Hemoglobin San Diego (β109 (G11) Val→Met)

CRYSTAL STRUCTURE OF THE DEOXY FORM

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ABSTRACT The three-dimensional structure of deoxyhemoglobin San Diego has been solved to 3.5 Å resolution using mixed crystals of the variant hemoglobin and hemoglobin A. The site of the amino acid replacement can be clearly located in the difference electron density map and is consistent with the chemical result (β109 Val→Met) discussed in the preceding paper. The abnormally high oxygen affinity and impaired cooperativity appear to result from perturbations of the $\alpha_1\beta_1$-subunit interface.

INTRODUCTION

The inseparable or electrophoretically silent hemoglobin mutants are difficult to characterize by chemical methods, as shown by Nute, Stamatoyannopoulos, Hermodson, and Roth (1) in the accompanying paper. X-ray analysis is a physical method which allows the three-dimensional structure of a protein to be determined without any knowledge of its chemical constitution (though not all amino acid side chains can be identified). Determination of an unknown structure can be very laborious, but once a structure has been found it is relatively easy to detect small differences between it and a chemically related one, provided the two structures form isomorphous crystals. Fortunately the hemoglobin molecule is so large that the substitution of one amino acid side chain for another rarely affects the arrangement of the molecules in the crystal, so that the condition of isomorphism is fulfilled in most variants.

Determination of the difference between the variant and the normal structure then requires the recording of the intensities of about 14,000 reflexions in the X-ray diffraction pattern from the variant crystals. From the differences between them and known intensities recorded from a normal crystal, a map can be calculated which presents the difference between the electron density of the variant and the normal form in a series of sections through the hemoglobin molecules, like microtome sections through a tissue only on a 10,000 times smaller scale. These maps are featureless where the two structures are alike, but where they differ peaks appear: positive ones where density has been added and negative ones where it has been taken away. When an atom or a side chain has moved, this will manifest itself by a negative peak away from, and a positive one towards, the direction of movement. The coordinates of all the nonhydrogen atoms in normal human deoxyhemoglobin are known. Therefore the coordinates of the peaks in the difference map give away both the site of the amino acid substitution and the stereochemical disturbances caused by it. The chemical nature of the substitution, on the other hand, is difficult to identify by X-ray analysis alone, for lack of sufficient resolution.

The nature of these disturbances often explains, at least qualitatively, abnormal functions such as a changed oxygen affinity, a diminished Bohr effect, or a low Hill's constant. The cooperative functions of hemoglobin can be represented most simply in terms of the allosteric theory of Monod, Wyman, and Changeux (2). This is based on the existence of two alternative structures, deoxy and oxy, one having a low and the other a high oxygen affinity, and it describes the oxygen equilibrium curve in terms of only two arbitrary constants denoted by L and c. L is the equilibrium constant between the two structures in an oxygen-free solution, and c the ratio of the oxygen dissociation constants of the two structures. Variant hemoglobins in which an interface between two subunits is altered may have a changed oxygen affinity, or a low Hill's constant, mainly because L has changed, while in variants with a replacement in one of the heme pockets the c might be the constant primarily affected. How-

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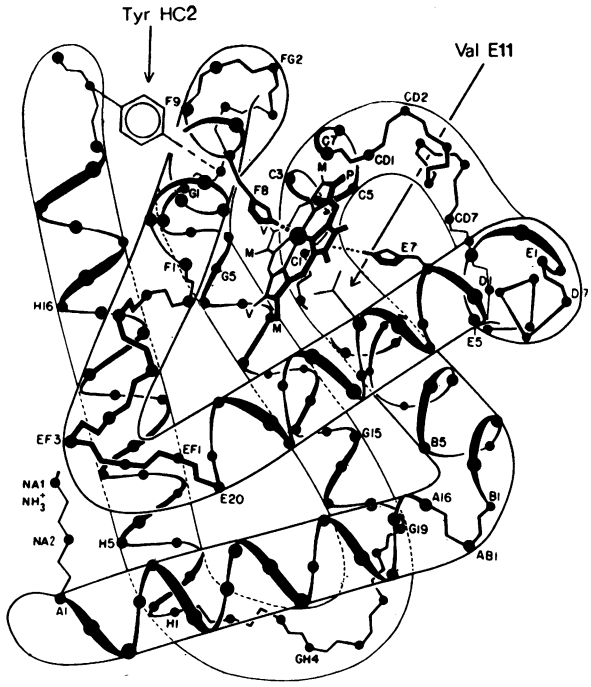

FIGURE 1 Schematic diagram showing the course of the polypeptide backbone in a single hemoglobin subunit. There are eight helical segments folded about the heme.

ever, it is important to realize that no variant or chemically modified hemoglobin has so far been found in which one of these constants has been altered without the other; in fact, their separation may be regarded as something in the nature of a mathematical abstraction.

I set out to try and locate the site of the amino acid substitution in Hb San Diego by X-ray analysis when it had not yet been chemically determined. I hoped that knowledge of the site of the mutation and the disturbances it produced in the normal structure might yield a stereochemical explanation of the mutant molecule's abnormal properties. As it turns out both the chemical and the X-ray crystallographic approaches have born fruit almost simultaneously, with comparable levels of effort. The results are consistent and complementary.

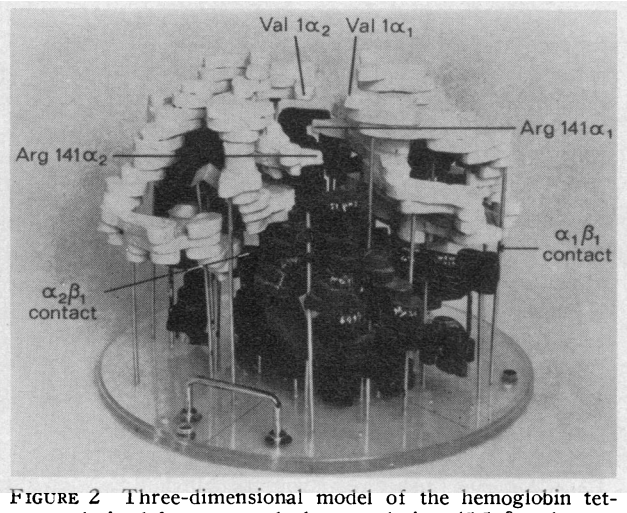

FIGURE 2 Three-dimensional model of the hemoglobin tetramer derived from an early low-resolution (5.5 Å) electron density map. The model is viewed from the side, with the molecular twofold axis running vertically. The α-subunits are white, the β are black, and the dark tilted discs are two of the four hemes. At this resolution only long tubes of density corresponding to the helices can be seen; atomic positions are obtained from higher resolution studies. The interface $\alpha_2\beta_1$ is equivalent to $\alpha_1\beta_2$.

METHODS

Crystallization. Hemolysate containing about 50% Hb San Diego, the gift of Dr. G. Stamatoyannopoulos, was crystallized under the conditions used for Hb A (3). Since all the material crystallized, it was assumed that the crystals used contained Hb San Diego in the same proportion as the hemolysate.

Data collection and processing. A full set of 3.5 Å data, including Friedel pairs, was collected on a computer-controlled Hilger and Watts diffractometer. The mean standard deviation in scaling together data from two crystals was 1.2% of mean structure amplitude, and the mean isomorphous difference, after scaling of mutant data to the native high resolution data, was 4%. The synthesized difference electron density map was symmetry-averaged about the molecular twofold axis, plotted, and enlarged photographically onto clear sheets for interleaving with the native 2.5 Å Fourier.[1]

RESULTS

The results that follow describe features in the difference map (Hb San Diego — Hb A). The observed changes in structure are small relative to the entire molecule, so that the normal molecular nomenclature can be meaningfully preserved. In this system, the amino acids in each type of chain (α and β; Hb = $\alpha_2\beta_2$) are numbered sequentially from the NH_2-terminus and in addition are identified with a position in an α-helix or interhelical region. Each of the four hemoglobin subunits (Fig. 1) is essentially a box built of eight α-helices (A–H) to contain the iron-porphyrin complex (heme) which binds oxygen. The complete hemoglobin tetramer (Fig. 2) has a twofold molecular symmetry axis and two types of important subunit contact, denoted $\alpha_1\beta_1$ and $\alpha_2\beta_1$. Inspection of models of the molecule in its two functional conformations (deoxy and oxy) has shown that the $\alpha_2\beta_1$-contact and the intersubunit salt bridges which are connected to it are functionally the most important regions of the structure (4). The $\alpha_1\beta_1$-interface is normally a passive con-

[1] Arnone, A., and L. F. Ten Eyck. 2.5 Å amplitudes and phases for human deoxyhemoglobin. Unpublished results.

tact, behaving somewhat like a bearing. In Hb San Diego, a mutation at the $\alpha_1\beta_1$-interface causes movements both of whole helices (bulk steric rearrangements) and of specific residues engaged in important hydrogen bond and salt bridge interactions.

The mutation. The replacement of valine by methionine entails the addition to the side chain of sulphur's 16 electrons, as well as a change in shape. The expected large positive electron density peak is seen in Figs. 3 and 4. Below it, on either side, are two small negative features (*a* in Fig. 3) which mark the disappearance of one of the valine methyl groups. The added methionine methyl forms part of the main positive peak. Hence the general character of the mutation is replacement of a short, stubby side chain by a long thin one, both hydrophobic.

Helix movements. The side chain at position $\beta 109$ acts as a spacer between the β-G helix, to which it is attached, and the β-B and α-H helices. Its contact with the α-chain H helix is part of the $\alpha_1\beta_1$-subunit interface. The difference Fourier shows that while the methionine's lesser width allows helix β-B to move closer to β-G from the side (*b* in Fig. 3, *a* in Fig. 4), its greater length pushes helices β-G and α-H above it farther apart (negative features along helix G in Fig. 3 indicate that it moves down perpendicular to the plane of the section). The E helix of the β-chain follows its neighbor β-B in moving closer to the $\alpha_1\beta_1$-interface (*c* in Fig. 3). The arrangement of these helices is shown schematically in Fig. 5.

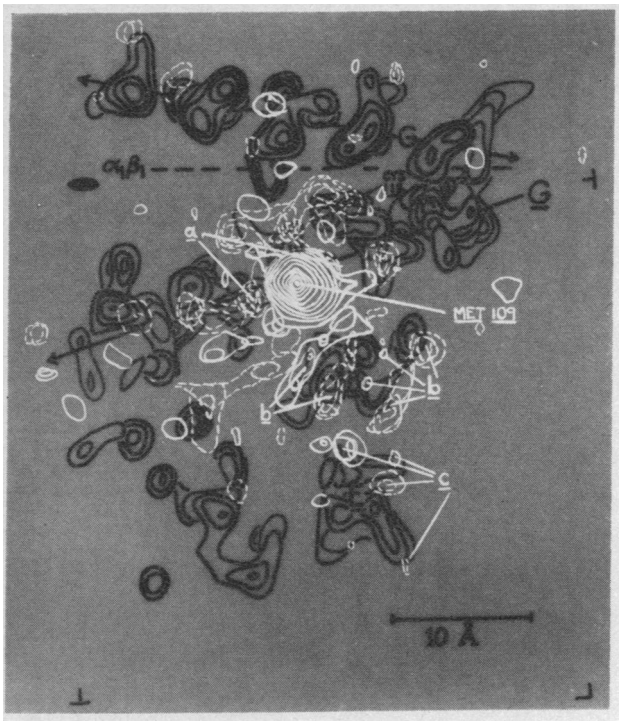

FIGURE 3 Lower half of mutation; $y = -3$ to $+2$. Black contours are native Hb at 2.5 Å, white contours are Hb San Diego minus Hb A (solid lines positive density, dotted lines negative) contoured at 0.012 e/Å^3 (not corrected for less than full occupancy or the Luzzati factor [10] of 2). Capital letters denote helices; underlined features refer to β-chain, otherwise α-chain. Black crystallographic symbol marks the molecular dyad axis. (*a*) negative peaks due to removal of one delocalized valine methyl. (*b*) pairs of negative and positive peaks showing movement of helix B towards helix G. (*c*) pairs of peaks showing movement of helix E in same direction. The left-hand end of helix G ($\alpha_1\beta_2$-interface) is virtually unperturbed.

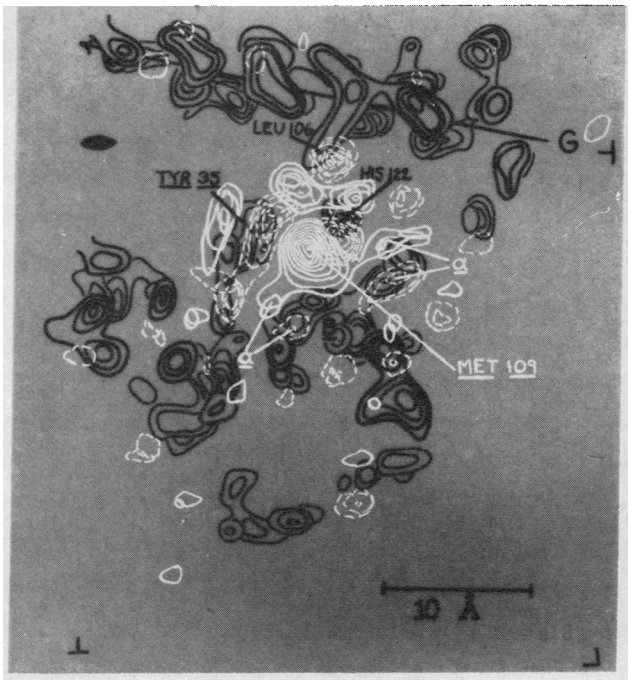

FIGURE 4 Upper half of mutation; $y = +3$ to $+5$. (*a*) pairs of negative and positive peaks showing movement of helix B as in Fig. 3. Tyr 35β and His 122α are strongly perturbed; Leu 106α is pushed before His 122. The positive peak above His 122 is continuous with one, to the left, indicating a slight shift of Asp 126 down from a higher section.

Perturbations of specific residues. The methionine replacing valine 109β perturbs directly three residues: Tyr 35(C1)β and His 122(H5)α above it, and Cys 112(G14)β on the next turn of the β-G helix. Cys 112β moves towards the methionine side chain from the side (Fig. 3), while Tyr 35β and His 122α move away from it as it protrudes further upward than the valine side chain. The latter movements (Figs. 4 and 6) are particularly important, since they cause a weakening of the interchain hydrogen bond Tyr $35\beta_1$ ... Asp 126(H9)α_1 and formation of a new intra-α-chain bond

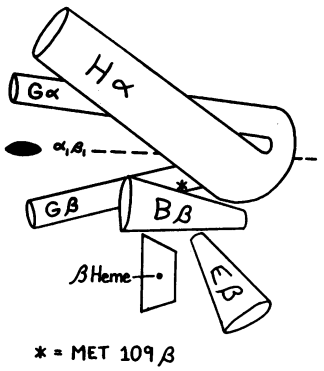

FIGURE 5 Schematic diagram of the region of the mutation from the same viewpoint as Figs. 3 and 4 (down y axis). Helices G and H are almost horizontal, while B and E slope downwards.

Asp 126α_1 ... His 122α_1. Apparently a rearrangement takes place in which the carboxylic oxygen of Asp 126 which normally interacts with Tyr 35β swings towards His 122α, making the new hydrogen bond. The second carboxylic oxygen of the aspartate side chain is probably now in a position to interact with Tyr 35β, but since this oxygen also participates in a salt bridge with Arg 141(HC3) of the other α-chain (characteristic of the deoxy state), its interaction with each residue ought to be weaker than the counterpart in Hb A. Unfortunately the limited resolution of the difference Fourier synthesis leaves the exact arrangement unclear.

The magnitude of the movement of His 122 is sufficient to push the side chain of Leu 106(G13)α away from the $\alpha_1\beta_1$-interface (Fig. 4); this is the only appreciable shift on the α-G helix.

Lack of movement at the hemes or the $\alpha_1\beta_2$-interface. Neither α- nor β-heme pockets show features large enough to be likely causes of changed oxygen affinity. Both the α- and β-hemes may increase in tilt slightly, but these features are at the limit of detectability and probably negligible. The β-G helix, whose extreme end is involved in the $\alpha_1\beta_2$-interface [principally through Asp 99(G1) in deoxy and Asn 102(G4) in oxy], exhibits major movements only at its middle. These motions are almost completely damped out at the $\alpha_1\beta_2$-interface (left-hand border of Fig. 3) and never reach the region of the β-chain salt bridges. The inter-α-chain salt bridges are only slightly perturbed as indicated earlier.

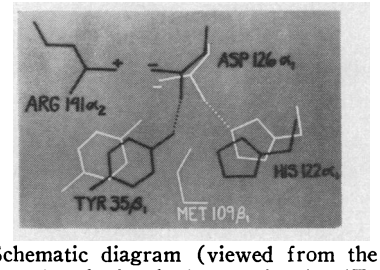

FIGURE 6 Schematic diagram (viewed from the side, perpendicular to y) of the hydrogen bonds (Tyr 35β_1 ... Asp 126α_1, His 122α_1 ... Asp 126α_1) and salt bridge (Arg 141α_2 ... Asp 126α_1) affected by the introduction of methionine at position 109β. A weak hydrogen bond may exist between Asp 126α and Tyr 35β in the mutant, but it has not been drawn. Black lines show the native structure, white lines the structure of Hb San Diego.

DISCUSSION

It is evident from these results, as well as from the chemical work, that the altered properties of Hb San Diego result from steric effects induced by the mutation. In the deoxy structure these include the rearrangement to a weaker configuration of one intersubunit hydrogen bond, creation of a new intra-subunit bond, and probable weakening of a salt bridge that normally stabilizes the deoxy state. The effect on the salt bridge is likely to be the smallest, since the component groups show little rearrangement, especially in terms of their relative distances. The disturbed hydrogen bond, on the other hand, is one of the very few (perhaps two or three) that stabilize the $\alpha_1\beta_1$ interface; the new hydrogen bond that is formed is between residues of the same α-chain and hence contributes nothing towards the stability of the interface. Complete loss of the Tyr 35β ... Asp 126α hydrogen bond in Hb Philly (Tyr 35$\beta \rightarrow$ Phe [5]) causes increased dissociation into monomers; weakening of the bond in Hb San Diego may produce a similar, though probably smaller, effect which might be detected by increased reactivity of cysteines 104α and 112β. In addition, since any change which destabilizes the hemoglobin tetramer is likely to unbalance particularly the tense deoxy structure, the weakening of this hydrogen bond can be said to be a factor tending to alter the allosteric equilibrium towards the oxy structure. Weakening of the Arg 141α_2 ... Asp 126α_1 salt bridge has been shown to cause a similar destabilization of the deoxy structure relative to that of oxyhemoglobin. Hence this mutant should belong to the class (4, 6–9) whose functional alterations can be interpreted as due to an alteration of the allosteric equilibrium in favor of the oxy structure (decreased allosteric constant L), which in turn implies higher affinity and lower cooperativity. The results show that the site of an amino acid substitution can be located from X-ray analysis of mixed crystals of a variant hemoglobin and Hb A.

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REFERENCES

1. Nute, P. E., G. Stamatoyannopoulos, M. A. Hermodson, and D. Roth. 1974. Hemoglobinopathic erythrocytosis due to a new electrophoretically silent variant, hemoglobin San Diego (β109(G11)Val$\rightarrow$Met). *J. Clin. Invest.* **53**: 320.
2. Monod, J., J. Wyman, and J.-P. Changeux. 1965. On the nature of allosteric transitions: a plausible model. *J. Mol. Biol.* **12**: 88.
3. Perutz, M. F. 1968. Preparation of haemoglobin crystals. *J. Cryst. Growth.* **2**: 54.
4. Perutz, M. F. 1970. Stereochemistry of cooperative effects in haemoglobin. *Nature* (*Lond.*). **228**: 726.
5. Perutz, M. F., and H. Lehmann. 1968. Molecular pathology of human haemoglobin. *Nature* (*Lond.*). **219**: 902.
6. Pulsinelli, P. D. 1973. Structure of deoxyhaemoglobin Yakima: a high affinity mutant form exhibiting oxy-like $\alpha_1\beta_2$ subunit interactions. *J. Mol. Biol.* **74**: 57.
7. Greer, J. 1971. Three-dimensional structure of abnormal human haemoglobins Kansas and Richmond. *J. Mol. Biol.* **59**: 99.
8. Morimoto, H., H. Lehmann, and M. F. Perutz. 1971. Molecular pathology of human haemoglobin: stereochemical interpretation of abnormal oxygen affinities. *Nature* (*Lond.*). **232**: 408.
9. Edelstein, S. J. 1971. Extensions of the allosteric model for haemoglobin. *Nature* (*Lond.*). **230**: 224.
10. Luzzati, V. 1952. Traitement statistique des erreurs dans la determination des structures cristallines. *Acta Crystallogr.* **5**: 802.