Chapter II

Methodology

II.A. Introduction to and characterization of Cp

Clostridium pasteurianum (Cp) is a Gram-positive bacterium with a low-G+C content from the class *Firmibacteria.*[46] It is a strict anaerobe found widespread in soil and a diazotroph capable of producing endospores.[46] The Rees group originally obtained lyophilized Cp (strain W5, accession number 6013) from the American Type Culture Collection (ATCC) in the early 1990's.[20] To verify the identity of the bacterium, I performed several experiments. Microscopy images of a liquid culture show the general rod-like shape of Cp when it is healthy and at the spore-producing phase (Figure II-1). The gel from polymerase chain reaction (PCR) using bacterial and universal primers for the RNA of the small ribosomal subunit (16S rRNA) gene (Figure II-2) shows one band at ~1500 nucleotides with the bacterial primer and one band at ~1000 nucleotides with the universal primer. Lack of additional bands indicates that the Cp culture is free from non-bacterial contamination. Also, RNA sequencing of the small ribosomal subunit gave an identity of 99% (589/596), confirming that the culture is Cp.

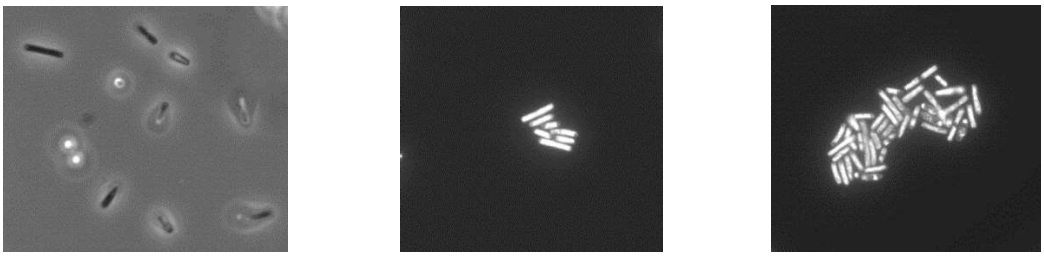


Figure II-1. All images are at 1000x magnification (left) Live view of Cp culture. Short rods and spherical objects are Cp cells orientated at an angle. (middle) DAPI stained Cp culture fixed on a glass slide. (right) DAPI stained Cp culture at the spore-producing phase.

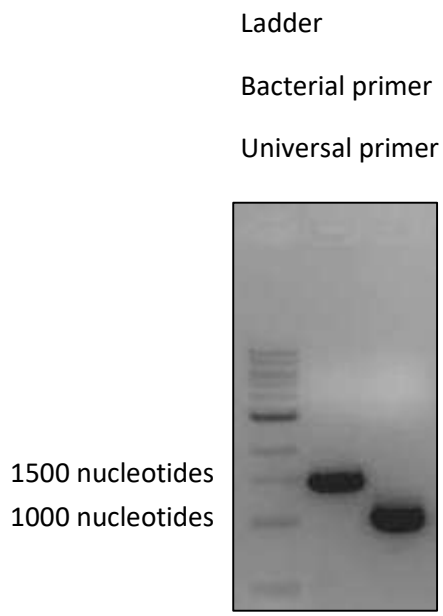


Figure II-2. Gel of PCR product using bacterial and universal primers for the RNA of the small ribosomal subunit (16S) gene. The gels show that there is no non-bacterial contamination.

II.B. Cell growths

II.B.i. Growth in mineral and potato media

Cp cultures were permanently stored in 1 mL aliquots (0.5 mL culture and 0.5 mL 50% sterilized glycerol) in liquid nitrogen. The frozen cell stock was replenished from 100 mL mineral medium cultures in the growth phase (<24 hours growth). Live cultures were maintained on mineral medium agar plates with no ammonium source at 4° C. New plates were streaked once per month. Every six months, new plates were streaked from frozen cell pellets; two consecutive generations of plate growths were required to obtain healthy cells. Agar plates were prepared with mineral medium according Table II-1. Contrary to liquid medium cultures, no ammonium chloride or calcium carbonate was added. 1.5% agar was added to the medium for plates prior to autoclaving. Calcium chloride was not added until after autoclaving, and a 100x calcium chloride stock was used rather than 1000x as for liquid medium cultures. This prevents calcium

phosphate precipitation, which appears as small white flakes in the cooled gel. Media for plates was generally made at volumes of 400 mL (20 plates).

To prepare cells for harvesting from a 15-L growth in a carboy, five new plates were streaked near an open flame with a sterilized loop. Plates were incubated upside-down at 30° C in a sealed jar kept anaerobic with an Oxoid AnaeroGen sachet for 36-48 hours. If plates were streaked from freshly grown plates at 30° C, only 36 hours was required for full growth. If plates were streaked from plates stored at 4° C, 48 hours was required for growth.

While the plates incubated, all media, long needles (~18 Gauge; used for purging the media of oxygen), rubber stoppers, and filters (made by inserting half a cotton ball into a Luer-Lok syringe) were prepared and autoclaved. Liquid mineral media were prepared in Erlenmeyer flasks with at least three times the headspace volume as liquid volume to allow room for gas production during cell growth. The openings of the media flasks were covered with aluminum foil and sealed with parafilm upon cooling to room temperature after autoclaving. Media stored at room temperature was not used if made more than three weeks in advance.

Once the plates were ready, two flasks of 100 mL mineral media were purged with filtered nitrogen gas. The flask opening remained completely covered with aluminum foil to avoid exposure to contaminants and air. The required amounts of phosphate, biotin, and ammonium chloride were added at the start of the purging. The phosphate was *slowly* added and while shaking the flask. After about 30 minutes, cells were inoculated into media from plates near an open flame. Upon opening the anaerobic jar, one plate was immediately sealed with parafilm and stored at 4° C as the new master plate. To the other four plates, working two plates at a time, 4 mL of the purged media was transferred to the plate. Cells were suspended by gently rubbing a cooled, flame-sterilized cell spreader around the plate. The suspended cells were inoculated into the purged media. The cultures continued to purge for another 20

minutes, after which the purging needle was slowly removed as a sterilized rubber stopper was simultaneously depressed into the flask on top of the foil. The stopper was pushed down as far as possible on top of the foil, and then the flask was completely sealed with parafilm and tape. Cultures were incubated at 30° C and 200 rpm for 18 - 24 hours. The optical density (OD) of the cultures was monitored visually or with a UV-vis spectrophotometer at 600 nm. Cultures grew to an OD_{600} of 6-10. Healthy cultures had a small layer of foam (0.5-1 cm). Generally, two liquid cultures were grown in duplicate. The pH range for optimum growth is 5.8 to 6.5.[47] The calcium carbonate and phosphate in the liquid mineral medium act as pH buffers.[47]

Cultures were scaled up to 250 mL by inoculating from the 100 mL starter cultures at a dilution of 1:10. 250 mL cultures were grown for ~12 hours. The culture was then scaled up to 1.5 L with a starting OD_{600} of 0.5-0.8. For the 1.5-L cultures, the media was purged using a tubed septum rather than needles, as illustrated in Figure II-3. 1.5 L cultures were grown for 8-9 hours.

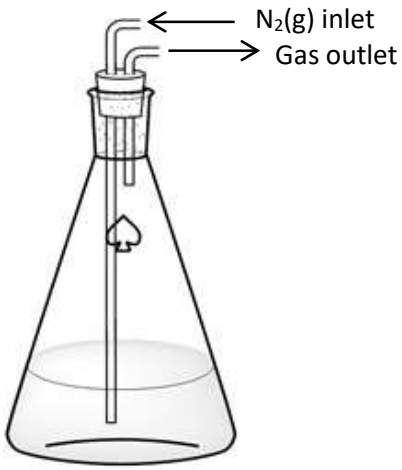


Figure II-3. 1.5 L cultures were purged through glass tubes fitted into the rubber stopper.

Figure II-4. The carboy setup for 15-L cell growths. Heating tape was wrapped around the carboy. Another layer of aluminum foil was wrapped around the outside of the carboy (not shown) to avoid heat loss to the atmosphere. A stir bar at 300 rpm was used to keep the culture agitated. A thermometer between the first aluminum foil layer and the carboy was used to monitor the approximate temperature of the culture. Continuous nitrogen was given to the carboy through rubber tubing. The nitrogen flow rate was monitored from bubbling the out-gas into a flask of water.

While the 1.5 L culture was growing, the 20 L carboy was prepared with 15 L of mineral medium. The setup is shown and described in Figure II-4. The carboy was purged with nitrogen gas for at least six hours prior to inoculation. During this time, the temperature stabilized at 30° C. One hour before inoculation, the appropriate amount of phosphate, biotin, and ammonium chloride was added. Only a 2.5 mM ammonium supplement was provided to activate nitrogenase expression. (The pre-cultures may also be grown on limited or no ammonium supplement; however, it was easier to keep cells alive with an ammonium supplement.) After adding all solutions to the carboy, the nitrogen flow rate was increased to a vigorous bubble: higher flow rates increased nitrogenase activity.

The OD_{600} of the carboy was measured at inoculation and every hour starting at 5 hours. Nitrogenase activity (section II.D.) was measured every hour starting at 5 hours. Just after the activity/OD_{600} peaked, cells were harvested by collecting the culture in 1-L centrifuge bottles on ice. (Stirring was turned off during collection to avoid collecting calcium carbonate.) The culture was centrifuged in several batches at 7000 rpm for 20 minutes and 4° C. The supernatant was discarded and the cell pellets were immediately frozen in liquid nitrogen. From a 15 L culture, it was common to obtain >50 g cell pellet, which was stored in liquid nitrogen or at -80° C. The cell pellets are spongy as a result of gas escaping from the cells and are light brown in color.

For all cell growths, the starting OD_{600} of each culture was controlled to avoid overgrowth. Overgrown cultures sometimes experienced an acid crash as a result of too much butyric and other acids. This causes the Cp metabolism to shift toward the production of alcohols and is characterized by a sweeter smell than the typical rancid smell of butyric acid.[49–52]

Table II-1. Recipe used to make mineral media

Components of mineral medium	Quantity needed for various culture sizes			
	100 mL	250 mL	1.5 L	15 L
Sucrose (g)	2	5	30	300
Water (mL)	90	225	1350	13.5 L
Mineral stock (1000x, mL)	0.1	0.25	1.5	15
FeMoCa (500x, mL)	0.2	0.5	3	30
$CaCl_2$ stock (1000x, mL)	0.1	0.25	1.5	15
$MgSO_4$ stock (1000x, mL)	0.1	0.25	1.5	15
$CaCO_3$ (g)	0.5	1.25	7.5	~17 **
Phosphate stock (10x, mL) *	10	25	150	1500
NH_4Cl (3M, mL) for 10 mM *	0.333	0.8325	4.995	12.5 (2.5 mM)
Biotin (100x, mL) *	1	2.5	15	150

* Chemicals added before inoculation when the media is purged with nitrogen.
** Limited $CaCO_3$ is added to the carboy media to prevent the stir bar from sticking in the solid.

Table II-2. Recipes for mineral media stock solutions

Chemical	Mol. Wt. (g/mol)	Conc. (mM)	Mass per volume (g)			Notes
			500 mL	1 L	1.5 L	
Mineral stock (1000x)						Do not autoclave
$CoCl_2 \cdot 6H_2O$	237.93	1		0.24		
$CuSO_4$	159.61	1		0.16		
$ZnCl_2$	136.28	1		0.14		
$MnSO_4 \cdot H_2O$	169.02	10		1.69		
$NiSO_4 \cdot 6H_2O$	262.86	1		0.26		
Phosphate stock pH6.4 (10x)						Autoclave separately
KH_2PO_4	136.09	800	54.44	108.87	163.31	
Na_2HPO_4	141.96	200	14.20	28.39	42.59	
3 M NH_4Cl stock						Autoclave separately; make batches 500 mL or less
NH_4Cl	53.49	3000	80.24			
0.9M $CaCl_2 \cdot 2H_2O$ (1000x)						Only make in 500-mL batches
$CaCl_2 \cdot 2H_2O$	131.02	900	66.2			
1.67M $MgSO_4 \cdot 7H_2O$ (1000x)						Only make in 500-mL batches
$MgSO_4 \cdot 7H_2O$	246.47	1670	205.8			
FeMoCa (500x)						Dissolve citric acid in water first, then add Fe and Mo
Citric Acid$\cdot H_2O$	210.14	90		18.91		
$FeSO_4 \cdot 7H_2O$	278.01	18		5.00		
$Na_2MoO_4 \cdot 2H_2O$	241.95	1		0.24		
Biotin (100x)						Dissolve in 100% ethanol. Store at 4° C.
Biotin	244.31	1		0.24431		
Citric Acid						Do not autoclave
Citric acid monohydrate	210.14	1 M	105	210	315	

Potato medium was sometimes employed to jumpstart Cp growth from agar plates to liquid media. Although Cp readily grow in potato medium, the subsequent growths in mineral medium were more difficult to control, likely due to potato contaminations. To prepare potato medium (Figure II-5), 3-4 mm layer of calcium carbonate was added to the bottom of each of ten test tubes (16 x 150 mm). A fresh (Russet) potato from the supermarket was sliced into small cubes about 0.5 x 0.5 x 0.5 cm^3. Each test tube was filled one-third (~5 cm) with potato cubes and then two-thirds (~10 cm) with 2% sucrose solution. Tubes were capped with a metal cap and autoclaved.

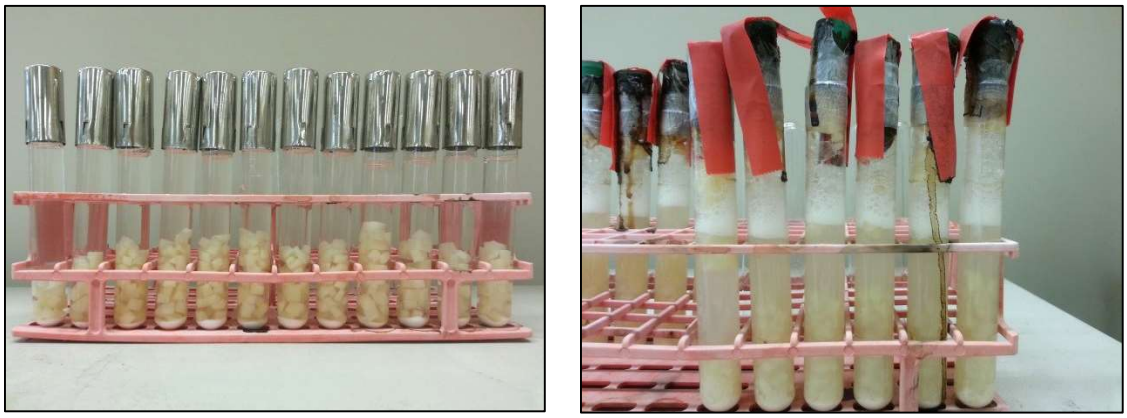


Figure II-5. (left) Potato tubes before autoclaving, showing the relative amounts of $CaCO_3$, potato cubes, and sucrose solution. (right) Potato tubes with fully grown Cp ready to be inoculated into mineral media.

Immediately after autoclaving, the metal caps were removed, and the top of each tube was plugged with half a sterile cotton ball. ~5 drops each of concentrated potassium carbonate and pyrogallol were added to each cotton ball to remove oxygen from the tube. Tubes were promptly and tightly capped with a rubber septum and then allowed to stand at room temperature to cool. While cooling, ~5 mL mineral medium was added to plates to suspend cells. To inoculate the potato tubes, the cotton plug was removed and 1 mL suspended cells was added to each tube. A new cotton plug was placed in each tube with the same treatment of alkaline pyrogallol. The rubber stoppers were placed back in each tube, and each tube was sealed with parafilm and tape. (The tape is necessary so the rubber stoppers do not eject during cell growth.) Cells were ready to inoculate into mineral media once vigorous bubbling was observed and the potato culture was opaque (Figure II-5). During inoculation into mineral media, care was taken to not transfer potato chunks.

II.B.ii. Growth curves

Cp growth patterns depend on the amount of ammonium supplied to the mineral media.[53] To determine the optimum level of ammonium, carboy growths were performed at different initial concentrations of ammonium chloride. The cultures were monitored until activity ceased. The results are shown in Charts II-1, 2, and 3. A description of activity calculation is provided in section II.D.ii. Although lack of ammonium supplement produces the highest activity, the peak activity occurs at a low OD (Chart II-1), which is unfavorable for harvesting large quantities of cells. The opposite is observed with 10 mM ammonium supplement: the peak activity is low and occurs at a high OD (Figure II-3). The best condition is with a limited ammonium supplement in which the peak activity per OD occurs at a reasonably high activity and OD (Chart II-2).[53]

All 15 L harvest cultures were provided with 2.5 mM ammonium supplement (Chart II-4). Growth increases at a steady rate while the initial ammonium supplement is consumed (~0-4 hours), after which the growth reaches a lag phase where the cells begin to express nitrogenase (~4-6 hours). Once a sufficient amount of active nitrogenase is available, cell growth resumes using of the ammonium produced by nitrogenase (~6-9 hours). Cells are harvested after the activity/OD peaks, ~9 hours.

Chart II-1. 15 L culture with no ammonium supplement

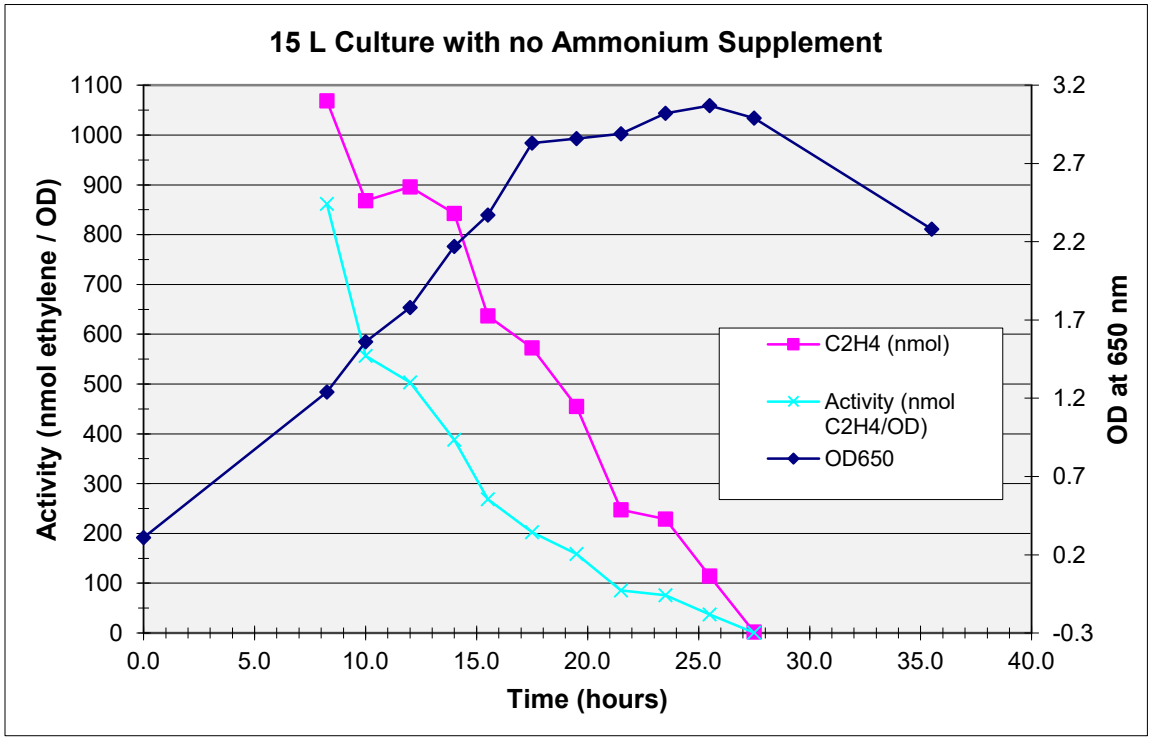


Chart II-2. 15 L culture with 2.5 mM ammonium supplement

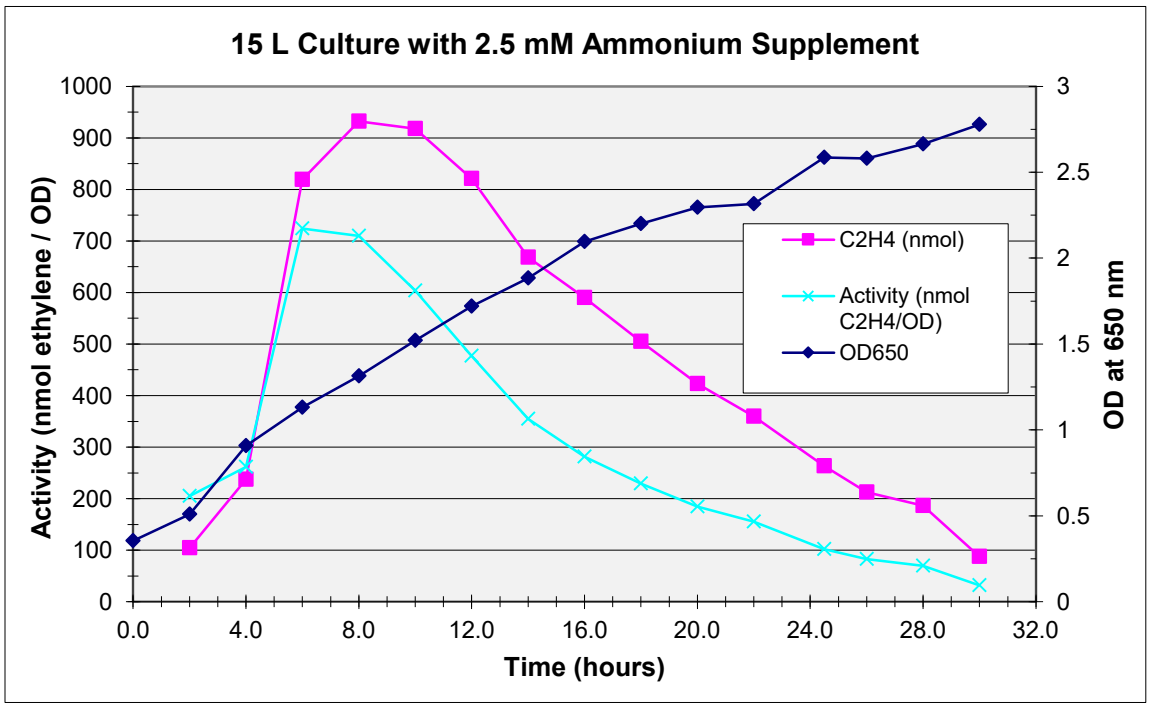


Chart II-3. 15 L culture with 10 mM ammonium supplement

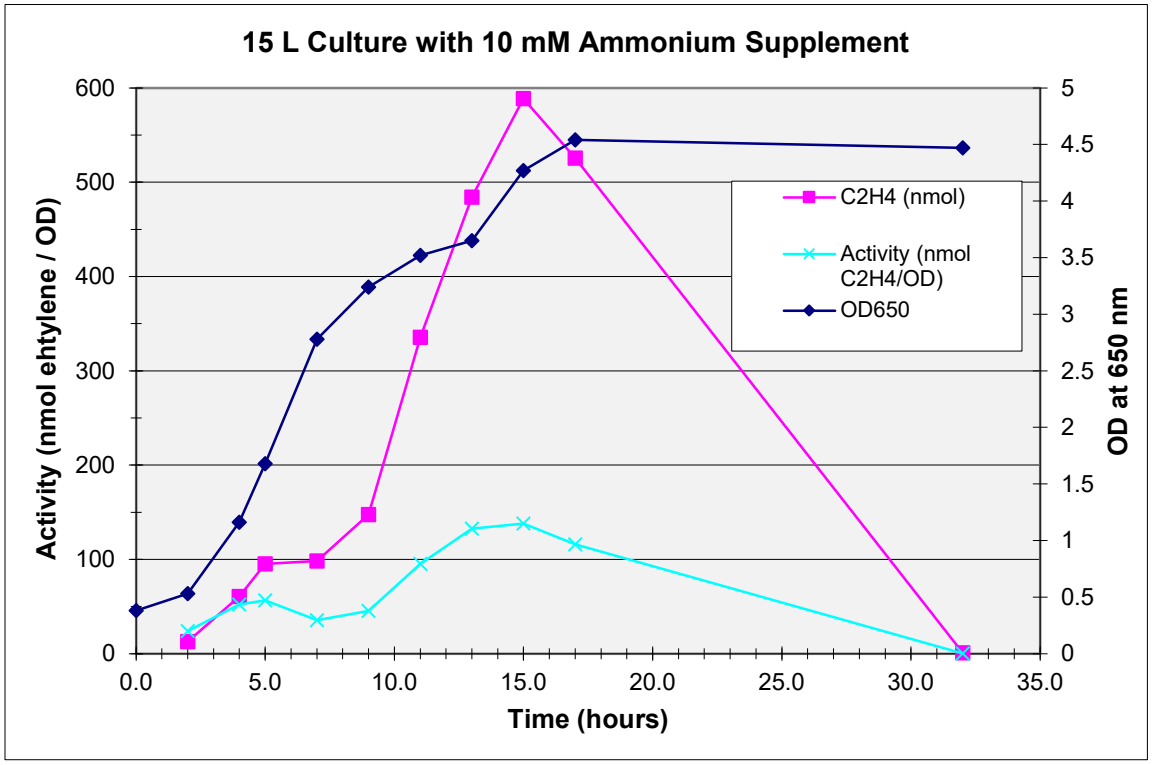
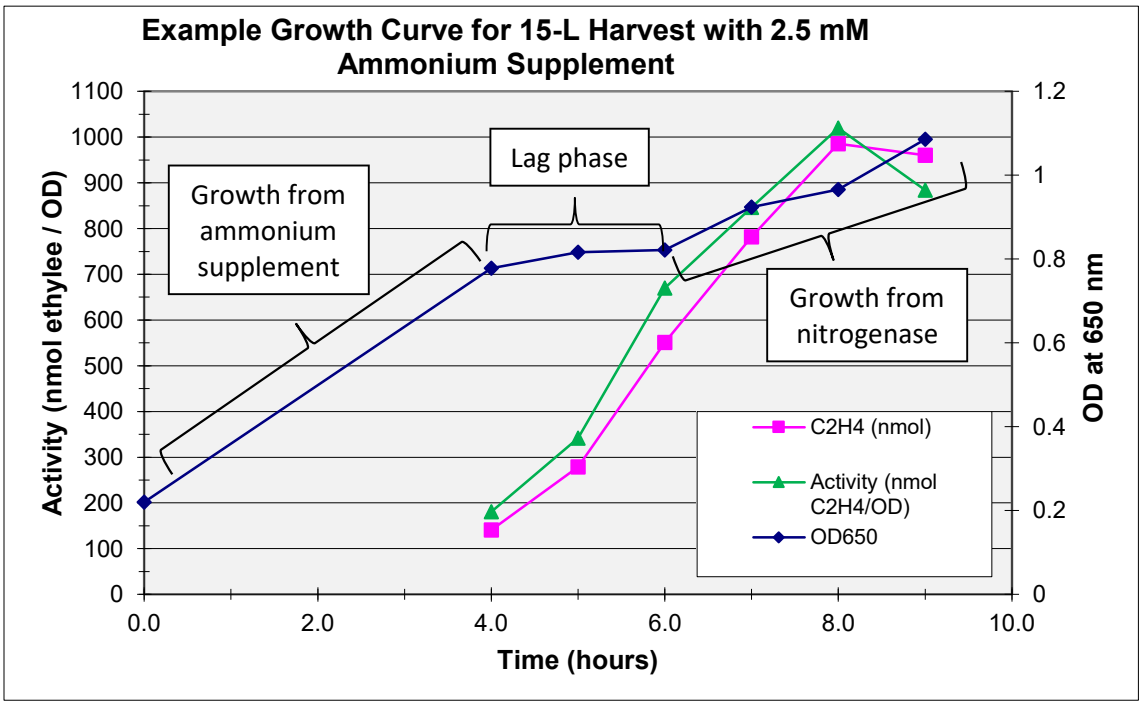


Chart II-4. Example growth curve for harvest cultures at 2.5 mM ammonium supplement



II.C. Protein purification

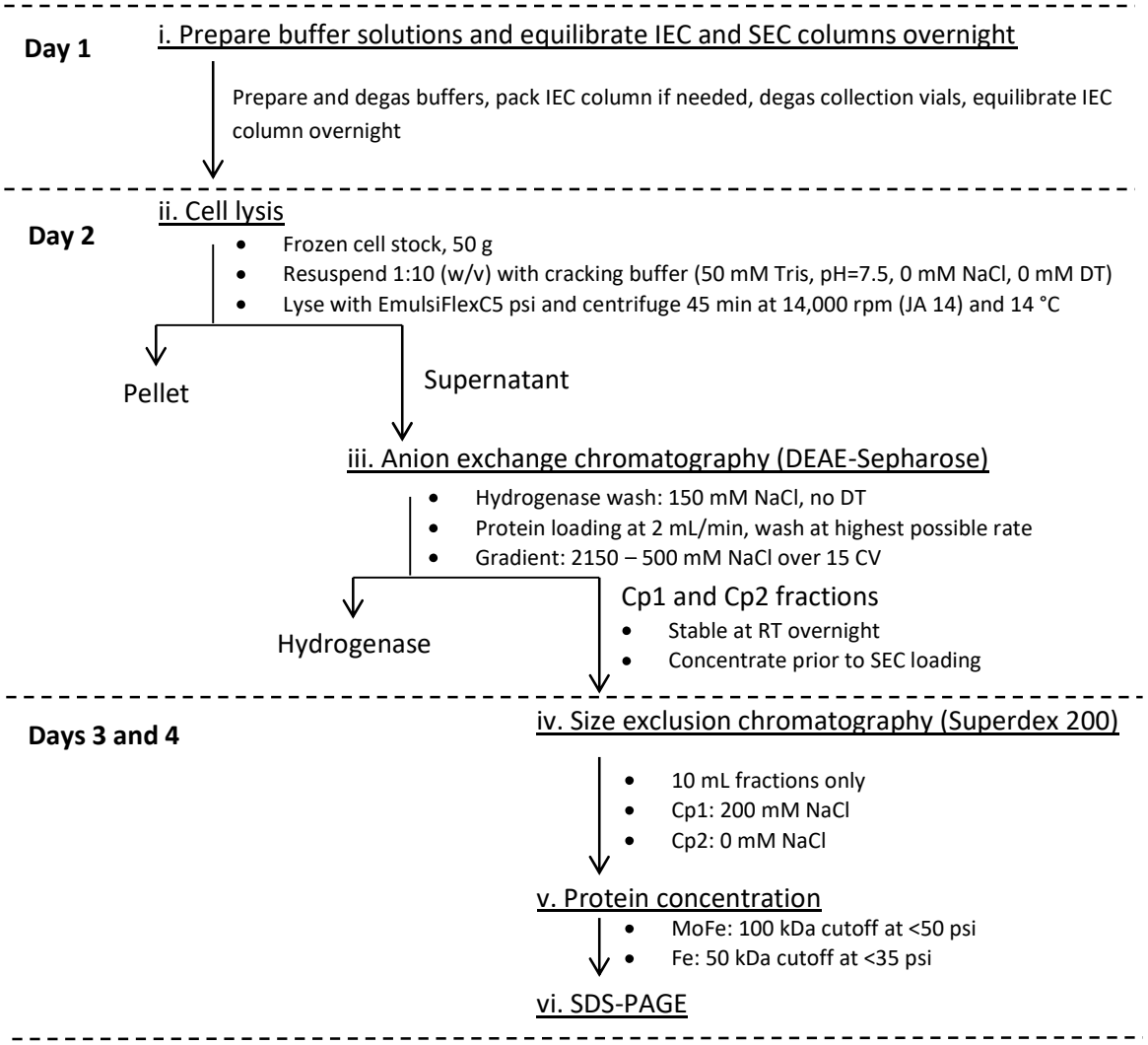


Figure II-6. Flow diagram of the Cp1 and Cp2 purification process.

II.C.i. Preparation of IEC and SEC columns and buffer solutions

II.C.i.a. IEC column

Diethylaminoethyl (DEAE) sepharose, a positively charged resin, was used for ion exchange column chromatography (IEC). If the column had no cracks and protein did not precipitate during the previous purification, the resin was recycled without unpacking the

column. In this case, 2-3 column volumes each of the following solutions (in order) were run through the column: 1 M NaCl, 100 mM NaCl, 1 M NaCl, 100 mM NaCl, water, 0.5 M NaOH, water, 0.5 M HCl. The column was stored in 20% ethanol if not in use for longer than a few days.

Unpacked resin was stored in a plastic bottle at 4° C in 30% ethanol with 50 mM Tris/HCl pH 8.0. To recycle the resin for use, the resin was soaked in 6 M urea for at least two hours. Next, the following washing procedure was executed: wash resin with 8 L water; suspend resin in 1 L of 0.5 M sodium hydroxide in a plastic beaker for 30 minutes; wash with 10 L water; suspend in 1 L of 0.5 M HCl for 30 minutes; wash with 12 L water. All wash cycles were performed using vacuum filtration and a large Büchner funnel with filter paper. The resin was never allowed to dry out while filtering. During the suspension stages, the resin was stirred occasionally with a glass rod. Prior to packing the column, the recycled resin was degassed on the vacuum manifold with eight cycles of 5-minute vacuum and 30-seconds argon. Instead of using a stir bar, which could sheer the resin particles, the flask was manually shaken. Generally, a column diameter of 2.5 cm was selected and packed no taller than 20 cm (~100 mL of packed resin). After packing, the IEC column was washed with water to remove any ethanol, which could cause NaCl in the buffer solutions to precipitate. After several column volumes of water were washed through the column, it was equilibrated overnight with a buffer of the same salt concentration as the cracking buffer (buffer A).

II.C.i.b. SEC column

A typical size exclusion column (SEC) included ~475 mL of Superdex S200 resin. After washing with water, the column was equilibrated overnight with the E_F (elution) buffer. After use, the column was flushed with more elution buffer, washed with several column volumes of water, and then stored under 20% ethanol. Flow rates never exceeded 2 mL/min.

II.C.i.c. Buffer solutions

Buffer solutions were prepared according to Table II-3. First, NaCl and Tris base were dissolved in water. After the solids completely dissolved, the pH was lowered with 6 M HCl. The buffers were diluted to the appropriate volume, after which all buffers (excluding the cracking buffer) were vacuum filtered. The cracking buffer and small volume of buffer A were transferred to 2 L and 1 L round bottom flasks, respectively, with a stir bar and capped with a septum. The other buffers were transferred to large round bottom flasks (3-5 L) and capped with an adapter that supports the inward flow of argon and the outward, anaerobic flow of buffer to the column. The buffers were degassed on the vacuum manifold with 10 cycles of 10-minutes vacuum and 2-minutes argon. Flasks were occasionally manually shaken during the vacuum cycles. Wheaton vials used to collect fractions eluted from the columns were also degassed on the Schlenk line.

After degassing was complete, the cracking buffer and small volume of buffer A were transferred to the anaerobic tent. The cracking buffer was divided into two portions: 500 mL and 250 mL. To the three 5-L flasks still on the Schlenk line, sodium dithionite ($Na_2S_2O_4$) solutions were added according to Table II-4, after which three additional cycles of vacuum and argon were performed.

The IEC column was equilibrated overnight using a combination of buffers A and B equal to the cracking buffer salt concentration. Also, the following materials were brought into the anaerobic tent in preparation for use: protein concentrating systems, four 250-mL centrifuge bottles, homogenizer, 100% glycerol (degassed), three 1000-mL round bottom flasks (for cell suspension, collecting lysed cells, and IEC loading protein), 1000 mL beaker and stir bar, 500 mL round bottom flask, assorted sizes of auto-pipettes, and a 500 mL graduated cylinder.

Table II-3. Buffer solutions for protein purification experiments

Buffer	Purpose	NaCl		Tris base		pH	Amount (L)	Filtered?
		mM	g	mM	g			
Cracking	Lyse cells	50	3.0	50	6.06	7.75	1	no
Buffer A	IEC	0	0	50	18.2	7.75	3	yes
Buffer A	Dilute Fe protein after IEC	0	0	50	3.03	7.75	0.5	yes
Buffer E_F	SEC, Fe protein	50	8.8	50	18.2	7.75	3	yes
Buffer E_M	SEC, MoFe protein	200	35.1	50	18.2	7.75	3	yes
Buffer B	IEC	500	58.5	50	12.1	7.75	2	yes

Table II-4. Sodium dithionite solutions added to buffers

Buffer	Amount	$Na_2S_2O_4$ (mM)	0.5 M Tris base (mL)	$Na_2S_2O_4$ (g)	Quantity
Cracking buffer & buffer A (for Fe protein dilution)	500 mL	5	2.5	0.435	2
Cracking buffer	250 mL	50	12.5	2.176	1
Buffers A, E_F, E_M	3 L	5	15	2.612	3
Buffer B	2 L	5	10	1.741	1

II.C.ii. Cell lysis

Cells were lysed under pressure with an EmulsiFlex C5 (Figure II-7). Frozen cell stock was thawed in cracking buffer (typically 50 g cells in 500 mL cracking buffer) with stirring until the suspension was homogenous (~20 minutes). 200 µL glycerol was added as an anti-foam reagent. In 50 mL portions, the cell suspension was homogenized and collected in a 1000-mL round bottom flask. The cell suspension, an empty 1000 mL flask, and 250 mL cracking buffer with 50 mM dithionite (flush solution for EmulsiFlex) were removed from the tent, put under positive argon pressure, and placed in an ice bath (to dissipate heat acquired from the EmulsiFlex during lysis). The EmulsiFlex was washed with water and the flush solution, after which the cell suspension was ruptured at ~2500 psi. The lysate was collected in the empty 1000 mL flask. After use, the EmulsiFlex was washed with flush solution and water and stored under 20%

ethanol. The cell suspension was centrifuged at 30,000 × g for 45 minutes at 15° C. A successful lysis yielded a dark brown supernatant and a pellet with three layers (lipids, cytosol, calcium carbonate). The supernatant was transferred to a round bottom flask for loading onto the IEC.

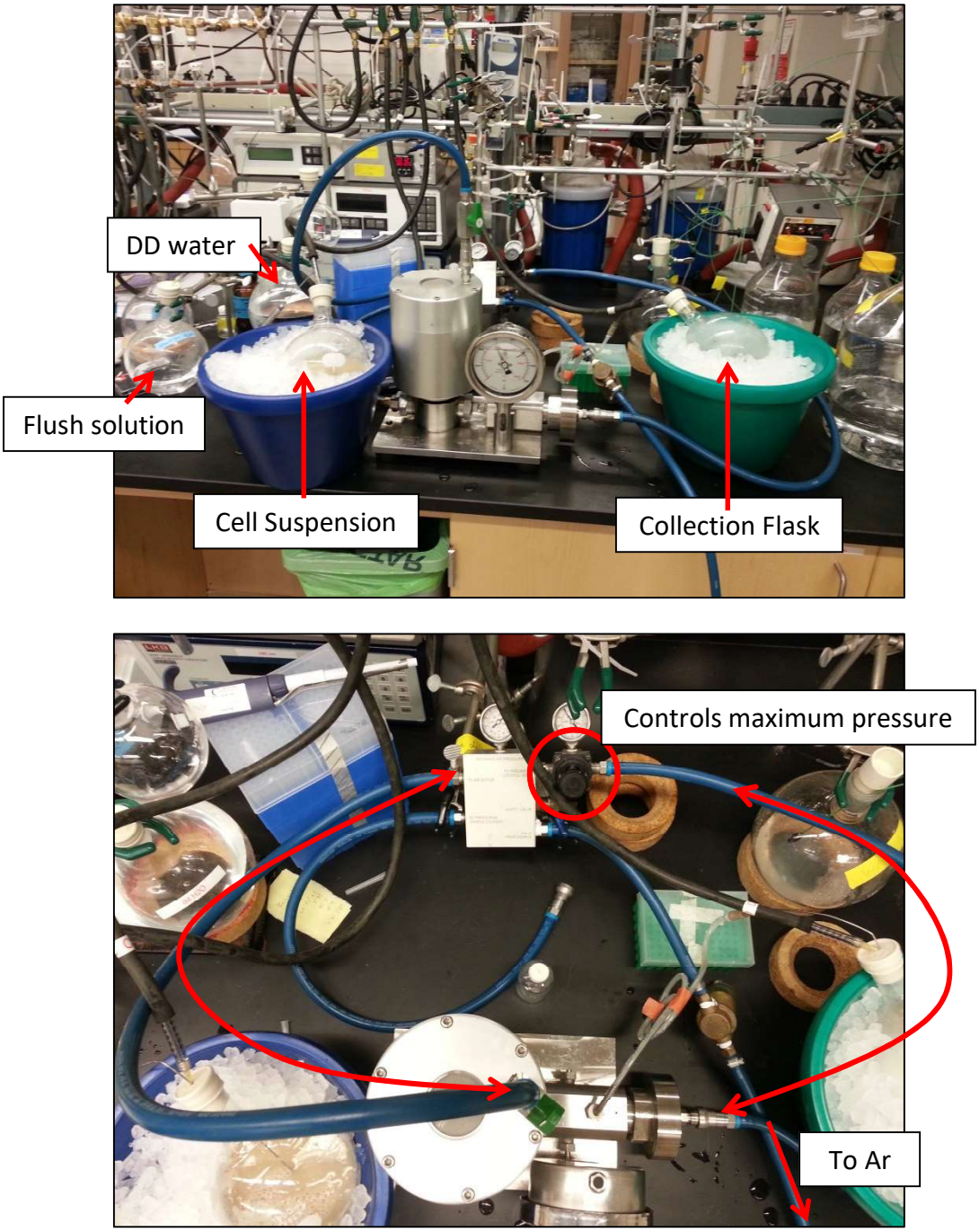
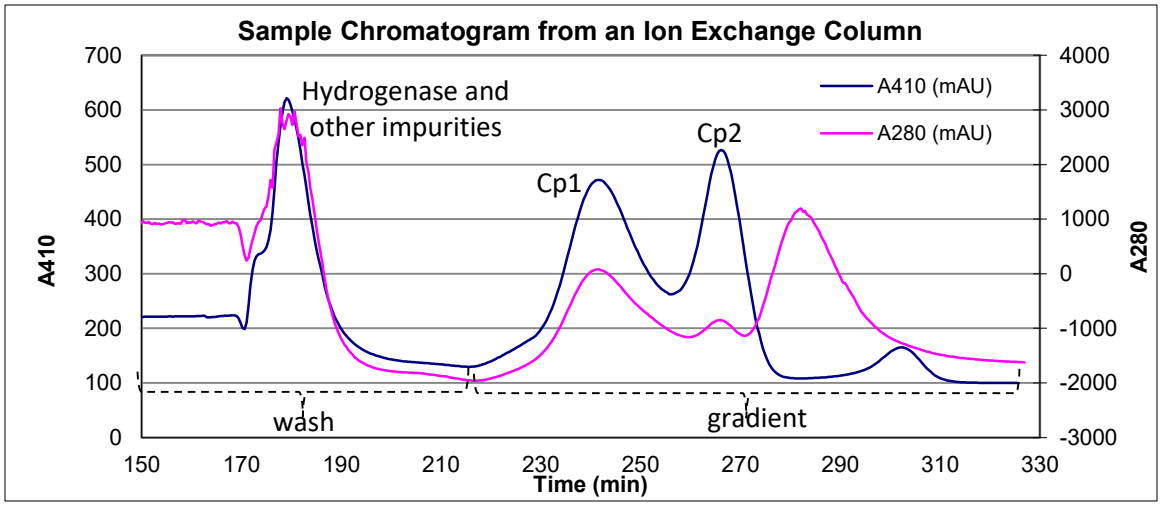


Figure II-7. Setup of cell lysis via the EmulsiFlex C5.

II.C.iii. Anion exchange chromatography

The flask containing protein was removed from the anaerobic tent and immediately put under positive argon pressure and placed on ice to avoid protein precipitation. Without introducing any air bubbles into the system, the protein was loaded onto the IEC at 2 mL/min through a long needle using an ÄKTA system (Pharmacia Biotech ÄKTA Explorer Air100). Since there is typically ~500 mL of protein solution, the loading process takes several hours. When experimenting with new buffers, the protein was loaded using an FPLC pump (which is less easily clogged compared to the ÄKTA) in case protein or lipids precipitated during loading.

Chart II-5. Sample chromatogram from the IEC



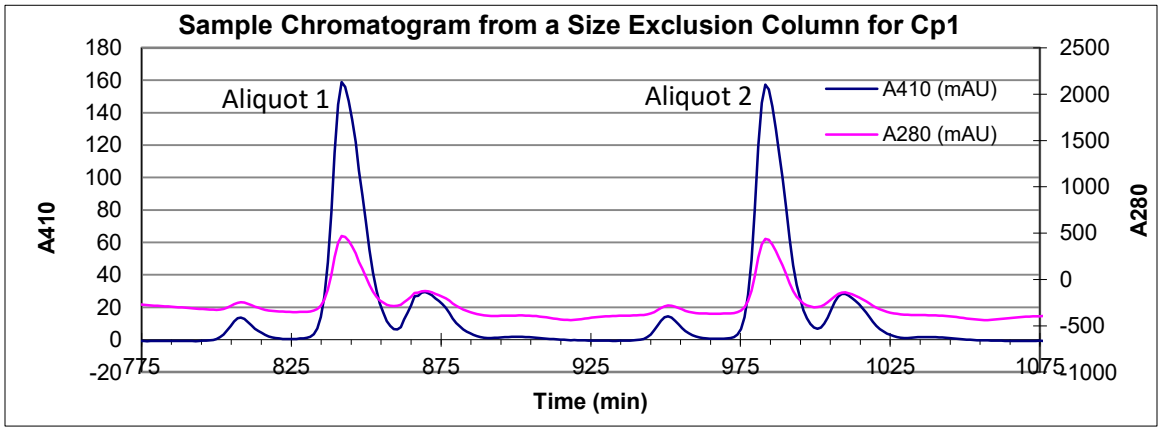
After protein loading was complete, elution was monitored at 280, 410, and 550 nm. For the column wash and elution, the flowrate was as fast as possible without exceeding a backpressure of 0.5 MPa (usually, 3.0-4.0 mL/min). The column was washed with 5-10 column volumes of 150 mM NaCl (mixture of buffers A and B) to elute hydrogenase and other unwanted material from the crude extract. After the column looked clean and no more peaks were visible in the chromatogram, a gradient was started using buffers A and B. A typical gradient was 150-

500 mM NaCl over 15 column volumes with flow rate of ~3-4 mL/min. Cp1 elutes first, typically ~250 mM NaCl; Cp2 follows closely behind at ~300 mM NaCl; ferredoxin elutes ~450 mM NaCl. A sample chromatogram is shown in Chart II-5. All bands were some shade of brown or black. Protein fractions were collected in anaerobic Wheaton vials. Cp1 and Cp2 were left overnight at room temperature if proceeding to SEC the following day; otherwise, the proteins were frozen and stored in liquid nitrogen. After running several column volumes of 500 mM NaCl over the IEC, it was washed according to the procedure previously described.

II.C.iv. Size exclusion chromatography

The SEC was washed with several column volumes of water and equilibrated overnight with E_F buffer. In the morning, the Cp1 and Cp2 solutions were concentrated in an Amicon Stirred Cell under argon pressure with a 100 and 50 kDa membrane, respectively. Concentrated protein was loaded on the SEC in 10-mL fractions at 1-1.5 mL/min and eluted at 2 mL/min using the buffers stated in Table II-3. The backpressure was not allowed to exceed 0.25 MPa. After collecting purified protein, the SEC column was flushed with additional elution buffer, washed with water, and stored in 20% ethanol. A typical SEC chromatogram is shown in Chart II-6.

Chart II-6. Sample SEC chromatogram



II.C.v. Protein concentration

Cp1 and Cp2 fractions were concentrated in an Amicon Stirred Cell under argon pressure using 100 kDa and 30 kDa cutoff filters, respectively. The pressure during Cp1 and Cp2 concentration was <50 psi and <35 psi, respectively. A Cary 300 Scan UV-visible spectrophotometer was used to determine the concentration of the purified protein. The instrument was first calibrated to the SEC elution buffer with 100% and 0% transmission. To 980 µL of the buffer, 20 µL of concentrated, purified protein was added. The cuvette was capped with a septum so maintain an anaerobic environment. The entire absorbance spectrum was collected. To determine the specific concentration, Beer's Law was employed: (sample dilution) * [(absorbance at 410 nm) - (absorbance at 280 nm)] * (extinction coefficient) * (path length, 1 cm) = protein concentration. The extinction coefficients for the Fe and MoFe proteins are 6.702 (at 430 nm) and 3.066 $mM^{-1}cm^{-1}$, respectively.[54] The target protein concentration was 25-35 mg/mL. The purified protein was frozen and stored in liquid nitrogen in 0.1 mL aliquots.

II.C.vi. SDS-PAGE

To confirm the purity and identity of the purified protein, sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was performed on small samples from all stages of the protein purification. Samples were prepared by adding 20-30 µL of loading dye to 50 µL of each sample. Highly concentrated samples, such as the purified protein, were diluted with water before adding loading dye. 1% SDS was added to any sample containing insoluble material, such as the cell pellet. 10 µL of each sample was loaded into 12% Tris/HCl gels as well as a protein ladder. Gels were run at 200 V for ~45 minutes, after which the gels stained for one hour and then de-stained for at least one hour. A typical gel is shown in Figure II-8.

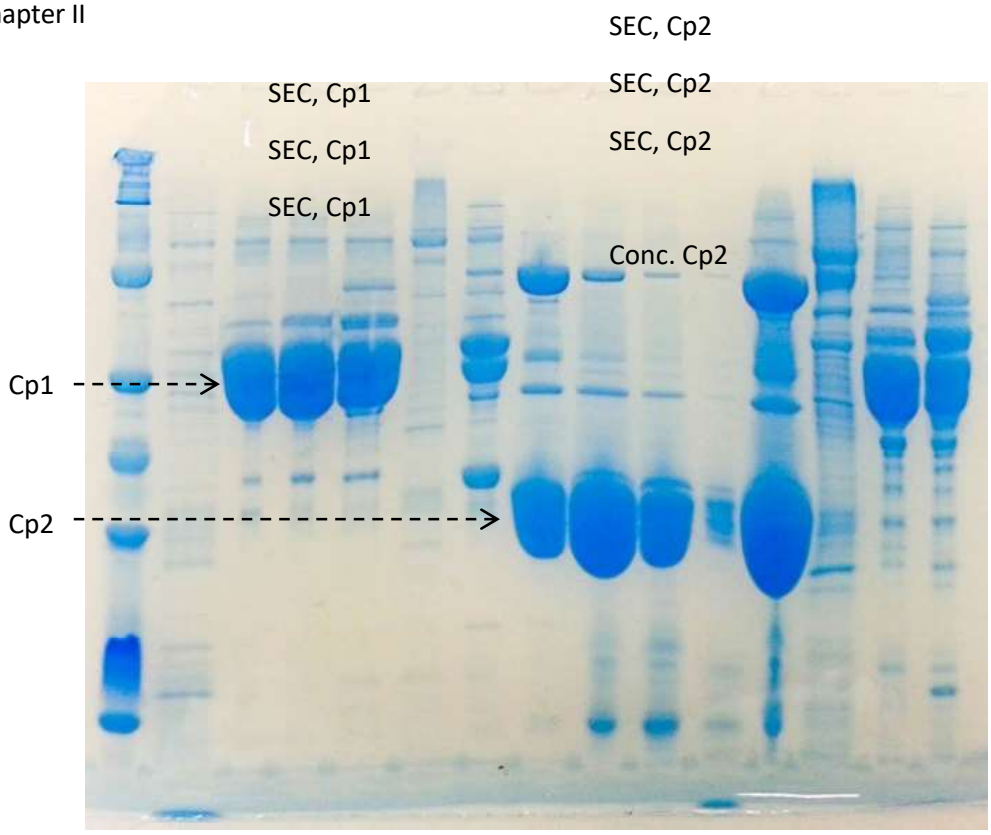


Figure II-8. Sample gel for protein purification.

II.D. Acetylene reduction activity assays

Although there are many substrates of nitrogenase,[55] acetylene reduction to ethylene is typically used to characterize the activity of nitrogenase because acetylene and ethylene are easily detected with high sensitivity using gas chromatography (GC).

II.D.i. Whole cell acetylene reduction assays

Whole cell assays were used to monitor nitrogenase activity during cell growth. Prior to starting the activity assays, a 500-mL sealed round bottom flask was filled with acetylene by evacuation of water with acetylene pressure. 2.5 mL of 5 mM sodium dithionite in 500 mM Tris base was injected into the flask. The dithionite solution was swirled around the flask, and the flask stood at room temperature for approximately one hour to remove excess oxygen.

Activity assay vials that have been in the anaerobic tent for at least 24 hours were capped with small rubber septa inside the anaerobic tent. Once removed from the tent, they were kept under positive argon pressure on the vacuum manifold. To perform the assays, 2 mL cell culture was injected into each of two assay vials. 1 mL acetylene was injected into the vials, and they were immediately placed in a water bath at 30° C and 120 rpm. After exactly 20 minutes, the assays were quenched with 0.25 mL concentrated citric acid. A negative control containing 2 mL water instead of cell culture was also prepared. A 50 µL sample of the headspace of each vial was injected into the GC. All GC runs were done at 110° C for 5 minutes. The area and percent area of the three peaks (methane/impurity at ~0.16 minutes, ethylene at ~0.8 minutes, and acetylene at ~2.3 minutes) were detected with a flame ionization detector (FID) and recorded. Calculations were done as described in section II.D.ii.

II.D.ii. Protein component acetylene reduction assays

Protein component acetylene reduction assays were performed to characterize the activity of purified protein and MoFe and Fe protein titrations. Each assay includes the MoFe protein, the Fe protein, an electron source (such as dithionite), ATP, an ATP regenerating system (creatine phosphokinase, phosphocreatine disodium, and magnesium chloride), and acetylene. The ATP regenerating system is required since ADP can inhibit nitrogenase.[56] Experimental details are provided in Table II-5. Solutions of 250 mM ATP, 2.5 mg/mL creatine phosphokinase (PCK), 100 mM phosphocreatine disodium (PC), and 500 mM $MgCl_2$ were made by dissolving the solid in assay buffer (0.5 M Tris/HCl at pH 7.5, 200 mM NaCl). ATP, PCK, and PC were kept on ice until added to the assay mixture in order to avoid degradation. Higher Tris concentration is used for the assays to ensure the pH is properly maintained, thereby avoiding decomposition of dithionite upon acidic conditions.

Experiment	Component ratio	Cp1 (mg)	Cp1 (nmole)	Cp2 (mg)	Cp2 (nmole)	Cp2/Cp1	Cp1 (µL)	Cp2 (µL)	Buffer (µL)
control	0	0.03	0.135	0	0	0	50	0	250
1	0.25	0.03	0.135	0.004	0.068	0.5	50	12.5	237.5
2	0.5	0.03	0.135	0.008	0.135	1	50	25	225
3	1	0.03	0.135	0.016	0.271	2	50	50	200
4	1.5	0.03	0.135	0.024	0.406	3	50	75	175
5	2	0.03	0.135	0.032	0.542	4	50	100	150
6	3	0.03	0.135	0.048	0.812	6	50	150	100
7	5	0.03	0.135	0.081	1.354	10	50	62.5 *	187.5
8	8	0.03	0.135	0.129	2.167	16	50	100 *	150
9	10	0.03	0.135	0.162	2.708	20	50	150 *	100
10	15	0.03	0.135	0.242	4.062	30	50	187.5 *	62.5
11	20	0.03	0.135	0.323	5.416	40	50	250 *	0

Table II-5. The following conditions lead to a total reaction volume of 1.300 mL (including 1.000 mL assay buffer and 0.300 mL protein and SEC buffer). Typically, experiments were performed in triplicate. The buffer used in the assays consists of 50 mM Tris/HCl and 200 mM NaCl at pH 7.5. Three protein stocks were prepared: Cp1 at 0.6 mg/mL, Cp2 at 1.29 mg/mL, and Cp2 at 0.32 mg/mL. Experiments requiring the more concentrated Cp2 stock solution are marked with an asterisk. A more concentrated Cp2 stock solution is necessary for the larger component ratios because otherwise an excessive amount of the more dilute Cp2 stock solution would be required in the assay mixture.

To 7.2 mL of a 50 mM Tris buffer (pH 7.5), 2 mL of the PC solution, 200 µL of the ATP solution, 100 µL of the $MgCl_2$ solution, and 500 µL of the PCK solution were added in the stated order (to avoid precipitation). 1 mL of this solution was added to each assay vial. Vials were purged on a Schlenk line with 12 cycles of 2.5-minutes vacuum and 0.5-minutes argon flush. During the purging, the acetylene flask was prepared as described in the previous section. The MoFe and Fe protein stock solutions were diluted in the anaerobic chamber as needed with a 50 mM Tris/HCl pH 7.5, 200 mM NaCl solution. The final protein dilutions contained 5 mM sodium dithionite. After purging, 50 µL of 500 mM sodium dithionite solution (dissolved in 0.5 M Tris/HCl pH 7.5, 200 mM NaCl) was added to each vial. The final composition of the assay mixture was 50 mM Tris/HCl at pH 7.5, 5 mM ATP, 5 mM $MgCl_2$, 20 mM PC, 2.5 mg/mL PCK, and ~19 mM sodium dithionite. The nitrogenase proteins are sensitive to salt concentration; therefore, the ionic strength should be kept constant (~at 48 mM NaCl) in each assay vial. Adjustments may be made using the salt concentration of the protein-free buffer as needed.

Prior to performing the titration experiments, a calibration curve was constructed by injecting 20, 30, and 40 µL of acetylene into the GC and determining the moles of acetylene in each measurement from the resulting peak area (Chart II-7). Three trials of each protein component ratio were typically run. For each assay vial, 1 mL headspace was replaced with 1 mL acetylene after which the vial was incubated at 30° C for 5 minutes. After incubating, MoFe protein and SEC buffer were added. Addition of Fe protein initiated the reaction. Assays were run for 10 minutes while incubating a water bath at 30° C and 120 rpm. After exactly 10 minutes, reactions were quenched with 0.25 mL concentrated citric acid. 50 µL of headspace was injected into the GC. All GC runs were done at 110° C for 5 minutes. The area and percent area of the methane/impurity, ethylene, and acetylene peaks were detected with a flame ionization detector (FID) and recorded.

To calculate the specific activity of the protein as a function of component ratio or dilution, ethylene formation is calculated for each vial by $v = (P/216.92)*((8.80-t)/0.05)/10$, where P is the original ethylene peak area reading and t is total volume (mL) of liquid in the vial (1.3 mL). 216.92 is the correction factor for converting the peak area to nanomoles ethylene; 8.80 refers to the total volume of the activity assay volume in mL; 0.05 refers to 50 µL headspace injected into the GC, and 10 is the reaction time in minutes. The ethylene peak area should be corrected by subtracting the ethylene peak area of a control assay to account for ethylene impurities in the acetylene. The maximum ethylene formation velocity is evaluated by Michaelis-Menton fitting of the resulting titration curve (titrant amount vs. v). The maximum velocity divided by the amount of titrated protein in the vial is the final specific activity presented in nanomole ethylene per minute per milligram protein. A sample titration curve is shown in Chart II-8.

Chart II-7. Sample calibration curve for acetylene reduction activity assays

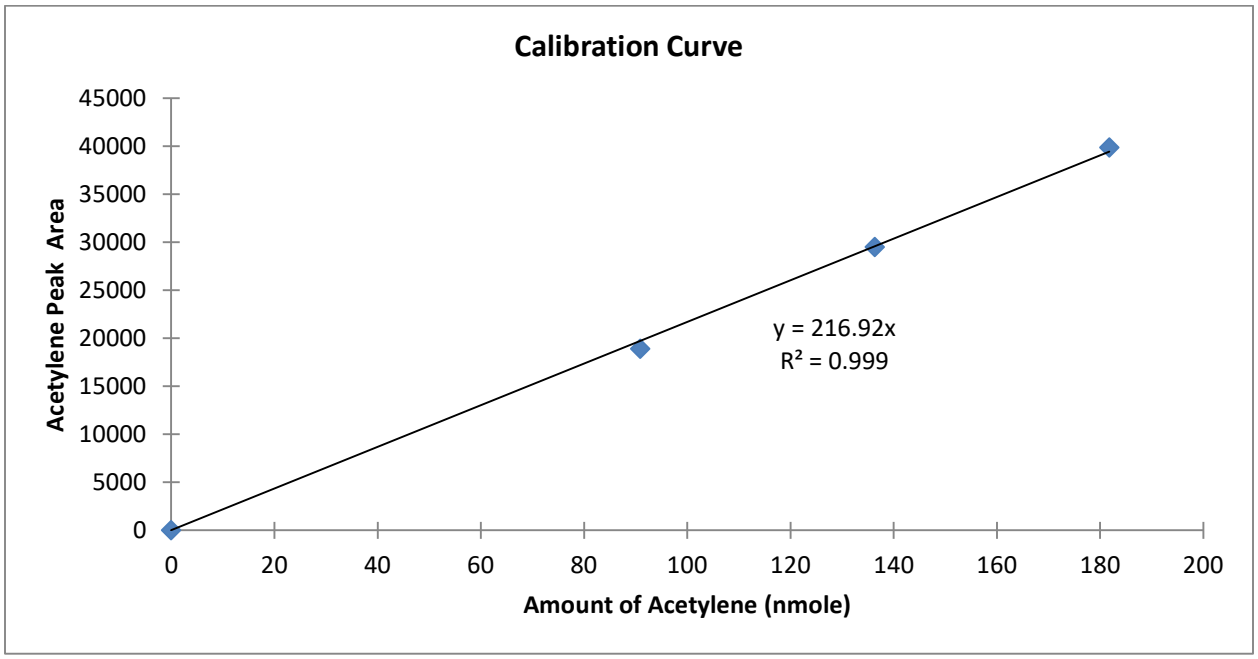
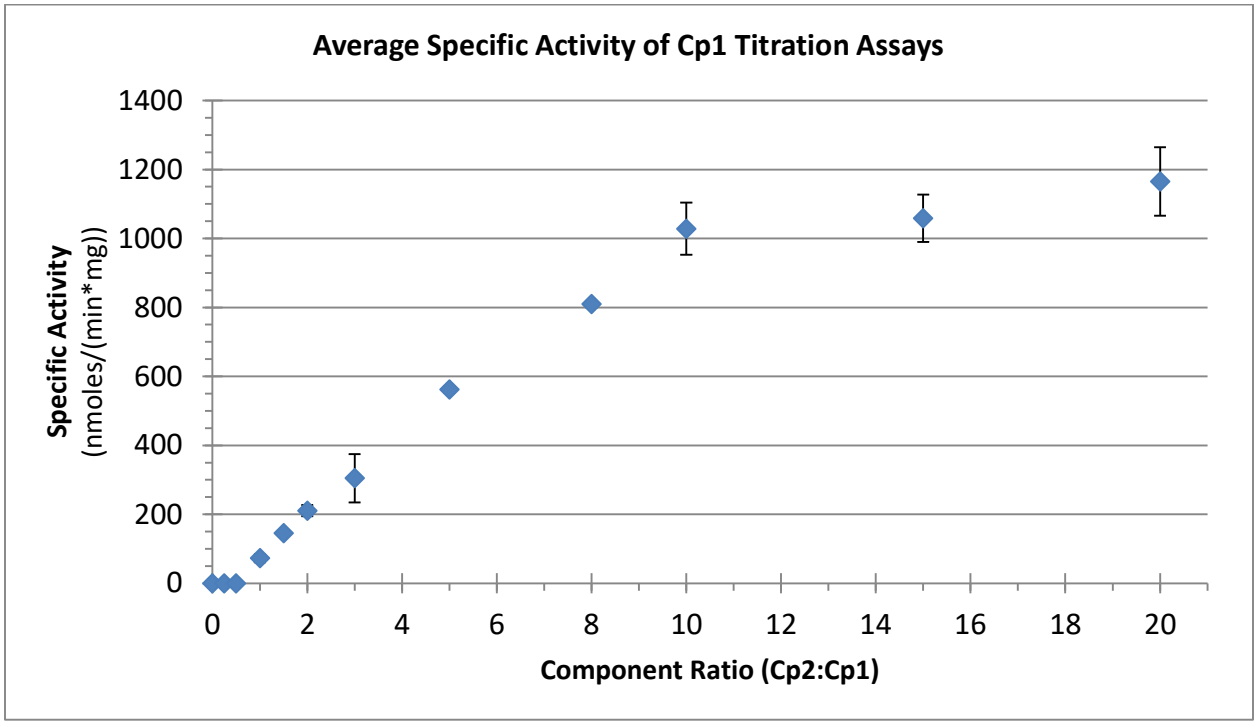


Chart II-8. Average specific activity of Cp1 titration assays



II.E. Crystallization

24-well Cryschem Plates were purchased from Hampton Research. Plates and 1.5-mL Eppendorf tubes were brought into the anaerobic tent (95% argon, 5% hydrogen) at least two days prior to use to allow all oxygen to diffuse out of the plastic. All solutions used in crystallization were filtered with a 0.22 µm, 50-mL Steriflip sterile centrifuge tube (EMD Millipore) and were then purged of oxygen using Schlenk lines (10 cycles of 3-minutes vacuum and 40-seconds argon) with stirring. Care was taken when subjecting poly(ethylene)glycol (PEG) solutions to vacuum so as not to suck up bubbles into the vacuum line.

In the anaerobic tent, crystallization solutions for each well were prepared in 1.5 mL Eppendorf tubes. The appropriate amount of double-distilled water and PEG was added to each tube. Tubes were then closed and inverted 20 times to create a homogenous solution. All remaining components, such as salt and Tris, were added. Additionally, freshly prepared 5 mM

sodium dithionite was added to each condition. Tubes were closed and inverted another 20 times. Tubes were allowed to sit for at least 45 minutes at room temperature before setting trays. During that time, protein was removed from the liquid nitrogen dewars and brought into the anaerobic tent to thaw.

Crystallization plates were setup using the sitting drop vapor diffusion method (Figure II-11). To each reservoir of the first 12 wells, 500 or 1000 µL crystallization solution was added. 0.5-2.0 µL protein was then added to the crystal well followed by an equal amount of reservoir solution. These 12 wells were then sealed with clear tape. The process was repeated for the remaining 12 wells. Plates stood undisturbed at room temperature. Most crystals started to form within 24 hours. Plates were checked for crystals using a low power setting on a microscope in the anaerobic tent to avoid heat or radiation damage to the crystals. Typical crystal morphologies observed included bars, triangular prisms, and hexagonal prisms (Figure II-9).

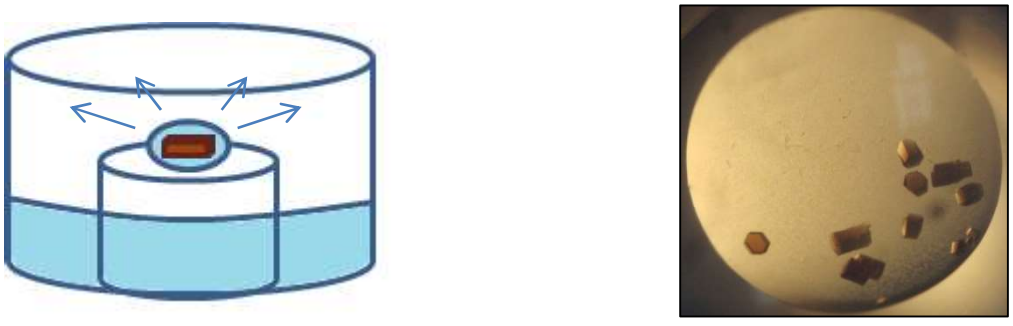


Figure II-9. (left) In sitting drop vapor diffusion crystallization, each well is sealed and water in the crystallization drop evaporates. At critical concentration, the protein precipitates or crystallizes. (right) Typical crystal morphologies observed include blocks, triangular prisms, and hexagonal prisms.

II.F. X-ray crystallography and structure solution

II.F.i. Crystal harvesting onto loops for cryogenic X-ray or neutron diffraction

The most reliable cryo-protection method was adding 2-methyl-2,4-pentanediol (MPD) and a few drops of Fomblin Y 16/6 oil directly to the crystallization drop. First, the sealing tape was sliced away. Under a microscope, the skin (a thin film of protein) of the crystal drop was removed by gently dragging a needle over the surface of the drop. Next, 10 µL reservoir solution and 1-2 µL degassed 100% MPD was added to the drop. Under a microscope, a needle was used to gently mix the solution around the crystal. A small layer of degassed Fomblin Y 16/6 oil (~3 drops) was added to the crystal drop, which remained on the top of the drop. Cryogenic loops were used to fish crystals out of the wells. Once looped, a crystal was slowly dragged through the oil layer. If the crystal was surrounded by an aqueous solution bubble, it was carefully dragged through the drop until the bubble disappeared. Once removed from the well, the looped crystal was immersed in liquid nitrogen as quickly as possible. After equilibrating to the liquid nitrogen temperature, it was carefully placed inside a cryo-vial. Crystals were stored in liquid nitrogen dewars.

II.F.ii. Crystal harvesting into capillaries for room temperature X-ray or neutron diffraction

For room temperature experiments, crystals must be in air-tight quartz capillaries. First, quartz capillaries were brought into the anaerobic tent at least 48 hours prior to harvesting to allow any oxygen in the capillary to diffuse out. At the time of harvesting, the tape on the crystallization well was removed, as was the skin on top of the crystal drop, as described in section II.F.i. 10 µL reservoir solution was added to the crystal drop. An additional 20 µL reservoir solution was transferred to a clean, random spot on the top of the plate. The crystal was fished with a loop and transferred to the drop on the top of the plate.

Next, the capillary, with a diameter about 1/3 larger than the crystal, was prepared. A tiny piece of the capillary was snipped off the small opening. If the opening was not even, it was gently tapped on a smooth surface or re-snipped. The larger opening of the capillary was inserted into piece of rubber tubing about 18 inches long, which was connected to a pipette tip with its small end snipped off by a few millimeters. Using a pipette, the crystal was *gently* sucked into the middle of the capillary.

Once the crystal was in the capillary, it was situated to roughly the middle of the capillary using the pipette. Next, most of the solution was removed from the capillary using wicks. The crystal remained in a drop of solution as small as possible (to minimize background scattering) with a nearby solution plug of at least a few millimeters in length to keep the capillary environment humid (Figure II-10). After the crystal and solution plug were positioned, the small opening of the capillary was sealed with wax. The large end was then snipped off and sealed. Care was taken to not heat the crystal while applying hot wax to the capillary. At this point, the capillaries could be removed from the tent. Capillaries were placed level in a small box and loosely held in place with a wadded Kim wipe.

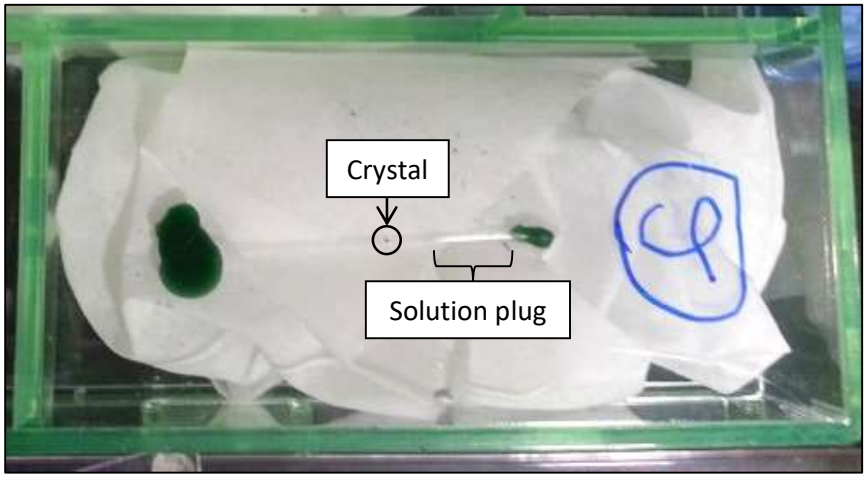


Figure II-10. The crystal is positioned in the middle with a small plug of solution nearby. The capillary is stored in a small box and is loosely held in place with a wadded Kim wipe.

II.F.iii. Data collection

Diffraction data was either collected in-house on a Rigaku MicroMax 007-HF X-ray generator with a Rigaku RAXIS-IV++ detector or remotely from the Stanford Synchrotron Radiation Lightsource (SSRL) on beamline 12-2 with a DECTRIS Pilatus 6M detector. For in-house experiments, 360 diffraction images were collected with an oscillation angle of 1° and 360° rotation. In a typical diffraction experiment at the SSRL, sets of 1440 diffraction images were collected at 12,999.97 eV with an oscillation angle of 0.25° (about half the mosaicity) and 360° rotation.

II.F.iv. Structure solution

Data sets were indexed and integrated with the XDS program package or MOSFLM.[57,58] Typically, MOSFLM was used to assess the quality of the reflections; indexing and integrating were performed with XDS. Scaling and merging were carried out with the CCP4 suite (POINTLESS and SCALA or AIMLESS), from which the resolution cutoff was determined by various metrics.[58] Phasing was calculated experimentally or by molecular replacement (PHASER or MOLREP). Initial structure refinement was carried out with CNS, and alternative conformations and isotropic *B*-factors were refined with REFMAC5.[59–61] For data sets near 1 Å resolution, anisotropic refinement was also performed. Simulated annealing was calculated using PHENIX.[62] Structures were further refined in COOT using the validation tools, including examining Ramachandran plot, searching for un-modeled blobs with electron density above 0.7 e/Å^3, and checking the difference density, waters, geometry, peptide omega angles, *B*-factors, Gln and Asn outliers, rotamer probability, and density fit. Figures were made in PyMOL.[63] For anomalous data, Friedel's Law was set to false during initial data processing. Following merging, structure factors were calculated using SFALL and Fourier transformation was carried out using FFT in CCP4.