Structural insights into GDP-mediated regulation of a bacterial acyl-CoA thioesterase

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Thioesterases catalyze the cleavage of thioester bonds within many activated fatty acids and acyl-CoA substrates. They are expressed ubiquitously in both prokaryotes and eukaryotes and are subdivided into 25 thioesterase families according to their catalytic active site, protein oligomerization, and substrate specificity. Although many of these enzyme families are well-characterized in terms of function and substrate specificity, regulation across most thioesterase families is poorly understood. Here, we characterized a TE6 thioesterase from the bacterium Neisseria meningitidis. Structural analysis with X-ray crystallographic diffraction data to 2.0-Å revealed that each protein subunit harbors a hot dog-fold and that the TE6 enzyme forms a hexamer with D3 symmetry. An assessment of thioesterase activity against a range of acyl-CoA substrates revealed the greatest activity against acetyl-CoA, and structure-guided mutagenesis of putative active site residues identified Asn24 and Asp99 as being essential for activity. Our structural analysis revealed that six GDP nucleotides bound the enzyme in close proximity to an intersubunit disulfide bond interactions that covalently link thioesterase domains in a double hot dog dimer. Structure-guided mutagenesis of residues within the GDP-binding pocket identified Arg93 as playing a key role in the nucleotide interaction and revealed that GDP is required for activity. All mutations were confirmed to be specific and not to have resulted from structural perturbations by X-ray crystallography. This is the first report of a bacterial GDP-regulated thioesterase and of covalent linkage of thioesterase domains through a disulfide bond, revealing structural similarities with ADP regulation in the human ACOT12 thioesterase.

Acyl-CoA thioesterases are a large family of enzymes that catalyze the hydrolysis of the thioester bond between a carboxyl group and a sulfur atom, producing fatty acids and CoA. These enzymes are conserved throughout evolution and are expressed ubiquitously in bacteria, fungi, plants, and mammals, and localized in a range of cellular organelles such as mitochondria, cytoplasm, endoplasmic reticulum, and peroxisomes (1). The structure and function of many thioesterases have been characterized recently from bacteria, plants, and animals (2–7), elucidating important roles in various lipid metabolic pathways through regulating the cellular concentrations of fatty acids, acyl-CoA, and CoASH (8, 9). The mechanistic details through which thioesterases carry out catalysis have also been described (10–18), utilizing either a two-step reaction mechanism involving nucleophilic attack by aspartate or glutamate to produce an enzyme–substrate intermediate (14, 17), or an alternative mechanism involving an acid/base-like catalytic reaction, where the carbonyl carbon atom is attacked directly by an aspartate-activated water molecule to cleave the thioester bond (17).

Although the structure, function, and catalytic reaction mechanisms are being unveiled for a range of thioesterase families, our knowledge of regulation within these enzyme families remains comparatively limited. In the human Them1 thioesterase, which hydrolyzes a range of fatty acyl-CoAs with preference for long chain acyl-CoA molecules, dimerization is induced by fatty acyl-CoAs, coenzyme A (CoASH), ATP, and ADP, and the catalytic activity enhanced by ATP and inhibited by ADP and CoA (19). In human ACOT12, a previous model of ADP and ATP regulation was proposed to be mediated through domain assembly but recently discounted with structural and biophysical evidence identifying a nucleotide-binding site located between two regulatory loop regions, one linking the hot dog domains, and the second involving the C terminus of the protein (4). Human ACOT9 has been shown to be negatively regulated by NADH and CoA, however, neither a structural nor biochemical basis for this regulation has been determined. Regulation of Paal1-type thioesterases revealed an induced fit regulatory mechanism and half-of-sites reactivity, with the binding of substrates inducing small rigid-body rearrangements of the hot dog domains that inhibit binding in two of four possible catalytic sites (10). Overall, these limited and varied snapshots of thioesterase regulation highlight the need for a greater understanding of how these enzymes are regulated to control the cellular concentrations of important metabolites including coenzyme A, and activated fatty acids.
Structural insights into thioesterase regulation

### Results and discussion

**Structure of NmACT**

Fatty-acyl CoA thioesterases play essential roles in lipid metabolism and a wide range of cellular functions, however, regulation of these enzymes remains poorly understood across most thioesterase families. To better understand the structure, function, and regulation of a TE6 family member, we expressed and purified a thioesterase from *Neisseria meningitidis* by affinity and size exclusion chromatography (supplemental Fig. S1), and characterized the enzyme by X-ray crystallography, substrate screening, enzyme kinetic assays, small angle X-ray scattering, and structure-guided mutagenesis.

Protein crystals grown in 100 mM Tris, pH 8.5, and 2 M ammonium phosphate, belongs to space group P22_3 diffracted to 2.0 Å at the Australian Synchrotron macromolecular crystallography beamlines, and the phases solved by molecular replacement using chain A from PDB\(^2\) code 1VPM (33% sequence identity (20)) as a search model. Following model rebuilding and refinement in COOT (21) and Phenix (22), respectively, the final model consisted of residues 5–158, \(R_{\text{work}}\) and \(R_{\text{free}}\) values of 18 and 20%, respectively, and good stereochemistry (Table 1).

The crystal structure revealed each protomer within the enzyme contained a “hot dog”-fold, composed of a five-stranded anti-parallel \(\beta\)-sheet wrapping a central \(\alpha\)-helix (Fig. 1A) (23, 24). The protein structure also revealed an additional C-terminal \(\alpha\)-helix that packs against the \(\beta\)-sheet on the opposite side of the central \(\alpha\)-helix (Fig. 1A). The overall topology of the protomer is \(\beta_1-\alpha_1-\beta_3-\beta_4-\beta_5-\alpha_2-\beta_2\) (Fig. 1B), with the \(\beta\)-strands varying in length from 8 to 16 amino acids, and the \(\alpha_1\) and \(\alpha_2\) helices composed of 22 and 21 residues, respectively.

The asymmetric unit (ASU) contained four protomers, all similar in structure with the greatest root mean square deviation (r.m.s. deviation) of 0.27 Å. The arrangement of these chains in the ASU (Fig. 2A) did not resemble the quaternary structure of any previously described thioesterase, and the contacts between the two hot dog dimers were unlikely to represent a stable biological unit. Examination of all possible oligomeric structures was assessed by characterizing the interfaces both within and outside of the asymmetric unit. Based on the extent of intermolecular interactions from this analysis, the most likely biological quaternary structure was a hexamer and dimer.

### Table 1

Data collection and refinement statistics of NmACT-WT and mutants

| Protein            | NmACT-WT   | NmACT-Cys158-X | NmACT-N24A   | NmACT-D39A   | NmACT-Cys158-X-R93E |
|--------------------|------------|----------------|--------------|--------------|---------------------|
| Wavelength (Å)     | 0.9557     | 0.9786         | 0.9537       | 0.9537       | 0.9762              |
| Resolution range (Å)| 38.1–2.0 (2.1–2.0) | 34.1–2.3 (2.4–2.3) | 36.0–2.0 (2.1–2.0) | 39.8–2.8 (2.9–2.8) | 48.5–2.8 (2.9–2.8) |
| Space group        | P2, 3      | P2, 3          | P2, 3        | P3, 2 1      | P2, 3               |
| Unit cell          | 152.4 152.4 152.4 | 152.6 152.6 152.6 | 152.8 152.8 152.8 | 226.1 226.1 68.3 90 90 120 | 153.2 153.2 153.2 |
| Unique reflections  | 79,515 (7,831) | 52,676 (5,239) | 80,089 (7,963) | 49,406 (4,911) | 28,972 (2,905)      |
| Multiplicity       | 43 (42.0)  | 22 (21)        | 12 (12.2)    | 5.5 (4.8)    | 3.5 (3.4)           |
| Completeness (%)   | 100.0 (100.0) | 100.0 (100.0) | 100.0 (100.0) | 100.0 (100.0) | 97.4 (99.7)         |
| Mean I/σ(I)        | 18.4 (3.8) | 40.4 (14.1)    | 23.6 (7.6)   | 13.9 (5.3)   | 6.5 (2.0)           |
| Wilson B-factor     | 24.4       | 23.0           | 22.1         | 38.5         | 29.7                |
| \(R_{\text{merge}}\) | 0.029 (0.20) | 0.013 (0.050) | 0.020 (0.096) | 0.041 (0.177) | 0.123 (0.574)       |

Here, we provide structural insights into the regulation of an acyl-CoA thioesterase from *Neisseria meningitidis*. Through combined experimental approaches including X-ray crystallography, small-angle X-ray scattering, structure-guided mutagenesis, substrate specificity, and enzyme-activity assays, we have elucidated the binding pocket and regulatory allosteric effect of GDP. Importantly, the structural integrity of all mutants within the active site and GDP binding determinants did not perturb the structure of the enzyme. Overall, this study presents the first structural basis for GDP-mediated regulation within any thioesterase family, and identifies a possible evolutionary link with regulatory mechanisms identified in human thioesterases.

2 The abbreviations used are: PDB, Protein Data Bank; ASU, asymmetric unit; r.m.s., root mean square; SAXS, small angle X-ray scattering.

\(^2\)
formed from three ASU’s each contributing two hot dog subunits (Fig. 2B). This is consistent with the structure analysis using the PDBe PISA server (25), the elution profile of the enzyme during size exclusion chromatography (supplemental Fig. S1) and biological assemblies obtained in different crystal forms (see Table 1). This proposed biological assembly also correlated well with small angle X-ray scattering data (Fig. 3), with the hexameric arrangement producing a $\chi^2$ value of 0.83 (Table 2).

The biological assembly buries a total surface area of 18,878 Å$^2$, and two types of intermolecular interfaces contribute the majority of these interaction interfaces. The first type of interface is between two hot dog subunits creating a “double hot dog,” with the two central $\alpha$-helices arranged parallel (Fig. 4). This interface buries ~2,840 Å$^2$ of surface area, and is mediated by ionic interactions between His$^{30}$:Asp$^{39}$ and Lys$^{141}$:Asp$^{155}$, and 10 hydrogen bonds (supplemental Table S1). The high resolution structure revealed two symmetry related disulfide interactions at the interface, involving Cys$^{157}$ of one hot dog subunit, and Cys$^{158}$ of the other hot dog subunit (Fig. 5). This is the first report of a disulfide bond bridging two hot dog subunits.
Interestingly, we identified a GDP molecule bound at each disulfide bond (Fig. 5), also representing the first report of a GDP-bound thioesterase. Structural analysis revealed the GDP-binding pocket to be composed of Ala\(^{78}\), Asn\(^{81}\), Arg\(^{93}\), Ser\(^{109}\), Tyr\(^{111}\), Arg\(^{138}\), and Lys\(^{141}\) from each hot dog protomer, Ser\(^{157}\) and Cys\(^{158}\) of another hot dog protomer within the double hot dog subunit, and Arg\(^{95}\) from another hot dog subunit outside of the double hot dog subunit (Fig. 6).

In addition to the two GDP molecules bound at each hot dog dimer interface, each dimer also contained two bound CoA molecules, positioned with the terminal sulfur in close proximity to putative active site residues Asn\(^{24}\) and Asp\(^{39}\). Each CoA makes H-bond interactions with Thr\(^{66}\), Phe\(^{64}\), Lys\(^{65}\), Arg\(^{85}\), Thr\(^{86}\), Ser\(^{87}\), Arg\(^{146}\), and Ser\(^{149}\) (supplemental Table S2).

The second of the two major interfaces involve two hot dog domains associating through /H\(^{9252}\) strand one, the central \(\alpha\)-helices, and various loop regions (Fig. 4). Each interaction interface buries 2,650 \(\AA^2\), and the interface is mediated by salt bridge interactions involving Arg\(^{85}\) and Glu\(^{95}\), and 8 hydrogen bonds (supplemental Table S1).

### Enzyme activity and substrate specificity

Because the specificity of the enzyme has not been determined previously, we screened a range of substrates using an established 5,5'-dithiobis(nitrobenzoic acid) assay (26, 27) to identify the substrate specificity. Substrates ranging in carbon chain length from two (C\(_2\)) to 20 (C\(_{20}\)) were screened, with the highest activity observed for acetyl-CoA (Fig. 7); \(K_m = 2.1 \text{ mM}\) and \(K_{cat} = 33 \text{ s}^{-1}\) (Table 3). Catalytic residues within thioesterases vary considerably across different family members (4, 20, 26–29), therefore to establish residues involved in Nm\(\text{ACT}\) activity, putative catalytic residues in close proximity to the terminal sulfur atom in CoA were mutated, and the proteins were assessed for activity. Residues Asn\(^{24}\) and Asp\(^{39}\) were mutated to Ala residues and tested for activity against acetyl-CoA. Both Nm\(\text{ACT-N24A}\) and Nm\(\text{ACT-D39A}\) variants displayed negligible thioesterase activity (Fig. 7), implying these residues are important for catalysis. To confirm that the mutations do not perturb the structural integrity of the proteins, the elution profiles of the mutant enzymes were shown to be identical to that of the wild-type enzyme and the structures were confirmed by crystallography. Both Nm\(\text{ACT-N24A}\) and Nm\(\text{ACT-D39A}\) exhibited the same structures, including subunit and biological assemblies as the wild-type enzyme (r.m.s.

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**Table 2**

**SAXS data statistics**

| Data collection parameters | Australian Synchrotron SAXS/WAXS beamline |
|----------------------------|------------------------------------------|
| Instrument                 | Australian Synchrotron SAXS/WAXS beamline |
| Beam geometry              | 120-\(\mu\)m point source |
| Wavelength                 | 1.033 (Å) |
| Q range                    | 0.009 to 0.54 Å\(^{-1}\) |
| Exposure time              | 18 \(\times\) 1 s exposures |
| Sample flow                | 4 ml/l |
| Concentration range        | 0.07–1.1 mg/ml |
| Temperature                | 283 K |
| Structural parameters      |                                           |
| \(I(0)\) (from P(\(r\))     | 5.45E-03 cm\(^{-1}\) |
| \(R_r\) (from P(\(r\))      | 30.08 (Å) |
| \(I(0)\) (from Guinier)    | 5.41E-03 (cm\(^{-1}\)) |
| \(D_{max}\) (from Guinier) | 81 (Å) |
| Porod volume estimate      | 167.97 (Å\(^{-3}\)) |
| Dry volume calculated      | 127.12 (Å\(^{-3}\)) |
| from sequence              |                                           |
| Molecular mass determination|                                           |
| Partial specific volume    | 0.736 (cm\(^2\) g\(^{-1}\)) |
| Contrast                   | 2.897 (\(\Delta\rho \times 10^{-3}\) cm\(^{-1}\)) |
| Molecular mass \(M_r\) (from I(0)) | 102.1 kDa |
| Protomer \(M_r\) from sequence, 17.3 (protomer) | 103.8 (hexamer) |
| Software employed          |                                           |
| Primary data reduction     | ScatterBrain (Australian Synchrotron) |
| Model intensities computation | CRYSOL |
| Graphics representations   | PyMOL |

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Figure 4. The biological unit of the Nm\(\text{ACT}\) is composed of a hexamer of thioesterase domain protomers exhibiting D3 symmetry (left). The two interfaces that mediate arrangement of the biological assembly are depicted middle and right panels.
deviation <0.50). These active site residues appear to be conserved across a wide range of thioesterase proteins (Fig. 8).

Assessing the role of the Cys\textsuperscript{137}:Cys\textsuperscript{158} disulfide bond and GDP

The presence of a disulfide bond that covalently tethers two hot dog domains in a double hot dog configuration has not been observed previously. The covalent linkage of thioesterase domains is a distinguishing feature between prokaryotic and eukaryotic thioesterases, with prokaryotic thioesterases generally encoding single thioesterase domain proteins, whereas eukaryotic thioesterases harbor fused thioesterase domains (Fig. 9). Therefore, the covalent linkage of thioesterase domains within a double hot dog domain was unexpected, and since GDP was also found to be in close proximity to the disulfide bond, we tested whether disruption of the disulfide interaction and GDP binding may affect activity. Each disulfide bond is composed of Cys\textsuperscript{137} from one hot dog chain, and Cys\textsuperscript{158} of another within the hot dog dimer across the type 1 interface, and since the three residues at the C terminus of the enzyme contained Cys\textsuperscript{158}, Gly\textsuperscript{159}, and Cys\textsuperscript{160}, we introduced a stop
be not essential for activity (29). It therefore remains unclear from an enzyme catalytic perspective, why some hot dog domains are fused, particularly eukaryotic thioesterases, whereas others can form the same oligomeric biological unit and carry out the same reactions from a single thioesterase domain.

The elucidation of a bound GDP molecule orientated with the terminal β-phosphate moiety toward the disulfide bond, suggested a possible role in regulation. Because the GDP co-purified with the enzyme, and high ionic strength buffers, pH trials, and denaturants failed to dissociate the nucleotide, to test the importance of GDP binding on activity, we designed a structure-guided mutation in the GDP-binding pocket to disrupt binding, and assessed for activity against acetyl-CoA. Because the residues that mediate disulfide bond formation also contribute to GDP binding, we examined whether GDP was present in the crystals of NmA CT-Cys$^{158}$-X. Strong density corresponding to GDP was observed (Fig. 10), indicating that this mutation alone was not sufficient to disrupt GDP binding. We therefore used the NmA CT-Cys$^{158}$-X mutant to create additional, structure-guided mutants to disrupt the GDP interaction. We found that whereas the NmA CT-Cys$^{158}$-X contained GDP in the crystal structure, an NmA CT-Cys$^{158}$-X:R93E mutation displaced GDP from the binding pocket (Fig. 10). This establishes Arg$^{93}$ as an important binding determinant in the interaction, and is consistent with the structure analysis identifying ionic interactions between the α-phosphate group of GDP and the positively charged Arg guanidinium group (Fig. 6).

### Table 3

| Enzyme                        | $K_w$ (mM) | $K_{cat}$ (s$^{-1}$) | $K_{cat}/K_w$ |
|-------------------------------|------------|----------------------|---------------|
| NmA CT-WT                     | 2.1 ± 0.5  | 33 ± 6               | 15.6 × 10$^{-3}$ |
| NmA CT-Cys$^{158}$-X          | 1.6 ± 0.5  | 24 ± 6               | 14.4 × 10$^{-3}$ |
| NmA CT-N24A                   | ND$^{a}$   | ND                   | ND            |
| NmA CT-D39A                   | ND         | ND                   | ND            |
| NmA CT-Cys$^{158}$-X:R93E     | ND         | ND                   | ND            |

$^{a}$ ND stands for not determined due to low activity.

codon at Cys$^{158}$ (NmA CT-Cys$^{158}$-X) to ensure complete abrogation of any disulfide formation, and preventing the possibility of Cys$^{160}$ forming a disulfide bond. The NmA CT-Cys$^{158}$-X mutant protein was expressed successfully and eluted from the size exclusion column with a profile similar to that of the wild-type enzyme, indicating that the quaternary structure was also maintained. Additionally, we crystallized NmA CT-Cys$^{158}$-X to examine whether any local differences were present (structural comparisons of mutants presented below). The overall structure was found to be highly similar to that of the wild-type enzyme, with an overall r.m.s. deviation of 0.29 Å. Notably, the C-terminal residues 153-SEDMS caused the NmA CT-Cys$^{158}$-X to be poor electron density, including a higher degree of flexibility in this region, however, the overall position of the C-terminal helices remained identical. We also noted a minor change in the β-bulge, particularly at Lys$^{61}$, which shifts 2.2 Å. Given these minimal local changes, we tested whether these could have an effect on enzyme activity. We found no significant difference in the enzyme activity of NmA CT-Cys$^{158}$-X (Fig. 7; see Table 3; $K_w$=1.6 mM, $K_{cat}$=24 s$^{-1}$), indicating that the disulfide bond does not appear to play a role for activity. This is not unexpected because the covalent fusion of hot dog domains in eukaryotic thioesterases also appears to

![Figure 7. Activity of NmA CT and mutants against acetyl-CoA substrate.](Image)
duced crystals in a different space group was the active site mutant NmACT-D39A, crystallizing in P3121. Details of all data collections and refinement statistics of structures are presented in Table 1. The structural alignment of variants NmACT-Cys158-X, NmACT-N24A, NmACT-D39A, and NmACT-Cys158-X:R93E with NmACT-WT showed high similarity (Fig. 11) in the quaternary structure with r.m.s. deviation values of 0.29, 0.48, 0.50, and 0.57 Å, respectively. We also ensured the elution profiles from analytical size exclusion experiments were identical between wild-type and all variants, which together with crystallography data, ensure that the observed differences in activity are specific rather than the result of perturbations in enzyme structure and/or oligomerization.

Comparison of NmACT with human ACOT12

This is the first report and structural characterization of a prokaryotic thioesterase bound with GDP. One other structure deposited to the PDB has been used to describe nucleotide-binding and regulation, and this was for the multidomain human thioesterase, ACOT12 (4). Although the domain organization in ACOT12, which contains two non-identical hot dog domains and a C-terminal steroidogenic acute regulatory protein-related lipid transfer (START) domain, is markedly different to NmACT, the structural assembly of the thioesterase domains are surprisingly similar (Fig. 12). Notably, in the ACOT12 structure, only three coenzyme A and ADP molecules are present from a possible six sites, which is likely due to the eukaryotic thioesterase containing two fused, non-identical hot dog domains. In contrast, NmACT, which harbors six identical domains in the biological unit, has six GDP and coenzyme A molecules bound at all symmetry related sites (Fig. 12). To test if the location of the coenzyme A and regulatory nucleotide molecules were conserved, the structures of NmACT and ACOT12 were superimposed. We found that both coenzyme A and nucleotide molecules were positioned and orientated in almost identical fashion in both bacterial and human thioes-

**Sequence alignment showing active site residues.**

![Figure 8. Structural alignment of thioesterases deposited to the PDB. The Asn²⁴ and Asp³⁹ residues, identified as catalytically important, are highly conserved.](image)

**Structures of TE6 Thioesterases**

**Prokaryotic**

- HD domain 3D6L
- HD domain 1YLI

**Eukaryotic**

- HD domain HD domain 42V3
- HD domain HD domain START domain 387K

**NmACT (this study)**

- HD domain HD domain 5V3A

![Figure 9. Domain organization of TE6 thioesterases. Prokaryotic thioesterases harbor single thioesterase domains, whereas eukaryotic ones contain fused thioesterase domains. The linkage of thioesterase domains by a disulfide bond may be analogous to a double hot dog domain fusion observed in eukaryotes.](image)

![Figure 10. Dimer of (A) NmACT-WT and (B) truncated version NmACT-Cys¹⁵⁸-X showing the presence of CoA and GDP supported by a 2Fo-Fc annealed omit map contoured at 2σ (green mesh), whereas (C) Cys¹⁵⁸-X:R93E has no GDP supported, by an absence of density from a 2Fo-Fc annealed omit map contoured at 2σ (green mesh).](image)
terases (Fig. 13 and 14). Significantly, residues important for mediating H-bond interactions in the ACOT12-ADP structure, Asn\textsuperscript{252}, Arg\textsuperscript{264}, Ser\textsuperscript{283}, Arg\textsuperscript{312}, and Arg\textsuperscript{313}, were highly conserved in the Nm\textit{ACT} structure (Fig. 14). Although the similar position and orientation of the bound nucleotides in Nm\textit{ACT} and ACOT12 is indicative of a conserved regulatory mechanism, there are notable differences between the two structures, which likely reflects the mechanism through which the two thioesterase domains are bound. In ACOT12, nucleotide regulation was reported to occur through the linker region between the two hot dog domains (residues 154–178), and the C-terminal hot dog domain (4). Interestingly, because Nm\textit{ACT} is a single thioesterase domain, it lacks an equivalent linker region. In this case, the hot dog domains are instead covalently linked through a disulfide interaction, which interacts with the nucleotide, however, we have shown that this disulfide interaction is not strictly required for GDPmediate regulation. Thus, both prokaryotic and eukaryotic thioesterases display nucleotide-mediated regulation but through different mechanisms, as well as contrasting methods of tethering double hot dog domains.

**Conclusion**

This study describes a unique fusion of hot dog domain dimers mediated by disulfide bonds, present within a hexameric thioesterase. A GDP molecule was found positioned at each of these disulfide interaction sites and through structure-guided-mutational analysis, we identified Arg\textsuperscript{93} as an important binding determinant. The R93D mutation prevented GDP binding, and abolished enzyme activity, whereas retaining the
same tertiary and quaternary structural features as the wild-type enzyme (confirmed by X-ray crystallography). This established a clear link between GDP binding and enzyme activity, not described previously in any other thioesterase to date. Mutational analysis of putative active site residues identified Asn^{24} and Asp^{39} as important for catalysis, and these catalytic residues are conserved across many thioesterases. Finally, structural comparisons with the recently elucidated human thioesterase, ACOT12, revealed structural similarities in nucleotide binding. Overall, our study reveal high resolution structural insights into nucleotide binding within thioesterases, which are important for enzyme activity.

**Experimental procedures**

*Expression, purification, and crystallization*

The expression, purification and crystallization of *NmACT*-WT, *NmACT*-Cys^{158}-X, *NmACT*-N24A, *NmACT*-D39A, and *NmACT*-Cys^{158}-X:R93E were carried out as described in our crystallization report (7). All constructs were co-crystallized with CoA in multiple crystallizing conditions as reported for *NmACT*-WT (7). Crystals grown in 100 mM Tris, pH 8.5, and 2 M ammonium phosphate led to complete datasets for *NmACT*-WT, *NmACT*-Cys^{158}-X, *NmACT*-N24A, *NmACT*-D39A, and *NmACT*-Cys^{158}-X:R93E, and diffraction to 2.0, 2.3, 2.0, 2.8, and 2.8Å, respectively. Crystals of *NmACT*-D39A were grown in 0.4 M ammonium phosphate monobasic and diffracted to 2.8 Å.

*Crystal structure determination*

Sparse matrix screening was performed using the hanging drop vapor diffusion method and a 1:1 ratio of protein:reservoir solution. Positive conditions were optimized by varying pH and...
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concentrations of precipitant and protein. Crystals were diffracted at the Australian Synchrotron, and reflections indexed, integrated, scaled, and merged in MOSFLM and AIMLESS (31). Phases were determined by molecular replacement using Bacillus halodurans PDB code 1VPM (33% identity) as a search model for NmACT wild-type (NmACT-WT) and the latter to determine structures of NmACT mutants. Model building and refinement were performed using COOT (21) and Phenix (22), producing models with \( R/R_{	ext{free}} \) of 0.17–0.19 and 0.20–0.21, respectively. The structures have been deposited to the PDB and issued codes 5V3A, SSZZ, 5S2Y, 5T02, and SSZU.

Substrate specificity assay

NmACT substrate specificity was determined spectrophotometrically at 412 nm against a wide range of commercially available acyl-CoA substrates. The enzymatic activity was recorded as the increase in the formation of 2-nitro-5-thiobenzoate anion (TNB\(^2^-\)), a chromogenic substrate that can be measured at 412 nm \( (\varepsilon = 13,600 \text{ M}^{-1} \text{ cm}^{-1}) \) by the reaction of 5,5’-dithiobis(nitrobenzoic acid) with free CoASH (27). Reactions were started by the addition of substrate, and the absorbance at 412 nm followed for 20 min at 25 °C over a range of substrate concentrations. The calculation of specific activity of the enzyme was done using Prism software, where the readings of two independent experiments performed in triplicate were used. The enzyme specific activity is expressed in mmol/min/mg.

Small angle X-ray scattering (SAXS)

Data were collected at the Australian Synchrotron on the SAXS/WAXS beamline using the Pilatus 1M detector. Data were collected over concentration ranges of 0.07–1 mg/ml. For each sample, 50 \( \mu \)l of sample was drawn through a 1.5-mm quartz capillary and exposed to the X-ray beam while moving. To control for radiation damage, 18 \times 1-s exposures were made and compared for evidence of systematic change. The scattering data were collected at 10 °C with a beam energy of 11 KeV and in a Q range from 0.009 to 0.541 \( \text{Å}^{-1} \), multiple images were averaged together and background subtracted. Detector images for each concentration were averaged using Scatterbrain to generate a number of SAXS data sets for subsequent analysis using ATSAS (version 2.4.3) software (32). PRIMUS was used to subtract background scattering from data files and Guinier fits and \( P(r) \) distribution plots were generated using GNOM. CRYSoL was used to generate theoretical curves and compare scattering data with crystal structure data.

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