

# Structure, Substrate Complexation and Reaction Mechanism of Bacterial Asparaginases

Mario Sanches<sup>1,#</sup>, Sandra Krauchenco<sup>1</sup> and Igor Polikarpov<sup>\*,1</sup>

<sup>1</sup>Grupo de Cristalografia, Instituto de Física de São Carlos, Universidade de São Paulo, Av. Trabalhador São-carlense, 400, CEP 13560-970, São Carlos, SP, Brazil

Current address: Brazilian Synchrotron Light Laboratory (LNLS), Structural Molecular Biology Center, Crystallography and Spectroscopy of Biomolecules, Caixa Postal 6192, CEP 13084-971, Campinas, SP, Brazil

**Abstract:** Asparaginases and glutaminases are enzymes that catalyze the hydrolysis of asparagine or glutamine to the corresponding acid and ammonia. Based on their biochemical properties and sequence homology, this group of proteins, common to various organisms, can be divided into three families: bacterial asparaginases, plant asparaginases and enzymes similar to *Rhizobium etli* asparaginase. Bacterial L-asparaginases can be further subdivided into two types: type I, which are expressed constitutively and display enzymatic activity towards both L-asparagine and L-glutamine, and type II, induced by anaerobic conditions, which have high specific activity towards L-asparagine. Type II L-asparaginases (e.g. *E. coli* L-asparaginase) have been used in the treatment of acute lymphoblastic leukemia for many years, but their medical applications are limited by severe side effects and by the development of resistant tumors in a fraction of the patients. In this paper we review available structural and biochemical information on bacterial L-asparaginases, and focus on a detailed mechanistic description of their reaction mechanism, including the structural basis for the preference of these enzymes for threonine residues as the primary nucleophiles. The L-asparaginase enzymatic mechanism involves two catalytic triads operating at distinct steps of the reaction pathway. The first triad, Thr12-Tyr25-Glu283 (*E. coli* asparaginase numbering), acts during the acylation step starting with a nucleophilic attack of the primary nucleophile (Thr12) on the substrate, which results in an intermediate covalently bound to the enzyme. The second triad, Thr89-Lys162-Asp90, acts by activating a water molecule, which releases the product through a second nucleophilic attack. A detailed comprehension of the enzymatic mechanism of these bacterial enzymes in structural terms might open the way to design modified L-asparaginases with improved biomedical and biotechnological properties.

**Keywords:** Asparaginase, glutaminase, enzymatic mechanism, substrate recognition, leukemia treatment.

## 1. INTRODUCTION

L-asparaginase/L-glutaminase is a generic denomination for enzymes that catalyze the transformation of L-asparagine or L-glutamine into their respective acids and ammonia. These enzymes can be specific for asparagine, with negligible activity against glutamine, and thus termed asparaginases (EC 3.5.1.1), or can catalyze both asparagine and glutamine conversion, in which case they receive the denomination of glutaminase-asparaginases (EC 3.5.1.38).

Based on sequence homology analysis [1, 2], as well as on biochemical [3-6] and crystallographic data [7-20], available asparaginase sequences can be divided into three families. The first family corresponds to the bacterial-type asparaginases, the second to plant-type asparaginases and the third to enzymes similar to *Rhizobium etli* asparaginase [1].

Bacterial-type L-asparaginases can be further classified into two subtypes: type I and type II. Two types of L-asparaginases, found in *E. coli*, have been designated EC1 and EC2 in *E. coli* B [4], and AsnI and AsnII in *E. coli* K-12

[3]. Type I was found to be expressed constitutively, whereas type II is induced by anaerobiosis [3]. The L-asparaginases have  $K_m$  values for L-asparagine of  $3.5 \times 10^{-3}$  M and  $1.2 \times 10^{-5}$  M, respectively [5]. Only the type II L-asparaginases present tumor inhibitory activity and, for this reason, have been extensively studied [3]. Tumor-inhibitory asparaginases have also been isolated from a number of bacterial sources (such as *Proteus vulgaris*, *Acinetobacter glutaminasificans*, *Pseudomonas putida*, *Wolinella succinogenes* and others), but only the enzymes from *Escherichia coli* and *Erwinia chrysanthemi* (previously known as *Erwinia carotovora*) have been and are being frequently used in cancer therapy [21-23].

It was demonstrated early on that the main physiological function of asparaginases is to make cell growth possible in ammonia-deficient media by regulation of the utilization of asparagine and glutamine as a nitrogen source for cell nutrition [24, 25]. Several studies demonstrated a high activity of glutaminase and asparaginase during cell growth [3, 22, 23, 26]. Additionally, recent research has demonstrated that *Pseudomonas putida* KT2440 is unable to utilize glutamine in the absence of its functional asparaginase [25].

The expression of the asparaginases/glutaminases is strongly and specifically activated by their substrates, glutamine and asparagine, and/or reaction products, glutamic and aspartic acid [3, 25]. It has also been shown that many

\*Address correspondence to this author at the Grupo de Cristalografia, Instituto de Física de São Carlos, Universidade de São Paulo, Av. Trabalhador São-carlense, 400, CEP 13560-970, São Carlos, SP, Brazil; Tel: +55(16)3373-9874; Fax: +55(16)3373-9881; E-mail: ipolikarpov@ifsc.usp.br

preferred carbon sources such as glucose or mono- and dicarboxylic acids exert efficient carbon catabolite repression of asparaginase expression [25]. The biochemical reason for this seems to reside in the fact that Gln and Asn are eventually degraded to intermediates of the tricarboxylic acid cycle (2-oxoglutarate and fumarate, respectively) [3]. If intermediates of the cycle or metabolites are readily available, there is no need for the utilization of amino acids for this end. Additional regulation might come from the increase in the level of glutamine synthetase as a result of ammonia deprivation, which stimulates the production of asparaginases [25]. In the case of *Klebsiella aerogenes* this stimulus is necessary and sufficient for L-asparaginase expression [24].

In this paper we briefly review biomedical and biotechnological applications of bacterial asparaginases and focus on structural information and a detailed description of the mechanism of action of these enzymes.

## 2. BIOMEDICAL AND BIOTECHNOLOGICAL APPLICATIONS OF L-ASPARAGINASES

L-asparaginases are known chemotherapeutic agents against cancer, such as acute lymphoblastic leukemia and lymphosarcoma, which are used mainly in the treatment of children. Several recent reviews are available concerning the use of L-asparaginase in cancer therapy (see, for example [22, 23]). Leukaemic cells lacking the mammalian asparagine-synthetase enzyme depend on exogenous sources of asparagine for protein synthesis and survival. Theoretically, the deamination of serum asparagine selectively kills leukaemic cells, leaving normal cells, which have the ability to synthesize asparagine intracellularly, unaffected [21]. Moreover, studies of the action of asparaginase upon neoplastic cells with respect to the nutritional requirements caused by the lack of asparagine, led to the introduction of new drugs as well as the combination of asparaginases with drugs with similar modes of action in the clinical treatment of lymphoblastic leukemia [27].

L-asparaginases have been found in many mammalian and bacterial species, but only the enzymes from *E. coli* and *Erwinia chrysanthemi* have been produced on an industrial scale. Although the drugs from both sources have identical mechanisms of action and toxicities, their pharmacokinetic properties are different, and patients allergic to one drug are frequently resistant to the other. Though important in cancer therapy, the clinical utility of L-asparaginase is often limited by three factors [28-30]. First, the broad variety of side effects associated with L-asparaginase administration, including immunosuppression and pancreatitis [28]. The toxic effects fall into two main categories, those related to immunologic sensitization to a foreign protein and those related to the inhibition of protein synthesis [28]. Second, about 10% of successfully treated patients suffer a relapse with the appearance of tumors that are resistant to further L-asparaginase therapy. Lastly, prolonged treatment with L-asparaginase may improve the growth of resistant tumors and increase their metastatic activity [29]. In spite of the considerable amount of ongoing research, the molecular basis of L-asparaginase resistance, which is a major clinical problem, remains poorly understood [26]. Two possible mechanisms for L-asparaginase resistance have been proposed [29]. The first is related to an increase in asparagine

synthase levels, which has been found in the blasts cells of patients with acute lymphoblastic leukemia clinically resistant to the drug [26]. Another mechanism appears to be the induction of a host response leading to the production of anti-asparaginase antibodies, which neutralize L-asparaginases impeding their enzymatic activity [30]. A PEG conjugated or PEG *E. coli* asparaginase was found to be a useful alternative in patients with prior clinical hypersensitivity to native *E. coli* asparaginase [31]. PEG-asparaginase reduces the immunogenicity of the protein, increases its stability in plasma and provides a drug that is suitable for use in heavily pretreated patients [31].

Another potentially important application to asparaginases is in the food industry. Acrylamide in industrialized foods is largely derived from heat-induced reactions between the  $\alpha$ -amino group of the free amino acid asparagine and carbonyl groups of reducing sugars such as glucose [32]. Therefore, lowering either the asparagine or glucose content can be expected to result in reduced acrylamide formation. The available information on adverse manifestations of acrylamide and its major metabolite glycinamide indicates that neurotoxicity is a documented effect in human epidemiological studies. On the other hand, reproductive toxicity, genotoxicity, and carcinogenicity are further potential human health risks, on the basis of animal studies [32]. Therefore the use of L-asparaginases to hydrolyze asparagine in food as well as in the development of an immunoassay (ELISA) for the analysis of asparagine content could be a useful approach to reduce the extent of acrylamide formation [32].

## 3. AMINO ACID SEQUENCES

Sequence homology analyses [1] demonstrated that the amino acid sequences of all known type I and II asparaginases are very similar. The most conserved regions of their amino acid sequences are those around the active site residues. All bacterial-type sequences studied possess two highly conserved amino-acid motifs [2], [LIVM]-x(2)-T-G-G-T-[IV]-[AGS] and G-x-[LIVM]-x(2)-H-G-T-D-T-[LIVM], where the PROSITE [33] notation -x(2)- stands for -x-x- and a bracketed position shows the variability at this site. The first includes the residue corresponding to EcA Thr12 and the second includes the residues corresponding to EcA Thr89 and Asp90 which are part of the enzyme active site [7]. Another residue that shows absolute conservation is the lysine residue corresponding to EcA Lys162, which also participates in the catalytic mechanism of bacterial L-asparaginases [14]. Larger differences are mostly confined to the loops and the regions located on the molecular surface of the enzymes.

## 4. STRUCTURAL INFORMATION

Crystallographic research of asparaginases has been going on for almost forty years now. The early investigations include the crystallization and preliminary crystallographic studies of *Erwinia chrysanthemi* asparaginase [34], *Proteus vulgaris* asparaginase [35], *E. coli* asparaginase [36], *Acinetobacter glutaminasificans* glutaminase-asparaginase [37], *Pseudomonas* 7A glutaminase-asparaginase [38] and *Wolinella succinogenes* asparaginase (previously called *Vibrio succinogenes*) [39]. The first reported crystallographic model was that of *Acinetobacter glutaminasificans* glu-

taminase-asparaginase [40], a low-resolution (3Å), partially correct structure determined in the absence of a complete amino acid sequence of the enzyme.

To date the crystallographic structures of five bacterial type II L-asparaginases and L-glutaminase-asparaginases, both wild type and mutants, have been solved in the presence a number of different ligands. The wild type structures of asparaginases from *Erwinia chrysanthemi* [15, 18], *Pseudomonas 7A* [9, 13, 14], *Acinetobacter glutaminasificans* [10], *Wolinella succinogenes* [11] and *Escherichia coli* [8, 12, 19] are available at the Protein Data Base (PDB). Table 1 summarizes some pertinent data for the wild type structures of the enzymes from each of these organisms. According to their substrate specificity the enzymes have been classified as either asparaginases or glutaminase-asparaginases. The *E. chrysanthemi* enzyme has previously been described as an L-asparaginase [7], even though its activity against L-glutamine is not negligible.

Until now, the structure from the hyperthermophilic archaeon *Pyrococcus horikoshii* (PhA) is the only reported 3D X-ray model of a type I L-asparaginase [20].

#### 4.1. The Quaternary Organization

These extensive structural studies of asparaginases have led to detailed descriptions of their common structural features. All bacterial type II L-asparaginases are active as homotetramers containing approximately 330 amino acids per monomer and four identical non-cooperative active sites. According to the nomenclature introduced for the *E. coli* L-asparaginase structure [8, PDB code 3eca], the four subunits are labeled A, B, C and D. Two active sites are formed at the dimerization interface between the A and C subunits (as well as between B and D), the so-called intimate dimer, and two such intimate dimers unite to form a tetramer with 222 symmetry (Fig. 1a, b). The 222 symmetry of the homotetramer has been observed in various crystal structures of type II L-asparaginases solved to date. This is true of cases in which only one independent subunit was present in the asymmetric unit and the functionally-relevant tetramer generated by crystallographic 222 symmetry, (such as PGA [9] and ErA [16],) as well as cases in which the symmetry of the tetramer is only approximate, e.g. EcA structure [8, 16].

Although apparently the enzymes should be enzymatically competent as dimers, the formation of tetramers appears to be essential for the catalytic competence of type II L-asparaginases. Recent X-ray analysis of the EcA-II enzyme makes clear some structural reasons for why this enzyme functions as a stable tetramer and not as separate dimers [19]. The inspection of the intimate dimer in the structures of type II L-asparaginases shows that the role of tetramer formation is to guarantee proper folding of the enzyme, since the stability of the tetramer relies on the interface interactions between subunits. The assembly of the intimate dimers is mainly hydrophobic in nature and presents a number of the characteristics typical of permanent physiologically relevant complexes [41]. The tetramer keeps the most hydrophobic part of the protein buried inside its core, away from contact with solvent, providing stabilization to the structure and rendering a globular shape to the molecule, with the polarizable surface oriented towards the external medium [41]. The very robust active sites are buried at the interface between the intimate dimers and are not directly affected by pH changes.

In contrast, the recent crystal structure of the type I L-asparaginase PhA, shows it to only form an intimate dimer and not to associate as a homotetramer [20]. A clear hydrophobic patch is absent from the region of the PhA surface which corresponds to the tetramerization interface of the type II L-asparaginases. Furthermore, several polar residues located at the interface between intimate dimers, such as Arg116, Asp152 and Asp158 of EcA-II, which are conserved in all type II L-asparaginases, are missing from the sequences of PhA and EcA-II (Fig. 1c). The dimeric structure of type I L-asparaginase is, therefore, consistent with the sequence comparison analysis [1].

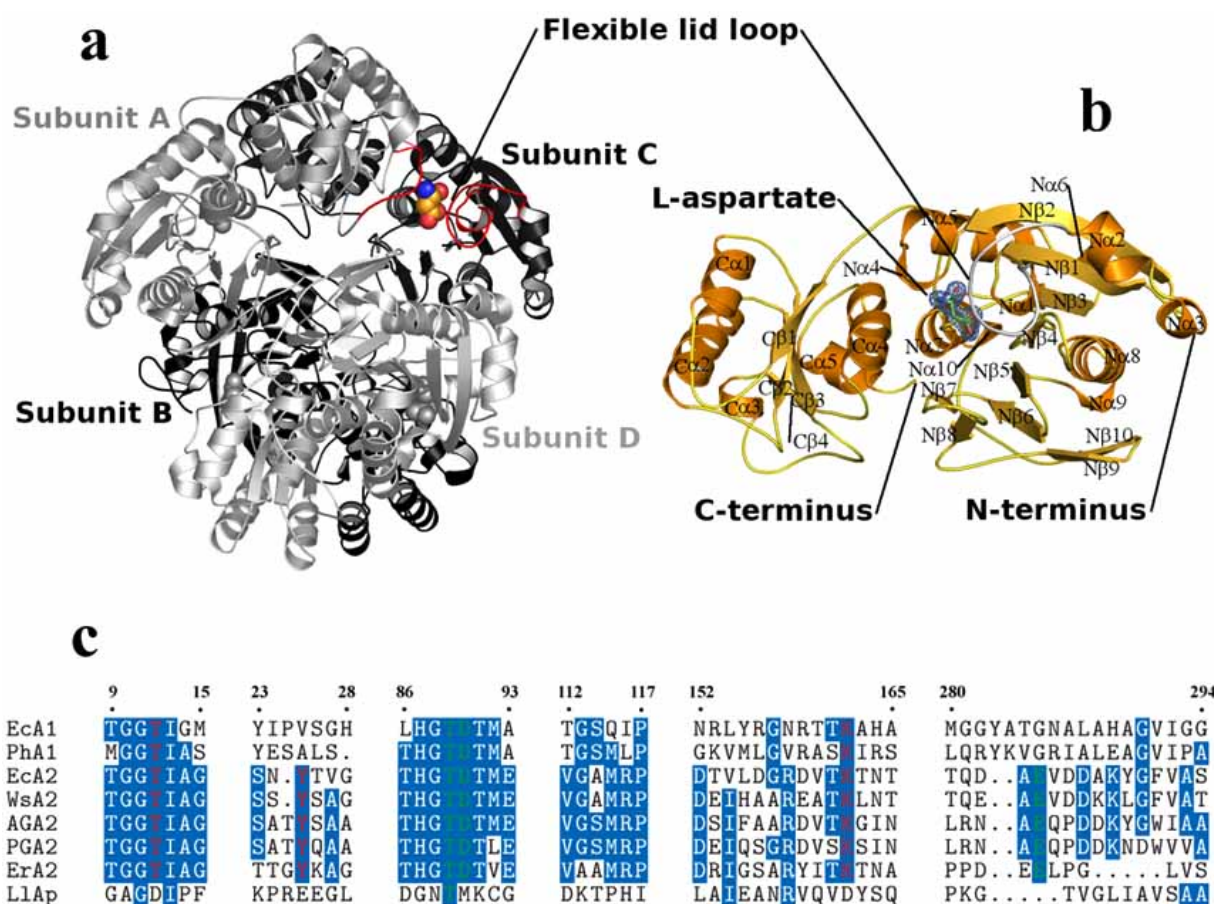
#### 4.2. The Overall Architecture of a Monomer

Each asparaginase subunit consists of two easily identifiable  $\alpha/\beta$  domains, a larger N-terminal and a smaller C-terminal domain, connected by a loop consisting of about 20 residues (Fig. 1b). The N-terminal domain contains an eight-stranded mixed  $\beta$ -sheet. There is a topological switch-point between strands N $\beta$ 1 and N $\beta$ 3 where adjacent  $\beta$ -strands continue in opposite directions, folding into  $\alpha$ -helices on either side of the sheet. This directional change creates a cleft characteristic of all parallel  $\alpha/\beta$  structures that serves as a pocket

**Table 1. Wild Type Asparaginase Structures Deposited at the RCSB Protein Data Bank**

Specificity	Organism	PDB Code	Ligand
L-Asn	<i>Escherichia coli</i> (EcA2)	3ECA/1NNS	L-Asp/L-Asp
L-Asn	<i>Wolinella succinogenes</i> (WsA2)	1WSA	-
L-Asn(L-Gln)	<i>Erwinia chrysanthemi</i> (ErA2)	1HFJ/1HFK/1HFW/1HG0/ 1HG1/1O7J/1JSL/1JSR	SO <sub>4</sub> <sup>2-</sup> /SO <sub>4</sub> <sup>2-</sup> /L-Glu/Suc/ D-Asp/SO <sub>4</sub> <sup>2-</sup> /DDO/LDO
L-Asn/L-Gln	<i>Acinetobacter glutaminasificans</i> (AGA2)	1AGX	-
L-Asn/L-Gln	<i>Pseudomonas 7A</i> (PGA2)	3PGA/4PGA/1DJO/1DJP	-/NH <sub>4</sub> <sup>+</sup> , SO <sub>4</sub> <sup>2-</sup> /DONV/DON
L-Asn	<i>Pyrococcus horikoshii</i> (PhA1) (type I)	1WLS	-

The ligand modeled in the active site of each structure is indicated, as well as the protein substrate specificity for L-asparagine or L-glutamine. Although the *E. chrysanthemi* enzyme is considered an L-asparaginase, its activity against L-glutamine is higher than that usually observed for other L-asparaginases. The structure from the hyperthermophilic archaeon *Pyrococcus horikoshii* (PhA) is the only reported 3D X-ray model of a type I L-asparaginase. Abbreviations: L-Asn: L-asparagine, L-Gln: L-glutamine, L-Asp: L-aspartate, L-Glu: L-glutamate, Suc: succinic acid, D-Asp: D-aspartate, DDO: 6-hydroxy-D-norleucine, LDO: 6-hydroxy-L-norleucine, DONV: 6-diazo-5-oxo-L-norvaline, DON: 6-diazo-5-oxo-L-norleucine.



**Fig. (1).** Three-dimensional models of *E. coli* L-asparaginase with L-aspartate bound together with an amino acid sequence alignment of five asparaginases. **(a)** Tetrameric assembly showing the L-Asp as spheres. The flexible lid loop, containing the catalytic residues Thr12<sub>EcA</sub> and Tyr25<sub>EcA</sub>, as well as the loop containing the residue Glu283<sub>EcA</sub> in the other subunit of the intimate dimer, are shown in red, with the L-Asp shown in colors. Both loops are in close contact. **(b)** An *E. coli* L-asparaginase monomer. L-Asp bound to the active site is shown as balls-and-sticks with correspondent  $2|F_{\text{obs}}| - |F_{\text{calc}}|$ ,  $\alpha_{\text{calc}}$  electron density contoured at  $1\sigma$  **(c)** Amino acid sequence alignment of representative parts of type-I and type-II L-asparaginases. Type I L-asparaginases from *E. coli* (EcA1) and *Pyrococcus horikoshii* (PhA1) are aligned with type-II *E. coli* (EcA2), *W. succinogenes* (WsA2), *A. glutaminasificans* (AGA2), *Pseudomonas* 7A L-asparaginase/L-glutaminase (PGA2), *E. chrysanthemi* (ErA2), and with plant-type L-asparaginase from *Lupinus luteus* (LIAp) [54]. The numbering on the top of the figure refers to the *E. coli* L-asparaginase sequence of the fragment. Residues of the first catalytic triad are colored in red while the ones of the second triad are colored in green. The blue background indicates conserved residues with 50% threshold.

utilized in ligand binding [8]. The  $\beta$ -strand connectivity for the parallel part of the sheet is similar to that of flavodoxin [8]. The helices in flavodoxin superimpose on the four N-terminal helices in L-asparaginases, but in a different order due to the occurrence of a left-handed crossover between the fourth and the fifth  $\beta$ -strands [7]. This type of motif is rarely observed in proteins, but when found always serves as an important structural determinant of their activity [42]. This also applies to the L-asparaginases in which the left-handed crossover forms part of the active site. Two antiparallel  $\beta$ -strands, N $\beta$ 7 and N $\beta$ 8, leave the main sheet and form a  $\beta$ -hairpin, which is positioned near to the interior of the tetramer and may play a role in subunit adhesion. This domain also contains four  $\alpha$ -helices with two of them, N $\alpha$ 1 and N $\alpha$ 4, exposed to solvent on one side of the  $\beta$ -sheet, and the remaining two, N $\alpha$ 2 and N $\alpha$ 3, on the opposite side, near to the domain interface. A flexible loop at the beginning of the N-terminal domain (residues 15-30 in EcA-II), which is partially or completely disordered in most reported structures of

L-asparaginases, contains two important catalytic residues and forms a lid over the active site. The smaller C-terminal domain (residues 213-326 in EcA-II) consists of a four-stranded parallel  $\beta$ -sheet and four  $\alpha$ -helices. Helices C $\alpha$ 1 and C $\alpha$ 2 are on the side distal to the domain interface while C $\alpha$ 3 and C $\alpha$ 4 are on the interdomain side of the sheet.

The structural differences observed between the structure of PhA and the type II L-asparaginases help to explain the lower affinity for the substrate observed in the type I L-asparaginases [20]. There are three regions in the PhA structure where the main-chain is not well superimposed with the type II enzymes. The first is the linker connecting the two domains, which in PhA is highly extended due the deletion of seven residues compared with the sequence of type II L-asparaginases. Second, PhA has nine extra residues at the C-terminal end that protrude from the globular body of the PhA monomer. The third structural difference is in the flexible loop of the active site of PhA, which is stabilized by the formation of a  $\beta$ -hairpin and by elaborate interactions with

the type-I-specific  $\alpha$ -helical region derived from the other subunit of the dimer [20].

### 4.3. The Enzyme Active Site

The location of the active site of type II L-asparaginase has been elucidated in studies of the binding of ligands such as the product L-aspartate [8, 17, 19], the alternative products D-aspartate, L-glutamate and succinic acid [15, 17], inhibitors such as diazo analogues of glutamine, 6-diazo-5-oxo-L-norleucine and asparagines, 5-diazo-4-oxo-L-norvaline [14], or suicide inhibitors such as the L and D stereoisomers of 6-diazo-5-oxy-norleucine [15]. In the structures determined in the absence of ligands, the active site is frequently occupied by ions (such as sulfate anions) originating from the crystallization solution [13, 16, 18]. The binding pocket assembly of asparaginases involves residues of both subunits of an intimate dimer, e.g. subunits A and C in EcA-II (Fig. 1). The residues Thr12<sub>EcA</sub>, Tyr25<sub>EcA</sub>, Ser58<sub>EcA</sub>, Gln59<sub>EcA</sub>, Thr89<sub>EcA</sub>, Asp90<sub>EcA</sub>, Ala114<sub>EcA</sub> and Lys162<sub>EcA</sub> from one subunit, and Asn248<sub>EcA</sub> and Glu283<sub>EcA</sub> from the other intimate subunit form the binding pocket.

The residues Thr12 and Tyr25 are located in the flexible loop region while the rest of the binding pocket is more rigid and located in the core of the molecule. It has been suggested [9, 10] that the flexible loop has an important role during the catalytic reaction. In the open conformation, it assists in substrate recognition. Once the substrate is bound, the loop undergoes a series of conformational changes allowing several residues to interact with the substrate molecule and to determine its proper orientation with respect to the rigid part of the active site. The closed conformation of the loop traps the substrate molecule, maintaining it in a fixed position [12, 14]. The closed conformation of the loop is maintained by interactions of Tyr25/Thr12-Substrate/Product-Binding Pocket. The structures lacking a ligand or with a mutation of the lid loop tyrosine either have the lid loop in the open conformation or simply lack interpretable electron density for the lid loop due to disorder. On the other hand, in the structures refined with the closed lid loop, the interactions between the main chain atoms of the ligand with the atoms of the binding pocket residues are those responsible for the stabilization of closed-loop conformation. After reaction, the conversion of the amide group of the substrate to a carboxylic acid group is proposed to generate electrostatic repulsion causing the flexible loop to change conformation and return back to the open state, and the products to leave the reaction center [9, 10]. The opening of the lid loop occurs around three fixed points, or hinges (Fig. 2a). These three hinges divide the whole movement into two: the opening of the small and more rigid loop, containing Thr12, and the opening of the longer lid loop, containing Tyr25. In the PGA structure, with the lid loop open, the distance between the hydroxyl groups of the catalytic threonine and tyrosine residues is as large as 6.9Å whereas in the EcA-II structure, with lid loop closed, this distance is significantly reduced to 2.9Å (Fig. 2a).

Therefore, loop flexibility is considered to be fundamentally involved with the enzymatic activity of the type II L-asparaginases. Conversely, the structure of type I L-asparaginase, exemplified by PhA, reveals that the corre-

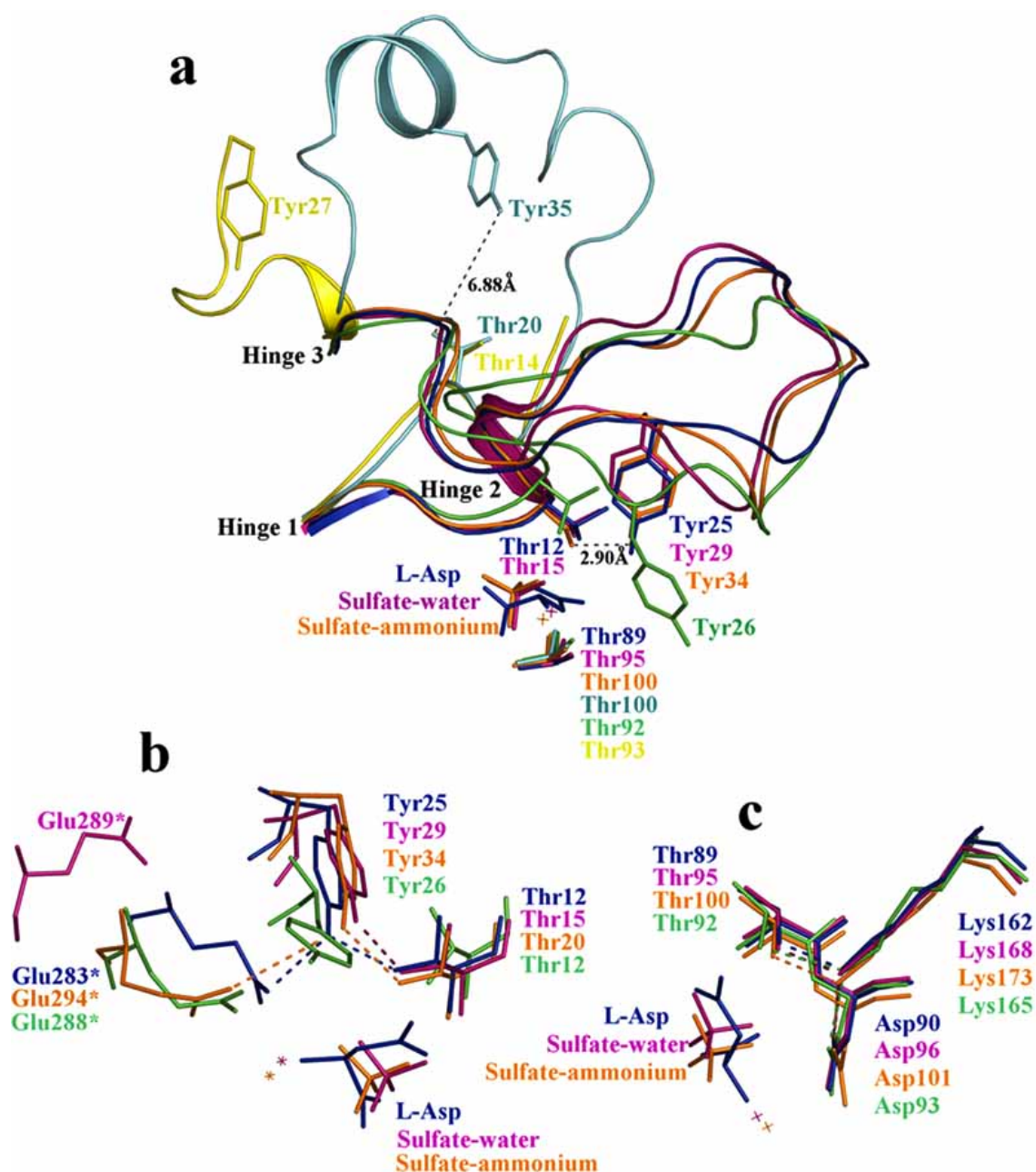
sponding region is stabilized by the formation of a  $\beta$ -hairpin and by intricate interatomic interactions between the first  $\beta$ -strand of this hairpin and the  $\alpha$ -helix C $\alpha$ 3 from the other subunit of the dimer. In the structures of the type II enzyme, there is a deletion of two to six residues in the region corresponding to C $\alpha$ 3. Thus, the  $\beta$ -hairpin formation as well as the interaction between the  $\beta$ -hairpin and C $\alpha$ 3\* seems to be specific to the type I enzyme.

An additional integral part of the active site architecture is the conserved network of water molecules, which have a significant participation in the catalytic properties of L-asparaginases. Analysis of all high-resolution structures of bacterial L-asparaginases with well-defined solvent shows that all of them contain a conserved water molecule (W1 in the EcA structure [19]) occupying the same site. This water molecule systematically presents a characteristically low B-factor and plays an active role in stabilizing the conformation of the flexible region of the active site as well as the substrate molecule itself *via* a hydrogen bond made with the O atom of its amide group. Other active site water molecules playing a role in stabilizing particular conformations of surrounding residues, form a well conserved network of hydrogen bonds which can serve for rapid proton/electron transfer during the enzymatic reaction.

### 4.4. Enzyme-Substrate Interactions

A detailed analysis of the interactions within the independent L-asparaginase active sites indicates the elements crucial for binding of different ligand molecules. Comparison of the structures of ErA and EcA with bound L-Asp [7, 8, 17, 19] and ErA in its complex with L-succinate [15] shows that the networks of hydrogen bonds formed between enzyme and substrates are very similar. The side chain atoms of substrates are located between the hydroxyl groups of Thr12<sub>EcA</sub> (15<sub>ErA</sub>) and Thr89<sub>EcA</sub> (95<sub>ErA</sub>), and close to the main-chain atoms of Ala114<sub>EcA</sub> (120<sub>EcA</sub>). The oxygen atom O $\delta$ 1 from the  $\gamma$ -carboxylate group of the substrate is sandwiched between Thr12<sub>EcA</sub> (15<sub>ErA</sub>) and Thr89<sub>EcA</sub> (95<sub>ErA</sub>), located on the opposite sides of the  $\gamma$ -carboxylate plane of the ligand, forming two hydrogen bonds with the main chain nitrogens of these residues and a third hydrogen bond with a water molecule [15, 17]. This structural arrangement is similar to the 'oxyanion hole' seen in serine proteases and was suggested to act in stabilizing the partial negative charge that develops in the tetrahedral intermediate on formation of the acyl-enzyme complex [7, 14]. Presumably, when asparagine is a substrate, the carbonyl oxygen occupies the position of O $\delta$ 1, while the amide nitrogen occupies the position of O $\delta$ 2 [7, 8]. The structures of the complexes of PGA with the suicide inhibitors DON and DONV [14] show the presence of strong continuous electron density connecting both residues Thr20<sub>PGA</sub> (12<sub>EcA</sub>) and Tyr34<sub>PGA</sub> (25<sub>EcA</sub>) to the inhibitors, indicating the covalent linkage between them.

The second oxygen atom of the  $\gamma$ -carboxylate group in these two ligands interacts with the  $\gamma$ -OH group of Thr89<sub>EcA</sub> (95<sub>ErA</sub>), the main chain oxygen of Ala114<sub>EcA</sub> (120<sub>ErA</sub>), and a water molecule. The carboxyl oxygens of the substrate are almost coplanar with the carbonyl of Ala114<sub>ErA</sub> (120<sub>EcA</sub>), which can act as an acceptor in a hydrogen bond with the O $\delta$ 2, indicating that the side-chain carboxyl group of the



**Fig. (2).** (a) Cartoon representation of the active site and flexible lid loop of the six known class II asparaginase structures: *E. coli*-L-Asp (in dark blue), *Pseudomonas* 7A (no ligand, light blue), *Pseudomonas* 7A-SO<sub>4</sub><sup>2-</sup>-NH<sub>4</sub><sup>+</sup> (in orange) *E. chrysanthemi*-SO<sub>4</sub><sup>2-</sup> (in magenta), *A. glutaminasificans* (no ligand, in green), *W. succinogenes* (no ligand, in yellow). The structure of *W. succinogenes* was refined with the lid loop only partially built, which indicates its flexibility and disorder. The structural alignment indicates that the open conformation might be caused by the lack of ligand. *A. glutaminasificans* is also an apo structure, which could explain its unusual conformation for the lid loop. In order to compare the differences between the closed and open conformations, both structures of *Pseudomonas* 7A are shown. It is interesting to note that the sulfate ion in the ErA and PGA structures occupies an equivalent position to the main chain carboxyl group of the EcA-II structure and that the amino group position of EcA-II is occupied by an ammonium in PGA and a water molecule in ErA. Since all three structures are in the closed conformation and the ligands make equivalent contacts in the active site, it likely that these are key interactions responsible for keeping the loop in the closed conformation. (b) Schematic representation of the catalytic triads involved on the reaction mechanism of asparaginases: *E. coli*-L-Asp, *Pseudomonas* 7A-SO<sub>4</sub><sup>2-</sup>-NH<sub>4</sub><sup>+</sup>, *E. chrysanthemi*-SO<sub>4</sub><sup>2-</sup>, *A. glutaminasificans* (no ligand). The same color-coding scheme as in Fig. 1a was used. The *W. succinogenes* structure was excluded because of its open conformation and the lack of part of the lid loop. Thr12<sub>ECA</sub> is the primary nucleophile of the first catalytic triad, acting during the acylation step. Residue Glu283<sub>ECA</sub> of another subunit of the intimate dimer (marked by an asterisk) polarizes Tyr25<sub>ECA</sub>, which acts as a transitory base, enhancing the nucleophilicity of Thr12<sub>ECA</sub>. Note that Glu289<sub>ECA</sub> is inserted in the tip of a flexible loop on the surface of the ErA molecule [15] and is mobile/disordered as judged from its very high B-factors, which could explain the distinct position and orientation of this particular residue. (c) The second catalytic triad with Thr89<sub>ECA</sub> as the nucleophile. The triad is important for the correct orientation and activation of the catalytic water molecule that releases the product and regenerates the enzyme during the deacylation step.

ligand is protonated [7, 15]. It has been proposed that after enzymatic hydrolysis, the interaction of the negatively charged carboxylate group of the product generates a strong repulsion with the main chain carbonyl oxygen of Ala114<sub>ErA</sub> (120<sub>ErA</sub>) which helps to release the product by triggering the change in the flexible loop conformation to the open state [7, 9, 10].

In all cases, the  $\alpha$ -carboxyl group of the ligand molecule and the  $\gamma$ -OH group of Ser62<sub>ErA</sub> (58<sub>ErA</sub>) appear to be fundamental for binding [15]. Furthermore, interactions with the main-chain nitrogen atoms of Ser58<sub>ErA</sub> (62<sub>ErA</sub>) and Asp90<sub>ErA</sub> (96<sub>ErA</sub>) indicate that the fold of the protein fragments containing these residues should be invariant for bacterial L-asparaginases in order to ensure productive ligand recognition [15]. This is probably the reason behind the greater rigidity of these fragments, for which low B-factors for the contributing atoms were observed for all structurally studied L-asparaginases [7-12, 15].

The  $\alpha$ -amino group of the L-Asp, important for interaction with the enzyme binding site [43], interacts with the residues Glu59<sub>ErA</sub> (63<sub>ErA</sub>) and Asp90<sub>ErA</sub> (96<sub>ErA</sub>), implying this group to be protonated [7, 8]. Furthermore, in the case of ErA complexed with D-Asp and L-Glu, despite the differences in the sizes and stereochemistry of these molecules, the  $\alpha$ -amino groups of both ligands participate in two hydrogen bonds with the carboxyl oxygen atoms of Asp96<sub>ErA</sub> (90<sub>ErA</sub>) and Glu63<sub>ErA</sub> (59<sub>ErA</sub>), respectively, plus with a water molecule, highly conserved in L-asparaginase active sites [15]. This water molecule is found in an equivalent position in all known asparaginase structures and is hydrogen bonded to the main chain atoms of the enzyme [7], in a tetrahedral coordination to O $\delta$ 1 of the substrate (O $\epsilon$ 1 of the aspartyl group in EcA<sub>T89V</sub>), the carbonyl oxygen of His87<sub>ErA</sub> (93<sub>ErA</sub>) and the main chain nitrogen atoms of Ile13<sub>ErA</sub> (16<sub>ErA</sub>) and Ala114<sub>ErA</sub> (120<sub>ErA</sub>) that identifies it an unambiguous proton donor. The lack of an  $\alpha$ -amino group, however, does not affect the binding mode of L-succinate to ErA, however the above mentioned conserved water molecule is absent in the X-ray structure of this complex [15].

The binding of L-Glu to ErA causes structural distortions of the enzyme's active site [15]. The side chain of L-Glu is larger than that of L-Asp, which forces the  $\delta$ -carboxylate plane to be almost perpendicular to the  $\gamma$ -carboxylate plane of L-Asp, and this results in suboptimal orientation of the nucleophile Thr15<sub>ErA</sub> (12<sub>ErA</sub>) whose side chain points away from the ligand molecule [15]. One of the side-chain oxygens of L-Glu forms two hydrogen bonds with Thr95<sub>ErA</sub> (89<sub>ErA</sub>), one with its  $\gamma$ -OH group and the second with the main-chain nitrogen atom [15]. The second oxygen atom of the side chain in this ligand also makes two hydrogen bonds, one with the main-chain nitrogen of Thr15<sub>ErA</sub> (12<sub>ErA</sub>), and the other with a water molecule [15]. Furthermore, no interaction with the main chain oxygen of Ala120<sub>ErA</sub> (114<sub>ErA</sub>) was observed and the overall number of contacts between the side chain of L-Glu and the enzyme was smaller than in the case of L-Asp [15].

A quite different situation was found for D-Asp [15]. Its side chain points toward the active site flexible loop due to its inverted stereochemistry, preventing the formation of a stable active conformation, such as the one observed for L-Asp. There are no interactions observed between the D-Asp

side chain and either of the two active site threonine residues [15]. One of the oxygen atoms of this side chain forms two hydrogen bonds with the main chain nitrogen of Ser62<sub>ErA</sub> (58<sub>ErA</sub>) and with a water molecule [15]. The second oxygen atom interacts only with one water molecule and this interaction is conserved in all complexes [15]. The side chain of D-Asp forms a small overall number of interactions with the enzyme and, as is the case of L-Glu, no hydrogen bond with Ala120<sub>ErA</sub> (114<sub>ErA</sub>) is observed [15].

In the PGA structure solved in the presence of a sulfate and an ammonium bound to the active site [9], these two ions are located, respectively, at the equivalent positions of the carboxyl and amine groups of the L-Asp (Fig. 2b and 2c). Additionally, the N and O $\delta$ 2 sites of aspartate are occupied in the PGA structure [9] by water molecules. This is also true for the ErA X-ray crystallographic model containing a sulfate ion, where a water molecule was modeled in the position equivalent to the amine group [18]. These small molecules generate a hydrogen-bonding pattern that is comparable to that of the aspartate main chain atoms in the L-Asp complex, thus preserving the same set of interactions in the active site. The binding of a ligand with a large negative charge such as sulfate to the active site of the enzyme can be readily understood in terms of interactions with the hydroxyl group of Thr15<sub>ErA</sub> (12<sub>ErA</sub>) [18]. In the case of the enzyme-aspartate complex, the side chain of Thr15<sub>ErA</sub> presents the electron pair of its hydroxyl O atom towards the C $\gamma$  atom of the ligand, while the hydroxyl proton contributes to the hydrogen bond with Tyr29<sub>ErA</sub> (25<sub>ErA</sub>). On the other hand, in complex with a sulfate anion, the hydroxyl group of Thr15<sub>ErA</sub> forms a strong hydrogen bond with the O4 atom of the ligand providing additional stabilization of the complex [18].

## 5. THE MECHANISM OF ACTION

There is general agreement that a detailed understanding of the catalytic mechanism of L-asparaginases might permit the design of modifications to the wild type proteins that could lessen the severe side effects of their application in cancer therapy [26].

The currently accepted mechanism for the transformation of L-asparagine into L-aspartate and ammonia by L-asparaginases proceeds *via* a covalently bound intermediate involving a  $\beta$ -aspartyl enzyme [7, 12, 14, 15], as opposed to the model of an enzyme-bound aspartic anhydride as the reaction intermediate [44, 45]. This implies a double displacement, or 'ping-pong', mechanism where at first a nucleophilic group of the enzyme attacks the C $\gamma$  of the substrate (L-asparagine or L-glutamine) leading to a tetrahedral intermediate which subsequently breaks down to form an acyl-enzyme intermediate with the enzyme covalently bound to the substrate. This is followed by the elimination of ammonia. This intermediate is then attacked by a second nucleophile, normally water, resulting in the hydrolysis of the acyl-enzyme intermediate yielding the acidic product and free enzyme.

Activated substrate analogs [8-19] have been used to identify residues located at the active site, and based on these results it has been proposed that this family of hydrolases is mechanistically similar to the serine proteases. If this is the case one would expect to find a catalytic triad formed in the

same way as in serine proteases. It has been shown, however, that none of the three histidines in *E. coli* L-asparaginase are required for catalysis [46], thus eliminating the possibility of a classical catalytic triad.

### 5.1. The Two Catalytic Triads

The residues Thr89<sub>EcA</sub>, Asp90<sub>EcA</sub> and Lys162<sub>EcA</sub> were initially suggested to be the catalytic triad [47], with the threonine acting as the primary nucleophile, based on collective evidence from the crystallographic studies of EcA bound to L-Asp [8], ErA bound to L-Asp [7] and PGA determined in the open conformation [9]. Thus Thr89 would be active as the attacking nucleophile while a proximal Lys162, which is stabilized by Asp90, would act as a base to enhance the nucleophilicity of the catalytic Thr89 residue [8, 9].

However, the presence of another threonine residue, Thr12<sub>EcA</sub>, in the enzyme active site instigated an alternative hypothesis, which proposes Thr12 to be the attacking nucleophile [7] (Fig. 2b and 2c). This hypothesis has encountered resistance based on the fact that in the structure of the enzyme determined with the active site in the open conformation [9], Thr12 is far from the other residues that make up the active site [7, 8]. On the other hand, the rearrangement of the flexible loop over the active site in the structures of enzyme:ligand complexes brings this residue into close proximity to the substrate and to the residues of the active site pocket [7, 8].

The almost symmetric location of these two threonine residues, Thr12<sub>EcA</sub> or Thr89<sub>EcA</sub>, above and below the C $\gamma$  of the substrate suggests that one of them must be the nucleophile of the acylation step<sup>1</sup>. This statement is supported by the location of both threonines within two absolutely conserved regions of amino acid sequence among all bacterial asparaginases, being located at residues 9<sub>EcA</sub> to 15<sub>EcA</sub> and 86<sub>EcA</sub> to 91<sub>EcA</sub> [2] (Fig. 1c). Furthermore, it has been experimentally demonstrated that both the threonines participate directly in the L-asparaginase catalytic mechanism [12, 48, 49]. Mutation of either Thr12<sub>EcA</sub> or Thr89<sub>EcA</sub> diminishes enzymatic activity to about 0.01% relative to the wild-type enzyme [48, 49]. Mutation of T12A<sub>EcA</sub> resulted in a marked decrease in the catalytic activity, but the affinity of the enzyme for aspartate was practically unaffected. This evidence suggests that Thr12<sub>EcA</sub> is not required in the aspartate binding process [48] and most probably acts as the primary nucleophile of the enzyme.

The superposition of the available models of bacterial L-asparaginases reveals that a large portion of the active site, which includes the initially proposed catalytic triad (Thr89<sub>EcA</sub>, Asp90<sub>EcA</sub> and Lys162<sub>EcA</sub>) does not change its conformation upon interaction with different ligands (substrates, ions or water) and thus presumably is fairly rigid (Fig. 2c). This is further evidenced by the relatively low temperature factors in these regions [14, 15]. The starting position and optimum orientation of nucleophiles is of crucial importance

in enzymes controlled by molecular mechanics [13]. As perceived by Jakob and collaborators [13], the position of Thr89<sub>EcA</sub> with respect to the  $\gamma$ -carboxylate group of L-Asp yields an unfavorable geometry for the nucleophilic addition to the  $\gamma$ -carbon of the substrate. In order to form a covalent tetrahedral intermediate, the  $\gamma$ -O of Thr89<sub>EcA</sub> would have to reach the  $\gamma$ -C of the substrate, which requires a large main chain shift within the active site, inconsistent with its observed rigidity. In contrast, Thr12<sub>EcA</sub> is located in the flexible loop of the active site and has ideal geometry to attack the electrophilic  $\gamma$ -C of L-Asp. In this case, the optimum positioning of Thr12<sub>EcA</sub> and the covalent bond formation could be achieved by a simple change of the  $\chi$ 1 torsion angle [13]. Additional support to the notion that Thr12<sub>EcA</sub> is the attacking nucleophile comes from the structural study of the mutant T89V<sub>EcA</sub> [12]. In this X-ray structure of a complex, the substrate is covalently bound to the Thr12<sub>EcA</sub> which corroborates the hypothesis of a  $\beta$ -aspartyl-enzyme and the action of Thr12<sub>EcA</sub> as the primary nucleophile in the acylation step. The authors proposed that the inactivation of the mutant is probably caused by the lack of a water molecule in the active site due to the low polarity of the valine methyl group at position 89<sub>EcA</sub>. In addition, a structural study of the PGA complexes with the suicide inhibitors DON and DONV, diazo analogs of glutamine and asparagine, respectively, also supports the role of Thr20<sub>PGA</sub> (equivalent to Thr12<sub>EcA</sub>) as the primary nucleophile [14].

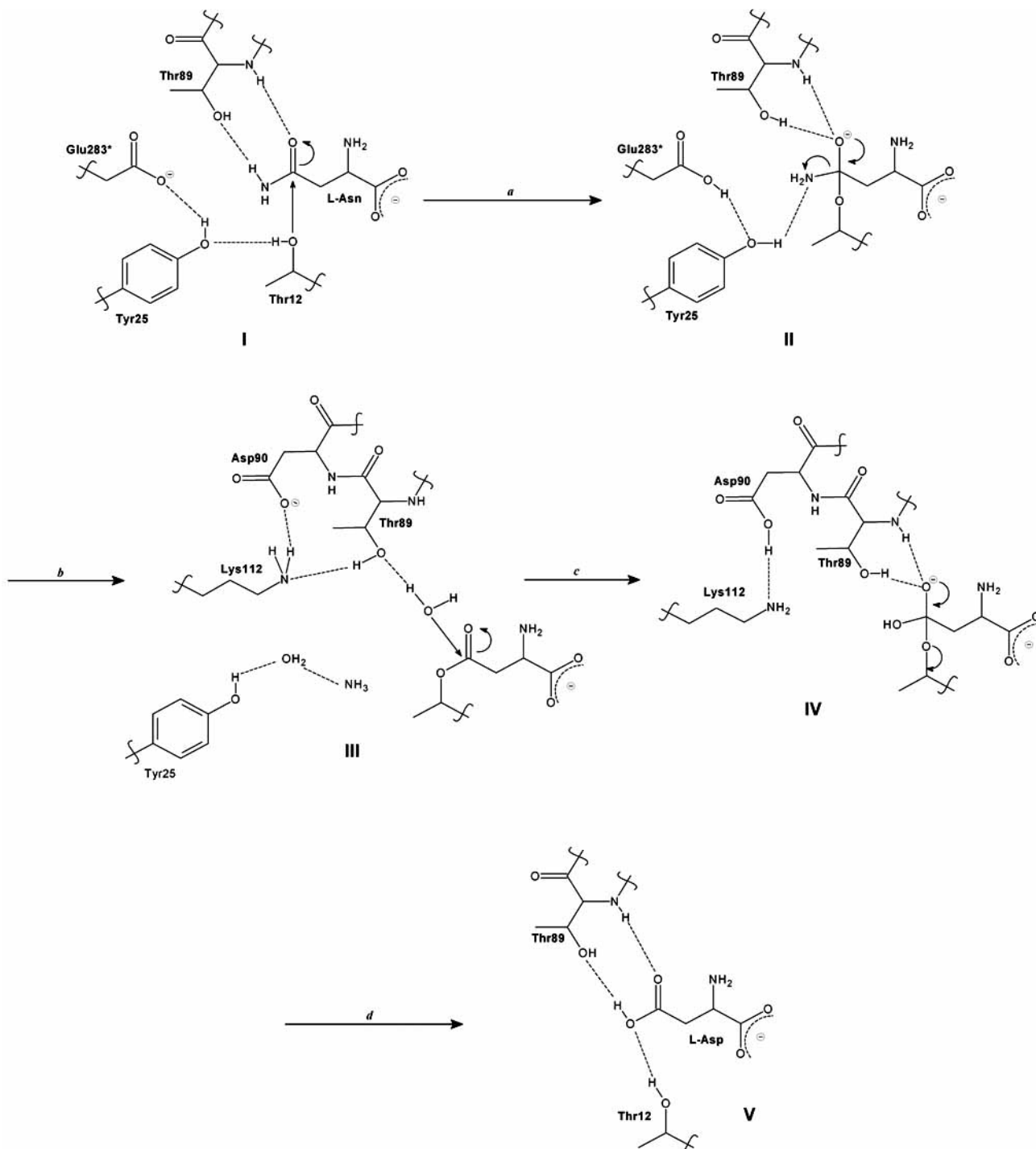
Structural studies indicate that the activation of Thr12 involves Tyr25 [14], the catalytic importance of which has been clearly demonstrated [49-51]. It is the central residue in the catalytic triad formed, in addition, by the primary nucleophile, Thr12, and Glu294 (from subunit B). Average distances from the Tyr25OH to Thr12O $\gamma$  and to the Glu294 side-chain carboxyl have typical hydrogen-bonding lengths of 2.85 and 2.90 Å, respectively, which are observed in the catalytically competent conformation of the L-asparaginases in the presence of substrate [14]. Ligand-triggered closure of the flexible loop, which brings into position not only Tyr25 but also the attacking nucleophile Thr12, is required for molding the enzyme into a productive conformation. Indeed, even before crystal structures were available, NMR studies revealed that in EcA a histidine and a tyrosine residue, subsequently identified as H183 and Y25, are both highly mobile in the free enzyme but become immobilized upon aspartate binding [52]. Furthermore, it has been shown that replacement of Y25 with Ala, His or Phe reduces  $k_{cat}$  to about 1% of the wild-type value without markedly affecting  $K_m$  [49]. This indicates that only a phenolic group in position 25<sub>EcA</sub> can preserve normal catalytic rates. It was also proposed that the catalytic role of Tyr25 is to position Thr12 in a way that is optimal for nucleophilic attack on the scissile bond [53]. Finally, based on the PGA glutaminase-asparaginase X-ray structures solved as a complex with the suicide inhibitors DON and DONV, it was suggested that the catalytic triad could involve a glutamic acid residue, Glu294<sub>PGA</sub> (Glu283<sub>EcA</sub>), which would assist in abstracting the proton from the Tyr34<sub>PGA</sub> (Tyr25<sub>EcA</sub>) [14]. Thus the first stage of the L-asparaginases catalytic mechanism (the acylation reaction) involves the participation of the Thr12<sub>EcA</sub>-Tyr25<sub>EcA</sub>-Glu283<sub>EcA</sub> catalytic triad, with Thr12<sub>EcA</sub> acting as the primary nucleophile. Fig. 2b shows the superposition of this first catalytic triad with the representative structures of

<sup>1</sup> Acylation is the denomination given to the nucleophilic attack by the oxygen of the nucleophile to the carbonyl carbon of the substrate, leading to the formation of a tetrahedral acyl intermediate. Deacylation, on the other hand, refers to the second nucleophilic attack that regenerates the enzyme and releases the product.

asparaginases of the five bacterial organisms that were analyzed.

In contrast with the great effort that has been made in identifying the catalytic triad that participates in the acylation step, the second triad, which acts during the deacylation of the covalently bound  $\beta$ -aspartyl enzyme, has been consistently neglected. The isolation of the intermediate for the

mutant T89V<sub>EcA</sub> has shown the importance of Thr89<sub>EcA</sub> in activating the water molecule that performs the second nucleophilic attack [12]. In the same way that an isolated threonine residue is not a nucleophile strong enough to attack the carbonyl of the substrate without the formation of a triad, it appears that the Thr89<sub>EcA</sub> alone is not sufficient to convert a water molecule into a nucleophile adequate for product release [12]. This water molecule could be located



**Fig. (3).** Reaction mechanism scheme for the transformation of L-Asn into L-Asp by L-asparaginases. The stages *a* and *b* represent the acylation step to form the  $\beta$ -aspartyl-enzyme (III) followed by stages *c* and *d* of deacylation, leading to the product (V). Dashed lines represent hydrogen bonds, while straight and curved arrows indicate nucleophilic attacks and electronic movements, respectively. The amino acid numbering is based on the *E. coli* L-asparaginase sequence.

on either side of the plane formed by the ester group. One of these sides is buried by the hydrophobic side chain of Val27<sub>EcA</sub> and the aspartyl group. In all other asparaginase structures with substrate the side chain of the homologous residue (Val or Ala) occupies the same position. There is no base available to activate a water molecule on this side of the ester plane. A water molecule on the other side, by contrast, would be close to the  $\gamma$ -OH of Thr89<sub>EcA</sub>, a residue conserved in all bacterial L-asparaginases [2]. Furthermore it has been postulated that the lack of this water molecule in EcA<sub>T89V</sub> is an important factor for the mutant's inefficiency in the deacylation reaction [12]. Fig. 2c shows the structural alignment of the triad Thr89<sub>EcA</sub>-Lys162<sub>EcA</sub>-Asp90<sub>EcA</sub> in representative structures of asparaginases. Historically proposed as the primary triad of the enzyme [47], it might instead play the role of the second catalytic triad responsible for activating the water molecule.

## 5.2. The Detailed Reaction Mechanism

Based on the aforementioned data, the reaction mechanism of bacterial L-asparaginases most probably involves two catalytic triads acting independently at distinct reaction steps (Fig. 3). This enzymatic mechanism is based on the reaction scheme proposed by Ortlund *et al.* [14] and is in agreement with the available structural data and the site-directed mutagenesis studies.

During step (a) the nucleophilicity of the primary nucleophile is enhanced by the formation of the catalytic triad Thr-Tyr-Glu (Fig. 3). The tyrosine residue acts as a transitory base, abstracting the proton from the Thr residue. In the EcA structure (as well as in the T89V<sub>EcA</sub> structure and other structures with the modeled lid loop), Tyr25<sub>EcA</sub> is the only residue able to form a hydrogen bond with the Thr12<sub>EcA</sub> hydroxyl. Glu283<sub>EcA</sub>, residing in the other subunit of the intimate dimer, completes the first catalytic triad by withdrawing the proton from the tyrosine. It is also likely that this tyrosine residue has a role in the correct orientation of the threonine side chain by providing a hydrogen bond between these two residues. At the same time as the Thr-Tyr-Glu triad is formed, the coordination between another threonine in the active site (Thr89<sub>EcA</sub>) and the substrate enhances the substrate side chain electrophilicity (I) and also stabilizes the oxyanion formed on the tetrahedral intermediate (II). The  $\beta$ -aspartyl enzyme is formed in step (b), by an electronic movement that regenerates the carbonyl and eliminates an ammonia molecule (III). It is feasible that the catalytic tyrosine helps to export the ammonia molecule, intermediated by the constellation of water molecules surrounding the hydroxyl of the tyrosine. These waters are present in all analyzed structures in which the flexible loop is found in a closed conformation. The deacylation step begins with the nucleophilic attack of a water molecule to the carbonyl carbon of the  $\beta$ -aspartyl intermediate (III), which forms the tetrahedral oxyanion intermediate IV, similar to intermediate II. This water molecule, which has been shown to participate in the deacylation of the  $\beta$ -aspartyl enzyme by <sup>18</sup>O exchange experiments [45], has its nucleophilicity enhanced by interaction with the second catalytic triad formed by residues Thr-Lys-Asp. The regeneration of the carbonyl double bond eliminates the main reaction product, regenerating the enzyme (V). It is highly probable that, during both nucleophilic

attacks, the oxyanion intermediates II and IV are stabilized by hydrogen bonds with Thr89<sub>EcA</sub>.

## 5.3. A Threonine Acting as a Nucleophile

The unusual preference of the asparaginases for threonine and not serine in this first catalytic triad is a fact that deserves special consideration. Since these two residues differ by only a methyl group (C $\gamma$ 2), the reason for this preference must reside in the interaction of this methyl group with its environment. In fact, the EcA L-asparaginase structure reveals that this methyl group is encapsulated in an essentially apolar cavity, composed mainly of the aromatic ring of the residue Tyr25<sub>EcA</sub>, Pro117<sub>EcA</sub> and the main chain of the residues Arg116<sub>EcA</sub> and Met115<sub>EcA</sub>. This insertion of the C $\gamma$ 2 into this hydrophobic cavity restrict rotational freedom of the Thr12<sub>EcA</sub> side chain, thus rendering a nucleophile that is properly oriented with respect to the substrate, in contrast to a free-to-rotate serine residue in which the methyl group is absent. This is supported by the finding that the mutant T12S<sub>EcA</sub> yields an enzyme with only 20% activity towards L-Asn comparing with the wild type [49].

## ABBREVIATIONS

NMR	= Nuclear magnetic resonance
PDB	= Protein Data Base
D-Asp	= D-aspartic acid
DDO	= 6-hydroxy-D-norleucine
DON	= 6-diazo-5-oxo-L-norleucine
DONV	= 6-diazo-5-oxo-L-norvaline
L-Asp	= L-aspartic acid
LDO	= 6-hydroxy-L-norleucine
L-Glu	= L-glutamic acid
Suc	= Succinic acid
EcA	= <i>Escherichia coli</i> L-asparaginase
WsA	= <i>Wolinella succinogenes</i> L-asparaginase
ErA	= <i>Erwinia chrysanthemi</i> L-asparaginase
AGA	= <i>Acinetobacter glutaminasificans</i> L-asparaginase/L-glutaminase
PGA	= <i>Pseudomonas</i> 7A L-asparaginase/L-glutaminase
PhA	= Hyperthermophilic archaeon <i>Pyrococcus horikoshii</i> L-asparaginase.

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## REFERENCES

- [1] Bonthron DT, Jaskólski M. Why a "benign" mutation kills enzyme activity. Structure-based analysis of the A176V mutant of *Saccharomyces cerevisiae* L-asparaginase I. *Acta Biochim Polon* 1997; 44: 491-504.

- [2] Borek D, Jaskólski M. Sequence analysis of enzymes with asparaginase activity. *Acta Biochim Polon* 2001; 48: 893-902.
- [3] Cedar H, Schwartz JH. Localization of the two L-asparaginases in anaerobically grown *Escherichia coli*. *J Biol Chem* 1968; 242: 3753-3755.
- [4] Campbell HA, Mashburn LT, Boyse EA, Old LJ. Two L-asparaginases from *Escherichia coli* B. Their separation, purification and antitumor activity. *Biochemistry* 1967; 6: 721-30.
- [5] Ho PPK, Milikin EB, Bobbitt JL, et al. Crystalline L-asparaginase from *Escherichia coli* B, I. Purification and chemical characterization. *J Biol Chem* 1970; 245: 3708-15.
- [6] Dunlop PC, Meyer GM, Roon RJ. Reactions of Asparaginase II of *Saccharomyces cerevisiae*. *J Biol Chem* 1980; 255:1542-6.
- [7] Miller M, Rao JKM, Wlodawer A, Gribskov MR. A left-handed crossover involved in amidohydrolysis catalysis - crystal-structure of *Erwinia-chrysanthemi* L-asparaginase with bound L-aspartate. *FEBS Letters* 1993; 328: 275-9.
- [8] Swain AL, Jaskólski M, Housset D, Rao JKM, Wlodawer A. Crystal-structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer-therapy. *PNAS* 1993; 90: 1474-8.
- [9] Lubkowski J, Wlodawer A, Housset D, Weber IT. Structural characterization of *Pseudomonas 7a* glutaminase-asparaginase. *Biochemistry* 1994a; 33: 10257-65.
- [10] Lubkowski J, Wlodawer A, Ammon HL, Copeland TD, Swain AL. Refined crystal-structure of *Acinetobacter glutaminasificans* glutaminase-asparaginase. *Acta Crystallogr D* 1994b; 50: 826-32.
- [11] Lubkowski J, Palm GJ, Gilliland GL, Derst C, Röhm KH. Crystal structure and amino acid sequence of *Wolinella succinogenes* L-asparaginase. *Eur J Biochem* 1996; 241: 201-7.
- [12] Palm GJ, Lubkowski J, Derst C, Schleper S, Röhm KH, Wlodawer A. A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: Crystal structure of a Thr-89-Val mutant. *FEBS Lett* 1996; 390: 211-6.
- [13] Jakob CG, Lewinski K, LaCount MW, Roberts J, Lebioda L. Ion binding induces closed conformation in *Pseudomonas 7A* glutaminase-asparaginase (PGA): Crystal structure of the PGA-SO42--NH4+ complex at 1.7 angstrom resolution. *Biochemistry* 1997; 36: 923-31.
- [14] Ortlund E, Lacoount MW, Lewinski K, Lebioda L. Reactions of *Pseudomonas 7A* glutaminase-asparaginase with diazo analogues of glutamine and asparagine result in unexpected covalent inhibitions and suggests an unusual catalytic triad Thr-Tyr-Glu. *Biochemistry* 2000; 39: 1199-1204.
- [15] Aghaiypour K, Wlodawer A, Lubkowski J. Structural basis for the activity and substrate specificity of *Erwinia chrysanthemi* L-asparaginase. *Biochemistry* 2001; 40: 5655-64.
- [16] Jaskólski M, Kozak M, Lubkowski J, Palm G, Wlodawer A. Structures of two highly homologous bacterial L-asparaginases: a case of enantiomeric space groups. *Acta Crystallogr D* 2001; 57: 369-77.
- [17] Kolyani KA, Wlodawer A, Lubkowski J. Structural basis for the activity and substrate specificity of *Erwinia chrysanthemi* L-asparaginase. *Biochemistry* 2001; 40: 5655-64.
- [18] Lubkowsky J, Dauter M, Aghaiypour K, Wlodawer A, Dauter Z. Atomic resolution structure of *Erwinia chrysanthemi* L-asparaginase. *Acta Crystallogr D* 2003; 59: 84-92.
- [19] Sanches M, Barbosa JARG, Oliveira RT, Abrahão-Neto J, Polikarpov I. Structural comparison of *Escherichia coli* L-asparaginase in two monoclinic space groups. *Acta Crystallogr D* 2003; 59: 416-22.
- [20] Yao M, Yasutake Y, Morita H, Tanaka I. Structure of the type I L-asparaginase from the hyperthermophilic archaeon *Pyrococcus horikoshii* at 2.16 angstroms resolution. *Acta Crystallogr D* 2005; 61: 294-301.
- [21] Broome JD. Studies on the mechanism of tumor inhibition by L-asparaginase: effects of the enzyme on asparagine levels in the blood, normal tissues, and 6C3HED lymphomas of mice: differences in asparagine formation and utilization in asparaginase-sensitive and -resistant lymphoma cells. *J Exp Med* 1968; 127: 1055-72.
- [22] Muller HJ, Boos J. Use of L-asparaginase in childhood ALL. *Crit Rev Oncol Hematol* 1998; 282: 97-113.
- [23] Avramis VI, Panosyan EH. Pharmacokinetic/Pharmacodynamic Relationships of Asparaginase Formulations. *Clin Pharmacokin* 2005; 44: 367-93.
- [24] Resnick AD, Magasanik B. L-Asparaginase of *Klebsiella aerogenes*. Activation of its synthesis by glutamine synthetase. *J Biol Chem* 1976; 251: 2722-8.
- [25] Sonawane A, Kloppner U, Derst C, Rohm KH. Utilization of acidic amino acids and their amides by pseudomonads: role of periplasmic glutaminase-asparaginase. *Arch Microbiol* 2003; 179: 151-9.
- [26] Asselin BL. The three asparaginases. Comparative pharmacology and optimal use in childhood leukemia. *Adv Exp Med Biol* 1999; 457: 621-9.
- [27] Ronghe M, Burke GA, Lewis SP, Estlin EJ. Remission induction therapy for childhood acute lymphoblastic leukaemia: clinical and cellular pharmacology of vincristine, corticosteroids, L-asparaginase and anthracyclines. *Cancer Treat Rev* 2001; 27: 327-37.
- [28] Wang B, Relling MV, Storm MC, et al. Evaluation of immunologic crossreaction of anti-asparaginase antibodies in acute lymphoblastic leukemia (ALL) and lymphoma patients. *Leukemia* 2003; 17: 1583-8.
- [29] Woo MH, Hak LJ, Storm MC, et al. Hypersensitivity or development of antibodies to asparaginase does not impact treatment outcome of childhood acute lymphoblastic leukemia. *J Clin Oncol* 2000; 18: 1525-32.
- [30] Chakrabarti R, Schuster SM. L-asparaginase: perspectives on the mechanisms of action and resistance. *Int J Pediatric Hematol Oncol* 1997; 4: 597-611.
- [31] Inada Y, Furukawa M, Sasaki H, et al. Biomedical and biotechnological applications of PEG- and PM-modified proteins. *Trends Biotechnol* 1995; 13: 86-91.
- [32] Friedman M. Chemistry, biochemistry, and safety of acrylamide. *J Agric Food Chem* 2003; 5116: 4504-26.
- [33] Hofmann K, Bucher P, Falquet L, Bairoch A. The PROSITE database, its status in 1999. *Nucleic Acids Res* 1999; 27: 215-9.
- [34] North ACT, Wade HE, Cammack KA. Physicochemical Studies of L-Asparaginase from *Erwinia carotovora*. *Nature* 1969; 224: 594-5.
- [35] Lee B, Yang HJ, Henry GM, et al. Crystallographic studies on L-asparaginase from *Proteus vulgaris*. II. Symmetry and location of the tetrameric molecule. *J Biol Chem* 1973; 248: 7620-3.
- [36] Itai A, Yonei M, Mitsui Y, Iitaka Y. Crystallographic study on the orthorhombic crystal of L-asparaginase from *Escherichia coli* HAP. *J Mol Biol* 1976; 105: 321-5.
- [37] Wlodawer A, Hodgson KO, Bensch K. Studies of two crystal forms of L-glutaminase-asparaginase from *Acinetobacter glutaminasificans*. *J Mol Biol* 1975; 99: 295-9.
- [38] Wlodawer A, Roberts J, Holcenberg JS. Characterization of crystals of L-glutaminase-asparaginase from *Acinetobacter glutaminasificans* and *Pseudomonas 7A*. *J Mol Biol* 1977; 112: 515-9.
- [39] Ammon HL, Murphy KC, Chandrasekhar K, Wlodawer A. Preliminary crystallographic study of an L-asparaginase from *Vibrio succinogenes*. *J Mol Biol* 1985; 184: 179-81.
- [40] Ammon HL, Weber IT, Wlodawer A, et al. Preliminary crystal structure of *Acinetobacter glutaminasificans* glutaminase-asparaginase. *J Biol Chem* 1988; 2631: 150-6.
- [41] Jones S, Thornton JM. Principles of protein-protein interactions. *PNAS USA* 1996; 93: 13-20.
- [42] Richardson JS In: *Advances in Protein Chemistry*. New York, Academic 1981; 34: 196-7.
- [43] Kelo E, Noronkoski T, Stoineva IB, Petkov DD, Mononen I. Beta-aspartylpeptides as substrates of L-asparaginases from *Escherichia coli* and *Erwinia chrysanthemi*. *FEBS Lett* 2002; 528: 130-2.
- [44] Ehrman M, Cedar H, Schwartz JH. L-asparaginase-II of *Escherichia coli* - Studies on enzymatic mechanism of action. *J Biol Chem* 1971; 246: 88-94.
- [45] Röhm KH, van Etten RL. The O-18 isotopic effect in C-13 nuclear magnetic resonance spectroscopy: Mechanistic studies on asparaginase from *Escherichia coli*. *Arch Biochem Biophys* 1986; 244: 128-136.
- [46] Wehner A, Harms E, Jennings MP, et al. Site-specific mutagenesis of *Escherichia coli* asparaginase-II - none of the 3 histidine-residues is required for catalysis. *Eur J Biochem* 1992; 208: 475-80.
- [47] Dodson G, Wlodawer A. Catalytic triads and their relatives. *Trends Biol Sci* 1998; 23: 347-52.
- [48] Harms E, Wehner A, Aung HP, Röhm KH. A catalytic role for threonine-12 of *Escherichia coli* asparaginase-II as established by site-directed mutagenesis. *FEBS Letters* 1991; 285: 55-8.

- [49] Derst C, Wehner A, Specht V, Röhm KH. States and functions of tyrosine residues in *Escherichia-coli* asparaginase-II. *Eur J Biochem* 1994; 224: 533-40.
- [50] Derst C, Henseling J, Röhm KH. Probing the role of threonine and serine residues of *E. coli* asparaginase II by site-specific mutagenesis. *Protein Eng* 1992; 5: 785-9.
- [51] Derst C, Henseling J, Rohm KH. Engineering the substrate specificity of *Escherichia coli* asparaginase. II. Selective reduction of glutaminase activity by amino acid replacements at position 248. *Protein Sci* 2000; 9: 2009-17.
- [52] Bagert U, Rohm KH. On the role of histidine and tyrosine residues in *E. coli* asparaginase. Chemical modification and 1H-nuclear magnetic resonance studies. *Biochim Biophys Acta* 1989; 9991: 36-41.
- [53] Aung HP, Bocola M, Schleper S, Röhm KH. Dynamics of a mobile loop at the active site of *Escherichia coli* asparaginase. *Biochim Biophys Acta* 2000; 14812: 349-59.
- [54] Mishalska M, Bujacz G, Jaskolski M. Crystal structure of plant asparaginase. *J Mol Biol* 2006; 360: 105-16.

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