

The Absence of Proximal Strain in the Truncated Hemoglobins from *Mycobacterium tuberculosis*

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Hemoglobin (Hb) is considered the paradigm for allosteric proteins.² Recently, many new classes of Hb's have been discovered in bacteria, algae, plants, invertebrates, and vertebrates.^{3–6} These new Hb's are made of myoglobin (Mb)-like subunits that vary from the conventional three-over-three α -helical sandwich motif found in most Hb's to a novel two-over-two α -helical domain discovered in truncated hemoglobins (TrHb's).^{7–9} The quaternary assemblage of the subunits in these Hb's is remarkably diverse, ranging from the monomeric Hb of bacteria to the 144 subunits of gigantic annelid extracellular Hb.^{3,5}

Among the newly discovered Hb's, the two TrHb's from *Mycobacterium tuberculosis*, HbN and HbO, are especially interesting.^{8–11} On the basis of the crystallographic data, HbN is a head-to-tail homodimer.⁸ HbO, on the other hand, is a dodecamer that consists of six pairs of asymmetric dimers,⁹ although it exists as a dimer under the solution conditions applied here.¹² The oxygen binding affinity of HbN is extremely high, making the determination of the oxygen binding cooperativity a nontrivial task. Initial measurements of the Hill coefficient indicated a very high level of cooperativity,¹⁰ but it was not confirmed in later experiments.¹³ However, another way of determining cooperative interactions is to study the effect on the heme in one subunit when a ligand binds to the other. We did such measurements and determined that there is a strong heme–heme communication in HbN that is mediated by interactions between residues in the distal pocket and the bound ligand.¹⁴ To evaluate whether proximal interactions also play a role in the intersubunit communication, as is observed in most other allosteric globins, resonance Raman spectroscopy was employed to study the structural properties of the nanosecond photoproduct of the CO-bound derivative of HbN. The result was compared to that of HbO and human hemoglobin (HbA).

The resonance Raman spectrum of the deoxy-derivative of HbN is shown in Figure 1. The peaks at 307, 350, and 678 cm^{-1} are assigned to the γ_7 , ν_8 , and ν_7 modes of the porphyrin ring, respectively.¹⁵ The strong peak at 226 cm^{-1} is assigned to the iron–histidine (Fe–His) stretching mode.¹⁶ Figure 2 shows the resonance Raman spectra of the deoxy-derivative of HbO. As in HbN, the line at 226 cm^{-1} is assigned to the Fe–His stretching mode,¹¹ and the 307, 349, and 679 cm^{-1} peaks are assigned to the γ_7 , ν_8 , and ν_7 modes of the porphyrin ring, respectively.¹⁵ On the other hand, the 322 and 416 cm^{-1} peaks in HbO are assigned to the γ_6 and $\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\beta)$ vibrational modes, respectively.¹⁵ The enhancement of the out-of-plane γ_6 porphyrin mode is consistent with a slightly distorted heme,¹⁷ evident in the crystal structure.⁸ The development of the $\delta(\text{C}_\beta\text{C}_\alpha\text{C}_\beta)$ mode, on the other hand, may reflect the distinct structure and/or environment of the heme group in HbO, since the heme group in HbO is rotated by 180° along the methinic α – γ meso axis, relative to HbN (and Mb), as shown in Chart 1.⁹

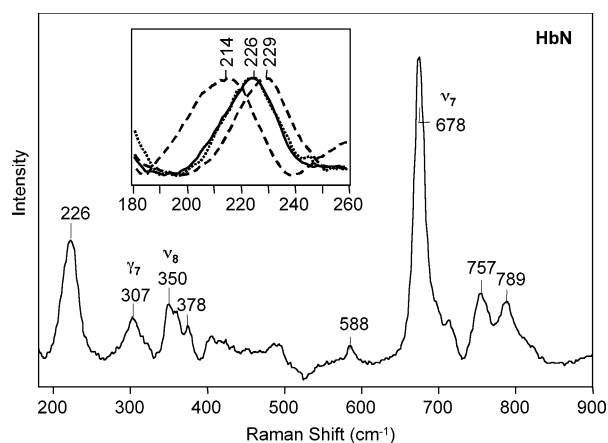


Figure 1. Resonance Raman spectrum of the deoxy-derivative of HbN (0.2 mM) in pH 7.5 Tris buffer, obtained with 406.7 nm excitation from a Kr^+ laser. Insert: The Fe–His stretching mode, the photoproduct of the CO-bound form (the dotted line), and that of the deoxy-derivative of HbN (the solid line). The dash lines show the Fe–His stretching modes of the deoxy-derivative (214 cm^{-1}) and the nanosecond photoproduct (229 cm^{-1}) of HbA. The spectra of the photoproducts were obtained with an 8 ns laser pulse (435.8 nm) from a frequency-doubled Nd:YAG laser that was frequency-shifted via a hydrogen shifter. Here the laser was used to photodissociate CO from the protein as well as the excitation light source for the Raman measurements. For details of the experimental system, see ref 1.

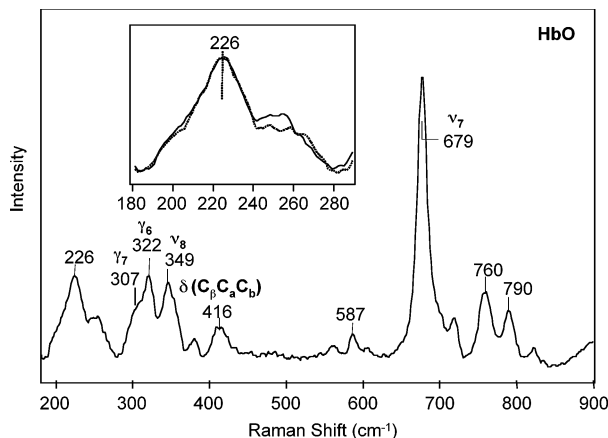


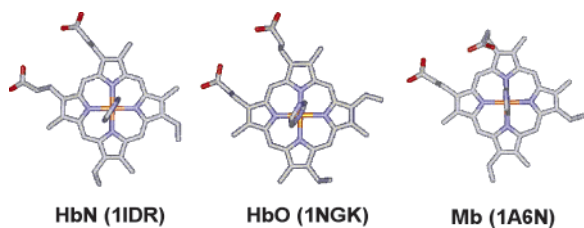
Figure 2. Resonance Raman spectrum of the deoxy form of HbO (0.2 mM) in pH 7.5 Tris buffer. Insert: The Fe–His stretching modes of the 8 ns photoproduct of the CO-bound form (the dotted line) and that of the deoxy-derivative of HbO (the solid line). The experimental conditions are the same as those in Figure 1.

The similar Fe–His stretching frequencies identified at 226 cm^{-1} in HbN and HbO suggest that the proximal Fe–His bond strengths in these two TrHb's are comparable, and they are stronger than those in HbA and Mb, which exhibit Fe–His stretching frequencies

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Chart 1



at 214 and 220 cm^{-1} , respectively. In HbA or Mb, the imidazole ring of the proximal His is in an eclipsed orientation with respect to the pyrrole nitrogen atoms. In contrast, that in the HbN or HbO is in a staggered geometry (Chart 1). The higher frequency of the Fe–His stretching modes thus could in part be a consequence of the reduction of the repulsive interactions between the imidazole ring and the pyrrole nitrogen atoms.

Photodissociating CO from the CO derivative of the dimeric HbN with an 8 ns laser pulse produces a five-coordinate deoxy-species that has an Fe–His stretching frequency at 226 cm^{-1} , which is identical to that of the equilibrium ligand-free form as shown in the insert in Figure 1. Interestingly, the Fe–His stretching frequency of the nanosecond photoproduct of HbO is also identified at 226 cm^{-1} ; again, it is identical to that of the equilibrium ligand-free form (insert in Figure 2). These surprising results indicate that within 8 ns the photoproduct has the same structure as that of the equilibrium deoxy derivative for both of these TrHb's.

In sharp contrast to HbN and HbO, in the equilibrium deoxy derivative of HbA (the tense T state), the bond between the iron atom and the proximal His is strained by the F-helix due to the quaternary constraints imposed by intersubunit interactions, which weaken the Fe–His bond resulting in the low frequency of the stretching mode at 214 cm^{-1} (insert in Figure 1). In the nanosecond photoproduct of the CO derivative of HbA, the iron atom moves out of plane in a direction toward the proximal His leading to a shorter and stronger Fe–His bond with a frequency at 229 cm^{-1} , 15 cm^{-1} higher than that in the equilibrium T state, as shown in the insert in Figure 1. The final structural transition from this R quaternary structure to the T structure does not occur until tens of microseconds later.^{18,19} The nanosecond photoproduct, therefore, retains the relaxed R structure as characterized by the high frequency of the Fe–His stretching mode.^{18,19} This type of behavior is characteristic of all globins in which the allosteric linkage is mediated through the Fe–His bond, and it is absent in the monomeric Mb.

The data presented here for HbN and HbO indicate that movement along the Fe–His bond following the dissociation of CO does not trigger a quaternary structural transition in these two TrHb's. They also suggest that either no significant structural rearrangement of the polypeptide on the proximal side of the heme is required upon photodissociation when the iron moves to an out-of-plane position (it should be noted that in a series of five-coordinated heme model compounds, the out-of-plane distortion of the iron atom ranges from 0.3 to 0.6 Å)²⁰ or if there is any movement of the polypeptide, it must occur on a time scale faster than 8 ns, orders of magnitude faster than the quaternary structural transition observed in other allosteric globins.

On the basis of the photolysis results in dimeric HbN, it is concluded that the intersubunit communication in HbN triggered by ligand binding is not coupled with the conformational changes

on the proximal side of the heme as observed in HbA. HbO is also a dimeric protein under the conditions applied here. It is unclear whether there is structural communication between the two subunits of the dimer in HbO; nonetheless, if there is communication between the two subunits, it is anticipated that the intersubunit communication, just as in HbN, cannot be mediated by the structural movement on the proximal side of the heme. This may be a functional consequence of the unique structure of TrHb's in which the F helix is floppy with only one single helical turn in contrast to the rigid F helix with three helical turns in HbA.

Three decades ago, Perutz proposed that the allosteric structural transition in hemoglobin is triggered by movement of the Fe–His bond upon ligand binding.²¹ This model has served as the foundation for our thinking about the allosteric mechanism of Hb's since then. The current findings in HbN delineate new cases in which allosteric structural transitions are not triggered by proximal movement. A related mechanism has been suggested for sea lamprey Hb, in which the allosteric transition is a result of distal steric hindrance instead of a conformational change on the proximal side of the heme.²² The absence of proximal strain in both HbN and HbO may be an important integral part of the structural features that determine the wide spectrum of physiological functions postulated for the many novel bacterial Hb's discovered in recent years.

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