Purification of Proteorhodopsin by Using Citrate and Phosphate to Induce Selective Precipitation

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Abbreviations

pR, proteorhodopsin; bR, baceriorhodopsin; OG, β-octyl-D-glucoside; PMSF, phenylmethylsulphonyl fluoride; AMP, ampicillin; SDS-PAGE, sodium dodecyl sulfate polyacrylamide

Introduction

Proteorhodopsin (pR), a homologue of archaeal bacteriorhodopsin (bR), is a 249-amino-acid, seven-helix, transmembrane protein bound to a retinal molecule that functions as a light-driven proton pump. The retinal chromophore of pR absorbs a photon and isomerizes, which induces conformational changes in the protein structure [1]. Proteorhodopsin was discovered in the year 2000 from the DNA sequences of several uncultured species of marine γ-proteobacteria, which is a component of marine planktons present in ocean surface water [2, 3]. Since the discovery of pR, additional studies have found that pR is widely distributed in the world. In fact, it has been projected that 10^{28} light-driven pR expressing bacteria exist in the world [4, 5]. Proteorhodopsin is believed to play an important role in the energy balance of the Earth due the large marine biomass of bacterioplanktons [6].

Understanding the structure of pR, along with the major active site amino acid residues, will help us explore the important functions of this protein. The majority of active site amino acid residues as well as the amino acid residues that

form retinal binding pockets in archaeal bacteriorhodopsin are also highly conserved in pR [2]. Like bacteriorhodopsin, pR has conserved arginine, aspartate, and lysine residues, which are important in the proton transport mechanism. Specifically, the active site residues Arg^{82}, Asp^{85}, Asp^{212}, and Lys^{216} in bR are conserved as Arg^{94}, Asp^{97}, Asp^{227}, and Lys^{231} in pR [7]. Furthermore, the retinal chromophore of pR covalently binds as a protonated Schiff's base at Lys^{231}, while Asp^{97} plays a significant role in proton transfer from the photoactivated retinal as it is the primary proton acceptor [2, 7].

The ultimate goal of this project is to form a crystallized pR in order to obtain a well-defined three-dimensional structure, which is essential to enhance and facilitate a greater understanding between the structure of pR and its function. Furthermore, an understanding of the purification procedures of pR could provide better methods for purifying and crystallizing other transmembrane proteins, in particular G protein-coupled receptors. Crystallization requires a high protein purity of approximately 95% or greater.

Proteorhodopsin had been previously purified with affinity chromatography using Phenylsepharose™, hydroxyapatite and/or Ni-NTA resin [8, 9]. However, the last technique requires pR to be cloned into the pBAD-TOPO vector with a 6xHig -tag at the C-terminus [2]. The use of columns also requires substantial cost and time, especially for purifying quantities of more than ~ 1-5 mg. Previously, it was shown that proteorhodopsin (pR) is selectively precipitated using 100mM citrate

pH 5.5 containing 3% octyl-glucoside [6]. Applying this method, we have been able to precipitate pR selectively from a detergent extract of *E. coli* cells. In fact, this method alone yields a pR purity typically approaching 32%. The current experiments mainly concentrate on establishing an optimized purification pR yield by controlling specific conditions such as concentration, solubility, pH, and pR-specific precipitating agent. Most important, we determined that phosphate helps with selectively precipitating the impurities from pR, and its use thereby can increase the purity of pR. We systematically explored the optimal concentrations of phosphate, citrate, and octylglucoside to obtain with the most favorable higher pR purity than has ever previously been obtained without the use of column purification methods, i.e. in excess of 50% purity.

Methods

Enriched Luria-Bertani (LB) Media

We added 10 g of Bacto® Tryptone, 5 g of Yeast Extract, 6 g of sodium phosphate monobasic ($NaH_2PO_4 \cdot H_2O$), 8.7 g of potassium phosphate dibasic (K_2HPO_4) and 10 mL of glycerol per 1-liter of distilled water into a beaker. After dissolving, concentrated NaOH was used to adjust the pH to 7. Once made, the solution was sterilized by autoclaving (15 min) and then stored in a cold room (approximately 4°C).

Protein Expression

Proteorhodopsin was expressed in *E. coli* strain UT5600 using a pBAD TOPO plasmid [6]. 1 mL of LB media was transferred into a small tube along with 50 μg/mL of ampicillin. Then, an average-size transfected *E. coli* colony was collected with a sterile pipette tip and transferred into the 1 mL of LB/Amp media, which was then incubated overnight at 37°C. This starter culture was transferred into a flask that contains 500 mL enriched LB media containing 50 μg/mL ampicillin. The flask was placed in the shaker bath at 37°C for approximately 4-5 hours. After 4-5 hours of growth, the heater was turned off and the flask was cooled quickly to room temperature, using a bucket of ice added to the shaker bath. Once the temperature reached ~20°C, 1/100 volume of 20% L-arabionose was added, along with ~10 μg/liter of all *trans*-retinal dissolved in acetone. The flask remained shaking (~30 rpm) in the shaker bath overnight in a dark room at 15-20°C. The flask was then moved to the cold room and left overnight (without shaking).

Cell Harvest

The cells were collected by 30 min of centrifugation at 6000 rpm (Beckman® JA-10 rotor). The supernatant usually immediately separated from the pellet. We determined the wet weight of the pellet by taring an empty centrifuge tube, then weighing it along with the pellet formed by centrifugation.

Cell Lysis

The pellet was completely resuspended in approximately 5x volume of lysis buffer (10 mM HEPES pH 7.1 containing 1 mM PMSF protease inhibitor, 3% β-octyl-D-glucoside, 0.08% lysozyme and 0.01 mg/mL DNAse). The lysed cells in lysis buffer were centrifuged at 3900 rpm in Beckman® tabletop centrifuge at 4°C using GA-6 rotor. The reddish colored supernatant was immediately separated from the pellets. If the pellets were still colored pinkish, then the pellets were re-extracted in lysis buffer. Ultimately, the grayish pellet containing impurities, i.e. undesired proteins, was discarded, and colored supernatants were combined. This combined solution is referred to as raw detergent extract of *E. coli*.

pR Purification

1/3 volume of 100 mM sodium citrate pH 5.5 was added to the raw detergent extract and then left to sit for 15 minutes. The solution was then spun down for 15-30 minutes at 3900 rpm at 4°C. If any white pellet was obtained at this time, these impurities were discarded; the colored supernatant was separated from the impurities, and another 1/3 volume of 100 mM sodium citrate was added to the colored supernatant. The centrifugation was repeated, and any colored pellets were saved; the supernatant was further diluted each time until the supernatant became nearly colorless. All the pink-colored pellets obtained from the previous

citrate steps were recombined by solubilizing into a minimal (~50μL) volume of 50 mM Tris-Cl pH 9.0 containing 3% OG. Once the pellets were completely resuspended, they were centrifuged (15 min, 3900 rpm). The supernatants were then transferred to a new tube and any pelleted impurities were discarded. A UV/Visible spectrometry measurement determined the degree of purity at this particular stage.

The colored supernatant was then repelleted using the citrate precipitation methods described above. Any remaining colored supernatant was repeatedly precipitated by further addition of 100 mM citrate, until all the color was transferred from the supernatant to pellets. We made sure to save any colored pellets while removing impurities. Once the supernatant became clear in color, we recombined all the colored pellets by solubilizing them into a small volume of 50 mM Tris-Cl pH 9.0 containing 3% OG, and spun it down. Again separating the pink-colored pR-containing supernatant from impurities in the colorless pellet. Afterwards, we saved 50 μl of pR solubilized in Tris-Cl for additional UV/Visible spectrometry measurements.

Use of Phosphate as a Citrate competitor in Purification
After two full cycles of citrate-induced precipitation and resolubilization in 3% OG Tris pH 9.0, we added 1/3 volume of 600 mM phosphate, 30 mM sodium citrate pH 5.5, containing 0.4% OG, and incubated for 15 minutes at room temperature. Then, we spun it down for 15 minutes at 3900 rpm at 4°C. If any

impurities were precipitated, they were discarded. Then a similar volume of 30

mM citrate/ 600 mM phosphate pH 5.5, containing 0.4% OG, was added,

followed by centrifugation. The addition citrate/phosphate/OG solution, followed

by centrifugation, was repeated until the pR precipitated out of solution as a

colored pellet. Once a pR pellet was obtained, the supernatant was further diluted

with 10 mM citrate pH 5.5 containing 0.4% OG alone (without phosphate), until

the supernatant became nearly colorless. All the colored pellets were recombined

by solubilizing them into a small volume of 50 mM Tris-Cl pH 9.0 with 3% OG

and was again spun down. The pink-colored supernatant was separated and

collected from any impurities. 50 μl of pR solubilized in Tris-Cl was saved for

UV/Visible spectrometry measurements. pR was reproduced from the colored

supernatant using a combination of citrate and phosphate precipitation method

described above.

Absorption Spectroscopy

Absorption spectra were measured on a Shimadzu UV-265 spectrophotometer at

2nm resolution over the range 700-250 nm. A sample volume of 0.6ml was used

in a masked quartz micro cuvette for all measurements. An absorbance ratio

A_{280}:A_{520} of ~2.0 indicates 100% pure pR, as shown previously [9].

Result and Discussion
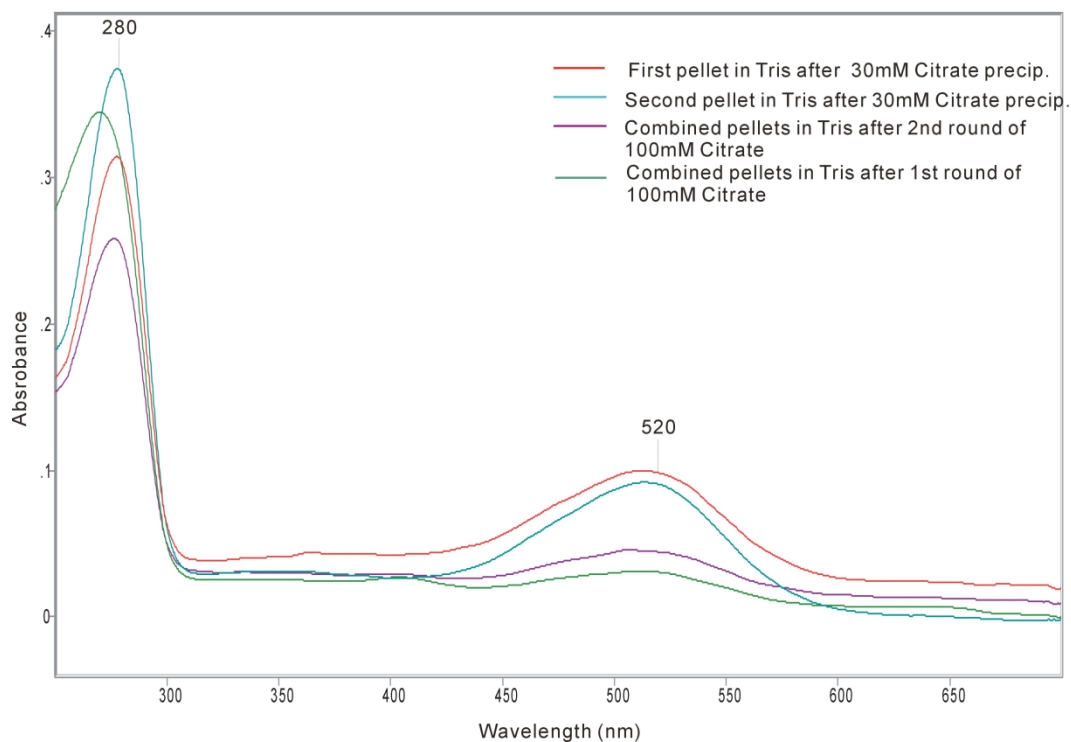
Purification of pR with Citrate Precipitation

Figure 1. UV/Visible Absorption Spectra of pR in 50 mM Tris-Cl pH 9.1 containing 3% octylglucoside solution at three rounds of purification. All spectra were measured in the presence of OG at pH 9.1. Green Spectra indicates the preliminary purification of pR using 100 mM citrate pH 5.5; Purple Spectra indicates the second round of the same method; Red, spectra for the first pellet treated with 30 mM citrate pH 5.5 resuspended in Tris; Blue, the same method – 30 mM citrate pH 5.5 – was applied.

As shown in Figure 1, the preliminary purification of pR using 100 mM citrate pH 5.5 yielded approximately 11:1 ratio of $A_{280}:A_{520}$. The same method of purification with 100 mM citrate was repeated for the second round resulting in approximately 6:1 absorbance ratio. Within two rounds of 100 mM citrate precipitation, we were able to purify pR and reduce the ratio from 11:1 to 6:1. After two full cycles of citrate, pR was treated with 30 mM citrate pH 5.5

containing 0.4% OG, in which we measured the UV spectra of pellets separately, instead of combining them, at each addition of citrate. The ratio of the first pellet in Tris was nearly 3:1 while the second pellet in Tris was 4:1 (Figure 1). The color of pellets at this stage was very bright with high intensity in pR pellets. The result indicates that a high concentration of citrate already had precipitated the impurities as well as other undesired proteins by citrate binding to numerous proteins. The color also indicates that pR has one or more binding sites for citrate; thus, a lower concentration of citrate is enough to specifically bind pR to precipitate more effectively once the impurities are removed.

Finding a Better Concentration of Citrate in the Presence of 600mM Phosphate
After a few rounds of citrate precipitation including both 100 mM concentration and a lower concentration, pR was treated with 30 mM citrate and 600 mM phosphate pH 5.5 containing 0.4% OG. This particular concentration of phosphate along with citrate brings down more impurities, while leaving pR solubilitzed. We believe that phosphate prevents precipitation by competing for the citrate binding site, thereby blocking citrate-induced aggregation. This particular method could be a good transition in purification as it removes more impurities and increases the purity of pR. In the presence at constant 600 mM concentration of phosphate, we attempted to explore a better concentration of citrate. The goal was to find the lowest citrate concentration that can induce aggregation in the presence of phosphate.
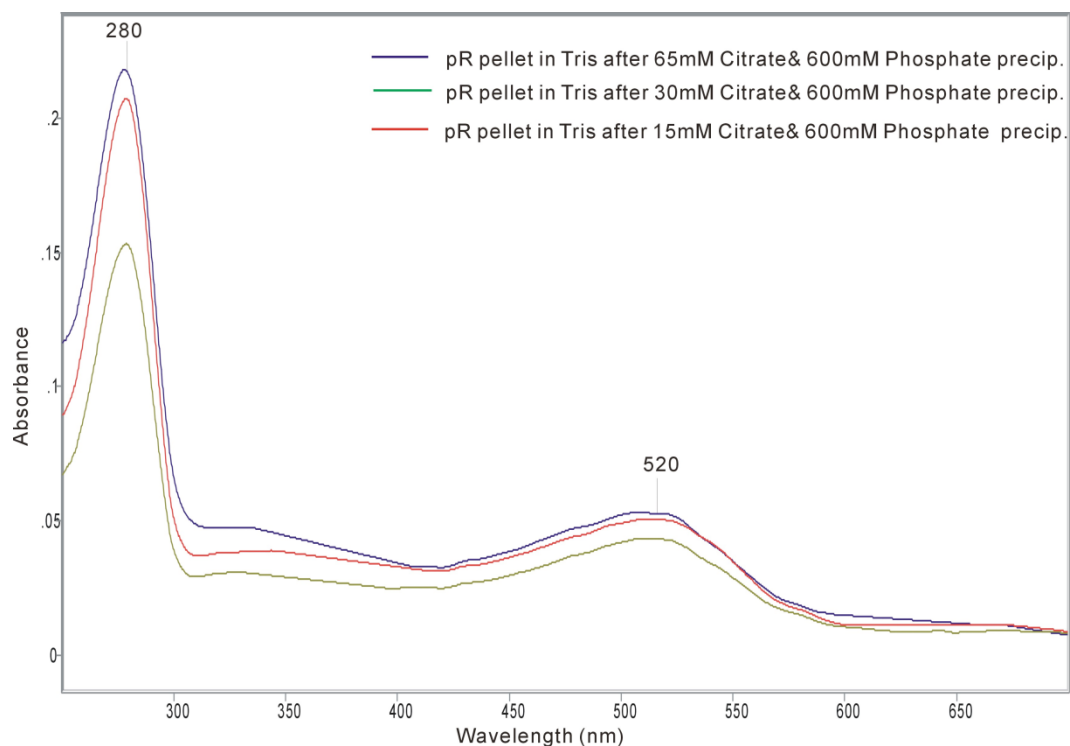
Figure 2. UV/Visible Absorption Spectra of pR resuspended in Tris-Cl pH 9.1 with 3% OG after treated with different concentrations of citrate at pH 5.5 while have the same 600 mM phosphate present. Purple Spectra indicates pR treated with 65 mM citrate; Green, 30 mM citrate; Red, 15 mM citrate.

We tested three different citrate concentrations – 65 mM, 30 mM and 15 mM – at pH 5.5 with the presence of 600 mM phosphate. Among different concentrations, the ratio of A_{280}:A_{520} of 30 mM citrate along with 600 mM phosphate gave approximately 3:1, one of the best purity levels we have ever obtained in the laboratory without columns, while 65 mM and 15 mM both yielded approximately 4:1 ratio (Figure 2). It was interesting to note that 30 mM, which is neither the lowest nor the highest concentration of citrate, could bring down the pR to the highest purity level so far achieved.

Based on these results we investigated the most favorable conditions that would increase the degree of purity of pR and have the ability to recover pR in high yield. We examined several different ways of purification of pR after a few rounds of purification steps with both 100 mM and 30 mM citrate pH 5.5.

Use of Phosphate in pR Purification

We tried using 600 mM phosphate with 30 mM citrate pH 5.5 containing 0.4% OG, only without switching back to citrate alone after 2-3 citrate precipitation rounds. Using a mixture of citrate and phosphate, we were able to remove more impurities, and then precipitated pR. The pellets were resuspended in Tris for the UV spectra measurements. The first pellet in Tris resulted approximately 7:1 ratio of purity, whereas a ratio of the second and third pellets in Tris diminished to 4:1 and 3:1 respectively (Figure 3). As more volume of phosphate along with citrate was added, we were able to obtain purer pellets. However, one problem that occurred in this particular method was that it was difficult to recover the pR completely, meaning there was still color left in the supernatant and we were not able to completely bring the color down into a pellet. This might suggest that 600 mM phosphate may be too high in pR precipitation, as the binding sites may have been occupied by phosphate; thus, phosphate blocks citrate-induced aggregation.
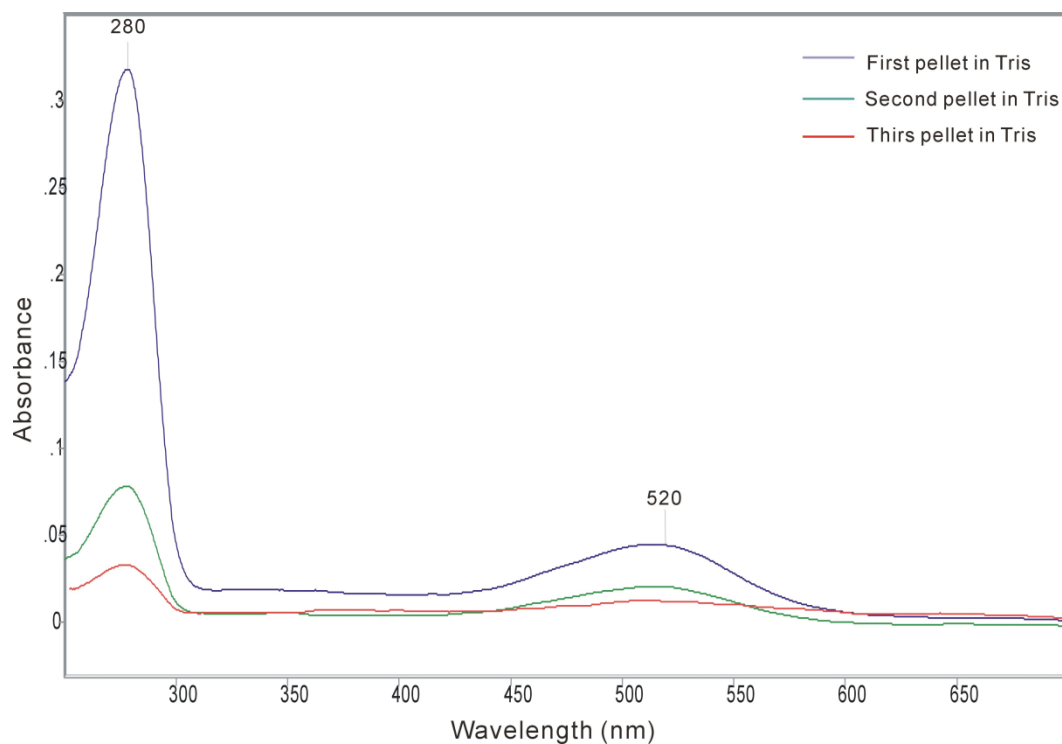
Figure 3. UV/Visible Absorption Spectra of pR in 50 mM Tris-Cl pH 9.1 containing 3% OG at 30 mM citrate and 600 mM phosphate pH 5.5 w/ 0.4% OG purification. Blue, first precipitated pR pellet in Tris; Green, second pR pellet obtained; Red, third precipitation of pR pellet resuspended in Tris for spectrum. There was still color left in the supernatant even after third precipitation and could not complete reproducing pR.

Use of a Lower Concentration of Citrate Alone

After the preliminary citrate precipitation procedures including both 100 mM and 30 mM citrate at pH 5.5, we tried an even lower concentration of citrate, 10 mM citrate pH 5.5 containing 0.4% OG, in a small amount of pR in Tris and repeated the same addition/centrifugation procedure steps. The low concentration of citrate precipitation without any phosphate maintained approximately a 4:1 ratio of pR purity at the first addition of 10 mM citrate. In the second addition, we brought all the color down from the solution into a pellet; the purity of the pellet was increased to 6:1 due to a precipitation of pR, even with other undesired proteins (Figure 4).
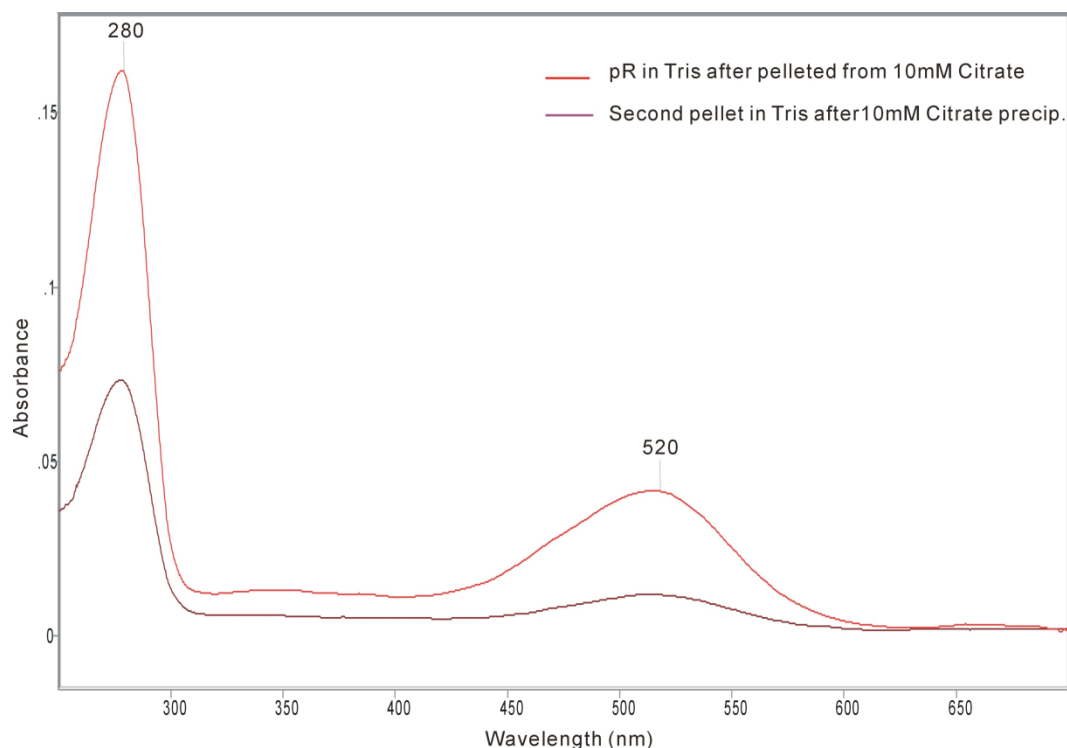
Figure 4. UV Spectra of pR resuspended in Tris-Cl pH 9.1 w/ 3% OG after 10 mM citrate pH 5.5 containing 0.4% precipitation. Red, first precipitation; Purple, second precipitation of pR that had precipitated all color into a pallet from the supernatant.

Alternating Treatment with Citrate Alone and with both Citrate and Phosphate

The results from applications of 30 mM citrate and 600 mM phosphate and 10 mM citrate alone has led us to treat pR with 30 mM citrate and 600 mM phosphate pH 5.5 contaning 0.4% OG first after the preliminary citrate precipitation, and then switching to 10 mM citrate pH 5.5 containing 0.4% OG, and *vice versa*.

pR that was initially treated with a mixture of citrate and phosphate resulted in approximately 7:1 A_{280}:A_{520} ratio. Once the first pR pellet was obtained from citrate and phosphate solution, we treated pR with 10 mM citrate alone without having phosphate in the solution. The second pellet obtained from addition of 10

mM citrate pH 5.5 containing 0.4% OG reduced the purity down to 5:1. Finally,

the third pR pellet treated with 10 mM citrate pH 5.5 was the last pellet

precipitated, which brought all the color from the supernatant down; the purity of

third pR pellet decreased as the ratio rose to 6:1 ratio of purity (Figure 5).
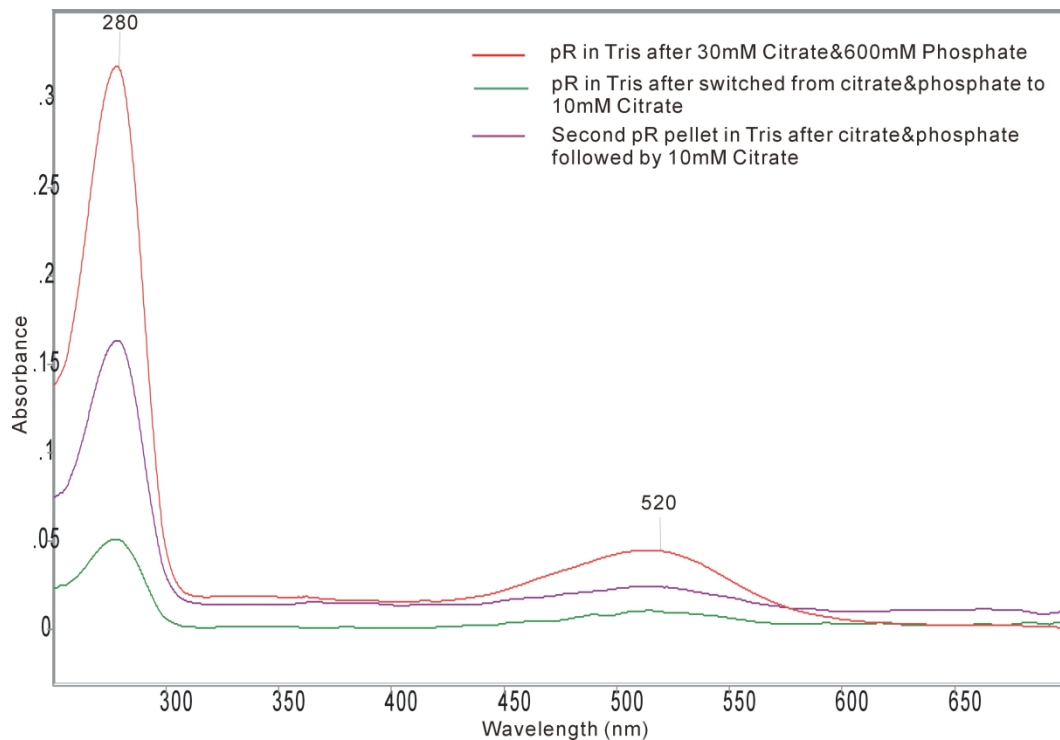

Figure 5. UV spectra of pR, which was initially treated with 30 mM citrate along with 600 mM phosphate then switched to 10 mM citrate alone, resuspended in 50 mM Tris-Cl pH 9.1 containing 3% OG. Red, spectra of pR precipitated from use of citrate and phosphate; Green, pR pellet precipitated from 10 mM citrate after switched from citrate and phosphate solution; Purple, second pR pellet obtained from 10 mM citrate precipitation.

Moreover, we tried reversing the order of steps. We initially treated with 10 mM

citrate pH 5.5 containing 0.4% OG, and then switched to 30 mM citrate and 600

mM phosphate pH 5.5 with 0.4% OG. This method was problematic because it

was difficult to precipitate pR completely in the presence of phosphate. We tested

the idea of raising the concentration of citrate, which would allow to compete

with phosphate as it would precipitate pR. The difficulty in recovering pR was finally solved by gradually reducing the citrate and phosphate concentrations, while keeping OG concentration constant. We added 400 mM citrate and 600 mM phosphate pH 5.5 containing 0.4% OG. Then we added an equal volume of 0.4% OG solution itself and let it sit over a long period of time (~10 days). The constant OG concentration, with a high concentration of citrate, selectively precipitated pR. The UV absorption spectrum indicated that the ratio of purity was nearly 5:2, which was the best result obtained to date (Figure 6), and likely indicative of a final pR purity level of ~50% of protein by weight.

Overall, the use of phosphate along with citrate is promising in pR purification. This particular purification procedure has brought us a step closer in achieving crystallization through efficient use of inexpensive materials.
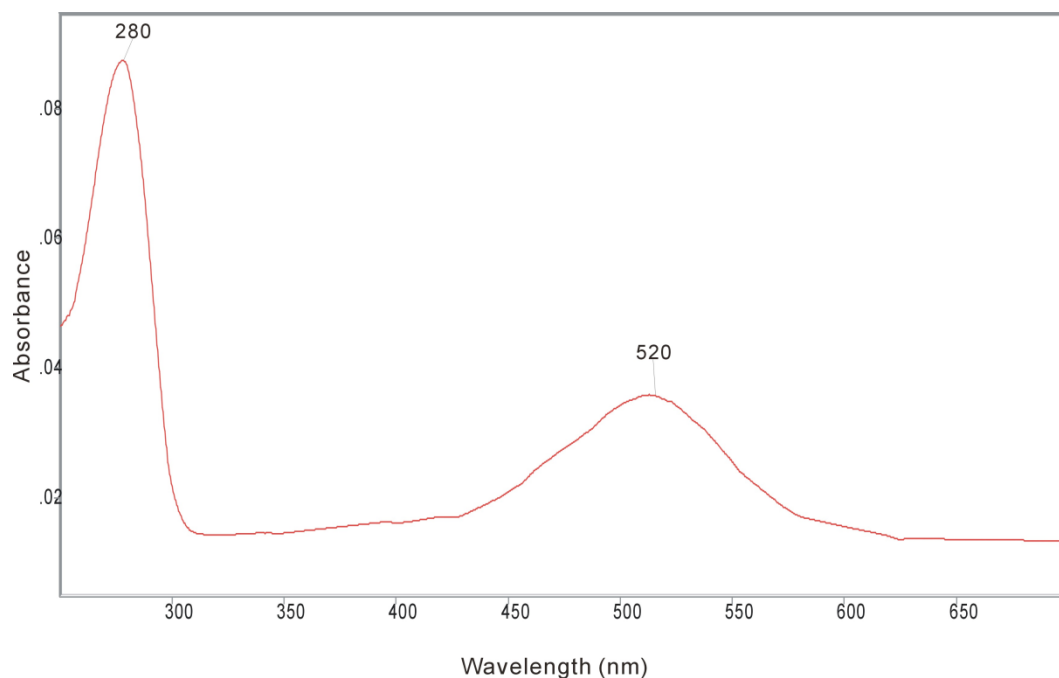
Figure 6. UV/Visible Spectrum of pR, which has been precipitated from 400 mM citrate and 600 mM phosphate pH 5.5 containing >0.4% OG, dissolved in 50 mM Tris-Cl pH 9.1 w/ 3% OG. The spectrum indicates a purity of pR after the pR purification in alternating 10 mM citrate pH 5.5 w/ 0.4% OG and 400 mM citrate and 600 mM phosphate pH 5.5 w/ > 0.4% OG.

Phosphate and pK$_a$ value

During this whole project, I have noticed one very interesting result. When I

added 30 mM citrate and 600 mM phosphate pH 5.5 containing 0.4% OG into pR

resuspended in 50mM Tris-Cl pH 9.1 with 3% OG, the color clearly changes from
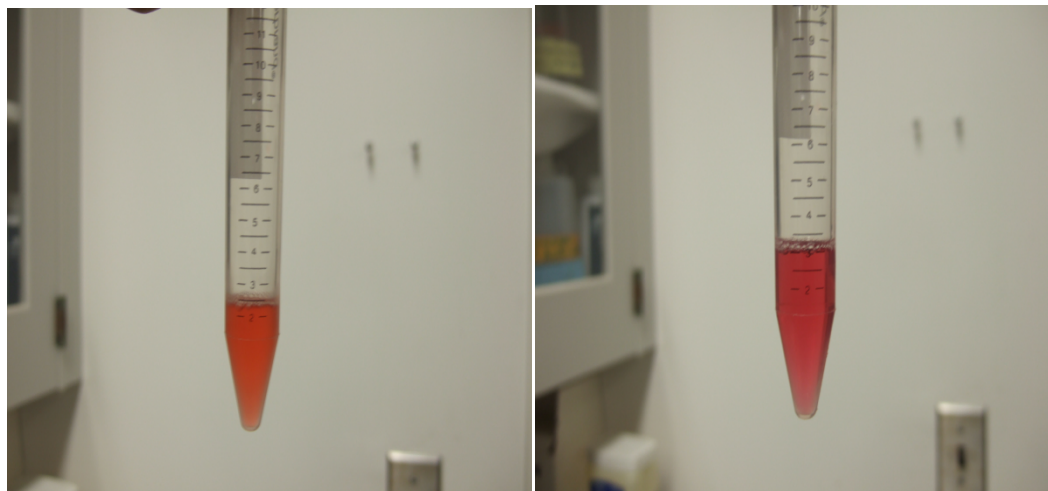
reddish to purple (Figure 7).

Figure 7. The color shift in pR depends on the presence of phosphate. Left, 10 mM citrate pH 5.5 containing 0.4% OG added into pR dissolved in 50 mM Tris-Cl pH 9.1 containing 3% OG; Right, 30 mM citrate and 600 mM phosphate pH 5.5 containing 0.4% OG added into pR resuspended in 50 mM Tris-Cl pH 9.1 containing 3% OG.

That is, the color of pR is red at basic pH (Tris-Cl pH 9.1 containing 3% OG).

When we added citrate, pH 5.5 to such a pH 9 solution, there was no notable

change in the color of pR. However, a different result was obtained when 30 mM

citrate and 600 mM phosphate pH 5.5 was added into the same pR sample,

originally at pH 9. When the mixture of citrate and phosphate was added into pR,

the color consistently turned into an intense purple and was clearly

distinguishable from the color of pR that was phosphate-free though they were at

similar pH levels (Figure 8 Left). Then, subsequent switching of the solutions,

i.e. addition of phosphate into the solution that originally had citrate only, yielded

the same intense purple color (Figure 8 Right). This indicates that phosphate

could raise the pK_a value of pR, which is due to the titration of Asp[97] the
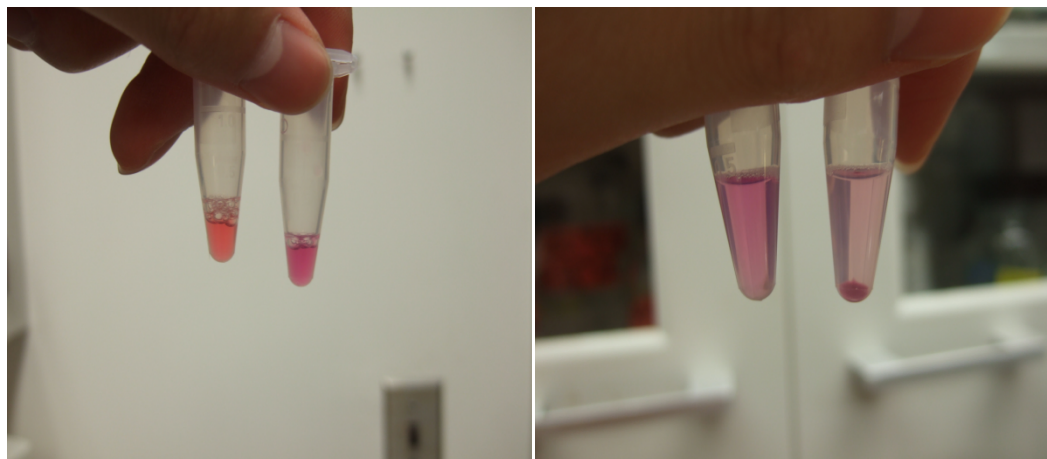
chromophore's counter ion.

Figure 8. Comparison in color of pR with the presence of phosphate and without phosphate. *Left*, 10 mM citrate pH 5.5 containing 0.4% OG is added into pR dissolved in 50 mM Tris-Cl pH 9.1 w/ 0.4% OG on the left, whereas 30 mM citrate and 600 mM phosphate pH 5.5 containg 0.4% OG is added into pR dissolved in Tris on the right; *Right*, a solution of citrate and phosphate is added into pR on the left while 10 mM citrate is added into pR instead of a mixture of phosphate and citrate on the right.

References

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Summary

Proteorhodopsin (pR) is a transmembrane protein bound to a retinal molecule that function as light-driven proton pump. In other words, when proteorhodopsin is bound to a retinal molecule and is exposed to light, a conformational change occurs as it generates energy. Retinal is a derivative of vitamin A. Proteorhodsopin is believed to serve as a possible alternative energy source that could play an important role in the energy balance of the Earth. Proteorhodopsin is widely distributed, and the bacteria containing it are some of the most prevalent organisms on Earth. Proteorhodopsin has many potential uses and benefits in commercial biotechnological applications and other industries. Also, understanding the roles of pR in the oceanic food chain might help us to study some significant impacts of global changes on marine communities and in the ecosystem.

Unfortunately, ever since this particular protein has been discovered in 2000, it has been difficult and challenging to obtain a well-defined three-dimensional structure. Knowing the 3-D crystal structure of proteorhodopsin would tremendously enhance a greater understanding in its functions and specific physiological roles.

In order to obtain a well-defined three-dimensional structure, we have to form a crystallized pR to perform x-ray crystallography – a method of determining the

arrangement of atoms within a crystal – and crystallization requires a high pR purity approximately 95% or greater.

Therefore, this project focused on establishing an optimized purification pR yield by controlling specific conditions such as concentration, solubility, pH, and pR-specific precipitating agents. An understanding of the purification procedures of proteorhodopsin could provide a better understanding in other various transmembrane proteins, in particular the G protein-coupled receptors, which constitute a large protein family of seven transmembrane receptors that activate signal transduction pathways and cellular responses to pharmaceuticals.

Proteorhodopsin had been previously purified with columns and with other materials that require both time and high cost. Professor Braiman and his laboratory developed and established an invention for purifying pR using a simple salt, sodium citrate, to precipitate pR selectively from a detergent extract of *E. coli* cells. This particular method gave approximately 20-30% purity in a single step, and is suitable for the preliminary purification.

In this project, we continued to explore a method that would increase the purity as well as have the ability to reproduce a large quantity of pR. We expected that another simple salt, phosphate, could help with selectively precipitating the impurities from pR, which may increase the purity of pR. We have obtained evidence that alternating treatment between citrate alone, and with citrate along

with phosphate, may be a useful set of purification steps throughout this project. The pR obtained from this purification procedure using citrate and phosphate yields adequate quantity and purity of proteins that are suitable in techniques such as SDS-PAGE (sodium dodecylsulfate polyacrylamide gel electrophoresis) and UV/Visible absorption spectrum. This purification procedure of proteorhodopsin is simple; it includes adding solution then centrifugation in order to selectively precipitate impurities or pR. Purple pellets contain pR, and should be saved for UV/Visible Spectroscopy for the examination of the degree of pR purity. The advantage of using citrate and phosphate in pR purification is that they are inexpensive materials, which reduce cost tremendously as it eliminates the use of a column, but still has the ability to provide the same level of purity. This simple purification method to purify pR proved less costly and more efficient. Also, this method could possibly purify other transmembrane proteins (G protein-coupled receptors), which would be enormously significant for pharmaceutical purposes.

This project should be continued to seek better conditions that would increase the quantity of pR as well as purity. The pR purification procedure described in the Capstone Project has shown potential, but needs further work. The ultimate goal of the project is a well-defined three-dimensional structure of proteorhodopsin and this work has brought us a step closer in achieving crystallization through citrate and phosphate precipitation of pR.