Effects of Ethylenediaminetetra-acetate and Deoxycholate on Kinetic Constants of the Calcium Ion-Dependent Adenosine Triphosphatase of Human Erythrocyte Membranes

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EDTA and deoxycholate have been applied successfully in the isolation and purification of enzymes, in particular of Na^+, K^+ -ATPase,* since these compounds are able to degrade membrane structures (Charnock & Post, 1963; Schoner *et al.*, 1967; Jørgensen, 1968; Jørgensen & Skou, 1969).

However, the application of these agents results in a severe problem: when they are able to degrade a membrane structure, the structure of a membrane-bound enzyme may be altered simultaneously. This may result not simply in an inactivation of an enzyme (e.g. Mg^{2+} -ATPase; Schoner *et al.*, 1967; Jørgensen & Skou, 1969) but rather in an alteration of some, e.g. kinetic, properties.

The present paper describes some results on the side effects of EDTA and deoxycholate on the kinetic properties of Ca^{2+} -ATPase (EC 3.6.1.3), from human erythrocyte membranes, produced when these compounds were used in the purification of this enzyme. The studies were performed to test the suitability of EDTA and deoxycholate as an aid in the purification procedure for Ca^{2+} -ATPase of human erythrocyte membranes in comparison with a method published previously (Wolf, 1970, 1972).

Materials and methods

Chemicals. ATP (disodium salt) was from C. F. Boehringer und Soehne G.m.b.H., Mannheim, German Federal Republic, and E. Merck A.G., Darmstadt, German Federal Republic; *trans*-cyclohexane-1,2-diamine-*NNN'*-tetra-acetate (Chel-CD) was from Fluka A.G. Buchs, Switzerland; N_2 (purified) was from Linde A.G., Mainz-Kostheim, German Federal Republic; all other chemicals were from E. Merck A.G. In all cases the metal ions were used as chlorides. The water was twice redistilled in a quartz apparatus.

Erythrocyte membrane preparations. Preparation procedure E_1 was carried out as follows. All steps were done at 2–4°C. The erythrocytes spun down from 1 litre of human blood (acid–citrate–glucose-stabilized, group O, Rh⁺, 1–2 days old) were resuspended in about 1 litre of a solution containing

(final concentrations) 150 mM-NaCl, 5 mM-KCl and 1 mM-MgCl₂. The cell suspension was centrifuged for 30 min at 5000g, and the supernatant as well as the leucocytes were carefully sucked off and discarded. This washing procedure was repeated twice.

Packed erythrocytes (120 ml) were lysed at pH 7.4–7.5 at 2–4°C in a medium containing (final concentrations) about 14 mM-NaCl, 10 mM-KCl, 20 mM-tris-HCl buffer and 20 mM-sucrose. The final volume was 1200 ml. After sedimentation at 22000g for 30 min (MSE High Speed 18, 6 × 250 ml rotor) the supernatant and the small ‘button’ of unhaemolysed erythrocytes were discarded. During the subsequent washing of the ‘ghosts’ the osmolarity of the original haemolysing medium was lowered by applying a solution containing (final concentrations) 1 mM-NaCl, 1 mM-KCl, 2 mM-tris-HCl buffer and 2 mM-sucrose, pH 7.7. After five or six washings a slightly pink ‘ghost’ suspension was obtained, which was frozen, after addition of the same volume of 0.5 M-sucrose, at –80°C and stored at –20°C.

The haemoglobin content of the ‘ghosts’ was less than 0.1% of that of the untreated erythrocytes [determined by the method of Rimington (1942) as modified by Arnold (1970)].

Protein determinations were carried out by a micro-Kjeldahl method after the removal of the membrane-bound phospholipids with methanol–chloroform (1:1, v/v) (Reed *et al.*, 1960; Dodge *et al.*, 1963).

The following preparation methods are modifications of the procedure for preparation E_1 . In preparation procedure E_2 2 mM-EDTA was present during haemolysis and subsequent three of the five or six washings. In preparation procedure E_3 2 mM-EDTA and 0.06% sodium deoxycholate were present during haemolysis and subsequent three of the five or six washings. In preparation procedure E_4 1 mM-EDTA and 0.2% sodium deoxycholate were present during haemolysis and subsequent three of the five or six washings.

The determination of enzyme activity and the application of Ca^{2+} –EDTA buffer (0.4 mM-EDTA) were performed as described previously (Wolf, 1972).

Results

To demonstrate changes in kinetic properties of the enzyme produced by different purification procedures,

* Abbreviations: Na^+, K^+ -ATPase, Mg^{2+} -ATPase and Ca^{2+} -ATPase, $\text{Na}^+ + \text{K}^+$ -stimulated, Mg^{2+} -dependent and Ca^{2+} -dependent adenosine triphosphatase respectively.

Table 1. Comparison of the kinetic constants of different Ca-ATPase preparations

The K_m values were taken from Lineweaver-Burk plots of $1/v$ versus $1/[MgATP]$ and the K_{Ca} values were taken from a plot of $1/(v_{[Ca^{2+}]} / v_{[Ca^{2+}] = 0} - 1)$ versus $1/[Ca^{2+}]$.

Preparation no.	Additions to haemolysis and three washing solutions	Specific activity (nmol of P_i /min per mg of protein)	K_m (μM)	K_{Ca} (μM)
E ₁		9.1	40	2.9
E ₂	2 mM-EDTA	13.7	36	8.0
E ₃	2 mM-EDTA + 0.06% deoxycholate	16.1	38.5	9.0
E ₄	1 mM-EDTA + 0.2% deoxycholate	2.2	222	No activation by Ca^{2+}
E ₅ *	2 mM- Mg^{2+} + 0.025 mM- Ca^{2+}	146	40	2.3

* Wolf (1970).

each of the preparations E₂–E₄ was compared with the preparation E₁ on the basis of the K_m value and the apparent Ca^{2+} dissociation constant at pH 7.0.

K_m values. The K_m values were determined under the following conditions: $[Na^+]$ 100 mM, $[Mg^{2+}]$ 2 mM, $[Ca^{2+}]$ 0.036 mM (adjusted with 0.4 mM- Ca^{2+} -EDTA buffer), pH 7.0, temperature 30°C. The MgATP concentration was determined by use of $K = 2.15 \times 10^{-4} M$ as the dissociation constant of the complex (Wolf, 1969).

Whereas 2 mM-EDTA or 2 mM-EDTA + 0.06% deoxycholate does not change the K_m value from the value of 40 μM obtained with preparation E₁, 1 mM-EDTA + 0.2% deoxycholate increases it to 310 μM . The K_m value of the preparation described in previous papers (Wolf, 1970, 1972) amounts to 40–50 μM , in good agreement with the value obtained with the untreated enzyme preparation E₁. The K_m values obtained are summarized in Table 1.

Degree of activation by Ca^{2+} and apparent Ca^{2+} dissociation constants. The degree of activation by Ca^{2+} and the apparent Ca^{2+} dissociation constant (Wolf, 1972) were measured under the following conditions: $[Na^+]$ 100 mM, $[Mg^{2+}]$ 2 mM, $[MgATP]$ 1 mM, pH 7.0, temperature 30°C.

To allow direct comparison of the degrees of activation of different enzyme preparations by Ca^{2+} , the relative activity, defined by the ratio $v_{[Ca^{2+}]} / v_{[Ca^{2+}] = 0}$, was plotted versus $[Ca^{2+}]$. As shown in Fig. 1, the affinity between Ca^{2+} and the enzyme is significantly decreased in preparations E₂ and E₃ compared with preparation E₁, whereas the enzyme of preparation E₄ is not any further activated by Ca^{2+} .

According to the results given in Table 1, preparations E₂ and E₃ differ from preparation E₁ with respect to the apparent Ca^{2+} dissociation constant. The value is increased from 2.9 μM to about 8–9 μM . An enzyme preparation described previously (Wolf,

Fig. 1. Dependence on Ca^{2+} concentration of the relative reaction rate $v_{[Ca^{2+}]} / v_{[Ca^{2+}] = 0}$ for Ca^{2+} -ATPase

Conditions were as follows: $[Na^+]$ 100 mM, $[Mg^{2+}]$ 2 mM, $[MgATP]$ 1 mM, pH 7.0. The Ca^{2+} concentration was adjusted with 0.4 mM- Ca^{2+} -EDTA buffer (Wolf, 1972). $\circ$, Preparation E₁; $\square$, preparation E₂; $\triangle$, preparation E₃; ∇ , preparation E₄.

1970) showed a value of $2.3 \mu\text{M}$, which again is in good agreement with the value obtained with preparation E_1 . The values are summarized in Table 1.

Discussion

The results presented here indicate that: (1) the exposure of the Ca^{2+} -ATPase to EDTA or EDTA + deoxycholate results in an irreversible increase of the K_m value (with 1 mM-EDTA + 0.2% deoxycholate) and of the apparent Ca^{2+} dissociation constant; (2) the apparent Ca^{2+} dissociation constant is more sensitive to the action of these agents than is the K_m value; (3) the response of the enzyme to Ca^{2+} is lost completely after treatment with 1 mM-EDTA + 0.2% deoxycholate, although the enzymic activity is not completely lost; (4) particularly, the Ca^{2+} dissociation constant strongly depends on the preparation conditions. This constant is therefore a sensitive indicator for modifications of the Ca^{2+} -ATPase that are produced during the purification procedures.

The application of EDTA and deoxycholate for the purification of Ca^{2+} -ATPase of human erythrocyte membranes at concentrations used for the purification of Na^+, K^+ -ATPase (Schoner *et al.*, 1967; Jørgensen & Skou, 1969) yields some increase in specific activity of the enzyme, when 1 mM-EDTA + 0.06% deoxycholate is applied during haemolysis and the subsequent three washings. However, these compounds are not suitable for the purification of Ca^{2+} -ATPase (in agreement with the results obtained with Mg^{2+} -ATPase; Schoner *et al.*, 1967; Jørgensen & Skou, 1969), since they cause an alteration of the kinetic properties of Ca^{2+} -ATPase even at concentrations at which an increase of the specific activity is obtained.

The decrease of the affinity between Ca^{2+} and the enzyme and the complete loss of response to Ca^{2+} apparently has to be related to the removal of Ca^{2+} from specific Ca^{2+} -binding sites of the membrane-bound Ca^{2+} -ATPase (Wolf, 1972) by EDTA. This interpretation is consistent with considerations mentioned by Tanford (1968), who stated that specific binding of enzymes may disappear when the protein is exposed to unfavourable conditions (i.e. extreme pH values, high temperature, treatment with detergents) in the absence of the specific effector. The result may be an irreversible conformational change, which is designated as a 'partial denaturation'. This conformational change is indeed irreversible in the case of erythrocyte membrane Ca^{2+} -ATPase, as shown by the results given in Fig. 1.

Schatzmann & Rossi (1971) have described a Ca^{2+} -ATPase preparation from human erythrocyte membranes exhibiting a Ca^{2+} dissociation constant of $4.08 \mu\text{M}$, which is in agreement with results reported previously (Wolf, 1970, 1972) and here. They used a

purification procedure proposed by Garrahan *et al.* (1969), which includes the application of 1 mM-EDTA during haemolysis, but not during the washing steps. Apparently the presence of 1 mM-EDTA in only one step of the isolation procedure is not sufficient for the Ca^{2+} concentration to reach the 'critical' value at which the partial denaturation of the enzyme occurs.

EDTA in the absence or in the presence of 0.06% deoxycholate affects the Ca^{2+} -binding site but not the substrate-binding site (the K_m value remains constant). The increase in the concentration of the ionic detergent deoxycholate to about 0.2%, however, causes a change in the K_m value as well as the complete loss of the response to Ca^{2+} , although the enzyme activity is not completely lost. This change in the K_m value indicates that the increased concentration of deoxycholate must cause more severe changes in the membrane architecture than does EDTA with or without low concentrations of deoxycholate.

Since a fraction of the Ca^{2+} cannot be removed from the membrane by EDTA (Durruti-Cubria *et al.*, 1967), it appears reasonable that an incomplete loss of the membrane-bound Ca^{2+} results in a change of the ATPase properties, i.e. the disappearance of the response to Ca^{2+} , but not in a complete loss of activity.

It may be suggested that the phenomenon of increased specific activity combined with altered kinetic properties is not limited to the Ca^{2+} -ATPase of human erythrocytes. At present it seems possible that similar effects could occur in the purification procedures for other enzymes as a result of the application of chelators and detergents, although a significant increase in the specific activity of the respective enzyme is obtained. Certainly, this point of view has to be emphasized in the discussion on the suitability of enzyme purification procedures and the reagents applied to them.

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