EFFECT OF TEMPERATURE AND ORGANIC PHOSPHATE*  

Photodissociation of Ligands from Heme and Heme Proteins  

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The effect of temperature on ligand photodissociation from protoheme and the heme proteins hemoglobin (Hb) and myoglobin (Mb) has been examined. The quantum yield of photodissociation (Φ) is greater at 40°C than at 0°C; in general, larger increases are seen in the less photosensitive complexes, while Φ does not change in the most photosensitive complexes. The ratio of Φ at 40°C to Φ at 0°C is 1.5 for HbCO, 2.3 for n-butyl isocyanide Hb, 2.7 for HbO2, and 1.3 for HbNO, with initial Φ values of 0.38, 0.26, 0.028, and 0.083, respectively. This pattern of quantum yield increases is seen in protoheme as well as Hb and Mb ligand photolysis.

The allosteric effector inositol hexaphosphate increases the quantum yield of ligand photolysis from hemoglobin. As with temperature, inositol hexaphosphate addition has a larger effect on complexes of low quantum yield; Φ increases 1.2-fold for HbCO and 2.2-fold for HbO2 at 0°C.


Most ferrous heme ligands are photodissociable to some extent. The original observations were made by Haldane and Lorrian Smith on the highly photosensitive derivative carbon monoxide hemoglobin (1) and have been extended by subsequent investigators to include O2, NO, cyanide, and isocyanides (2-4). Quantum yield determinations on some heme complexes have shown considerable differences, both among the different ligands and, for each ligand, with changing solution conditions such as pH, ionic strength, and protein concentration.

Bücher and Negelein (5), in careful measurements of the absolute quantum yield, reported a pH- and ionic strength-dependent value of about 0.25 for HbCO tetramers in 0.02 M pyrophosphate buffer at 4°C. MbCO had an invariant quantum yield of almost 1.0. In a later study, Noble et al. (6), while confirming the variability of HbCO photosensitivity, found higher quantum yields, of about 0.4 for tetramers. They suggested that this discrepancy might perhaps be due to the higher temperature (20°C) of their experiments, but did not make any measurements at other temperatures. An investigation into the effect of temperature on the photosensitivity of the single site heme proteins myoglobin and horseradish peroxidase made by Bonaventura et al. found no change in quantum yield between 20 and 30°C (7). However, this study was restricted to one ligand, CO, and did not include hemoglobin. The derivatives tested all had high quantum yields, in which little or no increase with temperature would be expected, as the value cannot exceed a maximum of 1.0.

In the present work we have investigated the effect of temperature on the quantum yields of hemoglobin, myoglobin, and protoheme, studying derivatives covering a wide range of photosensitivity.

In addition, we have studied the influence of the allosteric effector inositol hexaphosphate on the photolability of hemoglobin derivatives.

EXPERIMENTAL PROCEDURES

Materials—Human hemoglobin solutions were prepared as described previously (8) and stripped by passage through a column of Sephadex G-55 (fine) equilibrated with 0.005 M [piperazine(2-hydroxyethyl)]]bis(2-ethanesulfonic acid), pH 7.4. Sperm whale metmyoglobin (Sigma) was reduced to the ferrous form just prior to each experiment. Inositol hexaphosphate (Sigma) was reduced to the ferric form just prior to each experiment by a few crumbs of dithionite (Manox). Concentrations of the solutions were determined spectrophotometrically.

The buffer used was 0.05 M phosphate, pH 7.0. Air-saturated buffers were used for the O2 experiments. Oxygen-free solutions were prepared by bubbling a syringe filled with buffer with N2 (Airco), and CO-saturated solutions by bubbling with CO (Matheson). NO solutions were prepared by injecting a syringe of deoxy buffer into a tonometer filled with NO (Matheson), shaking well to assure equilibration, and withdrawing into the syringe. N-Butyl isocyanide (Aldrich) solutions were prepared as described by Olson and Gibson (9). Inositol hexaphosphate (Sigma) stock solutions were made by dissolving the sodium salt in water and adjusting the pH to 7.0 with HCl.

Flash Photolysis Apparatus—The flash apparatus used was similar to that described previously, with some modifications (10). A water-jacketed cell holder was used for temperature regulation. The aperture for the observing beam in this cell holder was offset toward the side of the flash tube to provide an average 2.5-mm path length for the photolysing beam. This minimized any errors due to light intensity attenuation through absorption by the sample solution. The path length of the observing beam was 1.0 cm.

The time course of ligand dissociation was measured during the light flash. Light from the flash was excluded from the photomulti-
Photolysis of the sample by the observing beam, a problem with $k_{\text{FeX}}$ to MbCO.

Quantum Yield Calculations—In flash photolysis, a brief pulse of light is used to dissociate ligand from the heme; the ligand then rebinds in a dark reaction, as shown in Equation 1:

$$\text{FeX} \xrightarrow{\text{light}, k_{\text{light}}} \text{Fe} + X \xrightarrow{k_{\text{dark}}} \text{FeX}$$ (1)

The rate of ligand photodissociation is proportional to the light intensity $I$, the quantum yield $\phi$, and the extinction coefficient.

$$\frac{d\text{Fe}}{dt} = k_{\text{light}} (\text{FeX}) + k_{\text{dark}} (\text{FeX}) - k_{\text{dark}} (\text{Fe}) (X)$$ (2)

where $k_{\text{light}}$ and $k_{\text{dark}}$ are the ligand rebinding and dissociation rates in the dark, respectively.

The time course of the lamp flash was measured without the heme sample in place, and the tabulated values of the light intensity were used for $I(t)$ in Equation 2. The rate of ligand rebinding to heme in the dark was measured after the flash had ended for each sample and used as the value for $k_{\text{dark}}$. Photodissociation of the ligands was much faster than their dark dissociation; the observed rates of photolysis ranged from 1,000 to 80,000 s$^{-1}$, depending on the photosensitivity of the complex and the flash lamp used. The fastest dark dissociation rate, that of HbO$_2$, is only about 30 s$^{-1}$, the term $k_{\text{dark}}(\text{FeX})$ in Equation 2 is therefore negligible. The quantity $k_{\text{light}}$ was calculated from the time course of the photolysis reaction by a least squares fit to Equation 2. The resulting number is a measurement proportional to the absolute quantum yield. In absolute measurements, Büchner et al. (5, 11) found a quantum yield of nearly unity for MbCO. MbCO was thus assigned a quantum yield of 1.0; all values of $\phi$ for other derivatives reported here are calculated relative to MbCO.

This method can lead to inaccuracies in the measurement of quantum yield of different ligands, as the overlap between the flash spectrum and heme protein absorption spectrum is not the same for all derivatives; however, we were primarily interested in measuring the changes in photosensitivity of the heme ligand complexes rather than their absolute quantum yields.

Two different flash lamps were used in these experiments to test if the observed value of $\phi$ depended on the delivery rate of photons. One flash was of relatively high intensity and short duration, with a time to 1/e of 200 $\mu$s, the other having low intensity and long duration, with a time to 1/e of 850 $\mu$s. While the measured rates of photolysis were higher with the fast, high intensity flash than with the slow flash, the calculated quantum yields, relative to that of MbCO, were the same for both flash lamps.

Temperature A typical experiment is shown in Fig. 1. The time course of HbCO photolysis was followed spectrophotometrically at 0, 20, and 40°. It should be noted that change in temperature alters the spectrum of both deoxy and carbon monoxide hemoglobin (12), and at the observation wavelength (430 nm), the total absorbance excursion increases at higher temperature. The quantum yield calculations therefore took into account these differences in excursions. The normalized time courses of CO dissociation at the three temperatures are shown in Fig. 1; the rate steadily increases with temperature. The lines through the experimental points are calculated using Equation 2, with $\phi$ values of 0.35, 0.46, and 0.69 and $k_{\text{dark}}$ values of 3, 6, and $12 \times 10^3$ s$^{-1}$ for 0, 20, and 40°, respectively.

A wavelength of 436 nm was chosen for the HbCO observations to exclude absorbance change contributions from quaternary conformational transitions. Before the light flash, the hemoglobin is fully liganded and in the R state, and after complete photodissociation the fully deoxy hemoglobin is in the T state, but during photolysis a transient, spectrally distinct species, denoted Hb*, is formed (11). Hb* is interpreted to be unliganded R state hemoglobin, the immediate product of photodissociation, which subsequently undergoes conformational transition to the T state. CO dissociation from hemoglobin was followed at the deoxy T-deoxy R isosbestic point, 436 nm (13).

The time course of the R$\rightarrow$T transition was followed at the Hb-HbCO isosbestic, 425 nm (Fig. 2). The conformational change substantially lags behind the CO removal. Photolysis observations at wavelengths other than 436 nm therefore contain greater contributions from the R$\rightarrow$T transition in the latter part of the reaction than at the beginning and the form of the apparent time course of ligand removal would be altered.

The other hemoglobin derivatives tested also show temperature-dependent photolability (Table I). The quantum yield of HbCO goes from 0.38 to 0.70, n-butyl isocyanide Hb from 0.26 to 0.40, HbO$_2$ from 0.028 to 0.076, and HbNO from 0.003 to 0.004 as the temperature is increased from 0 to 40°. Except for the least photosensitive ligand, NO, the general pattern is of decreasing change in quantum yield as the initial value of $\phi$ is raised; the ratio of $\phi$ at 40°/$\phi$ at 0° is 2.7, 2.3, and 1.8 for 0° of 0.028, 0.26, and 0.38 respectively.

In contrast to HbCO, MbCO has a temperature-invariant quantum yield (Table I); the time course of photolysis is the same at 0° as at 40°. However, the less photosensitive deriv-
A model of photolysis which includes a photoexcited intermediate of the heme complex can account for the pattern of quantum yield changes observed here (15). The simplest such scheme is:

$$\text{FeX} \xrightarrow{k_1} \text{FeX}^* \xrightarrow{k_2} \text{Fe} + X$$

(3)

where FeX* is a photoexcited intermediate, $k_1$ is the rate constant for its formation, proportional to light intensity and extinction coefficient, $k_2$ is the constant for quenching or decay back to the liganded ground state, and $k_2$ is the constant for ligand dissociation from the excited state.

The quantum yield is proportional to the rate of appearance of unliganded heme. If it is assumed that the system is in a steady state, and the concentration of FeX* is constant, it follows that

$$\frac{d\phi}{dt} = \frac{k_1 k_2}{k_1 + k_2} \langle \text{FeX} \rangle = \frac{k_1}{(k_1/k_2) + 1} \langle \text{FeX} \rangle$$

(4)

The value of $\phi$ is controlled by $k_1$ and $k_2$, the rates of quenching and dissociation; there is a hyperbolic relationship between the ratio $k_1/k_2$ and the quantum yield. Any factor which changes this ratio will affect the quantum yield in the manner observed in these experiments; complexes with initial high values of $\phi$ (small $k_1/k_2$) will show little change, while complexes with low values of $\phi$ (large $k_1/k_2$) will show greater change.

The observed temperature-induced increases in quantum yield of the hemoglobin derivatives were fit to the model as shown in Fig. 3. The points are experimental values of $\phi$ at 0 and 40°, plotted against each other, for each ligand. The increase in $\phi$ values between 0 and 40°, calculated according to Equation 4 and given by the solid line, can be produced by a 3.9-fold decrease in the ratio $k_1/k_2$.

This model can be applied to myoglobin and protoheme as well as to hemoglobin. MbCO and heme CN, with quantum yields of close to 1.0, show no temperature dependence, but the less photosensitive n-butyl isocyanide derivatives of both show increased quantum yields at higher temperatures. The decreases in the ratio $k_1/k_2$ required to produce the changes
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seen are different for each protein. The temperature effect, although modified by the protein, is a property of the heme itself.

Similar agreement with the model is found for the inositol hexaphosphate-induced changes in $\phi$, with calculated decreases in $k_1/k_2$ of 1.5-fold. Inositol hexaphosphate shifts the hemoglobin conformational equilibrium toward the T state, and its effect on the quantum yield of the hemoglobin derivatives studied may reflect differences in photosensitivity between the T and R conformations. However, Brunori et al. (4) found the quantum yield of trout Hb I, a cooperative, tetrameric hemoglobin, to be independent of initial saturation with CO, suggesting that the T and R states have equal photosensitivity. In addition, the shape of the CO equilibrium curve of human hemoglobin is unaffected by light, as is the Bohr effect (16).

This contradiction may perhaps be resolved by noting that inositol hexaphosphate binds to R as well as to T state hemoglobin (17) and the changes we have observed here may take place primarily within the R state. HbO$_2$ exhibited the largest increase in quantum yield upon inositol hexaphosphate addition, yet the total extent of O$_2$ dissociation was only 10 to 20%, leaving the hemoglobin still highly saturated. Even under equilibrium conditions, in the presence of inositol hexaphosphate, HbO$_2$ at this level of saturation is predominantly in the R state.

HbCO, on the other hand, is much more photolabile and virtually all of the ligand is removed, leaving unsaturated hemoglobin which, under equilibrium conditions, is in the T state. However, in kinetic experiments such as these, the rate of conformational transition to the T state is slower than the CO dissociation rate, as shown in Fig. 2. Sawicki and Gibson (13) have suggested that the $T \rightarrow R$ transition rate may decrease by a constant factor for each ligand molecule remaining on the hemoglobin. The major contribution in conformational change seen therefore comes from unsaturated hemes, and photolysis occurs primarily from molecules still in the R state.

The situation is different for HbNO. Fully liganded HbNO is converted by inositol hexaphosphate to the T state at low pH (18). Photolysis therefore takes place from the R state in the absence of inositol hexaphosphate and from the T state in its presence. However, HbNO showed a smaller increase in quantum yield than the derivatives which did not change conformation.

HbNO was an exception to the pattern of quantum yield changes in both temperature and inositol hexaphosphate experiments. Although it was the least photosensitive derivative tested, the quantum yield increases measured were smaller than for other, more photosensitive derivatives.

The factors other than temperature and organic phosphate which influence the quantum yield include pH and ionic strength. HB-CO becomes more photosensitive as the pH is lowered, or as ionic strength is raised by the addition of NaCl (5, 6).

The quantum yields of different heme protein derivatives are not correlated with heme affinity for the ligands; the affinity of hemoglobin increases in the order n-butyl isocyanide < O$_2$ < CO < NO, but the order of quantum yields is NO < O$_2$ < n-butyl isocyanide < CO. However, examination of the conditions affecting the photolability of an individual ligand indicates that changes in quantum yield are correlated with changes in affinity for each derivative. Increasing temperature lowers the affinity of both myoglobin and hemoglobin for ligands, and raises their quantum yields. For hemoglobin, adding IHP or NaCl or lowering the pH decrease ligand affinity and increase quantum yields.

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