A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: Crystal structure of a Thr-89-Val mutant

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Abstract ***Escherichia coli*** **asparaginase II catalyzes the hydrolysis of L-asparagine to L-aspartate via a threonine-bound acyl-enzyme intermediate. A nearly inactive mutant in which one of the active site threonines, Thr-89, was replaced by valine was constructed, expressed, and crystallized. Its structure, solved at 2.2 Å resolution, shows high overall similarity to the wild-type enzyme, but an aspartyl moiety is covalently bound to Thr-12, resembling a reaction intermediate. Kinetic analysis confirms the deacylation deficiency, which is also explained on a structural basis. The previously identified oxyanion hole is described in more detail.**

Key words: Asparaginase II; Acyl-enzyme intermediate; Threonine amidohydrolase; Enzymatic mechanism

1. Introduction

Asparaginases catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia. The enzymes isolated from *Escherichia coli* (EcA) and *Erwinia chrysanthemi* (ErA) have been utilized as anti-leukemia drugs for many years [1]. Treatment with asparaginases is often accompanied by severe side effects, which are partially attributed to the glutaminase activity of these enzymes [2]. In order to understand the enzyme specificity and to ultimately eliminate the glutaminase activity, the mechanism of action must be elucidated. Several members of a larger family of homologous L-asparaginases have thus been thoroughly investigated over many years.

Results of kinetic measurements [3,4] indicated that the enzymatic reaction proceeds via a covalent intermediate, probably a β-aspartyl enzyme (Fig. 1). This mechanism was confirmed by NMR studies with aspartate through the oxygen exchange reaction with bulk solvent at the side chain carboxylate [5]. These experiments showed that at pH < 5 L-aspartate with a protonated side chain binds to the enzyme as tightly as L-asparagine and it can also form an acyl-enzyme intermediate, subsequently hydrolyzed to L-aspartate. Thus, at low pH both L-aspartate and L-asparagine can function as substrates. The nature and identity of the primary nucleophile of the enzyme participating in the formation of the acyl-enzyme intermediate have been major subjects in the investigations of the mechanism of asparaginases.

Chemical modification with substrate analogs has identified Thr-119 or Ser-120 in EcA [6] and Thr-12 in *Acinetobacter glutaminasificans* glutaminase-asparaginase (AGA) [7] as reactive residues. Mutational studies on EcA showed no significant importance of Thr-119 and Ser-122 [8] but revealed Thr-12, Tyr-25, Thr-89, Asp-90, and Lys-162 as essential [9]. Structural data showed that all these essential residues are located in the active site.

The crystal structures of five bacterial asparaginases have been solved (EcA [10], ErA [11], AGA [12], *Pseudomonas* 7A glutaminase-asparaginase (PGA) [13], and *Wolinella succinogenes* asparaginase (WsA) (Lubkowski et al., submitted for publication). Each has the same tetrameric quaternary structure as it has in solution, a homotetramer of approx. 4×330 residues. The tetramer is more accurately described as a dimer of dimers. Each of two identical active sites in each dimer is formed by both monomers. In the structure the active sites have been observed with and without aspartate as ligand. Part of the active site is covered by a flexible loop that contains the two important residues Thr-12 and Tyr-25. The hydroxyl groups of Thr-12 and Thr-89 are closest to the side chain carboxylate of an aspartate bound in the active site. These residues are the most likely candidates for the primary nucleophile. Bacterial L-asparaginases were the first threonine amidohydrolases described in the literature. Only recently, two other threonine amidohydrolases, 20S proteasome [14] and aspartylglucosaminidase [15], have been reported. Both enzymes belong to the nonrelated superfamily of N-terminal nucleophile (Ntn) hydrolases [16].

So far, neither kinetic experiments nor structural data could unambiguously identify which of the two threonine residues in asparaginase adjacent to the substrate is the primary nucleophile. Here we present the structure of *E. coli* asparaginase II T89V mutant with aspartate covalently bound to Thr-12, strongly suggesting that Thr-12 assumes the role of the primary nucleophile.

2. Materials and methods

The oligonucleotide-directed mutagenesis of the *ans*B gene in M13mp19 [17,18] and the overexpression of EcA [19] have been described in detail elsewhere. Mutants T89V and T89S were constructed via the degenerate oligonucleotide 5′-ACC CAC GGT **(G,A)(T,G)**C GAC ACG ATG-3′.

Identification of mutant phages by DNA sequencing, subcloning of the mutant genes into the *Eco*RI/*Hin*dIII site of plasmid pTWE1, and expression in an EcA-free strain of *E. coli*, CU1783, followed the published protocols [19].

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Abbreviations: EcA, *Escherichia coli* asparaginase II; ErA, *Erwinia chrysanthemi* asparaginase; PGA, *Pseudomonas* 7A glutaminase-asparaginase; AGA, *Acinetobacter glutaminasificans* glutaminase-asparaginase; WsA, *Wolinella succinogenes* asparaginase; AHA, L-aspartic β-hydroxamate; MES, 2-[*N*-morpholino]ethanesulfonic acid; rmsd, root mean square deviation.

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All kinetic experiments were performed with the asparagine analog L-aspartic β-hydroxamate (AHA) as the substrate. The activities of EcA toward its natural substrate, L-asparagine, and AHA are comparable. The latter substrate was preferred for the sensitive and reliable assay of its product, hydroxylamine (NH_2OH), with 8-hydroxyquinoline [8].

In 'initial burst' experiments, the time course of the appearance of NH_2OH and aspartate was followed at very high enzyme concentrations. The enzyme was first dialyzed against a volatile buffer (20 mM NH_4HCO_3 at pH 8) and lyophilized. The resulting preparations, almost salt-free, were reconstituted in the minimum volume of 50 mM MES-NaOH, pH 5.0, to give EcA concentrations of 30–50 mg/ml. The reaction was started by addition of AHA solution. At various times, 10-μl aliquots were withdrawn and analyzed for NH_2OH and aspartic acid. NH_2OH was determined as above, while aspartic acid was assayed with a coupled enzymatic test involving aspartate transaminase and malate dehydrogenase [20].

Crystals were grown by the vapor diffusion method. The protein was concentrated to 17 mg/ml in 10 mM Tris-HCl, pH 7.0. Drops (protein solution:well solution 1:2) were equilibrated against 36% MPD, 100 mM sodium aspartate, 100 mM sodium acetate, pH 5.0, at 15°C. Thin plates appeared after 2–10 days, growing slowly in thickness.

X-ray data were collected at −170°C (MSC low-temperature unit) using an R-axis II image plate detector mounted on a Rigaku RU-200 rotating anode generator with mirrors operating at 50 kV and 100 mA. X-ray data, extending to 2.2 Å, were collected from one crystal of size 0.5×0.5×0.15 mm. The protein crystallized in the space group $P2_12_12_1$ (unit cell: $a = 95.0$ Å, $b = 126.2$ Å, $c = 155.7$ Å). Data processing for 197,314 observations was performed with DENZO and SCALEPACK [21], resulting in 68 197 unique reflections ($R_{merge} = 10.2\%$, 72% complete).

All calculations related to either structure solution or refinement of the model were performed with X-PLOR [22], using the parameters of Engh and Huber [23]. Model rebuilding was performed using the FRODO computer graphics package [24]. The structure was solved by molecular replacement using the EcA tetramer [10] as a model. Rotation and translation functions gave very clear solutions for different sets of parameters. Data with a cutoff of $2.5\sigma(F)$ (60 324 reflections) were used. At the final stages of rebuilding and refinement, water molecules were added and Thr-12 was replaced by β-(β-aspartyl)threonine (Fig. 2). The final crystallographic R factor was 18.2% with good geometry (rmsd from parameter set: bonds, 0.015 Å; angles, 1.9°; dihedral angles, 25.0°; improper angles, 1.8°). Structural superpositions were performed with the program ALIGN [25].

3. Results

The mutant proteins EcA(T89V) and EcA(T89S) were purified by standard procedures developed for EcA(wt). Their isoelectric points (determined by isolectric focusing), molecular size (determined by analytical gel filtration and SDS-polyacrylamide gel electrophoresis), and thermodynamical stabilities (derived from denaturation curves in guanidine hydrochloride solutions) were almost the same as those found with wild-type EcA (data not shown).

Despite the different crystal forms ($P2_12_12_1$ vs. $P2_1$ for EcA(wt), crystallized from MPD/PEG at pH 5.0), the overall structure of EcA(T89V) closely resembles that of EcA(wt). The rmsd between the monomers in the asymmetric unit is 0.24 Å (averaged for all combinations, 1270 of 1305 atoms used in each alignment) for main chain atoms in EcA(T89V), 0.22 Å (1220 atoms) in EcA(wt), and 0.30 Å (1250 atoms) between EcA(wt) and EcA(T89V). Pairs of monomers and the whole tetramers align equally well (0.31 Å, 2470 of 2610 main chain atoms and 1260 of 1304 Cα, respectively). Thus, not only is the structure of the monomers highly conserved with respect to EcA(wt), but also the arrangement of the subunits within the tetramer is preserved.

The active site in EcA(T89V) is very similar to that in EcA(wt). Average rmsd values are 0.33 and 0.44 Å for the four independent monomers of EcA(T89V) and EcA(wt), respectively. All atoms of the ligand, all residues with atoms closer than 4.0 Å to the ligand, and Lys-162 were used for these superpositions. EcA(wt) and EcA(T89V) align with an rmsd of 0.55 Å (the same atom set as before, but without ligand) (Fig. 3). B factors of all active site residues are comparable to or below the average value for the entire molecule (20.8 $Å^2$) except for residues on the flexible loop, including Tyr-25 and Val-27 (average B factors 35.4 and 45.6 $Å^2$, respectively), which are close to the tip of this loop.

The most important feature of this structure is an aspartyl moiety covalently bound to Thr-12. Electron density maps clearly showed the covalent linkage and allowed unambiguous modeling of the aspartyl. The density for the amino group orients the aspartyl such that the ester linkage must be through the aspartate side chain carboxyl group. Several residues contribute to the binding of the aspartyl moiety in the acyl enzyme. The α-amino group of the aspartyl is hydrogen bonded to Oε1 of Glu-59, Oδ2 of Asp-90, and Oε2 of Glu-283* (residues marked with an asterisk belong to the second monomer forming the active site), while the α-carboxyl group is coordinated by main chain nitrogens of Asp-90 and Ser-58, and Oγ of Ser-58 (Fig. 4). The main chain nitrogen of Val-89

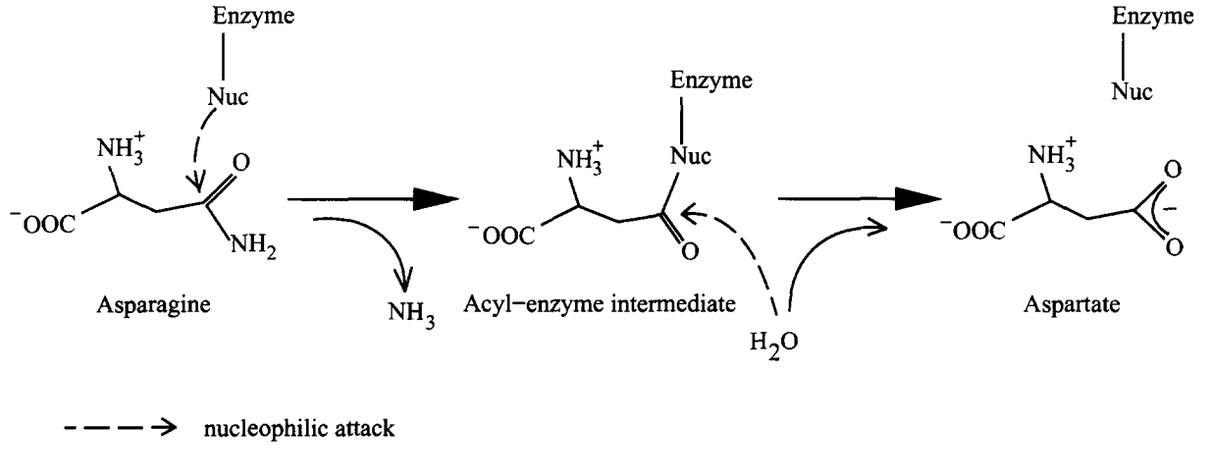

Fig. 1. Schematic illustration of the reaction catalyzed by asparaginases. The proposed covalent intermediate is formed by the EcA residues Thr-12 or Thr-89 as the nucleophile (Nuc).

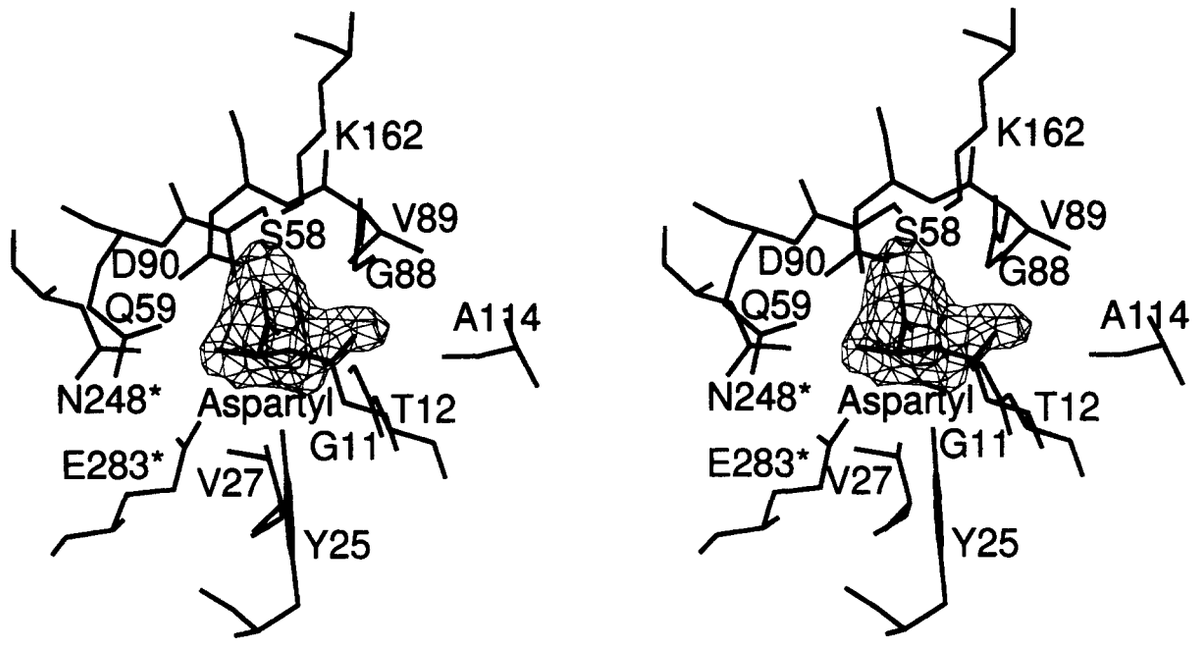

Fig. 2. Stereo diagram of the final model in the active site (monomer 3). The electron density ($|F_o|-|F_c|$, contoured at 2.7σ) was created from the model before introducing the covalently bound aspartyl group. Residues marked with an asterisk belong to the second monomer forming the active site.

and a water molecule form an oxyanion hole for the carbonyl oxygen of the ester group (Oε1), in the same way that Oδ1 of the aspartate is coordinated in EcA(wt). We identified a water molecule in an equivalent position in all known asparaginase structures. The tetrahedral coordination of this water molecule (Oε1 of the aspartyl group in EcA(T89V) or Oδ1 of the aspartate in EcA(wt); main chain nitrogens of Gly-13 and Ala-114; carbonyl oxygen of His-87) makes it an unambiguous proton donor toward Oε1 of the ester group.

Because of the formation of the acyl enzyme, a few structural changes in the active site are visible. To create the ester bond, Oγ1 of Thr-12 and Cδ of the ligand move toward each other (by 0.6 and 1.1 Å, respectively, compared with EcA(wt)). One of the two ligand side chain oxygens in EcA(wt), Oδ1, is retained in the ester group of the acyl enzyme as Oε1, while the other one (Oδ2) is cleaved off.

Exchange of the hydroxyl group in Thr-89 by a methyl group in Val-89 destroys the hydrogen bond to the amino

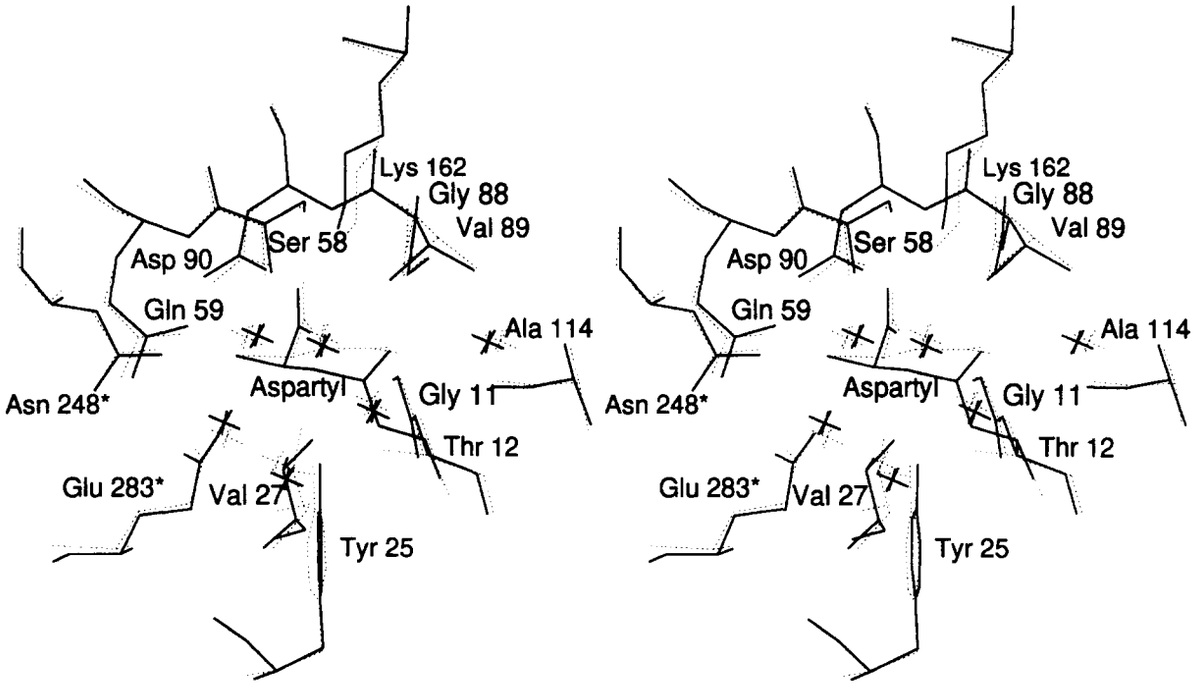

Fig. 3. Superposition of the active sites of EcA(T89V) and EcA(wt). Only one monomer of each structure is shown for clarity. EcA(T89V) is represented by solid lines, EcA(wt) by dashed lines. Water molecules are represented by crosses.

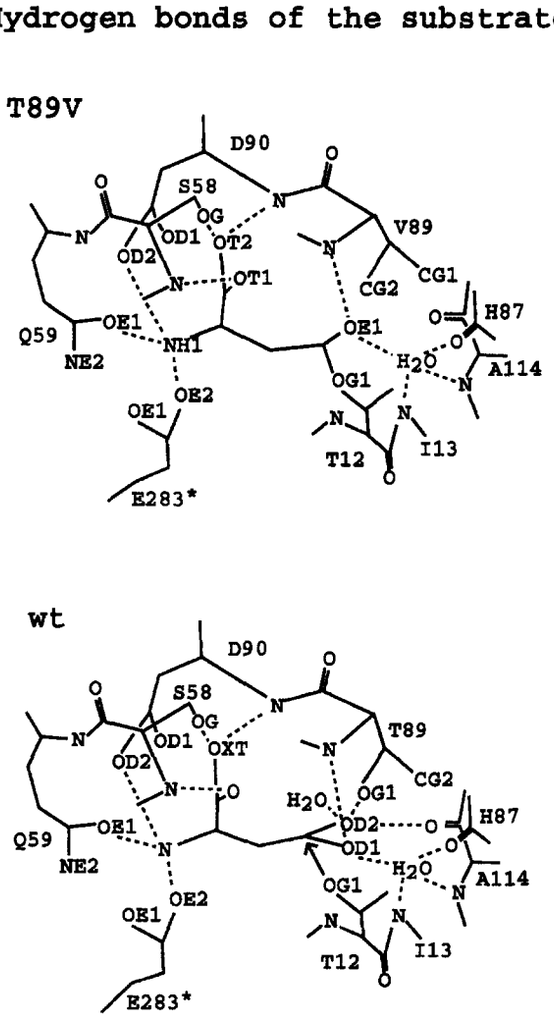

Fig. 4. Hydrogen-bonding network in the active site. Hydrogen bonds (dotted lines) stabilize the conformation of aspartate in EcA(wt) and of aspartyl in EcA(T89V). The proposed nucleophilic attack is indicated by an arrow.

group of Lys-162, resulting in interaction of the amino group with the main chain oxygens of Asn-164 and Thr-165, as well as with a water molecule. This change disrupts the characteristic asparaginase triad Asp-90–Lys-162–Thr-89.

The refined structure of the asparaginase mutant contains 719 water molecules. Those on monomer-monomer interfaces are less mobile (average B factor is 21.9 Å^2 for 147 water molecules, compared to 25.2 Å^2 for all 719 water molecules), while the average B factor for water molecules located more than 3.6 Å from any protein atom (73 water molecules) is 35.4 Å^2. Most of the water molecules (75%) were identified in equivalent sites of independent monomers.

Ten water molecules near the active site are conserved in all known structures of asparaginases. One of these molecules is mentioned above as part of the oxyanion hole. A ring of five water molecules connects all important active site residues by hydrogen bonds. Two of the five water molecules might be part of the path for exchanging ammonia for water during the reaction. Another set of four water molecules and Glu-93, Glu-94, and Arg-272 stabilize the main chain nitrogen and oxygen of Lys-162, an important residue for the reaction.

The maximum velocity (V) of EcA(T89V) was decreased by a factor of 25 000, whereas the activity of EcA(T89S) reached about 20% of that of the wild type (Table 1). Both enzyme forms exhibited Michaelis constants (K_m) comparable to, or even lower than, that of EcA(wt). To examine whether the residual activity of EcA(T89V) could be due to contamination with the wild-type enzyme, we expressed several independent batches of the mutant and purified the enzyme by different protocols (i.e. with or without re-chromatofocusing, with or without gel filtration). All of the resulting preparations had essentially the same kinetic properties. The ratio of activity toward L-asparagine and L-glutaminase activity of the mutant is at the detection limit (data not shown).

The unique kinetic properties of EcA(T89V) are apparent from the results shown in Fig. 5. Addition of the substrate AHA resulted in the rapid release of an approximately stoichiometric amount of aspartate. In the first minutes of the reaction this amount increased only slowly, whereas hydroxylamine was formed initially at a rate higher than that in the steady state. This finding indicates the existence of an initial burst of hydroxylamine formation (the reason for the initial rapid appearance of aspartate is discussed below). Initial bursts are typical of enzymes that use a double-displacement, or 'ping-pong' mechanism and occur only when the rate of cleavage of the covalent intermediate is significantly lower than its rate of formation. As expected on the basis of its known kinetic properties, EcA(wt) at high dilution liberated hydroxylamine and aspartate at the same rate.

4. Discussion

The structural study of EcA(wt) is part of our current interest in understanding the mechanism of action of asparaginases on a molecular level. Five residues (Thr-12, Tyr-25, Thr-89, Asp-90, and Lys-162) were shown to be important for catalytic activity [9]. Among them, only the threonines are in favorable positions for the nucleophilic attack on the substrate, the proposed first step of the reaction.

In the structure of EcA(T89V) an aspartate covalently bound to Thr-12 might resemble the acyl-enzyme intermediate. Clearly, the inactivity of this mutant is not due to its improper folding or to quaternary structure changes. The stability of EcA(T89V) and its main chain conformation are highly similar to those of EcA(wt). Furthermore, residue 89 shows virtually unaltered conformation in both structures, with the methyl group of the valine exactly replacing the hydroxyl group of the threonine (Fig. 3). The principal changes are the orientation of the side chain nitrogen of Lys-162 and the acylated Thr-12. Upon mutation, the hydrogen-bonding network of the asparaginase triad (Thr-89, Lys-162, Asp-90) is completely disrupted. This disruption is triggered by the loss of the hydrogen bond between Oγ1 of Thr-89 and the amino group of Lys-162, indicating the importance of the latter residue. Although the normal pK_a of lysine would predict that the amino group of Lys-162 is protonated, kinetic evidence of

Table 1
Kinetic parameters of EcA(wt) and Thr-89 mutants (in 50 mM MES-NaOH, pH 5.0, at 25°C)

Enzyme form	V (U/mg)	K_m (μM)
EcA(wt)	50 ± 2	155 ± 10
EcA(T89V)	0.0021 ± 0.0003	120 ± 30
EcA(T89S)	11 ± 1	90 ± 8

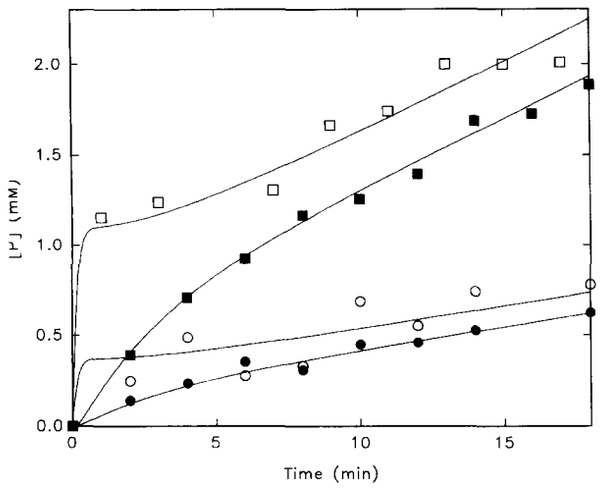

Fig. 5. Initial burst of NH_2OH formation by EcA(T89V). The experiment was performed at pH 5.0 and 25°C as described in Section 2 at an enzyme concentration of 0.35 mM (circles) or 1.1 mM (squares). The concentration of NH_2OH (closed symbols) and aspartate (open symbols) is plotted vs. reaction time. The solid lines are based on a kinetic model described in the text with the following constants: $k_1 = 5\ M^{-1}\ s^{-1}$; $k_2 = 3.5 \times 10^{-3}\ s^{-1}$; $k_3 = 1.7 \times 10^{-3}\ s^{-1}$; $K_d = 1 \times 10^{-5}$ M.

Lys-162 mutants is in support of this residue acting as a base (unprotonated) in the rate-determining step (data not shown). This lysine might thus play the role of a proton buffer for Thr-89.

The conformation of the acyl enzyme fits well into the reaction path. While the presumed nitrogen of the substrate asparagine [11] is cleaved off, the carbonyl oxygen of its ester group is stabilized by an oxyanion hole just as is the corresponding oxygen in EcA(wt).

In our current model of the mechanism of action, a water molecule is required as the nucleophile in the deacylation step. This water molecule could be located on either side of the plane formed by the ester group. One of these sides is buried by the hydrophobic side chain of Val-27 and the Cζ atom of the aspartyl group. In all other asparaginase structures with substrate the side chain of the homologous residue (Val or Ala) occupies the same place. Although Val-27 is part of a flexible loop, this conformation seems representative for the enzyme with substrate. A water molecule on this side could not be activated by any base. A water molecule on the other side would be close to Oγ1 of Thr-89 in the case of EcA(wt). In EcA(T89V), no density was found in this position in the map of any monomer; presumably, the methyl group of the valine repels any water molecule. In structures with substrate (EcA(wt), ErA), one oxygen (Oδ2) of the substrate side chain carboxylate occupies this position. However, the active sites with no ligands (PGA, WsA) show that water can bind to this position. Only Thr-89 can function as a base to activate this water molecule. We postulate that the lack of this water molecule in EcA(T89V) is an important factor for the mutant's inefficiency in the deacylation reaction. Other conserved water molecules are expected to play a role in the ammonia-water exchange and the stabilization of Lys-162.

Our kinetic results show that Thr-89, a strictly conserved residue in all known microbial asparaginases, is absolutely necessary for catalysis. The apparent first-order rate constant for AHA hydrolysis by EcA(T89V) is in the same range as the nonenzymatic hydrolysis of AHA at neutral pH. EcA with serine in position 89 exhibits activities in the same order of magnitude as wild-type EcA, indicating that the hydroxyl group in position 89 is indeed required.

The 'burst' behavior of EcA(T89V), shown in Fig. 5, is in full agreement with the existence of a covalent acyl-enzyme intermediate demonstrated by crystallography. Typically, such burst events (known, for example, from serine proteinases) take place in the millisecond to second range. The burst observed with EcA(T89V) is much slower, indicating that the mutation affects both the acylation and deacylation rates. Another unexpected feature is the initial rapid release of stoichiometric amounts of aspartate without concomitant liberation of NH_2OH. Obviously, despite dialysis the purified enzyme contained aspartate in each of the active sites, which was displaced upon addition of AHA (20 mM, more than $100 \times K_m$). As shown by Jayaram et al. [26], commercial asparaginase preparations isolated from *E. coli* cells contain bound aspartate (up to 3.2 molecules per tetramer), which could be removed only by prolonged dialysis at slightly alkaline pH or by depleting aspartate in the medium. Our results suggest that mutant T89V binds aspartate even more tightly than does the wild-type enzyme, such that the dialysis at pH 8 was not sufficient to remove it. Since Val-89 is probably not the cause of tight binding, the only other major change in the active site, at Lys-162, is the most likely reason. The movement of its amino group decreases repulsion to the amino group of the substrate. This effect underscores the involvement of Lys-162 in catalysis rather than in binding.

On the basis of the following simplified model (where E = enzyme, F = acyl-enzyme intermediate, A = AHA, D = aspartate, and N = NH_2OH),

$$\begin{array}{ccccccccc} & & k_1 & & k_2 & & k_3 & & K_d & \\ ED + A & \rightarrow & EA & \rightarrow & F & \rightarrow & ED & \leftrightarrow & E + D & \\ & & \downarrow & & \downarrow & & & & & \\ & & D & & N & & & & & \end{array}$$

the solid lines in Fig. 5 were calculated by numerical simulation with the program KINSIM [27]. The rate constants that yielded the curves shown in the figure are given in the legend. The value of K_d applied in the simulation corresponds to the dissociation constant of the enzyme-aspartate complex at pH 5, while k_3 equals the turnover number of AHA hydrolysis by EcA(T89V). The magnitude of k_1, being much higher than k_2 and k_3, was not critical. Although the fit has not been fully optimized, the results show that the model is entirely compatible with the data.

The aspartylthreonine structure represents a good model for the acyl-enzyme intermediate: the active site needs to rearrange only a little, the aspartyl is embedded in a sufficient hydrogen-bonding network, and hydrolytic cleavage in the case of the wild-type enzyme can be modeled. Although the acyl enzyme in EcA(T89V) is unambiguous, an alternate acyl-enzyme formed by Thr-89 cannot be excluded. The action of Thr-12 could be due to the inactivity of residue 89. Moreover, an acyl enzyme such as that observed in EcA(T89V) requires Thr-12 to be a strong nucleophile, which cannot be explained easily. The presence of a hydrogen bond between the hydroxyl groups of Thr-12 and Tyr-25 suggests that the tyrosine is involved in the abstraction of a proton from Thr-12. Although Tyr-25 is less likely to act as a base, it may relay the proton to further residues, possibly involving active site

water molecules. Further kinetic and structural evidence is necessary to understand the function of these residues and to characterize additional intermediates of the reaction.

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