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Intermediate Structure of Normal Human Haemoglobin : Methaemoglobin in the Deoxy Quaternary Conformation

LEIGH ANDERSON

Medical Research Council Laboratory of Molecular Biology
Hills Road, Cambridge, England

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This paper describes a new method of producing a crystalline intermediate between the unligated and ligated states of haemoglobin, suitable for X-ray analysis, by the use of a lattice strengthening reagent. Acrylamide is polymerized in the liquid of crystallization after the crystal has grown, forming a stiff supporting gel between the haemoglobin molecules, but not covalent bonds with them. The structure of human haemoglobin A crystallized in the deoxy quaternary structure (T-state†) and then oxidized by air after lattice strengthening (tertiary structure made met, or r-state) was determined to 3·5 Å resolution by the difference Fourier technique. Marked changes in tertiary structure in the region of the haem pockets and the contacts between the subunits ($\alpha_1\beta_2$) are observed. The iron is seen to move towards the plane of the porphyrin, causing a change of tilt of the haem. This appears to act as a lever setting in train stereochemical changes that loosen several hydrogen bonds within and between subunits, on which the stability of the tertiary and quaternary deoxy structures depend. The liganding water molecule itself causes a slight opening of the haem pocket in the α subunit, and a substantial one in the β subunit. The structural changes seen here in going from the tertiary deoxy to the aquomet state within the quaternary T-structure are similar, but opposite, to those seen earlier in going from aquomet to deoxy in the quaternary R-structure of BME-haemoglobin. Changes in tertiary structure associated with addition of ligand to the T-structure or the removal of ligand from the R-structure are thus seen to be complementary. Electron density maps show the α haems to undergo autoxidation more readily than the β haems, just as the β haems were reduced more easily than the α haems in BME-haemoglobin.

1. Introduction

The stereochemical basis of haemoglobin function has been elucidated by a number of stages, beginning in 1960 with the solution to 5·5 Å resolution of the molecular structure (Perutz *et al.*, 1960). Comparison of oxy and deoxy structures† at low resolution revealed a well-defined change in the relation of the subunits to one another (the change of quaternary structure) but no alteration in the tertiary configuration of the

† Abbreviations used: a notation based on that of Monod *et al.* (1965) for allosteric proteins is used throughout. T (for tense) refers to the deoxy quaternary arrangement, while R (for relaxed) refers to the oxy or met type arrangement, in which the subunits normally have tertiary structures more similar to those of dissociated monomers. Lower-case letters refer to tertiary structural states associated with absence (t) or presence (r) of ligand. BME, bis-(*N*-maleimidomethyl)-ether; MA, met-acryl; DA, deoxy-acryl.

subunits (Muirhead & Perutz, 1963). Later comparison at 2·8 Å resolution did show changes in tertiary structure, but failed to unravel the mechanism by which these brought about the change in quaternary structure. Since X-ray data represent an average over time and over many molecules, a better stereochemical understanding of the pathway(s) by which conformational changes take place can only be had by studying crystals of stable intermediates. Correlation of functional properties with structure in mutant haemoglobins has identified some of the crucial regions of the molecule, mutations which lock the quaternary structure (some haemoglobins M) being especially useful (Greer, 1971). In such cases the effect of ligand binding on the α or β subunits can be observed in the absence of a change of quaternary structure. In BME-Hb, a chemically modified haemoglobin, the situation is similar, but here the lock is a bifunctional chemical reagent which cross-links the Cys F9(93) and His FG4(97) of each β chain (Simon *et al.*, 1967; Moffat, 1971). A permanent oxy quaternary structure results. Early attempts to cross-link normal haemoglobin crystals with glutaraldehyde in the manner of Quiocho & Richards (1964), were unsuccessful.

Interpretation of movements associated with ligand binding would be easier if the work could be carried out on unmodified Hb A, the normal human component, but the ligand-linked change in quaternary structure characteristic of Hb A normally causes its crystals to break up when their ligation state is altered. Apparently the crystal lattice forces, amounting to no more than about five kilocalories per mole for haemoglobin, cannot easily maintain the molecules in their original quaternary conformation (though this is in some cases possible; see Materials and Methods, section (g)). It occurred to me that use might be made of the crystal's water spaces (about 50% of the crystal volume) to incorporate some inert and amorphous polymeric material that would mechanically clamp each molecule in its original quaternary structure, while allowing various small tertiary movements as on ligand binding. Initial attempts to soak acrylamide monomer into deoxy-Hb A crystals failed, presumably because low-level spontaneous polymerization clogged the water channels at the crystal surface and prevented further diffusion. Later it was found that co-crystallization of deoxy-Hb A with small concentrations of monomer allowed the latter to be polymerized inside the crystals, yielding a restrained but unaltered deoxy structure. Oxidation of these crystals with air gave the met-acryl (T;t $\rightarrow$ r) crystals used in this investigation. Similar attempts have produced horse met crystals capable of being fully reduced (R;r $\rightarrow$ t) without breaking up. These have not yet been studied crystallographically but should soon provide a check of the BME-Hb map.

In future other, perhaps preferable, restraining matrix materials may be found; the various styrenes and methacrylates in particular deserve to be tried. Acrylamide has been used here because it was available in the laboratory.

2. Materials and Methods

(a) *Crystallization*

The addition of acrylamide to the standard human deoxy-haemoglobin crystallization mixture of Perutz (1968) yields no crystals, since the protein's solubility is greatly increased. Therefore the following modification of the usual recipe was used (contents of the best vial from a range of salt concentrations are described): 1·0 ml saturated ammonium sulphate, 0·6 ml distilled water, 4·2 ml solution C and 0·1 ml acrylamide solution I, to which is added (under pure N_2) 0·7 ml human oxy-haemoglobin (6%) and 0·1 ml ferrous citrate. All solutions are as normally used (Perutz, 1968; solution C is made of 0·8 vol.

4 M-$(NH_4)_2SO_4$, 0·05 vol. 2 M-$(NH_4)H_2PO_4$ and 0·15 vol. 2 M-$(NH_4)_2HPO_4$), except acrylamide solution I, which is made from 30 g acrylamide and 0·8 g bis-acrylamide (BDH Chemicals, Ltd) dissolved in 93 ml solution C and 5 ml water. This mixture was biphasic and required shaking before use. It was later observed that crystals of slightly better morphology were obtained through use of fresh solution containing acrylamide recrystallized from ethyl acetate.

(b) *Treatment of crystals*

Deoxy crystals prepared in the above manner were rather tightly stuck to the sides of their vials and had to be broken off; this seldom damaged the removed portion, but did produce small conchoid surface fractures as in chipped glass. These crystals were soaked for about 6 h in a 1:1 mixture of mother liquor and a substitute containing an equivalent concentration of buffered ammonium sulphate saturated with riboflavin. They were then exposed to u.v. light (3 cm from a 30 W Hanovia Chromatolite) for 12 to 18 h while lying beneath 2 mm of the same fluid in a quartz cuvette. Polymerization of the interstitial acrylamide was largely completed by this procedure as evidenced by the residual crystal-shaped gel remaining when the crystal's haemoglobin had been removed by exposure to low salt. The mother liquor produced only a little polyacrylamide precipitate on irradiation.

(c) *Fingerprinting of haemoglobin from polymerized crystals*

A straightforward fingerprinting procedure (pH 6·5 electrophoresis followed by chromatography in a 15:3:10:12 (by vol.) mixture of butanol/acetic acid/water/pyridine; Kilmartin & Clegg, 1967) was used to compare the tryptic peptides of haemoglobin eluted from polymerized crystals with those from normal Hb. No significant differences were found.

(d) *Addition of ligand*

On exposure to air, polymerized deoxy crystals were oxidized in 10 to 18 h, as judged by their colour under the microscope. Attempts to get more reproducible oxidation with ferricyanide were unsuccessful because the crystals broke up. Met-Hb crystals were mounted (in air) in quartz capillaries and used as soon as possible. A continuous, slow degradation followed that was made worse by exposure to X-rays and by the presence of bulk liquid. Deoxy crystals were exposed to CO in an attempt to produce ferrous liganded T-state crystals, but these always broke up, for reasons explained below.

(e) *Data collection and processing*

Two sets of 3·5 Å data (including Friedel related reflections) were collected on a Hilger–Watts four-circle diffractometer controlled by a Ferranti Argus 400 computer. The first was from the deoxy-acryl structure (not exposed to riboflavin or u.v., but presumably polymerized by the X-rays); the second from the met-acryl. Deoxy-acryl crystals seemed to show much sharper peaks in the omega-scan mode than normal; otherwise they were quite ordinary. Neither their temperature factors nor their longevity were remarkably improved. Met-acryl crystals were comparatively short-lived and tended to die in variable fashions, sometimes by the normal linear degradation and sometimes by abrupt dissolution after hours of uniform data collection. These effects necessitated extensive editing and partial re-collection of the met-acryl data. The over-all mean standard deviations in scaling different crystals together were 2·6% of mean structure amplitude for deoxy-acryl and 5·0% for met-acryl, reflecting the poorer quality of the latter crystals.

Difference Fourier syntheses of deoxy-acryl minus native (based on 4700 of 7000 independent reflections, mean isomorphous difference of 5·4%) and met-acryl minus deoxy-acryl (5165 reflections, 10·5% mean difference) were computed using the native amplitudes and phases obtained by Muirhead & Greer (1970). After orientation to the standard skew planes axes, the MA—DA map was symmetry averaged about the molecular 2-fold axis and both were transferred to clear plastic sheets (by projection) in sections at 1 Å intervals perpendicular to y for interleaving with the native Fourier. Selected sections were drawn larger and superimposed on the horse deoxy-haemoglobin model (2 cm = 1 Å) in a Richards-type optical comparator (Richards, 1968).

(f) *BME map*

The met-BME minus deoxy-BME difference Fourier was calculated by Perutz and Greer. The map has been described in part before (Perutz & Ten Eyck, 1972; Perutz, 1970).

(g) *Oxidation of native deoxy crystals (no acrylamide)*

Human deoxy-haemoglobin crystals prepared in the usual way (Perutz, 1968; without acrylamide) were exposed to air to check Perutz's observation (personal communication) that most of the diffraction pattern of deoxy-haemoglobin crystals disappears after they have been exposed to air. Abrupt exposure to air, as in the case of acrylamide, yielded intact crystals diffracting to 4 or 5 Å. Slow oxidation, achieved by opening vials of crystals to air (crystals on vial bottom beneath 2 cm mother liquor) and inverting vials 1 month later (crystals suspended in air), yielded, after a further month, T-state met crystals diffracting to better than 3·5 Å. Intensities were similar to those observed with met-acryl, which was proved to be in the T-state. The native T-met crystals were not as stable under X-rays as crystals containing acrylamide, but their existence indicates that purely crystal forces are very nearly able to maintain metHb in the T-state.

3. Results

The deoxy-acryl minus native difference Fourier (Plate I) shows no significant features and supports the conclusion that the interstitial gel network is not covalently bound to the protein, or at least not with appreciable frequency at any particular sites. This is further reinforced by the observations that practically all the haemoglobin contained in polymerized crystal can be eluted from the gel by exposure to low salt and that the eluted material produces a normal tryptic fingerprint. Hence it seems justifiable to consider the deoxy-acryl structure as having the full structural integrity of Hb A which is being held in a form-fitting molecular net.

The met-acryl minus deoxy-acryl difference Fourier (Plates II to V) displays a number of interesting features, the most obvious of which are the large ligand peak near the α-haem iron (Plates II and III; marked $\underline{H_2O}$) and much smaller ligand peak at the analogous β-haem position (Plate V). The ratio of integrated densities is roughly 1·4 to 1, with a conservatively estimated 4·8 new electrons, instead of the expected ten present at the α-haems (the estimate of new electrons includes the necessary factor of 2; Luzzati, 1953). This indicates that most of the β haems were not oxidized at the time of data collection, despite checks of large difference reflections to ensure this, and that associated structural changes in the β-haem pocket are probably seen at reduced relative amplitude. However, it also provides direct evidence that the α haems of Hb A in the deoxy quaternary structure are more easily oxidized on exposure to air than the β haems, though this finding does not strictly require a greater ligand affinity for the α chains. A similar result is obtained when trying to reduce BME-haemoglobin (oxy structure); the β chains are more easily reduced (Perutz, 1970).

A number of structural features also parallel the BME difference map, particularly in regions removed from the BME substituent. This is fortunate, since there are many statistically significant features dispersed widely throughout the molecule; correlation between two maps of similar processes (addition and removal of ligand) helps to discard some of the noise and points to important features, particularly those of a locally reversible nature.

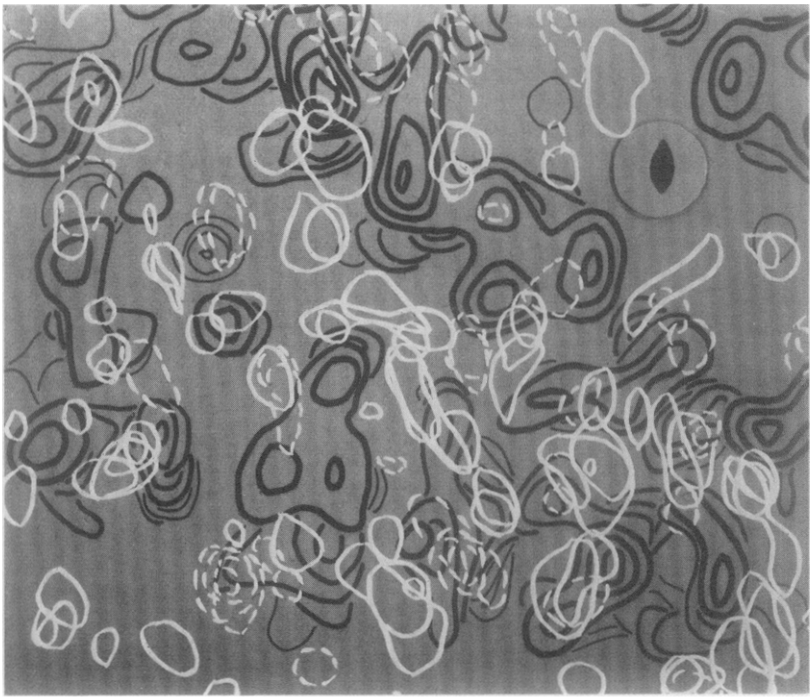

PLATE I. Difference Fourier synthesis of deoxy-acryl minus native deoxy-haemoglobin (white contours at 0·015 e/Å^3 intervals, negative dashed, zero omitted, sections $y = +13$ to $+8$) superimposed on the native Fourier (black, higher contours at 0·15 e/Å^3 intervals obscuring those below, width also proportional to height). The solid black crystallographic symbol marks the molecular dyad axis. There are virtually no features as high as 2 contours (first level of MA − DA, Plates III to VI); these few would be substantially flattened by symmetry averaging. Hence no features comparable to those in MA − DA are seen; features apparently showing several contours are merely the result of stacking 6 difference map sections.

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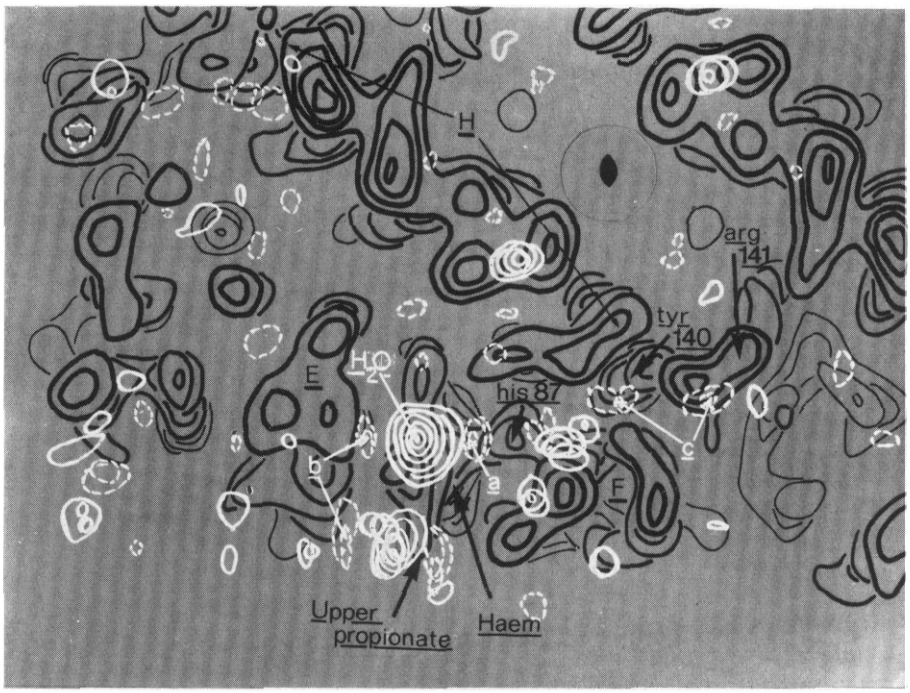

PLATE II. Sections +13 to +8 of the symmetry averaged difference Fourier synthesis of met-acryl minus deoxy-acryl haemoglobin (white, 0·013 e/Å^3 intervals, zero and first level omitted) superimposed on the native Fourier. This is the upper half of the α-haem pocket. Labels which are underlined refer to the α chains; otherwise β chain. Capital black letters are helices. a, Negative peak behind the iron, showing its movement into the porphyrin plane. b, Negative peaks indicating a movement of E helix residues back from the haem. c, Negative peaks indicating a slight delocalization of the penultimate tyrosine HC2(140) and the attached main chain. H_2O marks the water molecule ligand, some of whose contours have been eliminated for clarity.

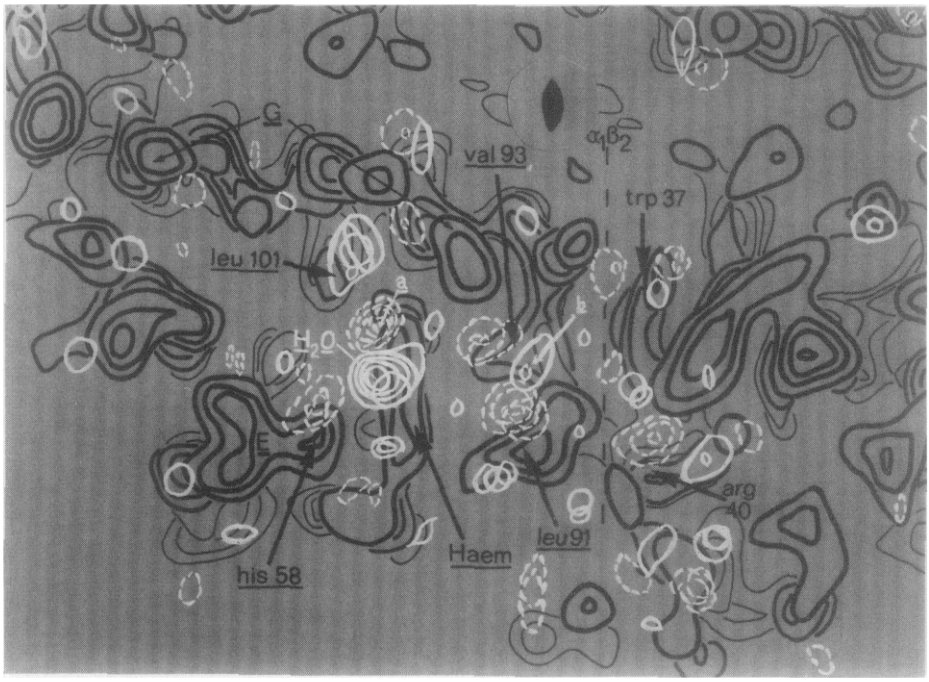

PLATE III. Lower half of the α-haem pocket ($y = +7$ to $+3$) in MA − DA. H_2O marks the lower portion of the same water molecule as in Plate II. a, Negative peak indicating a movement of the haem's bottom inside corner away from Leu G8(101) and toward Val FG5(93). Also near Val 93 is a positive peak (b), indicating an upward movement of Tyr C7(42)α (Plate IV).

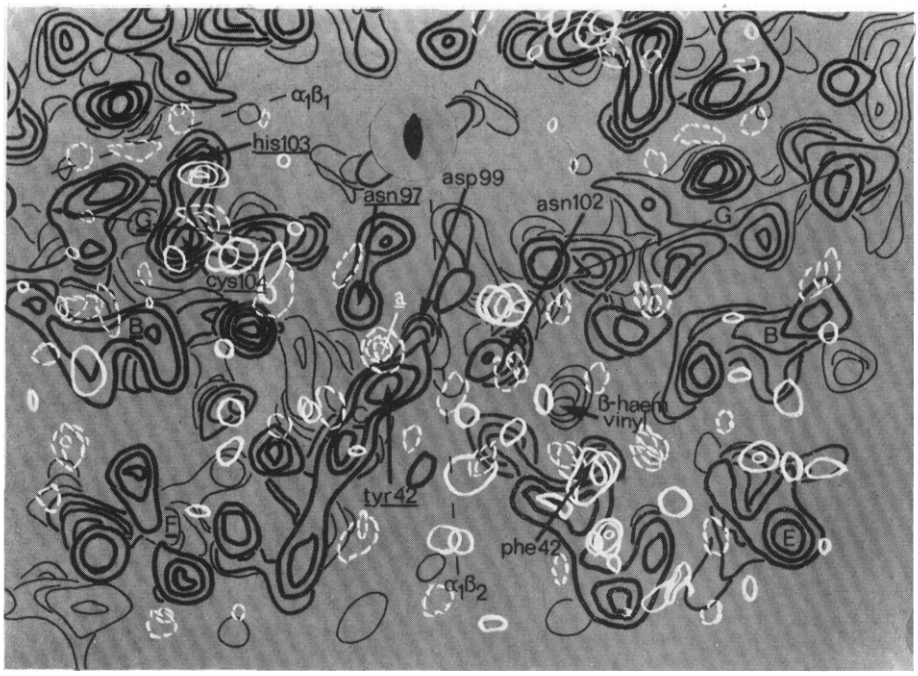

PLATE IV. Main contact regions between α and β subunits ($y = +1$ to -4) in MA — DA. Pushing and sliding movements are visible at the $\alpha_1\beta_1$ interface but no large well localized movements are discernable except for His G10(103)α and Cys G11(104)α. At the $\alpha_1\beta_2$ interface, a movement of Tyr C7(42)α away from its haem and upward toward Val FG5(93)α (b in Plate III) weakens the T-state intersubunit hydrogen bond to Asp G1(99)β. Feature (a) shows the separation (H-bond breaking) between Tyr C7(42) and Asn G4(97); Asn G4(102)β swings upward approaching its position in the R-state hydrogen bond to Asp G1(94)α. The β-haem vinyl (top inside haem corner) pushes (or follows) Asn 102.

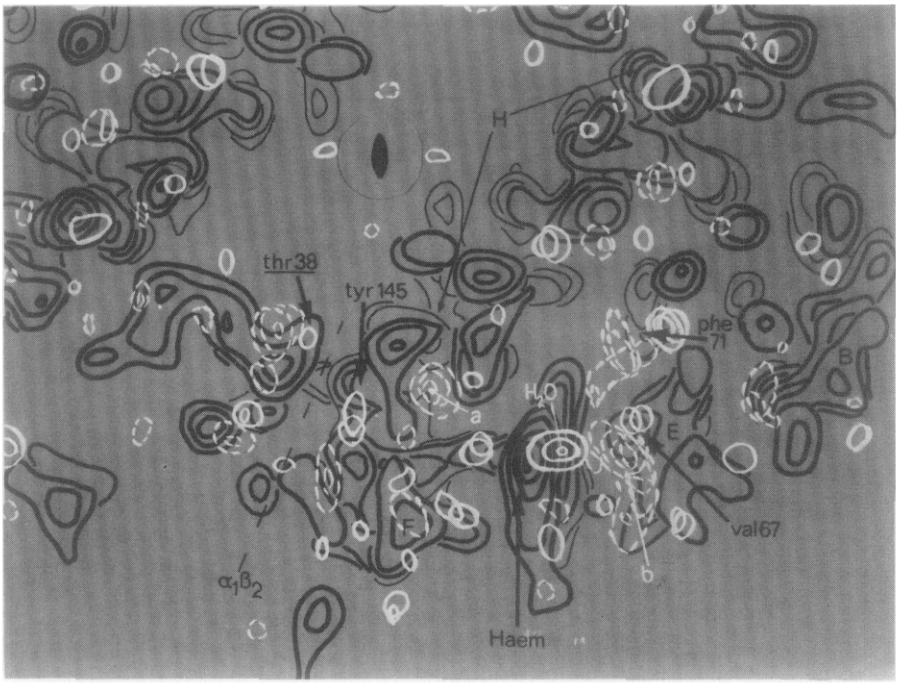

PLATE V. Central portion of the β-haem pocket in MA — DA. H_2O marks the smaller β-haem water ligand peak. a, Negative peak between the β-chain penultimate tyrosine and Phe G5(103), possibly indicating partial delocalization of the tyrosine. b, Large negative region indicating an opening of the β-haem pocket.

(a) *The α-haem region*

There are four important movements of the α haem itself (Plates II and III). The first is a shift of the iron atom towards the plane of the porphyrin ring (a in Plate II) corresponding to the expected decrease in the iron's atomic radius on transition from high spin ferrous to high spin ferric (Hoard, 1968; Perutz, 1970). The displacement of the iron from this plane is normally 0·75 Å in deoxy (T), 0·3 Å in met (R) and very near zero in CO (R) haemoglobin (Huber *et al.*, 1970; E. Heidner, personal communication); evidently oxidation to met has pulled the iron substantially toward the porphyrin plane, though probably not by the full 0·45 Å deoxy (T)— met (R) difference. The iron movement peak is small, consistent with the notion that tension at the haem, characteristic of the T-structure, prevents its approaching as close to the haem plane as in met (R). The even larger movement, almost into the plane, which ought to occur on combination with CO, cannot be observed in this T-state system since the crystals break up. Presumably a transition to the R quaternary structure takes place in this case despite the restraining polyacrylamide gel. A second feature is the strong movement of the haem's bottom inside corner toward the haem-proximal side (a in Plate III) pushing Val FG5(93) before it and pulling Leu G8(101) behind. Val FG5(93) and its immediate neighbours are important in shaping the $\alpha_1\beta_2$ interface, which suggests that this movement may be the beginning of a crucial rearrangement in that area. Finally, there are the shifts observed in the haem propionate groups. The upper group shows a large movement toward Lys E10(61) (b in Plate II), which is pushed back, while the lower one shifts slightly in the opposite direction (Plate III). The upper propionate may be displaying a magnified movement of the haem corner to which it is attached, since this is diagonal to the lower one first mentioned and might be expected to move in the opposite sense during rotation about the other two, fairly rigid, haem corners. These movements and their relation to the $\alpha_1\beta_2$ interface are shown schematically in Figure 1.

The BME difference map tells substantially the same story in reverse (since ligand is being removed), save that the iron's movement is not seen and that the lower propionate's movement is much larger. If it is only the quaternary T-structure

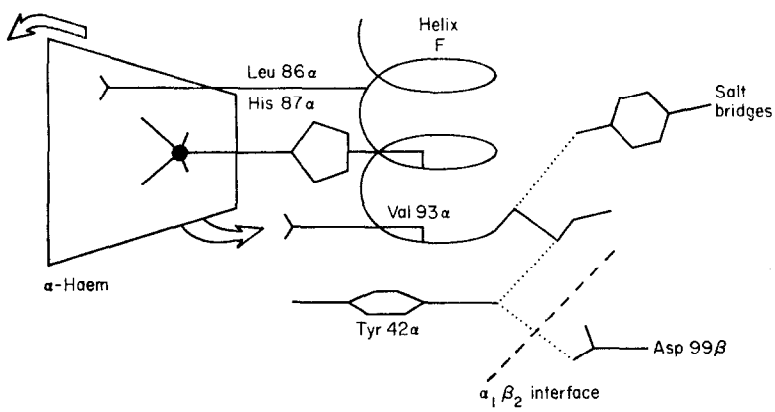

FIG. 1. Schematic diagram showing the proposed mechanism of α-chain tertiary structure change on ligation. A decrease in porphyrin–His F8(87) separation causes helix F to move closer. The iron acts as a ball and socket joint. Leu F7(86) is rigidly attached to His 87 and pushes strongly on the upper outside haem corner, while Val FG5(93), less securely fixed to His 87, is displaced by the opposite haem corner, and the nearby main-chain hydrogen-bonding network loosened.

that exerts tension on the haem, lengthening the iron–nitrogen bonds significantly (Perutz, 1972), then the lack of a visibly large iron movement might, in fact, be expected; in BME (R-state), the transition from ferric to ferrous ought to lead to a smaller displacement of the iron than in the T-structure. However, the evidence on this point is still ambiguous, since in the BME structure a smaller movement of the iron may be compensated for by a larger one of the porphyrin ring.

In both met-acryl and BME, the haem pockets are seen to rearrange themselves in almost identical ways to conform to the modified haem configuration. The steric influence of the ligand is seen as well, pushing the haem-facing residues of helix E away from the haem in MA—DA (b in Plate II; His 58 in Plate III), while its loss brings them closer together in BME. The α-chain F helices of the two structures behave rather differently due to a large change in the configuration of Tyr HC2(140)α in BME, but not in MA—DA. Nevertheless, it appears that the inclination of helix F over the haem changes analogously in both difference maps. Addition of ligand causes the top of helix F to move towards the haem, and the bottom of the helix (Val FG5(93)α) away from it. Inspection of the model shows that this type of movement could easily be produced by a shortening of the haem–histidine distance, as observed in this structure and known to occur generally on ligation of deoxy-Hb. The resulting greater haem tilt is consistent with recent detailed comparisons of oxy and deoxy structures (A. D. McLachlan, personal communication). Removal of ligand brings about the opposite movement.

(b) *The $\alpha_1\beta_2$ interface*

The $\alpha_1\beta_2$ subunit interface, and not $\alpha_1\beta_1$, is probably the one responsible for initiating haem–haem interaction, since it is the site of the largest differences between oxy and deoxy structures, and is on the shortest line connecting a pair of interacting haems. The met-acryl minus deoxy-acryl difference map supports this conclusion.

In this intermediate structure the expulsion of tyrosines HC2(140)α from their pockets between helices F and H has not yet occurred (Fig. 2). The delocalization of the α-chain C-terminus and consequent breakage of inter-α-chain salt bridges which such an expulsion implies has been identified by Perutz (1970) as a necessary step in the transition from the deoxy to the oxy quaternary structure. Hence the bonding pattern in the region of the $\alpha_1\beta_2$ interface and the integrity of the salt bridges identifies it as a deoxy quaternary structure. The beginnings of the changes to the quaternary oxy structure should nevertheless be visible, since the α haems have taken on ligand, and, in fact, they are. Tyrosine HC2(140)α is seen to move slightly towards freedom (c in Plate II) but is stopped by one or a combination of several effects: the steric interference of Trp C3(37)β, just across the interface; a hydrogen bond with the main chain of Val FG5(93)α; or the restraint exerted on the exposed C-terminus by the acrylamide network. Probably all these are involved. As a result of the small movement allowed, the main chain of Tyr (140)α becomes slightly more delocalized. Arginine HC3(141)α_1, on the other hand, remains completely stable, as do its salt bridges to Val NA1(1)α_2 and Asp H9(126)α_2.

The β chain's penultimate Tyr HC2(145)β moves slightly away from the haem and Phe G5(103)β (negative peak a in Plate V), but neither it nor the attached His HC3(146)β is appreciably delocalized.

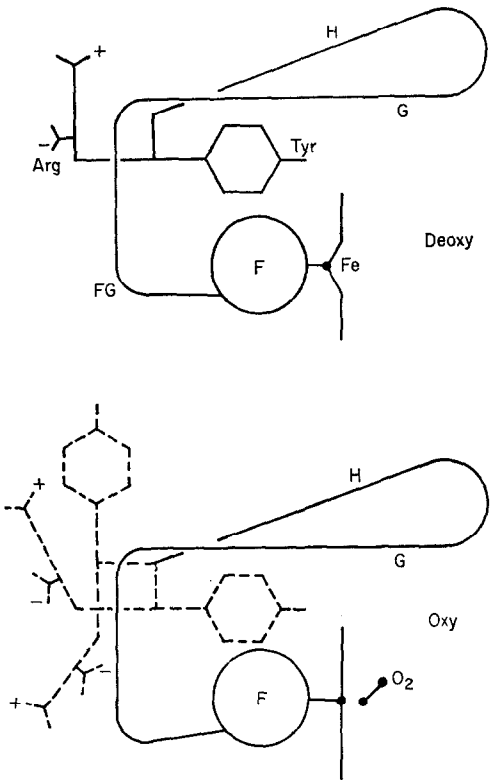

FIG. 2. Schematic diagram of the configuration of the α-chain penultimate tyrosine (HC2(140)) and adjoining salt bridge in the normal deoxy and oxy quaternary structures.

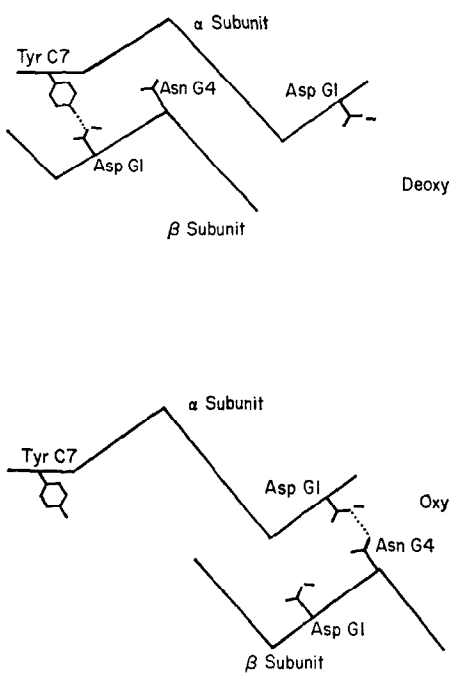

FIG. 3. Schematic diagram of the configurations of the $\alpha_1\beta_2$ interface associated with deoxy and oxy quaternary structures.

Two other stereochemical aspects of the $\alpha_1\beta_2$ interface seem to be pointing to the imminence of quaternary structure change. First, there are the beginnings of a changeover from the deoxy to the normal oxy hydrogen-bonding network (Fig. 3). The movement of Val FG5(93)α and with it Tyr C7(42)α (hydrogen-bonded to the Val FG5 main chain; b in Plate III) appears to break the intra-α-chain hydrogen bond Tyr C7(42)α—Asn G4(97)α (a in Plate IV) and to appreciably loosen the interchain hydrogen bond Tyr C7(42)α—Asp G1(99)β_1, though Asp G1β does not itself move far. Asn G4(102)β has broken its intra-β-chain hydrogen bond to the main chain of Asp G1(99)β and has swung upwards, nearer to Asp G1(94)α, its interchain hydrogen bond partner in the oxy configuration. This latter movement is particularly interesting since Asn G4β is in van der Waal's contact with the top inside corner β-haem vinyl. This group seems to follow (or cause) the asparagine's movement in the present structure. Asp G1(94)α, toward which Asn G4β swings, has adopted a more randomized configuration and spends less time pointing toward the internal cavity. The bond Asn G4β—Asp G1α has probably not yet formed. Thus there is a net loss of perhaps one characteristic T-state hydrogen bond across the interface and a movement toward formation of a new, R-state, bond.

In addition, there are changes in the dovetailing of the β chain F-G corner with the C-D corner of the α chain. In the normal deoxy (T-state) structure, Thr C6(41)α fits like a knob in the indentation formed by Val FG5(98)β's main chain, whereas in normal oxy (R-state), the next knob along, Thr C3(38)α, fits in this same notch. In the met-acryl structure, the knob in question is still Thr C6α, since the quaternary structure has not changed, but it can be seen to have moved towards the outside face of the notch, the root of His FG4(97)β. Further, the notch itself is being pulled away and down by the movement of helix F-β in response to the pull on the haem-linked histidine. Thus the dovetailing also appears ready to shift toward the R-state arrangement.

The BME map also shows several movements at the $\alpha_1\beta_2$ interface, chief among them a large shift in Asn G4(102)β, indicating the rupture of its oxy-structure hydrogen bond to Asp G1(99)β. However, other features in this region are of little value for the present comparison, since the starting configurations of deoxy and oxy are so different.

(c) *The β-haem pocket*

Even the low ligand occupancy observed results in a marked expansion of the β-haem pocket. The whole lower portion of the E helix, in particular residues His E7(63), Lys E10(66), Val E11(67) and Phe E15(71), is pushed back and down (b in Plate V). The B helix behind it is also forced back. On the proximal side, there is a slight shift of helix F toward the haem and downward. The haem itself shows only a shift of its top inside vinyl group toward the $\alpha_1\beta_2$ interface (Plate V) and a movement of its upper propionate in the opposite direction. Both haem movements appear to affect the $\alpha_1\beta_2$ interface, the vinyl by interaction with Asn G4(102)β as mentioned previously, and the propionate by interaction with Phe CD1(42)β, which hangs down above it. The importance of these effects, in comparison with movements of the α haems, is difficult to assess because of their relatively lower amplitude. Nevertheless, it seems clear that ligand binding markedly widens the β-haem pocket, whereas it only widens the α-haem pocket very slightly. This confirms the results expected from comparison of met and deoxy models (Perutz, 1970), and also the changes seen in

BME. A more detailed comparison of the changes in the present structure with those in BME in the region of the F helix and the F-G corner yields few significant similarities, due probably to the proximity of the BME substituent (at Cys F9(93)β).

(d) *The $\alpha_1\beta_1$ interface*

By comparison to $\alpha_1\beta_2$, the $\alpha_1\beta_1$ interface is expected to be a passive contact, acting somewhat like a bearing surface at which sliding and rotational movements can occur during the change of quaternary structure. This view is based on the relative harmlessness of mutations at this interface, on its variability in different species and on the absence of any major differences between the intersubunit bonding pattern in the oxy and deoxy structures. The present map supports that picture. The only significant movements that occur are on the α-chain G helix and the β-chain G and H helices. Residues His G10(103)α and Cys G11(104)α (Plate IV) show the largest individual shifts; whether this is a result of interaction across the interface or through the neighbouring Leu G8(101)α (Plate III) in contact with the haem, cannot be judged. Interaction with a β-chain residue, Asn G10(108)β, may be important in the movement of His G10α, but otherwise movements on the G and H helices of the β subunit are small and delocalized. In general, this region points out the difficulties encountered in interpreting large clusters of features; the directions of movement cannot be determined. Important features are identified by the size of the associated peaks. Thus it is not possible to state with any certainty the direction of movements at the $\alpha_1\beta_1$ interface. With the exception of the ones around His G10, most of them are probably movements of entire helices, characterized by long tubes of negative and positive density. Eventually, the application of real-space refinement techniques might allow sense to be made of this chaos. But to apply them a resolution higher than 3·5 Å will be needed.

4. Discussion

The results of this investigation support several of Perutz's earlier conclusions about the mechanism of haem–haem interaction. In the first place, they confirm that the changes of tertiary structure that occur on ligation can be accounted for as being a purely mechanical consequence of the contraction of the iron–nitrogen bonds and, especially in the β subunits, of the steric effect of the ligand itself. The different roles assigned to the two types of subunit contact are also well illustrated. The $\alpha_1\beta_1$ interface, though it shows large collective movements, appears to be primarily a passive contact. No interpretable set of linked movements is found to connect α and β haems across this interface. The $\alpha_1\beta_2$ interface is different in that the total number of relative movements is smaller, but those that do occur are concentrated on individual side chains. A series of interactions can be traced involving, at each end, residues in van der Waal's contact with α and β haems and mediated between subunits by a specific rearrangement of hydrogen bonds.

The structure of met-acryl, when compared to BME, reveals, in addition, something about the simplicity of ligand-linked tertiary structural changes, and allows a simple mechanical model of the α-chain movements to be formulated. At least at the α haems, those tertiary movements involved in reducing met-BME haemoglobin (quaternary

R-state) are reproduced in the opposite sense in the oxidation of deoxy-acryl haemoglobin (quaternary T-state). Transition from either endpoint (R;r or T;t) toward a configuration intermediate between the two (R;t or T;r) produces similar changes in opposite directions. These tertiary changes are presumably the cause of the quaternary structure transitions that follow. Whether or not the tertiary changes that are caused by quaternary transitions (the second half of haem–haem interaction) are similar to those observed here cannot be said.

The simple mechanism (Fig. 1) that can be constructed to explain these ligand-linked movements works, in the α chain, roughly in the following way. Binding of ligand causes shortening of the iron–pyrrole nitrogen bonds, which pulls His F8 closer to the plane of the porphyrin ring. Leucine F7, which is rigidly linked to His F8, is in van der Waal's contact with the upper propionate side chain of the haem and acts as a pressure point, while the iron atom fixed to the centre of the haem acts as a ball and socket joint. Consequently, the upper outside corner of the porphyrin moves to the left, and the diametrically opposite corner moves to the right; little movement is observed at the outside bottom corner because this is sandwiched between Leu FG3 and Phe CD1. The tilting of the haem appears to provide the leverage for the other stereochemical changes in the α chain, including the loosening of several hydrogen bonds crucial to the stability of the deoxy quaternary structure. These include the bond between Tyr C7α and Asp G1β, which stabilizes the quaternary T-state, and the bond between Tyr HC2α and the main chain Val FG5α, which stabilizes directly the tertiary t-state. The latter is of special importance, since Perutz has suggested that its rupture may lead to the breakage of four of the six salt bridges peculiar to the quaternary T-structure. If the tertiary mechanism outlined here were observed, on the electron density map, to cause complete disruption of this bond, a plausible mechanism for the change of quaternary structure would be established as well. However, the bond is only loosened; ligand binding might act through some subsidiary mechanism to actually dislodge the tyrosines HC2 and break the salt bridges. In particular, let us consider the original model (Perutz, 1970) involving contraction of the tyrosine pockets. The BME difference Fourier showed the α-chain tyrosines inserting themselves between helices F and H, causing a marked displacement of the whole F helix toward the outside of the molecule. Measurements of the pocket's width in the oxy and deoxy models also showed a definite decrease in size on going from the T to the R structure (Perutz, 1970), since confirmed by refinement of atomic co-ordinates (A. D. McLachlan, personal communication). Hence it would seem reasonable to postulate an additional simple relation between ligation of each haem and the size of the adjacent tyrosine pocket, ignoring for the moment the additional effect of quaternary structure on tyrosine configuration. This simple hypothesis is not, however, consistent with the met-acryl structure, since the α haems, at least, are largely liganded and only a small negative peak is seen near the OH of the tyrosines. Rather, it is Val FG5 that is being pulled away from Tyr HC2. I take this to mean that a squeezing together of helices F and H is probably not the major cause of tyrosine expulsion, but more likely a result of it.

Since, in fact, the tyrosines (α chain, at least) occupy their pockets in three of the four simple haemoglobin conformations (T;t, T;r, R;t but not R;r) it follows that aspects of both the tertiary (haem-linked) and quaternary changes must be produced for loosening or expulsion to occur. Both types of change are, of course, caused by ligand binding but one is presumably the direct expression of it inside a subunit,

while the other is an interactive expression at the contact. The likely seat of a complex phenomenon such as this is, as suggested, at the $\alpha_1\beta_2$ interface; a contact between surfaces which show large relative movements on change of quaternary structure, yet are also closely linked to the tilt of the haem and associated tertiary changes caused by ligation of the irons. Both tertiary and quaternary structural strain produced at the $\alpha_1\beta_2$ interface would be relieved by loosening of the tyrosines and consequent breakage of the salt bridges.

What remains unclear in this picture is, how quaternary structure affects the oxygen affinity of the subunits. In deoxy BME, with the oxy quaternary structure, the tyrosines HC2(140)α are in their pockets. If the quaternary change alone has not removed the tyrosines, then either a tyrosine in its pocket has a very different effect on tertiary structure in the R-state than in the T-state, or subunit affinity must be affected by quaternary structure in some other way to produce co-operativity. The $\alpha_1\beta_2$ interface seems a more likely candidate. Expulsion or loosening of penultimate tyrosines appears to be an intermediate step in the transitions between T- and R-states; their action can be seen best as a mechanism for amplifying through salt bridges events around the $\alpha_1\beta_2$ interface.

The conclusions arrived at here are tentative as yet, for several reasons. The transition from deoxy- to met-Hb shifts the iron atom only about half way from its out-of-plane position in deoxy to its in-plane position in oxy-haemoglobin, so that only the first stages of the transition from the unliganded to the liganded tertiary structure are seen (i.e. perhaps r met is not identical to r oxy). At 3·5 Å resolution it was not possible to measure the atomic shifts or to detect the smaller ones. A firmer and more certain understanding of the tilting of the haems and the transmission of the stereochemical changes from the haem to the subunit interfaces will have to await higher resolution and a system where the transition from deoxy- to CO-haemoglobin can be studied in the R- and T-structures. Nevertheless, the precedence of the $\alpha_1\beta_2$ interface in determining quaternary structure seems well-established.

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REFERENCES

Greer, J. (1971). *J. Mol. Biol.* **59**, 107–126.
Hoard, J. L. (1968). In *Structural Chemistry and Molecular Biology* (Rich A. & Davidson, N. eds), pp. 571–594, Freeman, San Francisco.
Huber, R., Epp, O. & Formanek, H. (1970). *J. Mol. Biol.* **52**, 349–354.
Kilmartin, J. V. & Clegg, J. B. (1967). *Nature (London)*, **213**, 269–271.
Luzzati, V. (1953). *Acta Cryst.* **6**, 142–152.
Moffat, J. K. (1971). *J. Mol. Biol.* **58**, 79–88.
Monod, J., Wyman, J. & Changeux, J.-P. (1965). *J. Mol. Biol.* **12**, 88–118.
Muirhead, H. & Greer, J. (1970). *Nature (London)*, **228**, 516–519.
Muirhead, H. & Perutz, M. F. (1963). *Nature (London)*, **199**, 633–638.
Perutz, M. F. (1968). *Journal of Crystal Growth*, **2**, 54–56.
Perutz, M. F. (1970). *Nature (London)*, **228**, 726–734.
Perutz, M. F. (1972). *Nature (London)*, **237**, 495–499.
Perutz, M. F. & Lehmann, H. (1968). *Nature (London)*, **219**, 902–909.
Perutz, M. F. & Ten Eyck, L. F. (1972). *Cold Spring Harbor Symp. Quant. Biol.* **36**, 295–310.

Perutz, M. F., Rossman, M. G., Cullis, A. F., Muirhead, H., Will, G. & North, A. C. T. (1960). *Nature (London)*, **185**, 416–422.

Quiocho, F. A. & Richards, F. M. (1964). *Proc. Nat. Acad. Sci., U.S.A.* **52**, 833–839.

Richards, F. M. (1968). *J. Mol. Biol.* **37**, 225–230.

Simon, S. R., Konigsberg, W. H., Bolton, W. & Perutz, M. F. (1967). *J. Mol. Biol.* **28**, 451–454.