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The kinetics of adenine nucleotide binding to chloroplast ATPase, CF_0-CF_1, during the illumination and post illumination period in isolated pea thylakoids

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The steady-state binding of [2-^{3}H]ADP to thylakoid membrane protonmotive ATPase (CF_0-CF_1) was studied using a quench technique similar to that developed by Strotmann, Bickel-Sandkotter and Shoshan (FEBS Lett. 101 (1979) 316–320). The amount of adenine nucleotide bound in the light and post-illumination dark period is shown to depend on the efficacy of uncoupling when the reaction is quenched. Previous observations that post-illumination binding of [2-^{3}H]ADP displays a fast and a slow phase of binding were confirmed only when uncoupling efficiency was relatively low during quenching. When uncoupling efficiency is increased, the fast phase of dark [2-^{3}H]ADP binding is abolished, and correspondingly more nucleotide appears to be bound upon quenching in the light. The previous assignment of the observed kinetic phases to different species of enzyme-nucleotide complex has been reassessed in the light of this new data.

Introduction

Net ATP synthesis in isolated chloroplasts is catalysed by the protonmotive ATPase, CF_0-CF_1, when an electrochemical potential difference in protons, $\Delta\tilde{\mu}_{H^+}$ is generated across the thylakoid membrane. It is generally assumed that $\Delta\tilde{\mu}_{H^+}$ is required for ATP synthesis in two quite distinct ways: (i) kinetically, to activate the enzyme, presumably via induced conformational changes; (ii) thermodynamically, to provide the free energy necessary for net synthesis [1,2].

There is now considerable evidence in support of these ideas, much of which has come from studies of bound nucleotides on the enzyme. Strotmann and colleagues [3,4] have shown that illumination of thylakoids causes the release or exchange of one adenine nucleotide/CF_1. Prior to illumination, the exchangeable nucleotide is tightly bound to CF_1 in a non-exchangeable form. Both the rate and extent of light-dependent nucleotide release correlate with the rate and extent of appearance of ATPase [5,6] or ATP $\leftrightharpoons$ P_i exchange [7] reactions measured in the subsequent dark period. The initial rate of release of bound nucleotide is as fast as the initial rate of ATP synthesis [8] when $\Delta\tilde{\mu}_{H^+}$ is generated artificially by electrical pulses. The extent of nucleotide release correlates with the amount of ATP synthesis induced by acid/base transitions [9]. These observations suggest that the kinetic activation of the

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Abbreviations: CCCP, carbonylcyanide *p*-trichloromethoxyphenylhydrazone; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; $\Delta\tilde{\mu}_{H^+}$, ΔpH, difference in electrochemical potential of protons or pH between the intrathylakoid space and the suspending medium; CF_0-CF_1, chloroplast protonmotive ATPase; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone.

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enzyme by $\Delta\tilde{\mu}_{H^+}$ correlates with the release or exchange of the tightly bound adenine nucleotide on membrane-bound CF_0-CF_1.

In the absence of medium nucleotides, release of the exchangeable nucleotide is complete upon illumination of thylakoids. The preilluminated membranes retain the ability to rebind ADP in the ensuing dark period, and, unlike release, rebinding does not require $\Delta\tilde{\mu}_{H^+}$ [3]. In the presence of medium nucleotides, a steady-state level of nucleotide remains tightly bound in the light, and this can be measured by quenching simultaneously with an uncoupler (to abolish $\Delta\tilde{\mu}_{H^+}$) and ADP (to prevent rebinding of labelled ADP via isotopic dilution) [4]. The amount of nucleotide that remains tightly bound in the light decreases with increasing $\Delta\tilde{\mu}_{H^+}$ [10] and the rebinding of ADP in the ensuing dark period is biphasic [4,5]. It is now generally assumed that the ADP that remains tightly bound in the light represents those enzymes that remain inactive [1–12]. The fast kinetics of binding in the following dark period are thought to be due to a class of 'loosely bound' adenine nucleotide that rapidly converts to tightly bound nucleotide upon dissipation of $\Delta\tilde{\mu}_{H^+}$ [4,5,11,12]. The slow kinetics of dark binding represent activated ATPase from which the loosely bound ADP has dissociated [3–5].

The relationship between the dissociated and loosely binding ADP forms of the enzyme and those enzymes that are catalytically active is unclear [10–12]. We have been interested in using the tightly bound ADP as an independent assay of enzyme activation before and after reductive modulation of ATPase activity [1,9]. During these studies, we observed that the steady-state binding of ADP in the light appeared to depend on the conditions of quenching. In this paper, we report our investigations of this effect, and this leads us to question the assignment of various enzyme species to the kinetic components of nucleotide binding that has up to now been made.

Materials and Methods

Intact chloroplasts were isolated from dark-adapted *Pisum sativum* (variety Feltham First), lysed and washed as described [1]. Experimentation was completed within 3 h of chloroplast isolation. Unless stated otherwise, determinations of bound nucleotides and of ΔpH were made simultaneously using 20 μg thylakoids suspended in 1 ml of a medium containing 20 mM KCl, 5 mM $MgCl_2$, 0.1 mM methyl viologen, 15 mM glucose, 5 μM 9-aminoacridine, 1000 U catalase, 10 U hexokinase, 5 μg diadenosine pentaphosphate, 5 μM ADP containing 37 kBq [2-^{3}H]adenosine diphosphate and 30 mM Tricine-KOH (pH 8.1). The suspension was contained in a magnetically stirred 10 mm tube inserted in the well of Hansatech DW2 oxygen electrode (Hansatech, Kings Lynn, Norfolk, U.K.). Actinic illumination was provided by a halogen lamp filtered through a Schott RG665 red-pass filter. 9-Aminoacridine fluorescence was detected as described [1].

Bound nucleotides were detected by the quench techniques developed in the laboratory of Strotmann et al. [4]. Unless otherwise stated, the reaction was terminated in the light or the dark by the rapid addition of 0.5 ml of quench medium containing 15 mM ADP/30 μM carbonylcyanide *p*-trichloromethoxyphenylhydrazone (CCCP)/0.3 M NH_4Cl/25 mM KCl/12.5 mM $MgCl_2$/25 mM Tricine-KOH (pH 8.1). The quenched samples were centrifuged and the pellets were washed three times by resuspension and centrifugation in 1 ml of wash medium containing 20 mM KCl/5 mM $MgCl_2$/5 mM NH_4Cl/25 mM Tricine-KOH (pH 8.1). The final pellet was resuspended in 0.5 ml of wash medium and the bound nucleotides were quantitatively released from the membranes by addition of 0.1 ml of 20% trichloracetic acid. After centrifugation to remove denatured membranes, 0.5 ml of the supernatent was added to 4 ml of scintillation fluid ('Ecoscint', National Diagnostics) and counted on an LKB 1219 liquid scintillation counter.

Results

Fig. 1A shows the amount of tightly bound ADP that can be detected on thylakoids using a quench method developed by Strotmann et al. [4]. Thylakoids were illuminated in the presence of 2 or 20 μM [2-^{3}H]ADP and the reaction was quenched in the light or dark by addition of excess unlabelled ADP and an uncoupler (5 μM

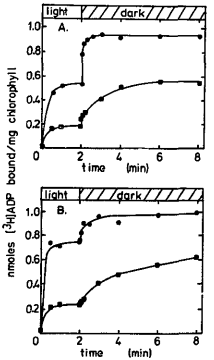

Fig. 1. The binding kinetics of [2-^{3}H]ADP to thylakoids in the light and a subsequent dark period. The data points indicate the times at which the reaction was stopped by addition of quench medium. The quench medium was either (A) modified by lowering CCCP to 15 μM and omitting NH_4Cl, or (B) not modified, containing 30 μM CCCP and 0.3 M NH_4Cl as described in Materials and Methods. The assay medium contained either 2 μM (■) or 20 μM (●) [2-^{3}H]ADP during illumination.

CCCP). A steady-state level of labelling is observed to be reached within 1 min in the light and further binding of ADP with biphasic kinetics is observed upon transition to darkness. These observations are essentially identical with those originally reported [4], although the experimental conditions are slightly different in that lower chlorophyll concentrations were used, chloroplasts were derived from peas rather than spinach and CCCP was used in place of FCCP. The general shapes of the binding curves have been confirmed in a number of other reports [5,10–12].

The rapid phase of nucleotide binding in the dark was suggested to reflect the inactivation of complexes to which ADP is loosely bound during the light. If this explanation is correct, then increasing the concentration of uncoupler in the quench medium should not decrease the amplitude of the fast phase of dark binding (defined as binding occurring in the first 2 s of terminating illumination). This has been observed by Schumann [11] in a study where the concentration of FCCP in the quench medium was varied. Fig. 1B shows that our observations are in apparent disagreement with this report. When the efficacy of the quench medium is increased by adding 0.3 M NH_4Cl together with CCCP and ADP, then the fast phase of dark binding is abolished, the slow phase being largely unaffected. The disappearance of the fast phase appears to be due to an increase in the level of nucleotides apparently bound when the membranes are quenched in the light.

Fig. 2 shows in more detail the effect of uncoupler concentration in the quench medium and provides a partial explanation for the discrepancy in the data presented here with that reported previously [4,5,11]. Fig. 2A depicts the levels of bound ADP detected upon quenching in the light and after 2 s dark when the quench medium contains only CCCP and unlabelled ADP. The difference between the curves represents the fast phase of dark binding, which is clearly maximal at low concentrations of uncoupler. Increasing the CCCP concentration decreases the extent of the

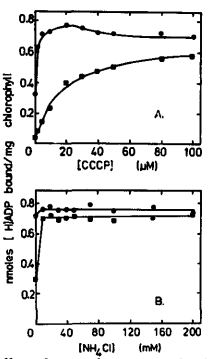

Fig. 2. The effect of uncoupler concentration in the quench medium on the amounts of [2-^{3}H]ADP bound to thylakoids. The reaction was quenched in the light after 2 min illumination (■) or after 2 min light and a subsequent 2 seconds dark period (●). (A) NH_4Cl was omitted from the quench medium and the CCCP concentration was varied as indicated. (B) The CCCP concentration in the quench medium was maintained at 30 μM and the NH_4Cl concentration was varied as indicated.

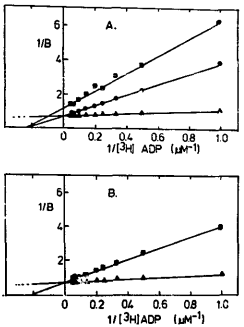

Fig. 3. Double reciprocal plots of the amount of [2-^{3}H]ADP bound (1/B) as a function of the [2-^{3}H]ADP concentration in the assay medium. 1/B has units of nmol [2-^{3}H]ADP^{-1}. mg chlorophyll. Quench medium was added after 2 min illumination (■), 2 min illumination and 2 s dark (○) or 2 min illumination and 10 min dark (▲). The quench medium contained (A) 15 μM CCCP and NH_4Cl was omitted, or (B) 30 μM CCCP and 0.3 M NH_4Cl.

fast phase, again by increasing the light level of bound ADP, but the effect is not complete and a smaller fast phase of binding is clearly visible even at very high concentrations of CCCP. In contrast, Fig. 2B shows that addition of a relatively low concentration of NH_4Cl to the quench medium (which also contains 30 μM CCCP) abolishes the fast phase of dark binding by increasing the level of nucleotides detected when thylakoids are quenched in the light. Thus, increasing the concentration of CCCP is much less effective than the additional inclusion of NH_4Cl. The observation that 10 mM NH_4Cl is as effective as 0.3 M (Fig. 2B) rules out any non-specific effect of salt addition as the explanation for the effect of this uncoupler in the quenching medium.

The dependence of the different binding phases on the medium [2-^{3}H]ADP concentration has been studied using the two quenching regimes. These data have been plotted in double reciprocal form and they yield straight lines, as shown in Fig. 3. This allows estimation of the apparent binding constants and maximal binding capacities, which have been summarised in Table I. Several features are clearly evident from these data:

(i) when NH_4Cl is omitted from the quench medium (Fig. 3A), a fast phase of post-illumination dark binding is obvious at all ADP concentrations. When NH_4Cl was included in the quench medium (Fig. 3B), there is no detectable difference in the levels of nucleotides bound when the reaction is quenched in the light or at 2 s dark.

(ii) using either quenching medium, the apparent K_d for nucleotides bound at 2 s dark is the same as that observed in the light, both being considerably higher than the value observed if binding is allowed to proceed for 10 min in the dark, as observed by others [4,5,11].

(iii) the data obtained when only CCCP and unlabelled ADP were used to quench the reaction (Fig. 3A) are very similar to those reported by Schumann [11]. The maximal binding capacity for ADP is significantly smaller when the reaction is quenched in the light than when it is quenched at either 2 s or 10 min dark. In other words, increasing ADP in the incubation does not affect the fast phase of dark binding (relative to binding in the light) but abolishes the slow phase. This effect can be seen in the data of Fig. 1.

(iv) in contrast to (iii), when NH_4Cl is included in the quench medium, the amount of ADP bound after 2 min illumination is the same as that bound after illumination and 2 s dark, and the curves extrapolate to give the same binding capacity as

TABLE I

ESTIMATION OF THE APPARENT DISSOCIATION CONSTANTS AND BINDING CAPACITIES FOR ADP DURING A LIGHT-DARK TRANSITION

Values were calculated from the data of Fig. 3.

Incubation conditions	Apparent dissociation constant (μM)		Binding capacity (nmol/mg Chl)	
	quench A	quench B	quench A	quench B
2 min light	4.1	4.9	0.8	1.5
2 min light +2 s dark	4.5	4.1	1.3	1.3
2 min light +10 min dark	0.5	0.7	1.4	1.5

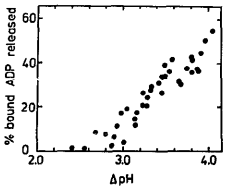

Fig. 4. The net release of bound [2-^{3}H]ADP from thylakoids as a function of the steady-state ΔpH estimated from quenching of 9-aminoacridine fluorescence. Thylakoids were preincubated in assay medium for 2 min in the light then left for 6 min in the dark to load CF_0-CF_1 with labelled ADP. Various concentrations of nigericin were then added. A second illumination period was given, ΔpH was estimated, and the membranes were then quenched in the light for determination of bound nucleotides.

quenching after illumination and 10 min dark. Thus, *net* release of tightly bound nucleotides from CF_1 observed upon illumination becomes progressively smaller as the ADP concentration in the incubation medium is raised. This occurs because increasing the ADP concentration abolishes the slow phase of dark binding and because there is no fast phase under these conditions. At saturating ADP, there is no net release of bound nucleotides upon illumination of thylakoids (but there is exchange of bound ADP with medium nucleotides).

In an earlier study, the enhanced quenching conditions were used to estimate the level of activation of CF_0-CF_1 from the steady-state level of ADP detected upon quenching in the light [1]. We were concerned whether the result noted in (iii) affected the validity of using this parameter as a measure of activation. However, Fig. 4 shows that, when a non-saturating concentration (5 μM) of [2-^{3}H]ADP is used, the net release of bound nucleotides still responds to the steady-state ΔpH in a way similar to that usually observed for ATP synthesis [13]. It appears therefore that, even though the kinetics are markedly different upon quenching the system with saturating uncoupler, the level of ADP that remains tightly bound in the light is at least qualitatively related to the fraction of enzymes that are inactive. This relationship has been explored in detail and will be reported elsewhere.

Discussion

Although more complex schemes can easily be devised and perhaps may ultimately be more relevant, discussion of the data presented here will be restricted to the following simple scheme, which has commonly been used in the literature [2]:

$$E_i = ADP \overset{k_1 \cdot \Delta\tilde{\mu}_{H^+}}{\underset{k_2}{\rightleftharpoons}} E_a \ldots ADP \overset{k_3}{\underset{k_4}{\rightleftharpoons}} E_a + ADP$$

The nucleotide that appears to be tightly bound when membranes are quenched in the light has generally been assigned to those complexes that are in the catalytically inactive E_i = ADP form at the time of quenching [4]. This requires the assumption that $k_3 > k_2$. The fast phase of dark binding was thought to represent rapid conversion of loosely bound ADP to tightly bound nucleotide as $E_a \ldots$ADP converts to E_i = ADP via k_2 [4], and the slow phase was thought to be due to [^{3}H]ADP binding to nucleotide-depleted E_a (which, although not shown, may also be in equilibrium with an inactive E_i form [11]). However, this assignment of the kinetics does not predict the observation that increasing the concentration of uncoupler in the quench medium abolishes the fast phase of dark binding, since k_2 should be independent of $\Delta\tilde{\mu}_{H^+}$. The simplest explanation for this observation is that k_2 is in fact greater than k_3, and by increasing the efficiency of uncoupling during the quench, both E_i = ADP and $E_a \ldots$ADP are trapped when membranes are quenched in the light. The remaining dark binding is slow, and represents [2-^{3}H]ADP binding to E_a. This form is less prevalent at higher concentrations of [2-^{3}H]ADP in the incubation medium (because of the equilibria k_3/k_4 and hence the slow phase of dark binding is abolished at saturating concentrations of ADP in the assay medium.

The above explanation implies that when the uncoupling efficiency of the quench medium is reduced (the conditions used in many published studies [4,5,11,12]), the fast phase of dark binding that arises is due to those $E_a \ldots$ADP complexes that can exchange with unlabelled ADP during quenching in the light. Such an exchange would be possible if the mixing of unlabelled ADP were rapid during quenching, but the dissipation of

$\Delta\bar{\mu}_{H^+}$ was rather slower. The fraction of $E_a \ldots ADP$ that exchanges would depend upon the extent to which E_i = ADP can reactivate during the quenching process, and thus on the lifetime of $\Delta\bar{\mu}_{H^+}$ during this period. Although it was assumed that uncoupling is rapid upon addition of FCCP [3], it has recently been shown that CF_o-CF_1 can turnover several times under similar conditions [12]. Thus, although the fast phase is probably derived from those complexes in the $E_a \ldots ADP$ form, it does not quantitatively measure this fraction. This interpretation is consistent with previous observations showing that the fast phase of dark [2-^{3}H]ADP binding is not affected by isotopic dilution of medium [2-^{3}H]ADP during the quench [11]. It does not rule out the possibility that exchange of $E_a \ldots ADP$, when it occurs, is not via the k_3/k_4 equilibrium but by binding of unlabelled ADP to an alternative site with a high K_d [12].

Thus, the above interpretation seriously questions the idea that the net level of nucleotide that remains apparently tightly bound during steady-state illumination is a direct measure of the fraction of inactive complexes, as generally assumed. This is most obviously incorrect at saturating [2-^{3}H]ADP in the incubation and using an efficient quenching regime. Under these conditions, all the enzymes apparently bind an ADP tightly in the light, yet at least some must be in the catalytically active $E_a \ldots ADP$ form. At lower concentrations of ADP, and using an efficient quenching regime, a decrease in the tightly bound nucleotide in the light is observed. This probably reflects the accumulation of nucleotide-depleted forms due to the higher value of the apparent dissociation constant for ADP bound in the light. As indicated in Fig. 4, *net* release of bound nucleotide under these conditions appears to show a similar dependence on ΔpH as ATP synthesis, and thus may still be a valid indicator of the extent of enzyme activation. The same would be true of pre-steady-state methods, where unidirectional release of ADP was observed from prelabelled membranes in the absence of labelled nucleotide in the medium [8]. However, we conclude that the level of bound nucleotide detected in the light, or in the fast phase of dark binding, if present, cannot be reliably related to a single class of bound nucleotides.

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