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Biochemical and structural analysis of N-terminal acetyltransferases

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Abstract

N-terminal acetylation is a co- and post-translational modification catalyzed by the conserved N-terminal acetyltransferase (NAT) family of enzymes. A majority of the human proteome is modified by the human NATs (NatA-F and H), which are minimally composed of a catalytic subunit and as many as two auxiliary subunits. Together, NATs specifically regulate many cellular functions by influencing protein activities such as their degradation, membrane targeting, and protein-protein interactions. This chapter will describe methods developed for their preparation, and their biochemical and structural characterization. This will include methodologies for expression and purification of recombinant NAT protein, kinetic assays, biochemical and biophysical assays, and strategies for structural studies.

1. Introduction

The N-terminal acetylation (NTA) of proteins is both a co- and posttranslational process responsible for marking approximately 80% of the human proteome (Ree, Varland, & Arnesen, 2018). The modification correlates with the regulation of various cellular functions by influencing such protein activities as their degradation, membrane targeting, and protein-protein interactions (Behnia, Panic, Whyte, & Munro, 2004; Dikiy & Eliezer, 2014; Setty, Strohlic, Tong, Boone, & Burd, 2004; Shemorry, Hwang, & Varshavsky, 2013).

During NTA, N-terminal acetyltransferases (NATs) transfer the acetyl-group from the acetyl coenzyme A (acetyl-CoA) co-factor to an N-terminal substrate. These NATs (NatA-F and H) are a conserved family of enzymes in metazoans containing a catalytic subunit, featuring a GCN5-related N-acetyltransferase (GNAT) fold, and up to two additional auxiliary subunits for ribosomal anchoring and/or the regulation of enzymatic activity (Fig. 1). These enzymes are further classified based on their substrate specificity as designated by the first or first two N-terminal residues. The NATs have a conserved range of substrate profiles: for example, the promiscuous NatA complex modifies N-termini with small residues, such as Ser-, Gly-,

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and Thr-; while NatD only modifies the Ser-Gly-Arg-Gly N-termini of the histones H2A and H4 (Drazic et al., 2018; Ree et al., 2018).

Unsurprisingly, this ubiquitous N-terminal mark appears to play a role in human biology and disease. For example, mutations arising in genes corresponding to the subunits of the NatA complex, the catalytic subunit *NAA10* and auxiliary subunit *NAA15*, appear to contribute to some developmental disorders, including the fatal X-linked Ogden Syndrome (Aksnes, Ree, & Arnesen, 2019; Cheng et al., 2018; Rope et al., 2011). Similarly, some studies suggest a connection between NAT protein expression, such as the NatA-C complexes and some cancers (Kalvik & Arnesen, 2013). NTA may even play a role in Parkinson's disease through its modification of the protein implicated in the neurodegenerative disease, α -synuclein (Dikiy & Eliezer, 2014; Kang et al., 2012).

Although the first eukaryotic NAT was first described in 1989 (Lee, Lin, & Smith, 1989), it was not until the last decade that the structure-function relationship of the NATs were described in relation to their catalytic mechanisms and substrate specific activities. Like the lysine acetyltransferase, GCN5, the NAT catalytic subunits use an acetyl-CoA co-factor to mediate N-terminal protein substrate acetylation (Evjenth et al., 2012). However, the architecture of the catalytic fold does not promote the binding of a lysine side chain, as a result of an obstructing conserved $\beta 6$ - $\beta 7$ hairpin loop (Liszczyk, Arnesen, & Marmorstein, 2011; Magin, March, & Marmorstein, 2016). In addition to the key roles played by NAT enzyme auxiliary subunits in allosterically regulating the catalytic subunit, recent studies have reported the regulation of the human and *C. thermophilum* NatA complexes by an intrinsic binding partner, HYPK, as well the structural basis for the binding of the NatA complex to the ribosome to facilitate its co-translational activity (Gottlieb & Marmorstein, 2018; Knorr et al., 2018; Weyer et al., 2017). With the emergence of the NATs as a family of enzymes with substrate-specific activities that influence cellular functions in human biology and disease, the necessity for additional studies of NAT regulation and function grows.

This chapter describes the methods developed for the production, and biochemical and structural characterization of NAT enzymes. In particular, this chapter will contain protocols focused on the preparation of recombinant NAT proteins, enzyme assays for the measurement of their kinetic parameters, assays for their biophysical characterization, and strategies for structural studies.

2. Preparation of recombinant NAT proteins

2.1 Monomeric catalytically competent NATs

For the monomeric catalytically competent NATs (Naa10, Naa40, Naa50, Naa60, and Naa80), we typically follow a previously described method (Liszczyk et al., 2013) with some modifications.

2.1.1 Materials and buffer recipes

- Expression *E. coli*: Rosetta (DE3)pLysS (Millipore)
- LB media (Millipore)

- Antibiotics, such as kanamycin, ampicillin, or streptomycin (GoldBio)
- IPTG (GoldBio)
- Nickel-charged NTA resin (Thermo Scientific)
- TEV protease
- 5mL SP Ion-Exchange column (GE Healthcare)
- Typical Sizing Buffer: 25 mM HEPES, pH 7.0, 200mM NaCl, 1 mM TCEP
- S75prep gel filtration column (GE Healthcare)
- HiLoad Superdex 200 column (GE Healthcare)
- *Sp*Naa10 buffers:
 - Lysis: 25mM Tris, pH 8.0, 500mM NaCl, 10mM β -ME and complete, EDTA-free protease inhibitor tablet (Roche)
 - Wash: 25mM Tris, pH 8.0, 500mM NaCl, 25mM Imidazole, 10 mM β -ME
 - High Imidazole: 25 mM Tris, pH 8.0, 500 mM NaCl, 500 mM Imidazole, 10 mM β -ME
 - Low salt: 25mM sodium citrate monobasic, pH 5.5, 100mM NaCl, 2 mM DTT
 - High salt: 25 mM sodium citrate monobasic, pH 5.5, 750 mM NaCl, 2 mM DTT

2.1.2 Construct design—Deletion of the less conserved and typically disordered C-terminal region in NAT catalytic subunits can facilitate their expression, improve their stability as well as their propensity to crystallize. To aid in the production of these proteins, we recommend engineering them with a cleavable N-terminal affinity tag, such as 6xHis, 6xHis-SUMO, GST and MBP. For example, we successfully applied this strategy for the production and *in vitro* characterization, and crystallization of the *S. pombe* (Sp)Naa10p, in which we designed a C-terminally truncated construct (156 residues of 177) with a TEV-cleavable 6xHis N-terminal tag.

2.1.3 Overexpression and purification

1. **Overexpression:** The plasmid containing the NAT monomer of choice is expressed by transformation into Rosetta (DE3)pLysS competent cells using recommended procedures followed by inoculation of a ~ 100 mL overnight culture (minimally 8mL overnight per liter of large culture). Large cultures of LB are inoculated with the overnight stock which are then shaken at 37 °C until reaching an OD₆₀₀ of ~0.7. The protein is then overexpressed by induction with 0.5 mM IPTG and shaking overnight at 16°C for ~ 16h. In general, we have found that, depending on the NAT; the yield; and the experiment, we need to prepare roughly 3–12L at a time.

2. *Pellet resuspension:* The cells are harvested and resuspended into *SpNaa10* lysis buffer (20 mL per liter of LB culture). The resulting solution can be flash-frozen for storage at -80°C or lysed at 4°C . In general, all purification steps are performed at 4°C .
3. *Nickel affinity:* The supernatant is isolated by centrifugation at $28,000 \times g$ for 25 min, passed over nickel resin and then washed with 20 column volumes (CV) of *SpNaa10* wash buffer. To elute the protein, an imidazole gradient by addition of the high imidazole buffer (25–500 mM) is applied and the fractions containing the target protein are pooled.
4. *6xHis-tag cleavage and reverse nickel:* The 6xHis-tag is subsequently cleaved by addition of TEV protease during a 14-h dialysis into lysis buffer. This solution is passed over an additional nickel column to remove the 6xHis-tagged protease as well as any uncut protein, which is washed with 7 CV of *SpNaa10* wash buffer and pooled with the flow-through.
5. *Cation exchange:* The resulting solution is dialyzed into a buffer containing *SpNaa10* low salt buffer and loaded onto a 5-mL HiTrap SP ion-exchange column (GE Healthcare), where the low pH improves *SpNaa10p* binding to the SP column. The protein is eluted with the same buffer supplemented with a salt gradient (100–750mM NaCl).
6. *Storage conditions:* Peak fractions are concentrated to 8.0mgmL^{-1} (UV_{280} absorbance), flash-frozen, and stored at -80°C .

Although the purification of some NAT catalytic subunits requires the incorporation of a size-exclusion chromatography (SEC) polishing step, we have found that we can directly use ion-exchange-purified *SpNaa10p* protein for crystallization trials. In the case of Naa40, Naa50, and Naa80, we use a S75prep gel filtration column (GE Healthcare), while for Naa60, we use a HiLoad Superdex 200 column due to its propensity to dimerize.

2.2 Auxiliary subunits

2.2.1 Materials and buffer recipes

- Expression *E. coli*: Rosetta (DE3) pLysS (Millipore)
- LB media (Millipore)
- Antibiotics, such as kanamycin, ampicillin, or streptomycin (GoldBio)
- IPTG (GoldBio)
- Cellfectin (Invitrogen)
- DH10 cells, bac-to-bac system (Invitrogen)
- *Spodoptera frugiperda* (*Sf9*) cells (Invitrogen)
- SFM II medium (Invitrogen)
- Nickel-charged NTA resin (Thermo Scientific)
- TEV protease

- 5 mL SP Ion-Exchange column (GE Healthcare)
- Superdex 200 column (GE Healthcare)
- *Sp*NatA buffers:
 - *Sp*Naa10 buffers
 - SEC: 25mM HEPES, pH 7.0, 200mM NaCl, 1 mM TCEP
- hNatA buffers:
 - Lysis: 25mM Tris, pH 8.0, 500mM NaCl, 10mM imidazole, 10mM β -ME, PMSF, complete, EDTA-free protease inhibitor tablet, and DNase
 - Wash: 25 mM Tris, pH 8.0, 500mM NaCl, 10 mM imidazole, 10mM β -ME
 - Elution: 25mM Tris, pH 8.0, 200mM imidazole, 10mM β -ME
 - Dilution: 25mM Tris, pH 8.0, 10mM β -ME
 - Low salt: 25mM Tris, pH 8.0, 200mM NaCl, 10mM β -ME
 - High salt: 25mM Tris, pH 8.0, 1M NaCl, 10mM β -ME
 - SEC: 25mM HEPES, pH 7.0, 200mM NaCl, 1 mM TCEP

2.2.2 General construct design—Unlike the catalytic subunits, the large auxiliary subunits of the NAT complexes (Naa15, Naa25, and Naa35) share low sequence identity with one another. Therefore, when developing a strategy for their expression, we use a combination of sequence alignments and secondary structure predictions generated by a server such as Phyre2 or PsiPred (Buchan, Minneci, Nugent, Bryson, & Jones, 2013; Kelley, Mezulis, Yates, Wass, & Sternberg, 2015). Together, we use this data to identify several tagged auxiliary subunit candidates for co-expression trials with the corresponding untagged catalytic subunit. The catalytic/auxiliary complexes are then evaluated based on expression, solubility, SEC profiles, enzymatic activity (discussed below), and structural trials.

When preparing a NAT complex, we generally engineer the gene of the auxiliary subunit, such as *Sp*Naa15p of the NatA complex, so that it linked to an N-terminal 6xHis tag with an intervening TEV recognition site. For co-expression with *Sp*Naa15p, the untagged *Sp*Naa10 catalytic subunit (residues 1–156) is engineered into the second multiple cloning site of a pET-Duet vector. With the exception of the human and other metazoan NAT complexes, we express the NAT complexes using *E. coli*. We found that inositol hexaphosphate (IP₆), which is produced in *Sf9* cells and not in *E. coli*, co-purified and co-crystallized with human (h)NatA, suggesting that it plays a role as a scaffolding molecule for hNatA (Cheng et al., 2019; Gottlieb & Marmorstein, 2018). The other human NAT complexes are also overexpressed by (co-)transfection of *Sf9* cells (Gottlieb & Marmorstein, 2018; Scott et al., 2017). Additionally, we have found that, when visualizing these complexes by SDS-PAGE gels stained with Coomassie Brilliant Blue G-250, the larger auxiliary units, such as Naa15, tend to stain more strongly than that of the catalytic subunit (or other smaller subunits) of the complex (Fig. 2).

2.2.3 Overexpression and purification (non-metazoan NAT complexes)—For the expression and purification of the *Sp*NatA complex, we follow the same scheme as the *Sp*Naa10p outlined above, with a few changes.

1. *SEC*: The first of two peaks from the HiTrap SP-ion exchange column is concentrated and spun down or spin-filtered at 13.3 krpm for 5 min to remove particulate matter. Finally, the sample is injected onto an S200prep gel-filtration column.
2. *Storage conditions*: The target protein is concentrated to 15mgmL⁻¹ for use directly in crystallization trials, but can be flash-frozen for storage at -80 °C.

2.2.4 Overexpression and purification (metazoan NAT complexes)—We use the same strategy for the expression of the human (h)NatA complex, except both subunits are engineered into a pFAST BAC DUAL vector. The plasmid is generated for overexpression of hNatA by transfection of *Sf9* cells. In order to prepare the virus, a bacmid is generated by transposition into DH10 bac competent *E. coli* cells using the standard protocols of the bac-to-bac system.

1. *Overexpression*: *Sf9* cells cultured in SFM II medium are transfected with the resulting bacmid using cellfectin to produce the desired baculovirus, which is amplified until reaching a high titer. *Sf9* cells are grown to a density of 1×10^6 cells mL⁻¹ and infused with the amplified baculovirus to an MOI (multiplicity of infection) of ~ 1–2. The cells are grown at 27 °C and harvested 48 h post-infection.
2. *Pellet resuspension*: The cells are resuspended into hNatA lysis buffer (20 mL per liter of *Sf9* culture). The resulting solution can be flash-frozen for storage at -80 °C or lysed gently at 4 °C. In general, all purification steps are performed at 4 °C.
3. *Lysate clarification*: The supernatant is isolated by centrifugation at 28,000 $\times g$ for 40min and incubated on nickel resin for 30min to an hour.
4. *Nickel affinity in batch*: We have found that *Sf9* lysate can adhere to the resin and clog the column, which results in the use of a slightly different protocol than described above with *E. coli* lysate.
 - a. Following incubation, the resin is pelleted at 800 $\times g$ for 5 min and the supernatant is removed by decanting.
 - b. The resin is resuspended with lysis buffer (5 CV), pelleted, and the supernatant removed a second time.
 - c. The resin is then applied to a gravity flow column and washed with ~ 120 CV lysis buffer and batch eluted with 10 CV of elution buffer.
5. *Cation exchange*: The eluent is diluted to a final salt concentration of 200 mM NaCl and loaded onto a 5mL HiTrap SP-ion exchange column. We have found that SP-ion exchange of hNatA complexes using a buffer with a higher pH yields a cleaner product, likely due to the positively charged residues found in the N-

terminus and the core of hNaa15 (residues 9–41 and 590–639, respectively), which are reportedly responsible for ribosome binding (Knorr et al., 2018; Magin, Deng, Zhang, Cooperman, & Marmorstein, 2017). The protein is eluted using the same low salt buffer with a salt gradient (200 mM–1M NaCl) over the course of 20 CV.

6. *SEC*: Peak fractions are pooled, concentrated, and injected onto a Sup-erdex 200 Increase 10/300 gel filtration column in the same SEC buffer.
7. *Storage conditions*: Peak fractions are pooled, concentrated to 4.8mgmL⁻¹, and flash-frozen to be stored at –80 °C.

We have found that for hNatA, similar to *Sp*NatA, a single freeze-thaw cycle does not impact its enzymatic activity and propensity to crystallize. However, it is recommended to perform initial enzymatic assays to evaluate if the enzyme is active as well as set up any crystallization trials with fresh monodisperse protein. Subsequent studies can then be performed comparing the freeze/thawed protein and fresh protein side-by-side.

3. Kinetic analysis

3.1 NAT-specific kinetic properties and considerations

NAT-catalyzed NTA proceeds by an ordered BiBi mechanism (Evjenth et al., 2012; Liszczak et al., 2013), where acetyl-CoA binding precedes peptide binding. As a result, we typically prepare a reaction solution so that it contains all of the reaction components (buffer, peptide substrate, and NAT enzyme, in that order), and initiate the reaction by the addition of the acetyl-CoA co-factor. NATs can range in their Michaelis Menten constant (K_M) concentrations. The NATs have a narrow range of acetyl-CoA K_M values, from as low as ~2 μ M (human NatA/HYPK) to ~50 μ M (*C. albicans* NatB and *S. pombe* NatA). Unfortunately, K_M acetyl-CoA values have not been reported for all of the NATs. On the other hand, we have found that NAT complexes tend to have peptide K_M values ranging from ~30 μ M (human NatA) to ~700 μ M (*C. albicans* NatB) (Gottlieb & Marmorstein, 2018; Hong et al., 2017; Liszczak et al., 2013). With the exception of the metazoan-conserved Naa60 and Naa80, the monomeric NATs tend to have higher K_M values, as demonstrated by the ~2mM K_M reported for *Sp*Naa10p (Goris et al., 2018; Liszczak et al., 2013). It is also noteworthy that only k_{cat}/K_M values have been reported for human Naa40, human Naa50 and the archaeal NAT orthologue, ssNAT (Liszczak et al., 2011; Liszczak & Marmorstein, 2013; Magin, Liszczak, & Marmorstein, 2015), which may be suggestive of high K_M values, similar to *Sp*Naa10p, which are not amenable to the experimental setup. The synthetic peptides used for the enzymatic assays are prepared using trifluoroacetic acid with a final concentration ranging from 10% to 45% of the TFA salt (Genscript, 2019). The presence of high concentrations of TFA, particularly those necessary to achieve a concentration for steady-state kinetics studies ($5\text{--}10 \times K_M$), may lead to the destabilization of the enzyme of interest. It is important to keep this in mind when designing your kinetics experiment and synthesizing peptide for the studies, where the reaction must have sufficient buffering capacity and/or the experiment may be limited to the calculation of specificity constant, k_{cat}/K_M .

Some NATs are prone to either substrate or product inhibition (Montgomery et al., 2016). The human NatA is particularly sensitive to product inhibition due to its affinity to CoA (Fig. 3). In order to generate a Michaelis-Menten curve that could be properly fit, we instead use saturating acetyl-CoA at concentrations no higher than $\sim 100 \mu\text{M}$ (Gottlieb & Marmorstein, 2018).

The specific interaction of HYPK with the metazoan NatA requires particular care. HYPK has inherent inhibitory activity towards NatA-mediated activity. Due to the nature of the tight-binding interaction, $K_i = 0.7 \pm 0.3 \text{ nM}$, the association of the complex is on the order of a second (Copeland, 2005). As a result, when performing assays incorporating HYPK, it is important to ensure that the final concentration is accurate. Because HYPK has a low extinction coefficient ($\epsilon = 1490 \text{ M}^{-1} \text{ cm}^{-1}$), we incorporated the use of the uncut, MBP-tagged HYPK to ensure an accurate concentration determination ($\epsilon = 69,330 \text{ M}^{-1} \text{ cm}^{-1}$).

When working with NATs at low-nanomolar concentrations, it may be necessary to incorporate a reagent to prevent protein sticking to the walls of the reaction tube (blocking agent), such as bovine serum albumin, BSA ($0.5\text{--}2 \text{ mg mL}^{-1}$) or Tween-20 ($0.005\text{--}0.1\%$) in the reaction buffer to prevent non-specific interactions that can reduce the amount of available enzyme in the reaction ("Reducing Non-Specific Binding," 2014). In the case of the NatA complex, we found that we were able to observe consistent levels of activity at 100 nM *Sp*NatA without a blocking agent, but our studies using the human NatA at 10 nM required the addition of BSA at a final concentration 2 mg mL^{-1} (Gottlieb & Marmorstein, 2018; Liszczak et al., 2013). On the other hand, we have also conducted studies with the human NatB at 10 nM without the addition of a blocking agent. It is therefore important to screen both reagents to determine if blocking agent incorporation is beneficial and, if so, the minimum necessary for a robust signal-to-noise ratio. It is also important to keep in mind that BSA has free thiols, making the native protein incompatible with a CoA-detection assay.

Regardless of the assay used, monitoring of peptide substrate chemical acetylation is imperative. The N-alpha amino group of a peptide has a $\text{p}K_a$ of 7.7 ± 0.5 (Grimsley, Scholtz, & Pace, 2009), making it particularly susceptible to chemical acetylation in the presence of buffers with a high pH as well as at high concentrations of acetyl-CoA, due to the labile acetyl group. Moreover, we have observed that this effect is enhanced for lysine residues ($\text{p}K_a = 10.5 \pm 1.1$) in the presence of neighboring positively-charged residues, such as a polyArg tag (Olia et al., 2015), which we use as a tag for peptide capture on phosphocellulose filter paper (P81) with our radioactive acetyltransferase assay (see below). We control for potential chemical *N*-acetylation of the peptide or the enzyme by conducting a control reaction at the corresponding concentration of acetyl-CoA and in the absence of enzyme or peptide, respectively. The higher signal of the two is then subtracted as background from the corresponding reactions of interest.

3.2 Radioactive kinetic assays

We typically use radioactivity assays as a readout for N-terminal acetyltransferase activity. Not only are these assays used for direct-detection of NTA, they also have a low detection limit. Here we provide the reaction buffer used for the study of the human NatA. However,

we recommend successively screening pH, salt, and blocking agent followed by enzyme concentration in conjunction with linear range determinations.

3.2.1 Materials, buffer recipes, instrumentation, and software

- NAT enzyme
- Potential Reaction Buffer: 100mM HEPES, pH 8.0, 50mM NaCl 2mgmL⁻¹ BSA*
- ¹⁴C-radiolabeled acetyl-CoA (4 mCi/mmol; PerkinElmer Life Sciences)
- Synthetic peptide (Genscript)
 - First 7 residues of a corresponding biological substrate
 - C-terminal poly-Arg tag (RWGRPVGRRRRP; 12.7pL)
- P81 filter paper (Oakhill Lab; Saint Vincent's Institute of Medical Research, Australia)
- PerkinElmer Life Sciences Tri-Carb 2810 TR liquid scintillation analyzer
- GraphPad Prism

3.2.2 ¹⁴C NAT radioactive acetyltransferase assay—To study the acetyltransferase activity of the human NatA complex we used a radioactivity-based assay, which we have outlined below.

1. *Reaction conditions:* human NatA is mixed at a final concentration of 10nM in buffer with the peptide, histone H4 at a final volume of 30 μ L. Depending on the substrate being studied, the concentration of ¹⁴C-acetyl-CoA is either kept constant at saturating levels (100 μ M) or varied (0.73–94 μ M).
 - *Note:* To circumvent the strong substrate inhibition that we had observed with the canonical SASEA- peptide, we use the noncanonical substrate, H4, for our kinetic studies. This allowed us to generate Michaelis-Menten curves for both acetyl-CoA and the H4 peptide while minimizing the effects of CoA-mediated product inhibition (described above).
2. *Reaction timing and initiation:* Due to its sensitivity, it is important to ensure consistency when working with a radioactive assay. For this reason, we set up the reactions to be initiated by the addition of ¹⁴C-acetyl-CoA using intervals spaced out by 15 s (or 20 s, or 30 s, *etc.* to for ease) so that the duration of each reaction is exactly the same. In the case of the human NatA, the reactions are initiated at room temperature using 15-s intervals (0:15, 0:30, 0:45, and so on).
3. *Reaction termination:* 20 μ L of the reaction solution are carefully blotted onto the negatively charged P81 filter paper, capturing the positively charged peptide, after 12min (12:15, 12:30, 12:45, and so on), which we had previously determined to be within the linear range of catalysis (<10% substrate turnover).

4. *Sample preparation and readout:* The paper is then washed with 20 mM HEPES, pH 7.5 buffer $\times 3$ for 5 min to remove unreacted ^{14}C -acetyl-CoA, subsequently placed in acetone, then on a paper towel to air-dry to ensure stringent removal of water, which can interfere with the signal, and then into scintillation tubes. The tubes are filled with scintillation fluid, and read by a scintillation counter as counts per minute (CPM), which has a direct correlation with the amount of ^{14}C -acetyl-H4 captured on the paper transferred during acetylation.
5. *Kinetics fitting:* The signal is converted from CPM to molar units using a standard curve previously generated with known concentrations of ^{14}C -acetyl-CoA in scintillation fluid using the same scintillation counter. These values are used to generate a Michaelis-Menten curve in GraphPad Prism using a non-linear regression fit (kcat equation) that is constrained with the concentration of the hNatA enzyme (10 nM) in corresponding molar units used on the x -axis (0.01 μM).

The radioactivity assay does have its drawbacks: radiation work requires additional safety precautions and approval, the disposal and proper handling of radiation material (RAM) can be costly, and the P81 papers used for peptide capture are no longer in mass production. More recently; however, we have begun purchasing our P81 paper from a lab (Dr. Jon S. Oakhill) at Saint Vincent's Institute of Medical Research in Australia, which has produced results comparable to previous results.

3.3 Coupled kinetics assays

When unable to work with radioactive assays, the other standard assay used for the study of NAT kinetics is a CoA-detection coupled assay using 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), which is covered elsewhere (Foy, Thompson, & Arnesen, 2017). In the presence of a neutral to alkaline buffer, the DTNB reacts with the resulting free thiol group in coenzyme A to produce a compound has measurable absorbance at 412 nm. The assay, however, is limited due to the coupling of readout to the production of CoA, which can be chemically hydrolyzed. Moreover, DTNB will react with free thiols in the NAT and any contaminants present, requiring additional appropriate controls.

4. Biochemical and biophysical characterization

4.1 General considerations

We advise the use of inhibitors or co-factors, such as acetyl-CoA, CoA and the non-hydrolyzable acetyl-CoA, for these studies because they may introduce a conformational change that blocks or facilitates protein-protein interactions. We often conduct initial assays using acetyl-CoA because it is a stabilizing co-factor for NATs and KATs alike (Friedmann & Marmorstein, 2013). In the case of our studies of the human NatA with HYPK, we used 100 μM acetyl-CoA to be consistent with our kinetic studies for the biochemical and biophysical characterization of the complex (pull-downs, sedimentation equilibrium analytical ultracentrifugation, and differential scanning calorimetry). Moreover, in the case of yeast NatA or NatC produced recombinantly with *E. coli*, we recommend the inclusion of IP₆ (~ 15 μM or greater) due to its reported role as a scaffolding molecule and because the

biological concentration has been reported to be 5–15 μM (Szwergold, Graham, & Brown, 1987).

4.2 Analytical ultracentrifugation (AUC)

Analytical ultracentrifugation (AUC) is a powerful technique that exploits gravity for mass- and shape-dependent separation to calculate a protein's mass and deconvolute the composition of a protein solution (Schuck, Perugini, Gonzales, Howlett, & Schubert, 2002). Since there are numerous resources available for the analysis of both sedimentation-equilibrium (SE-AUC) and sedimentation velocity (SV-AUC) experiments (see Balbo & Schuck, 2002; Cole et al., 2008), this text will only discuss the experimental setups used thus far for the study of human NatA.

4.2.1 Materials, buffer recipes, instrumentation, and software

- Buffer: 25mM HEPES, pH 7.0, 200mM NaCl, 1 mM TCEP
- Protein (0.1–0.9 A_{280})
- Beckman Optima XL-I ultracentrifuge
- Quartz windows
- Four-hole rotor
 - SE-AUC: six-channel centerpieces
 - SV-AUC: two-channel centerpieces
- Heteroanalysis
- SEDPHAT
- SEDFIT

4.2.2 Sedimentation equilibrium—For the study of both human NatA and human Naa60 (NatF) (Gottlieb & Marmorstein, 2018; Stove et al., 2016), SE-AUC was used to deconvolute structures observed in the asymmetric unit cell. In the case of human NatA (Fig. 4A and B), which appeared as a dimer in the crystal asymmetric unit. Here we describe the conditions used for the human NatA complex.

1. *Sample preparation and setup:* The protein is prepared at three concentrations (A_{280} = 0.8, 0.5, and 0.2) in sample buffer, which is confirmed by nanodrop. The sample is then spin-filtered to remove particulates prior to cell assembly.
2. *AUC Settings:* SE-AUC experiments at 4 °C using three speeds (6, 9, and 12krpm), absorbance optics at 280nm.
3. *Theoretical mass calculation:* Using the three speeds and three concentrations, a theoretical molecular weight is calculated using the program HeteroAnalysis or SEDPHAT.

4.2.3 Sedimentation velocity—Because SV-AUC is both shape and weight-dependent, for the study of hNatA wild-type and mutant proteins (Y834F and Y834A) were analyzed

using SV-AUC to confirm that the mutants were folded correctly and mono-disperse (Fig. 4C).

1. *Sample preparation and setup:* The protein is prepared by dilution in sample buffer to an A_{280} of 0.6 by nanodrop. The sample is then spin-filtered to remove particulates prior to cell assembly.
2. *AUC settings:* Data is set to be obtained by spinning at 42 krpm over ~8h of centrifugation at 20 °C.
3. *Data analysis:* Data is analyzed using SEDFIT to calculate a continuous $c(s)$ distribution.

4.3 Pull-down assays

Some NATs are able to form multi-protein complexes and have been reported to interact with non-NAT proteins, but not all of these interactions have been fully characterized (Arnesen et al., 2005; Knorr et al., 2018; Kramer, Boehringer, Ban, & Bukau, 2009; Magin et al., 2017). For the characterization of these interactions, we use pull-down assays and, in the case of studies probing the NATs and their interactions with the ribosome, we employ the use of a sucrose cushion (Magin et al., 2017). Generally, these experiments are performed at 4 °C and can be supplemented to include co-factors.

4.3.1 Affinity pull-down materials and buffer recipes

- Pull-down buffer: 25 mM HEPES, pH 7.0, 200mM NaCl, 1mMTCEP
- Bait protein (MBP- or GST-tagged at 2 μ M final)
- Prey protein (untagged protein at 6 μ M final)
- 70 μ L affinity resin slurry
 - Amylose agarose (New England BioLabs)
 - Glutathione agarose (Gold Biotechnology)
- Elution buffer
 - 25 mM HEPES, pH7.0, 200mM NaCl, 40mM Maltose, 1 mM TCEP
 - 25mM HEPES, pH7.0, 200mM NaCl, 40mM Glutathione, 1 mM TCEP
 - SDS Loading Buffer

4.3.2 Affinity-based pull-down assays

Here, we provide an example using the protocol described for the study of the interaction between HYPK and human NatA.

1. *Pull-down:* The prey (MBP-tagged protein HYPK) is incubated with the prey (human NatA) in a total volume of 150 μ L for 30min and subsequently subjected to pull-down by incubation for additional 30min on amylose agarose resin.
2. *Resin wash and elution options:* The resin is washed with 80 CV pull-down buffer and eluted by incubation for 15 min. Results of the pull-down are analyzed

by visual inspection by comparison of the input and pull-down using 15% SDS-PAGE gels stained with Coomassie Brilliant Blue G-250. Following initial studies confirming the ability to elute the positive control (human NatA with MBP-tagged HYPK), we use boiling elution, where the resin is incubated with SDS gel-loading buffer.

4.3.3 Sedimentation assay materials and buffer recipes

- Ribosomes (1 μ M final)
- NatA (1.2 μ M final)
- Resuspension buffer: 10mM HEPES pH 7.5, 50mM KOAc, 10mM NaCl, 2mM DTT, 5mMMg(OAc)₂
- Cushion: 30% sucrose in resuspension buffer
- S120-AT2 rotor (ThermoFisher Scientific)
- Anti-His antibody, 1:2000 final dilution (GE Healthcare LifeSciences, monoclonal from mouse)

4.3.4 Sedimentation assay—When interrogating the NAT-ribosome interaction, we use a sedimentation assay that uses a sucrose gradient (Magin et al., 2017). Here, we describe the protocol used with the *S. pombe* ribosome and *Sp*NatA.

1. *Sample sedimentation:* Ribosomes are combined with NatA in a total volume of 35 μ L. This solution is added to 80 μ L of 30% sucrose in resuspension buffer containing and pelleted by ultracentrifugation at 120krpm.
2. *Sample visualization:* The supernatant is removed, and the ribosomal pellet is resuspended using 110 μ L resuspension buffer. The samples are analyzed using either SDS-PAGE or Western blot against the 6xHis- tagged *Sp*Naa15 subunit using an anti-His antibody.

4.4 Thermal stability assays

The evaluation of a protein's thermostability can provide insight into the biochemical details governing its structure and function. We employ differential scanning calorimetry (DSC) to determine a protein's melting temperature. Here, we describe the protocol outlined for the study of hNatA and hNatA/HYPK.

4.4.1 DSC materials, buffer recipes, instrumentation, and software

- 0.22 μ m vacuum or spin filters (Millipore)
- Sample buffer/SEC Buffer (25mM HEPES, pH 7.0, 200mM NaCl, 1 mM TCEP)
- Protein stock
- 10kDa MWCO MINI Dialysis Device (ThermoFisher Scientific)
- MicroCal VP-Capillary DSC instrument (Malvern Instruments, Malvern, United Kingdom)

- Origin 7.0 (OriginLab Corporation, Northampton, MA)

4.4.2 Differential scanning calorimetry

1. *Buffer preparation:* All buffers and diluted samples are prepared either by vacuum filtration or by spin filtration by centrifugation at 13 krpm for 10 min, respectively. These are then degassed for 5 min under low atmosphere with stirring.
2. *Buffer scans:* A buffer that matches the sample buffer, such as the dialysis buffer, is scanned such that buffer is in both the reference and sample cells. These scans are performed in triplicate and are inspected to ensure that the thermogram overlays well and is relatively flat.
3. *Sample preparation:* Protein is dialyzed as concentrated stocks ($\sim 4 \text{ mg mL}^{-1}$) overnight at 4 °C into SEC buffer. The concentration of the resulting protein is measured by UV₂₈₀ using a nanodrop spectrophotometer, diluted, and measured again. In the case of the hNatA and hNatA/HYPK, experiments were performed using $\sim 0.9\text{--}1 \text{ mg mL}^{-1}$ protein ($7\text{--}8 \text{ }\mu\text{M}$ hNatA and $6\text{--}8 \text{ }\mu\text{M}$ hNatA/HYPK).
4. *DSC settings:* DSC scans are recorded from 10 °C to 90 °C at a scan rate of 1 °C/min with a pre-scan thermostat of 10 min, mid feedback mode, and a filtering period of 30 s.
5. *Data analysis:* DSC data were analyzed using Origin such that raw DSC data is corrected for the instrument baseline by subtracting the buffer reference scan. A simple two-state unfolding model is used to determine the melting temperature (T_m) and associated thermodynamic parameters, such as change in heat capacity (ΔC_p), enthalpy (ΔH), entropy (ΔS), and Gibbs free energy (ΔG) (Durowoju, Bhandal, Hu, Carpick, & Kirkitadze, 2017).

4.4.3 Differential scanning fluorimetry—If differential scanning calorimetry is not an option due to equipment access and/or limited protein yield, another option is to perform differential scanning fluorimetry (DSF) or a Thermal shift assay (He, Hogan, Latypov, Narhi, & Razinkov, 2010; Lang & Cole, 2017). As opposed to DSC, which requires $\sim 300 \text{ }\mu\text{L}$ per assay at $0.2\text{--}1 \text{ mg mL}^{-1}$ (Huynh & Partch, 2015; Milev, 2016), DSF requires only a total of $20 \text{ }\mu\text{L}$ per assay at $\sim 0.1 \text{ mg mL}^{-1}$ and can be performed in a high-throughput set-up (Milev, 2016). This fluorescence-based assay uses a signal that is generated by the interaction of the SYPRO orange dye with hydrophobic regions in a target protein as it is unfolded, which is measured using a real-time qPCR instrument. For information about experimental setup, data read-out, and extrapolation of T_m values, we recommend further reading (Huynh & Partch, 2015), which includes extrapolation using GraphPad Prism or Microsoft Excel. For the NATs, we have found that, using this method of interpretation, acetyl-CoA-bound hNatA melting generally agrees with the DSC-derived T_m (Fig. 5).

Unfortunately, DSF data is only beneficial if the transition between folded to unfolded occurs as a two-state event, causing non-two-state events to be ambiguous. It is also limited in its ability to calculate thermodynamic parameters and, therefore, it is advised that

interpretation of DSF-derived thermodynamic values should be done with caution (Niesen, Berglund, & Vedadi, 2007; Wright, Stewart, Page, & Konkolewicz, 2017). Additionally, when studying protein-protein interactions using DSF, it is important to remember that the activity of the dye depends on the exposure of hydrophobic patches and, therefore, it is possible that a primarily hydrophobic interaction could yield ambiguous results. Finally, if a protein exists in multiple states, including being partially aggregated, there may be little data to resolve from the signal as a result of low fluorescence.

5. Structural studies

5.1 NAT structural overview

The NAT class of enzymes has been studied primarily using X-ray crystallography and a variety of bound molecules, such as co-factors or bisubstrate inhibitors (Table 1) (Deng et al., 2019; Goris et al., 2018; Gottlieb & Marmorstein, 2018; Hong et al., 2017; Liszczak et al., 2011, 2013; Liszczak & Marmorstein, 2013; Magin et al., 2015, 2016; Stove et al., 2016; Weyer et al., 2017). Although the NAT catalytic subunits share a high level of sequence identity (>25%), they each have distinct structural elements that contribute to their functional diversity (Fig. 6A). The topology of the catalytic GNAT fold features 4 α -helices and 7 β -strands and contains an acetyl-CoA-binding motif, Q/RxxGxG/A that is located in the α 3- β 4 segment (Salah Ud-Din, Tikhomirova, & Roujeinikova, 2016). Studies indicate that the length of the α 1-helix determines the ability of the catalytic subunit to bind to an auxiliary subunit (Liszczak & Marmorstein, 2013). The substrate binding groove formed between the α 1- α 2 and β 6- β 7 loops varies in depth, depending on the NAT. For example, the Naa10 catalytic subunit forms a more narrow groove when in complex with the Naa15 auxiliary subunit in order to accommodate N-termini with small residues while the human Naa80 features a relatively wide binding groove (Fig. 6B and C) (Goris et al., 2018; Liszczak et al., 2013). NatD, on the other hand, features both a narrow groove and an electronegative surface for the specific modification of the SGRG-N-terminus of the histones H2A and H4 (Magin et al., 2015). The α 1- α 2 loop, rather than using the canonical β 6- β 7 hairpin loop, occlude the substrate binding pocket while the β 6- β 7 loop is turned outward (Fig. 6D). Interestingly, although the NAT catalytic subunits all feature a GNAT fold and use a general base for catalysis, they appear to use different catalytic residues (Table 2).

Structures determined of NAT complexes demonstrate that the auxiliary subunits feature unique scaffolds (Fig. 7). The NatA Naa15 auxiliary subunit serves as a scaffold of protein-protein interactions, such as with HYPK, Naa50, and, similar to NatB and NatC, the ribosome to facilitate its co-translational activity (Gottlieb & Marmorstein, 2018; Knorr et al., 2018; Neubauer, 2012; Weyer et al., 2017). Additionally, some NAT complexes, such as NatA and NatE, have also been found to interact with IP₆. To date, six NatA and NatE-related structures have been determined containing the IP₆ molecule or an IP₆-like molecule, guanosine-5',3'-tetraphosphate (ppGpp), which co-purified from the *E. coli* lysate (Deng et al., 2019; Gottlieb & Marmorstein, 2018; Neubauer, 2012).

Here, we present a general protocol for the implementation of X-ray crystallization for the study of NAT enzymes.

5.2 Materials and buffer recipes

- Protein stock (3–45mgmL⁻¹) in sizing buffer (25 mM HEPES pH 7, 200 mM NaCl, 1 mM TCEP)
- 96-well crystallization hanging drop plate
- View plate seal (Hampton Research)
- 96-well crystallization screens, such as:
 - Peg/Ion (Hampton Research)
 - Wizard Series (Molecular Dimensions)
 - Crystal 1/2 (Hampton Research)
- Small molecule for co-crystallization
 - acetyl-CoA, CoA, or acetyl-CoA
 - Peptide substrate (4–7 residues; ± N-terminally acetylation)
 - Bisubstrate inhibitor
 - IP₆ (for work with NatA or NatC)

5.3 Crystallization and structure determination

The NATs crystallize over a range of concentrations (3–45mgmL⁻¹) using hanging drop vapor diffusion at 20 °C. We therefore recommend screening with a combination of co-factors and substrate peptides of varying lengths ranging from 4 to 7 residues. Another consideration is to screen with the reaction products: an N-terminally acetylated peptide and CoA. Finally, a bisubstrate inhibitor (transition-state mimic), which is typically synthesized through the coupling of a peptide to CoA with an acetamide linker (Foyn et al., 2013), may be useful in stabilizing the NAT enzyme and improving the crystal quality. In accordance with standard crystallization practices for the improvement of crystallization and diffraction, we would like to stress that it may also be necessary to introduce a binding partner to stabilize the NAT, explore alternative constructs, or switch to another species.

In the case of the 6xHis-tagged hNatA, an initial screen yielded one hit with PEG/ION, while screening with 6xHis-tagged NatA/HYPK_{full-length} dramatically increased the hit rate to 25 hits across three screens (Fig. 8A and C). Addition of an additive dramatically improved the diffraction for hNatA from 5 to 2.85 Å. On the other hand, truncation of the likely disordered N-terminal region of HYPK (residues 1–33), cleavage of the N-terminal 6xHis-tag on hNaa15 and micro-seeding using a 1:100,000 dilution significantly improved crystal diffraction quality from having no observable diffraction to diffraction out to 3.15 Å (Fig. 8B, D, and E).

When determining a NAT structure, it is important to keep in mind that the distinct NAT catalytic subunit architectures may reduce the likelihood for success when implementing a molecular replacement (MR) strategy. As a result, it is best to screen different NAT catalytic subunits when initially performing MR. However, due to the apparent diversity of the NATs,

it is best to attempt a brute-force strategy by using each of the PDB files from the NATs determined to date in order to identify a reasonable solution. The auxiliary subunits, however, are significantly distinct in their architecture from one another, making molecular replacement more difficult. As a result, the NAT complexes have been traditionally solved using selenomethionine-labeled protein and single-wavelength anomalous diffraction (SAD) or heavy-metal soaking for single isomorphous replacement with anomalous diffraction (SIRAD). Therefore, if MR does not yield a solution with an electron density map suitable for model-building, it may be necessary to turn to derivatization of the complex with either heavy metals or by selenomethionine-labeling.

6. Prospects and conclusion

The N-terminal acetyltransferase family of enzymes is an emerging class of regulatory enzymes that contribute to human health, development, and disease. In this chapter, we have outlined protocols used for the expression and purification of recombinant NATs, particularly NatA complex-related proteins, which can be adapted for the production of other NAT proteins. We have also discussed the methodologies used to date for the evaluation of NAT activity and function using enzymatic, biochemical and biophysical assays. Finally, we have described protocols for crystallizing NAT proteins and their complexes for X-ray structure determination, which should be useful for characterizing the structures of other NATs and their complexes. Although significant progress has been made in detailing NAT modes of catalysis and the interactions involved in substrate binding, a comprehensive study of NatC has not yet been published. More recently, advances have been made in our understanding of NatA co-translational activity through the structural determination of a *S. cerevisiae* ribosome in complex with Naa50-bound NatA (Knorr et al., 2018). The mechanism of co-translational activity of the other NATs and, particularly, how these details apply to metazoan NAT function has not yet been described. Furthermore, the intricacies of NAT involvement in human disease, such as cancer and some neurodegenerative diseases, remain unknown. Taken together, the study of the NATs is at a crossroads where an understanding of their *in vitro* behavior will likely aid in untangling their mechanisms of activity and regulation in human biology.

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Further reading

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Fig. 1.

The metazoan-conserved NATs, shown as cartoons, depicting their composition as well as their substrate specificity, as indicated by the first one or two residues. Asterisk (*) refers to Naa10 noncanonical substrates (unprocessed β - and γ -actin). Parenthesis indicate corresponding canonical substrates name for NATs that are highly specific. With the exception of the NATs outlined with a dashed line (NatF and NatH), all of the indicated NATs are able to interact with the ribosome and are found to localize throughout the cell.

Fig. 2. Coomassie-stained SDS-PAGE gel of SEC-purified human NatA/HYPK₃₄₋₁₂₉ showing the uneven staining of the Naa15 auxiliary subunit with respect to the other stoichiometric components of the NatA/HYPK complex.

Fig. 3. Human NatA Michaelis-Menten plot showing product inhibition at concentrations greater than $\sim 100 \mu\text{M}$. The fit to a conventional Michaelis-Menten curve is shown (red dotted line).

Fig. 4.

Plots from analytical ultracentrifugation (AUC) studies of human NatA. (A) A sedimentation equilibrium AUC (SE-AUC) experiment used to show that NatA is a monomer in solution. The log plot shows the calculated slopes of a theoretical monomer (blue) and a theoretical dimer, which is compared with the slope of NatA (black circles) from a representative concentration ($\text{OD}_{A280}=0.8$) and speed (12 krpm). (B) The quality of the data observed in (A) is indicated by the corresponding residuals for all speeds. (C) A sedimentation velocity AUC (SV-AUC) experiment used to show that NatA mutants ($\text{OD}_{A280} = 0.6$) are minimally perturbing to the structure of the complex.

Fig. 5. Melting temperature comparison of hNatA using differential scanning calorimetry (DSC, left)— 46.47 ± 0.011 $^\circ\text{C}$ —and differential scanning fluorimetry (DSF, right)— 46.5 ± 0.1 $^\circ\text{C}$ (mean $\pm$ S.D.). Melting temperatures (T_m) are calculated using a two-state unfolding model using either Origin 7.0 (DSC) or GraphPad Prism and a Boltzman sigmoidal analysis (DSF).

Fig. 6. NAT catalytic subunits. (A) *S. pombe* (*Sp*)NatA catalytic subunit, (left) *SpNaa10* (cyan;ribbon), depicted in its canonically active conformation with $\beta 6-\beta 7$ hairpin loop (purple), $\alpha 1-\alpha 2$ loop (black), and acetyl-CoA binding motif (sky blue) with bound acetyl-CoA (stick) (PDB: 4KVO). (Right) Transparent surface representation of *SpNaa10*, showing the topology of the narrow substrate binding groove with acetyl-CoA removed for clarity. (C) Human (*h*)Naa80 (orange) depicted in ribbon and transparent surface representation with the loops, $\beta 6-\beta 7$ hairpin (purple) and $\alpha 1-\alpha 2$ (black) loops, indicated to emphasize the width of the Naa80 substrate binding groove (PDB: 5WJD). (D) Human (*h*)Naa40 (yellow) depicted with $\alpha 1-\alpha 2$ loop (dark blue), which forms a unique interaction to occlude the Naa40 active site and form a narrow substrate binding groove (PDB: 4U9W).

Fig. 7.
Ribbon diagrams of HYPK-bound human NatA (PDB: 69C5, left) and *C. albicans* NatB (PDB: 5K04, right) depicted to emphasize the differences in auxiliary subunit scaffolding.

Fig. 8. Human NatA and NatA/HYPK crystals. (A) Initially grown using PEG/ION crystallization screen, and (B) following optimization. (C) Crystals of human NatA/HYPK_{full-length} and (D) NatA/HYPK₃₄₋₁₂₉ initially grown using PEG/ION crystallization screen, and (E) following optimization.

Table 1.

NAT Structures Determined by X-Ray Crystallography to Date

NAT	Subunits	Species	Co-crystallized with	PDB	Publication
ssNAT	ssNAT	<i>S. solfataricus</i>	acetyl-CoA	2X7B	(Liszczak & Marmorstein, 2013)
NatA	Naa10	<i>S. pombe</i>	acetyl-CoA	4KVX	(Liszczak et al., 2013)
	Naa10 Naa15		acetyl-CoA	4KVO	
			Bisubstrate inhibitor	4KVM	
	Naa10 Naa15	<i>S. cerevisiae</i>	apo	4HNY	(Neubauer, 2012)
			ppGpp	4HNX	
			IP ₆	4HNW	
	Naa10 Naa15 HYPK	<i>C. thermophilum</i>	CoA *	5NNR	(Weyer et al., 2017)
			carboxymethyl CoA	5NNP	
Naa10 Naa15	<i>H. sapiens</i>	acetyl-CoA *	6C9M	(Gottlieb & Marmorstein, 2018)	
			6C95		
NatB	Naa20 Naa25	<i>C. albicans</i>	CoA MES	5K04	(Hong et al., 2017)
			Bisubstrate inhibitor	5K18	
NatD	Naa40	<i>H. sapiens</i>	acetyl-CoA	4U9V	(Magin, Liszczak, & Marmorstein, 2015)
			CoA H2A/H4 peptide	4U9W	
		<i>S. pombe</i>	acetyl-CoA	4UA3	
NatE	Naa50	<i>H. sapiens</i>	CoA peptide	3TFY	(Liszczak, Arnesen, & Marmorstein, 2011)
			CoA Acetylated peptide	4X5K	N/A
			CoA	2PSW	N/A
			acetyl-CoA	2OB0	
	Naa10 Naa15 Naa50	<i>S. cerevisiae</i>	ppGpp Bisubstrate inhibitor	4XPD	(Neubauer, 2012)
			Bisubstrate inhibitor * IP6	6O07	(Deng et al., 2019)
NatF	Naa60	<i>H. sapiens</i>	CoA	5ICW	(Stove et al., 2016)
			Bisubstrate inhibitor	5ICV	
			CoA	5HH0	(Chen et al., 2016)
	Naa60 _{F34A}		5HH1		
	Naa60		acetyl-CoA	5HGZ	
NatH	Naa80	<i>H. sapiens</i>	acetyl-CoA	5WJD	(Goris et al., 2018)
			Bisubstrate inhibitor	5WJE	

* denotes that density for the corresponding added co-factor was not observed

Table 2.

Proposed NAT Catalytic Residues

NAT	Reported Catalytic Residues	Ordered Water?
ssNAT	1. H137 and E176 (NatE-type) 2. E84 and E176 (NatA-type)	-
NatA	E24; potentially Y138/Y139	-
NatB	Not determined	-
NatC	Not determined	-
NatD	Y85/E139	Yes
NatE	Y73/H112	Yes
NatF	Y97/H138	Yes
NatH	Not determined	Yes