Contributions of Asparagine at α97 to the Cooperative Oxygenation Process of Hemoglobin†

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ABSTRACT: According to the X-ray crystallographic results from human deoxyhemoglobin, β99Asp at the $\alpha_1\beta_2$ interface forms hydrogen bonds with α42Tyr and α97Asn. To clarify the structural and functional roles of the hydrogen bond between α97Asn and β99Asp, we have engineered a recombinant hemoglobin in which α97Asn is replaced by Ala, and have investigated its oxygen-binding properties, and have used proton nuclear magnetic resonance spectroscopy to determine the structural consequences of the mutation. Recombinant Hb (α97Asn→Ala) shows a milder alteration of functional properties compared to the severely impaired β99 mutants of the human abnormal hemoglobins. The addition of inositol hexaphosphate, an allosteric effector, causes recovery of the functional properties of recombinant Hb (α97Asn→Ala) almost to the level of human normal adult hemoglobin without this allosteric effector. r Hb (α97Asn→Ala) shows very similar tertiary structure around the heme pockets and quaternary structure in the $\alpha_1\beta_2$ interface compared to those of human normal adult hemoglobin. The proton nuclear magnetic resonance spectrum of the deoxy form of this recombinant hemoglobin shows the existence of an altered hydrogen bond which is believed to be between α42Tyr and β99Asp at the $\alpha_1\beta_2$ interface. Thus, the present results suggest that the intersubunit hydrogen bond between α97Asn and β99Asp at the $\alpha_1\beta_2$ interface is not as crucial as the one between α42Tyr and β99Asp in the deoxy quaternary structure. Preliminary molecular dynamics simulations have been used to calculate the contributions of specific interactions of several amino acid residues in r Hb (α97Asn→Ala) to the free energy of cooperativity of this recombinant hemoglobin. The results of these calculations are consistent with the experimental results.

X-ray crystallographic analyses of oxy- and deoxyhemoglobin (Hb)[1] have shown that the intersubunit contacts at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ subunit interfaces undergo a major alteration when the oxygenation state of the Hb molecule changes (Perutz, 1970; Baldwin & Chothia, 1979; Shaanan, 1983; Fermi et al., 1984). Human abnormal Hbs with amino acid substitutions at the $\alpha_1\beta_2$ subunit interface generally show very high oxygen affinity and greatly reduced cooperativity in O_2 binding [as measured by the Hill coefficient (n_{max})] compared to those of human normal adult hemoglobin (Hb A) (Dickerson & Geis, 1983; Bunn & Forget, 1986). X-ray crystallographic studies of Hb A (Fermi et al., 1984) 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 and thus to provide the free energy of cooperativity in binding oxygen. The importance of β99Asp in the cooperative oxygenation has also been demonstrated by the functional defects of human mutant Hbs with an amino acid substitution at the β99Asp position, such as Hb Kempsey (β99Asp→Asn) (Reed et al., 1968), Hb Yakima (β99Asp→His) (Jones et al., 1967), Hb Radcliffe (β99Asp→Ala) (Weatherall et al., 1977), Hb Hôtel Dieu (β99Asp→Gly) (Thillet et al., 1981), and Hb Ypsilanti (β99Asp→Tyr) (Glynn et al., 1968), which possess greatly reduced cooperativity and increased oxygen affinity relative to those exhibited by Hb A.

To investigate the functional role of α42Tyr at the $\alpha_1\beta_2$ subunit interface of deoxy-Hb, two recombinant Hb (r Hb) mutants, r Hb (α42Tyr→His) and r Hb (α42Tyr→Phe), have been constructed (Ishimori et al., 1989; Imai et al., 1991). r Hb (α42Tyr→Phe) exhibits essentially no cooperativity in binding oxygen ($n = 1.2$ at pH 6.8) and possesses very high oxygen affinity. However, r Hb (α42Tyr→His) exhibits considerable cooperativity ($n = 2$ at pH 6.8) and moderate oxygen affinity. These authors have attributed the milder functional impairment of r Hb (α42Tyr→His) to the presence of a new weak hydrogen bond between α42His and β99Asp in the deoxy state of r Hb (α42Tyr→His) but not in deoxy-r Hb (α42Tyr→Phe), suggesting that the hydrogen bond between α42Tyr and β99Asp in deoxy-Hb A plays a key role in stabilizing the deoxy quaternary structure and consequently in the cooperative oxygen-binding process of Hb.

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[1] Abbreviations: Hb, hemoglobin; Hb A, human normal adult hemoglobin; r Hb, recombinant hemoglobin; deoxy-Hb, deoxyhemoglobin; HbCO, carbonmonoxyhemoglobin; met-Hb, methemoglobin; IHP, inositol hexaphosphate; MD, molecular dynamics; MAP, methionine aminopeptidase; N-terminal, amino-terminal; Tris, tris[hydroxymethyl]aminomethane; TETA, triethylenetetraamine hydrochloride; EDTA, ethylenediaminetetraacetate; Mb, myoglobin; DSS, 2,2-dimethyl-2-silapentane-5-sulfonate.

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We have constructed a plasmid (pHE2) (Shen et al., 1993) in which synthetic human α- and β-globin genes (Hoffman et al., 1990) are coexpressed with the *Escherichia coli* methionine aminopeptidase (MAP) gene (Ben-Bassat et al., 1987) under the control of separate *tac* promoters. In *E. coli* cells harboring the pHE2 plasmid, the amino-terminal (N-terminal) methionine residues of the expressed Hb A have been effectively cleaved by the coexpressed MAP, and this expressed Hb A lacking an N-terminal methionine is identical to the native Hb A in a number of structural and functional properties (Shen et al., 1993). Recently, we have used molecular dynamics (MD) simulations to design compensatory amino acid substitutions in an abnormal Hb, Hb Kempsey (β99Asp→Asn), which substantially restore its allosteric properties (Kim et al., 1994). Hb Kempsey (β99Asp→Asn) has a high oxygen affinity and exhibits essentially no cooperativity in binding oxygen (Reed et al., 1968; Bunn et al., 1974). Computer simulations indicate that a new hydrogen bond involving β99Asn can be induced by replacing α42Tyr by a stronger hydrogen-bond acceptor such as Asp. The resulting double-mutant recombinant hemoglobin, r Hb (β99Asp→Asn, α42Tyr →Asp), was produced by site-directed mutagenesis (Kim et al., 1994). The oxygen affinity of r Hb (β99Asp→Asn, α42Tyr →Asp), while still high, is significantly lower than that of Hb Kempsey, and very substantial cooperativity has been restored, showing that the hydrogen bond involving the α42 and β99 amino acid positions in the $\alpha_1\beta_2$ interface is essential for the functional properties of Hb.

Although there are two hydrogen bonds involving β99Asp in the $\alpha_1\beta_2$ interface, so far most of the investigations have been focused only on the hydrogen bond between the α42 and β99 positions. Hb Dallas (α97Asn→Lys), which is the only known human mutant Hb with a mutation at the α97 position, exhibits very high oxygen affinity (Dysert et al., 1982). However, the introduction of a charged amino acid, lysine, at the α97 position may not be suitable for the investigation of the hydrogen bond between α97Asn and β99Asp. Thus, to investigate the specific functional role(s) of the hydrogen bond between α97Asn and β99Asp, we have applied site-directed mutagenesis to produce a new recombinant hemoglobin, r Hb (α97Asn→Ala), which disrupts the hydrogen bond between α97Asn and β99Asp but is found to cause only minimal structural perturbations.

In the present work, we have determined the oxygen-binding properties of r Hb (α97Asn→Ala) and have used ^{1}H-NMR spectroscopy to investigate the tertiary structure around the heme groups as well as the quaternary structure. Preliminary MD simulations have been used to calculate the free energy difference between native Hb A and r Hb (α97Asn→Ala). By using a thermodynamic integration method (Kirkwood, 1935), the contributions of specific interactions to the free energy change of cooperativity are estimated.

EXPERIMENTAL PROCEDURES

Plasmids, Strains, and Media. The Hb expression plasmid pHE2 (Shen et al., 1993) containing appropriately arranged and modified expression cassettes of synthetic α- and β-globin genes (kindly provided by Somatogen as pDLIII-13e) and the *E. coli* MAP gene (kindly provided by Cetus as pSYC1174) was used (after appropriate modifications) to produce mutant Hbs. Phagemid pTZ18U and *E. coli* JM109 were obtained from Bio-Rad and Promega, respectively. *E. coli* cells were grown in 2× YT medium (Miller, 1972) supplemented with 50 μg of ampicillin/mL. TB medium used for the expression of Hb contained bactotryptone (1.2%), bactoyeast extract (2.4%), glycerol (0.4%), KH_2PO_4 (17 mM), K_2HPO_4 (72 mM), and 100 μg of ampicillin/mL.

Site-Directed Mutagenesis. Somatogen's pDLIII-13e was inserted into phagemid pTZ18U (Bio-Rad). Site-directed mutagenesis was performed as previously described (Kunkel, 1985; Shen et al., 1993). Synthetic oligonucleotide 5′CAGTTTGAAGGCAACCGGATC3′ was used as a primer to introduce the mutation, α97Asn→Ala. The human normal α-globin gene in plasmid pHE2 was then replaced by the mutated α-globin gene to produce pHE226.

Growth of Cells. The plasmid pHE226 was transformed into *E. coli* BL21-DR, which is derived from strain BL21-DE3 (kindly provided by Dr. Maureen Gilmore-Hebert), is resistant to common phages, and contains more copies of I^q, and the cells were grown in TB medium in a 10-L Microferm fermentor (New Brunswick Scientific, model BioFlo 3000) at 30 °C until the optical density at 600 nm reached 10.

Expression of r Hb (α97Asn→Ala) was induced by adding isopropyl β-thiogalactopyranoside to 0.2 mM. The culture was then supplemented with hemin (50 mg/L), and the growth was continued for at least another 4 h. The cells were harvested by centrifugation and stored frozen at −80 °C until needed for purification (Shen et al., 1993).

Purification of Recombinant Hb. The r Hb (α97Asn→Ala) was purified as previously described (Shen et al., 1993; Looker et al., 1994) with minor modifications. The frozen cell paste was suspended in lysis buffer [40 mM Tris-[hydroxymethyl]aminomethane (Tris)-base (Sigma)/1 mM benzamidine hydrochloride (Sigma)] at 3 mL/g of cell paste and stirred until the mixture was smooth. Lysozyme [(Sigma) 1 mg/g of cell paste in 40 mM Tris-HCl at pH 8.0] was added. $MgCl_2$ and $MnCl_2$ to final concentrations of 1 and 0.1 mM, respectively, and DNAseI [(ICN Biochemicals) 2−3 μg/mL] were added to the cell paste prior to sonication. After sonification, the sonicate was saturated with CO gas and centrifuged to pellet cell membranes, etc. The supernatant was adjusted to pH 8.0 with 1 M Tris-base, and polyethyleneimine (Sigma) was added to a final concentration of 0.3%−0.5% to precipitate nucleic acids. After centrifugation, the supernatant was put through a Millipore Minitan Acrylic Ultrafiltration System to concentrate the protein. Then, the sample was dialyzed in 20 mM Tris-HCl/0.1 mM triethylenetetraamine hydrochloride (TETA; Sigma) at pH 8.3 overnight with one change of buffer. The sample was kept at 4 °C throughout the above procedures and maintained in the CO form throughout. We used two columns in the final purification process: (i) a Q-Sepharose fast-flow column (Pharmacia anion exchanger) was used to bind Hb. After the sample was loaded onto the column, it was washed with the running buffer (20 mM Tris-HCl/0.1 mM TETA at pH 8.3), and the pass through was monitored at 260 nm until the contaminating nucleic acids had eluted. Then, the Hb fraction was eluted from the column with 20 mM Tris-HCl/0.1 mM TETA at pH 7.2. After concentration, the Hb fraction was oxidized and reduced as described in Shen et al. (1993). (ii) a Mono S column (Pharmacia cation

exchanger HR16/10) with a gradient of 10 mM sodium phosphate/0.1 mM ethylenediaminetetraacetate (EDTA) at pH 6.8 (eluent A) to 20 mM sodium phosphate/0.1 mM EDTA at pH 8.3 (eluent B) was used to purify the r Hb (α97Asn→Ala).

Analytical Procedures. Automated cycles of Edman degradation were performed with an Applied Biosystems gas/liquid-phase sequencer (model 470/900A) equipped with an on-line phenylthiohydantoin amino acid analyzer (model 120A).

The mass spectrometric analyses were performed on a VG Quattro-Bio-Q mass spectrometer (Fisons Instruments, VG Biotech, Altrincham, U.K.). The instrument was set in the positive ion mode. The multiply-charged ion peaks from myoglobin (Mb; molecular mass, 16 591 Da) were used as an external reference for mass scale calibration (Zaia et al., 1992). Mb at one-fourth of the Hb subunit concentration was included in each sample as an internal standard. Scanning was in the multichannel analyzer mode from *m*/*z* 500 to 1500 at 8 s/scan. The collected data were processed by the MaxEnt program (Ferrige et al., 1992). The molecular weights calculated from the amino acid sequences of normal α and β chains of Hb A, α chain + methionine, and β chain + methionine are 15 126.4, 15 867.2, 15 257.6, and 15 998.6, respectively. The VG computer program uses the following average elemental atomic weights: C = 12.011; H = 1.00794; N = 14.00674; O = 15.994; and S = 32.066.

Oxygen Binding of Hb Samples. The oxygen dissociation curves of r Hb (α97Asn→Ala) and Hb A (0.1 mM of heme each) were measured by a Hemox-Analyzer (TCS Medical Products, Huntington Valley, PA) at 29 °C in 0.1 M sodium phosphate buffer in the pH range 6.2−8.4. 15 μL of a stabilizer [hexamethylphosphoramide (Sigma)] and 15 μL of antifoam [SAG-10 silicon antifoam emulsion (OSI Specialties)] were added to each 3-mL sample. After each measurement, the sample was checked for the presence of methemoglobin (met-Hb). Those with met-Hb greater than 5% were discarded. If necessary, a met-Hb reductase system (Hayashi et al., 1973) was added (120 μL) to each sample in order to prevent the formation of met-Hb. Partial O_2 pressure at 50% saturation (P_{50}) and the Hill coefficient (n_{max}) were determined from each oxygen dissociation curve.

NMR Measurements. ^{1}H-NMR spectra were obtained on a Bruker AM-300 spectrometer operating at 300 MHz at 29 °C. All Hb samples were in 0.1 M sodium phosphate buffer (in 100% H_2O) at pH 7.0. The Hb concentration was about 4% (2.5 mM in terms of heme). The water signal was suppressed by using a jump-and-return pulse sequence (Plateau & Guéron, 1982) except in the water presaturation experiment. The ^{1}H-NMR spectra of the HbCO and deoxy-Hb samples were obtained by using the proton decoupling coil of a 5-mm multinuclear probe with 90° pulses of 9.7 μs. Proton chemical shifts are referenced to the methyl proton resonance of the sodium salt of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) indirectly by using the water signal, which occurs at 4.76 ppm downfield from that of DSS at 29 °C, as the internal reference.

MD Simulations.[2] MD simulations were carried out with a stochastic boundary method (Brooks & Karplus, 1989) using CHARMM 22 with standard parameters for the polar hydrogen model (param19). The Hb molecule was partitioned into MD and Langevin regions with radii of 10 and 15 Å, respectively, which were centered on the center-of-mass of coordinates of the C_β of the α97Asn side chain in the crystal structures of Hb A in the deoxy and oxy forms (Shaanan, 1983; Fermi et al., 1984). The inside sphere was filled with CHARMM-adapted pre-equilibrated TIP3P water molecules (Jorgensen et al., 1983). The deoxy simulation included 88 water molecules, and the oxy simulation included 78 water molecules.

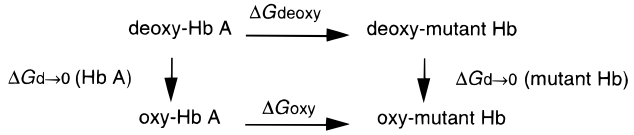
The transformation between wild-type and mutant proteins can be achieved by using a hybrid potential function $V_\lambda = (1 - \lambda)V_A + \lambda V_B$ (Gao et al., 1989; Tidor & Karplus, 1991), where λ is a coupling factor between 0 and 1. V_A and V_B are potential energy functions for Hb A and for mutant Hb, respectively. Simulations were done at nine values of λ_i (λ = 0.1, 0.2, ..., 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. Nonbonded interactions were truncated to zero at 8.5 Å, and a dielectric constant, $\epsilon = 1$, was used. All bonds involving hydrogen atoms were constrained with the SHAKE algorithm (Ryckaert et al., 1977). A 10-ps simulation takes about 2 h on a single-CPU SunSparc Workstation 10 with 1-fs integration time.

The free energy of simulations can be obtained from the trajectory files of MD simulations for both deoxy and oxy forms of Hb using the thermodynamic integration method (Kirkwood, 1935) with the following equation:

$$\Delta G = G_B - G_A = \int_0^1 \langle \Delta V \rangle_\lambda \, d\lambda \approx \sum_i \langle \Delta V \rangle_\lambda \, \Delta\lambda$$

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 from the thermodynamic cycle (Scheme 1) as shown by Gao et al. (1989):

Scheme 1



RESULTS

Biochemical Properties of r Hb (α97Asn→Ala). r Hb (α97Asn→Ala) from the sonicated cells of *E. coli* BL21-DR harboring plasmid pHE226 shows two major peaks (peaks a and b) on Mono S column chromatography (Figure

[2] It should be mentioned that our present approach to applying MD simulations and thermodynamic cycle calculations to r Hb (α97Asn→Ala) follows closely that used by Karplus and co-workers on a similar mutant Hb, Hb Radcliffe (β99Asp→Ala) (Gao et al., 1989). We recognize that there are both advantages and limitations inherent in this kind of approach to proteins as discussed by several researchers in the field [for example, refer to Mitchell and McCammon (1991), van Gunsteren and Mark (1992), Hermans et al., (1992), Shi et al. (1993), and Boresch et al. (1994)]. In our present study, we merely make use of the MD simulations and thermodynamic cycle calculations to gain some insights into our oxygen binding and ^{1}H-NMR results and to suggest new experiments.

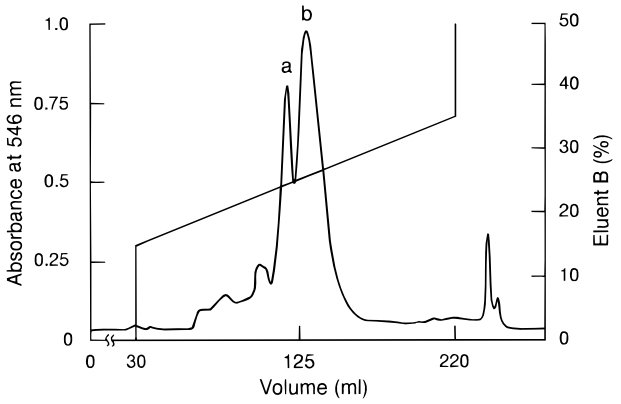


FIGURE 1: Purification of r Hb (α97Asn→Ala) through a Mono S column with a gradient of 10 mM sodium phosphate/0.1 mM EDTA at pH 6.8 (eluent A) to 20 mM sodium phosphate/0.1 mM EDTA at pH 8.3 (eluent B) at room temperature. Fractions were monitored at 546 nm.

Table 1: Mass Spectrometric Analysis of r Hb (α97Asn→Ala)

	molecular mass (Da)		
subunit	peak a	peak b	predicted[a]
α chain	15 083 ± 1 (70%)[b]	15 083 ± 1	15 083.4
α chain + met	15 214 ± 1 (30%)[b]	ND[c]	15 214.4
β chain	15 870 ± 2 (30%)[b]	15 867 ± 1	15 867.2
β chain + met	15 998 ± 1 (70%)[b]	ND	15 998.2

[a] See Experimental Procedures for details on calculating the expected or predicted molecular weight. [b] The number inside the parenthesis indicates the percent of the total protein in the peak with or without the N-terminal methionine as determined from the mass spectrum. [c] Not detected.

1). Both peaks in the CO form show a visible optical spectrum (over the range 350−700 nm) identical to that of Hb A (results not shown).

Electrospray mass spectrometry shows that the N-terminal methionine residues of peak b have been effectively cleaved by the coexpressed MAP (results not shown). For peak b, both α and β chains have the correct masses with undetectable (<2%) N-terminally added methionine. For peak a, about 30% of the α chain and about 70% of the β chain have the mass corresponding to that of normal α and β chains plus a methionine residue (Table 1). These mass spectrometric results have been confirmed by Edman degradation. Amino acid sequencing shows that 48% of the combined α and β chains for peak a contains N-terminally added methionine, whereas only 4% of the total α and β chains for peak b contains N-terminal methionine. Table 1 summarizes the mass spectrometric results obtained for r Hb (α97Asn→Ala). From the mass spectrometric data and the N-terminal sequence analysis, it is clear that the protein isolated from peak b represents the correct r Hb (α97Asn→Ala). r Hb (α97Asn→Ala) from peak b has been used for all further experiments, unless otherwise specified.

^{1}H-NMR Studies of r Hb (α97Asn→Ala). ^{1}H-NMR spectroscopy has been shown to be an excellent tool to investigate the tertiary and quaternary structural features of Hb (Ho, 1992). Figure 2A compares the exchangeable proton resonances of r Hb (α97Asn→Ala) and Hb A in the CO form. The resonance at ∼10.7 ppm from DSS has been assigned to the intersubunit hydrogen bond between α94Asp and β102Asn at the $\alpha_1\beta_2$ subunit interface, a characteristic feature of the oxy quaternary (R) structure (Fung & Ho,

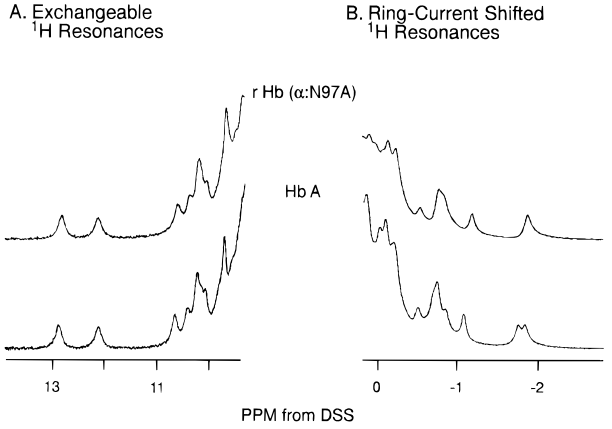


FIGURE 2: 300-MHz exchangeable proton resonances (A) and ring-current shifted proton resonances (B) of 4% r Hb (α97Asn→Ala) and 4% Hb A in the CO form in 0.1 M phosphate in H_2O at pH 7.0 and 29 °C.

1975). This resonance seems to be shifted slightly upfield to ∼10.6 ppm in r HbCO (α97Asn→Ala). Other exchangeable resonances which have been assigned to the $\alpha_1\beta_1$ interfacial hydrogen bonds between α126Asp and β35Tyr (∼12.9 ppm) and between α103Asp and β108Asn (∼12.1 ppm) (Russu et al., 1987) are essentially the same in the two samples.

Figure 2B compares the ring-current shifted resonances of Hb A and r Hb (α97Asn→Ala). The ring-current shifted ^{1}H resonances are known to be sensitive to the heme orientation and environment of Hb A in the CO form (Ho, 1992; Shen et al., 1993; Kim et al., 1994, 1995). The ^{1}H resonances from 0 to −2.0 ppm from DSS arise from some of the protons of the amino acid residues located in the vicinity of the heme pockets of the Hb molecule and of the porphyrins (Ho, 1992). There is considerable similarity in the ring-current shifted ^{1}H resonances of r HbCO (α97Asn→Ala) and those of HbCO A. However, the resonances from −1.7 to −1.8 ppm, which have been assigned to the γ_2-methyl groups of the E11Val (distal valine) of the α and β chains of HbCO A (Lindstrom et al., 1972; Dalvit & Ho, 1985) merge into one peak in the spectrum of r HbCO (α97Asn→Ala). This has also been observed for HbCO Kempsey (Lindstrom et al., 1973) and the compensatory mutant of Hb Kempsey, r HbCO (α42Tyr→Asp, β99Asp→Asn) (Kim et al., 1994).

Very low-field ^{1}H resonances of Hb A and r Hb (α97Asn→Ala) in the deoxy form are compared in Figure 3A. The resonance at ∼63 ppm has been assigned to the hyperfine-shifted $N_\delta H$ exchangeable proton of the proximal histidine residue (α87His) of the α chain of deoxy-Hb A and the one at ∼77 ppm has been assigned to the corresponding residue of the β chain (β92His) of deoxy-Hb A (Takahashi et al., 1980; La Mar et al., 1980). The chemical shift positions of these two proximal histidyl resonances in r deoxy-Hb (α97Asn→Ala) are exactly the same as those of deoxy-Hb A, indicating no perturbations around the proximal histidines of r Hb (α97Asn→Ala).

The ferrous hyperfine-shifted and exchangeable proton resonances of r Hb (α97Asn→Ala) and Hb A in the deoxy form are shown in Figure 3B. The hyperfine-shifted resonances arise from the protons on the heme groups and their nearby amino acid residues due to the hyperfine interactions between protons and unpaired electrons of Fe(II)

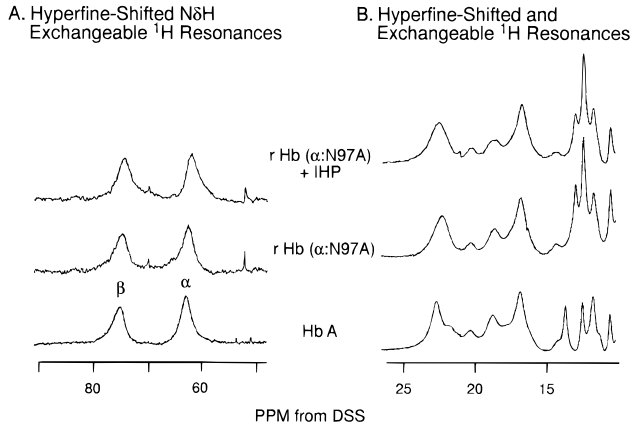


FIGURE 3: 300-MHz hyperfine-shifted $N_\delta H$ exchangeable proton resonances of the proximal histidine residues (A) and hyperfine-shifted and exchangeable resonances (B) of 4% r Hb (α97Asn→Ala) and 4% Hb A in the deoxy form in 0.1 M phosphate in H_2O at pH 7.0 and 29 °C. The spike at ~52 ppm was due to an instrumental artifact.

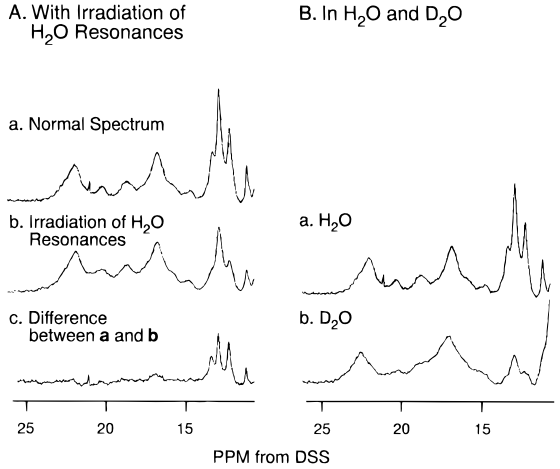


FIGURE 4: 300-MHz hyperfine-shifted and exchangeable resonances of 4% r Hb (α97Asn→Ala) in the deoxy form: (A) presaturated by irradiating the water resonance with a weak radio frequency in 0.1 M phosphate in H_2O at pH 7.0 and 29 °C and (B) in H_2O (a) and after the medium was exchanged with 0.1 M phosphate in D_2O at pD 7.0 and 29 °C (b). pD = pH (pH meter reading) + 0.4. The spike at ~21 ppm was due to an instrumental artifact.

in the heme iron atoms. The hyperfine-shifted resonances of r deoxy-Hb (α97Asn→Ala) are relatively similar to those of Hb A except that the resonance at ~22.8 ppm and a shoulder resonance at ~21.7 ppm from DSS of deoxy-Hb A [assigned to the protons associated with the β chains of deoxy-Hb A (Takahashi et al., 1980)] show a single broad resonance at ~22.2 ppm from DSS for r deoxy-Hb (α97Asn→Ala).

The exchangeable ^1H resonances over the spectral region from 11 to 14 ppm from DSS of deoxy-Hb A are known as excellent markers for the deoxy quaternary structure and of the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interface of Hb A (Ho, 1992). The resonance at ~14 ppm has been assigned to the intersubunit hydrogen bond between α42Tyr and β99Asp, a characteristic feature of the deoxy quaternary (T) structure (Fung & Ho, 1975). This resonance disappears in the spectrum of r deoxy-Hb (α97Asn→Ala) (Figure 3B). Instead a new resonance at ~13.2 ppm from DSS has appeared. The addition of 10 mM inositol hexaphosphate (IHP) does not cause any noticeable changes in either hyperfine-shifted or exchangeable resonances.

To determine the nature of this new resonance at ~13.2 ppm, the water peak was presaturated by irradiating the resonance with a weak radio frequency (Figure 4Ab) (Fung & Ho, 1975). The relative intensity of the resonances at ~12.9 and ~12.1 ppm, which are exchangeable resonances, and the resonance at ~13.2 ppm have all decreased (Figure 4Ab). The difference spectrum (Figure 4Ac) between this spectrum (Figure 4Ab) and that obtained with the jump-and-return pulse sequence (Figure 4Aa) (Plateau & Guéron, 1982) clearly indicates that the resonance at ~13.2 ppm from DSS is exchangeable. The origin of the resonance at ~13.2 ppm from DSS can also be confirmed by D_2O exchange. When a sample was prepared in D_2O buffer, the resonance at ~13.2 ppm from DSS as well as the other known exchangeable resonances at ~12.9 and ~12.1 ppm from DSS diminished (Figure 4Bb), clearly showing that the resonance at ~13.2 ppm from DSS is exchangeable rather than hyperfine shifted.

Oxygen-binding Properties of r Hb (α97Asn→Ala). When r Hb (α97Asn→Ala) at a concentration of 0.1 mM (in terms of heme) is applied to a Sephadex G-75 gel-filtration column, it elutes in a symmetrical peak at the same position as that of HbO_2 A (results not shown). Both P_{50} and n_{max} values for this r Hb (α97Asn→Ala) are invariant over the Hb concentration range 0.05−0.16 mM (results not shown). Thus, there is no unusual dissociation of this r Hb (α97Asn→Ala) into dimers during our O_2-binding studies, as compared to Hb A. The O_2-binding properties of r Hb (α97Asn→Ala) and Hb A are summarized in Figure 5. In 0.1 mM sodium phosphate at 29 °C, r Hb (α97Asn→Ala) exhibits higher oxygen affinity than Hb A. For example, at pH 7.0, P_{50} = 1.5 mmHg for r Hb (α97Asn→Ala) versus P_{50} = 14.5 mmHg for Hb A. r Hb (α97Asn→Ala) exhibits 60% of the Bohr effect of Hb A over the pH range 6.6−7.8 ($\Delta\log P_{50}/\Delta pH = -0.33$ for r Hb (α97Asn→Ala) versus $\Delta\log P_{50}/\Delta pH = -0.55$ for Hb A). r Hb (α97Asn→Ala) exhibits substantial cooperativity in binding of oxygen, especially at low pH; for example, n_{max} = 1.8 at pH 6.6 for r Hb (α97Asn→Ala) versus n_{max} = 3.0 for Hb A. A marked change occurs when 2 mM IHP is added to r Hb (α97Asn→Ala). The oxygen affinity is reduced significantly [P_{50} = 8.8 mmHg at pH 7.0 for r Hb (α97Asn→Ala)], and the cooperative oxygenation process for r Hb (α97Asn→Ala) in 2 mM IHP approaches the normal value for Hb A as manifested by the Hill coefficient, with an n_{max} value of 2.1−2.8 over the pH range from 6.6 to 7.8 compared to $n_{max} \approx$ 3.0 for Hb A over the pH range from 6.5 to 8.4. For details, see Figure 5.

From an analysis of the oxygen binding data, we can estimate the free energy of cooperativity (or interaction), ΔG, for r Hb (α97Asn→Ala) and compare it with that of Hb A. In 0.1 M phosphate at pH 7.4 and 29 °C, ΔG for r Hb (α97Asn→Ala) is −0.2 kcal/mol *vs* ΔG = −3.1 kcal/mol for Hb A. For r Hb (α97Asn→Ala) in the presence of 2 mM IHP at pH 7.4, the corresponding ΔG is changed to −0.5 kcal/mol.

MD Simulation. The free energy simulation results are shown in Table 2. Both deoxy and oxy tetramers are destabilized by the mutation α97Asn→Ala (by 13.7 and 11.4 kcal/mol per $\alpha_1\beta_2$ interface, respectively). The free energy of cooperativity for r Hb (α97Asn→Ala) is much smaller than that of Hb A (−0.2 kcal/mol *vs* −3.1 kcal/mol). The MD calculated value for the difference in the free energy of cooperativity between r Hb (α97Asn→Ala) and Hb A is −2.3

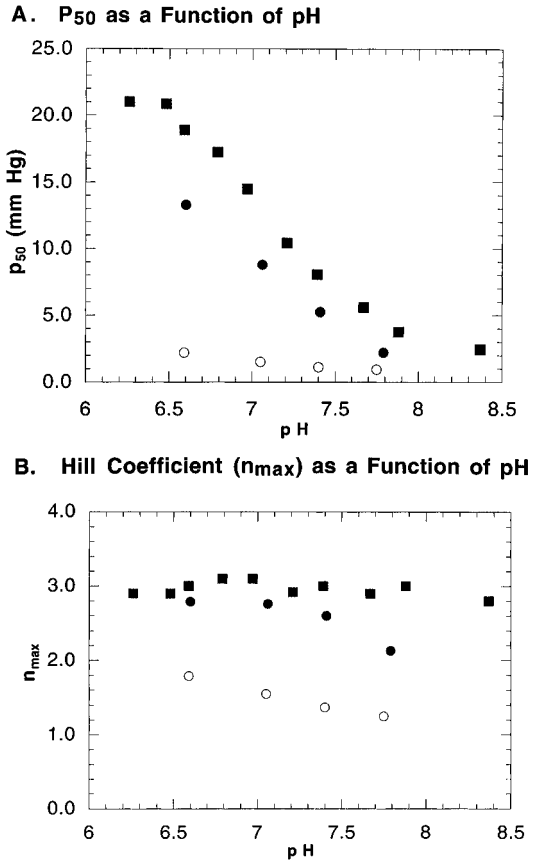


FIGURE 5: pH-dependence of oxygen affinity (A) and the Hill coefficient (n_{max}) (B): (■) Hb A; (○) r Hb (α97Asn→Ala); and (●) r Hb (α97Asn→Ala) in the presence of 2 mM IHP. Oxygen dissociation data were obtained with 0.1 mM concentration of Hb in 0.1 M sodium phosphate buffer in the pH range 6.2−8.4 at 29 °C. P_{50} (in mmHg) and n_{max} were determined from each curve.

kcal/mol per interface (Table 2), which is in good agreement with our experimentally determined value, −2.9 kcal/mol, and is about the expected value for an Hb which has a somewhat lower cooperativity than Hb A (Turner et al., 1992). Thus, our present MD simulation results may be used to obtain information about the specific interactions which contribute to the total free energy of cooperativity. The contributions of several individual amino acids to the total free energy of cooperativity are also shown in Table 2. Most individual amino acids contribute less than 1.0 kcal, indicating that the mutation introduced into the $\alpha_1\beta_2$ interface does not cause big conformational changes. The individual amino acid that experiences the largest free energy change due to the mutation is α94Asp. In both deoxy and oxy tetramers, α94Asp shows an unfavorable free energy of interaction. The destabilization of the deoxy structure is larger, resulting in a negative contribution to the free energy of cooperativity. These results indicate that there is a significant stabilizing interaction between α94Asp and α97Asn in both deoxy and oxy forms of Hb A, but the interaction is more stabilizing in the deoxy form. The X-ray crystallographic structures also indicate that the distance between the side chains of α94Asp and α97Asn in the deoxy tetramer (4.3 Å) is less than that in the oxy tetramer (5.7 Å). The mutation, α97Asn→Ala, gives β99Asp, its hydrogen bond partner in the deoxy structure, an unfavorable interaction in both deoxy and oxy tetramers, but the calculated destabilization is much larger in the deoxy structure than in the oxy one, resulting

Table 2: Free Energy Computed for the Mutation of r Hb (α97Asn→Ala)[a]

contribution	ΔG_{deoxy}[b]	ΔG_{oxy}	$\Delta\Delta G$[c]
solvent	1.2	−1.0	−2.2
hemoglobin[d]			
heme (α_1)[e]	−1.4	0.2	−1.6
intra (α_1)[f]	13.6	13.2	−0.4
α38Thr	1.8	2.5	0.7
α39Thr	1.9	1.1	−0.8
α42Tyr	2.2	1.3	−0.9
α93Val	−1.1	−0.5	0.6
α94Asp	10.6	8.4	−2.2
α96Val	−1.0	−0.5	0.5
inter (β_2)[g]	2.2	0.5	−1.7
β99Asp	3.2	1.4	−1.8
self (α97Asn→Ala)[h]	−1.8	−1.6	0.2
total	13.7	11.4	−2.3

[a] In kcal/mol. [b] The positive sign given in ΔG signifies a contribution to destabilize Hb (α97Asn→Ala) relative to Hb A. [c] $\Delta\Delta G$ is ΔG(oxy) − ΔG(deoxy), which corresponds to the difference of the free energy of cooperativity between Hb (α97Asn→Ala) and Hb A. [d] Only those amino acid residues that contribute more than 1.0 kcal/mol in either the deoxy- or the oxy-Hb molecule are listed in the above table. [e] A 15-Å sphere contains only that portion of the heme of the α_1 subunit. However, the MD simulations include the entire heme of the α_1 subunit. [f] The total contribution from the amino acids in the α_1 subunit. [g] The total contribution from the amino acids in the β_2 subunit. [h] The self-energy term is defined as the energy contribution which involves only those atoms in the mutant or the wild-type side chains at the α97 position (C_α is considered to be part of the side chain).

in a negative contribution to the total free energy of cooperativity. The mutation also gives an unfavorable interaction to α42Tyr, which forms a hydrogen bond with β99Asp in the $\alpha_1\beta_2$ interface of the deoxy structure, resulting in a negative contribution to the total free energy of cooperativity. Other amino acids in the C_α region, α38Thr (C3) and α39Thr (C4) also become somewhat destabilized by the mutation. Unlike the MD simulation results of Hb Radcliffe (β99Asp→Ala) (Gao et al., 1989), a mutation which involves the deletion of a charged side chain, the contribution of solvent is minimal in the MD simulation of Hb (α97Asn→Ala), i.e., 1.2 kcal for the deoxy form and −1.0 kcal for the oxy form of Hb (α97Asn→Ala) versus 46.0 kcal for the deoxy form and 68.5 kcal for the oxy form of Hb Radcliffe (β99Asp→Ala) (Gao et al., 1989).

DISCUSSION

The oxygen-binding properties of r Hb (α97Asn→Ala), Hb A, r Hb A, Hb Kempsey (β99Asp→Asn) (Bunn et al., 1974), r Hb (α42Tyr→Asp, β99Asp→Asn) (Kim et al., 1994), and the two α42 mutants of Ishimori et al. (1989) and Imai et al. (1991) are compared in Table 3. Hb Kempsey and r Hb (α42Tyr→Phe) show markedly increased affinities for oxygen. However, r Hb (α42Tyr→His) and r Hb (α42Tyr→Asp, β99Asp→Asn), which are believed to have a weak hydrogen bond in the $\alpha_1\beta_2$ interface, show oxygen-binding properties similar to those of r Hb (α97Asn→Ala). They all exhibit oxygen-binding affinities intermediate between β99 mutants and Hb A. When the allosteric effector, IHP, is added to r Hb (α42Tyr→His), r Hb (α42Tyr→Asp, β99Asp→Asn), and r Hb (α97Asn→Ala), the oxygen affinities of all of these r Hbs are reduced significantly and the cooperative oxygenation process approaches that of Hb A in the absence of IHP (Table 3). As there is no longer a hydrogen bond between α97Asn and

Table 3: P_{50} and n_{max} Values of Hb A and r Hbs in the Presence and the Absence of IHP in 0.1 M Phosphate at pH 7.4 and 29 °C Unless Otherwise Specified

Hb	P_{50}, mmHg		n_{max}		reference
	−IHP	+IHP[a]	−IHP	+IHP[a]	
Hb A	8.0	35.5	3.1	2.6	present work
r Hb A	7.8	37.1	3.0	2.6	Shen et al. (1993)
r Hb (α97Asn→Ala)	1.1	5.2	1.4	2.6	present work
r Hb (α42Tyr→Asp, β99Asp→Asn)	1.9	10.0	1.7	2.4	Kim et al. (1994)
r Hb (α42Tyr→His)[b]	1.4	15	1.9	2.1	Imai et al. (1991)
r Hb (α42Tyr→Phe)[b]	0.6	1.0	1.1	1.4	Imai et al. (1991)
Hb Kempsey (β99Asp→Asn)[c]	0.2	1.1[d]	1.1	1.7[d]	Bunn et al. (1974)

[a] IHP concentration was 2 mM unless otherwise specified. [b] In 0.05 M Bis-Tris (pH 7.4) containing 0.1 M Cl^- at 25 °C. [c] In 0.05 M Bis-Tris (pH 7.2) containing 0.1 M Cl^- at 20 °C. [d] IHP concentration was 1 mM.

β99Asp in the r Hb (α97Asn→Ala), only the hydrogen bond between α42Tyr and β99Asp is likely to exist in the $\alpha_1\beta_2$ interface of the deoxy structure. The moderately impaired oxygen-binding properties of r Hb (α97Asn→Ala) compared to those of Hb A suggest that the presence of the single hydrogen bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface of the deoxy structure may not be completely sufficient to maintain the normal deoxy quaternary structure of the Hb molecule. Thus, the hydrogen bond between α97Asn and β99Asp in the $\alpha_1\beta_2$ interface of the deoxy structure is believed to provide the additional force necessary to maintain the deoxy structure of an Hb molecule.

The presence of the hydrogen bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface of the deoxy structure can, in principle, be illustrated by ^{1}H-NMR spectroscopy. The exchangeable ^{1}H resonances over the spectral region from 11 to 14 ppm from DSS are known as excellent markers for the deoxy quaternary structure as well as the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces of Hb A (Ho, 1992). The resonance at ∼14 ppm has been assigned to the intersubunit hydrogen bond between α42Tyr and β99Asp, a characteristic feature of the deoxy quaternary (T) structure (Fung & Ho, 1975). The ^{1}H-NMR spectrum of r Hb (α97Asn→Ala) shows that there is a new resonance at ∼13.2 ppm from DSS which replaces the resonance at ∼14 ppm from DSS (Figure 3B). A decrease in the intensity of this resonance after water presaturation and the disappearance of the resonance after exchange of H_2O for D_2O clearly indicate that this resonance at ∼13.2 ppm from DSS is exchangeable (Figure 4). ^{1}H-NMR spectroscopy shows that the tertiary and quaternary structures of r deoxy-Hb (α97Asn→Ala) are very similar to those of deoxy-Hb A (Figures 2 and 3), indicating that the mutation, α97Asn→Ala, does not introduce major conformational changes in the subunit interface. Also, the mutation introduced, i.e., Asn→Ala, cannot induce a new hydrogen bond. These results suggest that the exchangeable resonance at ∼13.2 ppm from DSS of r deoxy-Hb (α97Asn→Ala) most likely is the resonance at ∼14 ppm from DSS, which is from the hydrogen bond between α42Tyr and β99Asp, shifted upfield.

As the tertiary and quaternary structures of r Hb (α97Asn→Ala) are very similar to those of Hb A, MD simulations may be applied successfully for the transformation from the wild-type (Hb A) to mutant Hb (α97Asn→Ala). Preliminary MD simulation results are consistent with the results of ^{1}H-NMR spectroscopy. The most affected amino acids due to the mutation α97Asn→Ala are α42Tyr, α94Asp, and β99Asp (Table 2). β99Asp forms hydrogen bonds with α97Asn and α42Tyr. The destabilizing effect of the mutation on β99Asp is expected to be caused by the absence of a hydrogen bond partner at α97Asn. In addition, the absence of a hydrogen bond between α97 and β99Asp can cause α42Tyr to be destabilized. This may be the reason for the upfield shift of the exchangeable resonance representing the hydrogen bond between α42Tyr and β99Asp from ∼14 to ∼13.2 ppm (Figure 3B). The amino acid most affected by the mutation is α94Asp. In both the deoxy and oxy tetramers of r Hb (α97Asn→Ala), α94Asp is destabilized, but the destabilization in the deoxy tetramer is larger, giving a negative contribution (−2.2 kcal) to the total free energy of cooperativity. Thus, the slight downfield shift of the exchangeable resonance at ∼12.6 ppm from ∼12.7 ppm from DSS in the r HbCO (α97Asn→Ala) (Figure 2A) could be explained by the MD simulation results.

It should be mentioned that the movement of the heme iron atoms and the sliding motion of the $\alpha_1\beta_2$ subunit interface and the presence of the intra- and intermolecular salt bridges, as well as the presence of the hydrogen bonds at the $\alpha_1\beta_2$ subunit interface, are all among the central features of the stereochemical basis for the cooperative oxygenation of Hb (Perutz, 1970; Dickerson & Geis, 1983). In particular, the high oxygen affinity and complete loss of cooperativity of r Hb (α42Tyr→Phe) (Ishimori et al., 1989; Imai et al., 1991) due to the absence of the hydrogen bond between α42Tyr and β99Asp at the $\alpha_1\beta_2$ interface indicate that the hydrogen bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface of the deoxy structure is crucial for the cooperative oxygenation process. As shown from the present investigation of r Hb (α97Asn→Ala), the absence of the hydrogen bond between α97Asn and β99Asp, i.e., the presence of the single hydrogen bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ interface of the deoxy structure, results in oxygen-binding properties that are only mildly impaired compared to r Hb (α42Tyr→Phe) or Hb Kempsey (Table 3). Thus, it can be concluded that the role of the hydrogen bond between α97Asn and β99Asp at the $a_1\beta_2$ interface of the deoxy structure is not so crucial as the role of the hydrogen bond between α42Tyr and β99Asp at the $\alpha_1\beta_2$ interface of the deoxy form of the Hb molecule. However, the presence of both hydrogen bonds at the $\alpha_1\beta_2$ interface of the deoxy form is essential to maintain the normal cooperative oxygenation process of Hb A.

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