OXYGEN RECOMBINATION KINETICS FOLLOWING LASER PHOTOLYSIS OF OXYHEMOGLOBIN

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SUMMARY: Oxygen recombination kinetics following laser flash photolysis of human oxyhemoglobin have been measured. Photolysis levels of 60-70% have been achieved with a 1 joule, 1 microsecond, 580 nanometer Rhodamine 6-G-ethanol liquid dye laser. The ratio of fast to slow rate constants was found to be 7 with the fast phase having a value of $5 \times 10^7 M^{-1} sec^{-1}$ and the slow phase a value of $7 \times 10^6 M^{-1} sec^{-1}$. Both values were obtained with 40μM heme in 0.1 M phosphate at pH 7 and 23°C. The quaternary conformational change in hemoglobin at pH 7 occurs in less than a few microseconds.

In order to be able to obtain a consistent picture of the dynamics of oxygen binding to hemoglobin one must combine the results of equilibrium and kinetic experiments. The values for the affinities of various hemoglobins for oxygen have made equilibrium oxygen experiments a common practice. However, the high rate of combination of oxygen with hemoglobin has made it difficult to obtain stop flow kinetic data for this process. Stop flow rapid mixing instruments with resolving times of the order of milliseconds require low oxygen concentrations so that the back reaction (oxygen dissociation) must be considered (1). A fast stop flow rapid mixing instrument with a resolving time of the order of several hundred microseconds has been used to study oxygen combination with deoxyhemoglobin (2). In contrast the slowness of the carbonmonoxide-deoxyhemoglobin reaction makes stop flow measurements relatively easy to perform compared with oxygen experiments.

A different approach to the kinetic problem is found in the method of flash photolysis in which the ligand is dissociated from the hemoglobin-ligand complex by a pulse of light. In this case, however, carboxyhemoglobin has been relatively easy to dissociate with conventional light sources (for example xenon flash lamps) while it was found that the quantum efficiency for photolysis of oxyhemoglobin was only one ninetieth of that of carboxyhemoglobin (3). This fact combined with the intrinsic pulse width of xenon flashes has prohibited flash photolysis experiments of oxyhemoglobin up to this time. In situations where a single monophasic reaction is to be expected, such as myoglobin and single α and β chains, it has been possible to photo-dissociate these oxy-

hemoproteins directly and to study oxygen recombination with single chains by this method (3,4,5).

Although kinetic results may be easily obtained from rapid mixing and flash photolysis experiments for carbonmonoxide combination with hemoglobin, the high affinity of hemoglobin for carbonmonoxide (about 200 times that for oxygen) makes routine equilibrium experiments difficult.

Experiments are reported here in which oxygen is directly dissociated from oxyhemoglobin and the subsequent recombination kinetics determined. The flash photolysis experiments may be performed with high oxygen concentration so that the back reaction (oxygen dissociation) may be neglected. This now makes it feasible to perform consistent equilibrium and rapid mixing and flash photolysis experiments with the physiological ligand oxygen. These experiments are made possible by utilizing the intense, narrow, monochromatic outputs of several laser systems to selectively excite the α and β bands of oxyhemoglobin. The same oxyhemoglobin sample may be flashed many times with no appreciable hemoglobin degradation. This is due to the high specificity of the laser radiation for the heme group and the high quantum efficiency for ligand dissociation. The 120 millijoule, 40 nanosecond, 530 nanometer frequency-doubled output of a neodymium-glass laser was first used to excite the β band of oxyhemoglobin which has maximum absorption at 542 nanometers in conjunction with the 220 millijoule, 400 nanosecond, 580 nanometer output of a cylindrical flashlamp pumped Rhodamine 6-G-ethanol liquid dye laser which excited the α band of oxyhemoglobin near the maximum absorption at 576 nanometers. The 530 nanometer pulse was separated from the 1060 nanometer primary pulse by the use of a dispersing prism, and the 1060 nanometer pulse was detected with a photodiode and used to trigger the liquid dye laser so that the 530 nanometer pulse and the 580 nanometer pulses were coincident to within a few microseconds. The two pulses were focused into a 5 mm by 5 mm cuvette. A tungsten iodide lamp was used for measuring light, and transmission changes occuring upon and subsequent to photolysis were detected with two photomultipliers (with appropriate interference filters) following a fifty percent beam splitter. The output of both photomultipliers were connected to a difference amplifier and the resulting wave-form photographed on an oscilloscope. This detection system offers one the opportunity of decreasing the laser artifact by subtraction if one of the wavelengths is chosen to be an isosbestic point for the reaction. Single beam operation may be obtained by replacing the above input to the difference amplifier with a battery off-set voltage. The rise time of the electronic detection system was 1 microsecond.

A typical oscilloscope trace showing the transmission change due to oxygen recombination following oxygen photolysis of horse heart oxymyoglobin is

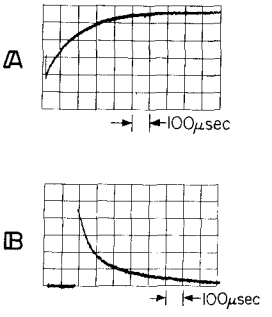

Figure 1. a) Transmission change which occurs upon 530 nanometer laser photolysis of 30μM oxyheme horse heart oxymyoglobin, in 0.1 M phosphate, 278μM oxygen, pH 7 at 21°C and measured at 436-456 nanometers. b) Transmission change which occurs upon 530+580 nanometer laser photolysis of 37μM oxyheme human oxyhemoglobin (HbA), in 0.1 M phosphate, 255μM oxygen, pH 7, 25.6°C and measured at 430-456 nanometers.

shown in Figure 1a. At 21°C, 0.1 M phosphate and pH 7.4 a second order rate constant of 2.1 x $10^7 M^{-1} sec^{-1}$ is obtained from the log plot of the corresponding absorbance change given in Figure 2 and is to be compared with a value of 1.9 x $10^7 M^{-1} sec^{-1}$ at 20°C and pH 7.4 obtained from a continuous flow experiment by Millikan (6) for horse myoglobin, a value of 1.2 x $10^7 M^{-1} sec^{-1}$ at 22°C, pH 7.1 given by Gibson (3) for whale myoglobin, a value of 1.4 x $10^7 M^{-1} sec^{-1}$ at 20°C and pH 7 given by Antonini (4) for horse myoglobin and 1.9 ± 0.04 x $10^7 M^{-1} sec^{-1}$ at 20°C and pH 7 for sperm whale myoglobin given by Bunori and Schuster (7). The latter value was derived from temperature jump experiments in the millisecond time range. A comparison of oxygen recombination kinetics following

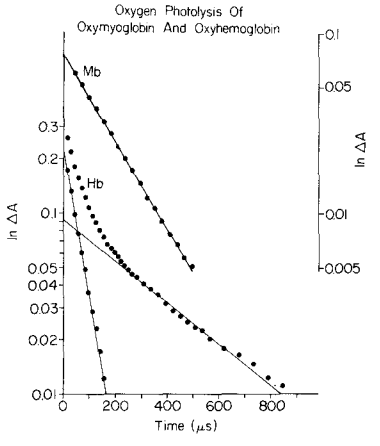

Figure 2. Logarithm delta absorption plots of the transmission changes shown in Figure 1. The left hand scale refers to HbA and the right hand scale to Mb.

either laser flash photolysis or temperature jump excitation of oxyhemoglobin is important because of the intrinsic differences in the two methods of excitation. The corresponding results for oxygen recombination following oxygen photolysis of human oxyhemoglobin is shown in Figure 1b. In this case it can be seen that recombination is biphasic. A value of 7 x $10^7 M^{-1} sec^{-1}$ at pH 7 and 25.6°C is found from this data for the "quickly reacting component" (8) and a value of 1 x $10^7 M^{-1} sec^{-1}$ is found for the apparent rate constant of slow phase. These values were obtained with 37μM heme human oxyhemoglobin prepared by the toluene method similar to Drabkin's (9) and diluted in 0.1 M sodium phosphate buffer. The results then should be comparable with previous work using hemoglobin samples prepared in the same way.

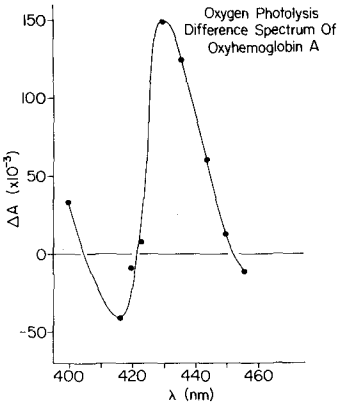

Figure 3. Photolysis difference spectrum for human oxyhemoglobin A of total transmission changes which occur at an approximately constant photolysis level of 20% with single beam operation. The experiment was performed with 32μM oxyheme, in 0.1 M phosphate, pH 7 at 21°C.

In order to verify that direct oxygen dissociation from oxyhemoglobin was taking place a photolysis spectrum was obtained at constant percent photolysis and is shown in Figure 3. In addition the experimentally determined linear variation of the pseudo-first-order rate constants with respect to oxygen concentration for both fast and slow components for human hemoglobin is shown in Figure 4. Solubility coefficients and partial gas pressures were used to calculate the oxygen concentrations in solution. The percent slow component was obtained from the values of the slow and fast absorbance changes found by extrapolation to zero time and is plotted as a function of oxygen concentration in Figure 5.

From the data given in this figure, i.e. the absence of an oxygen dependence for the initial amplitude of the slow phase (8) it may be concluded that

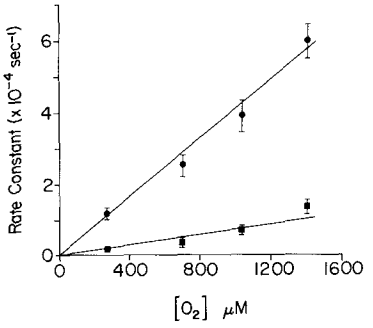

Figure 4. Variation of pseudo-first-order fast and slow rate constants with oxygen concentration for oxygen combination to human hemoglobin A following an approximately constant level of 20% for laser photolysis of human oxyhemoglobin. The experiment was performed with 33μM oxyheme, in 0.1 M phosphate, pH 7 at 20.5°C. The data were obtained at 430-456 nanometers.

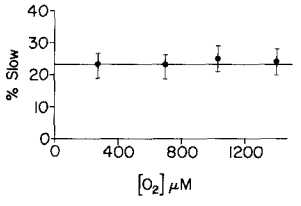

Figure 5. Variation of the percent slow delta absorbance change with oxygen concentration for oxygen combination to human hemoglobin A following an approximately constant level of 20% for laser photolysis of human oxyhemoglobin. The data is from the experiment shown in Figure 4.

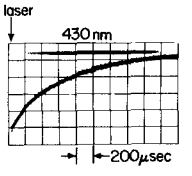

Figure 6. Transmission change which occurs upon photolysis of human oxyhemoglobin A by a 1 joule, 1 μsec, 580 nanometer Rhodamine 6-G-ethanol liquid dye laser. The experiment was performed with 40μM oxyheme, 272μM oxygen, pH 7 at 23°C and measured with single beam operation at 430 nanometers. The total oxy-to-deoxy transmission change corresponds to five vertical divisions on the above figure. The horizontal line corresponds to the transmission of the initial oxyhemoglobin sample. The photolysis level reached in this experiment was 60%. The large absorbance changes monitored in this experiment result in a distortion of the fast phase transmission change.

the rate of oxygen binding to the hemes in the oxyquaternary state is still not competitive with the rate of quaternary conformational change and thus that the rate of the quaternary conformational change in hemoglobin at pH 7, 0.1 M

phosphate and 20.5°C is of the order of or less than a few microseconds. The fractional photolysis level reached in the hemoglobin experiments described above was determined to be 22%.

In order to be able to study oxygen binding to deoxyhemoglobin it is necessary to achieve a higher degree of photolysis. In addition the value of the isosbestic point used for the above experiments was not exactly right so that the values of apparent rate constants quoted above are slightly fast. The following data were therefore taken with single beam operation. Figure 6 shows the transmission changes obtained at 430 nanometers following laser photolysis of human oxyhemoglobin at pH 7, 0.1 M phosphate and 23°C. The laser system used in this experiment was a 1 joule, 1 μsec, 580 nanometer cylindrical flashlamp-pumped Rhodamine 6-G-ethanol liquid dye laser, (Candela). The 3/4 inch diameter output was focused into a 5 mm cuvette which was backed by a reflector. By this means relatively uniform photolysis could be achieved throughout the sample volume. With the above system photolysis levels for oxyhemoglobin of 60-70% have been obtained.* The apparent rate constant for the slow phase may be most reliably obtained from the data at high photolysis while the rate constant for the fast phase is more reliably obtained from data at low photolysis. The slow phase absorption change corresponding to Figure 6 amounted to about 60%. The average values for the apparent rate constants obtained from data from several such experiments for oxygen combination with human hemoglobin A at 430 nanometers, pH 7, in 0.1 Molar sodium phosphate and at 23°C are $5 \times 10^7 M^{-1} sec^{-1}$ for the fast phase and $7 \times 10^6 M^{-1} sec^{-1}$ for the slow phase. It should be noted that the ratio of fast to slow rates for oxygen kinetics is about a factor of 7 while the ratio of fast to slow rates for carbonmonoxide kinetics is about 19. A scaling law thus does not exist for ligand binding to hemoglobin and each ligand must be investigated separately.

It is interesting to note that the rate of the "quickly reacting component" found in this experiment is similar to that for oxygen recombination to single chains (5). It is also to be compared with a value for k_4' of $3.3 \times 10^7 M^{-1} sec^{-1}$ at 21.5°C found by Gibson (1) by photolyzing carboxyhemoglobin in the presence of oxygen. Under the conditions of this experiment, part of the fast phase will be due to oxygen recombination with $\alpha_1 \beta_1$ dimers. The oxyhemoglobin used in these experiments (40μM) can be calculated to be about 24% dissociated into dimer if a tetramer to dimer equilibrium dissociation constant of 3μM heme is assumed (11). The slow component should be compared with

* Recently Alpert et al. (10) have reported a photolysis value of 30% with a 0.1 to 1 joule, 50 nanosecond, 529 nanometer frequency doubled neodymium glass laser.

the value obtained for oxygen combination with deoxyhemoglobin. The range of values given by Berger et al. in 1967 (2) was 2-4.5 x $10^6 M^{-1}sec^{-1}$ at 23°C, but more recent data (12) indicate that a value of about 4.3 x $10^6 M^{-1}sec^{-1}$ is now obtained over the 10 to 60% range. It should be noted that the value of the rate constant for the slow phase obtained by laser flash photolysis corresponds to the later rate limiting slow stages of ligand binding since any initial differences in slow phase are masked by the fast phase. A close comparison of these two results on the same sample under the same conditions may make it possible to determine at what point the quaternary transition takes place.

The small sample size needed for laser photolysis of oxyhemoglobin experiments, 0.25 ml of 40-200μM heme, makes it possible to extend the study of structure-function relationships in oxygen binding to hemoglobin by looking at oxygen kinetics of many artificial and mutant hemoglobins.

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