Short Communications

Activation of Membrane-Bound High-Affinity Calcium Ion-Sensitive Adenosine Triphosphatase of Human Erythrocytes by Bivalent Metal Ions

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The Ca^{2+} -sensitive ATPase (adenosine triphosphatase) of human erythrocyte membranes is activated, not only by Ca^{2+} ions, but also by a series of other bivalent metal ions including Sr^{2+} , Ba^{2+} , Mn^{2+} , Ni^{2+} , Co^{2+} , Cd^{2+} , Cu^{2+} , Zn^{2+} and Pb^{2+} . The degree of activation is dependent on the radius of the ion rather than on its nature, in contrast with the dissociation constant of the enzyme-metal ion complex.

The high-affinity Ca^{2+} -sensitive ATPase† (ATP phosphohydrolase, EC 3.6.1.3) of erythrocyte membranes has become an enzyme of particular interest, since Schatzmann (1973) could demonstrate that it is responsible for active Ca^{2+} transport outward across the erythrocyte membrane. It thus maintains the steep gradient between the low free Ca^{2+} concentration inside the cell and the high concentration outside.

Besides Ca^{2+} and Mg^{2+} , human erythrocytes contain other bivalent metal ions, often at concentrations considerably higher than the respective concentrations in plasma (Long & Harrison, 1969; Documenta Geigy, 1968). On the other hand, some enzymes [e.g. phosphoglucosutase (Ray, 1969) and alkaline phosphatase (Lazdunski *et al.*, 1969)] are activated, not only by one, but by a series of bivalent metal ions. Therefore we decided to investigate whether any bivalent metal ions other than Ca^{2+} had an effect on the membrane-bound Ca^{2+} -sensitive ATPase. We studied the effects of Sr^{2+} , Ba^{2+} , Mn^{2+} , Ni^{2+} , Co^{2+} , Cu^{2+} , Cd^{2+} , Zn^{2+} , Pb^{2+} and Hg^{2+} on this enzyme. Since most of these ions show an inhibitory effect at moderate and unphysiologically high concentrations on other ATPases (Rifkin, 1965; Voth, 1967), we used metal ion buffers described by Wolf (1973) to adjust very low concentrations of free metal ions. By means of these buffers it was possible to investigate bivalent metal ion concentrations in the range 10^{-14} – 10^{-7} M for heavy-metal ions.

Materials and methods

The enzyme was prepared and the activity measurements were performed essentially as described by Wolf (1972). All metal ions were used as chlorides,

except Cd^{2+} , Zn^{2+} and Hg^{2+} , which were used as acetates. Sucrose and Tween 20 were obtained from Serva, Heidelberg, W. Germany, and all other chemicals were obtained from Merck, Darmstadt, W. Germany. The water was twice-distilled over quartz.

The bivalent metal ion buffers (Wolf, 1973) consisted of 0.4 mM-EDTA and various total concentrations of Mg^{2+} and the metal ion to be buffered.

In all experiments, the concentration of free (non-complexed) Mg^{2+} was maintained at 2 mM, which was necessary for the formation of the active substrate MgATP (cf. Wolf, 1972) and for the proper function of the bivalent metal ion buffers. This could be achieved by calculating the total concentrations of Mg^{2+} and of the metal ion Me^{2+} , which had to be buffered, at given concentrations of free Mg^{2+} (2 mM), free Me^{2+} (variable) and total EDTA (0.4 mM) by means of eqns. (29) and (30) proposed by Wolf (1973). In a similar way, the total concentration of ATP was calculated by using 2 mM for the concentration of free Mg^{2+} , 1 mM for MgATP and 0.215 mM for K_{MgATP} (cf. Wolf, 1972). Finally, the value of the total Mg^{2+} concentration obtained by eqn. (30) had to be corrected for the amount of ATP-bound Mg^{2+} .

Results and discussion

Except for Hg^{2+} , all metal ions under investigation exert a stimulatory effect on the Ca^{2+} -sensitive ATPase of human erythrocyte membranes. As shown in Fig. 1, the degree of maximum activation, which was evaluated in a plot of $1/v - 1/[\text{Me}^{2+}]$ at $1/[\text{Me}^{2+}] = 0$, is dependent on the radius of the unhydrated ion, optimum activation being achieved by Ca^{2+} and Sr^{2+} . Metal ions with larger or smaller ion radii show a decreased potency of activation. The dependence of the relative maximum reaction rate on the ion radius

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† Abbreviation: ATPase, adenosine triphosphatase.

forms a bell-shaped curve for all metal ions except for Cd^{2+} and Hg^{2+} . Cd^{2+} shows a degree of activation that is much lower than would be expected from its ion radius. This decrease might be due to a possible reaction of Cd^{2+} with essential SH groups of the enzyme, thus decreasing its maximum activity. The hypothesis of the existence of essential SH groups is supported by the fact that Hg^{2+} completely inhibits the enzyme, and, further, by the finding that the enzyme is inhibited by a series of SH-group reagents such as 5,5'-dithiobis-(2-nitrobenzoate) and *N*-ethylmaleimide (H. U. Wolf, unpublished work).

The bell-shaped curve of Fig. 1 indicates that the activation potency of any ion (except for Cd^{2+} and Hg^{2+}) is dependent on the ion radius rather than on the nature of the activating ion. Apparently only Ca^{2+} and Sr^{2+} ions, with radii of 0.099 and 0.112 nm (0.99 and 1.12 Å) respectively, are able (by binding to the enzyme) to induce an optimum conformation of the catalytic residues of the enzyme. A deviation from this optimum value (in both directions) leads to a considerable decrease in the catalytic power of the enzyme. Thus the Ca^{2+} -sensitive ATPase of human erythrocytes might be an example of the well-known fact that enzyme catalysis is very sensitive to small shifts in the relative position of catalytic residues (Koshland & Neet, 1968).

There is obviously no correlation between the metal ion-enzyme dissociation constant K_{MeE} and the ion radius (Table 1). However, an interesting feature of the bivalent-metal-ion-binding behaviour can be noticed by comparing the values of K_{MeE} and the respective values of K_{MeEDTA} : the ratio of $K_{\text{MeE}}/K_{\text{MeEDTA}}$ varies (the only exception being Ba^{2+}) within one and a half orders of magnitude, whereas the values of the constants K_{MeE} vary within nine

Fig. 1. Dependence of the activation potency of bivalent metal ions on the ion radius

The activation potency is expressed as $V_{\text{max}}(\text{Me}^{2+})/V_{\text{max}}(\text{Ca}^{2+})$, where $V_{\text{max}}(\text{Me}^{2+})$ is the maximum activation by any bivalent metal ion and $V_{\text{max}}(\text{Ca}^{2+})$ the respective value for Ca^{2+} . The V_{max} values were obtained from plots of $1/v - 1/[\text{Me}^{2+}]$ as ordinate intercepts of the straight lines. The point marked Mg^{2+} represents the reaction rate in the presence of 2mM- Mg^{2+} +0.4mM- MgEDTA . $[\text{Na}^+] = 100\text{mM}$, $[\text{Mg}^{2+}] = 2\text{mM}$, $[\text{MgATP}^{2-}] = 1\text{mM}$, pH = 7.0, temperature = 30°C. Values of ion radii are taken from D'Ans-Lax (1970).

Table 1. Dissociation constants of the bivalent metal ion-enzyme complex

The values were obtained at pH 7.0 and 30°C. $[\text{Na}^+] = 100\text{mM}$, $[\text{Mg}^{2+}] = 2\text{mM}$, $[\text{MgATP}^{2-}] = 1\text{mM}$. Values of the ion radii are taken from D'Ans-Lax (1970). For comparison, the fourth column contains the values of the dissociation constants of the respective MeEDTA complexes as obtained by Hughes & Martell (1953) (Ni^{2+} , Co^{2+} , Cu^{2+} , Zn^{2+} , Cd^{2+} and Pb^{2+}) and by Sarma & Ray (1956) (all other bivalent metal ions).

Bivalent metal ion	Ion radius (nm)	K_{MeE} (M)	K_{MeEDTA} (M)	$\frac{K_{\text{MeE}}}{K_{\text{MeEDTA}}}$
Ni^{2+}	0.069	6.5×10^{-13}	3.2×10^{-18}	2.2×10^5
Co^{2+}	0.072	1.0×10^{-10}	4.0×10^{-16}	2.5×10^5
Cu^{2+}	0.072	2.9×10^{-13}	1.6×10^{-19}	1.8×10^5
Zn^{2+}	0.074	4.8×10^{-11}	5.0×10^{-16}	0.96×10^5
Mn^{2+}	0.080	1.2×10^{-8}	1.6×10^{-14}	7.5×10^5
Cd^{2+}	0.097	2.4×10^{-11}	1.0×10^{-15}	0.24×10^5
Ca^{2+}	0.099	9.2×10^{-7}	2.0×10^{-11}	0.46×10^5
Sr^{2+}	0.112	1.5×10^{-4}	2.0×10^{-9}	0.75×10^5
Pb^{2+}	0.120	2.0×10^{-12}	1.0×10^{-17}	2.0×10^5
Ba^{2+}	0.134	4.2×10^{-5}	1.2×10^{-8}	0.04×10^5

orders of magnitude. It is tempting to suppose that there might be some structural similarities between the metal ion-binding site of the Ca^{2+} -sensitive ATPase and EDTA. These results are somewhat reminiscent of the relationship between $K_{(\text{carboxypeptidase-Mg}^{2+})}$ and $K_{(\text{sulphur-nitrogen-ligand-Mg}^{2+})}$ as described by Vallee & Williams (1968).

The fact that Ca^{2+} -sensitive ATPase is activated by a great number of bivalent metal ion species indicates an unusually high tolerance of the activating ion radius. Although there are some other examples of enzyme that are able to bind a variety of bivalent metal ions [e.g. phosphoglucomutase (Ray, 1969) and alkaline phosphatase (Lazdunski *et al.*, 1969)], the number of metal ion species leading to an active enzyme is considerably smaller in most cases.

In some cases, the occurrence of an Mg^{2+} -dependent ATPase activity in the erythrocyte membranes is described (Bond & Green, 1971). From the results of Fig. 1 the question arises whether the activity in the presence of Mg^{2+} alone is due to a distinct enzyme or to the activity of the high-affinity Ca^{2+} -sensitive ATPase, in which the Ca^{2+} -binding site is occupied by Mg^{2+} in analogy to the binding of all other activating bivalent metal ions. In fact, if the Ca^{2+} -sensitive ATPase is able to bind a variety of different ions including heavy-metal ions, there is, in our opinion, no reason to suppose that Mg^{2+} is an exception with respect to the existence of a distinct ' Mg^{2+} -dependent ATPase'. However, this question cannot be answered finally by experimental investigations of the membrane-bound enzyme. Since the Ca^{2+} -sensitive ATPase from human erythrocyte membranes has been solubilized (Wolf & Gietzen, 1974), we consider that the solubilized purified enzyme will be useful in finding the answer to this question.

The finding that Sr^{2+} is a very potent activator of the Ca^{2+} -sensitive ATPase is not very surprising, since Olson & Cazort (1969) demonstrated that Sr^{2+} is transported actively out of the erythrocyte by a mechanism similar to that of Ca^{2+} transport. However, the physiological significance for this enzyme,

if there is any at all, of the effect of all other bivalent cations (especially of those that are present in the human erythrocyte in considerably high amounts) is unclear and remains to be investigated.

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- Bond, G. H. & Green, J. W. (1971) *Biochim. Biophys. Acta* **241**, 393–398
- D'Ans-Lax (1970) in *Taschenbach für Chemiker und Physiker* (Schäfer, K. & Synowietz, C., eds.), 3rd edn., vol. 3, pp. 258–259, Springer-Verlag, Berlin, Heidelberg and New York
- Documenta Geigy (1968) *Wissenschaftliche Tabellen* (Diehm, C. & Lentner, C., eds.), 7th edn., pp. 561–564, J. R. Geigy A. G., Pharma, Basel
- Hughes, V. L. & Martell, A. E. (1953) *J. Phys. Chem.* **57**, 694–701
- Koshland, D. E. & Neet, K. E. (1968) *Annu. Rev. Biochem.* **37**, 359–410
- Lazdunski, C., Petitclerc, C. & Lazdunski, M. (1969) *Eur. J. Biochem.* **8**, 510–517
- Long, C. & Harrison, D. G. (1968) *J. Physiol. (London)* **199**, 367–381
- Olson, E. J. & Cazort, R. J. (1969) *J. Gen. Physiol.* **53**, 311–322
- Ray, W. J. (1969) *J. Biol. Chem.* **244**, 3740–3747
- Rifkin, R. J. (1965) *Proc. Soc. Exp. Biol. Med.* **120**, 802–804
- Sarma, B. D. & Ray, P. (1956) *J. Indian Chem. Soc.* **33**, 841–848
- Schatzmann, H. J. (1973) *J. Physiol. (London)* **235**, 551–569
- Vallee, B. L. & Williams, R. J. P. (1968) *Chem. Brit.* **4**, 397–402
- Voth, D. (1967) *Brain Res.* **4**, 60–80
- Wolf, H. U. (1972) *Biochim. Biophys. Acta* **266**, 361–375
- Wolf, H. U. (1973) *Experientia* **29**, 241–249
- Wolf, H. U. & Gietzen, K. (1974) *Hoppe-Seyler's Z. Physiol. Chem.* **355**, 1273