Crystallization of Human Oxyhaemoglobin from Poly(Ethylene Glycol) Solutions

By MIECZYSLAW J. GRABOWSKI, ANDRZEJ M. BRZOSOWSKI,
ZYGMUNT S. DEREWENDA, TADEUSZ SKARŻYŃSKI, MIROSŁAW CYGLER,
ANDRZEJ STĘPIEŃ and ANNA E. DEREWENDA*

*Department of Crystallography, Institute of Chemistry,
University of Łódź, Nowotki 18, 91-416 Łódź, Poland*

(Received 9 January 1978)

Crystals of human oxyhaemoglobin were obtained from poly(ethylene glycol) solutions. Spectroscopic and spectrophotometric measurements on the solutions during crystallization and on the dissolved crystals indicate that the method yields stable crystals of oxyhaemoglobin. Preliminary X-ray studies showed that the crystals obtained are isomorphous with those of deoxyhaemoglobin obtained from poly(ethylene glycol) solutions [Ward, Wishner, Lattman & Love (1975) *J. Mol. Biol.* **98**, 161–177].

It has been argued that lattice forces and properties of the solvent may to some extent influence the molecular structure of proteins in the crystalline state. Ward *et al.* (1975) have described the structure of human deoxyhaemoglobin A crystals grown from poly(ethylene glycol) solutions. Their results indicate that the solvent used causes structural changes near the C-terminus of the β_1 -chain and in the vicinity of the haem groups, as compared with the refined structure of human deoxyhaemoglobin (Fermi, 1975) crystallized as described by Perutz (1968). These changes seem to resemble those that accompany the T→R transition and may be due to the rupture of certain salt bridges stabilizing the deoxy (T) structure (Perutz, 1970), especially the salt bridge linking histidine-146 and aspartate-94 of the β_1 -chain. To elucidate the problem further we have undertaken structural studies of human oxyhaemoglobin crystals grown from poly(ethylene glycol) solutions. The first stage of the work, described below, was the preparation of oxyhaemoglobin crystals suitable for X-ray studies.

Materials and Methods

Crystallization

Human oxyhaemoglobin was prepared from whole blood obtained from the local blood bank, by the method of Drabkin (1946), with cells lysed with distilled water. The haemolysate was centrifuged at 5000g for 45 min and dialysed against double-distilled water for 120 h at 4°C.

Oxyhaemoglobin was crystallized as described by Ward *et al.* (1975). The initial solution of oxyhaemoglobin was stirred in the atmosphere of air to ensure proper oxygenation. Crystallization was carried out

in sealed 2 ml glass tubes under conditions listed in Table 1. Toluene was used in half of the crystallization tubes to prevent bacterial infection.

Spectrophotometric measurements of the solutions and of the dissolved crystals were carried out with a Pye-Unicam SP.1800 spectrophotometer. Spectroscopic studies of the solutions during crystallization were done with a Carl Zeiss Jena optical spectroscope. To exclude the possibility of deoxygenation caused by bacterial infection we have performed additional microbiological tests.

X-ray studies

Precession and oscillation X-ray photographs were taken by using Cu K α radiation filtered through nickel foil.

Results

Crystallization

Crystals grew over a wide range of conditions, but best monocrystals were obtained from tubes where poly(ethylene glycol) composition was 19–20% (v/v) of the stock solution (Table 1, Plate 1). The crystals liquify when removed with the mother liquid from crystallization tubes. The nature of this phenomenon remains at present unknown. It seems possible that the steric exclusion mechanism of crystallization from poly(ethylene glycol) solutions suggested by Laurent (1963) may play a significant role. Our crystals were therefore strengthened before being sealed in capillary tubes for X-ray studies by immersion in the stock solution of poly(ethylene glycol). A similar method was proposed by Wishner *et al.* (1975) and used by Fermi & Perutz (1977) in their studies of human fluoromethaemoglobin with inositol hexaphosphate crystals, which tended to dissolve when exposed to the X-ray beam.

* Present address: Department of Immunology, Institute of Pathology, Medical Academy, Nowotki 137, 90-235, Łódź, Poland.

Table 1. *Crystallization conditions*

	Range of conditions attempted	Actual conditions for crystals used in X-ray studies
Final oxyhaemoglobin concentration (mg/ml)	30.0–42.4	35.0–35.5
Final poly(ethylene glycol) concentration (v/v)*	5–30%	19–20%
Buffer (Na ₂ HPO ₄ /NaH ₂ PO ₄)	0.01 M	0.01 M
pH	6.1–7.2	6.7
Temperature (°C)	+4	+4

* Poly(ethylene glycol) was actually added as a dilution of a stock solution prepared by dissolving 50 g of poly(ethylene glycol) (mol.wt. 6000; J. T. Baker Chemical Co., Philipsburg, NJ, U.S.A.) in 100 ml of water; v/v, percentage calculated as stock solution volume per total oxyhaemoglobin solution volume in the crystallization tube.

Spectroscopic studies of the solutions during the crystallization period (2–4 weeks) indicated full oxygenation: there were two absorption bands, at 542 and 578 nm.

Spectrophotometric studies of the mother solutions of the crystals did not reveal the presence of methaemoglobin in significant amounts. Some crystals were dissolved and examined spectroscopically 2 months after crystallization was started. The results indicated that the crystals kept in their mother liquid contained less than 3% of methaemoglobin, whereas the same crystals immersed for an additional 7 days in the stock solution of poly(ethylene glycol) contained less than 5% of methaemoglobin. Additional studies of the e.s.r. spectra of our crystals (M. Koter, unpublished work) confirmed these results.

Microbiological tests were performed after crystallization had been completed. No bacterial infection was detected in tubes kept at 4°C with toluene. Small amounts of bacteria were found in tubes at 4°C without toluene, but they seemed to have a negligible effect on the process of crystallization. As a control we tested tubes without toluene kept at room temperature (20°C). Large amounts of bacteria were found in those tubes, but crystals appeared in them after 1 week. In spite of their suitable parameters, these crystals were not used in further studies.

X-ray studies

Human oxyhaemoglobin crystallizes from poly(ethylene glycol) solutions in an orthorhombic system. The crystals exhibit the symmetry of space group P2₁2₁2 (Plate 2). The lattice constants obtained from oscillation and precession photographs are: $a = 96 \text{ \AA}$, $b = 98 \text{ \AA}$, $c = 66 \text{ \AA}$. There is one tetramer per asymmetric unit. The value of V_m (average crystal

volume per unit molecular weight) is $2.39 \text{ \AA}^3/\text{dalton}$ (Matthews, 1968).

Discussion

The studies by Perutz (1968) and Makinen (1975) indicated that under their experimental conditions during 2 weeks of the crystallization period at least 50% of oxyhaemoglobin had been oxidized to methaemoglobin. The lability of oxyhaemoglobin solutions is one of the reasons why the structure of oxyhaemoglobin is not yet known in detail. The structural data have been derived rather from analogues, such as the oxygen adduct of the 'picket-fence' complex of Collman *et al.* (1974). Our results indicate that it is possible to obtain stable oxyhaemoglobin crystals from poly(ethylene glycol) solutions.

The second point of interest arises from the results of the X-ray studies. It has been observed that, for crystals grown from high-ionic-strength solvents, crystals of deoxy- and oxy-haemoglobin vary considerably: oxyhaemoglobin crystallizes in the quaternary R structure and forms tetragonal crystals, whereas deoxyhaemoglobin forms monoclinic crystals belonging to space group P2₁ (Fermi, 1975). On the contrary, our crystals exhibit the same space-group symmetry as those of deoxyhaemoglobin obtained by Ward *et al.* (1975). The cell constants are also similar. We have compared the intensities taken from the precession photographs with the diffraction data for deoxyhaemoglobin, which we have obtained from Dr. J. C. Hanson from Johns Hopkins University, Baltimore, MD, U.S.A., and found them to be similar.

It must be noted that the crystals of human methaemoglobin with inositol hexaphosphate obtained by Fermi & Perutz (1977) are also iso-

EXPLANATION OF PLATE I

Crystals of human oxyhaemoglobin

Crystals of human oxyhaemoglobin immersed in the stock solution of poly(ethylene glycol) are seen in polarized light (magnification $\times 15$).

EXPLANATION OF PLATE 2

Precession photograph of the h01 zone of human oxyhaemoglobin crystal

Precession photograph of the h01 zone of human oxyhaemoglobin crystal grown from poly(ethylene glycol) solution was taken by using Cu K α radiation filtered through nickel foil; crystal-to-film distance was 60mm.

morphous with those of deoxyhaemoglobin crystallized from poly(ethylene glycol).

These results may indicate that, owing to the rupture of certain salt bridges, the quaternary structures of deoxy- and oxy-haemoglobin in crystals grown from poly(ethylene glycol) solutions are almost identical.

We are grateful to Dr. J. C. Hanson of the Thomas C. Jenkins Department of Biophysics, Johns Hopkins University, Baltimore, MD, U.S.A., for supplying us with the diffraction data of deoxyhaemoglobin crystallized from poly(ethylene glycol) (Ward *et al.*, 1975), and to Dr. M. Koter, of the Department of Biophysics, University of Łódź, for the preparation of e.s.r. spectra. This research was supported by the Ministry of Science, Technology and Higher Education under contract R.III.13.1.4.

References

- Collman, J. P., Gagne, R. R., Reed, C. A., Robinson, W. T. & Rodley, G. A. (1974) *Proc. Natl. Acad. Sci. U.S.A.* **71**, 1326–1329
- Drabkin, D. L. (1946) *J. Biol. Chem.* **164**, 703–723
- Fermi, G. (1975) *J. Mol. Biol.* **97**, 237–256
- Fermi, G. & Perutz, M. F. (1977) *J. Mol. Biol.* **114**, 421–431
- Laurent, T. C. (1963) *Biochem. J.* **89**, 253–257
- Makinen, M. W. (1975) in *Techniques and Topics in Bio-inorganic Chemistry* (McAuliffe, ed.), p. 44, William Clowes and Sons, London
- Matthews, B. W. (1968) *J. Mol. Biol.* **58**, 79–88
- Perutz, M. F. (1968) *J. Cryst. Growth* **2**, 54–56
- Perutz, M. F. (1970) *Nature (London)* **228**, 726–734
- Ward, K. B., Wishner, B. C., Lattman, E. E. & Love, W. E. (1975) *J. Mol. Biol.* **98**, 161–177
- Wishner, B. C., Ward, K. B., Lattman, E. E. & Love, W. E. (1975) *J. Mol. Biol.* **98**, 179–194