Biochimica et Biophysica Acta, 973 (1989) 78–85
Elsevier

BBA 42916

Sulfite-stimulated release of [^{3}H]ADP bound to chloroplast thylakoid ATPase

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(Received 6 June 1988)
(Revised manuscript received 29 August 1988)

Key words: CF_1; Thylakoid; Chloroplast ATPase; Sulfite; Activation; Bound ADP

The release of [^{3}H]ADP, previously bound to spinach thylakoids, is shown to occur during rapid ATP hydrolysis stimulated by sulfite. Release was exponential in the presence of saturating uncoupler, permitting an estimation of rate constants. The release process required conditions similar to those needed for ATPase: substrate, Mg^{2+}, Na_2SO_3 and prior activation of the thylakoids by reduction. However, release also occurred, although at a lower rate, when ADP replaced the ATP. It was then still inhibited by NaN_3, a strong ATPase poison, even though no ATP hydrolysis occurred. The hyperbolic response of the rate constant to variations in substrate or Na_2SO_3 concentration permitted kinetic analysis by reciprocal plots, and direct comparison with ligand effects on rates of ATP hydrolysis. The concentrations of MgATP or MgADP, and of Na_2SO_3, necessary for the release of ADP are lower than those significant for ATP hydrolysis. Rates of ADP release are three orders of magnitude lower than those of ATP hydrolysis. These results seem not to be consistent with the blockage of an obligate alternating catalytic site by the bound ADP, but do not rule out a regulatory role for it as an inhibitor of ATPase.

Introduction

The coupling factor, CF_1, of thylakoid membranes from chloroplasts is a latent ATPase, activated to permit hydrolysis in vivo by a combination of the high-energy state of the thylakoid membranes, and reduction of the disulfide bridge on its γ subunit [1,2]. Activity decays in darkness, due to loss of the high-energy state of the thylakoids [1]. Correlated with activation of CF_1 ATPase in vitro is the loosening of the bond between one tightly bound ADP and CF_1, as indicated by its loss, or induced exchangeability [3,4]. This fact, together with the observation that adding ADP to the medium accelerates dark decay of ATPase activity [4,5] has led to the proposal that tightly bound ADP serves a regulatory function, and is required as part of the system to maintain CF_1 in its latent state in the dark.

A second proposed role for tightly bound ADP is that of an adenylate at an alternate catalytic site. In the 'binding change' mechanism, the bound ADP at one site is releasable only when ATP binds at a second site, under energized conditions. Alternation between the sites is obligate, in this scheme [6,7]. Unless release of bound ADP occurs, the next ATP cannot be hydrolyzed.

According to the binding change mechanism, the release of previously bound ADP should be kinetically consistent with the rate of turnover of the ATPase. Also, if the bound ADP keeps the ATPase inhibited, its release should be rapid enough to account for the rise time of increase of the enzymatic activity. Challenges to these concepts come from studies in which net ADP release has seemed to be too slow to account for newly occurring ATP hydrolysis [8–10,11]; and these results are supported a fortiori by results reported here. Some of these challenges have been resolved in a recent study using a rapid stop-quench technique (Ref. 12; and see Discussion below).

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Abbreviations: CF_1, coupling factor one from thylakoid membranes; DL thylakoids, thylakoids activated by dithioerythreitol in the light; DTE, dithioerythreitol; Hepes, 4-(hydroxyethyl)-1-piperazineethanesulfonic acid; PMSF, phenylmethylsulfonyl fluoride; TDL thylakoids, thylakoids activated by trypsin exposure in the dark following light + dithioerythreitol activation; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

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In earlier work from this laboratory, methanol was found to induce both a rapid ATPase and release of bound ADP. However, the release of the ADP seemed to be several thousand-fold slower than ATP hydrolysis. Also, some release occurred with non-hydrolyzable substrates, and very little release when GTP was hydrolyzed at rates equal to those for ATP [13]. The kinetic discrepancy could be questioned, however, since it was conceivable that the observed activity was due to a small fraction of the CF_1 in the system, kept at a low but steady level by the balance between an activation no faster than ADP release, and possible permanent denaturation by the high levels of methanol used.

Recently, we have found ways to generate unusually rapid rates of hydrolysis of MgATP hydrolysis, using sulfite (accompanying paper [18]). With this system rapid hydrolysis rates continue for 15 min or longer, so potential denaturation is no longer a consideration. It seemed appropriate, therefore, to reexamine the question of the rate of release of bound ADP in relation to its proposed functions. Our results again question the validity of the proposed roles for bound ADP.

Materials and Methods

Tritiated ADP was obtained from Amersham; most other biochemical reagents were from Sigma.

Isolation of thylakoid membranes from market spinach, activation by dithioerythritol plus light (DL thylakoids) and preparation of trypsin-treated DL thylakoids (TDL thylakoids), along with standard assays for ATPase were as described in the accompanying paper [18].

Labeling of thylakoids with [^{3}H]ADP was accomplished by substituting [^{3}H]ADP for unlabeled ADP (total ADP concentration of 10 μM) during the light period plus DTE activation of thylakoids. It was assumed that the isolated thylakoids released 1 nmol endogenous ADP for every mg chlorophyll present during illumination. Illumination usually occurred with 1 μM [^{3}H]ADP (22 Ci · mmol^{-1}), 8 μM carrier ADP and 1 μM endogenous ADP. Occasionally, the [^{3}H]ADP concentration was increased to 2–3 μM, with commensurate decrease in carrier ADP.

After illumination, the unbound [^{3}H]ADP was removed by washing the (now) DL thylakoids twice in 50 mM Tricine (pH 8.0), 50 mM KCl and 10 mM DTE. The thylakoids (approx. 1 mg chlorophyll) were resuspended in 1.5 ml of the wash solution with the addition of NH_4Cl to 5 mM and gramicidin to 10 μM. When TDL thylakoids were being prepared, treatment with trypsin in the dark occurred at this point.

Stoichiometry of ADP bound to the DL and TDL thylakoids was estimated by sampling a 10-μl aliquot of thylakoids (approx. 10 μg chlorophyll), with vortexing, into 800 μl of a stopping solution containing 25 mM Tricine (pH 8.0), 10 mM NaN_3, 10 mM $MgCl_2$ with or without the addition of trichloroacetic acid to 5%. The membranes were sedimented for 5 min at 13000 × g in an Eppendorf centrifuge and 700 μl of the supernatant was counted in 4 ml of Triton-toluene scintillation fluid. The difference between the counts in the supernatant of the tube with vs. that without 5% trichloroacetic acid was the amount of [^{3}H]ADP which could be pelleted. This quantity, along with the specific activity of [^{3}H]ADP (usually at 2.2 Ci · mmol^{-1}) and the amount of chlorophyll pelleted, was used to estimate the ratio of bound ADP per mg chlorophyll. Counts were corrected (approx. 10%) for quenching by trichloroacetic acid when needed. This protocol yielded thylakoids labeled with 1.5–5 μCi [^{3}H]ADP per mg chlorophyll, representing between 0.8 and (the more usual) 1.2–1.4 nmol ADP per mg chlorophyll. An approximate value of 1 nmol of CF_1 per mg of chlorophyll was reported in previous quantitative analyses [14], hence the usual bound ADP level is about one per CF_1.

The [^{3}H]ADP which could not be pelleted usually amounted to 8–10% of the total counts released by 5% trichloroacetic acid. This percentage was remarkably constant and unyielding to procedures attempting to decrease it. Neither the addition of 1 mM ADP to the washing solutions after illumination, nor increasing the number of washes, affected the counts in the supernatant or altered the estimate for pelletable [^{3}H]ADP. The values for total bound and unpelletable [^{3}H]ADP were not changed by omitting DTE from the illumination reaction. Similarly, trypsin treatment of light and DTE-activated thylakoids did not increase the amount of unpelletable radioactivity and it was assumed in this work that CF_1 was bound to the pelletable membranes at all times.

The time-courses for release of bound ADP, concurrent with ATPase, involved adding 200 μl of labeled thylakoids to 1.8 ml of a reaction mixture containing 50 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, 100 U · ml^{-1} creatine kinase, 5 mM phosphocreatine, 5 mM NH_4Cl and 10 μM gramicidin at 37 °C, but no Na_2SO_3. Without sulfite neither ATPase activity nor release of bound ADP occurred. Thylakoids were maintained in this preincubation condition for 5 min, to allow sampling of aliquots for determining blanks for unpelletable [^{3}H]ADP and (ATPase product) phosphate. After Na_2SO_3 addition (usually to 60 mM), sampling continued for 10–30 min. Aliquots were 90 μl and were sampled into 1.6 ml of the stopping solution. Unpelletable [^{3}H]ADP was estimated from 700 μl and the remainder (800 μl) was used to determine inorganic phosphate with the LeBel reagent [15]. Prior to the addition of Na_2SO_3, several aliquots were also sampled into stopping solution with 5% trichloroacetic acid. The amount of bound ADP per mg chlorophyll estimated from these aliquots was close to the bound ADP per mg

chlorophyll stoichiometry determined during the [^{3}H]ADP loading process (above).

ATPase and [^{3}H]ADP release depended on the three components, $MgCl_2$, ATP and Na_2SO_3. Alternate protocols, including the substitution of ADP for ATP, varied the components in the pre-incubation period with activity initiated by adding the missing third component. If ADP was substituted for ATP, the adenylate kinase inhibitor, diadenosine pentaphosphate, was added to a concentration of 50 μM, and the ATP regenerating system (creatine kinase and phosphocreatine) was omitted. When ATPase and [^{3}H]ADP release activities were started by ATP addition, the first aliquot of 90 μl sampled, after ATP addition, was used as the blank for the inorganic phosphate level.

For kinetic measurements of bound ADP release without simultaneous measurement of ATP hydrolysis, 10 μl of [^{3}H]ADP-loaded thylakoids were added to 300 μl of a reaction mixture at 37°C containing 50 mM Tricine (pH 8.0), 5 mM NH_4Cl, 10 μM gramicidin with $MgCl_2$, ATP or ADP, Na_2SO_3 and NaN_3 as indicated. When ATP was the varied component, 50 $U \cdot ml^{-1}$ creatine kinase and 2 mM phosphocreatine were added; when ADP was substituted for ATP, 50 μM diadenosine pentaphosphate was added. After 10 s to 10 min, 500 μl of the stopping solution was added and unpelletable ADP was determined. Across a series of tubes, one of the components (ADP or ATP and Na_2SO_3) was varied such that the response of the release rate to the varied component could be determined. One tube did not contain the varied substrate and served as a blank for unpelletable ADP. Similarly, the total amount of ADP that could be released under physiological conditions was determined in a second tube which always contained 50 mM Tricine (pH 8.0), 5 mM NH_4Cl, 10 μM gramicidin, 10 mM $MgCl_2$, 10 mM ATP and 100 mM Na_2SO_3. This tube was incubated for 5 min, which was estimated to be at least 15-times the release, k (see Results below).

By varying the concentration of Na_2SO_3, ADP and ATP it was determined that the value for k responded to altered substrate concentrations in a hyperbolic fashion (see below). Accordingly, reciprocal plots of the data were used to estimate substrate concentrations needed for half-maximal effect and maximal k values, as in the Michaelis-Menten treatment of enzyme rate data. We do not intend to imply a mechanism for these effects, but for comparative purposes it is useful to obtain apparent K_m and k_{max} numbers for the ADP release reaction.

Results

Measurement of the remaining [^{3}H]ADP as a function of time showed apparent exponential decay kinetics (Fig. 1), when ATPase was active. Initiation of both

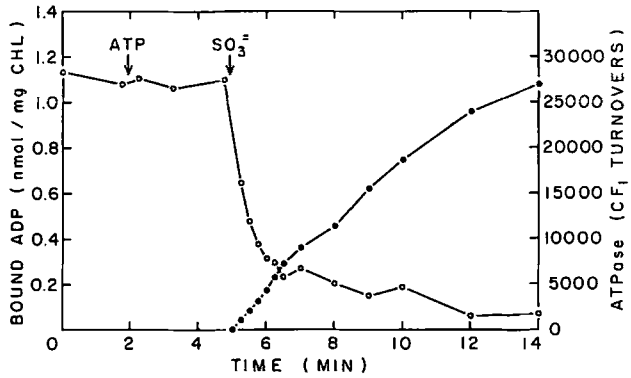

Fig. 1. Comparison of ATP hydrolysis and [^{3}H]ADP release in the same reaction mixture. DL thylakoids with 240 μg of chlorophyll pre-loaded with [^{3}H]ADP were incubated in 2 ml of 50 mM Tricine (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 60 mM Na_2SO_3, 100 U/ml creatine kinase, 5 mM phosphocreatine, 5 mM NH_4Cl and 10 μM gramicidin. ATP and Na_2SO_3 were added at the time points indicated. The ATPase rate is expressed as turnover, assuming 1 nmol CF_1 per mg chlorophyll. The initial ATPase rate was 260 μmol per mg chlorophyll per h, or 4333 turnovers per min. The apparent first-order rate constant for [^{3}H]ADP release was 2.10 per min.

ATP hydrolysis and [^{3}H]ADP release required addition of sulfite in the experiment shown in Fig. 1, and, in other experiments (not shown), also required the simultaneous presence of ATP and Mg^{2+}. ADP could substitute for ATP, at a lower rate (1/5 of that with ATP in Fig. 2, 1/10 or 1/20 in other experiments). When the data were plotted semi-logarithmically (Fig. 3) they

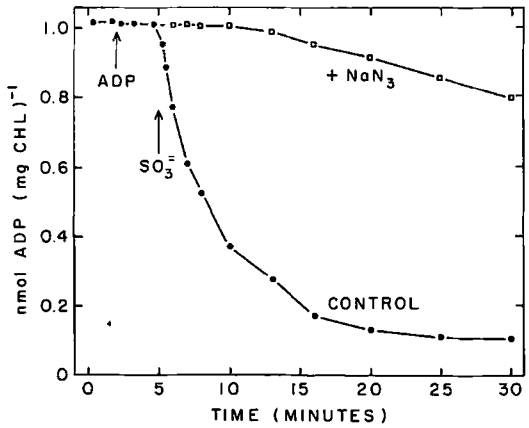

Fig. 2. Release of [^{3}H]ADP with medium ADP replacing ATP; inhibition by azide. DL thylakoids, pre-loaded with [^{3}H]ADP, were incubated in a medium similar to that described in Fig. 1, except that the $MgCl_2$ concentration was 1 mM instead of 10 mM, 1 mM ADP replaced the 10 mM ATP and phosphocreatine and creatine kinase were omitted. Sulfite and ADP were added at time points shown. Sodium azide was absent from the controls (solid circles); present at 200 μM in the inhibited tubes (solid squares). Concurrent with the release experiment, aliquots of the control tube were removed and used to measure ATPase in the same medium as that described in Fig. 1. The ATPase rate was constant at 250 μmol per mg chlorophyll per h (using the reaction mixture of Fig. 1) during the 30 min incubation with ADP.

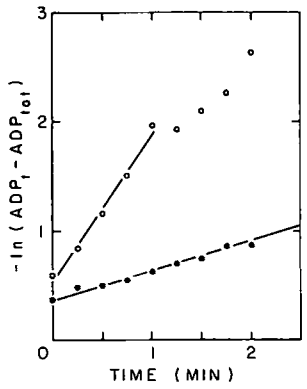

Fig. 3. Semi-logarithmic plot of time course of [^{3}H]ADP release. DL thylakoids, pre-loaded with [^{3}H]ADP, were incubated as in Fig. 1 with either 10 mM ATP (open circles) or 10 mM ADP (closed circles). The counts in the zero substrate tube were subtracted from those found at each time point (indicated as ADP_t on the ordinate); then divided by the total releasable counts (indicated as ADP_{tot}; also with zero substrate tube counts subtracted). The log of the resulting number is plotted vs. time. The apparent first-order rate constant calculated for MgATP was 1.36 · min^{-1} and for MgADP was 0.278 · min^{-1}.

fitted a straight line, whose slope permitted estimation of the release constant, k. Examination of Fig. 1 will show the approx. 10% of counts not released even after prolonged times in the reaction mixture. These residual counts had to be subtracted to obtain accurate kinetic data for the releasable nucleotides.

Part of the requirement for ADP or ATP could be as a substrate for the exchange of bound nucleotides. To test this, thylakoids previously exposed to sulfite, Mg^{2+} and ADP or ATP, were quenched with azide, washed twice and extracted with perchloric acid. The neutralized extracts were assayed both by HPLC (DEAE column) and by luciferase (see Ref. 13) for total ADP and ATP. The sum of these adenylates was generately between 4 and 5 nmol per mg chlorophyll, or 4 to 5 per mol of CF_1. This number did not change due to ATP hydrolysis, at a time when the bound [^{3}H]ADP was leaving. Thus, the apparent 'loss' of bound ADP must really be an exchange reaction. For convenience, we will describe the exchange as release of the bound [^{3}H]ADP, since that was the function actually measured.

In the experiment shown in Fig. 1, with DL thylakoids, the exponential rate constant for [^{3}H]ADP release was 2.1 · min^{-1} with MgATP; in that seen in Fig. 3, it was 1.26 · min^{-1} for MgATP and 0.278 · min^{-1} for MgADP. In Fig. 1, the rate of ATPase was 260 μmol/mg chlorophyll per h, which converts to a turnover of 4333 · min^{-1}, assuming 1 nmol CF_1/mg chlorophyll [14]. Note that no lag is seen in the onset of ATP hydrolysis, within the accuracy of these measurements.

Results from several experiments measuring ATPase and ADP release concurrently are shown in Table I. In these experiments, thylakoid ATPase had been preactivated by light plus DTE, then stored in the dark. ATPase was inactive until sulfite was added. It is apparent that rates of ATP hydrolysis are in the order of several thousand-fold greater than the amount of ADP released during the first minute. Calculating the rate of release during the first 5 s shows between 1000- and 4000-times more ATP hydrolyzed than ADP released in the same period. (These calculations assume one tightly bound light-exchangeable ADP per CF_1. Our measurements of bound ADP ranged from 0.8 to 1.2 nmol/mg chlorophyll – see Materials and Methods.)

Day-to-day variability was noted in the release constant k; so the differences between the experiments in Table I are probably not significant. However, constants determined with an individual preparation of thylakoids were stable, allowing critical comparisons between treatments.

The response of the rate constant for ADP release to varying concentrations of components needed for the reaction (sulfite, Mg^{2+}, ATP or ADP) seemed to follow a hyperbolic curve. Double reciprocal plots could, therefore, be used to estimate maximal k values (rates) as a function of the necessary components, and apparent affinities for Mg, ATP and sulfite. Re-calculating the data this way permitted a direct comparison between the effects of the required compounds on ADP release and on ATP hydrolysis.

The sulfite concentration ($K_s$obs) necessary to activate ATPase by 50% differed significantly from that needed for a 50% effect on the rate constant for release of bound [^{3}H]ADP. In the experiment shown with TDL thylakoids and MgATP (Fig. 4), the $K_s$obs for sulfite was 31 mM when measuring ATPase, while the half-

TABLE I

Representative release rate constants and ATPase rates during concurrent measurements as in Fig. 1

The reaction mixture contained 50 mM Tricine (pH 8.0 at 37 °C), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 100 U creatine kinase, 5 mM phosphocreatine, 5 mM NH_4Cl, 10 μM gramicidin and 60 mM Na_2SO_3. The Na_2SO_3 was added last, except for the results on the last line where ATP was added last and the Na_2SO_3 concentration was 80 mM. Rate constants for release were calculated as indicated in Materials and Methods. Data on the third line are from the experiment shown in fig. 1. Values are given in nmol per mg chlorophyll per min.

Release k (min^{-1})	Initial ADP release rate	Average ATPase rate		Ratio of ATPase/release [b]
		0–2 min	3–5 min	
0.97	0.92 [a]	4000	3200	4347
1.7	1.56	2200	1480	1410
2.1	1.89	4300	3800	2275
1.0	0.95	3700	3500	3894

[a] Calculated for the release during the first 0.1 min and assuming 1 nmol CF_1 per mg chlorophyll with 1 mol ADP per mol CF_1.

[b] Column 3/Column 2.

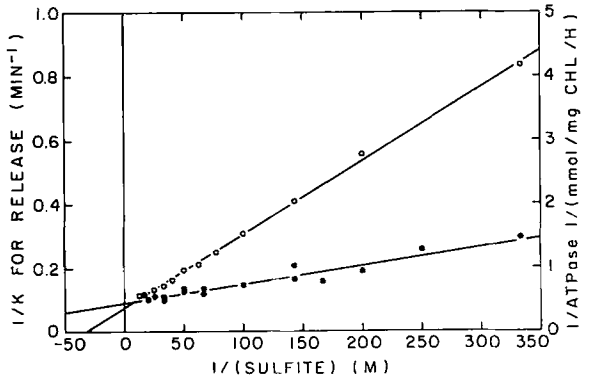

Fig. 4. Reciprocal plot to estimate the kinetic constants for ATPase (open circles) and [^{3}H]ADP release (closed circles) with respect to sulfite concentration. TDL thylakoids pre-loaded with [^{3}H]ADP were incubated in the same medium as that described in Fig. 1 for 15 s to assay [^{3}H]ADP release, and for 3 min to measure ATP hydrolysis. At the lowest Na_2SO_3 concentration in this experiment (3 mM), approx. 27% of the releasable ADP as released: at the highest concentration (30 mM), 68% was released. The ATPase V_{max} with respect to sulfite was 2704 μmol per mg chlorophyll per h and the K_s for sulfite was 31 mM. For [^{3}H]ADP release the k_{max} was 9.3 per min^{-1} and the K_s for sulfite was 4.7 mM.

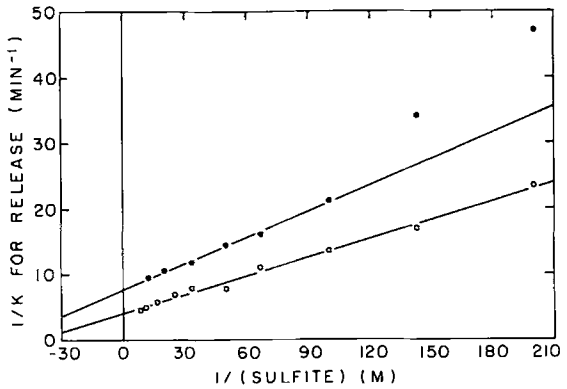

Fig. 5. Kinetic analysis of [^{3}H]ADP release with respect to sulfite when using medium ADP instead of ATP. Incubation of DL thylakoids, pre-loaded with [^{3}H]ADP, was for 3 min in the same medium as that described in Fig. 1 at pH 8.0 (open circles), or the same but using 50 mM Hepes at pH 7.3 (closed circles). In both experiments, 10 mM ADP was used instead of 10 mM ATP. The k_{max} was 0.25 · min^{-1} at pH 8.0 and 0.13 · min^{-1} at pH 7.3; the respective K_s values were 23 mM sulfite at pH 8.0 and 17 mM at pH 7.3.

maximal effect on the ADP-release constant occurred at only 4.7 mM. Similar measurements with DL thylakoids gave values of 160 mM for ATPase vs. 48 mM during [^{3}H]ADP release (data not shown).

The kinetics with respect to sulfite were also determined during the ADP-supported ADP release/exchange with DL thylakoids (Fig. 5). Values estimated for the maximum ADP release rate constant, k_{max}, were only 0.246 · min^{-1} at pH 8, and 0.103 · min^{-1} at pH 7.3, or 10- to 20-times lower than those noted for release when ATP was present (above). The K_s for sulfite was 23 mM at pH 8.0 and 17 mM at pH 7.3. The effect of pH was not fully consistent with bisulfite (HSO_3^-) as the active form of sulfite when ADP was the adenylate. However, with release supported by ATP, DL thylakoids had a K_s for sulfite of 16 mM at a pH of 7.3 (Table II).

TABLE II

Kinetic constants for sulfite and for azide when affecting release of [^{3}H]ADP

The reaction mixture contained 50 mM Tricine (pH 8.0) or 50 mM Hepes (pH 7.3), 50 mM KCl, 10 mM $MgCl_2$, 5 mM NH_4Cl, 10 μM gramicidin and 10 mM of either ADP or ATP with a range of Na_2SO_3 concentrations sufficient to estimate K_s. When ADP was used, 50 μM diadenosine pentaphosphate was also added. The K_i for azide was computed assuming competitive inhibition with sulfite.

Thylakoid activation	Adenylate	Assay pH	K_s for sulfite (mM)	k_{max} (min^{-1})	K_i for azide (μM)
DL	ATP	8.0	48	3.9	–
DL	ATP	8.0	40	3.2	32
DL	ATP	8.0	42	4.3	34
DL	ATP	7.3	16	2.5	25
TDL	ATP	8.0	4.7	9.3	15
DL	ADP	8.0	24	0.25	–
DL	ADP	8.0	14	0.17	64
DL	ADP	7.3	17	0.13	18

TABLE III

Estimates of the half-maximal ADP and ATP concentrations (K_m) necessary for maximal release (k_{max}) of bound ADP

The reaction mixture contained 50 mM Tricine (pH 8.0) or 50 mM Hepes (pH 7.3), 50 mM KCl, 2 mM $MgCl_2$, 5 mM NH_4Cl, 10 μM gramicidin and Na_2SO_3 as indicated, with a range of either MgADP (plus 50 μM diadenosine pentaphosphate) or MgATP (plus 50 U · ml^{-1} creatine kinase and 2 mM phosphocreatine) sufficient to estimate K_m. The K_i for azide was computed assuming non-competitive inhibition with the adenylate.

Thylakoid preparation	Na_2SO_3 (mM)	Assay pH	Adenylate	K_m (μM)	k_{max} (min^{-1})	K_i NaN_3 (μM)
DL	60	8.0	ATP	110	2.3	–
DL	50	8.0	ATP	43	1.4	56
DL	60	8.0	ATP	78	2.0	23
DL	30	7.3	ATP	74	1.6	44
DL	60	8.0	ADP	63	0.42	–
DL	50	8.0	ADP	74	0.15	52
TDL	60	8.0	ATP	38	8.6	–

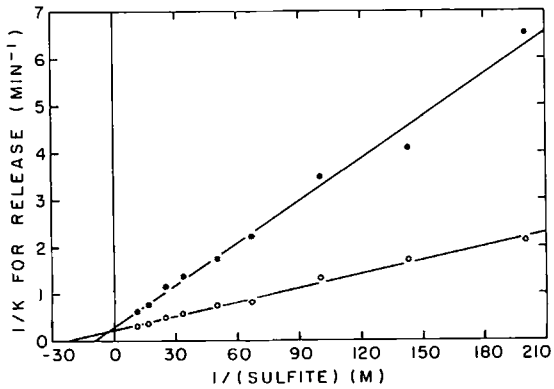

Fig. 6. Azide inhibition is competitive with sulfite. DL thylakoids were incubated for 30 s in the same medium as that described in Fig. 1, either without (open circles) or with (closed circles) 50 μM NaN_3 at sulfite concentrations ranging from 5 to 100 mM. The calculation of rate constant for [^{3}H]ADP release was performed as in Fig. 3; its reciprocal is plotted on the ordinate.

This 3-fold reduction in K_s from pH 8.0, is consistent with the approx. 4-fold increase in the proportion of bisulfite at pH 7.3 and suggests that bisulfite is the active species.

The inhibition of [^{3}H]ADP release by azide turned out, as in ATPase (accompanying paper [18]), to be 'competitive' with sulfite (Fig. 6). This was the case using MgATP with either DL thylakoids (Fig. 6) or TDL thylakoids; and also in the slower MgADP-supported release. K_i values for azide ranged from 15 to 65 μM in this reaction (Table II), which is similar to the azide inhibition of ATPase (accompanying paper [18]).

Unlike its effect on ATPase in which it is a competitive inhibitor of sulfite activation, 1 mM MgADP (plus 2 mM $MgCl_2$), did not alter the apparent kinetic constants under the conditions of Fig. 6 (data not shown). If medium ADP inhibited bound ADP release as it does

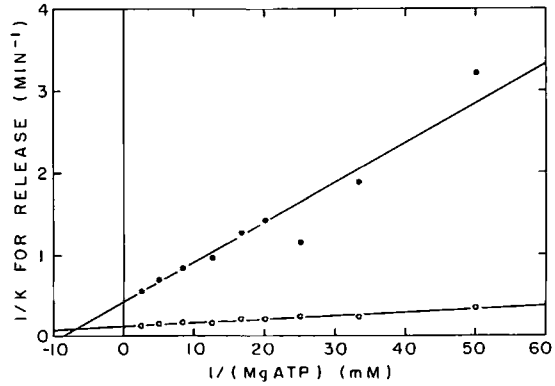

Fig. 7. Kinetics of [^{3}H]ADP release with respect to MgATP concentration, comparing DL thylakoids (closed circles) with TDL thylakoids (open circles). The two kinds of thylakoid, both pre-loaded with [^{3}H]ADP, were incubated for 10 s in the medium described in Fig. 1 then assayed for released radioactivity. Respective K_m values for DL and TDL thylakoids were 113 and 38 μM MgATP; k_{max} values were 2.33 and 8.65 $\cdot$ min^{-1}.

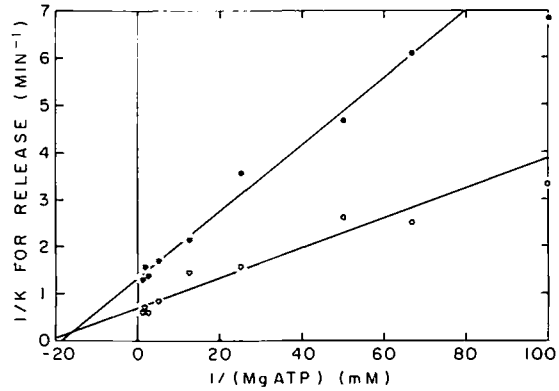

Fig. 8. Azide inhibition is non-competitive with respect to MgATP concentration. DL thylakoids, loaded with [^{3}H]ADP, were incubated as described in Fig. 1 with (closed circles) or without (open circles) 50 μM NaN_3. The K_m values for MgATP were 45 μM without azide and 54 μM with azide: k_{max} values were 1.43 and 0.78 $\cdot$ min^{-1}, respectively.

ATPase, the K_s for sulfite should have increased 2-fold – it did not.

The effect of MgATP concentrations inducing [^{3}H]ADP release is documented in Fig. 7, comparing DL with TDL thylakoids. As for ATP hydrolysis, the rate of release of ADP is much faster with TDL thylakoids than with DL thylakoids. The maximum release rate constant (k_{max}) was 2.33 $\cdot$ min^{-1} for DL thylakoids, and 8.65 $\cdot$ min^{-1} for TDL thylakoids. At the same time, the half-maximal concentration of MgATP (K_m) for the maximal release rate dropped from 113 to 38 μM, going from DL to TDL thylakoids. These values are considerably less than those for the K_m of MgATP during ATPase under similar conditions: approx. 900 μM with DL thylakoids and 1100 μM for TDL thylakoids (see accompanying paper [18]).

The interaction of azide as an inhibitor of [^{3}H]ADP release, with MgATP (Fig. 8) or MgADP (Table II) was apparently non-competitive. This would be expected if the primary effect of azide is in relation to the sulfite effect. Inhibition of ADP release by high concentrations of azide was so efficient that it was adopted for use in the stopping reagent. This allowed relatively mild conditions in quenching the ADP release reaction, as well as ensuring complete inhibition of ATPase.

Discussion

Two aspects of this work have implications for possible mechanisms of the CF_1 function. The first is the fact that the apparent loss of tightly bound [^{3}H]ADP occurred with thylakoids maximally uncoupled by the combination of gramicidin and NH_4Cl. Unlike the case with methanol ATPase [13], this 'loss' is actually an exchange with medium ATP or ADP. Induced exchangeability of the tightly bound ADP has been taken, to a very large extent, as one of the indicators of an energy-dependent conformational change in thylakoid-

bound CF_1 [1]. The effects of methanol, on the one hand, and sulfite, on the other, must duplicate those of the high-energy state in permitting loosening of the ADP-CF_1 association.

The second point is the very large discrepancy between the rate of release of bound ADP, and that of hydrolysis of ATP by the same enzyme. As this has important implications for the role of bound ADP, it is necessary to point out the limited conditions that had to be used. With maximal rates of ATPase using TDL thylakoids, (i.e., using 100 mM sulfite, so the release rate constant $k = 9.3 \cdot \text{min}^{-1}$: Fig. 4) approx. 90% of the bound ADP would have been released by 10 s. This prevented the use of TDL thylakoids in concurrent experiments to compare bound ADP release with ATPase. Similarly, monitoring release under conditions for maximal ATPase of DL thylakoids is precluded due to the high K_s for sulfite (160 mM) in ATPase. With DL thylakoids, at less than optimal sulfite concentrations (Fig. 1), the ATPase rate was 260 μmol per mg chlorophyll per h, or about 18% of that possible with saturating sulfite (1.4 mmol per mg chlorophyll per h) and the release rate constant was $2.1 \cdot \text{min}^{-1}$. Under these conditions, approx. 18% of the bound ADP should have been released by 5 s, and 41% was released by the first sampling point at 15 s. Numerically, this means that during the first 5 s, for instance, 361 nmol of ATP were hydrolyzed as 0.18 nmol of ADP was released, for a ratio of 2000/1. This calculation assumes the ATPase rate was linear, and ADP release was log-linear from the start. Our extrapolated curves did not suggest the appearance of any lags, but studies with a stop-flow apparatus will be needed to obtain truly accurate data about ATPase rates during the first few seconds.

The very large discrepancy between initial rates of ADP release and of ATP hydrolysis does not have to be qualified by a potentially rapid rate of CF_1 denaturation, as was the case when studying methanol-activated ATPase [13]. This observation means that the bound ADP, during the initial release as measured here, is not kinetically competent to be an intermediate at an obligate alternating site [7] for the concurrent ATP hydrolysis. However, it is important to note that this conclusion is at variance with those obtained on studying the location of 2 azide-ADP, replacing ADP as the inhibitory adenylate for ATPase [16]. The chemical evidence from that work, showing the inhibitory 2-azido-ADP covalently attached to a catalytic site, is at least as critical as our kinetic data. In addition, it is suggestive of ADP release and ATPase being similarly accelerated in TDL thylakoids, compared to DL thylakoids (Table II). Resolution of the meaning of the kinetic results requires further work.

One possibility is that the steady-state rate of ADP release may be faster than the initial rate. This was demonstrated recently for the Ca^{2+}-dependent ATPase of soluble CF_1 [12], where steady-state rates of ADP release and ATPase were found to be equivalent, even though the initial ADP release rate was 10-fold slower. However, the net rates in that system [12] are much slower than those observed here. With thylakoids, Mg^{2+} and sulfite, it would require a 2000-fold difference between initial and steady-state ADP release rates to support the binding change mechanism if the inhibitory ADP was at an alternate catalytic site. Our data also failed to show any sign of an initial lag in ATP hydrolysis, which might be correlated with the sluggish initial loss of enzyme-bound ADP. Nevertheless, it will be interesting to discover whether analysis similar to that in Ref. 12 in the present system, will continue to indicate much more rapid catalysis than exchange during the steady state.

The present data are not sufficient to rule out a role for bound ADP as an inhibitor of catalysis, if one postulates, as did Strotmann and Bickel-Sandkötter [9], that the ADP may be tightly or loosely bound, without actually coming off the enzyme. Only the tightly bound form might be inhibitory for ATPase. Transition to the loosely bound, non-inhibitory form could occur rapidly, but would not be detected by the kind of experiment performed here.

The requirement for MgATP (or MgADP) in ADP release suggests that the added nucleotide causes a conformational change in CF_1 as it binds to an alternative site. This binding seems to require all the other conditions needed for active ATPase, since release did not occur with thylakoids that had not been pre-reduced in the light, or in the absence of Na_2SO_3 or of Mg^{2+}; and release was prevented by NaN_3, which is a powerful ATPase inhibitor (Ref. 17; and accompanying paper [18]). Also, the K_i of azide for inhibition of the two reactions was very similar, at about 30 μM (Table II).

The discrepancies between the conditions needed for ATPase and for ADP release are very large. The concentrations of sulfite necessary for half-maximal stimulation of release by Na_2SO_3 (Figs. 4 and 5) are about 6-fold less than those determined for stimulation of ATPase (accompanying paper [18]). Similarly, the apparent K_m for MgATP during release was about 8-fold less (Fig. 8) for DL thylakoids and 30-fold less for TDL thylakoids (Fig. 7) than values for ATPase. Finally, ADP can replace ATP in this function, although at a lower rate. This was especially unexpected, because ADP could not replace ATP when measuring release of bound ADP in the methanol-activated system [14].

It seems reasonable to speculate that the kinetic constants determined during bound ADP release reflect the minimal conditions necessary to activate CF_1. Possibly a conformational change is induced which weakens the bond between ADP and CF_1 allowing the ADP to be released or exchanged.

Acknowledgements

Supported in part by National Science Foundation grant DMB-85-15207, and Hatch grant 185417.

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