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Engineering the substrate specificity of *Escherichia coli* asparaginase II. Selective reduction of glutaminase activity by amino acid replacements at position 248

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Abstract

The use of *Escherichia coli* asparaginase II as a drug for the treatment of acute lymphoblastic leukemia is complicated by the significant glutaminase side activity of the enzyme. To develop enzyme forms with reduced glutaminase activity, a number of variants with amino acid replacements in the vicinity of the substrate binding site were constructed and assayed for their kinetic and stability properties. We found that replacements of Asp248 affected glutamine turnover much more strongly than asparagine hydrolysis. In the wild-type enzyme, N248 modulates substrate binding to a neighboring subunit by hydrogen bonding to side chains that directly interact with the substrate. In variant N248A, the loss of transition state stabilization caused by the mutation was 15 kJ mol^{-1} for L-glutamine compared to 4 kJ mol^{-1} for L-aspartic β-hydroxamate and 7 kJ mol^{-1} for L-asparagine. Smaller differences were seen with other N248 variants. Modeling studies suggested that the selective reduction of glutaminase activity is the result of small conformational changes that affect active-site residues and catalytically relevant water molecules.

Keywords: L-asparaginase; *Escherichia coli*; glutaminase activity; site-directed mutagenesis; substrate specificity

Bacterial asparaginases have been employed in the treatment of acute lymphoblastic leukemia (ALL) for almost 30 years (Wriston & Yellin, 1973; Gallagher et al., 1989). Among the clinically useful asparaginases, isoenzyme II from *Escherichia coli* (EcA2) and an asparaginase isolated from *Erwinia chrysanthemi* (ErA) are now most widely used. Asparaginases, in combination with other drugs, are first administered to induce a remission of the disease (induction therapy). At a later stage, the continued application of asparaginase helps to prevent further outbreaks (maintenance therapy; for a recent review see Müller & Boos, 1998). The biochemical mechanism of asparaginase action in ALL therapy is simple: the transformed cells responsible for the disease have an absolute requirement for L-asparagine due to a decreased or missing asparagine synthetase activity. Depletion of asparagine in the blood, therefore, inhibits their proliferation. However, asparaginase therapy is often accompanied by serious side effects. In addition to hypersensitivity reactions, long-term treatment may lead to liver damage, acute pancreatitis, and other disturbances. It is thought that many of these effects are caused by the glutaminase activity of EcA2, which amounts to about 2% of its activity toward asparagine (Ollenschläger et al., 1988). Glutamine is the major transport form of amino nitrogen in the blood and also an amino group donor for many biosynthetic reactions. A prolonged decline of plasma glutamine levels, therefore, impairs a variety of biochemical functions, especially those of the liver.

In view of these facts, enzymes best suited for ALL treatment would be those with a high activity, a low K_m, and a strong preference for asparagine over glutamine. EcA2 and ErA best meet these criteria, whereas other asparaginases preferentially hydrolyze glutamine (see Fig. 2). In the 1980s, an asparaginase from *Wolinella succinogenes* (WsA) appeared to be promising, because in vitro studies indicated that its glutaminase activity was negligible (Durden & Distasio, 1981; Distasio et al., 1982). However, the crystal structure of WsA (Lubkowski et al., 1996) showed that its active site is almost identical to that of EcA2. In addition, WsA overexpressed in *E. coli* had a glutaminase side activity considerably higher than that reported in the literature (M. Liesert & K.H. Röhm, unpubl. obs.).

All known glutaminases/asparaginases of microbial origin have almost the same tertiary and quaternary structures and closely related active sites. The *E. coli* type II enzyme (EcA2) has been

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Abbreviations: EcA2, *Escherichia coli*; L-asparaginase, isoenzyme II (EC 3.5.1.1); ErA, *Erwinia chrysanthemi* asparaginase; WsA, *Wolinella succinogenes* asparaginase; AHA, L-aspartic β-hydroxamate.

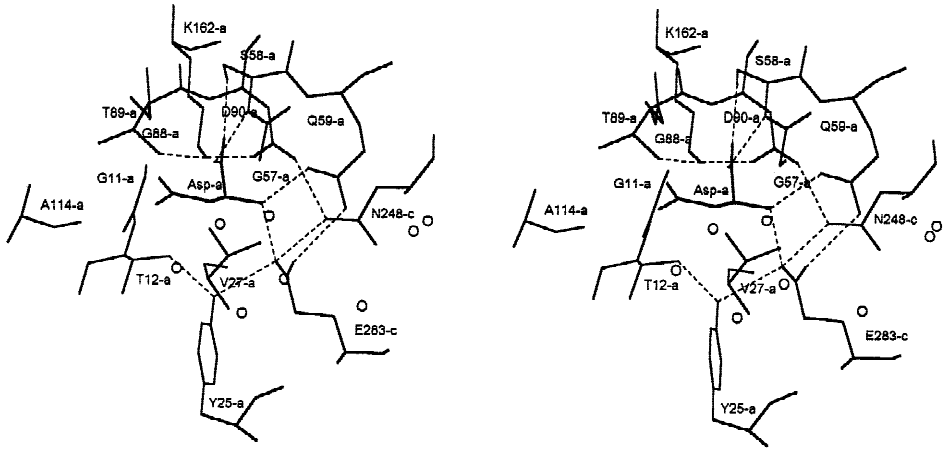

Fig. 1. Stereoview of the EcA2 active site with bound aspartate (Asp). Residues with suffix "–a" belong to subunit A, those with suffix "–c" to the neighboring subunit C. Hydrogen bonding interactions relevant for substrate binding or catalysis are indicated by dashed lines. A number of conserved water molecules are depicted as circles.

studied most extensively. Crystal structures of the EcA2/aspartate complex (Swain et al., 1993) and of variant EcA2 T89V (Palm et al., 1996) together with kinetic data collected in our laboratory (Wehner et al., 1994; Schleper, 1999) allowed the identification of residues involved in substrate binding and catalysis (see Fig. 1). Most of these amino acids are strictly conserved within the family (Fig. 2). A pair of threonine residues, T12 and T89, are located at either side of the scissile bond of the substrates. T12 and the adjacent Y25 are components of a mobile loop that closes over the active site during catalysis (Aung et al., 2000), while T89, D90 and K162 are all located in a rigid part of the structure. The relationship between the latter residues, which resemble the catalytic triads of serine hydrolases, has led to the suggestion of an analogous "asparaginase triad" (Dodson & Wlodawer, 1998). However, a number of results from our laboratory are not consistent with this notion. First, the crystal structure of variant EcA T89V showed a covalently bound β-aspartyl residue at T12, suggesting that T12 rather than T89 is the primary nucleophile (Palm et al., 1996). Second, our kinetic data indicate that the role of the putative "triad" is fundamentally different from the situation in serine enzymes (Schleper, 1999). Recently, an alternative "triad," made up of residues T12, Y25, and E283, was proposed (Ortlund et al., 2000). Such a scheme would imply a function of Y25 as general base, which is unlikely for chemical reasons. Second, E283, a crucial residue in such a mechanism, assists in substrate binding but is largely dispensable for catalysis (Schleper, 1999). Thus, the catalytic mechanism of the bacterial asparaginases remains a matter of debate.

The aim of the present study was to design and construct EcA2 variants with significantly reduced glutaminase activity. The use of such enzymes in ALL therapy may mitigate the side effects associated with wild-type EcA2.

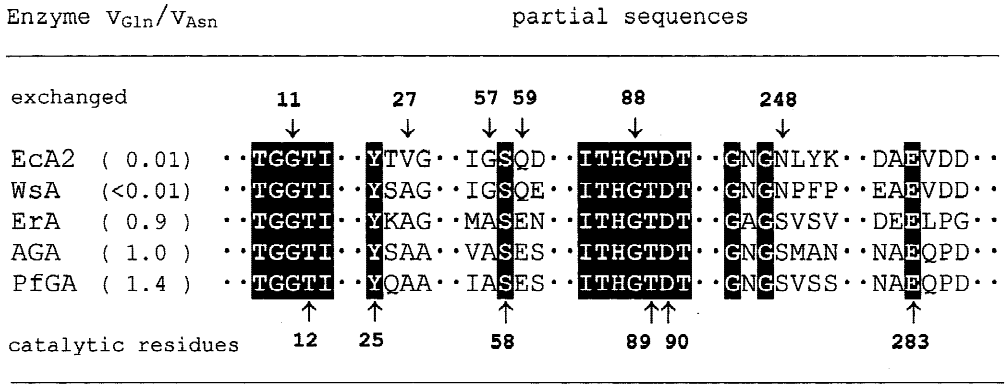

Fig. 2. Sequence alignment of type II asparaginases. The partial sequences shown include catalytical residues and the residues mutagenized in the present study ("exchanged"). Residues conserved in all type II enzymes are printed in reverse. The sequences shown are for asparaginase II from *E. coli* (EcA2; Jennings & Beacham, 1990), *W. succinogenes* asparaginase (WsA; Lubkowski et al., 1996), and glutaminases/asparaginases from *E. chrysanthemi* (ErA; Filpula et al., 1988), *Acinetobacter glutaminasificans* (AgA; Tanaka et al., 1988), and *Pseudomonas fluorescens* (PfGA; Hüser et al., 1999). Approximate ratios of the glutaminase and asparaginase activities of the enzymes are given in column v_{Gln}/v_{Asn}.

Results

Choice of residues for mutagenesis

In the context of the present study, we were interested in variants with low activity toward glutamine, but retaining high asparaginase activity. Therefore, residues required for catalysis (i.e., T12, Y25, T89, D90, and K162) were not taken into account. S58 and E283 were also excluded, because they are necessary for efficient substrate binding. On the other hand, a number of residues that surround the entrance to the active site without directly interacting with the substrate, appeared to be good candidates for a targeted modification of substrate specificity. These amino acids and their relationship with other residues and with bound aspartate are shown in Figure 1. Aspartate is both an inhibitor and a substrate of EcA2 (Röhm & Van Etten, 1986).

In the EcA2-aspartate complex, the side-chain carbonyl group of Q59 is hydrogen bonded to the ammonium function of bound aspartate. A network of further hydrogen bonds interconnects Q59 with E283 and Y25, and with the putative catalytic nucleophile, T12. The amide group of N248 bridges the side-chain carboxylates of D90 and E283, which, in turn, are involved in electrostatic interactions with the ammonium function of the substrate (note that both N248 and E283 belong to a neighboring subunit). The substrate carboxylate group is held in position by S58 via hydrogen bonds to the side-chain hydroxyl group and the main-chain NH, respectively. Glycines 11, 57, and 88 are immediate neighbors of catalytic residues (T12, S58, and T89), while V27 is part of the hydrophobic "back wall" of the substrate binding pocket.

Additional information was provided by the aligned sequences of type II asparaginases (Fig. 2). Glycines G11 and G88 (all sequence data given in EcA2 numbering) are conserved in all microbial asparaginases. V27 is close to Y25, an active-site residue, which is also present in all type II enzymes. Residues G57 and Q59 are immediately adjacent to S58, while N248 is preceded by a pair of invariant glycine residues (G245 and G247). Note that the enzymes with high glutaminase activity contain serine at position 248. In the present study, glycines 11, 57, and 88 as well as V27 were replaced with apolar amino acids of varying size to restrict the space available for the larger glutamine substrate. Positions 59 and 248 were substituted with both polar and nonpolar amino acids to allow for modifications of the hydrogen bonding network that interconnects these residues with neighboring amino acids and the substrate. In the following, asparaginase variants where residue X at position nnn is replaced with residue Y are referred to as "EcA XnnnY."

Expression and purification

All asparaginase variants described here were expressed and purified by our standard protocol (Harms et al., 1991a). The resulting preparations were at least 95% pure. Yields varied between 5 and 20 mg of purified enzyme per liter of *E. coli* culture, depending on the stability of the respective proteins. All variants had the normal tetrameric structure, as verified by analytical gel filtration.

Features of EcA2 variants

The variant enzymes were thoroughly characterized with respect to their catalytic and structural properties. In addition to kinetic parameters for several different substrates, thermodynamic binding constants K_d for aspartate were determined from the dependence of fluorescence quenching on ligand concentration. Stability parameters $\Delta G(H_2O)$ and T_m were derived from guanidine·HCl denaturation curves and thermal denaturation experiments, respectively (Derst et al., 1994).

Kinetic properties

For detailed kinetic analyses, L-asparagine, L-glutamine, and the substrate analog L-aspartic acid β-hydroxamate (AHA) were used as substrates. AHA was chosen, because the K_m values for Asn of wild-type EcA2 and many variants are in the micromolar range, and thus are difficult to determine with sufficient precision, while for AHA a highly sensitive and reliable assay is available (see Materials and methods). Activities toward L-asparagine were usually determined at a constant substrate concentration of 5 mM, which is almost saturating for most mutants. Kinetic parameters determined in this way are summarized in Tables 1 and 2.

Figure 3 compares K_m values for AHA with binding constants, K_d, for L-aspartate. Both parameters were determined at pH 6, because aspartate binding at higher pH is too weak to be easily measured by our assay. K_m(AHA) and K_d(Asp), when plotted against each other, exhibited a more or less linear correlation (Fig. 3), i.e., the mutations affected both parameters in the same way. This is especially obvious for series of homologous mutants, for example, for variants with exchanges at position 248 (dashed line).

To visualize the differential effects of a given mutation on the turnover of AHA and Gln, respectively, Figure 4 compares selected $\Delta\Delta G^*$ values for these substrates. $\Delta\Delta G^*$ is calculated from the specificity constants k_{cat}/K_m of wild-type enzymes and mutants,

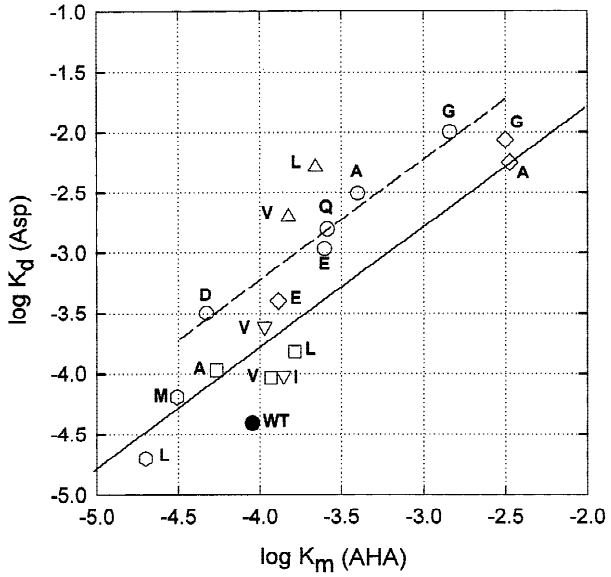

Fig. 3. Michaelis constants for AHA and binding constants for Asp of wild-type EcA2 and EcA2 variants. The data (measured at pH 6.0 and 25 °C and shown on logarithmic scales) are for wild-type enzyme (●) and variants with amino acid replacements at position 11 (△), 27 (⬡), 57 (□), 59 (◇), 88 (▽), and 248 (○). The residues introduced by mutagenesis are indicated in one-letter amino acid code adjacent to the symbols.

Table 1. *Kinetic parameters for EcA2 variants*[a]

	L-Aspartic acid-β-hydroxamate				L-Glutamine				
Substrate	k_{cat} (s^{-1})	K_m (mol L^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	$\Delta\Delta G$ (kJ mol^{-1})	k_{cat} (s^{-1})	K_m (mol L^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	$\Delta\Delta G$ (kJ mol^{-1})	L-Asparagine
Wild-type	$2.9(\pm0.1)\cdot10^{1}$	$3.5(\pm0.4)\cdot10^{-5}$	$8.3\cdot10^{5}$	—	$3.3(\pm0.2)\cdot10^{1}$	$3.5(\pm0.5)\cdot10^{3}$	$9.3\cdot10^{1}$		$4.9(\pm0.3)\cdot10^{1}$
G11V	$1.1(\pm0.1)\cdot10^{-1}$	$7.0(\pm1.5)\cdot10^{-5}$	$1.6\cdot10^{3}$	15.5	—	—	—	—	$1.2(\pm0.1)\cdot10^{-1}$
G11L	$<1\cdot10^{-2}$	$1.3(\pm0.4)\cdot10^{-4}$	$<8\cdot10^{1}$	23.0	—	—	—	—	$<1\cdot10^{-2}$
V27L	$1.03(\pm0.03)\cdot10^{1}$	$1.1(\pm0.3)\cdot10^{-5}$	$9.4\cdot10^{5}$	−0.3	$9.1(\pm1.1)\cdot10^{-2}$	$4.4(\pm2.4)\cdot10^{-3}$	$2.1\cdot10^{1}$	3.7	$8.0(\pm0.5)\cdot10^{0}$
V27M	$1.08(\pm0.06)\cdot10^{1}$	$1.5(\pm0.3)\cdot10^{-5}$	$7.2\cdot10^{5}$	0.4	$3.2(\pm0.3)\cdot10^{-2}$	$4.0(\pm0.5)\cdot10^{-3}$	$8\cdot10^{0}$	6.1	$3.4(\pm0.4)\cdot10^{0}$
G57A	$1.54(\pm0.08)\cdot10^{1}$	$6.9(\pm1.9)\cdot10^{-5}$	$2.2\cdot10^{5}$	3.3	$1.0(\pm0.1)\cdot10^{-2}$	$5.7(\pm2.6)\cdot10^{-3}$	$1.8\cdot10^{1}$	4.1	$1.8(\pm0.2)\cdot10^{1}$
G57V	$8.6(\pm0.5)\cdot10^{-1}$	$5.0(\pm1.1)\cdot10^{-5}$	$1.7\cdot10^{4}$	9.7	$6(\pm1)\cdot10^{-3}$	$2.4(\pm0.4)\cdot10^{-3}$	$2.5\cdot10^{0}$	9.0	$5(\pm0.5)\cdot10^{-1}$
G57L	$2.0(\pm0.1)\cdot10^{-1}$	$8.2(\pm0.9)\cdot10^{-5}$	$2.4\cdot10^{3}$	14.5	—	—	—	—	$5(\pm1)\cdot10^{2}$
Q59G	$1.43(\pm0.11)\cdot10^{1}$	$1.8(\pm0.4)\cdot10^{-3}$	$7.9\cdot10^{3}$	11.6	$1.0(\pm0.2)\cdot10^{-2}$	$5.0(\pm1.0)\cdot10^{-2}$	$2\cdot10^{-1}$	15.3	$7.7(\pm1.0)\cdot10^{0}$
Q59A	$9.7(\pm0.6)\cdot10^{0}$	$1.9(\pm0.4)\cdot10^{-3}$	$5.1\cdot10^{3}$	12.6	$1.0(\pm0.3)\cdot10^{-3}$	$1.0(\pm0.3)\cdot10^{-2}$	$1\cdot10^{-1}$	17.0	$8.0(\pm1.5)\cdot10^{0}$
Q59E	$2.0(\pm0.06)\cdot10^{0}$	$3.7(\pm0.4)\cdot10^{-5}$	$5.4\cdot10^{4}$	6.8	$2.4(\pm0.2)\cdot10^{-3}$	$2.3(\pm0.5)\cdot10^{-2}$	$1.0\cdot10^{0}$	11.3	$5.7(\pm0.5)\cdot10^{0}$
G88A	$4(\pm1)\cdot10^{-3}$	$4.0(\pm1.0)\cdot10^{-5}$	$1.0\cdot10^{2}$	22.4	—	—	—	—	—
G88I	$<1\cdot10^{-2}$	$5.0(\pm1.0)\cdot10^{-5}$	—	>20	—	—	—	—	—
N248A	$2.7(\pm0.1)\cdot10^{1}$	$1.9(\pm0.1)\cdot10^{-4}$	$1.4\cdot10^{5}$	4.4	$2.9(\pm0.3)\cdot10^{-3}$	$1.6(\pm0.1)\cdot10^{-2}$	$2\cdot10^{-1}$	15.3	$1.2(\pm0.1)\cdot10^{1}$
N248G	$2.3(\pm0.06)\cdot10^{1}$	$2.1(\pm0.2)\cdot10^{-4}$	$1.1\cdot10^{5}$	5.0	$4.6(\pm0.6)\cdot10^{-3}$	$6(\pm2)\cdot10^{-3}$	$8\cdot10^{-1}$	11.8	$1.3(\pm0.2)\cdot10^{1}$
N248D	$4.6(\pm0.3)\cdot10^{0}$	$5.6(\pm0.2)\cdot10^{-5}$	$8.2\cdot10^{4}$	5.8	$6.8(\pm1.1)\cdot10^{-3}$	$3.5(\pm1.5)\cdot10^{-3}$	$1.9\cdot10^{0}$	9.7	$3.0(\pm0.5)\cdot10^{0}$
N248Q	$2.1(\pm0.1)\cdot10^{1}$	$1.5(\pm0.2)\cdot10^{-4}$	$1.4\cdot10^{5}$	4.4	$3.2(\pm0.5)\cdot10^{-2}$	$2.1(\pm0.3)\cdot10^{-2}$	$1.5\cdot10^{0}$	10.3	$1.5(\pm0.1)\cdot10^{1}$
N248E	$2.6(\pm0.1)\cdot10^{1}$	$1.4(\pm0.1)\cdot10^{-4}$	$1.9\cdot10^{5}$	3.7	$1.9(\pm0.1)\cdot10^{-2}$	$7(\pm1)\cdot10^{-2}$	$2.7\cdot10^{0}$	8.8	$1.5(\pm0.2)\cdot10^{1}$

[a]The data were measured with the substrates indicated at pH 7.0 and 25 °C. $\Delta\Delta G^{*}$ measures the loss of transition state stabilization caused by the mutation (see text).

Table 2. *Kinetic constants for the hydrolysis of* L*-asparagine and* L*-glutamine by wild-type EcA2 and variant EcAN248A at pH 7.0 and 25°C*

	L-Asparagine				L-Glutamine			
	k_{cat} (s^{-1})	K_m (mol L^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	$\Delta\Delta G$ (kJ mol^{-1})	k_{cat} (s^{-1})	K_m (molL^{-1})	k_{cat}/K_m ($M^{-1}\,s^{-1}$)	$\Delta\Delta G$ (kJ mol^{-1})
Wild-type	$2.4(\pm0.1)\cdot10^{1}$	$1.5(\pm0.2)\cdot10^{-5}$	$1.6\cdot10^{6}$	—	$3.3(\pm0.2)\cdot10^{-1}$	$3.5(\pm0.5)\cdot10^{-3}$	$9.4\cdot10^{1}$	—
EcA N248A	$7.0(\pm0.1)\cdot10^{0}$	$9.5(\pm0.6)\cdot10^{-5}$	$7.4\cdot10^{4}$	7.6	$2.9(\pm1)\cdot10^{-3}$	$1.6(\pm0.2)\cdot10^{-2}$	$3.1\cdot10^{-1}$	15.3

and measures the loss of transition state stabilization resulting from an amino acid exchange.

Glycines 11 and 88

Both residues are immediately adjacent to the catalytic threonines T12 and T89 (see Fig. 1). Not unexpectedly, their replacement with large hydrophobic residues almost completely abolished catalysis. AHA binding, on the other hand, was not markedly impaired. As shown by Figure 3, the changes made at position 11 interfered with aspartate binding more strongly than the mutations at position 88.

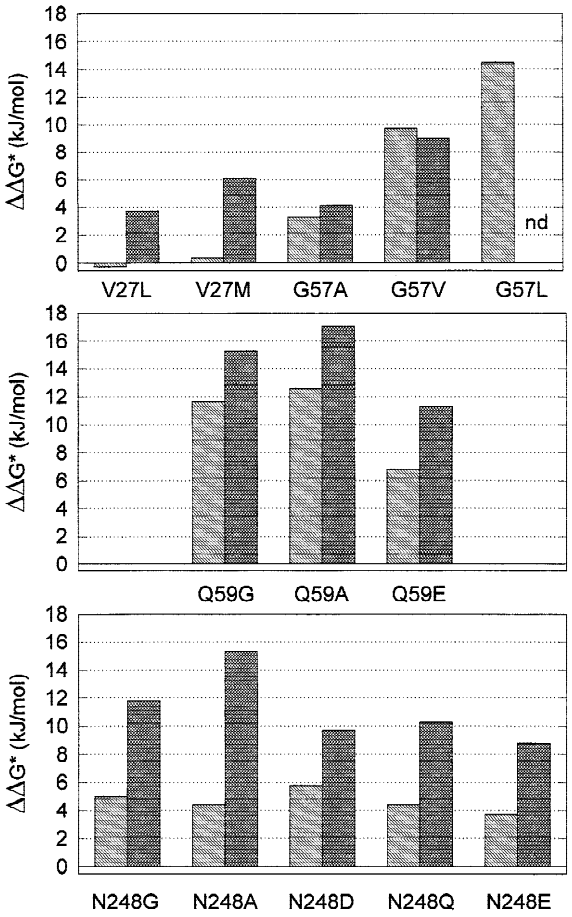

Fig. 4. Loss of transition state stabilization due to amino acid replacements in EcA2. Values of $\Delta\Delta G^*$ were calculated from the data in Table 1 for the substrates L-aspartic β-hydroxamate (light color) and L-glutamine (dark color). n.d., Not determined.

Valine 27

V27 is one of the amino acids lining a channel that leads into the active site. Valine at position 27 is typical of EcA2, while all other type II asparaginases have alanine at that position (Fig. 2). Substitution of V27 with leucine and methionine significantly improved AHA binding, as indicated by lower K_m values, while the k_{cat} values for either variant were only moderately reduced. In fact, the Michaelis constant for AHA of EcA V27L (11 μM at pH 7) is the lowest of all EcA2 variants known, and its "specificity constant" k_{cat}/K_m is higher than that of wild-type EcA2 (leading to a negative $\Delta\Delta G^*$). However, the changes made did not sufficiently discriminate between asparagine and glutamine to make the respective enzyme variants attractive in the context of this work (see Fig. 4).

Glycine 57

All known type II asparaginases with high glutaminase activities have alanine at position 57, while in EcA2 and WsA it is glycine (Fig. 2). Nevertheless, the replacement of G57 with alanine had little effect on substrate specificity. The activities with all three substrates (AHA, Asn, and Gln) were reduced to 30–40%, while the K_m values as well as K_d for aspartate were essentially unaffected. The exchange of G57 with bulky residues (Val and Leu) strongly reduced k_{cat} for all substrates, again without significant effects on substrate binding. As above, these modifications did not alter the substrate specificity in the desired manner, i.e., the activity toward asparagine was reduced by almost the same factor as that toward glutamine.

Glutamine 59

Only EcA2 and WsA have glutamine at position 59, while glutamate is found in the other class II enzymes. The corresponding replacement in EcA2 (i.e., Q59 with E) reduced k_{cat} for all three substrates to about 10% of the wild-type value without significantly discriminating between the asparaginase and glutaminase activities. On the other hand, the removal of the side chain at position 59 (EcA Q59G) or its reduction in size (EcA Q59A) affected glutaminase activity to a much larger extent than the turnover of AHA or Asn. Both mutations strongly increased the K_m values for AHA and the binding constants for aspartate (see Table 1; Fig. 5), confirming that Q59 is indeed important for efficient substrate binding.

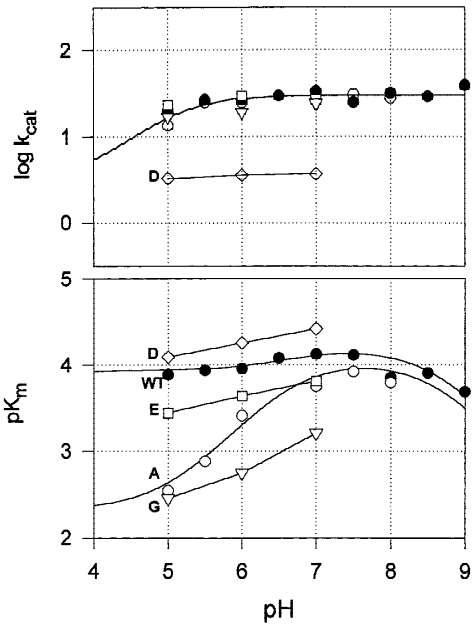

Fig. 5. pH Profiles of kinetic parameters for wild-type EcA2 and N248 variants. The data were measured at pH 7.0 and 25 °C with wild-type enzyme (●) and variants N248G (▽), N248A (○), N48D (◇), and N248E (□).

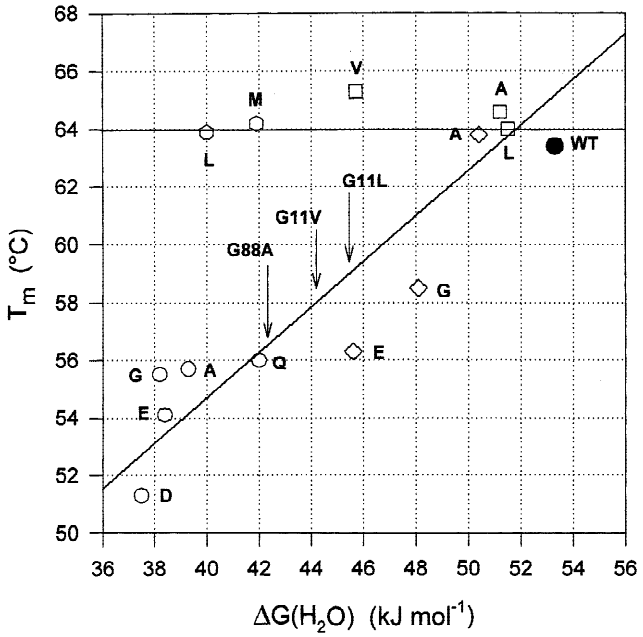

Fig. 6. Stabilities of EcA2 variants. Stability parameters $\Delta G(H_2O)$ and T_m were as described in Materials and methods for wild-type enzyme (●) and variants with amino acid replacements at position 27 (○), 57 (□), 59 (◇), and 248 (○). The residues introduced by the mutation are given in one-letter amino acid code adjacent to the symbols. Variants with replacements at positions 11 and 88 were unsuitable for thermal denaturation studies due to low residual activities. For these variants, $\Delta G(H_2O)$ is indicated by vertical arrows.

Asparagine 248

EcA2 and WsA differ from the other type II asparaginases with respect to amino acid 248. While the former have asparagine at that position, the latter contains a serine (Fig. 2). With the exception of EcA N248D, all amino acid substitutions made at position 248 affected the substrate specificity of the enzyme in the same general way: The activity toward AHA remained essentially the same; the maximum velocity of asparagine hydrolysis was moderately reduced, and the turnover of glutamine was strongly impaired (see Table 1). This was most notable with variant EcA N248A, where the loss of transition state stabilization of Gln hydrolysis (15.3 kJ mol^{-1}) was twice as high as that for asparagine (7.6 kJ mol^{-1}; see Table 2), and more than three times higher than the value for AHA (4.4 kJ mol^{-1}).

Figure 5 summarizes the pH dependence of the kinetic parameters of AHA hydrolysis by wild-type EcA2 and four N248 mutants. With all of these enzymes, k_{cat} was almost independent of pH between 5 and 8. EcA N248G and EcA N248A showed a marked decrease of pK_m (i.e., an increase of K_m) at low pH, which was not seen with wild-type enzyme and mutants N248D and N248E. The pK_m vs. pH profile of EcA N248A suggests that the protonation of a residue with an apparent pK_a between 6 and 7 is responsible for the increase in K_m at low pH.

Stabilities

Two independent methods were used to estimate the effects of the mutations on EcA2 stability, i.e., equilibrium denaturation in guanidine·HCl (monitored by the fluorescence of the only tryptophan of EcA2, W66), and thermal denaturation (monitored by activity measurements). The results of both approaches are compared in Figure 6. Note that $\Delta G(H_2O)$ values are true thermodynamic constants, while T_m values are purely empirical "melting temperatures," i.e., the temperatures where half of the activity was lost under the conditions of the experiments. Due to the different probes used, equilibrium denaturation only detected gross changes of conformation (W66Y is at a distance of 14 Å from the active site aspartate), while the loss of activity upon thermal denaturation may also reflect small local changes in the active site. For most of the EcA2 forms studied here, an approximately linear correlation between $\Delta G(H_2O)$ and T_m was observed. Exceptions were the mutations at positions 27 and 57, which led to a decrease of $\Delta G(H_2O)$, but not of T_m. As confirmed by Figure 6, none of the mutations seriously compromised the overall stability of the enzyme. On both scales, the loss of stabilization energy did not exceed 20%. Thus, under physiological conditions at 37 °C, all variants described here should be sufficiently stable for therapeutic applications.

Discussion

In the present study we show that the substrate specificity of type II glutaminases/asparaginases, i.e., their relative preference for one of the alternate substrates glutamine and asparagine, is modulated by amino acid side chains that interact with the α-ammonium group of the substrates. Among these residues, N248 appears to be especially important. Any one of a number of mutations made at position 248 affected the turnover of Gln to a much geater extent than that of Asn or the Asn analog AHA. Our kinetic data indicate that the reduction of glutaminase activity is mainly due to a reduced k_{cat}, while substrate binding is only moderately impaired.

Kinetic significance of k_{cat} and K_m

When interpreting kinetic parameters, one has to keep in mind that k_{cat} and K_m are inherently complex kinetic constants. The available evidence indicates that EcA2 employs a double-displacement, or "ping-pong" mechanism with a covalent β-aspartyl enzyme intermediate (Ehrman et al., 1971; Röhm & Van Etten, 1986). A simplified scheme of such a mechanism is

$$E + A \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} EA \overset{k_2}{\underset{P_1}{\downarrow}} E' \overset{k_3}{\underset{H_2O}{\uparrow}} EP_2 \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + P_2$$

where A is the substrate, E' is the acyl enzyme, P_1 is NH_3 (or NH_2OH in the case of AHA), and P_2 is aspartate. With $k_3' = k_3$ $[H_2O]$ and $K_S = k_{-1}/k_1$, the kinetic parameters k_{cat} and K_m are given by

$$k_{cat} = \frac{k_2 \cdot k_3'}{k_2 + k_3'}; \qquad K_m = K_S \cdot \frac{k_3'}{k_2 + k_3'}.$$

Consequently, K_m may not be taken as a direct measure of substrate binding unless $k_2 \ll k_3'$, i.e., unless acylation is rate limiting. With EcA2, there is firm evidence that this is indeed the case. First, a number of asparagine derivatives with substituted amide nitrogen are hydrolyzed at widely different rates that inversely correlate with the chemical stability of the respective amide bond (Herrmann et al., 1974). If deacylation was rate limiting, k_{cat} would be independent of the type of the amide bond of the substrate. Second, as shown by Figure 4, the K_m values for AHA and the binding constants for aspartate K_d, which are true equilibrium constants, exhibit a linear correlation over several orders of magnitude. Third, the inhibition constants K_i for aspartate are similar to the respective binding constants K_d (data not shown). Thus, in the case EcA2, K_m is a reliable indicator of binding affinity, while the magnitude of k_{cat} is governed by k_2.

Role of active site residues

Not unexpectedly, replacements of the glycines located in the immediate neighbourhood of the catalytic threonines 12 and 89 almost completely abolished EcA2 activity. Modeling studies showed that valine at position 11 and alanine at position 88 can be accommodated without seriously disturbing the active site geometry (data not shown). Indeed, these mutations had little effect on enzyme stability (Fig. 6) and on AHA binding (Table 1). In summary, glycine residues at positions 11 and 88 appear to be an absolute prerequisite for the optimum orientation of the neighboring catalytic side chains.

Residue 27, located in the rigid part of the active site behind the substrate, is apparently not crucial for either catalysis or binding. With the exception of EcA2, most type II enzymes have alanine there (see Fig. 2), and the present study shows that Leu and Ile are accepted as well. Interestingly, these mutations do not affect thermal denaturation but significantly decrease the stability in guanidine hydrochloride (Fig. 6). Their effects on catalysis depend on the substrate used: AHA turnover is almost unchanged, while the hydrolysis of both Asn and Glu is reduced to about 20% of the wild-type value, due to a decreased k_{cat}.

As judged by the localization of G57 (see Fig. 1), one should expect that mutations at that position affect substrate binding rather than catalysis. Our data show that the opposite is true: the introduction of increasingly larger side chains into position 57 (Ala, Val, Leu) progressively reduced k_{cat} without markedly increasing K_m for AHA or glutamine (Table 1). Hydrophobic side chains at position 57 may interact with the charged groups of the substrate and thus interfere with one or more step of the catalytic cycle. Rotations or translations of the substrate during catalysis may result in even closer distances. Protein stability was not appreciably affected by mutations at position 57 (see Fig. 6).

The predictions made for Q59, on the other hand, were borne out by experiment. Deletion of the side chain as in EcA2 led to a 50-fold increase of K_m for AHA, accompanied by a moderate reduction of k_{cat}. This is consistent with Q59 assisting in substrate binding by hydrogen bonding to the substrate ammonium group. Replacement of Q59 with glutamate, which is capable of a similar interaction, did not affect K_m (at least that for AHA). On the other hand, the negative charge introduced in this variant appears to interfere with catalysis as indicated by a 10-fold decrease of k_{cat}. These effects were more pronounced with glutamine as the substrate, leading to higher $\Delta\Delta G^*$ values for all of the variants examined. The greatest loss of stability was seen with EcA Q59E, probably due to electrostatic repulsion of its side chain with the side-chain carboxylates of D90 and E283. The stabilizing effect of the interactions of the Q59 side-chain amide group with neighboring residues seems to be insignificant, as indicated by wild-type values for EcA Q59A on both scales (Fig. 6).

While Q59 is primarily involved in substrate binding, N248 plays a pivotal role in stabilizing both the ground state and transition state of catalysis. This contribution of N248 is especially important for the turnover of the poor substrate glutamine, as indicated by much higher $\Delta\Delta G^*$ with all variants examined. The importance of N248 for catalysis is most noticeable with enzymes where the amide group is missing (i.e., EcA N248G and EcA N248A). Unexpectedly, glutamine at position 248 could not substitute for asparagine, indicating that strict sterical requirements have to be met. The acidic residues Asp and Glu were also unable to maintain efficient turnover. In the case of EcA N248D, it is probably the negative charge that interferes with catalysis, decreasing k_{cat} by an order of magnitude. On the other hand, Asp and Glu at position 248 both prevented the increase of K_m at low pH seen with EcA N248G and EcA N248A (Fig. 5). We assume that this effect is due to the protonation of D90, the anionic form of which binds to the substrate ammonium group (see Fig. 1). An additional negative charge introduced at position 248 apparently compensates for the loss of that interaction.

In addition to its catalytic role, N248 is also a significant stabilizing factor for the EcA2 tetramer. All mutations at position 248 destabilized the enzyme to about the same extent, decreasing $\Delta G(H_2O)$ by 12–14 kJ mol^{-1}, or T_m by 6–10 °C, respectively. This is not surprising, in view of the fact that N248 is involved in hydrogen-bonding interactions between different subunits (see Fig. 1).

To examine possible structural consequences of amino acid replacements at position 248, we modeled several such variants as complexes with glutamate and subjected their active-site regions to energy minimization (see Materials and methods). In the calculations, none of the mutations led to significant shifts of the ligand (glutamate) with respect to the catalytic residues or of the catalytic residues with respect to each other (data not shown). This suggests that the catalytic contributions of N248 are rather subtle and probably affect transition states rather than ground states, i.e., the enzyme–substrate and the enzyme–product complexes. Possibly, it

is the dynamics of the active site during catalysis that is primarily affected by the mutations. In that case, the static picture provided by our energy minimizations would not contribute at all to an understanding of the kinetic data.

Possible application of N248 variants in ALL therapy

Although physiological studies with the variants described here have not been performed yet, our kinetic data allow some predictions as to their performance in the treatment of ALL. In normal blood serum, the concentrations of asparagine and glutamine are about 50 and 600 μM, respectively (Ollenschläger et al., 1988).

Using these values and the data shown in Table 2, we find that the asparaginase activity of EcA N248A in vivo would amount to 12% of the wild-type activity, whereas the activity toward glutamine would be reduced to 0.2% of the wild-type value. Considering the amounts of enzyme typically administered in ALL therapy (10,000 U in a single dose), this activity ratio should still allow the depletion of asparagine without markedly reducing the much higher serum concentration of glutamine. The somewhat lower stability of the variant may or may not affect its half-life in vivo. The mechanism by which the enzyme is cleared from the circulation is unknown; however, it appears that the antigenicity of the protein is more important in this respect than its thermodynamic stability (Müller & Boos, 1998). Clearly, this point and the general performance of the variants in vivo have to be elucidated by further experiments.

Materials and methods

Site-directed mutagenesis

Site-directed mutagenesis of the *ansB* gene encoding EcA2 was performed by the phosporothioate method (Sayers et al., 1988) as described previously (Wehner et al., 1992). Mutations were introduced by means of the following degenerate oligonucleotide primers (mutagenic base changes shown in boldface)

Enzyme variants	Primer
G011V,L	5′-GCA ACC GGC (G,**C**)**T**G ACC ATT GCC-3′
V027L,M	5′-AAC TAC ACA (**C**,**A**)TG GGT AAA GTT-3
G057A,L,V	5′-GTG AAT ATC (G,**C**)(T,**C**)C TCC CAG GAC-3′
Q059A,E, G	5′-ATC GGC TCC **G**(**C**,A,**G**)G GAC ATG AAC-3′
G088V,I,A	5′-ATT ACC CAC (G,**A**)(**T**,**C**)T ACC GAC ACG-3′
N248A,D,G	5′-GGT AAC GGC **G**(**C**,A,**G**)C CTG TAT AAA-3′
N248Q,E	5′-GGT AAC GGC (**C**,**G**)A**G** CTG TAT AAA-3′

Mutant clones were identified by DNA sequencing. Then the mutant *ansB* genes were subcloned into the expression vector pT7-7 (Tabor & Richardson, 1985). Expression of EcA2 variants in *E. coli* CU1783 and purification of the recombinant enzymes were carried out as described before (Harms et al., 1991a). In each case, the mutant genes were completely sequenced to exclude the presence of additional, unwanted base changes.

Kinetic experiments

EcA2 activities were determined at 25 °C with L-aspartic acid-β-hydroxamate (AHA, Sigma, St. Louis), L-asparagine, and L-glutamine as substrates essentially as described by Derst et al. (1992). Enyzme and substrates were incubated in 50 mM Mes (pH 5–7), Mops (pH 6–8), or Tris (pH 7–9). In the discontinuous AHA assay, hydroxylamine released upon hydrolysis of the hydroxamate is reacted with 8-hydroxyquinoline under oxidative conditions to yield an intensely green oxindol dye with an absorption coefficient of about $1.7 \cdot 10^4$ L^{-1} mol^{-1} cm^{-1} at 705 nm. Owing to the high sensitivity and excellent repoducibility of the assay, Michaelis constants down to 20 μM and turnover constants as low as 0.01 s^{-1} can be determined without difficulty. The activities of most EcA2 variants toward AHA are comparable to or higher than those toward asparagine. In the alkaline range, NH_2OH becomes increasingly unstable, which has to be taken into account when working at pH values above 8.

The hydrolysis of L-asparagine and L-glutamine is traditionally monitored by measuring the formation of ammonia with Nessler's reagent. This assay is much less sensitive than the AHA method and generally less reliable. Therefore, the formation of NH_3 from Asn or Gln was usually followed with a coupled enzymatic assay using glutamate dehydrogenase as the indicator enzyme (Wehner et al., 1992). The method monitors the absorption change of NADH at 340 nm ($\epsilon = 6.22 \cdot 10^3$ L mol^{-1} cm^{-1}) and can be applied in a continuous manner. It is only useful at higher pH values, and thus unsuitable for studies of pH dependencies.

Estimates of the kinetic parameters k_{cat} and K_m and their standard deviations were obtained by nonlinear regression using the program SigmaPlot. The effect of a given modification on transition state stabilization $\Delta\Delta G^*$ is calculated from

$$\Delta\Delta G^* = -R \cdot T \cdot \ln \frac{k_{cat}/K_m(\text{mutant})}{k_{cat}/K_m(\text{wild-type})}.$$

Protein concentrations were determined from ultraviolet spectra (molar absorption coefficient of EcA2 at 280 nm: $\epsilon_{280} = 1.07 \cdot 10^5$ L mol^{-1} cm^{-1}).

Aspartate binding

The binding of L-aspartate to EcA2 was studied at 25 °C and pH 6.0 by titration experiments using the fluorescence of the single tryptophan residue of the enzyme as spectroscopic probe (see Harms et al., 1991b). The data were corrected for dilution and dissociation constants for aspartate K_d were estimated by fitting the following equation to the corrected curves

$$\Delta F = (\Delta F_t / 2[E]) \cdot \{([E] + [L]_t + K_d) - \sqrt{([E] + [L]_t + K_d)^2 - 4[E] \cdot [L]_t}\}$$

where ΔF is the fluorescence change observed at a total aspartate concentration of $[L]_t$, ΔF_t is the fluorescence change at saturating aspartate concentrations, and $[E]$ is the concentration of enzyme active sites.

Conformational stability

The thermodynamic stability of our EcA2 variants was estimated by denaturation experiments in guanidine hydrochloride solutions as detailed by Derst et al. (1994), again using the enzyme fluorescence as the spectroscopic probe. For most EcA2 variants, the titration data were consistent with the simple two-state model

$$N \overset{K_{NU}}{\rightleftharpoons} U$$

where N denotes the native, and U the unfolded state, and $K_{NU} = [U]/[N]$. According to Pace et al. (1989), the titration curves (fluorescence F_{obs} vs. denaturant concentration $[D]$) were normalized by introducing the fractional fluorescence change $F_{app} = (F_{obs} - F_N)/(F_U - F_N)$ where F_N is the fluorescence of the native state, F_U the fluorescence of the unfolded protein. F_{app} is related to K_{NU} by $F_{app} = K_{NU}/(1 + K_{NU})$. Assuming that the free energy of unfolding $\Delta G_u = -R \cdot T \cdot \ln(K_{NU})$ is linearly dependent on $[D]$ (i.e., $\Delta G_u = \Delta G(H_2O) - m \cdot [D]$), the free energy of unfolding in the absence of denaturant, $\Delta G(H_2O)$, can be estimated by extrapolation of the concentration dependence of ΔG_u to zero denaturant concentration, while m is given by the slope of the line in the transition region.

Thermal denaturation of EcA2 variants was monitored by activity measurements. Enzyme samples (50 μg/mL in 50 mM Tris/HCl, pH 8.0) were continuously heated in a thermocycler (heating rate: 1 K min^{-1}). At 1 min intervals, aliquots were withdrawn and immediately assayed for activity using AHA as the substrate. Empirical "melting temperatures" T_m were read at the inflection points of the resulting activity vs. T profiles.

Molecular modeling

To detect possible conformational effects of mutations at position 248, we modeled the active site of several variants. The calculations were performed using the AMBER force field (Cornell et al., 1995) as implemented in the HyperChem program package (HyperCube, Inc.). The EcA2-aspartate complex was used as the starting structure. Briefly, the aspartate ligand present in the structure was converted to glutamate, and an approximately spherical substructure encompassing all residues within 10 Å of the bound glutamate, including active-site water, was energy minimized using a full-atom approach, while the remainder of the structure was kept "frozen." Subsequently, asparagine 248 was replaced with other residues, and the resulting variant structures energy minimized as above.

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