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Quantifying the reversibility of ATP hydrolysis in rabbit skeletal myosin subfragment 1 using a luciferase assay

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Abstract

Background It is known that the adenosine triphosphate (ATP) hydrolysis in a number of ATPases is reversible. Radioactive methods are standard in measuring ATPase reversibility. We used myosin as a well-studied model enzyme to develop a luciferase-based assay to quantify the reversibility of ATP hydrolysis. In myosin, ATP bound to the active site is hydrolyzed into adenosine diphosphate (ADP) and inorganic phosphate (Pi) and recombined back into ATP multiple times before products of hydrolysis are released. The reversibility of ATP hydrolysis by skeletal myosin was previously confirmed using radioactively labeled isotopes. Transient kinetics studies indicated that the ATP hydrolysis step is temperature-sensitive, with a dissociation equilibrium constant of 1.6 at 3 °C. Consequently, the association equilibrium constant at this temperature is 0.63. The goal of our work was two-fold, (a) to develop a luciferase-based assay to measure the equilibrium constant of enzymatic ATP hydrolysis, eliminating the need for radioactively labeled Pi, and (b) refine the value of the association equilibrium constant of the myosin ATPase hydrolysis step.

Methods In this assay, a reaction mixture containing myosin and saturating levels of ADP and Pi was incubated to reach equilibrium, then the reaction was terminated, and the amount of ATP produced by myosin was quantified using the luciferase assay. The equilibrium constant of ATP hydrolysis was defined as the ratio of ATP to ADP bound to myosin.

Results We obtained a value of 0.78 ± 0.14 for the association equilibrium constant of ATP hydrolysis at 0 °C. 50 μM ADP bound to myosin is turned into 21.9 ± 3.0 μM ATP. Our result is in excellent agreement with the literature data, supporting the viability of the new methodology.

Discussion This methodology allows a more accessible and safe option to measure ATP production and reversibility of ATPase enzymes. Standard laboratory equipment is utilized in the assay, and the number of steps in the developed assay is reduced significantly compared to the radioactive method.

Keywords ATP hydrolysis, ATPase, Luciferase, Myosin, Equilibrium constant, Reversibility

Introduction

ATPases are a class of enzymes that hydrolyze ATP. Many of these enzymes are known to be reversible, meaning that they can make ATP in addition to cleaving it [1–9]. It is common to use radioactive isotopes, such as ^{32}P, to study ATPase enzyme activity, where radioactive phosphate binds to or is abstracted from the nucleotide. However, the use of radioactive isotopes poses a safety hazard

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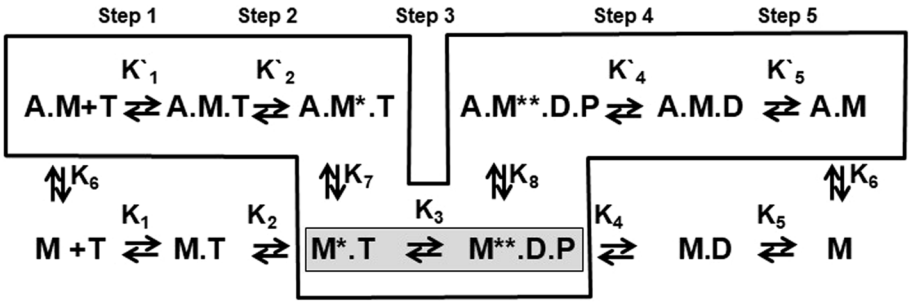

Scheme 1 Acto-myosin ATPase cycle reaction scheme. A = actin, M = myosin (M* and M** - myosin states with increased intrinsic fluorescence), T = ATP, D = ADP, P = phosphate. Asterisk indicates reaction rate constants describing acto-myosin kinetics. Boxed = generally accepted pathway of acto-myosin interaction. Highlighted = the reaction step studied in the paper. Step 3 is the reversible step of ATP hydrolysis by myosin. For rabbit skeletal myosin II k_{+3}=144s^{-1} and k_{-3}=16s^{-1} [1]. Rates and equilibrium constants of the cycle steps are myosin isoform specific. For myosin II the rates can be found elsewhere [24–28]

and necessitates an elaborate scheme for separating the reaction products to quantify the reaction output, potentially increasing the error of the experiment. We sought to develop a non-radioactive luciferase-based assay to determine the equilibrium constant of ATP hydrolysis using rabbit skeletal myosin subfragment 1 (S1) as a reversible ATPase model system. Luciferase is an enzyme that uses ATP to produce luminescence. This luminescent signal increases linearly with ATP concentration and can be used to quantify the amount of ATP present at equilibrium, allowing ATP to be counted without radioactive labeling. Luciferase-based assays are widely used and well-characterized. Most commonly they are used to determine cell viability, but there are some reports of using luciferase with purified proteins to measure ATP consumption by ATPases [10–12]. Luciferase assays offer a quick, easy, safe, accessible, and cost-effective alternative to radioactive isotope methodology.

Myosin is an ATPase molecular motor that utilizes ATP hydrolysis to fuel force-producing conformational change in the enzyme. Myosin works in a cycle (Scheme 1), interacting with two ligands, actin and nucleotide, and changes conformation two times in the cycle. ATP binding and subsequent hydrolysis result in myosin conformational change, or recovery stroke, when myosin transitions into the pre-power stroke structural state. Without actin, myosin stays in the pre-power stroke structural state for a relatively long time, actin significantly speeds up the cycle. ATP hydrolysis and release of the abstracted phosphate increases myosin affinity to actin, and strong actin binding results in myosin power stroke when myosin conformational change produces force. The reversibility of ATP hydrolysis has been observed and well documented in myosin, meaning that products of hydrolysis can be turned back into reactants within the active site using ^{32}P and ^{3}H isotope labeled ATP and Pi [1, 13–17]. A number of ^{18}O isotope studies confirmed that phosphate abstraction from ATP and ATP synthesis occurs multiple times at the myosin active site [18–23]. Determining how much ATP is present in the active site at equilibrium is necessary to fully characterize the kinetics of the actomyosin force production cycle. The equilibrium constant for reversible ATP hydrolysis at the myosin active site is defined as the ratio of ATP to ADP bound to myosin at equilibrium.

It is well established that the equilibrium constant for ATP dissociation at the active site of rabbit skeletal myosin II (K_3) is 9 at room temperature in the absence of actin [1], corresponding to an association equilibrium constant of 0.11. The constant was initially estimated using a radioactive method and later confirmed using transient kinetics methods by measuring the forward and reverse rates of the reaction [1, 26]. Subsequent transient kinetics chase experiments further validated the temperature dependence of this step, revealing that the equilibrium shifts toward the substrate (ATP) at lower temperatures. At 3 °C, the equilibrium constant for ATP dissociation was determined to be 1.6, resulting in an association equilibrium constant of 0.63 [29]. Analysis of literature data from ^{32}P isotope studies at room temperature yields a wide range for the association equilibrium constant, varying from 0.017 to 0.9 [1, 13–17]. This wide range is likely due to the methodologies used in these investigations, which include quenching the reaction with acid and the complex procedures required for separating ATP, ADP, and Pi.

Similar to previous myosin experiments, our approach is to supply myosin with saturating levels of ADP and Pi, allow equilibrium to be reached, and then measure ATP produced. Levels of ADP and Pi need to be saturating to ensure the active site of all myosins is occupied. Based on the literature data, we selected a temperature of 0 °C for our experiments to shift the equilibrium of ATP

hydrolysis toward the substrate, thereby increasing the sensitivity of our measurements. Myosin ATPase activity is slow without actin, it has been shown that a single ATP turnover is complete in one minute [1]. We incubated the reaction for five minutes, and after the incubation, the reaction is terminated by boiling to denature myosin and release all nucleotides present in the active site. After termination, the amount of ATP can be measured using luciferase and an ATP standard curve. The amount of ATP produced from ADP will tell us the equilibrium constant of ATP hydrolysis, defined as the ratio of ATP to ADP in the myosin active site at equilibrium.

Here we demonstrate that a luciferase-based assay is a viable alternative to radioactive methodologies, providing a safer, simpler, and more accessible solution to measuring ATP hydrolysis equilibrium. We determine the equilibrium constant of ATP hydrolysis to be 0.78 ± 0.14 at 0 °C. This means that at equilibrium, 43.8% of the ADP and Pi in the myosin active site is turned into ATP. This number falls well in the range of numbers determined from previous studies. Our experiments confirm that myosin is in this reversible state for a relatively long time in the absence of actin.

Methods

Materials

MgATP: Adenosine triphosphate stock solution with equimolar $MgCl_2$, pH 7–7.5: Sigma-Aldrich Adenosine 5' triphosphate sodium salt hydrate CAS number 34369–07-8.

MgADP: Adenosine diphosphate stock solution with equimolar $MgCl_2$, pH 7–7.5: Sigma-Aldrich Adenosine 5' diphosphate sodium salt CAS number 20398–34-9.

$MgCl_2$ stock solution for nucleotides, VWR Magnesium chloride hexahydrate CAS number 97061–352.

Pi: Phosphate stock solution, pH 7–7.5: Fisher Chemical Sodium phosphate monobasic dihydrate CAS number 13472–35-0, Fisher Chemical Sodium phosphate dibasic dihydrate CAS number 10028–24-7.

Reaction Buffer: 50 mM Tris–HCl, 50 mM KCl, 1 mM $MgCl_2$, pH 7.5

Boiling Buffer: 25 mM Tricine, 400 mM KCl, 5 mM $MgCl_2$, pH 7.8

Luciferase: Promega CellTiter-Glo® 2.0 Cell Viability Assay CAS number G9242.

Opaque flat bottom 96 well plates: ThermoScientific Nunc™ MicroWell™ 96-Well, Nunclon Delta-Treated, Flat-Bottom Microplate CAS number 136101.

Clear flat bottom 96 well plates: Greiner Bio-one UV-STAR® MICROPLATE, 96 WELL, HALF AREA CAS number 675801.

Heating block: ThermoScientific Digital Dry Baths/Block Heater, CAS number 88870003.

Microcentrifuge: Eppendorf® Microcentrifuge 5415R.

Plate Reader: Molecular Devices SpectraMax® iD5 plate reader.

Concentrations of myosin, ATP, and ADP were determined spectrophotometrically assuming the extinction coefficient $e_{280nm} = 0.74\ (mg/ml)^{-1}\ cm^{-1}$ for myosin and $e_{260nm} = 15.4 * 10^3\ M^{-1}\ cm^{-1}$ for ATP and ADP.

Methods

Myosin S1 preparation

Full-length skeletal myosin was prepared from rabbit muscle tissue according to Margossian and Lowey [30]. The catalytic subfragment 1 was prepared according to Margossian and Lowey [30] by an alpha-chymotryptic digest for ten minutes sharp at room temperature ($T = 21 \pm 1$ °C) in 100 mM Tris–HCl, pH 7.5, 120 mM NaCl, 1 mM EDTA, 1 mM DTT. The cleavage reaction was terminated with PMSF. Concentrations of alpha chymotrypsin and PMSF in the final solution were 30 µg/ml and 2 mM respectively. Digested myosin was clarified by centrifugation at 100,000 g at 4 °C for 2 h. Purified S1 was dialyzed into the reaction buffer overnight and characterized using SDS-PAGE (Figure S1). Freshly prepared myosin S1 was stored on ice and used for up to 2 consecutive days. Before each experiment, the myosin solution was clarified by centrifugation at 100,000 g at 4 °C for one hour to remove denatured protein. The concentration of myosin S1 was determined after clarification before each experiment from optical absorption using serial dilutions and averaging results of measurements. We report results obtained from eight different preparations of myosin S1, using the muscle tissue of two rabbits.

Assay

Reactions were carried out at 0 °C in an ice slurry. Final concentrations in 120 µl reaction volume were as follows: myosin 50 µM, MgADP 1 mM, Pi 50 mM. 1 mM ADP and 50 mM Pi are the saturating concentrations, taking the dissociation constant of rabbit skeletal myosin S1 and ADP as 0.95 µM and the constant of Pi dissociation from myosin S1 and ADP complex as 1.5 mM [24]. A master mix containing ADP and Pi was made to reduce errors between samples. The master mix was aliquoted into each tube and kept on ice. Ice-cold myosin was added to start the reaction and was allowed to reach equilibrium for 5 min. While the reaction was reaching equilibrium, the termination tubes containing 900 µl boil buffer were placed in a heating block at 100 °C. We kept tubes closed to prevent evaporation. The temperature of the boil buffer in a tube was 96 °C at the time of reaction termination. After 5 min, 100 µl was removed from the reaction and terminated in 900 µl boiling buffer for 1 min. The addition of 100 µl ice-cold sample to 900 µl of boil buffer

reduced the temperature to 85 °C. After boiling for one minute, tubes were transferred to ice while the remaining samples were run. It is worth noting that ATP was not affected by boiling. We found that boiling buffer should have a high salt concentration to aid in nucleotide release from denatured myosin. Finally, samples were centrifuged at 16,000 g for 20 min to spin out denatured myosin, leaving released nucleotide in the supernatant. This supernatant was used for plating in an opaque 96-well plate. We selected a five-minute incubation time of the reaction mixture for convenience. Preliminary tests indicated that the reaction reaches equilibrium within one minute of incubation (Figure S2).

Luciferase plating

100 µl of the terminated sample was plated in triplicate on an opaque flat bottom Nunc™ MicroWell™ 96-Well plate and mixed 1:1 with luciferase, thawed in 20℃ water bath prior. A 10-min incubation period preceded the luminescence measurement of all wavelengths with SpectraMax® iD5 plate reader by Molecular Devices. All technical repeats were represented as the average of triplicates of terminated samples. A total of 7 technical repeats were run in each of the 17 sets of assays done, giving 119 total assays.

Control experiments

To confirm that the source of luciferase luminescence is ATP produced by myosin during the ATP synthesis reaction, rather than ATP contamination, we mixed luciferase with the reaction components both separately and in combinations: myosin, phosphate, and ADP; myosin and phosphate; myosin and ADP; and ADP and phosphate. Luciferase luminescence was observed only in reactions that included ADP. Solutions containing only myosin, phosphate, or a myosin-phosphate mixture showed only the background signal of the buffer.

Standard curve

The standard curve was measured for each performed assay. ATP dilutions were prepared in the same boiling buffer that samples were terminated in. 100 µM ADP, corresponding to the maximum possible level of ADP present in the sample, was included in each dilution of the curve to account for the signal acquired from high concentrations of ADP. When ADP is included in the ATP standard curve, the intercept increases, and the curve remains linear (Fig. 1). The standard curve is highly reproducible with an average coefficient of determination R^2 value of 0.99 ± 0.01. The same plating parameters were used for the standard curve. The amount of ATP present in the sample at equilibrium was calculated by the standard curve containing both ATP and ADP.

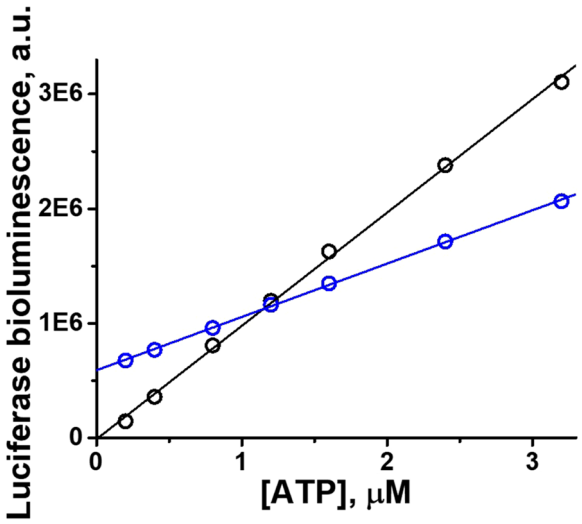

Fig. 1 Representative standard curves containing only ATP (black) and ATP + 100 µM ADP (blue). The difference in slopes indicates that ADP is the substrate for luciferase and needs to be accounted for. The ATP + ADP curve was used to quantify ATP concentrations in samples

Nucleotide concentration variance

A quality check was performed on each terminated sample to ensure all samples contained equal levels of nucleotide by measuring absorbance at 260 nm with SpectraMax® iD5 plate reader by Molecular Devices. 75 µl of the terminated sample was plated in triplicate and mixed 1:1 with boil buffer in a 96-well UV-STAR® MICROPLATE. The absorbance of triplicates was averaged to represent the sample. Any samples containing significantly high or low levels of a nucleotide may not give an accurate reflection of ATP produced and therefore were eliminated from analysis. Outliers were determined for each technical repeat, defined as absorbance at 260 nm falling outside 1.5×the inter-quartile range (IQR). The variance of nucleotide for each set is shown in Fig. 2. Five outliers were found and removed from the analysis, leaving 114 assays for analysis.

Acquisition of fluorescent transients

In the ATP-induced myosin S1 conformational change experiment, 2 µM myosin S1 was rapidly mixed with ATP solutions of varying concentrations. Transient intrinsic fluorescence of myosin S1 was measured using a Bio-Logic SFM-300 stopped-flow transient fluorimeter (Bio-Logic Science Instruments SAS, Claix, France), equipped with an FC-15 cuvette. Myosin S1 fluorescence was excited at 297 nm and detected using a 320 nm cutoff filter. Multiple transients were acquired and averaged to improve the signal-to-noise ratio. A total of 8000 points

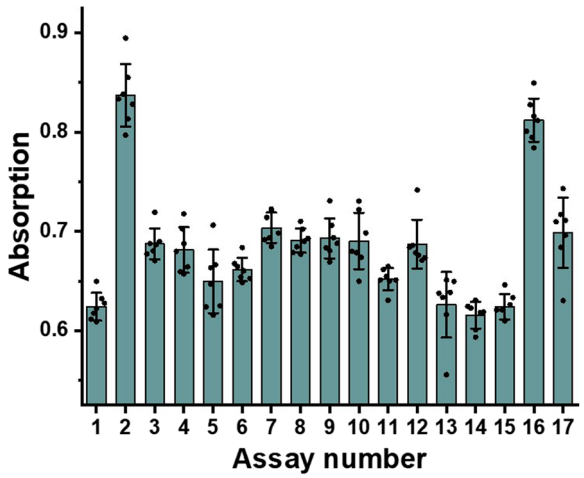

Fig. 2 Quality check on terminated samples to ensure similarity of nucleotide concentration in samples using absorbance at 260 nm. 17 total assay sets, each with 7 technical repeats. Terminated samples were diluted two times to give absorbance values within the range of 0.1–0.9. Each sample was measured in triplicate and reported as the triplicates average, shown here by dots. The bar height represents the mean of all technical repeats within a set, error bars represent the standard deviation

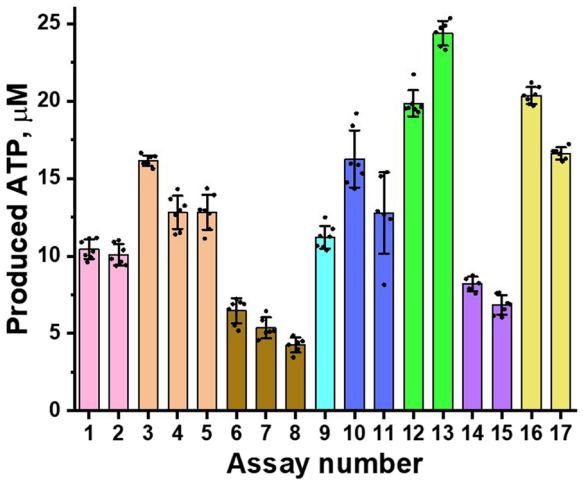

Fig. 3 Variation in ATP production in performed assays. Each bar represents the average ATP concentration in one assay set including seven technical repeats. Individual reactions are represented by dots and error bars are the standard deviation of the mean. The same color of bars represents reactions using myosin from the same S1 preparation. The figure illustrates the error of the assay and the error introduced by various protein activity in independent preparations

were collected in each experiment. All experiments were conducted at 20 °C.

Analysis of fluorescence transients

The obtained transients were fitted with a single-exponential function, $S(t) = S_0 + A \cdot \exp(-k_{obs} \cdot (t - t_0))$, where S(t) is the observed signal at time t, A is the signal amplitude, t_0 is the time before the flow stops, and k_{obs} is the observed rate constant. We achieved a good single-exponential fit for the transients of the ATP-induced myosin S1 conformational change. The dependence of the observed rates (k_{obs}) on the ATP concentration was fitted with a hyperbolic function, $k_{obs} = v_{max} \cdot [ATP] / (K_D + [ATP])$, which allowed for the determination of the maximum rate of the reaction, v_{max} (the horizontal asymptote). K_D is the equilibrium dissociation constant of the collision complex formation. All exponential fits were performed using Origin 9 (OriginLab Corp, Northampton, MA). All transients from the same protein preparation were fitted simultaneously, as they all exhibited the same intensity of myosin S1 fluorescence at the origin (the mixing point) and the end of the transient (due to complete myosin S1 conformational change upon ATP binding).

Results

We performed 119 assays, seventeen sets of seven technical repeats, using myosin from eight preparations from the muscle tissue of two rabbits. Five outliers were removed based on the absorbance quality check. On average of all myosin S1 preparations, the amount of ATP produced in the reaction is 12.7 ± 5.6 μM. The deviation of ATP measurements is likely due to the variability of myosin activity, and not the assay itself since the standard curve is highly reproducible. Despite our effort to standardize myosin S1 preparations, including myosin solution ultracentrifugation before each experiment, different myosin S1 preparations yield various levels of activity, as shown in Fig. 3. The activity of S1 was confirmed using transient kinetics control experiments, which consistently showed fully functional myosin, interacting with ATP with $v_{max} = 199 \pm 14\ s^{-1}$ and $K_D = 64 \pm 11$ μM (Fig. 4). Transient kinetics assay reports the activity of functional myosin S1 but does not report the absolute concentration of functional enzymes in solution. Therefore, it is likely that the amount of fully functional S1 in each preparation varies; this leads to the increased standard deviation of ATP production, averaged from all performed assays. To accurately assess the standard deviation of the assay and address differences in myosin activity across various preparations, we calculated the mean ATP production for every set of assays and normalized each technical replicate within each assay set to the set with maximal ATP production, assuming that the preparations with the highest ATP production should be the closest to 50 μM active sites. This process was repeated for all 114 assays, ensuring that variations in myosin activity were appropriately accounted for. After correction, the maximum ATP

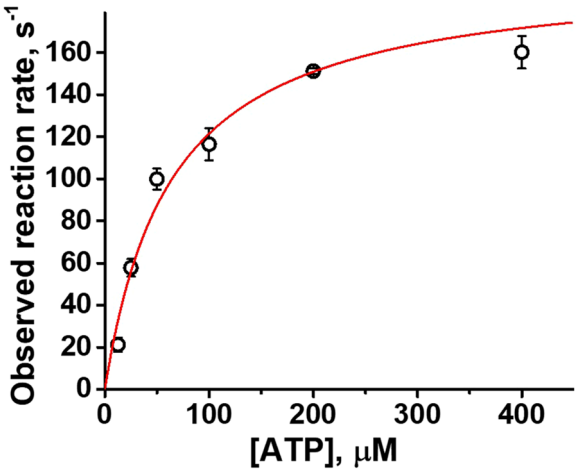

Fig. 4 Myosin S1 transient kinetics. Typical relationship between the observed rate constant of association of 2 μM myosin S1 with variable concentration of ATP. N = 3, T = 20 °C. The fit with a hyperbola gives the maximal rate of the reaction $v_{max} = 199 \pm 14\ s^{-1}$ and the dissociation constant $K_D = 64 \pm 11$ μM

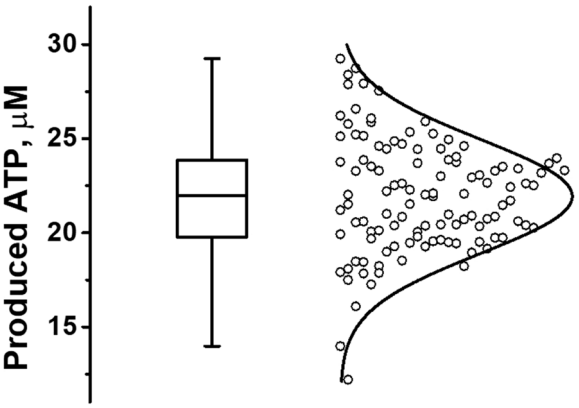

Fig. 5 Assay statistics. Distribution of results of the total number of assays reported ($N = 114$) after removing outliers from the absorbance quality check and after normalization of myosin S1 activity in all preparations. The data is represented as a box and whiskers plot (left) and individual points as open dots (right). The line (right) shows the normal distribution of data

concentration produced in the reaction is 21.9 ± 3.0 μM (Fig. 5). The range and spread of the data satisfy to normal distribution according to the Shapiro–Wilk normality test with $p = 0.36$, confirming that normality cannot be rejected.

Since 50 μM myosin was saturated with ADP and Pi, the amount of nucleotide bound to myosin would also be 50 μM, yielding 21.9 μM ATP and 28.1 μM ADP at equilibrium. The ratio of ATP to ADP gives an equilibrium constant of 0.78 ± 0.14, in excellent agreement with the previous data [29]. At equilibrium, 43.8% of the ADP and Pi in the active site is converted into ATP. We assume that produced ATP was present only at the myosin active site and not in the solution, since prolonged incubation did not result in the increased amount of ATP.

Discussion

Radioactive isotope assays are a well-established method. However, the accessibility of the assays is a hurdle. Administrative controls, materials, and safety must be taken into account with radioactive material. Radioactive methods require a scintillation counter to count the isotopes and a Geiger counter to monitor the contamination of the workspace and workers. Adequate setup for containment and monitoring are also required when working with radioactive materials. Proper disposal of radioactive materials, which can be challenging with large volumes, is also necessary. The safety hazards are a glaring drawback of radioactive methodologies. Using these methodologies requires diligent and careful workflow with precautions and controls in place to prevent exposure. Working with ^{32}P specifically requires shielding due to the emissions' capability to penetrate the skin. Aside from the radioactive material, the solvents used are toxic, and composed of aromatic organics such as toluene. Additionally, the steps required to isolate the labeled nucleotide are time-consuming and laborious. The numerous hurdles faced in working with radioactive methods limit researchers' ability to characterize ATPase enzymes.

The accepted value for the equilibrium constant of ATP dissociation at the myosin active site, as determined from transient kinetics studies at room temperature, is 9 [24], corresponding to an association equilibrium constant of 0.11. The reversibility of myosin ATP hydrolysis was studied using the radioactive ^{32}P isotope method, and the literature data can be used to calculate the corresponding equilibrium constant. Assuming that ATP produced in the reverse reaction remains bound to the myosin active site and does not release into the solution, and using the equilibrium constants for ADP and Pi dissociation from myosin and the myosin-ADP complex, which are 0.95 μM and 1.5 mM, respectively [24], the ratio of ATP to ADP bound at the active site can be determined. This ratio yields the association equilibrium constant.

Walcott and Boyer [17] reported the production of 129 pmol of ATP by 2.1 mg of myosin (likely heavy meromyosin) when mixed with saturated ATP (5 mM) and 30 nmoles of 32Pi and incubated for 15 min at room temperature. These data allow for the calculation of an equilibrium constant of 0.9. Goody et al. [13] studied the dependence of reverse ATP hydrolysis on Pi concentration in solution and determined that 86 μM myosin S1, mixed with saturated ADP and Pi (2.5 mM and 40 mM, respectively), produced 1.4 μM ATP at 23 °C. These data

give an equilibrium constant of 0.0172. Sleep and Hutton [16] found that 1.2% of myosin S1 had ATP bound when 50 μM S1 was mixed with 0.33 mM Pi and 100 μM ADP and then chased with actin to release the formed ATP into the solution. These data yield an equilibrium constant of 0.0741 at 21 °C.

All these results were obtained at pH 7.0–8.0, with 5–60 mM KCl, and 3–5 mM $MgCl_2$. In each case, the hydrolysis reaction was quenched with 0.35–0.5 M perchloric acid.

When we tested the reaction quenching with perchloric acid, we found significant variability in detecting ATP production by myosin in technical replicates. We conclude that quenching the reaction with acid results in uncontrollable adhesion of nucleotides and denatured protein, preventing nucleotides from entering the solution. This effect was noted in several reports [13, 16], and to address the problem, an excess of unlabeled ATP was added during quenching [16]. We found that quenching the reaction in a high salt boiling buffer produced less error and did not affect ATP integrity. We conclude that the high variability in the equilibrium constant observed in previous studies is likely due to the quenching method used.

Another factor contributing to variability in previous studies may be the method of separation of ATP, ADP, and Pi. Different chromatography methods were employed, such as 2D thin-layer chromatography [16] and HPLC with various column types, including charcoal and ion exchange [15–17]. Such elaborate nucleotide separation procedures may introduce errors in nucleotide quantification, affecting the precision of ATP determination in the reverse reaction.

Standard curves with and without 100 μM ADP were generated for each assay. The curves are highly reproducible and show that 100 μM ADP alters the slope of the standard curve. This suggests that ADP may act as a substrate for luciferase, competing with ATP. While the inhibition of luciferase by ADP is outside the scope of our work and requires further investigation, it is important to note that the standard curve remains linear across the entire range of ATP concentrations used in our assay. However, the presence of high ADP concentrations in the assay must be taken into consideration.

The determination of the equilibrium constant from myosin concentration and the concentration of produced ATP relies on the assumption that all myosin molecules are active in the assay. However, our results indicate that this assumption is not necessarily true. The preparations show variation in absolute activity, which we attribute to differing numbers of active myosin molecules, despite our efforts to standardize preparation and eliminate non-functional protein through ultracentrifugation. The variation in equilibrium constants for the reversible ATP hydrolysis by myosin may be due to differences in the absolute activity of myosin in the preparations.

The equilibrium constant determined by the luciferase assay agrees well with the literature data [29] and aligns with previous results determined from radioactive labeled isotopes. When considering the safety hazards and resources necessary to measure ATP hydrolysis reversal with radioactive methods, our assay provides a more appealing option due to its simplicity, safety, common measurement technology, and cost efficiency. This methodology should be tested with other ATPases that are known to be reversible to further validate its merit in characterizing ATP reversibility.

Conclusion

We developed a luciferase-based assay to determine the equilibrium constant of the reversible ATP hydrolysis by myosin S1. The determined equilibrium constant is in excellent agreement with the literature data. We conclude that this assay is a viable alternative to using radioactively labeled isotopes to measure the reversibility of myosin ATP hydrolysis, in addition to being safer, simpler, and more accessible. Using rabbit skeletal myosin ATPase as a model system for reversible ATPases, we have shown that this assay can be used to measure the kinetics of other reversible ATPases.

Abbreviations

ATP	Adenosine triphosphate
ADP	Adenosine diphosphate
Pi	Inorganic phosphate
IQR	Interquartile range
S1	Rabbit skeletal myosin subfragment 1

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s44330-024-00016-1.

Supplementary Material 1.

Acknowledgements

NA.

Authors' contributions

YN and AJ designed research, AJ and AN performed research, AJ and YN analyzed data, AJ, AN, YN wrote the paper.

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Data availability

All raw numerical data is available in the MS Excel format upon request. All numerical data include metadata on experimental conditions, such as buffer composition, pH, etc.

Declarations

Ethics approval and consent to participate
Myosin S1 and actin were produced from rabbit skeletal tissue. All experimental protocols were approved by the Institutional Animal Care and Use Committee of UNC Charlotte and all preparations were performed in accordance with relevant guidelines and regulations. The animals involved in our study were obtained from a local rabbitry. Informed consent from the owner to use the animals in our study was obtained prior to the commencement of our research. This process was in strict accordance with ethical guidelines and institutional regulations governing the use of privately owned animals in research.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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