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Crystallization and preliminary X-ray crystallographic analysis of the GluR0 ligand-binding core from *Nostoc punctiforme*

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GluR0 from *Nostoc punctiforme* (*Np*GluR0) is a bacterial homologue of the ionotropic glutamate receptor. The ligand-binding core of *Np*GluR0 was crystallized at 294 K using the hanging-drop vapour-diffusion method. The L-glutamate-complexed crystal belongs to space group $C222_1$, with unit-cell parameters $a = 78.0$, $b = 145.1$, $c = 132.1$ Å. The crystals contain three subunits in the asymmetric unit, with a V_M value of 2.49 Å^3 Da^{-1}. The diffraction limit of the L-glutamate complex data set was 2.1 Å using synchrotron X-ray radiation at beamline BL-4A of the Pohang Accelerator Laboratory (Pohang, Korea).

1. Introduction

Ionotropic glutamate receptors (iGluRs) are integral membrane proteins that play a central role in excitatory neurotransmission by forming ligand-gated ion channels (Mayer & Westbrook, 1987; Dingledine *et al.*, 1999). iGluRs exist as tetramers (dimers of dimers; Laube *et al.*, 1998; Rosenmund *et al.*, 1998), with each subunit composed of an amino-terminal domain (ATD), a ligand-binding core (S1 and S2), three membrane-spanning domains (TM1, TM2 and TM3), a cytoplasmic re-entry loop and a C-terminal domain (CTD). Note that two discontinuous segments, S1 and S2, are responsible for neurotransmitter binding. iGluRs have been conventionally classified into three subtypes according to their sensitivity to the agonists AMPA (α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid), kainate (a structural analogue of glutamate) or NMDA (*N*-methyl-D-aspartate) (Hollmann & Heinemann, 1994). All three types are also activated by glutamate, the physiological neurotransmitter. These ligand-gated ion channels transduce chemical signals into electrical impulses by opening a transmembrane pore in response to the binding of neurotransmitter molecules.

The structural components of the various iGluRs that have been resolved so far include the ligand-binding cores of GluR2 (AMPA receptor; Armstrong *et al.*, 1998; Armstrong & Gouaux, 2000; Hogner *et al.*, 2002, 2003; Jin *et al.*, 2002; Kasper *et al.*, 2002; Lunn *et al.*, 2003; Sun *et al.*, 2002), GluR5 (kainate receptor; Mayer, 2005; Naur *et al.*, 2005), GluR6 (kainate receptor; Mayer, 2005; Nanao *et al.*, 2005) and NR1 (NMDA receptor; Furukawa & Gouaux, 2003). In addition, a prokaryotic iGluR that exhibits significant sequence homology with the eukaryotic receptor has been discovered (Chen *et al.*, 1999) and studied on the ligand-binding core structure of GluR0 (a bacterial homologue) from *Synechocystis* (Mayer *et al.*, 2001). In contrast to the eukaryotic receptor, *Synechocystis* GluR0 (*S*GluR0) is not gated by classical glutamate receptor agonists such as AMPA, kainate or NMDA, but is activated by a large number of L-α-amino acids, including glutamate, serine and glutamine. Moreover, electrophysiological measurements indicate that *S*GluR0 receptors form transmembrane ion channels that are selectively permeable to K^+ ions, whereas the eukaryotic receptors form channels that are permeable to both Na^+ and K^+ ions. *S*GluR0 shares a common membrane topology, having two transmembrane domains (TM1 and TM2) and a re-entry loop similar to the P loop found in K^+ channels (Chen *et al.*, 1999).

By searching microbial sequence databases, other putative bacterial glutamate receptors have been identified from *Synechococcus* species, *Prochlorococcus marinus*, *Trichodesmium erythraeum*

IMS101, *Nostoc punctiforme*, *Magnetospirillum magnetotacticum* and *Silicibacter pomeroyi* (Kuner *et al.*, 2003). These bacterial homologues could be invaluable for structural analysis of glutamate receptors as bacterial proteins can often be isolated in quantity.

However, in order to resolve some of the remaining questions about glutamate receptors (*e.g.* the mechanisms of tetramer formation and desensitization), further resolution of the structure will be required. As a first step toward determining the structure of GluR0 from *N. punctiforme* (*Np*), we report here the overexpression, purification and crystallization of the *Np*GluR0 ligand-binding (LB) core and its preliminary X-ray characterization.

2. Materials and methods

2.1. Expression and purification

The construct for expression of the *Np*GluR0 LB core was made as follows. The S1 and S2 segments constituting the LB core were amplified by the polymerase chain reaction (PCR) from full-length *Np*GluR0 using the primers S1-forward (CGATAA CATATG CTCCAACAACCGTTATTAGTAGCT), S1-reverse (CGATAA GGATCC ACTCTCTAGATTGCGTACCATAAT), S2-forward (CGATAA GGATCC GGCGATATCAGGAGTATAGACGAT) and S2-reverse (CGATAA CTCGAG AGAATTTTTAGGATC-GAACCACTT). The PCR products were digested with restriction enzymes and ligated into a pET-23a vector (Novagen, USA) in a three-point ligation (*Nde*I, *Bam*HI and *Xho*I). With this vector, the expressed protein contains a C-terminal His tag and two *Np*GluR0 sequences (71-LQQP . . . NLES-169 and 281-GDIR . . . PKNS-405) separated by a Gly-Thr linker (Chen *et al.*, 1998), which was verified by sequencing. After transforming *Escherichia coli* with the vector, the recombinant LB core of *Np*GluR0 was induced by treating the bacteria with 1 m*M* isopropyl β-D-thiogalactopyranoside (IPTG) for 6 h at 310 K. The bacteria were then collected, resuspended in lysis buffer (50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl and 5 m*M* imidazole) and lysed by sonication. The lysate was centrifuged at 14 000*g* for 30 min, after which the supernatant was collected and centrifuged at 16 000*g* for an additional 30 min. The supernatant was again collected and loaded onto a gravity-flow column (Bio-Rad) packed with Ni–NTA affinity resin (Peptron) pre-equilibrated with lysis buffer (2 ml bed volume per litre of culture). After washing the column and matrix with ten bed volumes of lysis buffer, the C-terminal His-tag fusion protein was eluted using a solution consisting of 50 m*M* sodium phosphate pH 8.0, 300 m*M* NaCl and 300 m*M* imidazole. The fractions containing *Np*GluR0 LB core were collected and the recombinant protein was further purified by gel filtration using a Superdex 200 column (Pharmacia Biotech) pre-equilibrated with a solution of 20 m*M* HEPES–NaOH pH 7.5 and 150 m*M* NaCl. The fractions containing the *Np*GluR0 LB core were again collected and concentrated to 21.8 mg ml^{-1} by ultrafiltration (Amicon Centricon 10).

2.2. Crystallization and data collection

The *Np*GluR0 LB core was crystallized at room temperature (294 $\pm$ 1 K) using the hanging-drop vapour-diffusion method. Crystals were grown on a siliconized cover slip by equilibrating a mixture containing 1 µl protein solution (21.8 mg ml^{-1} protein in 20 m*M* HEPES–NaOH pH 7.5 and 150 m*M* NaCl) and 1 µl reservoir solution [16%(*w*/*v*) PEG 8000, 0.1 *M* sodium citrate pH 5.0, 5 m*M* imidazole, 5 m*M* L-glutamate] against 0.5 ml reservoir solution. Crystals formed from the precipitate after 3 d and grew to a largest dimension of 0.3 mm. We tried to grow crystals of *Np*GluR0 LB core in the absence of L-glutamate, but no crystals suitable for X-ray diffraction measurement were obtained without adding L-glutamate.

For cryogenic experiments, a suitable cryoprotectant was determined to be reservoir solution plus 20%(*v*/*v*) glycerol. Successful flash-freezing was achieved when the crystals were transferred directly from the drop to the cryoprotection solution and allowed to

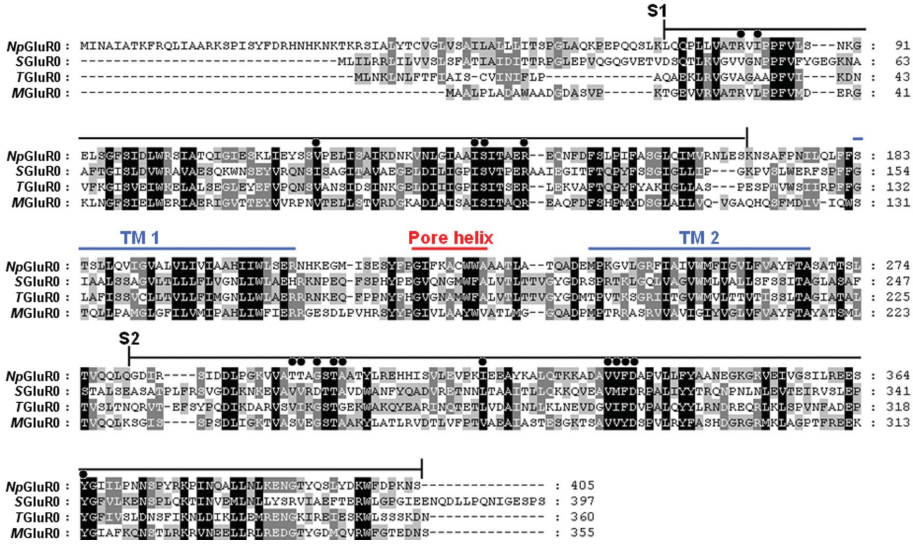

Figure 1
Sequence alignment of *Np*GluR0 and other bacterial iGluR homologues. The amino-acid sequences of GluRs from *N. punctiforme* (*Np*GluR0), *Synechocystis* (*S*GluR0), *T. erythraeum* IMS101 (*T*GluR0) and *M. magnetotacticum* (*M*GluR0) were aligned using *ClustalX* (Thompson *et al.*, 1997). Highly conserved residues are shaded grey. Bacterial GluRs have two transmembrane segments (TM1 and TM2) and a re-entry loop (pore helix). The TM helices were predicted using a consensus of the results from the programs *TMPred* (http://www.ch.embnet.org/software/TMPRED_form.html), *PHDhtm* (http://www.embl-heidelberg.de/predictprotein/) and *SOSUI* (http://sosui.proteome.bio.tuat.ac.jp/sosuiframe0.html). The two discontinuous segments, S1 and S2, comprise the LB core. The expressed *Np*GluR0 LB core contains the sequences 71-LQQP . . . NLES-169 and 281-GDIR . . . PKNS-405 separated by a Gly-Thr linker. Residues constituting the L-glutamate-binding site are indicated by filled circles.

Table 1
Data-collection statistics.

Values in parentheses are for the last shell.

X-ray source	PAL-4A
Space group	$C222_1$
Unit-cell parameters (Å)	a = 78.0, b = 145.1, c = 132.1
Wavelength (Å)	1.00
Resolution range (Å)	50–2.1
No. of observed reflections	84618
No. of unique reflections	44121
Completeness (%)	99.7 (99.0)
$R_{\rm sym}$†	0.061 (0.240)
$I/\sigma(I)$	24.8 (5.5)

† $R_{\rm sym} = \sum_h \sum_i |I(h)_i - \langle I(h)\rangle| / \sum_h \sum_i I(h)_i$, where I is the intensity of reflection h, $\sum_h$ is the sum over all reflections and $\sum_i$ is the sum over i measurements of reflection h.

equilibrate for 1 min. An L-glutamate complex data set was collected at beamline BL-4A of the Pohang Accelerator Laboratory (Pohang, Korea) using the X-ray beam at a single wavelength (1.0000 Å); diffraction was obtained to 2.1 Å. The data set was indexed and processed using *HKL*2000 (Otwinowski & Minor, 1997). Data-collection statistics are given in Table 1.

3. Results

The conserved residues constituting the L-glutamate-binding site in the amino-acid sequence alignment are indicated in Fig. 1; the ligand-binding properties of *Np*GluR0 LB core were determined using the isothermal titration calorimetry method. Comparison of the binding affinity showed a higher affinity for L-glutamate ($K_{\rm d}$ value of 25 μM) compared with other amino acids (L-serine, L-glutamine, L-aspartate, L-cysteine, L-alanine, L-glycine, L-threonine, D-glutamate, D-serine and D-glutamine) and known iGluR agonists (AMPA, kainate and NMDA; unpublished data).

The *Np*GluR0 LB core crystal (Fig. 2) belongs to space group $C222_1$ and has unit-cell parameters a = 78.0, b = 145.1, c = 132.1 Å. Assuming the presence of three molecules per asymmetric unit, the Matthews coefficient ($V_{\rm M}$) was calculated to be 2.49 Å^3 Da^{-1}, which corresponds to a solvent content of 50.6%. These values are within the range previously observed for protein crystals (Matthews, 1968).

The molecular-replacement program *MOLREP* (Vagin & Teplyakov, 1997) was used for structural determination of the *Np*GluR0 LB core; the 2.0 Å crystal structure of the *S*GluR0 LB core (Mayer *et al.*, 2001; PDB code 1ii5) was used as the model (monomer model) for the cross-rotation search. The amino-acid sequences of the *S*GluR0 and *Np*GluR0 LB cores share about 28% identity and 50% homology (Fig. 1). The cross-rotation function for this model was calculated using data in the resolution range 15–4 Å and the first three peaks in the output appeared as distinct solutions. These three peaks grew more distinct in the translation calculations, with an overall correlation coefficient of 37.4% and an R factor of 47.6%. Rigid-body refinements, simulated annealing, overall anisotropic B factor and individual restrained B factor refinements were performed using *CNS* (Brünger *et al.*, 1998). After these refinement steps, the resultant electron-density map showed three protein molecules and three L-glutamate-binding sites in an asymmetric unit and the quality of the initial map was high enough to build most of the residues. Model building and further refinement is ongoing.

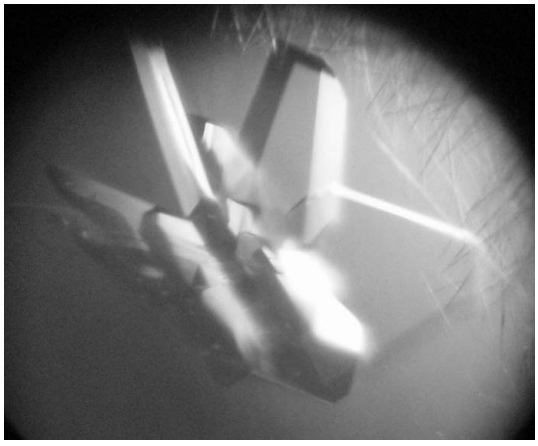

Figure 2
A crystal of the *Np*GluR0 LB core grown for 3 d using 0.1 *M* sodium citrate pH 5.0, 16%(*w*/*v*) PEG 8000, 5 m*M* L-glutamate and 5 m*M* imidazole. Its approximate dimensions are 0.3 × 0.2 × 0.1 mm. Before data collection, adjoined crystals were broken into smaller and clearer pieces.

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References

Armstrong, N. & Gouaux, E. (2000). *Neuron*, **28**, 165–181.
Armstrong, N., Sun, Y., Chen, G. Q. & Gouaux, E. (1998). *Nature (London)*, **395**, 913–917.
Brünger, A. T., Adams, P. D., Clore, G. M., DeLano, W. L., Gros, P., Grosse-Kunstleve, R. W., Jiang, J.-S., Kuszewski, J., Nilges, N., Pannu, N. S., Read, R. J., Rice, L. M., Simonson, T. & Warren, G. L. (1998). *Acta Cryst.* D**54**, 905–921.
Chen, G. Q., Cui, C. H., Mayer, M. L. & Gouaux, E. (1999). *Nature (London)*, **402**, 817–821.
Chen, G. Q., Sun, Y., Jin, R. & Gouaux, E. (1998). *Protein Sci.* **7**, 2623–2630.
Dingledine, R., Borges, K., Bowie, D. & Traynelis, S. F., (1999). *Pharmacol. Rev.* **51** 7–61.
Furukawa, H. & Gouaux, E. (2003). *EMBO J.* **22**, 2873–2885.
Hogner, A., Greenwood, J. R., Liljefors, T., Lunn, M.-L., Egebjerg, J., Larsen, I. K., Gouaux, E. & Kastrup, J. S. (2003). *J. Med. Chem.* **46**, 214–221.
Hogner, A., Kastrup, J. S., Jin, R., Liljefors, T., Mayer, M. L., Egebjerg, J., Larsen, I. K. & Gouaux, E. (2002). *J. Mol. Biol.* **322**, 93–109.
Hollmann, M. & Heinemann, S. (1994). *Annu. Rev. Neurosci.* **17**, 31–108.
Jin, R., Horning, M., Mayer, M. L. & Gouaux, E. (2002). *Biochemistry*, **41**, 15635–15643.
Kasper, C., Lunn, M.-L., Liljefors, T., Gouaux, E., Egebjerg, J. & Kastrup, J. S. (2002). *FEBS Lett.* **531**, 173–178.
Kuner, T., Seeburg, P. H. & Guy, H. R. (2003). *Trends Neurosci.* **26**, 27–32.
Laube, B., Kuhse, J. & Betz, H. (1998). *Neuroscience*, **18**, 2954–2961.
Lunn, M.-L., Hogner, A., Stensbøl, T. B., Gouaux, E., Egebjerg, J. & Kastrup, J. S. (2003). *J. Med. Chem.* **46**, 872–875.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Mayer, M. L. (2005). *Neuron*, **45**, 539–552.
Mayer, M. L., Olson, R. & Gouaux, E. (2001). *J. Mol. Biol.* **311**, 815–836.
Mayer, M. L. & Westbrook, G. L. (1987). *Prog. Neurobiol.* **28**, 197–276.
Nanao, M. H., Green, T., Stern-Bach, Y., Heinemann, S. F. & Choe, S. (2005). *Proc. Natl Acad. Sci. USA*, **102**, 1708–1713.
Naur, P., Vestergaard, B., Skov, L. K., Egebjerg, J., Gajhede, M. & Kastrup, J. S. (2005). *FEBS Lett.* **579**, 1154–1160.
Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
Rosenmund, C., Stern-Bach, Y. & Stevens, C. F. (1998). *Science*, **280**, 1596–1599.
Sun, Y., Olson, R., Horning, M., Armstrong, N., Mayer, M. L. & Gouaux, E. (2002). *Nature (London)*, **417**, 245–253.
Thompson, J. D., Gibson, T. J., Plewniak, F., Jeanmougin, F. & Higgins, D. G. (1997). *Nucleic Acids Res.* **25**, 4876–4882.
Vagin, A. & Teplyakov, A. (1997). *J. Appl. Cryst.* **30**, 1022–1025.