amine.

Protecting groups were removed (a) by liquid HF,[11] yielding at best 13% β-EP, and (b) by sodium in liquid NH_3,[12] providing up to 27.5% β-EP. For the HF cleavage, compound X (153 mg, 30 μmol) was exposed for 30 min at 0 °C to anhydrous liquid HF–anisole–diethyl sulfide (6:1:2). Typical workup[13] yielded 100 mg (84%) of a white powder. Reversed-phase HPLC (76 mg, 0.9 × 500 mm Partisil ODS-2 column, Pyr–AcOH–CH_3CN–*i*-C_3H_7OH–H_2O (5.9:1.9:13:13:66.2, v/v), isocratic mode, *o*-phthalaldehyde-fluorescence monitoring) produced directly homogeneous β-EP, Ia (12.0 mg, 13.2% based on X[14]), besides a multitude of side-product peaks (total peptide recovery was 55 mg (61%)). For the Na–liquid NH_3 cleavage, a suspension of compound X (51 mg, 10 mol) in anhydrous refluxing liquid NH_3 (125 mL) was treated for 30 min with a 10-fold excess of Na (60 mg, 2.6 mmol). Addition of NH_4Cl (0.13 g, 2.5 mmol), evaporation, Sephadex G-15 desalting (0.5 M AcOH), and lyophilization yielded 27 mg (68.7%) of white powder. Reversed-phase HPLC, as above, produced β-EP, Ib (10.8 mg, 27.5%, of a total peptide recovery of 24 mg in a few peaks).

Physicochemical and biological properties of human β-endorphin samples Ia and Ib were identical and indistinguishable from authentic material: AAA, $Lys_{5.00}$, $Asp_{2.08}$, $Thr_{2.97}$, $Ser_{1.90}$, $Glu_{3.13}$, $Pro_{0.95}$, $Gly_{3.2}$, $Ala_{2.08}$, $Val_{0.95}$, $Met_{1.03}$, $Ile_{1.70}$ (72-h hydrolysis), $Leu_{2.05}$, $Tyr_{1.92}$, $Phe_{1.93}$; analytical HPLC (5 μ, 0.46 × 15 cm ES Industries C-8 column, system as above), single symmetrical peak, $K' = 2.8$. ORD and CD spectroscopy of I in CH_3OH–H_2O (3:1) produced spectra superimposable with authentic human β-EP:[2,4] $[\alpha]^{270}_{\lambda}$ −206° at 400 nm, −538° at 300 nm, −2190° at 240 nm. The opioid activity of synthetic human β-EP was identical with that of authentic material in the guinea pig ileum assay[15] (50% inhibition at 1.8×10^{-9} M) and was blocked by the specific opiate antagonist naloxone (complete reversal at 3.0×10^{-8} M).

Acknowledgment. The authors thank Mrs. I. D. Kulesha, Mr. J. Michalewsky, and Dr. F. Scheidl for their help in this work.

Supplementary Material Available: Elemental analysis of peptides II–X (1 page). Ordering information is given on any current masthead page.

References and Notes

(1) Abbreviations used: AAA, amino acid analysis; AcOH, acetic acid; Boc, *tert*-butyloxycarbonyl; Bzl, benzyl; DCC, dicyclohexylcarbodiimide; DMF, dimethylformamide; β-EP, β-endorphin; Et, ethyl; *i*-C_3F_6HOH, hexafluoroisopropyl alcohol; *i*-C_3H_7OH, isopropyl alcohol; HOBt, 1-hydroxybenzotriazole; HPLC, high performance liquid chromatography; OBzl, benzyl ester; Pyr, pyridine; Z, benzyloxycarbonyl.
(2) Li, C. H. *Arch. Biochem. Biophys.*, **1977**, 183, 592–604.
(3) Li, C. H.; Yamashiro, D.; Tseng, L. F.; Loh, H. H. *J. Med. Chem.*, **1977**, 20, 325–328. Atherton, E.; Caviezel, M.; Over, H.; Sheppard, R. *J. Chem. Soc., Chem. Commun.*, **1977**, 819–821. Coy, D. H.; Gill, P.; Kastin, A. J.; Dupont, A.; Cusan, L.; Labrie, F.; Britton, D.; Fertel, R. *ibid.*, **1977**, 107–110. Segal, D. S.; Browne, R. G.; Bloom, F.; Ling, N.; Guillemin, R. *Science*, **1977**, 198, 411–414.
(4) Li, C. H.; Lemaire, S.; Yamashiro, D.; Doneen, B. A. *Biochem. Biophys. Res. Commun.*, **1976**, 71, 19–25. Synthetic β-EP prepared in this work behaved identically in CD and ORD spectroscopy and in vitro bioassay with synthetic material of Li et al. which was shown to be indistinguishable from isolated natural material.[2]
(5) Honzl, J. and Rudinger, J. *Collect. Czech. Chem. Commun.*, **1961**, 26, 2333–2344.
(6) Most effective and selective Boc group cleavage (see Schnabel, E.; Klostermeyer, H.; Berndt, H. *Justus Liebigs Ann. Chem.*, **1971**, 749, 90–108) of II was obtained by BF_3–OEt_2, of IV, VI, and VIII by HCOOH.
(7) Dupuis, G.; Finn, F. M.; Storey, H. T.; Yanaihara, C.; Yanaihara, N.; Hofmann, K. *J. Am. Chem. Soc.*, **1971**, 93, 5526–5539.
(8) Correct elemental analyses were obtained for all protected peptide intermediates; see paragraph at end of paper regarding supplementary material.
(9) König, W. and Geiger, R. *Chem. Ber.*, **1970**, 103, 788–798.
(10) Geiger, R.; Jäger, G.; König, W.; Volk, A. *Z. Naturforsch.*, **1969**, 24B, 999–1004.
(11) Sakakibara, S.; Shimonishi, Y.; Kishida, Y.; Okada, M.; Sugihara, H. *Bull. Chem. Soc. Jpn.*, **1967**, 40, 2164–2167.
(12) Sifferd, R. H.; du Vigneaud, V. *J. Biol. Chem.*, **1935**, 108, 753–761. Roberts, C. W. *J. Am. Chem. Soc.*, **1954**, 76, 6203–6204. Na–liquid NH_3 cleavage (under nonideal conditions, i.e., use of excess Na because of the presence of ammonolysable benzyl ester groups) was used because of the very high degradative loss during liquid HF cleavage.
(13) See, e.g., Watanabe, H.; Ogawa, H.; Yajima, H. *Chem. Pharm. Bull.*, **1975**, 23, 375–383. Evaporation of HF, dissolution of residue in 1 M AcOH (20 mL), extraction with ether, lyophilization (white powder), Sephadex G-15 gel filtration using 0.5 N AcOH, and lyophilization.
(14) Molecular weight of lyophilized I ~3935, based on a peptide content of ~88%.
(15) Kosterlitz, H. W.; Lydon, R. J.; Watt, A. J. *Br. J. Pharmacol.*, **1970**, 39, 398–413. IC_{50} was determined by plotting log molar concentration against mean percent inhibition. The peptides were assayed twice at five different concentrations (2–10 μg/mL). Dose response curves were superimposable.

Chrysa Tzougraki, Raymond C. Makofske
Thomas F. Gabriel, Su-Sun Wang
Rostyslaw Kutny, Johannes Meienhofer*
Chemical Research Department, Hoffmann-La Roche Inc.
Nutley, New Jersey 07110

Choh Hao Li
Hormone Research Laboratory, University of California
San Francisco Medical Center
San Francisco, California
Received May 26, 1978

A Kinetic α-Deuterium Isotope Effect for the Binding of Purine Nucleosides to Calf Spleen Purine Nucleoside Phosphorylase. Evidence for Catalysis by Distortion

Sir:

Kinetic α-deuterium isotope effects, k_H/k_D, for the calf spleen purine nucleoside phosphorylase (E.C. 2.4.2.1) catalyzed phosphorolysis of adenosine at pH 7.2 and inosine at pH 7.5 are 1.047 ± 0.017 and 1.043 ± 0.004, respectively, Table I. As detailed below, these values suggest that binding of the nucleoside to the active site of the enzyme occurs with significant change of the geometry of these substrates at C-1′.

Kinetic α-deuterium isotope effects originate almost wholly from differences between the zero-point energies of the two isotopic species in the ground state and transition state resulting from the sensitivity of the α-hydrogen bending mode vibrations to isotopic substitution.[1] Thus, such isotope effects can provide information about changes in bonding which occur as the reactant proceeds from ground to transition state and have been successfully exploited in mechanistic studies of organic reactions[2] as well as enzymic ones.[3,4] In certain enzymatic cases, the α-deuterium effect reflects only binding of the substrate to the enzyme and thus may reveal vibrational perturbations at the isotopically substituted atom resulting from enzyme-induced substrate distortion.

The potential utility of kinetic α-deuterium isotope effects as probes of substrate distortion for enzymic reactions was investigated for calf spleen purine nucleoside phosphorylase (PNPase):[5]

$$\text{purine nucleoside} + P_i \rightleftharpoons \text{purine base} + \text{ribose 1-phosphate} \quad (1)$$

PNPases are specific for purine nucleosides having the β configuration at C-1′ and the reaction proceeds with inversion at this center. The nature of kinetic plots for all PNPases studied suggests a sequential rather than ping-pong mechanism; that is, catalysis occurs through a ternary complex of PNPase, nucleoside, and orthophosphate.[5]

Specifically, the reaction catalyzed by PNPase from calf spleen proceeds through an ordered Bi-Bi kinetic mechanism with nucleoside adding to the enzyme before phosphate.[6] Northrop has demonstrated that for enzymes having such a kinetic mechanism the observed kinetic isotope effect, determined by a competitive method, is for binding of the first

0002-7863/78/1500-6249$01.00/0

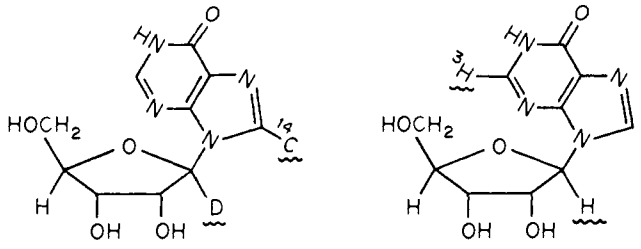
Table I. Kinetic α-Deuterium Isotope Effects for the Calf Spleen Purine Nucleoside Phosphorylase Catalyzed Phosphorolysis of Adenosine and Inosine at 25 °C

	adenosine[a]	inosine[b]
1	1.057	1.047
2	1.055	1.045
3	1.028	1.042
4		1.037
Av	1.047 ± 0.017[c]	1.043 ± 0.004[c]

[a] [Phosphate] = 0.2 M, pH 7.2. [b] [Phosphate] = 0.06 M, pH 7.5. [c] Standard deviation.

substrate to the enzyme if this substrate contains the isotopic label and the second substrate is at saturating concentrations.[7] Accordingly, the kinetic α-deuterium isotope effects reported herein were measured with levels of phosphate 100 to 400 times greater than the K_m value for phosphate ($K_m = 5 \times 10^{-4}$ M[6]) for calf spleen PNPase.

Kinetic isotope effects were determined using the competitive double-label technique developed by Raftery.[8,9] A mixture of substrate labeled with deuterium at C-1′ and ^{14}C at purine C-8 and protio substrate labeled only with ^{3}H at purine C-2 was allowed to react with calf spleen PNPase for a predetermined time to a yield of 5% or less. At the end of the reaction



the purine product and unreacted nucleoside were separated chromatographically and counted in a scintillation counter to determine the $^{14}C/^{3}H$ ratios for these materials. The isotope effects were calculated according to

$$\frac{k_H}{k_D} = \frac{(^{14}C/^{3}H)_{purine}}{(^{14}C/^{3}H)_{unreacted\ nucleoside}} \quad (2)$$

Details of the methodology have been presented elsewhere.[8-11]

Calf spleen PNPase was purchased from Sigma Chemical Co. as a crystalline suspension in 3.2 M $(NH_4)_2SO_4$, pH 6.0. The specific activity of the preparation used was 24 U/mg of protein.

The syntheses of the labeled substrates have been described.[10-12] Deuterium label was incorporated at the C-1′ position to an extent of no less than 95% as ascertained by ^{1}H NMR spectroscopy (HA-220). For the deuterio substrates no resonance corresponding to a proton on C-1′ was observed, despite repeated scanning of the appropriate region of the spectrum.

The measured isotope effects (Table I) for PNPase-catalyzed nucleoside degradation are small, but distinctly different from unity as evidenced by the following: (i) reproducibility of the measured effect (standard deviations of 2% or less are obtained for these measurements and isotope effects >1.02 can confidently be said to be different from unity); (ii) measurement of isotope effects of unity (this laboratory has reproducibly measured kinetic α isotope effects equal to 1.00[10,13] including those for the phosphorolysis of adenosine (1.00 ± 0.02) and inosine (1.009 ± 0.001) catalyzed by *E. coli* PNPase at pH 7.3[13]); and (iii) carefully conducted control experiments[11-13] designed to detect potential problems such as tritium exchange out of substrate or product and scrambling of ^{14}C and ^{3}H purine bases by enzyme-catalyzed base exchange.

The value of k_H/k_D of 1.04-1.05 for binding of inosine and adenosine to calf spleen PNPase requires that the vibrations associated with the α hydrogen have been loosened in going from the unbound reactant molecules to the transition state for binding. Thus, as the nucleoside binds to the active site of the enzyme, distortion of the ribose ring occurs causing the 1′ carbon to undergo a geometry change characteristic of partial $sp^3 \rightarrow sp^2$ rehybridization.[14] The energy required to bring about this distortion is presumably supplied through some process by which the enzyme utilizes binding energy derived from favorable interactions with parts of the substrate other than the reacting group.

The minimum energy needed to bring about this distortion can be calculated by application of the Streitweiser approximation:[15]

$$\frac{k_H}{k_D} = \exp\left[\frac{0.187}{T}\sum_i (\nu_{Hi} - \nu_{\ddagger Hi})\right] \quad (3)$$

For a kinetic isotope effect of 1.04, the sum of the differences between the isotopically sensitive frequencies in the ground and transition states is equal to 63 cm^{-1} or 1.9×10^{12} s^{-1}. This corresponds to an energy change of 1.25×10^{14} erg/mol or 0.18 kcal/mol. This represents a lower limit to the portion of the total binding energy utilized by the enzyme to bring about substrate distortion.

Based only on the measured isotope effects, it is impossible to know what vibrational changes, other than those involving the α hydrogen, occur as the nucleoside undergoes binding to enzyme. As C-1′ assumes its new geometry, the C–N bond may be stretched and the annular oxygen may be repositioned to optimize orbital overlap with the incipient carbonium ion, but such coupling has not been demonstrated. If there is a lack of coupling, distortion of the ribose ring, perhaps effected through interactions of the enzyme with the three hydroxyls, would perturb the vibrational modes associated with the α hydrogen on C-1′ without appreciably altering the bonding of the aglycone or annular oxygen to the C-1′.

The phenomenon of binding-induced substrate distortion has been suggested previously for the enzyme lysozyme. Evidence for the distortion of the saccharide bound in subsite D into a half-chair conformation during the binding of oligosaccharide substrates to lysozyme comes from several sources: (i) X-ray crystallographic studies of the nonproductive binding of chitotriose to the A, B, and C subsites of lysozyme and model building based on these studies;[16,17] (ii) thermodynamic studies of substrate binding which estimate that the contribution to the free energy of binding of oligosaccharides from the saccharide bound in subsite D is unfavorable by 3-6 kcal/mol;[18-22] and (iii) ^{1}H NMR studies which examined the change in the C-1′-C-2′ coupling constant between the free trisaccharide substrate and substrate bound in the C, D, and E subsites.[23]

Binding-induced substrate distortion may be a general phenomenon, accounting for the frequent observation of the manifestation of substrate specificity in V_{max} rather than in K_m.[24,25]

Although kinetic isotope effects provide a sensitive probe of binding-induced substrate distortion, rather few isotope effects for binding have been measured. Such an effect has been measured for the binding of a mixture of NADH and [A-4-^{2}H]-NADD to equine liver alcohol dehydrogenase,[26,27] an enzyme which specifically transfers the A hydrogen. This effect was measured independently in two laboratories, under somewhat different conditions, with the results that $K_H/K_D = 1.00 \pm 0.02$[26] or $K_H/K_D = 1.72 \pm 0.26$[27] where the effect is for the binding constant. The discrepancy here remains to be resolved.

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.

References and Notes

(1) M. Wolfsberg and K. J. Stern, *Pure Appl. Chem.*, **8**, 225 (1964).
(2) (a) V. J. Shiner, Jr., in "Isotope Effects in Chemical Reactions", C. J. Collins and N. S. Bowman, Ed., Van Nostrand-Reinhold Co., Princeton, N.J., 1970; (b) *ACS Symp. Ser.*, **No. 11** (1975).
(3) J. Hogg in "Transition States of Biochemical Processes", R. D. Gandour and R. L. Schowen, Ed., Plenum Press, New York, N.Y. 1978.
(4) E. H. Cordes and H. G. Bull in ref 3.
(5) R. E. Parks, Jr., and R. P. Agarwal in "Enzymes", Vol. 7, 3rd ed, P. D. Boyer, Ed., Academic Press, New York, N.Y. 1972, p 468.
(6) T. A. Krenitsky, *Mol. Pharmacol.*, **3**, 526 (1967).
(7) D. B. Northrop in "Isotope Effects of Enzyme Catalyzed Reactions", W. W. Cleland, M. H. O'Leary, and D. B. Northrop, Ed., University Park Press, Baltimore, Md., 1977. Although this point can easily be demonstrated algebraically, it follows directly from the fact that saturating concentrations of the second substrate make addition of the first one irreversible. Thus isotopic discrimination, if any, reflects an isotope effect on the rate of the binding of the first substrate.
(8) F. W. Dahlquist, T. Rand-Meir, and M. A. Raftery, *Proc. Natl. Acad. Sci. U.S.A.*, **61**, 1194 (1968).
(9) F. W. Dahlquist, T. Rand-Meir, and M. A. Raftery, *Biochemistry*, **8**, 4214 (1969).
(10) H. G. Bull, J. Ferraz, E. H. Cordes, A. Ribbi, and R. Apitz-Castro *J. Biol. Chem.*, **253**, 5186 (1978).
(11) R. Romero, Ph.D. Thesis, Indiana University, 1978.
(12) R. Romero, R. L. Stein, H. G. Bull, and E. H. Cordes, *J. Am. Chem. Soc.*, in press.
(13) R. L. Stein, Ph.D. Thesis, Indiana University, 1978.
(14) There is no compelling reason to rule out an extreme variant of this conclusion: that the binding of nucleoside to the enzyme involves fission of the C–N bond to generate an EA complex containing a carboxonium ion. A referee was kind enough to point out that this represents a terminal case of "catalysis by distortion".
(15) A. Streitweiser, R. H. Jagow, R. C. Fahey, and S. Suzuki, *J. Am. Chem. Soc.*, **80**, 2326 (1958).
(16) C. C. F. Blake, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. R. Soc. London, Ser. B*, **167**, 365 (1967).
(17) C. C. F. Blake, L. N. Johnson, G. A. Mair, A. C. T. North, D. C. Phillips, and V. R. Sarma, *Proc. R. Soc. London, Ser. B*, **167**, 378 (1967).
(18) D. M. Chipman, V. Grisaro, and N. Sharon, *J. Biol. Chem.*, **242**, 4388 (1967).
(19) D. M. Chipman and N. Sharon, *Science*, **165**, 454 (1969).
(20) D. M. Chipman, *Biochemistry*, **10**, 1714 (1971).
(21) L. N. Johnson, D. C. Phillips, and J. A. Rupley, *Brookhaven Symp. Biol.*, **21**, 120 (1968).
(22) J. A. Rupley and V. Gates, *Proc. Natl. Acad. Sci., U.S.A.*, **57**, 496 (1967).
(23) B. D. Sykes, S. L. Patt, and D. Dolphin, *Cold Spring Harbor Symp, Quant. Biol.*, **36**, 29 (1971).
(24) W. P. Jencks, "Catalysis in Chemistry and Enzymology", McGraw-Hill, New York, NY 1969.
(25) W. P. Jencks *Adv. Enzymol.*, **43**, 219 (1975).
(26) E. deJuan and K. B. Taylor, *Biochemistry*, **15**, 2523 (1976).
(27) K. Bush, H. R. Mahler, and V. J. Shiner, Jr., *Science*, **172**, 478 (1971).

R. L. Stein, R. Romero, H. G. Bull, E. H. Cordes*
Department of Chemistry, Indiana University
Bloomington, Indiana 47401

Received May 25, 1978

Resonance Coherent Anti-Stokes Raman Scattering Evidence for Out-of-Plane Heme Iron Displacement within 6 ns of CO Dissociation in CO Hemoglobin[1]

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 displacement[2]) within 6 ns following photolysis of the CO ligand.

CARS is a nonlinear optical effect[3] 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 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.

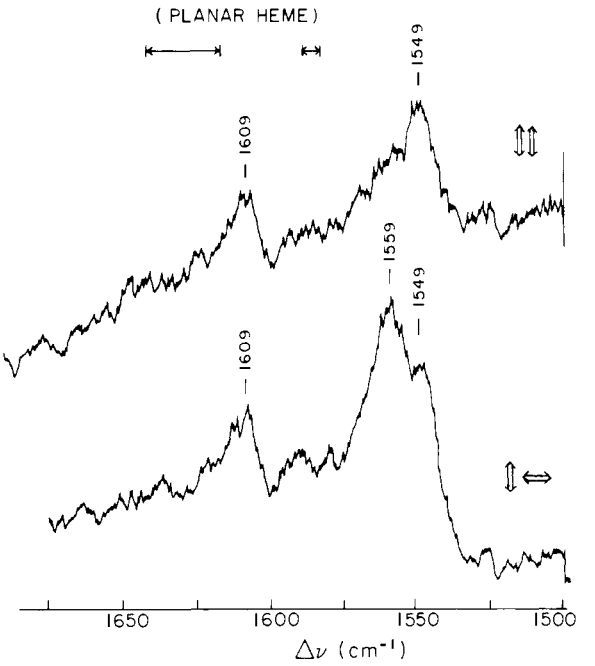


Figure 1: Polarized CARS spectrum of COHb: ⇕⇕, dye lasers polarized in the same direction; ⇔, dye lasers polarized orthogonal to each other; ω_1 = 569 nm (pulse energy ~6 μJ); ω_2 = 622-629 nm (pulse energy ~20 μJ); [COHb] = 0.69 mM; laser repetition rate = 10 pulses/s; 100-pulse average; ω_2 scan rate = 0.75 nm/min. Sample in Teflon-stoppered 1-mm path length visible cell.

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 $\rho_{CARS} \approx \rho^2_{Raman}$ = 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 *deoxy*hemoglobin[8] 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 Hb[9] 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 ~25 μJ/pulse, while we calculate that only 4μJ are needed to photolyze all of the COHb molecules in the CARS interaction volume (estimated to be 8×10^{-6} mL)

0002-7863/78/1500-6251$01.00/0