ELECTRON ACCEPTANCE AT PHOTOSYSTEM II IN UNCOUPLED SPINACH THYLAKOIDS

Resolution of two sites of electron acceptance prior to the DBMIB block with melittin, a new peptide inhibitor

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1. Introduction

Our understanding of photosystem II electron transport has been greatly facilitated through the use of specific photosystem I inhibitors and the use of class III oxidants [1]. However, all class III oxidants do not accept electrons at the same place near photosystem II. The sensitivity of the 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB)-mediated photosystem II reaction toward 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) was dependent on light intensity [2] while quinonediimines accepted electrons more directly from a photosystem II carrier, prior to the plastoquinone pool [2]. Based on steady-state kinetic analyses of photosystem II activities catalyzed by lipophilic electron acceptors, quinones and quinonediimines were concluded to accept electrons from two different sites on the reducing side of photosystem II.

These conclusions are important because some properties of photosystem II electron transport depend on the type of lipophilic electron acceptor which is employed. For instance, photosystem II electron transport to various quinonediimines is inhibited by uncouplers [4–7], while the electron transport to the quinones is not [5,7,8]. An additional complexity indicated by kinetic studies [3] is the apparent presence of two photoreduction reactions in the quinonediimine-catalyzed reaction. Here we introduce a new, linear peptide inhibitor (melittin) which selectively inhibits between the sites of quinone and quinonediimine reduction.

Melittin is a lytic peptide which contains 26 amino acids in a linear array [9]. It is isolated from the venom of the honeybee, *Apis mellifera*, which contains a complex mixture of peptides, enzymes and other substances [10]. The amino acid sequence is known [9] and it is characterized by having 6 highly hydrophilic residues (2 Lys, 2 Arg, 2 Gln) at the C-terminus. This peptide is water soluble, but it interacts spontaneously with biological membranes, increasing the bilayer permeability to ions or molecules [11].

2. Materials and methods

Spinach thylakoids were prepared by grinding fresh market spinach (*Spinacea oleracea*) in a Waring Blender as in [12], except that the final resuspension media contained: 200 mM sucrose; 20 mM *N*-[tris-(hydroxymethyl)methyl]glycine; Tricine/NaOH (pH 7.5); 3 mM $MgCl_2$; 50 mM KCl and 2 mg/ml bovine serum albumin. These suspensions were adjusted to a final chlorophyll (chl) concentration [13] of 0.5 mg chl/ml, and were frozen at −70°C for later use. Upon thawing, the thylakoids displayed rates of electron transport equivalent to the fresh preparations. KCN/Hg inhibited thylakoids were prepared as in [7]. These inhibited thylakoids were washed once in the media described above and were frozen at −70°C.

The electron transport reactions were measured as either O_2 production or as methylviologen mediated O_2 uptake using a Clark-type oxygen electrode. The white light intensity just outside the water-jacketed electrode cell (1.7 ml) was 400 kErgs . s^{-1} . cm^{-2}. All electron transport reactions reported herein were uncoupled with 10 mM methylamine.

Tricine; phenylenediamine (PD); bovine serum albumin; 2,6-dichlorophenolindophenol (DCIP); melittin and methylviologen were obtained from Sigma. 2,6-Dichloro-*p*-benzoquinone (DCBQ); 2,5-dimethyl-*p*-benzoquinone (DMQ); *N,N,N',N'*-tetramethyl-*p*-phenylenediamine dihydrochloride (TMPD) were obtained from Eastman. Diaminodurene (DAD) was obtained from Research Organics/Inorganics. DAD, PD, and TMPD were recrystallized prior to use [14]. DMQ and DCBQ were dissolved in 50% ethanolic ethylene glycol. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) was obtained from Pfaltz and Buaer (Stanford, CT) and recrystallized prior to use. 2,5-Dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) was a generous gift from Professor A. Trebst, Bochum.

3. Results

Fig.1 shows that the small, water-soluble peptide melittin is capable of interacting with the thylakoid membrane in such a way that electron transport between water and methylviologen is completely inhibited at ~25 μM. However, electron transport from water to ferricyanide does not appear to be severely inhibited at the concentrations tested. Since there have been reports of ferricyanide reduction by photosystem II [15], we were prompted to see if melittin potentiated this reduction. Thus fig.1 also shows that the presence of 1 μM 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB) almost completely inhibits the water to ferricyanide reaction in the absence of added melittin. As the melittin concentration increases, the rate of ferricyanide mediated O_2 production also increases until reaching apparent saturation at ~25 μM melittin. The methylviologen reaction is similarly inhibited by DBMIB, but added melittin does not relieve the observed inhibition.

The data in fig.1 can be best interpreted by assuming that melittin is capable of inhibiting sequential electron transport but that there is a parallel increase in the ability of ferricyanide to accept electrons at photosystem II. Therefore, we examined the reduction of various other photosystem II acceptors in the presence of melittin.

Fig.2 shows the effects of melittin on two lipophilic acceptors, oxidized phenylenediamine (PD^{ox}) and dichlorobenzoquinone (DCBQ). The DCBQ-mediated reaction is insensitive to melittin. The

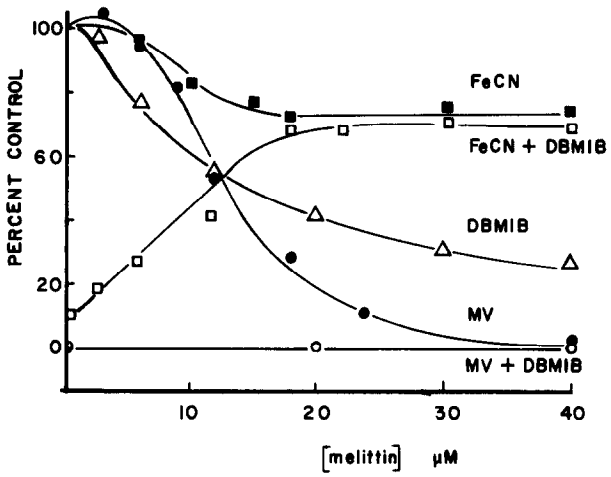

Fig.1. Melittin inhibition of uncoupled photosystem I and photosystem II electron transport. The photosystem I electron acceptors used are potassium ferricyanide (FeCN), methylviologen (MV) and 2,5-dibromo-3-methyl-6-isopropyl-*p*-benzoquinone (DBMIB). To assay photosystem II activity DBMIB was added to 0.88 μM final conc. Electron transport was assayed as O_2 production or uptake as in section 2. Reaction mixture at 18°C contained: 100 mM sucrose; 25 mM KCl; 15 mM Hepes/NaOH (pH 7.5); 1.5 mM $MgCl_2$; 10 mM methylamine; thylakoids equivalent to 30 μg chl/ml; and where indicated, 0.4 mM $K_3Fe(CN)_6$; 0.88 μM DBMIB (as an inhibitor); 0.85 mM methylviologen; and 0.5 mM DBMIB (as an acceptor). Melittin was added as a 200 μM aqueous solution. Assays were begun immediately after the addition of melittin. Control rates of the indicated reactions were (in μequiv. . h^{-1} . mg chl^{-1}): (●–●) $H_2O \rightarrow MV$, 280; (■–■) $H_2O \rightarrow FeCN$, 380; (△–△) $H_2O \rightarrow DBMIB$, (□–□) $H_2O \rightarrow FeCN(+DBMIB)$, 53; (○–○) $H_2O \rightarrow MV(+DBMIB)$, O.

PD^{ox}-mediated reaction is at first inhibited by melittin up to 5 μM followed by a range of apparent stimulation. The range of stimulation coincides closely with the stimulation observed in the melittin stimulated water to ferricyanide reaction seen in fig.1. Since excess ferricyanide is present to maintain the PD in the oxidized state it is likely that the observed stimulation is due to melittin potentiated ferricyanide reduction.

Fig.3 shows the effects of melittin on a number of other photosystem II reactions. The various acceptors seem to exhibit one of two basic types of behavior. The quinonediimine (PD^{ox} and DAD^{ox}) mediated reactions show initial sensitivity to melittin which is maximal at ~5 μM. The quinones (DMQ and DCBQ) are insensitive to melittin in the indicated concentration range. This insensitivity is apparent whether or not ferricyanide is present to keep the quinones in

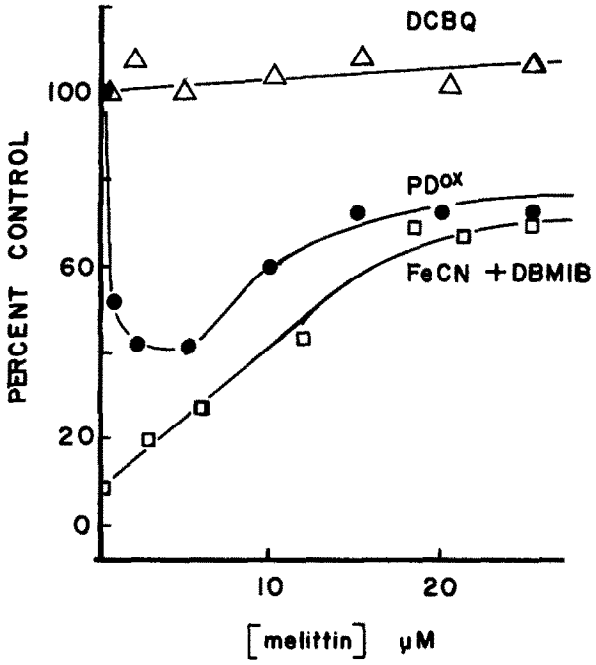

Fig.2. Melittin inhibitor of photosystem II electron transport to class III acceptors. The acceptors used were phenylenediimine (PD^{ox}) and 2,6-dichlorobenzoquinone (DCBQ) and ferricyanide (FeCN). Reactions all contained 0.88 μM DBMIB and were like those in fig.1 except: DCBQ was present where indicated (△–△) at 0.5 mM; PD^{ox} was present where indicated (●–●) at 0.5 mM and kept oxidized with 1.8 mM FeCN; FeCN was present where indicated (□–□) at 0.4 mM. Thylakoids were inhibited as in section 2 with KCN/Hg prior to use to inhibit sequential electron transport through both photosystems. Thylakoids were present equivalent to 30 μg chl/ml. The control rates were (in μequiv. . h^{-1} . mg chl^{-1}): DCBQ, 390; PD^{ox}, 780; FeCN + DBMIB, 53.

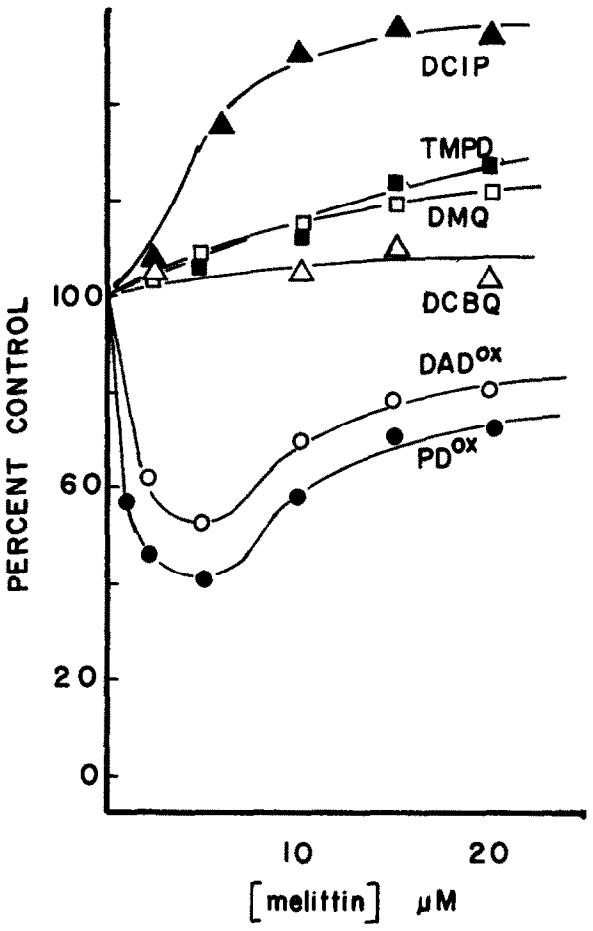

Fig.3. Melittin inhibition of photosystem II electron transport. Thylakoids were inhibited with KCN/Hg prior to use. Reactions were like those in fig.2. The assays catalyzed by phenylenediimine (PD^{ox}), diiminodurene (DAD^{ox}), and *N,N,N',N'*-tetramethylphenylenediimine ($TMPD^{ox}$) all contained acceptors at 0.5 mM and 1.8 mM ferricyanide (FeCN) to keep the acceptors oxidized. No FeCN was present with the DCIP, DMQ or DCBQ, although the presence of FeCN made no substantial difference in the rates or the shape of the curves. The control rates were, (in μequiv. . h^{-1} . mg chl^{-1}): (▲–▲) DCIP, 270; (■–■) $TMPD^{ox}$, 490; (□–□) DMQ, 390; (△–△) DCBQ, 390; (○–○) DAD^{ox}, 430; (●–●) PD^{ox}, 480.

the oxidized state. The 2,6-dichlorophenolindophenol (DCIP) catalyzed reaction is also insensitive. Strangely, oxidized *N,N,N',N'*-tetramethylphenylenediamine ($TMPD^{ox}$) mediates an insensitive reaction in spite of its structural similarity to DAD^{ox} and PD^{ox}.

Finally, it can be seen in fig.4 that all the photosystem II reactions we have dealt with here, either in the presence or the absence of melittin are sensitive to inhibition by 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) in the same way.

4. Discussion

We have exposed thylakoid membranes to melittin and we report here striking effects on photosystem II electron-transport reactions. The main significance of this inhibitor is that it allows one to distinguish between the electron transport mediated by quinones and quinonediimines. Thus a new site of inhibition may be described which further clarifies electron transport to class III acceptors. Since the inhibition of the quinonediimine reduction occurs at concentrations far lower than those needed to inhibit sequen-

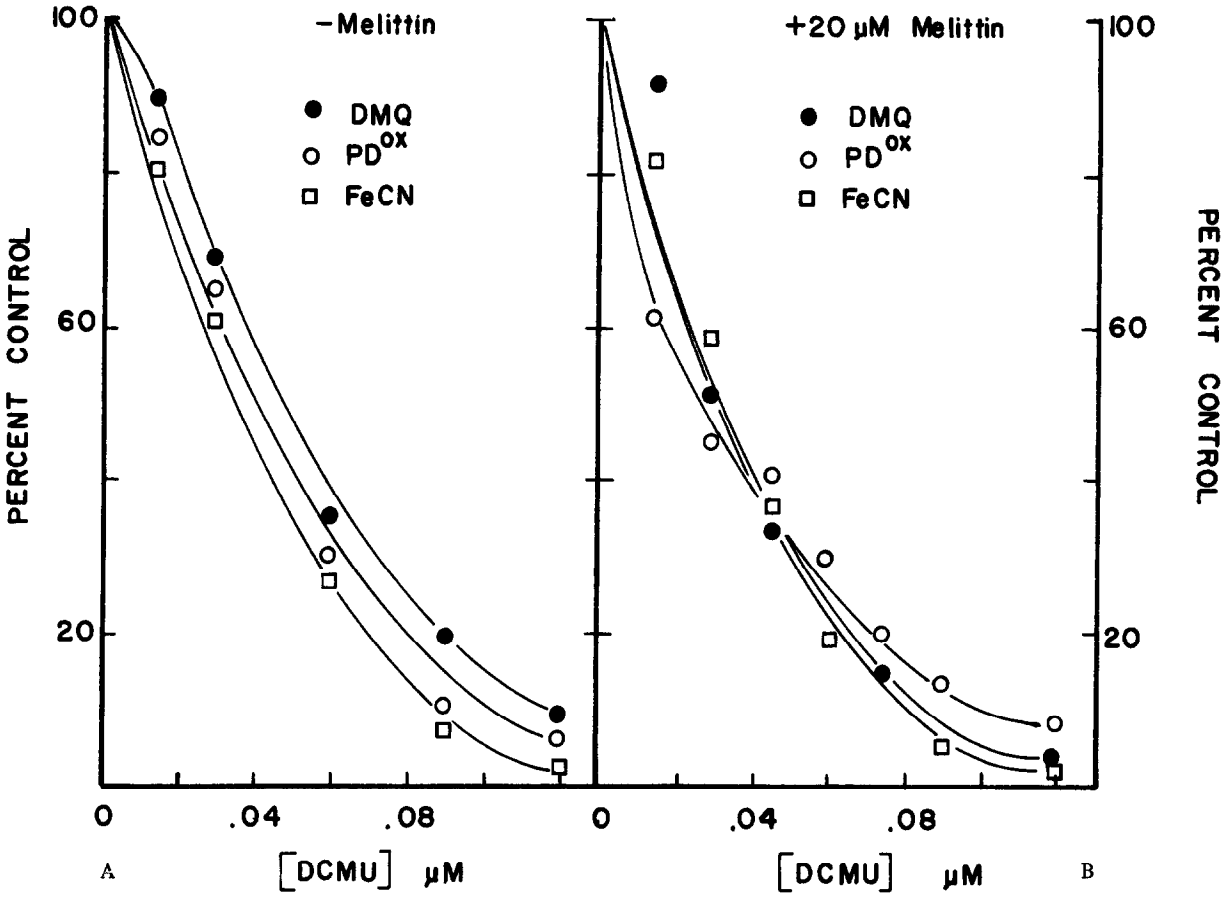

Fig.4. DCMU inhibition of photosystem II in the presence of melittin. Reactions were like those in fig.3 except that control thylakoids were used and were equivalent to 30 μg chl/ml. 3-(3,4-Dichlorophenyl)-1,1-dimethylurea (DCMU) was added to the indicated concentrations. (B) contained 20 μM melittin. All reactions are fully inhibited by DCMU at the same concentrations required for the control reactions.

tial electron transport through both photosystems to methylviologen, we must conclude that the inhibition occurs very close to the actual site of quinonediimine reduction, without interfering with electron transport either past the block or to the insensitive acceptors such as the quinones, ferricyanide and DCIP. The simplest interpretation of our findings as well as those in [2–8] is that there must be some electron carrier(s) which is capable of reducing the class III acceptors at two sites. Melittin interferes with reduction at one site (the quinonediimine site) but not the other. This new site of inhibition is schematically portrayed in fig.5.

At higher melittin concentrations, the water to DBMIB reaction is also inhibited. The light intensity experiments in [2] clearly show that DBMIB accepts electrons from the plastoquinone pool and that the quinonediimines accept electrons closer to photosystem II, before those electrons are pooled. It was suggested by these workers that the quinonediimines react directly with photosystem II units. Thus melittin could inhibit by interacting with these same photosystem II units in such a way that quinones (and other acceptors) could still be reduced while the quinonediimines could not. Presumably, the transfer of electrons to the plastoquinone pool is not interrupted at low concentrations of melittin but at higher concentrations the reduction of DBMIB is inhibited. Since the melittin concentration needed to observe this inhibition is about that needed for the inhibition of sequen-

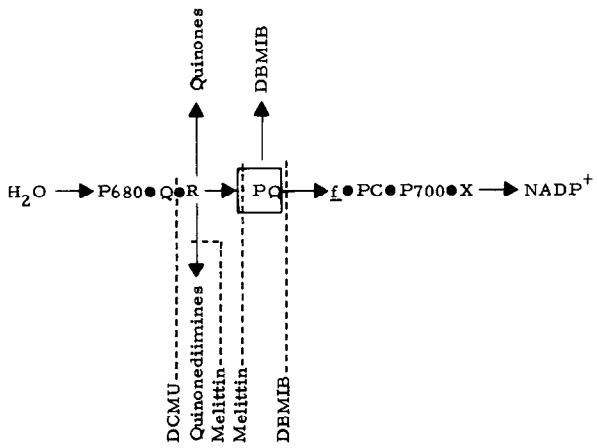

Fig.5. Sites of inhibition by melittin. Scheme indicates that melittin inhibits the quinonediimine reduction without inhibiting electron transport between O_2 and PQ. Melittin also inhibits the $H_2O \rightarrow$ DBMIB reaction without inhibiting the reduction of quinones. This may be the site at which melittin inhibits sequential electron transport between the photosystems. Arrows indicate electron transport, broken lines indicate inhibition. Components separated by (•) are thought to occur as part of a photosystem unit. P680, photosystem II photoact; Q, primary acceptor of photosystem II; R, postulated carrier(s) which reduces quinones and quinonediimines; PQ, plastoquinone pool; *f*, cytochrome *f*; PC, plastocyanin; P700, photosystem I photoact; X, primary acceptor of photosystem I.

tial electron transport to methylviologen, we conclude that the sequential electron transport may be inhibited at the plastoquinone pool. We are further characterizing this reaction with fluorescence studies and studies of the intensity on the inhibition process.

There is no simple explanation for melittin selectivity. The melittin-insensitive reaction systems include hydrophilic species such as ferricyanide, as well as hydrophobic systems such as those mediated by DMQ. The melittin-sensitive photosystem II reaction systems include the more hydrophilic quinonediimines and the highly hydrophobic DBMIB. The inhibition of the quinonediimine catalyzed reactions is not just the uncoupler inhibition of these reactions in [4–7], since all the assays reported here were done in the presence of 10 mM methylamine which completely uncoupled photophosphorylation (not shown). Neither do the midpoint potentials offer any clue to selectivity. Both the melittin sensitive and the melittin insensitive acceptors offer similar, overlapping ranges of midpoint potentials [16]. With regard to selectivity, it is of interest to note that the $TMPD^{ox}$ reaction was not inhibited by melittin. Its behavior was more like that of the quinone-type acceptors. Apparently the presence of the methyl groups on the amines allows $TMPD^{ox}$ to accept electrons where PD^{ox} and DAD^{ox} cannot. It would be of interest to see if $TMPD^{ox}$ mediates a photosystem II reaction which is kinetically similar to the quinones or the quinonediimines. Such experiments are underway in our laboratory.

The mechanism of inhibition is probably related to the ability of melittin to enter the lipid bilayer. This has been described [17] process in terms of a small water soluble tetramer which can spontaneously insert its hydrophobic N-terminus into the membrane lipid, while leaving the highly polar C-terminus at the aqueous interface. In the thylakoid, the hydrophobic portion of the peptide must enter the bilayer to interfere with smooth electron transfer between some electron carrier and the oxidized quinonediimine or DBMIB. Interference with this carrier does not inhibit the reduction of all acceptors as evidenced by the melittin-insensitive electron transport reactions. As well as inhibiting, the melittin seems to expose the photosystem II site of ferricyanide reduction. However, it is not possible at this time to conclude that the inhibiting interference is directly related to the exposure of the ferricyanide site. Thus ferricyanide may be as likely to accept electrons before or after the site of quinone reduction as it is to be reduced at the same site with the quinones.

We are in the process of determining the location and orientation of melittin in the thylakoid membrane and phospholipid vesicles. We expect that it will lie within the outermost 10–15 Å of the lipid bilayer with its hydrophilic portion exposed to solvent. If this is the case, then the inhibition described here can be located in this same region. Thus the inhibitor melittin may prove useful, not only as a unique inhibitor of electron transport, but also as a probe for locating some specific sites within the membrane.

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