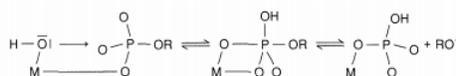


## 2.6: Ester Hydrolysis and Phosphoryl Transfer

Hydrolysis of carboxylic and phosphoric esters is also a slow process at neutral pH, and is catalyzed by acids and bases by mechanisms similar to those involved in amide and peptide hydrolysis. Metal ions are also good catalysts of both carboxylic and phosphoric ester hydrolysis, typically with rate increases much higher than those observed for hydrolysis of amides or peptides (Table 2.8). The ability of metal ions to coordinate to the carbonyl oxygen—which is higher in amides than in esters—is inversely correlated with their catalytic properties, perhaps because the main role of the metal ion is not in polarizing the carbonyl group, but in providing a metal-coordinated hydroxide as the attacking nucleophile.<sup>108</sup> For the hydrolysis of phosphate esters, it is difficult to draw conclusions based on experience with carboxylic esters, because, although the coordinating ability of the phosphoric oxygen may be higher, thus favoring the polarizing role of the metal, the nucleophilic attack is also likely to be easier, because the energy of the trigonal bipyramidal intermediate is probably rather low. Base-catalyzed hydrolysis of phosphate esters occurs with inversion of configuration, and this supports the existence of a trigonal bipyramidal intermediate.<sup>140</sup> The metal acts both as activator of substrate through binding and as Lewis acid to provide the OH moiety for the nucleophilic attack:



(2.20)

As with peptide hydrolysis, several enzyme systems exist that catalyze carboxylic and phosphoric ester hydrolysis without the need for a metal ion. They generally involve a serine residue as the nucleophile; in turn, serine may be activated by hydrogen-bond formation—or even proton abstraction—by other acid-base groups in the active site. The reaction proceeds to form an acyl- or phosphoryl-enzyme intermediate, which is then hydrolyzed with readdition of a proton to the serine oxygen. Mechanisms of this type have been proposed for chymotrypsin.<sup>141</sup> In glucose-6-phosphatase the nucleophile has been proposed to be a histidine residue.<sup>142</sup>

Again by analogy with peptide hydrolysis, metalloenzymes catalyzing ester hydrolysis may take advantage of additional chemical features provided by amino-acid residues present in the active-site cavity. This situation occurs with carboxypeptidase,<sup>143</sup> which shows esterase activity *in vitro*. Although the rate-limiting steps for carboxylic esters and peptides may differ, several features, such as the pH dependences of  $k_{\text{cat}}$  and  $K_{\text{m}}$  and the presence of two spectroscopically observable intermediates, point to substantially similar mechanisms. On the other hand, carboxylic ester hydrolysis catalyzed by carbonic anhydrase seems to rely on fewer additional features of the active-site cavity, perhaps only on the presence of a metal-coordinated hydroxide that can perform the nucleophilic attack on the carbonyl carbon atom.<sup>47</sup>

Metalloenzyme-catalyzed phosphoric ester hydrolysis can be illustrated by alkaline phosphatase, by far the most-investigated enzyme of this class. The protein is a dimer of 94 kDa containing two zinc(II) and one magnesium(II) ions per monomer, and catalyzes, rather unspecifically, the hydrolysis of a variety of phosphate monoesters as well as transphosphorylation reactions. The x-ray structure at 2.8 Å resolution obtained on a derivative in which all the native metal ions were replaced by cadmium(II) reveals three metals in each subunit, all located in a single binding region (Figure 2.32). In the native enzyme  $M_1$  and  $M_2$  sites are occupied by zinc and  $M_3$  by magnesium.<sup>144</sup>  $M_1$  was first reported to be coordinated to three histidine residues (His-331, 372, and 412 in Figure 2.32). Further refinement indicated that Asp-327 could be a ligand to  $M_1$ , in the place of His-372.<sup>145</sup>  $^1\text{H}$  NMR spectroscopy of the enzyme with cobalt substituted in the  $M_1$  site shows that there are three exchangeable protons sensing the paramagnetic metal ion.<sup>146</sup> They could come from three histidine NHs, or from two histidine NHs and another group containing the exchangeable proton very close to the metal ion, like an arginine. Protein ligands to  $M_2$  are Asp-369, His-370, and Asp-51, the latter probably bridging  $M_2$  to  $M_3$  with the other carboxyl oxygen.  $M_3$  is coordinated, in addition, to Asp-51, to Asp-153, to Thr-155, and to Glu-322. Several spectroscopic pieces of evidence on the native and metal-substituted derivatives indicate that  $M_1$  is five-coordinate, but  $M_2$  and  $M_3$  are six-coordinate, probably with water molecules completing the coordination spheres.<sup>28</sup>

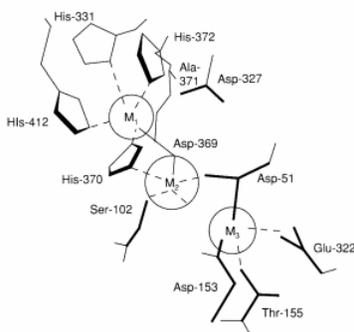


Figure 2.32 - Schematic drawing of the active-site cavity of a subunit of alkaline phosphatase.<sup>28,144,145</sup> The catalytic metal is labeled  $M_1$ . The  $M_1$ - $M_2$  distance is = 4 Å, the  $M_2$ - $M_3$  distance is = 5 Å, and the  $M_1$ - $M_3$  distance is = 7 Å.<sup>144,145</sup>

$M_1$  is essential for activity, but full catalytic efficiency is reached only when all metal ions are present. These data suggest that maximum activity is the result of fine-tuning several chemical properties of the active site as a whole, including the nature of the  $M_1$  metal, which can be only zinc or cobalt (Table 2.4).

A further key feature of the active site is the presence of a serine residue (Ser-102), the oxygen atom of which is close to the  $M_1$  -  $M_2$  pair (especially to  $M_2$ ), although not at direct binding distance according to the crystal structure. There is ample and direct evidence that Ser-102 is reversibly phosphorylated during the course of the catalytic reaction, and that  $M_1$  is able to coordinate a phosphate ion.<sup>28</sup>

Another crucial piece of information obtained by physico-chemical techniques is that the lability of the phosphoseryl intermediate and the catalytic activity increase with pH, depending on the state of ionization of an active-site group, which is most likely a water molecule coordinated to  $M_1$ .<sup>147</sup> Thus the active form of the enzyme is again a metal-hydroxide species. Furthermore, an inactive derivative with copper ions in the  $M_1$  and  $M_2$  sites shows evidence of magnetic coupling between the metal ions, of the magnitude expected if the two metals shared a common donor atom.<sup>148</sup> Likely candidates are a bridging hydroxide ion or Ser-102, which thus might be somewhat mobile relative to the position occupied in the x-ray structure, and demonstrate its potential ability to be activated for the nucleophilic attack by coordination to a metal ion. Such a mechanism would be an "inorganic" version of the type of activation postulated for chymotrypsin and other hydrolyases.

A possible mechanism for alkaline phosphatase-catalyzed phosphoric ester hydrolysis could involve the following steps (Figure 2.33):

1. Binding of the phosphate group to  $M_1$ —in the place of a water molecule—by one of the nonprotonated oxygens, and subsequent activation of the phosphorus atom for nucleophilic attack. The binding of the substrate may be strengthened by interaction with the positively charged Arg-166 residue<sup>149</sup> (not shown). The steric alteration in the active site could cause movement of Ser-102 toward  $M_2$ , with deprotonation upon binding.
2. Nucleophilic attack on phosphorus by the coordinated serine alkoxide, cleaving the ester bond and liberating the alcohol product.
3. Formation of the phosphoseryl intermediate with cleavage of the  $M_1$ - phosphate bond, decreasing the  $pK_a$  of the second coordinated water molecule, the proton of which could be taken up by the leaving alcohol.
4. Attack by the metal-coordinated hydroxide on the phosphoryl derivative, possibly with  $M_2$  again polarizing the seryl oxygen, yielding a free phosphate ion coordinated to  $M_1$ . A further water molecule could aid in the liberation of phosphate via an associative mechanism.

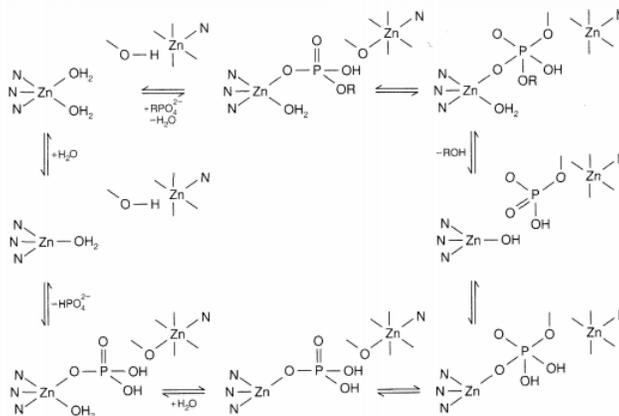


Figure 2.33 - Possible catalytic cycle of AP.

In the presence of alcohols, alkaline phosphatase displays transphosphorylation activity, i.e., hydrolysis of the starting ester and esterification of the phosphate group with a different alcohol. This ability is easily understood if one keeps in mind that the reaction depicted above is reversible, and that a different alcohol may be involved in the formation of the ester bond. Most group-transfer reactions catalyzed by metalloenzymes are likely to proceed through the same elementary steps proposed for hydrolytic reactions.

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