The calcium, copper and zinc content of some annelid extracellular haemoglobins

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The extracellular haemoglobins of *Lumbricus* and *Tylorrhynchus* contain 50 and 61 tightly bound calcium atoms per molecule, respectively. In addition, they contain one to four tightly bound copper and zinc atoms. Although the role of the latter is unknown, that of calcium is probably structural, assisting in the maintenance of the native hexagonal bilayer structure.

INTRODUCTION

The annelid extracellular haemoglobins and chlorocruorins represent one of four groups of extracellular invertebrate haemoglobins classified according to their quaternary structure (Vinogradov, 1985); they exhibit a characteristic hexagonal bilayer shape in electron micrographs and have a low iron content of approx. 0.24% (w/w). The haemoglobins of the oligochaete *Lumbricus terrestris* and of the polychaete *Tylorrhynchus heterochaetus* appears to have identical dimensions by scanning transmission electron microscopy, 30 nm × 20 nm (Vinogradov *et al.*, 1982). Both consist of about 200 polypeptide chains with substantial amino acid sequence homology between the two molecules (Suzuki & Gotoh, 1986a,b; Gotoh *et al.*, 1986). It is known that the haemoglobins of *Tubifex* and of another oligochaete, *Octalasion complanatum*, contain substantial amounts of calcium (Rokosz & Vinogradov, 1982; Chiancone *et al.*, 1976) and that the dissociation at alkaline pH of several annelid extracellular haemoglobins and chlorocruorins is decreased by the presence of Ca(II) and Mg(II) ions (Chung & Ellerton, 1979; Vinogradov *et al.*, 1980). In a few cases the integrity of the native hexagonal bilayer structure at neutral pH is dependent on the presence of these ions: examples are the haemoglobin of *Amphitrite ornata* (Chiancone *et al.*, 1980) and the chlorocruorin of *Myxicola infundibulum* (Vinogradov *et al.*, 1985). In addition, in the case of *Lumbricus*, *Tubifex* and *Octalasion* haemoglobins, Ca(II) promotes re-association to the hexagonal bilayer structure at neutral pH subsequent to dissociation at alkaline pH (Kapp *et al.*, 1984; Polidori *et al.*, 1984; Chiancone *et al.*, 1982).

EXPERIMENTAL PROCEDURES

Live *Lumbricus terrestris* were obtained from Forest City Bait Farm (London, Ontario, Canada); the haemoglobin was prepared as described earlier (Shlom & Vinogradov, 1973). Live *Tubifex tubifex* were obtained from G. Schue Co. (Chino, CA, U.S.A.); the haemoglobin was prepared as described earlier (Vinogradov *et al.*, 1979). *Tylorrhynchus* haemoglobin was prepared as described elsewhere (Gotoh & Kamada, 1980). The haemoglobin solutions (10–60 mg·ml⁻¹) in distilled deionized water were dialysed against 10 mM-EDTA at

neutral pH. The concentrations of the *Lumbricus* and *Tubifex* haemoglobin solutions were determined spectrophotometrically using the known absorbances of the cyanmet derivatives at 540 nm: 0.435 (cm·mg/ml)⁻¹ (Shlom & Vinogradov, 1973) and 0.390 (cm·mg/ml)⁻¹ (Polidori *et al.*, 1984), respectively. The absorbance of *Tylorrhynchus* haemoglobin was assumed to be the same as that of *Lumbricus* haemoglobin. Metal analysis was carried out in a Varian model AA 1475 atomic absorption spectrophotometer with programmable sample changer and a DS-15 Hewlett Packard data collection system. The standards were from Fisher Scientific Co. and Banco-Andersen Co.

RESULTS AND DISCUSSION

The results presented in Table 1 show that the number of Ca(II) bound by one molecule of the annelid haemoglobin with an association constant higher than the equilibrium constant for the formation of the Ca(II)–EDTA complex at neutral pH, log *K'* = 7.49 (Portzehl *et al.*, 1964), varies between 40 and 65, in agreement with earlier results (Chiancone *et al.*, 1976; Rokosz & Vinogradov, 1982). The discrepancy between the two values for the calcium content of *Tubifex* haemoglobin could be due to the fact that the worms were from different sources: from Michigan in the earlier study and from Mexico in the present one. The substantial content of calcium is consonant with a structural role for this metal favouring the native, hexagonal bilayer structure.

The haemoglobin of *Lumbricus* is known to consist of at least two types of polypeptide chains: four haem-containing chains of approx. 17 kDa and at least three chains of approx. 35 kDa which contain very little or no haem (Mainwaring *et al.*, 1986; Walz *et al.*, 1987). *Tubifex* haemoglobin is very similar to *Lumbricus* haemoglobin (Vinogradov *et al.*, 1979; Polidori *et al.*, 1984). We have recently proposed a 'bracelet' model of annelid haemoglobins, in which the chains of approx. 35 kDa form a circular scaffolding, or 'bracelet', decorated with twelve complexes of the remaining, haem-containing polypeptide chains (Vinogradov *et al.*, 1986). It is tempting to speculate that the calcium is involved in assisting the 35 kDa subunits to act as 'linkers', i.e. in the formation of the 'bracelet' structure.

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Table 1. Metal content of annelid haemoglobins

Samples were dialysed exhaustively against 10 mM-EDTA at neutral pH. The number of metal atoms per molecule was calculated assuming a molecular mass of 3.8×10^6 (Vinogradov *et al.*, 1977, 1980).

Species	Content (atoms/molecule)		
	Ca	Cu	Zn
<i>Lumbricus</i>	49.5	0.72	1.5
<i>Tylorrhynchus</i>	61	3.9	1.0
<i>Tubifex</i>	41 (65)*	—	—
<i>Octalasmus</i>	46 ± 4†	—	—

*Determined from X-ray fluorescence measurements of haemoglobin from *Tubifex* collected in the Rouge River (Dearborn, MI, U.S.A.) (Rokosz & Vinogradov, 1982).

† Calculated from one calcium per 3.2–3.8 haem groups, obtained from n.m.r. quadrupole relaxation measurements (Chiancone *et al.*, 1976) and 160 haem groups, obtained from the known iron content, 0.24% (w/w), and a molecular mass of 3.8×10^6 .

The content of copper and zinc in all three haemoglobins is very low, i.e. one to four atoms per molecule of $M_r 3.8 \times 10^6$, and the role, if any, of these metals remains to be discovered. It is possible that among the 200 or more polypeptide chains constituting these giant haemoglobin molecules, there are one or more chains containing copper and/or zinc which have an enzymic function. Another possibility is that these represent a contamination by a very minor protein present in the plasma of the annelids. We are presently attempting to determine which of the subunits of the haemoglobins bind the calcium, copper and zinc.

This research was partially supported by United States Public Health Service grant HL/DK 38674.

REFERENCES

Chiancone, E., Bull, T. E., Norne, J. E., Forsen, S. & Antonini, E. (1976) *J. Mol. Biol.* **107**, 25–34

- Chiancone, E., Brenowitz, M., Ascoli, F., Bonaventura, C. & Bonaventura, J. (1980) *Biochim. Biophys. Acta* **623**, 146–162
- Chiancone, E., Vecchini, P., Ascoli, F. & Antonini, E. (1982) *Life Chem. Rep. Suppl.* **1**, 205–209
- Chung, M. C. M. & Ellerton, H. D. (1979) *Prog. Biophys. Mol. Biol.* **35**, 53–102
- Gotoh, T. & Kamada, Y. (1980) *J. Biochem. (Tokyo)* **87**, 557–562
- Gotoh, T., Shishikura, F., Snow, J. W., Erefej, K. I., Vinogradov, S. N. & Walz, D. A. (1986) *Biochem. J.* **241**, 441–445
- Kapp, O. H., Polidori, G., Mainwaring, M. G., Crewe, A. V. & Vinogradov, S. N. (1984) *J. Biol. Chem.* **259**, 628–639
- Mainwaring, M. G., Lugo, S., Fingal, R., Kapp, O. H. & Vinogradov, S. N. (1986) *J. Biol. Chem.* **261**, 10899–10908
- Polidori, G., Mainwaring, M. G., Kosinski, T. F., Schwarz, C., Fingal, R. & Vinogradov, S. N. (1984) *Arch. Biochem. Biophys.* **233**, 800–814
- Portzehl, H., Caldwell, P. C. & Ruegg, J. C. (1964) *Biochim. Biophys. Acta* **79**, 581–591
- Rokosz, M. J. & Vinogradov, S. N. (1982) *Biochim. Biophys. Acta* **707**, 291–293
- Shlom, J. M. & Vinogradov, S. N. (1973) *J. Biol. Chem.* **248**, 7904–7912
- Suzuki, T. & Gotoh, T. (1986a) *J. Biol. Chem.* **261**, 9257–9267
- Suzuki, T. & Gotoh, T. (1986b) *J. Mol. Biol.* **190**, 119–123
- Vinogradov, S. N., Shlom, J. M., Hall, B. C., Kapp, O. H. & Mizukami, H. (1977) *Biochim. Biophys. Acta* **492**, 136–155
- Vinogradov, S. N. (1985) *Comp. Biochem. Physiol.* **82B**, 1–15
- Vinogradov, S. N., Hersey, S. L., Frohman, C. & Kapp, O. H. (1979) *Biochim. Biophys. Acta* **578**, 216–222
- Vinogradov, S. N., Shlom, J. M., Kapp, O. H. & Frossard, P. (1980) *Comp. Biochem. Physiol.* **67B**, 1–12
- Vinogradov, S. N., Kapp, O. H. & Ohtsuki, M. (1982) in *Electron Microscopy of Proteins* (Harris, J. R., ed.), vol. 3, pp. 135–164, Academic Press, London
- Vinogradov, S. N., Standley, P. R., Mainwaring, M. G., Kapp, O. H. & Crewe, A. V. (1985) *Biochim. Biophys. Acta* **828**, 43–50
- Vinogradov, S. N., Lugo, S. D., Mainwaring, M. G., Kapp, O. H. & Crewe, A. V. (1986) *Proc. Natl. Acad. Sci. U.S.A.* **83**, 8034–8038
- Walz, D. A., Snow, J. W., Mainwaring, M. G. & Vinogradov, S. N. (1987) *Fed. Proc. Fed. Am. Soc. Exp. Biol.* **46**, 2266

Received 13 August 1987/4 November 1987; accepted 20 November 1987