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STEADY-STATE KINETIC ANALYSES OF PHOTOSYSTEM II ACTIVITY CATALYZED BY LIPOPHILIC ELECTRON ACCEPTORS

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Summary

Steady-state kinetic analyses of the Photosystem II activity elicited by quinones and quinonediimines in KCN/Hg-inhibited chloroplasts reveal that:

1. Quinones generate a single reaction in which one quinone competes with another for photoreduction by Photosystem II.

2. Quinonediimines also compete with one another for photoreduction, but the electron transport activity elicited by quinonediimines is the composite of two separate reactions, one of which is sensitive to ionic strength and atebrin-binding.

3. The relationship between a quinone and a quinonediimine with respect to photoreduction by Photosystem II is non-competitive rather competitive.

These findings are interpreted to indicate that quinones and quinonediimines accept electrons from two separate sites near the reducing site of Photosystem II.

Introduction

Exposure of isolated spinach thylakoid membranes to DBMIB, KCN, or KCN/Hg has been shown to block the photoreduction of methylviologen and

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCBQ, 2,6-dichloro-*p*-benzoquinone; DMQ, 2,5-dimethyl-*p*-benzoquinone; PD, *p*-phenylenediamine; DAD, 2,3,5,6-tetramethyl-*p*-phenylenediamine; TMPD, *N,N,N',N'*-tetramethyl-*p*-phenylenediamine; Tricine, *N*-Tris(hydroxymethyl)-methylglycine. The subscript $_{ox}$ following the abbreviation for a *p*-phenylenediamine refers to the oxidized, or diimine, form of these acceptors.

ferricyanide [1—3]. Electron transport can be restored in these inhibited chloroplast preparations by addition of oxidized lipophilic electron acceptors (*p*-benzoquinones, quinonediimines) which elicit non-cyclic electron transport dependent upon Photosystem II. Energy coupling in these Photosystem II reactions, with a P/e_2 of 0.25—0.35 [4], is presumed to occur by the liberation of protons from the oxidation of water [5] on the interior of the thylakoid membrane [6].

Certain properties of these Photosystem II electron transport reactions are independent of the lipophilic electron acceptor used and of the procedure employed to inhibit Photosystem I. For example, a higher light intensity is required for maximal Photosystem II electron flow than is required for Photosystem II plus I reactions [7]. Whereas the pH optimum for Photosystem II electron transport occurs at approximately pH 7—7.5, the P/e_2 values obtained for these reactions under varying pH conditions demonstrate a broad pH optimum [8]. Since these properties of Photosystem II reactions are independent of the *p*-benzoquinone or quinonediimine electron acceptor used, they are characteristic of the electron transport and energy coupling endogenous to Photosystem II.

One property of Photosystem II electron flow, however, depends upon the nature of the lipophilic electron acceptor employed to restore activity in Photosystem I-inhibited chloroplasts. Several investigators [9—12] have shown that Photosystem II electron transport activity supported by quinonediimines is depressed upon addition of uncouplers. Photosystem II electron transport reactions supported by *p*-benzoquinones, on the other hand, are unaffected by addition of uncouplers [10,12,13]. Despite extensive investigations of the several phenomena associated with Photosystem II electron transport supported by lipophilic oxidants, there is insufficient evidence to critically evaluate the various mechanisms proposed to explain the interaction of these electron acceptors with the thylakoid membrane. We have therefore further investigated this problem; steady-state kinetic analyses using KCN/Hg-poisoned chloroplasts indicate that quinonediimines and *p*-benzoquinones utilize different sites of reduction within the thylakoid membrane. Although *p*-benzoquinones appear to be reduced at a single site within the thylakoid membrane, two sites of quinonediimine reduction have been kinetically resolved.

Materials and Methods

Chloroplast thylakoid membranes of spinach (*Spinacia oleracea* L.) were isolated [12] and subjected to KCN/Hg treatment [13] as described previously. The chloroplast preparations were frozen in aliquots at −70°C and thawed immediately prior to assay. Frozen and thawed chloroplasts retained electron transport activities equivalent to the activities obtained prior to freezing [3].

Electron transport activities were assayed by measuring light-induced changes in O_2 concentration. White light (10^6 ergs · cm^{-2} · s^{-1}) was provided by an Oriel model 6325 light source and passed through 5 cm 0.2% $CuSO_4$ solution serving as a heat filter. Light-induced changes in O_2 concentration were measured with a Clark-type oxygen electrode (YSI) fitted to a 1.6 ml thermostatted (25°C) cuvette. The reaction suspension contained, unless otherwise

stated, 20 mM Tricine (pH 8), 62.5 mM NaCl, KCN/Hg-inhibited chloroplasts (25—30 μg chlorophyll/ml), 2.5 mM $K_3Fe(CN)_6$, and class III electron acceptors as noted in Results.

Electron acceptors used to support Photosystem II non-cyclic electron transport in these experiments were recrystallized prior to use [14]. DMQ and DCBQ were prepared as ethanolic solutions; the concentration of organic solvent in the assay suspensions never exceeded 1.5%. The other class III electron acceptors used in these studies (PD, DAD, TMPD) were dissolved in H_2O. In experiments with varying concentrations of lipophilic electron acceptors, additions (from serial dilutions of 20 mM stock solutions) were made using microliter syringes.

Tricine, bovine serum albumin, and PD were obtained from Sigma. All other electron acceptors were obtained either from Eastman (DMQ, DCBQ, TMPD) or Research Organics/Inorganics (DAD). Atebrin was a gift from Dr. F.L. Hoch.

Results

Rate saturation effects of lipophilic electron acceptors

As shown in Fig. 1A, optimal rates of electron flow supported by Photosystem II in KCN/Hg-poisoned chloroplasts depend upon the presence of lipophilic electron acceptors, as has been previously shown with KCN- [15] and DBMIB-poisoned [16] chloroplasts. The stimulation of electron flow in these chloroplasts by two lipophilic acceptors (DCBQ, PD_{ox}) is shown in Fig. 1A. These data represent the average of several experiments with chloroplasts

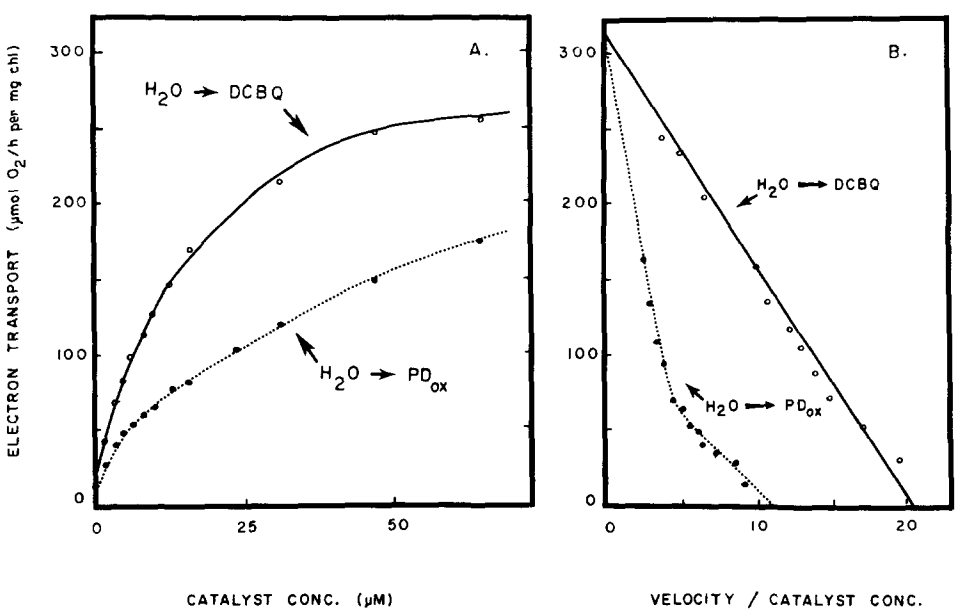

Fig. 1. Electron transport supported by Photosystem II in KCN/Hg-treated chloroplasts as a function of lipophilic acceptor concentration. (A) Saturation curves for acceptors PD_{ox} and DCBQ. (B) Transformation of the data in (A) to a Woolf-Augustinsson-Hofstee plot. A basal rate of 12 μmol oxygen evolved/h per mg chlorophyll was subtracted from the rates observed in the presence of acceptors.

possessing a very low level of electron transport in the absence of lipophilic acceptors. The low basal rate observed here could be due either to direct reduction of ferricyanide by Photosystem II or to a residual level of electron flow through plastocyanin, and it represents a contaminating contribution to the activity supported by PD_{ox} and DCBQ. Thus, the effects of these two catalysts on the rate of Photosystem II electron transport were examined by first subtracting the basal rate of electron flow obtained in the absence of the catalysts [17] and by the subsequent replotting of the corrected data on a Woolf-Augustinsson-Hofstee reciprocal transformation (Fig. 1B). Fig. 1B shows that, whereas the stimulation of electron transport with DCBQ elicits linear saturation kinetics, the stimulation of electron flow observed with increasing concentrations of PD_{ox} is distinctly biphasic in nature.

Quinonediimine electron acceptors other than PD_{ox} were also assayed for rate saturation characteristics in KCN/Hg-treated chloroplasts; DAD_{ox} and $TMPD_{ox}$ yielded biphasic saturation kinetics similar to those obtained with PD_{ox}. These biphasic kinetics show that, at low catalyst concentrations, the rate of electron transport supported by Photosystem II is faster than the rate predicted from simple Michaelis-Menten kinetics. Kinetics similar to those shown for PD_{ox} can be derived as the sum of two Michaelis-Menten reactions [17]. Complex kinetics such as these are often found in biological systems and have been interpreted to indicate a two enzyme system in which each enzyme, although catalyzing the same reaction, possesses a different set of kinetic parameters [17—19].

Kinetic relationships between p-benzoquinone and quinonediimine electron acceptors

The catalyst concentration effects of lipophilic electron acceptors were next examined under conditions in which the basal rate of Photosystem II electron transport was artificially increased. The effect of catalyst concentration was examined for one electron acceptor in the presence of a fixed, subsaturating concentration of a second acceptor. The presence of this second acceptor increased the rate of electron flow to a higher level than that of the basal rate. This rate (in the presence of the second acceptor alone) represented a new, increased contamination of the basal electron flow. Fig. 2 shows the results of such an experiment in which the effects of PD_{ox} concentration on the rates of Photosystem II electron transport in KCN/Hg-poisoned chloroplasts were assayed in the presence of a fixed concentration of DAD_{ox}. The rate of electron flow obtained in the presence of DAD_{ox} alone was subtracted from these rates obtained in the presence of varied concentrations of PD_{ox} and the resulting data were transformed to a reciprocal plot as shown in Fig. 2. Note that, although the apparent maximum velocity obtainable under these conditions does not shift, a difference can be noted in the apparent K_m for PD_{ox}. Thus, it is evident from Fig. 2 that PD_{ox} and DAD_{ox} interact in a competitive manner; the presence of DAD_{ox} in the reaction cuvette competitively reduces the amount of electron transport which can be attributed to a given concentration of PD_{ox}. A competitive interaction was characteristic of any such experiment involving two different quinonediimines.

An experiment similar to that shown in Fig. 2 was also performed using two

p-benzoquinones as electron acceptors. Fig. 3 shows the results obtained when a fixed subsaturating concentration of DMQ was used to raise the contaminating rate of electron flow in the presence of increasing concentrations of DCBQ. Since optimal concentrations of DMQ support rates of electron flow which are substantially less than those obtainable with many other lipophilic acceptors [3], the concentration of DMQ required in this experiment was comparatively high (125 μM). The data were treated as described above for experiments involving quinonediimines; the rate of electron flow in the presence of DMQ alone was subtracted from the rates obtained in the presence of DMQ plus DCBQ. Fig. 3 shows the resulting reciprocal plot. As was true with quinonediimines, quinones, when assayed together, interact in a competitive manner; the presence of a fixed concentration of DMQ competitively reduces the amount of electron transport which can be attributed to a given concentration of DCBQ.

If, however, the concentration effects of a quinone on Photosystem II electron flow are examined in the presence of a quinonediimine, the relationship between the two acceptors is not competitive in nature. Fig. 4 shows the results of an experiment in which PD_{ox} was used to increase the contaminating rate of electron flow during assays examining the effects of the concentration of DCBQ on electron transport. The data were treated in a manner similar to that used in Figs. 2 and 3. Fig. 4 shows that the relationhip between PD_{ox} and DCBQ is non-competitive in nature; the presence of PD_{ox} in the reaction cuvette non-competitively reduces the rate of electron transport which can be

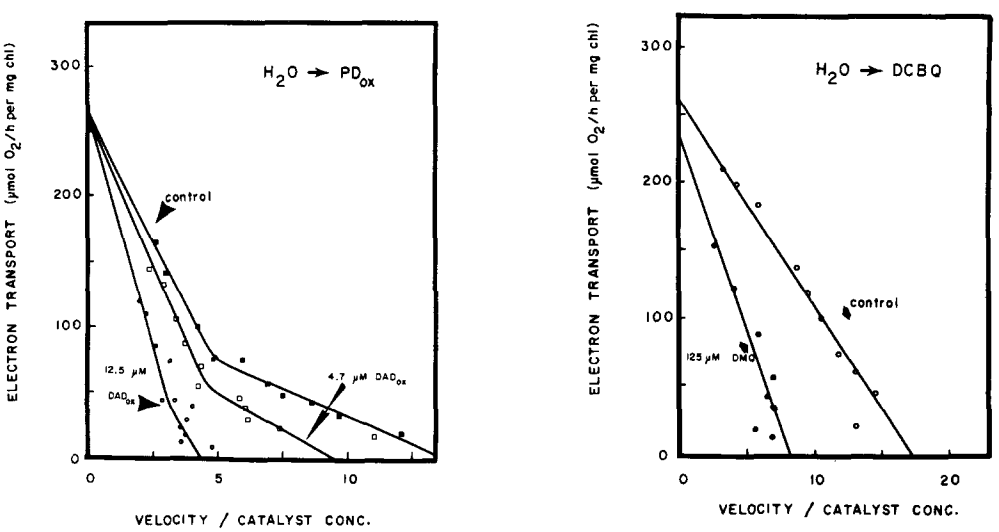

Fig. 2. Effect of the presence of DAD_{ox} on the rate saturation characteristics of PD_{ox}, in KCN/Hg-treated chloroplasts. The results are presented as a Woolf-Augustinsson-Hofstee plot. The following basal contaminanting rates were subtracted from the rates with PD_{ox}: 0 μM DAD_{ox}, 12; 4.7 μM DAD_{ox}, 21.3; 12.5 μM DAD_{ox}, 52.8.

Fig. 3. Effect of the presence of DMQ on the substrate saturation characteristics of DCBQ in KCN/Hg-treated chloroplasts. The results are presented as a Woolf-Augustinsson-Hofstee plot. The following contaminating rates were subtracted from the rates with DCBQ: 0 μM DMQ, 12; 125 μM DMQ, 68.2.

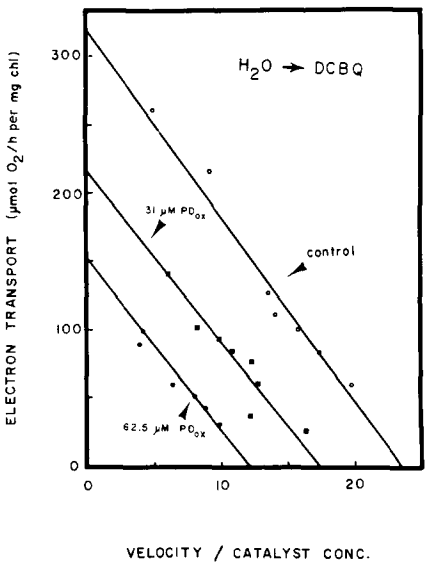

Fig. 4. Effect of the presence of PD_{ox} on the rate saturation characteristics of DCBQ in KCN/Hg-treated chloroplasts. The results are presented as a Woolf-Augustunsson-Hofstee plot. The following rates were subtracted from the rates in the presence of DCBQ: 0 μM PD_{ox}, 12; 31 μM PD_{ox}, 126; 62.5 μM PD_{ox}, 185.

attributed to a given concentration of DCBQ. A non-competitive relationship was found whenever a quinone (DMQ, DCBQ) was assayed in the presence of a quinonediimine (DAD_{ox}, PD_{ox}, $TMPD_{ox}$).

Effects of NaCl concentration on the biphasic saturation kinetics observed with quinonediimines

Since maximal rates of quinonediimine-supported electron flow depend upon the uncoupler-sensitive proton gradient [12], treatments which influence either the extent of proton uptake (pyridine) or the extent of the proton gradient (ADP plus P_i) were assayed for possible effects of quinonediimine saturation kinetics. Pyridine enhances the extent of the proton uptake in illuminated chloroplasts by buffering the internal aqueous space of the thylakoid membranes [20]. ADP plus P_i increase the rate at which accumulated protons are returned to the exterior of the thylakoid membrane [21]. Neither of these treatments, however, appreciably influenced the biphasic nature of the saturation kinetics observed with quinonediimines (data not shown).

The ionic environment in the reaction mixture, however, profoundly influenced the biphasic nature of quinonediimine saturation kinetics. As shown in Fig. 5, high NaCl concentrations increased the rate of electron transport supported by low concentrations of PD_{ox} but failed to similarly stimulate electron flow supported by high PD_{ox} concentrations. Thus, as shown in Fig. 5, the biphasic nature of the reciprocal transformation of the PD_{ox} data is enhanced by high salt concentrations(60—95 mM). Intermediate NaCl concentrations (45 mM), however, depressed rates supported by low concentrations of PD_{ox} and therefore attenuated the biphasic nature of PD_{ox} saturation kinetics. Con-

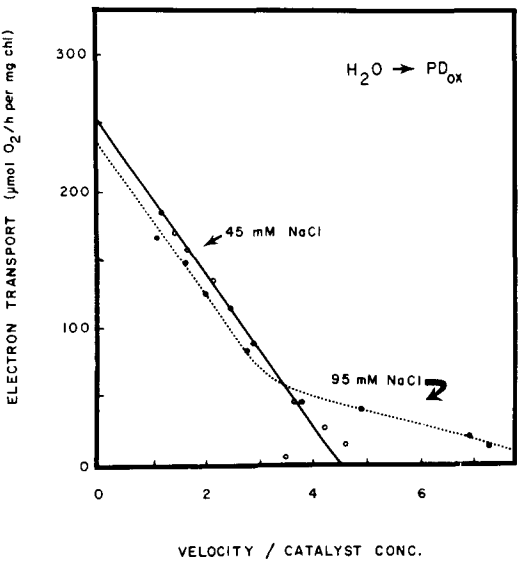

Fig. 5. Effects of NaCl concentrations on the rates of electron transport supported by PD_{ox} in KCN/Hg-treated chloroplasts. The results are presented as a Woolf-Augustinsson-Hofstee plot. The following contaminating rates were subtracted from the rates occurring in the presence of PD_{ox}: 45 mM NaCl, 20; 95 mM NaCl, 16.

centrations of NaCl less than 45 mM served to further decrease the rates of electron flow observed with low concentrations of PD_{ox} or DAD_{ox}, and produced complexities which resulted in sigmoidal, rather than linear saturation kinetics (data not shown). Although quinonediimine electron transport activity was influenced by these variations in NaCl concentrations, Table I shows that NaCl had little effect on the electron transport activity supported by DCBQ.

It should be noted that the ability of NaCl to attenuate biphasic PD_{ox} saturation kinetics is due to changes in the ionic, rather than osmotic, conditions in the reaction mixture. Experiments performed in the presence of NaCl, plus sucrose added in amounts calculated to compensate for the osmotic differential between low and high concentrations of NaCl, yielded results similar to those obtained in Table I.

Effects of atebrin on the biphasic saturation kinetics observed with quinonediimines

Atebrin presented a special case among the compounds assayed for effects on quinonediimine activity. A derivative of 9-aminoacridine, atebrin possesses uncoupler activity [22], but does not competitively inhibit quinonediimine-supported Photosystem II electron flow [12]. However, as shown in Fig. 6, the presence of atebrin in the reaction cuvette enhances the biphasic nature of the PD_{ox} saturation kinetics.

The rates of electron flow given in Fig. 6 are those which occur after the reaction mixture, containing chloroplasts, atebrin, and PD_{ox}, was illuminated for approximately 1 min; when atebrin and low concentrations of PD_{ox} are assayed together, maximum rates of electron flow occurred only after a lag

TABLE I

EFFECTS OF SALT CONCENTRATION ON THE RATE OF ELECTRON TRANSPORT SUPPORTED BY LOW CATALYST CONCENTRATIONS IN KCN/Hg-TREATED CHLOROPLASTS

The reaction conditions, other than NaCl concentration, are those given in Materials and Methods. Rates of electron transport are expressed as μmol O_2/h per mg chlorophyll.

Catalyst (μM)	Electron transport rates		
	no NaCl	31 mM NaCl	63 mM NaCl
None	9	19	11
DCBQ			
5	67	72	70
10	115	117	126
62	245	245	252
DAD_{ox}			
5	13	16	26
10	15	27	32
62	72	85	100
PD_{ox}			
5	16	37	46
10	42	—	64
62	186	179	175

time. This is shown in the oxygen electrode tracings presented in Fig. 7. As the concentration of PD_{ox} was increased, the lag time before the onset of maximal electron transport rates was decreased. As the concentration of atebrin was increased, the lag time was increased. The lag period occurred in the presence of either white or red illumination, and no such lag period occurred when

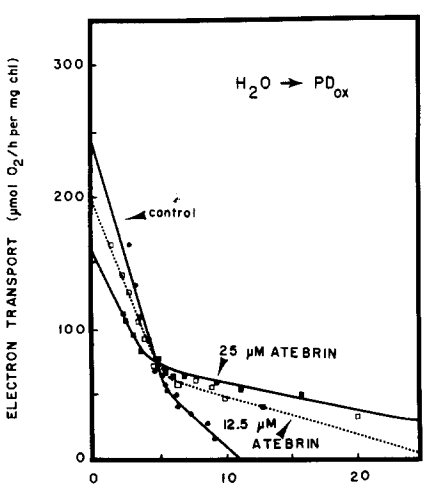

Fig. 6. Effect of atebrin on the rates of electron transport supported by PD_{ox} in KCN/Hg-treated chloroplasts. The rates shown were those obtained after the reaction suspensions were illuminated for 1 min. The results are presented as a Woolf-Augustinsson-Hofstee plot. The contaminating, basal rate in the absence of PD_{ox}, which was subtracted from the rates in the presence of PD_{ox}, was the same in the presence and absence of atebrin [12].

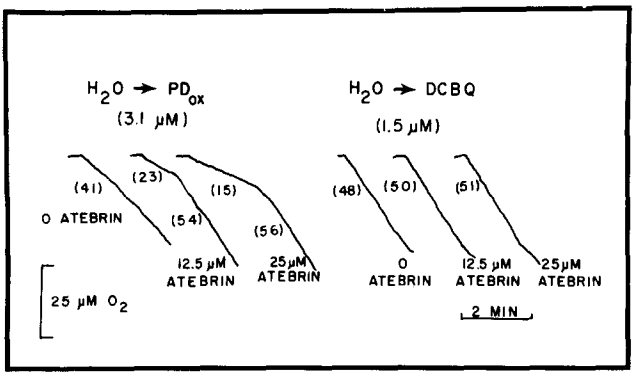

Fig. 7. Effect of atebrin on electron transport supported by low concentrations of either PD_{ox} or DCBQ, in KCN/Hg-treated chloroplasts. The numbers in parenthesis represent rates of electron transport expressed as μmol of oxygen evolved/h per mg chlorophyll. Note that the rate of electron transport supported by PD_{ox} is decreased initially by the presence of atebrin, and then stimulated by increasing time of illumination.

atebrin and DCBQ were assayed together (see Fig. 7). Thus, atebrin interferes with the initial activity supported by low concentrations of PD_{ox}, and when this interference is overcome by illumination atebrin subsequently promotes PD_{ox}-supported electron flow, as evidenced by the enhanced biphasic response shown in Fig. 6.

Discussion

The results presented here further document the complexities associated with catalysis of Photosystem II electron transport by quinones and quinonediimines. Quinones elicit a single reaction exhibiting Michaelis-Menten kinetics, and one quinone (DMQ) competes with another (DCBQ) for reduction by Photosystem II. A similar pattern of competitive interactions can be demonstrated among quinonediimines, but the activity elicited by these compounds is far more complex. A Woolf-Augustinsson-Hofstee plot (Fig. 1) of Photosystem II activity produced by increasing concentrations of PD_{ox} reveals the existence of two reactions whose kinetic parameters differ substantially. In addition, the data in Figs. 5—7 show that the reaction obtained with low concentrations of quinonediimines is sensitive to ionic strength and to the presence of atebrin.

There is no simple explanation for these observations. Although the acceptors used in the present investigation differ from one another in both redox potential and lipophilicity, no dependence of Photosystem II activity on either of these chemical properties of acceptors has emerged from our data. We have considered the possibility that our results simply reflect a difference in the mechanisms of photoreduction of quinones and quinonediimines by a single membrane-bound electron donor. For example, one of these acceptor groups might require a pair of one electron donations, while the other set of acceptors might be reduced by a single, two electron transfer. If these mechanisms were to involve a single component of the photosynthetic electron transport chain, however, we would expect to have observed competitive, rather than noncompetitive kinetics in the experiments shown in Fig. 4.

In view of these considerations, the most likely interpretation of our results is that quinones and quinonediimines are photoreduced at different sites near the reducing side of Photosystem II as proposed originally by Cohen et al. [10]. According to this interpretation, unique electron donors must exist to transfer electrons from Photosystem II to either quinones or quinonediimines. It is not possible to predict from our data the relationship of these electron donor sites to one another. They may be present as part of the linear sequence of carriers utilized to transfer electrons from Q to Photosystem I. Alternatively, one set of reduction sites may be disconnected from the main path of electron transport between Photosystems II and I. The existence of one such pathway has been proposed to account for the photoreduction of cytochrome *b*-559 by Photosystem II [23].

All of the quinonediimines examined in our experiments produced biphasic kinetics of the type shown in Fig. 1. Such kinetics are seen in other biological systems [17—19] and are interpreted to indicate the presence of two simultaneously occurring reactions involving a single substrate. In our assay system, such a result implies that quinonediimines are accepting electrons from two different electron donors. Support for this contention is shown in Figs. 5—7. Both ionic strength and atebrin influence the kinetics of only the reaction obtained with low concentrations of a quinonediimine catalyst. Other investigators have shown that NaCl can influence Photosystem II light-harvesting efficiency [24], activate Photosystem II units [25,26], and influence the ability of ferricyanide to approach the thylakoid membrane [25]. It is unlikely, however, that these phenomena account for our observations. We have intentionally used high light intensities (10^6 ergs $\cdot$ cm^{-2} $\cdot$ s^{-1}) to compensate for any reduced efficiency of Photosystem II light harvesting. If the effect of NaCl were to influence ferricyanide approach to the thylakoid membrane, then the salt effects reported here should vary with the concentration of ferricyanide present in our assay system. We have not observed this effect (data not shown). The most probable explanation is therefore that the effect of salt we have observed is to regulate the electron donor site involved in photoreduction of low concentrations of quinonediimines.

The effect (Fig. 7) of atebrin on photoreduction of low concentrations of quinonediimines is more difficult to understand. Atebrin, at our assay pH (8.0), is charged [22], uncouples photophosphorylation in KCN/Hg-inhibited chloroplasts (Guikema, J.A., unpublished observations), and therefore must bind to our membrane preparations. Although atebrin is both photoactive [27,28] and chemically reactive [29], our results are not a reflection of these properties of atebrin. The data shown in Fig. 7 could be obtained with either red or white light, and a reaction of atebrin with quinonediimines (to form an unreactive complex) is unlikely since (a) the effect is reversed by prolonged illumination and (b) Kraayenhof et al. [30] obtained atebrin fluorescence quenching by energization of KCN-inhibited thylakoid membranes in the presence of high concentrations of quinonediimines. We feel, therefore, that the effect of atebrin on photoreduction of low concentrations of quinonediimines is related to the ability of cations to displace acridine dyes from the thylakoid membrane [31]. Binding of atebrin to the thylakoid membrane would initially depress the activity of the salt-sensitive reaction with low quinonediimine concentrations. With

prolonged illumination, however, the atebrin might be partially displaced by cations to restore this quinonediimine photoreduction reaction.

In summary, our data indicate that part of the complexity associated with catalysis of Photosystem II electron transport by quinones and quinonediimines is due to the existence of different sites of photoreduction of these compounds on the reducing side of Photosystem II. Photoreduction of quinonediimines is further complicated by the apparent presence of two photoreduction reactions unique to these compounds. One of these photoreduction reactions is sensitive to ionic strength and cation (atebrin) binding to the thylakoid membrane.

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