ON THE MECHANISM OF THE Zn^{2+} AND Co^{2+}-ALKALINE PHOSPHATASES OF E. COLI. NUMBER OF SITES AND ANTICOOPERATIVITY

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Received September 20, 1969

Two active centers have been found per molecule of Co^{2+} and Zn^{2+}- alkaline phosphatase of E. Coli. They are both phosphorylated at acidic pH with ^{32}P-labelled orthophosphate, AMP or ATP. The pH-dependence of the covalent phosphorylation shows that only one site is phosphorylated at alkaline pH with organic substrates. Equilibrium binding studies have shown a strong anticooperativity in the non covalent binding of orthophosphate to the enzyme.

The alkaline phosphatase of E. coli is a dimeric molecule formed of 2 identical subunits (1). This enzyme appears to possess 4 Zn atoms per molecule ; 2 of them are functional and the 2 others have a structural role (2,3). However the determination of the number of active centers is still an unresolved problem. Evidences for 1 or 2 active centers have been obtained with each one of the main techniques used so far : isolation of the phosphoserine of the active center (4 - 6), rapid kinetics (7 - 10), spectrophotometric techniques using the Co^{2+}-enzyme (2,11). The results presented in this paper indicate 2 active centers per molecule which are both phosphorylated at acidic pH, and a strong anticooperativity in the non-covalent binding of orthophosphate to the enzyme.

Results and discussion

A- Covalent binding

The time and concentration dependences of the covalent phosphorylation of alkaline phosphatase are presented in fig. 1.

The maximal amount of covalent phosphorylation at different pH's and with various substrates is presented in fig. 2. Several interesting remarks or conclusions can be drawn from these results.

1) There are 2 active sites which can be phosphorylated both in Zn^{2+} and Co^{2+}-phosphatases. The maximal phosphorylation of these sites

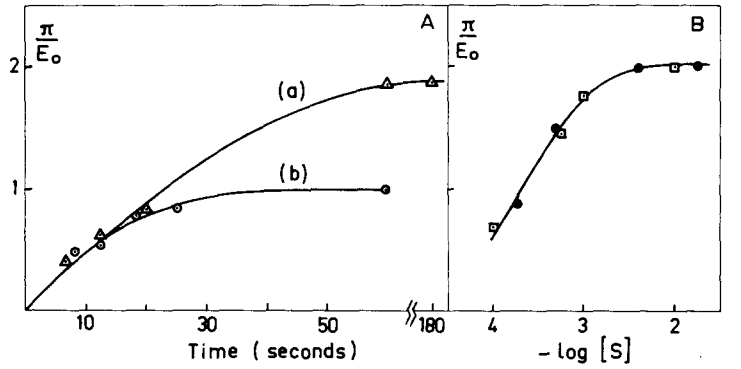

Fig. 1 A. Time dependence for the covalent phosphorylation of the active centers with orthophosphate (10^{-2}M) at 0° in 0.5 M NaCl. (a) pH 4 ; (b) pH 5. B. The concentration dependence for the covalent phosphorylation of the active centers with orthophosphate (□) and AMP (●). pH 4, 0°.

E_o and π are the concentrations of the enzyme and the covalently bound phosphate respectively. The enzyme was phosphorylated by incubation with radioactive substrates such as ^{32}P-AMP, ATP (γ), or orthophosphate. The isolation of the phosphoryl-enzyme after quenching at acidic pH was done according to Lazdunski (18) and Petitclerc et al. (13). Control experiments have shown that neither a denatured phosphatase nor the apoenzyme incorporate radioactive phosphate. No radioactivity was incorporated after an incubation with ^{14}C-AMP.

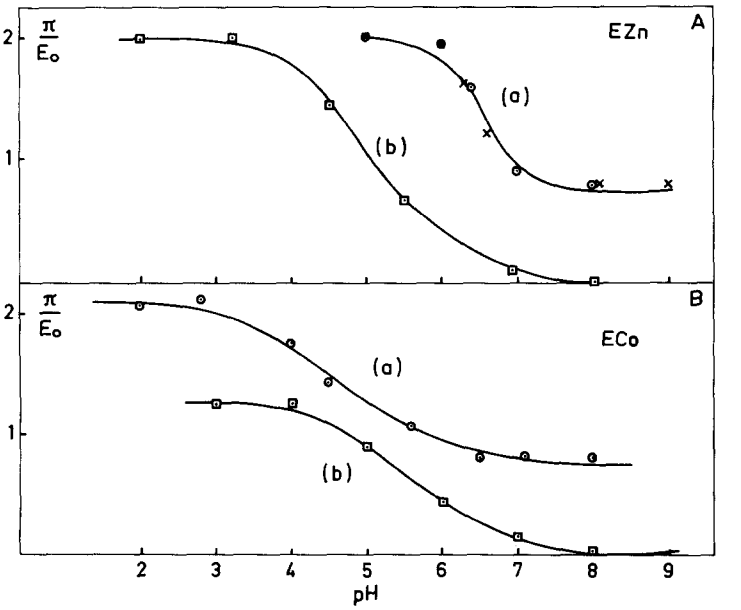

Fig. 2 pH-dependence of phosphorylation of the active centers of alkaline phosphatase at 0°. A. Zn^{2+}-phosphatase (a) ^{32}P-AMP (o) and ^{32}P-ATP (×) ; (b) ^{32}P-orthophosphate. B. Co^{2+} phosphatase. (a) ^{32}P-AMP ; (b) ^{32}P-orthophosphate. The concentrations of the phosphorylating agent were chosen to obtain the maximal amount of phosphorylation at all pH's.

can only be obtained at very acidic pH's with all substrates. Under t conditions the dephosphorylation of the diphosphorylated derivative i clearly the rate determining step in the reaction mechanism.

2) Labeling of Zn^{2+} or Co^{2+}-phosphatase with ^{32}P-ATP or ^{32}P-AMP shows that only one site can be phosphorylated in the alkaline pH-ran where the enzyme is normally functional.

3) The pH-profiles for the covalent incorporation of phosphate from ^{32}P-ATP and ^{32}P-AMP are nearly identical. This was expected from substrates with the same kinetic properties (12). The pH profile is quite different with orthophosphate ; the phosphorylation of both sit is still possible at acidic pH, but no covalently bound phosphate can be obtained at alkaline pH. Thus, phosphorylation of the active cente is much slower with orthophosphate than with organic phosphates at hi pH's. The mid-point of the curves in fig. 2 is displaced by 1.5 pH un with the Zn^{2+}-phosphatase on replacement of organic substrates by orthophosphate. It is shifted from pH 4.65 to pH 5.6 for the Co^{2+}-enzym A more detailed discussion of the enzyme mechanism with an interpreta tion of the pH-profiles will be published elsewhere (13).

4) The replacement of Zn^{2+} by Co^{2+} in the native enzyme does no change the values of phosphorylation plateaus at acidic or alkaline pH's with organic phosphates as substrates. However the mid-point of the π/E_o - pH curves is shifted considerably from pH 6.6 to pH 4.65 when Co^{2+} is substituted for Zn^{2+}. Conversely a small shift from pH 5 to pH 5.6 can only be observed with orthophosphate as the phosphoryla ting agent. However, no more than 1.25 covalently bound phosphate can be incorporated at acidic pH with saturating concentrations of the substrate. It is probable that under these conditions, in the system Co^{2+}-phosphatase-orthophosphate, the dephosphorylation of the diphosphorylated phosphatase is no longer the determining step in the catalysis.

The catalytic properties of the Zn^{2+} and Co^{2+}-phosphatases have already been compared and discussed for pH's higher than 5.5 (12,14). Some kinetic data obtained with both metalloenzymes at acidic pH's are shown in fig. 3. The activity of the Co^{2+}-phosphatase is only 10 of that of the Zn^{2+}-enzyme at alkaline pH. The situation is completel reversed at pH 3-4 where the Co^{2+}-phosphatase is a much more active enzyme. We previously reported (12) that the exchange of Zn^{2+} for Co^{2} decreased the phosphorylation rate of the active serine by a factor c about 16 for most substrates at alkaline pH. The exchange increases the dephosphorylation rate of the diphosphorylated derivative by a fa

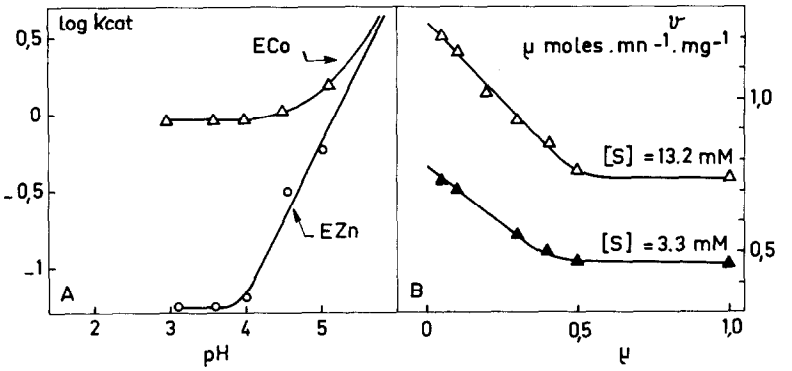

Fig. 3 A. pH dependence of k_{cat} (sec^{-1}). Vm = k_{cat} $[E_o]$. 25°. B. Ionic strength (μ) dependence of the activity of Co^{2+}-phosphatase at pH 3.4, 25°. Similar results have been obtained with the Zn^{2+}-enzyme.
Substrate : p-nitrophenylphosphate.

tor of about 20 at pH 3-4. These observations seem to be a first step toward an understanding of the role of the functional metals. Other metallophosphatases are presently being studied.

An increase of the ionic strength decreases considerably the dephosphorylation rate of the diphosphorylated derivative both for the Zn^{2+} and Co^{2+} phosphatases (fig. 3). The situation is very different from that which has been observed at alkaline pH where the ionic strength increases considerably the maximal rates of both metalloenzymes (12).

B- Non-covalent binding

Since orthophosphate forms no covalent phosphorylated derivative with the enzyme at pH higher than 7.5-8, it was of a great interest to determine the number of substrate molecules bound in the non-covalent (Michaelis) phosphatase-orthophosphate complex. Scatchard's representations of equilibrium dialysis experiments are shown in fig. 4. An important result appears immediately : the adsorption of 2 phosphate molecules on the enzyme is an anticooperative process. A similar result has been found with the Zn^{2+}-enzyme in Vallee's laboratory (14). One site binds phosphate strongly and its saturation occurs at very low phosphate concentrations ; the saturation of the second site is then much more difficult and much higher concentrations of phosphate are necessary. This anticooperative character of the alkaline phosphatase has also been shown (13) using Hummel and Dreyer's technique (15). Fig. 4B shows that the variation of K_I with pH determined from Scatchard's plots for the

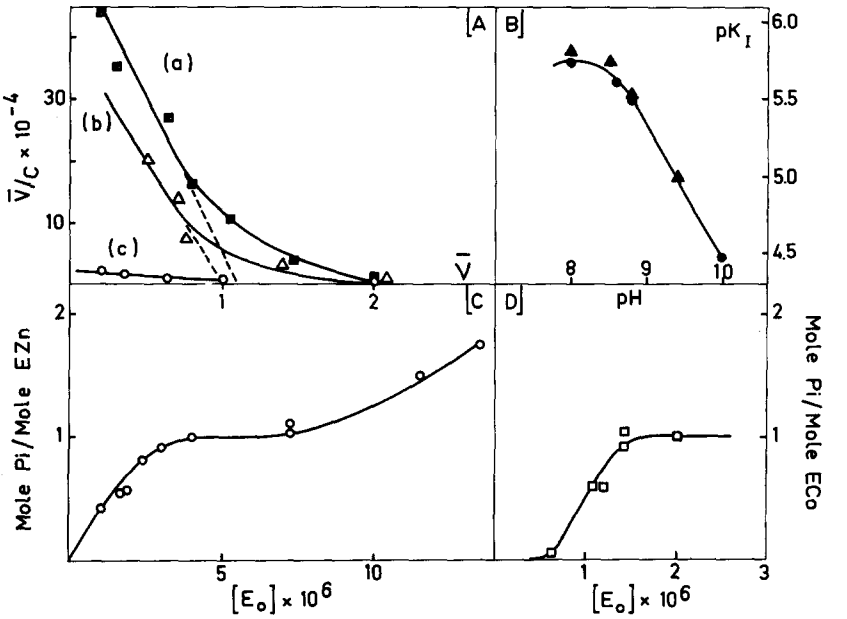

Fig. 4 A. Scatchard's plots for the binding of orthophosphate (Pi) to the Zn^{2+}-phosphatase. $\bar{v}$: mole binding ratio Pi/dimer ; c : equilibrium concentration of orthophosphate. If the 2 sites were identical, a single straight line should be obtained with an extrapolation at $\bar{v} = 2$. Equilibrium dialysis was carried out according to Gilbert and Müller-Hill (17) at 25° with 0.4 M NaCl. (a) pH 8, (b) pH 8.8, (c) pH 10. B. Variation of pK_I with pH. (Δ) kinetic determinations (16). (●) pK_I was calculated from the slope ($1/K_I$) of the $\bar{v}/c$ - $\bar{v}$ plots for the saturation of the first site. C and D. Enzyme concentration dependence of the orthophosphate-phosphatase complex. The enzyme-phosphate complex was formed by incubation of high concentrations of Zn^{2+} (c) or Co^{2+}-phosphatase (d) with radioactive orthophosphate (10^{-2} M). 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.

saturation of the first site is identical to that previously obtained from kinetic data (16). The non-covalent complexes can be isolated in the stable form provided that high enough concentrations of enzyme ar used (fig. 4C and D). With the Zn^{2+}-phosphatase a concentration of 4-5 x 10^{-6} M of enzyme is sufficient to prepare the complex with only one bound phosphate. A concentration higher than 2 x 10^{-5} M would be necessary to obtain the complex with 2 non-covalently bound phosphate Only the complex with one phosphate has been obtained with 2 x 10^{-6} M Co^{2+}-phosphatase. It is probably worthwile to underline that the binding of the functional Zn^{2+}-atoms to the apophosphatase also appears to be an anticooperative process (3).

The authors are greatly indebted to Mr A. Moulin and C. Gache for assistance in performing equilibrium dialysis and kinetic experiments

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