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On the Mechanism of Sulfite Activation of Chloroplast Thylakoid ATPase and the Relation of ADP Tightly Bound at a Catalytic Site to the Binding Change Mechanism†

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ABSTRACT: Washed chloroplast thylakoid membranes upon exposure to [^{3}H]ADP retain a tightly bound [^{3}H]ADP on a catalytic site of the ATP synthase. The presence of sufficient endogenous or added Mg^{2+} results in an enzyme with essentially no ATPase activity. Sulfite activates the ATPase, and many molecules of ATP per synthase can be hydrolyzed before most of the bound [^{3}H]ADP is released, a result interpreted as indicating that the ADP is not bound at a site participating in catalysis by the sulfite-activated enzyme [Larson, E. M., Umbach, A., & Jagendorf, A. T. (1989) *Biochim. Biophys. Acta 973*, 75-85]. We present evidence that this is not the case. The Mg^{2+}- and ADP-inhibited enzyme when exposed to MgATP and 20-100 mM sulfite shows a lag of about 1 min at 22 °C and of about 15 s at 37 °C before reaching the same steady-state rate as attained with light-activated ATPase that has not been inhibited by Mg^{2+} and ADP. The lag is not eliminated if the enzyme is exposed to sulfite prior to MgATP addition, indicating that ATPase turnover is necessary for the activation. The release of most of the bound [^{3}H]ADP parallels the onset of ATPase activity, although some [^{3}H]ADP is not released even with prolonged catalytic turnover and may be on poorly active or inactive enzyme or at noncatalytic sites. The results are consistent with most of the tightly bound [^{3}H]ADP being at a catalytic site and being replaced as this Mg^{2+}- and ADP-inhibited site regains equivalent participation with other catalytic sites on the activated enzyme. The sulfite activation can be explained by sulfite combination at a P_i binding site of the enzyme-ADP-Mg^{2+} complex to give a form more readily activated by ATP binding at an alternative site.

The chloroplast, mitochondrial, and bacterial ATP synthases and the corresponding F_1 ATPases, as isolated or after exposure to ATP or ADP, retain bound ADP after removal of medium nucleotides by gel filtration. The ADP present on the enzyme has been conventionally designated as tightly bound ADP. Labeling studies with 2-azidoadenine nucleotides [see Lunardi et al. (1987), Wise et al. (1987), and Guerrero et al. (1988)] have shown that there are two types of nucleotide binding sites, catalytic and noncatalytic, and that tightly bound ADP can be present on both types of sites. Although the chloroplast F_1 ATPase (CF_1), like other F_1 ATPases, likely has three noncatalytic and three catalytic nucleotide binding sites, uncertainty remains as to whether all three catalytic sites function in an equivalent manner or whether one or more has a type of regulatory function.

One catalytic site on CF_1, like other F_1 ATPases, has a much higher affinity for ATP or ADP than the other two catalytic sites. CF_1 can be obtained with about one tightly bound [^{3}H]ADP present either by its exposure to medium [^{3}H]ADP present either by its exposure to medium [^{3}H]ADP for 1-3 h (Bruist & Hammes, 1981) or by the cleavage of [^{3}H]ATP and the removal of medium nucleotides (Wu & Boyer, 1986). Similarly, about one tightly bound [^{3}H]ADP per synthase on chloroplast thylakoid membranes can be obtained by short exposure to light and medium [^{3}H]ADP or [^{3}H]ATP [see Shavit and Strotmann (1980), McCarty and Nalin (1986), Du and Boyer (1989)]. Observations of Smith and Boyer (1976) showed that such tightly bound ADP on chloroplast thylakoid membranes was released during the initial turnover of the synthase induced by an acid-base transition. This was interpreted as resulting from the participation of the tight ADP binding site as an alternating site in the binding change mechanism. A catalytic site location for the tightly bound ADP was also indicated by the demonstration that such ADP on CF_1 (Feldman & Sigman, 1982), on thylakoid membranes (Feldman & Sigman, 1983), or on the mitochondrial enzyme (Sakamoto & Tonomura, 1983) formed bound ATP upon exposure to high concentrations of medium P_i. A catalytic site location also has been supported by several other studies (Carmeli et al., 1981; Feldman & Boyer, 1985; Drobinskaya et al., 1985) and demonstrated by labeling studies with 2-azido-ADP and 2-azido-ATP (Xue et al., 1987).

Other studies have raised the possibility that catalysis might involve other sites on the enzyme and not include the site where the readily replaceable ADP discussed above is bound. For example, in a series of studies from Hammes' laboratory the ADP was regarded as being at noncatalytic sites [e.g., see

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Bruist and Hammes (1981)], but more recent reports concur with a catalytic site location (Leckband & Hammes, 1987). Binding of ADP is well-known to be associated with an inhibition of the ATPase activity of CF_1 or of thylakoid membranes. One possibility suggested is that this ADP is located at noncatalytic regulatory sites [see Shavit (1980) and Strotmann (1984)]. However, recent studies have given convincing evidence that the inhibition results from the binding of ADP at catalytic sites (Zhou et al., 1988; Du & Boyer, 1989). Even if the tightly bound ADP is at a site that has a potential for catalytic activity, the possibility has not been eliminated that the site could somehow be shifted to a regulatory function and remain occupied by the ADP while the two other catalytic sites were activated and carry out catalysis.

A possible regulatory role of the tightly bound ADP was probed by Larson et al. (1989) in their recent studies of the pronounced sulfite activation of the ATPase in chloroplast thylakoid membranes. They found that in the presence of sulfite many molecules of ATP could be hydrolyzed before extensive replacement of the tightly bound [^{3}H]ADP on the synthase occurred. They favored participation of a regulatory site that retained the [^{3}H]ADP while other sites were activated for catalytic turnover. Their report served as the stimulus for the investigations reported by us in this paper.

Our results show that when chloroplast thylakoid membranes, with [^{3}H]ADP tightly bound in the presence of Mg^{2+}, are exposed to MgATP and sulfite, there is a considerable lag in the onset of rapid ATP hydrolysis. During the lag time any enzyme freed of the inhibitory [^{3}H]ADP can hydrolyze many molecules of ATP while the relatively slow release of tightly bound [^{3}H]ADP from other enzyme molecules continues until near maximal activity is attained. Our data further show that the replacement of [^{3}H]ADP from catalytic sites correlates with the attainment of a maximum hydrolysis rate. Some [^{3}H]ADP that is not readily replaced is considered to be on inactive or poorly active enzyme or at noncatlytic sites. The results are consistent with the tightly bound [^{3}H]ADP, which is at a catalytic site and which is necessary for the Mg^{2+}-induced inhibition, being released by sulfite activation in the presence of MgATP. The site previously occupied by the [^{3}H]ADP can then participate equally with other sites in an alternating site catalysis as in the binding change mechanism. Some considerations are included about the basis of the sulfite activation.

Experimental Procedures

Isolation of Chloroplast Thylakoid Membranes. The chloroplast thylakoid membranes were isolated essentially as described by Shavit and Strotmann (1980). Fresh market spinach leaves were processed in a Waring blender in grinding solution which contained, at pH 8, 50 mM Tricine, 0.4 M sucrose, 0.2 M choline chloride, and 5 mM $MgCl_2$. The membranes were collected by centrifugation for 4 min at 3000*g* and washed once with grinding solution. The membranes were washed once more with a solution containing, at pH 8.0, 2 mM Tricine and 10 mM NaCl, collected by centrifugation for 2 min at 12000*g*, washed three times with a solution containing, at pH 8.0, 2 mM Tricine, 50 mM NaCl, and 1 mM $MgCl_2$, and collected by centrifugation for 2 min at 3000*g*. Finally, the membranes were washed once again with grinding solution and resuspended in grinding solution to a chlorophyll concentration of 3–4 mg/mL. The thylakoid membranes were used directly or stored in liquid N_2.

Preparation of ATPase-Active Thylakoid Membranes. The thylakoid membranes, isolated as described above, were illuminated for 5 min in a 10-mL volume containing, at pH 8.0, 50 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 0.1 mM phenazine methosulfate, 50 mM dithiothreitol, and 0.2–0.4 mg/mL chlorophyll. The sample was diluted just before the light was turned off with 8–10 volumes of a solution containing, at pH 8.0, 4 mM Tricine, 100 mM KCl, and 0.5 mM EDTA, centrifuged for 2 min at 3000*g*, and washed once with the same solution. The pellet was resuspended in 1–2 mL of the same solution containing 50 mM dithiothreitol. The thylakoid membranes prepared in this way have the ability to hydrolyze ATP in the dark without further light activation (Du & Boyer, 1989) and are stable for hours on ice. Such preparations were used for all the experiments reported in this paper.

Measurement of ATP Hydrolysis. ATP hydrolysis by ATPase-active thylakoid membranes was usually carried out as follows: the membranes (about 0.4 mg/mL chlorophyll) were first incubated for 2 min in a 1-mL volume containing, at pH 8.0, 40 mM Tricine, 50 mM KCl, and 5 mM $MgCl_2$, with or without 2 μM ADP. Then an equal volume of a solution containing, at pH 8.0, 50 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 10 mM ATP, 20 mM phosphoenolpyruvate, and 0.2 mg/mL pyruvate kinase, with or without sulfite, was added. Aliquots were removed at the indicated times and quenched with trichloroacetic acid, and the P_i produced was measured.

Incorporation of [^{3}H]ADP into Thylakoid Membranes. The ATPase-active thylakoid membranes were incubated for 5 min in the dark in a 20-mL volume, at pH 8, containing 50 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, 2 μM [^{3}H]ADP ($0.7–3 \times 10^6$ cpm/nmol), and about 0.2 mg/mL chlorophyll. The thylakoid membranes were collected by centrifugation for 2 min at 3000*g*, washed three times with a 50-mL volume containing, at pH 8, 2 mM Tricine, 50 μM NaCl, and 1 mM $MgCl_2$; and resuspended in 1–2 mL of the same solution to give a final chlorophyll concentration of 2–3 mg/mL. The thylakoid membranes usually contain about 0.6–0.8 mol of [^{3}H]ADP/CF_1 (assuming 1 nmol of CF_1/mg of chlorophyll).

Release of Tightly Bound [^{3}H]ADP from Thylakoid Membranes. The thylakoid membranes containing tightly bound [^{3}H]ADP, prepared as described above, were incubated for 2 min in the dark in a 3-mL volume containing, at pH 8, 50 mM Tricine, 50 mM KCl, 5 mM $MgCl_2$, and about 0.4 mg/mL chlorophyll. After incubation, an equal volume of a solution containing, at pH 8.0, 50 mM Tricine, 200 mM sulfite, 50 mM KCl, 5 mM $MgCl_2$, 10 mM ATP, 20 mM phosphoenolpyruvate, and 0.2 mg/mL pyruvate kinase was added. Aliquots (0.4 mL) were removed and quenched at the times indicated by adding to 0.1 mL of 250 mM EDTA to give a final concentration of 50 mM. Samples were centrifuged in an Eppendorf microcentrifuge. The phosphate produced and the [^{3}H]ADP released in the supernatant were measured by standard procedures. Values were corrected for the amounts in the supernatants from zero-time samples; these usually contained less than 10% of the total bound [^{3}H]ADP.

Results

For our experiments we used thylakoid membranes that had been thoroughly washed, exposed to dithiothreitol and light, diluted in the light, and harvested by centrifugation. Such preparations have relatively high ATPase activity after storage in the dark, without reexposure to light (Du & Boyer, 1989). Nearly complete inhibition of the ATPase activity results when such membranes are exposed to 2 μM ADP and 5 mM Mg^{2+} for 2 min. This provided a convenient means of obtaining fully inhibited preparations to test for sulfite activation and for comparison of activities with and without the pronounced MgADP inhibition.

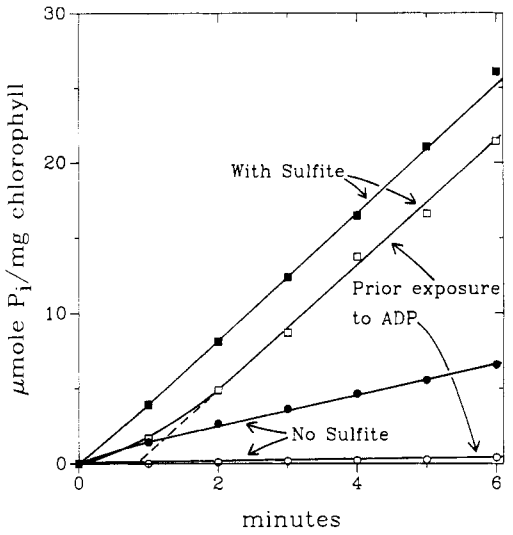

FIGURE 1: Activation of thylakoid membrane ATPase by sulfite. The ATPase-active thylakoid membranes were incubated at 22 °C for 2 min in a solution containing, at pH 8.0, 50 mM Tricine, 50 mM KCl, and 5 mM $MgCl_2$, with or without 2 μM ADP. After this exposure to Mg^{2+}, ATP was added and hydrolysis measured as described under Experimental Procedures with or without a final concentration of 100 mM sulfite present.

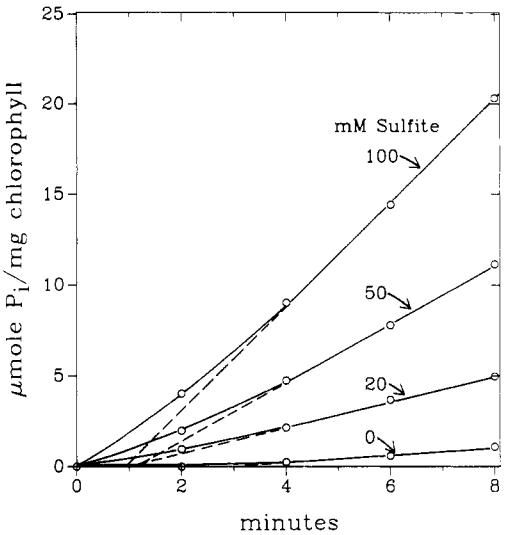

FIGURE 2: Effect of sulfite concentration on the activation of thylakoid membrane ATPase. The ATPase-activated thylakoid membranes were incubated for 2 min with 2 μM ADP, and then ATP was added with or without sulfite as described in Figure 1. The final sulfite concentrations are indicated on the figure.

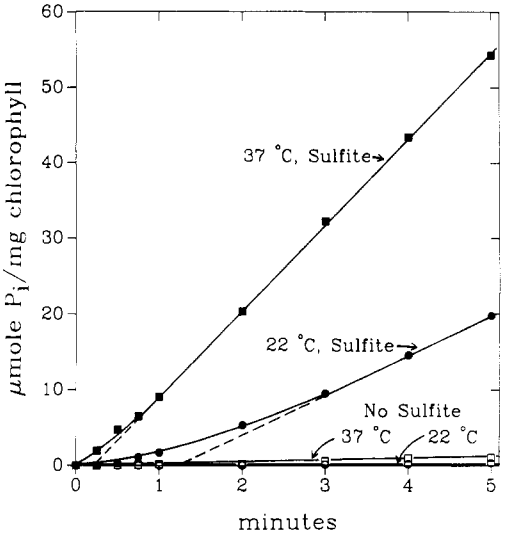

FIGURE 3: Effect of temperature on the lag in thylakoid membrane ATPase activation. The ATPase-active thylakoid membranes were exposed at 22 or 37 °C to 5 mM Mg^{2+} and 2 μM ADP, and then ATP hydrolysis was measured with or without 100 mM sulfite present as described in Figure 1 and under Experimental Procedures.

A Pronounced Lag Occurs in the Sulfite Activation. Results presented in Figure 1 show the effect of 100 mM sulfite on the ATPase activity of the MgADP-inhibited thylakoids measured at 22 °C. As has been noted previously, with prior exposure to Mg^{2+} and ADP and without sulfite addition the ATPase activity is very low. If the prior exposure to ADP is omitted, there is considerable ATPase activity. The presence of sulfite, as reported by Larson and Jagendorf (1989), markedly increases the ATPase activity. The steady-state rate attained in the presence of sulfite for thylakoid membranes inhibited by prior exposure to Mg^{2+} and ADP is as great as the rate when the inhibition was not induced. Thus, the sulfite completely obliterates whatever extent of inhibition was induced by the prior exposure to Mg^{2+} and ADP. The most important finding shown by the data is the existence of a definite lag in the sulfite activation when the membrane ATPase is initially inhibited by prior exposure to ADP and Mg^{2+}. As indicated by the dashed extension of the steady-state rate to the abscissa, about 1 min was required to attain the maximal rate in the presence of 100 mM sulfite. With a pyruvate kinase trap present to remove ADP, the rate is linear for more than 10 min after the maximal rate is attained.

Activation by Sulfite Requires the Presence of ATP. To test whether exposure to sulfite would activate the enzyme without ATP present, experiments were conducted, under the conditions reported for Figure 1 but with up to 200 mM sulfite present for 5 min before addition of ATP. The same extent of delay in the onset of ATPase activity was found with or without exposure to sulfite prior to addition of ATP. This result is of distinct interest as it demonstrates a need for ATP binding for the sulfite activation to take place.

A Lag Occurs at Lower Sulfite Concentrations. In the experiments of Figure 1, 100 mM sulfite was added. Additional measurements were made at lower sulfite concentrations. Figure 2 shows the effect of the presence of 0, 20, 50, and 100 mM sulfite on the ATPase activity of thylakoid membranes after prior exposure to Mg^{2+} and ADP to inhibit the initial activity. A concomitant increase in ATPase activity with an increase in sulfite concentration is noted as expected. As shown by the dashed extensions to the abscissa, a lag is observed without sulfite addition and all sulfite concentrations tested. The highest sulfite concentration tested (100 mM) is considerably above that used by Larson et al. (60 mM) for their experiments on the correlation of P_i formed with bound [^{3}H]ADP released.

An additional point is shown by comparison of the data of Figures 1 and 2. They illustrate a variability in the response to sulfite that we have noted with different thylakoid membrane preparations. In five similar experiments with different preparations, the average lag time was 56 ± 19 s. In addition, some variability was observed in the tendency for the ATPase activity to increase without sulfite addition. For example, the preparation used in Figure 2 showed a somewhat greater increase in activity than the preparation used for Figure 1, as well as a shorter lag with addition of 100 mM sulfite. Our experience appears to be akin to that of Larson et al. (1989), who noted variability in the rates of [^{3}H]ADP replacement with different preparations.

A Lag Is Also Observed at 37 °C. The experiments of Larson and Jagendorf (1989) were conducted at 37 °C, and

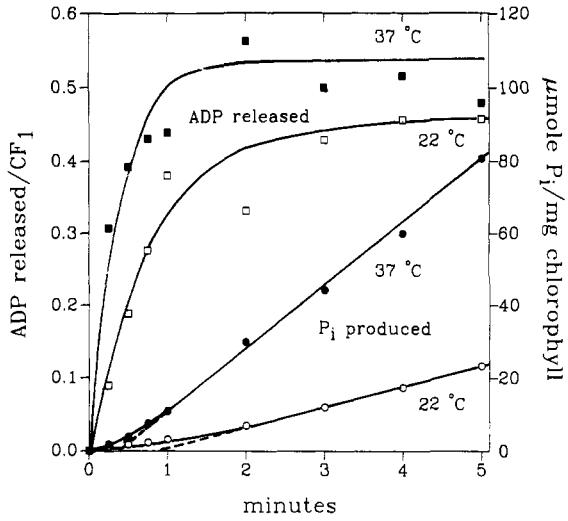

FIGURE 4: Correlation between the activation of thylakoid membrane ATPase and the release of the tightly bound [^{3}H]ADP. Thylakoid membranes were labeled with [^{3}H]ADP and washed, and ATP hydrolysis and bound [^{3}H]ADP release in the presence of 100 mM sulfite were measured as described under Experimental Procedures. The points for the [^{3}H]ADP release and ATPase activity are experimental. The lines for ATPase activity are drawn to meet the points. The lines for the bound [^{3}H]ADP are theoretical for a first-order process, as described in the text.

it was thus desirable to check if a lag was also evident at this temperature. The results presented in Figures 3 and 4 show that this is indeed the case. The rate at 37 °C is, as expected, considerably faster, and a lag of about 25% of that observed at 22 °C occurs.

Bound [^{3}H]ADP Is Released as the ATPase Activity Increases. If the presence of one inhibited catalytic site stops essentially all ATPase activity and if all three sites must participate sequentially for maximal activity, then the release of [^{3}H]ADP should parallel the increase in activity. The experiments reported in Figure 4 were designed to test this possibility. The ATPase activity and the release of bound [^{3}H]ADP as observed at two temperatures for the same preparation are shown in the figure. The activity curves are similar to those reported earlier (Du & Boyer, 1989). The extent of [^{3}H]ADP release rises to a maximal value as activity is regained. The lines for [^{3}H]ATP release given in the figure are theoretical plots assuming that release up to the observed maximum follows a first-order process with half-times of 35 and 17 s at 22 and 37 °C, respectively. Such half-times would give lags in the range of those observed. Within reasonable experimental error, the results show that the release of [^{3}H]ADP parallels closely the gain in catalytic activity. However, not all the bound [^{3}H]ADP is rapidly released. As shown in Figure 1, the presence of sulfite completely overcomes the inhibitions induced by prior exposure to Mg^{2+} and [^{3}H]-ADP. This means that full potential activity is attained even though a fair portion of the enzyme still retains tightly bound [^{3}H]ADP. This behavior is considered further under Discussion.

Discussion

Three characteristics of the sulfite activation that relate to the function of the tightly bound ADP at a catalytic site of the ATP synthase in thylakoid membranes are shown by the data reported here. One is that, with 100 mM sulfite present, exposure of the enzyme with tightly bound [^{3}H]ADP to Mg^{2+} before ATP addition results in a lag of about 1 min at 22 °C and of about 15 s at 37 °C before maximum activity is attained (Figure 3). A second is that with the sulfite-activated preparation the release of the tightly bound [^{3}H]ADP follows a first-order process that is closely correlated with the gain in catalytic activity at both temperatures (Figure 4). A third is that, with thylakoid membranes whose ATPase activity is inhibited by exposure to ADP and Mg^{2+}, incubation with sulfite before ATP addition does not overcome the lag.

The observation of a pronounced lag in the sulfite activation of the F_1-F_o ATPase activity of chloroplast thylakoids was foreshadowed by previous data. The isolated CF_1 ATPase with tightly bound ADP at catalytic sites when exposed to Mg^{2+} before ATP addition shows a well-documented lag in attaining the steady-state ATPase rate (Carmeli et al., 1981; Feldman & Boyer, 1985). More pertinently, a lag in sulfite activation has been seen previously with the intact F_1-F_o ATPase from another enzyme source; Moyle and Mitchell (1975) found a lag in the sulfite activation of the ATPase activity of liver submitochondrial particles.

For the experiments on the correlation between bound [^{3}H]ADP removal and activity, account needs to be taken of the portion of the tightly bound [^{3}H]ADP that remains after essentially full activation is attained. There are several reasons why enzyme that shows sluggish [^{3}H]ADP replacement may be present. The γ-subunit sulfhydryl groups of a portion of the enzyme may be partially reoxidized, resulting in a loss of catalytic capacity. Some partially damaged or incompletely active enzyme may be present that can still bind ADP at catalytic sites. This is the probable basis for why some CF_1 preparations have a portion of their tightly bound ADP at catalytic sites that are difficult to replace (Feldman & Boyer, 1985; Leckband & Hammes, 1987). Such a characteristic is readily understandable in that difficulty may be expected in attaining 100% active enyzme. A sluggish enzyme may be one that has lost capacity for full catalytic site cooperativity but retains some capacity for un-site or bi-site catalysis. Mutant forms of the *Escherichia coli* F_1 ATPase are known that readily bind ATP and form tightly bound ADP at catalytic sites and that have lost the cooperativity necessary for rapid turnover (Wise et al., 1984; Duncan & Senior, 1985). In addition, there may be small amounts of the [^{3}H]ADP at noncatalytic sites. We regard the tightly bound [^{3}H]ADP that is not readily replaced (for example, see Figure 4) as being at sites that do not have a sulfite-activatable capacity to participate in rapid catalysis.

An important result for understanding the basis for the sulfite activation is that incubation of the thylakoid membranes with sulfite prior to the addition of ATP did not eliminate the lag in the onset of full activity after ATP addition. If removal of the tightly bound ADP is necessary for overcoming the lag, then the incubation with sulfite would not be expected to remove the ADP. This is as observed by Larson et al. (1989). Earlier, Vasilyeva et al. (1982) noted that the inhibition induced by Mg^{2+} and ADP with beef heart submitochondrial particles was not overcome by incubation with sulfite. It is clear that the inhibited form of the thylakoid ATPase induced by Mg^{2+} and ADP in the dark (Du & Boyer, 1989) is not reactivated by sulfite unless MgATP is present. Because the inhibitory ADP is at one catalytic site and all noncatalytic sites very likely have bound nucleotide present under the conditions used (Xue et al., 1987), the effect of MgATP must be due to MgATP binding at another catalytic site. In accord with the mechanism of bicarbonate activation of CF_1 ATPase (Guerrero and Boyer, unpublished results), we propose that sulfite combines in place of P_i with the enzyme–ADP form and makes it easier for the conformational change induced by MgATP

binding at another catalytic site to cause release of the tightly bound ADP.

The inability of incubation of the inhibited enzyme–ADP–Mg^{2+} with sulfite to overcome the lag indicates that the activation does not result from the combination of sulfite with the inhibited enzyme but must await the addition of ATP. The addition and release of sulfite are probably rapid, with the slow step responsible for the lag being the conformational changes following MgATP binding at another catalytic site.

The above-suggested mode of sulfite activation differs from earlier suggestions. For example, Vasilyeva et al. (1982) showed that sulfite favored reversal of the ADP inhibition but suggested that sulfite shifted an equilibrium that existed between inactive and active enzyme forms. Larson et al. (1989) recognized that sulfite loosens an ADP–CF_1 association but suggest that sulfite duplicates the effect of a high-energy state.

Our results are consistent with the binding change mechanism in which the catalytic site occupied by the tightly bound [^{3}H]ADP joins the other two catalytic sites for continued steady-state catalysis, but they do not prove that this is the case. In contrast, if it should be established that fully active ATPase can be obtained while the catalytic site occupied by [^{3}H]ADP remains inactive, then only two active catalytic sites are required for rapid steady-state catalysis. The patterns of ^{18}O exchange have given convincing evidence that catalysis by all participating sites follows the same pathway (Boyer, 1987). For this reason we are reluctant to accept suggestions of catalysis limited to two sites because it is difficult to understand how two sites could carry out catalysis identically when their interactions with the single-copy subunits and the inactive β-subunit are very likely not identical. Consideration thus needs to be given to why Larson et al. (1989) did not find release of the tightly bound [^{3}H]ADP to be correlated with the development of ATPase activity.

Probably no single explanation accounts for the discrepancy between our results and the interpretation of those of Larson et al. (1989). It must be emphasized that there is likely considerable heterogeneity of the bound [^{3}H]ADP, as is evident from their Figure 1 and shown also by our data in this paper. A considerable portion shows a slow or even very slow rate of replacement. The 10-min period that they used for the [^{3}H]ADP labeling could have sufficed for labeling of quite sluggish as well as fully activated synthases on thylakoid membranes, and the subsequent two washes to remove medium [^{3}H]ADP could have damaged the enzyme further. In addition, the well-washed thylakoid membranes could have a small fraction of empty noncatalytic sites that were filled with poorly replaced [^{3}H]ADP. Prior experience with isolated CF_1 is instructive in this regard. Bruist and Hammes (1981) regarded the tightly bound [^{3}H]ADP on CF_1 as not at catalytic sites on the basis of the same reasoning used by Larson et al. (1989), namely, that after ATP addition many molecules of ATP per enzyme could be hydrolyzed while considerable tightly bound [^{3}H]ADP remained. Later kinetic studies of Feldman and Boyer (1985) suggested but did not prove that the replacement of the [^{3}H]ADP was sufficiently rapid to account for the appearance of increased ATPase activity. Leckband and Hammes (1987) used experimental procedures better suited for rapid kinetic measurements and demonstrated that most of the bound [^{3}H]ADP was removed at a kinetically competent rate. Both Feldman and Boyer (1985) and Leckband and Hammes (1987) note that a fair portion of the [^{3}H]ADP appeared to be on sluggish enzyme and was not rapidly replaced.

Figure 1 of Larson et al. (1989) is the only experiment they report in which the initial rate of bound [^{3}H]ADP release can be assessed. This experiment needs to be examined more closely because it does not, as they noted, suffice to eliminate an initial quite rapid replacement. In their Figure 1, the line for the amount of bound [^{3}H]ADP present appears to be drawn from the last measured point prior to sulfite addition and not from the time of addition. This makes the initial rate appear slower. The first measurement after sulfite addition appears to be at 15 s, and the measurement does not eliminate the possibility that most of the release observed, after a short lag, occurred in less time. Perhaps more importantly, they calculate their [^{3}H]ADP release rates from semilogarithmic plots of $-\ln ([ADP]_t - [ADP]_{tot})$ vs time. This could be misleading because about half of their total bound [^{3}H]ADP appears to be more slowly replaced than the initial portion. The value obtained from the semilog plots will reflect the slowly replaced better than any rapidly replaced [^{3}H]ADP.

Figure 1 of Larson et al. (1989) also does not provide convincing evidence for a lack of a lag. As we note, a lag is more easily observed at a lower temperature than they used. If their data points are considered to be precise, there is an upswing in the rate during the first 90 s suggestive of a lag. This makes the 15-s point important for assessment of whether or not a lag occurred. The amount of P_i present at this time is not given. Their extensive wash without Mg^{2+} and ADP present, by reducing medium ADP and Mg^{2+} to very low levels, could have activated some ATPase, and during the about 3 min that ATP was present before sulfite was added, sufficient P_i could have been formed to reduce the accuracy of the indicated increase at 15 s. In addition, a fraction of activated ATPase that gives only very slow P_i formation in the absence of sulfite could start hydrolyzing ATP much more rapidly as soon as sulfite is added, adding to P_i formation without bound [^{3}H]ADP release.

In addition to the above considerations, it is pertinent that the 60 mM concentration of sulfite used for the experiment of Larson et al. under discussion is considerably less than that they found necessary for half-maximal activation of their thylakoid preparation. Without sulfite present, MgATP does not induce an active ATPase—it is as if any lag were very long. It is thus logical that sulfite concentrations that partially activate the enzyme would not eliminate all lag behavior. Although in our limited experiments we did not note any appreciable change in the lag time with an increase of sulfite concentration up to 100 mM, considerably higher sulfite concentrations might reduce the lag time.

Consideration is also appropriate for the interesting observations of Larson et al. (1989) on the slow ADP-promoted release of the tightly bound [^{3}H]ADP. Two explanations may be offered. There is considerable adenylate kinase activity associated with thylakoid membranes, and the inhibition by diadenosine pentaphosphate may have not been complete. Thus, the [^{3}H]ADP release could result from a very slow rate of ATP formation and hydrolysis. A second possibility is that medium ADP may promote a slow direct exchange with the tightly bound ADP. Release of the ADP may not occur in one step, and with partial release there may be partial occupancy of a binding region by medium ADP, thereby promoting further release. The ADP-promoted exchange thus does not provide evidence against the suggested role for ATP in the binding change mechanism.

Our observations and the preceding considerations are regarded as adequately removing the concern expressed by Larson et al. (1989) that their results appeared not to be

consistent with the site retaining the tightly bound [^{3}H]ADP participating in an obligatory alternating- or sequential-site catalysis. To have a form of the F_1 ATPases with tightly bound ADP at a catalytic site that becomes very sluggish in presence of Mg^{2+} is not an expected property for an enzyme. This behavior has caused considerable past confusion because insufficient recognition has been given to the potential presence of two types of tightly bound ADP, at catalytic and at noncatalytic sites, and because when the ADP is at catalytic sites an inhibited form arises from exposure to Mg^{2+}. These and other factors have delayed the recognition that present information is consistent with the catalytic site that binds ADP tightly becoming an equal participant with other catalytic sites in steady-state catalysis.

The results of this paper show that the exchangeable and tightly bound ADP on active ATP synthase of chloroplast thylakoids is at a site that participates with other catalytic sites in the onset of rapid photophosphorylation. This is in harmony with early results of Smith and Boyer (1976) and of Gräber et al. (1977), showing that with onset of photophosphorylation the tight ADP is released during the first enzyme turnover.

Registry No. ADP, 58-64-0; ATP synthase, 37205-63-3; ATPase, 9000-83-3; MgATP, 1476-84-2; sulfite, 14265-45-3.

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