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A catalytic role for threonine-12 of *E. coli* asparaginase II as established by site-directed mutagenesis

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A threonine-12 to alanine mutant of *E. coli* asparaginase II (EC 3.5.1.1) has less than 0.01% of the activity of wild-type enzyme. Both tertiary and quaternary structure of the enzyme are essentially unaffected by the mutation; thus the activity loss seems to be the result of a direct impairment of catalytic function. As aspartate is still bound by the mutant enzyme, Thr-12 appears not be involved in substrate binding.

Asparaginase II; Mechanism; Catalysis; Thr-12; Mutagenesis; (*E. coli*)

1. INTRODUCTION

The asparaginases (EC 3.5.1.1) catalyze the hydrolysis of L-asparagine to L-aspartate and ammonia. Asparaginase isoenzyme II of *Escherichia coli*, an enzyme noted for its use as an anti-leukemia agent [1], has been extensively studied (for reviews see [2,3]). Kinetic evidence suggests that catalysis by asparaginase II involves a covalent intermediate, probably a β-aspartyl enzyme [4,5]. However, the reactive group in the enzyme is still not known with certainty. Handschumacher and his colleagues [6–8] showed that L-5-diazo-4-oxo-norvaline (DONV) covalently binds and inactivates *E. coli* asparaginase. This effect was attributed to modification of a serine residue in position 120 of the recently corrected sequence [9]. However, the assignment was not unequivocal. At about the same time, Holcenberg et al. [10] demonstrated that *Acinetobacter* glutaminase-asparaginase, which is highly homologous to *E. coli* asparaginase II, is modified by DON (the norleucine analogue of DONV) at threonine-12. An 8 amino acid sequence encompassing this residue is conserved in most asparaginases examined so far; this motif is, therefore, considered an asparaginase sequence 'signature'. However, the role of threonine-12 in the asparaginases remained unclear. Here we describe the site-directed replacement of threonine-12 of *E. coli* asparaginase II with alanine, and its effects on the structure and function of the enzyme.

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2. MATERIALS AND METHODS

2.1. *Mutagenesis*

The construction of expression systems for *E. coli* asparaginase II is described in detail elsewhere [11]. Before mutagenesis the 1.35 kB *Eco*RI/*Hin*dIII fragment from expression vector pTWE1 (a derivative of pT7-7 carrying the wild type *ans*B gene) was cloned into the multiple cloning site of M13mp19, using standard procedures [12]. Oligonucleotide-mediated mutagenesis was performed according to the method of Eckstein and coworkers [13] with a commercial kit (Amersham, Amersham, UK). The codon for amino acid 12 was changed from ACC (Thr) to GCC (Ala) by introducing the synthetic 18-mer primer 5'-ACCGGCGGCGCCATTGCC-3'. The additional base exchange at position 9 of the primer replaces the triplet for Gly-11 (GGG) with another glycine codon (GGC). This change introduced a new *Nar*I restriction site (underlined) in order to facilitate identification of the mutant asparaginase gene. Recombinant phage plaques obtained in the mutagenesis experiment were propagated and screened for a new *Nar*I restriction site.The 1.35 kb *Eco*RI/*Hin*dIII fragment from such a a mutant phage was excised, isolated by agarose gel electrophoresis, and re-inserted into *Eco*RI/*Hin*dIII-cut vector pT7-7 for expression of the mutant enzyme. The resulting plasmid is called pTWE5.

2.2. *Expression and purification*

Wild type and mutant asparaginases were expressed from pTWE1 or pTWE5, respectively, with the asparaginase-free *E. coli* strain CU1783 [11] as the host. Both enzymes were separated from cell pellets by osmotic shock and purified from the resulting supernatant fluid by ammonium sulfate fractionation and chromatofocusing as decribed [11].

2.3. *Protein chemistry and activity assays*

Protein concentrations were determined from UV spectra by assuming an absorption coefficient for asparaginase of $A_{278}^{0.1\%} = 0.74$. Amino acid analyses were performed by reversed-phase HPLC of PTC-amino acids obtained by pre-column treatment of hydrolysates with phenyl isothiocyanate. Hydrolysis, derivative formation and analysis were carried out essentially as detailed in [14]. N-terminal sequence analysis was performed by automated Edman degradation on an Applied Biosystems gas phase sequencer. The activity of wild type asparaginase was determined by direkt UV spectrophotometry at 225 nm [2]. Another, more sensitive assay is based on the determination of hydroxylamine liberated from L-aspartic-β-hydroxamate [15]. The

latter method, which reliably detects asparaginase activities down to a few μU, was used to assay solutions of mutant enzyme. Both types of assay were conducted in MES or MOPS buffers at pH 7.0 and 25°C.

2.4. *Spectroscopy*

Fluorescence measurements were performed at 25°C on a Jasco FP-770 spectrofluorimeter. For all experiments enzyme solutions in 50 mM MOPS/NaOH, 100 mM NaCl, pH 7.0, were employed. Fluorescence quenching by L-aspartic acid was studied by titrating 1.5 ml volumes of enzyme solution (0.5–3 mg protein/ml) with 10 μl volumes of 10 mM aspartic acid, pH 7.0 (excitation at 285 nm, emission detected at 323 nm). NMR spectra were recorded at 500 MHz on a Bruker AMX-500 spectrometer. Sample preparation and experimental details were similar to those described previously [16].

3. RESULTS AND DISCUSSION

3.1 *Structural properties of mutant T12→A*

The T12→A mutant of asparaginase was purified by the same protocol as described for wild type enzyme. In both cases the yields of enzyme protein were comparable (10–15 mg/l of culture volume). Owing to the very low activity of the mutant, asparaginase-containing fractions were identified by SDS-gel electrophoresis.

The mutant enzyme was indistinguishable from wild type asparaginase with respect to a number of properties. Its isoelectric point, as estimated by chromatofocusing was 4.8–4.9 (wild type = 4.8). Both enzyme species showed the same relative mobility during gel filtration on Sephacryl S-300 and comigrated in SDS gel electrophoresis. This suggests that the mutant, like wild-type enzyme, is a tetramer of 35 kDa subunits.

The amino acid composition of the mutant and wild type asparaginase were identical within experimental error, except for the values for Thr and Ala which displayed the expected shift towards a higher alanine content (data not shown). However, with 34 threonine and 32 alanine residues per subunit of wild type enzyme, the expected change is difficult to determine quantitatively, since Thr is partially destroyed during hydrolysis. Therefore, the presence of the T12→A change was confirmed by sequence analysis of the N-terminus of the mutant protein. The first 16 residues were as expected [9], including a glycine residue in position 11 and an alanine in position 12.

The present evidence further suggests that the gross conformation of the enzyme is not detectably affected by the mutation. So, for instance, the CD-spectra of both species were similar , as were their denaturation temperatures T_m, measured by differential scanning calorimetry (B.Z. Chowdhry and K.H. Röhm, unpublished data).

3.2. *Catalytic features*

The activity of the T12→A mutant is extremely low. With L-aspartic acid-β-hydroxamate as the substrate, our preparation exhibited a specific activity of less than 0.01 U/mg. In contrast, wild type asparaginase shows a

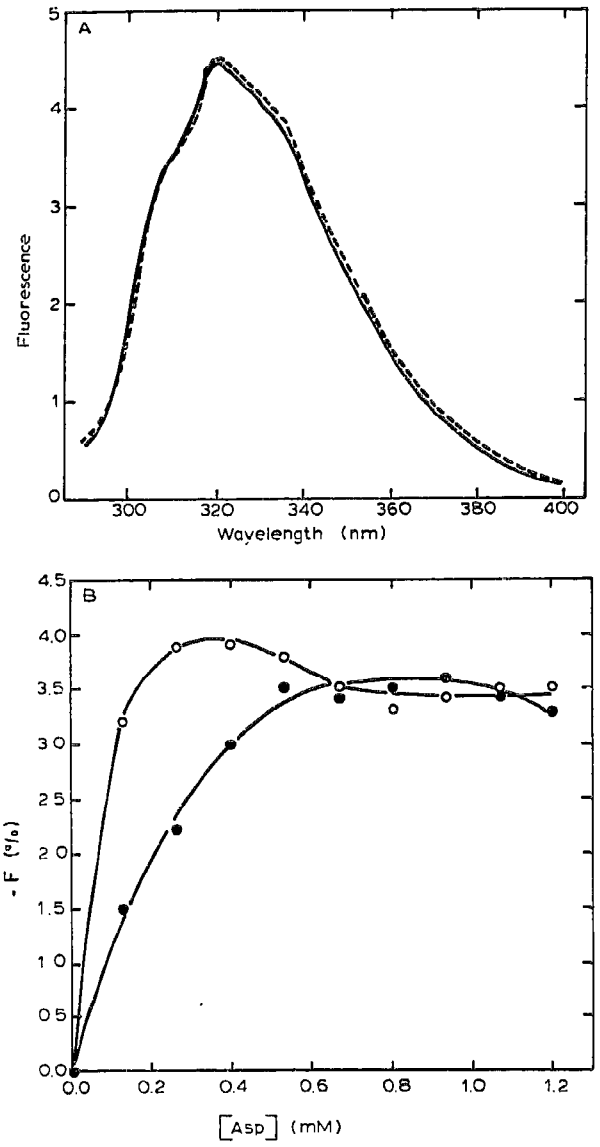

Fig. 1. Fluorescence quenching by aspartate. (A) Fluorescence emission spectra of wild type asparaginase II (——) and mutant T12→A (– – –) at the same protein concentration. Excitation was at 285 nm, fluorescence is given in arbitrary units. (B) Percent change of emission intensity at 323 nm upon titration with aspartate at pH 7.0. Again, the data are for wild type enzyme (○), and for mutant T12→A (●).

specific activity of 150 U/mg under the same conditions. Although the *E. coli* host strain used, CU 1783, has been made asparaginase II-negative by genetic manipulations [11], we cannot entirely exclude that the observed residual activity is due to contamination with host enzyme activity. At present, detailed kinetic studies are in progress to clarify this point.

In order to ascertain whether the loss of activity is due to impaired substrate binding, we examined the interaction of the mutant enzyme with L-aspartic acid. Aspartate, a product of the hydrolysis reaction, is also

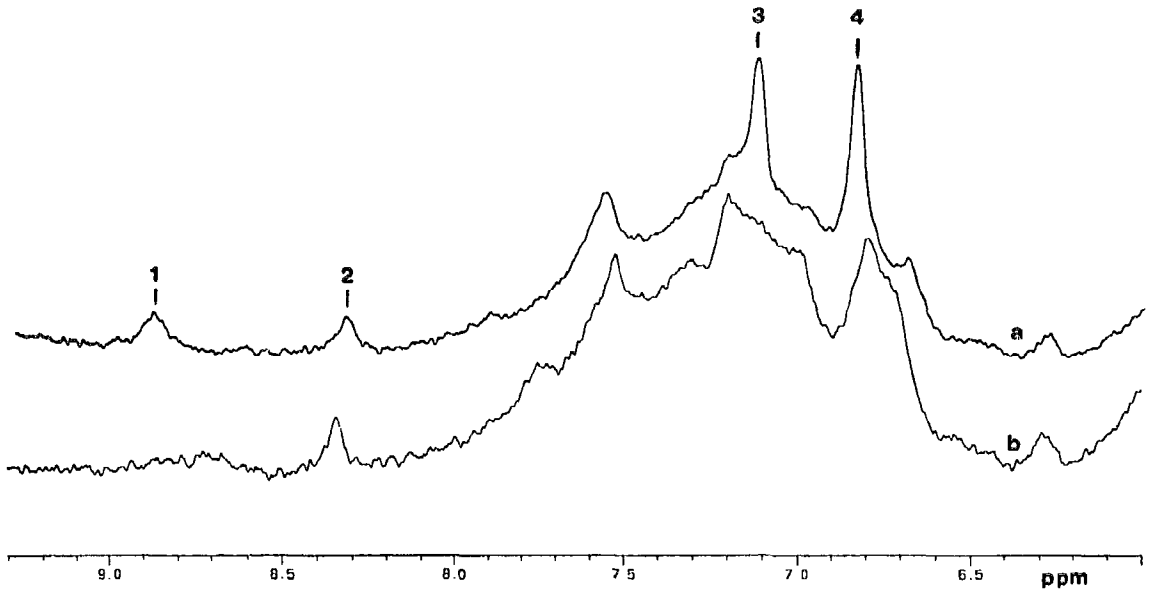

Fig. 2. Effect of aspartate on the ^{1}H-NMR spectrum of mutant T12→A. A 1.8 mM enzyme solution in unbuffered 350 mM NaCl, pH 5.0, was used. (a) (Upper trace) without aspartate; the marked signals correspond to 2 different histidine residues (1,2) and a tyrosine residue, respectively (3,4). (b) (Lower trace) the same solution with 1 mol of aspartate added per mol subunit. Signals 1, 3 and 4 have broadened out.

a competitive inhibitor as well as as a substrate of asparaginase-catalyzed oxygen-exchange [5]. Aspartic acid protonated at the β-carboxyl group binds at least as tightly to asparaginase II as L-asparagine.

Two spectroscopic approaches were used. The first one is based on the observation by Homer [17] that aspartate binding slightly quenches the fluorescence of the single tryptophan residue of asparaginase II. The fluorescence emission spectra of wild type asparaginase and the Thr12→Ala mutant are shown in Fig. 1A. Except for a minor redshift of the spectrum of the mutant enzyme the curves are identical. Again, this suggests comparable protein conformations of both enzyme species. In contrast, the aspartic acid titration curves of wild type and mutant enzyme are different (Fig. 1B). Although the overall reduction of fluorescence upon aspartate binding was about the same in either case (3.5% at pH 7.0), the aspartate concentrations necessary to attain the maximal effect were 3-4-fold higher with the mutant enzyme. The shapes of both curves indicate that at least 2 different concentration-dependent effects are involved in fluorescence quenching, and a quantitative analysis is difficult with our present data. Nevertheless, it appears justified to conclude that the mutant binds aspartic acid, although with somewhat reduced affinity.

A second approach to study aspartate binding relied on changes in the aromatic region of the ^{1}H-NMR spectrum previously observed with wild-type enzyme. During progessive saturation with aspartate, one of the histidine C_ϵ signals as well as 2 additional peaks (attributed to a tyrosine residue) broaden and eventually disappear [16]. Fig. 2 shows that exactly the same effect is exhibited by the T12→A mutant, indicating that aspartate is bound with close to normal affinity.

From these findings we infer that Thr-12 of asparaginase II is not required for substrate binding, but is involved in catalysis. It is conceivable that Thr-12, like the active-site serine residues of the serine hydrolases, participates in the covalent acyl enzyme intermediate. Such a role of a threonine residue would be rather uncommon. On the other hand, a hydrogen bond formed by the hydroxyl group of Thr-12 might be required for transition-state stabilization or some subsequent step in the catalytic cycle. Experiments to elucidate the role of Thr-12 are under way in our laboratory.

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