LIPOPHILICITY AND CATALYSIS OF PHOTOPHOSPHORYLATION I
Sulfonated phenazonium compounds are ineffective in mediating cyclic photophosphorylation in photosystem-I-subchloroplast vesicles

G. HAUSKA
Lehrstuhl für Biochemie der Pflanzen, Ruhr-Universität Bochum, Germany

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1. Introduction

N-methyl-phenazonium methosulfate (PMS)* and its 1-oxy-derivative, pyocyanine, are the most efficient mediators of cyclic photophosphorylation [1, 2], provided a proper redox balance is maintained in the chloroplast preparation [3, 4].

The mechanism of coupling phosphorylation to membrane electron transport is still under debate, but evidence is accumulating that a transmembrane electron flow, as postulated by the chemiosmotic hypothesis [5], is indeed existing in membranes of chloroplasts [6] and mitochondria [7]. This is thought to be responsible for the observed transmembrane proton flux. In view of a chemiosmotic coupling mechanism it has been suggested** that in artificial cyclic photophosphorylation the mediator, like PMS, itself acts as the transmembrane electron and proton carrier. Such an action would depend on the permeability of the oxidized and reduced forms of PMS, i.e. on the lipophilicity of the compounds.

This communication shows that sulfonated analogs of PMS and pyocyanine are not able to catalyze cyclic photophosphorylation in photosystem-I-subchloroplast vesicles, despite the very similar chemical properties to those of their parent compounds. The poor solubility of the sulfonates in organic solvents suggests that they are not able to penetrate the lipophilic regions of the chloroplast membrane.

* *Abbreviations*:
PMS, *N*-methyl-phenazonium methosulfate; MPS, *N*-methyl-phenazonium-3-sulfonate; pyocyanine-S, pyocyanine-3-sulfonate; Tricine, Tris (hydroxymethyl) methylglycine; DCMU, dichlorophenyl-1,1-dimethylurea.

** H.T. Witt personal communication.

2. Results and discussion

Fig. 1 shows the structure of the four phenazonium compounds investigated. PMS is a cation with delocalized charge. On reduction it becomes electroneutral. Pyocyanine is depicted in its zwitter ionic form. The charges can combine by mesomerism, which is responsible for the low pK of this phenol and its solubility in organic solvents. The fully reduced form is an amino-phenol with a pK around 9 (cf. [8], p. 418) and should also be predominantly uncharged at physiological pH. The SO_3^--group in MPS will exert strong hydrophilicity in both the oxidized and reduced form of the compound. Illumination of MPS under aerobic conditions results in formation of a blue compound, similar to the reaction of PMS [2] which yields pyocyanine. We, therefore, conclude that the product formed is pyocyanine-3-sulfonate (fig. 1). The reaction of illuminated MPS with oxygen is about three times faster than that of PMS.

The spectra of the sulfonates resemble closely those of PMS and pyocyanine, the SO_3^--group exerting a bathochromic effect. The main absorbance peaks of MPS are found at 392 and 266 nm compared to 388 and 260 nm for PMS; for pyocyanine-S they are at 710, 322 and 245 nm instead of 690, 312 and 240 nm for pyocyanine [9, 10].

As expected the midpoint potential of MPS is slightly higher than that of PMS (130 and 80 mV, cf.

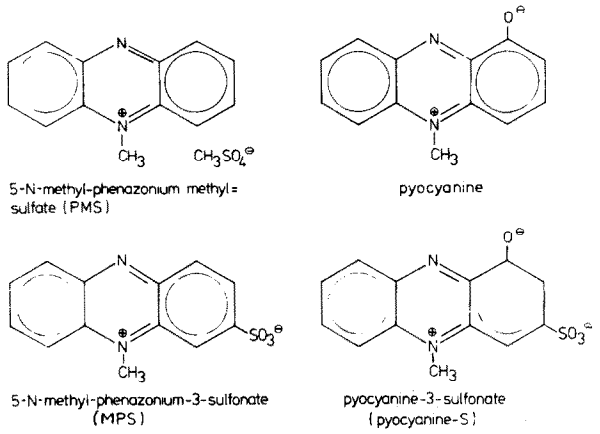

Fig. 1. Structure formulae of the phenazines investigated.

[9], p. 419). Both can be fully reduced by excess ascorbate at pH 8.0 under anaerobic conditions [10]. The reduced forms are both auto-oxidizable, as shown in fig. 2, but the reduced MPS reacts slower. The reason could be sought in the electronegative effect of the sulfonate group lowering the susceptibility of the aromatic ring for an electrophilic attack of oxygen. A more feasible explanation might be that in the case of PMS even in the presence of excess ascorbate under the conditions in the oxygraph cell there is always a minute amount of phenazyl radical present [10] which rapidly reacts with oxygen. MPS might be more completely reduced so that less phenazyl is present.

Anaerobic illumination of MPS and PMS [9] under argon at pH 8.0 results in a photoreduction with water as the electron donor similar to the photoreduction of FMN [11]. Again the reaction of MPS is faster than that of PMS like the reaction to pyocyanine under air.

The redox potentials of the pyocyanines are too low for reduction by ascorbate. Reduction observed as decolorization can be achieved with dithionate or borohydride under anaerobic conditions.

Table 1 shows the partition coefficients for the oxidized and reduced phenazine compounds between water at pH 8.0 and organic solvents. It can be seen that although oxidized PMS distributes in favor of the water phase it is much more lipophilic than oxidized MPS. Reduced PMS is insoluble in aqueous media [10] and accordingly very easily extracted into organic solvents. In contrast reduced MPS remains in

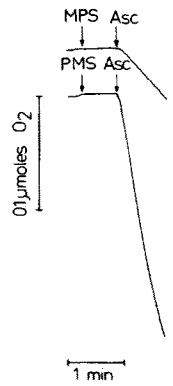

Fig. 2. Reaction of PMS and MPS with oxygen after reduction with ascorbate. The reaction was measured in 20 mM Tris-HCl, pH 8.0, with an oxygen electrode using a Gilson oxygraph. PMS and MPS were added to a final conc. of 5×10^{-5} M and ascorbate to a concentration of 1 mM. Oxygen uptake with ascorbate alone could be neglected.

Table 1

Partition coefficients for the phenazine derivatives in various water/organic solvent systems.

Compound	P_{H_2O}–organic solvent			
	Benzene	Chloroform	n-Octanol	n-Butanol
PMS	13.3	4.5	6.5	3.6
MPS	>100	>100	9.3	6.5
PMS_{red}	<0.02	<0.02	<0.02	<0.02
MPS_{red}	>100	>100	4.5	0.54
Pyocyanine	—	<0.05	0.70	—
Pyocyanine-S	—	>100	6.2	—

Three ml of an aqueous solution of the phenazines (3×10^{-4} M for PMS and MPS, and 10^{-4} M for pyocyanine and pyocyanine-S), buffered at pH 8.0 with 20 mM Tris-HCl, were mixed with an equal volume of organic solvent for 30 sec in a test tube on a Vortex shaker. The absorbancies (at 388 nm for PMS, 392 nm for MPS, 690 nm for pyocyanine and 710 nm for pyocyanine-S) of the aqueous phases were recorded before and after extraction in a Cary Model 15 spectrophotometer. In the cases of reduced PMS and MPS the extraction was carried out under argon in Thunberg tubes after addition of excess ascorbate. After removal of the organic phase the aqueous layer was aerated to reoxidize the phenazines. Complete reoxidation was achieved by addition of a drop of 3% H_2O_2. As a reference in these cases served the absorbance of the aqueous PMS- or MPS solution after reduction and reoxidation without extraction. The partition coefficients are expressed as the ratio of the remaining absorbance over the lost absorbance after extraction.

Table 2

Comparison of PMS and pyocyanine with their sulfonated analogs in cyclic photophosphorylation of photosystem-I-subchloroplast vesicles.

Compound	Phosphorylation
	μmoles P_i esterified per mg chlorophyll and hr
PMS	350
PMS + ascorbate	455
MPS	< 10
MPS + ascorbate	< 10
Pyocyanine	< 10
Pyocyanine + BH_4^-	280
Pyocyanine-S	< 10
Pyocyanine-S +BH_4^-	< 10

The assay for photophosphorylation was carried out as described by McCarty and Racker [14]. The reaction mixture contained in 1 ml 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, subchloroplast vesicles corresponding to 10 μg chlorophyll, 5×10^{-5} M phenazine compound and where indicated 3 mM ascorbate or a few crystals of borohydride. The samples were illuminated in a water bath at room temp. for 2 min with saturating white light. PMS and MPS were tested under air, pyocyanine and pyocyanine-S under argon [4].

the water phase. Similarly pyocyanine is easily extracted into organic solvents while pyocyanine-S is not.

To summarize, the redox properties of the phenazonium compounds and their sulfonated derivatives are very similar, while the lipophilicity is highly decreased for the sulfonates, especially for the reduced form of MPS compared to PMS. It is, therefore, justified to compare the ability of these compounds to mediate cyclic phosphorylation in view of their lipophilicity (cf. [12] for a detailed approach of this kind to predict biological effects of substances).

Table 2 shows that sulfonated phenazines are inefficient in catalyzing cyclic photophosphorylation in contrast to their parent compounds. Photosystem-I-subchloroplast vesicles [13] prepared by the action of digitonin were used because they exhibit high rates of cyclic phosphorylation [4] but have lost non-cyclic photosystem-II-linked phosphorylation which could

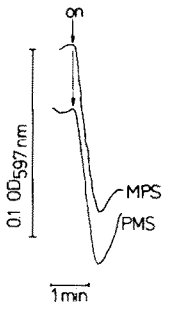

Fig. 3. Reduction of oxidized plastocyanin by PMS or MPS during illumination with white light. The reaction was measured by the decrease of absorbance at 597 nm. A Ziess spectrophotometer Model PMQ2 modified for illumination from the side was employed. PMS and MPS were 5×10^{-5} M.

interfere. Using white light PMS is active in the presence or absence of ascorbate, as shown previously [4], while MPS is inactive in either case. Pyocyanine has to be reduced with borohydride before phosphorylation can be observed in these vesicles [4]. Pyocyanine-S remains inactive even after reduction with borohydride.

In fig. 3. it is seen that reduced PMS and MPS are able to reduce plastocyanin in a rapid reaction. After initial decrease of absorbance at 597 nm a rise of absorbance is seen from the traces in fig. 3. This is explained by the assumption that after complete reduction of plastocyanin pyocyanine formation starts. Reduction of mammalian cytochrome *c* can also be observed with either compound. Previous results suggest that plastocyanin in the chloroplast is located inside the thylakoids [6] as a primary electron donor for P_{700} in photosystem-I while the reducing end of photosystem-I is located on the outer surface [15]. It is feasible to suggest that in cyclic electron flow PMS and pyocyanine are reduced at the outer surface and oxidized inside the vesicles possibly by plastocyanin. This redox reaction would constitute an artificial proton translocating loop according to the chemiosmotic hypothesis of energy transduction [5], if the radical form of the reduced phenazines could be neglected. Alternatively one could state in a more general way that phenazines have to donate their electrons to an acceptor in a hydrophobic environment which stabilizes the energy rich precursors of ATP resulting from the redox reaction.

The high efficiency of PMS-dependent cyclic phosphorylation can be explained by the high lipophilicity of reduced PMS which impregnates the hydrophobic parts of the chloroplast membrane shortening diffusion distances.

It should be mentioned that MPS and pyocyanine-S are able to catalyze a photosystem-II-linked noncyclic phosphorylation in broken chloroplasts like FMN [16] (Hauska et al., in preparation). This type of reaction is highly sensitive to DCMU and dibromothymoquinone [17] and does not seem to rest upon the lipophilicity of the artificial redox system, although some interesting influences of lipophilicity have been described [18] for various Hill-reactions.

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