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Rapid Report

Redox titration of multiple protein phosphorylations in pea chloroplast thylakoids

Todd Silverstein [1], Lüling Cheng and John F. Allen *

Plant Cell Biology, University of Lund, Box 7007, S-220 07 Lund (Sweden)

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Redox titrations were carried out on the protein kinase reactions of isolated pea chloroplast thylakoid membranes. Of the 13 phosphoproteins observed by autoradiography, all were found to titrate with the same midpoint potential of around +40 mV. Eleven proteins, including LHCII, D1, D2, CP43, a 9 kDa and a 55 kDa protein, were phosphorylated under *reducing* conditions, with a midpoint potential of $E_m = +38 \pm 4$ mV, $n = 0.95 \pm 0.06$. Two other proteins, including one at 63 kDa, were phosphorylated only under *oxidizing* conditions, $E_m = +33 \pm 11$ mV, $n = 0.67 \pm 0.09$. These midpoint potentials and n values suggest that either cytochrome b_6 or else a semiquinone associated with the cytochrome b_6f complex may be the controlling redox sensor. The 'reverse' redox dependence of phosphorylation of the 63 kDa and 46 kDa bands suggests that more than one redox-controlled protein kinase (or phosphatase) functions in the thylakoid membrane. We also suggest that a protein kinase (or phosphatase) is itself regulated by phosphorylation in a redox-controlled reaction.

The redox control of LHCII phosphorylation by a 64 kDa kinase associated with the cytochrome b_6f complex [1,2] in the thylakoid membrane is implicated in the regulation of excitation energy distribution between Photosystems I and II [3–5]. This protein kinase may be autophosphorylated on an N-terminal serine or threonine residue [6–8], though the relationship between this process and kinase activity remains unresolved [7]. In addition to LHCII and the 64 kDa kinase itself, several other thylakoid proteins are known to be phosphorylated [4,5], including the D1 and D2 reaction center polypeptides, the CP43 core LHC polypeptide, a 9–11 kDa and a 55 kDa protein (Horton, P. and Allen, J.F., unpublished observations) and the cytochrome b_6 polypeptide [7], among others. Here we present the results of redox titrations of all 13 pea thylakoid protein phosphorylation reactions that we were able to follow by phosphorimaging. Our objective was to see if a single redox-controlled kinase could be responsible for all of them, and to attempt to characterize the controlling redox sensor.

Thylakoid membranes were isolated from pea chloroplasts as described previously [9]. Reaction medium (1 ml) containing 0.1 M sorbitol, 50 mM Hepes (pH 7.6) and $MgCl_2$, NaCl and NH_4Cl, all at 5 mM, was added to a redox cuvette. Four additions of redox mediators (see Table I) were introduced into the cuvette as follows: (1) a combination of all five aqueous mediators; (2) a combination of seven of the nine ethanol-dissolved mediators; (3) 2,5-dihydroxy-1,4-benzoquinone; and (4) 2-hydroxy-1,4-naphthoquinone. The last two were added separately as they were unstable in solution with the other mediators. Total ethanol concentration in the cell was kept below 2% (v/v). Finally, thylakoid membranes were added to give a final chlorophyll concentration of 30 μg/ml.

The cuvette was then stoppered and placed in a temperature-controlled, magnetically stirred cell-holder. Argon was delivered through copper tubing, and additions were made with a Hamilton syringe through a side port with fitted silicon rubber septum. The combination platinum redox electrode (ABB Kent-Taylor 1143–400) was connected to a pH meter (Philips PW9420) and calibrated with known concentration ratios of ferri-/ferrocyanide. All measurements were made at 23.5°C in the dark. The redox potential was maintained (±10–25 mV) at the desired potential

* Corresponding author: Fax +46 46 104009.
[1] Permanent address: Chemistry Department D-125, Willamette University, 900 State Street, Salem, OR 97301 USA.

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

TABLE I

Mediators used to perform the redox titrations

BQ = benzoquinone; NQ = naphthoquinone; AQ = anthraquinone; DAD = 2,3,5,6-tetramethyl phenylene diamine; TMPD = *N,N,N',N'*-tetramethyl phenylene diamine.

Mediator		$E°$ (mV)	Solvent	Stock concentration	Final concentration
1	1,4-BQ	+280	EtOH	2.5 mg/ml	2.5 μg/ml
2	TMPD	+260	EtOH	3.0 mg/ml	3.0 μg/ml
3	DAD (daminodurene)	+220	EtOH	2.4 mg/ml	12 μg/ml
4	1,2-NQ	+135	EtOH	0.6 mg/ml	3.0 μg/ml
5	PMS (phenazine methosulfate)	+80	H_20	1.5 mg/ml	7.0 μg/ml
6	5-Hydroxy-1,4-NQ	+50	EtOH	3.0 mg/ml	3.0 μg/ml
7	DQ (duroquinone = Me_4BQ)	+5	EtOH	4.0 mg/ml	4.0 μg/ml
8	2,5-dihydroxy-1,4-BQ	−60	EtOH	0.7 mg/ml	3.5 μg/ml
9	AQ	−100	EtOH	saturated	1:500 dilution
10	2-Hydroxy-1,4-NQ	−145	EtOH	3.0 mg/ml	3.0 μg/ml
11	AQ-2,6-disulfonate	−185	H_2O	1.5 mg/ml	7.5 μg/ml
12	AQ-2-sulfonate	−225	H_2O	1.5 mg/ml	7.5 μg/ml
13	Benzul viologen	−311	H_2O	0.2 mg/ml	1.0 μg/ml
14	Methyl viologen	−430	H_2O	0.2 mg/ml	1.0 μg/ml

by additions of 2–10 μl of sodium dithionite (5, 20 or 40 mM, prepared freshly in nitrogen-bubbled 4 M Tris (pH 8)) or potassium ferricyanide (10, 20 or 50 mM). The protein kinase reactions were initiated by addition of [γ-^{32}P]ATP to a final concentration of 0.15 mM, 3 μCi/ml. After 10 min, 0.8 ml of the mixture was

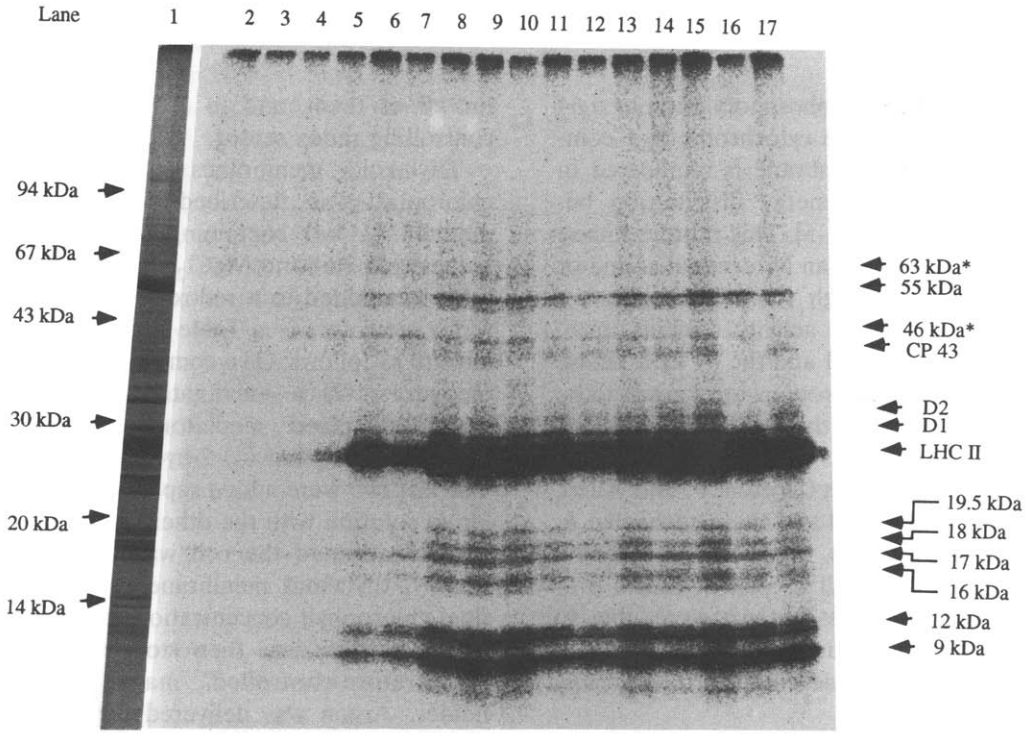

Fig. 1. SDS-PAGE of pea thylakoid membrane proteins. Membranes were incubated with reaction medium and redox mediators in the dark, T = 23.5°C, at specific redox potentials. 10 min after addition of γ^{32}P-ATP phosphorylation was quenched by TCA precipitation. Lane 1 shows the Coomassie blue-stained gel for the +200 mV incubation. Stained gels for all other potentials were essentially identical. Lanes 2–17 are phosphoimages showing the extent of ^{32}P labelling at various redox potentials from +200 to −137 mV (left to right). Lane 2: +200 mV; lane 3: +163; lane 4: +128; lane 5: +105; lane 6: +75; lane 7: +66; lane 8: +51; lane 9: +39; lane 10: +31; lane 11: +27; lane 12: +2; lane 13: −30; lane 14: −64; lane 15: −98; lane 16: −113; lane 17: −137 mV. Arrows to the left-hand side of lane 1 indicate the positions of molecular mass markers. Arrows to the right-hand side of lane 17 identify the bands whose labelling was quantified. The heavily labelled band at 26–28 kDa is LHCII. Other prominent bands are the 9 kDa protein, a 12 kDa and a 55 kDa protein (which may be the CF_1 α or β subunit). Other less heavily labelled bands are at 15, 16, 17, 18, 19.5, 30 (D1), 32 (D2), 42 (CP43), 46 and 63 kDa.

withdrawn from the cell, precipitated in Eppendorf tubes containing 0.16 ml of 30% (w/v) trichloroacetic acid, and sedimented at 16000 × *g* 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 1 h, and SDS-PAGE and autoradiography were performed [10]. Measurement of ^{32}P-labelling of each band was performed by phosphorimaging with a Fuji BAS2000 Bio-Imaging Analyzer. Background labelling was subtracted for each gel lane.

In Fig. 1 we present the polypeptide pattern of thylakoid membrane proteins resolved by SDS-PAGE

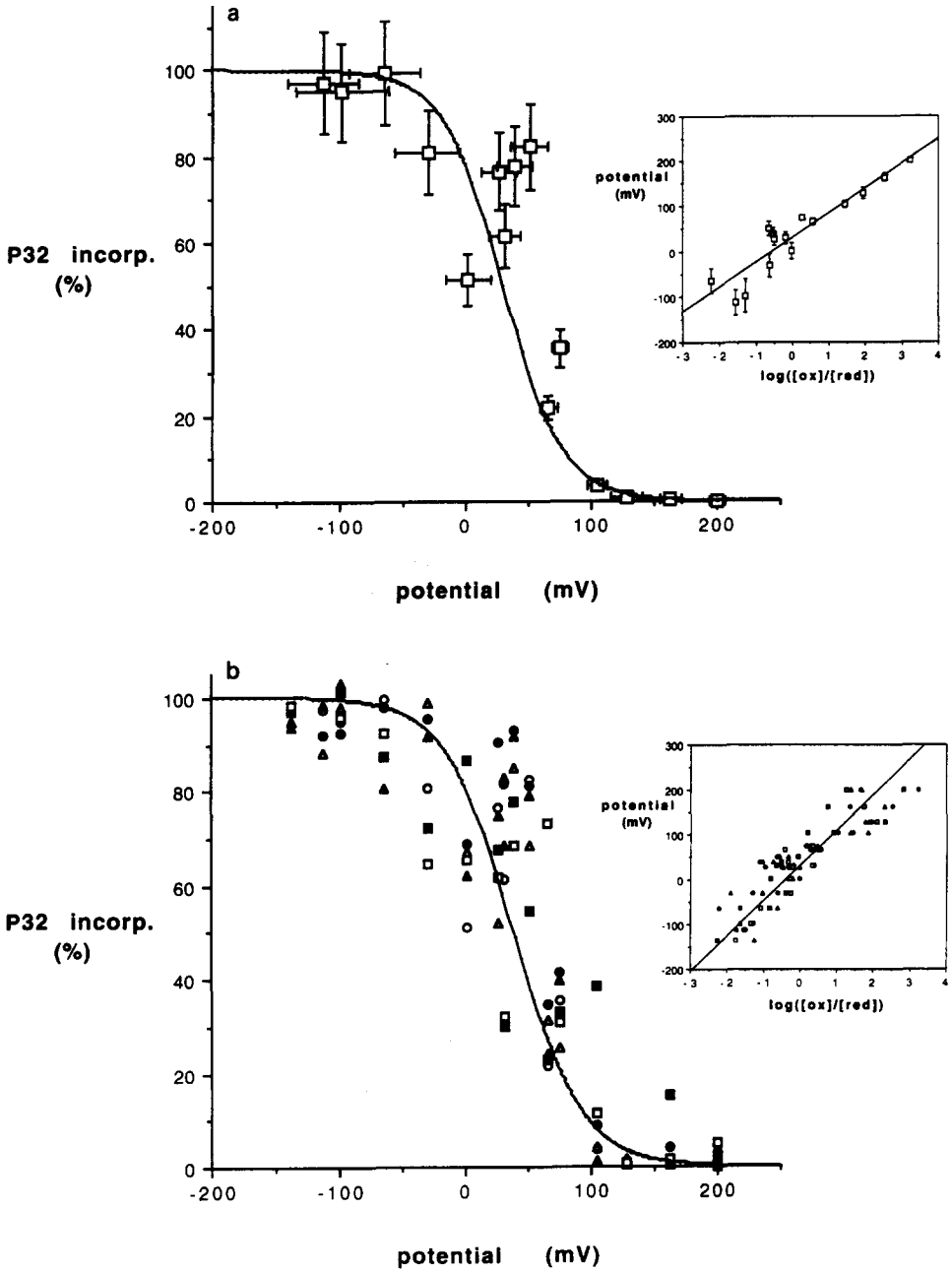

Fig. 2. Redox titration of ^{32}P incorporation into thylakoid phosphoproteins. (a) The intensity of the LHCII band, quantified by phosphoimage analysis, is plotted against potential. The inset shows a semi-log Nernst plot of the data; linear regression gave a best fit for intercept (E_m) = +30±9 mV, slope = +55±6 mV (n = 1.07±0.12), R^2 = 0.864. The solid lines in both the inset and in the full figure are calculated from this best fit. (b) Combined data for LHCII (empty circles), D1 (filled circles), D2 (empty squares), CP43 (filled squares), 9 kDa (empty triangles) and 55 kDa (filled triangles) proteins treated as in (a), above. The solid lines are calculated from the semi-log linear regression best fit (see inset): intercept (E_m) = +39±4 mV, slope = +62±3 mV (n = 0.95±0.05), R^2 = 0.794. 2c: Data for the 63 kDa protein treated as in (a) above. The solid lines are calculated from the semi-log linear regression best fit (see inset): intercept (E_m) = +44±8 mV, slope = −91±8 mV (n = [−]0.65 ±0.06), R^2 = 0.901. Note that this phosphoprotein exhibits nearly the same midpoint potential as those in (b), but has a reverse redox dependence: it is maximally phosphorylated under *oxidizing* (E > 44 mV) conditions.

(see lane 1). The phosphorimage (lanes 2–17) clearly shows that most of the polypeptides increase in ^{32}P labelling as redox potential decreases from +200 to −140 mV. However, two proteins at 63 and 46 kDa show *decreased* labelling as the potential decreases.

^{32}P incorporation measured by phosphoimaging analysis is presented in Fig. 2. Fig. 2a shows the redox behavior of LHCII phosphorylation. From the semi-log Nernst plot (see inset), we calculate a mid-point potential (E_m) of $+30 \pm 9$ mV, and $n = 1.07 \pm 0.12$ electrons. These values are consistent with data reported previously for pea thylakoids [11] ($E_m = +7 \pm 3$ mV, and $n = 0.95 \pm 0.06$) and also for *Acetabularia* thylakoids [2] ($E_m = +119 \pm 4$ mV, and $n = 1.14 \pm 0.13$). Of the 13 phosphoproteins discernible by autoradiography, 11 showed redox control identical to that of LHCII. In Fig. 2b we have plotted data for six of these proteins, those most conspicuous in the autoradiograph. For these six proteins, $E_m = +39 \pm 4$ mV and $n = 0.95 \pm 0.05$. Results for all of the thylakoid phosphoproteins are presented in Table II.

The two remaining phosphoproteins, at 63 and 46 kDa, titrate with roughly the same midpoint potential as the others but with reversed redox dependence. These two bands are maximally phosphorylated under *oxidizing* conditions and completely dephosphorylated under reducing conditions. The 63 kDa protein shows this trend most clearly (Fig. 2c), though it is only lightly phosphorylated even at +200 mV. From this titration curve we find that $E_m = +44 \pm 8$ mV and $n = (-)0.65 \pm 0.06$. This unexpected redox dependence has not yet been reported for thylakoid phosphoproteins, though we have observed similar behavior in intact chloroplasts (Allen, C.A. and Silverstein, T., unpublished observations). A previous report [12] demonstrated phosphorylation of a 63 kDa and a 42 kDa protein in PS II particles in the dark (non-reducing conditions). The corresponding bands were unlabelled (63 kDa) or only lightly labelled (42 kDa) in the light (reducing conditions), which is consistent with our observation of phosphorylation of two similar bands under oxidizing conditions in thylakoids.

TABLE II

Summary of results for each of the 13 thylakoid phosphoproteins

Protein	E_m (mV)	n	R^2
LHCII	+30± 9	1.07±0.12	0.864
9 kDa	+40±12	0.93±0.15	0.799
55 kDa	+40±12	1.0 ±0.2	0.754
CP43	+38±11	0.95±0.15	0.800
D1	+56±11	0.95±0.15	0.808
D2	+31±11	0.75±0.10	0.841
12 kDa	+44±17	1.0 ±0.3	0.561
16 kDa	+30±13	0.69±0.13	0.800
17 kDa	+50±11	0.59±0.08	0.870
18 kDa	+43±11	0.91±0.15	0.810
19.5 kDa	+55±16	1.0 ±0.3	0.699
All (11) above:	+38± 4	0.95±0.06	0.790
'Reverse' titrators:			
63 kDa	+44± 8	(−)0.65±0.06	0.901
46 kDa	+15±26	(−)0.6 ±0.3	0.407
Both above:	+33±11	(−)0.67±0.09	0.790

We suggest that our results are consistent with phosphorylation of all 13 thylakoid proteins being under the redox control of a single endogenous agent with a midpoint potential of about +40 mV and $n = 1$ (see

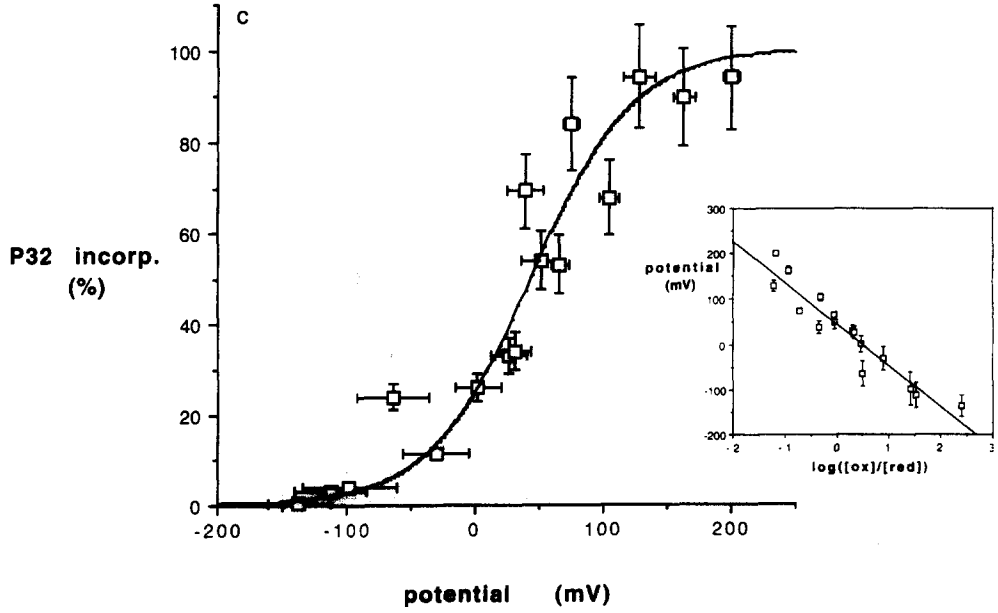

Fig. 2 (continued).

Table II). Three thylakoid membrane one-electron transfer reactions are possible candidates: low potential cytochrome *b*-559 (E_m = +20 to +65 mV [13,14]), high potential cytochrome b_6 (E_m = −15 to −45 mV [15]) and plastoquinone redox couples. Ohad and coworkers have implicated the cytochrome b_6f complex in redox regulation [2] and shown that a purified membrane preparation consisting of only the cytochrome b_6f complex and the 64 kDa LHCII kinase exhibits redox control in vitro [1]. Hence, the endogenous redox sensor is likely to lie somewhere within the cytochrome b_6f complex. This would exclude both cytochrome b_{559} and PQ_B, which are located in the PS II reaction center.

A careful examination of plastoquinone redox behavior in the cytochrome b_6f complex allows further characterization of the possible redox sensor involved. Since reduced plastoquinone (PQH_2) bound at the oxidizing Q_o site loses its two electrons in a highly co-operative manner with no detectable semiquinone intermediate [15,18], *n* should effectively be 2 for this reaction, thus ruling it out as a candidate. Regarding PQ reduction at the reducing Q_i site, of the two one-electron steps, $PQ/PQ\cdot^-$ and $PQ\cdot^-/PQH_2$, the midpoint potential of the latter (+200 to +300 mV) is probably too high to be considered. This leaves only the $PQ/PQ\cdot^-$ couple at the Q_i site ($E_m \approx 0$ to +100 mV) and the high-potential cytochrome b_6 ($E_m \approx -15$ to −45 mV) as possible candidates for the redox sensor implicated in controlling thylakoid protein phosphorylation.

Recent results from two laboratories have demonstrated that PS II photoinhibition is under redox control. Gong et al. [16] described a syndrome they called 'photodamage', constituting light-induced inhibition of CO_2 uptake and electron transfer rates, showing that this process increased 3- to 5-fold in pure nitrogen compared to air. They found the affected sites to be in the PS II reaction center (namely, P680/Pheo/Q_A/Q_B), as well as an unknown component in PS I. Nedbal et al. [17] redox-titrated the photoinhibition of oxygen evolution and fluorescence yields, and found enhanced photoinhibition under reducing conditions. Their titration points to a one-electron process with E_m around +25 mV. Recalculation of their results shows $n = 0.37 \pm 0.05$ and $E_m = +5 \pm 15$ and -21 ± 10 mV. Their low values of *n* can probably be ascribed to poor equilibration between the redox electrode and the medium [11], due to their use of only one redox mediator at each redox potential. Both groups report that photoinhibition is enhanced under reducing conditions [16–17], with a midpoint potential [17] not far from that which we measure for thylakoid protein phosphorylation. Since we have demonstrated that the phosphorylation of several PS II proteins (e.g., D1, D2 and CP43) is under similar redox control, it is possible that these redox dependent kinase reactions play a role in photoinhibition.

We wish to draw attention to the implications of the data in Figs. 2b and 2c. First, the fact that there are two sets of proteins whose phosphorylations titrate in opposite directions suggests that there is more than one redox-controlled kinase in the thylakoid membrane. It has been proposed that the LHCII kinase is a 64 kDa membrane protein which is activated in the light and also under reducing conditions (for a review see Ref. 4). In addition, this kinase is autophosphorylated in the light [7,8]. In order for a set of proteins to become phosphorylated under *oxidizing* conditions (Fig. 2c), there must be either an additional kinase that is redox activated under oxidizing conditions ($E_h > 40$ mV), or an additional, constitutively active kinase with a phosphatase that is activated under reducing conditions ($E_h < 40$ mV). We are currently performing experiments to test whether the phosphatase reactions are under redox control.

We must consider the obvious possibility that the 63 kDa reverse-titrating phosphoprotein in Fig. 2c is the 64 kDa LHCII kinase. From this it would follow that the *dephosphorylated* form of the kinase is active, while its phosphorylated form is inactive. This contradicts previous suggestions that the LHCII kinase is active in its autophosphorylated form [7,8]. We are presently conducting experiments to identify the 63 kDa phosphoprotein and to test directly the relationship between its phosphorylation and LHCII kinase activity.

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