Biochimica et Biophysica Acta, 443 (1976) 206–218

BBA 77393

Ca^{2+} ACTIVATION OF MEMBRANE-BOUND (Ca^{2+}+Mg^{2+})-DEPENDENT ATPase FROM HUMAN ERYTHROCYTES PREPARED IN THE PRESENCE OR ABSENCE OF Ca^{2+}

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(Received December 5th, 1975)

SUMMARY

The kinetics of Ca^{2+} activation of membrane-bound (Ca^{2+}+Mg^{2+})-dependent ATPase (ATP phosphohydrolase EC 3.6.1.3) from human erythrocytes was studied.

The ATPase from membrane prepared in the presence of 0.7–500 μM Ca^{2+} showed positively cooperative behaviour and a K_m for Ca^{2+} of between 1 and 4 μM. If the membranes were prepared in the absence of Ca^{2+} the K_m increased, and an enzyme model with at least four calcium-binding sites accounted for the kinetic change assuming that one calcium-binding site decreased its affinity.

Mg^{2+} or Mg-ATP could not replace Ca^{2+}.

Continuous-flow centrifugation involving a shear stress on membranes was necessary to obtain the high affinity ATPase activity. Using ordinary centrifugation the Ca^{2+}-prepared membranes behaved as membranes prepared in the absence of Ca^{2+}.

The Ca^{2+}-stimulated ATPase from membranes prepared without Ca^{2+} showed reduced maximum activity, but dialyzed, membrane-free hemolysates, whether prepared with Ca^{2+} present or not, recovered the activity when the hemolysate was present during the ATPase assay.

It is suggested that the different Ca^{2+}-affinities of the Ca^{2+}-stimulated ATPase correspond to two different states of the calcium-pump.

INTRODUCTION

Erythrocyte ghosts prepared in the presence of divalent metal ions show reduced (Ca^{2+}+Mg^{2+})-dependent ATPase activity compared to ghosts prepared in the presence of metal chelating agents [1]. However, when the ATPase sites are made accessible, ghosts prepared in the presence of Ca^{2+} show higher (Ca^{2+}+Mg^{2+})-dependent ATPase activity than ghosts prepared with chelating agents [2, 3]. Furthermore, the ATPase of the former ghosts is characterized by a low Michaelis constant for Ca^{2+} whereas ghost ATPase prepared in the absence of Ca^{2+} shows diverging kinetics apparently with two different affinities for Ca^{2+} [2, 4]. Recently, Quist and

Roufogalis [5] reported that a high-affinity and a low-affinity ($Ca^{2+}+Mg^{2+}$)-dependent ATPase occurred simultaneously in all their membrane preparations whereas previous investigations demonstrated the existence of preparations with only high-affinity ATPase [2, 4, 6].

In the present work properties of ghosts prepared in the presence of Ca^{2+}, Mg^{2+}, ATP, and in the absence of these ATPase effectors have been further investigated, especially in relation to the accessibility of ($Ca^{2+}+Mg^{2+}$)-dependent ATPase and the differences of enzyme kinetics between membrane preparations hemolyzed in the presence of Ca^{2+} and in the absence of Ca^{2+}, respectively.

METHODS

Preparation of erythrocyte membranes

The membranes were prepared as described previously [2] with the following modifications. Erythrocytes were sedimented from recently outdated bank blood (63 ml citrate phosphate dextrose+450 ml blood) by centrifugation for 10 min at $2000 \times g$, and the plasma and buffy coat were removed by aspiration. The erythrocytes were washed three times at room temperature in 310 mOsm sodium phosphate buffer, pH 8.0 (cf. Dodge et al. ref. 7). Each blood specimen was made up of the washed erythrocytes from two bags of bank blood.

EGTA-hemolyzed membranes. The erythrocytes were hemolyzed at 8 °C in 9 vol. of a buffer containing 6.7 mM sodium phosphate and 1.0 mM ethyleneglycol bis(β-aminoethylether)-*N,N'*-tetraacetic acid (EGTA, Sigma), pH 7.4 (approx. 25 mOsm) and stored overnight. Next day the ghosts were collected by centrifugation (see below) and washed twice in 10 vol. of 10 mM Tris · HCl (pH 7.7 at 22 °C). The temperature during preparation varied between 4 and 8 °C. The final membrane suspension contained 10–20 g dry membrane per l suspension and was stored at −25 °C.

Ca^{2+}-hemolyzed membranes. In these preparations 1.5 mM $CaCl_2$ was added to the hemolyzing buffer. This resulted in the presence of Ca^{2+} during the whole preparation which was performed as above.

Centrifugation of membrane suspensions.

The centrifugations were performed in a refrigerated Sorvall RC2-B centrifuge at 20 000 rev./min in two different ways. I. In capped polycarbonate tubes for 10 min. II. In a Szent-Györgyi-Blum continuous-flow device with a flow of about 50 ml/min. The last centrifugation involves a shear stress on the membranes when the suspension passes the border between the stationary and the rotating parts of the flow system [8]. Centrifugation of type II was used throughout the investigation, unless otherwise stated.

Determination of ATPase activity

The Mg^{2+}-dependent activity was assayed [2] by measuring P_i liberated at 37 °C in a basal medium of 3 mM Tris · ATP, 4 mM $MgCl_2$, 1 mM EGTA, 70 mM Tris · HCl, and 0.5–1.0 g dry membrane per l medium, pH 7.25. The enzyme reaction was stopped by $HClO_4$ and P_i was measured by an isobutanol extraction method [9]. Tris · ATP was prepared by cation-exchange of $Na_2ATP \cdot 3\,H_2O$ (Sigma) and sub-

sequent elution with 0.1 M Tris. The Ca^{2+}-stimulated activity was determined as the difference between the activity measured in the basal medium supplemented with $CaCl_2$, i.e. ($Ca^{2+}+Mg^{2+}$)-dependent activity, and the Mg^{2+}-dependent activity. The unit of ATPase activity is $\mu mol \cdot min^{-1}$ per g, dry membrane exclusive of hemoglobin. Protein constituted 51.1±0.4 % (S.E., 49 preparations) of the dry membrane (exclusive of hemoglobin). Max. 10 % of ATP was hydrolyzed and velocity of hydrolysis was constant during the enzyme reaction.

Ca^{2+} concentrations in solutions containing $CaCl_2$+EGTA

The Ca^{2+} concentrations were measured by a calcium ion selectrode (Radiometer F 2112 Ca). The selectrode was calibrated at 8, 20, and 37 °C with various calcium buffers at an ionic strength of 0.1 M according to Růžička et al. [10] and with modifications of these buffers adjusted to $I = 0.03$ M. The calibration curves (mV vs. pCa) were straight lines when the Ca^{2+} concentration varied between $10^{-6.6}$ and $10^{-2.0}$ M.

Methods of analysis

The determination of pH, dry matter, protein, hemoglobin, ATP, and calcium (atomic absorption) were performed as previously described [2, 8].

RESULTS

Kinetics of the Ca^{2+} activation of Ca^{2+}-stimulated ATPase

Ca^{2+}-hemolyzed membranes. It was shown previously [2] that a Lineweaver-Burk plot of the Ca^{2+}-stimulated ATPase activity vs. the Ca^{2+} concentration during the ATPase reaction could be fitted by a linear regression if the erythrocyte membranes were prepared in the presence of Ca^{2+}. However, including ATPase determinations at Ca^{2+} concentrations below 2 μM the enzyme of Ca^{2+}-hemolyzed membranes showed positively cooperative behaviour as indicated by a curved Lineweaver-Burk plot concave up (Fig. 1, curve B). This type of kinetics imply the existence of at least two calcium-binding sites and will be referred to as type B.

EGTA-hemolyzed membranes. The Ca^{2+}-stimulated ATPase of EGTA-hemolyzed membranes showed also positively cooperative behaviour at low Ca^{2+} concentrations. In addition, another curvature could be detected (Fig. 1, curve A). This curvature which is concave down was demonstrated previously [2] and corresponds to the plateau of the saturation curve A in Fig. 2 at Ca^{2+} concentrations between 10 and 100 μM. A similar plateau has been described by Schatzmann and Rossi [11] investigating Ca^{2+}-stimulated ATPase from erythrocyte membranes prepared in the presence of EDTA. Sigmoid Lineweaver-Burk plots as curve A in Fig. 1, were obtained in all our experiments (about 15, not shown) which include EGTA-hemolyzed membranes.

Teipel and Koshland [18] showed that an intermediary plateau in saturation curves appears when the affinity for substrate first decreases, then increases again with increasing substrate concentration. In the case of EGTA-hemolyzed membranes such two shifts of affinity imply at least three calcium-binding sites but the existence of one more site must be assumed to explain the positive cooperativity at the lowest Ca^{2+} concentrations. This type of kinetics which imply the existence of at least four calcium-binding sites will be referred to as type A.

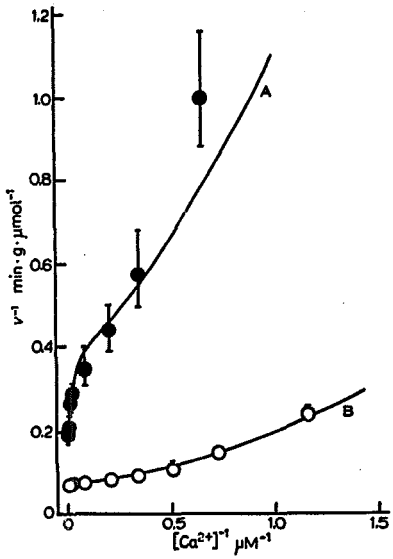


Fig. 1. Lineweaver-Burk plot of Ca^{2+}-stimulated ATPase activity (v) vs. Ca^{2+} concentration. Three experiments with different blood specimens. ATPase medium, 3 mM Tris · ATP, 4 mM $MgCl_2$, 1 mM EGTA, 70 mM Tris · HCl, 0.5–1.0 g dry membrane per 1 medium, various concentrations of $CaCl_2$, pH 7.25. ●, EGTA-hemolyzed membranes. Curve A depicts the reciprocal of v calculated from Eqn. 2 (see text) with the stability constants $K_1 = K_2 = K_4 = 0.6$, $K_3 = 4 \cdot 10^{-4}$ μM^{-1}, $V = 5.40$ μmol · min^{-1} · g^{-1}. ○, Ca^{2+}-hemolyzed membranes. Curve B depicts the reciprocal of v calculated from the constants $K_1 = K_2 = K_3 = 0.85$, $K_4 = 0.25$ μM^{-1}, $V = 14.25$ μmol · min^{-1} · g^{-1}. Vertical bars, ±S.E.

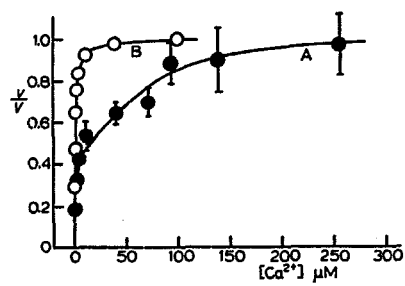
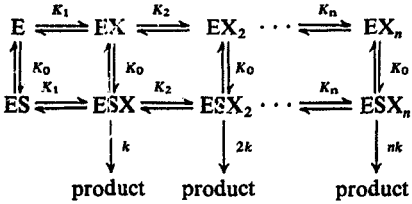


Fig. 2. Saturation curve of the experiments from Fig. 1. v/V vs. Ca^{2+} concentration. Curves A and B depict v/V calculated as in Fig. 1.

Enzyme model. The used enzyme model with n calcium-binding sites is shown in Eqn. 1:



(1)

E, S, and X refer to the enzyme, the substrate (Mg-ATP), and the activator (Ca^{2+}), respectively. K_1–K_n and K_0 are stability constants, e.g. $K_n = [EX_n]/[X] \cdot [EX_{n-1}] = [ESX_n]/[X] \cdot [ESX_{n-1}]$ and $K_0 = [ES]/[E] \cdot [S]$. The brackets refer to concentrations, ES, EX_n, and ESX_n refer to complexes between enzyme, substrate, and activator. For simplicity it is assumed that only one mol substrate is bound per mol enzyme but this is not necessarily true. According to Schatzmann [13] the activator: substrate ratio is one rather than any higher value. However, this is a minor point, as the model implies that substrate and activator bind to the enzyme independently of each other (cf. Wolf ref. 6). The rate constants $k, 2k, \cdots, nk$ are proportional to the number of mol X bound per mol enzyme. If it is assumed that the rates of equilibration between enzyme and substrate or activator are rapid relative to the rate of catalysis, the following expression can be derived (cf. Teipel and Koshland ref. 12):

$$v = \frac{V}{n} \frac{K_1[X] + 2K_1K_2[X]^2 + \cdots + nK_1K_2 \cdots K_n[X]^n}{1 + K_1[X] + K_1K_2[X]^2 + \cdots + K_1K_2 \cdots K_n[X]^n} \tag{2}$$

v is the initial velocity of the enzyme reaction, and V is maximum velocity at a given substrate concentration. If one mol S is bound per mol E and E_t refers to the total enzyme concentration, V is

$$V = \frac{nkE_tK_0[S]}{1 + K_0[S]} \tag{3}$$

Curve fitting. In the present work the kinetic data of the Ca^{2+}-stimulated ATPase have been fitted by the most simple enzyme model which is able to fit the results from both EGTA-hemolyzed and Ca^{2+}-hemolyzed membranes, i.e. the model with $n = 4$. The curve fitting was performed by choosing appropriate values of stability constants, calculating v/V from Eqn. 2, and minimizing the sum of squares of the deviations between observed and calculated values by changing the constants.

Figs. 1 and 2 show that the two calculated v-curves fit the data within the standard error of the means (a single point fits within two times S.E.). It is noticed that the four stability constants used for the calculation of curve B are all of the same magnitude. K_4 has to be 3–4 times smaller than the other constants to fit the points around a Ca^{2+} concentration of 10 μM. Three of the constants used for curve A are of the same magnitude as the constants of type B, whereas the magnitude of K_3 has to be much lower to fit the points along the plateau of Fig. 2. V is determined from Lineweaver-Burk plots as the interception on the vertical axis obtained by extra-

polation. Fig. 1 confirms that V of EGTA-hemolyzed membranes is significantly lower than V of Ca^{2+}-hemolyzed membranes [2]. As shown previously [2] the ATPase from Ca^{2+}-hemolyzed membranes was inhibited by Ca^{2+} concentrations above 100 μM (not shown in Figs. 1–5). The enzyme model does not account for this phenomenon.

However, the enzyme model with $n = 5$ and $n = 6$ fitted the data just as well as the 4-site model, and in the case of Ca^{2+}-hemolyzed membranes even a 2-site model fitted the results. It is therefore not possible to conclude anything about the exact number of calcium-binding sites except that there is more than one site per mol of enzyme and that it is necessary to assume the existence of at least four sites to explain the results obtained with EGTA-hemolyzed membranes. The use of the 6-site model made the determination of the stability constants rather uncertain because identical saturation curves were obtained whether the low calcium-affinity was attributed to one or to two sites.

Accessibility of membrane-bound ATPase

The type of centrifugation influenced both the maximum activity (V) and the kinetics of membrane-bound Ca^{2+}-stimulated ATPase (Fig. 3). Centrifuging Ca^{2+}-

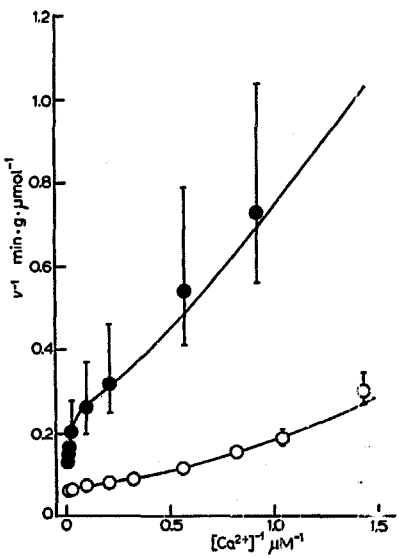


Fig. 3. Lineweaver-Burk plot of Ca^{2+}-stimulated ATPase activity (v) vs. Ca^{2+} concentration in Ca^{2+}-hemolyzed membranes prepared by two types of centrifugation. Five experiments with different blood specimens. ATPase medium as in Fig. 1. ●, centrifugation in capped tubes. Curve, reciprocal of v calculated from Eqn. 2 with the constants $K_1 = K_2 = K_4 = 0.6$, $K_3 = 3 \cdot 10^{-4}\ \mu M^{-1}$, $V = 8.0$ μmol · min^{-1} · g^{-1}. ○, centrifugation in continuous-flow system. Curve, reciprocal of v calculated from the constants $K_1 = K_2 = K_3 = 0.85$, $K_4 = 0.15\ \mu M^{-1}$, $V = 16.0$ μmol · min^{-1} · g^{-1}. Vertical bars, ±S.E.

TABLE I

Ca^{2+}-STIMULATED ATPase ACTIVITY AND GLYCERALDEHYDE 3-PHOSPHATE DEHYDROGENASE ACCESSIBILITY IN RELATION TO TYPE OF CENTRIFUGATION DURING MEMBRANE PREPARATION

ATPase was determined as in Fig. 1. Glyceraldehyde 3-phosphate dehydrogenase was determined according to Steck and Kant [14] and the highest activity in each experiment was fixed at 100. Three and six experiments with capped tubes and continuous flow system, respectively, with different blood specimens. Mean ±S.E.

	EGTA-hemolyzed membranes		Ca^{2+}-hemolyzed membranes	
	Capped tubes	Continuous flow	Capped tubes	Continuous flow
Ca^{2+}-stimulated ATPase $\mu mol \cdot min^{-1} \cdot g^{-1}$	7.7±1.9	8.1±0.9	8.9±3.2	16.5±0.8
Glyceraldehyde 3-phosphate dehydrogenase				
−Triton X-100	81 ±3	91 ±4	58 ±6	63 ±6
+Triton X-100	75 ±9	100	68 ±6	68 ±5
% Accessible	108	91	85	93

hemolyzed membranes in capped tubes during the membrane preparation, the Ca^{2+}-stimulated ATPase showed a reduced V and enzyme kinetics quite similar to the kinetics of EGTA-hemolyzed membranes prepared by centrifugation in the continuous-flow system (Fig. 1 curve A). The ATPase kinetics (not shown) and V (Table I) of the EGTA-hemolyzed membranes were not changed by centrifugation. Preliminary experiments showed that treatment with an Ultra-Turrax homogenisator (20 000 rev./min for 30 s) of Ca^{2+}-hemolyzed membranes prepared in capped centrifuge tubes increased the maximum ATPase activity and changed the enzyme kinetics from type A to B.

It has been suggested that the accessibility of the erythrocyte ATPase is reduced by the presence of divalent cations during hemolysis [1, 2]. The effect of the con-

TABLE II

THE EFFECT OF BUFFER SHIFT DURING PREPARATION AND FREEZE-THAW TREATMENT OF MEMBRANES ON THE (Ca^{2+}+Mg^{2+})-DEPENDENT ATPase ACTIVITY.

The activities include the Mg^{2+}-dependent ATPase activity. Treatments: no buffer shift, hemolysis succeeded by two washings in 6.7 mM sodium phosphate (pH 7.4). Two experiments. Buffer shift, hemolysis succeeded by two washings in 10 mM Tris · HCl (pH 7.7 at 22 °C). Three experiments. Freeze-thaw treatment: membrane suspensions stored at −25 °C for 20 h. All the preparations were centrifuged in the continuous-flow system. The figures represent maximum activities (V). S.D. 2.61 calculated by Bartlett's test.

Membranes	No buffer shift		Buffer shift	
	No freezing	Freezing	No freezing	Freezing
EGTA-hemolyzed $\mu mol \cdot min^{-1} \cdot g^{-1}$	7.7	9.7	8.0	7.6
Ca^{2+}-hemolyzed $\mu mol \cdot min^{-1} \cdot g^{-1}$	7.4	15.8	17.3	16.5

tinuous-flow centrifugation or the Ultra-Turrax treatment might be to establish the accessibility of ATPase in the Ca^{2+}-hemolyzed membranes. However, the type of centrifugation did not influence the accessibility of glyceraldehyde 3-phosphate dehydrogenase (Table I) which serves as a marker of the inner surface of the erythrocyte according to Steck and Kant [14]. Concerning the effectors of this enzyme the four types of membranes are all permeable. The assumed variations of ATPase accessibilities could therefore not be due to unspecific variations in membrane permeability. The difference of dehydrogenase activity between EGTA-hemolyzed and Ca^{2+}-hemolyzed membranes (Table I) might be due to differences in extraction of the enzyme during the membrane preparation (cf. Shin and Carraway ref. 15).

However, the effect of continuous-flow centrifugation on the maximum ATPase activity (V) of Ca^{2+}-hemolyzed membranes was only found in preparations which include either freeze-thaw treatment or a shift from P_i to Tris washing buffer (Table II). The freeze-thaw treatment is a well-known means to activate latent ATPase activity

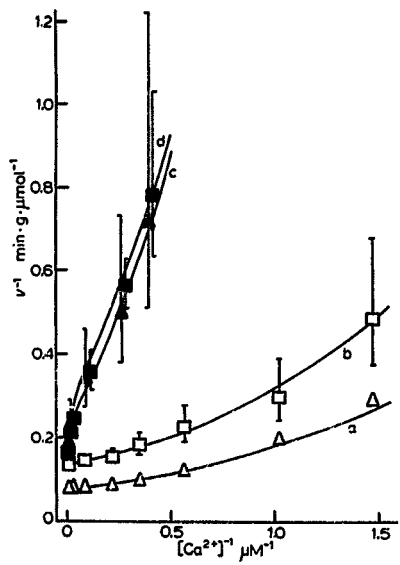


Fig. 4. Lineweaver-Burk plot of Ca^{2+}-stimulated ATPase activity (v) vs. Ca^{2+} concentration during ATPase assay in membranes prepared as EGTA-hemolyzed membranes (cf. Methods) but including various additions during hemolysis. The Ca^{2+} concentrations were measured in the hemolysates (mean ±S.E.). ATPase medium as in Fig. 1. Additions: ■, 1.5 mM Mg^{2+}. $V = 6.4$ μmol · min^{-1} · g^{-1}. Two experiments with different blood specimens. ▲, 1.5 mM Mg^{2+} + 1.5 mM Mg-ATP. $V = 8.0$ μmol · min^{-1} · g^{-1}. The loss of ATP during storage overnight was insignificant. Same experiments as previous addition. □, 0.7±0.1 μM Ca^{2+}. $V = 7.6$ μmol · min^{-1} · g^{-1}. Three experiments with the same blood specimens as in Fig. 1. △, 24±7 μM Ca^{2+}. $V = 13.7$ μmol · min^{-1} · g^{-1}. Same experiments as previous addition. The curves depict reciprocal of v calculated from Eqn. 2 with the constants (expressed as μM^{-1}): a and b, $K_1 = K_2 = K_3 = 1.0$, $K_4 = 0.25$. c, $K_1 = K_2 = K_4 = 0.25$, $K_3 = 4 \cdot 10^{-4}$. d, $K_1 = K_2 = K_4 = 0.3$, $K_3 = 6 \cdot 10^{-4}$. Vertical bars, ±S.E.

[16, 17]. The effect of buffer shift is probably to make the membranes permeable, just as a shift from P_i to Tris buffer makes the erythrocyte membranes permeable to ferritin in the presence of Ca^{2+} [18]. As mentioned in Methods all preparations in this investigation included both buffer-shift and freezing-thawing (except in Table II). It might be pointed out that these treatments could not replace the continuous-flow centrifugation (Fig. 3).

Presence of Ca^{2+}, Mg^{2+}, and ATP in addition to EGTA during hemolysis

Fig. 4 shows no effect of the presence of Mg^{2+} and ATP during hemolysis (cf. Fig. 1, curve A) on the maximum activity and the enzyme kinetics of membrane-bound Ca^{2+}-stimulated ATPase. However, the presence of about 25 μM Ca^{2+} during hemolysis was sufficient to obtain high V and enzyme kinetics of type B (Fig. 4). The presence of 0.7 μM Ca^{2+} during hemolysis was sufficient to produce enzyme kinetics of type B but V was strongly reduced (Fig. 4). The differences between the kinetics of membranes hemolyzed in the presence of 0.7 μM Ca^{2+} and in the presence of 1.5 mM Mg^{2+}+1.5 mM Mg-ATP, respectively, is remarkable, in as much as the maximum activities are equal. In addition, the kinetic differences are obvious from the Hill plot (Fig. 5) which shows a maximum slope (n_H) of 1.5 in the membrane preparations prepared in the presence of 0.7–500 μM Ca^{2+}, whereas n_H does not exceed 1.0 in membranes prepared in the absence of Ca^{2+}. In two other experimental series including Ca^{2+}-hemolyzed membranes (not shown) n_H was 2.2 and 2.3, respectively. These values of n_H do not conflict with a multisite enzyme model.

The reduction of the maximum ATPase activity of membranes hemolyzed in the absence of Ca^{2+} may be due to a solubilization of the Ca^{2+}-stimulated ATPase as reported by Weidekamm and Brdiczka [19], incubating erythrocyte membranes in the presence of EDTA and Mg-ATP. In addition, by the absence of Ca^{2+} the kinetics of the residual membrane-bound ATPase changed from type B to A in contrast to the solubilized ATPase which showed Michaelis-Menten kinetics [19]. The need of

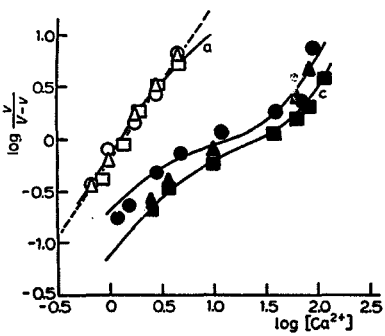


Fig. 5. Hill plot of the experiments from Fig. 1 and 4. Identical symbols. In Ca^{2+}-hemolyzed membranes from Fig. 1 the Ca^{2+} concentration during hemolysis was 500 μM. The curves depict log ($v/V-v$)) calculated from Eqn. 2 with the constants (expressed as μM^{-1}): a, $K_1 = K_2 = K_3 = 1.0$, $K_4 = 0.25$. c, $K_1 = K_2 = K_4 = 0.25$, $K_3 = 4 \cdot 10^{-4}$. e, $K_1 = K_2 = K_4 = 0.6$, $K_3 = 4 \cdot 10^{-4}$. Dotted line, regression line with a slope of 1.5 based on the preparations hemolyzed in the presence of Ca^{2+} (0.7–500 μM).

25 μM Ca^{2+} to prevent reduction of V but only 0.7 μM Ca^{2+} to prevent the change of kinetics suggests that the kinetics of membrane-bound ATPase is determined by calcium binding to a site with higher affinity than the site(s) responsible for the maintenance of high V.

Changing the properties of EGTA-hemolyzed membranes

The addition of Mg^{2+} and ATP during the first Tris-washing of EGTA-hemolyzed membranes showed no significant effect on Ca^{2+}-stimulated ATPase (Table III) in accordance with the lacking effect of Mg^{2+} and ATP during hemolysis (Fig. 4). However, all the additions including Ca^{2+} lead to membrane preparations which showed ATPase kinetics of type B in spite of the hemolysis in the absence of Ca^{2+} (Table III). The maximum ATPase activity (V) of EGTA-hemolyzed membranes increased significantly only when Ca^{2+} was added in the presence of Mg^{2+}, ATP, or high concentrations of hemolysate (Table III), but regarding V the addition of Ca^{2+} to membranes stored overnight in the absence of Ca^{2+} (EGTA-hemolyzed membranes) could never replace the addition of Ca^{2+} during the hemolysis (Ca^{2+}-hemolyzed membranes).

However, the presence of dialyzed, membrane-free hemolysate during the ATPase assay increased the maximum activity of membranes hemolyzed in the presence of a calcium chelating agent, as previously demonstrated by Bond and Clough [20], leading to identical values of V in EGTA-hemolyzed and Ca^{2+}-hemolyzed membranes (Table IV). The enzyme kinetics of the EGTA-hemolyzed mem-

TABLE III

EFFECT OF ADDITION OF ATP, Mg^{2+}, AND Ca^{2+} DURING THE PREPARATION OF EGTA-HEMOLYZED MEMBRANES ON Ca^{2+}-STIMULATED ATPase

ATPase medium as in Fig. 1. S.D. 1.66, calculated by Bartlett's test.

Additions	Number of experiments	V μmol · min^{-1} · g^{-1}	Enzyme kinetics
Added during first Tris-washing			
None	8	5.8	A
1.5 mM ATP	2	6.2	A
0.5 mM Mg^{2+}	2	5.9	A
2.0 mM Mg^{2+} + 1.5 mM ATP	3	8.1	A
0.5 mM Ca^{2+}	4	8.1	B
2.0 mM Ca^{2+} + 1.5 mM ATP	2	11.5	B
0.5 mM Ca^{2+} + 1.5 mM Mg^{2+}	2	11.0	B
0.5 mM Ca^{2+} + 1.5 mM Mg^{2+} + 1.5 mM ATP	4	10.6	B
Other additions			
First washing with membrane-free hemolysate including 1.5 mM Ca^{2+}, instead of Tris-washing	2	9.7	B
1.5 mM Ca^{2+} added before first centrifugation to EGTA-hemolysate which was stored overnight	4	11.7	B
Ca^{2+}-hemolyzed membranes	7	14.6	B

TABLE IV

EFFECT OF ADDITION OF DIALYZED, MEMBRANE-FREE HEMOLYSATES ON Ca^{2+}-STIMULATED ATPase DURING ATPase DETERMINATION

ATPase medium as in Fig. 1. In the cases of additions the hemolysate made up 10 % of the medium. The hemolysates were prepared according to Bond and Clough [20]. S.D. 1.67 calculated by Bartlett's test.

Additions	Number of experiments	V μmol · min^{-1} · g^{-1}	Enzyme kinetics
EGTA-hemolyzed membranes			
None	3	6.5	A
Membrane-free EGTA-hemolysate	2	14.3	*
Membrane-free Ca^{2+}-hemolysate	2	15.3	*
Ca^{2+}-hemolyzed membranes			
None	3	14.9	B
Membrane-free EGTA-hemolysate	1	14.4	B
Membrane-free Ca^{2+}-hemolysate	1	15.2	B

* Enzyme kinetics was neither typically A nor B and more experiments are needed for elucidation.

branes activated by hemolysate was neither typically A nor B but seems to be influenced by the hemolysate (not shown). The effect of membrane-free hemolysate was the same whether the hemolysate originated from EGTA-hemolysis or from Ca^{2+}-hemolysis (Table IV). The occurrence of activator [20] in the hemolysate seems therefore to be independent of the kinetic state of membrane-bound ATPase. The hemolysate showed no effect on the ATPase of Ca^{2+}-hemolyzed membranes (Table IV).

DISCUSSION

The preparations of Ca^{2+}-stimulated ATPase showing enzyme kinetics of type A (cf. Results) include erythrocyte membranes prepared in the absence of Ca^{2+} (Figs. 1 and 4) and membranes prepared in the presence of Ca^{2+} using ordinary centrifugation in capped tubes (Fig. 3). ATPase preparations with similar kinetic properties have been described previously [2, 5, 11, 21, 22]. The characteristic features of this kinetics have been ascribed to the presence of two different ATPase activities with high and low calcium-affinity, respectively. Quist and Roufogalis [5] suggested that the high and low affinity originate from two distinct erythrocyte ATPases, whereas Schatzmann [4] considered that the low affinity may represent an artifact. Figs. 1–4 demonstrate that the kinetic behaviour could be explained by the existence of one enzyme with four calcium-binding sites, one site having lower affinity than the others. In the enzyme model with six calcium-binding sites the low affinity might be attributed either to one or to two sites (cf. Results). However, the kinetics could also be due to a mixture of two or more enzymes, or subunits, as emphasized by Teipel and Koshland [12].

The preparations of Ca^{2+}-stimulated ATPase showing enzyme kinetics of type B (Figs. 1–4) include membranes prepared in the presence of Ca^{2+} using continuous-flow centrifugation which involves a shear stress on the membranes. The

B-kinetics implies that the calcium-binding sites all show high affinity leading to K_m for Ca^{2+} varying from 1 to 4 μM in different preparations. This high-affinity Ca^{2+}-stimulated ATPase is membrane-bound and shows positively cooperative behaviour at low Ca^{2+} concentrations (Figs. 1–5), in contrast to the solubilized high-affinity Ca^{2+}-stimulated ATPase (Wolf et al. [23, 24], Weidekamm and Brdiczka [19]) which shows Michaelis-Merten kinetics. In addition, the membrane-bound ATPase is inhibited by high Ca^{2+} concentrations [2] contrary to the solubilized enzyme [19]. However, the solubilized ATPase is probably identical with the membrane-bound enzyme, the properties of which may depend on its location in the membrane.

The Ca^{2+}-stimulated membrane ATPase prepared by Wolf [6] did neither show positively cooperative behaviour nor inhibition by high Ca^{2+} concentrations. However, the specific activity of Wolf's ATPase was equal to that of Weidekamm and Brdiczka's solubilized ATPase, i.e. 4–5 times higher than V found in our preparations, and Wolf's ATPase may therefore be more loosely attached to the membrane than our ATPase.

Referring to the enzyme model the effect of the removal of Ca^{2+} during membrane preparation could be explained by assuming that the affinity of one of the calcium-binding sites is reduced about 10^3 times. The question is whether the observed shift of affinity is an artifact or whether it represents a physiological response. A shift in vivo could be explained in two ways.

Firstly, the ATPase may shift between a low-affinity or resting state characterized by reduced activity of the calcium pump (cf. Schatzmann ref. 4) and a high-affinity or active state with maximum pump activity. In the resting state the intracellular Ca^{2+} concentration may rise to a certain level, e.g. caused by some external stimulus, before the ATPase shifts to the active state and subsequently rapidly re-establishes the low intracellular Ca^{2+} concentration. This shift might be provoked by Ca^{2+}. The whole reaction may represent a brief intracellular Ca^{2+}-signal triggering various cell reactions as suggested previously [8].

Secondly, the shift of affinity may correspond to the phenomenon emphasized by Schatzmann [13] that the calcium pump must reduce its affinity for Ca^{2+} during the outward transport through the membrane at least by a factor of 700. However, this shift could hardly be controlled by low Ca^{2+} concentrations.

In the membrane preparations the shift of affinity depends not only on Ca^{2+} but also on type of centrifugation (Fig. 3), i.e. Ca^{2+} and continuous-flow centrifugation alike are necessary during preparation to obtain the high-affinity state. The effect of centrifugation suggests that the shift of affinity is related to the concept of accessibility. It has been proposed that EDTA reduces the accessibility of the ATPase sites in erythrocytes because Ca^{2+} enhanced the sensitivity of $(Ca^{2+}+Mg^{2+})$-dependent ATPase to heat, trypsin and *N*-ethylmaleimide in EDTA-hemolyzed membranes [25], and because EDTA reduced the sensitivity of ATPase to dinitrophenol stimulation [26]. If the ATPase is in the low-affinity state in both EGTA-hemolyzed and Ca^{2+}-hemolyzed membranes before centrifugation, it is possible that the continuous-flow centrifugation is necessary to make a regulatory ATPase site accessible for Ca^{2+}, resulting in the Ca^{2+}-dependent shift of affinity if Ca^{2+} is present. However, it is difficult to understand the effect of centrifugation in relation to the function of the intact erythrocyte, but the dependence of centrifugation may be caused by the exposure to buffer-solutions of low ionic strength during preparation. Quist and Roufogalis [5]

reported that the low-affinity Ca^{2+}-stimulated ATPase activity was favoured when ghosts were subjected to low ionic strength.

The reduction of V, as demonstrated in EGTA-hemolyzed membranes, may be in significant for the intact erythrocyte, because V was not reduced when the ATPase reacted in the presence of hemolysate (Table IV).

The strong influence of the preparation method indicates that the kinetic data from Ca^{2+}-stimulated ATPase have to be interpreted cautiously. It cannot be concluded whether one or more Ca^{2+}-stimulated ATPases occur in the erythrocyte membrane until the nature of the low-affinity ATPase activity has been clarified. However, it seems certain that Ca^{2+} controls the properties of the membrane-bound Ca^{2+}-stimulated ATPase in vitro, but the results suggest that this ATPase should be studied under more physiological conditions, e.g. concerning ionic strength and presence of cellular constituents, in order to understand the function of the enzyme in vivo, including the role of Ca^{2+}.

ACKNOWLEDGEMENTS

I wish to thank Mrs. Jytte Möller for excellent technical assistance and Dr. Elo H. Hansen for guidance on preparing the calcium buffers.

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