Acta Crystallographica Section D
Biological Crystallography
ISSN 0907-4449

Crystallization and preliminary X-ray structure determination of jack bean urease with a bound antibody fragment

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Received 17 July 2001
Accepted 14 December 2001

Urease allows organisms to use exogenous and internally generated urea as a nitrogen source, by catalyzing the hydrolysis of urea to form ammonia and carbon dioxide. Urease may also participate in the systemic nitrogen-transport pathways and possibly acts as a toxic defence protein. Jack bean urease (JBU) was the first nickel-metalloenzyme identified and was crystallized as early as 1926. Despite this, the structure has not yet been determined. An antibody fragment, Fv, that has a high affinity for JBU has been used to aid crystallization. The complex, which retains full enzyme activity, forms very small crystals that diffract weakly to 3.3 Å. The crystals belong to the rhombohedral space group $R32$, with unit-cell parameters $a = b = 228.6$, $c = 130.9$ Å. The structure of the urease molecule has been solved by molecular replacement using the structure of homogenous enzyme from *Klebsiella aerogenes* as a search model.

1. Introduction

Ureases catalyze the hydrolysis of urea to form ammonia and carbamate. The carbamate then spontaneously decomposes to form carbon dioxide and ammonia. Non-enzymatic breakdown of urea produces ammonia and cyanic acid (Blakeley *et al.*, 1982), but urea is very stable in solutions that have a pH in the range 2–12 and has a half-life of 3.6 y at 311 K (Zerner, 1991). Urease accelerates the reaction rate of hydrolysis of urea by at least 10^{14} compared with the spontaneous reaction (Hausinger, 1993). Medically, urease is one of the key enzymes used by pathogenic bacteria causing gastric ulcers and urinary stone formation (Mobley & Hausinger, 1989). In agriculture, ureases within the soil can decompose urea-based fertilizers, resulting in both nitrogen deficiency and ammonia toxicity, leading to plant damage (Mobley & Hausinger, 1989). In 1926, jack bean urease (JBU) was crystallized by James Sumner (Sumner, 1926). These were the first crystals of a characterized enzyme and it enabled the demonstration that enzymes were proteins. 50 y later, urease was shown to be a nickel-metalloenzyme (Dixon *et al.*, 1975), the first of its kind. JBU exists as monomers, trimers and hexamers of identical 91 kDa subunits, each containing two nickel ions per subunit (Hirai *et al.*, 1993). Although JBU was one of the first proteins to be crystallized, its structure has yet to be determined. These first crystals were shown to diffract weakly to 3.5 Å using a synchrotron source and belong to the complex cubic space group $F4_132$, with very large unit-cell lengths, $a = 364$ Å (Jabri *et al.*, 1992).

The structure of the *K. aerogenes* urease (KAU) has been determined crystallographically to 2.2 Å (Jabri *et al.*, 1992). Although KAU contains three polypeptide chains, these appear fused in JBU so that the two proteins are 54% identical over the full length of the latter.

Antibodies have been used to aid the crystallization and structure determination of previously unsolved proteins. Heterogeneity, insolubility, molecular flexibility or polydispersity of a solution can inhibit crystallization (Laver, 1990). The use of an antibody fragment–antigen complex can overcome some of these problems, as they are reasonably soluble and bind specifically to selected antigens, thus potentially transforming aggregated material into a soluble monodisperse sample that is suitable for crystallization (Kovari *et al.*, 1995). A Fab fragment has been used to help crystallize the insoluble p24 protein of HIV capsid protein (Prongay *et al.*, 1990). This had not previously been crystallized owing to the capsid protein forming oligomers of variable sizes in solution. Other attempts to try and crystallize this protein, including the addition of detergents, had proved unsuccessful. Fab fragments have also been used successfully to stabilize the flexible HIV reverse transcriptase to obtain diffracting crystals (Jacobo-Molina *et al.*, 1991, 1993). In the structure determination of the membrane protein cytochrome *c* oxidase, an Fv was used to increase the polar

surface of the protein for crystallization purposes (Ostermeier *et al.*, 1995).

An Fv has been used to help crystallize JBU in a different space group so that its structure can be determined.

2. Experimental

2.1. Cloning, expression and extraction

The gene fragment encoding the anti-urease FvU4 with a hydrophilic hydrophil-II tail (sequence GSGSGNSGKGYLK) and a hexahistidine tag was cloned into the vector pPIC9K (Invitrogen). Expression of this 27 kDa protein was in *Pichia pastoris* (Invitrogen). The cells were grown in buffered glycerol-complex medium (BMGY) at 303 K until an OD_{600} of between 2 and 6 was reached. The biomass was then harvested by centrifugation at 2000*g* for 5 min at room temperature. The cells were then re-suspended in buffered methanol-complex medium (BMMY) (which induces expression) and grown to an OD_{600} of approximately 1. The cells were incubated for 48 h at 303 K with an additional feeding with methanol to a final concentration of 0.5% after 24 h. The supernatant was cleared of cells by centrifugation at 2000*g* for 5 min at room temperature.

The urease was extracted from jack bean meal (Sigma) using a method based on that described by Blakeley *et al.* (1969). 1750 ml of 1 m*M* DTT was heated to 312 K, after which 750 ml of acetone was added and the mixture was removed from the heat. 500 g of jack bean meal was added slowly whilst stirring and this was left stirring for a further 5 min. The mixture was then centrifuged at 3000*g* for 10 min. The supernatant was left to stand at 277 K for 48 h, after which it was centrifuged at 3000*g* for 30 min at 277 K. The pellet was resuspended in 20 ml of 0.02 *M* phosphate buffer pH 7.1, 1 m*M* EDTA, 1 m*M* DTT and then centrifuged at 35 000*g* for 30 min. The supernatant was retained and the pellet was discarded.

2.2. Protein purification and crystallization

4 l of the FvU4 supernatant was filtered through a 0.2 µm sterile filter and then loaded onto an Ni-NTA column (Pharmacia) that had been equilibrated in phosphate-buffered saline (PBS). A gradient of 0–500 m*M* imidazole in PBS was used to elute the bound protein. The eluate was analyzed by SDS–PAGE and the pooled fractions were dialyzed into PBS and loaded onto a Mono-Q column (Pharmacia). The bound materials were then eluted from the column with a 0–1 *M* NaCl gradient in PBS. The fractions that proved to contain more than 95% FvU4 as analyzed by SDS–PAGE were pooled together. A total of 20 mg of Fv was purified, which was then dialyzed into PBS buffer.

The urease was dialyzed into PBS and then loaded onto a S200 gel-filtration column (Pharmacia). The sample was eluted in PBS and the fractions were analyzed by SDS–PAGE. A total of 60 mg of urease was purified from the gel-filtration system as determined by SDS–PAGE analysis.

Preparation of the urease–Fv complex was achieved by mixing urease monomer and the Fv fragment in a molar ratio of 1:4 to ensure that all the Fv binding sites of the urease were occupied. Any excess Fv that remained in solution was then washed away in a 50 kDa molecular-weight cutoff concentrator, so that a pure single species of complex remained. The sample was run on a 4–15% gradient gel using native buffer strips on a PhastSystem (Pharmacia) to determine complex formation (Fig. 1) prior to concentrating to 10 mg ml^{-1}.

Initial crystallization trials were set up using a sparse-matrix crystallization screen (Jancarik & Kim, 1991; Hampton Research) and the hanging-drop technique. All drops consisted of 2 µl of the concentrated complex and 2 µl of the reservoir solution; the trials were stored at 295 K. Conditions that yielded microcrystals of the urease–Fv complex were identified and optimization of the conditions produced larger crystals using 2 *M* ammonium sulfate, 100 m*M* sodium acetate pH 5.6. The obtained crystals were triangular rods, but were only 50 µm in the longest dimension (Fig. 2). A native gel showed that the JBU–Fv complex was present in the crystals. Further optimization and attempts at seeding did not improve the size of the crystals.

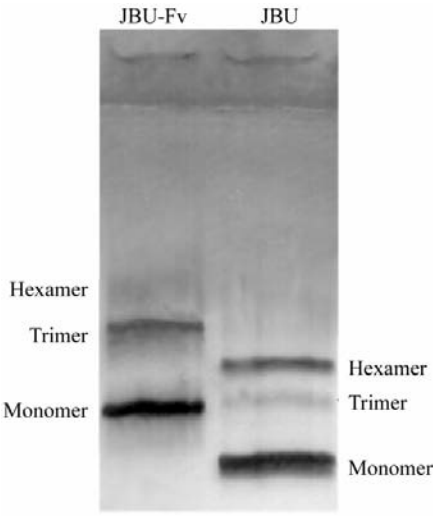

Figure 1
A native gel of urease and urease–Fv complex. Samples can be seen to be present as monomers, trimers and hexamers. The sample containing both urease and Fv can be seen to run with a differing mobility to the urease alone sample, indicating that the Fv is bound to the urease.

Table 1
Data-collection statistics for crystals of the JBU–Fv complex.

Values in parentheses are for the highest resolution shell (3.55–3.35 Å).

Space group	*R*32
Unit-cell parameters (Å, °)	$a = b = 228.6$, $c = 130.9$, $\alpha = \beta = 90$, $\gamma = 120$
Resolution range (Å)	30–3.35
Total No. of reflections	31426
No. of unique reflections	14725
Completeness (%)	77.5 (51.2)
$I/\sigma(I)$	3.2 (2.4)
R_{merge} (%)	20.0 (26.1)

2.3. X-ray data collection and molecular replacement

Attempts to freeze the crystals using a variety of cryoprotectants led to complete loss of diffraction. For data collection, crystals were mounted in thin-walled capillaries of diameter 0.3 mm (Glas) and were cooled to 277 K using the air flow from a Cryostream. A data set was collected at station 9.6, Daresbury Laboratory, England at a wavelength of 0.96 Å using a MAR CCD detector. Although diffraction initially extended to 3 Å in many cases, the diffraction decayed rapidly, sometimes to below 4 Å after a single image. The crystals also diffracted with low intensity and therefore no image is shown here. The final data set was derived from the five best crystals and

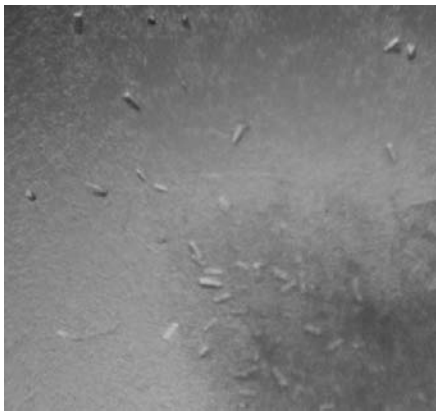

Figure 2
Crystals of the urease–Fv complex obtained using the hanging-drop method. The size of these crystals is approximately 50 × 20 × 20 µm.

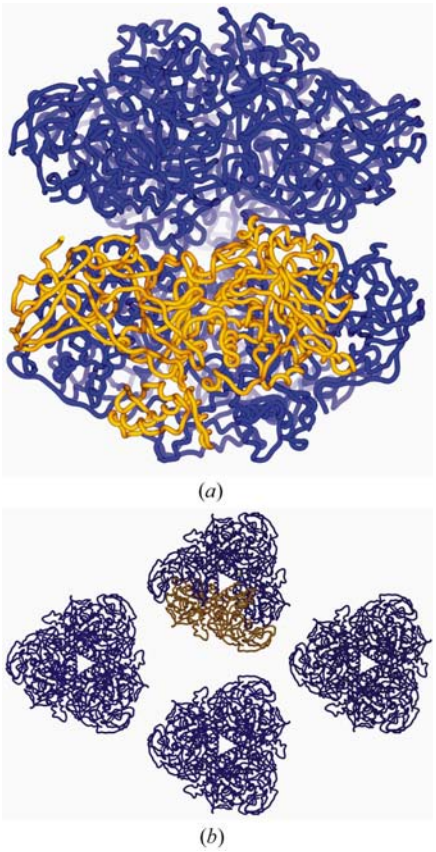

Figure 3
Molecular-replacement solutions for urease, with one urease monomer coloured yellow. (*a*) The urease hexamer viewed along the *x* axis; the interface between the two trimers can easily be seen. (*b*) Packing of the urease molecules viewed down the *z* axis. The gaps between the molecules leave enough space for the Fv. These diagrams were made using *SPOCK* (Copyright 1998, Jon A. Christopher).

data extended to only 3.3 Å. The data were processed using *MOSFLM* (Leslie, 1992) and scaled using *SCALA* from the *CCP*4 suite of programs (Collaborative Computational Project, Number 4, 1994). The data were indexed in a *R*32 unit cell (Table 1), with an $R_{\rm merge}$ that was rather higher than expected, possibly owing to the weakly diffracting crystals and the high level of decay of the diffraction pattern. The unit-cell parameters are $a = b = 228.6$, $c = 130.9$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$ and the unit-cell volume is 5.9×10^6 Å^3. The $V_{\rm M}$ value of 2.8 Å^3 Da^{-1} for one urease molecule and one Fv molecule per asymmetric unit lies within the range given by Matthews (1968). The solvent content in the crystals for one Fv–urease complex in the asymmetric unit would be 56%; if, however, there were two molecules per asymmetric unit, then the solvent content would be only 12%.

The program *AMoRe* implemented in the *CCP*4 suite of programs (Navaza, 1994) was used to find a molecular-replacement solution for JBU. The coordinates for *K. aerogenes* urease (PDB code 1kau) were used as a search model in the resolution range 20–4 Å for the rotation function. This result was then incorporated into the translation search over the resolution range 10–4 Å. A correlation coefficient of 35.4% and an *R* value of 47.3% indicated a probable solution. The next highest peak had a correlation coefficient of 27.7% and an *R* value of 49.7%. Rigid-body refinement based on the most probable solution was then carried out using *X-PLOR* 3.1 (Brunger, 1992), reducing the *R* value to 42.2%. The packing of the molecule was inspected using the program *O* (Jones *et al.*, 1991) and the urease hexamer could easily be identified (Fig. 3*a*). The crystal packing showed no bad contacts between the urease molecules; however, there were large spaces in the packing which could accommodate the Fv fragment. In particular, the urease hexamers were organized into isolated columns along *z*, indicating that the Fvs must bridge between them to complete the crystal packing (Fig. 3*b*), although their positions and orientations are not immediately obvious in the initial maps.

Further work on the molecular-replacement solution for the Fv and refinement is in progress.

We would like to show our appreciation to the Biorecognitions Unit at Unilever, especially to John Windust, Samantha Williams and Sandra Hemmington, for their help with the cloning and purification of the Fv. Thanks also go to all members of the Astbury Centre for Structural Molecular Biology group at Leeds, especially Joachim Jæger and Maire Convery, for their help with the molecular replacement. This work was funded by the BBSRC and Unilever Research.

References

Blakeley, R. L., Treston, A., Andrews, R. K. & Zerner, B. (1982). *J. Am. Chem. Soc.* **104**, 612–614.
Blakeley, R. L., Webb, E. C. & Zerner, B. (1969). *Biochemistry*, **8**, 1984–1990.
Brunger, A. T. (1992). *X-PLOR, Version* 3.1. *A System for X-ray Crystallography and NMR.* New Haven, CT, USA: Yale University Press.
Collaborative Computational Project, Number 4 (1994). *Acta Cryst.* D**50**, 760–763.
Dixon, N. E., Gazzola, C., Blakeley, R. L. & Zerner, B. (1975). *J. Am. Chem. Soc.* **97**, 4131–4133.
Hausinger, R. P. (1993). *Biochemistry of Nickel*, ch. 3. New York: Plenum Press.
Hirai, M., Kawaihirai, R., Hirai, T. & Veki, T. (1993). *Eur. J. Biochem.* **215**, 55–61.
Jabri, E., Lee, M. H., Hausinger, R. P. & Karplus, P. A. (1992). *J. Mol. Biol.* **227**, 934–937.
Jacobo-Molina, A., Clark, A. D. Jr, Williams, R. L., Nanni, R. G., Clark, P., Ferris, A. L., Hughes, S. H. & Arnold, E. (1991). *Proc. Natl Acad. Sci. USA*, **88**, 10895–10899.
Jacobo-Molina, A., Ding, J., Nanni, R. G., Clark, A. D. Jr, Lu, X., Tantillo, C., Williams, R. L., Kamer, G., Ferris, A. L., Clark, P., Hizi, A., Hughes, S. H. & Arnold, A. (1993). *Proc. Natl Acad. Sci. USA*, **90**, 6320–6324.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Jones, T. A., Zou, J. Y., Cowan, S. W. & Kjeldgaard, M. (1991). *Acta Cryst.* A**47**, 110–119.
Kovari, L. C., Momany, C. & Rossmann, M. G. (1995). *Structure*, **3**, 1291–1293.
Laver, W. G. (1990). *Methods*, **1**, 70–74.
Leslie, A. G. W. (1992). *Jnt CCP4/ESRF–EAMBC Newsl. Protein Crystallogr.* **26**.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–493.
Mobley, H. L. T. & Hausinger, R. P. (1989). *Microbiol. Rev.* **53**, 85–108.
Navaza, J. (1994). *Acta Cryst.* A**50**, 157–163.
Ostermeier, C., Iwata, S., Ludwig, B. & Michel, H. (1995). *Nature Struct. Biol.* **2**, 842–846.
Prongay, A. J., Smith, T. J., Rossmann, M. G., Ehrlich, L. S., Carter, C. A. & McClure, J. (1990). *Proc. Natl Acad. Sci. USA*, **87**, 9980–9984.
Sumner, J. B. (1926). *J. Biol. Chem.* **69**, 435–441.
Zerner, B. (1991). *Bioorg. Chem.* **19**, 116–131.