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ACTION OF ALKYL CATIONS AND THE NATURAL ATPase INHIBITOR FROM MITOCHONDRIA ON SOLUBLE MITOCHONDRIAL ATPase*

M. TUENA DE GÓMEZ PUYOU, A. GÓMEZ PUYOU and M. SALMÓN

Departamento de Biología Experimental, Instituto de Biología, U.N.A.M., Apartado Postal 70-600, México, D.F. (México)

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SUMMARY

The effect of the natural ATPase inhibitor and octylguanidine on the ATPase activity of soluble oligomycin-insensitive mitochondrial F_1 were compared. Both compounds induced a maximal inhibition of 60–80 % in various preparations of F_1 studied. The inhibition was of the uncompetitive type with respect to MgATP, and the action of the compounds was partially additive. The data suggest that octylguanidine reproduces the action of the natural ATPase inhibitor. Alkylammonium salts also affect the ATPase activity in a similar form. F_1 bound to Sepharose-hexylammonium is largely inactive, whilst free hexylammonium at higher concentrations induces only a partial inhibition of the activity. This suggests that the degree of immobilization of F_1 is related to the magnitude of inhibition of ATPase activity induced by alkyl cations. The binding of F_1 to Sepharose-hexylammonium is prevented by high concentrations of Na^+ or K^+.

INTRODUCTION

The isolation from mitochondria of a low molecular weight protein that inhibits ATP hydrolysis was originally described by Pullman and Monroy [1]; the findings of these authors have been confirmed in various laboratories [2–5]. This protein, known as the natural ATPase inhibitor, is heat stable and sensitive to incubation with trypsin [1, 6]. In addition to inhibiting ATP hydrolysis, this compound also inhibits all the ATP-driven energy-linked reactions of submitochondrial particles [7]. Moreover, it has been reported [2] that the binding of the inhibitor to mitochondrial ATPase is very much enhanced by Mg^{2+}+ATP, and Van de Stadt et al. [8] observed that the action of the inhibitor critically depends on the ADP/ATP ratio. In the light of these data, it has been suggested that the inhibitor exerts a regulatory function on mitochondrial oxidative phosphorylation.

Recently, it was reported that octylguanidine inhibited the ATPase activity of

* Contribution No. 459 of the Instituto de Química, U.N.A.M.

particulate and soluble mitochondrial ATPase [9, 10]; also octylguanidine [10] was found to protect soluble mitochondrial ATPase against its inactivation by low temperatures [11, 12]. As the natural inhibitor also exerts these two actions [1], it was thought that octylguanidine might mimic the action of the natural inhibitor. The results described in this work are consistent with this idea. Also some studies with alkyl cations covalently bound to Sepharose beads were conducted to further explore the behavior of the soluble F_1 component [11] of the mitochondrial ATPase complex.

MATERIALS AND METHODS

Mitochondria from bovine heart were prepared as described elsewhere [12], submitochondrial particles were prepared as described by Lee et al. [13]. The detailed procedure for the isolation and purification of soluble F_1 will be elaborated elsewhere, but briefly the procedure consists of solubilization of the enzyme from the particles according to Hortsman and Racker [2], followed by centrifugation of the suspension at 105 000 $\times$ *g* for 60 min. The supernatant is applied to a column of Sepharose-hexylammonium (Pharmacia) and eluted with 0.15 M sucrose, 2 mM EDTA and 2 mM ATP (adjusted to pH 7.3) until no protein is detected in the eluate. Following the application of the same elution mixture supplemented with 1.0 M KCl to the column, almost the totality of the enzyme elutes in the void volume. The enzyme is precipitated with an equal volume of saturated $(NH_4)_2SO_4$ as described by Hortsman and Racker [2] and maintained at 4 °C until further studies.

For the assay of ATPase activity, the $(NH_4)_2SO_4$ suspension is centrifuged at 10 000 $\times$ *g* for 10 min; the supernatant is decanted and any solution adhering to the tube is wiped off. The precipitate is dissolved in 0.15 M sucrose, 2 mM EDTA and 2 mM ATP at pH 7.3 and centrifuged again. Any precipitate that appears is discarded; the supernatant is used for measurements of ATPase activity. The enzyme thus prepared is about 80 % pure on the basis of its specific activity and analysis by sodium dodecyl sulfate gel electrophoresis [2, 14, 15].

The natural ATPase inhibitor was prepared according to the procedure described by Nelson et al. [16] for the F_1 inhibitor. The procedure was followed up to the precipitation step with trichloroacetic acid. The trichloroacetic acid precipitate is dissolved in water and adjusted to pH 5.5 with KOH, the suspension is centrifuged at 105 000 $\times$ *g* for 60 min. The supernatant is adjusted to pH 7.4 and the inhibitor precipitated with 0.83 M $(NH_4)_2SO_4$. The suspension is centrifuged at 105 000 $\times$ *g* for 60 min. The precipitate is dissolved in 0.25 M sucrose and maintained at 4 °C.

ATPase activity was measured by incubating soluble F_1 in the conditions described under Results. After incubation, the reaction was stopped with 6 % trichloroacetic acid (final concentration) and inorganic phosphate was determined according to Sumner [17]. The activity of the natural ATPase inhibitor was measured as described by Pullman and Monroy [1] unless otherwise stated. Protein was measured according to Lowry et al. [18].

Chemicals. Octylguanidine (sulfate) was prepared as previously described [10]. Hexylammonium covalently bound to Sepharose 4B (Sepharose-hexylammonium) was purchased from Pharmacia. ATP, ADP, phosphoenolpyruvate and pyruvate kinase were obtained from Sigma Chemical Co. Ethylamine, butylamine, hexylamine and octylamine were obtained from Eastman Chemical Co.

RESULTS

Comparison between the natural inhibitor and octylguanidine

As the natural ATPase inhibitor [1–8] and octylguanidine [9, 10] inhibit the ATPase activity of soluble and particulate F_1, and in addition, both compounds protect soluble F_1 [1, 10] against the detrimental action of low temperatures, it was thought that octylguanidine might reproduce the action of the natural inhibitor. Accordingly their action on soluble F_1 was further compared.

In agreement with Pullman and Monroy [1], the natural inhibitor did not induce complete inhibition of the ATPase activity of soluble F_1; maximal inhibition ranged between 60 and 80 %, depending on the preparation of F_1 employed (Fig. 1). Horstman and Racker [2] reported a higher sensitivity in their particles. Octylguanidine, similarly to the inhibitor, induced an inhibition which in several preparations ranged between 60 and 70 %.

Fig. 2 shows the effect of octylguanidine and the natural inhibitor on the ATPase activity of F_1 incubated at various concentrations of Mg ATP. In contrast to Van de Stadt et al. [8], who found that the natural inhibitor exerted a non-competitive type of inhibition in their F_1 preparation, in the conditions we used the natural inhibitor induced an uncompetitive inhibition. The reason for the discrepancy is not clear, but the different experimental conditions and the different F_1 preparation employed could be responsible for the observed differences. Octylguanidine, similarly to the inhibitor, exerted an uncompetitive type of inhibition (Fig. 2).

In agreement with the suggestion that alkylguanidines and the natural inhibitor act on the same site, it was found that the action of the natural inhibitor and octylguanidine are partially additive (Fig. 3). It is to be noted that the combination of the two compounds induced a maximal inhition of 60–70 % in the preparation of

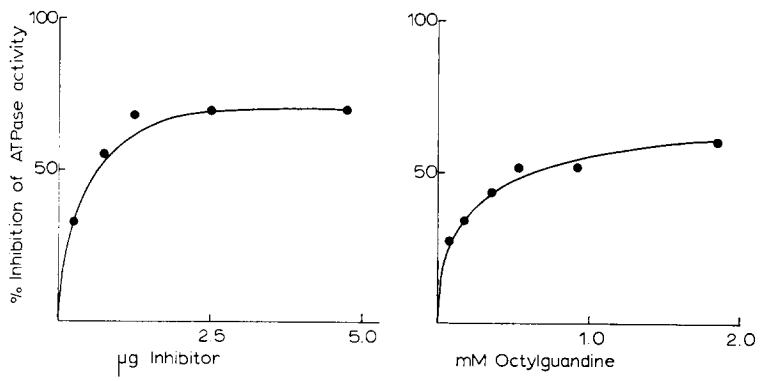

Fig. 1. Effect of octylguanidine and the natural ATPase inhibitor on the ATPase activity of F_1. ATPase activity was measured in the presence of an ATP-regenerating system: 10 mM Tris · HCl (pH 7.7), 2.0 mM ATP; 4.0 mM $MgCl_2$, 5 mM phosphoenolpyruvate and 10 μg of pyruvate kinase. The concentrations of the natural inhibitor shown were preincubated with F_1 (50 μg) for 10 min in 20 mM Tris · HCl (pH 6.7), 4 mM $MgCl_2$, 0.08 mM ATP, 0.1 M sucrose, and 0.08 mM EDTA in a final volume of 50 μl; at the end of this time an aliquot (5 μl) was withdrawn and added to the mixture for measuring ATPase activity. The control for the latter experiments was F_1 treated in identical conditions, but in the absence of the natural inhibitor. In the case of octylguanidine, the assay mixture contained the indicated concentrations of octylguanidine.

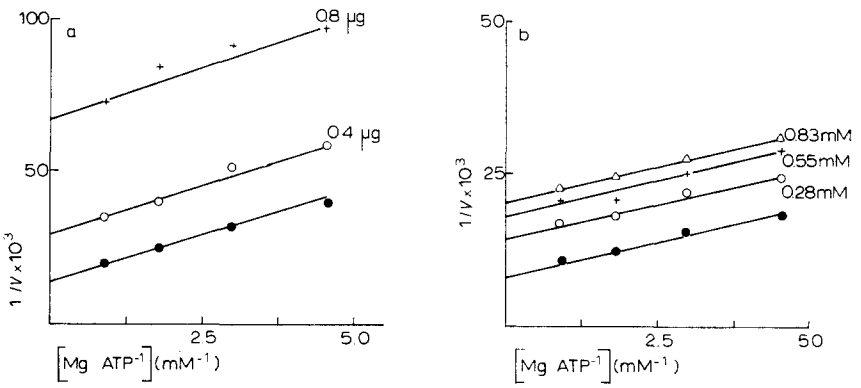

Fig. 2. Effect of the natural inhibitor and octylguanidine on the ATPase activity of soluble F_1 at various concentrations of Mg ATP; Lineweaver-Burk plot. The experimental conditions were as in Fig. 1, except that the preincubation mixture contained 60 μg of F_1, the indicated concentrations of the natural inhibitor (a) or of octylguanidine (b); ●–●, control. The incubation mixture contained Mg ATP/Mg^{2+} free = 1. V = μmol $P_i \cdot mg^{-1} \cdot min^{-1}$.

F_1 shown; this corresponds to the maximal inhibition obtainable with either of the two compounds. The maximal inhibition induced by either or by a combination of the inhibitor and octylguanidine was never 100 %, in one preparation the maximal inhibition was approx. 80 % (the maximal inhibition that was obtained in five different preparations of F_1).

Effect of alkylammonium salts on the ATPase activity of soluble F_1

Table I shows the effect of various alkyl-substituted ammonium salts on the ATPase activity of F_1. This experiment was carried out to explore whether the guanidinium moiety is specific for inhibiting the activity, or whether other positive

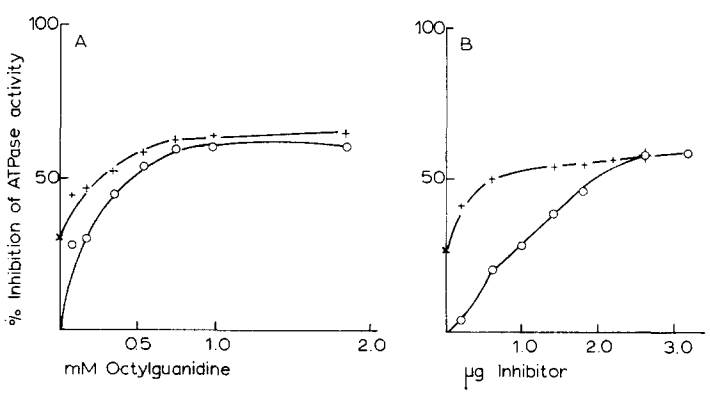

Fig. 3. Additive inhibiting action of octylguanidine and the natural inhibitor on the ATPase activity of F_1. The experimental conditions were as in Fig. 1 except that the preincubation and incubation mixtures contained octylguanidine and the inhibitor as shown. In A, +–+ indicates that F_1 was preincubated with 0.5 μg of inhibitor as in Fig. 1; thereafter an aliquot of the preincubation mixture was added to tubes that contained the indicated concentration of octylguanidine. In B, +–+ indicates that F_1 preincubated with the indicated concentration of the inhibitor was added to tubes that contained 0.180 μM octylguanidine.

TABLE I

EFFECT OF VARIOUS ALKYLAMMONIUM SALTS ON THE ATPase ACTIVITY OF SOLUBLE F_1

The incubating conditions were 5.5 mM ATP, 10 mM Tris · HCl (pH 7.7), 4.0 mM $MgCl_2$, 20 mM of each of the indicated chloride salts and 2 μg of F_1. Final volume, 1.0 ml; temperature, 30 °C; incubation time, 10 min.

Additions	ATPase (μmol $P_i \cdot mg^{-1} \cdot min^{-1}$)
–	80
Ethylammonium	70
Butylammonium	60
Hexylammonium	39
Octylammonium	14

TABLE II

BINDING OF SOLUBLE F_1 TO SEPHAROSE-HEXYLAMMONIUM

F_1 (40 μg) was incubated with Sepharose-hexylammonium which contained the indicated concentration of hexylammonium covalently bound to Sepharose 4B and 4 mM Tris · HCl (pH 7.3) in a final volume of 1.0 ml for 5 min. At this time the suspension was centrifuged for 5 min and an aliquot of the supernatant was withdrawn to measure ATPase activity in a mixture that contained 5.5 mM ATP, 10 mM Tris · HCl (pH 7.7) and 4.0 mM $MgCl_2$ in a final volume of 1.0 ml; temperature, 30 °C, incubation time, 10 min.

Hexylammonium (mM)	F_1 bound (%)
0.4	68
0.8	88
1.2	96

TABLE III

ATPase ACTIVITY OF F_1 BOUND TO SEPHAROSE-HEXYLAMMONIUM

F_1 (38 μg) was incubated with Sepharose-hexylammonium (2.0 mM of hexylammonium), 2 mM ATP, 2 mM EDTA and 0.15 mM sucrose (pH 7.3) in a final volume of 1.0 ml for 5 min. At the end of this time the mixture was centrifuged and in an aliquot of the supernatant ATPase activity was measured. The Sepharose pellet was washed twice by suspending the pellet in the same media as above and ATPase activity was measured in the corresponding supernatants (first and second wash). After the second wash the mixture for the assay of ATPase activity was added to the Sepharose pellet (free of supernatant by decantation) and ATPase activity was measured. The KCl supernatant of the Sepharose pellet refers to F_1 that had been treated under identical conditions, except that after the second wash the Sepharose pellet was suspended in the mixture mentioned above that contained 1.0 M KCl, after centrifugation an aliquot of the supernatant was withdrawn for measurement of ATPase activity. ATPase activity was measured as in Table II.

	Activity (%)
Bound	100
First wash	10
Second wash	7
Sepharose pellet	22
KCl supernatant of Sepharose pellet	77

charged groups attached to an alkyl chain could also exert this type of action. Although guanidines are much more effective than amines on a concentration basis, the inhibiting action of alkyl cations is not exclusive of guanidines, since hexylammonium and particularly octylammonium inhibit ATPase activity. Also it seems that an alkyl chain of more than six carbons is a requisite for an alkyl cation to inhibit ATPase activity.

On the assumption that alkyl cations reproduce the action of the natural ATPase inhibitor, some insight into the relationship between the inhibitor and F_1 can be obtained if some of the conditions that affect the interactions of alkyl cations and F_1 are ascertained. To this purpose, the interactions between F_1 and Sepharose-bound hexylammonium and some of the properties of the bound enzyme were studied. In the experiment shown in Table II, various quantities of Sepharose-bound hexylammonium were incubated with F_1; after 5 min of incubation, the suspension was centrifuged and an aliquot of the supernatant was withdrawn to measure ATPase activity. The difference between the activity added and that found in the supernatant may be considered as the amount of enzyme bound to Sepharose-hexylammonium. As expected from the results of the method of purification of F_1 that has been employed a sufficient quantity of Sepharose-hexylammonium may bind almost the totality of the enzyme added (Table II).

In a second step of an identical experiment, the ATPase activity of the Sepharose pellet to which F_1 was bound was measured. The results showed that the enzyme bound to Sepharose-hexylammonium is largely inactive (Table III). Nevertheless, elution of the enzyme from the Sepharose by high concentrations of K^+ results in an enzyme that is fully active (see preparative procedure). It is to be noted that concentrations of free hexylammonium much higher than those that were employed in the experiment of Table III induced only a partial inhibition of ATPase activity (See Table I). Apparently hexylammonium bound to a solid phase is much more effective than free hexylammonium in inhibiting the ATPase activity of soluble F_1.

The ATPase activity of F_1 may be stimulated by K^+ [19, 20]; therefore, it was considered interesting to study whether release of F_1 from alkyl cations could be induced by K^+ and whether this was related to the stimulation of the activity by this cation. In this respect, it should be recalled that Hortsman and Racker [2] reported that 50 mM K^+ and Na^+ induced full separation of the natural inhibitor from F_1. As shown in Table III, the K^+-induced release of the enzyme from Sepharose-hexylammonium requires relatively high concentrations of K^+. Moreover, release of the enzyme is also induced by Na^+. As stimulation of ATPase activity is induced by K^+, but not by Na^+ [20], most probably dissociation of inhibitor from F_1 and stimulation of ATPase activity as induced by monovalent cations are two different processes.

DISCUSSION

The results of this work, as well as those described previously [10] indicate that certain organic cations, such as the alkyl derivatives of guanidinium and ammonium ions affect the properties of F_1. In some aspects their action resembles that of the natural ATPase inhibitor, i.e. both inhibit the ATPase activity of submitochondrial particles and soluble F_1, protect against cold-induced inactivation of F_1, and affect very similarly the kinetics of enzyme activity when tested as a function of

the concentration of MgATP. In addition the action of octylguanidine and the natural inhibitor are partially additive. These findings although not conclusive suggest that alkyl cations and the natural inhibitor inhibit ATPase activity by acting on a common site.

In favor of this suggestion, it should be recalled that the inhibitors from heart and yeast [3, 5], regardless of their different net charge, possess an important number of basic residues which may well represent the chemical groups through which the inhibitor affects the ATPase activity of F_1.

It has also been shown that alkyl cations require a carbon chain of at least six carbons in order to induce inhibition of ATPase activity [10]. Presumably the carbon chain is required for a more stable interaction between the enzyme and the cation through hydrophobic bonding. Related to this previous finding is the observation that Sepharose-bound alkyl cations are more effective than the free cations in inhibiting ATPase activity. Indeed in the conditions of the experiment of Table II, 1.5 mM of hexylammonium bound to Sepharose beads bound the totality of the added enzyme with a very significant inactivation of the enzyme (Table III), whilst 20 mM free hexylammonium inhibited ATPase activity by approx. 50–60 % (Table I).

These observations indicate that binding of free hexylammonium to F_1 does not suffice to induce total inactivation of the enzyme. It has been reported that ligands attached to Sepharose are more effective than the free ligand [21]; apparently this is due to a stronger immobilization of the protein in the Sepharose-bound ligand. Thus it is conceivable that a similar mechanism operates in the phenomenon presently described. That is, complete inhibition of enzyme activity requires binding of the inhibitor plus immobilization of the enzyme. Moreover, the strong binding between F_1 and Sepharose-hexylammonium would perhaps explain why very high concentrations of salt are required to prevent binding of F_1 to Sepharose-hexylammonium.

The question is whether a similar phenomenon operates in the membrane with the ATPase inhibitor. From the data available, it would seem that the natural inhibitor is more effective in particles than in soluble F_1. In addition Van de Stadt et al. [8] reported variations in the extent to which the natural ATPase inhibitor affects the activity of submitochondrial particles. Thus it is conceivable that the quantitative effect of the inhibitor depends on the degree of mobility or the conformation of F_1 in the membrane. Indeed it should be recalled that the question of why the natural inhibitor inhibits ATPase activity and the ATP-driven energy-linked reactions of submitochondrial particles without affecting oxidative phosphorylation [7] is not settled.

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