Chapter 13

Analysing the ATP Turnover Cycle of Microtubule Motors

Claire T. Friel, Clive R. Bagshaw, and Jonathon Howard

Abstract

Proteins of the kinesin superfamily share a conserved motor domain, which both hydrolyses adenosine-5′-triphosphate (ATP) and binds microtubules. To determine the mechanism of action of a kinesin, it is necessary to relate the chemical cycle of ATP turnover to the mechanics of microtubule interaction. In this chapter, a number of methods are outlined by which the ATP turnover cycle of a kinesin can be analysed with a particular focus on the use of fluorescently labelled ATP and ADP analogues as a means of isolating individual steps in the cycle. By analysing the ATP turnover cycle of a kinesin, both in solution and in the presence of microtubules, the change in nucleotide state triggered upon microtubule binding can be determined. This provides information vital to understanding the coupling of the chemical and mechanical cycles that is integral to the action of members of the kinesin superfamily.

Key words: Kinesin, ATP hydrolysis, Enzyme kinetics, Mant-ATP, HPLC, Stopped flow

1. Introduction

Kinesins are a large family of proteins that use turnover of adenosine-5′-triphosphate (ATP) to regulate their interaction with the microtubule cytoskeleton (1). Changes in the nucleotide state of the kinesin motor domain are stimulated by interaction with the microtubule, and in turn the affinity of a kinesin motor domain for the microtubule is altered according to its nucleotide state. The exact nature of the nucleotide-dependent interaction with microtubules varies among members of the kinesin superfamily producing kinesins tailored to different functions, such as cargo transport (2), microtubule sliding (3), and microtubule depolymerisation (4, 5). However, for all kinesins, ATP turnover is closely coupled to the interaction with microtubules which stimulates the ATP hydrolysis rate of a kinesin often by several

Anne Straube (ed.), *Microtubule Dynamics: Methods and Protocols*, Methods in Molecular Biology, vol. 777,
DOI 10.1007/978-1-61779-252-6_13, © Springer Science+Business Media, LLC 2011

1,000-fold above the basal ATP turnover rate. The precise nature of the way in which a kinesin hydrolyses ATP (chemical cycle) as well as the nature of its interactions with microtubules (mechanical cycle) must be known in order to fully comprehend the action of an individual kinesin. In this chapter, a selection of protocols are described by which the ATP turnover cycle of a kinesin can be studied, particularly focusing on identifying the rate-limiting step in the basal ATP turnover cycle and how this is affected by interaction with microtubules.

2. Materials

1. 100 mM Mg-ATP: The disodium salt of ATP (Roche #10519979001) is made up to a concentration of 100 mM in 100 mM $MgCl_2$ and the pH adjusted to 6.9 with KOH. The concentration should be checked by absorption at 260 nm ($\varepsilon = 15{,}400/\text{M cm}$) (6) and the solution stored in aliquots at −20°C.
2. 2′/3′-(*N*-methylanthraniloyl)-adenosine-5′-triphosphate (mant-ATP) (Jena biosciences NU-202).
3. 2′/3′-(*N*-methylanthraniloyl)-adenosine-5′-diphosphate (mant-ADP) (Jena biosciences NU-201).
4. 3′-(*N*-methylanthraniloyl)-2′-deoxy-adenosine-5′-triphosphate (mant-dATP) (Jena biosciences NU-203).
5. G25 sephadex resin. It is convenient to use pre-packed columns, for example NAP-5 column (GE Healthcare #17-0853-01).
6. C-18 column. For example, the Luna 3 μ C18(2) 100Å (Phenomenex).
7. C-18 column running buffer: 100 mM potassium phosphate pH 6.5, 10 mM tetrabutylammonium bromide (TBABr) (Fluka #86860), plus acetonitrile, HPLC grade (Perbio #51101) as required, 10% (Subheading 3.1.1) or 30% (Subheading 3.2.2). Filter the buffer using 0.45-μm nylon filters prior to use.
8. BIOMOL green reagent (Enzo Life Sciences AK-111).
9. 0.6 M perchloric acid, pre-cool on ice (Sigma-Aldrich #24425-2).
10. 1 M Tris, 3 M KOH.
11. 2 M HCl.
12. 96-well plate (Greiner #655101).
13. Ethylenediaminetetraacetic acid (EDTA): 0.5 M stock solution in H_2O, pH 8.0.

14. Dithiothreitol (DTT): 1 M stock solution in H_2O, make fresh from powder for use on the same day.
15. BRB80 buffer: 80 mM PIPES, KOH pH 6.9, 1 mM $MgCl_2$, and 1 mM EGTA (see Note 4). This buffer is normally made up as a 5× stock solution (note that PIPES buffer is not very soluble in water until the pH is raised to ~6).

---

3. Methods

3.1. Analysis of Overall Cycle

The starting point for an analysis of the ATP turnover cycle (Fig. 1) of a kinesin is to determine the complete ATP turnover cycle rate: the rate at which ATP is hydrolysed under steady-state conditions. To do this, the rate of product production (either ADP or P_i) is determined under saturating substrate (ATP) concentrations. This rate is governed by the slowest step in the ATP turnover cycle and as such is a measure of the rate-limiting step in the cycle. When the steady-state rate is normalised for the kinesin concentration, e.g. μmole Pi/μmole kinesin/second, it becomes equivalent to a first order rate constant for the reaction. This value is referred to in the literature by a number of different terms (e.g. ATPase rate and turnover rate); this variation in nomenclature can cause confusion (see Note 1). This rate constant may also be determined directly by transient-state methods (see Subheading 3.2) allowing identification of the rate-limiting step in the cycle.

In order to measure the rate of ATP turnover under steady-state conditions, the substrate (ATP) must be in large excess over the concentration of kinesin motor domains (see Note 2) such that the reaction proceeds under saturating ATP concentrations. High concentrations of ATP are required for a linear initial rate not only to minimise the depletion of ATP, but also to ensure that ATP remains competitive as ADP concentrations build up.

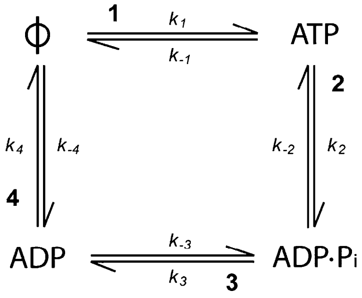

Fig. 1. The minimal ATP turnover cycle for an ATP-hydrolysing enzyme, such as a kinesin. Each kinesin motor domain can exist in four states with respect to nucleotide: nucleotide-free (ϕ), ATP-bound, ADP.P_i-bound, and ADP-bound.

In principle, to ensure that the ATP concentration is saturating, a titration of ATP should be carried out to determine the point at which the rate of ATP turnover no longer increases with increasing concentration of ATP. In practice, however, when working with micro-molar concentrations of kinesin, 2 mM ATP is generally found to be sufficient. Two methods are described by which the overall hydrolysis cycle rate can be determined: by following the production of ADP and the production of phosphate.

3.1.1. Measuring Production of ADP

There is no optical spectroscopic signal by which ADP can be distinguished from ATP. Therefore, in order to directly monitor the production of ADP, it is necessary to take samples as the reaction progresses and separate the product (ADP) from the substrate (ATP), for example by HPLC as described below (see Note 3).

1. Set up reaction mix with 2 mM Mg-ATP in the chosen buffer (see Note 4).
2. Start reaction by mixing kinesin (see Note 5) in chosen buffer with ATP-containing reaction mix (see Note 6).
3. Incubate reaction at chosen temperature (normally, either 25 or 37°C is used).
4. Take samples at selected time points (see Note 7), and quench reaction by mixing with an equal volume of ice-cold 0.6 M perchloric acid.
5. Neutralise samples by addition of the necessary volume of 1 M Tris, 3 M KOH, which is determined by trial reactions (e.g. if using 20 μl sample + 20 μl 0.6 M perchloric acid, 8 μl 1 M Tris, 3 M KOH is required). Neutralised samples can be stored at −80°C until all samples are ready for analysis.
6. Spin samples at 17,000 g 13,000 rpm, Heraeus Biofuge Microcentrifuge for 10 min to remove precipitate.
7. To separate ADP from ATP, run each reaction sample on a C-18 column (see Note 8) using isocratic flow with a 100 mM potassium phosphate, 10 mM TBABr, and 10% acetonitrile buffer (see Subheading 2, item 7). The flow rate used depends upon the pressure limits of the particular HPLC system and column used, but flow rates of ~1 ml/min are generally achievable.
8. The presence of both ADP and ATP is measured by absorbance at 260 nm. The integrated area under the respective peaks provides a measure of the quantity of each nucleotide present in the sample (Fig. 2a).
9. For each time point, calculate the proportion of ADP as a fraction of total nucleotide (area of ADP peak/[area of ADP + ATP peaks]).

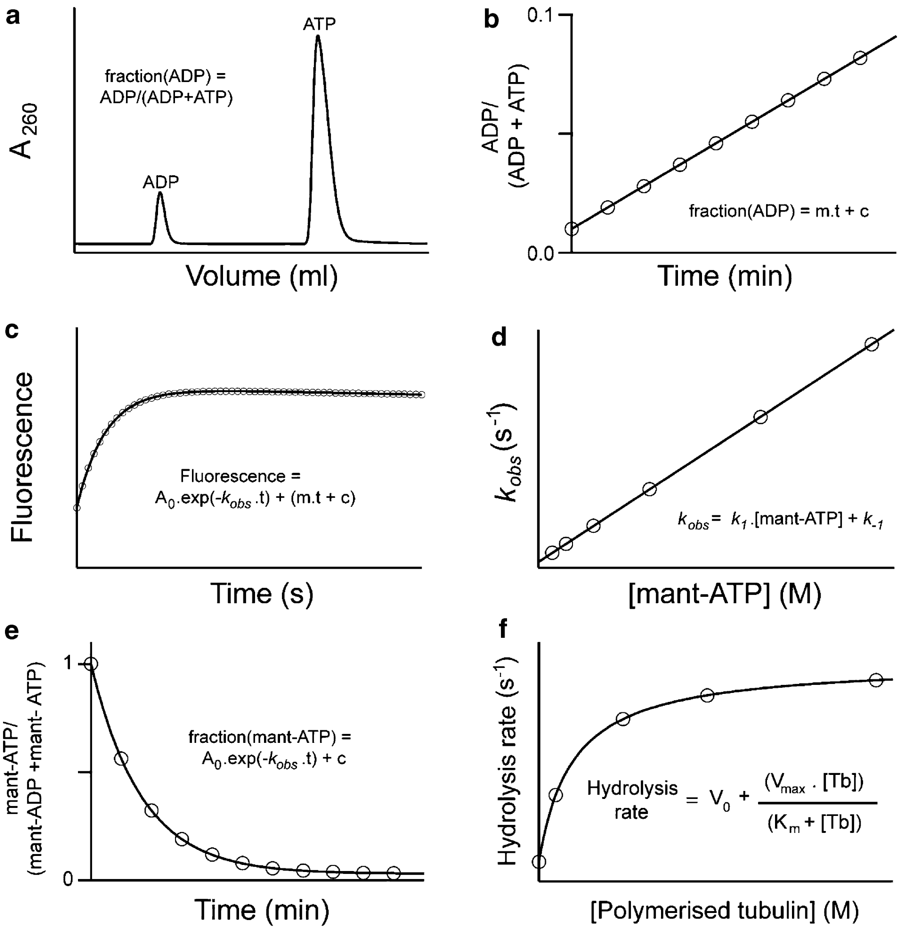

Fig. 2. (**a**) Example HPLC trace from a sample of the reaction of a kinesin with a saturating concentration of ATP. From this trace, ADP as a fraction of total nucleotide is calculated from area under the ADP peak divided by the sum of the areas under the ADP and ATP peaks. (**b**) Simulated data points (*open circles*), ADP as a fraction of total nucleotide [fraction(ADP)] versus time. The fraction(ADP) at each time point is determined from HPLC traces of the type shown in (**a**). The gradient (m) of a linear fit to these data gives the change in fraction(ADP) per unit time, from which the rate constant for ATP turnover can be calculated. (**c**) Simulated data points (*open circles*), fluorescence signal upon binding of mant-ATP to nucleotide-free kinesin. The fluorescence data is fit to an exponential function to obtain k_{obs} at each concentration of mant-ATP. A line of constant negative slope (m.t + c) is included, if necessary, in the fitting function to account for photo-bleaching of the mant group. (**d**) Simulated data points (*open circles*), k_{obs} [obtained from traces, such as (**c**)] versus the concentration of mant-ATP. Fitting this data to a linear function gives k_1 (gradient, association rate constant) and also k_{-1} (*y*-axis intercept, dissociation rate constant) (Fig. 1, step 1, when $k_{-1} > k_2$). (**e**) Simulated data points (*open circles*), mant-ATP as a fraction of total nucleotide [fraction(mant-ATP)] plotted against time. Data expected from the reaction of a kinesin with a sub-stoichiometric concentration of mant-ATP. The fraction(mant-ATP) is fitted to an exponential function to obtain k_{obs} for the hydrolysis of ATP. It is necessary to confirm whether this rate constant represents ATP binding or cleavage (Fig. 1, steps 1 and 2, see Note 18). (**f**) Simulated data points (*open circles*) showing the dependence of the complete ATP turnover cycle rate constant of a kinesin on the concentration of polymerised tubulin (microtubules). To obtain the ATP turnover rate (V_{max}) at saturating concentrations of polymerised tubulin ([Tb]), the data is fit to the function shown – V_0 is the ATP turnover rate in the absence of tubulin and K_m is the concentration of polymerised tubulin for which the half-maximal ATP turnover rate is observed.

10. Plot the fraction of ADP against time (Fig. 2b). The change in the fraction of ADP is linear if the reaction is in the steady state (see Note 9).
11. Fit the linear region of the data to a linear function, the slope of which gives the change in fraction of ADP per unit time.
12. To convert the change in fraction of ADP to change in concentration of ADP per unit time, multiply by the initial concentration of ATP (in this case, 2 mM).
13. Convert this into the rate constant for hydrolysis by dividing by the concentration of kinesin motor domains (see Note 10).
14. The value obtained has units of reciprocal time (normally quoted in s^{-1}) and represents the number of molecules of ATP hydrolysed per motor domain per second.

3.1.2. Measuring Production of Inorganic Phosphate

The progress of an ATP hydrolysis reaction can also be observed by monitoring the production of inorganic phosphate (P_i). As there is no signal by which phosphate can be directly observed, a secondary molecule is generally used via which P_i is detected. The most commonly used are molybdate/malachite green reagents that show an increase in absorbance at 620 nm when bound to inorganic phosphate. Such reagents can be prepared in the lab (7); however, there are a number of commercially available reagents (see Subheading 2, item 8).

1. Set up reaction mix with 2 mM Mg-ATP in the chosen buffer (see Note 4).
2. Start reaction by mixing kinesin (see Note 5) in chosen buffer with ATP-containing reaction mix (see Note 6).
3. Incubate reaction at chosen temperature (normally, either 25 or 37°C is used).
4. Take samples at suitable time points (see Note 7) and quench the reaction by addition to an equal volume of 2 M HCl.
5. Neutralise samples by addition of the necessary volume of 1 M Tris, 3 M KOH, which is determined by trial reactions (e.g. if using 25 μl sample + 25 μl 2 M HCl, 17.5 μl 1 M Tris, 3 M KOH is required).
6. When all samples are completed, spin at 17,000 g 13,000 rpm, Heraeus Biofuge Microcentrifuge for 10 min to remove precipitate.
7. To analyse sample using a plate reader, transfer 50 μl of the sample supernatant to a 96-well plate. Add 50 μl BIOMOL Green Pi detection solution.
8. Allow 20 min at room temperature for colour development (see Note 11).
9. Measure absorbance at 620 nm in a plate reader.

10. To convert the absorbance values to concentration of phosphate, a standard curve is required. Make a titration of an appropriate concentration range of phosphate standards (up to ~80 μM). Treat phosphate standards as the reaction samples (Subheading 3.1.2, steps 4–9).
11. Plot the concentration of phosphate against A_{620}, and fit a linear function to the data to obtain the relationship between A_{620} and phosphate concentration. Use this to convert absorbance data into phosphate concentration.
12. Plot phosphate concentration against time over the time course of the reaction.
13. Fit the linear region of the data to a linear function, the slope of which is the change in phosphate concentration per unit time (see Note 9).
14. Convert this into the rate constant for ATP turnover by dividing by the concentration of kinesin motor domains (see Note 10).
15. The value obtained has units of reciprocal time (normally quoted in s^{-1}) and represents the number of molecules of ATP hydrolysed per motor domain per second.

3.2. Analysis of Individual Cycle Steps

In order to determine which step in the cycle (Fig. 1) is responsible for the rate constant for ATP turnover of the complete cycle (Subheading 3.1), each step must be individually analysed. It is important to identify the rate-limiting step in the absence of microtubules, as this step must be accelerated by interaction with microtubules to produce the microtubule-stimulated increase in the ATP turnover rate generally observed for kinesins. Also, when the rate-limiting step in the cycle is known, the predominant intermediate is known, and therefore the predominant nucleotide-bound state in the absence of microtubules. This allows one to identify the change in nucleotide state of the kinesin that is triggered by interaction with microtubules.

One method used to determine rate constants for individual steps in the ATP turnover cycle (Fig. 1) is to take advantage of the properties of nucleotides labelled with the fluorescent group methylanthraniloyl (mant) (8). The mant group is most often used because being one of the smallest available fluorescent groups, when conjugated to the ribose it generally has little or no effect on the kinetics of ATP hydrolysis (9–11).

3.2.1. Association of ATP

The kinetics of ATP binding can be measured using mant-ATP taking advantage of the increase in fluorescence of the mant group that generally occurs upon nucleotide binding to the kinesin motor domain. To observe the kinetics of ATP binding, the ADP that remains trapped in the kinesin nucleotide-binding pocket in the absence of microtubules must be removed, allowing step 1 in the ATP turnover cycle to be observed in isolation (Fig. 1).

1. To a solution of kinesin in any appropriate storage buffer (preferably Mg^{2+}-free), add 1 mM (final concentration) EDTA pH 8 (see Note 12) and 1 mM (final concentration) DTT. The EDTA chelates Mg^{2+} ions causing the Mg^{2+} to be removed from the nucleotide-binding pocket. This in turn causes the nucleotide to be released (11).
2. Incubate at 25°C for 15 min.
3. Separate the free nucleotide from the protein by buffer exchanging into an appropriate Mg^{2+}-free buffer (see Note 13) using G25 sephadex resin (see Subheading 2, item 5).
4. Immediately after buffer exchange, add 1 mM $MgCl_2$ to the solution of nucleotide-free protein (see Note 14).
5. Make up a concentration series of mant-ATP in the appropriate reaction buffer. The range of concentrations of mant-ATP used depends on the concentration of kinesin available (see Note 15).
6. To simplify the observed kinetics, the reaction is carried out under pseudo-first-order conditions: the concentration of mant-ATP is in at least fivefold excess over the concentration of nucleotide-free protein. Therefore, it is assumed that the concentration of free mant-ATP remains constant over the course of the reaction; for further information on binding and dissociation kinetics, see (12).
7. Starting with the lowest mant-ATP concentration, rapidly mix the reactants (mant-ATP and nucleotide-free kinesin) in a 1:1 v/v ratio using a stopped-flow fluorimeter (see Note 16). The fluorescence of the mant-ATP is excited at 365 nm, and the emitted fluorescence above 400 nm is collected via a long-pass filter (GG400, Schott).
8. The observed rate constant (k_{obs}) at each mant-ATP concentration is determined by fitting the observed fluorescence increase to an exponential function (Fig. 2c) (see Note 17).
9. Plot the rate constants determined against the concentration of mant-ATP. There is a linear relationship between the observed rate constants and the concentration of mant-ATP, the gradient of which represents the association rate constant for mant-ATP (Fig. 1, k_1) with units of M^{-1}/s (Fig. 2d).

3.2.2. Cleavage of ATP

In order to determine whether the rate-limiting step is before or after the hydrolysis step, it is necessary to follow the kinetics of the ATP cleavage (hydrolysis) reaction (Fig. 1, step 2). This can be done using an excess of molar concentration of ATP over kinesin and determining if there is a burst of liberated phosphate when the reaction is terminated by an acid quench (which releases any bound phosphate) (13). It is also useful to determine the kinetics of ATP hydrolysis under single turnover conditions, i.e. mixing a molar concentration of motor domain in excess of the molar concentration of substrate (ATP). Multiple cycles of hydrolysis are prevented allowing transient intermediate species to be observed. However, in order that the ATP-binding phase is rapid compared with the rate-limiting step, it is necessary to use a kinesin concentration greater than the K_m for ATP, which may be difficult to achieve in practice (see Note 18).

As the reaction progresses, samples are quenched, denaturing the kinesin and releasing the protein-bound nucleotide. By separating the products using HPLC as described below, it is possible to observe the disappearance of the kinesin–ATP complex and thereby determine at least a lower limit for the rate constant for ATP cleavage (see Note 18).

When the reaction is carried out under single turnover conditions, the concentration of ATP used is limited by the concentration of kinesin available. Since most kinesins can be expressed and purified only at low micro-molar concentrations, it may be necessary to use mant-ATP and fluorescence detection to increase the sensitivity of the method. Alternatively, radioactive ATP with a labelled γ-phosphate can be used. However, using a more sensitive detection method will not overcome the problems associated with a kinesin, which has an inherently large K_m, because the observed kinetics will be limited by the binding reaction. Whether the observed reaction is limited by binding or cleavage should be checked (see Note 19).

1. Start reaction by mixing 1 μM nucleotide-free kinesin (Subheading 3.2.1, steps 1–4) motor domain (see Note 20) with 0.5 μM mant-deoxyATP (mant-dATP) (see Note 21).
2. Quench reaction at various suitable time points (see Note 22) by injection onto a C-18 HPLC column (see Note 23).
3. To separate mant-dATP from mant-dADP, run each sample over a C-18 column (see Note 24) using isocratic flow with a 100 mM potassium phosphate, 10 mM TBABr, and 30% acetonitrile buffer (see Subheading 2, item 7). The flow rate used depends upon the pressure limits of the particular HPLC system and column used, but flow rates of ~1 ml/min are generally achievable.
4. The presence of both mant-dADP and mant-dATP is measured using the fluorescence of the mant group ($\lambda_{ex} = 355$ nm, $\lambda_{em} = 448$ nm). The integrated area under the respective peaks provides a measure of the quantity of each nucleotide present in the sample.
5. For each time point, calculate the proportion of mant-dATP remaining as a fraction of total nucleotide (area of mant-dATP peak/[area of mant-dATP + mant-dADP peaks]).

6. Plot the fraction of mant-dATP/total nucleotide against time and fit the data to an exponential function in order to determine the rate constant for ATP cleavage (Fig. 2e).

3.2.3. Dissociation of Phosphate

For all kinesins studied to date, the dissociation of phosphate (Fig. 1, step 3) is faster than the cleavage of ATP (Fig. 1, step 2), so only a lower limit to the rate constant for the dissociation of inorganic phosphate can generally be determined. Detailed methods for the study of this step are, therefore, not described here. Information on observing P_i dissociation, by coupling the presence of P_i to the fluorescence signal of the phosphate-sensing protein, can be found in refs. 10, 14, 15.

3.2.4. Dissociation of ADP

To provide a signal by which the dissociation of ADP from a kinesin (Fig. 1, step 4) can be observed, ADP labelled with the mant fluorophore is used, again taking advantage of the signal difference generally observed between free mant-ADP and mant-ADP bound to the kinesin motor domain.

1. The unlabelled ADP bound to the kinesin motor domain in the absence of microtubules is exchanged for mant-ADP by the following method: to a solution of the kinesin of interest, add mant-ADP to a concentration in at least 25-fold molar excess over the concentration of kinesin motor domains.
2. Incubate at 25°C for 30 min.
3. Remove excess mant-ADP and other free nucleotides from the protein by buffer exchanging into the chosen reaction buffer using G25 sephadex resin (see Subheading 2, item 5).
4. The mant-ADP-loaded kinesin has a fluorescence signal, the loss of which is used to observe the dissociation of mant-ADP.
5. Rapidly mix the mant-ADP–kinesin complex (~1 μM) with a molar excess (50-fold or greater) of unlabelled ATP in a 1:1 v/v ratio using a stopped-flow fluorimeter (see Note 25).
6. The fluorescence of mant-ADP is excited at 365 nm, and the emitted fluorescence above 400 nm is collected via a long-pass filter (GG400, Schott).
7. The decrease in fluorescence, observed as the mant-ADP dissociates from the kinesin, is fit to an exponential function from which the rate constant for dissociation of ADP (Fig. 1, k_{-4}) is determined with units of s^{-1} (see Note 26).

3.3. Effect of Microtubules

To determine the effect of microtubules on the ATP turnover cycle, one must determine the rate constant of the complete ATP turnover cycle in the presence of microtubules. This can be done by either of the methods described in Subheading 3.1. The rate-limiting step in the microtubule-stimulated ATP turnover cycle can then be determined by analysing the individual steps in the cycle (Subheading 3.2) in the presence of microtubules.

1. Measure the rate constant of the complete cycle (as described in Subheading 3.1) at a range of concentrations of polymerised tubulin (microtubules) (see Note 27).
2. To determine the maximum ATP turnover rate constant at saturating concentrations of polymerised tubulin (microtubules), plot the rate constant for ATP turnover against the concentration of polymerised tubulin. Fit the data to a hyperbolic function which allows the determination of the V_{max} (i.e. the rate constant of the complete cycle for the kinesin under study in the presence of saturating polymerised tubulin) (Fig. 2f).
3. The rate constants for individual steps in the ATP turnover cycle (Fig. 1) can then be measured (according to the methods in Subheading 3.2) in the presence of a saturating concentration of polymerised tubulin (microtubules) in order to determine how the rate constants are affected by interaction of the kinesin with microtubules. At this point, the focus should be on steps in the cycle that are accelerated by the interaction with microtubules (i.e. have a rate constant in the absence of microtubules lower than the microtubule-stimulated ATP turnover rate).
4. The same procedure can be carried out to determine the effect of unpolymerised tubulin (free α/β-heterodimers) on ATP hydrolysis. This information can also be useful in uncovering the molecular mechanism of the action of a kinesin.

4. Notes

1. Much confusion in the literature has been caused by the tendency for the term “rate constant” to be shortened to “rate”. These terms are not equivalent. A rate refers to a change in concentration with time and has units of concentration (or amount) per unit time. A rate constant is the coefficient that relates the rate to the concentration of reactants – effectively, it is the rate at unitary concentration(s) of the reactants (analogous to a molar absorption coefficient). The units of a rate constant depend on the molecularity of the reaction, e.g. s^{-1} for uni-molecular, M^{-1}/s for bi-molecular reactions. Rates can be positive or negative and generally increase with increasing concentrations (the law of mass action). Rate constants are positive coefficients, but observed rate constants can increase or decrease with increasing concentration.
2. In the context of studying the reaction of a kinesin with ATP, it is convenient to determine the molar concentration

as the concentration of ATP-binding domains (i.e. the monomer concentration). Kinesin molecules consist of different numbers of nucleotide-binding domains: monomeric, dimeric, and tetrameric.

3. It is possible to indirectly monitor the production of ADP by linking it to the spectroscopic signal of another molecule. This has the advantage of keeping the steady-state ADP concentration very low and preventing product inhibition of the kinesin, and also allowing the reaction to be continuously monitored (7).
4. The choice of reaction buffer is dependent on the kinesin under study. However, in general, a buffer is chosen that is suitable for microtubules, and therefore facilitates further studies in the presence of microtubules. One of the most commonly used buffers for this purpose is BRB80 (Subheading 2, item 15). Extra salt (e.g. KCl) is often added to BRB80 to modulate the interaction of the kinesin under study with microtubules. The solubility of many kinesins is limited in buffers suitable for microtubule stability. Therefore, for long-term storage, a more suitable buffer is generally used and the kinesin exchanged into the reaction buffer directly prior to use.
5. The concentration of kinesin used is chosen depending on its particular kinetics. If the rate of product production is slow, the concentration of kinesin used may be increased to produce detectable levels of product over a practical time period. It may be necessary to carry out some test reactions to determine the most suitable conditions, but make sure that the steady-state criteria remain satisfied and the reaction is monitored for a time corresponding to two or more turnovers.
6. The method of mixing used to start the reaction depends on the kinetics of the reaction. For a reaction under steady-state conditions, manual mixing is generally fast enough to allow observation of the subsequent reaction. If the reaction kinetics are too fast for manual mixing, a more rapid mixing technique, such as quench flow, may be used (16).
7. The time points selected depend on the rate of product production (see Note 5). Choose times points that give a good number of data points (>4) over the linear range of the reaction (see Note 9).
8. The Luna 3 µ C18(2) 100 Å, 100 × 4.6 mm (Phenomenex), gives excellent separation of ADP from ATP with relatively short run times (~10 min).
9. Depending on the kinetics of hydrolysis and the time points taken, it is possible that a non-linear phase may be observed at the start of the reaction as the system approaches the steady state. Also, as substrate is consumed, the kinetics of the reaction

may become non-linear due to both substrate depletion and product inhibition; this generally occurs when as little as 10% of the substrate has been consumed.

10. Make sure to match the units of kinesin motor domain concentration with the concentration of nucleotide.
11. If samples are to be measured separately, as when using a spectrophotometer, stagger the addition of Biomol green reagent such that all samples have the same time for colour development between addition of the reagent and measurement of absorbance.
12. The exact concentration of EDTA is not vital, but should be in at least 50-fold molar excess of the kinesin motor domain concentration. Also note that the concentration of EDTA must be in excess of any Mg^{2+} present in the kinesin storage buffer.
13. The kinesin should be exchanged into the buffer that is used for the reaction. If the chosen buffer contains Mg^{2+} (see Note 4), an Mg^{2+}-free version should be used at this stage and the Mg^{2+} added after the buffer exchange step. To reduce loss of kinesin on the resin during buffer exchange, add 0.05% Tween-20 to the reaction buffer.
14. Re-addition of Mg^{2+} helps to stabilise the nucleotide-free kinesin. However, the apo-motor domains are still relatively unstable and the nucleotide-free kinesin should be kept on ice and used within a few hours. There are reports of nucleotide-free kinesin remaining active for up to 2 weeks when stored frozen in a high-salt glycerol buffer (17).
15. The concentration of the kinesin is generally changed in accordance with the concentration of mant-ATP so that it remains at between five- and tenfold molar excess over the concentration of kinesin motor domains. This is so the signal change due to the binding of mant-ATP to kinesin is not swamped by the fluorescence from free mant-ATP. The maximum concentration of mant-ATP at which the reaction can be monitored is, therefore, limited by the concentration of protein available.
16. It is important to account for the dilution of the reactants that occurs upon mixing. The concentration in the syringes prior to mixing is twice that in the reaction. It is the reaction (post mixing) concentrations of mant-ATP that should be used to determine the ATP association rate constant. It is worth noting both concentrations in the lab book/file tag, e.g. 10/5 μM ATP so that in later reference it is clear what was used (it is easy to divide by two twice if one's memory fades!).
17. It may be necessary to include a linear component with a negative slope to the exponential fit function to account for

photo-bleaching of the mant-labelled nucleotide. The extent of photo-bleaching (and also mixing artefacts) can be determined by carrying out a control reaction in which mant-ATP is mixed with reaction buffer without kinesin. Also, temporary closing of the excitation shutter can be used to check if a drift is due to photo-bleaching or some other cause.

18. Single turnover conditions require that the binding step is rapid compared with the subsequent steps, if the latter are to be resolved. This is achieved by using concentrations of reactants which are greater than the K_m. For the scheme in Fig. 1, k_1. [kinesin] should be $>k_{-1}+k_2$, for the hydrolysis step to be resolved from the binding step.

19. One method of distinguishing between these possibilities is to monitor the reaction continuously via the fluorescence signal of the mant group in conjunction with the quenching-type assay described. If the rate constant for the increase in fluorescence associated with the binding of mant-ATP to nucleotide-free kinesin matches the rate constant determined by the quenching assay, then ATP binding is slow and therefore the rate constant determined for the dissipation of the ATP–kinesin complex represents binding rather than cleavage of ATP.

20. Since it is often difficult to determine the exact concentrations of active protein, a diagnostic test can be carried out to ensure that a reaction is proceeding under single turnover conditions. Double the concentration of protein and keep the concentration of ATP the same; if operating under single turnover conditions, the rate constants determined for steps following binding should be unaffected. This also provides a method to confirm which step the rate constant measured represents; if the rate constant measured represents the binding of ATP to the nucleotide-free kinesin, it will double upon doubling the protein concentration.

21. 3′-mant-2′-deoxyATP (mant-dATP) is used rather than regular mant-ATP which is a mixture of 2′/3′-mant-ATP, and therefore gives two peaks for both ATP and ADP when the nucleotides are separated on a C-18 column.

22. It is necessary to collect a number of time points (preferably ten or more) over a reasonable timescale to get a good description of the progress of the reaction. It may be necessary to carry out some test reactions to get an idea of the timescale over which the reaction proceeds.

23. As mant-labelled nucleotides can be sensitive to harsh quenching conditions, quenching can be carried out by injection of reaction samples directly onto the column. In this case, it is essential to use a pre-column/pre-filter to protect the resolving column from denatured protein and other precipitates.

24. The mant label means that nucleotides bind more strongly to the column and are more difficult to separate. Therefore, a longer column may be required than for unlabelled nucleotides [e.g. Luna 3 μ C18(2) 100 Å, 250 × 4.6 mm (Phenomenex)].
25. Excess unlabelled ATP is included to prevent rebinding of mant-ADP, therefore ensuring that only the dissociation of mant-ADP is observed. For further information on binding and dissociation kinetics, see (12).
26. It may be necessary to include a linear component with a negative slope to the exponential fit function to account for photo-bleaching of the mant-labelled nucleotide. In the case of the mant-ADP–kinesin complex, the extent of photo-bleaching can be determined by performing a control reaction in which mant-ADP–kinesin is mixed with mant-ADP–kinesin.
27. In these assays, microtubules stabilised using the non-hydrolysable GTP analogue, GMPCPP, or the drug taxol are used. For information on how to prepare stabilised microtubules, see refs. 18–20.

Acknowledgements

We acknowledge with thanks Roger Goody and Aymelt Itzen for assistance in developing the protocol described in Subheading 3.2.2, and also Christopher Gell for critical reading of the manuscript. This work was supported by the Alexander Von Humboldt Foundation and the Max Planck Society.

References

1. Miki, H., *et al.* (2005) Analysis of the kinesin superfamily: insights into structure and function *Trends Cell Biol* **15**, 467–476.
2. Hirokawa, N., *et al.* (2009) Kinesin superfamily motor proteins and intracellular transport *Nat Rev Mol Cell Biol* **10**, 682–696.
3. Peterman, E. J., and Scholey, J. M. (2009) Mitotic microtubule crosslinkers: insights from mechanistic studies *Curr Biol* **19**, R1089–1094.
4. Howard, J., and Hyman, A. A. (2007) Microtubule polymerases and depolymerases *Curr Opin Cell Biol* **19**, 31–35.
5. Wordeman, L. (2005) Microtubule-depolymerizing kinesins *Curr Opin Cell Biol* **17**, 82–88.
6. Burton, K. (1969) *in* "Data for Biochemical Research" (Dawson, R. M., *et al.*, Eds.), Oxford university Press.
7. Hackney, D. D., and Jiang, W. (2001) Assays for kinesin microtubule-stimulated ATPase activity *Methods Mol Biol* **164**, 65–71.
8. Bagshaw, C. (2001) ATP analogues at a glance *J Cell Sci* **114**, 459–460.
9. Lisal, J., and Tuma, R. (2005) Cooperative mechanism of RNA packaging motor *J Biol Chem* **280**, 23157–23164.
10. Gilbert, S. P., *et al.* (1995) Pathway of processive ATP hydrolysis by kinesin *Nature* **373**, 671–676.
11. Sadhu, A., and Taylor, E. W. (1992) A kinetic study of the kinesin ATPase *J Biol Chem* **267**, 11352–11359.

12. Goodrich, J. A., and Kugel, J. F. (2007) Binding and Kinetics for Molecular Biologists, Cold Spring Harbor Laboratory Press, New York.
13. Johnson, K. A. (1983) The pathway of ATP hydrolysis by dynein. Kinetics of a presteady state phosphate burst *J Biol Chem* **258**, 13825–13832.
14. Brune, M., *et al.* (1994) Direct, real-time measurement of rapid inorganic phosphate release using a novel fluorescent probe and its application to actomyosin subfragment 1 ATPase *Biochemistry* **33**, 8262–8271.
15. Cochran, J. C., *et al.* (2009) ATPase cycle of the nonmotile kinesin NOD allows microtubule end tracking and drives chromosome movement *Cell* **136**, 110–122.
16. Johnson, K. A. (1986) Rapid kinetic analysis of mechanochemical adenosinetriphosphatases *Methods Enzymol* **134**, 677–705.
17. Ma, Y. Z., and Taylor, E. W. (1997) Kinetic mechanism of a monomeric kinesin construct *J Biol Chem* **272**, 717–723.
18. Desai, A., and Walczak, C. E. (2001) Assays for microtubule-destabilizing kinesins *Methods Mol Biol* **164**, 109–121.
19. Hyman, A., *et al.* (1991) Preparation of modified tubulins *Methods Enzymol* **196**, 478–485.
20. Gell, C., *et al.* (2010) Microtubule Dynamics Reconstituted in vitro and Imaged by Single-Molecule Fluorescence Microscopy *Methods in Cell Biology* **95**, 221–245.