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A left-handed crossover involved in amidohydrolase catalysis

Crystal structure of *Erwinia chrysanthemi* L-asparaginase with bound L-aspartate

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The crystal structure of L-asparaginase from *Erwinia chrysanthemi* in the presence and absence of L-aspartate was determined at 1.8 Å resolution. Conserved residues in a left-handed crossover (a rare occurrence in protein structures) link pairs of dimers into the catalytically active tetrameric form of the enzyme. The structure of ErA containing bound aspartic acid shows that this unusual strand connectivity is an essential part of the active site architecture, responsible for releasing the product of the enzymatic hydrolysis. The orientation of the bound aspartate indicates for the first time a threonine residue as a catalytic nucleophile.

L-Asparaginase; Left-handed crossover; Crystal structure; L-Aspartate-bound amidohydrolase; Threonine as nucleophile; *Erwinia chrysanthemi*

1. INTRODUCTION

L-Asparaginase belongs to a family of homologous amidohydrolases that deamidate L-asparagine and L-glutamine. Although much of the detailed enzymological study of L-asparaginases has focused on *Escherichia coli* asparaginase (EcA), it is clear that the enzymatic mechanisms of all type II amidohydrolases are similar. Kinetic studies show that the deamidation reaction takes place in two stages. First a nucleophilic group of the enzyme attacks the C_γ of the substrate forming an acyl–enzyme intermediate, and releasing the amine group. This intermediate is then attacked by a secondary nucleophile, normally water, releasing the product [1,2]. Activated substrate analogs [3,4] have been used to identify residues located at the active site, and based on these results it has been proposed that this family of enzymes are serine hydrolases mechanistically similar to the serine proteases. Recent crystallographic determination of the structures of bacterial L-asparaginases [5] (also, Gribskov et al., in preparation; Lubkowski et al., in preparation) allowed the location of the active site to be predicted by comparison of the parallel sheet motif in the N-terminal domain of L-asparaginase with nucleotide binding motifs such as those found in flavodoxin [6] and liver alcohol dehydrogenase [7]. The active site is located primarily in the parallel subdomain of the N-terminal domain in a cleft between the N- and C-terminal parallel sheet motifs belonging to *different* monomers. The occurrence of the active site between two α/β domains in L-asparaginase is similar to the situation in arabinose binding protein [8] wherein a cavity between two domains of the *same* molecule forms the arabinose binding site. The location of the L-asparaginase active site between the N-terminal domain of one molecule and the C-terminal domain of another implies that the active enzyme must be at least a dimer. Bacterial and mammalian asparaginases are known to function as tetramers and may thus be described as dimers of dimers. On the other hand, plant asparaginases are active as dimers [9–11], suggesting that a dimer is sufficient for activity. All three L-asparaginases studied by us crystallographically show a rare feature, a left-handed crossover (explained later) connecting two parallel β strands. Its importance for catalysis is discussed here based on the 1.8 Å resolution structure of *Erwinia chrysanthemi* (ErA) enzyme with bound aspartate.

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2. MATERIALS AND METHODS

ErA was provided by Shwu-Mann Lee of Program Resources Inc., Frederick, MD, USA. Crystals of ErA were grown in hanging and/or sitting drops from 50% ammonium sulphate in the pH range 8–9 (0.1 M CHES buffer) with 2% (w/v) of PEG 400. ErA crystalizes in the space group *C2* in a monoclinic unit cell of dimensions $a = 107.7$, $b = 91.4$, $c = 128.7$ Å and $\beta = 91.8°$ with the asymmetric unit containing an asparaginase tetramer. The X-ray crystallographic data on the native crystals were collected on a Siemens area detector. Data were measured for 537,875 reflections resulting, after merging and scaling, in 117,409 unique reflections extending to 1.71 Å resolution.

The structure of ErA was obtained using the molecular replacement technique starting with a preliminary model of EcA [5]. Details of the

ErA structure solution will be presented elsewhere (Gribskov et al., in preparation). The structure has been refined using the X-PLOR program [12] to an *R*-factor of 0.155 in the resolution range 10–1.8 Å with good geometry (e.g the rms deviation of bond lengths and angles from ideal values are 0.014 Å and 2.6°, respectively).

Crystals of ErA containing bound L-aspartic acid were obtained as follows. The native crystals were first lightly fixed with 0.02% glutaraldehyde in a synthetic mother liquor buffered to pH 5.5 with acetate buffer, as attempts to transfer ErA crystals to solution containing PEG or MPD instead of ammonium sulphate were unsuccessful. The cross-linked crystals were soaked overnight in a solution of 30% PEG 400 and 0.01 M L-aspartic acid in 0.1 M acetate buffer at pH 5. In comparison with the native crystals, there was practically no change either in the unit cell dimensions or in the quality of the diffraction pattern. For these crystals, data were collected to 1.8 Å resolution using the RAXIS imaging plate and were processed with the Molecular Structure Corporation software system. Data were collected for 191,721 reflections that finally resulted in 81,855 unique reflections. The native structure, with no solvent in the region of the active site and with other water molecules having B factors less than 30 Å^2, was refined as a rigid body followed by a simulated annealing protocol using the X-PLOR package. The aspartates in the active sites were modeled to an (F_o–F_c) electron density map and solvent molecules were added in steps and refinement was continued. The current *R*-factor is 0.16 for the 10–1.8 Å resolution data with good geometry (e.g. the rms deviation in bond lengths and bond angles from known values of 0.016 Å and 2.6°, respectively.).

3. RESULTS AND DISCUSSION

ErA is a homotetramer of 327 residue subunits with approximate 222 symmetry. Fig. 1 shows the structure of the ErA monomer. Each ErA subunit consists of two domains connected by an extended linker (residues 197–218). The larger N-terminal domain comprises two sub-domains. The first 156 residues form a parallel α/β structure similar to dehydrogenases [7], flavodoxin [6], and the two domains of arabinose binding protein [8], with a strand connectivity of [−1x, +2x, +1x, +1x]. Residues 157–196 of the N-terminal domain form a two-layered antiparallel β-sheet. Three of the five strands in this sub-domain continue the β-sheet from the N-terminal subdomain giving rise to an eight-stranded mixed β-sheet with the the first five strands being parallel and the last four being antiparallel. The smaller C-terminal domain consists of a four-stranded parallel β sheet with [+1x, +1x, +1x] connectivity. This parallel sheet, as in the N-terminal case, has two α-helices on either side.

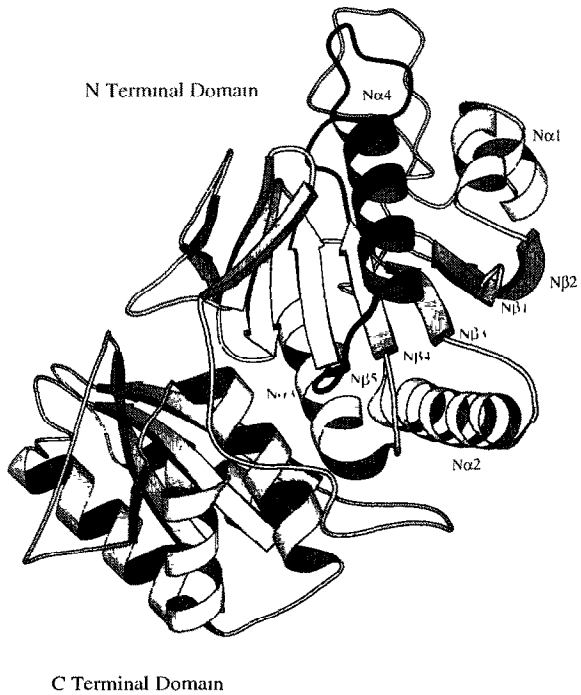

Fig. 1. Ribbon diagram [23] of an ErA monomer The active site lies at the top of the N-terminal domain. The left-handed crossover (heavily shaded) lies between Nβ4 and Nβ5.

In α/β structures, when one views two adjacent strands arranged in such a way that the first strand is on the left side and the second is on the right and the N-terminal end of the parallel strands is towards the observer and the C-terminal end goes away from observer, the connecting helix (or loop) from the first to the second strand (irrespective of the presence of any intervening strand) is above the plane of the two strands for a right-handed crossover and below the plane for a left-handed crossover [13]. In L-asparaginases, the crossover between Nβ4 and Nβ5 in the N-terminal domain is left-handed. Such crossovers have been observed only rarely in protein structures, for example, in subtilisin [14], acetylcholine esterase [15], and steroid dehydrogenase [16]. In subtilisin, residues in the left-handed crossover are essential for the function and the activity of the enzyme [17]. This also appears to be the case in the type II L-asparaginases in which the left-handed crossover forms part of the active site (see below). Furthermore, the amino acid residues that comprise the left-handed crossover are evolutionarily conserved (Fig. 2), probably because residues 128–132 provide a substantial part of the interface between the pairs of dimers in the tetramer. These residues are in contact with their symmetry-related cognates from the opposite dimer (i.e. are related by a non-crystallographic dyad axis).

Kinetic studies showed that at pH 5 aspartic acid binds to L-asparaginase equally well as asparagine [2], and electron density resembling aspartate was found in the active site of EcA [5]. Oxygen exchange experiments indicated that L-asparaginase catalyzes relatively rapid exchange between the β-carbonyl and water [2], and that aspartate is therefore a true substrate the binding of which depends on pH.

The difference Fourier map (Fig. 3) computed with the data collected on crosslinked ErA crystals soaked in aspartic acid solution buffered at pH 5.0, revealed the location and orientation of the bound aspartate, and the subsequent refinement proved that the substrate was present in the active site at high occupancy. At the current stage of refinement, the mean atomic temperature factors for the bound aspartates in the four monomers are, respectively, 29, 27, 28, and 25 Å^2. The struc-

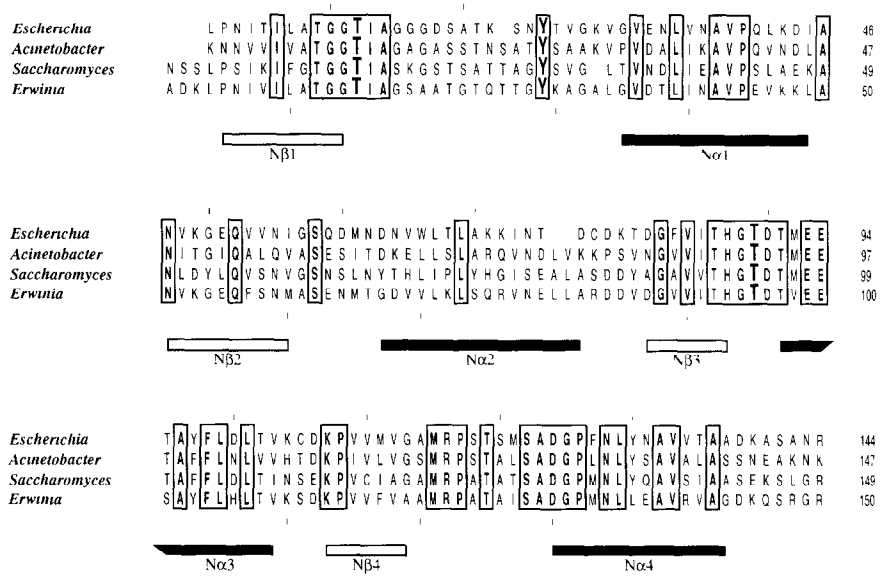

Fig. 2. Sequence alignment of the catalytic domain of type II amidohydrolases. Residues identical in all four sequences are boxed, and the catalytically important Thr-15 and Thr-95 are shown in large type. Open bars under the sequence show the locations of β-strands, and filled bars show the location of α-helices.

ture of ErA with bound aspartic acid thus confirms the location of the active site, and permits the groups involved in catalysis to be described.

As shown in Fig. 3, the side chain atoms of the bound aspartic acid are located between the hydroxyl groups of Thr-15 (12_{EcA}[1] and Thr-95 (89_{EcA}), and near to the main chain atoms of Ala-120 (114_{EcA}), the first residue of the left-handed connection between Nβ4 and Nβ5. The catalytic portion of the active site is therefore made up of the three loops linking Nβ1 to Nα1 (residues 14–35), Nβ3 to Nα3 (residues 93–97), and Nβ4 to Nα4 (residues 119–130). The β-carboxyl oxygens of the substrate are almost co-planar with the carbonyl of Ala-120 (114_{EcA}), which can act as an acceptor in an hydrogen bond with the aspartate $O_{\delta 2}$, suggesting that the bound aspartate is protonated. At neutral pH, the apposition of the charged aspartate carboxylate group and the main chain carbonyl oxygen of Ala-120 (114_{EcA}) generates a strong repulsion that helps to release the product of the enzymatic hydrolysis. Two water molecules are

[1] To simplify comparions between ErA and EcA, the EcA reside numbers are given in parentheses.

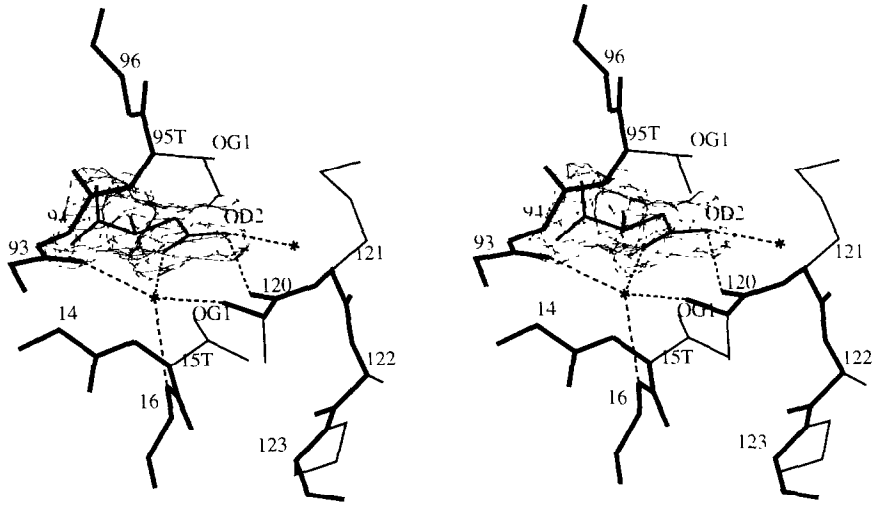

Fig. 3. Stereo diagram of the bound aspartic acid in the *initial* (F_o–F_c) electron density contoured at 3σ level. Selected hydrogen bonds between the reactive end of the substrate and its environment are shown as dashed lines, the two water molecules as asterisks, and the enzyme main chain is drawn in thick lines. For clarity not all side chains (thin lines) are shown.

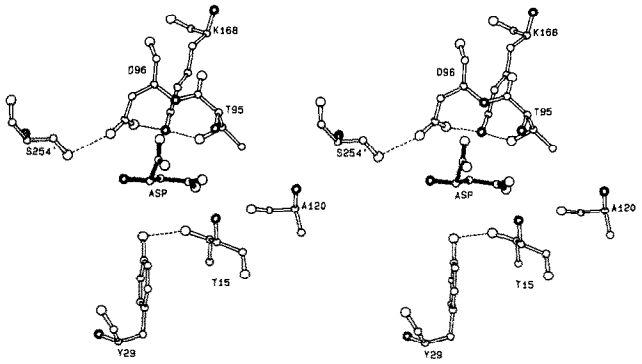

Fig. 4. Constellation of residues forming the ErA active site surface. The bound aspartic acid is shaded with the β-carbonyl extending towards the right. Note the location of the Oγ of Thr-15 and Thr-95 below and above the Cγ of the substrate. No other side chains closely approach the Cγ of the substrate.

also located in the same plane, forming an hydrogen bond network aiding in the proper positioning of the substrate. One of them anchors the β-carboxylate oxygen of the substrate to the main chain atoms of the enzyme by hydrogen bonds: as a proton donor to $O_{\delta 1}$ of the substrate and carbonyl oxygen of His-93 (87_{EcA}); as an acceptor from main chain NH groups of Ile-16 (13_{EcA}) and Ala-120 (114_{EcA}). $O_{\delta 1}$ of the substrate side chain is sandwiched between the main chain nitrogen of residues Thr-15 (12_{EcA}) and Thr-95 (89_{EcA}). This is similar to the 'oxyanion hole' seen in serine proteases [18] and presumably acts to stabilize the partial negative charge that develops in the tetrahedral intermediate of the formation of the acyl–enzyme complex. When asparagine is a substrate, the carbonyl oxygen presumably occupies the position of $O_{\delta 1}$, while the amide nitrogen occupies the position of $O_{\delta 2}$.

The α-carboxylate end of the substrate is held in position by a network of hydrogen bonds and charge interactions. The carboxylate group is oriented by hydrogen bonds to the main chain nitrogens of Ser-62 (58_{EcA}) and Asp-96 (90_{EcA}), and to the side chain O_γ of Ser-62 (58_{EcA}). The α-amino group interacts with the acidic residues Glu-63 (59_{EcA}) and Asp-96 (90_{EcA}). This interaction probably accounts for the observation that the substrate α-amino group must be protonated [19,20].

The amino acid residues that form the surface of the active site are shown in Fig. 4. Thr-95 (89_{EcA}), Lys-168 (162_{EcA}) and Asp-96 (90_{EcA}) are connected by strong hydrogen bonds. Asp-96 (90_{EcA}) also forms an hydrogen bond with Ser-254 (248_{EcA}) from another subunit. As a result of this string of interactions, this side of the active site is rigid. In contrast, both Thr-15 (12_{EcA}) and Tyr-29 (25_{EcA}) moved slightly from the positions observed in the native structure, indicating that that side of the active site cleft is somewhat flexible. In addition to its close proximity to the substrate, the hydroxyl of Thr-15 (12_{EcA}) might also act as a donor in an hydrogen bond with Tyr-29 (25_{EcA}). Although none of the side chains of the left-handed crossover are part of the active site surface, Met-121 (115_{EcA}) and Pro-123 (117_{EcA}) provide hydrophobic contacts for the methyl groups of Thr-95 (89_{EcA}) and Thr-15 (12_{EcA}), thus preventing rotation.

Mutation of either Thr-95 (89_{EcA}) or Thr-15 (12_{EcA}) diminishes enzymatic activity to about 0.01% of the wild-type enzyme's activity [21,22] (Röhm, personal communication). The positions of the amino acid residues in the active site suggest several possible pathways for the nucleophilic catalysis, but the almost symmetric location of these threonine residues above and below the Cγ of the substrate suggests that one of them must be the catalytic nucleophile. This is supported by the location of both threonines within two absolutely conserved regions of amino acid sequence located at residues 12 (9_{EcA}) to 16 (13_{EcA}) and 93 (87_{EcA}) to 97 (91_{EcA}) (Fig. 2). Site-directed mutagenesis experiments coupled with X-ray structural studies are necessary to completely elucidate the mechanism of the reaction.

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