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NUCLEOTIDE SPECIFICITY OF CF_1-ATPase IN ATP SYNTHESIS AND ATP HYDROLYSIS

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1. Introduction

Coupling factor (CF_1) from chloroplasts is known to catalyze the reversible formation of ATP from ADP and P_i. In chloroplasts and with the isolated enzyme no ATPase activity is observed unless the protein is activated. Activation can be achieved by artificial modification (trypsin treatment [1], heat [2], DTT [3]), which yields a Ca^{2+}-dependent ATPase. Physiologically, the membrane-bound enzyme can be activated by light [4–7]. Light-triggered ATPase is stimulated by thiol reagents. Most likely a light-dependent activation of CF_1 is also involved in the process of photophosphorylation [8–11].

From the differential inhibition of partial reactions by Fab fragments of antibodies against CF_1, it was concluded [12] that CF_1 contains two catalytic sites, one specialized for ATP synthesis and one for ATP hydrolysis and related reactions, like ATP–P_i exchange. The same was concluded [13] on the basis of studies with 1,N^6-etheno analogs of ADP/ATP and CDP/CTP. The nucleoside diphosphates were found to replace ADP in phosphorylation, but the nucleoside triphosphates were only poor substitutes in the ATPase reactions. The occurrence of two separate sites responsible for the catalysis of the forward and back reaction, respectively, would be of unique interest in enzymology. Therefore, we re-investigated the problem by determining the nucleotide specificities of the reactions. To avoid misinterpretation, it is important to determine the kinetic parameters (V_{max}, K_m) under comparable conditions and to consider their meaning critically.

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The results indicate that the actual relative nucleotide specificities are in fact very similar in photophosphorylation, light-triggered ATPase and Ca^{2+}-dependent ATPase reaction. However, the results also suggest that probably two different conformations of the chloroplast ATPase complex are involved in the catalysis of the physiological forward and back reaction, respectively.

2. Experimental

Chloroplasts were isolated from spinach leaves as in [14]. Photophosphorylation was measured as in [15] in a medium containing: 25 mM tricine buffer (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 5 mM $^{32}P_i$, 50 μM PMS and the indicated nucleoside diphosphates at varying concentrations: chlorophyll (chl) content was ~25 μg/ml; total vol. 0.5 ml; temp. 20°C; light intensity (white light) 0.118 W/cm^2. For measurement of light-triggered ATP hydrolysis, the chloroplasts were pre-illuminated for 3 min (white light, 0.118 W/cm^2) in a medium containing: 25 mM tricine buffer (pH 7.8), 5 mM $MgCl_2$, 10 mM DTE, 60 μM PMS and ~50 μg chl/ml. Immediately after turning off the light, the γ-^{32}P-labeled nucleoside triphosphates were added: total vol. 0.5 ml; temp. 20°C. After 25 s the reaction was stopped by addition of 50 μl 3 N $HClO_4$. $^{32}P_i$ contents were assayed in the isobutanol/benzene extracts of the phosphomolybdate complex [16].

For measurement of Ca^{2+}-dependent ATPase, CF_1 was isolated [17]. The enzyme was activated by treatment with trypsin (0.31 mg/mg CF_1) for 15 min in a medium containing 25 mM tricine buffer (pH 8.0) and 0.25 mM EDTA. Activation was stopped by the addition of trypsin inhibitor (1.88 mg/mg CF_1). After

5 min the ATPase reaction was started by adding the γ-^{32}P-labeled nucleoside triphosphates together with $CaCl_2$ (final conc. 5 mM): total vol. 0.11 ml; temp. 36°C. After 5 min the reaction was stopped by the addition of 10 μl 40% trichloroacetic acid. $^{32}P_i$ contents were assayed as above.

γ-^{32}P-Labeled nucleoside triphosphates were synthesized by photophosphorylation of the corresponding nucleoside diphosphates [18] and separated by column chromatography on PEI cellulose [19]. Elution was performed with a linear LiCl gradient (0–3 M). The radioactive products were identified by co-chromatography with the authentic compounds on thin-layer sheets [20]. Labeling in the γ-position was ascertained by degradation with hexokinase/glucose.

3. Results and discussion

In table 1 K_m and V_{max} values for ADP and a few analogs in PMS-mediated photophosphorylation are shown. Compared to the reported values determined in a methylviologen system [20], higher K_m values are obtained. This is due to the fact that both, V_{max} and K_m increase proportionally with increasing the efficiency of the electron-transport system [11,20]. Under the same experimental conditions, the ratio $K_{m(NuDP)}/K_{m(ADP)}$ may be regarded as an approximate relative measure for the affinity of an analog to the active site, provided that the V_{max} values are the same [20]. If V_{max} is altered, the actual relative affinity is approximately given by:

Relative affinity =

$$K_{m(NuDP)} \cdot V_{max(ADP)}/K_{m(ADP)} \cdot V_{max(NuDP)}$$

This simple relationship may be theoretically incorrect but practically useful. It presumes that K_d of the enzyme–substrate complex is very small compared to K_m and that K_m is linearly related to V_{max}. Both assumptions have been proven experimentally in the case of ADP [11].

In the same way relative nucleotide specificities are determined for light-triggered ATP hydrolysis. These results are shown in table 2. Comparison with the data from table 1 indicates that phosphorylation and ATP hydrolysis exhibit very similar relative affinities for the employed nucleotide analogs. 2′-Deoxy-ADP, GDP and 2′-deoxy-GDP shows the same V_{max} as ADP in photophosphorylation and the corresponding nucleoside triphosphates likewise exhibit the same V_{max} as ATP in light-triggered ATPase. However, N^6-dimethyl-ADP, 1,N^6-etheno-ADP and arabino-ADP as well as the triphosphates of those analogs are converted at a reduced V_{max} in both reactions. Athough the reduction in V_{max} is more pronounced in ATP hydrolysis, the calculated relative affinities are quite similar to those obtained in phosphorylation.

Table 1
Nucleotide specificity of PMS-cyclic photophosphorylation

Nucleotide	V_{max} (%)[a]	K_m (μM)	K_m (rel.)[b]
ADP	100	40	1
N^6-Dimethyl-ADP	70	125	4
1,N^6-Etheno-ADP	70	100	4
2′-Deoxy-ADP	100	40	1
Arabino-ADP	60	80	3
GDP	100	160	4
2′-Deoxy-GDP	100	300	8

[a] 100% = 500 μmol P_i incorporated . mg chl^{-1} . h^{-1}
[b] Computed as indicated in the text

Table 2
Nucleotide specificity of light-triggered ATPase

Nucleotide	V_{max} (%)[a]	K_m (μM)	K_m (rel.)[b]
ATP	100	70	1
N^6-Dimethyl-ATP	25	50	3
1,N^6-Etheno-ATP	40	125	4
2′-Deoxy-ATP	100	70	1
Arabino-ATP	25	70	4
GTP	100	310	4
2′-Deoxy-GTP	100	560	8

[a] 100% corresponds to 80–140 μmol P_i released . mg chl^{-1} . h^{-1}
[b] Computed as indicated in the text

Table 3
Nucleotide specificity of soluble Ca^{2+}-dependent ATPase

Nucleotide	V_{max} (%)[a]	K_m (μM)	K_m (rel.)[b]
ATP	100	520	1
1,N^6-Etheno-ATP	60	1140	4
2′-Deoxy-ATP	190	840	1
Arabino-ATP	30	630	4
GTP	90	4140	9
2′-Deoxy-GTP	30	1310	8

[a] 100% corresponds to 80–100 μmol P_i released . mg protein^{-1} . h^{-1}
[b] Calculated as indicated in the text

The phosphate chain-modified analogs ADPαS, B-form (R configuration at α-phosphate [21]) and ADPβS which both contain a sulfur instead of a non-bridging oxygen atom in α- and β-phosphate, respectively, are no substrates in photophosphorylation, and ATPαS, B-form as well as ATPβS, B-form (R configuration [22]) are not hydrolyzed by light-triggered ATPase [23]. These two analogs are relatively poor competitive inhibitors in light-triggered hydrolysis of ATP, the K_i values being 200 μM (ATPαS, B-form) and 90 μM (ATPβS, B-form), respectively. This corresponds to the low competitive effect of ADPαS, B-form, and ADPβS on photophosphorylation [16]. The A-form of ATPβS is a substrate in light-triggered ATPase but inactive in the trypsin-activated Ca^{2+}-dependent ATPase [23].

In trypsin-activated ATPase reaction the K_m values for ATP and the employed analogs are in general by one order of magnitude higher than in the light-triggered ATPase (table 3). Moreover the V_{max} values are quite different for the single analogs. Surprisingly, 2′-deoxy-ATP is found to be an even better substrate with regard to V_{max} than ATP itself. The relative affinities calculated as indicated above, are however similar to those obtained in light-triggered ATPase and phosphorylation. The only exception is GTP which shows a much slower affinity.

The above results demonstrate that the assumption of separate active sites for ATP synthesis and ATP hydrolysis is unnecessary from the standpoint of nucleotide specificity. However, ATP synthesis proceeds almost linearly with time even against progressive accumulation of the reaction product ATP. Actually, the competitive effect of added ATP on photophosphorylation is marginal, K_i being as large as 4.4 mM (fig.1).

Accordingly, the active site employed in ATP synthesis appears to have only a low affinity to medium

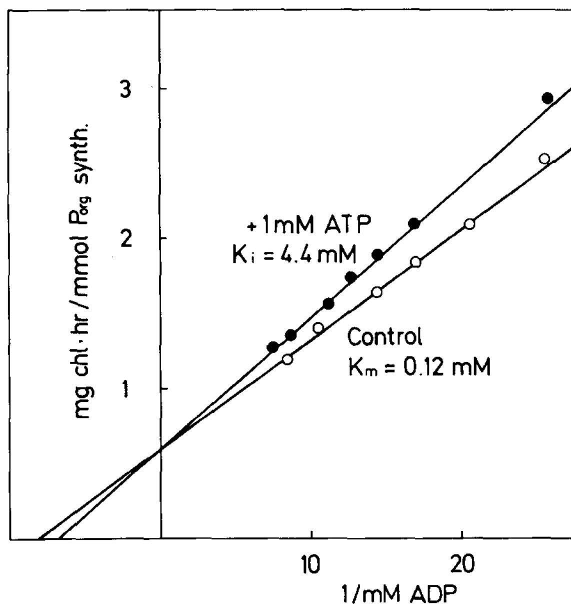
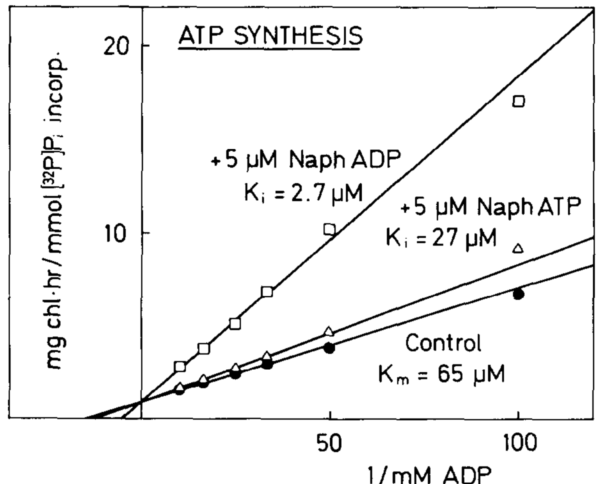


Fig.1. Effect of ATP on the rate of photophosphorylation.



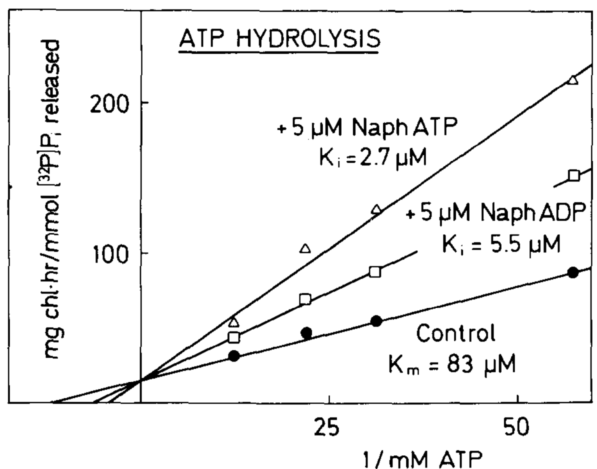


Fig.2. Effects of Naph-ADP and Naph-ATP on ATP formation and ATP hydrolysis. For measurement of ATP hydrolysis different concentrations of ADP were completely phosphorylated to [γ-^{32}P]ATP by pre-illumination of a chloroplast suspension for 5 min. Initial rates of ATP hydrolysis were determined by the disappearance of [γ-^{32}P]ATP in the following dark period. Additions of Naph-ADP and Naph-ATP, respectively, were made together with turning off the light.

ATP. This is further supported by comparison of the effects of 3′-naphthoyl esters of ADP and ATP, respectively, on photophosphorylation. Naph-ADP as well as other aryl- and acyl-esters of ADP are not substrate analogs in photosynthetic and oxidative phosphorylation but act as powerful competitive inhibitors of ATP synthesis [24–26], indicating that they occupy the active site of the corresponding ATPases with high affinity. The ATP analogs are likewise no substrates in ATP hydrolysis catalyzed by CF_1 or F_1 [25].

Fig.2 (top) shows that Naph-ADP exerts a strong competitive inhibition on ADP phosphorylation, while Naph-ATP is a very poor inhibitor. The K_i values differ by a factor of 10. In fig.2 (bottom) the effects of the two analogs on light-induced dark hydrolysis of ATP is shown. Compared to phosphorylation, the K_i for Naph-ADP is increased by a factor of 2, while K_i for the ATP analog is decreased dramatically by a factor of 10. Unquestionably the changes in the K_i values of the analogs reflect the changes in the affinities of the parental compounds ADP and ATP, respectively. Thus we conclude a considerable increase in the affinity of CF_1 to ATP when the conditions are changed from phosphorylation to ATP hydrolysis. The essential difference concerns the energy state of the chloroplasts. In the experiments described phosphorylation is measured by the initial rate of $^{32}P_i$ incorporation into ADP. For measurement of ATP hydrolysis, the added ADP is completely phosphorylated to $[\gamma\text{-}^{32}P]$ATP in the light and the decomposition of labeled ATP is followed after turning off the light.

CF_1 has been shown to undergo a conformational change when the chloroplasts are transferred from energized to de-energized conditions [27]. This is probably related to the observed change from ADP to ATP specificity.

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