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A photoacoustic calorimetric study of horse myoglobin

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The dynamics of the enthalpy and volume changes for the photodissociation of CO from horse myoglobin has been studied by time-resolved photoacoustic calorimetry which measures the dynamics of enthalpy and volume changes on the nanosecond time scale. The role of the Lys 45 salt bridge in the ligand dissociation is discussed.

1. Introduction

One of the fundamental questions relevant to the oxygen transport proteins myoglobin and hemoglobin is how the protein structure controls the thermodynamics of ligand binding. To begin to answer this question it is necessary to understand how the protein controls the dynamics of ligand association and dissociation which requires that the pathway for ligand diffusion between the heme pocket and the solvent be known. Surprisingly, the pathway has not been fully elucidated [1–6]. Examining the equilibrium structure of myoglobin reveals the absence of channels between the solvent and the heme pocket which could accommodate a molecule the size of dioxygen or carbon monoxide [7,8]. Consequently, ligand association/dissociation must be accompanied by significant structural reorganization of the protein matrix [1,9].

One experimental approach that has recently been employed in the investigation of the dynamics and energetics of ligand dissociation in sperm whale carboxymyoglobin is photoacoustic calorimetry [6,10]. This technique is sensitive to the dynamics of enthalpy and volume changes on the nanosecond-microsecond time scales. In this paper, we will discuss the application of time-resolved photoacoustic calorimetry to the photoinduced dissociation of carbon monoxide from horse carboxymyoglobin at pH 8.0. The results of this study will be discussed within the framework that is being developed for ligand dissociation in sperm whale carboxymyoglobin [10].

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2. Experimental

2.1. Materials

Horse heart metmyoglobin (Sigma) was dissolved in 0.1 M phosphate and in 0.1 M Tris buffers (pH 8.0), at a concentration of approx. 100 μM protein. The samples were filtered through a 0.45 μm Millipore filter and held overnight at 4°C. The solutions were then deoxygenated by stirring under N_2 for 1 h followed by reduction with a 10-fold excess of sodium dithionate. The sample was then divided into two; one sample was exposed to CO to form carboxymyoglobin and the

other was maintained as deoxymyoglobin, serving as the calibration compound.

2.2. Apparatus

The details of the photoacoustic calorimeter have previously been presented [6]. Briefly, a PRA nitrogen-pumped dye laser (LN1000/LN102), operating at 1 Hz, 510 nm, 500 ps pulse width and energy of approx. 15 μJ, induced the dissociation. The sample was maintained under anaerobic conditions in a thermostated cuvette to within ± 0.1° C. The acoustic waves were detected with a 0.5 MHz piezoelectric transducer, composed of lead zirconate-lead titanate. The output of the transducer was passed through a preamplifier (Panametric ultrasonic preamp) and time-resolved by a Gould 4072 digital oscilloscope, 100 MHz. The data were stored in an IBM XT computer.

2.3. Data analysis

The photoacoustic signal S is produced by the expansion or contraction, ΔV, of the irradiated sample which follows the relationship

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

where K is a function of the instrument response. There are two processes which can contribute to the change in volume of the sample. The first arises from the release of energy, ΔE, to the solvent by the reacting molecules. This gives rise to an increase in the temperature of the solvent surrounding the reacting molecule. The temperature increase causes the solvent to expand, ΔV_{th}, which generates an acoustic wave. The relationship between ΔV_{th} and ΔE is

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

where β is the thermal expansion coefficient, C_p the heat capacity, and ρ the density for the system [10]. In addition to thermal decays, there is another process which can produce acoustic waves. If during the course of reaction the molecular system increases or decreases its volume due to a change in configuration, ΔV_{con}, a sound wave will be generated. Therefore

$$S = K(\Delta V_{th} + \Delta V_{con}) = K\left[(\beta/C_p\rho)(\Delta E + \Delta V_{con})\right] \tag{3}$$

In order to eliminate K, a calibration compound is employed whose photochemistry converts the entire photon energy into heat on a time scale faster than the time resolution of the instrument (< 100 ns). The photoacoustic signal from the calibration compound is

$$S_{cal} = K'(\beta/C_p\rho)E_{h\nu} \tag{4}$$

where $E_{h\nu}$ is the photon energy. The ratio of the acoustic waves for the sample and the calibration compound, ϕ, is

$$\phi = S/S_{cal} = (\Delta E/E_{h\nu}) + \left(\Delta V_{con}/\left[(\beta/C_p\rho)E_{h\nu}\right]\right) \tag{5}$$

Defining

$$F(T) = \beta/C_p\rho \tag{6}$$

and rearranging leads to

$$E_{h\nu}\phi = \Delta E + \Delta V_{con}/F(T) \tag{7}$$

The quantity $F(t)$ is highly temperature dependent for proteins in aqueous media while ΔE and ΔV_{con} are assumed to be temperature independent over a narrow temperature range of approx. 10 °C. Thus, correlating $E_{h\nu}\phi$ with $1/F(T)$ yields ΔE and ΔV_{con}. The overall reaction enthalpy for the first intermediate is then

$$\Delta H = E_{h\nu} - \Delta E \tag{8}$$

Photoacoustic calorimetry is sensitive not only to enthalpy and volume changes but also to the dynamics of these processes. For a reaction that involves two sequential decays

$$A \xrightarrow{k_1} B \xrightarrow{k_2} C$$

the observed time-dependent voltage produced by the transducer, $E(t)$, results from the convolution of a time-dependent heat source, $H(t)$, with the instrument response function $T(t)$.

$$E(t) = H(t)T(t) \tag{9}$$

For this simple kinetic scheme $H(t)$ is expressed as

$$H(t) = \phi_1 \exp(-t/\tau_1) + [\phi_2 k_1/(k_2 - k_1)] \times [\exp(-t/\tau_1) - \exp(-t/\tau_2)] \quad (10)$$

where $\tau = 1/k$. The quantities ϕ_1 and ϕ_2 are defined as in eq. 5 and represent the ratios of the volume changes for the first and second kinetic event to the volume change for the calibration compound. Again ϕ_1 and ϕ_2 have contributions from both thermal and configuration changes, ΔV_{th} and ΔV_{con}, which are separated through the temperature dependence of ϕ_1 and ϕ_2. The process for obtaining the parameters ϕ_1, τ_1, ϕ_2 and τ_2 involves assuming a set of parameters, calculating $H(t)$ which is convoluted with $T(t)$, obtained from the calibration compound, to produce a calculated $E(t)$. The calculated $E(t)$ is then compared to the experimental $E(t)$ through analysis of residuals. The fitting parameters are then optimized by minimizing the residuals. Numerous algorithms have been presented for deconvolution of the acoustic wave data [10].

From the temperature dependence of τ, the enthalpy of activation, ΔH^+, and the entropy of activation, ΔS^+, are obtained from activated complex theory

$$\ln(k/\{k_b T/h\}) = \Delta S^+/R + (-\Delta H^+/RT) \quad (11)$$

where $k = 1/\tau$.

2.4. Temperature dependence of F(T)

To obtain ΔE and ΔV_{con}, $E_{h\nu}\phi$ is correlated with $F(T)$ which is temperature dependent. In order to measure $F(T)$ for 0.1 M phosphate or 0.1 M Tris protein buffer solutions, the photoacoustic wave amplitudes of bromocresol purple in distilled water at dilute concentration (< 1 μM) and deoxymyoglobin in the buffered solutions are compared as a function of temperature. The ratio of the acoustic wave amplitudes for the two solutions directly reflect the ratio of $F(T)$ for the two solutions. Since the temperature dependence of $\beta/C_p\rho$ is known for distilled water, $F(T)$ can be obtained for the protein solution.

3. Results

The temperature dependence, ranging from 29.7 to 15.1° C, of the photoacoustic spectra for horse deoxymyoglobin (Mb) and carboxymyoglobin (MbCO) in 0.1 M phosphate buffer (pH 8.0) is shown in fig. 1. The amplitudes of the MbCO waves are larger than those of the corresponding Mb waves. Furthermore, the MbCO waves are shifted in time relative to Mb, indicating that kinetic events are occurring on the time scale of the instrument resolution, 100 ns to 20 μs.

Deconvolution of the MbCO waves in either 0.1 M phosphate or 0.1 M Tris requires, as a minimum, a two-step kinetic model (eq. 10). During the deconvolution τ_1 was held constant at 1 ns for all excited states have relaxation processes on the picosecond time scale. Only ϕ_1, ϕ_2 and τ_2 were allowed to vary. The correlation of $E_{h\nu}\phi_1$ and $E_{h\nu}\phi_2$ with $C_p\rho/\beta$ for carboxymyoglobin in 0.1 M phosphate (pH 8.0) is shown in fig. 2. The resulting enthalpy and volume changes are given in table 1. The corresponding values for carboxymyoglobin in 0.1 M Tris (pH 8.0) are also listed in table 1.

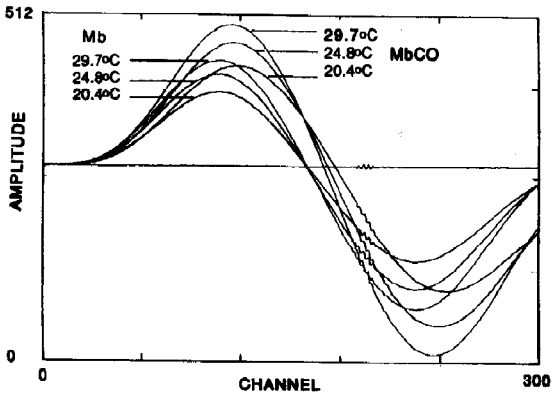

Fig. 1. Photoacoustic spectra of horse deoxymyoglobin (Mb) and carboxymyoglobin (MbCO) at 29.7, 24.8 and 20.4° C. Sample concentration: 100 μM, 0.1 M phosphate (pH 8.0), $\lambda_{exc} = 510$ nm.

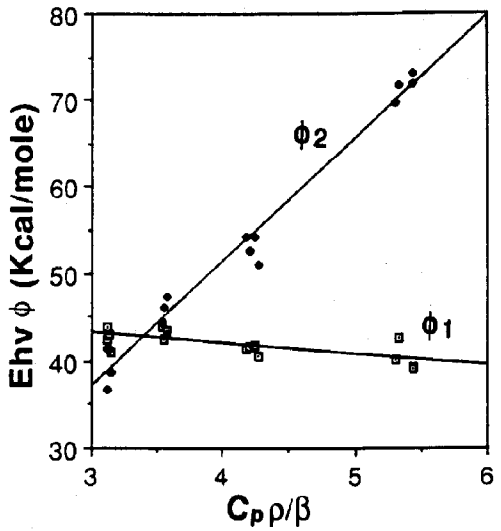

Fig. 2. Correlation of $\phi_1 E_{h\nu}$ and $\phi_2 E_{h\nu}$ vs $C_p\rho/\beta$ for horse carboxymyoglobin in 0.1 M phosphate at pH 8.0.

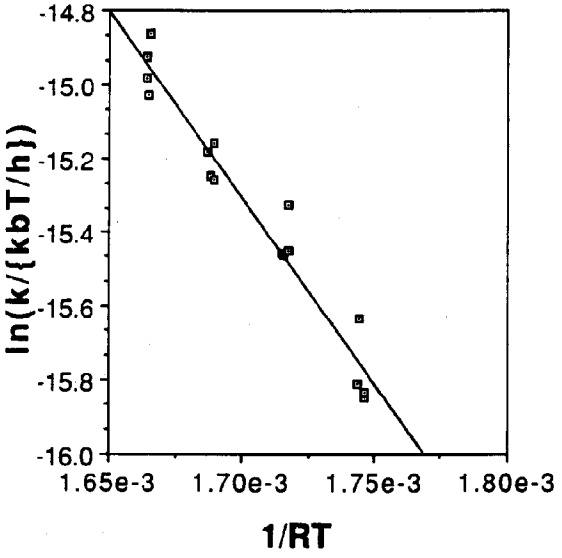

Fig. 3. Plot of $\ln(k/\{k_bT/h\})$ vs $1/RT$ for the second kinetic process, τ_2, for horse carboxymyoglobin in 0.1 M phosphate at pH 8.0.

Table 1

Enthalpy and volume changes resulting from photodissociation of CO from horse carboxymyoglobin

Buffer (pH 8.0)	ΔH_1 (kcal/mol)	$\Delta H_{(1+2)}$ (kcal/mol)	ΔV_1 (ml/mol)	$\Delta V_{(1+2)}$ (ml/mol)
0.1 M phosphate	8.9±1.3	14.4±2.8	−1.2±0.3	13.0±0.7
0.1 M Tris	7.4±2.0	14.3±2.9	−1.7±0.5	13.8±0.7

The enthalpy and entropy of activation of the second kinetic process are obtained from the temperature dependence of τ_2 (eq. 11), whose correlation is shown in fig. 3 for carboxymyoglobin in 0.1 M phosphate (pH 8.0). The resulting values for 0.1 M phosphate and 0.1 M Tris are given in table 2.

Table 2

Thermodynamic activation parameters from the temperature dependence of τ_2 for the decay of the intermediate in horse carboxymyglobin

Buffer (pH 8.0)	ΔH^+ (kcal mol^{-1})	ΔS^+ (cal K^{-1} mol^{-1})
0.1 M phosphate	10.0±0.7	3.7±2.1
0.1 M Tris	10.2±0.7	4.0±2.2

A reaction profile of the enthalpy and volume changes for horse carboxymyoglobin in 0.1 M phosphate is shown in fig. 4. For the purpose of comparison, the corresponding values for sperm whale myoglobin under the same conditions are also displayed.

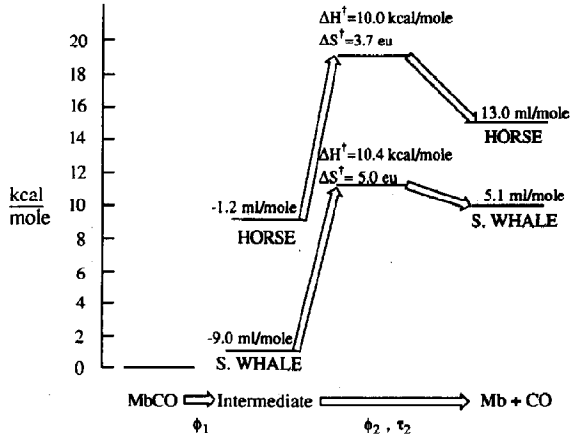

Fig. 4. Enthalpy and volume changes for photolysis of horse and sperm whale carboxymyoglobin in 0.1 M phosphate at pH 8.0.

4. Discussion

The pathway by which a ligand in myoglobin diffuses between the heme pocket and the solvent has been the subject of numerous studies [1–6]. Most of the discussion to date has been focused upon ligand dissociation in sperm whale myoglobin due to availability of crystallographic structures of ligated and unligated sperm whale myoglobin [7,8]. These studies reveal that in both metmyoglobin and carboxymyoglobin there is a salt bridge linkage between the Arg 45 and the propionate side chain of the heme's pyrrole. The binding of ligands larger than CO, such as an imidazole [11] or a phenyl group [5], results in the displacement of the distal histidine, His-E7, causing the rupture of the salt bridge so that the propionate and the guanidinium group of Arg 45 become solvated. The breaking of the salt bridge and the displacement of the distal histidine create a channel between the solvent and the heme pocket through which a ligand can diffuse.

In our photoacoustic investigation of CO dissociation in sperm whale carboxymyoglobin, we found evidence that the Arg 45 propionate salt bridge does rupture during ligand dissociation [6,10]. Upon absorption of a photon by sperm whale carboxymyoglobin in 0.1 M phosphate (pH 8.0), an intermediate is produced within the time resolution of the experiment (< 100 ns), whose enthalpy change relative to carboxymyoglobin is $\Delta H_1 = 0.8 \pm 1.4$ kcal/mol with a volume change that is negative, $\Delta V_1 = -9.0 \pm 0.3$ ml/mol. The intermediate then decays on the 700 ns time scale at 20 °C to produce an overall change of $\Delta H_{(1+2)} = 9.9 \pm 2.0$ kcal/mol and $\Delta V_{(1+2)} = 5.1 \pm 0.5$ ml/mol. The activation parameters for this latter process are $\Delta H^+ = 10.4 \pm 0.4$ kcal/mol and $\Delta S^+ = 5.0 \pm 1.6$ cal/K per mol. The values for ΔH_1 and ΔV_1 were surprising, since it was anticipated that ΔH for CO dissociation from Fe may range from 15 to 20 kcal/mol [12]. Clearly, other processes are occurring that are exothermic upon the formation of the intermediate and endothermic upon decay of the intermediate.

One possible contribution to the observed enthalpy and volume changes associated with the formation of the intermediate is the breaking of the salt bridge. Upon exposure of the charges to solvent, the water will undergo electrostriction, producing a negative volume change, to solvate the charges which leads to a decrease in enthalpy (exothermic) and entropy [13]. Unfortunately, there are virtually no data regarding the magnitude of the changes in the enthalpy and entropy for the breaking of salt bridges. To study the involvement of the salt bridge in the ligand dissociation process, we examined [10] sperm whale myoglobin whose salt bridge had been removed through site-directed mutagenesis where the Arg 45 has been replaced by a Gly (R45G). This substitution causes the enthalpy change for the formation of the intermediate to increase ($\Delta H_1 =$ 8.7 kcal/mol) and reduces the magnitude of the negative volume change ($\Delta V_1 = -4.7$ ml/mol). The overall enthalpy and volume changes as well as the activation parameters are only slightly modified ($\Delta H_{(1+2)} = 12.3$ kcal/mol, $\Delta V_{(1+2)} = 5.5$ ml/mol; $\Delta H^+ = 11.3$ kcal/mol, $\Delta S^+ = 5.6$ cal/K per mol). Comparison of the values for wild-type sperm whale myoglobin and R45G mutant strongly suggests that the salt bridge is broken upon formation of the intermediate.

From these photoacoustic studies, the model that emerges for ligand dissociation in sperm whale myoglobin is that upon dissociation of CO there is significant restructuring within the protein that causes the Arg 45 salt bridge to break within 100 ns. Following diffusion of CO out of the protein, the protein undergoes a rate-limiting 700 ns restructuring followed by re-formation of the salt bridge. The re-formation of the salt bridge is not involved in the rate-determining step for the decay of the 700 ns intermediate as the activation parameters for this decay are the same in wild-type sperm whale myoglobin and the R45G mutant which does not have a salt bridge. The nature of the protein restructuring that has activation parameters of $\Delta H^+ = 10.4$ kcal/mol and $\Delta S^+ = 5$ cal K^{-1} mol^{-1} still remains to be elucidated. Furthermore, although the salt bridge is broken upon the formation of the intermediate, it has not been demonstrated that the ligand diffuses through the resulting channel.

Comparing the amino acid sequence of sperm whale and horse myoglobin, of the 153 amino

acids only 19 differ. In the region of the heme pocket, the residues Arg 45 and Ala 74 in sperm whale are replaced by Lys 45 and Gly 74 in horse [14]. Regrettably, there is no available X-ray structure for horse myoglobin so that the state of the salt bridge between Lys 45 and the propionate side chain is not known. The X-ray structure of unligated seal myoglobin in the met state, which has a Lys 45, reveals the existence of a salt bridge [15]. The structure for seal carboxymyoglobin has not been determined. The overall enthalpy change for the dissociation of CO from horse carboxymyoglobin has been previously determined [16] as $\Delta H = 17$ kcal/mol. Within the error of the experiment, the overall binding enthalpy determined by photoacoustic calorimetry, ($\Delta H = 14.4 \pm 2.8$ kcal/mol) is the same.

The most striking difference between sperm whale and horse carboxymyoglobin are the enthalpy and volume changes for the formation of the intermediate: $\Delta H = 0.8$ kcal/mol, $\Delta V = -9$ ml/mol for sperm whale and $\Delta H = 8.9$ kcal/mol, $\Delta V = -1.2$ ml/mol for horse. The difference in enthalpy is very similar to that observed in wild-type sperm whale and R45G mutant which lacks a salt bridge. A model that serves to rationalize the difference in these values is one in which Lys 45 in horse carboxymyoglobin does not form a salt bridge, but instead is solvated by water while in horse deoxymyoglobin, Lys 45 does form a salt bridge. If the formation of the horse intermediate does not involve salt bridge cleavage due to its absence, the volume change associated with the horse intermediate should be more positive than that with the sperm whale intermediate. The difference between the horse and sperm whale intermediates is more positive by 7.8 ml/mol. Once the CO ligand has departed the protein, the Lys 45 should then form the salt bridge in horse deoxymyoglobin. The net change in horse myoglobin, carboxy to deoxy, would be removal of a pair of charges from the solvent to form a salt bridge while there is no net change in the salt bridge for sperm whale. Therefore, the overall volume change for carboxymyoglobin to deoxymyoglobin should be larger in horse than sperm whale and the approximate difference should be the same as that observed between the two intermediates for horse and sperm whale (7.8 ml/mol). Indeed, the difference in the overall volume change for the two species is 7.9 ml/mol.

Surprisingly, the activation parameters for the decay of the 700 ns transient are very similar in horse, wild-type sperm whale and the sperm whale R45G mutant [10]. This again supports the previous proposal that the rate-determining step for the decay of the intermediate does not involve the re-formation of a salt bridge [10]. It is also unlikely that this decay is the diffusion of the ligand out of the protein as the entropy of activation for this process should be negative, based upon molecular dynamic calculations [9]. It is more probable that the rate-determining step for the decay of the intermediate is the reorganization of the protein structure leading to the deoxy form. When the CO dissociates from the heme iron, the molecular volume of the ligand will increase due to an increase in the van der Waal radius. This will produce a structural change within the protein, possibly a partial unpacking at the α-helices' interfaces. This process would be accompanied by an increase in solvation of the protein surface. Once the ligand escapes the protein, the matrix will reorganize towards the deoxy form liberating water to the solvent; the entropy for this process is positive [17] and should have a positive entropy of activation.

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