X-ray and Functional Studies of Hemoglobins Nancy and Cochin-Port-Royal*

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The mutations in hemoglobin Nancy β145(HC2) Tyr→Asp and hemoglobin Cochin-Port-Royal β146(HC3) His→Arg involve residues which are thought to be essential for the full expression of allosteric action in hemoglobin. Relative to the structure of deoxyhemoglobin A, our x-ray study of deoxyhemoglobin Nancy shows severe disordering of the β chain COOH-terminal tetrapeptide and a possible movement of the β heme iron atom toward the plane of the porphyrin ring. These structural perturbations result in a high oxygen affinity, reduced Bohr effect, and lack of cooperativity in hemoglobin Nancy.

In the presence of inositol hexaphosphate (IHP), the Hill constant for hemoglobin Nancy increases from 1.1 to 2.0. But relative to its action on hemoglobin A, IHP is much less effective in reducing the oxygen affinity and in increasing the Bohr effect of hemoglobin Nancy. This indicates that IHP does not influence the R = T equilibrium as much in hemoglobin Nancy as in hemoglobin A, and this probably is due to the disordering of His 146β which is known to be part of the IHP binding site. IHP is also known to produce large changes in the absorption spectrum of methemoglobin A, but we find that it has no effect on the spectrum of methemoglobin Nancy.

In contrast to the large structural changes in deoxyhemoglobin Nancy, the structure of deoxyhemoglobin Cochin-Port-Royal differs from deoxyhemoglobin A only in the position of the side chain of residue 146β. The intrasubunit salt bridge between His 146β and Asp 94§ in deoxyhemoglobin A is lost in deoxyhemoglobin Cochin-Port-Royal with the guanidinium ion of Arg 146β floating freely in solution. This small difference in structure results in a reduced Bohr effect, but does not cause a change in the Hill coefficient, the response to 2,3-diphosphoglycerate, or the oxygen affinity at physiological pH.

A number of studies have shown that mutations and chemical modifications of human hemoglobin involving the COOH-terminal dipeptide of either the β chains (tyrosine 145β, histidine 146β) or the α chains (tyrosine 140α, arginine 141α) lead to major changes in one or more of the parameters which measure hemoglobin function (1–7). From crystallographic studies of normal and cross-linked hemoglobin, Perutz (3) found these dipeptides to be highly solvated, and therefore randomly positioned, in methemoglobin whereas they assume well defined positions in deoxyhemoglobin with the penultimate tyrosines locked in pockets between the F and H helices and the polar COOH-terminal residues forming strong salt bridges. Perutz proposed that these salt bridges and the immobilized tyrosines are required to constrain the structure of the deoxyhemoglobin tetramer (the T state) so that the oxygen affinity of the heme groups is reduced relative to their oxygen affinity in oxyhemoglobin (the R state). In this paper we report solution and single crystal studies of two abnormal hemoglobins involving mutations of the β chain COOH-terminal dipeptide: hemoglobin Cochin-Port-Royal β146(HC3) His→Arg (6) and hemoglobin Nancyβ145(HC2) Tyr→Asp (7).

EXPERIMENTAL PROCEDURE

Solution Studies-Blood from heterozygous patients was collected on heparin and the lysate purified by the standard toluene method. The abnormal component was separated from normal hemoglobin A by DEAE-Sephadex chromatography using 0.05 M Tris.HCl buffer to form a linear pH gradient varying from 7.90 to 7.35 in the case of hemoglobin Cochin-Port-Royal and from 7.80 to 6.80 in the case of hemoglobin Nancy. The lysate and pure components were stripped by chromatography on a Dintzis column (10). The oxygen affinity was measured spectrophotometrically by the method of Benesch et al. (11) at 25° in 0.05 M bis-Tris* buffer with or without 0.1 M NaCl, or in 0.1 M NaCl plus known amounts of neutralized DPG or IHP.

Proton titration was performed on stripped hemoglobin as described by Kilmartin (12), and in the presence of a 10-fold excess of IHP (13). Hemoglobin Osler (8) and hemoglobin Fort Gordon (9) also have been shown to have the mutation β145(HC2) Tyr→Asp.

The abbreviations used are: bis-Tris, 2,2-bis(hydroxymethyl)-2,2',2'-nitroriatriethanol; DPG, 2,3-diphosphoglycerate; IHP, inositol hexaphosphate.
Difference spectra of oxidized hemoglobin in the presence and absence of IHP were recorded on a Cary 118 spectrophotometer. The hemoglobin was oxidized by the nitrate procedure of Kilmartin (12).

Diffraction data were collected to a resolution of 3.5 Å on an Enraf-Nonius CAD4 diffractometer using the Ω scan mode. An empirical correction was made for absorption (14) and a linear degradation factor was estimated from repeated intensity measurements of four standard reflections. This degradation factor never exceeded 8% of the initial intensities. Two crystals were used for a complete set of data for each mutant hemoglobin.

Table I

| pH | \(\log p_5\) -IHP | \(\log p_5\) +IHP | \(\Delta \log p_S\) | \(\Delta \log p_5\) -IHP | \(\Delta \log p_5\) +IHP |
|----|-----------------|-----------------|-----------------|-----------------|-----------------|
| 7.20 | -0.50 | -0.08 | 0.42 | 1.1 | 2.0 |
| 6.60 | -0.36 | 0.31 | 0.67 | 1.1 | 2.1 |
| 6.40 | -0.28 | 0.35 | 0.63 | 1.1 | 2.0 |
| 5.90 | -0.36 | 0.35 | 0.71 | 1.9 | 9.0 |

FIG. 1. Bohr effect of stripped hemoglobin A -- - ; stripped hemoglobin Nancy, - - - - ; hemoglobin Nancy + 10-fold excess of IHP, C --- O. Conditions, hemoglobin concentration = 1.55 x 10^{-6} M, 0.10 M KCl at 25 °C.

FIG. 2. Difference spectrum. A, [methemoglobin A + IHP] - [methemoglobin A]; B, [methemoglobin Nancy + IHP] - [methemoglobin Nancy]. Conditions, hemoglobin concentration = 2.0 x 10^{-6} M, IHP concentration = 1.6 x 10^{-4} M, in 0.05 M bis-Tris + 0.10 M NaCl at pH 7.2, 25 °C.
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Lys 40α and Asp 94β are not disordered in deoxyhemoglobin Nancy, but remain fixed in the positions found in deoxyhemoglobin A.

In addition to these strong features, the deoxyhemoglobin Nancy difference map contains two weaker ones which may be very significant. A pair of positive and negative peaks straddle the native density of Cys 93β (the positive peak may be diminished and distorted by the massive negative peak adjacent to it at the position of Tyr 145β) indicating a movement of Cys 93 toward the space normally occupied by Tyr 145β. Another weak pair of peaks flank the iron atom of the β heme group indicating a small movement of the iron toward the heme plane, or possibly a small shift of the entire heme group with the movements of the light atoms unobserved at the resolution of this map. Anderson (17) has observed a similar movement upon oxidation of deoxyhemoglobin A, but in that case the presence of the water ligand produced a massive positive peak (relative to the weak negative peak on the opposite side of the iron atom) not seen in our difference map.

In contrast to the large structural changes caused by the mutation in deoxyhemoglobin Nancy, the difference electron density map of deoxyhemoglobin Cochin-Port-Royal (Fig. 4C) shows only one major feature, a very intense negative peak which is at the position of the imidazole group of His 146β in deoxyhemoglobin A. A few very weak positive contours (not shown) indicate that the guanidinium group of Arg 146β assumes a number of random conformations on the surface of the β subunit.

**DISCUSSION**

**Hemoglobin Cochin-Port-Royal**

**Bohr Effect**—In deoxyhemoglobin A, His 146β contributes to the formation of two salt bridges. One is intrasubunit linking the imidazole group with Asp 94β and the other is intersubunit linking the α-carboxyl group with Lys 40α (3). From NMR experiments on carbonmonoxy- and deoxyhemoglobin A, Kilmarbin et al. (18) found that the pK of the imidazole group is increased to 8.1 in deoxyhemoglobin from the normal value of 7.1 found in carbonmonoxyhemoglobin. They estimate from this change in pK that the His 146-Asp 94 salt bridge is responsible for about 40% of the alkaline Bohr Effect.

Our X-ray studies of deoxyhemoglobin Cochin-Port-Royal show that the guanidinium ion of Arg 146β floats freely in solution and does not interact with Asp 94β to reform the intrasubunit salt bridge. Remarkably, the changes in electron density are confined entirely to the side chain of residue 146β. No other structural perturbations are observed. In particular, the intrasubunit salt bridge is completely intact in deoxyhemoglobin Cochin-Port-Royal.

The functional studies of Wajcman et al. (6) showed that hemoglobin Cochin-Port-Royal has a normal Hill coefficient, a normal response to DPG, and (at physiological pH) a normal oxygen affinity. The only functional difference is a reduced Bohr effect, 0.35 proton/heme compared with 0.48 proton/heme for hemoglobin A; a decrease of 27%. It is not clear why the full 40% decrease is not realized in this case, but it may be that the guanidinium ion of 146β decreases the pK of some basic group (e.g., His 22, His 136, or the α-amino group of Val 113) in oxyhemoglobin Cochin-Port-Royal and thereby generates some additional Bohr protons to partially compensate for the loss of the salt bridge in the deoxy structure.

**Cooperativity**—When both the inter- and intrasubunit salt bridges associated with His 146β are removed (as in des-His 146β) hemoglobin (19) where residue 146β is specifically eliminated by digestion with carboxypeptidase B, or in N-ethylsuccinimide hemoglobin (2) where the bulky N-ethyldiim
FIG. 4. Fourier maps showing contiguous sections of electron density immediately below those shown in Fig. 3. This region of the β chains includes the lower half of the H helices and residues His 146 and Asp 94. The label x marks the position of the α carbon atom of His 143β.

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mide group attached to Cys 93β prevents the salt bridges from forming), the Hill coefficient is reduced to about 2.5 (see Table II). Our x-ray and functional studies of hemoglobin Cochin-Port-Royal show that the intrasubunit salt bridge is not required to full cooperativity (n = 3.0). However, it is not true that the presence of the intersubunit salt bridge implies full cooperativity. In the case of hemoglobin Hiroshima, replacement of His 146β by aspartate causes a decrease in n to 2.4 (21), but x-ray studies have shown that the intersubunit salt bridge is not broken in the deoxy state (22).

Hemoglobin Nancy

Stability of T State—In addition to hemoglobin Nancy, an enzymatically modified hemoglobin and two mutant hemoglobins involving Tyr 145β have been studied in some detail; des-(His 146β, Tyr 145β) hemoglobin (4, 23), hemoglobin Bethesda 145β Tyr→His (24, 25), and hemoglobin Rainier 145β Tyr→Cys (24, 26). In every case, the oxygen affinity is close to the average value for free α and β chains, cooperativity is virtually eliminated with n = 1.0 to 1.2, and the Bohr effect is reduced by about 40% to about 0.062 e Å⁻³. The large negative peak shows the displacement of the imidazole group of His 146β.

Removing the salt bridges, however, does not totally explain the very high oxygen affinity or the corresponding lack of cooperativity in these four hemoglobins because other hemoglobins, namely des-(His 146β) hemoglobin and N-ethylsuccinimide (NES) hemoglobin (27), which lack these salt bridges but retain Tyr 145β have moderately low oxygen affinities and n values of 2.5. Tyr 145β stabilizes the T state of deoxyhemoglobin by forming van der Waals contacts with portions of helices F and H and a hydrogen bond between its phenolic hydroxyl group and the carbonyl group of Val 98β (3). Using the allosteric model of Monod et al. (28), Baldwin (29) has shown that the parameter L (the ratio of T to R structure in deoxyhemoglobin) must be reduced from a normal value of 10⁵ to about 300 to account for the higher oxygen affinity and lower n value of des-(His 146β) hemoglobin (4, 23). Using the allosteric model of Monod et al. (28), Baldwin (29) has shown that the parameter L (the ratio of T to R structure in deoxyhemoglobin) must be reduced from a normal value of 10⁵ to about 300 to account for the higher oxygen affinity and lower n value of des-(His 146β) hemoglobin (27). Using the allosteric model of Monod et al. (28), Baldwin (29) has shown that the parameter L (the ratio of T to R structure in deoxyhemoglobin) must be reduced from a normal value of 10⁵ to about 300 to account for the higher oxygen affinity and lower n value of des-(His 146β) hemoglobin (27).
The affinity of IHP for deoxyhemoglobin Nancy must have decreased and/or it must have increased for oxyhemoglobin Nancy. The observed disordering of His 143β in deoxyhemoglobin Nancy implies that IHP would probably have a decreased affinity for the deoxy structure since His 143β is part of the IHP binding site (31). This is consistent with the very small increase of only 0.13 proton/heme in the Bohr effect of hemoglobin Nancy (compared with 0.45 proton/heme for hemoglobin A) after the addition of a 10-fold excess of IHP.

The small increase in the Bohr effect also indicates that the His 146β-Asp 94β salt bridge is not reformed in the IHP ‘deoxy-hemoglobin Nancy complex. The observed disordering of His 143β in deoxyhemoglobin A and abnormal deoxyhemoglobin Nancy must have its action on hemoglobin A. It can be shown (29) to a good approximation that the value of \( \log p_{50} \) due to the binding of any heterotopic ligand is related only to the association constants of that ligand (in this case IHP) with deoxy and fully oxygenated hemoglobin. Therefore, relative to hemoglobin A the affinity of IHP for deoxyhemoglobin Nancy must have decreased and/or it must have increased for oxyhemoglobin Nancy. The observed disordering of His 143β in deoxyhemoglobin Nancy implies that IHP would probably have a decreased affinity for the deoxy structure since His 143β is part of the IHP binding site (31). This is consistent with the very small increase of only 0.13 proton/heme in the Bohr effect of hemoglobin Nancy (compared with 0.45 proton/heme for hemoglobin A) after the addition of a 10-fold excess of IHP.

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In the presence of IHP the value of \( L \) for deoxyhemoglobin A increases from 10^4 to 10^6 (29). The ratio of T to R structure in aquomethemoglobin is given by \( L^* = Lc^* \) (where \( c = K_T/K_R \) is the ratio of equilibrium constants for the reduction reaction in the R and T states). Perutz et al. (32) have estimated that for aquomethemoglobin A the value of \( L^* \) changes from <1 to >1 with the addition of IHP. This inversion of population in favor of the T structure is thought to result in an increase in tension on the heme iron which in turn leads to changes in the adsorption spectrum of aquomethemoglobin (32). If, however, the value of \( L \) is much lower than 10^4, as it is in deoxyhemoglo-

| Hemoglobin                  | Functional properties | Structural differences between deoxyhemoglobin A and abnormal deoxyhemoglobin |
|-----------------------------|-----------------------|--------------------------------------------------------------------------------|
|                            | \( \log p_{50} \)     | \( \pi \) |    | Conditions | Ref. |                          | Ref.                                        |
| Hemoglobin A                | 0.80                  | 3.0 | 100 | 25°, pH 7.1, 0.05 M bis-Tris, 0.10 M CI^- | 6 | Only the intrasubunit salt bridge between His 146β and Asp 94β is lost; no other structural changes are detectable. |
| Cochin-Port-Royal (β146(HC3) His→Arg) | 0.80                  | 3.0 | 73 | 25°, pH 7.1, 0.05 M bis-Tris, 0.10 M CI^- | 6 |                                                |
| Nancy (β145(HC2) Tyr→Asp)  | -0.50                 | 1.1 | 50 | 25°, pH 7.2, 0.05 M bis-Tris, 0.10 M CI^- | 5 | Severe disordering of the β chain COOH-terminal tetrapeptide results in the loss of both salt bridges associated with His 146β as well as the intrasubunit hydrogen bond between Tyr 146β and Val 98β. |
| Hiroshina (β146(HC3) His→Asp) | 0.30                  | 2.4 | 60 | 20°, pH 7.2, 0.10 M phosphate | 20, 21 | Only the intrasubunit salt bridge between His 146β and Asp 94β is lost; other structural changes are small. |
| des–(His 146β) (His 146β(HC3) removed) | 0.40                  | 2.5 | 60 | 25°, pH 7.2, 0.20 M phosphate | 27 | Both salt bridges associated with His 146β are lost; other structural changes are small. |
| N-Ethylmaleimide hemoglobin | 0.56                  | 2.4 | 60 | 25°, pH 7.2, 0.20 M phosphate | 27 | Both salt bridges associated with His 146β are lost; other structural changes are small. |
| des–(His 146β, Tyr 145β) (His 146β(HC3) and Tyr 145β(HC2) removed) | -0.42                 | 1.0 | 33 | 30°, pH 7.2, 0.10 M bis-Tris | 4 | Both salt bridges associated with His 146β are lost as well as the intrasubunit hydrogen bond between Tyr 146β and Val 98β; the COOH-terminal dipeptide (Lys 144β and His 143β) is disordered. |
| Rainier (145β(HC2) Tyr→Cys) | -0.48                 | 1.1 | 68 | 20°, pH 7.2, 0.10 M phosphate | 24 | Both salt bridges associated with His 146β are lost as well as the intrasubunit hydrogen bond between Tyr 146β and Val 98β; a disulfide bridge between Cys 145β and Cys 93β is formed. |
| Betheda (145β(HC2) Tyr→His) | -0.30                 | 1.2 | 52 | 20°, pH 7.2, 0.10 M phosphate | 24 |                                                |

50% in hemoglobin Nancy is consistent with the loss of the His 146β-Asp 94β salt bridge (2, 19). On the other hand, Kilmartin et al. (5) have shown that only 10% of the Bohr effect is related to changes in the R structure upon ligation, the remainder is generated by the R → T transition. Thus, retention of one-half of the Bohr effect is evidence for the R → T transition in solution and this is consistent with the crystallization of deoxyhemoglobin Nancy in the T state.

**Effect of IHP—**Subsequent to the discovery of Benesch et al. (30) of IHP as an extremely potent effector of hemoglobin oxygen affinity, the dimished or absent allosteric properties of several abnormal hemoglobins have been partially (or even fully) restored to normal levels by the addition of IHP (4, 5).

We find that a 10-fold excess of IHP increases \( \pi \) from 1.1 to 2.0 for hemoglobin Nancy, but that it is only about 60% as effective in raising the log \( p_{50} \) of hemoglobin Nancy relative to its action on hemoglobin A. It can be shown (29) to a good approximation that the value of \( \log p_{50} \) due to the binding of any heterotopic ligand is related only to the association constants of that ligand (in this case IHP) with deoxy and fully oxygenated hemoglobin. Therefore, relative to hemoglobin A the affinity of IHP for deoxyhemoglobin Nancy must have decreased and/or it must have increased for oxyhemoglobin Nancy. The observed disordering of His 143β in deoxyhemoglobin Nancy implies that IHP would probably have a decreased affinity for the deoxy structure since His 143β is part of the IHP binding site (31). This is consistent with the very small increase of only 0.13 proton/heme in the Bohr effect of hemoglobin Nancy (compared with 0.45 proton/heme for hemoglobin A) after the addition of a 10-fold excess of IHP. The small increase in the Bohr effect also indicates that the His 146β-Asp 94β salt bridge is not reformed in the IHP-deoxyhemoglobin Nancy complex.
hin Nancy, then the value of $I_6$ may be so low that addition of excess IHP will not produce a significant concentration of T structure. Moreover, as discussed above, IHP does not shift the $R \rightarrow T$ equilibrium as much in hemoglobin Nancy as in hemoglobin A. These two factors together with a reduction of tension in the $\beta$ hemes of the hemoglobin Nancy T structure (see below) would explain the inability of IHP to change the visible absorption spectrum of aquomethemoglobin Nancy (Fig. 2).

Release of Heme Tension—High spin heme iron in the ferrous state, and to a smaller degree in the ferric state, is out of the plane of the porphyrin ring because of its large radius (3, 33). Perutz (34, 35) and Perutz et al. (15, 16, 32) have presented evidence to support the idea that the low oxygen affinity of deoxyhemoglobin A is due in part to constraints on the globin heme group and adjacent atoms which would produce differences in electron density too weak to be observed for the lighter triflavin. Therefore consistent with Perutz’s hypothesis. However, at the present resolution of our map we cannot rule out the possibility that this movement reflects a very small shift of the entire heme plane would be expected. Our difference map of deoxyhemoglobin Nancy indicates such a movement and is therefore consistent with Perutz’s hypothesis. However, at the present resolution of our map we cannot rule out the possibility that this movement reflects a very small shift of the entire heme group and adjacent atoms which would produce differences in electron density too weak to be observed for the lighter atoms.

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