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The Application of the Molecular Replacement Method in Studies on the Quaternary Structure of Haemoglobin

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Abstract

It has been found that the crystallization of oxyhaemoglobin from polyethylene glycol solutions [Grabowski *et al.* (1978). *Biochem. J.* **171**, 277–279] yields at least three different forms of isomorphous crystals ($P2_12_12$, $a = 96{\cdot}66$, $b = 97{\cdot}88$, $c = 65{\cdot}40$ Å). 3·5 Å resolution data have been collected for two of the three identified forms. The orientations and positions of the $\alpha\beta$ haemoglobin dimers within the asymmetric units of these crystals have been established by the molecular replacement method with computing techniques different from those used by Ward, Wishner, Lattman & Love [*J. Mol. Biol.* (1975), **98**, 161–171] in their studies of human deoxyhaemoglobin crystals obtained from polyethylene glycol solutions. The calculations have been performed also with diffraction data from deoxyhaemoglobin and fluoromethaemoglobin + inositol hexaphosphate crystallized from polyethylene glycol by Fermi & Perutz [*J. Mol. Biol.* (1977), **144**, 421–431]. In all cases, the individual dimers have been positioned independently and it is shown that, in the methods using a fast rotation function, three- and multidimensional residual-type translation functions may be directly applied to the structure determination of those complex structures in which the structure of only one of the two subunits present in the asymmetric unit is known. It is also shown that in all crystals studied the haemoglobin dimers are arranged in the $\mathscr{T}$ conformation, which seems to exclude the possibility of the full oxygenation of haemoglobin crystallized from polyethylene glycol solutions.

Introduction

In spite of the fact that various forms of haemoglobin isolated from numerous species have been studied extensively, the knowledge of the structure of oxyhaemoglobin and the chemical nature of the structural changes accompanying the oxygenation of the protein is not complete. The determination of the structures of unliganded native (Fermi, 1975) and abnormal (*e.g.* Tucker & Perutz, 1977; Anderson, 1975) haemoglobins, various forms of methaemoglobin (*e.g.* Anderson, 1973; Fermi & Perutz, 1977), as well as such liganded forms as human carboxyhaemoglobin (Baldwin & Chothia, 1979) and nitric oxide haemoglobin (Deatherage & Moffat, 1979), which seems to be the closest structural analogue to oxyhaemoglobin, have provided adequate data to formulate a general picture of structural changes accompanying the binding of ligands to haemoglobin (Baldwin & Chothia, 1979). The main feature of these changes is a transition in the quaternary structure from the $\mathscr{T}$ (tense) to the $\mathscr{R}$ (relaxed) conformation. This transition, which may be described as the change in the mutual orientation of the two $\alpha\beta$ dimers, is triggered primarily by the movement of the iron atom of the haem group towards the ligand, and the structural rearrangements due to the steric effects of the ligands (Perutz, 1970). The structure of the oxyhaem complex has been known from the studies of the so-called 'picket-fence' complexes (Collman, Gagne, Reed, Robinson & Rodley, 1974) and the results were later confirmed by Phillips (1978), who succeeded in the determination of the structure of oxymyoglobin. However, the details of the chemical changes underlying the physiological function of haemoglobin remain unclear, as the structure of oxyhaemoglobin is not yet known.

The method of crystallization of haemoglobin proposed by Grabowski *et al.* (1978) opened the way to our investigations. We have found, however, that this method yields at least three different forms of isomorphous crystals, identified by marked differences in their diffraction patterns, all of which were also different

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from the diffraction pattern of deoxyhaemoglobin crystallized from polyethylene glycol solutions.

The structure of deoxyhaemoglobin from polyethylene glycol has been solved by Ward, Wishner, Lattman & Love (1975) using the molecular replacement method. However, these authors encountered some difficulties, pointing out that the rotation function maps (Lattman & Love, 1970) gave convincing answers only when the whole haemoglobin tetramer was used as the search unit. These difficulties could have been caused, at least in part, by the use of a coarse sampling grid in the early stages of the rotation searches, and by the use of horse deoxyhaemoglobin atomic parameters, instead of human, unavailable at that time, in the preliminary stages of structure solution. Fermi & Perutz (1977) have also observed that the molecule of fluoromethaemoglobin + inositol hexaphosphate, in the crystals they obtained from polyethylene glycol, may be rotated slightly with respect to deoxyhaemoglobin, but they were unable to evaluate that rotation.

The crystals we obtained are not exactly isomorphous with those of deoxyhaemoglobin and fluoromethaemoglobin. It seemed therefore necessary to establish the orientation and position of the molecule in the asymmetric unit from the beginning. There was some possibility that the quaternary structure of haemoglobin in these crystals was not the $\mathscr{T}$ conformation owing to the presence of oxygen. We therefore decided to find out whether methods other than those used by Ward *et al.* (1975), *i.e.* the fast rotation function (Crowther, 1972) and residual-type translation function (Nixon & North, 1976), would allow for the independent positioning of the $\alpha\beta$ dimers, and hence for the direct study of the quaternary structure of haemoglobin. In order to have a full comparison of the results obtained using our methods, we have also used in our calculations the diffraction data of deoxyhaemoglobin (kindly provided by Dr J. C. Hanson) and of fluoromethaemoglobin + inositol hexaphosphate crystallized from polyethylene glycol solutions (kindly provided by Dr J. Baldwin).

Materials and methods

(*a*) *Crystallization and data collection*

The crystals were grown and treated as described by Grabowski *et al.* (1978). Their quality was checked by X-ray photography. Three characteristic diffraction patterns have been identified and compared with that of deoxyhaemoglobin crystals grown from the same solvent. (The precession photographs of deoxyhaemoglobin were kindly provided by Dr C. Beddell.) Neither of the forms we identified was identical with deoxyhaemoglobin, and the differences in the diffraction patterns were especially pronounced in the medium- and high-resolution range. All crystals exhibit $P2_12_12$ space-group symmetry, but the cell constants of the crystals we obtained (a = 96·66, b = 97·88, c = 65·40 Å) are somewhat smaller than those of the isomorphous crystals of deoxy- and fluoromethaemoglobin (a = 97·07, b = 99·42, c = 65·98 Å).

Diffraction data to 3·5 Å resolution for crystals of two of the identified forms (Fig. 1) were collected on a Hilger–Watts four-circle computer-controlled diffractometer with an ω–2θ scan. Short background scattering measurements were taken on either side of all peaks (each one 10% of the peak counting time). The backgrounds for the weakest portions of the data were used to generate an average background function tabulated against θ, χ and φ. This follows a method suggested by Krieger, Chambers, Christoph, Stroud & Trus (1974) and programmed by P. E. Nixon (1976). Each unique set of data was collected from a single crystal in overlapping shells with approximate resolution limits: 25, 8, 6, 5, 4 and 3·5 Å. Radiation damage was monitored by following the intensities of three standard reflections measured every 100 general reflections. The fall-off in intensity was approximated as a polynomial, but was found to be insignificant. The crystals seem to be particularly resistant to X-rays. Lorentz and polarization corrections were applied, and the intensities were corrected for absorption following the method of North, Phillips & Mathews (1968).

(*b*) *Rotation searches*

The orientation of the two $\alpha\beta$ dimers was studied for all of the investigated haemoglobin forms with the fast rotation function (Crowther, 1972). In all cases, the $|F_o|$ intensities were compared to an $|F_c|$ set calculated with the fast-Fourier transform for the haemoglobin $\alpha\beta$ dimer or tetramer in a dummy $P1$ cell, with axes corresponding to the orthogonal axes of the standard haemoglobin coordinate system (Fermi, 1975) and with cell constants twice the maximum dimensions of the molecule used. (Atomic parameters were kindly provided by Dr O. Kennard of the Protein Data Bank.)

Theoretically, the rotation function should exhibit a maximum for matrix $[R]$, such that $[R].[X]$ (where matrix $|X|$ denotes the coordinates of the atoms in the model molecule) is the best approximation of the atomic coordinates in the investigated structure. Matrix $[R]$ may be defined in many ways; in terms of Eulerian angles

$$[R] = \begin{bmatrix} \cos\alpha\cos\beta\cos\gamma - \sin\alpha\sin\gamma & -\cos\alpha\cos\beta\sin\gamma - \sin\alpha\cos\gamma & \cos\alpha\sin\beta \\ \sin\alpha\cos\beta\cos\gamma + \cos\alpha\sin\gamma & -\sin\alpha\cos\beta\sin\gamma + \cos\alpha\cos\gamma & \sin\alpha\sin\beta \\ -\sin\beta\cos\gamma & \sin\beta\sin\gamma & \cos\beta \end{bmatrix}.$$

If there are two identical subunits in the asymmetric unit and their relative orientation is defined by matrix $[R_0]$, then the rotation function will exhibit another maximum, equal in magnitude, for matrix $[R_1] = [R].[R_0]$. In deoxyhaemoglobin tetramer the two $\alpha\beta$ dimers are related by a twofold axis (y axis of the reference coordinate system) which is a non-crystallographic diad in the $P2_12_12$ cell. It is easy to show, with the above R matrix definition, that if R is generated by a set of Eulerian angles α,β,γ, then $\pi+\alpha, \pi-\beta, \pi-\gamma$ will generate $[R_1]$ for deoxyhaemoglobin. It may also be shown that the haemoglobin pseudodiad relating the α and β chains (x axis of the reference coordinate system) should produce an approximate fit between the data sets for angles $\pi+\alpha, \pi-\beta, -\gamma$ and $\alpha, \beta, \pi+\gamma$. The rotation function should therefore exhibit two additional equivalent maxima, lower in magnitude than the previous pair. The $P2_12_12$ space-group symmetry generates the following equivalent sets of Eulerian angles:

$$\begin{array}{ll} S_1 & \alpha,\beta,\gamma \\ S_2 & \alpha,-\beta,\gamma+\pi \\ S_3 & \pi-\alpha,\pi-\beta,\gamma+\pi \\ S_4 & \pi-\alpha,\pi+\beta,\gamma \\ S_5 & \pi+\alpha,-\beta,\gamma+\pi \\ S_6 & \pi+\alpha,\beta,\gamma \\ S_7 & -\alpha,\pi+\beta,\gamma \\ S_8 & -\alpha,\pi-\beta,\gamma+\pi. \end{array}$$

With these symmetry operations we would expect the rotation function to show maxima for the following sets of Eulerian angles:

1. α,β,γ
2. $(\pi+\alpha,\pi-\beta,\pi-\gamma) \equiv (\pi-\alpha,\beta,-\gamma)$ by S_8
3. $(\pi+\alpha,\pi-\beta,-\gamma) \equiv (\pi-\alpha,\beta,\pi-\gamma)$ by S_8
4. $\alpha,\beta,\pi+\gamma$.

The rotation function program calculates in the ranges α 0–π, γ 0–2π, β 0–$\pi/2$, and, as shown above, we would expect all the maxima to occur on one section of constant β value.

If the quaternary structure of haemoglobin molecules in any of the studied crystals is of the $\mathscr{R}$(liganded) type, then the $[R_0]$ matrix relating both dimers would be different and would result from the reorientation of the $\alpha_2\beta_2$ dimer with respect to the $\alpha_1\beta_1$ dimer, as described by Baldwin & Chothia (1979). The maxima of the corresponding rotation functions would be displaced.

For every data set the following computing procedure was used.

1. Calculation of the rotation function maps with 10–6, 6–5 and 5–4 Å data.

At this stage we computed the values of the rotation function of a 5° grid for α, β and γ, and with the maximum order of the Bessel functions equal to 30. The values of F_c were the molecular transforms of a single $\alpha\beta$ dimer.

2. 10–4 Å search, through a 36 Å radius sphere on a 2·5° grid (maximum order of the Bessel functions is 60).

3. Calculations as in 1 and 2 but with the F_c being the molecular transforms of the deoxyhaemoglobin tetramer.

(*c*) *Translation searches*

The translation function we used was based on the residual-type translation function used by Cutfield, Cutfield, Dodson, Dodson & Sabesan (1974) and described fully by Nixon & North (1976). Structure factor calculation was with the Agarwal fast Fourier transform procedure (Agarwal, 1978) in space group $P1$. The individual contribution of each molecule and its symmetry equivalents were summed with phase modifications corresponding to the different translations. The rotation parameters were held fixed in these calculations. The conventional R factor $\sum||F_o| - |F_c||/\sum F_o$ was used to detect the correct translation.

This procedure was used to establish the positions of whole tetramers, treated as rigid bodies, as well as for separate dimers. In the latter case the values of the molecular transforms were calculated only for one of the dimers in each search.

In order to position the dimers independently in one search calculation, the molecular transforms were treated as a function of six translational parameters, three for each dimer.

The following procedure was used for each of the four data sets investigated:

1. Calculation of a full translation function map for 7 Å resolution data over the whole unit cell using a coarse grid of 3 Å and treating the haemoglobin tetramer as a rigid body (oriented in agreement with the results of rotational searches). As the calculation of the translation function map is rather time consuming, all further searches were performed round the minimum obtained at this stage.

2. Calculation of a translation function map for the 5 Å resolution data (still treating the haemoglobin tetramer as the search unit) on a 0·5 Å grid. This stage was essentially a refinement of the results obtained in the previous search.

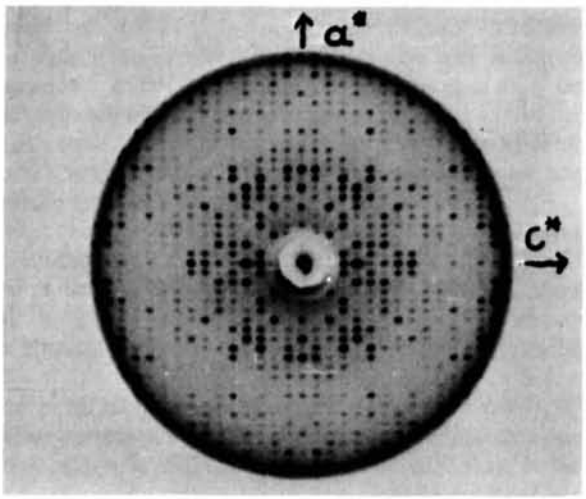
3. At this point, the calculations of stage 2 were repeated for each dimer separately.

4. The positional parameters for both dimers were refined with a six-dimensional translation function program. The calculations were performed on a fine grid (0·1 Å) with 4·5 Å data excluding the weakest 20% of the structure amplitudes.

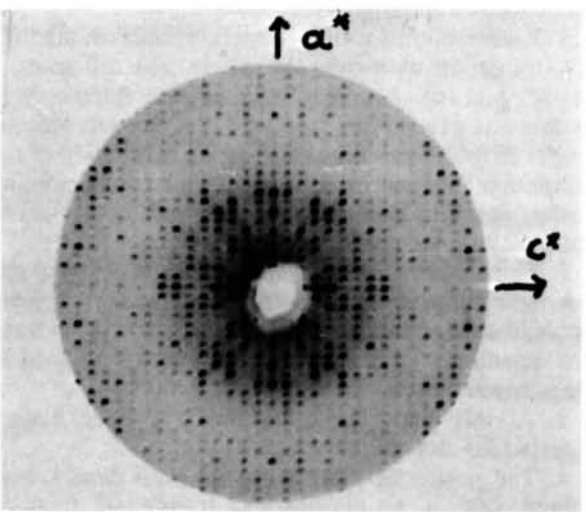
5. The 4·5 Å search was then repeated for the whole tetramer and for each of the dimers separately.

Results

The *h*0*l* zone precession photographs of the crystals (Fig. 1) demonstrate the differences in the diffraction patterns. The agreement analysis (Table 1) shows that out of the four investigated haemoglobin forms, deoxyhaemoglobin and fluoromethaemoglobin + inositol hexaphosphate have the most similar structures (lowest *R* factor value). In the haemoglobin crystals grown from polyethylene glycol, the haemoglobin structure differs from the model deoxyhaemoglobin molecule as much as it differs from deoxyhaemoglobin crystallized from polyethylene glycol solutions. The structural differences between the two forms of haemoglobin also seem to be significant.



(*a*)



(*b*)

Fig. 1. *h*0*l* zone precession photographs of (*a*) form I of native haemoglobin, and (*b*) form II of native haemoglobin obtained by the method of Grabowski *et al.* (1978); the photographs have been taken under slightly different conditions, and have been brought to the same scale photographically.

Table 1 *Agreement analysis*

	1	2	3	4
1. Native haemoglobin, form II	–	0·307	0·314	0·252
2. Native haemoglobin, form I	–	–	0·327	0·313
3. Fluoromethaemoglobin	–	–	–	0·192
4. Deoxyhaemoglobin	–	–	–	–

The conventional *R* factor has been calculated for 10–3·5 Å resolution data. All crystals were grown from polyethylene glycol.

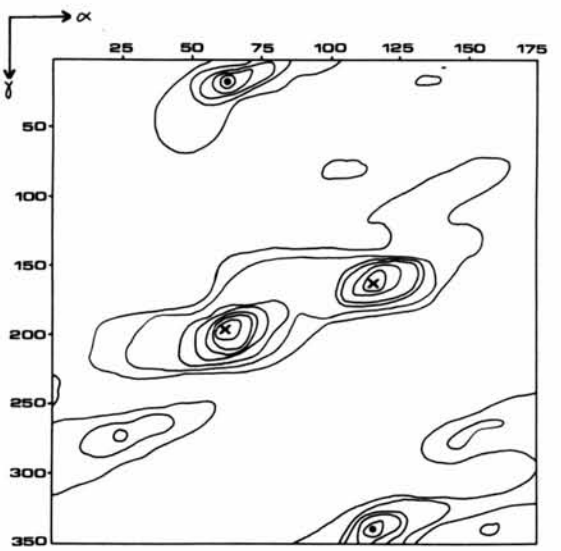


Fig. 2. A section through a rotation function map of native haemoglobin, form II. 10–4 Å data has been used; a single $\alpha\beta$ dimer was used as the search unit. The value of β on this section is 55°. × denotes the true maxima, ● denotes the pseudosolutions.

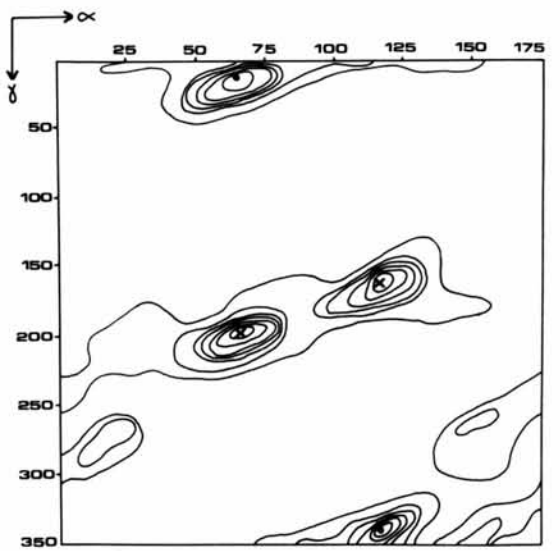


Fig. 3. A section through a rotation function map of deoxyhaemoglobin. Other details as for Fig. 2.

The results of the rotation searches are shown in Table 2. The orientation of the haemoglobin dimers is, within the computing accuracy, identical to the $\mathscr{E}$ quaternary conformation. All the maps are very clear (Figs. 2 and 3). It is interesting to note, however, that while each shell of the data confirmed the solution, in several ranges one of the pseudosolutions referring to the molecule rotated 180° around the molecular pseudodiad gave the highest overlap peak. The answer was unambiguous in higher-resolution calculations and in the tetramer search, which clearly distinguishes between the true and pseudo peaks.

Table 3 summarizes the results of the final stage of the translation searches. Interestingly, the results of the

Table 2. *Orientation of αβ dimers in crystals of four forms of haemoglobin crystallized from polyethylene glycol solutions*

Deoxyhaemoglobin (data from Ward *et al.*, 1975)				
Resolution limits (Å)	10–6	6–5	5–4	10–4
Search sphere radius (Å)	3–35	3–30	3–24	3–36
Number of $\|F_o\|$	878	799	1174	778
Number of $\|F_c\|$	702	876	387	856
Peak 1: α, β, γ (°)	−115,122,−17·5	−110,120,−15	−110,125,−15	−115,122·5,−17·5
height	58	46	59	45
Peak 2: α, β, γ (°)	63,57,197	65,55,197	65,55,195	66·5,57·5,196
height	68	71	61	51
Peak 3: α, β, γ (°)	65,56,17	67,52,15	70,55,15	60,55,17
height	48	31	53	38
Peak 4: α, β, γ (°)	−115,125,162	−120,128,165	−110,110,160	−115,122·5,161·5
height	59	59	36	44
Fluoromethaemoglobin + inositol hexaphosphate (data from Fermi & Perutz, 1977)				
Resolution limits (Å)	10–6	6–5	5–4	10–4
Search sphere radius (Å)	3–35	3–30	3–24	3–36
Number of $\|F_o\|$	586	453	321	477
Number of $\|F_c\|$	702	876	387	856
Peak 1: α, β, γ (°)	−115,120,−17·5	−115,125,−20	−110,125,−20	−116·5,121·5,−18·5
height	83	78	130	51
Peak 2: α, β, γ (°)	67·5,57,195	64,55,198	70,60,190	69,56·5,193·5
height	93	115	156	57
Peak 3: α, β, γ (°)	67·5,57·5,15	70,55,20	70,55,25	62·5,52·5,15
height	62	70	122	37
Peak 4: α, β, γ (°)	−110,122,165	−120,125,160	−120,125,160	−112·5,123·5,162·5
height	82	83	127	45
Native haemoglobin form I				
Resolution limits (Å)	10–6	6–5	5–4	10–4
Search sphere radius (Å)	3–35	3–30	3–24	3–36
Number of $\|F_o\|$	896	652	986	645
Number of $\|F_c\|$	713	874	359	856
Peak 1: α, β, γ (°)	−114,122·5,−18	−112·5,122·5,−17·5	−112,125,−15	−113·5,122·5,−18
height	62	62	56	51
Peak 2: α, β, γ (°)	62·5,57·5,197	65,55,195	65,57,195	66,57·5,196
height	73	54	39	50
Peak 3: α, β, γ (°)	68,57,15	70,57·5,12·5	67·5,55,15	66,57·5,16
height	46	38	51	34
Peak 4: α, β, γ (°)	−115,123,162	−115,115,170	−110,110,165	−115,122·5,161
height	46	38	36	44
Native haemoglobin form II				
Resolution limits (Å)	10–6	6–5	5–4	10–4
Search sphere radius (Å)	3–35	3–30	3–24	3–36
Number of $\|F_o\|$	728	514	1453	666
Number of $\|F_c\|$	713	874	359	856
Peak 1: α, β, γ (°)	−114,122·5,−18	−112,125,−17	−112,124,−16	−114,124,−19
height	54	63	69	44
Peak 2: α, β, γ (°)	65,55,196	65,55,200	67·5,55,195	68,56,196
height	67	68	52	50
Peak 3: α, β, γ (°)	68,55,15	65,50,15	70,55,15	70,55,15
height	45	42	58	32
Peak 4: α, β, γ (°)	−112·5,122·5,162	−125,105,155	−110,110,165	−115,124,160
height	55	69	43	39

Table 3. *Results of the translation searches*

The values of the shifts are given with respect to the standard origin in $P2_12_12$ space group.

Deoxyhaemoglobin (data from Ward *et al.*, 1975)

	$\alpha_1\beta_1$	$\alpha_2\beta_2$
Orientation (°)	$\alpha=-115\ \beta=122{\cdot}5\ \gamma=-16{\cdot}4$	$\alpha=65\ \beta=57{\cdot}5\ \gamma=196{\cdot}4$
Number of reflections	3578	3578
Independent search (Å)	$x=27{\cdot}28\ y=26{\cdot}52\ z=15{\cdot}55$	$x=27{\cdot}78\ y=26{\cdot}45\ z=15{\cdot}61$
R value (%)	47·97	49·25
6–*D* search (Å)	$x=27{\cdot}76\ y=26{\cdot}49\ z=15{\cdot}47$	$x=27{\cdot}63\ y=26{\cdot}36\ z=15{\cdot}47$
R value (%)	35·09	
Tetramer search (Å)	$x=27{\cdot}76\ y=26{\cdot}49\ z=15{\cdot}47$	
R value (%)	35·24	

Fluoromethaemoglobin + inositol hexaphosphate (data from Fermi & Perutz, 1977)

	$\alpha_1\beta_1$	$\alpha_2\beta_2$
Orientation (°)	$\alpha=-116{\cdot}5\ \beta=121{\cdot}5\ \gamma=-18{\cdot}5$	$\alpha=69\ \beta=56{\cdot}5\ \gamma=193{\cdot}5$
Number of reflections	3692	3692
Independent search (Å)	$x=28{\cdot}31\ y=26{\cdot}38\ z=15{\cdot}35$	$x=27{\cdot}78\ y=26{\cdot}49\ z=15{\cdot}61$
R value (%)	49·72	50·22
6–*D* search (Å)	$x=28{\cdot}30\ y=26{\cdot}63\ z=15{\cdot}09$	$x=27{\cdot}89\ y=26{\cdot}49\ z=15{\cdot}47$
R value (%)	37·82	
Tetramer search (Å)	$x=28{\cdot}16\ y=26{\cdot}63\ z=15{\cdot}34$	
R value (%)	38·47	

Native haemoglobin form I

	$\alpha_1\beta_1$	$\alpha_2\beta_2$
Orientation (°)	$\alpha=-115\ \beta=122{\cdot}5\ \gamma=-16{\cdot}4$	$\alpha=65\ \beta=57{\cdot}5\ \gamma=196{\cdot}4$
Number of reflections	2546	2546
Independent search (Å)	$x=27{\cdot}39\ y=25{\cdot}97\ z=15{\cdot}6$	$x=26{\cdot}99\ y=25{\cdot}97\ z=15{\cdot}6$
R value (%)	52·61	54·09
6–*D* search (Å)	$x=27{\cdot}39\ y=26{\cdot}24\ z=15{\cdot}6$	$x=26{\cdot}99\ y=25{\cdot}97\ z=15{\cdot}47$
R value (%)	35·93	
Tetramer search (Å)	$x=27{\cdot}26\ y=25{\cdot}97\ z=15{\cdot}6$	
R value (%)	36·40	

Native haemoglobin form II

	$\alpha_1\beta_1$	$\alpha_2\beta_2$
Orientation	$\alpha=-114\ \beta=124\ \gamma=-19$	$\alpha=68\ \beta=56\ \gamma=196$
Number of reflections	2435	2435
Independent search (Å)	$x=28{\cdot}06\ y=25{\cdot}83\ z=15{\cdot}22$	$x=27{\cdot}66\ y=25{\cdot}69\ z=15{\cdot}6$
R value (%)	51·52	51·89
6–*D* search (Å)	$x=28{\cdot}06\ y=25{\cdot}69\ z=15{\cdot}22$	$x=27{\cdot}53\ y=25{\cdot}83\ z=15{\cdot}6$
R value (%)	37·38	
Tetramer search (Å)	$x=27{\cdot}79\ y=25{\cdot}83\ z=15{\cdot}47$	
R value (%)	38·81	

searches with a single dimer as the search unit matched accurately those obtained from the six-parameter translation search. The positions of minima produced by the single dimer are in agreement with the results of the multidimensional search. In the deoxyhaemoglobin tetramer, constructed by rotation of the $\alpha\beta$ dimer about the y axis the dimers have a common origin and the coordinates of the R factor minima should be identical (Ward *et al.*, 1975). The agreement between this structure and deoxyhaemoglobin data fully confirmed these expectations. The remaining three sets exhibit quite interesting regularity. Although the dimers remain close to the tetramer position there is a slight but *consistent* shift of each dimer, especially along the crystallographic a axis. Note the almost perfect agreement between the positions of the dimers and tetramers in deoxyhaemoglobin. The value of the R factor for the tetramer as a rigid body exceeds the value obtained from the multi-dimensional search.

Discussion

Our calculations have demonstrated that all the haemoglobin molecules that we examined, prepared from polyethylene glycol solutions, are in the $\mathcal{E}$ conformation. The difference in the positions of the $\alpha\beta$ dimers in crystals which are assumed to contain liganded haemoglobin are small, but the consistency of the results suggests these are real. Although both

extreme quaternary structures ($\mathscr{T}$ and $\mathscr{R}$) have been described in detail (Baldwin & Chothia, 1979) the structures of intermediate forms of haemoglobin have never been refined. The slight displacement of one of the dimers in relation to the other upon ligation may be caused by changes in the tertiary structure, which, judging from the agreement analysis, must be significant. The analysis of the difference Fourier maps of the two forms we observed is under way.

We have also demonstrated that the difficulties encountered by Ward *et al.* (1975) in their studies of deoxyhaemoglobin may be easily overcome by different computing procedures as these authors themselves have foreseen. The use of the fast rotation function, and the fast Fourier method for structure factor calculation means that the computing time needed for calculating rotation functions at quite high resolution is not excessive. We had no difficulties in determining the orientation of the individual subunits. The results of the translation searches with a single dimer as the search unit were also unambiguous and confirmed by the multidimensional version of the program. It must be noted that we were successful in the determination of the rotational and translational parameters separately for both of the subunits in the asymmetric unit. We believe that the computing methods we used may prove very helpful in the determination of complex structures such as complexes of proteins and nucleic acids or enzymes and macromolecular inhibitors in which the molecular structure of one of the components is known from previous studies.

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