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THE PHOSPHORYLATION SITE ASSOCIATED WITH THE OXIDATION OF EXOGENOUS DONORS OF ELECTRONS TO PHOTOSYSTEM I

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SUMMARY

1. The Photosystem I-mediated transfer of electrons from diaminodurene, diaminotoluene and reduced 2,6-dichlorophenolindophenol to methylviologen is optimal at pH 8–8.5, where phosphorylation is also maximal. In the presence of superoxide dismutase, the efficiency of phosphorylation rises from $\leqslant$ 0.1 at pH 6.5 to 0.6–0.7 at pH 8–8.5, regardless of the exogenous electron donor used.

2. The apparent K_m (at pH 8.1) for diaminodurene is $6 \cdot 10^{-4}$ M and for diaminotoluene is $1.2 \cdot 10^{-3}$ M. The concentrations of diaminodurene and diaminotoluene required to saturate the electron transport processes are $>$ 2 mM and $>$ 5 mM, respectively. At these higher electron donor concentrations the rates of electron transport are markedly increased by phosphorylation (1.5-fold) or by uncoupling conditions (2-fold).

3. Kinetic analysis of the transfer of electrons from reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) to methylviologen indicates that two reactions with very different apparent K_m values for $DCIPH_2$ are involved. The rates of electron flux through both pathways are increased by phosphorylation or uncoupling conditions although only one of the pathways is coupled to ATP formation. No similar complications are observed when diaminodurene or diaminotoluene serves as the electron donor.

4. In the diaminodurene → methylviologen reaction, ATP formation and that part of the electron transport dependent upon ATP formation are partially inhibited by the energy transfer inhibitor $HgCl_2$. This partial inhibition of ATP formation rises to about 50 % at less than 1 atom of mercury per 20 molecules of chlorophyll, then does not further increase until very much higher levels of mercury are added.

Abbreviations: $DCIPH_2$, reduced 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N'*-ethanesulfonic acid; P/e_2, ratio of the number of molecules of ATP formed to the number of pairs of electrons transported; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea. DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; HEPPS, *N*-2-hydroxyethylpiperazine-*N*-propanesulfonic acid; EDAC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide.

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5. It is suggested that exogenous electron donors such as diaminodurene, diaminotoluene and $DCIPH_2$ can substitute for an endogenous electron carrier in donating electrons to cytochrome *f* via the mercury-sensitive coupling site (Site I) located on the main electron-transporting chain. If this is so, there would seem to be no reason for postulating yet another coupling site on a side branch of the electron transport chain in order to account for cyclic photophosphorylation.

INTRODUCTION

The transport of electrons from water to conventional Hill reaction oxidants such as ferricyanide or methylviologen is coupled to ATP formation at two sites [1–4]. By the use of appropriate combinations of exogenous electron donors, electron acceptors and electron transport inhibitors it is possible to divide the complete electron transport process into two partial reactions each of which involves one of the two coupling sites [5]. This approach makes possible the study of segments of the electron transport chain and therefore the study of the functionally isolated coupling sites without physical disruption of the chloroplast lamellae by detergents or mechanical treatments. Through the use of these partial reactions it has been discovered that the two coupling sites in chloroplasts behave differently in many respects [5, 13, 42].

Activities of the recently discovered coupling site [1–4] believed to be associated with water oxidation (see ref. 6), Coupling Site II, can best be separated from activities of the well-known coupling site between plastoquinone and cytochrome *f* [7, 8], Coupling Site I, by using lipophilic oxidants as acceptors of the electrons from Photosystem II. These "Class III" acceptors (e.g. oxidized *p*-phenylenediamine) intercept electrons primarily at a point between the photosystems [9] (presumably before the plastoquinone pool [10]). Thus when a Photosystem-I component of the reduction of these acceptors is eliminated by inactivating plastocyanin with KCN [11] or poly-L-lysine [12], or by blocking electron transport at plastoquinone with 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) [1–3], the resulting pure Photosystem II reaction includes only Coupling Site II. Studies of Coupling Site II isolated in this manner have revealed the following characteristics: (a) Site II does not control the rate of electron transport since the rate is independent of phosphorylating or uncoupling conditions (refs 9, 10 and 13; but see refs 3 and 14); (b) Site II exhibits a characteristic phosphorylation efficiency (P/e_2) of 0.3–0.4 [2–5, 10, 11] which is independent of pH over the range 6–9 [5, 10]; (c) a light-driven reversible H^+-uptake reaction is associated with the partial reaction [5]. The efficiency of this proton uptake (H^+/e) is 0.4–0.5 over the pH range 6–8.5 [15]; finally (d) phosphorylation supported by Coupling Site II is not inhibited by the chloroplast energy transfer inhibitor $HgCl_2$ [16, 17].

Similarly, it is possible to isolate Coupling Site I from Site II by inhibiting electron flow from Photosystem II with 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) while using an appropriate exogenous source of electrons for Photosystem I. In an earlier paper it was reported that the Photosystem I-dependent transport of electrons from reduced 2,6-dichlorophenolindophenol ($DCIPH_2$) to methylviologen utilizes Coupling Site I but not Coupling Site II [5]. Site I is probably a primary rate-determining step for the overall Hill reaction ($H_2O \rightarrow$ methylviologen) [7]. In fact, many of the characteristics of the $DCIPH_2 \rightarrow$ methylviologen and the water $\rightarrow$ methylviologen reactions are similar. For instance, the rate of electron flux from $DCIPH_2$ to methylviologen is markedly stimulated by phosphorylation or by uncoupling conditions [5, 18–20] as is the overall Hill reaction. ATP formation supported by the transfer of electrons from $DCIPH_2$ to methylviologen is partially inhibited by $HgCl_2$, as is phosphorylation in the overall Hill reaction [16, 17]. Moreover, the efficiency of phosphorylation (P/e_2) is strongly pH dependent in the $DCIPH_2 \rightarrow$ methylviologen reaction [5] as is the efficiency of phosphorylation in the overall Hill reaction.

There are, however, some characteristics of the $DCIPH_2 \rightarrow$ methylviologen reaction which detract from its usefulness as a system for studying isolated Coupling Site I. For example, $DCIPH_2$ donates electrons to the transport chain in at least two places. Most of the electrons from $DCIPH_2$ are donated on the Photosystem II side of cytochrome *f* [21, 22]. This electron transport pathway is coupled to phosphorylation (via Site I) and is sensitive to inhibition by KCN [5]. A second, smaller portion of the electrons from $DCIPH_2$ are donated to P_{700} via a KCN-insensitive pathway which is not coupled to phosphorylation [23, 24]. Furthermore, the proportion of the electron transport which is KCN-insensitive is a function of the physical state of the chloroplasts, being much greater in damaged or "leaky" preparations [5].

In an effort to avoid some of the difficulties encountered with $DCIPH_2$ as electron donor, we have used other compounds which have long been known to be donors of electrons to Photosystem I (for a more complete discussion of Photosystem I reactions, see reviews by Trebst, contained in references 39 and 41). Several investigators have reported that diaminodurene is an extremely efficient donor of electrons to Photosystem I, supporting very high rates of ATP formation [18, 25, 26], but the properties of the coupling site responsible for this phosphorylation have not previously been characterized, nor has the coupling site been definitely located. Trebst and coworkers have recently postulated that lipophilic exogenously added donors of electrons catalyze phosphorylation via an "artificial" coupling site formed when protons are released to the inner phase of the thylakoid upon oxidation of the donor by plastocyanin or P_{700} [39, 40]. In this paper we arrive at a slightly different conclusion: that the electron-transport pathway from diaminodurene (or from the chemically related compound diaminotoluene) to methylviologen very likely includes the coupling site located just before cytochrome *f* (Site I). If so, the high rates of electron transport and ATP formation associated with these partial reactions, as well as the absence of secondary electron pathways, make these systems superior to the $DCIPH_2 \rightarrow$ methylviologen reaction for the isolation and study of Coupling Site I in chloroplasts.

MATERIALS AND METHODS

Chloroplasts were prepared from leaves of fresh market spinach (*Spinacia oleracea* L.) as described earlier [5]. However, for experiments with $HgCl_2$ it was necessary to avoid the use of tricine buffer since this compound strongly complexes Hg^{2+}. Chloroplasts for use in these experiments were isolated in a similar manner, substituting *N*-2-hydroxyethylpiperazine-*N′*-propanesulfonic acid (HEPPS)/NaOH for the tricine or *N*-2-hydroxyethylpiperazine-*N*'-ethanesulfonic acid (HEPES) where appropriate. In addition, EDTA was omitted from the grinding medium and bovine serum albumin was omitted from the suspension medium when $HgCl_2$ was used.

Electron transport was measured as the oxygen uptake resulting from the aerobic reoxidation of reduced methylviologen [18]. A membrane-covered Clark-type oxygen electrode was used. Reactions (2.0 ml final volume) were run in thermostatted vessels at 19 °C. Orange actinic illumination ($>$ 600 nm; $>$ 500 $\text{kerg} \cdot \text{cm}^{-2} \cdot \text{s}^{-1}$) was supplied by a 500-W tungsten projector lamp. The beam was passed through a 1-l round-bottom flask, containing a dilute $CuSO_4$ solution, which served both as a condensing lens and as a heat filter. ATP formation was determined as the $^{32}P_i$ incorporation into ATP as described elsewhere [27]. Radioactivity was measured as Cerenkov radiation by the technique of Gould et al. [28].

Diaminodurene (Research Organic/Inorganic Chemical Corp.) and 2,5-diaminotoluene (Aldrich) were dissolved in 0.1 M HCl, treated with Norit A, and recrystallized as the dihydrochlorides from concentrated HCl. Sodium 2,6-dichlorophenolindophenol (Matheson) was dissolved in ethanol and filtered. Fresh stock solutions of diaminodurene, diaminotoluene and DCIP were made before each experiment. Diaminodurene and diaminotoluene were dissolved in 0.01 M HCl. Ethanolic solutions of DCIP were further diluted with 1 mM Na_2HCO_3 so that the final concentration of ethanol in the reaction mixture did not exceed 0.5 %. The concentration of the DCIP solution was determined from the absorbance at 600 nm using an extinction coefficient (ε) of $2.1 \cdot 10^4$.

Superoxide dismutase was prepared from fresh bovine erythrocytes by the procedure of McCord and Fridovich [29]. The specific activity, assayed as the inhibition of cytochrome *c* reduction with xanthine oxidase, was $>$ 3000 units mg protein.

RESULTS

Diaminodurene → methylviologen and diaminotoluene → methylviologen

Earlier studies of the Photosystem I-catalyzed transport of electrons from diaminodurene to methylviologen indicated that very high rates of electron transport can occur. However, a tight coupling between electron transport and phosphorylation was not apparent in these studies: the rate of electron transport being only slightly increased by phosphorylation or uncoupling conditions [18, 25]. Furthermore, the efficiency of phosphorylation (P/e_2) was found to be rather low, usually about 0.3 [18].

In this paper we have re-examined the diaminodurene → methylviologen reaction in an effort to better characterize the relationship between electron transport and phosphorylation. Recently several workers have shown that the photooxidations of a number of exogenous electron donors can be associated with misleadingly high rates of oxygen uptake when methylviologen serves as the electron acceptor [30–33]. This is because significant (and variable) portions of the superoxide radicals ($\cdot O_2^-$) generated by the aerobic reoxidation of the methylviologen radical react directly with the exogenous electron donor (AH_2) and are reduced to peroxide ($2 \cdot O_2^- + AH_2 \rightarrow 2\,HO_2^- + A$). This leads to an exagerated rate of O_2 uptake since some of the $\cdot O_2^-$, which normally dismutates to regenerate 1/2 of the consumed O_2 ($2 \cdot O_2^- + 2H^+ \rightarrow H_2O_2 + O_2$), becomes trapped as additional H_2O_2; to the extent that superoxide

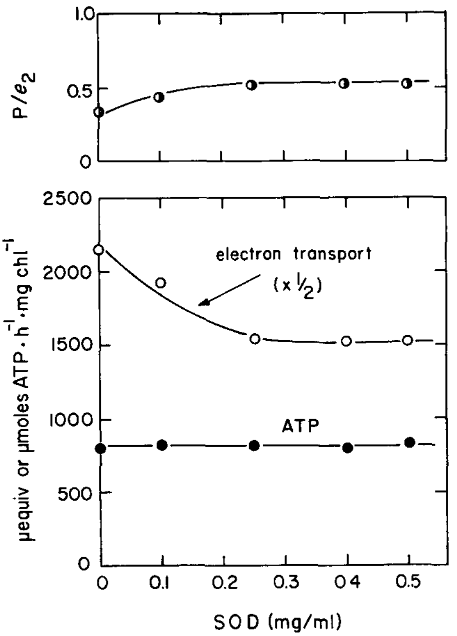

Fig. 1. Effect of bovine erythrocyte superoxide dismutase on the electron transport and phosphorylation associated with the diaminodurene → methylviologen reaction. The 2.0-ml reaction mixture consisted of: 0.1 M sucrose, 2 mM $MgCl_2$, 50 mM tricine/NaOH (pH 8.1), 0.75 mM ADP, 5 mM $Na_2H^{32}PO_4$, 2.5 µM DCMU, 2.5 mM ascorbate, 2.5 mM diaminodurene, 100 µM methylviologen, chloroplasts containing 5 µg chlorophyll and the indicated amount of superoxide dismutase (SOD). Note that although the oxygen uptake is lowered approximately 30 % by SOD, the rate of ATP formation is unaffected and therefore there is an increase in the apparent P/e_2 ratio from 0.3 to 0.5. For explanation see the text and ref. 30.

reacts with the donor, the transport of a single electron results in the uptake of a whole molecule of oxygen. However, if the dismutation of $\cdot O_2^-$ is greatly enhanced with superoxide dismutase, the reaction with the electron donor is eliminated and the rate of O_2 uptake becomes a reliable measure of the actual rate of electron transport, the uptake of a molecule of oxygen representing the transport of exactly two electrons ($O_2 = e_2$). This is, of course, only true if the chloroplast preparation is free of catalase activity (which was the case with the chloroplasts used in this study).

Fig. 1 shows the effect of bovine erythrocyte superoxide dismutase on the Photosystem I reaction diaminodurene → methylviologen. As increasing amounts of the enzyme compete more effectively for $\cdot O_2^-$, the rate of O_2 uptake is lowered by about 30 %, beyond which further addition of the enzyme has little effect. However, as might be expected, the rate of phosphorylation and therefore presumably the actual rate of electron transport is independent of superoxide dismutase. Because of this decrease in O_2 uptake the apparent P/e_2 (P/O_2) ratio increases from 0.3 to about 0.5. These data confirm the observation of Ort and Izawa [30], and indicate that accurate determinations of electron transport rates in the diaminodurene → methylviologen reaction can only be obtained in the presence of a suitable amount of superoxide

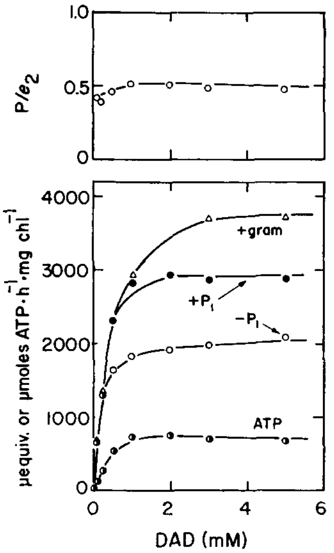

Fig. 2. Effect of diaminodurene (DAD) concentration on the rate of electron transport and phosphorylation in the diaminodurene → methylviologen reaction. The reaction mixture was as described in Fig. 1, except that the diaminodurene concentration was varied as indicated. The concentration of superoxide dismutase was 0.4 mg/ml. When added, gramicidin was 4 µg/ml.

dismutase. Very similar effects of superoxide dismutase were also observed when 5 mM diaminotoluene or 2,6-dichlorophenolindophenol served as the electron donor.

Since electron transport from diaminodurene (or diaminotoluene) to methylviologen exhibits many of the characteristics of the complete Hill reaction (H_2O → methylviologen), both reactions probably share the same rate-limiting step (i.e. Coupling Site I). At high diaminodurene concentrations (> 2 mM), the electron transport from diaminodurene to methylviologen is considerably accelerated by phosphorylation or to an even greater extent by uncoupling (Fig. 2). The failure of some earlier investigators to detect these large stimulations is perhaps due in part to the low concentrations of diaminodurene generally employed in the study of this reaction: typically < 0.5 mM. At these lower concentrations the donation of electrons by diaminodurene can be rate-limiting, and the effects of phosphorylation or uncoupling on electron transport may be largely concealed. At the higher diaminodurene concentrations used here, however, the diaminodurene → methylviologen reaction resembles very much both the $DCIPH_2$ → methylviologen and the H_2O → methylviologen reaction in its response to phosphorylating or uncoupling conditions, even though all rates are much higher in the diaminodurene system.

The kinetics of the diaminodurene → methylviologen reaction are shown by a double-reciprocal plot in Fig. 3. The rate-limitation imposed by the energy coupling mechanism is clearly revealed in this plot as departures from linearity. The data also indicate that this rate limitation can only be completely relaxed in the presence of an uncoupler.

The reaction diaminotoluene → methylviologen exhibits very similar characteristics to the diaminodurene → methylviologen reaction, except (a) electron trans-

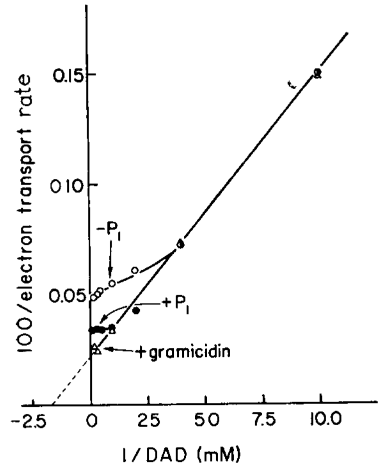

Fig. 3. Double-reciprocal plot showing the effect of diaminodurene concentration on electron transport in the diaminodurene → methylviologen reaction. The data are replotted from Fig. 2. (Electron transport rates are in μequiv · h^{-1} per mg chlorophyll.) Note that, at very low diaminodurene concentrations, electron transport in the presence or absence of phosphate (P_i) or the uncoupler germicidin is limited by the same rate-determining step. As the concentration of diaminodurene is increased, different rate-limitations become evident unless the reaction is uncoupled by gramicidin.

port supported by diaminotoluene saturates at slightly higher concentrations (> 5 mM), and (b) the absolute rates of electron transport and phosphorylation are about 30 % lower under optimal conditions with diaminotoluene as the electron donor. Some of the characteristics of Photosystem I reactions catalyzed by diaminodurene, diaminotoluene and $DCIPH_2$ are summarized in Table I.

TABLE I

DIAMINODURENE, DIAMINOTOLUENE AND REDUCED 2,6-DICHLOROPHENOLINDOPHENOL AS DONORS OF ELECTRONS TO PHOTOSYSTEM I

Reactions were run as described in Fig. 2 when diaminodurene and diaminotoluene were the electron donors, or as in Fig. 5 when $DCIPH_2$ served as the electron donor.

Electron donor	Apparent K_m (mM)	Approximate concentration required to saturate rates (mM)	Approximate V (μequiv · h^{-1} per mg chlorophyll)*
Diaminodurene	0.6	3	4000
Diaminotoluene	1.2	5	3000
$DCIPH_2$	0.03** 0.5**	>0.6	>1000

* Determined in the presence of 4 μg/ml gramicidin D. The rates are those obtained at the saturating or nearly saturating light intensities used in this study (see Methods).

** High- and low-affinity components determined as described in ref. 34. See Fig. 5 and text for further explanation.

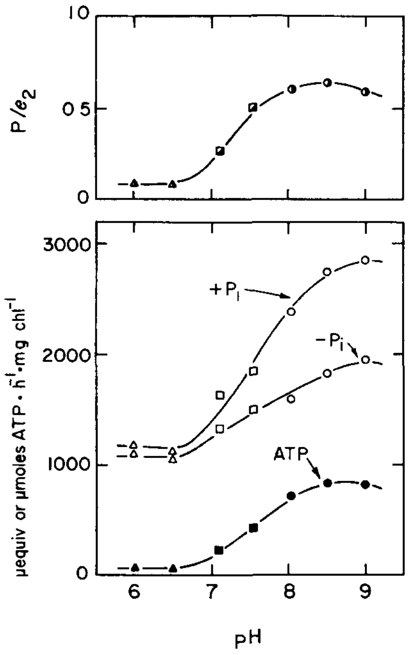

Fig. 4. Effect of pH on the rate of electron transport and phosphorylation associated with the diaminodurene → methylviologen reaction. The reaction mixture was as described in Fig. 1, except that the concentration of diaminodurene was 3 mM. The concentration of superoxide dismutase was 0.37 mg/ml. The buffers (50 mM) employed were 2-(*N*-morpholino)-ethanesulfonic acid/NaOH (triangles), HEPES/NaOH (squares) and tricine/NaOH (circles). Note that the efficiency of phosphorylation (P/e_2) is strongly pH-dependent, being optimal at pH 8–9, and that a large stimulation of electron transport by phosphorylation is also seen at the higher pH values. These characteristics resemble very closely the characteristics of Coupling Site I in chloroplasts (see Introduction and ref. 5).

Effects of pH on the diaminotoluene → methylviologen reaction are shown in Fig. 4. The rates of both electron transport and ATP formation are optimal at pH 8.5 or higher, with little or no phosphorylation occuring below pH 7. The P/e_2 ratio, therefore, shows the strong pH-dependence characteristic of Coupling Site I [5]. Indeed, the effects of pH on the H_2O → methylviologen, $DCIPH_2$ → methylviologen and diaminodurene → methylviologen reactions are strikingly similar (compare Fig. 4 and ref. 5, Fig. 1).

$DCIPH_2$ → methylviologen

The widely used Photosystem I reaction transporting electrons from reduced $DCIPH_2$ to methylviologen probably utilizes the coupling site after plastoquinone and before cytochrome *f* (i.e. Site I) [21, 22]. However, it has been shown that the donation of electrons to Photosystem I by $DCIPH_2$ has two components [5, 23, 24]. One component is coupled to phosphorylation (presumably via Site I) and is sensitive to inactivation of plastocyanin by KCN treatment. The other component is not coupled to phosphorylation and is insensitive to KCN (ref. 5, see below).

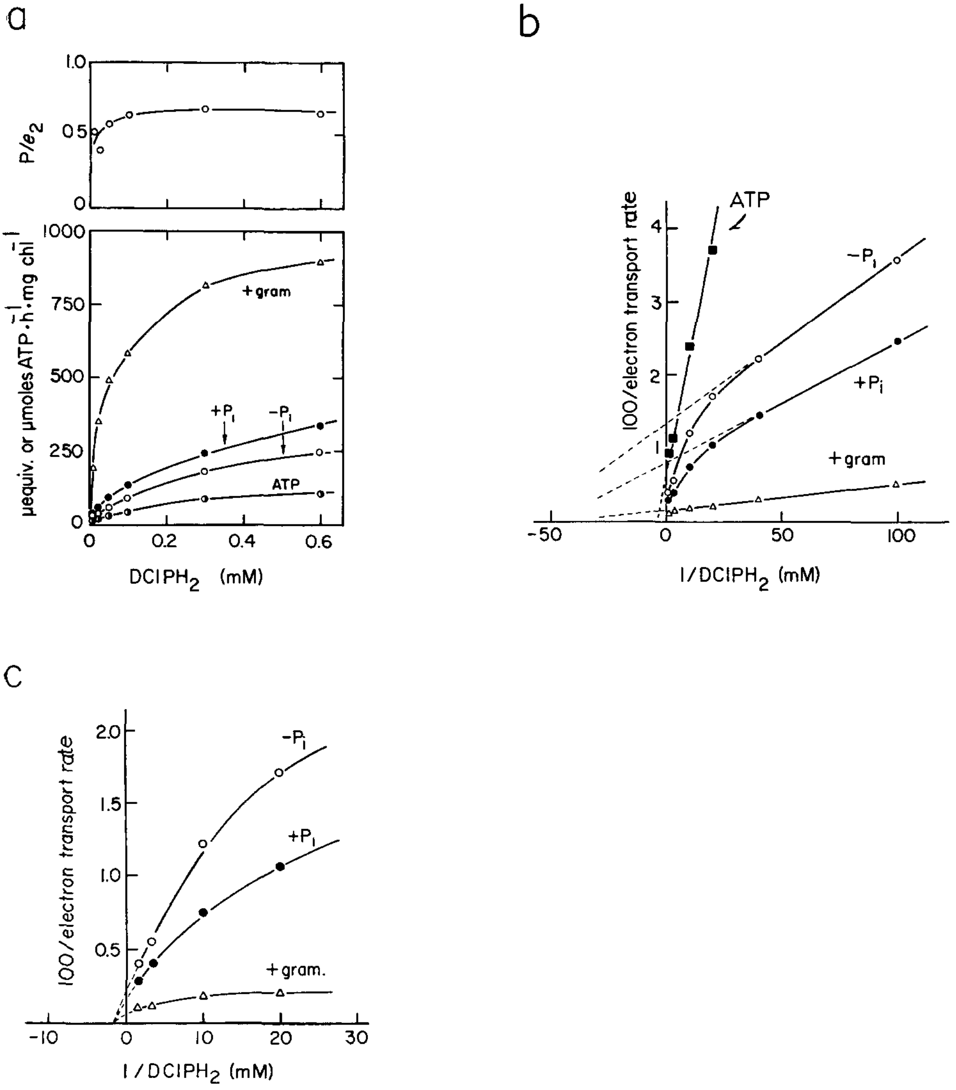

Fig. 5. a. Effect of DCIPH$_2$ concentration on electron transport and phosphorylation in the DCIPH$_2$ → methylviologen reaction. The reaction mixture (2.0 ml) contained 0.1 M sucrose, 2 mM MgCl$_2$, 50 mM tricine/NaOH (pH 8.1), 0.75 mM ADP, 2.5 µM DCMU, 100 µM methylviologen, 2.5 mM ascorbate, 250 µg superoxide dismutase, chloroplasts containing 40 µg chlorophyll, and the indicated amount of DCIPH$_2$. When added, Na$_2$H^{32}PO$_4$ (P$_i$) was 5 mM; gramicidin was 4 µg/ml. Note that even at the lowest levels of DCIPH$_2$ tested, where the rates of coupled electron flow are very low, a large stimulation of electron transport by gramicidin can be observed. b. Double-reciprocal plot of the data presented in a. Electron transport (circles, triangles) is in µequiv · h^{-1} per mg chlorophyll; ATP formation (squares) is in µmol ATP · h^{-1} per mg chlorophyll. Note the biphasic nature of the plots, indicating that two different reactions with different affinities for DCIPH$_2$ are competing for DCIPH$_2$ as a substrate [34]. Note also that the rate of electron transport from DCIPH$_2$ to methylviologen is increased by phosphorylation and uncoupling conditions in both reactions. c. Replot of some of the data presented in b showing the component of the DCIPH$_2$ → methylviologen reaction with the higher apparent K_m.

The two-component nature of this reaction, also noted by Arntzen et al. [24], is illustrated in the double reciprocal plots in Figs 5b and 5c. The biphasic nature of the plots is characteristic of a system in which two reactions compete for the same substrate [34], $DCIPH_2$ in this case. It is important to note, however, that both reactions appear to be dependent on the coupling state of the chloroplast, although only the reaction with the lower affinity for $DCIPH_2$ seems to support ATP formation (Fig. 5b); even at the lowest concentrations of $DCIPH_2$, where the high affinity, non-phosphorylating reaction predominates, significant stimulations of electron transport by uncouplers and by $ADP+P_i$ are observed. Perhaps the access of the reduced indophenol to its electron donation sites within the lipid membrane is limited to some extent by the high energy state (proton gradient?) associated with the membrane.

$HgCl_2$ inhibition

$HgCl_2$ has been shown to act as a unique energy transfer inhibitor in chloroplasts at extremely low levels [35]. Concentrations of 50 nmol Hg^{2+} per mg chlorophyll or less inhibit ATP formation in the Hill reaction to a 50 % inhibition plateau. It has recently been shown that $HgCl_2$ inhibits phosphorylation associated with $H_2O \rightarrow$ methylviologen (Coupling Sites II and I) and $DCIPH_2 \rightarrow$ methylviologen (Site I only)

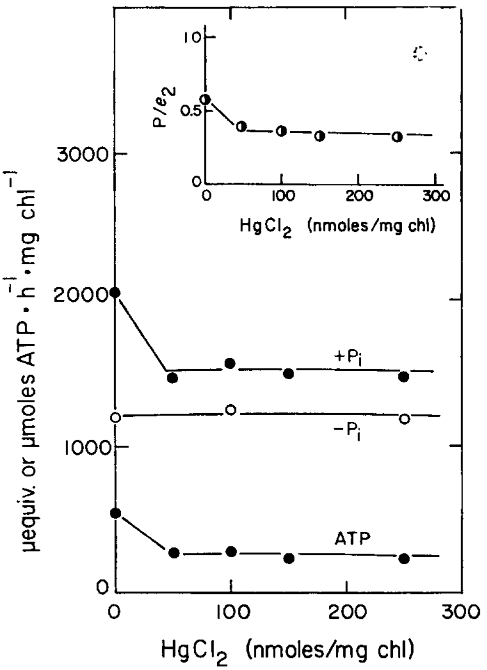

Fig. 6. Effect of the energy transfer inhibitor $HgCl_2$ on electron transport and phosphorylation associated with the diaminodurene → methylviologen reaction. The reaction mixture was as in Fig. 1, except the buffer employed was 50 mM HEPPS/NaOH (pH 8.0). The concentration of superoxide d smutase was 0.37 mg/ml. Chloroplasts were incubated with the indicated amount of $HgCl_2$ for 20 s in the dark before addition of the remainder of the reaction mixture. Note that ATP formation and that portion of the electron transport dependent upon phosphorylation are inhibited to a plateau of about 50 % by low concentrations of $HgCl_2$.

but not phosphorylation associated with H_2O → Class III acceptors (Site II only) [16]. The implication is that $HgCl_2$ acts as a specific inhibitor of Coupling Site I. (The reason for the peculiar 50 % inhibition plateau is not at all understood.) Fig. 6 shows that the diaminodurene → methylviologen reaction also includes the Hg-sensitive coupling site.

The effects of $HgCl_2$ on the diaminodurene → methylviologen reaction presented here are somewhat at variance with those reported by Bradeen and Winget [17]. They found that phosphorylation-coupled electron transport from diaminodurene to methylviologen was insensitive to low concentrations of $HgCl_2$ (250 nmol/mg chlorophyll) although phosphorylation itself was inhibited about 30 % by 50–250 nmol $HgCl_2$ per mg chlorophyll. (Higher concentrations of mercury result in electron transport inhibition due to plastocyanin inactivation [36].) However, it should be pointed out that a small inhibition of a very fast reaction can often be difficult to detect when the electron transport is measured with the slow-responding, Clark-type oxygen electrode.

DISCUSSION

There are a variety of compelling reasons for believing that the primary pathway of electron transport from $DCIPH_2$ to methylviologen includes the coupling site located between plastoquinone and cytochrome *f*, which we have called Site I. Izawa [21] and Larkum and Bonner [22], on the basis of spectral evidence, and Neumann et al. [19], on the basis of uncoupler studies, have suggested that $DCIPH_2$ donates electrons to the transport chain at a point before a phosphorylation-dependent rate-limiting reaction on the Photosystem II side of cytochrome *f*. It has also been shown that the phosphorylation-coupled $DCIPH_2$ → methylviologen reaction resembles the coupled overall H_2O → methylviologen reaction in its response to pH, to $ADP+P_i$, to uncouplers, to $HgCl_2$, to other energy transfer inhibitors and to KCN [5]. It seems highly probable therefore that both reactions share the same rate-determining coupling site. This conclusion is further strengthened by the fact that the sums of the phosphorylation efficiencies of the two partial reactions (H_2O → Photosystem II → Class III acceptors, and $DCIPH_2$ → Photosystem I → methylviologen are very close to the efficiencies observed for the overall H_2O → methylviologen reaction over the wide pH range 6–9 (see refs 5 and 42).

Unfortunately the $DCIPH_2$ → methylviologen reaction is not altogether satisfactory for the study of Coupling Site I. As we have seen, the reaction is complex. The electron transport has a non-phosphorylating component which utilizes a different pathway and the magnitude of this component varies with the state of the chloroplast membrane (see Fig. 5 and refs. 5, 22–24).

The results presented in this paper seem to indicate that the photooxidations of diaminodurene or diaminotoluene are partial reactions of the electron transport chain which also involve Coupling Site I. Again the effects of pH, of $ADP+P_i$, of uncouplers, of $HgCl_2$ and of KCN are similar in the diaminodurene (or diaminotoluene) → methylviologen, in the $DCIPH_2$ → methylviologen and in the overall H_2O → methylviologen reactions. Therefore, the criteria of Site I involvement applied to the $DCIPH_2$ reaction may also be applied to the diaminodurene (or diaminotoluene) → methylviologen reactions. This conclusion is strongly supported by the recent finding

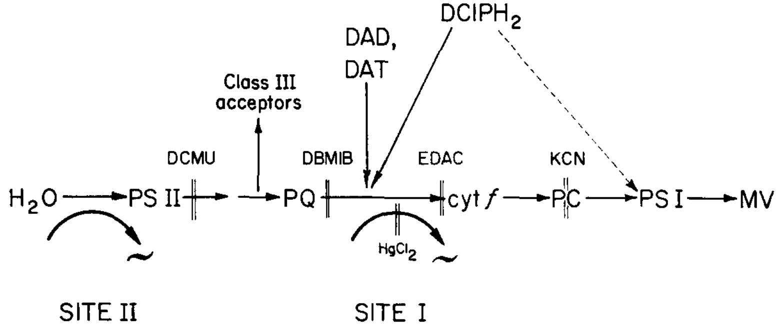

Fig. 7. A scheme for electron transport pathways in isolated chloroplasts showing the two sites of energy transduction (~). PS II, Photosystem II; PS I, Photosystem I; PQ, plastoquinone; cyt *f*, cytochrome *f*; PC, plastocyanin; DAD, diaminodurene; DAT, diaminotoluene; MV, methylviologen; Class III acceptors include lipophilic strong oxidants such as oxidized *p*-phenylenediamines or *p*-benzoquinones. Note that in this scheme diaminodurene, diaminotoluene and $DCIPH_2$ donate electrons directly to the main electron transport chain at a point before the rate-determining Coupling Site I, which also limits the rate of electron transport from H_2O to methylviologen. Note also the two component pathways of the $DCIPH_2 \rightarrow$ methylviologen reaction, one KCN-sensitive and one KCN-insensitive [5]. For further explanation see refs 2–6, 8–11, 16, 23 and 37.

of McCarty [37] that the electron transport inhibitor 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDAC), which apparently blocks electron flow from plastoquinone to cytochrome *f* at some point after the DBMIB inhibition site, inhibits the transport of electrons from diaminodurene to methylviologen. This suggests that, while the chemiosmotic model for Photosystem I phosphorylation may be basically correct, the actual acceptor of electrons from diaminodurene is not plastocyanin (as suggested by Trebst [39, 40]) but cytochrome *f*, as is clearly the case with the coupled $DCIPH_2 \rightarrow$ methylviologen reaction [21, 22]. This is consistent also with the fact that no energy control over electron transport can be detected between cytochrome *f* and $NADP^+$ [7, 8].

A scheme summarizing these observations and conclusions is presented in Fig. 7.

One important implication of the results summarized above concerns the probable location of the coupling site associated with Photosystem I cyclic phosphorylation reactions such as those catalyzed by pyocyanine, diaminodurene/oxidized diaminodurene, or $DCIPH_2$/DCIP. It has long been postulated that cyclic photophosphorylation is associated with a separate coupling site not on the main electron transport chain (for a review, see ref. 38). In light of the evidence presented here it seems more likely that the reduced form of the cycling cofactor (i.e. $DCIPH_2$, diaminodurene or reduced pyocyanine) donates electrons directly to the main electron transport chain at a point before Coupling Site I (i.e. before cytochrome *f*, but see ref. 40) while the oxidized form of the cycling cofactor (DCIP, oxidized diaminodurene or pyocyanine) accepts electrons from Photosystem I. Trebst and co-workers have recently presented a similar view [4, 39, 40]. Such schemes eliminate altogether the need to postulate a special "cyclic" coupling site associated with a separate electron-transport side chain. Instead these schemes have cyclic phosphorylation resulting from the proton translocating properties of the exogenously added lipophilic electron donor: i.e. the donor is reduced (consuming protons) on the outside and oxidized (releasing protons) on the inside of the thylakoid, thereby generating a transmembrane proton gradient which may drive phosphorylation. The scheme presented in Fig. 7 is more specific in that cytochrome *f* is suggested to be the acceptor of electrons from the exogenous (artificial) electron donor (e.g. diaminodurene) as well as the endogenous (natural) electron donor plastoquinone. Studies with electron transport inhibitors also lend support to the scheme. Ouitrakul and Izawa [11] found that KCN inactivation of plastocyanin inhibited cyclic phosphorylation catalyzed by $DCIPH_2$/DCIP, diaminodurene/oxidized diaminodurene, pyocyanine and low concentrations of *N*-methylphenazonium methosulfate (PMS)*, showing that cyclic electron transport shares at least this carrier with non-cyclic electron transport. Furthermore, McCarty [37] has shown that pyocyanine-mediated cyclic phosphorylation is inhibited by EDAC. Thus it seems clear that cytochrome *f* (and therefore probably Coupling Site I) is involved in the cyclic reaction [37].

If we are to equate the site of cyclic photophosphorylation and other Photosystem I-mediated phosphorylation reactions with the rate-limiting Coupling Site I of the non-cyclic electron transport system, a problem of relative rates arises, since the Photosystem I photophosphorylation can be several times faster than the non-cyclic photophosphorylation associated with the Hill reaction. This apparent conflict, however, may be easily explained in terms of the differences in the concentrations and reactivities of the different electron donors (e.g. diaminodurene vs. natural electron donor) at the common site of oxidation, where energy conservation takes place. Electron transport rates of all of these reactions can be under comparable degrees of energy control, regardless of the electron flux, since the steady-state level of the high-energy intermediate or state (proton gradient?), and therefore the back pressure it imposes on electron transport through the coupling site [13], may well increase or decrease in approximate proportion to the level of electron flux. The result would be a similar degree of dependence of electron flow on phosphorylation and on uncoupling regardless of the absolute level of electron flux.

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* Cyclic phosphorylation reactions catalyzed by PMS are complex, the properties apparently varying with the PMS concentration. See refs 11, 17 and 23.

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