Ferredoxin Catalyzed Cyclic Photophosphorylation: Reversal of Dibromothymoquinone Inhibition by N, N, N′, N′-Tetramethyl-*p*-phenylenediamine

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Conditions for washed, spinach thylakoid membranes to catalyze cyclic phosphorylation using ferredoxin as the cofactor for electron transfer have been re-examined. It was found necessary to "redox poise" the system; however, the best method to accomplish the poising seemed to be the use of a reductant (glucose-6-phosphate) and not by optimizing the rate of phosphorylation with 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU). Under these conditions, ferredoxin catalyzed cyclic phosphorylation was found to be sensitive to the inhibitors antimycin A and dibromothymoquinone (DBMIB). The inhibition of ferredoxin catalyzed cyclic phosphorylation by DBMIB, but not by antimycin A, was completely reversed by N, N, N′, N′-tetramethyl-*p*-phenylenediamine (TMPD). These data are taken as further support for the function of plastoquinone in ferredoxin catalyzed cyclic phosphorylation. The effect of TMPD in reversing the DBMIB inhibition is interpreted as the formation of a TMPD bypass on the internal side of the thylakoid membrane around the DBMIB site of inhibition.

Introduction

The selective use of electron transfer inhibitors, electron donors, and electron acceptors has facilitated the study of reactions catalyzed by the various segments of the photosynthetic electron transfer chain [1]. Dibromothymoquinone (DBMIB), a plastoquinone antagonist [2], has proven to be an extremely useful tool in the elucidation of those segments coupled to phosphorylation [3]. There now appear to be two "sites" of energy conservation in higher plant chloroplasts; one "site" associated with the release of protons from water into the internal space of the thylakoid membrane and a second "site" associated with proton translocation coupled to vectorially directed reduction-oxidation reactions of plastoquinone [4].

Low concentrations of DBMIB have been shown by Böhme and Cramer [5] to completely block the reoxidation of plastohydroquinone in non-cyclic electron transfer and hence completely block coupled phosphorylation. Trebst and Reimer [3] and Selman [6] have demonstrated that under these conditions, DBMIB inhibition of both electron transfer and coupled phosphorylation can be reversed by the addition of catalytic amounts of substituted phenylenediamines, the most effective being N,N,N′,N′-tetramethyl-*p*-phenylenediamine (TMPD). The TMPD reversal of DBMIB inhibition has been interpreted as indicating that TMPD generates a bypass of electron flow around the DBMIB inhibition site on the internal side of the thylakoid membrane, thus preserving the native "site" of energy conservation [3, 6].

Photosystem I driven cyclic phosphorylation can be catalyzed by a large number of artificial cofactors, some of which are sensitive to inhibition to DBMIB (*e. g.* menadione) and some of which are relatively insensitive to DBMIB (*e. g.* diaminodurene and phenazine methosulfate) [2]. Arnon and co-workers [7, 8] were the first to demonstrate that the *in vivo* oxidant for photosystem I, ferredoxin, can also serve as a cyclic cofactor with naked thylakoid lamellae, albeit at very high concentrations and under anaerobic conditions. Subsequently, Böhme *et al.* [2] showed that this reaction is sensitive to inhibition by DBMIB, implicating a role for plastoquinone as the proton carrier coupling cyclic electron transfer to phosphorylation.

In this paper, we have re-examined the conditions needed to clearly demonstrate ferredoxin catalyzed cyclic photophosphorylation and have investigated

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Abbreviations: TMPD, N,N,N′,N′-tetramethyl-*p*-phenylenediamine; DCMU, 3-(3,4-dichlorophenyl)-1,1-dimethyl urea; DBMIB, dibromothymoquinone.

the influence of two inhibitors, antimycin A and DBMIB, on this reaction. In addition, we also show that DBMIB inhibition, but not antimycin A inhibition, of ferredoxin catalyzed cyclic phosphorylation can be reversed by catalytic amounts of TMPD.

Methods and Materials

Naked thylakoids were prepared from spinach as previously described [9] and resuspended in buffer containing 20 mM tricine-KOH (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, and 0.3 M sucrose. Chlorophyll concentration was determined as described by Arnon [10]. Reaction mixtures contained in 1.0 ml: 20 mM tricine-KOH (pH 8.0), 10 mM KCl, 5 mM $MgCl_2$, 3.0 mM ADP, 3.0 mM [^{32}P]phosphate (containing approximately 3×10^6 CPM), 0.1 mM $NADP^+$, 3.0 mM glucose-6-phosphate, 0.1 units of glucose-6-phosphate dehydrogenase (Sigma Chemical Co.), and ferredoxin as indicated in the legends to the figures. 3-(3,4-dichlorophenyl)-1,1-dimethyl urea (DCMU), DBMIB, and antimycin A were dissolved in methanol and added to reaction mixtures as methanolic solutions. The concentration of methanol never exceeded 2% of the reaction mixture volume. Reaction mixtures were added to 9.0 ml serum vials, stoppered, and depleted of oxygen by swirling during alternating cycles of gas evacuation followed by gassing with nitrogen over an 8.0 min period (4 cycles). Chloroplasts were degassed separately and added to reaction mixtures just prior to illumination at a concentration equivalent to 10 μg chlorophyll per ml. Reaction vials were placed on top of a 5 cm glass heat sink containing a 1% $CuSO_4$ solution and were illuminated from below by three 150 W incadescent floodlamps. The light intensity at the surface of the vials was approximately 3200 ft-candles. After illumination for 5.0 min, reactions were stopped by the addition of 0.2 ml of 1.0 N $HClO_4$. ATP formation was measured as previously described [11].

Spinach ferredoxin was prepared essentially as described by Keresztes-Nagy and Margoliash [12] as modified by Petering and Palmer [13]. In addition, after the second DEAE column the preparation was rechromatographed on a Sephadex G-50 column (2.5 cm $\times$ 100 cm) equilibrated with buffer containing 0.05 M Tris-Cl (pH 8.0) and 0.5 M NaCl. Fractions containing ferredoxin were pooled and concentrated to 2 mg protein per ml by ultrafiltration.

Results

DCMU is an extremely potent inhibitor of non-cyclic electron transfer with complete inhibition of either the ferricyanide or $NADP^+$ Hill reaction occurring at approximately 1.0 μM. The effect of DCMU, however, on photosystem I catalyzed cyclic phosphorylation depends on the cofactor. If properly poised (*i. e.* at an optimal ratio of reduced to oxidized mediator), cyclic phosphorylation catalyzed by either diaminodurene (DAD) or phenazine methosulfate (PMS) is relatively insensitive to inhibition at high concentrations of DCMU [14]. On the other hand, cyclic phosphorylation catalyzed by ferredoxin is extremely sensitive to DCMU. Tagawa *et al.* [7] have demonstrated that in white light low concentrations of DCMU stimulate ferredoxin catalyzed cyclic phosphorylation, whereas higher concentrations of DCMU inhibit. This presumably reflects the need to establish the proper ratio of reduced to oxidized ferredoxin in the reaction mixture. A similar dependence on the concentration of DCMU for maximal rates of cyclic phosphorylation in intact chloroplasts has also been observed [15, 16]. Because the concentration of DCMU needed for optimal rates of ferredoxin catalyzed cyclic phosphorylation in white light is generally not sufficient to completely inhibit non-cyclic electron transfer under the same conditions, the results obtained are often difficult to interpret.

Fig. 1 shows the results of a DCMU titration for phosphorylation supported either by ferredoxin or

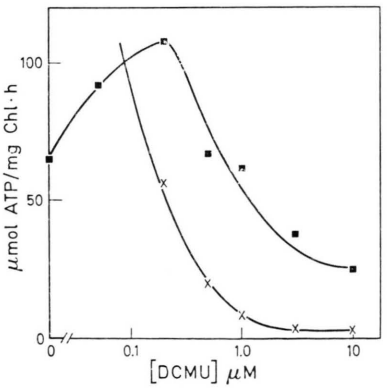

Fig. 1. DCMU inhibition of ferricyanide and ferredoxin supported phosphorylation. Conditions as described in Methods. The concentration of ferredoxin (■) was 0.3 mg protein/ml and ferricyanide (×) 1.0 mM. Ferricyanide rates that were off the scale of the graph were 262 and 190 μmol ATP per mg chlorophyll per h at 0 and 0.05 μM DCMU, respectively.

ferricyanide under identical conditions. A marked two fold stimulation of phosphorylation is observed with ferredoxin at 0.2 μM DCMU. This is in general agreement with the existing data in the literature [15, 16]. However, under these same conditions, ferricyanide reduction supports about 50% of the rate of phosphorylation obtained with ferredoxin. Clearly then, the total phosphorylation observed with ferredoxin at 0.2 μM DCMU cannot be attributed to a purely cyclic reaction, but rather phosphorylation resulting from a mixture of both cyclic and non-cyclic electron transfer. The difference in rates between ferredoxin and ferricyanide supported phosphorylation remains about constant at DCMU concentrations above 0.2 μM. Whereas the rate of phosphorylation supported by ferricyanide reduction approaches zero at 1.0 μM DCMU, the rate of ferredoxin catalyzed phosphorylation remains constant between 1 and 10 μM DCMU. It is only in this concentration range that we believe that the reaction catalyzed by ferredoxin can be considered to be due to cyclic electron transfer. Thus, all succeeding experiments have been performed in the presence of 3.0 μM DCMU to completely block photosystem II and a combination of glucose-6-phosphate, $NADP^+$, and glucose-6-phosphate dehydrogenase to reduce ferredoxin.

Böhme *et al.* [2] were the first to demonstrate that the ferredoxin catalyzed cyclic reaction can be inhibited by DBMIB. Fig. 2 shows that 1.0 μM DBMIB is sufficient to inhibit ferredoxin supported phosphorylation about 80 – 90% or to the level obtained in the absence of exogenously added ferredoxin (compare data at 0 μM TMPD). Increasing the DBMIB concentration above 1.0 μM does not increase this level of inhibition (data not shown). Addition of TMPD to these chloroplasts results in a complete reversal of the DBMIB inhibition. The TMPD reversal of the DBMIB inhibition saturates at about 40 μM or at about the same concentration of TMPD required to reverse the DBMIB inhibition of non-cyclic electron transfer [6]. Addition of TMPD to chloroplasts in the absence of DBMIB has little effect on the rate of ferredoxin supported phosphorylation. Fig. 2 also demonstrates that ferredoxin is required for the TMPD reversal of the DBMIB inhibition. If ferredoxin is omitted from the reaction mixture, TMPD has no effect on the rate of phosphorylation up to 40 μM although it does seem to stimulate the rate at higher concentrations.

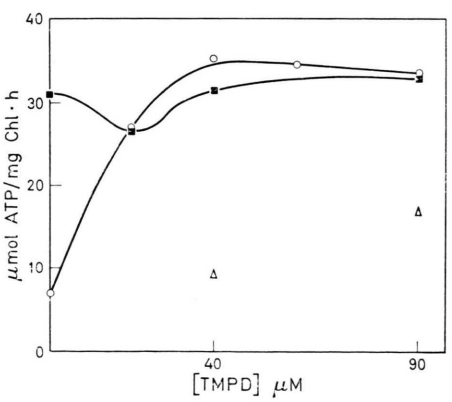

Fig. 2. TMPD reversal of DBMIB inhibition. Conditions as described in Methods. (■) without DBMIB, 0.3 mg ferredoxin/ml; (○) 1.0 μM DBMIB, 0.3 mg ferredoxin/ml; (△) 1.0 μM DBMIB without added ferredoxin. All measurements were made in the presence of 3.0 μM DCMU.

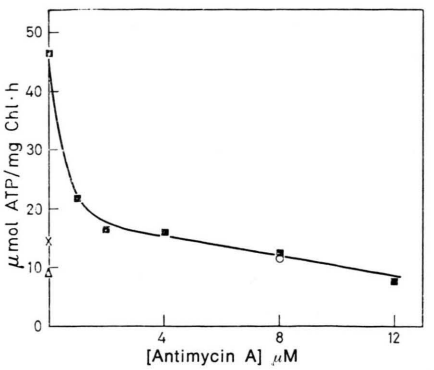

Fig. 3. Antimycin A inhibition of ferredoxin catalyzed cyclic phosphorylation. Conditions as described in Methods. (■) 0.4 mg ferredoxin/ml; (×) 0.4 mg/ml ferredoxin without glucose-6-phosphate dehydrogenase; (△) without ferredoxin; (○) 0.4 mg ferredoxin/ml plus 40 μM TMPD. All measurements were made in the presence of 3.0 μM DCMU.

Fig. 3 shows the sensitivity of ferredoxin supported phosphorylation to the inhibitor antimycin A. The inhibition curve appears to be biphasic with respect to the concentration of antimycin A. A rapid drop of about 80% of the rate of phosphorylation occurs between 0 and 2 μM antimycin A followed by a slower loss of activity which extends out to about 8 to 12 μM and results in complete inhibition of phosphorylation. At no point along this curve is TMPD capable of reversing the inhibition of antimycin A (data shown only for 8 μM antimycin A). (Note also the point which shows the dependence of ferredoxin supported phosphorylation on the addition of glucose-6-phosphate plus glucose-6-phosphate dehydrogenase.)

Discussion

The ability of ferredoxin to participate as a co-factor for cyclic phosphorylation driven by photosystem I was first demonstrated by Arnon and co-workers [7, 8]. Although the conditions generally used to measure this reaction are quite stringent; anaerobiosis, a "catalytic" amount of DCMU, and relatively high concentrations of ferredoxin, Arnon and Chain [17] recently reported that the system can function in air at much lower ferredoxin concentrations than we have used. Indeed, other studies using intact chloroplasts and algae have shown that both have the capacity to drive a photosystem I catalyzed cyclic reaction [14 – 16]. Most of these systems have two features in common, namely the stimulation of ferredoxin supported phosphorylation by DCMU and the inhibition of this phosphorylation by relatively low concentrations of antimycin A.

The function of DCMU appears to be one of "redox poising" as Hauska *et al.* [18] have shown to be necessary for other mediators of cyclic phosphorylation. We also find a stimulatory effect of low concentrations of DCMU on the rate of ferredoxin dependent phosphorylation in agreement with previously reported results [15, 16]; however, upon comparison of this rate with the rate of phosphorylation supported by ferricyanide under the same conditions, it is apparent that the ferredoxin supported rate cannot be attributed completely to cyclic electron transfer (0.3 to 0.4 mg ferredoxin per ml is sufficient to support a Hill reaction from water to ferredoxin at approximately 40 μ equivalents per mg chlorophyll per hr for five minutes). Thus, we have found it necessary to add more DCMU in order to completely block photosystem II. Under these conditions, ferredoxin is incapable of catalyzing cyclic phosphorylation unless the reaction mixture is supplemented with a reductant (see Fig. 3).

Tagawa *et al.* [7], Huber and Edwards [16], and Kaiser and Urbach [15] have all reported that cyclic phosphorylation can be completely inhibited by low concentrations of antimycin A. Taking this as criteria for cyclic phosphorylation and the observation that our system is also completely sensitive to low concentrations of this inhibitor, we would conclude that, under our conditions, ferredoxin is indeed catalyzing a cyclic reaction coupled to phosphorylation.

In agreement with the initial findings of Böhme *et al.* [2] and other [16, 17], we also find that ferredoxin catalyzed phosphorylation can be completely inhibited by DBMIB. The concentration of DBMIB required for complete inhibition, however, is about 1 μM or three times more than that needed to completely block non-cyclic phosphorylation [2, 6]. The reason for this discrepancy is not immediately apparent and deserves further investigation.

As is the case with non-cyclic phosphorylation [3], a catalytic amount of TMPD completely reverses the inhibition by DBMIB. TMPD is, however, incapable of reversing antimycin A inhibition. Fig. 4 shows a model for what we believe might be the

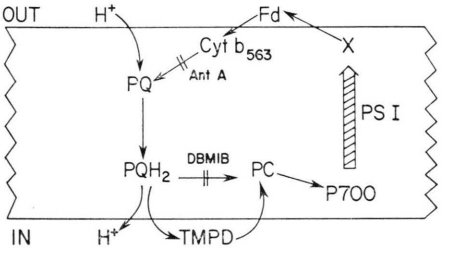

Fig. 4. Model for electron transfer in ferredoxin catalyzed phosphorylation. Abbreviations: Fd, ferredoxin; Cyt b_{563}, cytochrome b_{563}; PQ, plastoquinone; PQH_2, plastohydroquinone; PC, plastocyanin; PS I, photosystem I; X, electron acceptor for photosystem I; Ant A, antimycin A.

pathway of cyclic electron transfer under our conditions. We believe that TMPD bypasses the DBMIB block of electron transfer on the internal side of the thylakoid membrane, bridging electron transfer from plastohydroquinone to photosystem I. The protons that have been brought across the membrane by plastohydroquinone are then deposited inside the thylakoid membrane where they are used to develop a transmembrane pH gradient which provides the driving force for phosphorylation [4]. This model must be considered as tentative because, as yet, there is only indirect evidence supporting an interaction of ferredoxin with cytochrome b_{563} [19].

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