Sedimentation velocity through sucrose gradients for determination of molecular mass of integral membrane proteins

This technique is useful for gross approximation of relative molecular mass of protein complexes. By varying both the sucrose concentration in the gradient and the time of centrifugation, one can separate a variety of distinct molecular weight components. The mass of the protein complex can be estimated from its sedimentation constant, which itself is estimated from the migration through the sucrose gradient. The sedimentation constant is dependent on the partial specific volume of the protein and of the detergent, the density of the aqueous medium, and the temperature. I have used this technique to approximate the molecular weight of invariant chain and class II/ invariant chain complexes, and to demonstrate aggregation of newly synthesized MHC class II α and β chains.

Because sedimentation constants cannot be directly converted to molecular mass, the technique is only useful in determining relative molecular mass; thus, one needs to use standards of known molecular mass with which to compare the size of your experimental sample. For integral membrane proteins, this can be done in one of two ways. If characteristics of the detergent are known, such as micelle size and partial specific volume, one can calculate the effects of the detergent on migration and its contribution to molecular mass; then one can use typical soluble globular proteins as molecular weight standards, such as albumin (Mr=66,000), aldolase (complex Mr=158,000), and thyroglobulin (complex Mr~670,000). However, this assumes that only a single micelle is bound to the complex, and the calculations of partial specific volumes and micelle size may be tricky. Thus, I prefer to compare migration to other integral membrane proteins as molecular weight standards in order to simplify the analyses. While one still has to assume that the same number of micelles bind to the standards and to the experimental sample, other assumptions are minimized.

The following is the protocol I have used for monitoring sizes of metabolically labeled or surface labeled MHC class II complexes. For standards, I always run one gradient with globular standards so as to easily standardize one experiment to the next. In addition, for each experiment I usually use MHC class I (Mr~60,000) and CD45 (Mr~200,000) as integral membrane protein molecular mass standards, if that is easy to do. Be careful, when choosing other proteins as standards, to pick only ones that span the membrane once; who knows how multiple spans will affect detergent binding.

Protocol

1. Prepare metabolically labeled or surface labeled cells as desired. For pulse/chases, I used about 8 spleens per experiment, labeling with 2mCi/ml ^{3}H-leucine or ^{35}S-methionine and then dividing this into a pulse and 3 chases, so 2 spleens per gradient. For cell lines, I used about 1-2x10^{7} cells per gradient, labeled similarly. I forget what I used for surface labeling - I think just one or two spleens worth of cells.

2. Prepare detergent lysates. I did all of the published work with Trtion X-100, using 1% to solubilize the cells and 0.1% in the gradients. For the earlier work, in which I tried to correlate sedimentation with gel filtration data, I used either CHAPS or octyl glucoside, because they have

known small micelle sizes and known partial specific volumes. If you plan only on comparing to things like class I and CD45, the triton X-100 should be fine. I always include a slew of protease inhibitors, including: PMSF or AEBSF (0.25mM); leupeptin (10μg/ml); aprotinin (33μg/ml); pepstatin A (5μg/ml); E-64 (5μg/ml); and iodoacetamide (20mM). The iodoacetamide also prevents artifactual disulfide bond formation in the lysate; if you're really concerned, as I was, to eliminate this artifact, I pretreat the cells, while still alive, with 20mM N-ethyl maleimide in PBS prior to freezing or lysis.

3. Do any necessary pre-clearing before running the gradients. For the spleen cells, I pre-cleared 4 times: twice with rabbit anti-mouse Ig + protein A sepharose, once with Pansorbin, and once again with protein A-sepharose + protein G-sepharose (if I was planning to use protein G-sepharose for any of the precipitations). For cell lines, you need to use your experience.

4. Prepare gradients. I used varying sucrose concentration gradients, depending on the requirement. For analysis of the aggregates, I used a 10-40% sucrose gradient with a 16hr spin - this puts most of the fractions above the Mr = 250,000 range. For I chain trimers and $\alpha\beta$I complexes, I used 4-21% sucrose with a 15hr spin - this placed these complexes around the middle of the gradient. For $\alpha\beta$ dimers at the surface, I used a 4-15% gradient with a 32 hr spin - this placed the dimers in the middle of the gradient. I am careful to monitor the sucrose concentration in a sucrose stock solution by using a refractometer - it's never what it's supposed to be! The buffer I used was:

0.15M NaCl
50mM Tris-HCl pH 7.4
0.02% NaN3
0.1% Triton X-100

I prepared stock top and bottom sucrose solutions in this buffer from a 70% stock sucrose solution and concentrated stocks of the other components. I then took as much as I needed of each of these top and bottom solutions, and added protease inhibitor cocktails to each. If you're not using something protease sensitive (like invariant chain - class II dimers are pretty protease resistant), this is probably unnecessary, but a nice precaution.

To be *really* valid, *i.e.* if you are doing the sedimentation constant calculation, the gradients should be the in the 5.1ml tubes to be used with a SW50.1 rotor - something to do with the geometry of the tubes and minimizing friction. This is how I did the calculations with I and $\alpha\beta$I complexes. However, for decent comparative rates, the 12ml tubes and the SW41 rotor are easier to use and provide sufficiently valid results. The gradients are not self-forming, so you need to prepare them with a gradient maker. BioComp sells a beautiful gradient maker that makes it really easy to do the gradients simply by layering the two stocks one on top of the other; it then rotates the tubes at an appropriate angle and speed to generate a linear gradient. This is an expensive machine however; thus, most people do it the old fashioned way with the typical manual gradient maker, such as that from BioRad, which requires that each tube is prepared separately.

Finally, I chill the prepared gradients at 4°C for at least 1 hr (up to several hrs) while the samples are being prepared.

5. Final sample preparation. After pre-clears, I spin the hell out of the lysates - 2 x 14,000 RPM x 10 minutes in the microfuge. This is to ensure that I've removed all particulates and don't have crap running through the gradient to the bottom, causing turbulence and other nastiness. Then I carefully layer the sample onto the gradient - 0.5ml for the 12 ml tubes; 100-250μl for the 5.2ml tubes.

6. Centrifugation. All spins are done at about the same speed - 39,000 RPM for the SW41, 35,000 RPM for the SW50.1. There is a rumor that it should be done at 10°C, again having something to do with turbulence and geometry, but I have always done it at 4°C with no problems. The time will vary for the separation that you need. For the larger complexes, like the aggregates and I and $\alpha\beta$I complexes, I spin 15-16hrs (pick one and be consistent). For the cell surface complexes, I double it to 32 hrs. I have altered the speeds and times slightly to get the same time constant without too much variation in migration, so this is possible to make the timing more convenient.

7. Elution. There are two ways of doing this. The *best* way is to poke a hole in the bottom of the tube, using a needle connected to tubing, and drip out the bottom with minimal turbulence and the shortest amount of tubing to your fraction collector. This is not easy unless you have an appratus to do it - there are some old apparati around with a screw/needle hooked up to a tube holder, but these are becoming increasingly difficult to find. The next best way is to elute from the bottom using a peristaltic pump hooked up to tubing and a capillary tube to go down into the tube to the bottom of the gradient. You cause some turbulence this way and the tubing from the gradient is longer, allowing some mixing to occur; I haven't had too many problems, but I have been looking at gross differences, and it is possible that you may get sharper bands by eluting directly from the bottom with the needle method. In any case, I use a fraction collector to collect fractions based on time (using the pump - if you collect by drop, the drop-size will vary depending on the sucrose concentration, but if you collect by time, you will get even volumes throughout the gradient). I try to go for 15 fractions from the 12ml tubes, about 0.8ml each; I try to get 12 fractions, ~ 0.4ml each, from the 5.1ml tubes.

8. Analysis. Now is the painful part - immunoprecipitating from each fraction. The nice thing is that you don't have to pre-clear, but it's still a pain-in-the-butt. For my experiments, I precipitated each fraction first for the experimental sample (e.g. class II molecules) and then for the standard proteins (e.g. class I and CD45) in a sequential manner. If your standards are well separated on subsequent SDS-PAGE, you can co-precipitate multiple ones together in the same tubes to save time.

After SDS-PAGE and analysis of the peak distribution of experimentals and standards, you can figure out the relative M_r by the following formula:

$$d_1/d_2 = (M_{r1}/M_{r2})^{2/3}$$

where d_1 and d_2 are the distances travelled by the unknown and known standard proteins, respectively, and M_{r1} and M_{r2} are the relative molecular weights of the unknown and known proteins, respectively. Remember that fraction 1 is the origin, and so is considered to represent

distance traveled of 0. Otherwise, the fraction #s-1 can be used as relative distances.

References:

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