Enhanced Resolution of Hemoglobin Dynamics Provided by Subunit-Specific Resonance Raman Signals

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Hemoglobin (Hb), a tetrameric protein containing four protohemes, binds O_2 and other exogenous ligands cooperatively. A two state model proposed by Monod, Wyman, and Changeaux[1] and further supported by the X-ray crystallographic studies of Perutz[2] states that deoxy Hb exists in one quaternary structure while that of ligated Hb is different. The reaction coordinate between these states remains largely undefined due to the high degree of cooperativity which ensures that the intermediate states are very sparsely populated at equilibrium and accessible only by kinetic methods[3] or by studying chemically modified derivatives which mimic the partially ligated tetramer.[4] Resonance Raman (RR)[5] and time-resolved resonance Raman (TR^3) spectroscopy[6−8] provide the capability of monitoring the detailed structure and dynamics (TR^3) of various molecular fragments throughout the Hb tetramer. However, all previous TR^3 studies of the photoproduct at 10 ns of native Hb were plagued by the interpretational ambiguity which arises from the fact that the observed shifts are actually an average shift; i.e., a conglomerate of signals arising from the four individual hemes present in the tetramer. Here, transient RR studies are reported for unique hemoglobin hybrids containing deuterated hemes in either the α or β subunits, which yield separate signals for the ν_{19} modes of the two hemes (occurring $\sim$20 cm^{-1} apart). Comparison of the spectra of hybrids shows that subunit heterogeniety exists in the equilibrium deoxy form but not in the photoproduct.

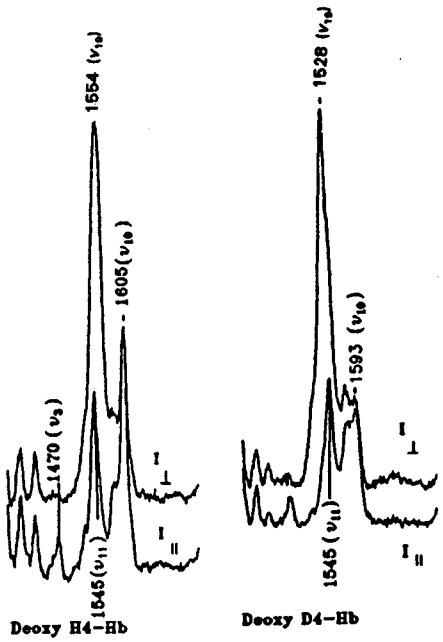

Figure 1. Parallel and perpendicular polarized resonance Raman spectra of deoxy Hb tetramers (native Hb and Hb-d4). Excitation 532.1 nm, 1.8 mJ/pulse, 20 Hz, 0.2 mM heme, pH 7.4.

The protoheme-d4, hemoglobin, and hybrids used in this study were prepared according to established procedures.[9 a−f,10] Both native Hb and the Hb-d4 were subjected to a subunit separation procedure, the details of which (as performed in our laboratory) are fully described elsewhere.[10] The resulting isolated subunits (i.e., α_d with β_h and α_h with β_d) were recombined in an appropriate manner[10] to produce both hybrids i.e., $(\alpha_d\beta_h)_2$ and $(\alpha_h\beta_d)_2$, where the subscript d indicates that the subunit contains protoheme-d4.

The RR spectra of the deoxy and photolyzed forms of the CO ligated derivatives of both hybrids (as well as the native and Hb-d4 parents) were acquired on a Spex Model 1269 single monochromator equipped with a 532 nm notch filter (Kaiser Optical, Ann Arbor, MI) and a Princeton Instruments Model ST-130 intensified CCD detector. Excitation for all spectra was provided by the second harmonic (532.1 nm) line from a Spectra Physics GCR-11 pulsed Nd-YAG laser.

The RR spectra of deoxy-Hb and deoxy Hb-d4 (Figure 1) exhibit a number of structure sensitive "marker" modes, labeled ν_{10} (dp), ν_{11} (dp), ν_{19} (ap), and ν_3 (p),[7c] some of which are sensitive to deuteration (ν_{10} and ν_{19}). It has been previously shown[6−8,11,12] that, upon photolysis of $Hb(CO)_4$ with $\sim$10 ns laser pulses, a fully deligated initial photoproduct (Hb)* is formed whose transient RR spectrum exhibits vibrational frequencies which are slightly downshifted relative to those of the equilibrium T-state deoxy derivative. Dasgupta and Spiro[7c] have carefully documented the precise magnitudes of these frequency shifts (Δ) between Hb and Hb* and have shown that the frequency of ν_{19} for Hb is 3 cm^{-1} higher than for ν_{19} of Hb*.

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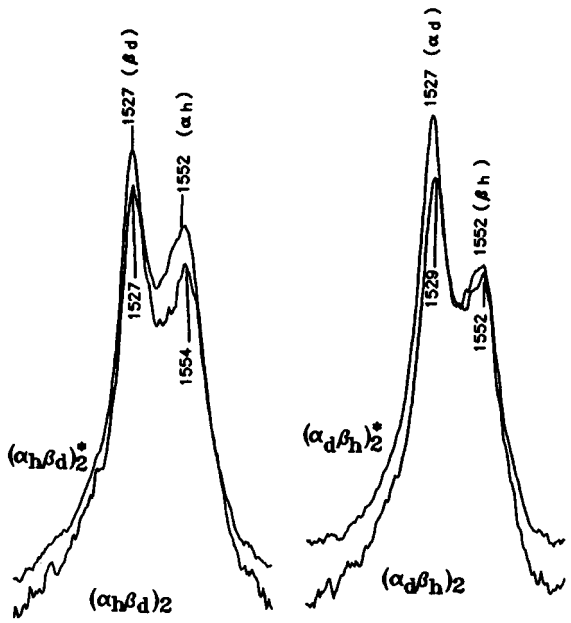

Figure 2. Difference spectra (perpendicular-parallel) comparing the deoxy and photolyzed species for each of the two hybrids, measurement conditions as in Figure 1.

The above observations indicate that heme core marker modes, ν_{10} and especially ν_{19} are most suitable for investigating subunit-specific structural alterations of deoxy-Hb vs Hb*. Attention in the present study is focussed on documentation of the frequency shifts for the ν_{19} modes of the heme and heme-d4 groups of the hybrids; i.e., $(\alpha_h\beta_d)_2$ and $(\alpha_d\beta_h)_2$, in the deoxy and photolyzed forms.

In order to isolate the contributions from the two ν_{19} (ap) modes, the spectrum acquired with parallel polarization is interactively subtracted from that acquired with perpendicular polarization to the point where ν_{10} and other isolated depolarized modes disappear. The effect is to cancel out the contribution from the depolarized ν_{11} modes which overlap, to some extent, the ν_{19} modes. There have been reports on the effects of depolarization ration dispersion which can, in principle, affect peak frequencies. Such effects are generally considered to be insignificant for the types of measurements that were done here.[7c−e] Spectra ($I_\perp - I_{||}$) of both the equilibrium deoxy and photolyzed forms for both hybrids are given in Figure 2. Comparison of the frequencies of the ν_{19} modes associated with the α_h subunits of the $(\alpha_h\beta_d)_2$ hybrids ($\sim$1550 cm^{-1}) yields a 2−3 cm^{-1} downshift in the spectrum of $(\alpha_h\beta_d)_2$*. On the other hand, the ν_{19} modes associated with the β_d subunits ($\sim$1527 cm^{-1}) in each spectrum exhibit only a $\leq$1 cm^{-1} difference. A comparison of the corresponding ν_{10} frequencies in each of the parallel and perpendicular spectra (not shown) also yields a shift for the α_h subunits of $\sim$2 cm^{-1}.

Also shown in Figure 2 are the observed spectra for the partner hybrid; i.e., $(\alpha_d\beta_h)_2$ and $(\alpha_d\beta_h)_2$*. In this case the ν_{19} mode associated with the α_d subunit of $(\alpha_d\beta_h)_2$* is shifted to lower frequencies compared to that of the α_d subunit of $(\alpha_d\beta_h)_2$. The ν_{19} frequencies for the natural abundance heme are essentially identical (within 1 cm^{-1}) in the two spectra, indicating there is no significant shift for the β_h subunit. Also, comparison of the ν_{10} frequencies in the parallel and perpendicular spectra yields an insignificant shift for the β_h subunits. Thus, careful comparison of the ν_{19} modes in the spectra of the deoxy and photolyzed hybrid indicate that the α_d subunit mode shifts to lower frequency by $\sim$2.5 cm^{-1} while that of the β_h subunit shifts by $\leq$1 cm^{-1}. The data from these spectral comparisons are summarized in Table 1.

The spectral comparisons summarized above document an $\sim$2.5 cm^{-1} shift to lower frequency for the α subunits of $(\alpha\beta)_2$* relative to deoxy $(\alpha\beta)_2$. As can be seen from Table 1, comparison of the two photolyzed hybrids (i.e., $(\alpha_h\beta_d)_2$* vs $(\alpha_d\beta_h)_2$*) indicates no significant differences between the α and β subunits (i.e., ν_{19} of α_h occurs at the same frequency as ν_{19} of β_h).

Table 1. Observed Frequencies and Shifts for ν_{10} and ν_{19}

	ν_{19} (h4)	ν_{19} (d4)	ν_{10} (h4)	ν_{10} (d4)
Hemoglobin Tetramer				
Hb and Hb-d4	1554	1528	1605	1593
Hb* and Hb-d4*	1552	1527	1603	1591
Hemoglobin Hybrids				
$(\alpha_{h4}\beta_{d4})_2$	1554	1527	1604	*a*
$(\alpha_{h4}\beta_{d4})_2$*	1552	1527	1602	
Δ(deoxy-photolyzed)	2.3 ± 0.8	≤1.0	2.5 ± 1.2	
$(\alpha_{d4}\beta_{h4})_2$	1552	1529	1600	
$(\alpha_{d4}\beta_{h4})_2$*	1552	1527	1600	
Δ(deoxy-photolyzed)	1.2 ± 0.6	2.5 ± 0.5	0.3 ± 0.5	
$\Delta(\alpha_{d4} - \beta_{d4})$* and $(\alpha_{h4} - \beta_{h4})$*		0.7 ± 0.3 and 0.4 ± 0.3		
$\Delta(\alpha_{d4} - \beta_{d4})$ and $(\alpha_{h4} - \beta_{h4})$		2.0 ± 0.4 and 2.1 ± 0.7		

[a] Band was unresolved and exact frequency could not be determined. Difference spectra were plotted and viewed using Spectracalc@ software and the peak positions determined by visual estimation of the band center. The reported values from such spectral comparisons represent the average values and standard deviation for six separate experimental sessions employing five different preparations of each hybrid.

Significant differences, however, are observed upon comparing the spectra of the two deoxy hybrids. The ν_{19} mode of the α_h subunit of $(\alpha_h\beta_d)_2$ is 2−3 cm^{-1} higher than the ν_{19} mode of the β_h subunit of $(\alpha_d\beta_h)_2$. Comparison of the corresponding heme-d4 modes [i.e., α_d of $(\alpha_d\beta_h)_2$ compared to β_d of $(\alpha_h\beta_d)_2$] also yields an $\sim$2 cm^{-1} difference (Table 1).

The present results provide unambiguous spectral evidence for heme structural heterogeniety in the equilibrium deoxy form which is much smaller or absent in the 10 ns photoproduct. Thus, upon photolysis of the CO adduct, the heme structures in both the α and β subunits are essentially identical and differences must arise at some point during evolution of the R-state quaternary structure to the T-state. It is noted that previously reported transient RR studies of the ν(Fe-His) mode in Fe/Co hybrids (at pH 7, 3 mM IHP, "T-state" and pH 9, "R-state") yielded a distinctly lower frequency for only the α_{Fe} subunit of the $(\alpha_{Fe}\beta_{Co})_2$ species at pH = 7 ("T-like" state).[13a] Furthermore, it is important to point out that the results of recent TR^3 studies of low frequency heme modes in Hb and Hb-d4 are consistent with the present results.[13b] Thus, a low frequency out-of-plane heme mode (γ_7, $C_\alpha C_m$ wagging),[13c] which is observed as a relatively narrow band (at $\sim$300 cm^{-1}) in the RR spectrum of the photoproduct, broadens or splits into two bands (296, 305 cm^{-1}) at longer times (0.5−17 μs). While in the previous study subunit-specific marker bands were not available, the present results indicate that it is apparently the hemes of the α subunits which experience this structural change.

In summary, the exploitation of the deuterium sensitivity of certain heme core marker bands permits spectral resolution of the α and β subunit signals in RR and TR^3 studies of Hb. The results of this initial study document essentially identical heme structures for both subunits in the $\sim$10 ns photoproduct but significant differences in the equilibrium deoxy form. Subsequent studies will focus on the temporal evolution of the subunit heterogeniety and on definitive identification of the sites of geminate[14] and second-order recombination with CO.[11]

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