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IDENTIFICATION OF CHLOROPLAST THYLAKOID PHOSPHOPROTEINS. EVIDENCE FOR THE ABSENCE OF PHOSPHORYL-POLYPEPTIDE INTERMEDIATES IN THE ATPase COMPLEX

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Summary: About 20 chloroplast thylakoid membrane polypeptides in the mol. wt. range from 5000 to 70,000 are phosphorylated under in vivo conditions. The major phosphoproteins were identified as the apoprotein b of the LHCP complex and the smallest subunit of cytochrome b 563. Radioactivity was also associated with polypeptide bands containing the apoprotein a of the LHCP complex, cytochrome f, the apoprotein of the reaction center chlorophyll-protein of PS II, the ferredoxin-$NADP^+$-reductase and the proteolipid of the ATPase complex. The subunits of the stromal and membrane-bound RuDP carboxylase as well as the ATPase complex including the proteolipid subunits were not phosphorylated.

Chloroplast thylakoid protein phosphorylation is involved in the regulation of energy distribution between the photosystems (1). Light-dependent phosphorylation by a membrane-bound protein kinase which uses ATP as phosphoryl group donor (2) occurs mainly in the components of the LHCP complex and a 9,000 molecular weight polypeptide (3,4).From recent experiments it has been concluded that the DCCD-reactive proteolipid subunits of the ATPase complex becomes phosphorylated on exposure of chloroplasts to light (5,6). Phosphorylation also occurs in the mitochondrial proteolipid fraction (7-9) or the proteolipid subunits of the oligomycin-sensitive ATPase complex (10) as well as polypeptides belonging to the animal cell transport ATPases (11-13) and the H^+-translocating *Neurospora* plasma membrane ATPase (14). It has therefore been speculated that phosphoryl-polypeptide intermediates of the ATPase complexes of energy-transducing membranes could play a role in ATP synthesis or the regulation of this process (15). Because of the significance of this conception for a better understanding of the mechanism of ATP synthesis the *in vivo* phosphorylation of thyla-

Abbreviations: DCCD, dicyclohexylcarbodiimide; LHCP, light-harvesting chlorophyll a/b-protein; RuDP, ribulosediphosphate; SDS, sodium dodecylsulfate;

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koid membrane polypeptides with special regard to the ATPase complex subunits has been reexamined in the work described here.

MATERIALS AND METHODS

Plants of Vicia faba were grown for 3 weeks in the glashouse as described in (16). The upper part of a plant with 6 leaves was captured at the stem and stored in 5 ml solution of carrier-free [^{32}P]orthophosphate with 35 MBq/ml and incubated for 8 h in the dark and than 4 h in the light. Chloroplasts were isolated as fast as possible and the thylakoid membranes purified as described previously (17). The isolated thylakoid membranes were immediatly desintegrated with SDS buffer for gelelectrophoretic separation of the membrane polypeptides or with Triton X-100 at a chlorophyll to detergent ratio (mg/ml) of 50 in order to immunoprecipitate the ATPase complex and the RuDP carboxylase. Immunoprecipitation of these proteins was carried out as described in (18) using monospecific antisera prepared against the coupling factor CF_1 from Vicia faba and the carboxylase from Nicotiana tabaccum.

SDS/urea acrylamide gradient gel electrophoresis was performed as recently described (18). The gels stained with Coomassie brillant blue G 250 were directly exposed to X-ray sensitive film for autoradiography.

RESULTS AND DISCUSSION

Exposure of Vicia faba leaves to carrier-free [^{32}P] orthophosphate in the light results in the incorporation of radioactivity into at least 20 chloroplast thylakoid membrane polypeptide bands the molecular weights of which are in the range from about 5,000 to 70,000 (Fig. 1). Compared on the basis of molecular weights and the polypeptide labeling patterns, the components which become phosphorylated in vivo are very probably identical with those being identified as the products of in vitro phosphorylation of pea chloroplasts (2-4). This indicates that membrane protein phosphorylation does not depend on the cellular integrity of chloroplasts. The dominating thylakoid phosphoproteins have previously been identified as a polypeptide doublet belonging to the light-harvesting chlorophyll a/b-protein (LHCP) complex and a 9,000 molecular weight component (3,4,6). Since the LHCP complex contains two major subunits termed a and b which form common apo- and holoprotein bands during SDS polyacrylamide gel electrophoresis in the absence of urea (19) it remained to be established which of them are really phosphorylated. SDS/urea acrylamide gradient gel electrophoresis allows a successfull separation of all LHCP complex polypeptides (18,19) which was a prerequisite for the identification of the phosphorylated protein moieties. The results show that most

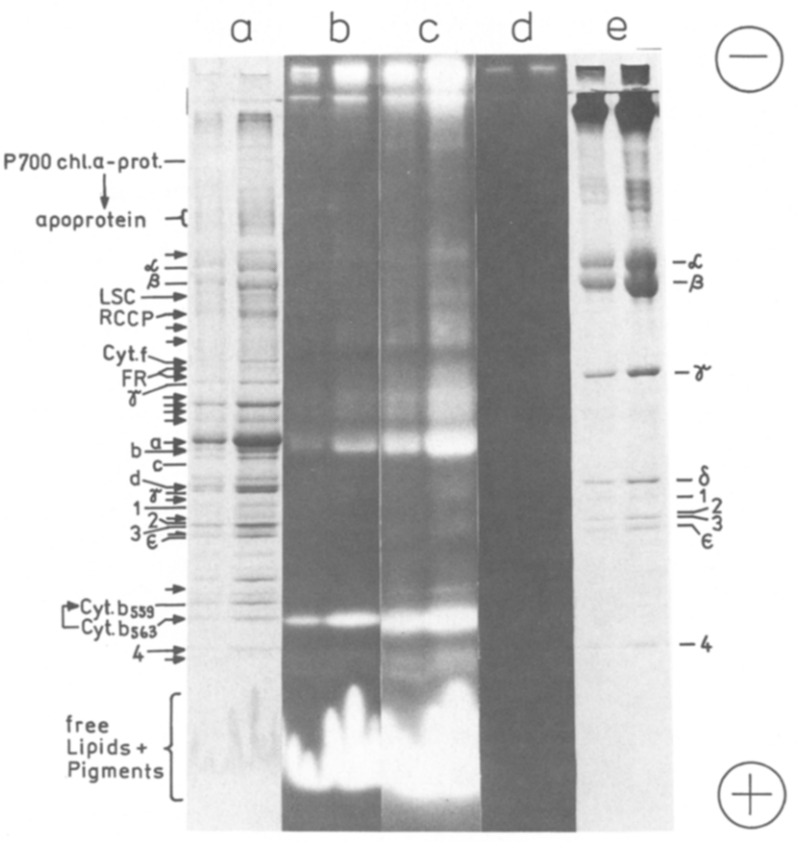

Figure 1. Identification of chloroplast thylakoid membrane polypeptides phosphorylated in vivo.
Plants were incubated with [^{32}P] orthophosphate in the light and chloroplast thylakoid membranes isolated as described under "MATERIALS AND METHODS". Thylakoid proteins (a) or the ATPase complex (e) which had been immunoprecipitated from Triton X-100 membrane extracts were fractionated by SDS/urea acrylamide gradient gel electrophoresis and the stained gel (a, e) was exposed to X-ray sensitive film for autoradiography. b, c, autoradiograms of a (expositon time: 30 h and 120 h, respectively); d, autoradiogram of e (exposition time: 120 h). The radiolabeled bands are indicated with arrows. α - ϵ (CF_1) and 1-4 (CF_0), ATPase complex (CF_1-CF_0); 4, proteolipid of the ATPase complex; LSC, large subunit of the RuDP carboxylase; P700 chl.a-prot. and RCCP, reaction center chlorophyll-proteins of photosystem I and II, respectively; a-d, polypeptides of the LHCP complex; FR, ferredoxin-NADP$^+$-reductase polypeptides.

of the [^{32}P] radiolabel was associated with the polypeptide b, while polypeptide a contained comparable less radioactivity. However, it remains an open question whether the radioactivity is indeed associated with subunit a or with the apoprotein of another chlorophyll-protein recently termed CP III or CP 29 (20,21)

having a similar electrophoretic mobility but does not belong to the LHCP complex (22). Of the other LHCP components previously termed c and d which have molecular weights of 23,000 and 22,000 only the latter is located in a band containing radioactivity.

On the basis of previous results which concern the identification of polypeptide bands in the gelelectrophoretic spectra of thylakoid membranes (18,23-25) it had been possible to identify some further components as phosphoproteins. [^{32}P] radiolabel was present in zones containing cytochrome f, the apoprotein of the reaction center chlorophyll-protein of PS II, the both polypeptides of ferredoxin-$NADP^+$-reductase preparations, the proteolipid of the ATPase complex and the large subunit of the RuDP carboxylase. It has been found, however, using the method of immunoprecipitation that both subunits of the stromal and membrane-bound RuDP carboxylase are not phosphorylated (not shown) indicating that another phosphopolypeptide comigrates with the large subunit of the enzyme during SDS gel electrophoresis of thylakoid polypeptides.

The major phosphopolypeptide of the thylakoid membrane has a molecular weight of about 8,000 under our conditions and is believed to be identical with the small component phosphorylated in vitro (2,4). It shows the same electrophoretic mobility as the smallest subunit of cytochrome b_{563} (18,25) which is an oligomeric lipoprotein containing 2 or 3 kinds of polypeptides (25,27). This component moves more slowly during SDS/urea polyacrylamide gel electrophoresis as compared with the proteolipid subunit of the ATPase complex. The band containing the proteolipid also shows incorporation of [^{32}P] under our conditions. There are experimental results which led to the conclusion that the ATPase complex proteolipid becomes phosphorylated on exposure of chloroplasts to light (5,6). These authors obtained [^{32}P] incorporation into the smallest polypeptide band of a crude ATPase complex preparation which had been extracted with octylglucoside/cholate from thylakoid membranes. Because of the significance of this finding the presence of radioactivity in the subunits of the ATPase complex which had been isolated by immunoprecipitation from Triton X-100 thylakoid extracts was investigated. As shown in Fig. 1 none of the nine ATPase complex subunits were found to be phosphorylated, although a high portion of radioactivity was still asso-

ciated with the purified immunoprecipitates. This [^{32}P] appears therefore to be incorporated into bound adenine nucleotides or is noncovalently attached to the protein complex. This led to the conclusion that in the preparation of (6) the proteolipid components formed a common band with the labeled cytochrome b_{563} subunit or the phosphorylated polypeptides comigrating with the proteolipid during SDS gel electrophoresis. This conclusion is supported by the fact that cytochrome b_{563} is released together with the ATPase complex and other proteins during membrane extraction with cholate (28). The results described here evidently show that the DCCD-binding proteolipid or any of the other subunits of the ATPase complex is not phosphorylated. It can therefore be excluded that protein phosphorylation is involved in opening and/or closing the H^+-ion channel of the chloroplast ATPase complex as previously speculated (15). Moreover, the absence of stable, long-living phosphoryl-polypeptide intermediates in the ATPase complex *in vivo* is contradictory to the phosphoenzyme intermediate hypothesis (29).

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