Differential phosphorylation of the light-harvesting chlorophyll–protein complex in appressed and non-appressed regions of the thylakoid membrane

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Received 8 October 1982

It is now established that the light-harvesting chlorophyll–protein complex (LHCP) of chloroplasts becomes phosphorylated in the light. In this study subfractionation of phosphorylated intact chloroplasts has been carried out to compare the phosphorylation of LHCP in non-appressed and appressed thylakoid regions. The results show around 10-times higher relative phosphorylation in the non-appressed regions than in the appressed ones. Since the non-appressed thylakoids also contain almost all photosystem 1, this region is likely to be the site for energy transfer from LHCP to photosystem 1 under phosphorylated conditions.

Protein phosphorylation *Thylakoid organization* *Chloroplast subfractionation*
Light-harvesting chlorophyll–protein complex *Energy transfer* *Membrane lateral heterogeneity*

1. INTRODUCTION

The light-harvesting chlorophyll *a/b* complex (LHCP) is the major constituent of the chloroplast thylakoid membrane [1]. Polypeptides of this complex become phosphorylated in the light by a membrane-bound protein kinase that is controlled by the redox state of the plastoquinone pool [2–4]. The phosphorylated LHCP can be dephosphorylated by a phosphatase [5]. This reversible phosphorylation has been suggested to regulate the distribution of light energy captured by LHCP to ensure a balanced input of energy to the two photosystems [6] so that unphosphorylated LHCP will canalize energy to PS2 and the phosphorylated form to PS1. It is not clear how a phosphorylation of LHCP would regulate the distribution of excitation energy. Any explanation, however, has to take into account a spatial separation of the two photosystems implied by fractionation [7–10] and ultrastructural [11] studies. Thus PS1 is mainly excluded from the appressed grana regions and concentrated in the non-appressed thylakoid regions, while the opposite holds for PS2. Moreover, the non-appressed regions contain only a minor portion (15–20%) of the LHCP whose main location is with PS2 in the grana partitions [9]. This lateral heterogeneity in the arrangement of the photosystems in stacked thylakoids suggests that interactions between phosphorylated LHCP and PS1 would occur primarily in the non-appressed regions. To investigate this possibility we have compared the degree of light-induced phosphorylation of LHCP in subfractions derived from non-appressed and appressed thylakoid regions. This study suggests that LHCP in the non-appressed thylakoids is about 10-times more phosphorylated than LHCP in the appressed thylakoids.

Abbreviations: Chl, chlorophyll; LHCP, light-harvesting chlorophyll *a/b*–protein complex; PS, photosystem; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis

Published by Elsevier Biomedical Press
001457593/82/0000—0000/$2.75

2. MATERIALS AND METHODS

2.1. *[^{32}P]Phosphate labelling of intact chloroplasts*

Intact chloroplasts were prepared from 600 g of dark-adapted spinach (*Spinacia oleracea* L.) leaves [12]. Chloroplasts were 80% intact according to the ferricyanide test [13]. Incubation with $^{32}P_i$ was based on the procedure of Bennett [5]. Chloroplasts (10 mg Chl in 40 ml of: 0.33 M sorbitol, 50 mM HEPES–KOH (pH 7.6), 10 mM KCl, 2 mM EDTA) were illuminated (500 $\mu E \cdot m^{-2} \cdot s^{-1}$) in the presence of carrier-free $^{32}P_i$ (3–5 mCi). After 5 min 360 ml of cold 5 mM $MgCl_2$ was added to break the intact chloroplasts. After a further 15 s 100 ml of cold 0.5 M NaF, 25 mM EDTA, 50 mM sodium phosphate (pH 7.4) was added to inhibit phosphatase activity. Thylakoids were sedimented at 4000 × *g* for 5 min, and washed twice in 100 mM NaF, 5 mM EDTA, 10 mM sodium phosphate (pH 7.4).

2.2. *Fractionation of thylakoids*

Washed thylakoids were disintegrated and fractionated [8,14] with some modifications to allow for the addition of the phosphatase inhibitor NaF. The first Yeda press medium contained 150 mM NaF, 50 mM sodium phosphate (pH 7.4). The homogenate was centrifuged for 30 min at 40000 × *g* and the resulting supernatant for another 1 h at 100000 × *g* yielding the Y-100 material. The 40000 × *g* pellet was resuspended in 100 mM sucrose, 5 mM sodium phosphate (pH 7.4), 10 mM NaF, followed by the second Yeda press treatment and phase partition. The phase system was changed to: 5.7% Dextran T500, 5.7% polyethylene glycol 4000, 20 mM sucrose, 20 mM sodium phosphate (pH 7.4) and 10 mM NaF. The membrane vesicles of the B3 fraction were collected by centrifugation.

2.3. *Electrophoresis and analysis of [^{32}P]phosphate*

Chlorophyll–protein complexes were resolved by mild SDS–PAGE [15] using 10% acrylamide gels and an SDS/chlorophyll ratio of 5 during solubilization. The gels were scanned at 675 and 650 nm to quantify the complexes [15]. Polypeptides were analyzed by denaturing SDS–PAGE using a 12–20% acrylamide gradient [16]. Gels were stained with Coomassie brilliant blue and scanned using a laser densitometer. LHCP apopolypeptides were quantified from their peak areas. All gels were sliced, solubilized in H_2O_2/perchloric acid and counted for radioactivity in Aquasol.

3. RESULTS

Washed thylakoids and the subfractions enriched in non-appressed (Y-100) and appressed (B3) thylakoids [9] were isolated from phosphorylated intact chloroplasts. The relative amounts of chlorophyll–protein complexes in these fractions were determined by a mild SDS–PAGE [15] that resolves two PS1 complexes (CPI + CPIa), one PS2 complex (CPa) and three bands of the light-harvesting complex ($LHCP^{1-3}$). As shown in table 1 the Y-100 fraction was markedly depleted in LHCP and the PS2 complex. In contrast the B3 fraction was enriched in these complexes while depleted in the PS1 complexes. This is in agreement with [9] and demonstrates that the lateral heterogeneity of the thylakoid membrane largely persists after phosphorylation.

Table 1

Relative chlorophyll distribution of chlorophyll–protein complexes from phosphorylated thylakoids and subfractions derived from non-appressed (Y-100) and appressed (B3) regions (% of total Chl)

Fraction	PS1 CPI + CPIa	PS2 CPa	$LHCP^{1-3}$	Free Chl.
Thylakoids	25	8	48	19
Y-100	61	3	16	20
B3	10	10	63	17

In all fractions most of the incorporated [^{32}P]-phosphate radioactivity was recovered under the LHCP bands, while only small amounts were detected under the CPa band and virtually none under the CPI and CPIa bands. Table 2 compares the specific phosphorylation of LHCP (cpm/μg Chl) in the three fractions. The phosphorylation of LHCP in the Y-100 fraction was considerably higher than in unfractionated thylakoids. In contrast there was a quite low phosphorylation of LHCP in the B3 fraction. Thus, the specific radioactivity of LHCP in the Y-100 fraction was on

average 7-times higher than in the B3 fraction. The high degree of phosphorylation in Y-100 compared to B3 was seen for all three LHCP bands (table 2).

The phosphorylation of LHCP in the Y-100 and B3 fractions was also analyzed by SDS–PAGE under denaturating conditions, thereby resolving the individual apo-polypeptides of LHCP. As revealed from parallel electrophoresis of pure LHCP [17] this complex contained one major M_r 25000 and one minor M_r 23000 polypeptide (fig. 1).

Table 2

Phosphorylation of LHCP in thylakoids and subfractions derived from non-appressed (Y-100) and appressed (B3) regions

Fraction	Exp.	LHCP$^{1-3}$	LHCP^1	LHCP^2	LHCP^3
Thylakoids	1	1701	2315	1640	1341
	2	2668	2886	2434	2568
Y-100	1	7234	12037	6099	5067
	2	9126	13191	6578	8261
B3	1	874	897	574	953
	2	1766	2004	1320	1747
Y-100/B3		6.8	10	7.8	5.0

The specific phosphorylation of LHCP, from two independent experiments, is expressed as cpm/μg Chl. The Y-100/B3 ratios are calculated from the two experiments

1). These two polypeptides comprised a prominent portion of the total staining both in starting thylakoids (28%) and the B3 fraction (42%), while they

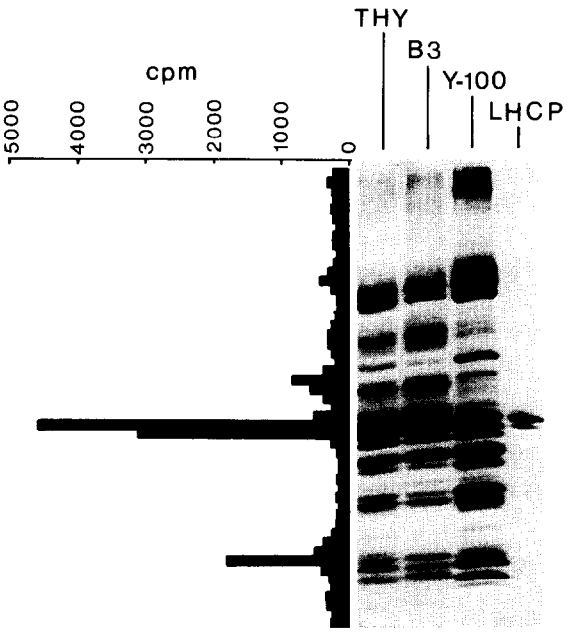

Fig. 1. Polypeptide composition of thylakoids, Y-100 and B3 fractions as resolved by denaturating SDS–PAGE. Pure LHCP was used to identify the apo-polypeptides of the dissociated complex. The histogram shows the [^{32}P]phosphorylation pattern of the original thylakoids (THY).

Table 3

Phosphorylation of LHCP in thylakoids and subfractions derived from non-appressed (Y-100) and appressed (B3) regions

Fractions	Expt	LHCP apo-polypeptides (M_r 25000 + 23000)	M_r 25000 polypeptide	M_r 23000 polypeptide
Thylakoids	1	2280	1566	5107
	2	3770	3226	5916
Y-100	1	7177	6203	8100
	2	9996	9335	10660
B3	1	1239	1076	1963
	2	1531	1344	2186
Y-100/B3		6.2	6.6	4.5

The specific phosphorylation of LHCP, from two independent experiments, is expressed as cpm/relative protein units. The relative amount of LHCP protein was determined from the peak area after scanning of Coomassie brilliant blue-stained gels

were less abundant in the Y-100 fraction (9%).

Most of the radioactivity in the thylakoids comigrated with the two apo-polypeptides of LHCP, and only little radioactivity was observed in the M_r 10000, 32000–34000 and 45000 regions (fig. 1) in agreement with previous work [5]. This was also the case for the Y-100 and B3 fractions (not shown). Table 3 compares the specific incorporation of [^{32}P]phosphate into the M_r 25000 and 23000 apo-polypeptides of LHCP (cpm/relative protein units) in the thylakoid, Y-100 and B3 fractions. There was a much higher degree of phosphorylation in the M_r 25000 and 23000 polypeptides of the Y-100 fraction than in the starting material. These polypeptides were also much less phosphorylated in the B3 fraction. Thus, the specific phosphorylation of LHCP in the Y-100 fraction was on average more than 6-times higher than in the B3 fraction, calculated on both apo-polypeptides.

4. DISCUSSION

This investigation presents evidence that the small pool of LHCP in non-appressed thylakoids is considerably more phosphorylated after illumination than the majority of LHCP located in the grana appressions. The subfraction derived from non-appressed membranes (Y-100) showed 6–7-times higher specific phosphorylation of LHCP than the B3 subfraction enriched in inside-out vesicles derived from grana partitions. The LHCP phosphorylation ratio between the two fractions was essentially the same, both when the undissociated complex and its apo-polypeptides were examined (table 2,3). The differences in the phosphorylation ratio between the three chlorophyll *a/b* bands comprising LHCP (table 2) may be due to co-migration of non-pigmented phosphoproteins or differential loss of free chlorophyll. Similarly, the somewhat less pronounced difference in phosphorylation ratio between the Y-100 and B3 fractions seen for the M_r 23000 compared to the M_r 25000 polypeptide (table 3), is probably caused by an overestimation of the amount of the M_r 23000 LHCP apo-polypeptide in the Y-100 fraction (fig. 1), due to a co-migration M_r 23000 polypeptide originating from the PS1 complex [15] which is enriched in this fraction.

The value for the B3 fraction should be taken as a maximum for the specific phosphorylation of LHCP in the native grana appressions, since this fraction is contaminated with about 25% right-side-out vesicles [18]. These are probably derived from non-appressed thylakoids and therefore carry highly phosphorylated LHCP. Such a contamination can be estimated to contribute to at least ⅓ rd of the radioactivity associated with LHCP in the B3 fraction. Therefore the specific phosphorylation of LHCP in the non-appressed thylakoids can be estimated to be about 10-times higher than in the appressed grana partitions. This means that about 70% of the total phosphorylated LHCP is located in the non-appressed thylakoids, although only about 20% of the LHCP is located there. It should also be stressed that the sum of LHCP phosphorylation in the two regions is equal to that of the starting thylakoids. Differential dephosphorylation of the appressed and non-appressed thylakoid material during preparation cannot, therefore, be the reason for the observed differences.

The high degree of phosphorylation of LHCP in non-appressed thylakoids together with their very high content of CPI suggest that this region is the site for increased energy transfer from LHCP to PS1 under phosphorylated conditions. This energy transfer is less likely to occur in the appressed grana partitions due to their low degree of LHCP phosphorylation and extreme scarcity of PS1 reaction centres [19].

The high specific phosphorylation in non-appressed thylakoids compared to appressed ones could be due to the location of the protein kinase in the former region or to an inability of ATP to penetrate into the partitions. Alternatively, as in [20], the extra negative charge introduced through phosphorylation would cause LHCP-PS2 of the appressed grana to move out into the non-appressed region. This view has gained support from very recent freeze-fracture studies ([21]; Simpson, D.J., personal communication) showing particle movements from appressed to non-appressed regions following phosphorylation. In contrast, a comparison of chlorophyll *a/b* ratios in subfractions isolated from unphosphorylated and phosphorylated thylakoids gave no evidence of migration of complexes [22]. However, as shown by linear dichroism [23] only a small degree of destacking occurs due to phosphorylation, and differences in

the composition of thylakoid subfractions may be quite small. Thus, in order to establish whether the observed high phosphorylation of LHCP in non-appressed thylakoids is the result of lateral migration or a higher kinase activity in this region, sensitive analysis of the chlorophyll–protein composition in fractions obtained from parallel subfractionation of phosphorylated and unphosphorylated chloroplasts is required.

ACKNOWLEDGEMENTS

We thank Dr John Bennett, Dept. of Biological Sciences, Warwick, for encouragement and advice concerning this study. The skilful technical assistance of Mrs Agneta Persson and Maria Jönsson is appreciated. This study was sponsored by the Swedish Natural Science Research Council.

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