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Assessing ATP binding and hydrolysis by NLR proteins

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Summary

Nucleotide-binding and leucine rich repeat domain-containing proteins (NLR) are central to the formation of many inflammasome complexes. Several inflammasome forming NLR proteins are known to be ATPases, but the nucleotide binding specificity of many remains to be characterized. The oligomerization of NLR proteins and assembly of inflammasomes require the ATP (or other nucleotide) binding activity of the NLR proteins. Quantitative and qualitative studies of the nucleotide binding properties of these proteins are useful tools in studying the regulation of inflammasome activity, and will be outlined in this Chapter.

Keywords

Nucleotide binding; leucine rich repeat domain-containing protein; ATPase; nucleotide binding

1. Introduction

NLR proteins were the first class of proteins recognized to function in assembling Caspase-1 activating complexes known as inflammasomes [1,2]. Since that original observation, other proteins have also been found to assemble inflammasome complexes [3]. The assembly of the inflammasome depends on the regulated oligomerization of the complex's primary scaffolding protein. In reported studies of NLR protein activity, the oligomerization of NLR proteins is dependent on their nucleotide binding activity [4,5].

NLRs have a conserved tripartite primary structure consisting of an amino-terminal effector domain, central nucleotide-binding domain, and carboxyl-terminal series of leucine rich repeats [6]. These structural elements are similar to a subfamily of proteins encoded by plant disease resistance genes (R genes), which generally have an alternative amino-terminal domain motif, and to the mammalian Apoptotic Protease Activating Factor-1 (APAF-1), which carries a series of carboxyl-terminal WD-40 motifs rather than leucine-rich repeats. The central, nucleotide-binding domain is homologous to the AAA-ATPase superfamily. Similar to observations with APAF-1 and plant R genes, nucleotide binding and/or hydrolysis appear to play an important regulatory role in NLR protein function [7–10].

Many NLR-encoding genes have strong genetic association with human diseases [11]. The importance of nucleotide binding in NLR protein function was further supported by the discovery that a number of highly penetrant, inherited, auto-inflammatory disorders are

associated with missense mutations in the nucleotide binding domains of these genes [12–17]. In particular, mutations in the gene encoding NLRP3 that lead to alterations in the nucleotide binding domain are associated with three dominantly inherited periodic fevers: Familial Cold Autoinflammatory Syndrome (FCAS), Muckle-Wells Syndrome (MWS), and Neonatal Onset Multisystemic Autoinflammatory Disease (NOMID) [16,18–21]. Patients with any of the three syndromes have elevated IL-1 β levels in their serum, which is thought to result from constitutive assembly of the NLRP3 inflammasome [22,23].

The role of nucleotide binding in NLR protein function has been explored in a handful of NLR proteins. These studies have demonstrated that most NLR proteins appear to be ATP or dATP binding proteins with two notable exceptions: 1) the MHC class II transcriptional activator (CIITA) was found to bind to GTP and its GTP binding activity controls trafficking of the protein into the nucleus and 2) all triphosphate nucleotides were found to support *in vitro* inflammasome assembly by NLRP1 [24,5]. Direct nucleotide binding studies by NLR proteins requires a source of purified protein. There are no published studies in which a native NLR protein has been purified from an endogenous source using affinity or conventional chromatography. Isolated NLR protein nucleotide-binding domains have been expressed as fusion proteins with Glutathione-S-Transferase (GST) and Maltose Binding Protein (MBP) in *E. coli* expression systems and can be purified to near homogeneity [25,26]. Further, the purification of affinity-tagged, recombinant, full-length NLR proteins has now been reported using a variety of mammalian and insect cell expression systems [4,5,27,28]. Both isolated nucleotide-binding domains and purified full length proteins can be used for studies of nucleotide binding and hydrolysis. We show the partial purification of NLRP3 using tandem affinity tag purification strategy that we recently described for NOD2 (*see* Figure 1) [28].

Many assays for studying protein-nucleotide interactions are now available, where nucleotide ligands can be modified with either radioactive isotopes (most commonly ^3H , ^{32}P , and ^{35}S) without altering their chemical structure or with small molecule labels (biotin or fluorescent moieties) through chemical linkers. Fluorescence anisotropy, which measures changes in molecular tumbling that result from a fast tumbling small molecule (like a fluorescently labeled nucleotide analog) binding to a large slower tumbling molecule (like a protein), has previously been used to characterize nucleotide binding by NLRP1 and NOD1 [5,27]. We have utilized radiolabeled nucleotide ligands to study nucleotide-binding properties of NLR proteins, including NLRP3, NLRP12, and NOD2 [26,4,28]. Herein, we review the methodology for three assays: 1) binding of a non-hydrolyzable ATP analog, Adenosine-5'-O-(3-thiotriphosphate) or ATP γS ; 2) binding of a photo-activated ATP analog; and 3) steady-state ATPase activity assessed by measuring release of the terminal orthophosphate from ATP.

2. Materials

2.1 Purified NLR proteins

There is not a consensus, generalizable methodology for NLR protein isolation. Expression of NLR proteins fused with two different affinity tags in insect cell culture using

recombinant baculovirus followed by tandem affinity purification has been particularly successful in our hands [4,28] (*see Note 1*).

1. NLR protein storage buffer: 50 mM NaHEPES, pH 7.5, 500 mM NaCl, 1 mM DTT, and 0.1% (w:v) CHAPS.

2.2. Measurement of Adenosine-5'-O-(3-thiotriphosphate) (ATP γ S) binding by NLR proteins

1. House vacuum system or vacuum pump (*see Note 2*).
2. Laboratory bench and fume hood designated for use of radioactivity materials (*see Note 3*).
3. Portable heat lamp.
4. Liquid scintillation counter.
5. Adjustable temperature water bath.
6. 10 place vacuum manifold with 25 mm filter holder (Hoefer, San Francisco, CA).
7. Varipet Syringe Dispenser, 10 mL capacity adjustable repeating syringe dispenser (Bel-Art Products, Wayne, NJ)
8. MF-Membrane HA filter (0.45 μ m pore size, 24 mm diameter, Millipore).
9. [35 S]-ATP γ S (1250Ci/mmol, PerkinElmer, Waltham, MA).
10. 5 mL plastic liquid scintillation vials.
11. ScintiSafe Econo 2 cocktail (Fisher Scientific) or other liquid scintillation fluid
12. 2x ATP binding buffer: 100 mM Tris-HCl, pH 7.5, 300 mM NaCl, 4 mM DTT, 50 mM MgCl₂ and 10% Glycerol (*see note 4*).
13. Wash buffer: 20 mM Tris-HCl, pH 7.5, 120 mM NaCl, 25 mM MgCl₂ and 0.05% Triton X-100.
14. 9" $\times$ 9" pyrex pan.
15. 15 mL conical screw cap tubes.
16. 1.5 mL microfuge tubes.
17. Aluminum foil.

2.3. Photo-affinity labeling of NLR proteins with 8-azido-ATP analogs

1. UV light (254 nm).
2. Savant SpeedVac (Thermo Scientific) or other centrifugal evaporator system.
3. FluorChemE multimode, CCD based imaging system (Protein Simple, Santa Clara, CA)
4. 8-N₃-ATP[γ]biotinpentylamine (Affinity Photoprobes, Lexington, KY).
5. Cross-linking buffer: 50 mM Tris-HCl, pH 7.5, 0.1% CHAPS, 150 mM KCl and 5% glycerol.

6. 1x TBST: 20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.5% (v:v) Tween-20.
7. Blocking Buffer: 5% (w:v) dry milk in 1x TBST.
8. Streptavidin-Horse Radish Peroxidase conjugate (G.E. Healthcare).
9. SeeBlue® Plus2 Protein size markers (Invitrogen, Life technologies) or other protein size markers.
10. Precast NuPAGE® 4–12% (1.0 mm) Bis-Tris gel and NuPAGE® MOPS running buffer (Invitrogen, Life Technologies)
11. NuPAGE® SDS-PAGE gel running apparatus and power supply (Invitrogen, Life Technologies).
12. Nitrocellulose iBlot® transfer stacks and iBlot® gel transfer device (Invitrogen, Life Technologies).
13. SuperSignal West Pico Chemiluminescent Substrate (Pierce, Thermo Scientific).

2.3. Measurement of steady state ATPase activity of NLR proteins

1. Laboratory bench and fume hood designated for use of radioactivity materials (*see note 3*).
2. Activated charcoal (Sigma-Aldrich).
3. [γ - 32 P]-ATP, 3000Ci/mmol (PerkinElmer).
4. Liquid scintillation counter.
5. 5 mL plastic liquid scintillation vials (RPI, Mt. Prospect, III).
6. Scintillation liquid: ScintiSafeEcono 2 LSC-cocktail (Fisher Scientific).
7. Water bath.
8. Microcentrifuge.
9. 1.5 mL microfuge tubes.
10. 100 mL graduated cylinder.
11. Parafilm.
12. Acidic phosphate solution: 100 mM HCl/100 mM Na₂HPO₄. Weigh 13.7 g Na₂PO₄·7H₂O, add to a beaker containing 400 mL dH₂O. Stir to dissolve the chemical and add 4.2 mL concentrated HCl to the solution. Continue to stir. Make up to 500 mL with dH₂O (*see note 5*).
13. 1x ATP binding buffer specified in step 12 of section 2.2.

3. Methods

3.1 Measurement of Adenosine-5'-O-(3-thiotriphosphate) (ATP γ S) binding by NLR proteins

In this assay, purified NLR protein mixtures of proteins containing NLR proteins are incubated with a radiolabeled, non-hydrolyzable analog of ATP, [35 S]-ATP γ S. After the

binding reaction is complete, the protein bound nucleotide is isolated from the free nucleotide by filtering the solution through a protein-binding nitrocellulose filter. The filter-associated label is then detected using liquid scintillation counting. Variations of this assay can be performed to assess the relative nucleotide binding stoichiometry of different proteins (or preparations of proteins), the relative preference of a particular NLR protein for a nucleotide base by including titrations of competitor nucleotides, or relative rates of association or dissociation of an NLR protein from ATP γ S (*see* Figure 2). Additionally, the optimal buffer pH, NaCl concentration, and Mg²⁺ ion concentration may vary from protein to protein. We recommend optimizing these when exploring ATP γ S-binding activity of uncharacterized NLR proteins. The description of the assay that follows reflects the first of these assays, a comparison of relative nucleotide binding by different NLR containing protein preparations. The assay is carried out at a single concentration of ATP γ S for a fixed time of binding (a time that has previously been demonstrated achieves maximum binding for NLRP3). In the described assay, we compare the ATP γ S binding of NLRP3 at 4 stages of protein preparation (Figure 1 and 3).

1. Determine the number of filters required for the assay (*see note 6*):
 - a. 3 for counting total CPM in the binding reactions. +
 - b. 3 for determining nonspecific filter binding (no protein control) +
 - c. $3 \times \#$ of samples or time points to be tested
2. Soak MF membrane filters in Wash Buffer, ~200 mL buffer in 9" $\times$ 9" pyrex pan. To wet membrane evenly, place filters in pan and rock or shake slowly for at least 30 min at room temperature (*see note 7*).

While membranes are soaking, set up binding reactions:

3. Thaw and prepare 1 μ L diluted [³⁵S]-ATP γ S for each filtered reaction. Dilute [³⁵S]-ATP γ S 1:16 in 2x ATP binding buffer. Use 1 μ L of the diluted isotope in each 100 μ L binding reaction, yielding a final concentration of 5 nM [³⁵S]-ATP γ S in the reaction mix.
4. Turn on water bath and set temperature to 30 °C.
5. Prepare 2x reaction mix (2x ATP binding buffer with [³⁵S]-ATP γ S). Place 49 μ L 2x binding buffer $\times$ (# of prepared filters) in 15 mL conical screw cap tube (for a low number of binding reactions a 1.5 mL microfuge tube can be substituted) and add 1 μ L $\times$ (# of prepared filters) diluted [³⁵S]-ATP γ S (*see note 8*).
6. Label three 1.5 mL microfuge tubes for each sample to be tested and three additional tubes for a no protein control.
7. Add 50 μ L of 2x binding buffer with [³⁵S]-ATP γ S to each reaction tube.
8. Dilute protein samples being tested for assay. We typically use ~100 ng purified NLR protein for each binding reaction. We dilute purified protein preparations to 2 ng/ μ L in NLR Protein Storage Buffer (*see note 6*).

9. At timed intervals add 50 μ L protein solution (or Protein Storage Buffer) into the appropriate reaction tube, vortex and place reaction in a 30°C water bath. Start all reactions for one sample type in 40 second intervals then wait 2 min and initiate the next reaction. Allow the binding reaction to proceed 60 minutes. See Table 1 for starting and completion time example for assay run to generate Figure 3. (*see Notes 9 & 10*)
10. Approximately 5 min before the end of the hour, turn on the vacuum for the filtration system and close the individual valves for each filter holder spot and prepare to process the reactions:
11. Lay three wet filter membranes on filter support grids of the vacuum manifold and place manifold chimneys in place over the filters (*see Note 11*)
12. Add 4 mL ice-cold 1x ATP binding buffer to filter each holder (*see Note 12*).
13. Add 100 μ L (all) of the reaction mixture to the buffer in a filter holder and open the filter valve to begin the filtration of that sample.
14. Repeat the transfer of reaction mixtures to filter holders for all three reactions of a single sample at 20 s intervals.
15. Wash 3x by immediately adding 8 mL wash buffer to each holder after all buffer passes through the membrane using the Varipet Syringe Dispenser (for a total of 8 mL $\times$ 3) (*see Note 13*).
16. Turn off vacuum and remove filter membranes from the filtration system to a 20 cm $\times$ 20 cm sheet of aluminum foil.
17. Repeat steps 11–16 of section 3.1, for each reaction sample.
18. To determine the total cpm placed in each reaction and the specific activity (cpm/pmol) of the labeled [35 S]-ATP γ S, run a set of three filters through steps 11–16 skipping the addition of reaction (step 13).
19. Dry membranes thoroughly using a heat lamp (*see Note 14*).
20. For the 3 filters prepared in step 18, pipette 2.5 μ L of label containing 2x binding buffer (remaining from step 5) onto each filter and allow filters to completely dry. (The cpm detected on these filters represents 1/20 the total placed in the reaction.)
21. Put the filters into 5 mL scintillation vials and add 5 mL scintillation liquid.
22. Count using a liquid scintillation counter using a program suitable for 35 S detection.

3.2 Photo-affinity labeling of NLR proteins with 8-azido-ATP analogs

Most NLR proteins are not efficiently purified to homogeneity and ATP γ S binding stoichiometries tend to be a fraction of the expected number of molecules of NLR protein in the assay described above, thus, a secondary assay demonstrating that the NLR protein in the assay is responsible for the detected ATP binding is recommended. Photo-affinity

labeling with an ATP-analog like 8-N₃-ATP[γ]biotinpentylamine allows visualization of the ATP-analog-associated protein after separation by SDS-PAGE. This method allows confirmation of ATP association with the expected protein in the preparation.

1. Place 2 μ L of 50 μ M 8-N₃-ATP[γ]biotinpentylamine into a 1.5 mL microfuge tube.
2. Place the tube in SpeedVac or other brand of vacuum assisted centrifugal concentrator, dry for 10 min (*see Note 15*).
3. Add 25 μ L cross-linking buffer to the tube to dissolve 8-N₃-ATP[γ]biotinpentylamine.
4. Add 25 μ L of protein-containing solution (between 300–600 ng of protein in the reaction) and mix thoroughly.
5. Irradiate samples for 3 min with UV light (254 nm). We do this by placing open tubes in a UV crosslinking apparatus (UV StrataLinker 1800, Stratagene).
6. Add 15 μ L 4 $\times$ NuPAGE loading buffer and 6.6 μ L 10x reducing agent to the reaction and incubate in a 95°C heat block for 5 min (*see Note 16*).
7. Resolve the protein on NuPAGE® 4–12% Bis-Tris gel run in MOPS running buffer according to the manufacturers protocol.
8. Transfer the proteins to nitrocellulose according to the manufacturers protocol.
9. Incubate the membrane in blocking buffer at room temperature for 1 h with shaking.
10. Dilute Streptavidin-Horse Radish Peroxidase conjugate 1:1500 in blocking buffer, (e.g., 10 μ L in 15 mL).
11. Discard the blocking buffer on membrane and replace it with the diluted Streptavidin-Horse Radish Peroxidase conjugate.
12. Incubate for 2 h at room temperature with shaking.
13. Discard the conjugate antibody and replace with 15 mL TBST
14. Incubate for 10 min at room temperature with shaking.
15. Repeat steps 11–14 of section 3.2, three additional times for a total of 4x 10 min washes.
16. Prepare SuperSignal West Pico chemiluminescent substrate by mixing 500 μ L of each component.
17. Discard the last TBST wash and add 1 mL of chemiluminescent substrate to surface of the membrane.
18. Allow detection reaction to proceed at room temperature for 3 min.
19. Visualize ATP-biotin bound proteins using FluorEChem CCD imaging system set for luminescent image capture or by X-ray film exposure.

3.3 Measurement of steady state ATPase activity of NLR proteins

Measurement of ATP hydrolysis by NLR proteins can be assessed by measurement of released ^{32}P orthophosphate from $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ substrate. The reaction is stopped by addition of a phosphoric acid/activated charcoal solution. ATP (and other nucleotides bind to activated charcoal under these conditions while the orthophosphate product is free, allowing for separation of the substrate and product. Steady state hydrolysis is dependent on the affinity of the NLR protein for substrate, the concentration of NLR and substrate in the reaction, the rate of catalysis, and the rate of product/substrate exchange. By utilizing substrate concentrations several orders of magnitude greater than the K_m of the protein for ATP, the rate limiting steps of the reaction are the catalytic rate and exchange rates. This reaction can initially be performed across a range of substrate concentrations to define the K_m for any given NLR protein and to define the concentration of ATP to be used in future studies. The exchange rate is rapid for the only proteins of this family in which this has been measured and may be a point of regulation in the biological action of NLR proteins. Therefore, NLR protein steady state ATPase activity may be a useful assay in identifying the biochemical activity of NLR protein regulatory factors.

3.3.1 De-fining Charcoal solution—In this assay, activated charcoal is used to separate nucleotide-associated ^{32}P ($[\gamma\text{-}^{32}\text{P}]\text{-ATP}$), which binds activated charcoal, and released $[\text{}^{32}\text{P}]\text{-orthophosphate}$. The following procedure is used to prepare de-fined charcoal stop solution (*see Note 17*):

1. Place ~25 ml (packed powder volume) of activated charcoal powder in a 100 mL graduated cylinder.
2. Add ~75 mL (three times charcoal volume) of acidic phosphate solution. Cover the cylinder with parafilm and mix by inversion.
3. Let the cylinder sit at room temperature for 10 min to allow the charcoal to settle.
4. Carefully decant the liquid, which contains small charcoal particles (it is ok to lose a little charcoal powder).
5. Repeat steps 2–4 of section 3.3.1, five times. If small particles remain visible in the decanted solution, repeat an additional 5 times.
6. Estimate the volume of remaining charcoal powder in the cylinder and add acidic phosphate solution to the charcoal to reach the ratio of 3:1 (v/v). This charcoal stop solution is now ready for use.

3.3.2 Measuring ATPase activity

1. Dilute $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ 1/200 in 2x ATP binding buffer (*see Note 18*).
2. Make a 2x reaction buffer master mix: for each planned reaction time point, add 48 μL of 2x ATP binding buffer, 1 μL of diluted $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$, and 1 μL of unlabeled ATP (100 μM , *see Note 19*).
3. Dilute proteins to be tested to appropriate concentration (40 – 160 ng/ μL) in their normal storage buffer.

4. Prepare two 1.5 mL microfuge tubes with 50 μL of diluted protein/time point to be studied. (250 μL for the assay conditions outlined in step 2).
5. Prepare two 1.5 mL microfuge tubes with 100 μL of protein storage buffer to be used as no protein control at the first and last time point.
6. Prepare stop reaction tubes (one for each time point to be studied, 14 for the experiment outlined above in step 2): 1.5 mL microfuge tube with 500 μL defined, phosphoric acid charcoal suspension (*see Note 20*).
7. Place stop reaction tubes in a rack with lids open.
8. Initiate each reaction at 30 s intervals by pipetting a volume equal to the volume of protein solution (or no protein buffer control) of 2x reaction buffer, mix briefly in a vortex mixer, then place the reactions in a 30°C water bath.
9. At $t = 5$ min, remove 100 μL of each reaction (protein containing reactions and no protein controls) and add immediately to one stop reaction tube containing acidified charcoal at 30 s intervals.
10. Close the stop reaction tube lid and mix with a single flick.
11. Once all 5 min time point samples have been stopped in charcoal, quickly vortex each sample.
12. Repeat steps 9–11 for only the protein containing samples at the following times: $t = 15$ min, 25 min, and 35 min.
13. Repeat steps 9–11 for both protein containing samples and no protein control at $t = 65$ min.
14. Centrifuge all stopped reactions at $13,000 \times g$ for 5 min.
15. Remove 120 μL of each supernatant to a scintillation vial for counting.
16. Add 5 mL scintillation cocktail and count on program appropriate for detection of ^{32}P (total cpm released = $5 \times$ cpm in scintillation vial).

3.3.3. Determining the specific activity of ATP in the reaction

1. Prepare two stop reactions tubes (as described in step 6 of section 3.3.2) and add an additional 100 μL H_2O to each tube.
2. Centrifuge each tube in microfuge for 5 min at $13,000 \times g$.
3. Transfer 120 μL of supernatant from each tube into separate scintillation vials.
4. Dilute 2 μL of the reaction buffer master mix in 198 μL H_2O .
5. Spike 10 μL of diluted reaction buffer master mix from 3.3.3 step 4 (containing a total of 200 fmol ATP) into each scintillation vial, add 5 mL scintillation fluid, and count the vials on a program appropriate for detection of ^{32}P . The specific activity of $[\gamma\text{-}^{32}\text{P}]\text{-ATP}$ is calculated as mean CPM from two vials/200 fmol.
6. Analyze data by plotting the release of ^{32}P against time (*see Note 21*).

4. Notes

1. The quantity of proteins utilized in these assays is based on our recent experience with the NLR protein NOD2 [28]. Depending on the system utilized to express and isolate the protein, there may be great variability in the quantity of protein available and the specific ATP binding of the proteins in those preparations.
2. Because the filtered solutions will contain radioactive waste, ensure the vacuum system is appropriately isolated from the house vacuum using filters and waste flasks.
3. Utilize β emitting isotopes (^{35}S and ^{32}P) only in appropriately approved laboratory areas in compliance with your institution's regulations regarding safe handling of radioactivity. Utilize adequate plexiglass based shielding and appropriate personal safety protection.
4. We recently optimized these buffer conditions including pH, NaCl concentration, and MgCl_2 concentration for NOD2. For investigations which will assess NLR proteins for which no conditions have been published, these parameters may require pilot testing to identify optimal conditions.
5. Use normal safety precautions when diluting strong acids.
6. The assay depicted in Figure 3 required 18 filters.
7. We have not noted a change in assay performance for filters soaked as long as 2 hours
8. For example, if testing binding of 4 protein preparations, as in Figure 3, one would mix 882 μL of 2x ATP binding buffer + 18 μL diluted [^{35}S]-ATP γS
9. If pilot experiments indicate the buffering and/or salt concentrations for the protein storage or optimized ATP γS binding are greatly different from those reported for this assay, the content of the 2x binding buffer may be adjusted to achieve the desired reaction conditions.
10. Precise timing of reaction initiation and stopping the reaction are not required when incubation time is extended beyond when maximum binding is achieved under the conditions of the assay (i.e. when equilibrium binding has been achieved).
11. To avoid leaking, you need to align membrane very well with the support grid, and ensure that when the chimney is placed the entire circumference of the filter is in contact with the chimney.
12. Background ^{35}S binding to the filters is erratic if reaction mixtures are pipetted onto drying filters, it is important the reaction is diluted into a significant volume of wash then filtered immediately.
13. Keep the wash buffer bottle on ice in an ice bucket to keep the buffer chilled.
14. Variability in the moisture content of filters will lead to variable quenching during scintillation counting.

15. 8-N₃-ATP[γ]biotinpentylamine comes dissolved in methanol which might denature active NLR proteins if used without drying.
16. We routinely utilize the Invitrogen NuPAGE and iBlot systems for protein separation and immobilization on nitrocellulose. It is expected that other SDS-PAGE systems and electrophoretic transfer protocols can be used with similar efficiency.
17. Charcoal dust or fines are present in commercially available activated charcoal powder and will not readily pellet with centrifugation. This leads to suspended ³²P-labeled nucleotide contaminating the ortho-phosphate containing supernatant and unreasonably high background counts in the ortho-phosphate release assay.
18. This dilution typically yields enough cpm/reaction to allow reasonable detection (but this will vary with the age of the isotope). Liquid scintillation counting of the reaction mixture is required to determine the specific activity of the substrate in this assay.
19. We typically use a 5-point time course and run each reaction in duplicate. We also include a no protein control reaction run at the first and last time point also run in duplicate. This experimental set up requires 700 μL of 2x reaction buffer master mix.
20. Mix charcoal suspension well by inverting the bottle several times before time pipetting suspension or placing the bottle on a stir plate with constant stirring while pipetting contents. Also, cut off 2–3 mm of a 1 mL pipette tip to facilitate pipetting charcoal suspension.
21. If the data reasonably fits to a straight line, the slope of the line represents the steady rate of hydrolysis. If substrate is exhausted in this assay, the release of [³²P]-orthophosphate will slow down at later times as the concentration of substrate drops below ten times the K_m. If this is the case, the data will not fit linear equation well as the slope shifts towards horizontal.

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Figure 1. Partial purification of recombinant NLRP3 for ATP binding studies

Sf9 cells were infected with recombinant baculovirus expressing Halo-NLRP3-6xHIS, as previously described for expression of recombinant NOD2 [28]. The cells were lysed (**Lys**) and Halo-NLRP3-6xHIS was enriched by elution from an immobilized metal chromatography resin (**Ni(1) E**). The enriched Halo-NLRP3-6xHIS was captured using Halo-ligand chromatography (Promega) and NLRP3 free of the Halo and 6xHIS tags was eluted by cleavage with recombinant 6xHIS-TEV protease (**Halo E**). TEV protease was removed from the preparation by passing the recombinant NLRP3-containing through a second immobilized metal affinity chromatography resin (**Ni(2) FT**). 25 ng of protein from each stage of purification (lanes as indicated) was subjected to SDS-PAGE and total protein was visualized with silver staining. The location of Halo-NLRP3-6xHIS and NLRP3 are marked with arrows. The location of 6xHIS-TEV protease is indicated by and asterisk (*) and contaminating proteins in the preparation are noted by the hash symbol (#).

Figure 2. Experimental outlines for studies of NLR proteins utilizing ATP γ S filter binding method

(A) Comparison of ATP binding stoichiometry between protein preparations: binding reactions for each protein to be compared are set up in parallel and allowed to proceed to equilibrium prior to measurement. (B) Comparison of inhibitors (small molecule inhibitors, potential regulatory proteins, or competitive binding with different nucleotides) of NLR protein nucleotide binding: inhibitors are mixed with [35 S]-ATP γ S prior to starting binding reaction. (C) Rate of ATP γ S binding to NLR proteins: NLR protein(s) is added to [35 S]-ATP γ S binding reaction and aliquots of the reaction are removed at specified time points and analyzed for bound [35 S]-ATP γ S. (D) Rate of ATP γ S disassociation from NLR proteins: the NLR protein is incubated with [35 S]-ATP γ S until equilibrium is reached, an excess of unlabeled-ATP γ S (usually 100 to 1000 fold) is added, and NLRP3 bound [35 S]-ATP γ S is measured at specified time points after addition of unlabeled nucleotide.

Figure 3. [35 S]-ATP γ S binding by recombinant NLRP3 at different stages of purification

100 ng of protein from each stage of purification indicated in Figure 1 was incubated with [35 S]-ATP γ S for 60 minutes as indicated in the text. The amount of protein bound [35 S]-ATP γ S was assessed for each preparation. The bars indicate mean of triplicate binding reactions and error bars show + S.D.

Table 1

Experimental layout for testing [^{35}S]-ATPYS-binding capacity of recombinant NLRP3 at various stages of purification shown in Figure 1.

Tube #	Sample	Start time	Stop time
1	No protein (protein storage buffer)	0:0:0	1:00
2	No protein (protein storage buffer)	0:0:20	1:00:20
3	No protein (protein storage buffer)	0:0:40	1:00:40
4	NLRP3-expressing Sf-9 lysate	0:2:0	1:02
5	NLRP3-expressing Sf-9 lysate	0:2:20	1:02:20
6	NLRP3-expressing Sf-9 lysate	0:2:40	1:02:40
7	Ni ²⁺ elution	0:4:0	1:04
8	Ni ²⁺ elution	0:4:20	1:04:20
9	Ni ²⁺ elution	0:4:40	1:04:40
10	Halo elution	0:6:0	1:06
11	Halo elution	0:6:20	1:06:20
12	Halo elution	0:6:40	1:06:40
13	$^{2\text{cd}}\text{Ni}^{2+}$ FT	0:8:0	1:08
14	$^{2\text{cd}}\text{Ni}^{2+}$ FT	0:8:20	1:08:20
15	$^{2\text{cd}}\text{Ni}^{2+}$ FT	0:8:40	1:08:40