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Sulfite stimulation of chloroplast coupling factor ATPase

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The latent ATPase of spinach chloroplasts is activated by the thylakoid high-energy state, plus reduction of the disulfide bond on the γ subunit. If the high-energy state decays, activity disappears. We find that ATPase activity can be restored not only by a second illumination, but also by inorganic sulfite in the dark. With sufficient sulfite present, high concentrations of uncoupler no longer inhibit ATP hydrolysis. However, sulfite does not replace light in permitting rapid reduction of the disulfide bond on the γ subunit by dithioerythreitol. Sulfite together with saturating levels of the uncouplers, NH_4Cl and gramicidin, stimulates very high rates of Mg^{2+}-dependent ATPase. Using thylakoids treated with trypsin following reduction in the light, the consequent rates of ATPase can be as high as 3000 μmol/mg chlorophyll per h, or 5-times faster than any previously reported. The system follows hyperbolic kinetics with respect to sulfite, and extensive kinetic characterization of thylakoid-bound ATPase became possible. Kinetic analyses indicated competitive relationships between ADP and both ATP and sulfite, and between azide (a high-affinity inhibitor in this system) and sulfite. There was a mutual interdependence of kinetic constants for ATP and for sulfite: as the concentration of one of these decreased, the values for both K_m and V_m of the other decreased.

Introduction

Chloroplast coupling factor synthesizes ATP using energy from a transmembrane proton gradient (ΔpH) that develops with illumination during photophosphorylation [1]. ATP is synthesized on an extrinsic membrane protein (CF_1) containing five subunits [2] in a probable stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$ [3,4]. An intrinsic membrane component (CF_o) serves as the binding site for CF_1 and conducts protons across the thylakoids [5,6].

CF_1 is light-regulated [7], undergoing a conformational change in the light [8] that exposes a disulfide in the γ subunit for reduction in vivo [9,10] or in vitro by sulfhydryl-reducing reagents [11]. Isolated thylakoids are usually latent for the reverse reaction of ATPase, but a dark-phase, Mg^{2+}-dependent, activity can be elicited after reduction [12]. ATP hydrolysis in this system is coupled to reverse proton pumping, as indicated by stimulation on addition of limited concentrations of uncouplers [13,14], showing that an excessive ΔpH restricts continuing ATPase. Also indicative of tight coupling with proton pumping, is the inhibition caused when the trans-membrane movement of protons is prevented by DCCD modification of CF_o [5]. While reduction in the light is sufficient for initial activation of ATPase, a continuing ΔpH is needed to maintain activity in the dark. Thus, the ATPase decays when illuminated thylakoids are stored in darkness without ATP, and can be restored by a brief light flash to form a ΔpH [15,16]. Earlier evidence included the decay of ATPase by delaying ATP addition after darkening [17], or by adding saturating levels of uncoupler to destroy the ΔpH [13].

Trypsin treatment of unmodified thylakoids in the light also potentiates a Mg^{2+}-dependent ATPase [18]. This ATPase is similarly dependent on the maintenance of a ΔpH for activity, but requires continuous illumination since trypsin treatment partially uncouples [19]. A light-dependent cleavage site in the γ subunit has been

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Abbreviations: Caps, 3-(cyclohexylamino)-1-propanesulfonic acid; CF_1, coupling factor one from spinach thylakoid membranes; Ches, 2-(cyclohexylamino)ethanesulfonic acid; DCCD, *N,N'*-dicyclohexylcarbodiimide; DL thylakoids, thylakoids activated by dithioerythreitol in the light; DTE, dithioerythreitol; FCCP, carbonyl cyanide *p*4-trifluoromethoxy-phenylhydrazone; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; Mes, 4-morpholineethanesulfonic acid; Pipes, 1,4-piperazine-diethanesulfonic acid; SDS, sodium dodecyl sulfate; Taps, tris(hydroxymethyl)methylaminopropanesulfonic acid; TDL thylakoids, thylakoids activated by trypsin exposure in the dark following light + dithioerythreitol; TL thylakoids, thylakoids activated by trypsin in the light; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine.

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characterized as identical to one of two sites cleaved when reduced thylakoids are treated with trypsin in the dark [20].

We report that the oxyanion sulfite (HSO_3^-) is able to substitute for the ΔpH requirement requirement for maintenance of ATPase – once thylakoids have been potentiated for ATPase by treatments affecting the γ subunit of CF_1. Stimulation of ATPase by sulfite is hyperbolic in the presence of saturating uncoupler, side-stepping the difficulties in characterizing the kinetics of CF_1 when a trans-membrane ΔpH is necessary for maintenance of ATPase.

Materials and Methods

Latent thylakoids were prepared by grinding deveined spinach in a Waring Blender for 30 s with 300 mM sorbitol, 25 mM Tricine-NaOH (pH 7.5), 10 mM NaCl, 5 mM ascorbate, 1 mM EDTA, 200 μM phenylmethylsulfonyl fluoride, and 1 $mg \cdot ml^{-1}$ bovine serum albumin. The brei was filtered through 12 layers of cheesecloth and centrifuged at $5000 \times g$ for 5 min in a Sorvall SS-34 rotor. The pellet was resuspended in 1 mM Taps (pH 8.0) and 10 mM NaCl, centrifuged at $5000 \times g$ for 5 min and resuspended in the same medium at a chlorophyll concentration of 1.5 $mg \cdot ml^{-1}$. Chlorophyll was determined as in Ref. 21.

Trypsin-treated latent thylakoids were prepared by incubating latent thylakoids with 1 μg TPCK-trypsin/10 μg chlorophyll for 10 min at 0 °C in 1 mM Taps (pH 8.0) and 10 mM NaCl, followed by a 10-fold (w/w) excess of lima bean trypsin inhibitor.

DTE plus light (DL) thylakoids were prepared by suspending latent thylakoids as a film between the inner and outer surfaces of two close-fitting test tubes (0.023 mg chlorophyll per cm^2 of glass surface area) with 50 mM Tricine (pH 8.0), 50 mM KCl, 200 μm *N*-methylphenazonium methosulfate, 2 mM $MgCl_2$, 20 mM Dithiothreitol, 10 μM ADP, 0.882 mM Taps (pH 8.0) and 8.82 mM NaCl. The double test tube apparatus was stoppered, submerged in a 16 °C water bath, and illuminated with 3 $mmol \cdot m^{-2} \cdot s^{-1}$ photosynthetically active radiation for 10 min. After illumination, the thylakoids were stored on ice in the dark with additional DTE to a final concentration of 40 mM. Unless coupled thylakoids were necessary, NH_4Cl to 5 mM and gramicidin to 10 μM were added after illumination as the saturating uncoupler.

For preparation of trypsin plus light (TL) thylakoid, the same procedures were followed as for DL thylakoids, except that DTE was omitted, trypsin was added to 1 μg trypsin/20 γg chlorophyll (75 $\mu g \cdot ml^{-1}$) and the illumination time was decreased to 5 min. Immediately after illumination, a 10-fold (w/w) excess of lima bean trypsin inhibitor, NH_4Cl to 5 mM and gramicidin to 10 μM were added. The uncouplers ensured rapid loss of the high-energy state in these thylakoids. These procedures were adapted from Ref. 20.

Trypsin after DTE plus light (TDL) thylakoids were prepared by exposing DL thylakoids (containing the saturating uncoupler) to 1 μg trypsin/10 μg chlorophyll for 10 min at 0 °C in the dark, followed by the addition of trypsin inhibitor. This protocol was also adapted from Ref. 20.

Phosphate from hydrolyzed ATP was determined colorimetrically using a modification of the method described in Ref. 22. The phosphate reagent was prepared by mixing 3 volumes of A (3.66 M acetic acid, 0.66 M sodium acetate, 20 mM $CuSO_4$, 1% SDS) with 1 volume of B (5% $(NH_4)_6(MoO_4)_{24}$) then adding 1 volume of C (2% *p*-methylaminophenol sulfate, 10% Na_2SO_3).

ATPase was initiated by adding 2–10 μl of thylakoids (approx. 3–13 μg chlorophyll) to 0.5 ml of reaction mixture containing additions as indicated. The reaction was stopped by 1 ml of the phosphate reagent and further development of phosphate-dependent color was halted after 5 min by 0.5 ml of 10% sodium citrate (a modification of the color-stop solution used in Ref. 23). All ATPase measurements were referenced to a blank without enzyme that contained MgATP, but not sulfite. The assays were carried out at 37 °C for 2–10 min. The assay pH was controlled by titrating concentrated reagent stocks with 10 M HCl or NaOH until a dilution of the stock, at the highest assay concentration, did not vary from the desired pH by more than 0.03 units.

ATPase measurements for kinetic analysis were collected as a series of treatments. Across the series of treatments MgATP was altered, but within each treatment, MgATP was constant while sulfite was varied. Once each treatment had been analyzed to determine the kinetic constants for sulfite ($K_s$obs and $V_s$obs), data for a constant sulfite concentration, but from treatments across the series with varying MgATP concentrations, were plotted to estimate the kinetic constants for MgATP ($K_m$obs and $V_m$obs). An apparent weak chelation between Mg^{2+} and sulfite was compensated for, if indicated, by adding 2 mM $MgCl_2$ in excess of the MgATP concentration. In view of their hyperbolic response, ATPase data were analyzed using the non-linear least-squares minimization routine in [24].

The ATPase response to sulfite stimulation was hyperbolic, and so the same mathematical analysis could be used (similar to the Michaelis-Menten treatment), as if sulfite were another substrate. We realize that it is probably not consumed in the reaction, but the mathematical treatment simplifies the presentation and interpretation of a large amount of data, and facilitates comparisons between sulfite and true substrates. The terms 'competitive', 'K_i', etc. are used as mathematical abstractions, not as components of a formal kinetic

model. Since the values for these constants for sulfite varied depending on the ATP concentration, they are usually designated as '$K_s$obs' and '$V_s$obs'. Numerical constants for sulfite and substrates are reported in the figures and tables.

All reagent grade chemicals, biochemicals and enzymes were purchased from Sigma except for anhydrous $MgCl_2$ (Aldrich), TPCK-trypsin (Cooper Biomedical; Worthington Division) and *p*-methylaminophenol sulfate (Kodak). ATP solutions (Grade I) were calibrated assuming an extinction coefficient of 15.4 mM^{-1} at 259 nm [25]. The $MgCl_2$ was assumed to be 98% pure (Aldrich literature) and stock solutions were prepared from supernatants after centrifugation at 50000 × *g* for 30 min. The pK_a for the dissociation of HSO_3^- to SO_3^{2-} was estimated to be 6.85 at 25°C by titration of a 100 mM Na_2SO_3 solution with additions of 10 M HCl.

Results

Thylakoid membranes with ATPase activated by light and DTE ('DL' thylakoids) lose this activity on storage in the dark. In preliminary experiments [26], we found that a very active ATPase could be restored by adding sulfite to these completely inactive preparations, entirely without the usually necessary [15,16] second illumination. While sulfite replaced light in bringing activity back to already potentiated thylakoids, it could not substitute for light in the initial activation. Treatment of latent thylakoids in the presence of MgATP and sulfite with either DTE or low levels of trypsin did not elicit substantial ATPase. Nor did these treatments potentiate ATPase that could be expressed later in the light period, following washing to remove the sulfite and DTE or trypsin. Thus sulfite was apparently unable to replace the light-formed ΔpH causing conformational changes that expose the γ subunit to reduction by sulfhydryl-reducing reagents or to cleavage by trypsin. However, once ATPase was potentiated through the light-mediated treatments, sulfite was found to stimulate ATPase substantially, even in the presence of saturating uncoupler.

The sulfite-stimulated ATPase of DL thylakoids is most probably coupled to the development of a ΔpH. This is seen clearly by the requirement of the presence uncoupler in order to achieve maximal ATPase rates with sulfite (Fig. 1). In addition, the presence of enough sulfite caused insensitivity to the usual inhibition by excess uncoupler. With 80 mM sulfite, even 20 mM NH_4Cl failed to inhibit ATPase. This reinforces the idea that when sulfite is present, the high-energy state that is initially formed by light but then maintained by ATP hydrolysis, is no longer necessary to maintain ATPase. Substitution of gramicidin or FCCP for NH_4Cl gave very similar uncoupler profiles (data not shown). The ATPase of TL and TDL thylakoids also did not

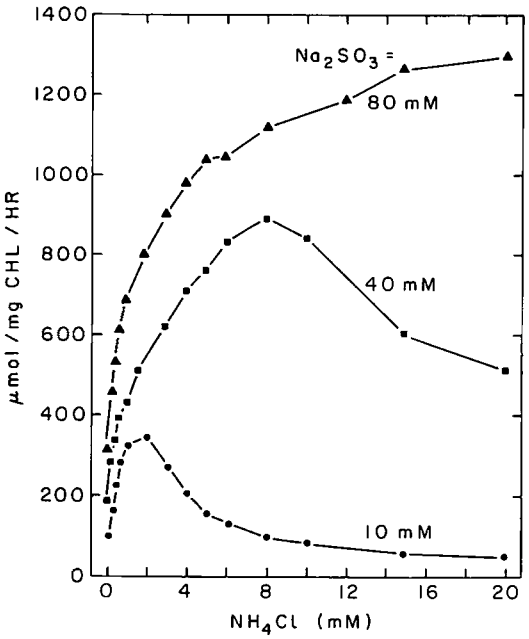

Fig. 1. Effect of partial uncoupling on ATPase of DL thylakoids in the presence of sulfite. The reaction mixture contained 50 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP plus the indicated concentration of NH_4Cl and 10 mM Na_2SO_3 (filled circles), 40 mM Na_2SO_3 (filled squares) or 80 mM Na_2SO_3 (filled triangles).

respond to uncoupler addition in the presence of 80 mM sulfite.

A further criterion for generation of a ΔpH during ATPase with sulfite was persistence of activity after dilution of the sulfite to ineffective levels (Table I). If sulfite-stimulated ATPase failed to pump protons, its activity should decay immediately on dilution of sulfite to below effective concentrations. This immediate loss of activity was seen, for instance, in the case of the methanol-stimulated thylakoid ATPase [27]. On the other hand, formation of a ΔpH during sulfite-stimulated ATPase should be detectable as continued ATPase after dilution. This was found (Table I, condition 1) even when uncouplers were present (condition 2) after the transfer to medium without sulfite (0–5 min rate). The presence of sulfite permits some ATPase activity even with truly saturating levels of uncoupler present (conditions 3 vs. 4, after transfer, Table I). The activity of stored thylakoids during the first exposure to MgATP (see legend to Table I) also illustrates the activation of ATPase by sulfite in otherwise inactive, pre-reduced thylakoids.

Attempts were made to determine the kinetic parameters of stimulation by sulfite. While the stimulation by sulfite was apparently hyperbolic, the presence of a concurrent trans-membrane ΔpH caused a decidedly non-linearity in Lineweaver-Burke plots of 1/rate vs. 1/sulfite concentration (Fig. 2). The addition of 5 μM gramicidin and 5 mM NH_4Cl caused near linearity of the plot. To ensure that only sulfite-dependent ATPase

TABLE I

Transfer experiments demonstrating the formation of a ΔpH during sulfite stimulated ATPase

DL thylakoids were first incubated in 25 mM TAPS (pH 8.4), 50 mM KCl, 5 mM $MgCl_2$, 5 mM ATP, 100 mM Na_2SO_3 for 30 s. After 30 s, 10 μl (5 μg chlorophyll) was transferred to 1.0 ml of fresh reaction mixture (Second ATPase), without Na_2SO_3, but with the additions indicated. The entire experiment was performed twice. ATPase during the first 30 s incubation with Na_2SO_3 was 210 μmol ATP per mg chlorophyll per h for Expt. 1 and 160 μmol ATP per mg chlorophyll per h for Expt. 2. The incubation in the second reaction was divided into two time periods to separate any transient effects caused by the transfer (0.–5 min) from steady-state ATPase (5–10 min).

Condition	Additions to the second ATPase	Exp. No.	ATPase rate (μmol ATP·mg chlorophyll·h)	
			0–5 min	5–10 min
1	no addition	1	83	10
		2	110	76
2	10 μM gramicidin and 5 mM NH_3Cl	1	65	23
		2	81	0
3	100 mM Na_2SO_3, 5 mM NH_4Cl, 10 μM gramicidin	1	200	130
		2	72	120
4	100 mM Na_2SO_3	1	380	350
		2	480	440

would be characterized, saturating uncoupling with 5 mM NH_4Cl plus 10 μM gramicidin (Fig. 3) and subtraction of ATPase measured in the absence of sulfite was adopted for the kinetic analyses. From these

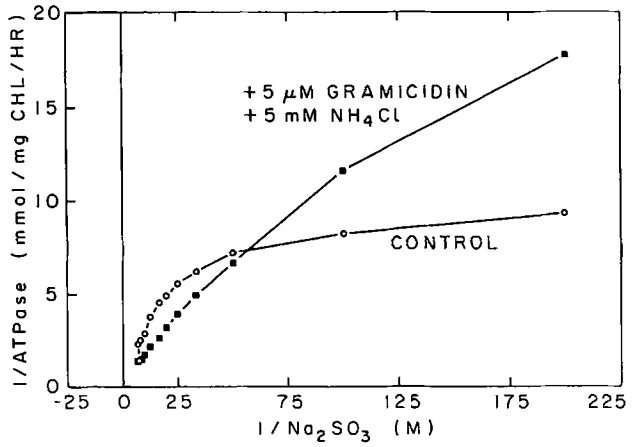

Fig. 2. Reciprocal plot to estimate the kinetic constants for sulfite in the presence and absence of saturating uncoupler. DL thylakoids were incubated in 50 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP with no additions (open circles) or with 5 μM gramicidin and 5 mM NH_4Cl (filled squares). The data were not adjusted for ATPase measured with 0 mM Na_2SO_3 which was 92 μmol ATP per mg chlorophyll per h in the absence of uncoupler (open circles) and 17 μmol ATP per mg chlorophyll per h with saturating uncoupler (closed circles).

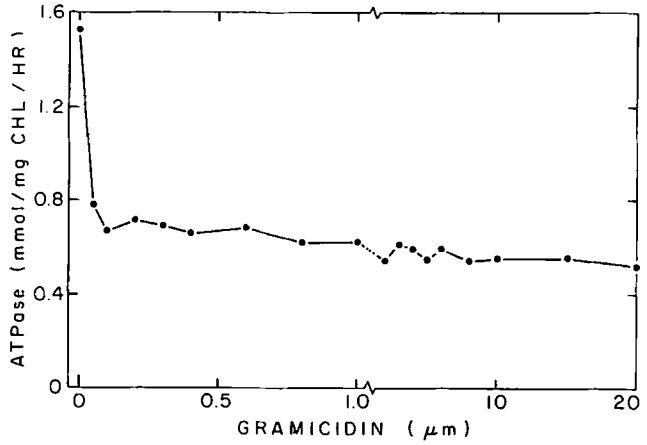

Fig. 3. Uncoupler concentration profile for DL thylakoids. The reaction mixture contained 50 mM Tricine (pH 8.0), 10 mM $MgCl_2$, 10 mM ATP, 80 mM Na_2SO_3 and 5 mM NH_4Cl with the indicated concentration of gramicidin.

analyses, values were obtained equivalent to K_m and V_m for a substrate or activator, but are designated $V_s$obs and $K_s$obs for use with sulfite data (Table II). This nomenclature is used because the values for sulfite vary with ATP concentration, and vice versa (see below), so any one determination is not an absolute constant.

The response of ATPase to sulfite concentrations is shown in Fig. 4. ATPase of latent and trypsin-treated latent thylakoids did respond to sulfite addition (Fig. 4a), but these rates were less than 5% of those determined with potentiated thylakoids (Fig. 4b). While the kinetic constants for sulfite (Table II) were somewhat variable from day to day, in general the $K_s$obs determined for both TL and TDL thylakoids was 10-fold less than that estimated for DL thylakoids. The TDL thylakoids consistently had the highest $V_s$obs (3 mmol ATP per mg chlorophyll per h) followed by the DL (1.5 mmol ATP per mg chlorophyll per h^{-1}) and lastly the

TABLE II

Estimation of the kinetic constants for sulfite ($K_s$obs and $V_s$obs) in the presence and absence of DCCD

The reaction mixture contained 50 mM Tricine (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 5 mM NH_4Cl and 10 μM gramicidin with varying concentrations of Na_2SO_3 between 5 and 120 mM in ten steps. DCCD, where indicated, was added to a concentration of 200 μM to the reaction mixtures and pre-incubated with the stock thylakoids for at least 15 min. $K_s$obs values are given in mM and $V_s$obs in μmol ATP per mg chlorophyll per h.

Thylakoid preparation	Control		+200 μM DCCD	
	$K_s$obs	$V_s$obs	$K_s$obs	$V_s$obs
Latent	35	39	170	170
Trypsin-treated latent	22	120	140	99
DL	270	1500	360	920
TL	32	710	76	640
TDL	22	3000	120	2000

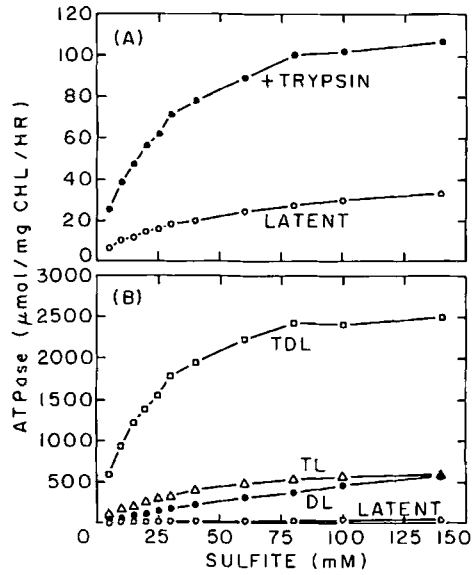

Fig. 4. Response of ATPase sulfite concentration. The reaction mixture contained 50 mM Tricine (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 5 mM NH_4Cl and 10 μM gramicidin with the indicated concentration of Na_2SO_3. (A) Latent thylakoids (open circles), trypsin-treated latent thylakoids (closed circles). (B) Latent thylakoids (open circles) DL thylakoids (filled circles), TL thylakoids (open triangles), TDL thylakoids (open squares). The plotted data were adjusted for ATPase occurring in the absence of Na_2SO_3 which was 9.2 (latent), 11 (trypsin treated latent), 11 (DL), 29 (TL), and 22 μmol ATP per mg chlorophyll per h (TDL).

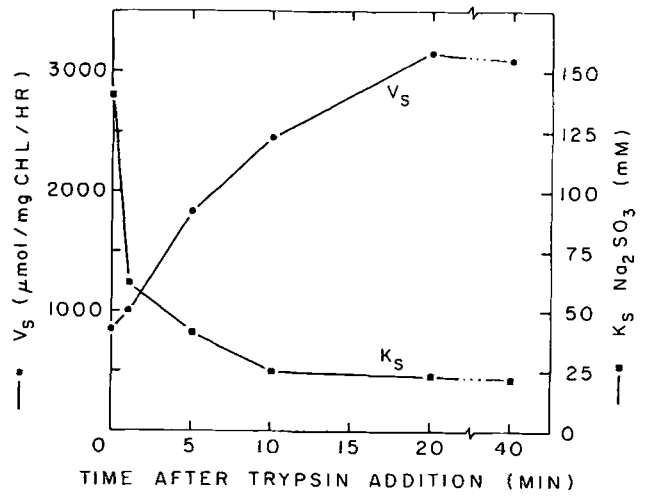

Fig. 5. Alterations of the kinetic constants for sulfite during trypsin treatment. DL thylakoids were treated with 1 μg trypsin/20 μg chlorophyll for the indicated periods of time then a 10-fold excess of trypsin inhibitor was added. The kinetic constants for sulfite ($K_s$obs, filled squares: $V_s$obs, filled circles) were determined in 50 mM Tricine (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 10 μM gramicidin and 5 mM NH_4Cl with 10 concentrations of sulfite between 10 and 120 mM.

TL thylakoids (0.7 mmol ATP per mg chlorophyll per h). In the case of the DL thylakoids, the $K_s$obs was so high (at pH 8.0) that concentrations in excess of 200 mM sulfite would have been needed to determine its value accurately. However, such high concentrations also began to inhibit the ATPase, thus it was not possible to determine the kinetic values precisely for DL thylakoids.

Examination of the time-course of trypsin treatment on the apparent kinetic constants for sulfite (Fig. 5) suggests there may be two effects. Specifically, the remarkable decrease in $K_s$obs caused by trypsin is much more rapid than the large increase in $V_s$obs. The former was 50% complete after 1 min of exposure to trypsin; the latter only after 6 min.

Surprisingly, 200 μM DCCD treatment caused only a slight inhibition of ATPase (Table II). Inhibition of DCCD has been ascribed to covalent modification of CF_0, which prevents the movement of protons through CF_0 (see Ref. 28). Under conditions similar to those of Table II, the ATPase of thylakoids activated solely by DTE plus light was inhibited by greater than 95% [5]. Relative failure to inhibit here should not have been due to interference with the DCCD-binding reaction, since thylakoids were pre-incubated with the reagent prior to addition of sulfite.

The mono-anionic form of sulfite (HSO_3^- = bisulfite) was reported to be the species active in stimulating ATPase of mitochondrial F_1 [29]. This was apparently confirmed with TDL thylakoids (Fig. 6). The $K_s$obs for HSO_3^- varies 3-fold over the 2.5 pH unit range, while that for total sulfite changes over 300-fold. These data make it much more probable that HSO_3^-, rather than SO_3^{2-}, is the species responsible for stimulating ATPase. Similar results were found for DL thylakoids (data not

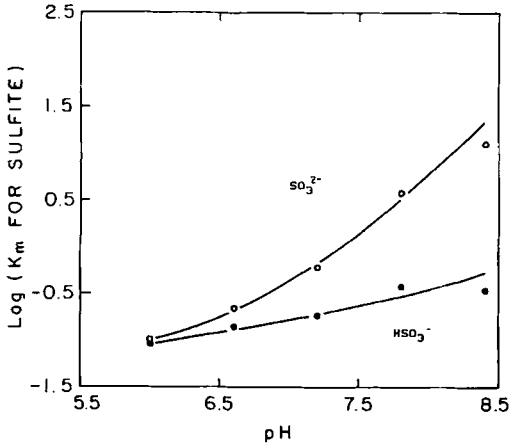

Fig. 6. Dependence of the $K_s$obs for (total) sulfite and the $K_s$obs for HSO_3^- on pH. TDL thylakoids were incubated in a reaction mixture containing 100 mM of a combined buffer stock (composed of equal parts of: Mes, Pipes, Hepes, Tricine, Taps, Ches and Caps) 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 10 μM gramicidin and 5 mM NH_4Cl adjusted to the indicated pH. The $K_s$obs for Na_2SO_3 is plotted as the $\log_{10}(K_s\text{obs})$ (indicated as $\log(K_m$ for sulfite)) in the units of log(mM) using open circles. A plot of the $\log_{10}(K_s\text{obs})$ for the concentration of monoprotonated HSO_3^- species is indicated by the filled circles. To estimate the concentration of HSO_3^- the Henderson-Hasselbalch equation was used at the indicated pH assuming a pK_a of 6.85 for H_2SO_3.

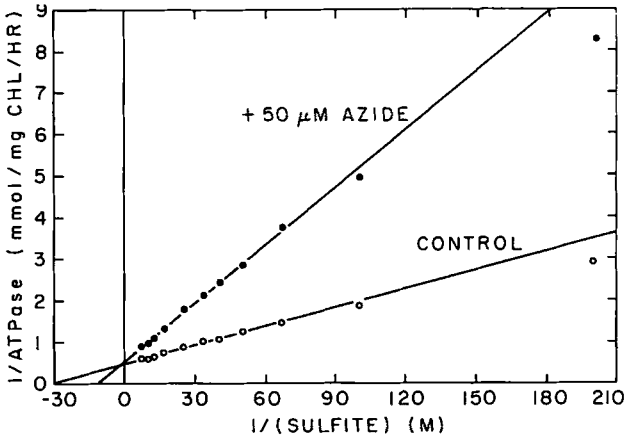

Fig. 7. Effect of azide on the kinetic constants for sulfite. TDL thylakoids were incubated in 50 mM Tricine (pH 8.0), 50 mM KCl, 10 mM $MgCl_2$, 10 mM ATP, 10 μM gramicidin, 5 mM NH_4Cl and the indicated concentration of sulfite with no additions (open circles) or with 50 μM azide (filled circles).

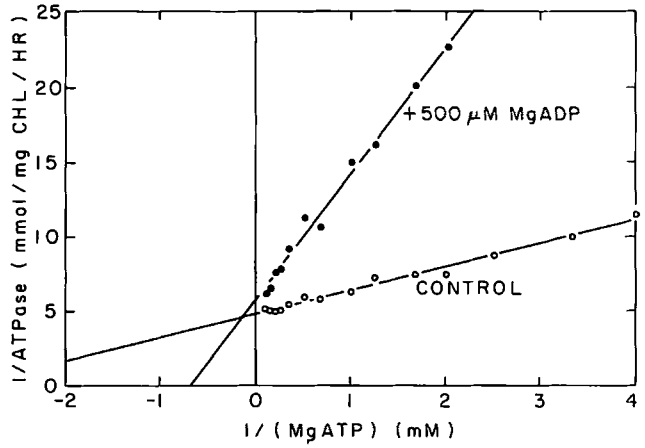

Fig. 8. Competitive inhibition between ADP and ATP in the presence of sulfite. DL thylakoids were incubated in 50 mM Hepes (pH 7.3), 50 mM KCl, 60 mM Na_2SO_3, 2 mM $MgCl_2$, 5 mM NH_4Cl, 10 μM gramicidin and the indicated concentration of Mg^{2+}/ATP with no additions (open circles) or with 500 μM Mg^{2+}ADP (filled circles).

shown). The dependence of stimulation on the HSO_3^- concentration was exploited in subsequent kinetic analyses of DL thylakoids. By decreasing the pH of the assay to 7.3, which increases the relative concentration of HSO_3^- 4-fold vs. that at a pH of 8.0, accurate determinations of the $K_s$obs for (total) sulfite with DL thylakoids could be determined (see below).

Stimulation of ATPase by bisulfite (HSO_3^-) rather than sulfite may explain why azide, also a mono-anion, was an apparent competitive inhibitor vs. sulfite (Fig. 7). Under these conditions, the kinetic constants for sulfite, with the TDL thylakoids, indicated a competitive K_i for azide of 28 μM (Fig. 7). The K_i for azide was not altered when TL (K_i = 27 μM) or DL (K_i = 33) thylakoids were substituted. Similarly, at a pH of 7.3, azide was also a competitive inhibitor of sulfite stimulation, and with DL thylakoids a K_i of 38 μM was determined. Competitive inhibition between azide and bicarbonate (HCO_3^-) toward the bicarbonate-stimulated ATPase of mitochondrial F_1 has been shown previously [29].

An apparent competitive interaction between ADP and ATP was observed in the presence of sulfite (Fig. 8). The kinetic constants for ATP indicated a competitive K_i for ADP of 130 μM with the DL thylakoids at a pH of 7.3 (Fig. 8). At a pH of 8.0, the K_i for ADP did not change and was similar for the DL, TL and TDL thylakoids (data not shown). However, demonstration of competitive inhibition between ADP and ATP required the addition of 2 mM $MgCl_2$ in excess of that added stoichiometrically with both ATP and ADP. In the absence of excess Mg^{2+}, the reciprocal plots were not linear and indicated apparent positive cooperativity with respect to ATP. This requirement for excess Mg^{2+} may indicate that high levels of sulfite chelate the metal, thereby altering the actual MgATP and MgADP concentrations. Uncertainties in determining actual species concentrations prevented a rigorous analysis of inhibition of ADP. A similar K_i for ADP was reported with soluble CF_1 in the presence of methanol and sulfite [30].

There was also an apparent competitive interaction between ADP and sulfite for stimulation of ATPase (Fig. (). In this case, the kinetic constants for sulfite indicated a K_i for ADP of 1.33 mM which was unaltered (K_i = 1.2 mM) when the experiment was repeated at a pH of 8.0. The slight diminution of $V_m$obs (Fig. 8) or $V_s$obs (Fig. 9) in the presence of ADP is expected if ADP is a competitive inhibitor of both ATP and sulfite. The 10-fold difference between the K_i values for ADP when competing against ATP (130 μM) vs. those when competing against sulfite (1.3 mM) suggests different sites of interaction for ADP. Alternatively, sulfite may

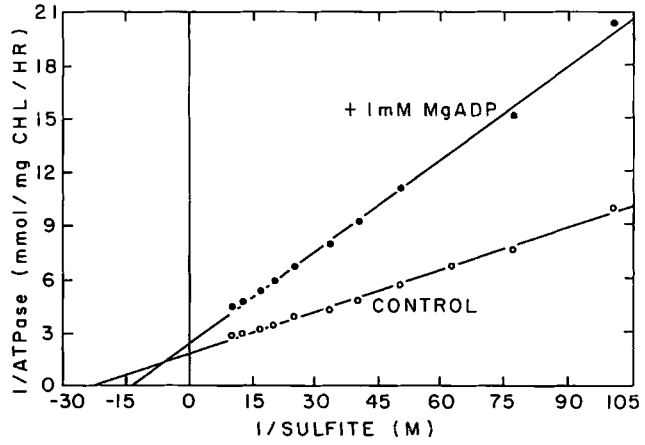

Fig. 9. Competitive inhibition between ADP and sulfite in the presence of ATP. DL thylakoids were incubated in 50 mM Hepes (pH 7.3), 50 mM KCl, 12 mM $MgCl_2$, 10 mM ATP, 5 mM NH_4Cl, 10 μM gramicidin and the indicated concentration of Na_2SO_3 with no additions (open circles) or with 1 mM Mg^{2+}ADP (filled circles).

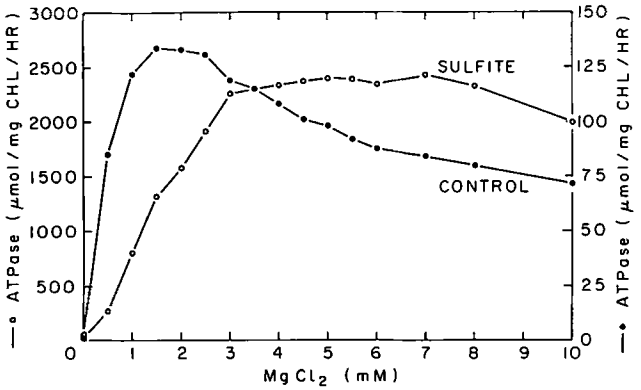

Fig. 10 Effect of Mg^{2+} concentration on ATPase. TDL thylakoids were incubated in 50 mM Tricine (pH 8.0), 50 mM KCl, 5 mM ATP, 5 mM NH_4Cl, 10 μM gramicidin and the indicated concentration of $MgCl_2$ in the absence (solid circles) and presence of 80 mM Na_2SO_3 (open circles). Note the different scales for the two reactions, indicated on the two ordinates of the graph.

alter the affinity of the CF_1 for ADP as it does for ATP (see below).

Magnesium had little effect on the sulfite-stimulated ATPase of potentiated thylakoids beyond its requirement as a substrate component (Fig. 10). Without sulfite, a small degree of inhibition by free Mg^{2+} could be seen (Fig. 10) although not nearly as much as that found with the methanol-stimulated ATPase of thylakoids, or of heat plus DTE-activated soluble CF_1 [27,31].

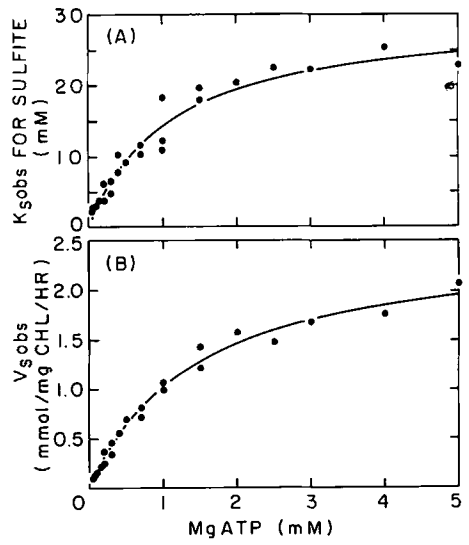

Fig. 11. Response of the kinetic constants for sulfite ($K_s$obs and $V_m$obs) to ATP concentration. TDL thylakoids were incubated in 50 mM Tricine (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 20 U·ml^{-1} creatine kinase, 1 mM phosphocreatine, 5 mM NH_4Cl, 10 μM gramicidin and the indicated concentration of MgATP and a range of Na_2SO_3 concentrations sufficient to estimate the kinetic constants for sulfite ($K_s$obs and $V_m$obs) for each concentration of sulfite. (A) Response of $K_s$obs to Mg^{2+}ATP concentration. Each point represents a determination of the $K_m$obs for sulfite at the indicated concentration of MgATP. The hyperbolic curve was drawn using the Michaelis-Menten equation assuming a numerical value of 30.3 for V_m, which should be equal to the upper limit of the $K_s$obs for sulfite in mM, and a value of 1.51 for K_m, which should equate the upper limit of the $K_m$obs for MgATP in mM. (B) Response of $V_s$obs MgATP concentration. The hyperbolic curve was drawn assuming a V_m of 2.56, which should equal the upper limit of $V_m$obs for MgATP in mmol per mg chlorophyll per h and a K_m of 1.51, which should equal the upper limit of the $K_m$obs for MgATP in mM.

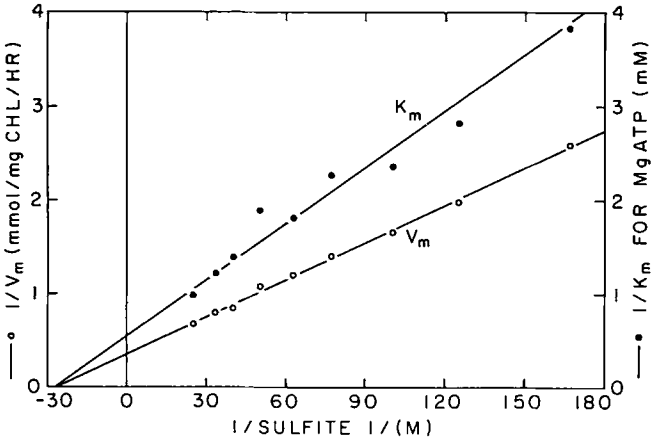

Fig. 12. Reciprocal plot of the kinetic constants for MgATP at varying sulfite concentrations. Here, the kinetic constants for MgATP ($K_m$obs and $V_m$obs) were determined at different concentrations of sulfite, then plotted vs. MgATP concentration as a reciprocal. TDL thylakoids were incubated in 50 mM Tricine (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 5 mM NH_4Cl and 10 μM gramicidin with the indicated concentration of Na_2SO_3 and a range of MgATP concentrations sufficient to estimate the kinetic constants at each sulfite level. Each determination of $V_m$obs (open circles) and $K_m$obs (closed circles), at the indicated sulfite concentrations, potentially used 24 levels of MgATP concentration between 50 μM and 5 mM. The lines were drawn using the reciprocal form of the Michaelis-Menten equation where the kinetic constant determined (either $V_m$obs or $K_m$obs) was substituted for V_m and the sulfite concentration was substituted for K_m. In the reciprocal plot of $V_m$obs (open circles) vs. sulfite concentration, the line was drawn using a numerical value of 2.93 for V_m, which should equal the upper limit of $K_s$obs for sulfite in mmol per mg chlorophyll per h and a K_m of 36.4, which should be the maximum value of the $K_s$obs for sulfite in mM. The plot of $K_m$obs at different concentrations of sulfite (closed circles), used a V_m value of 1.83 which should equate the maximal $K_m$obs for MgATP in mmol ATP per mg chlorophyll per h and a value of 39.4 for K_m, which should be the maximal $K_s$obs for sulfite.

A large interdependence was found between the kinetic constants for sulfite and for ATP. Increasing ATP levels caused continuous increases in values in both $K_s$obs and $V_s$obs for sulfite (Fig. 11) and increasing concentrations of sulfite similarly raised the values of the kinetic constants for ATP (Fig. 12). There seemed to be a reciprocal dependence between these two compounds in determining the kinetic constants of the other. When the kinetic constants for sulfite were determined at different MgATP concentrations, a hyperbolic relationship between $K_s$obs or $V_s$obs vs. MgATP was found (Fig. 11). If the curves in Fig. 11A and B were replotted on a reciprocal basis, straight lines were found as in Fig. 12. A similar dependence of the kinetic constants for MgATP on the sulfite level, plotted here

as a double reciprocal (Fig. 12), was also found. Results qualitatively similar to these were also found with DL thylakoids.

When the ATP concentrations were above 100 μM, the ratios of $V_s$obs/$K_s$obs, determined by varying sulfite levels at each level of ATP, were relatively constant. Similarly, when sulfite concentrations were greater than 10% of the maximal $K_s$obs (above 30 mM for DL thylakoids and 3 mM for TDL thylakoids), the ratios of $V_m$obs/$K_m$obs (i.e., the kinetic constants for MgATP), determined at each sulfite level, were also relatively constant. When either ATP or sulfite levels dropped below the minima noted above, values for both K_m (of MgATP) and K_S (of sulfite) dropped faster than the respective V_m values. However, technical difficulties at the very low substrate level prevented determination of whether these reductions were real or artifacts.

Discussion

In an operational sense, it is clear that sulfite replaces light and a ΔpH in permitting continuing catalytic activity by previously activated thylakoids whose ATPase has become inactive in a subsequent dark period. The failure of high levels of uncoupler to inhibit ATPase, once enough sulfite is present (Fig. 1), is another indication that the membrane high-energy state is no longer needed to maintain activity.

A further resemblance between light and sulfite is found in the striking interaction between the kinetic constants for sulfite and ATP (Fig. 11). In determining the kinetic constants for ADP in photophosphorylation [32], both K_m and V_m decreased as the light intensity decreased; and also the ratio V_m/K_m for ADP was independent of illumination. Exactly that pattern is found for the respective interactions between sulfite concentration and V_m/K_m for the ATPase substrate, ATP. This similarity further strengthens the analogy that sulfite acts as a substitute for the ΔpH necessary to maintain ATPase. It is also interesting that the values suggested to be lower limits for $K_m$obs and $K_s$obs are quantitatively similar to kinetic constants determined to be the half-maximal concentrations of sulfite and Mg^{2+}-ATP necessary to stimulate the release of ADP that was previously bound to CF_1 in the light (accompanying paper [55].

The activating effect (i.e., sulfite permitting activity with previously reduced thylakoid-bound CF_1, become inactive due to lack of a proton-motive force) may be different from the very large stimulation of rates of ATPase by sulfite (Figs. 1 and 4, etc.). Stimulation of mitochondrial F_1 ATPase by oxyanions was noted earlier [29], and ascribed to the product release step [33]. Sulfite stimulates the ATPase of F_1 from *Rhodospirillum rubrum* strongly and, as with chloroplast thylakoids, overcomes an inhibition imposed by high levels of uncouplers [34]. Webster et al. concluded that "...anions such as sulfite convert the chromatophore ATPase into a form which is a more efficient energy transducer..." [35]. The action of sulfite on chloroplast thylakoids CF_1 may be unique in permitting initial activation that ordinarily requires the high-energy state.

Since illumination (i.e., a ΔpH) is needed for both the initial activation of thylakoid ATPase, and for maintaining its activity, it could easily have been assumed that the same process was involved in both phenomena. Our results indicate this is not the case, since sulfite can substitute for the 'maintenance' function, but not for the primary activation by light, which exposes the γ subunit disulfide to reduction.

The sulfite-stimulated ATPase of (uncoupled) potentiated thylakoids is, in some ways, a greatly improved model system for studying the kinetics of thylakoid-bound CF_1. Unlike the usual ATPase, its activity is neither dependent on a degree of ΔpH for initial stimulation, nor inhibited by an excessive ΔpH during activity and therefore, is not sensitive to the delicate balance between those two effects. In the absence of uncoupler, the sulfite-stimulated ATPase of DL thylakoids was coupled to the formation of a ΔpH (Table I, Fig. 1), suggesting a normal enzyme system. In the presence of saturating uncoupler, the response of ATPase to sulfite concentration was hyperbolic (Fig. 4), facilitating kinetic analysis of the system.

The sulfite functional effect is almost certainly not due to chemical modification of the cysteines. First, its effects are shared by selenite, which has a different chemistry. Second, the sulfite stimulation is almost completely reversible on dilution, as long as the proton-motive force is destroyed (Table I); and that would not be true for an effect dependent on chemical modification.

An effect of prior reduction of the disulfide on the γ subunit, and the accessibility of this subunit to cleavage by trypsin, was noted earlier [20]. One site was accessible to cleavage under all circumstances, but a second site was cleaved only after the disulfide bond had been reduced. Our procedures for treating thylakoids with trypsin were very similar to those described in the earlier paper, and our results provide evidence for differing functional effects of cleavage at the two sites. In our case, the TL and TDL thylakoids would have a trypsin cleavage at the common site, and only the γ subunit of TDL thylakoids would be cleaved at the unique site. Both TL and TDl thylakoids showed a 10-fold decrease in $K_s$obs for sulfite compared to DL thylakoids (Fig. 4), so this change is a result of cleavage at the common site. Correspondingly, cleavage at the unique site after disulfide reduction, is probably responsible for the very large increase in $V_s$obs, unique to TDL thylakoids. Also suggestive of two trypsin cleavage

sites is the differential rate of change in $K_s$obs vs $V_s$obs (measured (measured subsequently) during the time course of trypsin treatment of DL thylakoids, with the disulfide bond already reduced (Fig. 5).

ATPase rates for TDL thylakoids with saturating sulfite (up to 3000 μmol/mg chlorophyll per h) must be the highest (constant) rates measured with CF_1 to date, which may indicate that the combination of tryptic cleavage at the second site, plus sulfite, overcomes all mechanisms that limit ATPase. We would like to note that this rate amounts to 833 turnovers of $CF_1 \cdot s^{-1}$, assuming 1 nmole CF_1 per mg chlorophyll. It is equal to the highest rates of photophosphorylation ever reported [36], which were those found for chloroplasts of Swiss chard grown in Israel. Maximal rates with ordinary spinach chloroplasts have not exceeded 50% of the ATPase rates noted here.

The failure of DCCD to cause major inhibition of sulfite-stimulated ATPase (Table II) implies that movement of protons through CF_0 is not a critical part of the catalytic cycle for thylakoid ATPase. Because sulfite-stimulated thylakoid ATPase does not depend on a trans-membrane ΔpH, any constraints imposed on ATPase by the state of CF_0 may be much diminished. It seems unlikely that the DCCD-poisoned ATPase is able to form a ΔpH; and the kinetic results would be consistent with a slight decoupling of CF_1 from CF_0 which allowed ATPase in the absence of proton conduction.

Except for 1 recent report from this laboratory [37], azide was not known as an inhibitor of thylakoid ATPase. This is, in part, because it fails to inhibit either the Ca^{2+}-dependent ATPase or ATP hydrolysis by fully coupled thylakoids [37]. The competitive interactions between sulfite and azide (fig. 7) superficially suggest that both of these anions are binding to a single site. However, in studies with mitochondrial F_1, arguments have been advanced that sulfite and azide do not bind to the same site. For instance, sulfite stimulation of F_1 ATPase can be eliminated by *p*-chloromercuribenzene sulfonate treatment, while azide is still an effective inhibitor [38,39].

Isolated F_1, unlike CF_1, exhibits substantial ATPase in the absence of sulfite, possibly as an intrinsic property of the conformational state of the enzyme. If so, azide inhibition may pertain to residual activity in the absence of sulfite activation just as much as to sulfite-stimulated ATPase. Alternatively, 'maintenance' of F_1 ATPase may be necessary just as with CF_1, but with this function carried out by ATP itself. This might explain the highly curved Lineweaver-Burks plots found when estimating the ATP kinetic constants with F_1 [29] and with methanol-activated CF_1 [27]. Methanol-activated CF_1 is functionally close to F_1, since it exhibits substantial intrinsic ATPase, which can be further stimulated by sulfite. Azide inhibition of the H^+-ATPase of *Escherichia coli* [40] was shown to affect multi-site, but not uni-site catalysis. In view of the specificity of the azide effects with CF_1, additional factors are likely to be involved in its action with this enzyme.

The mechanism by which sulfite stimulates ATPase is unknown, but reversal of Mg^{2+} inhibition is strongly suggested. With isolated CF_1, Mg^{2+} is a strong inhibitor [41,42], which can be reversed by stimulatory anions [43]. Also, with methanol-activated ATPase of thylakoids [27,31], both inhibition of ATPase by micromolar levels of free Mg^{2+}, and reversal of this inhibition by sulfite were demonstrated. With both thylakoid-bound and free CF_1, sulfite was by far the most effective anion.

The proton-pumping thylakoid ATPase activated by light and DTE (i.e., DL thylakoids with a ΔpH) is relatively insensitive to inhibition by low levels of Mg^{2+}. Mg^{2+} even failed to inhibit uncoupled DL thylakoids without sulfite present (Fig. 10); and in this respect chloroplasts differ from chromatophores of *R. rubrum* [34]. However, the inability to observe inhibition by added Mg^{2+} does not entirely rule out the suggestion that sulfite may stimulate by alleviating Mg^{2+} inhibition. In the absence of sulfite, activity may already be inhibited by Mg^{2+}, which would preclude further inhibition by exogenous Mg^{2+}.

There are strong precedents in the literature for expecting inhibitory effects of Mg^{2+} on the F_1/F_0 enzymes, possibly linked to the presence of bound adenylates. Formation of Mg^{2+}-inhibited conformations of F_1 [44] and CF_1 have been suggested [45]. The demonstration in Ref. 46 that pre-treatment of F_1 with EDTA plus ATP stimulates transiently higher ATPase, while addition of Mg^{2+} before ATP induces a lag in ATPase, which suggests that slow conformational changes induced by Mg^{2+} do occur. Of great relevance in this study was the observation [46] that sulfite was not an effective stimulator of ATPase, not was azide an effective inhibitor, during the period of transiently higher levels of ATPase. Additionally, stimulation by sulfite, and inhibition by azide, was immediate if the enzyme was pre-treated with Mg^{2+}. Similar MgATP concentrations were needed to shorten the lag phase, as in its function as substrate for ATPase. Qualitatively similar results have been found with CF_1 [47].

Taking into account the above inferences, and in view of the replacement of the light effect by sulfite, it seems possible that a major role of ΔpH in maintaining ATPase is to avert inhibition by Mg^{2+}. The lack of ΔpH generation during methanol-activated ATPase, or with soluble ATPase, would be the reason for their sensitivity to Mg^{2+}, following this concept.

Considering that sulfite is a phosphate analog, coupled with our observations that sulfite and ADP interact in a competitive fashion (Fig. 9), effects of phosphate on the binding of ADP to CF_1 may be relevant. The addition of phosphate with ADp, after illumina-

tion, but before ATP addition, prevents the dark decay of thylakoid ATPase usually observed [48]. Phosphate also enhances the ability of ATP to reactivate ATPase of darkened thylakoids that are devoid of nucleotides [49]. These effects of phosphate are probably due to the prevention of the binding of ADP [50], which may be a regulator of ATPase [51]. The demonstration that phosphate is able to stimulate the release of ADP that was previously bound to thylakoids [52] strengthens the possibility that interactions with bound ADP may be an important component in stimulation of ATPase by sulfite. With F_1 [53], pre-treatment with Mg^{2+} did not inhibit subsequent ATPase if the enzyme had been stripped of bound nucleotides. However, if a single ADP was bound before pre-treatment with Mg^{2+}, then inhibition by Mg^{2+} was apparent. Substitution of ATP for ADP did not result in sensitivity toward Mg^{2+} unless in situ hydrolysis to ADP was allowed. Phosphate reversed this ADP-dependent Mg^{2+} inhibition. The apparent linkage of Mg^{2+} inhibition with the presence of bound ADP has not been tested yet for CF_1.

With thylakoids, sulfate (H_2SO_4), chromate (H_2CrO_4), phosphite (H_3PO_3) and arsenate (H_3AsO_4), all reasonable phosphate analogues which are competitive with phosphate in photophosphorylation, failed to duplicate the sulfite effect [54]. Only selenite (H_2SeO_3) showed the same strong stimulation of thylakoid ATPase. If sulfite is binding to a phosphate site, the lack of stimulation by alternate anions, including phosphate, may be due to a greater stringency on structure required for stimulation vs. competition. Of the group, sulfite and selenite are the only trigonal anions which contain a carbonyl, hydroxyl and hydroxylate anion at physiological pH. These attributes are paralleled by small organic anions that stimulate an apparently similar Mg^{2+}-dependent ATPase of soluble CF_1 [43]. Most efficacious of the organic anions were dicarboxylic acids, of which, those with the highest pK_a for deprotonation of the second carboxylic acid stimulated the highest ATPase. While a hydroxyl and hydroxylate cannot occur simultaneously with the carbonyl, for a single carboxyl, sequential formation through reversible proton loss is possible. Notable for its absence as a stimulator of thylakoid CF_1 is the bicarbonate anion (H_2CO_3). So far, attempts to stimulate high rates of ATPase which bicarbonate have failed.

Sulfite, when placed at the end of an ADP, results in a composite structure that seems to simulate an ATP chemically. We might suggest that sulfite activates, and azide inhibits, by binding next to a bound ADP. This bound ADP might exert its effects on ATPase by affecting the regulation of ATPase by Mg^{2+}. As a corollary, if sulfite is a true replacement for the ΔpH necessary to maintain ATPase, then the function of this ΔpH may be to prevent inhibition by Mg^{2+}.

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