Characteristics of the Chloroplast ATP Synthase As Revealed by Reaction at Low ADP Concentrations†

Steven D. Stroop‡ and Paul D. Boyer*

Molecular Biology Institute and Department of Chemistry and Biochemistry, University of California—Los Angeles, Los Angeles, California 90024

Received July 19, 1984

ABSTRACT: The chloroplast ATP synthase at low ADP concentrations has reaction characteristics that are in accord with the binding change mechanism for ATP synthesis with sequential participation of catalytic sites. The dependence of the photophosphorylation rate on ADP concentration is distinctly biphasic, with apparent K_m values of 0.62 and 31 μM and V_m values of 37 and 907 μmol (mg of chlorophyll)$^{-1}$ h^{-1}. As ADP concentration is decreased to below 1 μM, the extent of water–oxygen incorporation into ATP reaches a maximum, indicating over 50 reversals of the formation of bound ATP prior to its release; as ADP concentration is increased, reversals decrease to an average of about 0.8 per ATP released. The distributions of [γ-^{18}O]ATP species formed at low, intermediate, and high ADP concentrations are as predicted for single reaction pathways. The amount of ATP transiently bound to a catalytic site decreases to less than one per synthase as the ADP concentration drops below 4 μM. These data support a mechanism in which a catalytic site, largely filled at 1–4 μM ADP, continues to reversibly form ATP which is released very slowly unless P_i and ADP bind to another catalytic site on the synthase. The release of ATP still contributes appreciably to rate limitation even at saturating ADP concentrations.

The mechanism of action of the ATP synthase from chloroplasts, mitochondria, and microorganisms continues to be the focus of many studies. This paper reports experiments designed to further evaluate the binding change mechanism for ATP synthesis by chloroplast thylakoids during photophosphorylation. Previous measurements of the dependence of the rate of photophosphorylation on ADP concentration have been interpreted as showing simple hyperbolic behavior [see Schlodder et al. (1982)]. Such behavior is not as expected if there is prominent cooperativity of catalytic sites on the enzyme. Evidence for catalytic site cooperativity is given by observations of increased water–oxygen incorporation into each ATP formed as ADP concentration is lowered as if ATP is not released from one catalytic site until ADP and P_i bind at another site (Hackney et al., 1979; Kohlbrenner & Boyer, 1983). These results suggest that, at substrate concentrations considerably below those required for half-maximal velocity, a slow photophosphorylation rate from single-site catalysis might be observed. This would be in accord with the binding change mechanism for ATP synthesis with sequential participation of catalytic sites on the synthases.[1] Initial velocity behavior and other reaction characteristics should be evident at low ADP concentrations which would not be expected for an enzyme showing Michaelis–Menten behavior.

Experiments are reported here on the rate of photophosphorylation as a function of ADP concentration, on the levels of bound catalytic ATP, and on water–oxygen exchange characteristics as a function of ADP concentration. The data obtained are consistent with site–site interactions resulting in prominent negative cooperativity of substrate binding and positive cooperativity of catalysis as in the binding change mechanism.

Materials and Methods

Materials. Chloroplast thylakoid membranes were prepared from market spinach as described by McCarty & Racker (1967) with an additional wash step to reduce endogenous ADP concentrations. The grinding and washing mix 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 dialyzed against *N*-[tris(hydroxymethyl)methyl]glycine 37.5 mM (tricine) (pH 8) before use. Highly enriched [γ-^{18}O]ATP was prepared from [^{18}O]carbamoyl phosphate and ADP by an adaptation of the method of Mokrasch et al. (1960) for the synthesis of [^{32}P]-ATP. To minimize phosphate contamination during these studies, acid-washed glassware and disposable plastic ware were used for all experimental procedures, and HCl solutions were prepared from glass-distilled HCl. All solutions were made with reverse osmosis deionized water.

Photophosphorylation. The reactions were carried out at pH 8 and room temperature in a mixture containing 7.5 mM $MgCl_2$, 37.5 mM NaCl, 10 mM or 1.5 mM [^{18}O,^{32}P]P_i, 33 μM phenazine methosulfate, 37.5 mM tricine/NaOH, 37.5 mM glucose, approximately 20 units/mL hexokinase, and varying concentrations of ADP. This system regenerates ADP at a constant concentration as glucose 6-phosphate is formed. Assay mixtures contained approximately 40 μg of chlorophyll/mL (about 50 nM synthase) with volumes ranging from 1 to 3 mL and were illuminated up to 5 min by a projector lamp providing about 300 W of heat-filtered white light. A typical rate of photophosphorylation, measured by [^{32}P]P_i appearance in glucose 6-phosphate, was 600–800 μmol (mg of chlorophyll)$^{-1}$ h^{-1}. The chlorophyll content was determined by the method of Arnon (1949). ATP synthase concentrations

† Supported in part by Department of Energy Contract DE-AT03-76ER70102.

* Address correspondence to this author at the Molecular Biology Institute.

‡ U.S. PHS Predoctoral Trainee.

[1] The designation as sequential participation instead of alternating participation of catalytic sites may be preferable because it is likely that three sites participate in catalysis and the term alternating can be interpreted as involving only two sites.

0006-2960/85/0424-2304$01.50/0

were taken as 1.3 nmol of synthase/mg of chlorophyll as estimated by Strotmann et al. (1973). The amount of unenriched P_i contamination introduced by the isolation and degradation of the glucose 6-phosphate was estimated by comparing the specific activity of the final isolated $^{32}P_i$ with that of the original substrate medium $^{32}P_i$. $^{32}P_i$ was assayed by using Cerenkov counting in water. Inorganic phosphate was measured by the catalyzed phosphate assay of Ohnishi & Gall (1978). ADP was measured by using a pyruvate kinase/NADH coupling assay (Pullman et al., 1960). Contamination of added [^{18}O]P_i by nonisotopic P_i averaged only 6 nmol of P_i per sample for the entire procedure. Corrections for this small contamination were not necessary.

Separation of Medium P_i and Glucose 6-Phosphate. Reactions were quenched with an equal volume of 1 M perchloric acid and put on ice. The precipitate was removed by centrifugation, and the nucleotides were adsorbed to acid-washed charcoal. The bulk of the charcoal was removed by centrifugation and the supernatant filtered through 0.45-μm disposable filters. Glucose 6-phosphate was isolated and degraded to yield P_i as described by D. Hackney (personal communication). Medium P_i was separated from glucose 6-phosphate by three extractions of the phosphomolybdate complex using 2-methyl-1-propanol/benzene (1:1 v:v) (Hackney et al., 1980). The lower aqueous layer containing the glucose 6-phosphate was applied to a 0.7 × 5 cm column of Bio-Rad AG 1-X4 100–200 mesh anion-exchange resin. Samples with reaction volumes greater than 2 mL required larger columns. The column was washed with 8 mL of 0.5 M HCl and then with 50 mM tris(hydroxymethyl)aminomethane (Tris)/10 mM HCl until the eluate reached pH 7. Glucose 6-phosphate was eluted with 6 mL of 50 mM Tris/10 mM HCl/200 mM KCl. P_i was cleaved from the glucose 6-phosphate by boiling in 0.15 M KOH for 10 min. The solution was neutralized, acidified to about 0.4 M, and made 10 mM in molybdate, and the phosphomolybdic acid was removed by three extractions with solvent. The [^{32}P]P_i was back-extracted into 3 mL of 0.2 M Tris, diluted to 15 mL with H_2O, and absorbed on a 0.5 × 3 cm column of AG 1-X4 100–200 mesh anion-exchange resin. The column was washed with H_2O, 2 mL of 20 mM $MgCl_2$, 2 mL of 20 mM KCl, and then 10 mM HCl until the eluate reached pH 3. The P_i was eluted with 3 mL of 30 mM HCl, the solution lyophilized, and the P_i analyzed for ^{18}O content.

^{18}O Measurements. [^{18}O]P_i was converted to triethyl phosphate with diazoethane and analzyed with a Hewlett-Packard 5995 gas chromatograph/mass spectrometer (O'Neal et al., 1983). 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. Calculations were made as described elsewhere (Hackney et al., 1980; O'Neal et al., 1983). All statistical variations were calculated as standard deviation.

Estimation of the Number of Water Oxygens Incorporated into Each ATP Released. The expected ^{18}O enrichment for ATP produced by photophosphorylation from ADP and [^{18}O]P_i if no oxygen exchange had occurred was determined theoretically from the distribution of ^{18}O in the medium [^{18}O]P_i. Correction was made for the random loss of one P_i oxygen as required for ATP synthesis, taking into account the probabilities of that loss changing the distribution of ^{18}O in the [^{18}O]ATP produced if there were no oxygen exchange. For example, if 20% of the medium P_i consisted of the species with three ^{18}O atoms, $P^{18}O_3{}^{16}O_1$, the fraction of ATP derived from this P_i containing two ^{18}O atoms would be $0.2 \times {}^3/_4$. A computer algorithm was written for this calculation. This distribution was compared with the experimental distributions to determine the amount of water–oxygen exchange.

Hexokinase-Inaccessible ATP. This was determined essentially as described by Rosen et al. (1979). The medium phosphate concentration used was 150 μM in order to limit the rate of synthesis and ensure efficient hexokinase trapping. Reactions were pulsed for 10 s with [^{32}P]P_i and then quenched. A P_i chase of 14 μmol was added for less than 5 s to some reactions before quenching.

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. Rates were shown to be linear for the time periods of measurement. The glucose 6-[^{32}P]phosphate and [γ-^{32}P]ATP were isolated from [^{32}P]P_i by the molybdate–triethylamine precipitation method of Grubmeyer & Penefsky (1981).

RESULTS

Some ^{18}O Exchange Controls. Our objective was to carefully quantitate the extent of *intermediate* ATP ⇄ HOH oxygen exchange (that occurring when medium P_i is converted to medium ATP). Therefore, it was necessary to check whether any *medium* P_i ⇄ HOH or *medium* ATP ⇄ HOH oxygen exchange occurred under the conditions used for photophosphorylation.

To check for medium P_i ⇄ HOH oxygen exchange photophosphorylation was done with 1.5 mM [^{18}O]P_i (98.4 ± 0.5 atom % ^{18}O) and 0.4 μM added ADP in a volume of 2 mL. Under these conditions extensive intermediate ATP ⇄ HOH oxygen exchange occurs. The medium P_i isolated from a zero-time control contained an average enrichment of 93.3 ± 2.4%; the slight decrease in total ^{18}O results from dilution by nonisotopic P_i from all reaction components. A sample of medium P_i analyzed after reacting for 5 min, sufficient time to give the amount of glucose 6-phosphate required for ^{18}O analysis, had an average enrichment of 93.7 ± 1.1%. Medium P_i ⇄ HOH oxygen exchange was thus negligible under our experimental conditions.

To check for medium ATP ⇄ HOH oxygen exchange, photophosphorylation was carried out with 10 μM ADP and 10 mM unenriched P_i in a volume of 1 mL. As photophosphorylation proceeded, [γ-^{18}O]ATP was added from a syringe pump for 1 min. The amount of [^{18}O]ATP added was about 14% of that formed by photophosphorylation. It was anticipated that the hexokinase–glucose trap would remove both the nonenriched ATP and the added [^{18}O]ATP rapidly enough so that no measurable medium ATP ⇄ HOH exchange would occur. Such exchange, based on the observed P_c at 10 μM ADP (Table I) would be expected to cause a significant change in the ratios of glucose 6-phosphate species containing one or two ^{18}O atoms to the species with three ^{18}O atoms present; i.e., $P^{18}O_3$ would decrease while $P^{18}O_1$ and $P^{18}O_2$ would increase. No change in these ratios was observed. We thus conclude that neither medium P_i ⇄ HOH nor medium ATP ⇄ HOH oxygen exchange made any significant contribution to the total ^{18}O exchange observed. All exchange was thus attributable to the intermediate ATP ⇄ HOH oxygen exchange accompanying net ATP synthesis.

Extent of Oxygen Exchange as ADP Concentration Is Lowered. If an upper limit of ^{18}O exchange is reached as ADP concentration is lowered, we needed to know how much of the ADP present in the reaction mixture components might participate in photophosphorylation. This could not be assessed by measuring the ADP released upon acid denaturation and extraction as some ADP bound to the thylakoids, or even to the hexokinase present, might not be available for photo-

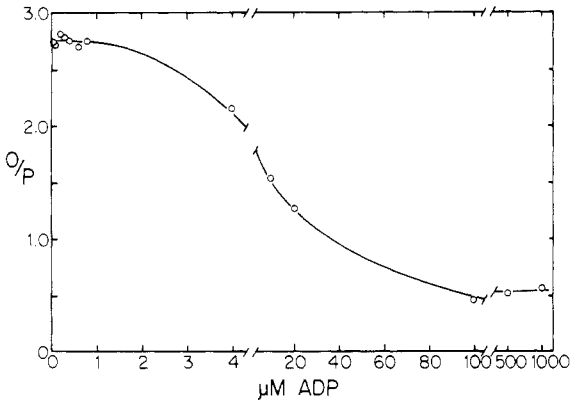

FIGURE 1: Increase of water–oxygen incorporation into ATP formed by the chloroplast ATP synthase as the ADP concentration is decreased. Experimental conditions are given in the text.

phosphorylation. An estimation of the amount of available ADP was made by measurement of the rate of photophosphorylation with 0, 0.2, or 0.4 μM added ADP. The rates were 0.01, 0.21, and 0.45 nmol/s. Other experiments showed that rates were linear for at least 5 min, but with very low ADP net photophosphorylation ceased after about 10 min. The approximate doubling in rate with doubling of the added ADP concentration and the observed rate without added ADP (less than $^1/_{10}$ of that with only 0.2 μM added ADP) indicate that less than 0.02 μM ADP was contributed by the reaction components. This amounts to less than 0.5 mol of ADP per CF_1 ATPase on the thylakoids. The added ADP could thus be taken as the effective ADP concentration for our experimental conditions. The extent of oxygen exchange at different ADP concentrations was measured in a series of experiments on different days and with different thylakoid preparations. This was done because individual experiments are time consuming, and it was established by using thylakoid preparations of differing activities that the essential characteristics being studied, the amount of oxygen exchange per ATP made, appeared to be independent of the maximum rate of photophosphorylation of a given chloroplast thylakoid preparation. At the lowest substrate concentrations tested, a reaction volume of 4 mL and a 5-min reaction time were needed to give the about 35 nmol of glucose 6-phosphate required for ^{18}O analysis. At saturating ADP concentrations a 1-mL reaction volume and 1 min of photophosphorylation gave more than sufficient glucose 6-phosphate for analysis.

Figure 1 shows the extent of oxygen exchange at increasing ADP concentrations, plotted as the O/P ratio (the average number of water oxygens in the γ-phosphoryl of each ATP made). Data for the lowest ADP concentrations indicate that an upper limit for the O/P value of 2.75 ± 0.04 is being reached. At high substrate concentration the O/P ratio decreases to a limit of 0.52 ± 0.05.

Table I gives these O/P values together with several parameters that were calculated from the measurements of oxygen exchange and net rate of ATP formation. Agreement within an experimental series at a given ADP concentration is in general better than that between different ADP concentrations, but the dominant trend of ADP concentration effects is clearly evident. The results show that as ADP concentration is lowered, the ATP formed at the catalytic site is increasingly cleaved and synthesized up to a maximum of 50 times before being released. This upper limit of oxygen exchange is reached when the ADP concentration is reduced to less than 1 μM. The oxygen exchange at low ADP levels becomes independent of ADP concentration as if single site

Table I: Effects of ADP Concentration on Parameters Calculated from Oxygen Exchange Measurements[a]

[ADP] (μM)	O/P	P_c	R	ρ[b]
1000	0.57 ± 0.02	0.48	0.9	780
500	0.52 ± 0.02	0.45	0.8	680
100	0.47 ± 0.02	0.43	0.7	570
20	1.27 ± 0.05	0.75	2.9	1150
10	1.53 ± 0.01	0.81	4.1	900
4	2.15 ± 0.02	0.91	10	1000
0.8	2.75 ± 0.01	0.98	50	980
0.6	2.70 ± 0.08	0.97	36	610
0.4	2.75 ± 0.03	0.98	50	650
0.3	2.78 ± 0.01	0.98	50	620
0.2	2.81 ± 0.03	0.98	50	530
0.1	2.71 ± 0.03	0.97	36	190
0.05	2.74 ± 0.02	0.98	50	110

[a] Data shown were calculated from three or more analyses. O/P is the average number of water oxygens in the γ-phosphoryl of each ATP synthesized. The partition coefficient, P_c, is the probability of bound ATP undergoing oxygen exchange prior to release. R is the number of reversals of bound ATP formation prior to release of ATP. The P_c and R values are calculated from the distribution of [^{18}O]ATP species formed as described previously (Hackney et al., 1980). The rate of reversal, ρ, is the product of the reversals, R, and the rate of ATP synthesis from Figure 3. [b] Units: μmol (mg of chlorophyll)$^{-1}$ h^{-1}.

catalysis is occurring. At near saturating ADP and P_i there is still nearly one reversal of formation of bound ATP before its release. This is in agreement with earlier findings (Shavit et al., 1967; Avron et al., 1965; Kohlbrenner & Boyer, 1983).

The rate of bound-ATP formation and cleavage, ρ (Table I), also shows the striking fact that the rate of this reaction reversal remains approximately constant although there is an over 90-fold increase in the net reaction velocity as the ADP concentration is increased from 0.2 to 1000 μM. This is consistent with each synthase always having a bound ATP present that is subject to a constant rate of cleavage. The fall in the observed rate of reaction reversal, as the concentration of added ADP is lowered to 0.1 μM or less, could represent the decreased occupancy of single cataltyic sites. These results together with the data on hexokinase-inaccessible ATP are considered further under Discussion.

Distribution of [^{18}O]*ATP Species Formed.* If catalytic sites participate sequentially as suggested for the binding change mechanism, the distribution of [γ-^{18}O]ATP species formed should be homogeneous as predicted for a single reaction pathway at all ADP concentrations. This was shown to be the case for photophosphorylation at 20 μM ADP in the experiments of Hackney et al. (1979). The distributions observed for photophosphorylation at a low (0.4 μM) and a relatively high (500 μM) ADP concentration are shown in Figure 2. They correspond to those expected for a single catalytic pathway. The results allow approximation of the expected distributions at various ADP levels if ATP hydrolysis was occurring by action of two independent catalytic sites with characteristics as indicated by initial velocity data given in the following section. Such independent sites should give rise to detectable heterogeneity of [^{18}O]ATP species for the 0.4 and 500 μM ADP samples. But a more sensitive test for two pathways of P_i formation is provided by an assay at an intermediate ADP level. At 10 μM ADP about the same amounts of ATP should be formed by each postulated independent catalytic site. Figure 3 shows the observed pattern of [^{18}O]ATP species formed, that predicted for the binding change mechanism with cooperative catalytic sites and that predicted for independent catalytic sites with exchange characteristics as given for the low and high ADP in Figure 2. The observed distribution corresponds well to that predicted for a single reaction pathway, as in the binding change

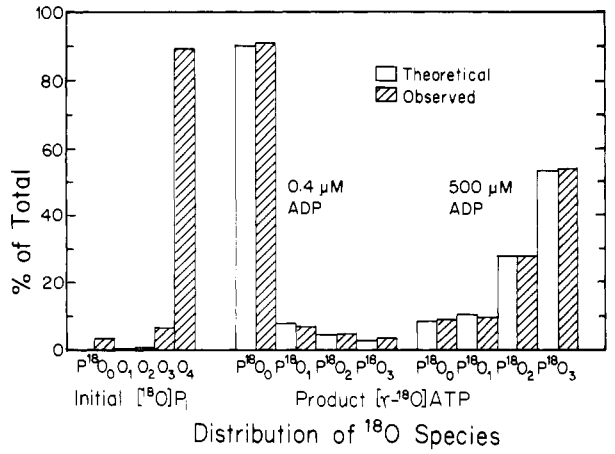

FIGURE 2: Observed and theoretical distributions of ^{18}O in ATP formed by the chloroplast ATP synthase at low and high ADP concentrations where upper and lower limits of oxygen exchange are found. The ATP species formed from $[^{18}O]P_i$ at 0.4 and 500 μM ADP are disignated as $P^{18}O_0$, $P^{18}O_1$, $P^{18}O_2$, and $P^{18}O_3$ according to the number of ^{18}O atoms present in the γ-phosphoryl group.

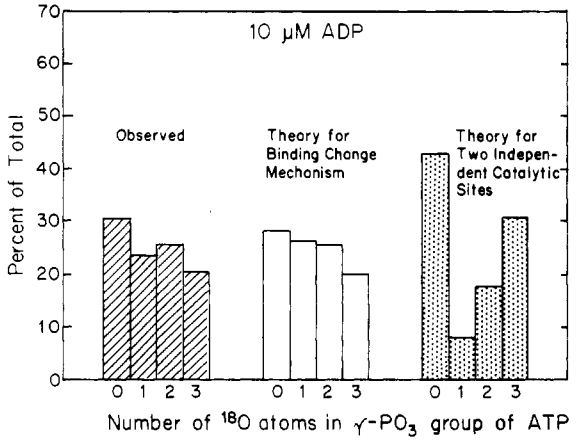

FIGURE 3: Observed and theoretical distributions of ^{18}O in ATP formed at an intermediate ADP concentration where oxygen exchange is between the upper and lower limit.

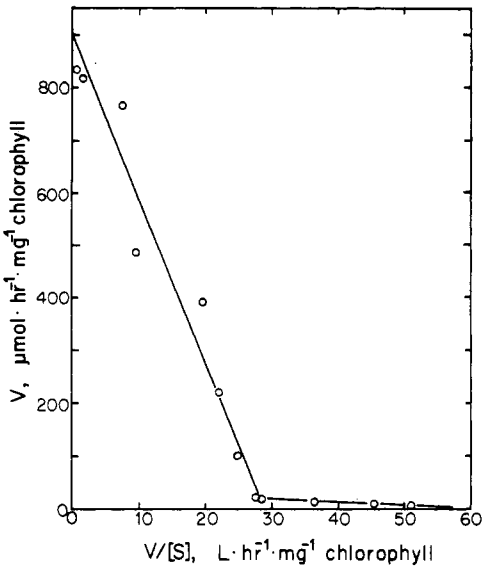

FIGURE 4: Effect of a wide range of ADP concentrations on the rate of photophosphorylation as demonstrated by an Eadie–Hofstee plot.

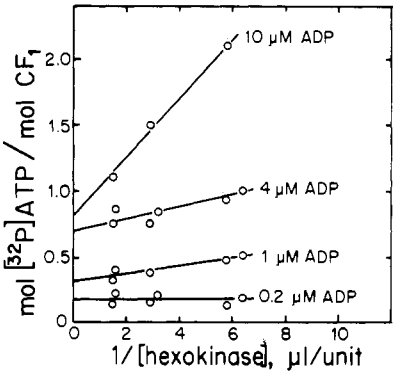

FIGURE 5: Measurement of the retention of bound ATP at catalytic sites during photophosphorylation at low ADP concentrations. Reactions were pulsed with $^{32}P_i$ approximately 10 s prior to quenching. Extrapolation to 1/[hexokinase] equals zero gives a measure of the bound ATP at catalytic sites (Rosen et al., 1979).

mechanism. This confirms and extends the earlier data of Hackney et al. (1979). There is a quite small deviation of the distribution at 10 μM ADP from the theoretical prediction. The nature of the deviation suggests that there could be a small amount of photophosphorylation occurring with a high level of exchange as in single-site catalysis. However, additional experimentation would be necessary to determine if this is the case.

ADP Concentration–Velocity Relationships. If slow single-site catalysis occurs when the ADP concentration is lowered below 1 μM, as indicated by the preceding results and as predicted by the binding change mechanism, then substrate-binding properties should be reflected by initial velocity measurements. To our knowledge, initial velocity measurements at ADP concentrations far below those required for half-maximal velocity have not been reported.

Figure 4 is an Eadie–Hofstee plot of velocity measurements over a 10 000-fold range of ADP concentrations from 0.1 μM to 1 mM. As predicted, distinctly biphasic behavior is observed. From this plot we estimate an $S_{0.5}$ of 31 μM for the high substrate range and 0.62 μM for the low substrate range. Similar values are obtained from $1/v$ vs. $1/S$ plots. Corresponding V_{max} values are 907 and 37 μmol (mg of chlorophyll)$^{-1}$ h^{-1}.

The higher $S_{0.5}$ value of 31 μM corresponds to the apparent K_m for photophosphorylation that has been frequently observed (Bennum & Avron, 1965; Harvey & Brown, 1969; Ryrie & Jagendorf, 1971). The lower $S_{0.5}$ points to the presence of catalytic sites with a high affinity for ADP. These findings are as expected for a negative cooperativity of ADP binding. At the lower ADP concentrations reversal of bound ATP formation at catalytic sites continues, but net catalysis is slow because the ATP is released slowly. With higher ADP concentrations the rate of ATP release is rapid. The negative cooperativity of ADP binding is accompanied by a positive cooperativity of catalysis.

Level of Bound, Catalytic ADP at Low ADP Concentrations. If the low $S_{0.5}$ and V_{max} reported in this paper for less than micromolar ADP concentrations reflect single-site catalysis of tightly coupled alternating sites, then when ADP concentrations are above 1 μM, at least one catalytic site per synthase should be filled with bound ADP or ATP. As the ADP concentration is reduced, to less than the $S_{0.5}$ value of 0.6 μM, a corresponding decrease in the fraction of catalytic sites retaining bound ATP and ADP should be evident. To measure the amount of ATP that is transiently tightly bound during net ATP synthesis, we used the hexokinase-accessibility technique as described by Rosen et al. (1979). This is based on the measurement of the total amount of ATP in the reaction medium (after an acid quench) as the hexokinase concentration is increased to high levels and extrapolated to infinite hexokinase.

The results given in Figure 5 demonstrate that when the ADP concentration is 1 μM or less, the level of hexokinase-inaccessible ATP fails. This is as expected if the enzyme shows a negative cooperativity of ADP binding and has a high-affinity cataltyic site with a K_d for ADP of about 1 μM.

Under some conditions, a portion of the newly formed ATP released from catalytic sites appears to bind to sluggish or to noncatalytic sites in preference to reacting with hexokinase (Shavit, 1980; Smith et al., 1983). Thus, all the hexokinase-inaccessible ATP may not be at the sites responsible for most of the net catalysis. Transfer of ATP to sluggish or noncatalytic sites is expected to be small at the low photophosphorylation rate and high hexokinse concentrations used in these experiments. Rosen et al. (1979) demonstrated that several seconds were required for labeling of such sites even with 1 mM ADP. We observed that with 4 μM ADP that over 85% of the hexokinase-inaccessible ATP disappeared within a few seconds after a 50-fold excess of unlabeled P_i was added. The total amount of bound ATP decreases markedly when medium ADP drops below 1 μM, and the amounts observed are an upper limit. The conditions of the formation of this bound ATP and its properties give assurance that it is mostly at sites involved in the principal catalytic pathway.

Discussion

The measurements at low ADP concentrations, of the net rate of photophosphorylation, of the amount of ATP bound at catalytic sites, and of the water–oxygen incorporation into ATP formed reveal a behavior of the chloroplast ATP synthase consistent with the predictions based on the binding change mechanism with sequential catalytic site participation. Catalytic sites with low turnover capacity are present on the synthase and approach saturation at around 1 μM ADP. The reversible interconversion of bound ADP and P_i to bound ATP continues at this site at about the same rate as when additional catalytic sites are filled at much higher ADP concentrations. The release of a tightly bound ATP is definitely rate limiting.

The chloroplast ATPase likely has an $\alpha_3\beta_3\gamma\delta\epsilon$ subunit stoichiometry (Merchant et al., 1983; Moroney et al., 1983) and thus three potential catalytic sites. The results reported here can be accommodated by sequential participation of only two catalytic sites. Possibly only two of the three potential catalytic sites on the synthase are operative, but for reasons of symmetry of function we feel it is likely that all sites participate equally. A more reasonable possibility is that the rate with two sites occupied is not much less than that with three sites occupied. In addition, negative cooperativity of binding may offset the effects of positive catalytic cooperativity on the reaction velocity. Differences in apparent K_m values for the filling of the second and third sites would not be revealed by our limited data.

The behavior when the ADP concentration is 1 μM or less could reflect single-site catalysis, but our data are insufficient to establish this possibility. Although the fall in the level of hexokinase-inaccessible ATP when ADP is reduced below 4 μM is consistent with the occurrence of single-site catalysis, present measurements can only be regarded as approximations of the number of participating sites per synthase. There is uncertainty not only in the extrapolation to infinite hexokinase but also in the equilibrium between bound ATP and ADP + P_i and in the total concentration of potentially active sites present. Similar data with the mitochondrial F_1 ATPase indicated that single-site catalysis might occur with 0.1–1 μM ATP (Gresser et al., 1982), but the remarkably high affinity of the ATPase for the first ATP bound (Grubmeyer et al., 1982) makes it likely that two-site catalysis was being observed.

The upper limit of oxygen exchange found at low ADP concentrations can be used to estimate the change in the ATP off-rate that occurs when ADP + P_i binding allows protonmotive force energy to be used. The ATP formed at the catalytic site is being released about 50 times more slowly at low ADP concentrations than at high ADP concentrations as shown by the change in reversals in Table I. This is still a much more rapid release than that of the tightly bound ATP at the catalytic site of deenergized thylakoids as observed by Smith et al. (1983). The higher rate with the energized thylakoids may represent the ability of protonmotive force to drive the energy-linked binding change without substrates at an alternate site. This would lead to a waste of energy because bound ADP and P_i would not be converted to bound ATP when the ATP is released from an alternate site. However, a control system that allowed energy-using steps to be nonproductive at these very low substrate concentrations would still satisfy biological requirements, in particular, because photophosphorylation would be occurring in vivo at ADP concentrations above 250 μM (Santarius & Heber, 1965).

When isolated CF_1 ATPase cleaves low concentrations of ATP, the rate of reversal of cleavage of bound ATP, from the data of Kohlbrenner & Boyer (1983), is about 2 mol s^{-1} (mol of $CF_1)^{-1}$. However, during synthesis (see Table I) the rate of reversal is about 190 mol s^{-1} (mol of membrane-bound $CF_1)^{-1}$. It is apparent that the combination of the CF_1 with the membrane and energization of the membrane considerably increase the rate of interconversion of bound substrate. The poorly understood factors that contribute to the relatively low ATPase activity of CF_1 likely also limit its rate of oxygen exchange.

Although substrate binding and product release are the principal energy-requiring steps, energization may affect the interconversion step some. The net rate of ATP synthesis for a chloroplast thylakoid preparation at high substrate is about 800 nmol (mg of chlorophyll)$^{-1}$ h^{-1} and is equivalent to about 170 mol s^{-1} (mol of ATP synthase)$^{-1}$, or about the same as the rate of reversal of bound ATP cleavage; therefore, the energy-linked conformational transition leading to the release of bound ATP must occur only when ATP and not ADP + P_i is present at the catalytic site, or the equilibrium must be shifted in favor of ATP during the conformational transition. Some effect on the equilibrium interconversion by energization is indicated by the data of Sherman & Wimmer (1983) showing a decrease in oxygen exchange during ATP hydrolysis by chloroplast thylakoids in the presence of uncouplers. This decrease could, however, result from the factors that decrease CF_1 ATPase activity in contrast to ATP synthase activity.

The behavior reported here for chloroplast photophosphorylation is similar to related observations with mitochondrial oxidative phosphorylation. For example, Hackney & Boyer (1978) noted that lowering the ADP concentration to 5 μM with the mitochondrial enzyme increased the number of reversals of bound ATP formation to about 80, and Gresser et al. (1979) showed that nearly 0.6 ATP/mol of bound F_1 ATPase on submitochondrial particles remains inaccessible to hexokinase during net oxidative phosphorylation at 1 μM ADP.

The ^{18}O exchange data in this paper could by themselves be explained by the presence of two types of synthase, one fully active at low ADP concentration giving lots of exchange and another type capable of much more rapid reaction but requiring high ADP concentrations. But such a possibility is ruled out by the earlier experiments of Hackney et al. (1979) showing that a single reaction pathway was involved at ADP

concentrations giving intermediate levels of oxygen exchange and also is shown by the results presented here.

Besides the ATP that is transiently tightly bound at catalytic sites, ATP synthase preparations also have a tightly bound ATP that is labeled too slowly from medium P_i to be on the main catalytic pathway (Rosen et al., 1979; Shavit, 1980; Alflalo & Shavit, 1984). Such slowly labeled ATP may be either at noncatalytic sites or at catalytic sites of sluggish or inactive synthase. The presence of at least two types of slowly labeled ATP that remain tightly bound in isolated thylakoids has led to some confusion. It is thus pertinent to briefly summarize the evidence that an important portion of this tightly bound ATP represents a catalytic intermediate. Although much supportive evidence has been derived from the study of the isolated F_1 ATPases and synthase preparations not actively making ATP, we regard evidence obtained during actual net photophosphorylation as most definitive and emphasize such evidence in this discussion.

Three different types of experimental findings give evidence that tightly bound ATP serves as a transient, catalytic intermediate during net photophosphorylation. The demonstration of the increased incorporation of water oxygen into each ATP formed as the ADP concentration is lowered is extended to lower ADP concentrations in this paper. Not only does the extent of reaction reversal at the catalytic site increase, but also, as first shown by Hackney et al. (1979) and extended herein, the distribution of [γ-^{18}O]ATP species formed is homogeneous as predicted by the binding change mechanism (Figures 2 and 3).

An additional important facet of the oxygen exchange measurements shown in this paper is that the rate of formation and cleavage of bound ATP remains about the same even though the total number of reversals decreases and the net rate of ATP formation increases markedly with increasing substrate concentration. This is as expected if the synthase complex retains a tightly bound ATP at a catalytic site even when medium ADP concentrations are as low as micromolar. The experiments of Wimmer & Rose (1979) showing the extensive exchange that each ATP molecule undergoes during the light-driven ATP $\rightleftarrows$ HOH oxygen exchange without added ADP or P_i are another manifestation of this property.

A second type of evidence for the participation of tightly bound ATP during net photophosphorylation comes from the rapid mixing and quenching experiments of Rosen et al. (1979). They demonstrated that an ATP which serves as a catalytic intermediate remains tightly bound when the chloroplast thylakoids are deenergized by addition of ammonia or photophosphorylation is stopped by addition of ethylenediaminetetraacetic acid (EDTA).

A third type of evidence comes from the measurement of the amount of bound catalytic ATP present when the ADP or P_i concentrations are reduced to far below the concentration required for half-maximal velocity. This was shown by the demonstration of the presence of hexokinase-inaccessible ATP that behaved as a catalytic intermediate and remained bound at about one ATP per ATP synthase molecule at an ADP concentration of 20 μM (Rosen et al., 1979), or as shown in Figure 5, with ADP as low as 4 μM. When the net reaction rate is lowered, but the concentration of an intermediate remains high, it follows that its rate of turnover must decrease; i.e., there is a decrease in the off-rate constant for the bound ATP.

Recently reported experiments with deenergized thylakoids and with isolated CF_1 ATPase significantly add to the evidence for the participation of a tighly bound ATP in catalysis. Smith et al. (1983) showed that ATP is very tightly bound to the synthase when net photophosphorylation is blocked by addition of ammonia or EDTA and it continues to undergo slow oxygen exchange. The tight ATP is thus at a catalytic site. In experiments with both isolated and thylakoid-bound CF_1 ATPase, Feldman & Sigman (1982) have shown that a tightly bound ATP is slowly formed from enzyme-bound ADP at a catalytic site in the presence of high medium P_i concentrations. This is in accord with the energy-driven conformational change being necessary to allow both the competent binding of P_i and ADP and the release of ATP.

The oxygen exchange data show that even at high ATP an average of about 0.8 reversal of ATP formation occurs before ATP release. This continued oxygen exchange could logically be the result of some synthesis by a modified or partially inactivated enzyme form that gave extensive change with the bulk of the synthesis occurring with little or no exchange. But the distribution of [^{18}O]ATP species shows that only one reaction pathway is involved. Thus, release of ATP still contributes appreciably to rate limitation even under optimal photophosphorylation conditions. This could reflect the maximum rate of the energy-linked binding change.

ACKNOWLEDGMENTS

We thank Drs. Teri Melese and Rajendra Kandpal for valuable discussions, Kerstin Stempel for ^{18}O analyses, editorial assistance, and preparation of the figures, and George Lopatin for ^{18}O analyses.

Registry No. ADP, 58-64-0; ATP synthase, 37205-63-3.

REFERENCES

Aflalo, C., & Shavit, N. (1984) in *Advances in Photosynthesis* (Sybesma, C., Ed.) Vol. II, pp 559–562, Martius Nijhoff/Dr. W. Junk, The Hague.

Avron, M., Grisaro, V., & Sharon, N. (1965) *J. Biol. Chem. 240*, 1381–1386.

Bennum, A., & Avron, M. (1965) *Biochim. Biophys. Acta 109*, 117–127.

Boyer, P. D., & Kohlbrenner, W. E. (1981) in *Energy Coupling in Photosynthesis* (Selman, B. R., & Selman-Reimer, S., Eds.) pp 231–240, Elsevier/North-Holland, New York.

Bruist, M. F., & Hammes, G. G. (1982) *Biochemistry 21*, 3370–3377.

Feldman, R. I., & Sigman, D. S. (1982) *J. Biol. Chem. 257*, 1676–1683.

Gresser, M., Cardon, J., Rosen, G., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 10649–10653.

Gresser, M. J., Myers, J. A., & Boyer, P. D. (1982) *J. Biol. Chem. 257*, 12030–12038.

Grubmeyer, C., & Penefsky, H. S. (1981) *J. Biol. Chem. 256*, 3728.

Grubmeyer, C., Cross, R. L., & Penefsky, H. S. (1982) *J. Biol. Chem. 257*, 12092–12100.

Hackney, D. D., & Boyer, P. D. (1978) *J. Biol. Chem. 253*, 3164–3170.

Hackney, D. D., Rosen, G., & Boyer, P. D. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3646–3650.

Hackney, D. D., Stempel, K. E., & Boyer, P. D. (1980) *Methods Enzymol. 64*, 60–83.

Harvey, M. J., & Brown, A. P. (1969) *Biochim. Biophys. Acta 180*, 520–528.

Kohlbrenner, W. E., & Boyer, P. D. (1983) *J. Biol. Chem. 258*, 10881–10886.

McCarty, R. E., & Racker, E. (1967) *J. Biol. Chem. 242*, 3435–3439.

Merchant, S., Shaner, S., & Selman, B. A. (1983) *J. Biol. Chem. 258*, 1026-1031.
Mokrasch, L. C., Caravaca, J., & Grisolia, S. (1960) *Biochim. Biophys. Acta 37*, 442.
Moroney, J. V., Lopresti, L., McEwen, B. F., McCarty, R. E., & Hammes, G. G. (1983) *FEBS Lett. 158*, 58-62.
Ohnishi, S. T., & Gall, R. S. (1978) *Anal. Biochem. 88*, 347-356.
O'Neal, C. C., Bild, G. S., & Smith, L. T. (1983) *Biochemistry 22*, 611-617.
Pullman, M. E., Penefsky, H. S., Datta, A., & Racker, E. (1960) *J. Biol. Chem. 235*, 3322-3329.
Rosen, G., Gresser, M., Vinkler, C., & Boyer, P. D. (1979) *J. Biol. Chem. 254*, 10654-10661.
Ryrie, I., & Jagendorf, A. T. (1971) *J. Biol. Chem. 246*, 582-588.
Santarius, K. A., & Heber, U. (1965) *Biochim. Biophys. Acta 102*, 39-54.
Schlodder, E., Graber, P., & Witt, H. T. (1982) in *Electron Transport and Photophosphorylation* (Barber, J., Ed.) pp 105-167, Elsevier Biomedical Press, Amsterdam.
Shavit, N. (1980) *Annu. Rev. Biochem. 49*, 111-138.
Shavit, N., Skye, G. E., & Boyer, P. D. (1967) *J. Biol. Chem. 242*, 5125-5130.
Sherman, P. A., & Wimmer, M. J. (1983) *Eur. J. Biochem. 136*, 539-543.
Smith, L. T., Rosen, G., & Boyer, P. D. (1983) *J. Biol. Chem. 258*, 10887-10894.
Strotmann, H., Hesse, H., & Edelmann, K. (1973) *Biochim. Biophys. Acta 314*, 202-210.
Wimmer, M. J., & Rose, I. A. (1977) *J. Biol. Chem. 252*, 6769-6775.

Distance between the Visible Copper and Cytochrome *a* in Bovine Heart Cytochrome Oxidase†

Gay Goodman* and John S. Leigh, Jr.
Department of Biochemistry and Biophysics, School of Medicine G4, University of Pennsylvania, Philadelphia, Pennsylvania 19104
Received May 9, 1984

ABSTRACT: Electron paramagnetic resonance (EPR) at 15 K was used to probe the magnetic interaction between the visible copper Cu_A^{2+} and ferric cytochrome *a* in the carbon monoxide compound of beef heart cytochrome oxidase. At pH 8.6, the midpoint potentials (E_m's) for one-electron oxidation of Cu_A^+ and cytochrome a^{2+} were found to be 195 and 235 mV, respectively. Because the E_m of Cu_A is well below that of cytochrome *a* under these conditions, the microwave power saturation of Cu_A could be measured as a function of percentage cytochrome *a* oxidized. Although progressive power saturation data directly provide only the product of the spin-lattice and transverse relaxation rates $\Delta[1/(T_1T_2)]$, Castner's theory for the saturation of inhomogeneously broadened lines [Castner, T. G., Jr. (1959) *Phys. Rev. 115* (*6*), 1506-1515], along with our own theoretical formulation of the dipolar T_2, enabled us to determine the change in T_1 of Cu_A due to dipolar relaxation by cytochrome *a*. The orientation of the principal *g* values of Cu_A with respect to those of cytochrome *a* was evaluated in partially oriented membranous multilayers. When allowance was made for uncertainties in the relative Cu_A-cytochrome *a* configuration and in the dipolar axis-magnetic field orientation, a range for the spin-spin distance *r* was calculated on the basis of the dipolar T_1 of the g_x component of Cu_A. This distance range was further restricted by consideration of T_1 for the nonunique orientations of Cu_A giving rise to the g_y signal. Only those values of *r* are possible for which the calculated T_1 ratio (g_x/g_y) is equal to the experimentally determined ratio. To allow for T_1 effects due to scalar exchange interaction, a small correction to the dipolar-only distance was calculated. No line broadening consistent with either dipolar or exchange coupling was detected. Taken together, all of the available data lead us to conclude that the Cu_A-cytochrome *a* distance falls within the range 8 Å $\leq r \leq$ 13 Å.

Cytochrome oxidase (ferrocytochrome *c*:O_2 oxidoreductase; EC 1.9.3.1), the terminal enzyme of the mitochondrial electron-transfer chain, is responsible for the concerted transfer of four electrons to molecular oxygen in an energy-yielding redox reaction, resulting ultimately in ADP phosphorylation. Four metal ions are present, each capable of one-electron oxidation/reduction: the two heme iron atoms of cytochromes *a* and a_3 and two copper atoms, Cu_A and Cu_B.

Stopped-flow optical absorbance experiments revealed the sequence of events in the reaction with cytochrome *c* to be reduction of cytochrome *a* (Gibson & Greenwood, 1965) with a second-order rate constant of $k = 10^7$ M^{-1} s^{-1} (Andréasson et al., 1972) followed by much slower transfer to cytochrome a_3 and, presumably, Cu_B (Brunori et al., 1979). Gibson & Greenwood (1965) monitored the 820-nm band and observed a rapid decrease in absorbance, slightly less rapid than the change at 605 nm but much more rapid than the slower phase. They concluded that copper (i.e., Cu_A) is involved in the initial stages of electron transfer from cytochrome *c*. Using a rapid-freeze technique, Beinert et al. (1976) followed changes in the electron paramagnetic resonance (EPR) signals of Cu_A and cytochromes *a* and a_3 upon reaction with reduced cytochrome *c*. They found that both Cu_A and cytochrome *a* are reduced in the rapid phase of electron transfer to cytochrome

† This work was supported by NIH Grant GM-12202.
* Address correspondence to this author at the Department of Molecular Neurogenetics, E. K. Shriver Center, Waltham, MA 02254.

0006-2960/85/0424-2310$01.50/0