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A Cubic Form of Ox Haemoglobin

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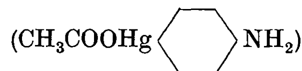
The crystals of a new form of ox oxy-haemoglobin are cubic, of space group $P4_132$ or $P4_332$. Measurements of density and water content of the wet crystals show that the unit cell, of edge 142·2 Å, contains 12 haemoglobin molecules. This figure is confirmed by measurement of the cell dimensions of air-dried crystals. The molecules, unlike those in the normal orthorhombic form of ox haemoglobin, must lie on dyad axes, as in horse haemoglobin I.

The normal crystal form of ox oxy-haemoglobin is orthorhombic, the space group being $P2_12_12_1$. This form was first studied by Crick (1956), who derived the packing arrangement of the molecules within the crystal lattice. Subsequently, (Green & North) the isomorphous-replacement method has been used to investigate the electron-density distribution by means of similar techniques to those originally applied to horse haemoglobin by Green, Ingram & Perutz (1954). In the course of the work on ox haemoglobin, attempts have been made to combine the protein with various reagents containing suitable heavy atoms. In addition to a number of different heavy-atom compounds, other experimental conditions such as protein concentration and the composition of the salt used to precipitate the protein from solution during crystallisation have also been varied. Generally, the crystals which have grown have been isomorphous with the normal native protein form or with slightly shrunk modifications of it. In three separate experiments, however, crystals of an entirely different type have been produced, and these form the subject of the present note.

Preparation of crystals

In each case, iodo-acetic acid had first been added to the haemoglobin solution. This substance is known to combine with the highly reactive sulphydryl groups in the protein and so to prevent a heavy-atom compound, to be added subsequently, from becoming attached to them. (Several protein derivatives with heavy atoms at the sulphydryl sites had already been prepared and the object of the present experiments was to induce heavy atoms to go elsewhere in the protein.) The method used was to mix the protein solution with excess iodo-acetic acid solution and to dialyse away the excess against glass-distilled water. In one experiment, the iodo-acetic acid blocked haemoglobin had then been mixed with an equimolar solution of mercuric acetate, in the second case with an equimolar solution of *p*-aceto mercuri-aniline



and in the third case, no heavy-atom compound had been added. X-ray photographs from all three types of crystal are practically identical, and so neither of the heavy-atom compounds actually combined with the protein to any appreciable extent.

In all three cases, the protein was finally precipitated from solution by means of a salt solution consisting of 7 parts of $4M$ ammonium sulphate and 3 parts of saturated di-ammonium phosphate. The crystals which grew from these preparations were found to be non-birefringent and to be octahedral in habit.

X-ray diffraction data

X-ray precession photographs were taken with the beam along the 4-fold, 3-fold and 2-fold symmetry axes of the octahedra and these show that the crystals, in fact, have 432 cubic symmetry. As there are no systematic absences of general reflections, the unit cell must be primitive; since proteins are known to be made up of L-amino acids only, symmetry elements in protein crystals are restricted to rotational and screw axes, so that the space group of the present crystals must be $P432$, $P4_132$, $P4_232$ or $P4_332$.

The only $h00$ reflections observed out to $h = 40$ are those with $h = 4$, 8, 12, 20, 28 and 32. The axial reflections are thus restricted to those with $h = 4n$; the space group is therefore $P4_132$ or $P4_332$, which, of course, differ only in being mirror images of each other.

There are 24 general positions in the unit cells of these space groups or 12 positions on 2-fold axes. The large number of different orientations of the asymmetric unit of structure and high symmetry of the cell cause this crystal to be a most unsuitable one to use for the solution of the molecular structure. However, being the first cubic haemoglobin crystal, it is of interest to compare it with the other known forms. The question of greatest interest, though, is

Table 1. *Crystal data*

(Based on Kendrew 1954: additional data from Blow 1958)

Species	Symmetry	Space group	No. of molecules in unit cell	Volume per molecule
human (reduced) II	monoclinic	$P2_1$	2	$1{\cdot}36\times10^5$ Å^3
human III	tetragonal	$P4_12_1$	4	1·39
human I	orthorhombic	$P2_12_12_1$	4	1·41
rabbit	monoclinic	$C2$	2	1·42
ox	orthorhombic	$P2_12_12_1$	4	1·43
sheep (foetal) II	monoclinic	$P2_1$	2	1·43
pig II	monoclinic	$P2_1$	2	1·44
human (sickle cell)	orthorhombic	$P2_12_12$	8	1·44
pig I	orthorhombic	$P2_12_12_1$	4	1·45
human (reduced) III	monoclinic	$P2_1$	4	1·54
horse II	orthorhombic	$P2_12_12_1$	4	1·60
horse (reduced)	hexagonal	$C3_12$	6	1·61
hamster	orthorhombic	$P2_12_12_1$	4	1·62
horse III	monoclinic	$P2$	4	1·66
sheep (foetal) I	orthorhombic	$B22_12$	4	1·69
horse I	monoclinic	$C2$	2	1·75
human (reduced) I	monoclinic	$P2_1$	4	1·76
sheep (adult)	monoclinic	$C2$	4	1·89

whether or not the molecules lie on 2-fold axes in this form of ox haemoglobin. In several of the known haemoglobin forms, including horse haemoglobin type I, which has been studied so extensively by Perutz and his collaborators, the molecules do, in fact, lie on 2-fold axes. The normal orthorhombic crystals of ox haemoglobin contain no 2-fold rotation axes, so that if ox haemoglobin molecules possess such axes, they do not make use of them in the crystal. There are reasons, however, for supposing that the ox molecules do have symmetry, both on grounds of the general chemical similarity of different haemoglobins and because heavy atoms appear to become attached in pairs symmetrically related to the outline of the molecule in the orthorhombic crystals. The evidence is discussed in greater detail elsewhere (Green & North). It is clear, though, that it is of some interest to determine the size of the asymmetric unit in the cubic ox crystals.

The length of the unit-cell edge was determined by measurement of the precession photographs and found to be 142·2 Å. Thus, the volume of the unit cell is $2{\cdot}88\times10^6$ Å^3, so that the volume per asymmetric unit is $1{\cdot}20\times10^5$ Å^3. Table 1 lists the previously discovered forms of haemoglobin, with details of crystal symmetry and the volume of crystal per protein molecule; the types are listed in order of the latter quantity.

Thus, if the asymmetric unit of structure of cubic ox haemoglobin is a whole molecule, the molecules are more closely packed than in any other haemoglobin; if the unit is a half molecule, then the packing is much less close than in other forms. It is interesting, and unhelpful, that Table 1 indicates no correlation between the closeness of packing and the degree of symmetry of the crystal lattices.

Measurement of density and water content

The density of the crystals was measured by use of a bromobenzene–xylene gradient column, as described by Low & Richards (1952). Crystals of dimensions $\frac{1}{2}$–1 mm. were blotted free of surplus mother liquor and were then dropped into the column, which was calibrated at the beginning and end of the experiment by weighing a glass bead at different heights in the column, in air and in water. The density measured in this manner corresponds to the crystals as grown and used for the X-ray photographs, i.e. with the interstices between the protein molecules filled with salt solution.

The water content of the crystals was found from the loss in weight of a number of large crystals (10–20 mg. total weight) blotted free of surplus mother liquor and then dried to constant weight at 115 °C. In another experiment the crystals were dried in a vacuum desiccator; the two methods gave similar results. Work on protein crystals (Haurowitz, 1950) has shown that crystals air-dried in this manner still retain 5–10% of their own weight in water, so in the present work $7\frac{1}{2}$% has been subtracted from the apparent weight of protein.

The air-dried crystals still contained a substantial amount of salt, and it was, of course, necessary to allow for this. Perutz' work on horse haemoglobin has shown that not all of the space between molecules is available to salt (Perutz, 1946); in fact, there seems to be a monomolecular layer of bound water associated with each haemoglobin molecule and this is apparently not permeable to salt ions. The remaining unbound water is available to salt ions and may be expected to have the same salt concentration as the mother liquor outside the crystal. Perutz found that the bound

water amounted to 0·3 g. per g. of protein. These observations have been made use of in the present work; it has been assumed that, of the estimated water content, 0·3 g. per g. of protein is salt-free water and the remainder has the same concentration of salt as the crystal mother liquor. The salt content of the mother liquor was estimated by weighing a crystal-free sample of mother liquor removed from the crystallising tube; this was then placed in a vacuum desiccator and dried to constant weight.

These data have been used to calculate the protein content of the crystals and hence, from the density and cell dimensions, the molecular weight of the asymmetric unit of structure.

Density = $1{\cdot}240 \pm 0{\cdot}002$ g.cm.$^{-3}$.
Loss of weight on air drying = $36{\cdot}7 \pm 1{\cdot}0$% of original weight.
Weight of salt in mother liquor = 66% of weight of water.
Water content of air-dried crystals assumed to be 7·5% of weight of protein.
Bound water impermeable to salt assumed to be 30% of weight of protein.
Molecular weight of protein per asymmetric unit of structure = 37,000.

The molecular weight of haemoglobin is about 68,000, so there seems little doubt that the asymmetric unit of cubic ox haemoglobin is a half molecule. The principal sources of error in the above measurements arise from the determinations of salt and water contents. The water content is unlikely to have been overestimated, so that, in this respect, the figure for molecular weight is an upper limit. The quantity of salt may be incorrect in either direction; even if it is double the true value, however, the protein molecular weight would only be increased to 40,000, and so the conclusions are not invalidated.

Cell dimensions of air-dried crystals

The volume per molecule of air-dried haemoglobin crystals has proved to be much more constant than that of wet crystals, the values found varying between 97,000 and 104,000 Å^3 (Bragg, Howells & Perutz, 1954). The cell dimensions of air-dried crystals cannot be measured nearly as accurately as those of wet crystals, as disorder is so great that only a few diffraction spectra are visible.

The cell edge of air-dried cubic ox haemoglobin was found to be 108–112 Å. Taking the above figures for the volume per molecule of the other air-dried forms, there are 13 ± 1 molecules in the unit cell of cubic ox haemoglobin, thus confirming the deductions from the above data on the wet crystals.

Discussion

In cubic ox haemoglobin, the asymmetric unit of structure is a half molecule, so the molecules possess dyad axes of symmetry. Since the cubic crystals were produced merely by the addition of iodo-acetic acid and the use of ammonium sulphate/phosphate mixture for salting out, it is reasonable to conclude that the protein itself consists of two identical halves in ox as in some other haemoglobins, but that other molecular features normally give rise to a form of packing in which the dyad axes are not utilised.

The packing of the molecules in the cubic lattice is so loose that it does not yield fresh information on the shape of the haemoglobin molecules. The shape which has been derived from studies of packing in other forms of haemoglobin and of salt–water changes, i.e. roughly spheroidal having dimensions of about $55 \times 55 \times 65$ Å (Bragg, Howells & Perutz, 1954) may be fitted into the cell quite readily. Taking the coordinates of the centres of the molecules as those given for space group $P4_132$, in *the International Tables for Crystallography*, vol. I, i.e. $\frac{1}{8}, x, \frac{1}{4}+x$ together with related points, a value of $x = 0{\cdot}20$ seems to give satisfactory distances between neighbouring molecules, but without defining the orientations of the molecules very closely.

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