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CATION REQUIREMENT FOR THE RECONSTITUTION OF OLIGOMYCIN-SENSITIVE ATPase BY MEANS OF SOLUBLE F_1-ATPase AND F_1-DEPLETED SUBMITOCHONDRIAL PARTICLES

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Bovine heart submitochondrial particles depleted of F_1 by treatment with urea ('F_1-depleted particles') were incubated with soluble F_1-ATPase. The binding of F_1 to the particles and the concomitant conferral of oligomycin sensitivity on the ATPase activity required the presence of cations in the incubation medium. NH_4^+, K^+, Rb^+, Cs^+, Na^+ and Li^+ promoted reconstitution maximally at 40–74 mM, guanidinium$^+$ and Tris$^+$ at 20–30 mM, and Ca^{2+} and Mg^{2+} at 3–5 mM. The particles exhibited a negative ζ-potential, as determined by microelectrophoresis, and this was neutralized by mono- and divalent cations in the same concentration range as that needed to promote F_1 binding and reconstitution of oligomycin-sensitive ATPase. It is concluded that the cations act by neutralizing negative charges on the membrane surface, mainly negatively charged phospholipids. These results are discussed in relation to earlier findings reported in the literature with F_1-depleted thylakoid membranes and with submitochondrial particles depleted of both F_1 and the coupling proteins F_6 and oligomycin sensitivity-conferring protein.

Introduction

In a previous paper from this laboratory [1], it was reported that the binding of soluble F_1-ATPase to bovine heart submitochondrial particles depleted of F_1, oligomycin sensitivity-conferring protein and F_6 requires the presence of monovalent cations NH_4^+, K^+, Rb^+ and Cs^+ were more efficient than Na^+ and Li^+ in promoting F_1 binding, whereas divalent cations were without effect. The monovalent cations were also able to replace F_6 in promoting F_1 binding, but F_6 was needed, together with oligomycin sensitivity-conferring protein, to confer maximal oligomycin sensitivity on the bound F_1.

In order to investigate further these cation effects it was felt necessary to choose a simpler system, consisting of submitochondrial particles depleted only of F_1. In this paper, Sephadex-treated EDTA particles were depleted of F_1 by treatment with urea, and the effects of cations on the rebinding of soluble F_1 to these particles and on the concomitant conferral of oligomycin sensitivity on the bound F_1 were investigated. As will be shown, the presence of cations is obligatory also in this system, but is not as specific with respect of the cations used as earlier found to be the case with the F_1-oligomycin sensitivity-conferring protein-F_6-depleted particles. Evidence will be presented indicating that the cation effect consists of a neutralization of negative charges on the surface of

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Abbreviations F_1, coupling factor 1, F_6, coupling factor 6

the F_1-depleted particles. These results are similar to those earlier reported by Telfer et al. [2] with F_1-depleted thylakoid membranes.

Material and Methods

Preparations EDTA particles were prepared as described earlier [3]. By passing these particles through Sephadex G-50 and then treating them with 2 M urea [4], submitochondrial particles depleted of F_1 were obtained [5].

F_1 was prepared according to the method of Horstman and Racker [6] and stored as a saturated $(NH_4)_2SO_4$ suspension. The ATPase activity of F_1 was approx 80 μmol/min per mg protein at 30°C. F_1 was dissolved at a protein concentration of 4 mg/ml in a solution containing 0.25 M sucrose, 10 mM Tris-sulfate and 0 25 EDTA, pH 8 (buffer A) and, when indicated, dialyzed against the same buffer for 3 h at room temperature before use.

Binding experiments. These experiments were carried out by incubating dialysed F_1 and F_1-depleted particles in small plastic tubes containing buffer A and, when indicated, in the presence of various cations, for 15 min at room temperature (approx. 22°C) in a final volume of 150 μl. For measuring the percentage of binding the samples were centrifuged at room temperature for 15 min at 15 000 × *g* in a Beckman J21 centrifuge using a JA20 rotor

ATPase activity was measured both before and after centrifugation, in the latter case both in the supernatants and in the resuspended pellets. The extent of binding F_1 to the particles was expressed as the percentage of the activity that disappeared from the supernatant after centrifugation.

Oligomycin sensitivity was determined using 3 μg oligomycin/mg particle protein, which gave maximal inhibition.

ATPase activity. ATPase activity was determined spectrophotometrically by coupling the reaction to the pyruvate kinase and lactate dehydrogenase reactions and following the oxidation of NADH at 340 nm [7]

Protein determination Protein content was estimated either by the biuret method [8] or according to the method of Lowry et al. [9].

Measurement of ζ-potential. For measuring the ζ-potential of the particles the microelectrophoresis method of Bangham et al. [10] was used. Electrokinetic mobilities were measured at 25°C with a Rank Brothers Mark II microelectrophoresis apparatus using a cylindrical cell, with two palladium electrodes and two platinum 'probe' electrodes (Bottisham, Cambridge, U K.) Measurements were done at the stationary level in the cell

The ζ-potential (mV) was calculated from the measured value of the electrophoretic mobility, u ($\mu m \cdot cm \cdot V^{-1} \cdot s^{-1}$), by the Helmoltz-Smoluchowski equation.

$$\zeta = \frac{u\eta}{\varepsilon_r \varepsilon_0}$$

where η is the viscosity of the aqueous phase ε_r the dielectric constant and ε_0 the permittivity of free space (see Ref. 11 for a review).

Results and Discussion

Incubation of F_1-depleted particles with soluble F_1 (0.065 mg F_1/mg particle protein) resulted in virtually complete binding of F_1 to the particles and a concomitant appearance of oligomycin sensitivity of ATPase activity (Table I).

When F_1 was dialyzed before incubation with the particles, thus removing the $(NH_4)_2SO_4$

TABLE I

BINDING OF F_1 AND CONCOMITANT CONFERRAL OF OLIGOMYCIN SENSITIVITY ON F_1-DEPLETED SUBMITOCHONDRIAL PARTICLES

F_1-depleted submitochondrial particles (230 μg protein) were incubated, as described in Materials and Methods, in the presence of F_1 (15 μg) and, when indicated, NH_4^+ (75 mM) The extent of binding was determined by centrifuging the particle and determining the remaining ATPase activity in the supernatant Oligomycin sensitivity was tested by measuring the ATPase activity in the total sample before centrifugation, in the absence and presence of 3 μg oligomycin mg particle protein For other details, see Materials and Methods

Additions	% bound	% oligomycin sensitive
F_1 (undialyzed)	92	95
F_1 (dialyzed)	38	35
F_1 (dialyzed)+NH_4^+ (75 mM)	93	93

originating from the stock solution of F_1 (see Materials and Methods), the extent of both binding and oligomycin sensitivity decreased to 35–40%

A similar effect observed when F_1 was passed through a Sephadex G-50 column instead of dialysis (not shown)

When dialyzed F_1 was incubated with the F_1-depleted submitochondrial particles in the presence of 75 mM NH_4^+, maximal extents of binding and oligomycin sensitivity were found (see Table I). When the reconstituted particles were dialyzed, F_1 remained bound and oligomycin sensitive, indicating that once F_1 was bound, the presence of cations in the medium was no longer needed.

Since the binding of F_1 and the conferral of oligomycin sensitivity occurred in parallel, when the above particles were incubated with F_1, in the following experiments oligomycin sensitivity was used as a measure of the reconstitution.

As shown in Fig. 1A, NH_4^+, Na^+, K^+, Li^+, Rb^+ and Cs^+ were equally efficient in promoting reconstitution, all being maximally efficient at or above a concentration of these cations of about 50 mM Organic monovalent cations such as $Tris^+$ or $guanidinium^+$ also promoted reconstitution (Fig. 1B), and maximal effect in this case occurred at a concentration of 20–30 mM. It should be noted that the incubation medium in all these experiments contained 10 mM Tris, which probably explains the 30–40% reconstitution found without any further addition of cations. In fact, when Tris was altogether omitted from the incubation medium, there occurred virtually no binding of F_1 and oligomycin sensitivity.

In all the above experiments, the cations were added as the chlorides. When the anion was changed to sulfate or citrate, the concentrations of the cations required for reconstitution remained unchanged, showing that it is the cation and not the anion that promotes the reconstitution

Fig. 2 shows that the reconstitution was also promoted by divalent cations such as Ca^{2+} or Mg^{2+}. In this case the concentration needed for maximal effect was about 3 mM, i.e, much lower than in the case of monovalent cations.

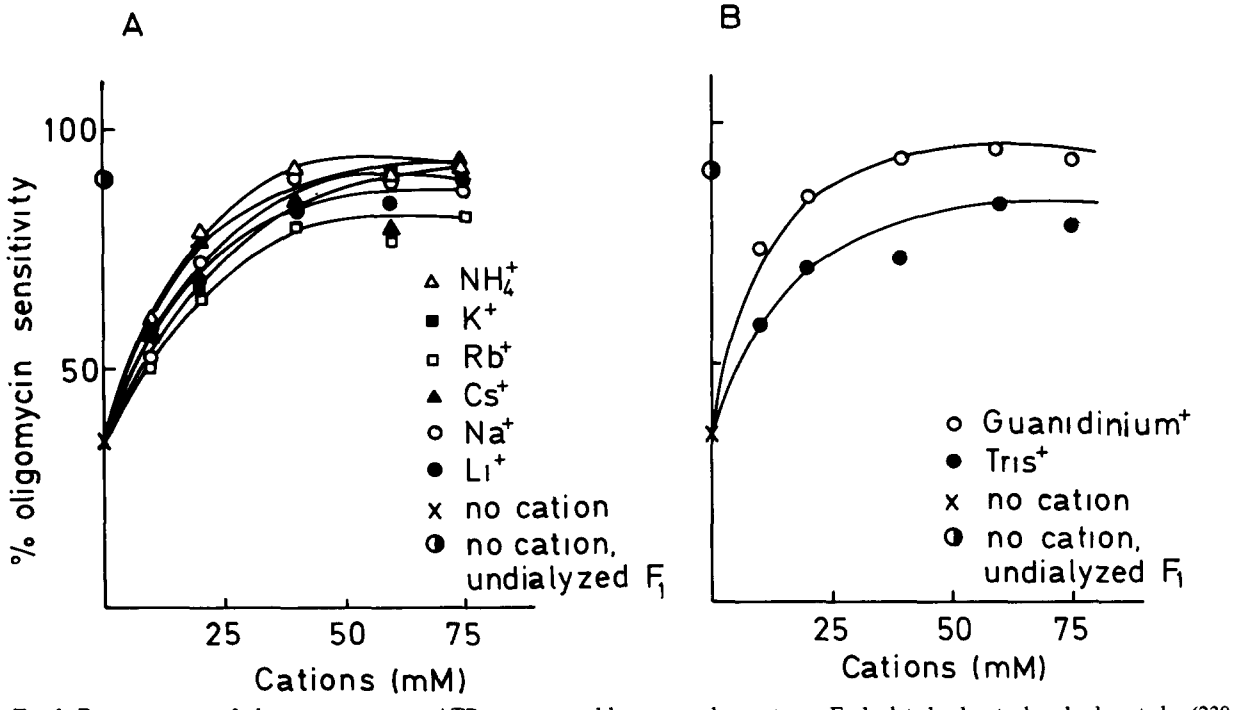

Fig 1 Reconstitution of oligomycin-sensitive ATPase promoted by monovalent cations F_1-depleted submitochondrial particles (230 μg) were incubated, as described in Materials and Methods, in the presence of F_1 (15 μg) and, when indicated, in the presence of monovalent cations Dialyzed F_1 was used unless otherwise indicated

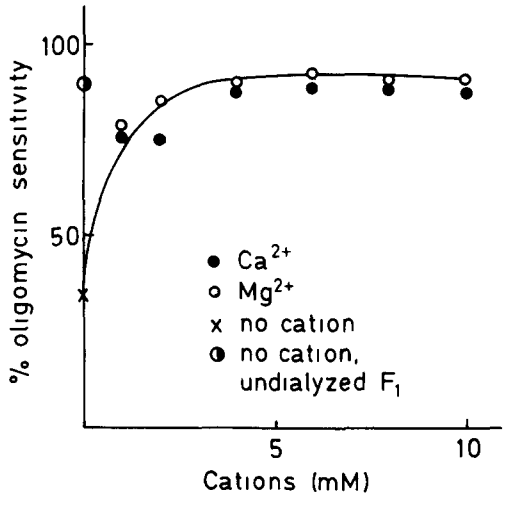

Fig 2 Reconstitution of oligomycin-sensitive ATPase promoted by divalent cations F_1-depleted submitochondrial particles (230 μg) were incubated, as described in Materials and Methods, in the presence of F_1 (15 μg) and, when indicated, in the presence of divalent cations Dialyzed F_1 was used unless otherwise indicated

Fig. 3 compares the extent of reconstitution obtained on varying the F_1/particle protein ratio. The maximal amount of F_1 that could be bound and rendered oligomycin sensitive by the F_1-depleted submitochondrial particles was about 0.065 mg F_1/mg particle protein (Fig. 3A), in accordance with previous findings [12]. When comparing the NH_4^+ requirement at different F_1/particle protein ratios, it was found (Fig 3B) that the less

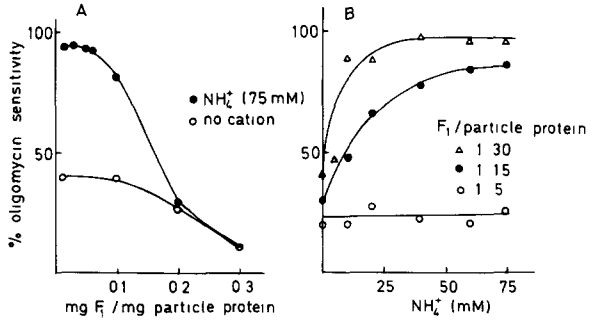

Fig 3 Reconstitution of oligomycin-sensitive ATPase at varying F_1/particle protein ratios and varying concentrations of NH_4^+ F_1-depleted submitochondrial particles (230 μg) were incubated, as described in Materials and Methods, with different amounts of dialyzed F_1 and, when indicated, in the presence of NH_4^+

F_1 was used per particle protein, the lower was the concentration of NH_4^+ needed for maximal reconstitution

We interpreted the above effect of cations in terms of a neutralization of negative membrane charges, probably negatively charged phospholipids (e g , cardiolipin), on the membrane surface (Fig. 4). F_1 is a negatively charged polyelectrolyte In the absence of added cations, the negative surface charges on the F_1-depleted particles will repel F_1 from the interface, hence preventing a recombination with oligomycin sensitivity-conferring protein and F_6. The cationic screening will lower the surface potential and the Debye length The more complete reconstitution with added F_1 we intend to achieve, the higher is the cation concentration needed to promote maximal extent of F_1 binding. The time course of the recombination might be governed by the electrostatic barrier of F_1 in the aqueous environment, as well as by the probability of its collision with its binding sites on F_6 and oligomycin sensitivity-conferring protein at the membrane surface. Once bound, F_1 is not dissociated upon lowering the salt concentration, probably because of a strong, specific electrostatic attraction between negative charges of F_1 and the positive charges of F_6 and oligomycin sensitivity-conferring protein.

To test this interpretation we have measured

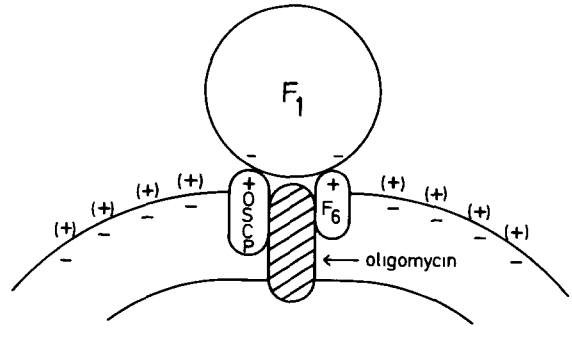

Fig 4 Schematic illustration of the notion that, when F_1 is removed from submitochondrial particles by urea treatment, positive charges are exposed on oligomycin sensitivity-conferring protein (OSCP) and F_6, which interact with negative charges on the membrane surface, mainly negatively charged phospholipids Added cations (+) neutralize these negative charges and thereby promote the rebinding of F_1 and the concomitant conferral of oligomycin sensitivity

the ζ-potential as an indicator of the potential of the particles. Measurements are made in the absence and presence of added cations by using the microelectrophoresis method described in Materials and Methods. It may be seen in Table II that, in the absence of added cations, the particles exhibited a negative ζ-potential, which markedly decreased upon the addition of cation. The potential dropped to virtually zero upon addition of 75 mM Na^+ or NH_4^+ or 4 mM Ca^{2+}, which is in good agreement with the concentrations of these cations needed for the promotion of maximal reconstitution of F_1 to the particles The higher efficiency of the divalent as compared to the monovalent cations is in agreement with the Gouy-Chapman theory. These findings thus support the above interpretation of our results concerning the cation effects on the rebinding of F_1, to the F_1-depleted particles.

A similar interpretation was earlier proposed for the cation requirement of the rebinding of F_1 to F_1-oligomycin sensitivity-conferring protein-F_6-depleted particles [1]. However, in that case, NH_4^+, K^+, Rb^+ and Cs^+ were more efficient than Na^+ and Li^+, and Ca^{2+} and Mg^{2+} were ineffective. This more selective cation requirement may be explained by the fact that in the reconstitution of oligomycin-sensitive ATPase activity requires the reinsertion of F_6 and oligomycin sensitivity-conferring protein which may be hindered by certain cations, possibly by a restriction of phospholipid mobility. Interestingly, a similar restricted pattern of cation requirement has been observed by Nalęcz and Wojtczak [13] for the relief of NAD^+ inhibition of NADH oxidation by submitochondrial particles. They pointed out the possible importance of intramitochondrial K^+ for the activity of the respiratory chain. Our previous [1] and present results indicate a general role of intramitochondrial cations in controlling the surface charge of the matrix side of the inner mitochondrial membrane and thereby the activity and, perhaps even more importantly, the assembly of the ATP-synthesizing enzyme system. Evidently, the electrostatic control of this assembly is similar to that of the chloroplast ATPase, as reported by Telfer et al. [2], except that in that case F_6 and oligomycin sensitivity-conferring protein do not seem to be involved in the binding of F_1 to the membrane sector of the system.

TABLE II

EFFECTS OF CATIONS ON THE ζ-POTENTIAL OF SUBMITOCHONDRIAL PARTICLES

F_1-depleted submitochondrial particles (500 μg) were diluted in 10 ml of a medium containing 10 mM Tris-sulfate, pH 8 0, and 0 25 mM EDTA The ζ-potential was measured as described in Material and Methods

Addition	ζ-potential (mV)
None	−14 4
Na^+ 10 mM	−7 7
30 mM	−4 9
75 mM	≈ 0
NH_4^+ 10 mM	−8 8
30 mM	−6 0
75 mM	≈ 0
Ca^{2+} 0 5 mM	−10 7
1 mM	−8 2
4 mM	≈ 0

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