THE ROLE OF MEMBRANE POTENTIAL IN THE TIGHT BINDING OF ADENINE NUCLEOTIDES OF CHLOROPLAST CF_1

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1. Introduction

Light induced binding of adenine nucleotides to chloroplast membrane bound CF_1 has been shown to be uncoupler sensitive [1–3] and insensitive to energy transfer inhibitors [1,4]. It has been suggested that a conformational change in the coupling factor molecule may be responsible for the light-induced nucleotide exchange [1,2]. This suggestion is supported by evidence for a light-induced uncoupler sensitive conformational change in CF_1 as monitored by both tritium exchange and incorporation of *N*-ethyl maleimide [5,6]. Since the nucleotide exchanges can also be induced by an acid–base transition, it was concluded that the transmembrane ΔpH is the driving force for this process [7].

Because of the similar energization requirement for tight nucleotide binding and photophosphorylation, the possibility that tightly bound nucleotides are intermediates on the pathway of photophosphorylation has been suggested by several investigators [1,2,8]. A catalytic site on the main route of phosphorylation should rapidly turn over substrate ADP during steady state phosphorylation. Complete turnover of nucleotides bound at such a site should occur with each turnover of the catalytic site, i.e., the kinetics of tight nucleotide binding should be as fast as phosphorylation.

In this communication, we will present evidence indicating that a transmembrane electrical potential is necessary for the rapid binding of nucleotides to CF_1. Agents that collapse the membrane electrical potential can prevent rapid nucleotide exchange even after the coupling factor has gone through several turnovers of photophosphorylation, indicating that tightly bound nucleotides are not intermediates on the main pathway of phosphorylation.

2. Materials and methods

Chloroplasts were isolated from market spinach as previously described [9]. Nucleotide binding was measured according to the method of Magnussen and McCarty [2], using centrifugation through silicone oils as the means for separating the thylakoids from the media. Prior to the centrifugation step, the reaction mixture (0.5 ml) was given timed light treatments in a waterjacketed vessel.

The reaction mixture used for all experiments described here consisted of 50 mM KCl, 20 mM tricine NaOH (pH 8.0), 5 mM $MgCl_2$, 0.2 mM methylviologen and about 100 μg/ml chlorophyll at 18°C. Illumination was with heat-filtered (dilute $CuSO_4$) white light at an intensity of 6×10^6 ergs cm^{-2} s^{-1}. Illumination times were controlled by a Uniblitz model 225-0 electromagnetic shutter used with a Uniblitz model 310 electronic timing unit.

Beckman microfuge tubes were filled with 0.1 ml 10% sucrose followed by 0.1 ml of a mixture of Versilube F-50 and SF-96 (50) in a ratio of 3:1 (v/v). After the illumination described above, 0.1 ml samples of the chloroplast reaction mixture were placed on top of the silicone layer and centrifuged for 20 s in a Beckman model 152 microfuge. The tubes were then placed in solid CO_2 and later the bottom phase was cut off and placed in 1.0 ml 30% H_2O_2, 2% Triton X-100 and bleached for 5 h at 75°C.

Using companion tubes as controls, the trapped space was determined using [^{14}C]sorbitol and appropriate corrections were made on the experimental samples based on the [^{14}C]sorbitol control. Chlorophyll determinations were carried out on the lower phase according to the method of Arnon [10]. The scintillation cocktail used was Tritosol [11]. When error bars are shown, the given point is the average amount of label found in four microfuge tubes prepared from a common sample.

Photophosphorylation was measured according to the method of Saha and Good [12]. The reaction mixture contained approximately 4 μCi/ml $^{32}P_i$ and phosphorylation was measured using single flashes of light.

Valinomycin, ADP and ATP were purchased from Sigma. [^{14}C]ATP was from Schwartz-Mann, [^{14}C]ADP, [^{3}H]ADP and [^{14}C]sorbitol were from Amersham-Searle. [^{3}H]ATP and $H_3^{32}PO_4$ were from ICN.

3. Results

The kinetics for the onset of the light-induced nucleotide exchange may reflect either the kinetics of a conformational change in CF_1 exposing the nucleotide exchangeable site, or actual binding kinetics of nucleotides exchanging into this site. Figure 1 shows the amount of nucleotide bound to the thylakoids after short flashes of light (20–1000 ms). The onset of binding in the light has a halftime of about 30–40 ms and appears to be independent of whether ATP or ADP is used. The reaction medium contains 50 mM KCl, and addition of the potassium-specific ionophore, valinomycin, appears to severely inhibit adenine nucleotide binding, especially at illumination times shorter than 200 ms. There is a day-to-day variation in the approach to saturation in the nucleotide binding curve for both ADP and ATP (compare fig.1 and 2), the reason for which is not understood. In spite of such variation, the valinomycin effects are very consistent.

The capacity for post-illumination ATP binding decays with a half-time of approximately 2 min (data not shown). Because of this we tested the possibility that most of the nucleotide binding was taking place in the dark time between illumination and centrifugation, with valinomycin exerting its effect in this period by causing a rapid decay of the exchangeable state. Table 1 shows that the presence of valinomycin added 5 s after termination of illumination does not significantly decrease the capacity for ATP binding when the nucleotide is added 30 s after illumina-

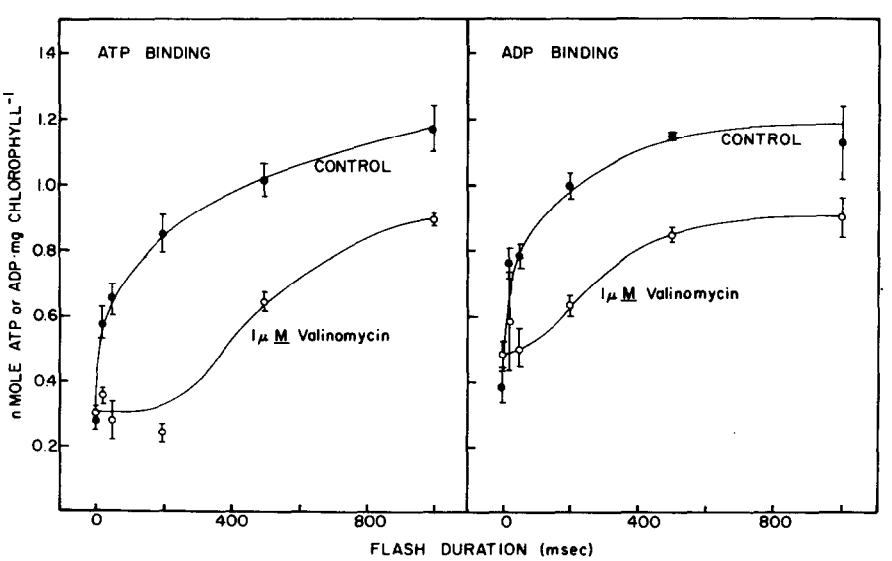

Fig.1. Effect of valinomycin on the binding of [^{14}C]ATP and [^{14}C]ADP to thylakoids as a function of illumination times. The reaction medium was described in Materials and methods. The nucleotide concentration was 10 μM with about 2×10^5 cpm · ml^{-1}. A single flash was given each sample.

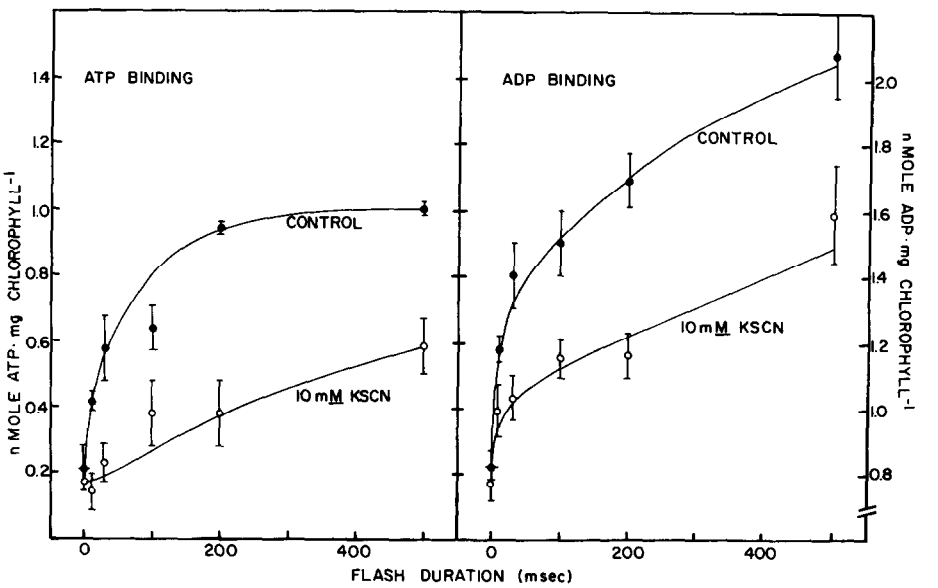

Fig.2. Effect of 10 mM KSCN on the binding of [^{3}H]ATP and [^{3}H]ADP to thylakoids as a function of illumination time. Additional KCl (final concentration of 60 mM) was added to the controls. The nucleotide concentration was 10 μM with about 3 X 10^5 cpm · ml^{-1}. Single flashes given as for fig.1.

tion, compare the second and third columns; although it does decrease the binding when present during illumination (column one). We can infer from this that valinomycin is exerting its effect on the formation of the nucleotide exchangeable state, with no detectable effect on the decay of that state. The uncoupler gramicidin, also does not decrease the amount of ATP bound after illumination, provided the gramicidin is added after the light is turned off. Added during illumination (column one), gramicidin inhibits nucleotide binding. This is consistent with the results of Strotmann and Bickel-Sandkötter using the uncoupler, carbonyl cyanide-*p*-trifluoromethoxyphenylhydrazone [3].

If the effect of valinomycin plus potassium on the kinetics of nucleotide binding is because of a dissi-

Table 1
Effect of ionophores on post-illumination ATP binding

	Amount [^{14}C]ATP bound (nmol ATP · mg chl^{-1})		
	ATP and ionophore present during illumination	ATP added after illumination	ATP and ionophore added after illumination
Dark (no addition)	0.7 ± 0.1		
Light (no addition)	1.7 ± 0.1	1.5 ± 0.1	
+2 μM Gramicidin	1.0 ± 0.1	1.0 ± 0.1	1.3 ± 0.2
+1 μM Valinomycin	1.2 ± 0.1	1.3 ± 0.1	1.3 ± 0.1

The reaction mixtures were as described in Materials and methods. ATP concentration was 10 μM, containing about 2 X 10^5 cpm · ml^{-1} and the illumination time used was 10 s. After illumination, the experiments given by columns two and three were treated with either ATP, given at 30 s dark time; or ionophore, given at 5 s dark time, followed by ATP given at 30 s dark time. The experiment represented by column one had ATP and ionophore present during the illumination

pation of transmembrane electrical potential, then other permeant ions [13,14] should elicit the same effect. Figure 2 shows the effect of SCN^- on ATP and ADP binding kinetics. Although the effect is not identical to that of valinomycin, qualitatively it is similar in that the rapid onset of nucleotide binding does not occur.

If the effect of permeant ions is on the formation of a nucleotide exchangeable state, and not directly on nucleotide binding, then the light-dependent release of previously bound nucleotide should also be inhibited. Figure 3 shows that effects of valinomycin plus potassium on the release of previously bound [^{3}H]ADP. Valinomycin clearly inhibits the release of the previously bound nucleotide. Since valinomycin inhibits both the binding and debinding of nucleotide, we can conclude that it is exerting its effect on the formation of a nucleotide exchangeable state.

Ort et al. [14,15] have shown that photophosphorylation in multiple flashes of 1–1000 ms duration exhibits a lag of a few milliseconds before initiation of the steady state rate. This lag is extended to about 40 ms in the presence of permeant ions. They concluded that a transmembrane electrical potential was necessary for the synthesis of ATP at flash times up to ~40 ms. It is relevant to ask if the inhibition of nucleotide binding to CF_1 by valinomycin could be responsible for the lag in the onset of photophosphorylation. We therefore measured the lag in the onset of phosphorylation under our conditions using single flashes of light and compared it to the amount of ADP bound under identical conditions. As fig.4 shows, the lag in phosphorylation was about 50 ms, in contrast to the valinomycininduced inhibition in ADP binding, which was extended considerably longer. So it would appear that the inhibition of ADP binding by valinomycin is not responsible for the lag in the onset of phosphorylation.

4. Discussion

The use of single, short (10–1000 ms) flashes enables us to estimate the formation kinetics of the nucleotide exchangeable state. It has been proposed

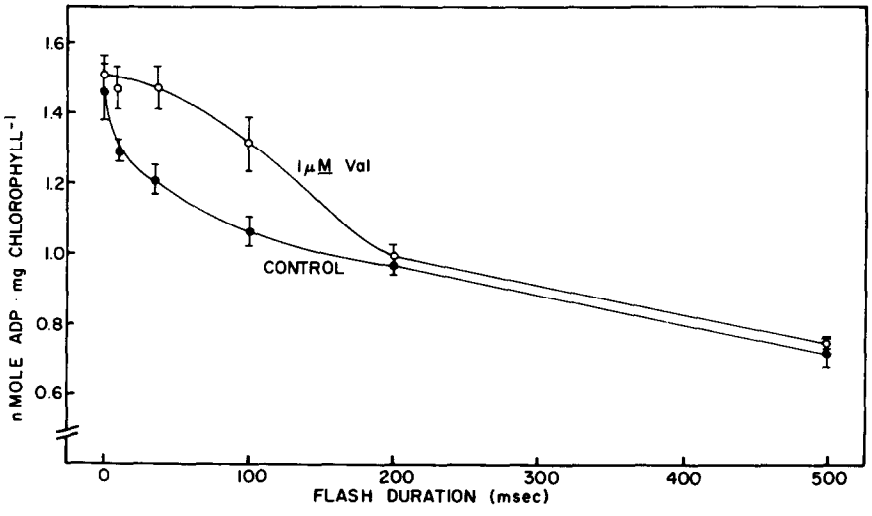

Fig.3. Effect of valinomycin on the release of bound [^{3}H]ADP. Preillumination was carried out in 30 ml reaction mixture containing ≈ 100 μg/ml chlorophyll, 0.2 M sucrose, 20 mM tricine · NaOH (pH 8.0), 5 mM $MgCl_2$, 0.5 mg/ml bovine serum albumin, 0.2 mM methyl viologen and 10 μM ADP containing about 7×10^5 cpm · ml^{-1}. Illumination time was 30 s at 4°C. The reaction mixture was then centrifuged at 2500 × *g* for 6 min, washed once (without methyl viologen or ADP) and resuspended in the same media. The reaction mixture for the nucleotide debinding experiment is described in Materials and methods and also contained 10 μM 'cold' ADP. Aliquots of this chloroplast suspension (with or without 10 μM valinomycin) containing membrane-bound, labeled nucleotides, were given various lengths of single-flash illumination, transferred to the microfuge tubes and centrifuged through the silicon layer.

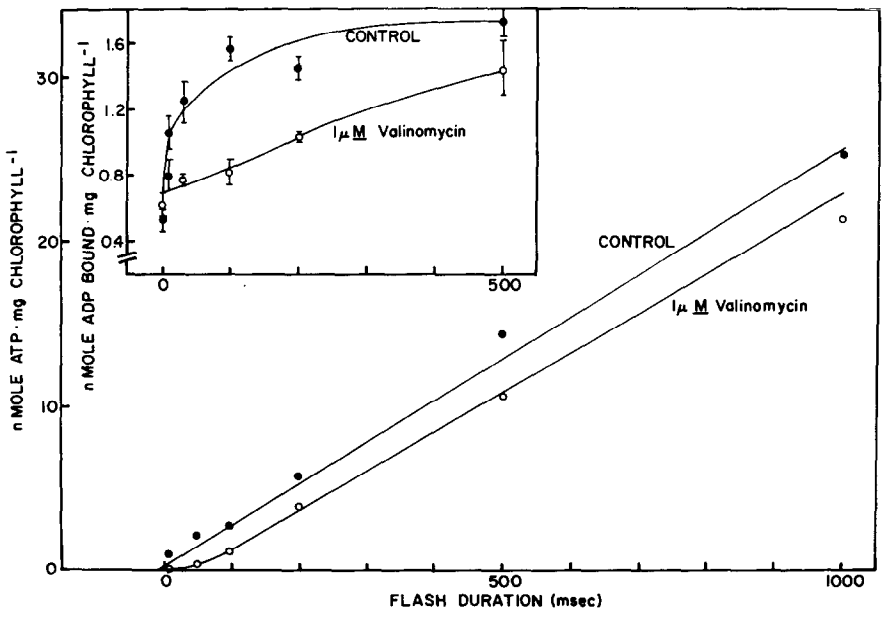

Fig.4. Effect of valinomycin on phosphorylation and [^{3}H]ADP binding (inset) as a function of single-flash illumination time. The reaction mixture also contained 0.9 mM P_i in both experiments. In the phosphorylation experiment the reaction was terminated by the addition of 0.1 ml 10% perchloric acid.

that these kinetics provide a measurement of the rate of a conformational change in CF_1, provided that the exchange itself is not rate-limiting [2]. We have found that agents which will dissipate a transmembrane electrical potential (valinomycin plus K^+, or SCN^-) also prevent the rapid formation of the nucleotide exchangeable state. This infers that an electrical potential may be involved in the rapid onset of a conformational change in CF_1.

It is not clear why valinomycin should inhibit nucleotide exchange after longer periods of illumination (10 s, see table 1). It is also not clear why SCN^-, which should provide a virtually inexhaustible supply of permeant anions, should not prevent the nucleotide exchange completely if an electrical potential is a critical factor. It is quite possible that the nucleotide exchange can somehow also use ΔpH as a driving force for its formation. Perhaps a transmembrane electrical potential is necessary for the rapid formation of the binding state, but a ΔpH, which would not form as fast, can also cause the formation of the binding state (see ref. [7]). However, since an energized state sizeable enough to drive steady state phosphorylation cannot initiate the formation of the nucleotide exchangeable site (see fig.4), this possibility is also troublesome.

Ort et al. [14,15] have shown that the lag in the synthesis of ATP in the presence of permeant ions is not extended by addition of permeant buffers. They concluded that protons produced by electron transport within the membrane are used directly for phosphorylation before they equilibrate with the inner osmotic space, i.e., there is not a definite requirement for a transmembrane ΔpH. It is possible that the very slow onset kinetics of nucleotide binding in the presence of permeant ions is driven by the developing transmembrane ΔpH. This could explain the apparent discrepancy between the development of an energized state sufficient to drive phosphorylation (protons utilized directly) and the development of a state that drives the nucleotide exchangeability (protons from a transmembrane ΔpH). It should be pointed out that there is no contradiction in the concept of an electrical potential contributing to the rapid onset of nucleotide binding and our hypothesis that the more slowly developing ΔpH drives the tight binding in the absence of a membrane potential. In both cases we can attribute the molecular mechanism

to proton–CF_1 interactions, rather than supposing some ill-defined 'effect' of an electrical potential on membrane or CF_1 macromolecules in one case and proton–macromolecule interactions in another case. Following the initial suggestion of Mitchell (ref. [19]) concerning the 'proton well', it is physically reasonable that a transmembrane electrical potential, positive on the side having the higher H^+ concentration, should increase the concentration of protons in the proton-conducting well significantly above the proton concentration in the inner bulk phase.

The data presented here clearly show that the nucleotide exchangeable site exhibits significant differences from the catalytic site. We use the value of 1.3 nmol $CF_1 \cdot$ mg chl^{-1} [16]. It is quite apparent that turnover at the catalytic site is kinetically distinguishable from tight nucleotide binding in the presence of valinomycin and potassium (cf. fig.4). For example, after 200 ms the catalytic site on the CF_1 has turned over at least 3 times, yet the increment of nucleotide bound over the dark level is only about 0.2 nmol · mg chl^{-1}; or about 0.15 nucleotides per CF_1 (see fig.4). Because the turnover of nucleotides at the catalytic site in the presence of valinomycin is much faster than the exchange into the nucleotide tight binding site, it seems unlikely that the tightly-bound nucleotides we measure are of catalytic significance in the mechanism of phosphorylation. Similar conclusions have also been reached by Shavit et al. [17] and Smith et al. [18].

If the tight nucleotide binding involves a process distinct from the net formation of ATP, the question remains as to the function of the tight binding. One possibility is that the observed tight binding is part of a regulatory function of CF_1. It is known that CF_1 has at least two states that differ in proton conductance and that ADP or ATP are effectors that bestow the low conductance state [20]. If the tight binding reflects such an involvement, it may be only a laboratory curiosity, since with the K_m near 10 μM for either ADP or ATP, it is likely that in vivo there always is a nucleotide occupying the binding site given that stroma nucleotide levels nearly always will exceed 10 μM. A more productive hypothesis would envisage the tight binding site interacting, in the light, with free ADP and ATP adjusting the relative amounts of bound nucleotide in accordance with their proportions in the stroma phase. A shift to a higher ATP-binding state could, for instance, lead to an increased Mg^{2+} release, stimulating ATP utilization by the appropriate Calvin-Benson cycle enzymes [21,22].

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