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Subunit Disassembly Pathway of Human Hemoglobin Revealing the Site-Specific Role of Its Cysteine Residues

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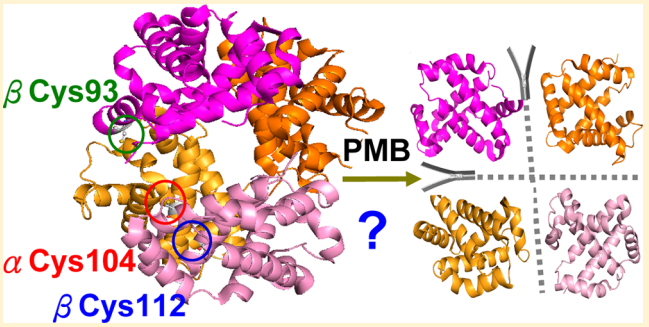
Supporting Information

ABSTRACT: Cysteine residues play a unique role in human hemoglobin (Hb) by affecting its cooperative oxygen binding behavior and the stability of its tetrameric structure. However, how these cysteine residues fulfill their biophysical functions from the molecular level is yet unclear. Here we study the subunit disassembly pathway of human hemoglobin using the sulfhydryl reagent, *p*-hydroxymercuribenzoate (PMB) and investigate the functional roles of cysteine residues in human hemoglobin. We show evidence from the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry that all three types of cysteine residues, including the surface-exposed βCys93 and the shielded αCys104 and βCys112 are reactive to PMB, resolving an issue long under debate. It is demonstrated that all three types of cysteine residues must be blocked by PMB to accomplish the subunit disassembly, and the PMB-cysteine reactions proceed in a stepwise manner with an order of βCys93, αCys104, and βCys112. The PMB reactions with the three different cysteine residues demonstrate strong site-specificity. The possible influence of PMB-cysteine reactions to the stability of various intersubunit salt bridges has been discussed based on the crystallographic structure of hemoglobin, providing insights in understanding the hemoglobin subunit disassembly pathway and the site-specific functional role of each cysteine residue in hemoglobin.



1. INTRODUCTION

Cysteine is unique among all amino acids because of the highly reactive sulfhydryl (-SH) group at its side chain. It often serves as the active site for biolabeling[1−3] and has been widely utilized as the molecular probe for conformational changes in proteins.[4−7] The significance of cysteine residues in human adult hemoglobin has been recognized previously.[6−10] Structurally, hemoglobin (Hb) of normal human adults is a heterotetramer $\alpha_2\beta_2$, comprised of two α and two β subunits arranged tetrahedrally. Because each α subunit has one cysteine residue at αCys104 and each β subunit has two cysteine residues at βCys93 and βCys112, there exist three types (and totally six) of cysteine residues in each hemoglobin molecule. The most studied among the three types of cysteine residues is βCys93 due to its several important biological and physiological functions. This cysteine residue has been found to play an active role in the allosteric transition of hemoglobin and affect the redox potential and oxygen affinity of the heme iron.[11−13] Recently, βCys93 has been suggested as the binding site for S-nitrosylation, an important process in the vascular control.[14−17] Its possible effect in inhibiting the sickle cell fiber formation has also been investigated.[18] αCys104 and βCys112, on the other hand, reside in the relatively interior portion of hemoglobin. While these two cysteine residues are often considered inaccessible for chemical reactions, they appear to exert substantial impacts in the quaternary structure of hemoglobin. Waterman[19] has performed the subunit recombination experiment and suggested that αCys104 may have to be free if the dislike subunits are to be combined stoichiometrically to form a hemoglobin tetramer. Alben and Bare have utilized αCys104 to probe the tertiary structure of α chain, the ligation state of heme$^{\alpha}$ group and its broader impact on the quaternary structure of hemoglobin.[20] The importance of βCys112 in stabilizing the $\alpha\beta$ heterodimer has also been suggested.[8] Yamaguchi et al. further explored the functionality of βCys112 by examining four β112 substituents in vitro.[21] However, despite these effects of cysteine residues in hemoglobin, a detailed molecular mechanism illustrating how cysteine residues carry such substantial impacts remains unavailable.

Here we study the hemoglobin subunit disassembly pathway, with an aim to illustrate the mechanistic origins responsible for the significance of cysteine residues in human hemoglobin. The sulfhydryl reagent, *p*-hydroxymercuribenzoate (PMB) has been used to perturb the cysteine residues, which eventually breaks hemoglobin into subunits. Owing to the high affinity between the mercury on PMB and the sulfur on the cysteine residue, the sulfhydryl (-SH) group of cysteine residues is blocked, resulting into a heavy and bulky tail of -S-Hg-C_6H_4-COO^-, as illustrated in Scheme 1.

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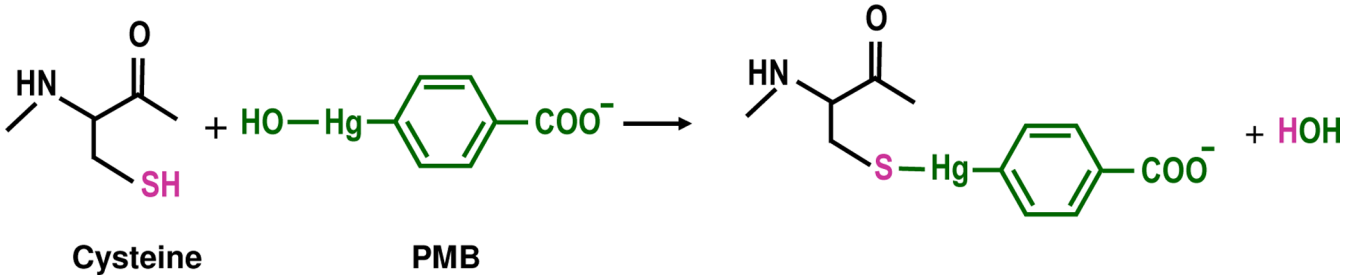
Scheme 1. PMB-Cysteine Reaction Scheme



PMB has been generally used to prepare isolated α and β globins since its introduction by Bucci and Frontcelli.[22] This procedure allows ones to characterize the properties of the two dissimilar subunits separately and makes the study of recombinant hemoglobin (rHb) possible.[23−26] For instance, Mizutani and co-workers have recently adapted this procedure to probe the protein dynamics of isolated subunits via the time-resolved resonance Raman spectroscopy.[23] In that work, the authors demonstrated the spectral changes of isolated α subunits occur faster than those of isolated β globins and pointed out the crucial role of intersubunit interactions in affecting the structural change rate in the heme pocket.

Despite this common usage of PMB, however, little is known about how the PMB reaction with cysteine leads to the subunit disassembly of hemoglobin. Issues such as how many cysteine residues are reactive to PMB and whether they are all necessarily involved in the disassembly process are unsettled. Among these cysteine residues, only βCys93 is generally accepted as the active site, whereas the reactivity of αCys104 and βCys112 to PMB remains an open issue. Rosemeyer et al.[27] and Chiancone et al.[28] have suggested that both αCys104 and βCys112 need to react with PMB to fully dissociate hemoglobin; however, Ioppolo et al.[29] have considered βCys112 not essential to react with PMB to break hemoglobin apart. Attempts have been made by Mawjood et al.[30] more recently to clarify the number and position where PMB is attached on hemoglobin using the electrospray ionization mass spectrometry. However, no signs of PMB-bound globins were observed from that work due to an acid-acetone treatment adapted which removed the bound PMB from globins. Latest efforts were made by Lu et al.[31] who reinvestigated the number of reactive cysteine residues to PMB using the nanoelectrospray ionization quadrupole time-of-flight mass spectrometry. The authors from that work suggested that only the surface-exposed βCys93 is accessible to PMB, whereas the shielded cysteine residues are nonreactive to PMB unless other denaturants, such as urea, is added. However, this result is inconsistent with the previous studies.[27,28] Moreover, Knee et al.[18] recently utilized *N*-ethylmaleimide to probe the role of βCys93 in antisickling and found out that the chemical modification occurs not only at βCys93, but also moderately at αCys104. This result hints that βCys93 is not the only reactive cysteine residue to PMB. Nevertheless, considering that there exist totally five subunit−subunit interfaces, α_1/β_1, α_1/β_2, α_2/β_1, α_2/β_2, and α_1/α_2 (there is no β_1/β_2 intersubunit contact) in each hemoglobin with strictly specific spatial arrangements at the quaternary level, it is certainly remarkable that such a complex structure can be completely disassembled simply by attacking its cysteine residues. This fact clearly speaks for the profound functional roles of cysteine residues in hemoglobin.

Here we first clarify the number of cysteine residues reactive to PMB and their reaction sequence via the matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry. We then demonstrate that the three types of cysteine residues must all react with PMB if a complete subunit disassembly is to be achieved, and each PMB-cysteine reaction is site-specific with distinct binding strength. By evaluating the intersubunit interactions and their possible correlations with the cysteine residue through the delicate salt bridge network from the crystallographic structure of hemoglobin, a possible subunit disassembly pathway and the site-specific functional role of each cysteine residue in hemoglobin are suggested.

2. EXPERIMENTAL SECTION

2.1. Materials. Hemoglobin was prepared from the freshly drawn human blood.[32] The erythrocytes after separated from plasma were washed four times with 0.9% NaCl solution. The packed erythrocytes were mixed with one volume of water and dialyzed overnight at 4 °C. Purified hemoglobin solution was obtained by centrifuging the dialyzed hemoglobin solution at 18000 × g for 20 min to remove any particulate matters. The concentration of hemoglobin was 0.042 mM, as determined spectrophotometrically at 576 nm with an extinction coefficient of 10.07 L/mmol·cm.[33] PMB was purchased from Sigma-Aldrich in the form of *p*-hydroxymercuribenzoic acid sodium salts. The PMB solution was prepared by dissolving 50 mg PMB in 10 mL of 0.1 M KOH and back-titrated with 1 M acetic acid until a very slight precipitate remained.[38] To track the PMB reaction evolution with hemoglobin, the PMB-to-Hb molar ratio was adjusted by mixing 1 mL of 0.042 mM purified Hb with varying amounts of PMB solution, ranging between 2 and 600 μL.

The subunit isolation and -SH regeneration closely follows the procedures by Yip et al.[34] The PMB-Hb mixture was first centrifuged at 12000 × g for 10 min to remove any extra PMB sediments. A half portion of this PMB-Hb solution was then purified and buffer-exchanged via the gel filtration by passing through a P6 (Bio-Rad) column equilibrated with 50 mM Tris·HCl buffer at pH 8.4. Isolated α globins were obtained by passing this PMB-treated Hb solution through a DEAE-MacroPrep (Bio-Rad) column equilibrated with the same buffer. The other half portion of PMB-treated hemoglobin solution was passed through another P6 column equilibrated with 20 mM potassium phosphate at pH 6.4. Isolated β globins were obtained by passing the eluent through a CM-MacroPrep (Bio-Rad) column equilibrated with the same buffer. To regenerate the -SH groups on the PMB-bound isolated globins, the isolated globins were passed through a P6 column equilibrated with 0.1 M potassium phosphate at pH 7.4, containing 25−50 mM 2-mercaptoethanol (Sigma-Aldrich). Excess mercaptoethanol was removed by immediately passing

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the regenerated subunits through a P6 column equilibrated with 0.1 M potassium phosphate at pH 7.4.

2.2. MALDI-TOF Mass Spectrometry Measurements. All mass spectra were obtained via the MALDI-TOF MS system (Autoflex 1, Bruker Daltonics Inc., Germany). While several matrix materials have been tested, including sinapinic acid (SA, Sigma-Aldrich), 4-hydroxy-α-cyanocinnamic acid (α-CHC, Sigma-Aldrich) and 2,5-dihydroxy benzoic acid (2,5-DHB, Sigma-Aldrich), SA appears to give the most satisfactory resolution. Therefore, all mass spectra presented here were obtained using SA as the matrix material. For each measurement, the sample of interest was well mixed with the matrix material and deposited onto a conductive sample support, which was then placed into the desiccator to eliminate water before the MALDI-TOF mass spectrometric measurements. The desorption was triggered by a 50 Hz, 337 nm N_2 laser, which vaporized the sample and matrix and lead to the ionization of the analyte molecules during the process. The ionized molecules were steered and accelerated by a set of ion optics and were allowed to drift down to a flight tube, where they were separated according to their mass-to-charge ratio. The laser power was kept at ~85 μJ/pulse during all MALDI-TOF mass spectrometric measurements. The ion extraction delay and other relevant parameters were optimized to achieve the optimal resolutions.

3. RESULTS AND DISCUSSION

3.1. All Three Types of Cysteine Residues Are Reactive to PMB. To clarify the existing debate of whether αCys104 and βCys112 are reactive to PMB besides βCys93, we first examine the reactivity of hemoglobin to PMB upon the PMB-treatment. Before mixing with any PMB, the MALDI-TOF mass spectrum of the purified hemoglobin (Figure 1a) shows two intense features at 15126 and 15867 Da which represent the typical α and β globins of human hemoglobin. A number of minor features are also observed at 15288, 15742, 16029, and 16483 Da, which can be assigned to glycated-α globin (α_g), heme-bound α globin (α_h), glycated-β globin (β_g), and heme-bound β globin (β_h), respectively.[35]

After treating with an excess amount of PMB, two new intense features appear at 15446 and 16507 Da at the expense of the native α and β globins (Figure 1b). The 15446 Da feature is 320 Da more than α globin. Because this mass shift matches exactly the mass difference when the sulfhydryl group is modified by PMB, this feature can be viewed as the direct signature of α globin bound with PMB (α^{PMB}) via its only cysteine residue, αCys104.

On the other hand, the feature of 16507 Da is 640 Da more than the native β globin, corresponding exactly twice the mass increase when PMB-cysteine reaction occurs. Therefore, this feature can only be ascribed to two PMB molecules bound with β globin (β^{2PMB}). Because each β globin has two -SH binding sites at βCys93 and βCys112, respectively, this β^{2PMB} feature thus offers the unambiguous evidence that both cysteine residues on β globin are reactive to PMB. Together with the α^{PMB} feature at 15446 Da, the two new intense PMB-labeled features provide the explicit evidence that βCys93 is not the only reactive cysteine residue of human hemoglobin. Instead, all three types of cysteine residues are reactive to PMB, with no need to add any other extra denaturants. This result not only puts the long-existing debate to an end, but also embarks the beginning in identifying the site-specific roles of cysteine residues in dissembling human hemoglobin into subunits.

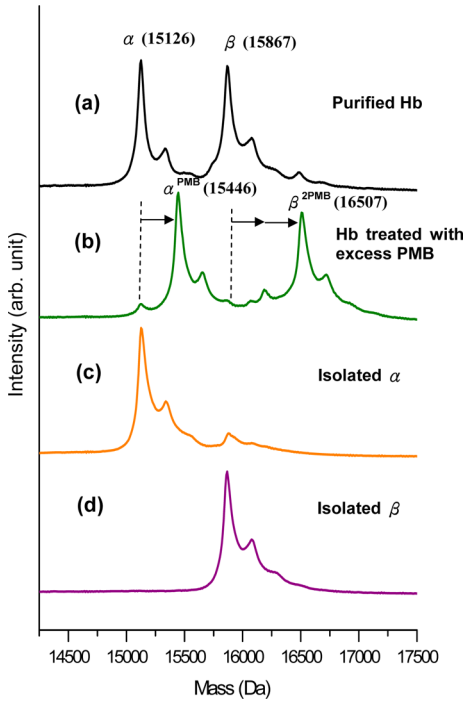

Figure 1. Reactive cysteine residues upon PMB treatment and the subunit isolation efficiency. MALDI-TOF mass spectrum of (a) purified hemoglobin. Major peaks at 15126 and 15867 Da correspond to α and β globins; (b) Hemoglobin treated with excess PMB. Major peaks at 15446 and 16507 Da correspond to α^{PMB} and β^{2PMB}, providing the direct evidence that all three types of cysteine are reactive to PMB; (c) Isolated and regenerated α-SH globin; (d) Isolated and regenerated β-SH globin.

The solution of Hb mixed with excess PMB was allowed to stand at 4 °C for several hours to disassemble hemoglobin into subunits. Isolations of dissimilar subunits are subsequently performed via the ion-exchange chromatography, followed by the sulfhydryl (-SH) functional group regeneration. To ensure the capability and the effectiveness of PMB in disassembling hemoglobin, which serves as a critical reference for later investigations, we have obtained the MALDI-TOF mass spectra for the isolated α globins (Figure 1c) and β globins (Figure 1d). These two spectra explicitly show that dislike subunits are well separated after the PMB-treatment and the desired type of globins can be readily collected after the ion-exchange chromatography with the other type of globins efficiently removed. The sulfhydryl groups on α and β globins are both regenerated successfully, as evidenced from the mass peak position. The major mass feature of the isolated α globin returns back to its nascent mass at 15126 Da, and that of isolated β globins also shifts back to its original value of 15867 Da.

To eliminate the potential effect of PMB on the stability of the hemoglobin complex, that is to rule out the possibility that PMB may simply act as a denaturant which dissociates the hemoglobin complex and exposes the buried cysteine residues for reactions, we have performed a set of control experiments by studying the stability of hemoglobin when it is treated with varying amounts of *p*-hydroxybenzoate (PB), where the Hg atom is absent. The excessively PB-treated hemoglobin was let go through the same subunit isolation procedures as that performed on the PMB-treated hemoglobin. The result shows that the hemoglobin complex remains stable after treated with

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PB and the subunit cannot be separated (see Supporting Information). Therefore, the possibility that buried cysteine residues react with PMB only after hemoglobin has been denatured can be excluded. It further proves that cysteine residues are indeed essential and operative in dissociating the hemoglobin complex

3.2. PMB Reacts with the Different Types of Cysteine Residues via a Stepwise Manner. It is instructive to learn whether PMB reacts with the three different types of cysteine residues consecutively, or in parallel. To reveal the PMB reaction evolution in detail, we have systematically regulated the amount of PMB mixed with hemoglobin, characterized by the PMB-to-Hb molar ratios. Figure 2a–f shows the MALDI-TOF mass spectra of PMB-modified hemoglobin with an increasing PMB-to-Hb molar ratio ranging from 0.29, 0.58, 0.87, 2.61, 6.98 to 10.17, respectively.

At low PMB-to-Hb molar ratios, such as that shown in Figure 2a (PMB-to-Hb ratio of 0.29), the obtained MALDI-TOF mass spectrum closely resembles that of the purified hemoglobin. No obvious PMB-induced effect can be observed until a PMB-to-Hb molar ratio of 0.58 is reached (Figure 2b). From Figure 2b, it appears that PMB attack one of the cysteine residues on β globin first, as evidenced from the onset of the feature of 16187 Da, which corresponds to β globin bound with one PMB molecule (β^{PMB}).

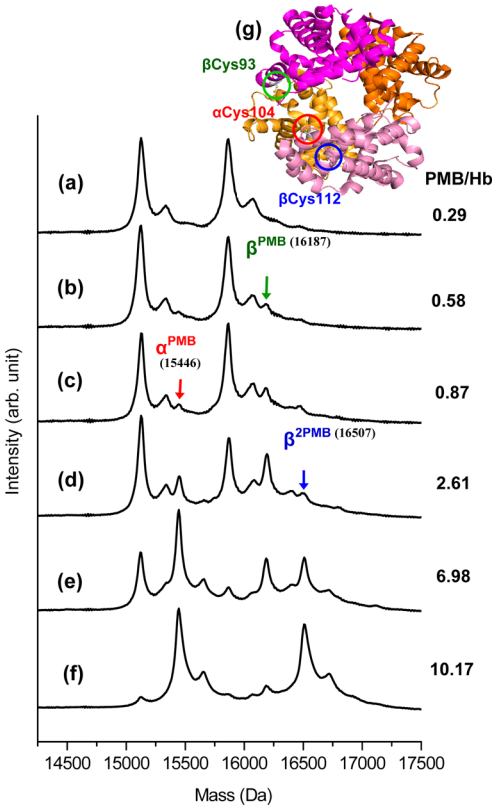

Figure 2. PMB reacts with different cysteine residues in a stepwise manner. (a–f) MALDI-TOF mass spectra of PMB-modified hemoglobin with an increasing PMB-to-Hb ratio from 0.29, 0.58, 0.87, 2.61, 6.98 to 10.17, respectively. (a) Hb mixed with minimal PMB, where no obvious PMB-induced effect is yet seen; (b) Onset of β^{PMB} feature at 16187 Da (green arrow) indicating the binding of PMB to βCys93; (c) Onset of α^{PMB} feature at 15446 Da (red arrow) representing the binding of PMB to αCys104; (d) Onset of β^{2PMB} feature at 16507 Da (blue arrow) indicating that PMB begin to bind to βCys112 besides βCys93; (e) PMB-bound globins become dominant over the nascent globins; (f) Hb mixed with an excess amount of PMB where most cysteine residues are bound with PMB; (g) Hemoglobin structure (PDB code: 2DN2[36]) with its three types of cysteine sites highlighted in the colored circles: green, βCys93; red, αCys104; and blue, βCys112.

As the PMB-to-Hb ratio continues to rise, the second PMB binding site reveals. The onset of α^{PMB} at 15446 Da (Figure 2c, PMB-to-Hb ratio of 0.87) provides the firm evidence that PMB begins to react with α globin via its only cysteine residue, αCys104. Increasing the PMB-to-Hb ratio further, the β^{2PMB} feature at 16507 Da begins to emerge in addition to the continuously rising peaks of β^{PMB} and α^{PMB} (Figure 2d, PMB-to-Hb ratio of 2.61). With the continuously rising PMB-to-Hb ratio, the PMB-bound globins gradually become dominant over the native globins (Figure 2e, PMB-to-Hb ratio of 6.98). Eventually, nearly all cysteine residues become bound with PMB (Figure 2f, PMB-to-Hb ratio of 10.17).

Although the binding site for the PMB bound α subunit can be unambiguously ascribed to αCys104, it is not immediately clear whether the singly-PMB bound β subunit is originated from that modified at βCys93 or βCys112, or even a mixture of both species. However, insights can be extracted by considering the intrinsic structural characteristics of hemoglobin tetramer and the properties of the two β cysteine residues.

βCys112 is well shielded inside the protein (Figure 2g) and is nonreactive.[31] In order to become reactive, βCys112 must first be exposed and become accessible for PMB. As we have ruled out the possibility that PMB may simply denature the protein from the control experiment on the PB-treated hemoglobin (section 3.1 and Supporting Information), this excludes the possibility that the denatured hemoglobin exposes βCys112 for PMB reaction. Therefore, the βCys112-PMB reaction can only take place after some prior PMB modifications that already occur and expose the βCys112 to PMB.

Because the first PMB-bound feature appears at the β subunit, the singly-PMB bound β subunit thus cannot be that bound at βCys112. The possibility that the singly-PMB bound β subunit is at βCys112, or a mixture of that modified both at βCys93 and βCys112 can be excluded. Also, considering the relative location of the two β-cysteine residues, βCys93 resides at a position much closer to the hemoglobin surface than βCys112 (Figure 2g). It is energetically unfavorable for PMB to bypass βCys93 to react with the more buried βCys112. As a result, the first PMB reacting site should be ascribed to βCys93, and PMB reacts with the three distinct types of cysteine residues in a stepwise manner, with a specific order of βCys93, αCys104, and βCys112.

3.3. PMB-Cysteine Bindings Demonstrate Strong Site Dependence. To extract more insights regarding the PMB-cysteine reactions from the resulting MALDI-TOF mass spectra, we have examined the degrees of PMB-modification at the three different cysteine sites in terms of the PMB fractional saturation ratios as a function of the free PMB concentration, as shown in Figure 3.

The PMB fractional saturation for each cysteine residue can be defined as

$$\theta_{\beta Cys93} = \frac{\beta Cys93^{PMB}}{\beta Cys93 + \beta Cys93^{PMB}}$$

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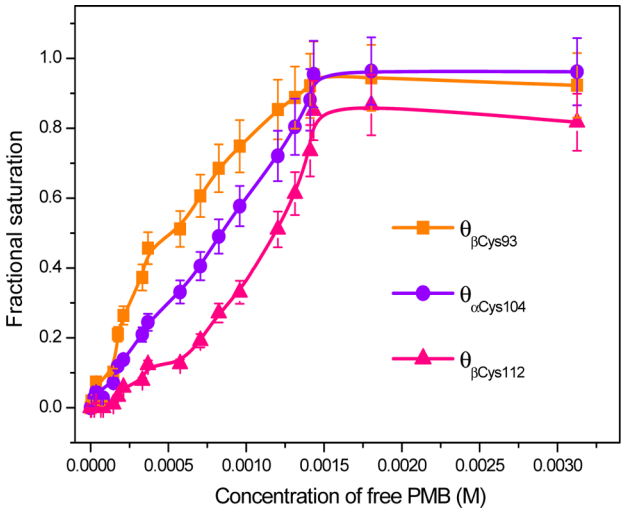

Figure 3. Fractional PMB saturation ratio of the three cysteine sites as a function of the free PMB concentration: −■−, $\theta_{\beta Cys93}$; −●−, $\theta_{\alpha Cys104}$; and −▲−, $\theta_{\beta Cys112}$. The three curves demonstrate strong site-dependence for the PMB-cysteine reactions.

$$\theta_{\alpha Cys104} = \frac{\alpha Cys104^{PMB}}{\alpha Cys104 + \alpha Cys104^{PMB}}$$

$$\theta_{\beta Cys112} = \frac{\beta Cys112^{PMB}}{\beta Cys112 + \beta Cys112^{PMB}}$$

While all three cysteine residues eventually saturate, the PMB-modification proceeds markedly different with the three different cysteine residues, demonstrating a strongly site-dependent behavior. βCys93 rapidly becomes bound with PMB at low free PMB concentrations, as manifested from the steep rise of the saturation ratio of $\theta_{\beta Cys93}$, while $\theta_{\beta Cys112}$ grows faster at the later stage. The saturation behavior of αCys104 appears to be the intermediate between the two β-cysteines residues.

For each PMB-cysteine binding there exists an association constant K_a. Or the more conventionally used is the dissociation constant K_d when the reverse reaction direction is considered. We may thus describe the PMB-cysteine reactions and their dissociation constants as

$$\beta Cys93^{PMB} \rightarrow \beta Cys93 + PMB \quad K_{d1} = \frac{\beta Cys93 \cdot PMB}{\beta Cys93^{PMB}}$$

$$\alpha Cys104^{PMB} \rightarrow \alpha Cys104 + PMB \quad K_{d2} = \frac{\alpha Cys104 \cdot PMB}{\alpha Cys104^{PMB}}$$

$$\beta Cys112^{PMB} \rightarrow \beta Cys112 + PMB \quad K_{d3} = \frac{\beta Cys112 \cdot PMB}{\beta Cys112^{PMB}}$$

The fractional saturation ratio of βCys93 can be rewritten as

$$\theta_{\beta Cys93} = \frac{\beta Cys93^{PMB}}{\beta Cys93 + \beta Cys93^{PMB}} = \left[\left(\frac{\beta Cys93 \cdot PMB}{K_{d1}}\right) \Big/ \left(\beta Cys93 + \frac{\beta Cys93 \cdot PMB}{K_{d1}}\right)\right]$$

and thus

$$\theta_{\beta Cys93} = \frac{PMB}{K_{d1} + PMB}$$

At the point where half of βCys93 are bound with PMB,

$$\theta_{\beta Cys93} = \frac{1}{2} = \frac{PMB}{K_{d1} + PMB}$$

and K_{d1} = PMB.

The dissociation constant K_{d1} of 4.2×10^{-4} can thus be obtained as the value of K_{d1} equals the free PMB concentration at the half-saturated point of βCys93. Similarly, the dissociation constants K_{d2} and K_{d3} can also be obtained to be 8.6×10^{-4} and 1.3×10^{-3}, respectively. Because a lower dissociation constant value means tighter binding, it readily indicates that the binding strength between PMB and the three cysteine residues decreases progressively as the PMB reaction proceeds, with the binding strength between PMB and βCys93 being the strongest and that between PMB and βCys112 the weakest. The free energy change associated with PMB binding to βCys93, αCys104, and βCys112 can also be derived to be −19.2, −17.5, and −16.4 kJ ± 10%, respectively, according to $\Delta G = -RT \ln K_a = RT \ln K_d$.

3.4. All Cysteine Residues Must React with PMB To Achieve Complete Subunit Disassembly. To clarify the necessities of the three PMB-cysteine modifications in accomplishing the subunit disassembly, the subunit isolation efficiency for incompletely PMB-treated hemoglobin has been examined. We demonstrate here the condition where only 33% of α globins are bound with PMB, while only about 23% of β globins have both their -SH groups bound with PMB (Figure 4a). This partially PMB-treated hemoglobin was let go through the same subunit isolation procedure. It was found that under such a condition the dislike globins cannot be separated

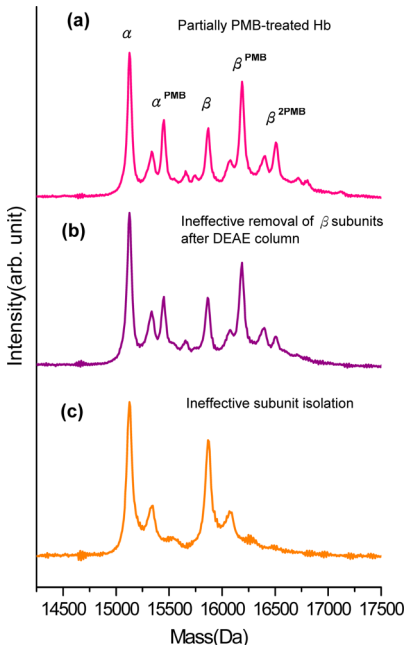

Figure 4. Incomplete PMB-Hb reaction vs uneffective subunit isolation. MALDI-TOF mass spectrum of (a) partially PMB-treated Hb; (b) uneffective subunit isolation resulted from the above condition, manifested from the incomplete removal of unwanted β globins when collecting α globins; (c) after -SH regeneration, α and β globins cannot be separated cleanly.

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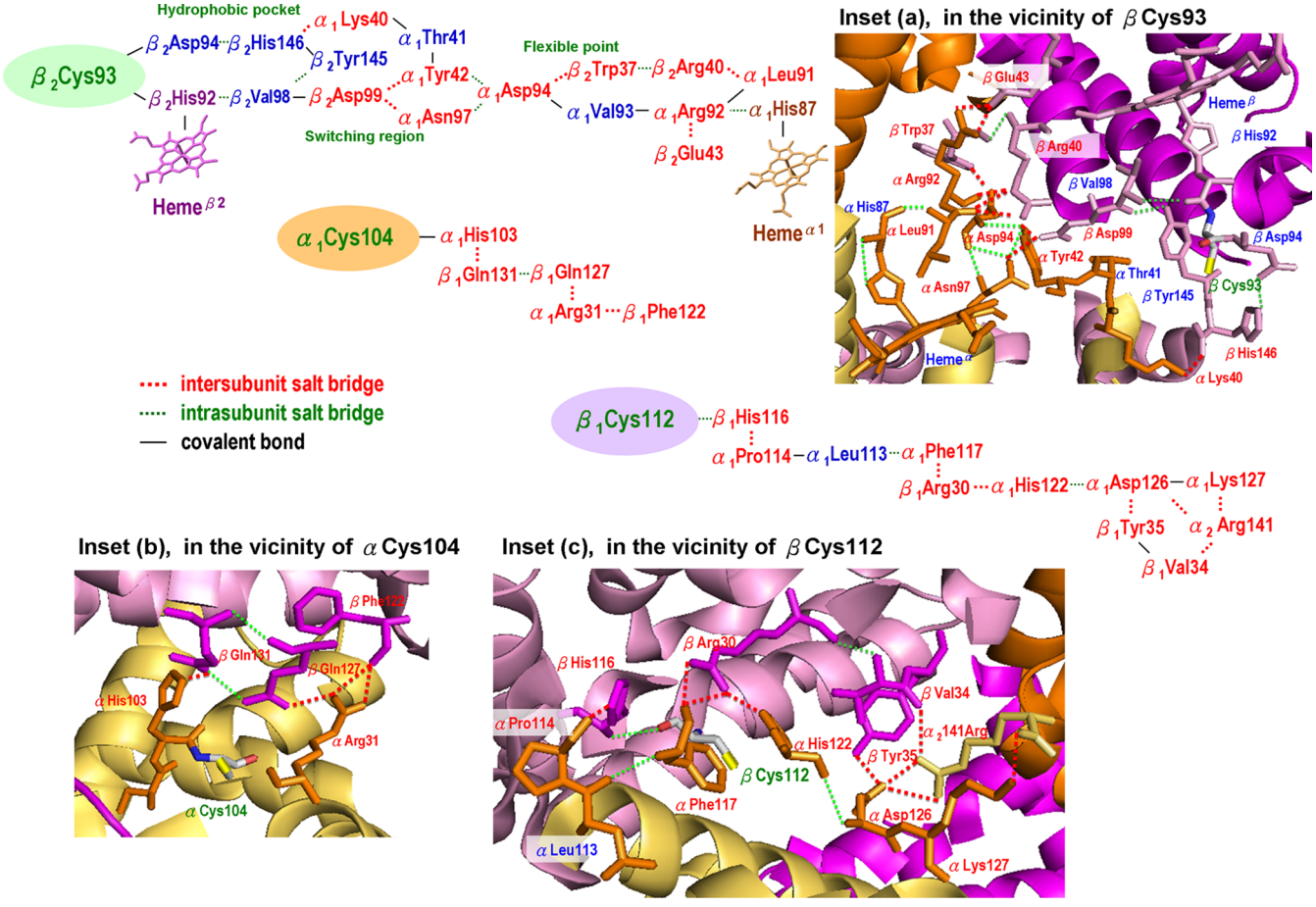
Chart 1. Correlations between Each Cysteine Residue and the Various Intersubunit Salt Bridges of Hemoglobin According to the Crystallographic Coordinate of Deoxy-Hemoglobin (PDB: 2DN2)[36a]



[a]To view the delicate intermolecular interaction network with clarity, all intersubunit salt bridges are designated as red dash lines, while all intrasubunit salt bridges are designated as green dash lines. The covalent bonds are denoted as black solid lines. Notation color on residues: Cysteine residues: green, critical residues involved in the intersubunit salt bridges: red; other relevant residues: blue. The crystallographic images illustrating the vicinity of interacting cysteine residues, βCys93, αCys104 and βCys112 are shown in inset (a), (b) and (c) accordingly, with the intersubunit salt bridges designated as the red dash lines and the intrasubunit salt bridges as green dash lines.

satisfactorily at all (Figure 4b). The β-associated components were to be removed after eluting through the DEAE-MacroPrep column when collecting the isolated α^{PMB} globins. However, the mass spectrum of the collected eluent shows that most β-associates came out together with α^{PMB} globins with nearly no loss (Figure 4b), except β^{2PMB} whose intensity was markedly reduced. Specifically, the fractions of the unbound β subunit and that eluted out after the DEAE column are nearly the same, both around 0.3 ± 10%, suggesting that nearly all unbound β subunits come out together with the α subunit. Similarly, the fractions of the singly-PMB bound β subunit and that eluted through the DEAE column are both around 0.5 ± 10%. In contrast, the fraction of the doubly-PMB bound β subunit is 0.2 ± 10%, while that passed through the DEAE column is only 0.1 ± 10%. In comparison with the other two β subunit species, including the unbound β subunit and the singly-PMB bound β subunit, the doubly-PMB bound β subunit is eliminated by the DEAE column much more effectively, suggesting that the doubly-PMB bound β subunit is more detached from the α subunit. After the -SH regeneration, the amount of β subunits remains significant (Figure 4c), indicating that the subunit isolation is inefficient for the partially PMB-treated Hb.

Nevertheless, it is intriguing to tackle why β^{2PMB} was the only relatively effectively filtered out species among all β-associated components. Considering that it is only when the substances carry the correct polarity can they pass through the ion-exchange resin, β globins should in principle be trapped in the anion-exchange column if isolated properly. The only possibility to interpret such ineffective subunit isolation is that β and β^{PMB} are not yet completely detached from α and α^{PMB} globins and were therefore eluted out together with α^{PMB}. The relatively effective removal of β^{2PMB} globins and the nearly unchanged β and β^{PMB} globins peaks (Figure 4b) therefore suggest that the second cysteine residue of β subunit needs to react with PMB to achieve the complete subunit disassembly. Because βCys112 is also the last cysteine residue to react with PMB (section 3.2), this result implies that all cysteine residues must react with PMB to completely disassemble hemoglobin into subunits.

3.5. Possible Hb Subunit Disassembly Pathway via Disruption of Intersubunit Contacts. Because hemoglobin is broken down into subunits, this indicates all intersubunit contacts are eliminated. The significance of intersubunit salt bridges in regulating the allostery and functions in proteins[37−40] and specifically in hemoglobin has been addressed.[41−43] In the

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case of hemoglobin, intersubunit interactions are substantial not only in sustaining the quaternary structure of hemoglobin, but also in regulating its allostery and heme−heme communication. To learn how subunits can be disintegrated from the tetramer upon the PMB modifications, it is instructive to investigate how PMB-cysteine reactions can possibly affect the intermolecular interactions, particularly those along/near the subunit interface. We investigate the intersubunit salt bridges of hemoglobin at each subunit interface from the crystallographic structure of hemoglobin[36,44] (see Supporting Information, 2). As each PMB-cysteine modification can be considered as a structural perturbation, the most vulnerable residues to experience this perturbation are most likely its adjacent residues and the residue(s) it associates with via some intermolecular interactions, including hydrogen bonds and electrostatic interactions. As the stability of a salt bridge is regulated by its geometry, minor structural modification may cause a profound impact to destroy the salt bridges in its vicinity.[45] We thus tentatively map out the correlations between each specific cysteine residue and the intersubunit salt bridges in hemoglobin by analyzing its crystallographic structure (PDB code: 2DN2),[36,44] shown in Chart 1.

From discussions in section 3.2, βCys93 is ascribed as the first reacting cysteine residue to PMB. Being closest to the hemoglobin surface, βCys93 is also the most accessible cysteine residue for PMB to encounter and react with. βCys93 resides at a special location. Its immediate neighboring residue, βAsp94, forms a crucial salt bridge with βHis146, the C-terminus of β globin. The βAsp94···βHis146 salt bridge is substantial, accounting for nearly 40% of the Bohr effect.[46] The possible impact of modified βCys93 on the βAsp94···βHis146 salt bridge has been addressed by Fronticelli and co-workers who have specifically pointed out the structural role of βCys93 in regulating this important salt bridge.[6] Once βCys93 is modified by PMB, the bulky of PMB may inevitably rupture βAsp94···βHis146 and break the hydrophobic pocket sustained by it. The loss of βAsp94···βHis146 may alter the local environment of βHis146, destabilizing the intersubunit βHis146···αLys40 salt bridge at the α_1/β_2 (and α_2/β_1) interface. βCys93 is also adjacent to the heme-binding histidine, βHis92, which coordinates β globin to the heme functional group. The PMB-modification at βCys93 may transmit to βHis92 and, through an internal βHis92···βVal98 salt bridge, further destabilize several α_1/β_2 intersubunit salt bridges, including βAsp99···αTyr42, βAsp99···αAsn97, αAsp94···βTrp37, βArg40···αLeu91, and αArg92···βGlu43. It is intriguing to note that these intersubunit salt bridges are all cross-linked with one another, either through an intrasubunit salt bridge (green dash line in Chart 1) or via an influenced residue (black solid line in Chart 1). A crystallographic image illustrating the intimate salt bridge network in the vicinity of βCys93 is shown as the inset (a) in Chart 1. As a result of the α_1/β_2 interface destabilization, it is likely that hemoglobin is disassembled into a pair of $\alpha\beta$ heterodimers upon the PMB modification at βCys93.

αCys104 situates in a vicinity of intense α_1/β_1 intersubunit salt bridges. Once αCys104 is perturbed by PMB, it may impact a number of α_1/β_1 intersubunit salt bridges that are associated with αCys104. The most vulnerable residue may be its immediate neighbor residue, αHis103 and the intersubunit salt bridge formed by it, αHis103···βThr131. Propagated through two intrasubunit βThr131···βGln127 salt bridges, three other α_1/β_1 intersubunit salt bridges, including βGln127···αArg31 and two αArg31···βPhe122 may also be affected. A crystallographic image illustrating the salt bridge network in the vicinity of αCys104 is shown in the inset (b) of Chart 1. αCys104 has been reported to be significant in stabilizing hemoglobin.[47] It has been pointed out that modification at αCys104 impairs the cooperative oxygen binding capability of hemoglobin, likely by eliminating the contribution of α_1/β_1 interfacial contacts to the cooperativity.[47] From Chart 1, αCys104 appears to be a key in sustaining nearly half of the α_1/β_1 (and α_2/β_2) intersubunit salt bridges. Upon blocking the -SH group of αCys104, these α_1/β_1 interfacial contacts will likely be lost.

βCys112 also resides near the α_1/β_1 interface. Adachi et al.[8] have pointed out that βCys112 is important for the formation of stable $\alpha\beta$ heterodimers. βCys112 is internally salt-bridged with βHis116, as shown in the inset (c) of Chart 1, which shows a crystallographic image in the vicinity of βCys112 and the relevant salt bridges in this region. As βHis116 forms an important intersubunit salt bridge with αPro114,[48] once βCys112 is modified by PMB, the perturbation on βCys112 may propagate to disrupt βHis116···αPro114. Through αPro114···αPhe117, it may affect the rest of α_1/β_1 interfacial contacts, including two αPhe117···βArg30, and one βArg30···αHis122. Through another internal salt bridge, αHis122···αAsp126, the only remaining α_1/β_1 intersubunit salt bridge, αAsp126···βTyr35 may also be lost. αAsp126 and its adjacent residue, α_1Lys127, constitute the only two α_1/α_2 intersubunit contacts with the opposite α globin via α_2Arg141. The α_1Asp126···α_2Arg141 and α_1Lys127···α_2Arg141 intersubunit salt bridges, and thus, the α_1/α_2 interface may also be eliminated when αAsp126 experience perturbation. From this salt bridge network associated with βCys112, it reveals a possible mechanism for one to comprehend why the doubly-PMB bound β subunit is more detached from the α subunit and thus more efficiently eliminated by the DEAE column, as shown in Figure 4.

To this point, all intersubunit contacts at each interface are likely disrupted upon the structural perturbation originated from the PMB-cysteine modifications. The tetrameric hemoglobin could have now been fully disassembled into individual α and β monomers (Figure 1c,d), completing the subunit disassembly process.

3.6. Site-Specific Biophysical Function of Each Cysteine Residue in Hemoglobin. By investigating the subunit disassembly pathway of hemoglobin, it reveals that the -SH groups of all three types of cysteine residues must be blocked by PMB to accomplish the hemoglobin subunit disassembly process, and the entire hemoglobin disassembly is achieved by the stepwise and hierarchical blocking of all six sulfhydryl groups at the three different types of cysteine residues. By further analyzing the intercorrelations between each cysteine residue and the various intersubunit interactions based on the crystallographic structure of hemoglobin, it appears that each cysteine residue exhibits a unique functional role in the entire hemoglobin. βCys93 situates at a critical position by neighboring with βAsp94, an important amino acid residue responsible for the Bohr effect and the heme-binding proximal histidine, βHis92. It appears to be critical in sustaining the intersubunit interactions at the α_1/β_2 (and α_2/β_1) interface via the salt bridge network. We also note that a possible heme$^{\beta 2}$−heme$^{\alpha 1}$ communication pathway is manifested via the intermolecular interaction network, as seen in Chart 1. Within this pathway, the βAsp99···αTyr42 and αAsp94···βTrp37 intersubunit salt bridges have already been identified as the

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critical salt bridges in the allosteric control. The βAsp99···α-Tyr42 intersubunit salt bridge has been termed "the switching region", and the αAsp94···βTrp37 intersubunit salt bridge has been called "the flexible point".[49] The two salt bridges have been studied in detail by vibrational spectroscopic methods.[26,43,50−52] From the close association of βCys93 with this potential heme−heme signaling pathway, one may better appreciate the origin why βCys93 exhibits such tremendous impacts in affecting the heme activity and the cooperative oxygen binding capacity. On the other hand, αCys104 appears to be the key to sustain nearly half of the α_1/β_1 (and α_2/β_2) intersubunit contacts which it associates with via the salt bridge network. At last, the most shielded cysteine residue, βCys112 is likely responsible for stabilizing the other half of the α_1/β_1 (and α_2/β_2) intersubunit contacts as well as those on the α_1/α_2 interface.

4. CONCLUSIONS

In this work, we have demonstrated that all three kinds of cysteine residues are reactive to PMB, resolving a long-debated issue. This clarification is a crucial step in understanding the hemoglobin subunit disassembly pathway and the site-specific roles of all cysteine residues. We show that PMB modifications proceed with an order of βCys93, αCys104, βCys112. By investigating the subunit isolation efficiency of partially PMB-treated hemoglobin, we find that all cysteine residues must be blocked by PMB to accomplish the complete Hb subunit disassembly. By tracking the possible correlations between each cysteine residue and the various intersubunit contacts from the crystallographic structure of hemoglobin, the site-specific functional roles of cysteine residues are revealed. The biophysical role of βCys93 is outstanding due to the critical location it situates. Upon the PMB modification at βCys93, it may first alter the hydrophobic local environment and follow by hierarchically disrupting the intersubunit salt bridges along the α_1/β_2 (and α_2/β_1) interface. αCys104 is important in stabilizing nearly half of the α_1/β_1 (and α_2/β_2) intersubunit salt bridges from the salt bridge network of hemoglobin. βCys112, which also resides near the α_1/β_1 (and α_2/β_2) interface, plays a crucial role in maintaining the stabilities of the other half of the α_1/β_1 (and α_2/β_2) intersubunit salt bridges, as well as those on the α_1/α_2 interface.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Additional details and data for (1) Stability of hemoglobin upon the PB treatment; and (2) Intersubunit salt bridges of hemoglobin (PDB code: 2DN2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Pizano, A. A.; Lutterman, D. A.; Holder, P. G.; Teets, T. S.; Stubbe, J.; Nocera, D. G. Photo-Ribonucleotide Reductase β2 by Selective Cysteine Labeling with a Radical Phototrigger. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 39−43.

(2) Johnson, C. P.; Tang, H. Y.; Carag, C.; Speicher, D. W.; Discher, D. E. Forced Unfolding of Proteins within Cells. *Science* **2007**, *317*, 663−666.

(3) Kim, Y. G.; Ho, S. O.; Gassman, N. R.; Korlann, Y.; Landorf, E. V.; Collart, F. R.; Weiss, S. Efficient Site-Specific Labeling of Proteins Via Cysteines. *Bioconjugate Chem.* **2008**, *19*, 786−791.

(4) Lemmon, C. A.; Ohashi, T.; Erickson, H. P. Probing the Folded State of Fibronectin Type III Domains in Stretched Fibrils by Measuring Buried Cysteine Accessibility. *J. Biol. Chem.* **2011**, *286*, 26375−26382.

(5) Burgoyne, J. R.; Madhani, M.; Cuello, F.; Charles, R. L.; Brennan, J. P.; Schroder, E.; Browning, D. D.; Eaton, P. Cysteine Redox Sensor in PKGIα Enables Oxidant-Induced Activation. *Science* **2007**, *317*, 1393−1397.

(6) Vasquez, G. B.; Karavitis, M.; Ji, X. H.; Pechik, I.; Brinigar, W. S.; Gilliland, G. L.; Fronticelli, C. Cysteines β93 and β112 as Probes of Conformational and Functional Events at the Human Hemoglobin Subunit Interfaces. *Biophys. J.* **1999**, *76*, 88−97.

(7) Alben, J. O.; Bare, G. H. Fourier Transform Infrared Spectroscopic Study of Molecular Interactions in Hemoglobin. *Appl. Opt.* **1978**, *17*, 2985−2990.

(8) Adachi, K.; Yamaguchi, T.; Pang, J.; Surrey, S. Effects of Increased Anionic Charge in the β-Globin Chain on Assembly of Hemoglobin in Vitro. *Blood* **1998**, *91*, 1438−1445.

(9) Juszczak, L. J.; Manjula, B.; Bonaventura, C.; Acharya, S. A.; Friedman, J. M. UV Resonance Raman Study of β93-Modified Hemoglobin A: Chemical Modifier-Specific Effects and Added Influences of Attached Poly(Ethylene Glycol) Chains. *Biochemistry* **2001**, *41*, 376−385.

(10) Khan, I.; Dantsker, D.; Samuni, U.; Friedman, A. J.; Bonaventura, C.; Manjula, B.; Acharya, S. A.; Friedman, J. M. β93 Modified Hemoglobin: Kinetic and Conformational Consequences. *Biochemistry* **2001**, *40*, 7581−7592.

(11) Riggs, A. Molecular Adaptation in Haemoglobins - Nature of the Bohr Effect. *Nature* **1959**, *183*, 1037−1038.

(12) Riggs, A. Binding of N-Ethylmaleimide by Human Hemoglobin and Its Effect Upon Oxygen Equilibrium. *J. Biol. Chem.* **1961**, *236*, 1948−1954.

(13) Storz, J. F.; Weber, R. E.; Fago, A. Oxygenation Properties and Oxidation Rates of Mouse Hemoglobins That Differ in Reactive Cysteine Content. *Comp. Biochem. Physiol., Part A: Mol. Integr. Physiol.* **2012**, *161*, 265−270.

(14) Jia, L.; Bonaventura, C.; Bonaventura, J.; Stamler, J. S. S-Nitrosohaemoglobin: A Dynamic Activity of Blood Involved in Vascular Control. *Nature* **1996**, *380*, 221−226.

(15) Stamler, J. S.; Jia, L.; Eu, J. P.; McMahon, T. J.; Demchenko, I. T.; Bonaventura, J.; Gernert, K.; Piantadosi, C. A. Blood Flow Regulation by S-Nitrosohemoglobin in the Physiological Oxygen Gradient. *Science* **1997**, *276*, 2034−2037.

(16) Bonaventura, C.; Godette, G.; Ferruzzi, G.; Tesh, S.; Stevens, R. D.; Henkens, R. Responses of Normal and Sickle Cell Hemoglobin to S-Nitroscysteine: Implications for Therapeutic Applications of NO in Treatment of Sickle Cell Disease. *Biophys. Chem.* **2002**, *98*, 165−181.

(17) Singel, D. J.; Stamler, J. S. Chemical Physiology of Blood Flow Regulation by Red Blood Cells: The Role of Nitric Oxide and S-Nitrosohemoglobin. *Annu. Rev. Physiol.* **2005**, *67*, 99−145.

(18) Knee, K. M.; Roden, C. K.; Flory, M. R.; Mukerji, I. The Role of β93 Cys in the Inhibition of Hb S Fiber Formation. *Biophys. Chem.* **2007**, *127*, 181−193.

(19) Waterman, M. R. Role of Cysteine Residues in Hemoglobin Structure and Function - Transfer of *para*-Mercuribenzoate from α Subunits to β Subunits During Tetramer Formation. *Biochim. Biophys. Acta* **1974**, *371*, 159−167.

(20) Alben, J. O.; Bare, G. H. Ligand-Dependent Heme-Protein Interactions in Human-Hemoglobin Studied by Fourier-Transform Infrared-Spectroscopy - Effects of Quaternary Structure on α-Chain Tertiary Structure Measured at the α-104(G11) Cysteine-SH. *J. Biol. Chem.* **1980**, *255*, 3892−3897.
(21) Yamaguchi, T.; Pang, J.; Reddy, K. S.; Surrey, S.; Adachi, K. Role of β112 Cys (G14) in Homo- (β_4) and Hetero- ($\alpha_2\beta_2$) Tetramer Hemoglobin Formation. *J. Biol. Chem.* **1998**, *273*, 14179−14185.
(22) Bucci, E.; Fronticelli, C. A New Method for the Preparation of α and β Subunits of Human Hemoglobin. *J. Biol. Chem.* **1965**, *240*, PC551−PC552.
(23) Yamada, K.; Ishikawa, H.; Mizutani, Y. Protein Dynamics of Isolated Chains of Recombinant Human Hemoglobin Elucidated by Time-Resolved Resonance Raman Spectroscopy. *J. Phys. Chem. B* **2012**, *116*, 1992−1998.
(24) Birukou, I.; Maillett, D. H.; Birukova, A.; Olson, J. S. Modulating Distal Cavities in the α and β Subunits of Human HbA Reveals the Primary Ligand Migration Pathway. *Biochemistry* **2011**, *50*, 7361−7374.
(25) Birukou, I.; Schweers, R. L.; Olson, J. S. Distal Histidine Stabilizes Bound O_2 and Acts as a Gate for Ligand Entry in Both Subunits of Adult Human Hemoglobin. *J. Biol. Chem.* **2010**, *285*, 8840−8854.
(26) Hu, X. H.; Spiro, T. G. Tyrosine and Tryptophan Structure Markers in Hemoglobin Ultraviolet Resonance Raman Spectra: Mode Assignments Via Subunit-Specific Isotope Labeling of Recombinant Protein. *Biochemistry* **1997**, *36*, 15701−15712.
(27) Rosemeyer, M. A.; Huehns, E. R. On the Mechanism of the Dissociation of Haemoglobin. *J. Mol. Biol.* **1967**, *25*, 253−273.
(28) Chiancone, E.; Currell, D. L.; Vecchini, P.; Antonini, E.; Wyman, J. Kinetics of the Reaction of the "Masked" and "Free" Sulfhydryl Groups of Human Hemoglobin with *p*-Mercuribenzoate. *J. Biol. Chem.* **1970**, *245*, 4105−4111.
(29) Ioppolo, C.; Chiancone, E.; Antonini, E.; Wyman, J. Dissociation of Fetal Hemoglobin by *p*-Chloromercuribenzoate and Preparation of Native γ Chains. *Arch. Biochem. Biophys.* **1969**, *132*, 249−254.
(30) Mawjood, A. H. M.; Miyazaki, G.; Kaneko, R.; Wada, Y.; Imai, K. Site-Directed Mutagenesis in Hemoglobin: Test of Functional Homology of the F9 Amino Acid Residues of Hemoglobin α and β Chains. *Protein Eng.* **2000**, *13*, 113−120.
(31) Lu, M. L.; Li, X. F.; Le, X. C.; Weinfeld, M.; Wang, H. L. Identification and Characterization of Cysteinyl Exposure in Proteins by Selective Mercury Labeling and Nano-Electrospray Ionization Quadrupole Time-of-Flight Mass Spectrometry. *Rapid Commun. Mass Spectrom.* **2010**, *24*, 1523−1532.
(32) Benesch, R. E.; Benesch, R. The Influence of Oxygenation on the Reactivity of the -SH Groups of Hemoglobin. *Biochemistry* **1962**, *1*, 735−738.
(33) Zijlstra, W. G.; Buursma, A.; Meeuwsenvanderroest, W. P. Absorption-Spectra of Human Fetal and Adult Oxyhemoglobin, De-Oxyhemoglobin, Carboxyhemoglobin, and Methemoglobin. *Clin. Chem.* **1991**, *37*, 1633−1638.
(34) Yip, Y. K.; Waks, M.; Beychok, S. Reconstitution of Native Human Hemoglobin from Separated Globin Chains and Alloplex Intermediates. *Proc. Natl. Acad. Sci. U.S.A.* **1977**, *74*, 64−68.
(35) Wada, Y. Advanced Analytical Methods for Hemoglobin Variants. *J. Chromatogr., B* **2002**, *781*, 291−301.
(36) Park, S.-Y.; Yokoyama, T.; Shibayama, N.; Shiro, Y.; Tame, J. R. H. 1.25 Resolution Crystal Structures of Human Haemoglobin in the Oxy, Deoxy and Carbonmonoxy Forms. *J. Mol. Biol.* **2006**, *360*, 690−701.
(37) Sackin, H.; Nanazashvili, M.; Li, H.; Palmer, L. G.; Walters, D. E. An Intersubunit Salt Bridge near the Selectivity Filter Stabilizes the Active State of Kir1.1. *Biophys. J.* **2009**, *97*, 1058−1066.
(38) Binter, A.; Staunig, N.; Jelesarov, I.; Lohner, K.; Palfey, B. A.; Deller, S.; Gruber, K.; Macheroux, P. A Single Intersubunit Salt Bridge Affects Oligomerization and Catalytic Activity in a Bacterial Quinone Reductase. *FEBS J.* **2009**, *276*, 5263−5274.
(39) Biter, A. B.; Lee, S.; Sung, N.; Tsai, F. T. F. Structural Basis for Intersubunit Signaling in a Protein Disaggregating Machine. *Proc. Natl. Acad. Sci. U.S.A.* **2012**, *109*, 12515−12520.
(40) Nishida, M.; Okamoto, M.; Ohno, A.; Okumura, K.; Hayashi, K.; Ikeda, K.; Inoue, S. Inhibitory Activities of the Heterotrimers Formed from Two Alpha-Type Phospholipase A(2) Inhibitory Proteins with Different Enzyme Affinities and Importance of the Intersubunit Electrostatic Interaction in Trimer Formation. *Biochim. Biophys. Acta, Proteins Proteomics* **2010**, *1804*, 2121−2127.
(41) Yusuff, O. K.; Babalola, J. O.; Bussi, G.; Raugei, S. Role of the Subunit Interactions in the Conformational Transitions in Adult Human Hemoglobin: An Explicit Solvent Molecular Dynamics Study. *J. Phys. Chem. B* **2012**, *116*, 11004−11009.
(42) Yeh, S. R. A Novel Intersubunit Communication Mechanism in a Truncated Hemoglobin from Mycobacterium Tuberculosis. *J. Phys. Chem. B* **2004**, *108*, 1478−1484.
(43) Nagatomo, S.; Nagai, M.; Kitagawa, T. A New Way to Understand Quaternary Structure Changes of Hemoglobin Upon Ligand Binding on the Basis of UV-Resonance Raman Evaluation of Intersubunit Interactions. *J. Am. Chem. Soc.* **2011**, *133*, 10101−10110.
(44) Fermi, G.; Perutz, M. F.; Shaanan, B.; Fourme, R. The Crystal Structure of Human Deoxyhaemoglobin at 1.74 Å Resolution. *J. Mol. Biol.* **1984**, *175*, 159−174.
(45) Kumar, S.; Nussinov, R. Salt Bridge Stability in Monomeric Proteins. *J. Mol. Biol.* **1999**, *293*, 1241−1255.
(46) Kilmartin, J. V.; Fogg, J. H.; Perutz, M. F. Role of C-Terminal Histidine in the Alkaline Bohr Effect of Human Hemoglobin. *Biochemistry* **1980**, *19*, 3189−3193.
(47) Waterman, M. R. Effect of Carbamidomethylation of Cysteine Residues G11(104)α on Properties of Hemoglobin A. *Biochim. Biophys. Acta* **1976**, *439*, 167−174.
(48) Adachi, K.; Yang, Y.; Lakka, V.; Wehrli, S.; Reddy, K. S.; Surrey, S. Significance of β116 His (G18) at $\alpha1\beta1$ Contact Sites for $\alpha\beta$ Assembly and Autoxidation of Hemoglobin. *Biochemistry* **2003**, *42*, 10252−10259.
(49) Baldwin, J.; Chothia, C. Haemoglobin: The Structural Changes Related to Ligand Binding and Its Allosteric Mechanism. *J. Mol. Biol.* **1979**, *129*, 175−220.
(50) Balakrishnan, G.; Tsai, C. H.; Wu, Q.; Case, M. A.; Pevsner, A.; McLendon, G. L.; Ho, C.; Spiro, T. G. Hemoglobin Site-Mutants Reveal Dynamical Role of Interhelical H-Bonds in the Allosteric Pathway: Time-Resolved UV Resonance Raman Evidence for Intra-Dimer Coupling. *J. Mol. Biol.* **2004**, *340*, 857−868.
(51) Kavanaugh, J. S.; Rogers, P. H.; Arnone, A.; Hui, H. L.; Wierzba, A.; DeYoung, A.; Kwiatkowski, L. D.; Noble, R. W.; Juszczak, L. J.; Peterson, E. S.; Friedman, J. M. Intersubunit Interactions Associated with Tyr42α Stabilize the Quaternary-T Tetramer but Are Not Major Quaternary Constraints in Deoxyhemoglobin. *Biochemistry* **2005**, *44*, 3806−3820.
(52) Tolbert, T. J.; Wong, C. H. New Methods for Proteomic Research: Preparation of Proteins with N-Terminal Cysteines for Labeling and Conjugation. *Angew. Chem., Int. Ed.* **2002**, *41*, 2171−2174.