A Time-Resolved Photoacoustic Calorimetry Study of the Dynamics of Enthalpy and Volume Changes Produced in the Photodissociation of Carbon Monoxide from Sperm Whale Carboxymyoglobin†

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ABSTRACT: The dynamics of the enthalpy and volume changes produced in the photodissociation of carbon monoxide from sperm whale myoglobin is investigated by time-resolved photoacoustic calorimetry. The enthalpy and volume changes for the formation of the geminate pair, which occurs within 50 ns of photolysis, are $\Delta H = -2.2 \pm 2.8$ kcal/mol and $\Delta V = -10.0 \pm 1.0$ mL/mol relative to carboxymyoglobin. The enthalpy and volume changes associated with formation of deoxymyoglobin and solvated carbon monoxide, formed with a half-life of 702 ± 31 ns at 20 °C, are $\Delta H = 14.6 \pm 3.4$ kcal/mol and $\Delta V = 5.8 \pm 1.0$ mL/mol relative to carboxymyoglobin.

The mechanism of the binding and dissociation of carbon monoxide to sperm whale myoglobin has been extensively investigated. This subject has been addressed by a variety of experiments and theoretical studies (Antonini & Brunori, 1971), yet two questions are unresolved: (i) the pathway by which a ligand diffuses between the heme pocket and the solvent and (ii) how the protein structure modulates the binding energy of the ligand. The structures of deoxymyoglobin and carboxymyoglobin reveal no accessible channels through the protein matrix for ligand diffusion. In order to create a channel that would allow for ligand diffusion, substantial reorganization of the protein structure is required. On the basis of recent molecular dynamic calculations (Case & Karplus, 1979) and X-ray diffraction studies (Ringe et al., 1984; Kuriyan et al., 1986) of sperm whale myoglobin, several pathways, each requiring large amplitude motions of the amino acid side chains, have been proposed. In this paper, we report a time-resolved photoacoustic calorimetry study of sperm whale myoglobin and the implications of the results upon the mechanism of ligand diffusion.

Time-resolved photoacoustic calorimetry monitors the dynamics of enthalpy and volume changes associated with reactive intermediates produced photochemically (Rudzki et al., 1985). The present experiment measures the sum of enthalpy and volume changes occurring on a time scale of less than 50 ns. For events between 50 ns and 50 μs, both the dynamics and amplitudes of enthalpy and volume changes are resolved. We have applied this methodology to a variety of organic (Goodman & Peters, 1986; Goodman et al., 1986a) and organometallic reactions (Goodman et al., 1986b; Yang et al., 1986). This report is our first application of time-resolved photoacoustic calorimetry to a problem of biochemical interest. Ort and Parsons (1979) have developed a photoacoustic method for determining enthalpy and volume changes on time scales longer than 100 μs and have applied it to the photochemical cycle of bacteriorhodopsin.

Experimental Procedures

Apparatus. A PRA nitrogen-pumped dye laser (LN1000/LN102) operating at 2 Hz, 510 nm, pulse width 500 ps, 19 μJ, is used for excitation. The sample is held in a 1 cm × 1 cm cuvette embedded in an aluminum block thermostated by a Haake A80 temperature controller. The acoustic waves are detected by a 1-MHz lead zirconate–lead titanate piezoelectric transducer whose output voltage is amplified (Panametric ultrasonic preamp and PAR 113 amplifier) and recorded by a transient digitizer (LeCroy WD 8256). The data are transferred to a Digital MINC laboratory computer where each acoustic wave is normalized to the laser energy measured by a Laser Precision, Rj-7000, pyrolytic probe. Each acoustic wave for a given sample represents the average of 100 laser shots. The deconvolution of the experimental acoustic waves is performed by the computer program DECON on a Vax 11/750. A more extensive description of the experiment and the method of deconvolution has recently been reported (Rudzki et al., 1985).

Sample. Sperm whale metmyoglobin (Sigma) is dissolved in a 0.1 M tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl) buffer, pH 8.0, to produce a 1.6×10^{-5} M solution. The solution is filtered through a 0.45-μm Millipore filter. Deoxygenation of the metmyoglobin solution is achieved by stirring under a flow of N_2 at 22 °C for 1 h. The sample is then reduced with a tenfold excess of ascorbic acid (Sigma). Carboxymyoglobin is obtained by bubbling CO through the deoxymyoglobin solution. The ratio of absorbances for deoxymyoglobin (556 nm/434 nm) and carboxymyoglobin (579 nm/542 nm) are reported by Hardmann et al. (1966) to be 0.105 and 0.872, respectively. Only samples whose absorbance ratios are within 1% of these values are employed in the experiments.

Methods and Results

The principles of the photoacoustic effect are well established (Patel & Tam, 1981). When a molecule absorbs a photon from a monochromatic light pulse, the absorbed photon may be converted into thermal energy, E_{TH}, which will give rise to an increase in temperature along a cylinder defined by the path of the laser beam through the sample. This increase in temperature, ΔT, in turn will give rise to an increase in

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volume of the solution, ΔV, along the irradiated cylinder, generating an acoustic wave that propagates radially outward. Assuming that all the photon energy is converted into thermal energy, the increase in E_{TH} along the laser beam is expressed as

$$E_{TH} = E_0(1 - 10^{-A}) \quad (1)$$

where A is the absorbance of the sample and E_0 is the energy of the laser pulse. The increase in temperature along the irradiated cylinder, with initial volume V_0, is

$$\Delta T = E_{TH}/C_p\rho V_0 \quad (2)$$

where C_p is the heat capacity of the solution and ρ is its density. Finally, the volume change is related to the temperature change through the thermal expansion coefficient of the solution, β.

$$\Delta V = \beta V_0 \Delta T \quad (3)$$

For the chemical systems of interest, photolysis gives rise to metastable species whose energetics relative to the reactant are unknown. The energetics of the intermediate are established by comparison of the acoustic wave generated by the decay of the excited state to create the intermediate, producing thermal energy E_{TH}(sample), with that of a reference compound whose excited-state decay converts the entire photon energy into heat, E_{TH}(ref). The ratio of acoustic wave amplitudes ϕ represents the fraction of the photon energy that is converted into heat.

$$\phi = \Delta V_{sample}/\Delta V_{ref} = E_{TH}(\text{sample})/E_{TH}(\text{ref}) \quad (4)$$

Thus, the enthalpy of the intermediate relative to the reactant is

$$\Delta H = E_{photon}(1 - \phi) \quad (5)$$

The acoustic wave detector, a piezoelectric transducer, is sensitive not only to the amplitude of the acoustic wave, which carries the enthalpic information, but also to the temporal profile of the acoustic wave, revealing the dynamics of the decay process providing that the decay dynamics are on the time scale of the transducer response. The time-dependent voltage produced by the transducer, $E(t)$, is the result of the convolution of a time-dependent heat source, $H(t)$, with the instrument response function $T(t)$.

$$E(t) = H(t)*T(t) \quad (6)$$

For a kinetic process that involves two intermediates with sequential decays

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C \quad (7)$$

the time dependence of the concentrations for A and B is

$$[A] = A_0 \exp(-t/\tau_1)$$

$$[B] = \frac{A_0 k_1}{k_2 - k_1}[\exp(-t/\tau_1) - \exp(-t/\tau_2)] \quad (8)$$

This leads to a the time-dependent heat source that can be expressed (Rudzki et al., 1985) as

$$H(t) = \phi_1 \exp(-t/\tau_1) + \frac{\phi_2 k_1}{k_2 - k_1}[\exp(-t/\tau_1) - \exp(-t/\tau_2)] \quad (9)$$

where $k = 1/\tau$. The values ϕ_1 and ϕ_2, which relate to the enthalpy changes for kinetic processes 1 and 2, and the associated relaxation times τ_1 and τ_2 are obtained by a deconvolution procedure. A set of values for the four fitting parameters are used to calculate $H(t)$, which is then convoluted with the instrument response function $T(t)$, obtained from a calibration compound, to generate a calculated experimental wave, $E_{exptl}(t)$. The calculated wave is compared to the experimental wave by the sum of the square of the residuals. The four fitting parameters are then varied to minimize the residuals.

The fitting parameter ϕ is the ratio of the volume change for the sample to the volume change of the reference. In previous experiments (Goodman & Peters, 1986; Goodman et al., 1986a,b) it was assumed that the volume changes are produced only by thermal expansion due to an increase in temperature induced by the reacting molecules. However, if changes in molecular conformations are associated with the relaxation process, a second source of volume change will then contribute to the production of the acoustic wave (Ort & Parson, 1979). Consequently, the acoustic wave may be produced by two types of volume changes, thermal and conformational.

$$\Delta V = \Delta V_{TH} + \Delta V_{CON} \quad (10)$$

These two contributions are separable through the temperature dependence of the acoustic wave amplitude. From eq 3, the overall volume change is expressed as

$$\Delta V = \beta V_0 \Delta T + \Delta V_{CON} \quad (11)$$

The thermal expansion coefficient, β, of water is temperature dependent and has a value of zero at 3.9 °C. On the other hand, it will be shown that ΔV_{CON} is approximately temperature independent. The calibration compound deoxymyoglobin undergoes no net photochemistry on a time scale of 1 ns and thus serves only to convert the photon energy into heat. Therefore

$$\phi = \Delta V_{sample}/\Delta V_{ref} = \frac{\beta(T)V_0\Delta T_{sample}}{\beta(T)V_0\Delta T_{ref}} + \frac{\Delta V_{CON}}{\beta(T)V_0\Delta T_{ref}} \quad (12)$$

$$= \phi_{TH} + \Delta V_{CON}/[\beta(T)V_0\Delta T_{ref}] \quad (13)$$

where ϕ_{TH} represents the thermal contribution of ϕ. By setting $X = \beta(T')/\beta(T)$, then

$$\phi = \phi_{TH} + \Delta V_{CON}X/[\beta(T')V_0\Delta T_{ref}] \quad (14)$$

Defining

$$\phi_{CON}(T') = \Delta V_{CON}/[\beta(T')V_0\Delta T_{ref}] \quad (15)$$

then

$$\phi = \phi_{TH} + \phi_{CON}(T')X \quad (16)$$

where $\phi_{CON}(T')$ represents a ratio of volume changes at a reference temperature T'. Consequently, a plot of ϕ vs X should be linear with a slope of $\phi_{CON}(T')$ and an intercept of ϕ_{TH}. Both ϕ and X are obtained experimentally as a function of temperature. Deconvolution of the carboxymyoglobin wave form yields ϕ, and the ratio of the amplitudes of the deoxymyoglobin wave form at the reference temperature T' and temperature T yields X.

The temperature dependence of the thermal expansion coefficient of the solvent is illustrated in Figure 1. The 510-nm irradiation of 1.6×10^{-5} M deoxymyoglobin in 0.1 M Tris-HCl, pH 8.0, produces acoustic waves whose amplitudes are temperature dependent and approach zero amplitude at approximately 4 °C. The waves above 4 °C represent an overall positive volume of expansion as the thermal expansion coefficients are positive. In order to test the relationship between the thermal expansion coefficient of 1.6×10^{-5} M deoxymyoglobin in 0.1 M Tris-HCl and the thermal expansion coefficient of distilled water, the acoustic wave amplitudes at

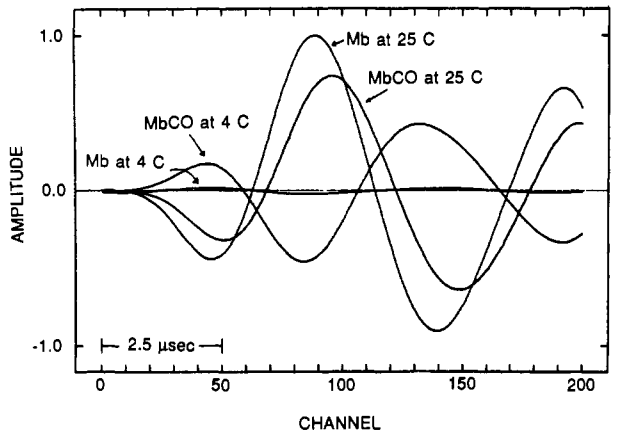

FIGURE 1: Temperature dependence of the photoacoustic signal from deoxymyoglobin (Mb) and carboxymyoglobin (MbCO), 1.6×10^{-5} M, in 0.1 M Tris-HCl, pH 8.0; λ_{exc} = 510 nm at 25 and 4 °C.

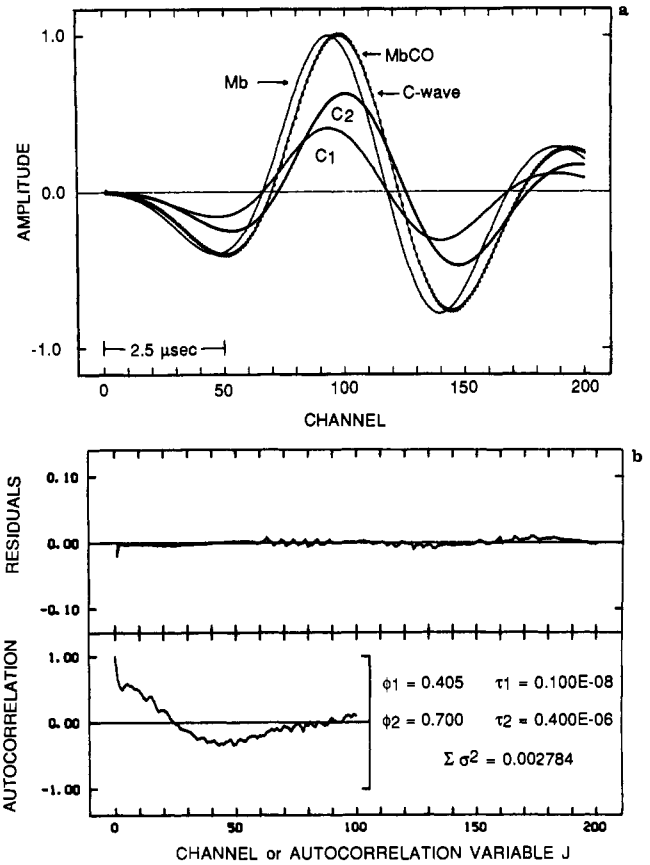

FIGURE 2: Deconvolution of the carboxymyoglobin wave form at 30 °C; λ_{exc} = 510 nm. (a) T-wave, deoxymyoglobin (Mb), 1.6×10^{-5} M; E-wave (solid line), carboxymyoglobin (MbCO); C-wave (dotted line), sum of C_1 (τ_1 = 1 ns, ϕ_1 = 0.405) and C_2 (τ_2 = 400 ns, ϕ_2 = 0.700). (b) Diagnostics of the deconvolution.

the various temperatures, ranging from 30 to 4 °C, were correlated with the corresponding distilled water values and were found to yield a slope of 0.98 ± 0.02. The acoustic wave amplitudes were scaled so that the 30 °C value for the buffer was identical with the 30 °C value for distilled water. Since the temperature dependence of the acoustic amplitudes in 0.1 M Tris-HCl is identical with the thermal expansion coefficient of distilled water and goes to zero at approximately 4 °C, it is concluded that the thermal expansion coefficient of a 0.1 M Tris-buffered myoglobin solution is virtually identical with that of distilled water at a given temperature.

The temperature dependence of the acoustic wave amplitudes for carboxymyoglobin is shown in Figure 1. As the

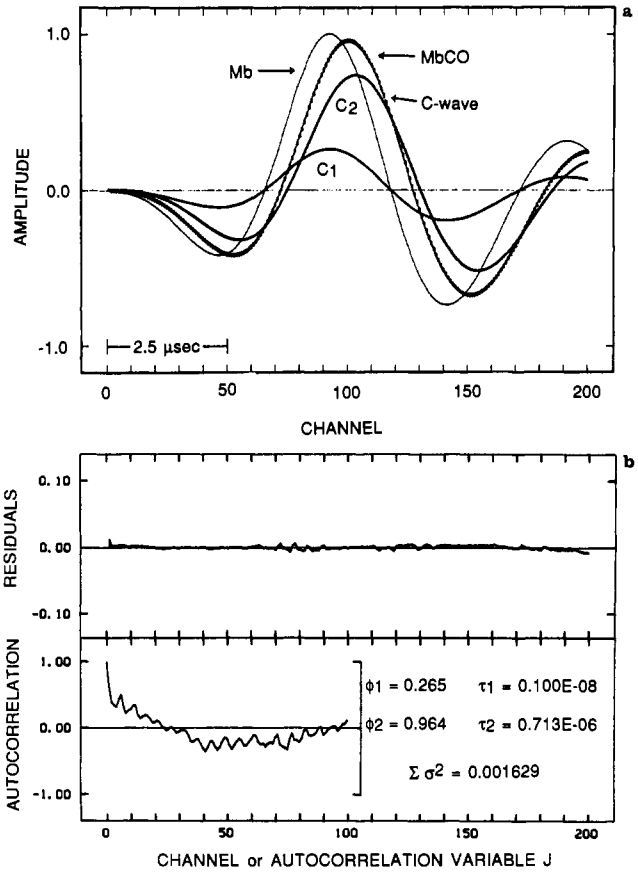

FIGURE 3: Deconvolution of the carboxymyoglobin wave form at 20 °C; λ_{exc} = 510 nm. (a) T-wave, deoxymyoglobin (Mb), 1.6×10^{-5} M; E-wave (solid line), carboxymyoglobin (MbCO); C-wave (dotted line), sum of C_1 (τ_1 = 1 ns, ϕ_1 = 0.265) and C_2 (τ_2 = 713 ns, ϕ_2 = 0.964). (b) Diagnostics of the deconvolution.

temperature is decreased from 30 °C, there is a reduction in the wave amplitude and a pronounced shift in the wave. The acoustic wave at 4 °C represents almost entirely the volume change due to a change in conformation of the protein following irradiation. The sign of amplitude of the wave at 4 °C is opposite to that produced in the irradiation of deoxymyoglobin at temperatures above 4 °C and therefore reveals a component of the volume change for carboxymyoglobin that is negative.

The deconvolution of the carboxymyoglobin acoustic wave forms was carried out by using the computer program DECON (Rudzki et al., 1985). The instrument response function, $T(t)$, was obtained from deoxymyoglobin irradiation. In order to achieve an acceptable fit between the calculated and experimental waves, it was necessary to employ a kinetic scheme involving a minimum of two sequential decays for $H(t)$ (eq 9). In addition, the fitting parameter τ_1 was held constant at 1 ns. Following absorption of a 510-nm photon by carboxymyoglobin, vibrational relaxation within the excited singlet state as well as dissociation of CO occurs on a time scale shorter than the instrument response, 50 ns. The instrument will respond to the sum of these enthalpic changes, manifested as ϕ_1. Consequently, for the deconvolution, only three parameters were varied: ϕ_1, ϕ_2, and τ_2. The results of the deconvolution of five separate experiments are given in Table I. Examples of the deconvolution for two data sets at 30 and 20 °C are shown in Figures 2 and 3. For the deconvolutions, both ϕ_1 and ϕ_2 were varied independently from −0.5 to 1.5 in increments of 0.01. Also, τ_2 was varied from 50 ns to 10 μs. Five separate experiments were performed, encompassing four temperatures, 30, 25, 20, and 15 °C, and comprising a total

Table I: Temperature Dependence of the Fitting Parameters for Carboxymyoglobin with τ_1 = 1 ns

	T (°C)	ϕ_1	ϕ_2	τ_2 (ns)
run 1[a]	30	0.431 ± 0.027[c]	0.664 ± 0.028	383 ± 21
	20	0.258 ± 0.005	0.954 ± 0.007	695 ± 16
run 2	30	0.463 ± 0.024	0.628 ± 0.022	371 ± 25
	20	0.262 ± 0.007	0.943 ± 0.010	654 ± 34
run 3	30	0.451 ± 0.022	0.627 ± 0.041	345 ± 43
	20	0.251 ± 0.003	0.960 ± 0.007	692 ± 17
run 4[b]	30	0.456 ± 0.043	0.657 ± 0.042	439 ± 10
	25	0.351 ± 0.011	0.787 ± 0.010	541 ± 8
	20	0.211 ± 0.010	1.00 ± 0.011	723 ± 10
run 5[d]	30	0.421 ± 0.010	0.665 ± 0.012	379 ± 32
	25	0.360 ± 0.003	0.787 ± 0.002	540 ± 3
	20	0.191 ± 0.005	1.033 ± 0.002	747 ± 5
	15	−0.066 ± 0.016	1.457 ± 0.005	1035 ± 15

[a] Each run represents the average of four data sets. [b] Average of three data sets each. [c] ±1 standard deviation. [d] Average of two data sets each.

Table II: Fitting Parameters from the Linear Least-Squares Analysis of ϕ vs X

	$\phi_{TH}(1)$	$\phi_{TH}(1+2)$	$\phi_{CON}(1)$[d]	$\phi_{CON}(1+2)$
run 1[a]	1.05	0.71	−0.59	0.37
run 2	0.98	0.80	−0.51	0.29
run 3	1.05	0.67	−0.60	0.40
run 4[b]	1.14	0.83	−0.67	0.28
run 5[e]	0.99	0.74	−0.55	0.33
av	1.04 ± 0.05[c]	0.75 ± 0.06	−0.58 ± 0.05	0.33 ± 0.05

[a] Each run represents the average of four data sets for each of two temperatures: 30 and 20 °C. [b] Run 4 consists of three data sets for each of three temperatures: 30, 25, and 20 °C. [c] ±1 standard deviation. [d] At 30 °C. [e] Run 5 consists of two data sets for each of four temperatures: 30, 25, 20, and 15 °C.

of 41 data sets. The average values of ϕ_1 and ϕ_2 from each of the five experiments were correlated with X, which is calculated from the temperature dependence of the acoustic wave amplitude of deoxymyoglobin, to obtain the slopes $\phi_{CON}(1)$ and $\phi_{CON}(1+2)$ and the intercepts $\phi_{TH}(1)$ and $\phi_{CON}(1+2)$. The enthalpic $\phi_{TH}(1)$ and volume $\phi_{CON}(1)$ parameters, relating transient 1 to MbCO, and the enthalpic $\phi_{TH}(1+2)$ and volume $\phi_{CON}(1+2)$ parameters, relating transient 2 to MbCO, are given in Table II for each of the five experiments.

The assumption that the volume change resulting from a change in protein conformation, ΔV_{CON}, is independent of temperature is supported by the data shown in Figure 4. Equation 16 predicts that if ΔV_{CON} is independent of temperature, then there should be a linear correlation for both ϕ_1 and ϕ_2 with X. This is observed over the 15–30 °C temperature range.

The thermal fitting parameters for transient 1, $\phi_{TH}(1)$, and transient 2, $\phi_{TH}(1+2)$, are converted into enthalpic changes through the relationship expressed by eq 5. A photon at 510 nm has an energy of 55.9 kcal/mol. This leads to an apparent enthalpic change between carboxymyoglobin and transient 1 of $\Delta H_1 = -2.2 \pm 2.8$ kcal/mol and an enthalpic change between carboxymyoglobin and transient 2 of $\Delta H_2 = 13.9 \pm 3.4$ kcal/mol.

The conformation parameters $\phi_{CON}(1)$ and $\phi_{CON}(1+2)$ can be converted into absolute volume changes. From eq 1–3, the volume change for the reference compound, deoxymyoglobin, due to thermal relaxation is

$$\Delta V = \beta E_0(1 - 10^{-A})/(C_p\rho)$$

where $\beta = 303 \times 10^{-6}$ K^{-1} at 30 °C for water, $E_0 = 19 \times 10^{-6}$ J, $C_p = 1$ cal g^{-1} K^{-1}, $A = 0.80$, and $\rho = 1$ g mL^{-1}. The absolute volume change produced as a result of the thermal

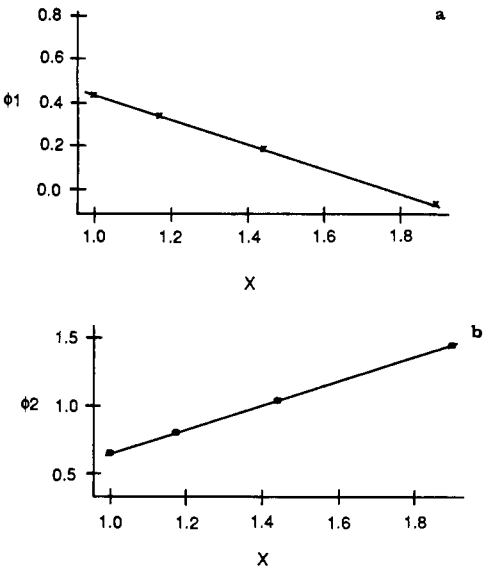

FIGURE 4: (a) Correlation of ϕ_1 vs X from run 5 (Table I). The slope $\phi_{CON}(1)$ is −0.555 ± 0.015 and the intercept $\phi_{TH}(1)$ is 0.989 ± 0.021. The correlation coefficient is 0.996. (b) Correlation of ϕ_2 vs X from run 5 (Table I). The slope $\phi_{CON}(2)$ is 0.893 ± 0.015 and the intercept $\phi_{TH}(1)$ is −0.241 ± 0.020. The correlation coefficient is 0.999.

relaxation of deoxymyoglobin is $\Delta V = 1.17 \times 10^{-9}$ mL. Normalizing this value to the moles of photons absorbed yields a molar volume change of $\Delta V = 17$ mL/mol. Consequently, from eq 15, the conformational volume change upon the formation of the first transient from MbCO is $\Delta V_1 = -10.0 \pm 1.0$ mL/mol and the formation of the second transient from MbCO is $\Delta V_2 = 5.6 \pm 0.8$ mL/mol.

Discussion

The temperature-dependent acoustic waves resulting from the photolysis of carboxymyoglobin produce parameters that relate to kinetics, enthalpy, and volume changes for the dissociation of carbon monoxide from the protein. In order to develop a model for the interpretation of these data, it is necessary to first briefly review previous structure, kinetic, and thermodynamic studies of carboxymyoglobin.

The kinetics of carbon monoxide association and dissociation within sperm whale myoglobin has been well characterized by both transient absorption and resonance Raman spectroscopies. Photolysis of carboxymyoglobin gives 100% cleavage of the iron–carbon monoxide bond in 350 fs (Martin et al., 1983) to generate the geminate pair. The relaxation kinetics of the geminate pair has been studied by nanosecond flash photolysis (Henry et al., 1983). The relaxation kinetics were found to be biphasic, leading the authors to propose the kinetic scheme

$$\text{MbCO} \underset{k_{21}}{\overset{k_{12}}{\rightleftharpoons}} \text{Mb:CO} \underset{k_{32}}{\overset{k_{23}}{\rightleftharpoons}} \text{Mb} + \text{CO}$$

The geminate pair Mb:CO, where CO is thought to be within the protein matrix, decays by either geminate pair recombination to form carboxymyoglobin, $k_{21} = 2.3 \times 10^5$ s^{-1}, or ligand diffusion out of the protein, $k_{23} = 5.2 \times 10^6$ s^{-1}. At 22 °C, ligand dissociation dominates with only 4% of the geminate pair undergoing recombination. Importantly, the decay kinetics of the geminate pair were well fit, employing only a single exponential at 22 °C.

An extensive flash photolysis study that varied both temperature and viscosity lead Frauenfelder and co-workers (Austin et al., 1975) to a more complex kinetic model involving five species.

$$\underset{A}{MbCO} \underset{k_{BA}}{\overset{k_{AB}}{\rightleftharpoons}} \underset{B}{Mb{:}CO} \underset{k_{CB}}{\overset{k_{BC}}{\rightleftharpoons}} \underset{C}{Mb{::}CO} \underset{k_{DC}}{\overset{k_{CD}}{\rightleftharpoons}} \underset{D}{Mb{:::}CO} \underset{k_{ED}}{\overset{k_{DE}}{\rightleftharpoons}} \underset{E}{Mb + CO}$$

The rate coefficients for each of the kinetic processes as well as the corresponding enthalpy and entropy of activation were derived. From the known overall enthalpy change for CO binding to Mb, E → A, and the enthalpy of activation for each kinetic process, obtained from Eyring transition-state theory, the enthalpy change for the formation of each intermediate was calculated: A → B, ΔH = 18 kcal/mol; A → C, ΔH = 25 kcal/mol; A → D, ΔH = 18 kcal/mol.

A second quantity measured in the photoacoustic calorimetry experiment is changes in enthalpy. The overall enthalpy of CO binding with sperm whale myoglobin has been determined by gas–liquid microcalorimetry according to Rudolph et al. (1972). At 25 °C, pH 8.0, in either 0.1 M Tris-HCl or 0.1 M potassium phosphate buffer, the enthalpy of reaction is −17.7 ± 0.4 kcal/mol.

$$Mb(aq) + CO(aq) \rightarrow MbCO(aq)$$
$$\Delta H = -17.7 \pm 0.4 \text{ kcal/mol}$$

This quantity is independent of buffer, thus supporting the conclusions of Rossi-Fanelli and Antonini (1958) that ionizable amino acids do not play an important role in the overall binding of ligand.

The channel through which the ligand enters the heme pocket from the solvent is a question that is unresolved. The initial X-ray structures of deoxymyoglobin and carboxymyoglobin reveal a tightly packed protein with no channels connecting the heme pocket to the solvent (Kendrew et al., 1960; Perutz & Mathews, 1966; Takano, 1977a,b). This observation is supported by molecular dynamic calculations where, in the absence of conformation changes, the barrier for ligand diffusion from the heme pocket to the solvent is in excess of 90 kcal/mol (Case & Karplus, 1979). To facilitate ligand diffusion, large amplitude motions of the amino acid side chains are required.

A pathway for ligand dissociation has been proposed on the basis of comparison of the structures for metmyoglobin and metmyoglobin treated with phenylhydrazine (Ringe et al., 1984). This chemical modification covalently binds a phenyl group to the iron on the distal side of the heme which, due to the bulky size of the phenyl ligand, causes a distortion of the protein structure away from metmyoglobin structure. Importantly, the displacement of His-64 appears to cause the rupture of the salt bridge formed between Arg-45(CD3) and the propionate group of the pyrrole. The net result of this displacement is to produce a channel from the heme pocket to the solvent.

The kinetics, thermodynamics, and volume changes obtained from time-resolved photoacoustic calorimetry will be discussed within the framework developed by Ringe et al. (1984) for ligand diffusion. An important component of the ligand dissociation process is the breaking of the Arg-45–propionate salt bridge. Both the enthalpic and volume changes associated with the rupture of the salt bridge will be manifested in the acoustic data. Unfortunately, a quantitative measure of the enthalpic and volume changes associated with the breaking of an arginine–propionate salt bridge is unknown. However, the signs for the changes of these quantities are known (Kauzmann, 1959). When a salt bridge breaks, both a negative charge and a positive charge are exposed to water; the water becomes strongly oriented about the charges and compresses in order to increase the solvation of the charges. This leads to the liberation of the heat due to a decrease in enthalpy and the ordering of the water produces a decrease in entropy for the system. In addition, there will be a decrease in the overall volume of the system as a result of the electrostriction of the water about the charges.

The analysis of the acoustic data from carboxymyoglobin reveals that two kinetic events occur on the time scale of the instrument response. The kinetics are modeled as two sequential decays, each with an exponential relaxation. The first event occurs in less than 50 ns while the second event has a half-life of 702 ± 31 ns at 20 °C. On the basis of prior transient absorption studies, the first kinetic process is attributed to the formation of the geminate pair from the excited state. The assignment of the second kinetic process is less straightforward. Henry et al. (1983) found the decay of the geminate pair takes place with a time constant of 180 ns at 22 °C, which is significantly shorter than the 702 ns observed by photoacoustic calorimetry.

The enthalpic change associated with the formation of the geminate pair, MbCO → Mb:CO, is ΔH_1 = −2.2 ± 2.8 kcal/mol, assuming that the quantum yield for the formation of the geminate pair from the excited state is 1.0. This assumption is supported by picosecond and nanosecond transient absorption studies (Henry et al., 1983; Martin et al., 1983). As the result of the second decay process, the overall change in enthalpy relative to carboxymyoglobin is ΔH_2 = 13.9 ± 3.4 kcal/mol. This value does not correspond to the enthalpy change for the formation of the second state, for account must be taken of the quantum yield for the formation of the second state from the geminate pair. The quantum yield for the decay of the geminate pair to produce deoxymyoglobin and CO is 0.96, and the quantum yield for geminate pair recombination is 0.04. Thus the enthalpy change manifested in the second decay, 16.1 kcal/mol [13.9 kcal/mol − (−2.2) kcal/mol] reflects both relaxation processes. The enthalpy change for the decay of the geminate pair to form Mb + CO, ΔH_2*, is

$$16.1 \text{ kcal/mol} = 0.04(-2.2 \text{ kcal/mol}) + 0.96(\Delta H_2^*)$$

or ΔH_2^* = 16.8 kcal/mol. Therefore, accounting for the quantum yield for the formation of Mb + CO, the overall enthalpy change for the dissociation of CO from carboxymyoglobin is 14.6 ± 3.4 kcal/mol. This quantity is in close agreement with the value of Rudolph et al. of 17.7 ± 0.4 kcal/mol. Therefore, it appears that the second relaxation process corresponds to the decay of the geminate pair to give solvated CO and deoxymyoglobin.

Determining the volume changes associated with the formation of the geminate pair and deoxymyoglobin is also dependent upon the quantum yields for their formation. As the quantum yield for geminate pair formation is 1.0, the corresponding volume change relative to carboxymyoglobin is −10.0 ± 1.0 mL/mol. Similarly, with a quantum yield of 0.96 for the formation of deoxymyoglobin and CO, the overall volume change relative of carboxymyoglobin is 5.8 ± 1.0 mL/mol.

The most surprising finding of this photoacoustic study is the change in enthalpy upon geminate pair formation. It might be anticipated that the enthalpy of the geminate pair relative to MbCO would be of the order of the Fe–CO bond energy, probably in excess of 18 kcal/mol. The observed value of −2.2 kcal/mol must reflect other contributions from changes within the protein. One possible source contributing to the decrease in enthalpy for geminate pair formation is from the rupture of the Arg-45–propionate salt bridge. The solvation of the charges by water leads to a decrease in enthalpy for the system. In addition, the breaking of a salt bridge results in electrostriction of the water about the charges. This proposal is supported by the observed volume change of −10 mL/mol, which is attributed to electrostriction of water about the ex-

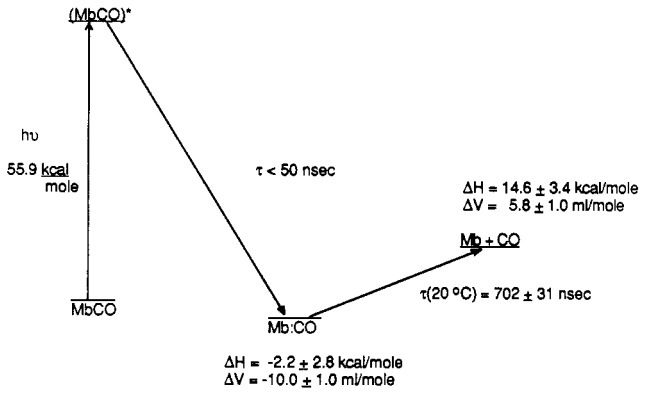

FIGURE 5: Dynamics, enthalpy, and volume changes for the photolysis of sperm whale carboxymyoglobin in 0.1 M Tris-HCl, pH 8.0. All values for enthalpy and volume changes are relative to carboxymyoglobin.

posed charges. These findings suggest that the photochemical cleavage of the Fe–CO bond induces the rupture of the salt bridge in less than 50 ns.

The cleavage of the Arg-45–propionate salt bridge upon geminate pair formation also explains the increase in enthalpy, 16.1 kcal/mol, and volume change, 15.8 mL/mol, associated with the decay of the geminate pair to form deoxymyoglobin. Since the Arg-45–propionate salt bridge is intact in deoxymyoglobin, the salt bridge must be re-formed once CO departs the protein. The formation of a salt bridge is driven by an increase in entropy but at the expense of an increase in enthalpy. Therefore, an increase in both enthalpy and volume is expected for the re-formation of the salt bridge.

From nanosecond flash photolysis studies (Henry et al., 1983) the decay time of the geminate pair is 180 ns at 22 °C, while the second kinetic event measured in photoacoustic calorimetry has a decay time of 702 ns at 20 °C. Clearly, two different processes are being monitored. The flash photolysis experiment measures the fraction of deoxymyoglobin after photolysis as a function of time. The decay of the geminate pair is determined by the kinetics of recombination, loss of deoxymyoglobin, and the fraction that undergoes recombination. Once the ligand diffuses away from the heme pocket into the protein matrix so that there is no further loss of deoxymyoglobin, the optical experiment becomes insensitive to subsequent events within deoxymyoglobin. The photoacoustic calorimetry experiment, on the other hand, is only sensitive to volume and enthalpy changes. It appears that the dominant enthalpy change on a time scale greater than 100 ns is associated with the re-formation of the salt bridge, an event occurring with a time constant of 702 ns. Photoacoustic calorimetry does not resolve the change in enthalpy associated with the decay of the geminate pair, presumably due to a small change in both enthalpy and volume.

The enthalpy profile for MbCO → Mb:CO → Mb + CO derived from photoacoustic calorimetry is substantially different from that deduced from the enthalpies of activation obtained through the temperature dependence of the kinetics of association and dissociation (Austin et al., 1975). The enthalpy change for the geminate pair formation is approximately 18 kcal/mol, as determined by flash photolysis, compared to the −2.2 kcal/mol from photoacoustic calorimetry. This discrepancy may be attributed to differences in experimental conditions. To obtain the activation parameters for the kinetic processes associated with the geminate pair, a glycerol–water matrix is utilized over a wide in temperatures, as low as 40 K. At the lower temperatures, the salt bridge may remain intact following photolyhsis of MbCO as a result of the requirement of significant restructuring of the solvent to accommodate the pair of changes that may not be energetically feasible. Consequently, the geminate pair produced in the matrix may have the Arg-45–propionate intact, thus accounting for the discrepancy between the two results.

In summary, the results of the photoacoustic study of carboxymyoglobin are depicted in Figure 5. The data are consistent with the model proposed by Ringe et al. (1984) that involves the rupture of the Arg-45–propionate salt bridge to facilitate ligand diffusion. Experiments are in progress to further explore the role of the salt bridge as well as the possible involvement of proton release or uptake in the formation of the geminate pair, which might lead to the −2.2 kcal enthalpy change at pH 8.0.

REFERENCES

Antonine, E., & Brunori, M. (1971) *Hemoglobin and Myoglobin in Their Reactions with Ligands*, North-Holland, Amsterdam.

Austin, R. H., Beeson, K. W., Eisenstein, L., Frauenfelder, H., & Grunsalus, I. C. (1975) *Biochemistry 14*, 5355–5373.

Case, D. A., & Karplus, M. (1979) *J. Mol. Biol. 132*, 343–368.

Goodman, J. L., & Peters, K. S. (1986) *J. Am. Chem. Soc. 108*, 1700–1701.

Goodman, J. L., Peters, K. S., Misawa, H., & Caldwell, R. A. (1986a) *J. Am. Chem. Soc. 108*, 6803–6805.

Goodman, J. L., Peters, K. S., & Vaida, V. (1986b) *Organometallics 5*, 815–817.

Henry, E. R., Sommer, J. H., Hofrichter, J., & Eaton, W. (1983) *J. Mol. Biol. 166*, 443–451.

Kauzmann, W. (1959) *Adv. Protein Chem. 14*, 1–63.

Kendrew, J. C., Dickerson, R. E., Strandberg, B. E., Hart, R. G., Davies, D. R., Phillips, D. C., & Shore, V. C. (1960) *Nature (London) 185*, 422–427.

Kuriyan, J., Wilz, S., Karplus, M., & Petsko, G. A. (1986) *J. Mol. Biol. 192*, 133–154.

Martin, J. L., Migus, A., Poyart, C., Lecarpentier, Y., Astier, R., & Antonette, A. (1983) *Proc. Natl. Acad. Sci. U.S.A. 80*, 173–177.

Ort, D. R., & Parson, W. W. (1979) *Biophys. J. 25*, 355–364.

Patel, C. K. N., & Tam, A. C. (1981) *Rev. Mod. Phys. 53*, 517–550.

Perutz, M. F., & Matthews, F. S. (1966) *J. Mol. Biol. 21*, 199–202.

Ringe, D., Petsko, G. A., Kerr, D. E., & Ortiz de Montellano, P. R. (1984) *Biochemistry 23*, 2–4.

Rossi-Fanelli A., & Antonini, E. (1958) *Arch. Biochem. Biophys. 77*, 478–485.

Rudolph, S. A., Boyle, S. O., Dresden, D. F., & Gill, S. J. (1972) *Biochemistry 11*, 1098–1101.

Rudzki, J. E., Goodman, J. L., & Peters, K. S. (1985) *J. Am. Chem. Soc. 107*, 7849–7854.

Takano, T. (1977a) *J. Mol. Biol. 110*, 537–568.

Takano, T. (1977b) *J. Mol. Biol. 110*, 569–584.

Yang, G. K., Peters, K. S., & Vaida, V. (1986) *Chem. Phys. Lett. 125*, 566–569.