NUCLEOTIDE BINDING AND REGULATION OF CHLOROPLAST ATP SYNTHASE

Susanne BICKEL-SANDKÖTTER and Heinrich STROTMANN*

Abteilung für Biochemie der Pflanzen, Botanisches Institut der Tierärztlichen Hochschule Hannover, Bünteweg 17, 3000 Hannover, FRG

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1. Introduction

The CF_0–CF_1 complex catalyzes the reversible formation of ATP coupled to transmembrane proton translocation in chloroplasts. The enzymatic activity of membrane-bound and isolated CF_1 is however latent. In chloroplasts, induction of the ATP hydrolyzing activity requires pre-illumination of the membranes (light-triggered ATPase) [1–5]. Recently, it has been demonstrated that light activation is related to release of tightly-bound ADP from CF_1 [6]. However, the induced ATPase is known to be deactivated by ADP [7]. This reaction is quantitatively related to re-binding of ADP to the previously depleted sites to form the tight CF_1–ADP complex again [6]. Moreover, it has been shown that under different conditions the rate of ATP hydrolysis is controlled by the fraction of CF_1 molecules which are free from tightly bound ADP [6].

In ATP synthesis, energy-dependent activation of CF_1 is also most likely a prerequisite, as concluded from the induction and threshold phenomena in photophosphorylation [8]. Furthermore, pre-treatments of the chloroplasts which induce the ATP hydrolyzing activity of CF_1 increase the yield of ATP formation in single turnover flashes [9]. In [10] it was suggested that the physiological activation process may be monitored by energy-dependent exchange of tightly bound adenine nucleotides on CF_1.

Under phosphorylating conditions tightly bound adenine nucleotides are exchanged against medium ADP [11–14]. The energy-dependent reaction is the release step facilitated by a conformational change of CF_1, whereas the binding step does not require further energy [14]. The light steady state is characterized by a relatively low amount of tightly bound nucleotides, but after switching off the light, this fraction increases to a maximal value [15].

Here, steady state measurements of tightly bound as well as loosely bound adenine nucleotides are described. In analogy to the results obtained in light-triggered ATPase, we may assume that the portion of tightly bound nucleotides represents inactive CF_1 molecules. If it is further assumed that the loosely bound nucleotides are essentially catalytic nucleotides, we can conclude from the results that:

(i) The steady state fraction of active ATPase is regulated by energy input;

(ii) The catalytic site of an inactive enzyme molecule is virtually inaccessible to medium nucleotides.

This is supported by results from another side. In the second part of this paper phosphorylation as a function of ADP concentration is shown at varying efficiencies of electron transport. It is demonstrated that the kinetic parameters V_{max} and K_m proportionally decrease if energy input is reduced. They both become zero in the dark. This can be expressed by a theoretical model which is in complete accordance with the conclusions derived from the binding studies.

2. Experimental

Chloroplasts were prepared from freshly harvested spinach leaves as in [14]. For measurements of steady state binding of ADP, the chloroplasts were incubated in a medium consisting of: 25 mM Tricine buffer

* New address: Lehrstuhl für Biochemische Pflanzenphysiologie (Botanik II), Universität Düsseldorf, Universitätsstr. 1, 4000 Düsseldorf, FRG

(pH 8.0); 50 mM NaCl; 5 mM $MgCl_2$; 5 mM P_i (pH 8.0); 50 μM PMS; 10 mM glucose; 30 units/ml hexokinase (type F-300, purchased from Sigma Chemical Co.) and 0.1–10 μM [^{14}C]ADP (Amersham Buchler, C-8-labeled, spec. act. 50 μCi/μmol). Chlorophyll was ~0.4 mg/ml; the total volume of reaction mixture 1 ml. The experiments were done at room temperature.

After addition of the chloroplasts, 0.25 ml reaction mixture was filled into small semi-transparent centrifugation tubes (Beckman) which then were inserted into a centrifuge (Microfuge 152, Beckman) with illumination equipment from the top. After 90 s preincubation, light was switched on (white light, maximum intensity 1.5×10^{-2} W/cm^2 inside the tubes). After 30 s the chloroplasts were spun down without turning off the light. Dark samples were treated correspondingly.

The supernatant (A) as well as another aliquot of the complete reaction mixture (B) (each 0.2 ml) were mixed with 20 μl 40% perchloric acid and centrifuged at 15 000 × *g*. The amount of totally bound adenine nucleotides was calculated from the difference in the ^{14}C contents between B and A.

For measurements of the fraction of tightly bound nucleotides a third aliquot of the reaction mixture was filled into the centrifugation tubes which contained 20 μl quenching solution in their perforated hat-shaped covers. The quenching solution consisted of 135 mM unlabeled ADP and 67.5 mM FCCP [15]. By turning on the centrifuge, the drop is injected from the cover into the tubes. This leads to homogenous mixing with the chloroplast suspension in a rather short time.

Photophosphorylation was performed as in [16]. The incubation medium contained: 25 mM Tricine buffer (pH 8.0); 50 mM NaCl; 5 mM $MgCl_2$; 5 mM $^{32}P_i$; 25 mM glucose, 15 units/ml hexokinase; ADP as indicated; and 50 μM PMS or 0.1 mM methylviologen, respectively. Chlorophyll was ~25 μg/ml; reaction was at 20°C; maximum light intensity was 0.12 W/cm^2.

3. Results

3.1. *Steady state ADP binding*

Fig.1 shows steady state binding of ^{14}C-labeled adenine nucleotides by chloroplasts in a system containing [^{14}C]ADP, unlabeled P_i and an ADP regenerating hexokinase trap. Nucleotide binding as determined by difference measurements includes all types of interactions, e.g., tight and loose binding to CF_1 and a small unspecific (membrane) binding [14]. In an illuminated chloroplast suspension, CF_1-bound nucleotides can be expected to consist of [^{14}C]ADP as well as [^{14}C]ATP. The technique described allows a sufficiently exact measurement up to 10 μM [^{14}C]-ADP. With the highest ADP concentration, binding is almost saturated in the light (fig.1, top). Maximum binding is ~1 mol/mol CF_1, if based on a ratio of

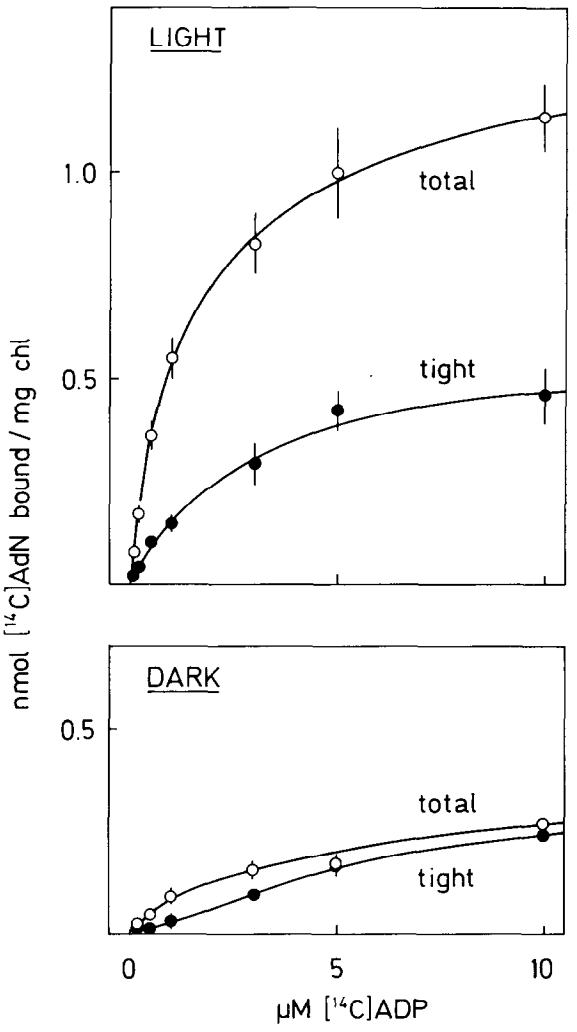

Fig.1. Steady state binding of [^{14}C]ADP by broken chloroplasts. Experimental conditions and procedures in section 2. The values are averages from 6 (upper curve) and 3 (lower curve) different experiments, respectively. The bars indicate the standard deviations.

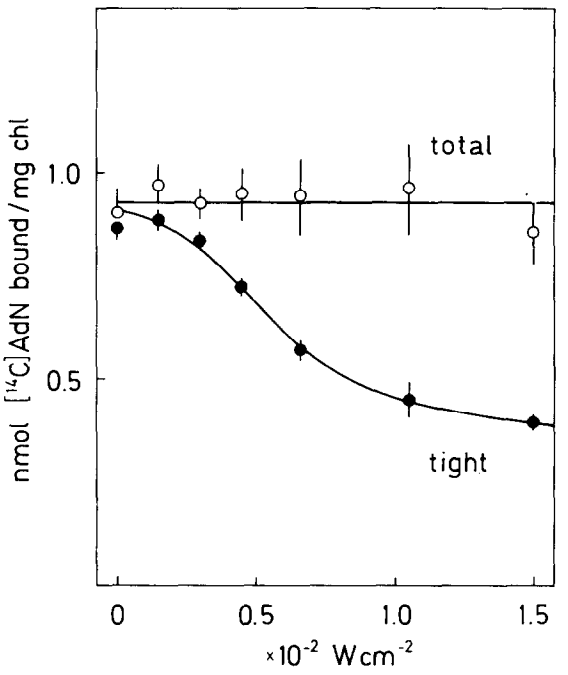

Fig.2. Steady state binding of [^{14}C]ADP as a function of light intensity. [^{14}C]ADP was 5 μM. After pre-illumination (30 s) at 1.5 × 10^{-2} W/cm², the indicated light intensities were employed for 60 s. Two experiments were averaged.

1 CF_1/860 chl [17]. By using the FCCP/ADP quenching technique in [15], tightly bound nucleotides can be discriminated from the loosely bound ones. Under the employed experimental conditions, ~40% of the nucleotides are found to be tightly bound.

In the dark, nucleotide binding is comparably low (~0.2 mol/mol CF_1 at 10 μM ADP), and virtually no loose binding takes place (fig.1, bottom).

In fig.2 steady state binding as a function of light intensity is shown. Added [^{14}C]ADP was 5 μM. To attain quantitative exchange of the endogenous bound nucleotides with the added labeled ADP, the chloroplasts are illuminated with full light for 30 s before the intensity is adjusted to the indicated values. The total amount of bound labeled nucleotides is largely independent of the light intensity. However, the fraction of tightly bound nucleotides increases with decreasing light intensity. If a dark period is employed after pre-illumination, nearly all the bound nucleotides are present in a tightly bound form. CF_1 molecules, which contain a tightly bound ADP are catalytically inactive in ATP hydrolysis [6]. If the same is considered in ATP synthesis, we have to conclude from the preceding results that:

(i) The fraction of active ATPases in steady state photophosphorylation is regulated by light intensity.

If the loosely bound nucleotides are assumed to represent essentially the catalytic ADP and ATP, we have to conclude that:

(ii) The inactive enzyme molecules are unable to perform catalytic ADP binding. Accordingly enzyme activation would mean an opening of the catalytic site to medium ADP (and perhaps P_i).

Both conclusions are supported by a completely different experimental approach as shown in the following.

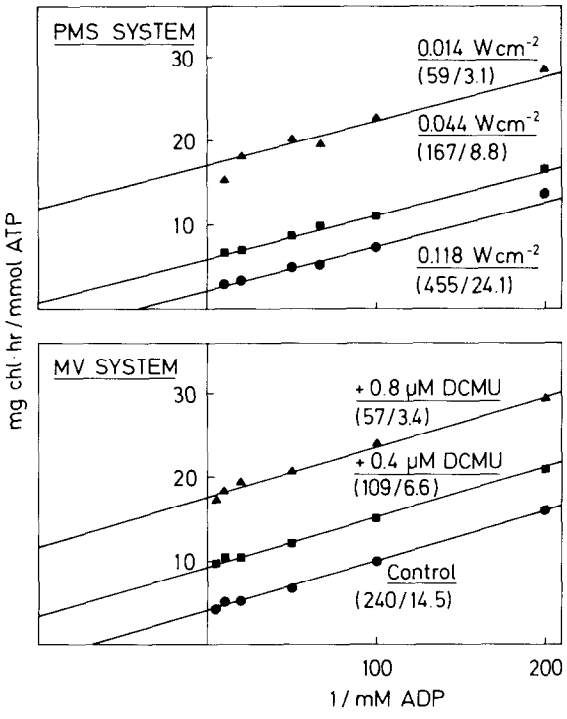

Fig.3. Photophosphorylation as a function of ADP concentration at 3 light intensities (PMS system) and 2 different DCMU concentrations (methylviologen system), respectively. In the latter series the light intensity was 0.118 W/cm². Experimental conditions as in section 2. The figures in brackets mean the calculated V_{max} (in μmol ATP . mg chl^{-1} . h^{-1}) and $K_{m(ADP)}$ (in μM).

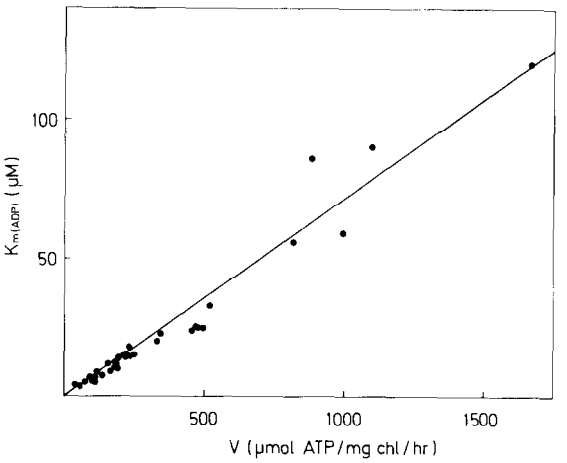

Fig.4. $K_{m(ADP)}$ as a function of V_{max}. Experimental results from 10 expt as shown in fig.3. The line is a computed curve (details in section 4).

3.2. *Steady state photophosphorylation as a function of ADP concentration and electron transport*

In fig.3 phosphorylation as a function of [ADP] is shown in a PMS system at 3 different light intensities (upper part) and in a methylviologen system at 3 different concentrations of DCMU (lower part). The Lineweaver-Burk plots reveal that upon lowering the efficiency of electron transport, both V_{max} and K_m proportionally decrease. Formally this leads to the same results as if an uncompetitive inhibition takes place.

In fig.4 a plot K_m *vs* V_{max} is shown from different experiments of the same type as in fig.3. The diagram demonstrates a virtually linear relationship between the two parameters. At $V_{max} = 0$, the value for K_m is likewise zero.

4. Discussion

The experimental results can be explained by a model derived from the Briggs-Haldane form of an enzyme reaction. With regard to the substrate ADP, the reaction sequence includes reversible binding and practically irreversible product (=ATP) formation. The latter reaction is of course complex and consists of ATP formation on the enzyme as well as ATP release. However, since the experiments are performed at excess P_i, Mg^{2+} and in the presence of a hexokinase trap, the single steps may be summarized in the indicated way. Energy dependency of the process is marked by the arbitrary factor e (variable from 0–1). Moreover the model contains a side reaction which leads to the formation of tightly bound ADP from the active enzyme–ADP complex. As mentioned above, this form of the enzyme is assumed to be catalytically inactive. The reverse reaction is energy-dependent.

$$E + ADP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E - ADP \xrightarrow[(P_i, Mg^{2+})]{e \cdot k_2} E + ATP$$

$$k_3 \downarrow \quad \uparrow e \cdot k_{-3}$$

$$E = ADP_i$$

The model is justified by the following experimental results:

(i) The sun of tightly bound and loosely bound adenine nucleotides is $1/CF_1$ at ADP saturation (fig.1).

(ii) The ratio between the two species of bound nucleotides is variable and dependent on light intensity (fig.2).

(iii) Release of tightly bound adenine nucleotides from CF_1 is energy-dependent [11–15], the energy-requiring step is the transformation of the tight complex into a loose one [14,15].

(iv) After de-energization the equilibrium of the reaction would be shifted completely towards the tight complex (fig.2). Thus further ADP binding would be prohibited (fig.1).

From the reaction scheme follows:

$$K_m = \frac{e \cdot k_{-3}(k_{-1} + e \cdot k_2)}{k_1(k_3 + e \cdot k_{-3})}$$

and

$$V_{max} = [E_t] \frac{e \cdot k_2 \cdot e \cdot k_{-3}}{k_3 + e \cdot k_{-3}}$$

The model can explain the following results obtained in steady state phosphorylation measurements:

(i) If the energy parameter e is set to zero, both V_{max} and K_m become zero (fig.4). (In a simple linear scheme K_m would yield $k_{-1}/k_1 = K_d$ at $e = 0$.)

(ii) The relationship between K_m and V_{max} is:

$$K_m = V_{max} \frac{k_{-1} + e \cdot k_2}{[E_t] \cdot k_1 \cdot e \cdot k_2}$$

It is linear, if k_{-1} is small compared to k_2. The line drawn in fig.4 is a computed curve obtained by variation of e from 0–1 assuming the following rate constants: $k_1 = 3\ \mu M^{-1} \cdot s^{-1}$; $k_{-1} = 4.5\ s^{-1}$; $k_2 = 800\ s^{-1}$; $k_3 = 40\ s^{-1}$ [15]; $k_{-3} = 40\ s^{-1}$ [15].

(iii) Linearity between K_m and V_{max} on one hand, and intersection of the K_m axis at zero on the other, fulfill the characteristics of an uncompetitive type of inhibition of phosphorylation which has been observed upon lowering the energy input (fig.3).

The model implies that the tight nucleotide binding site is not separate but only a different form of the active site of CF_1. This may be the easiest, although not the only, way to interpret the obtained results. One could arrive to the same conclusions if tight ADP binding is assumed to occur on a separate site of the active enzyme–ADP complex. In order to explain the measured stoichiometry it has then to be assumed that the substrate ADP is displaced from the catalytic site when the 'tight' site is going to be occupied. The finding that tightly bound ADP equilibrates with medium ADP at the onset of energization [18] may even speak in favor of such an interpretation.

Reversible activation of chloroplast ATPase attained by the antagonism of tight binding of ADP and its energy-dependent release, could explain the high efficiency of energy utilization in photophosphorylation. If the active ATPase molecules were able to drain protons through the thylakoid membrane, one could expect a shift of the ATPase equilibrium towards net ATP synthesis even at low energy input.

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