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# Inhibition of the ATPase activity of the catalytic portion of ATP synthases by cationic amphiphiles

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**Abstract**

Melittin, a cationic, amphiphilic polypeptide, has been reported to inhibit the ATPase activity of the catalytic portions of the mitochondrial (MF1) and chloroplast (CF1) ATP synthases. Gledhill and Walker [J.R. Gledhill, J.E. Walker. Inhibition sites in F1-ATPase from bovine heart mitochondria, Biochem. J. 386 (2005) 591–598.] suggested that melittin bound to the same site on MF1 as IF1, the endogenous inhibitor polypeptide. We have studied the inhibition of the ATPase activity of CF1 and of F1 from *Escherichia coli* (ECF1) by melittin and the cationic detergent, cetyltrimethylammonium bromide (CTAB). The $Ca^{2+}$- and $Mg^{2+}$-ATPase activities of CF1 deficient in its inhibitory ε subunit (CF1-ε) are sensitive to inhibition by melittin and by CTAB. The inhibition of $Ca^{2+}$-ATPase activity by CTAB is irreversible. The $Ca^{2+}$-ATPase activity of F1 from *E. coli* (ECF1) is inhibited by melittin and the detergent, but $Mg^{2+}$-ATPase activity is much less sensitive to both reagents. The addition of CTAB or melittin to a solution of CF1-ε or ECF1 caused a large increase in the fluorescence of the hydrophobic probe, *N*-phenyl-1-naphthylamine, indicating that the detergent and melittin cause at least partial dissociation of the enzymes. ATP partially protects CF1-ε from inhibition by CTAB. We also show that ATP can cause the aggregation of melittin. This result complicates the interpretation of experiments in which ATP is shown to protect enzyme activity from inhibition by melittin. It is concluded that melittin and CTAB cause at least partial dissociation of the α/β heterohexamer.
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*Keywords:* Chloroplast ATP synthase; ATPase activity; Melittin; Cationic detergent; Subunit dissociation

## 1. Introduction

The coupling membranes of mitochondria, bacteria and chloroplasts contain an enzyme, the ATP synthase, which couples the synthesis and hydrolysis of ATP to the movement of protons down their electrochemical proton gradient established by electron transport [1]. The ATP synthases are comprised of two readily separable parts: F1 which contains the catalytic sites of the enzyme and Fo, which is an integral membrane protein complex. ATP synthases are sometimes referred to as F1Fo or as F1Fo-ATPase. F1 consists of five different polypeptide chains in the stoichiometry of $\alpha_3\beta_3\gamma_1\delta_1\varepsilon_1$.

Although the ATP synthases are similar in their structure and in mechanism of ATP formation, they differ in the manner in which their activity is regulated. Mitochondria contain a polypeptide, IF1 [2], that binds to mitochondrial F1 in the presence of ATP and under conditions where the electrochemical proton gradient is dissipated as in ischemic muscle tissue. The binding of IF1 essentially locks in ATP bound to a catalytic site, thereby preventing wasteful ATP hydrolysis. There is no analog of IF1 in bacteria or chloroplasts, but the ε subunit is an inhibitor of the activity of the bacterial [3] and chloroplast [4] ATP synthases. The activity of the chloroplast ATP synthase is stimulated in the light by reduction of the disulfide bond in the γ subunit [5,6]. This reductive activation is unique to the ATP synthase in chloroplasts.

The ε subunit of the chloroplast ATP synthase is not only an inhibitor, but also, paradoxically, is required for ATP synthesis [4]. The C-terminal domain, which by analogy to this domain in the ε subunit from the ATP synthase of *Escherichia coli* [7] should assume a helix-loop–helix structure, is not needed for ATP synthesis [8], but is required for the inhibition of ATPase

*Abbreviations:* ANS, 1-anilinonaphthalene-8-sulfonic acid; F1, the catalytic part of the ATP synthases; CF1, chloroplast F1; CF1-ε, CF1 deficient in its ε subunit; ECF1, F1 from *Escherichia coli*; MF1, mitochondrial F1; CTAB, cetyltrimethylammonium bromide; NPA, *N*-phenyl-1-naphthylamine; CMC, critical micellar concentration

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activity of chloroplast F1 (CF1) deficient in its ε subunit (CF1-ε) [9].

IF1 binds to MF1 selectively at an interface between one of the three pairs of α and β subunits ($\alpha_{DP}$ and $\beta_{DP}$) [10]. As predicted from cross-linking studies [11], there are extensive contacts between the inhibitor protein and MF1at β subunit residues 394–495. The sequence identity between bovine MF1 β subunit and spinach CF1 β subunit in this region is 74%, with an additional 11% of the residues strongly similar. Gledhill and Walker [12] pointed out that both IF1 and melittin, a polypeptide in bee venom, are cationic amphiphiles that have a helix-turn–helix structural motif. From inhibitor titration studies they concluded that melittin bound to the same site on MF1 as IF1.

These considerations prompted us to examine the inhibition of the ATPase activity of CF1 by melittin that had first been described by Davis and Berg [13] in 1981. Since basic amphiphiles, including CTAB, inhibit the ATPase activity of CF1 [14], it seemed possible that melittin could act in a similar manner to that of CTAB. Therefore, the effects of CTAB were compared to those of melittin. Melittin and CTAB inhibited the $Ca^{2+}$- and $Mg^{2+}$-ATPase activities of CF1-ε. In contrast, the $Ca^{2+}$-ATPase activity of ECF1, but not its $Mg^{2+}$-ATPase activity, was inhibited by both CTAB and melittin. Melittin and CTAB likely act by causing at least partial dissociation of the $\alpha_3\beta_3\gamma$ structure.

## 2. Materials and methods

CF1-ε was prepared from CF1 from spinach thylakoids [4]. The enzyme was stored as an ammonium sulfate precipitate and was desalted before use by the Sephadex G-50 centrifuge column method [15]. ECF1 was the generous gift of Drs. T. Duncan and R. Cross of the Upstate Medical Center, Syracuse, NY and was desalted as described for CF1. Protein concentrations were determined by the Bradford method [16]. Melittin and CTAB were from Sigma, St. Louis, MO. NPA, from Molecular Probes, was a gift from Drs. D. Toptygin and L. Brand of the Department of Biology, Johns Hopkins University.

The ATPase activity of CF1-ε was determined at 37 °C in the following reaction mixtures (1.0 ml): $Ca^{2+}$-ATPase; 50 mM Tris–HCl (pH 8.0), 5 mM $CaCl_2$ and 5 mM ATP. $Mg^{2+}$-ATPase; 50 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 4 mM ATP and, usually, 25 mM $Na_2SO_3$. The reactions were stopped by the addition of 1.0 ml of 0.5 N trichloroacetic acid. Pi was determined spectrophotometrically [17]. All assays were done in duplicate and the duplicates generally agreed within 5% or less. Similar methods were used to determine the ATPase activity of ECF1, except that sulfite was not present in the $Mg^{2+}$-ATPase reaction mixture. The $Ca^{2+}$-ATPase activity of CF1-ε ranged from 11 to 21 μmol $min^{-1}$ mg $protein^{-1}$ and the $Mg^{2+}$-ATPase, from 13 to 18 μmol $min^{-1}$ mg $protein^{-1}$. The $Ca^{2+}$-ATPase activity of ECF1 ranged from 38 to 50 μmol $min^{-1}$ mg $protein^{-1}$, and the $Mg^{2+}$-ATPase activity, 23 to 30 in the same units.

The inhibition of $Ca^{2+}$-ATPase activity of CF1-ε by CTAB increased with time of incubation with CF1-ε prior to assay. For example, the inhibition of $Ca^{2+}$-ATPase activity of CF1-ε (5 μg in 0.5 ml) by 10 μM CTAB increased from 20% with no incubation prior to assay to 50% after a 30 min incubation at room temperature. Therefore, unless otherwise stated, the enzymes in 25 mM Tris–HCl (pH 8.0) were incubated for 30 min at room temperature with either CTAB or melittin before assay. ECF1 lost from 36% ($Ca^{2+}$-ATPase activity) to 54% ($Mg^{2+}$-ATPase activity) when incubated under these conditions. Therefore, ECF1 was added directly to the ATPase reaction mixtures that contained the indicated concentrations of melittin and CTAB.

The critical micellar concentration (CMC) of CTAB was determined from the fluorescence of *N*-phenyl-1-naphthylamine (NPA) [18] at room temperature. A value of 0.6 mM was obtained in pure water and in 25 mM Tris–HCl (pH 8.0), the CMC of CTAB was determined to be 0.16 mM.

CTAB was determined by a spectrophotometric assay patterned after the sulfatide assay of Keen [19]. The anionic dye, erythrosine, when complexed with the cationic CTAB, becomes extractable from aqueous solution into $CHCl_3$. The dye solution (0.1% in 0.5 mM KOH) was prepared by dissolving 0.1 g of the dye in 5 ml of 10 mM KOH and diluting the solution to 100 ml with water. CTAB (0 to 30 nmol) was added to 13 × 100 mm glass tubes, followed by 1.0 ml of 10 mM KOH and 0.2 ml of the dye solution. After 5 min at room temperature, 1.0 ml of a $CHCl_3$:$CH_3OH$ mixture (1:1 by volume) was added and the tubes were vigorously mixed on a vortex mixer for 10 s. The tubes were centrifuged for 2 min in a clinical centrifuge and the upper layer was removed and discarded. One ml of $CHCl_3$ was added to the lower layer and the absorbance of the samples determined at 537 nm, the peak of the CTAB–erythrosine complex. The standard curve was linear ($R$= 0.993) and had a slope of 0.029 $A_{537\ nm}$/nmol CTAB.

The fluorescence of *N*-phenyl-1-naphthylamine (NPA) is enhanced when it binds to hydrophobic surfaces of proteins [20]. Neutral NPA is more suitable for use with the cationic melittin and CTAB, than the anionic fluorophore, ANS, which has similar properties [21]. NPA (4 μM in 25 or 50 mM Tris–HCl (pH 8.0) at room temperature) fluorescence was excited at 360 nm and determined at 430 nm.

The inhibition of the $Ca^{2+}$-ATPase activity of CF1-ε by CTAB was compared to the enhancement of NPA fluorescence by addition of CF1-ε to (25 μg) to 1.5 ml of 20 mM Tris–HCl (pH 8.0) that contained various concentrations of CTAB. After the fluorescence had stabilized (1.5 to 2 min), 0.2 ml aliquots were taken for the assay of $Ca^{2+}$-ATPase activity.

## 3. Results

In agreement with the results of Davis and Berg [13], the $Ca^{2+}$-ATPase activity of CF1-ε is inhibited by melittin (Fig. 1A). CF1-ε in 20 mM Tris–HCl (pH 8.0) was incubated with melittin at the indicated concentrations for 30 min at room temperature prior to the ATPase activity assay. The concentration of melittin in the incubation mixtures with 5 μg of CF1-ε in 0.5 ml that gave 50% inhibition ($I_{50}$) in five separate experiments was 2 +/− 1 μM (mean and standard deviation). The $I_{50}$ increased at higher CF1-ε concentrations. For example, when the incubation with the melittin was carried out at 50 μg $ml^{-1}$ of CF1-ε, the $I_{50}$ was 7 μM. The $Mg^{2+}$-ATPase activity of CF1-ε, assayed in the presence of 25 mM sulfite to relieve inhibition by $Mg^{2+}$ and $Mg^{2+}$-ADP [22], was also inhibited by melittin with an $I_{50}$ of 10 μM (Fig. 1A).

Reduction of the disulfide bond in the γ subunit of CF1-ε [23,24], as well as cleavage of the γ subunit by trypsin [25], reduces the effectiveness of the ε subunit as an inhibitor of the ATPase activity of the enzyme. Thus, if melittin were to inhibit the ATPase activity of CF1-ε in a manner similar to that of the ε subunit, these treatments of the enzyme should decrease melittin inhibition. This was not the case. The $Ca^{2+}$-ATPase activity of the reduced or trypsin-treated enzyme was inhibited by low melittin concentrations. For example, the $Ca^{2+}$-ATPase activity of trypsin-treated CF1-ε was inhibited 80% by 2.5 μM melittin (data not shown).

Fig. 1B shows that CTAB inhibits the $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase activities of CF1-ε over a concentration range that is somewhat higher than that for melittin inhibition. In four separate experiments, the $I_{50}$ for CTAB inhibition of $Ca^{2+}$-ATPase activity was 5 +/− 2 μM (mean and standard deviation). It is unlikely that micelles play a role in inhibition by CTAB since the critical micellar concentration of CTAB, determined under the conditions the enzyme was incubated with the detergent, was 0.16 mM. The sulfite-activated $Mg^{2+}$-ATPase activity of CF1-ε was also inhibited by CTAB, with an $I_{50}$ of approximately 10 to 15 μM (Fig. 1B).

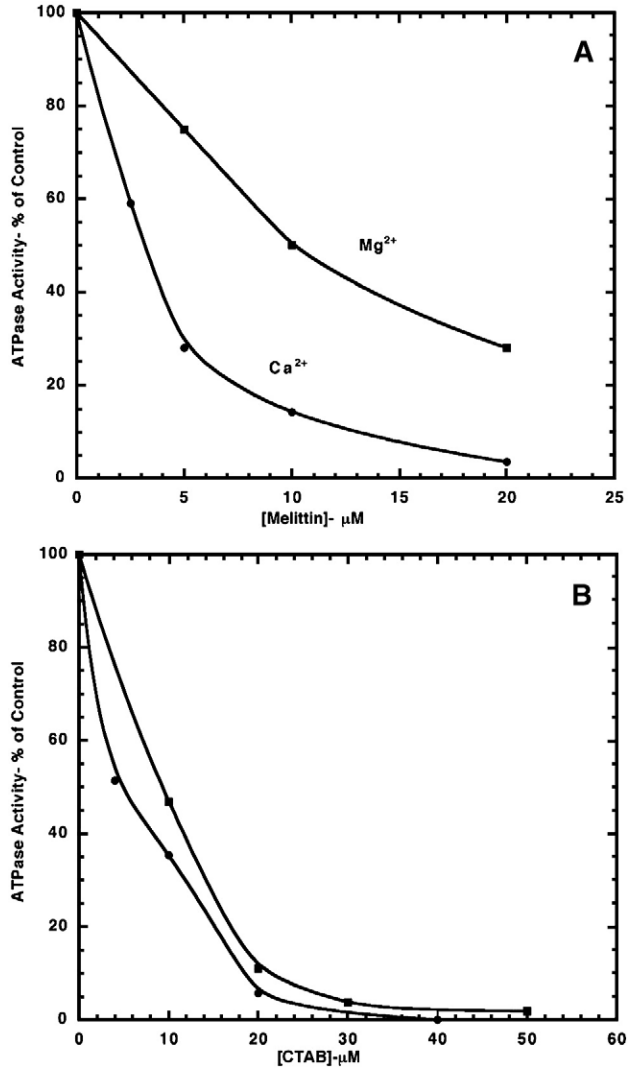


Fig. 1. Inhibition of the ATPase activities of CF1-ε by melittin and CTAB. CF1-ε (5 μg in 0.5 ml of 25 mM Tris–HCl, pH 8.0) was incubated with the indicated concentrations of melittin (A) or CTAB (B) for 30 min at room temperature and $Ca^{+}$- and $Mg^{2+}$-ATPase (with 25 mM sulfite) reaction mixtures added in 0.5 ml. The symbols in B are: squares, $Mg^{2+}$-ATPase and circles, $Ca^{2+}$-ATPase.

Gledhill and Walker [12] reported that the ATPase activity of F1 from the thermophilic bacterium PS3 was not inhibited by melittin. We were surprised to find that the $Mg^{2+}$-ATPase activity of ECF1 is also very resistant to inhibition by melittin (Fig. 2A) and CTAB (Fig. 2B). In these experiments the melittin and CTAB were added directly to the reaction mixtures. Lower concentrations of CTAB stimulated $Mg^{2+}$-ATPase activity by ECF1. No sulfite was added to the $Mg^{2+}$-ATPase reaction mixture. Thus, the only significant difference between the $Mg^{2+}$- and $Ca^{2+}$-ATPase reaction mixtures is the divalent cation. In contrast, the $Ca^{2+}$-ATPase activity of ECF1 was inhibited by melittin (Fig. 2A) and CTAB (Fig. 2B) over concentration ranges similar to those at which the $Ca^{2+}$-ATPase activity of CF1-ε is inhibited.

The $Ca^{2+}$-ATPase activity of CF1-ε is somewhat more sensitive to CTAB than $Mg^{2+}$-ATPase activity when the reagents are added directly to the assay mixtures, but the difference is not as dramatic as that for ECF1. The $I_{50}$ for $Ca^{2+}$-ATPase was about 12 μM and for $Mg^{2+}$-ATPase, it was 25 μM (data not shown).

The inhibition of the $Ca^{2+}$-ATPase activity of CF1-ε by CTAB was irreversible. CF1-ε (0.1 mg $ml^{-1}$) was incubated with 50 μM CTAB for 30 min at room temperature and then the CTAB was removed by centrifugal gel filtration. No CTAB was detected by the sensitive colorimetric assay described under "Materials and methods"; yet, the ATPase activity was still inhibited. The addition of β-cyclodextrin which can assist the folding of CTAB-treated proteins [26] also did not reverse the inhibition.

Melittin and CTAB may cause at least partial dissociation of CF1-ε, thereby inhibiting the ATPase activity of enzyme. Subunit dissociation has been correlated to the inactivation of the ATPase activity of CF1-ε in the cold. The fluorescence of ANS is a convenient indicator of the cold dissociation of CF1 [27]. ANS fluorescence increases as hydrophobic surfaces on CF1 are exposed to the solvent during dissociation. A substitute for

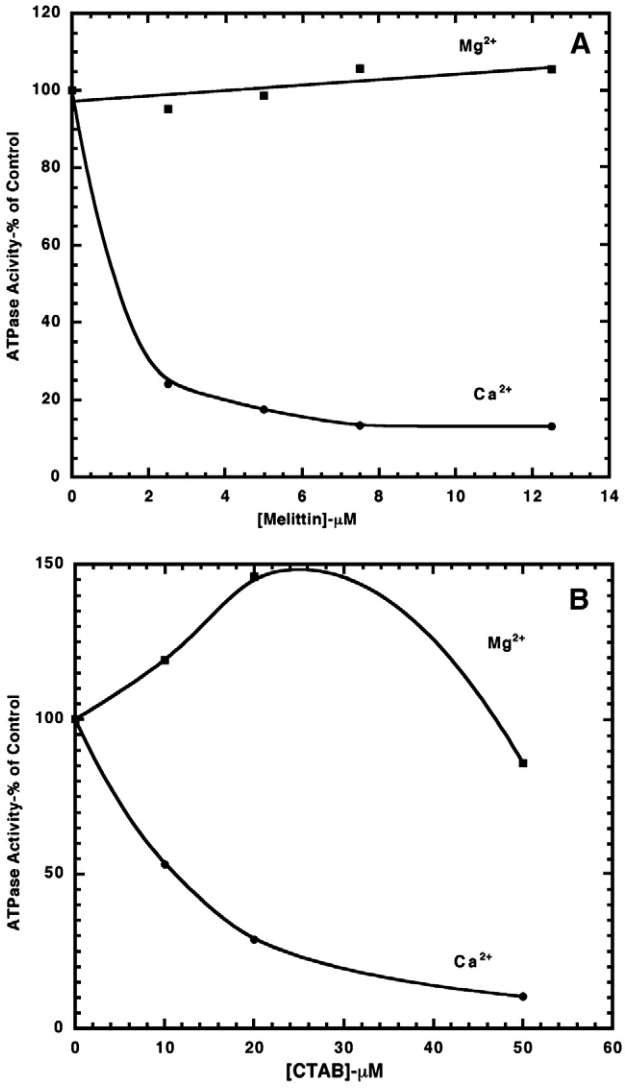


Fig. 2. Inhibition of the ATPase activities of ECF1 by melittin and CTAB. Melittin and the CTAB were added directly to the reaction mixtures.

Table 1
NPA is a suitable probe for monitoring CF1-ε subunit dissociation

| Incubation conditions | NPA fluorescence |
|---|---|
| No CF1-ε | 55 |
| CF1-ε, no ATP, room temperature | 190 |
| CF1-ε, +ATP, room temperature | 200 |
| CF1-ε, no ATP, on ice | 390 |
| CF1-ε, +ATP, on ice | 210 |

CF1-ε (50 μg) was incubated in 50 mM Tris–HCl (pH 8.0), 200 mM NaCl and 4 μM NPA for 30 min under the conditions shown. The ATP concentration was 0.5 mM. NPA fluorescence is given in arbitrary units.

ANS was needed to determine if CTAB and melittin cause dissociation of CF1-ε because of the probable interaction between the basic amphiphiles and the anionic ANS. NPA is a suitable substitute. The fluorescence of NPA was enhanced by the addition of CF1-ε (Table 1). NPA fluorescence of a solution containing CF1-ε was further enhanced after incubation for 30 min in the cold under conditions known to cause an increase in ANS fluorescence [27]. ATP prevented the increase in fluorescence of either ANS [27] or NPA (Table 1) in the cold.

As shown in Fig. 3, CTAB at 50 μM, a concentration below the CMC, had little effect on the fluorescence of NPA. The NPA fluorescence more than doubled when CF1-ε (33 μg $ml^{-1}$) was added. A much larger increase in NPA fluorescence was seen when both CTAB and CF1-ε were present, indicating that CTAB can at least partially dissociate the enzyme. A similar increase in NPA fluorescence was seen when CF1-ε was added together with 20 μM melittin (Fig. 3). The fluorescence emission maximum of NPA in the presence of CF1-ε was 465 nm. When CTAB (or melittin) and CF1-ε were present together, the emission maximum shifted to between 430 and 435 nm. This blue shift of NPA fluorescence is consistent with the probe being in a more hydrophobic environment. Melittin and CTAB had quite similar effects on NPA fluorescence in the presence of ECF1 (Fig. 3).

The increase in NPA fluorescence caused by the addition of CTAB to CF1-ε was correlated to the inhibition of the $Ca^{2+}$-ATPase activity of the enzyme (Fig. 4). CF1-ε was added to a stirred fluorometer cuvette that contained the indicated concentration of CTAB and NPA in 20 mM Tris–HCl (pH 8.0). The fluorescence was recorded and aliquots taken for the determination of $Ca^{2+}$-ATPase activity. It is evident that the inhibition of ATPase activity and increase in NPA fluorescence occur over the same concentration range of CTAB.

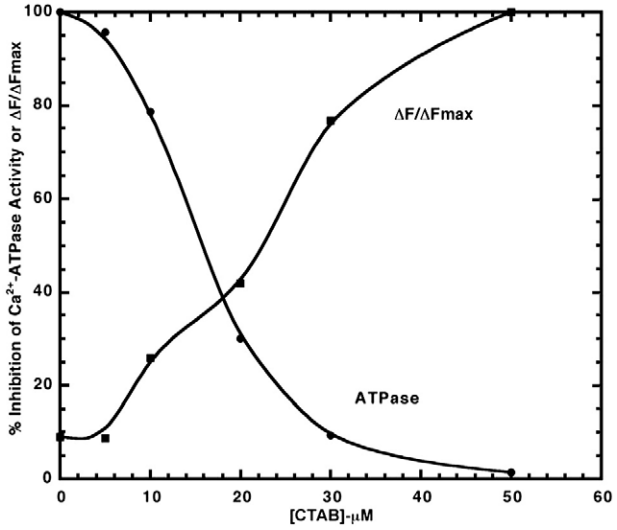


Fig. 4. CTAB concentration dependence for $Ca^{2+}$-ATPase activity inhibition and increase in NPA fluorescence. CF1-ε (25 μg) in 1.5 ml of 20 mM Tris–HCl (pH 8.0) and 4 μM NPA was incubated at room temperature with the CTAB concentrations shown until the NPA fluorescence (squares) stabilized. Aliquots were then taken for assay of $Ca^{2+}$-ATPase activity (circles). The maximum change in fluorescence was 420 U.

The incubation of CF1-ε with 0.5 mM ATP prior to the addition of CTAB partially protected the $Ca^{2+}$-ATPase activity from inhibition by the detergent (Fig. 5). A similar experiment could not be carried out with melittin because ATP appears to cause aggregation of the polypeptide. Melittin in aqueous solution exists as monomers and tetramers [28]. The monomer–

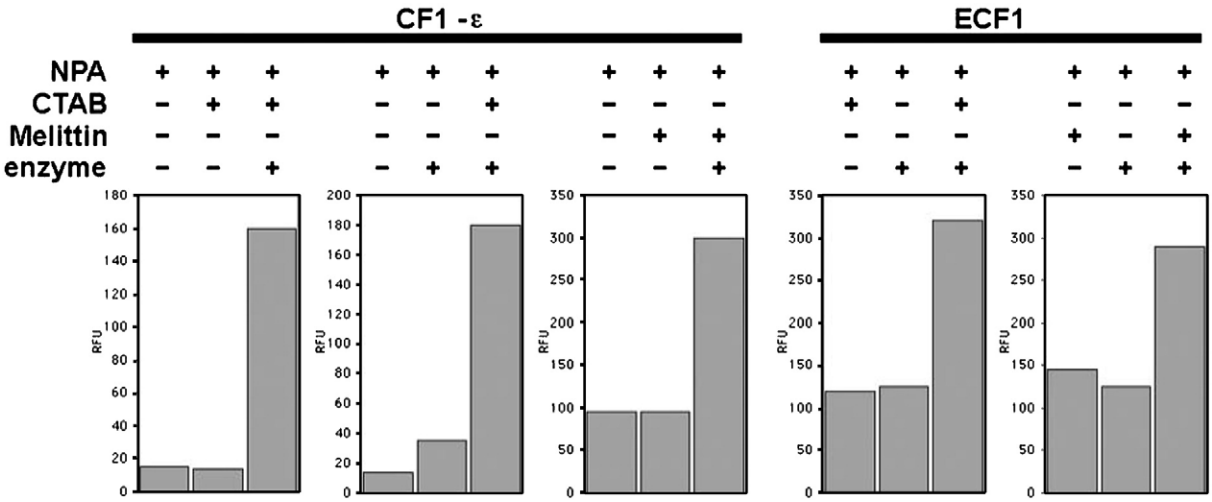


Fig. 3. Effects of melittin and CTAB on NPA fluorescence in the presence or absence of CF1-ε or ECF1. NPA (4 μM) fluorescence was determined in 20 or 50 mM Tris–HCl at room temperature. The CF1-ε concentration was 33 μg $ml^{-1}$ and that of ECF1, 15 μg $ml^{-1}$. The CTAB concentration was either 50 μM (CF1-ε) or 20 μM (ECF1) and melittin was present at 12.5 μM (CF1-ε) or 17 μM (ECF1). RFU stands for relative fluorescence units.

tetramer equilibrium is sensitive to ionic strength. The tetramer concentration would increase upon ATP addition. Moreover, it was noticed that when a 50 μl aliquot of a 1 mM melittin solution was added to a stirred 1.5 ml solution of 50 mM Tris–HCl (pH 8.0) and 1 mM ATP, a precipitate formed. GTP had a similar effect. If, however, the same amount of ATP was added to a solution of 30 μM melittin, no precipitate formed. The final concentrations of ATP and melittin are the same in both cases, but the concentration of tetramers in the 1 mM solution, as calculated from the tetramer dissociation constants in reference [29], would be about 500 times higher than in the 10 μM solution. It seems likely that ATP causes aggregation of the melittin tetramers.

The aggregation of melittin by ATP was studied further using NPA fluorescence. A concentration of melittin of 10 μM was chosen because large changes in NPA fluorescence could be seen without the formation of a visible precipitate. The addition of 15 μl of a 1 mM melittin solution to 1.5 ml of a buffered solution containing 4 μM NPA and 1.0 mM ATP, caused a rapid, large increase in NPA fluorescence (Fig. 6A). The fluorescence decreased over the next 90 s. In contrast, when ATP (1.0 mM) was added to a solution that contains NPA and 10 μM melittin, a smaller fluorescence increase was seen (Fig. 6B). The fluorescence emission maximum for NPA shifted from 480 nm in the absence of melittin to 425 nm in the presence of melittin and ATP. The blue shift and fluorescence yield enhancement of NPA by melittin in the presence of ATP are consistent with ATP promoting melittin tetramer formation. Although no visible precipitate was formed, there was significant light scattering by the samples to which melittin had been added to ATP solutions. For example, the fluorescence of a sample at 380 nm (excitation 360 nm) that contained ATP and melittin (ATP added first) was more than twenty times that of a sample that contained just melittin.

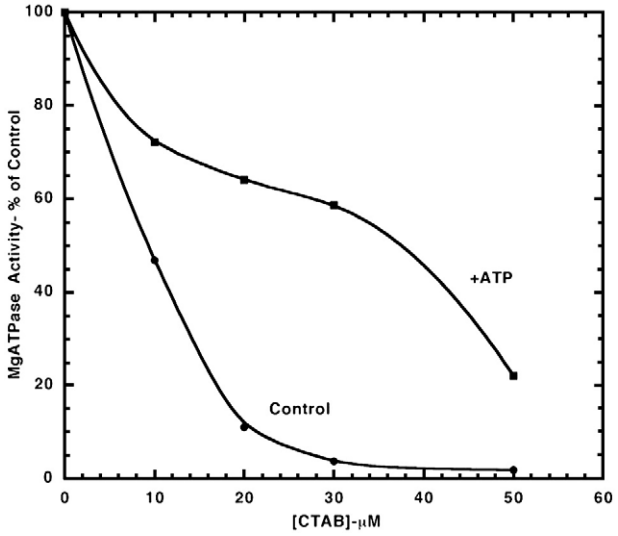


Fig. 5. ATP protects the ATPase activity of CF1-ε from inhibition by CTAB. CF1-ε (25 μg) was incubated in 0.5 ml of 20 mM Tris–HCl for 10 min at room temperature with and without 0.5 mM ATP. Aliquots (0.1 ml) of the incubations were diluted to 0.5 ml with 20 mM Tris–HCl (pH 8.0) containing the concentrations of CTAB shown. After 30 min at room temperature, sulfite-activated $Mg^{2+}$-ATPase activity was determined.

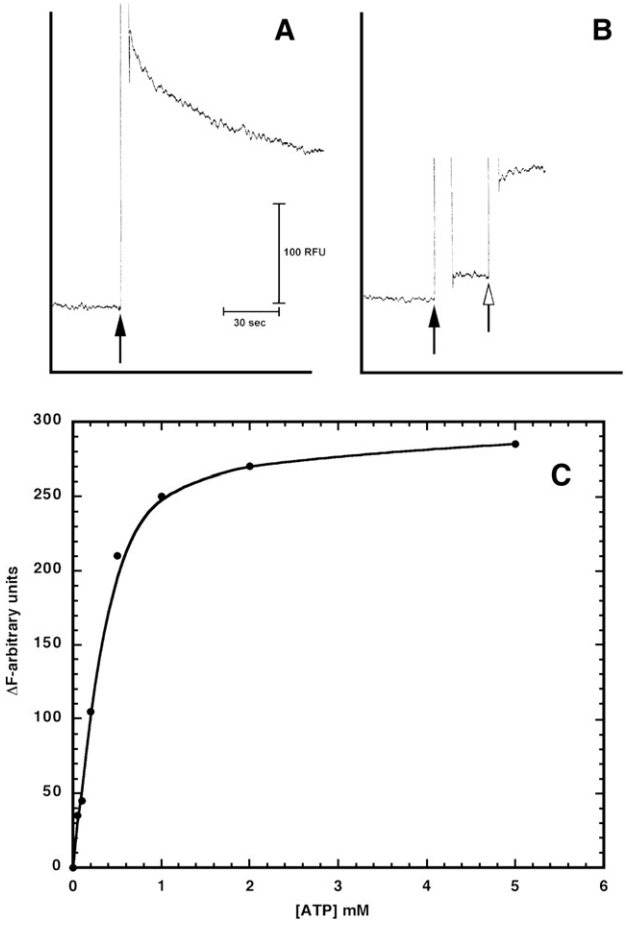


Fig. 6. Effects of ATP and melittin on NPA fluorescence. NPA (4 μM) in 1.5 ml of 50 mM Tris–HCl (pH 8.0) fluorescence was determined at room temperature and the mixtures were stirred . In A, 15 μl of a 1 mM solution of melittin was added (dark arrow) to a solution that contained 1 mM ATP in addition to the Tris buffer and NPA. In B, 15 μl of a 1 mM solution of melittin was added (dark arrow) to the Tris/NPA solution, followed by 15 μl of a 100 mM ATP solution (open arrow). In C, 15 μl of a 1 mM melittin solution was added to solutions that contained 4 μM NPA in 50 mM Tris–HCl (ph 8.0) and the indicated concentrations of ATP. $\Delta F$ is the difference between the fluorescence directly after melittin addition from that before melittin addition.

As little as 0.05 mM ATP gave an easily discernable increase in the fluorescence of NPA upon melittin addition (Fig. 6C). Half-maximal effect was at about 0.3 mM and saturation was approached at 2 to 5 mM ATP. ADP at 1 mM had a much smaller effect than ATP on NPA fluorescence in the presence of melittin and AMP had no effect (data not shown). Also, AMP partially blocked the increase in NPA fluorescence by ATP in the presence of melittin. The four negative charges of ATP and the adenine moiety seem to be involved in the aggregation of melittin.

## 4. Discussion

The action of melittin on CF1 and ECF1 resembles that of CTAB. Both are amphipathic, basic molecules that inhibit $Ca^{2+}$-ATPase activity at micromolar concentrations. Both melittin and

CTAB seem to cause at least partial dissociation of the F1 oligomeric structure and it is likely that this dissociation is the cause of the inhibition of activity. The irreversibility of the inhibition of the ATPase activity in CF1-ε by CTAB supports the notion that CTAB causes subunit dissociation.

The ATPase activity of F1 from beef heart mitochondria is inhibited by melittin [12] Both melittin and CTAB were found to increase the fluorescence of NPA in the presence of MF1 (R. E. McCarty, unpublished observations), suggesting that the amphiphiles can cause dissociation of MF1. It is interesting to note that Barzu et al. [30] found that the inhibition of the ATPase activity of MF1 by CTAB was substantially decreased by dilution of the CTAB concentration from 200 μM to 40 μM with ATPase reaction mixture. In our experiments, CF1-ε was incubated with 5 μM CTAB for 30 min prior to assay. $Mg^{2+}$-ATP may also protect the ATPase activity of MF1 from inhibition by CTAB.

Based on inhibitor titration data, Gledhill and Walker [12] concluded that melittin bound to the same site on MF1 as the inhibitor polypeptide, IF1. It is known [10] from the X-ray structure of the MF1–IF1 complex that IF1 binds at an α/β interface. Melittin could also bind at an α/β interface of CF1-ε, resulting in a loosening of the structure and, possibly, subunit dissociation. CTAB mimics the action of melittin. The observation that ATP can protect the $Ca^{2+}$-ATPase activity of CF1-ε from inhibition by CTAB is consistent with the hypothesis that CTAB can cause dissociation of the enzyme. ATP protects CF1 from dissociation in the cold [27] and from thermal denaturation [31].

It is tempting to conclude from the markedly different effects of melittin and CTAB on the $Ca^{2+}$-ATPase and $Mg^{2+}$-ATPase activities of ECF1 that these two activities differ in their mechanisms. Certainly, the proton-linked activities of membrane-bound CF1 work with $Mg^{2+}$-nucleotide complexes [32]. However, the hydrolysis of $Ca^{2+}$-ATP by a hybrid F1 assembly that contained recombinant α and β subunits from *Rhodospirillum rubrum* F1 and chloroplast γ subunit was shown [33] to support rotation of the γ subunit. This result indicates that $Ca^{2+}$-ATP hydrolysis can occur by the multisite binding change mechanism [1].

Mutated forms of ECF1 [34] and of the *R. rubrum* F1 [35] have been found in which the $Mg^{2+}$-ATPase activity of the mutant was enhanced and the $Ca^{2+}$-ATPase activity, suppressed. ATPase activities are determined at dilute protein concentrations and, often, at 37 °C. If the mutated F1 assemblies were less stable than the wild-type, they could dissociate during assay. $Mg^{2+}$-ATP stabilizes CF1 from dissociation, probably by binding to non-catalytic sites at α/β interfaces. $Ca^{2+}$-ATP may be less effective in stabilizing the enzyme against dissociation. $Mg^{2+}$-ATP could also protect ECF1 from the action of melittin and CTAB by tightening the a/β heterohexamer structure. Occupancy of a non-catalytic site or sites by $Mg^{2+}$-ATP may also prevent the binding of the amphiphiles to α/β interfaces.

ATP has been reported to protect the activity of a number of enzymes, including the $Na^+$, $K^+$-ATPase and $Ca^{2+}$-ATPase of sarcoplasmic reticulum [36], from inhibition by melittin. If the melittin monomer is the active species, protection could arise at least in part from the promotion of the formation of melittin tetramers by mM concentrations of ATP. ATP also causes further aggregation of melittin as shown here. Thus, the effects of ATP on the inhibition of enzyme activity by melittin must be interpreted with caution.

In conclusion, melittin and CTAB, both cationic amphiphiles, inhibit the ATPase activities of CF1-ε and ECF1. This inhibition likely results from alterations of the structure of the enzymes that expose hydrophobic surfaces of the enzyme to the solvent. Some dissociation of the enzyme by melittin and CTAB seems likely.

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