Involvement of Glu G3(101)β in the Function of Hemoglobin

COMPARATIVE O₂ EQUILIBRIUM STUDIES OF HUMAN MUTANT HEMOGLOBINS*

(Received for publication, July 6, 1984)

Daniel T.-b. Shih‡, Richard T. Jones‡, Kiyohiro Imai‡, and Itiro Tyuma‡

From the ‡Department of Biochemistry, School of Medicine, Oregon Health Sciences University, Portland, Oregon 97201 and the ‡Department of Physicochemical Physiology, Medical School of Osaka University, Nakanoshima, Osaka 530, Japan

The glutamyl residue at G3(101)β of normal hemoglobin (Hb A) is one of the αβ2 subunit contacts which are vital to O₂ binding properties of the molecule. The O₂ equilibrium properties of the four mutants with different substitutions at this site are studied in order to elucidate the role of this residue. Under stripped conditions with minimum chloride the order of O₂ affinities is: Hb A (Glu) ≪ Hb Rush (Gln) ≪ Hb British Columbia (Lys) ≪ Hb Potomac (Asp) ≪ Hb Alberta (Gly). The first Adair constants, K₁, for the mutant hemoglobins are greater than that for Hb A whereas the fourth, K₄, are similar, indicating that the allosteric constants (L) of these mutants are greatly reduced. Therefore, the G3(101)β residue contributes intrinsically to the strengthening of the structural constraints that are imposed upon the deoxy (T) forms but not the oxy (R) form. On addition of 0.1 M Cl⁻ and further addition of 2,3-diphosphoglycerate or inositol hexaphosphate, their O₂ affinities and cooperativities are altered, reflecting different responses to anionic ligands. Hb Rush exhibits a stronger chloride effect than Hb A and the other variants and, as a result, an increased Bohr effect and a smaller heat of oxygenation at pH 6.5. These changes are consistent with an increased positive net charge in the central cavity of Hb Rush and subsequent extra anion binding in the deoxy form. The tetramer to dimer dissociation constants are estimated to be greater than normal for Hb British Columbia and less than normal for Hb Alberta. This comparative study of the G3(101)β mutants indicates that the size and the charge of this residue may influence the switching of two neighboring interchain hydrogen bonds that occur during oxygenation of normal hemoglobin.

A structure-based understanding of the O₂ binding mechanism of the hemoglobin tetramers has been developed by Perutz and his co-workers (1–4) using x-ray crystallography. According to these studies structural sites for O₂-linked allosterism are grouped into three specific regions: 1) O₂ binding sites (heme and its contacts), 2) allosteric binding sites (H⁺, CO₂, 2,3-diphosphoglycerate, Cl⁻ binding sites), and 3) subunit interfaces (mainly the αβ2). Simultaneously with the x-ray crystallographic investigations, extensive studies of the functional properties of abnormal hemoglobins as well as chemically modified hemoglobins in solution state have been carried out by other techniques. Conclusions from these results are complementary to those drawn from x-ray crystallographic studies in many ways, but they are not entirely in agreement. Therefore, further studies of abnormal and chemically modified hemoglobins are continuing in order to gain a better understanding of the ligand binding mechanism of the hemoglobin tetramer.

According to the Monod, Wyman, and Changuey two-state theory (5) the quaternary structures of the two states, R (relaxed) and T (tense), are different in the number and energy of the noncovalent bonds between the subunits. These stabilizing contacts are known to be primarily at αβ₂ subunit interfaces where striking differences have been demonstrated to occur upon ligation (1–3). Mutant hemoglobins which have substitutions at this interface generally manifest dramatic changes in their functional properties (6, 7). Typical examples are mutations that occur at Asp G1(99)β which results in a shift of the allosteric equilibrium towards the R state while mutations at Asn G4(102)p result in a T' shift. These changes in the allosteric equilibrium are due to alterations of some of the contacts of these residues which stabilize either the T form or the R form of the quaternary structure of normal hemoglobin.

In this paper we describe a comprehensive, comparative study of four mutant hemoglobins that affect the residue 191 (G3) of the β chain. This is located between G1(99)β and G4(102)β at the αβ₂ subunit interface. Earlier reports of G3(101)β hemoglobin mutants indicate that substitution for the glutamyl residue in normal hemoglobin results in variable changes of the functional properties of the hemoglobin molecule. Hb British Columbia (Lys (101)β) (8) and Hb Alberta (Gly (101)β) (9) were found to have high O₂ affinities while Hb Rush (Gln (101)β) (10) was reported to exhibit a normal function. The remaining mutant, Hb Potomac (Asp (101)β), had been detected because of an abnormal O₂ affinity of the blood of the affected individuals rather than by electrophoresis because its amino acid substitution does not involve a charge difference (11). Explorations of these abnormal functional properties based upon x-ray crystallographic data are not yet available (12). Furthermore, the earlier reports were individual studies that were carried out under different experimental conditions and therefore cannot be compared easily. To elucidate the functional differences of these mutants as well as to evaluate the importance of the glutamyl residue of G3(101)β in the normal allosteric oxygen binding, we have done comparative studies of these four mutants under the same experimental conditions.

**MATERIALS AND METHODS**

Heparinized blood was obtained from the propositus or affected relative for whom the abnormal hemoglobins were first described. In...
the case of Hb British Columbia, a sample was also obtained from a second apparently unrelated person from Alaska. Unfractionated hemolysates obtained from washed, lysed erythrocytes were then freed from organic phosphates by passage through an ion-exchange (Diiatid) column at 4 °C (13). These were stored in the CO form on ice. The CO derivative of abnormal hemoglobin was isolated from normal CO hemoglobins on a DEAE-Sephadex (Pharmacia) column at 4 °C. A nine-chamber pH gradient system from pH 8.1 to 7.4 of 0.05 M Tris-HCl was used for the chromatography. Hb Potomac was isolated using an anaerobic chromatography system as described earlier (14). The bound CO was removed by a photoconversion method (15). Tris, bis-Tris, inositol hexaphosphate, 2,3-diphosphoglycerate, and all the components of a methemoglobin reducing system (16) were purchased from Sigma. Reagents of NaCl, HCl, K2HPO4, and KH2PO4 were products of J. T. Baker Chemical Co. Oxygen equilibrium curves were measured with an automatic recording apparatus of Imai et al. (17–19). A Gilford 290 spectrophotometer and a polarographic oxygen electrode of Beckman Instruments (No. 39065) were employed in this apparatus. Data acquisition and reduction were accomplished using a PDP 11/20 computer (Digital Equipment Corp.), as previously reported (19–21). The concentration of hemoglobin samples was 80 μM on a heme basis. To maintain the methemoglobin formation at minimum levels, the methemoglobin reducing system (16) was added to each sample before measurement.

The Adair constants (K1 to K4), i.e. the intrinsic association equilibrium constants for four-step oxygen binding, were evaluated by a least-square curve-fitting method (19, 21). Overall oxygen affinity and cooperativity were measured by median oxygen pressure (P50) and maximal slope of the Hill plot (nmax), respectively, which were calculated from the Adair constants. Oxygen equilibrium data obtained for various hemoglobin concentrations were analyzed in terms of the Hill scheme as described by Imai and Yonetani (22) to evaluate the tetramer-dimer dissociation constant and other related parameters.

RESULTS

Oxygen equilibrium curves for Hb A isolated from the four hemolysate samples showed good agreement. The percentage of methemoglobin was less than 3% before the equilibrium measurements and not more than 7% after.

At pH 7.4 in the presence of minimum amounts of anions (approximately 7 mM Cl−, phosphate free) all of the abnormal hemoglobins presented intrinsic properties of high O2 affinity (1/P50) and impaired cooperativity (nmax) (Fig. 1A). The order of O2 affinity is: Hb A < Hb Rush < Hb British Columbia < Hb Potomac < Hb Alberta. The differences in oxygen affinity among the abnormal hemoglobins are amplified to different degrees on the addition of 0.1 M chloride (Fig. 1B). At this condition the O2 binding properties of Hb Rush resemble those of Hb A. On the addition of stronger allosteric effectors such as 2 mM 2,3-diphosphoglycerate or 2 mM inositol hexaphosphate, the O2 binding of these abnormal hemoglobins is partially restored to normal in varying degrees (Table I). The order of O2 affinity among the abnormal hemoglobins are amplified to different degrees on the addition of 0.1 M chloride (Fig. 1B). At this condition the O2 binding properties of Hb Rush resemble those of Hb A. On the addition of stronger allosteric effectors such as 2 mM 2,3-diphosphoglycerate or 2 mM inositol hexaphosphate, the O2 binding of these abnormal hemoglobins is partially restored to normal in varying degrees (Table I). The order of O2 affinity among the abnormal hemoglobins are amplified to different degrees on the addition of 0.1 M chloride (Fig. 1B). At this condition the O2 binding properties of Hb Rush resemble those of Hb A.

Table I summarizes values of K1 (i = 1 to 4), 1/P50, and nmax which were obtained under various anionic conditions at pH 7.4. The coefficient of variation was less than 10% for K1 and K4 values but varied over relatively wide ranges for K2 and K3. The K1 values for the β101 mutants except for Hb Rush are greater than that for Hb A while the K4 values are not very different from that for Hb A. For Hb British Columbia, K4 in the stripped conditions is somewhat smaller than that of the other hemoglobins. For Hb Rush, K1 in the presence of organic phosphate is similar or smaller than that of Hb A and always smaller than that of the other mutants.

The decrease in O2 affinity or the increase in log P50 in response to the increase of chloride concentration is similar

1 The abbreviation used is: bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.
Involvement of Glu G3(101)β in the Function of Hemoglobin

TABLE I
Calculated values of Adair constants (K, (mm Hg)^{-1}), median O₂ pressure (P₅₀ (mm Hg)), and maximal slope of the Hill plot (n₅₀)

| G3(101)β Mutant Hbs | Control (Hb A) | Hb Alberta | Hb BC* | Hb Rush | Hb Pt* (Asp) |
|---------------------|----------------|------------|--------|---------|--------------|
| (60 μmol/hemoglobin) | (Glu)          | (Gly)      | (Lys)  | (Gln)   | (Asp)        |
| In the presence of ~7 mM Cl⁻ |                |            |        |         |              |
| K₁              | 18 × 10^{-2}  | 11 × 10^{-2} | 94 × 10^{-2} | 62 × 10^{-2} |              |
| K₂              | 47 × 10^{-2}  | 26 × 10^{-2} | 88 × 10^{-2} | 15 × 10^{-2} |              |
| K₃              | 41 × 10^{-2}  | 17 × 10^{-2} | 30 × 10^{-2} | 90 × 10^{-2} |              |
| K₄              | 47 × 10^{-2}  | 70 × 10^{-2} | 29 × 10^{-1} | 50 × 10^{-1} |              |
| 1/P₅₀           | 63 × 10^{-2}  | 24 × 10^{-2} | 16 × 10^{-1} | 14 × 10^{-1} | 21 × 10^{-1} |
| n₅₀             | 2.6           | 1.4        | 1.5    | 1.5     |              |
| In the presence of 0.1 M Cl⁻ |                |            |        |         |              |
| K₁              | 54 × 10^{-3}  | 71 × 10^{-2} | 24 × 10^{-2} | 89 × 10^{-3} |              |
| K₂              | 65 × 10^{-3}  | 16 × 10^{-2} | 45 × 10^{-3} | 27 × 10^{-3} |              |
| K₃              | 10 × 10^{-2}  | 61 × 10^{-2} | 14 × 10^{-1} | 14 × 10^{-2} |              |
| K₄              | 66 × 10^{-1}  | 35 × 10^{-1} | 30 × 10^{-1} | 54 × 10^{-1} |              |
| 1/P₅₀           | 22 × 10^{-2}  | 13 × 10^{-3} | 46 × 10^{-3} | 22 × 10^{-2} | 11 × 10^{-1} |
| n₅₀             | 2.9           | 2.0        | 2.3    | 2.7     | 1.6          |
| In the presence of 0.1 M Cl⁻ and 2 mM DPG |                |            |        |         |              |
| K₁              | 15 × 10^{-3}  | 13 × 10^{-2} | 32 × 10^{-3} | 13 × 10^{-3} |              |
| K₂              | 11 × 10^{-3}  | 20 × 10^{-2} | 36 × 10^{-3} | 70 × 10^{-4} |              |
| K₃              | 49 × 10^{-3}  | 68 × 10^{-3} | 67 × 10^{-3} | 45 × 10^{-3} |              |
| K₄              | 39 × 10^{-4}  | 50 × 10^{-3} | 34 × 10^{-1} | 62 × 10^{-1} |              |
| 1/P₅₀           | 76 × 10^{-3}  | 52 × 10^{-3} | 13 × 10^{-2} | 70 × 10^{-3} | 48 × 10^{-2} |
| n₅₀             | 3.1           | 2.4        | 2.7    | 3.2     | 1.4          |
| In the presence of 0.1 M Cl⁻ and 2 mM IHP |                |            |        |         |              |
| K₁              | 90 × 10^{-4}  | 27 × 10^{-3} | 15 × 10^{-3} | 50 × 10^{-4} |              |
| K₂              | 11 × 10^{-3}  | 57 × 10^{-3} | 25 × 10^{-3} | 15 × 10^{-3} |              |
| K₃              | 50 × 10^{-4}  | 43 × 10^{-3} | 70 × 10^{-4} | 20 × 10^{-4} |              |
| K₄              | 11 × 10^{-2}  | 50 × 10^{-2} | 21 × 10^{-1} | 13 × 10^{-1} |              |
| 1/P₅₀           | 28 × 10^{-3}  | 13 × 10^{-3} | 47 × 10^{-3} | 20 × 10^{-3} | 16 × 10^{-2} |
| n₅₀             | 2.5           | 2.7        | 2.4    | 2.4     | 1.7          |

*BC, British Columbia.
*Pt, Potomac (this sample is not adequate for the calculation of K₅₀ values because of the contamination of about 15% Hb A (see text)).

be neglected as will be described later. The pH dependences of n₅₀, for the mutant hemoglobins except for Hb Alberta are similar to that for Hb A while Hb Alberta shows a somewhat larger pH dependence of n₅₀.

Heats of oxygenation (ΔH) were obtained by using the Van't Hoff equation from oxygen equilibrium curves determined at different temperatures (20, 25, and 30 °C) in the presence of 0.1 M Cl⁻. All of the hemoglobins studied showed similar ΔH values at pH 9.0, while only Hb Rush shows a smaller ΔH value at pH 6.5 (Table II).

Oxygen equilibrium curves were determined at different hemoglobin concentrations ranging from 2 to 120 μM in 0.05 M bis-Tris buffer (pH 7.4) containing 0.1 M Cl⁻ at 25 °C. Values of log P₅₀ and n₅₀ (the Hill coefficient at half-saturation) are plotted against the inverse of protein concentration in Fig. 4. Hb British Columbia shows greater concentration dependences than the other hemoglobins. The decrease in both parameters upon going to low protein concentrations is attributed to partial dissociation of the tetramer to the αβ dimers that bind oxygen with a high affinity but without cooperativity. The concentration-dependent oxygenation data were analyzed by the method of Adair and Yonetani (22) in which oxygen saturation of a given hemoglobin solution is expressed by a linear combination of Hill equations for the tetramer and for the dimer. The fractions of the tetramer and dimer are treated as functions of oxygen saturation. Table III summarizes values of the parameters which are contained in the scheme described above. Lines drawn in Fig. 4 were calculated from these parameter values. The fits of the calculated lines to the experimental plots are good except for the log P₅₀ data of Hb Rush and the n₅₀ data of Hb Alberta. The parameter values indicate that: the free αβ dimers of all the hemoglobins listed in Table III exhibit the same oxygen affinity; the order of oxygen affinity for the tetramer is Hb Rush < Hb A < Hb British Columbia < Hb Alberta; the cooperativity of Hb Alberta is lower than those of the other hemoglobins; and the tetramer-dimer dissociation constant of Hb Rush is normal, but the subunit dissociations of Hb Alberta and Hb British Columbia are less and more, respectively, compared to that of Hb A. From the present analysis, the P₅₀ and n₅₀ values for purely tetrameric hemoglobin can be inferred. The parameter values calculated for 60 μM hemoglobin solutions (P₅₀ (60 μM)) are somewhat different from the inferred values for the tetramer (P₅₀ (4)), being most marked in Hb British Columbia (Table III). However, these differences are not so great that the qualitative conclusions drawn in the present study are seriously affected.

**DISCUSSION**

X-ray crystallographic studies of the hemoglobin tetramer show that the most drastic changes between the liganded and the unliganded forms occur along the αβ subunit interface (23-26). These changes are related to the relative movement of the two αβ dimers that involves: (a) the shift of the H bond...
Involvement of Glu G3(101)β in the Function of Hemoglobin

from Tyr C7(42)α1-Asp G1(99)β2 in the deoxy form to Asp G1(94)α1-Asn G4(102)β2 in liganded form, (b) the slipping of the FGβ2 corner relative to the C helix of the α1 subunit (His FG4(97)β2 nests between the side chains of Pro CD2(44)α1 and Thr G6(41)α1 in deoxyhemoglobin, whereas in the liganded form this His slips past the Thr (C6(41)α1 and sits between it and Thr C3(38)α1, and (c) the involvement of carboxy termini of both α and β chains in salt bridges in the deoxy form but not in the liganded form. A comparative study of hemoglobin mutants occurring at these regions by Shi et al. (27) indicated that the loss of the H bonds that occur in Asp G1(99)β2 mutants causes a drastic R shift which is greater than that observed for the substitutions occurring at the switching point, His FG4(97)β or at the carboxyl terminus His HC3(146)β. These results indicate that the H bond trade-offs along the αβα interfaces may serve as key factors in the quaternary structural switching from the T to the R during ligation which is responsible for the cooperativity of hemoglobin. In normal hemoglobin A, the glutamyl residue G3(101)β is located between these two H-bonds listed under a above. Results of the present comparative study of β101 mutants demonstrate the consequence of substituting for Glu G3(101)β on the affinity and cooperativity of the mutant hemoglobins. Comparison of the Adair constants, K1 and K4, for the normal and mutant hemoglobins in Table I indicates that the oxy conformation is similar for these hemoglobins while the deoxy conformation is not, and hence the Glu G3(101)β residue of Hb A contributes to maintaining the structural constraints imposed upon the deoxy form and stabilizing the deoxy tense structure relative to the oxy relaxed structure.

The relatively large decreases of O₂ affinity for Hb Rush on the addition of anions, such as Cl⁻, 2,3-diphosphoglycerate, or inositol hexaphosphate, indicate an increased O₂-linked anion effect in this variant. Dr. Perutz indicated to us that the negatively charged Glu G3(104)β is neutralized by the positively charged Arg G6(104)β in the central cavity of deoxy-Hb A.² The substitution of Glu→Gln in Hb Rush would

² M. F. Perutz, personal communication.

---

**Table II**

| Average heat of oxygenation | ΔH kcal/mol heme |
|----------------------------|------------------|
| pH 6.5                     |                  |
| Hb A                       | −13.8            |
| Hb Rush                    | −8.1             |
| Hb British Columbia        | −13.8            |
| Hb Alberta                 | −13.8            |
| pH 9.0                     |                  |
| Hb A                       | −17.1            |
| Hb Rush                    | −16.8            |
| Hb British Columbia        | −17.7            |
| Hb Alberta                 | −17.4            |

² In 0.05M bis-Tris buffer containing 0.1M Cl⁻ (pH 6.5).
³ In 0.05M Tris buffer containing 0.1M Cl⁻ (pH 9.0).
Involvement of Glu G3(101)β in the Function of Hemoglobin

The Bohr effect of hemoglobin is a typical heterotropic effect that arises from differences in the proton binding due to changes of the pK of specific ionizable groups that occur during oxygenation. Alteration of the alkaline Bohr effect of hemoglobin can be expected in cases of 1) direct or indirect modification of the Bohr groups, 2) modification of the binding sites of cofactors (28-30), and 3) extreme R-shift of the allosteric equilibrium with essential loss of subunit cooperativity (27). Due to the low pK and negative charge of the Glu G3(101)β, this residue is not likely to be a binding site for a Bohr proton or for other anion cofactors. Although increases to the O2 affinity are found in most of the 101 mutants, the subunit interactions remain in cooperative modes. Therefore, with the exception of Hb Rush it is not surprising that β101 mutants display a normal Bohr effect under the experimental conditions used. The increase of the Bohr effect in Hb Rush can be explained by its enhanced chloride binding because part of Bohr proton binding is associated with anion binding during deoxygenation.

Besides the non-heme ligands, there are other factors which can affect the oxygen equilibrium of hemoglobin such as temperature and hemoglobin concentration. As the concentration of hemoglobin decreases the average heat of 02-linked proton becomes larger. This results in an enhanced dimer dissociation and a relatively large R shift of the allosteric equilibrium with essential loss of subunit cooperativity (27). The increase of the Bohr effect in Hb Rush can be explained by its enhanced chloride binding because part of Bohr proton binding is associated with anion binding during deoxygenation.

The average heat of oxygenation of hemoglobin is pH dependent and can be determined indirectly by using the Van't Hoff equation. The difference of the heat of oxygenation, ΔH, between neutral pH and alkaline pH has been attributed to the heat of release of Bohr protons and anions upon oxygenation (32). In the present study, all of the β101 mutants except Hb Rush exhibit a normal heat of oxygenation at both pH 6.5 and 9.0. Hb Rush exhibits a smaller than normal heat of oxygenation at pH 6.5. This can be explained by the greater extent of cancellations of the average heat of O2 binding to hemoglobin by the greater heat of O2-linked proton and anion release of Hb Rush due to its excess binding of anion and proton.

It is clear that various amino acid residue substitutions that occur at the Glu G3(101)β site result in dramatic changes in the functional properties of hemoglobin. This presents a question of what factor contributes most towards maintenance of normal hemoglobin function: charge, hydrophilicity, or size of this amino acid residue. The O2 affinities of the β101 mutants relative to Hb A are in increasing order: Hb A (Glu) < Hb Rush (Gln) < Hb British Columbia (Lys) < Hb Potomac (Asp) < Hb Alberta (Gly), when measured under the stripped condition with minimal chloride. The data suggest that the size of the amino acid residue is the single most critical factor in determining the relative oxygen affinities of this set of hemoglobins. However, the negative charge of the normal β101 Glu is also important in maintaining normal function. In the case of Hb British Columbia, the increase in O2 affinity is amplified by its enhanced dimer dissociation which is probably the result of the extra lysyl positive charges along the αβ2 interfaces. The substantial increase in the O2 affinity of Hb Potomac (Asp), which has similar charge and hydrophilic properties as Hb A (Glu), can only be explained by the change in size of the residue side chain or location of its carboxyl group. Hb Rush (Gln), which has the least change in size but has lost the negatively charged carboxyl group, shows the least change in O2 binding properties compared to Hb A (Glu). The increased chloride effect of Hb Rush is most

![Fig. 4. The effect of hemoglobin concentration on the O2 binding properties of isolated β101 mutant hemoglobins in 0.05 M bis-Tris buffer, pH 7.4, 25°C. Cooperativity (nH) above and oxygen affinity (log P50) below. The arrow on the abscissa indicates the concentration used in the present study for comparison of functional properties.](image_url)

**Table III**

| Experimental conditions as in the legend to Fig. 4. |
|---------------------------------------------------|
| **Hb A** | **Hb Alberta** | **Hb British Columbia** | **Hb Rush** |
| Kd(αβ dimer) | 3 | 3 | 3 | 3 |
| Kd(tetramer) | 0.17 | 1.1 | 0.35 | 0.14 |
| m' | 3.1 | 2.2 | 3.1 | 3.2 |
| Kd(αβ dimer) | 6 × 10^-4 | 5 × 10^-7 | 3 × 10^-3 | 6 × 10^-5 |
| P50 (60 μM) | 5.1 | 0.86 | 2.1 | 6.2 |
| P50 (tL) | 5.9 | 0.91 | 2.9 | 7.1 |
| n50 (60 μM) | 2.7 | 2.0 | 2.3 | 2.8 |
| n50 (tL) | 3.1 | 2.2 | 3.1 | 3.2 |

*Oxygen association constant for αβ dimer (mm Hg^-1).

*Oxygen association constant for hemoglobin tetramer (mm Hg^-1).

*Hill coefficient for hemoglobin tetramer.

*Hill coefficient for hemoglobin tetramer.

*Hill coefficient for oxyhemoglobin (M).

*Calculated value of oxygen pressure at half-saturation for 60 μM hemoglobin solution (mm Hg).

*Calculated value of oxygen pressure at half-saturation for purely tetrameric hemoglobin (mm Hg).

*Calculated value of oxygen pressure at half-saturation for purified tetrameric hemoglobin.

*Calculated value of Hill coefficient for purely tetrameric hemoglobin.

Prevent the neutralization of the positive Arg G6(104)β. According to this explanation we could account for the extra Cl⁻ binding by deoxy-Hb Rush by postulating the attraction of the Cl⁻ by a positively charged cluster formed along the αβ2 contact in the cavity. Following from this postulation, one would expect the chloride effect on the function of the 101 mutants to be in the order of Hb British Columbia > Hb Rush > Hb Alberta > Hb A = Hb Potomac. However, the chloride effect on oxygen affinity (expressed by Δ log P50/Δ log [Cl⁻]) for these hemoglobins was found to be of the same order, therefore, for these properties, therefore, would cancel out the extra Cl⁻ binding.

The Bohr effect of hemoglobin is a typical heterotropic effect that arises from differences in the proton binding due
likely due to the extra allosteric binding of chloride anion due to the increase in the net positive charge in the central cavity along the $\alpha_1 \beta_2$ interface due to the loss of the negative charges of the $\beta 101$ Glu. Thus, the size and charge of Glu $\beta 101$ apparently are both involved in maintaining normal $O_2$ affinity and allosteric properties, respectively. Therefore, the contribution of the $\beta 101$ site to the mechanism underlying the molecular function does not seem to be the result of any single factor alone.

Although the complete role of Glu G3(101)$\beta$ in the functional properties of hemoglobin remains uncertain, the data presented here do provide further insight into the involvement of this important residue in the allosterism of hemoglobin. As mentioned in the Introduction, mutant hemoglobins which have substitutions at the $\alpha_1 \beta_2$ interface manifest dramatic changes in their functional properties. The most drastic changes in both homotropic and heterotropic properties of $\alpha_1 \beta_2$ mutants yet found occur from substitutions for the normal $\beta 99$ residue (27). All of the $\beta 99$ mutants appear to disrupt  a critical hydrogen bond which stabilizes the normal T conformation (7). This leads us to believe that the H bonds at the $\alpha_1 \beta_2$ subunit interface are more important than other electrostatic or van der Waals forces in maintaining the normal T conformation of the molecule. The $\beta 101$ residue is located between this critical $\beta 99$ site and the $\beta 102$ residue that also forms a H bond which stabilizes the normal R conformation. We postulate that any change in the size or charge of the normal $\beta 101$ residue adversely affects the switching of the molecule between its two normal quaternary structures (T and R).

Acknowledgments—We express our appreciation to Dr. M. F. Perutz for his valuable comments and to Dr. H. Morimoto for using his atomic model with helpful discussion.

REFERENCES
1. Muirhead, H., and Greer, J. (1970) Nature 228, 516-519
2. Perutz, M. F. (1970) Nature 228, 726-730
3. Perutz, M. F. (1976) Br. Med. Bull. 32, 195-208
4. Ferri, G. (1975) J. Mol. Biol. 97, 237-256
5. Monod, J., Wyman, J., and Changeux, J. P. (1965) J. Mol. Biol. 12, 88-118
6. Perutz, M. F., and Lehman, H. (1968) Nature 219, 902-909
7. Morimoto, H., Lehmann, H., and Perutz, M. F. (1971) Nature 232, 408-413
8. Jones, R. T., Brimhall, B., and Gray, G. (1976-77) Hemoglobin 1, 171-182
9. Mant, M. J., Salkie, M. L., Cope, N., Appling, F., Bolch, K., Jayakalashmi, M., Gravelly, M., Wilson, J. B., and Huisman, T. H. J. (1976-77) Hemoglobin 1, 183-194
10. Adams, J. G., III, Winter, W. P., Tausk, K., and Heller, P. (1974) Blood 43, 261-269
11. Charache, S., Jacobson, R., Brimhall, B., Murphy, E. A., Hathaway, P., Winslow, R., Jones, R., Rath, C., and Sunkovich, J. (1978) Blood 51, 331-338
12. Fermi, G., and Perutz, M. F. (1981) in Atlas of Molecular Structures in Biology: (2) Haemoglobin-Myoglobin (Phillips, D. C., and Richards, F. M., eds) p. 77, Clarendon Press, Oxford
13. Nozaki, Y., and Tanford, C. (1987) Methods Enzymol. 11, 715-733
14. Shiib, T.-B., and Jones, R. T. (1980) Hemoglobin 4, 541-550
15. Shiib, T.-B., Jones, R. T., and Johnson, C. S. (1982) Hemoglobin 6, 1553-167
16. Hayashi, A., Suzuki, T., and Shin, M. (1973) Biochim. Biophys. Acta 310, 309-316
17. Imai, K., Morimoto, H., Kotani, M., Watari, H., Hirata, W., and Kuroda, M. (1970) Biochim. Biophys. Acta 200, 189-196
18. Imai, I. (1981) Methods Enzymol. 76, 438-489
19. Imai, K. (1982) Allosteric Effects in Hemoglobin, Cambridge University Press, Cambridge
20. Shiib, T. B., Jones, R. T., Bonaventura, J., Bonaventura, C., and Schneider, R. G. (1984) J. Biol. Chem. 259, 967-974
21. Imai, K. (1981) Methods Enzymol. 76, 470-486
22. Imai, K., and Yonetani, T. (1977) Biochim. Biophys. Acta 490, 164-170
23. Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. G. C. (1968) Nature 219, 131-139
24. Muirhead, H., and Greer, J. (1970) Nature 228, 516-519
25. Fermi, G. (1975) J. Mol. Biol. 97, 237-256
26. Baldwin, J., and Chothia, C. (1979) J. Mol. Biol. 129, 175-220
27. Shiib, T. B., and Jones, R. T. (1984) Brussels Hemoglobin Symposium (Schneik, A. G., and Paul, C., eds), pp. 103-115, Editions de l'Université de Bruxelles, Brussels
28. Van Beeck, G. G. M., Zuiderveld, Z. R. P., and de Bruin, S. H. (1979) Eur. J. Biochem. 99, 379-383
29. O'Donnell, S., Mandaro, R., Schuster, T. M., and Arnone, A. (1979) J. Biol. Chem. 254, 12204-12208
30. Ferret, M. P., Kilmartin, J. V., Nishikura, K., Floss, J. H., Butler, P. J. G., and Rollema, H. S. (1980) J. Mol. Biol. 138, 649-670
31. Ackers, G. K., Johnson, M. L., Mills, F. C., and Ip, S. H. C. (1976) Biochem. Biophys. Res. Commun. 69, 135-142
32. Autorini, E., Wyman, J., Brunori, M., Fronticelli, C., Bucci, E., and Rosai-Faneli, A. (1965) J. Biol. Chem. 240, 1066-1103