A Thermodynamic Study of Azide Binding to Myoglobin

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The requisite first step in the function of a variety of biological macromolecules is binding of a smaller molecule (the ligand). Examples include substrate association with an enzyme, antibiotic binding to a nucleic acid, and antigen interaction with an antibody. Characterization of ligand binding can provide insight into the natural function of the ligand and the mechanism of action associated with the macromolecule. Fundamental properties of interest in characterizing ligand binding include the strength of binding, number of binding sites, interaction between sites, and the energetics of binding (changes in free energy, entropy, and enthalpy). All these properties can be derived from evaluation of the equilibrium thermodynamics associated with the system. This expanded treatment of equilibrium binding is a natural extension to that presented in most introductory courses.

We have designed a set of simple experiments to introduce chemistry and biochemistry students to the experimental determination of dissociation constants for exogenous ligand binding to biological macromolecules. Students characterize the equilibrium thermodynamic properties of azide binding to the ferric form of myoglobin, metmyoglobin (metMb). The experimental design and execution are straightforward and readily adapted to other protein systems. The initial set of experiments can be completed in two 3-hour laboratory periods. A third laboratory period is required for students to probe the temperature dependence, which allows them to characterize further the thermodynamic properties of this system. No previous knowledge of biochemistry is assumed, and therefore these experiments have served as a useful introduction to protein biochemistry for our chemistry students.

The objective of these experiments is to determine the dissociation constant associated with azide binding to the Fe(III) form of myoglobin. Myoglobin is the oxygen storage protein isolated from the muscle tissues of a wide variety of organisms ranging from molluscs to mammals (1, 2). It is a single-subunit protein possessing a single heme iron, the site of reversible oxygen binding. The heme cofactor comprises a square planar iron atom coordinated to four nitrogen atoms of the tetrapyrole macrocyclic porphyrin ring, which is held within the confines of the protein by a combination of hydrophobic interactions and a single metal–protein coordinate bond. Oxygen binds to the Fe(II) form of the protein (deoxyMb). The metMb binds a variety of exogenous ligands including azide, fluoride, cyanide, and thiocyanate and is the focus of this laboratory project (3).

To meet the objectives of this project, students must first develop a method for quantifying the myoglobin–azide adduct (M bN3). They are expected to have completed the design aspect before attending the first lab period. Review of their proposed protocol assures that they get off to an effective start. Their first laboratory period is devoted to executing a saturation study as monitored by changes in the visible absorption upon azide binding. From these data students are able to assess the maximum change in absorption, which occurs when the protein binding sites are saturated, and to determine the molar extinction coefficient for the M bN3 adduct. They are also able to select an appropriate concentration range for their more detailed titration. The second lab period is devoted to the careful execution of this titration.

A point of intersection between two overlapping spectra for a solution containing only two species contributing to the absorption is called an isobestic point. Every spectrum recorded during the titration should pass through these isobestic points, as shown in Figure 1, providing immediate feedback on the quality of the experimental data. Data from this titration are used to generate a Scatchard plot (4), from which the dissociation constant and number of binding sites are extracted. The values obtained by students in our laboratories, Kd = 7.8 μM and n = 1.03, are comparable to values recently reported (5). Development of the principles associated with this method of analysis are addressed in the associated lab documentation. A final lab period can be devoted to repeating this titration at various temperatures, allowing evaluation of other thermodynamic properties.
Understanding ligand binding to a single site in myoglobin is the first step toward appreciating the complexity of the multiple equilibria associated with the four interacting sites of hemoglobin (Hb) and the methods required to address this more complicated case. Developing the ideas associated with assessment of multiple equilibria (e.g., Hill plot analysis) (4) is a natural extension to this project. An interesting result that will initially puzzle students is that the Hill coefficient for azide binding to metHb is reported to be $n_H = 1.0$ (3), which suggests no interaction between binding sites! Based on class readings, students are familiar with the fact that oxygen binding to human deoxyHb displays positive cooperativity, having a Hill constant between 2.8 and 3.0 (2). They know that various structural changes have occurred in the protein following the binding of oxygen, including the movement of the iron into the plane of the porphyrin ring. Exploration of metMb and metHb structures (2, 6) illustrates that the ferric ions in the met forms of these proteins have already adopted the in-plane position, similar to that of the oxygenated forms. It is perhaps a consequence of this movement that the “tension” is relieved and no cooperativity is observed, suggesting that the four sites in metHb are acting independently.

**Materials and Methods**

Stock solutions of 2.0 mM metmyoglobin ($M_r$ 18,000, $e_{540} = 8.8$ mM $^{-1}$cm$^{-1}$ [3]), 20 mM NaN$_3$, and 2.0 M NaCl are prepared in a 0.1 M borate buffer, pH 9.5. MetMb is prepared by dialyzing commercially available horse heart Mb (Sigma Chemical Company) against an excess of K$_3$Fe(CN)$_6$, which is subsequently removed by exhaustive dialysis against buffer. Samples are prepared by adding NaN$_3$ solution to the M b sample using NaCl to maintain a constant ionic strength. Final myoglobin sample volumes should be ca. 1.5 mL and concentrations ca. 50 mM. Samples are allowed to equilibrate until no change is seen in the absorption at 540 nm, typically 60 min at 20 °C. Absorption spectra are collected on a Hewlett-Packard 8452A UV–vis spectrophotometer using 1.0 cm quartz microcuvettes. A circulating water bath connected to the cuvette holder allows for temperature regulation. Unless otherwise indicated, temperature was held constant at 20 °C.

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**Literature Cited**

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