Chapter 5

Effect of Guanidine Hydrochloride on Stability and Dynamics of Myoglobin

5.1 Abstract

Laser flash photolysis and visible optical methods have been used to study the dynamic events in horse myoglobin-CO complex in the subdenaturing range of guanidine hydrochloride concentration at pH 7, 20°C. Increments in denaturant concentration from strongly native to unfolding leads to increase in rates of both rebinding and the replacement of CO, but the increase in the bimolecular CO rebinding rate is larger than the CO replacement rate. This produces a parallel increase in the apparent equilibrium binding constant, \( K_{\text{app}} = \frac{k_{\text{on}}}{k_{\text{off}}} \) across the unfolding transition. The results indicate that subdenaturing concentrations of GdnHCl change the dynamics and structure of myoglobin-CO complex. A more open active site structure of myoglobin-CO complex is observed in the presence of the denaturant with respect to the native sample. The increase in \( K_{\text{app}} \) is a direct indication of gradual structure loss with increments of denaturant, since the rate-limiting barrier in the rebinding process is known to be due to the protein matrix (202). The change in the global protein structure in the subdenaturing limit of GdnHCl has also been examined. Analysis of the denaturant dependence of \( \Delta G_{D} \) shows the appropriateness of the denaturant binding model rather than the linear free energy model.
5.2 Introduction

Myoglobin is a small protein that stores and transports dioxygen in muscles. In the reduced state, myoglobin reversibly binds small gaseous ligands such as O₂, NO, and CO through the remaining Fe²⁺ coordination position on the distal side of the heme (203). The covalent bond between the iron and the CO can be broken by using visible light (by a laser flash or extended illumination). If the photodissociated ligand diffuses into the solvent, rebinding to myoglobin will follow bimolecular kinetics. However, the fraction of the photodissociated ligand that remains momentarily ‘trapped’ within the protein matrix can recombine by intramolecular diffusion and collision with iron—a process called geminate rebinding (202). In geminate rebinding at 293K heme relaxations take place on a picosecond time scale (204-205), and protein relaxation takes place on the same time scale as the ligand escapes (206). The photodissociation occurs with unity quantum yield within a picosecond producing a geminate pair. From the geminate pair 96% of the CO molecule exits the protein matrix into the solvent whereas 4% rebind rapidly to the heme iron (207). Ligand rebinding to the heme occurs on the millisecond time scale (208-209). Ligand replacement dynamics in myoglobin-CO complex is a widely studied area (208, 210-214).

In the present photolysis work, ligand rebinding in the presence of subdenaturant concentration of GdnHCl are considered. GdnHCl-induced unfolding of myoglobin has been reported (215-216). Bismuto et al. measured denaturation curves monitored by CD and Soret optical absorption, and found that both curves are very similar (217). The results indicate that the local structure
around the heme is controlled by the destruction of the secondary structure, and is complete at GdnHCl>2.0 M. The effectiveness of the neutron-scattering and high-resolution wide-angle X-ray scattering techniques in revealing the denaturant-induced effects on protein structure and dynamics has already been demonstrated (10, 218). Recently Natali et al. have used X-ray absorption spectroscopy to demonstrate small but significant variations in the active site pocket of myoglobin-CO complex induced by GdnHCl (10, 21). They found a sizable change in Fe-CO bonding angle as well as in the CO-rebinding rates occurring after Fe-CO dissociation in GdnHCl containing myoglobin-CO solution. The native and unfolded-state hydrogen and thiol-disulfide exchange studies of ferric aquomyoglobin have shown that thiol group exchange (SX) occurs by a novel denaturant-dependent process (219). Thus, structure and dynamics of myoglobin are strongly GdnHCl dependent. In particular, highly denatured state of myoglobin presents a more open active site structure and overall increased H-atom motions with respect to the native case (10).

In spite of these advances, laser photolysis and ligand recombination reactions have not been carried out systematically in the presence of subdentauring to unfolding concentrations of denaturants. In an earlier study Sharma et al observed no appreciable change in the CO recombination rate with increments of urea (220). Choi and Terazima used GdnHCl, instead of urea, and reported that the rate of the bimolecular CO recombination with deoxymyoglobin increases as the denaturant concentration increases up to 4.0 M GdnHCl (221). From these rates they calculated hydrodynamic radius of unfolded myoglobin through diffusion coefficients and reported that unfolded carboxymyoglobin has a hydrodynamic radius 4-times larger then that of the native protein. In the present
study, kinetics of ligand exchange and rebinding of myoglobin-CO reactions have been measured in the 0–1.75 M range of GdnHCl. As the protein is taken from the native state to denaturant-induced destabilized state, the enthalpic barrier becomes smaller. The conformational entropy loss, $\Delta S$, is also found smaller in magnitude under unfolding conditions. The global protein structural changes are due to the binding of GdnHCl to the protein.

### 5.3 Materials and Methods

Horse heart myoglobin (typeIII) was from Sigma. Denaturants were obtained from Gibco BRL. All experiments were done in 50 mM sodium phosphate buffer at pH 7, 22°C.

#### 5.3.1 Equilibrium unfolding measurements of myoglobin and carboxymyoglobin

Samples of myoglobin (10 µM) were prepared in the 0-4.0 M range of GdnHCl. To prepare carboxymyoglobin the solutions were deaerated and reduced under nitrogen with 1.5 mM sodium dithionite. The dithionite-reduced samples were saturated with CO by passing a slow stream of the dry gas through the solutions for a minute. Samples were then incubated in tightly capped quartz cuvettes or rubber capped small glass tubes for ~15 minute. The sealed tubes were shaken gently in dark for 5-10 minute at room temperature. Tryptophan fluorescence excited at 280 nm was measured at 350 nm. The reported GdnHCl concentrations and pH values are those determined after measurement.
5.3.2 Measurement of CO replacement rates in myoglobin-CO complex by hexacyanoferrate (III) ion

Myoglobin initially dissolved in the buffer containing a desired concentration of GdnHCl was deaerated, reduced by the addition of sodium dithionite, and liganded with CO. 1 ml of this solution was diluted into 1 ml of degassed K₃[Fe(CN)₆] (800 µM) buffer solution containing the matching concentration of GdnHCl. This procedure allows complete replacement of CO by hexacyanoferrate (III) ion. Kinetics of CO replacement was monitored by 421-nm Soret absorbance. Final concentration of protein, carbonmonoxide, K₃[Fe(CN)₆], and sodium dithionite were ~10 µM, ~500 µM, ~400 µM, and ~ 0.5 mM, respectively.

5.3.3 Laser photolysis and nano-to-millisecond rate measurements

10-12 µM myoglobin solution prepared in 50 mM phosphate buffer in desired concentration of GdnHCl, was deaerated and reduced by the addition of sodium dithionite (1.5 mM). The reduced samples were incubated for 15-30 minutes at 22°C under 1 atmosphere CO gas pressure in tightly capped 1-cm cuvettes. CO photolysis was achieved by irradiation with 50 (±10) mJ pulses of the 532-nm second harmonic light of a Spectra Physics Q-switched Nd/YAG laser (10 Hz). Spectral changes following each photolysis pulse were recorded with a pulsed Xe lamp. The measurements employed an Applied Photophysics laser flash photolysis spectrometer. The sample temperature was maintained by using an external circulating water bath. Single-wavelength kinetic traces were analyzed by single-exponential functions as described under results section.
5.3.4 Thermal denaturation of oxidized myoglobin in the presence of subdenaturing concentrations of GdnHCl

Oxidized myoglobin was dissolved ~15 minutes before the experiment in buffers containing different concentrations of GdnHCl (0.0, 0.1, 0.3, 0.5, 0.8, 1.1 and 1.35M) at pH 6.2. Thermal denaturation was followed by monitoring optical density at 409 nm using a Cary 100 (Varian) spectrophotometer. The heating rate used was 0.5 K/min in peltier. The temperature in the cuvette was monitored during measurement using a digital thermometer. The accuracy of the probe was ± 0.5 K.

5.4 Results

5.4.1 Kinetics of geminate and bimolecular CO rebinding after photolysis

CO photolysis was carried out in aqueous solutions of myoglobin liganded with CO at pH 7.0, 20°C. Figure 5.4.1a shows the observed changes in 434 nm (the $\lambda_{\text{max}}$ in the difference spectrum of the protein in the absence and the presence of CO) absorbance from 15 ns to 1.8 µs after the CO is photolyzed in the presence of 0.35 M GdnHCl. The data are best described by a single exponential fit with geminate recombination rate constant, $k_{\text{gem}} = 5.95 \times 10^{-7}$ s$^{-1}$. After ~1.1 µs, the OD starts to decrease relatively slowly in a single-exponential decay due to CO rebinding with a rate, $k_{\text{on}} = 0.487 \times 10^{-6}$ M$^{-1}$ s$^{-1}$.
5.4.2 Determination of CO replacement rate constants of myoglobin-CO complex

The replacement reaction can be written as

\[
\text{Mb} + \text{CO} + \text{R} \xrightleftharpoons[k_{\text{off}}]{k_{\text{on}}} \text{MbR} + \text{CO} \xrightleftharpoons[k_{\text{R}}]{k_{\text{on}}^{-1}} \text{Mb} + \text{CO} + \text{R}
\]

where R is the replacing ligand, \(k_{\text{on}}\) and \(k_{\text{on}}^{-1}\) are the association constants for the binding of CO and R, respectively, to the unliganded protein, and \(k_{\text{off}}\) and \(k_{\text{R}}\) are the corresponding dissociation rate constants. For the present results a pseudo-first-order rate constant can be used because both ligand concentrations (~ 400
µM) are much larger than the protein concentration (~10 µM). For this scheme, the apparent rate constant $k_{app}$ is given by (222).

$$\frac{k_{app} = \frac{k_{off} k_{iR} + k_{R} k_{on} + k_{off} k_{R}}{k_{on} + k_{iR} + k_{R}}}{(2)}$$

The hexacyanoferrate (III) ion binds much faster and dissociates much slower than CO. Thus, $k_{iR} \gg k_{off}$, and $k_{R} \ll k_{off}$, and since $k_{R}$ is far smaller than $k_{iR}$, eq (2) simplifies to $k_{app} \sim k_{off}$. Therefore, the apparent rate constant obtained from the CO/[Fe(CN)$_6$]$^{3-}$ replacement reaction is a very good approximation for the overall CO dissociation rate constant. Figure 5.4.2 shows the single–phase replacement of CO from myoglobin-CO complex by hexacyanoferrate (III) ion in 0.35 M GdnHCl with a rate constant, $k_{off} = 0.021$ s$^{-1}$.

**Figure 5.4.2** The single–phase replacement of CO from myoglobin-CO complex by hexacyano- ferrate (III) ion with time constant $\tau = 46.7$ s.

### 5.4.3 Determination of apparent equilibrium binding constant, $K_{app}$ (M$^{-1}$) of myoglobin-CO complex

The simple sequential three-state model (207) has been used for the detailed analysis of the geminate ligand-binding process

$$\begin{align*}
\text{Mb} – \text{CO} & \overset{k_{21}}{\rightleftharpoons} \text{Mb : CO} \overset{k_{23}}{\rightleftharpoons} \text{Mb + CO}
\end{align*}$$

(3)
The observable geminate rate constants \( (k_{gem}) \), geminate yield \( (\phi_{gem}) \), bimolecular rebinding rate constants \( (k_{on}) \), and dissociation rate constants \( (k_{off}) \) are related to the rate constants for the elementary processes of the three-state sequential scheme by the following equations (208):

\[
k_{on} = k_{32} \times \phi_{gem}
\]
\[
k_{off} = k_{12} \times (1 - \phi_{gem})
\]

The apparent binding constant is given by

\[
K_{app} = \frac{k_{on}}{k_{off}}
\]  

(4)

5.4.4 GdnHCl dependence of global stability, rebinding \( (k_{gem}) \), bimolecular rebinding \( (k_{on}) \), and ligand exchange rates \( (k_{off}) \)

Figure 5.4.3a shows GdnHCl-induced equilibrium unfolding transition of carboxymyoglobin analyzed using the standard two-state equations already described in preceding Chapters. Figure 5.4.3b presents the rate coefficients for geminate \( (k_{gem}) \) and bimolecular CO rebinding \( (k_{on}) \) in the 0-1.75 M range of GdnHCl. The rate coefficients for CO replacement reaction \( (k_{off}) \) in the same range of the denaturant concentration is presented in figure 5.4.3c. The rates at different concentrations of the denaturant are listed in Table 1. The results show that as the denaturant concentration increases from strongly native condition to unfolding condition, the rates of both CO rebinding and replacement increase. However, the gradient for the bimolecular CO rebinding is larger than that for CO replacement. It leads to a parallel increase in apparent equilibrium binding constants, \( K_{app} = \frac{k_{on}}{k_{off}} \). These observations have three implications. (i) Both geminate and bimolecular rebinding rates increase, the latter to a greater extent, in the presence of subdenaturing concentration of GdnHCl, so relaxation and
diffusion are involved in these rebinding processes. (ii) Bimolecular CO rebinding rates and CO replacement rates increase as GdnHCl concentration increases, indicating structural and dynamical changes in myoglobin even in the subdenaturing limit, consistent with earlier observation of some dynamical changes in the presence of lower concentration of the denaturant \((10)\). A more open active site protein structure presumably has higher rates of CO rebinding and replacement \((210)\). (iii) The denaturant-induced increase in the equilibrium binding constants, \(K_{\text{app}}\), suggests uncommon path for entry and exit of CO.

**Figure 5.4.3** Equilibrium stability and rate–denaturant profiles. (a) GdnHCl-induced melting of myoglobin-CO complex at 22\((\pm1)\)°C. Values of \(\Delta G^{\circ}_{\text{m}}\) and \(m_g\) obtained are 5.75\((\pm0.87)\) kcal mol\(^{-1}\) and 3.31\((\pm0.5)\) kcal mol\(^{-1}\) M\(^{-1}\), respectively. (b) GdnHCl dependence of nanosecond geminate rebinding rates \((k_{\text{gem}} \text{s}^{-1})\), (c) GdnHCl dependence of CO replacement rates \((k_{\text{off}} \text{s}^{-1})\).
Table 1. Observed and derived kinetic constants for CO with myoglobin in different GdnHCl concentrations at pH 7, 20(±1) °C.

<table>
<thead>
<tr>
<th>GdnHCl (M)</th>
<th>(k_{\text{gem}} \times 10^{-8}) (s(^{-1}))</th>
<th>(k_{\text{on}} \times 10^{-6}) (M(^{-1}) s(^{-1}))</th>
<th>(k_{\text{off}} \times 10^{2}) (s(^{-1}))</th>
<th>(K_{\text{app}} \times 10^{7}) (M(^{-1}))</th>
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</thead>
<tbody>
<tr>
<td>0.000</td>
<td>0.526</td>
<td>0.417</td>
<td>1.84 ± 0.12</td>
<td>2.43</td>
</tr>
<tr>
<td>0.025</td>
<td>-</td>
<td>-</td>
<td>1.83</td>
<td>-</td>
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<td>0.050</td>
<td>-</td>
<td>-</td>
<td>1.81</td>
<td>-</td>
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<td>0.100</td>
<td>0.547 ± 0.02</td>
<td>0.348</td>
<td>2.03 ± 0.04</td>
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<td>0.200</td>
<td>0.585 ± 0.02</td>
<td>0.362</td>
<td>1.98 ± 0.03</td>
<td>1.83</td>
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<tr>
<td>0.300</td>
<td>0.641 ± 0.14</td>
<td>0.401</td>
<td>2.17 ± 0.17</td>
<td>1.84</td>
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<td>0.400</td>
<td>0.676 ± 0.13</td>
<td>0.485</td>
<td>2.00 ± 0.17</td>
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<td>0.500</td>
<td>0.705 ± 0.08</td>
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<td>2.10 ± 0.17</td>
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<td>0.600</td>
<td>0.643 ± 0.03</td>
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<td>2.18 ± 0.16</td>
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<td>0.750</td>
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<td>0.668</td>
<td>2.17 ± 0.02</td>
<td>3.17</td>
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<td>0.672 ± 0.02</td>
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<td>1.750</td>
<td>0.726 ± 0.09</td>
<td>1.450</td>
<td>2.56</td>
<td>5.69</td>
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</tbody>
</table>

*a*The errors in \(k_{\text{gem}}\) and \(k_{\text{off}}\) are calculated from standard deviations of two and three measurements, respectively.

5.4.5 Analysis of thermal and denaturant-induced unfolding parameters of myoglobin in the presence of subdenaturing concentrations of GdnHCl

To examine the protein stability and the thermodynamic properties of the N ⇔ U transition, Soret absorbance (409 nm)-monitored thermal transition of
myoglobin was studied in the presence of different concentrations of GdnHCl (Figure 5.4.4a). GdnHCl shifts the transition to lower temperatures. The unfolding transitions were analyzed using the standard equations for two-state transition. Both transition temperature ($T_m$) and van’t Hoff enthalpy ($\Delta H_m$) decreases linearly with increase in denaturant concentration, (Figure 5.4.4b and inset). An important thermodynamic parameter characterizing the thermal unfolding transition of proteins is the difference in the heat capacity between the folded and unfolded protein ($\Delta C_P$). One of the reliable methods for obtaining an accurate value of $\Delta C_P$ is to measure the temperature dependence of the van’t Hoff enthalpy of transition at different denaturant concentrations (223, 224). Figure 5.4.4d (inset) shows the dependence of van’t Hoff enthalpy on the transition temperature. The value of $\Delta C_P$ ($= 2.31\pm0.1$ kcal K$^{-1}$ mol$^{-1}$) was determined by linear least-squares fit of eq (5) to $T_m$ and $\Delta H_m$

$$\Delta H_m = \Delta C_P T_m + b$$

where $b$ is the y-intercept. Using a $\Delta C_P =2.31\pm0.1$ kcal K$^{-1}$ mol$^{-1}$, the free energy of thermal denaturation ($\Delta G_T$) at temperature $T$ (K) was determined from the integrated form of Gibbs-Helmholtz relation (eq 6) with temperature-independent $\Delta C_P$.

$$\Delta G_T = \Delta H_m \left(1 - \frac{T}{T_m}\right) - \Delta C_P \left(T_m - T\right) + T \ln \left(\frac{T}{T_m}\right)$$

Observed and derived thermodynamic parameters for thermal denaturation of myoglobin in different GdnHCl concentrations are summarized in Table 2. Thermal denaturation free energy data are mapped on to the $\Delta G_D$ vs GdnHCl plot presented in Figure 5.4.4d. Also presented in Figure 5.4.4d are unfolding free energy data obtained at 22°C in the transition region of Figure 5.4.4c along with
linear extrapolation to 0.0 M GdnHCl (solid line). The linear dependence appears to hold rather well from the highest GdnHCl concentration down to about 1.35 M. In concentrations lower than 1.35 M, $\Delta G_D$ deviates in a manner that would result in a more positive value for the intercept ($\Delta G_D^0$) then would be predicted by the linear extrapolation model. This direction of curvature is consistent with that expected for both the denaturant binding model and Dill’s model of protein unfolding (65, 215, 225-227). The data were therefore fitted to eq 7 (215),

$$\Delta G_D = \Delta G_D^0 - (\Delta n)RT \ln (1 + k[GdnHCl]) \ (7),$$

where $\Delta n$ is the number of specific binding sites on the protein for the denaturant which binds with an average association constant $k$. Results of the least squares analysis of the $\Delta G_D$ versus GdnHCl concentration plot are shown in Figure 5.4.4d (dotted curve). The values extracted are $\Delta n=16$, $k=1.08$, and $\Delta G_D^0=9.6 \text{ kcal mol}^{-1}$, very close to those reported by Pace and Vanderburg (1979) and others (215, 227).
Figure 5.4.4 (a) Thermal unfolding of oxidized myoglobin at pH 6.2. Transitions were monitored by the decrease in optical density at 409 nm. Curves labeled from 1 to 7 correspond to thermal transitions in the presence of 0.0, 0.1, 0.3, 0.5, 0.8, 1.1 and 1.35 M GdnHCl, respectively. The solid curves represent non-linear least–squares fits to the two-state unfolding model. (b) Variation of $\Delta H_m$ with denaturant concentration. Temperature midpoint of unfolding ($T_m$) in different GdnHCl concentration is shown in the inset. (c) GdnHCl-induced equilibrium unfolding transition of myoglobin at pH 6.2, 22°C. The iterated fit parameters are $\Delta G_D^\circ = 6.21(\pm 0.42) \text{ kcal mol}^{-1}$ and $m_g = 4.35(\pm 0.32)$. (d) Evaluation of unfolding free energy changes as a function of GdnHCl. Points in the transition region are calculated from the equilibrium curve shown in panel c. Points below 1.35 M GdnHCl have been calculated from thermal denaturation data in the presence of different concentrations of GdnHCl, at 22°C, shown in panel a. The free energy values were calculated by the use of Gibbs-Helmholtz equation (eq 6). The solid line represents the results of the application of the linear extrapolation model. The dotted line represents the results of the application of the binding model. The inset shows the enthalpies of the thermal transition of myoglobin as a function of transition temperture in the presence of different GdnHCl concentrations. The solid line represents linear least–square fit to eq (5) with slope=2.31(± 0.1) kcal K$^{-1}$ mol$^{-1}$. 
**Table 2.** Observed and derived thermodynamic parameters of myoglobin at pH 6.2, 22°C

<table>
<thead>
<tr>
<th>GdnHCl (M)</th>
<th>$\Delta G$ (kcal mol$^{-1}$)</th>
<th>GdnHCl (M)</th>
<th>$\Delta H_m$ (kcal mol$^{-1}$)</th>
<th>$T_m$ (°K)</th>
<th>$\Delta G_f$ (kcal mol$^{-1}$)</th>
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<td>0.30</td>
<td>109.50</td>
<td>347.53</td>
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<td>1.50</td>
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<td>342.80</td>
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<td>0.80</td>
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<td>335.70</td>
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</tr>
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<td>1.58</td>
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<td>1.8106</td>
</tr>
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<td>1.62</td>
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</table>

**5.4.6 Denaturant dependence of activation enthalpy and conformational entropy of myoglobin-CO reactions**

The observation that $k_{gem}$, $k_{on}$ and $k_{off}$ varies with increment of subdenaturing concentration of GdnHCl up to 1.75 M (Figure 5.4.3b and 5.4.3c) warrants a thermodynamic analysis of the reaction. The pertinent reaction monitored is (207),
and the analysis is based on the thermodynamic formulation of the conventional transition state theory (30), with the assumption that GdnHCl has no effect at the transition state level. As argued in Chapter 1, if the increases in rate coefficients is due to destabilization of the reactant state, the increase in entropy of the ground state in the presence of denaturant must be compensated by a decreases in activation enthalpy of the respective reaction. This follows from

\[
\frac{H_{\text{gem/on/off}}}{RT} = \ln \left( \frac{v}{k_{\text{gem/on/off}}} \right) + \frac{S_{\text{gem/on/off}}}{R}
\]

where \(v\) is the vibrational frequency, \(H_{\text{gem/on/off}}\) and \(S_{\text{gem/on/off}}\) are changes in enthalpy and entropy, respectively, between the reactant and transition states. The temperature dependent of the rate coefficient is given by Arrhenius law,

\[
k_{\text{gem/on/off}} = A \exp(-E_a/RT)
\]

where \(A\) is the front factor, \(E_a\) is the activation energy. Figure 5.4.5a shows these plots for a strongly refolding condition (0.5 M for bimolecular rebinding and 0.0 M GdnHCl for geminate rebinding and CO replacement reactions) and a weakly unfolding condition (1.75 M GdnHCl). By comparing eq (9) with the Arrhenius equation the following relations are obtained:

\[
E_a = H_{\text{gem/on/off}}
\]

\[
\ln(A/v) = S_{\text{gem/on/off}}/R
\]

Figure 5.4.5b shows the denaturant distribution of \(H_{\text{off}}\) determined from a series of temperature dependent CO replacement study in the 0-1.75 M range of GdnHCl. For geminate and bimolecular CO rebinding, \(H_{\text{gem}}\) and \(H_{\text{on}}\) were determined only in strongly native and weakly unfolding conditions (Table 3).
GdnHCl concentration is increased from strongly native condition to unfolding conditions the enthalpic barriers for all myoglobin-CO reactions are lowered. The conformational entropy loss, \( \Delta S \), relative to the entropy of protein in the absence of the denaturant, were calculated according to

\[
\Delta S = k_B \ln \left( \frac{A_x}{A_{\text{ref}}} \right)
\]

(12),

where \( A_{\text{ref}} \) and \( A_x \) are the Arrhenius front factors for temperature dependence of the reactions—\( A_{\text{ref}} \) in the absence of GdnHCl, and \( A_x \) in the presence of \( x \) concentration of GdnHCl. \( \Delta S \) is expressed in Boltzmann units (\( k_B \)). The calculated values of \( \Delta S \) are listed in table 3. For CO replacement reactions of myoglobin-CO complex, the plot of \( \Delta S \) as a function of GdnHCl concentration is shown in figure 5.4.5c. The lowered values of \( H_{\text{gem/on/off}} \) and \( \Delta S_{\text{gem/on/off}} \) of myoglobin-CO reactions in the presence of GdnHCl strongly suggest that in the subdenaturing limit of GdnHCl the free energy and conformational entropy of the protein increase, leading to decrease in \( k_{\text{gem/on/off}} \).

**Figure 5.4.5** (a) The upper fits show temperature dependence of the geminate recombination rate, \( k_{\text{gem}} \), in the presence of 0.0 M (\( \circ \), \( H_{\text{gem}} = 3.46 (\pm 0.3) \text{kcal mol}^{-1} \)) and 1.75 M GdnHCl (\( \bullet \), \( H_{\text{gem}} = 2.59 (\pm 0.09) \text{kcal mol}^{-1} \)). The lower fits show temperature dependence of CO replacement rates, \( k_{\text{off}} \), in 0.0 M GdnHCl (\( \square \), \( H_{\text{off}} = 21.54 (\pm 0.59) \text{kcal mol}^{-1} \)) and 1.75 M GdnHCl (\( \blacksquare \), \( H_{\text{off}} \)).
= 18.93 (± 0.99) kcal mol\(^{-1}\)). The fits in the middle of panel (a) show temperature dependence of CO rebinding rates, \(k_{on}\) in the presence of 0.5 M (◊, M\(^{-1}\) s\(^{-1}\), \(H_{on} = 9.76(±1)\)) and 1.75 M GdnHCl (◇, M\(^{-1}\) s\(^{-1}\), \(H_{on} = 7.81(±1)\) kcal mol\(^{-1}\)). Panels (b) and (c) show GdnHCl dependence of enthalpic barriers \(H_{off}\) (kcal mol\(^{-1}\)) (▲) and conformational entropy loss by the protein in the presence of denaturants relative to the entropy of the protein in the absence of the denaturant (△).

**Table 3** Arrhenius parameters for CO and myoglobin reaction at pH 7, in different GdnHCl concentration

(a) For CO rebinding:

<table>
<thead>
<tr>
<th>GdnHCl (M)</th>
<th>(H_{off}) (kcal/mol)</th>
<th>(lnA)</th>
<th>(\Delta S_{off}) (in k(B))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>21.54 ± 0.59</td>
<td>32.43 ± 1.1</td>
<td>0.0 ± 0.0</td>
</tr>
<tr>
<td>0.2</td>
<td>22.63 ± 0.69</td>
<td>34.27 ± 2.1</td>
<td>1.84 ± 1.0</td>
</tr>
<tr>
<td>0.4</td>
<td>22.36 ± 0.78</td>
<td>33.84 ± 1.3</td>
<td>1.41 ± 0.2</td>
</tr>
<tr>
<td>0.6</td>
<td>20.38 ± 0.79</td>
<td>30.59 ± 1.4</td>
<td>-1.83 ± 0.3</td>
</tr>
<tr>
<td>1.0</td>
<td>20.31 ± 0.59</td>
<td>31.01 ± 1.8</td>
<td>-1.41 ± 0.7</td>
</tr>
<tr>
<td>1.75</td>
<td>18.93 ± 0.99</td>
<td>28.88 ± 0.9</td>
<td>-3.56 ± 0.2</td>
</tr>
</tbody>
</table>

(b) For CO replacement:

<table>
<thead>
<tr>
<th>GdnHCl (M)</th>
<th>(H_{gem}) (kcal/mol)</th>
<th>(lnA)</th>
<th>(\Delta S_{gem}) (k(B))</th>
<th>GdnHCl (M)</th>
<th>(H_{on}) (kcal/mol)</th>
<th>(lnA)</th>
<th>(\Delta S_{on}) (k(B))</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.0</td>
<td>3.46 ± 0.3</td>
<td>23.21 ± 0.5</td>
<td>0.0</td>
<td>0.5</td>
<td>9.76 ± 1</td>
<td>29.74 ± 2</td>
<td>0.0</td>
</tr>
<tr>
<td>1.75</td>
<td>2.59 ± 0.09</td>
<td>22.57 ± 0.1</td>
<td>-0.64</td>
<td>1.75</td>
<td>7.81 ± 1</td>
<td>27.96 ± 1</td>
<td>-1.8</td>
</tr>
</tbody>
</table>
5.6 Discussion

The myoglobin-ligand binding results clearly show that the reactivity of the protein is significantly modulated even under mild denaturing conditions. Explicably, this arises from alteration in the protein structure and conformation. However, a detailed description of the structural changes, especially those that directly influence the ligand entry and exit, is not possible in the absence of crystal structure with the denaturant. There have been indications that the tertiary structure around the heme is not denatured completely even after the secondary structure vanishes (221). A recent wide-angle X-ray scattering study of myoglobin shows that as GdnHCl concentration increases from 0 to 2.5 M, the tertiary structure remains almost intact even though the secondary structure is destroyed to a very large extent (218). Neutron scattering data have provided evidence for substantial changes in the dynamics of myoglobin in the presence of GdnHCl. Highly denatured state of myoglobin presents a more open active site structure and higher overall H-atom motion with respect to the native state (10). A recent report indicates a relation between bimolecular CO rebinding rates of reduced myoglobin and diffusion coefficient of carboxymyoglobin. Under unfolding conditions, Choi and Terazima (221) observe lower diffusion coefficient and higher hydrodynamic radius. The higher hydrodynamic radius of the protein is related to a more open form. According to Champion et al. (210) a more open active site myoglobin structure displays higher rates for both CO rebinding and replacement processes. The mechanism whereby GdnHCl induces an opening of the active site structure of myoglobin remains elusive. It is known that the movement of the side chain of His 64, and a salt bridge between Arg 45 (or Lys 45 in horse myoglobin) and heme-6 propionate control the outside access
to the distal cavity around the heme (228-229). It is possible that the imidazole side chain of His 64 is swung out and/or the Lys 45–heme propionate interaction ceases to exist even under mild denaturing conditions rendering possible uncontrolled access of ligands to the heme site. This speculation remains to be tested by crystallography in the presence of the denaturant.

There has been considerable debate about how chemical denaturants destabilize or unfold proteins. Although the most popular ‘linear free energy model’ (LFEM) that holds that the unfolding free energy can be linearly extrapolated from the transition region to the absolute native condition, has been theoretically formalized by Schellman (11-12), unfolding data can also be treated by invoking stoichiometric binding of denaturant molecules to protein groups (64). While the LFEM has been experimentally validated by numerous studies (65, 68, 227, 230-231, for example), some of the most detailed solution-state studies of the interaction of chemical denaturants with proteins validate the binding model (34, 68, 227, for example). Protein structures determined from crystals soaked in denaturants (31-33) also lend substantial support to the binding model. The results for myoglobin presented here validate further the binding model, and are consistent with similar such studies reported earlier (215, 227, for example). The observation that the ΔG_D-denaturant plot deviates systematically upward along the ordinate suggests that a linear extrapolation of the ΔG_D-denaturant gradient backward to the ordinate would suggest an underestimated value of ΔG_D° (Figure 5.4.4d). The total number of guanidinium binding sites in myoglobin is ~16. This result is very close to those reported by Pace and Vanderburg (1979) and others (215, 227).
As already discussed in Chapter 1, X-ray structures of several proteins in the presence of denaturants indicate multiple hydrogen bonding interactions of GdnH\(^+\) ions and urea with protein groups. The results of ferrocyt c–CO association and dissociation have shown that subdenaturing concentrations of GdnHCl stabilize the protein through the ‘stiffening effect’. One of the objectives of the myoglobin-ligand experiments presented here was to check if low concentrations of GdnHCl stabilize this protein as well. The results though do not directly show such entropic stabilization (Figure 5.4.5 and Table 3). However, they do not negate the denaturant-induced stiffening effect. The reason for the observed increase in rates with denaturant increments (Figure 5.4.3) could be that the subglobal modes of motions, and therefore collisions, which influence the rates of myoglobin-ligand reactions are little constrained by the protein-denaturant interactions.

### 5.6 Summary and conclusion

Subdenaturing concentration of guanidine hydrochloride affects both protein structure and dynamics. A more open active site structure is observed with increasing denaturant concentration, which leads to a parallel increase in magnitudes of the geminate rebinding, bimolecular rebinding and ligand replacement rates of the myoglobin-CO reaction. The data for equilibrium denaturation induced by GdnHCl validate the stoichiometric binding model for protein unfolding, suggesting that the structural and dynamical changes across the folding-unfolding transition are due mainly to GdnHCl binding to the protein, although denaturant-mediated alterations in protein-solvent interactions are also likely to contribute. The protein-denaturant binding interactions in the presence of