Bound ATP in Chloroplast Membranes: Formation and Effect of Different Inhibitors on the Labelling

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Abstract

Chloroplast membranes contain firmly bound nucleotides. Their synthesis seems not to be dependent on energy. The amount of labelled firmly bound ATP extracted from membranes after incubation in the light of the presence of $^{32}P_i$ is only slightly affected by uncouplers such as desaspidin and CCCP or energy transfer inhibitors as phlorizin at concentrations where steady state phosphorylation is completely abolished. With Dio-9 or NEM, however, the labelling of firmly bound ATP is lowered to a similar extent as the steady state phosphorylation. These effects can be explained assuming a direct modification of the coupling factor.

The results of a two stage incubation experiment using a rapid filtration technique support our earlier hypothesis that the γP in the liberated ATP does not origin from the previously built phosphorylated intermediate.

Introduction

Most of the proposed schemes on the mechanism of phosphorylation of ADP coupled to electron transport show two different high-energy intermediates, a non-phosphorylated one, usually called $X \sim Y$, and a phosphorylated one, called $X \sim P$ [1].

Abbreviations:
   CCCP = carbonylcyanid-3-chlorophenylhydrazon; NEM = N-ethylmaleimide;
$^{32}P_i$ = labelled inorganic phosphate.

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The presence of these intermediates follows from kinetic experiments as shown by Eisenhardt [2], Shen and Shen [3] and Hind and Jagendorf [4]. Biochemical isolation has not been successful so far [see e.g. 5]. In bacterial chromatophores besides membrane bound phosphate [6], a membrane bound high energy intermediate $X \sim ADP$ [7] have been postulated. Recently, membrane bound ATP was demonstrated in different electron transport supporting membranes [8-11]. It was postulated that this ATP which is firmly bound to the membrane is the precursor of the free ATP synthesized during phosphorylation. Several characteristics of the membrane bound ATP point to its identity with the kinetically demonstrated phosphorylated high energy intermediate.

In this paper three different kinds of ATP in membranes of chloroplasts are differentiated on the basis of their solubility and the effects of several inhibitors of phosphorylation on the formation of these types of ATP are demonstrated.

Methods

A two stage incubation method was employed to separate the nucleotide carrying membranes from any components present in the incubation medium [12]. In the experiments with chloroplasts this separation was achieved most completely and in the shortest time by immobilizing the membranes on a filter system. We used membrane filters SM 113 (Sartorius) on glass fibre filter CF/C (Whatman). For details see [13].

Preparation of the chloroplast membranes. Whole spinach chloroplasts were prepared by a method similar to that described by Cockburn *et al.* [14]. The isolation medium contained 0.1 M NaCl, 10^{-3} M MgCl$_2$, 0.05 M Tris-HCl pH 8.0. Twice washed chloroplasts were resuspended in a hypotonic medium containing 0.05 M Tris-HCl pH8.0, 0.035 M NaCl, 10^{-3} M MgCl$_2$ and 2.10^{-5} M Na-ascorbate. Then the particles were sonicated for 20 sec and centrifuged for 10 min at 45,000 × g. The pellets were washed 1-3 times and resuspended in the same medium to give a final concentration of 1 to 1.5 mg chlorophyll/ml.

EDTA-uncoupled membranes were produced using a method similar to Shosham and Shavit [15]. The incubation of the chloroplast fragments (0.3 mg chlorophyll/ml) was performed in 1.25 mM EDTA at room temperature.

NEM-treatment was performed similar to the method of McCarty [16]. Once washed chloroplast membranes (0.33 mg chlorophyll per ml) were preincubated at room temperature in 5 mM NEM in the light for 2 min. The preincubation mixture was the same as shown on Table III(b), without phosphate. After illumination DTT was added in excess (3 mM). Then the mixture was centrifuged for 10 min 36,000 × g.

Chlorophyll was determined by the method of Arnon [17].

Incubation and separation. The suspended chloroplast membranes

were incubated at room temperature on the filters. Details of the incubation media are given in the legends to the figures and tables.

A Philips Comptalux 300 W lamp was used as the light source at a distance of 30 cm from the samples, giving a light intensity of 28 mW/cm^2. In order to absorb the radiant heat a $CuSO_4$-filter was placed between the lamp and the sample.

The free reaction products in the medium were separated from the membrane bound ones by sucking the incubation mixture through the filters by a controlled vacuum. The immobilized chloroplast fragments on the filter were then washed with the buffer mixture. Bound products were extracted with perchloric acid (0.5%) first and SDS (2%) in perchloric acid (5%) in a second step.

Steady state phosphorylation. The reaction buffer was 0.05 M Tris-HCl pH 8.0 or 0.05 M HEPES pH 8.0 respectively and contained 0.05 M KCl, 10^{-3} M $MgCl_2$, 10^{-4} M ADP, 10^{-4} M Na-ascorbate, 10^{-4} M pyocyanin and a rate limiting concentration of phosphate of 10^{-4} to 5.10^{-4} M $^{32}P_i$ giving an activity of 1 to 2.10^6 cpm/incubation. One ml of the chloroplast membranes was incubated in 4 ml of the reaction mixture on the filters for 2 min in the light or the dark. The reaction was stopped with perchloric acid of a final concentration of 5%. The incubation mixture then was forced through the filters quickly and analysed subsequently.

Measurement of radioactivity. The total amount of $^{32}P_i$ esterified was determined according to Avron [18]. Radioactivity was measured in a scintillation counter (Nuclear Chicago 724) in aqueous solution using Cerenkov emission [19].

Chromatographic separation of the nucleotides. Nucleotides were separated on DEAE-Sephadex A-25 columns with a chloride gradient as reported previously [8].

Solubilization of the chloroplast membranes and separation of proteins from free nucleotides. The firmly bound nucleotides were released from the membranes which had been washed with buffer and with weak perchloric acid by solubilizing the membranes with 2% SDS in 5% $HClO_4$. Sodium acetate (2.5 M) was then added to the solution which changes the pH to 3.0. The samples were immediately frozen in liquid air and stored at $-20°C$ until analysed.

The proteins were separated from the low molecular weight components using a G-50 Sephadex column according to Mardh and Zetterquist [20] and then prepared for ion-exchange chromatography as before [8].

Determination of the total amount of nucleotides in chloroplast membranes. ATP measurements were done by the luciferase method according to Chapman [21]. ADP was first converted into ATP using phosphoenolpyruvate and pyruvate-kinase. The amount of ADP was then calculated from the difference of the two determinations.

Determination of the labelling pattern of ATP. After separation of the nucleotides and determination of the radioactivity in the ATP fraction an aliquot of it was incubated with glucose and hexokinase. The reaction products were separated again on a DEAE-A-25 column. Determination of the radioactivity in glucose-6-phosphate, ADP and ATP allows the calculation of the labelling pattern of the original ATP.

Chemicals. ADP, ATP, phosphoenolpyruvate, hexokinase and pyruvatekinase were purchased from Boeringer, Mannheim, BRD. $^{32}P_i$ was obtained from Eidg. Institut für Reaktorforschung, Würenlingen. Dio-9 was a gift from Gisl-Brocadec, Delft.

Results

As shown in Fig. 1 and Table I, three different kinds of ATP can be distinguished by their extraction characteristics. While the amount of acid-soluble ATP (called ATP_{as}) and SDS soluble ATP (called ATP_{fb} = firmly bound ATP) are relatively constant, the pool of buffer
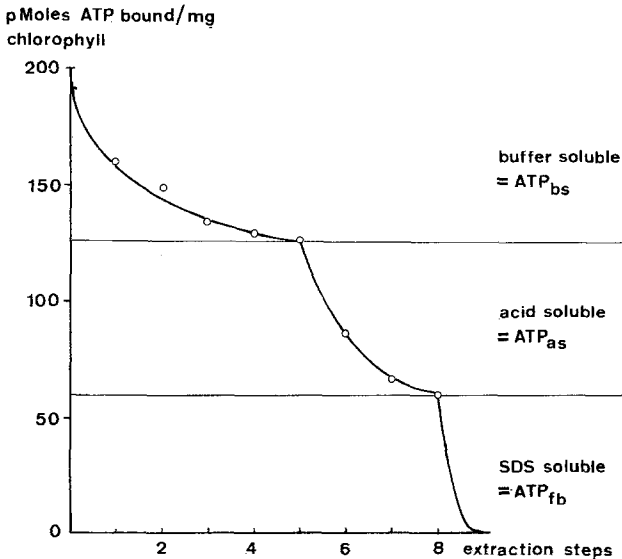

Figure 1. Characterization of the binding of ATP by extraction with buffer, $HClO_4$ and SDS. The chloroplast membranes (0.6 mg chlorophyll/ml) were incubated in 5 ml of a reaction medium containing 50 mM HEPES (ph 7.0), 0.2 mM sodium ascorbate, 0.5 mM pyocyanin, 50 mM KCl, 1 mM $MgCl_2$, 0.0312 mM $^{32}P_i$ 10.2 x 10^6 cpm/ml. The suspended membranes were exposed to light for 2 min. The labelled nucleotides were extracted with buffer (5 steps with 7 ml each of Tris-HCl, pH 8.0), with acid (3 steps with 7 ml each of perchloric acid, 0.5%) and SDS (2% in 5% perchloric acid).

TABLE I. Relation between the number of washings of chloroplast membranes and the labelling of free and bound ATP with ^{32}P in the absence of exogenous ADP

No. of washings	1	2	3
	pmoles/mg chlorophyll		
ATP_{bs}	194	96	26
ATP_{as}	102	114	119
ATP_{fb}	54	55	61

The once, twice or three times washed chloroplast fragments, end-concentration 0.34 mg chlorophyll/ml, were incubated in 5 ml of a reaction mixture containing 50 mM Tris-HCl buffer (pH 8.0), 0.1 mM sodium ascorbate, 0.1 mM pyocyanin, 50 mM KCl, 0.5 mM $MgCl_2$, 0.53 mM $^{32}P_i$, 8.6 x 10^6 cpm/ml. Light exposure: 2 minutes.

TABLE II. Labelling of acid soluble and SDS soluble ATP and ADP after different incubation conditions, and ratios of ATP to ADP

Conditions	Acid soluble nucleotides			SDS soluble nucleotides		
	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
	pmoles/mg chl.			pmoles/mg chl.		
Dark 60/sec	20	14	1.8	17	8	2.1
Light 1/sec	28	12	2.3	11	4	2.8
Light 10/sec	36	13	2.8	16	6	2.6
Light 60/sec	78	26	3.0	21	8	2.8

The reaction conditions were as given on Fig. 1 except for the $^{32}P_i$-activity (0.048 mM, 8.2 x 10^6 cpm/ml).

extractable labelled ATP varies greatly depending on the amount of endogenous nucleotides. Repeated washing of the chloroplast fragments before the experiment reduced the labelling of the free ATP practically to zero, while both the ATP_{as} and the ATP_{fb} showed only small changes.

In Table II the labelling of the two bound kinds of ATP after incubation in the light and in the dark are compared. While almost no buffer soluble ATP gets labelled in the dark, the dark labelling of ATP_{as} reaches approximately 25% and that of the ATP_{fb} as much as 80% compared with the light experiment (each after 60 seconds). A similar high dark labelling of the ATP_{fb} in membranes of Rhodospirillum rubrum was seen before [8]. The same table gives data on the labelling of the ADP. While the ATP/ADP ratio increases with time in the acid soluble and even more in the buffer soluble nucleotides (not shown), the values for the SDS-soluble nucleotides stay fairly constant around 2.6 to 2.8 between 1 sec and 60 sec light incubation and at 2.1 in the dark

TABLE III. Effects of the uncouplers desaspidin and CCCP and the energy transfer inhibitor phlorizin on the labelling of bound and free ATP

	(a) Desaspidin (pmoles/mg chl.) control + desaspidin		(b) CCCP (pmoles/mg chl.) control + CCCP		(c) Phlorizin (pmoles/mg chl.) control + phlorizin	
ATP_{bs}	152	8	—	—	7.5	1
ATP_{as}	34	5	163	106	58	59
ATP_{fb}	48	32	124	94	25	26
Percentage inhibition of steady state phosphorylation	0	95	0	98	0	92

The reaction mixtures contained:

(a) Conditions as described in Fig. 1, except that the labelled phosphate (0.036 mM) contained 12.02×10^6 cpm/ml. The end-concentration of desaspidin was 0.01 mM.

(b) 50 mM Tris-HCl (pH 8.0), 0.1 mM sodium ascorbate, 0.1 mM pyocyanin, 50 mM KCl, 1 mM $MgCl_2$, 0.048 mM $^{32}P_i$, 17.8×10^6 cpm/ml, 0.01 mM CCCP. The concentration of the chloroplast fragments was 0.18 mg chlorophyll/ml.

(c) Conditions as given under (b) except that the labelled phosphate (0.064 mM) contained 13.5×10^6 cpm/ml, phlorizin was added in an end-concentration of 4 mM.

The inhibitors were added in the dark to the chloroplast fragments suspended in 50 mM Tris-HCl buffer 2 min before the incubation. The light exposure was 2 min.

The conditions for steady state phosphorylation are given in "Methods".

TABLE IV. Effect of Dio-9 and light on the labelling of free and bound ATP

		Free nucleotides	ATP_{as}	ATP_{fb}	$^{32}P_i$ esterified in steady state phosphorylation (%)
		pmoles/mg chlorophyll			
Light	− Dio-9	98	64	26.4	100
Light	+ Dio-9	23	8	3.2	12
Dark	− Dio-9	28	23.2	16.4	4.5
Dark	+ Dio-9	20	7.5	3.0	4

The chloroplast membranes (0.12 mg chlorophyll/ml) were incubated in 5 ml of a medium containing 50 mM HEPES (pH 8.0), 0.5 mM sodium ascorbate, 0.05 mM PMS, 50 mM KCl, 1 mM $MgCl_2$, 0.144 mM $^{32}P_i$, 11.3×10^6 cpm/ml. The end-concentration of Dio-9 was 100 μg/ml. The application of the inhibitor is described in Table 3. Incubation time was 2 min.

The values of ATP_{as} and ATP_{fb} were determined after separation of the nucleotides by ion exchange chromatography, the free nucleotides are calculated after isobutanol-benzene-extraction [18].

TABLE V. Effect of EDTA and NEM on the amount of free and bound ATP

	EDTA			NEM		
	Control	+ EDTA	Inhibition (%)	Control	+ NEM	Inhibition (%)
	pmoles/mg chl			pmoles/mg chl		
ATP_{bs}	82	17	80	132	52	60
ATP_{as}	90	27	70	143	54	62
ATP_{fb}	50	14	72	35	11.4	67
Percentage inhibition in steady state phosphorylation			81			55

The preparation of uncoupled membranes and the conditions for steady state phosphorylation are described in "Methods". The medium for incubation of the uncoupled membranes was the same as given in Table 3b), except for $MgCl_2$ (5 mM) and $^{32}P_i$ (0.156 mM). Light exposure was 2 min. NEM-treatment of the membranes is given in "Methods". Main incubation and extractions were the same as shown in Table 3b and Fig. 1. The incubation time was 2 min. The absolute amounts of $^{32}P_i$ esterified in steady state phosphorylation under the described conditions (see "Methods") were 271 nMoles/mg chl.h and 309 nMoles/mg chl.h resp. in the control experiments.

experiment. This value suggests an equilibration between ADP and ATP and also between the γ and the β phosphate group in the ATP (see also Table V).

The effect of some uncouplers and inhibitors of phosphorylation on the labelling of the different ATPs is given in Tables III and IV. The inhibitors were used in concentrations reducing the formation of ATP in a steady state phosphorylation assay to 2-10% of the control. A similar inhibition is shown for the buffer soluble ATP (when detected in once washed chloroplasts), but a much smaller effect is seen for the acid soluble and the SDS soluble ATP. In the case of the energy transfer inhibitor phlorizin, no effect is seen on the labelling of the two kinds of bound ATP. Dio-9, another energy transfer inhibitor (Table IV), has a completely different effect on the labelling of ATP_{as} and ATP_{fb}. The formation of all three types of ATP is diminished drastically.

Experiments using agents reacting with the coupling factor or removing it, suggest that the binding of the nucleotides occurs at the coupling factor itself. This is demonstrated in Table V. With appropriate concentrations of EDTA, which removes the coupling factor from the membrane, an inhibition of photophosphorylation of 81% was observed, at the same time both the ATP_{as} and the ATP_{fb} are reduced by approximately 70%. Similarly a treatment of the chloroplasts with NEM in the light, which reduced the overall phosphorylation by 55% gave a parallel decrease in the ATP_{as} and ATP_{fb}.

TABLE VI. Absolute amounts of extractable ATP, labelling pattern in ATP and ATP/ADP labelling ratio

	(a) Extractable ATP pmoles/mg chlorophyll	(b) Labelling in the γP	(c) ATP/ADP labelling ratio
ATP_{bs}	140	96%	20
ATP_{as}	2160	58%	1-3
ATP_{fb}	7400	58%	1-3

(a) Conditions for phosphorylation are similar as described in Table 3. The extraction procedure is given in Fig. 1, except that the volumes of the extraction medium were each 2 ml for buffer and acid and 5 ml for SDS solution.

TABLE VII. Esterification of $^{32}P_i$ to acid soluble ATP in a two stage incubation after addition of $^{32}P_i$ in the dark or in the light

Conditions		^{32}P label in acid soluble ATP
Phase 1 (light, 60 sec)	Phase 2 (dark, 120 sec)	pM/mg chlorophyll
^{32}P	^{32}P	144
^{31}P	^{32}P	86
^{32}P	^{31}P	30

The preincubation was done in 50 mM HEPES (pH 6.8), 0.1 mM sodium ascorbate, 0.1 mM pyocyanin, 50 mM KCl, 0.5 mM $MgCl_2$, 0.01 mM phosphate. The incubation was performed in a medium containing 50 mM HEPES (pH 8.0), 50 mM KCl, 0.5 mM $MgCl_2$, 5 μM phosphate, 5 μM ADP. Between the two incubations on the filter, the free reaction products were drained and the membranes rinsed 5 times with 5 ml aliquots of a buffer solution containing 50 mM Tris-HCl (pH 7.0).

A direct measurement of the different species of ATP (Table VI) shows that much more firmly bound ATP can be extracted than after calculation from its labelling with $^{32}P_i$, suggesting a poor equilibration between the added $^{32}P_i$ with the bound unlabelled P_i. On the other hand the distribution of the P-label in the three species shows an almost complete equilibration between the β and the γP in the bound forms of ATP while the buffer soluble ATP is mostly γ labelled. This equilibration is further demonstrated by the low ATP/ADP ratio of the labelling for the bound forms compared with the high value for the buffer soluble form.

An experiment with chloroplast membranes similar to the isotopic concentration experiments with Rhodospirillum [8, 22] showed that also in chloroplasts the liberated ATP after a second incubation with
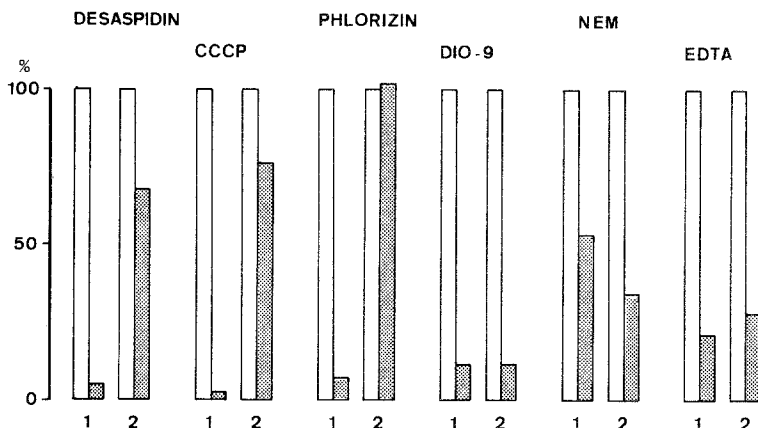
Figure 2. The reaction conditions are the same as described in the legends to the appropriate tables.

1 stands for steady state phosphorylation in the presence of 1 mM ADP, 0.5 mM phosphate.

[_____] control without inhibitor.

[▓▓▓▓▓▓▓] experiment with inhibitor.

The amounts of nucleotides are calculated after isobutanol-benzene extraction.

2 stands for ATP_{fb} in a phosphorylation experiment without exogenous ADP. The conditions for incubation are given in the related tables.

[_____] control without inhibitor.

[▓▓▓▓▓▓▓] experiment with inhibitor.

ATP_{fb} was determined after chromatographic separation of the SDS-extracted nucleotides (see Fig. 1).

ADP, P_i and Mg^{2+}, the specific activity of the ATP is more correlated to the specific activity of the radioactive phosphate used in the second stage than to the one in the first incubation (Table VII). This supports the hypothesis proposed earlier that the last step of the phosphorylation is not a phosphate transfer from a phosphorylated high energy inter-mediate to ADP but a condensation of free ADP with free P_i with the use of the energy of hydrolysation of the bound high energy phosphate intermediate, possibly the ATP_{fb}. In Fig. 2 the results of the effects of the different inhibitors are summarized.

Discussion

With differential extraction of the chloroplast membrane, three different species of ATP—buffer soluble, acid soluble and SDS soluble—were demonstrated. The simple method of distinguishing these types of attachment to the membrane by sequential extraction raises the question

whether the differentiation of the two types of bound ATP (ATP_{as} and ATP_{fb}) may be an artefact. Indeed the results of the labelling of the γP and the ratio of ATP/ADP in Table V do not show differences between ATP_{as} and ATP_{fb}. The same holds true for the effect of EDTA and to a smaller extent of NEM (Table IV). On the other hand one would not expect to see a difference after removal or modification the coupling factor where both types of ATP are supposed to be bound. Similarly, the ratios of ATP/ADP as well as ATP_{as}/ATP_{fb} in Tables II and III would point to different kinds of binding of the ATP due to different and separate environments.

While the absolute amounts of liberated ATP (Table V) are well in accordance with calculations of the concentration of the coupling factor in the membrane and support the assumed relation of 1 or 2 bound nucleotides per coupling factor, the data calculated from the incorporation of ^{32}P are much too low in the order of 1 to 3 times 10^2. This large difference can be explained partially by the isotopic dilution of the added $^{32}P_i$ with unlabelled bound P_i. On the other hand the possibility that only a fraction of the coupling factor may equilibrate with the medium in the absence of free ADP cannot be ruled out.

As shown by the effect of chemical uncouplers or of energy transfer inhibitors (Table III) the formation or at least the labelling of the bound ATP (ATP_{as} and ATP_{fb}) does not seem to need energy and proceeds in the dark as well as in the presence of the inhibitors. The partially lowered values of bound ATP with uncouplers may be due to the induction of an ATPase as seen in mitochondria and are less pronounced when the uncoupler is added a few seconds before the light instead of 60 sec before. Similar results for uncouplers and especially for energy transfer inhibitors are demonstrated for the conformational change of the coupling factor detected by the ^3H_2O exchange [23]. On the other hand the modification or removal of the coupling factor reduced the phosphorylation and the amount of bound ATP about equally. A comparison of the effect of the two energy transfer inhibitors phlorizin and Dio-9 on the formation of bound ATP point to a completely different site of action of the two inhibitors. These two agents lead to a strong reduction of the steady state phosphorylation in the concentrations used. While the labelling of the firmly bound ATP is not affected by phlorizin, it is drastically diminished by Dio-9. We can therefore assume that Dio-9 directly interferes with the coupling factor possibly at the active site. This mechanism is proposed as an explanation of the effect of NEM [16]. A similar conclusion of different mechanisms of action of Dio-9 and phlorizin has been reached by Walz (personal communication) on the basis of a completely different experimental approach.

The ratio of ATP/ADP for the firmly bound form remains relatively constant in the range of 2-3, even after short illumination time of only

1 sec followed by a dark reaction time of a few seconds. On the other hand the ratio is increasing with time in the acid soluble form indicating a preferential incorporation of the label into the ATP_{as}.

In the last few years several mechanisms for the formation of ATP in chloroplasts have been proposed [9, 10, 24, 25]. According to Moudrianakis [24] the coupling factor contains 2 ADP, one formed by a phosphorylation of AMP, the other bound directly to the coupling factor as ADP. Labelled ATP is then formed as a result of a transphosphorylation. Our results do not agree with this hypothesis because the P_i of the liberated ATP does not seem to originate from the previously formed phosphorylated intermediate (in Moudrianakis hypothesis the AD*P). In the most recent formulations of the mechanism by Boyer [10] and Slater [9], a firmly bound ATP is a precursor of the liberated ATP. Its formation is independent of energy.

This idea is very similar to ours, but their hypotheses lack to explain the different origin of the phosphate of the soluble ATP and the firmly bound ATP and the different labelling pattern in the nucleotides. The data from chloroplasts reported in this paper can be best explained by our hypothesis based on the work on the phosphorylation in Rhodospirillum rubrum chromatophores [8]. According to this hypothesis, the coupling factor has two binding sites for adenine nucleotides. At site I firmly bound ATP is formed by a previously energized coupling factor. This ATP is hydrolysed in a subsequent second conformational change while simultaneously at the second site acid soluble ATP is formed which may be liberated in the presence of excess of free ADP. A rapid equilibration in the dark can take place when one assumes only small energy differences between ATP present at site I (ATP_{fb}) and at site II (ATP_{as}). A further indication of a direct participation of ADP in the last step of the phosphorylation, the formation of free ATP, is given by the fact that the amount of buffer soluble ATP depends strongly on the concentration of available ATP, whereas for the formation of bound nucleotides the concentration of the endogenous ADP has no effect.

Acknowledgment

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