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Regulation of Electron Transport in Isolated Chloroplasts by Sequential Binding of Adenine Nucleotides to the Coupling Factor Protein*

Yasuo Mukohata, Takao Yagi, Yasuo Sugiyama, Akemi Matsuno, and Mitsuhiro Higashida

Department of Biology, Faculty of Science, Osaka University, Toyonaka, Japan 560

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Abstract

Changes in the ferricyanide-reducing activity in isolated spinach chloroplasts by adenine nucleotides were measured in the presence or absence of phosphate, arsenate or pyrophosphate at 15°C. The activity changes were analyzed and ascribed to the interaction between the nucleotides and the chloroplast coupling factor (CF_1).

ADP and ATP (but not AMP) partially inhibited ferricyanide reduction through a 1:1 binding to the inhibition site on CF_1.

When the ferricyanide reduction was coupled to either phosphorylation or arsenylation, the inhibition by ADP was released through a 1:1 binding of the second ADP molecule to the coupling site on the CF_1 with which the first ADP had been associated.

The association constants of ADP for the inhibition and the coupling site were found to be approximately 5×10^5 and 6×10^4 M^{-1}, respectively. The *Km* value of ADP for arsenylation (pH 8.3) was around 17 μM.

The ADP-regulated electron transport was defined based on these results. The ADP-regulated ferricyanide reduction, when coupled with phosphorylation, revealed a stoichiometry of $P/\Delta e = 1$ between the amounts of esterified phosphate and reduced ferricyanide.

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Introduction

Avron *et al.* [1] reported that the Fecy (ferricyanide) reduction in isolated chloroplasts was partially inhibited by ATP (or ADP) at 3 μM. McCarty *et al.* [2] reported that light-induced proton uptake was stimulated by the presence of ATP (or ADP, both in the 1-10 μM range). Telfer and Evans [3] showed that ADP or ATP (at 27 μM) decreased the proton permeability of the thylakoids. Shavit and Hercovici [4] reported that ATP formation by acid-base transition was inhibited by ATP (above 10 μM) added in the acid but not the basic stage.

Racker's school isolated and purified [5] CF_1, and found it to be composed of five different subunits [6], two of which were capable of hydrolyzing ATP when activated [7]. Roy and Moudrianakis [8] reported that there were two binding sites on CF_1 for ADP but not for AMP (added externally). Girault *et al.* [9] confirmed this finding by showing that two ADP molecules were bound stepwise by purified CF_1 as the ADP concentration increased.

Meanwhile, chloroplasts prepared in a choline medium were found to be superior to the other preparations in biological activities [10, 11], and suitable for quantitative analysis of the regulation pattern (inhibition and restoration process) of Fecy reduction by adenine nucleotides. We attempted to analyze the regulation pattern through a model of a sequential binding of these nucleotides by CF_1.

Experimental Procedures

Chloroplasts were prepared from market spinach leaves by a method described previously [10]. The preparation medium contained 0.5 M choline chloride, 5 mM $MgCl_2$ and 20 mM Tricine (tris(hydroxymethyl)methylglycine) at pH 7.8. The reaction mixture contained 0.1 M sucrose, 5 mM $MgCl_2$, 10 mM Tricine (pH 8.3), 600 μM Fecy and chloroplasts equivalent to 20 μg/ml in the chlorophyll concentration determined according to the method of Arnon [12]. In experiments on the pH dependence of Fecy reduction, a mixed buffer composed of citrate, piperazine-N-N'-bis(2-ethane sulfonic acid), Tricine and sarcosine (10 mM each) was used instead of a single Tricine buffer. Nucleotides (from Sigma Chemical Co.), inorganic phosphate (^{32}Pi for phosphorylation assay), PP*i*, A*s* (arsenate) and methylamine hydrochloride were added to the reaction mixture as required.

Fecy reduction was determined from the difference in the absorbance at 420 nm before and after actinic illumination (5×10^4 lux, white light) given for a few minutes in a water bath at $15 \pm 0.1°$ C. The amounts of ^{32}Pi esterified during Fecy reduction was determined by a modification of the method of Asada *et al.* [13].

Results

Figure 1 illustrates the effects of adenine nucleotides on Fecy reduction and phosphorylation. ADP and ATP almost equally inhibited Fecy reduction. The inhibition became largest at ADP (or ATP) concentrations above 50 μM. In contrast, AMP and IDP inhibited Fecy reduction only at concentrations higher than 100 μM, possibly due to contaminating ADP in these nucleotide reagents. When P*i*, A*s*, PP*i* or adenosine was added separately (up to 1 mM), Fecy reduction was not inhibited (data not shown). However, as Fig. 1 shows, addition of P*i* (or A*s*, e.g. 1 mM) along with ADP (or ATP) increased the extent of inhibitions. On the contrary, addition of PP*i* decreased the extent of inhibition by ADP (or ATP; not shown).

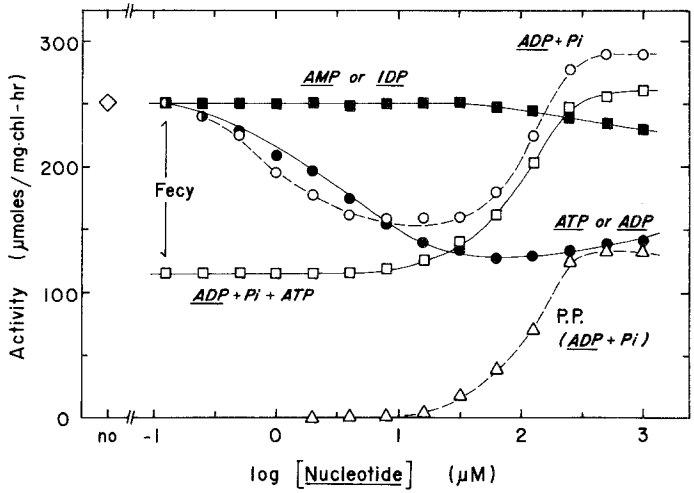

Figure 1. Effects of nucleotides on Fecy-reducing activity in isolated spinach chloroplasts (pH 8.3, 15° C). The concentration of the underlined nucleotides was changed, and that of the other reagent(s) was 1 mM. The activity profiles obtained in the presence of IDP and ADP were, within the range of experimental error, identical with those of AMP (■) and ATP (●), respectively. The effect of ADP on phosphorylation activity (△, P.P.) is also shown. The plotted rates of the reactions under phosphorylation condition (△, ○ and □) at 1 μM < ADP < 100 μM were lower than the estimated initial rates because of ADP shortage in the reaction mixture during actinic illumination. For further details, see text.

When P*i* (or A*s*) was present along with ADP at the concentrations higher than 10 μM, inhibition was seemingly released and Fecy-reducing activity was restored, reaching a maximum level of ADP concentrations above 500 μM. The observed rates of Fecy reduction (and phosphorylation) were less than the true initial rates for phosphorylating conditions

due to exhaustion of ADP at concentrations below 100 μM. The extent of restoration of Fecy reduction (Δe as equimolar electrons) from the ADP-inhibited activity level could thus be compared with the extent of phosphorylation (P; as *Pi* esterified) only at ADP concentrations above 100 μM. Then a stoichiometric relationship between P and Δe was found to be close to 0.9 from Fig. 1. ADP above 1 mM in the simultaneous presence of *Pi* (or *As*) depressed the restoration (not shown).

We also found that Mg^{2+} was required for the inhibition by ADP or ATP as well as for the restoration coupled with phosphorylation or arsenylation (reported elsewhere).

Figure 2 shows a Hill plot [14] for the inhibition of Fecy reduction by ADP or ATP. For this plot, the following assumptions were made: (1) one electron transport chain involves several binding sites for ADP (and ATP), and some of them are critical for the electron transport activity; (2) when ADP (or ATP) molecules bind to all of these critical sites, they cooperate to inhibit the activity of

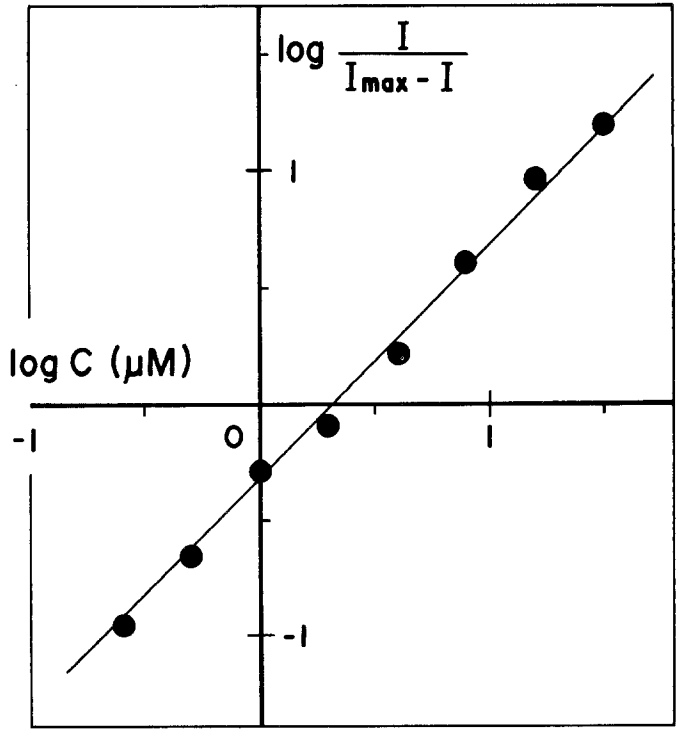

Figure 2. Hill plot of the inhibition process of Fecy reduction caused by ADP (= that by ATP in Fig. 1; ●). C = ADP concentration. For details, see text.

the chain; (3) at an ADP (or ATP) concentration high enough to cause maximum inhibition (extent $= I_{max}$) in the Fecy reduction measured, all of the critical sites in chloroplasts in the reaction mixture are occupied by ADP (or ATP). From the plot, the slope n_i, i.e. the number of cooperative critical binding sites, was found to be 1 (1.0-1.2, depending on the chloroplast preparation and experimental accuracy) with an association constant K_i of 4.8×10^5 $((2.2\text{-}8.0) \times 10^5)\,M^{-1}$.

The extent of inhibition largely depended on the chloroplast preparation. Inhibition was observed distinctly with choline-prepared chloroplasts, but only to a small extent with chloroplasts prepared in an ordinary (0.5 M sucrose) medium (these chloroplasts had lower phosphorylation activity than choline-prepared ones [10, 11]). Uncouplers or EDTA-washing (removal of CF_1 [15]) diminished both inhibition and restoration. Figure 3 shows the effect of methylamine on the Fecy-reducing activities measured in the presence of 100 μM ADP ($Fecy_{ADP}$), or 1 mM ADP and 1 mM Pi ($Fecy_{ADP,Pi}$) or without either ($Fecy_{none}$). As the methylamine concentration increased, the extent of inhibition ($\Delta Fecy_{inhibited}$ = $Fecy_{none} - Fecy_{ADP}$) decreased almost parallel to the extent of restoration ($\Delta Fecy_{coupled} = Fecy_{ADP,Pi} - Fecy_{ADP} = \Delta e$).

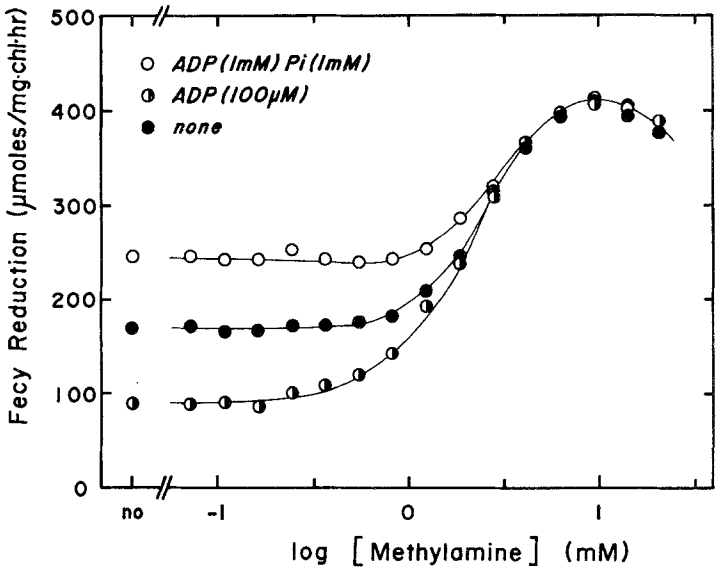

Figure 3. Effects of methylamine on Fecy-reducing activities in isolated spinach chloroplasts (pH 8.3, 15°C) in the presence or absence of ADP and/or Pi. ◑, $Fecy_{ADP,Pi}$ in the presence of both 1 mM ADP and 1 mM Pi; ○, $Fecy_{ADP}$ in the presence of 100 μM ADP only; ●, $Fecy_{none}$ in the absence of both.

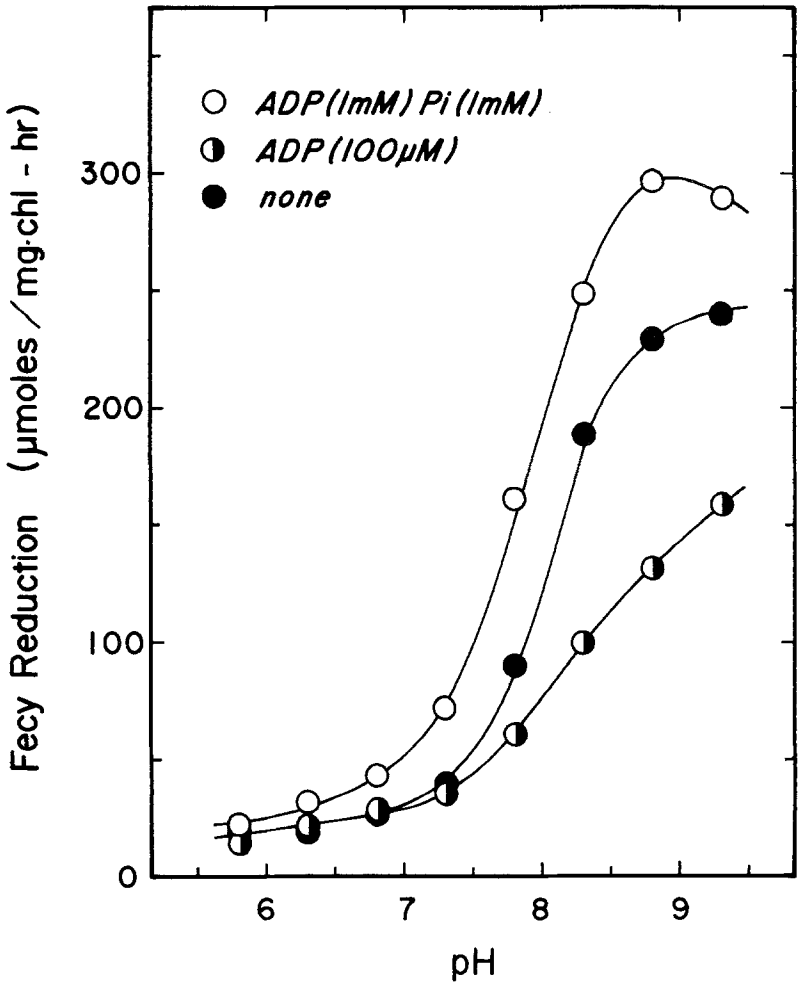

Figure 4. (A) pH Dependence of Fecy-reducing activities in isolated spinach chloroplasts (15°C) in the presence or absence of ADP and/or Pi. Symbols are the same as in Fig. 3. (B) pH Dependence of the difference in the activities shown in Fig. 4A. △ $Fecy_{coupled}$ (●) = $Fecy_{ADP,Pi}$ − $Fecy_{ADP}$ and △ $Fecy_{inhibited}$ (○) = $Fecy_{none}$ − $Fecy_{ADP}$. Phosphorylation activity (△; coupled with $Fecy_{ADP,Pi}$) is also plotted.

Figure 4A shows the pH dependence of $Fecy_{ADP}$, $Fecy_{ADP,Pi}$ and $Fecy_{none}$. Figure 4B shows the pH dependence of the activity of phosphorylation coupled with $Fecy_{AD,Pi}$, and $\Delta Fecy_{inhibited}$ and $\Delta Fecy_{coupled}$ obtained from Fig. 4A. Phosphorylation activity and

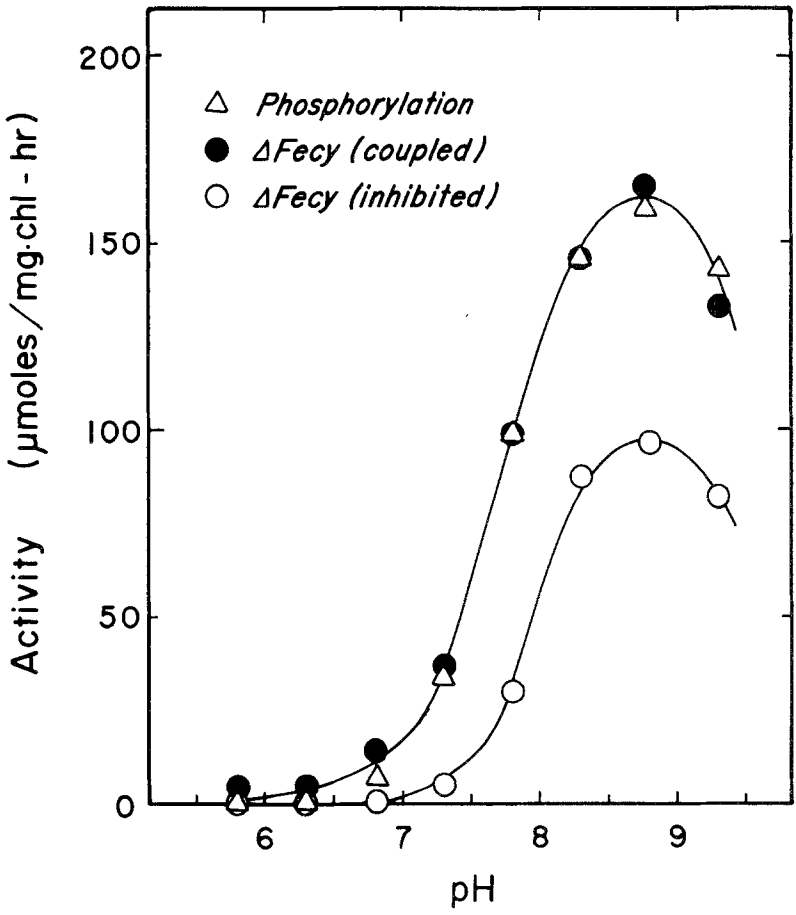

Fig. 4(B).

these ΔFecy activities exhibited similar bell-shaped pH dependence curves with the same pH optimum of about 8.5. Note that there was again an obvious stoichiometric relationship, $P/\Delta e = 1$ within the pH range measured.

Figure 5 represents the Fecy-reducing activities measured in the presence of ADP and 1 mM As ($Fecy_{ADP,Pi}$) or of ATP and 1 mM As ($Fecy_{ATP,As}$) *vs.* the nucleotide concentration. As the ADP concentration increased, as in the case of $Fecy_{ADP,Pi}$ in Fig. 1, $Fecy_{ADP,As}$ was first inhibited then restored under the condition of arsenylation. $Fecy_{ATP,As}$ was only inhibited as the ATP concentration increased. The difference, the extent of restoration coupled with arsenylation was taken to be $\Delta Fecy_{restored}$ =

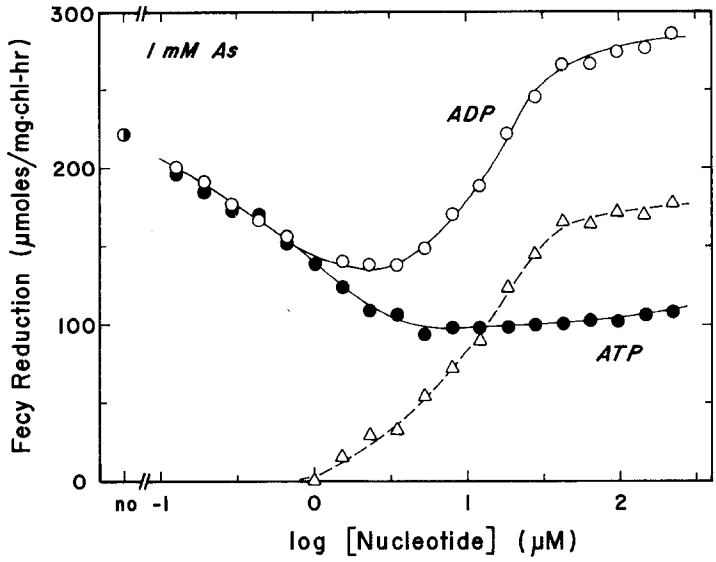

Figure 5. Fecy-reducing activities in isolated spinach chloroplasts (pH 8.3, 15°C) at various ADP or ATP concentrations in the presence of As. ○, Fecy$_{ADP,As}$ in the presence of both ADP and 1 mM As; ●, Fecy$_{ATP,As}$ in the presence of both ATP and 1 mM As. The difference of these activities, △ Fecy$_{restored}$ = Fecy$_{ADP,As}$ — Fecy$_{ATP,As}$, is also plotted (△).

Fecy$_{ADP,As}$ — Fecy$_{ATP,As}$ and is also plotted in Fig. 5 (the broken line).

In these experiments, we confirmed that the time course of Fecy$_{ADP,As}$ was linear as described by Avron and Jagendorf [16]. The rate of Fecy$_{ADP,As}$ was, within experimental error, identical* with the asymptotic initial rate of Fecy$_{ADP,Pi}$ (the time courses were curved at ADP concentrations below 100 μM because of ADP exhaustion as described above) at the same ADP concentration. The rates of Fecy$_{ATP,As}$ and Fecy$_{ATP,Pi}$ (both had linear time courses) agreed with each other. Therefore, it appeared adequate to approximate Fecy$_{ADP,As}$ as Fecy$_{ADP,Pi}$ and ΔFecy$_{restored}$ as ΔFecy$_{coupled}$ over the ADP concentration range measured.

Figure 6 represents a Lineweaver-Burk plot for ΔFecy$_{restored}$ shown in Fig. 5. Here, we assumed that restoration takes place in the presence

* Polarographic traces of oxygen evolution under our experimental conditions indicated that the rates of Fecy$_{ADP,As}$ were about 10% larger than the asymptotic initial rates of Fecy$_{ADP,Pi}$ throughout an ADP concentration range of 0.5 to 500 μM. Therefore, the V_{max} value of Fecy$_{ADP,As}$ could differ from that of Fecy$_{ADP,Pi}$, while the K_m values would be close to each other.

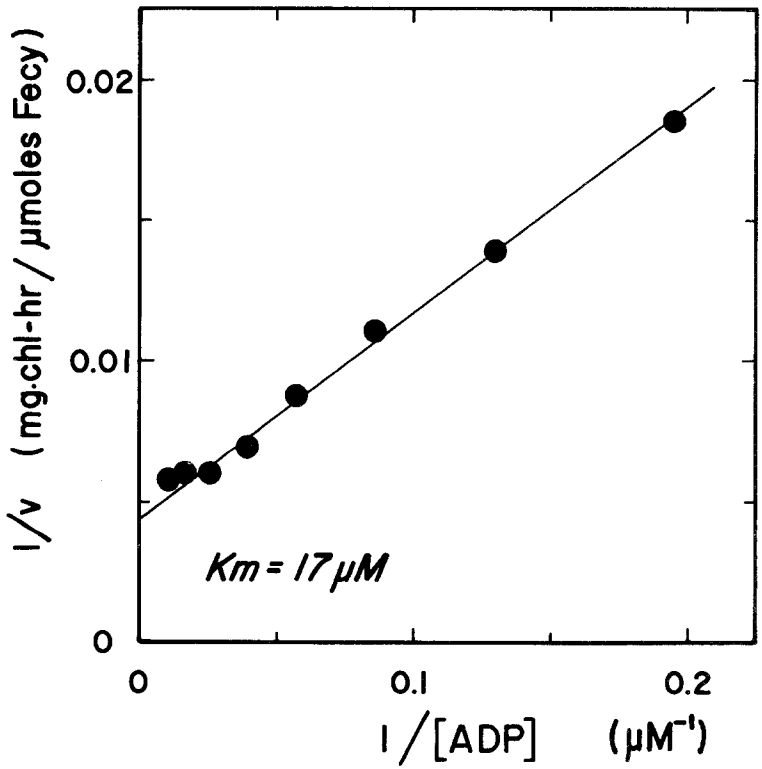

Figure 6. Lineweaver-Burk plot of Δ Fecy$_{restored}$ shown in Fig. 5 ($\triangle$). For details, see text.

of As (or Pi) as the second ADP molecule(s) bind(s) to the site(s) on the once-inhibited electron transport chain. The plot appeared to be straight and the V_{max} value was estimated to be 230 (145-270) μmoles Fecy reduced/mg chlorophyll hr. The *Km* value for ADP was 17 (11-20) μM and agreed with the value for the acid-base transition phosphorylation (12-15 μM) reported by Yamamoto and Tonomura [17]. Therefore, the number of assumed cooperative binding sites for ADP in the restoration process, n_c, would be close to 1 and the association constant for this second ADP, K_c, would be around 6 (5-9) x 10^4 M^{-1}.

Discussion

Figure 1 confirms the observation of Avron *et al.* [1] and shows that non-cyclic electron transport is inhibited by not only ATP but ADP

itself.* Figure 1 also shows that neither AMP nor IDP inhibited Fecy reduction, and that the Fecy-reducing activity under ATP inhibition was restored as phosphorylation proceeded.

Similarity in the effects of an uncoupler (Fig. 3) and pH (Fig. 4) on both inhibition and restoration of Fecy reduction strongly implies the involvement of CF_1 not only in the (phosphorylation-coupled) restoration, but also in the inhibition mechanism, as suggested by Avron *et al.* [16]. The effect of ATP (and ADP) at such low concentrations (around 10 μM) on the proton permeability of the thylakoid membrane has been ascribed to the interaction of ATP (and ADP) and CF_1 [2, 3].

Roy and Moudrianakis [8] and Forti *et al.* [18] showed that the binding of two ADP molecules by one CF_1 is an essential step for photophosphorylation. Girault *et al.* [9] supported this scheme by a study of the interaction between purified CF_1 and ADP (and ATP) using a technique of circular dichroism spectropolarimetry. They found that one CF_1 bound two ADP molecules stepwise with an increase in the ADP concentration, and estimated the association constants of this two-step binding to be 8×10^5 and approx. 4×10^5 M^{-1} for the first and second ADP, respectively. They also reported that the binding of AMP (externally added) was fairly weak, and that PP_i inhibited the binding of ADP by CF_1.

The n_i or n_c value of around 1 obtained here from Fig. 2 or 6 simply meant that the binding of ADP molecule(s) to the assumed site(s) in the inhibition and restoration process was not cooperative but independent. However, the K_i (2.2 to 8×10^5 M^{-1}) and K_c (5 to 9×10^4 M^{-1}) values obtained here agreed fairly well with the values determined physicochemically [9]. The nucleotide specificity (ADP and ATP but not AMP nor IDP) in the inhibition process and the effect of PP_i were also consistent with the results of Girault *et al.* [9]. The restoration process to which the second ADP binding was assigned, paralleled the phosphorylation process.

Therefore, when these circumstances are taken into account, it becomes likely that on CF_1, at least one binding site is responsible for each one of the two processes, interacts with ADP in a 1:1 ratio and apparently regulates electron transport. Of these two independent sites, one for the first ADP (or ATP) would be the inhibition site and the other for the second ADP (or AMP, reported in the succeeding paper) would be the coupling site.

The inhibition site, which would be located on one (probably the α-subunit [6]) of the two (latent ATPase) subunits [7] of CF_1, is specific

* The electron transport, e.g. from water to 2,6-dichlorophenolindophenol (DPIP) and from reduced DPIP to methyl viologen, was also inhibited partly and then restored with increasing ADP concentration in the presence of *Pi* or *As*.

for adenosine with at least a pyrophosphate group. When the inhibition site is occupied by ADP or ATP, a conformation change will be induced in CF_1. Such conformation changes have been suggested by McCarty *et al.* [2], Telfer and Evans [3] and Girault *et al.* [9] and would cause inhibition of electron transport, probably as a result of the decrease in proton permeability (leakage) of the thylakoids [2, 3].

The coupling site, seemingly exposed through the conformation change, would be on the other subunit. When the coupling site is occupied by ADP in the presence of P_i (or A_s), phosphorylation (or arsenylation) would take place, accompanying a stoichiometric translocation of protons [19]; $P/\Delta H^+ = 0.5$ [20], which are supplied by a proton pump driven by electron transport ($\Delta H^+/\Delta e = 2$ [21, 22]). Consequently, the extent of restoration of Fecy-reducing activity from the once-inhibited level is stoichiometric with the amount of *Pi* esterified ($P/\Delta e = 1$). For the turnover of phosphorylation, there would be another, light-dependent, conformation change that has been suggested by Ririe and Jagendorf [23] and McCarty and Fagan [24]. For the $P/\Delta H^+$ value, Schröder *et al.* [25] reported the value of 0.33 that was supported by Portis and McCarty [26]. If this is the case, the $P/\Delta e$ value should not exceed 0.67 which is much smaller than the value obtained on the basis of the regulation mechanism described above.

This is one possible explanation for the mechanism regulating photosynthetic activities through the sequential binding of adenine nucleotides by CF_1, although the mechanism of phosphorylation itself still is unknown. AMP in the presence of *As* uniquely affected the restoration of Fecy reduction inhibited by ATP (or ADP, reported in the succeeding paper). This suggests that AMP could also be involved in the photophosphorylation mechanism [8].

The overall electron transport in isolated chloroplasts apparently consists of the electron transport regulated by ADP (or ATP) and that independent of ADP (or ATP; basal). The ADP-regulated electron transport activity is defined as the incremental activity above the activity level inhibited maximally by ADP or ATP. The remaining part of the activity is thus ADP-independent and, furthermore, Mg^{2+}-independent (reported elsewhere). Good *et al.* [27] proposed a stoichiometry of $P/\Delta e = 1$ (according to our notation, for full length of non-cyclic electron transport) using the electron transport activity measured in the absence of *Pi* as the basal activity. Reeves and Hall [28] showed the results similar to ours, in terms of photosynthetic control ratio using the ATP-independent activity as the basal. Here, we have shown a convincing basis for the ADP-regulated electron transport. We pointed out that the ADP concentration required for maximum inhibition was restricted to a limited range (around 100 μM for 100 μg chlorophyll/5 ml), and that ADP could be replaced by ATP within this concentration range.

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