Acta Biochimica Polonica

Vol. 47 No. 3/2000

807–814

QUARTERLY

Preliminary crystallographic studies of Y25F mutant of periplasmic *Escherichia coli* L-asparaginase✪

Maciej Kozak[1, 2✉], Mariusz Jaskólski[2, 3] and Klaus H. Röhm[4]

[1]*Department of Macromolecular Physics, Faculty of Physics, A. Mickiewicz University, Poznań, Poland,* [2]*Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznań, Poland,* [3]*Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznań, Poland,* [4]*Institut für Physiologische Chemie, Philipps Universität, Marburg, Germany*

Received: 12 May, 2000; accepted: 19 June 2000

Key words: amidohydrolase, leukemia, protein crystallography, mutagenesis

Periplasmic *Escherichia coli* L-asparaginase II with Y25F mutation in the active-site cavity has been obtained by recombinant techniques. The protein was crystallized in a new hexagonal form ($P6_522$). Single crystals of this polymorph, suitable for X-ray diffraction, were obtained by vapor diffusion using 2-methyl-2,4-pentanediol as precipitant (pH 4.8). The crystals are characterized by a = 81.0, c = 341.1 Å and diffract to 2.45 Å resolution. The asymmetric unit contains two protein molecules arranged into an AB dimer. The physiologically relevant ABA'B' homotetramer is generated by the action of the crystallographic 2-fold axis along [1, -1, 0]. Kinetic studies show that the loss of the phenolic hydroxyl group at position 25 brought about by the replacement of Y with F strongly impairs k_{cat} without significantly affecting K_m.

L-Asparaginases (EC 3.5.1.1) hydrolyze L-asparagine to L-aspartate, with the release of ammonia (Fig. 1). L-Asparaginase activity was first discovered in the blood plasma of guinea pig by Clementi (1922). Later, enzymes with analogous activities were isolated from bacteria and plants. The studies on mice performed by Kidd (1953) showed antitumor activity of the guinea pig serum against certain lymphomas. Broome (1961) correlated this effect with Clementi's observations and ascribed it to asparaginase activity. Since then the

✪The research of Mariusz Jaskólski was supported in part by an International Research Scholar's award from the Howard Hughes Medical Institute. Some of the calculations were performed using the Poznań Supercomputing and Networking Center.

✉Corresponding author: Maciej Kozak, Department of Macromolecular Physics, A. Mickiewicz University, Umultowska 85, 61-614 Poznań, Poland; tel: (48 61) 827 3266; fax: (48 61) 825 7758; e-mail: mkozak@main.amu.edu.pl

Abbreviations: MPD, 2-methyl-2,4-pentanediol; PDB, Protein Data Bank.

antitumor activity of asparaginases has been the subject of numerous studies.

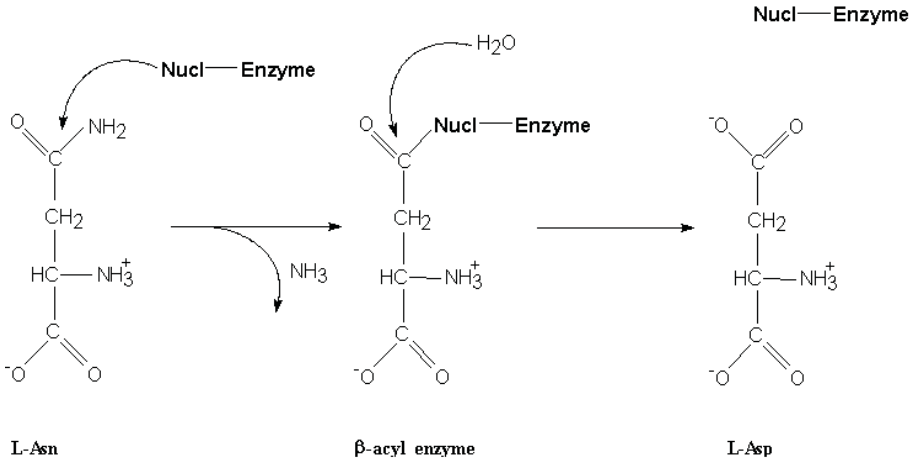

Figure 1. The asparaginase II reaction.

In *Escherichia coli* two forms of this enzyme, termed type I (cytosolic) and type II (periplasmic) have been found. They are characterized by, respectively, high and low K_M for asparagine. Only some L-asparaginases, those with high affinity towards the substrate (K_M about 10^{-5} M), show antitumor activity. Type I and type II isoenzymes have been found in both eukaryotic and prokaryotic organisms but their evolutionary relation is obscure (Bonthron & Jaskólski, 1997). Type II enzymes isolated from *E. coli* (EcAII) and *Erwinia chrysanthemi* (ErA) are successful drugs in the treatment of acute lymphoblastic leukemia, leukemic lymphosarcoma and lymphosarcoma (Hill *et al.*, 1967; Chakrabarti, 1997). Their therapeutic effect is related to depleting the circulating pools of asparagine and, in consequence, to decreasing the source of asparagine for the tumor cells, which (in contrast to normal cells) are incapable of intracellular asparagine synthesis and therefore are asparagine-dependent. However, the clinical advantages of asparaginases are limited because of toxic side effects and instances of spontaneous resistance of the tumor cells (Alberts *et al.*, 1999; Ettinger *et al.*, 1997).

The crystal structures of several type II bacterial asparaginases are known. They include the enzyme from *E. coli* – EcAII with bound aspartate (Swain *et al.*, 1993; Protein Data Bank, PDB, entry 3ECA), and its active-site T89V mutant with covalently bound product (Palm *et al.*, 1996; PDB entry 4ECA) as well as enzymes from *E. chrysanthemi* – ErA (Miller *et al.*, 1993), *Wolinella succinogenes* – WsA (Lubkowski *et al.*, 1996; PDB entry 1WSA), *Acinetobacter glutaminasificans* – AGA (Lubkowski *et al.*, 1994b; PDB entry 1AGX) and *Pseudomonas 7A* – PGA (Lubkowski *et al.*, 1994a; PDB entry 3PGA; Jakob *et al.*, 1997; PDB entry 4PGA). Type II asparaginases are active as homotetramers with nearly ideal 222 symmetry. The identical subunits (in EcAII 326 amino acids each) composing a homotetramer are denoted A, B, C, D (Fig. 2). The active site is created by subunits A and C (or B and D). Therefore, the asparaginase tetramer is more accurately described as a dimer of dimers. The molecules of the reaction product, L-aspartate, found in the crystal structures of the enzymes, define the location of the active site (Fig. 3) and the key surrounding residues: T12, Y25, S58, T89, D90, and K162 (in EcAII sequence). The role of these residues has been confirmed by mutagenesis and kinetic studies (Röhm & Van Etten, 1986; Bagert & Röhm, 1989; Derst *et al.*, 1992; Derst *et al.*, 1994). One of the proposed mechanisms of action suggests similarity to the reaction catalyzed by serine proteases (Rao *et al.*,

1996). The role of the S-H-D catalytic triad of serine proteases can be played in L-asparaginases by a similar triad, T89-K162-D90. This triad is conserved in the sequences (Bonthron & Jaskólski, 1997) and in all known three-dimensional structures of the bacterial enzymes (Dodson & Wlodawer, 1998). Alternatively, T12 has been implicated as the primary nucleophile (Palm *et al.*, 1996; Ortlund *et al.*, 2000). However, for its activation a hydrogen abstracting group is required. The mutated residue, Y25, is involved in the native enzyme in binding of the reaction product in the active site through its side-chain OH group. The presence of a hydrogen bond between the hydroxyl groups of Y25 and T12 suggest that Y25 could be involved in the activation of T12 (Palm *et al.*, 1996). Crystallographic studies of covalent complexes between *Pseudomonas 7A* glutaminase-asparaginase and diazo analogs of natural substrates also suggest that residue Y35 (equivalent to Y25 in EcAII) can be directly involved in the catalysis (Ortlund *et al.*, 2000; PDB codes 1DJO and 1DJP). On the other hand, the kinetic properties of enzyme variants with amino acid replacements in position 25 do not support the direct involvement of Y25 in catalysis. Several variants of this type showed residual activities as high as 1% of the wild-type value, and little effect on K_m was seen (Derst *et al.*, 1994). Moreover, mutants lacking E283, which would be necessary to activate Y25, have almost normal activities (Schleper *et al.*, unpublished data). Another important role of

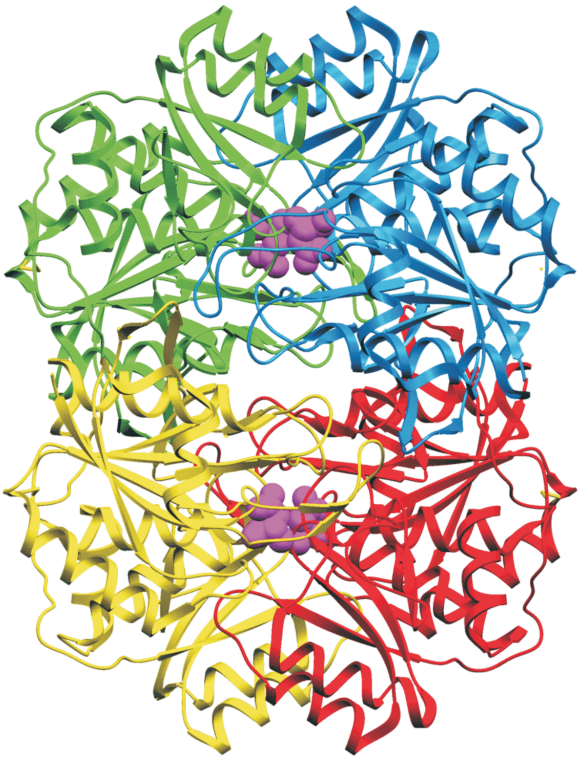

Figure 2. The *E. coli* asparaginase II homotetramer (PDB entry 3ECA).

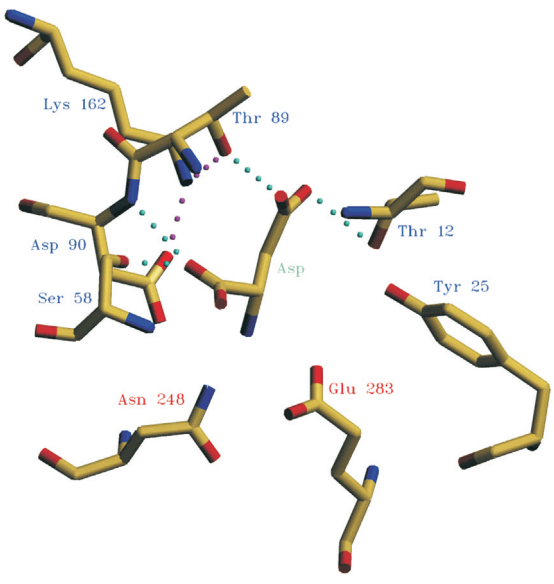

Figure 3. Active site of *E. coli* L-asparaginase II (Swain *et al.*, 1993).

Y25 in EcAII catalysis may be due to the fact, that this residue is part of a mobile loop that closes upon the active site after substrate binding. This structural element is disordered in nearly all asparaginase structures but is well-defined in PGA and especially in the covalent intermediate of mutant T89V (Palm *et al.*, 1996). Recent stopped-flow studies with EcAII indicate that the rate of loop closure decreases by three orders of magnitude when Y25 is replaced with other residues (Aung *et al.*, 2000).

The crystal structure of the Y25F mutant is of interest because it could be helpful in explaining the mechanism of substrate recognition and docking and the role of this residue in catalysis.

MATERIALS AND METHODS

Protein expression and purification. The Y25F mutant of EcAII was constructed and purified as described previously for the native form (Harms *et al.*, 1991). For mutagenesis of the *ansB* gene subcloned in M13mp19, the oligonucleotide 5′-AAA TCT AAC TTC ACA GTG GGT-3′ was employed to replace Y25 with F (mutagenic changes underlined).

Crystallization. A new polymorphic form of *E. coli* L-asparaginase II has been obtained for the Y25F mutant using new crystallization conditions. The crystallization experiments were conducted at 20°C using the vapor diffusion method and the hanging or sitting drop technique (McPherson, 1982). The protein fraction was desalted and then concentrated using Centricon-10 concentrators. The protein concentration, determined by UV absorption at 280 nm, was 10–15 mg/ml in 10 mM sodium citrate buffer, pH 4.8. Initial crystallization conditions (precipitant and pH) were established by the sparse-matrix method (Jancarik & Kim, 1991) using Crystal Screen II (Hampton Research, California, U.S.A.). Protein samples, 5 μl, were mixed on siliconized cover slips (for hanging drop experiments) or on polypropylene bridges (for sitting drop experiments) with equal amounts of reservoir solutions. The droplets were equilibrated against 1 ml reservoir solution in 24-well cell culture plates. The best crystals were obtained when the reservoir contained 46–48% 2-methyl-2,4-pentanediol (MPD), 100 mM sodium citrate buffer, pH 4.8, and 10–20 mM $CaCl_2$. Prismatic hexagonal crystals (Fig. 4) appeared after about 2 days and reached maximum dimensions of 0.6 × 0.3 × 0.3 mm within one week. The crystals for X-ray diffraction experiments were mounted in thin-walled quartz capillary with a small amount of mother liquor.

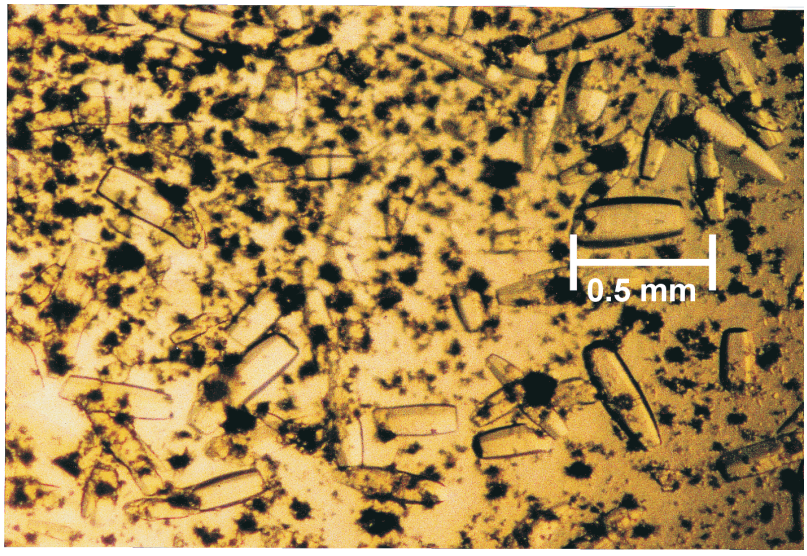

Figure 4. Single crystals of the Y25F mutant of *E. coli* L-asparaginase II.

Diffraction experiments. X-ray diffraction data were collected at room temperature using synchrotron radiation at λ = 0.98 Å

(EMBL, Hamburg Outstation c/o DESY, beamline X11) and a 345-mm MarResearch image plate scanner. The crystal-to-detector distance was 372 mm and the oscillation range 1.5°. 74,462 reflections (with $I/\sigma(I) > 0.0$) were collected to 2.45 Å resolution (Fig. 5). They were merged to give a unique data set of 23,517 reflections characterized by

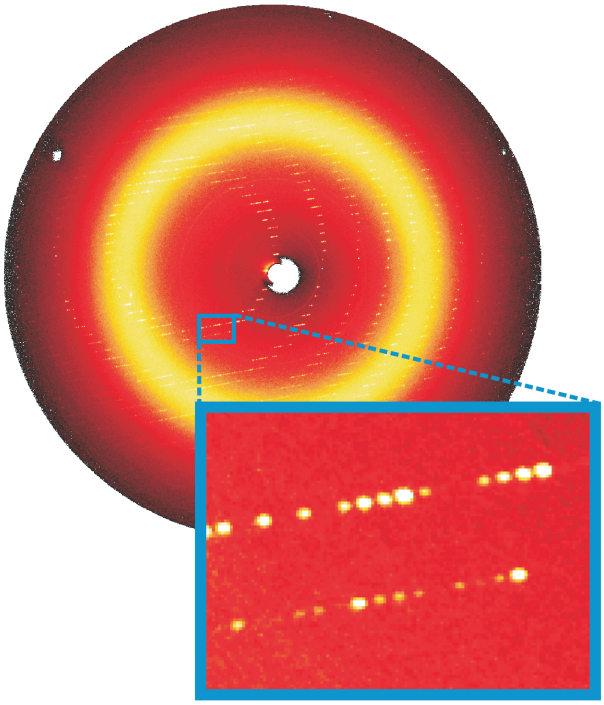

Figure 5. Typical diffraction pattern and its enlargement (oscillation range 1.5°).

The edge of the detector corresponds to 2.45 Å resolution.

R_{int} = 0.097 and $< I/\sigma(I) >$ = 7.9 (Table 1). Indexing and integration of the images was done in Denzo and scaling of the intensity data in Scalepack of the "HKL" program package (Otwinowski & Minor, 1997).

Structure solution. The crystal structure was solved by molecular replacement using the AMoRe program package (Navaza, 1994). X-ray diffraction data between 15–3.5 Å resolution were used and the AB dimer of native EcAII (Swain *et al.*, 1993) served as molecular probe. The rotation function was calculated using a Patterson radius of 45 Å. The best rotation peak had correlation coefficient of 0.112 and was 1.62 times higher than the next peak. In the next step, a translation search was performed for these peaks using the Crowther & Blow (1967) translation function. In the $P6_522$ space group, it produced for the first rotation peak a solution with very high correlation coefficient (0.716) and low R-factor (0.314). This best molecular replacement solution after rigid-body optimization is characterized by a correlation coefficient 0.769 and R-factor 0.288. In contrast, no translation solution could be found in the enantiomorphic $P6_122$ space group.

RESULTS AND DISCUSSION

Kinetic features of the Y25F mutant

The loss of the phenolic hydroxyl group at position 25 brought about by the replacement of Y with F strongly impairs k_{cat} without significantly affecting K_m (Derst *et al.*, 1994). With L-Asn as the substrate, k_{cat} decreases by a factor of 2000 while with AHA the decrease is only 200. Other Y25 mutants (Y25A, Y25G) have comparable kinetic properties indicating that it is indeed the OH group which is required. Recent stopped-flow studies with the double mutant W66Y/Y25W show that Y25 is crucially involved in the closure of the active-site loop (Aung *et al.*, 2000) which brings in position another essential residue, i.e. T12. This function of Y25 alone could account for the observed loss of activity in Y25F. The evidence for a role of Y25 as a general base (Ortlund *et al.*, 2000) is still rather circumstantial, and further experiments are needed to substantiate or refute such an assumption.

Crystallography

The crystal was very stable in the X-ray beam allowing collection of a complete data set from a single specimen. Our attempts to collect diffraction data at low temperature were unsuccessful. Flash freezing (Teng, 1990) increased

the mosaicity of the crystals and spot size, which in combination with the huge c parameter (341.1 Å) limited the accessible d-spacings. The experimental data represented 92.0% of the theoretically possible reflections (85.4% in the last resolution shell 2.54–2.45 Å) (Table 1). So far, seven different crystal forms have been reported for *E. coli* asparaginase II and its mutants. A summary of their crystal parameters and of asymmetric unit composition is presented in Table 2. The crystals of the present form belong to the 622 class and have a unit cell characterized by the following lattice parameters: a = 81.0, c = 341.1 Å. The space group enantiomorph, $P6_522$ was confirmed by molecular replacement calculations. The 222 point symmetry of the EcAII homotetramer and the crystallographic symmetry of the space group preclude odd numbers of EcAII molecules in the asymmetric unit. An analysis of the Matthews volume (Matthews, 1968) for this new hexagonal form of EcAII indicates that only two monomers could be accommodated in the asymmetric unit, corresponding to a Matthews coefficient of 2.33 $Å^3$/Da. There are three different subunit-subunit combinations within a 222-type EcAII tetramer (AC, AB, AD). In the nomenclature of the EcAII structure (PDB code 3ECA), only the AC dimer has all the components necessary for the formation of the active-site. In situations where the asparaginase tetramer has only one perfect two-fold symmetry axis, instead of asking what subunits constitute the asymmetric unit, it is better to ask which of the possible dimers has crystallographic symmetry and should not, therefore, be used to describe the unique element of the crystal architecture. In this new hexagonal form of EcAII it is the intimate AC dimer that is located across a crystallographic two-fold axis (along [1,–1,0]). The active sites of the tetrameric molecule are, therefore, created

Table 1. Summary of data collection

Resolution [Å]	I/σ(I)	R_{int}*	Completeness [%]
20.00–5.25	18.8	0.042	89.4
5.25–4.18	14.5	0.058	95.4
4.18–3.65	9.5	0.088	95.8
3.65–3.32	6.9	0.125	97.0
3.32–3.08	4.8	0.167	94.9
3.08–2.90	3.5	0.239	94.3
2.90–2.76	2.7	0.280	91.4
2.76–2.64	2.3	0.310	89.5
2.64–2.54	2.1	0.349	86.5
2.54–2.45	2.0	0.364	85.4
All data	7.9	0.097	92.0

*$R_{int} = \Sigma (I-\langle I\rangle) / \Sigma I$

Table 2. Crystallographic data for different polymorphic forms of *E. coli* L-asparaginase II

Form	Space group	a [Å]	b [Å] β [°]	c [Å]	Resolution [Å]	Asymm. unit contents	Reference
Native	*C*2	154.4	62.9 62.3	142.9	7.5	tetramer	Epp *et al.* (1971)
Native	$P2_12_12$	116.7	86.6	62.9	4.5	dimer	Itai *et al.* (1976)
Native	*I*222	62.1	73.7	140.9	3.5	monomer	Wlodawer (unpublished data)
Native	$P2_1$	76.7	96.1 97.1	111.4	2.3	tetramer	Swain *et al.* (1993)
Native	*C*2	76.3	134.6 110.5	64.8	1.95	dimer	Polikarpov *et al.* (1999)
S58A	$P2_12_12$	226.9	128.3	61.9	2.3	tetramer + dimer	Kozak & Jaskólski (2000)
T89V	$P2_12_12_1$	95.0	126.2	155.7	2.2	tetramer	Palm *et al.* (1996)
Y25F	$P6_522$	81.0	81.0	341.1	2.45	dimer	this work

from symmetrically dependent monomers. As indicated by the successful performance of molecular replacement, the asymmetric unit of the crystal can be conveniently described as containing the AB dimer.

REFERENCES

Alberts, S.R., Bretscher, M., Wiltsie, J.C., O'Neill, B.P., Mokri, B. & Witzig, T.E. (1999) Thrombosis related to the use of L-asparaginase in adults with acute lymphoblastic leukemia: A need to consider coagulation monitoring and clotting factor replacement. *Leuk. Lymphoma* **32**, 489–496.

Aung, H.P., Bocola, M., Schleper, S. & Röhm, K.H. (2000) Dynamics of a mobile loop at the active site of *E. coli* asparaginase II. *Eur. J. Biochem*. (submitted).

Bagert, U. & Röhm, K.H. (1989) On the role of histidine and tyrosine residues in *E. coli* asparaginase. Chemical modification and ^{1}H-nuclear magnetic resonance studies. *Biochem. Biophys. Acta* **999**, 36–41.

Bonthron, D.T. & Jaskólski, M. (1997) Why a "benign" mutation kills enzyme activity. Structure-based analysis of the A176V mutant of *Sacchromyces cerevisiae* L-asparaginase. *Acta Biochim. Polon.* **44**, 491–504.

Broome, J.D. (1961) Evidence that the L-asparaginase activity of guinea pig serum is responsible for its antilymphoma effects. *Nature* **191**, 1114–1115.

Chakrabarti, R. (1997) L-Asparaginase perspectives on the mechanisms of action and resistance. *Int. J. Pediatr. Hematol. Oncol.* **4**, 597–611.

Chibnall, A.C. (1930). *Protein Metabolism in the Plant*. Yale University Press, New Haven.

Clementi, A. (1922) La desamidation enzymatique de l'asparagine chez les différentes espéces animales et la signification physiologique de sa présence dans l'organisme. *Arch. Intern. Physiol.* **19**, 369.

Crowther, R.A. & Blow, D.M. (1967) A method for positioning a known molecule in an unknown crystal structure. *Acta Cryst.* **23**, 544–549.

Derst, C., Henseling, J. & Röhm, K.H. (1992) Probing the role of threonine and serine residues of *E. coli* asparaginase II by site-specific mutagenesis. *Protein Eng.* **5**, 785–789.

Derst, C., Wehner, A., Specht, V. & Röhm, K.H. (1994) States and functions of tyrosine residues in *Escherichia coli* asparaginase II. *Eur. J. Biochem.* **224**, 533–540.

Dodson, G. & Wlodawer, A. (1998) Catalytic triads and their relatives. *Trends Biochem. Sci.* **23**, 347–352.

Epp, O., Steigemann, W., Formanek, H. & Huber, R. (1971) Crystallographic evidence for the tetrameric subunit structure of L-asparaginase from *Escherichia coli. Eur. J. Biochem.* **20**, 432–437.

Ettinger, L.J., Ettinger, A.G., Avramis, V.I. & Gaynon, P.S. (1997) Acute lymphoblastic leukaemia: A guide to asparaginase and pegaspargase. *Biodrugs* **7**, 30–39.

Harms, E., Wehner, A., Jennings, M.P., Pugh, K.J., Beacham, I.R. & Röhm, K.H. (1991) Construction of expression systems for *Escherichia coli* asparaginase II and two-step purification of the recombinant enzyme. *Protein Exp. Purif.* **2**, 144–150.

Hill, J.M., Roberts, J., Loeb, E., Khan, A., MacLellan, A. & Hill, R.W. (1967) L-Asparaginase therapy for leukemia and other malignant neoplasms. Remission in human leukemia. *J. Am. Med. Assoc.* **202**, 116–122.

Itai, A., Yonei, M., Mitsui, Y. & Itaka, Y.J. (1976) Crystallographic study on the orthorhombic crystal of L-asparaginase from *Escherichia coli* HAP. *J. Mol. Biol.* **105**, 321–325.

Jakob, C.G., Lewinski, K., Lacount, M.W., Roberts, J. & Lebioda, M. (1997) Ion binding induces closed conformation in *Pseudomonas 7A* glutaminase-asparaginase (pga): Crystal structure of the PGA-SO_4^{2-}-$NH4^{+}$ complex at 1.7 Å resolution. *Biochemistry* **36**, 923–931.

Jancarik, J. & Kim, S.-H. (1991) Sparse matrix sampling: A screening method for crystallization of proteins. *J. Appl. Cryst.* **24**, 409–411.

Kidd, J.G. (1953) Regression of transplanted lymphomas induced *in vivo* by means of normal guinea pig serum. *J. Exp. Med.* **98**, 565–605.

Kozak, M. & Jaskólski, M. (2000) Crystallization and preliminary crystallographic studies of a new crystal form of *Escherichia coli* L-asparaginase II (Ser58Ala mutant). *Acta Cryst.* **D56**, 509–511.

Lubkowski, J., Palm, G.J., Gilliland, G.L., Derst, C., Röhm, K.H. & Wlodawer, A. (1996) Crystal structure and amino acid sequence of *Wolinella succinogenes* L-asparaginase. *Eur. J. Biochem.* **241**, 201–207.

Lubkowski, J., Wlodawer, A., Ammon, H.L., Copeland, T.D. & Swain, A.L. (1994a) Structural characterization of *Pseudomonas 7A* glutaminase-asparaginase. *Biochemistry* **33**, 10257–10265.

Lubkowski, J., Wlodawer, A., Housset, D., Weber, I.T., Ammon, H.L., Murphy, K.C. & Swain, A.L. (1994b) Refined crystal structure of *Acinebacter glutaminaficans* glutaminase-asparaginase. *Acta Cryst.* **D50**, 826–832.

Matthews, B.W. (1968) Solvent contents of protein crystals. *J. Mol. Biol.* **33**, 491–497.

McPherson, A. (1982) *The Preparation and Analysis of Protein Crystals*. John Wiley, New York.

Miller, M., Rao, J.K.M., Wlodawer, A. & Gribskov, M. (1993) A left-handed crossover involved in amidohydrolase catalysis. Crystal structure of *Erwinia chrysanthemi* L-asparaginase with bound L-aspartate. *FEBS Lett.* **328**, 275–279.

Navaza, J. (1994) AMoRe: An automated package for molecular replacement. *Acta Cryst.* **D50**, 157–163.

Ortlund, E., Lacount, M.W., Lewinski, K. & Lebioda, L. (2000) Reactions of *Pseudomonas 7A* glutaminase-asparaginase with diazo analogues of glutamine and asparagine result in unexpected covalent inhibitions and suggests an unusual catalytic triad Thr-Tyr-Glu. *Biochemistry* **39**, 1199–1204.

Otwinowski, Z. & Minor, W. (1997) Processing of X-ray diffraction data collected in oscillation mode. *Methods Enzymol.* **276**, 307–327.

Palm, G., Lubkowski, J., Derst, C., Schleper, S., Röhm, K.H. & Wlodawer, A. (1996) A covalently bound catalytic intermediate in *Escherichia coli* asparaginase: Crystal structure of a Thr-89-Val mutant. *FEBS Lett.* **390**, 211–216.

Polikarpov, I., de Oliveira, R.T. & Abrahão-Neto, J. (1999) Preparation and preliminary X-ray diffraction studies of a new crystal form of L-asparaginase from *Escherichia coli*. *Acta Cryst.* **D55**, 1616–1617.

Rao, J.K.M., Gribskov, M., Lubkowski, J., Miller, M., Swain, A.L. & Wlodawer, A. (1996) A comparison of the crystal structures of bacterial L-asparaginases. *Techniques in Protein Chemistry VII* (Marshak, R.M., ed.) Academic Press, San Diego.

Röhm, K.H. & Van Etten, R.L. (1986) The ^{18}O isotope effect in ^{12}C nuclear magnetic resonance spectroscopy: Mechanistic studies on asparaginase from *Escherichia coli*. *Arch. Biochem. Biophys.* **244**, 128–136.

Swain, A.L., Jaskólski, M., Housset, D., Rao, J.K.M. & Wlodawer, A. (1993) Crystal structure of *Escherichia coli* L-asparaginase, an enzyme used in cancer therapy. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 1472–1478.

Teng, T.Y. (1990) Mounting of crystals for macromolecular crystalography in free-standing thin film. *J. Appl. Cryst.* **23**, 387–391.