Biochimica et Biophysica Acta, 387 (1975) 335-348

BBA 46933

TIGHTLY BOUND NUCLEOTIDES OF THE ENERGY-TRANSDUCING ATPase OF CHLOROPLASTS AND THEIR ROLE IN PHOTOPHOSPHORYLATION

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(Received December 5th, 1974)

SUMMARY

1. Like other energy-transducing membranes, chloroplast membranes bear a coupling ATPase with especially tight binding sites for adenine nucleotides. Membranes washed several times still contain 2.5 nmol ATP and 1.3 nmol ADP bound per mg chlorophyll, which is equivalent to 1.9 ATP and 1.0 ADP per coupling ATPase.

2. In de-energized membranes, these nucleotides exchange to only a limited extent with added nucleotides. In membranes illuminated in the presence of pyocyanine, however, complete exchange of the bound nucleotides occurs rapidly, irrespective of whether ATP or ADP is present in the medium.

3. P_i can exchange into these nucleotides at both the β and γ positions when the membranes are energized in the presence of Mg^{2+}. Equilibrium with the β and γ groups of the bound nucleotides is, however, not complete.

4. The inhibitors and uncouplers Dio-9, S_{13} and EDTA have different effects on the exchange of nucleotides, the exchange of inorganic phosphate and photophosphorylation.

5. The bound ATP level on the membrane is stable to a wide variety of conditions. The ADP level, however, drops to near zero under conditions of maximal activation of the membrane ATPase.

INTRODUCTION

It has been reported previously that beef-heart mitochondrial ATPase (coupling factor 1 [1]), as isolated, contains tightly bound adenine nucleotides [2] and that these are also found on the membrane-bound ATPase [3]. We have suggested that the tight binding of nucleotides is involved in phosphorylation in such a way that energy is not required for ATP synthesis itself, but for release of tightly bound ATP from the

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Abbreviation: S_{13}, 5-chloro-3-*t*-butyl-2′-chloro-4′-nitrosalicylanilide.

ATPase [4–6]. A similar hypothesis has been put forward by Boyer [7, 8], who has also proposed an analogous mechanism for the myosin ATPase [9].

Tightly bound nucleotides have been demonstrated on all energy-transducing membranes so far investigated. They are found in beef-heart and rat-liver submitochondrial particles, chloroplast thylakoid membranes and membranes from *Escherichia coli* [3], in chromatophores from *Rhodospirillum rubrum* [10] and in membranes from the anaerobe, *Streptococcus faecalis* [11]. The amount of nucleotide is of the same order of magnitude (mol/mol) as the amount of coupling ATPase, where this is known. In the case of *S. faecalis*, *E. coli* and chloroplasts, the ATPase can be removed from the membrane by mild treatments, and the bound nucleotides are lost in parallel, indicating that the bound nucleotides are bound to the ATPase on the membrane. They do, in fact, remain bound to the coupling ATPase after its isolation, as has been previously shown with mitochondrial ATPase [2] and bacterial ATPase [11]. In this paper the same is shown to be true for the chloroplast ATPase. The wide distribution of tightly bound nucleotides on energy-transducing membranes from a variety of species lends support to the idea that they are involved in a process so universal as energy-linked phosphorylation. In the case of *R. rubrum*, mechanisms in which the bound nucleotides are involved in phosphorylation have indeed been proposed [10, 12].

It is shown in this paper that nucleotides are bound to the chloroplast coupling ATPase, and that the bound nucleotides exchange both their adenine and phosphate moieties with, respectively, adenine nucleotides and phosphate in the medium, when the membrane is energized. Under some circumstances, the total amount of nucleotides bound to the membrane may also be altered. This indicates that a conformational change in the coupling ATPase does occur during electron transfer in chloroplasts, leading to a change in the nucleotide-binding properties of this enzyme. A preliminary report of these findings has been given elsewhere [13].

MATERIALS AND METHODS

'Class II' chloroplasts (lacking an outer membrane) were prepared from depetiolated spinach leaves according to the procedure of Kraayenhof [14, 15] and resuspended in a medium containing 50 mM KCl, 50 mM NaCl, 10 mM Tricine/NaOH buffer, 5 mM $MgCl_2$ at pH 8.0, or 10 mM sodium pyrophosphate adjusted to pH 7.4 with HCl. They were 'stripped' of coupling ATPase where required by washing with 100 mM sorbitol, 2 mM Tricine/NaOH at pH 7.8 [16]. Before an experiment, the chloroplast membranes were freed from any nucleotides present in solution, by centrifugation 2–4 times (see legends to tables and figures) through the suspension medium. Chloroplasts incubated with ADP or ATP were prepared in either the pyrophosphate or the $MgCl_2$ medium. Chloroplasts incubated with P_i, or used in measurements of the total phosphorylation capacity were prepared in $MgCl_2$ medium, to avoid contamination with P_i present in the pyrophosphate.

Chloroplast suspensions were deproteinized using 4 % $HClO_4$ by the method of Rosing and Slater [17]. Before neutralization EDTA was added to a final concentration of 2 mM in excess of Mg^{2+} in order to prevent adenylate kinase activity. Nucleotide assays and radioactive counting for ^3H were performed on the deproteinized extract neutralized with 0.25 M Tris, 10 % (w/v) KOH to pH 7–8, after removal of $KClO_4$ by freezing and thawing, followed by centrifugation. This procedure led to a loss of less

than 12 % of the nucleotides at a concentration of 1 μM and less than 2 % at 10 μM, as shown by controls where radioactive ATP was used. This compares with the 40 % loss observed at 1.5 μM by Wiener et al. [18]. This may be due to the higher concentrations of $HClO_4$ and KOH used in the latter case.

Incorporation of $^{32}P_i$ into organic phosphate was estimated after extraction of unchanged inorganic phosphate as the molybdate complex from the unneutralized $HClO_4$ extract of labelled chloroplasts, according to Avron [19]. Chromatography of nucleotides was performed using the neutralized perchlorate extract, after desalting by adsorbing the nucleotides onto activated charcoal followed by elution with ethanol/ammonia (1 : 1, v/v) according to Roos and Loos [20]. Chromatography was performed on polyethylenimine-cellulose sheets by a modification of the methodof Randerath and Randerath [21] using 0.85 M LiCl/1 mM EDTA (pH 3.5) as eluant. The individual spots were localized by ultraviolet light, cut out of the sheet and tested for radioactivity as described below.

Nucleotides were assayed enzymatically as described by Bergmeyer [22] and low concentrations of ATP by the method of Stanley and Williams using luciferase [23]. Radioactivity was measured using a Nuclear Chicago liquid-scintillation counter ISOCAP 300. For ^3H, a scintillation liquid containing 4 g 2,5-diphenyloxazole and 50 mg 1,4-bis-(5-phenyloxazolyl-2-) benzene per l of toluene/96 % ethanol (19 : 6, v/v) was used. The volume of the aqueous sample was 25 μl or less in 10 ml scintillation fluid. ^{32}P was counted in aqueous solution using Cerenkov light [24]. Traces of ADP were removed from radioactive ATP solutions as described previously [2].

Photophosphorylation by isolated chloroplasts was measured as described by McCarty [25] using pyocyanine as mediator of cyclic electron transfer, except that bovine serum albumin was omitted from the reaction mixture. Rates of 6–7 μmol P_i esterified per min per mg chlorophyll were routinely obtained. Chlorophyll was estimated as described by Whatley and Arnon [26]. The ATPase activity of chloroplast membranes was measured with a sensitive pH meter in a medium containing 2.5 mM Tris, 50 mM KCl, 1 mM EDTA, 6 mM $MgCl_2$ and 2 mM Mg ATP brought to pH 8.0 with HCl. Soluble protein was measured by the method of Lowry et al. [27].

Carrier-free $^{32}P_i$, obtained from Philips-Duphar, was boiled in 1 M HCl for 1 h before use. [^{3}H]ATP and [^{3}H]ADP (about 20 Ci/mmol) were obtained from the Radiochemical Centre, Amersham.

S_{13} was kindly donated by Dr P. C. Hamm, Monsanto Comp., St. Louis, Mo., U.S.A., and Dio-9 by Mr H. A. F. Schenkels, Koninklijke Nederlandse Gist- en Spiritus Fabriek, Delft, The Netherlands. Pyocyanine was purchased as the perchlorate salt from Mann Research Laboratories, Inc. Polyethylenimine-cellulose sheets were obtained from Baker Chem. Co.

RESULTS

Bound nucleotides on chloroplast membranes

Table I shows the amounts of adenine nucleotide tightly bound to chloroplast membranes, expressed as nmol nucleotide per mg chlorophyll in the intact membrane. In Table I it is shown that the ATP and ADP levels are fairly constant at around 2.5 nmol ATP/mg chlorophyll and 1–3 nmol ADP/mg chlorophyll, irrespective of whether the chloroplasts are washed with 10 mM pyrophosphate (which removes non-

TABLE I

BOUND NUCLEOTIDE LEVELS IN WASHED CHLOROPLAST MEMBRANES

"Class II" chloroplasts [14, 15] were prepared in solutions containing either 10 mM sodium pyrophosphate at pH 7.4 ("PP_i medium"), or 50 mM KCl, 50 mM NaCl, 10 mM Tricine/NaOH buffer and 5 mM $MgCl_2$ at pH 8.0 ("$MgCl_2$ medium"). Where indicated, the chloroplasts were illuminated at a concentration of 200 μg chlorophyll/ml (total volume 10 ml) for 5 min at 10 °C, in the presence of 10 μM pyocyanine, and 200 μM ATP (in PP_i medium), 200 μM ADP (in PP_i medium) or 500 μM P_i (in $MgCl_2$ medium). Both the illuminated and non-illuminated chloroplasts were washed four times by repeated centrifugation through the same medium, resuspended at a chlorophyll concentration of 1.5–2.0 mg/ml, and extracted with 4 % $HClO_4$. Nucleotides were measured on the neutralized $HClO_4$ extract, and chlorophyll on the final chloroplast suspension.

The range over a number of assays (the number being shown in parentheses) is shown. The results were unaltered when the incubation with ADP, ATP or P_i took place in the dark.

Treatment	Concn (nmol/mg chlorophyll) of:		
	ATP	ADP	AMP
Washed with PP_i medium	2.2–2.5 (4)	1.1–1.5 (4)	0–1.1 (3)
Washed with $MgCl_2$ medium	2.1–2.9 (2)	1.5 (2)	<0.1 (2)
Illuminated with ATP before washing	2.2	—	—
Illuminated with ADP before washing	3.0	—	—
Illuminated with P_i before washing	2.1	—	—
Washed with low salt	1.1	0.7	<0.1
Boiled	0.1	—	—

specifically bound ATP very efficiently; Kemp, Jr, A., personal communication) or with a medium containing $MgCl_2$. Further, illumination with redox mediator in the presence of ATP, ADP or P_i causes little variation in the ATP level found on the membrane, from which it may be concluded that replacement of bound ATP by ADP, or phosphorylation of all the bound ADP to ATP with phosphate, does not occur irreversibly during this treatment (see also Table II, Section ii). These values are equivalent to 1.9 mol ATP and 1.0 mol ADP per mol ATPase, using the ATPase: chlorophyll ratio measured by Strotmann et al. [16]. AMP levels are lower and more variable, being absent from several preparations. Thus, it seems that comparable tight binding sites for AMP are not present.

The lower part of Table I indicates that when the coupling ATPase is removed from the membrane by a mild procedure (washing with low salt concentrations) or denatured by boiling, the nucleotides are also lost. The low-salt wash removes quite pure ATPase as shown by disc electrophoresis [16], making it likely that the membrane-bound nucleotides are in fact bound to the ATPase.This could be confirmed directly by concentrating this protein from the wash fluid, either by pressure dialysis or precipitation by ammonium sulphate. The protein thus isolated, with an ATPase activity measured after trypsin activation [28] of about 6 μmol/mg per min, contained 0.8 mol of ATP and 0.7 mol of ADP (no AMP) per mol ATPase, assuming a molecular weight of 325 000 [29] and that the isolated ATPase is completely pure. The reason for the departure of these values from 1.9 ATP, 1.0 ADP may be due to some denaturation of the enzyme during its isolation [2, 30].

TABLE II

CHANGES IN BOUND NUCLEOTIDE LEVELS IN CHLOROPLAST MEMBRANES

Chloroplasts were prepared as in Table I, and washed three times. Where indicated illumination took place after the third wash under the conditions given in Table I except that, where indicated, 5 mM dithioerythritol (DTE) were included in the illumination mixture. A further two washes took place after illumination, and the non-illuminated chloroplasts were washed twice more in parallel. After washing, the chloroplasts were extracted with 4 % $HClO_4$. Results are given as in Table I.

Treatment	Concn (nmol/mg chlorophyll) of:		
	ATP	ADP	AMP
(i) Washed with PP_i medium	2.2–2.5 (4)	1.1–1.5 (4)	0–1.1 (3)
as (i), illuminated	2.6–3.7 (2)	0.6	0.5
as (i), illuminated +5 mM DTE	2.4–2.9 (2)	0–0.2 (2)	0–0.7 (2)
(ii) Washed with $MgCl_2$ medium	2.1–2.9 (2)	1.5–1.6 (2)	<0.1 (2)
as (ii), illuminated +5 mM DTE+5 mM P_i	2.4–2.9 (3)	1.5–1.6 (3)	—

Changes in the nucleotide content of chloroplast membranes are observed when the membranes are illuminated in the presence of pyocyanine in a medium containing no Mg^{2+}. This is shown in Table II. This is particularly the case when 5 mM dithioerythritol is added to the incubation medium. Under these conditions, the ATPase activity of the chloroplasts increases by about 300 % owing to an alteration of the interaction between the ATPase protein and its inhibitor [31, 32]. Under optimal conditions, virtually all the ADP is lost while the ATP level remains unchanged. This loss of ADP does not occur if Mg^{2+} is included in the incubation medium, and is less if Mg^{2+} is present at any previous stage in the preparation of the chloroplasts (not shown).

Exchangeability of bound nucleotides on chloroplast membranes

Table III shows the results of incubating washed chloroplasts (containing only the tightly bound nucleotides) with labelled nucleotides or orthophosphate. Practically all the nucleotide bound exchanges with added [^{3}H] ATP or [^{3}H]ADP when the chloroplasts are illuminated with the labelled nucleotide in the presence of a redox mediator without added P_i. The amount of label incorporated is independent of which nucleotide is added, and chromatography confirms that, in the case of ADP at least, some of the incorporated label is present as ATP and some as ADP.

There is, thus, an exchange of the type $ADP_{free} \rightleftharpoons ATP_{bound}$ on the coupling factor under energized conditions, as well as the simple $ADP_{free} \rightleftharpoons ADP_{bound}$ exchange, and it seems likely from these data that both the corresponding exchanges with free ATP, viz. $ATP_{free} \rightleftharpoons ADP_{bound}$ and $ATP_{free} \rightleftharpoons ATP_{bound}$, occur. Free Mg^{2+} is not necessary for these exchanges, since the values given were obtained in a medium lacking in Mg^{2+}. The presence of Mg^{2+} does not affect the exchange against [^{3}H]ADP or [^{3}H] ATP (not shown).

If, after incubation in the medium containing pyrophoshate, the membranes are stripped of the coupling ATPase, the label is also lost from the membrane (Table III, line 3), showing that its binding site is indeed the coupling ATPase. Little or no exchange of

TABLE III

EXCHANGE OF BOUND NUCLEOTIDES IN CHLOROPLAST MEMBRANES

Thrice-washed chloroplasts were incubated in the dark or light as shown with radioactive nucleotides or P_i at the concentration given in the legend to Table I. The [^{3}H]ADP added had a specific activity of about 2000 cpm/nmol, the [^{3}H]ATP of 5000 cpm/nmol and the $^{32}P_i$ of 12 000 cpm/nmol under the counting conditions specified in Methods. After incubation with the label, the chloroplasts were washed until no more counts were present in the supernatant after centrifugation (spun down four times from a volume of 20 ml). In the experiment shown in the third line, chloroplasts were illuminated in the presence of [^{3}H]ATP and after two post-illumination washes in PP_i medium, the labelled chloroplasts were twice washed with 100 mM sorbitol, 2 mM Tricine/NaOH (pH 7.5) to remove the ATPase from the membrane. A $HClO_4$ extract of the washed chloroplasts was prepared and the radioactive nucleotides identified by chromatography and assayed as described in Methods. At the end of these experiments, the tightly bound ATP in the membranes was between 2.2–3.2 nmol/mg chlorophyll. Results are given as in Table I.

Exchange against	Treatment	Label incorporated (nmol/mg chlorophyll)	Incorporated as:
[^{3}H]ATP	–	0.6	
	Illuminated	2.4–3.2 (3)	Not tested
	Illuminated, stripped	0.8	
[^{3}H]ADP	–	0.3	
	Illuminated	2.0–3.2 (3)	ATP, ADP
$^{32}P_i$	–	0.1	
	Illuminated	2.4–4.0 (3)	ATP (β, γ positions) ADP (β position)

the nucleotides occurs in the dark over the same period, as shown in the table. Exchange is also low when incubation is carried out in the light but in the absence of redox mediator (Table IV), suggesting that electron transport is indeed needed for exchange.

With $^{32}P_i$ also, incorporation of label occurs only in the light in the presence of pyocyanine. $AP_{i\ free} \rightleftharpoons ATP_{bound}$ and a $P_{i\ free} \rightleftharpoons ADP_{bound}$ exchange were observed, as demonstrated by chromatography of the neutralized and the partially hydrolysed $HClO_4$ extract of the membrane, as shown in Table V. Before hydrolysis, twice as

TABLE IV

EFFECT OF REDOX MEDIATOR ON EXCHANGE OF BOUND NUCLEOTIDES IN ILLUMINATED CHLOROPLASTS

Chloroplasts were illuminated as in Table III in the presence of [^{3}H]ATP or $^{32}P_i$ except that, where indicated, pyocyanine was absent from the incubation medium. They were then washed, and the radioactivity in the bound nucleotides measured.

Exchange against:	Pyocyanine (μM)	Label incorporated (nmol/mg chlorophyll)
[^{3}H]ATP	0	1.4
	10	3.0
$^{32}P_i$	0	0.3
	10	2.5

TABLE V

DEMONSTRATION OF β-LABELLED ADP AND β,γ-LABELLED ATP IN BOUND NUCLEOTIDES

The neutralized $HClO_4$ extract of membranes labelled with ^{32}P was prepared as in Table III. Aliquots were taken and incubated at 0 °C for 30 min, and at 100 °C for 30 min after the addition of 40 mM HCl. The latter solution was then neutralized and chromatography performed on both extracts as described under Methods.

Spot on chromatogram	Radioactivity (cpm*)		Change on hydrolysis
	Before hydrolysis	After partial hydrolysis	
ATP	800	405	−395
ADP	428	656	+228

* Corrected for background radiation.

much label was present in the ATP spot as in the ADP spot, suggesting a ratio of 1 β,γ-labelled ATP:1β-labelled ADP. After partial hydrolysis, counts disappear from the ATP spot while about half as many appear in the ADP spot, consistent with this explanation, since it is known that the terminal phosphate is preferentially cleaved from ATP. In no experiment was complete labelling of all the β and γ phosphate groups, which would yield a total of 5–6 nmol ^{32}P/mg chlorophyll, observed in contrast to the almost complete exchange against ^{3}H-labelled adenine nucleotides. The labelling by ^{32}P tended to be more variable (results between 1.3 and 4.0 nmol incorporated per mg chlorophyll have been recorded) between different chloroplast preparations than the labelling by [^{3}H] adenine nucleotides. The reason for this is uncertain.

Phosphate pathway under phosphorylation conditions

Table VI shows the distribution of ^{32}P in the bound and free nucleotides when illumination takes place with added $^{32}P_i$ alone (as above), and with both added ADP and $^{32}P_i$ (net photophosphorylation conditions). Addition of ADP has no effect on the total amount or distribution of label in the bound nucleotides, i.e. both ATP and ADP become labelled with about twice as much label in ATP as in ADP. However, the nucleotides formed in solution during photophosphorylation show a very different pattern of labelling from the bound nucleotides, less than 2 % of the ^{32}P being present in the β-position of ATP or ADP.

When the labelled free nucleotides formed during phosphorylation in the experiment shown in Table VI were boiled in 0.1 M HCl, 80 % of the ^{32}P label disappeared from the organically-combined ^{32}P fraction within 30 min. However, if the nucleotides were first incubated with glucose, hexokinase and Mg^{2+}, the organic ^{32}P was completely protected against acid hydrolysis. This confirms that γ-labelled ATP is the major product of continued phosphorylation in these chloroplasts in the presence of ADP and $^{32}P_i$, in agreement with Avron [33], just as in mitochondria [34]. Thus, during photophosphorylation β-labelled nucleotides are released to the solution very slowly relative to the formation of γ-labelled nucleotides, and a mechanism of phosphorylation where $AMP_{free} \xrightarrow{P_i} ADP \xrightarrow{P_i} ATP_{free}$ as a major route (cf. ref. 38) is ruled out.

TABLE VI

DISTRIBUTION OF ^{32}P LABEL AFTER ILLUMINATION OF CHLOROPLASTS WITH $^{32}P_i$

Washed chloroplast membranes were prepared in $MgCl_2$ medium as described in Table I, and illuminated: (i) in a volume of 10 ml $MgCl_2$ medium, at a chlorophyll concentration of 200 μg/ml, in the presence of 10 μM pyocyanine and 500 μM $^{32}P_i$ at a specific activity of about 12 000 cpm/nmol; (ii) as (i), with the addition of 1 mM ADP (unlabelled); (iii) in a volume of 1 ml $MgCl_2$ medium, at a chlorophyll concentration of 20 μg/ml, in the presence of 10 μM pyocyanine, 1 mM ADP and 500 μM $^{32}P_i$, at a specific activity of about 1000 cpm/nmol. After illumination for 5 min, chloroplasts from samples i and ii were washed and a neutralized $HClO_4$ extract prepared as described in Table I. This extract contained only the labelled bound nucleotides of the membranes. After 2-min illumination, chloroplasts from sample iii were precipitated in the light by $HClO_4$ and a neutralized, deproteinated extract prepared. This extract contained almost entirely the free nucleotides labelled during photophosphorylation. Chromatography was carried out on the three extracts as described in Methods. The results are expressed for each of the three samples as cpm in a spot of a chromatogram divided by total cpm in the ATP+ADP spots of the same chromatogram (in percent).

Chloroplasts incubated with:	Radioactivity in spot (percent of cpm in ADP+ATP)			
	Bound nucleotides		Free nucleotides	
	ATP	ADP	ATP	ADP
500 μM $^{32}P_i$	70	30	(no free nucleotides)	
500 μM $^{32}P_i$+1 mM ADP	66	34	99	1

Inhibition of the exchange reactions

Fig. 1 shows a titration curve of the three exchange reactions and photophosphorylation with the uncoupler S_{13}. All four processes are about equally sensitive to uncoupler, 50 % uncoupling lying in region of 7 nmol S_{13}/mg chlorophyll. This is in

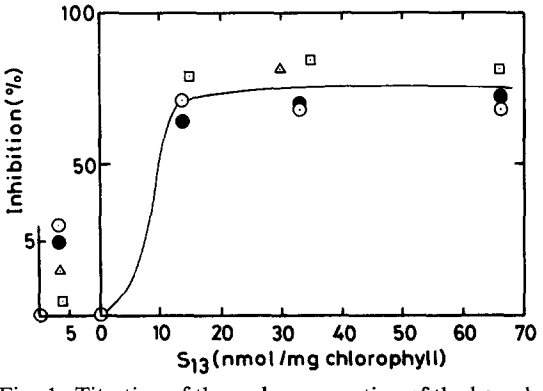

Fig. 1. Titration of the exchange reaction of the bound nucleotides with the uncoupler S_{13}. Washed chloroplast membranes were illuminated with [^{3}H]ATP, [^{3}H]ADP or $^{32}P_i$ as described in the legend to Table III, or under conditions of net phosphorylation as in Table VI (sample iii), except that varying amounts of the uncoupler S_{13} were added before illumination as indicated. The label in the bound nucleotides and total phosphorylation were measured as described, and expressed as a percentage of the value obtained without uncoupler. The percentage inhibition is plotted. ○, exchange against [^{3}H]ATP; ●, exchange against [^{3}H]ADP; △, exchange against $^{32}P_i$; ⊡, total phosphorylation. The inset on the left-hand side shows the effect of low concentrations of uncoupler, plotted on larger scales.

contrast to the results obtained in submitochondrial particles by Boyer et al. [8], who showed that, in this system, the total phosphorylation was much more sensitive to the uncoupler dinitrophenol than the $^{32}P_i$ incorporation into bound nucleotides.

In chloroplasts, the $^{32}P_i$ incorporation can be distinguished from the [^{3}H]ATP and [^{3}H]ADP incorporation by its greater sensitivity to EDTA (at concentrations too low to alter the amount of coupling ATPase on the membrane). This is shown in Table VII. While the [^{3}H]ATP and [^{3}H]ADP exchanges are unaffected by 5 mM EDTA (and can take place in a medium lacking in Mg^{2+}, as shown above), both photophosphorylation and $^{32}P_i$ exchange show an absolute requirement for Mg^{2+} and are abolished if excess EDTA is present. $^{32}P_i$ incorporation must therefore occur at a different step in the overall phosphorylation mechanism from the [^{3}H]adenine nucleotide exchange.

TABLE VII

EFFECT OF INHIBITORS OF PHOTOPHOSPHORYLATION ON THE EXCHANGE REACTIONS OF THE BOUND NUCLEOTIDES

Washed chloroplast membranes were illuminated as in the legend to Table VI except that the uncoupler and inhibitors of photophosphorylation were present as indicated. The label in the bound nucleotides and the total phosphorylation were measured as described, and expressed as a percentage of the value obtained without uncoupler or inhibitor. The absolute values of the controls, expressed as nmol label/mg chlorophyll, are within the ranges given in Table III for the exchange reactions, and under Methods for the total phosphorylation.

Exchange against	Label incorporated (% of control)		
	S_{13} (30 nmol/mg chlorophyll)	Dio-9 (5 μg/ml)	EDTA (5 mM)*
[^{3}H]ATP	28	73	91
[^{3}H]ADP	32	90	83
$^{32}P_i$	16	90	11
Overall phosphorylation rate	18	1	3

* In excess of Mg^{2+}.

Dio-9, while inhibiting photophosphorylation (possibly by inhibiting energy transfer from the electron transfer chain to the ATPase [35]) does not inhibit any of the three exchanges. This is again in contrast to the results obtained on submitochondrial particles by Boyer et al. [8], where oligomycin (an analogous inhibitor) inhibits $^{32}P_i$ incorporation into tightly bound ATP, but it is consistent with the results of Yamamoto et al. [10] on *R. rubrum* chromatophores, where oligomycin inhibits photophosphorylation but not $^{32}P_i$ incorporation into bound nucleotides.

DISCUSSION

These results demonstrate that the bound nucleotides found on the chloroplast coupling ATPase are indeed active during the process of energy-linked phosphorylation. Both their adenine and phosphate moieties become exchangeable with those of

nucleotide and inorganic phosphate in solution. The simplest interpretation is that a conformational change occurs in the ATPase during electron transfer. For, if we consider the bound adenine nucleotides as a label for the coupling ATPase, their change from a non-exchangeable to an exchangeable form during electron transport must indicate a conformational change in the coupling factor in direct analogy with the tritium exchange studies of Ryrie and Jagendorf [36]. Kraayenhof and Slater [37] have observed a rapid light-induced conformational change in chloroplast ATPase, covalently labelled with the fluorophore fluorescamine, and reconstituted with ATPase-depleted chloroplasts.

The change in ADP levels of the chloroplast ATPase during electron transport in the presence of dithioerythritol and the absence of Mg^{2+} suggests that the ATPase in chloroplasts isolated after this treatment has a stable conformation different from that in non-illuminated or Mg^{2+}-treated chloroplasts. The work of Bakker-Grunwald and Van Dam [31] has shown that this is indeed the case. They found that chloroplasts illuminated with dithioerythritol and a redox mediator have a raised ATPase activity which is stable for a considerable time (up to 1 h), but which decays rapidly (over about 5 min) if Mg^{2+} is present in the incubation mixture. The increased activity, corresponding to a change of the ATPase to an active conformation, thus correlates with the loss of ADP from the coupling factor. The reason for this is unknown; it may be that the alteration of the ATPase-inhibitor interaction causes a change at the (distant) ADP-binding site, but it could also be that the bound ADP in fact occupies the active site of the ATPase, and that its removal is necessary for ATPase activity.

Tightly bound nucleotides seem to be a common feature of coupling ATPases in a wide variety of energy-transducing membranes [2, 3, 10–12]. This suggests that they are involved in the phosphorylation mechanism itself. We have proposed a mechanism for phosphorylation linked to electron transfer [3–6] where energy is required, not for the ATP synthesis itself, but for the release of tightly bound nucleotides from the coupling membrane. The fact that energy, whether present in electrochemical or conformational form, is not necessarily required for ATP synthesis on an enzyme has only been realized recently [2–9]. It can be simply demonstrated by a consideration of the following equilibria:

$$ADP_f + P_{i\,f} \rightleftharpoons ATP_f + H_2O \qquad K_1 = \frac{[ATP]_f\,[H_2O]}{[ADP]_f\,[P_i]_f}$$

$$ATP_b \rightleftharpoons ATP_f + Pr \qquad K_2 = \frac{[ATP]_f\,[Pr]}{[ATP]_b}$$

$$ADP_b \rightleftharpoons ADP_f + Pr \qquad K_3 = \frac{[ADP]_f\,[Pr]}{[ADP]_b}$$

$$P_{i\,b} \rightleftharpoons P_{i\,f} + Pr \qquad K_4 = \frac{[P_i]_f\,[Pr]}{[P_i]_b}$$

$$ADP_b + P_{i\,b} \rightleftharpoons ATP_b + H_2O \qquad K_1' = \frac{[ATP]_b\,[H_2O]}{[ADP]_b\,[P_i]_b}$$

The subscripts b and f refer to bound and free, respectively, and Pr refers to protein. K_2, K_3 and K_4 are dissociation constants of the protein-ligand complexes.

From the relation, $K_1' = K_1\,(K_3K_4/K_2)/[Pr])$, we see that if $K_2 \ll K_3K_4$ (ATP

binds very strongly to the enzyme), $K_1' \gg K_1/[Pr]$ and therefore, at reasonable protein and P_i concentrations (less than molar) $[ATP]_b/[ADP]_b > [ATP]_f/[ADP]_f$, i.e. the ratio of ATP and ADP on the enzyme is greater than that in solution. It should be noted that the concentration of water at the active site of the enzyme need not be less than that in solution during this step.

If ATP is made in this way we would expect to find (1) very strong binding sites for nucleotides, especially ATP, on the coupling ATPase; (2) release of tightly bound nucleotides when the membrane is energized; and (3) no energy requirement for P_i incorporation into ATP on the coupling membrane.

The first two predictions are shown here to be verified in chloroplasts. The de-energized membranes, like other energy-transducing membranes [3, 10–12], contain very tightly bound ATP (Tables I and II). On energization, these nucleotides become less tightly bound, i.e. exchangeable with added nucleotides (Tables III and IV). Further, the labelling of both bound ADP and ATP by either added [^{3}H]ADP or added [^{3}H]ATP indicates that an equilibrium does indeed exist between ATP and ADP on the enzyme surface (as suggested above).

The third prediction is clearly not supported by the results reported. Incorporation of P_i into the bound nucleotides of chloroplast ATPase does require energy (Table III), as is seen also in submitochondrial particles [7]. This is not accommodated in the minimum hypothesis of phosphorylation proposed by ourselves [3–6] and Boyer [7–9] but it may reflect the necessity for a single 'priming' turnover of the coupling system (possibly linked to a shift of the inhibitor protein on the ATPase [39, 31] before phosphorylation can begin). Such a priming step is also required, according to Young et al. [40], to explain the characteristics of the $P_i \rightleftharpoons H_2O$ exchange in mitochondria.

We would predict, therefore, that a bout of phosphorylation begins with a single turnover of the respiratory chain which sets the system in a state in which it can begin ATP synthesis, and incorporation of P_i into ATP after this initial step does not require turnover of the respiratory chain. This hypothesis, although suggested by the other work quoted, remains to be tested. The inclusion of this step would explain the sensitivity of the exchanges to uncoupler, which dissipates the primed ('energized') state of the membrane, and their insensitivity to Dio-9, which does not. These conclusions are schematically presented in Fig. 2.

Here, the priming step (IV) brings ATP and ADP on the enzyme into equilibrium with each other, and with the solution, an equilibrium that can be displaced towards ATP by the addition of P_i (I) (if Mg^{2+} is also present). The single energy-requiring step in each phosphorylation cycle is the release of at least one molecule of ATP (II) to form an unstable, nucleotide-deficient form that spontaneously binds ADP (III) to form the original complex. The three asterisks shown here represent the three nucleotide-binding sites of CF_1. At least one of these (originally containing ATP) will be empty, but the remaining two may, or may not, still contain nucleotide, as the scheme requires only that one more molecule of ATP than ADP is released per cycle. The cycle (I+II+III) can then be repeated many times, but if the system is de-energized (e.g. by switching off the light, or addition of uncoupler) the initial 'frozen' state is regained.

Clearly, this scheme is incomplete in that it does not explain the observation that $^{32}P_i$ becomes incorporated into both β and γ positions of the bound ATP and ADP,

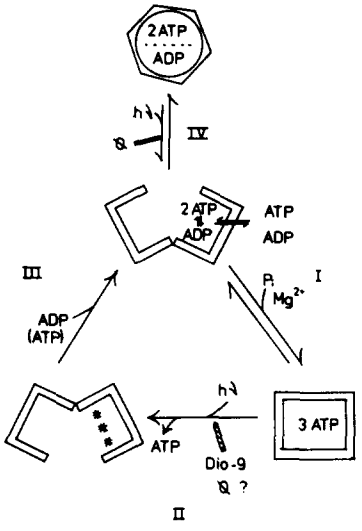

Fig. 2. Scheme for photophosphorylation via the chloroplast ATPase (for explanation, see text). ø, represents uncoupler.

while only γ-labelled ATP is released during photophosphorylation. A similar situation is observed in *R. rubrum* [12], but not in submitochondrial particles [8] where only γ-labelled ATP is observed. For want of further information, we have placed the β-labelling of ATP and ADP on a side path, but this is not yet established and a comparison of the rates of the [^{3}H]adenine nucleotide and $^{32}P_i$ exchanges has become an urgent experiment. However, whatever the exact details of the final scheme may be, it seems clear that bound nucleotides on coupling membranes do play a part in energy-linked phosphorylation, and must be taken into account when investigating this process.

Roy and Moudrianakis [38] have previously suggested the involvement in photosynthetic phosphorylation of nucleotides tightly bound to the coupling ATPase. Their scheme involves AMP as the initial acceptor of P_i. We have shown above that chloroplasts washed in $MgCl_2$ medium, which contain virtually no bound AMP (Table I), can readily incorporate $^{32}P_i$ into ADP and ATP without addition of AMP (Tables III and VI). Thus, it seems unlikely that AMP is itself the initial phosphate acceptor of photosynthesis. The results of Roy and Moudrianakis [38] are compatible with ours when it is considered that both the membrane-bound and the free coupling ATPase contain bound ATP and ADP, so that the binding of radioactive nucleotide in the experiments of these authors represents exchange reactions rather than de novo binding or synthesis.

ACKNOWLEDGEMENTS

We wish to thank Dr J. Rosing, Dr R. Kraayenhof, Dr O. A. Bakker-Grunwald and Dr A. Kemp, Jr for assistance, both theoretical and practical, during the course of this work. This work was supported in part by grants from the Netherlands Organization for the Advancement of Pure Research (Z.W.O.) under the auspices of the Netherlands

Foundation for Chemical Research (S.O.N.), and the N.A.T.O. research grant No. 589. During this work, D.A.H. was the recipient of a CIBA-Geigy European Fellowship.

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