Millisecond Time Resolution Electron Cryo-microscopy of the M-ATP Transient Kinetic State of the Acto-Myosin ATPase

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ABSTRACT The structure of the AM-ATP transient kinetic state of the acto-myosin ATPase cycle has been examined by electron microscopy using frozen-hydrated specimens prepared in low ionic strength. By spraying grids layered with the acto-S1 complex with ATP immediately before freezing, it was possible to examine the structure of the ternary complex with a time resolution of 10 ms. Disordered binding of the S1 was observed, suggesting more than one attachment geometry. This could be due to the presence of more than one biochemical intermediate, or to a single intermediate binding in more than one conformation.

INTRODUCTION

The widely accepted "tilting cross-bridge" model of force generation in muscle postulates a gross structural change in the myosin head while attached to actin (Huxley, 1969; Rayment et al., 1993). This mechanism requires two or more head attachment geometries; however, to date only one geometry has been described at moderate (~3 nm) resolution (Milligan and Flicker, 1987). This is the strongly attached state occurring with bound ADP or in the absence of nucleotide, which is characterized by binding to actin at ~45° and probably corresponds to the end of the cross-bridge power stroke. Other attached head states, such as AM-ATP and AM-ADP-Pi, have been difficult to study because the myosin-S1 is weakly bound to actin. Using low ionic strength to stabilize these states, we recently obtained electron micrographs of acto-myosin-S1 frozen during steady-state ATP hydrolysis (Walker et al., 1994). The pictures showed disordered S1 attachment, which suggests that the myosin head adopts more than one conformation while going through the ATPase cycle. The steady-state data in ATP, therefore, are compatible with a tilting mechanism but do not prove it.

One way to test the tilting-bridge hypothesis further is to obtain micrographs of the acto-S1 complex in defined kinetic states of the ATPase cycle. This will allow exploration of whether the different kinetic states are associated with particular structural geometries. In principle, freezing offers the fast time resolution necessary to separate kinetic intermediates, because cooling rates in small specimens can be ~10[7]°C/s. If it is assumed that the decrease in temperature necessary to arrest a specimen is ~100°C, the theoretical time resolution attainable by freezing is ~10 μs (Mayer and Astl, 1992). In practice, the time resolution achievable with transmission electron microscope specimens is much slower and is governed by the delays in mixing the specimen and

then thinning it to ~100 nm. Until recently, these manipulations required several seconds, but new methodology involving spraying the microscope grid with a reactant immediately before freezing has reduced the delay to 5–10 ms. This approach is widely applicable, because small molecules, proteins, or protein assemblies can be sprayed from solution. The present paper describes our initial experiments using this method, which have mainly involved spraying mant-ATP (2'-(3')-O-(n-methylanthraniloyl)-ATP (Woodward et al., 1991) onto grids coated with acto-S1. The micrographs obtained show disorder in S1 attachment similar to that seen during steady-state hydrolysis. We conclude that the M-ATP state of the ATPase cycle is dissimilar to the ADP state and is not characterized by a single attachment geometry.

MATERIALS AND METHODS

The spray method involves layering a microscope grid with one reactant and then spraying on a second reactant immediately before freezing (Berriman and Unwin, 1994). A schematic diagram of the apparatus we have built is shown in Fig. 1. 5 μl of acto-S1 is applied to a holey carbon coated grid that was glow-discharged in an amylamine atmosphere. The acto-S1 was previously incubated for 5 min in 5 μg/ml apyrase (Sigma Grade 7) to hydrolyze any ADP or ATP present. The grid is then blotted for 1 s by gentle squeezing from both sides with filter papers attached to two solenoids (see also Trinick and Cooper, 1990). 50 ms after the withdrawal of the filter papers, a pneumatic piston to which the forceps holding the grid are attached is activated. This propels the grid at speeds of 1–2 m/s into approximately 10 ml of liquid ethane in a metal thimble in contact with liquid nitrogen. The grid passes through a spray containing substrate (e.g., mant-ATP) at a distance of 1 cm (or less) above the ethane. The timing of the solenoids that activate the blotting, spraying, and plunging is under software control using a program written in TurboBASIC. The control signals pass through the printer port (data lines 2–9) of a personal computer and through an interface with an optical decoupler that electrically isolates the computer and solenoid circuits. The velocity of the grid is determined from a LED/photodiode pair interrupted by the sprocket holes of an exposed piece of 35-mm film attached to the pneumatic piston. Output from the LED is determined by the change in voltage to an input data line (pin #14) of the printer port.

To demonstrate that the area being viewed in a micrograph had received a spray droplet, an easily identifiable marker is included in the spray. In the present experiments, the marker particles were 10 or 5 nm colloidal gold (coupled to BSA, Biocell, Cardiff, U.K.). The frozen grids were viewed unstained in the electron microscope using low dose (~10 e/Å^2) to minimize radiation damage, which is the main factor limiting information recovery
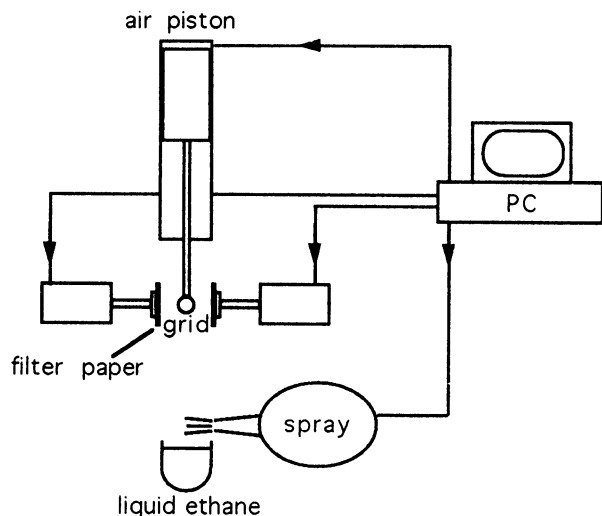
FIGURE 1   Diagram of spray-freezing apparatus.

FIGURE 2   (*top*) Electron micrograph of acto-S1 10 ms after spraying with mant-ATP. The microscope grid was coated with 3.4 μM actin and 4.5 μM S1 in 5 mM MOPS, 2 mM MgCl$_2$, pH 7 and then sprayed in the cold with a solution containing 5 mM Mg-mant-ATP and 1.6 $\times$ 10^{13} gold particles/ml in the same buffer. The arrowhead indicates a 10-nm colloidal gold particle. The other larger round objects visible are ice contamination and were clearly distinguishable from the gold by their lower brightness in the negative. (*bottom*) Specimen prepared as in (*top*) but in the absence of mant-ATP and using 5-nm colloidal gold (1.6 $\times$ 10^{14} particles/ml, see *arrows*). Magnification 304,000$\times$.

from frozen-hydrated specimens. The areas photographed were over holes in a carbon film, so the proteins were not attached to a substrate. Micrographs were recorded ~3 μm underfocus, which limits the spatial resolution to ~5 nm. This defocus was chosen empirically to observe individual S1 molecules with reasonable contrast. (Provided the freezing rate is sufficiently fast so that the water is vitreous, the spatial resolution attainable with an aperiodic object closer to focus at lower contrast is 2–3 nm.) Actin was prepared from rabbit skeletal muscle by the method of Pardee and Spudich (1982). Subfragment-1 containing both light chains was prepared from porcine cardiac myosin by papain digestion in the presence of Mg^{2+} and was a gift from R. Smith and I. Rayment. Other details were as described previously (Walker et al., 1994).

We used the ATP analog mant-ATP because at 4°C the rate of dissociation of M-mant-ATP from actin at saturating mant-ATP is 150 s^{-1}, which is at least fivefold slower than the rate of dissociation of M-ATP from actin. Therefore, 10 ms after mixing with acto-S1, approximately 25% of the S1 should remain bound to actin as AM-mant-ATP. At 4°C the rate of hydrolysis of ATP bound to myosin is ~7 s^{-1}. Although we have not yet measured the rate of hydrolysis of M-mant-ATP, it is unlikely to be fast enough for significant hydrolysis to occur in 10 ms. The intermediate bound to actin, therefore, should be >90% M-mant-ATP and <10% M-mant-ADP-Pi.

RESULTS

Fig. 2 (*top*) shows a micrograph of a mixture of actin and porcine cardiac S1 sprayed with 5 mM mant-ATP 10 ms before being frozen. In the background are many small particles whose size is compatible with their being unbound S1 molecules. Also visible are actin filaments, and it is clear that a substantial fraction of the S1 is bound to actin. This is best visualized by viewing the micrograph from the edge and sighting along individual filaments. The fraction of the S1 bound appears compatible with the expected estimate of 25%. The binding of the S1 to the actin appears to be disordered. This disorder is probably due to the partial occupancy of the available actin binding sites, but also appears to result from the S1 binding to the actin with variable geometry. In some cases, the S1 appears to be attached to the actin filament but separated
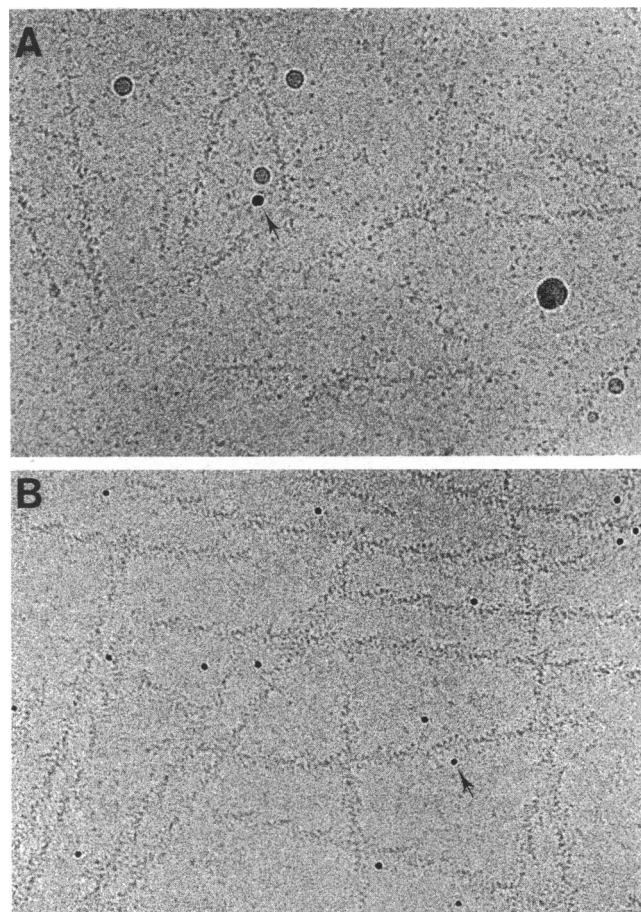
from it by a region of reduced density, which was also observed during steady-state hydrolysis (Walker et al., 1994).
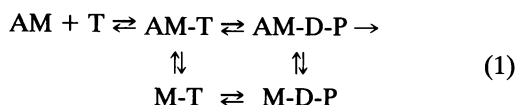
Also visible in Fig. 2 (*top*) is a 10-nm gold particle (*arrow*). Colloidal gold with particles of this size was included in the spray, and the presence of the particle in the micrograph confirms that the area of the grid photographed had received a spray droplet and, therefore, had been exposed to the mant-ATP. The droplets produced by the simple atomizer used are mostly 1–2 μm in diameter, although some are larger. Because the water layer has to be <100 nm to be suitable for microscopy, only edges of the regions to where the droplets had spread were sufficiently thin. The time for which these areas of the grid were exposed to the mant-ATP, therefore, may have been significantly less than the 10 ms between spraying and freezing.

Fig. 2 (*bottom*) shows a micrograph of a specimen sprayed with buffer containing gold but no nucleotide. The amount of free S1 in the background in this micrograph is much less than in the presence of nucleotide. The actin filaments have the "arrowhead" appearance characteristic of S1 tightly bound in the absence of nucleotide. In some of the filaments in this figure, the arrowheads have a one-sided appearance that we assume arose from interaction with the surface of the thin (60–80 nm) water layer. The arrowhead appearance was also observed in micrographs from grids layered with the acto-S1 complex in the absence of nucleotide and not sprayed (see also Milligan and Flicker, 1987; Walker et al., 1994). Also visible in the figure are gold particles 5 nm in diameter, demonstrating that this area of the grid was exposed to the sprayed buffer. 5-nm gold was used rather than 10-nm gold because it diffuses faster. The edge of the region to where the gold particles have spread, therefore, will be closer to the edge of the region to where small molecules in the sprayed buffer diffused.
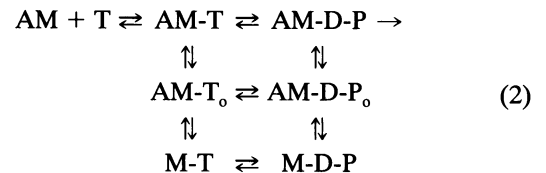
DISCUSSION

Comparison of Fig. 2, (*top* and *bottom*) shows that the appearance of the acto-S1 complex 10 ms after spraying with mant-ATP is quite different from that in the absence of nucleotide. Under the low ionic strength conditions used, a substantial fraction of S1-mant-ATP is attached to actin. This complex is disordered, and the appearance of the micrograph is superficially similar to micrographs obtained during steady-state ATP hydrolysis (Walker et al., 1994). The short time of exposure to substrate (<10 ms) ensures that the observed complex is AM-T rather than a mixture of AM-T and AM-D-P because the bound substrate will not have had time to hydrolyze. The data, therefore, suggest that the AM-T state is structurally different from the AM state and is not characterized by a single binding geometry.

A key question concerns which biochemical/structural intermediate(s) are being observed in Fig. 2 (*top*). The two predominant biochemical states will be M-T and AM-T. According to the simplest models (e.g., Lymn and Taylor, 1971; Eisenberg and Greene, 1980), which relate actomyosin ATP hydrolysis and muscle contraction, a single biochemical state (such as AM-T, see Eq. 1) was implied to have a single predominant structure.

$$AM + T \rightleftarrows AM\text{-}T \rightleftarrows AM\text{-}D\text{-}P \rightarrow$$
$$\text{\Updownarrow} \qquad \text{\Updownarrow} \qquad (1)$$
$$M\text{-}T \rightleftarrows M\text{-}D\text{-}P$$

More recent kinetic and structural models explicitly include additional intermediates (Geeves, 1995; Holmes, et al. 1995) as shown in Eq. 2. According to the terminology used by these workers, the intermediates $AM\text{-}T_o$ and $AM\text{-}D\text{-}P_o$ would be either collision complexes (Geeves, 1995) or nonspecific ionic intermediates (Holmes, 1995). The nomenclature used may be somewhat prejudicial because the kinetic character-

ization of the additional intermediates is much less well characterized than that for simpler models.

$$AM + T \rightleftarrows AM\text{-}T \rightleftarrows AM\text{-}D\text{-}P \rightarrow$$
$$\text{\Updownarrow} \qquad \text{\Updownarrow}$$
$$AM\text{-}T_o \rightleftarrows AM\text{-}D\text{-}P_o \qquad (2)$$
$$\text{\Updownarrow} \qquad \text{\Updownarrow}$$
$$M\text{-}T \rightleftarrows M\text{-}D\text{-}P$$

An extra intermediate such as $AM\text{-}T_o$ is inferred from slower than diffusion-limited rates of binding of myosin and myosin intermediates to actin (White and Taylor, 1976; Geeves, 1984) and from structural considerations (Rayment, 1993). We are unable to tell whether the multiple modes of binding that we have observed at low ionic strength are due to a mixture of states ($AM\text{-}T_o$ and AM-T) or multiple attachment modes in a single state (e.g., $AM\text{-}T_o$ or AM-T). The same question is pertinent to the "weak binding states" that have been observed by centrifuge binding experiments (Chalovitch and Eisenberg, 1982), ESR measurements (Fajer et al., 1991), and muscle mechanics (Brenner and Yu, 1993) at low ionic strength.

A second controversial point is whether complexes observed at low ionic strength are additional intermediates that are produced only at low ionic strength and are not part of the normal ATP hydrolysis and force-producing mechanisms. There are several pieces of experimental evidence to indicate that the low ionic strength intermediate, whether it is an initial intermediate ($AM\text{-}T_o$ in Eq. 2) or the "weakly" bound cross-bridge intermediate (AM-T in Eq. 1), is an intermediate that is specifically bound and is a part of the normal reaction pathway of myosin binding to actin. For an alternative view, see Holmes et al. (1995).

(1) The observed second-order rate constants for binding of S1 or S1-ADP to actin and the steady-state rate of ATP hydrolysis (S1-ADP-Pi binding to actin) have a dependence on ionic strength that can be modeled by a simple Debye-Huckel interaction between opposite charges (Siemankowski and White, 1984; White and Taylor, 1976). The slopes in Fig. 3, which are proportional to the charge product of the ionic interaction, are the same within experimental error for myosin-S1, myosin-S1-ADP, and steady-state actin-activated MgATP hydrolysis. There is an increase of approximately two orders of magnitude in the rate constants of binding on decreasing ionic strength. Rate constants measured at very low ionic strength (conditions of Walker et al., 1994), which are shown on the left side of Fig. 3, extend the linear plot of rate constants measured at higher ionic strength. The parallel dependences of S1 and S1-ADP binding to actin and dependence of steady-state ATP hydrolysis upon ionic strength over a wide range of ionic conditions are evidence for a common ionic component of the binding of myosin and myosin intermediates to actin. This interaction occurs over a wide range of ionic strengths from physiological to low ionic strength and is independent of ligands bound to myosin-S1. If an additional intermediate were present during steady-state ATP hydrolysis at low ionic strength, then a reduced
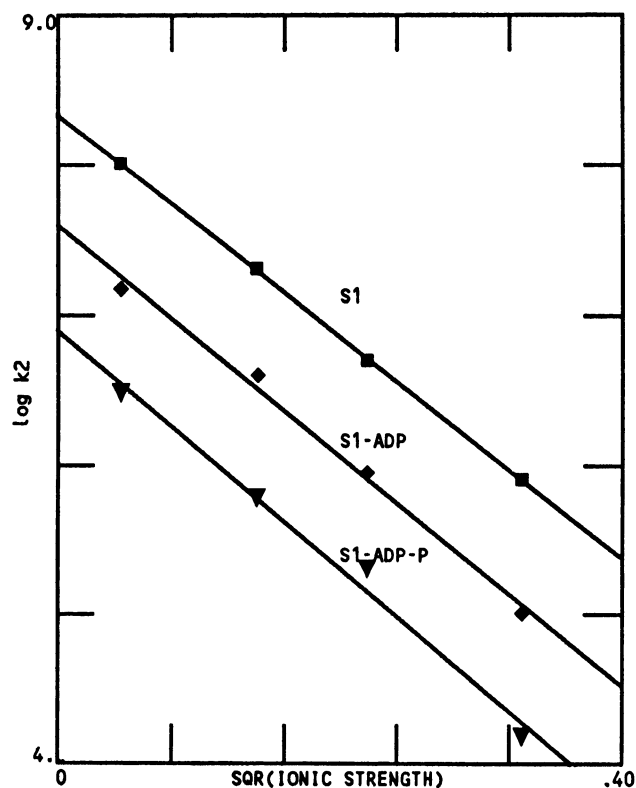
FIGURE 3 Dependence of the second-order rate constant of binding of S1, S1-ADP, and S1-ADP-Pi to actin upon ionic strength. Second-order rate constants were determined from the dependence of observed rate constants of binding upon the concentration of S1 or S1-ADP to actin in the presence and absence of 1 mM MgADP, or the dependence of the rate of steady-state hydrolysis of 1 mM MgATP upon actin concentration. Lines drawn through the data were obtained were by nonlinear least-square fits of log k_2 vs. (ionic strength)$^{1/2}$. The slopes and intercepts obtained were as follows. S1, -7.4 ± 0.5, 8.3 ± 1; S1-ADP: -7.7 ± 0.4, 7.6 ± 0.2; S1-ADP-Pi (steady-state ATP hydrolysis), -7.9 ± 0.6, 7.0 ± 0.2. Experimental conditions: 5 mM MOPS, 2 mM $MgCl_2$ pH 7 at 20°C with KCl added to give the indicated ionic strength (except for the lowest ionic strength points, which contained 1 mM MOPS, 0.2 mM $MgCl_2$, and either 0.1 mM ADP or ATP).

slope would be expected in the plot of the dependence of ATP hydrolysis on ionic strength. This is not observed. These data indicate that the ionic component of S1 and S1 products binding to actin is part of the normal reaction pathway and that the primary effect of low ionic strength is to increase the rate of binding of S1 and S1-ATP hydrolysis intermediates to actin.

(2) There is evidence for the specificity of the ionic interaction. A part of the myosin molecule implicated in the ionic component of acto-myosin binding is the loop between residues 626 and 647, which makes contact with the N-terminal portion of actin (Rayment, 1993). Exchanging this segment of the myosin sequence between smooth skeletal and Dictyostelium myosin produces chimeric myosins with maximal rates of actin-activated ATP hydrolysis dependent on the origin of the 626–647 sequence (Uyeda et al., 1994). This indicates that the sequence of residues involved in the ionic interaction is also an important determinant of the maximum hydrolysis rate.

In summary, there is a large change in the appearance of actomyosin-S1 by frozen-hydrated electron cryomicroscopy only 10 ms after mixing with mant-ATP. The S1-mant-ATP that remains bound to actin has a disordered appearance that is similar to that observed during steady-state ATP hydrolysis at low ionic strength. The multiple modes of binding may either be due to more than one type of AM-T intermediate or multiple binding geometries of a single intermediate. The ionic strength dependences of S1 binding to actin and actin activation of myosin ATP hydrolysis indicate that the primary affect of low ionic strength is to increase the rate of myosin-S1 binding to actin, and that low ionic strength intermediates are on the catalytic pathway.

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DISCUSSION

Session Chairperson: Ivan Rayment
Scribe: Willy Wriggers

BERNHARD BRENNER: This was unregulated actin, so wouldn't you expect from Ken Holmes' contribution this morning, that the weak interaction should be nonspecific?

JOHN TRINICK: What makes us believe that our results are representative in some way for high salt concentrations are the binding and ATPase rates as a function of salt: if you plot the ionic strength of binding of S1 or S1.ADP we don't find anything new at low salt compared to high salt.

MASATAKA KAWAI: Do you have an estimate of the rate constants of the kinetic state transitions you showed in the last slide, especially the fast two: A.M. to A.M.ATP and the detaching transition?

TRINICK: My collaborator Howard White can comment on that.

HOWARD WHITE: The maximum rate of dissociation is 150 per second for mant-ATP, which could either be assigned to the rate of the M.ATP coming off or, probably more likely, to a transition before it comes off.

DAVE THOMAS: We have apparently an example here of a single biochemical state associated with a wide range of angles. Spectroscopic experiments have been suggesting this for a long time: Pjotr Fajer will present some work later, where you have a multi-angular state that should be a single biochemical state. It has been suggested that the disorder is due to many different states. At least in biochemical terms that is not true. Of course there could be many different structural states within one biochemical state.

If you now identify the weak binding, early states with the A.M.ATP state you show on the transparency, and on the right you draw a state like the one you drew for A.M., the transition going from an disordered to an ordered state will produce an average tilt of about 45 degrees of the myosin S1 and should be able to produce force without any other stereospecific state other than the final.

GERALD POLLACK: Did you perform a control experiment to make sure it was not the spray that induced the disorder?

TRINICK: Yes. For example if one just sprays buffer, one only gets the normal arrowhead configuration.

ROGER COOKE: Some years ago Tom Pollard did a somewhat similar experiment, in which he decided that it looked like rigor in steady state hydrolysis. What is the difference between his result and yours? Why is his wrong?

TRINICK: One difference between our experiment and Tom's is, he froze his specimen, and then he cleaved it into two and etched it in vacuum. And in order to etched it you have to raise the temperature to about −100 degrees. Then he shadowed it with metal. Our results are different from his, possibly because something happened during the warming, drying, and shadowing procedure, which was not necessary in our case: our specimens were never warmed up above −170 degrees.

COOKE: You could test this hypothesis by warming your probe up, cooling it off, and see if it would change the angle.

TRINICK: Yes, we could warm it and then shadow it. Technically, it probably wouldn't be an easy experiment.

IVAN RAYMENT: These are very challenging results. Are you really confident that you get uniform mixing on that timescale?

TRINICK: I don't think the mixing is uniform. We have a grid with many droplets on it. With a small molecule like mant-ATP, the question is how to determine whether the spray has arrived at the observed region. We put 10-nm colloidal gold in the spray. This particle can be then detected in the image; in fact, it gives a very dense spot on the negative. So we are absolutely certain that the spray has arrived at the observed region. But it will not be uniformly mixed; we rather get a mant-ATP gradient. Locally, however, the ATP concentration will be very large.

HOWARD WHITE: I also want to answer this. The dimension of the picture is roughly 1 micron. The rate of diffusion is a few microns per millisecond. So the nucleotide concentration should be homogeneous within the field we are looking at. However, we can also find different fields on the same grid with rigor conformations, where the specimen has not been exposed to mant-ATP.