Mechanism for Oxygen Exchange in the Chloroplast Photophosphorylation System*

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The oxygen exchange that occurs between water and the γ-PO₃ of ATP in light-activated chloroplast lamellae was found to proceed with close to full equilibration of the oxygens before ATP returned to the medium. This is in contrast to the entry of approximately one water oxygen when ATP is synthesized from ADP and Pᵢ in the same system. In the latter case, the limitation is kinetic, however, not steric, as shown by the presence of some molecules containing more than one water-derived oxygen in the γ-PO₃. The different extents of exchange can be explained by a relatively faster rate of dissociation of ATP from the chloroplast coupling factor during synthesis from ADP and Pᵢ, relative to its dissociation in the absence of net phosphorylation. To determine the mechanism of γ-PO₃:H₂O exchange at full equilibration, its rate was compared with the rate of reversible ATP hydrolysis on the chloroplast coupling factor, i.e., [ATP·H₂O = ADP·Pᵢ].

Two mechanisms have been put forth to explain the incorporation of water oxygens into the γ-PO₃ of ATP synthesized during oxidative phosphorylation in mitochondria or photophosphorylation in plant chloroplasts. Either mechanism can account for the ATP:H₂O oxygen exchange that occurs in the absence of net phosphorylation. The scheme introduced by Shavit et al. (1) involves the dynamic reversal of ATP formation at the catalytic site of the coupling factor as depicted in Scheme 1. It is postulated that in H₁₈O, bound ATP is cleaved to bound ADP and bound [¹⁸O]Pᵢ; if the oxygens of Pᵢ become equivalent on the enzyme, [¹⁸O] will become incorporated into the γ-PO₄ of ATP upon its resynthesis on the enzyme. Net ATP hydrolysis to ADP and Pᵢ and ATP:ADP exchange do not seem to occur in the intact chloroplast system.

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Experimental Procedures

Materials—Disodium ATP and disodium ADP were purchased from Sigma Chemical Co., and [γ-32P]ADP from New England Nuclear, and [18O]-enriched water from Miles-Yeda. All other materials were obtained or prepared as previously described (6).

Methods—Spinach chloroplasts were isolated from fresh spinach leaves by the same double centrifugation, and finally suspended in a few milliliters of homogenizing medium. These washed whole chloroplasts were used the same day.

Osmotically shocked chloroplasts (type D, lamellae (13)) were prepared by suspending 2.5 ml of whole chloroplasts in 25 ml of 1 mM Tris (pH 7.0, for 6 min at 0°C, then adding 7.5 ml of 50% sucrose) using a Waring Blender at a Variac setting of 20 for 20 s. The slurry was suspended in 8 layers of cheesecloth, then quickly centrifuged at 7000 g for 3 min; the supernatant was removed by centrifugation at 27,000 g for 5 min; and the pellet was washed once with homogenizing medium, recovered, and was used the same day.

In-line displacement exchange.

The βγ bridge oxygen would remain fixed in the bridge throughout the exchange.

The nonbridge scrambling.

A calculation can be made of the rate of Pβγ-Pγ bond cleavage and γ-PO₂:H₂O exchange in ATP exposed to chloroplast lamellae in light—

Comparison of Rates of Pβγ-Pγ Cleavage and γ-PO₂:H₂O Exchange in ATP Exposed to Chloroplast Lamellae in Light—

A calculation can be made of the rate of Pβγ-Pγ bond cleavage during the incubation of [γ-32P]ATP with chloroplast lamellae (6). The m/e 143 peak heights, corrected for the 32O ion.
natural abundance contribution by subtracting 0.81% of the respective peaks at m/e 141, are measured for two trimethyl phosphate samples derived as follows: one from the γ-PO₃ unit of ATP as recovered from the incubation (Scheme 4, Route B) and one from the γ-PO₃ of the same ATP that has been treated to give ATP:PP₄ exchange (Scheme 4, Route A) and thus consists of a 50:50 mixture of the β-PO₃ and γ-PO₃ units of the untreated ATP. Correcting the percentage of m/e 143 of the latter trimethyl phosphate for the contribution of the γ-PO₃, by subtracting one-half the former, one has a measure of the m/e 143, above natural abundance, which represents ¹⁸O enrichment in the P₀ nonbridge position. Values calculated in this way are given in Table I. The increases seen over the control can be attributed to exchange of the P₀ nonbridge oxygens with the βγ bridge oxygen, and this indicates that the involved ATP at one time must have existed in the form of ADP → O- · P₁ (or ADP-X-PO₃) on the enzyme. That this observed reversible ATP cleavage is intimately associated with the mechanism of ATP synthesis during photophosphorylation is shown by dependence of the cleavage reaction on light and its sensitivity to NH₄Cl, a known inhibitor of photophosphorylation (16). The portion of the overall βγ bridge to β nonbridge scrambling reaction which is not light-dependent or NH₄Cl-sensitive (12%) probably results from contaminating enzymes. The existence of such enzymes is indicated by a light-independent scrambling reaction in the whole chloroplast system which proceeds at least 40 times faster than the light-dependent scrambling. Most of this light-independent activity has been removed by preparing the washed lamellae used in these studies.

The rate of light-dependent reversible ATP cleavage proceeded at 13% of the net rate of ATP synthesis determined at the same time under conditions, in the synthesizing system, that differed only in replacement of ATP (0.4 mM) by ADP (2 mM) and Pi (4 mM). This rate of reversible cleavage is obtained from the equation micromoles of ATP = - (ATP) ln | 1 - F |, where F is the fraction of equilibrium attained in the βγ bridge to β nonbridge scrambling reaction as determined from the m/e 143 of the β-PO₃ (6). At zero time, 0.99% of the ATP contains ¹⁸O in the β nonbridge positions, whereas 78.2% contains ¹⁸O in the βγ bridge (0.77% will contain ¹⁸O in both the bridge and nonbridge positions). As the ATP is reversibly cleaved, ¹⁸O will become distributed between the β nonbridge and βγ bridge positions until, at equilibrium, 52.03% of the ATP will contain one ¹⁸O in the β nonbridge position. (A small portion (0.51%) of the ATP will have ¹⁸O in both β nonbridge positions; this will not affect monitoring the equilibrium by the m/e 143.)

To distinguish between the two proposed mechanisms for γ-PO₃:γ-H₂O exchange, a comparison was made between the rate of P₀,γ-P₂ bond cleavage as measured in Table I and the rate of ATP:γ-H₂O exchange detected by ¹⁸O washout from the γ-PO₃ of the [γ-¹⁸O]ATP. In Table II are presented the analyses of trimethyl phosphate derived from the γ-PO₃ of the original ATP samples described in Table I prior to βγ interchange by acetyl-CoA synthetase. Because the integrity of the γ-PO₃ group is maintained in the mass spectral analysis, it can be said that, of those ATP molecules that underwent a loss of ¹⁸O, this loss was virtually complete. That is, in the complete system plus light, the decreases in m/e 145 and 147 can be accounted for largely by the increase in m/e 141, which represents only trimethyl [¹⁸O]phosphate. Because of this extensive ATP:H₂O exchange, one may calculate the portion of the ATP pool that has undergone γ washout from the extent, F, to which the m/e 147 peak has approached its equilibrium value of zero; thus ATP_washout = -(ATP)total ln
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[1 - F]. Referring to Tables II and I, 2.25 µmol of ATP experienced γ-washout in an incubation in which 1.75 µmol had undergone Pᵢ-O-Pᵢ bond cleavage. Both of these processes are light-dependent and NH₄Cl-sensitive. The apparently lower rate of cleavage relative to washout has been reproduced in two independent experiments and the ratios agree to within 2%.

Comparison of γ-PO₃ Water Exchange Patterns in ATP Synthesized de novo and in Added ATP—Previous work by Avron et al. (17) and by Chaney and Boyer (17) demonstrated that during ATP synthesis in H₂¹⁸O, approximately one water oxygen is found in the ATP formed. This is in contrast to the finding in Table II, in which added ATP was shown to have undergone almost complete equilibration with H₂O in the

| Table I |

ATP γ₈ bridge = β nonbridge "O scrambling catalyzed by spinach chloroplast lamellae

Reactions were carried out as described under "Experimental Procedures." Each incubation included 4 µmol of [γ-¹⁸O]ATP (Tris salt) and lamellae (specific activity 133) containing 600 µg of chlorophyll in a total reaction volume of 10.0 ml. Where indicated, 6 mM NH₄Cl was present. Incubation time was 10 min during which 13.3 µmol of ATP were synthesized in a parallel photophosphorylation assay. In the control incubation, lamellae were added at the end after addition of acid. The work up (Scheme 4, Route A) and analyses of purified ATP samples were done as described under "Experimental Procedures."

<table>
<thead>
<tr>
<th>Incubation conditions</th>
<th>Calculated m/e 143 of β-PO₃P of ATP</th>
<th>Fraction of equilibrium value (F,%)</th>
<th>β-scrambled ATP</th>
<th>ATP-synthesizing capacity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control, light</td>
<td>0.99</td>
<td>0.00</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Complete, dark</td>
<td>4.01</td>
<td>0.059</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>Complete, light + NH₄Cl</td>
<td>3.77</td>
<td>0.055</td>
<td>0.22</td>
<td></td>
</tr>
<tr>
<td>Complete, light</td>
<td>20.88</td>
<td>0.390</td>
<td>1.98</td>
<td>0.132</td>
</tr>
</tbody>
</table>

* Increase in m/e 143 due to "O natural abundance (0.81% of m/e 141) and the contribution of the γ-PO₃ is shown in Table II.
* F = m/e 143 - m/e 143 = m/e 143 - 0.99
* Micromoles of β-scrambled ATP = (ATP) ln [1/F₁], where F₁ = fraction of equilibrium value.

When ATP is synthesized in H₂¹⁸O from ADP and Pₐ, a totally different pattern of δ⁻¹⁸O content is found (Table III, Experiment 2). In this case, in contrast to the experiment done in the absence of net phosphorylation, very little or no ATP production has had the opportunity to experience water-oxygen incorporation, and if the γ-PO₃H₂O exchange is very high, the mass spectrum of trimethyl phosphate from the γ-PO₃ unit would approach that signifying complete equilibration with 61.0% "O-enriched water. The results indicate that incorporation of water oxygens into the synthesized ATP has occurred only to a small extent. In fact, the average incorporation of oxygen from water per ATP molecule is calculated to be 0.7 atom, [1.3 x (δ⁻¹⁸O in γ-PO₃/δ⁻¹⁸O in H₂O)]; this value is in agreement with the previously reported results of 0.7 to 1.0 (7, 17).

If, as seems likely, the exchanges that are seen in added ATP and in ATP generated during net synthesis occur by the
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Reactions were carried out in the light in a total volume of 1.0 ml as described under "Experimental Procedures": each incubation also included lamellae (specific activity 141) containing 123 μg of chlorophyll, H₂¹⁸O with 64.5 atom % ¹⁸O enrichment as reported, and 1 μmol of ATP or 4 mM ADP plus 4 mM ³²P (specific activity 1.41 × 10⁶ cpm/μmol). Reaction times were 12 min for the incubation with ATP alone, and 8 min for the system involving net synthesis during which 1.86 μmol of ATP were synthesized. The analyses of the purified ATP samples were done as described under "Experimental Procedures." m/e values are reported as percentages of the total signal area.

### TABLE III

<table>
<thead>
<tr>
<th>Experiment and additions</th>
<th>H₂¹⁸O enrichment</th>
<th>ATP γ-PO₃ pattern</th>
<th>m/e</th>
<th>Probability calculation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. ATP</td>
<td>84.4</td>
<td>Calculated for reacted ATP*</td>
<td>141</td>
<td>0.00 9.72 27.82 62.46</td>
</tr>
<tr>
<td>2. ADP + ³²P₃</td>
<td>61.0</td>
<td>Observed for reacted ATP</td>
<td>141</td>
<td>6.38 16.16 33.34 60.12</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Predicted for complete equilibration*</td>
<td>143</td>
<td>67.84 23.17 7.13 1.76</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>145</td>
<td>5.93 27.83 43.54 22.70</td>
</tr>
</tbody>
</table>

* The contribution of unreacted ATP has been subtracted. (All of m/e 141 and 0.81% × m/e 141 have been subtracted from the observed m/e 141 and 143, respectively. This approximation is a valid one because reacted ATP exhibiting complete γ-PO₃-H₂¹⁸O exchange would contribute only 0.38% of the total signal area as m/e 141.) The m/e values are reported as percentages of the remaining total signal area.

* Prediction based on the following:

### TABLE IV

Effect of ADP on lamellae-catalyzed ATP ⇌ H₂¹⁸O exchange

Reactions were carried out in the light in a total volume of 1.0 ml as described under "Experimental Procedures": each incubation included lamellae (specific activity 141) containing 123 μg of chlorophyll, H₂¹⁸O water (64.5 atom % ¹⁸O), and 1 μmol of ATP in the presence or absence of 0.2 μmol of [¹⁷¹H]ADP (specific activity 3.96 × 10⁶ cpm/μmol). Reactions were stopped after 12 min and the purified ATP samples analyzed as described under "Experimental Procedures." At that time 0.1 μmol of [¹⁷¹H]ATP was formed in the [¹⁷¹H]ADP incubation. m/e values are reported as percentages of the total signal area and include the contributions to m/e 141 and 143 due to unreacted ATP.

<table>
<thead>
<tr>
<th>Additions</th>
<th>m/e</th>
<th>Observed 145/147</th>
<th>Total ¹⁸O exchanged into γ-PO₃ (4R)*</th>
<th>ATP experiencing exchange</th>
<th>μmol</th>
</tr>
</thead>
<tbody>
<tr>
<td>ATP</td>
<td>141</td>
<td>83.57 4.45 6.69 5.30</td>
<td>1.26 33.52 0.180</td>
<td>0.180</td>
<td></td>
</tr>
<tr>
<td>ATP, [¹⁷¹H]ADP</td>
<td>141</td>
<td>91.21 3.32 3.22 2.25</td>
<td>1.43 16.52 0.088</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* The ratio of m/e 145/147 predicted for complete equilibration with 64.5 atom % ¹⁸O-enriched water is 1.65 (Table III, Footnote b).

* R is defined in Table II, Footnote a, as the percentage of ¹⁸O distributed among the four oxygens of trimethyl phosphate.

* Micromoles of ATP = -(ATPI In 11 - F₁₁₁₁), where

\[
P_{\text{in}} = \frac{m/e \ 141_a - m/e \ 141_i}{m/e \ 141_a - m/e \ 141_o} = \frac{99.2 - m/e \ 141_i}{99.2 - 4.5}
\]

**DISCUSSION**

The present studies indicate that, in the absence of apparent ATPase activity in spinach chloroplasts, ATP undergoes reversible cleavage to ADP at a minimum of 78% of the time it experiences exchange of water oxygen with the γ-phosphoryl group. This observation favors a reversible hydrolysis pathway for the ATP-H₂O exchange according to Scheme 1 and indicates that an in-line oxonium ion displacement mechanism can make only a minor contribution. In the myosin ATPase system, reversible ATP hydrolysis has also been proposed as the mechanism for the observed ATP-H₂O and P₁-H₂O exchanges, and the extent of exchange per molecule of ATP that predicted for complete equilibration of the γ-PO₃ with water oxygens. (The small portion of the ATP pool (0.1 out of 1.1 μmol) produced from [¹⁷¹H]ADP is probably due to P₁ contamination of the reaction mixture and would be expected to have a negligible effect on the mass spectrum, especially in the highly enriched regions, m/e 145 and 147.) The effect of P₁ on the ATP-H₂O exchange pattern is presently under investigation.

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ATP or P, was found to be high due to rate-limiting breakdown of the myosin product complex and a slow off rate of ATP (18, 19). One might speculate that these mechanistic features are shared by the energy-transducing systems of oxidative phosphorylation in mitochondria and membrane ATPases of bacteria as well.

The fact that the rate of ATP-H2O exchange appears to be faster than the rate of reversible ATP cleavage by a factor of 1.29 can be rationalized if the β-Po, unit of ADP is not torsionally symmetrical because ADP binding to the coupling factor, which is presumed to be tight, hinders rotation about the P-PO,P, bond. This possibility is currently being investigated. Nonequivalence of the oxygens of γ-Po, of tightly bound ATP and of P, has been demonstrated in the myosin ATPase system, in which one oxygen of P, exchanges with water oxygen much more slowly than the others (19). Such an observation suggests that one γ-oxygen of ATP is anchored to the enzyme during the entire exchange process including the [ADP, P] stage. The pattern of oxygen exchange catalyzed by chloroplast lamellae seems to preclude such a γ-Po, restriction, i.e. all the oxygens appear to be equivalent. However, this does not preclude binding of the β-Po, of ADP that prevents complete equivalence of these three phosphoryl oxygens during the whole period in which the P-PO,P, bond is cleaved. Such a binding effect may make postulation of an in-line displacement contribution to the γ-Po, exchange unnecessary.

By Bridge to β nonbridging 180 scrambling of γ-180 [ATP] does not proceed at a rate faster than γ 18O washout although, as discussed above, the scrambling rate may be underestimated if rotation about the P-PO,P, bond of ADP is hindered. If the true scrambling rate, now under investigation, remains less than that of exchange, the results would militate against the formation of a phosphorylated intermediate, or at least, would place restrictions on the rate constants involved in formation of an X-Po,.

From Equation 3, the fact that the reversible formation of bound ADP does not occur

\[ K = \frac{[\text{ATP}]_{180}}{[\text{ATP}]_{180}} = \frac{[\text{X-PO}_4]}{[\text{P}_4]} \]

to proceed at one-seventh the ATP synthesizing capacity (1), but this value does not distinguish between multiple cycles of exchange and single events. It is apparent from the studies reported here that added ATP which undergoes exchange does so to a much higher extent than ATP that is being synthesized from ADP and P,; this difference is not inconsistent with the occurrence of both exchange processes at the same site on the coupling factor protein if one considers the conditions involved:

\[ E + \text{ADP} + P_4, \frac{1}{2} \rightarrow E \cdot \text{ADP} \cdot P_4 \rightarrow \frac{3}{6} \rightarrow H_2O \]

During synthesis of ATP from ADP and P, the system is in a dynamic steady state (Equation 4) with E saturated with respect to ADP (4 mM) and P, (4 mM). (During the time of reaction, less than half of the ADP and P, were used.) Once ADP and P, bind, each exhibits a high commitment toward net ATP synthesis as shown by the pulse-chase experiments of Smith and Boyer (20), i.e. k3 \( \ll \) k,. Since approximately one water oxygen appears in the resulting ATP to undergo reversible hydrolysis before dissociation from E, and yet is fast enough to limit the extent of exchange to an average of one per oxygen per ATP. With the assumption that all four oxygens of the P, are equivalent in Step 3, an incorporation of 0.7 water oxygen makes the ratio k3/k1(H2O) = 1.0. Thus in the synthesizing system, the rate of reversible hydrolysis is approximately equal to the synthetic rate.

Starting from exogenous ATP and noting the virtual absence of net ATPase activity by the chloroplasts, an equilibrium state described by Equation 5 is established.

\[ E \rightarrow \text{X-PO}_4 \rightarrow \frac{H_2O}{3} \rightarrow E \cdot \text{ADP} \cdot P_4 \]

To account for the much greater extent of γ 18O washout observed per ATP molecule, k1(H2O) must be \( \gg \) k3; i.e. multiple reversible cleavages, not detectable using the β-bridging technique, must have occurred before the ATP dissociates. If one assumes that the equilibrium constant k1/k3 is the same in both the equilibrium and the steady state cases, then it can be determined that the major difference between the two systems is a faster rate of ATP dissociation in the ATP-synthesizing system, i.e. k3 > k5, by a factor of approximately 6 to 12.

The apparent increase in the off rate of ATP during its synthesis from ADP and P, relative to that in the absence of net phosphorylation may reflect an interaction between centers of ATP synthesis on the coupling factor. An "alternating site" mechanism for ATP synthesis has recently been proposed.

1 From Equation 4, if E is saturated with respect to ADP and P,,

\[ \text{v}_{\text{synthesis}} = k_1 \text{[E-ATP]} \leftrightarrow \frac{h_0 k_3 E}{k_1 + k_1 + k_5} \]

1 From Equation 4, if E is saturated with respect to ADP and P,,

\[ \text{v}_{\text{synthesis}} = k_1 \text{[E-ATP]} \leftrightarrow \frac{h_0 k_3 E}{k_1 + k_1 + k_5} \]

From Equation 5, if E is saturated with respect to ATP,

\[ \text{v}_{\text{washout}} = k_1 \text{[E-ATP]} \leftrightarrow \frac{k_3 E}{k_1 + k_3} \]

If k3/k1 is the same in both cases, and because k1(H2O) = k1, it follows that

\[ 6 \leq \frac{k_5}{k_3} \leq 12 \]

depending on the relative magnitudes of k1 and k5.
by Boyer and co-workers in which an energy-induced conformational change at one catalytic site promotes productive binding of ADP and P\(_i\), i.e. net reaction, is required to enable a loosening of bound ATP, an explanation would be afforded for the decrease in dissociation rate of ATP bound in a system in which net phosphorylation could not take place. Studies are in progress to further investigate this point, including the effect of P\(_i\) alone on the off rate of ATP.

In conjunction with the above hypothesis, inhibition of the ATP: H\(_2\)O exchange by ADP alone occurs without an effect on the high extent of exchange per molecule. The inhibition can be explained by a decrease in concentration of E·ATP, whereas partitioning of E·ATP to E·ADP·P\(_i\) and free ATP + E, governed by k\(_{\text{on}}\)/k\(_{\text{off}}\)(H\(_2\)O), remains the same. A previous study reported this ADP inhibition not to be strictly competitive (1). Cantley and Hammes (22) have demonstrated that two tight noncatalytic ADP binding sites exist on the solubilized chloroplast coupling factor, both before and after treatments that elicit the enzyme's latent ATPase activity. Inhibition of the ATPase by ADP also occurred and was suggested to be allosteric, involving promotion of an inactive state upon binding of the inhibitor. A similar mechanism could account for the ADP inhibition of the ATP: H\(_2\)O exchange in intact chloroplast lamellae.

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