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The Structure of Human Carbonmonoxy Haemoglobin at 2·7 Å Resolution

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The structure of human carbonmonoxy haemoglobin has been determined to 2·7 Å resolution using X-ray data for the native protein only. The atomic co-ordinates were refined from those of an initial model based on the co-ordinates of the closely related protein horse methaemoglobin whose structure is known at high resolution (Ladner *et al.*, 1977). The space group of the new unit cell ($P4_12_12$) is such that the location of the haemoglobin molecules is specified by two parameters only, and the values of these were found through a search for the best initial *R*-factor. The refined structure of human carbonmonoxy haemoglobin is, as expected, generally similar to that of horse methaemoglobin. The root-mean-squared shift for all atoms between the initial model and the final co-ordinates was 1·35Å.

The new structure confirms that the CO ligand lies off the normal to the haem plane in both α and β subunits, as indicated previously by a difference Fourier map of CO *versus* horse methaemoglobin (Heidner *et al.*, 1976). The Fe–C–O group, assumed to be linear, makes an angle of about 13° with the haem normal and it points towards the inside of the haem pocket. In the α subunit the iron atom lies in the mean plane of the haem within experimental error. In the β subunit the situation is less clear in that unconstrained refinement put the iron atom 0·22 Å from the haem plane, a distance that is 3/2 times the expected error. The new structure also confirms that in carbonmonoxy haemoglobin the side chain of cysteine β93(F9) points away from the surface of the molecule into the pocket between helices F, G and H that, in deoxyhaemoglobin, is occupied by the side chain of tyrosine β145(HC2). A detailed comparison of the structures of the deoxy and liganded forms from the same species is now possible and the conclusions drawn from this comparison are given in a separate publication (Baldwin & Chothia, 1979).

1. Introduction

The structures of human deoxy haemoglobin and of horse methaemoglobin are known from X-ray diffraction studies to 2·5 Å and 2·0 Å resolution, respectively (Fermi, 1975; Ladner *et al.*, 1977). In both forms the molecule is tetrameric, having two α and two β subunits related by a molecular 2-fold axis (see Fig. 1(a)). The tertiary structures of the subunits are similar in the two forms, consisting of seven helical regions in the α subunits and eight helical regions in the β subunits, with non-helical regions between them and at the amino and carboxy-termini. The structures of the $\alpha_1\ \beta_1$ and $\alpha_1\ \beta_2$ dimers are illustrated schematically in Figure 6(a) and (b). There is a difference in

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quaternary structure between deoxy- and methaemoglobin, the change in packing between the subunits occurring at the interface between the α_1 β_1 dimer and α_2 β_2 (see Fig. 6(b)). Each α and β subunit contains a haem group at the centre of which is an iron atom to which oxygen or another ligand (e.g. carbon monoxide or the water molecule in methaemoglobin) binds reversibly. The haem group is linked to the protein by a covalent bond to the proximal histidine residue (His(F8)). In deoxyhaemoglobin the iron atom is bonded to the imidazole nitrogen (N_ε) of His(F8) and to the four pyrrole nitrogens (N_1 to N_4) of the haem. In liganded haemoglobin, it is also bonded in the sixth position to the haem ligand.

The ligand binding affinity of each subunit of haemoglobin rises as the other haems become saturated with ligand, and a structural explanation of this phenomenon has been sought in comparisons of the structures of human deoxyhaemoglobin and horse methaemoglobin. It has been established that the co-operativity of ligand binding to haemoglobin is associated with the quaternary structure change that occurs between the deoxy and liganded forms, and the larger structural differences accompanying the quaternary change have been correlated with the function of haemoglobin by Perutz and co-workers (see reviews by Perutz, 1976,1979; Baldwin, 1975). Some more detailed comparisons of the tertiary structures of human deoxyhaemoglobin and horse methaemoglobin have been made (Takano, 1977; Ladner *et al.*, 1977) but the important differences due to ligation state may be small and might be obscured by changes in structure that are the consequence of the amino acid sequence differences between the two species. It is therefore necessary to know either the structure of horse deoxyhaemoglobin or that of human liganded haemoglobin to an accuracy comparable with that of the structures already known. Horse deoxyhaemoglobin was studied to 2·8 Å resolution by Bolton & Perutz (1970) but the structure was not refined as the data were poor. The structure of human liganded haemoglobin would in any case be the more useful of the two in that it would also enable some of the interesting abnormal human haemoglobins to be studied by difference Fourier methods.

The early attempts to study human liganded haemoglobin were frustrated by a lack of isomorphous derivatives (Muirhead, 1963). In recent structural determinations at high resolution, however, the final stages of refinement have abandoned the multiple isomorphous replacement phases and followed procedures in which the current atomic model of the protein is refined against the native data alone (Steigemann *et al.*, 1976; Jensen, 1976). The success of these methods suggested that a protein structure could be determined entirely from native data if a good starting model could be obtained, and high resolution structures, for example those of bovine β-trypsin (Bode & Schwager, 1975) and carboxypeptidase-B (Schmid & Herriott, 1976), have since been determined by refinement from models on related proteins. Horse methaemoglobin should be a good initial model for human liganded haemoglobin as the deoxy forms have been shown to be similar (Fermi, 1975). Therefore, provided the location of the molcules in the human unit cell could be found, the determination of the structure should be possible. Patterson search techniques (Rossmann, 1972) were used to find the location of the molecules in the structure determinations of carboxypeptidase-B (Schmid *et al.*, 1974) and bovine β-trypsin (Fehlhammer & Bode, 1975).

Perutz *et al.* (1951) showed that human methaemoglobin crystallised in space

group $P4_12_12$ or $P4_32_12$, with four tetrameric molecules in the unit cell. Prothero & Rossmann (1964) calculated the rotation function between the 5·5 Å X-ray amplitudes of horse methaemoglobin (Cullis *et al.*, 1961,1962) and those of a 5·5 Å data set measured for human methaemoglobin by Muirhead (1963), and found a value for the orientation of the molecule in the human unit cell. Baldwin used a two-parameter search method (previously unpublished but described below) using the X-ray amplitudes for human methaemoglobin and the electron density map of horse methaemoglobin, and determined both the position and orientation of the molecules and hence a set of low resolution phases. These phases were used by Greer (1971) to calculate a difference Fourier map of an abnormal haemoglobin at 5·5 Å resolution. The present study took as its starting point some of the earlier work using the low resolution data for human methaemoglobin. Higher resolution data were collected for carbonmonoxy haemoglobin. It would have been preferable to study the oxy form, but the problem of oxidation to the met form during data collection, although overcome recently for myoglobin (Phillips, 1978) and erythrocruorin (Weber *et al.*, 1978), has not yet been overcome for haemoglobin. Carbonmonoxy haemoglobin is a stable form of liganded haemoglobin in which the iron atoms are in the low-spin ferrous state, and it is therefore more closely related to the oxy form than is methaemoglobin in which the iron atoms are in the ferric state. The co-operativity, Bohr effect and the 2,3 diphosphoglycerate effect are similar for the binding of oxygen or carbon monoxide to haemoglobin.

The first part of this paper describes the analysis of the X-ray diffraction data of human liganded haemoglobin to determine the position and orientation of the haemoglobin tetramers in the crystallographic unit cell. Then the refinement of the atomic co-ordinates from an initial model based on the structure of horse methaemoglobin is described. The last section of this paper describes the structure of the haem groups in carbonmonoxy haemoglobin, in particular the orientation of the ligand molecule, and the conformation of the reactive cysteine residue β93(F9). A detailed comparison between the final co-ordinates of human carbonmonoxy haemoglobin and those of human deoxyhaemoglobin and horse methaemoglobin is presented in a separate publication (Baldwin & Chothia, 1979).

2. Crystallisation and Data Collection

Two sets of X-ray diffraction data from crystals of human liganded haemoglobin were used in the structure determination. Low resolution data for human methaemoglobin were used in the early stages of the analysis and higher resolution data for human carbonmonoxy haemoglobin were used for the eventual determination of the structure.

(a) *Human methaemoglobin data at 5·5 Å resolution*

The crystallisation of human methaemoglobin and the measurement of its X-ray diffraction data to 5·5 Å resolution were described by Muirhead (1963). Crystals were grown from 2·2 M-phosphate solution buffered to pH 6·6. The space group was determined as either $P4_12_12$ or $P4_32_12$ and the number of molecules per unit cell as 4. The diffraction data were recorded on precession photographs and the intensities of the spots were measured with a Joyce–Loebl microdensitometer 3CS. The data from 11 zero-layer and 4 first-layer photographs were processed and scaled together by standard methods. Data were obtained for about 750 independent reflections with spacings out to 5·5 Å.

(b) *Human carbonmonoxy haemoglobin data at 2·7 Å resolution*

Crystals of human carbonmonoxy haemoglobin were grown by N. L. Anderson (unpublished work), using a method based on that described by Perutz (1968). Each crystallisation tube was bubbled full of carbon monoxide before sealing. Glass tubes with greased, ground glass stoppers were used to prevent the escape of carbon monoxide or the introduction of air. Large crystals grew in 4 to 6 weeks. They were re-equilibrated with carbon monoxide in 3·5 M-phosphate buffer at pH 6·8 for 18 h before being mounted in quartz capillaries between plugs of cotton. These plugs were necessary to prevent crystal slippage, but as they only touched the sharp ends of the tetragonal-bipyramidal crystals they did not interfere with the diffracted radiation. Each capillary was flushed with carbon monoxide just before being sealed with wax.

The X-ray photographs were taken by Anderson using an Arndt–Wonacott rotation camera. The use of such a camera and the methods of measuring and processing data recorded on rotation photographs are described fully by various authors in the book edited by Arndt & Wonacott (1977). A monochromated X-ray beam was used and because the separation of the diffraction spots along the c^*-axis is small a collimator of 0·2 mm diam. was needed. A crystal-to-film distance of 90 mm allowed 2·7 Å resolution data to be collected. The c-axis of the crystal was parallel to the rotation axis of the camera, and a total rotation of 45° allowed measurement of all the independent reflections, except for those in the cusp along the c-axis. Fifteen exposures, each for a 3-deg. rotation, were taken, with 3 films in each pack. The exposure time for each rotation step was 6 h and all the data finally used were collected from one crystal. The cusp data were not collected so about 2% of the total data to 2·7 Å resolution (Arndt & Wonacott, 1977) were not included in the data set.

The intensities of the fully recorded and the partially recorded spots were measured on a flat-bed scanner (Mallett *et al.*, 1977). The intensities from each film were corrected for Lorentz, polarisation and absorption factors and then scaled together by means of symmetry-related reflections recorded on different films. The symmetry R-factor computed for the data set is given with other statistics in Table 1. Reflections that were split between 2 contiguous rotation photographs were not used in the scaling but were included in the R-factor calculation. Data were obtained for 8020 reflections with spacings out to 2·7Å.

TABLE 1

Statistics on data processing of 2·7 Å data

Total number of reflections measured	27,806
Number of reflections fully recorded	23,880
Number of reflections recorded in parts	3926
Number of independent reflections	8020
R-factor	0·049

$$R\text{-factor} = \frac{\sum_i |\bar{F} - F_i|}{\sum_i F_i},$$

where F_i = amplitude of ith reflection and $\bar{F}$ = average amplitude for all reflections symmetry-related to the ith reflection.

3. Determination of the Structure

This analysis used the fact that the shape of the haemoglobin molecule is expected to be as similar in horse and human liganded haemoglobins as it has been shown to be in the two deoxy forms (Bolton & Perutz, 1970; Fermi, 1975). This enables one to

use the structure of horse haemoglobin, which is known (Cullis *et al.*, 1961,1962; Ladner *et al.*, 1977), to find the location of the human haemoglobin molecule in its unit cell, and then to use it as an initial model for further refinement.

(a) *Determination of the space group and the location of the molecule in the unit cell*

(i) *Analysis of 5·5 Å human methaemoglobin data*

The space group of human liganded haemoglobin could be either $P4_12_12$ or $P4_32_12$. In either case the symmetry and the number of molecules per unit cell limit the number of parameters describing the position and orientation of the tetrameric molecule to two. Figure 1(a) and (b) illustrates the two parameters (q and θ) that must be determined. The origin of the molecular co-ordinate system is taken at the centre of mass of the four iron atoms, and this lies on the molecular dyad axis (Y).

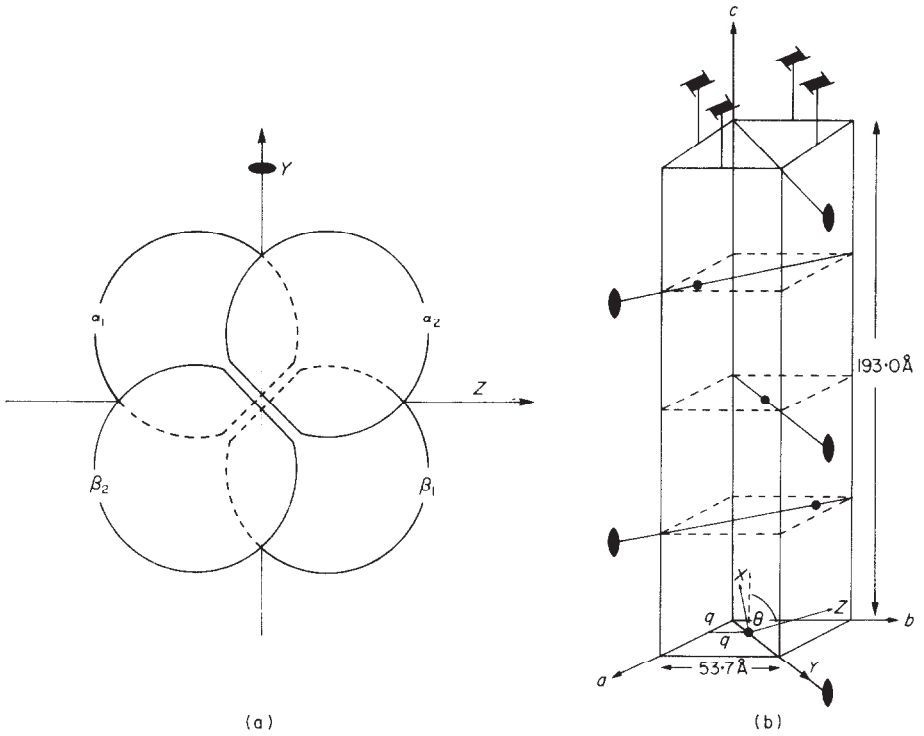

FIG. 1. (a) Schematic diagram of the haemoglobin tetramer showing the molecular axes. Y is the dyad axis relating the $\alpha_1\beta_1$ dimer to $\alpha_2\beta_2$. X (perpendicular to the paper) and Z are the pseudo-dyad axes relating α and β subunits.

(b) The unit cell of human liganded haemoglobin. The centres of the molecules lie on the diagonal dyad axes. There are 4 molecules per unit cell. This Figure shows the cell dimensions of human carbonmonoxy haemoglobin and shows space group $P4_12_12$ in which the molecular centres lie at positions $q,q,0$; $-q+1/2, q+1/2, 1/4$; $-q, -q, 1/2$; $q+1/2, -q+1/2, 3/4$. (Space group $P4_32_12$ would have molecular centres at $q,q,0$; $q+1/2, -q+1/2, 1/4$; $-q, -q, 1/2$; $-q+1/2, q+1/2, 3/4$.) θ is the angle between the molecular Z-axis and the c-axis of the unit cell. The positive direction of θ is chosen in the sense that rotates X into Z.

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