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CYCLIC PHOTOPHOSPHORYLATION REACTIONS CATALYZED BY FERREDOXIN, METHYL VIOLOGEN AND ANTHRAQUINONE SULFONATE

USE OF PHOTOCHEMICAL REACTIONS TO OPTIMIZE REDOX POISING

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Summary

The flavin analogue 5-deazariboflavin is a convenient catalyst for the photoreduction of low-potential redox compounds. In an anaerobic medium with Tricine buffer as the electron donor, 5-deazariboflavin is capable of photoreducing both ferredoxin and methyl viologen. We have used this method to conduct a comparative study of the Photosystem I photophosphorylation activities supported by the reduced forms of ferredoxin, methyl viologen and anthraquinone sulfonate. All of these catalysts are capable of generating high rates (200—500 μmol ATP/h per mg chlorophyll) of cyclic photophosphorylation, but only the activity dependent on ferredoxin exhibits sensitivity to antimycin A. This finding suggests that the size of the catalyst and its ability to approach the thylakoid membrane, rather than low-redox potential, governs antimycin A sensitivity. Ferredoxin-catalyzed activity is, however, less sensitive to inhibition by dibromothymoquinone than are the activities supported by methyl viologen and anthraquinone sulfonate. This discrepancy is due to binding of the inhibitor by ferredoxin.

Introduction

Lipophilic compounds such as diaminodurene and phenazine methosulfate will generate high rates of PS I cyclic photophosphorylation, in broken chloro-

Abbreviations: Chl, chlorophyll; DBMIB, 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethylurea; PS, photosystem; Tricine, *N*-tris(hydroxymethyl)methylglycine.

plasts, that are insensitive to inhibition by either DBMIB or antimycin A [1]. Ferredoxin and sulfonated quinones, on the other hand, produce phosphorylation reactions which generate lower rates of ATP synthesis, but these reactions are sensitive to DBMIB [1,2] and, in the case of ferredoxin, to antimycin A [3]. Assay of ferredoxin- or quinone-dependent cyclic reactions requires anaerobiosis to prevent autooxidation of the reduced mediator. Initiation of cyclic electron transport activity in these reaction systems depends on the presence of reduced mediator at the onset of illumination. Reduction of low-potential cyclic mediators can be accomplished in anaerobic systems by utilizing non-cyclic electron transport catalyzed by illuminated chloroplasts [1,4]; although PS I cyclic photophosphorylation activity is elicited by this method, it has the disadvantage of exposing the chloroplasts to prolonged illumination times, and accurate redox poising of the reaction mixture cannot be achieved since an oxidant, O_2, is produced.

The use of chloroplast non-cyclic electron transport for mediator reduction may be avoided if alternate methods for mediator reduction are employed. For example, anthraquinone sulfonate can be photoreduced anaerobically using Tricine as the electron donor [5]; this photoreduction procedure serves to produce high rates of anthraquinone sulfonate-catalyzed PS I cyclic photophosphorylation activity. The utility of this method suggested to us that it might be applied to other cyclic reactions catalyzed by compounds such as ferredoxin or methyl viologen if a suitable photochemical reductant could be found. Such a reductant has been derived from studies by Massey and Hemmerich [6], who showed that the flavin analogue 5-deazariboflavin could be used to photoreduce a number of low-potential redox proteins if a suitable electron donor such as EDTA was provided.

In this communication we describe the use of 5-deazariboflavin to photoreduce ferredoxin and methyl viologen in PS I cyclic assay systems, and present the results of experiments showing that ferredoxin can elicit high rates of PS I cyclic electron transport and photophosphorylation. Our data from comparative studies with anthraquinone sulfonate and methyl viologen indicate that only ferredoxin-catalyzed reactions are sensitive to inhibition by antimycin A.

Materials and Methods

Chloroplasts were prepared from market spinach by homogenizing 100 g of depetiolated leaves with a Waring blender in a medium (250 ml) containing 0.4 M NaCl, 20 mM Tricine (pH 8.0), 2 mM $MgCl_2$ and 0.2% bovine serum albumin. The homogenate was filtered through four layers of cheesecloth, debris was removed by centrifugation at $300 \times g$ for 1 min, and the broken chloroplasts were then pelleted by centrifugation at $4000 \times g$ for 10 min. These and all subsequent operations were carried out at 0—4° C. The chloroplast pellets were resuspended in a medium (150 ml) containing 0.15 M NaCl, 20 mM Tricine (pH 8.0), 5 mM $MgCl_2$ and 0.2% bovine serum albumin, and were then centrifuged at $4000 \times g$ for 10 min. The washed pellets were resuspended in a small volume of a medium containing 0.4 M sucrose, 15 mM NaCl, 20 mM Tricine (pH 8.0) and 0.2% bovine serum albumin. The chlorophyll concentration was adjusted to 2—3 mg/ml by dilution with the same medium

and the suspension was distributed into glass vials (0.5 ml aliquots) and frozen at −70° C prior to assay [7]. Ferredoxin was prepared as described by Petering and Palmer [8]. Fractions possessing A_{420}/A_{275} ratios greater than 0.48 were pooled, dialyzed against 20 mM Tricine (pH 8.0), and stored frozen at −70° C in 0.5 ml aliquots.

Assays of Photosystem I cyclic photophosphorylation activity were carried out in the reaction mixture previously described [5], under anaerobic conditions imposed by an oxygen-trapping system comprised of 6.7 mM glucose, 30 μg glucose oxidase and ethanol (0.3%, v/v); 3 μM DCMU was present in all assays. The anaerobic reaction mixture minus chloroplasts was first illuminated with white light ($3 \cdot 10^6$ ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}) to generate photoreduced mediator. 10 μl chloroplasts were added to the cuvette in the dark, and after a 1 min incubation period in the dark, the complete system was illuminated for 1 min with red light (10^6 ergs $\cdot$ cm$^{-2} \cdot$ s^{-1}) to catalyze cyclic photophosphorylation activity. Reaction mixtures were acidified with trichloroacetic acid, and ATP was determined by the method of Avron [9] after extraction of unreacted phosphate.

The concentrations of photoreduced mediators in reaction mixtures were determined using the method of Avron and Shavit [10] for measuring ferrocyanide concentration. Mediators were photoreduced for varying periods of time in the anaerobic reaction medium, chloroplasts were added, and an anaerobic solution (10 μl) of 20 mM ferricyanide was then added. For anthraquinone sulfonate and methyl viologen, the concentration of ferrocyanide (generated by oxidation of reduced mediator) was determined. Since acidification of ferredoxin generates H_2S, an interfering reductant, the extent of ferredoxin photoreduction by 5-deazariboflavin was determined by omitting the addition of trichloroacetic acid. Samples were centrifuged to remove chloroplasts, and 1 ml of the supernatant was transferred to the assay system to detect ferrocyanide.

Chlorophyll was determined by the method of Arnon [11]. Glucose oxidase was obtained from Worthington Biochemical Corp.; anthraquinone sulfonate was obtained from Eastman Organics and recrystallized. Antimycin A, ADP, bovine serum albumin, catalase, phenazine methosulfate and Tricine were obtained from Sigma. 5-Deazariboflavin was provided by Professor Vincent Massey, F.R.S., DBMIB was a gift from N.E. Good. All other chemicals used in these studies were of the purest grades commercially available.

Results

5-Deazariboflavin photochemistry with Tricine as the electron donor

Massey and Hemmerich [6] utilized EDTA as an electron donor for 5-deazariboflavin photoreduction reactions. Tricine can also serve to donate electrons to flavins in photochemical reactions [12]. Since we have shown [5] that Tricine can be used for anthraquinone sulfonate photoreduction in photophosphorylation reactions, we sought to determine whether Tricine could replace EDTA in our reaction system as the electron donor to 5-deazariboflavin. Table I presents the results of an experiment in which anaerobic solutions of either methyl viologen or ferredoxin plus Tricine and 5-deazaribo-

TABLE I

PHOTOREDUCTION OF FERREDOXIN AND METHYL VIOLOGEN BY 5-DEAZARIBOFLAVIN WITH TRICINE AS THE ELECTRON DONOR

The reaction mixtures contained glucose, glucose oxidase, catalase and ethanol at the concentrations described in Materials and Methods. The concentrations of Tricine, ferredoxin and methyl viologen were 50 μM, 66 μM and 100 μM, respectively. The concentration of 5-deazariboflavin was 10 μM in the ferredoxin experiment and 0.5 μM in the methyl viologen experiment. The light intensity was 400 000 ergs · $cm^{-2} \cdot s^{-1}$.

White light illumination time (s)	Ferredoxin reduction (ΔA_{463})	Methyl viologen reduction (ΔA_{396})
0	0	0
20	−0.04	+0.026
40	−0.09	+0.052
60	−0.13	+0.077
80	−0.17	+0.101

flavin were subjected to illumination for varying times with white light. As shown in the table, ferredoxin and methyl viologen are both photoreduced under these conditions, indicating that Tricine is an adequate substitute for EDTA as an electron donor to 5-deazariboflavin. In addition, the data in Table I show that the photoreduction of both methyl viologen and ferredoxin proceed in a linear fashion for up to 80 s, indicating that a direct proportionality exists between illumination time and the resulting concentration of photoreduced product.

Properties of PS I cyclic photophosphorylation reactions catalyzed by photoreduced mediators

In order to study PS I cyclic reactions catalyzed by photoreduced mediators, we first sought to determine the optimum photoreduction time with 5-deazariboflavin plus either 66 μM ferredoxin or methyl viologen. Fig. 1 shows the results of a typical experiment; photoreductive illumination with white light for about 30 s is sufficient to produce high rates of photophosphorylation with either ferredoxin or methyl viologen. We noted in these experiments that methyl viologen-catalyzed activity showed an apparent lag (Fig. 1); 8—10 s of preillumination appeared not to produce any reductant to initiate cyclic activity. Since methyl viologen is highly autooxidizable, we considered the possibility that some of the photoreduced dye was reoxidized by O_2 present in the added chloroplast suspension. This hypothesis was tested by a series of experiments in which the rate of ATP synthesis was determined as a function of the amount of reduced mediator present in the reaction mixture after chloroplast addition. The results of these experiments are presented in Fig. 2. When activity is plotted against the concentration of reduced, rather than total, mediator present, small amounts of reduced ferredoxin and anthraquinone sulfonate are relatively ineffective in producing ATP synthesis. However, as little as 5 nmol (3.3 μM) of reduced methyl viologen is sufficient to saturate the cyclic reaction; ferredoxin and anthraquinone sulfonate require 15 and 40 nmol, respectively.

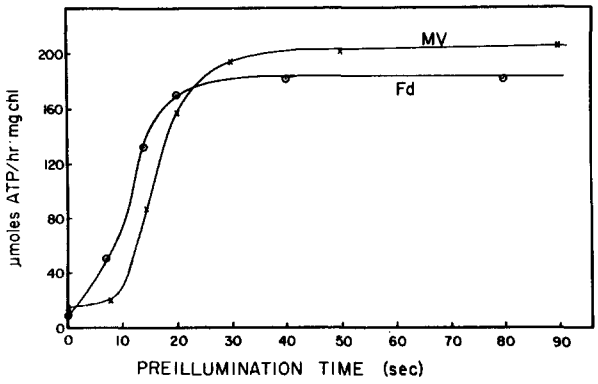

Fig. 1. Time course for poising of PS I cyclic photophosphorylation mediated by ferredoxin or methyl viologen. Conditions are given in Materials and Methods with the exception that the NaCl concentration in the experiment with ferredoxin (Fd) was 100 mM. The total concentrations of ferredoxin and methyl viologen (MV) were 66 µM; the 5-deazariboflavin concentrations were 5 µM (ferredoxin) and 0.6 µM (methyl viologen). Chloroplasts equivalent to 23 µg Chl were present.

It was apparent from these results that 5-deazariboflavin-photoreduced ferredoxin and methyl viologen, as well as anthraquinone sulfonate (photoreduced directly in the presence of Tricine), are all effective mediators of PS I cyclic activity. We next sought to determine what differences existed between the activities catalyzed by these mediators. Fig. 3 shows the results of experiments where the intensity of the red actinic light used to catalyze cyclic electron transport was varied; phenazine methosulfate-catalyzed activity is included for purposes of comparison. In the region from 0—50 000 ergs · $cm^{-2} \cdot s^{-1}$, ferredoxin, methyl viologen and anthraquinone sulfonate activities

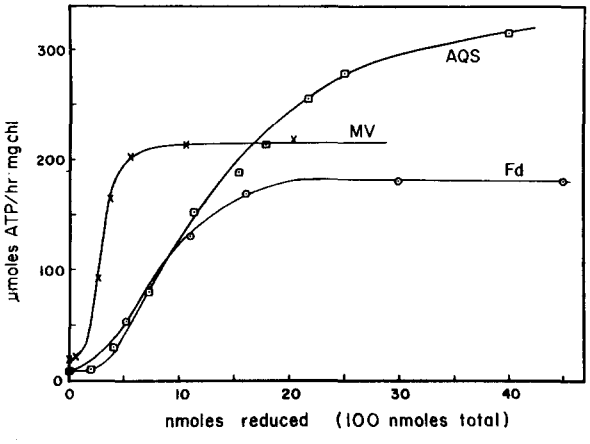

Fig. 2. Rate of PS I cyclic photophosphorylation plotted as the concentration of reduced mediator present during assay. Conditions as in Fig. 1. The anthraquinone sulfonate (AQS) concentration was 66 µM.

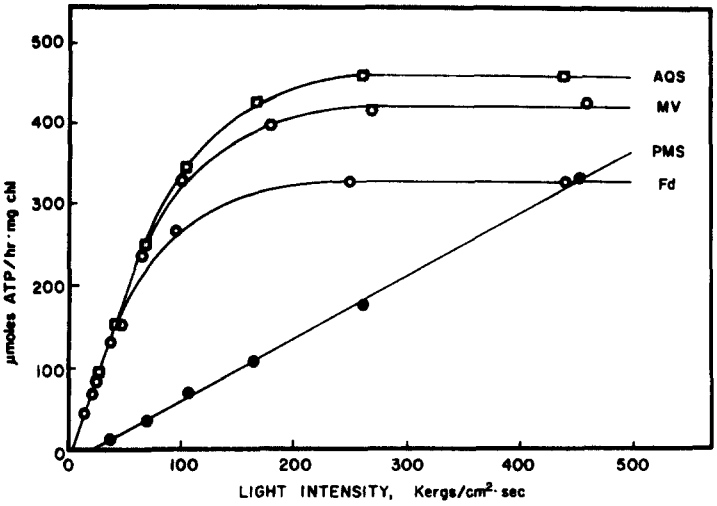

Fig. 3. Light intensity saturation curves for PS I cyclic photophosphorylation activity mediated by ferredoxin (Fd), methyl viologen (MV), anthraquinone sulfonate (AQS) and phenazine methosulfate (PMS). Conditions are given in Materials and Methods. Low-potential mediators were all present at a concentration of 100 μM; PMS was present at a concentration of 66 μM. The 5-deazariboflavin concentrations were 6.7 μM (ferredoxin) and 0.6 μM (methyl viologen). For the ferredoxin reactions, the NaCl concentration was raised to 100 mM, and for the PMS reaction system the O_2-trapping system was omitted and a 20 s preillumination with white light was given. Chloroplasts equivalent to 24 μg Chl were present in all assays.

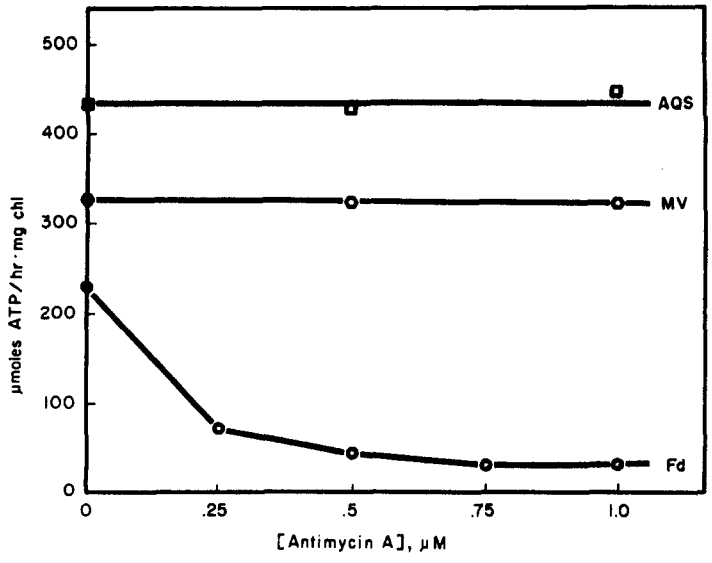

Fig. 4. Effect of antimycin A on PS I cyclic photophosphorylation reactions. Conditions are as described in Materials and Methods, except that in the ferredoxin (Fd) reactions the NaCl concentration was 100 mM and a dark incubation period of 5 min was inserted between addition of chloroplasts (24 μg Chl) and the red light illumination step. The 5-deazariboflavin concentrations were 3.3 μM (ferredoxin) and 0.6 μM (methyl viologen (MV)); the concentrations of anthraquinone sulfonate (AQS) and methyl viologen were 100 μM, the concentration of ferredoxin was 50 μM.

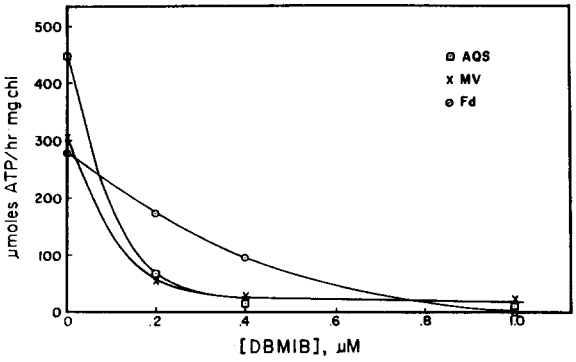

Fig. 5. Effect of DBMIB on PS I cyclic photophosphorylation reactions. Conditions are given in the legend to Fig. 4, with the exception that a 1 min dark incubation period preceded illumination with red light, and the concentration of ferredoxin (Fd) was 100 μM.

are all limited by light intensity and all three mediators show identical relative quantum efficiences; above 50 000 ergs · cm^{-2} · s^{-1} differences appear. Ferredoxin-catalyzed activity approaches saturation at lower intensities than do the reactions supported by anthraquinone sulfonate and methyl viologen. Phenazine methosulfate, on the other hand, is light limited at all intensities used in these experiments, and shows a greatly reduced relative quantum efficiency.

We next determined the effects of antimycin A and DBMIB on the photosynthetic phosphorylation activities supported by ferredoxin, methyl viologen and anthraquinone sulfonate. Fig. 4 shows that only ferredoxin-dependent activity is decreased by antimycin A; neither the methyl viologen nor the anthraquinone sulfonate-dependent reactions show any sensitivity to antimycin A. Results obtained with DBMIB are shown in Fig. 5. Although the activities catalyzed by methyl viologen and ferredoxin are sensitive to DBMIB, ferredoxin-supported activity appears to be least sensitive, a finding also reported by Binder and Selman [13].

TABLE II

EFFECT OF FERREDOXIN CONCENTRATION ON DBMIB INHIBITION OF CYCLIC PHOTOPHOSPHORYLATION

Conditions were as described in Materials and Methods, except that 0.6% (v/v) ethanol and 100 mM NaCl were present in the reaction mixture. The 5-deazariboflavin concentrations used were 3.3 μM (50 μM ferredoxin (Fd)) and 13 μM (100 μM ferredoxin). Chloroplasts equivalent to 24 μg Chl were present in all assays. The control rates (0% inhibition) were 260 and 280 μmol ATP synthesized/h per mg Chl for 50 and 100 μM ferredoxin, respectively.

DBMIB (μM)	Inhibition (%) of ATP synthesis catalyzed by	
	50 μM Fd	100 μM Fd
0	0	0
0.2	48	39
0.4	77	61
1.0	100	90

Berg and Izawa [14] and Guikema and Yocum [15] have shown that DBMIB inhibition can be prevented, or attenuated, by bovine serum albumin, which binds DBMIB (Siedow, J.N., personal communication). If DBMIB were binding to ferredoxin, as it does to bovine serum albumin, then the result in Fig. 5 should depend on the ferredoxin concentration in the assay system. This is in fact true, as shown in Table II, where DBMIB titrations were carried out at two ferredoxin concentrations. Note that DBMIB produces a stronger inhibition at the lower ferredoxin concentration, which indicates that DBMIB binding to ferredoxin is responsible for the result shown in Fig. 5.

Discussion

The use of photochemical techniques for the redox poising of low-potential mediators of PS I cyclic photophosphorylation reactions offers two distinct advantages over other methods. First, the extent of catalyst reduction may be accurately controlled by illumination time, and second, the chloroplasts used for assay are exposed to a single illumination step, that which is necessary for photophosphorylation to occur. The efficacy of the photochemical poising method is evidenced by the rates of ATP synthesis obtained with ferredoxin, methyl viologen, and anthraquinone sulfonate. These rates, ranging from 200 to 300 μmol ATP/h per mg Chl for ferredoxin, to 500 μmol ATP/h per mg Chl with anthraquinone sulfonate, are substantially higher than those previously reported [1,2,4,13], and this observation suggests that the exceptional difficulties associated with redox poising by chloroplast non-cyclic electron transport are alleviated by the photochemical poising method. If we assume that site I energy transduction occurs with an efficiency (P/e_2) of 0.65 [16], and that this is the only functional coupling site in our assay system, then PS I cyclic electron transport can be computed to occur in our reactions at rates of from 600–1500 μequiv./h per mg Chl, depending on the catalyst present.

Our data comparing the effects of DBMIB and antimycin A on the ATP synthesis supported by low-potential catalysts clearly show that a DBMIB-sensitive component, perhaps plastoquinone itself, is a common intermediate of the electron transport pathways utilized by ferredoxin, methyl viologen, and anthraquinone sulfonate to support photophosphorylation. We have confirmed the observation [13] that ferredoxin-catalyzed ATP synthesis is less sensitive to DBMIB; our exploration of this phenomenon (Table II) indicates that attenuation of inhibitory potency is due to binding of DBMIB by ferredoxin. However, concentrations of antimycin A which strongly inhibit ferredoxin-catalyzed photophosphorylation are ineffective in blocking activity catalyzed by either methyl viologen or anthraquinone sulfonate. This finding demonstrates that catalyst redox potential is not a factor in conferral of antimycin A sensitivity on photophosphorylation activity, since ferredoxin and methyl viologen possess equivalent redox potentials. This observation in turn suggests that ferredoxin, perhaps by virtue of its size, is restricted to an electron donation site which is antimycin A sensitive, a site which is not required for methyl viologen to donate electrons to the interphotosystem electron transport chain. A number of investigators [3,4,13] have proposed that cytochrome b_6 is involved in antimycin A-sensitive reactions catalyzed by ferre-

doxin. If this is so, then b_6 must be located near the exterior surface of the thylakoid membrane, rather than on the interior surface, as proposed by Cramer and Whitmarsh [17].

The sites to which methyl viologen and anthraquinone sulfonate donate electrons in our assay system cannot be identified from our data. Although it is unlikely that ferredoxin crosses the thylakoid membrane to donate electrons, we cannot be certain that anthraquinone sulfonate and methyl viologen, by virtue of their molecular weights, do not penetrate the membrane to some extent. The DBMIB sensitivity of the activities supported by these catalysts argues that they do not participate in a proton-electron shuttle such as has been described for phenazine methosulfate [2], the presence of charges on both mediators suggests that membrane permeation cannot be extensive and the relative quantum efficiences of phenazine methosulfate, anthraquinone sulfonate, and methyl viologen (Fig. 3) presents further evidence against extensive membrane permeation by the latter two mediators. It is therefore likely that these compounds must donate their electrons at or near the surface of the thylakoid membrane, to a component in close proximity to plastoquinone.

Other investigators [16,18] have used durohydroquinone to elicit DBMIB-sensitive non-cyclic electron transport through site I. The concentrations (greater than 200 μM) of this donor which are required for optimal activity are in substantial excess of those required for maximum rates of cyclic photophosphorylation (Fig. 2). Ferredoxin and methyl viologen are one-, rather than two-electron donors, and it is therefore possible that donation to the interphotosystem chain by added mediators occurs preferentially by a one-electron step. We have tested this assumption using the data of Ilan et al. [19] and Burstein and Davidson [20] to calculate concentrations of the semiquinone species of durohydroquinone and anthrahydroquinone sulfonate. These computations suggest that the semiquinone species of both quinones would be present under our conditions of assay in a range of concentrations similar to those of reduced methyl viologen and ferredoxin. This observation suggests, but by no means proves, that one-electron reductants are the species which serve as donors to the interphotosystem chain.

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