TIGHT AND LOOSE METAL BINDING SITES IN THE APOALKALINE PHOSPHATASE OF E. COLI

Reconstitution of the Cd^{2+}-phosphatase from the apoenzyme EPR study of the Mn^{2+}-phosphatase

C. LAZDUNSKI, C. PETITCLERC, D. CHAPPELET, * F. LETERRIER
* P. DOUZOU, M. LAZDUNSKI

Centre de Biologie Moléculaire du C.N.R.S.
31, Chemin Joseph Aiguier, Marseille, France

* Institut de Biologie physico-chimique
25, rue Pierre Curie, Paris, France.

Received June 19, 1970

SUMMARY

The titration of active centers during the reconstitution of the Cd^{2+}-phosphatase from the apophosphatase and the EPR analysis of the Mn^{2+}-enzyme demonstrate the existence of two tight and two loose metal binding sites in the Cd^{2+}-and Mn^{2+}-phosphatases. Direct evidence has been obtained for the participation of metals bound to loose sites both in the binding of substrates and phosphorylation of the active center.

The alkaline phosphatase of E. Coli is a dimeric protein with 2 identical subunits (1), 2 active centers (2) and a very characteristic feature, namely an anticooperative mechanism (2,3). It is a metalloenzyme with 4 g. atoms of zinc per mole of protein (4,5). Zinc may be replaced on the apoprotein by cobalt, cadmium, manganese, copper or nickel (5,6). We have recently demonstrated that Cd^{2+} - and Mn^{2+} - phosphatases have a very low catalytic activity towards organic phosphates. However they still contain 4 g. atoms of metal per mole of enzyme (5,7).

Two techniques have been used in this paper to demonstrate the existence of tight and loose metal binding sites in the Cd^{2+} - and Mn^{2+}- phosphatase. They are the titration of active centers during the reconstitution of the Cd^{2+}- phosphatase from the apophosphatase and the analysis of electron paramagnetic resonance (EPR) of the Mn^{2+} - enzyme.

Results

The Cd^{2+} -phosphatase like the other so-called "inactive" phosphatases, namely the Mn^{2+} - and the Cu^{2+}-phosphatases, is still capable of being phosphorylated on its active sites by substrates such as inorganic phosphate, pyrophosphate or AMP (7). Figure 1 presents reconstitution experiments of the Cd^{2+}-enzyme from the apophosphatase. It shows that the maximal phosphorylation of the active

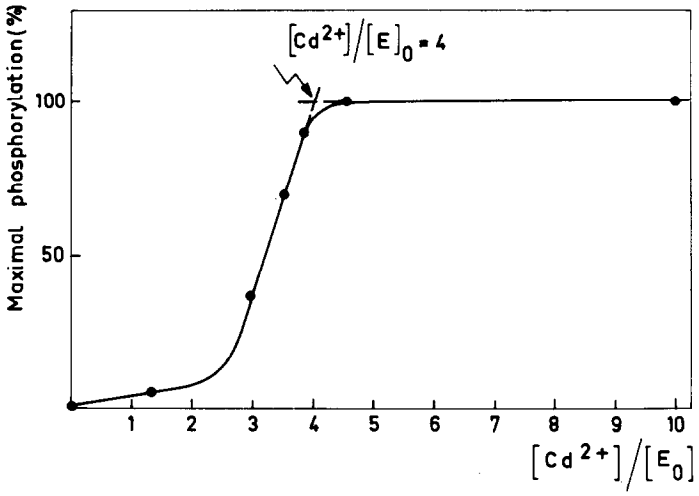

Fig. 1 : Reconstitution of the Cd^{2+}-enzyme from the apoenzyme. Aliquots of spectroscopically pure cadmium sulphate were added to the apoenzyme and the pH was maintained at 6.4 (no buffer) by adding the necessary amounts of NaOH. E_o = 50 µM, the dissociation constant for the most loosely bound Cd^{2+} being of 0.3 - 0.4 µM at pH 7.6 (5). After each addition of Cd^{2+} and an incubation of 5 minutes, the extent of phosphorylation of the active site is measured with ^{32}P-pyrophosphate (10 mM) at pH 5. Under these conditions, the Cd^{2+}-phosphatase with its full cadmium content incorporates 2 covalent phosphates per mole of protein.
Methods for preparing the apophosphatase and for labelling the active sites have been previously described (2,3).

---

sites under the influence of pyrophosphate only occurs when the enzyme contains 4 g. atoms of cadmium per mole of protein. The reconstitution profile is identical to the one found for the Zn^{2+}-phosphatase.

Zinc, which is a common metal for many metalloenzymes has unfortunately no unpaired electron but it can be replaced by manganese which permits EPR studies at room temperature. The functional properties of the Mn^{2+}-phosphatase have been described previously (7) and the enzyme has been shown to contain 4 manganese per mole of protein.

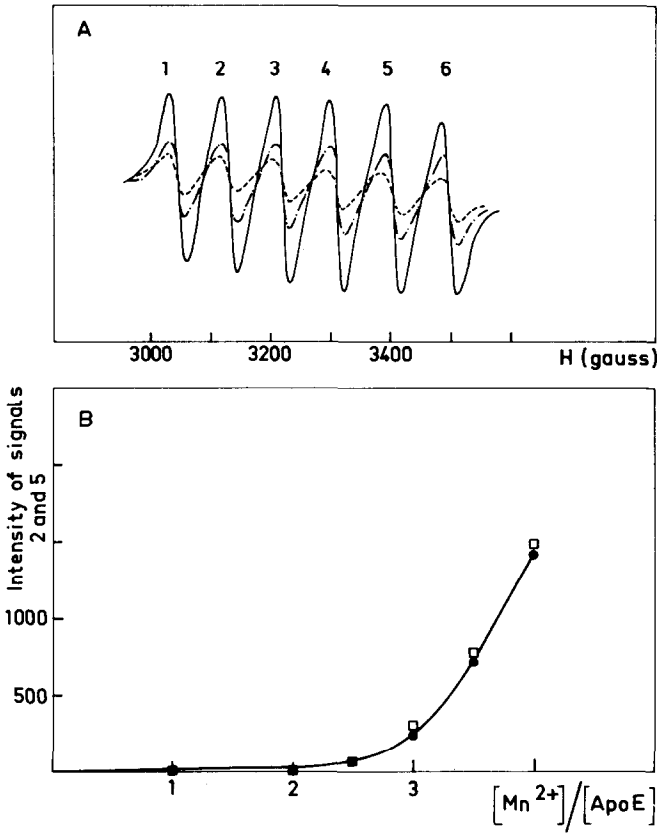

Fig. 2A : EPR spectra at pH 8, 20°C of the Mn^{2+}-phosphatase (apoenzyme + 4 g. atoms of Mn per mole of protein) ——— , of this Mn^{2+}-phosphatase + 1 equivalent of inorganic phosphate —.—, of this Mn^{2+}-phosphatase + 2 equivalent of inorganic phosphate - - - - . Protein concentration : 420 µM. Under these conditions both equivalents of orthophosphate are stoechiometrically bound to the enzyme (7). These spectra were recorded in a Varian E-3 spectrometer at 9.16 GHZ. Modulation amplitude 2.5 gauss, receiver gain 5 x 10^5, microwave power 10 mW.

Fig. 2B : Mn^{2+}/apoprotein ratio dependence for the intensities of lines 2 and 5 at pH 8, 20°C. Protein concentration 500 µM. Modulation amplitude 10 gauss, receiver gain 5 x 10^5, microwave power 10 mW. When the $[Mn^{2+}]/[ApoE]$ ratio is increased from 4 to 6, the intensity of signal 2 varies from 1400 to 5750 (Δ = 4350) ; the intensity of line 5 varies from 1480 to 6030 (Δ = 4550). An equivalent concentration of free Mn^{2+} (10^{-3} M of Mn^{2+} in the absence of apoenzyme) gives intensities of 7600 and 8100 for lines 2 and 5 respectively.

A typical EPR spectrum of a sample of phosphatase containing 4 g. atoms of Mn per mole is presented in figure 2A. It presents the same pattern of six characteristic lines which is observed with free Mn^{2+}. The variation of the intensity of lines 2 and 5 with the

$[Mn^{2+}]$ / [apoenzyme] ratio is presented in figure 2B. No signal was observed up to 2 g. atoms of Mn per mole of apoprotein. All signals appear at higher concentrations of Mn^{2+}. Both classes of metal sites are saturated after 4 g. atoms have been added to the apoenzyme. The intensity of lines 2 and 5 then increases considerably on addition of two more manganese per mole of apoenzyme (from $[Mn^{2+}] / [E_o] = 4$ to $[Mn^{2+}] / [E_o] = 6$). However this increase is less than what one would expect if these additional Mn^{2+} were unbound (see legend of fig. 2). A necessary conclusion is that they are at least partially bound to the phosphatase molecule under our experimental conditions. These additional manganese have nothing to do with the active structure or the function of the enzyme (7).

Fig. 2A also shows the influence of inorganic orthophosphate on the EPR spectrum of the Mn^{2+}-phosphatase.

Discussion

Figure 1 clearly shows the existence of 2 classes of metal binding sites in the apoenzyme. The binding of the first 2 Cd^{2+} to the tight sites (type I) does not allow the formation of phosphorylated derivatives. The formation of an enzyme which can be maximally phosphorylated on both sites at acidic pH is associated with the binding of the last 2 cadmium ions to the sites of type II.

Despite the fact that the Mn^{2+}-phosphatase displays very little activity, the replacement of Zn^{2+} for Mn^{2+} impairs neither the binding of substrates nor the phosphorylation and dephosphorylation of the active center (7). Electron paramagnetic resonance of the Mn^{2+}-phosphatase provides a direct evidence for the existence of 2 types of metal binding sites. Mn^{2+} is so strongly liganded to the sites of type I that no EPR spectrum appears after the addition of the first Mn^{2+} to the apoenzyme (figure 2). Signals are observed with a metal content of 3 g. atom of manganese per mole of protein, their intensity being higher after the addition of a fourth Mn^{2+}. The binding constants of the two manganese loosely bound to each site of type II might not be identical. This possibility is suggested by the fact that the intensities of lines 2 and 5 do not vary linearly between 2 and 4 g. atoms of Mn^{2+} per mole of apoprotein (figure 2B). Previous studies have also shown that the Mn^{2+}-phosphatase easily looses one of its four Mn^{2+} by simple chromatography on Sephadex G-25 (7). No such dissociation occurs however on chromatography of the Mn^{2+}-phosphatase -(orthophosphate)$_2$ complex (7). A similar situation might exist for the Zn^{2+}-

phosphatase since several authors have reported metal contents of only 3 g. atoms of zinc per mole of protein (8 - 10), while the Zn^{2+}-phosphatase - $(orthophosphate)_2$ complex contains 4 g. atoms of zinc per mole of protein under similar conditions (5).

At pH 8, the Mn^{2+}-phosphatase forms non-covalent complexes with an unusually high stability (7). The non-covalent binding of orthophosphate has been investigated by the EPR technique (fig. 2A). The intensities of lines 2 and 5 decrease by a factor of 2.1 on addition of 1 mole of orthophosphate per mole of Mn^{2+}-enzyme, they decrease by a factor of 4.3 on addition of a second mole of substrate.

At pH 5.5, orthophosphate forms stable covalent derivatives with the Mn^{2+}-enzyme (7). An EPR analysis of the Mn^{2+}-phosphatase at pH 5.5 has shown that both manganese of type II were less firmly bound to the apoprotein than they were at pH 8.0. However phosphorylation of the active sites by orthophosphate under acidic conditions also decreases considerably both intensities of lines 2 and 5.

All these results taken together can be considered as a direct evidence that the manganese atoms bound to the sites of type II are implicated both in the binding of orthophosphate and in the phosphorylation of the essential serine residue. They are therefore part of the active center. This conclusion is in agreement with previous results which have shown that the apophosphatase is unable to bind substrates (2,7). Previous data obtained in this laboratory have suggested that each one of the two active sites of the Zn^{2+} - enzyme is formed of one serine residue and of 2 zinc atoms bound to one site of type I and one site of type II respectively (3). Spectrophotometric and EPR studies of the Cu^{2+}-phosphatase also confirm this view (11).

We are grateful to Dr. P. Tordo for stimulating discussions.

(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) Petitclerc, C. Lazdunski, C. Chappelet, D., Moulin, A., and Lazdunski, M., Europ. J. Biochem. in press 14 (1970)
(4) Simpson, R.T., and Vallee, B.L., Biochem. 7, 4343 (1968)
(5) Lazdunski, C., Petitclerc, C., and Lazdunski, M., Europ. J. Biochem. 8, 510 (1969)
(6) Plocke, D.J., and Vallee, B.L., Biochem. 1, 1039 (1962)
(7) Chappelet, D., Lazdunski, C. Petitclerc, C. and Lazdunski, M., Biochem. Biophys. Res. Commun. in press.
(8) Tait, G.H., and Vallee, B.L., Proc. Nat. Acad. Sci. 56, 1891 (1966)
(9) Reynolds, J.A., and Schlesinger, M.J., Biochem. 7, 2080 (1968)
(10) Applebury, M.L., and Coleman, J.E., J. Biol. Chem. 244, 308 (1969)
(11) Lazdunski, C., Petitclerc, C. Chappelet, D., Leterrier, F., Douzou, P., and Lazdunski, M., to be published.