Kinetics of Iron(III) Reduction by Hemoglobin and Its Isolated α-Subunits

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Hb has long been assigned the principal responsibility of oxygen transport and delivery but more recently has been shown to act as a reducing agent.[1,2] Rifkind observed that Fe(II)–HbO_2 can reduce Cu(II) through cys-93 on the β-chain.[3,4] Eguchi and Saltman further characterized the kinetics and mechanisms of both Cu(II) and Fe(III) reductions.[1,2] This study extends the previous kinetic work by quantitating differences in the ability of tetrameric Hb ($\alpha_2\beta_2$) and its isolated α-subunits to reduce Cu(II) and Fe(III) complexes.

Materials and Methods

Reagents. Hb was isolated from fresh human red cells according to the method of Geraci et al.[5] and was further purified to remove HbA_2 by the method of Reiss et al.[6] α^{SH}-subunits were prepared from α^{PMB}-subunits by the method of Geraci et al.[5] Purity of the α^{SH}-subunits was verified by cellulose acetate electrophoresis. Purified Hb and α-chains were dialyzed at 4 °C in a stock buffer solution which contained 30 mM Hepes/140 mM NaCl, pH 7.0.

Preparation of Metal Chelates. Stock solutions (0.1–0.5 M) of Fe(III)·NTA (1:1; NTA = nitrilotriacetate), Ni(II)·Gly (1:4; Gly = glycine), Cu(II)·His (1:2; His = histidine), and Cu(II)·NTA (1:1) in buffer solution were freshly prepared as described previously.[2] All chemicals were the best commercial grade.

Kinetic Measurements. All experiments were carried out at 17 ± 0.5 °C to avoid precipitation of the α-subunits at higher temperatures in the presence of a large excess of Fe(III)·NTA. A typical test solution contained 60 μM Hb or α-subunits (measured as oxy-heme at 577 nm, ϵ = 14 600 M^{-1} cm^{-1}) and varying concentrations of Fe(III)·NTA (1.2–18 mM) or Cu(II)-chelating ligand (1.2 and 6 mM) in buffer solution. Reaction mixtures were scanned and pH was checked before and after the reaction to ensure that hemichrome formation did not occur. K_m and V_{max} were determined by weighted least squares linear regression.

Competition experiments were performed by incubating the α-subunits with various concentrations of Ni(II)·Gly (1.5, 6, and 60 mM) for 3 min prior to the addition of Fe(III)·NTA.

Spectrophotometric measurements were performed on a Hitachi 110A dual-beam spectrophotometer. Heme oxidation was determined by first measuring the 590-nm isosbestic wavelength and then monitoring the decrease in absorbance of the 577-nm peak of the oxygenated form of Hb and of the α-subunit. Absorbance measurements for the reduction of Fe(III)·NTA were taken at 10-s intervals for the first 2 min and at 30-s intervals for an additional 8 min. Cu(II) chelate reduction was measured at 1-min intervals for 60 min. The concentration of met-heme formed was calculated using the dual-wavelength method of van Kampen and Zijlstra.[7]

Results

Initial Rates of Fe(III)·NTA Reduction. A double-reciprocal plot of the initial velocities of the reduction of Fe(III)·NTA by Hb and isolated α-subunits is presented in Figure 1. The K_m value for Hb (24.1 mM) was more than 3 times greater than that of the α-subunits (7.2 mM). Little difference was observed in the V_{max} of the two proteins (Hb, 39 μM/min; α-subunits, 33 μM/min).

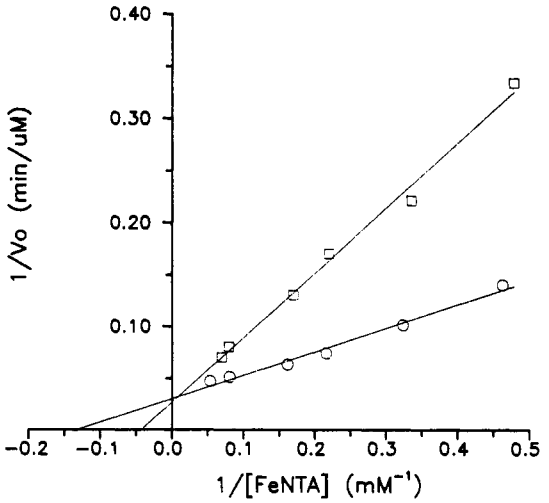

Figure 1. Double-reciprocal plot of initial velocities of Fe reduction by Hb (□) and α-subunits (○). Each point is the mean of three kinetic experiments with CV <3%. [heme] = 60 μM in Hepes/NaCl buffer, pH 7.0.

Pseudo-First-Order Rate Constant. Previous work with Hb has shown that Fe(III)·NTA is reduced through a site-specific mechanism. Derivations of two possible kinetic models of electron transfer have been described.[2] Under pseudo-first-order conditions, the formation of the protein–metal–chelator ternary complex is not rate limiting, and the rate expression simplifies to

$$k_{obs} = \frac{k_1 k_3}{k_{-1}[O_2] + k_3} \quad (1)$$

where k_1 and k_{-1} are the respective O_2 dissociation and association rate constants, k_3 is the electron-transfer rate constant, and $[O_2]$ is the concentration of O_2 in solution.

The pseudo-first-order rate constants of Fe(III)·NTA reduction were measured at high Fe(III) concentrations, where the values of V_{max} for Hb and α-subunits are 6.5×10^{-7} and 5.5×10^{-7} M/s, respectively. Under these conditions, $k_{obs} = v/[\text{heme-}O_2]$. Substituting V_{max} values for v and the appropriate $[\text{Heme-}O_2]$, k_{obs} for both Hb and the α-subunits is 0.01 s^{-1}. Rearranging eq 1 and solving for k_3, the electron-transfer rate is 6.35 s^{-1} for Hb and 3.69 s^{-1} for the α-subunits. Since $k_{obs} \ll k_1$ and $k_{obs} \ll k_3^2$, neither the O_2 dissociation step nor the electron-transfer step is rate limiting. Rather, the rapid formation of heme-O_2 ($k_{-1} = 5.5 \times 10^7$ M^{-1} s^{-1} for Hb and 5.0×10^6 M^{-1} s^{-1}) for the α-subunits) competes with the electron-transfer step for deoxy-heme, thereby limiting the reaction rate.

Ternary Complex Equilibrium Constant. The equilibrium constant for the formation of the ternary complex (O_2-heme–Fe(III)–NTA) may be derived from the rate expression[2]

$$\text{d[met-heme]}/dt = \frac{k_1 k_3 K[\text{heme-}O_2][\text{Fe(III)·NTA}]}{(k_1[O_2] + k_3)(1 + K[\text{Fe(III)·NTA}])} \quad (2)$$

From the initial velocity data for the reduction of Fe(III)·NTA, d[met-heme]/dt = 5.54×10^{-8} M/s for Hb and 1.22×10^{-7} M/s for the α-subunits at 17 °C, 2.1 mM Fe(III)·NTA, and 60 μM heme. Substituting of these values into eq 2 and solving for K, the equilibrium constant for the formation of the ternary complex is 41 M^{-1} for Hb and 142 M^{-1} for α-subunits.

Effect of Ni·Gly on Reduction of Fe(III)·NTA. To determine the mechanism by which Fe(III)·NTA was reduced by the α-subunit, we used the redox-inactive Ni·Gly chelate to compete

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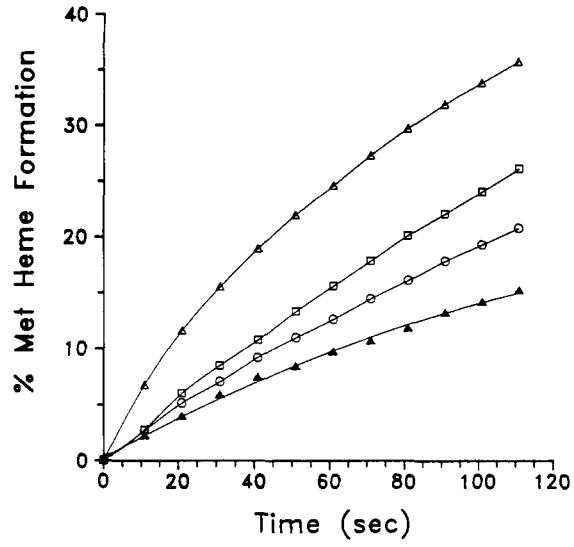

Figure 2. Competition plot of effect of Ni concentration on Fe reduction by α-subunits, showing met-heme formation as a function of time. Each point is the mean of three experiments with CV <2%. [α-subunit heme] = 60 μM; Fe:heme = 300:1. Ni:Fe ratios: 0 (△); 0.08 (□); 0.33 (○); 3.33 (▲). All reaction mixtures were in Hepes/NaCl buffer, pH 7.0.

for the Fe(III)-binding site on the protein. At the lowest Ni:Fe ratio (0.08), 26% inhibition of Fe(III)·NTA reduction was observed in the first 2 min of the reaction; at the highest ratio (3.33), almost 60% inhibition was observed (Figure 2). Previously, Ni(II) was shown to inhibit the reduction of Fe(III)·NTA by Hb in a site-specific manner.[2] Attempts to fit the competition data to traditional double-reciprocal plots were unsuccessful, so we cannot precisely determine the mechanism of competition. Control experiments using appropriate amounts of free glycine in the presence of Fe(III)·NTA and α-subunits were performed to ensure that ligand exchange and excess chelator did not bias the results. Additionally, no change was observed in either the solubility or the spectral properties of the α-subunits in the presence of Ni(Gly).

Discussion

Reduction of Fe(III)·NTA. Comparison of K_m, K (the ternary complex equilibrium constant), and k_3 (the electron-transfer rate) values for tetrameric Hb and α-subunits clearly demonstrates that subunit assembly has a significant effect on the electron-transfer rate and on the interaction of Fe(III)·NTA with the binding site(s) on the protein. Tetrameric subunit assembly ($\alpha_2\beta_2$) increases K_m more than 3-fold (7.2 mM for α-subunits; 24.1 mM for Hb), while it decreases the affinity of Fe(III)·NTA for the binding site on the protein (K = 142 M^{-1} for α-subunits and 41 M^{-1} for Hb). Further, the electron-transfer rate is increased by 72% (k_3 = 3.69 s^{-1} for α-subunits and 6.35 s^{-1} for Hb). The differences observed in the values of K_m, K, and k_3 as a result of tetrameric subunit assembly may be attributed to conformational changes in the protein. These changes may limit the accessibility of Fe(III)·NTA to its binding site(s) on the protein, thereby increasing K_m while decreasing K. The increase in the value of k_3 may be explained by the work of Sutin[8] and Isied,[9] whose theoretical work identified several factors that govern electron transfer. Two of these factors—electronic effects, which include geometric and steric constraints, and the overall redox potential of the system—would be significantly affected by the conformational changes associated with formation of the tetrameric Hb ($\alpha_2\beta_2$).

The observation that the value of k_{obs} is unchanged for the reduction of Fe(III)·NTA by both Hb and the α-subunits (0.01 s^{-1}) is not unexpected. Both proteins reduce Fe(III)·NTA through the same site-specific mechanism, and the reaction rate of this mechanism is limited by the O_2 association constant (k_{-1}). Further, the term $k_{-1}[O_2]$ in eq 1 dominates the expression for k_{obs}.

Competition Experiments. The inhibition of Fe(III)·NTA reduction by the α-subunit in the presence of Ni(II) supports the conclusion that the isolated α-subunit reduces Fe(III)·NTA through the same site-specific mechanism as that of tetrameric Hb. The inability to fit the data from the competition experiments to traditional double-reciprocal plots indicates that Ni(II) inhibits reduction of Fe(III)·NTA in a "mixed competition" fashion. It should be noted that speciation plots of Fe(III)·NTA at pH 7.0 indicate that 80% of the Fe(III)·NTA is in the form of a dihydroxy dimer, while the other 20% has a single hydroxide ion bound.[10]

Tetrameric Hb reduces Cu(II) site-specifically only at β-cys-93. Cu(II) chelates were tested on isolated α-chains to confirm the inability of α-chains to reduce Cu(II). No Cu(II) reduction was observed.

In conclusion, tetrameric subunit assembly strongly influenced the parameters K, K_m, and k_3 in the reduction of Fe(III)·NTA by the isolated α-subunit, while k_{obs} was unchanged. The reduction of Fe(III)·NTA occurs through a site-specific mechanism in both the isolated α-subunit and tetrameric Hb. Ni(II) was able to inhibit Fe(III)·NTA reduction in a "mixed competition" manner.

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Registry No. Fe-NTA, 16448-54-7; Fe, 7439-89-6; Ni, 7440-02-0.

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