Interaction of Myosin with Blebbistatin Reported by Fluorescence Spectroscopy

H Rodriguez, S Sylvester, R Sakowicz
Cytokinetics, Inc. South San Francisco, CA

Abstract #1498

Abstract

Blebbistatin is a specific inhibitor of myosin II and a valuable tool for dissecting the roles of myosin in cellular pathways (1). There is significant interest in understanding the mechanism by which blebbistatin alters the enzymatic properties of myosin (2). We investigated the fluorescent properties of blebbistatin alone and in a complex with the myosin motor domain (S1). We discovered that these properties vary significantly between the free and S1-bound state of the molecule. Additionally, these spectral changes are sensitive to the various nucleotide states of myosin. The fluorescent spectrum of blebbistatin interacting with S1-nucleotide complex displays a fluorescence enhancement, as well as a significant blue shift whose magnitude of change is modulated by the conformation of myosin. These properties were used to measure the binding affinity and binding kinetics of blebbistatin for myosin under differing enzyme conformations. Our studies indicated that the fluorescent properties of blebbistatin can be used as a convenient reporter of myosin conformations and may provide insight into the binding site of the inhibitor.

Introduction

Blebbistatin is a recently discovered small molecule inhibitor of Myosin IIs (1). A detailed study on the mechanism of this inhibitor has already been carried out on skeletal muscle myosin and suggests that the inhibitor interferes with the phosphate release step of the enzymatic cycle (2). We have found that blebbistatin inhibits the basal as well as the actin activated ATPase of bovine cardiac myosin subfragment-1 (cS1). Additionally, we have also found that blebbistatin has fluorescent properties that are modulated by the conformation of myosin. The specificity of this inhibitor for myosin IIs in conjunction with the fluorescent properties that are reported here provide a valuable tool for probing the binding interactions of blebbistatin to other myosins and for monitoring conformational changes within these myosins.

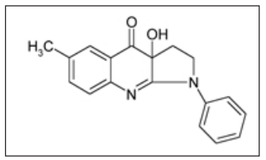


Figure 1: The structure of blebbistatin.

Materials & Methods

Materials
Blebbistatin was obtained from Toronto Research. Bovine cardiac myosin subfragment-1 (cS1) and actin were produced by the protein production group at Cytokinetics, Inc. (-)-S-blebbistatin was used for all studies.

Steady state kinetics
All steady state kinetic measurements were made using an NADH coupled assay system by monitoring the absorbance change at 340 nm in 10mM Pipes, 2mM $MgCl_2$, 1mM DTT pH 6.8 (PM10) buffering system. Temperature was set at 25 °C unless otherwise specified. All measurements were carried out in a Molecular Devices SpectraMax plate reader.

Fluorescence spectroscopy
Fluorescence measurements were carried out on a Spex FluoroMax fluorimeter. The excitation wavelength was set to 434 nm with a 10 nm bandpass. All spectra were corrected and buffer subtracted.

Rapid kinetic measurements
All rapid kinetic measurements were carried out on a Hi-Tech SF-61DX2 stopped-flow apparatus temperature controlled to 25° C. The excitation wavelength was set to 434 nm and emission was collected through a 455 nm long pass filter. On average, three transient traces were collected and averaged together. All data analysis was conducted using the KinetAsyst software.

Results

The ATPase of bovine cardiac subfragment-1 is inhibited by blebbistatin

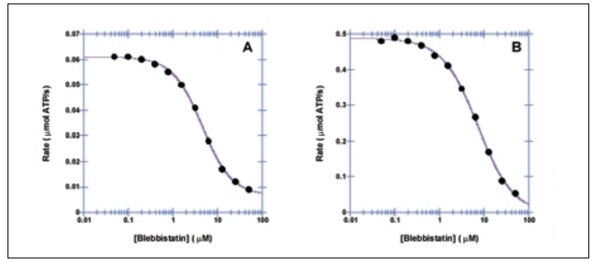


Figure 2: Inhibition curves of (-)-S-blebbistatin against the (**A**) basal ATPase of bovine cardiac S1 (IC50= 4.5 μM) and (**B**) actin stimulated ATPase of bovine cardiac S1 (IC50=7.3 μM). These data show that blebbistatin is capable of interacting with cS1 and acto-cS1.

Blebbistatin has fluorescent properties that are modulated by the conformations of myosin

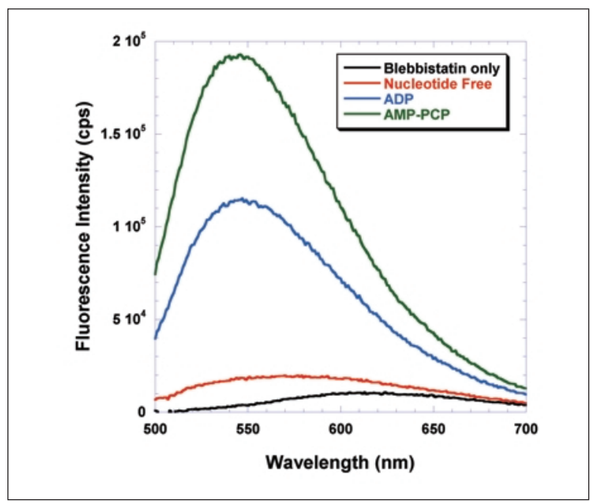


Figure 3: Emission spectra of (-)-S-blebbistatin in the presence of bovine cardiac S1 under differing nucleotide states. Experiment was conducted at 10 μM blebbistatin, 5 μM bovine cardiac S1 and 1mM nucleotide. Spectra are background corrected. An enhancement in fluorescence as well as a blue shift are noticeable when blebbistatin binds to cS1 and these properties are further enhanced in ADP and ATP-like states of myosin.

The fluorescent properties of blebbistatin can be used to monitor blebbistatin binding

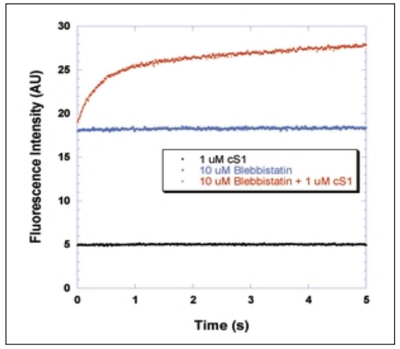


Figure 4: Representative trace of the binding kinetics of blebbistatin to cS1 measured by stopped-flow fluorescence spectroscopy. Emission was collected through a 455 nm long pass filter. Cardiac myosin was mixed with blebbistatin to give the final concentrations noted on figure. Data were fit to a single exponential time course with linear drift; use of double exponential did not improve the fit of the data.

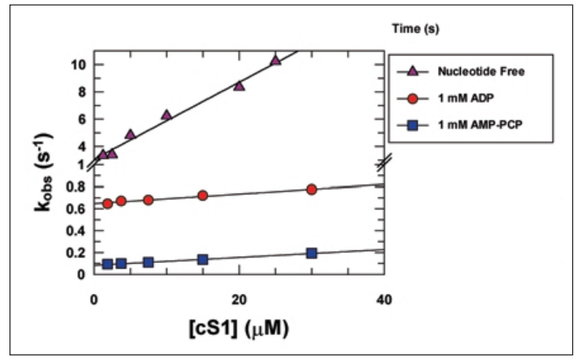
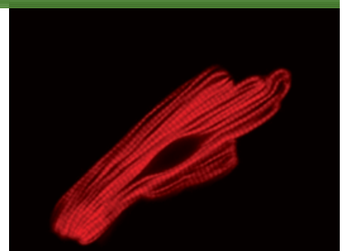


Figure 5: Figure 5. Binding kinetics of blebbistatin to cS1 under different nucleotide states. Blebbistatin solution (5 μM final) containing the indicated nucleotide (1mM) was rapidly mixed with varying concentrations of cS1 containing nucleotide at 1mM.

	k_{ON}	k_{OFF}	K_D
Nucleotide Free	$2.7x10^5$ M^{-1} s^{-1}	3.2 s^{-1}	12 μM
ADP State	$4.3x10^3$ M^{-1} s^{-1}	0.6 s^{-1}	139 μM
ATP State	$3.6x10^3$ M^{-1} s^{-1}	0.08 s^{-1}	22 μM

Table 1: Summary of the binding rates of blebbistatin to cS1 in different nucleotide states. The dissociation constants were computed from the ratio of the off and on rates.



Summary/Conclusion

We have shown that (-)-S-blebbistatin inhibits the basal as well as actin stimulated ATPase of bovine cardiac S1. It was also shown that blebbistatin has fluorescent properties that are modulated by the nucleotide state of myosin. We observed enhancements in the fluorescence emission as well as a blue shift, indicating that the fluorophore is sampling a more non-polar environment.

These fluorescent properties were used to measure the binding kinetics of blebbistatin. Our analysis shows that blebbistatin binds much more rapidly to cS1 in the absence of nucleotide.

The fluorescent properties of blebbistatin provide a convenient probe for studies of myosin II motors.

References

1. Straight, AF *et al*. Dissecting temporal and spatial control of cytokinesis with a myosin II Inhibitor. *Science* (2003).

2. Kovacs, M *et al*. Mechanism of blebbistatin inhibition of myosin II. *J Biol Chem* (2004).

Cytokinetics