THE NON-EQUIVALENCE OF THE ACTIVE SITES AND THE MECHANISM OF A MUTATIONALLY ALTERED E.coli ALKALINE PHOSPHATASE.

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Summary: The activated form of the U-47 mutant of E.coli alkaline phosphatase is a dimer with 4g atoms of zinc per mole. Its specific activity is only two-tenths that of the wild type enzyme. Analysis of the catalytic action by transient kinetics and quenching experiments shows that only one site per dimer (half-site reactivity) can be phosphorylated by substrates and $^{32}P_i$ at acidic pH and by substrates at alkaline pH. The rate constants of the phosphorylation (k_2) and of the dephosphorylation (k_3) of the active site are drastically changed by the mutation. The pH dependence of k_2 and k_3 is reported.

U-47 is an alkaline phosphatase negative mutant of E.coli.Under normal growth conditions it produces an inactive alkaline phosphatase deficient in zinc (1). Incubation of the inactive phosphatase with zinc restores some catalytic activity (1). The mutationally altered enzyme is an interesting phosphatase species for further investigation of the characteristic feature of the phosphatase mechanism,i.e. negative cooperativity and half-of-the sites reactivity (2-4).

EXPERIMENTAL PROCEDURES

Purification of the U-47 alkaline phosphatase: the mutationally altered alkaline phosphatase was obtained from U-47, a phosphatase-negative, CRM-forming mutant isolated by Levinthal (5) from strain K_{10} of E.coli. Culture of cells and purification of the enzyme were carried out as described previously (6).

Zinc content evaluation in the P_i-phosphatase complex, steady state kinetic measurements,determinations of non-covalent binding of $^{32}P_i$ as well as of covalent phosphorylation, and stopped-flow experiments were carried out as previously described for wild type E.coli and intestinal phosphatases (2-4, 7,8). For active site titration we used $A_{1cm}^{1\%}$=7.7 at 278 nm and a molecular weight of 86 000.

RESULTS

The phosphatase purified from the U-47 mutant has a specific activity (V_m) for p-nitrophenylphosphate of only 0.006 µmoles.min^{-1}.mg^{-1} (pH 8.5,25°, NaCl 0.4M). Incubation of the nearly inactive enzyme for 2 hours at 37° in 100mM TES[1], pH 6.8, with 3mM $ZnCl_2$ and 0.1mM inorganic phosphate increases V_m to 8µmoles.min^{-1}.mg^{-1}.The specific activity of the wild-type phosphatase measured under the same conditions is 37µmoles.min^{-1}.mg^{-1} .

Non-activated U-47 phosphatase is mostly in the monomeric form. This was demonstrated by immunoadsorption on a column of antidimer antibody coupled to a polyacrylamide gel (9). The antibody was made against the wild type dimeric enzyme; it does not cross-react with the monomeric subunit (9). Only 20% of the mutant phosphatase was recognized as a dimer. After activation in the presence of orthophosphate and zinc,the activated phosphatase is totally adsorbed on the column and is therefore reassociated into the dimeric form. The presence of a slow equilibrium between monomeric and dimeric forms of the non-activated phosphatase was confirmed by sedimentation velocity experiments. The inactive enzyme gives two peaks corresponding to the monomer ($S^{\circ}_{20,W}$=3.8S) and to the dimer ($S^{\circ}_{20,W}$=6S).

The inactive U-47 phosphatase contains only 1g atom of zinc per mole of 86 000 molecular weight. After activation, the U-47 phosphatase-inorganic phosphate complex contains 4 ± 0.2g atoms of zinc per mole of dimer, a zinc content identical to that of the wild type enzyme (7,10). These data confirm that zinc stabilizes the dimeric structure (11).

Complexes formed by U-47 phosphatase with $^{32}P_i$ and [^{32}P] AMP. Inorganic phosphate forms a phospho-intermediate with the active site of U-47 activated phosphatase. The pH dependence of the maximal covalent labelling is presented in Figure 1A. No more than 1 mole of covalent phosphate can be incorporated at acidic pH under saturating concentrations of inorganic phosphate (100mM). A diphospho-enzyme was obtained with the wild type enzyme under the same conditions (2).Non-covalent binding of $^{32}P_i$ to U-47 activated phosphatase occurs at alkaline pH. At enzyme concentrations between 0.5 and 1 µM,the non-covalent complex, which can be isolated in stable form, contains one non-covalently bound phosphate per mole of dimeric phosphatase (Figure 1B). Figure 1A also presents

[1] TES: N-tris (hydroxymethyl)methyl-2 aminoethanesulfonate-Cl.

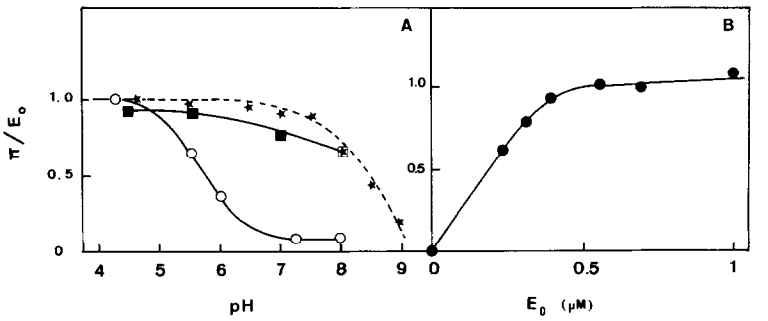

Fig. 1- A - pH dependence of the covalent phosphorylation of the U-47 phosphatase at 20° with $^{32}P_i$ (○), with [^{32}P]AMP (■) and with p-nitro or 2,4-dinitrophenylphosphates (★) (stopped-flow results). Curve (---) is a calculated curve of Π/E_o (burst) versus pH using values of k_2 and k_3 from Fig 2 and equation [3]. - B - Enzyme concentration dependence of the stability of the non-covalent $^{32}P_i$-phosphatase complex. The non-covalent complex was initially formed by incubation of 5 μM activated enzyme with 10mM $^{32}P_i$ at pH 8.0, 25°. It was isolated at different dilutions on Sephadex G25 as described for wild type enzyme (2). Π and E_o are the concentrations of bound phosphate or released phenols and of enzyme

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labelling results, at different pH, obtained after quenching the phosphoderivative formed in the steady state of [^{32}P]AMP hydrolysis. Only one of the two active sites of the dimer can be phosphorylated in U-47 activated phosphatase with saturating concentrations of AMP at acidic as well as at alkaline pH. Under the same conditions, the wild type enzyme incorporated covalently 2 phosphates per mole of dimer at acidic pH and only 1 phosphate per mole at alkaline pH. Covalent phosphorylation of the non-activated U-47 phosphatase does not occur with $^{32}P_i$ or [^{32}P]AMP.

Transient kinetics[2]. Typical analyses of transient kinetics obtained with U-47 activated phosphatase and 2,4-dinitrophenyl phosphate as substrate are presented in Figure 2. At pH 5.0 a burst of 1 mole of dinitrophenol

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[2] The simplest mechanism for alkaline phosphatase is:

$$E + S \rightleftharpoons E - S \xrightarrow{k_2} E - P \xrightarrow{k_3} E + P_i$$
$$\qquad\qquad\qquad\qquad\quad \searrow ROH$$

k_2 and k_3 are the first order rate constants for the phosphorylation and dephosphorylation of the active site.
$k_{cat} = k_2 k_3/k_2 + k_3$ [1] ; $k_o = k_2 + k_3$ [2] when $S > K_m$; k_o is the first order rate constant for the transient phase.
$\pi/E_o = (k_2/(k_2 + k_3))^2$ [3] is the burst amplitude when $S > K_m$.

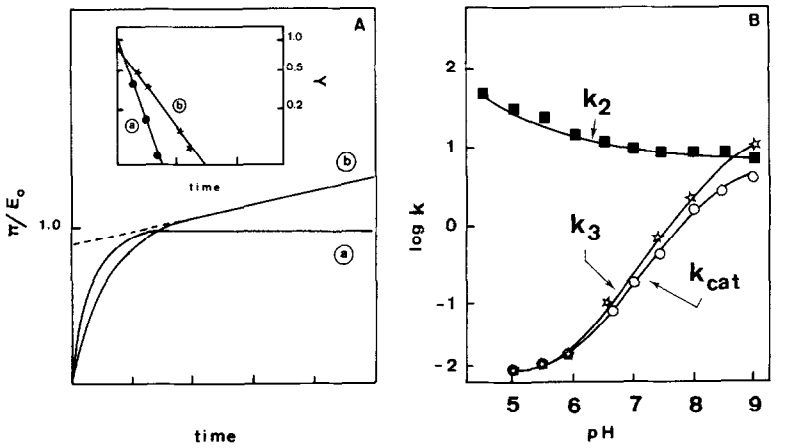

Fig. 2- A - Transient kinetics of 2,4-dinitrophenylphosphate hydrolysis by U-47 phosphatase at pH 5.0 (a) and pH 7.5 (b), 10°. $[E_o]$ = 8 µM and $[S_o]$ = 1 mM. Time scales : 80 msec per division (a) and 200 msec per division (b). Y is the difference between the extrapolation of the steady state and the experimental curve. Inset : log plot demonstrating first-order kinetics for the burst. - B - pH-dependence of k_{cat}, k_2, k_3.

per mole of dimeric phosphatase appears in the presteady state period. The first order rate constant for the phosphorylation of the active site is k_2 = 30 sec^{-1}, the first order rate constant for the dephosphorylation of the monophosphoenzyme (steady state) is k_3 = 0.015 sec^{-1}. Variations with pH of burst values, of k_2, of k_3 and of k_{cat} are given in Figures 1A and 2B. The decrease of the observed burst amplitude at alkaline pH is due to the fact that k_3 increases rapidly while k_2 remains approximatively invariant; k_3 becomes equal or higher than k_2 in the region of pH 8.5-9. Identical burst amplitude, k_2, k_3 and k_{cat} values are found with p-nitrophenyl phosphate and 2,4-dinitrophenyl phosphate at pH 7.5. Moreover, the mode of activation of the mutant enzyme does not affect transient kinetics. Identical data were obtained for U-47 phosphatase activated with Zn^{2+} alone or with Zn^{2+} and inorganic phosphate provided that the final inorganic phosphate concentration in the stopped-flow experiment was at least 100 times lower than the substrate concentration. The non-activated phosphatase does not give a burst at acidic or alkaline pH ($\Pi/E_o < 0.1$).

DISCUSSION

Wild type *E.coli* alkaline phosphatase is active in the dimeric form and contains 4g atoms of Zn^{2+} per mole. Activation of U-47 mutant phosphatase

restores these essential properties. Negative cooperativity was found previously for the wild type E.coli phosphatase for the non-covalent binding of P_i at alkaline pH. Negative cooperativity is also found for the activated mutant enzyme since it binds non-covalently only one P_i per dimer at pH 8.0. Although the two sites are not equivalent at acidic pH, they can both be phosphorylated with P_i or organic phosphates (AMP, nitrophenylphosphates) in the wild-type phosphatase. One site is phosphorylated very rapidly (in less than 2.5 msec) and the other slowly ($t_{1/2}$ = 10 to 30 m sec) (4). Absolute negative cooperativity, i.e. half-of-the sites reactivity, is observed under the same conditions with U-47 activated enzyme: only one phosphate is incorporated covalently from [^{32}P]AMP and a burst of only one mole of nitrophenol is observed in transient kinetics. Half-of-the sites reactivity is known for the wild type E.coli enzyme at pH 7-8(2,4). The same observation is made with the U-47 mutant phosphatase. Only one of the 2 sites is phosphorylated at a given time under steady state conditions both for the wild type and for the mutant enzyme. The mechanism for the U-47 activated phosphatase probably includes kinetic cooperativity between subunits i.e. a Flip-Flop type mechanism similar to those already proposed for wild type E.coli and intestinal phosphatases (3,8). The U-47 mutation not only alters the affinity of the essential zinc for the apophosphatase, but it also changes drastically the rate constants for phosphorylation and dephosphorylation of the active site. With the wild type phosphatase the first site that reacts at acidic pH is phosphorylated with a first order rate constant higher than 1000 sec^{-1} (4). Phosphorylation of the only site phosphorylated at pH 7-8 also occurs with a rate constant higher than 1000 sec^{-1} (4).The advantages of the U-47 activated mutant for these studies are that it displays half-of-the sites reactivity from pH 4.5 to pH 8.5 and that the phosphorylation of the active site is a fairly slow process, permitting easy analysis. The phoshorylation step (k_2) occurs at the same rate with 2 phosphates with very different leaving groups, p-nitrophenol and 2,4-dinitrophenol (pK_a are respectively 7.2 and 4.1).This is an indication that the rate of the phosphorylation step is limited by a conformational change affecting the enzyme-substrate complex structure. The rate of this conformational change is nearly independent of pH (Figure 2B). The mutation alters the rate constant for this conformational change at alkaline pH from $k_2 > 1000$ sec^{-1} for the wild type enzyme to $k_2 = 10$ sec^{-1} for the U-47 activated enzyme. Thus at pH 8.0, the rate of the conformational change is decreased by a factor at least 100, while the rate of dephosphorylation of the monophospho-derivative, k_3, is decreased only by a factor of 4.

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