Preliminary Studies of Shear Denaturation on Cytochrome-C

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ABSTRACT The properties of a protein can be seen through the folding of that protein. One-way to observe a protein fold is to unfold it first. Shear denaturation is one possible way to unfold a protein. The researchers attempted to unfold cytochrome-c (cyt-c) using shear denaturation. This was attempted by flowing cyt-c through different length, micron size capillaries at different flow velocities. The pressure gradient that drives the flow rate, the shear rate that the cyt-c undergoes, is also changing. Shining a UV laser on the capillary that the cyt-c is flowing through and watching for a fluorescence change with a charged coupled device (CCD) camera can reveal the unfolding. Tryptophan is used as a control experiment to test the system before flowing cyt-c. Using the system that the researchers designed, they were unable to witness any unfolding of cyt-c at different shear rates.

Introduction

A protein is a three dimensional structure made up of a long chain of amino acids. The difference between proteins is due to the difference in their amino acid sequences. The amino acid chains quickly fold by the attractions and repulsions between the positive and negative electric charges of the atoms making up the molecules [1]. The final shape will be the one in which the positive and negative charges are as close to one another, on the average, as they can get; in technical terms, the protein molecule has the lowest possible free energy [2]. The final shape or structure of a protein decides its function. The human body has thousands of proteins, each with a different structure and function. Watching a protein fold helps in determining its intermediate states, its time scale for folding, and its structure, which in turn helps to identify the function of that protein.

Proteins fold in steps and at different rates. Each step takes a different amount of time, short periods of time. Proteins fold in periods from microseconds to seconds,

which can be a problem if important steps are often too fast to be observed. In order to make the early stages of folding experimentally accessible, we need some means of initiating the process on a shorter timescale than the fastest structural event of interest [3]. Watching a protein fold completely leads toward its actual structure and function. This is why the folding process must be initiated on a shorter time scale. Also, watching proteins fold on a short time scale helps to understand what controls folding [4]. In this experiment, we will try to unfold a protein, cytochrome-c (cyt-c), using shear denaturation. Unfolding of cyt-c using shear denaturation would allow us to trigger a quick refolding of the protein, which will help to understand how proteins fold. Theoretically, shear denaturation can occur by passing a protein, cyt-c, through a micron size capillary at different pressures, which may cause very high shear rates. Cyt-c was chosen because it has been very well studied and is very well known. When it is passed through the capillary, theoretically it will be pulled apart due to shear flow (see Fig. 1).

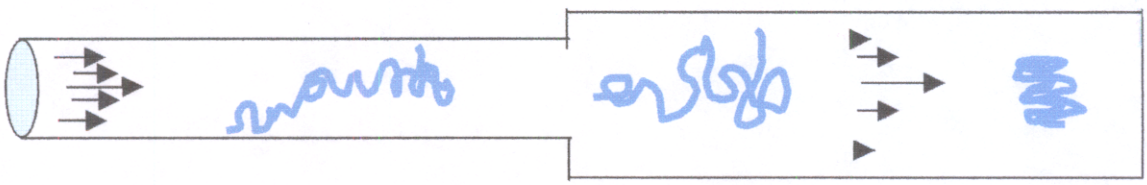

FIGURE 1. The fluid dynamics of a protein unfolding under shear flow is shown in the first cylinder. The velocity of the flow is greater in the center than on the sides. This force unfolds the protein, but then as the protein enters the second cylinder, it starts to refold because the shear flow is not as great. This is how shear denaturation is used to watch a protein fold.

When a fluid flows through a capillary, the velocity is higher in the center and zero on the sides, which is shear flow. Shear flow will allow the protein to be pulled apart. Shear denaturation of DNA molecules has already been accomplished and may indicate the possibility of seeing shear denaturation in proteins; however, DNA is a large molecule [5].

The technique that we use to determine if the cyt-c is unfolding is quite simple. We push the protein through the micron-sized tube by using a pressure gradient and then shine a UV laser on the protein. We watch for a change in the fluorescence of the protein using a CCD camera. If the fluorescence of the protein increases as the pressure gradient of the protein being passed through the tube increases, the cyt-c is unfolding. The fluorescence of cyt-c would increase if unfolded because cyt-c contains tryptophan and a heme group. When folded, the heme group is close to the tryptophan and it quenches the tryptophan fluorescence. When unfolding, the heme group stretches away from the tryptophan and the tryptophan fluorescence increases.

One reason for studying the unfolding of a protein through shear denaturation is to be able to see the protein fold again. Many experts believe that a protein can be shear denatured because fluid shear will unfold a polymer [6]. If a protein can be put under conditions to unfold, the conditions can be changed and the protein can be seen folding. This technique for unfolding the protein will enable researchers to watch the same protein refold in a short time scale. This is very important in studying the properties of different proteins, especially cyt-c. Cyt-c has never been studied under shear denaturation. We are undertaking the first systematic biophysical study of protein shear denaturation.

Materials and Methods

The experimental setup is a very important part of any experiment (see Fig. 2). The UV laser that is used for the experiment is a minilite laser which produces two wavelengths, a 532-nm beam and a 266-nm beam. We will utilize the 266-nm beam but not the 532-nm beam because the 266-nm beam excites tryptophan fluorescence. The 532-nm beam simply adds unwanted fluorescence or noise to the image. To get the 266-

nm beam we used a fused silica prism. The prism split the beam into a 266-nm beam and a 532-nm beam. Then using mirrors we deflected the 266-nm beam onto the capillary. The CCD camera is very sensitive to light so we placed filters inside to help keep unwanted light out. The Schott BG3 filter allows light up to about 450-nm to be seen by the camera; this cuts off scattered 532-nm beam light. The other filter used is the Schott WG320, which cuts off light from the 266-nm laser. The CCD detection is therefore optimized to observe tryptophan fluorescence, which peaks at about 350 nm. Also, to control and enhance the CCD picture we use a 10x microscope objective; this enables us to see the fluorescence and fiber more easily. Since the CCD camera is so sensitive to outside light, we designed a box to enclose the CCD camera and sample holder to keep stray light out but still allow the laser to shine on the capillary.

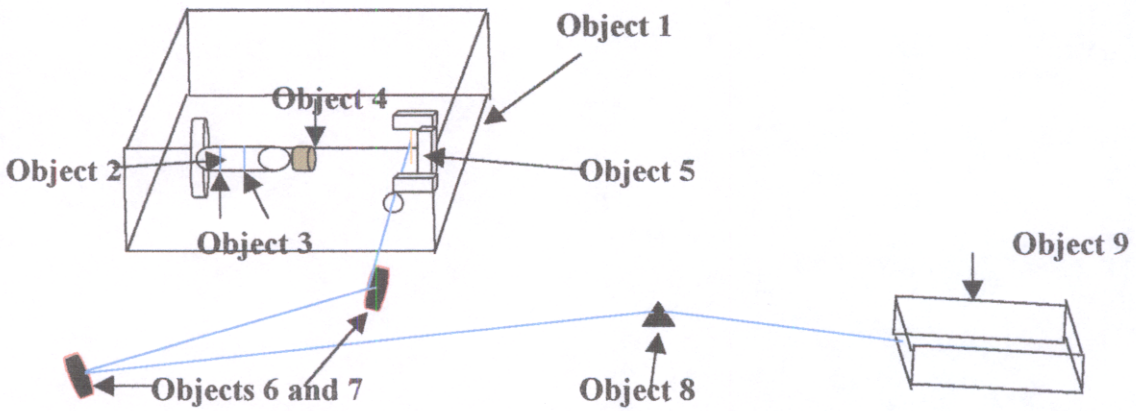

FIGURE 2. The experimental setup for shear denaturation. Object 1 is a box designed to keep stray light away from the CCD camera. Object 2 is the CCD camera, which is looking at the capillary holder. Object 3 is the two Schott filters that are part of the CCD camera. Object 4 is the microscope objective, 10 x, collecting the fluorescent light from the sample. Object 5 is the capillary holder. Object 6 and 7 are mirrors used to deflect the UV laser beam onto the sample through the hole in the box. Object 8 is a prism used to split the 532-nanometer light and the 266-nanometer light and dumps the 532-nanameter light. Object 9 is the minilite UV laser, which produces the 266-nm and 532-nm light.

It was difficult to design a device capable of holding the capillary and allowing a UV laser to shine on it and a charged coupled device (CCD) camera to take a picture of it. In order to hold the capillary, we designed a capillary holder (see Fig. 3).

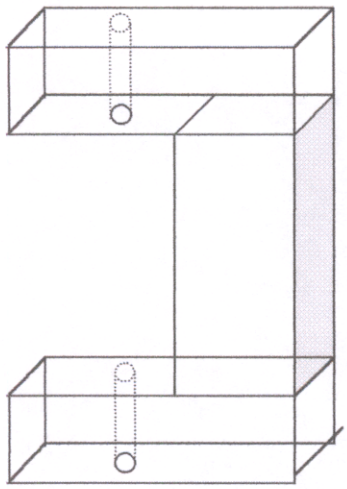

FIGURE 3. This is the diagram for the capillary holder. The fiber is held at the top hole by the needle and fluid is pumped through the hole into the fiber. There is 2 inches of freedom between the top and bottom holes to make the length of the capillary.

In order to make shear denaturation occur, we must have a very high shear rate. Shear rates depend on the applied pressure and the ratio between the channel length and diameter of the capillary (see Fig. 4). The shear rate is also different depending on what pressure is going to be used. In this experiment the highest pressure that will be used is 26 psi. The upper limit of the pressure in the system being 26 psi, our capillaries must be very small. The capillaries that we decided to use were fused silica. We ordered these capillaries from Polymicro Technologies. The only complication regarding the capillaries is that they come with a polyamide coating on them, which must be removed to reveal the fluorescence of the tryptophan (see Fig. 5). We ordered four different diameter sized capillaries for the experiment: the 20-micron inner diameter (ID) with a 90-micron outer diameter (OD), the 20-micron ID with a 150-micron OD, the 50-micron ID with a 363-micron OD, and the 150-micron ID with a 363-micron OD. Having these different diameter capillaries allows us to experiment with different shear rates.

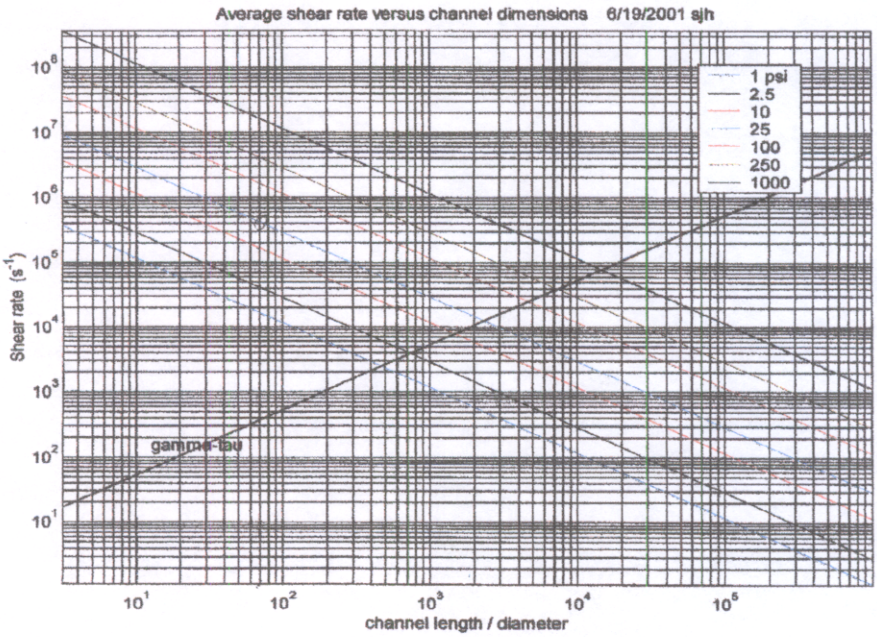

FIGURE 4. Table used to determine what shear rate a protein will undergo depending on the ratio between the channel length and diameter of the capillary. The shear rate is also different depending on what pressure is going to be used. In this experiment the highest pressure that will be used is 26 psi.

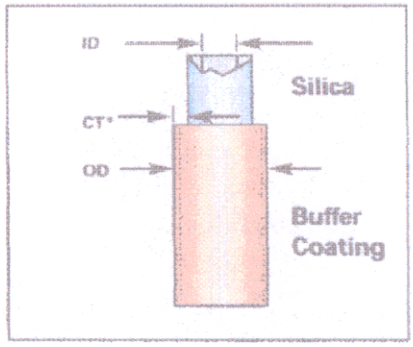

FIGURE 5. A capillary with and without the polyamide coating. The figure also shows where the ID and OD are measured from on the capillary.

The polyamide coating on the capillaries is a problem because the polyamide is fluorescent when excited by UV light (266 nm). So the only way we can use them is to take off the polyamide coating. We tried several different methods in order to achieve this task. We first used an electric coil, but we could not get the beam to pass through the

capillaries. A flame from a lighter was used; the flame worked, but it had some problems. We had to make sure to burn it carefully or the capillary would bend because it would get too hot. Also, we had to burn it so that no residue was left over from the burning of the lighter. This method proved to be burdensome. The best method we tried was heating concentrated sulfuric acid mixed with hydrogen peroxide. We mixed 98% sulfuric acid with 30% hydrogen peroxide, six parts to one. We heated this mixture to above 100° C. We then placed the capillary into it and watched the polyamide disappear in no more than 10 sec.

In order to hold the capillary while taking off the polyamide coating and while the capillary was in the sample holder, we had to epoxy it into a hypodermic needle. The epoxy we used is Devcon Five Minute Epoxy. The needles that were used were 25 gauge by 5/8 in. with a 0.5-mm diameter and 22 gauge by 1.5 in. with a 0.7-mm diameter. We cut the 22 gauge by 1.5 in. needle with wire cutters to make it fit into the capillary holder. Then we ground the end of the needle down using a sandstone until we could see the opening of the needle completely because the opening would close when the needle was cut. Before we could epoxy the capillaries into the needles we cut them to the desired length, which depends on the shear rate desired. We used a capillary cutter that was provided by Polymicro Technologies.

Once everything was set up, we flowed water, tryptophan, and cyt-c through tygon tubing to the sample using pressures from 1 psi to 25 psi. The tygon tubing that we used is 1/16 of an inch ID and 1/8 of an inch OD. The water used is deionized. The tryptophan is a 20-micromolar (μM) solution in 0.1 Molar phosphate buffer, and the pH is 7.0. The cyt-c that was used is two different mixtures. One was a 24-μM solution in

2.94-guanidinium chloride, and the other was 5.88-μM in 27.3% methanol. The cyt-c that we used is at the mid-point of its folding/unfolding process at room temperature. This allowed the fluorescence to have its greatest possible intensity if any unfolding should occur.

Experimental

The purpose in the experiment is to see cyt-c unfold using shear denaturation. In order to see cyt-c unfold, the fluorescence intensity has to increase as the pressure increases. Before we run cyt-c through the capillary that we decide to try, we want to flow water and tryptophan. The CCD camera should not show any fluorescence for water, but the tryptophan should. The tryptophan should have the same fluorescence intensity as the pressure increases. If it does, then we can proceed and run cyt-c through the capillary. Before flowing tryptophan or cyt-c we will take pictures with the CCD while water is flowing through the sample for background cancellation, to check how much stray light enters the system. We use both the tryptophan or cyt-c picture and the water picture (see Fig. 6) to find out how much fluorescence is going through the capillary by using image mathematics in the Win View program that is used to run the CCD.

We started the experiment using the 20-micron ID capillary with a length of 50 mm when we started looking for shear denaturation. The capillary is held by epoxy in the needle so that only 10 mm can be seen at the end of it. A few problems arose when we first started to do the experiment.

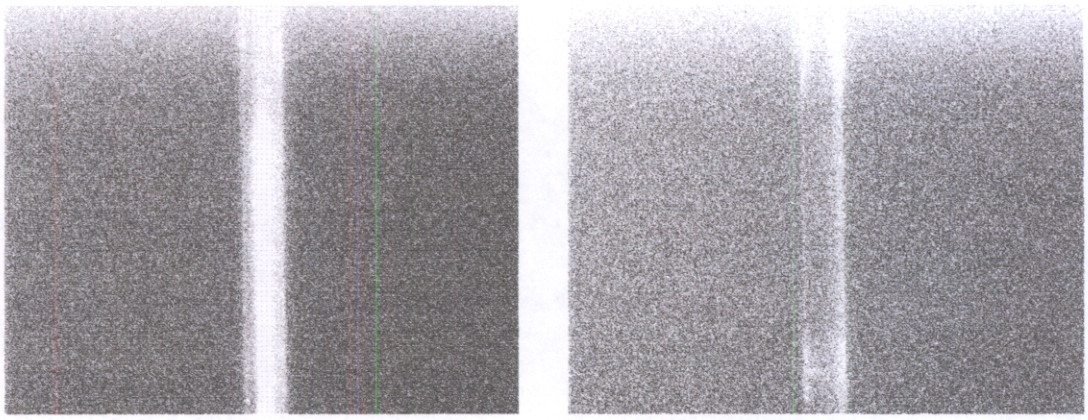

FIGURE 6. The first picture taken by the CCD is while tryptophan is flowing through the capillary. Any white seen in the picture is fluorescence. The white in the middle is the fluorescence from the tryptophan. The second picture taken by the CCD is while water is flowing through the capillary. The only florescence seen is scattered light, which is all that should be seen.

While doing the experiment, we fluctuated the pressure, but this was not a good thing to do. When the pressure is decreased from a high pressure to a low one, backflow would occur in the system. This is not consistent in doing this experiment, so we decided only to use increasing pressures. Another problem encountered was how to make the fluid at the end of the capillary flow and not make a bubble on the end of the capillary. This bubble hurts the experiment in different ways. When a bubble forms at the end of the capillary, it moves the fiber a little. This does not allow for a good background cancellation. To stop the bubble from flowing, we tried a piece of tygon tubing, but the fluid would not flow. We tried a glass pipette, but sometimes the capillary would not be touching it and the bubble at the end would form again and move the capillary. Putting it in the end of another capillary did not work; the fluid would not flow, but made a bigger bubble at the end. The best way to catch the fluid at the end of the capillary was with a q-tip and a Kim wipe touching the end of the capillary. This allowed the fluid somewhere to go; however, we could not recover any sample this way.

After fixing these problems that occurred while experimenting with the 20-micron, 50 mm long capillary, we ran tryptophan through at increasing pressures. The data for the tryptophan after subtracting the background was theoretically how it was supposed to look, the same fluorescence intensity with increasing pressure (see Fig. 7).

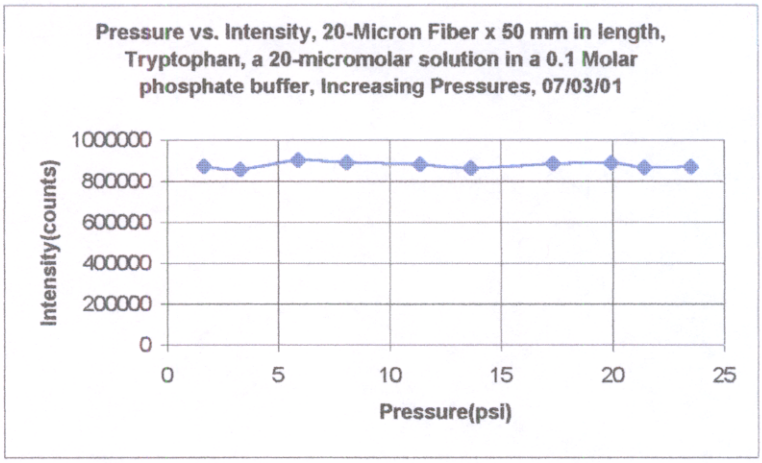

FIGURE 7. Graph of the 20-micron x 50 mm long capillary. Tryptophan, a 20-μM solution in 0.1Molar phosphate buffer is being run through the capillary. The pressures increase from 1 psi to 24 psi. The graph shows a straight line for the pressure vs. intensity, which is characteristic of tryptophan.

This allowed us to flow cyt-c, a 24-μM solution in 2.94-guanidinium chloride, through the system, the cyt-c behaved as the tryptophan (see Fig. 8).

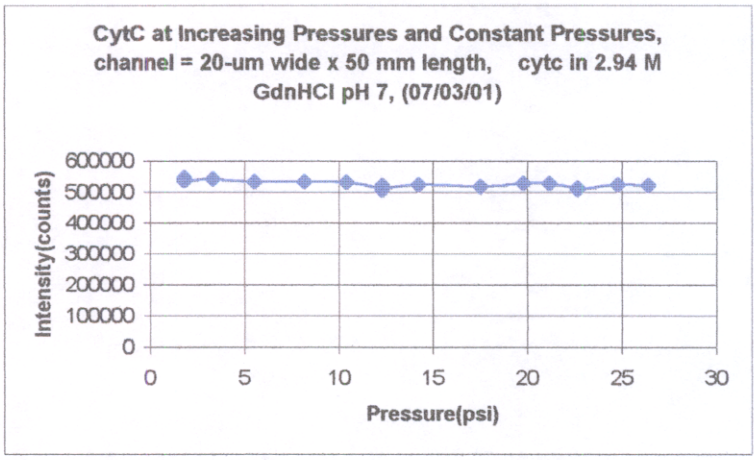

FIGURE 8. Graph of the cyt-c in a 2.94 GdnHCl solution, pH = 7.0. The capillary is 20-microns x 50 mm in length. The pressures increase from 1 psi to 26 psi and show no unfolding. No unfolding is present because there is no increase in the intensity of the cyt-c as the pressure increases.

This occurrence meant that cyt-c was not unfolding using a 20-micron, 50 mm long fiber at 26 psi. The maximum shear rate obtained using this size capillary was about 1 x 10^4 (s^{-1}).

The next shear rate that was tried was about 1 x 10^6 (s^{-1}) and was obtained by using a 50-micron fiber, 3 mm long. The process was very tedious while working with such a short capillary. The first fluid that we ran was tryptophan again and the data turned out normal, a straight line as the pressure increased (see Fig. 9).

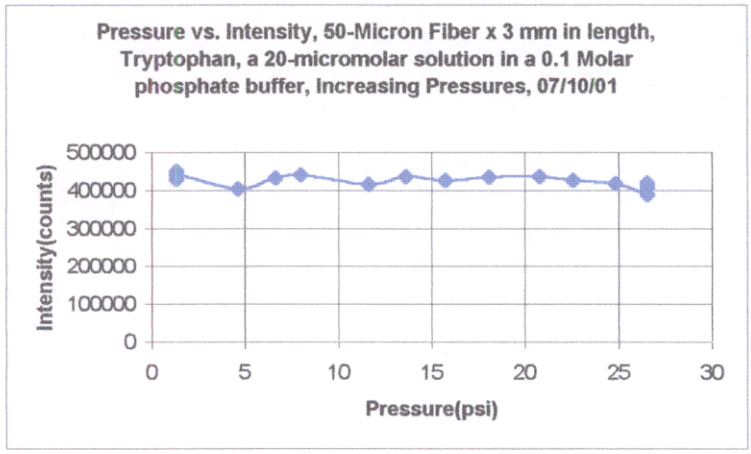

FIGURE 9. Graph of 50-micron x 3 mm long capillary. Tryptophan, a 20-μM solution in 0.1 Molar phosphate buffer is being run through the capillary. The pressures increase from 1 psi to 26 psi. The graph shows a straight line for the pressure vs. intensity, which is characteristic of tryptophan. The fluctuation in the graph may be due to noise in the system.

This allowed us to run cyt-c, a 5.88-μM in 27.3% methanol, and the same result was achieved, but there were little differences. The graph is not an exact straight line; it fluctuates a little. This may be due to noise in the system or unreliable background subtractions (see Fig. 10).

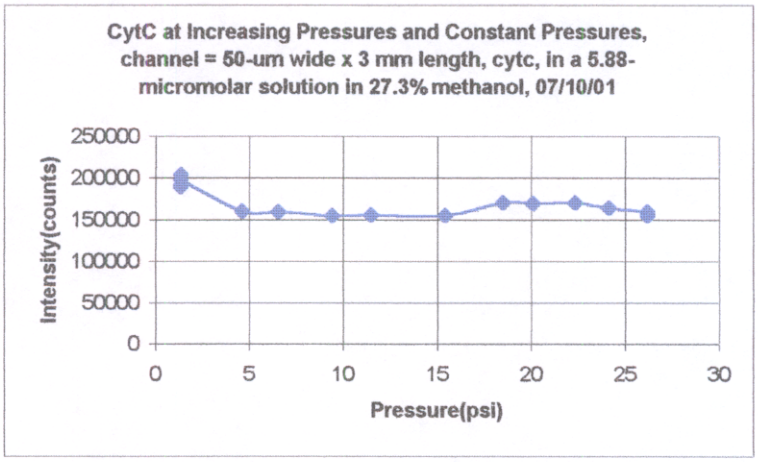

FIGURE 10. Graph of the cyt-c in a 5.88-μM solution in 27.3% methanol, pH = 7.0. The capillary is 50-microns x 3 mm in length. The pressures increase from 1 psi to 26 psi and show no unfolding. No unfolding is present because there is no increase in the intensity of the cyt-c as the pressure increases. Little fluctuation occurs due to noise in the system and unreliable background subtractions.

Results

This experiment revealed that high shear rates can be obtained in silica capillaries, but we were unable to unfold cytochrome-c using shear denaturation at shear rates of 1 x 10^4 (s^{-1}) and 1 x 10^6 (s^{-1}). We were able to see tryptophan and cyt-c fluorescence by eliminating all the background fluorescence and stray light. As seen in Figures 7-10, the fluorescence intensity did not change as the pressures increased. This was supposed to occur while flowing tryptophan because we were able to obtain the desired pressure-independent tryptophan fluorescence signal as a control experiment (see Fig. 7 and 9). The intensity did not change in Figures 8 and 10, which means that cyt-c did not unfold. This does not mean that cyt-c cannot be unfolded using shear denaturation. The shear rate needed to unfold a protein is not known, so we might have used shear rates that were too high or too low. We explored only a very small part of the many shear rates that may unfold a protein depending on the relaxation time. The relaxation time depends on the

solution conditions of the protein, or how much time it takes to see it fold and unfold (see Fig. 11).

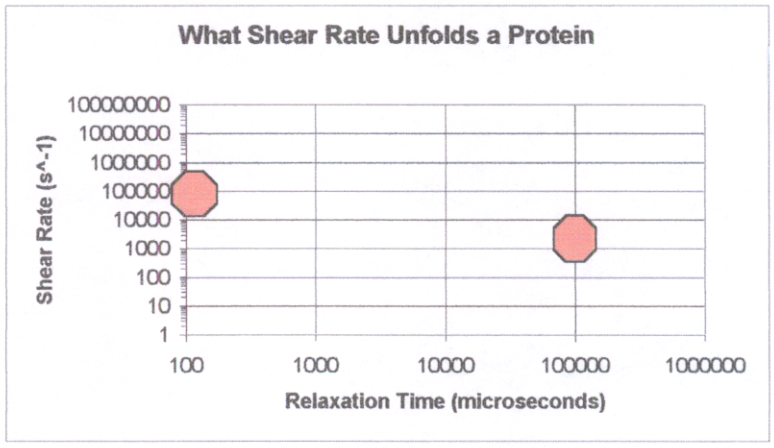

FIGURE 11. The graph shows, in red, the areas of shear we looked at for the different capillaries and solutions of cyt-c we used during the experiment. There are a lot of areas or different shear rates that may unfold a protein that we did not explore.

Also, during the experiment we ran into many different problems that had to be addressed, which we are not certain were completely remedied, due to complications in our system. The capillary moving during the experiment is a significant problem, especially when doing the image math to subtract out the background fluorescence. We used many different techniques to stop the capillary from moving, but none worked completely. Each technique would stop the capillary from moving a significant amount, but it still moved a little. From the q-tip to the micropipette, each remedy allowed some movement. We even tried to epoxy the capillary to another needle, but this clogged the capillary. So this flaw in the system was not addressed completely and leaves room for improvement and error.

More problems occurred as we ran water through the system. The water was fluorescent, which is should not occur. This means that tryptophan or cyt-c must stay behind in the tygon tubing or due to the dead volume in the capillary holder. To fix this

problem we had to flush the system with a lot of water and make sure that no fluorescence was seen when flowing water. After that, tryptophan or cyt-c could be run through the capillary, but this still allows for significant error in the data taken.

Discussion

Using the system we designed, shear denaturation of cyt-c using different shear rates could not be obtained. In the future, we should design a system that does not leave so much room for error. This design should include a way to hold the end of the capillary so it does not move at all and thus allows efficient background subtraction. The experiment shows that the system cannot have dead volume; there has to be a way to eliminate all tryptophan and cyt-c fluorescence after they flow through the capillary. Elimination of fluorescence will cut down on time and the use of materials. Also, in the future different shear rates should be used since it is not known which shear rate must be used to unfold a protein.

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