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PHOTOSYSTEM II ENERGY COUPLING IN CHLOROPLASTS WITH H_2O_2 AS ELECTRON DONOR

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Summary

NH_2OH-treated, non-water-splitting chloroplasts can oxidize H_2O_2 to O_2 through Photosystem II at substantial rates (100—250 μequiv · h^{-1} · mg^{-1} chlorophyll with 5 mM H_2O_2) using 2,5-dimethyl-*p*-benzoquinone as an electron acceptor in the presence of the plastoquinone antagonist dibromothymoquinone. This H_2O_2 → Photosystem II → dimethylquinone reaction supports phosphorylation with a P/e_2 ratio of 0.25—0.35 and proton uptake with H^+/e values of 0.67 (pH 8)–0.85 (pH 6). These are close to the P/e_2 value of 0.3–0.38 and the H^+/e values of 0.7—0.93 found in parallel experiments for the H_2O → Photosystem II → dimethylquinone reaction in untreated chloroplasts. Semi-quantitative data are also presented which show that the donor → Photosystem II → dibromothymoquinone (→O_2) reaction can support phosphorylation when the donor used is a proton-releasing reductant (benzidine, catechol) but not when it is a non-proton carrier (I^-, ferrocyanide).

Introduction

It is established that an energy conservation mechanism leading to ATP formation is associated with Photosystem II [1—3]. A membrane protein which seems to be specifically related to Photosystem II energy coupling has recently been detected [4]. From P/e_2 determinations for the whole-chain electron transport reactions in Tris- or NH_2OH-washed chloroplasts, it was suggested

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Abbreviations: DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; Mes, 2-(*N*-morpholino)ethanesulfonic acid.

earlier that Photosystem II can drive phosphorylation with artificial electron donors as efficiently as with the natural donor, water [5—7]. Photosystem II-driven, *p*-phenylenediamine-supported cyclic photophosphorylation and proton translocation have been demonstrated [8,9]. However, quantitative relationships among electron flow, phosphorylation and proton changes in donor-supported Photosystem II reactions have not been determined yet, because of the difficulty of finding donor-acceptor combinations which will allow one to observe donor-supported, non-cyclic Photosystem II electron flow.

The main objective of this paper is to describe experiments in which we have succeeded in measuring Photosystem II electron transport and coupled reactions in NH_2OH-treated chloroplasts using H_2O_2 as an electron donor and 2,5-dimethylquinone as acceptor. The ability of Photosystem II to oxidize H_2O_2 was first noted by Inoue and Nishimura [10]. Although Velthuys and Kok [11] concluded recently from their flash experiments that O_2 production from H_2O_2 was due in large part to a Photosystem II-mediated dismutation-like process, in our NH_2OH-treated chloroplasts the H_2O_2-supported O_2 production did represent the net oxidation of H_2O_2 to O_2 as documented below. In this study, however, no effort was made to elucidate the chemical mechanisms involved in the oxidation of H_2O_2.

Materials and Methods

Envelope-free chloroplasts were prepared from commercial spinach (*Spinacia oleracea* L.) and treated with NH_2OH to inactivate the O_2-evolving enzyme as described elsewhere [12]. All the reagents used were from Sigma except for H_2O_2 (Mallinckrodt), 2,5-dimethyl-*p*-benzoquinone (Eastman) and DCMU (ICN Rare Chemicals Division). DBMIB was a gift from Dr. N.E. Good. O_2 evolution or consumption was measured using a Clark-type O_2 electrode covered with a teflon membrane. The reaction cuvette was thermostated at 21°C with circulating water. The actinic light used was a rate-saturating orange light (550—700 nm, 600 W/m^2). The duration of illumination was varied by means of a mechanical shutter. Phosphorylation was assayed as the formation of ^{32}P-labeled ATP using a modification of the method of Avron [13]. Light-induced pH changes in the reaction medium were monitored using a conventional combination glass electrode and a Corning Digital 110 pH meter connected to a Heat/Schlumberger EU-200 amplifier-recorder system. The cuvette-bath assembly used was the same as that used for O_2 assay.

Results

H_2O_2 oxidation by NH_2OH-treated chloroplasts

If the O_2 evolution from H_2O_2 was due to oxidation ($H_2O_2 \rightarrow 2\ H^+ + O_2 + 2e^-$) rather than dismutation ($2\ H_2O_2 \rightarrow 2\ H_2O + O_2$), the evolution of 1 mol of O_2 should be accompanied by a release of 2 equiv. of protons ($\Delta H^+/\Delta O_2 = 2$) when an electron acceptor such as ferricyanide is used:

$$H_2O_2 + 2\ Fe(CN)_6^{3-} \rightarrow 2\ H^+ + O_2 + 2\ Fe(CN)_6^{4-}$$

This prediction was verified by the experiment of Fig. 1 which shows that

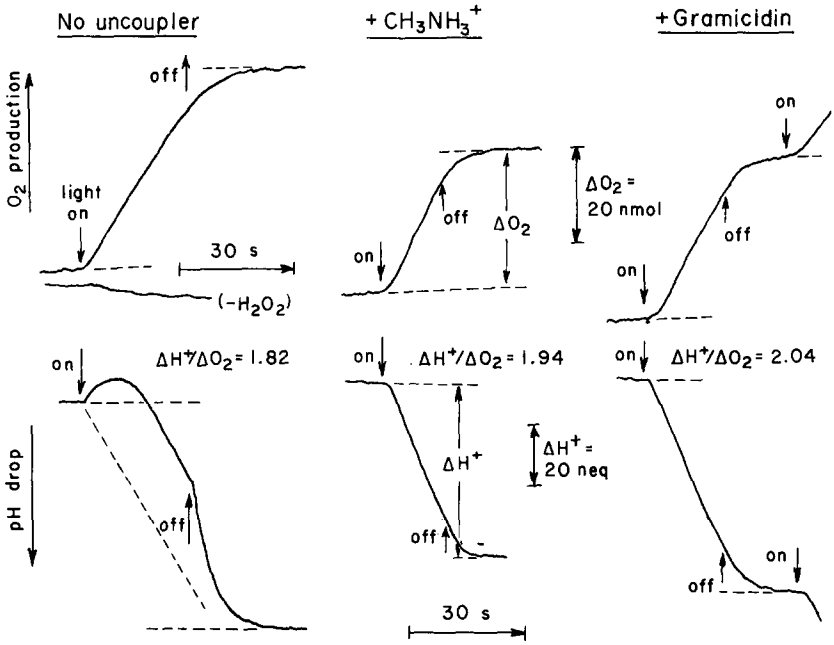

Fig. 1. O_2 and pH traces from experiments which demonstrated that H_2O_2 photooxidation in NH_2OH-treated chloroplasts follows the formula $H_2O_2 \rightarrow O_2 + 2\,H^+ + 2e^-$. O_2 and H^+ changes were assayed in duplicate experiments using identical reaction mixtures and the same cuvette-bath assembly. The reaction mixture (3 ml) contained 0.1 M sucrose, 0.5 mM Hepes/NaOH buffer (pH 7.5), 25 mM NaCl, 3 mM $MgCl_2$, 5 mM H_2O_2, 0.4 mM $K_3Fe(CN)_6$, 1 mM KCN (catalase inhibitor) and NH_2OH-treated chloroplasts equivalent to 50 μg chlorophyll/ml. When used, methylamine-HCl was 10 mM and gramicidin 5 μM. ΔH^+ was determined by titration with 1 mM HCl after turning off the light (cf. legend for Fig. 6).

illumination of NH_2OH-treated chloroplasts with H_2O_2 and ferricyanide caused an uncoupler-insensitive, irreversible pH drop in the weakly buffered medium and that the amount (equiv.) of H^+ produced was twice as much as the amount (mol) of O_2 produced during the same illumination period. It is also noteworthy that in the absence of uncouplers the H^+ trace clearly showed a superimposed, reversible H^+ uptake by chloroplasts indicative of energy coupling (Fig. 1).

Phosphorylation coupled to Photosystem II oxidation of H_2O_2

The following experiments were all conducted using 2,5-dimethylquinone, a well established Photosystem II electron acceptor (Class III acceptor) [14]. To eliminate the Photosystem I-dependent part of electron flow, the plastoquinone antagonist DBMIB [15] was routinely included in the reaction mixture (2—4 μM depending on the amount of chloroplasts). As seen in Fig. 2, the $H_2O_2 \rightarrow$ dimethylquinone reaction in NH_2OH-washed chloroplasts was only partially inhibited by DBMIB, and the large, DBMIB-insensitive component of the reaction clearly supported phosphorylation. This DBMIB-insensitive component was totally abolished by DCMU, indicating that it was indeed a Photosystem II-driven reaction. The concentration of H_2O_2 used in these and most of routine experiments was 5 mM. Although this concentration of H_2O_2 was suboptimal with respect to the reaction rate (Fig. 3), the use of higher concentrations was

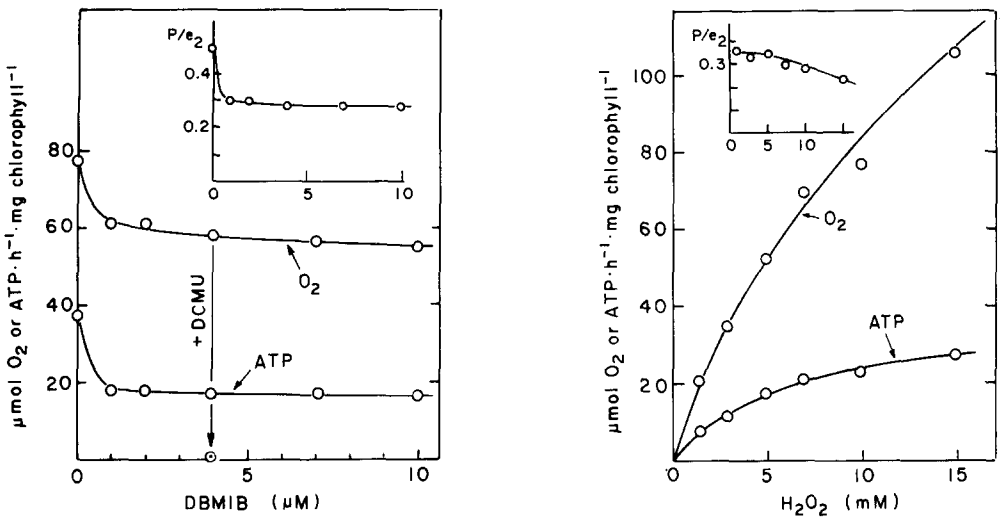

Fig. 2. Partial inhibition by DBMIB of electron flow (O_2 evolution) and ATP formation associated with the $H_2O_2 \rightarrow$ dimethylquinone reaction. The reaction mixture (2 ml) contained 0.1 M sucrose, 40 mM Hepes/NaOH buffer (pH 7.5), 3 mM $MgCl_2$, 0.8 mM ADP, 5 mM $Na_2H^{32}PO_4$, 5 mM H_2O_2, 0.5 mM 2,5-dimethyl-*p*-benzoquinone, 1 mM KCN (catalase inhibitor), NH_2OH-treated chloroplasts equivalent to 25 μg chlorophyll/ml and indicated concentrations of DBMIB. When used, DCMU was 1 μM. The reaction time was 30 s.

Fig. 3. The $H_2O_2 \rightarrow$ Photosystem II $\rightarrow$ dimethylquinone reaction and associated phosphorylation in NH_2OH-treated chloroplasts as a function of the H_2O_2 concentration. The reaction mixture was as in Fig. 2 except that the H_2O_2 concentration was varied and 2 μM DBMIB was always present to eliminate the Photosystem I-dependent part of the reaction.

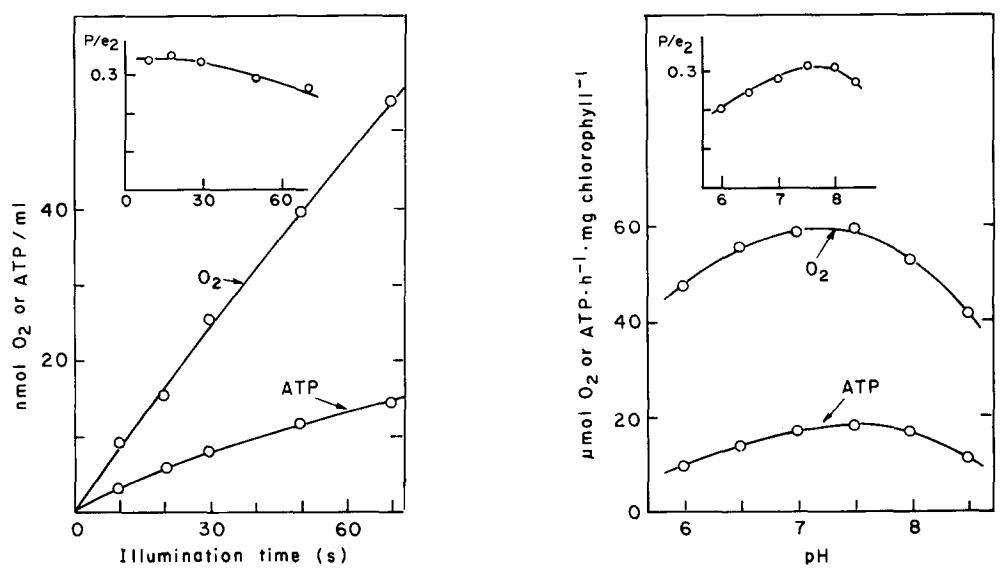

Fig. 4. The $H_2O_2 \rightarrow$ Photosystem II $\rightarrow$ dimethylquinone reaction and associated phosphorylation as a function of the reaction time. The experimental points were obtained from a series of identical reaction mixtures illuminated for the indicated periods of time. The reaction mixture used was as in Fig. 2 except that it contained 4 μM DBMIB and chloroplasts equivalent to 50 μg chlorophyll/ml.

Fig. 5. The $H_2O_2 \rightarrow$ Photosystem II $\rightarrow$ dimethylquinone reaction and associated phosphorylation in NH_2OH-treated chloroplasts as a function of the pH. The buffers used were Mes/NaOH (pH 6 and 6.5), Hepes/NaOH (pH 7 and 7.5) and Tricine/NaOH (pH 8 and 8.5) all at 30 mM. Other conditions were as in Fig. 2 except that 2 μM DBMIB was always present.

avoided because of their detrimental effects on phosphorylation (Fig. 3, inset). A further restriction to the use of H_2O_2 for phosphorylation experiments was the reaction time. As Fig. 4 shows, even with 5 mM H_2O_2, the phosphorylation efficiency (P/e_2) decreased rather quickly with the reaction time (less than 1 min). In determining the pH profiles of H_2O_2 oxidation and of the associated phosphorylation (Fig. 5), we therefore used a relatively short illumination time of 20—30 s to minimize the harmful effect of H_2O_2 or of its intermediate oxidation product(s) on phosphorylation. Both the O_2 production rate and the phosphorylation rate exhibited a very broad maximum in the pH 7—7.5 region. The relatively pH-independent coupling efficiency with the maximum P/e_2 value of 0.33 in the pH 7.5—8 region (Fig. 5, inset) is strongly reminiscent of standard Photosystem II phosphorylation in which water serves as the electron donor [16]. The maximum P/e_2 value ranged from 0.25 to 0.35 depending on the chloroplast preparation.

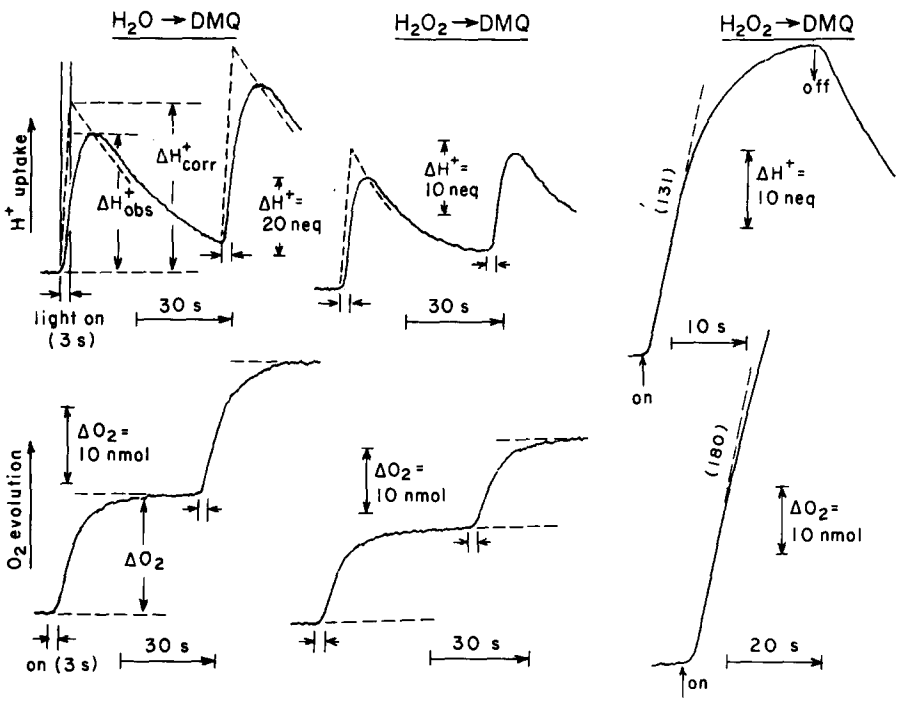

Fig. 6. pH and O_2 traces from experiments designed to determine the H^+/e ratios of proton translocation associated with the $H_2O \rightarrow$ Photosystem II $\rightarrow$ dimethylquinone reaction ($H_2O \rightarrow$ DMQ) in untreated chloroplasts and the $H_2O_2 \rightarrow$ Photosystem II $\rightarrow$ dimethylquinone reaction ($H_2O_2 \rightarrow$ DMQ) in NH_2OH-treated chloroplasts. pH and O_2 changes were assayed in duplicate reaction mixtures using the same cuvette-bath assembly. The reaction mixture (3 ml) contained 0.1 M sucrose, 0.5 mM Hepes/NaOH buffer (pH 7—7.1), 3 mM $MgCl_2$, 25 mM NaCl, 5 mM H_2O_2 (if used), 1 mM KCN, 0.5 mM 2,5-dimethyl-*p*-benzoquinone, 4 μM DBMIB, 0.8 mM ADP and chloroplasts equivalent to 50 μg chlorophyll/ml. In pH 6 and pH 8 experiments (not shown here, but see Table I), Hepes was replaced by Mes/NaOH and Tricine/NaOH buffer, respectively. ΔH^+ was determined by titration with 1 mM HCl in the light after a stationary state of proton uptake was achieved. (There was, however, no appreciable difference in titration value between light and dark periods.)

Estimation of H^+/e ratios

We found that the H_2O_2 → Photosystem II → dimethylquinone reaction in NH_2OH-washed chloroplasts supports proton translocation in much the same manner as does the H_2O → Photosystem II → dimethylquinone reaction in untreated chloroplasts (in both systems 4 μM DBMIB present in the reaction mixture). The H^+/e ratio was determined in two ways: (a) from the total amounts of H^+ and O_2 changes induced by brief illumination (3 s), and (b) from the initial slopes of H^+ and O_2 changes under continuous illumination (Fig. 6). In the first method, observed H^+ changes were 'corrected' for the dark decay using the graphical method suggested previously [17], since it was clear that a portion of H^+ taken up during the light period would have leaked out before the conventional pH recorder system used (response lag, approx. 1 s) registered a peak. Results are summarized in Table I together with the P/e_2 values obtained in a parallel experiment and the H^+/P ratios computed from the P/e_2 and H^+/e values. In both the H_2O_2 and H_2O system the corrected H^+/e ratios and those obtained from the initial slopes agreed fairly well, and tended to approach unity (0.7—0.9) showing a slight pH dependence. The H^+/P ratio was definitely a function of the pH, ranging approximately from 3—4 (at pH 8) to 7—8 (at pH 6).

TABLE I

H^+/e, P/e_2 AND H^+/P RATIOS OF THE H_2O_2 → PHOTOSYSTEM II → DIMETHYLQUINONE REACTION IN NH_2OH-TREATED CHLOROPLASTS AND OF THE H_2O → PHOTOSYSTEM II → DIMETHYLQUINONE REACTION IN UNTREATED CHLOROPLASTS

The two ΔH^+ and ΔO_2 values given for each pH represent yields from the first and the second illumination (each 3) and are expressed in μequiv./mg chlorophyll and μmol/mg chlorophyll, respectively. The P/e_2 values were determined using the same chloroplast preparation and the same reaction medium as used for the H^+/e determination except that ^{32}P-labeled Na_2HPO_4 (5 mM) was present and the reaction time was 10 s rather than 3 s. In computing H^+/e and P/e_2 ratios, the relation $O_2 = 2e$ was used for the H_2O_2 system and $O_2 = 4e$ for the H_2O system. For experimental details, see Fig. 6. ± indicates the maximum range of the H^+/P values computed from the Ht/e (corr.), H^+/e (from slope) and P/e_2 values shown.

Electron donor	pH	ΔH^+ (obs.)	Dark decay $t_{1/2}$ *	ΔH^+ (corr.)	ΔO_2	H^+/e from:			P/e_2	H^+/P
						ΔH^+ (obs.)	ΔH^+ (corr.)	Slopes		
H_2O_2	6.2	120	16	139	88	0.68	0.79	0.85	0.20	7.8 ± 0.8
		106	16	117	84	0.63	0.70			
	7.1	112	14	128	92	0.61	0.70	0.73	0.27	5.2 ± 0.2
		107	13	123	90	0.59	0.68			
	8.0	58	5	75	59	0.54	0.69	0.67	0.31	4.2 ± 0.2
		65	6	87	70	0.46	0.62			
H_2O	6.0	235	21	268	83	0.71	0.81	0.93	0.24	7.1 ± 0.7
		219	20	245	80	0.68	0.77			
	7.0	240	20	272	81	0.74	0.84	0.84	0.35	4.7 ± 0.1
		250	18	284	88	0.71	0.81			
	8.0	132	7	179	69	0.48	0.65	0.70	0.38	3.5 ± 0.1
		138	6	185	69	0.50	0.67			

* The half-time (s) of post-illumination H^+ efflux.

TABLE II

PHOTOSYSTEM II ELECTRON TRANSPORT AND PHOSPHORYLATION IN NH_2OH-TREATED CHLOROPLASTS AS OBSERVED IN THE DONOR → PHOTOSYSTEM II → DBMIB (→ O_2) SYSTEM

The reaction mixture (2 ml) contained 0.1 M sucrose, 30 mM Tricine/NaOH buffer (pH 8.3), 2 mM $MgCl_2$, 10 mM NaCl, 0.8 mM ADP, 5 mM $Na_2H^{32}PO_4$, 20 μM DBMIB (electron acceptor/inhibitor), chloroplasts equivalent to 100 μg chlorophyll, and the electron donor indicated: benzidine dihydrochloride (0.1 mM), catechol (0.1 mM), NaI (10 mM), or $K_4Fe(CN)_6$ (10 mM). The reaction time was 4 min.

Electron donor	H^+ release from donor upon oxidation	ATP formation (μmol/h per mg chlorophyll)	Electron transport (μmol O_2 taken up/h per mg chlorophyll)	P/O_2
Benzidine	yes	7.5	14.2	0.53
Catechol	yes	22.7	cycle	?
I^-	no	0	20.5	0
$Fe(CN)_6^{4-}$	no	0	6.8	0
None	—	0	0	—

Photosystem II phosphorylation with other donors

Table II summarizes attempts to demonstrate Photosystem II-mediated non-cyclic photophosphorylation using artificial electron donors other than H_2O_2. In this experiment (pH 8.3) a high concentration of DBMIB (20 μM) was used as the inhibitor/electron acceptor, and the electron flow was assayed as O_2 consumption taking advantage of the autooxidizability of reduced DBMIB at alkaline pH values [18]. For some unknown reason the reaction rates were very slow, but semi-quantitatively the results were quite clear: benzidine, a proton-carrying electron donor, supported both electron flow and phosphorylation while the non-proton-carrying donors I^- and ferrocyanide supported only electron flow. Catechol, a proton carrier, mediated a relatively rapid phosphorylation, but the electron flow was undetected clearly because catechol and its oxidation product(s) induced a cyclic electron flow around Photosystem II. All of these reactions were completely inhibited by 2 μM DCMU (data not shown) indicating that they were indeed supported by Photosystem II. This experiment substantiates the inference drawn earlier from the whole-chain donor reactions (donor → Photosystem II → Photosystem I → acceptor) that Photosystem II can drive phosphorylation only when proton-releasing reductants (including water) werve as electron donors [6,7].

Discussion

The finding that the H_2O_2 → Photosystem II → dimethylquinone reaction drives proton translocation with H^+/e ratios approaching unity (0.7—0.8) can be readily explained by assuming that H_2O_2 undergoing oxidation in the thylakoid discharges its protons internally and the dimethylquinone undergoing reduction consumes mostly external protons. Thus the finding provides an additional basis for the currently accepted vectorial model of Photosystem II, in which the components on the oxidizing side of Photosystem II are placed near the inner surface of the membrane and those on the reducing side near the external surface (for a review, see Ref. 19). Although H_2O_2 is a relatively polar substance, its non-ionic nature (pK_a 11.6) and small molecular size will

alow it to penetrate the membrane easily. The high H_2O_2 concentration requirement (apparent $K_m \approx 10$ mM, see Fig. 2) of the reaction should therefore be a reflection of the intrinsically low reactivity of H_2O_2 with its oxidation site, a fortunate fact in view of the many electron transport reactions that are most conveniently measured as the reduction of O_2 to H_2O_2 catalyzed by low-potential electron acceptors (e.g., methylviologen).

We are not certain about the reality of the slight pH dependence of the H^+/e ratio observed for both the $H_2O_2 \rightarrow$ dimethylquinone reaction and the $H_2O \rightarrow$ dimethylquinone reaction (approximately 0.7 at pH 8 to 0.8—0.9 at pH 6). Considering the fact that ΔH^+ determinations by our methods is liable to underestimation at higher pH values where the back flow of protons is relatively rapid, it seems reasonable to assume that in both reaction systems the H^+/e ratio is actually pH independent and is at least 0.8 at all pH values, or even 1.0, as has been reported for the $H_2O \rightarrow$ Photosystem II $\rightarrow$ high ferricyanide system [20] *. However, if one takes the chemiosmotic view [21] and assumes that ATP formation is supported by the efflux of accumulated protons with an overall H^+/P ratio of 3 [22,23], the H^+/e ratio of 0.8—1.0 should, in theory, be able to drive ATP formation with a P/e_2 value of 0.53—0.67. The observed P/e_2 values are only 0.3—0.4. In other words the overall minimum H^+/P ratio in these Photosystem II reactions may be as high as 5—6 (3.4—4.4 if directly computed from observed P/e_2 and H^+/e ratios, see Table I). It does not seem likely, however, that these apparent low efficiencies of proton utilization would be due to a common type of uncoupling (increased proton leakage) since the kinetics of post-illumination proton effluxes seemed quite normal ($t_{1/2}$ 15—20 s at pH 7). We suspect that the dimethylquinone used as the electron acceptor might interact with the hydrophobic region of the ATP synthetase complex in such a way as to curtail the efficiency of proton utilization. Possibly all quinoid oxidants useful as Photosystem II electron acceptors may tend to have similar effects, since the phosphorylation efficiencies of Photosystem II electron transport are in general appreciably lower than expected from the simple by-passing of one of the two sites of energy coupling (see, e.g. Ref. 1).

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* A relatively low H^+/e ratio of 0.5 has been reported for the $H_2O \rightarrow$ Photosystem II $\rightarrow$ DBMIB system [24]. This was interpreted to suggest that the highly membrane-soluble DBMIB (20 μM) might take up protons, as it undergoes reduction, non-directionally from both sides of the membrane.

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