Biochimica et Biophysica Acta, 314 (1973) 211–223

BBA 46589

STUDIES ON THE ENERGY COUPLING SITES OF PHOTOPHOSPHORYLATION

I. SEPARATION OF SITE I AND SITE II BY PARTIAL REACTIONS OF THE CHLOROPLAST ELECTRON TRANSPORT CHAIN

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(Received April 9th, 1973)

SUMMARY

1. The transport of electrons from H_2O to lipophilic oxidants such as oxidized *p*-phenylenediamines and 2,5-dimethylquinone, when observed in the presence of the plastoquinone antagonist dibromothymoquinone, has a pH optimum of approximately 7.5 and is independent of the presence or absence of ADP and phosphate. Nevertheless the electron transport supports phosphorylation with an efficiency (P/e_2) of 0.3–0.4 and this efficiency is practically pH independent. A reversible proton uptake is associated with the electron transport. The energy coupling site responsible for the phosphorylation, which must be before plastoquinone, we have designated Site II, while the well-known rate-determining coupling site after plastoquinone and before cytochrome *f* is referred to as Site I.

2. The transport of electrons from reduced 2,6-dichlorophenolindophenol (DCIP) to methylviologen, when observed in the presence of the Photosystem II inhibitor 3-(3,4-dichlorophenyl)-1,1-dimethylurea, is remarkably similar in most respects to the overall Hill reaction (*e.g.* $H_2O \rightarrow$methylviologen). The rate of electron flow is markedly stimulated by ADP and phosphate. Electron transport and phosphorylation have the same pH optimum of about 8.5. The P/e_2 ratio is also strongly pH dependent, showing a similar pH optimum of 8.0–8.5. However, the absolute value of the P/e_2 ratio observed for the partial reaction reduced DCIP→methylviologen is lower than the P/e_2 ratio observed for the overall reaction $H_2O \rightarrow$methylviologen at all pH values. The maximum P/e_2 value observed for the reduced DCIP→methylviologen raction is 0.5–0.6 at pH 8.0–8.5 while the maximum value for the $H_2O \rightarrow$methylviologen reaction under the same conditions is about 1.1.

3. When the P/e_2 ratios for the two partial reactions ($H_2O \rightarrow$dimethylquinone and reduced DCIP→methylviologen) are added together at all pH values from 6 to 9, the resulting curve is very close to the P/e_2–pH profile experimentally obtained for

Abbreviations: P/e_2, the ratio of the molecules of ATP formed per pairs of electrons transported; 4′-deoxyphlorizin, 4,6′-dihydroxy-2′-glucosidodihydrochalcone; DCIP, 2,6-dichlorophenolindophenol; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Tricine, *N*-tris(hydroxyethyl)methylglycine; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid.

the overall Hill reaction $H_2O \rightarrow$methylviologen. It seems probable, therefore, that the transport of electrons from reduced DCIP to methylviologen utilizes only the rate-determining coupling Site I while the overall transport of electrons from H_2O to methylviologen utilizes both Site I and Site II.

INTRODUCTION

The existence of two sites of energy coupling associated with noncyclic electron transport in isolated chloroplasts has been postulated for some time based on various lines of indirect evidence[1–7]. A new approach to this problem has recently been made possible with the use of the lipophilic "Class III" electron acceptors (such as oxidized *p*-phenylenediamines)[8]. In previous papers we have shown that the transport of electrons from water to these Class III acceptors is insensitive to the plastocyanin inhibitors KCN[9] and poly-L-lysine[10], and also to the plastoquinone antagonist dibromothymoquinone[11]. These inhibitors strongly inhibit the reduction of conventional (Class I[8]) acceptors such as ferricyanide or methylviologen, which require participation of both Photosystem II and Photosystem I. Furthermore, Class III acceptors are reduced at a point before the electrons from the independent Photosystem II units are pooled[9,12]. From these observations we have concluded that the reduction of Class III acceptors takes place predominately before plastoquinone[12]. Moreover, since the reduction of Class III acceptors is firmly coupled to phosphorylation even in the presence of these inhibitors, we have concluded further that there is a coupling site before plastoquinone (Site II) in addition to the coupling site believed to be located after plastoquinone and before cytochrome *f*[13] (Site I).

The phosphorylation reaction associated with Site II is characterized by (i) a pH optimum between 7 and 8, (ii) a low coupling efficiency ($P/e_2 = 0.3–0.4$) which is practically pH independent, and (iii) the lack of effect of ADP and phosphate or uncouplers on the rate of electron transport. The characteristics of conventional noncyclic photophosphorylation, which utilizes both Site II and Site I, are quite different. The pH optimum for both electron transport and ATP formation is 8.5 or above. The phosphorylation efficiency is also a strong function of pH, showing a similar optimum (where the P/e_2 is 1.0–1.1 in average chloroplast preparations). Furthermore, the rate of electron transport responds sharply to phosphorylating or uncoupling conditions. One may therefore reasonably deduce that these prominent features of conventional noncyclic photophosphorylation originate almost entirely from the coupling reaction at Site I. The existence of a rather inconspicuous coupling reaction at Site II must be largely masked, except for its contribution to the overall efficiency of phosphorylation.

In order to verify these deductions, however, it is essential to find a partial reaction of the electron transport chain which includes only coupling Site I. Such a reaction should very much resemble the complete noncyclic reactions except for its efficiency of phosphorylation (P/e_2), which should be approximately 0.6–0.7, instead of slightly above 1.0.

Larkum and Bonner[14] and Izawa[15], on the basis of spectral evidence, and Neumann *et al.*[16] on the basis of uncoupler studies, have postulated that reduced 2,6-dichlorophenolindophenol (DCIP) in the presence of 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) donates electrons to the electron transport chain on the Pho-

tosystem II side of the coupling site preceeding cytochrome *f*. The studies reported in this paper have provided strong evidence that the electron flow from reduced DCIP to methylviologen indeed constitutes a partial reaction which includes coupling Site I but not coupling Site II. In addition, we report here and in a subsequent paper[17] on further characterization of the coupling reaction at Site II including the demonstration of a "proton pump" driven by a partial reaction which involves only Site II.

MATERIALS AND METHODS

Chloroplasts were isolated in the cold (4 °C) by a technique similar to that used in previous studies[8,11,12]. Leaves of fresh market spinach (*Spinacia oleracea* L.) were washed in cold distilled water and ground briefly (3–7 s) in a Waring blendor containing a medium consisting of 0.3 M NaCl, 30 mM *N*-tris(hydroxyethyl)methylglycine (Tricine)–NaOH (pH 7.8), 3 mM $MgCl_2$ and 0.5 mM EDTA. After filtering the homogenate through multiple layers of cheesecloth, the chloroplasts were sedimented at $2500 \times g$ for 2 min. The pellet was resuspended in a medium containing 0.2 M sucrose, 5 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES)–NaOH (pH 7.5), 2 mM $MgCl_2$ and 0.05% bovine serum albumin. After a brief centrifugation to remove whole cells and debris (45 s at $2000 \times g$) the chloroplasts were sedimented at $2000 \times g$ for 4 min, resuspended in a fresh volume of the same medium, and again sedimented. The final pellet was taken up in a small volume of the suspension media.

The reduction of 2,5-dimethyl-*p*-benzoquinone, oxidized *p*-phenylenediamines and high concentrations of dibromothymoquinone was measured spectrophotometrically as described earlier[8] as the decrease in absorbance of the reaction mixture at 420 nm due to the reduction of excess ferricyanide. Methylviologen reduction (either water or artificial reductants as electron donor) was measured as oxygen uptake resulting from the reoxidation of reduced methylviogen[18]. A Clark-type membrane-covered oxygen electrode was used for assay. No catalase inhibitor was needed since our chloroplast preparations were free of catalase ativity.

Reactions were run in a final volume of 2.0 ml in thermostatted cuvettes at 19 °C. Actinic light (> 600 nm) was supplied by a 500-W slide projector and the appropriate filters.

ATP formation was determined for a 1-ml aliquot of the reaction mixture by extracting unreacted ^{32}P-labeled orthophosphate as phosphomolybdic acid into butanol–toluene (1:1, v/v) as detailed by Saha and Good[19]. Radioactivity in the final aqueous phase was measured by the Cerenkov technique of Gould *et al.* [20].

KCN-treated chloroplasts were prepared by incubating chloroplasts at 0 °C in a 30 mM KCN solution buffered at pH 7.8 as described by Ouitrakul and Izawa[9].

Stock solutions of 2,5-dimethyl-*p*-benzoquinone and dibromothymoquinone were made in ethanol–ethylene glycol (1:1, v/v). DCIP was dissolved in ethanol and diluted with glass distilled water. DCMU was dissolved in ethanol and further diluted with 0.01 M NaCl. At all times the concentration of organic solvent in the final reaction mixture was 1% or less.

RESULTS

The effect of pH on the rate of electron transport and phosphorylation using three different electron donor–acceptor systems is shown in Fig. 1. When electrons

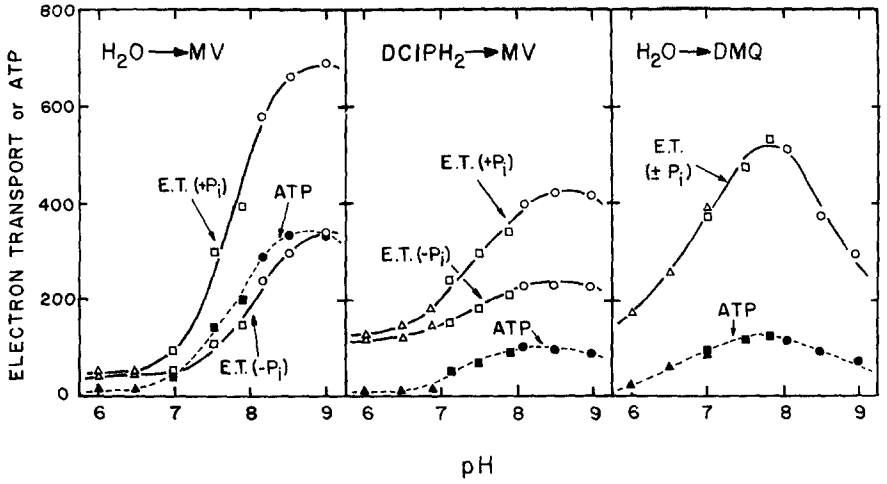

Fig. 1. Effect of pH on the rates of electron transport and phosphorylation associated with various electron donor–acceptor systems. The reaction mixture (2 ml) contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM buffer, 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$ (when added), chloroplasts containing 40 μg chlorophyll, and the indicated electron donor or acceptor system. These systems were: methylviologen, 50 μM; 2,5-dimethyl-*p*-benzoquinone, 0.5 mM *plus* 0.4 mM ferricyanide; reduced DCIP ($DCIPH_2$), 0.4 mM *plus* 2.5 mM ascorbate, 1 μM DCMU and 50 μM methylviologen. The buffers employed were 2-(*N*-morpholino)ethanesulfonic acid–NaOH (triangles), HEPES–NaOH (squares) and Tricine–NaOH (circles). Open symbols are for electron transport (E.T.) and solid symbols are for phosphorylation (ATP). When 2,5-dimethyl-*p*-benzoquinone was the electron acceptor 0.5 μM dibromothymoquinone was added to the reaction mixture to block the Class I component of 2,5-dimethyl-*p*-benzoquinone reduction. Rates are in μequiv or μmoles ATP/h per mg chlorophyll. Note the similarity between H_2O→methylviologen and reduced DCIP→methylviologen but not H_2O→2,5-dimethyl-*p*-benzoquinone. Abbreviations: MV, methylviologen; $DCIPH_2$, reduced DCIP; DMQ, 2,5-dimethyl-*p*-benzoquinone.

from water reduce the Class I acceptor methylviologen through the two sites of energy coupling, both electron flow and phosphorylation show a pH optimum around pH 8.5. The rate of electron transport in the absence of phosphate (basal rate) is much slower but shows a similar pH optimum. This is in good agreement with previous reports for ferricyanide reduction and the associated phosphorylation[1]. When reduced DCIP serves as electron donor and methylviologen as acceptor (in the presence of DCMU) the effect of pH is very similar to that observed for the H_2O→methylviologen reaction. Again the optimal pH for electron transport and phosphorylation is about 8.5 and a marked stimulation of electron transport by the concomitant phosphorylation is observed. Clearly the two reaction systems H_2O→methylviologen and reduced DCIP →methylviologen are governed by the same rate-limiting phosphorylation reaction. However, when the Class III acceptor 2,5-dimethyl-*p*-benzoquinone is reduced *via* Photosystem II by electrons from water, a different effect of pH is evident. 2,5-Dimethyl-*p*-benzoquinone reduction and the associated phosphorylation (which involves only coupling Site II) exhibit a considerably more acidic pH optimum than is observed for the H_2O→methylviologen system or the reduced DCIP→methylviologen system.

The effect of pH on the phosphorylation efficiency (P/e_2) of each of the three types of electron donor–acceptor systems mentioned above produced some striking results (Fig. 2). The P/e_2 ratio for the H_2O→methylviologen system is strongly pH

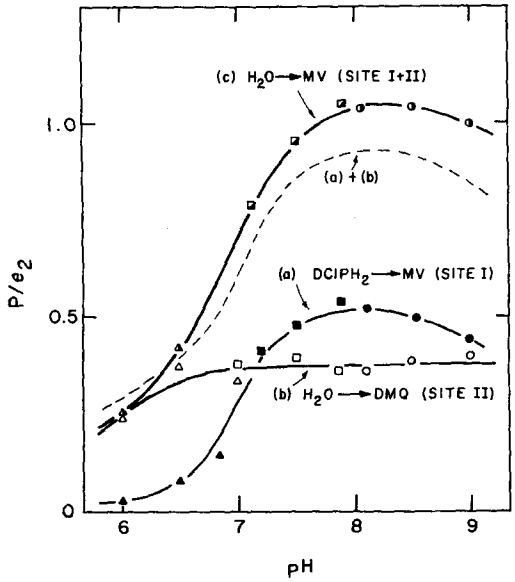

Fig. 2. Effect of pH on the phosphorylation efficiency (P/e_2) of three different electron donor–acceptor systems. P/e_2 values for each system were computed from the data presented in Fig. 1. Note that if the P/e_2 values for H_2O→2,5-dimethyl-*p*-benzoquinone (Photosystem II only) are added to the values for reduced DCIP→methylviologen (Photosystem I only), the sum (dashed curve) is close to the P/e_2 values obtained for H_2O→methylviologen (Photosystem II *plus* Photosystem I). Note also that the P/e_2 ratio associated with 2,5-dimethyl-*p*-benzoquinone reduction is practically constant over a wide pH range, whereas the P/e_2 ratios for H_2O→methylviologen and reduced DCIP→methylviologen are strongly pH dependent. Abbreviations: see Fig. 1.

dependent, showing a pH optimum of about 8 to 8.5. In contrast, the P/e_2 ratio for the H_2O→2,5-dimethyl-*p*-benzoquinone system, which utilizes only coupling Site II is essentially pH independent from pH 6.5 to 9. Thus the pH-dependent portion of the P/e_2 ratio for the H_2O→methylviologen system must be located after the site of 2,5-dimethyl-*p*-benzoquinone reduction. The effect of pH on the P/e_2 ratio associated with the reduced DCIP→methylviologen reaction once again resembles very closely the effect observed for the H_2O→methylviologen system. This similarity again suggests that the coupling site associated with the reduced DCIP→methylviologen system may be the same rate-limiting coupling site (Site I) associated with the H_2O→methylviologen reaction. However, the P/e_2 values observed for the reduced DCIP→methylviologen system are markedly lower than those for the H_2O→methylviologen system over the entire range of pH values tested, as though a pH-independent component is absent in the reduced DCIP system. Indeed, when the P/e_2 values for the two partial reactions (H_2O→2,5-dimethyl-*p*-benzoquinone and reduced DCIP→methylviologen) are added together, the resulting curve (Fig. 2, dashed line) is in fact very close to the experimentally obtained curve for the overall reaction H_2O→methylviologen. This implies very strongly that the partial reaction reduced DCIP→methylviologen is indeed utilizing only the coupling site that normally limits the rate of electron transport to Class I acceptors (*i.e.* Site I).

The curve derived by adding the P/e_2 values for the two partial reactions is

slightly lower than the observed curve for $H_2O \rightarrow$methylviologen, reflecting the fact that the P/e_2 ratios observed for the reduced DCIP→methylviologen reaction (maximum 0.55) are slightly lower than the values one would expect (0.6–0.7) from the difference in P/e_2 between the complete system $H_2O \rightarrow$methylviologen and the partial system $H_2O \rightarrow$2,5-dimethyl-*p*-benzoquinone. This discrepancy could be explained in two ways. If reduced DCIP had a secondary, uncoupling effect the P/e_2 values for the reduced DCIP→methylviologen reaction would be lowered. This seems unlikely, however, since it has already been shown that the reaction exhibits a constant P/e_2 over a wide range of reduced DCIP concentrations[21]. To further eliminate this possibility the effect of reduced DCIP on the post-illumination phosphorylation ("X_E") was examined. If reduced DCIP had an uncoupling action, then its presence in the dark (phosphorylation) stage of the experiment should decrease the yield of X_E. The high sensitivity of this method in detecting an uncoupling effect has been previously demonstrated by Hind and Jagendorf[22]. Table I shows that neither reduced DCIP nor 2,5-dimethyl-*p*-benzoquinone decreases the X_E yield to any significant extent, thus practically eliminating the possibility of these compounds having a significant uncoupling effect. These results are also important in that they ensure that the low efficiencies of phosphorylation observed for systems involving 2,5-dimethyl-*p*-benzoquinone ($P/e_2 = 0.3$–0.4) or reduced DCIP ($P/e_2 = 0.5$–0.6) are not due to an uncoupling effect of these compounds.

TABLE I

EFFECTS OF REDUCED DCIP *PLUS* ASCORBATE AND 2,5-DIMETHYL-*p*-BENZOQUINONE ON POST-ILLUMINATION ATP FORMATION (X_E)

Reduced DCIP (0.13 mM *plus* 0.8 mM ascorbate), 2,5-dimethyl-*p*-benzoquinone (0.17 mM) or methylamine (3.3 mM) were present only in the dark phosphorylation stage of the experiment. Chloroplasts containing 100 μg chlorophyll were illuminated for 20 s in a continuously stirred reaction mixture (2 ml) containing 0.1 M sucrose, 50 mM NaCl, 2 mM $MgCl_2$, 10 mM 2-(*N*-morpholino)ethanesulfonic acid–NaOH buffer (pH 6.0) and 5 μM pyocyanine. Immediately after shutting off the light 1 ml of a strongly buffered ADP–phosphate mixture (0.1 M Tricine–NaOH buffer (pH 8.2), 2 mM ADP, 10 mM $Na_2H^{32}PO_4$) containing the additions was quickly injected into the suspension to initiate ATP formation. After 20 s the dark phosphorylation was terminated by addition of 0.5 ml 1 M $HClO_4$. All reactions were run in a thermostated water bath at 19 °C. Note that both reduced DCIP (*plus* ascorbate) and 2,5-dimethyl-*p*-benzoquinone did not inhibit the yield of X_E, whereas the known uncoupler methylamine did.

Addition (dark stage)	*Expt*	*ATP formed (nmoles/100 μg chlorophyll)*	*Effect*
None	a	7.4	—
	b	7.7	
Reduced DCIP	a	9.2	Slight stimulation
	b	8.4	
2,5-Dimethyl-*p*-benzoquinone	a	6.9	None
	b	7.5	
Methylamine	a	2.6	Inhibition
	b	2.4	

TABLE II

EFFECT OF KCN TREATMENT ON ELECTRON TRANSPORT AND PHOSPHORYLATION ASSOCIATED WITH THE PHOTOSYSTEM I-DEPENDENT REACTION REDUCED DCIP→METHYLVIOLOGEN

Reaction conditions are as described in Fig. 1. KCN treatment of chloroplasts is described in Methods. The rates of electron transport (E.T.) and phosphorylation (ATP) are given in μequiv or μmoles ATP/h per mg chlorophyll. Electron transport from H_2O to methylviologen in the KCN-treated chloroplasts used here was completely inhibited.

Reaction pH	*Control chloroplasts*		*KCN-treated chloroplasts*	
	E.T.	*ATP*	*E.T.*	*ATP*
6.0	127	4	90	2
7.0	240	49	123	5
8.0	397	102	154	4
9.0	420	93	148	1

Alternatively, some of the electrons from reduced DCIP may be donated to Photosystem I *via* a secondary, nonphosphorylating pathway. In fact, there is already strong evidence for this possibility. Ouitrakul and Izawa[9] have shown that the phosphorylation associated with the reaction reduced DCIP→methylviologen is abolished by KCN treatment but the electron transport itself is only partially inhibited[9]. Recently, Izawa *et al.*[23] have shown by EPR studies that reduced DCIP can donate electrons directly to P_{700}, by-passing a KCN block at plastocyanin. They suggested that this portion of the donor reaction is not coupled to phosphorylation. Table II shows the effect of KCN treatment on the electron transport from reduced DCIP to methylviologen and the associated phosphorylation. Undoubtedly the reduced DCIP→methylviologen reaction contains a minor component which is KCN resistant and not coupled to phosphorylation. (It should be noted here that KCN treatment itself has no appreciable uncoupling or inhibitory effect on phosphorylation[9].) It therefore seems reasonable to conclude that the "true" P/e_2 values for this partial reaction are slightly higher than those shown in Fig. 2 (curve a). Thus the true sum of the P/e_2 values obtained for the partial reactions reduced DCIP→methylviologen (involving Site I only) *plus* H_2O→2,5-dimethyl-*p*-benzoquinone (involving Site II only) must indeed be very close to the values obtained for the overall reaction H_2O→methylviologen (Site II *plus* Site I).

The conclusion that the complete noncyclic electron transport H_2O→methylviologen and the partial electron transport reduced DCIP→methylviologen are governed by the same energy coupling reaction (Site I), is further strengthened by the experiments of Fig. 3 in which the effects of the energy transfer inhibitor 4′-deoxyphlorizin[31] on electron transport and phosphorylation were examined. In both systems ATP formation and that portion of the electron transport which is dependent upon the presence of ADP and phosphate are inhibited in a very similar manner. However, the phlorizin derivative has no effect on electron transport from H_2O to oxidized diaminodurene (a Class III acceptor[8]) either in the presence or absence of phosphate, although phosphorylation is inhibited. These observations are directly in line with the concept that the coupling site near Photosystem II (Site II) has no control over

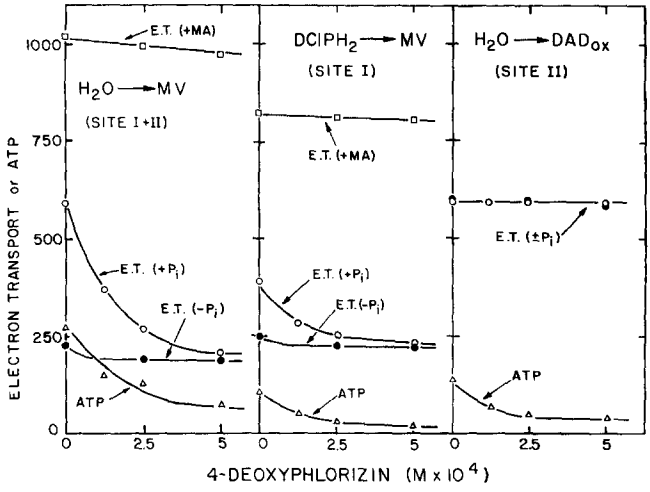

Fig. 3. Effect of the energy transfer inhibitor 4′-deoxyphlorizin on electron transport and phosphorylation associated with different electron donor–acceptor systems. Reaction conditions are essentially as described in Fig. 1. The buffer used was 50 mM Tricine–NaOH (pH 8.0). Methylamine (MA) was 10 mM when added. The chlorophyll concentration was 20 μg/ml. When oxidized diaminodurene (DAD_{ox}) was the electron acceptor the chlorophyll was 10 μg/ml and 0.5 μM dibromothymoquinone was added to block the Class I component of oxidized diaminodurene reduction. Rates of electron transport (E.T.) and phosphorylation (ATP) are in μequiv or μmoles ATP/h per mg chlorophyll. Note that for H_2O→methylviologen and reduced DCIP→methylviologen both ATP formation and that portion of the electron transport dependent upon phosphorylation are inhibited by 4′-deoxyphlorizin. However, when the Class III acceptor oxidized diaminodurene is being reduced, electron transport is not affected by the absence of phosphate or the presence of 4′-deoxyphlorizin, although phosphorylation is inhibited by the latter. Also note that ATP formation associated with all three electron donor–acceptor systems exhibits about the same sensitivity to 4′-deoxyphlorizin. Abbreviations: see Fig. 1.

electron transport while the site between plastoquinone and cytochrome *f* (Site I) does.

Relevant to these fiindings is the question of why the reduction of oxidized *p*-phenylenediamines (typical Class III acceptors) did not seem to be stimulated by ADP and phosphate at all[8], despite the fact that some portions of these acceptors are reduced *via* the complete electron transport pathway (as are Class I acceptors), utilizing both coupling sites (Site II and Site I)[9]. We have reinvestigated this problem and found that the concomitant phosphorylation does stimulate electron flow quite consistently (Table III). The stimulation may seem quite small, but this is simply because the very fast reduction of these compounds by Photosystem II. The absolute stimulation is in fact approximately equivalent to the stimulation observed when electrons flow from water to methylviologen. However, when this "Class I component" of the reduction of Class III acceptors is eliminated by the addition of low concentrations of dibromothymoquinone (or by KCN treatment[9]), the electron transport, now mediated only by Photosystem II and utilizing only coupling Site II, becomes completely independent of phosphorylating conditions. The genuine Photosystem II electron transport from H_2O to dibromothymoquinone (high concentration) is also not influenced by phosphorylation (Table III) or by uncouplers[12].

TABLE III

EFFECT OF PHOSPHORYLATING CONDITIONS ON ELECTRON TRANSPORT AS A FUNCTION OF THE ELECTRON ACCEPTOR

The 2.0-ml reaction mixture contained 0.1 M sucrose, 2 mM $MgCl_2$, 1 mM ADP, 5 mM $Na_2H^{32}PO_4$ (if added), 50 mM Tricine–NaOH (pH 8.1), chloroplasts, and the indicated acceptor. These acceptors were: methylviologen, 50 μM; oxidized *p*-phenylenediamine, 0.5 mM *plus* 1.5 mM ferricyanide; dibromothymoquinone, 4.5 μM *plus* 0.4 mM ferricyanide. The concentration of chlorophyll was 20 μg/ml when methylviologen or dibromothymoquinone acted as the electron acceptor and 15 μg/ml when oxidized *p*-phenylenediamine was the electron acceptor. Rates are given in μequiv or μmoles ATP/h per mg chlorophyll. Note that the reduction of oxidized *p*-phenylenediamine contains a phosphate-sensitive Class I component. If this component is abolished with a low concentration of dibromothymoquinone (which blocks electron transport to cytochrome f[30]) the effect of phosphate on oxidized *p*-phenylenediamine reduction is eliminated, even though a substantial rate of phosphorylation remains. Similarly, the Photosystem II-driven reduction of high concentrations of dibromothymoquinone, which accepts electrons from plastoquinone[12], is firmly coupled to ATP formation even though the electron transport shows no effect by phosphate.

Electron transport system	*Acceptor class**	*Coupling site involved***	*Electron transport rate*				
			$-P_i$	$+P_i$	$\Delta E.T.$	*ATP*	P/e_2
$H_2O \rightarrow$methylviologen	I	Site II + Site I	190	458	(268)	276	1.20
$H_2O \rightarrow$ox. *p*-phenylenediamine	III (+I)	Site II (+ Site I)	1370	1560	(190)	405	0.52
$H_2O \rightarrow$ox. *p*-phenylenediamine (+ $5 \cdot 10^{-7}$ M dibromothymoquinone)	III	Site II	860	860	(0)	185	0.43
$H_2O \rightarrow$dibromothymoquinone	III	Site II	198	200	(2)	34	0.34

* See ref. 8 and Introduction.

** Proposed sites of phosphorylation (see Fig. 5 and refs 9, 11, 12).

Fig. 4 shows a light induced pH rise ("proton uptake") associated with coupling Site II when 20 μM dibromothymoquinone was the electron acceptor. It can be seen that at pH 8.1, where reduced dibromothymoquinone is rapidly reoxidized by molecular oxygen[12], repeated periods of illumination induced the familiar reversible pH rise in the reaction medium. If DCMU is added (abolishing electron transport) the pH rise is also abolished. Similary, at pH 7.4 a brief illumination induces the pH rise. However, at this pH reduced dibromothymoquinone is not reoxidized by molecular oxygen. Thus, repeated illuminations do not induce a pH rise once all of the dibromothymoquinone is reduced and electron transport cannot proceed. This is comfirmed by the fact that addition of oxidized dibromothymoquinone restores the pH rise. The Photosystem II-dependent pH rise is also abolished by conventional uncouplers such as methylamine and gramicidin.

DISCUSSION

In previous papers[9,11,12] we have amply documented evidence that there is a site of energy coupling (Site II) near Photosystem II or, more specifically, before plastoquinone, in addition to the well-recognized coupling site (Site I) which lies between plastoquinone and cytochrome f and governs the rate of noncyclic electron

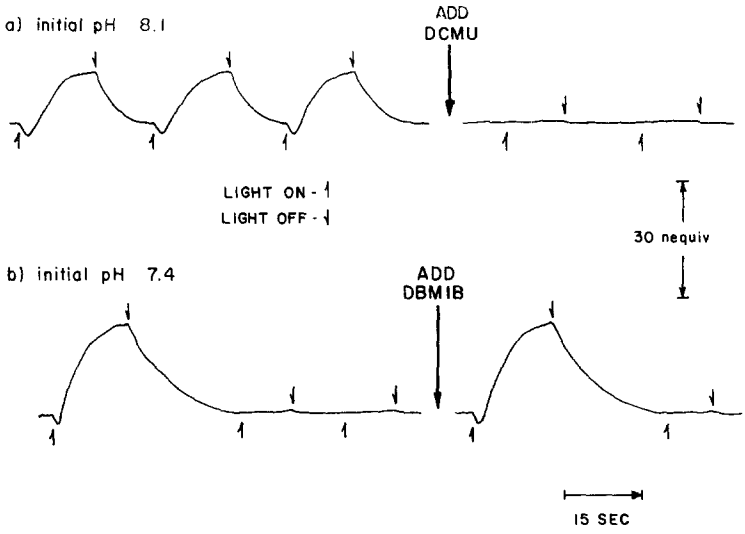

Fig. 4. Light-induced pH rise in the medium ("proton uptake") associated with the reduction of dibromothymoquinone by Photosystem II. The continuously stirred sample was illuminated in a thermostatted cuvette (19 °C) with strong white light. The change of pH of the medium was recorded using a Sargent miniature combination electrode and a Corning expanded scale pH electrometer. The scale was calibrated by adding in the light a known amount of HCl. The 2.0-ml sample contained 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM NaCl, 20 μM dibromothymoquinone, 0.5 mM Tricine–NaOH buffer at the indicated pH and chloroplasts containing 140 μg chlorophyll. When DCMU was added (Expt a) the final concentration was 2.1 μM. In Expt b 40 nmoles dibromothymoquinone (DBMIB) were added to restore the initial pH rise.

flow[13,24]. The main body of evidence comprises the fact a unique type of phosphorylation is observed when the lipophilic Class III oxidants (*e.g.* 2,5-dimethyl-*p*-benzoquinone, oxidized *p*-phenylenediamines, *etc.*) are reduced *via* Photosystem II alone. To observe these genuine Photosystem II reactions, the simultaneous reduction of the acceptors through Photosystem I needs to be eliminated (a) by blocking plastoquinone with dibromothymoquinone[25,26] or (b) by inactivating plastocyanin with KCN[9,10,23] or (c) by using dibromothymoquinone itself as a Class III acceptor (at high concentrations)[12]. The phosphorylation efficiency (P/e_2) of these Photosystem II reactions is always between 0.3 and 0.4, regardless of the acceptor used[9,11,12] and therefore regardless of the electron transport rate, which ranges from about 50 μequiv/h per mg chlorophyll when dibromothymoquinone is used as the acceptor to about 1500 when oxidized *p*-phenylenediamine is the electron acceptor. (This makes it extremely unlikely that the phosphorylation associated with the reduction of Class III acceptors is due to a Photosystem II-catalyzed cyclic electron flow.) The efficiency of phosphorylation supported by Site II is practically independent of pH over a wide range (ref. 12; see also Fig. 2, this paper). The electron transport is not stimulated by ADP and phosphate (ref. 12; see also Table III, this paper). When the electron transport chain is not blocked at plastoquinone or at plastocyanin, that is, when Class III acceptors are being reduced in part by Photosystem II alone and in part *via* both Photosystem II and Photosystem I, then the characteristics of the associated phosphorylation reactions become intermediate between those outlined above and those observed for

standard noncyclic photophosphorylation reactions such as with ferricyanide or methylviologen (Class I acceptors). There seems no doubt that we have succeeded, by the combined use of Class III acceptors and the inhibitors which block electron transport at or after plastoquinone, in disclosing and functionally "isolating" a coupling site (Site II) located before plastoquinone.

In this paper we have presented strong evidence which shows that the Photosystem I-dependent transport of electrons from reduced DCIP to methylviologen involves only Site I (Figs 1–3). The noninvolvement of Site II in this reaction is also consistent with the fact that the reaction is totally insensitive to dibromothymoquinone[25]. The lack of dibromothymoquinone inhibition indicates that plastoquinone, and therefore the coupling site before plastoquinone (Site II), does not participate in the reduced DCIP→methylviologen partial reaction.

Photosystem I-dependent reactions with reduced DCIP as electron donor have often been regarded as complex, involving a cryptic cyclic phosphorylation which could make phosphorylation appear completely unrelated to the observed rates of electron flow[32]. However, at least under the conditions employed in this study, the relation of phosphorylation to observed electron flow seems quite rigid, judging by the effect of ADP and phosphate, the uncoupler methylamine, and the energy transfer inhibitor 4′-deoxyphlorizin. Trebst and Pistorius[27] have presented brief data which led them to the same conclusion. Neumann *et al.*[16] have postulated, based on their uncoupler studies, that the reduced DCIP→methylviologen reaction and the H_2O→methylviologen reaction share the same rate-limiting phosphorylation site (our Site I). Shavit and Shoshan[28] have pointed out that high concentrations of ATP (where ATP acts as an energy transfer inhibitor) affect the reduced DCIP→$NADP^+$ system and the H_2O→$NADP^+$ system in a very similar manner. However, a detailed comparison of the phosphorylation reactions associated with the reduced DCIP system and the complete noncyclic system, such as we have presented here, has not been previously reported.

The failure of earlier investigators to detect significant phosphorylation associated with the photooxidation of reduced DCIP by Photosystem I may have been due largely to the nature of the chloroplast material used. Swollen, broken, or otherwise "leaky" chloroplast preparations give a high rate of electron transport (reduced DCIP→methylviologen) with very little, if any, phosphorylation. This electron transport is mostly KCN insensitive (unpublished data of J. M. Gould) and therefore probably represents increased access of reduced DCIP directly to P_{700} (ref. 23). Larkum and Bonner[14] have found that the reduced DCIP-induced cytochrome *f* response is also greatly diminished in broken chloroplasts.

The main conclusions we have drawn from this study are summarized in the scheme presented in Fig. 5. When Class I acceptors (*e.g.* methylviologen) are being reduced by electrons from water or reduced DCIP, the rate of electron flow is limited by the energy coupling reaction between plastoquinone and cytochrome *f* (Site I). The electron flux through Site I responds strongly to the addition of ADP and phosphate, uncouplers, or energy transfer inhibitors. The efficiency of phosphorylation (P/e_2) at this site is also pH dependent, having a maximum at pH 8.0–8.5 (observed maximum P/e_2, 0.5–0.6; predicted, 0.6–0.7). As mentioned above, Site II exerts no apparent control over electron flow. The efficiency of phosphorylation at Site II ($P/e_2 = 0.3$–0.4) is essentially pH insensitive. One possible explanation for this pH

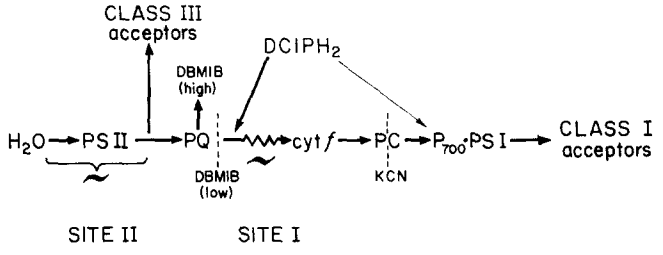

Fig. 5. A scheme for electron transport pathways in isolated chloroplasts showing the two sites of energy coupling (~). PS II, Photosystem II; DBMIB, dibromothymoquinone; PQ, plastoquinone; cyt *f*, cytochrome *f*; PC, plastocyanin; P_{700}, primary donor to Photosystem I (PS I). The zig-zagged line at Site I represents the primary rate-limiting step of electron transport to Class I acceptors. Class I acceptors include methylviologen, ferricyanide, flavins and ferredoxin–$NADP^+$. Class III acceptors include oxidized *p*-phenylenediamine, oxidized diaminodurene, 2,5-dimethyl-*p*-benzoquinone, *etc.* Since Class III acceptors can also act to some extent as Class I acceptors, genuine Photosystem II reactions are observed only when plastoquinone or plastocyanin is blocked (by dibromothymoquinone or KCN, respectively). Thus, in the presence of these inhibitors, the partial reaction H_2O→Class III acceptor includes coupling Site II but not Site I. Similarly, the partial reaction reduced DCIP→Class I acceptor includes coupling Site I but not Site II, whereas the overall reaction H_2O→Class I acceptor includes both Site II and Site I. Note also that the reduced DCIP→Class I acceptor contains both a KCN-sensitive and KCN-insensitive component.

insensitivity could be that coupling Site II is buried in a hydrophobic region of the membrane and therefore does not "see" the medium pH. This is consistent with the idea put forth by Böhme and Trebst[6] and Yamashita and Butler[7] that there is a coupling site on the water oxidizing side of Photosystem II, perhaps associated with the water-splitting reaction itself. It has been suggested that the protons lost during the oxidation of water are released to the inside of the thylakoid[29].

$$H_2O \rightarrow 2e^- + \tfrac{1}{2}O_2 + 2H^+_{(inside)}$$

The pH rise observed in the external medium when dibromothymoquinone (high concentration) is the electron acceptor (Fig. 4) would therefore represent the loss of protons from the medium for the reduction of the lipophilic dibromothymoquinone

$$2e^- + \text{dibromothymoquinone} + 2H^+_{(outside)} \rightarrow \text{reduced dibromothymoquinone}$$

in the thylakoid membrane. It is therefore probable that a transmembrane proton gradient is associated with the pathway H_2O→Photosystem II→dibromothymoquinone as well as with reduced DCIP→Photosystem I→methylviologen[21].

Finally, it should be mentioned that the relationship between Site I (see Fig.5) and the two coupling sites postulated by Neumann *et al.*[16] to be associated with Photosystem I is still unclear. Preliminary experiments have indicated that ATP formation coupled to the Photosystem I-dependent electron flow from diaminodurene to methylviologen may not utilize the coupling site which limits the H_2O (or reduced DCIP) →methylviologen reaction (unpublished data of J. M. Gould). Experiments are in progress to determine if further subdivision of Site I into two sites may be necessary.

ACKNOWLEDGEMENTS

The authors wish to thank Mr Don Ort and Dr N. E. Good for valuable discussions and Dr G. D. Winget for the gift of the 4'-deoxyphlorizin. Supported by a grant (GB 22657) from the National Science Foundation, U.S.A.

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