Acid/Base-Induced Exchange of Adenine Nucleotides on Chloroplast Coupling Factor (CF_1)

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Exchange of CF_1-bound adenine nucleotides was shown to take place when isolated broken chloroplasts were subjected to an acid/base treatment. This process was measured in forward (incorporation of ^{14}C-labeled nucleotides) as well as back exchange reactions (release of bound labeled nucleotides). In the presence of unlabeled ADP and P_i a high precentage of labeled ATP was found to be released into the medium.

Exchange is highly specific for exogenous ADP and ATP. Acid/base-induced adenylate exchange and acid/base-induced phosphorylation were measured in dependence of several external factors. The substrate constant for ADP in exchange was 1.6 μM, whereas an apparent K_m of 5 μM was determined for ADP in the phosphorylation process.

Acid/base-induced exchange of adenine nucleotides and acid/base-induced phosphorylation depend in a similar way on the pH values of the acid and base stages.

Acid/base-induced ATP formation was sensitive to prolonged treatment in the acid stage whereas exchange was not. Mg^{2+} was strictly required in phosphorylation but less important in exchange. The phosphorylation ability decreased within seconds after acid/base transition; however, the ability of [^{14}C]ADP incorporation persisted for minutes. The formation of ATP depended on an acid/base transition, whereas some adenylate exchange was also induced by an acid treatment alone.

The results suggest that appropriate pH values at the inner and outer side of the thylakoid membrane rather than energy from a pH gradient is sufficient for adenylate exchange to take place on membrane-bound CF_1.

Introduction

Coupling factor CF_1 is generally assumed to catalyze the terminal reactions of photophosphorylation by reversal of ATPase reaction [1–3]. In elucidating the mechanism of energy transduction two recent experimental results may be of particular significance: (I) CF_1 was shown to undergo a conformational change when the thylakoid membrane is energized [4–6] suggesting that energy from electron transport may be directed in some way to the coupling protein. (II) CF_1 contains tightly bound adenine nucleotides in the de-energized state of the membrane [7–20]. On energization they are transferred to a loosely bound, exchangeable form [10–14, 16, 17]. Most probably the two effects are closely related to each other: an energy-induced conformational change of CF_1 may be the reason for the differential binding properties to adenylates.

In a hypothesis developed by Boyer [12] and Slater [10, 13], the formation of tightly bound ATP in the de-energized phase of the phosphorylation cycle and the energy dependent exchange of bound ATP with free ADP and P_i was regarded to be an essential event in the terminal reactions of phosphorylation. This presumes adenylate exchange to take place at the catalytic site of ATP synthetase. More recently a modified concept was established by Smith *et al.* [14] who assumed a transfer of the catalytic site to a tight ATP binding site as an intermediate step ("dual site mechanism").

The attractiveness of the conformational hypothesis led us to an extensive investigation of the energy-induced adenine nucleotide exchange process on CF_1. Several experimental results have already been published in previous papers [11, 15, 16]. Energy required for adenylate exchange can be supplied either by light dependent electron transport or by an acid/base transition [15], suggesting that a pH gradient across the thylakoid membrane is the driving force in this process **. In the present paper

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** Recently it was shown by P. Gräber *et al.* [32] that exchange can also be induced by an external electrical field.

acid/base-induced adenine nucleotide exchange was studied in more detail and discussed with respect to the relationship to the mechanism of phosphorylation.

Methods

Isolation and pre-treatment of chloroplasts has been described in a preceding paper [15]. For chlorophyll determination the method of Arnon [21] was applied.

Acid/base-induced back exchange experiments were carried out with chloroplasts which were pre-labeled with [^{14}C]ADP in the light. Pre-labeling was performed in a medium containing 50 mM Tricine buffer pH 8.3, 50 mM NaCl, 5 mM $MgCl_2$, 1 mM methylviologen, and 25 μM [^{14}C]ADP (Amersham-Buchler, Braunschweig). The chlorophyll content was 1 mg/ml. The samples were illuminated for one minute (white light, 5×10^6 ergs per cm^2 and sec) at 20 °C and then centrifuged for two minutes at $15000 \times g$. The pellet was washed four times using a solution of 2 mM Tricine buffer pH 8.3, 50 mM NaCl, and 5 mM $MgCl_2$. The residual bound label is exclusively related to CF_1 [11, 15]. For acid/base-induced release of the bound ^{14}C-labeled nucleotides, aliquots of the pellets were suspended in acid medium, usually containing 10 mM sodium succinate pH 4.0 and 25 μM DCMU for 15 sec. 1 ml of this suspension was poured into 1 ml base medium consisting of 110 mM Tricine buffer pH 8.3, 50 mM NaCl, 5 mM $MgCl_2$, and free nucleotides or P_i as indicated. The samples were kept in the base stage for one minute at room temperature and then centrifuged for two minutes at $15000 \times g$. In aliquots of the supernatants the contents of labeled nucleotides were measured by liquid scintillation counting in Unisolve 1 Scintillator (Koch Light Laboratories Ltd.) using the liquid scintillation spectrometer "tricarb, model 3320" (Packard). Adenine nucleotides were separated employing ion exchange column chromatography on Dowex-Cl (Serva) as described elsewhere [22].

In forward exchange experiments chloroplast pellets were suspended in an acid medium containing 10 mM sodium succinate pH 4.0 and 25 μM DCMU. The pH was controlled using a glass electrode. After a given time (usually 15 seconds) 1 ml of this suspension was mixed with 1 ml base medium, consisting of 110 mM Tricine buffer pH 8.3, 50 mM NaCl, 5 mM $MgCl_2$, and 50 μM [^{14}C]ADP. After one minute at room temperature the samples were centrifuged for two minutes at $15000 \times g$. The pellets were washed four times using a solution of 10 mM Tricine buffer pH 8.3, 50 mM NaCl, and 5 mM $MgCl_2$. The contents of total membrane bound ^{14}C-labeled nucleotides were determined either directly or after extraction of the nucleotides by urea treatment as described elsewhere [15]. In the first case quench correction was performed using internal or external standards. – Acid/base-induced phosphorylation was measured under the same experimental conditions, except that unlabeled ADP (0.2 mM) was used and ^{32}P-labeled orthophosphate (2 mM) was added. Incorporation of $^{32}P_i$ into the organic phosphate fraction was determined as described elsewhere [23].

Results

I. Acid/base-induced release of tightly bound ^{14}C-labeled adenine nucleotides

In previous reports it was demonstrated that chloroplasts specifically incorporated [^{14}C]ADP into CF_1 in a light-dependent reaction [11, 15]. The bound label could not be removed by subsequent washes. However, on illumination of the membranes, the bound nucleotides were partly released or exchanged depending on the absence or presence of free ADP or ATP, respectively [10, 11, 15, 16]. In the experiments shown in this chapter, chloroplasts were pre-labeled with [^{14}C]ADP in the light and then subjected to acid/base treatment.

In an experiment shown in Table I, unlabeled ADP and P_i, respectively, were added either in the acid or in the base stage. Afterwards the released labeled nucleotides were separated by chromatography. No difference was observed whether the two substrates were present in the acid stage or only added to the base medium. A large amount of bound nucleotides was released in the presence of ADP, indicating an acid/base-induced exchange. In the absence of free ADP release was much lower and scarcely exceeded the acid control.

If P_i was omitted from the medium, mainly [^{14}C]ADP was liberated. In this case the pattern of released nucleotides closely resembled that of nucleotides present on the membranes before energization [11, 16]. The pattern was changed towards [^{14}C]ATP production, when the medium contained P_i. The result suggests that phosphorylation of [^{14}C]ADP took place either prior to release or in a consecutive reaction.

Under all conditions the acid controls gave a higher amount of released nucleotides than the base controls, even in the presence of P_i. However, most of the nucleotides liberated by acid treatment alone were shown to be [^{14}C]ADP.

Table I. Acid/base-induced release of bound [^{14}C]adenine nucleotides with different additions in acid or base step, respectively. Final chlorophyll content was 0.125 mg/ml. Where indicated, P_i at a final concentration of 5 mM or ADP at a final concentration of 0.2 mM were added. If ADP or P_i were added in the acid step, the base step medium contained the same additions in order to maintain the indicated concentrations.

	nmol [^{14}C]AdN released per mg chlorophyll			
	ATP	ADP	AMP	total
Acid/base, no addition	0.052	0.097	0.013	0.162
Acid contr., no addition	0.047	0.102	0.010	0.159
Base contr., no addition	0.017	0.068	0.005	0.090
Acid/base, $+P_i$ in base	0.122	0.076	0.013	0.211
Acid/base, $+P_i$ in acid	0.119	0.085	0.015	0.219
Acid contr., $+P_i$	0.066	0.102	0.017	0.185
Base contr., $+P_i$	0.025	0.067	0.008	0.100
Acid/base, +ADP in base	0.143	0.342	0.034	0.519
Acid/base, +ADP in acid	0.139	0.335	0.044	0.518
Acid contr., +ADP	0.062	0.172	0.025	0.259
Base contr., +ADP	0.018	0.075	0.008	0.101
Acid/base, $+P_i$+ADP in base	0.349	0.146	0.035	0.530
Acid/base, $+P_i$+ADP in acid	0.365	0.139	0.030	0.534
Acid contr., $+P_i$+ADP	0.113	0.220	0.030	0.363
Base contr., $+P_i$+ADP	0.026	0.071	0.010	0.107
Acid/base, no addition	0.052	0.102	0.014	0.168
Acid contr., no addition	0.048	0.108	0.020	0.176
Base contr., no addition	0.012	0.066	0.008	0.086

In early experiments on acid/base phosphorylation it was established that the presence of an appropriate membrane permeating anion during the acid stage was required for satisfactory ATP yields [24, 25]. In acid/base-induced adenine nucleotide exchange a similar requirement was found. Table II shows succinate to be the most suitable anion, yielding the highest exchange rate and also the largest amount of [^{14}C]ATP under the employed conditions (unlabeled ADP + P_i present).

The yield of [^{14}C]ATP greatly depends on the time of incubation in the acid medium, although the total exchange of adenine nucleotides was unaffected (Fig. 1). This result corresponds to the previous finding that acid/base phosphorylation is reduced with prolonged treatment at pH 4.0 [15]. Obviously the exchange reaction is less sensitive to the acid treatment than the ATP generating system.

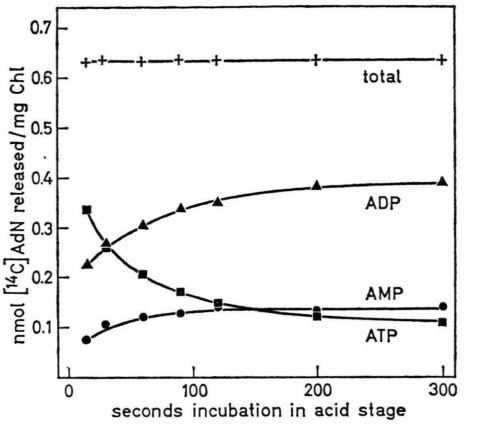

Fig. 1. Release of bound [^{14}C]adenine nucleotides as a function of incubation time in the acid step. The base step medium contained 0.2 mM ADP and 2 mM P_i. Final chlorophyll content was 0.25 mg/ml.

II. Acid/base-induced incorporation of [^{14}C]adenine nucleotides

Light induced electron transport can be replaced by an acid/base transition to induce [^{14}C]adenine nucleotide incorporation into the membranes (forward exchange reaction). In Table III the acid/base-induced incorporation of ^{14}C-labeled AMP, ADP, and ATP are shown. While AMP exhibited only a minor affinity to the membranes, much higher amounts of nucleotides were incorporated using [^{14}C]ADP and [^{14}C]ATP, respectively. In accordance with the back exchange experiments (see Table I) there was no difference whether the nucleotides were added in the acid or base step. In forward exchange the acid controls also yielded higher

Acid stage	Treatment	nmol [^{14}C]AdN released/mg chlorophyll
Succinate (pks_1=4.2 pks_2=5.6)	acid/base	0.56 (0.42 ATP + 0.11 ADP + 0.03 AMP)
	acid control	0.26
	base control	0.06
Malate (pks_1=3.4 pks_2=5.2)	acid/base	0.31 (0.12 ATP + 0.17 ADP + 0.02 AMP)
	acid control	0.11
	base control	0.06
Maleate (pks_1=1.9 pks_2=6.5)	acid/base	0.17 (0.06 ATP + 0.10 ADP + 0.01 AMP)
	acid control	0.06
	base control	0.06

Table II. Acid/base-induced back exchange of bound [^{14}C]-labeled adenine nucleotides as affected by the presence of organic acids in he acid stage. Base step contained additions of 0.2 mM ADP and 5 mM P_i (final concentrations). Final chlorophyll concentration was 0.250 mg/ml in the experiments employing succinate and malate, respectively, and 0.201 mg/ml in the experiment with maleate.

Table III. Acid/base-induced binding of ^{14}C-labeled AMP, ADP, and ATP (final concentrations: 25 μM). Chlorophyll content after acid/base transition was 0.250 mg/ml.

	Additions in acid or base stage, respectively	nmol [^{14}C]AdN bound/mg chlorophyll
[^{14}C]AMP	acid	0.036
	base	0.033
	acid control	0.014
	base control	0.026
[^{14}C]ADP	acid	0.611
	base	0.630
	acid control	0.216
	base control	0.066
[^{14}C]ATP	acid	0.574
	base	0.568
	acid control	0.241
	base control	0.104

amounts of incorporated nucleotides than the base controls.

The effect of Mg^{2+} on ADP binding is shown in Table IV. Mg^{2+} does not seem to be strictly required in the binding process; however, it stimulates the incorporation of ADP by about 50%. Previously a similar effect has been described in light induced adenylate exchange [16].

Table IV. Effect of Mg^{2+} on acid/base-induced binding of ^{14}C-labeled ADP (final concentration: 22.9 μM). Where indicated 5 mM $MgCl_2$ (final concentration) was added. Chlorophyll content in the incubation mixture was 0.40 mg/ml.

Present in acid stage	Present in base stage	nmol [^{14}C]AdN incorporated per mg chlorophyll
ADP+Mg^{2+}	ADP+Mg^{2+}	0.367
Mg^{2+}	ADP+Mg^{2+}	0.355
ADP	ADP+Mg^{2+}	0.345
	ADP+Mg^{2+}	0.334
ADP	ADP	0.236
	ADP	0.246
ADP+Mg^{2+}	acid control	0.180
ADP	acid control	0.124
base control	ADP+Mg^{2+}	0.072
base control	ADP	0.070

Fig. 2 shows the amount of [^{14}C]ADP incorporated as a function of [^{14}C]ADP concentration. The double-reciprocal plot reveals a monophasic concentration curve with a substrate constant (K_s) of 1.6 μM. In light induced ADP binding K_s values of 2 μM [11] and 5 μM [17], respectively, were found. In a parallel experiment the apparent K_m for ADP in

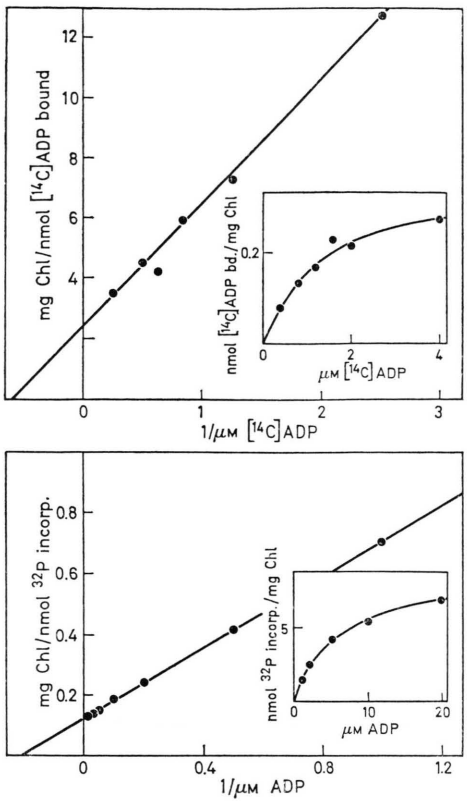

Fig. 2. Acid/base-induced [^{14}C]ADP binding and phosphorylation as a function of ADP concentration. For phosphorylation measurements the medium contained 2 mM [^{32}P]P_i. Chlorophyll contents after acid/base transitions were 0.275 mg/ml.

acid/base-induced phosphorylation was determined to be 5 μM. It differs from the apparent K_m reported for light dependent phosphorylation (60 μM [26, 27]).

In the following experiments comparative studies of [^{14}C]ADP incorporation and phosphorylation were performed. In Figs 3 and 4 the pH values of the base and acid stages, respectively, were varied. In Fig. 5, a constant pH jump of 3.5 units, starting from different pH values of the acid step, was employed. In all cases the two processes exhibited the same pH optima, although the shapes of the pH curves were not completely identical.

In a recent paper [11] we reported [^{14}C]ADP to be incorporated at a high rate by pre-illuminated chloroplasts. The ability of ADP binding was maintained for several minutes in the dark. A similar result was obtained, when energization of the membranes was carried out by an acid/base shift instead of illumination (upper part of Fig. 6). A subsequent

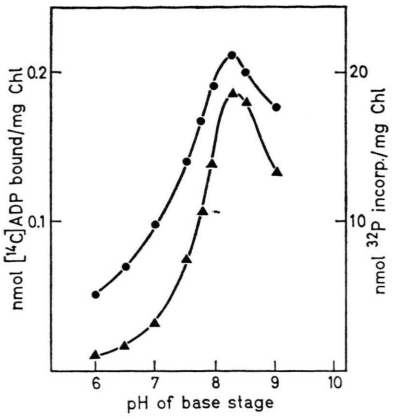

Fig. 3. Acid/base-induced [^{14}C]ADP binding (●) and phosphorylation (▲) in dependence of the pH of the base step. pH of the acid medium was 4.0. Chlorophyll content after acid/base transition was 0.50 mg/ml. [^{14}C]ADP concentration was 22.9 μM; in phosphorylation experiments ADP and P_i concentrations were 0.2 mM and 2 mM, respectively.

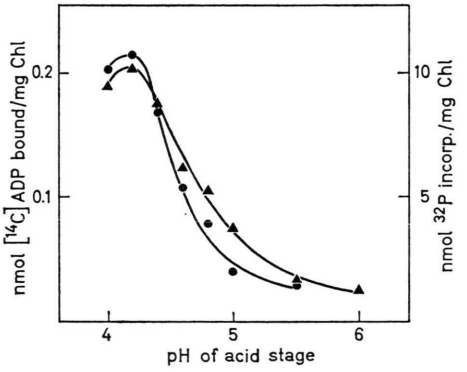

Fig. 4. Acid/base-induced [^{14}C]ADP binding (●) and phosphorylation (▲) in dependence of the pH of the acid step. pH of the base medium was 8.3.

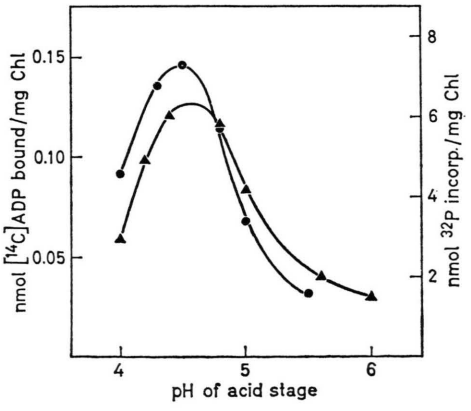

Fig. 5. Acid/base-induced [^{14}C]ADP binding (●) and phosphorylation (▲) mediated by a constant pH jump of 3.5 units. pH of acid and base steps were varied.

discharge of the energy state by FCCP did not change the slow decay kinetic. On the other hand, post-acid/base phosphorylation decreased within a few seconds after pH transition (lower part of Fig. 6).

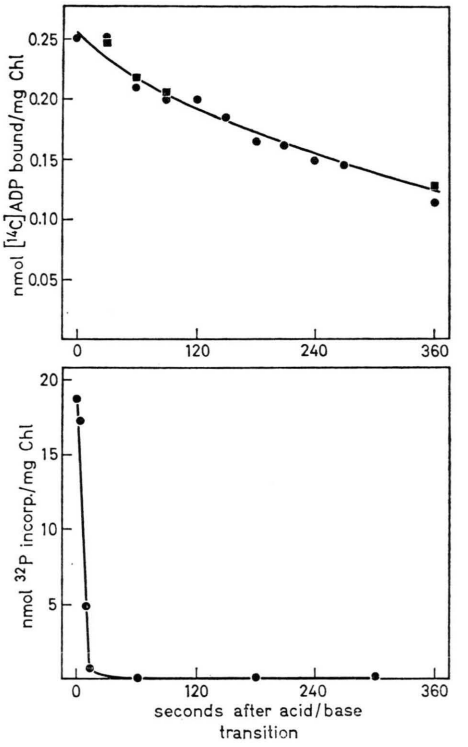

Fig. 6. [^{14}C]ADP binding (upper part) and phosphorylation (lower part) following a preceding acid/base transition. Acid/base transition was performed as described in methods, except that [^{14}C]ADP (19.7 μM) and ADP (0.2 mM) + [^{32}P]phosphate (2 mM) were omitted from the base step medium and added at indicated times. Chlorophyll content in the complete medium was 0.22 mg/ml.

(■) Addition of FCCP (final concentration 36 μM) at zero time.

Discussion

CF_1 contains firmly bound adenine nucleotides which are exchanged for free ADP or ATP on energization of the thylakoid membranes [10, 11, 15–17]. Most probably adenylate exchange is related to a conformational change of CF_1, which has been concluded to occur under similar conditions from several indirect evidences [4–6]. The data on adenylate exchange [10–17] and conformational change [4–6] of coupling factor can be taken as a hint that these reactions can be induced either by light or by an acid/base transition.

In all essential respects acid/base-induced nucleotide exchange resembles light induced exchange. Both, forward and back exchange are caused by light as well as by an acid/base shift. Similar substrate constants for ADP were obtained in the two systems. Comparable effects of Mg^{2+} have been found and the same slow decay of [^{14}C]ADP binding has been observed after a preceding energization of the membranes by light or by acid/base transition. This parallel behavior may indicate that light- and acid/base-induced nucleotide exchange are identical processes caused by two different treatments. Obviously a difference in pH at the inner and outer sides of the thylakoid membrane is the direct occasion for the conformational change of CF_1 which transfers the protein from a non-exchangeable to an adenine nucleotide exchangeable form [10, 11, 15, 18]. In the light system the asymmetrical proton distribution is established by the electron transport-driven unidirectional proton pump. There are two possibilities to explain the relationship between proton distribution and conformational change of CF_1: (1) the change in proton conformation needs energy in form of a transmembrane pH difference, (2) it is induced only by a proper pH milieu at the two sides of the membrane. Although not yet discussed, the latter possibility gains some probability from the experimental results presented in this paper. In each of our experiments it is obvious that acid treatment alone yielded a relatively effective exchange, although an acid/base transition was the optimum condition. Since acid-induced exchange was obtained in the forward as well as in the back reaction, an artifact due to protein denaturation is unlikely. On the other hand, ATP formation strictly requires a transmembrane pH gradient [28, 29]. Accordingly, in the presence of P_i, release of ATP from bound ADP was found on acid/base transition but not in acid controls.

The relationship between adenine nucleotide exchange and phosphorylation still is not clear. Comparison of acid/base-induced adenylate exchange and phosphorylation as affected by the pH of acid and base stages suggests that adenylate exchange might be involved in the process of phosphorylation as a partial reaction. This is one of the essential assumptions of the conformational hypothesis as put forward by Boyer [12] and Slater [10, 13]. It may be supported additionally by the finding that free ATP is formed from bound ADP in the presence of P_i. However, recently Rosing *et al.* [30] and Shavit *et al.* [31] demonstrated conclusively that tightly bound ADP is released first into the medium and then converted to ATP by phosphorylation, probably at a different site. Moreover, from our experiments on light induced adenylate exchange a direct participation of tightly bound ADP in phosphorylation is unlikely [11]. The rate of exchange of bound nucleotides is much too low as to account for the steady state rate of ATP formation. A critical evaluation of the available data on tightly bound adenine nucleotides favours the view that these are not related to the catalytic site of ATP synthetase.

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