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EVIDENCE FOR THE INVOLVEMENT OF $(Cu\text{-}ATP)^{2-}$ IN THE INHIBITION OF HUMAN ERYTHROCYTE $(Ca^{2+} + Mg^{2+})$-ATPase BY COPPER

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The effects of copper on the activity of erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase have been tested on membranes stripped of endogenous calmodulin or recombined with purified calmodulin. The interactions of copper with Ca^{2+}, calmodulin and $(Mg\text{-}ATP)^{2-}$ were determined by kinetic studies. The most striking result is the potent competitive inhibition exerted by $(Cu\text{-}ATP)^{2-}$ against $(Mg\text{-}ATP)^{2-}$ ($K_i = 2.8\ \mu M$), while free copper gives no characteristic inhibition. Our results also demonstrate that copper does not compete with calcium either on the enzyme or on calmodulin. The fixation of calmodulin on the enzyme is not altered in the presence of copper as shown by the fact that the dissociation constant remains unaffected. It may be speculated that $(Cu\text{-}ATP)^{2-}$ is the active form of copper, which could plausibly be at the origin of some of the pathological features of erythrocytes observed in conditions associated with excess copper.

Introduction

It has already been established that the lifespan of human erythrocytes is decreased and their rate of destruction increased in pathological conditions associated with high serum copper levels [1–7].

In the erythrocyte, copper decreases the intracellular concentration of reduced glutathione [2,8] and alters the function of hemoglobin [9]. It also reduces the activities of certain cytosolic key enzymes such as hexokinase [10]. It has been suggested that copper could directly oxidize the SH groups of human erythrocytes membrane proteins [11,12], a fact which results in the formation of high molecular weight membrane protein polymers. Copper has also been described as the origin of the production of superoxyde ions [13]. Furthermore, a copper-oxygen complex may be directly involved in the peroxydation of lipids [14]. Copper also modifies the permeability of the membranes to potassium [15]. As shown by Adams et al. [16], the filterability of erythrocytes incubated in copper-added nonplasmatic media is decreased. From their experiments they conclude that the surface-to-volume ratio of the cell is decreased, as would be the flexibility of the membrane skeleton. Since these factors which determine the capacity of erythrocyte to deform are modified, it would be consistent to think that these alterations could be related to a copper-induced inhibition of the erythrocyte membrane ATPases (EC 3.6.1.3) [17].

We recently brought to evidence, on membranes incubated with copper, both the inhibition of the $(Na^+ + K^+)$-ATPase and that of the $(Ca^{2+} + Mg^{2+})$-ATPase, this last enzyme being more sensitive to copper [18]. In the present paper, we report the results of experiments devoted to the

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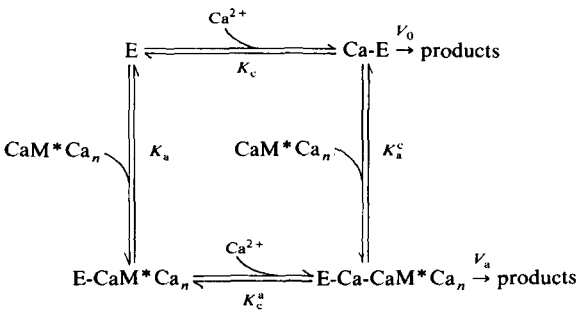
molecular effect of copper on $(Ca^{2+} + Mg^{2+})$-ATPase.

We have used the model proposed by Jarrett and Kyte [19] and completed by Cox et al. [20] to study the action of copper on the activity of the enzyme as a function of Ca^{2+} and of active species of calmodulin (CaM^*Ca_n). These authors suggest that the enzyme works according to the following scheme:



In all studies, appropriate experiments were done in order to determine the effect of copper on the fixation of Ca^{2+} on the enzyme and on calmodulin, as well as on the fixation of CaM^*Ca_n on $(Ca^{2+} + Mg^{2+})$-ATPase. Since the activity of $(Ca^{2+} + Mg^{2+})$-ATPase requires $(Mg\text{-}ATP)^{2-}$, the action of $(Cu\text{-}ATP)^{2-}$ and of free copper against $(Mg\text{-}ATP)^{2-}$ was equally investigated.

Materials and Methods

Membrane preparation. Human erythrocyte membranes were prepared as described by Scharff and Foder [21]: 'A' membranes (without calmodulin) and 'B' membranes (with calmodulin). B membranes were prepared with the following slight modifications: 10 μM of $CaCl_2$ were used and nitrilotriacetic acid was omitted. Membranes were stored overnight in liquid nitrogen.

Calmodulin preparation. Calmodulin was prepared according to the method of Luthra et al. [22]. The preparation was completed as follows: the eluate obtained on CM-Sephadex C-50 was heated at 85 °C for 5 min and further centrifuged at $20000 \times g$ for 15 min. The supernatant was then submitted to affinity chromatography on phenyl-Sepharose CL-4B [23]. The calmodulin fractions were lyophylized after 18 h dialysis against distilled water.

Assay of $(Ca^{2+} + Mg^{2+})$-ATPase. The enzyme activity was measured by the assay of P_i released at 37 °C using an adaptation of the method of Anner and Mossmayer [24]. The reaction medium (final volume: 500 μl) contained 70 mM Tris-HCl (pH 7.4), 200 μg membrane proteins/ml, $CaCl_2$, calmodulin, ATP and $MgCl_2$ in concentrations required by each experiment. After a 20 min preincubation, the reaction was started by addition of ATP or membranes. At 0, 10, 20, 30 and 40 min, 70 μl were removed and added to 830 μl of 15 mM ammonium heptamolybdate in 0.35 M H_2SO_4. After 10 min, 100 μl of a malachite green solution were added (74 mg malachite green in 200 ml water containing 4 g polyvinyl alcohol. The green colour which developed was read after 10 min in a Beckman model 35 spectrophotometer set at 630 nm. A standard curve was obtained by replacing the reaction medium with 70 μl of solutions containing, respectively, 14.3, 28.6, 71.4, 114.3 and 255.7 μM KH_2PO_4. The activities of appropriate blanks were substracted to obtain ATPase activation by Ca^{2+} or by Ca^{2+} and calmodulin. The results were expressed as nmol P_i released/min per mg protein. The protein concentrations were determined by the method of Lowry et al. [25].

Determination of final concentrations of Ca^{2+}, $(Mg\text{-}ATP)^{2-}$, CaM^*Ca_n, Cu^{2+}, $(Cu\text{-}ATP)^{2-}$. For each experiment, the concentrations of ATP, $CaCl_2$, $MgCl_2$, calmodulin (CaM) and $CuSO_4$ were adjusted to obtain the final amounts of Ca^{2+}, $(Cu\text{-}ATP)^{2-}$, CaM^*Ca_n required by using the following equations and equilibrium constants [26–29]:

$$H^+ + ATP^{4-} \leftrightharpoons HATP^{3-} \qquad K_a = 3.16 \cdot 10^6 M^{-1}$$
$$H^+ + HATP^{3-} \leftrightharpoons H_2ATP^{2-} \qquad K_a = 1.12 \cdot 10^4 M^{-1}$$
$$Ca^{2+} + ATP^{4-} \leftrightharpoons CaATP^{2-} \qquad K_a = 3.98 \cdot 10^3 M^{-1}$$
$$Ca^{2+} + HATP^{3-} \leftrightharpoons CaHATP^{-} \qquad K_a = 7.07 \cdot 10^4 M^{-1}$$
$$Mg^{2+} + ATP^{4-} \leftrightharpoons MgATP^{2-} \qquad K_a = 5.07 \cdot 10^4 M^{-1}$$
$$Cu^{2+} + ATP^{4-} \leftrightharpoons CuATP^{2-} \qquad K_a = 1.25 \cdot 10^6 M^{-1}$$
$$CaM + Ca^{2+} \leftrightharpoons CaM\,Ca_1 \qquad K_1 = 5.00 \cdot 10^5 M^{-1}$$
$$CaMCa_1 + Ca^{2+} \leftrightharpoons CaM\,Ca_2 \qquad K_2 = 1.66 \cdot 10^5 M^{-1}$$
$$CaMCa_2 + Ca^{2+} \leftrightharpoons CaM\,Ca_3 \qquad K_3 = 5.50 \cdot 10^4 M^{-1}$$
$$CaMCa_2 + Ca^{2+} \leftrightharpoons CaM\,Ca_4 \qquad K_4 = 5.00 \cdot 10^3 M^{-1}$$

K_1, K_2, K_3 and K_4 are the stoechiometric constants.

Action of copper. In all the experiments, copper ($CuSO_4$) was initially added into the assay medium. When the action of copper was studied against

Ca^{2+}, the reaction was started by ATP. In experiments devoted to study the action of copper against $(Mg\text{-}ATP)^{2-}$, the reaction was started by the addition of membranes, copper, $MgCl_2$ and ATP being simultaneously added in the cuvette. $CuSO_4$ was used as a source of copper since the sulfate ion has no effect on erythrocyte metabolism [10].

Reagents. Phenyl-Sepharose CL-4B and CM-Sephadex C-50 were obtained from Pharmacia and malachite green from Merck (Art 1398). All other reagents were of analytical grade.

Results and Discussion

Copper inhibition as a function of free Ca^{2+}

On A membranes, without or with calmodulin (Figs. 1 and 2), copper induces a noncompetitive inhibition, which suggests that it does not interfere with the fixation of Ca^{2+} on the enzyme. The K_{Ca} values found: 1.7 μM with calmodulin and 30 μM without calmodulin conform with published data [20,21,30]. Cox et al. [20] showed that the activation of $(Ca^{2+} + Mg^{2+})$-ATPase by calmodulin is due to the active species $CaMCa_3$ and $CaMCa_4$. This activation is exhibited by an increase of V_{max} and a decrease of K_m. There is no variation of K_m in the presence of both copper and calmodulin. This result is consistent with the fact that copper does not modify the concentration of active species of calmodulin, thus suggesting that there is no modification of calcium fixation on calmodulin sites.

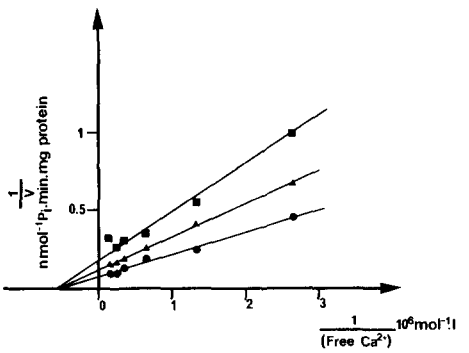


Fig. 2. Double-reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase kinetics on A membranes as a function of free calcium in the presence of calmodulin. The concentration of calmodulin was 40 nM. $(Mg\text{-}ATP)^{2-}$ was maintained constant at 1.79 mM. The concentrations of free calcium were 6.10, 4.55, 3.01, 1.50, 0.75 and 0.37 μM. ●, Without copper; ▲, 0.200 μM copper; ■, 0.400 μM copper.

On B membranes (Fig. 3), copper exerts a noncompetitive inhibition. When calmodulin is fixed on the ATPase before the action of copper, the inhibition is similar to the preceding one obtained with A membranes and added calmodulin.

It may be concluded from these results that copper does not interfere with the fixation of Ca^{2+} either on calmodulin or on the enzyme.

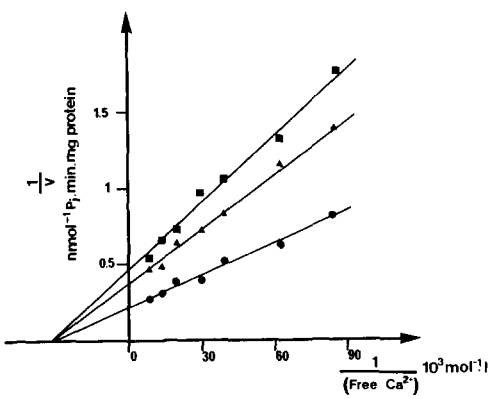


Fig. 1. Double-reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase kinetics on A membranes as a function of free calcium in the absence of calmodulin. $(Mg\text{-}ATP)^{2-}$ was maintained constant at 1.79 mM. The concentrations of free calcium were: 122.3, 75.0, 53.6, 33.9, 24.7, 16.0 and 11.8 μM. ●, Without copper; ▲, 0.125 μM copper; ■, 0.250 μM copper.

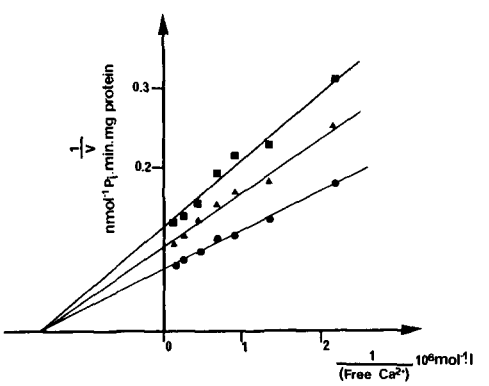


Fig. 3. Double-reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase kinetics on B membranes as a function of free calcium. $(Mg\text{-}ATP)^{2-}$ was maintained constant at 1.79 mM. The concentrations of free calcium were 7.80, 4.60, 2.30, 1.52, 1.14, 0.76 and 0.45 μM. ●, Without copper; ▲, 0.200 μM copper; ■, 0.400 μM copper.

Effect of copper as a function of calmodulin concentrations

Using the already proposed scheme, Jarrett and Kyte [19] and Cox et al. [20] have demonstrated the following relation between the initial rate and the active species of calmodulin:

$$V_i = \frac{V_{max}(CaM^*Ca_n)}{K_{app} + (CaM^*Ca_n)}$$

in which $V_i = V_a - V_0$

and

$$K_{app} = K_a^c \frac{K_c + (Ca^{2+})}{K_c^a + (Ca^{2+})}$$

K_{app} may be easily determined by kinetic studies and K_a^c deducted from the second equation, knowing the values of K_c and K_c^a.

The results of these kinetics are given in Fig. 4. the noncompetitive inhibition demonstrates that K_{app} is not affected and remains constant at 4 nM. It thus results that K_a^c does not vary under the action of copper. Taking the values of $K_c = 30$ μM and $K_c^a = 1.7$ μM found using the conditions described by Cox et al. [20], we obtain $K_a^c = 0.8 \cdot 10^{-3}$ μM, a value which agrees with that reported by these authors. This result argues in the favour of copper not modifying the fixation of CaM^*Ca_n on $(Ca^{2+} + Mg^{2+})$-ATPase.

Copper inhibition as a function of $(Mg\text{-}ATP)^{2-}$

This study was performed in order to investigate and discriminate between the inhibitory effect of $(Cu\text{-}ATP)^{2-}$ and of Cu^{2+} vs. $(Mg\text{-}ATP)^{2-}$.

Action of $(Cu\text{-}ATP)^{2-}$

The results of the kinetics are shown in Fig. 5. The inhibition is competitive and the apparent K_i is 2.8 μM. This inhibition could plausibly result from the conformational similarity of $(Cu\text{-}ATP)^{2-}$ and of $(Mg\text{-}ATP)^{2-}$.

Action of free copper

The results of the kinetics are shown in Fig. 6. The inhibition is noncharacteristic and therefore cannot be related to the effect of free copper. It should be noted that the decrease of this inhibition follows the decrease in the amounts of $(Cu\text{-}ATP)^{2-}$ as evident for the lower values of $(Mg\text{-}ATP)^{2-}$. This fact is also in favour of $(Cu\text{-}ATP)^{2-}$ as inhibitor.

In the two preceding experiments, the apparent K_m for $(Mg\text{-}ATP)^{2-}$ is, respectively, 310 and 210 μM, thus, in good agreement with the values re-

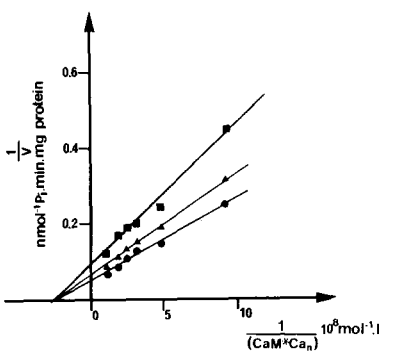


Fig. 4. Double-reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase kinetics on A membranes as a function of $(CaMCa_3 + CaMCa_4)$ concentrations. The concentration of free calcium was 6.1 μM. $(Mg\text{-}ATP)^{2-}$ was maintained constant at 1.79 mM. Calmodulin was varied to obtain the following concentrations of active species $(CaMCa_3 + CaMCa_4)$: 8.4, 5.4, 4.2, 3.2, 2.1 and 1.1 nM. ●, Without copper; ▲, 0.100 μM copper; ■, 0.200 μM copper.

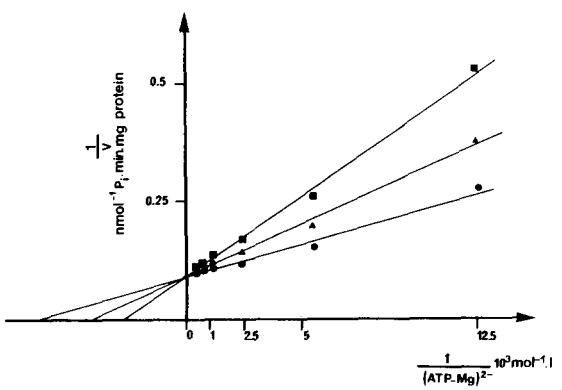


Fig. 5. Double-reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase kinetics on A membranes as a function of $(Mg\text{-}ATP)^{2-}$ concentrations with constant $(Cu\text{-}ATP)^{2-}$. Calmodulin concentration: 40 nM. Free calcium was maintained at 6.2 μM. $(Mg\text{-}ATP)^{2+}$ concentrations: 1.79, 1.34, 0.89, 0.45, 0.22, 0.11 and 0.07 mM. ●, Without $(Cu\text{-}ATP)^{2-}$; ▲, 2.49 μM $(Cu\text{-}ATP)^{2-}$; ■, 4.98 μM $(Cu\text{-}ATP)^{2-}$. Maximal levels of free copper: 11.5 and 23.0 nM, respectively, with 2.49 and 4.98 μM of $(Cu\text{-}ATP)^{2-}$.

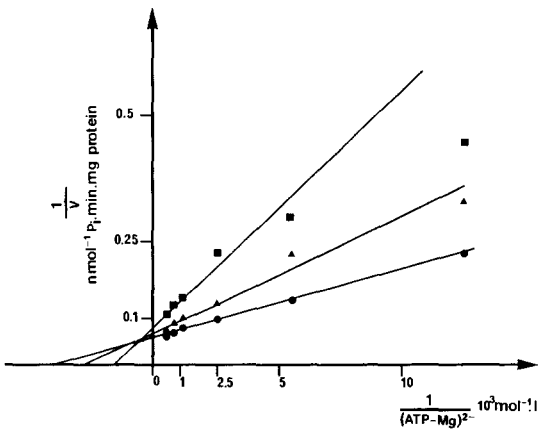


Fig. 6. Double-reciprocal plots of $(Ca^{2+} + Mg^{2+})$-ATPase kinetics on A membranes as a function of $(Mg\text{-}ATP)^{2-}$ concentrations with constant free copper. Calmodulin concentration: 40 nM. Free calcium was maintained constant at 6.2 μM. $(Mg\text{-}ATP)^{2-}$ concentrations: 1.79, 1.32, 0.86, 0.40, 0.18 and 0.08 mM. ●, Without free copper; ▲, 23 nM free copper; ■, 46 nM free copper. Concentrations of $(Cu\text{-}ATP)^{2-}$: 4.98, 4.33, 3.37, 2.32 and 1.57 μM (with 23 nM of free copper) and 9.95, 8.95, 6.75, 4.65 and 3.15 μM (with 46 nM of free copper).

ported by Muallem and Karlish [31].

Finally, our results show that excess copper complexed as $(Cu\text{-}ATP)^{2-}$ exerts a competitive inhibition against $(Mg\text{-}ATP)^{2-}$. The K_i of 2.8 μM shows the high sensitivity of $(Ca^{2+} + Mg^{2+})$-ATPase to this copper complex. If one considers the normal concentration of ATP [32] in human erythrocytes, together with the high affinity of ATP for Cu^{2+} and the pathological levels of copper actually known [1,6], it may well be predicted that concentrations of $(Cu\text{-}ATP)^{2-}$ above 2.8 μM should be associated with excess copper. Such a situation should normally results in $(Ca^{2+} + Mg^{2+})$-ATPase inhibition. It is known that impairment of $(Ca^{2+} + Mg^{2+})$-ATPase increases intracellular calcium which in turn initiates the following events: activation of a transglutaminase [33], activation of a proteinase [34] and activation of a phosphatidylinositol phosphodiesterase [35], facts which have been described at the origin of erythrocyte membrane alterations. Copper could then initiate erythrocyte lysis through such sequences. The high sensitivity of erythrocyte $(Ca^{2+} + Mg^{2+})$-ATPase to copper is consistent with this enzyme being a preferential factor in accelerated red-cell destruction associated with high copper levels.

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