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Assessment of Total Catalytic Sites and the Nature of Bound Nucleotide Participation in Photophosphorylation*

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Application of a new hexokinase accessibility method for the measurement of bound [^{32}P]ATP during steady state photophosphorylation by chloroplast thylakoids, together with mixing-quenching and filtration techniques has shown the following.

1. Two classes of bound ATP exist: a transitorily tightly bound class that represents a catalytic intermediate and a noncatalytic class of tightly bound ATP that is labeled much more slowly, presumably from medium components. Thylakoids filtered after a few seconds of photophosphorylation have some of both classes present. Thus, ATP may remain bound at a catalytic site on membranes in the absence of continued energization.

2. During steady state photophosphorylation, the sum of catalytically competent bound ATP plus P_i is considerably greater than 1/chloroplast ATP synthase complex even when substrate concentration limits the synthesis rate. At a given instant, therefore, most or all phosphorylation complexes are functional, with the simultaneous participation of more than one catalytic site/enzyme.

3. During photophosphorylation, with substrate concentration well below K_m values, most ATP synthase complexes retain a bound, catalytic ATP. This behavior is not consistent with independent catalytic sites.

These results give additional support to the view developed in this laboratory that ATP synthesis is accomplished by a mechanism in which two catalytic sites are functioning alternately on the ATP synthase. This mechanism proposes that energy-linked conformational changes in the ATP synthase promote binding of P_i and ADP in a mode competent for ATP synthesis at one catalytic site and concomitantly the release of tightly bound ATP from an alternate site.

Membrane-bound ADP and ATP are found in a wide variety of energy transducing systems (see Ref. 1 for review). In submitochondrial particles (2-4) and in chloroplasts (5, 6) tightly bound nucleotides have been shown to be associated with the ATP synthase complex[1] and are removed from the membrane when a portion of the complex called coupling factor, or CF_1, is removed (2, 5, 7-9).

Harris and Slater (5), Smith and Boyer (10), Magnusson and McCarty (8), and Strotmann *et al.* (11) have shown that tightly bound nucleotides on chloroplast thylakoid membranes are not readily exchangeable in the dark but become exchangeable in the light or during acid-base transition. This indicates an energy dependence of the nucleotide binding sites and suggests that bound nucleotides may be involved in the mechanism of oxidative and photophosphorylation. The nature and extent of their participation is a matter of controversy. Cantley and Hammes (12) consider the two nucleotide binding sites that they find on isolated chloroplast coupling factor to be "allosteric conformational switches," whereas Roy and Moudrianakis (13), Harris *et al.* (2), Beyeler and Bachofen (14), and Kozlov and Skulachev (15) postulate that tightly bound nucleotides found on thylakoid membranes or isolated CF_1 represent intermediate steps in the overall process of ATP synthesis. In contrast, results of Smith and Boyer (10) led them to suggest that catalytic nucleotides do not account for all of these bound nucleotides, with the implication that more than one class of bound nucleotides exists (16).

Studies in our laboratory on ATP synthesis catalyzed by mitochondria and chloroplasts have been guided by the view that the most meaningful mechanistic information about catalytic processes may come from approaches that allow determination of the nature and amount of catalytic intermediates, their rates of formation and disappearance, and factors that affect these rates. This means that the most useful data may need to be derived from experiments performed on systems actively catalyzing steady state ATP synthesis or hydrolysis. Approaches that have yielded information along this line include the measurement of rate of exchange of phosphate oxygens during net synthesis and hydrolysis of ATP (17, 18) and tracer studies of labeled ATP, ADP, and P_i in millisecond mixing-quenching and filtration studies (10).

In the present studies, we have extended the rapid mixing and filtration techniques as initially applied to chloroplast phosphorylation by Smith and Boyer (10). Their studies of ATP synthesis, induced by acid-base transition, showed: presence of bound ADP and P_i committed to ATP synthesis; presence, on thylakoids after an EDTA quench, of tightly bound ATP kinetically competent to be an intermediate in synthesis; and that most or all of the ATP synthase complex appeared to be simultaneously active during ATP synthesis. Objections can be made that the use of an acid-base transition to induce ATP synthesis may not be mechanistically analogous to light-driven phosphorylation. The studies reported in this paper were performed using continuous light to drive ATP synthesis and improved methods for estimating the nature and amount of enzyme-bound intermediates.

A troublesome point in the study of bound nucleotides has

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[1] The complex of CF_1 ATPase with other proteins on membranes that is capable of photophosphorylation is referred to as the ATP synthase complex, and the CF_1 portion is referred to as the ATP synthase.

been that the measurement of bound reactants on quenched thylakoids and submitochondrial particles even when filtration is accomplished within milliseconds may not reveal the actual level of bound intermediates during the steady state. A new approach which measures the amount of bound ATP during steady state ATP synthesis has been devised based on the inaccessibility of bound ATP to medium hexokinase. The major focus of this paper is the use of this and other techniques to measure the amount and catalytic properties of ATP bound to chloroplast membranes during steady state photophosphorylation.

EXPERIMENTAL PROCEDURES

Chloroplast thylakoid membranes were prepared from market spinach essentially as previously described (19). Replacement of NaCl in the grinding mix by 200 mM choline-Cl and 5 mM $MgCl_2$, as suggested by Mukohata *et al.* (20), improved the retention of phosphorylation capacity. Hexokinase (type C-300) was purchased from Sigma Chemical Co., St. Louis, MO.

The standard reaction mixture contained 10 mM $MgCl_2$, 25 mM NaCl, 50 mM Tricine[2]/NaOH (pH 8.0), 33 μM pyocyanine, 25 mM glucose and ADP, P_i, hexokinase, and thylakoids as indicated in figure legends. Mixing techniques using an electronic shutter or rapid mixer were essentially as described previously (19, 21). The methods of quenching and the incubation times are as described in figure or table legends. Perchloric acid-quenched samples[3] were treated to isolate [^{32}P]ATP as described in the preceding paper (22). Thylakoids quenched with NH_4Cl were filtered (in the dark), through two Gelman 0.45-μm UM-47 mm filters by vacuum aspiration. The thylakoids were then washed by three 2-ml aliquots of 200 mM EDTA, pH 8.0. Controls showed little or no loss of bound nucleotides by this washing procedure. Nucleotides were extracted from washed membranes with two 2-ml aliquots of 1 M perchloric acid, 10 mM EDTA, containing 1 μmol of carrier ATP. The extract was collected, put on a charcoal column, and treated to determine [^{32}P]ATP (19).

The light source used to illuminate chloroplasts provided about 1.6×10^6 erg $\times$ cm^{-2} $\times$ s^{-1} of heat-filtered white light to the reaction vessel surface. Maximum rate of [^{32}P]P_i incorporated into ATP was 900 to 1200 μmol/mg of chlorophyll/h. Chlorophyll was determined by the method of Arnon (23). All experiments were performed at room temperature (about 22°C).

ATP synthase concentrations were taken as 1.3 nmol of synthase/mg of chlorophyll as estimated by Strotmann *et al.* (24) using a molecular weight for CF_1 of 325,000 as determined by Farron (25) and confirmed by us for the chloroform extracted enzyme.[4]

RESULTS

ATP Labeled on Filtered Thylakoids after Illumination—Any ATP bound at the active site of synthase molecules during synthesis of ATP from ADP and [^{32}P]P_i must be labeled rapidly from [^{32}P]P_i. Any ATP bound on a noncatalytic site (16) would be labeled less rapidly. Experimental determination of the time course of bound ATP labeling during ATP synthesis, under conditions where all or nearly all ATP synthase molecules are active, might thus reveal the presence of two classes of bound ATP.

Fig. 1 is the result of an experiment designed to study the labeling pattern of ATP bound to thylakoid membranes after ATP synthesis was terminated by stopping illumination. Evidence will be presented later that under the conditions of this experiment most ATP synthase complexes are active. The figure shows a biphasic labeling curve and, thus, indicates that

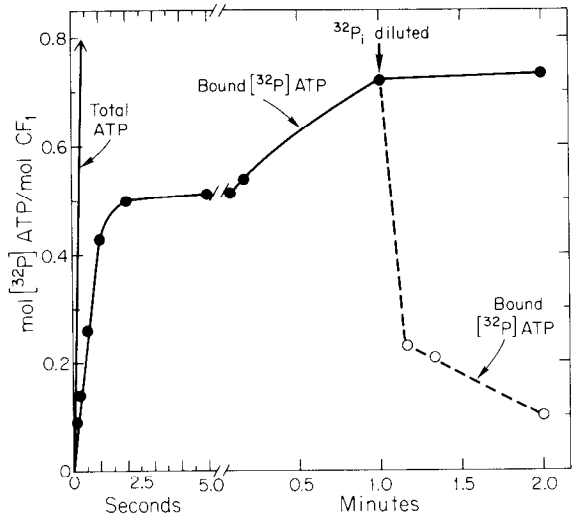

FIG. 1. **Time course of labeling of ATP tightly bound to quenched chloroplast thylakoids.** A 0.75-ml reaction mixture of 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 33 μM pyocyanine, and 1 mM ADP containing in addition 10 mM [^{32}P]P_i, 10 mM glucose, 80 units of hexokinase, and 0.1 mg of chloroplasts was illuminated using an electronically timed device for the indicated times. Samples then were mixed with an equal volume of 150 mM NH_4Cl, 50 mM Tris, pH 8.0, within 1 s after a shutter terminated the light. The chase was performed with 30 mM unlabeled P_i-containing reaction mixture (without additions) with continued illumination for the indicated times, then was followed by the addition of an equal volume of NH_4Cl/Tris. Net synthesis was measured by quenching with an equal volume of 1 M perchloric acid. Quenched samples were treated as described under "Experimental Procedures" to determine bound [^{32}P]ATP. The graphs are plotted as if the cold P_i chase provided an infinite dilution of [^{32}P]P_i. This allows better visualization of the amount of bound [^{32}P]ATP that is released.

there are two classes of bound [^{32}P]ATP based on the rate of thylakoid labeling: a more rapidly labeled class that reaches near maximal labeling within 2 s and a class which reaches near maximal labeling within 1 min of ATP synthesis.

In this experiment, ATP synthesis was initiated by turning on the light. Under similar conditions a lag in reaching maximal rates of ATP formation is observed (21). Thus, the bound [^{32}P]ATP that reaches a maximum level after about 2 s could represent labeling of ATP at catalytic sites.

Also shown in Fig. 1 is the decrease in bound [^{32}P]ATP that results when the specific activity of the [^{32}P]P_i is rapidly diluted after 1 min of labeling. The disappearance, or washout, of [^{32}P]ATP is biphasic as is the labeling curve. The slowly labeled and, therefore, noncatalytic bound ATP is washed out at about the same rate as it was formed. A catalytic class of bound ATP should wash out at a rate of about ½ the bound [^{32}P]ATP per 0.69 turnover time for the synthase. In this experiment turnover time was about 4 ms. Results of Fig. 1 are consistent with participation of the more rapidly labeled nucleotide as a catalytic intermediate. For a more critical assessment of the kinetic competency of this class rapid mixing techniques were used.

Rapid Mixing Experiments—The preceding experiments have the difficulty that the initial labeling is not under conditions of steady state ATP formation. Two mixing procedures have been used to measure the labeling of bound intermediates on thylakoids under steady state conditions. The first procedure involves illumination of the chloroplasts in the absence of substrate. Labeled substrate is rapidly mixed with the illuminated thylakoids so that ATP synthesis very quickly reaches it maximal steady state rate. In the second procedure, chloroplasts are illuminated in the presence of unlabeled

[2] The abbreviation used is: Tricine, *N*-[Tris(hydroxymethyl)-methyl]glycine.

[3] Use of an acid quench has been shown to satisfactorily stop catalytic events and completely extract ATP from chloroplast thylakoids (19).

[4] Comparison of the amount of total nucleotides (1, 2) and catalytic nucleotides (this paper) on chloroplast thylakoids with the amount of total nucleotide and catalytic binding sites on isolated CF_1 (12) support the conversion factor of Strotmann *et al.* (24) upon which our stoichiometry of bound intermediates/CF_1 ATP synthase are based.

substrate to attain steady state synthesis rates and then rapidly mixed with the radioactive tracer to follow the labeling of bound species. This latter procedure was used for the experiment shown in Fig. 2.

Fig. 2 indicates the labeling pattern of rapidly labeled, bound [^{32}P]ATP when steady state synthesis of [^{32}P]ATP from ADP and [^{32}P]P_i was terminated by quenching with the uncoupler NH_4Cl. The quenched thylakoids then were filtered, washed, and acid-extracted in darkness during a period of about 30 s. The turnover time for the coupling factor in this experiment was about 6 ms. The labeling of ATP bound to the filtered thylakoids reached a maximum of about 0.2 mol of [^{32}P]ATP/CF_1 shortly after the turnover time, consistent with participation of this ATP as an intermediate. Also shown in Fig. 2 is the loss of ^{32}P from bound ATP following rapid dilution with unlabeled P_i. The loss of label is rapid, corresponding to that expected for a catalytic intermediate.

The difference between the stoichiometry of about 0.5 mol of rapidly bound [^{32}P]ATP/mol of synthase, which is found in the hand mixing experiment shown in Fig. 1, and the amount of 0.2 mol/mol of synthase, found on the thylakoids after rapid mixing and quenching (Fig. 2), illustrates the inadequacies of these quench-filtration procedures for assessment of steady state levels of intermediates. It appears that different amounts of interconversion or exchange of bound substrates occurs after net phosphorylation has stopped.

These studies with light-energized chloroplasts confirm previous results, using acid-base transition for energization of thylakoids and EDTA as a quench (10), which showed kinetically competent, bound ATP on quenched thylakoids. We have also used an EDTA quench with light-driven phosphorylation (data not shown) and observed that [^{32}P]ATP binds in a pattern similar to that shown in Fig. 2, obtained with an NH_4Cl quench. The apparent stoichiometry of about 0.2 mol of catalytic ATP labeled/mol of synthase was also observed. The merits of different quenching procedures are considered under "Discussion."

Source of Slowly Labeled, Noncatalytic, Tightly Bound [^{32}P]ATP—If the slowly labeled and tightly bound [^{32}P]ATP is not a catalytic intermediate, then its most likely origin is from [^{32}P]ATP formed at the catalytic site and released to the medium before binding to a noncatalytic site. This would mean that the rate of labeling should be dependent upon the medium [^{32}P]ATP concentration which, at steady state, reflects the rate of ATP synthesis and the efficacy of hexokinase for regenerating ADP from ATP. Fig. 3 shows an experiment similar to that of Fig. 1 but in which the rate of ATP synthesis was decreased by lowering medium ADP concentration, while leaving the concentration of hexokinase at about the same level. These conditions would be expected to decrease the level of medium [^{32}P]ATP to about 1/15 of the level it was in Fig. 1.

A comparison of the labeling patterns of bound [^{32}P]ATP in Figs. 1 and 3 reveals that the rate of labeling of the catalytic site is unaffected by the change in conditions. In experiments reported in Fig. 1, the noncatalytic sites are maximally labeled by 1 min, whereas in Fig. 3, where medium [^{32}P]ATP is at a lower concentration during net synthesis, very little labeling of the noncatalytic site has occurred by 1 min. This result suggests that the labeling of the noncatalytic, tightly bound [^{32}P]ATP during steady state ATP formation from [^{32}P]P_i occurs via medium [^{32}P]ATP.

Hexokinase Accessibility Experiments—The experiments presented so far have all dealt with [^{32}P]ATP bound to quenched thylakoids. Uncertainty existed as to whether the observed amount of bound ATP corresponded to that present during steady state, prior to quenching. Therefore, a new method was developed to study the amount and properties of [^{32}P]ATP bound to thylakoids at steady state. The method is based on the premise that under appropriate conditions all [^{32}P]ATP not accessible to medium hexokinase is bound to the catalytic site of the coupling factor. It has been shown that the coupling factor contains the only sites for ATP binding on the thylakoid membranes (2, 5, 7–9). Also, our results mentioned above show that only a small portion of noncatalytic sites are labeled during short incubation times when medium [^{32}P]ATP concentration is kept low. Thus, if during steady state ATP formation from [^{32}P]P_i the concentration of hexokinase could be increased to infinity, an acid quench of the solution would show the amount of [^{32}P]ATP at the catalytic site. The hexokinase concentration obviously cannot be made infinite but the relationship of total [^{32}P]-ATP/CF_1 *versus* 1/(hexokinase) can be plotted and the hexokinase concentration can be extrapolated to infinity. The intercept on the ordinate will yield bound [^{32}P]ATP/CF_1 (see "Appendix" for the mathematical justification).

Fig. 4 shows the results of an experiment based on the hexokinase accessibility approach. The rate of ATP synthesis

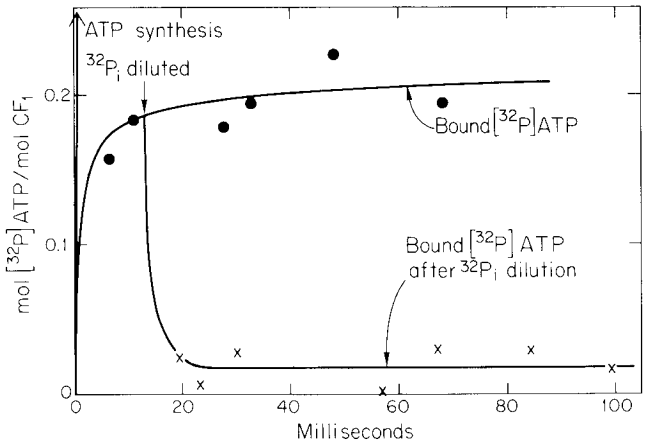

FIG. 2. **Demonstration of kinetic competence of labeled ATP bound to de-energized thylakoids.** A 1.0-ml reaction mixture of 1 mM ADP, 10 mM P_i, 10 mM $MgCl_2$, 50 mM NaCl, 50 mM Tricine/NaOH (pH 8.0), 33 μM pyocyanine, and 25 mM glucose containing, in addition, 80 units of hexokinase and 0.1 mg of chloroplasts was illuminated for 5 s. This reaction mix then was mixed rapidly with 1.0 ml of reaction mix (without additions), but containing radiolabel in the 10 mM P_i, for the indicated times and then quenched with an equal volume of 150 mM NH_4Cl, 50 mM Tris, pH 8.0, or chased with an equal volume of unlabeled 50 mM P_i-containing reaction mix (without additions) and then quenched. Net synthesis was measured and quenched samples were treated as in Fig. 1.

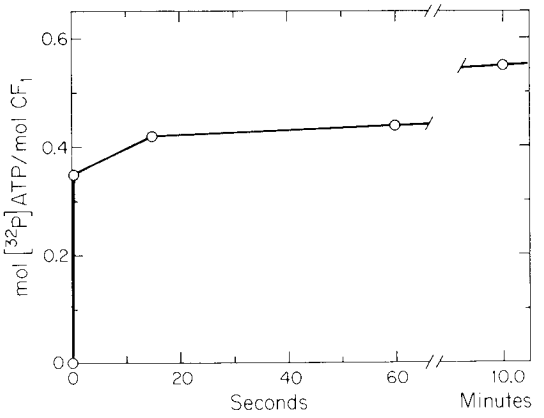

FIG. 3. **Time course of labeling ATP tightly bound to quenched chloroplast thylakoids at limiting concentration of ADP.** Experiment as Fig. 1 but using 2 μM ADP instead of 1 mM ADP, using 40 units of hexokinase/sample and without chase.

was reduced by lowering the concentration of P_i. The hexokinase concentration was varied by a factor of 20. After the chloroplasts had synthesized [^{32}P]ATP for 15 s, the reaction was acid-quenched and the [^{32}P]ATP present in the quenched reaction mixture was determined. The figure shows a plot of total [^{32}P]ATP/CF_1 *versus* 1/(hexokinase) in the reaction mix. The [^{32}P]ATP/CF_1 intercept at infinite hexokinase concentration is about 1.0. This is the amount of ATP presumably bound to the catalytic site during ATP synthesis in which the synthase is turning over about five times/s. The amount of free [^{32}P]ATP/CF_1 is the amount of total [^{32}P]ATP above 1.0. This amount varies by a factor of about 20, as does the hexokinase concentration.

Another hexokinase accessibility experiment was performed by allowing chloroplasts to synthesize [^{32}P]ATP from 100 μM ADP and with limiting concentrations of 400 to 200 μM [^{32}P]P_i. The concentration of hexokinase was varied with a maximum amount similar to that in the previous experiment. These reaction conditions give much higher free [^{32}P]ATP than in the previous experiment. After 15 s of ATP synthesis the reactions were quenched with perchloric acid and total [^{32}P]ATP/CF_1 was determined. A plot of the results is given in Fig. 5. The extrapolations to infinite hexokinase obviously have greater uncertainty than that of Fig. 4. The intercept at the total [^{32}P]ATP/CF_1 axis indicates that, at these higher P_i concentrations, the amount of bound [^{32}P]ATP may be greater than 1.0/CF_1. This could reflect the increase in the amount of free [^{32}P]ATP over that of the previous experiment resulting in some labeling of the noncatalytic sites during the 15 s of synthesis.

Kinetic Competence of [^{32}P]ATP Transitorily Tightly Bound to the Synthase during Steady State ATP Synthesis—The rapid mixing experiment shown in Fig. 3 demonstrates that a fraction of the [^{32}P]ATP remaining bound to de-energized thylakoids after ATP synthesis from ADP and [^{32}P]P_i is kinetically competent as an intermediate in synthesis. Table I gives the results of a similar rapid mixing experiment performed to determine if the approximately 1.0 mol of [^{32}P]-ATP/synthase, shown by the hexokinase accessibility method to be bound to the synthase during steady state, meets the kinetic requirements of an intermediate. The results show a ^{32}P-labeling pattern as expected for an intermediate. The bound ATP is synthesized at a rate at least equal to the net rate of free ATP synthesis and the amount of the bound [^{32}P]ATP remains constant with time.

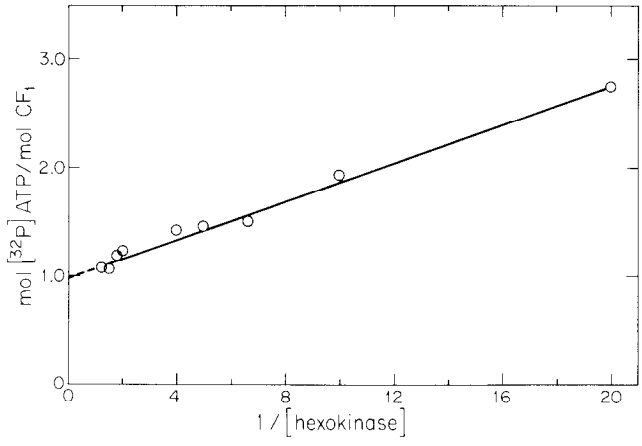

FIG. 4. **Determination of transitorily tightly bound [^{32}P]-ATP/synthase during steady state ATP synthesis using the hexokinase accessibility approach.** A 1.0-ml reaction mixture contained 10 mM $MgCl_2$, 50 mM Tricine/NaOH (pH 8.0), 25 mM NaCl, 25 mM glucose, 33 μM pyocyanine, hexokinase as indicated, and, in addition 100 μM ADP, 20 μM [^{32}P]P_i and 40 μg of chloroplasts. The maximum hexokinase concentration was 400 units. The reactions were quenched with 1.0 ml of 1 M perchloric acid after 15 s of illumination. Quenched samples were treated as in Fig. 1.

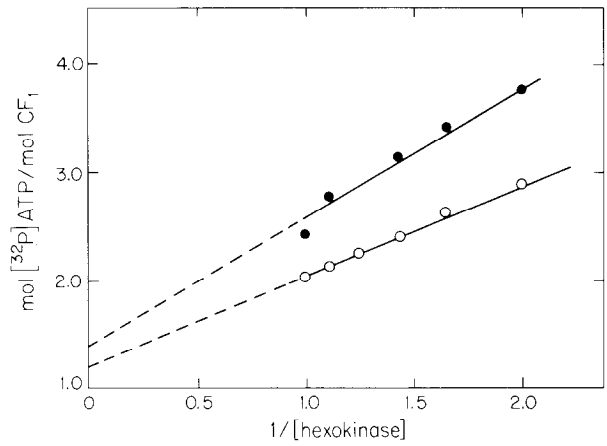

FIG. 5. **Studies of the noncatalytic, tightly bound ATP during steady state ATP synthesis using the hexokinase accessibility approach.** The procedures and reaction mix were as for Fig. 4 and contained, in addition: ○——○, 100 μM ADP, 200 μM [^{32}P]P_i, and 30 μg of chloroplasts; ●——●, 100 μM ADP, 400 μM [^{32}P]P_i, and 15 μg of chloroplasts.

TABLE I

Kinetic competence of transitorily tightly bound ATP under conditions of the hexokinase accessibility approach

A 1.0-ml reaction mix containing 10 mM $MgCl_2$, 25 mM NaCl, 33 μM pyocyanine, 50 mM Tricine/NaOH (pH 8.0), 25 mM glucose, 40 μg of chloroplasts, and hexokinase as indicated was illuminated and then mixed rapidly with 1.0 ml of reaction mixture, without chloroplasts and hexokinase but containing 10 mM [^{32}P]P_i and 8 μM ADP, for the indicated times. Reactions were quenched with 2 ml of 1 M perchloric acid. Quenched samples were treated as in Fig. 4. Some apparent scatter noted in results at longer reaction times may reflect limitation in mixing with the equipment used.

Quench time	Hexokinase units	[^{32}P]ATP/CF_1
ms		
288	0	2.4
	200	0.67
	240	1.07
	250	0.91
	320	1.36
	360	0.70
	400	0.76
160	0	1.3
	100	1.21
	120	1.04
	140	0.98
	160	0.89
	180	0.95
80	0	0.67
	100	0.55
	120	0.63
	160	0.54
	180	0.58
	200	0.51

Presence of Bound P_i Committed to ATP Synthesis—Previous work from this laboratory (10) has shown that chloroplast thylakoids energized by acid-base transition have approximately 0.7 mol of P_i and of ADP/mol of synthase committed to ATP synthesis. This gives a minimum value for the amount of participating catalytic sites. This amount and the amount of catalytic, bound ATP found in the hexokinase accessibility experiments implied that there is likely more than one participating catalytic site on each synthase during light-driven ATP formation. To test this possibility further, measurements of committed P_i during steady state photo-

phosphorylation were made. The concentrations of substrates, principally P_i, limited phosphorylation to about ⅕ of the maximal potential rate. This allowed correlation with the hexokinase accessibility experiments performed under similar conditions.

The data in Fig. 6 show the presence of about 0.3 mol of bound [^{32}P]P_i/mol synthase that is committed to ATP synthesis. The sum of this committed P_i and the kinetically competent, bound ATP (Figs. 4 and 5; Table I) is greater than 1.0 even though under the reaction conditions used the rate of ATP synthesis is limited by substrate concentration. These results require that there be at least two participating catalytic sites/chloroplast ATP synthase.

Fig. 7 shows a comparison of total ATP formation using an EDTA quench as compared to a perchloric acid-quench. The observed ATP formed after a given time period is somewhat higher with an EDTA quench. Results using an NH_4Cl quench (not shown) are similar to those with an EDTA quench. Also, earlier data with the acid-base transition (10) showed a similar pattern. An important feature of Fig. 7 is that with the EDTA quench, within experimental error, no committed P_i was detected. This is in contrast to the committed P_i detected with a perchloric acid quench (Fig. 6). A reasonable explanation is that EDTA may form a complex with free Mg^{2+}, but Mg^{2+} bound with P_i and ADP at the catalytic site may suffice to allow limited ATP formation to continue. This, and perhaps some time delay in the effectiveness of the EDTA quench in stopping net ATP formation, would account for the slightly higher level of total ATP observed with the EDTA as compared to the perchloric acid quench. Thus, the bound [^{32}P]ATP found on EDTA-quenched and washed membranes that appears to be kinetically competent as an intermediate may arise from two sources, [^{32}P]ATP present at a catalytic site at the time of EDTA addition and additional [^{32}P]ATP formed from bound [^{32}P]P_i at the catalytic site. It was these uncertainties about the EDTA- or NH_4Cl-quench method that served as stimuli for development of the hexokinase accessibility method reported earlier.

DISCUSSION

There has been accumulating evidence that energy transducing ATP synthases from various sources have bound ADP and ATP that are labeled slowly during ATP formation or cleavage (see review by Harris (1)). Although such results have been interpreted as indicating a noncatalytic role for such nucleotides, various models for oxidative phosphorylation and photophosphorylation have included these nucleotides as catalytic intermediates (2, 13–15). This has been deemed justified on the basis that, under the reaction conditions used, only a portion of the phosphorylation complexes may be active at a given time and, thus, labeling through catalytic participation would appear to be slow.[5] The approaches and results presented here allow the conclusion that under the conditions we used, most of the ATP synthase complexes in the thylakoid membranes are catalytically active at any given time during steady state, light-driven ATP synthesis. Yet, under these conditions our results show that a class of slowly labeled, bound nucleotides exists. Thus, these bound nucleotides do not participate as catalytic intermediates.

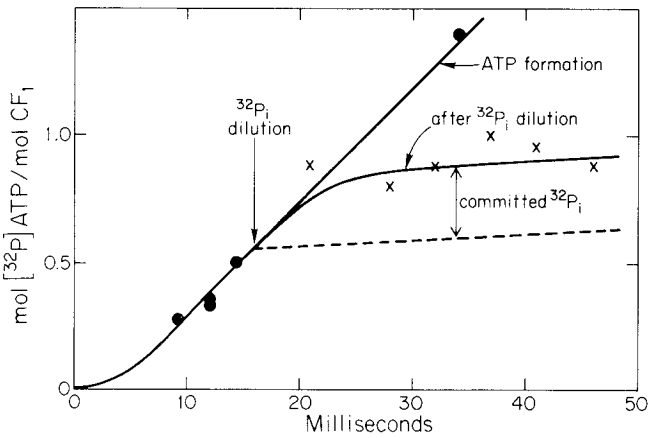

FIG. 6. **Measurement of committed P_i using an acid quench.** A 1.0-ml reaction mixture of 50 mM Tricine/NaOH (pH 8.0), 50 mM NaCl, 5 mM $MgCl_2$, 33 μM pyocyanine, and containing, in addition, 0.1 mg of chloroplasts was preilluminated for 10 s and then mixed rapidly with a 1.0-ml reaction mixture without additions but containing 200 μM ADP and 400 μM [^{32}P]P_i. The reaction was quenched with an equal volume of 1 M perchloric acid or chased with an equal volume of reaction mix, without addition, but containing 100 μM ADP and 4 mM unlabeled P_i and then quenched with perchloric acid.

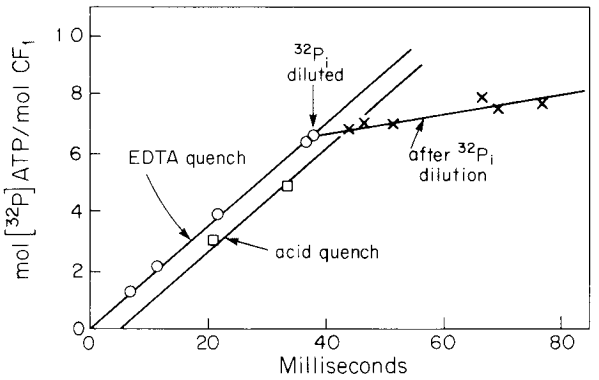

FIG. 7. **Apparent inadequacy of an EDTA quench for measurement of committed P_i.** Reaction conditions as in Fig. 2 except glucose and hexokinase were omitted. An equal volume of 200 mM EDTA, pH 8.0, or 1 M perchloric acid was used for the quench. The chase contained 50 mM unlabeled P_i. EDTA-quenched samples were treated with perchloric acid (0.5 M final) and these samples with the perchloric acid-quenched samples were treated to isolate [^{32}P]ATP as in Fig. 1.

The isolated chloroplast ATPase and related ATPases from other sources are composed of two major subunits α and β, and three minor subunits γ, δ, and ε (28). Two α-β pairs are present in each functional ATPase molecule (28). Chemical modification (12, 29–31) and bound nucleotide labeling results (32) suggest that the catalytic sites are on the β subunits. We interpret our present results on the supposition that this is the case and that the noncatalytic nucleotide binding sites are likely on the α subunit. Other subunits are regarded as participating in binding of the synthase to the hydrophobic membrane components and in transmitting conformational changes in the proton channel to the catalytic site (33).

The chloroplast ATP synthase on washed thylakoids has been observed to contain about 2 mol of ATP and 1 mol of ADP/mol (5). As noted in the present experiments and by Smith and Boyer (10) some ATP bound to catalytic sites is present in freshly labeled and filtered membranes. With storage, bound ATP has been observed to form bound ADP (8) and such bound ADP is released within a few milliseconds upon energization by acid-base transition (10). It, thus, seems likely that the bound ADP on the isolated ATPase or on well

[5] Lack of participation of all potentially functioning ATP synthase complexes has been suggested to occur with low light intensity (26, 27). Also, it may be relevant that, in a number of the reported studies with chloroplasts, the maximal photophosphorylation rate was considerably less than that observed with conditions as used for the present paper. This could place restrictions on interpretations.

washed membranes is largely on a β subunit catalytic site, with some catalytic sites empty.

The other nucleotide is noncatalytic bound ATP and, thus, would be expected to be on the α subunits. In the present experiments only a portion of the noncatalytic nucleotide binding sites were labeled with ATP. Various factors, such as medium ATP, ADP, and P_i concentrations, time of incubation, light intensity, pH, temperature, etc., may modify labeling patterns of these noncatalytic sites.

More relevant to our present interest is how during catalysis nucleotides and P_i are bound to the catalytic site, interconverted, and released, that is, how they are involved in the mechanism of catalysis. The results in this paper address mainly one aspect of this mechanism, namely the nature and amount of substrates that are transitorily bound to catalytic sites during steady state photophosphorylation. Measurement of the amount and nature of intermediate(s) is important because the alternating site model developed in this laboratory requires that more than one catalytic site/synthase be active at any one time and that not more than one site/synthase be at a particular stage in the catalysis. In addition, appreciable bound intermediates should remain even at low substrate concentrations. But before discussion of these aspects, evidence for the energy-linked binding change mechanism[6] will be briefly summarized to aid consideration of present data. A series of experiments over the past several years, largely from this laboratory, have led to this binding change mechanism for ATP synthesis by mitochondria and chloroplasts. In this mechanism, energy-linked conformational changes are driven by proton translocation across the membrane. The changes concomitantly promote ATP release at one site of the synthase and the binding of P_i and ADP in a mode competent for ATP synthesis at an alternate site. This model is depicted schematically in Fig. 8.

Evidence that energy input is not used for covalent bond formation but promotes the release of bound ATP and the binding of P_i and ADP so as to favor bound ATP formation comes from the following observations.

During ATP Hydrolysis

1. Incorporation of more than one water oxygen into each P_i formed by submitochondrial particles (intermediate $P_i \rightleftharpoons$ HOH exchange) is not abolished by uncouplers, as if reversal of bound ATP hydrolysis occurs without an energy source (36–38).

2. With submitochondrial particles uncouplers block the rapid exchange of oxygen of medium P_i with water. Bound P_i formed from ATP still undergoes exchange and release; thus, de-energization by uncoupler blocks P_i binding (36).

3. With submitochondrial particles uncouplers stop medium ATP $\rightleftharpoons$ HOH exchange even though the continuing intermediate $P_i \rightleftharpoons$ HOH exchange would label bound ATP with water oxygens (17). Thus, the binding of ATP still occurs, but release is blocked.

4. Intermediate $P_i \rightleftharpoons$ HOH exchange can be observed with various mitochondrial ATPase preparations of decreasing complexity (38) and, at low ATP concentration, even with the purified mitochondrial or chloroplast ATPase (38, 39). Reversible formation of ATP at the catalytic site, thus, does not require a transmembrane proton gradient or potential.

During ATP Synthesis

5. Uncouplers increase the apparent K_m for both ADP and P_i in oxidative phosphorylation by submitochondrial particles (40), as if energy is required for competent binding.

6. Chloroplast thylakoids can have a transitorily tightly bound ATP which is released to the medium when ATP synthesis results from acid-base transition (10).

[6] This may be conveniently referred to as the binding change mechanism for ATP synthesis; see Boyer, P. D., in *Bioenergetics of Membranes* (Lee, G. P., ed), Addison-Wesley, Reading, Mass., in press.

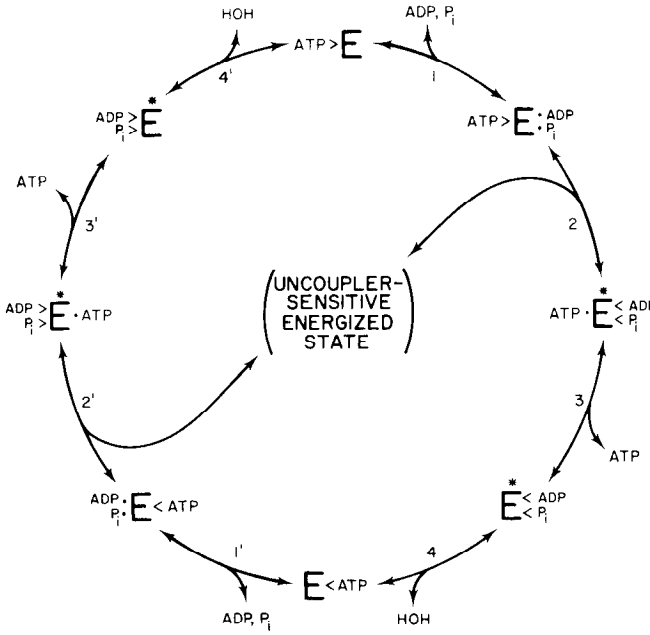

FIG. 8. **The energy-linked binding change mechanism for ATP synthesis.** For ATP formation as by chloroplasts or mitochondria reaction proceeds in a clockwise direction. In this model, taken from Hackney and Boyer (18), a bracket indicates slowly released ATP, or ADP + P_i capable of forming bound ATP without further energy input, and a center dot indicates relatively rapid release of substrate. The uncoupler-sensitive energized state includes Mitchell's protonmotive force (34, 35) and any conformational intermediates in the protein complex providing linkage between proton translocation and the catalytic site (33). The model depicts only minimal steps; for example, independent binding steps for P_i and ADP are not included nor are possible alternate routes or additional likely intermediate steps between depicted complexes.

Without ATP Formation or Hydrolysis

7. Submitochondrial particles do not catalyze $P_i \rightleftharpoons$ HOH exchange in the presence of ADP unless energy is provided by oxidation or ATP hydrolysis (36), as if energy is required for binding of P_i and ADP in a mode competent for ATP synthesis.

Evidence best explained by participation of alternating sites is as follows.

During ATP Hydrolysis

8. With submitochondrial particles removal of medium ADP by pyruvate kinase blocks medium $P_i \rightleftharpoons$ ATP and ATP $\rightleftharpoons$ HOH exchanges (17), as if ADP binding at an alternate site is necessary for ATP release.

9. Decrease of ATP concentration during ATP hydrolysis by submitochondrial particles increases the extent of water oxygen incorporation into each P_i formed (37, 38), as if ADP and P_i cannot be released at one site until ATP binds at another.

10. Results as given in Point 9 (above) have been obtained with purified mitochondrial ATPase (16, 38, 39), with measurements of ^{18}O-P_i species formed that eliminate explanations other than the alternating sites (39).

During ATP Synthesis

11. With submitochondrial particles removal of medium ATP blocks the medium $P_i \rightleftharpoons HOH$ exchange although intermediate ATP $\rightleftharpoons$ HOH exchange continues (18), as if ATP binding at an alternate site is necessary for bound P_i release.

12. Decrease of either ADP or P_i concentration during synthesis of ATP by submitochondrial particles greatly increases the extent of water oxygen incorporation into each ATP formed (intermediate ATP $\rightleftharpoons$ HOH exchange) (18), as if binding of P_i and ADP at one site is necessary for release of ATP formed at an alternate site.

13. Results similar to the above are given by chloroplast thylakoids. Measurement of ^{18}O-ATP species formed is consistent with alternating sites but eliminates enzyme hysteresis and other related explanations (41).

14. Chloroplast thylakoids maintain relatively high concentrations of bound ATP at catalytic sites even with low medium ADP and P_i concentrations (this paper), as if bound ATP is not released until ADP and P_i bind at an alternate site.

15. Submitochondrial particles with low medium ADP concentrations show appreciable steady state concentrations of transitorily bound ADP and ATP (22), as expected for the alternating but not a single site mechanism.

16. Binding of the antibiotic, efrapeptin, at the catalytic site competes with ADP and P_i during synthesis but not with ATP during hydrolysis (42). This behavior is consistent with alternating sites but not single site participation.

Other evidence consistent with or favoring the alternating site mechanism is as follows:

17. The mechanism explains how one energy input can accomplish the two demonstrated changes, promotion of ATP release and of competent ADP + P_i binding (17, 18).

18. More than one catalytic site/ATP synthase is active at one time in chloroplast thylakoids during net ATP synthesis (this paper).

19. Inactivation of catalysis by modification of 1 residue/ATPase (29, 30, 43, 44) is readily explainable by the mechanism.

20. With a bacterial ATPase, release of labeled, bound nucleotide is promoted by addition of medium nucleotide, as if interchange between tight and loose sites can occur (45). However, whether these nucleotides are at catalytic sites for oxidative phosphorylation is uncertain.

21. When ATP binds to chloroplast thylakoids in the light without added ADP, nearly complete exchange of γ-PO_3 oxygens with water results, as if binding of ADP and P_i at an alternate site promotes ATP release (46).

Other important aspects of data in this paper are readily interpretable by the model depicted in Fig. 8. This minimal model includes only four forms of enzyme with bound intermediates; for example, the forms ATP > E and E < ATP at the top and bottom of the scheme being equivalent in reactivity but with ATP on alternate sites. In photophosphorylation there is considerable incorporation of water oxygen into ATP molecules even under optimal conditions (41, 46, 47). This is explainable if some rate limitation is imposed after Step 4, allowing reversal of Step 4 and water incorporation into bound P_i which reforms bound ATP. Because this occurs with excess P_i and ADP present, an additional rate-limiting step would need to be imposed between Steps 1 and 4, or alternatively, the interconversion

$$\underset{P_i}{\overset{ADP}{\cdot}}E < ATP \xleftrightarrow[HOH]{} \underset{P_i}{\overset{ADP}{\cdot}}E \underset{< P_i}{< ADP}$$

(not depicted in the scheme) could occur with rate limitation imposed at Step 3.

The lack of any appreciable $P_i \rightleftharpoons HOH$ exchange with chloroplasts, even in the presence of medium ATP (46), suggests that Step 2 is essentially irreversible. This is in contrast to the behavior of mitochondria, as outlined in the previous paper (22). The result with chloroplasts is in harmony with the presence of bound P_i and ADP committed to go on to form free ATP, as demonstrated earlier for acid-base driven synthesis (10) and in this paper. In the presence of hexokinase, the principal forms for committed P_i and ADP would likely be

$$\overset{*}{E}\underset{<P_i}{<ADP} \text{ and } ATP\cdot\overset{*}{E}\underset{<P_i}{<ADP}.$$

Three of the four forms of enzyme depicted in the scheme of Fig. 8 have either a tightly or loosely bound ATP present. This is quite in accord with experimental results indicating that most synthases have bound ATP present during steady state ATP synthesis. Also, it is possible that a form such as $ATP\cdot\overset{*}{E} < ATP$ participates, resulting in up to or even more than 1 mol of bound ATP/enzyme.

The hexokinase-accessibility and committed-P_i approaches used in this paper are regarded as measuring steady state concentrations of some of these intermediates. Although the quench-filtration approaches do not give adequate measures of steady state concentrations, they do reveal minimum concentrations of bound intermediates, possibly with some interconversion occurring after quench addition. It is of interest that addition of EDTA, by removal of free Mg^{2+}, or of NH_4Cl, by dissipation of protonmotive force, or of stopping illumination give similar levels of bound intermediates. The presence of bound intermediates after de-energization can be accounted for by the alternating site model, with the quench procedures effectively stopping Step 2 (Fig. 8).

It is important to note that the binding change reaction scheme, as given, predicts that at relatively low ADP or P_i concentrations, or both, and with fully energized chloroplasts and a slowly reversible Step 2, the forms produced by Steps 2, 3, and 4 would still remain. This leads to the prediction that, even with ADP and P_i concentrations well below their K_m values, considerable bound, catalytic ATP should remain. This is as observed and noted earlier under "Discussion." Such a result is not readily explainable by the operation of independent catalytic sites.

In conclusion, on the basis of the findings in the present paper and the previous paper on submitochondrial particles (22), together with other information outlined above, we regard the broad features of the energy-linked binding change mechanism as being established. Many important aspects and details obviously remain for future investigation.

APPENDIX

Justification for Use of ATP/CF_1 Versus 1/(Hexokinase) Plots to Measure Bound [^{32}P]ATP

For chloroplasts:

$$\frac{d(ATP)}{dt} = \frac{k^c_{cat}E_t^c(ADP)}{(ADP) + K_m^c}$$

for hexokinase

$$\text{Rate of ATP synthesis} = \frac{k^h_{cat}E_t^h(ATP)}{(ATP) + K_m^h}$$

At high ADP concentrations and high hexokinase concentrations (ADP) will be essentially constant; therefore:

$$\frac{d(ATP)}{dt} = k^c_{cat}E_t^cC = k'^cE_t^c$$

at steady state,

$$\frac{d(\mathrm{ATP})}{dt} = \frac{d(\mathrm{ADP})}{dt}$$

and

$$k'^{c}E_t^{c} = \frac{k_{cat}^{h}E_t^{h}(\mathrm{ATP})}{(\mathrm{ATP}) + K_m^{h}}$$

and

$$(\mathrm{ATP})_{free} = \frac{k'^{c}E_t^{c}K_m^{h}}{k_{cat}^{h}E_t^{h} - k'^{c}E_t^{c}}.$$

Then

$$(\mathrm{ATP})_{total} = (\mathrm{ATP})_{bound} + (\mathrm{ATP})_{free} = \frac{k'^{c}E_t^{c}K_m^{h}}{k_{cat}^{h}E_t^{h} - k'^{c}E_t^{c}} + KE_t^{c}$$

where K is the moles of bound ATP/mol of ATP synthase and $(CF_1) = E_t^{c}$:

$$\frac{(\mathrm{ATP})_{total}}{CF_1} = \frac{(\mathrm{ATP})_{bound}}{CF_1} + \frac{(\mathrm{ATP})_{free}}{CF_1} = \frac{k'^{c}K_m^{h}}{k_{cat}^{h}E_t^{h} - k'^{c}\,CF_1} + K$$

Under conditions used in this experiment, $k_{cat}^{h}E_t^{h} \gg k'_{c}\,CF_1$ and a plot of $(ATP)_{total}/CF_1$ *versus* $1/E_t^{h}$ (or 1/hexokinase) will intercept the ordinate at K when the concentration of hexokinase $\rightarrow \infty$ since the $k'^{c}K_m^{h}/[k_{cat}^{h}E_t^{h} - k'^{c}\,CF_1]$ term drops out.

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