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LIPOPHILICITY AND CATALYSIS OF PHOTOPHOSPHORYLATION*

II. QUINOID COMPOUNDS AS ARTIFICIAL CARRIERS IN CYCLIC PHOTOPHOSPHORYLATION AND PHOTOREDUCTIONS BY PHOTOSYSTEM I

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SUMMARY

The topography of the chloroplast membrane has been studied using the following pairs of quinoid compounds with similar structure and chemical properties, but with different lipid solubility: phenazine/sulfophenazine, naphthoquinone/naphthoquinone sulfonate, indophenol/sulfoindophenol and lumiflavin/FMN.

All these compounds in the oxidized form are able to accept electrons from the photosynthetic electron transport chain in Hill reactions. However, only the lipophilic compounds in the reduced form can donate electrons to Photosystem I, when electron flow from Photosystem II is blocked by inhibitors. This is in agreement with the notation that the oxidizing site of Photosystem I (P_{700}^+) and the electron donors for Photosystem I (cytochrome *f* and plastocyanin) are located inside the lipid barrier of the inner chloroplast membrane. The reducing sites in the Hill reactions must be located on the outer surface, accessible from the suspending medium.

It has been known for a long time that *N,N'*-tetramethyl-*p*-phenylenediamine can donate electrons to Photosystem I, but contrary to diaminodurene (2,3,5,6-tetramethyl phenylenediamine) it does not induce ATP formation. Both compounds are lipophilic and have similar redox potentials, but only the latter carries hydrogens which are involved in the redox reaction. For energy conservation, coupled to electon flow in Photosystem I, it therefore seems necessary that the lipophilic redox compound in the reduced form can carry hydrogens through the chloroplast membrane.

Abbreviations: DBMIB, dibromomethylisopropyl-*p*-benzoquinone; DAD, diaminodurene; DCMU, dichlorophenyl-1,1-dimethylurea; DCIP, 2,6-dichlorophenolindophenol; HEPES, *N*-2-hydroxyethylpiperazine-*N*-2-ethane sulfonic acid; PMS, *N*-methylphenazonium methosulfate; pyocyanine, 1-hydroxy-*N*-methylphenazonium salt; DCIP sulfonate, 2,6-dichlorophenolindophenol-3'-sulfonate; PMS sulfonate, *N*-methylphenazonium-3-sulfonate; pyocyanine sulfonate, 1-hydroxy-*N*-methylphenazonium-3-sulfonate; TMPD, *N,N'*-tetramethyl-*p*-phenylenediamine; Tricine, tris(hydroxymethyl)methylglycine.

* For Paper I see Hauska, G. (1972) *FEBS Lett.* 28, 217–220.

INTRODUCTION

Quinones and quinoid compounds may be reduced by chloroplasts in the light, *i.e.* they are electron acceptors in Hill reactions[1,2]. This photoreduction of the compounds is stoichiometric with oxygen evolution and coupled ATP formation[3]. The reduced form of some of the compounds formed in the Hill reaction may react back with the oxygen evolved (pseudocyclic electron transport and pseudocyclic photophosphorylation)[4,5] or with an endogenous compound of the electron transport chain, *i.e.* they may be electron donors for Photosystem I (cyclic electron transport or cyclic photophosphorylation)[6].

Earlier results indicated that quinones are cofactors of cyclic photophosphorylation if their redox potential is more negative than zero voltage[4,7]. However, there are notable exceptions to this. PMS, which has a redox potential around 80 mV, is the most active cofactor of cyclic photophosphorylation found so far[8]. Also DCIP and DAD, with redox potentials around 200 mV, are active in cyclic photophosphorylation[4,9]. Reduced DCIP and DAD are also electron donors for photoreductions by Photosystem I as is TMPD[9,10,11]. Though all three compounds have comparable redox potentials, the TMPD system is not coupled to ATP formation[11,12]. This indicates that the redox potential is not the only decisive property of cofactors for photophosphorylation linked to Photosystem I.

Recent results indicated that lipophilicity and polarity of cofactors of cyclic photophosphorylation, of possible electron donors and of electron acceptors are indeed of importance for the site of their reaction with components of the electron transport system and for the stoichiometry of the reaction with ATP formation[13-15]. The present paper investigates cyclic photophosphorylation and photoreductions of several classes of quinoid compounds comparing pairs of specimens with different lipophilicity and polarity but similar redox potentials. The results can be explained by the assumption that only lipophilic, unpolar components can carry reducing equivalents into or through the membrane of the photosynthetic electron transport system of chloroplasts. This allows the reaction with the endogenous carriers involved in electron transport coupled to ATP formation.

METHODS

Chloroplasts were prepared either as described by McCarty and Racker[16] or according to Nelson *et al.*[17]. Phosphorylating digitonin subchloroplast vesicles were obtained by the method of Anderson and Boardman[18] including 0.4 M sucrose in the chloroplast suspension.

Cyclic and pseudocyclic photophosphorylation was routinely measured according to McCarty and Racker[16]. The reaction mixture contained in 1 ml final volume, 50 mM Tricine–NaOH, pH 8.5, 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 albumin and chloroplast membranes corresponding to 10 μg chlorophyll. To establish anaerobic conditions, Thunberg tubes were flushed 4 times with argon[19]. The samples were illuminated in a water bath at room temperature for 2 min with white light of $2 \cdot 10^6$ ergs$\cdot$cm$^2 \cdot$s^{-1} intensity. Further specifications are given in the legends to the tables.

The Hill reaction was measured either polarographically in a Gilson oxygraph

or manometrically in the Warburg apparatus. In the former case, the reaction volume was 1.5 ml and the same reaction mixture as above was employed containing 20 to 30 μg chlorophyll. In the latter case, the reaction mixture contained in 3 ml: 30 mM Tris–HCl buffer, pH 8.0, 3 mM $MgCl_2$, 3 mM ADP, 3 mM $^{32}P_i$, chloroplasts membranes corresponding to 100 μg chlorophyll and additions according to the legends. The vessels were flushed with nitrogen and illuminated with 35000 lux at 15 °C.

Oxygen uptake *via* electron flow through Photosystem I was also measured in the oxygraph using the same reaction mixture as above, which additionally contained 3 mM ascorbate, $5 \cdot 10^{-5}$ M indophenol, $1 \cdot 10^{-4}$ M methyl viologen, $1 \cdot 10^{-4}$ M azide, $2 \cdot 10^{-5}$ M DCMU and chloroplast membranes corresponding to 10 μg chlorophyll.

The partition coefficients for indophenols in water–organic solvent systems given in Table II were determined by measurement of the absorbance of the aqueous phase at 600 nm before and after extraction. The coefficients of the reduced forms were accordingly measured by reduction with ascorbate before, and reoxidation by excess ferricyanide after extraction (*cf.* ref. 13). The synthesis of DBMIB has been reported[20], as well as the one of the pyocyanine sulfonate[13]. We are indebted to Professor Hemmerich, Konstanz, for the gift of 3-methyllumiflavin.

Preparation of DCIP sulfonate: 2,6-Dichloroquinone chloroimide was prepared according to Gibbs[39] from 2,6-dichloro-4-aminophenol and NaOCl solution. 20.95 g (0.1 mole) of 2,6-dichloroquinone chloroimide were dispersed in 20 ml of water and a solution of 21.8 g (0.1 mole) of the disodium salt of 2-hydroxyphenylsulfonic acid in 20 ml of water was added with stirring. A blue-green solution was formed immediately. After filtration and cooling to 5 °C 50 ml of satd NaCl solution were added. The sodium salt of DCIP sulfonic acid was precipitated as green platelets. It contained 2 moles of NaCl. Yield: 12.4 g (26%). $C_{12}H_6Cl_2NNaO_5S + 2\,NaCl$ (mol. wt 487.0 + 116.9). Found: C 29.8, H 1.4, N 2.9, Cl 28.4; calcd C 29.6, H 1.2, N 2.9, Cl 29.0.

RESULTS

As has already been reported phenazine derivatives substituted by a sulfo-group (PMS sulfonate or pyocyanine sulfonate) are not cofactors of cyclic photophosphorylation in subchloroplast vesicles[13]. Further details of the comparison of PMS and PMS sulfonate in photophosphorylation by chloroplasts are presented in Table I. PMS-catalysed cyclic photophosphorylation is resistent to the inhibitor of Photosystem II DCMU[8] and the antagonist of plastoquinone, DBMIB[21], as is well known. PMS sulfonate on the other hand, is unable to support DCMU- or DBMIB-insensitive photophosphorylation. This is not due to an uncoupling or other effects on the ATP-forming system of the substituted PMS, because in the absence of the inhibitors PMS sulfonate is a perfectly good cofactor for phosphorylation of the pseudocyclic type. The same is found with the pair pyocyanine and pyocyanine sulfonate, *i.e.* the hydroxylated derivatives of PMS. Because of the importance of poising the redox state of a cyclic electron flow system, the property of pyocyanine as a cofactor of DCMU- and DBMIB-insensitive cyclic photophosphorylation becomes apparent only if the cofactor is in the reduced state at the

start of the reaction[4,19]. PMS is reduced chemically by white light; pyocyanine, however, has to be reduced by a reducing substance like $NaBH_4$. TMPD/ascorbate, as used in Table I, also reduces the cofactor *via* Photosystem I; TMPD/ascorbate alone yields no ATP[11]. Pyocyanine sulfonate remains ineffective also under these balancing conditions.

FMN has been described by Whatley *et al.*[6] as cofactor of cyclic photophosphorylation and Arnon *et al.*[5] have reported on the special experimental conditions required. Doubts remained whether flavins are indeed catalyzing an electron flow system solely dependent on Photosystem I (ref. 4). As Table II indicates, FMN,

TABLE I

PHOTOPHOSPHORYLATION WITH PMS DERIVATIVES IN ISOLATED CHLOROPLASTS

The assay for photophosphorylation is described under Methods. In addition, the basic reaction mixture contained $5 \cdot 10^{-5}$ M phenazine compound, 3 mM ascorbate and where indicated $5 \cdot 10^{-6}$ M DBMIB, $1 \cdot 10^{-5}$ M DCMU and $1 \cdot 10^{-4}$ M TMPD. The reactions were carried out anaerobically under argon.

Addition	*μmoles ATP formed/mg chlorophyll per h*			
	PMS	*PMS sulfonate*	*Pyocyanine*	*Pyocyanine sulfonate*
—	550	255	440	185
DBMIB	495	25	105	17
DCMU	505	< 5	55	< 5
DCMU + TMPD	—	—	370	< 5
DCMU + TMPD + DBMIB	—	—	395	< 5

TABLE II

PHOTOPHOSPHORYLATION WITH FLAVIN AND NAPHTHOQUINONE DERIVATIVES

The basic reaction mixture additionally contained $1 \cdot 10^{-4}$ M flavin or naphthoquinone and for cyclic phosphorylation $2 \cdot 10^{-5}$ M DCMU, 3 mM ascorbate and $1 \cdot 10^{-4}$ M TMPD. All reactions were carried out under argon.

Additions	E_0' (*mV*) (*cf. ref. 27*)	*μmoles ATP/mg chlorophyll per h*		
		Chloroplasts		*Digitonin subchloroplasts*
		Pseudocyclic	*Cyclic*	*Cyclic*
—		—	7	7
FMN	−187	127	11	12
Riboflavin	−195	129	24	15
3-Methyllumiflavin	−220	122	72	45
1,2-Naphthoquinone	127	192	285	125
1,2-Naphthoquinone-4-sulfonate	208	265	8	< 5
1,4-Naphthoquinone	50	254	193	104
1,4-Naphthoquinone-2-sulfonate	113	247	9	< 5

riboflavin and methyllumiflavin are cofactors of pseudocyclic electron flow (*i.e.* in the absence of inhibitors of electron flow through Photosystem II). However, only the lipophilic methyllumiflavin is appreciably active also in cyclic photophosphorylation (*i.e.* in argon *plus* DCMU, being poised by TMPD/ascorbate). The same is true for subchloroplasts. The redox potentials of the three alloxazine derivatives are rather similar, but they differ in lipophilicity. Not only phenazines and alloxazines, but also naphthoquinones are active in cyclic phosphorylation, only if unpolar. Whereas both naphthoquinones and naphthoquinone sulfonates are mediators of pseudocyclic photophosphorylation, the polar naphthoquinone sulfonates are inactive under argon in the presence of DCMU. The redox potential of *o*-naphthoquinone is high enough for reduction by ascorbate alone. Why TMPD also in this case is additionally needed to achieve cyclic phosphorylation is not yet understood. A detailed description of TMPD as a balancing agent for cyclic electron flow will be presented in a subsequent paper.

The results with cofactors of cyclic photophosphorylation described above show that the approach of compounds to the reducing site of Photosystem I is possible for uncharged and charged specimens because all compounds tested are cofactors of pseudocyclic electron transport. This is in agreement with the finding that this reducing site is accessible for antibodies on the outer surface of the thylakoids (*cf.* ref. 22). For cycling of electrons back into Photosystem I (presumably *via* plastocyanin and/or cytochrome *f*) however, the reduced specimen may not carry a charged, highly hydrated group. The lipophilic properties essential for cofactors of cyclic photophosphorylation are therefore required for their electron donor activity implying that the oxidizing end of Photosystem I, P_{700}, is buried in the hydrophobic region or is located on the inner surface of the chloroplast membrane. The latter has been suggested on theoretical grounds by Mitchell[22], because his chemiosmotic theory requires loops in the electron transport chain. Supporting this Schliephake *et al.*[23] produced evidence that Photosystem I (as well as Photosystem II) on illumination gives rise to a positive charge on the inside and a negative charge on the outside of the membrane. Furthermore it has been found[24] that plastocyanin, the primary electron donor for Photosystem I, is functioning in the hydrophobic region or on the inner surface of the membrane.

Therefore the importance of polarity and lipophilicity of electron donors for Photosystem I was investigated in the indophenol class by comparing DCIP and DCIP sulfonate.

Table III compares the principal properties of the two compounds. Both are electron acceptors in a Hill reaction. The reduction is coupled to ATP formation. The concentration of DCIP is in the order of the concentration where the oxidized form is an uncoupler[25,26]. However, the concentration of oxidized DCIP falls below the uncoupling range because of the photoreduction. The comparison of the chemical properties of the two compounds show that according to Clark[27], there is almost no difference in redox potential and pK values, but a marked difference in lipid solubility is observed, DCIP sulfonate preferring an aqueous environment. Table III indicates that the lipid solubility does not matter for the activity as Hill reagents.

Table IV compares the uncoupling properties of DCIP and DCIP sulfonate. In a ferricyanide Hill reaction (in which ferricyanide keeps the indophenols in the

TABLE III

PROPERTIES AND HILL REACTIONS OF DCIP AND DCIP SULFONATE

The Hill reactions were measured polarographically under phosphorylating conditions (see Methods). The basic reactions mixture additionally contained $1 \cdot 10^{-4}$ M indophenol and chloroplasts corresponding to 20 μg chlorophyll. The determination of the partition coefficients is described under Methods.

		DCIP	*DCIP sulfonate*
Hill reactions			
μatoms O evolved/mg chlorophyll per h		155	183
μmoles ATP formed/mg chlorophyll per h		124	160
P/e_2		0.8	0.87
Chemical properties			
E_0' (*cf.* ref. 27)		217 mV	240 mV
pK_{ox}		5.7	6.1
$pK_{red\ 1}$		6.0	6.0
$pK_{red\ 2}$		10.2	10.3
Absorbance peak of the oxidised form		600 nm	650 nm
P water/benzene	oxidised	0.79	100
	reduced	0.42	100
P water/chloroform	oxidised	0.23	100
	reduced	0.41	100
P water/1-butanol	oxidised	0.05	30
	reduced	0.03	0.15

oxidized state), as is well known[25,26], DCIP uncouples electron flow, *i.e.* the ratio of P/e_2 is lowered. DCIP sulfonate on the contrary, has very little uncoupling activity. In Table IV two experiments are shown; one which measured oxygen evolution manometrically, the other polarographically. Due to the difference in methods P/e_2 ratios in the latter case are lower. But the influence of the two indophenols is the same in both types of experiments, *i.e.* DCIP uncouples, DCIP

TABLE IV

UNCOUPLING OF FERRICYANIDE-HILL REACTION BY INDOPHENOLS

The manometric and polarographic measurements of oxygen production are described under Methods. In the former case ferricyanide was 3.3 mM, in the latter 1 mM.

	μatoms O/mg chlorophyll per h	*μmoles ATP/mg chlorophyll per h*	P/e_2
Expt 1			
—	200	213	1.07
+0.1 mM DCIP	140	67	0.48
+0.1 mM DCIP sulfonate	160	137	0.85
Expt 2			
—	183	123	0.65
+0.2 mM DCIP	101	27	0.17
+0.2 mM DCIP sulfonate	114	68	0.59

TABLE V

INDOPHENOLS IN CYCLIC PHOTOPHOSPHORYLATION OF ISOLATED CHLOROPLASTS

The basic reaction mixture additionally contained the indicated concentrations of indophenols, an equal amount of ascorbate and $2 \cdot 10^{-5}$ M DCMU. The reactions were carried out under argon.

Concentration (mM)	*μmoles ATP/mg chlorophyll per h*	
	DCIP	*DCIP sulfonate*
0.05	30	< 5
0.2	85	< 5
0.5	90	< 5

sulfonate does not. Presumably because of the high self-absorption of the oxidized indophenols, the stimulation of electron flow by uncoupling is not observed.

Table V indicates that DCIP sulfonate is not a cofactor of cyclic photophosphorylation, whereas DCIP is (if properly poised). This is in accordance with the behaviour of the other cofactors of cyclic photophosphorylation described in Tables I and II.

Table VI and Fig. 1 describe the electron donor activity of indophenols (kept reduced by ascorbate) in photoreductions by Photosystem I. Whereas $DCIPH_2$ is a very good electron donor for Photosystem I, as is well known[10], $DCIPH_2$ sulfonate is comparatively ineffective in broken chloroplasts as well as in fragmented ones. Photoreductions at the expense of $DCIPH_2$ show a small, but consistent ATP formation and in addition a marked stimulation of electron flow upon addition of

TABLE VI

INDOPHENOLS AS ELECTRON DONORS FOR PHOTOSYSTEM I

The assay was performed under air in the oxigraph with phosphorylating conditions (see Methods). The reaction mixture additionally contained 3 mM NH_4Cl where indicated.

	DCIP		*DCIP sulfonate*	
	O_2 uptake (μatoms/mg chlorophyll per h)	*ATP (μmoles/mg chlorophyll per h)*	*O_2 uptake (μatoms/mg chlorophyll per h)*	*ATP (μmoles/mg chlorophyll per h)*
Broken chloroplasts				
—	203	42	25	< 5
+ NH_4Cl	392	18	25	< 5
Digitonin vesicles				
—	465	39	104	< 5
+ NH_4Cl	910	156	104	< 5

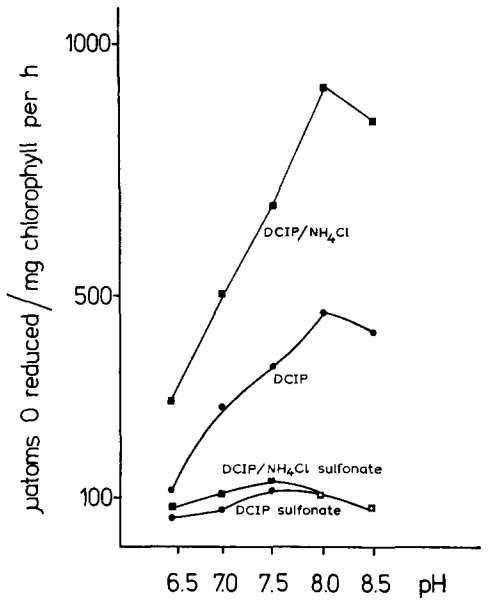

Fig. 1. pH dependence and stimulations by NH_4Cl of indophenol-catalysed electron flow through Photosystem I in digitonin subchloroplast vesicles. The reaction was carried out without ADP and P_i in the oxigraph as described under Methods and in Table VI, except that the pH was varied: HEPES replaced Tricine at pH 6.5 and 7.0.

an uncoupler[37,38]. This is shown in Table VI and Fig. 1. In broken chloroplasts, NH_4Cl uncouples electron flow, whereas in digitonin vesicles it stimulates ATP formation, as has been observed by Neumann *et al.*[28]. The DCIP-sulfonate system does not show this stimulation. The pH optimum of DCIP-sulfonate oxidation is lower than that for DCIP (Fig. 1) in digitonin subchloroplast vesicles.

DISCUSSION

The photosynthetic electron transport chain of chloroplasts may react with numerous exogenous redox compounds. Electron acceptors may be reduced in Hill reactions, electron donors may be oxidized at different sites along the chain. In relating the activity to the properties of the redox compound in the past, the main consideration was the redox potential of the compound in relation to the possible redox potential of the site in the electron transport system it may react with. In this way the redox potential of the reducing site of Photosystems I and II were estimated[29–33].

With the impact of Mitchell's theory of energy conservation[34], the influence of charge-, proton- and ion-carrying compounds on ion transport and ATP formation became apparent. But only recently the importance of lipophilicity and polarity for the reaction of electron acceptors and donors in chloroplast reactions were considered[13–15]. Saha *et al.*[14] grouped Hill reagents in classes according to polarity and the stoichiometry of electron flow to ATP formation. They suggested that polar quinones and ferricyanide may be reduced by Photosystem I and lipophilic quino-

diimines by Photosystem II. This concept was further substantiated by use of the plastoquinone antagonist DBMIB, which prevents electron flow from Photosystem II to Photosystem I (ref. 15). It appeared that lipophilic quinones in a certain redox potential range are reduced by Photosystem II with a small, but distinct ATP formation coupled to it[15]. The polar ferricyanide may also be reduced by Photosystem II (though in undisturbed chloroplasts preferentially by Photosystem I), but this reduction is even less coupled[15]. Furthermore in studying polar (PMS sulfonate) and unpolar (PMS) phenazines[13], it became clear that only the lipid-soluble compound PMS, which can penetrate into or through the lipophilic region of a lipid membrane[35], is catalyzing cyclic photophosphorylation.

This importance of the lipophilicity of cofactors of cyclic photophosphorylation and electron donors for photoreductions by Photosystem I is further substantiated by the results in the present paper. By comparing alloxazines, phenazines and naphthoquinones as possible cofactors of cyclic phosphorylation, it is observed that only an unpolar compound is active, whereas charged specimens, like FMN, naphthoquinone and PMS sulfonate are inactive.

Because all compounds tested are cofactors in pseudocyclic photophosphorylation, all compounds, whether charged or not, are reduced by Photosystem I. The reason for the necessity of high lipophilicity of a cofactor of cyclic photophosphorylation is therefore sought in the required permeation of the reduced form, through or into the membrane, to react back with the electron donor site of Photosystem I. This is corroborated by the effect of a polar substituent on an electron donor for photoreductions by Photosystem I. Whereas $DCIPH_2$, as is well known[10], is an excellent electron donor for Photosystem I, thus reversing DCMU inhibition of $NADP^+$ reduction or of the Mehler reaction, $DCIPH_2$ sulfonate is not an electron donor in broken chloroplasts and only weakly active in subchloroplast vesicles.

$DCIPH_2$ sulfonate should be able to react with accessible plastocyanin. Therefore it can be assumed that the endogenous plastocyanin is on the inside of the thylakoid membrane, as has been deduced already from other types of experiments[24,36]. This endogenous plastocyanin can react only with the lipid-soluble $DCIPH_2$, but not with $DCIPH_2$ sulfonate.

Photoreductions at the expense of $DCIPH_2$ are coupled to ATP formation and show a marked stimulation by the uncoupler NH_4Cl[37,38]. Furthermore oxidized DCIP is an uncoupler[25,26]. DCIP sulfonate shows neither of these effects. This points out again the requirement of lipid solubility for compounds involved in, or effecting energy conservation.

The difference between the two electron donors for Photosystem I, DAD and TMPD, described already in 1964 (refs 9 and 11) may now also be explained. Both phenylenediamines are lipid soluble and therefore can penetrate the membrane, thus being electron donors. In addition, DAD, a primary diamine may carry hydrogens across the membrane during the reduction–oxidation cycle contrary to TMPD, which cannot do this because this tertiary diamine only forms a radical instead of a chinoid diimine upon oxidation. Since photoreductions with DAD are coupled to ATP formation, whereas with TMPD they are not[9,11,12,38], one indeed arrives at the concluding postulate that artificial mediators of photophosphorylations by Photosystem I have to be lipophilic proton-carrying redox compounds which create an artificial site (loop) of photophosphorylation.

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