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Nanosecond Absorption Study of Kinetics Associated with Carbon Monoxide Rebinding to Hemoglobin S and Hemoglobin C Following Ligand Photolysis

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SUMMARY: The absorption spectra of photolysis intermediates of the CO complex of hemoglobin S and hemoglobin C, in the tetramer form, have been measured between 10 ns and 200 ms after excitation. These data were analyzed using singular value decomposition (SVD) and global analysis to determine kinetic lifetimes associated with various processes involved in CO recombination. The results of this analysis show that, in the tetramer (non-aggregated) form, hemoglobin S and hemoglobin C exhibit the same kinetics associated with CO recombination as hemoglobin A .

Hemoglobin in patients with sickle cell disease or with CC disease differs from hemoglobin A (HbA) of normal adults in the substitution of a single amino acid. In hemoglobin S (HbS) the substitution is from β 6 Glutamate to Valine (1), and in hemoglobin C (HbC) Lysine is substituted for β 6 Glutamate (2). Under physiological conditions, *deoxy*-HbS polymerizes to form polymers and polymer bundles and *oxy*-HbC forms crystals (3). Sickle cell disease results in substantial morbidity and mortality. The homozygote state for HbC results in a mild hemolytic anemia and normal survival.

In the polymer form, HbS has been shown to have a low oxygen affinity compared to HbA (4-9). However, in the unpolymerized form, HbS and HbA have the same binding equilibra (10). Furthermore, the bimolecular ligand rebinding kinetics are the same for unpolymerized HbS and HbA (11, 12). Thus, these kinetic processes appear unaffected by the amino acid substitution at β 6. The purpose of this work is to determine, with nanosecond resolution, whether these and earlier kinetic processes occuring after

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photolysis of unpolymerized forms of HbS-CO and HbC-CO differ from those of HbA-CO.

Materials and Methods

All blood samples were obtained following federal regulations and guidelines outlined by the National Institutes of Health. Hemoglobin S was prepared as described previously (13). Cells were washed in 1% NaCl and lysed by incubation in distilled water. Membranes were removed by centrifugation and the hemoglobin was dialyzed against distilled water and frozen in liquid nitrogen for storage. The percentage of HbF was determined to be 4.7% using the alkali denaturation method (14). Purified HbC was prepared from the hemolysates of a CC patient and an individual heterozygous for hemoglobins C and A. Hemolysates were made by freeze thawing red cells washed in isotonic saline and purified on CM-52 columns as previously described (15). The separation of HbC and HbA from an AC hemolysate ensures a proper control. Most of the HbA used in this study was prepared in the same manner as the HbS (13). In order to test for the effects on kinetic studies of other hemoglobins that may have been present in the HbA preparation, some measurements were made on purified HbA (as described for HbC). No difference was detected in the kinetics involving HbA prepared in either manner.

Time resolved optical density (TROD) measurements were performed on hemoglobin samples diluted to about 120 μM in heme in 0.1M sodium phosphate buffer, pH 7.3. The samples were maintained at one atmosphere CO . Less than 100 μM of sodium dithionite was added prior to measurements. The samples were flowed through a 1/2 mm path length cell.

The TROD instrument used in this study has been described previously (16, 17). A Xenon flashlamp produced probe pulses at 2 Hz focused on the sample to a diameter of about 2 mm. Actinic pulses of 17 mJ per pulse were produced by a Quanta Ray DCR-2 Nd:YAG laser, frequency doubled to 532 nm. The excitation pulses made an angle of about 150^0 with respect to the probe beam. The diameter of the laser beam was about 5 mm. A clean up Glan-Taylor polarizer was used to insure the polarization purity of the actinic beam. The probe beam was passed through a polarizer oriented along the magic angle to reduce artifacts from reorientation kinetics of the photolysed hemoglobin molecules. The probe beam was focused through a 100 μm slit into a Jarrel Ash spectrograph (150 grooves per mm) and detected with a EG&G OMA II detector. A Stanford Instruments DG535 delay/pulse generator was used to control the timing of the detector gate (10ns) and the firing of the flashlamp with respect to the laser.

Absorption spectra were measured at 72 times from 10 ns to 200 ms following laser photolysis. Data were collected from 300 nm to 700 nm but only data from 400 to 470 nm were used in the analysis of kinetic processes presented here. These data can be represented by a $m \times n$ matrix, A, corresponding to the m wavelengths and n time delays. The measurements were repeated several times for each hemoglobin type with separate preparations of the sample. The data was analyzed by singular value decomposition (SVD) (18-20). SVD rewrites the data matrix as the product of three matrices

$$A = USV^T. \tag{1}$$

U is a $m \times n$ matrix containing the optical density for n orthonormal basis spectra at m wavelengths. V^T denotes the transpose of V, a $n \times n$ matrix giving the amplitude of each basis spectrum at n time delays. S is an $n \times n$ matrix containing the singular values of A, a determinant of the contribution of each basis spectum to the measured spectrum at a given

time. In practice, only the largest singular values and time amplitudes are retained. The smaller values and amplitudes are discarded as noise. Thus SVD provides a concise, noise-filtered representation of the data. This truncated representation is used to fit to exponential lifetimes and amplitudes using a (non-linear) least square global analysis fitting technique (17).

Results

Transient Hb-HbCO absorption difference spectra taken at 72 times following photolysis are shown in Fig. 1 for HbA, HbC, and HbS. Each spectrum represents the difference in absorption between the photolysis product and the ligated ground state molecule at a given time. The positive peak around 430 nm corresponds to the increase in absorption of the transient photolyzed species and the negative peak around 417 nm corresponds to its decreased absorption relative to the ligated ground state. The return of the photolyzed product to ground state is evidenced by the approach of the difference spectra to zero at later times. The spectra were corrected for a stray light artifact that arises in detection around the narrow Soret absorption peak. At high protein concentrations, detected stray light in this absorption band tends to diminish the negative peak of the TROD spectra around 417 nm.

The spectra shown in Fig. 1 are very similar but some differences can be discerned upon close examination. In particular, the negative peak for the HbS is larger than that of HbA and HbC. This feature is most likely due to imperfections in the stray light correction. Of more concern here, however, is whether these three hemoglobin forms yield the same number of photolysis intermediates with the same kinetics. Thus the data was analyzed by SVD and global analysis. SVD was applied in order to obtain a concise, noise filtered representation of the data. The data matrices obtained by using the five highest singular values, their corresponding basis spectra, and time courses were fit using global kinetic analysis to obtain apparent lifetimes and amplitudes for exponential decays of intermediate species formed by HbCO photolysis. The global analysis uses a model that assumes that each intermediate species undergoes parallel, first-order decays. Although this assumption may appear unrealistic for photolyzed HbCO, the parallel process model is commonly applied in this case because the rate constants from such a parallel process model are the same as the observed rate constants of first-order exponential processes, which are model independent. However, since the amplitudes of the exponential processes are model dependent, their physical significance in this application is limited to a representation of the relative contribution of each rate constant to the overall spectral kinetics.

While we have shown (Paquette, S. J., Björling, S. C., Goldbeck, R. A., and Kliger, D.S., in preparation) that at least six processes are needed to explain HbA ligand recombination processes, it is useful to analyze our data with a five exponential fit for comparison to previous analyses present in the literature (21). The results of global kinetic analysis for the hemoglobin species using a fit to five exponential processes are summarized in Table 1. A summary of the results of a fit to six exponential processes is

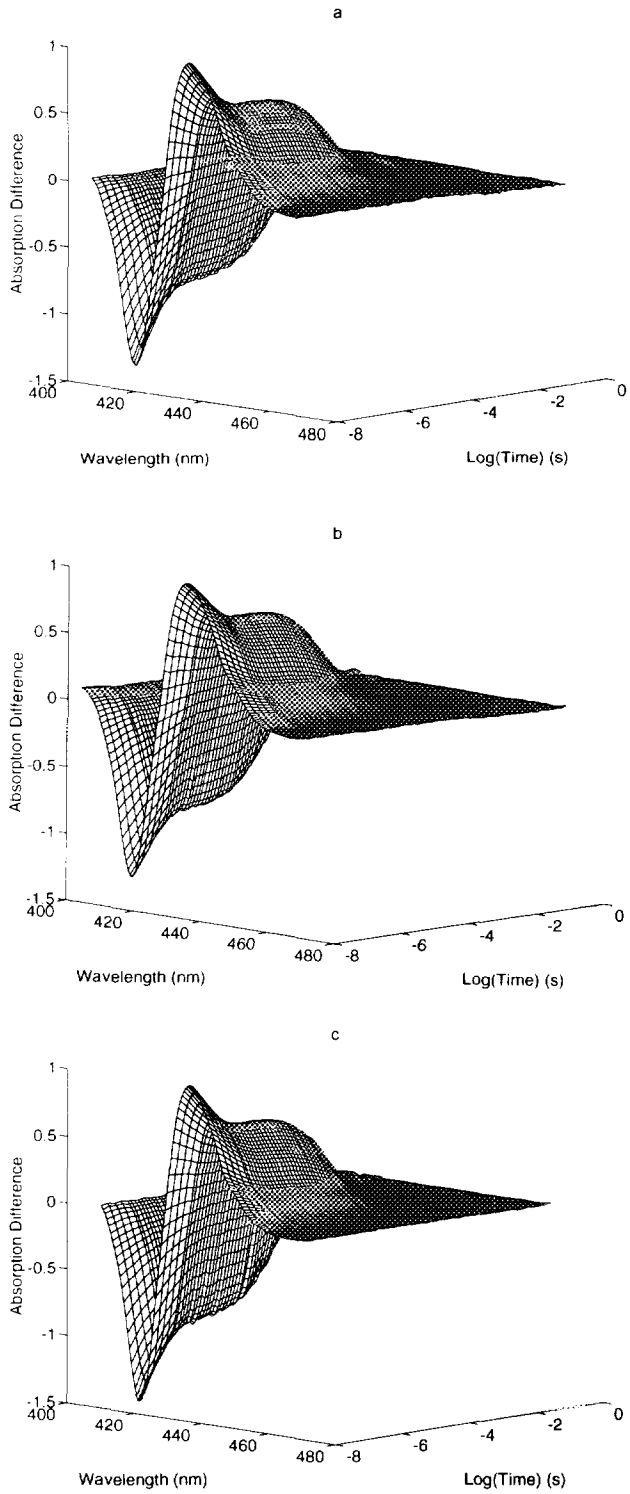

Figure 1. Transient Hb-HbCO absorption difference spectra. Spectra are shown at 73 times following laser photolysis. Each data set has been normalized by its maximum absorption difference. a) HbA. b) HbC. c) HbS.

Table 1

	HbA	HbC	HbS
τ_1 / Amp_1	28 ± 4 ns / 0.33 ± 0.02	31 ± 6 ns / 0.29 ± 0.04	27 ± 3 ns / 0.31 ± 0.01
τ_2 / Amp_2	130 ± 17 ns / 0.17 ± 0.02	130 ± 10 ns / 0.16 ± 0.03	130 ± 12 ns / 0.16 ± 0.02
τ_3 / Amp_3	24 ± 4 μs / 0.08 ± 0.07	32 ± 4 μs / 0.12 ± 0.02	27 ± 4 μs / 0.08 ± 0.01
τ_4 / Amp_4	158 ± 9μs / 0.31 ± 0.02	163 ± 9 μs / 0.33 ± 0.03	161 ± 8μs / 0.31 ± 0.02
τ_5 / Amp_5	3.6 ± 0.1 ms / 0.11 ± 0.01	3.3 ± 0.2 ms / 0.10 ± 0.02	3.4 ± 0.3 ms / 0.14 ± 0.03

given in Table 2. The tables summarize results from five measurements on HbA, three measurements on HbC, and five measurements on HbS. Each rate and amplitude is presented ± one standard deviation. The rates are not dependent on concentration, as a measurement on 78 μM HbA produced results similar to measurements at higher concentrations. A standard t-test showed that at a 95% confidence level there was no significant difference in the means given in Tables 1 and 2 except for those between HbC and HbS in Amp_3 using a five exponential fit.

Discussion

The lifetimes and relative amplitudes of exponential processes obtained from global analysis are remarkably similar for the three hemoglobins studied here. These results indicate that HbA, HbC and HbS are functionally identical in the tetramer form.

Table 2

	HbA	HbC	HbS
τ_1 / Amp_1	24 ± 3 ns / 0.30 ± 0.03	25 ± 1 ns / 0.25 ± 0.06	22 ± 4 ns / 0.27 ± 0.03
τ_2 / Amp_2	104 ± 6 ns / 0.20 ± 0.02	105 ± 7 ns / 0.20 ± 0.01	99 ± 18 ns / 0.20 ± 0.03
τ_3 / Amp_3	1.8 ± 0.4 μs / 0.020 ± 0.003	1.8 ± 0.9 μs 0.022 ± 0.002	1.9 ± 1μs 0.025 ± 0.003
τ_4 / Amp_4	42 ± 9 μs / 0.10 ± 0.01	42 ± 3 μs / 0.14 ± 0.03	50 ± 11 μs / 0.11 ± 0.02
τ_5 / Amp_5	180 ± 6μs / 0.27 ± 0.02	185 ± 15 μs / 0.29 ± 0.03	193 ± 26μs / 0.26 ± 0.03
τ_6 Amp_6	3.80 ± 0.1 ms / 0.11 ± 0.01	3.5 ± 0.2 ms / 0.10 ± 0.02	3.7 ± 0.2 ms / 0.13 ± 0.03

The fit of the TROD data to five exponential processes produced similar lifetimes as those found in an earlier study by Hofrichter et al. (21). By varying ligand pressure and the degree of laser photolysis, Hofrichter et al. were able to interpret the five exponential processes. The first process, τ_1, was interpreted as geminate recombination of the photolyzed CO molecule. This process is followed by a tertiary relaxation of the heme (τ_2). According to Hofrichter et al., the R→T transition occurs with a 20 μs lifetime (τ_3). The fourth and fifth processes were interpreted as the bimolecular recombination of CO to the R- and T-states. In the analysis of separate experiments, we have been able to fit hemoglobin TROD data to six exponential processes with reproducibility matching or exceeding that obtained in the five exponential fits (Paquette, S. J., Björling, S. C., Goldbeck, R. A., and Kliger, D.S., in preparation). The inclusion of a sixth exponential process results in the splitting of the R→T transition into a small 1 μs process and a larger 40 μs process. That the 1 μs process is related to the R→T transition is supported by measurements using time-resolved circular dichroism (Björling, S. C., Paquette, S. J., Milder, S. J., Goldbeck., R. A., and Kliger, D. S. in preparation) and time-resolved optical rotary dispersion (22). In any case, fitting hemoglobin TROD data to six exponentials requires a better signal to noise ratio and results in a more refined kinetic analysis than a fit to five exponentials. That the TROD of HbA, HbC, and HbS (in the tetramer form) have similar lifetimes and amplitudes even when fit to six exponentials is very strong evidence for their functional similarity. The discrepancy in Amp_3 between HbA and HbC using a five exponential fit was not confirmed with a fit to six exponentials. Futhermore, when comparing the number of quantities given in the tables, at the 95% confidence level, one would expect one of these to randomly come out as "significantly different." Therefore the null hypothesis that there are no significant differences in the kinetics associated with ligand rebinding to HbA, HbC, amd HbS holds.

The similarities found in the analysis of nanosecond TROD data for HbA, HbC , and HbS are consistent with earlier studies of these hemoglobin types in the tetramer form. Both the equilibrium binding (10) and bimolecular recombination and dissociation rates (11) were found to be identical for HbS and HbA tetramers. The present study shows that there is no difference in the kinetics associated with CO recombination between HbA, HbC, and HbS. These results suggest that there is no difference in kinetic cooperativity in these species. In addition, this study indicates that the ligand affinity of the heme and entry and exit rates of the CO molecule are similar. Thus the substitution of Valine or Lysine for Glutamate at the β 6 position has no effect on these kinetics.

The similarity in the kinetics measured in this study for HbA, HbC, and HbS is not surprising given that equilibrium ligand binding to these hemoglobins in the tetramer form is identical. However, polymerized forms of HbS and HbC have lower ligand affinity in equilibrium than HbA (4-9). This lower affinity could be due to differences in the entry or exit rates of the ligand or a lower affinity of the heme for the ligand due to conformational distortions caused by polymerization. We are currently investigating these possibilities by studying the kinetics associated with ligand rebinding to polymerized forms of HbS and crystal forms of HbC.

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