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Effect of a T81A mutation at the subunit interface on catalytic properties of alkaline phosphatase from *Escherichia coli*

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Abstract

Although alkaline phosphatase (APase) from *Escherichia coli* crystallizes as a symmetric dimer, it displays deviations from Michaelis–Menten kinetics supported by a model describing a dimeric enzyme with conformationally and kinetically non-equivalent subunits. The proposed model, explaining the mechanism of substrate hydrolysis, encompasses a conformational change mediated by subunit interactions [S. Orhanović, M. Pavela-Vrančič, Eur. J. Biochem. 270 (2003) 4356–4364]. The significance of interactions at the subunit interface and the involvement of the β-pleated sheet stretching from underneath the active site to the subunit surface, in the catalytic mechanism, has been probed by site-directed mutagenesis. The mutant APase, carrying alanine in place of Thr81, was analyzed in comparison to the wild-type protein. The T81A mutation, introduced at the subunit interface, significantly affected the protein kinetic properties, emphasizing the importance of subunit interactions in the catalytic process.

Keywords: Alkaline phosphatase; Thr81Ala mutation; Kinetic properties

1. Introduction

A kinetic behaviour, often described as negative cooperativity, or in the extreme case as half-of-the-sites reactivity, is a frequent kinetic feature of oligomeric enzymes [1–5]. Alkaline phosphatase (APase, E.C. 3.1.3.1.) is one of many enzymes that display deviations from Michaelis–Menten kinetics resembling negative cooperativity [6,7]. A possible advantage of such a kinetic behaviour is not well understood. Investigation of competitive inhibition led to the conclusion that pronounced deviations from Michaelis–Menten kinetics, in the presence of a competitive inhibitor, could only be assessed by a kinetic model assuming kinetically non-equivalent subunits [7]. APase from *Escherichia coli* is a dimer composed of identical monomers (449 residues, MW 47 199). According to the crystal structure determined at 2 Å resolution, the most prominent structural feature of the monomer is a 10-stranded β-sheet with an active site at the carboxyl side of the β-sheet. APase from *E. coli* has three metal binding sites in the vicinity of the active site residue Ser102 [8]. Both, positive cooperative interactions for binding of Zn^{2+} ions to the M1 binding site, and negative cooperative interactions for binding of activating cations to the M3 binding site have been detected [9,10]. Kinetic data, obtained from examining the influence of metal ions on the catalytic properties of APase and the observed asymmetry, indicate that asymmetry is not induced by variable metal ion content. The mode of APase activation with Mg^{2+} emphasized the importance of allosteric interactions and conformational changes in the reaction mechanism of APase from *E. coli*. A model describing APase as an asymmetric dimer, activated by Mg^{2+} ions, has been proposed [11]. Since both substrate (phosphomonoester) and product (inorganic phosphate; P_i) bind in the same manner to the active site, increased affinity for the substrate simultaneously increases the affinity for the product, resulting with reduced k_{cat}. According to the suggested model, the problem of high-affinity substrate binding, and an easy or controlled release of a valuable product has been resolved by alteration between high-affinity/low k_{cat} and low-affinity/high k_{cat} subunit conformations. An advantage of such an asymmetric dimer configuration would be a possibility of enhancing the reaction rate with a ligand capable of inducing

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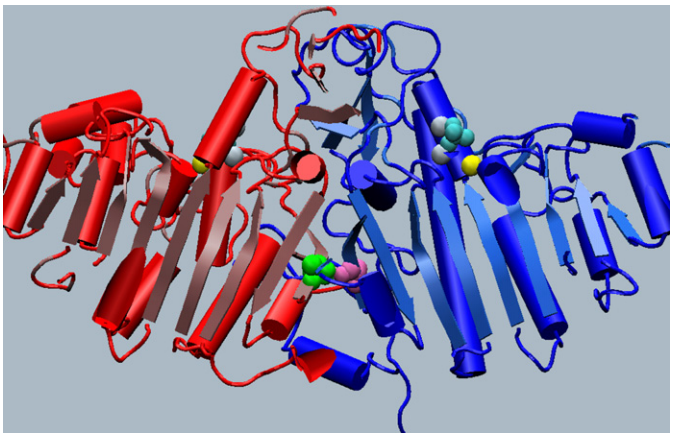

Fig. 1. Crystal structure of dimeric alkaline phosphatase from *E. coli*, showing Thr81 at the subunit inteface (green, pink). Metal ions and phosphate bound in the active site region are displayed: Zn^{2+} (grey), Mg^{2+} (yellow), and phosphate (tan).

the same conformational change as the substrate. At elevated ligand concentration, the conformational change would occur at a higher rate. It is possible, as suggested from the crystal structure determined by Stec et al. [12], that the conformational difference between the subunits is small and restricted to the active site region. In absence of large conformational changes, conformational information could be transferred between active sites and across the subunit interface by means of rigid segments in the polypeptide structure, such as the β-pleated sheet stretching from underneath the active site to the subunit interface. β-Pleated sheets from neighbouring subunits are in contact *via* hydrogen bonds between pairs of Thr81 and Gln83 residues from adjacent subunits (Fig. 1). The contribution of these bonds to the catalytic mechanism has been probed by site-directed mutagenesis. Upon replacement of Thr81 with alanine, stability of the dimeric structure of metal-free APase, susceptibility to thermal denaturation and kinetic properties of the T81A mutant protein were assessed, and compared to the wild-type enzyme.

2. Materials and methods

2.1. Site-directed mutagenesis

The wild-type APase gene (*phoA*) was PCR amplified using bacterial DNA isolated from the *E. coli* strain AB1157 as the template. PCR was performed using primers complementary to the 5′-terminal end of the *phoA* open reading frame and the region downstream of the *phoA* termination signal, except for several base changes introduced to create *Rca*I and *Bam*HI restriction sites. The PCR product was directly cloned into the pCR®2.1-TOPO vector (Invitrogen). The *Rca*I–*Bam*HI fragment, containing the *phoA* gene, was subcloned into the pET11d expression vector (Novagene) and transformed into the *E. coli* strain JM109. Site-directed mutagenesis was conducted using the GeneEditor™ mutagenesis system (Promega). The mutagenic oligonucleotide (37-mer) contained two mutations of the *phoA* sequence, one to replace Thr81 with alanine and one to create a *Nae*I restriction site, without altering the amino acid sequence. Mutant plasmids were identified by *Nae*I restriction analysis. The mutation was verified by sequencing the entire *phoA* gene at Mycrosynth, Balgach, Switzerland.

2.2. Expression and purification

The *E. coli* strain E15, carrying a deletion in the *phoA* gene, was transformed with the pET11d expression vector containing the *phoA* gene, under control of the T7 promotor, encoding either wild-type APase or the T81A mutant protein. The E15 strain was co-transformed with the pGP1-2 plasmid containing the gene for T7 RNA polymerase under control of a thermolabile repressor. Bacterial cultures were grown in LB medium (50 ml), containing ampicillin (100 μg/ml) and kanamycin (50 μg/ml), at 30 °C until an OD of 0.5 was achieved. Induction of APase production was accomplished by incubating the culture in a water bath at 42 °C for 45 min. The culture was subsequently grown overnight at 37 °C. Bacterial cells were harvested by centrifugation at 6500 × *g* for 15 min and subjected to cold osmotic shock. Prior to thermal denaturation of sensitive proteins at 75 °C for 15 min, a solution of 31 μM $ZnCl_2$ in 0.62 M Tris/HCl, pH 7.6 (1 ml) was added to the periplasmic fraction. Measurements of APase activity have shown no activity change during thermal denaturation. Denatured impurities were separated by centrifugation at 9000 × *g* for 20 min. The protein sample was applied to an anion exchange Q6 column (BioRad). The fraction displaying the highest activity contained essentially pure APase, as revealed by SDS–PAGE. The protein concentration in purified samples was determined according to Bradford [13] using BSA (bovine serum albumin) as a standard.

2.3. Stability of the dimeric structure of apo APase (metal-free APase)

The enzyme preparation was dialyzed against three changes of 50 mM tris(hydroxymethyl)aminomethane (Tris)/HCl, pH 8, containing 20 mM EDTA, followed by five changes of the same buffer without EDTA. Following dialysis, the enzyme preparation was incubated for 24 h in 20 mM Tris/HCl, pH 7.6, supplemented with Zn^{2+} and Mg^{2+} ions. Upon incubation, catalytic activity was measured and the enzyme preparation was subjected to gel filtration chromatography on a Superdex 75 column (Amersham Biosciences).

2.4. Kinetic analysis

Enzymatic activity was determined by measuring the absorbance change at 405 nm and 25 °C, due to an increasing concentration of the reaction product, *p*-nitrophenol (*p*NP), using the Lambda 40 Bio spectrophotometer (Perkin-Elmer, Norwalk, USA). Activity was measured in a reaction mixture, containing 3 ml 1 M Tris/HCl, pH 8, or 2.3 ml 0.35 M 2-amino-2-methyl-1-propanol (2A2M1P) buffer, pH 10.5, 100 μl of enzyme solution in 20 mM Tris/HCl, pH 7.6, and 100 μl of substrate solution (*p*-nitrophenyl phosphate hexahydrate, disodium salt; *p*NPP) of an appropriate concentration. Kinetic analysis was performed using *p*NPP as substrate at concentrations ranging