Electrochemical Investigation of Redox Thermodynamics of Immobilized Myoglobin: Ionic and Ligation Effects

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In this study, we investigated redox thermodynamics of myoglobin as well as the ionic (phosphate ions) and ligation (imidazole) effects via a dynamic electrochemical approach. We employed a previously established system that features nanomediated, direct electrochemistry of myoglobin and myoglobin in an immobilized state (i.e., diffusionless electrochemistry). Thermodynamics parameters were obtained by measuring redox potential ($E^\circ$) of myoglobin at varied temperature ($T$), in the presence and in the absence of specific ions or axial ligands. As a step further, we evaluated contributions from allosteric effect and axial iron ligation by partitioning $E^\circ$ changes into entropic and enthalpic terms. Compensation phenomena between the entropic and enthalpic changes were observed in all these cases. On the basis of these studies, we also correlated these phenomena to possible structural variations.

1. Introduction

The thermodynamics of a redox protein is highly tunable via ion/ligand binding or formation of protein complexes.1–5 It has been one of nature’s strategies to optimize redox thermodynamics of electron-transfer (ET) proteins in living systems (e.g., respiratory chains). Therefore, considerable efforts have been taken to understand these tuning effects in in vitro model systems.6–8 Dynamic electrochemistry (e.g., cyclic voltammetry) is a particularly useful tool for such studies, which has proven superior to the more traditional static electrochemistry (e.g., potentiometry).9

Sola and co-workers have presented comprehensive electrochemical studies on redox thermodynamics as well as ligand-binding effects of a variety of small ET proteins.1–3,10–14 However, their recent effort to develop a dynamic approach to a large molecule, metalloenzyme, horseradish peroxidase (HRP), has been hampered due to slow electron communications of the HRP with electrodes. In their work, they instead employed the traditional potentiometric titration to obtain the redox potentials of HRP in different environments.15 Myoglobin (Mb), as a non-ET metalloprotein, has also been well-known to exhibit very poor electrochemistry.4,15 In an attempt to establish electron communication between Mb and electrodes, Rusling et al. entrapped Mb in a biomembrane-like film and obtained well-defined electrochemistry of Mb.16 Following their pioneering work, we and others have achieved direct electrochemistry of proteins with a variety of film materials, including inorganic materials, polyelectrolytes and biopolymers,17–18 However, to study protein—ligand binding and have systematically investigated influence of polyelectrolytes on the ligation of Mb and imidazole.23 Although redox thermodynamics and kinetics of Mb in the immobilized state deviate slightly from that in the solution phase, it may serve as a convenient model system to investigate interactions between Mb and ligands or modulators. For example, it eliminates problems arising from diffusion of proteins to the interface, thus significantly simplifying the research system. We hereby employed this established system to

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study the thermodynamics of film-entrapped Mb. Since redox potentials of proteins are sensitive indicators of peptide structural alterations surrounding the active site,\textsuperscript{24} we also interrogated effects of specific ions and/or ligands on redox thermodynamics of Mb with this method.

2. Materials and Methods

2.1. Reagents. Horse heart myoglobin was purchased from Sigma Chemical Company, and 3-[N-morpholino]propanesulfonic acid (MOPS) was obtained from Amresco. Other reagents used were of analytical reagent grade. Water was purified with a Milli-Q purification system to a specific resistance (\(\geq 16 \text{ M}\Omega \text{ cm}^{-1}\)) and used to prepare all solutions.

2.2. Preparation of Electrodes. The working electrode was a pyrolytic graphite (PG) disk electrode (edge plane, \(A = 5.88 \text{ mm}^2\)). A saturated calomel electrode (SCE) was used as the reference electrode, while all potentials reported here are referred to a standard hydrogen electrode (SHE). A platinum wire electrode served as the counter electrode. The substrate PG was first polished using rough and fine sand papers. It was then polished to a mirror smoothness with an alumina (particle size of about 0.05 mm\(^3\)/water slurry on silk. After that, it was ultrapure-d in water for ca. 5 min. DMSO was mixed with 8 mg/mL Mb aqueous solutions at 1:1 ratio. Twenty microliters of such a mixed solution was evenly spread onto the PG electrode surface. Electrodes were dried overnight at room temperature and then thoroughly rinsed with water.

2.3. Electrochemical Measurements. Electrochemical experiments were performed using a model 263A potentiostat/galvanostat (PAR, EG&G, USA). Prior to the experiment, the phosphate buffer (pH 7.0) was bubbled thoroughly with high-purity nitrogen, then a stream of nitrogen was blown gently across the surface of the solution in order to maintain the solution anaerobic throughout experiments.

The temperature dependence of redox potentials (\(E^{\circ}\)) of Mb was measured in a nonisothermal electrochemical cell.\textsuperscript{25} In this setup, the reference electrode is held at a constant temperature and connected to working solutions with a salt bridge, while the half-cell containing the working electrode is under precise thermostatic control with a water bath. The salt bridge was formed by injecting a heated mixture of 3% agar and 4.2 M KCl solution into a U-shaped tube, which was then cooled to form gel in the tube. The liquid junction between the working compartment and the salt bridge was formed using a porous glass, which prevented mixing of the two solutions. The cell arrangement employed in the present work can be written as follows:

\[
\text{PG|Hg|HgCl}_2(\text{sat}), \text{KCl( sat)}||\text{KCl (4.2 M)|KCl (4.2 M)|M}^\text{III}|\text{M}^\text{II}|\text{PG}
\]

We chose 4.2 M KCl in the salt bridge since the solubility of KCl is slightly greater than 4.2 M even at the lowest temperature that was employed in our measurements. In this experiment, reaction entropy (\(\Delta S^{\circ}\)) was obtained from the plot of \(E^{\circ}\) vs temperature (\(T\)), while reaction enthalpy (\(\Delta H^{\circ}\)) was obtained according to the Gibbs–Helmholtz equation.\textsuperscript{2}

3. Results

3.1. Electrochemical Characterization of Mb/DMSO Films on PG Electrodes. In our previous studies, we have proven that charged polyelectrolytes that were employed to form biomembrane-like Mb films perturb ligand-binding properties of Mb.\textsuperscript{23} To minimize this effect, a neutral molecule, dimethyl sulfoxide (DMSO), was employed to form Mb films in this work. As well as other film materials, we and others have proven that heme protein can directly exchange electrons with electrodes.\textsuperscript{26,27}

More importantly, various spectroscopic techniques have provided evidence that while protein structures may be more or less perturbed, they may retain compact tertiary structure and substantial integrity of the active site microenvironment in solution containing up to 60% DMSO.\textsuperscript{28} These two findings form the basis to employ the Mb/DMSO/PG system to study redox thermodynamics of Mb.

Consistent with previous reports, we have observed a pair of well-defined redox peaks for Mb-decorated electrodes in a 0.05 M phosphate buffer (pH 7.0), with an \(E^{\circ}\) of \(-0.068 \text{ V}\) (Figure 1).\textsuperscript{16,17} This pair of peaks has been attributed to the reduction and oxidation of heme iron.\textsuperscript{16,17} The \(E^{\circ}\) of surface-confined Mb is lower than that obtained in solutions, which is typical for proteins entrapped in membranes or films and reflects the effect of surface immobilization.\textsuperscript{28,29} Similarly, well-defined Mb electrochemistry was obtained for the same electrode in a 0.05 M MOPS buffer (pH 7.0), with a slightly lower \(E^{\circ}\) of \(-0.144 \text{ V}\). The variation of \(E^{\circ}\) in different mediums is possibly attributed to the ionic effect (vide post). Note that Mb is electrochemically silent in the absence of DMSO, which dearly shows the ET-facilitation effect of DMSO.\textsuperscript{16,17} The peak currents of Mb reduction and oxidation are linearly proportional to scan rates (Figure 1 inset), which suggests that this corresponds to the electrochemistry of a surface-confined species. It is also worthwhile to point out that Mb-decorated electrodes are stable under experimental conditions employed in this work, without significant signal (peak current) loss after temperature cycles or by varying ionic strength of electrolytes (data not shown).

3.2. Redox Thermodynamics of Surface-Confined Mb. The redox potential of Mb shifts to the negative along with the elevated temperature. This reflects the progressive opening of heme crevices and thus enhanced solvent accessibility upon temperature increase.\textsuperscript{10} We have observed a linear dependence for the \(E^{\circ} - T\) plot, the slope of which gives \(\Delta S^{\circ}/\text{R}\) (Figure 2). \(\Delta H^{\circ}\) can be computed from the Gibbs–Helmholtz equation. We have thus obtained the reaction entropy and enthalpy (Table 1) of Mb in different states. It is possible to establish a correlation between \(E^{\circ}\) and structural properties of redox proteins by determining corresponding enthalpic and

\begin{figure}[h]
\centering
\includegraphics[width=0.5\textwidth]{figure1.png}
\caption{Cyclic voltammograms (CVs) of Mb-modified PG electrodes in pH 7.0, 0.05 M PBS (solid line) and MOPS (dash line): temperature, 298 K; scan rate, 50 mV/s. Inset is the relation between wave currents and scan rate in PBS (\(\bullet\)) and MOPS (\(\bullet\)) buffer.}
\end{figure}

Table 2. Variations in Thermodynamics Parameters for Mb and Mb in Different Ionic Medium, and with or without Imidazole Ligand

<table>
<thead>
<tr>
<th></th>
<th>ΔE°</th>
<th>ΔTAS°</th>
<th>ΔH°</th>
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<tbody>
<tr>
<td>(Mb−Im)−(Mb), phosphate</td>
<td>−97</td>
<td>406</td>
<td>−503</td>
<td></td>
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<tr>
<td>(Mb−Im)−(Mb), MOPS</td>
<td>−16</td>
<td>484</td>
<td>−500</td>
<td></td>
<td></td>
</tr>
<tr>
<td>(Mb−phos)−(Mb-MOPS)</td>
<td>80</td>
<td>−96</td>
<td>176</td>
<td></td>
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<tr>
<td>(Mb−phos)−(Mb-MOPS), imidazole</td>
<td>−1</td>
<td>−174</td>
<td>173</td>
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* Difference between redox potentials in different conditions, with an average error of 7.3 mV. Average errors for ΔTAS°, and ΔH°° are 13 and 20 mV, respectively. This term stands for entropic contribution to redox potentials. This term stands for enthalpic contribution to redox potentials.

buffer differ significantly from those obtained in the MOPS buffer. The decrease in ΔS°° (−30 J mol⁻¹ K⁻¹) suggests that Mb reduction leads to more pronounced conformational changes upon phosphate binding. This observation is consistent with the fact that specific anion (e.g., phosphates) binding to Mb allosterically modulates the microenvironment of heme sites. We also note that ion-binding alters the surface electrostatic potential of proteins, which might affect solvent reorganization on reduction. This effect may also contribute to the reduction thermodynamics of Mb. We thus speculate that Mb reduction might be simultaneously influenced by these two effects.

3.3. Redox Thermodynamics of Mb−Imidazole Complex. The binding of axial ligands to heme sites is known to alter the microenvironment surrounding the redox reaction center. Correspondingly, we have observed a decrease in $E^\circ$ (Figure 2), as well as significant entropy and enthalpy gain for Mb redox in the presence of imidazole ligand. The decrease in the redox potential is consistent with the fact that imidazole binds much more strongly to oxidized than to reduced state of hemes. As illustrated in Table 2, the lowered $E^\circ$ mainly originates from the enthalpic contribution. The enthalpic gain is an effect arising from the enhanced stabilization of the oxidized heme by imidazole, a much stronger electron donor than water.

\[
\text{Mb Fe(III)} + \text{H}_2\text{O} + \text{Im} \leftrightarrow \text{Mb Fe(III) Im} + \text{H}_2\text{O} \quad (1)
\]

\[
\text{Mb Fe(III) Im} + e^- \leftrightarrow \text{Mb Fe(II) Im} \quad (2)
\]

\[
\text{Mb Fe(II) Im} \leftrightarrow \text{Mb Fe(II) + Im} \quad (3)
\]

where Mb Fe(III) and Mb Fe(II) are defined as the oxidized and reduced state of Mb, respectively, and Im stands for the strong field ligand, imidazole.

The entropic term actually plays a reversed role on the change of the redox potential of Mb (Table 2). However, we note that the enthalpic effect of axial ligations prevails over the entropic contribution, which results in the observed $E^\circ$ decrease. It thus seems that ligand-binding related enthalpy−entropy compensation phenomenon exists in a variety of heme proteins.

3.4. Implications for Interactions between Anion and Ligand Bindings. Phosphate anions modulate the microenvironment surrounding the heme site as a kind of allosteric effector. Therefore, it is possible that phosphate ions may influence the ligand binding processes. Apparently, in our experiment, $E^\circ$ decreases by 97 mV upon imidazole binding in phosphate buffers, while only by 16 mV in MOPS buffers (Table 1). This difference


55x64](Table 2). Both entropic term partially compensates the enthalpic effect (Table 2). However, the entropic contribution to redox potentials is mainly due to the enthalpic contribution, while the entropic term partially compensates the enthalpic effect (Table 2). Both ΔS°° and ΔH°° of Mb in the phosphate

entropic components. In both phosphate and MOPS buffers, the reduction of Mb is accompanied by negative changes of both entropy and enthalpy. While the exact mechanism of these entropy and enthalpy losses remains ambiguous, it is possible to postulate that the entropy loss upon reduction reflects conformational variations upon Mb reduction, i.e., limited protein flexibility in the reduced state; the enthalpy loss arises from the stabilization of the reduction Mb over its oxidized counterpart and from limited solvent accessibility in the reduction state. We note that other effects such as variations in hydrogen bonds or solvent reorganization might also contribute. It is also interesting to note that the negative redox potential of Mb is mainly of entropic origin. The enthalpic term favors Mb reduction. However the entropic term disfavors Mb reduction, which prevails over the entropic contribution and results in the negative shift in $E^\circ$ of Mb. Finally, we should point out that the analysis of redox thermodynamics might deviate from the state of Mb in aqueous solutions due to the fact that surface immobilization significantly alters its thermodynamic properties (e.g., shifts in $E^\circ$).

It is of interest to investigate ionic effects on Mb redox thermodynamics. MOPS is known to be a nonbinding buffer, whereas phosphate ions have specific binding sites on Mb. Evidently, $E^\circ$ of Mb is more positive in the phosphate buffer, as compared to that in the MOPS buffer. This suggests that phosphate-binding favors Mb reduction. In this case, the increased $E^\circ$ in the presence of phosphate is mainly due to the enthalpic contribution, while the entropic term partially compensates the enthalpic effect (Table 2). Both ΔS°° and ΔH°° of Mb in the phosphate

Figure 2. Linear plot of redox potentials ($E^\circ$) of Mb versus temperature (T) at pH 7.0: 0.05 M phosphate buffer (a) and MOPS buffer (b). Scan rate was 50 mV/s.

Table 1. Redox Thermodynamic Parameters for Mb and Mb−Imidazole

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suggests that phosphate-binding allosterically significantly stabilizes the imidazole binding to the heme. It is possible to quantitatively evaluate this difference by comparing the dissociation constant ($K_d$) of Mb–imidazole in different conditions. The relationship between the dissociation constant of Mb–Im and the redox potential is defined by the following equation

$$E^{\circ}(L) = E^{\circ} + \frac{RT}{2nF} \ln \frac{D_M^{n+}}{D_{ML}^{n+}} - \frac{RT}{nF} \ln K_d - pRT/nF \ln [L] \quad (4)$$

where $E^{\circ}(L)$ and $E^{\circ}$ stand for the redox potentials of Mb before and after binding to the ligand and $D_M^{n+}$ and $D_{ML}^{n+}$ represent the diffusion coefficients in the absence and presence of ligand, which were assumed to be the same in our system. $K_d$ represents the apparent dissociation constant, $p$ is the coordination number ($p = 1$), and $[L]$ is the concentration of ligand. All other symbols have their normal meanings. According to this equation, the presence of phosphate ions increases the $K_d$ of Mb–Im complex by as much as 23.5-fold. This clearly shows the stabilization effect of phosphate ions on ligand binding. We also note that in the presence of imidazole, $E^{\circ}$ in the phosphate buffer is not different from that in the MOPS buffer, in contrast to a 80 mV decrease upon phosphate binding in the absence of imidazole. These phenomena imply that interactions between anion-binding and ligand-binding exist; these anions and ligands might have a synergic effect on the redox thermodynamics of Mb.

4. Conclusions

In summary, we have investigated redox thermodynamics of Mb with Mb/DMSO decorated electrodes and dynamic electrochemical means. We have shown that this method provides a convenient approach to interrogate ionic and ligation effects on thermodynamics. Changes in the redox potential have been evaluated with both enthalpic and entropic terms and have shown certain relevance to structural variations of proteins. On the basis of thermodynamic analysis, we have also suggested that phosphate ions and ligand binding might interact via allosteric effect and synergically influence redox thermodynamics of Mb.

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