THE Mn^{2+}- ALKALINE PHOSPHATASE OF E. COLI

D. Chappelet, C. Lazdunski, C. Petitclerc
M. Lazdunski
Centre de Biologie Moléculaire du C.N.R.S.
31, Chemin Joseph Aiguier, Marseille, France.

Received May 23, 1970

SUMMARY

The Mn^{2+}-phosphatase has a very low catalytic activity as compared to the Zn^{2+}-or Co^{2+}- alkaline phosphatases. It has 2 active sites which can be phosphorylated at acidic pH with ^{32}P-pyrophosphate or ^{32}P-inorganic orthophosphate. The phosphorylation is low at alkaline pH. The non-covalent complex Mn^{2+}-phosphatase-(orthophosphate)$_2$ has been isolated at pH 7.6 and its stability has been carefully studied. Under the same conditions the apophosphatase is unable to bind inorganic phosphate. The complex Mn^{2+}-phosphatase -(orthophosphate)$_2$ contains 4 g. atoms of Mn per mole.

The alkaline phosphatase of E. Coli is an enzyme with 2 identical subunits (1), 2 active centers (2), 4 g. atoms of zinc per mole of protein (3,4) and an anticooperative mechanism (2,5). Zinc is an essential part of the enzyme and its removal involves a complete loss of activity (4,6). Zinc may be replaced on the apoprotein by several other metals like cadmium, manganese, nickel, cobalt or copper. The ORD spectra of the different metallophosphatases are very similar suggesting little change in protein structure (4). However only the cobalt and zinc-alkaline phosphatases were found to be fairly active enzymes. Their catalytic properties have been studied with great detail (7,8). Nickel, manganese and cadmium-phosphatases have a negligible catalytic activity (4,6). The results presented in this paper are part of our studies concerning the role of the metal in the mechanism of the alkaline phosphatase of E. Coli. EPR studies of the Mn^{2+}-phosphatase are presently being carried out in this laboratory.

Results

The maximal activity (Vm) of the Mn^{2+}-phosphatases is 0.05 µmoles/mn/mg at 25°C, pH 8.5 in 0.4 M NaCl, as compared to

35 µmoles/mn/mg for the Zn^{2+}-phosphatase. The low activity of the Mn^{2+}-phosphatase could even be due to a contamination by traces of Zn^{2+}-phosphatase.

Inorganic phosphate forms covalent derivatives with the Mn^{2+}-enzyme as it does with the much more active Zn^{2+} or Co^{2+}-phosphatases. The pH-dependence of the maximal labelling with ^{32}P-orthophosphate and ^{32}P-pyrophosphate is presented in figure 1A. The main features of the labelling with ^{32}P-orthophosphate resemble very much what has been previously found for the Zn^{2+} or Co^{2+}-phosphatases. The maximal phosphorylation occurs at acidic pH while only a small amount of radioactive phosphate is incorporated at more alkaline pH. The mid-point of the variation is at pH 6.2-6.3 while it was at pH 5.1 and 5.6 for the Zn^{2+}-and Co^{2+}-phosphatases respectively (2). No more than 1.3 covalently bound phosphate can be incorporated in the Mn^{2+}-phosphatase at acidic pH under saturating conditions of orthophosphate while a diphosphorylated derivative of the Zn^{2+}-phosphatase is easily formed under the same conditions (2). The Mn^{2+}-phosphatase behaves exactly as does the Co^{2+}-phosphatase which also incorporates no more than 1.3 phosphates from ^{32}P-orthophosphate while both sites were phosphorylated under the same conditions with ^{32}P-AMP (2).

A direct proof of a turnover of the phosphoryl-enzyme was obtained as follows. The Mn^{2+}-alkaline phosphatase was treated at pH 5 with 10 mM unlabelled orthophosphate under conditions known to give an incorporation of 1.3 covalent phosphates per mole. After an incubation of 5 minutes, minute amounts of carrier-free ^{32}P-orthophosphate were added to the mixture. The unlabelled covalent phosphate is easily replaced by ^{32}P-orthophosphate. The reaction is first-order and is completed after 30 minutes.

The extent of the phosphorylation with ^{32}P-pyrophosphate is also higher at acidic pH than at alkaline pH. Only 0.2 phosphates are incorporated at alkaline pH as compared to about 1 for the Zn^{2+} or the Co^{2+}-phosphatase (5). Pyrophosphate is a bad substrate which does not saturate the enzyme at pH 4.0 even at a concentration of 20 mM. This explains the low incorporation at acidic pH. The substrate concentration dependence of the phosphorylation with ^{32}P-pyrophosphate at pH 4.0, 25°C, gives an extrapolation of 1.8 sites phosphorylated under saturating conditions (fig.1B). The Km value is 16 mM.

Fig.2 indicates the enzyme concentration dependence of the total amount (covalent + non covalent) of ^{32}P-orthophosphate bound

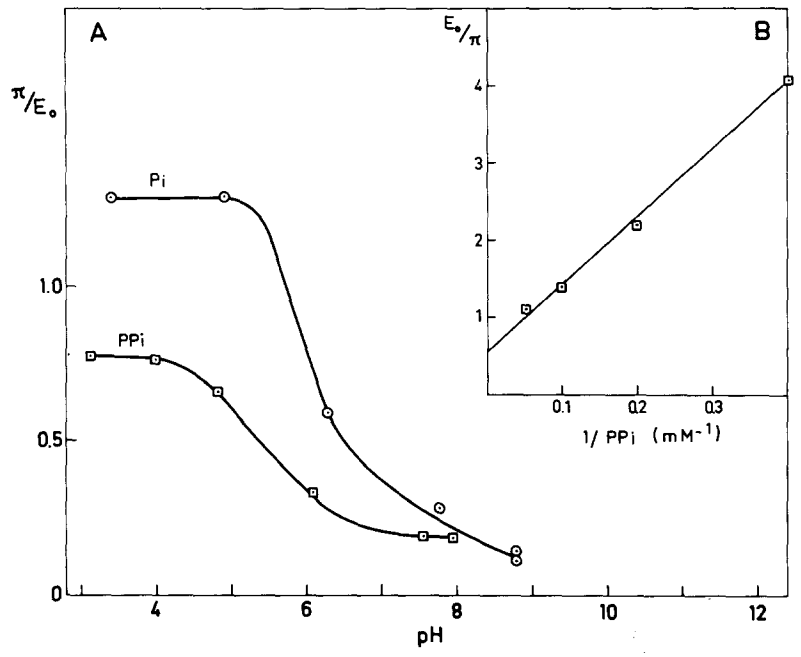

Fig. 1A - pH-dependence of the phosphorylation of the Mn^{2+}-phosphatase by ^{32}P-inorganic phosphate (Pi = 10 mM) and ^{32}P-pyrophosphate (PPi=10mM) at 25°C. Eo and π are the concentrations of the enzyme and the covalently bound phosphate respectively. The labelling is completed after 1 minute. Experiments were carried out in the presence of excess Mn^{2+} (0.25 mM) in order to prevent any dissociation or exchange. Eo : 18.5 to 28.5 µM. Covalent phosphorylation was estimated as previously described (2,5). The Mn^{2+}-phosphatase was prepared as described in fig.3.
Fig. 1B - Concentration dependence of the covalent phosphorylation of the Mn^{2+}-phosphatase by ^{32}P-pyrophosphate at pH 4.0, 25°.
Eo = 18.5 µM, 0.25 mM of spectrographically pure manganese sulfate.

---

per mole of Mn^{2+}-phosphatase at pH 7.6, 25°C. The complex was initially formed by incubation of 24 µM of Mn^{2+}-phosphatase with 10 mM ^{32}P-orthophosphate. It was isolated on Sephadex G 25 and it contains 1.9 moles of ^{32}P-orthophosphate per mole of enzyme. The stability of this complex Mn^{2+}-phosphatase-(orthophosphate)$_2$ on dilution was tested as shown in fig.2. It remains quite stable at a concentration of 2 µM. The dissociation constants are then very low for both phosphates, probably lower than 0.5 µM. This is an interesting result since the complex Zn^{2+}-phosphatase -(orthophosphate)$_2$ has been shown to be stable only at concentrations higher than 20 µM at pH 8 (2). It dissociates at lower concentrations to give rise to a complex Zn^{2+}-phosphatase-(orthophosphate)$_1$ stable at concentrations around 5 µM. The complex Co^{2+}-phosphatase-(orthophosphate)$_1$ is stable at concen-

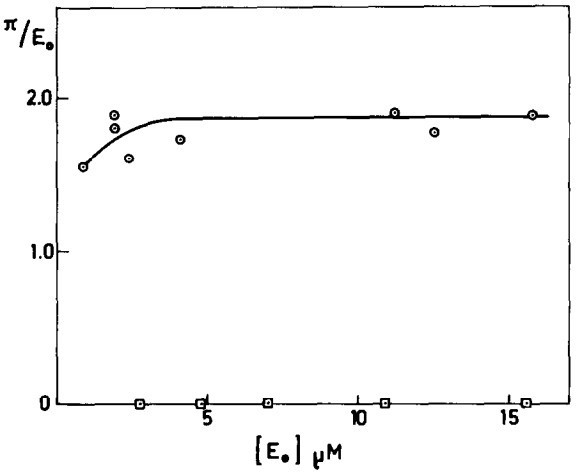

Fig. 2 - Enzyme concentration dependence of the total amount of ^{32}P-orthophosphate bound per mole of phosphatase (⊙). pH 7.6, 25°C. The enzyme-phosphate complex was formed by incubation of high concentrations (see text) of Mn^{2+}-phosphatase with ^{32}P-orthophosphate (10 mM) in the presence of 0.44 mM manganese sulfate. The complex was isolated on Sephadex G 25 and analysed for radioactivity content. The same operation was repeated at different dilutions of the isolated complex. The same type of experiment was carried out with the apophosphatase at pH 6.0 in a Tris-HCl buffer (10 mM) in the presence of 0.01 mM EDTA (⊡). The stability of the complex Mn^{2+}-phosphatase-^{32}P-orthophosphate)$_2$ was tested in exchange experiments as follows. The complex (13.5 μM) was incubated with 100 mM unlabelled orthophosphate during 2 minutes then passed through a column of Sephadex G 25 equilibrated at pH 7.6 with 0.5 mM manganese sulfate. The amount of covalently bound ^{32}P-orthophosphate was then evaluated as usual (2,5).

---

trations of the order of 2 μM (2). The stability of the complex containing 1.9 moles of ^{32}P-orthophosphate per mole of Mn^{2+}-phosphatase was also tested in exchange experiments in the presence of unlabelled phosphate (see fig.2). Most of the radioactivity, due to non covalent binding, is readily displaced and only 0.2 mole of covalently bound ^{32}P-orthophosphate remains attached to the enzyme. This value is in agreement with the results in fig. 1A. Fig.2 shows that the apophosphatase is unable to bind ^{32}P-orthophosphate.

The number of Mn^{2+} per mole of protein has been evaluated as previously described for the Zn^{2+}-phosphatase (4). It is clear from fig.3 that the Mn^{2+}-enzyme like the Zn^{2+}-phosphatase contains 4 g. atoms of metal per mole of protein when associated in the Me^{2+}-phosphatase-(orthophosphate)$_2$ complex. The same experiment as in fig.3 done without orthophosphate results into the loss of about 1 Mn^{2+} since only 3.2 - 3.3 Mn^{2+} remain attached to the enzyme. It has been

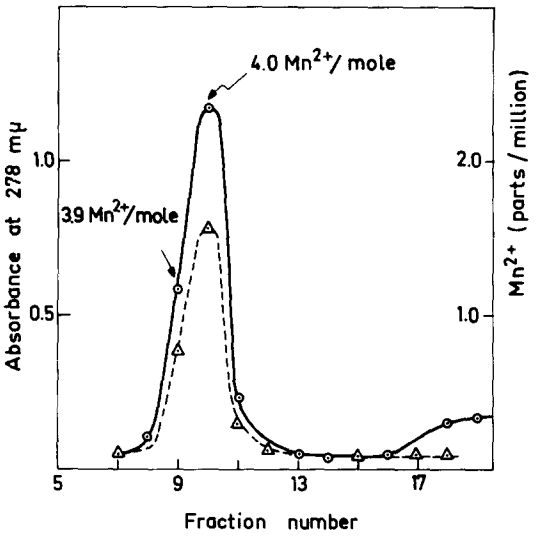

Fig. 3 - Number of manganese atoms per molecule of Mn^{2+}-phosphatase. The apoenzyme was prepared as previously described (5). To obtain the Mn^{2+}-phosphatase, the inactive apoenzyme was passed through Sephadex G-25 (column 18 x 1.5 cm) equilibrated with 0.5 mM spectrographically pure manganese sulfate at pH 7.5, 25°C. This Mn^{2+}-phosphatase solution (27 μM) containing 0.5 mM Mn^{2+} was incubated in 10 mM orthophosphate during 6 hours at 20°, then chromatographed in a Sephadex G-25 column equilibrated at pH 7.6 with 10 mM orthophosphate to eliminate the excess of Mn^{2+}. The manganese content (_____) was estimated by atomic absorption at 279.5 nm. Protein concentration was evaluated at 278 nm ($E^{0.1\%}_{1\ cm} = 0.77$) (-----).

previously found that there exists at least 2 sets of zinc in the Zn^{2+}-phosphatase (2,3,5). One set is tightly bound to the apoprotein while the other set is much more loosely bound in the absence of phosphate. The situation is apparently similar in the Mn^{2+}-enzyme.

Discussion

The Mn^{2+}-phosphatase contains 4 Mn atoms per molecule. The enzyme has a very low catalytic activity toward p.nitrophenyl phosphate. However it is perfectly able to bind orthophosphate, a reaction product, as well as pyrophosphate, a substrate. There are 2 active sites per mole of enzyme. Non covalent complexes containing about 2 moles of orthophosphate per mole of Mn^{2+}-phosphatase can be easily isolated. They do not dissociate at concentrations as low as 2 μM. They are much more stable than the corresponding complexes with the active Co^{2+}- or Zn^{2+}-phosphatases. The replacement of Zn^{2+} for Mn^{2+} impairs neither the binding of substrates nor the phosphorylation and dephosphorylation of the active center. It does not change the general

property found with actives enzymes that is to be phosphorylated on the essential serine residue more easily at acidic than at alkaline pH. The Cd^{2+} and Cu^{2+}-phosphatases, which have a very low catalytic activity, also incorporate nearly 2 covalent phosphates per mole at acidic pH while the extent of phosphorylation is much lower at alkaline pH. A detailed discussion of these data will be given elsewhere.

The apophosphatase with a conformation apparently identical to that of the Zn^{2+}-phosphatase (9) is unable to bind orthophosphate. (fig.2) or to incorporate phosphate covalently from ^{32}P-AMP (2). The metal is thus obviously necessary for the binding of substrates. However the nature of the metal is not crucial in the adsorption process since the Mn^{2+}-phosphatase binds orthophosphate even better than the Zn^{2+} or Co^{2+}-enzymes. The nature of the metal is essential for the control of the rates of phosphorylation and/or dephosphorylation steps. One of these steps or both of them are considerably slowed down when Zn^{2+} or Co^{2+} are replaced by Mn^{2+}.

The authors thank Mr. A. Moulin for excellent technical assistance.

(1) Rothman, F. and Byrne, R., J. Mol. Biol. 6, 330 (1963)
(2) Lazdunski, C., Petitclerc, C., Chappelet, D., and Lazdunski, M., Biochem. Biophys. Res. Commun. 37, 744 (1969)
(3) Simpson, R.T., and Vallee, B.L., Biochem. 7, 4343 (1968)
(4) Lazdunski, C. Petitclerc, C., and Lazdunski, M., Europ. J. Biochem. 8, 515 (1969)
(5) Petitclerc, C., Lazdunski, C., Chappelet, D., Moulin, A., and Lazdunski, M., Europ. J. Biochem. in press. 14, (1970)
(6) Plocke, D.J., Levinthal, C., and Vallee, B.L., Biochem. 1, 373 (1962).
(7) Lazdunski, C. and Lazdunski, M., Europ. J. Biochem. 7, 294 (1968)
(8) Gottesman, M., Simpson, R.T., and Vallee, B.L., Biochem. 8, 3776 (1969)
(9) Appleбury, M.L., and Coleman, J.E., J. Biol. Chem. 244, 308 (1969)