Video Article

Biochemical Assays for Analyzing Activities of ATP-dependent Chromatin Remodeling Enzymes

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Abstract

Members of the SNF2 family of ATPases often function as components of multi-subunit chromatin remodeling complexes that regulate nucleosome dynamics and DNA accessibility by catalyzing ATP-dependent nucleosome remodeling. Biochemically dissecting the contributions of individual subunits of such complexes to the multi-step ATP-dependent chromatin remodeling reaction requires the use of assays that monitor the production of reaction products and measure the formation of reaction intermediates. This JOVE protocol describes assays that allow one to measure the biochemical activities of chromatin remodeling complexes or subcomplexes containing various combinations of subunits. Chromatin remodeling is measured using an ATP-dependent nucleosome sliding assay, which monitors the movement of a nucleosome on a DNA molecule using an electrophoretic mobility shift assay (EMSA)-based method. Nucleosome binding activity is measured by monitoring the formation of remodeling complex-bound mononucleosomes using a similar EMSA-based method, and DNA- or nucleosome-dependent ATPase activity is assayed using thin layer chromatography (TLC) to measure the rate of conversion of ATP to ADP and phosphate in the presence of either DNA or nucleosomes. Using these assays, one can examine the functions of subunits of a chromatin remodeling complex by comparing the activities of the complete complex to those lacking one or more subunits. The human INO80 chromatin remodeling complex is used as an example; however, the methods described here can be adapted to the study of other chromatin remodeling complexes.

Video Link

The video component of this article can be found at https://www.jove.com/video/51721/

Introduction

SNF2 family chromatin remodeling complexes include a central SNF2-like ATPase subunit [1,2]. Some SNF2-like ATPases function as single subunit enzymes, while others function as the catalytic subunit of larger multi-subunit complexes. Elucidating the molecular mechanisms by which each of the subunits of chromatin remodeling complexes contribute to their activities requires the ability to perform biochemical assays that dissect the remodeling process.

ATP-dependent nucleosome remodeling by the human INO80 complex and other chromatin remodeling enzymes can be envisioned as a multi-step process that starts with binding of the remodeling enzyme to nucleosomes, followed by activation of its DNA- and/or nucleosome-dependent ATPase, translocation of the remodeling enzyme on nucleosomal DNA, and eventual repositioning of nucleosomes [1,2]. Understanding the molecular details of the ATP-dependent chromatin remodeling process requires dissection of the remodeling reaction into its individual steps and definition of the contributions of individual subunits of the chromatin remodeling complex to each step of the reaction. Such analyses require the ability to analyze nucleosome remodeling and other activities using defined molecular substrates *in vitro*.

In a previous JOVE protocol, we described procedures used to generate INO80 chromatin remodeling complexes and subcomplexes with defined subunit compositions [3]. Here, we present three biochemical assays that enable quantitative analysis of the nucleosome binding, DNA- and nucleosome-activated ATPase, and nucleosome remodeling activities associated with such complexes.

Protocol

1. ATP-dependent Nucleosome Remodeling Assays

To measure ATP-dependent nucleosome remodeling activities, immunopurified INO80 or INO80 subcomplexes are incubated with ATP and a mononucleosomal substrate, which contains a single nucleosome positioned at one end of a 216-bp, ^{32}P-labeled DNA fragment. The reaction products are then subjected to electrophoresis in native poly-acrylamide gels.

1. To generate the ^{32}P-labeled, '601' DNA fragment, amplify from pGEM-3Z-601[4] a 216 bp DNA fragment containing an end-positioned 601 nucleosome positioning sequence, using the oligonucleotides 5′-ACAGGATGTATATATCTGACACGTGCCTGG and 5′-AATACTCAAGCTTGGATGCCTGCAG as forward and reverse primers.
    1. Set up a 100 µl PCR reaction as described in **Table 1**.
    2. Perform the PCR reactions in a thermal cycler using the following program: 1 min at 96 °C, followed by 45 sec at 94 °C, 30 sec at 57 °C, 60 sec at 72 °C for 30 cycles; ending with 7 min at 72 °C.
    3. After the PCR reaction is finished, remove the unincorporated nucleotides by passing the reaction products twice through nuclease-free spin columns.
    4. Run 5 µl of the purified PCR product in a 1.2% agarose gel to confirm that the PCR reaction generated the desired ~216 bp DNA fragment.
    5. Dilute 5 µl of the purified PCR product 20-fold, and measure the DNA concentration using a UV spectrophotometer. The average yield is ~40 ng/µl.
    6. Measure the radioactivity of 1 µl of the purified PCR product in a scintillation counter. Estimate the labeling efficiency by calculating the cpm/ng. A successful labeling reaction is expected to yield ~15,000 cpm/ng of 601 DNA fragment.

2. Transfer Hela cell nucleosomes onto the labeled 601 DNA using a serial dilution method [5].
    1. Mix 2 pmol of ^{32}P-labeled 601 DNA fragment with 6 µg of Hela nucleosomes (prepared as described [5]) in 50 µl of a buffer containing 1.0 M NaCl, 10 mM Tris-HCl, pH 8.0, 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT and incubate at 30 °C for 30 min.
    2. Sequentially dilute the mixture to 0.8 M, 0.6 M, and 0.4 M NaCl by dilution with 12.5 µl, 20.8 µl, and 41.6 µl, respectively, of 10 mM Tris-HCl (pH 8.0), 1 mM EDTA, 0.1 mM PMSF, and 1 mM DTT, with a 30 min incubation at 30 °C after each dilution.
    3. Further dilute the mixture to 0.2 M and then to 0.1 M NaCl by addition of 125 µl and then 250 µl of the same buffer containing 0.1% Nonidet P-40, 20% glycerol, and 200 µg/ml BSA, with a 30 min incubation at 30 °C between the final two dilutions. Store the mononucleosome substrate at 4 °C for up to 3 months.

3. Perform ATP-dependent nucleosome sliding reactions in a total volume of 10 µl. The reaction components are listed in **Table 2**. Since the optimal NaCl concentration for INO80 nucleosome remodeling activity is ~50 mM NaCl (unpublished results), adjust the total concentration of NaCl in each reaction to 50 mM, taking into account the amount of NaCl contained in preparations of nucleosomes and enzyme.
    1. Before beginning to set up the assays, cast native poly-acrylamide gels (18 x 16 cm). To prepare a single gel containing 5% acrylamide (acrylamide:bisacrylamide 37.5:1), 0.5x TBE (45 mM Tris borate, 1 mM EDTA), 0.01% ammonium persulfate (APS), and 0.001% *N,N,N´,N´*-tetramethylethylenediamine (TEMED), mix the ingredients listed in **Table 3**. Allow the gel to polymerize for at least 2 hr at RT.
    2. Meanwhile, for each reaction to be performed, combine in a pre-chilled siliconized 1.5 ml microcentrifuge tube ~20 nM INO80 or INO80 subcomplex (prepared as described [3]) with an amount of EB100 buffer (**Table 4**) sufficient to give a volume of 4.75 µl. Immediately re-freeze any remaining INO80-containing fractions in powdered dry ice or liquid nitrogen.
    3. Set up a master cocktail with the rest of the ingredients, scaling up by a factor of 'X' (where X = total number of reactions +3). The amount of each ingredient needed for a single 10 µl reaction is listed in **Table 5**; the recipe should be scaled up according to the number of reactions to be performed. Mix well by tapping the tube or by pipetting up and down with a Pipetman and spin the tube for a few seconds in a benchtop microcentrifuge.
    4. Dispense 5.25 µl of the master cocktail to each of the reaction tubes set up in step 1.3.2. Mix well by pipetting up and down. Start the reactions by transferring reaction tubes to a 30 °C heat block or water bath and incubate for 2 hr.
    5. Meanwhile, prepare 'removing mix' cocktail containing competitor DNA and nucleosomes, scaling up by a factor of X (X = total number of reactions + 4). The amount of each ingredient needed to prepare 1.5 µl removing mix for a single reaction is listed in **Table 6**; the recipe should be scaled up depending on the number of assays to be performed.
    6. Terminate reactions by adding 1.5 µl of the removing mix. Mix well, spin down, and incubate at 30 °C for a further 30 min.
    7. Meanwhile, pre-run the native polyacrylamide gel in a vertical electrophoresis unit at 100 V for 30 min at 4 °C, using 0.5x TBE as running buffer with a magnetic stir bar inside the lower chamber to maintain constant buffer circulation.
    8. To load the sample, add 2.5 µl of loading dye containing 3x TBE, 30% glycerol, 0.25% Bromophenol Blue, and 0.25% Xylene Cyanol FF. Mix well, briefly spin the samples, and load onto the gel using loading tips.
    9. Run the gel at 200 V for 4.5 hr at 4 °C with buffer circulation.
    10. To detect the signal, transfer the gel to a stack of two sheets of filter paper. Wrap the filter paper with the gel on top using clear plastic wrap, and then expose it to a storage phosphor screen at 4 °C for the desired time.
    11. Scan the screen with an isotope imaging scanner system and analyze the data using suitable software.

2. Mononucleosome Binding Assays

To assay the binding affinity of a given INO80 complex for mononucleosomes, perform an Electrophoretic Mobility Shift Assay (EMSA) using the mononucleosomal substrate generated in Step 1.2.

1. Set up the reaction mixes for binding assays as described for nucleosome remodeling assays but omit the ATP and removing mix from the reactions; incubate at 30 °C for 30 min.
2. Add 2.5 µl of loading dye to each reaction mixture, and apply to a native polyacrylamide gel containing 3.5% acrylamide (acrylamide:bis 37.5:1), 1% Glycerol, 0.5x TBE, 0.01% APS, and 0.001% TEMED.
3. Using 0.5x TBE as running buffer, run the gel at 200 V for 2.5 hr at 4 °C with buffer circulation and expose to a storage phosphor screen.

3. DNA- and Nucleosome-dependent ATPase Assays

Perform ATPase assays in 5 µl reaction mixtures containing 20 mM Tris-HCl (pH 7.5), 60 mM NaCl, 6.6 mM $MgCl_2$, 0.8 mM EDTA, 0.015% Nonidet P-40, 2.5% glycerol, 0.1 mg/ml BSA, 1 mM DTT, 0.1 mM PMSF, 2 mM ATP, 2 µCi of [α-^{32}P] ATP (3,000 Ci/mmol). For each INO80 complex or amount of INO80 complex to be assayed set up three parallel reactions, one containing EB100 buffer to measure DNA- or

nucleosome-independent ATPase, one containing closed circular plasmid DNA (5,000 bp, ~30 nM) to measure DNA-dependent ATPase, and one containing Hela oligonucleosomes (~185 nM) to measure nucleosome-dependent ATPase. Set up all reactions on ice.

1. For each reaction, combine 10-50 nM of the immunopurified INO80 or INO80 subcomplexes with an amount of EB100 buffer sufficient to give a volume of 2.2 µl in pre-chilled lubricated 1.5 ml microcentrifuge tubes. Immediately re-freeze any INO80-containing fractions in powdered dry ice or liquid nitrogen.
2. Set up a master cocktail. The amount of each ingredient needed for a single reaction is listed in **Table 7**. Scale up the recipe by scaling up by a factor of 3(X+2) +1, where X = the number of INO80 preparations to be assayed.
3. To prepare 'sub-cocktails' containing buffer only, DNA, or nucleosomes, dispense 2.5(X+2) µl of the master cocktail into three separate tubes. Add 0.3(X+2) µl of either EB100, closed circular plasmid DNA (1.5 µg/µl), or Hela oligonucleosomes (1.5 µg/µl) and mix well.
4. Dispense 2.8 µl of the appropriate sub-cocktail to the enzyme-containing reaction tubes set up in step 3.1. Gently pipette up and down to mix; avoid introducing bubbles.
5. To start reactions, transfer the reaction tubes to a 30 °C heat block.
6. After 5, 15, 30, and 60 min of incubation, spot 0.5 µl of each reaction mixture onto a cellulose polyethyleneimine thin layer chromatography (TLC) plate (20 x 10 cm) in a straight line at least 1.5 cm away from the bottom edge. Immediately return reaction tubes to the 30 °C heat block so multiple time points can be taken from a single tube. After spotting, dry the TLC plates using a blow dryer.
7. Transfer the TLC plates to a glass chamber containing enough 0.375 M potassium phosphate (pH 3.5) to allow the bottom 0.5 cm of the TLC plate to be submerged in the solution.
8. Cover the chamber, and develop until the front of the liquid phase reaches the top of the TLC plates. Immediate dry the plates thoroughly using a blow dryer.
9. Expose the dried TLC plates to a storage Phosphor screen at RT. Scan the screen with an isotope imaging scanner system and determine the amount of radioactive ATP substrate and ADP product.
10. To calculate the amount of ATP hydrolyzed, multiply the % ATP hydrolyzed by the amount of ATP present in the starting reaction mixture using the following formula: pmol ATP hydrolyzed = 10 pmol ATP in starting reaction x [ADP/(ATP+ADP)]

Representative Results

The figures show representative results of biochemical assays used to characterize INO80 activities, including nucleosome sliding (**Figure 1**) and binding (**Figure 2**) assays and DNA- or nucleosome-dependent ATPase assays (**Figure 3**).

The experiment shown in **Figure 1** compares the ability of intact INO80 complexes purified through FLAG-Ies2 or FLAG-INO80E and of INO80 subcomplexes purified through either FLAG-Ino80ΔN or Ino80ΔNΔHSA to catalyze remodeling of mononucleosomes assembled on a 216 bp, radiolabeled DNA fragment. The position of the nucleosome on the DNA fragment affects electrophoretic mobility; laterally positioned nucleosomes run faster in the gel than more centrally positioned nucleosomes. Since INO80 chromatin remodeling complexes preferentially move mononucleosomes toward the center of a piece of DNA[6,7,8], remodeling activity is monitored by the emergence of a population of nucleosomes that exhibit decreased electrophoretic mobility. At the end of the reaction, an excess of Hela oligonucleosomes and salmon sperm or other DNA must be added to the reaction mix as competitor to remove any substrate-bound INO80 or INO80 subcomplexes, since bound remodeling enzyme will change the electrophoretic mobility of the nucleosome substrate. Complexes purified through FLAG Ino80ΔN lack the metazoan-specific subunits INO80D, INO80E, Amida, MCRS1, NFRKB, and UCH37, while complexes purified through Ino80ΔNΔHSA also lack Arp4, Arp8, and YY1. Complexes purified through FLAG-Ies2, FLAG-INO80E, and FLAG-Ino80ΔN have similar activities, indicating that the metazoan-specific subunits are dispensable for nucleosome remodeling by the INO80 complex. In contrast, complexes purified through FLAG-Ino80ΔNΔHSA are inactive in this assay, indicating that remodeling activity depends on the HSA domain of the Ino80 ATPase and/or its associated subunits.

The experiments in **Figure 2** compare the nucleosome binding activities of INO80 and INO80 subcomplexes using electrophoretic mobility shift assays. These assays are performed similarly to the nucleosome remodeling assays, except that there is no addition of oligonucleosomes and DNA at the conclusion of the reaction to remove nucleosome-bound complexes. Accordingly, incubation of nucleosomes with increasing amounts of intact INO80 complexes purified through the INO80E subunit leads to a dose-dependent disappearance of the band corresponding to free mononucleosomes and appearance of a new 'shifted' species that migrates near the top of the gel (lanes 6-8). In contrast, when nucleosomes are incubated with smaller complexes that had been purified through Ino80ΔN and that lack a subset of INO80 subunits, the shifted species migrates more rapidly (lanes 2-5 and 9-11), suggesting that the relative mobility of the super-shifted band is determined by the size of the complexes assayed.

Figure 3 shows results of an assay comparing the DNA- and nucleosome-activated ATPase activities of two different INO80 subcomplexes. The more slowly migrating spots correspond to the starting α-^{32}P labeled ATP, and the more rapidly migrating species are ADP reaction products; arrows indicate the direction of solvent migration.

One of the complexes assayed here, INO80ΔN, includes an Ino80 ATPase subunit that extends to the protein's normal C-terminus, while the other, INO80ΔNC, lacks the Ino80 C-terminal region. Although these two complexes are otherwise identical, the rate of ATP hydrolysis (measured by conversion of radio-labeled ATP to ADP) is greater in the presence of INO80ΔNC, suggesting the C-terminus of the Ino80 ATPase may negatively regulate its activity. Note that the rate of ATP hydrolysis by both complexes is greater in the presence of nucleosomes than DNA, suggesting the INO80 nucleosome remodeling complexes prefer nucleosomal substrates for ATP hydrolysis.

In the assay shown in the figure, there is only a very low level of ATP hydrolysis in reactions that lack DNA or nucleosomes (ranging from undetectable to <5% of that observed in the presence of DNA/nucleosomes). Because the ATPase activity of INO80 and other SNF2 family chromatin remodeling enzymes is strongly stimulated by DNA or nucleosomes, the presence of substantial DNA- and/or nucleosome-independent ATPase activity in the purified preparations of the INO80 complex would suggest the presence of contaminating cellular DNA, or alternatively, contaminating non-INO80 ATPases that were not successfully removed during purification. Several steps can be taken to minimize introducing unwanted DNA and/or ATPase during the purification.

1) Increase the salt concentration (NaCl) in the binding and washing steps during the purification. Use of less than 200 mM NaCl in binding and washing steps usually yields preparations of remodeling complexes with unacceptable levels of contaminating DNA and/or ATPases. We routinely purify INO80 complexes using a buffer containing 450 mM NaCl to minimize contaminants (described in [3]). We have used buffers containing as much as 1 M NaCl to purify active INO80 complexes; however, we obtain a decreased yield of active complexes under these conditions;

2) Decrease the ratio of FLAG agarose to cell lysate during immunopurification; the optimal amount of FLAG-agarose should be determined by titration;

3) ATP-dependent chaperones may remain bound to FLAG-tagged proteins during immunopurification. These can often be removed by including 1 mM ATP in the washing buffer during immunopurification;

4) We have successfully removed contaminating DNA by including benzonase at a concentration of 25 units/ml during incubation of extract with FLAG-agarose beads. CAUTION: It is essential to make sure benzonase is removed during the subsequent washing steps, as residual DNase will degrade substrate DNA or nucleosomes during assays for INO80 activity.

The interpretation of these ATPase assays could, in principle, be complicated by the fact that INO80 chromatin remodeling complexes contain several potential ATPases, including the SNF2-like core ATPase Ino80, actin-like proteins Arp5, Arp8, Baf53a, actin, and the AAA+ ATPases Tip49a and Tip49b. Despite the physical presence of multiple ATPases, however, previous studies have shown that only complexes containing catalytically active Ino80 can support DNA- or nucleosome-activated ATP hydrolysis; complexes containing the catalytically inactive E653Q form of Ino80 ATPase fail to exhibit detectable DNA- or nucleosome-stimulated ATPase activity under any conditions tested[8]. Thus, DNA- and/or nucleosome-stimulated ATPase activity of INO80 or INO80 subcomplexes are mainly contributed by the Ino80 ATPase subunit.

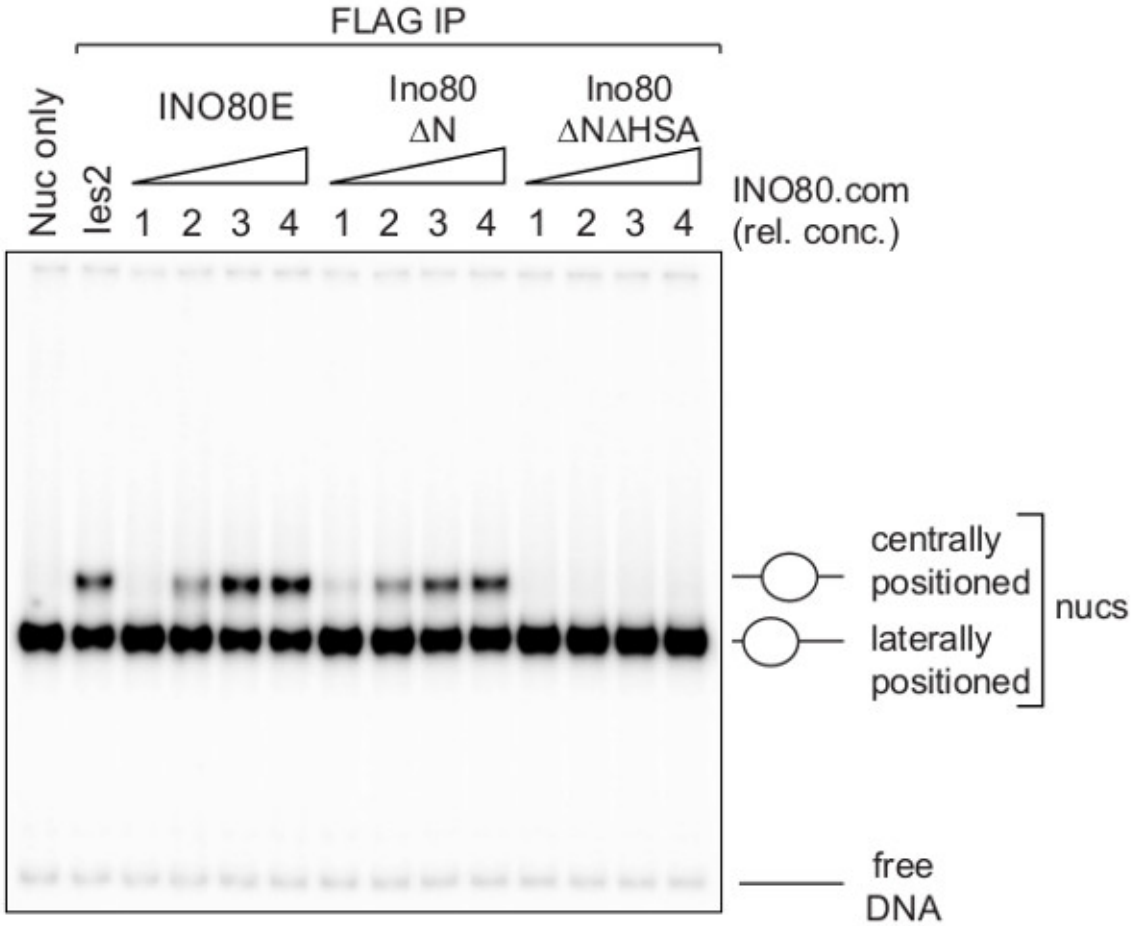

Figure 1. INO80 nucleosome remodeling activity depends on the Ino80 HSA domain and/or associated subunits but is independent of the Ino80 NTD and metazoan-specific subunits. Nucleosome remodeling assays were performed with FLAG-immunopurified complexes from nuclear extracts prepared from cell lines expressing FLAG-tagged versions of wild type or mutant INO80 subunits. Intact INO80 complexes were purified from cell lines expressing FLAG-Ies2 or FLAG-INO80E; INO80 subcomplexes were purified from cell lines expressing FLAG-Ino80ΔN or FLAG-Ino80ΔNΔHSA. A relative concentration (rel. conc.) of 1 corresponds to ~10 nM INO80 complex. Please click here to view a larger version of this figure.

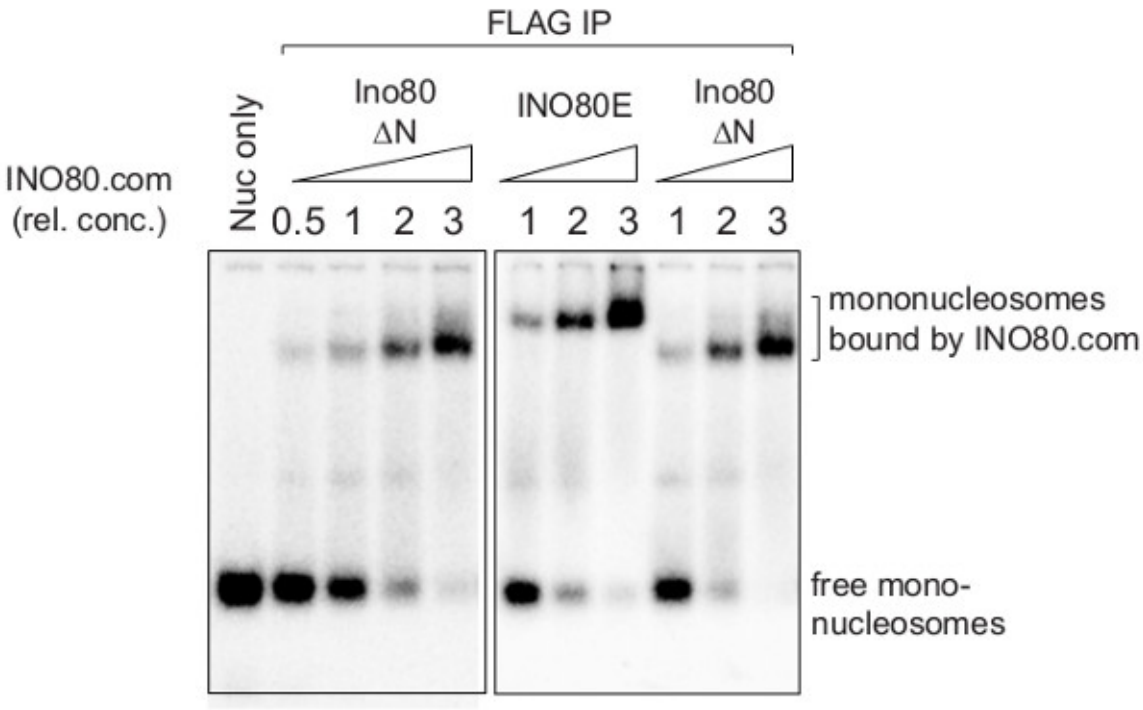

Figure 2. Nucleosome binding by the INO80 complex is independent of the Ino80 NTD and metazoan-specific subunits. Nucleosome binding assays were performed in the presence of varying amounts of the indicated FLAG-immunopurified INO80 complex. Binding of INO80 or INO80 subcomplexes to mononucleosomes results in the emergence of slow-migrating "super-shifted" bands corresponding to mononucleosomes stably bound by INO80 or INO80 subcomplexes. Please click here to view a larger version of this figure.

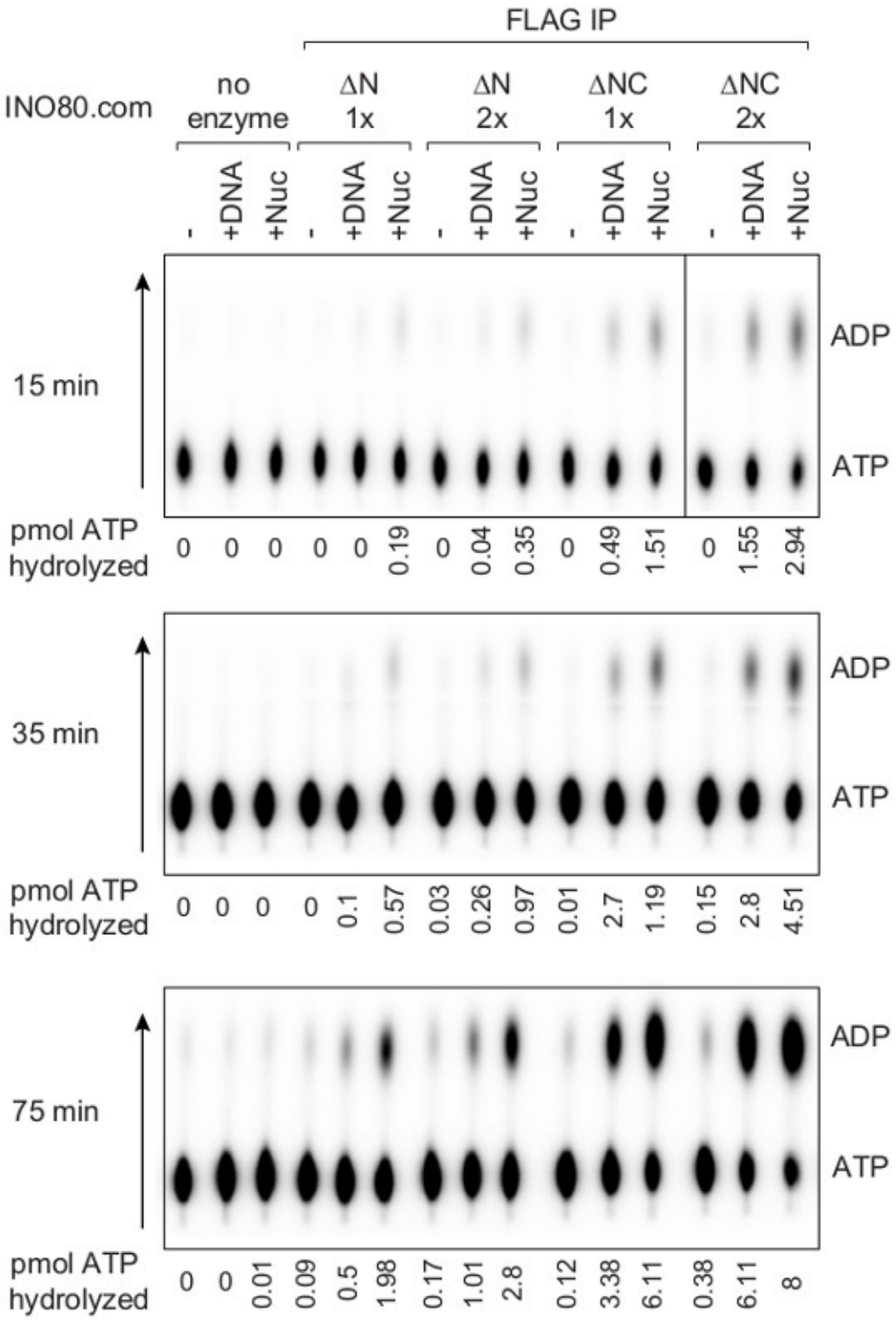

Figure 3. DNA- and nucleosome-dependent ATPase assays. TLC (thin layer chromatography)-based ATPase assays were performed to measure the rate of ATP hydrolysis by INO80 subcomplexes purified through FLAG-Ino80ΔN (ΔN) or FLAG-Ino80ΔNC (ΔNC) in the presence of saturating amounts of DNA or nucleosomes. Assays were performed using two different concentrations of each complex (5 nM and 10 nM) and for three different reaction times. The amount of ATP hydrolyzed (in pmol) in the reactions is indicated under each panel. Please click here to view a larger version of this figure.

67.5 µl	H_2O
10 µl	10x PCR reaction buffer (see Materials List)

1 µl	pGEM-3Z-601 (10 ng/µl)
5 µl	forward primer (10 µm)
5 µl	reverse primer (10 µm)
0.5 µl	dNTP stock solution containing 10 mM each of the 4 dNTPs
1 µl	Taq DNA Polymerase (5 units/µl)
10 µl	[α-^{32}P] dCTP (6,000 Ci/mmol, 3.3 µM)

Table 1. Reaction mix for PCR amplification of radiolabeled '601' DNA fragment.

20 nM	INO80 or INO80 subcomplexes
2.8 nM	nucleosomes (consisting of a mixture of mononucleosomes on the ^{32}P-labeled 601 DNA fragment and Hela cell nucleosomes)
1 mM	ultrapure ATP
20 mM	HEPES-NaOH (pH 7.9)
50 mM	NaCl
5 mM	$MgCl_2$
1 mM	dithiothreitol (DTT)
0.1 mM	phenylmethanesulfonyl fluoride (PMSF)
0.1 mg/ml	bovine serum albumin (BSA)
5%	glycerol
0.02%	Nonidet P-40
0.02%	Triton X-100

Table 2. Components of ATP-dependent nucleosome remodeling assays.

32.6 ml	H_2O
5 ml	40% acrylamide/Bis (37.5:1)
2 ml	10x TBE (900 mM Tris-borate, 20 mM EDTA)
Add the following ingredients immediately before pouring the gel:	
0.4 ml	10% ammonium persulfate
0.1 ml	TEMED

Table 3. Preparation of native polyacrylamide gel for ATP-dependent nucleosome remodeling assays.

10 mM	HEPES pH 7.9
10%	glycerol
100 mM	NaCl
1.5 mM	$MgCl_2$
0.05%	TritonX-100
Add just before use:	
1 mM	DTT
200 µM	PMSF
1:1,000 dilution	Protease Inhibitor Cocktail (see Materials List)

Table 4. EB100 buffer.

3.9 µl	H_2O
1 µl	10x Remodeling Buffer (200 mM HEPES-NaOH (pH 7.9), 0.2% NP-40, 0.2% Triton X-100, 50% Glycerol, 50 mM $MgCl_2$, 1 mg/ml BSA)
0.1 µl	100 mM ATP
0.01 µl	1 M DTT
0.01 µl	100 mM PMSF

0.25 µl	reconstituted mononucleosome substrate from Step 1.2

Table 5. Components of the master cocktail for ATP-dependent nucleosome remodeling assays.

0.33 µl	400 nM Hela nucleosomes
0.75 µl	100 nM sonicated salmon sperm DNAs
0.01 µl	1 M DTT
0.01 µl	100 mM PMSF

Table 6. Removing mix cocktail.

2 µl	H_2O
0.25 µl	20x ATPase Buffer containing 400 mM Tris-HCl (pH 7.5), 200 mM NaCl, 132 mM $MgCl_2$, 16 mM EDTA, 0.3% Nonidet P-40, 50% glycerol, 2 mg/ml BSA
0.1 µl	100 µM ATP
0.005 µl	1 M DTT
0.005 µl	100 mM PMSF
0.1 µl	[α-^{32}P] ATP (3,000 Ci/mmol)

Table 7. Components of the master cocktail for ATPase assays.

Discussion

To ensure that nucleosome remodeling and ATPase activities we observe in assays depend on the catalytic activity of INO80 complexes, and not on contaminating remodeling and/or ATPase enzymes, we routinely assay nucleosome remodeling and ATPase activity of catalytically inactive versions of INO80 complexes, purified in parallel with wild type INO80 using the same procedure. A negative control reaction lacking ATP should also be performed when assaying nucleosome remodeling activity to test for the presence of contaminating ATP and/or ATP-independent remodeling activities, which could complicate interpretation of experiments comparing the activities of different preparations of INO80 complex or subcomplexes. Catalytically inactive versions of INO80 or INO80 subcomplexes should exhibit no nucleosome remodeling or ATPase activities. In addition, detection of DNA- or nucleosome-independent ATPase activity indicates the presence of contaminating ATPase(s) in the enzyme fraction. If activity is still detected in the presence of catalytically inactive Ino80 or if there is substantial DNA- or nucleosome-independent ATPase activity even after optimizing purification as described in 'Representative Results,' complexes can be further purified using additional steps such as ion exchange or gel filtration chromatography or gradient sedimentation.

When measuring nucleosome remodeling and ATPase activities, it is advisable to perform assays for varying lengths of time and with more than one concentration of INO80 or INO80 subcomplexes to ensure measurements are being taken when product-time and dose-response curves are linear. Similarly, nucleosome binding assays should be performed using several concentrations of INO80 complex(es); otherwise, one cannot reliably compare the activities of different INO80 preparations.

Nucleosome sliding and binding assays should always include control reactions that include mononucleosome substrate alone as a marker to show the electrophoretic mobility of the starting nucleosome population. In this way, one can easily identify changes due specifically to the presence of the enzyme fraction. Such control reactions also assess the integrity of the reconstituted mononucleosomes.

In order to conduct easily interpretable nucleosome sliding and binding assays, it is useful to generate homogenous mononucleosomal substrates; for that reason, the assay described in this protocol uses nucleosomes assembled on a DNA fragment containing a strong nucleosome positioning sequence. Although the INO80 complex tends to reposition laterally positioned nucleosomes to a more central position on a DNA fragment, other chromatin remodeling enzymes, such as the ISWI-containing NURF complex[9], move nucleosomes from more central positions toward the ends of DNA fragments. Thus, during characterization of any chromatin remodeling enzyme it is useful to prepare mononucleosome substrates in which the nucleosome positioning sequence is at different locations. Alternatively, one can employ substrates in which nucleosomes have been assembled on DNA fragments without a nucleosome positioning sequence. In this case, the starting nucleosome population will include a mixture of more centrally and laterally positioned nucleosomes.

The ATP-dependent nucleosome remodeling process can lead to various remodeling outcomes, including complete nucleosome displacement from the DNA, nucleosome movement on the DNA, or transient alterations in nucleosome structure that may not lead to changes in the positions of nucleosomes once the reaction reaches equilibrium but are nevertheless functionally significant. The nucleosome sliding assay described can monitor the first two reactions, but may not be able to detect transient alterations in nucleosome structure. One assay that can detect some such transient alterations takes advantage of the fact that nucleosomal DNA on the surface of the nucleosome octamer is largely inaccessible to cutting by a restriction enzyme, whereas linker DNA that has been displaced from the octamer surface by nucleosome sliding or partial unwinding of nucleosomal DNA is susceptible to cutting [10,11,12]. Such an assay can also be particularly useful in the event that the nucleosome remodeling enzyme binds so tightly to its substrate that it cannot be removed by competitor [13].

Our nucleosome sliding and binding assays measure INO80 activities using mononucleosomal substrates prepared with native Hela cell histones. However, remodeling activities of INO80 or other chromatin remodeling enzymes can in principle be affected by the presence or absence of specific post-translational modifications or histone variants. In addition, mononucleosomes may stimulate INO80 complex's activities

differently than di-nucleosomes, or an array of nucleosomes. Thus, measuring INO80 complex's activities using more complicated and diverse nucleosomal substrate may provide insight into the mechanism by which INO80 chromatin remodeling complexes are regulated.

As an alternative to the use of radiolabeled DNA fragments in nucleosome remodeling and binding assays, some investigators visualize nucleosomes and/or DNA by staining nucleosomal DNA with ethidium bromide [14]; however, assays performed using non-radioactive detection methods can be considerably less sensitive than the one described in this protocol, typically requiring the use of considerably more enzyme.

Monitoring the progress of ATPase reactions as a function of time using the ^{32}P-based assay described here requires separate rounds of sample handling and analysis at each time point. As an alternative approach, ATPase activity can be measured using fluorescence-based assays [15,16], which take advantage of the fact that the change of fluorescence signal can be easily monitored in real time, thus providing better opportunity to track conversion of substrate into product in real time. Such assays can, however, be technically challenging and are in general less sensitive than the assay described here.

The procedures we described in this JOVE paper have been used to characterize three different biochemical properties of INO80 or INO80 subcomplexes in the process of ATP-dependent nucleosome remodeling. By using various INO80 subcomplexes or INO80 mutants in these assays, it is possible to dissect the functional contributions of various INO80 subunits and/or domain structures to the INO80 nucleosome remodeling activity and to define step(s) in the remodeling reaction at which INO80 subunits and/or domains may be important. These procedures can be adapted for study of other nucleosome remodeling and binding enzymes and ATPases with both DNA- and nucleosome-dependent or independent activities. We note that different chromatin remodeling enzymes may have higher or lower intrinsic ATPase or nucleosome remodeling activities than the human INO80 complex. Hence, when adapting these protocols for analysis of other chromatin remodeling enzymes, one should vary concentrations of enzyme, ATP, DNA or nucleosomes, and reaction times and/or temperatures in order to optimize the assays for the particular enzymes being studied.

Disclosures

The authors declare that they have no competing financial interests.

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