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The structure and peroxidase activity of myoglobin in alcoholic solvents

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ABSTRACT

We present data on the peroxidase activity of equine skeletal myoglobin in water, methanol, ethanol, 1-propanol, 2-propanol, 1-butanol, and 1-pentanol. For experimentation in non-aqueous solvents the protein structure was stabilized through lyophilization from a solution of a buffer and 98% (dry weight) KCl. Myoglobin showed some solubility in methanol and its structure was probed with circular dichroism and UV–Vis absorption spectroscopies. Peroxidase activity of myoglobin toward the oxidation of *o*-phenylenediamine to 2,3-diaminophenazine was observed and measured in each of the listed solvents. The system in methanol showed the fastest initial rate of reaction. This initial rate in the other organic solvents was similar to the rate in water. However, myoglobin showed stable catalysis for a longer period of time in water. These results provide a first step forward in guiding the utilization of re-engineered myoglobin variants in non-aqueous solvents.

1. Introduction

Enzymes are noted for their ability to facilitate reactions while being discriminant over substrate selectivity and generating products with tight regio- and stereo-control. In these processes, the protein structure controls access to the active site and defines the geometry of the resultant molecules. One of the primary goals of protein engineering is to generate industrially relevant catalysts that maintain the selectivity of enzymes for non-biological processes [1]. In this way, proteins can be repurposed for uses outside of those required by Nature.

There are multiple approaches for developing novel enzymes, including *de novo* protein design [2–5], directed evolution [6–9], and employing a protein as a modular ligand framework [10–13]. In the final example, synthetic inorganic complexes replace metal cofactors, such as heme, and bring new functionality to the protein. Myoglobin is one of the most studied proteins in these re-engineering experiments [11]. There are a number of reasons why this protein is exploited for these studies. Myoglobin was one of the first proteins to be re-engineered; replacing its native iron porphyrin with another transition metal porphyrin is a relatively straightforward process [14,15]. Subsequently, the extent to which myoglobin has been altered has become more extensive [11]. Included in these efforts are myoglobin variants where the heme has been replaced with a Mn^{2+}(salen) complex and a Cr^{3+}(Schiff base) complex, respectively [16,17]. In both of these re-engineered myoglobin complexes, the protein structure has been shown to facilitate a more stereo-selective sulfoxidation reaction than the corresponding metal complex on its own.

For many re-engineered proteins, the intended substrates and products are more soluble in organic solvents than in aqueous solution. To make industrial-scale production feasible, they must be functionally active in these same organic solvents. While facilitating enzyme activity in non-aqueous solvents may seem like a counter-intuitive process, there are examples going back to 1985 that help to inform the best practices for doing so [18–21]. The most important step for preserving enzyme function in these non-natural environments is lyophilization of the protein from an aqueous solution that contains an excipient [19]. Excipients are a class of molecules that help to preserve protein structure in harsh, non-aqueous environments. Another observation from experiments of proteins in organic solvents is that enzyme activity is increased for solvent systems with an absence of water over systems that contain a mixture of organic solvents and water [22,23].

There have been a number of successful industrial applications of these systems outside of the fundamental research [20,24,25]. However, many of the lessons learned from studying non-aqueous enzymes have yet to be applied to engineered proteins. The research presented here seeks to make a first step toward this goal by exploring the peroxidase activity of myoglobin in organic solvents. The outcomes of this research will be applied to re-engineered myoglobin variants.

In this manuscript, we discuss the ability of myoglobin to catalyze the conversion of *o*-phenylenediamine to 2,3-diaminophenazine in multiple alcoholic solvents (methanol, ethanol, 1-propanol,

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2-propanol, 1-butanol, 1-pentanol). The protein is prepared for these experiments by lyophilizing it from water in the presence of KCl. The protein structure was studied with UV–Vis and circular dichroism spectroscopies. The *o*-phenylenediamine conversion was measured and analyzed using UV–Vis spectroscopy. We make further suggestions on how to improve the catalysis of myoglobin, and myoglobin variants in non-aqueous solvents.

2. Materials and methods

Equine skeletal myoglobin, *o*-phenylenediamine, sodium phosphate monobasic, and sodium phosphate dibasic were purchased from Sigma–Aldrich. Sodium citrate, citric acid, sodium acetate, acetic acid, 30% hydrogen peroxide, HPLC grade water, methanol, and 2-propanol were purchased from Fisher Scientific. 1-Propanol, 1-butanol, and 1-pentanol were purchased from Alfa Aesar. Tris was purchased from Amresco. Ethanol (200 proof) was purchased from Koptec.

The following buffers were prepared: 10 mM citrate (pH 3), 10 mM acetate (pH 5), 10 mM phosphate (pH 7), and 10 mM Tris (pH 9). 5 mg of myoglobin and 495 mg of KCl were dissolved in 20 mL of buffer and placed in a 50 mL, conical, polypropylene centrifuge tube. A corresponding sample containing only KCl in the buffered solution was also prepared as a control. These solutions were flash frozen in liquid nitrogen and lyophilized in a LabConco FreeZone freeze dry system.

Samples for analysis of protein structure were prepared by suspending or dissolving 15 mg of lyophilized protein/KCl mixture in a solvent. Before the protein mixture was added to the solvent, the powder was ground with a mortar and pestle to increase the available surface area of the composite particles. The suspension was added to a quartz cuvette (either 10 mm or 1 cm thick). Circular dichroism spectra were recorded on a Jasco 715 spectrometer. UV–Vis spectra were recorded on a Shimadzu UV-2550 spectrophotometer.

Kinetics measurements were performed using an Ocean Optics Jaz spectrometer with an Ocean Optics DH-2000 light source. The cuvette, containing a sample and a micro stir bar, were placed in a qpod 2e cuvette holder from Quantum Northwest. The sample temperature was set to 25 °C and the stir function of the holder was set to the maximum setting. For data collection, the integration time was set to 1 ms, 200 scans were averaged, and one measurement was recorded every 10 s. Several stock solutions were made for the kinetics measurements. 15 mg of the lyophilized protein mixture was suspended in the solvent of interest. 3 mg of *o*-phenylenediamine was dissolved in 3.0 mL solvent. 0.114 mL of 30% H_2O_2 was dispersed in 0.886 mL of solvent. When performing the measurement, 2.025 mL of the *o*-phenylenediamine and 0.75 mL of the H_2O_2 solutions were added to the cuvette. Data recording was initiated followed by the addition of 0.225 mL of protein dispersion to the cuvette. A corresponding set of measurements where 0.225 mL of KCl and buffer, dispersed in the solvent, were added to the cuvette in place of the protein. The kinetics data were processed by subtracting the time(0) trace (just before the protein was added) from all of the spectra. The spectra were then background corrected to adjust for fluctuations in lamp intensity over the course of the experiment. Finally, from each spectrum at some time, *t*, the corresponding spectrum from the non-protein measurement was subtracted to remove any absorbance from non-catalyzed *o*-phenylenediamine oxidation.

3. Results and discussion

Myoglobin was chosen for this work because it has been the focus of a number of experiments where its native heme has been replaced with some non-natural cofactor [11,13]. In preparation for working with these myoglobin variants in non-aqueous solvents, it is sensible to first study the native protein. While myoglobin functions as an oxygen storage protein, its peroxidase activity (Fig. 1) has been noted [26–30]. The oxidative chemistry of heme proteins, which includes peroxidase and monooxygenase catalysis, is a hallmark of these enzymes and represents an important class of reactions to study in organic solvents. Horseradish peroxidase and other peroxidase proteins are likely to have a much higher activity in non-aqueous environments (as they do in aqueous ones). However, as this work will be applied to re-engineered myoglobin enzymes, the catalytic activity of natural myoglobin remains the focus of this study.

The structure and function of myoglobin in organic solvents has been previously studied [32,33]. These experiments looked to determine the amount of solvent (in a water:solvent mixture) that would be required to weaken the myoglobin structure and diminish its activity. However, none of these studies follow the best practice-protocols for working with enzymes in organic solvents (preparing the enzyme by lyophilizing it from a buffered solution containing excipient molecules) [19]. As such, this work represents the first study of myoglobin activity and structure while using optimal experimental environments.

3.1. Myoglobin structure

One of the primary tasks for preparing active enzymes in organic solvents is maintaining and supporting protein structure in non-aqueous environments [19]. Because organic-phase function can be linked to protein structure, increased activity can often be correlated to increased structural stability. As these experiments are meant to inform future studies with re-engineered myoglobin variants, we posit that environments leading to increased peroxidase function in myoglobin will be the same that support activity in the variants.

When we initiated this project there were a number of excipients that we could have used. Typical excipients used include

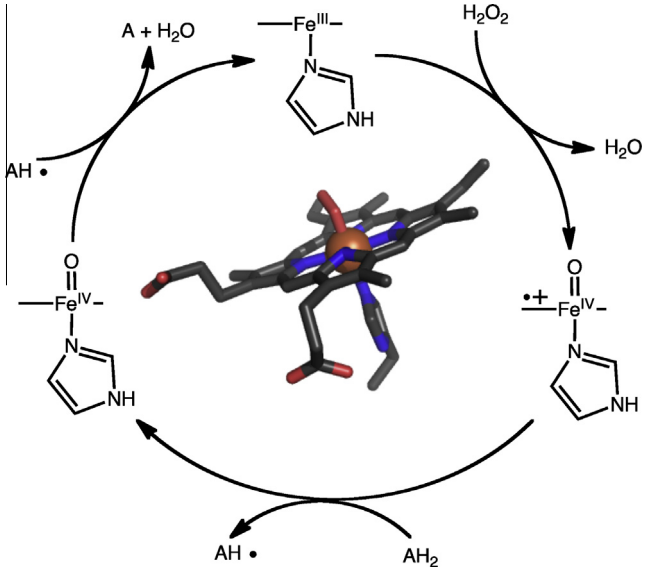

Fig. 1. Peroxidase catalytic cycle and myoglobin active site. In a typical peroxidase [31], the resting state (a histidine ligated Fe^{III}-porphyrin complex) is oxidized to a ferryl complex with a radical porphyrin cation (known as compound **I**). Compound **I** oxidizes a substrate (AH_2) and is converted into a ferryl complex (known as compound **II**). Compound **II** performs a second oxidation step on the substrate, and the catalyst returns to the resting state. In myoglobin, this cycle is complicated by the fact that the protein's resting state is an Fe^{II}-dioxygen complex.