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Properties of the catalytic ($\alpha\beta$)-core complex of chloroplast CF_1-ATPase

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The $CF_1(\alpha\beta)$-core complex previously isolated from the spinach chloroplast CF_0F_1-ATP synthase contains equal amounts of $CF_1\alpha$- and β-subunits, functions as a soluble Mg^{2+}-ATPase and forms a hybrid F_0F_1-ATPase when incorporated into β-less *Rhodospirillum rubrum* membrane-bound F_0F_1 (Avital, S. and Gromet-Elhanan, Z. (1991) J. Biol. Chem. 266, 7067–7072). Here, we demonstrate that this soluble spinach $CF_1(\alpha\beta)$-Mg^{2+}-ATPase, unlike its latent parent CF_1-ATPase, does not respond to activation by octyl glucoside, is only slightly stimulated by sulfite and not inhibited by free Mg^{2+}, azide or tentoxin. The $CF_1(\alpha\beta)$-ATPase does however bind tentoxin rather tightly and is stimulated by it at concentrations that inhibit the parent CF_1-ATPase. Unlike this soluble $CF_1(\alpha\beta)$-ATPase, the hybrid Mg^{2+}-ATPase formed by incorporation of the same $CF_1(\alpha\beta)$ preparation into β-less *R. rubrum* F_0F_1, is markedly stimulated by sulfite and completely inhibited by azide and tentoxin. These results indicate that (a), for stimulation by tentoxin the presence of α- and β-subunits from a sensitive CF_1 is enough, whereas for inhibition by it other F_1-subunits are required and these can come from a tentoxin resistant F_1-species, such as *R. rubrum* and (b), although the single-copy subunits are not required for F_1-ATPase activity, their presence results in increased rates and large changes in the catalytic properties.

Introduction

All living cells contain a membrane embedded F_0F_1-ATP synthase-ATPase complex that couples proton-translocation to ATP synthesis and hydrolysis. The catalytic F_1 component can be readily solubilized as a functional ATPase, that consists of five different polypeptide subunits with a stoichiometry of $\alpha_3\beta_3\gamma\delta\epsilon$. It has up to six catalytic and regulatory nucleotide binding sites residing on the large α- and β-subunits [1,2]. These large subunits form a symmetric hexamer, but the presence of single copies of the smaller γ, δ and ϵ-subunits breaks this symmetry [3–6]. The resulting complicated asymmetric structure leads also to an asymmetry in the nucleotide binding-sites that gives rise to a functional asymmetry [7–9].

Earlier experiments seemed to indicate that an asymmetric structure is a prerequisite for F_1-ATPase activity, since the minimal combination of subunits reported to form an active ATPase was either $\alpha_3\beta_3\gamma$ or $\alpha_3\beta_3\delta$. An $\alpha_3\beta_3\gamma$-ATPase complex was reconstituted from purified subunits of EcF_1 [10], TF_1 [11] and StF_1 [12]. A similar complex was also obtained by a stepwise removal of the δ and ϵ-subunits from CF_1 [13]. An $\alpha_3\beta_3\delta$-ATPase was assembled up to now only from purified subunits of TF_1 [11], but its properties have been reported as significantly different from those of TF_1 [14].

Recently, however, various active F_1-ATPase complexes containing only α- and β-subunits have been reported for TF_1 [15–17] and CF_1 [18]. The $TF_1(\alpha\beta)$ complexes have been prepared from a 1:1 mixture of isolated α- and β subunits obtained by over expression of their genes. They have been shown to assemble in the absence of nucleotides and Mg^{2+} into an $\alpha_3\beta_3$ complex [15,16], but in the presence of about 30 μM MgATP(D)P the $\alpha_3\beta_3$ hexamer dissociates into $\alpha_1\beta_1$ dimers. Both hexamers and dimers are active ATPases [17,19].

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Abbreviations: CF_1, EcF_1, RrF_1, StF_1 and TF_1, the soluble F_1-ATPase from chloroplasts, *Escherichia coli*, *Rhodospirillum rubrum*, *Salmonella typhimurium* and the thermophilic bacterium PS3; $TF_1(\alpha\beta)$ and $CF_1(\alpha\beta)$, the $F_1(\alpha\beta)$-core ATPase complexes that contain an equal number of α- and β-subunits; $CF_1\alpha$, $CF_1\beta$ and $RrF_1\beta$, α- and β-subunits of CF_1 and RrF_1; Tricine, *N*-[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]glycine; AMP-PNP, adenylyl-β,γ-imidodiphosphate; Bchl, bacteriochlorophyll.

A direct assembly of $CF_1\alpha$- and β-subunits into active $CF_1(\alpha\beta)$ complexes is as yet impossible, since all attempts to isolate pure $CF_1\alpha$ in a soluble active form have failed [20]. An assembled active $CF_1(\alpha\beta)$ preparation could however be isolated and purified from the spinach membrane-bound CF_0F_1 by a procedure that involves extraction of coupled spinach thylakoids with 2 M LiCl in presence of 4 mM MgATP [21] and fractionation of the resulting extract on FPLC ion-exchange columns [18]. The purified $CF_1(\alpha\beta)$ preparation contains an equimolar ratio of α- and β-subunit, is active as a soluble Mg^{2+}-ATPase and can also form a membrane-bound hybrid F_0F_1-ATPase [18], when incorporated into β-less *Rhodospirillum rubrum* F_0F_1 [22]. Gel filtration of the $CF_1(\alpha\beta)$-ATPase in presence of MgATP revealed a mixture of $CF_1\alpha_3\beta_3$ and $\alpha_1\beta_1$ complexes but here, unlike in the $TF_1(\alpha\beta)$-ATPase [17,19], removal of MgATP resulted in complete loss of ATPase activity (Sokolov, Avital and Gromet-Elhanan, unpublished observations).

The isolation of active $F_1(\alpha\beta)$-core complexes suggests that F_1-ATPase activity is not dependent on the presence of single-copy subunits. The properties of these complexes might however be different from those of the intact F_1-ATPases, or their $\alpha_3\beta_3\gamma$ or $\alpha_3\beta_3\delta$ complexes and could, thus, enable us to document changes in both structure and function of the core complex that are imposed by the presence of single copy subunits.

In this report, we describe the catalytic properties of the $CF_1(\alpha\beta)$-ATPase, which in several aspects differ from those of the parent CF_1-ATPase, as well as from those of the hybrid F_0F_1-ATPase formed by incorporation of the same preparation of $CF_1(\alpha\beta)$ into β-less *R. rubrum* F_0F_1.

Materials and Methods

Preparations. The $CF_1(\alpha\beta)$-core complex was prepared from a 2 M LiCl-extract of washed spinach thylakoids as described by Avital and Gromet-Elhanan [18]. The purified complex was concentrated by ultrafiltration on a YM10 membrane (centriprep device) to 2–5 mg/ml and stored in liquid nitrogen. Control and β-less *R. rubrum* chromatophores and the $RrF_1\beta$-subunit were prepared as described [23,24]. Spinach CF_1 was a generous gift of Dr. R.E. McCarty, The Johns Hopkins University, Maryland.

Assays. Soluble ATPase activity was assayed for 5 min at 35°C in a 0.3 ml reaction mixture that contained, unless otherwise stated, 50 mM Tricine (pH 8.0), 5 mM $MgCl_2$, 5 mM ATP and 2–20 μg of protein. The reaction was stopped by 0.3 ml of 0.5 M trichloroacetic acid and the released P_i estimated [25].

Reconstitution of the β-less *R. rubrum* chromatophore F_0F_1 by incorporation of $RrF_1\beta$ or $CF_1(\alpha\beta)$ was carried out by incubating chromatophores equivalent to 3 μg of bacteriochlorophyll with 5–10 μg of the incorporated protein for 1 h at 35°C, in a final volume of 0.2 ml containing 50 mM Tricine (pH 8.0), 23 mM $MgCl_2$, 4 mM ATP, 1 mM DTT and 7.5% glycerol. When stated, the same procedure was applied on control chromatophores. The reconstituted chromatophores were centrifuged [26] and the pellets resuspended in 0.22 ml of 50 mM Tricine. Their restored ATPase activity was assayed on duplicate samples of 0.1 ml which, unless otherwise stated, were preincubated for 10 min at 35°C in 0.6 ml of 50 mM Tricine (pH 8.0) with the inhibitors or stimulators mentioned in the text. The reaction was then started by adding 0.1 ml of a solution giving a final concentration of 2 mM $MgCl_2$ and 4 mM ATP and stopped by 0.1 ml of 2 M trichloroacetic acid. The released P_i was measured after centrifugation as described in Ref. 25.

Binding of tentoxin to the $CF_1(\alpha\beta)$ complex was carried out by incubating 100–200 μg of the complex with 10–20 μM tentoxin for 10 min at room temperature. The unbound tentoxin was removed by elution-centrifugation through Sephadex G-50 columns [27] as outlined in Ref. 28. In the absence of the $CF_1(\alpha\beta)$ complex tentoxin was fully retained on the Sephadex columns, since the eluant lost completely its capacity to inhibit CF_1-ATPase activity.

Protein was determined by the Bradford method [29] using bovine serum albumin as the standard. Bacteriochlorophyll was measured according to Ref. 30.

Results

Various isolated purified $F_1\beta$-subunits were reported to have either no measurable [10,11,18,22,26] or very low ATPase activity that required hours of incubation for assay [31,32]. Even the $TF_1\alpha$- and β-subunits, that were obtained by overexpression and found to be more active than those purified from dissociated TF_1 [15], were reported to have no meaningful ATPase activity [16]. On the other hand all isolated $F_1(\alpha\beta)$ complexes containing equimolar ratios of the α- and β-subunits were found to have easily measurable ATPase activities [15–19].

The $TF_1(\alpha\beta)$-ATPase activities range between 4–20% of their parent TF_1-ATPase activity [15–17,19]. CF_1 is, however, a latent ATPase showing very low Ca^{2+}-ATPase and even lower Mg^{2+}-ATPase activities [33]. It can be activated by various treatments that have been correlated either with removal of its ϵ-subunit [34,35] or with the reduction of a disulfide bond in its γ-subunit [36]. It is, therefore, not surprising that the $CF_1(\alpha\beta)$-core complex, containing neither γ nor ϵ [18], shows with no additions a higher Mg^{2+}-ATPase activity than its parent latent CF_1-ATPase (Table I).

This $CF_1(\alpha\beta)$-Mg^{2+}-ATPase activity is linear with time and enzyme concentration (not shown). On addition of octyl glucoside the latent CF_1-Mg^{2+}-ATPase has been shown to be activated [37] by both removal of the ϵ-subunit and relief of the inhibitory effect of free Mg^{2+} [35]. As is illustrated in Table I, this last effect is only partial, since even in presence of octyl glucoside the CF_1-Mg^{2+}-ATPase activity is lower at a $MgCl_2$/ATP ratio of 1 as compared to 0.5. On the other hand, the basic activity of the $CF_1(\alpha\beta)$-Mg^{2+}-ATPase is rather higher at a $MgCl_2$/ATP ratio of 1 and is not affected by octyl glucoside under both tested $MgCl_2$/ATP ratios.

Table I shows also the different sensitivities to tentoxin of CF_1 and its isolated $(\alpha\beta)$-core complex. At 3 μM, tentoxin inhibits by 40–60% the CF_1 latent and octyl-glucoside-activated Mg^{2+}-ATPase, whereas the $CF_1(\alpha\beta)$-Mg^{2+}-ATPase is not affected by tentoxin in the presence of octyl glucoside and rather stimulated by it in the absence of octyl glucoside. Tentoxin is a species-specific effector of CF_1-ATPase [38] which at 0.1–10 μM blocks [37,38], but above 100 μM stimulates, both its trypsin-activated Ca^{2+}-ATPase [39] and octyl-glucoside-activated Mg^{2+}-ATPase [40]. Furthermore, at 300 μM, tentoxin has been shown to stimulate by itself, in the absence of octyl glucoside, the Mg^{2+}-ATPase activity of CF_1 isolated from various sensitive sources [40]. The observed stimulation of the $CF_1(\alpha\beta)$-Mg^{2+}-ATPase by a tentoxin concentration that inhibits the CF_1-Mg^{2+}-ATPase, could be due to a general downshift in the tentoxin concentrations required to inhibit or stimulate the $CF_1(\alpha\beta)$-ATPase. Fig. 1 illustrates however that this is not true, since no inhibition of the $CF_1(\alpha\beta)$-Mg^{2+}-ATPase activity was observed even at 300-fold lower concentrations of tentoxin. On the other hand, the stimulation of $CF_1(\alpha\beta)$-Mg^{2+}-ATPase activity by tentoxin was very clear, amounting already to 300% of control at 10 μM.

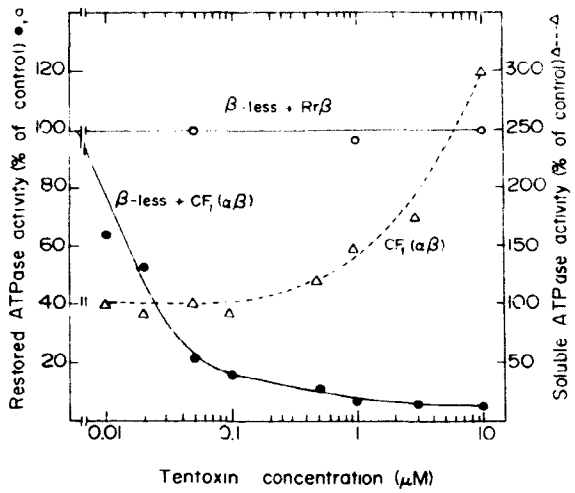

Fig. 1. Effect of tentoxin on the soluble Mg^{2+}-ATPase activity of the $CF_1(\alpha\beta)$-core complex as compared to the Mg^{2+}-ATPase activity restored to β-less *R. rubrum* chromatophores by their reconstitution with the same $CF_1(\alpha\beta)$ complex or with Rrβ. Soluble ATPase activity was assayed as described in Materials and Methods, except that the enzyme was preincubated for 10 min. at 35°C with the stated concentrations of tentoxin and the assay was started by addition of a solution giving a final concentration of 5 mM $MgCl_2$ and ATP. The reconstitution and restored ATPase activity were carried out as described in Materials and Methods. The reconstituted chromatophores were preincubated for 10 min at 35°C with the stated concentrations of tentoxin and the assay was started by addition of a solution giving a final concentration of 2 mM $MgCl_2$ and 4 mM ATP. Control rates were 0.18 μmol P_i released per min per mg protein for the soluble ATPase (△ — — — △) and 129 (●———●) or 242 (○———○) μmol P_i released per h per mg bacteriochlorophyll for the restored ATPase.

TABLE I

Properties of the soluble Mg^{2+}-ATPase activity of CF_1 and its $(\alpha\beta)$-core complex

The soluble ATPase activity was assayed as described in Materials and Methods, except for the stated concentrations of $MgCl_2$ and ATP. When stated, 40 mM octyl glucoside and/or 3 μM tentoxin were added before the reaction was started by addition of either latent CF_1 or $CF_1(\alpha\beta)$.

Additions to the assay	Mg^{2+}-ATPase activity (μmol P_i released /min per mg protein) measured with			
	$MgCl_2$ + ATP (2+4 mM)		$MgCl_2$ + ATP (5+5 mM)	
	CF_1	$CF_1(\alpha\beta)$	CF_1	$CF_1(\alpha\beta)$
None	0.10	0.16	0.02	0.20
Octylglucoside	9.90	0.17	7.50	0.21
Tentoxin	0.04	0.26	0	0.28
Octylglucoside + tentoxin	5.65	0.17	4.50	0.19

Besides its activity as a soluble Mg^{2+}-ATPase, the isolated $CF_1(\alpha\beta)$ complex can also form a membrane-bound hybrid F_0F_1-ATPase when incorporated into inactive β-less *R. rubrum* F_0F_1 [18]. When the same preparation of $CF_1(\alpha\beta)$, whose soluble Mg^{2+}-ATPase activity was only stimulated by tentoxin, was incorporated into β-less F_0F_1 a tentoxin sensitive hybrid Mg^{2+}-ATPase activity was restored (Fig. 1). On the other hand, reincorporation of the native $RrF_1\beta$ into the β-less *R. rubrum* F_0F_1 restored the native Mg^{2+}-ATPase activity that is completely resistant to tentoxin. These results demonstrate that the $CF_1(\alpha\beta)$-core complex is responsible for conferring tentoxin sensitivity to the hybrid F_0F_1-Mg^{2+}-ATPase. However, since the $CF_1(\alpha\beta)$ soluble Mg^{2+}-ATPase activity is stimulated, but not inhibited, by tentoxin it is clear that inhibition by tentoxin requires the presence of other F_1-subunits.

TABLE II

Effect of tentoxin presence during reconstitution or assay on the restored Mg^{2+}-ATPase activity

The restored Mg^{2+}-ATPase activity was assayed as described in Materials and Methods, except that 20 mM Na_2SO_3 was added to the preincubation step.

System tested		Mg^{2+}-ATPase activity (μmol P_i released/h per mg Bchl)		
chromatophores	added subunit	without tentoxin	with tentoxin present only during	
			reconstitution [a]	assay [b]
Control	none	730	750	720
β-less	none	20	17	19
β-less	Rrβ	511	490	520
β-less	$CF_1(\alpha\beta)$	500	24	40

[a] Reconstitution of $CF_1(\alpha\beta)$ into β-less chromatophores was carried out in presence of 3 μM tentoxin. The reconstituted chromatophores were centrifuged, resuspended in buffer without tentoxin and assayed for restored ATPase activity.

[b] Reconstitution was carried out in the absence of tentoxin which was added, to a final concentration of 3 μM, only to the assay of restored ATPase activity.

In the hybrid these subunits are provided by the tentoxin resistant *R. rubrum* F_0F_1. Their effect must therefore be indirect, probably via changes in conformation induced in $CF_1(\alpha\beta)$ upon its interaction with one or more of the single-copy subunits.

Table II illustrates that tentoxin inhibition is irreversible, since the same degree of inhibition of the $CF_1(\alpha\beta)$ containing hybrid Mg^{2+}-ATPase was obtained when tentoxin was present only during the reconstitution and washed out before the assay, or only during the assay. Table II also demonstrates that under all tested conditions control *R. rubrum* chromatophores, as well as β-less chromatophores reconstituted with $RrF_1\beta$, are completely resistant to tentoxin. Indeed, ATP synthesis and hydrolysis by *R. rubrum* chromatophores and the soluble ATPase activity of isolated RrF_1 are unaffected by tentoxin concentrations ranging between 0.01 μM to 1 mM (Weiss, S. and Gromet-Elhanan, Z., unpublished results).

The stimulatory effect of low tentoxin concentrations on the soluble spinach $CF_1(\alpha\beta)$-Mg^{2+}-ATPase suggest that this $CF_1(\alpha\beta)$ complex must bind tentoxin as tightly as its parent spinach CF_1. In the absence of labeled tentoxin direct binding tests could not be carried out. But, as can be seen in Table III, elution-centrifugation through a Sephadex G-50 column [26] was found to serve as an indirect but sensitive assay for demonstrating that tentoxin does indeed bind tightly to the $CF_1(\alpha\beta)$ complex. $CF_1(\alpha\beta)$ was not retained on this Sephadex column and after column-centrifugation its full capacity to reconstitute β-less chromatophores and restore a hybrid F_0F_1 Mg^{2+}-ATPase appeared in the eluant (Table III, Expt. 1). Tentoxin, on the other hand, was fully retained on the Sephadex and after column-centrifugation of tentoxin by itself or in the presence of BSA, its inhibitory effect disappeared from the eluant (Table III, Expts. 2 and 3). But when tentoxin was applied to the Sephadex after incubation in presence of $CF_1(\alpha\beta)$ most of its inhibitory effect appeared in the eluant (Table III, Expt. 4). These results demonstrate that tentoxin binds to $CF_1(\alpha\beta)$ in a tight enough manner so that most of it remains bound during elution-centrifugation. Indeed the soluble Mg^{2+}-ATPase activity of the $CF_1(\alpha\beta)$ + tentoxin used in Expt. 4 of Table III was 3–4-fold higher than that of the $CF_1(\alpha\beta)$ used in Expt. 1–3 of Table III, due to stimulation by the bound tentoxin and this stimulation was retained after column centrifugation (not shown).

Further characterization of the soluble $CF_1(\alpha\beta)$-Mg^{2+}-ATPase as compared to the membrane-bound restored hybrid F_0F_1 Mg^{2+}-ATPase activity obtained with the same $CF_1(\alpha\beta)$ preparation, revealed additional differences in their response to various effectors, such as sulfite (Fig. 2) and azide (Fig. 3). Sulfite has been reported to stimulate by several-fold the Mg^{2+}-ATPase activity of membrane-bound and isolated F_1-complexes from mitochondria [41], chloroplasts [42]

TABLE III

Demonstration of the tight binding of tentoxin to the $CF_1(\alpha\beta)$-core complex by its inhibition of restored hybrid Mg^{2+}-ATPase activity in reconstituted β-less chromatophores

The compounds listed under A and B were preincubated separately for 10 min at room temperature and either used directly for reconstitution with β-less *R. rubrum* chromatophores ("no treatment"), or centrifuged through a G-50 Sephadex column and the eluate used for reconstitution.

Expt. No.	Additions to β-less chromatophores during reconstitution		Mg^{2+}-ATPase activity (μmol P_i released/h per mg Bchl)	
	A	B	no treatment	column-centrifugation
1	$CF_1(\alpha\beta)$	none	579	544
2	$CF_1(\alpha\beta)$	tentoxin	107	542
3	$CF_1(\alpha\beta)$	BSA + tentoxin [a]	116	584
4	$CF_1(\alpha\beta)$ + tentoxin [a]	none	81	189

[a] Tentoxin at 10 μM was preincubated alone (Expt. 2), or with BSA (Expt. 3) or in Expt. 4 with $CF_1(\alpha\beta)$ and its final concentration when added directly to the reconstitution mixture was 0.4 μM.

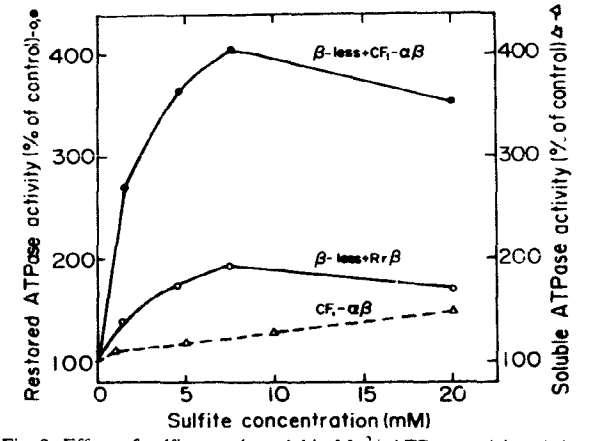

Fig. 2. Effect of sulfite on the soluble Mg^{2+}-ATPase activity of the $CF_1(\alpha\beta)$-core complex as compared to the Mg^{2+}-ATPase activity restored to β-less chromatophores by the same $CF_1(\alpha\beta)$ complex and by $Rr\beta$. Conditions of reconstitution, preincubation and assays of soluble and restored ATPase were as described in Fig. 1.

and *R. rubrum* chromatophores [43]. As can be seen in Fig. 2, sulfite stimulates the Mg^{2+}-ATPase activity restored to β-less F_0F_1 by incorporation of $CF_1(\alpha\beta)$ or $RrF_1\beta$ by 4- and 2-fold, respectively, whereas the soluble Mg^{2+}-ATPase shows only a 50% stimulation by 20 mM sulfite. The presence of sulfite did not change the effect of tentoxin on these ATPase activities. As is shown in Table II, addition of 3 mM tentoxin in presence of 20 mM sulfite has no effect on the Mg^{2+}-ATPase activity restored to β-less F_0F_1 by incorporation of $RrF_1\beta$ but fully inhibits the activity restored by incorporation of $CF_1(\alpha\beta)$. The soluble $CF_1(\alpha\beta)$-Mg^{2+}-

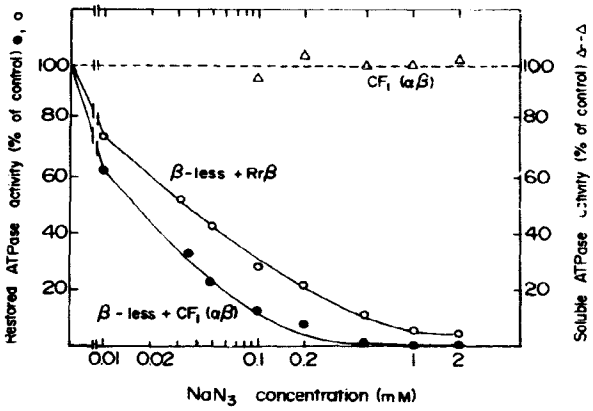

Fig. 3. Effect of azide on the soluble Mg^{2+}-ATPase activity of the $CF_1(\alpha\beta)$-core complex as compared to the Mg^{2+}-ATPase activity restored to β-less chromatophores by the same $CF_1(\alpha\beta)$ complex and by $Rr\beta$. Conditions of reconstitution, preincubation and assays of soluble and restored ATPase were as described in Fig. 1.

ATPase that was stimulated in presence of either tentoxin (Fig. 1) or sulfite (Fig. 2) was stimulated in presence of both reagents to a somewhat higher, although not completely additive, extent (not shown).

Azide, unlike tentoxin, is a rather general potent inhibitor of the membrane-bound and soluble F_1-Mg^{2+}-ATPases from all tested sources [44–47]. As is illustrated in Fig. 3, azide inhibits to about the same extent the Mg^{2+}-ATPase restored to β-less F_0F_1 by incorporation of either $RrF_1\beta$ or $CF_1(\alpha\beta)$. But the soluble Mg^{2+}-ATPase activity of the same $CF_1(\alpha\beta)$ preparation is completely resistant to azide. The recently isolated $TF_1(\alpha\beta)$-ATPase has also been described as insensitive to azide [15,16] and much less sensitive to sulfite [16] than its parent TF_1-ATPase. Thus, all the results obtained up to now with the various isolated $F_1(\alpha\beta)$-core complexes indicate that, although the single-copy subunits are not required for F_1-ATPase activity, their presence change dramatically not only its structure but also its catalytic properties.

Discussion

F_1-$\alpha_3\beta_3$ and $\alpha_1\beta_1$ complexes, that function as the catalytic core of F_1-ATPase, have first been reported for the thermophilic TF_1-ATPase [15–17]. The enzymic properties of these $TF_1(\alpha\beta)$-core complexes were in some respects similar to their native TF_1, as in substrate specificity for nucleotide triphosphates [16] and sensitivity to AMP-PNP [15], but in others different from those of TF_1, as in a less stringent divalent cation specificity [16] and insensitivity to azide [15,16].

We have recently succeeded in isolating the first mesophilic $CF_1(\alpha\beta)$-ATPase as well as a pure $CF_1\beta$ from a 2 M LiCl extract of spinach chloroplasts [18]. The $CF_1(\alpha\beta)$ is composed of equivalent amounts of α- and β-subunits, functions as a soluble Mg^{2+}-ATPase and also forms a membrane-bound hybrid F_0F_1-ATPase when incorporated into β-less *R. rubrum* F_0F_1, whereas the pure $CF_1\beta$ has neither of these activities [18]. Further experiments have demonstrated that only $CF_1(\alpha\beta)$ preparations containing equimolar amounts of $CF_1\alpha$ and β-subunits show soluble ATPase activity, since several other preparations, containing only 0.2 or 0.5 mol of $CF_1\alpha$ per mol of $CF_1\beta$, that were isolated from tobacco or lettuce chloroplasts, could form hybrid F_0F_1-ATPases when incorporated into β-less F_0F_1, but showed no soluble ATPase activity [48].

As in the case of the $TF_1(\alpha\beta)$-core complex, the enzymic properties of the spinach $CF_1(\alpha\beta)$-Mg^{2+}-ATPase presented here were in some respects similar to, but in others different from, those of its parent spinach CF_1-ATPase as well as of the membrane-bound hybrid it forms with β-less *R. rubrum* F_0F_1. Thus, the basic property of binding tentoxin, a specific effector of CF_1-ATPases from sensitive plants such as spinach or

lettuce [38], was retained by the isolated spinach $CF_1(\alpha\beta)$-core complex (Table III). However, the soluble $CF_1(\alpha\beta)$-Mg^{2+}-ATPase was stimulated, but never inhibited, even by relatively low tentoxin concentrations that inhibit all sensitive CF_1-ATPases (Table I and Fig. 1). These results suggest that for inhibition by tentoxin the presence of one or more of the single copy F_1 subunits is required. An indication that the F_1-subunit responsible for the appearance of tentoxin inhibition is γ came from recent observations that spinach CF_1 nearly completely devoid of its δ and ϵ-subunits is inhibited by tentoxin as the whole native spinach CF_1 (McCarty, R.E., personal communication). Further experiments have now identified $CF_1\beta$ as the tentoxin sensitive subunit [49].

The soluble $CF_1(\alpha\beta)$-Mg^{2+}-ATPase differed from both the membrane-bound hybrid it forms with β-less *R. rubrum* F_0F_1 and its parent, latent CF_1-Mg^{2+}-ATPase also in its much lower stimulation by sulfite (Fig. 2) and resistance to inhibition by azide (Fig. 3). The insensitivity to azide, which has also been observed in the $TF_1(\alpha\beta)$-core complex [15,16] is especially interesting. Azide was reported to inhibit multisite, but not unisite catalysis of EcF_1 [50] and mitochondrial F_1 [51] and was, therefore, suggested to block catalytic cooperativity. Thus, the observation that all isolated $F_1(\alpha\beta)$ core complexes are insensitive to azide, could indicate the absence of cooperative interactions. But, although the $TF_1\alpha_3\beta_3$-ATPase was not inhibited by azide [15,16], it was reported to exhibit cooperative kinetics as a function of ATP concentration [16]. These results were, however, obtained under conditions that are now known to induce a quick dissociation of the $TF_1\alpha_3\beta_3$ hexamer into $\alpha_1\beta_1$ dimers [17,19]. Since both complexes are active ATPases, their MgATP-dependent interconversion complicates the interpretation of kinetic measurements under catalytic conditions. Two reports have recently established the presence of a one-hit one-kill phenomenon in $TF_1\alpha_3\beta_3$ inactivated by 7-chloro-4-nitrobenzofurazan [52] or 3′-*O*-(4-benzoyl)benzoyl adenosine 5′-diphosphate [53]. These results suggest the possible presence of functional heterogeneity in $TF_1\alpha_3\beta_3$, but direct measurements of MgATP induced three-site cooperativity are not available as yet for this hexamer.

Unfortunately, it is also as yet impossible to search directly for cooperative kinetics in the $CF_1\alpha_3\beta_3$-ATPase. Although all our preparations of $CF_1(\alpha\beta)$ exhibit similar rates of ATP hydrolysis and identical responses to tentoxin, sulfite and azide, they elute from gel filtration columns in presence of MgATP as mixtures of $\alpha_3\beta_3$ and $\alpha_1\beta_1$ in changing ratios (Sokolov, Avital and Gromet-Elhanan, unpublished observations). This behavior could either result from enhanced dissociation of $CF_1\alpha_3\beta_3$ due to dilution, or indicate the presence of discrete $\alpha_3\beta_3$ and $\alpha_1\beta_1$ complexes, in varying amounts, in the different preparations. Our attempts to separate these complexes and stabilize the $CF_1\alpha_3\beta_3$, by removal of MgATP led to loss of ATPase activity. We are now searching for conditions that will stabilize the $CF_1\alpha_3\beta_3$-complex in an active state and, thus, enable direct measurements of its kinetic parameters.

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