Biochimica et Biophysica Acta 893 (1987) 342–348
Elsevier

BBA 42604

An ATP/ADP γ-phosphate exchange catalyzed by chloroplast ATPase

Susanne Bickel-Sandkötter and Heike Schüll

Botanisches Institut der Universität Düsseldorf, Düsseldorf (F.R.G.)

(Received 3 April 1987)

Key words: ATPase; ATP–ADP exchange; Phosphate exchange; Photophosphorylation; (Spinach chloroplast)

Chloroplast thylakoids show a light and Mg^{2+}-dependent ATP/ADP γ-phosphate-exchange activity in the absence of inorganic phosphate. This exchange activity is inhibited by the energy-transfer inhibitor phlorizin, indicating that it is catalyzed by the chloroplast ATPase. In contrast to ATP/inorganic phosphate exchange no ATP/ADP exchange is observed in the dark after preillumination in presence of dithiothreitol. No net hydrolysis is measured in the light which could account for apparent ATP/ADP exchange. The results suggest that two cooperating catalytic sites approach each other during the catalytic process in a way that a transfer of phosphate from bound ATP on site 1 to bound ADP on site 2 occurs without intermediate inorganic-phosphate release, and might proceed without intermediate ATP hydrolysis and resynthesis. In the present paper evidence is given that the exchange process requires less energy than photophosphorylation.

Introduction

Chloroplast ATPase catalyzes a couple of exchange reactions reflecting partial steps of the overall enzymatic reaction, the reversible production of ATP from ADP and inorganic phosphate, described by the equation

$$3\ H^+_{int} + ADP + P_i \rightleftharpoons 3\ H^+_{ext} + ATP + H_2O$$

Originally chloroplasts appeared not to have any phosphorylation-related exchange reactions [1] like that found in mitochondria. But the later use of more sensitive methods showed that ATP-P_i exchange occurs during net ATP synthesis in the light [2] and with much faster rates in the dark after light-triggering in presence of thiol compounds [3,4]. More detailed experimental approach of the ATP-P_i exchange showed that it results from rephosphorylation of medium ADP formed by ATP hydrolysis, driven by the ATP-induced $\Delta\mu_{H^+}$ [5].

In the light chloroplasts catalyze an exchange of oxygen atoms between $H_2{}^{18}O$ and ATP [2,6] and with lower rates between $H_2{}^{18}O$ and P_i [2]. In the absence of ADP a more effective incorporation of ^{18}O into ATP as well as into P_i could be measured than in its presence [2]. These observations indicate reversible cleavage of ATP by $H_2{}^{18}O$ to ADP and $P^{18}O_4^{3-}$ which rephosphorylates enzyme-bound ADP to give ATP which is ^{18}O-labelled in γ-phosphate. These results, together with the fact that ATP-P_i exchange under energized conditions is much slower than the H_2O-ATP exchange [2], led to the conclusion that ADP and P_i remain bound during the exchange process, whereas H_2O at the active site is exchanged rapidly

Abbreviations: CF_1, chhloroplast coupling factor 1; Chl, chlorophyll; P_i, inorganic phosphate; PMS, *N*-methylphenazonium methosulfate; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine.

Correspondence: S. Bickel-Sandkötter, Botanisches Institut der Universität Düsseldorf, Universitätsstrasse 1, D-4000 Düsseldorf, F.R.G.

against labelled H_2O from the medium. The occurrence of these exchange reactions indicated the reversibility of the ATPase reaction at the active site of energized CF_1.

Another exchange reaction which has been described to occur in mitochondria, is an ADP/ATP exchange [7]. Kahn and Jagendorf [8] reported an energy-independent ADP/ATP exchange in chloroplasts. They isolated an enzyme from chloroplasts which catalyzed this exchange. However, this reaction was obviously not related to photophosphorylation. In broken chloroplasts this exchange activity which could be measured in the dark disappeared with the number of washings, while most of the ability to catalyze photophosphorylation had been retained [9]. No exchange activity could be found in washed broken chloroplasts in the dark [1,9] as well as in the dark after preillumination in presence of dithiothreitol [3]. We recently reported a light-dependent phosphate exchange between ATP and ADP in four times washed broken chloroplasts. This exchange can be observed when no inorganic phosphate is present in the medium [10].

The present paper extends our investigations on this ATP/ADP exchange which can be discriminated from accompanying reactions like phosphorylation by endogenous phosphate and a slow residual adenylate kinase reaction.

Methods

Spinach chloroplasts were isolated and washed four times in order to remove most of the adenylate kinase as described earlier [11]. Measurements of ATP/ADP exchange were carried out in a medium containing 25 mM Tricine (pH 8), 50 mM NaCl, 5 mM $MgCl_2$, 50 μM PMS and the indicated concentrations of ADP and ATP. The reactions were carried out in small glass vials which were inserted into a temperature-controlled water bath on a magnetic stirrer. The temperature was 20 °C, and the standard light intensity (white light) 400 W/m^2. After a preillumination time of 5 min, carrier-free [8-^{14}C]ADP was added. The reaction was stopped by addition of perchloric acid to give a final concentration of 0.5 M.

The pattern of nucleotides were analyzed by TLC (PEI-Cellulose sheets, CEL 300 PEI/UV_{254}, Macherey-Nagel) after neutralization of the samples. The spots corresponding to AMP, ADP and ATP were scraped out and their radioactivity measured in Opti-Fluor scintillator (United Technologies Packard) in a liquid scintillation counter.

Photophosphorylation was measured parallel to exchange under comparable conditions. If not indicated otherwise, instead of [^{14}C]ADP a mixture of phosphate (5 mM) and [^{14}C]ADP was added after the preillumination time and the [^{14}C]ATP increase determined by TLC analysis.

ATP hydrolysis was measured by the formation of inorganic phosphate, which was determined colorimetrically, as described in Ref. 12.

Results

Isolated thylakoid membranes contain a low level of inorganic phosphate even after extensive washing. In the presence of ADP this endogenous P_i gives rise to ATP formation via photophosphorylation.

In Fig. 1 photophosphorylation by endogenous phosphate is shown. In this experiment three-times washed chloroplasts were either illuminated or kept in the dark in the presence of [^{14}C]ADP for 10 min. The results show a linear increase of [^{14}C]ATP in the dark which is obviously due to adenylate kinase reaction, and a superimposed increase of [^{14}C]ATP due to photophosphorylation in the light which is terminated after about 4

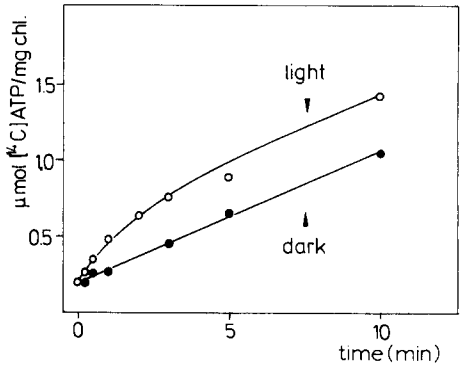

Fig. 1. [^{14}C]ATP formation by broken chloroplasts in the light (○) and in the dark (●). The reaction medium contained 250 μM [^{14}C]ADP and chloroplasts equivalent to 25 μg chlorophyll/ml. The chloroplasts were washed three times before use.

min. The calculated endogenous phosphate is 0.4 μmol/mg Chl. In order to avoid interference of photophosphorylation by endogenous P_i with ATP/ADP exchange, in the following experiments thylakoids were preilluminated for 5 min in the complete ADP- and/or ATP-containing medium before carrier-free [^{14}C]ADP was added. By the pretreatment all endogenous and possible contaminating phosphate of the ADP and ATP solutions is used up by photophosphorylation before the exchange reaction is measured.

Fig. 2 shows the kinetics of the increase of [^{14}C]ATP in the following light period after addition of [^{14}C]ADP. The upper curve shows the formation of [^{14}C]ATP with 500 μM ATP and 200 μM ADP in the medium. The initial rate of [^{14}C]ATP formation in this case is 13.5 μmol/mg Chl per h. Deflection of the curve may be explained by the decrease of the specific activity of [^{14}C]ADP and the increase of specific activity of [^{14}C]ATP during the course of the reaction. A slower increase of [^{14}C]ATP can be observed when ATP is omitted from the medium (bottom curve). Under these conditions the rate of [^{14}C]ATP formation is 1.8 μmol/mg Chl per h and an equal rate of [^{14}C]AMP formation is measured. The same rate of [^{14}C]AMP formation is observed in the sample containing ADP + ATP. In the absence of ATP, equal rates of formation of [^{14}C]ATP and [^{14}C]AMP are observed in the light as well as in the dark, as shown in Table I.

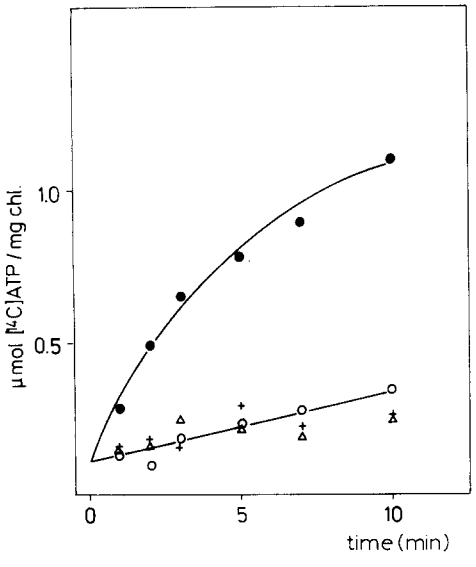

Fig. 2. Kinetics of [^{14}C]ATP (●, ○) and [^{14}C]AMP (△, +) formation in the light in presence of ATP (upper curve) and when ATP is omitted from the medium (bottom curve). The reaction medium containing 200 μM ADP +/− 500 μM ATP and 20 μg Chl/ml was preilluminated for 5 min before carrier-free [^{14}C]ADP was added.

As the sum of the increase of ATP and AMP corresponds to the decrease of ADP, and the reaction is not energy-dependent, this [^{14}C]ATP surely has its origin in the activity of the adenylate kinase. When [^{14}C]ATP formation by adenylate kinase reaction is subtracted from total [^{14}C]ATP formation in the experiment shown in Fig. 2, a residual rate of 12 μmol [^{14}C]ATP/mg Chl per h may be ascribed to light-dependent ATP/ADP γ-phosphate exchange. K_m(ADP) for the exchange reaction is 57 μM, whereas K_m(ATP) is 400 μM (Fig. 3). V_{max} varies between 12 and 25 μmol/mg Chl per h.

Fig. 4 shows inhibition of the exchange reaction by the energy-transfer inhibitor phlorizin, in comparison with inhibition of photophosphorylation. The I_{50} value is 0.75 mM in both reactions. Phlorizin is known to interact with the CF_1 sector of chloroplast ATPase. Hence participation of CF_1 in the ATP/ADP γ-phosphate exchange reaction is likely.

The Mg^{2+}-dependence of the reaction is shown in Fig. 5, it corresponds to the Mg-dependence of photophosphorylation.

Fig. 6 shows the inhibition of exchange and photophosporylation, respectively, by the uncoupler nigericin. By 0.5 μM nigericin photophos-

TABLE I

ADENYLATE KINASE ACTIVITY OF WASHED BROKEN CHLOROPLASTS

Conditions as in Fig. 2. Carrier-free [^{14}C]ADP was added after 5 min preillumination in the presence of 200 μM ADP either in the light or 15 s after switching the light off. Chlorophyll content was 20 μg/ml.

Conditions	increase (μmol/mg Chl per h)		decrease
	AMP	ATP	ADP
Light	2.4	2.2	4.2
Dark	2.0	2.2	4.4

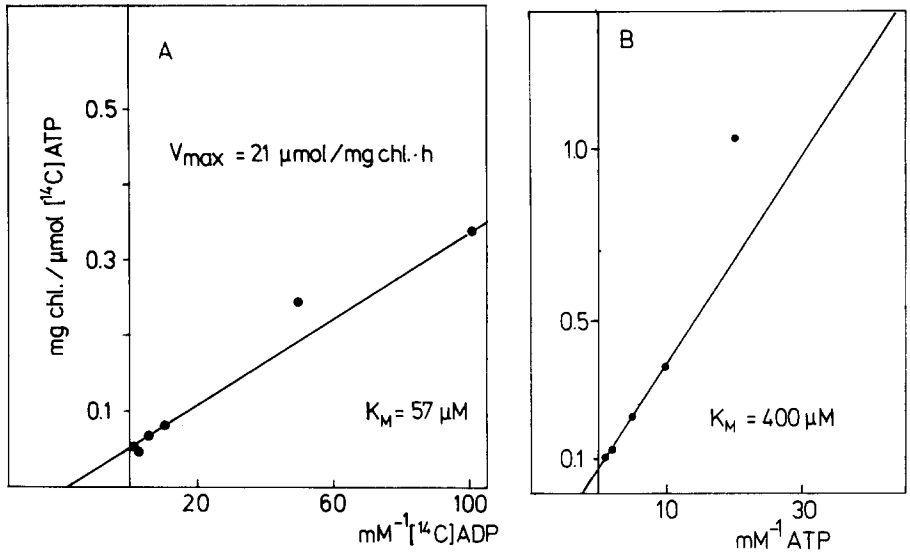

Fig. 3. Determination of the kinetic constants for ADP (A) and ATP (B) in the ATP/ADP phosphate exchange reaction. Nucleotide concentrations were varied from 0 to 1 mM, the concentration of the other nucleotide was 0.2 mM. Chlorophyll content 20 µg/ml.

phorylation is decreased to about 9%, whereas 20% of the exchange rate are left at the same uncoupler concentration.

Comparison of light-intensity curves of exchange and photophosphorylation shows significant differences. While ATP formation exhibits a sigmoidal intensity dependence with the known 'low light lag' [14], the light intensity curve of

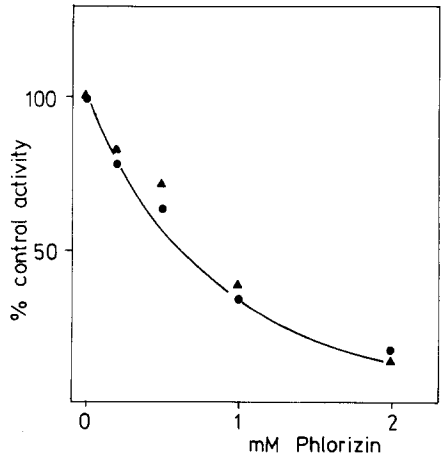

Fig. 4. ATP/ADP phosphate exchange (▲) and photophosphorylation (●) as affected by phlorizin, measured under comparable conditions. The assay medium contained 20 µg chlorophyll/ml, 200 µM [^{14}C]ADP, 500 µM ATP and in case of phosphorylation additional 5 mM P_i. Control activities: (▲) 7.8 µmol [^{14}C]ATP/mg Chl per h, (●) 179 µmol [^{14}C]ATP/mg Chl per h.

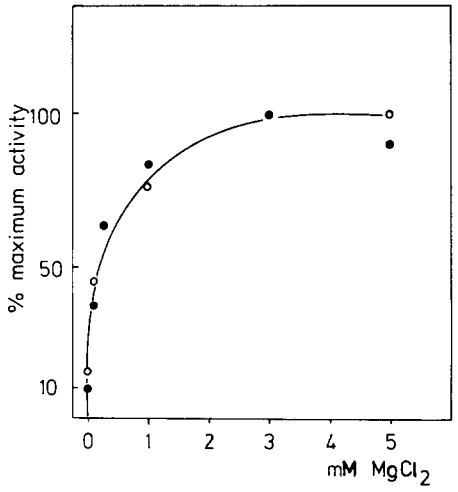

Fig. 5. ATP/ADP exchange and photophosphorylation as a function of $MgCl_2$ concentration. Exchange (●): 200 µM [^{14}C]ADP, 500 µM ATP, control activity 9.7 µmol [^{14}C]ATP/mg Chl per h. Phosphorylation (○): control activity 140 µmol/mg Chl per h, the assay medium contained 5 mM P_i instead of ATP.

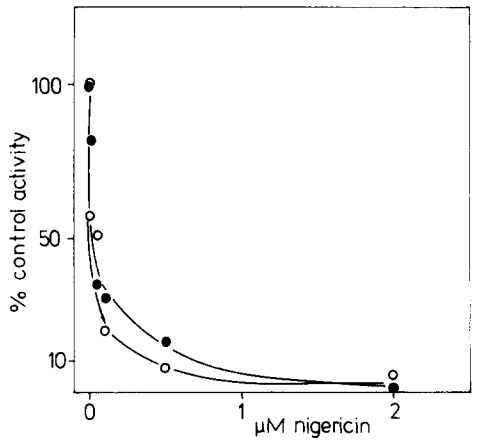

Fig. 6. ATP/ADP exchange and photophosphorylation as affected by the uncoupler nigericin. Conditions: 3-min preillumination in the presence of 200 μM ADP and 500 μM ATP, followed by addition of carrier-free [^{14}C]ADP with or without 5 mM P_i. Exchange (●): The rates were determined by following kinetics from 1 to 5 min, control activity was 7.2 μmol/mg Chl per h. Phosphorylation (○): kinetics from 15 to 60 s, control activity 390 μmol/mg Chl per h.

ATP/ADP γ-phosphate exchange is hyperbolic (Fig. 7). Both reactions were measured under the same conditions, except that in the case of phosphorylation 5 mM P_i was added after the standard time of preillumination, carried out in both cases in the presence of 200 μM ADP and 500 μM ATP.

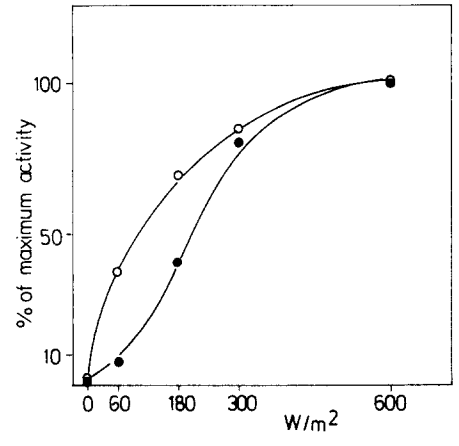

Fig. 7. ATP/ADP exchange and photophosphorylation as affected by light intensity. Conditions as described in Fig. 6. Control activities: exchange (○): 7 μmol/mg Chl per h, phosphorylation (●): 267 μmol/mg Chl per h.

TABLE II

FORMATION OF PHOSPHATE FROM [^{14}C]ATP AND CONCOMITANT [^{14}C]ADP FORMATION BY DITHIOTHREITOL-ACTIVATED THYLAKOIDS IN THE ABSENCE AND PRESENCE OF ADP

The reaction medium contained 5 mM dithiothreitol, 200 μM [^{14}C]ATP and 100 μM ADP, when indicated. The nucleotides were added after 2 min preincubation in the light. The rates were obtained by measuring kinetics from 30 s to 5 min. The chlorophyll content was 20 μg/ml.

Conditions	(μmol P_i/ mg Chl per h)		(μmol [^{14}C]ADP/ mg Chl per h)	
	+ADP	−ADP	+ADP	−ADP
Light	0	0	13.0	0
Dark after preillumination	4.5	33.0	3.75	30.0
Dark	1.0	1.5	4.5	3.0

In order to examine whether the measured ATP/ADP exchange can be interpretated as the result of ATP hydrolysis and simultanous resynthesis of ATP by liberated phosphate, exchange and net formation of phosphate were measured under various conditions, as shown in Table II. In order to activate ATP hydrolysis, the chloroplasts were illuminated in the presence of dithiothreitol. At variance with the experiments shown above, exchange was measured by using the substrate couple [^{14}C]ATP and ADP according to

$$[^{14}C]ATP + ADP \overset{\Delta\mu_{H^+}}{\rightleftharpoons} [^{14}C]ADP + ATP$$

In the light, in the absence of ADP as well as in its presence, no accumulation of inorganic phosphate could be detected, indicating that net ATP hydrolysis is blocked under these conditions. Nevertheless, formation of [^{14}C]ADP from [^{14}C]ATP is found in the presence of unlabeled ADP, which may be referred to ATP/ADP γ-phosphate exchange. In the dark after preillumination, phosphate and [^{14}C]ADP is formed at the same rate from [^{14}C]ATP when ADP is omitted from the medium, indicating light-triggered ATP hydrolysis. When ADP was added simultaneously with ATP, the formation of P_i and [^{14}C]ADP, respectively, was largely prevented. Very low rates of P_i

and [^{14}C]ADP formation were obtained with control chloroplasts (no preillumination), both in the absence and presence of ADP.

Discussion

The above results demonstrate that in the absence of P_i isolated thylakoids carry out a light-dependent exchange between ATP and ADP which most likely includes a transfer of γ-phosphate from ATP to ADP:

$$[^{14}C]ADP + ATP \overset{\Delta\mu_{H^+}}{\rightleftharpoons} [^{14}C]ATP + ADP$$

From the inhibitory effect of phlorizin it may be concluded that this exchange activity is catalyzed by the chloroplast ATPase. Interfering reactions like adenylate kinase activity or photophosphorylation by endogenous phosphate can be subtracted or experimentally excluded (Fig. 1, Table I). Participation of an "ADP/ADP-transphosphorylation" process as described by Moudrianakis and Tiefert [15] to occur in isolated CF_1 can be ruled out by measuring the exchange reaction with the substrate couples [^{14}C]ATP and ADP (as demonstrated in Table II and Ref. 10). Moreover, the reaction observed by Moudrianakis and Tiefert was described to require no Mg^{2+}, whereas a strong Mg^{2+} dependency of the ATP/ADP phosphate exchange is shown here. Many properties of the here described ATP/ADP exchange reaction are similar to known properties of photophosphorylation, like the requirement for Mg^{2+} (Fig. 5), the inhibition by phlorizin, as well as the K_m(ADP) for the exchange (57 μM) which is comparable to the K_m for ADP in photophosphorylation under the employed conditions [16]. For ATP, as the phosphate donor in the exchange reaction a much higher K_m of 400 μM has been determined. These results coincide with the previously shown fact that in energized chloroplasts catalytic binding is highly specific for ADP [17] and that ATP is only a poor competitive inhibitor of ATP synthesis with a K_i of about 4 mM [18].

During the exchange reaction the nascent phosphate obviously is not released into the medium before it is rebound to [^{14}C]ADP. No net ATP hydrolysis is measured in the light (Table II) which could account for apparent ATP/ADP exchange. Moreover, the high $K_m(P_i)$ compared to the low K_m(ADP) in photophosphorylation [16] and exchange (Fig. 3) would make P_i rebinding from the medium unlikely. For the same reason it seems improbable that P_i formed by ATP hydrolysis remains bound to the catalytic site, while bound ADP undergoes exchange with medium ADP. A covalent phosphorylated intermediate has been excluded experimentally [19]. These considerations, together with the finding that arsenate has only a small inhibitory effect on the exchange [10] lead to the assumption that two of the cooperating catalytic sites [20] approach each other during the exchange process, so that a transfer of phosphate from ATP on site 1 to ADP on site 2 occurs without intermediate P_i release.

The energy requirement of photophosphorylation obviously is stronger than that of ATP/ADP phosphate exchange, as demonstrated by the light intensity curves and the effect of uncoupling. If assumed that in the process of photophosphorylation energy is needed for binding and displacement of the substrates, and that further energy is needed for the formation of the phosphoanhydride bond [21] which includes the nucleophilic reaction of $ADPO^-$ with protonated phosphate, we might explain the smaller energy requirement of the exchange reaction compared with net ATP formation in the following way. If the transfer of the γ-phosphate in ATP/ADP exchange proceeds directly without intermediate ATP hydrolysis and resynthesis, energy might be required for binding and release of the substrates only. This assumption gets support by the early observation of Shavit et al. [2] that ADP inhibits the light-dependent ATP/$H_2{}^{18}O$ exchange, which might reflect the phosphate transfer between ATP and ADP.

If this interpretation is correct, the question rises, why no exchange could be found in the dark after preillumination in presence of dithiothreitol, as presented in Table II. The $\Delta\mu_{H^+}$ induced by ATP hydrolysis in the dark after preillumination in presence of dithiothreitol obviously is sufficient to drive the rephosphorylation of ADP, in the process of ATP-P_i exchange [5] and should be sufficient to drive ATP/ADP exchange as well. The conditions for ATP/ADP exchange differ from those for ATP γ-phosphate exchange on

principle in the added amount of ADP. ADP causes rapid decay of the active state of the ATPase established by preillumination [22]. This deactivation is due to tight binding of ADP [23] to a site located in the β-subunit of CF_1 [20]. This explains the very low rate of ATP hydrolysis (13% of the control rate) observed in the presence of ADP (Table II). As a consequence a low $\Delta\mu_{H^+}$ is established, which probably is insufficient to drive the exchange reaction.

Kahn and Jagendorf [8] isolated and characterized an enzyme from chloroplasts which catalyzed an energy-independent ATP/ADP-phosphate exchange. This exchange reaction obviously is not related to the here described energy-dependent ATP/ADP γ-phosphate exchange. As shown by Ben-Yehoshua and Avron [9] this dark exchange most likely is related to phosphoglycerate kinase contaminations of the chloroplast preparations.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft. The authors thank Prof. H. Strotmann for helpful discussions and correction of the manuscript and Dr. H. Bickel for helpful discussions concerning the lower energy dependence of the ATP/ADP exchange.

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