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THE POSSIBLE ROLE OF TIGHTLY BOUND ADENINE NUCLEOTIDES IN OXIDATIVE AND PHOTOSYNTHETIC PHOSPHORYLATION

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The tightly bound nucleotides of the beef-heart mitochondrial ATPase are released during cold inactivation followed by ammonium sulfate precipitation. During incubation at 0°C the sedimentation coefficient (s_{20w}) of the ATPase first declines from 12.1 S to 9 S. Prolonged incubation or precipitation with ammonium sulfate leads to dissociation of the 9 S component into subunits with s_{20w} of 3.5 S. The 9 S component still bears bound nucleotides which exchange more extensively and rapidly with added nucleotides than those bound to the active 12.1 S component. The bound nucleotides are lost when the 9 S form dissociates into the smaller subunits. Thus, firm binding of nucleotides is a property of the quarternary structure of the enzyme.

The exchangeability of the nucleotides bound to the ATPase of chloroplast membranes is greatly increased in membranes illuminated in the presence of pyocyanine. P_i can exchange into both the β and γ positions of the bound nucleotides when the membranes are energized in the presence of Mg^{2+}. The exchange of the nucleotides and the incorporation of P_i are insensitive to the inhibitor Dio-9 but are inhibited by the uncoupler S_{13}.* This inhibition by S_{13} parallels that of the inhibition of photosynthetic phosphorylation. These findings are discussed with regard to our hypothesis that electron transfer causes release of preformed tightly bound ATP from the ATPase by inducing a conformational change.

INTRODUCTION

Tightly bound adenine nucleotides (ATP and ADP) have been demonstrated on isolated mitochondrial (1), chloroplast (2), and bacterial (2, 3) ATPase. Preparations of mitochondrial, chloroplast, and bacterial membranes contain firmly bound nucleotides in amounts to be expected from the concentration of ATPase in the membranes (2). Removal of the ATPase from the membranes causes a parallel loss of the bound nucleotides, indicating that these nucleotides are bound to the ATPase.

We have suggested that these nucleotides are involved in the mechanism of oxidative phosphorylation in such a way that energy input is required not for ATP formation, but for the release of preformed ATP from the ATPase (2, 4). A similar mechanism has also

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

been proposed by Boyer et al. (5) who suggested further that related mechanisms can describe energy transduction in muscle contraction, active transport, and pyrophosphate formation (6, 7).

Implicit in this mechanism is that the tightly bound nucleotides become more loosely bound during conformational changes in the ATPase induced by energization of the membranes.

The nucleotides present on isolated beef-heart mitochondrial ATPase exchange very slowly with added nucleotides (1). In this paper we shall show that the rate and extent of exchange is increased when the ATPase is kept at 0°C in the presence of high salt concentrations, conditions that cause a decline in the sedimentation coefficient of the ATPase from 12S to 9S. Ammonium sulfate precipitation of the 9S component leads to dissociation into 3S subunits and concomitant release of the bound nucleotides, indicating that firm binding of nucleotides is a property of the quarternary structure of the ATPase.

It is further shown that the exchange of nucleotides tightly bound to chloroplast membranes is very slow under de-energized conditions. However, in membranes illuminated in the presence of an electron mediator, there is a rapid and complete exchange of bound nucleotides with added ATP or ADP. P_i can exchange into both the β and γ position of the bound nucleotides under those conditions.

The effects of cold treatment on the conformation of the ATPase and the reactivity of the bound nucleotides in the different conformations and the effect of energization on the exchangeability of the nucleotides tightly bound to chloroplast membranes are discussed with regard to the proposed mechanism of oxidative phosphorylation. Reports of these findings are given elsewhere (8, 9).

METHODS

Mitochondrial ATPase (F_1) was prepared by the method of Knowles and Penefsky (10). Protein was estimated by the method of Lowry et al. (11) using bovine serum albumin or egg albumin as a standard. The absorbance coefficient of these proteins at 280 nm was taken as 0.895 $(mg/ml)^{-1}$ cm^{-1}. The specific activity directly after the preparation was about 100 μmoles phosphate released per min per mg protein, measured in the presence of an ATP regenerating system by the method of Pullman et al. (12). The molecular weight of the protein was taken to be 360,000 (13).

Since the enzyme is stored in a buffer containing 0.25 M sucrose, 10 mM Tris acetate, 2mM EDTA, and 4 mM ATP at pH 7.5, it was freed from ATP by precipitation by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution (pH 7.2). After standing in ice for 10 min, the protein was spun down at 0°C, the supernatant discarded, and the dried pellet was carefully dissolved in the sucrose-Tris-EDTA medium at room temperature. The preparations used in the experiments described in this paper were washed four times by this procedure and if necessary, finally clarified by centrifugation at room temperature. It was possible, by taking care to avoid cold or surface denaturation during this procedure, to restrict the loss in specific activity to less than 10%.

"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 $MgCl_2$, pH 8.0, or 10 mM sodium pyrophosphate adjusted to pH 7.4 with HCl. They were "stripped" of the coupling ATPase where required by washing with 100 mM sorbitol, 2 mM Tricine-NaOH, pH 7.8 (16). Before an experiment, the chloroplast membranes were freed from any nucleotides present in solution by centrifugation two to four times (see legends to Tables and figures) through the suspension medium.

Chloroplast and ATPase suspensions were deproteinized with 4% $HClO_4$ by the method of Rosing and Slater (17). 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. In the chloroplast experiments, EDTA to a final concentration of 2 mM in excess of Mg^{2+} was added, in order to prevent adenylate kinase activity.

Incorporation of $[^{32}P]\,P_i$ into organic phosphate was estimated after extraction of unchanged inorganic phosphate as the molybdate complex from the unneutralized $HClO_4$ extract of labeled chloroplasts, according to Avron (18). Chromatography of nucleotides was performed with 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 (19). Chromatography was performed on polyethyleneminecellulose sheets by a modification of the method of Randerath and Randerath (20) using 0.05 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.

ATP was determined with hexokinase (EC 2.7.1.1) and glucose-6-phosphate dehydrogenase (EC 1.1.1.49), ADP with pyruvate kinase (EC 2.7.1.40), and lactate dehydrogenase (EC 1.1.1.27) as described by Bergmeyer (21) and low concentrations of ATP by the method of Stanley and Williams using luciferase (22). 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 liter 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 (23). Traces of ADP were removed from radioactive ATP solutions as described previously (1).

The ATPase activity of the mitochondrial ATPase was measured with an ATP-regenerating system at 30°C. The protein was first diluted in the sucrose-Tris-EDTA medium described above to a protein concentration of about 0.2 mg/ml. An aliquot of this solution containing about 2 μg protein was added to a cuvette containing 33 mM Tris-acetate, 83 mM sucrose, 10 mM $MgCl_2$, 1 mM EDTA, 2 mM ATP, 1.5 mM phosphoenol pyruvate, 0.17 mM NADH, 6 units (μmole/min) pyruvate kinase (EC 2.7.1.40), and 12 units lactate dehydrogenase (EC 1.1.1.27) at pH 7.2. ATPase activity was calculated from the rate of NADH oxidation followed at 340 nm in a Zeiss spectrophotometer. The activity of the washed enzyme measured by this method usually was less than 100 μmoles min^{-1} mg $protein^{-1}$, the activity given for the freshly prepared enzyme. This lower activity is due to the lower pH (7.2 instead of 7.5) at which the activity was measured and to some inactivation during washing of the ATPase.

Photophosphorylation by isolated chloroplasts was measured as described by McCarty (24) using pyocyanine as mediator of cyclic electron transfer, except that bovine serum

albumin was omitted from the reaction mixture. Rates of 6–7 μmole P_i esterified per min per mg chlorophyll were routinely obtained. Chlorophyll was measured as described by Whetley and Arnon (25).

Ultracentrifugation was performed in a MSE analytical ultracentrifuge, equipped with a four-hole rotor using a single-sector cell with wedge windows. Viscometry at 20°C was carried out with a rotating-cylinder viscometer designed by Zimm and Crothers (26).

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

S_{13} was kindly donated by Dr. P. C. Hamm, Monsanto Co., St. Louis, Mo., 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 Chemical Co.

RESULTS

Nucleotide-Binding and Exchange Properties of Different Species Obtained after Cold Treatment of Purified Beef-Heart Mitochondrial ATPase

Beef-heart mitochondrial ATPase (12) and corresponding enzymes isolated from chloroplast (27) and Escherichia coli membranes (28) are cold labile, that is, they lose activity when kept at 0°C. Penefsky and Warner (29) have shown that during incubation at 0°C the active enzyme (11.9S) is transformed into 9.1 S and 3.5 S species, and that the transformation to the latter species is promoted by high concentrations of salt. The cold-inactivated enzyme can be reactivated by warming the protein solution to 30°C.

The adenine nucleotides firmly bound to the mitochondrial ATPase are not removed by repeated precipitation of the enzyme by ammonium sulfate, but precipitation of the cold-treated enzyme leads to loss of the bound nucleotides (1).

In an earlier publication (8) we have shown that during cold inactivation the active ATPase is transformed into 9 S and 3 S species, and that ammonium sulfate precipitation at this stage leads to disappearance of the 9 S species.

Precipitation of the enzyme by addition of ammonium sulfate after inactivation at 0°C but prior to reactivation by warming the protein solution leads to irreversible loss of ATPase activity (Fig. 1).

The behavior of the tightly bound ATP during cold inactivation and the effect of ammonium sulfate precipitation is shown in Fig. 2. During cold inactivation, the activity of the ATPase was followed and after various time intervals two samples were taken, one of which was treated with charcoal to remove free nucleotides and the other precipitated with ammonium sulfate and redissolved in buffer. The ATP content of acid extracts of both samples was determined and plotted vs the specific activity of the ATPase. From this figure it is clear that most of the ATP remains tightly bound to the species arising during cold inactivation but that it is removed by ammonium sulfate precipitation of the enzyme.

From the above mentioned experiments we conclude that during cold inactivation the active ATPase (11.9 S) is transformed into a 9 S form, still bearing bound nucleotides, which subsequently decomposes into the 3 S subunits which do not bind adenine nucleotides. This last step is promoted in the presence of high salt concentrations, for example,

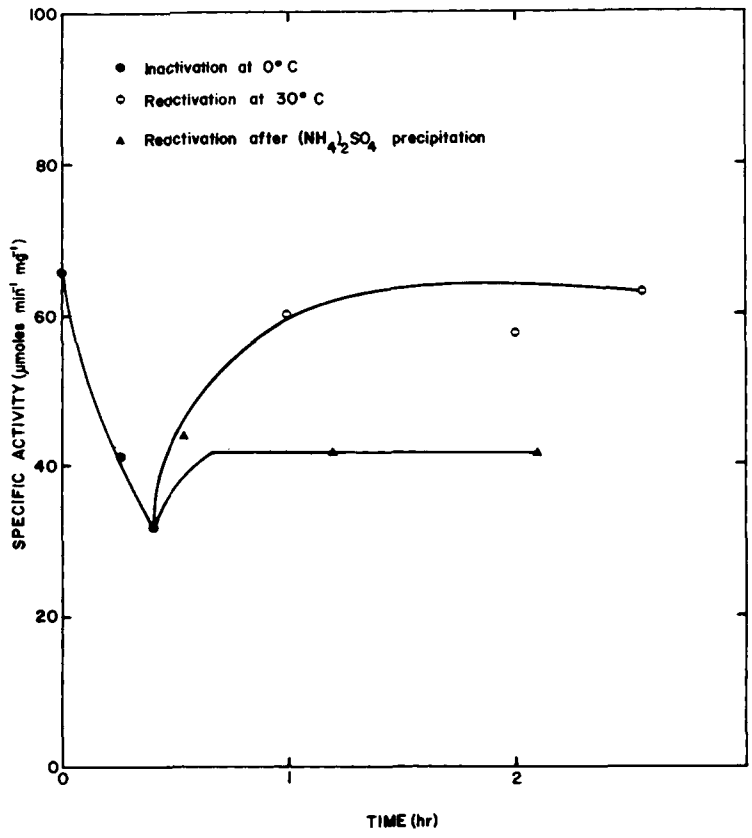

Fig. 1. Reactivation of ATPase after ammonium sulfate precipitation of cold-inactivated enzyme. Washed ATPase was inactivated at 0°C in a buffer containing 10 mM Tris maleate and 50 mM KNO_3 (pH 7.5), at a protein concentration of 4.3 mg/ml. After 25 min two samples were taken. The protein from one sample was precipitated immediately by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and dissolved in 10 mM Tris maleate and 50 mM KNO_3 (pH 7.5) at room temperature. Samples of the nonprecipitated and the cold-inactivated enzyme were diluted with 3 vol of a buffer containing 40 mM Tricine-NaOH, 4 mM ATP, and 34% (w/v) glycerol at pH 7.8 and incubated at 30°C. ○, nonprecipitated; △, precipitated.

by precipitating the enzyme with 50% ammonium sulfate.

The tightly bound nucleotides bound to the active ATPase exchange slowly and partly with added nucleotides at room temperature (1). Only 0.8 mole of labeled ATP and 0.4 mole of labeled ADP are exchanged after a 2-hr period. The experiment described in Fig. 3 shows that during inactivation-reactivation, up to 1.4 moles of labeled ATP can be incorporated per mole of ATPase. Total bound ATP and [^{3}H] ATP are plotted against activity during the reactivation. Since the lines corresponding to total and labeled ATP incorporated are approximately parallel, it is concluded that all the "newly incorporated" ATP during reactivation comes from the solution, i.e. that the ATP on the 9S form is

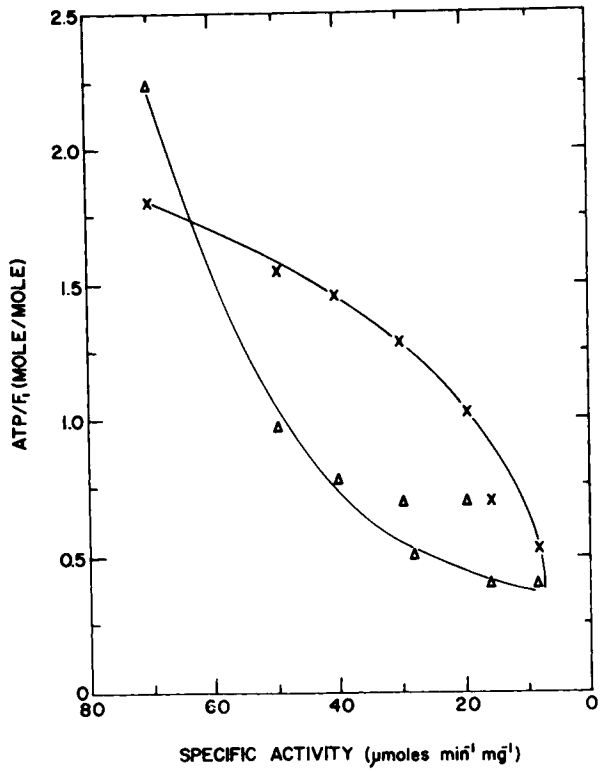

Fig. 2. The amount of bound ATP as a function of the specific activity of cold-inactivated F_1 precipitated with $(NH_4)_2SO_4$ or treated with charcoal. Washed ATPase was dissolved in a buffer containing 10 mM Tris maleate and 50 mM KNO_3, pH 7.5, and inactivated at 0°C, at a protein concentration of 1.5 mg/ml. At intervals the activity was measured and two samples were taken, one of which was treated with charcoal (about 20 mg/ml sample). The charcoal was removed from the solution by centrifugation. In the other, the protein was precipitated by addition of an equal volume of saturated $(NH_4)_2SO_4$ solution. The precipitated protein was spun down and redissolved in sucrose-Tris-EDTA buffer. The ATP content was determined on a $HClO_4$ extract of both solutions by the luciferase method. △ –△, $(NH_4)_2SO_4$-precipitated sample; X—X, charcoal-treated sample.

freely exchangeable, in contrast to that bound to the 12S form. Also, the exchange against added ADP is promoted under those conditions (not shown here).

Role of Nucleotides Tightly Bound to the Chloroplast ATPase in Photophosphorylation

In Table I it is shown that washed chloroplast membranes contain tightly bound adenine nucleotides in amounts equal to 2.5 nmole ATP and 1.3 nmole ADP per mg chlorophyll. With the ATPase-to-chlorophyll ratio measured by Strotmann et al. (16), these values correspond to 1.9 moles ATP and 1.0 moles ADP per mole ATPase. Since the AMP levels are lower and more variable (in some preparations AMP is even absent) it is concluded that no comparable binding sites for AMP are present in chloroplast membranes (cf. ref. 30).

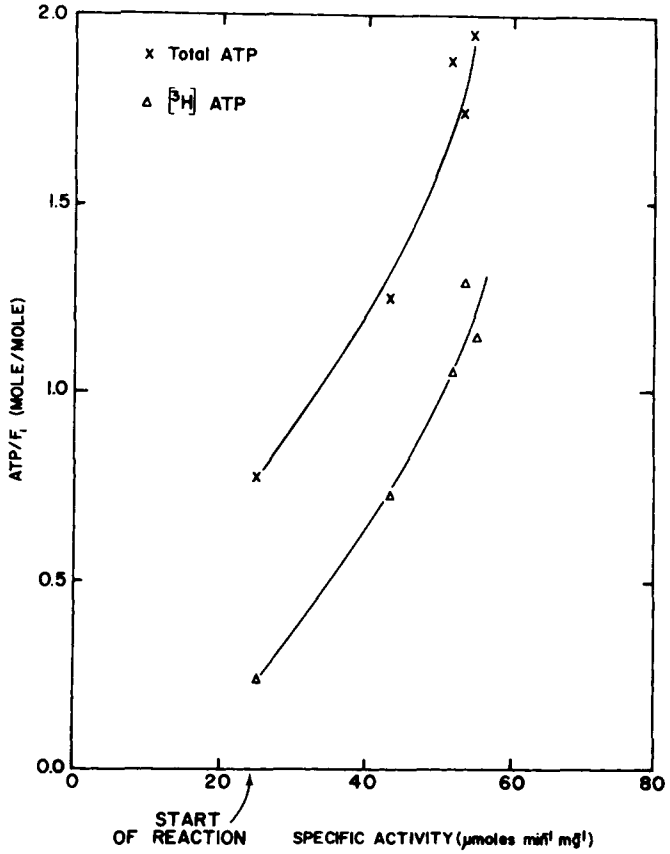

Fig. 3. ATP bound to ATPase during reactivation in the presence of ATP. Washed ATPase was inactivated at 0°C and reactivated as described in the legend to Table II Expt. A. Total ATP (X) and [^{3}H]ATP (△) bound to the washed enzyme were determined on $HClO_4$ extracts of the protein taken at various times during the reactivation.

The ATP and ADP levels are fairly constant irrespective of whether the chloroplasts are washed with 10 mM PP_i (which removes nonspecifically bound adenine nucleotides. A. Kemp, Jr., personal communication) or with a medium containing $MgCl_2$. When the coupling ATPase is removed from the membranes by washing with low salt concentrations (16) or denatured by boiling, the bound nucleotides are also lost. In fact, the nucleotides can be recovered from the fairly pure ATPase obtained by the former procedure, indicating that they are indeed bound to the ATPase.

The exchange of the bound nucleotides against added nucleotides is very slow in nonenergized chloroplast membranes (i.e., chloroplasts incubated in the dark) as shown in Table II A. Energization of the chloroplasts by illuminating them in the presence of a redox mediator without added P_i results in nearly complete exchange of the bound nucleotides with added [^{3}H]ATP or [^{3}H]ADP. In the case of an ADP exchange, the label bound to the membranes can be recovered as both ADP and ATP, which means that ex-

TABLE I. Bound Nucleotide Levels in Washed Chloroplast Membranes

Treatment	ATP	ADP	AMP
	(nmoles/mg chlorophyll)		
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	–	–

"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 $MgCl_2$, pH 8.0 ("$MgCl_2$ medium"). Where indicated, the chloroplasts were illuminated at a concentration of 200 μg/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 nonilluminated 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 was measured 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.

changes of the type $ADP_{free} \rightleftharpoons ADP_{bound}$ and $ADP_{free} \rightleftharpoons ATP_{bound}$ can occur. Although not tested, it seems likely that the corresponding exchanges with added ATP viz. $ATP_{free} \rightleftharpoons ATP_{bound}$ and $ATP_{free} \rightleftharpoons ADP_{bound}$ can also take place. Since the label is removed from the membranes after stripping of the ATPase, it is concluded that the labeled nucleotides are indeed bound to the ATPase. Electron transport is a necessary condition to promote exchange, since illumination in the absence of an electron donor gives rise to a low exchange (Table II B).

Even in the absence of ADP or ATP, $[^{32}P]P_i$ can be incorporated into the bound nucleotides during illumination in the presence of pyocyanine. Label occurs in this case both in the β and γ position of ATP and in the β position of ADP. Since γ-labeled ATP is the major product in photophosphorylation (31), it is concluded that release of β-labeled nucleotides to the solution is slow under those conditions.

In contrast to the complete labeling with $[^3H]$ nucleotides, the labeling with $[^{32}P]P_i$ is variable and incomplete (1.3–4 moles incorporated per mg chlorophyll, while 5–6 moles are available for labeling).

Figure 4 shows the effects of various inhibitors of photophosphorylation on the ex-

TABLE II A. Exchange of Bound Nucleotides in Chloroplast Membranes

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

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 2,000 cpm/nmole, the [^{3}H]ATP of 5,000 cpm/nmole, and the [^{32}P]P_i, of 12,000 cpm/nmole 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 postillumination washes in PP_i medium, the labeled chloroplasts were twice washed with 100 mM sorbitol, 2 mM Tricine-NaOH (pH 7.5) to remove the ATPase from the membrane. An $HClO_4$ extract of the chloroplasts was then 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 and 3.2 nmol/mg chlorophyll. Results are given as in Table I.

TABLE II B. Effect of Redox Mediator on Exchange of Bound Nucleotides in Illuminated Chloroplasts

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

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

change reactions. The exchange reactions and the phosphorylation reaction are equally affected by the uncoupler S_{13}, which differs from the results obtained by Boyer et al. (5) with submitochondrial particles, who found that labeling of bound ATP with [^{32}P]P_i is less sensitive to the uncoupler dinitrophenol than is oxidative phosphorylation.

Addition of 5 mM EDTA has more effect on the P_i incorporation and photophosphorylation than on the nucleotide exchanges, which shows an absolute requirement for Mg^{2+} and leads to the conclusion that the pathway of [^{32}P]P_i incorporation is different from that of [^{3}H] adenine nucleotide incorporation into the bound nucleotides.

Dio-9, an inhibitor of photophosphorylation (probably acting like oligomycin in

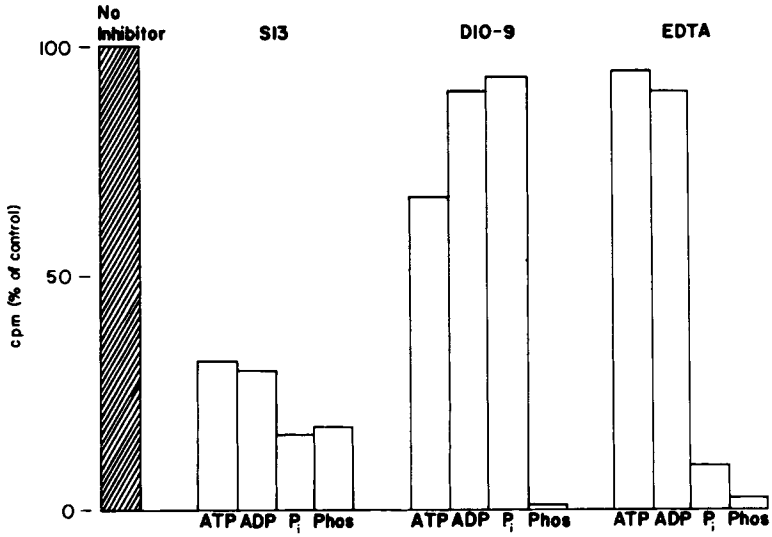

Fig. 4. Effect of inhibitors of photophosphorylation on the exchange reactions of bound nucleotides. Washed chloroplast membranes were illuminated with [^{3}H] ATP, [^{3}H] ADP, or [^{32}P] P_i as described in the legend to Table II A or under conditions of net phosphorylation, except that the uncoupler and inhibitors of photophosphorylation S_{13} (at 30 nmole/mg chlorophyll), Dio-9 (at 5 μg/ml), or EDTA (at a concentration of free EDTA [excess above Mg^{2+}] of 5 mM) were present as indicated. The label in the bound nucleotides and the total phosphorylation were measured as described and expressed as percent of the value obtained without uncoupler or inhibitor.

mitochondrial preparations) does not inhibit either of the three exchanges, which is in line with the results of Yamamoto et al. (32) on R. rubrum chromatophores, who found that oligomycin, an inhibitor of photophosphorylation in this system, does not affect [^{32}P] P_i labeling of bound nucleotides. Our results are, however, in contrast to those of Boyer et al. (5) who found an inhibition of labeling of mitochondrially bound ATP by oligomycin.

DISCUSSION

In the first part of this paper we studied the effect of cold treatment of the ATPase purified from beef-heart mitochondria on the behavior of the tightly bound nucleotides. The nucleotides bound to the ATPase are released during cold inactivation followed by ammonium sulfate precipitation. This loss of nucleotides is not a result of the transition of the active ATPase into the inactive 9S component, but is caused by the subsequent transformation of the 9S form into 3S subunits, a step accelerated by precipitation with ammonium sulfate.

These findings can be reconciled in the following scheme:

$$\underset{(12.1\,S)}{F_1\text{-Adn}} \xrightarrow[(1)]{0^\circ C} \underset{(9S)}{9S\ \text{form-Adn}} \xrightarrow[(2)]{\text{irreversible}} \underset{(3.5S)}{\text{Subunits + Adn}}$$

$$\uparrow \quad \_\_\_\_\_ \quad \downarrow$$

$$30^\circ C$$

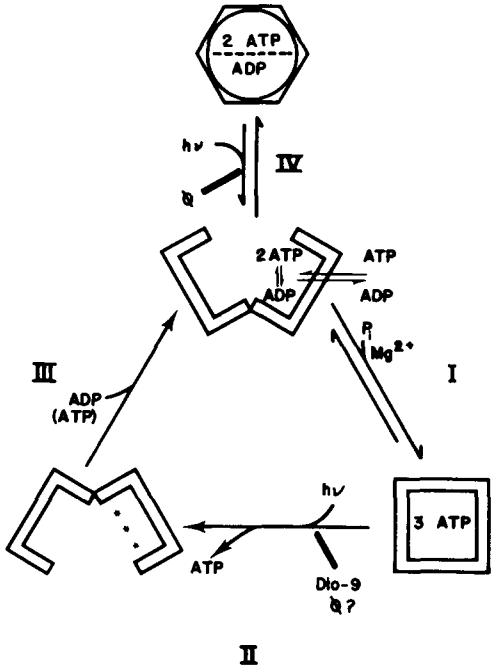

Fig. 5. Scheme for photophosphorylation via the chloroplast ATPase (for explanation, see text). Φ represents uncoupler.

Upon incubation at 0°C the active ATPase (12.1S) containing tightly bound nucleotides is transformed into a 9S form still bearing bound nucleotides. This first step is reversible by warming the protein solution to 30°C. In contrast to the nucleotides on the 12S form those on the 9S species are readily exchangeable against added nucleotides. Prolonged incubation at 0°C or precipitation with ammonium sulfate leads to irreversible decomposition of the 9S form into 3.5S subunits with concomitant loss of the bound nucleotides.

We have proposed that the prominent function of energy derived from electron transfer is to bring about release of preformed tightly bound ATP by inducing a conformational change in the ATPase (2, 4). This makes the 9S form of considerable theoretical interest since it is possible that it represents a different conformation (less streamlined) of the active 12S component, which may be equivalent to the postulated high energy form of the enzyme bound to the membrane. Especially, the increased exchangeability of the bound adenine nucleotides on the 9S form encouraged us in considering this possibility, since this is a property predicted for the high energy form of the ATPase in our phosphorylation mechanism.

In the second part of this paper we described the effect of energization of the chloroplast membranes on the exchangeability of the adenine nucleotides and the phosphate moieties of the nucleotides tightly bound to the coupling ATPase.

It is shown that: (a) a rapid exchange of the bound nucleotides against added nucleotides and incorporation of $[^{32}P]P_i$ into the bound nucleotides can occur only during

light-induced electron transport, conditions giving rise to conformational changes in the chloroplast ATPase (33, 34); (b) these exchanges have the same sensitivity for the uncoupler S_{13} as photophosphorylation; (c) in contrast to photophosphorylation, the exchanges are insensitive to the inhibitor Dio-9; (d) both the incorporation of $[^{32}P]P_i$ into bound nucleotides and photophosphorylation require magnesium, while the exchange against added nucleotides does not; (e) bound nucleotides can be labeled with $[^{32}P]P_i$ in both the β and γ positions, while only γ-labeled ATP is released during photophosphorylation.

That energy is needed for the exchange of bound nucleotides is in line with our original hypothesis. The finding that electron transport is necessary for P_i incorporation into the bound nucleotides (which is also seen in submitochondrial particles [5]) is not accommodated in the minimum hypotheses brought forward by ourselves (2, 4) and Boyer (5, 6, 7). This may reflect either a need for some energy input for the ATP formation at the catalytic site (a possibility left open by Boyer et al. [7]) or the necessity for a single priming turnover of the coupling system, which may be linked to dissociation of the inhibitor protein (35, 36) from the ATPase. Young et al. (37) required such a priming step for the explanation of certain characteristics of the P_i-HOH exchange. Uncoupler inhibits all three exchanges probably by dissipating the primed (energized) state of the membrane, a state not affected by Dio-9, which explains the insensitivity of the exchanges to the latter.

These findings are schematically represented in Fig. 5. As a result of the priming step (IV), bound ATP and ADP can equilibrate with each other and with nucleotides in solution. Addition of P_i leads to displacement of the equilibrium in the direction of ATP formation, a step that requires Mg^{2+}. The next step is the release of at least one molecule of ATP promoted by energy derived from electron transport, leaving an unstable nucleotide-deficient form that spontaneously binds ADP (step III) to form the original complex. The cycle (I + II + III) can proceed until the system is de-energized, in which case the initial frozen state is restored.

We are aware of the fact that this scheme is incomplete (it does not explain, for instance, the P_i incorporation into the β positions of the bound nucleotides), but in our opinion, it is clear that nucleotides bound to energy-conserving membranes play an important role in the mechanism of the phosphorylation reaction.

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