The Effects of Inositol Hexaphosphate on the Allosteric Properties of Two β-99-substituted Abnormal Hemoglobins, Hemoglobin Yakima and Hemoglobin Kempsey

Masako Nagai, Machiko Nishibu, Yoshihito Sugita, and Yoshimasa Yoneyama

From the Biological Laboratory, Kanazawa University School of Paramedicine, and the Department of Biochemistry, Kanazawa University School of Medicine, Kanazawa, Japan

Richard T. Jones

From the Department of Biochemistry, University of Oregon Medical School, Portland, Oregon 97201

Susan Gordon

From the Department of Haematology, Sydney Hospital, Sydney, Australia

Hemoglobins (Hb) Yakima and Kempsey were purified from patients' blood with diethylaminoethyl cellulose column chromatography. The oxygen equilibrium curves of the two hemoglobins and the effects of organic phosphates on the function were investigated. In 0.1 M phosphate buffer, Hill's constants n for Hb Yakima and Hb Kempsey were 1.0 to 1.1 at the pH range for 6.5 to 8.0 and the oxygen affinities of both the mutant hemoglobins were about 15 to 20 times that of Hb A at pH 7.0. The Bohr effect was normal in Hb Yakima and one-fourth normal in Hb Kempsey. In the presence of inositol hexaphosphate, the oxygen affinities of Hb Yakima and Hb Kempsey were greatly decreased, and an interesting result revealed that these hemoglobins showed clear cooperativity in oxygen binding. Hill's constant n in the presence of inositol hexaphosphate was 1.9 for Hb Kempsey and 2.3 for Hb Yakima at pH 7.0. The cooperativities of these mutant hemoglobins were pH-dependent, and Hb Kempsey showed high cooperativity at low pH (n = 2.1 at pH 6.6) and low cooperativity at high pH (n = 1.0 at pH 8.0). Hb Yakima showed similar pH dependence in cooperativity. In the presence of inositol hexaphosphate, Hb A showed a pH-dependent cooperativity different from those of Hb Yakima and Hb Kempsey, namely, Hill's n was the highest in alkaline pH (n = 3.0 at pH 8.0) and decreased at lower pH (n = 1.5 at pH 6.5). 2,3-Diphosphoglycerate bound with the deoxygenated Hb Yakima and Hb Kempsey, however, had no effect on the oxygen binding of these abnormal hemoglobins. The pH-dependent cooperativity of αβ₂ contact anomalous hemoglobin and normal hemoglobin was explained by the shifts in the equilibrium between the high and low ligand affinity forms.

Materials and Methods

Materials—Pentacyclohexylammonium salt of 2,3-P₃-glycerate was obtained from Boehringer, converted to the acid form by treatment.
with Dowex 50 resin, and neutralized to pH 7 with sodium hydroxide solution. IHP and bis-tris were purchased from Sigma.

Separation of Hb Yakima and Hb Kempsey from Hb A—Hemolysate from heterozygotes contained approximately 40% Hb Yakima and 60% Hb A. These two hemoglobins were separated by chromatography on DEAE-cellulose (Whatman DE32) as follows. Glycerinated erythrocytes containing Hb Yakima (stored at 80° before use) were hemolyzed by dialysis against deionized water. After removing the ghosts by centrifugation, the hemoglobin solution was equilibrated with 0.01 M Tris-HCl buffer, pH 8.3, by passage through a Sephadex column, and applied to a DE32 column (1.5 x 40 cm) which had been previously equilibrated with the same buffer. Elution was performed by a linear gradient from 0 to 0.1 M NaCl in 0.01 M Tris-HCl buffer, pH 8.3. A typical elution pattern is shown in Fig. 1. Hb Kempsey was separated from Hb A by the same procedure as that used for Hb Yakima. The completeness of separation was verified by acrylamide gel electrophoresis in the discontinuous system of Poulik (5). No trace of Hb A was found in any of the preparations used.

Stripped hemoglobin was prepared according to the method of Benesch et al. (6) but with a slight modification. A Sephadex G-25 column was used which was equilibrated with 0.1 M NaCl in 0.05 M Tris buffer, pH 7.0, instead of 0.1 M NaCl solution.

Oxygen Equilibrium—Oxygen equilibrium curves were determined by a spectrophotometric method according to Sugita and Yoneyama (7). Concentrations of hemoglobin were between 4 and 6 x 10^{-8} M (in heme) in 0.1 M phosphate buffer. The effects of organic phosphates on the oxygen equilibrium curves were measured in 0.05 M bis-tris buffer containing 0.1 M NaCl. The pH of the hemoglobin solution was determined at the end of each experiment. Deoxygenation was carried out by repeating alternate evacuation and flushing with Q gas (helium-isobutane, 99.05:0.95) in a Thunberg type cell with a 1-cm light path and then small quantities of sodium borohydride were added to ensure the complete deoxygenation of the sample. As Hb A in the presence of IHP could not be completely oxygenated by air at pH below 7.0, 100% oxygenation of hemoglobin was performed by using pure oxygen.

Measurements of 2,3-P,,-glycerate and IHP Binding—The amount of 2,3-P,-glycerate bound to hemoglobin was measured by equilibrium dialysis according to De Bruin and Janssen (8) and the number of moles bound per hemoglobin tetramer calculated. The gel filtration method of Gray and Gibson (9) was adopted for the determination of IHP bound to hemoglobin because IHP did not elute across a Visking dialysis membrane. The amount of hemoglobin was determined spectrophotometrically by the absorption of the oxygenated form at 580 nm, and that of phosphate was determined by the method of Gray and Gibson (9). This latter method is suitable for the samples containing large amounts of organic materials.

RESULTS

Oxygen Equilibrium of Hb Yakima and Hb Kempsey—The oxygen affinities of Hb Yakima and Hb Kempsey as a function of pH are shown in Fig. 2. Oxygen affinities of these abnormal hemoglobins were much higher than that of Hb A. The p50 values, which correspond to the oxygen pressure at which one half of the hemoglobin is in the oxygenated form, were 10.5 mmHg for Hb A, 0.7 mmHg for Hb Yakima, and 0.5 mmHg for Hb Kempsey at pH 7.0. The values of n, which represent the interaction coefficient in Hill equation Y = Kp^n/(1 + Kp^n), were 1.0 to 1.1 for both Hb Yakima and Hb Kempsey and 2.6 to 2.8 for Hb A, and the results showed the lack of cooperative interactions in Hb Yakima and Hb Kempsey. Plots of Y/1 - Y versus p50 were linear over the pH range examined.

The Alog p50/ΔpH values calculated between pH 7.3 and 7.8, are 0.65, 0.50, and 0.18 for Hb A, Hb Yakima, and Hb Kempsey, respectively. The Bohr effect was almost normal for Hb Yakima and one-fourth for Hb Kempsey compared with that for Hb A.

Effects of Organic Phosphates on Oxygen Equilibrium—Fig. 3 shows the oxygen equilibrium curves of Hb Kempsey and Hb A in the presence and absence of organic phosphates. The oxygen affinity of stripped Hb A was decreased to about 2 times and 10 times in p50 value by the addition of 2,3-P,-glycerate and IHP, respectively. However, as already reported by Benesch and Benesch (10), the values of Hill's n for stripped Hb A did not change in the presence and absence of these organic phosphates at neutral pH. When Hb Kempsey was stripped, the oxygen affinity increased to about twice that of non-striped hemoglobin. 2,3-P,-glycerate had no effect on the oxygen affinity and heme-heme interaction of stripped Hb Kempsey, but in the presence of IHP the oxygen affinity of Hb Kempsey showed a 5-fold increase in p50. The Hill's coefficient was increased from 1.0 to 1.9 at pH 7. This result shows that...
cooperativity loss by amino acid substitution in Hb Kempsey can be partially restored in the presence of IHP. Fig. 4 shows the effects of 2,3-P₃-glycerate and IHP on stripped Hb Yakima. 2,3-P₃-glycerate had no effect on the oxygen affinity and heme-heme interaction of Hb Yakima. IHP reduced the oxygen affinity of Hb Yakima and the Hill's coefficient n was increased from 1.0 to 2.3 at pH 7. These results indicate that IHP has a great effect on the cooperativity of these α₁β₂ contact anomalous hemoglobins. The changes in pₐ₀ of Hb Kempsey, Hb Yakima, and Hb A as a function of pH in the presence of IHP are shown in Fig. 5 together with those of nonstripped hemoglobins in 0.1 M phosphate buffer solution for comparison. As shown in the previous section, the Bohr effect of Hb Kempsey in the absence of IHP was one-fourth that for Hb A, and that of Hb Yakima was nearly normal. By the addition of IHP, changes in pₐ₀ with pH of both Hb Kempsey and Hb Yakima were greatly increased, whereas the Bohr effect of Hb A was scarcely changed. The curves of log pₐ₀ versus pH for Hb A in the presence of IHP were shifted to the upper region, indicating that the oxygen affinity was greatly decreased by binding with IHP. The Bohr effect (Δlog pₐ₀/ΔpH) calculated between pH 7.3 and 7.8 are given in Table I. Hb A showed about the same magnitude of the Bohr effect both in the presence and absence of IHP, whereas the two abnormal hemoglobins exhibited larger Bohr effects in the presence of IHP. However, the Hill's n in the presence of IHP was greatly changed with pH in both normal and abnormal hemoglobins. Therefore, Δlog pₐ₀/ΔpH of each hemoglobin in Table I is an apparent Bohr effect.

By measuring 2,3-P₃-glycerate binding to hemoglobin by equilibrium dialysis (8) and IHP binding to hemoglobin by gel filtration (9), we confirmed that under the present experimental conditions, both the organic phosphate compounds were stoichiometrically bound to Hb Yakima and Hb Kempsey in the deoxygenated state.

Change of Hill Coefficient n at Function of pH—The Hill’s n of Hb A, Hb Yakima, and Hb Kempsey in the presence of IHP varied with the change in pH. The relationship between pH and the Hill coefficient of Hb A and Hb Kempsey are shown in Fig. 4. The cooperativity of Hb A was enhanced by increasing pH (n = 3.0 at pH 8.0) and was dropped at a lower pH range (n = 1.5 at pH 6.5). On the contrary, the n values of Hb Kempsey were increased by lowering pH (n = 2.1 at pH 6.5) and were decreased at higher pH (n = 1.0 at pH 8.0). IHP was most effective in restoring the cooperativity of Hb Kempsey at the acidic pH. The Hill’s n of Hb Yakima changed from 1.6 at pH 7.9 to 2.3 at pH 6.7. Accordingly, the effects of pH on the Hill coefficient of abnormal hemoglobins Yakima and Kempsey were quite different from that of Hb A in the presence of IHP.

DISCUSSION

Structural integrity at α₁β₂ contact region is of great importance for normal hemoglobin function, because most of the amino acid residues in this region are invariants in all of the known hemoglobins of vertebrate and almost all mutants

![Fig. 4. Oxygen equilibrium curves of stripped Hb Yakima in the presence and absence of organic phosphates. Conditions were the same as in Fig. 3.](http://www.jbc.org/)

![Fig. 5. pH-dependent shift in log pₐ₀ of Hb A, Hb Yakima, and Hb Kempsey. pₐ₀ curves in the absence of IHP were derived from the data of Fig. 2. Ten moles of IHP per 1 mol of heme were added to the hemoglobin solution (50 μM) in 0.05 M bis-tris-0.1 M NaCl at the pH lower than 7.5. For the pH higher than 7.5, 0.1 M bis-tris—0.1 M NaCl was used. Temperature, 22°.](http://www.jbc.org/)

![Fig. 6. pH-dependent changes of Hill’s n for Hb A and Hb Kempsey in the presence of 10 mol of IHP per mol of hemoglobins (in heme). Conditions are the same as in Fig. 5.](http://www.jbc.org/)
with replacements at the contact region show impaired heme-heme interaction (11). Such mutants also possess abnormal affinity for ligands whereas most of them retain a normal Bohr effect.

The oxygen equilibrium curves of purified Hb Yakima and Hb Kempsey in 0.1 M phosphate buffer are hyperbolic, indicating significant impairment of heme-heme interaction. Hill's $n$ over the pH range examined in the present experiment are 1.0 to 1.1 for both Hb Yakima and Hb Kempsey. These values are similar to those of Novy et al. (2) for Hb Yakima ($n = 1.0$) and of Reed et al. (3) for Hb Kempsey ($n = 1.1$) which were obtained from the oxygen equilibrium curves of mixed hemolysate. They also showed the normal Bohr effect for Hb Yakima and the decreased Bohr effect for Hb Kempsey by comparing the changes of pH during oxygenation and deoxygenation. From the present measurements of oxygen equilibrium with spectrophotometric method, the Bohr effect for Hb Yakima was approximately the same as that for normal hemoglobin and that for Hb Kempsey was one-fourth that for Hb A. 2,3-P$_2$-glycerate was bound to both Hb Yakima and Hb Kempsey in the deoxygenated state, but it had no effect on the ligand binding properties of two $eta_2$-substituted hemoglobins. When IHP was bound to these abnormal hemoglobins, it had great effects on the ligand affinity, the Bohr effect, and the cooperativity. The deoxygennated Hb Yakima and Hb Kempsey do not seem to convert to a constrained form by binding with 2,3-P$_2$-glycerate but by binding with a more potent allosteric effector, IHP, the conformations of the abnormal hemoglobins may change and their oxygen affinity decreases. In the presence of IHP, the oxygen affinity of Hb Yakima and Hb Kempsey were greatly decreased ($P_{50}$ at pH 7.0; 0.28 (IHP) and 1.4 (+IHP) for Hb Kempsey, 0.36 (IHP) and 2.25 (+IHP) for Hb Yakima) and the Hill's $n$ at pH 7.0 was increased from 1.0 to 2.3 in Hb Yakima and to 1.9 in Hb Kempsey. Hb Kempsey and Hb Yakima showed the greatest cooperativity at acidic pH. In the presence of IHP, the cooperativity in Hb A was the greatest at alkaline pH. The result concerning Hb A is similar to those obtained by Bunn and Guidotti (12).

The ligand affinities and the Hill's $n$ for Hb Yakima, Hb Kempsey, and Hb A in various conditions are plotted on the same figure as shown in Fig. 7, and the over-all dependence of cooperativity on ligand affinity is found to be hyperbolic with $n$ being decreased at both the extremely high and low values of $P_{50}$. In the range between 2 and 50 mmHg of $P_{50}$, the $n$ values of hemoglobins are maintained above 2.0 in both normal and abnormal hemoglobin.

According to the two-state model of Monod et al. (13), the degree of cooperativity of hemoglobin depends on $L$, the equilibrium constant between the R (relaxed) and T (tense) states in the absence of ligand, and $c = K_r/K_t$, where $K_r$ and $K_t$ represent the microscopic dissociation constants for ligand in the R and T states, respectively. The values of $L$ and $c$ are obtained by fitting oxygen saturation data to the equation:

$$Y = \frac{(1 + a)^3 + L \alpha (1 + \alpha a)^3}{(1 + \alpha)^4 + L (1 + \alpha c)^4} \quad \alpha = \frac{P_{50}}{X_r}$$

where $Y$ is the fractional saturation and $\alpha$ is the normalized ligand concentration. Rubin and Claudeux (14) and Edelstein (15) had shown through numerical analysis that the dependence of Hill's constant on $L$ follows a bell-shaped curve, that is, the degree of cooperativity is largest at an optimum value of $L$ and decreases on either side. They had also shown that $L$ is directly related to $c$. Bunn and Guidotti (12) and Minton (16) also found a dependence of $n$ on $P_{50}$. They showed, however, that no single value of $c$ could fit the calculated curve to all of the data and suggested the change in the intrinsic affinity of the T state.

The two-state model postulates that the conformation of hemoglobin with high $P_{50}$ is almost frozen in the T state and with low $P_{50}$ in the R state. In accordance with the model, hemoglobin variants with $P_{50}$ of less than 2 mmHg, such as Hb Chesapeake ($P_{50} = 0.4$ mmHg at pH 7.2, $n = 1.3$) (17) and Hb Rainier ($P_{50} = 0.5$ mmHg at pH 7.1, $n = 1.3$) (18), usually show decreased heme-heme interaction. Hemoglobin variants with very low oxygen affinity, such as Hb M Iwate ($P_{50} = 55$ mmHg at pH 7.0, $n = 1.0$) (19) and Hb Kansas ($P_{50} = 40$ mmHg at pH 7.0, $n = 1.3$) (20), have also decreased cooperativity. Hb A and other hemoglobin variants which show normal ligand affinity are considered to take a suitable equilibrium constant, $L$ for high $n$ value.

In Fig. 7, each set of points for Hb A, Hb Yakima, and Hb Kempsey at various pH values is on a calculated curve based on two-state model with a fixed c value according to Bunn and Guidotti (12), and the results can be interpreted to mean that proton produces a change in $L$ without affecting the value of $c$. IHP appeared similarly to shift the equilibrium substantially towards the T state, but it seems also to decrease $c$ slightly, namely the ligand affinity in the T state was a little lowered. Both the left ends of the curves formed by the data for Hb Yakima and Hb Kempsey join with the straight line representing $n = 1$ at the point corresponding to the mean $P_{50}$ value for the isolated $\alpha$ and $\beta$ chains of Hb A ($P_{50} = 0.4$ mmHg). As shown in Fig. 7, the data for the abnormal hemoglobins fit fairly well with the curve obtained by assuming $P_{50}$ of the R state as 0.4 mmHg and $c$ as 0.04. The curve formed by the data for Hb A in the presence of IHP joins with the line representing $n = 1$ at $P_{50}$ of 100 mmHg which is considered to be the ligand affinity of the T state. If we assume that the $P_{50}$ of the R state is the same as that of isolated chains (0.4 mmHg), then the value for $c$ becomes 0.004. The calculated curve using these parameters agrees well with the data for Hb A in the presence of IHP. Similarly, the data for Hb A in the absence of IHP can be explained by a simple two-state model assuming the same $P_{50}$ for the R state as above and a little smaller $P_{50}$ value for the T state than that in the presence of IHP ($c = 0.01$). The assumption that normal hemoglobin and abnormal hemo-
gloerins which have amino acid substitutions at \( \alpha_2 \beta_2 \) contact region, all have the same oxygen affinity in the R state as that for isolated chains is consistent with the models proposed by Monod et al. (13) and Perutz (21) that the subunits in the R state suffer the least constraints. The result that the effect of proton on ligand affinity is only to change the allosteric equilibrium constant \( L \), agrees with the mechanism proposed by Perutz (21). IHP is supposed to bind to hemoglobin in the same way as does 2,3-P\(_3\)-glycerate and it actually appears to stabilize the T state. However, IHP seems to decrease the intrinsic ligand affinity in the T state, and the data for Hb A in the presence and absence of IHP cannot be fitted with a single \( c \) value as pointed out by Bunn and Guidotti (12).

Amino acid substitutions in Hb Yakima and Hb Kempsey are in the position of 890 Asap, which is located at \( \alpha_2 \beta_2 \) contact region and forms an important hydrogen bond with 924 Tyr in the deoxygenated form and stabilizes deoxy conformation. Consequently substitution of the acidic aspartic acid residue by basic histidine in Hb Yakima and asparagine in Hb Kempsey, breaks this hydrogen bond and the allosteric equilibrium is shifted extremely toward the R state. In the presence of a potent allosteric effector, IHP, the equilibrium is shifted toward the T state and the shift is further strengthened by hydrogen ions. So, the cooperativity of Hb Kempsey and Hb Yakima is increased in the presence of IHP at low pH. According to Edelstein, \( L \) is proportional to the fourth power of \( \alpha(1/2) \), and allosteric equilibrium constant for normal hemoglobin is calculated as \( L = 4 \times 10^9 \). This value is larger than that originally reported by Monod et al. \( (L = 9 \times 10^9) \) (13) and is nearly equal to that proposed by Edelstein \( (L = 3 \times 10^9) \) (15).

As reported above, the abnormal function of \( \alpha_2 \beta_2 \) contact anomalous hemoglobins is not a fixed one but in suitable conditions the lost cooperativity by amino acid substitution is restored. The cooperativity of Hb A is lost in certain conditions, where the allosteric equilibrium is shifted extremely. In both normal and abnormal hemoglobins, these changes in Hill’s \( n \) were observed only when the value for \( L \) was changed considerably by the concurrent effect of proton and IHP.

Acknowledgments—Thanks are due to Dr. R. D. Koler of the University of Oregon Medical School, for his aid in obtaining Hb Yakima blood, and to Dr. T. Saito of the University of Tokyo Faculty of Medicine for transporting blood specimens.

REFERENCES
The effects of inositol hexaphosphate on the allosteric properties of two beta-99-substituted abnormal hemoglobins, hemoglobin Yakima and hemoglobin Kempsey.

M Nagai, M Nishibu, Y Sugita and Y Yoneyama