Mechanisms by Which Reactions Catalyzed by Chloroplast Coupling Factor 1 Are Inhibited: ATP Synthesis and ATP–H_2O Oxygen Exchange†

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ABSTRACT: The ATP–H_2O back-exchange reaction catalyzed by membrane-bound chloroplast coupling factor 1 (CF_1) in the light is known to be extensive; each reacting ATP molecule nearly equilibrates its γ-PO_3 oxygens with H_2O before it dissociates from the enzyme. P_i, As_i, ADP, and GDP, alternate substrates of photophosphorylation, each inhibit the exchange reaction. At all concentrations of these substrate/inhibitor molecules tested, the high extent of exchange per molecule of ATP that reacts remains the same, while the number of ATP molecules experiencing exchange decreases. Thus, these inhibitors appear to act in a competitive-type manner, decreasing ATP turnover, as opposed to modulating the rate constants responsible for the partitioning of E·ATP during the exchange reaction. This is consistent with the identity of CF_1 catalytic sites for ATP–H_2O back-exchange and ATP synthesis. Carbonyl cyanide *m*-chlorophenylhydrazone and NH_4Cl (uncouplers of photophosphorylation) and phloridzin (an energy-transfer inhibitor) also lower the rate of ATP–H_2O back-exchange; they too are found to act by decreasing the turnover of the ATP pool, not the extent of exchange per reacting ATP molecule. The extent of ATP–H_2O forward oxygen exchange, which occurs during net ATP synthesis prior to product dissociation, is unaffected by uncouplers, whether catalyzed by native CF_1 (ATPase latent) or the dithiothreitol/light-activated ATPase form. The mode of NH_4Cl inhibition of the ATP synthesis reaction, therefore, is not through a change in the partitioning of the E·ATP complex. This is important in view of the uncoupler-modulated partitioning of E·ADP·P_i observed during ATP hydrolysis in the same system [Sherman, P. A., & Wimmer, M. J. (1983) *Eur. J. Biochem. 136*, 539–543]. Varying light intensity has no effect upon the extent of ATP–H_2O forward oxygen exchange during ATP synthesis catalyzed by native CF_1. However, once the enzyme has been ATPase activated, light intensity is found to modulate the partitioning of E·ATP, in contrast to its lack of effect on the partitioning of E·ADP·P_i measured during ATP hydrolysis.

Membrane-bound chloroplast coupling factor (ATPase latent) catalyzes a light-dependent exchange of oxygen between the γ-PO_3 moiety of ATP and H_2O in the absence of net hydrolysis of that ATP (Avron & Jagendorf, 1959; Shavit et al., 1967). Using $\beta\gamma$-bridge–β-nonbridge positional isotope exchange in $\beta\gamma$-bridge ^{18}O-labeled ATP, Wimmer & Rose (1977) observed reversible cleavage of the CF_1-bound ATP, which was light dependent and uncoupler sensitive and which correlated to the γ-PO_3–H_2O oxygen exchange. Reversible hydrolysis of bound ATP to bound ADP/P_i was thereby postulated as the mechanism for the cryptic ATP–H_2O back-exchange catalyzed by CF_1,[1] consistent with its occurrence at the ATP synthesis/hydrolysis site.

To monitor the ATP–H_2O oxygen exchange in the Wimmer and Rose study, a new method was used for determining the $^{18}O/^{16}O$ isotopic composition of phosphate molecules. Formerly, the γ-PO_3 unit of ATP was isolated as P_i, and the $^{18}O/^{16}O$ content was measured by mass spectrometry after combusting the P_i to CO_2 (Boyer & Bryan, 1967). Because the P–O bonds of P_i are destroyed, it is not possible to distinguish oxygen-18 that is present to a low extent per molecule in a large fraction of the P_i pool or concentrated to a high extent in a small fraction of the total P_i. When one is analyzing either P_i formed during ATP hydrolysis or P_i derived from the γ-PO_3 unit of ATP produced during ATP synthesis, the CO_2 method is informative. With inclusion of product trapping systems, these reactions are rendered irreversible, and the isolated P_i is a homogeneous sample in which all molecules produced have experienced oxygen exchange to the same extent. Such experiments now have been reported demonstrating substrate modulation of oxygen exchange in the mitochondrial and chloroplast systems (Hackney & Boyer, 1978; Hackney et al., 1979). However, P_i isolated from the γ-PO_3 unit of ATP that has undergone CF_1-catalyzed ATP-H_2O back-exchange is a heterogeneous pool; at the end of the incubation period, only a certain fraction of the ATP has had time to react during the first-order approach to equilibrium. To fully analyze the reactant pool, determination must be made of how many ATP molecules underwent exchange (substrate turnover) and to what extent the exchange proceeded per molecule of ATP reacting. The only information that isotope ratio data from the $P_i \rightarrow CO_2$ analysis can provide here is how many H_2O oxygens are incorporated into the total ATP pool.

Conversion of P_i to volatile trimethyl phosphate, instead of to CO_2, with subsequent analysis by chemical ionization mass spectrometry maintains the integrity of the P–O bonds (Midelfort & Rose, 1976). When applied by Wimmer & Rose (1977) to the chloroplast photophosphorylation system, this method of analysis provided the first qualitative and quantitative information about an ATP–H_2O back (or "medium")

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[1] Abbreviations: CF_1, chloroplast coupling factor 1; PMS, phenazine methosulfate; CCCP, carbonyl cyanide *m*-chlorophenylhydrazone; DTT, *dl*-dithiothreitol; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride.

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oxygen exchange reaction. The exchange is extensive, with nearly full equilibration of the γ-PO_3 with water before each ATP dissociates, and the rate of turnover of the ATP pool is significant (approximately 15% of the ATP synthesis rate).

CF_1-catalyzed ATP-H_2O back-exchange is inhibited by ADP and P_i, substrates of photophosphorylation (Shavit et al., 1967; Avron et al., 1965; Chaney & Boyer, 1969). Because the isotopic composition of the ATP pool was determined by the traditional combustion method, these studies could not distinguish between inhibition due to a decrease in the extent of oxygen exchange per molecule of ATP reacting or to a decrease in the turnover of ATP during the incubation. The present study was conducted by using whole-molecule P_i analysis to determine how substrate molecules inhibit the ATP-H_2O back-exchange, especially in view of the kinetic modulation seen as substrate concentration is varied during ATP synthesis in the same system (Hackney et al., 1979). The mechanism by which uncouplers NH_4Cl and CCCP and energy-transfer inhibitor phloridzin effect the exchange reaction was also investigated. These compounds are known inhibitors of chloroplast photophosphorylation (McCarty & Racker, 1968), and uncouplers are known to inhibit the ATP-H_2O exchange (Wimmer & Rose, 1977).

The generally accepted hypothesis of energy coupling is that a light-induced electrochemical gradient of protons acts as the obligatory intermediate between the electron-transport chain and the ATP synthesis reaction catalyzed by F_1 complexes (Mitchell, 1961). Recent reports from this laboratory have examined the effects of light intensity and uncouplers on the P_i-H_2O forward oxygen exchange accompanying ATP hydrolysis by the activated CF_1 ATPase (Sherman & Wimmer, 1982, 1983). Those studies now have been extended to include the effect of light and uncouplers on the ATP-H_2O forward exchange reaction accompanying ATP synthesis in both the ATPase-latent and ATPase-activated systems.

Experimental Procedures

Materials and Methods. Na_2ADP, Na_2ATP, Na_2GDP, phenazine methosulfate, dithiothreitol, carbonyl cyanide *m*-chlorophenylhydrazone, and phloridzin were purchased from Sigma. P^1,P^5-Bis(5′-adenosyl) pentaphosphate (Ap_5A) and lyophilized hexokinase were from Boehringer Mannheim.

[γ-^{18}O]ATP, labeled to ≥90% oxygen-18 enrichment in the three γ-phosphoryl oxygens, was chemically synthesized, purified, and analyzed as previously described (Wehrli et al., 1965; Midelfort & Rose, 1976). [^{32}P]P_i was purchased from New England Nuclear and was used to enzymatically prepare [γ-^{32}P]ATP of high specific radioactivity (Scendel & Wells, 1973; Sherman & Wimmer, 1982). Radioactive substrates ([^{32}P]P_i or [γ-^{32}P]ATP) were added to the appropriate ^{18}O-labeled substrate in most experiments in order to (1) measure the forward velocity of both ATP synthesis and hydrolysis reactions and (2) detect any exogenous P_i contamination that would prevent accurate interpretation of isotopic data from the oxygen-18 studies. No detectable P_i contamination (≤2%) occurred in any of the experimental samples analyzed.

Osmotically shocked chloroplast lamellae were prepared from fresh market spinach as previously described (Wimmer & Rose, 1977) and used on the same day. The concentration of chlorophyll was determined by measuring the absorbance at 652 nm of a filtered solution of lamellae preparation dissolved in 80% acetone (Walker et al., 1971).

Exchange and control reactions were performed in 125-mL, long-necked Erlenmeyer flasks, positioned at various fixed distances from 75-W reflector lamps. A modified Warburg apparatus was used to provide steady shaking, constant 25 °C temperature, and uniform light intensity. Reactions were stopped by addition of 50 μL of 70% perchloric acid/mL of incubation. Precipitated protein was removed by centrifugation, and aliquots of the reaction supernatants were removed for forward velocity measurements.

Standard methods of assaying for [γ-^{32}P]ATP synthesis and hydrolysis were used. ATP and glucose 6-phosphate quantitation from an ATP synthesis experiment involved separating unreacted [^{32}P]P_i from product [γ-^{32}P]ATP or glucose 6-[^{32}P]phosphate by molybdate/2-methyl-2-propanol-benzene extraction and an aqueous phase wash with nonradioactive P_i, followed by liquid scintillation counting of the aqueous phase (Avron, 1960). P_i quantitation from an ATP hydrolysis experiment involved extraction of product [^{32}P]P_i as the phosphomolybdate complex into butyl acetate, followed by liquid scintillation counting of the organic phase (Sanru, 1974).

Reaction Conditions. ATP-H_2O back-exchange incubations contained 50 mM Tris-HCl, pH 7.8, 5 mM $MgCl_2$, 10 mM NaCl, 67 μM PMS, 0.67 mM [^{18}O]ATP, and 100–150 μg of chlorophyll/mL of final reaction volume. Photophosphorylation controls that accompanied all back-exchange incubations contained 50 mM Tris-HCl, pH 7.8, 8 mM $MgCl_2$, 10 mM NaCl, 67 μM PMS, 8 mM ADP, 8 mM [^{32}P]P_i (specific radioactivity 10^5–10^6 cpm/μmol), and 100–150 μg of chlorophyll/mL of reaction volume. These controls were necessary in order to ensure that the activity of this relatively unstable enzyme complex remained constant over the course of each exchange experiment. Also, 15–30% of the [γ-^{18}O]-ATP pool was desired to be turned over in exchange to accurately measure the micromoles of ATP reacted, while minimizing the correction necessary for multiple reactions, or recycling, of the ATP pool (see Oxygen Exchange Calculations). The ATP-H_2O back-exchange is known to proceed at 10–15% the turnover rate of a parallel photophosphorylation reaction under the conditions described. In uninhibited controls of inhibition sequences (Tables I, III, IV), a higher fraction of exchanged ATP was necessary to enable accurate measurement of inhibited samples.

Most studies of ATP-H_2O forward exchange that accompanies ATP synthesis were performed under the following conditions (see Tables for variations): 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 5 mM $MgCl_2$, 100 μM PMS, 5 mM [^{18}O]P_i, 25 μM ADP, and 200 μg of chlorophyll/mL of reaction volume. Hexokinase (60–100 units) and 10 mM glucose were added to recycle substrate ADP, keeping its (low) concentration constant during the reaction period and preventing any back-exchange of newly synthesized ATP.

In experiments where the latent ATP hydrolysis activity of CF_1 was elicited, the enzyme was preincubated in high light (55 700 lx) in the presence of 10 mM DTT for 5 min prior to the addition of [γ-^{32}P]ATP (Sherman & Wimmer, 1982). Conditions for most ATPase reactions were 50 mM Tris-HCl, pH 7.8, 50 mM NaCl, 5 mM MgCl, 100 μM PMS, 3 mM [γ-^{32}P]ATP, 10 mM DTT, and 200 μg of chlorophyll/mL of reaction volume. Because the ATPase reaction was studied in the light as well as in the dark, 5 mM Na_2HAsO_4 was included in all incubations to competitively trap product [^{32}P]P_i and prevent its use in the resynthesis of [^{32}P]ATP (Sherman & Wimmer, 1982).

Oxygen-18 Sample Workup. The γ-PO_3 unit of reacted and unreacted ATP from ATP-H_2O back-exchange incubations was isolated as previously described (Midelfort & Rose, 1976; Wimmer & Rose, 1977). Briefly, the γ-PO_3 unit of the purified ATP is transferred to dihydroxyacetone with glycerokinase, followed by base-catalyzed elimination of P_i from

the dihydroxyacetone phosphate product. The P_i is purified and converted to the free acid by passage over AG 1-X8 (Cl^- form); after evaporation to dryness, the sample is dissolved in 0.1 mL of absolute methanol for methylation, described below.

Because a glucose/hexokinase product trapping system is included in all ATP–H_2O forward exchange (ATP synthesis) incubations, the γ-PO_3 unit of product ATP is transferred to glucose, forming glucose 6-phosphate. Isolation of the γ-PO_3 moiety involves separating glucose 6-phosphate from substrate [^{18}O]P_i via molybdate/2-methyl-2-propanol–benzene extraction of P_i (Avron, 1960). After purification of glucose 6-phosphate by AG 1-X8 (Cl^- form) chromatography, base-catalyzed elimination of P_i is done by incubation in 150 mM KOH at 100 °C for 12 min (D. D. Hackney, personal communication); the P_i is then treated as above in preparation for methylation. Importantly, no change in the oxygen isotopic composition of the ATP γ-PO_3 moiety occurs in the transfer to glucose or elimination from glucose 6-phosphate as P_i. The P_i produced contains one constant ^{16}O from glucose plus the original γ-PO_3 unit.

Analysis of [^{18}*O*]P_i. The free-acid form of all P_i samples was converted to the volatile methylated derivative trimethyl phosphate by reaction with a freshly prepared ethereal solution of diazomethane (Midelfort & Rose, 1976). Analysis of the ^{18}O/^{16}O isotopic composition was performed on a Finnigan Model 4021 gas chromatograph/mass spectrometer at the WVU Mass Spectrometry Center, Department of Biochemistry, under the following conditions: 15-m SE30 capillary column, isothermal elution at 125 °C, and chemical ionization using methane. Multiple-ion detection and data manipulation was made possible through the use of an interfaced Incos data system. Monitoring of the mass spectrum at mass to charge (*m/e*) ratios of 141, 143, 145, 147, and 149 reflects trimethyl phosphate molecules containing zero to four ^{18}O atoms/molecule, respectively.

Oxygen Exchange Calculations. (*A*) *ATP–H_2O Back-Exchange.* Qualitative analysis of the mass spectral data of the γ-PO_3 unit of ATP at the end of an ATP–H_2O back-exchange incubation indicates that there are basically two types of [^{18}O/^{16}O]ATP: (1) *unreacted ATP*, the majority (≥85%) of which has all of its γ-PO_3 oxygens ^{18}O enriched (*m/e* 147), and (2) *reacted ATP*, with nearly all of its γ-PO_3 oxygens unenriched (*m/e* 141). Thus, the number of micromoles of ATP experiencing back-exchange can be calculated from the measured decrease in the *m/e* 147 component of the mass spectral data, as previously described (Wimmer & Rose, 1977). This calculation involves a correction for the statistical probability of multiple reactions of a single ATP molecule (ATP pool recycling) as turnover increases.

(*B*) *ATP–H_2O Forward Exchange.* Unlike the back-exchange reaction, the product of ATP–H_2O forward exchange (ATP) is chemically different from that of the substrate (P_i), and all synthesized ATP molecules have gone through exchange to the same extent (homogeneous ATP pool). The extent of ATP–H_2O forward exchange represents the difference in ^{18}O/^{16}O isotopic content between γ-PO_3 moieties from P_i and ATP. Thus, the number of water oxygens incorporated into each ATP formed by CF_1 is calculated as follows:

$$H_2O \text{ oxygens/ATP formed} = (\text{total } {}^{16}O/P_i \text{ derived from ATP}) - [(3/4)(\text{total } {}^{16}O/\text{reaction } P_i) + 1]$$

where

$$\text{total } {}^{16}O/P_i \text{ molecule} = 4 \times \frac{\text{total } {}^{16}O \text{ atoms}}{\text{total } {}^{18}O + {}^{16}O \text{ atoms}}$$

Table I: Inhibition of the ATP–H_2O Back-Exchange Reaction by Inorganic Phosphate[a]

conditions	*m/e* 141	143	145	147	μmol exchanged[b]
dark control	2.79	2.28	4.09	100	
dark control + 5 μmol of P_i	3.49	2.19	4.34	100	
no additions	48.08	11.89	10.78	100	0.90
0.3 μmol of P_i	42.38	9.01	9.04	100	0.77
0.6 μmol of P_i	32.78	7.00	8.92	100	0.62
1.5 μmol of P_i	24.27	4.23	5.02	100	0.41
5.0 μmol of P_i	11.72	2.97	5.31	100	0.19

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 67 μM PMS, 0.67 mM [γ-^{18}O]ATP, and 100 μg of chlorophyll in a total volume of 3.0 mL. Light intensity for all reactions is 55 700 lx. Incubation time is 2.5 min. [b] Micromoles of ATP experiencing γ-PO_3–H_2O oxygen exchange.

Total ^{16}O atoms = 4(*m/e* 141) + 3(*m/e* 143) + 2(*m/e* 145) + *m/e* 147; total ^{18}O atoms = 3(*m/e* 147) + 2(*m/e* 145) + *m/e* 143.

Results

Kinetic Effect of Substrate Molecules on the ATP–H_2O Back-Exchange Reaction. Results presented here confirm and extend the observation that certain substrate molecules inhibit the CF_1–catalyzed ATP–H_2O back-exchange reaction (eq 1).

$$E + ATP \rightleftharpoons E{\cdot}ATP \underset{H_2O}{\overset{H_2O}{\rightleftharpoons}} E{\cdot}ADP{\cdot}P_i \qquad (1)$$

The question is asked whether the inhibition results from (a) complete inhibition of a fraction of the enzyme catalyzing the reaction through a competitive-type interaction, with no change in reaction rate constants, or (b) inhibition of the catalyzed exchange process itself through an alteration of enzymatic reaction rate constants. By maintaining the integrity of the γ-PO_3 unit in the analyzed trimethyl phosphate samples, these two modes of inhibition can be distinguished. As discussed in the introduction, the two inhibitory mechanisms will manifest themselves in either a decrease in turnover of the ATP pool or a decrease in the extent of exchange per molecule that reacts, respectively.

The first substrate/inhibitor molecule tested was inorganic phosphate, with results in Table I. For comparison, the data have been normalized to the *m/e* 147 isotopic species. In the light and in the absence of inhibitors, the extent of exchange per molecule of ATP reacting is high, as previously reported (Wimmer & Rose, 1977), with the decrease in *m/e* 147 accompanied by the major increase in *m/e* 141. Therefore, rate-limiting dissociation of ATP is indicated. The presence of P_i, at all concentrations tested, does not alter the extensive exchange pattern of each molecule of reacted ATP but does decrease the number of micromoles of ATP that experience exchange.

In testing the substrate/inhibitor ADP, the known presence in chloroplast preparations of a small amount of adenylate kinase (Cohn, 1953) complicated this experiment. ATP synthesized from ADP by the adenylate kinase reaction will look identical with [^{18}O]ATP that has equilibrated its γ-PO_3 with $H_2{}^{16}O$ by oxygen exchange. However, addition of the specific inhibitor of this enzyme, Ap_5A (Lienhard & Secemski, 1973), can reduce the reaction to a level that makes correction to the light-dependent back-exchange performed in the presence of ADP minimal (Table II). Ap_5A is thus included in all ADP incubations.

Table II: Control of Contaminating Adenylate Kinase Activity with the Inhibitor Ap_5A[a]

conditions	m/e 141	143	145	147	μmol of synthesized [^{16}O]ATP
dark control	1.69	2.23	3.69	100	
dark control + 2 μmol of ADP	14.98	2.91	3.58	100	0.24
dark control + 2 μmol of ADP + 20 μM Ap_5A	6.64	2.87	4.21	100	0.08

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 67 μM PMS, 0.67 mM [γ-^{18}O]ATP, and 100 μg/mL chlorophyll in a total volume of 3.0 mL. All reactions are performed in the dark. Incubation time is 3 min.

Table III: Inhibition of the ATP–H_2O Back-Exchange Reaction by ADP[a]

conditions[b]	m/e 141	143	145	147	μmol exchanged[c]
dark control	6.53	1.59	13.11	100	
dark control + 3 μmol of ADP	15.62	1.45	13.87	100	0.15
no additions – Ap_5A	43.59	8.03	17.90	100	0.58
no additions	43.98	7.35	16.52	100	0.58
0.2 μmol of ADP	21.74	4.37	15.26	100	0.26
0.5 μmol of ADP	18.18	4.17	15.48	100	0.19
1.0 μmol of ADP	16.16	3.55	15.48	100	0.13
2.0 μmol of ADP	17.19	2.68	14.08	100	0.09
3.0 μmol of ADP	17.87	2.57	13.78	100	0.04

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 10 mM NaCl, 67 μM PMS, 5 mM $MgCl_2$, 0.67 mM [γ-^{18}O]ATP, and 100 μg/mL chlorophyll in a total volume of 3.0 mL. All reactions are performed under a light intensity of 55 700 lx. Incubation time is 3 min 15 s. [b] Except where indicated, all incubations include 16.7 μM Ap_5A. [c] Micromoles of ATP experiencing γ-PO_3–H_2O oxygen exchange. Data are corrected for light-independent adenylate kinase activity in incubations containing ADP in the light.

Table IV: Inhibition of the ATP–H_2O Back-Exchange Reaction by GDP[a]

conditions	m/e 141	143	145	147	μmol exchanged[b]
dark control	1.69	2.23	3.69	100	
dark control + 2 μmol of GDP	1.89	2.72	3.53	100	
no additions	67.45	12.98	10.30	100	1.15
0.3 μmol of GDP	52.05	11.13	8.62	100	0.94
0.6 μmol of GDP	45.62	10.61	8.37	100	0.85
1.2 μmol of GDP	39.47	9.24	7.61	100	0.75
2.0 μmol of GDP	30.76	8.94	8.06	100	0.63
6.0 μmol of GDP					0.49[c]

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 10 mM NaCl, 67 μM PMS, 5 mM $MgCl_2$, 0.67 mM [γ-^{18}O]ATP, and 100 μg/mL chlorophyll in a total volume of 3.0 mL. Light intensity for all reactions is 55 700 lx. Incubation time is 4 min. [b] Micromoles of ATP experiencing γ-PO_3–H_2O oxygen exchange. [c] Results from a separate experiment.

In Table III, despite the presence of Ap_5A (which is shown to have no effect on the ATP–H_2O exchange reaction), a small amount of adenylate kinase activity remains detectable when ADP is added to the dark control incubation. (The increase seen in this background reaction from Table II, 0.15 vs. 0.08 μmol, is due to the presence of a higher [ADP] and lower [Ap_5A].) Correction for that [γ-^{16}O]ATP synthesized by adenylate kinase during the back-exchange incubations is done at all ADP concentrations tested, assuming linearity from zero to the highest [ADP]. This assumption is a logical one at the ADP/ATP concentrations used considering substrate K_ms. The high extent of oxygen exchange per molecule of ATP is maintained in the presence of ADP; however, as the concentration of ADP is increased, the micromoles of ATP experiencing exchange decreases.

Inhibition by GDP of ATP–H_2O back-exchange was also examined (Table IV). GDP and GTP are substrates for CF_1-catalyzed NTP synthesis and hydrolysis reactions (Franek & Strotmann, 1981), and the effect of GDP here is similar to that of ADP or P_i. The inhibition that this and the other substrate/inhibitors show on the ATP–H_2O back-exchange is achieved through lowering turnover, the number of ATP molecules that bind, react, and dissociate from CF_1.

Kinetic Effect of Nonsubstrate Inhibitors on the ATP–H_2O Back-Exchange Reaction. CCCP and NH_4Cl are uncouplers of chloroplast photophosphorylation. They are reported to exert their inhibitory effect on that reaction by dissipating the light-induced, electrochemical proton gradient (McCarty, 1980). Phloridzin is an energy-transfer inhibitor that appears to interact directly with CF_1 to inhibit photophosphorylation (Winget et al., 1969). In testing the effects of the three compounds on ATP–H_2O back-exchange, inhibitor concentrations were used that gave less than complete inhibition of photophosphorylation and of exchange to allow distinction between effects on turnover and on extent of exchange per molecule of reacting ATP. The results are shown in Table V. Although their modes of inhibition of photophosphorylation are different, CCCP/NH_4Cl and phloridzin have strikingly similar inhibitory effects on the ATP–H_2O back-exchange reaction. The high extent of oxygen exchange is maintained, and calculations again show that it is turnover of ATP that is decreased in the presence of these inhibitors.

Uncoupler and Light-Intensity Effects on ATP–H_2O Forward Exchange during ATP Synthesis Catalyzed by Latent and ATPase-Activated CF_1. ATP–H_2O forward exchange accompanies CF_1-catalyzed ATP synthesis (eq 2), resulting

$$E + ADP + P_i \rightleftharpoons E \cdot ADP \cdot P_i \underset{H_2O}{\overset{H_2O}{\rightleftharpoons}} E \cdot ATP \longrightarrow E + ATP \quad (2)$$

in incorporation of water oxygens into the γ-PO_3 moiety of synthesized ATP, despite net dehydration during synthesis. The mechanism by which uncoupler NH_4Cl inhibits ATP

Table V: Effect of NH_4Cl, CCCP, and Phloridzin on the ATP–H_2O Back-Exchange Reaction[a]

conditions	relative ATP synthesis rate (%)[b]	m/e 141	143	145	147	μmol exchanged[c]
dark control		8.25	4.12	13.38	100	
no additions	100	34.67	6.70	15.31	100	0.44
10 μM CCCP	69	22.86	6.11	14.08	100	0.26
0.5 mM NH_4Cl	62	15.80	4.92	12.99	100	0.12
0.5 mM phloridzin	44	13.66	3.64	13.57	100	0.08

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 10 mM NaCl, 5 mM $MgCl_2$, 67 μM PMS, 0.67 mM [γ-^{18}O]ATP, and 100 μg/mL chlorophyll in a total volume of 3.0 mL. Light intensity of all reactions was 55 700 lx. Incubation time is 4 min. [b] ATP synthesis conditions: 8 mM $MgCl_2$, 8 mM ADP, and 8 mM [^{32}P]P_i. Light intensity = 55 700 lx. [c] Micromoles of ATP experiencing γ-PO_3–H_2O oxygen exchange.

Table VI: ATP–H_2O Forward Exchange during ATP Synthesis by Native CF_1 in the Absence and Presence of NH_4Cl[a]

conditions	relative ATP synthesis rate (%)	m/e 141	m/e 143	m/e 145	m/e 147	water oxygen atoms per molecule of ATP formed
no additions	100	33.00	22.47	25.06	19.45	1.01
0.75 mM NH_4Cl	67	30.61	22.90	27.25	19.24	0.96
1.5 mM NH_4Cl	39	33.11	22.31	27.25	17.26	1.04

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 100 μM PMS, 10 mM glucose, 25 μM ADP, 5 mM [^{18}O]P_i, 50 units of hexokinase, and 200 μg/mL chlorophyll in a total volume of 2.0 mL. Light intensity of all reactions is 55 700 lx. Incubation times are 2.5 min, 4 min, and 6 min 10 s, respectively. No detectable change in the isotopic composition of the P_i pool occurs during each incubation.

Table VII: ATP–H_2O Forward Exchange during ATP Synthesis by ATPase-Activated CF_1 in the Absence and Presence of NH_4Cl[a]

conditions	relative synthesis rate (%)[b]	relative hydrolysis rate (%)[c]	m/e 141	m/e 143	m/e 145	m/e 147	water oxygen atoms per molecule of ATP formed
medium light	32	42	51.00	18.20	16.67	14.16	1.50
medium light + 1.33 mM NH_4Cl	7	72	54.13	16.69	16.50	12.59	1.56

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 100 μM PMS, 10 mM glucose, 25 μM ADP, 5 mM [^{18}O]P_i, 10 mM DTT, 70 units of hexokinase, and 200 μg/mL chlorophyll in a total volume of 3.0 mL. Enzyme was activated for ATPase activity by preincubation at 55 700 lx for 5 min. Incubation times are 45 s and 1.5 min, respectively. No detectable change in the isotopic composition of the P_i pool occurs during each incubation. [b] Relative to high light control. [c] Relative to dark control (ATPase conditions: 2 mM [γ-^{32}P]ATP, 5 mM $NaAs_i$); medium light = 21 000 lx.

Table VIII: Effect of Light Intensity on the ATP–H_2O Forward Exchange Reaction That Accompanies ATP Synthesis[a]

conditions	relative synthesis rate (%)	relative hydrolysis rate (%)[b]	m/e 141	m/e 143	m/e 145	m/e 147	water oxygen atoms per molecule of ATP formed
native enzyme							
high light	100		26.21	19.86	29.21	24.74	0.89 (0.99)[c]
medium light	70		26.58	22.98	27.27	23.20	0.96 (1.00)
low light	49		23.73	20.91	30.71	24.71	0.85 (0.95)
ATPase activated							
high light	79	0	38.65	22.88	22.29	16.20	1.30 (1.32)
medium light	32	42	51.00	18.20	16.67	14.16	1.50 (1.57)
low light	16	58	63.36	17.52	9.97	9.13	1.77 (1.77)

[a] Reaction mixtures contain 50 mM Tris-HCl (pH 7.8), 50 mM NaCl, 5 mM $MgCl_2$, 100 μM PMS, 10 mM glucose, 25 μM ADP, 10 mM DTT, 5 mM [^{18}O]P_i, 70 units of hexokinase, and 200 μg/mL chlorophyll in a total volume of 3.0 mL. Enzyme was activated for ATPase activity by preincubation at 55 700 lx for 5 min. Incubation times for the native enzyme are 35 s, 45 s, and 1.5 min, respectively, and for the activated enzyme are 30 s, 45 s, and 1.5 min, respectively. No detectable change in the isotopic composition of the P_i pool occurs during each incubation. [b] Relative to dark control (ATPase conditions: 2 mM [γ-^{32}P]ATP, 5 mM As_i). High light = 57 000 lx; medium light = 21 000 lx; low light = 11 000 lx. [c] The average of three independent experiments is shown in parentheses.

synthesis was examined by observing its effect on the oxygen exchange reaction, which directly reflects the forward and reverse partitioning of E·ATP. The results with native enzyme (Table VI) show that in the presence of NH_4Cl, at a level that inhibits the synthesis reaction up to 61% relative to that of the uninhibited control, the extent of oxygen exchange per molecule of ATP formed remains constant at 1.0. Therefore, in lowering the rate of the ATP synthesis reaction, uncoupler does not act by changing the partitioning of E·ATP.

The latent ATPase activity of CF_1 can be elicited by a number of procedures (Petrack & Lipmann, 1961; Petrack et al., 1965; Kaplan et al., 1967; McCarty & Racker, 1968; Binder et al., 1978). With the fully reversible light/DTT method, the CF_1 system retains its ATP synthesizing activity. Accompanying ATP hydrolysis is a P_i–H_2O forward oxygen exchange involving incorporation of water oxygens into product P_i in excess of the 1.0 chemically necessary for hydrolysis. This exchange reflects the forward and reverse partitioning of the enzyme products complex E·ADP·P_i and has been used to study the mechanism of light and uncoupler modulation of the ATPase system (Sherman & Wimmer, 1982, 1983). The partitioning of E·ADP·P_i is uneffected by changing light intensity but is shifted toward product dissociation in the presence of uncoupler.

In view of the lack of effect of NH_4Cl on ATP–H_2O forward exchange in the native CF_1 system, the experiment was repeated on the light/DTT-activated ATPase (Table VII). A light intensity was chosen for the ATP synthesis reaction such that a parallel ATPase incubation would proceed at a significant rate compared to a dark control ATPase reaction. Under the conditions used (low[ADP]), 1.50 water oxygens are incorporated into each ATP molecule formed in the absence of NH_4Cl. (Thus, ATPase activation itself appears to decrease the forward partitioning of E·ATP, perhaps through a change in ADP/P_i concentration dependence.) When added at a concentration that inhibits the ATP synthesis reaction by nearly 80% and stimulates the parallel ATP hydrolysis reaction by greater than 70%, NH_4Cl does not alter the number of water oxygens incorporated into synthesized ATP. This lack of effect was confirmed in a second separate experiment and suggests that activation of the CF_1 ATPase does not change the mechanism of uncoupler action.

To complete the overall study of modulators of CF_1-catalyzed reactions, the effect of light intensity on the ATP–H_2O forward exchange in both the latent and ATPase-activated CF_1 systems was examined (Table VIII). As with uncoupler addition, marked changes in the rate of ATP synthesis and hydrolysis can be achieved by varying light intensity. In the latent ATPase system, the decrease in the ATP synthesis rate by decreased light intensity is not accompanied by a change in the incorporation of water oxygens into ATP. However, once the enzyme is activated for ATP hydrolysis, the number of water oxygens incorporated into product ATP increases as the light intensity is raised.

Discussion

There are two general ways in which the rate of an enzyme-catalyzed reaction can be varied: (1) the effective amount of enzyme can be changed, or (2) rate-limiting rate constants that govern the overall reaction can be altered relative to those of other steps. In most enzyme mechanisms, information concerning individual rate constants is not easy to obtain. The ATP synthesis/hydrolysis systems afford a unique opportunity for study because partitioning of intermediate complexes is directly reflected in oxygen exchange reactions.

Whole-molecule phosphate analysis is a recently developed technique for assaying exchange products. By analyzing P_i in the form of trimethyl phosphate instead of CO_2, CF_1-catalyzed reactions can be fully characterized in terms of extent of exchange and turnover. The method is especially important when analyzing back-exchange reactions in which only a fraction of the substrate reacts, producing a heterogeneous pool of molecules. Such is the case for the light-dependent ATP–H_2O back-exchange catalyzed by native CF_1.

This reaction qualifies in many respects as being a catalytic site event (Wimmer & Rose, 1977). The kinetic features that govern the exchange can be examined in view of the reversible hydrolysis mechanism

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ATP \underset{H_2O}{\overset{H_2O\ k_2}{\underset{k_{-2}}{\rightleftharpoons}}} E{\cdot}ADP{\cdot}P_i \xrightarrow{\text{ATPase latent}} \quad (3)$$

The extent of exchange in reacted ATP is determined by the ratio of rate constants $k_2(H_2O)/k_{-1}$. k_{-1} must be rate limiting under normal conditions because of the high extent of exchange per molecule of ATP that reacts. The substrate/inhibitor molecules ADP, P_i, As_i, and GDP do not alter the extent of exchange, indicating no detectable change in the rate constants governing the partitioning of E·ATP. These substrate molecules appear to inhibit the back-exchange reaction by a competitive-type process. That the catalytic ATP–H_2O exchange site is the same site at which ATP and GTP synthesis occur is further suggested by the fact that GDP is found to be only slightly less effective an inhibitor of the back-exchange than ADP at the same ATP concentration. This corresponds to its slightly lower apparent affinity as a substrate (ADP K_m = 40 μM; GDP K_m = 160 μM). The K_m for P_i (0.65 mM) is also reflected in its relatively lower effectiveness as an inhibitor compared to nucleotide molecules.

A complete competitive inhibition study varying ATP concentration cannot be done accurately in this light/volume-dependent system. Volume changes alter the light-dependent CF_1 specific activity for photophosphorylation, the necessary control to which exchange rates are normalized. In addition, ample ATP to work up without significant [^{16}O]P_i contamination and an accurately detectable percent turnover in the back-exchange reaction are major considerations. The apparent tight binding of ATP would preclude use of a feasible ATP concentration range. [The large value of $k_2(H_2O)/k_{-1}$ does not necessarily indicate "tight binding" as assumed by many authors; however, that the exchange rate is essentially the same from 0.4 to 3.0 mM ATP (M. J. Wimmer, unpublished observations) does suggest a low K_m value.]

Uncouplers NH_4Cl and CCCP do not effect the extent of exchange in ATP undergoing back-exchange but act, again, by inhibiting turnover of the ATP pool. Phloridzin behaves in a similar manner. Thus far, no kinetic effect involving detectable altered partitioning of E-ATP has been observed for the back-exchange reaction. This is consistent with the lack of uncoupler effect on the partitioning of E·ATP in the ATP–H_2O forward exchange during ATP synthesis (Tables VI and VII), despite inhibition of the synthesis reaction, further supporting identity of catalytic sites.

The lack of kinetic modulation of the ATP–H_2O back-exchange reaction actually provides support for proposed cooperative mechanisms of ATP synthesis in the same system. In the synthesis reaction, as substrate ADP or P_i concentration is lowered, the extent of the accompanying forward exchange in product ATP increases. This is explained by ADP/P_i binding at one catalytic site enhancing ATP dissociation at an adjacent interacting site, presumably by conformational change (Cross, 1981). In the back-exchange reaction, the substrate and product both are ATP, and the exchange is already extensive at high substrate concentration. (Any increase in extent as [substrate] is lowered would be difficult to detect.) This difference between the two reactions is not surprising considering that the ATP–H_2O back-exchange is a *partial* reaction, with no ADP/P_i binding step to allow modulation of the partitioning of E·ATP. The light dependence of the back-exchange implicates an energy-requiring step in this partial reaction, apparently independent of substrate modulation effects. Further clarification of the role of energy in F_1 systems is needed.

Recent studies from this laboratory (Sherman & Wimmer, 1982, 1983) illustrate that the P_i–H_2O forward oxygen exchange catalyzed by activated membrane-bound CF_1 ATPase is decreased in extent by the uncouplers NH_4Cl and FCCP. The extent of this exchange is determined by the ratio of rate constants k_3/k_{-2}, which govern the forward and reverse partitioning of the E·ADP·P_i complex:

$$E + ATP \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E{\cdot}ATP \underset{H_2O}{\overset{H_2O\ k_2}{\underset{k_{-2}}{\rightleftharpoons}}} E{\cdot}ADP{\cdot}P_i \xrightarrow{k_3} E + ADP + P_i \quad (4)$$

Uncouplers have been found to increase the rate of the overall ATPase reaction, and their effect on the accompanying P_i–H_2O oxygen exchange is consistent with ATPase stimulation caused by the increased forward partitioning of the E·ADP·P_i complex. Importantly, the uncoupler inhibition of ATP synthesis also can be explained by the decreased forward partitioning of E·ADP·P_i in the synthesis direction, and no change in partitioning of E·ATP need occur, as is the case.

A proposed mechanism for the inhibitory action of uncouplers is concomitant reduction of the rates of both ATP dissociation and ADP/P_i binding during the ATP synthesis reaction (Cross, 1981). The studies from this laboratory provide direct kinetic evidence in support of the ADP/P_i binding effect but are inconsistent with uncoupler modulation of the partitioning of E·ATP.

Membrane-bound chloroplast coupling factor 1 is an oligomeric enzyme that is proposed to include energy-dependent conformational changes in its overall catalytic pathway (Cross, 1981). It has been suggested that an energy-dependent equilibrium of different enzyme forms might exist once CF_1 is activated for ATP hydrolysis (Franek & Strotmann, 1981; Sherman & Wimmer, 1982). Results presented in this study indicate that the ATP–H_2O forward exchange accompanying ATP synthesis under native conditions is uneffected by light intensity (Table VIII). However, once the enzyme is activated for ATP hydrolysis, light-induced alterations in the partitioning of E·ATP are observed (k_2/k_{-1} increases with decreasing light); no effect is seen, or required, on the P_i–H_2O oxygen exchange reaction accompanying ATP hydrolysis in the same system, governed by k_3/k_{-2} (Sherman & Wimmer, 1982). The in-

creased partitioning of E·ATP to E·ADP·P_i with decreasing light could explain the rate modulations by decreased light intensity: ATP synthesis decreases, ATPase increases.

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Registry No. ATP, 56-65-5; GDP, 146-91-8; ADP, 58-64-0; H_2O, 7732-18-5; O_2, 7782-44-7; ATPase, 9000-83-3; phosphate, 14265-44-2.

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Prothrombin Biosynthesis: Characterization of Processing Events in Rat Liver Microsomes†

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ABSTRACT: Plasma and hepatic microsomal forms of rat prothrombin have been compared by sodium dodecyl sulfate–polyacrylamide electrophoresis and isoelectric focusing. The major prothrombin species that accumulated in the microsomes of rats treated with warfarin had a molecular weight of 78 500 and a p*I* in 8 M urea of 6.3–6.5. Plasma prothrombin had a molecular weight of 83 500 and a p*I* of 5.3–5.7. Microsomes from normal rat liver contain a second pool of precursor with a molecular weight of 83 500, and digestion with the glycosidase Endo H indicated that this form has been processed to contain complex carbohyrates, while the M_r 78 500 form is a high mannose form and is the substrate for the vitamin K dependent carboxylase. Treatment of rats with tunicamycin revealed that glycosylation was not essential for carboxylation or secretion from the liver. Comparison of the aglyco forms of prothrombin and its precursors suggests that the intracellular forms contain a basic, M_r ~1500 peptide that is missing from the plasma form of prothrombin.

Prothrombin, clotting factors VII, IX, and X, protein C, and protein S are plasma proteins that require vitamin K for their formation (Suttie, 1983). The vitamin functions as a cofactor for the posttranslational carboxylation of specific glutamyl residues in microsomal precursor proteins to form γ-carboxyglutamyl (Gla)[1] residues in biologically active completed proteins (Suttie, 1980). In the rat, administration of the anticoagulant warfarin or vitamin K deficiency causes plasma prothrombin levels to decline and precursor forms to accu-

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[1] Abbreviations: Gla, γ-carboxyglutamic acid; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate; DTT, dithiothreitol; Endo H, endoglycoside H; PMSF, phenylmethanesulfonyl fluoride; IEF, isoelectric focusing; ECV, *Echis carinatus* venom; TCA, trichloroacetic acid.

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