosphate incorporated into cytochrome *b6* is somewhat lower than that of the 64 kDa and 14 kDa polypeptides, the rate of phosphorylation is very similar (Fig. 1C). These results indicate that phosphorylation of cytochrome *b6* constitutes one of the stages creating a functional association between the LHCII kinase and the cytochrome *b6/f* complex [5]. This is commensurate with the recent finding that a fraction of the cytochrome complex migrates from grana to stroma lamellae during state I to state II transition. It was also proposed that the redistribution of cytochrome complex may be related to the mechanism of cyclic electron flow activation around photosystem I [7]. The observations that the kinase is localized primarily at the fringe of grana stacks within a region of approximately 40 nm [5], Gal et al., in preparation) and associated with the cytochrome complex, suggests that its activity is involved in both,





← Fig. 1. Cytochrome *b6* is phosphorylated concomitant to the self-phosphorylation of the 64 kDa kinase in cytochrome *b6/f* complex fractions enriched in LHCII kinase (K-F). Panel A: the polypeptide pattern (A) and heme staining (B) of fractions enriched in the cytochrome *b6/f* complex containing LHCII kinase (K-F, cf. ref. 5). Panel B: autoradiogram of a phosphorylated K-F fraction. K-F fractions (0.3 mg protein/ml) were incubated in a reaction mixture (0.5 ml) containing [γ-^{32}P]ATP (0.25 mM, 1.000 cpm/pmol), 50 mM Tris-HCl, pH 8.0, 10 mM $MgCl_2$, at 25°C. At the desired time points samples were withdrawn, precipitated by 10% cold thrichloroacetic acid and subjected to SDS-PAGE followed by autoradiography. Lanes from left to right, samples incubated for 2, 5, 10 and 20 min, respectively. Panel C: phosphorylation kinetics of the 64 kDa polypeptide (LHCII kinase) and of cytochrome *b6*. K–F fractions were incubated as described in panel B. At the desired time points the samples were withdrawn for SDS-PAGE and autoradiography. The degree of phosphorylation was determined using scanning densitometry of the respective bands. The identification of the specific polypeptides was done either by Western blotting (kinase) or heme staining (cytochrome *b6*).

phosphorylation and lateral diffusion of LHCII as well as of cytochrome complex. The outlined localization of the kinase could therefore facilitate the redistribution of *both* protein complexes between grana and stroma thylakoids.

The 64 kDa polypeptide resolved by SDS-PAGE from the purified cytochrome complex was electroblotted on polyvinylidene-difluoride (PVDF) of Glassybond (Biometra, Göttingen) membranes [8] and subjected to N-terminal amino acid sequencing by Edman degradation using an Applied Biosystem 475A gas Phase Microsequencer. To assess the identity of the 64 kDa band, a phosphorylated sample was run in parallel with the bulk sample and used as an internal marker for the phosphorylated 64 kDa band. The result of six independent experiments (15–20 pmol protein/run) is the N-terminal sequence **A - P - I - L - P - D - V - E - K - S - T - L - S - D - A**. This sequence is identical to that of the kinase isolated from intact spinach thylakoids (G. Hind, personal communication). As judged from cross-reactivity of antibodies prepared against the spinach protein, the 64 kDa kinase from spinach should contain epitopes similar to those present in the putative 64 kDa LHCII kinase from thylakoids of other species including pea, *Lemna* [9], as well as *Chlamydomonas* and the prokaryote *Prochlorotrix* (data not shown).

The N-terminal sequence of the 64 kDa polypeptide presents three potential phosphorylation sites (Ser-10, Ser-13 and Thr-11) in agreement with the previously reported data indicating that the kinase contains phosphorylated Thr and Ser residues [9,10]. It is interesting to note in this context that LHCII apoprotein substrate can be phosphorylated on a Ser or Thr residue close to its N-terminus [12]. Considering the revised enumeration of amino acid residues, potential phosphorylation sites in the cytochrome *b6* polypeptide, in turn, could be Ser-23 and/or Thr-22 in the first, stroma-located

[13,14] hydrophilic loop of the N-terminal sequence, or, probably less likely, Thr-107 in the small central, and Ser-212 in the terminal stroma-located segments of the polypeptide chain. All these residues are remarkably conserved in the corresponding tobacco protein [15].

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