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Effects of Macromolecular Crowding on Ferredoxin and Ferredoxin-NADP+ Reductase Kinetics

Danielle Bautista
Duquesne University

David W. Seybert Ph.D.

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Danielle Bautista | Bayer School of Natural and Environment Sciences | Faculty Advisor: David W. Seybert, Ph. D.

Introduction

High concentrations of macromolecules may modify protein – protein interactions through a phenomenon known as macromolecular crowding. Literature studies have suggested that crowding affects a variety of biochemical functions, including conformational protein folding[1], diffusion rates[2,3], RNA conformations[4], and enzymatic reaction rates[5-7]. Our studies focus on understanding the effects of macromolecular crowding on electron transfer reactions between protein complexes *in vitro.*

Background

Cellular interiors contain macromolecules that range up to 20 to 30% by weight percent[1], and these concentrations may vary depending upon the intracellular location. For example, chloroplast thylakoids membranes may contain up to 70 to 80% proteins by weight percent[2]. Naturally, cells are crowded, which is why it is important to perform studies considering these conditions. The term macromolecule encompasses a variety of constituents, including: proteins, carbohydrates, nucleic acids, and lipids. Of these, proteins and nucleic acids are the most prevalent macromolecules within the intracellular matrix of the cell.

For the past fifty to seventy years, scientists have studied enzymes and proteins in extremely dilute solutions[1]. In the absence of added macromolecules, dilute solutions provide a matrix that allows the kinetics of biological reactions to be measureable. In the cell, these redox reactions occur on the timescale of nanoseconds, which is extremely fast. These dilute solutions do not represent the true nature of cellular interiors. Therefore, it is essential to study these systems under crowded conditions. Literature studies have demonstrated the wide range effects on biochemical functions[1-7]. Therefore, by modeling a

crowded environment, scientists can more accurately simulate the physiological conditions within the cell[8]. These studies may lead to a new approach in the methodology for understanding protein systems.

Macromolecular crowding can be conceptualized through the excluded volume theory[9]. The volume occupied by macromolecules is large considered substantial in comparison to the rest of the volume the cell. When considering excluded volume there is less available room for interactions to occur due to the presence of larger macromolecules. Steric repulsion is the major source of interaction between macromolecules, when considering the excluded volume theory[9]. Theoretically, macromolecular crowding predicts two outcomes depending on the nature of the reaction. These two reactions types are dependent on their kinetic and thermodynamic properties[10].

It is important to determine the effects of viscosity because macromolecule crowding may lead to viscous solutions. As the concentration of macromolecules increases there also tends to be an increase in the viscosity. Viscous solutions have more internal friction, which in turn, decreases the ability of solutes to move on fast timescales in solution[11]. This is because macromolecular crowding can give rise to viscous solutions. Also, macromolecular crowding is strongly influenced by the size and shape of the macromolecule[11]. Studies using polymers such as Ficolls and dextrans have vastly different results in comparison to studies with globular proteins[11]. Therefore it is important to understand the effects of differing types of macromolecular crowding agents.

To model macromolecular crowding we chose bovine serum albumin (BSA). In past studies, Ficoll and dextran were used as crowding agents. Although these two matrix modifiers are commonly used, the cellular interior has a larger concentration of proteins in comparison to polysaccharides. BSA is globular in nature and is speculated to be inert in regard to our system.

Ferredoxin (Fdx) and ferredoxin – nicotinamide adenine dinucleotide phosphate ($NADP^+$) reductase (FNR) were utilized as our model electron transfer system. Physiologically, they are found in the photosynthetic electron transport chain. During electron transfer Fdx transfers one electron from its iron sulfur center (Fe_2S_2)[12]. FNR, in turn, accepts two electrons from two Fdx via flavin adenine dinucleotide (FAD) prosthetic group[12]. The Fdx structure is considerably smaller in comparison to its

reductase counterpart weighing approximately 11,000 Daltons[13]. FNR is larger compared to the Fdx protein, with the molecular weight approximately 32,000 Daltons[13]. Figure 1 illustrates the two proteins prior to association.

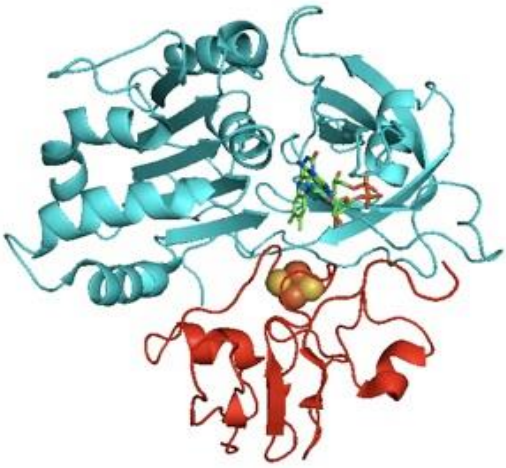

Figure 1: The crystal structure of the Fdx-FNR complex in the early stages of association during electron transfer. The top (blue) depicts the FNR, whereas the bottom (red) depicts the Fdx[14].

Physiologically, the electron transfer of Fdx and FNR proceeds with the reduction of $NADP^+$. Our reactions did not use this electron transfer system. Instead we utilized cytochrome *c* (cyt *c*) as a surrogate electron acceptor and NADPH as the reductant. Cyt *c* was chosen as a surrogate because PSI, the natural electron acceptor, is difficult to reconstitute *in vitro*. Although cyt *c* is not the physiological final acceptor, it enabled us to monitor the electron transfer between Fdx and FNR. Ultimately, reduction of cyt *c* can be measured through UV – Vis spectroscopy.

Materials and Methods

For electron transfer to occur numerous components are required. NADPH is created through a regenerating system consisting of glucose – 6 – phosphate (G6P), glucose –6 – phosphate dehydrogenase (G6PDH), and $NADP^+$. G6P and G6PDH were purchased from Calbiochem, whereas $NADP^+$ came from Sigma Aldrich. The FNR and Fdx proteins were extracted and purified from *spinacia oleracea*. Reduction

of cytc is monitored through change in absorbance at the wavelength of 550 nanometers at 25 degrees Celsius. A 10 mM phosphate buffer at pH 7 was used for all kinetic assays. To simulate crowding, the macromolecular crowding agent, BSA was dissolved in the 10 mM phosphate buffer by w/w% at pH 7.

Data was analyzed through the determination of two Michaelis – Menten constants, K_m and V_{max}. Our experiments follow hyperbolic kinetics as a function of Fdx concentration. K_m and V_{max} values were determined two ways. The first through a double reciprocal plot, otherwise known as a Lineweaver – Burk plot. Here the K_m and V_{max} values were calculated as the reciprocal of the x – intercept and y – intercept respectively. The second way to determine this was through nonlinear least squares analysis utilizing the software Prism.

Hypothesis and Specific Aims

Utilizing the Fdx-FNR we intend to mimic the enzymatic reaction occurring between the two complexes to understand how macromolecular crowding will impact the kinetics. Our hypothesis was to investigate whether or not macromolecular crowding will decrease the rate of electron transfer between ferredoxin and ferredoxin-$NADP^+$ reductase.

The first specific aim was to determine the K_m and V_{max} values in the presence of BSA. Through determination of these Michaelis - Menten constants we will have a deeper understanding of the effects of BSA on Fdx-FNR redox reactions. These K_m and V_{max} values will then be compared in the absence and presence of BSA. Through other calculations we presented the relative activity within the reactions. We will be able to compare the BSA experiments to the control experiments (without the presence of macromolecular crowders) and determine if the rate of reaction is increasing or decreasing.

The second specific aim was to determine if there was any effect of viscosity on the electron transfer reactions. It was important to verify that crowding, and not viscosity influenceed our experiments. Experiments utilizing viscous solutions suggest that electron transfer rates are altered due to

the overall medium, whereas crowding should alter rates due to excluded volume. These viscosity assays used glycerol as a viscogen.

Results and Discussion

To investigate the first specific aim the K_m and V_{max} values of the Fdx-FNR system, assays were performed using 0%, 5%, 10%, 15% and 20% BSA. All of these experiments were compiled into an composite plot (Figure 2). From this double reciprocal plot we were able to determine the K_m and V_{max} values. Table 1 contains the K_m and V_{max} values with the corresponding concentration of BSA. These results suggest that there is a primary effect on the V_{max} values, as the concentration of BSA increases. In these experiments the K_m value did not display any significant trends. Therefore the major kinetic effect is postulated to be a V_{max} effect and not a K_m effect. Through other calculations we calculated the relative V_{max}, or activity. These values are found in the far right column of Table 1.

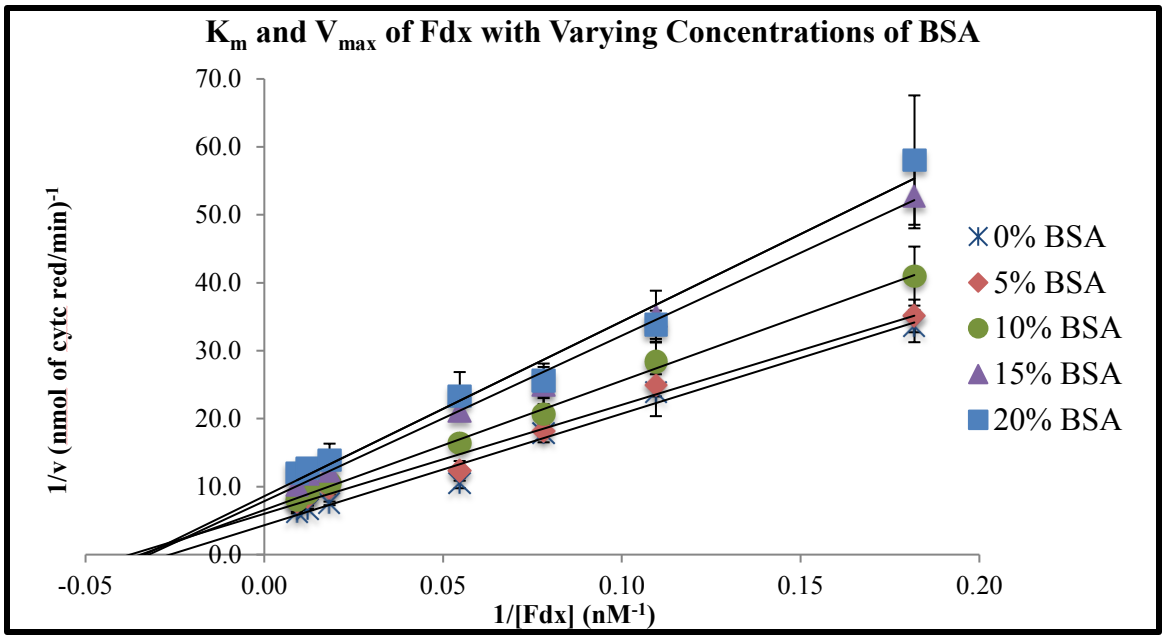

Figure 2: Effects of bovine serum albumin (BSA). Double reciprocal plots show a comparison of kinetic assays in the presence of 0%, 5%, 10%, 15%, and 20% BSA. Error bars represent a standard deviation.

BSA Concentration (%)	Km (nM^{-1})	Vmax $(\Delta Abs/min)^{-1}$	Relative V_{max} $(\Delta Abs\ /min)^{-1}$
0	38 ± 1	0.23 ± 0.01	100
5	27 ± 1	0.17 ± 0.01	85 ± 3
10	29 ± 2	0.15 ± 0.03	64 ± 5
15	31 ± 2	0.13 ± 0.02	50 ± 5
20	30 ± 3	0.12 ± 0.01	45 ± 6

Table 1: K_m and V_{max} values for Fdx as a function of BSA concentration. Error reported as a 95% confidence interval.

The second specific aim was to determine if viscosity had any effect on the Fdx-FNR system. Therefore studies were conducted to determine whether viscosity is affecting the kinetics of the Fdx and FNR. Previous studies have demonstrated that viscosity has minimal effects on redox active enzymes[3,5]. Our studies included glycerol as the viscogen, because of its small size our expectation that it would be inert with respect to the Fdx – FNR system.

Our data demonstrates that concentrations of glycerol up to 10% show no inhibition on the reaction rate. When the concentration is increased to 15% and 20% the electron transfer rate between the Fdx and FNR is reduced. Figure 3 represents the relative rate as a function of glycerol concentration. The data points in figure 4 show that between 0% and 10% glycerol the electron transfer rates remained essentially constant. When the concentration of glycerol was increased above 10% the electron transfer rate began to decrease. At 15% glycerol the rate decreased by about 16%. At 20% BSA the rate decreased by approximately 28%. These studies have decreased the reaction rate at higher concentrations of glycerol, therefore more experiments will be performed to compare the viscosity assays and BSA assays.

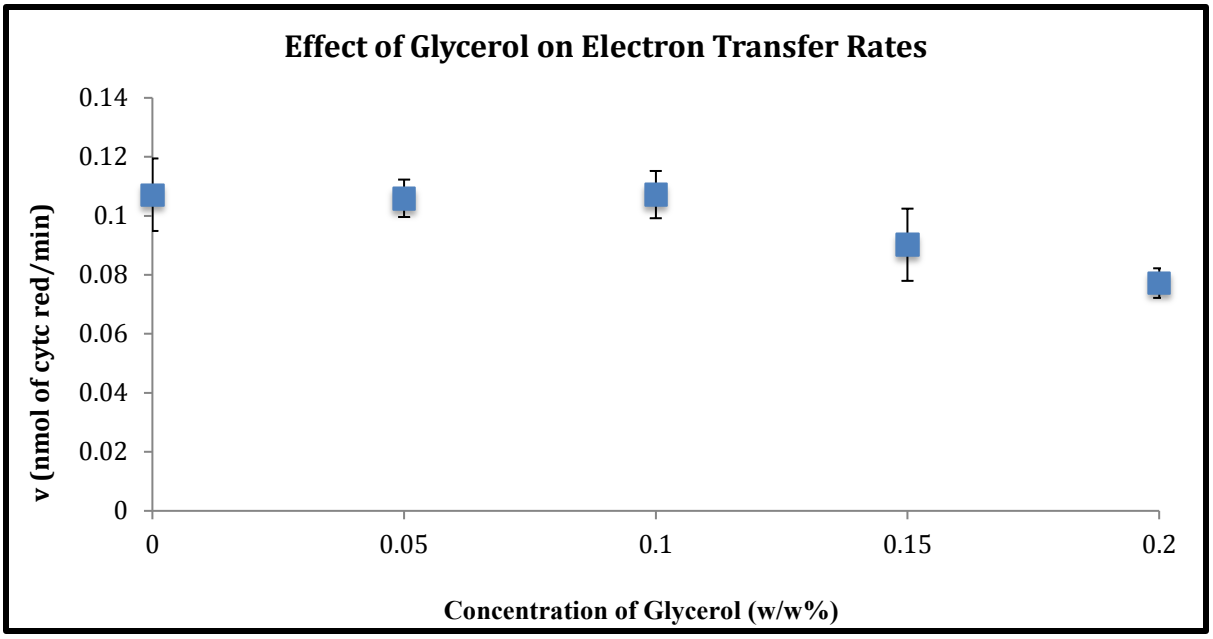

Figure 3: Effects of glycerol on kinetics of cytochrome *c* reduction. Error bars represent a standard deviation.

Our experiments thus far support our hypothesis. As the concentration of BSA increases, the rate of electron transfer between the Fdx and FNR complexes decreases. The primary affect is shown in V_{max}, whereas K_m stays essentially constant. Experiments focused on viscosity suggest that there is an effect on electron transfer at high concentrations of viscogens. These studies indicate that there may be an effect of both macromolecular crowding and viscosity.

Future Directions

To continue this project there several different ways to proceed. Our last assay was performed with BSA at 20%, but physiologically cellular conditions contain up to 20% and 30% of macromolecules by percent weight. Therefore, we will continue to increase the concentration of BSA to more closely mimic intracellular conditions. Also, limiting our crowding solutions to only one macromolecule does not entirely replicate intracellular conditions since cells are heterogeneous. Therefore we will attempt to create heterogeneous solutions of macromolecules. Experiments with heterogeneous solutions may have

synergistic effects on the electron transfer rates. We will also continue to perform studies on viscosity. We will determine K_m and V_{max} values using glycerol as the viscogen. These studies will then be compared with macromolecular crowding studies utilizing BSA.

Another direction is to correlate the studies between the ferredoxin, a plant-type system and adrenodoxin, a mammalian system. We intend to implement a fluorescence assay and use the physiological electron acceptor, cytochrome P450. Studying both systems in conjunction will yield valuable complementary information. Preliminary results show that the V_{max} of the Fdx-FNR system is affected, whereas the K_m is affected in the adrenodoxin – adrenodoxin reductase system in the presence of macromolecular crowders.

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