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Crystallization of the haptoglobin–hemoglobin complex

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Two orthorhombic forms of crystals of the haptoglobin–hemoglobin complex were obtained using polyethylene glycol as precipitant. These crystals did not diffract well enough for data collection and work on the complex is no longer continued. However, the description of the crystallization conditions may be useful in future endeavors to obtain suitable crystals.

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1. Introduction

Haptoglobin (Hp) is a mammalian serum glycoprotein which forms a stable complex with hemoglobin (Hb) with 1:1 stoichiometry and a molecular weight ~166 kDa. The formation of the complex prevents renal damage and may play a role in Hb metabolism. Hp is also an important acute phase protein and was shown to have a number of biological activities (Dobryszycka, 1993). Langlois & Delanghe (1996) reported that Hp is associated with prevalence and clinical evolution of many inflammatory diseases and may be involved in the immune response.

Early attempts of crystallization of Hp and Hp–Hb (Waks *et al.*, 1968; Haupt & Heide, 1970) gave microcrystals. The crystals of the Hp–horse Hb complex obtained by Szilágyi (unpublished work), using dialysis methods with 2.1 *M* phosphate buffer pH 9.0 and 2.0 *M* calcium chloride buffered with TRIZMA pH 8.4–9.0, were 1–2 mm in length, but were unsatisfactory for data collection. They were strongly birefringent, but the pinacoid end faces were convex, indicating disorder. The crystals of Hp–horse Hb obtained from ammonium sulfate (Yang & Przybylska, 1973) were hexagonal, but too small for X-ray examination. Hwang (1979) obtained much larger hexagonal crystals of Hp–human Hb which diffracted to 7 Å and their asymmetric unit contained two molecules of the complex. The use of polyethylene glycol (PEG) as the precipitant was subsequently investigated.

2. Experimental

2.1. Preparation of hemoglobin

The red cells were washed and lysed by a modified method of Brown *et al.* (1976). The solution containing $K_3Fe(CN)_6$, NaCN and KH_2PO_4 was then added to Hb. The resultant cyanmet–Hb solution was checked spectrophotometrically for the absence of oxy-Hb and met-Hb. Subsequently, it was passed through Sephadex G-25 (medium) column equilibrated with 0.01 *M* sodium phosphate buffer pH 7.0.

2.2. Preparation of the Hp–Hb complex

Haptoglobin of 1–1, 1S–1S phenotype (Pastewka *et al.*, 1973) was obtained from the ascitic fluid of one patient. After centrifugation, the fluid was extensively dialysed in 0.01 *M* sodium acetate pH 5.0, recentrifuged and dialysed in 0.01 *M* sodium phosphate pH 7.0. A calculated amount of Hb was then added and the solution stirred slowly for up to 24 h at 277 K.

Seiler (1972) found that binding of Hb to Hp lowers the stability of the cyanmet–Hb bond and the concentration of cyanide required to keep Hb fully liganded is higher for the complex than for Hb.

The complex was isolated by using a DE 52 (pre-swollen, Whatman) column equilibrated with 0.01 *M* sodium phosphate buffer pH 7.0. The molarity of the buffer was increased gradually to 0.06 *M*, but prior to this increase the yellow albumin band was removed from the top of the column as soon as Hp–Hb had separated. The eluted complex solution was treated with sodium *p*-chloromercuribenzoate or sodium *p*-chloromercuribenzenesulphonate and stirred for several hours before passing it through a G-25 (medium) column equilibrated with 0.01 *M* sodium phosphate buffer pH 7.0.

The purity of the complex was ascertained by disc electrophoresis or isoelectrofocusing methods.

2.3. Crystallization of the complex

Crystals of form *A* shown in Fig. 1 were obtained using the hanging-drop method with seeding. To 5.6 µl of solution containing 3.6% Gly-L-Ser, 0.02 *M* sodium acetate buffer pH 4.9 and 0.02% chloretone, 1.4 µl of 3.2% Hp–Hb in 0.001 *M* sodium phosphate buffer pH 7.0 was added, followed by 3 µl of 20%(*w*/*v*) PEG 1500 (Sigma). The final concentrations of Hp–Hb and PEG were 0.45%(*w*/*v*) and 6%,

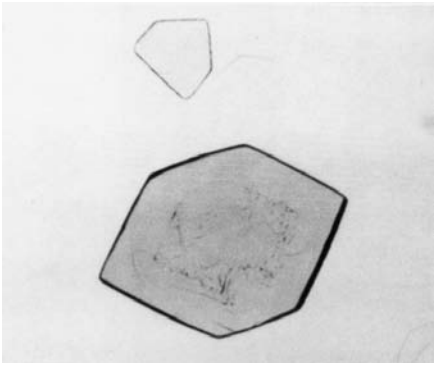

Figure 1
Orthorhombic crystal of the Hp–Hb complex, form *A*, obtained at pH 4.9 (length 0.4 mm).

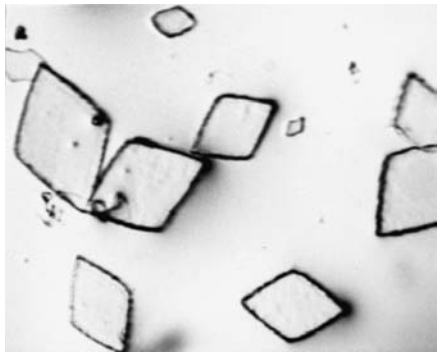

Figure 2
Orthorhombic crystals of the Hp–Hb complex, form *B*, obtained at pH 5.6 (lengths up to 0.2 mm).

respectively. The final pH was probably below the isoelectric point for Hp–Hb (5.1). The crystals appeared within hours.

Form *B* crystals (Fig. 2) were grown in bacteriological tubes using a batch method with similar concentrations of Hp–Hb, Gly-L-Ser and chloretone. The concentration of PEG 1500 was lower (4.3%). The Hp–Hb solution was in 1 m*M* sodium phosphate buffer pH 5.6 and no other buffer was added.

Tetragonal crystals were also obtained in similar trials, but using the complex solution dialysed in water and eliminating chloretone. The buffer was not added at all or only in very small amount (droplet: 1 m*M* sodium phosphate pH 5.8). This method, however, had to be abandoned because of the difficulty of controlling fungal growth.

3. Results and discussion

A full range of PEG (1500–20000) was used for different trials, with the best results obtained with PEG 1500. Because of the very high solubility of the complex, the ionic strength had to be kept as low as possible by decreasing the content of buffer. To maintain low pH, sodium azide was replaced by chloretone. In our early crystallization trials twinning was frequently observed, but this was prevented by including small amounts of various additives. Best results were obtained with Gly-L-Ser. In connection with this, it is interesting that Kurosky *et al.* (1980) reported the homology of the heavy chain of Hp with the chymotrypsinogen family of serine proteases.

The crystals of form *A* were orthorhombic with unit-cell parameters a = 70.7, b = 405, c = 71.5 Å. Since very thin plates were fragile, a novel method of mounting them in capillaries using bundles of milkweed fibres was developed (Przybylska, 1988). They diffracted very poorly and a feasibility study using the synchrotron radiation at Cornell High Energy Synchrotron Source (CHESS) was carried out, where the low resolution of 7–9 Å was confirmed. Owing to low resolution and the difficulty of screening *h*1*l* reflections from the *h*0*l* zone, the space group could not be determined, but the lattice was probably primitive with four molecules per unit cell and therefore one molecule per asymmetric unit. The calculated solvent content is 61% (Matthews, 1968).

Form *B* of the complex, shown in Fig. 2, was also orthorhombic with unit-cell dimensions a = 62, b = 210 and c = 289 Å and Z = 8. They diffracted to ∼8 Å and were not examined using synchrotron radiation. The space group was not unequivocally determined, but the presence of a face-centered lattice was indicated.

In 1986, the work on the complex was discontinued and at that time, because of low resolution, the publication of crystallization details did not seem justified. However, considering the steadily growing biological and clinical importance of Hp and the progress in protein crystallography methods (McPherson *et al.*, 1995), this challenging crystallographic investigation may be resumed. Of the new approaches, lowering the temperature (Murakami *et al.*, 1998) and crystallization in microgravity appear promising. Microheterogeneity of the Hp–Hb complex (Yang & Przybylska, 1973) arising from the variation in the sialic acid residue content may be responsible for the observed disorder. Since the crystals of the desialated complex components obtained at the time were too small for X-ray examination, more work on the effect of desialation would be warranted.

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