Oxygen binding by *Helix pomatia* α -haemocyanin studied by X-ray-absorption spectroscopy

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The X-ray absorption spectra of haemocyanin from *Helix pomatia* were obtained by using X-rays from synchrotron radiation. Cu *K*-edges were recorded at four conditions, namely fully oxygenated, 85% oxygenated, 12% oxygenated and fully deoxygenated. The percentage oxygenation calculated from the edge-shift of the partially oxygenated samples did not agree with the percentage oxygenation as determined by u.v. measurements. Two intermediates in the oxygenation process are presented to explain the observed dissimilarities.

Oxygen transport in several arthropods and molluscs is accomplished by the protein haemocyanin. This huge molecule contains copper, two copper atoms per oxygen molecule bound. Haemocyanin from the mollusc *Helix pomatia* consists of 20 very large polypeptide chains (Siezen & van Driel, 1974). These chains are composed of eight structural domains, each bearing one oxygen-binding site (Gielens *et al.*, 1977; Torensma *et al.*, 1980). So, 160 molecules of oxygen can be bound by one *Helix pomatia* haemocyanin molecule, with a molecular weight of 9×10^6 . The transport efficiency is greatly increased by interaction between the 160 binding sites forming the native molecule. These interactions can be divided into homotropic and heterotropic interactions. The former represent the interactions between identical ligands; this is known as co-operativity. The latter represent interactions between different ligands, e.g. the influence of protons on the oxygen affinity (Monod *et al.*, 1965). Positive co-operativity is observed for α -haemocyanin from *Helix pomatia* at pH 8.2 in the presence of 10 mM-CaCl₂. Oxygen-binding equilibria obtained under these conditions when plotted logarithmically show the following three features. (1) At low saturation oxygen binds with relatively low affinity; the slope in this region is unity. (2) At

medium saturation the slope of the binding curve is increased to about 4. (3) At high saturation oxygen binds with high affinity as compared with the affinity at low saturation; the slope of the binding curve in this region is 1. This behaviour has now been analysed within the framework of a two-state model (Colosimo *et al.*, 1977).

Important features of the model are as follows [complete description is published elsewhere (Monod *et al.*, 1965)]. The model postulates two states that are in equilibrium with each other. Co-operativity arises by a change in the equilibrium constant. The low-affinity state is called T(ense)-state and the high affinity state is called R(elaxed)-state. Both states are indicated in Fig. 1. Deoxygenated haemocyanin is almost completely in the T-state and oxygenated haemocyanin is almost completely in the R-state.

X-ray-absorption spectroscopy is a probe of the local environment of a metal atom in a complex environment of other atoms. The application of this technique to metalloproteins has been reviewed by Cramer & Hodgson (1979). Investigation of completely deoxygenated and oxygenated haemocyanin with this technique resulted in a description of the structure of the deoxy and oxy forms of mollusc haemocyanin (see Fig. 3) (Eccles, 1977; Brown *et al.*, 1980; Co *et al.*, 1981; Co & Hodgson, 1981). We have previously reported identical spectra for the oxy forms of mollusc and arthropod haemocyanins (Torensma & Phillips, 1981). This present paper deals with unexpected results of the analysis of the absorption edge structure not only from deoxy and oxy forms of

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haemocyanin but also of edges obtained at intermediate oxygenation values.

Experimental

Helix pomatia α -haemocyanin was isolated by the procedure of Heirwegh *et al.* (1961). Protein was regenerated before use as described by Lontie & Witters (1966). The percentage oxygenation at a given oxygen pressure was estimated by measuring absorbance changes at 346 nm, by the method of Konings *et al.* (1969). Concentrated protein solutions (30 mg/ml) were dialysed against several changes of 0.1 M-Tris/HCl buffer, pH 8.2, containing 10 mM-CaCl₂. Samples were equilibrated to the chosen oxygen pressure and placed in an air-tight box having the same partial oxygen pressure as the sample. The sample was transferred to a centrifuge tube, which was sealed and placed in the rotor. The box was opened and the sample was pelleted by centrifuging at 100 000 *g* for 4 h. After the run was ended the rotor was placed in the box. After the chosen oxygen pressure had been reached in the box, the rotor was opened and the pelleted protein was transferred to the measuring cell. The sample was stored in a glass vessel with the same partial oxygen pressure as the sample until use.

X-ray-absorption spectra were recorded at the European Molecular Biology Laboratory Outstation, Hamburg, with the use of synchrotron radiation from the storage ring DORIS. Spectra for four *Helix pomatia* haemocyanin samples were obtained: (1) fully oxygenated, (2) 85% oxygenated, (3) 12% oxygenated and (4) fully deoxygenated. Spectra were calibrated in energy by comparison with a copper-foil spectrum. Individual scans were checked for beam dumps and edge-shifts before averaging. The spectra showed no changes in features with time that would have indicated problems with air leaking into the cell or radiation damage to the protein. The absorption edge spectra shown in Fig. 2 were extracted from the average raw spectra by fitting the pre-edge region with a fourth-order polynomial, extrapolating the fit through the edge region and then subtracting the fit from the original data. The edges were normalized to the same height before any comparison, as the data-collection method gives no absolute scale for the absorption coefficient. At oxygen pressures where only some of the sites are oxygenated, the resulting X-ray-absorption spectrum is composed of a mixture of the spectra of fully oxygenated and of fully deoxygenated sites. The contribution of each spectrum (oxy or deoxy form) to the spectrum at intermediate oxygen pressures was calculated by fitting to linear combinations of the spectra of the fully oxygenated and fully deoxygenated forms by a least-squares method. At conditions where a mixture

of deoxygenated and oxygenated sites exists, the resulting X-ray-absorption spectrum was calculated from linear combinations of the fully oxygenated and fully deoxygenated spectra.

Results and discussion

Fig. 1 shows the oxygen-binding behaviour of *Helix pomatia* α -haemocyanin at the same experimental conditions as those at which the X-ray-absorption spectra were recorded. The oxygen pressures at which the spectra were recorded are indicated in this Hill plot. Also, the low-affinity (T-)state and the high-affinity (R-)state are indicated. The Cu *K*-edge spectra of all four *Helix pomatia* samples are shown in Fig. 2. Also shown are spectra calculated by using the oxygenation fraction obtained from the u.v. measurements and the best least-squares fits for the partially oxygenated samples. The spectra of the fully oxygenated and fully deoxygenated forms agree with previously published spectra obtained with *Megathura crenulata* (Eccles, 1977; Co *et al.*, 1981; Co & Hodgson, 1981), *Helix pomatia* (Co *et al.*, 1981) and *Busycon canaliculatum* (Brown *et al.*, 1980). There is, however, a discrepancy between the values of oxygenation obtained by u.v.-absorption and by X-ray-absorption spectroscopy. The sample

Fig. 1. Hill plot of oxygen binding by *Helix pomatia* α -haemocyanin

The low-affinity (T-)state and the high-affinity (R-)state are indicated. The arrows indicate the oxygen pressure at which the partially oxygenated samples were prepared. Experimental conditions: 0.1 M-Tris/HCl buffer, pH 8.2, containing 10 mM-CaCl₂. pO_2 is the oxygen pressure; Y is the fractional saturation with oxygen.

Fig. 2. Cu K-edge absorption spectra for *Helix pomatia* α -haemocyanin

Measured spectra obtained at four experimental conditions; ·····, spectra calculated from a linear combination of the experimentally obtained Cu K-edge of fully oxygenated and fully deoxygenated haemocyanin. Curve *a*, fully deoxygenated; curve *b*, 12% oxygenated; curve *c*, 12% oxygenated (—) or 33% oxygenated (·····); curve *d*, 85% oxygenated (—) or 60% oxygenated (·····); curve *e*, 85% oxygenated; curve *f*, fully oxygenated.

thought to be 12% oxygenated from u.v. measurements appears to be 33% oxygenated by X-ray-absorption spectroscopy. Also, the 85%-oxygenated sample (by u.v.-absorption spectroscopy) appears to be 60% oxygenated by X-ray-absorption spectroscopy. The only other report available at the moment appeared to obtain a good comparison between the u.v.-determined oxygenation and that obtained by X-ray-absorption spectroscopy (Eccles, 1977). However, the percentage oxygenation (by u.v. measurements) in that report (Eccles, 1977) was extracted from a paper by Miller & Van Holde (1974), which paper described the oxygenation behaviour of *Callinassa californiensis*, a species having a different oxygenation behaviour from that of the *Megathura crenulata* species used by Eccles (1977), and moreover was concerned with an arthropod haemocyanin rather than a mollusc haemocyanin. More recently oxygen-binding data for the latter species have become available (Senozan *et al.*, 1981). These data show that under the conditions used by Eccles (1977) over 90% of the sites will be occupied with oxygen when the protein is exposed to air. Therefore there is also a discrepancy between X-ray-absorption and u.v.-absorption measurements in Eccles's (1977) work. The magnitude of these differences is larger than any random error in the calculation. Moreover, the pattern of the discrepancies ruled out explanations such as reduction of samples by the X-ray beam (Chance *et al.*, 1980) or oxygen leaking into the cell. Considering the enormous amount of equilibrium

oxygen-binding data, sustained by kinetics data, it seems implausible to postulate non-linearity between the amount of oxygen bound and the concomitant u.v.-spectral changes. Therefore it appears that the amount of oxygen bound is non-linear to the changes in the accompanying X-ray-absorption spectra. We therefore seek a model for the binding site changes during oxygenation that would explain this non-linearity.

From Fig. 1 it is seen that the 12%-oxygenated sample still is in the low-affinity state. Under these conditions there are, however, two kinds of binding sites in the sample, one having bound oxygen and the other being empty, both being in the low-affinity (T-)state. Also, for the 85%-oxygenated sample there exist two kinds of binding sites in the sample, again one occupied with oxygen and the other empty, both sites being in the high-affinity (R-)state. Our results would be explained if the empty binding sites and/or the occupied binding sites change structure during the transition between the T-state and the R-state. The possibility where both change is illustrated in Fig. 3. The empty binding sites in the low-affinity (T-)state possess the deoxy structure (Fig. 3*a*), when oxygen is bound to an empty site in this state, it remains in the low-affinity (T-)state and the binding site will have the structure denoted in Fig. 3(*b*). On switching to the high-affinity (R-)state, both the empty (Fig. 3*c*) and the occupied (Fig. 3*d*) sites change structure. It is evident that X-ray-absorption spectra taken at intermediate oxygenation values cannot be fitted with a linear

Fig. 3. Structures of the active site of *Helix pomatia* α -haemocyanin

(a) Deoxygenated low-affinity site; (b) oxygenated low-affinity site; (c) deoxygenated high-affinity site; (d) oxygenated high-affinity site. The structure shown in (a) is in accordance with Co & Hodgson (1981). The structure shown in (d) is in accordance with Co *et al.* (1981). In (b) the bond between copper and oxygen is shown as ----- to indicate that more possibilities exist.

combination of the two structures shown in Figs. 3(a) and 3(d). This would also be true if only the empty site or the occupied site changed structure, but this seems less likely. Support for our proposal of four instead of two structures comes from kinetic data. Temperature-jump experiments with *Octopus vulgaris* haemocyanin indicated the existence of two oxygenated structures (Brunori, 1971). In our terminology these oxygenated forms are represented by the structures shown in Figs. 3(b) and 3(d). Furthermore, carbon monoxide bound to haemocyanin gives an edge-shift (Eccles, 1977), though no change from T-state to R-state takes place (Bonaventura *et al.*, 1974; Kuiper *et al.*, 1976), proving that an edge-shift is possible without altering the gross structure of the binding site, as we suggest for the structures shown in Figs. 3(a) and 3(b). It may be recalled that oxygenation is linear with spectral changes in the u.v.-spectral region. This implies that there exist no gross differences between the u.v. absorption at 346 nm of the structures shown in Figs. 3(a) and 3(c) and also between the structures shown in Figs. 3(b) and 3(d).

Summarizing, the present study indicates the existence of four structures involved in the oxygen-binding process (Fig. 3). More experiments are called for to determine the two structures present during partial oxygenation. With more certain knowledge of the number of structures and the fraction of each that is present, it will be possible to isolate the spectra of the intermediate states. If this can be done in the e.x.f.a.s. (extended X-ray-absorption fine structure) region of the spectra (difficult because of signal-to-noise considerations), then the structures of the intermediate sites would be elucidated.

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References

- Bonaventura, C., Sullivan, B., Bonaventura, C. & Bourne, S. (1974) *Biochemistry* **13**, 4784–4789
- Brown, J. M., Powers, L., Kincaid, B., Larrabee, J. A. & Spiro, T. G. (1980) *J. Am. Chem. Soc.* **102**, 4210–4216
- Brunori, M. (1971) *J. Mol. Biol.* **55**, 39–48
- Chance, B., Angiolillo, P., Yang, E. K. & Powers, L. (1980) *FEBS Lett.* **112**, 178–182
- Co, M. S. & Hodgson, K. O. (1981) *J. Am. Chem. Soc.* **103**, 3200–3201
- Co, M. S., Hodgson, K. O., Eccles, T. K. & Lontie, R. (1981) *J. Am. Chem. Soc.* **103**, 984–986
- Colosimo, A., Brunori, M. & Wyman, J. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., ed.), pp. 189–192, Springer-Verlag, Berlin, Heidelberg and New York
- Cramer, S. P. & Hodgson, K. O. (1979) *Prog. Inorg. Chem.* **25**, 1–39
- Eccles, T. K. (1977) Ph.D. Thesis, Stanford University
- Gielens, C., Préaux, G. & Lontie, R. (1977) in *Structure and Function of Haemocyanin* (Bannister, J. V., ed.), pp. 85–94, Springer-Verlag, Berlin, Heidelberg and New York
- Heirwegh, K., Borginon, H. & Lontie, R. (1961) *Biochim. Biophys. Acta* **48**, 517–526
- Konings, W. N., van Driel, R., van Bruggen, E. F. J. & Gruber, M. (1969) *Biochim. Biophys. Acta* **194**, 55–66
- Kuiper, H. A., Torensma, R. & van Bruggen, E. F. J. (1976) *Eur. J. Biochem.* **68**, 425–430
- Lontie, R. & Witters, R. (1966) in *The Biochemistry of Copper* (Peisach, J., Aisen, P. & Blumberg, W. E., eds.), pp. 455–463, Academic Press, New York
- Miller, K. & Van Holde, K. E. (1974) *Biochemistry* **13**, 1668–1674
- Monod, J., Wyman, J. & Changeux, J. P. (1965) *J. Mol. Biol.* **12**, 88–118

- Senozan, N. M., Landrum, J., Bonaventura, J. & Bonaventura, C. (1981) in *Invertebrate Oxygen Binding Proteins* (Lamy, J. & Lamy, J., eds.), pp. 703–717, Marcel Dekker, New York
- Siezen, R. J. & van Driel, R. (1974) *J. Mol. Biol.* **90**, 91–102
- Torensma, R. & Phillips, J. C. (1981) *FEBS Lett.* **130**, 314–316
- Torensma, R., van der Laan, J. M., van Bruggen, E. F. J., Gielens, C., Paemel, L., Verschuieren, L. J. & Lontie, R. (1980) *FEBS Lett.* **115**, 213–215