THE EFFECT OF VALINOMYCIN ON ELECTRON TRANSPORT IN INTACT SPINACH CHLOROPLASTS

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1. Introduction

Illumination of isolated, intact (type A [1]) chloroplasts, the leaves of higher plants, or algal cells results in a pronounced decrease in chlorophyll fluorescence yield, occurring over the course of several minutes [2–4]. This slow fluorescence quenching has been ascribed to efflux of Mg^{2+} from the thylakoids in response to light-induced H^+-uptake [3,5,6]. The proposed sequence of events culminating in quenching can be summarized as:

$$\begin{array}{l} e^-\text{transport} \rightarrow H^+\text{-uptake into} \rightarrow \\ \qquad\qquad\qquad \text{the thylakoids} \\ \\ Mg^{2+}\text{-efflux from} \rightarrow \text{Quenching} \\ \text{the thylakoids} \end{array} \qquad (1)$$

Thus, in intact chloroplasts, slow fluorescence quenching can be inhibited or reversed by:

(1) DCMU, under conditions that eliminate photosynthetic electron transport [3,5];
(2) Uncouplers, which dissipate the H^+ gradient across the thylakoid membrane [3,5];
(3) Ionophore A23187, which facilitates Mg^{2+} return to the thylakoid in exchange for H^+ [6].

Slow fluorescence quenching under aerobic conditions appears to reflect the simultaneous occurrence of cyclic and pseudocyclic electron flow. It has been shown [7] that inhibition of non-cyclic electron flow to CO_2 has little effect on slow quenching, but that diminution of electron flow to O_2 (low O_2 tensions) is severely inhibitory. Low concentrations of DCMU (0.1 μM) were reported [8] to overcome this inhibition, suggesting that cyclic electron flow can drive slow quenching. Essentially complete inhibition by low concentrations of antimycin A, under both aerobic and anaerobic conditions, with or without DCMU, has been taken as evidence that cyclic electron transport is, in fact, central to the quenching process [8]. Electron flow to O_2 is presumably required simply to maintain the cycle in a suitably oxidized state [9–11] (a requirement that can be met, under anaerobic conditions, by low DCMU concentrations). Measurements of slow fluorescence quenching can thus be used to identify potential inhibitors of cyclic and pseudocyclic electron flow.

The results reported here demonstrate that low concentrations (< 0.5 μM) of the K^+-specific ionophore valinomycin [12] completely inhibit slow fluorescence quenching in intact chloroplasts. Quenching can be restored in these preparations with low concentrations of DCMU or elevated O_2 tensions, suggesting that inhibition reflects a selective interference with the normal interaction between O_2 and reduced electron transport carriers.

2. Methods

Intact (type A) chloroplasts were prepared from greenhouse spinach as in [13]. Chloroplasts were 77–90% intact as determined by the ferricyanide reduction method [14,15] and fixed CO_2 at rates of 100–200 μmol .mg chl^{-1} $.h^{-1}$ [7].

Fluorescence was measured as in [13,16] in medium consisting of 0.33 M sorbitol and 50 mM Hepes, adjusted to pH 8.0 with KOH (~30 mM K^+).

Chlorophyll concentration was 7.5 μg .ml^{-1}.

Non-cyclic electron flow, with KNO_2 (1 mM) or oxaloacetate (2.5 mM) as acceptor, was measured polarographically [17] in saturating light at 20°C. Chlorophyll concentration was 12.8 μg .ml^{-1}.

Low-salt chloroplasts were resuspended and assayed in 0.33 M sorbitol, 10 mM Hepes, adjusted to pH 7.6 with Tris base [18].

Valinomycin was obtained from Lilly Labs, Indianapolis, IN, and was prepared as a 175 μM stock solution in methanol.

3. Results and discussion

As summarized in fig.1, low concentrations of valinomycin severely inhibit slow, light-induced fluorescence quenching in intact chloroplasts. Under the experimental conditions of fig.1 (sorbitol–Hepes–KOH medium, 7.5 μg chl .ml^{-1}, no added electron acceptor), quenching was completely eliminated with $\geqslant 0.4$ μM valinomycin; 50% inhibition occurred at 0.15 μM. Similar inhibition was observed with chloroplasts resuspended and assayed in the absence of inorganic cations or in a medium [7] capable of supporting rapid CO_2 fixation (data not shown). This finding was somewhat surprising since reports [18,20] had indicated that valinomycin was unable to reverse fluorescence quenching unless both K^+ and low concentrations of uncoupler were present.

The apparent discrepancy between the results of fig.1 and the reported lack of valinomycin effect on the fluorescence yield of intact chloroplasts [18,20] appears to be the consequence of differences in the time of valinomycin addition and/or valinomycin: chlorophyll ratios. As shown in fig.2, addition of 0.44 μM valinomycin to chloroplasts in which quenching has occurred (solid trace) provokes only

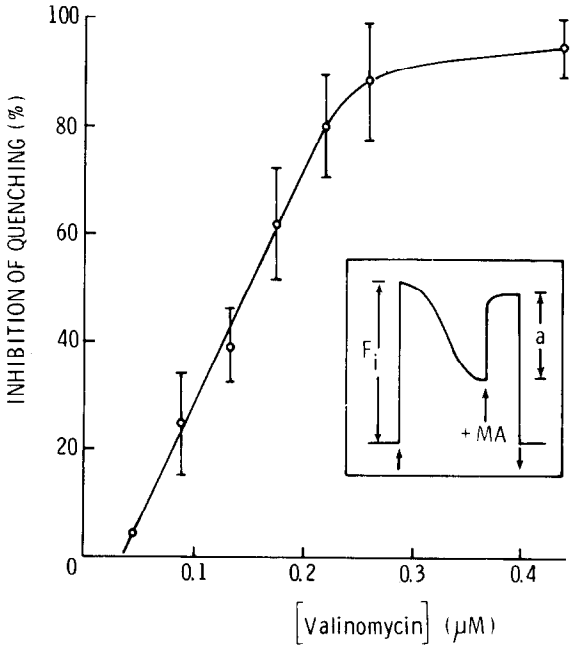

Fig.1. Inhibition of slow fluorescence quenching by low concentrations of valinomycin. Chloroplasts were illuminated for 4 min in the presence of varying valinomycin concentrations. Methylamine (MA, 30 mM) was then added to determine the amount of reversible quenching [19] that had occurred. [Reversible quenching = $(a/F_i \times 100\%)$; see inset. Actinic light on (↑) and off (↓).] The results shown are the means, with standard deviations, of three separate experiments.

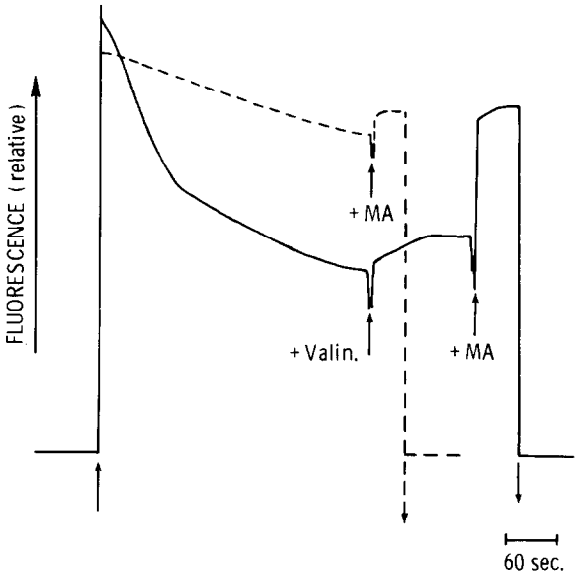

Fig.2. Dependence of fluorescence yield on the time of valinomycin addition. Valinomycin (0.44 μM) was added prior to illumination (broken trace) or after 5 min in the light (solid trace). Methylamine was added as indicated. Other details as in fig.1. The difference in F_i for the two samples most probably reflects a small difference in chlorophyll concentration. Valinomycin has no significant effect on F_i.

Table 1
Reversal of slow fluorescence quenching by valinomycin

Valinomycin (μM)	Quenching reversal elicited by valinomycin (% of total reversal)[a]
0.35	15.8
0.88	66
1.75	84

[a] The protocol was that shown in fig.2 (solid trace). Chloroplasts were illuminated for 4 min, then valinomycin was added. After the fluorescence had reached a steady state, 30 mM methylamine was added to obtain complete reversal of quenching. The reversal obtained with valinomycin is expressed as a percent of the total fluorescence increase observed. Other details as in fig.1

a small increase in fluorescence, i.e., reverses quenching poorly. In contrast, incubation of the chloroplasts with the same concentration of valinomycin for 1 min prior to illumination results in severe inhibition of quenching (broken trace). (Note that the uncoupler methylamine increases the fluorescence of both samples to the same value.) The data of table 1 demonstrate that valinomycin added during illumination can reverse fluorescence quenching. However, ~5-fold higher concentrations are required. Furthermore, the valinomycin concentration required to inhibit quenching is roughly proportional to chlorophyll concentration. Valinomycin:chlorophyll ratios in earlier investigations were significantly lower than those reported here.

Valinomycin at < 1 μM has been reported to have no effect on non-cyclic or menadione-catalyzed cyclic electron flow [21–23], on the extent of light-induced H^+-uptake [24], or on phosphorylation [21,22] in broken chloroplasts. The data in table 2 demonstrate that valinomycin (< 2 μM) is neither an uncoupler nor an inhibitor of non-cyclic electron flow in intact chloroplasts. Both NO_2^- [25] and oxaloacetate are reduced by intact chloroplasts in reactions that do not consume ATP. Valinomycin neither speeds electron flow to NO_2^- in the coupled system nor inhibits methylamine-uncoupled electron flow to NO_2^- or to oxaloacetate.

The data of table 3 show that the inhibition of quenching by valinomycin is analogous to the inhibition caused by low O_2 tensions. Slow fluorescence quenching is restored under anaerobic conditions by low concentrations of DCMU, as reported [8], and by oxaloacetate. Similarly, quenching that has been inhibited by valinomycin is restored by 0.25 μM

Table 2
Effect of valinomycin on electron flow in intact spinach chloroplasts

Additions[a]	Net O_2 evolution (μmol .h^{-1} .mg chl^{-1})
NO_2^- + DL-glyceraldehyde[b]	28.5
NO_2^- + DL-glyceraldehyde + 1 μM valinomycin	29.2
NO_2^- + DL-glyceraldehyde + 2 μM valinomycin	27.2
NO_2^- + methylamine	85.6
NO_2^- + methylamine + 0.58 μM valinomycin	83
NO_2^- + methylamine + 1.17 μM valinomycin	89.5
NO_2^- + methylamine + 1.75 μM valinomycin	96
Oxaloacetate + methylamine[b]	18.9
Oxaloacetate + methylamine + 1 μM valinomycin	25.2

[a] Chlorophyll concentration was 12.8 μg .ml^{-1}. Additions, where indicated, were made to the following final concentrations: KNO_2, 1 mM; DL-glyceraldehyde, 10 mM; methylamine, 30 mM; oxaloacetate, 2.5 mM

[b] In these experiments, the assay medium contained, in addition to sorbitol and Hepes buffer, 2 mM EDTA, 5 mM $Na_4P_2O_7$, and 0.25 mM K_2HPO_4. Valinomycin inhibition of quenching is as severe in this medium as in sorbitol–Hepes–KOH

Table 3
Effect of DCMU and oxaloacetate on valinomycin- or N_2-inhibited fluorescence quenching

Additions[a]	Atmosphere[b]	% Quenching (reversible)
None	air	42
Valinomycin	air	0
Valinomycin + DCMU	air	53.8
Valinomycin + DCMU + antimycin A	air	1.6
Valinomycin + oxaloacetate	air	21
Valinomycin + oxaloacetate + antimycin A	air	0
DL-glyceraldehyde	air	36.2
DL-glyceraldehyde	N_2	14.1
DL-glyceraldehyde + DCMU	N_2	44.5
DL-glyceraldehyde + oxaloacetate	N_2	32.3

[a] Additions, where indicated, were made to the following final concentrations: valinomycin, 0.44 μM; DCMU, 0.25 μM; oxaloacetate, 2.5 mM; antimycin A, 1 μM; DL-glyceraldehyde, 10 mM

[b] For measurements made under N_2, the medium was bubbled with N_2 for 15 min prior to chloroplast addition (O_2, ~25 μM). Other details as in fig.1

DCMU and by oxaloacetate. Note, however, that in both cases oxaloacetate is somewhat less effective that DCMU in restoring quenching.

As illustrated in fig.3, increasing valinomycin concentrations shift the DCMU-sensitivity curve for fluorescence quenching progressively to the right. Similar data have been reported for the effect of anaerobic conditions on the sensitivity of the *trans*-thylakoid pH gradient to DCMU [8]. Ongoing CO_2 fixation has been observed to shift the DCMU-sensitivity curve for fluorescence quenching to the left [11]. The shift in the DCMU-sensitivity curve produced by valinomycin implies an increasingly reduced electron transport chain. (It should be noted that at higher valinomycin concentrations, DCMU is unable to restore quenching completely. This correlates with the ability of higher valinomycin concentrations to reverse quenching (table 1) and may represent a secondary effect of the ionophore.)

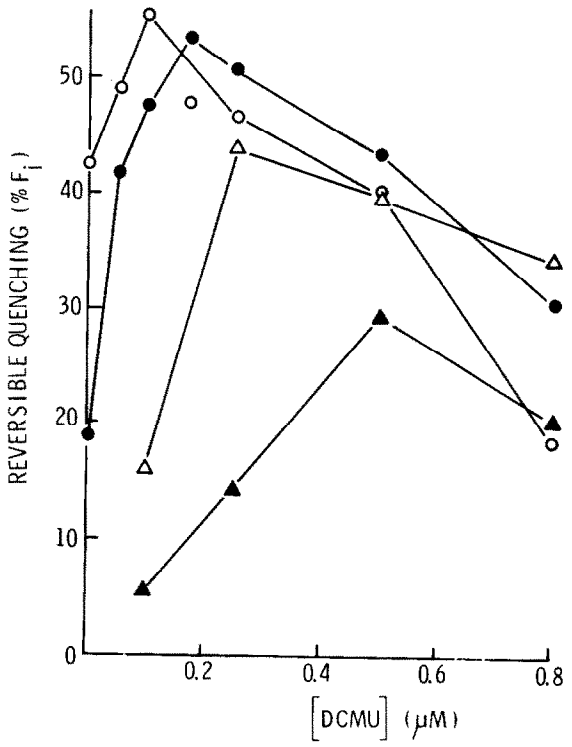

Fig.3. Effect of valinomycin on the DCMU sensitivity of slow fluorescence quenching. The extent of methylamine-reversible quenching was determined, as outlined in fig.1, as a function of DCMU concentration for the control (○) and for valinomycin at 0.44 μM (●), 0.88 μM (△), and 1.32 μM (▲).

Presumably the sole effect of low concentrations of DCMU is to reduce the rate at which electrons are supplied by photosystem II to the remainder of the electron transport chain. The restoration of quenching obtained with DCMU suggests that inhibition by valinomycin occurs at the level of electron flow. (DCMU does not overcome inhibition of quenching by A23187 or methylamine.) Furthermore, because DCMU completely restores quenching (table 3), we infer that other processes involved in the slow changes in fluorescence yield, e.g., H^+-uptake, cation movements, membrane conformational changes, are not affected by low concentrations of valinomycin. Polarographic measurements (table 2) suggest that non-cyclic electron transport from water to NADP is also unaffected by valinomycin under these conditions.

A straightforward interpretation of our data is that inhibition of quenching by valinomycin reflects:

(1) Inhibition of electron flow to O_2, occurring at the level of O_2 interaction with or access to the electron transport chain;

(2) subsequent over-reduction of chloroplast electron carriers and consequent inhibition of cyclic electron flow.

Accordingly, DCMU and oxaloacetate restore electron flow in the cycle, and thus quenching, by decreasing electron input and increasing electron withdrawal, respectively. (The cyclic nature of the restored electron flow is confirmed by the antimycin A sensitivity [26] of the quenching (table 3).)

An effect of valinomycin on electron flow to O_2 is supported by experiments in which the influence of O_2 tension on slow fluorescence quenching was determined. As shown in fig.4, increased O_2-concentrations overcome inhibition of quenching by valinomycin, as well as speeding quenching in the control samples. Valinomycin increases the O_2 concentration required for 50% of maximal quenching from $< 100\ \mu M$ to $\sim 800\ \mu M$. The inhibitory effect of valinomycin can thus be understood as an increase in the app. K_m of the electron transport chain for O_2.

The mechanism of valinomycin action remains to be resolved. At the concentrations employed, the sole reported effect of valinomycin on broken chloroplasts is enhanced membrane permeability to K^+ [22]. Addition of 0.1 μM valinomycin to intact chloroplasts was found [27] to result in loss of

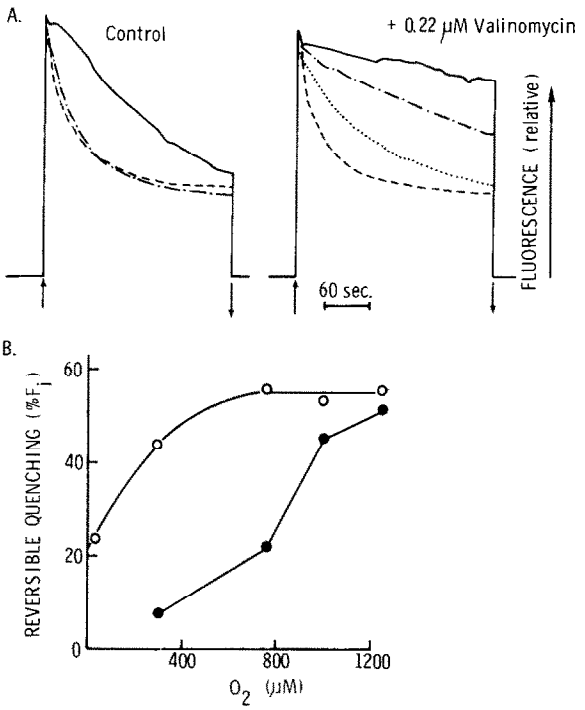

Fig.4. Effect of O_2 tension on slow fluorescence quenching measured in the presence and the absence of valinomycin. (A) Traces obtained at O_2 concentrations of 287 μM (——), 765 μM (– · –), 996 μM (· · · ·), and 1250 μM (– – –). (B) Methylamine-reversible quenching (expressed as % F_i) is plotted as a function of O_2 concentration in the absence (○) and in the presence (●) of 0.22 μM valinomycin. O_2 tension was varied by bubbling the assay medium with O_2 or N_2 prior to chloroplast addition. Other details as in fig.1.

20–30% of their internal K^+. Two types of models for valinomycin action can thus be proposed:

1. Valinomycin, by increasing thylakoid membrane permeability to K^+, could enhance K^+ efflux from the thylakoids in response to H^+-uptake at the expense of Mg^{2+} efflux [28,29]. To the extent that quenching reflects specifically Mg^{2+} movement, it would be inhibited. This possibility can be dismissed, since treatments whose only consequence is restoration of electron flow counteract the inhibitory effects of valinomycin.

2. By increasing envelope K^+ permeability, valinomycin might alter stromal K^+ concentration and/or the

potential difference across the chloroplast envelope. Either of these parameters could influence electron flow to O_2, the former, for example, by controlling the accessibility of ferredoxin (e.g., [30]), the latter by governing O_2 permeability of the envelope [31]. This issue is currently under investigation.

4. Conclusions

Valinomycin is a potent inhibitor of the slow fluorescence quenching phenomenon in intact chloroplasts. Inhibition by valinomycin is similar to that caused by low O_2 tensions in that it can be reversed by low concentrations of DCMU, by oxaloacetate, and by elevated O_2 tensions. It is therefore suggested that valinomycin alters the interaction of O_2 with the photosynthetic electron transport chain. By upsetting the redox poise normally maintained by electron flow to O_2, valinomycin acts indirectly to decrease the rate of cyclic electron flow. The known specificity of valinomycin for K^+ implicates cations other than H^+ and Mg^{2+} in the regulation of photosynthetic electron transport events.

Acknowledgements

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