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POSSIBLE ROLE OF FIRMLY BOUND ATP IN THE ENERGY TRANSDUCTION OF PHOTOSYNTHETIC MEMBRANES

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Chromatophores of Rhodospirillum rubrum and spinach chloroplasts contain firmly bound ATP that is rapidly labeled along with ADP in the presence of $^{32}P_i$ and endogenous nucleotides. The labeling is not entirely dependent on light. In chloroplasts three types of bound ATP can be defined methodologically by their extraction properties: buffer-soluble; acid-soluble; and SDS-soluble or firmly bound ATP. Extensive washing of the chloroplasts does reduce buffer-soluble but not acid-soluble and firmly bound ATP. Buffer-soluble [^{32}P] ATP is almost exclusively γ labeled while acid-soluble and firmly bound ATP are labeled in the β and γ position equally. CCCP, desaspidin, and phlorizin do not inhibit the labeling of firmly bound ATP, whereas the phosphorylation is almost abolished. However, EDTA and NEM pretreatments of the choloroplasts affect both reactions similarly.

The postillumination [^{32}P] ATP synthesis with chromatophores can be inhibited by adding ATP to the incubation mixture after illumination if $^{32}P_i$ is included only during the dark incubation, but is without effect if $^{32}P_i$ is present only during illumination. On the other hand, ADP added after illumination inhibits post-illumination [^{32}P] ATP formation in both chromatophores and chloroplasts only if $^{32}P_i$ is present during illumination. The data can be explained by a coupling factor having two sites, as proposed previously on the basis that firmly bound ATP does not transfer its phosphoryl group but seems to drive a synthesis of acid-soluble ATP which incorporates free phosphate (Lutz, H. U., Dahl, J. S., and Bachofen, R., Biochim. Biophys. Acta 347:359 [1974]).

INTRODUCTION

It was long thought that the electron transport chain contains a phosphorylated intermediate that transfers its phosphoryl group to added ADP (1, 2). Although scientists in many laboratories attempted to isolate such a precursor of ATP, none showed positive evidence that a phosphorylated compound is synthesized faster than ATP and is able to transfer its phosphoryl group to added ADP. In 1971 Roy and Moudrianakis (3, 4) proposed bound nucleotides as intermediates of free ATP synthesized during photophosphorylation of chloroplasts. Similar conclusions were drawn by Yamamota et al. (5) using Rhodospirillum rubrum chromatophores.

New information about membrane potentials and conformational changes of proteins led to models that do not need a phosphorylated intermediate prior to ATP, but favor the ATP synthesis over its hydrolysis simply by removal of water from the ADP and phosphate binding site (6). There is good evidence for the exclusion of water from the energized coupling factor of chloroplasts (7). However, there is little known about the

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formation of firmly bound ATP. Attempts have been made to characterize this entity in terms of solubilization properties (8–11).

Some recent studies focus on the role of firmly bound ATP. Boyer et al. (9) and Slater et al. (11) suggest that this firmly bound ATP is released in an energy-dependent reaction and thus does not represent a true intermediate. We have previously presented evidence suggesting that firmly bound ATP formed during illumination of chromatophores of Rhodospirillum rubrum is able to drive a synthesis of acid-soluble ATP in the dark (12). Using an isotope concentration method, we found that ATP synthesized in the dark period incorporates highly labeled $^{32}P_i$ added during the dark incubation. We concluded from this finding that firmly bound ATP does not transfer its phosphoryl group but most probably is hydrolyzed as it drives the esterification of ADP and free phosphate. On the basis of these findings we proposed a working hypothesis involving a coupling factor with two sites (Fig. 1).

In this communication we describe experiments that are designed to test this hypothesis in Rhodospirillum rubrum and in spinach chloroplasts. Since chloroplasts and chromatophores are yet such complex systems, results in favor of our hypothesis do not prove it, but render it possible among other suggested mechanisms. Part of this work has been published previously (12–14).

METHODS

Preparation of Chloroplast Membranes

Twice-washed, whole chloroplasts isolated according to Cockburn et al. (15) were sonicated for 20 sec in a hypotonic medium containing 50 mM Tris-HCl (pH 8.0), 35 mM

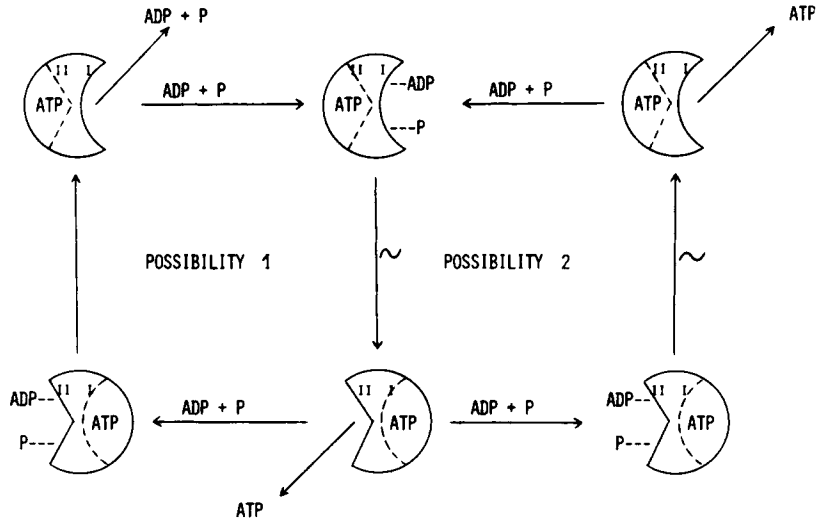

Fig. 1. Working hypothesis showing a coupling factor with two sites. The energization of the coupling factor leads to the formation of a firmly bound ATP at site I while the ATP at site II is released. The net production of one buffer-soluble ATP per energization can be achieved in two ways: possibility 1 takes into consideration that the two sites would be different and that site I, if containing ATP, is inaccessible to perchloric acid. In possibility 2, however, it is assumed that either site can once in a cycle become inaccessible to acid (further explanations in the text).

NaCl, 1 mM $MgCl_2$; and 0.2 mM sodium ascorbate and centrifuged for 10 min at 4,500 g_{av}. The pellets were washed one to three times and resuspended in the same medium to give a final concentration of 1–1.5 mg Chl/ml.*

Incubation of the Membranes and Isolation of Bound Nucleotides

The chloroplast membranes were added at room temperature to an incubation mixture (as given in the legends) and after 1 min of equilibration transferred to a glass fiber filter (CF Whatman) on top of a membrane filter (SM 113, Sartorius). The suspension was illuminated from above for 1 min (if not otherwise mentioned). A Philips Comptalux 300-W lamp was used as the light source, giving a light intensity of 28 W/cm^2. The sample was protected from infrared heating by a $CuSO_4$ filter. Immediately after illumination the free reaction products in the medium were separated from the membrane-bound products by drawing the incubation mixture through the filters by a controlled vacuum. The buffer-soluble nucleotides were then extracted from the immobilized chloroplast fragments by washing five times with 7 ml 0.5% perchloric acid (PCA). After each washing solution had reacted for 1 min with the immobilized chloroplasts, it was drawn through the filters under a controlled vacuum. The firmly bound nucleotides were then removed by addition of 7 ml 1% SDS in 5% perchloric acid.

Chromatographic Separation of the Nucleotides

This separation was done on DEAE-Sephadex A-25 columns. For the SDS-soluble fractions, the solubilized proteins were separated from the nucleotides with a G-50 Sephadex column as previously reported (12). All methodological details for the experiments with chromatophores not given in the legends to the appropriate Tables and Figures are described in our earlier work (12, 13).

RESULTS

Chromatophores of Rhodospirillum Rubrum

Chromatophores illuminated in the presence of unlabeled phosphate and then separated from the preincubation mixture form labeled ATP in the dark when ADP and $^{32}P_i$ are added. Figure 2 shows that a substantial amount of [^{32}P] ATP is formed immediately after the addition of ADP and $^{32}P_i$. Even in the "0 min incubation assay" the same level is reached as after an incubation for up to 2 min. The short time necessary to stop enzymatic activity with 0.5% perchloric acid is apparently long enough for the reaction to take place despite the low pH. For both the preilluminated and dark preincubated chromatophores, the amount of labeled ATP increases after 2 or 1 min, respectively, because of the cooperative action of adenylate kinase, the added ADP, and the ATP-$^{32}P_i$ exchange reaction. Since the same amount of [^{32}P] ATP is formed in the 0 min assay as after a short incubation, the postillumination ATP synthesis can be studied without further incubation.

If the coupling factor does have two sites and contains one firmly bound ATP upon energization, addition of unlabeled ATP and $^{32}P_i$ instead of ADP and $^{32}P_i$ should depress the amount of acid-soluble, labeled ATP formed in a postillumination ATP synthesis. The expected mechanism is shown in Fig. 3. The coupling factor is energized in the presence

*Abbreviations used: BChl, bacteriochlorophyll; Chl, chlorophyll; PCA, perchloric acid; Tris, Tris-hydroxymethyl amino-methane; HEPES, N-2-ethanesulfonic acid; NEM, N-ethyl-maleimide; CCCP, carbonylcyanid 3-chlorophenylhydrazin; DTT, dithiothreitol; P_i, inorganic phosphate.

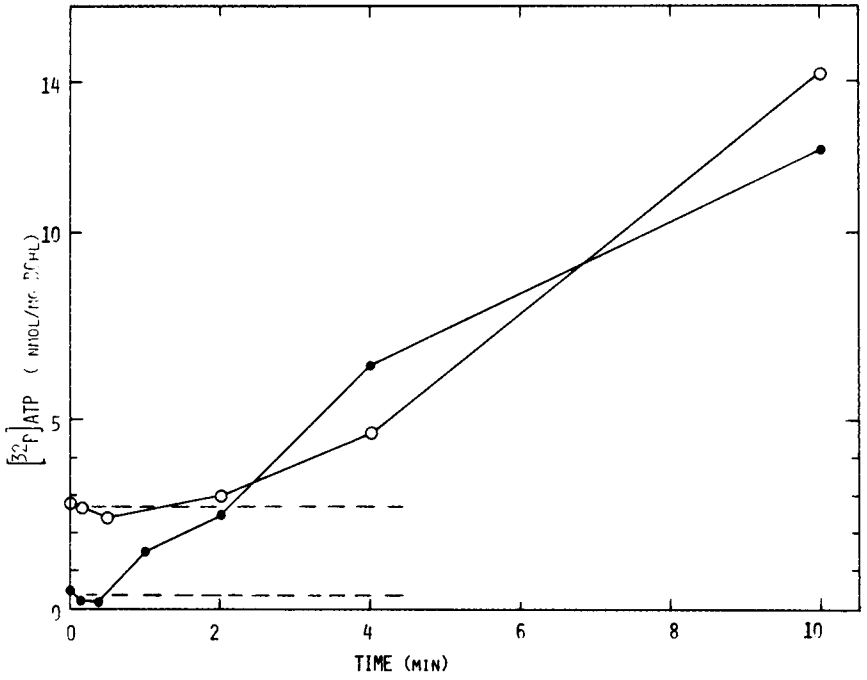

Fig. 2. Time course of the acid-soluble [^{32}P] ATP* formation in a postillumination ATP synthesis and the dark control using unlabeled phosphate during illumination and $^{32}P_i$ during dark incubation. Chromatophores of R. rubrum were preincubated for 5 min in light or dark at 30°C in a total volume of 5 ml, containing 5 mM KCl, 2 mM sodium succinate, 7.5 mM phosphate (pH 8.0), 0.1 M Tris-HCl (pH 8.0), and chromatophores 50 μg BChl/ml. Immediately afterwards the chromatophores were separated in the dark from this preincubation mixture by being passed through a Sephadex G 25 column (for details see reference 13). The chromatophore fraction (50 μg BChl/ml) was then incubated in the dark for varying times in a total volume of 2 ml, containing 2 mM $MgCl_2$, 0.1 mM sodium succinate, 1 mg/ml BSA, 2 mM ADP (pH 8.0), 0.1 mM HEPES-NaOH (pH 8.0), 0.2 mM $^{32}P_i$ (pH 8.0) (1.5×10^6 cpm/ml). The reaction was stopped by the addition of perchloric acid, 0.5% final concentration. ○—○ after preincubation in light; ●—● after dark preincubation.

*Acid-soluble [^{32}P] ATP is extracted from the chromatophores after preincubation and gel filtration. Gel filtration may not be as effective in removing buffer-soluble ATP as the multiple washings used with the immobilized chloroplast preparations.

of a low amount of ADP (0.02 mM) and unlabeled phosphate ($P \rightarrow {}^{32}P$ exp). It is assumed that an unlabeled, firmly bound ATP is formed at site I. Upon addition of $^{32}P_i$ and ADP firmly bound ATP is hydrolyzed, while at site II an acid-soluble, labeled ATP is formed. However, added ATP competes with ADP and $^{32}P_i$ at site II and thus inhibits the formation of labeled ATP. The extent of inhibition is limited by the residual amount of ADP still present in the chromatophores after separation from the preincubation mixture. If $^{32}P_i$ is added during preillumination and $^{31}P_i$ during the dark incubation, the effect of added ADP and ATP should be reversed (see Fig. 3, $^{32}P \rightarrow P$ exp). In Table I the actual results of the two types of experiments are listed. Although the inhibition by ATP in a $P \rightarrow {}^{32}P$ exp and that by ADP in a $^{32}P \rightarrow P$ exp are only 50%, there is a clear trend consistent with the predictions. However, an absolute quantitation of the results is difficult, since it is not known to what extent the added phosphate equilibrates with the phosphate

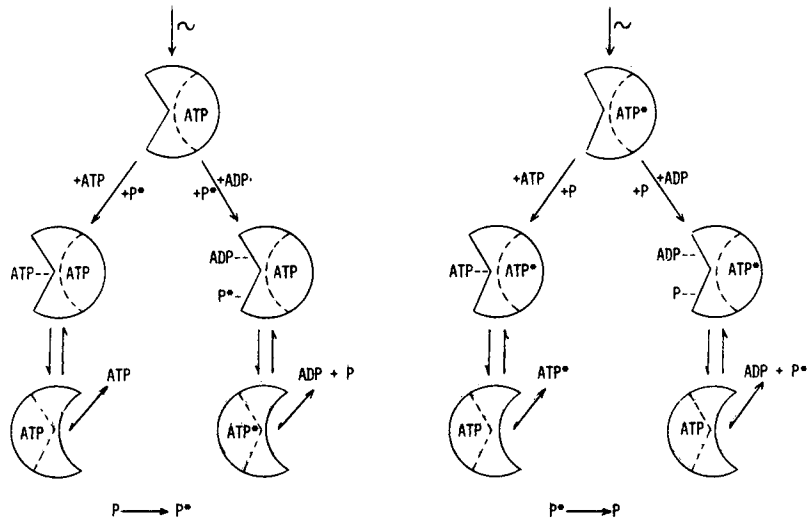

Fig. 3. Working hypothesis showing the postillumination [^{32}P] ATP synthesis if $^{32}P_i$ is added either during illumination ($^{32}P \rightarrow P$ exp) or during dark incubation ($P \rightarrow {}^{32}P$ exp). Explanation in the text.

TABLE I. Acid-Soluble [^{32}P] ATP Formed in Postillumination ATP Synthesis Assays at 0 Min Incubation[1]

		Percent of acid-soluble [^{32}P] ATP		
		Stopping mixture		
Light preincubation	Dark incubation	PCA	PCA + ADP	PCA + ATP
$^{31}P_i$	$^{32}P_i$	100[2]	77	46
$^{32}P_i$	$^{31}P_i$	100[2]	48	100

[1] Chromatophores of Rhodospirillum rubrum were preilluminated at 30°C for 5 min in the presence of 5 mM KCl, 0.02 mM ADP, 5 mM $^{31}P_i$, or $^{32}P_i$ as listed, 0.1 M Tris-HCl (pH 8.0), 1 mM sodium succinate, chromatophores 0.75 mg BChl/ml. Immediately afterwards the chromatophores were separated from the preincubation mixture (see Fig. 2 and reference 12). The chromatophore fractions so obtained were added to the stopping mixture containing (final concentrations) 0.5% perchloric acid (PCA), 5 mM $MgCl_2$, 0.1 mM sodium succinate, BSA 1 mg/ml, 0.05 mM $^{31}P_i$ of $^{32}P_i$, 0.1 M HEPES-NaOH (pH 8.0), and 2 mM ADP or ATP where listed. The phosphorylation activity of the chromatophores was 183 μmoles P_i esterified/mg BChl × h.

[2] Since it is impossible to determine the degree of exchange for the added P_i with the P_i still present after gel filtration in a 0 min assay, the data are given in percent of those values determined after stopping with PCA alone. The actual amounts calculated by neglecting exchange are 0.039 nmoles ATP/mg BChl for the $^{31}P \rightarrow {}^{32}P$ experiment and 6.2 nmoles ATP/mg BChl for the $^{32}P \rightarrow {}^{31}P$ experiment.

still present in the chromatophores after separation from the preincubation mixture.

In order to study the actual time course of the ATP-inhibitable postillumination [^{32}P] ATP synthesis, ATP was added to the 0 min assay and the value thus obtained taken as a control. The amount of [^{32}P] ATP formed after 15 and 60 sec from $^{32}P_i$ and ADP is shown in Fig. 4 for 30° and 0°C. Preilluminated chromatophores synthesize acid-

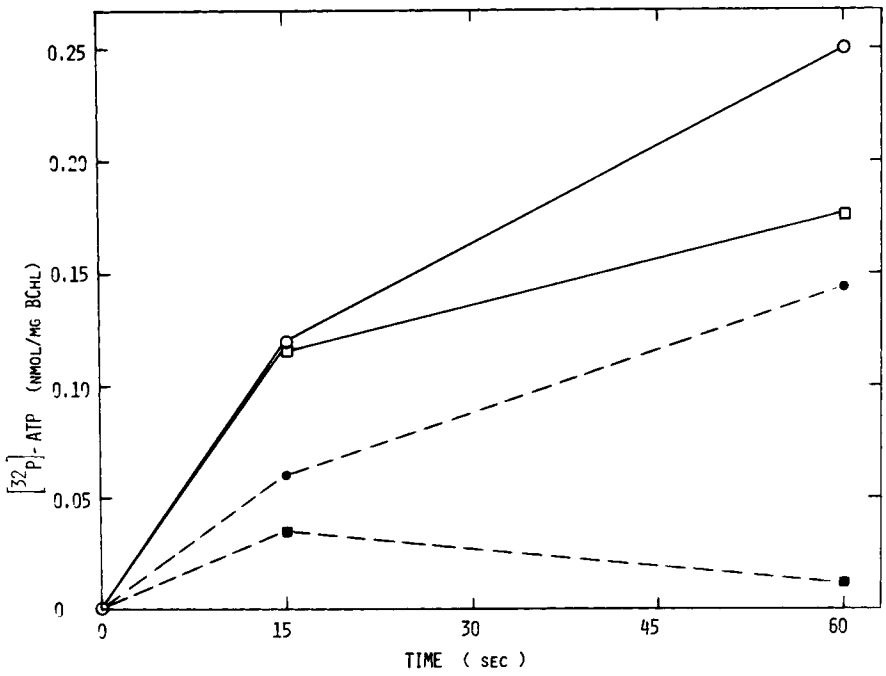

Fig. 4. Effect of temperature on the formation of acid-soluble [^{32}P] ATP* in postillumination ATP synthesis. All data shown represent differences between the amount of labeled ATP from ADP and $^{32}P_i$ found at time (X) minus that which has been recorded at time (0) after the addition of perchloric acid, $^{32}P_i$ plus ATP as an inhibitor. The conditions are as in Table I. ○—○, Preincubation in light, dark incubation at 30°C. □—□, Preincubation in light, dark incubation at 0°C. ●- - - -●, Dark preincubation, dark incubation at 30°C. ■- - -■, Dark preincubation, dark incubation at 0°C.

*Acid-soluble [^{32}P] ATP is extracted from the chromatophores after preincubation and gel filtration. Gel filtration may not be as effective in removing buffer-soluble ATP as the multiple washings used with the immobilized chloroplast preparations.

soluble [^{32}P] ATP as well at 30° as at 0°C. The light-triggered formation of [^{32}P] ATP is completed in less than 15 sec. The corresponding dark controls indicate that the amount of adenylate kinase and ATP-$^{32}P_i$ exchange is temperature dependent and almost negligible at 0°C.

Chloroplast Fragments

Chloroplast fragments were illuminated in the presence of $^{32}P_i$ under phosphorylating conditions but in the absence of added nucleotides. After the incubation the reaction mixture is removed by suction while the chloroplasts are immobilized on the filter. When these chloroplasts are washed several times with different solutes, three types of [^{32}P] ATP can be extracted (Fig. 5): buffer-soluble, acid-soluble, and SDS-soluble or firmly bound ATP. The amount of buffer-soluble [^{32}P] ATP extractable after illumination depends on the number of washings to which the chloroplasts are subjected prior to illumination as given in Table II. However, the amounts of [^{32}P] ATP extractable with acid and SDS remain virtually unchanged. The labeling of the acid-soluble and SDS-soluble nucleotides proceeds rapidly. 50% of the total labeling shown after 1 min is reached in the first second (Table III). Furthermore, the labeling of these nucleotides is only slightly dependent on

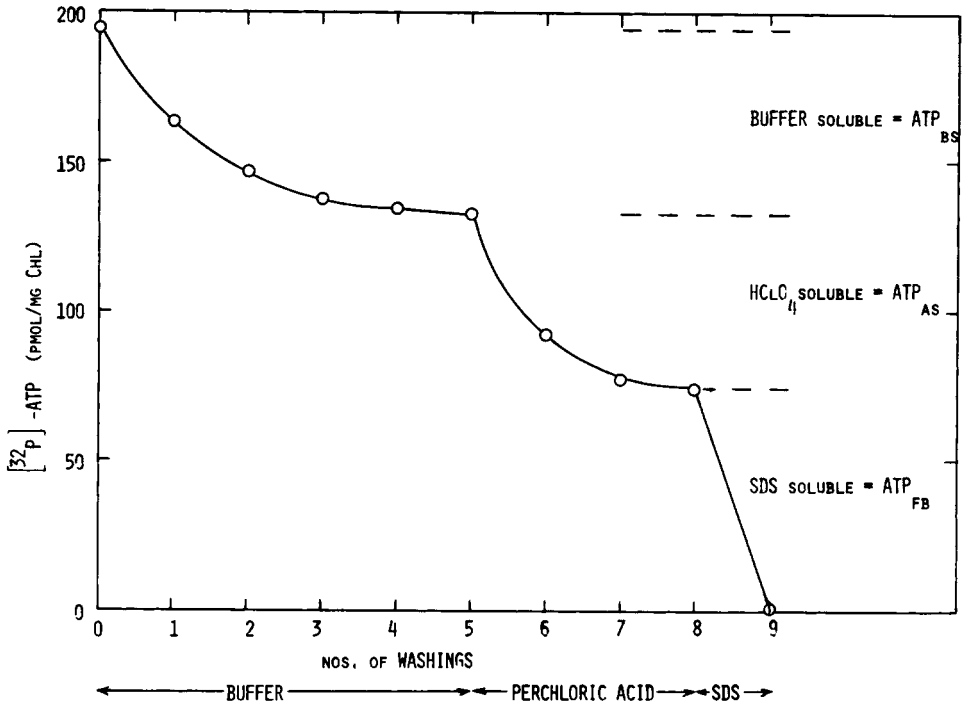

Fig. 5. Characterization of the binding of labeled ATP in chloroplast fragments by using different solvent systems to solubilize ATP. The graph represents the remaining [^{32}P] ATP in the chloroplasts after subsequent washings of the immobilized chloroplast fragments. The differently bound nucleotides were extracted as mentioned in Methods. The reaction mixture contained 0.6 mg Chl/ml, 50 mM HEPES-NaOH (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 × 10^6 cpm/ml).

TABLE II. Effect of Washing Chloroplast Fragments with Buffer before the Incubation on the Amounts of Buffer-Soluble, Acid-Soluble, and SDS-Soluble [^{32}P] APT[1]

Number of washings	[^{32}P] ATP (pmoles/mg Chl)		
	Buffer-soluble	Acid-soluble	SDS-soluble
1	194	102	54
2	96	114	55
3	26	119	61

[1] The experiments were performed as mentioned in Methods except that the chloroplast fragments were further washed as listed before a 2-min incubation in the light. The once-, twice-, or three-times washed chloroplast fragments (final concentration 0.34 mg chlorophyll/ml) were incubated in a total volume of 5 ml containing 50 mM Tris-HCl (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 × 10^6 cpm/ml).

light since the dark labeling of the acid-soluble ATP reaches 25% and the SDS-soluble ATP 80% of that in the light. In all the assays, the labeling ratio of ATP to ADP remains approximately constant (2:3).

Despite the fact that acid-soluble and firmly bound ATP are rapidly labeled, only a

TABLE III. ^{32}P Labeling of Acid-Soluble and SDS-Soluble ATP and ADP as a Function of Incubation Time[1]

Conditions	Acid-soluble nucleotides			SDS-soluble nucleotides		
	ATP	ADP	ATP/ADP	ATP	ADP	ATP/ADP
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
Dark 60 sec	20	14	2.3	17	8	2.1

[1] Conditions as in Fig. 5 except for $^{32}P_i$ 0.048 mM (8.2×10^6 cpm/ml).

small portion of the total amount of these species seems to be labeled, as is evident from quantitative measurements of the total amounts of extractable nucleotides with the luciferin-luciferase method (16) (Table IV). The radioactive portion of the buffer-soluble ATP is almost exclusively γ labeled, while both the acid-soluble and SDS-soluble [^{32}P] ATP seem to be equally β, γ labeled (Table IV). These results suggest an inherent difference only between buffer-soluble and both acid-soluble and firmly bound ATP.

The effect of uncouplers and energy transfer inhibitors on the formation of the differently bound [^{32}P] ATP was studied. The phosphorylation activities and the amount of firmly bound [^{32}P] ATP after treatment with inhibitors during the illumination are given in percent of the controls in Fig. 6. At the concentrations used, desaspidin, CCCP, and phlorizin almost abolish phosphorylation, yet the amount of SDS-soluble [^{32}P] ATP is only slightly affected. However, Dio-9 present in the reaction mixture and also an EDTA and NEM pretreatment (for details see Fig. 6) of the chloroplasts inhibit phosphorylation and the labeling of firmly bound [^{32}P] ATP to similar extents. Furthermore the changes of the amounts of acid-soluble [^{32}P] ATP parallel those of firmly bound [^{32}P] ATP for most inhibitors (19). Desaspidin, however, reduces acid-soluble ATP to 14% and firmly

TABLE IV. Absolute Amounts of Extractable ATP Measured by the Luciferase and Labeling Pattern of the Radioactive Portion[1]

ATP soluble in	Amount of ATP pmoles/mg Chl	Percent[2] labeling in γ position
Buffer	140	96
Acid	2160	58
SDS	7400	58

[1] The reaction mixtures are as given in Fig. 6 for the CCCP experiment (except for CCCP). In order to determine the absolute amounts of bound ATP with the luciferin-luciferase, the volume of the washing solution had to be cut down to 2.0 ml for buffer and acid and 5 ml for the SDS solution.

[2] The percent of ^{32}P labeling in the γ position was calculated from the results of the following treatment: after DEAE-A25 column chromatography an aliquot of the ATP fraction was incubated with hexokinase and glucose and the reaction products were again separated over a column.

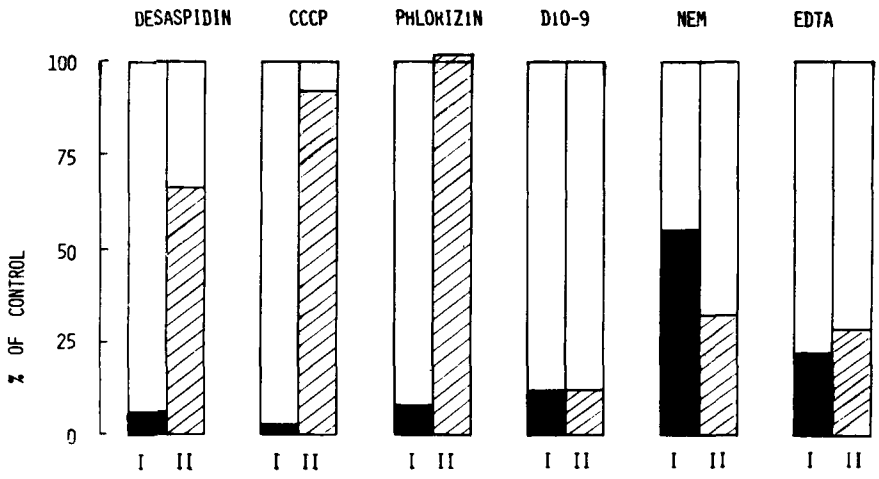

Fig. 6. Effects of uncouplers and energy transfer inhibitors on the phosphorylation (I) and formation of firmly bound [^{32}P] ATP (II) in the absence of added nucleotides. The bars numbered (I) represent the phosphorylation activities obtained in the presence of an inhibitor expressed in percent of the control. The bars numbered (II) represent the amount of [^{32}P] ATP extractable with SDS from illuminated, immobilized chloroplast fragments with inhibitor present during illumination (given in percent of that found in the absence of inhibitor). The phosphorylation activities were measured after incubation of the membranes on the filters in the reaction mixture as given in Fig. 5 but completed by 1 mM ADP. The firmly bound [^{32}P] ATP was determined as given in Methods. The final concentration of the reactants are given for each inhibitor experiment and its control: Desaspidin: reaction mixture as in Fig. 5, except $^{32}P_i$ 0.036 mM (12.02 × 10^6 cpm/ml), plus or minus 0.05 mM desaspidin. CCCP: 50 mM Tris-HCl (pH 8.0), 0.1 mM sodium ascorbate, 0.1 mM pyocyanin, 50 mM KCl, 1 mM $MgCl_2$, chloroplast fragments 0.18 mg Chl/ml, $^{32}P_i$ 0.048 mM (17.8 × 10^6 cpm/ml), plus or minus 0.01 mM CCCP. Phlorizin: reaction mixture as for the CCCP experiment, except for $^{32}P_i$ 0.064 mM (13.5 × 10^6 cpm/ml), chloroplasts 0.24 mg Chl/ml, plus or minus 4 mM phlorizin. Dio-9: 50 mM HEPES-NaOH (pH 8.0), 1 mM $MgCl_2$, 50 mM KCl, 0.05 mM PMS, 0.05 mM sodium ascorbate, chloroplasts 0.12 mg Chl/ml, $^{32}P_i$ 0.144 mM (11.3 × 10^6 cpm/ml), plus or minus 100 μg/ml Dio-9. EDTA: chloroplasts were prepared according to Shoshan and Shavit (17) (0.3 mg Chl/ml in 1.25 mM EDTA). The reaction mixture was as for the CCCP experiment, except for 5 mM $MgCl_2$ and chloroplasts (0.25 mg Chl/ml). NEM: control and test chloroplasts were treated according to McCarty et al. (18) either without or with 5 mM NEM (0.33 mgChl/ml) for 2 min in the light. DTT was added to a final concentration of 3 mM and the chloroplasts centrifuged for 10 min at 36,000 g_{av}. The pretreated chloroplasts were then subjected to the filter assay in the same reaction mixture as for the CCCP experiment, except for CCCP.

bound to 68% (14). On the other hand, the small amount of buffer-soluble [^{32}P] ATP extractable after the removal of the incubation mixture is inhibited in a way similar to the overall phosphorylations (19).

The postillumination ATP sysnthesis with chloroplasts yields a greater amount of labeled [^{32}P] ATP if $^{32}P_i$ has been added during the subsequent dark incubation ($P \rightarrow {}^{32}P$ exp) than if added during illumination ($^{32}P \rightarrow P$ exp) (Table V). In the $^{32}P \rightarrow P$ experiment, ADP added together with P_i in the dark incubation inhibits the amount of acid-soluble [^{32}P] ATP. These results are quite similar to those obtained with chromatophores.

DISCUSSION

The characterization of three pools of labeled ATP by solubilization properties is methodological and does not necessarily differentiate three types of bound ATP. A

TABLE V. Acid-Soluble [^{32}P] ATP Formed in Post-illumination ATP Synthesis Assays[1]

Light preincubation 1 min	Dark incubation 2 min	[^{32}P] ATP pmoles/mg Chl
$^{32}P_i$	$^{32}P_i$	144
$^{31}P_i$	$^{32}P_i$	86
$^{32}P_i$	$^{31}P_i$	30

[1] The assays were performed using the filtration technique. After preincubation in the light, the preincubation mixture was removed and the immobilized chloroplast fragments were washed five times with 5 ml 50 mM Tris-HCl (pH 7.0), 1 mM $MgCl_2$ (total time about 45 sec). Immediately afterwards the incubation mixture was added, removed after 2 min, and then the protocol was applied as given in Methods. The preincubation mixture (5 ml) contained 50 mM HEPES-NaOH (pH 6.8), 0.1 mM pyocyanine, 50 mM KCl, and 0.01 mM phosphate ($^{31}P_i$ or $^{32}P_i$ as given). The incubation mixture (5 ml) contained: 50 mM HEPES-NaOH (pH 8.0); 50 mM KCl, 0.05 mM $MgCl_2$; 0.005 mM ADP; and 0.005 mM phosphate ($^{31}P_i$ or $^{32}P_i$ as given).

difference between buffer-soluble ATP and both acid-soluble and SDS-soluble ATP is supported by differences in the labeling patterns of these pools and by their sensitivity to inhibitors. There is less evidence (effect of desaspidin, dark labeling, and the slightly increasing labeling ratio of acid-soluble nucleotides) suggesting a difference between acid-soluble and firmly bound ATP other than solubilization properties. Since our hypothesis allows for a rapid ATP-ADP and ATP-$^{32}P_i$ exchange, it does not predict a different labeling pattern for acid-soluble and firmly bound ATP.

Energization of the coupling factor in the absence of added nucleotides stimulates formation of firmly bound ATP and thus a chromatophore's capacity to synthesize [^{32}P] ATP in the dark. Incubation of chloroplasts with $^{32}P_i$ shows that not only bound ATP but also ADP is rapidly labeled in the light as well as in the dark (Table III). This phenomenon has been studied extensively in chloroplasts by Roy and Moudrianakis (3, 4) and similar observations have been made for R. rubrum chromatophores (12). The mechanism by which ADP is labeled during short incubations is still unresolved, but may be explained in terms of Roy's and Moudrianakis' scheme (3, 4). All [^{32}P] ATP values for chloroplasts were based on the concentration of the added labeled phosphate, neglecting the exchange with endogenous phosphate because it is impossible to quantify the phosphate exchange.

This is probably the main reason these values are up to 100 times smaller than those determined with luciferase or as obtained for R. rubrum chromatophores, for which a complete exchange was considered (12). However, it is possible that only a fraction of the bound nucleotides equilibrates with the added label. The rapid labeling of both the firmly bound and acid-soluble ATP in chloroplasts is compatible with the fact that the post-illumination ATP synthesis in R. rubrum is completed within the time necessary to denature the chromatophores. A similar rapid exchange of bound and added ATP during acid denaturation was previously observed for mitochondria by Cross and Boyer (8).

EDTA treatment that detaches the coupling factor from the chloroplast does indeed show a parallel inhibition of both the phosphorylation and the firmly bound [^{32}P] ATP

formation. The same results were previously found for chromatophores (12). NEM is supposed to interfere with the coupling factor only during illumination by blocking a terminal step of the energy transduction (18, 20). It is therefore expected and also found that the amount of firmly bound ATP is reduced to an extent similar to the overall phosphorylation activity. The energy transfer inhibitors phlorizin and Dio-9 show surprisingly different effects: while Dio-9 inhibits, phlorizin does not affect the synthesis of firmly bound [^{32}P] ATP. In contrast to this finding, not only phlorizin but also Dio-9 failed to inhibit the reaction taken as quasidirect evidence for a conformational change of the chloroplast coupling factor: i.e. the $^{3}H_2O$ uptake by the coupling factor during illumination (7).

The results obtained for the postillumination [^{32}P] ATP synthesis in both chloroplasts and chromatophores are consistent with our working hypothesis. However, there may be other ways to explain these results, because the observed inhibitions by ATP in the P $\rightarrow$ ^{32}P experiment and by ADP in the ^{32}P $\rightarrow$ P experiment are only partial. Although we have found for chromatophores that the formation of acid-soluble [^{32}P] ATP in a P $\rightarrow$ ^{32}P postillumination ATP synthesis proceeds as well at 30° as at 0°C, there is good reason to assume that a reaction involving a conformational change is temperature dependent (21). It is possible that the degree of the temperature dependence for the ATP synthesis is different from that for adenylate kinase and ATP-$^{32}P_i$ exchange.

The formulations of the energy-transducing mechanism by Boyer et al. (9) and Slater et al. (11) have several points in common with our working hypothesis, but both fail to explain the labeling pattern of the acid-soluble [^{32}P] ATP in a postillumination ATP synthesis. It could be argued that the total amounts being labeled in a postillumination ATP synthesis (P $\rightarrow$ ^{32}P experiment; or as in reference 12) are so small that artifacts are likely to occur in a multicomponent system, as chloroplasts or chromatophores are. However, some of the doubts can be eliminated by considering the specificity of the labeling as shown by using inhibitors. Our previous (12) and present results do not favor a transphosphorylation between nucleotides and added ADP, as suggested by Roy and Moudrianakis (3, 4), but could be explained by our working hypothesis. There is as yet no other hypothesis that is compatible with the above-mentioned finding.

The following paragraphs deal with entirely different hypotheses and their compatibilities with experimental data from various groups. Since the formation of ATP could be favored by the removal of water from the active site leading to a firmly bound ATP, Boyer's version involves an energy-consuming step to release this ATP. The results presented by Eisenhardt and Rosenthal (22), showing that the addition of ADP to uncoupled mitochondria yields approximately the same ATP jump as when added to coupled ones, do not favor the need of energy to release the ATP, but point to a requirement for binding of ADP.

An important aspect of certain inhibitors of coupling factor activities is that maximum inhibition does not exceed 50%, as found if chloroplasts are pretreated with NEM (18, 20), sulfate (23), or permanganate (24) in the light. If the permanganate pretreatment is performed in the dark, a much larger inhibition of phosphorylation is observed. Datta et al. (24) explained this effect as being nonspecific. An alternative explanation of these results involves a coupling factor with two sites, of which only one is inactivated when the coupling factor is kept in an energized state without being able to phosphorylate. The fact that pretreatment in the dark causes a greater inhibition could indicate that both sites are alternatively but equally inactivated when endogenous ATP at one site is hydrolyzed forming ATP at the other site.

Another line of evidence also suggests that the coupling factor has two sites for ADP or ATP as well as pyrophosphate. Girault et al. (25) have concluded from CD difference spectra of coupling factor-bound ADP minus free ADP that the coupling factor has two binding sites that are not independent. Keister and Minton (26) showed several years ago that pryophosphate added to chromatophores of R. rubrum drives an ATP synthesis that does not involve a phosphoryl transfer from pyrophosphate to ADP, but incorporates free phosphate exactly as described for the ATP synthesis driven by the firmly bound ATP (12). The existence of at least two active centers acting cooperatively has been proposed also by Nelson et al. (27). Furthermore, Repke et al. (28, 29) have recently published a model which is very similar to ours.

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