1

Equilibrium Binding of Proteins to F-Actin

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Summary

This chapter reviews some of the many available methods for measuring the binding of myosin and other proteins to actin. Binding to actin has special considerations because actin is a long lattice and the binding site of many of its binding partners consists of multiple actin protomers. The analysis of binding to a lattice cannot be done by standard methods such as a Scatchard plot. Rational methods of analysis are described.

Key Words: Myosin; S1; actin; G-actin; F-actin; cooperativity; kinetics; binding; binding analysis; equilibrium; association constant; parking problem; ATPase rate; equations; sedimentation; ATP.

1. Introduction

The physical sizes of F-actin and actin-binding proteins place restrictions on the types of assays that can be used for measuring equilibrium binding. This chapter describes variations of sedimentation and fluorescence methods that have broad applications to the study of actin. Sedimentation methods commonly consist of two steps: separation of free ligand from the ligand–actin complex by sedimentation and determination of the free and bound ligand. Fluorescence methods are based on the change in environment of tryptophan residues or covalently attached probes on either actin or the ligand protein. Pyrene on Cys 374 of actin is responsive to binding of myosin subfragment 1 (S1) and other proteins and changes the affinity of binding to S1 by less than a factor of 2 *(1)*. Fluorescein-labeled S1, 4-(iodoacetamido) salicylic acid (ISAL)-labeled S1, and *N*-((2-(iodoacetoxy)ethyl)-*N*-methyl)-amino-7-nitrobenz-2-oxa-1,3-diazole (IANBD)-labeled caldesmon are examples of fluorescent ligand proteins that have been used in actin-binding studies *(2)*.

From: *Methods in Molecular Biology, vol. 392: Molecular Motors*
Edited by: A. O. Sperry © Humana Press Inc., Totowa, NJ

Measurement of protein binding to actin has special requirements because of the unique properties of actin. Actin can exist either as a globular monomer (G-actin) or as a very large filamentous polymer (F-actin). Care must be taken to avoid changing the state of actin during the measurement, as the affinities of ligands for actin are normally dependent on the state of actin. The methods described in this chapter are for binding to F-actin. Other physical changes in actin can also affect the measurement of binding. For example, bundling of actin filaments causes increased light scattering that complicates fluorescence measurements.

The affinity of myosin for actin depends on the nucleotide bound to the active site of myosin. **Figure 1** shows the salt dependencies of S1-ATP and S1-ADP binding to F-actin. The large difference in affinity resulting from the substitution of ADP for ATP at the active site of S1 must be considered when measuring binding of S1-ATP to actin. Hydrolysis of a large fraction of ATP during the measurement can lead to grossly inaccurate results. **Figure 1** also illustrates

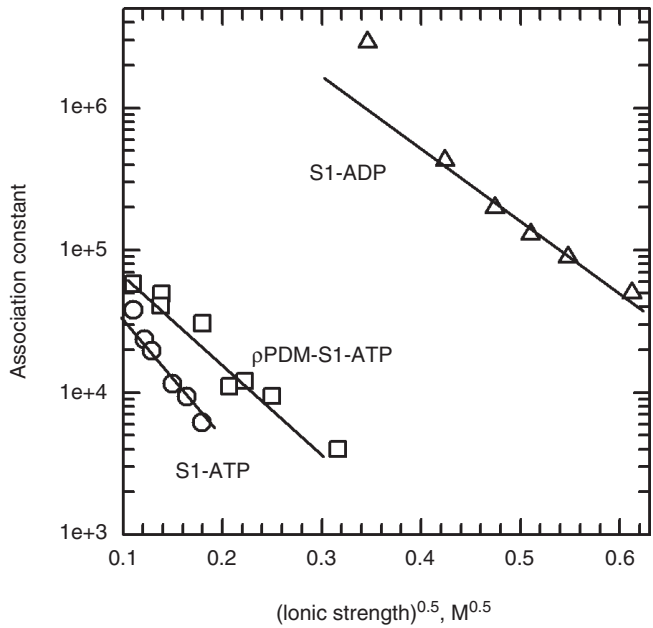

Fig. 1. Dependence of the affinity of myosin S1 to actin on the nucelotide bound to S1 and on ionic strength. Association constants (1/M) obtained at 25°C are shown as a function of the square root of the ionic strength according to the Debye–Hückel relationship (Castaneda-Agullo et al. 1961) ***(29)***. [Data from Gafurov et al. 2004 ***(24);*** Chalovich et al. 1981 (***30***), 1983 ***(28);*** Chalovich and Eisenberg 1982 ***(16)***.]

that the ionic strength can be adjusted to facilitate a particular binding study by bringing the affinity into a region that is most accurately measured.

The affinity between two proteins can be determined by keeping one protein fixed and varying the other. More information can be obtained for long lattices, such as actin, if the experiment is designed so that the occupancy of the lattice is varied. Furthermore, binding measurements are most accurate if the actin concentration is lower than the dissociation concentration of the actin–ligand complex.

The lower limit of actin concentration is set by the sensitivity of the measurement and by depolymerization of F-actin below its critical concentration ***(3)***. Phalloidin, a toxin of the angel of death mushroom, binds to actin and lowers its critical concentration by 30-fold (1 : 1 complex) or 90-fold (2 : 1 phalloidin: actin) ***(4,5)***. A 1 : 1 complex of phalloidin and actin has been used to determine dissociation constants as low as 10 n*M* ***(6)***.

Ligands vary widely in the stoichiometry of binding to actin. S1 binds to a single actin protomer, fesselin binds to three or four protomers ***(7)***, while tropomyosin ***(8)*** and caldesmon ***(9)*** bind to about seven protomers. One must use a general binding equation that is valid irrespective of the number of protomers that form a binding site. Valid methods of analysis are described below.

Temperature is an important variable for binding studies because it affects protein stability, the rate of reaching equilibrium, and the affinity. The fluorescence intensity may increase at lower temperature because of a decrease in collisional quenching ***(10)***. Although the choice of temperature is important, it is most important that the temperature be constant for all measurements.

2. Materials (*see* Note 1)

1. Actin depolymerization buffer: 0.5 m*M* ATP (Cat. #A7699, Sigma, St. Louis, MO), 2 m*M* tris-(hydroxymethyl)aminomethane (Tris)-HCl, pH 7.8, 0.1 m*M* $CaCl_2$.
2. Preparative ultracentrifuge with type 50 Ti and type 50.2 Ti rotors (Beckman Coulter, Fullerton, CA).
3. Column chromatography equipment.
4. Water bath sonicator.
5. Gel filtration resins such as Sephadex G100 or Superdex 200 resins (Pharmacia-LKB, Uppsala, Sweden) and Sephacryl S-300 HR (Sigma, St. Louis, MO).
6. Actin modification buffer: 100 m*M* Tris-HCl, pH 8.0, 1 *M* KCl, 20 m*M* $MgCl_2$, 1 m*M* $CaCl_2$, 0.1% NaN_3. Add 10 mL 1 *M* Tris-HCl pH 8, 50 mL 2 *M* KCl, 2 mL 1 *M* $MgCl_2$, 5 mL 20 m*M* $CaCl_2$, and 10 mL 1% NaN_3 to a 100-mL graduated cylinder. Add water to 100 mL. This stock can be stored in the refrigerator for several months.
7. Fluorescence probes such as *N*-(1-pyrene)iodoacetamide (Molecular Probes, Eugene, OR).

8. Dithiothreitol (Invitrogen, Carlsbad, CA).
9. UV spectrophotometer Bio-Spec-1601 (Shimadzu, Kyoto, Japan).
10. Phalloidin (Sigma, St. Louis, MO).
11. Beckman Optima TL ultracentrifuge with a TLA 120.1 rotor or a Beckman Airfuge (Beckman Coulter, Fullerton, CA).
12. Teflon beakers for mixing proteins, 3–5 mL capacity.
13. Highly purified bovine serum albumin or Tween 20.
14. 2X protein loading buffer: 0.5 *M* Tris-HCl, pH 6.8, 4.4% (w/v) sodium dodecyl sulfate (SDS), 20% (v/v) glycerol, 2% (v/v) 2-mercaptoethanol, and bromophenol blue in distilled/deionized water.
15. Circulating water bath model RTE-110 (Thermo-Neslab, Newington, NH).
16. Fluorescence spectrophotometer (Thermo Electron, Madison, WI).
17. Reducing buffer: 100 m*M* KCl, 10 m*M* phosphate buffer pH 6.5, 5 mM ethylenediaminetetraacetic acid (EDTA), 1 m*M* dithiothreitol.
18. ATP stock: Dissolve 0.12 g ATP (Cat. #A7699, Sigma, St. Louis, MO) in 2 mL water and adjust the pH to 7.0. Dilute 0.05 mL ATP solution to 50 mL using a volumetric flask. Measure the absorbance at 259 nm. The concentration of the undiluted ATP stock in m*M* units is then $64.9 \times A_{259}$ (*see* **Note 2**).
19. ATP-Tris solution: To 3 mL 200 m*M* Tris-HCl (pH 8.0) add about 100 mg solid ATP (*see* **18**, above). Adjust the pH to 8.0 with Tris base (NaOH or KOH should be avoided because the metal ions interfere with the subsequent assay). Dilute a 0.05-mL aliquot of the ATP to 50 mL in a volumetric flask. Measure the O.D. at 259 nm and calculate the ATP concentration as described above. Add enough of the ATP stock to make 2 mL 35 m*M* ATP. Add 0.2 mL ^{32}P-ATP and bring the total volume to 2.0 mL by the addition of 200 mM Tris-HCl pH 8.
20. ^{32}P-ATP stock: To 1 mCi ATP [γ-^{32}P] (Perkin Elmer-NEN, Boston, MA), add sufficient unlabeled ATP stock solution to make 4 mL 1 m*M* stock (final specific activity, about 250 mCi/mmole). Store frozen in 1-mL aliquots in a container rated for storage of ^{32}P such as an acrylic box (*see* **Note 3**). ^{32}P-ATP should be as fresh as possible and disposed of after 3–4 weeks because decreases in observed ATPase activity may occur.
21. Tris base, 2 *M* stock: Dissolve 24.2 g Tris base (F.W. 121.14) in water. Bring to 100 mL final volume.
22. Tris buffer, 200 mM, pH 8.0: Dissolve 4.8 g Tris base in 150 mL room temperature water. Adjust pH to 8.0 with HCl. Bring final volume to 200 mL with water.
23. Ammonium-EDTA reagent: Add 4 g NH_4Cl (F.W. 53.49) and 2.5 g EDTA disodium salt (372.24) to about 75 mL water. Adjust pH to 8.0 with Tris base. Adjust volume to 100 mL.
24. 1 *M* MOPS buffer: Dissolve 20.9 g MOPS [3-(*N*-morpholino)-propanesulfonic acid] (F.W. 209.26) in about 80 mL water. Adjust pH to 7.0 and volume to 100 mL.
25. ATPase quench solution: Add 64 mL 12 *N* HCl to 300 mL water (*see* **Note 4**). Add 0.11 g NaH_2PO_4 (F.W. 137.99). Adjust to 500 mL and store in a repipet dispenser bottle set to deliver 0.4-mL aliquots.

26. Silicotungstic acid solution: Add 20 mL 36 *N* H_2SO_4 to 300 mL water (*see* **Note 4**). Add 22 g silicotungstic acid (Cat. #A289, Fisher, Fair Lawn, NJ) and stir until dissolved. Bring volume to 500 mL. Store in a repipet dispenser bottle set to deliver 0.2-mL aliquots. The final solution is 1.4 *N* H_2SO_4, 4.4% silicotungstic acid.
27. Isobutanol:benzene (1 : 1): Add 250 mL isobutanol to 250 mL benzene and store in a repipet dispenser bottle set to deliver 1-mL aliquots. Use a hood and other normal safety precautions when using benzene (*see* **Note 5**).
28. Ammonium molybdate solution (5%): Dissolve 5 g ammonium molybdate (Cat. #A674, Fisher, Fair Lawn, NJ) in water to make 100 mL solution. Stir overnight or until completely dissolved. Filter. Store in a repipet dispenser bottle set to 0.2 mL. This solution should be discarded after 3–4 weeks or when a white precipitate is visible.
29. *N*-Pyrenyliodoacetamide stock: Weigh out between 1.4 and 2 mg pyrenyl iodoacetamide (Molecular Probes, Eugene, OR). Add dimethylformamide (Sigma, St. Louis, MO) to bring to 14 mg/mL. Vortex vigorously or sonicate in a water bath sonicator to dissolve the solute. Use immediately after preparation.
30. Hexokinase stock: To 2500 IU yeast hexokinase (Cat. #376811, Calbiochem, La Jolla, CA) add 2 mL 50 m*M* Tris-HCl, 5 m*M* $MgCl_2$. This stock solution is stable for 6 months at 4°C.
31. ADP stock: Dissolve 0.012 g ADP (Cat. #117105, Calbiochem, La Jolla, CA) in 2 mL water. Adjust to pH 7 with 5 *N* KOH. Dilute 0.05 mL of the ADP solution to 50 mL using a volumetric flask. Measure the absorbance at 259 nm. The concentration of the stock ADP (in m*M*) is then $64.9 \times A_{259}$.
32. ADP buffer for fluorescence measurements: This buffer contains ADP and ATP scavengers ***(11)***. To 1.66 mL 12 m*M* ADP, add 0.025 mL 2 *M* glucose, 0.14 mL hexokinase stock, 0.125 mg diadenosine pentaphosphate (Cat. #D4022, Sigma, St. Louis, MO) (0.031 mL 4 mg/mL diadenosine pentaphosphate stock), 1 mg bovine serum albumin (0.052 mL 38 mg/mL stock), and 0.59 mL water. This solution should be made on the day of the assay.
33. Ca^{2+} or ethylene glycol-bis(β-aminoethyl ether)-*N*,*N*,*N*′,*N*′-tetraacetic acid (EGTA) buffer for fluorescence measurements: Other components of the final reaction mixture are present in this buffer. We normally prepare this buffer at 1.54X the concentration that is required in the final fluorescence assay mixture (1.3 mL of this buffer is present in each 2 mL of the final solution). A typical buffer is 30.7 m*M* MOPS pH 7, 7.6 m*M* $MgCl_2$, 209 m*M* KCl (adjust this to the desired ionic strength), 1.54 m*M* EGTA (or 0.75 m*M* $CaCl_2$), and 1.54 m*M* dithiothreitol.
34. Actin-pyrene labeled ***(12,13)***: To 20 mg highly purified F-actin in 4 m*M* imidazole, 2 m*M* $MgCl_2$, add 1 mL actin modification buffer and water to 10 mL. Stir until the actin is homogeneous. Add 0.1 mL of 14 mg/mL solution of *N*-(1-pyrene)iodoacetamide in dimethylformamide with rapid stirring. Incubate 15 h at 10°C in the dark with slow stirring. Stop the reaction by adding a small flake of dithiothreitol. Centrifuge the actin in a Beckman 50 Ti rotor for 20 min at 60,000*g*. Save the supernatant containing the pyrenyl actin. Centrifuge the supernatant in a 50Ti rotor at 134,000*g* for 1 h. Remove the supernatant. Add 5 mL 4 m*M*

imidazole, 2 m*M* $MgCl_2$ to the pellet, and allow the tube to stand on ice in the dark for at least 1 h. Homogenize the softened pellet and dialyze against two changes of buffer of choice. Determine the concentration of actin by the Lowry assay and the concentration of bound pyrene by the absorbance at 344 nm using an extinction coefficient of $2.2 \times 10^4\, M^{-1}\, cm^{-1}$.

35. Phalloidin-F-actin: To 0.67 mL 100 m*M* NaCl, 10 m*M* MOPS pH 7.0, add 0.03 mL 1 m*M* phalloidin (Cat. #P2141, Sigma, St. Louis, MO) made in methanol and mix. Add 0.3 mL 100 μ*M* actin or pyrene-labeled actin and mix gently.
36. Actin-binding proteins of interest: The protein should be pure and homogeneous. The protein should be in a buffer that will not destabilize the protein and which will not interfere with subsequent analysis. Proteins used for co-sedimentation with actin should be centrifuged at 106,000*g* in a Beckman 50Ti rotor (or equivalent) for 1 h to remove aggregates.
37. Myosin S1: S1 prepared by enzymatic digestion should be purified by gel filtration chromatography on Sephacryl S-300 HR or an equivalent resin. Skeletal muscle S1 has 2 isoforms that bind differently to actin at low ionic strength ***(14)***. These isoforms may be resolved by ion-exchange chromatography on DEAE resin ***(15)***.
38. Ligand proteins modified with ^{14}C or fluorescent probes: This procedure may be optimized by adjusting the molar ratio of reagent to protein and the reaction time. Dialyze the protein against reducing buffer. After dialysis, add sufficient phosphate buffer, pH 6.0, to bring the final buffer concentration to 50 mM. Add dithiothreitol to bring the final concentration to 10 mM and incubate for at least 30 min at 37°C. Dialyze twice against a large excess of 100 m*M* KCl, 50 m*M* Tris-HCl, pH 7.5, 1 m*M* EDTA, 0.1 m*M* dithiothreitol, or run over a desalting column equilibrated with the same buffer. Adjust the protein concentration to about 2 mg/mL. Add the fluorescent reagent at a concentration equal to 0.1 m*M* plus three times that of the concentration of the protein. Incubate in the dark at 18°C for 4 h; stop the reaction with a small flake of dithiothreitol. Clarify the modified protein by centrifugation and remove unreacted probe by extensive dialysis or gel filtration chromatography. If the thiol groups of the protein are not particularly labile, the 0.1 m*M* dithiothreitol may be omitted from the dialysis buffer or column buffer. In that case, the amount of fluorescent reagent should be simply three times that of the concentration of the protein. Note that the amount of fluorescent reagent should be adjusted to obtain the desired extent of labeling for the particular protein studied.

3. Methods

3.1. Binding Methods Based on Pelleting Actin–Ligand Complexes

Protein mixtures are centrifuged. The actin–ligand complexes in the pellets and the supernatants are analyzed to determine the bound and free ligand, respectively. Measurement of both the ligand protein and actin may be done by several methods including polyacrylamide electrophoresis, capillary electro-

phoresis, HPLC, and enzymatic assays of the ligand protein. Enzymatic assays are useful when the ligand concentration is very low relative to the actin concentration, as in the case of S1 binding to actin during steady-state ATP hydrolysis.

3.1.1. Binding of Ligand to Actin

1. For moderate affinity complexes prepare 0.6-mL solutions containing various concentrations of the ligand protein and 0.5 μ*M* phalloidin-F-actin. It is best to use small Teflon beakers to stir the mixtures slowly at 5°C for 30 min or until the mixtures are homogeneous.
2. Transfer 0.5 mL of each mixture to a centrifuge tube and incubate for 5 min at the desired temperature.
3. Centrifuge the samples at the desired temperature for a sufficient time to produce a firm actin pellet (see below).
4. Remove all the supernatant and place into a small clean tube.
5. Carefully wash the pellet with 0.5 mL buffer to remove residual supernatant. Discard the wash.
6. Analyze the actin pellet to determine the ratio of bound ligand to actin monomer.
7. Analyze the supernatant to determine the amount of free ligand. It is also possible to determine the bound ligand from the free and vice versa using the conservation of mass equation: $[\text{Ligand}]_{\text{Bound}} = [\text{Ligand}]_{\text{Total}} - [\text{Ligand}]_{\text{Free}}$. Analyze both the supernatants and pellets whenever feasible.

3.1.2. Sedimentation of Actin–Ligand Complexes

Several combinations of centrifuges and rotors can be used for these assays. Sedimentation can be done in a Beckman Airfuge with rotor A-95 at 134,000 *g* for 20 min ***(16)*** with a TLA 120.1 rotor in a Beckman Optima TL ultracentrifuge at 43,500 *g* for 30 min, or with in a 50 Ti rotor in a preparative ultracentrifuge at 134,000 *g* for 30 min. The manufacturer of the rotor should be consulted for optimal volumes to be used in the respective centrifuge tubes. The rotor and centrifuge must be at the desired temperature before starting the binding reaction.

3.1.3. Controls for Sedimentation Assay

3.1.3.1. Sedimentation in the Absence of Actin

The most important control to run is the ligand in the absence of actin. Partial sedimentation of the ligand protein alone is normal. Ligand proteins may also be lost by adsorption to the walls of the vessel. The value of the amount of ligand in the control pellet should be added to the experimental supernatant and subtracted from the experimental pellet value prior to calculating binding constants. This correction can be minimized by using Teflon beakers and by adding

a carrier protein or a detergent to the assay mixture. Highly purified bovine serum albumin at 0.1 mg/mL or 0.05% Tween 20 works well with a number of actin-binding proteins.

3.1.3.2. Determination of the Optimum Additive Concentration

The optimum additive concentration maximizes the amount of ligand in the supernatant in the absence of actin and does not inhibit ligand binding to actin.

1. Prepare several centrifuge tubes each containing buffer and the pure ligand.
2. Prepare a duplicate set of tubes that also contain actin.
3. To each tube add a different concentration of a detergent or carrier protein.
4. Centrifuge all tubes and analyze the supernatants and pellets as in a normal binding assay. The optimum concentration of additive gives the maximum amount of bound ligand after correction for sedimentation in the absence of actin.

3.1.3.3. Corrections for Inactive Actin or Ligand

Other corrections that may be significant are for "dead" actin and ligand. The fraction of actin that is functional may be equated to that fraction that binds to S1 in the absence of nucleotide at moderate ionic strength. It is difficult to correct binding studies for the fraction of inactive actin unless the ligand binds to a single actin protomer. The best way to handle inactive actin is to eliminate it by further purification such as by going through another cycle of polymerization.

One can test the quality of the ligand protein by conducting a binding experiment at a low and constant ligand concentration with increasing actin concentrations. All of the ligand should be bound in the limit of infinite actin concentration. A Lineweaver–Burke plot (1/initial velocity vs. 1/[actin]) can be used to estimate the fraction of ligand that does not bind to actin. If the fraction of damaged ligand determined by this method is relatively small, it is possible to correct the total ligand concentration by multiplying the total concentration by (1 − fraction dead ligand).

3.1.3.4. Removal of Nonfunctional Ligand

Nonfunctional ligand can often be removed by employing a method of purification that selects for functional ligand. In the case of S1, it is possible to take advantage of the effect of ATP on actin affinity.

1. Prepare a buffer solution containing about 200 m*M* NaCl, 5 μ*M* S1, and 5 μ*M* actin.
2. Centrifuge for a sufficient length of time to produce a tight actin pellet and discard the supernatant.

3. Allow the pellet to swell in a small volume of the same buffer.
4. Homogenize pellet gently in a glass–Teflon homogenizer in a buffer containing 2 m*M* ATP to release the bound S1. This step must be done quickly so that the ATP does not become depleted.
5. Rapidly centrifuge again to form another actin pellet. The supernatant is enriched in functional S1.
6. Remove residual actin and ATP by column chromatography. A modification of this procedure can be used for other actin-binding ligands by employing a combination of conditions that alternatively favor binding and that promote dissociation.

3.1.4. Analysis of Pellets and Supernatants

3.1.4.1. Gel Electrophoresis

Supernatants from sedimentation experiments are readily analyzed by adding an SDS-containing sample buffer directly to the samples. Analysis of the pellets requires a little care.

1. Soften pellets by incubating on ice for 1 h after the addition of water equal to half of the initial reaction volume.
2. Add a volume of a 2X concentrated sample buffer equal to the volume of water added in step 1 and suspend the pellets using a bath sonicator.
3. Place supernatants and pellet solutions in a boiling water bath for 2 min to denature all proteins.
4. Following electrophoresis, scan the stained gels to determine the density of the actin and ligand bands. Densities can be converted into masses by the use of standard curves.
5. Prepare standard curves over a wide range of protein masses to ensure linearity over the range actually used in the assay. Run at least two concentrations of standards (a mixture of known amounts of actin and the ligand proteins) on each gel to correct for gel-to-gel differences in staining intensity.

3.1.4.2. HPLC

Reverse-phase HPLC is useful because the organic solvent keeps the actin in a monomeric state so that it can run readily on the column.

1. Mix protein pellets with 0.05 mL 20% acetonitrile, 0.1% trifluoroacetic acid in water.
2. Incubate the pellets in this solvent for a minimum of 1–2 h and then homogenize them mechanically or by using a water bath sonicator. It is critical that the proteins be totally solubilized.
3. Load the samples onto a C18 column (i.e., μBondapak; Waters Inc.) equilibrated with 20% acetonitrile and 0.1% trifluoroacetic acid.
4. Wash with the same buffer for 5 min at 1 mL/min.
5. Elute the proteins with a gradient to 70% acetonitrile over 25–30 min.

6. Monitor the protein peaks at 214 nm and analyze the peak areas either by weighing the cut-out peaks from chart paper or by utilizing a software package (i.e., Chrom Perfect Spirit Tiger II; Justice Laboratory).
7. Determine the amount of each protein in each mixture by comparing the areas of all peaks in the mixture with standards of the respective proteins. For an example of this procedure, see Fredricksen et al. (2003) ***(9)***.

3.1.4.3. Radioactively Labeled Ligand

Ligand proteins containing thiol groups may often be readily labeled with ^{14}C-iodoacetamide. The amount of ligand bound may be determined by dissolving the actin pellet after centrifugation and determining the radioactivity in a scintillation counter. One can also measure the loss of radioactivity from the supernatant as a measure of binding ***(17,18)***.

Radioactive probes simplify quantitation of binding and allow a wide range of ligand concentrations to be analyzed. However, the investigator must determine the extent to which modification has altered the affinity of the ligand protein for actin. Competitive binding studies are useful for determining the relative functionality of a modified ligand. The labeling procedure has not altered the affinity if binding of the radioactive ligand is reduced to 50% when the concentrations of unlabeled and labeled ligands are equal.

It is also important to confirm that the label (whether radioactive or fluorescent) is on the desired ligand.

3.1.4.4. Specific Ligand Assays: ATPase Activity

The concentration of free and bound ligand can be determined by an assay specific for the ligand. A common example of this approach is the use of ATPase rates to determine free myosin S1 concentrations when examining binding to actin during steady-state ATP hydrolysis (*see* **Subsection 3.1.3.3.**). Low S1 concentrations must be used to ensure that the ATP has not been exhausted during the mixing and sedimentation steps. (*See* **Note 6.**) Binding may be measured by varying the actin concentration at a constant low S1 concentration (typically 0.1 μ*M* for assays at 25°C). A good way to ensure that the binding is valid is to show that the same fraction bound is obtained when the S1 concentration is reduced.

The rate of ATP hydrolysis in the presence of ammonium ions may exceed the physiologically relevant actin activated rate and is well suited for determining the concentration of myosin in sedimentation assays. Metal ions interfere with this assay so Tris base should be used for adjusting the pH of stock solutions. F-actin also inhibits this ATPase reaction, so F-actin must be eliminated or depolymerized. F-actin is depolymerized in

the buffer described below by the combined effects of EDTA and high ionic strength.

1. The binding mixture consists of 1 mL of a solution containing 0.1–0.3 μ*M* S1, a variable amount of F-actin, 2 m*M* MgATP, 2 m*M* $MgCl_2$, 10 m*M* imidazole pH 7.0, 1 m*M* dithiothreitol, and ammonium chloride to obtain the desired ionic strength. Add all components, except the S1, into small Teflon beakers and stir on ice for 15 min.
2. Initiate binding reactions by adding S1 from a 2 μ*M* stock. Mix for 1 additional min.
3. Transfer known amounts of the mixtures to chilled centrifuge tubes. Incubate in a water bath for approximately 5 min to reach the desired temperature.
4. Centrifuge at 134,000 *g* for 25 min in a 50Ti rotor.
5. Remove the entire supernatant and place into ice-cold glass tubes.
6. Determine the S1 content of each supernatant using the ammonium-EDTA ATPase assay (see below).
7. Add 0.35 mL of each supernatant to a tube or Teflon beaker containing 0.5 mL ammonium-EDTA reagent. When measuring low levels of S1, it is helpful to add 1 mg/mL tropomyosin or bovine serum albumin as a carrier.
8. Incubate the samples in a water bath at 25°C. Start the reaction by adding 0.15 mL 35 m*M* γ-^{32}ATP-Tris.
9. Remove 0.2 mL reaction mixture at 2, 5, 10, and 20 min after initiating the reaction for determination of released phosphate. Place each 0.2-mL aliquot into a disposable tube (about 1 cm × 9 cm) containing 0.4 mL ATPase quench solution. Vortex briefly, and extract the ^{32}Pi by the modification of a standard method ***(19)*** described below.
10. To each tube add sequentially 0.2 mL silicotungstic acid solution, 1.0 mL 1:1 isobutanol: benzene (use in a hood), and 0.2 mL 5% ammonium molybdate. Vortex rapidly for exactly 30 s.
11. Allow the tube to rest for several minutes until the bright yellow organic phase separates from the lower clear aqueous phase. The upper phase contains the ^{32}Pi, which can be analyzed by scintillation counting. If the phases fail to separate well (i.e., when high protein concentrations are used), centrifuge the tubes in a hood for 10 s at low speed.
12. Remove 0.25 mL of upper yellow phase and add to a 10-mL scintillation vial. Add 8 mL scintillation fluid and count for sufficient time to collect at least 10^4 counts. (*See* **Note 7**.)

The ATPase rate of the no-actin control ($v_{A=0}$) is equivalent to 0% bound or 100% free S1. The fraction of bound S1 (FB) bound at a given actin concentration can be determined directly by Equation 1 where $v_{A=C}$ is the rate at some fixed actin concentration.

(1) $$FB = 1 - (v_{A=C}/v_{A=0})$$

3.2. Fluorescence-Based Actin Binding

Fluorescence assays can be used to measure binding when there is a change in the environment of a probe on either actin or the actin-binding protein. Environmentally sensitive probes are most easily utilized when placed on the lattice protein, actin. Changes in the fluorescence of a pyrene probe placed on Cys 374 of actin form the basis for several assays.

The following paragraph describes the measurement of binding of S1-ADP to pyrene-labeled actin. Variations of this method may apply to other actin-binding proteins.

3.2.1. Fluorescence Method

1. Into a 1 × 1 cm fluorescence cuvette, add 0.2 mL 3 mM phalloidin pyrene-actin, 1.3 mL EGTA buffer, and 0.5 mL 8 mM ADP buffer.
2. Incubate in the fluorimeter until the solution is at the desired temperature.
3. Set the excitation and emission wavelengths for 364 and 384 nm, respectively.
4. Record the initial fluorescence. Add S1 in a stepwise manner using a concentrated stock to minimize dilution. Subsequent additions should not be made until the fluorescence amplitude is stable; it may take several minutes for equilibrium to be reached. Begin by adding 1-µL aliquots of 50 µ*M* myosin S1. When the fluorescence changes become small, add 3- or 5-µL aliquots of S1. Finally, add 5-µL fractions of 200 µ*M* S1.
5. Continue the titration until there is no fluorescence change on addition of ligand. The experiment should be planned so that the plateau is reached when the added ligand volume is less than 0.2 mL so that the correction for the volume change will be small.

3.2.2. Fluorescence Data Corrections

Correct the data for dilution of the fluorescent probe and for changes in concentrations of the proteins. Corrections are imperfect, so it is preferable to avoid changes in volume greater than 10%. Spreadsheet programs are convenient for making these corrections. (*See* **Note 8.**)

3.3. Analysis of Actin-Binding Data

3.3.1. Noncooperative Binding with n = 1

A simple hyperbola can be fitted to the data to obtain the dissociation constant, K_{diss}, when there is no cooperativity when the binding site consists of one actin protomer ($n = 1$). Let θ be the ratio of the ligand-actin complex to total actin and let L_F be the free ligand concentration. θ is given by Equation 2.

$$\theta = (n \times L_F)/(K_{diss} + L_F) \quad (2)$$

A poor fit of a hyperbola to data may occur if the binding is cooperative, if the endpoint had not been determined accurately, or if there is nonspecific binding.

The dissociation constant can also be determined by an equation expressed in terms of total actin, $[A_T]$, and total ligand, $[L_T]$, concentrations ***(20)***:

$$\theta = \{([L_T] + K_{diss} + [A_T]) - \text{sqrt}(([L_T] + K_{diss} + [A_T])^2 - 4/[L_T][A_T])\}/2[A_T] \quad (3)$$

3.3.2. General Model

In cases where a ligand interacts with several actin protomers of the actin lattice, the relationship between theta and the free ligand concentration depends on additional factors. **Figure 2** illustrates the binding of a ligand (solid bars) to three actin protomers (open circles) within a long actin filament. The actin filament shown is partially decorated with ligand. Consider the possible fates of another ligand as it binds to actin. If the ligand were to bind to the region of actin designated as "A," the binding would be described with an association constant K. If that same ligand were to bind instead to region "B," the affinity would be equal to Kω. If $\omega > 1$, the binding would be stronger than to an isolated site and there would be positive cooperativity. If $0 < \omega < 1$, the binding would be weaker than to an isolated site, and there would be negative cooperativity. Binding to a doubly tandem site as in "C" would be described by $K\omega^2$. Binding is impossible if the gap between two bound ligands is less than the size of the binding site, as in "D." This last case illustrates a parking problem.

Saturation of the actin lattice with ligand is difficult when there is a parking problem, as the individual ligands must rearrange themselves to eliminate these gaps. An experimental way to minimize the parking problem is to mix the ligand with G-actin and initiate polymerization (*see* Fredricksen et al. 2003) ***(9)***. In that way the ligand can bind to the growing filament so that gap formation is unlikely. Obtaining full saturation and thus identifying the true number of protomers in a binding site, *n*, may be experimentally difficult when the value of *n* is large.

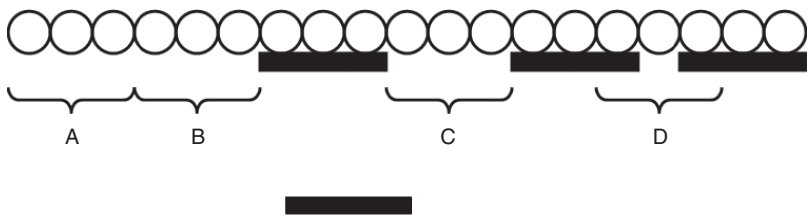

Fig. 2. Binding of a ligand (solid bar) where the binding site consists of three actin protomers: **(A)** binding to an isolated site; **(B)** binding to a singly contiguous site; **(C)** binding to a doubly contiguous site; **(D)** a gap too small for binding to occur.

Binding to an actin-like lattice of sites is correctly described by Equation 4 below ***(21)***. (*See* **Notes 9** and **10.**)

$$\frac{\theta}{L_F} = K \cdot (1-n\theta) \cdot \left(\frac{(2\omega-1)(1-n\theta)+\theta-R}{2(\omega-1)(1-n\theta)} \right)^{n-1} \cdot \left(\frac{1-(n+1)\theta+R}{2(1-n\theta)} \right)^{2} \quad (4)$$

Relationships of the binding curves to the parameters n and ω are shown in **Fig. 3**. Note that the ordinate of **Fig. 3** is theta*n (rather than theta) so that all curves reach a maximum of 1.0 for ease of comparison. The log of the concentration is shown so that the full binding profile can be examined. The curve with $n = 1$ is a simple S-shaped binding curve on the log plot and a hyperbola in the direct plot (see inset). Saturation of the actin filament becomes progressively more difficult as the value of n increases as a result of the parking problem. When $n = 7$, saturation is not reached even when the free ligand concentration is 10^4 times the dissociation constant.

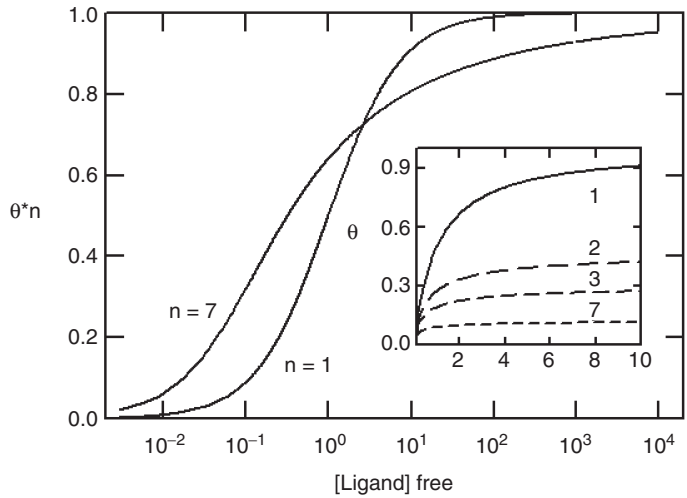

Fig. 3. Relationship of the binding curve to the number of actin protomers comprising a binding site. The ordinate is the product of theta (θ) and n so that both curves reach the same maximum value. Simulations used the Mc Ghee and von Hipple equation with values of K = 1 and ω = 1. Ligand concentration is on a log scale. Inset shows the low concentration region of binding. Data are plotted as theta (not theta*n) against the concentration (linear scale). Note that values of $n > 1$ give the appearance of negative cooperativity so that saturation is not reached even when the ligand concentration is 10^4 that of the binding constant, K.

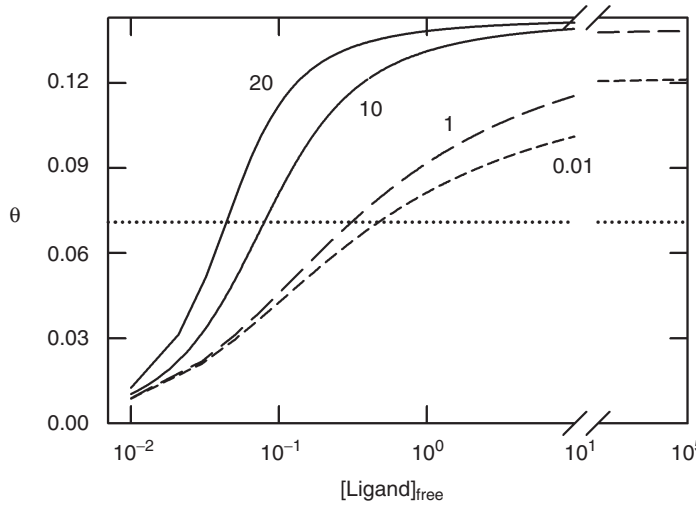

Fig. 4. Effect of the cooperativity parameter, ω, on the binding of a hypothetical ligand to actin having $n = 7$ and K = 1. Simulations used Equation 5. As in **Fig. 5**, the value of $n = 7$ caused saturation with the ligand to be difficult to achieve even at extreme ligand concentrations. However, the presence of positive cooperativity ($\omega > 1$) permits saturation to be achieved.

Positive cooperativity (negative free energy of interaction among adjacent ligands) can compensate for the parking problem. **Figure 4** shows a series of binding curves on a log plot each having K = 1 and $n = 7$ but with different values of the cooperativity parameter, ω. Values of $\omega < 1$ exaggerate the effect of the parking problem. However, when the value of ω is greater than 1, and particularly when $\omega > n$, the apparent negative cooperativity disappears.

An important point from **Figs. 3 and 4** is that it may be experimentally impossible to reach saturation with ligands having $n > 1$. This creates an ambiguity in the analysis because values of n and ω can compensate for each other and a unique fit may not be obtained. Another complication that can occur is that there may be different interactions of each part of a long ligand protein with each actin protomer. Some analytical approaches to this problem have been published earlier ***(9,22)***. Experimental approaches other than binding isotherms explored here are usually required in these cases.

3.3.3. Specific Model for Cooperative Binding of Myosin S1 to Actin-Tropomyosin

In the presence of tropomyosin or troponin-tropomyosin, the binding of S1-ADP or rigor S1 to actin exhibits apparent positive cooperativity. Plots of theta versus $S1_{free}$ are sigmoidal in the absence of Ca^{2+} (*see* Greene and Eisenberg, 1980) ***(17)***. The attachment of S1-ADP to actin-tropomyosin is thought to stabilize a conformation that binds more tightly to S1 ***(23)***. Two models that are based on somewhat different assumptions are commonly used to evaluate this special case. The Hill model describes the binding in terms of four parameters. K1 is the affinity of S1 to the inactive state of actin-tropomyosin. K2 is the affinity of S1 to the active state of actin-tropomyosin. Y is a cooperativity parameter. In the case of negative cooperativity $1 > Y > 0$, whereas for positive cooperativity $Y > 1$. The final parameter, L′, describes the tendency of an entire actin filament to exist in the inactive state in the absence of bound S1. The dependence of theta on the free ligand concentration, c, is given by the Hill relationship shown in Equation 5 below. Additional details about that model can be found elsewhere ***(24)***. (*See* **Note 11.**)

$$(5)\qquad \begin{cases} \theta = p_1 \dfrac{K_1 c}{1+K_1 c} + p_2 \dfrac{K_2 c}{1+K_2 c} \\ p_1 = \dfrac{2aY^{-1}}{\delta(a-1+\delta)} \qquad p_2 = \dfrac{2aY^{-1}}{\delta(1-a+\delta)} \\ \delta = \left[(1-a)^2 + 4aY^{-1}\right]^{1/2} \\ a = (1+K_2 c)^7 / L'(1+K_1 c)^7 \end{cases}$$

Figure 5 shows the effect of changes in L′ and Y on the simulated binding of a ligand (such as S1) to actin-tropomyosin-troponin. The plots are shown both on a direct scale and on a log scale for comparison to the other figures. A large value of L′ means that the inactive state is stabilized. **Figure 5** shows that as L′ increases, the free S1 concentration required to stabilize the active state increases (compare curves a, b, and c). Increasing the value of Y increases the sensitivity of the switch so that the transition from the inactive state to the active state occurs over a narrower range of free S1 concentrations. Curves c and d have the same value of L′ but d has a larger value of Y. Note that curve d rises more sharply than curve c.

An alternative description of the binding of S1 to actin-tropomyosin has been proposed by Geeves and co-workers. Details of this model have been described elsewhere ***(24,25)***.

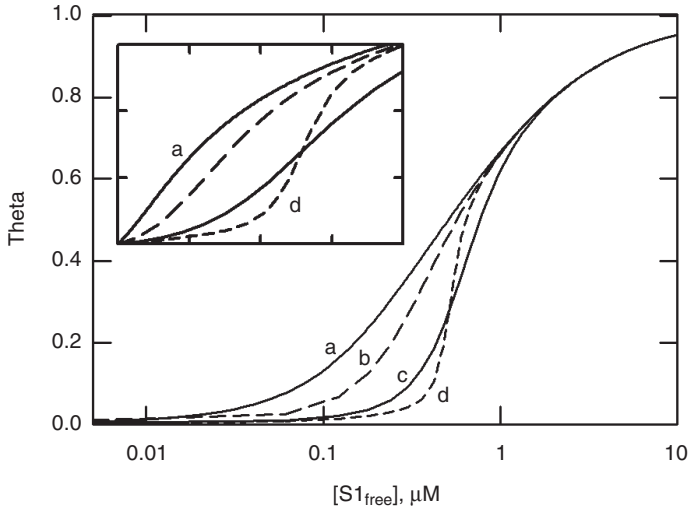

Fig. 5. Simulations of the Hill model for binding of S1 to actin. Each S1 binds to a single actin protomer. Values of theta are shown as a function of the free S1 concentration shown on a log scale. Inset shows the initial part of the curves plotted on a direct scale. The abscissa of the inset is from 0 to 0.8 μ*M* in steps of 0.2 μ*M*. The ordinate of the inset has values of theta from 0 to 0.6. Curves a through d are simulations with K1 = 0.1 and K2 = 2 μM^{-1}. Values of L′ and Y (L′, Y) varied: a (1,1), b (10,1), c (100,1), d (100,10).

4. Notes

1. All chemicals must be A.C.S certified reagent grade or better. Water must be distilled or otherwise purified. Proteins must be pure and the concentrations must be accurate. Most proteins are determined by absorbance measurements corrected for light scattering. Protein concentrations may also be measured by the Lowry assay with a bovine serum albumin standard. The Lowry assay is somewhat dependent on the amino acid composition of the protein and does not necessarily give the absolute protein concentration. Several methods are available for determining the absolute protein concentration directly. We have had success with the *o*-phthalaldehyde method *(26)*. One must hydrolyze the protein and compare the fluorescence of the product formed by reaction with *o*-phthalaldehyde with a standard curve made using alanine *o*-phthalaldehyde complex fluorescence as a standard.
2. The extinction coefficient for adenosine, AMP, ADP, and ATP is 15,400 $M^{-1}\,cm^{-1}$.
3. For added shielding, we slide the individual vials into blocks of acrylic plastic with dimensions 2 × 2 × 4 cm containing a hole of appropriate size to accommodate

the vial. For common cryogenic tubes we use holes 12–23 mm in diameter and 3.5 cm deep.

4. Use normal safety precautions when diluting strong acids.
5. Ethyl acetate or isobutanol alone are not as effective as isobutanol:benzene but may be used when safe handling conditions for benzene are not available.
6. An alternative method to study S1-ATP-"like" complexes is to modify S1 with ρ-phenylenedimaleimide to reduce the rate of ATP hydrolysis *(27,28)*.
7. The HCl in the quench stops the reaction while the excess Pi acts as a carrier for the ^{32}Pi. The silicotungstic acid solution precipitates the proteins. Molybdate forms a complex with Pi that is soluble in isobutanol:benzene. It is essential to extract the same fraction of ^{32}Pi in each assay.
8. Theta and free ligand concentrations can be determined from fluorescence measurements by the use of a spreadsheet as shown below. Let the initial volume of solution before addition of ligand be v_i.
   col(1): cumulative volume of ligand added.
   col(2): concentration of the ligand stock.
   col(3): total volume equal to v_i + col(1).
   col(4): total concentration of ligand = [col(1) × col(2)]/col(3).
   col(5): insert the raw fluorescence readings for each point of col(2).
   col(6): corrected fluorescence = col(5) × [col(3)/v_i].
   At this point determine the minimum (min) and maximum (max) values of col(6).
   col(7): normalized fluorescence = [col(6) − min]/[max–min].
   col(8): theta, moles of ligand bound/moles of actin total = col(7) × (1/number of actin protomers in a ligand-binding site). Note that if either the initial point or the endpoint is inaccurate the values of theta will be inaccurate. The assumption here is that the fluorescence reaches its maximum when the actin is saturated with ligand. It is a good idea to confirm that assumption with another type of measurement.
   col(9): bound ligand concentration = col(8) × c_A where c_A is the total actin concentration.
   col(10): free ligand concentration = col(4) − col(9).
   Now produce a plot of theta versus free ligand concentration or col(8) versus col(10). This is the curve that must be analyzed to obtain equilibrium binding parameters.
9. Equation 15 in the original article contains an error; the term $(2\omega + 1)$ should be $(2\omega - 1)$.
10. A sample program for analysis of Equation 4 is given here using the modeling program MLAB (Civilized Software, Bethesda, MD).
    /*This program fits the McGhee and von Hippel model to data pairs where column 1 is the free ligand concentration in micromolar units and column 2 is the value of theta. K is the association constant in micromolar. ww is used as the symbol for the cooperativity parameter in this routine. ww > 1 indicates positive cooperativity and 1 > ww > 0 indicates negative cooperativity. The program may fail with ww, = 1 but ww = 1.01 is generally fine. *n* is the number of actin protomers forming a single ligand-binding site. Note that the symbol v is used for theta in this subroutine.*/
    DELETE W

```
/*Make initial guesses for n, K, and ww. It is best to have good data at high ligand
concentrations so that the value of n is well constrained.*/
n = 4; K = 2; ww = 1.01
/*Create a data set of two columns with values of free ligand concentration (L)
in micromolar in column 1 and corresponding values of ligand bound/total actin
(v) in column 2. Name the file with a suffix .txt or .dat. The Read statement calls
the data file and defines it as having 36 rows and 2 columns.*/
DD=READ(dataname.txt,36,2)
CONC=DD COL(1)
/*Place reasonable limits on all values in the constraints statement.*/
CONSTRAINTS Q={K>0,K<100, ww >0, ww<100,n>4,n<8}
/*The McGhee and von Hipple equation is defined below. Each function is associ-
ated with a variable given in parentheses after the function name. The function
v(L) contains the ROOT operator that determines the roots of the equation
E(Z) – Z/L = 0. Z is a dummy variable used for theta in place of the v.*/
FUNCTION F(v) = 1-n*v
FUNCTION G(v) = v*(n+1)
FUNCTION R(v) = SQRT((1-G(v))*(1-G(v))+(4*ww*v*F(v)))
FUNCTION H(v) = ((2*ww-1)*F(v)+v-R(v))/(2*(ww-1)*F(v))
FUNCTION J(v) = (1-G(v)+R(v))/(2.0*F(v))
FUNCTION E(v) = K*F(v)*(H(v)^(n-1))*J(v)*J(v)
FUNCTION v(L) = ROOT(Z,0.001,1/n-0.001,E(Z)-Z/L)
/*The following statements define the range over which the functions are evaluated.
POINTNUM is the number of points you wish to include in the simulated curve.*/
STARTPOINT=0.5*MINV(CONC)
ENDPOINT=1.1*MAXV(CONC)
STEP=ENDPOINT/POINTNUM
/*The fit statement below defines which parameters are to be adjusted. FIT(K,
ww, n) allows all three variables to be adjusted while FIT(K, ww) allows only K
and ww to float.*/
FIT(K, ww, n), v TO D, CONSTRAINTS Q
/*The POINTS operator generates a matrix of points of theta and L using the
parameters obtained from the fit.*/
CV=POINTS(v,STARTPOINT:ENDPOINT:STEP)
/*The following statements produce a graph containing the original data, D, and
the fitted curve, CV.*/
DRAW D pt circle lt none color yellow
DRAW CV color yellow lt (.01,.0075,.013,0,0,.0075,0)
top title "McGhee & vonHippel Simulation" font 7 color yellow
bottom title "Free Ligand in microMolar" font 16
left title "Bound Ligand/Total Protein" font 16
frame color grey
imagebox color yellow
view
```

11. MLAB (Civilized Software, Bethesda, MD) routine for the Hill model.

```
/*The model of Hill, Eisenberg, and Greene for the binding of myosin S1 to actin-
tropomyosin or actin-tropomyosin-troponin. Concentrations are in units of μM
and association constants are in units of μM^-1.*/
DELETE W
STARTCONC=0.001
ENDCONC=5
STEPS=(ENDCONC-STARTCONC)/1000
/*Enter initial guesses for all parameters.*/
K1=0.1; K2=2; Y=1.01; LPRIME=1.01
/*Enter constraints or limits for the parameter values.*/
CONSTRAINTS Q = {Y>1,Y<1000,LPRIME>1,LPRIME<100,K2>0.1,K1>0}
/*The binding function is defined below.*/
FUNCTION A(C) = (1 + K2*C)^7/(LPRIME *((1+K1*C)^7))
FUNCTION S(C) = SQRT ( (1-A(C))^2 + 4*A(C)/Y )
FUNCTION P2(C) = 2*A(C)/Y /(S(C)* (1-A(C) + S(C)))
FUNCTION P1(C) = 1-P2(C)
FUNCTION THETA1(C) = K1*C/(1+K1*C)
FUNCTION THETA2(C) = K2*C/(1+K2*C)
FUNCTION THETA(C) = THETA1(C)*P1(C) + THETA2(C)*P2(C)
/*The following statement reads a set of data with 1000 rows and 2 columns.
Column 1 contains free S1 concentrations and column 2 contains corresponding
values of theta.*/
D1 = READ(dataname.txt,1000,2)
/*The following statement fits the Hill model to the data. Values in parentheses
are allowed to float. Fits are insensitive to K1 so the value of K1 is held
constant.*/
FIT(LPRIME,Y,K2), THETA TO D1, CONSTRAINTS Q
/*The following statement produces a matrix defining the best fit of the model to
the data.*/
cv1 = POINTS(THETA, STARTCONC:ENDCONC:STEPS)
/*The following statements produces plots of the data and of the fitted line
(D1).*/
DRAW D1, LINETYPE NONE, POINTTYPE CIRCLE color white
DRAW CV1, color white
/*The following statements define the plot.*/
top title "Fixed value of Y" font 7 color yellow
bottom title "[S1]free" font 16
left title "[S1]bound/[Actin]total" font 16
frame color grey
Frame 0.001 to 0.999, 0.001 to 0.999
framebox
imagebox color pink
view
```