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# Hydrogen Bonding to Trp $\beta$37 Is the First Step in a Compound Pathway for Hemoglobin Allostery

Robert A. Goldbeck,*,† Raymond M. Esquerra,‡ and David S. Kliger†

*Department of Chemistry and Biochemistry, University of California, Santa Cruz, California 95064, and Department of Chemistry and Biochemistry, San Francisco State University, San Francisco, California 94132*

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The kinetic mechanism of the R → T quaternary transition of human hemoglobin remains an open question. In the simplest kinetic picture, based largely on time-resolved optical absorption data for photolyzed HbCO,[1] the two α,$\beta$-chain heterodimers making up the tetramer change their quaternary structure (rotating relative to each other by 15°)[2] in a single, thermally activated rate process requiring tens of microseconds. However, recent time-resolved resonance Raman[3] and circular dichroism[4] results suggest that this reaction, which underlies hemoglobin's cooperative oxygen binding and serves as a prototype for protein allostery in general, may actually occur in more than one step.

We present here compelling kinetic evidence for the compound nature of the allosteric pathway in hemoglobin. We use time-resolved UV magnetic circular dichroism (MCD) spectroscopy of the tryptophan amino acid residues to show that formation of a Trp $\beta$37-Asp α94 hydrogen bond in the "hinge" region of the dimer−dimer interface is part of an obligatory R → T step preceding the kinetic step identified in absorption. Clarifying the kinetics of interdimer contact formation at Trp $\beta$37 (C3) is expected to be particularly important in understanding the mechanism of quaternary change because this residue, which is highly conserved in vertebrate hemoglobins, plays a key role in cooperativity and allostery.[5,6]

The tryptophan band position serves as a kinetic marker for quaternary conformation in the present study. The position of the tryptophan $L_1$ electronic transition in the aromatic region of the hemoglobin MCD spectrum depends on quaternary state, the T-state band being shifted to a slightly longer wavelength compared with that of the R state near 293 nm.[7] This red-shift arises from a hydrogen-bonding-induced perturbation of the electronic structure of the Trp37 residues contributed by the two $\beta$ chains. Of the six tryptophan residues present in the tetramer, only the $\beta$37 residues are located at the dimer−dimer interface, wherein the structural differences between quaternary states are largest. Moreover, the $\beta$37 residues are known to donate protons in hydrogen bonds to Aspα94 residues in the T crystal structure, an interaction that is missing in the R crystal structure.[8] The observation of the equilibrium UV MCD shift, along with other spectral evidence,[9] confirms that this structural difference between the R and T states also exists in solution. In the present work, we use near-UV time-resolved magnetic circular dichroism (TRMCD)[10] spectroscopy to monitor the tryptophan band position during the R → T transition of human hemoglobin for the status of a structural feature specific to the T quaternary conformation, the Trp $\beta$37-Asp α94 hydrogen bond.

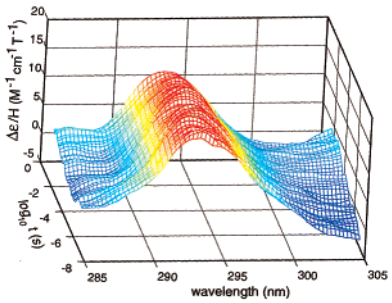


***Figure 1.*** Time-resolved tryptophan MCD spectra measured in human hemoglobin at 55 logarithmically spaced delay times ranging from 63 ns to 25 ms after photolysis of the CO complex (detection gate duration was 80 ns).[18]

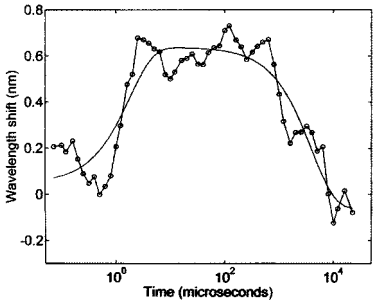


***Figure 2.*** Shift in tryptophan MCD band position vs delay time after photolysis. Shift values (circles) were obtained by nonlinear least-squares fitting of a Gaussian band shape with an offset baseline to each spectrum in Figure 1. Time constants of 1.9 ± 0.3 $\mu$s (amplitude = 0.58 ± 0.04 nm) and 4.0 ± 1.1 ms (amplitude = 0.70 ± 0.06 nm) were obtained for the growth and decay of the red shift, respectively, by application of a nonlinear least-squares procedure that used two exponential time functions and a constant term to fit (smooth line) the band shift evolution.[19]

We photolyzed HbCO to initiate quaternary relaxation from the R to the T conformation and monitored the spectral evolution during relaxation as described previously.[11] We observed that the center of the 293-nm tryptophan band apparent in the UV TRMCD spectra shown in Figure 1 shifted rapidly to the red from its R-state, non-H-bonded position by ∼0.6 nm (exponential time constant = 2 $\mu$s), as shown in Figure 2. This shift is similar to that observed for the difference between equilibrium T and R quaternary conformers,[7] and is consistent with the aggregate shift expected when two of six tryptophans donate a proton in a hydrogen bond (the individual signal from each H-bonded tryptophan being expected to shift by 2−5 nm to the red).[12]

We interpret the early time shift as evidence for the appearance of a T-state hydrogen bond with a time constant of 2 $\mu$s, much shorter than the time scale of the kinetic process conventionally

* To whom correspondence should be addressed. E-mail: goldbeck@chemistry.ucsc.edu.
† University of California.
‡ San Francisco State University.



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identified as the R → T transition (time constant of 20−40 $\mu$s under these conditions).[1b,13] The latter process is apparently tryptophan-MCD silent, there being no significant evolution of the band shift between 10 and 100 $\mu$s. The MCD band shift then decays with a 4-ms time constant to a value that is equal, within the experimental noise (∼10%), to the baseline value observed before the 2-$\mu$s process. The time constants observed here are in excellent agreement with time constants measured in heme band absorption studies under the same experimental conditions and assigned to protein structural relaxation (2 $\mu$s) and CO recombination with T conformers (4 ms).[1b,13] The TRMCD data presented here clarify the nature of the relaxation by providing direct evidence for the quaternary nature of the 2-$\mu$s process.[14] The observed correspondence between the time constants for band shift decay and T-state ligand recombination is expected because recombination of CO to T-state Hb breaks the Trp-Asp hydrogen bond, along with many other T-state dimer−dimer contacts, as it restores the original liganded R state.

The approximate equality of the magnitudes of the 2-$\mu$s shift and the reverse shift associated with the 4-ms process, in which all of the T-state conformers created by previous R → T processes are converted back to R, implies that the fast process converts the Trp-Asp dimer−dimer contact to its T-state H-bonded form nearly quantitatively.[15] The robust size of the tryptophan band shift observed at 2 $\mu$s thus indicates that the fast process is an obligatory and committed kinetic step in the conversion of R conformers to T.

The evidence presented here for a large and rapid change in a specific dimer−dimer contact that is clearly distinguishable from the slower process previously assigned to quaternary structural relaxation leads us to conclude that the R → T kinetic pathway requires at least two steps. The first step creates an obligatory allosteric intermediate in which a critical T-state contact, the Trp-Asp hydrogen bond, has been formed.[16] Completion of the dimer−dimer rotation and contact formation leading to the equilibrium T state is left to the later, rate limiting step in what can now be concluded unambiguously is a compound reaction pathway. The larger activation barrier, the appearance of heme−heme cooperativity effects,[15] and the larger perturbation of heme spectra[13,17] associated with the rate limiting step imply that it produces larger changes in protein conformation than occur in the primary step. This suggests in turn that the rate-limiting step is associated with changes at the switch region of the $\alpha_1\beta_2$ interface, wherein relative motion of the dimers is largest and steric interference arising from movement of the imidazole side chain of His $\beta_2$97 past the Thr $\alpha_1$41 residue is expected to present a substantial kinetic barrier to interconversion between the R and T conformations.

In summary, we find that stepwise progress of the protein along the allosteric pathway appears to start with a rearrangement of contacts at the hinge and end with rearrangements involving the switch region of the interface. By placing such constraints on the location and order of events in possible mechanistic models for the reaction pathway of hemoglobin allostery, the kinetic results presented here can aid in understanding how hemoglobin functions as a prototypical "molecular machine".

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(6) The hinge region $\alpha_1\beta_2$ contacts, wherein the C helix of a $\beta$ chain interacts with the FG corner of an α chain, are closer to the pivotal axis of dimer−dimer rotation than the $\beta$ FG corner to α C helix contacts in the "switch" region of the interface. Nevertheless, mutations at the hinge region, particularly $\beta$37 variants, can dramatically affect the allosteric control and cooperativity of ligand binding kinetics. See: Noble, R. W.; Hui, H. L.; Kwiatkowski, L. D.; Paily, P.; DeYoung, A.; Wierzba, A.; Colby, J. E.; Bruno, S.; Mozzarelli, A. *Biochemistry* **2001**, *40*, 12357−12368.

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(14) If the 2-$\mu$s MCD shift was connected only to tertiary and not to quaternary relaxation, then it would appear in the CO-dissociated R-state population as well as in the T (30−40% R → T tetramer conversion is expected from the kinetic model in ref 13) and its decay would reflect the ∼200-$\mu$s time constant characteristic of diffusive recombination (with 1 atm of CO) of the dominant R population (ref 1b), contrary to observation.

(15) A caveat is that some fraction of the deliganded tetramers acquiring an H bond in the 2-$\mu$s process probably recombines with CO before completely relaxing to the equilibrium T conformation. Recombination to this fraction is expected to proceed with the fast rate constant characteristic of the R state because allosteric perturbation of ligand binding rates appears to be communicated between subunits on a time scale >1 $\mu$s. See: Jones, C. M.; Ansari, A.; Henry, E. R.; Christoph, G. W.; Hofrichter, J.; Eaton, W. A. *Biochemistry* **1992**, *31*, 6692−6702. In light of this, the absence of appreciable band shift decay on the submillisecond time scale suggests that any population decay of the 2-$\mu$s intermediate through rapid ligand recombination is too small to detect or is offset by, and thus masks, an increase in band shift concomitant with the 20−40-$\mu$s R → T process.

(16) This conclusion is also consistent with resonance Raman results for the reverse reaction in sol−gel encapsulated Hb suggesting that Trp $\beta$37's T-state contacts are lost at the end of the (compound) T → R pathway. See: Juszczak, L. J.; Friedman, J. M. *J. Biol. Chem.* **1999**, *274*, 30357−30360.

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(18) Experimental Details: The samples contained 120 $\mu$M human hemoglobin (per heme basis), 1.0 atm of CO, and 0.10 M phosphate buffer, pH 7.3, maintained at 20 °C in a 2.5-mm path length cell. The sample was photolyzed with 7-ns, 20-mJ pulses of 532-nm light from a Nd:YAG laser, propagating perpendicularly to the probe flash lamp beam, to give essentially complete ligand dissociation (before geminate and bimolecular ligand recombination). Each time point represents the average of 6200 individual photolysis measurements. Fresh sample was flowed into the cell between measurements to avoid the accumulation of photodegraded or oxidized sample products. Magnetic field strength was 1.0 T. The spectra were smoothed as a function of time, using an 11-point Savitzky-Golay procedure. (Smoothing did not affect the kinetic analysis shown in Figure 2; analysis of the unsmoothed data gave time constants and amplitudes similar to those presented for the smoothed data.).

(19) Fitting with a third exponential time function was also tested, but the improvement in fit was small and not statistically significant.

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