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Structure of *Arabidopsis thaliana* At1g77540 Protein, a Minimal Acetyltransferase from the COG2388 Family ,[†,‡]

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Abstract

We describe X-ray crystal and NMR solution structures of the protein coded for by *Arabidopsis thaliana* gene At1g77540.1 (At1g77540). The crystal structure was determined to 1.15 Å with an *R* factor of 14.9% (R_{free} = 17.0%) by multiple-wavelength anomalous diffraction using sodium bromide derivatized crystals. The ensemble of NMR conformers was determined with protein samples labeled with ^{15}N and $^{13}C+^{15}N$. The X-ray structure and NMR ensemble were closely similar with r.m.s.d 1.4 Å for residues 8–93. At1g77540 was found to adopt a fold similar to that of GCN5-related N-acetyltransferases. Enzymatic activity assays established that At1g77540 possesses weak acetyltransferase activity against histones H3 and H4. Chemical shift perturbations observed in ^{15}N-HSQC spectra upon the addition of CoA indicated that the cofactor binds and identified its binding site. The molecular details of this interaction were further elucidated by solving the X-ray structure of the At1g77540–CoA complex. This work establishes that the domain family COG2388 represents a novel class of acetyltransferase and provides insight into possible mechanistic roles of the conserved Cys^{76} and His^{41} residues of this family.

As part of an ongoing effort to determine the three-dimensional structures of novel eukaryotic proteins, the Center for Eukaryotic Structural Genomics (CESG[1]) chose the gene product of

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[1]Abbreviations: APS Argonne National Laboratory Advanced Photon Source; At1g77540, the protein product produced from *Arabidopsis thaliana* gene At1g77540.1; CESG, Center for Eukaryotic Structural Genomics; CoA, coenzyme A, GNAT, GCN5-related N-acetyltransferases family; HATs, histone acetyltransferases; r.m.s.d., root-mean-square deviation.

Arabidopsis thaliana At1g77540.1 for structural characterization on the basis of its target selection algorithm. This protein (At1g77540) consists of 103-residues (11.6 kDa) and had less than 25% sequence identity to any other structure deposited in the Protein Data Bank. In addition, no biochemical function had been established for this protein or its close homologs. However, the *Superfamily* server (1) predicted a very weak relationship between At1g77540 and proteins of the acyl-CoA N-acyltransferase superfamily ($E = 2.5 \times 10^{-4}$). GNAT is an extensive superfamily of enzymes that are universally distributed in nature. This diverse set of enzymes uses acyl-CoA to acylate a variety of substrates and has been implicated in a wide range of physiological processes, including DNA transcription, histone acetylation, protein myristoylation, and antibiotic resistance (2). At present, structural information is available in the Protein Data Bank (3) for over forty members of the GNAT superfamily. Typically, these enzymes consist of ~160 amino acids and have a 3-layer ($\alpha\beta\alpha$) sandwich architecture consisting of 7 β-strands and 4 α-helices. The fold is conserved despite the rather low pairwise sequence identity (3–23%) of superfamily members (2).

Here we present the X-ray crystal structure at 1.15 Å resolution and the NMR solution structure of the At1g77540. The structures confirm that At1g77540 is a member of GNAT superfamily; specifically, the structures exhibit structural similarity to members of the GCN5-related N-acetyltransferase family of enzymes. We also show that At1g77540 binds acetyl coenzyme A (AcCoA) by mapping interactions of this cofactor with the protein in solution by NMR and by solving the X-ray structure of the At1g77540 protein-CoA complex at 2.06 Å resolution. Finally, we demonstrate that At1g77540 has a detectable level of acetyltransferase activity with histone substrates.

EXPERIMENTAL PROCEDURES

Expression and Protein Purification

Standard CESG pipeline protocols were used for cloning (4), protein expression (5), protein purification (6) and overall information management (7). Briefly, the At1g77540 protein was produced from 2 liters of chemically defined auto-induction medium (8). [U-^{13}C, ^{15}N]-protein for NMR investigations was produced by modifying the auto-induction growth components to include isotopically labeled compounds, $^{15}NH_4Cl$, [U-^{13}C]-glucose and [U-^{13}C]-glycerol, as the nitrogen and carbon sources. The details of the procedure have been described elsewhere (9).

Protein Crystallization

Crystals of At1g77540 were grown by the hanging drop method from 10 mg mL^{-1} protein solution in a buffer (50 mM NaCl, 3 mM NaN$_3$, 0.3 mM TCEP, 5 mM MES pH 6.0) mixed with an equal amount of well solution (29% MEPEG 5000, 100 mM sodium citrate, 100 mM PIPES pH 6.5) at 4 °C. Crystals typically grew as square plates with dimensions of about 200 μm × 200 μm × 30 μm. The crystals of At1g77540 belong to space group P2$_1$ with unit cell dimensions of a = 27.4 Å, b = 60.6 Å, c = 29.4 Å and β = 91.5°. Crystals were cryoprotected at 4 °C by soaking in a solution containing 30% MEPEG 5000, 100 mM sodium citrate, 100 mM PIPES pH 6.5 supplemented with increasing concentrations of glycerol, up to a final concentration of 20% glycerol. To prepare derivative crystals for phasing purposes, cryoprotected crystals were soaked for additional 40 seconds in the final cryoprotectant solution supplemented with 1 M NaBr.

X-ray Data Collection

X-ray diffraction data for bromide-soaked crystal were collected at sector 32-ID-B of the Argonne National Laboratory Advanced Photon Source (APS). An initial X-ray fluorescence scan of the crystal indicated a maximum of the bromide anomalous signal $E_{BULK-PEAK}$ =

13,479.2 eV and maximum dispersive differences at $E_{BULK-EDGE} = 13,477.3$ eV. These values, however, characterized the signal of the bulk bromide ions located in the solvent channels of the crystal. It was not immediately clear at what energy the maximal anomalous signal from ordered bromide ions could be obtained. The first dataset was collected at presumed peak energy 13,480 eV. Next, a dataset at high-remote energy of 13,580 eV was collected. Upon completion of data collection an analysis of the amount of anomalous signal in both datasets was performed by using the REFLECTION_STATISTICS utility of PHENIX (10). It became clear that the amount of anomalous signal was higher in the dataset collected at the high-remote energy than in that collected at the presumed peak energy (4.2% vs. 2.8%). Also, a larger number of (in retrospect, correct) anomalous sites were identified by HySS from the high-remote dataset than from the other (5 vs. 3) (11). This observation suggested that the energy maximizing the anomalous signal of ordered bromides was higher than E = 13,480 eV. Therefore, an additional dataset was collected at $E_{PEAK} = 13,484$ eV. The strategy proved successful: the new dataset had 5% anomalous signal, and 8 (in retrospect, correct) anomalous sites could be identified. Because the crystal showed no detectable radiation damage, additional datasets were collected at the low-remote energy E = 13,380 eV.

X-ray Structure Determination

The datasets of diffraction images were integrated and scaled using HKL2000 (12). The partial bromide substructure of derivatized At1g77540 crystals was determined using HySS (11). The protein structure was phased from 4-wavelength MAD data in CNS (13) using 5 consensus anomalous sites. Initial phase information obtained from CNS to 2.5 Å was further improved and extended to the 1.6 Å resolution of the low-remote dataset by electron density modification. The automatic tracing procedure of ARP/wARP (14) produced an initial model with 86% residues placed of which 93% had side-chains assigned. The structure was completed by alternate cycles of manual building in XFIT (15) and refinement in REFMAC5 (16) against another low-remote dataset extending to 1.15 Å resolution. All refinement steps were monitored by calculating an R_{free} value based on 5.1% of the independent reflections. The stereochemical quality of the final model was assessed by PROCHECK (17) and MOLPROBITY (18) (Table 1). Refined coordinates were deposited in RCSB protein databank (3) with accession number 1xmt. The figures were prepared using PYMOL (19).

Structure of At1g77540–CoA Complex

At1g77540 crystals were soaked overnight at 4 °C in 29% MEPEG 5K, 100 mM sodium citrate, 100 mM PIPES pH 6.5 supplemented with 9 mM acetyl-CoA. The crystals were cryoprotected with Fomblin 2500 (Aldrich, Saint Louis, MO). X-ray diffraction data for the complex were collected at NE-CAT 8BM beamline at APS and processed with HKL2000. The structure of the complex was solved by molecular replacement in MOLREP (20), manually adjusted with COOT (21) and refined in REFMAC5.

NMR Data Collection

NMR spectra used for the structure determination were collected at 25 °C on Varian INOVA 600 MHz instruments equipped with a 5 mm z-shielded gradient ^{1}H-^{13}C-^{15}N triple resonance probe at NMRFAM (University of Wisconsin, Madison). The NMR sample conditions for At1g77540 consisted of ≈1 mM protein in 10 mM KH_2PO_4, 50 mM KCl, 3 mM NaN_3, 10% D_2O, pH 6. NMR data sets used for backbone resonance assignments included: 2D ^{1}H-^{15}N HSQC, 3D HNCO, 3D HNCACB, 3D CBCA(CO)NH. NMR data sets used for side chain assignments included: 2D ^{1}H-^{13}C HSQC, 3D HBHA(CO)NH, 3D HC(CO)NH, 3D C(CO)NH, 3D H(C)CH TOCSY and 3D (H)CCH TOCSY. All NMR spectra were processed with NMRPipe (22) and with NMRView (23). Spectra used in the CoA binding study were recorded at 25 °C on a 500 MHz Bruker DMX spectrometer equipped with a triple-resonance

CryoProbe[TM] at NMRFAM. The spectra were processed and analyzed using the Bruker XINNMR software.

NMR Structure Determination

The initial ^1H-^{15}N HSQC spectrum revealed resonances of uniform shape and intensity consistent with a well folded protein. The At1g77540 protein studied consisted of 103 residues with 6 internal prolines. Of 97 backbone amide cross peaks expected in the ^1H-^{15}N-HSQC spectrum, 93 were observed; thus the protein was thus considered to be amenable to high-throughput NMR structure determination. Two residues, Gly[50] and Gly[52] displayed extremely weak cross-peak intensity in the ^1H-^{15}N-HSQC spectrum. The automated assignment program PISTACHIO (24) was used to assign 95% of backbone resonances and 70% of ^{13}C-side chain resonances. The remaining resonances were assigned by standard manual NMR methods. Secondary structural elements were identified by combined analysis of assigned chemical shifts and patterns of NOEs from the ^{15}N- and ^{13}C-edited NOESY spectra.

NMR Structure Calculation

Initial NOE assignments in the ^{15}N-edited NOESY-HSQC (τ_{mix} = 100 ms) and 3D ^{13}C-edited-NOESY-HSQC (τ_{mix} = 100 ms) data sets, including those indicative of secondary structural elements, were performed manually. These assignments were then used as preliminary input for automated NOE assignments. Structure calculations were performed with CNS (13) using ARIA 1.2 setup and protocols (25,26). Subsequent addition and/or correction of NOE assignments were performed manually. One-hundred conformers were generated initially, and the best 20 of these, selected by the ARIA algorithm on the basis their agreement with distance input restraints, were used for NOE calibration and assignment. This iterative process was repeated until a final calculation produced 20 converged, low energy structures. A total of 1925 unambiguous nuclear Overhauser effect derived restraints (NOEs) and 172 ambiguous NOEs, assigned by ARIA1.2 or by manual intervention, served as the foundation of the final structure calculation (Table 2). Additional terms in the calculation included 87 dihedral angle constraints derived from TALOS (27) and 56 hydrogen bond constraints derived from a combination of CSI and NOE analysis. These twenty structures were subjected to a final refinement protocol in which a physical force field and explicit water solvent were added to experimental restraints within the ARIA program. The final twenty structures of At1g77540 were validated by PROCHECK NMR (17). The NMR structure was deposited in the Protein Data Bank (3) under PDB # 2EVN; NMR data were deposited in the BioMagResBank (28) under BMRB # 6338.

CoA NMR Titration

A ^1H-^{15}N-HSQC spectrum of At1g77540 collected under the above solution conditions was used for reference. A 50 μl aliquot of a 6 mM stock solution of CoA was added to the protein solution to make the 1:1 (CoA:At1g77540) mixture. The pH was adjusted to 6.0 by addition of 3 μl of 0.1 M HCl. A ^1H-^{15}N-HSQC spectrum of this sample was then collected under conditions identical to the reference.

Acetyltransferase Assays

Aliquots of At1g77540 were dialyzed for 6 h into buffer containing 50 mM Tris pH 7.0, 150 mM NaCl, and 1 mM DTT. The protein concentration after dialysis was determined by Bradford assay to be 4.4 mg mL^{-1}. Aliquots were frozen in liquid nitrogen and stored at −20 °C until use. Acetyltransferase activity was measured by using a filter-binding assay as described previously (29). Briefly, reactions were carried out in 50 mM Tris, 50 mM Bis-Tris, 100 mM acetate buffer (1x TBA) pH 7.0 containing 1 mM DTT, 100 μM acetyl-CoA (4.95 μCi [^3H]-acetyl-CoA), and 6 μM–5 mM amino acid or peptide substrate. Enzyme concentration

in the final reaction mixtures was 0.08 mg mL^{-1}. Assays were performed at 25 °C. Data were visualized in KaleidaGraph (Synergy Software, Reading, PA).

RESULTS

At1g77540 Structure Statistics

The crystal structure of At1g77540 was solved to a resolution of 1.15 Å by four-wavelength multiwavelength anomalous diffraction (MAD) using sodium bromide derivatized crystals. Data collection, refinement and model statistics are summarized in Table 1. The final model describes a monomer in an asymmetric unit, containing residues 5–99. In addition, 11 bromide ions and 125 water molecules were built into the final structure.

Independently and in parallel to the crystal structure, we solved the solution NMR structure of At1g77540. A summary of experimental restraints and statistics for the final ensemble of conformers is shown in Table 2. The root-mean-square deviation (r.m.s.d.) for backbone atoms of residues 8–98 of the final ensemble relative to the average structure was 0.36 Å. Measurements of amide backbone ^{15}N-T$_2$ relaxation times (data not shown) gave an average value of 88 ± 15 ms. This value is consistent with At1g77540 being monomeric in solution; this fact is also corroborated an apparent monomeric state of At1g77540 in the crystals.

At1g77540 Fold

The three-dimensional structure of At1g77540 revealed that this protein belongs to the αβ-class of proteins with 2-layer (αβ) sandwich architecture (Figure 1A). The central feature of this protein is a mixed 5-stranded β-sheet formed by four antiparallel β-strands (*A*, *B*, *C* and *D*), and β-strand *E* parallel to β-strand *D* (Figure 1A). A single turn 3$_{10}$-helix *1* forms in the loop connecting β-strands *A* and *B*. A short α-helix *2* is located in the loop connecting β-strand *D* and the central "signature" α-helix *3*, which is grasped by the convex surface of the central β-sheet. Two additional helices *4* and *5* are located in the carboxyl-terminal part of the protein. Helix *4* is aligned at approximately 90° relative to helix *3*.

Comparison of X-ray and NMR Structures

A ribbon trace of the final ensemble of 20 NMR conformers chosen to represent the solution structure is shown in the same orientation as the crystal structure of At1g77540 (Figure 1B). Comparison of the X-ray structure and the representative NMR structure over all backbone heavy atoms in the structured residues 8–93 gave an r.m.s.d. of 1.4 Å. This indicates that the overall folds are in good agreement. Slight differences in the two structures were observed in the regions of defined secondary structure. Whereas all elements of secondary structure calculated in the NMR study were observed in the X-ray structure, the two single-turn helices *1* and *2*, and helix *5* seen in the X-ray structure (Figure 1B) were not defined in the NMR structure (Figure 1). These regions span residues 12–14 in the loop between β-strands *A* and *B*, residues 45–48 following the β-strands *D*, and residues 88–93 close to the carboxyl-terminus of the protein. (For better orientation a stereo ribbon trace of the X-ray At1g77540 structure with every 10th residue highlighted is presented in Figure 1C.) The lack of clear secondary structure in these regions of the NMR structure likely stems from backbone flexibility, as often is observed in loops and termini of proteins in solution. Recalculation of the backbone r.m.s.d. between the X-ray and NMR structures using only those residues in conserved secondary structural elements revealed a value of 1.2 Å, which supports the notion that the deviation in the backbone coordinates stems from inherent flexibility in the loops and the carboxyl-terminal region of the protein. Several amino- and carboxyl-terminal residues (1–5 and 99–103, respectively) are poorly defined in the NMR ensembles. These segments correlate well with highly disordered regions that lacked interpretable electron density in the At1g77540 X-ray map.

Sequence Homology

Analysis of the At1g77540 sequence by profile alignment methods revealed that this protein shows very weak similarity to the acyl-CoA N-acyltransferase superfamily ($E = 5.4 \times 10^{-5}$) (1). A *Conserved Domain Database* (30) search suggested that At1g77540 belongs to the COG2388 family of predicted acetyltransferases ($E = 6 \times 10^{-4}$). The COG2388 family is related to Pfam00583, which is known as the GCN5-like N-acetyltransferase (GNAT) family. The GNAT family members are important for the regulation of cell growth and development; many of them are involved in the histone acetylation and chromatin remodeling (2). Sequence alignment of twenty COG2388 family members revealed two absolutely conserved residues, Gly^{52} and Cys^{76}, and several highly conserved residues, including Phe^{17}, His^{41}, Thr^{42}, Val^{44}, Gly^{50}, and Pro^{74} (Figure 2). All these residues are shown in Figure 1*C* in ball-and-stick representation. Residue Gly^{52}, which corresponds to the key residue conserved in the GNAT superfamily, is located in the conserved (Arg/Gln)-*X*-*X*-**Gly**-*X*-(Gly/Ala) motif, which is implicated in the binding of acetyl-CoA (31). The motif is located in the loop connecting helices *2* and *3* (Figure 1A) of At1g77540 that contains residues Arg^{49}-Gly^{50}-Leu^{51}-Gly^{52}-Leu^{53}-Ala^{54}. The importance of Cys^{76} and His^{41} is discussed below.

Structural Homology

To confirm that At1g77540 belongs to the GNAT superfamily we submitted the 3D structure to the *DALI* (32) and *VAST* servers (33). The top homologs identified by both servers were, indeed, established or hypothetical members of the GNAT superfamily. The closest structural homolog found by *DALI* was a hypothetical Gcn5-like N-acetyltransferase from *Staphylococcus aureus* with *Z*-score of 10.7, r.m.s.d. 2.6 Å and 25% sequence identity over 87 aligned residues (PDB # 1r57). The top homolog identified by *VAST* was a histone acetyltransferase Hat1 from *Saccharomyces cerevisiae* with *VAST*-score of 9.3, r.m.s.d. 1.6 Å and 12.5% identity over 72 aligned residues (PDB # 1bob; (34)). At1g77540 showed significant structural similarity to more than forty additional established or putative acyltransferases. Interestingly, not a single residue within the structurally aligned regions of At1g77540 and its top ten homologs identified by *VAST* was fully conserved. This suggests that very diverse protein sequences can result in the fold adopted by the GNAT superfamily.

Structural Alignment

A structural alignment of At1g77540 and Hat1, the top homolog identified by *VAST*, revealed that both structures contain the "signature" helix wrapped by a central β-sheet (Figure 3a) characteristic of the GNAT superfamily (2). Also, both proteins contain a short carboxyl-terminal β-strand (*E* in At1g77540) juxtaposed in antiparallel direction to a β-strand (*D* in At1g77540), which possesses the universally conserved β-bulge (2). The backbone amide of the residue next to the β-bulge (analogous to Thr^{42} in At1g77540) was found to form a hydrogen bond to the carbonyl oxygen of the thioester group of bound acetyl-CoA. At1g77540 and Hat1 differ substantially in the amino- and carboxyl-terminal regions. The amino-terminal region of Hat1 (residues 1–121, not shown in Figure 3a) forms an independent domain attached to the central acetyltransferase domain by a β-strand antiparallel to the β-strand that corresponds to *A* in At1g77540. In comparison to At1g77540, Hat1 has a much longer carboxyl-terminal region, which forms a helical-bundle-like satellite domain (Figure 3a). In addition, structural elements corresponding to *motif C*, a conserved sequence previously defined for GNATs (35, 36), are missing in At1g77540. In Hat1 and other GNATs this motif forms a long helix that runs perpendicular to the strands of the central β-sheet. In At1g77540, this is represented only by a short connection between residues 13 and 15 (Figure 3a). The absence of structural elements corresponding to *motifC* and the additional upstream residues in At1g77540 leaves one face of the central β-sheet fully exposed (Figure 3a). The remaining sequence *motifs A*, *B*, and *D* defined for GNATs (35, 36) are structurally present in At1g77540 (Figure 2). It thus

appears that At1g77540 presents a "minimal" version of the acetyltransferase domain. In summary, fold analysis by *DALI* and *VAST* as well as the detailed inspection of structural features constitutive for acetyltransferases confirmed that At1g77540 is a member of the GNAT superfamily (2).

Structural alignment of At1g77540 with the only other COG2388 family member (PDB # 1r57) is displayed in Figure 3b. The two proteins share an identical fold and both reveal the common β-bulge, a structural trait which is common for GNAT superfamily members.

Acetyltransferase Activity Measurements

Both the sequence and structure homology searches suggested that At1g77540 is likely an acetyltransferase with unknown substrate specificity. To test whether At1g77540 functions as a protein acetyltransferase, we performed enzymatic assays utilizing radiolabeled acetyl-CoA and various substrates. The results showed that At1g77540 displays substrate specificity, because some substrates were acetylated while others were not. Observed acetylation of histones H3 and H4 as substrates (Figure 4) suggests that At1g77540 catalyzes protein acetylation. The rate determined for histone H3 was 0.04 nmol min^{-1} mg^{-1} enzyme and for histone H4 was 0.015 nmol min^{-1} mg^{-1} enzyme. At1g77540 did not exhibit significant acetyltransferase activity with a number of other substrates tested (data not shown): tubulin, lysine, BSA, histones H2A, and H2B, tubulin, and other small histone-based peptides.

Mapping of the CoA-binding Site

To provide evidence that At1g77540 binds (acetyl)-CoA in solution and to map the (acetyl)-CoA binding site, we compared chemical shifts from ^1H-^{15}N-HSQC spectra collected in the absence and presence of an equimolar amount of CoA. This strategy can be rationalized because *motif A* of the GNAT family, which is structurally conserved in At1g77540, supports the binding of acetyl-CoA through a network of hydrogen bonding interactions to backbone amide groups (37). Therefore, interactions between At1g77540 and CoA should be manifested by changes in resonance positions of backbone amide groups. The assigned HSQC spectrum of At1g77540 is shown in Figure 5A. Overlay of ^1H-^{15}N-HSQC spectra collected in the absence and presence of equimolar CoA revealed that the positions of 24 NH-backbone resonances are affected by CoA addition (Figure 5B). The affected residues were mapped onto the surface of At1g77540 (Figure 6A). The vast majority of these residues are located within the cleft formed by the carboxyl-terminal end of β-strand *D* (reside 42 and 44), the following loop (residues 47, 50–52), N-terminal region of helix *3* (residues 54, 55, 57 and 58), and helix *4* (residues 76, 78, 82–84, and 86).

At1g77540-CoA Complex

We subsequently characterized the complex by reference to the 2.06 Å resolution X-ray structure of At1g77540 crystals soaked in acetyl-CoA. Data collection, refinement and model statistics are summarized in Table 1. As expected, the cofactor bound within the cleft identified by the NMR experiment described above (Figure 6A). The bound cofactor adopts a *U*-like overall conformation that is stabilized by several hydrogen bonds to At1g77540, electrostatic interactions with charged residues, and hydrophobic contacts (Figure 6B). Specifically, the side chains of Ser^{77} and Thr^{82} form hydrogen bonds to the adenine ring; the side chain of Arg^{86} is in electrostatic contact with the 3′-phosphate group; the side chain of Tyr^{78} and the amide nitrogen of Gly^{52} and Ser^{55} form hydrogen bonds to the 5′-phosphate group; the amide nitrogens of Gly^{50} and Ala^{54} and a water molecule, which can form hydrogen bonds to the polar backbone atoms of any of residues Lys^{48}, Leu^{51}, Gly^{52}, and Leu^{53}, form hydrogen bonds to the phosphate of the 4-phosphopantothenic acid portion of the cofactor; the amide nitrogen of Val44 forms a hydrogen bond to the amide carbonyl of the 4-phosphopantothenic acid portion of the cofactor; and finally the carboxyl oxygen of Thr^{42} forms a hydrogen bond to the

nitrogen within the β-mercaptoethylamine portion of the cofactor. Comparison of the At1g77540 structure with that of At1g77540–CoA complex revealed a conformational change in the loop that spans residues 40–54 (Figure 6C). The largest displacement of the main chain occurs at Gly[50], and the sidechain of Arg[49] shows the most pronounced change in conformation. The electron density of the cofactor is well defined from the adenine ring to the C^β of the β-alanine portion of the 4-phosphopantothenic acid. From that point, however, the cofactor appears to adopt multiple conformations. We modeled two alternative conformations of a CoA into the residual electron density. Interestingly, acetyl-CoA used to prepare complex with At1g77540 was apparently hydrolyzed (or enzymatically converted to CoA) in the process of crystal derivatization. We could not identify electron density corresponding to the acetyl group of acetyl-CoA. The sulfur atom of the thiol group of CoA resides close (3.1 Å) to the sulfur atom of absolutely conserved Cys[76].

DISCUSSION

The X-ray and NMR solution structures of At1g77540 presented in this study clearly indicate that this protein adopts a fold similar to that previously observed in members of the GNAT superfamily. Primary sequence analysis of At1g77540 revealed that it belongs to the biochemically uncharacterized COG2388 family, which shows sequence relationship to the GNAT family of histone acetyltransferases (HATs) (30). We investigated this relationship in more detail and established that At1g77540 is indeed capable of binding acetyl-CoA as well as acetylating histones H3 and H4. The binding of acetyl-CoA is an absolute requirement for GNAT superfamily enzymes, and the preference for histones H3 and H4 is characteristic of eukaryotic GNAT family enzymes such as PCAF and GCN5 (38). The observed rate of histone acetylation by At1g77540 is, however, extremely low compared to other GNAT family HATs such as GCN5 (29) suggesting two possibilities: 1) histones are not the major substrate for At1g77540 and/or 2) At1g77540 requires other protein subunits or cofactors. In fact, nearly all known eukaryotic HATs function as large multiprotein complexes, which allows them to bind and recognize substrates more effectively than the catalytic subunit alone (39–41). At1g77540 is considerably shorter than other acetyltransferase domains, and its structure revealed what could be considered a "minimal" acetyltransferase fold. The main structural difference is that At1g77540 lacks the *motif C* features characteristic of GNATs. This "minimal" acetyltransferase thus may not provide the full range of interaction needed for specific binding and instead may require at least one partner to function efficiently.

There are two main general mechanisms of acetyl group transfer. One mechanism involves formation of an acetyl-enzyme intermediate and requires formation of two consecutive binary complexes to transfer the acetyl group to a substrate. The second mechanism requires formation of a ternary complex involving enzyme, acetyl-CoA and substrate. In the second case, the enzyme plays a role of a scaffold and provides residues that facilitate catalysis through direct attack of the substrate on the bound acetyl-CoA. Both types of mechanisms have been described for HATs: 1) yeast GCN5, a GNAT family enzyme, was shown to perform catalysis through a ternary complex, with Glu[173] being responsible for deprotonation of histone lysine residues in order to form a nucleophile for direct attack of acetyl-CoA (29); 2) MYST family acetyltransferase Esa1 was shown to form an acetyl-enzyme intermediate, with Cys[304] being acetylated. In addition, Glu[338], acting as a general base, was shown to be required for catalysis (42).

Inspection of the structure of At1g77540 and its complex with CoA revealed three interesting features in the putative active site of this enzyme that may be relevant to other members of the COG2388 family. 1) The protein backbone connecting strand E and helix 4 adopts a unique conformation compared to other acetyltransferases, such as Hat1 and Esa1 (PDB # 1bob and PDB # 1mja, respectively) (Figure 7). This unique conformation results in closing of the cavity

that usually accommodates the acetyl group of acetyl-CoA. 2) Instead, the strictly conserved Cys[76] of the COG2388 family is located in this area (Figure 2). 3) A highly conserved His[41] of the COG2388 family also maps to the putative active site (Figure 2). Whereas the proposed active site of At1g77540 exhibits obvious differences from those other known acetyltransferases, the loop connecting helices 2 and 3 of At1g77540 (highlighted by the black arrow in Figure 7) shares common structural features with other family members. This loop region, which is part of motif A, forms extensive hydrogen bonds with the pyrophosphate portion of CoA. Thus, it appears that local backbone conformations within this important CoA binding site are highly conserved.

We propose that At1g77540, and other COG23388 family enzymes, catalyze acetyl group transfer through formation of an acetyl-enzyme intermediate. According to our hypothesis, the strictly conserved Cys[76] acts as a nucleophile that is acetylated in the first step of the reaction cycle (Figure 8). Cys[76] is somewhat buried but may be deprotonated under physiological conditions. We found no residue in the vicinity of Cys[76] that could be involved in direct deprotonation of this cysteine. The hydroxyl oxygen of the highly conserved Thr[42] is located within 3.1 Å from the sulfur atom of Cys[76] and could provide further stabilization of the side chain conformation of Cys[76]. His[41] could be involved in the formation of the acetyl-enzyme intermediate. The imidazole nitrogen of His[41] is located 3.5 Å from the sulfur of CoA in the At1g77540-CoA complex. On the basis of this geometry, we propose that the protonated form of His[41] could be involved in the protonation of the acetyl-CoA on the sulfur atom, thus destabilizing the S–C thioester bond and making CoA a better leaving group (see Figure 8). Alternatively, at this stage of the reaction cycle, His[41] could be involved in the stabilization of the oxyanion of the tetrahedral intermediate. More importantly, however, His[41] may act as a general base during deprotonation of a substrate during the second transfer of the acetyl group. Depending on the identity of physiological substrate, His[41] could activate primary amines in a way analogous to the histone lysine activation by Glu[173] of GCN5, or it could activate hydroxyl groups as observed in the catalytic mechanism of chloramphenicol acetyltransferase (PDB # 3cla), where His[195] acts as a general base to abstract a proton from the primary hydroxyl group of chloramphenicol (43). It is of interest to note that the catalytically active Cys[304] of Esa1 is found in the location structurally equivalent to His[41] of At1g77540, and not Cys[76] of At1g77540. In addition, the location of Glu[338] of Esa1 corresponds approximately to that of Ser[75] of At1g77540, which is clearly not a conserved residue in COG2388. Similarly, the catalytically important Glu[173] of yeast GCN5 corresponds structurally to Asp[38] of At1g77540, another poorly conserved residue in COG2388. Taken together these comparisons suggest that At1g77540 likely represents a novel adaptation of the acetyltransferase fold tuned for a currently unknown substrate. Testing of the proposed mechanism will depend on identification of the true physiological substrate of At1g77540 and detailed kinetic studies to confirm or refute the presence of the acetylated intermediate and the roles of conserved Cys[76] and His[41]. To date the only other COG2388 family member that has been structurally characterized is a putative N-acetyltransferase from *Staphylococcus aureus* (PDB # 1r57). The fold of this protein is identical to that of At1g77540, and the protein contains cysteine and histidine residues in locations analogous to Cys[76] and His[41] (Figure 2).

Inspection of the At1g77540-CoA complex reveals that the CoA in the binding pocket displays a kinked shape, with the β-mercaptoethylamine portion of the cofactor splayed between parallel β-strands *D* and *E* and with extensive hydrogen bonds to conserved residues in *motif A*. Similar binding patterns have been observed in other CoA complexes of GNAT superfamily enzymes (34,44). The structure of the At1g77540-CoA complex can rationalize the conservation of Arg[86] (or lysine at the same position) seen in multiple sequence alignment of the COG2833 family of proteins (Figure 2). The Arg[86] residue is involved in electrostatic interactions with 3′-phosphate group of CoA, and thus stabilizes the binding of the cofactor. Similar stabilization

was seen in recent structure of human GCN5 (PDB # 1z4r) with Lys^{624} providing the electrostatic stabilization.

In summary, we have solved the structure of *A. thaliana* At1g77540 protein by X-ray crystallography and solution NMR. Fold analysis confirmed that the protein is a new member of the GNAT superfamily with a minimal acetyltransferase fold. We have provided evidence that At1g77540 binds CoA in a manner similar to other members of the superfamily and established that At1g77540 is an acetyltransferase by enzymatic assays. Based on structural comparisons with other known GNAT family members we propose that At1g77540 acts through a unique mechanism that likely involves conserved His^{41} and the strictly conserved Cys^{76}, which serves as nucleophile and undergoes acetylation; further studies will be needed to test this proposed mechanism. The identity of physiological substrate remains elusive and will be the object of further investigation.

Acknowledgements

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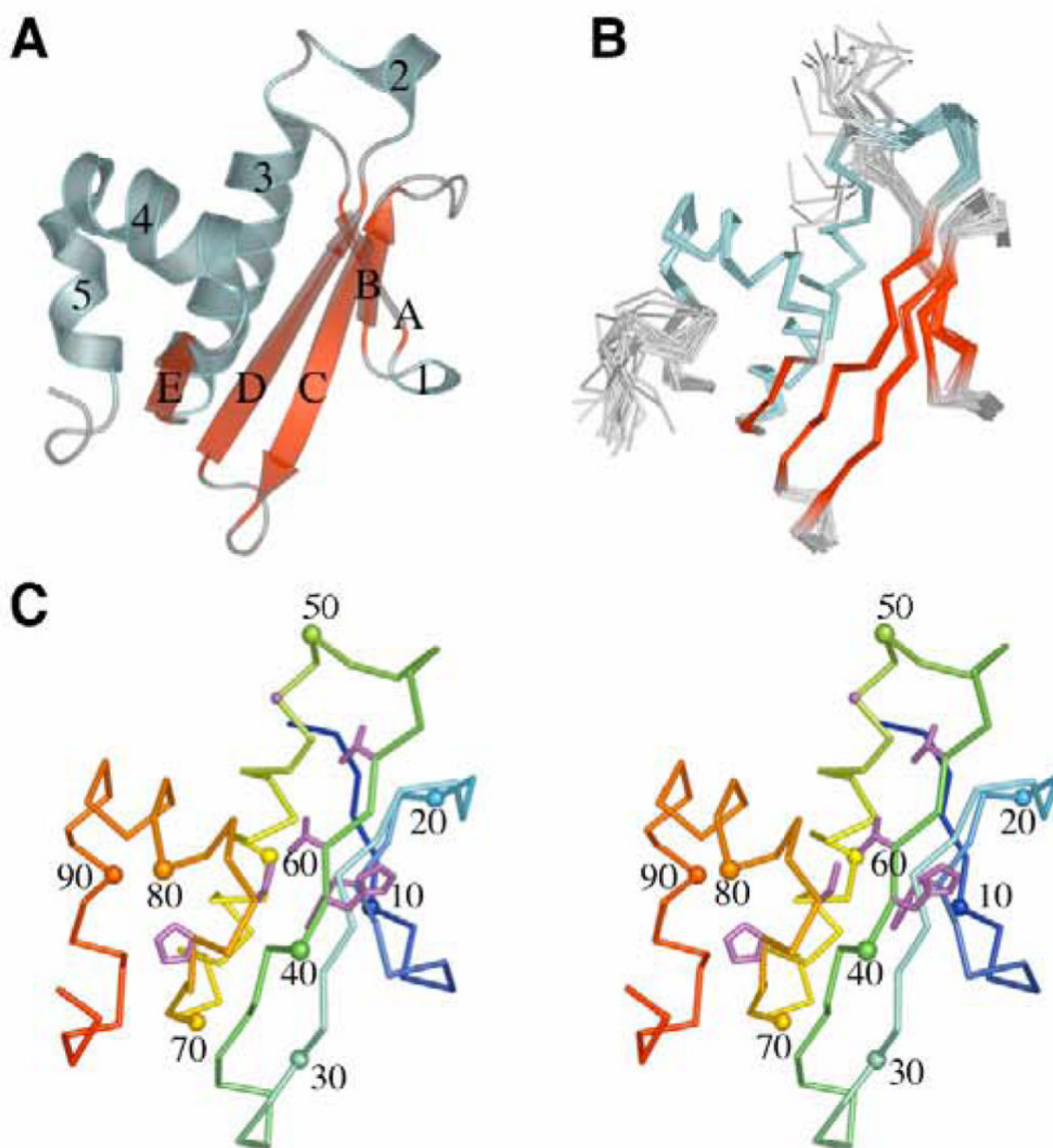
Figure 1.
Structure of At1g77540 from *A. thaliana.* (A) Ribbon diagram of the crystal structure of At1g77540 showing its 2-layer (αβ) sandwich architecture consisting of 5 β-strands (*A–E*), four α-helices (2–5), and one 3_{10}-helix (1). (B) Overlay of backbone traces from the top 20 conformers representing the NMR solution structure of At1g77540. (C) Stereoscopic view of the backbone of At1g77540 color coded from amino-terminus (blue) to carboxyl-terminus (red). Every tenth C^{α} is labeled and highlighted by a sphere of a color corresponding to that of the backbone. Highly conserved residues (shown as ball-and-stick, blue) include Phe[17], His[41], Thr[42], Val[44], Gly[50], Gly[52], Cys[76], and Pro[74].

Figure 2.
Sequence alignment of the COG2388 family. Multiple sequence alignment of At1g77540, a putative N-acetyltransferase from *Staphylococcus aureus* (PDB # 1r57), the histone acetyltransferase Hat1 from *Saccharomyces cerevisiae*, and 20 seed members of the COG2388 family from the *Conserved Domain Database* (30). The GenBank accession codes for these seed members are listed in the left column. Secondary structural elements of At1g77540 are represented by cylinders (helices) and arrows (β-strands). The ruler indicates every 5th (*) and 10th (|) residue of At1g77540 is provided for orientation. The number of proteins residues of selected proteins not included in the alignment is shown in brackets. Boxed and shaded areas correspond to *motif A* (yellow), *motif B* (pink), and *motif D* (green) defined for the GNAT

family proteins (35) from the structural alignment of At1g77540 and Hat1 (PDB # 1bob). Fully and highly conserved residues are highlighted by bold red letters.
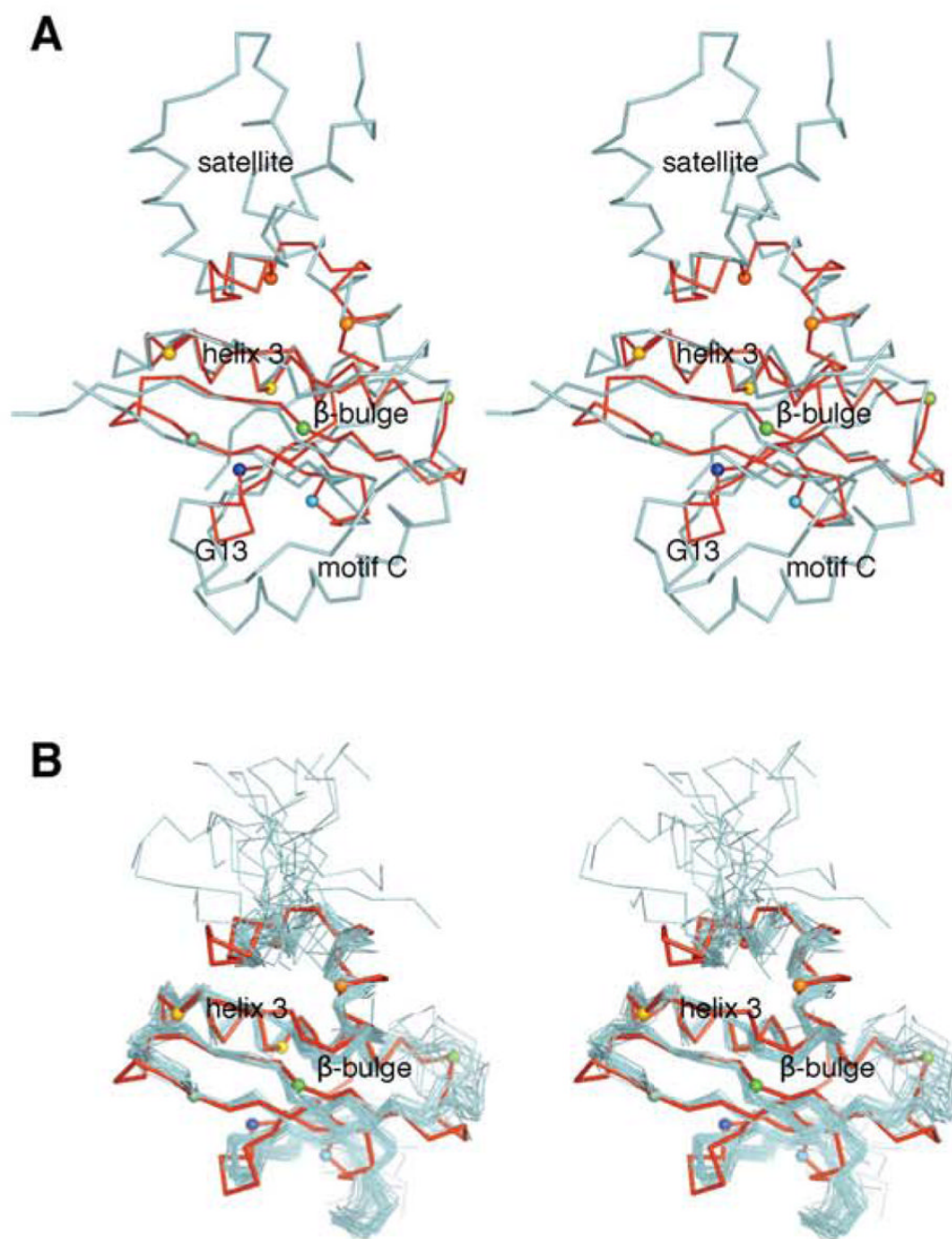
Figure 3.
Structural alignment of At1g77540 with (A) Hat1. Stereo diagram showing the aligned structural models for At1g77540 (red; PDB # 1xmt, this study) and histone acetyltransferase Hat1 from *Saccharomyces cerevisiae* (cyan; residues 122–300; PDB # 1bob). (B) COG2388 family member 1r57. Stereoscopic diagram showing the aligned structural models for At1g77540 (red; PDB #1xmt) and closest structural COG2388 family member from *Staphylococcus aureus* (PDB # 1r57). The view is rotated approximately 90° clockwise compared to that in Figure 1. Selected C$^\alpha$ atoms are highlighted by spheres, with colors consistent with those used in Figure 1C. At1g77540 contain only a minimal acetyltransferase fold with the conserved "signature" helix *3*, central β-sheet containing functionally important

β-bulge, and the CoA-binding loop (residues 43–53). Important structural features present in Hat1 but not present in At1g77540 include 1) *motif C*, which forms a helix that runs perpendicular to the β-strands of the central β-sheet; 2) the independent amino-terminal domain of Hat1 (residues 1–121 of Hat 1, not shown); 3) the helix-bundle-like satellite domain at the carboxyl-terminus of Hat1.
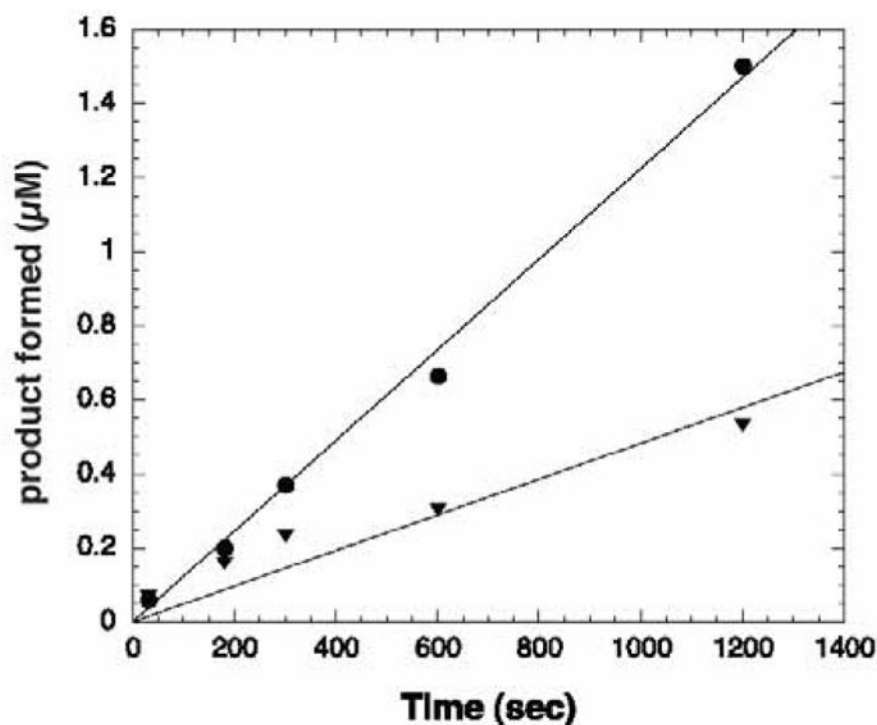
Figure 4.
Acetyltransferase activity assay of At1g77540. At1g77540 displayed acetyltransferase activity on histones H3 and H4. Data were generated using a filter binding assay (29). 0.08 mg/mL enzyme was mixed with 1 mM DTT, 100 μM acetyl-CoA (4.95 μCi [^{3}H]-acetyl-CoA), and 100 μM peptide substrate in 1× TBA buffer pH 7.0. Data were graphed as product formed vs. time, and a linear fit was applied to determine the rate. At1g77540 acetylates H3 (circles) at a rate of 0.04 nmol min^{-1} mg^{-1} of enzyme and H4 (triangles) at a rate of 0.015 nmol min^{-1} mg^{-1} of enzyme. The rate of non-enzymatic acetylation was determined from a matching reaction that contained enzyme buffer instead of enzyme. The background rate was determined and subtracted from the enzymatic rate. KaleidaGraph was used to analyze and plot the data.
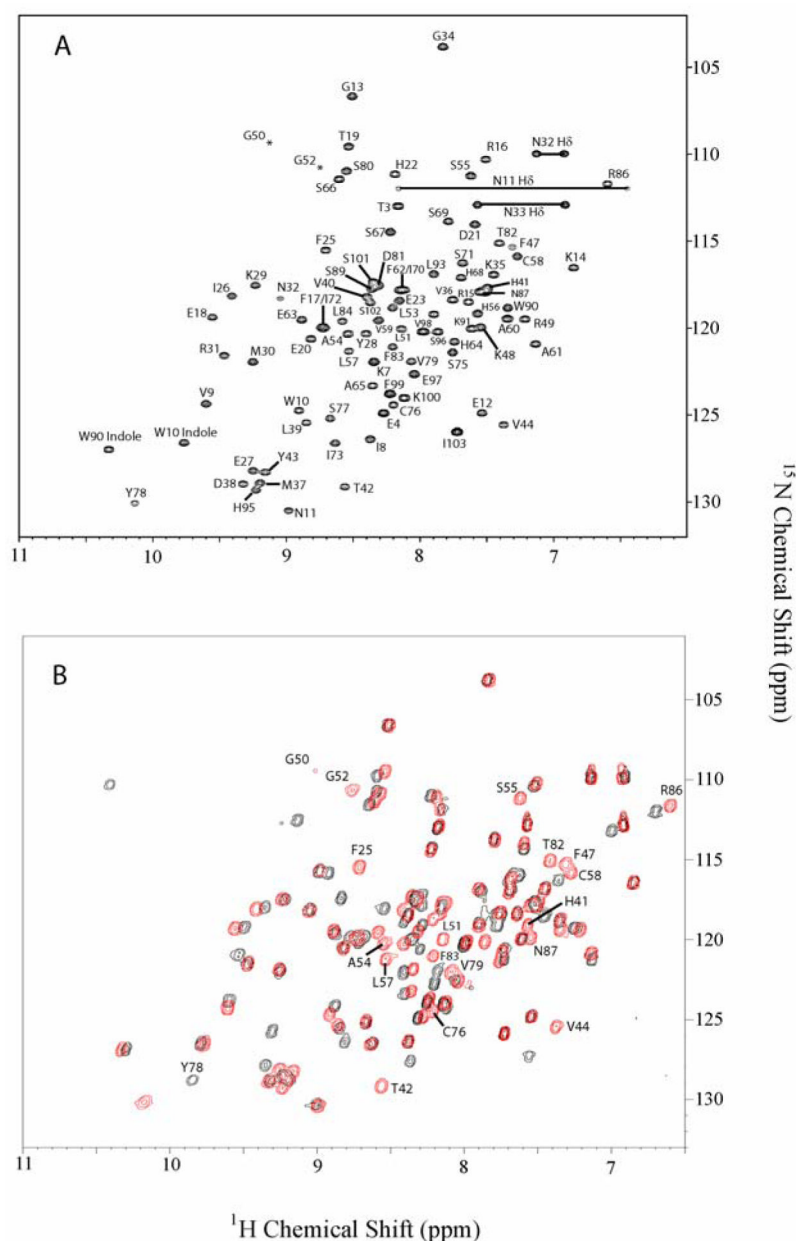
Figure 5.
^1H-^{15}N-HSQC spectrum of free and CoA-bound At1g77540. *A*, Spectrum of uniformly ^{15}N-labeled At1g77540 recorded at 25°C in 10 mM KH_2PO_4, 50 mM KCl, 3 mM NaN_3, and 90% H_2O/10% D_2O at pH 6.0. Assigned backbone amide resonance peaks are labeled with the single letter amino acid abbreviation and residue number corresponding to the primary sequence. Side chain indole resonances for Trp[10] and Trp[90] are shown in the lower left hand corner of the spectrum. Horizontal lines in the upper right hand corner display positions of side-chain amide groups of residues (Asn[11], Asn[32], and Asn[33]). *B*, Overlay of ^1H-^{15}N-HSQC spectra At1g77540 (red) and At1g77540–CoA complex (black). The most prominent chemical shift changes of residues affected by the addition of equimolar CoA to the protein solution are labeled as single letter abbreviation.
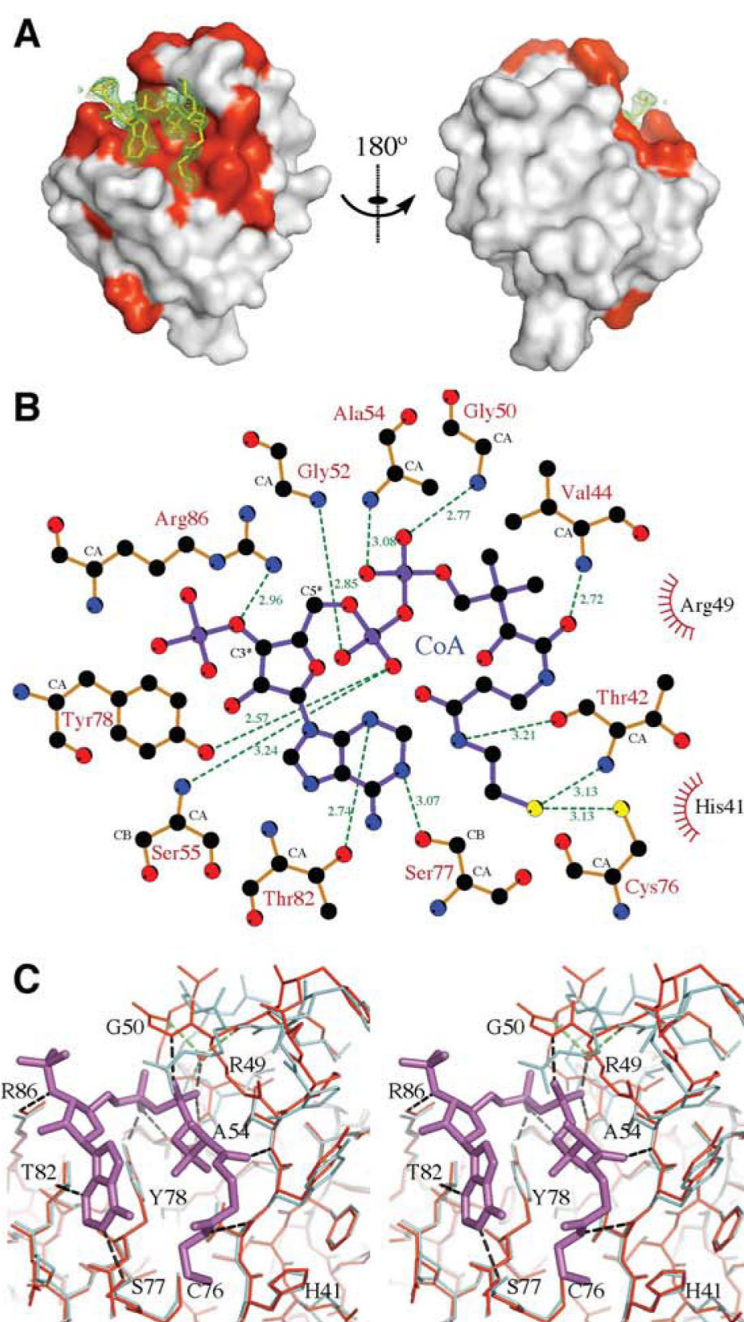
Figure 6.
Structural details of CoA binding to At1g77540. (A) The 25 residues significantly affected by the addition of CoA to At1g77540 in solution, as judged from spectral changes in ^{1}H-^{15}N-HSQC spectra, are highlighted in red and mapped onto the surface of the X-ray derived model for the At1g77540-CoA complex. CoA in the complex is indicated by yellow sticks. The omit map for CoA, contoured at a level of 0.6 σ, is shown by green mesh. (B) Schematic diagram of At1g77540 interactions with CoA. Hydrogen bonds are represented by dashed lines; interatomic distances are given in angstroms. "Radiating" spheres indicate hydrophobic contacts between the cofactor and the surrounding residues. The scheme was generated by LIGPLOT (45). (C) Overlay of the X-ray structural models of At1g77540 (cyan) and

At1g77540-CoA complex (red). CoA is shown in magenta. Hydrogen bonds are represented by black dashed lines. Selected residues are labeled for orientation.
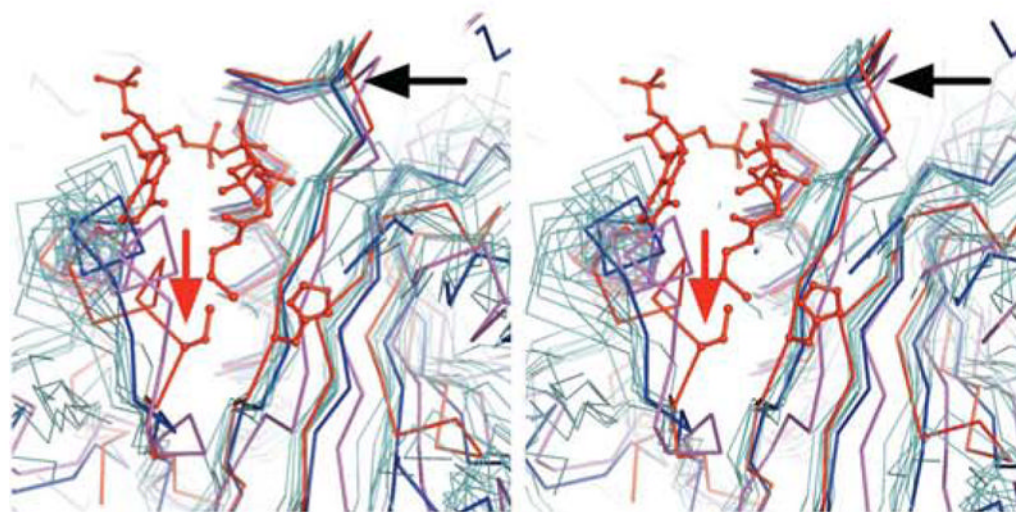
Figure 7.
Structural alignment of the At1g77540/CoA complex with other acetyltransferases. The X-ray structure of the At1g77540-CoA complex is displayed in red. Other structures shown are: PDB # 1bob in blue, PDB # 1mja in magenta, and other members of the GNAT superfamily (PDB # 1bo4, 1cm0, 1i12, 1j4j, 1qsm, 1qsn, 1s5k, and 1tiq) in light blue. The sidechains of His[41] and Cys[76] are displayed as sticks. The black arrow denotes the approximate location of the loop between helices 2 and 3 of At1g77540, which contains the CoA binding motif. The red arrow denotes the location of the unique backbone conformation around Cys[76].
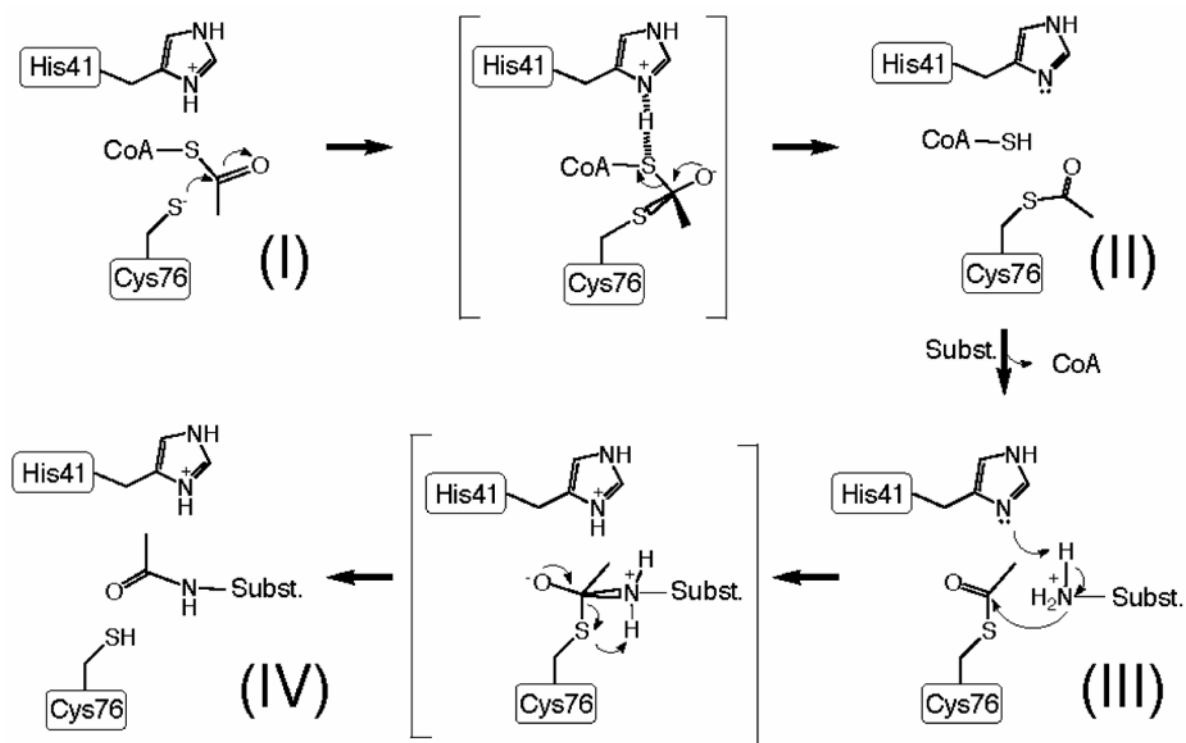
FIGURE 8.
Proposed catalytic mechanism for substrate acetylation by At1g77540.

Table 1

Crystal Parameters, Data and Refinement Collection Statistics

	Low Remote #2	Peak	Edge	High Remote	Low Remote #1	AcCoA complex
Spacegroup	$P2_1$	$P2_1$	$P2_1$	$P2_1$	$P2_1$	P21
Unit Cell parameters (Å, deg)	a=27.3, b=60.6, c=29.4, β=91.5	a=27.5, b=61.0, c=29.6, β=91.5	a=27.5, b=61.0, c=29.6, β=91.5	a=27.7, b=61.5, c=29.8, β=91.5	a=27.3, b=60.6, c=29.4, β=91.5	a=27.9, b=63.9, c=29.5, β=90.9
Data Collection statistics						
Energy (keV)	13.380	13.484	13.480	13.580	13.380	12.658
Wavelength (Å)	0.92668	0.91953	0.91980	0.91302	0.92668	0.97949
Resolution range (Å)[a]	20.29–1.15 (1.18–1.15)	30.53–1.75 (1.79–1.75)	30.50–1.60 (1.64–1.60)	30.73–1.60 (1.64–1.60)	30.28–1.6 (1.64–1.60)	31.97–2.05 (2.10–2.05)
Reflections (measured/unique)	113509/32713	75129/9963	97899/12942	98688/13263	96220/12701	27770/6473
Completeness (%)	95.9 (70.7)	99.9	99.9 (99.5)	99.9 (99.4)	99.9 (99.5)	99.2 (98.0)
R_{merge} [b]	0.039 (0.345)	0.049 (0.202)	0.033 (0.156)	0.042 (0.212)	0.027 (0.099)	0.052 (0.251)
Redundancy	3.5 (1.9)	7.5 (7.2)	7.6 (7.3)	7.4 (6.1)	7.6 (7.5)	4.3 (3.9)
Mean I/σ (I)	16.5 (2.2)	17.8 (6.7)	26.5 (8.4)	19.7 (5.8)	33.6 (13.4)	18.3 (5.0)
Refinement						
No. reflections (work/test)	31030/1658					5822/636
R_{cryst} [c]	0.148 (0.290)					0.160 (0.180)
R_{free} [d]	0.170 (0.318)					0.220 (0.245)
R.m.s.d. bonds (Å)	0.021					0.016
R.m.s.d. angles	2.022					1.664
Average B factor (Å²)	17.8					34.0
No. water molecules	125					61
No. ions/cofactors	11 bromides					1 CoA
PDB code	1XMT					2GDB
Ramachandran plot, residues in						
Most favorable region (%)	91.6					89.9
Additional allowed region (%)	8.4					10.1
Generously allowed region (%)	0.0					0.0
Disallowed region (%)	0.0					0.0

[a] Values in parentheses are for the highest resolution shell.

[b] $R_{merge} = \Sigma_h \Sigma_i |I_i(h) - \langle I(h) \rangle| / \Sigma_h \Sigma_i I_i(h)$, where $I_i(h)$ is the intensity of an individual measurement of the reflection and $\langle I(h) \rangle$ is the mean intensity of the reflection.

[c] $R_{cryst} = \Sigma_h |F_{obs}| - |F_{calc}| / \Sigma_h |F_{obs}|$, where F_{obs} and F_{calc} are the observed and calculated structure-factor amplitudes, respectively.

[d] R_{free} was calculated as R_{cryst} using 5.0% of the randomly selected unique reflections that were omitted from the structure refinement.

Table 2

Statistics for the 20 Conformers Representing the NMR Solution Structure of At1g77540

Root-mean-square-deviations relative to average structure (Å)	
Backbone (C^α, C′, N) atoms in 2nd structure	0.23 ± 0.04
Backbone all residues	1.18 ± 0.51
Heavy atoms in 2nd structure	0.57 ± 0.08
Heavy atoms in all residues	1.47 ± 0.41
Number of experimental restraints	
Intra residue NOEs	823
Inter residue sequential NOEs ($|i-j| = 1$)	486
Inter residue medium range NOEs ($1 < |i-j| < 5$)	333
Inter residue long range NOEs ($|i-j| > 4$)	455
Total NOEs	2097
Dihedral angle restraints	87
H-bond restraints	52
Restraint violations	
NOE distances with violations > 0.3Å	0.45 ± 0.67
dihederal with violations > 3°	1.57 ± 0.74
H-bond with violations > 0.3Å	0.0 ± 0.0
Final energies from simulated annealing (kcal/mol)	
F_{vdw}	−931 ± 25
F_{ele}	−3785 ± 70
Deviation from idealized geometry	
Bonds (Å)	0.0045 ± 0.001
Angles (deg)	0.60 ± 0.02
Impropers (deg)	1.90 ± 0.06
Ramachandran analysis (% of all residues)	
Residues in most favored regions	76
Residues in additional allowed regions	17.9
Residues in generously allowed regions	3.52
Residues in disallowed regions	2.57