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KINETICS OF HEME BINDING TO SEMI-ALPHA-HEMOGLOBIN*

Rah Youl Park and Melisenda J. McDonald**

Biochemistry Program, Chemistry Department,
University of Lowell, Lowell, MA 01854

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The binding of carbonmonoxyheme to semi-alpha-hemoglobin and to an apohemoglobin control was investigated using stopped-flow techniques in 0.025 M potassium phosphate buffer, pH 7 and 10°C. The resultant second order kinetic data were analyzed by the classical model which assumes the existence of an intermediate complex which either redissociates to reactants or undergoes an irreversible conversion to form hemoglobin. The rate constants for the latter unimolecular process were apparently not experimentally different for semi-alpha-hemoglobin and apohemoglobin (360 (± 100) s^{-1} and 480 (± 60) s^{-1}, respectively). However, the equilibrium dissociation constant for the intermediate of semi-alpha-hemoglobin (Kd = 9.3 (± 2.6) micromolar) was approximately two fold greater than that of apohemoglobin (Kd = 4.1 (± 0.5) micromolar). The reduced stability of the semi-alpha-hemoglobin complex was postulated to be due to the lower affinity of the beta pocket for heme. The studies reported here address the possible role of semi-alpha-hemoglobin as an intermediate in the assembly of hemoglobin *in vivo*.

The study of the post-translational modification of proteins is an area of contemporary biochemistry that is eliciting great interest. Human hemoglobin, a tetramer consisting of two alpha and two beta heme containing subunits, is often cited as a model for multisubunit assembly (1). In fact, two important post-synthetic events occur during the formation of hemoglobin. One essential process involves the proper assembly of nascent globin or heme-containing polypeptide chains (2-6). Another equally important post-translational step is the insertion of the heme moiety. Indeed, several laboratories (7-9) have extensively investigated the kinetic mechanism of the interaction *in vitro* between heme and apohemoglobin, a non-heme-containing alpha-beta dimer. The existence of this species *in vivo* implies that it may be a physiological intermediate in the assembly of hemoglobin.

Another plausible intermediate is semihemoglobin, consisting of a heme-containing subunit paired with a globin chain. Semi-

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** To whom correspondence should be addressed.

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alpha-hemoglobin, in which the heme group is associated with the alpha chain, has been found in normal erythrocytes (10). Elegant studies have characterized the structural and functional properties of semi-alpha-hemoglobin from normal adult hemolysate (11-14). The kinetics of the interaction of semi-alpha-hemoglobin with heme, however, has not been addressed. In this report, we have kinetically evaluated the insertion of heme into semi-alpha-hemoglobin and our findings are consistent with the premise that semi-alpha-hemoglobin may be a possible intermediate in the *in vivo* assembly of the hemoglobin tetramer.

Experimental Procedures

The method of Bucci & Fronticelli (15) with modifications (16) was employed to isolate alpha heme chains from normal adult hemolysate. Apohemoglobin was obtained by the acid-acetone method (17, 18). Cellulose acetate electrophoresis and heme titration confirmed the integrity of each preparation. The concentration of apohemoglobin was determined at 280 nm using a millimolar extinction coefficient of 12.7 $mM^{-1}cm^{-1}$ per peptide chain. Semi-alpha-hemoglobin was prepared by the chain transfer method of Cassoly (19, 20). An equimolar mixture, 1 mM each of apohemoglobin and carbonmonoxy heme-containing alpha chain, was incubated in 0.1 M potassium phosphate buffer, pH 7 for 48 hours at 4°C. Following desalting on Sephadex G-25, we employed anion exchange chromatography on DEAE-Sephadex A-50 to isolate semi-alpha-hemoglobin (Figure 1). The purified semi-alpha-hemoglobin was characterized electrophoretically on cellulose acetate (Figure 1, inset) and by spectrophotometric titration with heme. Results were obtained from a minimum of 5 independent preparations of apohemoglobin and semi-alpha-hemoglobin.

All static visible and ultraviolet measurements were made using a Cary 2200 recording spectrophotometer with the cuvette compartment temperature-controlled at 10°C. For all kinetic studies, a heme derivative shown to be monomeric (7), carbonmonoxy heme (CO-heme), was prepared from bovine hemin (Sigma Chemical Co. St. Louis, MO). The concentration of CO-heme solution was determined at 408 nm using a millimolar extinction coefficient of 145 mM^{-1} cm^{-1}. The time-dependent absorbance changes following rapid mixing of CO-heme with either apohemoglobin or semi-alpha-hemoglobin were monitored using a Kinetic Instruments stopped-flow device, thermostated at 10°C, equipped with a 20mm cuvette and integrated by 4120AT microcomputer based software (On-Line Instrument Systems, Inc., Jefferson, GA).

Results and Discussion

We have monitored the reaction of carbonmonoxy heme with either apohemoglobin or semi-alpha-hemoglobin in a stopped-flow apparatus at 420 nm in 0.025 M potassium phosphate buffer, pH 7 and 10°C. Second order plots reveal departures from linearity prior to 8 msec for apohemoglobin (Figure 2A) and 12 msec for semi-alpha-hemoglobin (Figure 2B). This deviation from homogeneous second order kinetics is greater at higher reactant concentrations. In addition, the apparent second order rate constant varied over the protein concentration range from 1 to 5 micromolar. Similar results have been noted previously for the

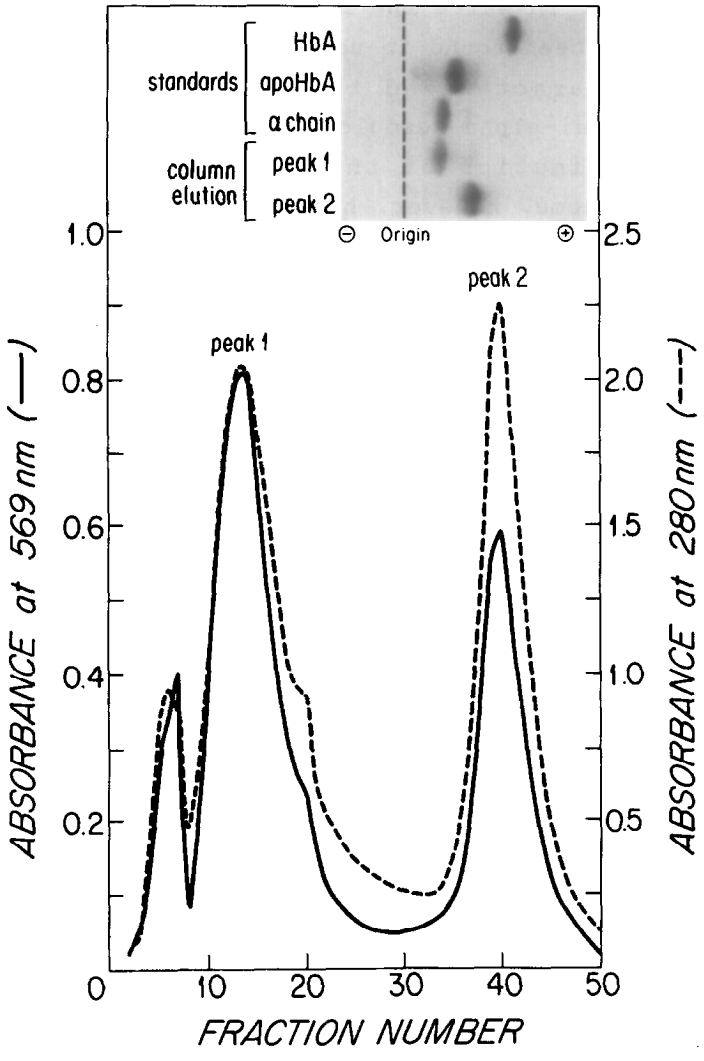

Figure 1: Elution profile for chromatographic purification of semi-alpha-hemoglobin derived from the incubation of heme-containing alpha chains with apohemoglobin. The mixture (50 mg total) was loaded on a DEAE-Sephadex A-50 anion exchange column (1x20 cm) previously equilibrated with 0.05 M Tris-HCl, pH 8.6. One column volume of the starting buffer was used to remove any unbound protein, followed by a stepwise change to 0.05 M Tris-HCl buffer, pH 8.15 to separate peaks 1 and 2. The ratio of the absorbance at 569 nm to that at 280 nm was monitored for each eluted species. Inset: Cellulose acetate electrophoresis showing three standard hemoglobin species and samples of each of the two peaks. Peak 1 corresponds to the alpha heme chain standard, while the mobility of peak 2 is characteristic of semi-alpha-hemoglobin.

reaction of apohemoglobin with CO-heme (7-9), *but constitute a novel finding for semi-alpha-hemoglobin.*

The finding that the deviations from second order kinetics are comparable for both proteins suggests that the mechanism of heme insertion into semi-alpha-hemoglobin is analogous to that of apohemoglobin. In the model proposed by Gibson & Antonini (7), a

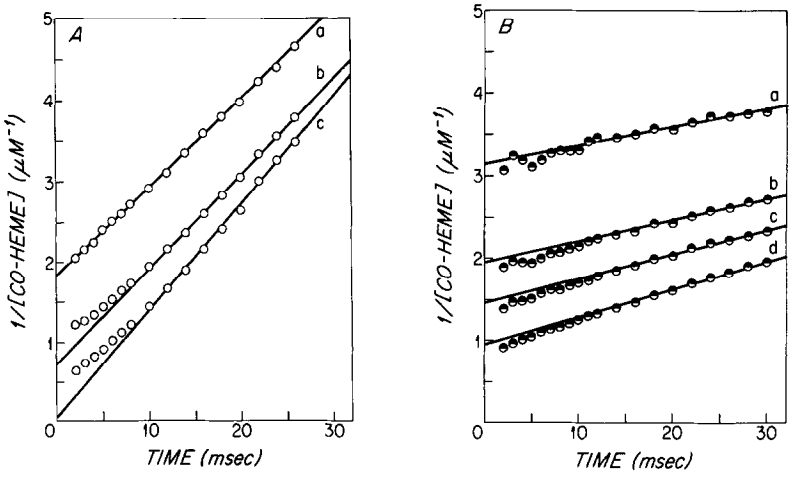

Figure 2: Second order rate plots for the CO-heme binding reaction. Conditions were 0.025 M potassium phosphate buffer, pH 7 and 10°C. A: The concentration of monomeric CO-heme was equivalent to that of apohemoglobin (on a subunit basis). The protein and CO-heme concentrations after mixing were: a, 1.1 micromolar; b, 2.1 micromolar; c, 5.3 micromolar. B: In semi-alpha-hemoglobin, only one of two heme binding sites is available; the concentration of CO-heme was made equivalent to that of semi-alpha-hemoglobin on a subunit basis (i.e., one half the protein concentration). The concentrations of CO-heme after mixing were: a, 1.0 micromolar; b, 1.5 micromolar; c, 2.0 micromolar; d, 3.0 micromolar.

transient encounter complex is postulated which is in equilibrium with the heme species and the apohemoglobin (or semi-alpha-hemoglobin) pool. This intermediate undergoes a unimolecular, irreversible rearrangement to form native carbonmonoxyhemoglobin. These workers derived a quantitative method to evaluate their

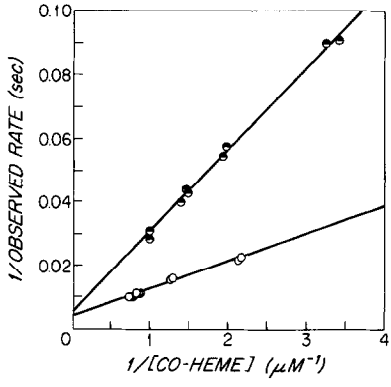

Figure 3: Double reciprocal plots for the reaction of CO-heme with apohemoglobin (○) and semi-alpha-hemoglobin (◓) based on the method of Gibson & Antonini (7). The observed rates were from pseudo-first order rate plots over the interval 3-8 msec for apohemoglobin and 3-12 msec for semi-alpha-hemoglobin. The CO-heme concentration was computed from the delta absorbance measurement at 3 msec after mixing. Each point represents an average of at least 4 independent determinations. The lines were fit by linear regression analysis; k_3 was determined from the intercept of the y-axis and K_d was calculated from the slope.

model utilizing double reciprocal plots similar to those shown in Figure 3. Using this technique, Rose & Olson (9) calculated a value of the equilibrium dissociation constant for the intermediate species (K_d) of 6.2 (± 2.0) micromolar and of the first order rate constant for the conformational transition (k_3) of 500 (± 150) s^{-1} for apohemoglobin. Our studies under similar experimental conditions revealed values of 4.1 (± 0.5) micromolar and 480 (± 60) s^{-1} for K_d and k_3, respectively.

The apparent rates of heme binding to semi-alpha-hemoglobin were observed to be slower than those of apohemoglobin over the protein concentration range studied. A double reciprocal plot (Figure 3) for heme binding to semi-alpha-hemoglobin enabled determination of values for K_d and k_3 of 9.3 (± 2.6) micromolar and 360 (± 100) s^{-1}, respectively. Since the k_3 values for apohemoglobin and semi-alpha-hemoglobin agree within experimental error, the stability of the intermediate appears to account for the difference in the observed rates. The value of K_d is approximately two-fold greater for semi-alpha-hemoglobin than for apohemoglobin, suggesting a lower equilibrium concentration of intermediate and hence a slower overall rate of heme binding to semi-alpha-hemoglobin. A plausible explanation for the less favorable formation of this intermediate may be that the only available site, the beta heme site, is less accessible than the alpha heme site. Indeed, recent proton nuclear magnetic resonance spectroscopy studies revealed intrinsic differences between the alpha and beta heme pockets (21, 22) which appear to support this premise. In addition, static titration studies with limiting concentrations of heme have shown that the alpha subunit of apohemoglobin has a higher affinity than the beta subunit for heme (23). This difference in the affinity of the subunits for heme may be reflected in the kinetics of heme binding; however studies to date (7-9) have not addressed this heterogeneity.

The sequence of post-translational events involved in human hemoglobin formation remains unknown (24). The hemoglobin molecule can be assembled into a functional tetramer either by heme chain combination or by globin chain combination followed by heme insertion. The investigations reported here address a third possible mechanism, that is assembly through a semihemoglobin intermediate. The kinetics of heme binding to semi-alpha-hemoglobin appear comparable to those of apohemoglobin and therefore, semi-alpha-hemoglobin may be a viable intermediate for hemoglobin assembly *in vivo*.

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