TITLE:

Anaerobic Protein Purification and Kinetic Analysis via Oxygen Electrode for Studying DesB Dioxygenase Activity and Inhibition

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SUMMARY:

Here we present a protocol for anaerobic protein purification, anaerobic protein concentration, and subsequent kinetic characterization using an oxygen electrode system. The method is illustrated using the enzyme DesB, a dioxygenase enzyme which is more stable and active when purified and stored in an anaerobic environment.

ABSTRACT:

Oxygen-sensitive proteins, including those enzymes which utilize oxygen as a substrate, can have reduced stability when purified using traditional aerobic purification methods. This manuscript illustrates the technical details involved in the anaerobic purification process, including the preparation of buffers and reagents, the methods for column chromatography in a glove box, and the desalting of the protein prior to kinetics. Also described are the methods for preparing and using an oxygen electrode to perform kinetic characterization of an oxygen-utilizing enzyme. These methods are illustrated using the dioxygenase enzyme DesB, a gallate dioxygenase from the bacterium *Sphingobium* sp. strain SYK-6.

INTRODUCTION:

Enzymes that utilize iron or other metals to activate oxygen are often susceptible to inactivation during the purification process because of their removal from the reducing environment of a cell. Therefore, these proteins must be used as cell lysates, be subjected to external reducing agents, or be purified anaerobically to ensure that they have optimal enzymatic activity[1-4]. For those enzymes that are oxygen-sensitive (specifically iron-containing enzymes), performing all the

purification and characterization steps while maintaining anaerobic conditions is necessary to fully characterize them. This has led researchers to develop entire laboratory set-ups within the confines of anaerobic chambers for studies ranging from protein expression through crystallography[5-8].

Herein, we report methods for the anaerobic purification and kinetic characterization of the enzyme DesB using an oxygen electrode system. DesB is a gallate dioxygenase from the bacterium *Sphingobium* sp. strain SYK-6 that is related to LigAB, a protocatecuate dioxygenase from the same organism. Both enzymes belong to the type II protocatechuate dioxygenase (PCAD) superfamily which has not been extensively studied to date[9], likely in part due to enzymes of this superfamily being susceptible to inactivation when purified using standard aerobic protein purification methods. Since some of the PCAD enzymes display substrate promiscuity while others are substrate-specific[2,10], further characterization of this superfamily is necessary to identify specificity determinants. As has been observed in several enzyme superfamilies[11-15], small molecules can alter activity via direct competitive inhibition or the binding of molecules to separate allosteric pockets which causes an increase or decrease in enzymatic activity[16]. While kinetics alone cannot differentiate the binding location of a modulator, determining the magnitude of an activity change is important for understanding the effects. As such, methods for kinetic characterization of native DesB activity and its activity in the presence of 4-nitrocatechol (4NC), a compound commonly used to characterize and inhibit dioxygenase enzymes[2,17,18], are shown.

DesB is able to break down gallate, a lignin-derived aromatic compound, via an extradiol dioxygenase (EDO) reaction in which ring opening is catalyzed using oxygen as one of the substrates[10,19]. This enzymatic reaction occurs within the context of the breakdown of lignin, an aromatic heteropolymer found in the cell wall of plants. Lignin can be depolymerized, yielding a variety of aromatic compounds that can be further broken down into central metabolites[3,20-33]. Extradiol dioxygenases (EDO) catalyze a ring opening reaction on dihydroxylated aromatic compounds, where cleavage occurs adjacent to a metal-coordinated diol; in contrast, intradiol dioxygenases cleave analogous aromatic compounds between the two hydroxyl groups (**Figure 1**). EDOs, like many other metalloenzymes, have a divalent metal center for coordinating Fe(II) composed of a two-histidine, one-carboxylate triad[9,34,35]. These metalloenzymes become oxidized, through either autoxidation or mechanism-based inactivation, whereas the enzyme is rendered inactive[2,36-38].

In the experimental procedures described in this manuscript, we utilize DesB, a member of the PCAD superfamily from the bacterium *Sphingobium* sp. SYK-6, to catalyze the addition of oxygen across the C4-C5 bond of gallate (**Figure 2A**). The regiochemistry of this cleavage is analogous to LigAB, which is a protocatechuate-4,5-dioxygenase (**Figure 2B**). Thus far, investigations of this gallate dioxygenase include no reports of compounds that inhibit DesB[10,19,39]. With the use of aerobic purification methods, DesB exhibited variable activity, while with the use of anaerobic methods we were able to consistently obtain protein with reproducible activity. The kinetic studies described here show the methods for anaerobic purification of DesB, kinetic

characterization of the reaction of DesB with gallate, and the inhibition of DesB by 4-nitrocatechol (4NC).

PROTOCOL

1. General materials and methods

1.1. Prepare all the required media as described in **Table 1.** Autoclave at 120 ˚C for 15 min. Sterile filter the SOC solution, after the addition of $MgCl_2$ and glucose, by passing it through a 0.2 µm filter. Adjust the pH of the Miller's Lysogeny Broth (LB media) solution prior to autoclaving. Supplement the LB-Amp media solution after autoclaving with sterile solutions of 0.2 mM L-cysteine, then 0.1 mM ferrous ammonium sulfate to enhance protein expression and solubility.

1.2. Prepare the Laemmli buffer and running buffer for polyacrylamide gel electrophoresis (PAGE) as described in **Table 2**. Adjust the pH of the solutions (using 1 M HCl and Tris base for buffer preparation) before diluting the components to their final volumes.

1.3. Prepare the buffers for protein purification as described in **Table 3**. Adjust the pH of the solutions (using 1 M HCl and Tris base for buffer preparation) before diluting the components to their final volumes.

1.4. Transfer the prepared buffers to a bottle that can be sealed with a one-hole rubber stopper and contains a glass tube.

1.4.1. Use a hose to connect the glass tube in the stopper to a vacuum source. Allow the solution to degas under negative pressure for approximately 30 min while stirring at medium speed.

1.4.2. Break the vacuum seal first, then turn off the vacuum. Next, pierce through the rubber stopper with two 20G needles (one that is long enough to reach the bottom of the bottle, and one shorter needle that can be used as a gas escape vent). Bubble in a steady flow of nitrogen while stirring at medium speed for 30 min.

1.4.3. Remove the needles and repeat the degassing and bubbling in nitrogen for a total of three cycles. When finished, properly seal the bottle with a solid rubber stopper. Further secure the stopper with paraffin film and copper wire (20G), and place them into the glovebox.

NOTE: All materials are placed into the glovebox and subjected to 3 cycles of purging the antechamber, followed by vacuuming out the oxygen and refilling it with nitrogen. Buffers used for purification and concentration can be capped and stored in the glove box indefinitely.

1.5. Prepare the sodium dithionate solution by dissolving approximately one spatula (0.31" x 2", the smaller end of a double-ended micro-tapered stainless-steel spatula) full of sodium dithionate in approximately 1 mL of degassed water in a 1.5 mL microcentrifuge tube. Seal the tube with paraffin film before removal from the glove box.

NOTE: Store solid sodium dithionate in the glove box to prevent oxidation.

2. Preparation of the amylose column for protein purification

2.1. Make a slurry from high flow amylose resin (15 mL) combined with 5 column volumes (75 mL) of amylose column buffer. Transfer the resin slurry into two 50 mL serum bottles and seal each bottle with a rubber septum. Puncture a 20G, 305 mm needle through the septa, and bubble nitrogen into the slurry for approximately 1 minute to remove oxygen from the suspension. Transfer the resin and protein-containing solution into the glove box.

2.2. In the glove box, pour the resin into a 2 x 30 cm borosilicate column fitted with a simple stopcock, allowing the resin to settle and create a level bed. Drain off the column buffer and equilibrate the column with 3 column volumes (~30 mL total) of degassed amylose column buffer using pressurized nitrogen gas to push the buffer through the resin at approximately 1 drop/s or 5 mL/min, until the solvent is just above the resin bed.

NOTE: After the protein is eluted (see step 3.5 below), the amylose column resin is regenerated by washing with 1 column volume (10 mL) of water, then 1 column volume of 1% sodium dodecyl sulfate (SDS), and finally, 3 column volumes (30 mL) of water. This step can be completed outside of the glove box. The column resin is stored at 4 °C in 20% ethanol and can be regenerated up to 5 times.

3. Protein expression and anaerobic purification of DesB

NOTE: The DesB gene was commercially synthesized, having been placed into pET-15b, pET-32a, and pMAL- c5x vectors using the NdeI and BamHI restriction cloning sites.

3.1. Complete the protein expression assays according to manufacturer's guidelines to determine the correct conditions for large scale expression (described in brief below).

3.1.1. Transform the DesB-containing plasmids into commercially made, chemically competent *E. coli* BL21 (DE3) cells, according to the manufacturer's instructions. In brief, thaw the cells on ice for 10 min, add approximately 10-50 ng of plasmid DNA to the cell mixture, heat shock the cells at 42 °C for 10 s, and incubate them on ice for an additional 5 min. Add SOC media to obtain a final volume of 1 mL and allow the cells to shake (~200 rpm) at 37 °C for 60 min. To an LB-Amp plate, add and spread 100 µL of the cell solution and incubate overnight at 37 °C.

3.1.2. Select a single colony-forming unit from the plate after overnight incubation and use this colony to inoculate 10 mL of LB-Amp media. Grow the cells overnight, with shaking (~200 rpm) at 37 °C.

3.1.3. To 10 mL of media, add 100 µL of the overnight growth solution, and shake the new media as described in step 3.1.2 at 37 °C. When the optical density at 600 nm (OD_{600}) is between 0.4-

0.8, add isopropyl β-D-1-thiogalactopyranoside (IPTG) to obtain a final solution concentration of 1 mM.

3.1.4. Immediately remove a 1 mL aliquot of the solution and transfer it to a 1.5 mL microcentrifuge tube with labeled time = 0 hours. Spin the tube at 15,000 x *g* for 10 min to pellet the cells. After spinning, remove the supernatant and store the cells at 4 °C.

3.1.5. Remove additional aliquots (1 mL each) at time intervals after the addition of IPTG (typically at 5, 10, 12, 18 and 24 h). As described in step 3.1.4, centrifuge each tube, decant the supernatant, and store the cells at 4 °C.

3.1.6. Once all the aliquots are obtained, resuspend the cells from each timepoint in 30 µL of bacterial protein extraction solution, incubate them at room temperature for 10-15 minutes, and centrifuge the tubes at 15,000 × *g* for 5 min to separate the soluble and insoluble proteins.

3.1.7. Transfer the soluble protein solution to a new microcentrifuge tube and resuspend the remaining insoluble pellets with 30 µL of bacterial protein extraction solution.

3.1.8. Combine the 30 µL of each solution (both soluble and insoluble solutions for each timepoint) with an equal volume of Laemmli buffer, then visualize on an SDS-PAGE gel[40]. Select conditions corresponding to the lanes containing the correct size bands in the soluble fraction for large scale protein expression and purification.

NOTE: Since minimal soluble protein was initially generated using the conditions above, various supplements were added to test for effects on the soluble protein expression, with 0.2 mM L-cysteine and 0.1 mM ferrous ammonium sulfate showing enhanced protein expression. According to this protein expression screen, an appropriate expression vector for soluble production of DesB was pMAL- c5x vector.

3.2. Following transformation into *Escherichia coli* BL21 (DE3), make a stock of DesB/pMal-c5x by combining 900 µL of bacterial culture (grown overnight with shaking at 200 rpm and 37 °C) with 100 µL of 80% glycerol. Store the stock at -80 °C in a cryogenic tube.

NOTE: Make a new frozen stock approximately every 6-8 months.

3.3. Use a pipette tip to scrape cells from the frozen stock and start a bacterial culture, growing overnight in 5 mL of LB-Amp media at 37 °C with shaking. Inoculate 1.5 liters of LB-Amp media in a 2 L flask at 37 °C and 200 rpm, using the overnight grown bacterial culture.

NOTE: Due to the observed sensitivity of the protein to oxygen, it was found that reduced headspace in the flask and moderate shaking speeds improved protein yield for large-scale expression.

3.4. Induce protein expression when the optical density at 600 nm (OD_{600}) is between 0.4-0.8 with 1 mM IPTG and supplemented with 0.2 mM L-cysteine and 0.1 mM ferrous ammonium sulfate. Lower the incubation temperature to 30 °C and shake at 200 rpm. Spin down the cells via centrifugation after 24 h post-induction at 4,700 x *g* for 10 min and discard the spent media.

3.5. Resuspend the cell pellet in 20 mL of amylose column buffer per every 1.5 L of growth, followed by the addition of 1 mg of lysozyme. Stir for 20 minutes on ice.

NOTE: More buffer can be added if the solution is too viscous, as protein solutions that are too viscous may disrupt the following homogenizing step.

3.6. Lyse the resuspended cell solution via high pressure homogenization with 3-5 passes at approximately 15,000 psi. Transfer the protein to a 50 mL centrifuge tube and flush the headspace with nitrogen for 1 min. Then, centrifuge the cell lysate at 20,000 x *g* for 40 min to remove the insoluble debris.

NOTE: Successful homogenizing causes the protein to form micelles as it flows through the tube and into the collection flask, and the solution appears as a translucent grey-brown color. Keep the homogenized protein on ice throughout the process.

3.7. After centrifugation, transfer the supernatant into a 50 mL tube (more than one may be necessary), cap the tube with a rubber septum, and with a 20-gauge needle, slowly bubble nitrogen gas through the solution for 2 min to displace oxygen. Wrap the septum with paraffin film and further secure it with copper wire, and bring the supernatant into the glove box along with the column materials.

3.8. Equilibrate the column (see step 2.3) and load the protein supernatant onto the column. Collect the flow-through in 5 mL fractions under moderately pressurized nitrogen (1 drop/second or approximately 5 mL/min). Next, wash the column using another 3 column volumes of amylose column buffer and manually collect 3 mL fractions in glass test tubes under moderately pressurized nitrogen (approximately 5 mL/min). Elute the protein using 5 column volumes (~50 mL) of elution buffer and manually collect 3 mL fractions under moderate nitrogen pressure (approximately 5 mL/min).

NOTE: The fractions can alternatively be collected using gravity filtration. Approximately 12 elution fractions should be collected.

3.9. Collect 30 µL aliquots of fractions and remove them from the glove box to allow the visualization of protein-containing fractions via SDS-PAGE analysis.

NOTE: The collected fractions remain in the glove box for the duration of this test. Purified DesB typically elutes from the column in fractions 3 through 5.

4. Anaerobic protein concentration/buffer exchange

4.1. Assemble the pressurized, stirred cell concentrator with a 76 mm reconstituted cellulose membrane filter (10 kDa cutoff). Add a sufficient amount of 20% ethanol into the concentrator to keep the membrane wet as the assembled concentrator is transferred into the glove box.

NOTE: Ensure that there are no tears in the membrane and that the O-ring is not wrinkled.

4.2. After bringing the stirred cell concentrator into the glove box, wash the ethanol solution off the membrane with water. Cap and pressurize the stirred cell to clean the tubing and membrane of any excess water.

4.3. Add all the fractions containing protein as determined by SDS-PAGE (**Figure 3)** to the concentrator. Add sufficient exchange buffer to yield 150 mL of total protein solution volume.

4.4. Pressurize the stirred cell chamber using an external N_2 line and concentrate the protein to 50 mL with moderate stirring speed.

NOTE: Achieving a concentration of 50 mL takes approximately 20 min.

4.5. Supplement 100 mL of exchange buffer with 0.1 mM dithiothreitol (DTT) and 0.1 mM ferrous ammonium sulfate, add the solution to the stirred cell, and concentrate the protein to a total volume of 50 mL with a moderate stirring speed.

4.6. After the solution reaches a volume of 50 mL, add in the supplemented exchange buffer once more, but concentrate the solution to a total volume of 10 mL with moderate stirring speed.

4.7. Filter the concentrated protein using a 0.45 µm pore size syringe filter and aliquot into individual 0.2 mL polymerase chain reaction (pcr) tubes (each containing approximately 150 µL of the protein solution). Remove the capped aliquots from the glove box and immediately flash freeze them with liquid nitrogen. Store the aliquots at -80 °C.

5. Desalting DesB

5.1. Flush the glove bag with nitrogen gas for approximately 1.5 hours before use.

5.2. Thaw a 150 µL aliquot of purified DesB protein inside of the glove bag, on ice.

5.3. While the protein thaws, prepare a small gravity, desalting column containing 3 mL of Sephadex G-25 coarse desalting gel in a 9 mL borosilicate column. Wash the column with 2 column volumes (~6 mL) of degassed desalt buffer.

NOTE: Replace the desalting gel when there is a change in gel color from white to yellow (after approximately 7 uses).

5.4. Load the thawed DesB onto the column via a gravity-controlled process. Discard the flow-through. Elute the protein with approximately 5 mL of desalting buffer.

NOTE: The pressure from the gas tank continually filling the glove bag will affect the rate at which the solutions drip from the column.

5.5.1 For initial determination of protein elution from the column, collect 12 vials/elutions (containing 3 drops per vial). Sealed with a rubber septum, remove them from the glove bag and test for the presence of protein either directly (through SDS-PAGE gel analysis) or indirectly (through examination of the activity of each fraction).

NOTE: Typically, the fractions are tested individually for activity, as described in step 7.1, with the greatest activity corresponding to the fraction with the greatest concentration of active protein. DesB usually elutes starting in the 7th drop, after which the following 9 drops of desalted protein are collected. Protein aliquots are stored on ice during the kinetics measurements (steps 7.1-7.4).

6. Preparing the oxygen electrode

6.1. Polish the silver anode ring and platinum cathode with electrode cleaner and a damp Q-tip until there are no visible signs of black oxide deposits.

6.2. With scissors, cut approximately one 1.5-inch square of spacer paper (or cigarette rolling paper, which works equally as well). Then, cut 1) small triangles onto each face of the square and 2) slits into the corners to aid wrapping of the electrode.

6.3. Add 5 drops of a 50% KCl solution onto the silver anode groove and connect them so they form one continuous ring of solution. Add 2 drops of the 50% KCl solution to the top of the platinum electrode. Carefully place the spacer paper on top of the platinum electrode and smooth out the spacer paper so there are no air bubbles.

NOTE: Use caution to avoid tearing the spacer paper.

6.4. Cut a piece of the S4 30m PTFE (polytetrafluoroethylene) membrane that is approximately 2 inches long and place it on top of the spacer paper. Cut off the edges of the membrane so they are aligned with the outer ring of the electrode.

6.5. Using the O-ring applicator, push the small O-ring over the electrode to secure the spacer paper and membrane onto the electrode. Place the larger O-ring onto the circular groove of the electrode, ensuring there is no membrane underneath it. Slide the top chamber of the electrode onto the base and screw the two together.

NOTE: The two are screwed on properly if no air bubbles stick to the sides of the chamber.

6.6. Place the assembled electrode on its base and connect the electrode to the monitoring system. Initiate the electrode control program and calibrate the electrode by performing the liquid phase calibration protocol (**Figure 4A**). The temperature variable should be the temperature of the room in which the experiment is performed, and the ambient pressure variable is dependent on the region and current weather conditions.

NOTE: Ambient pressure can be obtained from a weather report for the region.

6.7. Add 1 mL of 25 mM Tris buffer to the electrode chamber, insert the plunger, begin the calibration of an oxygen saturated solution, and adjust the stirrer speed to 100. After the electrode stabilizes, the calibration set-point for the oxygen saturated solution is determined (**Figure 4B**).

6.8. Without removing the plunger, use a 1.2 x 40 mm needle and 1 mL syringe to inject approximately 1 mL of sodium dithionite solution into the chamber, being careful to avoid adding any air bubbles (**Figure 4C**). Use a rubber stopper to seal the electrode chamber immediately after sodium dithionite addition.

6.9. Allow the calibration to continue until all the oxygen is utilized and the program is completed and save the calibration (**Figure 4D**). Aspirate the solution from the chamber and rinse the plunger and chamber with deionized water 5-6 times to ensure complete removal of the sodium dithionite. When rinsing, use a plastic tube equipped with a pipette tip connected to a side-arm vacuum flask. Avoid damaging the membrane.

7. **Kinetic assays using the oxygen electrode**

7.1. Identify the desalted aliquots containing active protein by serially testing 1 µL of enzyme solution for activity in a solution of 25 mM Tris buffer, with pH 7.5 and containing 100 µM gallate. Use the aliquot(s) with the greatest observed activity for the remaining experiments. (See step 5.5.1)

7.2. Determine the rate of the enzymatic reaction by measuring O_2 consumption using an O_2-sensitive Clark-type electrode with computer integration via the electrode control unit.

7.2.1. For each kinetics data point measurement of DesB, add 1 mL of 25 mM Tris buffer (pH 7.5) to the electrode chamber. Add a calculated volume of a 25 mM stock solution of gallate to the Tris buffer to achieve the desired final substrate concentration (0.05-25 mM).

NOTE: Prepare fresh stock solutions of gallate and inhibitory compounds with water daily and adjust the pH to 7.5 with the addition of 0.1 mM NaOH, if necessary.

7.2.2. After 10 s of stirring to allow for mixing of the solution, seal the chamber with the plunger slowly and screw the top down. A clean tissue may be used to soak up the excess liquid that is displaced from the chamber. Allow the electrode to equilibrate where it can produce a stable

measurement of the oxygen concentration in the solution for at least 1 minute before proceeding to the addition of enzyme (background O_2 consumption rate of ± 0.5 µM/min).

7.2.3. Using a gas tight 10 µL syringe, add approximately 1 µL of enzyme (approximately 3-7 µM enzyme) to the electrode chamber through the plunger.

NOTE: The amount of enzyme can be increased or decreased in response to the observed activity of the specific aliquot to allow for reaction rates to be sufficient.

7.2.4. Calculate the reaction velocity based on the slope of the first 30 s of linear data after enzyme addition and correct for background O_2 consumption using the 30 s of data immediately prior to enzyme addition. The rate of catalysis is equal to the rate of O_2 consumption (**Figure 5A**).

7.2.5. After collection of the rate data for a given substrate concentration, empty the electrode chamber through aspiration by using a plastic tube connected to a side-arm vacuum flask and rinsing repeatedly with water for 4-6 cycles. Set up the solution in the electrode as described in step 7.2.1 using a new substrate concentration.

7.2.6. Vary substrate concentrations in an unordered manner and replicate throughout the course of the experiment to account for decreases in enzyme activity with time (**Figure 5B**).

NOTE: When replicates of a specific concentration are run and demonstrate more than a 30% decrease in rate compared to an earlier run, no additional assays should be performed with that batch of enzyme. Typically, after 30-40 runs, the enzyme demonstrates a 30% decrease in activity.

7.2.7. Determine the kinetic parameters, k_{cat} (the catalytic constant for the conversion of substrate to product) and K_m (the Michaelis constant, operationally defined as the substrate concentration at which the initial rate is one-half of the maximum velocity), by a least-squares fitting of data from each of the substrate concentrations to the Michaelis−Menten equation (**Figure 6**) using GraphPad Prism.

7.3. Add 4-nitrocatechol (4NC) to test its impact on dioxygenation kinetics and determine the inhibition constant (K_i) with DesB. For each reaction condition, stock solutions of Tris (50 mM), gallate (25 mM), and 4NC (25 mM) are combined and diluted with water to yield a 2 mL solution of 25 mM Tris buffer (pH 7.5) with 1 mM gallate and varying concentrations of 4NC. The volume of 4NC was varied to achieve the desired final inhibitor concentration (0.05-20 mM).

7.3.1. After 10 seconds of stirring to allow mixing of the solution, place the plunger in the chamber slowly and screw the top down. A clean tissue may be used to soak up the excess liquid that is displaced from the chamber. Allow the electrode to equilibrate and produce a stable measurement of oxygen concentration in the solution for at least 1 minute before proceeding to the addition of enzyme (background O_2 consumption rate of ± 0.5 µM/min).

7.3.2. As described previously, add the protein (step 7.2.3), determine the reaction rate (step 7.2.4), and reset the electrode for the next condition.

7.3.3. As a part of this series of experiments, determine the reaction rate in the absence of inhibitor multiple times. Determine the inhibition by standardization of the rates of oxygen consumption in the presence of various inhibitor concentrations with 1 mM substrate and the absence of inhibitor (v/v_o).

7.3.4. Fit the inhibition of gallate dioxygenation to equation 7.3A and then the inhibition data to equation 7.3B using a graphing program (**Figure 7)**.

7.4. Determine the precise enzyme concentration for each experiment by performing a Bradford assay after completion of all kinetic runs.

NOTE: All rates were corrected for enzyme concentration. This step does not need to be done in a glove box or glove bag.

REPRESENTATIVE RESULTS:

Shown is the SDS-PAGE gel analysis of individual fractions from purification of the DesB-maltose binding protein (MBP) fusion construct (**Figure 3**). The gel reveals that the protein is pure (MW = 91.22 kDa), except for the presence of DesB (MW = 49.22 kDa) and MBP protein domain (42 kDa) cleaved from each other. Fractions E2 and E3 were selected for concentration (step 4.2).

Reproducible results from DesB kinetic assays depend on correct assembly, calibration, and experimental technique. It is necessary to input the correct ambient temperature and pressure, as these variables determine the percentage of O_2 expected to be dissolved in solutions during the assay (**Figure 4A**). The initial signal should be between 1800 and 2000 nmol/mL and should produce a stable rate close to zero, indicating that the oxygen saturation of the solution is minimally changing (**Figure 4B**). Once the sodium dithionate is added and the chamber is sealed, the rate of O_2 consumption should rapidly and steadily increase until there is minimal O_2 (<60 nmol/mL) left in the solution. A steady negative slope indicates that 1) the assembled electrode has no air leaks and 2) the electrode is adequately responding to changes in O_2 concentration (**Figure 4C,D**). If the O_2 remaining in solution is not sufficiently low, the calibration offset value will be incorrect. The calibration will have to be repeated with the electrode assembled correctly, and fresh sodium dithionite must be used.

When the electrode is correctly calibrated, kinetic assays can be performed. The first measurement establishes the activity of the freshly desalted enzyme using 100 µM gallate in 25 mM Tris buffer and 1 µL of enzyme. Before addition of the enzyme, the Tris buffer and gallate are stirred in the chamber with the plunger in position. This should produce an approximate initial signal between 250 and 350 nmol/mL, and the signal should stabilize (±5 nmol/mL/min) before the enzyme is added. A stable signal indicates that there are no variables *(*e.g., torn membrane, leftover sodium dithionite, dirty electrode) that may cause inconsistent measurements. When enzyme is added, the signal should rapidly and steadily decrease,

indicating that the reaction of DesB is proceeding, since O_2 is consumed in the reaction with gallate. The slope of the initial rate before enzyme addition and the catalytic rate were determined by using the rate tools and adjusting the window to 30 seconds (**Figure 5A**). After approximately 1-1.5 hours of use, the enzyme activity decreases by about 30-50%, at which point it should no longer be used (**Figure 5B**).

Once all kinetic measurements have been taken, the data can be plotted using a modeling program (**Figure 6**). The activity of DesB with gallate is obtained by measuring the rate of O_2 consumption in varying gallate concentrations. The background rate before enzyme addition is subtracted from the catalytic rate to obtain the corrected rate. The background corrected rate is

$$v = \frac{Vmax[S]}{\left([S]+K_M+\left(\frac{[S]^2}{K_{si}}\right)\right)}$$ **Equation 7.3A**

K_{si}= substrate inhibition constant

divided by the enzyme concentration and fitted to **equation 7.3A** (See step 7.3.3). A sufficient fit is dependent on the initial parameters for K_M and $K_{si,}$ (initial parameters: K_M = .125 mM, K_{si} = 1 mM). The result shows a hyperbolic curve that reaches a maximum plateau, then slopes downward slightly as [gallate] increases. This is a typical curve for an enzyme that exhibits substrate inhibition at high substrate concentrations.

$$v = \frac{Vmax[S]}{\left([S]+ K_M\left(1+\frac{[I]}{K_i}\right)\right)}$$ **Equation 7.3B**

The rate of inhibition of DesB with 4-nitrocatechol was determined by observing the reduction in oxygen consumption rate of DesB with 1 mM gallate in the presence of varying concentrations of 4-nitrocatechol (**Figure 7)**. The 4-NC concentration was plotted against the normalized activity (the rate in the presence of inhibitor was divided by the uninhibited rate) and fit to **equation 7.3B** (see step 7.3.3). The results indicate that 4-NC inhibits the consumption of oxygen, thus inhibiting the DesB reaction.

TABLE AND FIGURE LEGENDS:

Table 1: Ingredients for preparation of super optimal broth with catabolite repression (SOC media) and Miller's lysogeny broth with ampicillin (LB-Amp media).

Table 2: Ingredients for preparation of Laemelli and running buffers for SDS-PAGE analysis.

Table 3: Ingredients for preparation of buffers for protein purification.

Figure 1: Comparison of the reactions catalyzed by ring cleaving dioxygenases. The wavy lines show the corresponding carbon-carbon bond that is cleaved during the dioxygenase reaction,

where intradiol cleavage (red) breaks the bond between the two hydroxyl groups and extradiol cleavage (blue) breaks a carbon-carbon bond adjacent to the hydroxyl groups.

Figure 2: **The reactions catalyzed by DesB and LigAB, two related extradiol dioxygenases which belong to the type II protocatechuate dioxygenase (PCAD) superfamily**.

Figure 3: SDS-PAGE gel of DesB showing purification and concentration steps results. Lanes are labeled as follows: S = protein standard, FT1 and FT2 = the collected flow-through fractions, W1 and W2 = the collected wash fractions, E1-E4 = the collected elute fractions.

Figure 4: Generation of calibration curves for the oxygen electrode. (**A**) Prior to calibration, ambient temperature and pressure are entered into the program to determine oxygen concentrations for air-equilibrated solutions. (**B**) The oxygen saturated solution reaches equilibrium and generates a prompt. (**C**) Sodium dithionate solution is added to utilize all the oxygen, as indicated by the decrease in oxygen signal. (**D**) Calibration is obtained when the signal stabilizes at zero oxygen concentration.

Figure 5: Representative kinetic curves illustrating a typical loss of activity over time. (**A**) The reaction of 100 µm gallate using fresh DesB, with an O_2 utilization rate of -56.61 µM/min. (**B**) The reaction of 100 µm gallate using DesB after 2 h of data collection, with an O_2 utilization rate of -29.56 µM/min.

Figure 6: Plot of DesB activity with gallate, fit to the Michaelis-Menton equation.

Figure 7: Plot of 4-nitrocatechol inhibition of DesB dioxygenation of gallate (1 mM).

Figure 8: Coordination of gallate by DesB (pdb ID = 3wr3). As seen in many dioxygenases, the ligand coordinates to an active site iron(II) atom in a bidentate fashion. Based on the structural similarities between gallate and 4-nitrocatechol (4NC), the inhibition of DesB by 4NC was predicted.

DISCUSSION:

The critical steps in obtaining active, purified DesB protein involve the forming and maintaining of the reduced Fe(II) active site in the enzyme. As such, correct performance of the induction, purification, concentration, and desalting steps are essential to successfully obtaining active enzyme. Inducing protein expression in the presence of 1 mM ferrous ammonium sulfate ensures that Fe(II) is correctly incorporated into the active site of DesB. This method is inspired by studies like those with amidohydrolase metalloenzymes, which often require the addition of metal to growth media to allow proper folding and full occupancy of the metal binding site[6,41-43].

The anaerobic purification and concentration in the glove box are perhaps the most critical and technically difficult steps in this protocol. It is essential to maintain an oxygen-free atmosphere in the glove box. This requires maintaining the deoxygenation catalyst in the glovebox as prescribed by the manual, periodically changing the nitrogen tanks that are attached to the box

when they are low, ensuring that there are no holes in the gloves attached to the glovebox, and keeping a tray of fresh desiccant in the glovebox. Oxygen-sensitive strips that change color when exposed to ambient O_2 may be placed in the box to test for an O_2-free atmosphere. Furthermore, it is necessary to fully degas all buffers and solutions that will be used during the purification and concentration process. To ensure minimal O_2 in the buffers, take great care to limit exposure of the degassed buffers to air when switching between nitrogen bubbling and vacuuming cycles.

When running the column in the glove box, a good test to determine whether there is oxygen present in the buffers is to take a small portion of one of the wash fractions (approximately 0.1-0.5 mL) and place it in a microcentrifuge tube with a small portion of ferrous ammonium sulfate and DTT (less than the amount necessary for the concentration step). It is then important to mix the contents of the tube well and observe the color change. If the solution turns black, dark yellow, or orange, there is oxygen present in the protein fractions, and there will likely be reduced catalytic activity after the complete purification and concentration process. If the solution is a light lavender or very light-yellow color, there may be minimal O_2 present, but it is likely that the enzyme will still have high activity. The dark yellow to orange color change when oxygen is present is likely caused by oxidation of the iron in the ferrous ammonium sulfate to Fe(III), forming rust[44]. To fix this issue, it is recommended to degas the buffers and allow nitrogen to bubble through crude the protein solution for 1-2 extra minutes before placing them in the glove box. Also, it is important to ensure that the atmosphere in the glove box is truly O_2-free by using O_2-sensitive strips.

The last critical step, desalting the enzyme prior to kinetic characterization, requires an oxygen-free atmosphere and must be done on ice, as the purified protein is prone to denaturation at room temperature. Determining when the desalted protein comes off the column is essential to successful kinetic assays, as the most concentrated fractions give the most reproducible results. The fraction where the desalted protein is eluted must be determined every time a new batch of protein is purified and when the column is repacked with new resin. If activity is low during kinetic assays and the purification and concentration steps have been technically mastered, the issue may lie in the desalting step. When troubleshooting this step, it is crucial to check that the 50 mM Tris/10% t-butanol buffer is thoroughly degassed, repack the column with fresh Sephadex resin, and ensure that there are no holes in the glove bag.

After DesB has been successfully purified and desalted, kinetic assays using the oxygen electrode must be performed carefully to obtain data on catalytic activity of the enzyme. Kinetic measurements are typically reproducible when a catalytically active and concentrated protein is used. If data points are not reproducible when repeating a run for a substrate or inhibitor concentration data point, the issue may be that the protein has denatured or oxidized after extended use. Freshly desalted protein can be generated to allow continuation of data collection. It is important to retain all vials of the enzyme, so the exact protein concentration can be determined after completion of the data collection using a Bradford assay. This step is performed after the kinetics measurements because the enzyme loses activity over time, so performing it first may lead to lower activity and inaccurate kinetics measurements. The protein concentration is then used to convert observed catalytic rates into the reaction rates that are needed to

603 determine the turnover number. In addition to concerns about the loss of enzyme activity leading
604 to irreproducible kinetics results, degradation of the membrane covering the electrode after
605 extended use may also cause challenges when reproducing data. Membrane degradation is
606 typically indicated by an increase in the initial signal (from 250-350 nmol/mL to >350 nmol/mL)
607 or an inability to attain a stable background rate before enzyme addition (>±5 nmol/mL/min). If
608 either is observed, it is recommended to disassemble the electrode, clean any oxides off the
609 electrode using the supplied cleaning powder, reassemble the electrode, and recalibrate.
610
611 The method of anaerobic purification is very important for enzymes with a metal center that can
612 be oxidized, especially for those that have tightly bound metals which cannot be exchanged after
613 the protein folds. Although enzymes have evolved to protect themselves by coordinating oxygen
614 only after substrate coordination, they have done so in a cellular environment - the saturating
615 amounts of oxygen in an in vitro environment can lead to rapid oxidation of metals and the
616 conversion of Fe(II) into the inactive Fe(III) form[31]. This oxidation/inactivation can lead to skewed
617 results in which the enzyme is not in its catalytically active state. The kinetic assays using an
618 oxygen-sensitive electrode can be applied to enzymes that rely on oxygen as a substrate. Rather
619 than obtaining kinetics parameters using the change in intensity of substrate or product
620 absorption, this method allows for the visualization of oxygen consumption saturated in solution.
621 This method has been used previously with LigAB, another extradiol dioxygenase in the
622 protocatechuate dioxygenase superfamily that similarly relies on a Fe(II) in its active site to
623 coordinate and cleave its substrates.
624
625 This manuscript also provides additional information about the enzyme DesB from *Sphingobium*
626 sp. strain SYK-6. Following the work that defined the enzymatic function and structure of
627 DesB[10,19,39], it was determined herein that DesB is a competent catalyst of the dioxygenation of
628 gallate, with k_{cat} of 17.8 ± 1.0 s^{-1} and K_m of 45 ± 13 μM, resulting in k_{cat}/K_m of 3.98 x 10^5 M/s. These
629 rates are comparable to those determined for other dioxygenase enzymes, including LigAB (k_{cat}
630 of 51 s^{-1} and k_{cat}/K_M of 4.26 x 10^6 $M^{-1}s^{-1}$) and other dioxygenases which have k_{cat}/K_m values ranging
631 from 10^5-10^8 $M^{-1}s^{-1}$ [36,45-50].
632
633 The DesB active site was previously shown to be at the dimer interface, with residues from both
634 monomers contributing to the coordination of substrate [residues originating from the monomer
635 that binds the Fe(II) are indicated by their residue number, while residues from the other
636 monomer are indicated by their residue number and a prime (*i.e.,* Glu377')][6]. In the absence of
637 structural information showing the binding interactions of 4NC with DesB, the structural
638 similarities and differences between gallate and 4NC can provide insight into how DesB might be
639 inhibited by 4NC. Gallate has three hydroxyl substituents at C3, C4, and C5, with its C3 and C4
640 hydroxyls being coordinated to the Fe(II) center, and the C5 hydroxyl being coordinated by
641 Glu377' (**Figure 8**). The carboxylic acid group at C1 is coordinated by Tyr-391', Tyr-412', Thr-13,
642 and Thr-267 in the DesB active site. 4NC, which proved to be a modest inhibitor of DesB, has two
643 hydroxyls available to coordinate the Fe(II) center and one C1 nitro group that is isosteric to a
644 carboxylate (while also having two oxygens for coordination by residues 391, 412, 13, and 267),
645 but is much more electron-withdrawing than the carboxylic acid on gallate. Since 4NC displayed
646 only 36.6% inhibition of the DesB dioxygenation of gallate when the inhibitor was present in 5-

fold excess over substrate, it is unsurprising that it was not a very potent inhibitor (with a K_i of 2.3 ± 0.3 mM). This suggests that the C5 hydroxyl and C1 substituent play a significant role in promoting the enzyme-ligand complex. Since residues Glu377', Tyr-391', and Tyr-412' are all implicated in these interactions, this suggests that DesB active site contacts with adjacent monomers are important for the placement of a compound and structuring the active site.

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The authors have no competing financial interests or other conflicts of interest.

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