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Structures of Deoxy and Carbonmonoxy Haemoglobin Kansas in the Deoxy Quaternary Conformation

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Haemoglobin Kansas (Asn102(G4)β → Thr) is the only widely studied mutant or modified haemoglobin having four functional haems and displaying lower than normal oxygen affinity. Two forms of this mutant have been investigated by X-ray diffraction. The deoxy form, whose crystals are isomorphous with the native form, has been examined directly by the difference Fourier technique (3·4 Å). In addition to the replaced residue itself, the difference electron density map shows signs of slight movements throughout the region between α and β haem pockets. However, neither type of chain shows stereochemical evidence of a very abnormal oxygen affinity in the tetramer. The nature of the perturbation is different from that proposed in the earlier, low-resolution study of Greer (1971*a*). Exposure of deoxy crystals to carbon monoxide produces two new crystal forms similar but not identical to the native deoxy form. One of these structures has been solved by rotation and translation function methods and a difference map between carbonmonoxy haemoglobin Kansas in the deoxy quaternary structure and native deoxy haemoglobin has been calculated at 3·5 Å resolution. Carbon monoxide molecules are observed at three of the four haems, and two unidentified large peaks‡ appear next to the sulphydryl groups of Cys93β. None of the interchain salt bridges which stabilize the deoxy quaternary state appears to be broken, but large tertiary structural changes are seen in the liganded chains. It seems that when the molecule is subjected to the lattice constraints of the deoxy crystal, the salt bridges do not break upon ligand binding, even though the pH dependence of the first Adair constant and the linearity of proton release with ligand uptake both imply that this does happen to stripped HbA in solution.

1. Introduction

It has been known for some time that haemoglobin achieves its co-operativity by being able to lower (rather than raise) the affinity of its subunits under certain circumstances (Benesch *et al.*, 1961; Antonini *et al.*, 1965*a*). The mechanism involved has been shown, by a variety of methods, to be directly related to the quaternary structure of the protein (Muirhead & Perutz, 1963; Ogawa & Shulman, 1972; Cassoly *et al.*, 1971; Perutz *et al.*, 1972; Lindstrom *et al.*, 1972) in accordance with the allosteric theory of Monod *et al.* (1965). In the oxy or R quaternary structure†, subunits associate relatively loosely to yield a tetramer whose ligand binding and other properties are similar to those of a sum of two free α and two free β chains. However, in the deoxy or T quaternary structure, specific added structural constraints (the salt bridges) stabilize a slightly different arrangement of the subunits; one which strains

† The nomenclature of quaternary and tertiary conformations is based on that of Monod *et al.* (1965); R and T states are the normal oxy and deoxy quaternary structures, respectively.

‡ See note added in proof on p. 49.

each subunit so as to produce a "tension at the haems" opposing the binding of ligand (Perutz, 1972; Perutz *et al.*, 1974*a*,*b*,*c*). It is the change of structure from T to R during the course of ligand uptake which results in co-operativity. Because of the delicacy of the constraints stabilizing the T state, and the relative insensitivity of the loose R state to perturbation, most mutations and chemical modifications of haemoglobin shift the allosteric (R $\rightleftharpoons$ T) equilibrium by varying degrees toward the R state (Edelstein, 1971), resulting in a raised oxygen affinity.

Haemoglobin Kansas (Asn102(G4)β $\rightarrow$ Thr) (Reismann *et al.*, 1961; Bonaventura & Riggs, 1968) is of interest primarily because it is an exception to this rule: it has a low oxygen affinity.

Perutz & Lehmann (1968) discussed haemoglobin Kansas at a time when only the structure of horse methaemoglobin but not that of deoxyhaemoglobin was known in atomic detail. They suggested that in the R structure a short contact between the C_γ of Thr102β and the vinyl and methyl groups of pyrrol II might displace either the haem or helix G. They also pointed out that absence of the bond between Asp99(G1)α and Asn102β should favour the ready dissociation into dimers which had been discovered by Bonaventura & Riggs (1968). When it became known that this hydrogen bond stabilizes specifically the R but not the T structure, Morimoto *et al.* (1971) suggested that the absence of this bond in Hb Kansas would destabilize this structure and shift the allosteric equilibrium towards the T structure. This shift has since been observed experimentally by Ogawa *et al.* (1972), at least in concentrated haemoglobin solutions, while in dilute solutions the dissociation of the oxy structure into dimers drives the equilibrium in the opposite direction. However, the oxygen affinity even of dilute solutions of haemoglobin Kansas is abnormally low, and Riggs & Gibson (1973) showed that this may be due to an intrinsically low affinity of the abnormal β chains. Greer's X-ray analysis of deoxyhaemoglobin Kansas at 5·5 Å resolution appeared to show a displacement of the haem and the adjoining helical regions, consistent with Perutz & Lehmann's hypothesis of the steric effect of Thr102β upon the haem in methaemoglobin.

I have re-investigated the structure of deoxyhaemoglobin Kansas at 3·5 Å resolution, and am unable to confirm the large displacements seen in Greer's map. In retrospect these features of his difference Fourier appear to be due to poor data combined with errors in scaling. I have also used haemoglobin Kansas to attempt a crystallographic test of the role of the salt bridges in the allosteric mechanism.

Perutz (1970) suggested that the uptake of ligand by each subunit may be directly coupled to the rupture of the salt bridge linking that subunit to its neighbour in the T quaternary structure (Figs 3 and 4); since we now know the larger part of the Bohr effect is due to the salt bridges, such a mechanism follows necessarily from the linearity of proton release with ligand uptake (Antonini *et al.*, 1965*b*; Gray, 1970) and the pH dependence of the first Adair constant (Roughton, 1964; Imai & Yonetani, 1974). Therefore if a fully ligated haemoglobin could be crystallized in the T state, it ought to have these intersubunit salt bridges broken. There are no methods of doing this, but under certain conditions crystals of deoxyhaemoglobin A can be oxidized to methaemoglobin without a change of quaternary structure (Anderson, 1973). The salt bridges in these crystals remain intact, but it is argued that the iron–nitrogen bond lengths in methaemoglobin are about halfway between those of oxy and deoxy, and thus that the change in tertiary structure may not have been sufficient to break the salt bridges.

Recent nuclear magnetic resonance and kinetic studies have shown that allosteric effectors (in particular inositol hexaphosphate) can maintain haemoglobin Kansas in the T state throughout the course of ligation, an effect that does not occur in haemoglobin A (Hopfield *et al.*, 1972). This observation suggested that crystal lattice forces might be sufficient to maintain haemoglobin Kansas in the deoxy quaternary structure even when the molecules have been saturated with carbon monoxide. Experiments confirmed this but showed that the reaction was accompanied by a small lattice change and large intensity changes, which made the usual difference Fourier method inapplicable. Instead, the position and orientation of the tetramer of CO-Hb Kansas in the T state had first to be determined by rotation and translation function methods (Rossmann & Blow, 1962; Crowther & Blow, 1967). Structure amplitudes and phases were calculated on the assumption that the tetramers have the tertiary and quaternary structure of deoxyhaemoglobin A. A difference Fourier synthesis at 3·5 Å resolution was then calculated, using the difference between observed and calculated amplitudes as coefficients with the calculated phases. The difference electron density map shows large peaks at the ligand positions of three of the four iron atoms, and larger unidentified peaks near the SH groups of both cysteines 93β, indicating that the major features of the map must be correct. The map indicates marked changes in the tertiary structure of the subunits; some interpretable movements in the β chain appear analogous to the shifts seen in the α chains on oxidation of T state haemoglobin A (Anderson, 1973). However, none of the salt bridges was shown to break, nor were any of the adjacent penultimate tyrosines (140α, 145β) displaced from the pockets they occupy in deoxyhaemoglobin.

2. Materials and Methods

(a) *T state deoxyhaemoglobin Kansas*

(i) *Crystallization*

Purified haemoglobin Kansas was obtained as a gift from Dr R. G. Shulman and was crystallized as deoxyhaemoglobin by Dr M. F. Perutz in the normal way (Perutz, 1968), but with the addition of a small amount of inositol hexaphosphate. This ligand did not bind because of the high salt in the mother liquor. The resulting deoxy crystals were isomorphous with deoxy HbA. The crystal pH was between 6·6 and 6·9, as measured by phenol red.

(ii) *Data collection and processing*

Deoxy crystals were mounted in capillaries inside a nitrogen-filled glove box. A complete set of 3·35 Å data (including Friedel pairs) was collected on two crystals using a computer-controlled Hilger & Watts four-circle diffractometer. The data (in nine shells) were scaled together (mean standard deviation of 2·7% on $|F|$) and finally matched with the 2·5 Å native set of Arnone & Ten Eyck to yield difference Fourier coefficients of the form $|F_{\mathrm{Kan}}| - |F_{\mathrm{nat}}|$ (mean isomorphous difference of 8·4%). The resulting difference map was contoured, plotted, and photographically enlarged onto 10 in × 12 in sheet film for interleaving with the native Fourier (Arnone & Ten Eyck, unpublished results).

(b) *R state carbonmonoxy haemoglobin Kansas*

(i) *Precession photography*

A 10° precession photograph of R state CO-Hb Kansas (crystals grown in the same manner as CO-HbA (Perutz, 1968)) was compared to that of CO-HbA. To 5·5 Å resolution the intensity distributions are practically identical, indicating highly similar structures. Direct high-resolution comparison is impossible because of slight non-isomorphism between the crystal forms, such as is seen in virtually all mutant and modified haemoglobins in thi system.

(c) *T state carbonmonoxy haemoglobin Kansas*

(i) *Exposure of deoxyhaemoglobin Kansas crystals to carbon monoxide*

Inside a nitrogen-filled glove box, deoxy Hb Kansas crystals were mounted in capillaries, flushed with moist CO and sealed. In two experiments, crystals were placed on the diffractometer immediately after the introduction of CO, and sets of four reference reflections monitored over a period of time; the intensities changed for about 6 h in each case and then became constant. It seems likely that the process observed was one of slow structural change, although the possibility of some systematic artifact such as movement of mother liquor, is not excluded.

While surveying a number of crystals exposed to CO, it became apparent that two distinct results could occur. The first (F1) had a unit cell very similar to that of deoxy HbA, but quite different intensities (Plate I(a)). The second (F2) had a very similar intensity distribution, but with a unit cell twice as large and little scattering power beyond 4 Å resolution (Plate I(b)). Since good crystals of suitable size were scarce, it was decided to collect data on F1. Crystals of deoxy Hb Kansas crystallized in the presence of acrylamide (see Anderson, 1973) and exposed to CO produced yet another form (F3) whose cell was doubled along the original a axis.

The unit cell of F1 exhibits a slight contraction along the monoclinic b axis, but is otherwise very similar to the native $P2_1$ cell (values of the native cell are in parentheses): $a = 63{\cdot}4$ Å (63·4); $b = 81{\cdot}7$ Å (83·6), $c = 53{\cdot}7$ Å (53·9), $\beta = 100{\cdot}2°$ (99·25).

(ii) *Data collection and preliminary processing*

A complete set of 3·5 Å data (including Friedel pairs) was collected on a single crystal of T state CO-Hb Kansas F1 (same diffractometer as previously), during which standard reflections decreased by ~15% of their intensity. Peak width in the ω-scan mode was ~0·8°, indicating a high mosaic spread. Mean standard deviation in scaling 9 shells

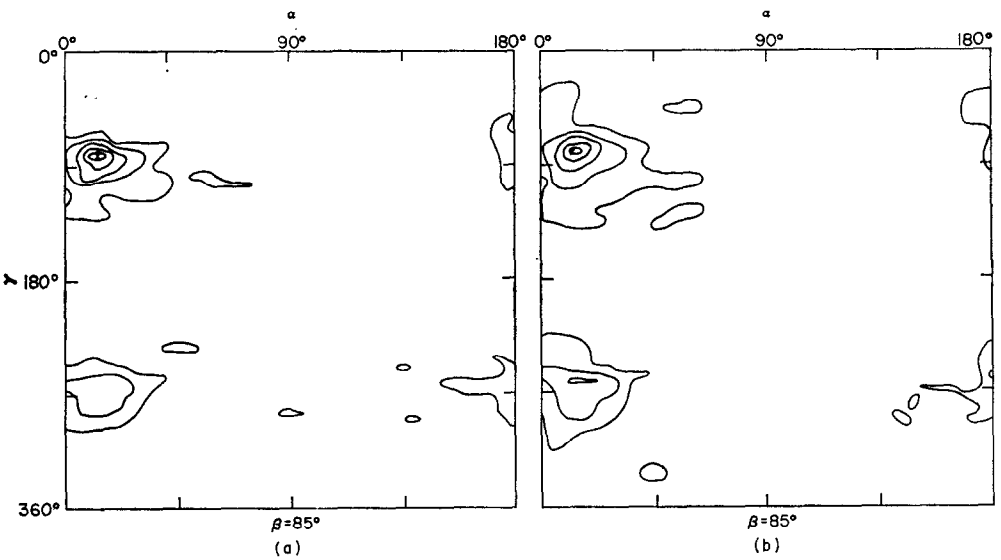


FIG. 1. Rotation function results, in terms of Euler angles, for (a) native deoxy haemoglobin *versus* a single native deoxy molecule in the standard skew orientation with neighbours removed, and (b) T state CO-Hb Kansas F1 *versus* the same single native molecule. The calculation was carried out with 1500 to 2000 large reflections between 25 Å and 4·5 Å resolution, expanding the Patterson over 30 orders of Bessel function and integrating overlaps out to a radius of 30 Å. The single molecule was placed with its dyad (y) along the crystal a^* and its x and z pseudo-dyads (horizontal and vertical in Plates II to IX) along the crystal c and b^*. The Euler rotations are: α about z, β about new y, γ about new z. A small x near the main peak indicates known orientation of the native molecule.

together by overlapping reflections was 7% of mean $|F|$. This data set has a mean $|F|$ of +15 (as compared with 26 for the native set) and a relative temperature factor of −15 when scaled directly against the native. Due to the large intensity changes and the diminished *b* axis, a direct difference Fourier synthesis showed no interpretable features.

(iii) *Paramercuribenzoate derivative*

Soaking in paramercuribenzoate yielded heavy atom deoxy Hb Kansas crystals of excellent quality (*h0l* Patterson based on 17° precession films was interpretable and very clean). However, upon exposure to CO, these crystals gave only F2 diffraction patterns and were thus useless in work with the F1 structure.

(iv) *Rotation function*

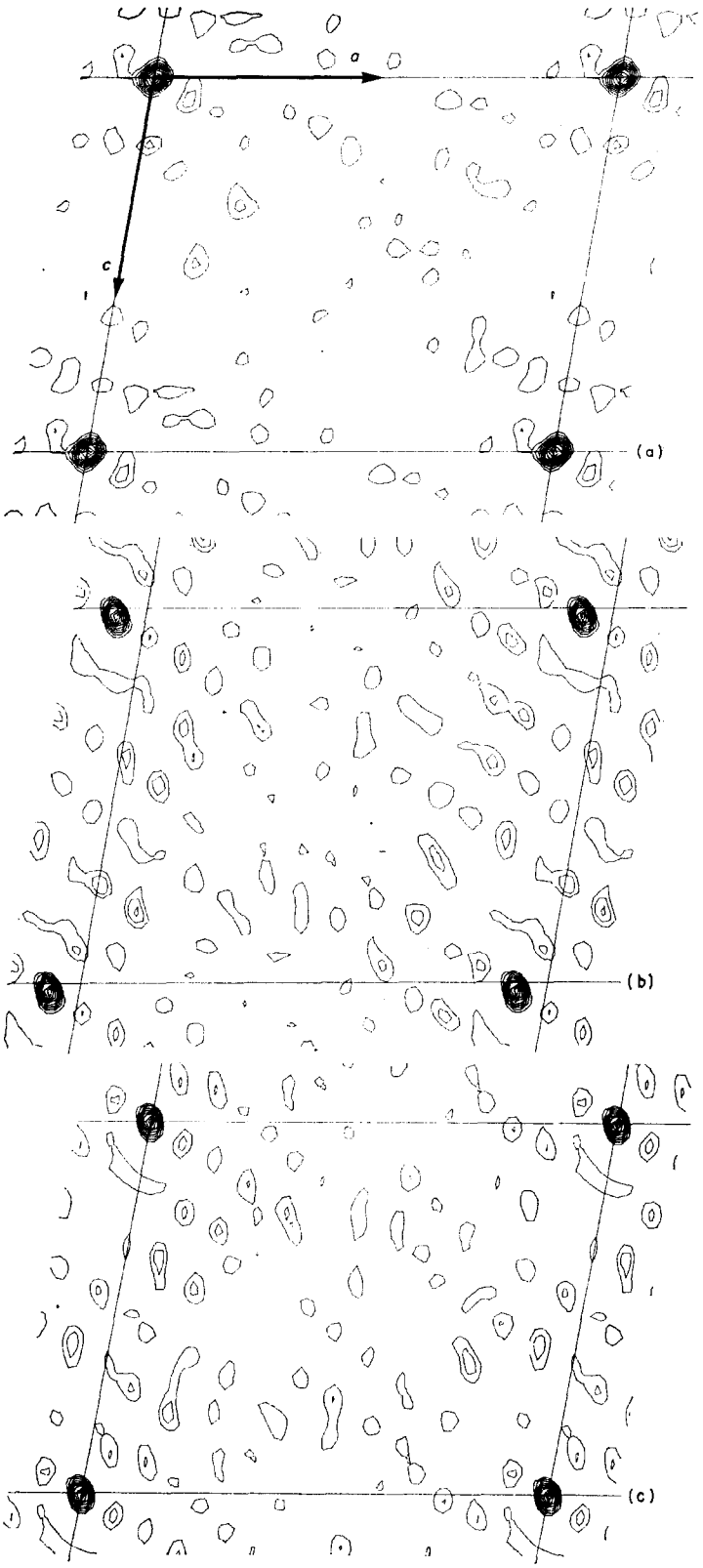
The rotation function is a mathematical technique for searching out orientational correlations between diffracted intensity distributions of two structures, and requires no knowledge of phases (Rossmann & Blow, 1962). The program used here, due to Crowther (1972) permits routine calculation with a large fraction of the total intensity on a very fine output grid. Figure 1 shows two such rotation function calculations, presented as asymmetric units in terms of Euler angles: Figure 1(a) shows the rotation function between the native ($P2_1$) deoxy Hb intensities and a computed set of intensities for a single molecule (made from the native 2·5 Å density, skewed to the dyad vertical orientation, with neighbours removed by a simple circular mask of varying radius for each section); Figure 1(b) shows the rotation function between the T state CO-Hb Kansas F1 intensities (also $P2_1$) and the same set of single native molecule intensities. In each case, the upper (stronger) peak represents the correct overlap of Hb tetramers, while the second (weaker) peak represents the superposition of α on β chains, and *vice versa*. The difference in orientation of the native and Kansas molecules is minimal (Table 1) and corresponds to a net rotation of $\chi = \sim 1{\cdot}5°$. Both rotation functions were calculated using ~1600 of the largest intensities for each set between 25·0 Å and 4·5 Å, with overlap in Patterson space integrated inside a sphere of 30 Å radius. The functions were generated on a grid of 2·5° in α, 1·0° in β, and 5·0° in γ, which is the useful limit of the present program in a situation of this symmetry. They are contoured in intervals of 10, where the maximum value is 55.

TABLE 1

Rotation function results

	Euler angles			Difference from angles in set	
	α	β	γ	(1)	(2)
(1) Angles predicted from known native orientation	~12·5°	~85·5°	~80·75°	—	1·3°
(2) Angles determined from native *versus* single molecule	13·25°	85·0°	81·0°	1·3°	—
(3) Angles determined from Kansas F1 *versus* single molecule	13·75°	84·5°	80·0°	1·5°	1·5°

Rotation functions were also calculated using only data between 4·0 Å and 3·0 Å resolution for native, and 4·0 Å and 3·5 Å for Kansas, with correspondingly reduced radius of integration in Patterson space. The native *versus* single molecule main peak was much sharper, and its position closer to that predicted, but the result for Kansas was inferior to the earlier, lower resolution calculation. This was due presumably to the poorer quality of the Kansas data and to the dissimilarity of Kansas and native (single) molecules at high resolution.


a
c
(a)
(b)
(c)

(v) *Translation function*

The translation function T_1 (Crowther & Blow, 1967) is a mathematical technique for determining the translation vector between two molecules (in a single crystal lattice) that are related by a known crystallographic rotational symmetry axis, provided the structure of the molecule is known. In this case the known structure of native deoxy Hb has been used to find the vector between two T state CO-Hb Kansas F1 molecules related by the crystallographic 2-fold screw axis. First a set of complex structure factors (F_s) was calculated for a Kansas unit cell containing a single native molecule (extracted from the native Fourier by a series of circular masks, one for each y section) in the known crystallographic orientation and position (i.e. phase origin at the native cell origin, on the 2_1 axis). Then the Fourier synthesis

$$T_1(t_x, t_y, t_z) = \sum_h \sum_k \sum_l (|F_{obs}(hkl)|^2 - |F_s(hkl)|^2 - |F_s^*(\bar{h}kl)|^2) \times F_s(hkl)F_s^*(\bar{h}k\bar{l}) \exp[-2\pi i(ht_x + kt_y + lt_z)]$$

was calculated between 8 Å and 4 Å, and the section at $y = \frac{1}{2}$ was plotted. (This section must contain the translation vector peak, since the crystallographic axis is a 2_1 axis.) Figure 2 shows $y = \frac{1}{2}$ sections from three such calculations: (a) native deoxy HbA *versus* single molecule (F_s) derived from native deoxy HbA, showing the expected vector of length $y = \frac{1}{2}$ extending vertically (perpendicular to the page) from the origin: (b) T state CO-Hb Kansas F1 *versus* single molecule (F_s) showing a lateral (in the page) relative displacement of the Kansas molecules of ~4·5 Å; (c) T state CO-Hb Kansas F1 *versus* a single molecule set identical to F_s except for a translation of the molecule by half the lateral vector seen in (b) (phase origin shifted), showing that the applied translation correctly represents the position of a single Kansas molecule with respect to the origin. The total translation vector between the Kansas molecules, in terms of native phase origins attached to each molecule (as in F_s), is: $x = -4{\cdot}0$ Å, $y = +40{\cdot}8$ Å ($\frac{1}{2}b$), $z = +1{\cdot}5$ Å.

(vi) *Construction of T state carbonmonoxy haemoglobin Kansas-like cell*

A knowledge of the orientations and relative positions of the two molecules in the T state CO-Hb Kansas F1 unit cell permitted the construction, in the computer, of an analogous cell containing native deoxy HbA molecules. This was accomplished using a set of very general programs for the manipulation of molecular densities and envelopes written by Gérard Bricogne. A single native molecule was removed from the native deoxy HbA Fourier, this time by a form-fitting three-dimensional envelope drawn from the Fourier on a tracing slate linked to a Modular One computer. The molecule was positioned correctly in the T state CO-Hb Kansas F1 cell, and all its symmetry related neighbours generated. No bad intermolecular contacts were observed. A set of 3·5 Å amplitudes and phases was calculated from this model cell, and these were used as the "native crystal data" in a normal difference Fourier against the Kansas F1 amplitudes. The crystallographic R factor between the two sets of data was 37% (as opposed to 48% between Kansas F1 and the real native data) and the resulting map is largely interpretable (Plates VI to IX).

(vii) *Crystal sulphydryl reactivity*

A large crystal of T state CO-Hb Kansas F1, prepared in the same way as those for data collection, was dissolved in water inside a nitrogen-filled glove box, and the solution transferred anaerobically to a Sephadex G50 column equilibrated with nitrogen-saturated buffer to separate the haemoglobin from the salt and ferrous citrate present in the crystal mother liquor. After treatment with CO, the sulphydryl reactivity of the haemoglobin was determined with paramercuribenzoate (Boyer, 1954). Based on calibration with a fresh,

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FIG. 2. Translation function results at $y = \frac{1}{2}$, for (a) native deoxyhaemoglobin *versus* a single native deoxy molecule in the crystallographic orientation and with phase origin on the $P2_1$ axis; (b) T state CO-Hb Kansas F1 *versus* the same native single molecule, showing displacement of Kansas molecules; (c) T state CO-Hb Kansas F1 *versus* a native single molecule translated relative to the phase origin by the amount determined in (b). All the data between 8 Å and 4 Å were used in each case. Black lines mark the boundaries of a single unit cell, with arrows a and c indicating the directions of these crystal axes.

pure, stripped CO-HbA solution, the Hb Kansas displayed 2·25 SH groups, while a control sample of HbA (obtained by dissolving, etc., a deoxy crystal as above) displayed 2·05 SH.

(viii) *Treatment of T state CO-Hb Kansas F1 with potassium ferricyanide*

When it became apparent that only three haems in Kansas F1 had bound CO, an attempt was made to dissolve crystals in the presence of potassium ferricyanide, oxidizing the single deoxy chain before it could react with ambient CO. Spectrophotometric examination of the result of this and similar control experiments with deoxy and CO HbA crystals showed a lack of reproducibility. Oxidation in the presence of only a small amount of protective ligand is apparently difficult to control.

3. Results

(a) *Deoxyhaemoglobin Kansas*

The difference Fourier of deoxyhaemoglobin Kansas (Plates II to V) is very clean and displays, in addition to the replaced residue 102β, a widely dispersed set of small movements interpretable as a slight contraction of the molecule towards the site of the amino acid substitution (Asn $\rightarrow$ Thr; net loss of one nitrogen atom).

(i) α *Haem pocket*

In the α-haem pocket (Plate II, above the level of the mutation) pairs of positive and negative peaks indicate movements of helices G and E, and of the F–G corner, toward the $\alpha_1\beta_2$ interface. The haem group also moves slightly in this direction, though the iron atom at its centre apparently does not. This indicates perhaps a small relaxation of the force normally pulling the iron atom away from the haem plane and lowering affinity. Histidine 58(E7)α swings slightly out from the haem pocket due to the haem's motion. Tryptophan 37(C3)β moves downwards towards the mutation, allowing tyrosine 140(HC2)α and valine 93(FG5)α to relax a little across the $\alpha_1\beta_2$ interface.

(ii) *The mutation site and the $\alpha_1\beta_2$ interface*

Plate III shows the large negative peak at the position of asparagine 102(G4)β due to the removal (by mutation to threonine) of one nitrogen atom, and the effective relocation of an oxygen atom at a lower level, closer to the main chain (Plate IV). Helices G, B and E of the α chain all show small shifts towards the mutation site, but it is tyrosine 42(C7)α and the adjacent residues of the C helix and C–D corner which undergo the clearest movements in this direction (a,b in Plate III). Tyrosine 42α is joined to aspartate 99β (Plate IV) by the only hydrogen bond to cross the $\alpha_1\beta_2$ interface in deoxyhaemoglobin. Individual residues of the β chain C helix and C–D corner are disturbed (particularly arginine 40(C6)β which is in contact with Tyr42α) but they do not show a movement bodily in any direction. Other β chain helices are relatively unperturbed at this level.

In Plate IV, showing the lower part of the mutation site, the large negative peak seen above (Plate III, Asn102) is continued, while beside and below it is a positive region corresponding to the relocated threonine oxygen atom. Asp99(G1)β, hydrogen bonded to Tyr42α in Plate III, moves toward the hole left by the mutation, as does the β F–G corner (extending down the page from Asp99). The αC helix (a in Plate IV) and adjacent residues (b,c) follow. Several negative peaks on the upper β haem indicate that this group is slightly disturbed by the mutation or by the movement of

PLATES II to V. Difference Fourier synthesis of deoxy Kansas minus deoxy native haemoglobin (white contours at 0·02 e/Å^3 intervals, negative dashed, zero omitted) superimposed on the native 2·5 Å Fourier (black contours at 0·15 e/Å^3; higher contours obscure those below). The Fourier is shown as stacks of 1 Å-thick y-sections (i.e. sections along the molecular dyad, which is marked by a black crystallographic symbol). Capital letters indicate helices; underlined labels refer to β chain, otherwise to α chain.

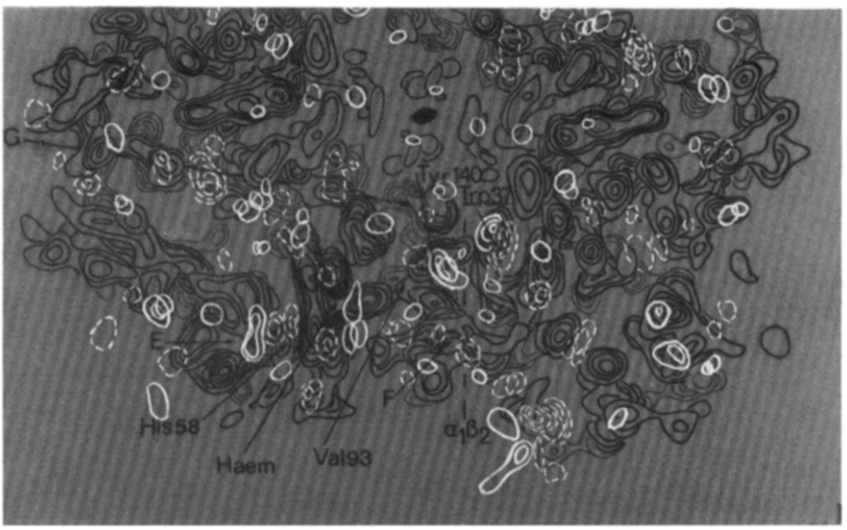

PLATE II. Region of the α haem (deoxy Kansas, $y = -4$ to $+10$). Haem moves very slightly towards the $\alpha_1\beta_2$ interface. G and E helices (His58) are slightly disturbed. Trp37β moves downwards allowing Tyr140α and Val93α to move a little to the right.

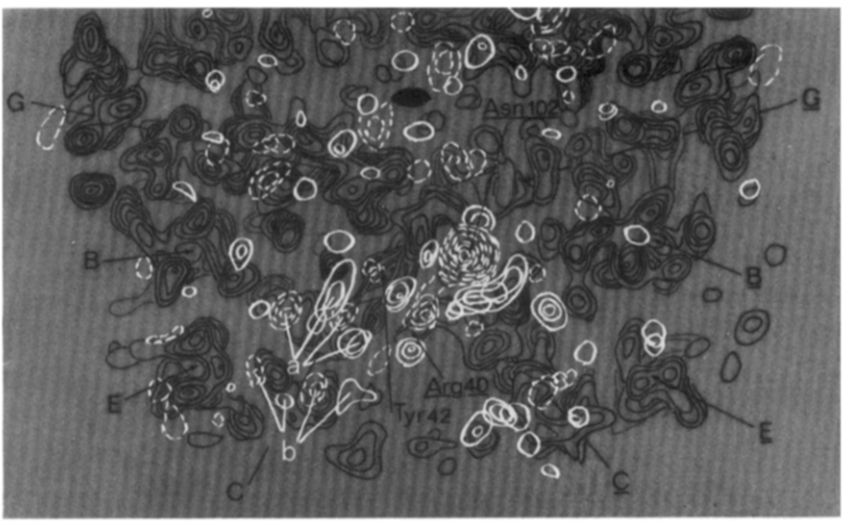

PLATE III. Upper portion of the mutation site (deoxy Kansas, $y = -1$ to $+3$). Negative density on Asn102β due to mutation causes Tyr42α and adjacent residues to move toward the right (pairs of negative and positive peaks a,b).

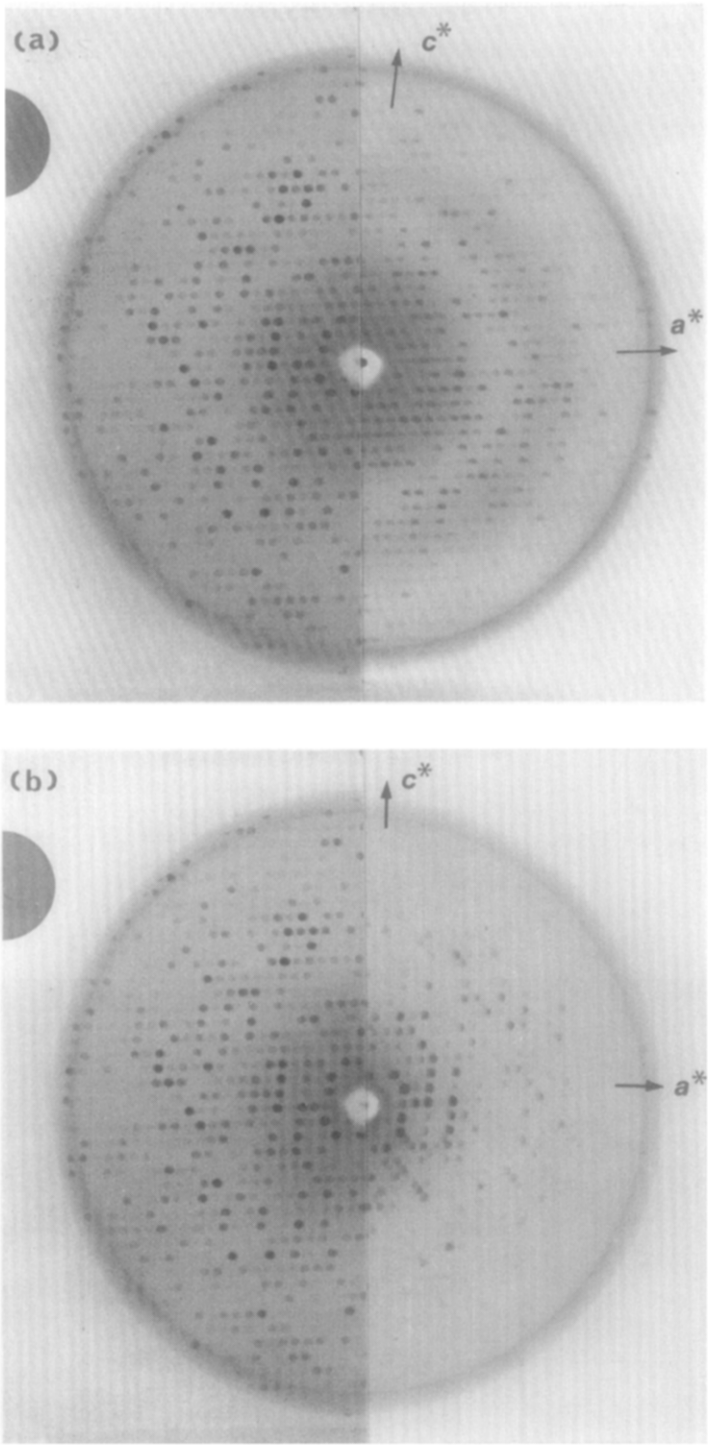

PLATE I. X-ray diffraction patterns ($h0l$) of haemoglobin Kansas: (a) deoxy (left half) compared to CO form 1 (F1; right half), and (b) deoxy (left half) compared to CO form 2 (F2; right half). Symbols a^* and c^* indicate the reciprocal lattice axes. The precession angle was 17°, so that the edge of the pattern corresponds to 2·7 Å resolution.

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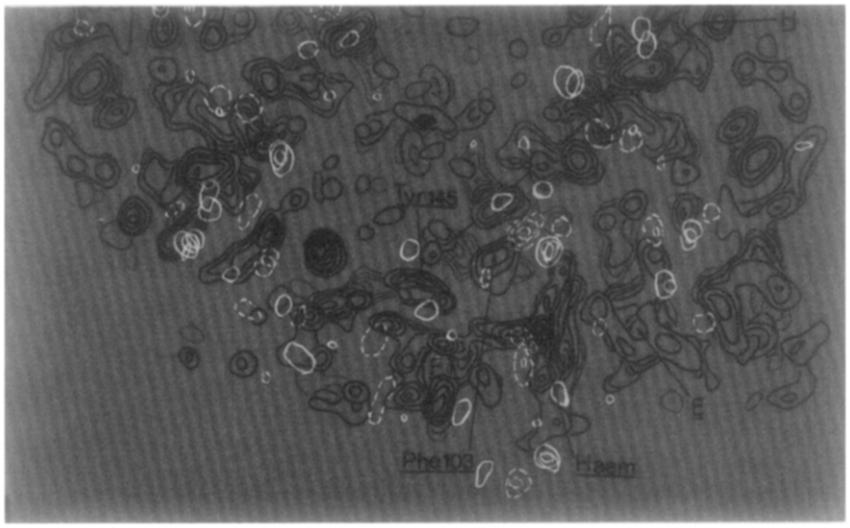

PLATE IV. Lower portion of the mutation (deoxy Kansas, $y = -6$ to -2). Clear movement of Asp99β (hydrogen bonded to Tyr42α; Plate III) toward negative peak at Asn102β. Positive density at Asn102β is threonine oxygen atom relocated closer to main chain. Various α chain residues shift toward mutation (a,b,c). Slight delocalization of top edge of β haem.

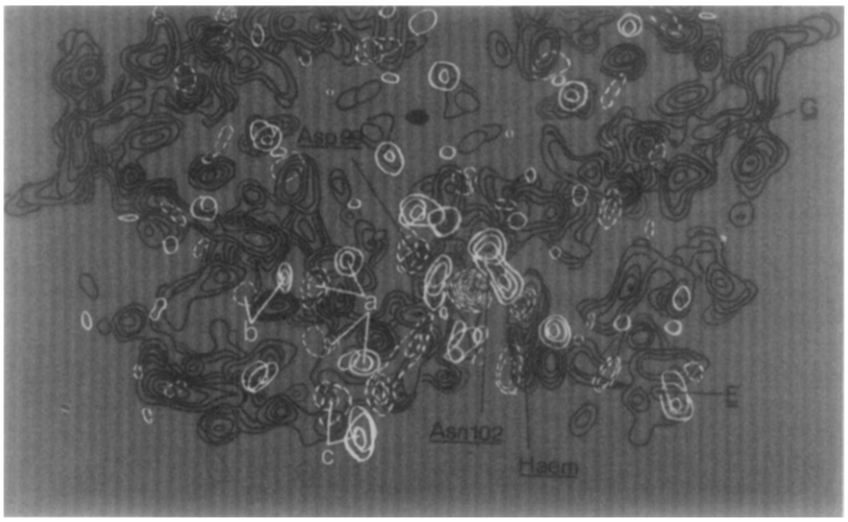

PLATE V. Region of β haem (deoxy Kansas, $y = -11$ to -7). Haem may move slightly toward E helix. F helix follows behind. Phe103 perturbed. Otherwise the lower β chain is unaltered.

PLATES VI to IX. Difference Fourier of T state CO-Hb Kansas (white) minus native deoxy haemoglobin (black), solved by rotation and translation function methods. Plates VI and VII show whole tetramer sections (two α chains, and tops of two β chains). Plates VIII and IX show half-tetramer sections, after averaging about the molecular dyad. Symbols as in Plates II to V.

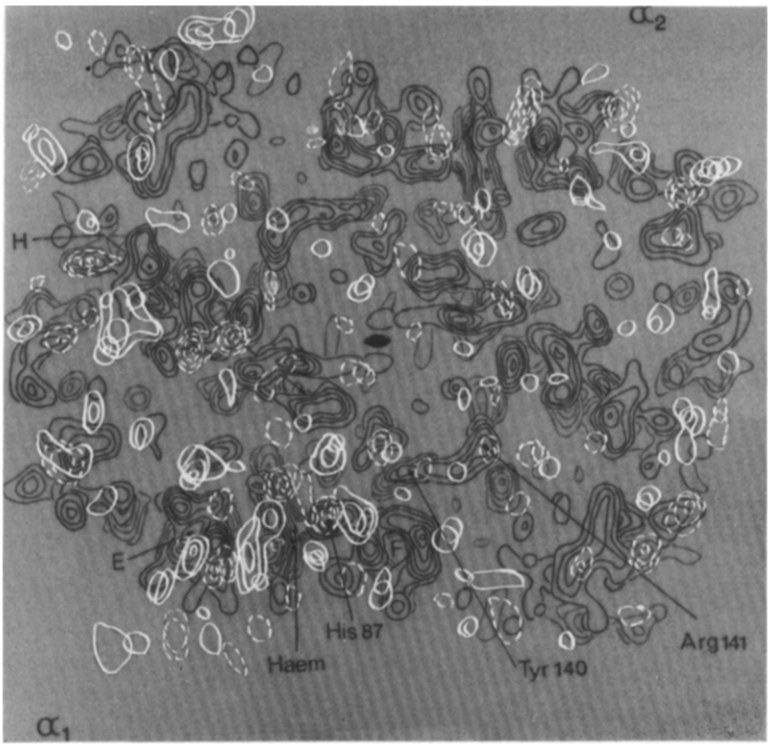

PLATE VI. Upper α haems (CO Kansas. y 9 to 12). Large disturbance of α_1 haem, indicating movement towards E helix. Tyr140α and Arg141α (inter-α-chain salt bridge) are not disturbed. Features in α_2 chain less pronounced.

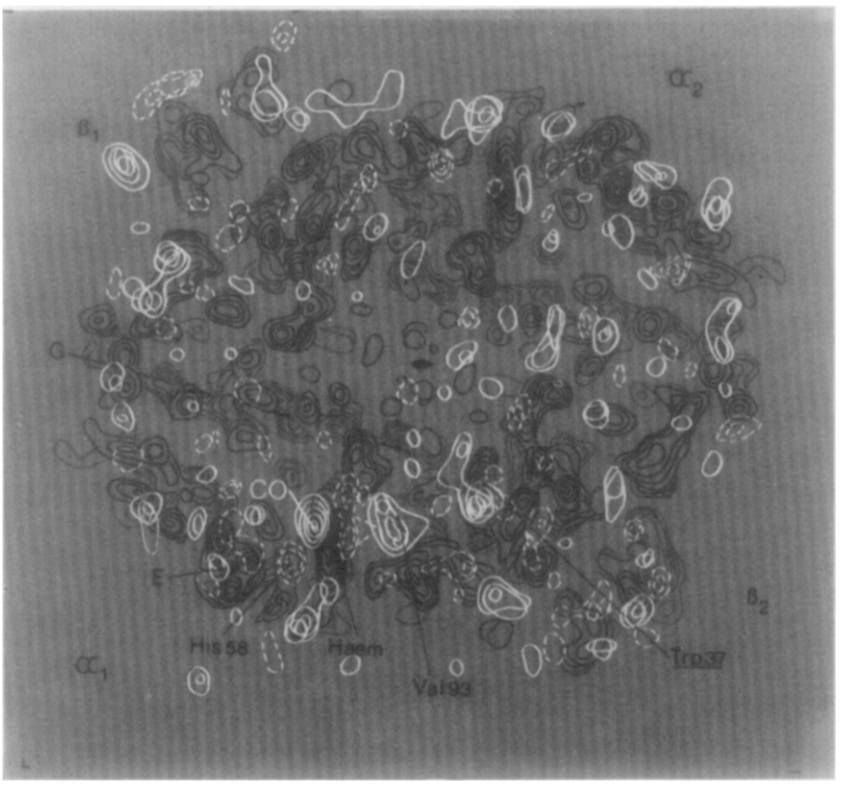

PLATE VII. Lower α haem (CO Kansas, $y = +5$ to $+8$) CO indicates bound ligand molecule in α_1 chain. Adjacent to it on opposite side of haem is small negative peak showing movement of iron atom toward CO; i.e. into haem plane. E helix (His58α) and F–G corner (Val93α) are perturbed, as well as Trp37β, across the $\alpha_1\beta_2$ interface. Smaller features in α_2 chain.

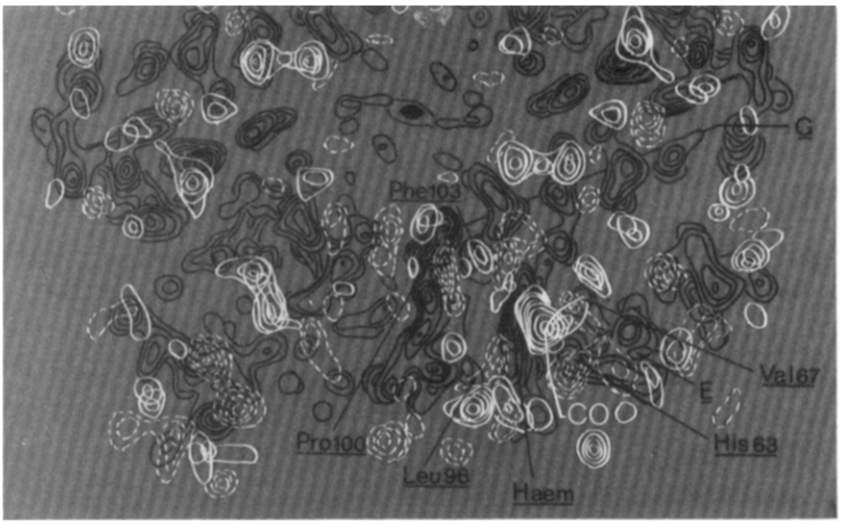

PLATE VIII. Upper β haem (CO Kansas, $y = -8$ to -5) CO indicates β haem ligand. Haem contacts at Phe103β, Leu96β and F–G corner (down the page from Pro100β) show movement to the left, implying a movement of the upper haem in this direction. E helix residues His63β and especially Val67β pushed strongly away by ligand. Large perturbations apparent in neighbouring α chain (left of Pro100β).

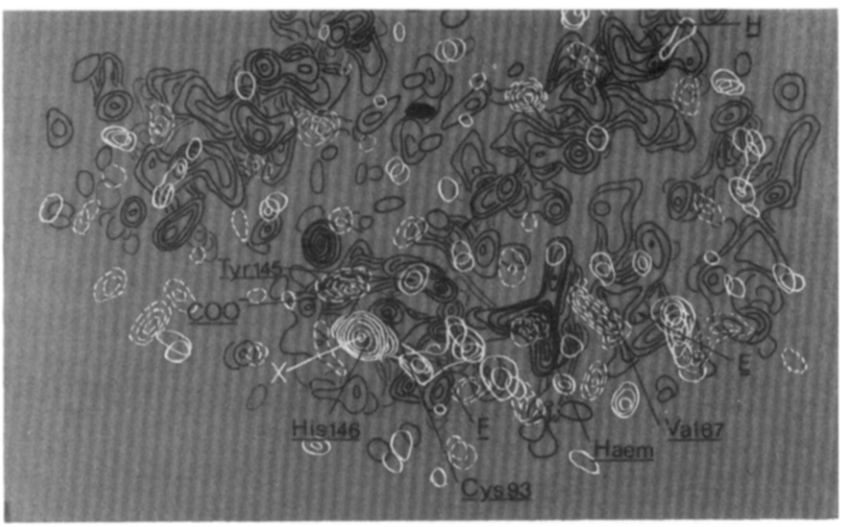

PLATE IX. Lower β haem (CO Kansas, $y = -15$ to -9). Small negative peaks near haem iron indicate movement upward and toward ligand molecule (Plate VIII); i.e. into haem plane. E helix, especially Val67β pushed away from haem. Positive features on F helix show movement toward haem, implying movement of lower haem to the right (i.e. tilt increases). Peak X shows binding of some atom(s) adjacent to reactive SH of Cys93β. Tyr145β and His146β (beneath peak X) are undisturbed. Negative peak between C terminus (COO^-) and Tyr145β probably indicates a delocalization of terminal peptide group.

the F–G corner. The lack of matching positive peaks means that instead of being simply pushed to one side, the haem is "loosened" or delocalized. The β chain G and E helices appear practically unperturbed.

(iii) *β Haem pocket*

The lower part of the β haem (Plate V) shows several more small negative peaks, again without matching positive density. From the location of these peaks on the proximal side of the haem (left in this Plate), and near the outward (down the page) side of the iron atom, it seems that the general direction of movement is probably toward the E helix and into the haem pocket. The F helix, attached to the haem by the proximal histidine, shows a hint of movement in this direction. Phenylalanine 103 may be slightly disturbed by such a shift. Otherwise the lower β chain shows no interpretable features.

(b) *T state carbonmonoxy haemoglobin Kansas*

The difference Fourier of T state CO-Hb Kansas (Plates VI to IX) is much less clear than that of the deoxy form primarily because of the extended and indirect nature of the calculation. As a result, only the larger features or groups of features are reliably interpretable. An additional complication lies in the fact that one α chain appears to be unliganded, making the pair of α chains unsymmetric about the molecular dyad axis. Hence in the α haem region, both α_1 and α_2 chains are shown. The β chains, both liganded, are presented after symmetry averaging to reduce noise. The region between α and β haem pockets, primarily the $\alpha_1\beta_2$ interface, contains no reliable features and is not shown.

(i) *α Haem region*

The region surrounding the α_1 haem (Plates VI and VII) shows several notable features. In addition to the large ligand peak (CO in Plate VII), there are clusters of positive and negative features on the distal and proximal sides of the haem, respectively. The negative peaks (primarily Plate VII) are near the lower inside haem corner, while the positive density (Plate VI) lies near the upper outside corner, indicating perhaps a net shift of the haem upwards and towards the outside of the E helix. Part of the negative density lies directly behind the iron (on the opposite side from the CO molecule in Plate VII), as expected if the iron moves into the haem plane. Though an accurate calculation is impossible, it seems unlikely that this relatively small negative peak could be associated with an iron movement of more than a few tenths of an Ångstrom unit. A movement of 0·75 Å, the difference between iron positions relative to haem plane in T state deoxy and R state CO structures, would produce positive and negative peaks each about as large as the CO molecule ($\sim$14 electrons).

Both the proximal and distal histidines (His87 in Plate VI, His58 in Plate VII) are disturbed by the binding of ligand. The E helix appears to be pushed away from the haem, but the F helix shows no interpretable bulk movement. Neither tyrosine 140α nor its attached salt bridge involving arginine 141α seems appreciably disturbed; the inter-α-chain salt bridges must still be intact.

The region surrounding the α_2 haem shows smaller features. A small ligand peak is accompanied by a smaller negative peak behind the iron, and by slight disturbance of His58α_2. Otherwise this haem pocket shows no reliably interpretable movements.

It appears that this anomaly of the T state CO-Hb Kansas crystal points to an interesting conclusion. If the effect is real, which is likely, then the degree of tertiary disturbance in a T state subunit is roughly related to the degree of ligation of that subunit, and not of its neighbours. Large effects are produced at α_1 by substantial ligation, but a small ligand occupancy at α_2 produces small tertiary effects, apparently not augmented by strain propagated from α_1. This conclusion is central to the allosteric theory, and has been demonstrated before by a number of techniques, though not by direct structural analysis. Unfortunately, it is difficult to rule out the possibility that phase errors, caused by a slight misalignment of the molecule, are responsible for a general loss of definition in some particular region (the α_2 haem pocket). However, since the other three haems seem well-phased, the one at α_2 ought to be fairly accurately positioned.

Although the crystallographic environments of the entrances to α_1 and α_2 pockets are different, they provide no simple explanation of the disparate ligand occupancies of the two haems.

(ii) *β Haem region*

Since both β haems are liganded, the difference Fourier in this region has been symmetry averaged to reduce noise (Plates VIII and IX). The ligand peak (CO in Plate VIII) is again associated with a smaller negative peak behind the iron (Plate IX). In addition, the general character of the tertiary rearrangement produced can be defined. The E helix residues facing the haem are pushed away by the ligand (His63 and Val67). This is most emphatic in the case of Val67, near the base of the haem, which in deoxyhaemoglobin partially obstructs the binding of ligand (Perutz, 1970). On the other (proximal) side of the haem, residues of and adjacent to the F–G corner are pushed back by the upper haem (Phe103, Pro100 and Leu96 in Plate VIII). The lower part of the F helix seems, however, to be drawn toward the haem by the pull of the proximal histidine (positive features on the haem side of helix F in Plate IX). These are the classic features associated with an increase in tilt of the haem, observed previously in the α chains of deoxygenated R state bis-maleimidomethyl ether-Hb (Moffat, 1971; Perutz & Ten Eyck, 1971) and in T state met-HbA (Anderson, 1973).

The region near the β chain salt bridges contains a large unexpected feature (X in Plate IX) complicating interpretation of this area. The nature of the peak is not known, but its location adjacent to cysteine 93β (the "reactive" cysteine) suggests that it could result from oxidation of the SH, from binding there of a metal ion, or conceivably from co-ordination of CO. Full normal sulphydryl reactivity was observed in haemoglobin from a similarly treated, though different, crystal. There is no room for a bound sulphate at this position, and the peak is too large to be water, a bound ammonium ion, or a movement of some residue in the area (no matching negative feature). Below peak X, the His146β · · · · Asp94β salt bridge appears unperturbed. Both tyrosine 145α and the β chain C-terminus, salt bridged to lysine 40α also appear undisturbed, although the terminal peptide group is delocalized (negative peak between COO^- and Tyr145β).

There is a general delocalization of α-chain residues facing the β F–G corner (negative density near Thr38α, opposite Pro100β) indicating perhaps some conformational mismatch at the $\alpha_1\beta_2$ interface. In general, α subunit features on these two Plates are not interpretable since they are the average of one liganded and one unliganded chain.

4. Discussion

(a) *Structure of deoxyhaemoglobin Kansas*

The difference Fourier of deoxy Hb Kansas illustrates primarily the response of the haemoglobin molecule to an internal vacancy the size of one nitrogen atom. By allowing neighbouring interfacial residues, especially Asp99β, to sink slightly into the β chain, the mutation provokes a widespread series of small movements across the $\alpha_1\beta_2$ interface and beyond, deep into the α subunit. The elegant stereochemical transmission of the disturbance emphasizes the complementarity of this interface and the tight internal packing of the subunits. Within the mutated β chain, a more restricted set of movements results. These are due to effects of the collapse towards the mutation, and perhaps also, with respect to the haem itself, to a close contact (steric interference) with the mutated residue.

Perutz & Lehmann (1968), on the basis of model building in the R quaternary structure, suggested this close contact as a likely source of tertiary disturbance, but not the collapse. Later, Greer (1971*a*) interpreted a 5·5 Å difference map of deoxy Hb Kansas as showing large disturbances at both haems, caused principally by the close contact of threonine with the β haem. The absence of these disturbances in either 5·5 or 3·5 Å difference Fouriers calculated with the present data indicates that they were due to inaccuracies in the measured intensities or mistakes in scaling.

On one important point the present and earlier investigations agree: the mutation produces some tertiary structural disturbance in much of the molecule. At this stage, however, the precise functional effects of these small alterations cannot be deduced. The movements at the $\alpha_1\beta_2$ interface may slightly increase or decrease the stability of that contact in the T state; but only slightly, since complementarity is maintained and the hydrogen bonding pattern is unchanged. The small shifts observed in both α and β haem pockets appear to indicate some relaxation of the tension at the haems; an effect which ought to raise the affinity of both subunits. Since this is not consistent with the slightly lowered T state affinity observed for stripped Hb Kansas (R. T. Jones & J. V. Kilmartin, personal communication), it must be concluded that subtle variations in affinity are the result of movements too small or too complex to be analyzed at 3·4 Å resolution.

The structural and functional abnormalities in the Hb Kansas T state are nevertheless significant, even if they cannot be simply correlated. Together, they provide a picture of limited changes in both α and β subunit affinity within an apparently normal T quaternary structure. The same seems to be true of Kansas in the R state, since its crystals are very similar to those of HbA, while at least one of its subunits has an abnormally low affinity (Riggs & Gibson, 1973; Gibson *et al.*, 1973). As has been noted by others, this situation is not a simple case of altered allosteric constant L (Morimoto *et al.*, 1971; Edelstein, 1971), but instead requires a full treatment allowing the ligand affinities of the α and β subunits to differ from normal in both quaternary structures (Ogata & McConnell, 1972).

(b) *Structure of T state carbonmonoxy haemoglobin Kansas*

Crystals of deoxyhaemoglobin Kansas are not destroyed by exposure to carbon monoxide, as are crystals of normal deoxyhaemoglobin. This behaviour provides a rare opportunity to view the tertiary effects of ligand binding within the T state, free of many of the difficulties encountered in previous intermediate model systems, such

as HbM Iwate (Greer, 1971*b*,*c*), T state met-HbA (Anderson, 1973) and for comparative purposes, horse deoxy R state bis-maleimidomethyl ether-Hb (Moffat, 1971; Perutz, 1970; Perutz & Ten Eyck, 1971). In each of these three earlier investigations, either only two haems could bind CO (Iwate), low-spin ligands (like CO) were unusable (T state met-HbA), or the system was not in the T state (bis-maleimidomethyl ether). T state CO-Hb Kansas presents its own problem, nevertheless, in the form of a small unit cell change on binding CO. This makes a normal difference Fourier synthesis impossible, and necessitates the use of rotation and translation function searches to solve the structure. Similar methods have been used before, notably by Schmid *et al.* (1974) in the solution of carboxypeptidase *B* at 5·5 Å resolution. The power of the technique now seems sufficient to allow solution to about 3·5 Å of any mutant or modified human haemoglobin. Unfortunately, the result can never be as sensitive as a direct difference Fourier synthesis, and this is apparent from the present map.

The plausibility of the T state CO-Hb Kansas structure, as solved by these methods, can be judged by three criteria; the appearance of difference peaks at the mutation site, the appearance of ligand peaks at the expected positions by the haems, and the 2-fold symmetry of important features about the molecular, non-crystallographic dyad axis. None of these criteria is completely satisfied; in particular no clearly defined negative density is found at the mutation site, indicating either that a feature of -5 electrons must be near the level of noise or that tertiary movements on CO binding have caused this hole to be filled. On the other hand, three haems show large ligand peaks, and in the β chains the major features are all symmetric about the dyad.

The anomalous absence of ligand from one α haem results in generally smaller features in that chain, which are unsymmetric with the other α chain. The source of this strange effect is not known; all haem pockets appear freely accessible from the solvent.

Two important pieces of evidence emerge from the interpretation of this structure. The first concerns the T state salt bridges, and the fact that they are not observed to break. In the single liganded α chain, such a disturbance would certainly have been observable, since it would involve removing Tyr140α from its pocket or at least shifting it. In the β chain, the situation is slightly complicated by the binding of an unknown entity at Cys93β. Nevertheless, since virtually all known additions at this site cause an increase in oxygen affinity symptomatic of T state destabilization (Antonini & Brunori, 1971), it can be inferred that peak X is likely to have a disruptive rather than a stabilizing effect on the His 146β $\cdots$ Asp94β hydrogen bond, and hence on the associated His146β(COO^-) $\cdots$ Lys40α salt bridge. Thus, these interactions, maintained in the present structure, would very probably exist in the absence of peak X. The structural evidence therefore seems to indicate an association between the salt bridge apparatus and the quaternary (but not tertiary) structure of the protein. A similar result was obtained from the structure of methaemoglobin A in the T state (Anderson, 1973), but the conclusions there were tentative, since in methaemoglobin the ligand does not draw the iron atom as far toward the haem plane as in CO haemoglobin (E. Heidner, personal communication).

The second piece of evidence concerns the direct tertiary structural effects of ligand binding. These effects can only be seen clearly in the β chain, to which symmetry averaging has been applied. The picture which emerges confirms the central fact known before from examination of the met and deoxyhaemoglobin models (Perutz, 1970); Val67(E11)β is pushed strongly away from the haem in the process of ligation.

In addition, it provides an overall view of the structural effects of ligand binding to the β chains in the T state. These are remarkably similar in character to those observed earlier in the α chain of T state methaemoglobin, and consist mainly of helix and side-chain rearrangements to allow an increase in tilt of the haem. Increased haem tilt thus emerges as the principal result of ligation in both types of chain, and the likely cause of the interfacial movements that bring about quaternary structural change (Anderson, 1973).

There are two final suggestions to be drawn from the map. The absence of very large negative features behind the iron atoms of liganded haems indicates that these atoms are probably not drawn completely into the haem plane, where they would lie in R state CO-haemoglobin (E. Heidner, personal communication). Evidence is thus provided for the existence of a considerable "tension at the haem" (Perutz, 1972; Perutz *et al.*, 1974*a*,*b*,*c*) exerted within the T quaternary structure regardless of ligation state. A second suggestion of theoretical importance comes from the observed rough relation between size of ligand peak and size of tertiary disturbance in the two dissimilar α chains. No large effects appear to have been propagated directly to the unliganded chain, in support of the allosteric theory of haemoglobin (Perutz, 1972). The possibility that this phenomenon is an artifact is not, however, completely ruled out.

(c) *Implications of intact salt bridges in T state carbonmonoxy haemoglobin Kansas*

Perutz *et al.* (1969) first put forward particular salt bridges between charged groups in the T state as possible sources of the Bohr effect. Since then the role of the salt bridge between His146β and Asp94β (Fig. 4) has been proved (Kilmartin *et al.*, 1973), and there is also strong, though indirect, evidence for a large change in the pK of the amino group of Val1α (Fig. 3) when the polar groups in its vicinity are rearranged

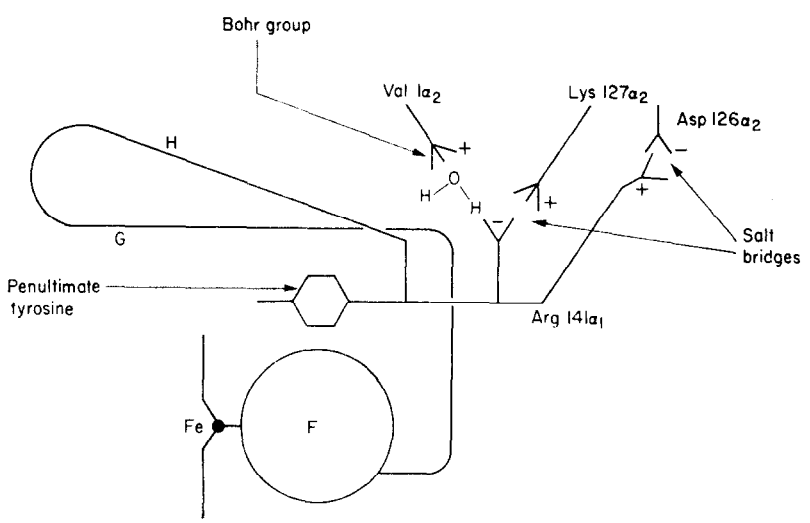

FIG. 3. Schematic diagram of the inter-α-chain salt bridges in the T state, including the pH-sensitive interaction of the NH_2 terminus of one α chain (not shown) with the COO^- terminus of the other (the α chain shown here) *via* a water molecule. The stereochemistry of the C-terminal residues is such that if the penultimate tyrosine(140(HC2)α) is forced out of its pocket between helices F and H, these salt bridges are torn apart. Transition to the R state also destroys them.

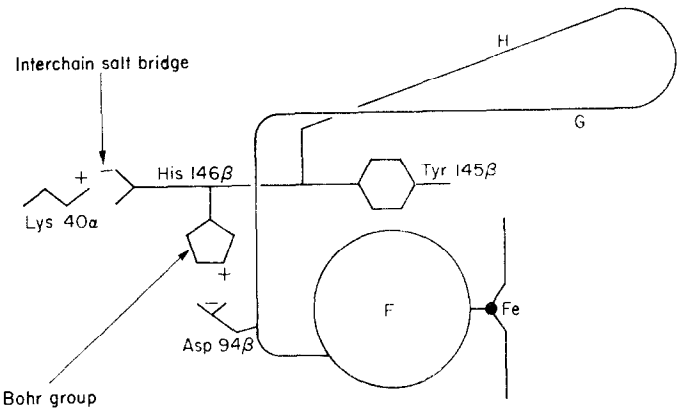

FIG. 4. Schematic diagram of β chain residues which are analogous to α chain groups shown in Fig. 3. Within the T state configuration shown, displacement of the penultimate tyrosine (145(HC2)β) ought to cause breakage of both interactions. Transition to the R state would certainly destroy the interchain salt bridge (COO^- terminus to Lys40α), but the state of the Bohr group interaction (His146β to Asp94β) may be determined primarily by the conformation of Tyr145β.

during oxygenation (Kilmartin & Rossi-Bernardi, 1969). In principle, the salt bridges in the T state could be affected by ligand binding in two different ways; each salt bridge could be broken when the subunit or units to which it is attached takes up ligand or, alternatively, the salt bridges between subunits could all break simultaneously when the quaternary structure changes. It has been shown that under conditions resembling those existing *in vivo*, the change in quaternary structure occurs mainly as the third oxygen molecule combines (Hopfield *et al.*, 1972). If the first model applied, salt bridges should be broken as each subunit combines with oxygen, and hence in kinetic experiments the release of Bohr protons should be a linear function of oxygen uptake; if the second model applied, Bohr protons should be released later during ligation when an appreciable fraction of the molecules change quaternary structure. The kinetic experiments of Antonini *et al.* (1965*b*), Gray (1970) and Olson & Gibson (1973) demonstrated that in stripped HbA proton release is synchronous with ligand uptake, thus favouring the first model. In equilibrium experiments, the first model requires the first Adair constant to be strongly pH-dependent, while within the second model it should be largely independent of pH. Imai & Yonetani (1974) have confirmed the pH-dependence of K_1, first demonstrated by Roughton (1964), thus adding further support to the first model. Based on difference Fourier maps of bis-maleimidomethyl ether deoxy-minus-methaemoglobin, Perutz (1970) proposed a specific stereochemical mechanism for the sequential rupture of the salt bridges, suggesting that the narrowing of the pocket between helices F and H displaced the penultimate tyrosines HC2 and thereby pulled the salt bridges apart.

While the chemical evidence obtained without the use of powerful allosteric effectors favours sequential rupture of the salt bridges, the crystallographic evidence presented here indicates that ligands can combine with haemoglobin Kansas in the T state without breaking the salt bridges. Furthermore, the crystals examined are very stable, indicating that this sort of structure may have some role in the behaviour of the protein in solution.

It seems unlikely that the state of these salt bridges can be explained by some

trivial peculiarity of the mutant, since most of the available data on haemoglobin Kansas in solution supports the assumption that its functional mechanism is nearly normal. These lines of evidence may be summarized as follows: (1) the kinetics of the release of a fluorescent 2,3-diphosphoglycerate analogue, and of the change in reactivity toward paramercuribenzoate during ligand uptake (Gibson *et al.*, 1973) are very similar to the results with HbA (MacQuarrie & Gibson, 1972; Gibson, 1973); (2) the equilibrium alkaline Bohr effect in the absence of phosphates is probably normal (J. V. Kilmartin, personal communication); (3) nuclear magnetic resonance spectra of stripped deoxy and CO forms are analogous to normal deoxy and CO spectra (Ogawa *et al.*, 1972), and (4) deoxy Hb Kansas crystallizes isomorphously with deoxy HbA, while CO-Hb Kansas (crystallized as the CO form) is apparently the same as CO-HbA except for a small change in unit cell size. It seems reasonable to conclude that haemoglobin Kansas forms essentially normal T and R structures and that its functional mechanism (including Bohr groups) behaves as in normal haemoglobin. The observed integrity of the salt bridges must then be explained in terms of properties peculiar to the crystal, and there are two of these: constraint of the quaternary structure by the lattice, and strengthening of the Bohr group interactions themselves at the crystal pH (6·6 to 6·9), relative to pH 7·0 or greater used in most of the other kinds of experiments. Quaternary constraint is probably the dominant effect, since in solutions of haemoglobin Kansas near the crystal pH (pH 7·0) salt bridges appear to be broken synchronously with ligand uptake (my preliminary experiments of the type performed by Antonini *et al.* (1965*a*)). An examination of the packing of molecules within the F1 crystals showed that the salt bridges are not directly affected by interactions with neighbouring tetramers. Thus the lattice constraint must act in a relatively non-specific way, preventing the small quaternary structure adjustments involved in the mechanism of salt bridge breakage in the T state.

In view of the evidence that the behaviour of the salt bridges may be influenced by the constraints imposed upon molecules in the T state, a closer examination of the inositol hexaphosphate-stabilized T state becomes important. Since it binds strongly at a single site between the β chains, this ligand causes structural perturbations in the tetramer quite different from any caused by the crystal lattice (Arnone & Perutz, 1974). Nevertheless, both act as constraints, preventing the T to R transition. If inositol hexaphosphate accomplishes this by frustrating small quaternary structure adjustments on ligand binding, then by analogy with the crystal, the salt bridges of liganded chains might not be broken. Solution experiments to date do not prove this analogy, though Kilmartin (1973) has observed that inositol hexaphosphate suppresses half the proton release accompanying oxidation of HbA. A more direct test would be to determine the pK of His146β (by nuclear magnetic resonance as in Kilmartin *et al.*, 1973) in CO haemoglobin Kansas with and without inositol hexaphosphate, thus detecting any change in the strength of the Bohr group salt bridge. An effect of inositol hexaphosphate on salt bridges in the T state might easily explain many of its "side-effects", such as lowering T state oxygen affinity (Tyuma *et al.*, 1973) and sulphydryl reactivity (Perutz *et al.*, 1974*b*).

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Note added in proof: The large positive peak near the Cys93β SH group of ^{T}HbCO Kansas (peak X in Plate IX) appears to be an artefact due to the presence at this site of a mercury atom in *p*-mercuribenzoate Hb (the most important heavy atom derivative used in determining the native structure). Since the native Fourier has a large negative peak here (due to small phasing errors), the reconstituted Kansas cell did also; when this structure was "subtracted" from the ^{T}HbCO Kansas crystal structure (having near zero density at this site) a large positive difference peak resulted. The effect was not noticed before in simple difference Fouriers (i.e. deoxyHb Kansas) because in that case neither set of |F|'s contains any information regarding heavy atoms or errors in phasing. The measured crystal sulphydryl reactivity is now seen to be correct (see Materials and Methods, section vii).