Journal of the Korean Magnetic Resonance Society **1997**, *1*, 112-125

Design of Novel Hemoglobins

Jong-Whan Choi, Seunho Jung[†] and Hyun-Won Kim[*]

Department of Biochemistry, Yonsei University Wonju College of Medicine
Wonju 220-701, Korea
[†]Department of Microbiological Engineering, College of Engineering, Konkuk University
Seoul 134-701, Korea
[‡]Medical Engineering Research Institute, Yonsei University, Wonju 220-701, Korea
Received October 22, 1997

Abstract: It has been demonstrated that mutant Hemoglobins (Hb) which have an altered $\alpha_1\beta_2$ subunit interface can be designed. A compensatory mutation for a naturally occurring abnormal human Hb, Hb Kempsey (β99Asp→Asn), has been designed, and this mutation allowed the molecule to regain its allosteric response. The calculated values for the difference in the free energy of cooperativity show excellent agreement with experimentally determined thermodynamic values, suggesting that the molecular dynamics simulation results can be used to obtain information about the specific interactions which contribute to the total free energy of cooperativity. These results provide encouragement to begin a systematic investigation of the molecular basis of the subunit interactions between the α_1 and β_2 chains of Hb A by designing appropriate r Hbs. These studies could lead to the design of Hbs with desired cooperativity in the oxygenation process and to the restoration of functional properties of abnormal hemoglobins associated with hemoglobinopathies. Thus, the present results also have the implications in using gene therapy to treat patients with hemoglobinpathies

INTRODUCTION

Human normal adult hemoglobin (Hb A), the oxygen carrier of blood, is a tetrameric protein consisting of two α chains of 141 amino acid residues each and two β chains of 146 amino acid residues each. Each Hb chain contains a heme group which is an iron complex of protoporphyrin IX. Under physiological conditions, the heme-iron atoms of Hb remain in the ferrous state. In the absence of oxygen, the four heme-irom atoms in Hb A are in the high-spin ferrous state [Fe(II)] with four unpaired electrons each. Each of the four heme-iron atoms in Hb A can combine with an O_2 molecule to give oxyhemoglobin (O_2) in which the iron atom is in a low-spin, diamagnetic ferrous state. The oxygen binding of Hb exhibits sigmoidal behavior, with an overall association constant expression giving a greater than first-power dependence on the concentration of O_2. Thus, the oxygenation of Hb is a

cooperative process, such that when one O_2 is bound, succeeding O_2 molecules are bound more readily. Hb is an allosteric protein, i.e., its functional properties are regulated by a number of metabolites [such as hydrogen ions, chloride, carbon dioxide, 2,3-diphosphoglycerate (2,3-DPG)] other than its ligand, O_2. It has been used as a model for allosteric proteins, and indeed, hemoglobins of vertebrates are among the most extensively studied allosteric proteins. Their allosteric properties are physiologically important in optimizing O_2 transport by erythrocytes. The large number of mutant forms of Hb available provides an array of structural alterations with which to correlate effects on function.[1-4]

There are two types of contacts between the α and β subunits of Hb. The $\alpha_1\beta_1$ (or $\alpha_2\beta_2$) contacts, involving B, G, and H helices, and GH corners, are called packing contacts. These contacts remain unchanged and hold the dimer together even when there is a change in the ligation state of the heme. The $\alpha_1\beta_2$ (or $\alpha_2\beta_1$) contacts, mainly involving C and G helices, and FG corners, are called sliding contacts, and undergo significant motion when there is a change in the ligation state of the heme. The movement of heme iron atoms and the sliding motions of the $\alpha_1\beta_2$ subunit interface, as well as the breaking of intra- and intermolecular salt bridges and hydrogen bonds as a result of the ligation of the Hb molecule, are among the most important features of the stereochemical mechanisms for the cooperative oxygenation of Hb.[5]

The Hill coefficient (n_{max}), which measures the cooperative oxygenation of Hb, provides a convenient measure of some of the allosteric properties of this protein.[1] Under usual experimental conditions, Hb A has an n_{max} value of approximately 3 in its binding with O_2. Human abnormal Hbs with amino acid substitutions in the $\alpha_1\beta_2$ subunit interface generally have high oxygen affinity and reduced cooperativity in O_2 binding compared to Hb A,[1,2] suggesting the importance of the $\alpha_1\beta_2$ subunit interface to the functional properties of Hb. In particular, mutant human Hbs with an amino acid substitution at the β99Asp, such as Hb Kempsey (β99Asp→Asn or β:D99N) (n_{max} = 1.1),[6] Hb Yakima (β99Asp→His) (n_{max} = 1.0),[7] Hb Radcliff (n_{max} = 1.1) (β99Asp→Ala),[8] Hb Hôtel Dieu (β99Asp→Gly) (n_{max} = 1.3),[9] and Hb Ypsilanti (β99Asp→Tyr) (n_{max} not reported),[10] possess greatly reduced cooperativity and increased oxygen affinity relative to those exhibited by Hb A. X-ray crystallographic studies of Hb A[5,11] have shown that β99Asp is hydrogen-bonded to both α42Tyr and α97Asn in the $\alpha_1\beta_2$ subunit interface of deoxy-Hb A, suggesting that the essential role of β99Asp is to stabilize the deoxy-Hb molecule by forming intersubunit hydrogen bonds.

Recently, two recombinant Hbs (r Hbs) with an amino acid substitution at the α42Tyr, r Hb (α42Tyr→His) and r Hb (α42Tyr→Phe), have been constructed.[12,13] r Hb (α42Tyr→Phe) exhibits essentially no cooperativity in binding oxygen (n=1.2) and

possesses very high oxygen affinity, whereas r Hb (α42Tyr→His) exhibits substantial cooperativity (n=2 at pH 6.8) and moderate oxygen affinity. These investigators have attributed the differences in function between these two mutants to the presence of a weak hydrogen bond between α42His and β99Asp in the deoxy form of r Hb (α42Tyr→His), and the lack of such a bond in deoxy-r Hb (α42Tyr→Phe). Since abnormal Hbs with an amino acid substitution at either β99Asp or α42Tyr, which lose the intersubunit hydrogen bonds in the deoxy form, also lose their functional properties, it has been suggested that these hydrogen bonds are crucial for the structure and function of the Hb molecule.

Molecular dynamics (MD) simulations have been used successfully to calculate the free energy difference between native and mutant proteins. Recent studies have involved the effects on protein stability,[14,15] on ligand binding,[16,17] and on cooperativity of Hb.[18-21] In most cases, MD-simulation results demonstrate excellent agreement with experimentally determined data. In particular, MD simulations of Hb Radcliff (β99Asp→Ala) show remarkable agreement between the measured thermodynamic value and the calculated cooperativity. The overall free energy change calculated is -5.5 kcal/mol, which has the same sign and is of the same order as the experimentally measured value of -3.4 kcal/mol, suggesting that MD-simulation results may be used to gain insights into specific interactions within a protein molecule.[18] The analysis of the free energy of simulation shows that the effect of the mutation, i.e. β99Asp→Ala, is more complex than the crystal structure suggests. Some of the contributions to the difference in the free energy of cooperativity are as large as 60 kcal/mol, indicating that essential thermodynamic elements are hidden in the measured value (-3.4 kcal). Thus, the partly canceling individual contributions in solvent-protein interactions and electrostatic interactions, which are not evident from a crystal structure, can be exposed only by a free-energy simulation, which could provide new insights into the origin of thermodynamic values.

Up to now, there have been no completely reliable approaches to predicting protein tertiary structure from its amino acid sequence. However, if only a few amino acid side chains in a protein are mutated, the conformational changes may, in some cases, be predictable by MD simulations. Recently, the calculated structures of mutant subtilins, in which the methionine at the 222 position has been replaced, i.e. 222Met→Ala, 222Met→Phe, and 222Met→Gln, have been compared with the X-ray structures of the respective mutants, and have shown good agreement between the predicted and X-ray results.[22] These calculations involved gradually changing methionine at the 222 position into the replacing amino acid using a thermodynamic integration method for free-energy determination.

Molecular dynamics simulations have been used to design mutant Hbs which have altered $\alpha_1\beta_2$ subunit interfaces. Since abnormal Hbs with an amino acid substitution at β99Asp not only lose the intersubunit hydrogen bonds in the deoxy form, but also lose their functional properties, the approach has been to design compensatory mutant Hbs by

additional mutations in the local environment of the β99 mutation in order to create new hydrogen bonds to compensate for the missing ones. Several novel Hbs have been designed in this way. In particular, for Hb Kempsey (β99Asp→Asn), which has a high oxygen affinity and exhibits essentially no cooperativity in binding oxygen,[23] our computer simulations indicate that a new hydrogen bond involving β99Asn can be induced in the deoxy form by replacing α42Tyr by a stronger hydrogen-bond acceptor, such as Asp. This suggests that a new Hb with the amino acid substitutions, β99Asp→Asn and α42Tyr→Asp (or β:D99N; α:Y42D), i.e. a mutant of Hb Kempsey, can regain the cooperativity lost in Hb Kempsey.[19]

To test the validity of the MD simulations, it is necessary to have the appropriate mutant proteins for experimental investigation. Synthetic human α- and β-globin genes which are coexpressed with the *Escherichia coli* methionine aminopeptidase (Met-AP) gene under the control of separate *tac* promoters has been constructed.[24] In *E. coli* cells harboring this plasmid, the N-terminal methionine residues of the expressed Hb A have been effectively cleaved by the coexpressed Met-AP, and this expressed r Hb A lacking an N-terminal methionine is identical to native Hb A in a number of structural and functional properties.[24] Using this *E. coli* expression system, site-directed mutagenesis have been applied to produce several new r Hbs which have replacements at the $\alpha_1\beta_2$ interface. The O_2 binding properties of these r Hbs have been determined, and proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) have been used to investigate the tertiary structures around the heme groups and the quaternary structure. In this article, r Hb (β99Asp→Asn, α42Tyr→Asp) has been used as an example of this approach.[19]

^{1}H-NMR spectroscopy has proven to be a powerful technique for investigating the structure-function relationship in Hb.[3] Due to the presence of the unpaired electrons in the high-spin ferrous atoms in deoxy-Hb and the highly conjugated porphyrins of the Hb molecule, the proton chemical shifts of various Hb derivatives cover a wide range. Resonances vary from about 20 ppm upfield from the proton resonance of a standard, 2,2-dimethyl-2-silapentane-5-sulfonate (DSS), to about 80 ppm downfield from DSS, depending on the spin state of the iron atoms and the nature of ligands attached to the heme groups. This unusually large spread of proton chemical shifts for deoxy-Hb A provides the selectivity and the resolution necessary to investigate specific regions of the Hb molecule.

MD SIMULATIONS

MD simulations of proteins involve treating each atom in the protein as a particle following the classical Newtonian equations of motion. By integrating Newton's equations of motion, atomic positions and velocities can be obtained as a function of time. In a protein consisting of many atoms, the total force acting on any one atom at any given time depends on the position of all the other atoms. Thus, a high-speed computer is needed to solve the

Newtonian equations in order to determine the positions and velocities of all the atoms in a protein molecule over a long period of time. Recent advances in computing technology have made it possible to investigate proteins in solution and to extend the simulation time in the range of nanoseconds.[25]

For the design of Hb which has an altered subunit interface, transformation between wild-type and mutant proteins using the thermodynamic integration method[26] has been employed. This method has been used for the calculation of free energy, i.e., gradually changing the potential from that representing wild-type Hb to the mutant form during a simulation. Energy minimized X-ray structures of the normal Hb tetramer were used to generate the starting configurations. MD simulations were carried out by a stochastic boundary method[27] using CHARMM 22 with standard parameters for the polar hydrogen protein model (param19). The molecule was partitioned into MD and Langevin regions with radii of 10 Å and 15 Å, respectively. The inside sphere was filled with charmm-adapted pre-equilibrated TIP3P water molecules.[28]

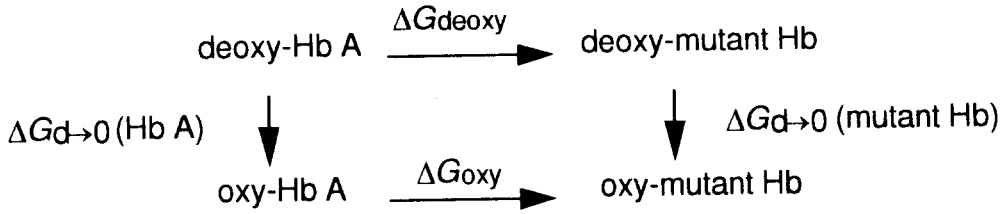
The transformation from wild-type to mutant proteins was achieved by using a hybrid potential function $V_\lambda = (1-\lambda)V_A + \lambda V_B$, where λ is a coupling factor between 0 and 1. V_A and V_B are potential energy functions for Hb A and mutant Hb, respectively.[18-21] Simulations were done at nine values of λ_i ($\lambda = 0.1, 0.2, \cdots, 0.9$), with 5-ps of equilibration followed by 5-ps of production dynamics, except at $\lambda = 0.1$ and $\lambda = 0.9$, where 10-ps of equilibration was employed. Non-bonded interactions which had been truncated to zero at 8.5 Å and a constant dielectric ($\varepsilon = 1$) were used. All bonds involving hydrogen atoms were constrained with the SHAKE algorithm.[29]

The free energy of simulation can be obtained from the trajectory files of the MD simulations for both deoxy and oxy forms of Hb using the thermodynamic integration method[26] with the following equation:

$$\Delta G = G_B - G_A = \int_0^1 \langle \Delta V \rangle_\lambda d\lambda \sim \sum_i \langle \Delta V \rangle_{\lambda_i} \Delta\lambda$$

where $\Delta V = V_B - V_A$, and the thermodynamic average $\langle \Delta V \rangle_\lambda$ indicates the average of V_λ over the hybrid system. The linear form of the thermodynamic equations shows that the total free energy of the simulations can be decomposed into individual additive contributions.

The change in the free energy of cooperativity resulting from the mutations can be indirectly obtained by the difference in the free energy of simulation between deoxy and oxy form from the thermodynamic cycle.[18-21]



For the purpose of a structural analysis, average structures for wild-type ($\lambda = 0$) and mutant systems ($\lambda = 1$) have been calculated from the nearest states that are simulated ($\lambda = 0.1$ and $\lambda = 0.9$, respectively) by using the exponential formula[30]

$$X_0 = \frac{< Xe^{+0.1\beta\Delta V} >_{\lambda = 0.1}}{< e^{+0.1\beta\Delta V} >_{\lambda = 0.1}} \quad \text{and} \quad X_1 = \frac{< Xe^{-0.1\beta\Delta V} >_{\lambda = 0.9}}{< e^{-0.1\beta\Delta V} >_{\lambda = 0.9}}$$

where X represents the Cartesian coordinates of the system, and X_0 and X_1 are the simulation-average wild-type and mutant coordinates, respectively, $\beta = 1/k_BT$, and $< >_\lambda$ is an ensemble average under the potential V_λ.

DESIGN AND EXPRESSION OF A COMPENSATORY MUTATION FOR HB KEMPSEY (β99ASP→ASN)

Design of a compensatory mutation

For a successful application of MD simulations to predict the structural changes in a mutant protein, it is essential that the local conformations of wild-type and mutant protein be similar. Deoxy-Hb Kempsey crystallizes in an oxy-like (or R-type) structure[31] and ^{1}H-NMR spectroscopic results also suggest that it has an oxy-like quaternary structure.[31,32] Thus, its quaternary structure is quite different from that of deoxy-Hb A. However, if new hydrogen bond(s) can be formed in the $\alpha_1\beta_2$ interface of Hb Kempsey by compensatory mutations, it would be expected that a stable deoxy-like quaternary structure could be maintained in this mutant. With this assumption, MD simulations have been used to design compensatory mutations in Hb Kempsey.[19]

Computer simulations indicate that a new hydrogen bond involving β99Asn can be induced in the deoxy form by replacing α42Tyr by a stronger hydrogen-bond acceptor, such as Asp. The average simulated structure of our mutant Hb (β99Asp→Asn, α42Tyr→Asp) in the deoxy form shows that new hydrogen bonds between β99Asn and α42Asp in the $\alpha_1\beta_2$ subunit interface can be formed. The side-chain angles (χ_1) of α42Asp and β99Asn in the averaged simulated structure are -77.3° and -171.3°, respectively. These angles are the

most frequently observed values in the rotamer library (48% and 33%, respectively),[33] indicating that these amino acids have a stable side-chain orientation. 100-ps MD simulations of the transformed mutant Hb (β99Asp→Asn, α42Tyr→Asp) in the deoxy form show that the new hydrogen bond between β99Asn and α42Asp is very stable, i.e. with an average simulated distance of 2.19 ± 0.16 Å.[19]

The change in the free energy of cooperativity resulting from the mutations (β99Asp→Asn, α42Tyr→Asp) can be indirectly obtained from the thermodynamic cycle. The measured thermodynamic value for Hb Kempsey is -3.4 kcal/mol per interface. If mutant Hb (β99Asp→Asn, α42Tyr→Asp) restores some cooperativity in binding oxygen, the change in the free energy of cooperativity is expected to be somewhere between 0 and -3.4 kcal.[34] The calculated value is -3.3 kcal/mol per interface, which is on the same order and same sign as the expected value. Considering that both mutations introduced involve charge changes, this calculated value seems to be in good agreement. The simulated results may be used to obtain information about the specific interactions which contribute to the total free energy difference.

Oxygen binding properties

The new r Hb (β99Asp→Asn, α42Tyr→Asp) suggested by our computer simulations has been produced. The O_2-binding properties of r Hb (β99Asp→Asn, α42Tyr→Asp), Hb Kempsey, and Hb A are shown in Fig. 1.[19] r Hb (β99Asp→Asn, α42Tyr→Asp) exhibits about 40% of the Bohr effect of Hb A over the pH range 6.8 to 7.9. In 0.1 M sodium phosphate at 29°C, r Hb (β99Asp→Asn, α42Tyr→Asp) exhibits intermediate oxygen affinity; for example, at pH 7.2, p_{50} = 2.5 mmHg for r Hb (β99Asp→Asn, α42Tyr→Asp) versus p_{50} = 10.5 mmHg for Hb A, and p_{50} = 0.2 mmHg at pH 7.2 for Hb Kempsey. However, r Hb exhibits significant cooperativity in binding of oxygen, especially at low pH; for example, n_{max} = 2.0 at pH 6.8 versus n_{max} = 3.0 for Hb A, and n_{max} = 1.1 at pH 7.2 for Hb Kempsey. A marked change occurs when the allosteric effector, inositol hexaphosphate (IHP), is added to r Hb (β99Asp→Asn, α42Tyr→Asp). The oxygen affinity is reduced significantly [p_{50} = 12.9 mmHg at pH 7.2 for r Hb (β99Asp→Asn, α42Tyr→Asp) versus p_{50} = 46.7 mmHg for Hb A and p_{50} = 1.1 mmHg at pH 7.2 for Hb Kempsey] and the cooperative oxygenation process for this double mutant approaches the normal value for Hb A as manifested by the Hill coefficient, with an n_{max} value of 2.3 to 2.5 over the pH range from 6.8 to 7.9.

The restoration of cooperativity in our r Hb (β99Asp→Asn, α42Tyr→Asp) relative to Hb Kempsey (β99Asp→Asn) can most likely be explained by the presence of a new hydrogen bond between α42Asp and β99Asn in the $\alpha_1\beta_2$ interface introduced by the

additional mutation α42Tyr→Asp. This new interfacial hydrogen bond can stabilize a deoxy-like (or T-type) structure, and thus, could provide the necessary free energy of cooperativity in binding oxygen. r Hb (α42Tyr→Asp) has been also expressd in *E. coli*

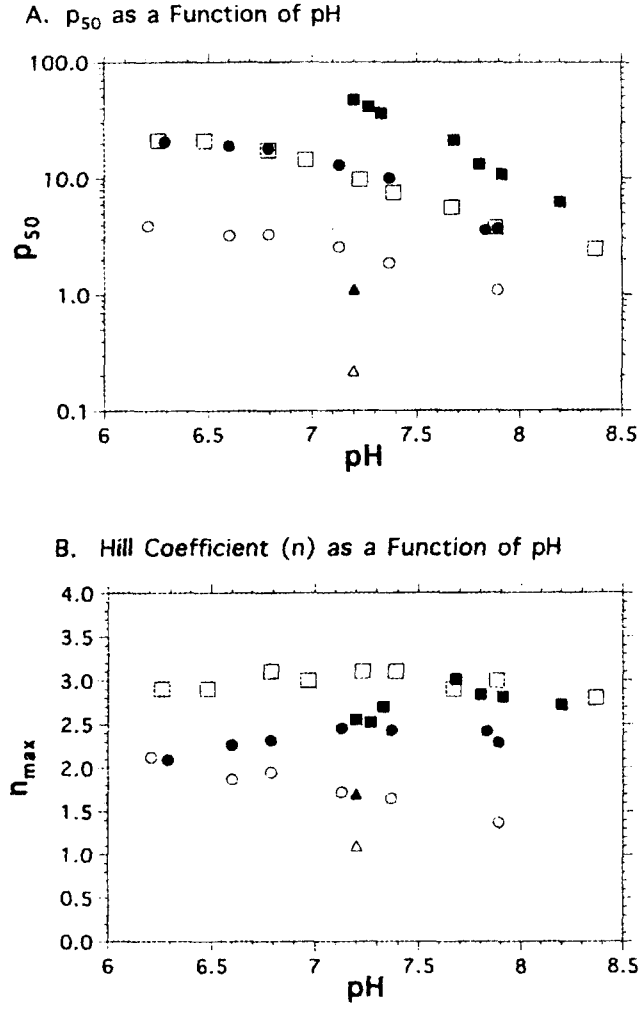


Fig. 1. pH dependence of oxygen affinity (**A**) and the Hill coefficient (**B**): (○) r Hb (β:D99N; α:Y42D); (●) r Hb (β:D99N; α:Y42D) with 2 mM IHP; (□) Hb A; (■) Hb A with 2 mM IHP; (△) Hb Kempsey β:D99N; and (▲) Hb Kempsey β:D99N with 1 mM IHP. Oxygen dissociation curves were measured by a Hemox-Analyzer, and p_{50} and the Hill coefficient were determined from each curve. Oxygen dissociation data were obtained with 0.1 mM Hb in 0.1 M sodium phosphate buffer in the pH range 6.5-8.0.

(unpublished reuslts). However, r Hb (α42Tyr→Asp) is an unstable hemoglobin which appears to lose hemes, i.e., the formation of Heinz bodies.[2] Hb Kempsey (β99Asp→Asn) exhibits very high oxygen affinity and greatly reduced cooperativity.[23] Thus, r Hb (β99Asp→Asn, α42Tyr→Asp) may be described as a case of "two wrongs making a right"!

Structural Investigation by ^{1}H-NMR Spectroscopy

^{1}H-NMR spectroscopy has been shown to be an excellent tool to investigate the tertiary and quaternary structural features of Hb. Low-field ^{1}H resonances of Hb A, Hb Kempsey, and r Hb (β99Asp→Asn, α42Tyr→Asp) are shown in Fig. 2 (unpublished results). The resonance at ~63 ppm from DSS has been assigned to the hyperfine-shifted

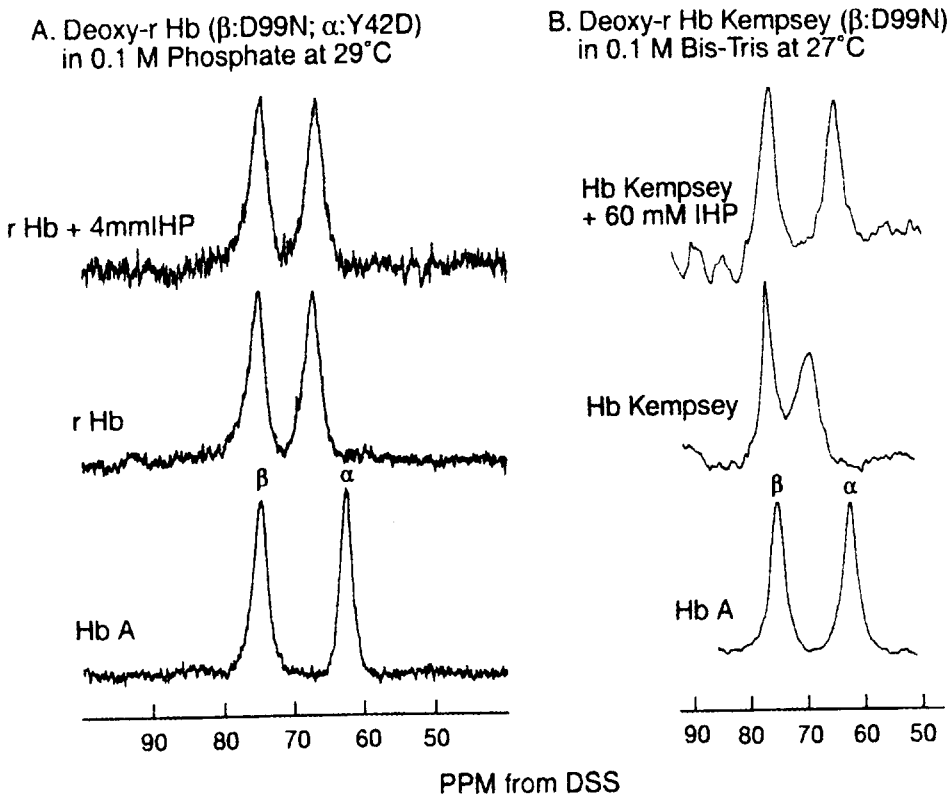


Fig. 2. Hyperfine-shifted NδH exchangeable proton resonances of the proximal histidine residues of Hb in the deoxy form: (**A**) r Hb (β:D99N; α:Y42D) and Hb A; and (**B**) Hb Kempsey (β:D99N) with and without IHP.

$N_{\delta}H$-exchangeable proton of the proximal histidine residue (α87His) of the α chain of deoxy-Hb A and the one at ~77 ppm from DSS has been assigned to the corresponding residue of the β chain (β92His) of deoxy-Hb A.[35,36] The chemical shift positions of the two proximal histidyl resonances in r Hb (β99Asp→Asn, α42Tyr→Asp) are very similar to those of Hb Kempsey, which shows a downfield resonance shift in the histidyl resonance of the α chain. In Hb Kempsey, the presence of IHP causes the proximal histidyl resonance from the α chain to be shifted upfield to a chemical shift position similar to that of deoxy-Hb A.[32] In contrast, IHP exerts no significant effect on r Hb (β99Asp→Asn, α42Tyr→Asp).

Fig. 3 shows the ring-current shifted resonances of Hb A, r Hb (β99Asp→Asn, α42Tyr→Asp), and Hb Kempsey (β99Asp→Asn) in the CO form. The proton resonances

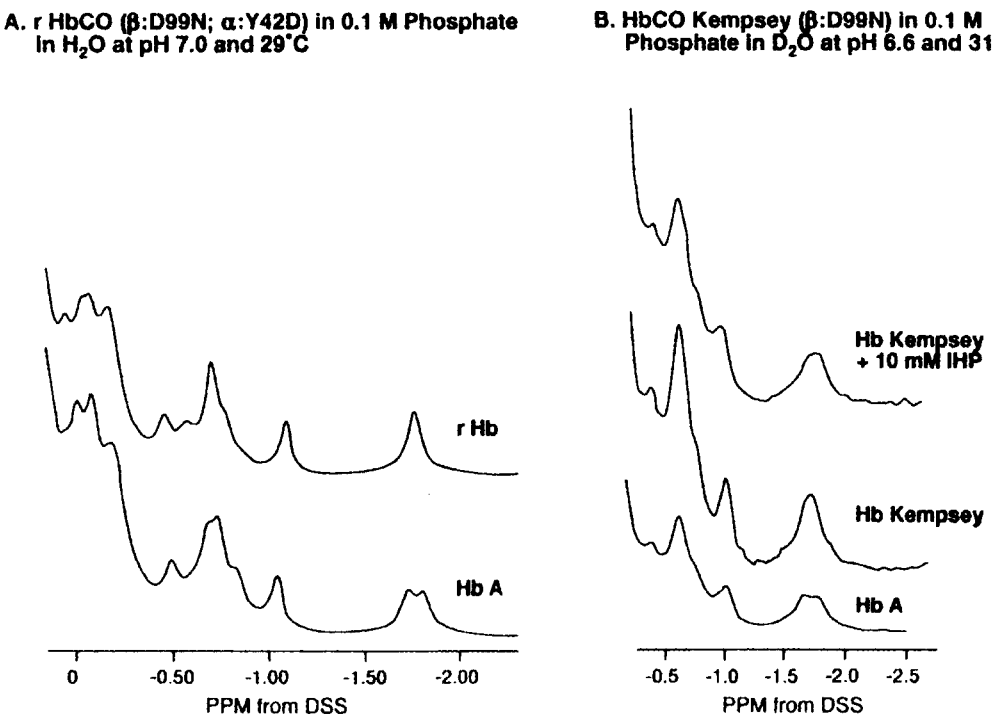


Fig. 3. Ring-current shifted proton resonances of Hb in the carbonmonoxy form: (**A**) r Hb (β:D99N; α:Y42D) and Hb A[19]; and (**B**) Hb Kempsey (β:D99N) with and without IHP.[38]

over the region from 0 to -2.0 ppm from DSS arise from the protons of amino-acid residues located in the vicinity of the heme groups and from those of the heme groups.[3] The resonances from -1.7 to -1.9 ppm from DSS, which have been assigned to the γ_1 and γ_2 methyl groups of β67E11Val (distal valine),[37] merge into one peak in the spectrum of the r Hb (β99Asp→Asn, α42Tyr→Asp). This has also been observed for Hb Kempsey in the CO form.[38] The exchangeable and ferrous hyperfine-shifted proton resonances of Hb A, Hb Kempsey (β99Asp→Asn), and r Hb (β99Asp→Asn, α42Tyr→Asp) in the deoxy form are shown in Fig. 4.[19] As most of the proton resonances appearing from ~9 to ~14 ppm from DSS have been assigned to the exchangeable proton resonances from interfacial hydrogen bonds, the presence of an extra hydrogen bond in the $\alpha_1\beta_2$ interface of r Hb (β99Asp→Asn, α42Tyr→Asp) can, in principle, be demonstrated by the appearance of a new resonance in this region. The resonance at ~14 ppm from DSS of Hb A has been assigned to the intersubunit hydrogen bond between α42Tyr and β99Asp of Hb A,[39] and is a key marker for the deoxy-quaternary structure of Hb A. This resonance is completely absent from the

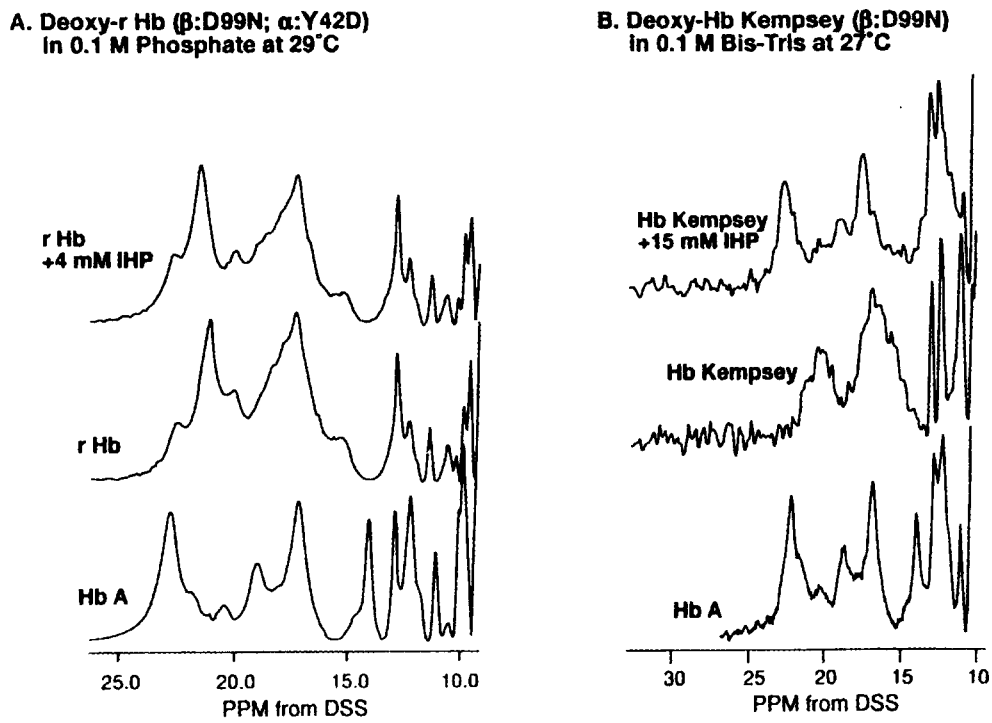


Fig. 4. Hyperfine-shifted and exchangeable proton resonances of Hb in deoxy form: (**A**) r Hb (β:D99N; α:Y42D) in the presence and absence of 4 mM IHP and Hb A;[19] (**B**) Hb Kempsey (β:D99N) in the presence and absence of 15 mM IHP and Hb A.[35]

spectra of Hb Kempsey and r Hb (β99Asp→Asn, α42Tyr→Asp), as expected. There are two broad, shoulder resonances around 15 and 23 ppm from DSS in the spectra of Hb Kempsey and r Hb (β99Asp→Asn, α42Tyr→Asp). However, additional work is still needed to ascertain the origin of these two resonances. The occurrence of an exchangeable proton resonance in the spectrum of a protein molecule depends on the exchange rate between the hydrogen-bonded proton and the protons of H_2O. Thus, the absence of resonances in the exchangeable proton resonance region does not necessarily mean the absence of interfacial hydrogen bonds. One of the $\alpha_1\beta_2$ interfacial hydrogen bonds (i.e, the one between α97Asn and β99Asp), shown in the crystal structure of deoxy-Hb A,[11] has also not been observed or identified in the exchangeable resonance region by ^{1}H-NMR spectroscopy.[3]

The hyperfine-shifted proton resonances of our deoxy-r Hb (β99Asp→Asn, α42Tyr→Asp) observed at 15 to 20 ppm downfield from DSS show significant changes from those of deoxy-Hb A and deoxy-Hb Kempsey (Fig. 4), indicating that there are structural differences among these three Hbs.[19] In contrast to deoxy-Hb Kempsey, where the addition of IHP can convert the spectrum to one similar to that of deoxy-Hb A in the hyperfine-shifted proton resonance region, the addition of IHP to deoxy-r Hb (β99Asp→Asn, α42Tyr→Asp) does not cause any noticeable changes, as shown in Fig. 4. It should also be noted that the addition of IHP does not cause any noticable changes in the hyperfine-shifted N_δH-exchangeable resonances of deoxy-r Hb (β99Asp→Asn, α42Tyr→Asp) (Fig 2), while the addition of IHP to Hb Kempsey causes a significant upfield shift in the histidyl resonance from the α chain (Fig. 2). A possible explanation is that deoxy-Hb Kempsey does not have the usual interfacial hydrogen bonds which stabilize the T-structure, i.e., deoxy-Hb Kempsey exists in an oxy-like (or R-type) quaternary structure.[31] Thus, the addition of a strong allosteric effector, IHP, can produce large conformational changes in the direction of the T-structure. These conformational changes are reflected in the hyperfine-shifted proton resonance region, as well as by an increase in the Hill coefficient from 1.1 to 1.7. Due to the presence of new interfacial hydrogen bonds, deoxy-r Hb (β99Asp→Asn, α42Tyr→Asp) can have a more stable deoxy-like structure, and the addition of IHP does not cause significant structural changes to affect the hyperfine-shifted proton resonances.

The existence of significant cooperativity in r Hb (β99Asp→Asn, α42Tyr→Asp) despite large alterations in the heme environment suggests that the $\alpha_1\beta_2$ interfacial hydrogen bonds are essential for maintaining cooperativity in the oxygenation process. It is likely that alterations in the heme environment are responsible for the high oxygen affinity of this mutant Hb.[19,38] Most of the known human abnormal Hbs which have high oxygen affinity also exhibit greatly reduced cooperativity. However, the present findings on r Hb

(β99Asp→Asn, α42Tyr→Asp) suggest that high oxygen affinity and reduced cooperativity may arise by different mechanisms.

Acknowledgement

This work is supported by Research Fund of Basic Medical Science, Ministry of Education, Korea, 1996.

REFERENCES

1. Dickerson, R.E. and Geis, I. "Hemoglobin: Structure, Function, Evolution, and Pathology". The Benjamin Cummings Publishing Co. Menlo Park, California, 1983.
2. Bunn, H. F. and Forget, B. G. "Hemoglobin; Molecular, Genetic, and Clinical Aspects." W. B. Saunders, Philadelphia, 1986.
3. Ho, C. *Advan. protein Chem.* **43**, 153-312 (1992).
4. Ho, C and Perussi, J. R. *Methods Enzymol.* **232**, 97 (1994).
5. Perutz, M. F. *Nature* (London) **228**, 726 (1970).
6. Reed, C. S., Hampson, R., Gordon, S., Jones, R. T., Novy, M. J., Brimhall, B., Edwards, M. J., and Koler, R. D. *Blood* **31**, 623 (1968).
7. Jones, R. T., Osgood, E. E., Brimhall, B., and Koler, R. D. *J. Clin. Invest.* **46**, 1840 (1967).
8. Weatherall, D. J., Clegg, J. B., Callender, S. T., Wells, R. M. G., Gale, R E., Huehns, E. R., Perutz, M. F., Viggiano, G., and Ho, C. *British. J. Haematol.* **35**, 177 (1977).
9. Thillet, J., Arons, N., and Rosa, J. B. *Biochim. Biophys. Acta.* **660**, 260 (1981).
10. Glynn,K.P., Penner,J.A., Smith,J.R., and Rucknagel, L. *Ann. Int. Med.* **69**, 769 (1968).
11. Fermi, G., Perutz, M.F., Shaanan, B., Fourme, R. *J. Mol. Biol.* **175**, 159 (1984).
12. Ishimori, K., Morishima, I., Imai, K., Fushitani, K., Miyazaki, G., Shih, D., Tame, J., Pagnier, J., and Nagai, K. *J. Biol. Chem.* **264**, 14624 (1989).
13. Imai, K., Fushitani, K., Miyazaki, G., Ishimori, K., Kitagawa, T., Wads, Y., Morimoto, H., Morishima, I., Shih, D. T.-B., and Tame, J. *J. Mol. Biol.* **218**, 769 (1991).
14. Prevost, M., Wodak, S. J., Tidor, B.,and Karplus, M. *Proc. Natl. Acad. Sci. U.S.A.* **88**, 10880 (1991).
15. Tidor, B. and Karplus, M. *Biochemistry* **30**, 3217 (1991).
16. Komeiji, Y., Uebayasi, M., Someya, J.-I., Yamamonto, I. *Protein Eng.* **5**, 759 (1992).
17. Lau, F. T. K., and Karplus, M. *J. Mol. Biol.* **236**, 1049 (1994).
18. Gao, J., Kuczera, K., Tidor. B., and Karplus, M. *Science* **244**, 1069 (1989).

19. Kim, H.-W., Shen, T.-J., Sun, D. P., Ho, N. T., Madrid, M., Tam, M. F., Zou, M., Cottam, P. F., and Ho, C. *Proc. Natl. Acad. Sci. U.S.A.* **91** 115471 (1994).
20. Kim, H.-W., Shen, T.-J., Sun, D. P., Ho, N. T., Madrid, M., and Ho, C. *J. Mol. Biol.* **248,** 867 (1995).
21. Kim, H.-W., Shen, T.-J., Ho, N. T., Zou, M., Tam, M. F., and Ho, C. *Biochemistry* **35,** 6620 (1996).
22. Heiner, A. P., Berendsen, H. J., van Gunsteren, W. F. *Protein Eng.* **4**, 397 (1993).
23. Bunn, H. F., Wohl, R. C., Bradley, T.B., Cooley, M., and Gibson, Q. H. *J. Biol. Chem.* **249**, 7402 (1974).
24. Shen, T.-J., Ho, N. T., Simplaceanu, V., Zou, M., Green, B. N., Tam, M.F., and Ho, C. *Proc. Natl. Acad. Sci. U.S.A.* **90**, 8108 (1993).
25. Karplus, M and Petsko, G. A. *Nature* **347**, 631 (1990).
26. Kirkwood, J. G. *J. Chem. Phys* . **3**, 300 (1935).
27. Brooks, C. L., III and Karplus, M. *J. Mol. Biol.* **208**, 159 (1989).
28. Jorgensen, W. L., Chandrasekar, J., Madura, J. D., Impey, R. W., and Klein, M. L. *J. Chem. Phys.* **83**, 3050 (1983).
29. Ryckaert, J.-P., Ciccotti, G., and Berendsen, H. J. C. *J. Comput. Phys.* **23**, 327 (1977).
30. Brooks, C. L., III *J. Phys. Chem.* 90, 6680 (1986).
31. Perutz, M. F., Ladner, J. E., Simon, S. R., and Ho, C. *Biochemistry* **13**, 2163 (1974).
32. Takahashi, S., Lin, A. K.-L., and Ho, C. *Biophy. J.* **39**, 33 (1982).
33. Ponder, J. W. and Richards, F. M. *J. Mol. Biol.* **193**, 775 (1987).
34. Turner,G.J., Galacteros, F., Doyle, M. L., Hedlund, B., Pettigrew, D. W., , Smith, F. R., Moo-Penn, W., Rucknagel, D. L., and Ackers, G. K. *Proteins* **14**, 333 (1992).
35. Takahashi, S., Lin, A. K.-L., and Ho, C. *Biochemistry* **19**, 5196 (1980).
36. Lar Mar, G. N., Nagai, K., Jue, T., Budd, D. L., Gersonde, K., Sick, H., Kagimoto, T., Hayashi, A., and Taketa, F. *Biochem. Biophys, Res. Commun.* **96**, 1172 (1980).
37. Lindstrom, T. R., Noren, I.E., Charache, S., Lehman, H., and Ho, C. *Biochemistry* **11**, 1677 (1972).
38. Lindstrom, T. R., Baldassare, J. J., Bunn, H. F., and Ho,C. *Biochemistry* **12**, 4212 (1973).
39. Fung, L-.W.-M and Ho, C. *Biochemistry* **14**, 2526 (1975).