Biochimica et Biophysica Acta, 357 (1974) 1–13

BBA 46767

NATIVE AND ARTIFICIAL ENERGY-CONSERVING SITES IN CYCLIC PHOTOPHOSPHORYLATION SYSTEMS

G. HAUSKA, S. REIMER and A. TREBST

Abt. Biologie, Ruhr-Universität Bochum (G.F.R.)

(Received December 28th, 1973)

SUMMARY

The influence of the plastoquinone antagonist, dibromothymoquinone, on cyclic photophosphorylation systems with various cofactors was studied in isolated chloroplasts. Three different groups of cofactors emerged: Lipophilic compounds insensitive to dibromothymoquinone, lipophilic quinones and lipid insoluble compounds with low redox potentials which were sensitive to dibromothymoquinone and lipophilic quinones with high redox potentials which were only active if *N*, *N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine (TMPD) was present. In the presence of TMPD all cyclic systems were insensitive to dibromothymoquinone. The TMPD effect on the systems is explained by a bypass of a rate-limiting step on the inside of the thylakoid membrane. It is concluded that cyclic photophosphorylation might be coupled to ATP formation either via the native energy-conserving site at plastoquinone or via an artificial energy-conserving site. An artificial energy-conserving site operates when the cofactor is a lipophilic compound, which carries hydrogens across the membrane and generates a proton gradient upon its oxidation inside the thylakoid, thus replacing native proton translocation.

INTRODUCTION

Evidence is accumulating that photosynthetic electron flow from water to $NADP^+$ in chloroplasts includes two sites of energy conservation, though these do not necessarily yield a stoichiometry of 2 ATP per 2 *e* transferred [1–3]. One energy-conserving site is associated with the oxidation of water and Photosystem II [1–5] and the other with plastoquinone [6, 7]. However, ATP formation in photo-reductions by Photosystem I at the expense of artificial donors and in some cyclic photophosphorylation systems is difficult to attribute to any of these sites for two reasons. Firstly, the redox potential of these compounds is too high for a reaction with plastoquinone and, secondly, there is no evidence for a control by energy coupling in the part of the electron transport from cytochrome *f* to $NADP^+$ [6, 8].

Abbreviations: DCIP, 2,6-dichlorophenolindophenol; DCMU, dichlorophenyl-1,1-dimethylurea; DMPD, *N*,*N*-dimethyl-*p*-phenylenediamine; PMS, *N*-methylphenazoniummethosulfate; TMPD, *N*,*N*,*N*′,*N*′-tetramethyl-*p*-phenylenediamine.

Early papers suggested a participation of plastoquinone in cyclic photophosphorylation. For example, it was found that only quinones with a redox potential more negative than zero volt catalyzed cyclic electron flow [9]. This was taken to indicate [10] that the cofactors were feeding into plastoquinone with a potential of about +60 mV. Experiments by Krogmann and Olivero [11] suggested the participation of plastoquinone in cyclic electron flow because extraction of plastoquinone from chloroplasts with heptane would inactivate *N*-methylphenazoniummethosulfate (PMS)-catalyzed cyclic photophosphorylation. Ont he other hand cofactors of cyclic photophosphorylation, like PMS or 2,6-dichlorophenolindophenol (DCIP), definetely have a more positive redox potential than plastoquinone. For the same reason donor systems like diaminodurene* or reduced DCIP for coupled electron flow in Photosystem I could not possibly react via plastoquinone. Also ultraviolet irradiation, which was shown to destroy plastoquinone [12], would only partly effect PMS-catalyzed photophosphorylation and coupled donor systems for Photosystem I [12, 13]. Schemes were therefore postulated for an ATP-coupling site in cyclic electron flow different from the one in non-cyclic electron flow [14, 15].

With the discovery of the plastoquinone antagonist, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone [16], a re-evaluation of the role of plastoquinone in cyclic photophosphorylation was possible [17]. Dibromothymoquinone completely inhibits non-cyclic electron flow from water to NADP [16]. It inhibits some cyclic photophosphorylation systems but not others [17], indicating that plastoquinone may or may not participate in cyclic systems. Therefore, the question as to the nature of the energy-conserving site in dibromothymoquinone-resistant cyclic electron flow remained to be answered.

According to the chemiosmotic hypothesis of energy conservation the redox cycle of plastoquinone/plastohydroquinone in the chloroplast membrane represents a transmembrane proton translocation [18, 19]. Recent investigations from our laboratory demonstrate that artificial quinoid compounds can replace this proton-translocating role of plastoquinone in energy conservation with Photosystem I, provided they are (i) lipid-soluble enough in both redox states to pass the chloroplast membrane and (ii) lose protons upon oxidation [20–22].

In this paper we re-investigate the sensitivity of cyclic photophosphorylation to dibromothymoquinone with different artificial redox systems. A classification of the compounds will be attempted, correlating redox potential and lipid solubility with catalysis of cyclic photophosphorylation and the effects of dibromothymoquinone. From this the concept is derived that artificial energy-conserving sites are operating in dibromothymoquinone-insensitive cyclic photophosphorylation systems. In dibromothymoquinone-sensitive cyclic photophosphorylations the native energy-conserving site via plastoquinone is responsible for ATP formation.

METHODS

Spinach chloroplasts were prepared as described by McCarty and Racker [23].

Cyclic photophosphorylation under anaerobic conditions in the presence of dichlorophenyl-1,1-dimethylurea (DCMU) was measured under two different conditions:

---

* Diaminodurene may be familiar as DAD.

Method 1. The reaction mixture, which was kept in Thunberg tubes flushed 4 times with argon, contained in 1 ml of the final volume: 50 mM Tricine–NaOH buffer (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 3 mM ADP, 2 mM P_i containing about 10^6 cpm ^{32}P, 1 mg defatted bovine serum albumine, $2 \cdot 10^{-5}$ M DCMU and chloroplasts corresponding to 10 μg chlorophyll. The samples were illuminated in a water bath at room temperature for 2 min with white light of 10^6 ergs $\cdot$ $cm^{-2} \cdot s^{-1}$ intensity.

Method 2. In this case the reaction mixture was kept in Warburg vessels under argon in a final volume of 3 ml. It contained 30 mM Tris–HCl buffer (pH 8.0), 3 mM $MgCl_2$, 3 mM ADP, 3 mM P_i containing about $2 \cdot 10^5$ cpm ^{32}P, $2 \cdot 10^{-5}$ M DCMU and chloroplasts corresponding to 200 μg chlorophyll. The samples were illuminated for 10 min at 15 °C with 35 000 lux of white light. Non-cyclic electron transport under aerobic conditions and accompanying phosphorylation was measured in the reaction vessel of a Gilson oxigraph in 1.5 ml final volume. The reaction mixture was the same as for Method 1 above, except that 5 mM ascorbate and 10^{-5} M methyl viologen were added.

Further details are given in the legends for the tables and figures. Esterified $^{32}P_i$ was assayed according to McCarty and Racker [23].

RESULTS

Numerous compounds have been described as catalysts of cyclic photophosphorylation in vitro [24]. In all these systems electron flow from water via Photosystem II is blocked by DCMU. Anaerobic conditions are desirable, many of the reduced forms of the mediators being autooxidizable. All compounds have to be added in the reduced form to provide a poised redox state of the cyclic system in order to be active in the presence of DCMU [25, 26]. Depending on the redox potential of the compound employed this can be achieved in several ways, such as pre-illumination in the absence of DCMU [25]. We wish to report that in every case reduction of the cofactor and poising of the system can be performed via electron flow through Photosystem I with TMPD and ascorbate as the electron source. Electron flow mediated with *N, N, N', N'*-tetramethyl-*p*-phenylenediamine (TMPD) and ascorbate as such is not coupled to ATP formation [27] as shown in the control of Table I. The cofactors employed in this study may be grouped into lipid-soluble and -insoluble redox compounds.

Lipid-soluble redox compounds

Table I summarizes our results with lipophilic catalysts for cyclic photophosphorylation; the reaction always runs in the presence of DCMU. The redox potentials [9, 28] are also shown for a correlation with activity (see also Fig. 1). The comparison of the values in Columns 1 and 2 shows the varying inhibition of the reaction by dibromothymoquinone. Column 3 shows the effect of TMPD, and Column 4 gives the values for the reaction rates in the presence of TMPD and dibromothymoquinone. It is clearly seen that in the presence of TMPD no inhibition of dibromothymoquinone is found in any case.

Cyclic photophosphorylation in the presence of reduced PMS or pyocyanine is not inhibited by dibromothymoquinone as has been shown before [17], neither in the presence nor in the absence of TMPD. TMPD stimulates slightly in the case of pyocyanine and has almost no effect in the case of PMS.

TABLE I

EFFECTS OF DIBROMOTHYMOQUINONE AND TMPD ON CYCLIC PHOTOPHOSPHORYLATION WITH LIPID-SOLUBLE ARTIFICIAL SYSTEMS

The conditions for the reaction with chloroplasts and the assay for ATP are described under Methods. Photophosphorylation with phenamzines, DCIP and *p*-phenylenediamines was measured according to Method 1 and the quinone-dependent reactions according to Method 2. The control gave the same value by either method. Reduction of the mediators: ascorbate was present 3 mM in the case of PMS and pyocyanine, 0.6 mM in the cases of DCIP and all the quinones. Pyocyanine, in the absence of TMPD, was reduced by addition of a grain of $NaBH_4$ [26] in the side arm of the Thunberg tube. All samples with quinones in the absence of TMPD were pre-illuminated for 1 min before DCMU and the phosphorylation substrates, ADP and P_i were added [25].

Mediator (10^{-4} M), reduced)	E'_0 (mV)	μmoles ATP/mg chlorophyll per h			
		Control	Dibromothymoquinone ($5 \cdot 10^{-6}$ M)	TMPD (10^{-4} M)	Dibromothymoquinone+TMPD
Phenazines:					
PMS	80	440	420	456	475
pyocyanine	− 38	280	255	360	372
Benzoquinones:					
p-benzoquinone	290	<5	<5	18	25
2-methyl-*p*-benzoquinone	230	<5	<5	32	38
2,6-dimethyl-*p*-benzoquinone	170	<5	<5	72	75
2,3,5-trimethyl-*p*-benzoquinone	107	<5	<5	111	82
duroquinone	60	30	<5	120	115
dibromothymoquinone	≈170	<5	—	125	—
Naphthoquinones					
1,2-naphthoquinone	127	<5	<5	125	123
1,4-naphthoquinone	50	39	<5	141	135
menadione	−10	57	<5	145	125
lawsone	−152	61	10	96	99
phthiokol	−180	58	<5	90	81
9,10-Anthraquinone	−238	21	<5	33	30
9,10-Phenanthrenequinone	28	75	17	147	123
p-Phenylenediamines					
p-phenylenediamine	363	7	9	45	40
2-methyl-*p*-phenylenediamine	≈310	21	28	110	130
diaminodurene	220	107	75	285	285
TMPD	220	<5	—	—	—
DMPD	310	<5	<5	<5	7
Indophenol: DCIP	217	31	22	106	140
Control: no mediator added	—	<5	<5	<5	8

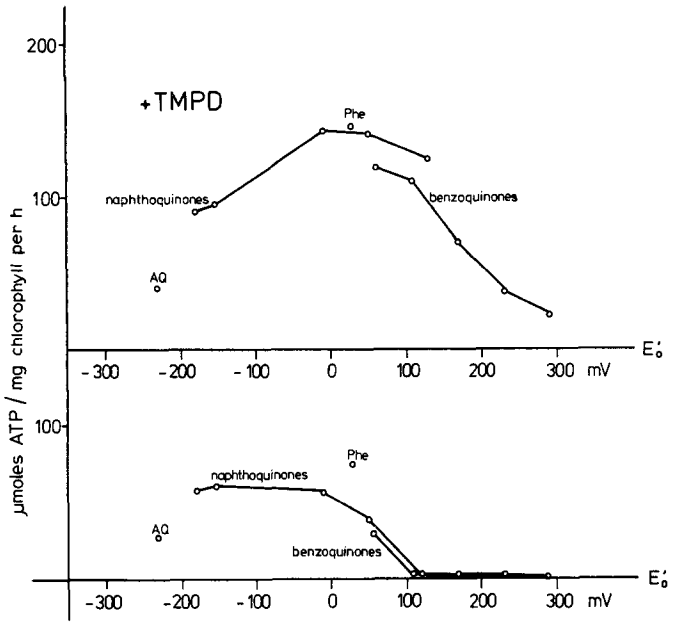

Fig. 1. Correlation between redox potential of quinones and their catalytic activity in cyclic photophosphorylation in the absence and presence of TMPD. The conditions for the reaction are given under Methods and in the legend for Table I. Phe stands for 9,10-phenanthrenequinone and AQ for 9,10-anthraquinone.

It appears that a more subtle poising of the electron transport system is required with quinones as cofactors, which is more reproducibly obtained at the longer illumination periods and higher chlorophyll concentrations of the second method. Poising in the absence of TMPD was achieved by a short pre-illumination in the absence of DCMU [25]. The behavior of the cyclic phosphorylation system with quinones resembles that of ferredoxin-dependent cyclic phosphorylation [29] and other lipid-insoluble redox compounds (see below).

As seen from Table I, first column, only quinones with a redox potential lower than about 100 mV catalyze cyclic photophosphorylation in the absence of TMPD; this system is inhibited by dibromothymoquinone (second column). All quinones are active in the presence of TMPD independently of redox potential, and the activity is then insensitive to dibromothymoquinone (Columns 3 and 4). Thus, the reaction with quinones having redox potentials higher than 100 mV is totally dependent on the presence of TMPD. This correlation of redox potential and activity has been previously reported [9] and is shown more clearly in Fig. 1. In the absence and presence of TMPD the activities drop with increasing redox potentials of the quinones, showing that the oxidations of the hydroquinone become limiting.

It is interesting to note that dibromothymoquinone, the antagonist of plastoquinone, at the rather high concentration of 10^{-4} M is also able to mediate cyclic electron transport efficiently in the presence of TMPD (Table I). This might also account for the small activity observed in the control (Table I) with $5 \cdot 10^{-6}$ M dibromothymoquinone.

DCIP and p-phenylenediamines

In spite of its high redox potential, DCIP, reduced by ascorbate, is able to catalyze cyclic phosphorylation [25]; this system is partially inhibited by dibromothymoquinone [17], as seen in Table I. In addition, Table I shows that TMPD greatly enhances the activity of DCIP as in the cases with quinones. Then the reaction is insensitive to dibromothymoquinone.

The same pattern is observed with *p*-phenylenediamines having free amino groups. Like DCIP they have rather high redox potentials. The reduced forms are employed in these cases, so no pre-reduction is afforded. *p*-Phenylenediamines with double-substituted amino groups, like TMPD and *N,N*-dimethyl-*p*-phenylenediamine, do not catalyze photophosphorylation [27, 30], as shown in Table I. Therefore, there is a synergistic action of C- and N-substituted *p*-phenylenediamines in cyclic phosphorylation. This is shown in more detail in Table II. No synergistic effect is found between two C- and N-substituted phenylenediamines.

TABLE II

SYNERGISM OF *p*-PHENYLENEDIAMINES IN DIFFERENT COMBINATIONS CATALYZING CYCLIC PHOTOPHOSPHORYLATION

The conditions for the reaction are given under Methods and in the legend for Table I. The concentration of the total *p*-phenylenediamines was 10^{-4} M, i.e., 10^{-4} M diaminodurene or $5 \cdot 10^{-5}$ M diaminodurene plus $5 \cdot 10^{-5}$ M TMPD, for example.

	μmoles ATP/mg chlorophyll per h			
	Diaminodurene	2-Methyl-*p*-phenylenediamine	TMPD	DMPD
Diaminodurene	125	122	285	215
2-Methyl-*p*-phenylenediamine	—	14	139	71
TMPD	—	—	5	5
DMPD	—	—	—	5

Table III shows that addition of ascorbate not only inhibits cyclic phosphorylation with *p*-phenylenediamines, as reported previously [26], but also abolishes the stimulatory effect of TMPD (first two columns of Table III). Aerobic conditions and addition of methyl viologen reverses the inhibition by ascorbate. The stimulatory effect of TMPD is also restored, suggesting that TMPD facilitates the oxidation of the *p*-phenylenediamines.

Inhibition by ascorbate is not observed in the case of DCIP (third column of Table III) or with quinones (not shown).

Fig. 2 demonstrates that the stimulatory effect of TMPD on the activities of diaminodurene and DCIP is indeed a stimulation of coupled electron flow through Photosystem I; O_2 uptake and phosphorylation are simultaneously stimulated under conditions which allow the measurement of both. The stimulatory effect of TMPD in electron flow through Photosystem I, cyclic or non-cyclic, decreases with increasing concentration of diaminodurene or DCIP (Fig. 3). This concentration effect is more pronounced with diaminodurene than with DCIP.

TABLE III

EFFECTS OF ASCORBATE AND METHYL VIOLOGEN ON THE STIMULATION BY TMPD OF CYCLIC PHOTOPHOSPHORYLATION WITH *p*-PHENYLENEDIAMINES AND DCIP

The conditions for the reaction are given under Methods and in the legend for Table I; diaminodurene, *p*-phenylenediamine and DCIP were at 10^{-4} M, ascorbate at 5 mM, methyl viologen at 10^{-4} M and TMPD at 10^{-4} M where indicated.

Additions	μmoles ATP/mg chlorophyll per h		
	p-Phenylenediamine	Diaminodurene	DCIP
—	7	115	5
TMPD	45	270	80
Ascorbate	5	24	21
Ascorbate + TMPD	5	14	153
Ascorbate + methyl viologen	10	122	35
Ascorbate + methyl viologen + TMPD	115	330	390

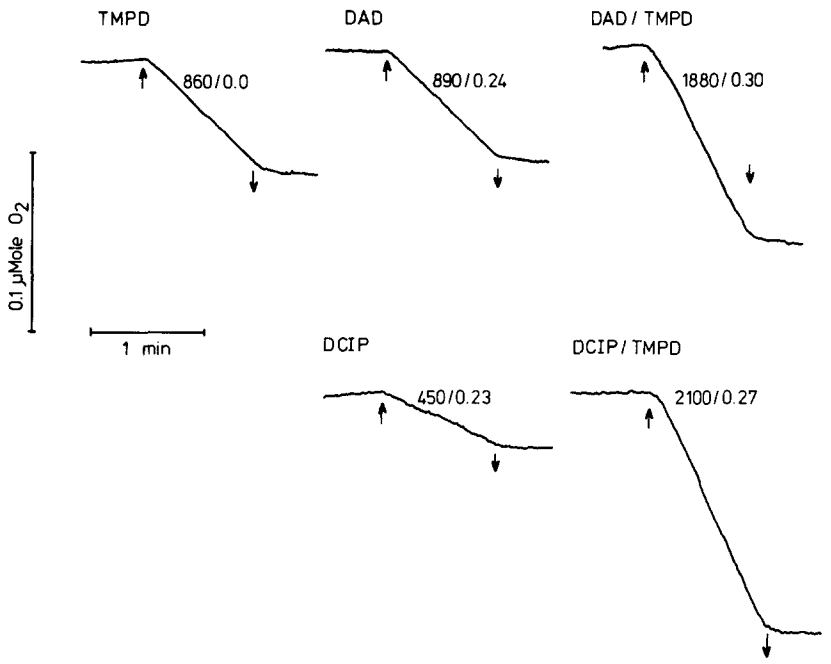

Fig. 2. Stimulation of ascorbate photo-oxidation by Photosystem I and coupled photophosphorylation in the presence of diaminodurene (DAD) or DCIP. The conditions for the aerobic reaction in the oxigraph are described under Methods. TMPD, diaminodurene and DCIP were added, where indicated, to a final concentration of 10^{-4} M. The first number, at every individual trace, represents the rate of O_2 uptake in 2 electrons/mg chlorophyll per h, the second number gives an apparent P/e_2 ratio.

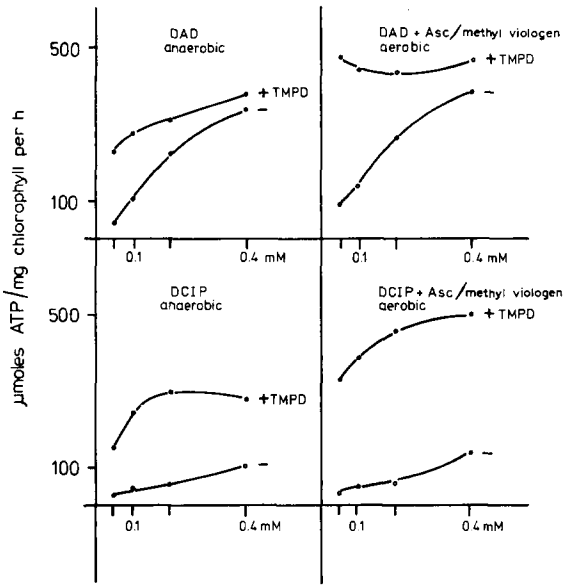

Fig. 3. Effect of TMPD (10^{-4} M) on Photosystem I dependent photophosphorylation at increasing concentrations of diaminodurene (DAD) or DCIP. The reaction conditions for cyclic photophosphorylation (Method 1) and for phosphorylation coupled to non-cyclic electron transport through Photosystem I under aerobic conditions are given in Methods. Asc stands for ascorbate.

TABLE IV

EFFECTS OF DIBROMOTHYMOQUINONE AND TMPD ON CYCLIC PHOTOPHOSPHORYLATION CATALYZED BY LIPID-INSOLUBLE ARTIFICIAL REDOX COMPOUNDS

Method 2 was employed for the reaction (see Methods). 0.6 mM ascorbate was present, except in the cases with ferrocyanide and *N*-methylphenazonium-3-sulfonate. Except in the cases with *N*-methylphenazonium-3-sulfonate, DCIP-3′-sulfonate and ferrocyanide the samples were illuminated for 1 min before addition of DCMU, ADP and P_i as described for Table I. In the case of *N*-methylphenazonium -3-sulfonate ascorbate was present in a concentration of 3 mM.

Mediator (10^{-4} M, reduced)	E'_0 (mV)	μmoles ATP/mg chlorophyll per h			
		Control	Dibromothymoquinone ($5 \cdot 10^{-6}$ M)	TMPD (10^{-4}M)	Dibromothymoquinone +TMPD
N-Methylphenazonium-3-sulfonate	130	<5	<5	<5	12
1,2-Naphthoquinone-4-sulfonate	208	<5	<5	<5	15
1,4-Naphthoquinone-2-sulfonate	113	<5	<5	<5	7
9,10-Anthraquinone-2-sulfonate	225	24	<5	60	72
DCIP-3′-sulfonate	240	<5	<5	<5	9
Ferrocyanide	430	<5	<5	<5	<5
Methyl viologen	−440	<5	<5	76	69

Lipid-insoluble redox compounds

As in the cases with quinones, these compounds were assayed according to Method 2 (see Methods) and were reduced by pre-illumination in the absence of DCMU.

Table IV shows that from the compounds tested only anthraquinone sulfonate, which has a low redox potential, yields cyclic phosphorylation in the absence of TMPD, which is then fully inhibited by dibromothymoquinone. For unknown reasons methyl viologen remains inactive as cofactor despite its low redox potential. In the presence of TMPD both compounds are active in cyclic ATP formation and no inhibition by dibromothymoquinone is observed.

TABLE V

EFFECTS OF DIBROMOTHYMOQUINONE AND TMPD ON CYCLIC PHOTOPHOSPHORYLATION CATALYZED BY FERREDOXIN

The reaction conditions are described in Table IV. 10^{-4} M ferredoxin and, where indicated, $5 \cdot 10^{-6}$ M dibromothymoquinone and 10^{-4} M TMPD were present.

Additions	μmoles ATP/mg chlorophyll per h
Control	55
Dibromothymoquinone	7
TMPD	45
Dibromothymoquinone+TMPD	39

Lipid-insoluble compounds with redox potentials higher than 100 mV remain inactive even in the presence of TMPD.

Ferredoxin

Ferredoxin is lipid insoluble and behaves similarly to anthraquinone sulfonate (Table V). Under poised conditions it is able to catalyze cyclic phosphorylation [29], which is sensitive to dibromothymoquinone [17]. Method 2 and pre-illumination were employed as with the quinones. In the presence of TMPD the reaction with ferredoxin becomes insensitive to dibromothymoquinone.

DISCUSSION

Cyclic phosphorylation in vitro can be sustained with a variety of artificial redox compounds [24], whose function is to connect electron flow from the primary acceptor of Photosystem I to an endogenous oxidant. This paper classifies cofactors of cyclic phosphorylation according to their redox potentials, their lipid solubility and the effect of dibromothymoquinone and TMPD on their reaction. Summarizing our data we can distinguish three major groups of active redox compounds by these criteria.

The first group of cofactors, represented by phenazonium salts, are lipid-soluble and are highly active in cyclic photophosphorylation. The cyclic system they catalyze is neither inhibited by dibromothymoquinone nor stimulated greatly by TMPD (Table I). In addition, it saturates only at high light intensities [31] and is

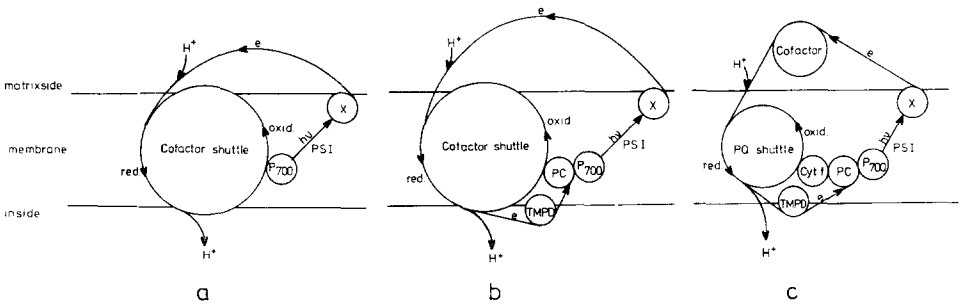

Fig. 4. Types of cyclic electron flow with artificial mediators. In Scheme a a native, whereas in Schemes b and c an artificial energy conserving site is operating. PS I stands for photosystem I, PC for plastocyanin, P_{700} for the reaction center chlorophyll of photosystem I, X for the primary acceptor of Photosystem I and Cyt f for cytochrome *f*.

only partially inhibited by KCN, an inhibitor of plastocyanin [2]. This reaction is therefore assumed to represent the shortest possible cycle around Photosystem I (Scheme a in Fig. 4). The function of the mediator in the cycle is to carry electrons and protons from the outside to the inside of the thylakoid.

The second group of cofactors is characterized by relatively high redox potentials and lipid solubility (Scheme b in Fig. 4). It can be subdivided into the *p*-phenylenediamines and DCIP on one hand, and the lipophilic quinones with redox potentials higher than 100 mV on the other. The cyclic phosphorylation system with the former is partially inhibited by dibromothymoquinone and is stimulated by TMPD. The cyclic phosphorylation system of the latter is totally dependent on TMPD (Table I). This is explained by the assumption that although these quinones can mediate a transmembrane shuttle of electrons and protons they cannot interact with endogenous carriers such as cytochrome *f* and plastocyanin, located inside the thylakoid [32]. *p*-Phenylenediamines can react with endogenous plastocyanin which, for example, also follows from the sensitivity of their reaction to KCN [2].

The third group of cofactors comprises lipid-insoluble redox compounds, including ferredoxin and also a few lipophilic quinones, both with low redox potentials (Tables I, IV and V). Because of the sensitivity to dibromothymoquinone cyclic electron flow catalyzed by these compounds obligatorily includes plastoquinone (Scheme c in Fig. 4). The function of this group of compounds is to bridge electron flow from the primary acceptor in Photosystem I to plastoquinone on the outer surface of the thylakoid membrane (Scheme c in Fig. 4). The only difference between the lipophilic quinones and the lipid-insoluble compounds of this group is that the quinones might penetrate into the membrane and intermingle with the plastoquinone pool.

In the first three papers of this series [20–22] we emphasized that in order to be active in cyclic phosphorylation, redox compounds require lipid solubility and must be hydrogen carriers. It follows from the results presented here that if plastoquinone is involved, lipid solubility is not decisive for catalytic activity as long as the redox potential is low enough to reduce plastoquinone. The hydrogen-carrying feature is also not required, as is seen in the case of ferredoxin. This is because plastoquinone itself can function as the transmembrane hydrogen carrier in these systems. There-

fore, we have to extend our conclusion of the preceding paper [21]: catalysts of cyclic photophosphorylation have to be lipophilic and hydrogen carriers if the endogenous hydrogen carrier plastoquinone cannot participate.

The best candidate for the cofactor of cyclic photophosphorylation in vivo is ferredoxin. This is concluded from the fact that cyclic photophosphorylation in vitro with ferredoxin, and in vivo, is sensitive to antimycin [29, 33, 34] and to dibromothymoquinone [35, 36]. This evidence suggests that this path of cyclic electron flow around Photosystem I includes plastoquinone and a native energy-conserving site.

The stimulatory effect of TMPD

The stimulating effect of TMPD/ascorbate might partly be due to a poising [25, 26, 29, 37, 38] of the redox state of the carriers involved in cyclic electron flow, because of its known properties as a non-coupled electron donor system for Photosystem I [27]. It might be superior to some of the earlier poising systems, because it is present throughout the experiment and therefore would also take care of drainage of electrons from the cycle during the light period. This would be particularly important in systems with highly auto-oxidizable cofactors such as methyl viologen, and would explain why TMPD/ascorbate is stimulating ATP formation in cyclic systems, even if the cofactor is added in the already reduced state. However, a simple feed-in of electrons from TMPD/ascorbate is not a sufficient explanation because ascorbate alone should be already effective with cofactors of appropriate redox potentials such as the phenylenediamines. We would like to propose that the TMPD-poising occurs in, or even inside the membrane, because the charged ascorbate will not be able to penetrate the thylakoid membrane, whereas TMPD can. Since the stimulation by TMPD is also observed in non-cyclic electron flow (Table III) through Photosystem I, the rate limit which is overcome by TMPD seems to be in the oxidation of the reduced mediator by Photosystem I rather than in its reduction. Otherwise methyl viologen should oppose the effect of TMPD (Table III). For quinones, the analogous conclusion can be drawn from the observed drop of activity with increasing redox potentials, which is shifted to more positive values if TMPD is present (Fig. 1).

The action of TMPD inside the membrane is in agreement with the present knowledge of the topography of the thylakoid membrane. In all possibility plastocyanin is the endogenous carrier connecting electron flow from TMPD to Photosystem I, and plastocyanin seems to be located inside the membrane [32]. A TMPD effect inside the membrane has already been inferred from recent experiments, in which TMPD bypasses the dibromothymoquinone inhibition of photosynthetic NADP reduction [39, 40]. Because the P/e_2 ratio of about 1 is also restored [39, 40], TMPD cannot bypass the dibromothymoquinone-inhibition site by just being an electron donor for Photosystem I from the outside, because this system is not coupled [27]. The same bypass of the dibromothymoquinone-inhibition site is assumed to take place in all the cyclic phosphorylation systems which are insensitive to dibromothymoquinone in the presence of TMPD. TMPD is stimulating by either bridging electron flow from reduced plastoquinone (Scheme c in Fig. 4) or from the mediator (Scheme b in Fig. 4) to the endogenous carriers of Photosystem I. This bridging inside represents a counterpart to the bridging outside, catalyzed by lipid-insoluble redox compounds such as ferredoxin (Scheme c in Fig. 4).

A more specic mechanism of this action of TMPD inside the thylakoid is,

however, necessary to explain the stimulatory effects in the systems with DCIP and *p*-phenylenediamines, which exhibit a dibromothymoquinone-insensitive, TMPD-independent part, i.e. they can react directly with endogenous oxidants different from plastoquinone (Table I). The facilitated photo-oxidation of DCIP and *p*-phenylenediamines by TMPD points to a different reaction mechanism, possibly involving radical forms in a chain reaction.

Native and artificial sites of energy conservation

According to Mitchell's chemiosmotic theory [18] an energy-conserving site in electron transport consists of a transmembrane electron-transferring step and a transmembrane hydrogen-transferring step in opposite directions. Photosynthetic electron flow in the chloroplast membrane seems to have two such loops. One is the combination of hydrogen transfer in the water-splitting system with electron transfer in the reaction center of Photosystem II and the other is the combination of hydrogen transfer in the redox reaction of plastoquinone with electron transfer in the reaction center of Photosystem I [19] (see [41] for a recent review). These are the two possible native coupling sites for ATP formation.

In cyclic electron flow the native site of energy conservation with plastoquinone and Photosystem I might operate in vivo, and is operative in vitro when ferredoxin, other lipid-insoluble redox compounds of low redox potential or lipophilic quinones of low redox potential are used as mediators (Scheme c in Fig. 4). A physiological role of this native cyclic phosphorylation is at least established for certain algae [42]. The involvement of a quinone and a reaction center in cyclic ATP formation is strikingly similar to the reaction in photosynthetic bacteria, where the cyclic system seems to represent the major path of electron flow [43].

With lipophilic artificial redox compounds, which lose protons upon oxidation, one can induce artificial sites of energy conservation in which plastoquinone as the transmembrane hydrogen carrier is replaced by the added cofactor. The other half of the loop, i.e. the electron transfer through the membrane in the reaction center of Photosystem I, remains the same as in native energy conservation.

ACKNOWLEDGEMENT

The technical assistance of Miss A. Hellmich is acknowledged. The work was supported by Deutsche Forschungsgemeinschaft.

REFERENCES

1 Trebst, A. and Reimer, S. (1973) Biochim. Biophys. Acta 305, 129–139
2 Ouitrakul, R. and Izawa, S. (1973) Biochim. Biophys. Acta 305, 105–118
3 Reeves, S. G. and Hall, D. O. (1973) Biochim. Biophys. Acta 314, 66–78
4 Böhme, H. and Trebst, A. (1969) Biochim. Biophys. Acta 180, 137–148
5 Junge, W. and Ausländer, W., (1974) Biochim. Biophys. Acta 333, 59–70
6 Böhme, H. and Cramer, W. A. (1972) Biochemistry 11, 1155–1160
7 Reinwald, E., Stiehl, H. H. and Rumberg, B. (1968) Z. Naturforsch. 23b, 1616–1617
8 Avron, M. and Chance, B. (1966) Brookhaven Symp. Biol. 19, 149–160
9 Trebst, A. and Eck, H. (1961) Z. Naturforsch. 16b, 44–49
10 Kandler, O. (1960) Annu. Rev. Plant Physiol. 11, 37–54

11 Krogmann, D. W. and Olivero, E. (1962) J. Biol. Chem. 237, 3292–3295
12 Trebst, A. and Pistorius, E. (1965) Z. Naturforsch. 20b, 885–889
13 Shavit, N. and Avron, M. (1963) Biochim. Biophys. Acta 66, 187–195
14 Gromet-Elhanan, Z. (1969) Progress in Photosynthesis Research, (Metzner, H., ed.), Vol. III, pp. 1197–1202, Tübingen
15 Arnon, D. I., Knaff, D. B., McSwain, B. D., Tsujimoto, H. Y., Chain, R. K., Malkin, R. and Bearden, A. J. (1972) in Structure and Function of Oxidation Reduction Enzymes, (Akeson, A. and Ehrenberg, A., eds), pp. 237–255, Pergamon Press, Oxford
16 Trebst, A., Harth, E. and Draber, W. (1970) Z. Naturforsch. 25b, 1157–1159
17 Böhme, H., Reimer, S. and Trebst, A. (1971) Z. Naturforsch. 26b, 341–352
18 Mitchell, P. (1966) Biol. Rev. 41, 445–502
19 Witt, H. T., Rumberg, B. and Junge, W. (1968) in Biochemie des Sauerstoffs (Hess, B. und Staudinger, H. J., eds), pp. 262–305, Springer Verlag, Berlin
20 Hauska, G. (1972) FEBS Lett. 28, 217–220
21 Hauska, G., Trebst, A. and Draber, W. (1973) Biochim. Biophys. Acta 305, 632–641
22 Hauska, G. and Prince, R. (1974) FEBS Lett., 41, 35–39
23 McCarty, R. E. and Racker, E. (1967) J. Biol. Chem. 242, 3435–3439
24 Arnon, D. I. (1961) Fed. Proc. 20, 1012–1022
25 Trebst, A. and Eck, H. (1961) Z. Naturforsch. 16b, 455–461
26 Hauska, G., McCarty, R. E. and Racker, E. (1970) Biochim. Biophys. Acta 197, 206–218
27 Trebst, A. (1964) Z. Naturforsch. 19b, 418–421
28 Mansfield Clark, W. (1960) in Oxidation Reduction Potentials of Organic Systems, Williams and Wilkins, Co., Baltimore
29 Tagawa, K., Tsujimoto, H. Y. and Arnon, D. I. (1963) Nature 199, 1247–1251
30 Wessels, J. S. C. (1964) Biochim. Biophys. Acta 79, 640–642
31 Avron, M. (1960) Biochim. Biophys. Acta 40, 257–272
32 Racker, E., Hauska, G. A., Lien, S., Berzborn, R. J. and Nelson, N. (1971) in Proc. 2nd Int. Congr. Photosyn. Res., Stresa, Vol. II, pp. 1097–1113, W. Junk N.V., The Hague
33 Tanner, W., Loos, E. and Kandler, O. (1965) in Currents in Photosynthesis (Thomas, J. B. and Goedheer, J. C., eds), pp. 243–251, AD. Donker, Rotterdam
34 Urbach, W. and Simonis, W. (1964) Biochem. Biophys. Res. Commun. 17, 39–45
35 Urbach, W. and Kaiser, W. (1971) in Proc. 2nd Int. Congr. Photosyn. Res., Stresa, Vol. II, pp. 1401–1411, W. Junk N.V., The Hague
36 Bothe, H. and Loos, E. (1972) Arch. Mikrobiol. 86, 241–254
37 Jagendorf, A. T. and Avron, M. (1959) Arch. Biochem. Biophys. 80, 246–257
38 Whatley, F. R. (1963) in Photosynthetic Mechanisms of Green Plants (Kok, B. and Jagendorf, A. T., eds), pp. 243–251, National Academy of Sciences, Washington
39 Trebst, A. and Reimer, S. (1973) Z. Naturforsch. 28c, 710–717
40 Trebst, A. and Reimer, S., (1973) Biochim. Biophys. Acta 325, 546–557
41 Trebst, A. and Hauska, G., Naturwissenschaften, in the press
42 Simonis, W. and Urbach, W. (1973) Annu. Rev. Plant Physiol. 24, 89–114
43 Frenkel, A. W. (1970) Biol. Rev. 45, 569–616