THE STIMULATION OF POST-ILLUMINATION ATP SYNTHESIS BY VALINOMYCIN

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1. Introduction

A high-energy state or intermediate (X_E) is formed in illuminated chloroplasts which can drive ATP formation in a dark reaction [1]. Since the characteristics of X_E and of the light-induced H^+ uptake [2] are similar, it has been proposed that X_E is a gradient in H^+ activity across chloroplast membranes. This view is substantiated by the finding that ATP formation may be driven in total darkness by an artificially-produced H^+ gradient [3].

Nelson et al. [4] reported that the treatment of lettuce chloroplasts with digitonin inhibited H^+ uptake and post-illumination ATP synthesis more severely than photophosphorylation. In our previous studies on phosphorylation in subchloroplast particles [5, 6], we concluded that H^+ uptake in these particles was electrogenic and, furthermore, that a membrane potential might play a role in photophosphorylation. To test this idea further, the effects of valinomycin and K^+ on post-illumination ATP synthesis and H^+ uptake in subchloroplast particles were tested. These reagents were found previously [7] to enhance H^+ uptake in *R.rubrum* chromatophores. In this paper, we report that valinomycin in the presence of K^+ stimulated H^+ uptake as well as the formation of X_E in subchloroplast particles.

2. Methods

Spinach chloroplasts were isolated as described [8], and subchloroplast particles (SCP) were prepared by sonic oscillation of chloroplasts [9]. Subchloroplast particles were also prepared with digitonin as described by Anderson and Boardman [10]. The fraction used was that sedimenting between 50,000 *g* and 144,000 *g* (D-144 particles). Post-illumination ATP synthesis was assayed as described previously [9], except that 50 μM *N*-methyl phenazonium methosolfate was used in place of pyocyanine. Changes in pH were monitored as previously outlined [5]. Chromatophores from *Rhodospirillum rubrum* were the generous gift of Dr. R.J. Guillory.

3. Results

Whereas H^+ uptake in chloroplasts is not affected by low concentrations of valinomycin [11], the extent of H^+ uptake in SCP and in D-144 particles was enhanced by valinomycin (table 1). This effect was dependent upon K^+ since valinomycin did not stimulate H^+ uptake in SCP [5] or D-144 particles suspended in choline-Cl. Valinomycin in the presence of K^+ increased the apparent rate of the pH rise in D-144 particles and in SCP. However, the rate of decay of the pH in the dark was less affected by valinomycin as was the rate of the pH rise. It should be mentioned that whereas the rates of cyclic photophosphorylation with *N*-methyl phenazonium methosulfate in D-144 particles were equivalent to those in chloroplasts [12], the extent of H^+ uptake in the absence of valinomycin was only one-tenth to one-fifth that in chloroplasts.

Post-illumination ATP synthesis in chloroplasts was slightly inhibited by valinomycin (table 2). In

Table 1
Effect of valinomycin on the light-induced H^+ uptake in subchloroplast particles.

Preparation	Salt added	Valinomycin (μg per ml)	H^+ uptake (μeq per mg of chlorophyll)	k_{rise} (sec^{-1})	k_{decay}
SCP	KCl	–	0.37	0.037	0.033
SCP	KCl	0.5	0.51	0.061	0.041
SCP	KCl	1.0	0.47	0.061	0.054
D-144	KCl	–	0.051	0.046	0.050
D-144	KCl	0.5	0.116	0.119	0.049
D-144	KCl	5.0	0.168	0.147	0.057
D-144	choline-Cl	–	0.038	0.073	0.067
D-144	choline-Cl	0.5	0.035	0.065	0.071

The reaction mixture contained, in a volume of 3 ml, 50 mM KCl or 50 mM choline-Cl, 10 μM *N*-methyl phenazonium methosulfate and SCP or D-144 particles equivalent to about 150 μg to 300 μg of chlorophyll. The samples were continuously flushed with argon. The temperature was 4° and the initial pH was adjusted to 6.2 ± 0.05. k_{rise} and k_{decay} are apparent first order constants calculated as described previously [8].

Table 2
Stimulation of post-illumination ATP synthesis in subchloroplast particles and chromatophores by valinomycin.

Preparation	Valinomycin (μg per ml)	^{32}P esterified (nmoles per mg of chlorophyll)
Chloroplasts	–	70.1
Chloroplasts	0.5	67.5
Chloroplasts	1.0	57.7
SCP	–	10.0
	0.5	15.9
	1.0	18.8
D-144 particles	–	1.4
	0.5	3.6
	1.0	5.8
	2.0	8.8
	5.0	9.3
Chromatophores	–	2.9
	1.0	10.2

The light stage reaction mixture contained in 0.5 ml, 10 mM morpholinoethane sulfonate-NaOH, pH 6.0, 50 mM KCl, 0.05 mM *N*-methyl phenazonium methosulfate and subchloroplast particles or chromatophores equivalent to 0.1 mg of chlorophyll. After illumination at 15 to 17° for 20 or 30 sec with white light (5×10^5 ergs/cm^2/sec) in a 0.5 ml syringe, the mixture was injected into a dark reaction mixture which was previously described [9]. Valinomycin was added to the light stage reaction mixture. Non-illuminated samples esterified less than 0.5 nmoles of $^{32}P_i$ per mg of chlorophyll.

Table 3
Effects of KCl and of valinomycin in the dark stage on X_E phosphorylation in D-144 particles.

Experiment	Additions	$^{32}P_i$ esterified (nmoles per mg chlorophyll)
I	50 mM choline-Cl	1.8
	50 mM choline-Cl + valinomycin	2.5
	25 mM choline-Cl + 25 mM KCl	2.2
	25 mM choline-Cl + 25 mM KCl + valinomycin	7.1
II	50 mM KCl	2.3
	50 mM KCl + valinomycin in light stage	8.5
	50 mM KCl + valinomycin in dark stage	3.6

The experimental conditions were the same as those given in the legend to table 2. The valinomycin was 4 μg per ml in experiment I and 2 μg per ml in experiment II.

Table 4
Formation rate of X_E in D-144 particles.

Illumination time (sec)	$^{32}P_i$ esterified (nmoles per mg of chlorophyll)	
	– valinomycin	+ valinomycin (1 μg/ml)
5	1.1	11.3
10	2.9	11.8
30	3.6	11.1
60	3.2	9.3

Conditions were as given in table 2.

contrast, valinomycin stimulated this reaction in SCP, D-144 particles and chromatophores of *R. rubrum*. As was the case for H^+ uptake, the stimulation of post-illumination ATP synthesis in D-144 particles by valinomycin was more pronounced than that in SCP. The stimulation of X_E formation by valinomycin in six different preparations of D-144 particles ranged from 4- to 7-fold, whereas in SCP this stimulation was generally less than two-fold.

In the absence of K^+, valinomycin had little effect on post-illumination ATP synthesis in D-144 particles (table 3). Maximal stimulation of X_E formation was observed at a KCl concentration of 25 mM. Furthermore, valinomycin only slightly stimulated post-illumination ATP formation when it was added to the dark stage reaction mixture. Post-illumination ATP formation in D-144 particles in the presence of valinomycin (1 μg per ml) and 50 mM KCl was abolished by 5 mM NH_4Cl, 0.4 mM octyl amine and 3 μM nigericin.

The rate of formation of X_E in D-144 particles was markedly accelerated by valinomycin, as was the case for H^+ uptake (table 4). X_E formation at 17° in D-144 particles supplemented with valinomycin was nearly completed in 5 sec, whereas in the absence of valinomycin, it was about 1/3 completed. The half-time for X_E formation in D-144 particles in the presence of 1 μg valinomycin per ml at 4° was about 5 sec. The half-time for H^+ uptake in D-144 particles in the presence of 1 μg per ml of valinomycin and at 4° was also about 5 sec. In chloroplasts, valinomycin did not affect the rate of X_E formation.

4. Discussion

The enhancement of the rate and extent of H^+ uptake in subchloroplast particles by valinomycin is similar to that observed in chromatophores of *R. rubrum* [7]. Thus, it is possible that H^+ accumulation in subchloroplast particles, as well as that in chromatophores, is limited by the membrane potential it develops [7]. In the presence of valinomycin and K^+, H^+ uptake would be counterbalanced by K^+ extrusion, thereby resulting in dissipation of the membrane potential and allowing the uptake of more H^+. If this is so, these experiments provide further evidence that H^+ uptake in subchloroplast particles develops not only a gradient in H^+ activity but also a membrane potential which persists at the steady state.

In view of the fact that valinomycin and K^+ affect H^+ uptake and post-illumination ATP synthesis in a similar manner, it is tempting to conclude that the H^+ activity gradient is the driving force for phosphorylation under these conditions. Although the membrane potential caused by H^+ uptake should be attenuated by valinomycin and K^+, it is not possible to rule out a contribution of a K^+ diffusion potential to the high-energy state [13]. In the presence of valinomycin, K^+ would be transported out of the interior space of the subchloroplast particles in response to H^+ uptake giving rise to a gradient of K^+ activity. The decay of this gradient in the dark could result in the formation of a K^+ diffusion potential. Such a diffusion gradient would decay more slowly than a membrane potential formed solely by H^+ uptake. The stimulation of post-illumination ATP synthesis by valinomycin may be rationalized by assuming that the high energy state in subchloroplast particles decays in the dark at a slower rate in the presence of valinomycin and K^+ than in their absence. Therefore, more of the high energy state would be conserved at ATP when valinomycin and K^+ were present. In the absence of valinomycin and K^+, the membrane potential developed by H^+ uptake may decay so quickly that it is largely dissipated by the time the subchloroplast particles come in contact with the phosphorylation reagents. Furthermore, the lack of effect of valinomycin and K^+ on H^+ uptake in chloroplasts may indicate that H^+ uptake in chloroplasts generates primarily

It should be pointed out that valinomycin and K^+ have no effect on the rate of phosphorylation in sub-

chloroplast particles under continuous illumination. Furthermore, whereas the extent of post-illumination ATP synthesis in D-144 particles is at best one-fifth of that in chloroplasts, the rates of cyclic phosphorylation are comparable to those in chloroplasts [12]. Thus, it is probable that the extent of post-illumination ATP synthesis is a measure of the capacity of the high energy state rather than of its potential.

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