Phytoglobin as catalytic enzyme for production of ammonia from hydroxylamine and hydrazine

by

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Phytoglobins are hexa-coordinated hemoglobin found in plants, whose physiological role is ambiguous. Phytoglobins are known to catalyze the reduction of inorganic nitrogenous compounds such as hydroxylamine. This reduction reaction has been studied extensively by single turnover reactions. However, currently there is no information available on the multi-turnover reactions catalyzed by phytoglobins in presence of exogenous electron source. In this study we present the results of using of dithionite as an external source of electron for the reduction of hydroxylamine, which gives a maximal rate of $2.8 \text{s}^{-1}$. Furthermore, we demonstrate that the rate-limiting step in this case is the electron transfer from dithionite to the substrate bound phytoglobin. This completely contrasts with what was observed previously in the single turnover assay.

Hydroxylamine reduction is the only known 2-electron reduction process catalyzed by phytoglobin to date. In the current study we also present a novel 2-electron reduction of hydrazine to ammonia catalyzed by phytoglobin. Hydrazine is an intermediate produced during the reduction of nitrogen to ammonia by nitrogenase enzyme. Hence, this discovery opens avenues for engineering phytoglobins for catalyzing the production of ammonia from dinitrogen. Interestingly, this reaction is also limited by electron transfer rate of the dithionite to hydrazine bound phytoglobins. Therefore catalytic rate of ammonia production by phytoglobins can be improved by introducing a more robust reducing system. We believe this can be achieved by using physiological proteins responsible for
reduction of phytoglobins. Hence, this presents a need for characterization and optimization of the physiological proteins responsible for phytoglobin reduction.
CHAPTER 1

INTRODUCTION: PHYTOGLOBINS A VERSATILE INORGANIC NITROGEN REDUCTASE

The development of human civilization can be traced back to a few key revolutions. One such revolution is the “Neolithic Revolution” which is marked by the shift towards agriculture for food rather than hunting and gathering. Ever since this transition towards agriculture, there has been a constant demand to improve crop yields, especially in the wake of an ever increasing population. This constant drive to improve yields resulted in the green revolution towards the end of 1960s, which saw an increase in the use of fertilizers to enrich the soil with essential nutrients required for growth of plants. The increasing use of fertilizers has led to an increase in demand for ammonia, which is primarily produced via the Haber Bosch process.

The Haber Bosch process requires N\textsubscript{2} and H\textsubscript{2} as raw materials for the production of NH\textsubscript{3}. The N\textsubscript{2} is extracted from the air and the H\textsubscript{2} is obtained from the steam reforming process, which is carried out at high temperature and pressure. To get the maximum yield, the reaction must be carried out at approximately 200 bars and 400-500°C. This makes the overall process highly energy intensive. In fact, even with the current advances towards minimizing energy losses, about 400 kJ is required for every mole of ammonia produce. This equates to 1% of total energy produced in the world being used towards production of ammonia. Hence, with the increasing demand for ammonia production and the inefficiency associated with existing processes used to produce it, there is a need to develop a novel method for sustainable production of ammonia, which does not rely on fossil fuels.
We believe that this problem can be addressed biologically by using enzymes to catalyze the production $\text{NH}_3$. In nature, the enzyme nitrogenase fixes the nitrogen to ammonia. However, this enzyme-catalyzed reaction has some major drawbacks. Firstly, these reactions are not energetically efficient as it utilizes 8 moles of ATP to make 1 mole of $\text{NH}_3$. This is about the same energy requirement as the synthetic Haber Bosch process\textsuperscript{6}. Secondly, nitrogenases are complex enzymes whose reaction mechanisms have yet to completely elucidated\textsuperscript{7}, making industrial scale application of nitrogenase difficult. Even with these drawbacks, they are the only known enzymes that can catalyze this reaction. Hence, they have been used as a template to model various catalysts with the aim of producing ammonia from dinitrogen.

Besides direct fixation of dinitrogen, the interest in nitrogenase also extends to its ability reduce other inorganic compounds such as hydrazine ($\text{HZ}$)$^8$ and hydroxylamine ($\text{HA}$)$^9$. The reduction of HA (reaction 1) and HZ (reaction 2) is significant because they are both reduced to ammonia by a $2e^-$ reduction process (Reaction 1 and 2). Furthermore, HZ is also an intermediate molecule produced by nitrogenase during reduction of nitrogen to ammonia\textsuperscript{10}. Thus it follows that any enzyme that is able to catalyze the reduction of these molecules could potentially be developed for ammonia production. Interestingly enough, phytoglobins (phyto, plant: globin, heme contain protein, structurally similar to sperm whale hemoglobin)\textsuperscript{11} are also able to catalyze the reduction of HZ(Chapter 3) and HA to ammonia\textsuperscript{12,13}.

\begin{align*}
1) \quad \text{NH}_2\text{OH} + 2e^- + 2H^+ & \rightarrow \text{NH}_4^+ + \text{OH}^- \\
2) \quad \text{NH}_2\text{NH}_2 + 2e^- + 4H^+ & \rightarrow 2\text{NH}_4^+
\end{align*}

Reactions 1 & 2: Inorganic nitrogen reduction.
Phytoglobin is a plant heme protein that is expressed in response to hypoxia, nitrate, nitrite, and nitric oxide in cells\textsuperscript{14}. Its exact physiological function in the plant is not yet clear. Some believe it is a nitric oxide scavenger\textsuperscript{15,16}, while others suggest that it might function in hypoxic energy metabolism through a mechanism such as fermentative ammonification\textsuperscript{12,17}. Regardless of its physiological function, phytoglobin is distinguished by its ability to efficiently reduce inorganic nitrogen compounds such as nitrite and hydroxylamine to form ammonia.

The phytoglobin active site consists of a heme prosthetic group in which a single iron is coordinated by a (proximal) histidine on one side, and reversibly coordinated by another (distal) histidine at the ligand-binding site (Figure 1). Thus, phytoglobin exists in rapid equilibrium between a pentacoordinate state capable of reacting with exogenous ligands, and a hexacoordinate state that can rapidly transfer electrons to the ligand-bound active site\textsuperscript{18}. This gives phytoglobins the ability to catalyse multi-electron reduction reactions such as reduction of Hydroxylamine\textsuperscript{12,13} and Hydrazine (chapter 3) to NH\textsubscript{3}. The mechanism of reduction of the nitrogenase complex mirrors that of the phytoglobin’s mechanism. The complex consists of the catalytic domain which binds substrate and the reducing domain which can transfer electrons to the substrate in the active center\textsuperscript{6}. Hence, we can draw parallels between phytoglobins and nitrogenase based on their ability to catalyze a 2e- reduction process of HZ and HA.

This leads us to hypothesize that phytoglobins can be engineered to catalyze the reduction of nitrogen to ammonia. Furthermore, phytoglobins can be reduced by reductants such dithionite\textsuperscript{18} and ferredoxin reductase\textsuperscript{14} without a need for concomitant ATP hydrolysis, as is the case with nitrogenase reduction by Fe-reductase\textsuperscript{6}. Thus making
it more energetically favorable than nitrogenase. As a first step towards this goal, we characterized the reduction of HA and HZ catalyzed by phytoglobins. Chapter 2 of this thesis describes the work done to determine the effectiveness of using phytoglobin to reduce HA in a catalytic manner. Chapter 3 describes the novel hydrazine reduction reaction catalyzed by phtyoglobins.

**Hydroxylamine Reduction by Phytoglobins**

In previous work, the quantitative analysis of reactions between phytoglobin and hydroxylamine were based on observing the oxidation of deoxyferrous phytoglobin\textsuperscript{12,13}. This meant that reducing capacity was consumed in the reaction and the reaction stops after all the reducing equivalents were spent. The rate of single turnover thus determined had a maximal rate of 80s\textsuperscript{-1}. However, to be used as a catalyst for production of ammonia we need to quantify multi-turnover reduction catalyzed by phtyoglobins. Hence, it is necessary to develop a steady-state assay that directly monitors ammonium production. This was achieved (Chapter 2) by 1) the use of dithionite (DT, reducing agent) as an exogenous electron source to continuously reduce oxidized protein, and by 2) using o-phthalaldehyde (OPA), which specifically reacts with ammonia to give a fluorescent signal\textsuperscript{19}, to detect amount of ammonia formed.

The k\textsubscript{cat} thus determined had a maximal value of 2.8s\textsuperscript{-1} (at the [DT] = 40mM), which is lower than what is observed in the single turnover assay of 80s\textsuperscript{-1}. Furthermore, the k\textsubscript{cat} (turnover) of this reaction was directly proportional to the concentration of DT used in the reaction and not dependent on the concentration of the protein. This is
indicative of the second electron required for the reduction of HA being obtained from DT and this being the rate-limiting step. So in theory we could improve the catalytic rate by using a better/alternate reducing system. This has a precedence rooted in the nitrogenase enzyme, which only catalyzes the reduction of nitrogen in the presence of Fe-reductase enzyme\textsuperscript{20}. Therefore, by utilizing the reduction systems employed by plants to reduce phytoglobins \textit{in-vivo}, we could achieve better reduction rates. Currently it is hypothesized that the phytoglobins are reduced by ferredoxins, which are coupled to NADPH oxidation via ferredoxin reductase\textsuperscript{21}. However, further study needs to be conducted in this area to confirm this hypothesis and determine if this would serve as better reductant than DT.

**Hydrazine Reduction by Phytoglobins**

The discovery of hydrazine as an intermediate during dinitrogen reduction has led to increases in catalyst efficiency with metallo-organic catalyzed reduction of hydrazine\textsuperscript{22,23}. However, not much has been studied in terms of using enzymes for the reduction of hydrazine, and thus far nitrogenase is the only enzyme that has been shown to catalyze reduction of hydrazine to ammonia\textsuperscript{8}. Hence, this discovery of hydrazine reduction by rice phytoglobin will open new avenues for exploration. We unequivocally demonstrate the ability of phytoglobins to reduce hydrazine to ammonia by using NMR and the OPA assay, which is used to detect ammonium. The rate of reduction was quantitatively determined by monitoring the oxidation of phytoglobin, which occurs concomitantly with the reduction of hydrazine. This gave a maximal rate of 0.169min\textsuperscript{-1}
for rice phytoglobin for a single turnover reaction. Furthermore, when the reaction was catalyzed in presence of DT it gave a kcat of 0.227 min\(^{-1}\). This suggests a common slow step in both single turnover and catalytic reactions, unlike in the case with HA.

The rates are comparable to turnover frequency (TOF) observed with metallo-organic catalysts, as determined by the turnover number of the catalyst in the reaction time. However phytoglobin has a 10-fold increase in turnover number (166) compared to 16-17 by metallo-organice catalysts\(^{22,23}\).These turnover numbers represent the minimum turnover observed in the lifetime of the reaction and could possibly be higher. However, they still pale in comparison to hydrazine kcat of 80 min\(^{-1}\) and TON > 10\(^8\) for reduction by nitrogenase\(^{8,24}\). This disparity could potentially be solved by enzyme reduction systems as proposed earlier, and by understanding the mechanism of this reaction.

NMR and OPA assays were performed to elucidate the mechanism of the reaction and determine the identity of the products formed. The product was confirmed to be \(NH_4^+\) as expected, based on NMR peak of 20 ppm and by formation of fluorescent isoindole molecule. However, the stoichiometry of the reaction was not as straightforward as described in reaction 2. Only 13\% of hydrazine consumed in the reaction was converted to ammonia, as determined by peak area in NMR. This disproportion in amount of HZ consumed to amount produced has been noted in other systems as well\(^{23}\). This disproportion reaction (reaction 3) is typically associated with reduction of catalyst when external reductant is not provided. Furthermore, this should still result in higher percent conversion of hydrazine to ammonia. This indicates that we need to further investigation to completely elucidate the mechanism of this reaction.
3) $3N_2H_4 \rightarrow 4NH_3 + N_2$

Reaction 3: Disproportionation reaction of hydrazine.
Figures

Figure 1. The molecular structure of phytoglobin in the pentacoordinate (green) and hexacoordinate (blue) states. The distal histidine binds reversibly to the heme iron while the proximal histidine remains coordinated. When the distal histidine is dissociated, the pentacoordinate protein can bind exogenous ligands such as hydrazine and hydroxylamine. (Structures from PDB codes 1d8u and 2oif.)

References

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CHAPTER 2

STEADY-STATE KINETICS OF PHYTOGLOBIN-CATALYZED HYDROXYLAMINE REDUCTION TO AMMONIUM

Introduction

Oxygen transport hemoglobins (Hbs) such as red blood cell Hb, myoglobin, and leghemoglobin have evolved to bind oxygen reversibly by avoiding chemical reactions such as superoxide formation and concomitant heme oxidation. Many Hbs including neuroglobin and cytoglobin in animals, and the plant phytoglobins do not transport oxygen, and exhibit interesting chemistry that could help to reveal their specific functions in signaling, detoxification, or energy metabolism under oxygen-limited conditions. Furthermore, the simplicity of the Hb fold with its clear ligand binding site presents an ideal platform for rational design of protein-based catalysts. However, our understanding of catalysis by Hbs is limited by a dearth of studies reporting reactions with multiple turnover; rather, most are confined to simple binding or single turnover events.

Reactions between ferrous ($\text{Fe}^{2+}$) Hbs and nitric oxide, nitrite, and hydroxylamine (HA) are important in anaerobic biology. The reaction with nitrite yields ferric ($\text{Fe}^{3+}$) Hb and nitric oxide, which rapidly binds unreacted ferrous Hb and inhibits subsequent reactions. For this reason, examinations of nitrite reduction by Hbs have been limited to single turnover reactions and do not allow investigations of mechanisms of catalysis. Ammonium, the product of hydroxylamine reduction, does not inhibit further reaction and thus can be produced catalytically by Hb in the presence of a reductant, although previous studies have been limited to pre steady-state, single-turnover experiments.
The plant Hb "Phytoglobin" (Phyt) is much faster than other Hbs at the single-turnover reduction of HA. The reaction requires two electrons, and previous work has shown that these electrons can originate solely from ferrous Phyt$^{2+}$ molecules. In the single turnover reaction two Phyt$^{2+}$ molecules are oxidized to form one ammonium, and the reaction rate is limited by HA binding (Reactions 1 and 2).

1) Phyt$^{2+}$ + HA $\rightleftharpoons$ Phyt$^{2+}$-HA  
   $K_{HA}$

2) Phyt$^{2+}$-HA + Phyt$^{2+}$ $\rightarrow$ 2Phyt$^{3+}$ + NH$_4^+$  
   $k_{ET}$

Phyt is particularly good at this reaction due to its higher affinity for hydroxylamine and rapid intermolecular electron exchange rate compared to other Hbs. A molecular attribute of Phyt implicated in these features is facile intramolecular reversible "hexacoordination" of the heme iron by a histidine side chain located near the ligand binding site. It has been proposed that weak hexacoordination might be necessary for allowing HA binding, and facilitating reduction of the heme iron. This hypothesis is supported in single turnover experiments, but has not been tested during steady state catalysis.

An exogenous source of electrons such as the reductant dithionite (DT, S$_2$O$_4^{2-}$, which breaks down to SO$_2^-$) can drive Phyt-catalyzed HA reduction, which could proceed by a few different mechanisms.

3) Phyt$^{3+}$ + SO$_2^-$ $\rightarrow$ Phyt$^{2+}$  
   $k_{red}$

4) Phyt$^{2+}$-HA + SO$_2^-$ $\rightarrow$ Phyt$^{3+}$ + NH$_4^+$  
   $k_{ET}$
1) Reactions 1 and 2 could be followed by reduction of Phyt$^{3+}$ to re-form Phyt$^{2+}$ (Rxn3),
2) DT could directly reduce Phyt$^{2+}$-HA (reaction 4), or both of these reactions could occur.

Multiple turnover of HA catalyzed by Phyt has been reported in the presence of DT but the reaction mechanism has not been characterized in detail$^{22}$. Nor has it been determined whether Phyt is distinguished by greater catalytic activity than other Hb, or whether if hexacoordination is important during catalysis.

The present work characterizes steady-state Phyt catalysis of ammonium production from HA and DT. Reaction velocities were monitored directly as ammonium formation using catalytic (nM) concentrations of Phyt. $K_m$ and $k_{cat}$ values were measured for rice Phyt, myoglobin, and a rice Phyt mutant protein in which the hexacoordinating histidine has been replaced by leucine (Phyt:H63L). Surprisingly, our results counter those from single turnover reactions$^{12}$; all of the Hbs have similar $k_{cat}$ values reflecting DT reduction of the HA-bound intermediate, and $K_m$ for Phyt is only two-fold lower than for myoglobin and Phyt:H63L. These results are a rare example of steady state catalysis by Hbs that reveals the importance of such reactions for drawing conclusions about hemoglobin physiological functions and the rational design of proteins.

**Materials and Method**

**Solution preparations for enzyme assay:** Rice wild type and H63L phytoglobin were expressed using a heterologous expression system as done previously$^{11}$. In short, the cDNA of the proteins were cloned into pet28 plasmid and expressed in BL21(DE3) cells. The cells were grown in terrific broth at 37°C with constant shaking at
200rpm for 24hrs. The cells were harvested, lysed by sonication and the protein was purified from the soluble fraction by His-Tag affinity purification. Anaerobic buffers (100mM Phosphate buffer) were prepared by boiling the buffers for 30min, followed by purging with nitrogen until the buffers cooled down to room temperature. Following which, the buffers were allowed to equilibrate in the anaerobic chamber O/N (95% argon, 5% Hydrogen COY Laboratory). The dithionite solution was prepared fresh before use in the hood and quantified by using an extinction coefficient of 8mM$^{-1}$cm$^{-1}$ at 314 nM$^{15}$. 1mM CO solution was prepared by purging CO gas through the 10mM Phosphate buffer for at least 30min.

**Solution preparations for ammonia Assay:** The O-phthalaldehyde (OPA) reagent was obtained from Sigma Aldrich. The assay was adapted based on OPA based ammonia quantification technique as reported previously$^{16}$. The ammonia detection reagent was freshly prepared just before use from a stock solution of 70mM OPA and 70mM Sodium Sulfite. The OPA stock solution of 70mM was prepared in HPLC grade methanol and 70mM sodium sulfite stock solution was prepared in 100mM pH 9.5 sodium borate buffer. The final composition of the ammonia detection reagent is 4mM OPA and 4mM sulfite in 100mM Borate buffer.

**Hydroxylamine Time course Assay:** The reduction of hydroxylamine was initiated by addition of dithionite solution to a premixed solution of phytoglobin and hydroxylamine. The reaction was stopped at different times (2, 5, 7.5, 10, 15 min) by mixing a 50uL aliquot of the reaction mixture to an equal volume of 1mM CO solution.
The NH$_4$CL (0 - 300uM) standards were prepared fresh for each assay. The concentration of ammonia in the reaction and standards were determined by reacting 40ul of each to 260uL of the ammonia detection reagent. This mixture was incubated for 2hrs at room temp and the fluorescence was recorded in TECAN Safire with an excitation wavelength of 360nM and an emission wavelength of 423nM. The fluorescence intensity of the standards was used to generate a standard curve. The concentration of ammonia in the reaction mixture was determined from the standard curve. The ammonia concentrations thus obtained were used to determine the initial velocities.

**Phytoglobin HA bound spectra:** A solution of ~9uM phytoglobin and hydroxylamine was prepared in the anaerobic chamber. The reaction was initiated by the addition of dithionite (10mM final concentration) to the above solution. This reaction was monitored via absorbance spectra collected at fixed interval (every 30sec for 2min, every 1min after that) for 20 min via an Agilent Cary 60 spectrophotometer.

**Results**

**Kinetic consideration of using dithionite as a reducing agent.** DT (S$_2$O$_4^{2-}$) dissociates in solution to form the radical anion $SO_2^{−}$ as described in the following Reaction:
Both $S_2O_4^{2-}$ and $SO_2^-$ could act as reducing agents in theory, but in practice the radical anion is the only species that reduces rice phytoglobin (Reaction 3)\textsuperscript{15}, and we hypothesize that it also acts as the reducing agent for Phyt$^{2+}$-HA (Reaction 4). A concern with using DT as the reducing agent is that the availability of $SO_2^-$ might be the rate-limiting step under certain conditions. DT dissociation to form $SO_2^-$ has equilibrium ($K_d$) and rate ($k_d$) constants of $10^{-9}$ M and 3.4 s\textsuperscript{-1}, respectively\textsuperscript{13}. Therefore $[SO_2^-]$ can be calculated from Equation 1.

$$[SO_2^-] = ([S_2O_4^{2-}] \cdot K_d)^{1/2}$$

20mM DT (a typical concentration in our reactions) produces 4.47μM of $SO_2^-$, but consumption of $SO_2^-$ beyond this concentration by Reactions 3 and 4 requires Reaction 5. The rate of change of $[SO_2^-]$ produced by these reactions is presented in Equation 2.

$$\frac{d[SO_2^-]}{dt} = 2k_d[S_2O_4^{2-}] - 2k_a[SO_2^-]^2 - k_{ET}[Phyt^{2+}:HA][SO_2^-] - k_r[Phyt^{3+}][SO_2^-]$$

With a simplifying assumption, we can estimate the conditions under which $SO_2^-$ production might become limiting. We know from previous studies that the reduction rate ($k_{red}$) of Phyt is 1.4 x 10$^8$ M$^{-1}$s$^{-1}$\textsuperscript{15}, and since $k_{ET}$ (reaction 4) is a electron transfer similar to $k_r$, we will assume for now that $k_{ET}$ has a similar value. This, along with the definition $\text{Phyttot} = \text{Phyt}^{2+}:\text{HA} + \text{Phyt}^{3+}$, produces Equation 3 from Equation 2.

$$\frac{d[SO_2^-]}{dt} = 2k_d[S_2O_4^{2-}] - [SO_2^-](2k_a[SO_2^-] - k_{ET}[Phyt\text{tot}])$$
Because $k_a \gg k_{ET}$ (by at least factor of 10), and when using a maximum [Phyt] of 100 nM, the relative consumption of $SO_2^-$ by Phyt will be minimal. Finally, under our experimental conditions $DT \gg [SO_2^-] \gg [Phyto_{total}]$, meaning that the velocity of $SO_2^-$ production will be $\sim = 2k_d[S_2O_4^{2-}]$. At 20 mM [DT], $SO_2^-$ is available at 124 mM/s, whereas the maximum rate of ammonium production observed in the present experiments is 0.16 µM/s. Thus $SO_2^-$ production from DT is not a rate limiting consideration under these conditions.

A steady state assay for HA reduction to ammonium. Previous quantitative investigations of HA reduction by Hbs have monitored Hb oxidation using visible absorbance spectroscopy. Ammonium was determined to be the product of the reaction$^{10}$, and it has been shown by $^{15}$N-NMR that the reaction is catalytic in the presence of DT$^6$. Quantification of catalysis requires accurate measurement of ammonium in µM concentrations. $^{15}$N-NMR is ideal for specificity but lacks the sensitivity necessary for these reaction velocities, so a chemical detection method using O-phthalaldehyde (OPA)$^{16,17}$ was tailored for this purpose (Figure 1A). One drawback to chemical detection of ammonium is cross-reaction with other nitrogenous compounds (such as HA), protein amino groups, and a potential for reaction with DT. The experiments in Figure 1 were designed to demonstrate the usefulness of the OPA assay for ammonium detection in the presence of HA, DT, and nM concentrations of protein.

Figure 1B is a standard curve for ammonium detection carried out in the presence of 2.5mM HA, demonstrating the specificity for ammonium. The conditions also include 50nM protein and 20mM DT to ensure that they present little cross-reactivity that
interferes with ammonium detection. The standard curve is linear up to 300µM ammonium, thus establishing the product concentration range available for our initial velocities. Figure 1C is an example of OPA fluorescence measurements associated with HA reduction by 50nM Phyt at increasing HA concentrations. HA results in an offset of fluorescence in OPA reaction, but does not influence the slopes of the curves associated with ammonium production. Figure 1D presents these data normalized to the change in ammonium concentration to demonstrate the quality of the velocity time courses.

**Kinetics of ammonium production by Phyt.** Initial velocities for ammonium production were collected as a function of HA concentration at three different Phyt concentrations, with [DT] fixed at 20 mM. Over this range of [HA], an asymptote was observed, and the curves were fit to the Michaelis-Menten model for enzyme catalysis. The observed $V_{\text{max}}$ value increased linearly with Phyt concentration (Figure 2B), at each point providing a $k_{\text{cat}}$ value of $1.96 \pm 0.15$ s$^{-1}$, and an average $K_m$ value of $318.2 \pm 63.6$ µM. The kinetic parameters of the enzymatic reaction are listed in Table 1.

The absorbance spectra accompanying single turnover reduction of HA by Phyt (Reactions 1 and 2) proceeds without formation of a measurable Phyt-HA intermediate leading to the conclusion that HA binding (Reaction 1) is the rate limiting step rather than reduction (Reaction 2). Figure 2C are absorbance spectra of single turnover HA reduction by Phyt demonstrating isosbestic points between the spectra of Phyt$^{2+}$ and Phyt$^{3+}$, showing that no intermediate is evident. On the contrary, catalytic reduction in the presence of DT proceeds via a distinct spectral intermediate. In the presence of DT (Figure 2D), a new spectrum is present that is different from those of the Phyt$^{2+}$ and Phyt$^{3+}$ proteins. The intermediate spectrum has a peak at 553 nm, lacks the 525 nm band
of Phyt$^{2+}$, and does not share isosbestic points with the spectra of Phyt$^{2+}$ and Phyt$^{3+}$. This spectrum persists during catalysis until HA is consumed, at which point the spectrum shifts to that of Phyt$^{2+}$.

The data in Figure 2 demonstrate that steady state catalysis of HA reduction by Phyt proceeds through the formation of an intermediate species. This intermediate could be Phyt-HA, and the $k_{\text{cat}}$ value observed at 20 mM DT could be limited by the rate of reduction by $SO_2^\cdot$. To determine if the rate of reduction is limiting catalysis, velocities were measured at fixed 50 nM [Phyt], and DT was varied from 10 to 40 mM (the maximum range possible for our assay conditions) (Figure 3A). $k_{\text{cat}}$ is linearly dependent on $[SO_2^\cdot]$ indicating that the anion radical is the reductant and that the reaction is limited by this reaction. The dependence of $k_{\text{cat}}$ on $[SO_2^\cdot]$ suggests a bimolecular rate constant for reduction of $4.2 \times 10^5 \text{ M}^{-1} \text{s}^{-1}$ (Figure 3B).

**Specificity of Phyt for catalytic Hydroxylamine reduction.** Interest in HA reduction by Phyt is motivated by its specificity for the single turnover reaction compared to other Hbs$^6$. One rationale for the current investigation is to determine if this specificity is also present during steady state catalysis. Figure 3C compares reaction rates for Phyt to those for horse heart myoglobin (Mb), which has been shown previously to be slow in the single turnover reaction$^6$. The $K_{\text{m}}$ value for the Mb-catalyzed reaction (770 µM) is ~2-fold higher than that of Phyt, but the Mb $k_{\text{cat}}$ value (1.8 s$^{-1}$) is nearly the same.

Another protein that exhibits greatly attenuated single turnover activity is the Phyt mutant protein H73L (Phyt:H73L), which lacks the histidine side chain that coordinates the ligand binding site in the wild type protein$^{11}$. The single turnover rate for Phyt:H73L
is nearly 1,000-fold slower than Phyt. Surprisingly, its $K_m$ and $k_{cat}$ values are similar to Mb. Its $K_m$ (740 µM) is ~ 2 fold larger than wild type Phyt, and its $k_{cat}$ (2.2 s$^{-1}$) is about the same as Phyt.

**The Rate limit to catalysis is reduction of the Phty-HA intermediate.** There are two routes that electrons from $SO_2^-$ could take to reduce HA. In one, reduction comes directly from Phyt$^{2+}$ (Reaction 2), and then the resulting Phyt$^{3+}$ is reduced by $SO_2^-$ (Reaction 3). In the second, the Phyt-HA complex is directly reduced by $SO_2^-$ (Reaction 4). These two reaction scenarios were simulated numerically using in Biokine Dynafit in an effort to distinguish between the two using our observed kinetic data.

The first scenario was simulated using reactions 1, 2, 3 and 5. Reactions 3 (re-reduction of Phyt$^{3+}$) and 5 (formation of $SO_2^-$) are not rate limiting, so the shape of the curve is dictated by Reactions 1 (HA binding) and 2 (electron transfer between Phyt$^{2+}$ and Phyt$^{2+}$-HA. In this case the rate limit is a bimolecular reaction between Phyt$^{2+}$ and Phyt$^{2+}$-HA molecules, and thus $k_{cat}$ would depend on [Phyt]. Figure 4A shows a simulation using $K_{HA}$, $K_{red}$ values of 133uM and 1.4x10$^8$M$^{-1}$s$^{-1}$ carried with a $k_{ET}$ value of 7x10$^7$M$^{-1}$s$^{-1}$, determined empirically to best fit the data at 100 nM [Phyt]. At lower [Phyt] the simulated rates decrease, but the experimental data do not. Thus the observed rates of catalysis are not consistent with reduction via protein intermolecular electron transfer.

Figure 4B shows a simulation of the second scenario, which encompasses Reactions 1, 3, 4, and 5. Again reactions 3 (re-reduction of Phyt$^{3+}$) and 5 (formation of $SO_2^-$) are not rate limiting, so the resulting curves are a function of Reaction 1 (HA binding) and reaction 4 (Phyt$^{2+}$-HA reduction by $SO_2^-$). In this simulation $K_{ET}$ is fixed at
the observed bimolecular rate constant for $SO_2^-$ reduction of Phyt (4.2x10^5 M^{-1}s^{-1} from Figure 3B), and the simulated curve fit well to the experimentally observed rates of reduction by varying only the equilibrium constant for HA binding. The fitted value for $K_{HA}$ is 133 µM. In this scenario the simulated curves match the experimental data at all [Phyt], and support hypothesized reduction of the Phyt-HA complex directly by $SO_2^-$. In the final simulation (Figure 4C), rates of reduction by Mb and Phyt:H73L were simulated using the same parameters as scenario two above, but fitting for different values of $K_{HA}$. Suitable fits are achieved in both cases with $K_{HA}$ of 800 µM for Mb and 428 µM for Phyt:H73L. Therefore, for each of the proteins investigated here, the rate-limiting step is reduction of the Hb-HA complex directly by $SO_2^-$. Furthermore, this value is the same for each, leading to similar steady state kinetic constants for ammonium formation from HA.

Discussion

**OPA-sulfite assay:** In this work we have shown a novel and relatively simple use of OPA-sulfite assay to do a high-throughput kinetic analysis of hydroxylamine reduction. The OPA-assay has been classically used with HPLC techniques to quantify the amount of ammonia. This is because the OPA assays were carried with mercapta-ethanol instead of sulfite, which formed a short-lived fluorophore\textsuperscript{18}. Furthermore, the OPA sulfite assay is robust enough to not be perturbed by presence of DT and HA. This assay will significantly aid in understanding kinetics that involve the formation of ammonia by phthyoglobins.
**Phytoglobins as a catalytic enzyme:** We demonstrate the ability of hemoglobin to be used as a catalytic enzyme with significant turnover. At the 40mM [DT], 600uM of ammonia was formed with a [phyto] of 50nM, which yields a turnover of $1.2 \times 10^5$. This to our knowledge has never been directly demonstrated for phytoglobins. Previous work on phytoglobins has been done in a single turnover fashion and their catalytic capabilities in in-vivo have been extrapolated based on it. This clearly doesn’t translate, especially in case of NO-dioxygenase reaction catalyzed by phytoglobins, where the reduction rate of phytoglobins dictates the overall rate in the cell. Similarly, phytoglobins have also been shown to catalyze multi electron HA reduction in a single turnover manner, but no study on the catalytic capability of the protein has been conducted. So, in this paper we set out to determine the catalytic potential of phytoglobin with DT as an exogenous electron source of the reaction. We demonstrate that DT can be used to drive the reaction in catalytic manner, albeit at slower rate of $\sim 2s^{-1}$ compared to the single turnover rate of $80s^{-1}$.

**Dithionite as an electron source for hydroxylamine reaction:** DT in solution exists in a equilibrium as described by reaction 5. Both species can technically act as a reductant, but $SO_2^-$ is primarily responsible for reduction of phytoglobins. Furthermore, we determine that radical anions are also the reductant responsible for donating electrons to HA bound phytoglobins (reaction 4). This is determined by the fact that kcat of the reaction was linearly dependent on the radical anion concentration figure 3b. This
bimolecular rate of reduction was determined to be $4.2 \times 10^5 M^{-1}s^{-1}$, which when used in numerical simulation was able to predict the observed rates of reduction (figure 4b).

Even though bimolecular rate is high, the observed rate is limited by the equilibrium concentration of $SO_2^-$ radical anion. The $[SO_2^-]$ is 6.3uM even at the highest concentration of 40mM DT used in the reaction. Higher concentration of DT resulted in reduced sensitivity of the assay and therefore could not be used in this study. The $SO_2^-$ concentration of 6.3uM yields an observed rate of 2.6s$^{-1}$, which is at par with the observed rate of 2.8s$^{-1}$ observed experimentally.

**Potential implication of catalytic activity of phytoglobins:** Single turnover assay performed previously$^6,12$ are good starting point for understanding HA reduction. These studies indicated that the rate-limiting step in the reaction was the rate of HA binding, which is in complete contrast to what is observed here. This exemplifies the argument made previously for the NO-dioxygenase activity of phytoglobin$^5$, wherein reduction of phytoglobin represent the true rate of reaction in-vivo and not the single turnover rate. Similarly in this case the kinetic limit of this reaction is set by overall concentration of reductant available at equilibrium. So in theory it is possible to achieve higher rates by increasing the concentration of $SO_2^-$. However, since the radical anion concentration is proportional to square of root DT concentration (equation 1), increasing the DT concentration only nominally increases the radical anion concentration.

In plants, it has been hypothesized that ferrodoxin and ferrodoxin reductase act in tandem to reduce phytoglobins$^{20,21}$. These proteins have not been previously characterized. A similar phenomenon is also observed in nitrogenase, which needs a
complementary Fe based reductase to show any discernable activity. This might well be the case for the reduction catalyzed by phytoglobin where in a coupled reductase is required to improve its activity.
Figures

A. Detection of ammonia by OPA and sulfite. The reaction between OPA and ammonia results in formation of an isoindole molecule. (Ex: 360nM, Em: 423nM).

B. Normalized standard curve showing linearity up to 300uM of ammonia. The standard curve is made in presence of 2.5mM HA, 20mMDT and 50nM phytoglobin.

C. Time course of increase in fluorescence with increasing NH4 concentration at varying HA concentration. The lines represent linear fit to the progress curve.

D. Normalized time course represented with amount of ammonia formed on the Y-axis.

Figure 1: Steady state OPA-Assay. A) Detection of ammonia by OPA and sulfite. The reaction between OPA and ammonia results in formation of an isoindole molecule. (Ex: 360nM, Em: 423nM). B) Normalized standard curve showing linearity up to 300uM of ammonia. The standard curve is made in presence of 2.5mM HA, 20mMDT and 50nM phytoglobin. C) Time course of increase in fluorescence with increasing NH4 concentration at varying HA concentration. The lines represent linear fit to the progress curve. D) Normalized time course represented with amount of ammonia formed on the Y-axis.
Figure 2: Kinetics of ammonia production by phytoglobin. A) The Michaelis-menten plot of catalysis with 100, 75 and 50nM phytoglobin concentration. The solid lines represent the lines fitted to a Michaelis-Menten equation B) Enzyme dependence plot, the Vmax increases with enzyme concentration as expected. C) Time course absorbance spectra of a single turnover assay, obtained previously\textsuperscript{12}. The spectra have a clear isosbestic points, which indicates no intermediate formation. D) Absorbance spectra of intermediate formed during catalysis in presence of DT. The spectra of oxidized and reduced phytoglobin are included for reference.
Figure 3: $SO_2^-$ reduction of phyto$^{2+}$:HA complex. A) Plot of $k_{cat}$ of the reaction at 10, 20 and 40mM of [DT]. B) Graph of $k_{cat}$ dependence on $[SO_2^-]$, the equilibrium concentration of $[SO_2^-]$ at each DT concentration was calculated using equation 1. C) The Michaelis-menten plot of catalysis of myoglobin (MB) and Rice phytooglobin H73L mutant. The solid lines in A) & C) represent the lines fitted to a Michaelis-menten equation.
Figure 4: Dynafit Simulation Plot, the lines represent the simulation data generated on Dynafit and markers represent the experimental data. A) Dynafit simulation based on reactions 1, 2, 3 and 5. The kcat of reaction simulation varies with [phytoglobin], doesn’t correlate with experimental data. B) Dynafit simulation based on reaction 1, 3, 4 and 5.
The kcat of simulation is independent of [phytoglobin] and reasonably predicts the observed data. C) Dynafit simulation of Myoglobin and Rice phytoglobin H73L mutant based on reaction 1, 3, 4 and 5. The simulated rate correlate to the experimental data. The \( k_{ET} \) values used for simulation are represented in the legend. The HA binding constant were determined empirically and differed for each protein.

**Tables**

Table 1: Kinetic parameters of rice phytoglobin. The values of Km and vmax were determined via curve fitting to Michealis-Menten equation in Igor Pro. The fitted curve and experimental rates are shown in figure 2

<table>
<thead>
<tr>
<th>Protein</th>
<th>Enzyme Conc</th>
<th>Vmax (uM/sec)</th>
<th>Km (uM)</th>
<th>kcat (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice HB</td>
<td>100nM</td>
<td>0.203 ± 0.019</td>
<td>312.9 ± 4.3</td>
<td>2.0</td>
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<tr>
<td>Rice HB</td>
<td>75 nm</td>
<td>0.145 ± 0.006</td>
<td>384.3 ± 27.2</td>
<td>1.9</td>
</tr>
<tr>
<td>Rice HB</td>
<td>50 nm</td>
<td>0.087 ± 0.002</td>
<td>257.4 ± 4.3</td>
<td>1.7</td>
</tr>
</tbody>
</table>

Table 2: Kinetic parameters of penta-coordinated protein. The values of Km and vmax were determined via curve fitting to Michealis-Menten equation in Igor Pro.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Enzyme Conc</th>
<th>Vmax (uM/sec)</th>
<th>Km (uM)</th>
<th>kcat (s(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rice H63L</td>
<td>100nM</td>
<td>0.223 ± 0.00</td>
<td>736.9 ± 78.47</td>
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</tr>
<tr>
<td>MB</td>
<td>100nM</td>
<td>0.181 ± 0.01</td>
<td>771.8 ± 23.60</td>
<td>1.8</td>
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</tbody>
</table>
References


(20) Wang, X.; Hargrove, M. S.


CHAPTER 3
REDUCTION OF HYDRAZINE TO AMMONIA CATALYZED BY
PHYTOGLOBINS

Introduction

Pythoglobins are a class of Hexa-coordinated hemoglobins whose function in plants have not yet been clearly defined. These proteins are over expressed in plants during hypoxic conditions and help to alleviate damage caused by reactive oxygen species\(^1\). However, their exact roles and functions have yet to be elucidated. *In-vitro* these proteins are able to catalyze reduction of inorganic nitrogen. These reactions include 1) Hydroxylamine reduction to ammonia\(^2\)\(^{-4}\) and 2) Nitrite reduction to Nitric Oxide\(^5\)\(^,6\). In this work we demonstrate the capability of pythoglobins to also catalyze the reduction of Hydrazine (NH\(_2\)-NH\(_2\)) to ammonia.

The reduction of HZ to ammonium is a unique reaction for two reasons. 1) It contains two nitrogen atoms rather than one, and 2) the bond between the two nitrogens must be broken. Hydrazine is not a common molecule in biological systems. In fact, its only roles are in the inter-conversion of dinitrogen and ammonium. It is an unreleased intermediate in nitrogen fixation and a metabolite in anaerobic ammonium oxidation, which converts ammonium into dinitrogen\(^7\). Furthermore, nitrogenase is the only known enzyme that will reduce hydrazine.\(^8\). The reduction of hydrazine is therefore a logical stepping-stone toward the design of new catalysts for dinitrogen activation and reduction.
The current synthetic method for making ammonium is the Haber-Bosch process, which relies intensively on fossil fuels for its energy and hydrogen requirements\(^9\). This has lead to a keen interest in developing better catalyst for the reduction of \(\text{N}_2\text{H}_4\) to \(\text{NH}_3\)^{10,11}. This reliance on fossil fuels is unsustainable, so it is necessary to develop alternative methods to produce ammonium from dinitrogen. However, most development efforts have been geared towards metallo-organic compounds, usually with Fe reaction centers. The discovery in the present study could potentially lead to engineered hemoglobins that can catalyze the reduction of nitrogen to ammonia. Hence in this work, we have characterized the reduction of hydrazine by pythoglobins as a first step towards this goal.

The reduction of the Hydrazine is a 2e- process similar to Hydroxylamine\(^2,12\). Therefore the reduction of hydrazine could be described as in Scheme 1 based on the reduction mechanism of hydroxylamine.

\[
\text{N}_2\text{H}_4 + 2\text{Phyto}^{2+} + 2\text{H}^+ \rightarrow 2\text{NH}_4^+ + 2\text{Phyto}^{3+}
\]

**Scheme 1:** Reduction of Hydrazine by deoxyferrous Hb.

The hydrazine reduction rates of Leghemoglobin (LBA), Neuroglobin (NGB), Rice Phyt and Tom Phyt were compared and phytoglobins were shown have the highest rates. They have maximal rates of 0.227min\(^{-1}\), which is on-par with turnover frequencies observed in metallo-organic catalyst\(^13\). Furthermore, stoichiometry studies using NMR and the OPA assay for ammonium reveal that the reaction mechanism is much more complex than that described in scheme 1. This could be indicative of a disproportionation reaction\(^11\) or a unspecific reaction of hydrazine with the amino acids of the protein. We are able to achieve catalytic turnover of hydrazine to ammonia in the presence of
dithionite, which gave a maximum turnover number of 166. This is higher than that observed by metallo-organic catalyst$^{11,13}$ by a factor of 10. This bolsters the hypothesis that phytoglobins are a viable catalyst for the production of ammonia.

Materials and Method

Preparations of reagents: All the proteins (phytoglobins, LBA, Ngb) were expressed and purified as outlined in chapter 2. Anaerobic buffers and solutions were made as described in chapter 2. N$^{15}$ hydrazine used for NMR was purchased from Cambridge isotopes. The concentration of dithionite (DT) was determined by using an extinction coefficient of 8mM$^{-1}$cm$^{-1}$ at 314nm$^{14}$.

Kinetic measurements of hemoglobin oxidation by hydrazine: Deoxyferrous hemoglobin were made via reduction with DT and running the protein on a buffer exchange column to remove excess DT. The protein (~10-15uM) thus obtained was sealed inside a cuvette with an airtight septa anaerobically. The reaction was initiated by introduction of hydrazine via a syringe outside the chamber. The absorbance was collected on Agilent Cary-60 UV–vis Spectrophotometer every 30 sec. The rate of oxidation was determined by the change of absorbance at a specific wavelength in the visible region (550 – 600 nM, unique for each protein) that showed the maximal change. The same procedure was followed, except the amount of hydrazine injected was modified to monitor the rate of oxidation at different concentration of hydrazine. The progress curves obtained were fit to exponential rate equation in Igor Pro. to determine the observed rate constants.
**Catalytic turnover of HZ.** The reduction of hydrazine by rice phytoglobin was carried out in the presence of DT (100uM). The progress of this reaction was monitored via the oxidation of DT by observing the change of absorbance at 314nm. The progress curve thus obtained was fit to a linear equation to determine the initial rate (M/min) of the reaction. These values were normalized by the protein concentration and used to determine the Michaelis Menten kcat (min⁻¹) and Km.

**NMR measurements:** The NMR measurements were taken in Burker 600 MHz NMR spectrometer at Iowa state central instrumentation facility. The N¹⁵ spectra were obtained via N¹⁵ direct detect using an inverse probe. N¹⁵ Glutamic acid (40ppm) at 10mM was added to the solution at the start the reaction. This was used as an internal standard for the NMR measurements. The areas of the respective peaks were used to determine the amount of HZ and ammonia. The NMR data was processed and quantified in MestReNova. NMR data was exported to Igor Pro. for plotting.

**O-phthalaldehyde (OPA) ammonia assay:** Catalytic assay at 5, 20 and 50mM hydrazine concentration were setup with 0, 100, 200, 300, 400, 500uM of dithionite and 3uM rice phytoglobin. The reaction was carried out in an anaerobic chamber at room temp for 48hrs, at which time all the DT in the reaction was consumed, as measured by absorbance at 314nm. The OPA assay for determination of ammonia formed was setup as mentioned in chapter 2. In brief, OPA detection reagents are prepared fresh by combining stock solution of OPA (70mM in HPLC grade methanol) and Sodium Sulfite (70mM in Borate Buffer) to get a final concentration of 4mM OPA and 4mM sulfite in 100mM.
Borate buffer pH 9.5. 20uL of the reaction mixture was added to 96 well plate followed by addition of 280uL of OPA detection reagent. The OPA reagent was incubated with the reaction mixture for 2hrs before detection of fluorescence intensity on the TECAN Safire instrument. The excitation wavelength of 360nm and an emission wavelength of 423nm were used to determine the amount of the fluorophore formed.

**Results**

**Specificity of hydrazine reduction:** The rates of hydrazine reduction by hemoglobin derived from different species were monitored via oxidation of the deoxyferrous hemoglobin. Figure 1 shows the time courses of reduction for different hemoglobins at 20mM hydrazine. The rates were determined by fitting the progress curves to exponential decays. (Table 1). The phytoglobin class of hemoglobins have the highest rates followed by Neuroglobin, and Myoglobin has the lowest rate. The coordination state of the globins has also been listed in Table 1. This shows a clear specificity for Hydrazine reduction by hexa-coordinated globin. Furthermore, activity of reduction by hexa-coordinated globins is proportional to the histidine off rate of the protein. These results are consistent with those observed for reduction of hydroxylamine by phytoglobins. Hence we can use reduction of hydroxylamine as a model for reduction of hydrazine by phytoglobins,

**Rice phytoglobin hydrazine reduction:** The above results indicate that the hexa-coordinated hemoglobins are able to catalyze the reduction of hydrazine better then penta-coordinated hemoglobins. However, they don't provide any information about the
kinetic limits of the reactions. Hence to determine the kinetic limit, reduction at different concentrations of HZ was measured for rice phytoglobin. Figure 2a shows the progress curves at different concentrations of hydrazine. The rates determined from these progress curves approach a saturation value of around 0.12 min\(^{-1}\) at high [HZ], which is lower than histidine off rate of 40 s\(^{-1}\) (Table 1). We know from our previous work\(^4\), that rates of reaction limited by binding would approach a rate limit equal to the histidine off rate i.e 40 s\(^{-1}\). Since that is not the case here, we can safely assume that binding is not the rate-limiting step. Furthermore, based on this assumption we can describe the relationship of the reduction rates and concentration of [HZ] by the following generalized equation 1.

\[
k_{\text{obs}} = \frac{k_{\text{prod}}[HZ]}{K_{HZ}+[HZ]}
\]

Fitting the observed concentration dependence of the reaction rate to Equation 1 yields a reduction rate \(k_{\text{prod}}\) with a maximum of 0.169 min\(^{-1}\) and binding constant \((K_{HZ})\) of 17.6 mM.

**Dithionite as the electron source for reduction:** In previous experiments, reactions were monitored via oxidation of hemoglobin. The oxidation of the protein causes the reaction to terminate as the protein loses its reducing equivalents. These reactions give a single turnover rate typical in the literature for reduction of inorganic nitrogen catalyzed by hemoglobins\(^2\text{-}^4,^6\). But for the effective use of hemoglobins as catalysts for production of ammonia, we need to determine the catalytic turnover rate. The reaction when catalyzed with excess of sodium dithionite (DT) occurs in a multiple turnover fashion. DT continuously reduces the oxidized hemoglobin back to
deoxyFerrous hemoglobin and also could provide the second electron required to catalyze reduction of HZ (Chapter 2). The reaction that occurs can be described as follows:

\[ DT \leftrightarrow 2 \cdot SO_2^- \]

\[ Phyto^{3+} + SO_2^- \rightarrow Phyto^{2+} \]

\[ Phyto^{2+} + HZ \rightarrow Phyto^{2+}:HZ \]

\[ Phyto^{2+}:HZ + Phyto^{2+} \rightarrow 2 \cdot Phyto^{3+} + 2 \cdot NH_4^+ \]

\[ Phyto^{2+}:HZ + SO_2^- \rightarrow Phyto^{3+} + 2 \cdot NH_4^+ \]

**Scheme 2.** Proposed mechanism of HZ reduction in presence of DT.

Since the consumption of DT happens concomitantly with the formation of HZ, we can monitor this reaction as a loss of the DT peak at 314nM. The initial rate \((k_{obs})\) of reaction was determined from the linear part of the progress curve at different concentrations of the HZ. The relation of initial rate to concentration of HZ can be described by equation 2

\[ k_{obs} = \frac{k_{cat} \cdot [HZ]}{K_m + [HZ]} \]

**Equation 2.** Michealis Menton equation.

The initial rate at various HZ concentrations is shown in figure 3, which when fit to equation 2 gives a \(k_{cat}\) of 0.227min\(^{-1}\) and \(K_m\) of 41mM.

**Determining the stoichiometry of the reaction.** The progress curves used to determine single turnover and multi-turnover rates were obtained by monitoring the oxidation state of the heme or DT. This is an indirect measure of the reaction and doesn’t give any information on the identity of the product. Hence, NMR and OPA based ammonia detection was used to determine the identity and amount of the product formed. Figure 4A, shows the formation of N\(^{15}\)-NH\(_4\) (20ppm) with the concomitant loss of N\(^{15}\)-
HZ (49ppm). However, when the amount of HZ loss is compared to amount of ammonia formed, we can only see 13% conversion of the HZ to ammonia (figure 4B). This would indicate a different stoichiometry of reduction from what is described in scheme 1.

To further understand the stoichiometry of this reaction, HZ reduction was setup with a range of DT concentration at Rice phytoglobin concentration of 3uM. The OPA assay was used to determine the amount of ammonia produced when all the DT was consumed (48hrs). The amount of the $NH_4^+$ formed at each concentration of DT is represented in the figure 5. The graph also indicates the amount of $NH_4^+$ that is expected if all the DT is consumed by the mechanism based on scheme 2. However, we only see 50% of the theoretically expected yield of ammonia at higher HZ concentration. This is consistent with the observed yield in NMR, wherein all the DT consumed is not utilized for reduction of hydrazine. The turnover number from reaction was determined to be 166 based on maximal the yield of 500uM $NH_4^+$ at Rice phytoglobin concentration of 3uM.

Discussion

Role of hexa-coordination in reduction of hydrazine: These results indicate that phytoglobins are able to catalyze the reduction of hydrazine to ammonia. Furthermore, phytoglobins are able to catalyze this reaction much faster than NGB and LBA at 20mM Hydrazine. The values in table 1 present a clear specificity for hydrazine reduction by hexa-coordinated globins with higher histidine off rate. This indicates that the histidine acts as a competitor of HZ binding to the active site. Similar effect has been observed with hydroxylamine reduction by phytoglobins, indicating that the reaction proceeds via binding of HZ to the heme center and not by an outer sphere mechanism.
Furthermore, the hexa-coordinated phytoglobins reactions are much faster than LBA a penta-coordinated hemoglobin. This indicates that phytoglobins with reversible hexa-coordination are more adept at this catalysis. The reversibility is a consequence of conformational flexibility of phytoglobins and presents itself as hexa-coordination since it puts the heme iron in the preferred low spin state. This flexibility perhaps offers phytoglobins an advantage for catalyzing the reduction of HZ in one of two ways. The histidine in the hexa-coordinate protein is able to position itself in the correct orientation for the catalyzing the reaction. The second being, this conformational flexibility allows for the protein to accept a wide range of ligands. In contrast, penta-coordinated hemoglobins like LBA are locked in a specific conformation, making them ideal for binding selectivity. However, this also reduces their versatility and these proteins show lower reactivity with hydroxylamine and hydrazine.

**The kinetic limit of hydrazine reduction by hemoglobin:** The hydrazine reaction at different concentrations of [HZ] was carried out to determine the kinetic limit of this reaction. The rate saturates at around 50mM HZ concentration, with a rate of 0.169min⁻¹. Ideally, if binding is the rate limiting step we expect to see a rate limit equal to the histidine off rate (k_{hist})¹²,²⁰. However, that is not the case here, which points to either a catalytic slow step or a slow electron transfer step. Furthermore, the reaction has a k_{cat} of 0.227min⁻¹ when setup in presence of DT showing a rate-limiting step of the same order as in the single turnover assay. This indicates both the reactions have a rate-limiting step of similar magnitude.
DT dissociates into $\text{SO}_2^-$, which acts as the reducing agent in the reaction. The dissociation occurs at rate 3.4 s$^{-1}$ and has equilibrium constant of $10^{39}$M$^{21}$. Thus, only 320nM of radical anion would be available for the reaction of hydrazine to ammonia under steady state conditions. If we assume the rate-limiting step (0.227min$^{-1}$) is the electron transfer from the radical anion to the HZ bound phytoglobin. We get an electron transfer rate of $1.2 \times 10^4$ M$^{-1}$s$^{-1}$, an order of magnitude lower than what is observed for electron transfer rate to HA bound phytoglobins (chapter 2) by the radical anion.

Hence, we hypothesize that in both single and multiple turnover assay, the rate of electron transfer to the HZ bound phytoglobin is the rate-limiting step. Further study is required to conclusively prove this hypothesis. Nevertheless, improving the rate of reduction is the key to improving the catalytic rate of phytoglobins. This holds true for reduction of both hydroxylamine (chapter 2) and hydrazine.

**Stoichiometry of the Hydrazine reduction:** NMR and OPA based assayed was used to determine the amount of ammonia formed by the reduction of Hydrazine. This was done to determine the stoichiometry and mechanism of the reaction. NMR was used to observe both the product formed and substrate consumed in reaction. So as to accurately determine the amount of hydrazine that was converted to ammonia (Figure 4). The amount of hydrazine consumed in the reaction was used to calculate the theoretical expected ammonia yield, based on scheme 1. However only 13% of the substrate consumed was obtained as product. This disproportion of hydrazine consumed to the amount of ammonia formed has been observed previously$^{11}$. However, the
disproportionation reaction (Scheme 3) only occurs in absence of an external source of reductant and therefore hydrazine acts as the reductant in these reactions. As a reductant HZ gets oxidized to dinitrogen and reduces the amount of hydrazine available for reduction. Furthermore, this disproportionate reaction would still convert 66% of consumed hydrazine to ammonia, which is not the case here.

\[ 3N_2H_4 \rightarrow 4NH_4^+ + N_2 \]

Scheme 3: Hydrazine disproportionation reaction.

We also determined the number of reducing equivalents required for the formation of 1 mole ammonia from hydrazine. This was achieved by measuring the amount of ammonia formed at different concentrations of DT using OPA assay. Each molecule of DT has two reducing equivalents. Hence based on scheme 2, each mole of DT would yield 4 moles of ammonium. However, this holds true only at lower concentrations of DT (figure 5). At highest concentration of DT tested (500uM) only 50% of reducing equivalents were utilized to make ~500uM of ammonia. This gives a turnover number of 166, which is higher than the turnover of 16-18 observed in metallo-organic catalyst\textsuperscript{11}. However, this pales in comparison to most enzymatic reactions which have >10\textsuperscript{8} turnovers.

This leads us to hypothesize that the protein is only capable of limited turnovers and gets inactivated in presence of hydrazine. This is not surprising considering hydrazine and its derivatives have been known to react with hemoglobins\textsuperscript{22}. Furthermore, the inactivation of organo-metallic catalyst in presence of hydrazine serves only to bolster this hypothesis\textsuperscript{11,13}. FT-ICR mass spectroscopy was used to determine if the protein was
modified by incubation with hydrazine (data not shown). However, the poor signal to noise ratio of the hydrazine incubated protein spectrum resulted in inconclusive results.

Nevertheless, the present study indicates phytoglobins can be used to catalyze the reduction of hydrazine with a turnover number of 166, higher than anything previously observed by metallo-organic catalyst. Furthermore, they are able to do so at a rate of 0.227 min⁻¹, which is comparable to the rate of reduction by other catalyst. This rate of reduction is limited by the rate of electron transfer. Therefore, to utilizes phytoglobin as catalyst we need to increase the catalytic efficiency by improving this electron transfer rate. This rate could be improved by using enzymatic reductant like ferrodoxin/ferrodoxin reductase, which are the native proteins responsible for reduction of phytoglobins in plants. We believe characterization and optimization of the reduction by these proteins could fundamentally transform the reaction capability of phytoglobins.
Figures

Figure 1: Hydrazine Reduction progress curves. Normalized progress curve for Lba, Ngb, Rice Phyt and Tom Phyt at 20mM Hydrazine (as markers). Solid curves are exponential fits to each progress curve. The rate constants thus determined are reported in Table 1.
Figure 2: Hydrazine reduction by rice Phyt. A) Normalized progress curve for rice Phyt with varying concentrations of Hydrazine. The line represents the exponential curve fit to the progress curve. B) Reduction rate determined from progress curve plotted against Hydrazine concentration, it shows a non-linear relationship.
Figure 3: Michealis Menten Plot. Initial rate of reaction was plotted against HZ concentration and represent michealis menten plot with a $k_{cat}$ of 0.227 min$^{-1}$ and $k_{m}$ of 41mM.
Figure 4: NMR based determination of products and stoichiometry. The reduction of $\text{N}^{15}$ HZ was monitored using direct-detect NMR. Product identity was determined by the position of peak and amount was calculated based on the volume of the peak. A) $\text{N}^{15}$ NMR spectrum of products of N15-HZ reduction by Rice HB(50uM) at 0hrs and 24hrs. B) A graph of amount of NH4 expected (solid color bar, left axis) based on the amount of HZ is consumed and amount of NH4 formed (Checkered bar, left axis) at different DT concentration. The marker on the graph represents the percent conversion of HZ to ammonia (Right axis)
Figure 5: Product stoichiometry at lower DT concentrations. Determination of NH4 concentration by OPA Assay. The bars represent the amount of ammonia detected using the OPA assay at different concentration of DT. The amount of ammonia expected based on scheme 2 is also represented on the graph. The markers on the graph represent the ratio of moles of DT consumed per mole of Ammonia formed. The graph also represents the amount of NH4 concentration produced from the reaction done at different concentrations of HZ.
Tables

Table 1: Hydrazine reduction rate. Pseudo first order rate constants ($k_{obs}$) of reduction determined by fitting oxidation progress curves to exponential curves at 20mM Hydrazine (Figure 1). The values of histidine off rates ($k_{his}$) have also been listed for comparison.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Coordination</th>
<th>$k_{his}$ ($s^{-1}$)</th>
<th>$k_{obs}$ ($min^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>LBA</td>
<td>penta</td>
<td>NA</td>
<td>0.0009</td>
</tr>
<tr>
<td>Ngb</td>
<td>hexa</td>
<td>1</td>
<td>0.01</td>
</tr>
<tr>
<td>RiceWt</td>
<td>hexa</td>
<td>40</td>
<td>0.07</td>
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<tr>
<td>Tom</td>
<td>hexa</td>
<td>185</td>
<td>0.11</td>
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</tbody>
</table>
References


(16) Olson, J. S.; Mathews, A. J.; Rohlf, R. J.; Springer, B. A.; Egeberg, K. D.; Sligar, S. G.; Tame, J.; Renaud, J.-P.; Nagai, K., Published online: 17 November 1988; [doi:10.1038/336265a0](10.1038/336265a0) **1988**, *336* (6196), 265–266.


CHAPTER 4
GENERAL CONCLUSIONS

Conclusions: Since the discovery of phytoglobins 20 years ago, there have been significant advances made towards understanding the role of these proteins in plants. Most studies are targeted towards understanding the inorganic reduction capabilities of these proteins, mainly nitrite and hydroxylamine reduction. The former forms nitric oxide during reduction which binds to phytoglobins and forms a dead complex, thus preventing the protein from catalyzing a multi-turnover reaction. In case of hydroxylamine (HA), multiple turnover reaction has been reported in presence of dithionite. However, these multi turnover reactions were never characterized. This creates a gap in our understanding of these proteins. In this study, we try to bridge this gap, by characterizing this multi-turnover reaction of hydroxylamine in presence of dithionite.

Furthermore, phytoglobins have the unique capability for reversible hexacoordination. This allows the protein to behave as ligand binders in the penta-coordinated state, similar to myoglobin. They also acts as electron donors in the hexa-coordinated state. Thus, these proteins are able to catalyze 2-electron reduction of hydroxylamine and hydrazine (chapter 3) and with one electron being donated from each heme iron. However, both these reactions when catalyzed in presence of dithionite are limited by the DT electron transfer rate to the substrate bound phytoglobin. This limit represents the amount of radical anion formed by equilibrium of dithionite dissociation.

Dithionite has been classically used for reducing protein due its large reduction potential, which again is a single reduction event. However, based on results in our study
we believe that dithionite is not a suitable reductant for multi-turnover reaction catalyzed by phytoglobins (chapter 2). Furthermore, in a single turnover reaction the phytoglobins were able to exchange electron at a bimolecular rate $2.3 \times 10^8 \text{M}^{-1}\text{s}^{-1}$. This suggests that higher reductions rates could be achieved by using reductase. This would in turn make it a viable catalyst for the production of ammonia. Towards this goal we believe ferredoxin and ferredoxin reductase, the putative reductase for phytoglobins, need to be characterized. The future studies will be motivated by this goal to develop a robust enzymatic reduction technique for ammonia production
References


