

The experimental approach used in this work is general and applicable to all those enzymes which react through an ordered mechanism and for which the first substrate to add can be synthesized in the appropriately labeled form.

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Resonance Coherent Anti-Stokes Raman Scattering Evidence for Out-of-Plane Heme Iron Displacement within 6 ns of CO Dissociation in CO Hemoglobin¹

Sir:

We report resonance CARS (coherent anti-Stokes Raman scattering) spectra of carbonmonoxyhemoglobin (COHb) which demonstrate that the iron atom moves out of the heme plane to its location in deoxyhemoglobin (0.60-Å displacement²) within 6 ns following photolysis of the CO ligand.

CARS is a nonlinear optical effect³ generated by crossing two laser beams, ω_1 and ω_2 , in the sample. The signal strength increases when the frequency difference, $\Delta\omega = \omega_1 - \omega_2$, matches a vibrational transition. A Raman spectrum is generated by plotting the CARS intensity as a function of $\Delta\omega$. The CARS cross section is proportional to the square of the ordinary Raman cross section, and is subject to enhancement via

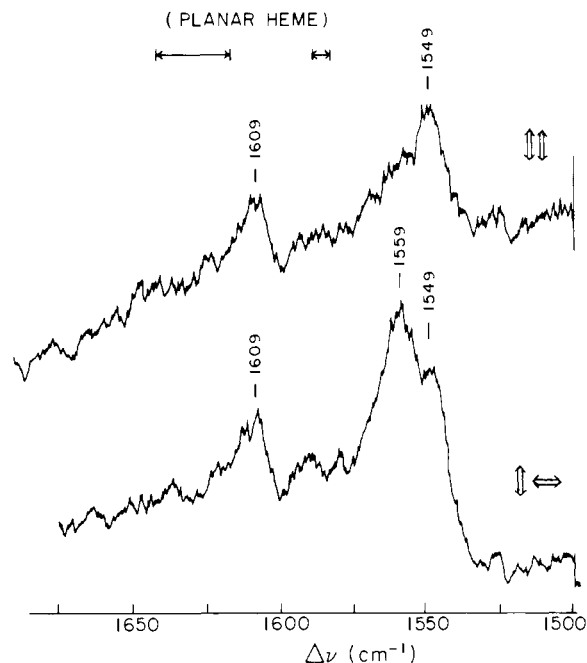


Figure 1: Polarized CARS spectrum of COHb: $\uparrow\uparrow$, dye lasers polarized in the same direction; \Leftrightarrow , dye lasers polarized orthogonal to each other; $\omega_1 = 569$ nm (pulse energy $\sim 6 \mu\text{J}$); $\omega_2 = 622\text{--}629$ nm (pulse energy $\sim 20 \mu\text{J}$); $[\text{COHb}] = 0.69$ mM; laser repetition rate = 10 pulses/s; 100-pulse average; ω_2 scan rate = 0.75 nm/min. Sample in Teflon-stoppered 1-mm path length visible cell.

electronic resonances.⁴ In our apparatus, ω_1 and ω_2 are the output of two tunable dye lasers pumped simultaneously by a 1 MW N_2 laser.⁵ The latter emits pulses of 10-ns duration, and the dye laser pulse width is 6 ns. Consequently the CARS experiment lasts 6 ns and is repeated many times to build up a spectrum.

Figure 1 shows the CARS spectrum of COHb obtained with ω_1 in resonance with the Q_0 transition at 569 nm. Q band, as opposed to Soret band resonance, was chosen because the depolarized and anomalously polarized Raman modes which are enhanced in this region have been demonstrated to be more sensitive to heme structural changes than are the polarized modes enhanced in the Soret region.⁶ CARS polarization measurements can be performed by aligning the polarizations of the two incident beams to be mutually parallel or perpendicular.⁵ The depolarization ratio, $\rho = I_{\perp}/I_{\parallel}$, has been shown to be approximately the square of the ordinary Raman depolarization ratio.⁷ Thus, depolarized, polarized, and anomalously polarized modes are expected to have $\rho_{\text{CARS}} \approx \rho_{\text{Raman}}^2 = 9/16$, $<9/16$, and $>9/16$, respectively.⁵

Although the polarized spectra of Figure 1 have not been corrected for the different incident laser powers inherent in the polarization experiment, it is apparent that they contain an anomalously polarized band at 1559 cm^{-1} and two depolarized bands at 1609 and 1549 cm^{-1} . These are exactly the features expected for deoxyhemoglobin⁸ and which we have observed in a CARS spectrum of deoxy-Hb itself, taken under the same conditions. (The quaternary structure has no influence on these frequencies, as has been demonstrated with carp Hb⁹ and with chemically modified human Hb.¹⁰) The resonance Raman frequencies of the first two of these bands in COHb are 1584 (ap) and 1631 cm^{-1} (dp).¹¹ We observe no discernible CARS peaks at these frequencies.

It is not surprising that most of the COHb molecules are photolyzed by the incident laser beams. Their combined energies are $\sim 25 \mu\text{J}/\text{pulse}$, while we calculate that only $4 \mu\text{J}$ are needed to photolyze all of the COHb molecules in the CARS interaction volume (estimated to be 8×10^{-6} mL)

Table I. Heme Marker Band Frequencies

	cm ⁻¹		
	III (dp) ^a	IV (ap) ^a	V (dp) ^a
COHb ^b		1584	1631
deoxy-Hb ^c	1546	1552	1607
(Im) ₂ Fe ^{II} (PP) ^d	1534	1583	1617
Fe ^{II} (MP) ^e	1570	1589	1642
COHb CARS ^f	1549	1559	1609

^a Band numbering from ref 15; dp = depolarized, ap = anomalously polarized. ^b From ref 11. ^c From ref 8. ^d Bis(imidazole) ferrous protoporphyrin IX (planar, low spin); from ref 15. ^e Ferrous mesoporphyrin IX dimethyl ester (four coordinate, intermediate spin); from ref 15. ^f This work; error in frequencies estimated as ± 3 cm⁻¹.

using the known absorptivities and dissociation quantum yield.¹² It is not possible to lower the laser energies sufficiently to reduce photolysis appreciably without losing the CARS signal (which varies linearly with the ω_2 power and as the square of the ω_1 power). The photolyzed COHb molecules have ample time to recombine in the 100 ms between the laser pulses as calculated using the second-order rate constant of 4.5×10^6 M⁻¹ s⁻¹.¹³ In order to ensure that our spectra were not artifacts due to changes which accumulate with repeated laser pulses, we redetermined them with the COHb solution circulating in a flow cell, with the estimated beam transit time being 20 ms. Although the signal/noise ratio deteriorated slightly, the spectral features were unaltered.

It is of interest that the CARS spectra show no evidence of peaks at the positions expected if the iron atom had stayed in the heme plane for a time comparable with 6 ns following the CO dissociation. The basis for this assertion is as follows. It is known that iron(II) tetraphenylporphyrin, without any axial ligands, exists in an intermediate spin, $S = 1$, ground state.¹⁴ The iron atom is in the porphyrin plane and the iron-nitrogen bonds are short,¹⁴ consistent with an empty $d_{x^2-y^2}$ orbital; the six iron(II) valence electrons occupy the four remaining d orbitals, with two electrons remaining unpaired. The resonance Raman spectrum of iron(II) mesoporphyrin,¹⁵ a close analogue of the protoporphyrin contained in Hb, is consistent with this picture. The three bands in the iron(II) mesoporphyrin of interest to this study are found at 1589 (ap) and at 1642 and 1570 cm⁻¹ (dp). When two imidazole ligands are bound to iron(II) protoporphyrin, the complex is low spin ($S = 0$) and the bands are at 1583 (ap) and 1617 and 1534 (dp). If CO dissociation from COHb leaves the iron atom transiently in the heme plane, with one axial imidazole ligand, the exact spin state is uncertain. However, the Raman frequencies can confidently be expected to be somewhere between those given above for four- and six-coordinate iron(II) heme (Table I summarizes the marker band frequencies expected for the various geometries along with the CARS data we have obtained). In particular, the ap band is expected at 1583–1589 cm⁻¹ and the high frequency dp band is expected at 1617–1642 cm⁻¹ for in-plane iron. As these bands are absent in our CARS spectra (see Figure 1), we conclude that the iron atom does not remain in the heme plane for an appreciable fraction of 6 ns.

Alpert et al.¹⁶ found an optical transient with a half-life of ~ 50 ns following laser photolysis of COHb which they suggested may be associated with a tilt of the porphyrin rings in the globin pocket. They rejected the possibility that the transient was associated with the iron out-of-plane displacement on the grounds that protein-free CO heme did not give the optical transient. This inference is open to the objection that the presence of protein restraints might slow down the iron displacement, but the present result confirms that the iron atom is out of the heme plane long before 50 ns. The photodissociation itself is very fast for COHb. Shank et al.¹⁷ reported at a time constant of < 0.5 ps for this process, while Rentzepis

and co-workers measured a value of 11 ps. Conceivably both CO dissociation and iron displacement could occur in a concerted process. Picosecond resonance Raman experiments will be needed to test this possibility.

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On the "Uncatalyzed" Oxidation of Sulfur(IV) in Aqueous Solutions

Sir:

Despite numerous reports on the rates of the uncatalyzed oxidations of aqueous sulfur(IV) solutions, we now report that the reactions are in fact trace-metal catalyzed. Using small amounts of chelating agents, EDTA and 1,10-phenanthroline, to complex trace metal ions, we have reduced the "uncatalyzed" oxidation rate up to four orders of magnitude at high pH and by a factor of 20 at low pH. We suggest that an analogous technique be used to test the degree to which other autoxidations, previously accepted as uncatalyzed, may be trace-metal catalyzed.

The oxidation of aqueous sulfur(IV) solutions to sulfate by molecular oxygen has attracted widespread attention since the early work of Bigelow,¹ with many studies²⁻⁸ concentrating largely on the kinetics of the previously assumed uncatalyzed reactions. There have been, however, major discrepancies in the reported kinetic parameters despite concerted efforts to eliminate contamination of the reactants from trace impurities. Owing to the extreme susceptibility of the oxidation reaction to catalysis by certain transition metal ions (Cu^{II} concentrations as low as 10^{-8} M have been reported as significantly affecting the sulfite reaction²), it appears that the reported anomalies may merely have been due to the varying levels of trace-metal impurities in the water and reactants used in the different investigations.

Rather than trying to achieve lower impurity levels than those in the previous studies, we masked the contribution to the reaction from trace-metal impurities by complexing with an effective chelating agent. EDTA was chosen in studying the