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Biophysical Chemistry 98 (2002) 15–25

Biophysical Chemistry

www.elsevier.com/locate/bpc

Recombinant hemoglobins with low oxygen affinity and high cooperativity

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Received 13 September 2001; accepted 18 October 2001

Abstract

By introducing an additional H-bond in the $\alpha_1\beta_2$ subunit interface or altering the charge properties of the amino acid residues in the $\alpha_1\beta_1$ subunit interface of the hemoglobin molecule, we have designed and expressed recombinant hemoglobins (rHbs) with low oxygen affinity and high cooperativity. Oxygen-binding measurements of these rHbs under various experimental conditions show interesting properties in response to pH (Bohr effect) and allosteric effectors. Proton nuclear magnetic resonance studies show that these rHbs can switch from the oxy (or CO) quaternary structure (R) to the deoxy quaternary structure (T) without changing their ligation states upon addition of an allosteric effector, inositol hexaphosphate, and/or reduction of the ambient temperature. These results indicate that if we can provide extra stability to the T state of the hemoglobin molecule without perturbing its R state, we can produce hemoglobins with low oxygen affinity and high cooperativity. Some of these rHbs are also quite stable against autoxidation compared to many of the known abnormal hemoglobins with altered oxygen affinity and cooperativity. These results have provided new insights into the structure–function relationship in hemoglobin.

Keywords: Recombinant hemoglobin; Low oxygen affinity; High cooperativity; Nuclear magnetic resonance

1. Introduction

Human normal adult hemoglobin (Hb A) is one of the most studied proteins and serves as a model for investigation of the structure–function relationship in multimeric, allosteric proteins as well as for research to understand the molecular details of intermolecular interactions in proteins. Hb A consists of four subunits i.e. two identical α-chains of 141 amino acids each and two identical β-chains of 146 amino acids each. The arrangement of the subunits of Hb A depends on the ligation state of the protein and is crucial in determining its function [1]. As first reported by Perutz [2], the four chains can be arranged into either the T (tense) quaternary structure for the low affinity deoxy-Hb or the R (relaxed) quaternary structure for the high affinity oxy-Hb. The transition from the T- to the R-state of hemoglobin involves a considerable change in the free energy upon oxygenation, which manifests itself in the cooperativity of oxygen

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0301-4622/02/$ - see front matter
PII: S0301-4622(02)00081-9

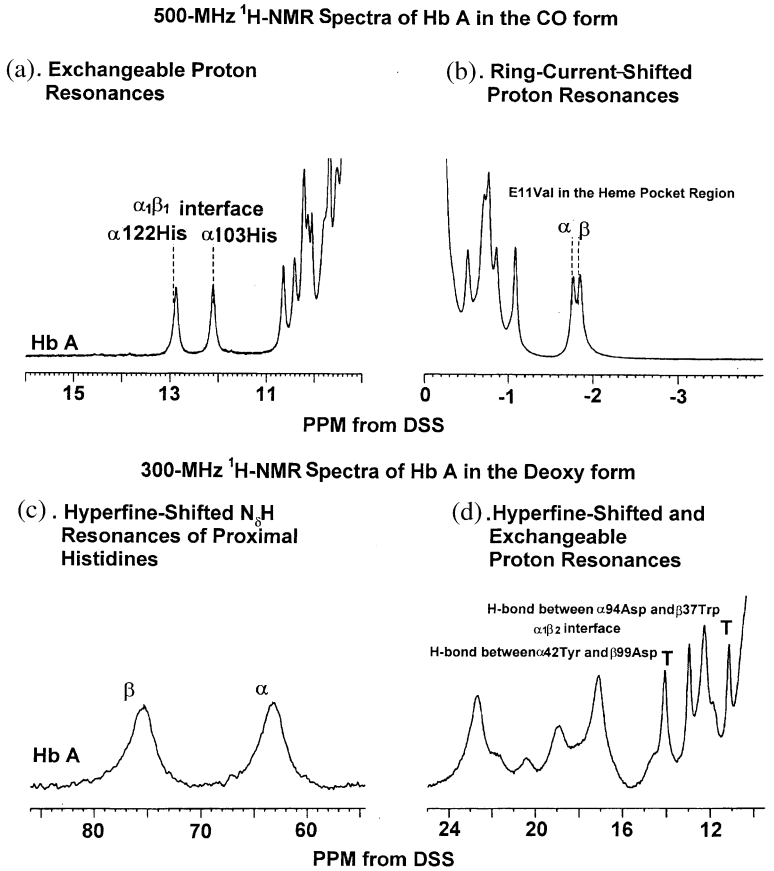

Fig. 1. A summary of 500- and 300-MHz ^{1}H-NMR spectra of 6–8% Hb A in 0.1 M phosphate at pH 7.0 in H_2O at 29 °C.

binding. Based on a comparison of the detailed structural features of Hb A in deoxy- and oxy-forms, Perutz and colleagues [2–5] have shown that upon ligation, the $\alpha_1\beta_2$ subunit interface undergoes a sliding movement, while the $\alpha_1\beta_1$ subunit interface remains essentially unchanged. Specific H-bonds, salt bridges, and non-covalent interactions characterize both types of subunit interfaces. Many of the naturally occurring human Hbs with mutations in the $\alpha_1\beta_2$ subunit interface are found to have increased oxygen affinity and diminished cooperativity [1,6].

Proton nuclear magnetic resonance (NMR) spectroscopy has been found to be an excellent tool for investigating the tertiary and quaternary structures and structural changes of Hbs in solution [7]. Several exchangeable proton resonances at 9–15 ppm downfield from the methyl proton resonance of 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) have been characterized as originating from the intersubunit H-bonds in the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in both the deoxy and CO (or oxy) states of Hb A (Fig. 1). These H-bonded protons observed by ^{1}H-NMR can be used as structural markers in structural and functional studies. In particular, the resonance appearing at ~14 ppm downfield from DSS has been assigned to the intersubunit H-bond between α42Tyr and β99Asp in the $\alpha_1\beta_2$ subunit interface of deoxy-Hb A [8], a characteristic feature of the T-quaternary structure of Hb A [2]. By observing this T-structural marker in both the deoxy and CO forms of Hbs under various experimental conditions, we can assess the stability of the T confor-

mation as well as monitor the T- to R-quaternary structural transition [7,9–13].

In our laboratory, we have developed an expression system to produce authentic Hb A in good yields in *Escherichia coli* [14,15]. With this expression system, we have designed and expressed a class of recombinant hemoglobins (rHbs) with low oxygen affinity and high cooperativity [9–13]. Our strategy for designing rHbs with low oxygen affinity and high cooperativity is to stabilize the T-quaternary structure without perturbing the R-quaternary structure. In this review, we will discuss the structural and functional properties of this class of rHbs with low oxygen affinity and high cooperativity caused by amino acid substitutions in the $\alpha_1\beta_2$ and $\alpha_1\beta_1$ subunit interfaces.

2. Low-oxygen-affinity rHbs with mutations in the $\alpha_1\beta_2$ subunit interface

The working hypothesis for our design of low-oxygen-affinity rHbs with high cooperativity is that if we can stabilize or provide extra stability to the T-quaternary structure of the Hb molecule without perturbing its R-quaternary structure, we should have Hbs with low oxygen affinity. This strategy was first demonstrated in rHb (αV96W) [9] and rHb (βL105W) [13] by introducing an additional H-bond in the $\alpha_1\beta_2$ subunit interface of the T structure. rHb (αV96W) and rHb (βL105W) both exhibit lower oxygen affinity as compared to Hb A and maintain high cooperativity as shown in Table 1 [9,13]. ^{1}H-NMR studies show that both rHbs exhibit quaternary and tertiary structures similar to those of Hb A in both the CO and deoxy forms [9,13]. The X-ray crystallographic studies of rHb (αV96W) in the T state show the existence of additional water-mediated H-bonds between α96Trp and β101Glu in the $\alpha_1\beta_2$ subunit interface and the central cavity of the Hb molecule [17], while the ^{1}H-NMR studies of rHb (βL105W) and rHb (αD94A, βL105W) confirm an additional H-bond formed between β105W and α94D in the T state [13]. According to ^{1}H-NMR studies, rHbCO (αV96W) and rHbCO (βL105W) can switch from the R- to the T-quaternary structure without changing their ligation states (as evidenced by the appearance of the T-state marker at 14 ppm), when the temperature is lowered and/or when a strong allosteric effector, such as inositol hexaphosphate (IHP), is added [9,13]. For example, as seen in Fig. 2, the intensity of the T-state marker in the spectra of rHbCO (αV96W) is much more prominent than in the spectra of rHbCO A at 11 °C and/or in the presence of IHP. The tendency of a ligated Hb to switch into the T-state conformation suggests that the equilibrium between the R- and the T-states of this rHb has been shifted toward the T state due to the mutation at the $\alpha_1\beta_2$ subunit interface [9,13]. In other words, these rHbs prefer to remain in the T-quaternary structure even when they are still ligated. The oxygen-binding studies as a function of temperature have further shown that upon decreasing temperature, the increase in oxygen affinity is much less pronounced in these two rHbs than in Hb A, suggesting a progressive stabilization of the T-quaternary structure of the mutants as the temperature is lowered [10,13]. Also, these rHbs exhibit a much less cooperative oxygen binding than Hb A when the temperature is lowered and/or IHP is added, suggesting that they prefer to remain in the deoxy state. Hence, we propose that the appearance of a T-structural marker in the ^{1}H-NMR spectra of rHbs in the CO

Table 1
Oxygen binding affinity (p_{50}) and cooperativity (n_{max}) for Hb A and rHbs in 0.1 M sodium phosphate buffer at pH 7.4 and 29 °C

Hemoglobin	p_{50} (mmHg)	n_{max}
Hb A[a]	8.0	3.1
rHb (αV96W)[a]	12.8	2.8
rHb (βL105W)[b]	28.2	2.6
rHb Presbyterian (βN108K)[a]	24.5	2.9
rHb Yoshizuka (βN108D)[a]	15.5	2.9
rHb (βN108Q)[c]	17.4	3.1
rHb (βN108R)[d]	28.9	3.1
rHb (βN108E)[d]	24.1	2.9
rHb (βN108A)[d]	23.1	3.1
rHb (αV96W, βN108K)[a]	48.8	2.3
rHb (αV96W, βN108D)[a]	18.6	2.6
rHb (αL29F, βN108Q)[c]	12.1	2.8

[a] Data taken from Ref. [11].
[b] Data taken from Ref. [13].
[c] Data taken from Ref. [12].
[d] Data taken from Ref. [16].

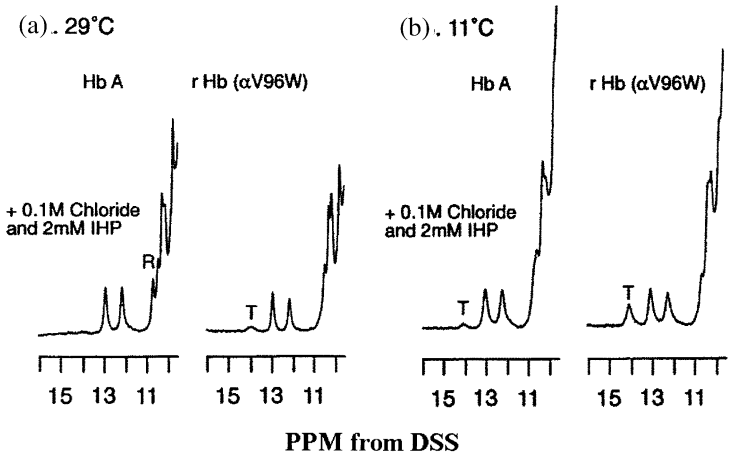

Fig. 2. Effects of temperature on the 300-MHz ^{1}H-NMR spectra of 4–6% solution of Hb A and rHb (αV96W) in the CO form in 0.1 M HEPES in H_2O at pH 7.0 and in the presence of 0.1 M chloride and 2 mM IHP; (a) at 29 °C; (b) at 11 °C.

form is an indication of a more stable T state, which serves as a molecular basis of the low oxygen affinity in rHb (αV96W) and rHb (βL105W) as well as other rHbs studied in our laboratory.

3. Low-oxygen-affinity rHbs with mutations in the $\alpha_1\beta_1$ subunit interface

We have further tested our working hypothesis on rHbs having amino acid substitutions at the β108 site, which is located in the $\alpha_1\beta_1$ subunit interface and in the central cavity of the Hb molecule. Hb Presbyterian (βN108K) and Hb Yoshizuka (βN108D) are two naturally occurring low-oxygen-affinity mutants [18–21], despite the fact that these two mutants have opposite charges. Previous NMR studies of rHb Presbyterian and rHb Yoshizuka have suggested that these two rHbs prefer to remain in the T state even when they are still ligated [11]. In an effort to investigate the functional role of the β108Asn site, we have constructed rHb (βN108Q), rHb (βN108E), and rHb (βN108R), which have been found to exhibit low oxygen affinity and high cooperativity as shown in Table 1 [12,16]. ^{1}H-NMR studies of these rHbs in the ligated form show the appearance of the T-state marker at 14 ppm upon decreasing the ambient temperature and adding 4 mM of IHP (Fig. 3). These results suggest that amino acid substitutions with either a positively charged (Lys/Arg), negatively charged (Asp/Glu), or a non-polar charged residue (Gln) at β108 can result in a more stable T state. It is noted that rHbs with amino acid substitutions at β108 are highly cooperative despite the fact that the resonance at 12.1 ppm, which is assigned to the H-bond between α103His and β131Gln in the $\alpha_1\beta_1$ subunit interface (C.K. Chang and C. Ho, unpublished results), is perturbed due to mutations at the β108 site (Figs. 3 and 4).

The side chain of β108Asn interacts freely with water molecules in both the deoxy and CO (or oxy) forms of the Hb molecule [3–5,22]. Previous studies on the oxygen-binding properties of rHb Presbyterian (βN108K) and rHb Yoshizuka (βN108D) have suggested that mechanisms other than the buildup of excess positive charges in the central cavity of Hb contribute to the lower oxygen affinity and high cooperativity of rHb Presbyterian [11]. Amino acid substitutions at β108Asn with either charged (Asp, Lys and Glu, Arg) or uncharged (Gln) residues result in rHbs with low oxygen affinity, further suggesting that the solvation effect can play a role in regulating the oxygen affinity of the Hb [23]. That is, as the β108Asn

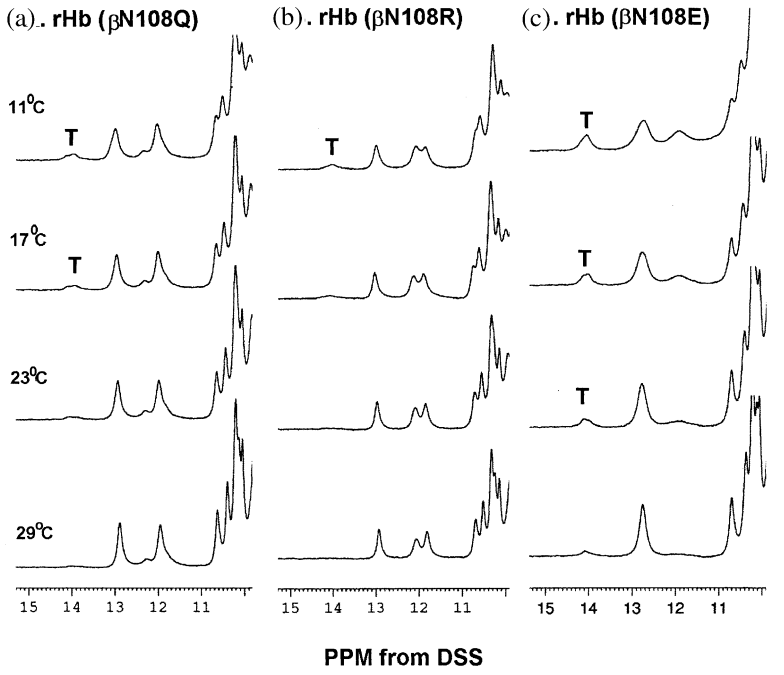

Fig. 3. Effects of temperature on the 500-MHz ^{1}H-NMR spectra of 4–6% solution of rHb (βN108Q) (a); rHb (βN108R) (b); and rHb (βN108E) (c) in the CO form in 0.1 M phosphate buffer in H_2O at pH 7.0 and in the presence of 4 mM IHP.

residue is replaced by Gln, Lys, Arg, Asp, or Glu, the electrostatic interactions between the β108 residue and the water molecules in the central cavity of the Hb become progressively stronger, which would increase the stability of the T state. The recent X-ray structure of rHb Presbyterian [24] has suggested that the extra H-bond formed between β108Lys and β35Tyr in the deoxy state can contribute to lowering the oxygen affinity of rHb Presbyterian by stabilizing the T state. An Ala mutation at β108, which is unlikely to introduce favorable solvation effects or to introduce an H-bond between β108Ala and β35Tyr, also exhibits a lowered oxygen affinity and good cooperativity (Table 1). Hence, the molecular basis of the low oxygen affinity found in rHbs with mutations at β108 appears to be removing the 'destabilizing' effect of asparagine in Hb A.

4. Allosteric interactions

Allosteric interactions are of central importance in biological systems [25–28]. Homotropic cooperative interactions are interactions between sites that bind the same ligand, such as in the case of Hb where the binding of one O_2 to one of the four hemes increases the affinity of the other sites towards O_2. The oxygenation of Hb is also regulated by interactions between individual amino acid residues and various solutes, such as hydrogen ions, chloride ions, inorganic phosphate, carbon dioxide, and organic polyanions, such as 2,3-bisphosphoglycerate (2,3-BPG) and IHP [1]. Such interactions are known as heterotropic allosteric interactions. The classical example of this type of interaction is the influence of pH on the oxygen-binding properties of Hb A. At pH above 6.5, the oxygen affinity of Hb A increases as the H^+ concentration decreases, known as the alkaline Bohr effect. Both homotropic and heterotropic allosteric interactions involve structural changes that are believed to mediate the allosteric effects. Determining the degree to which amino acid residues in the $\alpha_1\beta_2$ or $\alpha_1\beta_1$ interface and the central cavity of the Hb molecule can contribute to the

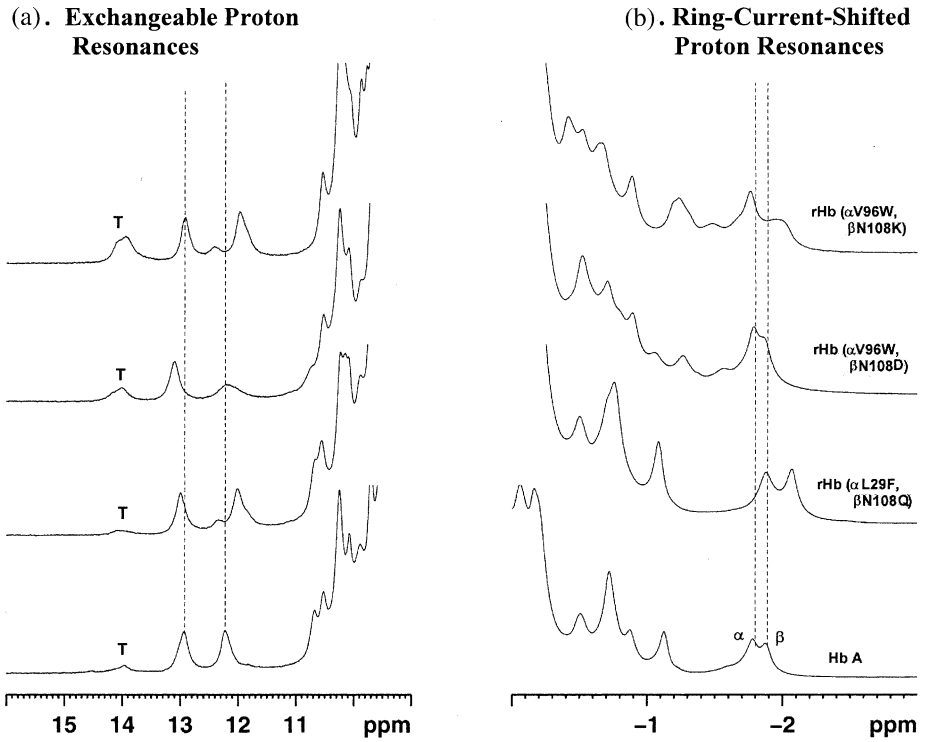

Fig. 4. 500-MHz ^{1}H-NMR spectra of 4–6% solution of Hb A, rHb (αL29F, βN108Q), rHb (αV96W, βN108D), and rHb (αV96W, βN108K) in the CO form in 0.1 M phosphate buffer in H_2O at pH 7.0 and in the presence of 2 mM IHP at 11 °C: (a) exchangeable proton resonances; (b) ring-current shifted proton resonances.

allostery in Hb would provide insights into how this protein can transmit ligand-binding information over a long distance. Previous studies have shown a marked difference of oxygen-binding properties among rHb (αV96W), rHb Presbyterian (βN108K), and rHb Yoshizuka (βN108D) in response to various heterotropic effectors, such as Cl^-, inorganic phosphate, and 2,3-BPG [11]. Table 2 shows the changes in free energy of the chloride or 2,3-BPG effect induced by mutation with either a positively charged (Lys), bulky non-polar (Trp), or a negative charged (Asp) residue in the $\alpha_1\beta_2$ or $\alpha_1\beta_1$ interface and the central cavity of the Hb molecule. rHb Yoshizuka exhibits significantly reduced chloride and 2,3-BPG effects, while rHb Presbyterian exhibits enhanced ones [11]. Amino acid substitution with a non-polar bulky residue, Trp, at α96, located in the $\alpha_1\beta_2$ interface and in the central cavity, results in a minor modification of the chloride/2,3-BPG effects. These results strongly suggest that an alteration of electrostatic interactions within the central cavity of the Hb molecule has a pronounced effect on the modulation of oxygen affinity by heterotropic effectors.

We have further investigated the sensitivity of oxygen binding to the ionic strength of the buffer (anion effect) by studying the effect of charge alterations in the central cavity of Hb molecules on the Bohr effect. As shown in Table 3, amino acid substitution at β108 with a positively charged residue i.e. Lys/Arg, results in an increased alkaline Bohr effect, while substitution with a negatively charged residue i.e. Asp/Glu, results in a decreased Bohr effect [11,16]. Thus, amino acid substitutions in the central cavity that increase the net positive charge enhance the Bohr effect. This

Table 2
Oxygen affinities of rHbs in 0.1 M HEPES and in 0.1 M HEPES plus 0.1 M chloride or 2 mM 2,3-BPG at pH 7.4 and 29 °C

	Hb A	rHb Presbyterian (βN108K)	rHb Yoshizuka (βN108D)	rHb (αV96W)
p_{50} (mmHg)				
−chloride	2.49	3.42	8.91	4.24
+chloride	6.50	13.49	12.66	9.07
+2,3-BPG	11.87	31.54	26.96	20.68
$\log[p_{50}(\text{mutant})/p_{50}(\text{Hb A})]$				
−chloride		0.14	0.55	0.23
+chloride		0.32	0.29	0.14
+2,3-BPG		0.42	0.36	0.24
$\Delta\Delta \log p_{50}$(chloride) [a]		+0.18	−0.26	−0.09
$\Delta\Delta \log p_{50}$(2,3-BPG) [a]		+0.28	−0.19	+0.01

Taken from Table 3 of Ref. [11].
[a] $\Delta\Delta \log p_{50} = \{\log[p_{50}(\text{mutant})/p_{50}(\text{Hb A})]$ (+chloride or 2,3-BPG)$\} - \{\log[p_{50}(\text{mutant})/p_{50}(\text{Hb A})]$ (−chloride or 2,3-BPG)$\} = \Delta \log p_{50}(\text{mutant}) - \Delta \log p_{50}(\text{Hb A})$. The Hb concentration was 0.1 mM (in terms of heme).

supports our model of the Bohr effect, in which the presence of anions alters the electrostatic distributions in the Hb molecule and thereby influences the microscopic mechanism of the Bohr effect [11,30–33].

It has been recognized that the $\alpha_1\beta_2$ subunit interface plays a very important role in the quaternary conformational changes of Hb upon oxygenation, and thus can affect its function. However, the role of the $\alpha_1\beta_1$ interface has often been overlooked. Though the $\alpha_1\beta_1$ subunit interface remains essentially unchanged based on a comparison of the crystal structures of Hb A in deoxy- and oxy-forms, amino acid substitutions in the $\alpha_1\beta_1$ subunit interface have been shown to affect the oxygen affinity and cooperativity of recombinant mutant hemoglobins [11,12]. Based on an analysis of the oxygen-binding curves, a substitution of Gln for Asn at β108 in the $\alpha_1\beta_1$ subunit interface has been shown to affect the oxygen affinity of the α-chain, especially in the initial stage of the oxygenation [12]. These results suggest that there is communication between the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces during the oxygenation process of the Hb molecule. This is yet another indication of multiple pathways for signal transmission in allosteric proteins [11,12,32–34].

5. Low-oxygen-affinity mutants with amino acid substitutions in both the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces or in the heme pocket region

Our working hypothesis has been further tested on double mutants with amino acid substitutions in both the $\alpha_1\beta_1$ and $\alpha_1\beta_2$ interfaces or in the heme pocket region of the α-chain, such as rHb (αV96W, βN108K), rHb (αV96W, βN108D), and rHb (αL29F, βN108Q). rHb (αV96W, βN108K), rHb (αV96W, βN108D), and rHb (αL29F, βN108Q) exhibit lower oxygen affinity than Hb A and maintain good cooperativity (Table 1) [11,12]. ^{1}H-NMR results show that the T state of these rHbs is more stable than that of Hb A (as evidenced by the appearance of the T-state marker at 14 ppm at low temperature and/or in the presence of IHP). rHb (αV96W, βN108K) is of particular interest because it exhibits the lowest oxygen affinity and the greatest tendency to switch to the T-quaternary structure when it is still ligated among all the low-oxygen-affinity rHbs studied in our laboratory (Fig. 4). Furthermore, the effect of αV96W (in the $\alpha_1\beta_2$ interface) and βN108K (in the $\alpha_1\beta_1$ interface) on the protein conformation is complementary or additive as shown by the higher intensity of the T marker of the double mutant

Table 3
Number of Bohr protons released upon oxygenation of rHbs at β108 and Hb A in 0.1 M HEPES and 0.1 M phosphate buffer at 29 °C

Hemoglobin	$\Delta \log p_{50}/\Delta$pH (per heme) in 0.1 M HEPES buffer	$\Delta \log p_{50}/\Delta$pH (per heme) in 0.1 M phosphate buffer
Hb A[a]	0.33 (pH 6.72–8.00)	0.52 (pH 6.53–8.00)
rHb (βN108Q)	0.23 (pH 7.18–8.01)[b]	0.56 (pH 6.79–8.09)[c,d]
rHb Presbyterian (βN108K)[a]	0.42 (pH 6.63–8.00)	0.62 (pH 7.00–8.01)
rHb (βN108R)	[e]	0.60 (pH 6.82–8.05)[b,d]
rHb Yoshizuka (βN108D)[a]	0.18 (pH 6.59–8.00)	0.29 (pH 6.50–8.28)
rHb (βN108E)[b]	0.13 (pH 6.80–8.12)	0.30 (pH 6.83–8.13)[d]

[a] Data taken from Table 1 of Ref. [11].
[b] Data taken from Table 4-2 of Ref. [16].
[c] Data taken from Table 1 of Ref. [12].
[d] Oxygen-binding measurements were conducted in the presence of the met-reductase system [29].
[e] Undetermined due to more than 8% of met-Hb formation during the oxygen-binding measurements.

compared to that of the single mutants [11]. Interestingly, rHb (αV96W, βN108K) still retains some cooperativity at low temperatures and in the presence of IHP i.e. $n_{max} = 1.3$, suggesting that there is considerable cooperativity in the oxygenation process of the T-state Hb in the solution [10,11]. This is in agreement with our early finding [35] and that of Ackers et al. [36].

Natural mutant Hbs with low oxygen affinity are known to exhibit an increased rate of autoxidation [1]. The oxidation rate appears to be inversely proportional to the oxygen affinity of Hbs [37]. rHb (βN108Q), however, exhibits a slower rate of autoxidation from Fe^{2+} to Fe^{3+} as compared to other low-oxygen-affinity rHbs that we have studied so far [12]. As suggested by Olson and coworkers [38,39], amino acid substitution of a bulky residue, such as Phe for Leu at the B10 position of myoglobin, can inhibit autoxidation and at the B10 position of the α-chain of hemoglobin can lower NO reaction in both the deoxy- and oxy-forms of Hb A. When we inserted the mutation αL29F into Hb A, rHb (αV96W, βN108K), and rHb (βN108Q), the autoxidation rates were decreased by 2-, 3-, and 2.8-fold, respectively [12,40]. It is noted that the formation of two sharp resonances at 23 and 37 ppm from DSS started during the first 30 min in the autoxidation process of rHb (αL29F, αV96W, βN108K) as seen in Fig. 5a. Resonances at 15 and 18 ppm started to build up together with the two resonances at 23 and 37 ppm after 3 h in the autoxidation process. The appearance of the proton resonances at 15, 18, 23 and 37 ppm from DSS in the ^{1}H-NMR spectra of met-rHb (αL29F, αV96W, βN108K) is suggestive of the formation of the anionic form of bishistidine hemichrome in rHb (αL29F, αV96W, βN108K) [40]. Hemichrome forms when met-Hb converts from the ferric high-spin form to the ferric low-spin form in which the distal imidazole displaces the H_2O ligand [41–43]. However, hemichrome-like spectra are not observed in the autoxidation process of rHb (αL29F, βN108Q) (Fig. 5b). It appears that the distal heme pocket structure of met-rHb (αL29F, βN108Q) may not be as hydrophobic as that of met-rHb (αL29F, αV96W, βN108K), and that H_2O may still be able to enter and to stay in the heme pocket of met-rHb (αL29F, βN108Q). rHb (αL29F, βN108Q) is stabilized against auto- and NO-induced oxidation as compared to rHb (βN108Q), but exhibits slightly lower oxygen affinity and good cooperativity as compared to Hb A [12].

Brunori and coworkers have selected Leu(B10) → Tyr and His(E7) → Gln as potentially relevant sites to limit the accessibility of the ligand and hence control ligand-binding parameters in the α- and β-chains of Hb A [44]. rHb ($\alpha_2^{YQ}\beta_2^{YQ}$) indeed exhibits very low oxygen affinity, reduced cooperativity, and slower NO and autoxidation reaction. These are interesting rHbs.

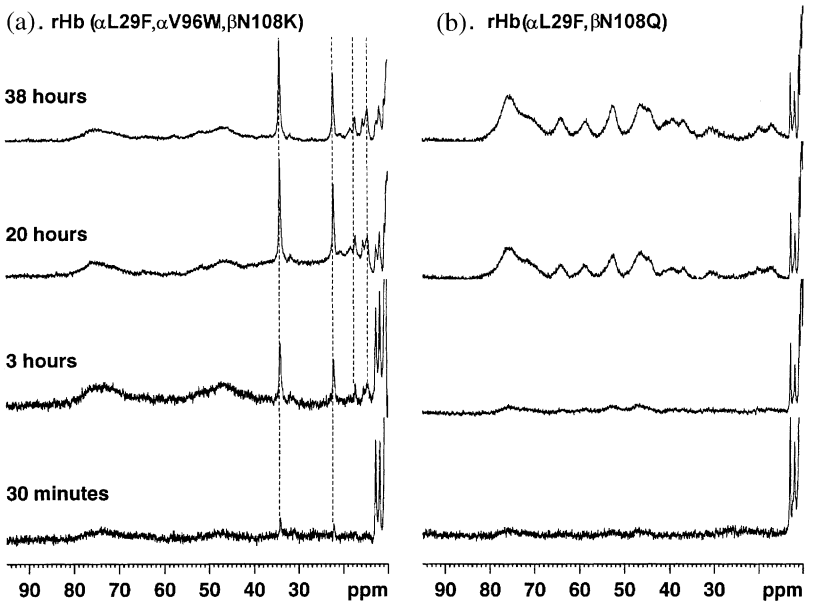

Fig. 5. 300-MHz ^{1}H-NMR studies of the effect of αL29F on the autoxidation process of oxy-rHb (αL29F, αV96W, βN108K) (a) and oxy-rHb (αL29F, βN108Q) (b) in Plasmalyte buffer at pH 7.4 and 37 °C.

6. Conclusion

Hemoglobins with low oxygen affinity and high cooperativity are a class of hemoglobins with interesting properties. The rHbs with low oxygen affinity and high cooperativity reported here appear to follow a similar mechanism in that they favor the T-quaternary structure even when they are fully ligated. Their structural and functional properties have provided new insights into the structure–function relationship of hemoglobin. Those rHbs that exhibit low oxygen affinity, high cooperativity, and stability against autoxidation are promising candidates as potential hemoglobin-based oxygen carriers.

It is most fitting at the occasion of the 65th birthday of our friend Maurizio Brunori to express our admiration as well as our friendship for him. He has made truly important contributions to the field of biophysical chemistry, especially in the study of the heme proteins and hemoglobin and myoglobin in particular. His work has made a major impact on our research on hemoglobin.

Acknowledgments

This work is supported by research grants from the National Institutes of Health (R01HL-24525 and R01HL-58249). We wish to thank Dr E. Ann Pratt for helpful discussions.

References

[1] R.E. Dickerson, I. Geis, Hemoglobin: Structure, Function, Evolution, and Pathology, Benjamin/Cummings, Menlo Park, 1983.
[2] M.F. Perutz, Stereochemistry of cooperative effects in haemoglobin, Nature 228 (1970) 726–739.
[3] J.M. Baldwin, The structure of human carbonmonoxy haemoglobin at 2.7 Å resolution, J. Mol. Biol. 136 (1980) 103–128.
[4] B. Shaanan, Structure of human oxyhaemoglobin at 2.1 Å resolution, J. Mol. Biol. 171 (1983) 31–59.
[5] G. Fermi, M.F. Perutz, B. Shaanan, R. Fourme, The crystal structure of human deoxyhaemoglobin at 1.74 Å resolution, J. Mol. Biol. 175 (1984) 159–174.
[6] H.F. Bunn, B.G. Forget, Hemoglobin: Molecular, Genetic and Clinical Aspects, WB Saunders, Philadelphia, 1986.
[7] C. Ho, Proton nuclear magnetic resonance studies on hemoglobin: cooperative interactions and partially ligat-

ed intermediates, Adv. Protein Chem. 42 (1992) 153–312.

[8] L.W.-M. Fung, C. Ho, A proton nuclear magnetic resonance study of the quaternary structure of human haemoglobins in water, Biochemistry 14 (1975) 2526–2535.

[9] H.-W. Kim, T.-J. Shen, D.P. Sun, N.T. Ho, M. Madrid, C. Ho, A novel low oxygen affinity recombinant hemoglobin (αV96W): switching quaternary structure without changing the ligation state, J. Mol. Biol. 248 (1995) 867–882.

[10] C. Ho, D.P. Sun, T.-J. Shen, N.T. Ho, M. Zou, C.-K. Hu, Z.-Y. Sun, J.A. Lukin, Present and future perspectives of blood substitutes, in: E. Tsuchida (Ed.), Recombinant Hemoglobins with Low Oxygen Affinity and High Cooperativity, Elsevier Science SA, Lausanne, Switzerland, 1998, pp. 281–296.

[11] C.-H. Tsai, T.-J. Shen, N.T. Ho, C. Ho, Effects of substitutions of lysine and aspartic acid for asparagine at β108 and of tryptophan for valine at α96 on the structural and functional properties of human normal adult hemoglobin: roles of $\alpha_1\beta_1$ and $\alpha_1\beta_2$ subunit interfaces in the cooperative oxygenation process, Biochemistry 38 (1999) 8751–8761.

[12] C.-H. Tsai, T.-Y. Fang, N.T. Ho, C. Ho, Novel recombinant hemoglobin, rHb (βN108Q), with low oxygen affinity, high cooperativity, and stability against autoxidation, Biochemistry 39 (2000) 13719–13729.

[13] T.-Y. Fang, V. Simplaceanu, C.-H. Tsai, N.T. Ho, C. Ho, An additional H-bond in the $\alpha_1\beta_2$ interface as the structural basis for the low oxygen affinity and high cooperativity of a novel recombinant hemoglobin (βL105W), Biochemistry 39 (2000) 13708–13718.

[14] T.-J. Shen, N.T. Ho, V. Simplaceanu, M. Zou, B.N. Green, M.F. Tam, C. Ho, Production of unmodified human adult hemoglobin in *Escherichia coli*, Proc. Natl. Acad. Sci. USA 90 (1993) 8108–8112.

[15] T.-J. Shen, N.T. Ho, M. Zou, D.P. Sun, P.F. Cottam, V. Simplaceanu, M.F. Tam, D.A. Bell, C. Ho, Production of human normal adult and fetal hemoglobins in *Escherichia coli*, Protein Eng. 10 (1997) 1085–1097.

[16] Tsai, C.-H., 2000. Ph.D. Thesis, Carnegie Mellon University.

[17] Y.A. Puius, M. Zou, N.T. Ho, C. Ho, S.C. Almo, Novel water-mediated hydrogen bonds as the structural basis for the low oxygen affinity of the blood substitute candidate rHb (αV96W), Biochemistry 37 (1998) 9258–9265.

[18] W.F. Moo-Penn, J.A. Wolff, G. Simon, M. Vacek, D.L. Jue, M.H. Johnson, Hemoglobin Presbyterian: β108 (G10) asparagine leads to lysine, A hemoglobin variant with low oxygen affinity, FEBS Lett. 92 (1978) 53–56.

[19] T. Imamura, S. Fujita, Y. Ohta, M. Hanada, T. Yanase, Hemoglobin Yoshizuka (G10(108)beta asparagine-asparatic acid): a new variant with a reduced oxygen affinity from a Japanese family, J. Clin. Invest. 48 (1969) 2341–2348.

[20] J.K. O'Donnell, P. Brich, C.T. Parsons, S.P. White, J. Okabe, M.J. Martin, C. Adams, K. Sundarapandiyan, B.N. Manjula, A.S. Acharya, J.S. Logan, R. Kumar, Influence of the chemical nature of side chain at β108 of hemoglobin A on the modulation of the oxygen affinity by chloride ions. Low oxygen affinity variants of human hemoglobin expressed in transgenic pigs: hemoglobins Presbyterian and Yoshizuka, J. Biol. Chem. 269 (1994) 27692–27699.

[21] T.H. Huisman, M.F.H. Carver, G.D. Efremov, A Syllabus of Human Hemoglobin Variants, The Sickle Cell Anemia Foundation, Augusta, GA, 1998.

[22] J.R. Tame, B. Vallone, The structure of deoxy human haemoglobin and the mutant Hb Tyrα42His at 120K, Acta Cryst. D56 (2000) 805–811.

[23] M.F. Colombo, D.C. Rau, V.A. Parsegian, Protein solvation in allosteric regulation: a water effect on hemoglobin, Science 256 (1992) 655–659.

[24] E.A. Brucker, Genetically crosslinked hemoglobin: a structural study, Acta Cryst. D56 (2000) 812–816.

[25] J. Monod, J. Wyman, J.P. Changeux, On the nature of allosteric transitions: a plausible model, J. Mol. Biol. 12 (1965) 88–118.

[26] D.E. Koshland, G. Némethy, D. Filmer, Comparison of experimental binding data and theoretical models in proteins containing subunits, Biochemistry 5 (1966) 365–385.

[27] J. Wyman, S.J. Gill, Binding and Linkage: Functional Chemistry of Biological Macromolecules, University Science Books, Mill Valley CA, 1990.

[28] M.F. Perutz, Mechanisms of Cooperativity and Allosteric Regulation in Proteins, Cambridge University Press, Cambridge, England, 1990.

[29] A. Hayashi, T. Suzuki, M. Shih, An enzymic reduction system for metmyoglobin and methemoglobin and its application to functional studies of oxygen carriers, Biochim. Biophys. Acta 310 (1973) 309–316.

[30] C. Ho, I.M. Russu, How much do we know about the Bohr effect of hemoglobin, Biochemistry 26 (1987) 6299–6305.

[31] M.R. Busch, J.E. Mace, N.T. Ho, C. Ho, Roles of the β146 histidyl residue in the molecular basis of the Bohr effect of hemoglobin: a proton nuclear magnetic resonance study, Biochemistry 30 (1991) 1865–1877.

[32] D.P. Sun, M. Zou, N.T. Ho, C. Ho, Contribution of surface histidyl residues in the α-chain to the Bohr effect of human normal adult hemoglobin: roles of global electrostatic effects, Biochemistry 36 (1997) 6663–6673.

[33] T.-Y. Fang, M. Zou, V. Simplaceanu, N.T. Ho, C. Ho, Assessment of roles of surface histidyl residues in the molecular basis of the Bohr effect and of β143 histidine in the binding of 2,3-bisphosphglycerate in human

normal adult hemoglobin, Biochemistry 38 (1999) 13423–13432.

[34] D. Barrick, N.T. Ho, V. Simplaceanu, F.W. Dahlquist, C. Ho, A test of the role of the proximal histidines in the Perutz model for cooperativity in haemoglobin, Nat. Struct. Biol. 4 (1997) 78–83.

[35] C. Viggiano, C. Ho, Proton nuclear magnetic resonance investigation of structural changes associated with cooperative oxygenation of human adult hemoglobin, Proc. Natl. Acad. Sci. USA 76 (1979) 3673–3677.

[36] G.K. Ackers, M.L. Doyle, D. Myers, M.A. Daugherty, Molecular code for cooperativity in hemoglobin, Science 255 (1992) 54–63.

[37] X. Ji, M. Karavitis, A. Razynska, H. Kwansa, G. Vásquez, C. Fronticelli, E. Bucci, G.L. Gilliland, α-Subunit oxidation in T-state crystals of a sebacyl cross-linked human hemoglobin with unusual autoxidation properties, Biophys. Chem. 70 (1998) 21–34.

[38] T.E. Carver, R.E. Brantley, E.W. Singleton, R.M. Arduini, M.L. Quillin, G.N. Phillips, J.S. Olson, A novel site-directed mutant of myoglobin with an unusual high O_2 affinity and low autooxidation rate, J. Biol. Chem. 267 (1992) 14443–14450.

[39] R.E. Brantley, S.J. Smerdon, A.J. Wilkinson, E.W. Singleton, J.S. Olson, The mechanism of autooxidation of myoglobin, J. Biol. Chem. 268 (1993) 6995–7010.

[40] S.T. Jeong, N.T. Ho, M.P. Hendrich, C. Ho, Recombinant hemoglobin (αL29F, αV96W, βN108K) exhibits low oxygen affinity and high cooperativity combined with resistance to autoxidation, Biochemistry 38 (1999) 13433–13442.

[41] A. Levy, P.K. Kuppusamy, J.M. Rifkind, Multiple heme pocket subconformations of methemoglobin associated with distal histidine interactions, Biochemistry 29 (1990) 9311–9316.

[42] W.E. Blumberg, J. Peisach, Low-spin compounds of heme proteins, in: R.F. Gould (Ed.), Advances in Chemistry Series 100, American Chemical Society, Washington, DC, 1971, pp. 271–291.

[43] A. Levy, V.S. Sharma, L. Zhang, J.M. Rifkind, A new model for heme–heme interactions in hemoglobin associated with distal perturbations, Biophys. J. 61 (1992) 750–755.

[44] A.E. Miele, S. Santancehe, C. Travaglini-Allocatelli, B. Vallone, M. Brunori, A. Bellelli, Modulation of ligand binding in engineered human hemoglobin distal pocket, J. Mol. Biol. 290 (1999) 515–524.