Photodissociation of the CO Complex of Horseradish Peroxidase Studied by Laser-Induced Optoacoustic Spectroscopy

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Photodissociation of the CO Complex of Horseradish Peroxidase Studied by Laser-Induced Optoacoustic Spectroscopy

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Laser-induced optoacoustic spectroscopy (LIOAS) was applied to the study of the photolysis of the CO-ligated heme protein horseradish peroxidase isoenzyme C (HRP). Laser photolysis produced structural volume changes faster than 50 ns. The photoreaction volume and enthalpy changes were determined by means of temperature-dependent measurements in the range 6−23 °C. The volume change (+29.6 mL/mol) can be mainly attributed to the displacement of CO to the bulk solvent. The enthalpy change is mainly related to the Fe−C bond energy with little contribution from the protein matrix. The results are interpreted in terms of the structural properties of HRP, which has a direct exit channel from the heme to the solvent, and compared to related studies on the CO complexes with myoglobin and hemoglobin.

Introduction

Heme proteins are involved in a variety of biological processes, including oxygen transport and storage and redox catalytic activity. In all the above biological functions, the protein active site includes the Fe atom of a porphyrin ring (in the case of globins and most peroxidases, a protoporphyrin IX ring), where small ligands are bound. Oxygen binds to the Fe(II) atom in globins, while hydrogen peroxide binds to Fe(III) in peroxidases in the first step of the catalytic reaction mechanism. The stability of these complexes, which characterizes the protein function and activity, is determined by the geometry and the chemical properties of the amino acids surrounding the ligand, which are arranged in the so-called distal cavity.[1] Carbon monoxide, although it is not a common physiological ligand, binds to the Fe(II) − thus probing the distal cavity − and forms stable complexes which can be investigated by both steady-state and time-resolved spectroscopical and structural techniques. Steady-state vibrational spectroscopy gives a very detailed insight of the geometry and force field of the Fe−C−O unit and indirectly of the environment surrounding the bound CO,[2] whereas UV−vis absorption probes the heme electronic structure and is only weakly sensitive to the surrounding. A peculiarity of the CO complexes is that the ligand can be efficiently photolyzed, allowing to probe the subsequent dynamics of both the ligand and the protein in the dissociation and recombination process. This has given rise to a wealth of time-resolved studies, including laser flash photolysis,[3] femtosecond transient absorption,[4] transient vibrational spectroscopy,[5,6] and time-resolved X-ray diffraction in the nanosecond range.[7]

Laser-induced optoacoustic spectroscopy (LIOAS) allows to measure the volume changes occurring in a medium following the excitation of an absorbing species by laser pulses.[8] The subsequent energy relaxation produces heating of the medium, thus generating a pressure wave which can be detected by piezoelectric transducers. Moreover, photochemically induced changes in the absorbing molecules may also lead to volume changes adding to the thermal volume changes.[9] The typical time scale for the detection of the pressure wave ranges from tenths of nanoseconds to some microseconds. In this respect, LIOAS is complementary to the optical techniques, since it measures properties of the bulk system and not of the chromophore only. The application of LIOAS to the study of the CO complexes with heme proteins allows the molecular rearrangement after photolysis to be characterized by the reaction volume change and the reaction heat. Among heme proteins, myoglobin,[10−13] hemoglobin,[14] and cytochrome P450[15] have been investigated by means of LIOAS. We have applied LIOAS to the study of horseradish peroxidase isoenzyme C (HRP), which is a prototypical heme peroxidase. The CO complexes of HRP have been previously characterized by means of vibrational spectroscopy[16−19] and laser flash photolysis.[20,21] Moreover, the X-ray structure of the Fe(III) resting state of the enzyme has been solved.[22] We will show that the photodissociation dynamics of HRP−CO is simple as compared to the CO complexes with myoglobin and hemoglobin, and we will discuss these features on the basis of the structural properties of the proteins.

Experimental Section

HRP isoenzyme C was purchased from Sigma and used without further purification. Phosphate buffer solutions with pH 7.0 and ionic strength 0.1 M were used for all measurements. The CO complexes were prepared by extensively purging the solutions with nitrogen, then with CO, and subsequently reducing the protein with an excess of sodium dithionite anaerobic solution. The absorption spectrum was immediately measured and the solution was anaerobically transferred to the measurement cuvette. The sample absorbance at 532 nm was within the range 0.15−0.25. The calorimetric reference was a solution of Fe(III) HRP of matched absorbance. We tested that this solution gave the same heat release as the calorimetric reference $KMnO_4$.

The excitation source for the LIOAS measurements was the second harmonic (532 nm) of a Nd:YAG laser with pulse width ≅ 10 ns operating at 0.5-Hz repetition rate. To overcome the pulse-to-pulse laser fluctuations, the LIOAS signal was simultaneously measured for the sample and for a reference solution. The laser beam was divided by a beam-splitter with ca. 50% reflectivity and simultaneously fed to two 1-cm optical path

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quartz cuvettes containing the sample solution and the reference solution. Two piezoelectric transducers (Panametrics V103-RM) with 1-MHz band-pass were clamped to a side of the cuvettes. The cuvettes and transducers were placed in a thermally insulated box containing a metal heat exchanger thermostated by the refrigerating liquid of a thermostat. The temperature was measured by copper−constantan thermocouples immersed in the reference solution. We estimated the accuracy in the determination of the temperature of the solutions to lie within ±0.05 °C by monitoring the thermostating bath temperature and the temperature gradients inside the box. The box was filled with nitrogen gas in order to prevent oxidation of the CO complex. The laser pulse energy was ≅8 μJ. We tested that the signals were linear with the incident laser pulse energy in the range 2−14 μJ. Due to the slow recombination of CO to HRP (250-ms time constant at 1 mM CO concentration, pH 8.5),[21] we observed a signal reduction in HRP−CO at higher repetition rates than 0.5 Hz. The signals from the piezoelectric transducers were amplified with Panametrics mod. 5660B preamplifiers and analyzed by a LeCroy 9344 digital oscilloscope operating at 50 Msample/s.

Results

The observed signal H in the LIOAS experiment is related to the volume changes following optical excitation.[8] It can have either a thermal or a structural origin. Thermal volume changes, ΔV_{th}, occur through the nonradiative pathways by which the absorbed energy can relax. Structural volume changes, ΔV_{st}, may also occur due to the rearrangement of the photoexcited molecule or of its environment. Both changes can additively contribute to the measured signal:

$$H = K(\Delta V_{th} + \Delta V_{st}) \quad (1)$$

where H is normalized for the number of the absorbed photons and K is a proportionality function which depends on the instrumental parameters and on the thermoelastic properties of the sample. Due to the presence of the constant K the LIOAS measurements are referred to the signal (taken at the same excitation wavelength and same temperature) of a calorimetric reference, i.e., a substance with $\Delta V_{st} = 0$, which is not fluorescent and relaxes all the absorbed energy through nonradiative pathways.

The thermal contribution ΔV_{th} strongly depends on the solvent, since it is proportional to the ratio of the thermoelastic parameters $\beta/(C_p\rho)$, where β is the volumetric expansion coefficient, C_p the heat capacity at constant pressure, and ρ the mass density. In the case of aqueous solutions, $\beta/(C_p\rho)$ is strongly dependent on temperature. Due to the peculiar properties of water, the volumetric expansion coefficient β is zero at 3.9 °C.[23] The LIOAS signal measured in aqueous solution at such temperature is therefore almost entirely due to the ΔV_{st} contribution, which can be assumed to be constant in relatively restricted temperature ranges (see below).

It can be shown[8] that the ratio of the signals of the sample (H^{sam}) and the calorimetric reference (H^{ref}) is given by:

$$\frac{H^{sam}}{H^{ref}} = \alpha + \frac{\Delta V_{st}}{E_\lambda}\left(\frac{C_p\rho}{\beta}\right) \quad (2)$$

where E_λ is the energy per photon at the laser wavelength λ and α is the fraction of the absorbed energy which is released as prompt heat. Equation 2 allows the calculation of ΔV_{st} from experimental measurements by plotting the ratio of the observed LIOAS signals as a function of the thermoelastic parameter ratio $(C_p\rho)/\beta$.

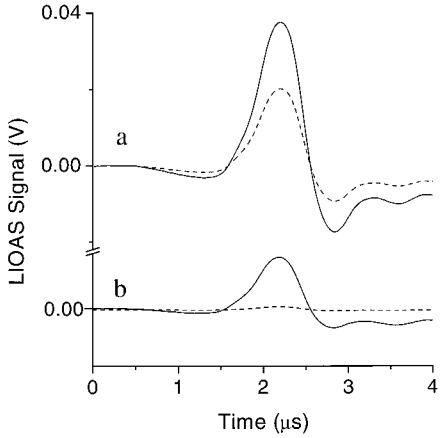

Figure 1. LIOAS signals of phosphate buffer solutions (pH 7.0, ionic strength 0.1 M) of HRP−CO (full line) and the calorimetric reference Fe(III) HRP (dashed line) at 22.2 °C (a) and 3.8 °C (b).

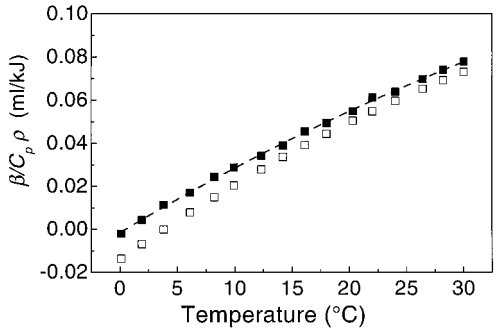

Figure 2. Temperature dependence of the ratio of thermoelastic parameters $\beta/(C_p\rho)$ for pure water (□) and for a 0.1 M phosphate solution (■), as obtained through eq 3 (see text). A polynomial fit (dotted line) is superimposed on the experimental values for phosphate.

Figure 1a shows the LIOAS signals of a solution of HRP−CO complex at 22.2 °C (full line) and of an Fe(III) HRP solution used as a calorimetric reference (dashed line). Figure 1b displays both signals at 3.8 °C. All the signals are normalized for the difference in the absorbed laser power. The time profile of both sample and reference LIOAS signals are the same at all temperatures within the time resolution of our setup. The identity of the shapes of all the measured signals allows for the use of the signal amplitude (difference between first maximum and minimum) for data analysis without deconvolution. The HRP−CO signal at 22.2 °C is about twice as large as the reference signal. At 3.8 °C, the reference signal almost disappears, while the HRP−CO signal stays relatively large and positive. The thermal contribution ΔV_{th} in the latter spectra is negligible since the expansion coefficient β is very low at this temperature. The excess signal of HRP−CO in Figure 1b is therefore due to a structural volume change ΔV_{st}.

To accurately determine ΔV_{st}, we need to determine the value of $(C_p\rho)/\beta$ for the buffer solution which we used for all measurements. Figure 2 compares the $\beta/(C_p\rho)$ values of pure water and 0.1 M phosphate buffer solution in the temperature range 0−30 °C. The water values were obtained from tables.[24] The values for 0.1 M phosphate solutions were obtained by the ratio of the energy-normalized amplitudes of the LIOAS signal of the calorimetric reference $KMnO_4$ in 0.1 M phosphate

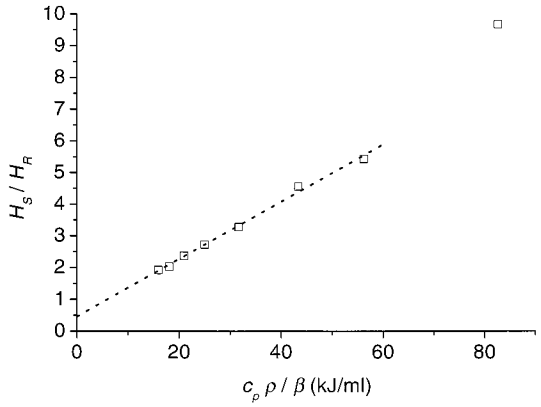

Figure 3. Ratio of the LIOAS signals of HRP−CO and Fe(III) HRP as a function of phosphate solution thermoelastic parameters. A linear fit (dotted line) for the data in the temperature range 23.3−6 °C is also displayed.

solution and in water solution, following the equation:[25]

$$\frac{H_{\mathrm{pho}}}{H_{\mathrm{wat}}} = \frac{(\rho v_a^2)_{\mathrm{pho}}}{(\rho v_a^2)_{\mathrm{wat}}} \left(\frac{\beta}{C_{\mathrm{p}}\rho}\right)_{\mathrm{pho}} \left(\frac{C_{\mathrm{p}}\rho}{\beta}\right)_{\mathrm{wat}} \qquad (3)$$

The density difference between water and phosphate solution was approximated to +0.5% for phosphate at all temperatures.[26] v_a (the sound velocity) was measured for the buffer solution by estimating the delay with respect to pure water in the arrival time of the acoustic wave, and it resulted to be 1.51×10^{-3} m s^{-1} at 20 °C (against 1.48×10^{-3} m s^{-1} for water). A polynomial (quadratic) fitting was performed to obtain the temperature dependence of $\beta/(C_{\mathrm{p}}\rho)$.

Figure 3 displays the ratio between the HRP−CO signal and the Fe(III) HRP signal as a function of the thermoelastic parameters, obtained by interpolation of the polynomial fitting in Figure 2. The temperature range was 23.3−4.4 °C. As already stated (eq 2), the slope of this plot yields ΔV_{st}.

Owing to the very strong variation of the thermoelastic parameters (especially β) between 6 and 4 °C, the data points at higher values of the ratio $(C_{\mathrm{p}}\rho)/\beta$ (>60 kJ/mL) have an intrinsic low accuracy. For this reason, only the data points in the range 23.3−6 °C were fitted to obtain the slope. This gives the value $\Delta V_{\mathrm{st}} = 20.3 \pm 0.63$ mL/mol. The intercept of the fitting curve yields $\alpha = 0.46 \pm 0.09$. α is related to the reaction enthalpy change through $\alpha = 1 - (\Delta H/E_\lambda)$.

Discussion

Both steady-state and time-resolved investigations have been performed on the HRP−CO complex by means of several methods. Although an X-ray structure of the ligand-bound protein is missing, the structure of the Fe(III) form of HRP[22] (see Figure 4a) can give information about the environment surrounding the ligated CO molecule. The porphyrin Fe atom is six-coordinated in HRP−CO, with the fifth position bound to the protein through the His170 residue and the bound CO molecule being located at the opposite side of the heme plane in the distal cavity of the protein. The key amino acids in the distal cavity are an arginine residue (Arg38) and a histidine residue (His42) which are involved in the catalytic function. Arg38 is hydrogen-bonded through a water molecule to one of the two propionate groups of the porphyrin, which have the same orientation according to the structural data. The propionate groups and Arg38 inhibit a direct access from the solvent to the Fe atom. The access is possible through an open side of the distal cavity, corresponding to the heme side which includes the C1 and C8 carbon atoms. The access channel is shown in Figure 4b.

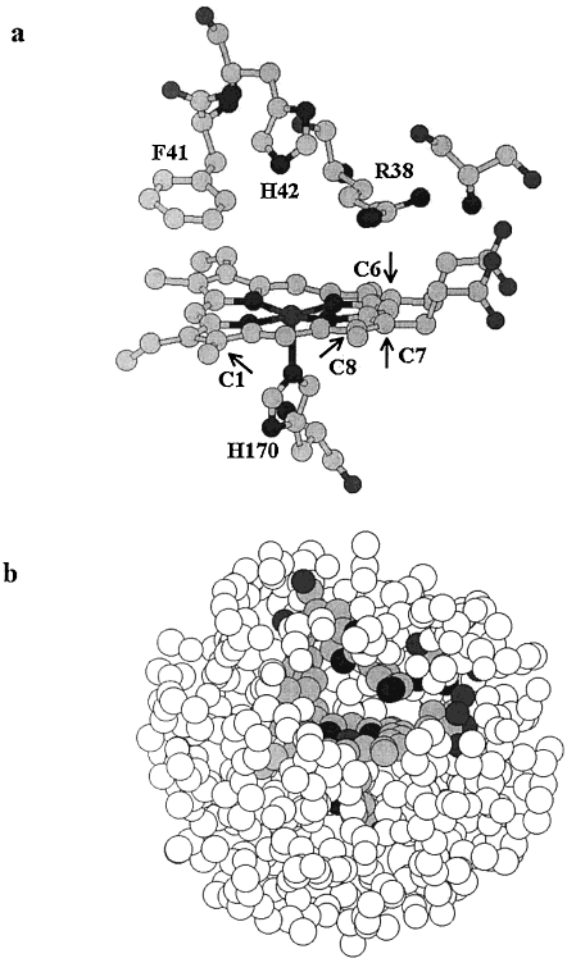

Figure 4. (a) Structure of the key amino acids in the distal cavity and of the heme in Fe(III) HRP. (b) Free access channel from the solvent to the heme in Fe(III) HRP. Only the amino acids which are inside a sphere of radius 14 Å − centered on the heme Fe − are shown. The evidenced atoms belong to the groups in part a, displayed with the same orientation. Structural data was obtained from the Brookhaven Protein Data Bank file 1ATJ.

The strongest interactions of the bound CO occur with the Arg38 and His42, as observed in the vibrational spectra of HRP−CO. In fact, the IR spectra displayed two CO stretching bands at a particularly low frequency, which were ascribed to conformers interacting with the distal cavity to a different extent.[17] Evidence for H-bonding was given by the H/D isotopic effect on both bands.[16] Site-directed mutagenesis and resonance Raman spectroscopy allowed to identify one conformer as a CO complex where CO is H-bonded to His42, while CO in the other conformer is H-bonded to Arg38.[18]

The dynamics of HRP−CO photolysis at room temperature appears relatively straightforward. The cleavage of the Fe−C bond occurs on a subpicosecond time scale and produces a five-coordinated Fe(II) heme which fully relaxes within 7 ns.[27,28] The recombination of CO to the photolyzed protein from the solvent has been widely characterized by laser flash photolysis and it follows a single-exponential law with a 250-ms time constant at room temperature, pH 8.5, 1 mM CO concentration.[21] Two reported quantum yield values for the photodissociation are 0.70 at 20 °C[29] and 0.65.[30] Both determinations were performed with microsecond laser pulses. Since the CO recombination time constant is several orders of magnitude

larger than microseconds, the reported quantum yield values can be also used for an analysis of the dynamics which we observed by LIOAS in the 50−1000 ns range.

The observed ΔV_{st} is related to the photolyzed fraction of the complex, hence to the photolysis quantum yield Φ, through the equation:

$$\Delta V_{st} = \Phi \, \Delta V_R \tag{4}$$

where ΔV_R is the reaction volume change. From the literature value[29] $\Phi = 0.7$ (the error limits are not reported), the reaction volume change is calculated to be: $\Delta V_R = [(20.3 \pm 0.63)/0.7]$ mL/mol = 29.6 mL/mol.

The dependence of Φ on temperature is not known. To assess the occurrence of this dependence, we measured the photoacoustic signal ratio H^{sam}/H^{ref} (see eq 2) as a function of temperature in a glycerol/water (47% v/v) mixture, where the thermoelastic parameters are very weakly dependent on temperature. The signal ratio (data not shown) was 1.17 ± 0.01 in the temperature range 6−30 °C, indicating that Φ changes less than ±5% if one considers that the limit H^{sam}/H^{ref} value is 1 for $\Phi = 0$. This ratio also confirms that the change with temperature of ΔV_R, if present, lies within these limits.

Our results show a single expansion which is faster than the time resolution of our setup. No evidence for the formation of intermediate species − of the kind of geminate pairs[31,11] − is observed within our time window. The origin of the expansion may lie in the Fe−C cleavage, the heme rearrangement, the protein matrix reorganization, and the escape of CO to the solvent. In the HRP−CO photolysis, both the Fe−C coordination bond and a H-bond are broken. For a simple molecule in solution, this should result in a volume expansion if only neutral products are formed. For instance, the cleavage of a C−C bond in organic solution gave a volume change of +10 mL/mol.[32] In the case of a complex system like a protein, the origin of the observed volume change needs a more subtle explanation. The overall molecular volume of the protein is not expected to change upon simple ligand dissociation, due to the presence of the distal pocket, where a small molecule can be arranged. Rather, a rearrangement of the protein matrix can be expected if polar or charged amino acids are displaced. The displacement of the dissociated CO to the bulk solvent − through the open access between the C1 and C8 carbon atoms of the porphyrin ring − can also contribute to the overall volume change. It is expected that the latter contribution is related to the van der Waals volume of the dissociated CO. Moreover, solvation of CO in the bulk water must be considered. The only experimental value for the partial molal volume of gaseous CO in liquid water known to us is 28.5 mL/mol at 0 °C,[33] which accounts for both the van der Waals volume and solvation volume. This value is very close to the expansion we measured for HRP−CO. On this basis, we can conclude that the reaction volume in the photolysis of HRP−CO is mainly due to the migration of CO to water and its solvation.

The activation volume for the binding of CO to HRP was determined in water and in various water/organic solvent mixtures.[34] The overall activation volume was −23.7 mL/mol in water at 20 °C, and it was mainly attributed to solvent reorganization. It is noteworthy that the reported value has opposite sign to our ΔV_R − which concerns a dissociation − and a similar modulus. This indicates that both the transition state and the final products experience a similar solvent influence.

A comparison can be made with previous time-resolved LIOAS studies of the photodissociation of the CO complexes

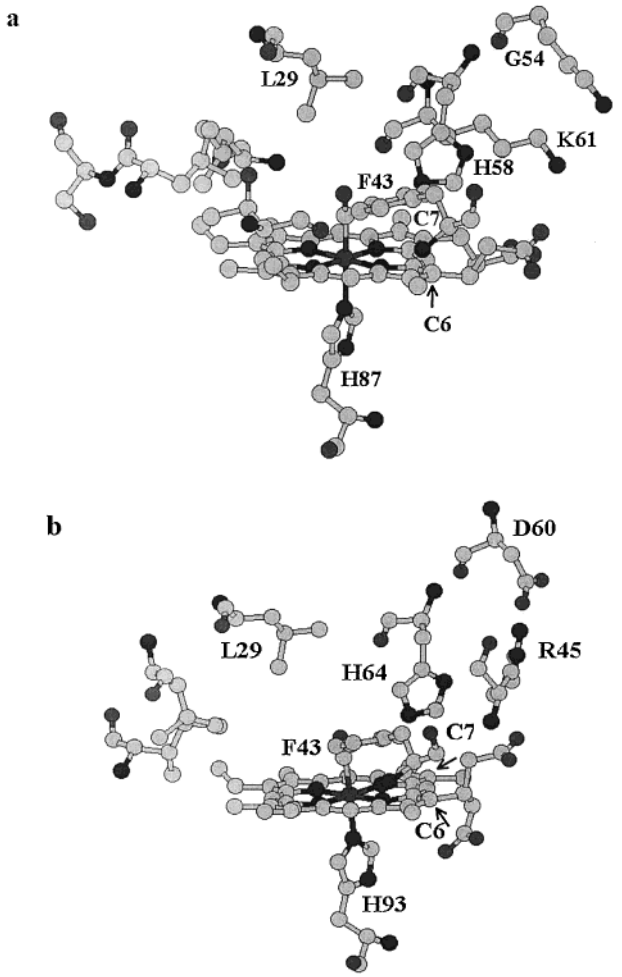

Figure 5. Structure of the key amino acids in the distal cavity and of the heme in the CO complexes with human hemoglobin, α-subunit (a), and with sperm whale myoglobin (b). Structural data was obtained from the Brookhaven Protein Data Bank files 1BBB and 1A6G, respectively.

of the related heme proteins, hemoglobin[14] and myoglobin.[11−13] A slightly smaller value of $\Delta V_R = +23.4$ mL/mol and the same time course for the LIOAS signal as for HRP−CO were found for the photolysis of the CO complex with human hemoglobin.[14] This may indicate that the reaction volume change in both cases is largely determined by the displacement of CO to the bulk water. A fast time constant for this process has been found by time-resolved UV resonance Raman spectroscopy (about 20 ns)[35] and by transient absorption spectroscopy (about 50 ns).[36] According to the X-ray structural data for the CO complex of human hemoglobin (see Figure 5a),[37] the Leu29 residue in the α-subunit or Leu28 in the β-subunit occupies the top region of the distal cavity where the distal histidine (His42) lies in HRP (see Figure 4a). The distal cavity space delimited by the HRP distal arginine (Arg38) is delimited by the distal histidine (His58α/63β) in CO−hemoglobin. The open channel observed for HRP is occupied by the Phe43α/42β residue. Therefore the only possible pathway for ligand escape from the cavity is through the heme side comprising the C6 and C7 atoms if the distal histidine can swing out of the cavity. The distal His swinging has been invoked as part of the mechanism for the creation of a ligand access/dissociation pathway in myoglobin (see below).[38,39] The different pathways between HRP−CO and hemoglobin−CO may give rise to different photodissociation dynamics, which would escape to our experimental observation due to the relatively slow time scale of the LIOAS measurements.

A multistep process has been detected by LIOAS in sperm whale myoglobin−CO photolysis with time resolution between 100 ns and 10 μs, including: a fast process (<100 ns), with a contraction $\Delta V_R = -10$ mL/mol, possibly leading to a geminate pair formation; and a slower, temperature-dependent process (700 ns at 20 °C), which is linked to CO migration to the bulk solvent and produces a positive $\Delta V_R = +5.8$ mL/mol with respect to the bound state, i.e., +15.8 mL/mol with respect to the intermediate.

If we focus on the volume changes, an apparent contrast exists between the measured values in the photodissociation of CO from HRP on one side and from myoglobins and hemoglobin on the other side. Actually, if we assume that most of the volume change is due to both van der Waals and solvation volume of CO, and that CO displacement occurs in the second slow photodissociation stage for myoglobin, then the slow volume change in CO−myoglobin photolysis should be interpreted as the sum of a similar expansion as for HRP of about 30 mL/mol and of a contraction of about 14 mL/mol. This contraction may be explained as a consequence of the formation of an open structure, which has been proposed to occur following CO photodissociation since a direct access channel to the solvent is absent in myoglobin.[38] The open form of CO−myoglobin has two main peculiarities with respect to the normal, closed form which is shown in Figure 5b:[40] The distal His (His64) is swung out of the cavity and H-bonded to the Asp60 residue (which is in turn H-bonded to Arg45), and a salt bridge is missing between Arg45 and heme-6-propionate.[39] Since the above groups are more exposed to the solvent, a contraction may be expected when open myoglobin is formed. In fact, it has been shown that solvation of polar amino acid side chains by water leads to a contraction ranging from 1 to 20 mL/mol.[41] This picture is at variance with the original interpretation of the LIOAS data for the photolysis of CO from sperm whale myoglobin, where the volume of solvated CO has not been taken into account. The different contraction of about 6 mL/mol (29.6 vs 23.4 mL/mol), which should accompany the photodissociation of CO−hemoglobin in our picture, could be due to different amino acids involved in the protein rearrangement taking place in the open structure. In conclusion, we tentatively assign the reduced expansion in both globins to the solvation of the open form. This process is absent in HRP, where CO can exit the protein through the access channel with minimal rearrangements of the protein matrix.

Our data also allow the evaluation of the HRP−CO photodissociation enthalpy change, following eq 2. We determined a reaction enthalpy change $\Delta H_R = \Delta H/\Phi = 176 \pm 33$ kJ/mol from the intercept of the fitting curve in Figure 3, using the reported quantum yield $\Phi = 0.7$. To our knowledge, no calorimetric data are available for the reaction between CO and HRP. On the other hand, an enthalpy of 146 kJ/mol (35 kcal/mol) was calculated for the cleavage of the Fe−CO bond in a model system CO−Fe porphyrin−imidazole.[42] This value is similar to our data if we consider that our experimental error limits are affected by the relatively large contribution of the volume change to the signal. The photodissociation enthalpy change observed for HRP−CO is significantly larger than the enthalpy change determined by LIOAS for myoglobin−CO (61 kJ/mol)[11] and hemoglobin−CO (75 kJ/mol).[14] This difference is parallel to the observed difference in the reaction volume change between HRP and globins. It appears that the additional processes due to protein rearrangements in myoglobin and hemoglobin − yielding the observed lower reaction volume change ΔV_R − also give an exothermic contribution, lowering the CO dissociation enthalpy from the calculated 146 kJ/mol to the observed 75−61 kJ/mol.

Conclusions

Time-resolved LIOAS allowed to measure the volume and enthalpy changes due to the CO dissociation following the laser photolysis of HRP−CO complex, which occurs within few nanoseconds. No evidence was found for the occurrence of intermediate species, found previously in the photodissociation of the CO complex of sperm whale myoglobin. The temperature-independent expansion of 29.6 mL/mol observed in phosphate buffer solution at pH 7.0 was attributed to the CO displacement to the bulk aqueous solution, in agreement with the partial molal volume of gaseous CO in water (28.5 mL/mol). Protein matrix rearrangements should contribute to the volume change only to a low extent. The X-ray structure of HRP, showing a free access for CO from the heme distal cavity to the solvent, is in agreement with this picture. The measured enthalpy change is close to the calculated volume for the cleavage of the Fe−CO bond in a model imidazole−heme−CO system.

We observed differences in the photolysis of the related CO complexes of myoglobin and hemoglobin with respect to both volume and enthalpy changes. The reduced expansion and the lower enthalpy change in globins than in HRP both point to additional protein matrix rearrangements, coupled to the photodissociation of CO. These rearrangements give a negative contribution to the volume change, adding algebraically to the volume change due to CO displacement, and an exothermic contribution lowering the reaction enthalpy. We correlate this difference with the absence of a free access channel to the heme in the globins.

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