

Preliminary X-ray diffraction studies of the external functional unit Rth2-e from the *Rapana thomasiana*M. Perbandt,^a V. Chandra,^b
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The 'external' oxygenated functional unit Rth2-e of the *Rapana* hemocyanin subunit RHSS2 was isolated and crystallized. X-ray intensity data to 3.3 Å resolution have been collected at 100 K and the structure has been solved using the molecular-replacement method. The space group is assigned to be the tetragonal $P4_32_12$, with unit-cell parameters $a = b = 105.5$, $c = 375.0$ Å.

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1. Introduction

Hemocyanins (Hcs) are respiratory proteins freely dissolved in the hemolymph of invertebrates of two phyla: Arthropoda and Mollusca. They bind dioxygen reversibly and transport it from the environment to the tissues. Hcs perform the same biological function as hemoglobins but differ in the active site, the length of the polypeptide chains and structural organization. Although similar in function, arthropodan and molluscan Hcs are completely different in molecular architecture. The proteins of the first group are hexamers (1×6) or multiples of hexamers ($n \times 6$, $n = 2, 4, 6, 8$) of approximately 75 kDa structural subunits. Each subunit represents a single polypeptide chain with one dioxygen-binding dinuclear copper-containing active site (Herskovits, 1988). It has been shown that the tertiary fold of the *Panulirus interruptus* and *Limulus polyphemus* Hc subunits is conserved and this is probably valid for all arthropodan Hcs (Volbeda & Hol, 1989; Hazes *et al.*, 1993). Molluscan Hcs are more complex proteins with molecular masses ranging from 3.6×10^6 (cephalopodan Hcs) to 43.4×10^6 Da (gastropodan aggregates). They form decameric or multi-decameric cylindrical aggregates with a diameter of approximately 300–350 Å and different lengths. The basic building block is a cylinder made up of ten 350–450 kDa structural subunits. Each subunit has seven (cephalopods) or eight (gastropods and some cephalopods) 50–55 kDa functional units (FUs) (Herskovits & Hamilton, 1991). Recently, cryoelectron microscopy studies of *Vampyroteuthis infernalis* and *Benthoctopus* cephalopod hemocyanins have been reported describing the subunit arrangement at 21 Å resolution (Mouche *et al.*, 1999). In principle, most of the FUs form the wall of the cylindrical aggregates and the rest, which can be classified as 'internal' units (e.g. the C-terminal FU of

the *Octopus* Hc), are folded inside the cylinders forming an 'arch' (Lambert *et al.*, 1994; Lambert, Boisset *et al.*, 1995; Lambert, Taveau *et al.*, 1995). The first complete amino-acid sequence of a molluscan Hc subunit, that from *Octopus dofleini* (cephalopod), has been reported (Miller *et al.*, 1998). Also, the crystal structure of the C-terminal FU of the same Hc, Odc, which is an 'internal' unit from the 'arch', has been analyzed (Cuff *et al.*, 1998).

Molluscan Hcs are powerful antigens probably owing to their high carbohydrate content and specific monosaccharide composition (Stoeva *et al.*, 1999). Keyhole-limpet (gastropod) Hc is used in laboratories and clinics as an immune stimulant (Jennemann *et al.*, 1994) and in the immunotherapy of bladder cancer (Lamm *et al.*, 1993) and renal cell carcinoma (Bichler *et al.*, 1990). The Hc of *R. thomasiana* is a glycoprotein with a carbohydrate content of 8.9% (Stoeva *et al.*, 1995). The Hc aggregates are composed of two structural subunits, RHSS1 and RHSS2 (Idakieva *et al.*, 1993). We have analysed and compared the sequence with those of other molluscan hemocyanin FUs of type e (Idakieva *et al.*, 2000; Perbandt *et al.*, 2001). In contrast to the 'internal' molluscan (cephalopod) hemocyanin functional units, the crystallographic structure of an 'external' unit or that of gastropod hemocyanin functional unit has not yet been analyzed. It has also been shown (Lamy *et al.*, 1998) that the decameric forms (the whole molecule of cephalopodan Hc and the half-molecule of gastropodan Hc) have different symmetries. This adds importance to the determination and refinement of the three-dimensional structure of the Rth2-e unit from the Hc cylinder wall. The structure will furthermore allow a detailed comparison of the tertiary structures of cephalopodan and gastropodan Hc FUs. Here, we report the preliminary X-ray results of the 'external' (from the wall of the Hc cylinders) FU Rth2-e of the *R. thomasiana* Hc subunit RHSS2.

2. Material and methods

2.1. Isolation of the *R. thomasiana* hemocyanin, the structural subunit RHSS2 and the functional unit Rth2-e

Marine snails, *R. thomasiana* grosse, were caught near to the Bulgarian coast of the Black Sea. Hemolymph was collected from specimens of 20–35 g. Hemocyanin was isolated by preparative ultracentrifugation as described before by Boteva *et al.* (1991) and the two subunits, RHSS1 and RHSS2, were isolated by the procedure specified in Idakieva *et al.* (1993). The order of FUs within the structural subunit was determined by comparison of the N-terminal sequences of RHSS2. Multiunit fragments as well as individual FUs were obtained through limited proteolysis by trypsin, endopeptidase Glu-C and plasmin, separated and purified by FPLC. All fragments and FUs were characterized by SDS-PAGE as

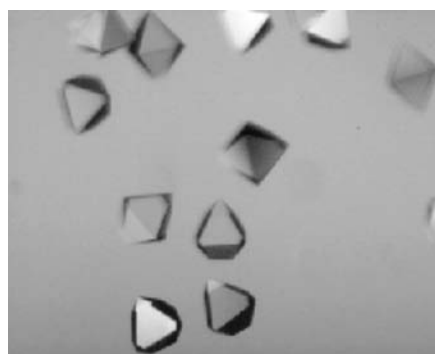


Figure 1
Bipyramidal crystals of the *R. thomasiana* hemocyanin functional unit R2e. The size of the crystals is approximately $0.2 \times 0.15 \times 0.2$ mm.

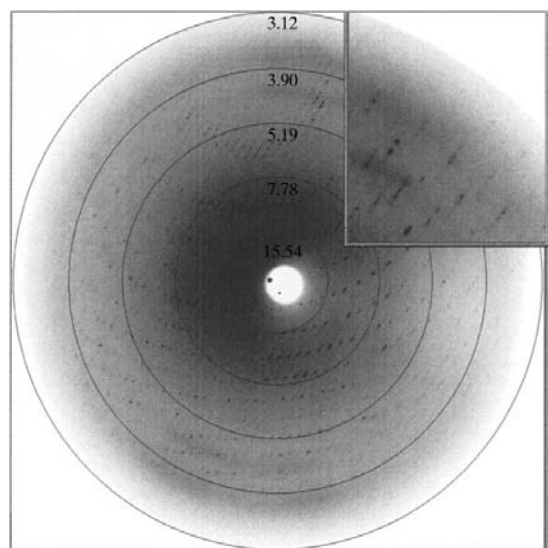


Figure 2
Diffraction pattern indicating the long cell axis.

described by Laemmli (1970). Comparison of the sequences of the whole subunit, multiunit fragments and FUs allowed determination of the order of FUs within the structural subunit (Gebauer *et al.*, 1999). The units were labeled 'a' to 'h' sequentially from the amino terminus. The 'external' FU Rth2-e from the Hc cylinder wall is a 50 kDa polypeptide chain. The protein used for crystallization experiments was homogeneous by SDS-PAGE as well as sequence analysis.

2.2. Crystallization and X-ray data collection

Crystals of the *Rapana* Hc 'external' FU Rth2-e were obtained within 6 d at room temperature by the hanging-drop vapour-diffusion method. Crystals suitable for X-ray analysis have a bipyramidal form with dimensions of about $0.2 \times 0.15 \times 0.2$ mm (Fig. 1). A purified sample of the protein was dissolved in water to an initial protein concentration of 20 mg ml^{-1} . The protein solution was mixed in a ratio of 1:1 with reservoir solution containing 0.2 M magnesium formate and 20 mM Tris-HCl buffer pH 7.8. Crystals were characterized and cryoconditions were optimized using synchrotron radiation at ELETTRA, Trieste and DESY/HASYLAB, Hamburg. Diffraction data to 3.3 Å were collected at 100 K at the National Synchrotron Light Source (BNL) beamline X9B (Fig. 2). The diffraction data were processed using *DENZO* and *SCALEPACK* (Otwinowski & Minor, 1997). Data-collection statistics are summarized in Table 1.

3. Results

The space group was finally assigned to be $P4_32_12$, with three molecules in the asymmetric unit. The packing parameter V_M was $3.1 \text{ Å}^3 \text{ Da}^{-1}$ (Matthews, 1968), with a solvent content of about 60%. The three molecules have been located by molecular-replacement methods using the program *AMoRe* (Navaza, 1997) with the coordinates of the 'internal' C-terminal FU Ogd from the cephalopod *Octopus dofleini* (Cuff *et al.*, 1998) as a search model. The correlation coefficient was 47.3%. The preliminary solution was confirmed by a primary rigid-body refinement procedure which reduced the *R* factor from

Table 1

Crystal parameters and data-collection statistics.

Values in parentheses are for the last resolution shell.	
Space group	$P4_32_12$
Unit-cell parameters (Å)	$a = b = 105.5,$ $c = 375.0$
V_M (Å ³ Da ⁻¹)	3.1
Resolution range (Å)	30.0–3.3
X-ray source	NSLS/X9B
Wavelength (Å)	0.98
No. of observations	792618
No. of unique reflections	33066
R_{sym} (%)	10.8 (37.3)
Average $I/\sigma(I)$	8.9 (2.1)
Completeness (%)	99.8 (97.8)

an initial value of 47.2% to 39.9% and R_{free} to 47.9% for all data in the resolution range 10.0–3.4 Å. It is an appealing fact that some kind of molecular self-assembly is evident from the crystal packing and symmetry. In the asymmetric unit the monomers interact *via* a 'head-to-tail' mode and assemble as a trimer which is stabilized by extensive intermolecular interactions. However, there is no obvious and clear symmetry between the monomers, as also earlier evidenced by a featureless self-rotation map. The individual trimers are further associated by the twofold crystallographic screw axis and arrange regular hexameric cylinders, the repeating structural element in the crystal.

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References

- Bichler, K.-H., Kleinknecht, S. & Strohmaier, W. L. (1990). *Urol. Int.* **45**, 269–283.
- Boteva, R., Severov, S., Genov, N., Beltramini, M., Filippi, B., Ricchelli, F., Tallandini, L., Pallhuber, M., Tognon, G. & Salvato, B. (1991). *Comp. Biochem. Physiol. B.* **100**, 493–501.

- Cuff, M. E., Miller, K. I., van Holde, K. E. & Hendrickson, W. A. (1998). *J. Mol. Biol.* **278**, 855–870.
- Gebauer, W., Stoeva, S., Voelter, W., Dianese, E., Salvato, B., Beltramini, M. & Markl, J. (1999). *Arch. Biochem. Biophys.* **372**, 128–134.
- Hazes, B., Magnus, K. A., Bonaventura, C., Bonaventura, J., Dauter, Z., Kalk, K. H. & Hol, W. G. J. (1993). *Protein Sci.* **2**, 597–619.
- Herskovits, T. T. (1988). *Comp. Biochem. Physiol. B*, **91**, 597–611.
- Herskovits, T. T. & Hamilton, M. G. (1991). *Comp. Biochem. Physiol. B*, **99**, 19–34.
- Idakieva, K., Severov, S., Svendsen, I., Genov, N., Stoeva, S., Beltramini, M., Tognon, G., Di Muro, P. & Salvato, B. (1993). *Comp. Biochem. Physiol. B*, **106**, 53–59.
- Idakieva, K., Stoeva, S., Pervanova, K., Genov, N. & Voelter, W. (2000). *Biochim. Biophys. Acta*, **1479**, 175–184.
- Jennemann, R., Gnewuch, C., Bosslet, S., Bauer, B. L. & Wienadt, H. (1994). *J. Biochem. (Tokyo)*, **115**, 1047–1052.
- Laemmli, U. K. (1970). *Nature (London)*, **227**, 680–685.
- Lambert, O., Boisset, N., Penczek, P., Lamy, J., Taveau, J.-C., Frank, J. & Lamy, J. N. (1994). *J. Mol. Biol.* **238**, 75–87.
- Lambert, O., Boisset, N., Taveau, J.-C., Preaux, G. & Lamy, J. N. (1995). *J. Mol. Biol.* **248**, 431–448.
- Lambert, O., Taveau, J.-C., Boisset, N. & Lamy, J. N. (1995). *Arch. Biochem. Biophys.* **319**, 231–243.
- Lamm, D. L., DeHaven, J. I., Riggs, D. R., Delgra, C. & Burrell, R. (1993). *Urol. Res.* **21**, 33–37.
- Lamy, J., You, V., Taveau, J.-C., Boisset, N. & Lamy, J. N. (1998). *J. Mol. Biol.* **284**, 1051–1074.
- Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
- Miller, K. I., Cuff, M. E., Lang, W. F., Varga-Weisz, P., Field, K. G. & van Holde, K. E. (1998). *J. Mol. Biol.* **278**, 827–842.
- Mouche, F., Boisset, N., Lamy, J., Zal, F. & Lamy, J. N. (1999). *J. Struct. Biol.* **127**, 199–212.
- Navaza, J. (1997). *Acta Cryst. A* **43**, 645–653.
- Otwinowski, Z. & Minor, W. (1997). *Methods Enzymol.* **276**, 307–326.
- Perbandt, M., Chandra, V., Idakieva, K., Pervanova, K., Rypniewski, W., Stoeva, S., Voelter, W., Genov, N. & Betzel, Ch. (2001). In the press.
- Stoeva, S., Rachev, R., Severov, S., Voelter, W. & Genov, N. (1995). *Comp. Biochem. Physiol. B*, **110**, 761–765.
- Stoeva, S., Schütz, J., Gebauer, W., Hundsdörfer, T., Manz, C., Markl, J. & Voelter, W. (1999). *Biochim. Biophys. Acta*, **1345**, 94–109.
- Volbeda, A. & Hol, W. G. J. (1989). *J. Mol. Biol.* **209**, 249–279.