Molecular basis for N-terminal acetylation by the heterodimeric NatA complex

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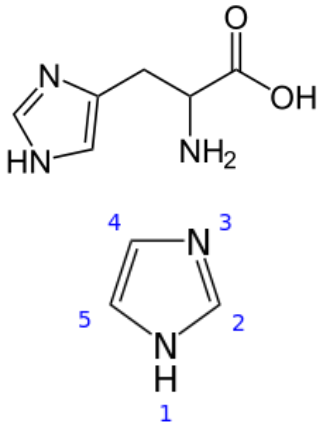
all the information and pictures on the following slides are derived from the above mentioned paper

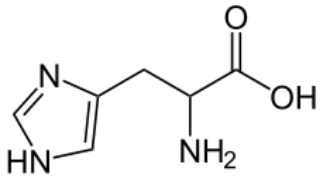
methods

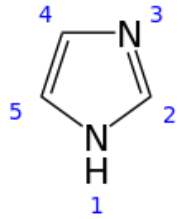


Naa10p expression and purification:

- full-length Naa10p (residues 1–177) and several C-terminal truncation constructs from *S. pombe*, were engineered into a modified pETDUET vector containing a TEV protease–cleavable His_6 tag
- constructs were transformed into Rosetta (DE3)pLysS, grown to an OD_{600} of 0.7, induced with 0.5 mM IPTG at 16 °C for ~16 h. Cells were lysed by sonication (25 mM Tris, pH 8.0, 500 mM NaCl, 10 mM β-ME and a complete, EDTA-free protease inhibitor tablet).
- Ni resin, washed with lysis buffer supplemented with 25 mM imidazole.
- eluted in the same buffer with an imidazole gradient (25–500 mM imidazole)
- TEV protease was added to fractions containing the target protein for the duration of a 14-h dialysis into lysis buffer
- additional nickel column to remove TEV protease as well as any uncut Naa10p
- protein fractions were dialyzed into a buffer containing 25 mM sodium citrate monobasic, pH 5.5, 100 mM NaCl and 2 mM DTT
- and loaded onto a 5-mL HiTrap SP ion-exchange column (GE Healthcare). The protein was eluted in the same buffer with a salt gradient (100–750 mM NaCl). Peak fractions were concentrated to 8.0 mg mL^{-1} (10-kDa concentrator; Amicon Ultra, Millipore), as measured by UV_{280} absorbance (extinction coefficient = 18,000), for crystallization trials.
- The selenomethionine derivative was prepared by expression of Naa10p (residues 1–156) in minimal medium (Molecular Dimensions) containing 50 µg L^{-1} of selenomethionine (Sigma)

methods





Naa10p–Naa15p (NatA) complex expression and purification.

- full-length Naa15p gene (encoding residues 1–729) was cloned into MCSI of a modified pETDUET vector
- DNA encoding residues 1–156 of Naa10p was engineered into MCSII
- binary protein complex was prepared essentially as described for Naa10p, except that the first of two peaks that eluted from the HiTrap SP ion-exchange column was concentrated to 1 mL, and loaded onto an s200prep gel-filtration column (GE Healthcare) in a buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl and 1 mM TCEP. This protein was concentrated to 15 mg mL−1, as measured by UV280 (extinction coefficient = 100,000) for crystallization trials
- Point-mutation proteins were generated with the Stratagene QuikChange protocol

methods

Naa10p crystallization

- Naa10p at a concentration of 8 mg mL−1 was mixed with acetyl CoA at a 1:3 molar ratio
- Initial crystallization hits were obtained from an Naa10p construct containing residues 1–161 (Naa10p (residues 1–161)) with hanging-drop vapor diffusion at 20 °C with a well solution containing 16% PEG 8000 (Hampton Research), 10% ethylene glycol and 0.1M HEPES, pH 7.5, and yielded poor diffraction
- These crystals were used to seed entire grid screens with Naa10p (residues 1–156), which did not crystallize in the absence of the seed crystals. Large crystals of Naa10p (residues 1–156) were obtained from this screen in a 1:1 mixture of protein and well solution (14% PEG 3350 and 0.1 M bis-Tris, pH 6.5) with hanging-drop vapor diffusion at 20 °C. An additive screen (Hampton Research) revealed that these crystals could be reproduced in the absence of seed crystals when 10% glycerol was present as an additive in the crystallization drop. Final crystallization conditions of the Naa10p protein (construct 1–156) were optimized and included a drop with a 2.5:0.5:2.0 ratio of protein (8 mg mL−1)/glycerol (50%)/well solution, mixed in that order. Selenomethionine crystals of this construct were obtained with identical conditions with a lower protein concentration (6.5 mg mL−1). Diffraction-quality crystals required 1–3 days to grow to maximum dimensions.

Naa10p–Naa15p crystallization

- NatA complex was concentrated to 12 mg mL^{-1}, mixed with 3× cofactor (acetyl CoA or bisubstrate inhibitor) and allowed to stand for 12–15 h at 4 °C. The initial crystallization hit was obtained with hanging-drop vapor diffusion at 20 °C in a well solution containing 10% PEG 4000, 10% isopropanol and 0.1 M HEPES, pH 7.0 (all reagents from Qiagen)
- best crystals were obtained by mixing protein/well at a 2.0-µL/2.0-µL ratio and grew to maximum dimensions in 7–10 d. These crystals were transferred into mother liquor supplemented with ethylene glycol in 5% increments (15%, 20% and 25%) for 1 min at each concentration and were finally flash-frozen in liquid nitrogen.
- The selenomethionine-labeled protein required that the protein buffer be supplemented with 1 mM reduced L-glutathione and 1 mM oxidized L-glutathione for crystallization to occur in conditions identical to those described for the native Naa10p–Naa15p complex

methods

structure determination.

- Data sets were collected at beamline X29A at the National Synchrotron Light Source (Brookhaven National Laboratory) and processed with HKL2000
- Initial molecular replacement trials with Phaser were performed with several truncated variants of the hNAA50 structure (PDB 3TFY) as a model but were unable to provide electron density maps suitable for model building
- A data set was then collected on the selenomethionine-labeled protein at the selenium peak wavelength (0.9795 Å), but selenium sites could not be identified with single-wavelength anomalous diffraction (SAD) or single isomorphous replacement with anomalous scattering (SIRAS)
- We located the selenium sites by performing molecular replacement on the selenomethionine-derivative data set, contouring the output electron density map to 3.0σ and placing selenium atoms at the resulting peaks in Coot
- These sites were input into Phaser along with the hNAA50 model, and an MR-SAD approach yielded high-quality electron density maps. Initial model building was carried out with ARP/wARP, and refinement was carried out with Phenix.refine (experimental phases excluded throughout refinement) while applying two-fold NCS

NatA

- Data sets were collected at beamlines X25 and X29A at the National Synchrotron Light Source (Brookhaven National Laboratory) and processed with HKL2000
- The two-wavelength MAD data were used by the Hybrid Substructure Search in Phenix to identify a total of eight heavy-atom sites in the asymmetric unit, and initial phases were obtained with SOLVE
- Initially, the Naa15p helices were built as polyalanine chains, and the Naa10p molecules were manually placed into the density-modified map with Coot.
- The resulting structure was used as a molecular replacement model for the selenomethionine-derivative data set that was collected at the selenium peak wavelength and processed to 3.35-Å resolution.

results

Table 1 Data collection and refinement statistics

	NatA–CoA-SASEA (native)[a]	NatA–CoA-SASEA (K_2PtBr_4)[a]		NatA–CoA-SASEA (SeMet)[a]	NatA–acetyl CoA (native)[a]	Naa10p–acetyl CoA (SeMet)[a]
Data collection						
Space group	*P*1	*P*1		*P*1	*P*1	$P2_1$
Cell dimensions						
a, *b*, *c* (Å)	81.439, 119.381, 134.063	81.021, 119.278, 133.520		80.388, 119.346, 133.054	80.739, 119.692, 132.024	40.974, 64.833, 60.723
α, β, γ (°)	80.200, 76.600, 70.425	80.305, 76.651, 70.384		79.265, 80.709, 70.450	80.284, 76.852, 70.651	90.000, 97.552, 90.000
		Peak	*Inflection*	*Peak*		*Peak*
Wavelength	0.9795	1.0717	1.0722	0.9791	0.9795	0.9795
Resolution (Å)	50.00–2.60 (2.69–2.60)[b]	50.00–3.50 (3.63–3.50)	50–3.50 (3.63–3.50)	50.00–3.35 (3.47–3.35)	50.00–3.15 (3.26–3.15)	30.00–2.00 (2.03–2.00)
R_{sym}	5.9 (57.8)	6.6 (22.1)	6.0 (23.7)	11.2 (55.0)	7.6 (60.2)	11.0 (50.4)
$I / \sigma I$	20.8 (1.7)	22.3 (8.8)	26.9 (7.3)	8.9 (2.0)	17.2 (2.4)	36.4 (5.6)
Completeness (%)	97.3 (87.0)	99.2 (98.8)	99.1 (97.6)	99.1 (98.7)	99.0 (98.4)	98.7 (84.4)
Redundancy	4.7 (4.5)	5.9 (5.9)	5.8 (5.7)	3.3 (3.3)	3.9 (3.9)	10.9 (9.0)
Refinement						
Resolution (Å)	50.00–2.60 (2.69–2.60)				50.00–3.15 (3.26–3.15)	30.00–2.00 (2.03–2.00)
No. reflections	137,695				77,244	21,216
R_{work} / R_{free}[c]	23.64 / 26.92				21.70 / 24.60	18.25 / 23.08
No. atoms	28,758				28,431	2,788
Protein	28,051				28,115	2,456
Ligand/ion	308				204	102
Water	330				107	230
B factors (Å^2)						
Protein	59.9				86.0	24.7
Ligand/ion	58.7				88.7	22.5
Water	45.8				52.4	31.5
r.m.s. deviations						
Bond lengths (Å)	0.003				0.003	0.008
Bond angles (°)	0.860				0.790	1.167

[a]One crystal was used for data collection and refinement when applicable. SeMet, selenomethionine. [b]Values in parentheses are for highest-resolution shell. [c]R_{free} was calculated with 5% of the

results - NatA structure

- Naa15: 37 α-helices (13 conserved TetratricoPeptideRepeats)
- interaction sites for Naa50 & HYPK?
- no putative ribosome binding motiv
- Naa15 stability depends on binding to Naa10
- hydrophobic interface of α1(Ile8, Leu11)-loop-α2(Leu28-Trp38) segment of Naa10 binds to Naa15

results - NatA structure

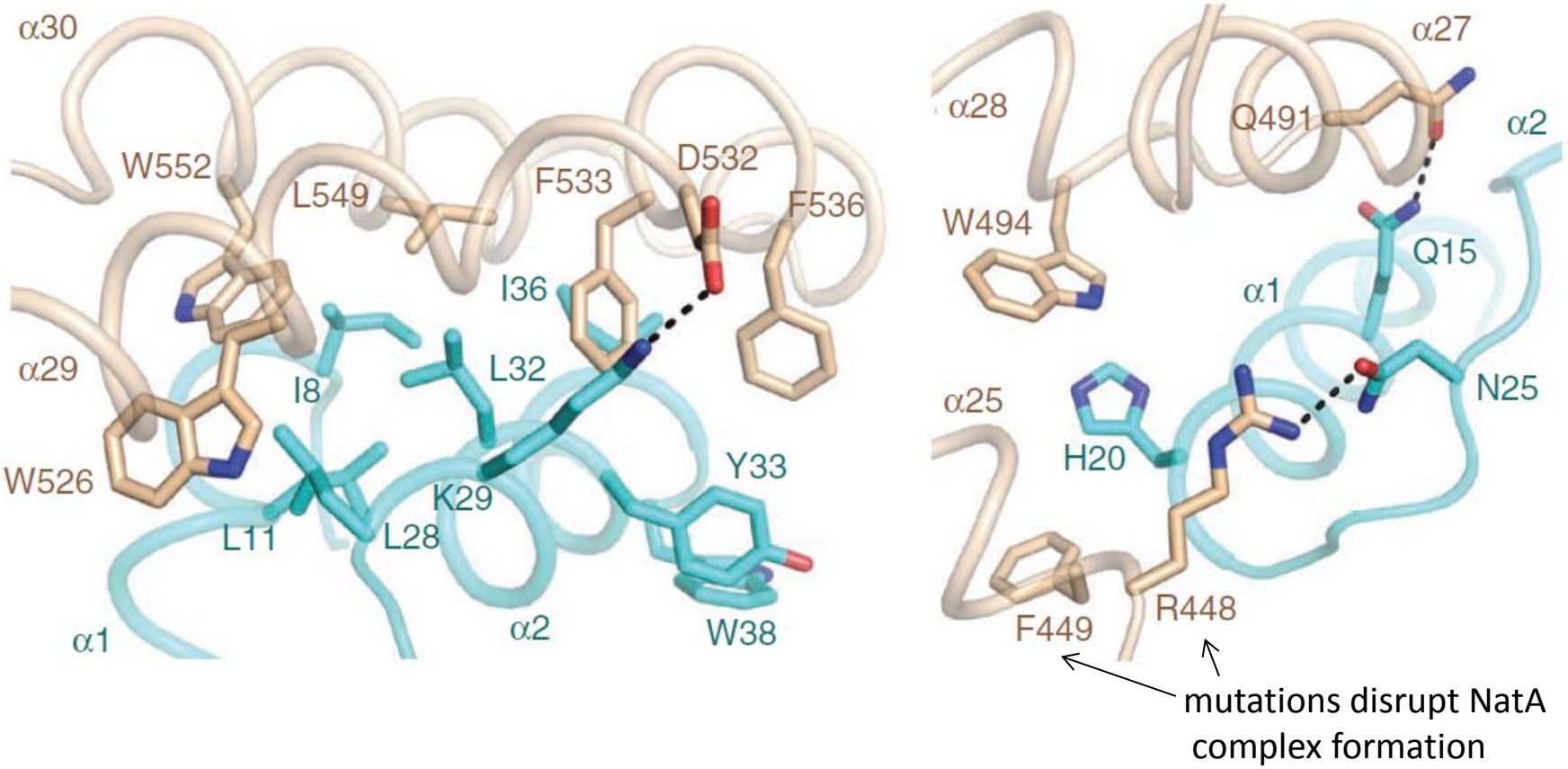
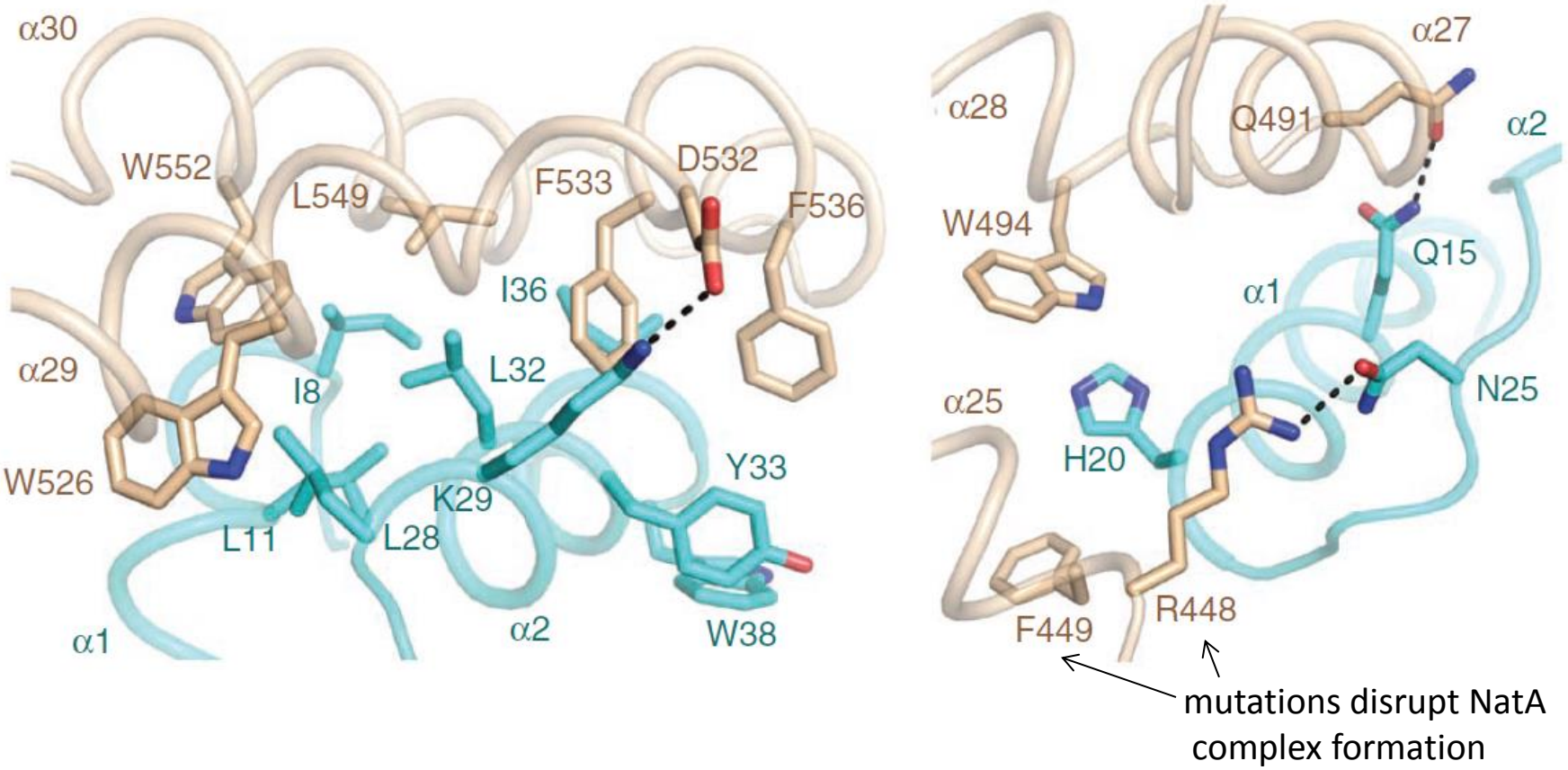


Figure 1 Ove (**c**) Zoom view highlighting key residues that compose the predominantly hydrophobic interface between Naa10p α1–α2 and Naa15p α29–α30. (**d**) Zoom view of the intersubunit interface at the C-terminal region of Naa10p α1 and the Naa15p α25–α27–α28 helices.

results - NatA structure



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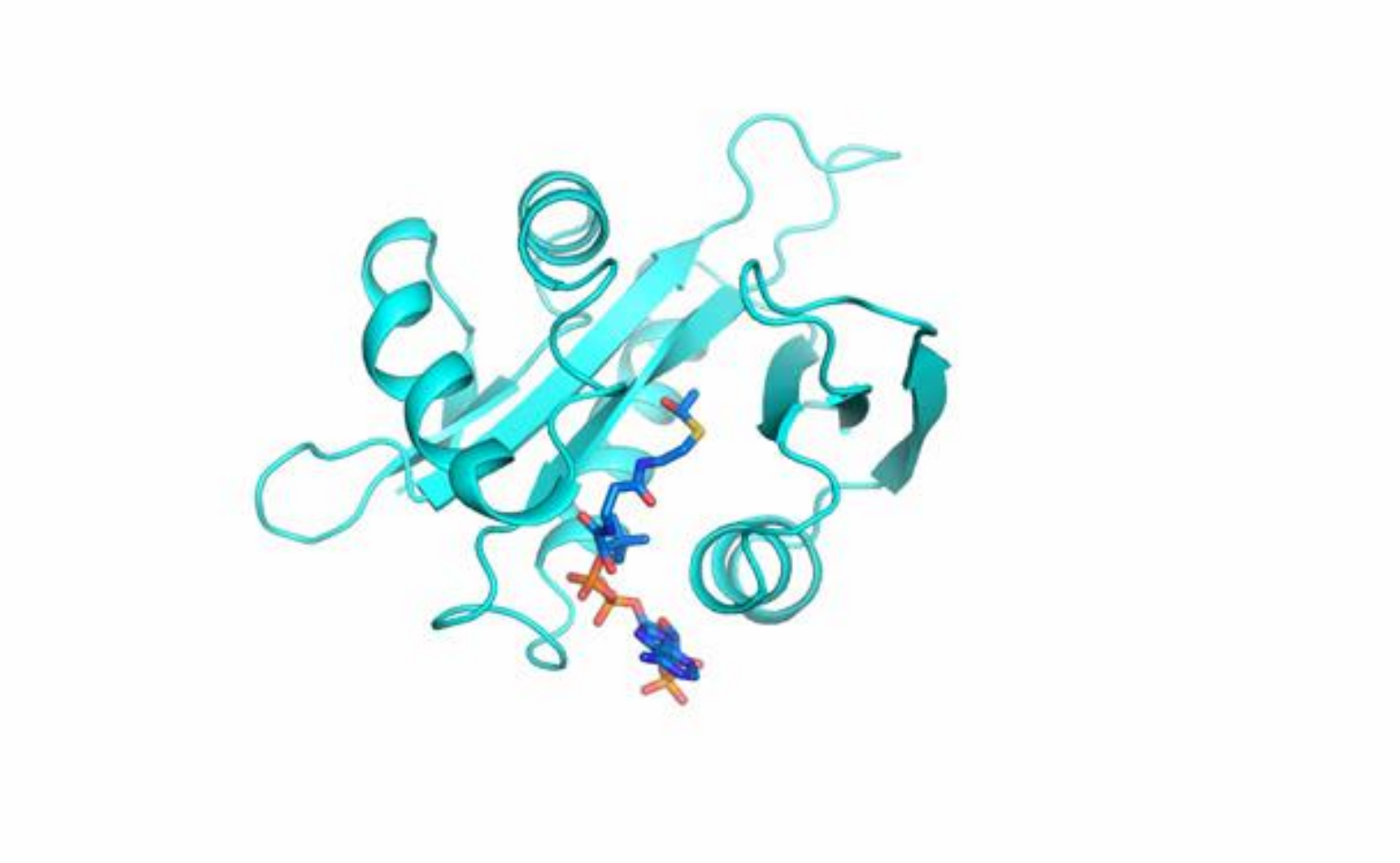
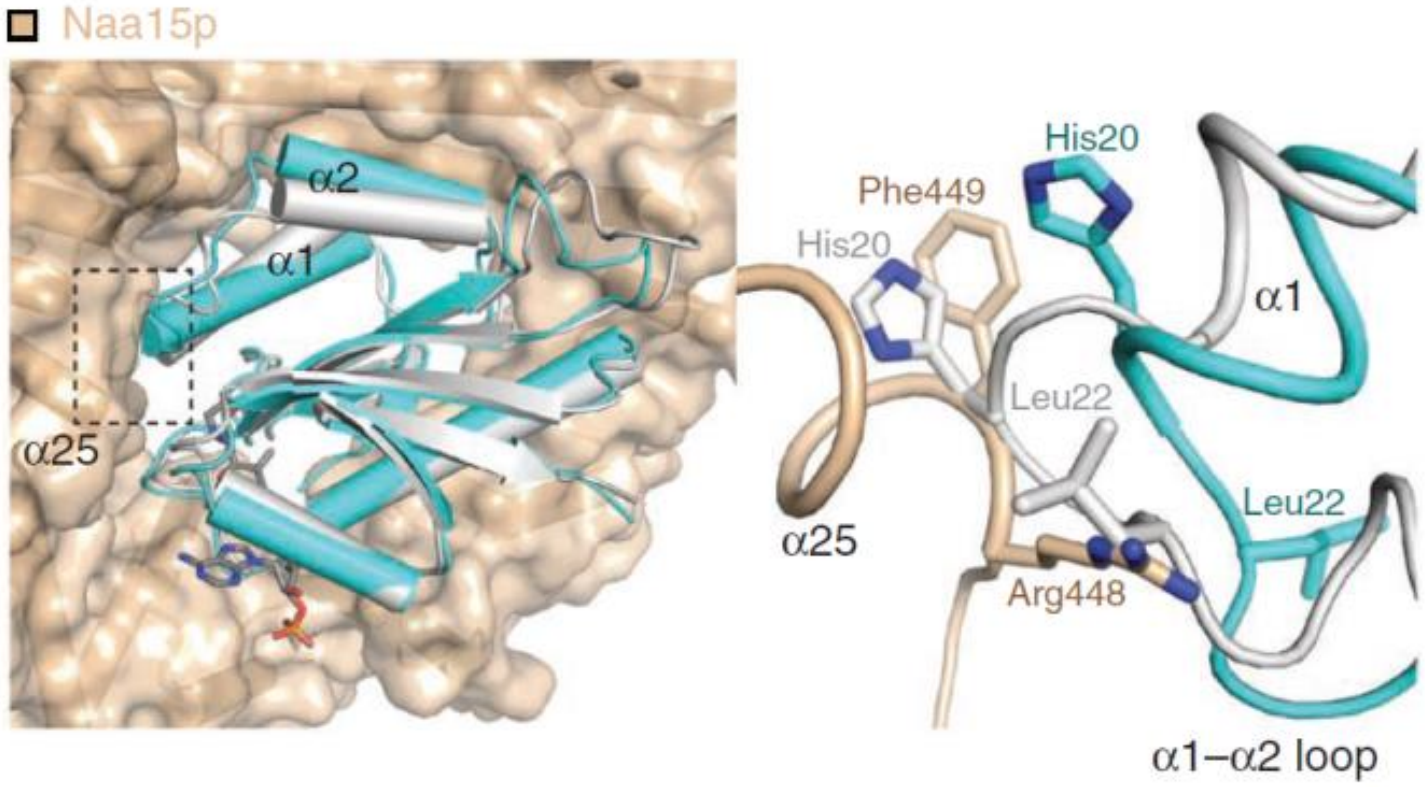
uncomplexed Naa10 vs complexed Naa10 structure

results - Naa10 structure (Naa15-interaction)

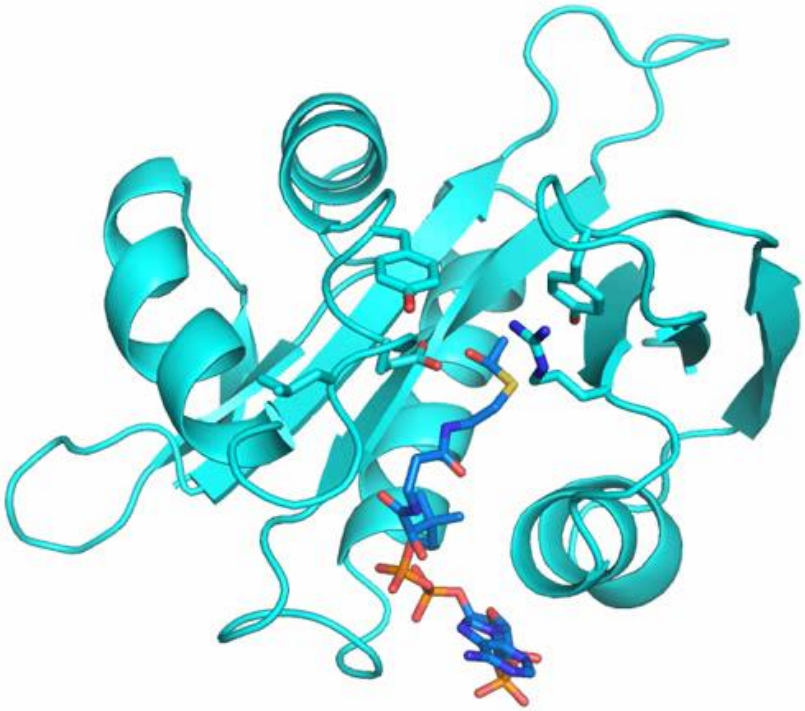


results - Naa10 structure (Naa15-interaction)

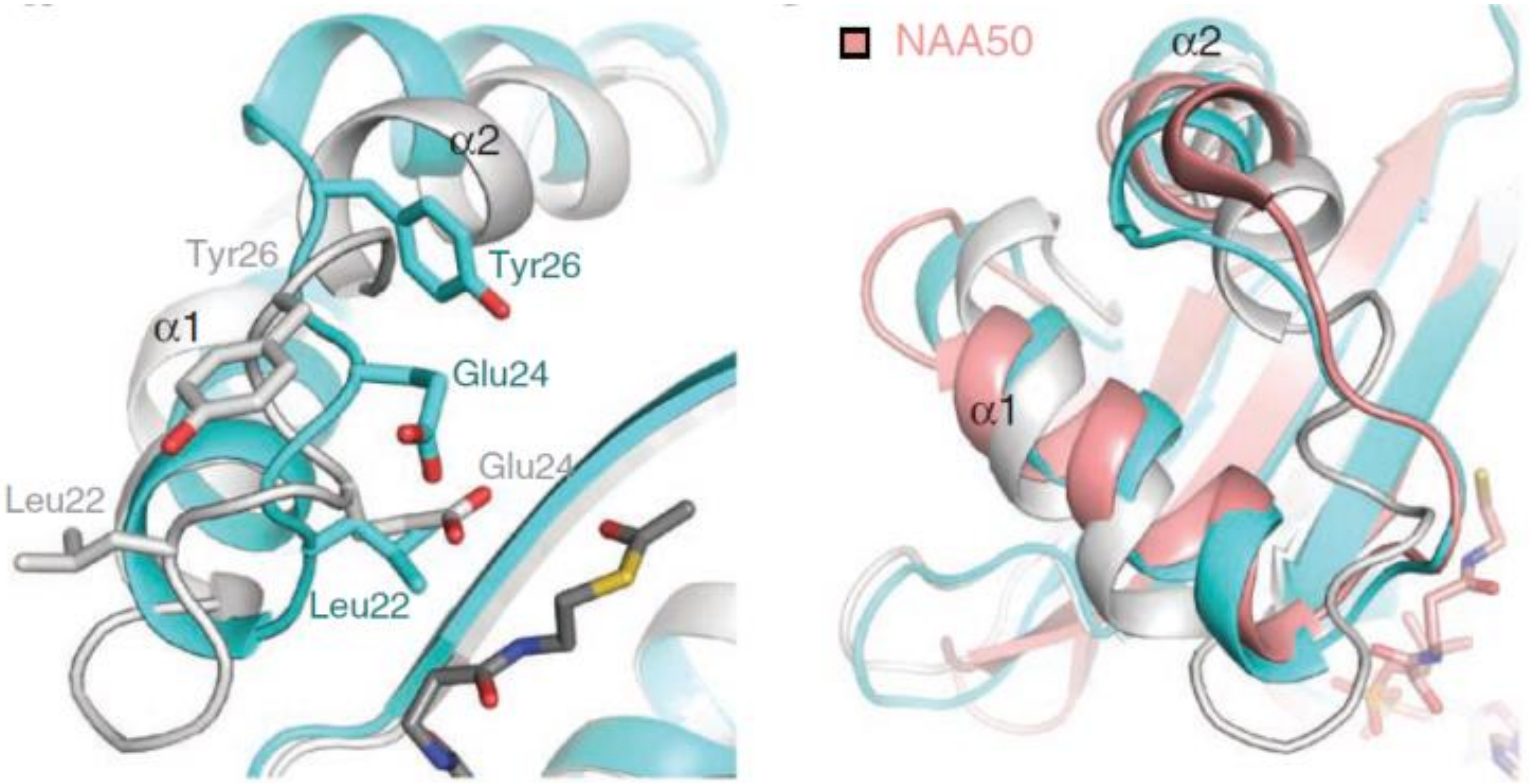


α1-loop-α2 clashes with Naa15 helices

results - Naa10 structure (active site)

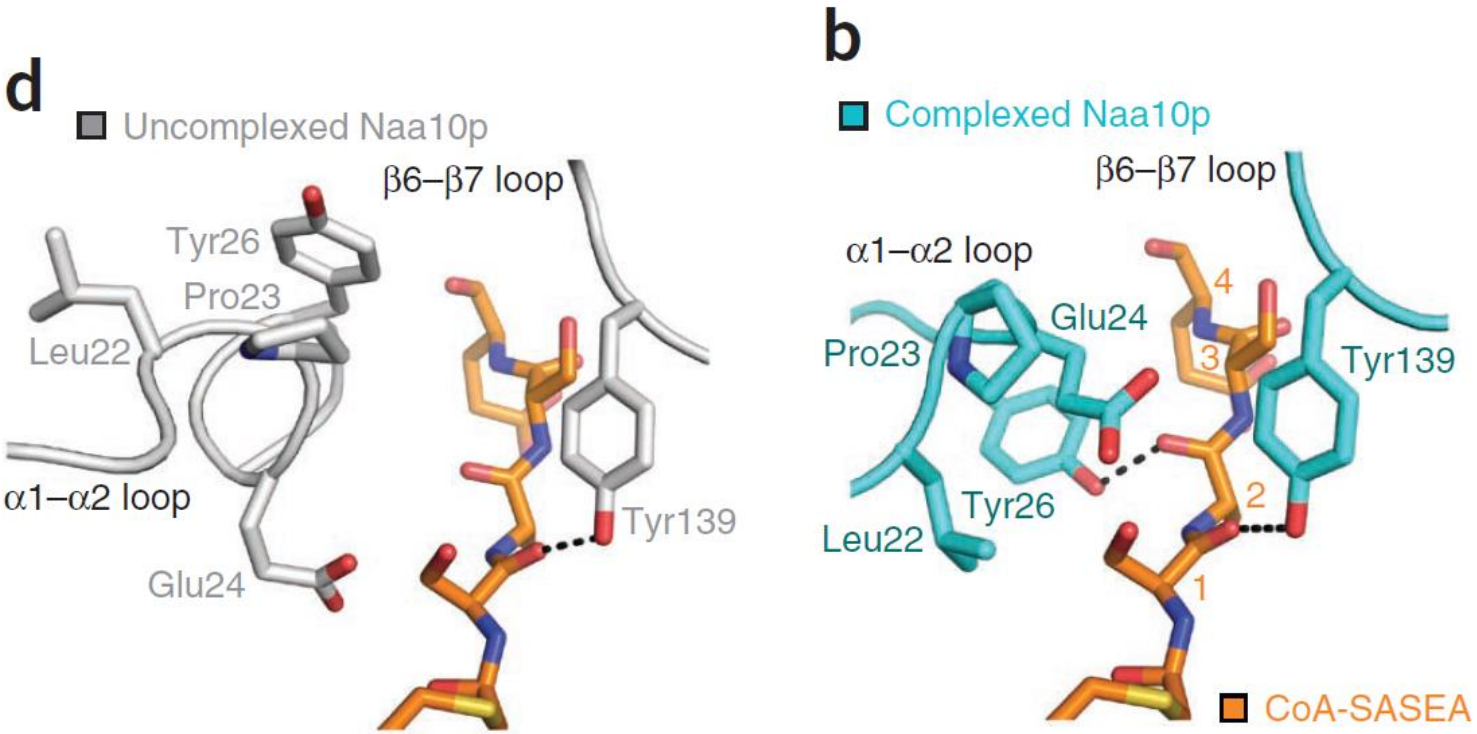


results - Naa10 structure (active site)



Leu22, Tyr26 and Glu24 shift (out) of active site
uncomplexed Naa10 shows higher similarity to Naa50 (pink)

results - enzymology



results - enzymology

Table 2 Catalytic parameters for wild-type and mutant NatA and wild-type monomeric Naa10p with various substrate peptides

Enzyme	Substrate (N terminus)	k_{cat} (s^{-1})	K_m[a] (μM)	K_m[a] (normalized to WT NatA[b])
NatA WT[d]	Ser1	3.0 ± 0.5	340 ± 50	1.0
	Met1	ND		
	Glu1	ND		
NatA-Naa10p mutations				
H20A	Ser1	1.5 ± 0.2	920 ± 110	2.7
L22A[d]	Ser1	0.081 ± 0.006	1,850 ± 190	5.4
P23A	Ser1	2.0 ± 0.1	740 ± 70	2.2
E24A	Ser1	ND		
	Met1	ND		
	Glu1	0.65 ± 0.08	250 ± 60	0.74
E24D	Ser1	0.42 ± 0.04	750 ± 40	2.2
E24Q	Ser1	0.025 ± 0.002	440 ± 50	1.3
Y26A	Ser1	0.047 ± 0.04	730 ± 70	2.1
K29A	Ser1	4.4 ± 0.6	300 ± 40	0.90
Y33A	Ser1	0.44 ± 0.02	510 ± 40	1.5
K59A	Ser1	1.5 ± 0.1	760 ± 70	2.2
E61A	Ser1	1.1 ± 0.1	410 ± 60	1.2
E62A	Ser1	1.1 ± 0.1	590 ± 70	1.7
H72A	Ser1	2.4 ± 0.3	600 ± 70	1.8
R80A	Ser1	0.85 ± 0.04	770 ± 70	2.3
H111A	Ser1	2.0 ± 0.2	330 ± 30	0.97
R113A	Ser1	0.19 ± 0.02	380 ± 30	1.1
Y139A	Ser1	NA	>2,000	>5.9
K29A Y33A	Ser1	0.61 ± 0.07	340 ± 80	1.0
K59A E61A	Ser1	1.7 ± 0.1	520 ± 60	1.5
K59A E62A	Ser1	0.73 ± 0.5	410 ± 30	1.2
NatA-Naa15p mutations				
R448A[e]				
F449A[e]				
F474A	Ser1	0.40 ± 0.04	350 ± 30	1.0
D532A	Ser1	0.86 ± 0.05	540 ± 60	1.6
F533A	Ser1	0.90 ± 0.08	330 ± 40	0.97
F536A	Ser1	1.8 ± 0.6	450 ± 60	1.3
F533A F536A	Ser1	3.0 ± 0.3	500 ± 50	1.5
Monomeric Naa10p WT	Ser1	ND		
	Met1	ND		
	Glu1	0.19 ± 0.02	1,720 ± 250	5.1

[a]K_m values are for the substrate peptide described in the Substrate column. [b]WT, wild type. [c]All normalizations are relative to wild-type NatA catalytic parameters generated with the Ser1 N-terminal substrate. [d]The acetyl CoA K_m was calculated for these variants (WT = 59 ± 5 μM; L22A = 53 ± 6 μM). [e]These mutations disrupted stable complex formation. Where k_{cat} is NA (not applicable), the K_m is >2,000 μM, thus a rate could not be calculated from our assay. Where k_{cat} is ND (not determined), activity could not be detected in our assay. Errors represent s.d. (n = 3).

results - enzymology