KINETIC ANALYSIS OF LIGHT-DEPENDENT EXCHANGE OF ADENINE NUCLEOTIDES ON CHLOROPLAST COUPLING FACTOR CF_1

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1. Introduction

Chloroplast coupling factor CF_1 contains tightly bound ADP which is exchanged against added ADP only on energization of the thylakoid membranes [1–6]. This ADP binding site is probably different from the catalytic ADP binding site in the process of photophosphorylation [5–9]. Nevertheless it may be involved in the mechanism of energy transduction. Light-induced nucleotide exchange and photophosphorylation show a parallel behavior in their dependence on several external factors, e.g., light intensity, pH, uncoupling and electron transport inhibition [10].

The mechanism of ADP exchange involves two consecutive reversible reactions and three different forms of CF_1 which can be characterized by different states of the ADP binding site [11]:

(i) A stable CF_1–ADP complex (tightly-bound ADP);
(ii) An unstable transitory CF_1–ADP complex (loosely bound ADP);
(iii) A metastable dissociated complex containing an unoccupied ADP binding site.

The energy-requiring step is thought to be related to a conformational change of the protein by which tightly bound ADP is transferred to the loosely bound form. The loosely bound ADP molecule can be replaced by dissociation and re-association with a free ADP molecule. The latter reaction does not require an energized state of the membrane [11]. During illumination of the chloroplasts all three forms of CF_1 can be expected to coexist in equilibrium. In the dark, the tight complex is the only form to occur if ADP is present in the medium [11].

As yet no method has been available to determine the amount of tightly bound ADP in the light stage or to follow the expected increase of the tight complex on de-energization. This can be achieved by employing the quenching technique described here. In this procedure incorporation of ADP into CF_1 is followed by using radioactively labeled ADP. The exchange reaction while in progress is quenched by injection of the uncoupler FCCP together with excess unlabeled ADP. Discharge of the energized state by FCCP stops the energy-dependent reaction step and at the same time labeled ADP, with the exception of the inaccessible tightly bound ADP, is displaced by isotope dilution. Thus the radioactivity retained after washing of the membranes corresponds to the amount of tightly bound ADP which is present at the moment when the reaction is stopped.

By this method steady state levels as well as transitory changes of tightly bound ADP can be measured. The results allow calculation of the rate constants and equilibrium constants of the reactions involved in the exchange process.

2. Methods

Chloroplasts from spinach leaves were isolated and washed as in [11]. For measurement of light-induced incorporation of ADP, the chloroplasts were incubated in a medium containing 25 mM Tricine buffer (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 0.5 mM methylviologen

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and 0.5–5 μM [^{14}C]ADP (Amersham-Buchler, labeled in C-8, spec. act. 50 μCi/μmol). The chlorophyll content was ~0.2 mg/ml. The total volume was 0.5 ml, the temperature 20°C. The reactions were performed in small glass vessels (diam. 18 mm). The reaction mixture was vigorously stirred by a magnetic stirrer. The reaction was started by illumination from the top (white light, usually 8×10^5 ergs cm^{-2} s^{-1}). The reaction was quenched by rapid injection of 0.2 ml of a solution containing FCCP and unlabeled ADP to give final concentrations of 5 μM and 5 mM, respectively.

In order to remove free label, after centrifugation the pellets were washed 3 times with washing solution (1 ml) [11]. The radioactivity of the washed resuspended pellets was measured by liquid scintillation counting [11].

In the experiment shown in table 1, chloroplasts were incubated with 1 μM [^{14}C]ADP in the light and quenched after 30 s with FCCP containing increasing concentrations of unlabeled ADP. A 1000-fold isotope dilution was sufficient to displace virtually all the accessible [^{14}C]ADP. Further increases in ADP concentration did not decrease the amount of bound label after washing which is therefore referred to as 'tightly bound [^{14}C]ADP'.

Uncouplers break down the energized state of chloroplasts within 2–5 ms [12] and hence the time required for the FCCP action is essentially determined by the mixing time. With the described technique complete mixing was attained in 200–400 ms. In order to get a higher time resolution, a quench flow system was also employed, which resembled the design in [13].

Table 1
Effect of unlabeled ADP in the quenching solution on the amount of tightly bound [^{14}C]ADP

ADP[a] (mM)	nmol [^{14}C]ADP tightly bound/mg chl
0	0.555
0.05	0.155
0.1	0.113
0.25	0.106
0.5	0.103
1	0.087
2.5	0.091
5	0.087

[a] Final concentration after addition of quenching solution

Chloroplasts were incubated in the presence of 1 μM [^{14}C]ADP in the light. After 30 s the reaction was stopped by injection of 5 μM FCCP containing the indicated concentrations of unlabeled ADP. Radioactivity related to the membranes was measured after removal of free nucleotides by washing

3. Results and discussion

In fig.1 the tight incorporation of [^{14}C]ADP into membrane-bound CF_1 in the light and following dark period and upon a second illumination is shown. In the first light period a steady state level was attained after ~30 s. When the light was turned off, a sudden increase followed by a slower increase of tightly bound [^{14}C]ADP occurred. When the chloroplasts were illuminated again, the previous light steady state was reached within 5 s.

At the light/dark transition the increase in tightly bound labeled ADP was clearly biphasic. However, when the chloroplasts were pre-illuminated and [^{14}C]ADP was added at the time of turning off the light, the rapid initial phase was absent (fig.2). The initial rapid phase was analyzed with a higher time

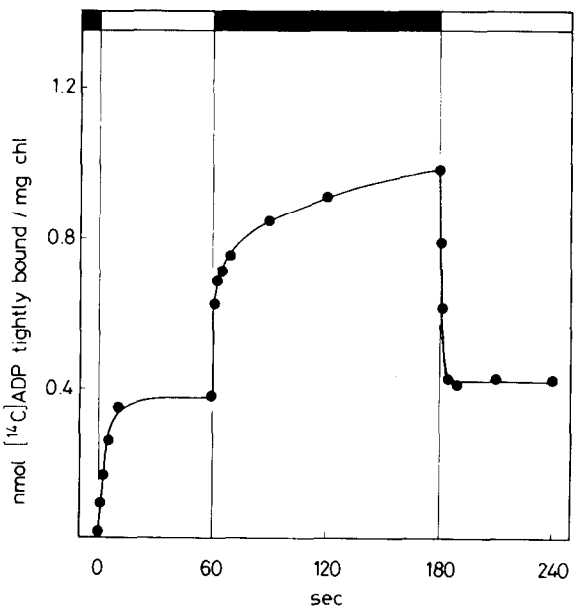

Fig.1. Tight binding of [^{14}C]ADP in a light–dark–light change. [^{14}C]ADP was 5 μM, chlorophyll 0.2 mg/ml.

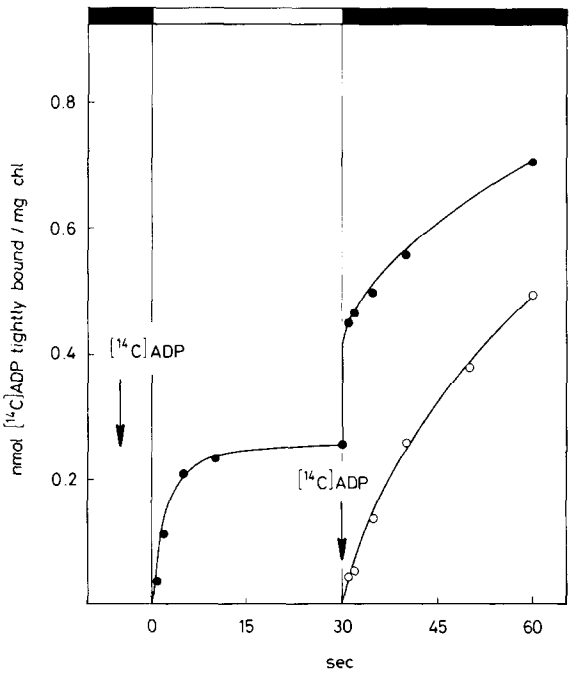

Fig.2. Tight binding of [^{14}C]ADP in the light and after pre-illumination. [^{14}C]ADP was added either prior to illumination or immediately after illumination in nucleotide free medium. Other experimental conditions as in fig.1.

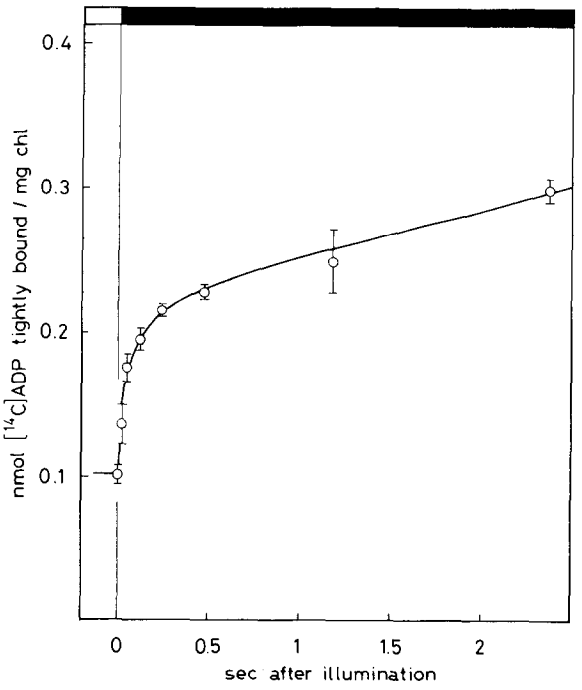

Fig.3. Resolution of the light/dark transient kinetics of tight binding of [^{14}C]ADP in a quench flow system. 0.2 ml incubation mixture containing chloroplasts (0.2 mg chl/ml) and 1 μM [^{14}C]ADP was placed in syringe I, 0.2 ml quenching solution in syringe II. After 30 s illumination of syringe I (diam. 4 mm) the contents of both syringes were pressed through darkened Y-shaped channels (diam. 0.5 mm, length 38 mm). Flow time was varied by changing the rate of forward movement of the plungers.

resolution by using a quench flow system (fig.3). From such experiments a half-time of the fast process of ~20 ms was estimated.

Energy-dependent ADP exchange at the 'tight binding site' can be described by a sequence of two reversible reactions [11]:

$$\text{X–ADP} \underset{k_{-1}}{\overset{k_1.E}{\rightleftharpoons}} \text{X*–ADP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} \text{X*} + \text{ADP}$$

(X–ADP, tight complex; X*–ADP, loose complex; X* + ADP, dissociated complex; E, arbitrary non-linear expression of the energy charge of the membrane).

The results shown in fig.1–3 could be explained in terms of this model if k_{-1} is assumed to be much larger than k_{-2}. In that case the fast increase of tightly bound ADP on light/dark transition would be due to the reconversion of the loose complex to tightly bound ADP and its amplitude should correspond to the light steady-state level of loosely bound ADP. The slow phase would include the whole sequence of the back reaction and would be limited by the binding of free ADP at the depleted binding site to form the loose complex. It is obvious that the first reaction would not take place when [^{14}C]ADP is added after pre-illumination in nucleotide-free medium.

The reaction scheme allows several predictions which can be scrutinized by experiment:

(1) The steady state distribution of the three forms of CF_1 in the light should depend on the energy charge of the membrane. The amount of tightly bound ADP should be higher at the expense of the other two forms, when the degree of energization is low and vice versa. This can be demonstrated experimentally by employing low and high light intensities (table 2).

Table 2
Tight binding of [^{14}C]ADP at low and high light intensities

Time of incubation	nmol [^{14}C]ADP tightly bound/mg chl	
	1.5×10^4 ergs cm^{-2} s^{-1}	4.0×10^5 ergs cm^{-2} s^{-1}
10 s light	0.095	0.202
30 s light	0.389	0.275
60 s light	0.501	0.272
120 s light	0.551	0.276
120 s light – 120 s dark	0.896	0.948

Chloroplasts (final conc. 0.182 mg chl/ml) were incubated in the presence of 5 μM [^{14}C]ADP and quenched at the indicated times by addition of 5 μM FCCP + 5 mM unlabeled ADP

(2) The steady state levels of the three CF_1 forms in the light should depend on the concentration of added [^{14}C]ADP. In accordance with the model, the light level of tightly bound ADP as well as the amplitude of the rapid phase at light/dark transition were both found to be smaller at the low than at the higher concentration of [^{14}C]ADP (fig.4).

(3) At a given light intensity the equilibrium constant K_1 ($= k_1.E/k_{-1}$) is equal to [X*–ADP]/[X–ADP]. If the amplitude of the fast increase of tightly bound ADP after light/dark transition is a measure of X*–ADP present in the light stage, K_1 can be calculated from the ratio of this amplitude to the amount of tightly bound ADP in the light. It has been found in a variety of experiments that this ratio is largely independent of the [^{14}C]ADP concentration and close to 1 at saturating light intensities.

(4) According to the scheme, the equilibrium (= dissociation) constant K_2 can be calculated from the concentration dependence of tightly bound ADP in the light equilibrium state. The plot 1/[ADP] versus 1/[X–ADP] gives a straight line with the intercepts $-2/K_2$ (abscissa) and $2/[X–ADP]_{max}$ (ordinate) at $K_1 = 1$.

The corresponding experiment is shown in fig.5. The computed dissociation constant K_2 was ~7 μM. In contrast to the variation in maximum amount of ADP incorporation in different experiments, K_2 was found to be almost invariable.

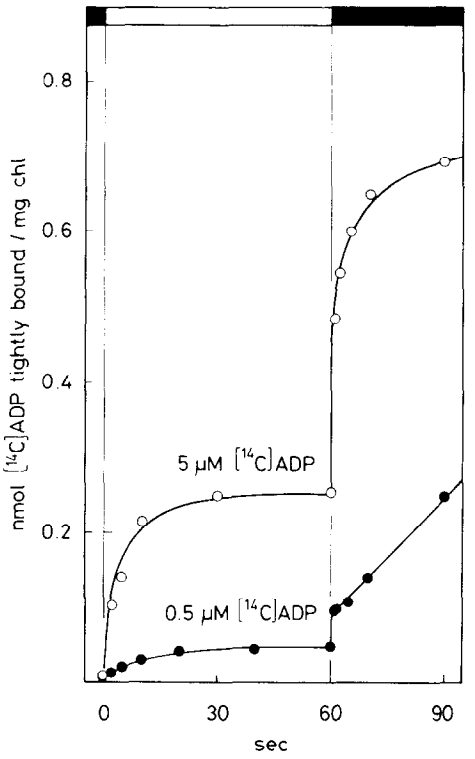

Fig.4. Tight binding of [^{14}C]ADP in the light and following dark period at two concentrations of [^{14}C]ADP.

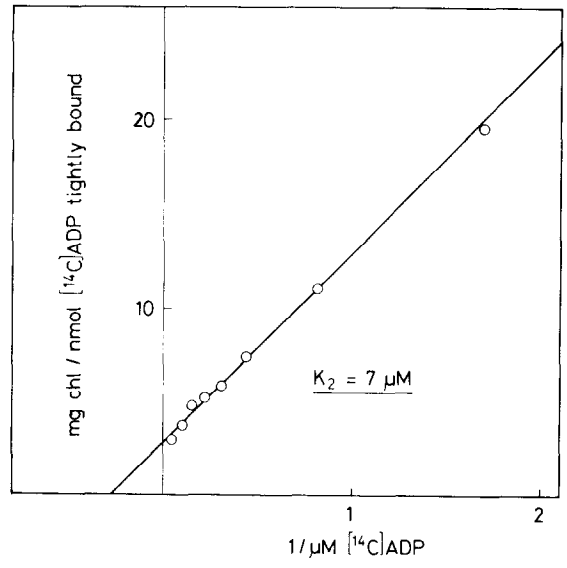

Fig.5. Tight binding of [^{14}C]ADP in the light steady state as a function of [^{14}C]ADP concentration (double reciprocal plot).

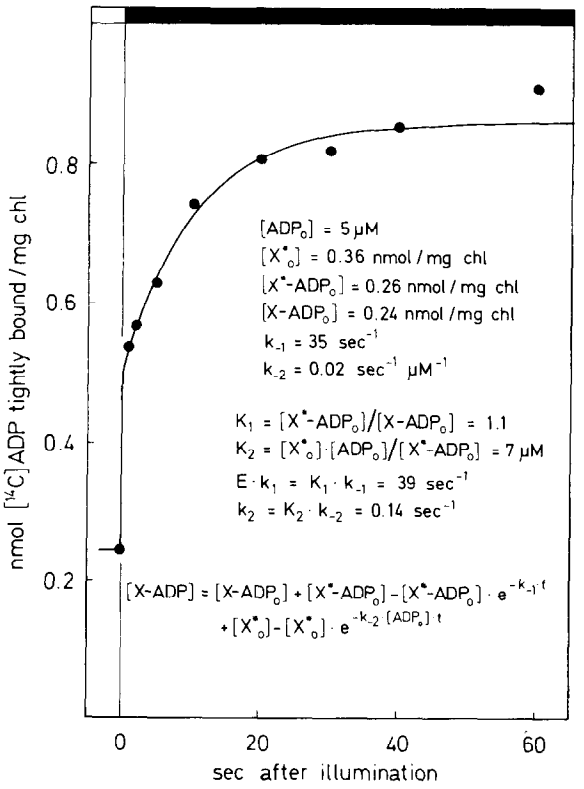

Fig.6. Mathematical simulation of the light/dark transient kinetics of tight binding of [^{14}C]ADP (curve) and experimental values.

(5) Using the proposed model, the kinetics of tight ADP binding at light/dark transition were simulated for different experimental conditions. An example is given in fig.6. On darkening the reaction sequence is practically unidirectional and can be described by the sum of the fast transition of X*–ADP and the slow transition of X* + ADP to X–ADP. The light steady state concentrations and the rate constants k_{-1}, k_{-2} were estimated from the experimental data presented, and optimized to give the best fit to the experimental curves. The resulting rate constants were k_{-1} = 35 s^{-1} and k_{-2} = 0.02 s^{-1} μM^{-1}. The calculated rate constants were $E.k_1 = 39\ s^{-1}$ and $k_2 = 0.14\ s^{-1}$. For K_2 a value of 7 μM was obtained, i.e., the same value as determined from equilibrium binding experiments (see fig.5).

The fast initial rise of tightly bound ADP at light/dark transition is probably not correlated to the decay of the transmembrane pH gradient which is of the order of several seconds under comparable conditions [14]. On the other hand, the rate of ADP binding by the depleted binding site is too low to account for the turnover of substrate nucleotides in the process of photophosphorylation. In accordance with earlier results [5–9] it may thus be concluded that this kind of energy-induced nucleotide exchange does not reflect an interaction of substrate ADP with the catalytic site of CF_1.

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