SPIN LABEL DETECTION OF INTERMOLECULAR INTERACTIONS IN CARBONMONOXY SICKLE HEMOGLOBIN

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ABSTRACT With recently developed spin label techniques for monitoring macromolecular rotational motion, heme-liganded sickle cell hemoglobin in the presence of inositol hexaphosphate is shown to exhibit restricted motional freedom as compared to liganded normal adult human hemoglobin. This motional restriction is dependent on both hemoglobin concentration and temperature.

INTRODUCTION

A great amount of interest has recently centered on determining the polymerization mechanism(s) of sickle cell hemoglobin (HbS) (1–11). The more significant results of this work include a partial thermodynamic characterization of the hydrophobic interactions that appear to dominate the polymerization mechanism (12, 13), and indications that polar interactions and allosteric effectors such as 2,3-diphosphoglycerate (DPG) also play roles in polymerization (8, 14). The contributions of allosteric effectors and the details of any nonhydrophobic contributions to polymerization, however, have been quite difficult to study in a system dominated by hydrophobic interactions (12). Furthermore, the gel phase of deoxy HbS appears to consist primarily of an equilibrium between monomer and polymer states (10), thus preventing any study of intermediate states.

One method, however, which may have utility in the study of interaction mechanisms involves a partial disruption of the intermolecular polymer contact sites through the use of Hb derivatives with specific structural alterations (15–18). Among the simplest structural alterations, of course, is heme ligation, where the quaternary structure of Hb changes from the deoxy to the oxy conformation. We report here the use of a recently developed spin label technique for monitoring protein rotational correlation times (19–20) to compare the rotational freedom of carbonmonoxy HbS and normal adult COHb (COHbA) in the presence and absence of organic phospate. Using this method, it is demonstrated that in the presence of inositol hexaphosphate (IHP), carbonmonoxy HbS (COHbS) exhibits a significant intermolecular interaction with a quadratic concentration dependence, indicating that organic phosphate may induce HbS aggregation even when in the fully liganded conformation. Furthermore, the in-

troduction of IHP results in a decrease in the energy barrier for COHbS rotational diffusion, again indicating the existence of liganded COHbS + IHP in an aggregated form.

MATERIALS AND METHODS

Blood was drawn into EDTA anticoagulant from normal adults and from homozygous sickle patients. Solutions of cell-free hemoglobin were prepared according to Abraham et al. (21). Electrophoresis of the HbS solution showed the composition to be ~95% HbS and ~5% fetal Hb. Lysates were used without further separation of Hb components. DPG was removed by passing the Hb solution over Rexyn I-300 (Fisher Scientific Co., Pittsburgh, Pa.). COHb was spin-labeled with 4-maleimido-2,3,6,6,-tetramethylpiperidinooxyl (Syva Corp., Palo Alto, Calif.) following the procedures of McCalley et al. (19). For concentration effect studies, spin-labeled COHbA and COHbS were concentrated in 0.05 M sodium phosphate buffer, pH 6.7, by ultrafiltration (Amicon Corp., Lexington, Mass.). This buffer was chosen for the temperature independence of its pH. Organic phosphate effects were studied by adding IHP (Sigma Chemical Co., St. Louis, Mo.) to separate stock solutions in an IHP:Hb molar ratio of 4:1. The concentration of each sample was checked spectrophotometrically. Samples were sealed into capillary pipettes under one atmosphere pressure of CO to ensure complete ligation during electron spin resonance (ESR) measurements. COHb rotational correlation times were determined from the apparent separation of the hyperfine extrema of the spin label which is immobilized within the protein matrix (19, 20). The relationship between Hb rotational motion and the magnitude of the hyperfine separation was empirically determined using sucrose solutions of known viscosity (M. Johnson, unpublished data). ESR spectra were measured at 5 mW microwave power and 1 G modulation on a Varian E-112 spectrometer equipped with a variable temperature accessory. Methemoglobin formation during experiments was found to be <5% of the sample in all cases.

RESULTS AND DISCUSSION

The variation of the rotational correlation time, τ, with Hb concentration at 35°–36°C is shown in Fig. 1 for the four cases, COHbA, COHbA + IHP, COHbS, and COHbS + IHP; general agreement is observed with the data obtained from water proton relaxation measurements (22). More specifically, the concentration dependence of the control COHbA sample, as determined from spin label measurements, is nearly identical with the behavior as determined from water proton relaxation. Likewise, in the presence of organic phosphate, the correlation time of COHbS increases significantly at high concentration when compared to that of COHbA, again in agreement with Lindstrom et al. (22). This agreement between the two methods clearly demonstrates the validity of the spin label technique used here, and thus permits an extension of the spin label methods to obtain a more quantitative description of the COHbS interaction as a function of concentration, temperature, and organic phosphate.

Recent work has shown that the translational diffusion coefficient is, to a first approximation, linearly dependent on Hb concentration, provided that only hydrodynamic considerations are of importance (23, 24). Rotational diffusion, which is inversely proportional to τ, exhibits behavior very similar to translational diffu-

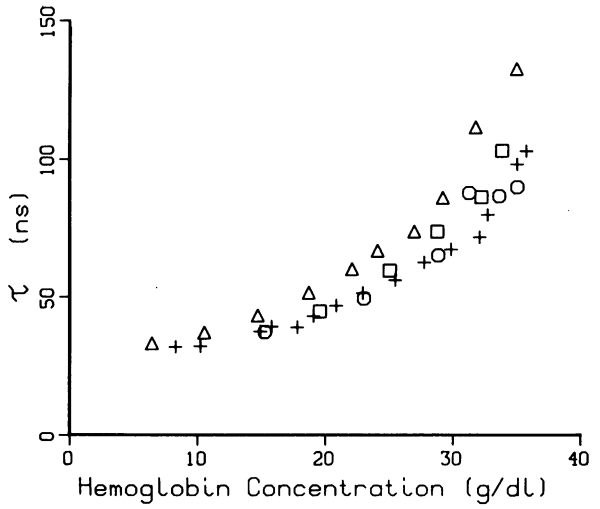

FIGURE 1 Hb rotational correlation times at 35°–36°C as a function of Hb concentration. (○) Stripped COHbA; (□) COHbA + IHP; (+) stripped COHbS; (△) COHbS + IHP.

sion (25). Thus, for a more quantitative analysis of our systems, we examine the concentration dependence of the inverse rotational correlation time, $1/\tau$. In Fig. 2 we observe that the $1/\tau$ concentration dependencies for COHbA, COHbA + IHP, and COHbS are linear and indistinguishable from each other at both 22° and 35°C. From this observation we make the empirical assumption that rotational diffusion for noninteracting Hb species will exhibit a linear concentration dependence. In contrast to the behavior of these three species, COHbS + IHP clearly exhibits a rate of rotational diffusion that is slower over the entire concentration range. Moreover, the inverse correlation time for HbS + IHP exhibits a quantitatively significant deviation from linearity over the concentration range covered. (The appropriate regression equations and parameters are given in Table I). The Stokes relation for rotational motion can be written in the form

$$1/\tau = \frac{kT}{V\eta}, \tag{1}$$

where k is Boltzman's constant, T is the absolute temperature, η is the effective solvent viscosity, and V is the volume of the diffusing molecule. Inasmuch as temperature and solution conditions are equivalent for all four samples, the slower rate of rotational diffusion exhibited by COHbS + IHP must be interpreted as resulting from COHbS + IHP aggregation. (Aggregation will increase the effective volume of the diffusing system and thus slow the rate of rotational diffusion due to the $1/V$ dependence of $1/\tau$.) The deviation from linearity exhibited by the COHbS + IHP concentration curves is further confirmation that IHP induces a concentration-dependent aggregation of COHbS. For an unbounded aggregation mechanism where the size of the aggregate increases indefinitely, one would expect the concentration curves of the aggregating

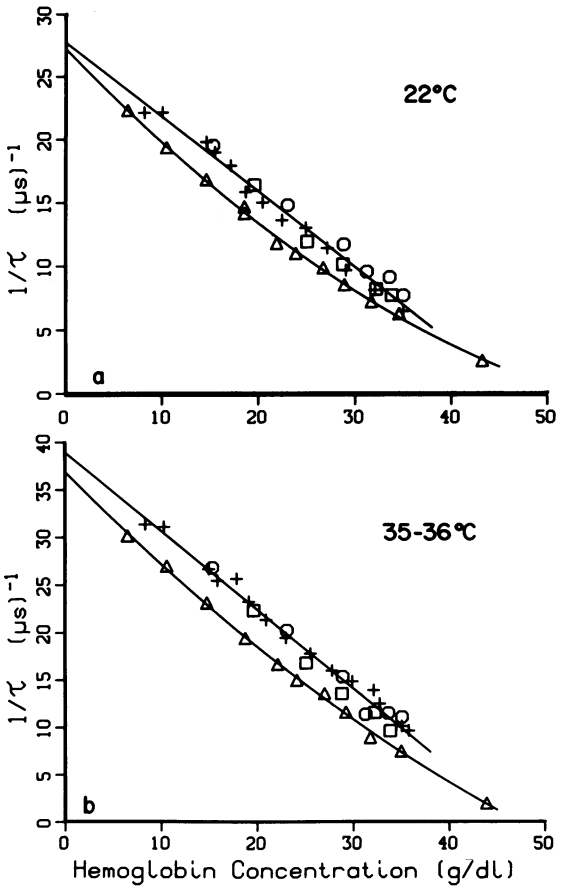

FIGURE 2

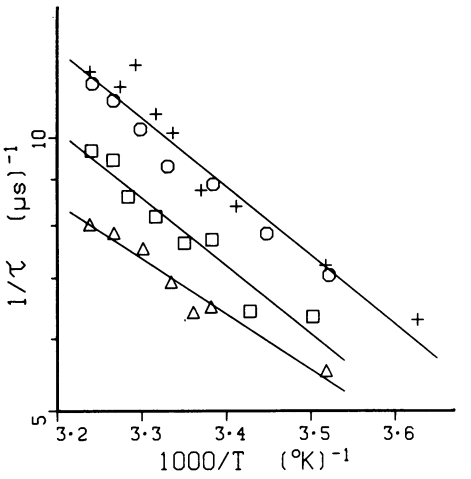

FIGURE 3

FIGURE 2 Inverse rotational correlation times as a function of Hb concentration (a) at 22°C, (b) at 35°–36°C. (○) stripped COHbA; (□) COHbA + IHP; (+) stripped COHbS; (△) COHbS + IHP. The straight lines in the figures are linear regressions of the combined COHbA ± IHP and COHbS – IHP data; data were combined because individual regressions did not differ significantly. The COHbS + IHP curves are the result of quadratic regressions of the form $1/\tau = d_0 + d_1 \cdot [\text{Hb}] + d_2 \cdot [\text{Hb}]^2$. The quadratic term is statistically significant ($P < 0.0001$) and appears to be independent of temperature. The difference in zero concentration intercepts between the COHbS + IHP curve and the combined regression for the other three species appears to be significant at 35°C, but not at 22°C. The relative uncertainty of one measurement with respect to another is approximately 2–3 μs^{-1}; the absolute uncertainty of individual measurements is approximately twice as large. The measurement uncertainties of $1/\tau$ are nearly independent of Hb concentration.

FIGURE 3 Temperature dependence of the rotational correlation time at a Hb concentration of 33.8 g/dl. (○) Stripped COHbA; (□), COHbA + IHP; (+) stripped COHbS; (△) COHbS + IHP. Straight lines shown are linear regressions of the logarithm of the inverse correlation times vs. inverse absolute temperature. The apparent activation energies are 3.4 ± 0.2 kcal/mol for the combined data set of COHbS, COHbA, and COHbA + IHP, and 2.8 ± 0.3 kcal/mol for HbS + IHP. Each point in this figure is an average of two measurements; the measurement uncertainties are thus approximately half the magnitude of those given in the caption to Fig. 2.

TABLE I

CONCENTRATION DEPENDENCE OF ROTATIONAL MOTION FOR Hb SYSTEMS*

Sample	T	d_0	d_1	d_2
	°C	μs^{-1}	$\mu s^{-1}\, g/dl^{-1}$	$\mu s^{-1}, g/dl^{-2}$
COHbA, COHbA + IHP and COHbS, combined data	22	27.7 ± 0.5	−0.59 ± 0.018	—‡
COHbS + IHP	22	27.2 ± 0.4	−0.801 ± 0.032	0.0054 ± 0.0006
COHbA, COHbA + IHP and COHbS, combined data	35–36	38.9 ± 0.6	−0.828 ± 0.022	—‡
COHbS + IHP	35–36	36.9 ± 0.5	−1.023 ± 0.045	0.0052 ± 0.0009

*Regressions were calculated from the equation, $1/\tau = d_0 + d_1 \cdot [\mathrm{Hb}] + d_2 \cdot [\mathrm{Hb}]^2$. The uncertainties listed are the calculated estimates of error from the regressions.

‡For the combined systems the quadratic coefficient was zero within the limits of uncertainty. Thus, only a linear regression was used for the combined data (the upper lines in Fig. 2 *a* and *b*).

species to diverge from those of the noninteracting species at all concentrations. For a bounded aggregation mechanism, however, where the maximum size of the aggregate is relatively small, one would expect divergence of the curves only at low concentration. At very high concentration, one would expect the two curves to converge again because the rate of rotational diffusion is then dictated primarily by the Hb-Hb intermolecular frictional coefficient, rather than solvent viscosity and molecular size. Thus, the positive quadratic concentration dependence of the COHbS + IHP curve in Fig. 2 indicates the existence of an aggregate of limited size. An equilibrium between monomers (one Hb molecule) and dimers (two Hb molecules) appears to be the most plausible aggregation mechanism.

A comparison of the curves in Fig. 2 indicates the existence of a small but real increase in the separation between the COHbS + IHP curve and the line for the group of COHbS, COHbA, and COHbA + IHP as the temperature is raised from 22° to 35°C. Because the changes are rather small, the temperature dependence is examined more explicitly in Fig. 3 at a constant Hb concentration (34 g/dl). Again the stripped HbA and HbS exhibit equivalent behavior over the full temperature range. However, at this high Hb concentration, the correlation time of HbA + IHP is significantly longer over the full temperature range than that of either of the stripped samples. This increased correlation time for HbA + IHP is probably the result of Hb intermolecular charge interactions which restrict rotational freedom after the highly charged IHP molecule complexes with Hb. The significant points that emerge from the data in Fig. 3, however, are (*a*) that COHbS + IHP again exhibits an even longer correlation time than COHbA + IHP, further confirming that addition of IHP induces a motionally restrictive interaction between COHbS molecules, and (*b*) that in the Arrhenius plot shown here, COHbS + IHP exhibits a slope, 2.8 kcal/mol, significantly smaller than that of the other three cases, 3.4 kcal/mol. The decrease in magnitude of the slope indicates that the activation energy for COHbS + IHP rotational diffusion is smaller than that for any of the other three cases. Such a decrease in the diffusional energy barrier again suggests that IHP induces COHbS aggregation at high concentration. A more

detailed quantitative interpretation, however, is probably not warranted with the present data.

Considering both the concentration and temperature studies together, we see that in comparison with any of the other three cases, COHbS + IHP exhibits a slower rate of rotational diffusion (or a longer rotational correlation time) over the full range of temperature and concentration considered in this study. This decreased rate of rotational diffusion must be interpreted as the result of an aggregation mechanism that increases the effective size of the diffusing species. The most likely mechanism for this aggregation of COHbS + IHP is that the Hb conformational change that occurs upon IHP binding (26) promotes a weak COHbS association.

Assuming that aggregation is in the form of a dimer, we can calculate an approximate association constant as follows. For a dimerization of the form $2Hb_1 \leftrightarrow Hb_2$, the association constant is

$$K = [Hb_2]/[Hb_1]^2. \tag{2}$$

At low concentration the observed rotational rate for COHbS + IHP will simply be the concentration weighted average of those due to the monomers and dimers, or

$$\frac{1}{\tau_{obs}} = \frac{[Hb_1]}{[Hb_1] + 2[Hb_2]} \cdot \frac{1}{\tau_M} + \frac{[Hb_2]}{[Hb_1] + 2[Hb_2]} \cdot \frac{1}{\tau_D}, \tag{3}$$

where $1/\tau_M$ and $1/\tau_D$ are, respectively, the monomer and dimer rotational rates. The dimer rotational rate can be approximated as

$$1/\tau_D \simeq \tfrac{1}{2}(1/\tau_M)$$

and Eq. 3 can be rewritten as $(1/\tau_{obs}) = \{[([Hb_T] - [Hb_2])/[Hb_T]\} \cdot 1/\tau_M$, where $[Hb_T] = [Hb_1] + 2[Hb_2]$ is the total Hb concentration. Substituting from Eq. 2 and rearranging, we have $K[Hb_1]^2/[Hb_T]\ \tau_M = 1/\tau_M - 1/\tau_{obs}$. At low concentration, most Hb molecules will be in the unaggregated form, and $[Hb_1] \simeq [Hb_T]$, thus $K \simeq (1/[Hb_T] \cdot (1/\tau_M - 1/\tau_{obs}) \cdot \tau_M$. Using the regression equations from Table I for the concentration dependencies of $1/\tau_{obs}$ and $1/\tau_M$, and keeping only the most important terms, gives $K \simeq (d_1^M - d_1^{obs})/d_0^M$, where d_0^M and d_1^M taken from the noninteracting species, COHbA, COHbA + IHP, and COHbS, and d_1^{obs} is the linear regression coefficient for COHbS + IHP. Using the appropriate values from Table I gives $K \simeq 40\ M^{-1}$ which would yield approximately 25% association at a COHbS concentration of 35 g/dl. Due to the number of approximations in this calculation, however, these numbers should probably be considered as order of magnitude estimates only.

CONCLUSIONS

The present study emphasizes the importance of organic phosphate in producing COHbS aggregation. The existence of an aggregated form of COHbS, even when fully liganded, further suggests that the erythrocyte DPG level may play an important role

in nucleation of the polymer state. Thus, the in vivo level of endogenous organic phosphate may well be an important variable in determining the clinical severity of sickle cell anemia in the individual.

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