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DELAYED LIGHT STUDIES ON PHOTOSYNTHETIC ENERGY CONVERSION

VIII. EVIDENCE FROM MILLISECOND EMISSION OF CHLOROPLASTS FOR TWO ADENYLATE BINDING SITES ON MEMBRANE-BOUND COUPLING FACTOR, CF_1

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SUMMARY

Effects of adenylates on chloroplast delayed light emission, at millisecond dark times, are inverse to the previously characterized effects of adenylates on electron transport rates. Either ADP alone or ATP alone increase intensity of delayed light, while ADP plus P_i decrease it. ADP alone requires the presence of an electron acceptor to have this effect on delayed light, but ATP does not.

All three adenylate effects are abolished by uncoupling with gramicidin, by partial removal of photophosphorylation coupling factor (CF_1) with EDTA, and by antibody to CF_1. Readdition of CF_1 re-established the adenylate effects in EDTA-stripped membranes. The three adenylate effects are differentially sensitive to pH, and pH differentially affected their abolition by antibody to CF_1. The two adenylate effects shown in the absence of P_i are exhibited at lower adenylate concentrations than the ADP plus P_i effect, and are also less sensitive to phloridzin.

These results are discussed in terms of probable adenylate effects on membrane-bound chloroplast coupling factor, CF_1. At least two ADP binding sites would differ with respect to adenylate concentration for half maximal binding; pH of optimal binding capacity; phloridzin sensitivity; and functional regulation of electron transport, proton uptake, and energy storage within the membrane as measured by delayed light emission. It remains unclear whether the high affinity ADP binding site is identical to a high affinity ATP binding site on CF_1.

Abbreviations: CF_1, chloroplast coupling factor; p.m.f., proton motive force; TES, *N*-tris (hydroxymethyl)-2-aminoethane sulfonic acid; Tricine, *N*-tris (hydroxymethyl) methyl glycine; Tris–HCl, tris (hydroxymethyl aminomethane)–HCl.

INTRODUCTION

Photosynthetic lamellae perform at least two types of energy conversion: quantum conversion to chemical potential, and coupling of electron transport to ATP formation. Both processes involve energy storage within the thylakoid membrane. Both types of energy storage are evident in different aspects of the back reaction of quantum conversion which results in millisecond delayed light emission from functional chlorophyll *a* of Photoreaction II: dark decay kinetics vs overall intensity of emission.

At one millisecond dark time after repeating flashes of exciting light, electron acceptors induce rapid decay kinetics in delayed light from chloroplasts [1]. This indicates that the millisecond dark decay kinetics reflect utilization by electron transport reactions of energy stored in primary photoproducts of Photoreaction II. On the other hand, the overall intensity of emission at 1ms was found by Mayne [8] to be reduced by phosphorylating conditions and uncouplers. Mayne suggested that the high energy state of photophosphorylation could be in equilibrium with Photoreaction II, and thus provide energy for delayed light emission. Nevertheless, Wells et al. [3] found that the dark decay kinetics of millisecond delayed light were not strongly affected by phosphorylating conditions. Thus it is possible to experimentally separate the effects of high energy coupling state (i.e. overall intensity of delayed light) from the effects due to depletion of photoproducts by electron transport (i.e. kinetics of dark decay).

The work of Wraight and Crofts [4], Ito et al. [5], Neumann et al. [6] and Cohen and Bertsch [7] has shown direct correlation between overall intensity of delayed light and total proton motive force (p.m.f.) across the thylakoid membrane. These observations are in agreement with Fleischmann's [8] suggestion that the primary photoact might provide charge separation across the membrane, and with Mitchell's [9] chemiosmotic hypothesis for p.m.f. as the driving force of phosphorylation.

Avron et al. [10] found inhibition of basal electron transport (i.e. absence of P_i) by ATP at concentrations considerably below the ADP concentrations required for maximal photophosphorylation (i.e. presence of P_i). The adenylate-induced inhibition of basal electron transport is apparently related to the ability of adenylates to increase the extent of light-induced proton uptake, as reported by McCarty et al. [11] and Telfer and Evans [12]. These authors suggest that the adenylates ADP and ATP, as well as energy transfer inhibitors such as DIO-9 and phloridzin, act directly on the photophosphorylation coupling factor (CF_1) [13, 14] and thereby decrease the rate of proton leakage through the thylakoid membrane. Neumann et al. [6] have attributed the adenylate-induced increase in delayed light emission, as well as the DIO-9 and phloridzin increases in delayed light, to the same mechanism. If CF_1 regulates membrane p.m.f., and if delayed light reflects p.m.f., the overall intensity of 1 ms delayed light might then provide a convenient tool for measuring adenylate binding to functional CF_1 in the thylakoid membrane.

We have investigated the pH dependence (pH 7–8.5), adenylate concentration requirements, and phloridzin sensitivity of three adenylate-induced effects on delayed

light and electron transport as defined by three experimental conditions: 1) ATP, 2) ADP, 3) ADP in the presence of P_i. Our results imply that adenylate-induced changes in overall intensity of millisecond delayed light emission can be taken as an indication of adenylate binding to CF_1, and that adenylates in the absence of P_i bind to one or more sites which are different from the phloridzin-sensitive site occupied by ADP in the presence of P_i.

MATERIALS AND METHODS

Spinach chloroplasts were essentially prepared as described by Jagendorf and Avron [18]. 50 g of washed and deveined spinach leaves were homogenized in a Waring blender for 10 s with 100 ml of 0.025 M Tricine buffer, pH 7.8, which contained 0.4 M sucrose and 0.010 M NaCl. Chloroplasts were washed once with an equal volume of the grinding medium and resuspended in 0.005 M TES buffer, pH 7.5 which contained 0.100 M sucrose and 0.010 M NaCl.

CF_1 was partially removed from thylakoids by EDTA washing of chloroplasts. Two degrees of CF_1 removal were achieved by using different chloroplast concentrations during the EDTA treatment. After "mild" stripping the EDTA-resolved thylakoids gave electron transport rates about equal to those of control chloroplasts in the presence of ADP and P_i. After "harsh" stripping the EDTA-resolved thylakoids gave electron transport rates about 50 % higher than those of control chloroplasts in the presence of ADP and P_i. The choroplast pellet was resuspended in 0.1 M sucrose, 0.005 TES–NaOH, pH 7.4, and washed once. The chloroplasts were then suspended in 0.001 M EDTA, pH 7.6, at either 0.2 μg chlorophyll/ml (mild stripping) or at 0.08 μg chlorophyll/ml (harsh stripping), allowed to stand 4 min at 3 °C, and collected by centrifugation at $20\,000 \times g$ for 10 min. The pellet was finally resuspended in the original grinding medium at a final concentration of 1 mg chlorophyll/ml. Chlorophyll concentration was determined by the method of Arnon [19].

A 25–45 % $(NH_4)_2SO_4$ fraction of CF_1 was further purified in the absence of ATP but in the presence of 50 mM Tris–HCl, pH 8, and 2 mM EDTA as previously described [14]. For reconstitution experiments, CF_1 was collected by centrifugation, dissolved in a minimal volume of buffer and desalted on a Sephadex G-50 column (1×15 cm). Protein concentration of CF_1 was determined spectrophotometrically [20].

Delayed light emission was measured as described previously [21, 22] with 250 exciting flashes per s. Ferricyanide reduction was measured as described by Bertsch and Lurie [22]. Aqueous stock phloridzin solutions were prepared by warming an aqueous phloridzin suspension (0.01 M) in a 50 °C bath for a few minutes until the solution cleared. Appropriate samples of it were then pipetted into reaction mixtures. A fresh stock solution was prepared for each experiment. Hexokinase was desalted on Sephadex G-50 column (1×15 cm) which had been previously equilibrated with 0.025 M sodium acetate buffer, pH 5.4, containing 0.05 M glucose. Disodium salts of ADP, ATP and crystalline ammonium sulfate suspension of yeast hexokinase were purchased from Sigma. Phloridzin was obtained from K & K Laboratories, Inc. Spinach was purchased from local markets.

RESULTS

Figs 1a and b give the pH dependence from pH 7–8.5 of 1 ms delayed light emission in the presence of ferricyanide, and of ferricyanide reduction rate, as affected by ATP, ADP, and ADP in the presence of P_i. The delayed light measurements were taken from oscilloscope photographs showing decay kinetics between 0.8 and 3 ms. None of these treatments strongly affected the rapid decay kinetics induced by the acceptor $K_3Fe(CN)_6$. ADP and ATP increased delayed light optimally in the more alkaline pH ranges, where they also showed the greatest effect on basal electron transport. The adenylates no longer increased delayed light at pH 7.5 or below. ADP in the presence of P_i was still effective in reducing delayed light at pH 7.5.

Certain differences between ADP and ATP effects are apparent from Figs 1a and b. ADP, at times, can be relatively less effective on both delayed light and electron flow at pH 8.0 than ATP. ATP, in contrast to ADP, continues to slightly inhibit basal electron transport at pH 7.5 and below. There is no effect of ATP or ADP on delayed light in this pH range.

Figs 2a, b, c and d give the adenylate concentration dependence at pH 8.5 for delayed light, and for rate of basal electron flow to ferricyanide. Maximal increase of delayed light was obtained with approx. 0.02–0.1 mM ADP or ATP, while maximal inhibition of basal electron transport occurred at about 0.05–0.1 mM ADP or ATP. ADP did not increase delayed light in the absence of acceptor, whereas ATP gave increased delayed light regardless of the presence or absence of acceptor.

AMP (1 mM), or P_i up to 3 mM, did not affect delayed light emission. In

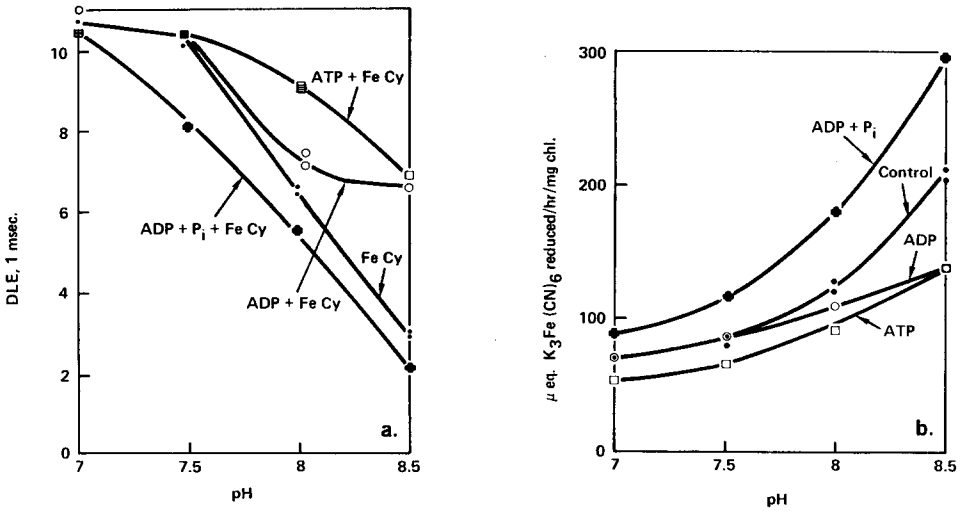

Fig. 1. Variation with pH of adenylate effects on delayed light emission and ferricyanide reduction. Duplicate 5 ml reaction mixtures contained 25 mM buffer (TES for pH 7 and 7.5, Tricine for pH 8 and 8.5), 25 mM NaCl, 4 mM $MgCl_2$, 0.3 mM $K_3Fe(CN)_6$, adenylates as indicated (0.2 mM ADP or ATP, 0.4 mM ADP+0.8 mM KP_i), and chloroplasts (10 μg Chl/ml of reaction mixture), and were preincubated for 1 min at room temperature before delayed light emission (DLE) and ferricyanide reduction were measured. Intensity of delayed light given in arbitrary units, rate of ferricyanide reduction given in microequivalents (μeq.).

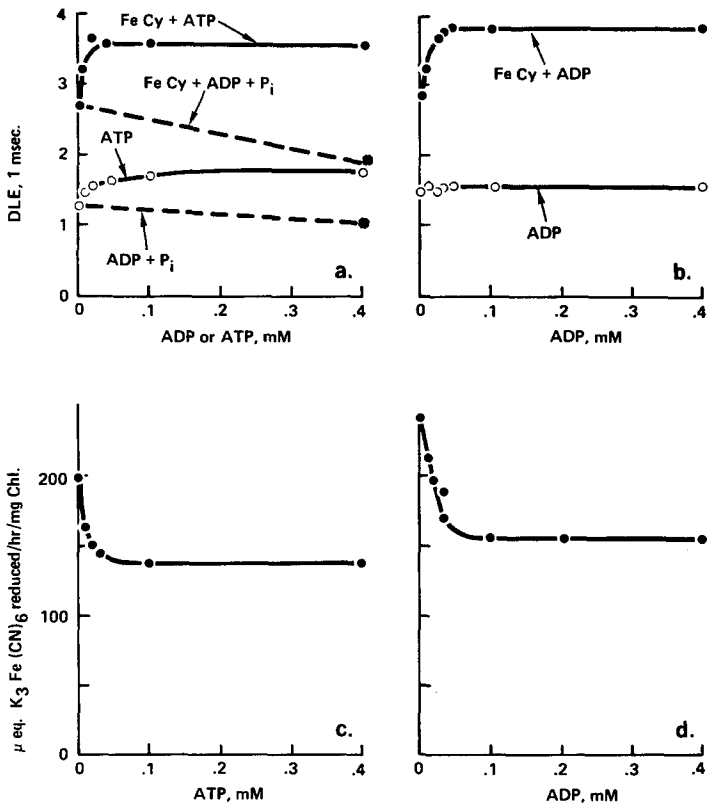

Fig. 2. Effect of ADP or ATP concentrations on the stimulation of delayed light emission and inhibition of ferricyanide reduction. Experimental conditions as in Fig. 1. Tricine buffer: pH 8.5.

order to exclude the possibility that our ADP solutions were significantly contaminated with ATP, we pretreated the reaction mixtures containing ADP for a few minutes with desalted hexokinase and glucose, before addition of chloroplasts. Inclusion of hexokinase and glucose in the reaction mixtures did not change the effect of ADP on delayed light. McCarty et al. [11] found, similarly, that hexokinase and glucose did not decrease the ability of ADP to stimulate H^+ uptake in chloroplasts.

Figs 3a and b compare the ADP concentrations required for increasing delayed light (absence of P_i) and decreasing delayed light (presence of P_i) with the concentrations of ADP required for the opposite effects on ferricyanide reduction. This experiment was carried out at pH 8, where both effects can be observed. Both optimal coupled electron transport, and optimal decrease in delayed light, occurred at about 0.3 mM ADP and 0.6 mM P_i. Avron et al. [10] similarly observed that maximal photophosphorylation and coupled electron transport occurred at the same ADP concentrations. P_i concentration in this experiment was arbitrarily set to twice that of ADP. ADP alone increased delayed light and decreased basal electron transport, both effects being shown at lower ADP concentrations than those required for the

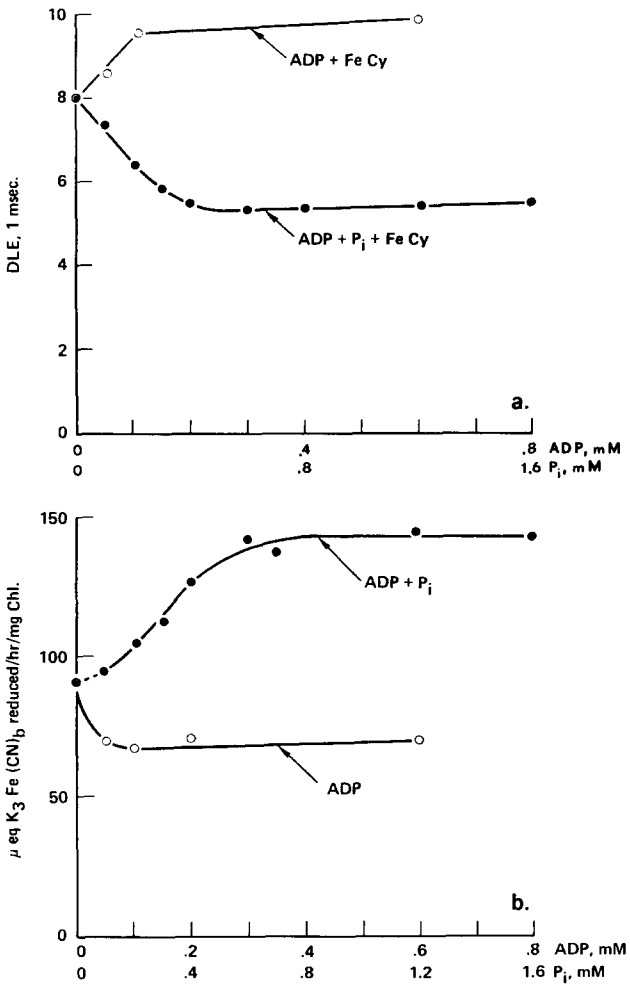

Fig. 3. Effect of ADP and ADP+P_i concentrations on delayed light emission and ferricyanide reduction. Experimental conditions as in Fig. 1. Tricine buffer: pH 8.0.

opposite effects found in the presence of P_i. More ADP appeared to be required at pH 8.0 than at pH 8.5 for maximal increase in delayed light, but this was not rigorously tested.

Table I gives the effect of the energy transfer inhibitor phloridzin on the adenylate-induced changes in delayed light, and on the adenylate-induced changes in ferricyanide reduction. As demonstrated previously [6], phloridzin completely inhibited the reduction in delayed light caused by phosphorylating conditions, and concomitantly reduced the rate of coupled electron transport to the basal level [23]. However, contrary to DIO-9 [6, 12], phloridzin did not stimulate delayed light in the presence of ferricyanide, nor did it affect basal electron transport. On the other hand, phloridzin was similar to ATP in that it stimulated delayed light in the absence of acceptor. Furthermore, the ATP and phloridzin increases in acceptorless delayed

TABLE I

EFFECT OF PHLORIDZIN ON DELAYED LIGHT EMISSION AND FERRICYANIDE REDUCTION IN THE PRESENCE AND ABSENCE OF ADENYLATES

Experimental conditions were as described in Fig. 1, except reaction mixtures minus adenylates preincubated 2 min at room temperature. Adenylates were then added and preincubated for 1 min before determination of delayed light or $K_3Fe(CN)_6$ reduction. Controls preincubated for 3 min. pH of Tricine buffer: 8.0.

Additions	Delayed light (mV at 1 ms)		$K_3Fe(CN)_6$ reduction (μequiv/h/mg chloroplasts)
	No acceptor	$+K_3Fe(CN)_6$	
Control	120	270	118
+0.2 mM ADP	120	355	80
+0.2 mM ATP	155	375	76
+0.4 mM ADP			
+0.8 mM KP_i	80	180	158
1 mM Phloridzin	150	250	118
+0.2 mM ADP	155	310	73
+0.2 mM ATP	182	310	76
+0.4 mM ADP			
+0.8 mM KP_i	130	300	70

light appear to be additive (ATP alone stimulated 35 units, phloridzin alone stimulated 29 units, both stimulated 62 units). Neumann et al. [6] reported that phloridzin increased delayed light in the presence of ferricyanide, but we did not find this effect. We do not understand the reason for this disagreement. Lack of phloridzin effect on basal electron transport was also reported by Izawa et al. [24].

Phloridzin effects on the ADP- and ATP-induced increases in delayed light were variable in the presence of ferricyanide. In different experiments we found the effect of phloridzin to range from almost no inhibition to almost complete inhibition of the ADP- and ATP-induced increases in delayed light. Yet phloridzin never affected the ADP or ATP inhibitions of basal electron transport, even in experiments where it strongly inhibited the ADP and ATP increases in delayed light (see Table I).

The uncoupler gramicidin at 1 μM concentration doubled electron transport while reducing the delayed light to about 25 % in either the presence or absence of ferricyanide. The uncoupler also abolished all adenylate effects on delayed light and electron transport. Partial removal of CF_1 with our "harsh" EDTA stripping increased ferricyanide reduction 2.5 times, which was about 50 % above that of control chloroplasts under phosphorylating conditions. Delayed light was reduced about 4–10 fold, and all adenylate effects on delayed light and electron flow were abolished. This harsh EDTA stripping, however, damaged thylakoids to the point that readdition of CF_1 would not reconstitute the system.

Table II gives the effect of our 'mild' EDTA stripping, and reconstitution by adding back CF_1. In these partially resolved thylakoids delayed light was reduced about 2–3 fold, rate of electron flow was increased approximately to that of control chloroplasts under phosphorylating conditions, and adenylate effects were either

TABLE II

EFFECT OF MILD EDTA STRIPPING, AND OF RECONSTITUTION WITH CF_1, ON DELAYED LIGHT EMISSION AND FERRICYANIDE REDUCTION IN THE PRESENCE AND ABSENCE OF ADENYLATES

Experimental conditions as in Table I. pH of Tricine buffer: 8.0. EDTA treatment: 0.2 μg chlorophyll/ml 0.001 M EDTA. For reconstitution: 50 μg chlorophyll of EDTA chloroplasts, 50 μmoles Tricine buffer, 4 μmoles of $MgCl_2$ to a final volume of 1 ml. Partially purified, desalted, CF_1 added as indicated. After 15 min on ice the reaction mixtures were diluted to 5 ml.

Additions	Delayed light (mV at 1 ms)		$K_3Fe(CN)_6$ reduction (μequiv/h/mg chloroplasts)
	No acceptor	$+K_3Fe(CN)_6$	
Control chloroplasts	370	3 200	111
+0.2 mM ADP	370	3 200	111
+0.2 mM ATP	390	3 500	99
+0.4 mM ADP			
+0.8 mM KP_i	350	2 600	144
Mild EDTA stripped chloroplasts	150	1 400	133
+0.2 mM ADP	145	1 350	138
+0.2 mM ATP	170	1 500	134
+0.4 mM ADP			
+0.8 mM KP_i	145	1 350	138
Mild EDTA + 500 μg CF_1	200	2 700	101
+0.2 mM ADP	200	2 700	102
+0.2 mM ATP	220	3 200	92
+0.4 mM ADP			
+0.8 mM KP_i	200	2 400	118

abolished or reduced in magnitude. In this particular experiment the adenylate effects on delayed light of control chloroplasts in the absence of acceptor were either small or absent. Furthermore, the ADP effect was completely absent in both delayed light and electron flow. This was not an unusual situation at pH 8.0, where we reliably observed reconstitution with CF_1, but which is not the optimal pH for observation of the ADP effects (see Fig. 1). In other experiments we found that our mild EDTA stripping reduced all these adenylate effects when they were present at pH 8.0, and that readdition of CF_1 increased the adenylate effects in such experiments. The data of Table II shows that readdition of CF_1 to partially resolved thylakoids reconstituted only those nucleotide effects which were present in the control chloroplasts.

Table III gives the effect of antibody to CF_1 on the three adenylate effects. Control serum had no effect on delayed light or electron transport in either the presence or absence of ADP, and control serum did not abolish any of the nucleotide effects. In other experiments we found that control serum also had no effect in the presence of either ATP or ADP plus P_i. Table III shows that in comparison to control serum, antiserum to CF_1 reduced delayed light, increased the rate of electron flow, and abolished or reduced all adenylate effects. Table III also shows that the effects of antiserum are pH dependent. Antiserum abolished all nucleotide effects at pH 7.95,

TABLE III

EFFECT OF ANTIBODY TO CF_1 ON DELAYED LIGHT EMISSION AND FERRICYANIDE REDUCTION IN THE PRESENCE AND ABSENCE OF ADENYLATES

Chloroplasts (50 ml) containing 50 μg of chlorophyll were mixed with 25 μl of either control serum or anti-CF_1 serum at about 3 °C. After about 15 s, reaction mixture (25 mM Tricine buffer containing 4 mM $MgCl_2$ and 25 mM NaCl) was added, and nucleotides where indicated, to a final volume of 5 ml. Reactions were performed in the order shown.

pH	Additions	Delayed light (mV at 1 ms) $K_3Fe(CN)_6$		$K_3Fe(CN)_6$ reduction (μequiv/h/mg chloroplasts)
8.4	chloroplasts	540	3 500	195
8.4	+0.2 mM ADP	460	4 400	165
8.4	+control serum	520	3 700	204
8.4	+control serum+0.2 mM ADP	520	4 600	171
8.4	+anti-CF_1	370	2 900	233
8.4	+anti-CF_1+0.2 mM ADP	380	3 400	306
7.95	+control serum	640	5 200	114
7.95	+control serum+0.2 mM ADP	680	5 600	94
7.95	+anti-CF_1	400	3 800	171
7.95	+anti-CF_1+0.2 mM ADP	370	3 750	165
8.4	+control serum	440	3 300	123
8.4	+control serum+0.4 mM ADP+0.8 mM P_i	300	2 450	150
8.4	+anti-CF_1	340	2 700	158
8.4	+anti-CF_1+0.4 mM ADP+0.8 mM P_i	270	2 300	168
7.95	+control serum	540	4 800	114
7.95	+control serum+0.4 mM ADP+0.8 mM P_i	500	3 800	152
7.95	+anti-CF_1	400	3 800	171
7.95	+anti-CF_1+0.4 mM ADP+0.8 mM P_i	350	3 700	173
8.4	+control serum	440	3 300	123
8.4	+control serum+0.2 mM ATP	520	3 800	100
8.4	+anti-CF_1	340	2 700	158
8.4	+anti-CF_1+0.2 mM ATP	350	2 600	158

but at pH 8.4 it only reduced effects of ADP and of ADP plus P_i. In different experiments we sometimes found no inhibition by antiserum of ADP and of ADP plus P_i effects at the higher pH, although in the same experiments the ATP effect was always abolished regardless of pH.

DISCUSSION

The close correlations between delayed light emission and rates of electron transport suggest that overall intensity of millisecond delayed light is reflecting regulation of electron transport by adenylates. Since gramicidin, removal of CF_1 with EDTA, and antibody to CF_1 (Table III) all abolished or strongly reduced the adenylate effects, the adenylate site of action is presumably CF_1. This view is strengthened by the fact that addition of CF_1 to EDTA-stripped thylakoids regenerates the ade-

nylate effects (Table II). Thus the present adenylate-induced changes in delayed light reflect adenylate binding to functional CF_1 in the thylakoid.

The different dependence on pH (Fig. 1) shown in both delayed light and electron transport suggests that the three adenylate effects are all acting through different mechanisms. On the other hand, the effects of ATP and ADP in the absence of P_i are shown at similar concentrations, and these concentrations are lower than the ADP concentration required for optimal effects in the presence of P_i (Figs 2 and 3). Thus, the adenylate concentration curves suggest only two different binding sites, with possibly ATP being the active agent in increasing delayed light and decreasing electron transport. In this case, the ADP effect would be due to conversion of ADP to ATP at the binding site, possibly by a myokinase-like reaction of the sort described by Roy and Moudrianakis [16]. This suggestion is in agreement with the lack of ADP effect on delayed light in the absence of electron transport, as contrasted to the ATP-induced increase in delayed light, which does not require electron transport (Figs 2a and b).

However, the inability of anti-CF_1 to abolish effects of ADP and of ADP plus P_i at pH 8.4, while completely abolishing ATP effects at this pH (Table III), lends support to the idea that the ATP binding site is not identical to either of the ADP binding sites. Thus, our evidence taken as a whole suggests at least two, and possibly three, adenylate binding sites on membrane-bound CF_1.

Phloridzin has been shown to completely inhibit photophosphorylation [23], ADP and orthophosphate increases in electron transport [23] and light-triggered ATPase [24]. However, the ADP- and ATP-induced increases in proton uptake are only partially inhibited by phloridzin [11], which is similar to the phloridzin effects we report on the ADP- and ATP-induced increases in delayed light in the presence of acceptor (Table I). The complete lack of phloridzin effect on the ATP-induced increase in delayed light in the absence of electron transport, and the complete lack of phloridzin effect on basal electron transport and its inhibition by ADP and ATP, suggest that the second binding site (as well as the hypothetical third site) is insensitive to phloridzin. In this respect, phloridzin is apparently quite different from DIO-9, which abolishes the adenylate effects [6, 11, 12].

Several other observations on CF_1 indicate the presence of two or more adenylate binding sites. Roy and Moudrianakis [16] found a biphasic binding profile for in vitro binding of [^{14}C]ADP to CF_1, with half-maximum bindings at about $2 \cdot 10^{-6}$ M and $3.5 \cdot 10^{-5}$ M ADP. Girault et al. [17] showed that at saturation of CF_1 in vitro, 2 moles of ADP were bound per mole of CF_1. McCarty et al. [26] found that the concentration of ADP required to protect photophosphorylation from inhibition by *N*-ethylmaleimide is lower than the ADP concentration required for maximal phosphorylation rates. Datta et al. [27] found that the half-maximum binding for ADP to membrane-bound CF_1 is about 10^{-5} M for the high-affinity binding site, based on experiments with permanganate or sulfate inactivation of CF_1 on functioning thylakoid membranes. This is in good agreement with our delayed light data (Fig. 2b) on functional membrane-bound CF_1, and contrasts with the in vitro measurements [16, 17]. Nelson et al. [15] have prepared antibodies against five subunits isolated from CF_1. Two antibodies inhibited photophosphorylation (anti-α and anti-γ) and the same two antibodies inhibited the ATP-induced stimulation of proton uptake in chloroplasts. Interestingly, Harris et al. [28] found beef

heart mitochondrial coupling factor (F_1) to contain five moles of bound adenylates: two moles of ADP and three moles of ATP. We know of no comparable studies with chloroplast coupling factor, CF_1, so the number of ATP binding sites on CF_1 is uncertain. The functional importance of the several adenylate binding sites remains unclear, although a regulatory role seems likely for the high affinity ADP binding site on CF_1.

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