Case 34
Kinetics of Alkaline Phosphatase

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Focus concept

Kinetic studies are carried out using the alkaline phosphatase, an enzyme that has an important role in skeletal mineralization.

Prerequisite

- Enzyme kinetics and inhibition.
- Basic enzyme mechanisms.

Background

Phosphatase enzymes are metalloenzymes that are widely distributed in nature, from bacteria to plants to humans and are characterized by their ability to catalyze the hydrolysis of phosphoric acid esters. The phosphatase family is a large one, and phosphatase enzymes can be divided up into four groups, depending on their preferred substrate. The particular phosphatase enzyme considered in this case, alkaline phosphatase (ALP), is a member of the phosphomonoesterase group, and it hydrolyzes monoesters, as its name suggests. Unlike some phosphomonoesterases, which are highly specific and act on only one substrate, alkaline phosphatase has a broad substrate specificity and is named alkaline phosphatase because its pH optimum is usually around 9. (Other broad-spectrum phosphoesterases with pH optima less than 7 are termed acid phosphatases.) Four isozymes[1] of ALP exist; three of which are found exclusively in intestine, placenta and in germ cells. A fourth isozyme has been termed tissue non-specific ALP (TNSALP)[2] because of its wide tissue distribution, but its distribution is hardly random, being found in abundance in hepatic, skeletal and renal tissues.

There has been a great deal of interest in the alkaline phosphatases since their discovery in 1923. But despite more than 80 years of study, there is much about the enzyme that remains unknown. At first its location in bone suggested a role in ossification, but ALP is also found in tissues that don't mineralize, indicating that the enzyme has a role in other physiological processes. Throughout the years, additional roles for ALP have been suggested, and the list is lengthy.

The TNSALP isozyme has received attention among those scientists who study bone disease because of its location in skeletal tissues. It is probably the TNSALP isozyme that is involved in mineralization, which is carried out by specialized bone cells called osteoblasts. These cells promote hydroxyapatite crystal growth and deposition on a collagenous matrix to form the bone. Growth of the calcium

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[1]Isozymes are forms of enzymes that have the same catalytic activity but differ in primary structure, usually by only a few amino acids.

[2]TNSALP is referred to as "TNAP" in some publications.

phosphate hydroxyapatite crystals first occurs in membrane-limited matrix vesicles (MV) of the osteoblasts, and TNSALP has been found in these vesicles. It has been hypothesized that the role of TNSALP is to provide phosphate for the hydroxyapatite mineral, but recent studies involving pyrophosphate have indicated that the role of TNSALP is likely to be far more complex.

The disease hypophosphatasia may yield some clues as to the function of ALP. Hypophosphatasia was first described by John Rathburn, a Canadian pediatrician, in 1948 who noted that the disease was prevalent among members of the Mennonite population in Manitoba. The severity of the disease varies widely, from death *in utero* as the most extreme case to mild cases in which the only symptom is premature loss of teeth. Serum ALP concentrations are decreased in individuals with hypophosphatasia; a mutated TNSALP gene has recently been shown to be responsible, and the severity of the disease is correlated with the extent of the mutation.

Interestingly, the physiological substrate for ALP has not been identified. But that hasn't stopped the development of clinical assays for ALP, since the enzyme readily hydrolyzes artificial substrates. Alkaline phosphatase assays are of clinical importance because increased or decreased levels of ALP are usually indicative of disease. These assays use an artificial substrate at high concentrations at nonphysiological alkaline conditions.

The kinetic studies described here are similar to the clinical assays for ALP; ie, the reactions are not carried out under physiological conditions. As the author of the studies states, "...the substrate is synthetic, the buffer contains Tris, which is also synthetic, and stimulates the activity of alkaline phosphatase in a concentration-dependent manner...the molar and ionic concentrations of the reaction mixtures are unphysiological.., the pH used may or may not reflect that occurring *in vivo*, and the temperature is ~12˚C too low for a mammalian enzyme."

That being said, the enzyme kinetic studies described here may still provide valuable insights concerning the kinetic behavior of enzymes. In this study, the intestinal form of calf ALP was used. This enzyme is a glycoprotein with a molecular weight of about 140,000 daltons. It is a dimer consisting of two nearly identical monomers. Each subunit contains a tightly bound zinc that is required to maintain the structural integrity of the ALP enzyme. In addition, there is a second less-tightly bound zinc that participates in the catalytic process. The enzyme has additional binding sites for Mg^{2+} ions, but these ions are less tightly bound than the zinc ions, and it's possible for the zinc ions to bind to these "magnesium binding sites" as well.

Questions

1. The activity of alkaline phosphatase was measured in the presence of the artificial substrate *p*-nitrophenylphosphate, as shown in Figure 34.1. ALP catalyzes the hydrolysis of the phosphate group to yield *p*-nitrophenol, which is bright yellow (the other reactants and products are colorless in aqueous solution); thus the formation of product can be monitored spectroscopically at 400 nm, the λ_{max} for *p*-nitrophenol.

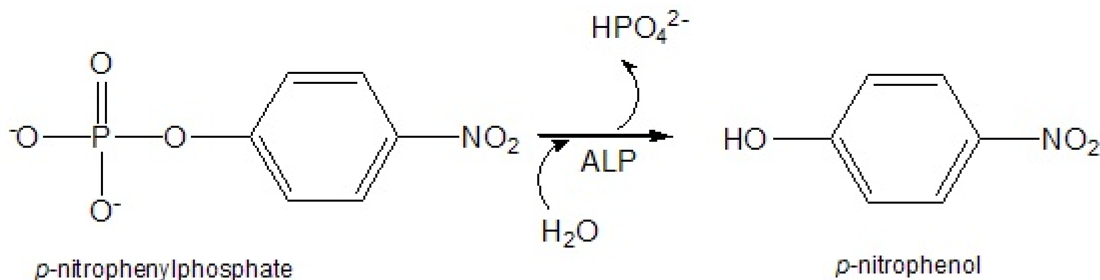

Figure 34.1: The reaction catalyzed by alkaline phosphatase.

The activity of the enzyme was measured in the absence and in the presence of 1 mM phosphate (one of the products of the reaction). The data are shown in Table 34.1. Prepare a Lineweaver-Burk plot from these data.

Table 34.1: Inhibition of mouse ALP by phosphate (data from Dean, 2002).

PNPP Concentration, mM	V_o, µmol/min(w/o inhibitor)	V_o, µmol/min(with inhibitor)
0.054	0.0065	0.0029
0.081	0.0083	0.0041
0.108	0.0105	0.0053
0.162	0.0133	0.0067
0.270	0.0167	0.0111
0.540	0.0208	0.0167

Reactions were run in a volume of 5.0 mL of 0.5 M Tris buffer (pH = 9.0) containing 5 mM $MgCl_2$ in the presence or absence of 1 mM monobasic sodium phosphate.

a. What are the K_M and V_{max} values for ALP in the absence of inhibitor? in the presence of the inhibitor?
b. What type of inhibitor is phosphate? Explain.
c. Calculate the values of α and/or α', if they are significantly different from 1. What kind of inhibitor is phosphate? What does the type of inhibition tell you about the structure of the binding site?
d. Calculate the K_i and/or K_i' (whichever is appropriate) for phosphate (Hint: You can obtain these values from α and α').

2. The activity of the enzyme was measured in the absence and in the presence of another inhibitor, 10 mM phenylalanine. The data are shown in Table 34.2. Prepare a Lineweaver-Burk plot from these data.

Table 34.2: Inhibition of ALP by phenylalanine (data from Dean, 2002).

PNPP Concentration, mM	V_o, μmol/min(w/o inhibitor)	V_o, μmol/min(with inhibitor)
0.081	0.0083	0.0061
0.108	0.0105	0.0067
0.162	0.0133	0.0078
0.270	0.0167	0.0093
0.540	0.0208	0.0100

Reactions were run in a volume of 5.0 mL of 0.5 M Tris buffer (pH = 9.0) containing 5 mM $MgCl_2$ in the presence or absence of 10 mM phenylalanine.

a. What are the K_M and V_{max} values for ALP in the absence of inhibitor? in the presence of the inhibitor?
b. What type of inhibitor is phenylalanine? Explain.
c. Calculate the values of α and/or α', if they are significantly different from 1. What kind of inhibitor is phenylalanine? Explain, including what is happening on a molecular level.
d. Calculate the K_i and/or K_i' (whichever is appropriate) for phenylalanine. (Hint: You can obtain these values from α and α').

3. The activity of the enzyme can be measured in the presence of Mg^{2+} and Zn^{2+}. The results are shown in Figures 34.2 and 34.3 below. Write a paragraph discussing the effect of the ions on ALP activity.

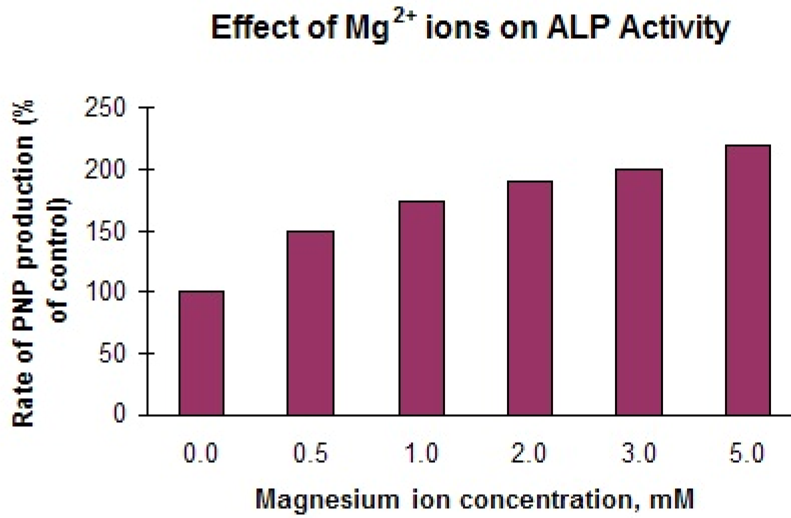

Figure 34.2: Magnesium ions affect ALP activity in a dose-dependent manner (from Dean, 2002).

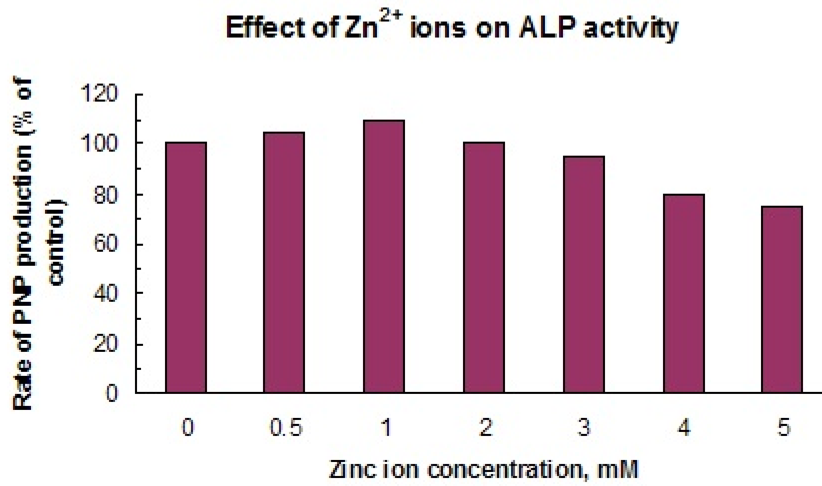

Figure 34.3. Zinc ions effect ALP activity (from Dean, 2002).

References

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