around the nonpolar solute, termed in the last 15 years as the "hydrophobic" effect. What has not been appreciated is that this by itself would result in a *negative* value for ΔC_p(solvn). To explain the observed positive value, we must assume that these clathrate cages are in dynamic equilibrium with less ordered cages so that the solute must be considered to exist in at least two different solvent environments, with the positive ΔC_p(solvn) arising from the "melting" of the ordered clathrates to a less ordered solvent cage. This would correspond to a heat capacity of isomerization.

A number of researchers have proposed that it is only the immediate solvent shell around a dissolved solute molecule that is needed to account for the anomalously large and positive values of ΔC_p(solvn). This is supported by data showing that ΔH(solvn) and ΔS(solvn) for a large number of non-H-bonding solutes correlate very well with the van der Waals surface estimated for these solutes.[19] However, none of these efforts have done better than about a 10-20% fit of ΔC_p(solvn) over a temperature range of 0-100 °C. Even this is done with the assumptions of rather arbitrary values of the H bond. Further, none of the efforts have employed discrete structures for liquid water in an effort to relate the ΔH(solvn) to specific structural changes.

One consequence of these very large positive values of ΔC_p(solvn) is that at higher temperatures near 90-100 °C the solution of argon or carbon tetrachloride in water is endothermic rather than exothermic and water appears to be much more of a "normal" solvent.[5]

Very similar arguments must apply to the solution of ions in water. About half or more of the solvation energy of ions comes from the first solvent shell,[14] while outside this highly ordered shell there are likely to be oligomeric structures. For ammonium ion and hydronium ion, recent investigations[15] have demonstrated the appearance of pentameric structures which form part of dodecahedral assemblies.

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Photoacoustic Calorimetry Study of Human Carboxyhemoglobin

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Abstract: Time-resolved photoacoustic calorimetry is used to study the dynamics of the enthalpy and volume changes associated with the formation of triply ligated carboxyhemoglobin from carboxyhemoglobin. The enthalpy for the formation is ΔH = 18.0 ± 2.9 kcal/mol, and the volume change is ΔV = 23.5 ± 0.5 mL/mol. All observed kinetic processes occur in less than 100 ns.

Introduction

Hemoglobin has been the most extensively studied allosteric protein, yet a dynamical molecular description for the mechanism of the allosteric effect remains to be fully elaborated.[1] Although there is a good description, in terms of structure and energetics, of the initial and final states for ligand binding, little is known regarding intermediate states that play a dominant role in cooperative ligand binding. This is attributed in part to low populations of intermediate states under equilibrium conditions. However, ligand dissociation induced by photophysical techniques has been successful in producing significant populations of intermediate species, which have been kinetically and vibrationally characterized.[2-7] Although kinetic techniques allow spectroscopic access to transient species, the associated energetics, which are fundamental to the development of an understanding of the allosteric effect, are exceedingly difficult to discern.

With the recent development of time-resolved photoacoustic calorimetry, it is now feasible to measure the dynamics of enthalpy and volume changes that accompany photoinduced ligand dissociation on the nanosecond-microsecond time scale.[8] This method has been applied to ligand dissociation in both sperm-whale and horse carboxymyoglobin where enthalpy changes associated with protein tertiary structural rearrangements are observed in the nanosecond time regime.[9-11] In this paper we report our first photoacoustic calorimetry study of human carboxyhemoglobin where the enthalpy and volume changes for ligand dissociation to produce triply ligated R-state carboxyhemoglobin are measured.

Experimental Section

Protein Purification. The A_0 component of human hemoglobin, removed of organic phosphates, was prepared by the method of Williams and Tsay[12] and stored as a frozen pellet in liquid nitrogen. Prior to the experiment it was dialyzed against 0.05 M phosphate buffer, pH 7.0, and diluted to a final concentration of 28 μM in hemoglobin. Carboxyhemoglobin was formed by bubbling CO through the solution.

Apparatus. The photoacoustic calorimeter has been described previously.[9] Briefly, a PRA nitrogen-pumped dye laser operating at 1 Hz is used to pass a beam with a wavelength of 500 nm, pulse energy of 15 μJ, and pulse width of 500 ps though the sample which is held under anaerobic conditions in a 1 cm × 1 cm thermostated cuvette. The acoustic waves are measured by a lead zirconate-lead titanate piezoelectric crystal operating at 0.5 MHz and are digitized by a 100-MHz Gould 4072

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oscilloscope. The data are stored in an IBM XT laboratory computer.

Data Analysis

The photoacoustic signal, S, is produced by an increase or decrease in the volume of the irradiated sample, ΔV:

$$S = K\Delta V \tag{1}$$

where the parameter K is a function of the instrument response. There are two processes that give rise to ΔV. First, if there is a release of heat, H_T, to the system, there will be a corresponding increase in the volume of the system, ΔV_{th}, where

$$\Delta V_{th} = (\beta/C_p\rho)H_T \tag{2}$$

Here β is the thermal expansion coefficient of the solution, C_p the heat capacity, and ρ the density. Second, if the molecules within the irradiated sample change their molecular size, ΔV_{con}, an additional contribution to the signal will be produced. These two contributions lead to the observed signal:

$$S = K(\Delta V_{th} + \Delta V_{con}) \tag{3}$$

In the photodissociation of carboxyhemoglobin, the quantum yield for dissociation must be taken into account. Upon photochemical cleavage, the ligand may diffuse out of the protein with a quantum yield Q or recombine with the Fe to re-form carboxyhemoglobin, i.e. geminate recombination, with a quantum yield to $(1 - Q)$. The net heat released from the decay of the excited state producing geminate recombination is just the energy associated with the photon, $E_{h\nu}$, scaled by the quantum yield for recombination. The net heat released from the decay of the excited state producing ligand dissociation will be defined as H scaled by the quantum yield for dissociation, Q. Thus the total heat released is

$$H_T = (1 - Q)E_{h\nu} + QH \tag{4}$$

Similarly, the net molecular volume change for photodissociation followed by geminate recombination is zero, since it occurs on a time scale faster than the instrument response, while the volume change associated with dissociation is $Q\Delta V_{con}$. Thus the observed photoacoustic signal is

$$S = K(\beta/C_p\rho)[(1 - Q)E_{h\nu} + QH] + KQ\Delta V_{con} \tag{5}$$

In order to eliminate K, a calibration compound is employed which converts the entire photon energy into heat on a time scale faster than the response of the instrument.

$$S_{cal} = K(\beta/C_p\rho)E_{h\nu} \tag{6}$$

The ratio of the acoustic wave amplitudes, ϕ, is determined by

$$\phi = S/S_{cal} = \{K(\beta/C_p\rho)[(1 - Q)E_{h\nu} + QH] + KQ\Delta V_{con}\}/[K(\beta/C_p\rho)E_{h\nu}] \tag{7}$$

Rearrangement leads to

$$E_{h\nu}(\phi - 1)/Q = -\Delta H + \Delta V_{con}/(\beta/C_p\rho) \tag{8}$$

The term $E_{h\nu} - H$ has been defined as ΔH, the enthalpy for the formation of the intermediate relative of carboxyhemoglobin. As the term $\beta/C_p\rho$ is dependent upon temperature, measuring ϕ and Q as a function of temperature allows for the correlation of $E_{h\nu}(\phi - 1)/Q$ with $C_p\rho/\beta$ yielding as the slope ΔV and the intercept $-\Delta H$.

Results

The photoacoustic spectra (acquired with a laser energy of 15 μJ) of 28 μM carboxyhemoglobin (HbCO) in 0.05 M phosphate buffer, pH 7.0, at 26.6 and 12.4 °C are shown in parts A and B of Figure 1, respectively. The photoacoustic signal of HbCO is found to be linear over the energy range 5–50 μJ. With a photolysis energy of 15 μJ, approximately 1% of the hemoglobin sample within the laser beam absorbs a photon. For the purpose of calibration, a 112 μM solution of horse metmyoglobin in 0.05 M phosphate buffer, pH 7.0, is used. Metmyoglobin converts the entire photon energy into heat on a time scale faster than the instrument response. We have found that metmyoglobin and deoxyhemoglobin give identical temperature-dependent acoustic waves. However, from a practical standpoint, it is more convenient to use metmyoglobin as a calibration compound, as it is difficult to maintain deoxyhemoglobin in its unligated state due to the presence of residual CO in the transfer lines to the cell. At 26.6 °C, the ratio of the integrated areas of the acoustic waves is $S(\text{HbCO})/S(\text{Mb}) = 1.73$. Furthermore, within the time resolution of the experiment, 100 ns, the HbCO acoustic wave does not shift in time relative to the Mb acoustic wave (Figure 1A). Thus, all of the observed kinetic processes occur in less than 100 ns and there are no measurable kinetic processes on the time scale of 100 ns–10 μs. When the temperature of the solution is reduced to 12.4 °C, there is a reduction in the acoustic wave amplitudes for both HbCO and Mb. The value of $S(\text{HbCO})/S(\text{Mb})$ increases to 2.35 (Figure 1B). The ratio of the two acoustic waves was examined at 19 temperatures ranging from 9.6 to 33.2 °C.

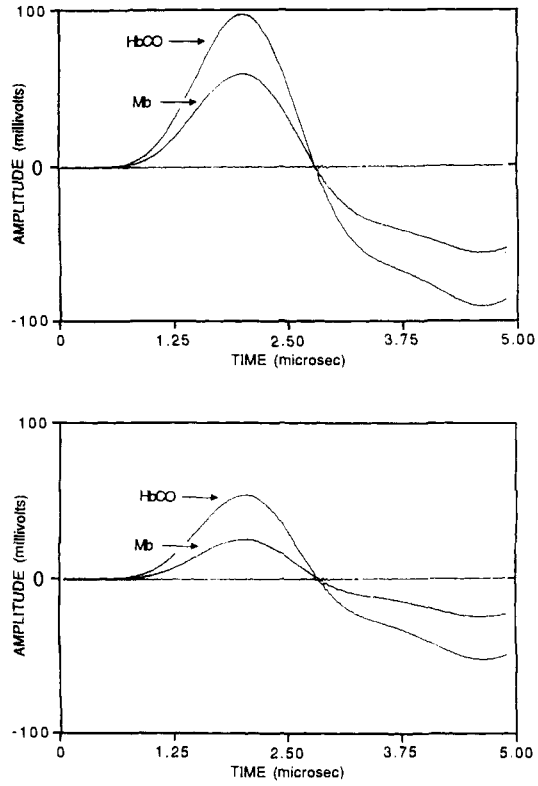

Figure 1. (A) Top: Photoacoustic spectrum of the calibration compound, 112 μM metmyoglobin (Mb), and the sample, 28 μM carboxyhemoglobin (HbCO), at 26.6 °C, in 0.05 M phosphate buffer, pH 7.0, λ_{exc} = 500 nm. (B) Bottom: Photoacoustic spectrum of the calibration compound, 112 μM metmyoglobin (Mb), and the sample, 28 μM carboxyhemoglobin (HbCO), at 12.4 °C, in 0.05 M phosphate buffer, pH 7.0, λ_{exc} = 500 nm.

In order to establish the values of $\beta/C_p\rho$ as a function of temperature, the photoacoustic wave amplitudes for bromocresol purple in distilled water at dilute concentrations (<1 μM) and metmyoglobin in the buffered solutions are compared as a function of temperature[9] (Figure 2). The ratio of the acoustic wave amplitudes for these two solutions reflects the ratio of $\beta/C_p\rho$ for the two solutions. Since these values are known as a function of temperature for distilled water, the values for the buffered solution can be derived.

The temperature dependence of the quantum yields for photodissociation of carboxyhemoglobin, Q, was deduced from the experiments of Saffran and Gibson.[13] For human carboxyhemoglobin in 0.05 M phosphate buffer at pH 7.0, the measured values for Q are 0.38 at 0 °C and 0.70 at 40 °C. To obtain Q for intermediate temperatures, we assumed that the quantum yield for production of the geminate pair is 1.0 and that the geminate pair decays by two pathways: dissociation from the protein with rate k_1 and geminate recombination with rate k_2. Thus Q is defined as

$$Q = k_1/(k_1 + k_2) \tag{9}$$

Assuming that k_1 may be expressed as $A_1 \exp(-E_1/RT)$ and k_2 may be expressed as $A_2 \exp(-E_2/RT)$, from the temperature dependence of Q the values of $E_1 - E_2$ and $\ln(A_1/A_2)$ may be

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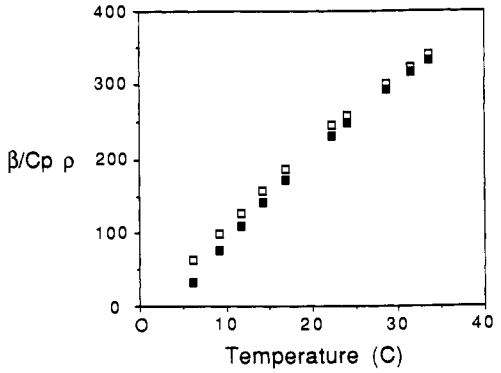

Figure 2. Thermal expansion coefficients ($\times 10^6$ mL/cal) for bromocresol purple (<1 μM) in distilled water (solid squares) and for metmyoglobin in 0.05 M phosphate buffer, pH 7.0 (open squares), as a function of temperature.

derived, from which it is then possible to calculate Q at intermediate temperatures. Given this extrapolation process for the determination of the quantum yields for photodissociation as a function of temperature, it is not possible to determine the error associated with Q. Thus, values for the enthalpy and volume changes associated with ligand dissociation do not take into account the error in the quantum yield for dissociation.

The correlation of $E_{h\nu}(\phi - 1)/Q$ with $C_p\rho/\beta$ is shown in Figure 3A, and resulting residuals for a linear fit are shown in Figure 3B, where $\Delta H = 18.0 \pm 2.9$ kcal/mol and $\Delta V = 23.4 \pm 0.5$ mL/mol.

Discussion

Under the conditions of the photoacoustic experiment, only 1% of the CO ligands undergo photodissociation, so that the predominant transient species is triply ligated carboxyhemoglobin. The dynamics of CO dissociation from R-state carboxyhemoglobin have been probed by femtosecond,[2] picosecond,[3] and nanosecond[4] absorption spectroscopy and picosecond and nanosecond resonance Raman spectroscopy.[5,7] Femtosecond kinetic studies[2] reveal that photolysis of HbCO produces $Hb(^5T_2) + CO(^1\Sigma)$ within 300 fs with nearly unit efficiency. As determined by nanosecond flash photolysis,[4] the geminate pair lifetime in R-state hemoglobin, which decays by either ligand dissociation from the protein or geminate recombination, is 44 ns at room temperature. On the time scale of 900 ns, a spectral shift occurs in the deoxy absorption spectrum which has been attributed to tertiary structural changes about the heme chromophore. Time-resolved resonance Raman studies support this kinetic description of ligand dissociation but with the modification that tertiary structural changes occur in a time regime ranging from 20 to 300 ns.[5-7] Quaternary structural changes have been observed in the single-ligand dissociation from HbCO producing triply ligated carboxyhemoglobin. Through modulation excitation,[14] the R → T transition for triply ligated carboxyhemoglobin occurs in 600 μs at 25 °C. Given the time resolution of the present photoacoustic calorimeter, this latter transition cannot be observed.

The enthalpy values for the formation of triply ligated carboxyhemoglobin from the fully ligated species are uncertain although values have been reported.[15] The uncertainty is attributed to the great difficulty in resolving the CO binding curves into the individual Adair constants because of the high ligand affinity. With a newly developed thin-layer microcalorimeter,[16] we have measured the total enthalpy associated with the binding of four CO's to deoxyhemoglobin in 0.1 M phosphate buffer at pH 7.0. The overall enthalpy, corrected for the heat of protonation of the buffer, is −60.1 kcal/mol, or an average of −15.0 kcal/mol per heme.

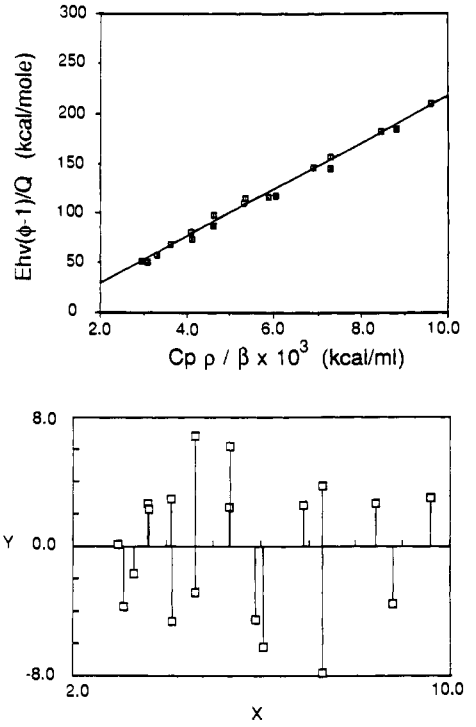

Figure 3. (A) Top: Plots of $E_{h\nu}(\phi - 1)/Q$ vs $C_p\rho/\beta$ for carboxyhemoglobin in 0.05 M phosphate buffer at pH 7.0. $\Delta H = 18.0 \pm 2.9$ kcal/mol, and $\Delta V = 23.4 \pm 0.5$ mL/mol. (B) Bottom: Residuals from a linear fit to the data displayed in part A.

Photoacoustic calorimetry reveals that, within 100 ns of photolysis, a transient species is produced whose changes in enthalpy and volume relative to fully ligated carboxyhemoglobin are 18 kcal/mol and 23.4 mL/mol, respectively. This volume change represents an increase of 0.02% of the total molecular volume of carboxyhemoglobin. On the time scale of 100 ns–10 μs, no kinetic events are detected. The sensitivity of the experiment is such that at 900 ns events with enthalpy changes of 3 kcal/mol or volume changes of 1 mL/mol are measurable. Thus the purported tertiary structural changes found at 900 ns in flash photolysis are not observed.[4] If the tertiary structural change is an entropy-driven process and there are no accompanying enthalpy and volume changes, photoacoustic calorimetry would not be sensitive to this process. However, it is difficult to imagine a protein structural change that does not involve a volume change. Thus it is difficult to reconcile this discrepancy between the flash photolysis and photoacoustic calorimetry studies.

On the basis of prior kinetic studies, the transient species formed within 100 ns corresponds to triply ligated carboxyhemoglobin. The enthalpy change associated with its formation, 18 kcal/mol, would then correspond to an enthalpy for CO binding to triply ligated species of −18 kcal/mol, which is greater than the −15 kcal/mol average ligand binding measured by microcalorimetry. This may reflect the greater enthalpy for CO binding to R-state as compared to T-state hemoglobin.

In our previous photoacoustic calorimetry studies of sperm-whale carboxymyoglobin,[9,10] ligand dissociation produced a protein structural rearrangement within 100 ns of photolysis, one component of which was the breaking of the Arg-45 salt bridge. Following escape of the ligand from the protein, the matrix relaxed toward the deoxymyoglobin equilibrium structure, re-forming the salt bridge in 700 ns, which was accompanied by an enthalpy change of 10 kcal/mol. Similar processes were observed in horse carboxymyoglobin.[11] For carboxyhemoglobin, the dominant enthalpy processes occur in less than 100 ns. Tertiary structural changes analogous to those in myoglobin are not observed.

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