PURIFICATION AND CHARACTERIZATION OF A THYLAKOID PROTEIN KINASE.

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1. INTRODUCTION

Although protein kinases are intimately involved in the control of metabolism in animal cells, little is known about the properties and functions of such enzymes in plants. The one exception is the well documented control of State transitions in the thylakoid by reversible phosphorylation of LHC-II (the light-harvesting chlorophyll a/b protein complex of photosystem II). The kinase catalyzing this phosphorylation is associated with the thylakoid membrane, and is regulated by the redox state of the plastoquinone pool (1).

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Lin et al. (2,3) reported the isolation and partial purification from spinach thylakoids of two protein kinases (CPK1, CPK2) of apparent molecular masses 25 kDa and 38 kDa. Neither enzyme could utilise isolated LHC-II as a substrate. The partial purification of a third protein kinase (LHCK) which can utilise both lysine-rich histones (IIIs and Vs) and isolated LHC-II as substrate was recently reported (4). This enzyme has now been purified to homogeneity (5) and characterized by SDS-polyacrylamide gel electrophoresis as a 64 kDa peptide.

From a comparison of the two isolation procedures used by ourselves and by Lin et al. we have concluded (6) that CPK1 is indeed a protein kinase, but has a low specific activity (between 100-200 times lower than that of LHCK) and accounts for w10% of total membrane kinase activity. CPK2, however, was identified as ferredoxin:NADP⁺ oxidoreductase (FNR) contaminated with LHCK.

2. MATERIALS AND METHODS

LHCK was extracted and purified from NaBr-treated spinach thylakoids as described by Coughlan and Hind (5). CPK1 and CPK2 were isolated as in (6).

Photoaffinity labelling of membranes and kinase-containing fractions with 8-azido-[γ -³²P]ATP was carried out as in Coughlan and Hind (6).

3. RESULTS AND DISCUSSION

LHCK was partially purified by selective membrane solubilisation in octylglucoside/cholate, ammonium sulfate precipitation, and desalting of the resolubilised pellet with a BioGel P300 column as in (4). The predominant proteins present were the four peptides of the cytochrome b/f complex and a 37 kDa peptide corresponding to FNR (7). When this mixture was fractionated on a sucrose density gradient as in (5), the peak of kinase activity sedimented to fractions 8-10. This peak was clearly separated from the plastoquinol:plastocyanin oxidoreductase activity of the cytochrome complex, but overlapped the zone containing diaphorase activity (Figure 1). The peaks of kinase and diaphorase activity were nonetheless distinct. SDS-polyacrylamide gel

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electrophoresis of the gradient fractions, followed by silver staining, revealed numerous peptides in the region associated with the peak of kinase activity (Figure 2). The 37 kDa peptide (FNR) was especially prominent.

FIGURE 1 (top). Enzymatic profile of density gradient fractions of P300 eluate. Procedures and assays are described in (5). Activities (as % of totals) are ●---●, plastoquinol:plastocyanin oxidoreductase; ○---○, FNR; ▲---▲, histone kinase. Δ---Δ, protein content; ---, sucrose concentration (w/v).

FIGURE 2 (bottom). Polypeptide composition of the density gradient fractions corresponding to Figure 1.

Fractions enriched in kinase activity were pooled and applied to a histone IIIs-Sepharose affinity column (5). All diaphorase activity and over 90% of the total applied protein was recovered in the throughput and initial washings. In contrast, all kinase activity was

bound to the column and was specifically eluted between 0.25-0.5M NaCl. SDS-polyacrylamide gel electrophoresis of these fractions revealed that all kinase activity was associated with a single peptide of apparent molecular mass 64 kDa (Figure 3).

FIGURE 3. Affinity chromatography of the kinase-enriched density gradient fractions on a histone IIIs-Sepharose column: denaturing gel electrophoretic analysis of column fractions, as described in (6).

The enzymatic characteristics of this kinase have been explored in some detail (5). In summary, the enzyme has a strong specificity for lysine-rich histones (IIIs, Vs) and will also phosphorylate casein. Isolated LHC-II is utilized as a substrate, but supports phosphorylation rates only 10-30% of those seen with histone IIIs. In common with many soluble kinases from animal cells, the enzyme also undergoes autophosphorylation (5).

The source of the discrepancies between the above findings and the work of Lin et al. (2,3) have been investigated. We found that the photoaffinity label 8-azido-ATP was ineffectual as a means of identifying thylakoid protein kinases in this system (6). Figure 4 shows the result of reacting spinach thylakoid membranes with the 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$, followed by denaturing gel electrophoresis, staining of the gel with Coomassie Brilliant Blue and autoradiography of the dried gel. Two major bands were labelled, of apparent masses 37 and 23 kDa. Labelling was light dependent and was abolished by preincubating with either 1 mM ATP or 8-azido-ATP. Treatment of membranes with NaBr had no effect on the labelling pattern, though incubation with the zwitterionic detergent CHAPS, which removes membrane bound FNR (8) abolished labelling at 37 kDa. LHCK was not labelled under any of the experimental conditions that we employed.

An explanation for this is provided by data on the inhibition of phosphorylation by 8-azido-ATP. This analog proved to be only a weak competitive inhibitor of LHCK ($K_i=200\text{ }\mu\text{M}$) whether the enzyme was purified or membrane-bound. In conjunction with the very low amounts of LHCK present in thylakoids (about 0.02% total membrane protein),

and the low cross-linking efficiency obtained with photoaffinity labels (typical values lie in the range 0.01-0.1%), this low an affinity for the ATP-binding site can readily account for the lack of detectable LHCK labelling by 8-azido-ATP.

FIGURE 4. Photoaffinity labelling of thylakoid membranes with 8-azido- $[\gamma\text{-}^{32}\text{P}]\text{ATP}$. Lanes 1 and 3, control membranes with MgCl_2 ; lane 2, control membranes without MgCl_2 ; lane 5, as lane 1 with 1 mM 8-azido-ATP present during photo-labelling; lane 6 membranes washed with NaBr ; lane 7, dark control of lane 1; lane 8, CHAPS-washed membranes.

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