Catalytic and Regulatory Effects of Light Intensity on Chloroplast ATP Synthase†

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Received September 9, 1986; Revised Manuscript Received November 5, 1986

ABSTRACT: The incorporation of water oxygens into ATP made by photophosphorylation is known to be increased markedly when either P_i or ADP concentration is lowered. The present studies show a similar increase in oxygen exchange when light intensity is lowered even with ample ADP and P_i present. The number of reversals of bound ATP formation prior to release increases about 1 to about 27 in the presence of dithiothreitol and to 5 in its absence. The equilibrium of the bound reactants still favors ATP at low light intensity, as shown by measurement of the amount of bound ATP rapidly labeled from [^{32}P]P_i during steady-state photophosphorylation. Changes observed in the interconversion rate in the absence of added thiol are likely involved in the regulation of the dark ATPase activity in the chloroplast. The interconversion rate of bound ATP to bound ADP and P_i in the presence of thiol is about the same at low and high light intensities. This rate of bound ATP formation is not sufficient, however, to account for the maximum rate of photophosphorylation. Thus, when adequate protonmotive force is present, the rate of conversion of bound ADP and P_i to bound ATP, and possibly that of bound ATP to bound ADP and P_i, must be increased, with proton translocation being completed only when bound ATP is present to be released. These observations are consistent with the predictions of the binding change mechanism with sequential participation of catalytic sites and are accommodated by a simplified general scheme for the binding change mechanism that is presented here. This scheme leads to separate, simple equations for oxygen exchange and net velocity of ATP synthesis. Their combination allows correlation of net velocity and oxygen exchange.

A binding change mechanism has been proposed for the ATP synthase of chloroplasts, mitochondria, and microorganisms (Boyer, 1979). Conformational changes accompanying proton translocation across the coupling membrane are regarded as promoting the release of an ATP at one catalytic site and changing the binding of ADP and P_i at another site in a manner that favors bound ATP formation. Three catalytic sites on the enzyme are thought to proceed in sequence through an identical series of conformational changes so that each site makes ATP by identical catalytic steps. A considerable number of experimental observations are consistent with this proposed mechanism, but additional tests of the mechanism are needed [for a review, see Cross (1981)].

Two unusual findings with the chloroplast ATP synthase that support the binding change mechanism are that even when substrate ADP or P_i concentrations are lowered far below those required for half-maximal velocity, a catalytic site retains a bound ATP (Rosen et al., 1979) and that this ATP is in dynamic equilibrium with bound ADP and P_i (Hackney et al., 1979). A consequence of this behavior is that each ATP made at low substrate concentrations is reversibly hydrolyzed many times at a catalytic site before being released to the medium. When ^{18}O-labeled P_i is used, such reversal is revealed by the extensive incorporation of water oxygens into the ATP made (Stroop & Boyer, 1985). In addition, the distribution of [^{18}O]ATP species (Hackney et al., 1979) made shows that synthesis occurs by a single catalytic pathway, in accord with the suggested mechanism.

In view of the above findings, it seemed probable that if the rate of photophosphorylation was reduced by lowering light intensity, a similar catalytic site retention of bound ATP undergoing a dynamic reversal of hydrolysis would result. Small increases in the extent of reversible hydrolysis of each bound ATP prior to release accompanying modest decreases in light intensity have been reported by Hackney et al. (1979) and by Spencer and Wimmer (1985).

Another point of interest is the role of the electrochemical potential and the reduction and oxidation of the γ-subunit disulfide in the regulation of the ATPase activity (see references under Discussion). The capacity of the chloroplast ATP synthase to hydrolyze ATP becomes activated in the light and decays back to a latent form in the dark. Additional factors, including Mg^{2+} ions, appear to have regulatory roles. The function of this activation in vivo is not known, but it may increase the efficiency of the ATP synthase.

The results reported in this paper show that if the rate of photophosphorylation is drastically reduced by lowered light intensity, a marked increase occurs in the extent of reversible hydrolysis of each bound ATP prior to its release. The equilibrium of the bound reactants still favors ATP at low light intensity as shown by measurement of the amount of bound ATP formed during steady-state photophosphorylation. The presence of dithiothreitol was found necessary to maintain a rapid interconversion rate of bound ATP to bound ADP and P_i at low light intensity. These results are consistent with the predictions of the binding change mechanism and give some additional insight into the role of the electrochemical potential and the γ-subunit disulfide in the regulation of the ATPase activity.

Materials and Methods

Materials. Chloroplast thylakoid membranes were prepared from market spinach as described by McCarty and Racker (1967). Thylakoids were prepared fresh from about 40 g of spinach and washed 3 times with 170 mL of grinding buffer to reduce levels of adenylate kinase. The grinding and washing

† This research was supported by Contract DE-AT03-76ER70102 from the U.S. Department of Energy and by U.S. Public Health Service Grant GM 119034.

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0006-2960/87/0426-1479$01.50/0

buffer contained 200 mM choline chloride and 5 mM $MgCl_2$ to improve the retention of photophosphorylation capacity (Rosen et al., 1979). Hexokinase (type C-300) purchased from Sigma Chemical Co., St. Louis, MO, was desalted on centrifuge columns equilibrated with 40 mM *N*-[tris(hydroxymethyl)methyl]glycine, pH 8, (Tricine). Diadenylate pentaphosphate was purchased from Boehringer-Mannheim. Acid-washed glassware and disposable plasticware were used for all experimental procedures, and HCl solutions were prepared from glass-distilled HCl.

Photophosphorylation. The reactions were carried out at room temperature and pH 8 in a mixture containing 8 mM $MgCl_2$, 38 mM NaCl, 1.5 mM [^{18}O,^{32}P]P_i, 33 μM phenazine methosulfate, 38 mM Tricine/NaOH, 38 mM glucose, approximately 20 units/mL hexokinase, 100 μM ADP, 40 μM diadenylate pentaphosphate, and 1 mM dithiothreitol with activated thylakoids. This system regenerates ADP as glucose 6-phosphate is formed. Each reaction contained approximately 40 μg of chlorophyll/mL (about 50 nM ATP synthase) in a volume of 3 mL and was illuminated for up to 15 min with a projector and a 300-W projector lamp. The light was filtered through a $CuSO_4$ solution. Fresh thylakoids were divided into two portions. One portion was treated with dithiothreitol. Reactions were carried out simultaneously so that results with and without dithiothreitol treatment could be compared. Reactions were quenched with 1 M perchloric acid. Voltage to the projector lamp was varied with a transformer, and light intensity was measured with a photometer. High light (100%) was approximately 5×10^6 erg^{-1} s^{-1} cm^{-2}. The chlorophyll content was determined by the method of Arnon (1949). ATP synthase concentrations were taken as 1.3 nmol of synthase/mg of chlorophyll as estimated by Strotman et al. (1973). Dithiothreitol-treated thylakoids were preilluminated for 5 min in the presence of 50 mM dithiothreitol then kept on ice in the dark before use. [^{32}P]P_i was assayed by Cerenkov counting in water. Inorganic phosphate was measured by the phosphate assay of Ohnishi and Gall (1978). Contamination of the [γ-^{18}O]P_i from ATP by nonisotopic P_i or from adenylate kinase activity was not significant, and correction of the $P^{18}O_0$ peak was not necessary.

^{18}O Measurements. Reaction mixtures were quenched and put on ice. The glucose 6-phosphate was isolated and degraded to P_i, and the P_i was purified as described previously (Stroop & Boyer, 1985). Low-light reactions typically yielded about 200 nmol of glucose 6-phosphate for analysis. The purified [^{18}O]P_i (originally the γ-phosphoryl of ATP) was converted to triethyl phosphate with diazoethane and analyzed with a Hewlett Packard 5995A gas chromatograph/mass spectrometer (Stempel & Boyer, 1986). P_i species containing zero to four atoms of ^{18}O were determined by specific ion monitoring of m/e ratios 155, 157, 159, 161, and 163 corresponding to the diethyl phosphate fragments. Analysis of the mass spectral data and calculations for the extent of reversible hydrolysis and the interconversion rate were made as described elsewhere (Stempel & Boyer, 1986; Hackney et al., 1980; O'Neil & Boyer, 1984; Stroop & Boyer, 1985). All statistical variations were calculated as standard deviations.

Inorganic medium P_i isolated from the reactions after quenching was used to determine the starting enrichment of [^{18}O]P_i and to calculate the amount of oxygen exchange occurring during synthesis of ATP. The ^{18}O distribution in the medium P_i did not change during the course of the reaction.

Hexokinase-Inaccessible ATP. This method is used to determine the levels of enzyme-bound product and is described by Rosen et al. (1979). Reactions were carried out in 2-mL volumes with 1 mM P_i and 150 or 400 units/mL hexokinase with a light intensity of 2% which limited the reaction rate by 98%. Reactions were illuminated for 1 min, then pulsed for 15 s with [^{32}P]P_i, and then quenched with 1 M perchloric acid. The resulting [γ-^{32}P]ATP was then isolated and counted (Vinkler et al., 1978). It was necessary to purify the [^{32}P]P_i prior to use by adding carrier P_i to about 5 mCi of [^{32}P]P_i and applying it to a 5×7 cm Dowex AG-1 column. The [^{32}P]P_i was eluted with 30 mM HCl after several washes with water.

Initial Velocity Determinations. Velocities were determined by measuring the amount of glucose 6-[^{32}P]phosphate formed after carrying out the reaction for 1–5 min with volumes of 1–3 mL. Reactions were quenched with 1 M perchloric acid, and the glucose 6-[^{32}P]phosphate was isolated from [^{32}P]P_i by the molybdate/triethylamine precipitation method (Grubmeyer & Penefsky, 1981).

RESULTS

Interpretation of Oxygen Exchange Data. The primary measurement made in these studies is of intermediate ATP $\rightleftarrows$ HOH oxygen exchange. As defined earlier (Hackney & Boyer, 1978), it is the exchange of water oxygens with phosphate oxygens which accompanies the net conversion of medium P_i to medium ATP. The extent of this oxygen exchange can be expressed in various ways. On convenient way is the number of water oxygens appearing in each ATP made; this can vary from 0 to 3. Another is the partition coefficient for exchange, the P_c, which is the probability that bound ATP formed at the enzyme catalytic site will undergo exchange prior to release; this can vary from 0 to 1.

The oxygen exchange is regarded as resulting from the reversal of hydrolysis of bound ATP to bound ADP and P_i prior to ATP release. From the measurement of oxygen exchange, the number of reversals of bound ATP formation prior to release can be calculated (Hackney et al., 1980) and is referred to in this paper as the number of reaction reversals. The rate of reaction reversal is given by the number of reaction reversals times the net rate of ATP synthesis. This rate of reaction reversal is sometimes conveniently referred to as the interconversion rate of bound ATP to bound ADP and P_i, or simply the interconversion rate. It is also equal to the rate of oxygen exchange that is occurring.

The net rate of ATP synthesis, or the rate of photophosphorylation, is always less than the total rate of ATP formation occurring at the catalytic sites because some reversal of bound ATP formation is always observed.

Oxygen Exchange with Dithiothreitol-Treated Thylakoids. Treatment of thylakoid membranes with dithiothreitol in the light activates the CF_1 ATPase (Petrack et al., 1965). This activation is associated with cleavage of a disulfide bond in the γ subunit. During photophosphorylation by chloroplasts, this disulfide bond likely is reduced by an endogenous reducing system involving thioredoxin (Mills et al., 1980). For measurement of the effect of light intensity on the oxygen exchange, it thus seemed preferable to have thiol present so that, except for reduced protonmotive force, the synthase would be operating under conditions of maximal photophosphorylation.

The extent of water oxygen incorporation into ATP, the oxygen exchange, was measured as a function of light intensity during net photophosphorylation with dithiothreitol-treated thylakoids. The ^{18}O distributions in the five species present in the initial P_i and in the four species expected in the γ-phosphoryl group of the ATP formed if no exchange occurs are shown in Figure 1. The distributions of [γ-^{18}O]ATP species formed at 100% and 2% light intensity are shown by the open bars in Figure 2. The solid bars represent the

Table I: Effect of Light Intensity on Kinetics and Intermediate ATP ⇄ HOH Oxygen Exchange Parameters during ATP Synthesis by Dithiothreitol-Treated and Untreated Thylakoids[a]

light intensity (%)	photophosphorylation rate	reaction reversals for each ATP released	interconversion rate	partition coefficient (P_c)	apparent K_m for ADP (μM)	hexokinase-inaccessible ATP (mol/mol of CF_1)
			Dithiothreitol Present			
100	461	1.1	507	0.52	13	ND
3	27	15	405	0.94	ND[d]	ND
2	16	27	432	0.97	7	0.67
			Dithiothreitol Absent			
100	571	0.79	451	0.45	31	1.0[c]
3	21	3.4	71	0.79	ND	ND
2	9.8	5.2	51	0.84	9[b]	0.96

[a] Light intensity at 100% equaled 5×10^6 erg s^{-1} cm^{-2}. The photophosphorylation and the interconversion rates are shown as micromoles of ATP synthesized per hour per milligram of chlorophyll. Reaction conditions were as given under Materials and Methods. [b] From Vinkler (1981). [c] From Rosen et al. (1979). [d] ND = not determined.

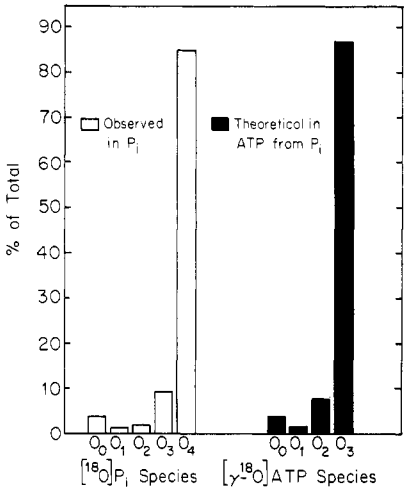


FIGURE 1: Distribution of ^{18}O as observed in the initial medium P_i and as expected (theoretical; Stroop & Boyer, 1985) in the ATP formed from this medium P_i if no oxygen exchange occurs. The designations O_0, O_1, O_2, O_3, and O_4 indicate the species containing 0–4 ^{18}O atoms.

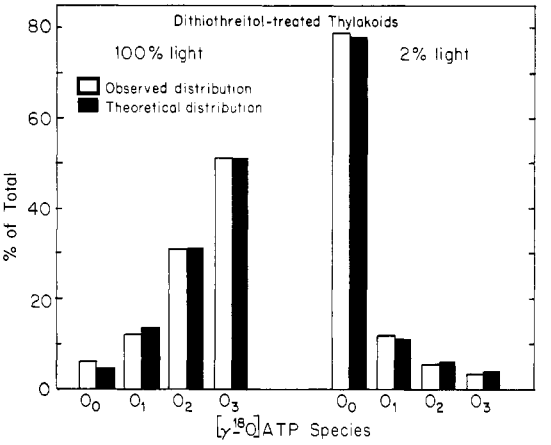


FIGURE 2: Observed and theoretical distributions of $[\gamma\text{-}^{18}O]$ATP species formed by dithiothreitol-treated thylakoids at 100% and 2% light intensity under reaction conditions as given under Materials and Methods. 100% light intensity equals 5×10^6 erg s^{-1} cm^{-2}.

theoretical homogeneous distribution for a single reaction pathway that would account for the total oxygen exchange observed. The observed distributions are close to the theoretical homogeneous distributions, indicating that ATP synthesis occurred essentially by a single mechanism at a given light intensity.

The first part of Table I summarizes the reaction and exchange data for 100%, 3%, and 2% light intensity with dithiothreitol-treated thylakoids. At 100% light, there is a slight exchange of oxygens equivalent to about 1 reaction reversal for each ATP released (row one of Table I). At 2% light, the synthesis rate drops by 97%, and there is a marked exchange of oxygens equivalent to about 27 reaction reversals. The partition coefficient, P_c, increases from 0.52 to 0.97, and from it, the number of reaction reversals can be calculated as mentioned above.

The interconversion rate of bound ATP to bound ADP and P_i with dithiothreitol-treated thylakoids remains relatively constant even though the number of reaction reversals for each ATP released increases almost 30-fold (Tables I and II). The interconversion rate remains relatively unchanged because neither the rate constant of bound ATP hydrolysis nor the bound ATP levels, as discussed below, change significantly with changes in light intensity.

The apparent K_m for ADP in thylakoids without thiol added has been shown to decrease with decrease in light intensity (Vinkler, 1981). The apparent K_m for ADP in dithiothreitol-treated thylakoids also decreases with decrease in light intensity as shown in Table I. The substrate ADP concentrations of 100 μM and 2 mM used in the experiments shown in Tables I and II would be saturating at both high and low light intensity both with and without dithiothreitol addition.

Table II: Effect of Light Intensity on ATP ⇄ H_2O Intermediate Oxygen Exchange during ATP Synthesis by Dithiothreitol-Treated Thylakoids at 2 mM ADP[a]

light intensity (%)	photophosphorylation rate[a]	reaction reversals for each ATP released	interconversion rate
100	568	0.8	454
2	18	27	486

[a] Definitions, units, and conditions are the same as in Table I, except 2 mM ADP, 1.5 mM $[^{18}O]P_i$, and 100 μM diadenylate pentaphosphate were used.

Similar modulation of oxygen exchange by light intensity was also oberved with 2 mM ADP (Table II) and 40 μM ADP (data not shown). An ADP concentration of 40 μM at 2% light increases the exchange (reaction reversals = 44) above that noted with 100 μM ADP (reaction reversals = 27). Lowered light intensity and decreased substrate concentration have an additive effect on the amount of oxygen exchange observed. Increased oxygen exchange observed with dithiothreitol-treated thylakoids during ATP synthesis at low light intensity is due to a hindered release of bound ATP from the catalytic site caused by decreasing protonmotive force.

Oxygen Exchange by Thylakoids without Dithiothreitol Addition. The effect of light intensity on water oxygen in-

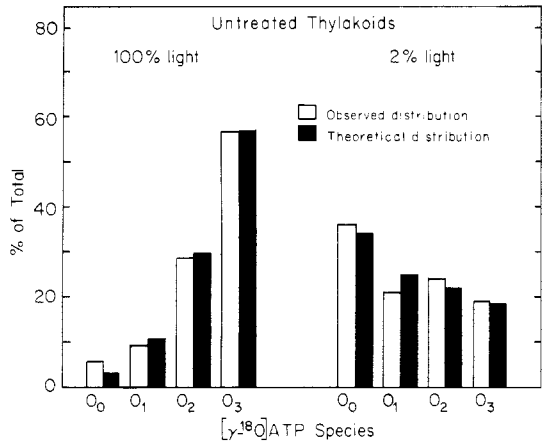


FIGURE 3: Observed and theoretical distributions of [γ-^{18}O]ATP species formed by untreated thylakoids at 100% and 2% light. Conditions were the same as for Figure 2.

corporation into ATP made by thylakoids without dithiothreitol addition is shown by the data in Figure 3 and Table I. The distribution of [γ-^{18}O]ATP species formed at 100% light is similar to that found for dithiothreitol-treated thylakoids (Figure 2). The observed distribution is essentially homogeneous, as predicted for ATP formation by a single reaction pathway. At 2% light, the synthesis rate drops by 98%, and oxygen exchange is increased only up to the equivalent of about 5 reaction reversals for each ATP released in contrast to what was found for dithiothreitol-treated thylakoids (Table I). In addition, there is a slight heterogeneity in the distribution. The most striking change is in the interconversion rate, which drops about 9-fold from 451 to 51 μmol·h^{-1}·mg^{-1}. This is discussed below in relation to the regulation of the APTase activity.

Levels of Bound ATP. The level of enzyme-bound ATP with dithiothreitol present at low light, as measured by the hexokinase-inaccessible ATP method, was 0.67 ± 0.1 (n = 4) per ATP synthase (Table I). This is approximately the same level found by Rosen et al. (1979) at high light intensity with untreated thylakoids. The level of ^{32}P-labeled ATP was measured about 15 s after [^{32}P]P_i addition. As noted by Rosen et al. (1979) and Stroop and Boyer (1985), very little of the slowly labeled, noncatalytic, bound ATP gains ^{32}P under these conditions, and the measurement is essentially that of the steady-state level of bound, catalytic ATP. Measurements of the level of bound ATP by the hexokinase-inaccessible method at low light intensity without dithiothreitol treatment gave a value of 0.96 ± 0.1 (n = 4), slightly greater than that found for dithiothreitol-treated thylakoids.

Discussion

Two characteristics of photophosphorylation are shown by these studies of intermediate ATP ⇄ HOH oxygen exchange at low light intensities. One is that when the rate of net ATP synthesis is markedly reduced by lowered light intensity, ATP formed at catalytic sites is reversibly hydrolyzed many times prior to release. The second is that even though the rate of ATP synthesis is drastically reduced, at least one site on the enzyme retains bound ATP recently formed from medium P_i. These results are in accord with predictions of the binding change mechanism with sequential site participation. When reduced light markedly lowers the protonmotive force, which is necessary for the release of bound ATP by the energy-linked binding change, the ATP remains at the catalytic site and continues to undergo reversible hydrolysis. In addition to these two characteristics, the results also help delineate the role of the enzyme disulfide bond reduction and oxidation in the regulation of the ATPase activity in the absence of light.

Earlier oxygen exchange studies of photophosphorylation showed an increase in the extent of reversible hydrolysis of bound ATP prior to release when ADP or P_i concentrations are lowered (Hackney et al., 1979). This substrate modulation of the oxygen exchange forms an important part of the evidence for the cooperative, sequential participation of catalytic sites as discussed previously (Stroop & Boyer, 1985). The possibility has also been considered that such substrate modulation might be due to separate proposed regulatory sites that cause increased oxygen exchange (Bruist & Hammes, 1982). However, this is made unlikely by the demonstration that only one single-reaction pathway is involved at a given substrate concentration, and not a combination of possible regulated and unregulated pathways. This has been shown for substrate modulation of intermediate ATP ⇄ HOH oxygen exchange both in ATP synthesis by photophosphorylation (Hackney et al., 1979) and in ATP hydrolysis by CF_1 ATPase (Kohlbrenner & Boyer, 1983). It is important to note that the increases in oxygen exchange at low light intensity reported here are observed at both low and high substrate concentrations. Especially relevant to this point are the data of Table II that show that light intensity has a pronounced effect on oxygen exchange even at an ADP concentration of 2 mM. Under such conditions, it seems extremely unlikely that regulatory sites left unoccupied by substrate would be involved in the increased oxygen exchange observed at low light intensity. In contrast, both substrate and light intensity modulation of the oxygen exchange can readily be explained by the binding change mechanism with sequential participation of catalytic sites.

Observations in various laboratories have amply demonstrated that light activates the capacity of the synthase for net photophosphorylation or for dark ATPase activity. A prominent factor in this activation is the cleavage of a disulfide on the γ subunit. Recent studies have focused on the role of light, the γ-subunit disulfide, and Mg^{2+} ions in the regulation of the ATPase activity (Vallejos et al., 1983; Nalin & McCarty, 1984; Schumann et al., 1985; Shahak, 1985, 1986). The reduction of the disulfide in vivo by thioredoxin may play an important role in the synthesis of ATP (Mills et al., 1980; Shahak, 1982). In their earlier studies, Spencer and Wimmer (1985) observed some increase in intermediate ATP ⇄ HOH oxygen exchange accompanying ATP synthesis at low light intensity only if the enzyme was activated by exposure to light and dithiothreitol. In the present studies, using much lower light intensities, we noted a definite stimulation of oxygen exchange at low light intensity even without dithiothreitol activation. With the activated thylakoids, a marked increase in the oxygen exchange was observed, similar to that found with high light intensity but low ADP concentration (Stroop & Boyer, 1985). In contrast to the effect of light intensity on oxygen exchange accompanying ATP synthesis reported here, Sherman and Wimmer (1982) showed no effect of light intensity on oxygen exchange accompanying ATP hydrolysis by activated thylakoids.

The increase in the number of reaction reversals with the activated thylakoids at low light intensity is accompanied by virtually no change in the interconversion rate of bound ATP to bound ADP and P_i from that observed at high light intensity where the net rate of ATP synthesis was 30 times faster. At low light intensity, bound catalytic ATP, as measured by the hexokinase-inacessible method, remains at high levels at catalytic sites and continues to be reversibly hydrolyzed at a constant rate even though the net rate of synthesis is greatly

decreased. Such behavior is similar to that documented more thoroughly in earlier studies for substrate modulation of oxygen exchange during photophoshorylation by chloroplast thylakoids (Hackney et al., 1979; Stroop & Boyer, 1985) or oxidative phosphorylation by submitochondrial particles (O'Neal & Boyer, 1984).

The increased oxygen exchange for each ATP released observed here at low protonmotive force results from a hindrance of the release of bound ATP from a catalytic site where it is in dynamic equilibrium with bound ADP and P_i. The binding change model predicts that energy is primarily required for the release of bound ATP; therefore, decreasing the available energy should hinder release. Our results do not support the interpretation of Spencer and Wimmer (1985) that the interconversion rate constant for bound ATP hydrolysis is increased at low light intensity.

Without added thiol, the increase in oxygen exchange observed with decreasing light intensity is accompanied by a considerable decrease in the interconversion rate of bound ATP to bound ADP and P_i, even though the level of bound ATP at the catalytic sites remains high. Our interpretation is that this decrease in the interconversion rate is related to the regulation of the ATPase activity by light intensity via an oxidized disulfide.

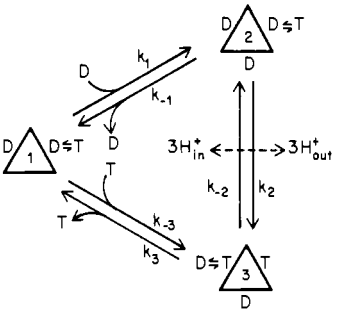
In studies of the effects of oxidants on the ATPase activity in intact chloroplasts, Shahak (1985) proposed a model where light intensity confers a differential sensitivity of the γ-subunit disulfide to oxidants and reductants. The disulfide is sensitive to reductants in the light and to oxidants in the dark. Her data indicate that there is an approximately 10-fold decrease in the dark ATPase activity due to oxidation of the disulfide. It may be significant that the interconversion rate of bound ATP reported here also decreases about 10-fold with deenergization of thylakoids without thiol addition. This decrease could be due either to a decrease in the amount of active synthase contributing to net ATP synthesis or to a decrease in the interconversion rate constant.

The amount of bound ATP labeled by [^{32}P]P_i in 10–15 s remains about constant with decreasing light. This suggests that all the enzyme may be potentially catalytically active. Graber et al. (1977) have proposed that in intact chloroplasts, a fraction of CF_1 (10–20%) works at a maximal rate but that this active fraction migrates statistically between all ATP synthases within about 1–3 s. Thus, at any one time, part of the enzyme may have relatively high activity and is responsible for most ATP synthesis while the remainder of the enzyme has little or no activity. With the disulfide form of the enzyme, a higher fraction may be inactive at low light intensity, accounting for most of the decreased total rate of net ATP synthesis and decreased interconversion rate. In the reduced form, that fraction would not change with light intensity. The other possibility is that all the enzyme is equally active at low light intensity. Then the decrease in the interconversion rate of bound ATP could be attributed to a decrease in the interconversion rate constant for hydrolysis of bound ATP with decreasing light intensity.

Our observations on the effects of dithiothreitol harmonize with those of others mentioned earlier and demonstrate that reduction of the γ-subunit disulfide bond causes a fundamental change in the interaction of the ATP synthase with the electrochemical potential.

An additional and important aspect of our data pertains to the total rate of ATP synthesis at the catalytic site. The total rate of ATP formation at the catalytic site is the sum of the interconversion rate and the rate of release of ATP to the medium as measured by net synthesis. As noted from the data in Table I, this total rate is at least twice as great at high as compared to low light intensity. An important requirement for the binding change mechanism is that proton translocation across the membrane cannot occur unless bound ATP is at a catalytic site ready to be released. The total rate of ATP formation can be accounted for if the synthase is induced by incipient proton translocation to increase the interconversion rate of bound ADP and P_i to bound ATP, and the conformational signals induced by the presence of bound ATP allow proton translocation to occur so that the binding changes leading to ATP release are completed. Such requirements for the synthesis of ATP by mitochondria have been noted earlier (O'Neal & Boyer, 1984).

Scheme I: Sequential Participation of Three Catalytic Sites in the ATP Synthase Reaction with Excess ADP and P_i Present[a]



[a] D = ADP + P_i; T = ATP; D ⇄ T = tightly bound, interconverting D and T. In this scheme, the site that becomes vacant after release of product ATP is arbitrarily put at the bottom of the triangle (form 1). At the end of each cycle, the enzyme will have an empty site different from that at the start of a cycle but will have the same reaction properties. Three cycles will be required to return a given site to its original status.

Simple depictions of events for sequential catalysis with continued oxygen exchange at low light intensity or low substrate concentration can be useful for better understanding of the catalytic process. Schemes presented earlier are useful to visualize principal reaction stages but need additional steps to account for the occurrence of oxygen exchange at all stages of catalysis, leading to complex kinetic equations. Another approach we have found useful is to formulate separate relationships for net reaction velocity and for oxygen exchange and then combine these for relationships governing oxygen exchange. One principal advantage of this approach is that even though each of the three catalytic sites is proposed to pass sequentially through catalytic steps, if all sites behave identically, the net velocity equation becomes strikingly simple.

Reaction Scheme I accommodating the above suggestions is depicted here, where the sides of the triangle depict the three catalytic sites that participate in sequence. The "T" denotes ATP, the "D" denotes ADP + P_i, and the "D ⇄ T" denotes the tightly bound ATP in dynamic equilibrium with ADP + P_i. The interconversion rate of D to T at the D ⇄ T site is considered to be constant, independent of substrate concentration or electrochemical potential. Excess substrate is considered to be present so that three-site catalysis prevails, and addition of P_i or ADP is not considered as separate steps. These assumptions simplify the kinetic equations and are satisfactory for the purpose of this paper. Constants k_1 and k_{-1} are the binding and dissociation rate constants for substrate. Constants k_2 and k_{-2} are the rate constants for the energy-linked binding change. They are not interconversion rate constants of bound ATP in this scheme. Constant k_3 and k_{-3} govern release and binding of ATP from a loose binding site. Note that in the binding change step, D ⇄ T at a catalytic

site is converted in a single step to T and that concomitantly D at another site is converted to tight-interconverting D $\rightleftarrows$ T. This accommodates the need for tightly bound ATP to be present at one site and loosely bound ADP and P_i at another for the binding change to be initiated and completed. In each cycle, a different catalytic site yields ATP to the medium, with a given catalytic site repeating its sequence every three cycles. Even though three sites are involved, the kinetics can be accommodated by this simple three-step mechanism. Not depicted in this minimal three-site scheme is the possible coupled, charged group migration that may occur in the F_0 portion during the binding change (Boyer, 1985). Such migration may mean that, for a more complete cycle, a step giving reorientation of such charged groups in the membrane may be necessary, corresponding to the E_1 and E_2 forms of the Na^+, K^+, and Ca^{2+} transport ATPases.

Scheme I readily accounts for the effect of light intensity on net ATP synthesis if decreased protonmotive force is regarded as decreasing the value of k_2 and increasing the value of k_{-2}. With respect to substrate concentration effects, only one apparent K_m is observed with ADP concentrations above about 5 μM (Stroop & Boyer, 1985), so that the simple Michaelis–Menten equation $v = V_{max}S/(K_m + S)$ applies, with $V_{max} = k_2k_3E_t/(k_2 + k_{-2} + k_3)$ and $K_m = (k_{-1}k_{-2} + k_{-1}k_3 + k_{-2}k_3)/(k_1k_2 + k_1k_{-2} + k_1k_3)$. When ADP concentrations are below about 5 μM, another apparent K_m with a much lower V_{max} becomes operative, and Scheme I would need to be expanded to include a reaction sequence with only 1 and 2, or possibly only 0 and 1, catalytic sites with reactants bound.

A separate relationship can be formulated to relate oxygen exchange parameters to net ATP formation. This is simply done if, as the data suggest, a rate constant, k_{ex}, for hydrolysis of bound ATP remains about constant even though substrate concentration or light intensity is varied. Thus, the rate of oxygen exchange is given by $k_{ex}E_t$ because exchange continues at one of the catalytic sites with all forms of the enzyme. The value for the partition coefficient (P_c, the probability of bound ATP entering into the exchange reaction) is given by $P_c = k_{ex}/(k_{ex} + v)$. When light intensity is lowered, or if substrate concentration is lowered, v will drop and P_c will increase with concomitant increase in oxygen exchange.

ACKNOWLEDGMENTS

We thank Drs. Chanock Carmeli and Richard Cross for valuable discussions and Kerstin Stempel for excellent technical assistance.

Registry No. ATP, 56-65-5; ADP, 58-64-0; ATP synthase, 37205-63-3; O_2, 7782-44-7; dithiothreitol, 3483-12-3.

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