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Chloroplast thylakoid protein phosphatase reactions are redox-independent and kinetically heterogeneous

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At least eleven thylakoid proteins become phosphorylated under reducing conditions, and redox titration has identified a common midpoint potential of $E_m = +38 \pm 4$ mV, $n = 0.95 \pm 0.06$. In the presence of the phosphatase inhibitor NaF (10 mM), the redox dependency of phosphorylation is found to be essentially unchanged: $E_m = +50 \pm 3$ mV, $n = 1.02 \pm 0.04$. Thylakoid membranes were phosphorylated in the light and then incubated at various redox potentials for 15 min in the dark; no redox dependency was observed in the dephosphorylation of any of the 17 bands then distinguishable by autoradiography and phosphorimaging. The phosphoprotein phosphatase reactions can be divided arbitrarily into four kinetic classes: the fastest, class I, includes LHC II; the moderate class II includes D1 and D2; the slow class III includes CP43 and the 9 kDa phosphoprotein; finally, a 19.5 kDa protein exhibited no loss of ^{32}P at all. In separate experiments we measured thylakoid protein dephosphorylation initiated by changing the redox potential from −140 to +200 mV, in the presence or absence of fluoride. In this case the results are consistent with at least two kinetically distinguishable classes of phosphoprotein phosphatase reactions. We conclude that thylakoid protein phosphatase reactions are kinetically heterogeneous and redox-independent. It follows that the redox dependency of thylakoid protein phosphorylation is a property of thylakoid protein kinase reactions. Our observed E_m and n values are consistent with a primary site of kinase redox control at the level of $PQ/PQ^{\cdot-}$ of the Q_i (Q_n) site of the cytochrome b_6/f complex.

Thylakoid protein phosphatases; Thylakoid protein kinase; Redox control; Redox sensor; Cytochrome b_6/f complex; LHC II

1. INTRODUCTION

The light-dependent phosphorylation of LHC II is under redox control, occurring only under reducing conditions, and the plastoquinone pool and cytochrome b_6/f complex have been implicated as sites of redox control [1–4]. LHC II phosphorylation is involved in regulation of excitation energy distribution between photosystems I and II [3–5]. Besides LHC II, several other thylakoid proteins are phosphorylated. Recently we have demonstrated that 13 phosphoproteins observed by autoradiography exhibit similar redox-controlled behaviour in which the midpoint potentials ranged from +30 to +55 mV, and n varied from 0.7 to 1.1 [6].

In studies of regulation of thylakoid protein phosphorylation it is usually assumed that the kinase is under redox control, while the phosphatase is constitutively active at a constant low level [3]. However, the converse could also be true, since activation of the phosphatase under oxidising conditions is also consistent with existing data. It has been suggested [4] that the LHC II phosphatase may be under redox control, thus serving to minimise net ATP hydrolysis during steady state photosynthesis. A previous study [7] of the thylakoid phosphatase did not examine redox control, but identified a pronounced kinetic heterogeneity among thylakoid phosphatase substrates. Specifically, LHC II was dephosphorylated most quickly, the 9 kDa protein most slowly, and D1, D2, and a 45 kDa protein (CP43?) at an intermediate rate [7]. Since there have been no previous reports on the regulation of the thylakoid protein phosphatases, we have performed experiments designed to test the redox dependency of these reactions.

2. MATERIALS AND METHODS

Thylakoid membranes were isolated from pea chloroplasts as described previously [8]. Redox titration was performed as in [6], at 22.5°C in the dark, with a final chlorophyll concentration of 30 $\mu g \cdot ml^{-1}$. The protein kinase reactions were initiated by addition of [γ-^{32}P]ATP to a final concentration of 0.15 mM, 3 $\mu Ci \cdot ml^{-1}$. After 10 min, 0.8 ml of the suspension was withdrawn from the cell, precipitated in Eppendorf tubes containing 0.16 ml of 30% (w/v) trichloroacetic acid, and sedimented at 12,000 rpm for 2.5 min. The pellets were washed once with 80% acetone that had been pre-cooled to −20°C, redissolved in 5% SDS-sample buffer at 70°C for one hour, and SDS-PAGE and autoradiography were performed [9]. Quantitation of the ^{32}P-labelled bands was performed by phosphorimaging with a Fuji BAS2000 Bio-Imaging Analyzer. Background labelling was subtracted for each lane.

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Abbreviations: PSI, photosystem I; PSII, photosystem II; LHCII, light harvesting complex II; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; CP43, 43 kDa PSII light harvesting core protein; D1 and D2, 31 and 32 kDa PSII reaction center polypeptides.

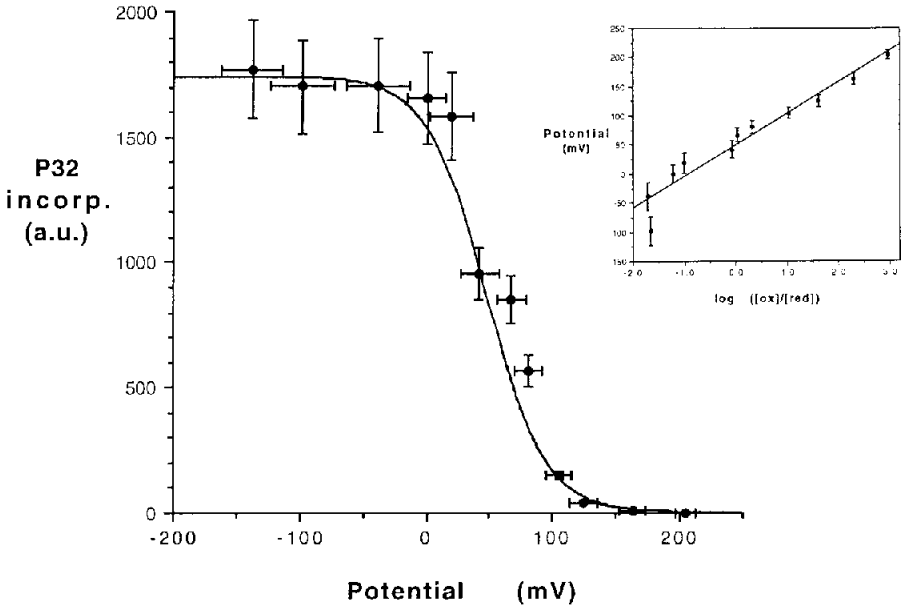

Fig. 1. Redox titration of ^{32}P incorporation into LHCII in the presence of NaF (10 mM). The intensity of the LHCII band, quantified by phosphorimage analysis, is plotted against potential. The inset shows a semi-log Nernst plot of the data; linear regression gave a best fit for intercept (E_m) = +48 ± 7 mV, slope = +54 ± 4 mV (n = 1.09 ± 0.08), r^2 = 0.937. The solid lines in both the inset and in the full figure are calculated from this best fit.

To distinguish between the kinase and phosphatase reactions, a suspension of thylakoid membranes was incubated in the light for 20 min with [γ-^{32}P]ATP (0.15 mM, 3 μCi · ml^{-1}) and NaF (10 mM). The membranes were then sedimented, washed twice, resuspended in medium containing NaF, and stored on ice. The phosphorylated thylakoids were then added to a fluoride-free medium in the redox cuvette for measurement of dephosphorylation. The final NaF concentration in the redox cuvette was 0.3 mM. Dephosphorylation was followed in the dark for 15 min at defined redox potentials. The level of phosphorylation in each sample was compared to that in a control sample precipitated at the start of each redox incubation.

3. RESULTS AND DISCUSSION

In Fig. 1 we present the redox titration data for LHC II phosphorylation in the presence of 10 mM NaF. From the semi-log Nernst plot (Fig. 1, inset), we obtain E_m = 48 ± 7 mV, n = 1.09 ± 0.07. In Table I we include results for all 13 bands that titrated with Nernstian behaviour (two bands were phosphorylated in a redox-independent manner; see below). In Fig. 2 the data for all of these bands is included in a single plot, yielding E_m = 50 ± 3 mV, n = 1.02 ± 0.04. These results compare favourably with those obtained previously in the absence of fluoride: E_m = 38 ± 4 mV, n = 0.95 ± 0.06 [6]. In particular, the ranges of values for E_m and n in the two experiments were strikingly similar: E_m = +30 to +55 mV ($-F^-$) vs. +40 to +55 mV ($+F^-$); n = 0.7 to 1.05 ($-F^-$) vs. 0.8–1.15 ($+F^-$). Since fluoride is a phosphatase inhibitor [7], and since inhibiting the phosphatase has no effect on the redox dependency of thylakoid protein phosphorylation, we can infer that the redox dependency does not reside in the phosphatase. The E_m and n values observed here and in [6] seem to rule out the bulk quinone pool as the primary site of redox control, and are consistent with a redox sensor [10] responding directly either to the PQ/PQ$^{\cdot-}$ couple at the Q_i (also termed Q_n) site (0 to +100 mV) or to low potential cytochrome b_{559} (+20 to +65 mV). We favour the Q_i site, since there is evidence ([2], reviewed in [4]) that the kinase is associated with the cytochrome *b/f* complex. High potential cytochrome b_6 (−15 to −45 mV) might also be considered a candidate.

In the absence of fluoride we observed two unique bands, at 46 and 63 kDa. Unlike all the other thylakoid phosphoproteins, which were phosphorylated under re-

Table I

Redox titration of thylakoid protein phosphorylation (+10 mM NaF)

Protein	E_m (mV)	n	r^2
LHC II	48	1.09	0.937
9.5 kDa	71	1.16	0.847
9(11) kDa	45	1.04	0.960
12 kDa	52	0.97	0.937
15 kDa	53	0.94	0.901
16 kDa	48	1.10	0.926
17 kDa	45	0.89	0.807
18 kDa	55	1.14	0.837
19.5 kDa	39	0.99	0.922
D1	49	1.03	0.943
D2	41	0.81	0.934
CP43	43	0.94	0.798
55 kDa	52	1.10	0.784
All bands	50 ± 3	1.02 ± 0.04	0.872

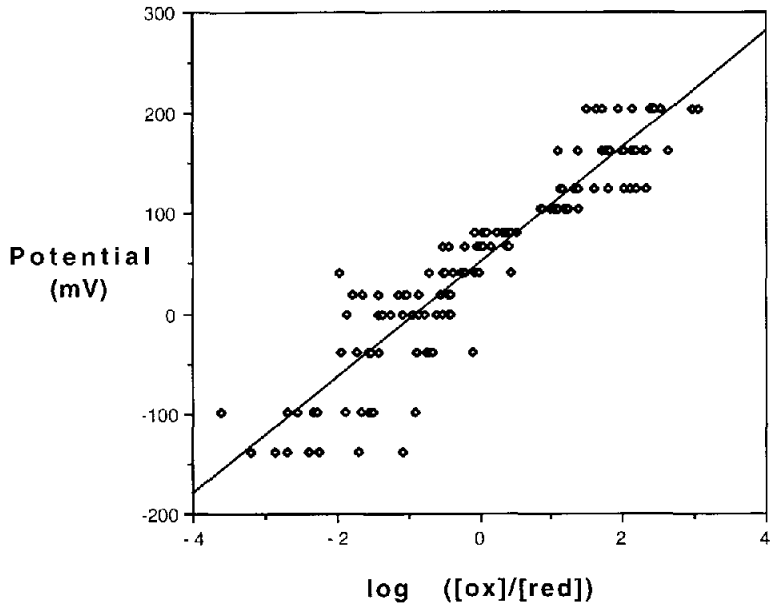

Fig. 2. Redox titration of ^{32}P incorporation into thylakoid phosphoproteins in the presence of NaF (10 mM). Combined data for all 13 titratable bands, treated as in Fig. 1 above. Linear regression gave a best fit (solid line) for: intercept (E_m) = +50 ± 3 mV, slope = +57 ± 2 mV (n = 1.02 ± 0.04), r^2 = 0.872.

ducing conditions (E_h <40 mV), these two proteins were phosphorylated only under oxidising conditions. In the presence of fluoride however, these two bands were labelled only lightly, and in a redox-independent manner (data not shown). It is possible that these proteins maintain a substantial level of endogenous phosphorylation throughout the initial dark incubation (oxidising conditions). In the absence of fluoride, the active phosphatase/kinase pair can catalyze exchange of unlabelled for labelled phosphate through phosphate hydrolysis followed by [γ-^{32}P]ATP-driven phosphorylation. If the phosphatase is inhibited by fluoride however, these two bands could remain stably phosphorylated with endogenous (unlabelled) phosphate, and thus show no ^{32}P labelling.

In order to separate the kinase and phosphatase reactions completely, we prepared phosphorylated thylakoid membranes by incubation in the light with [γ-^{32}P]-ATP. After removal of excess ATP by sedimentation and washing, we could follow the phosphatase reaction specifically. Fig. 3 shows, for the six phosphoproteins that constitute the fastest class of phosphatase substrates, the percentage of ^{32}P remaining after a 15 min incubation at defined redox potentials. For each potential, the control sample (100%) was precipitated by trichloroacetic acid at the start of the 15 min redox incubation (see section 2). For all 17 observable phosphoproteins, the phosphatase reaction was independent of redox potential. This is clearly seen for the six bands plotted in Fig. 3.

The redox-independence of the reaction allows us, for each band, to average the percentage ^{32}P remaining after 15 min over all redox potentials. The results are presented in Table II. From these data we find that the six bands plotted in Fig. 3 comprise a rapidly dephosphorylating class of phosphatase substrates, with only 10–25% of ^{32}P remaining after 15 min. This class in-

Table II

Percentage ^{32}P remaining in thylakoid phosphoproteins 15 min after preillumination

Protein	Protein	^{32}p remaining after 15 min
Class I	9.5 kDa	7 ± 13%
	LHC II	12 ± 3%
	17 kDa	18 ± 5%
	55 kDa	19 ± 5%
	18 kDa	20 ± 7%
	15 kDa	21 ± 8%
	16 kDa	25 ± 5%
Class II	D1	37 ± 17%
	60 kDa	55 ± 11%
	12 kDa	57 ± 13%
	68 kDa	57 ± 12%
	D2	60 ± 31%
Class III	CP43	75 ± 11%
	80 kDa	75 ± 22%
	9 kDa	76 ± 12%
	35 kDa	78 ± 15%
Class IV	19.5 kDa	130 ± 40%

Since the percentage ^{32}P remaining after 15 min was independent of redox potential (Fig. 3) for all bands, the values at all potentials are averaged for each band.

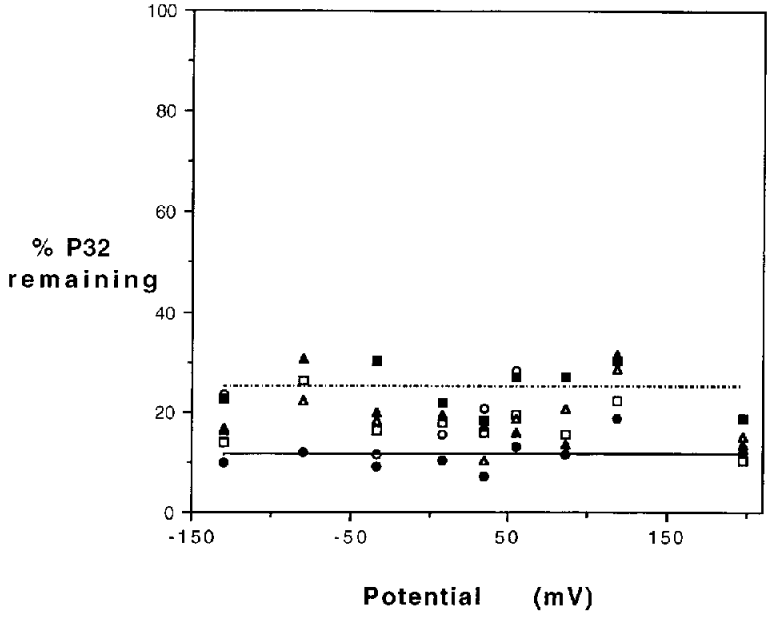

Fig. 3. Redox independence of thylakoid phosphoprotein phosphatase reactions. Percentage ^{32}P remaining after a 15 min dark incubation of light-phosphorylated thylakoid membranes at defined redox potentials is plotted for the fast class I substrates. (Filled circles) LHCII; (open circles) 15 kDa band; (filled squares) 16 kDa band; (filled triangles) 18 kDa band; (open triangles) 55 kDa band ($CF_1\alpha/\beta$ subunit?). The lower (solid) line is drawn through the average percentage ^{32}P for LHCII (12%), while the upper (dotted) line is drawn through the average percentage ^{32}P for the 16 kDa protein (25%).

cludes LHCII, a 55 kDa protein which may be the $CF_1\alpha$ or β subunit, plus five other low molecular weight bands (10–18 kDa).

The remaining bands in Table II may be grouped arbitrarily into three slower classes of phosphatase substrates: class II, an intermediate class of phosphatase substrates including D1, D2, and three other bands, with 40–60% of ^{32}P remaining; class III, a slow class including CP43, the 9 kDa protein, plus two other bands, with 75–80% of ^{32}P remaining; and finally a 19.5 kDa phosphoprotein which shows no dephosphorylation at all.

However, there is no obvious statistical delineation among the three slower classes. Standard deviations in class II range from 13% to 31%, those in class III range from 11% to 22%, and for the 19.5 kDa protein the percentage remaining ^{32}P is 130 ± 40%. Therefore these three slower classes may represent a single broad class of substrates with 50–100% of ^{32}P remaining after 15 min. In class I, however, standard deviations range from around 3% to 8%, so this class of rapidly-dephosphorylating proteins is statistically distinct from the others.

We also used a shift in redox potential to separate the kinase and phosphatase reactions. We initiated phosphorylation in the dark by a 10 min incubation at −140 mV in the presence of [γ-^{32}P]ATP, and then deactivated the kinase by adjusting the potential to +200 mV, maintaining this potential for 10 min. This was carried out both in the presence and absence of 10 mM NaF. The control for both of these samples was a thylakoid suspension incubated in the dark for 10 min at +200 mV in the presence of [γ-^{32}P]ATP, followed by a 10 min incubation at −200 mV. There was no fluoride present in the control sample.

The results of this experiment are presented in Table III. Both in the presence and absence of fluoride, the phosphoproteins break down into roughly two categories of phosphatase substrates: (a) rapidly dephosphorylating proteins, including LHC II, a 55 kDa protein, the two 'reverse titrators' (46 and 63 kDa), plus four low molecular weight proteins (10–18 kDa), which all have 10 ± 5% ^{32}P remaining after the 10-min, high-potential incubation; and (b) slowly dephosphorylating proteins, including D1, D2, CP43, the 9 kDa protein, plus three other low molecular weight proteins (12–20 kDa), which all have 36 ± 13% ^{32}P remaining. In the presence of fluoride the same two classes are still observed, but the remaining ^{32}P is of course higher: 40 ± 12% and 69 ± 10% for the fast and the slow classes, respectively.

The rapidly-dephosphorylating proteins in Table III correspond almost exactly to those of class I in Table II. The slowly-dephosphorylating proteins in Table III likewise correspond to those of classes II–IV in Table II. We note that 10 mM NaF may inhibit the phosphatase reaction slightly more effectively for the slowly-dephosphorylating proteins in Table III: 33 ± 12% inhibition for the rapidly dephosphorylating proteins, and 50 ± 20% inhibition for the slowly dephosphorylating proteins. These estimates of fluoride inhibition of the

Table III

Percentage ^{32}P remaining in thylakoid phosphoproteins after 10 min at +200 mV

	Protein	^{32}P left after 10 min ($-F^-$)	^{32}P left after 10 min ($+F^-$)	Phosphatase inhibition
Fast class	LHC II	5%	41%	38%
	9.5kDa	6%	42%	38%
	18 kDa	6%	29%	24%
	17 kDa	10%	47%	41%
	55 kDa	11%	57%	52%
	16 kDa	12%	40%	32%
	46 kDa	15%	17%	10%
	63 kDa	19%	44%	31%
	avg.	10 ± 5%	40 ± 12%	33 ± 12%
Slow class	D2	17%	73%	67%
	D1	29%	59%	42%
	15 kDa	30%	75%	64%
	35 kDa	–	68%	–
	CP43	31%	81%	72%
	9 kDa	38%	60%	35%
	19.5 kDa	47%	56%	17%
	12 kDa	58%	80%	52%
	avg.	36 ± 13%	69 ± 10%	50 ± 20%

Phosphorylation was initiated by a 10 min incubation with [γ-^{32}P]ATP in the dark at −140 mV. Dephosphorylation was followed after a shift to +200 mV. In column 2, the reaction medium contained 10 mM NaF. Percentage inhibition of the phosphatase by 10 mM NaF was calculated as follows: Since

100 − % ^{32}P (remaining) = % ^{32}P hydrolyzed (i.e. phosphatase activity),

then [100 − % ^{32}P ($+F^-$)]/[100 − % ^{32}P ($-F^-$)] = ratio of fluoride to fluoride-free phosphatase activity, therefore, % inhibition by fluoride = 100 − [100 − % ^{32}P ($+F^-$)]/[100 − % ^{32}P ($-F^-$)].

thylakoid phosphatase are consistent with previous results (Cheng, L. and Allen, J.F., unpublished), though they differ from a previously reported value of 91% inhibition [7].

The percentage of ^{32}P remaining is generally higher in Table II (after a 15 min incubation at different redox potentials) than in Table III (after a 10 min incubation at +200 mV). This difference probably stems from the inhibitory effect of the residual 0.3 mM NaF present in the dephosphorylation medium. Alternatively, the washing and resuspension steps involved could have partially depleted or denatured the thylakoid phosphatase, accounting for the lower dephosphorylation rate in Table II.

The difference in the apparent number of kinetic classes observed in the two experiments (four classes in Table II vs. two classes in Table III), may result from the different number of replicates in the two experiments. The redox-independence of the phosphatase provided us with nine replicate samples (Fig. 3, Table II), yielding reliable averages and standard deviations for each substrate dephosphorylation reaction. Experiments are currently underway to examine the complete time course of the dephosphorylation reactions in order to provide reaction rates for each phosphoprotein substrate, and thereby determine the number of kinetic classes of thylakoid protein phosphatase reactions.

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