Biochimica et Biophysica Acta, 1016 (1990) 55–62
Elsevier

BBABIO 43156

Promotion and inhibition of catalytic cooperativity of the Ca^{2+}-dependent ATPase activity of spinach chloroplast coupling factor 1 (CF1)

P. John Andralojc and David A. Harris

Department of Biochemistry, University of Oxford, Oxford (U.K.)

(Received 31 July 1989)

Key words: ATPase, F_1-; Co-operativity; Azide inhibition; ATPase inhibitor protein; (Chloroplast)

ATP- and ITP-stimulation of the Ca^{2+}-dependent hydrolysis of low concentrations of [γ-^{32}P]ATP was used as a direct demonstration of catalytic cooperativity in CF_1. CF_1 activated by ε-subunit removal or dithiothreitol, or by the presence of ethanol in the ATPase assay medium, shows pronounced catalytic cooperativity, with maximal stimulation of [γ-^{32}P]ATP hydrolysis at about 20 μM CaATP. Catalytic cooperativity is diminished by the presence of the ε-subunit or by pretreatment of either untreated or ε-depleted CF_1 with azide ($C_{1/2}$ = 30 μM). Both activated and untreated forms of CF_1 also exhibit hydrolysis of CaATP by a high-affinity, low-capacity mode of turnover, which is unaffected by any of the preceding treatments and shows normal Michaelis-Menten behaviour. We propose that this high-affinity mode represents unisite catalysis, and that the endogenous inhibitor, ε, and the exogenous inhibitor, azide, both act exclusively on cooperative interactions between the catalytic sites.

Introduction

The H^+-ATP synthases constitute a group of homologous enzymes responsible for ATP synthesis, linked to H^+-translocation, in mitochondria, chloroplasts and eubacteria. They comprise a membrane protonophore (F_0), and an extrinsic ATPase assembly, F_1. Even the F_1 portion has a complex structure, with a subunit stoichiometry $\alpha_3,\beta_3,\gamma,\delta,\varepsilon$, of which the three β subunits each bear a catalytic site (for a review, see Ref. 1).

The most intensely investigated enzyme in this group has been the mitochondrial F_1-ATPase. Here, the three catalytic ATP binding sites show decreasing affinities for ATP, the first ATP molecule binding with very high affinity ($K_d \approx 10^{-12}$ M) [2] and the last with a much lower affinity (K_m = 150–250 μM) [3,4]. This indicates either (a) that the enzyme is initially asymmetrical, and the binding sites fill in order of affinity, or (b) that the enzyme is initially symmetrical, but shows negative cooperativity in binding.

These alternative explanations have led to alternative models for ATP synthesis in which either (a) 1 site is specialised for ATP synthesis and 1 (or 2) other(s) is specialised for hydrolysis [5], or (b) all sites participate in both synthesis and hydrolysis, each passing through equivalent conformations during turnover [3,4].

Positive cooperativity in catalysis (stimulation of turnover of ATP at low concentration by higher ATP concentrations) can be demonstrated by measurement of P_i–H_2O (intermediate) exchange, the extent of which is inversely related to the rate of product release during the hydrolysis of ATP or of ATP analogues [6,7]. More recently, we have used ATP stimulation of the hydrolysis of low concentrations of [γ-^{32}P]ATP as a direct demonstration of catalytic cooperativity in mitochondrial F_1-ATPase [8], and have shown that azide specifically inhibits multisite turnover in mitochondrial F_1, leaving unisite catalysis unaltered [8]. Our results support a kinetic model in which ATP hydrolysis involves turnover at only one catalytic site ('unisite' catalysis), or cooperation between all three catalytic sites ('multisite' catalysis), according to the prevailing ATP concentration [8].

The situation is less clear for the isolated chloroplast ATPase (CF_1), partly because it is a latent ATPase as isolated (and must be activated before measurement), and partly because its major ATPase activity, which is Ca^{2+}-dependent, exhibits no P_i–H_2O exchange activity

Abbreviations: CF_1, chloroplast coupling factor 1; F_1, (mitochondrial) coupling factor 1; EF_1, soluble *E. coli* coupling factor 1; tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine.

Correspondence: D.A. Harris, Department of Biochemistry, University of Oxford, South Parks Road, Oxford, OX1 3QU, U.K.

[7]. However, catalytic cooperativity within this enzyme, during the hydrolysis of CaATP, is amenable to study using ATP stimulation of [γ-^{32}P]ATP hydrolysis as a probe.

The work described below demonstrates that CF_1, like mitochondrial F_1, can turn over (as a Ca^{2+}-ATPase) in 'unisite' and 'multisite' modes, the latter showing strong positive cooperativity in catalysis. Azide and the ε-subunit both inhibit CF_1 by specifically abolishing multisite catalysis. The significance of these findings with respect to the latency of isolated CF_1 is discussed.

Materials and Methods

Preparation of CF_1, its ε-subunit, and ε-depleted CF_1, and treatment of CF_1 with dithiothreitol were as described previously [9] except that, during ε isolation, the ε-subunit was eluted from the ion-exchange column in a buffer without ATP. ATP hydrolysis was measured on CF_1 preparations from which endogenous ATP had been removed by centrifuging through two consecutive Sephadex G-50 columns [10].

ATP hydrolysis was assayed in 0.5 ml of a buffer containing 20 mM tricine-NaOH (pH 8.0), 1 mM $CaCl_2$, 0.4 μCi · ml^{-1} [γ-^{32}P]ATP, 0.05–1000 μM ATP, at 37 °C. CF_1 was added to initiate the reaction and, after 2 and 4 min, 200 μl aliquots were quenched by addition to 200 μl ice-cold trichloroacetic acid (10%, w/v) containing 1 mM P_i and 2 mM ATP. (For a zero-time reference, the assay buffer was added to the quench medium prior to CF_1 addition.) Extraction and counting of free phosphate was carried out as previously [11]. Under these conditions, the rate of product release was constant for at least 4 min, during which time less than 10% of the added ATP was hydrolysed.

To measure azide inhibition, CF_1 (0.5 mg · ml^{-1}) was preincubated in 20 mM tricine (pH 8.0, NaOH) and sodium azide at the specified concentration. Where necessary, ATP was then removed by passage through two consecutive Sephadex G-50 columns pre-equilibrated in the same buffer. All subsequent dilutions, including that into the assay medium itself, were into buffers containing the specified concentration of azide. (Controls were treated in the same way except that azide was omitted.) Without this pretreatment inhibition developed only slowly in the assay medium, as occurs with mitochondrial F_1 [12]. Where indicated, ε-depleted CF_1 was reconstituted with the purified ε-subunit (to give more than 95% inhibition at [ATP] $\geqslant$ 100 μM) immediately prior to assay, by the method described previously [9].

Protein concentrations were determined using the dye binding method of Bradford [13], using bovine serum albumin as standard. [γ-^{32}P]ATP was supplied by Amersham, U.K., and ATP and ITP by Boehringer.

Results

Cooperativity and ATP hydrolysis by CF_1

Over the range 50–1000 μM CaATP, hydrolysis by CF_1 shows simple Michaelis-Menten kinetics (data not shown). This occurs in preparations depleted of the (inhibitory) ε-subunit (K_m = 200 μM, V_{max} = 3.5 μmol · min^{-1} · mg^{-1}), and in untreated CF_1 (K_m = 240 μM, V_{max} = 0.15 μmol · min^{-1} · mg^{-1}). The similarity in K_m values observed suggest similar species involved in turnover; indeed, these results suggest that the ATPase activity of untreated (latent) CF_1 (over this range of substrate concentrations) is mainly due to the presence of a small amount (about 4%) of ε-depleted material. This has been previously suggested [9].

However, over the range 0–20 μM CaATP, the kinetics of hydrolysis by CF_1 deviates considerably from this model. This is demonstrated most clearly if hydrolysis of a constant, low amount of [γ-^{32}P]ATP is measured at increasing concentrations of unlabelled ATP. Release of [^{32}P]P_i would be expected to stay constant (if $S \ll K_m$, so that v increased in proportion to [ATP]) or fall (if $S \geqslant K_m$), if Michaelis-Menten kinetics are followed. Fig. 1 (upper curve) shows that, for ε-depleted CF_1, [^{32}P]P_i release increases 3.5-fold as the unlabelled ATP concentration rises from 1–20 μM. This implies a stimulation of ATP hydrolysis by added ATP, which is not detected in the latent CF_1 preparation (lower curve).

The stimulation observed might be due to either (a) positive cooperativity of catalysis between the three active sites of CF_1, or (b) stimulation by ATP binding to a regulatory (non-catalytic) nucleotide binding site of CF_1. Since the non-catalytic binding sites of CF_1 are highly specific for adenine nucleotides [14,15], while the catalytic sites are more tolerant, we can distinguish

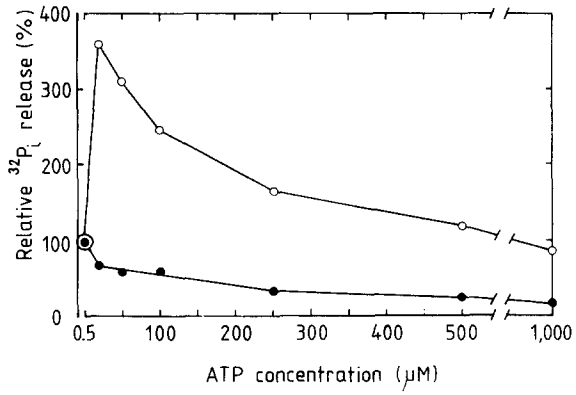

Fig. 1. Effect of removal of the ε-subunit on the ATPase activity of CF_1. ATP hydrolysis was measured, as described under Materials and Methods, CaATP concentration being varied as indicated. Untreated and ε-depleted CF_1 were present at 15 and 1.5 μg · ml^{-1}, respectively. 100% [^{32}P]P_i release corresponds to the level of [γ-^{32}P]ATP hydrolysis at 0.5 μM ATP, viz. $4.3 \cdot 10^{-4}$ and $2.04 \cdot 10^{-3}$ μmol · min^{-1} · mg^{-1} for native (●) and ε-depleted (○) coupling factor, respectively.

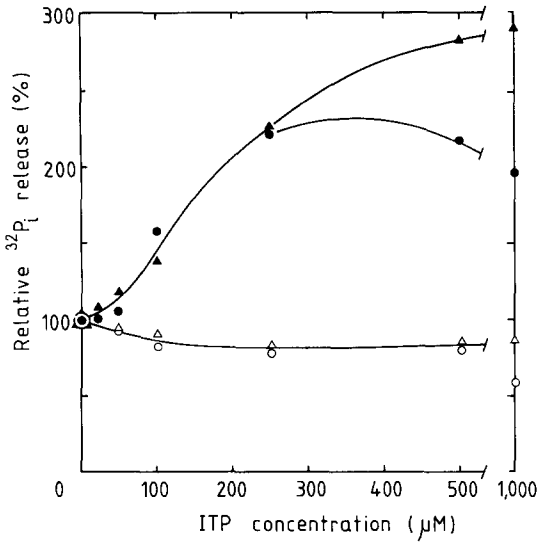

Fig. 2. Stimulation of ATP hydrolysis by ITP. ATP hydrolysis was assayed, as described, in the presence of either 1 μM ATP (●, ▲) or 50 μM ATP (○, △); and ITP concentrations between 0 and 1000 μM. Protein concentration was 1.5 $\mu g \cdot ml^{-1}$ for both dithiothreitol treated CF_1 (▲, △), and ε-depleted CF_1 (●, ○). 100% [^{32}P]P_i release corresponds to the ATPase activities in the absence of ITP, which were, ▲, $4.4 \cdot 10^{-3}$; △, $7.2 \cdot 10^{-1}$; ●, $4.7 \cdot 10^{-3}$; and ○, $6.2 \cdot 10^{-1}$ μmol/min per mg protein.

between these possibilities using ITP as a potential substrate.

Fig. 2 shows that, providing low concentrations of ATP are present (here 1 μM) ITP, too, stimulates the hydrolysis of [γ-^{32}P]ATP (upper curve). This effect is observed over a wide range of ITP concentrations (cf. Fig. 1), partly because CF_1 has a lower affinity for CaITP than for CaATP, but also partly because ITP does not itself dilute the specific radioactivity of ATP. As ITP binds to only the catalytic sites on CF_1, these results suggest that the stimulatory effects of ATP observed in Fig. 1 are due to positive cooperation between catalytic sites. This is confirmed in the lower curve of Fig. 2, which shows that at higher ATP concentrations (here 50 μM) ITP no longer stimulates turnover, as turnover is almost fully cooperative at this concentration.

Single and multisite catalysis by CF_1

Hydrolysis of ATP by various CF_1 preparations was investigated in detail over the range 0.05–15 μM ATP, where deviations from Michaelis-Menten behaviour were most marked (Fig. 3). As above, ε-depleted CF_1 shows an approx. 5-fold increase in turnover rate (beyond that expected from simply increasing substrate concentration) between 1 and 15 μM. However, the stimulatory effect of ATP is absent at very low concentrations, and in the range 0.05–1 μM ATP, the curve shows the expected downward trend (Fig. 3, inset). This can be explained if the positive effector site for ATP has a much lower affinity ($K_m \gg 1$ μM) than the primary catalytic site of CF_1 ($K_m < 1$ μM). Occupancy of the effector site would thus be minimal at [ATP] < 1 μM. These findings are consistent with the model for cooperative turnover proposed for MF_1 [3,4,8], where ATP bound at a high affinity catalytic site is hydrolysed only slowly until a second, lower affinity catalytic site is filled.

Fig. 3 shows that this pattern of [γ-^{32}P]ATP hydrolysis (fall, rise, fall) is also observed after CF_1 has been activated by reduction with dithiothreitol. Stimulation of hydrolysis by ITP also shows similar patterns in ε-depleted and dithiothreitol-reduced CF_1 (Fig. 2). Although the relative affinities of the ATP binding sites may differ slightly (see Fig. 3), we conclude that both activated preparations of CF_1 show similar cooperative properties.

In contrast, untreated CF_1 shows no such cooperative properties; No stimulation of [γ-^{32}P]ATP hydrolysis by

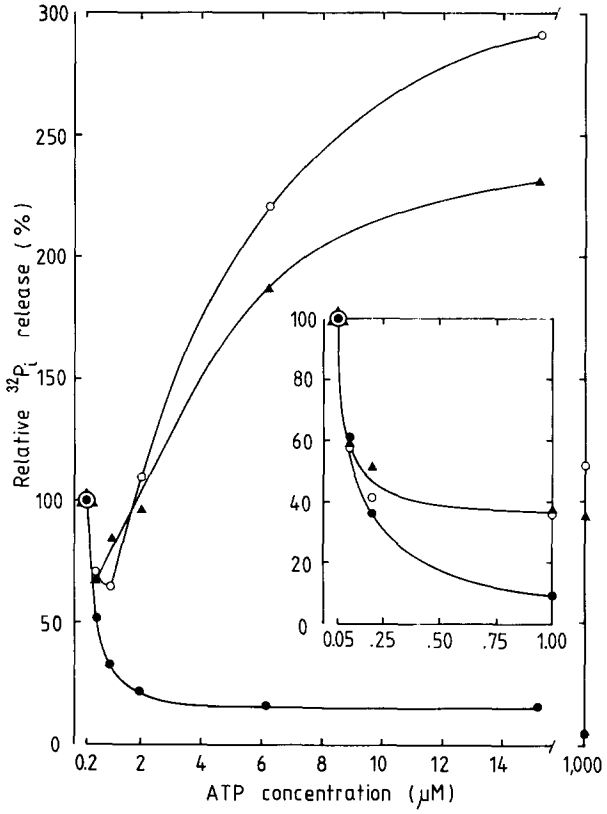

Fig. 3. Effect of removing ε or of dithiothreitol treatment on the hydrolysis of low concentrations of ATP. ATP hydrolysis was measured as described under Materials and Methods. (●) Native CF_1; (○) ε-depleted CF_1; (▲) dithiothreitol treated CF_1. The main figure shows the range 0.2–15 μM ATP, for which 100% [^{32}P]P_i release corresponds to ATPase activities of 0.93 (●); 0.96 (○); and 1.26 (▲) $nmol \cdot min^{-1} \cdot mg^{-1}$. Inset: in a separate experiment the response over the range 0.05–1.00 μM ATP was investigated, for which the corresponding ATPase activities are shown in Fig. 4. Protein concentration was 1.5 $\mu g \cdot ml^{-1}$, except for untreated CF_1 in the main figure, which was 15 $\mu g \cdot ml^{-1}$.

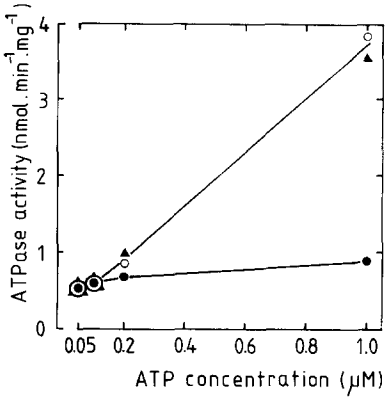

Fig. 4. ATPase activities of untreated and activated CF_1 at low ATP concentrations. Experimental details given in the legend to Fig. 3, using 1.5 μg $CF_1 \cdot ml^{-1}$ throughout. ●, Native CF_1; ○, ε-depleted CF_1; ▲, dithiothreitol-treated CF_1.

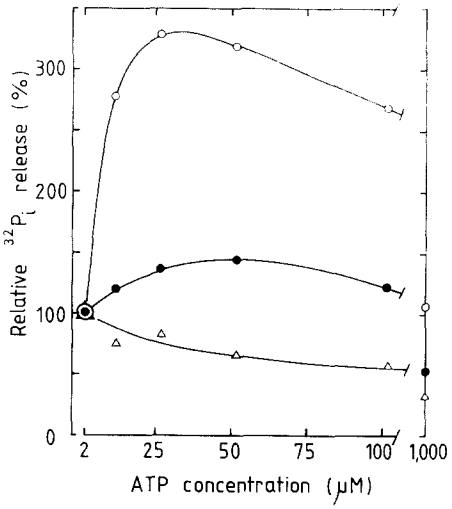

Fig. 5. Effect of ε on ATP hydrolysis by ε-depleted CF_1. Preincubation and ATPase assay as given in Materials and Methods. Untreated CF_1 was assayed at a protein concentration of 15 μg·ml^{-1}, ε-depleted CF_1 at 1.5 μg·ml^{-1}. ○, ε-depleted CF_1; ●, ε-depleted CF_1 plus ε; △, untreated CF_1. 100% [^{32}P]P_i release corresponds to the ATPase activities at 2 μM ATP, which were, 7.0 (○); 0.70 (●); and 0.93 (△) nmol·min^{-1}·mg^{-1}.

added ATP is observed over the range 0.05–15 μM (Fig. 3, cf. also Fig. 1). Therefore the activity of untreated CF_1 at very low ATP concentrations cannot reflect merely a contaminating activated species, as it would then simply mirror the properties of the other activated preparations. Untreated CF_1 thus appears to have its own low-capacity ATPase activity which is apparent only at low ATP concentrations.

This low capacity ATPase activity, in fact, appears to be present in both latent and activated CF_1 species. Fig. 4 shows that, as ATP levels fall, the rates of ATP hydrolysis by both activated and untreated CF_1 preparations converge, reaching a common value below about 0.1 μM ATP. This suggests that CF_1 possesses a high affinity ($K_m < 0.5$ μM), low capacity ($V_{max} \approx 1$ nmol·min^{-1}·mg^{-1}) mode of turnover that is unaffected by activation. ε-removal or dithiothreitol reduction activates CF_1 by allowing intersubunit cooperativity. Cooperativity leads to a 10^3–10^4-fold increase in V_{max} such that, at high ATP concentrations, only a small amount of contaminating activated CF_1 in the untreated preparation dominates the observed activity.

Removal of the ε-subunit from CF_1 results in an activated enzyme (rate of turnover about 35-times that of the untreated enzyme at 100 μM ATP) which shows marked cooperativity between catalytic sites (Figs. 1 and 3). Rebinding the ε-subunit leads to a preparation with low activity (> 95% suppression of activity at [ATP] = 100 μM, as in Ref. 9). Using the stimulation, by ATP, of [^{32}P]P_i release from [γ-^{32}P]ATP as a measure of cooperativity, it is seen from Fig. 5 that rebinding the ε-subunit also suppresses cooperativity. The rate of [^{32}P]P_i release in the reconstituted enzyme was only 35% higher at 25 μM ATP than at 1 μM ATP, whereas a stimulation of 330% was observed in the ε-depleted preparation. It will be noted that suppression of cooperativity was apparently incomplete (compare the lower two curves in Fig. 5). However, this residual cooperativity was apparently due to ethanol (2% v/v) carried into the assay from the buffer in which the ε-subunit was stored (see next section).

Effect of ethanol on CF_1 activity

The demonstration that cooperativity in ε-replete CF_1 was not fully suppressed in the presence of ethanol led to the reinvestigation of the effects of this solvent on CF_1 activity. Ethanol is known to stimulate Ca^{2+}-dependent ATP hydrolysis of CF_1 [16]. Fig. 6 shows that the increase in ATPase activity in untreated CF_1, induced by ethanol, is accompanied by the induction of catalytic cooperativity (compare upper and lower

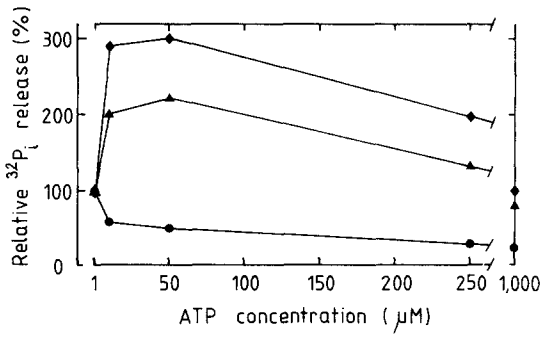

Fig. 6. Effect of ethanol on ATP hydrolysis by untreated CF_1. Experimental details given under Materials and Methods, except for the additional presence of ethanol in the assay medium and a CF_1 concentration of 15 μg·ml^{-1}. ●, 0%; ▲, 5%; and ◆, 10% (v/v) ethanol. 100% [^{32}P]P_i release was measured at 1 μM ATP, at which concentration the corresponding ATPase activities were, $9 \cdot 10^{-4}$ (●), $8 \cdot 10^{-4}$ (▲) and $2 \cdot 10^{-3}$ (◆) μmol·min^{-1}·mg^{-1}.

curves). The effect is fully reversible, disappearing if ethanol is diluted out (data not shown). At 1 μM ATP, hydrolysis by untreated CF_1 proceeds at an almost identical rate in the presence ($v = 0.8$ nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) or absence ($v = 0.9$ nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}) of 5% (v/v) ethanol. However, as the ATP concentration is raised, the rates with ethanol diverge sharply from those in the absence of ethanol. In the presence of ethanol, a biphasic curve, indicative of cooperativity, is observed (Fig. 6). At 10% (v/v) ethanol cooperativity is even more pronounced, although at this concentration some stimulation of rate is also observed at 1 μM ATP ($v = 2$ nmol $\cdot$ min^{-1} $\cdot$ mg^{-1}).

Effects of azide on CF_1 activity

Azide is a classical inhibitor of F_1-type ATPases from mitochondria and bacteria [12,17,18], and has recently been shown to have a limited effect on the Mg^{2+}-dependent, methanol-stimulated ATPase activity of purified CF_1 [19]. The Ca^{2+}-ATPase activity was unaffected by azide in these experiments. Such limited inhibition may be observed because azide acts slowly on CF_1, as it does on mitochondrial F_1, or because these measurements were made in the presence of methanol.

Fig. 7 shows that azide does inhibit the Ca^{2+}-ATPase activity of isolated, activated CF_1 in the absence of methanol. However, if ε-depleted CF_1 is not pretreated with azide, inhibition of its Ca^{2+}-ATPase activity by azide is not instantaneous but increases during the course of hydrolysis. In the absence of azide, a high constant rate is observed while, if ε-depleted CF_1 is pretreated with azide, a low constant rate is seen (Fig. 7). Fig. 7 also shows that this inhibition is at least partially reversible: if azide is present in the preincubation but omitted from the assay medium, inhibition is considerably reduced. (Recovery of activity is incomplete, however, due to the presence of traces of residual azide.) The presence or absence of CaATP during the preincubation with azide had no effect on the inhibition pattern observed (data not shown).

After such a preincubation, ε-depleted CF_1 is powerfully inhibited by azide when assayed at high ATP concentrations. $c_{1/2}$ for azide inhibition is about 30 μM, and at 1 mM azide the inhibition exceeds 98% (Fig. 8). However, at low ATP concentrations, the fractional inhibition falls until, with [ATP] < 100 nM, hydrolysis rates with and without azide converge (Fig. 9). It appears, therefore, that ε-depleted CF_1 has a small but significant ATPase activity insensitive to azide.

The same appears true for non-activated CF_1, although the effect here is more pronounced; at 100 μM ATP (Fig. 8) hydrolysis is highly sensitive to azide, while below about 1 μM ATP (Fig. 9) azide does not inhibit hydrolysis at all. These results suggest that azide inhibits CF_1 in the same way as its ε-subunit, having no effect on the high-affinity, low-capacity turnover (which predominates in untreated CF_1 at low ATP concentrations) but strongly inhibiting intersubunit cooperativity which dominates turnover at high concentrations.

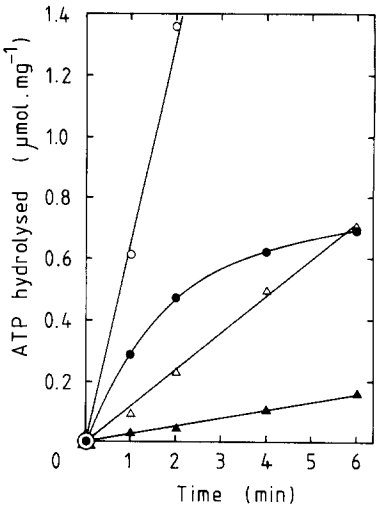

Fig. 7. Effect of azide pretreatment on the ATPase activity of ε-depleted CF_1. ε-depleted CF_1 at 0.5 mg $\cdot$ ml^{-1} was preincubated for 10 min at 25 °C with 20 mM tricine-NaOH (pH 8.0), 4 mM $CaCl_2$ and 4 mM ATP-NaOH (pH 8.0), with (▲, △) or without (●, ○) 2.5 mM NaN_3. It was then centrifuged through two consecutive G-50 Sephadex columns equilibrated in 20 mM tricine-NaOH (pH 8.0) $\pm$ 2.5 mM NaN_3. Ca^{2+}-ATPase activity was then measured, as described under Materials and Methods, at 50 μM ATP, in the presence (▲, ●) or absence (△, ○) of 2.5 mM NaN_3. Protein concentration in assay was either 5 μg $\cdot$ ml^{-1} (▲, ●, △) or 1.5 μg $\cdot$ ml^{-1} (○).

This is tested more directly in the experiment of Fig. 10, where intersubunit cooperativity is measured, as above, by the ability of unlabelled ATP or ITP to

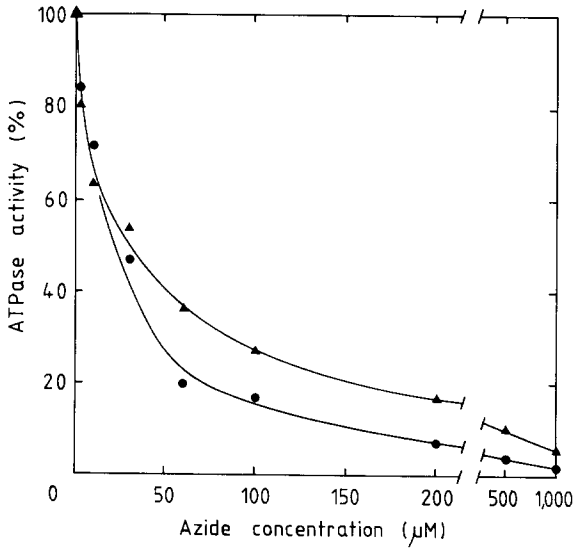

Fig. 8. Azide inhibition of untreated and ε-depleted CF_1 at high ATP concentrations. ATPase activity was determined using 100 μM ATP and CF_1 concentrations of either 2 μg $\cdot$ ml^{-1} for ε-depleted (●), or 40 μg $\cdot$ ml^{-1} for untreated CF_1 (▲). All other details given in Materials and Methods. 100% ATPase activity corresponds to $1.88 \cdot 10^3$ (●) or $2.5 \cdot 10^1$ nmol $\cdot$ min^{-1} $\cdot$ mg^{-1} (▲).

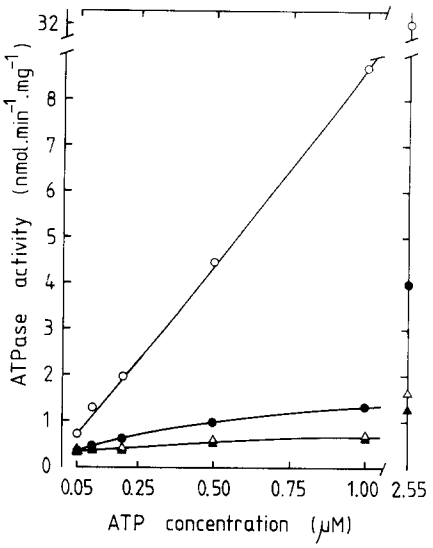

Fig. 9. Azide inhibition of untreated and ε-depleted CF_1 at low ATP concentrations. Azide treatment and ATPase assay as described in Materials and Methods, with both untreated (△, ▲) and ε-depleted CF_1 (○, ●) present at 2 $\mu g \cdot ml^{-1}$. Open symbols in the absence, filled symbols in the presence, of 0.2 mM sodium azide.

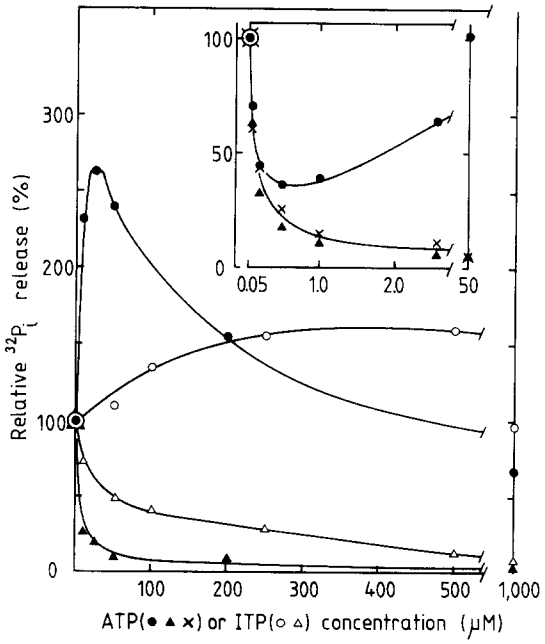

Fig. 10. Effect of azide on the ATP- and ITP-stimulated ATPase activities of ε-depleted CF_1. Filled symbols (●, ▲) show the effect of ATP, open symbols (○, △) the effect of ITP, on [γ-^{32}P]ATP hydrolysis. The effect of ITP was monitored in the presence of 1 μM ATP, as in Fig. 2. Triangles and circles are in the presence or absence of 2.5 mM azide, respectively, as described under Materials and Methods. CF_1 was assayed at a concentration of 1.5 $\mu g \cdot ml^{-1}$. 100% [^{32}P]P_i release corresponds to the ATPase activities at 1 μM ATP in the absence of ITP, which were: (●, ○) $1.24 \cdot 10^{-2}$; and (▲, △) $1.35 \cdot 10^{-3}$ $\mu mol \cdot min^{-1} \cdot mg^{-1}$. Inset: effect of azide on ATP-stimulated [γ-^{32}P]ATP hydrolysis by ε-depleted CF_1 at low substrate concentrations. ×, untreated CF_1. Other symbols and conditions as in the main figure. 100% [^{32}P]P_i release corresponds to ATPase activities of 0.68 (●), 0.35 (▲), and 0.34 (×) $nmol \cdot min^{-1} \cdot mg^{-1}$.

stimulate [γ-^{32}P]ATP hydrolysis. By this test, untreated CF_1 shows negligible cooperativity, while ε-depleted CF_1 gives the typical triphasic curve (as seen in Fig. 2). Azide-inhibited, ε-depleted CF_1, however, shows no cooperativity; a simple inhibition curve is obtained in the titration with unlabelled ATP (Fig. 10) and at [CaATP] < 1 μM, the curve obtained with azide inhibited, ε-depleted CF_1 is indistinguishable from that obtained with ε-replete CF_1 in the absence of azide (Fig. 10, inset). These effects are similar to, if more pronounced than, those observed with the mitochondrial F_1-ATPase, where azide again inhibits intersubunit cooperation [8].

Discussion

Unisite and alternating site catalysis

The work described above indicates that isolated CF_1 hydrolyses ATP by two mechanisms. In the presence of the natural inhibitor, the ε-subunit, or the exogenous inhibitor, azide, (Figs. 3, 9) turnover occurs in a high-affinity, low-capacity mode. This mode of turnover is also seen in CF_1 activated by ε-removal or dithiothreitol treatment, but only at very low ATP concentrations (Fig. 4).

As ATP concentration is raised, hydrolysis in these activated preparations becomes dominated by a high capacity, low affinity mode of turnover about $4 \cdot 10^3$-times faster. The presence of a small amount of ε-depleted CF_1 in samples of untreated CF_1 would account for the increased ATPase activities of the latter, at concentrations exceeding 20 μM ATP, i.e., observation of the slow, high-affinity mode of turnover is swamped by the small fraction of molecules exhibiting the high-capacity mode. This suggestion is supported not only by the similar K_m values of activated and untreated CF_1 for ATP hydrolysis in the concentration range 20–1000 μM ATP (see above), but also by the observation that azide inhibits the Ca^{2+}-ATPase of untreated and ε-depleted CF_1 at 100 μM ATP (Fig. 8).

Mitochondrial F_1 also exhibits a high-affinity, low-capacity, azide-insensitive mode of turnover which has been identified with unisite catalysis [8]. We propose that the low-capacity mode of ATP hydrolysis in CF_1 is also unisite catalysis. Mitochondrial unisite turnover has $K_m = 3$ μM and $V_{max} = 1$ $\mu mol \cdot min^{-1} \cdot mg^{-1}$ [4,8], both considerably higher than the values derived for CF_1 which are, respectively, 0.04 μM and 1 $nmol \cdot min^{-1} \cdot mg^{-1}$ (deduced from Figs. 4 and 9). However, the low value of V_{max} for CF_1 is comparable to the values obtained for hydrolysis of ATP by the isolated β-subunits of *R. rubrum* [11] and of CF_1 itself [31], consistent with the assignment of this activity to turnover in the absence of cooperative intersubunit interactions (unisite activity). Recently, activated, membrane-bound CF_1 has been shown to possess a comparable unisite mode of turnover [32].

As ATP concentrations are raised, cooperativity between the three catalytic sites becomes evident in activated CF_1. Further molecules of ATP bind with $K_m = 200\ \mu M$ (due to negative cooperativity in binding) and V_{max} rises to 3.5 $\mu mol \cdot min^{-1} \cdot mg^{-1}$ (due to positive cooperativity in catalysis). Above 50 μM, turnover rate is so high relative to the unisite rate that only this second class of site is observed and the enzyme follows apparent Michaelis-Menten kinetics (not shown). Below 0.2 μM ATP, less than 1% of the second class of site is occupied (Fig. 4) and again the enzyme behaves in a classical manner. Between 0.5 and 20 μM ATP, however, cooperativity is manifest by ε-depleted CF_1 in the biphasic plots of Figs. 1, 5 and 10, as the high-capacity, low-affinity mode of catalysis makes a variable but increasingly significant contribution to the overall rate.

Kohlbrenner and Boyer [7] inferred cooperativity in the Mg^{2+}-ATPase activity of CF_1 but the method they used, monitoring the ATP dependence of P_i–H_2O (intermediate) exchange, was not applicable to the Ca^{2+}-ATPase, which showed no exchange activity. This was shown by Wu and Boyer [20] to be due, not to an inability of the Ca^{2+}-dependent activity to promote the reversal of ATP cleavage, but to differences in overall size or ligand binding properties of the two divalent metal cations. Although the latter workers were able to demonstrate a slow P_i–H_2O exchange during CaATP hydrolysis in the presence of the detergent octyl glucoside, the Ca^{2+}-ATPase activity was not shown to be cooperative.

Perhaps surprisingly, the present work is, to our knowledge, the first demonstration of catalytic cooperativity in the Ca^{2+}-ATPase activity of CF_1. One advantage of our method is that it monitors cooperative effects directly at the level of ATP hydrolysis. The work described in this paper also reveals similarities in mechanism between the Mg^{2+}-ATPase and Ca^{2+}-ATPase activities of CF_1, not evident from the exchange studies. For example, catalytic cooperativity observed during Mg^{+}-dependent [7] and Ca^{2+}-dependent ATP hydrolysis is manifest over a common concentration range; 0.5–20 μM using ATP (Figs. 1, 3 and 10) and 20–500 μM using ITP (Figs. 2 and 10). As in previous studies [7,8] we have used ITP stimulation of ATP hydrolysis as a demonstration that stimulation of hydrolysis is due to active site–active site interaction (i.e., true cooperativity) and not due to interaction with the (highly specific) non-catalytic nucleotide binding sites.

There are three catalytic sites per CF_1 molecule [1,21]. We have considered two modes of catalysis, unisite catalysis (when only one out of the three sites is operative during each turnover) and multisite catalysis (when two or more sites cooperate to speed up turnover). Arguments against 'bisite' catalysis have been put forward previously [4,8], and it seems likely that all three catalytic sites participate in multisite turnover. However, the above work does not provide sufficient data to rule out a bisite mechanism in CF_1.

Effect of inhibitors of CF_1

The ε-subunit of CF_1 strongly inhibits multisite turnover in CF_1 (Figs. 1, 3) but not (azide insensitive) unisite catalysis (Fig. 4). It thus appears specifically to prevent cooperativity between the catalytic subunits of CF_1. In *E. coli*, too, removal of ε [22] or its digestion by trypsin [23] leads to an F_1 with increased catalytic cooperativity, which is manifest by [24] enhanced product (ADP, P_i) release during multisite ATP hydrolysis, and this was prevented by ε readdition.

This specific effect of the ε-subunit on cooperativity contrasts with the effect of the naturally occurring inhibitor of mitochondrial F_1. This protein inhibits both unisite and multisite hydrolysis equally [25,8]. Since both the ε-subunit (of EF_1) and the mitochondrial F_1 inhibitor (IF_1) interact with the carboxyl-terminal domain of the catalytic (β) subunit of F_1 [26,27], the reason for this difference in mode of action is unknown. Perhaps IF_1 has additional interactions with the active site of mitochondrial F_1. In this context, it is relevant that IF_1 and the ε-subunit of CF_1 do not cross-react; IF_1 will not bind to chloroplast F_1 even if its ε-subunit has been previously removed, while chloroplast ε does not affect mitochondrial F_1 (Andralojc and Harris, unpublished data).

Figs. 2 and 3 show that dithiothreitol-treated CF_1, which still contains bound ε-subunit [9], shows cooperative properties (and unisite catalysis) almost indistinguishable from ε-depleted CF_1. This is compatible with our previous proposal that dithiothreitol activates CF_1 by abolishing the effect of bound ε [9], but not with the suggestion that it does this by abolishing intersubunit (catalytic) cooperativity. The abnormal stoichiometry ($3\varepsilon/CF_1$) required for inhibition after dithiothreitol activation, which formed the basis of the latter suggestion, thus remains unexplained.

Our work suggests that organic solvents, too, stimulate CF_1 by promoting cooperativity. Fig. 6 shows that ethanol reveals cooperativity in untreated CF_1, leading to a 4-fold stimulation of hydrolysis at 1 mM ATP at levels of ethanol (5%, v/v) which do not affect turnover at 1 μM ATP (nor, therefore, unisite turnover rates). This can be explained if ethanol weakens the (hydrophobic) interaction between the β and ε subunits in CF_1. This view is supported by the requirement for ethanol in the physical removal of ε in the preparation of ε-depleted CF_1 [28].

The studies shown in Fig. 8 confirm that azide is an inhibitor of CF_1 and confirms its role as a general inhibitor of F_1-type ATPases [17,18]. The concentration of azide for half-maximal inhibition of CF_1 is about 30 μM, very similar to the value obtained for mitochondrial F_1 [17]. This value is also identical to the K_i for

azide inhibition of the sulphite-stimulated, Mg^{2+}-dependent ATPase activity of both dithiothreitol- and trypsin-treated thylakoids, reported by Larson et al. [29]. The observation that azide inhibits isolated CF_1 much less well than other F_1 species (compare [19] with [17,18]) could be due both to the need for preincubation for some minutes before inhibition is fully manifest (at least in the case of the Ca^{2+}-dependent activity, Fig. 7) and to the method used to activate the ATPase, it being highly susceptible in the untreated and ε-depleted forms (Fig. 8), but not in the presence of methanol [19].

Not only is azide a consistent inhibitor of homologous F_1 species, but it is confirmed here also to be a specific inhibitor of cooperative F_1 turnover, leaving unisite catalysis virtually untouched. This is consistent with previous observations on *R. rubrum* F_1, *E. coli* F_1 and mitochondrial F_1 [11,30,8]. How azide can exert such a specific effect, and why its inhibition should be time-dependent, are problems which require further investigation.

Acknowledgements

We would like to thank Lee Underwood of Toshiba, U.K., for the use of excellent word-processing facilities. This work was supported in part by the Agricultural and Food Research Council (grant No. AG 43/139).

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