MECHANISMS OF FORCE PRODUCTION

With an available crystal structure of myosin, we are coming closer to understanding the molecular details of the mechanism of force production. For myosin, it appears that a conformational change in the head is induced by ATP binding and then relaxed during ADP release to produce a force for movement, and a similar mechanism may operate for dynein. The kinesin ATPase may produce force by a similar mechanism, namely by coupling a change in protein structure to the interactions occurring at the nucleotide binding site. For example, in all three cases (myosin, dynein, and kinesin), the motors operate via a mechanism requiring a flexible linkage of the globular motor domain to its cargo. It has been argued that this flexible linkage is required to get unidirectional force from an ATPase cycle involving symmetrical conformational changes (Johnson, 1985). However, in the case of kinesin, the force is possibly produced at a different step of the cycle, following ATP hydrolysis and preceding the release of the kinesin from the microtubule as shown in Scheme 1. However, the slow release and fast rebinding open up the possibility that the fundamental mechanism of force production may involve an alternating activity of the two kinesin heads along the microtubule surface. It remains to be established how this cycle can be modulated to produce force in opposite directions.

Although we have established the elementary steps of the ATPase cycle and the pathway of coupling ATP hydrolysis to force production, the questions pertaining to the molecular details of the coupling pathway are by no means settled. The next few years promise to offer exciting new discoveries that will illuminate the mechanistic basis for kinesin-driven motility.

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DISCUSSION

Session Chairperson: Steven M. Block
Scribe: Kevin Hacker

YALE GOLDMAN: With the sequential release of two steps in series at 20/s, I think you would expect to see a lag in your turbidity signal when you mix in the ATP. Do you see that?

KENNETH JOHNSON: No, we don't. The answer to your question is a function of whether or not the intermediate species with one kinesin head dissociated contributes to the light scatter signal. If the kinesin molecule with both subunits dissociated was the only species contributing to the signal, then we would have seen a lag followed by a rate of 20/s. What we see is a rate which is a function of the sum of both species, which suggests that they both contribute equally. I think the interpretation of the turbidity signal is open to further investigation. The fact that the phosphate release kinetics, which is a signal proportional to the release of phosphate from each of these heads, gives a 13/s rate with no lag, I think really does substantiate our interpretation.

GOLDMAN: If one head is dissociated does phosphate release from that before the other head is dissociated?

JOHNSON: We don't know. I would guess that's quite likely to be correct. If the release is 100/s from the intermediate kinesin species with one head bound to the microtubule, then that phosphate will release faster than the second head. But that may not matter. Once the kinesin is off, then the release of the phosphate can occur randomly–I would expect–and the only cooperativity I would expect we are seeing is when the two kinesin heads are next to each other on the microtubule.

EMIL RIESLER: Is the dissociation an obligatory step in the release of phosphate in the pathway or can phosphate dissociate from an attached head?

JOHNSON: Well, we would have to cross-link the kinesin to the microtubule to test that, and even then that would be a questionable experiment. The kinetics show that phosphate release and microtubule release are occurring at the same rate, and neither one with a lag. So you can take the interpretation from there. The alternative pathway with phosphate release preceding kinesin dissociation from the microtubule is possible, but it requires a new intermediate.

DAVID HACKNEY: As you know we have a model which is not like your model, in that we think the predominant form

of the species during steady-state ATP hydrolysis likely only has one of the two heads bound. What that leads to is that you get many ATP molecules hydrolyzed during each time the head fuses on. In your model are you saying that you get only one each time a dimer binds?

JOHNSON: We have some evidence for processive hydrolysis of ATP at lower ionic strength. The question remains to be established whether that maintains at higher physiological ionic strength. We disagree on data quite a bit, and we have been friends for 15 years and we ought to be able to sort this out. I think just to summarize some of the differences and to keep this discussion in terms of the different observations [an overhead transparency of the differences was shown]. I anticipated this question, and this was not a plant. Well anyway, we see a structure with a stoichiometry of one kinesin head per tubulin dimer, and Dave has shown data where in the presence of AMPPNP, it looks like there is one kinesin dimer per tubulin dimer so they stack up with one head hanging off in solution. There is some difference in the conditions that we are doing those experiments [under] and that translates over into subsequent experiments I think as well, and we will have to resolve that. In addition, I described the kinetic studies showing that when the kinesin-ADP complex with two ADP bound (we have measured the stoichiometry and quantified that). When that complex binds to the microtubule, both ADPs are released at a fast rate, and there is no slow component. In addition our radioactivity experiments do not show that one of the two heads remains with ADP bound, whereas Dave has shown under his conditions that with radioactive ADP only half of it comes off fast and the other half comes off more slowly. So there is something different happening there. And finally, our model is supported by our phosphate burst amplitude as well as our ATPase burst amplitude. We observe two products per dimer or one per site. Whereas this [Dave's model] predicts that we should see one product per dimer rather than two. So I think we have to resolve this in terms of further experimentation.

HACKNEY: What is the fact in regards to the number that actually binds. What we did see is that the first head binds much more strongly than the second head. And we only see that kind of thing in the presence of AMPPNP, if we go to very high ratio of heads to the microtubules. So we see nothing wrong with having both heads bound. In fact we think that's one of the reasons we don't see the same thing. Since you add the heads first you will have both of them bound and your following the rate of release of having both of those heads coming off. We think that's not a normal intermediate that is formed during the sliding process, and the rates that we look at when we only have a single head bound relate more to what is going on during the sliding process. Most of your ADP release experiments have always been done with an excess of triphosphate, which is not the way we do the experiments at least as far as I know. You may have some that you have not shown which were done without an excess of triphosphate to remove the bound nucleotide and let it cycle. One of the things we show is that we don't see half of the sites if we allow triphosphate to bind and cause the net displacement. And just one last thing with your size of your phosphate burst. How does that relate to the fact that when you first looked at that burst by just plain total generation of phosphate you only saw half of the size there which you described as being due to the release rate of the triphosphate?

JOHNSON: The difference there is that experiment is based upon a different experiment and points to the power of Martin Webb's assay. As you know the binding of ATP to the microtubule-kinesin complex is quite weak, and in order to do single turnover above 500 μM ATP when you have 10 μM kinesin sites. And therefore what we measure is a burst amplitude as a function of ATP concentration which is extrapolating to 100 percent saturation if we could get the points high enough. In contrast, in the experiments that we have done with Martin Webb, the sensitivity of that assay is so good that it is very easy to do an experiment at several mM ATP and see the release of phosphate from 50-nM kinesin sites. There isn't any discrepancy between those two measurements. And in fact we expect that the burst amplitude is one per site.

JONATHON HOWARD: Does your K401 dimer move? And if it does at what speed does it move? And is the speed compatible with the ATPase rate?

JOHNSON: You guys are so predictable [an overhead transparency with the rate motility rates is shown]. The motility of this construct was reported to us as 400 nm/s/two heads, and that translates over into 10 mn/ATP step size given (200 nm/s/head)/20 s/head ATPase rate.

HOWARD: Now, does it move processively, because doesn't your light scattering indicate that in fact it's not moving processively, because after one cycle pretty much all of the motor has dissociated; and isn't it possible that part of the reason why you see the apparent slower rate of dissociation than the hydrolysis rate is that what you are seeing is dissociations over subsequent cycles so that you got a little bit of processivity which is causing an apparent release rate that is slower than the hydrolysis rate?

JOHNSON: That is not possible. I would worry about that interpretation without the phosphate release data, which is quantifiable in terms of its amplitude. So the amplitude is one turnover per site and that is occurring with that same rate of 13/s. And there is no other way around that measurement. I think that the other answer to that question is in terms of understanding the data that leads us to the notion that kinesin is processive. And what we are arguing is that kinesin doesn't have time to diffuse away, and if you strap onto its back this great huge bead and expect it to walk along, when it hops off for 50 μs it's seeing a local concentration of tubule of 1 mM and can rebind at 20,000/s. And therefore I think that we really have to consider two things. One, is there evidence for

processivity beyond the processivity which is seen in the light microscope experiments where the kinesin is constrained? That may be realistic biologically even, but the other question is whether or not there is processivity in dilute solution of a single kinesin molecule. And I think we have got a handle on that and will be able to measure that directly. But the proper experiments need to be done.

MICHAEL FERENCZI: How sensitive is the phosphate release step to phosphate concentration in the medium?

JOHNSON: Martin Webb is a very smart guy as you will find out when you read his paper. The assay works mainly because he uses his previous attempt at a phosphate assay as a "mop" in the system. So by preincubating the system with this mop you sequester all contaminating phosphate so that when you do your experiment the sensitivity is quite good.

FERENCZI: I'm very familiar with Martin's assay. My question really is the experiments that you have done using Martin's assay are done at very low phosphate concentrations, which is an very usual situation compared to the way that experiments are done normally. So do you think that working at very low phosphate concentrations, free phosphate may affect your kinetics and how?

JOHNSON: Well, I didn't really take time to describe the assay but the phosphate binding protein, which is engineered, binds phosphate at the rate of 10^8 M/s, and the signal is so good that we can work with large excess of the phosphate binding protein over the phosphate. In addition, the binding constant is 0.1 μM, so we are actually within a range of the K_d, and the concentration of the phosphate binding protein is itself higher than the K_d for phosphate. So with all of those things, and with the control experiments done properly that we did, I don't think there is any basis for thinking that the assay is not accurately measuring the rate of 13/s.

FERENCZI: No, what my question is, if you were not using this assay you would have a much higher phosphate medium and that much higher phosphate in the medium may gave you a different apparent phosphate release.

JOHNSON: I see, so what you're trying to suggest is that phosphate binding at some other site is somehow perturbing the kinetics by binding to another site on the protein. Is that what you are implying?

FERENCZI: Or the same site.

JOHNSON: The same site? Well, phosphate can't bind to a site until the bound phosphate leaves.

FERENCZI: The phosphate would come off and then come on but if you had phosphate binding protein present . . .

JOHNSON: I like to think of this in terms of Ralph Yount's backdoor model. The thing about release of a single phosphate molecule that you have to think about is that there are larger molecules than phosphate that release from enzymes at rates of thousands per s and bind at rates approaching diffusion. And there certainly is something structural which is controlling whether or not phosphate leaves. So that conformational change is what is controlling phosphate release and the structural transition then is what is rate limiting. Once that structural transition occurs, the phosphate is gone. I guess I still don't understand the nature of your concern.

SCOTT KUO: Could you put back on the first transparency? I was very struck by the picture you have drawn for the structure of the AMPPNP condition [The structure shown has only one head of kinesin bound to the microtubule]. I have some preliminary evidence that suggests that the kinesin head bound to the microtubule in the presence of AMPPNP can rotate. I have always thought that both heads are bound in the presence of AMPPNP, but I like your picture. It seems more constant with my data.

JOHNSON: That may have something to do with the coupling cycle so I think AMPPNP at a superficial level we can understand. But I think we need to understand how it is really interacting with the kinesin as a dimer.

ROGER COOKE: In the myosin world the location of the force-generating step has been largely defined by the effect of ligands, like phosphate, and ADP. I guess this is in some ways a followup to Mike Ferenczi's question. How many of those experiments have been done? Say the effect of either high ADP or high phosphate on either force or velocity and do they fit with your position for the force-generating step?

JOHNSON: Well, the experiments at high phosphate have not been done, but Ron Vale's lab has reported studies with beryllium fluoride and the effect there is to slow motility. But I don't think you can interpret that in terms of how it is effecting the pathway. Whether phosphate is coming off at a step subsequent to the force-producing step or at the force-producing step you couldn't tell. Phosphate could back the system up and slow motility by either way.

HOWARD: Can you respond to my question about the processivity? You said that the phosphate release rate was 13/s but in the study book you say that it is 20/s. Wait. The 13/s is for physical release of the heads from the filament but the phosphate release is 20/s. So why can't the phosphate release measure the turnover of the cycle, and this could be partially releasing over several cycles?

JOHNSON: No, the interpretation is that the phosphate release is fast following release of the kinesin-ADP-Pi from the microtubule. It still is not occurring over several cycles. The

observed rate of 13/s is due to a two-step sequential release from two heads at 20/s each. We will have to talk about that later at the poster session.

STEVEN BLOCK: Finally, I didn't get to ask my question or make a brief comment, and since this is going on the record and the workshop wasn't, those of you at the workshop yesterday know that at least from our physiological studies that we would love to see a mechanism in which there are two sequential rate-limiting steps in the reaction. This is one such example. It may not be unique but it leads to a class of mechanisms. I think a lot of us now are beginning to think about mechanisms for movement in which two heads might alternate to waddle this thing up the microtubule, and some of the data are beginning to come together to a certain extent on this.