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# The putative common mechanism for inactivation of alkaline phosphatase isoenzymes

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**Abstract**

Alkaline phosphatase (E.C. 3.1.3.1) is a family of dimeric metalloenzymes with a complex inactivation mechanism that still remains to be elucidated. We have put forward a novel mechanism of *Escherichia coli* alkaline phosphatase inactivation, based on experimental as well as structural data for this isoenzyme. It suggests several stages of disruption of the intersubunit contact before the loss of enzyme activity. Here we present initial evidence that the mechanism could also be valid for mammalian isoenzymes. The evidence includes thermal inactivation kinetics and the structural similarity of different alkaline phosphatases inferred from the alignment of their amino acid sequences. The suggested inactivation mechanism of alkaline phosphatases is supported by recent experimental data showing an important role of three intersubunit contact areas in determining the stability of alkaline phosphatase isoenzymes. 

*Keywords:* Alkaline phosphatase; Conformational lock; Stability; Structure; Denaturation; Oligomers; Isoenzymes

## 1. Introduction

Alkaline phosphatase (AP) is a widely occurring enzyme that catalyses the hydrolysis of monoesters of phosphoric acid. The isoenzyme from *Escherichia coli* is the only AP for which the crystal structure is available [1]. The molecule is a symmetrical dimer with a large intersubunit contact surface (Fig. 1). The active centers are situated in the clefts on each subunit. Considerable research has been devoted to the study of the thermal stability of APs. It was shown that alkaline phosphatase possesses a complex pattern of inactivation behavior. No universally accepted mechanism of the inactivation of APs has been suggested so far. In our previous paper [2], we were first to put forward a mechanism of *E. coli* AP denaturation based on structural data. According to that model, several hidden stages without any loss in enzyme activity should exist. These stages were explained by a sequential destruction of independent intersubunit contact sites in the dimeric

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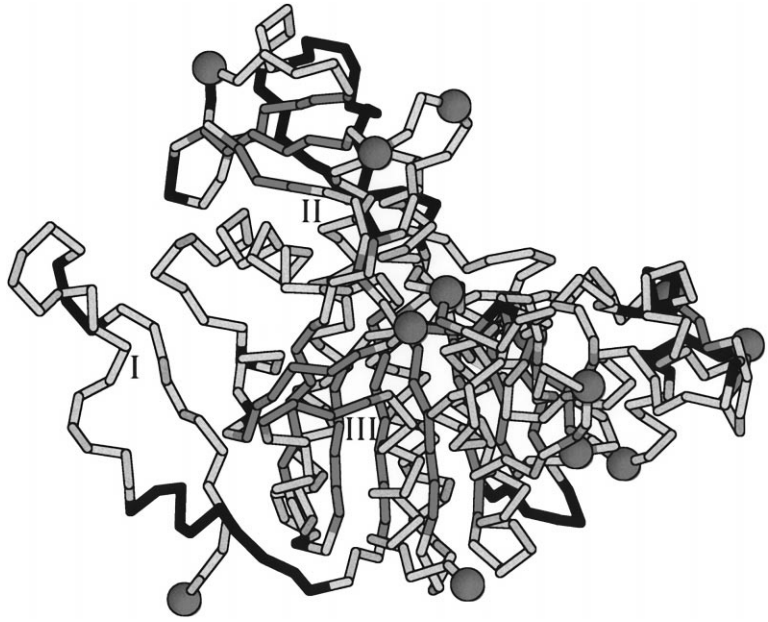


Fig. 1. Structure of alkaline phosphatase of *E. coli*. The C-α trace of one monomer is shown. The deletions, not found in bovine intestinal AP, are in black. The sites of insertions in bovine intestinal AP are shown with grey spheres. The β-sheet is dark grey. The separate contact sites of intersubunit interface, according to Ref. [2], are shown with roman numbers.

enzyme, leading to the dissociation of protein into inactive monomers (Eq. (1)). An approximate equation (Eq. (2)) was suggested for the assessment of the minimum number $n$ of hidden kinetic stages. This value, determined from the experimental data for the *E. coli* AP [2], agrees with the number of independent contact sites in the subunit interface of this enzyme (namely, three).

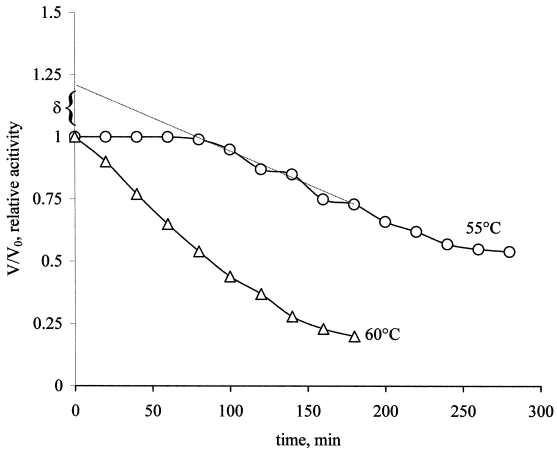


Fig. 2. The kinetics of thermal inactivation of bovine intestinal AP at 55°C and 60°C. $\delta = 0.2$, $n = 2.75$. Bovine intestinal AP was purchased from BDH, and was further purified as described [13]. Thermal inactivation was carried out at pH 7.5 in 0.05 M Tris–HCl buffer. The enzyme concentration was 2.6 μg/ml. The AP activity was measured using 6 mM *p*-nitrophenylphosphate as a substrate, in 0.05 M Tris–HCl buffer, pH 8.5, containing 1 mM $MgCl_2$. Changes in absorbance were measured at 400 nm. The initial enzyme activity was 0.07 μM/min.

$$E_2(3), \textit{stable} \leftrightarrow E_2(2) \leftrightarrow E_2(1), \textit{labile} \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} 2E_1 \xrightarrow{k_d} 2E_d \quad (1)$$

$$n = \frac{0.13 + \delta}{0.13 - 0.05\delta} \quad (2)$$

where $\delta$ is the excess over unity on the ordinate as shown in Fig. 2.

## 2. Discussion

It is well-known that the AP isoenzymes differ in their thermostability. However, they

still may share a common overall mechanism of inactivation. It is interesting to test whether the mechanism suggested for the *E. coli* isoenzyme is suitable for the APs from higher organisms. This hypothesis should be checked from both the experimental and theoretical (structural) point of view. We have chosen as a «model» mammalian AP the enzyme from bovine intestine because it (1) has the highest activity, (2) is commercially available, and (3) has a amino acid sequence which has recently been determined.

Depending on the specific experimental conditions, the mechanism (1) can result in completely different types of inactivation curves. However, the conditions must exist where one can observe a kinetic curve with the induction period. We have recently demonstrated that *E. coli* AP can show this behavior when inactivated in the presence of urea. Increasing the temperature can abolish the induction period. The mammalian APs are less stable than that of *E. coli*, so at similar temperatures, the inactivation goes without urea. The thermoinactivation curves for bovine intestinal AP, obtained at 55°C and 60°C, are presented in Fig. 2. The induction period is observed at 55°C. The minimum number $n$ of hidden stages, calculated by Eq. (2) using the $\delta$ value 0.2, is about 3. Thus, the experiment tends to support the suggested inactivation mechanism.

What can be the structural basis for such behavior? The three-dimensional structure is not available for APs of higher organisms and the level of similarity between bacterial and eukaryotic APs (about 30%) is not sufficient for a detailed homology modelling procedure. Nevertheless, some useful information can still be derived from the comparison of amino acid sequences of the enzymes. We have carried out the alignment of amino acid sequences of APs from *E. coli* and bovine intestine, as well as from several other sources (Fig. 3). The sequences were obtained from the database of amino acid sequences SwissProt [3]. The initial multiple alignment was obtained using the program Clustal [4], and was adjusted manually with the use of the structural information of *E. coli* enzyme. To improve the quality of alignment, the results of the prediction by the program PHDsec [5–7] of the secondary structure of bovine intestinal enzyme were also used. There is a substantial homology between mammalian APs, so the alignment presented here is largely similar to that in the paper [8] concerning the human AP isoenzymes. It is especially true for the core region of the molecule (the central β-sheet and flanking α-helices). The only exception in this part of the molecule is the strand F, which was misaligned in Ref. [8]. The secondary structure prediction also suggests that the strand E (which is on the edge of the β-sheet and far from the subunit interface) may replace a larger α-helical fragment in bovine intestinal AP.

All the insertions and deletions were made in the surfacial regions of the molecule. During the refinement of the alignment, all the glycosylation sites were also positioned at the surface of the molecule. There are only two such sites in the bovine intestinal AP, both in large surface insertion loops.

The bovine intestinal AP contains five cysteines, which are conserved within mammalian APs. The cysteines 101 and 121 are possible candidates for a disulfide bond formation, as well as two cysteines in the C-terminal part of the molecule (467 and 474). It is not likely that the fifth free cysteine is involved in the intersubunit contact.

The highly conserved β-sheet and helices suggest that the overall structure is the same for the whole AP family. However, some regions in the alignment present problems. For example, the amino-terminal loop 1–40, while clearly homologous in the bovine intestinal AP (10 conserved residues), contains a somewhat confusing deletion L33-K40. This can be partly compensated by the deletion Q13-D15. There is another possibility—a part of the amino-terminal loop in bovine intestinal enzyme may be α-helical, as is strongly suggested by PHDsec

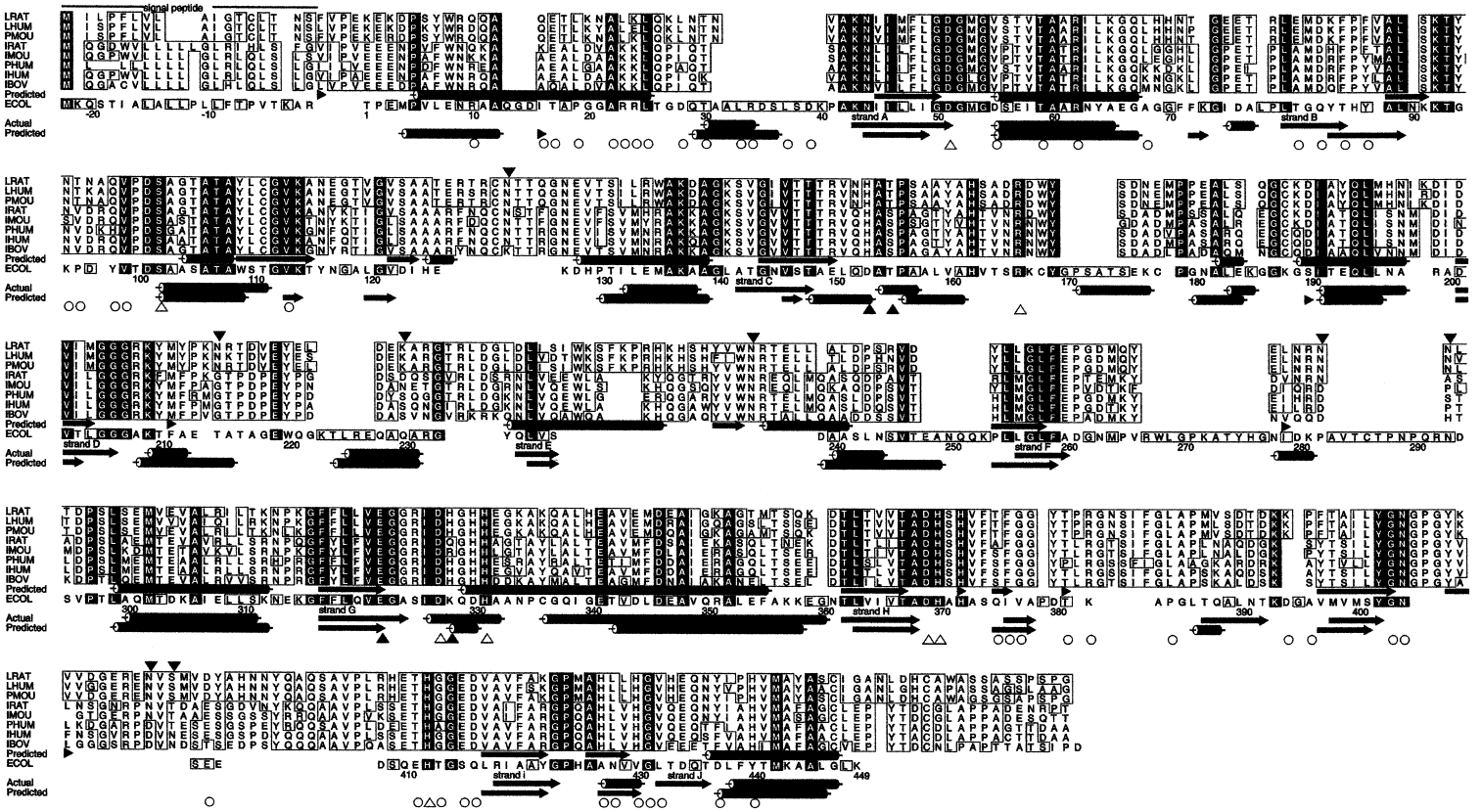


Fig. 3. Sequence alignment of alkaline phosphatases (APs). The following isoenzymes were compared: LRAT—tissue-nonspecific from rat (accession code in SWISSPROT P08289), LHUM—tissue-nonspecific from human (P05186), PMOU—mouse placental (P09242), IRAT—rat intestinal (P15693), IMOU—mouse intestinal (P24822), PHUM—human placental (P06861), IHUM—human intestine (P09923), IBOV—bovine intestinal (P19111), ECOL—*E. coli* (P00634). Strictly conserved residues are shadowed. The numbering is for *E. coli* AP. Under the IBOV sequence, the result of secondary structure prediction by the PHDsec program [5–7] for bovine intestinal AP is shown ('Predicted'). Under the ECOL sequence, the actual secondary structure of *E. coli* AP is shown ('Actual'). To evaluate the efficiency of the prediction method, the predicted secondary structure of *E. coli* AP ('Predicted') is also included under the actual structure. Other designations: upward hollow triangle—residues involved in the active site of *E. coli* AP, upward gray triangle—residues involved in magnesium binding site, downward black triangle—putative glycosylation sites in mammalian APs, hollow circle—residues on the subunit interface (those with a buried surface, upon dimer formation, of more than 10 Å$^2$, as calculated with the program WHATIF by G. Vriend). The carboxyl-terminal part of bovine intestinal AP, which is cleaved during the protein processing, is not shown. The graphical representation of the alignment is made with ALSCRIPT program [14].

program, and as it was first speculated [8]. It is also supported by the fact that, when modelled by an ideal α-helix, the segment 9–26 of bovine intestinal AP is amphiphilic, suggesting that one side of the helix is buried and the other exposed.

In the 372–412 region of *E. coli* AP (top minidomain), the homology with mammalian APs is weak, so the results of secondary structure prediction from PHDsec were used to improve the significance of the alignment. We suggest that the structural core of this domain, which is formed in *E. coli* AP by a three-stranded β-sheet, persists in mammalian APs, with the insertions forming surface loops without major secondary elements.

The top minidomain is of special interest because it contributes both to intersubunit interactions and (in mammalian isoenzymes) to the binding of various ligands, including collagen and some amino acids [9]. The domain is larger in mammalian APs as compared to *E. coli* enzyme due to several (a total of 32 residues) insertions in the region 381–408. However, in mammalian APs, the surface loop 267–292, which in *E. coli* enzyme forms the «back» part (as viewed from the subunit interface) of the top minidomain, is substituted by a shorter sequence. Interestingly, this five-residue sequence is homologous to the turn of the loop 267–292. Thus, the top domain of mammalian APs is only about 10 residues larger than that of *E. coli* AP. The insertions in the 381–408 region are likely to spatially compensate for the deletion of the 267–292 loop.

In our previous studies, we have suggested that the structural basis for the inactivation mechanism of *E. coli* AP (with several hidden stages preceding the dissociation of the dimer) can be a large intersubunit surface, with several independent contact sites. These three contact sites are shown on Fig. 1. The summary of structural differences between APs of *E. coli* and bovine intestine is also shown in Fig. 1.

The AP from bovine intestine retains a large contact area with several independent subcontacts. The contact site II is in fact created by the antiparallel β-strand (strand I), that moves the strands B and J out of the plane of the main β-sheet. This feature is conserved in mammalian enzymes. The N-terminal region is the most problematic; here the contact surface shrinks in mammalian enzymes compared to that of *E. coli*, and this could be the possible reason for mammalian APs being less stable. In Fig. 3, residues that have the buried surface larger than 10 $Å^2$ upon dimer formation are shown as white circles. The homology between the bovine intestinal AP and the *E. coli* enzyme in the subunit contact region is lower than the average. However, there are still 11 conserved residues out of the 49.

The experimental data available for different AP isoenzymes show that the enzyme has a complex mechanism of inactivation, which cannot be reduced to a simple one-stage scheme. The hypothesis that we have put forward can explain some data available in literature, including that obtained in recent studies. One of the implications of this hypothesis is that under certain conditions, several active isoforms of the enzyme should exist. Being equally active, these isoforms cannot be distinguished solely by measuring enzymatic activity, but they should differ in their biophysical properties, such as lability towards denaturing agents.

Other workers [10,11] have studied the reversible denaturation of *E. coli* AP by a variety of methods, including time-resolved room temperature phosphorescence from tryptophan residues. They found that when *E. coli* AP is inactivated by acid or guanidine chloride, active, but structurally less rigid intermediates are formed. Similar behavior was observed during renaturation experiments, when enzyme activity, on the one hand, and tryptophan phosphorescence and protein lability, on the other, recover their initial native level during completely different time scales.

In the context of our inactivation mechanism, these results could be explained as follows. The state of Trp109 of *E. coli* AP (from which the

phosphorescence is observed [10]), may be sensitive to the conformation of the loop 1–29 of the other subunit. The helix 102–111, where tryptophan 109 is situated, is hydrogen-bonded to the near 112–116 segment, which takes part in the contact site II (Fig. 1). The intermediate labile forms of the enzyme could correspond to dimeric AP molecules with partially disrupted contacts in the region of the 1–29 loop. This disruption may lead to more labile (but still active) enzyme isoforms with altered Trp109 phosphorescence due to changes in mobility of the amino acid chain near the contacts.

Another consequence of the suggested inactivation mechanism is the important role of the top domain in maintaining the native structure of AP. The disruption of intersubunit contact III (within or near the top domain) and the dissociation of dimer into monomers can disrupt the structure of the 410–416 loop (*E. coli* numbering), where His412 is a ligand to one of the active site zinc atoms. The influence of the top domain on the thermal stability of AP isoenzymes has been recently studied by swapping the surface loop in the top domains of these two APs using genetic engineering [9]. It is known that human placental AP is much more stable than the tissue-nonspecific enzyme. This surface loop (400–433 in placental AP numbering), as it is clear from sequence alignment, should be involved both in subunit interaction and binding the zinc of the active site (His432 of placental AP corresponds to His412 of *E. coli*). When the surface loop from tissue-nonspecific AP was introduced into placental enzyme, it acquired the stability similar to that of tissue-nonspecific AP. The reverse replacement increased the stability of tissue-nonspecific enzyme. Thus, the structure of the top domain is one of the determining factors in the stability of APs.

The important role of another intersubunit contact site (I in Fig. 1) for the stability of AP has been demonstrated [12]. It was shown that the proteolytic cleavage of an amino-terminal decapeptide from *E. coli* alkaline phosphatase results in an enzyme with a reduced affinity to zinc in one of the metal-binding sites. This modified enzyme easily dissociates into inactive monomers. In the context of our suggested mechanism, this data can be explained by a weaker intersubunit interaction in the N-cleaved enzyme. As a result, the binding of zinc atom by one of its ligands, for example His412, is reduced.

## 3. Conclusion

The data obtained so far on the inactivation of AP isoenzymes tend to support the hypothesis that the structure of subunit interface is an important factor determining the stability of AP. Taking into account experimental evidence for various isoenzymes, there may be a common mechanism for inactivation of APs. The structural foundation for this suggestion is based on the similarity of their amino acid sequences. As long as no X-ray structural data is available for the APs of higher organisms, the hypothesis about the common inactivation mechanism of APs can be useful in directing further experimental studies.

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