Letter to the Editor: ^{1}H, ^{13}C and ^{15}N resonance assignments and secondary structure of the cytotoxic RNase 3 from oocytes of bullfrog *Rana catesbeiana*

Yuan-Chao Lou[a], Yun-Ru Pan[a], Yi-Hsuan Ho[b], You-Di Liao[a] & Chinpan Chen[a,*]

[a]*Institute of Biomedical Sciences, Academia Sinica, Taipei 115, Taiwan;* [b]*Department of chemistry, National Taiwan University, Taipei 106, Taiwan*

Biological context

Several cytotoxic ribonucleases with antitumor activity have been identified from frog *Rana catesbeiana*, *Rana pipiens* and *Rana japonica* (Rosenberg et al., 2001). They are composed of 104-111 amino acids linked with four disulfide bridges and belong to the RNase A superfamily. The availability of these frog ribonucleases would be helpful to the investigation of these enzymes as potential agents for tumor therapy. A representative frog ribonuclease that is undergoing clinical trials for cancer treatment is Onconase isolated from *Rana pipien* (Newton et al., 2001). In addition to possessing cytotoxicity, the frog ribonucleases possess different catalytic activities, base specificities and optimal pH values for their activities. Also, the frog ribonucleases all contain a pyroglutamate residue (Pyr) at the N-terminus that has been identified to be crucial in the catalytic and cytotoxic activities (Hsu et al., 2003). On the contrary, Pyr, also found at N-terminus of human RNase 4 and RNase 5 (angiogenin), is not involved in catalytic activity (Terzyan et al., 1999). For NMR structural study, it is therefore prerequisite and interesting to develop a method for preparing the frog ribonuclease with the N-terminal Pyr to elucidate the structural role that pyr[1] plays as well as its three-dimensional structure. RC-RNase 3 is one of cytotoxic ribonucleases isolated from *Rana catesbeiana* and is composed of 105 residues with the pI value of 8.97. Among all known ribonucleases from *Rana catesbeiana*, RC-RNase 3 exhibits two unique properties. One is that RC-RNase 3 has base preference of CpU instead of CpG or UpG observed for other frog ribonucleases, and the other is that it has the lowest optimal pH of 5.0 for catalytic activity. Previously, we have reported the NMR resonances of the recombinant RC-RNase 2, RC-RNase 4 and RC-RNase L1 which all contain an extra Met residue at the N-terminus. To further gain insight into the structure/function relationship for the frog cytotoxic ribonucleases, in this work, we apply a new method, as explained in the next paragraph, to express the recombinant ^{13}C/^{15}N-labeled RC-RNase 3 with a Pyr at N-terminus and report the complete resonance assignments as well as secondary structure of this recombinant RC-RNase 3.

Methods and experiments

The gene encoding RC-RNase 3 was 5′ tagged with a *Nco*I-containing sequence CCATGGCT that encodes Met-Ala-amino acid residues, and fused with a modified *pel*B signal peptide sequence. It was then inserted into the pET11d expression vector through *Nde*I and *Bam*HI site (Huang et al., 1998). The Met-Ala-included signal peptide was removed *in vivo* in *Escherichia coli* before secreting into culture media. The gene was expressed in *E. coli* BL21(DE3) strain at 37 °C for 15 h, and then induced with IPTG for 8 h. After refolding, the uniformly ^{15}N- and/or ^{13}C-labeled proteins were purified by CM52 and Mono S column chromatographies. The authenticity of RC-RNase 3 sample was verified by SDS-PAGE and ES/MS analysis. Approximately 5 mg of the recombinant ^{13}C/^{15}N-labeled RC-RNase 3 was purified from one liter culture. All NMR expreriments were performed on a Bruker AVANCE 600 spectrometer equipped with a triple (^{1}H, ^{13}C and ^{15}N) resonance probe including shielded z-gradient. NMR samples were prepared in 50 mM phosphate buffer in 90% H_2O/10% D_2O at pH 3.5 and contained 0.3 ml of 1.5 mM protein in a Shigemi NMR tube (Allison Park. PA, USA). For the native RC-RNase 3, 2D ^{1}H NMR spectra (COSY, TOCSY and NOESY) were collected.

*To whom correspondence should be addressed. E-mail: bmchinp@ccvax.sinica.edu.tw
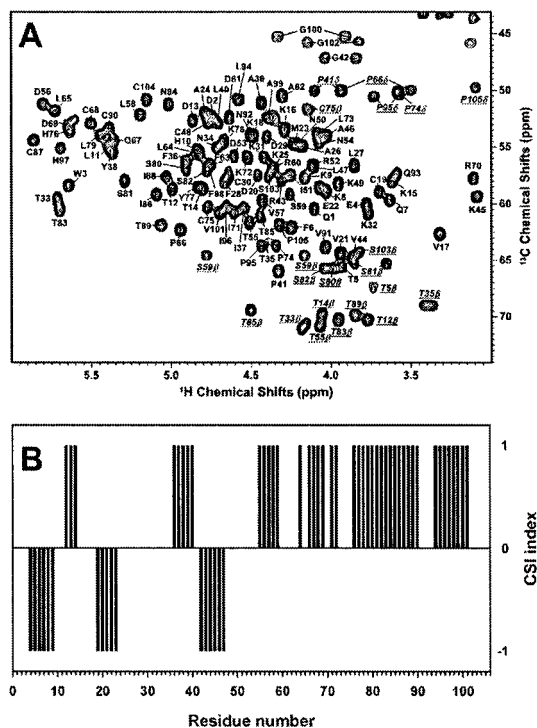
Figure 1. (A) A representative region of 2D ^{1}H-^{13}C HSQC spectrum shows C$^\alpha$/H$^\alpha$, C$^\beta$/H$^\beta$ (Ser and Thr), and C$^\delta$/H$^\delta$ (Pro) correlations for RC-RNase 3 recorded at 310 K and pH 3.5. The assignments are indicated with the one-letter amino acid code and residue number. For clarity, the correlations of C$^\beta$/H$^\beta$ and C$^\delta$/H$^\delta$ are shown with italic characters and underline. (B) The CSI consensus plot generated on the basis of ^{1}H$^\alpha$, ^{13}C$^\alpha$, ^{13}C$^\beta$ and ^{13}C$'$ chemical shifts clearly displays that RC-RNase 3 contains three α-helices and six β-strands secondary structures.

All heteronuclear NMR experiments were carried out as described in review articles (Clore and Gronenborn, 1994; Kay, 1995). All spectra were processed using XWIN-NMR and analyzed using AURELIA on SGI O$_2$ workstations. Linear prediction was used in the ^{13}C and ^{15}N dimensions to improve the digital resolution. Sequence-specific assignment of the backbone atoms was achieved by the independent connectivity analysis of CBCA(CO)NH, HNCACB, HNCO, HN(CA)CO and C(CO)NH. The ^{1}H resonances were assigned using TOCSY-HSQC, HAHB(CO)NH, HCCH-TOCSY and HC(CO)NH. A combined information from 2D ^{1}H-^{15}N HSQC and 3D NOESY-HSQC experiments yielded assignments for side-chain amide resonances of the Asn and Gln residues. Aromatic resonances were assigned using 2D ^{1}H-^{13}C HSQC, 2D NOESY and 2D TOCSY data.

Extent of assignments and data deposition

To demonstrate the high quality of NMR data, a representative region of 2D ^{1}H-^{13}C HSQC spectrum is shown in Figure 1A instead of a routine 2D ^{1}H-^{15}N spectrum. We have assigned all resonances of the backbone nuclei (^{1}H^N, ^{15}N, ^{13}C$^\alpha$, ^{1}H$^\alpha$ and ^{13}C$'$), with the exception of the H^N of Phe[98], and over 98% of the side-chain resonances. The functionally important residue, pyr[1], was unambiguously assigned, and its mobile ^{1}H^N was assigned at 8.38 ppm. The assignment of Pyr[1] may provide a clear view for how Pyr[1] structurally affects the biological functions. The side-chain NH$_2$ resonances of Gln[7] and Asn[34] were found to have unusual upfield chemical shifts at 6.19, 4.47 and 6.31, 3.68 ppm, respectively. Four distinct resonances were found for the aromatic protons of Tyr[77].

The consensus chemical shift index (CSI) plot shown in Figure 1B (Wishart and Sykes, 1994) reveals that RC-RNase 3 consists of 3 α-helices and 6 β-strands. The ^{1}H, ^{13}C and ^{15}N chemical shifts at 310 K and pH 3.5 have been deposited in the BioMagResBank (htto://www.bmrb.wisc.edu) under BMRB accession number 5787.

References

Clore, GM. and Gronenborn, A.M. (1994) *Meth. Enzymol.*, **239**, 349–363.

Hsu, C.H., Liao, Y.D., Pan, Y.R., Chen, L.W., Wu, S.H., Leu, Y.J. and Chen, C. (2003) *J. Mol. Biol.*, **326**, 1189–1201.

Huang, H.C., Wang, S.C., Leu, Y.J., Lu, S.C. and Liao, Y.D. (1998) *J. Biol. Chem.*, **273**, 6365–6401.

Kay, L.E. (1995) *Prog. Biophys. Mol. Biol.*, **63**, 277–299.

Newton, D.L., Hansen, H.J., Mikulski, S.M., Goldenberg, D.M. and Rybak, S.M. (2001) *Blood*, **97**, 528–535.

Rosenberg, H.F., Zhang, J., Liao, Y.D. and Kyer, K.D. (2001) *J. Mol. Evol.*, **53**, 31–38.

Terzyan, S.S., Peracaula, R., de Llorens, R., Tsushima, Y., Yamada, H., Seno, M., Gomis-Ruth, F.X. and Coll, M. (1999) *J. Mol. Biol.*, **285**, 205–214.

Wishart, D.S. and Sykes, B.D. (1994) *Meth. Enzymol.* **239**, 363–392.