Acetylation of lysine residues is an important posttranslational modification found in all domains of life. α-tubulin is specifically acetylated on lysine 40, a modification that serves to stabilize microtubules of axons and cilia. Whereas histone acetyltransferases have been extensively studied, there is no structural and mechanistic information available on α-tubulin acetyltransferases. Here, we present the structure of the human α-tubulin acetyltransferase catalytic domain bound to its cosubstrate acetyl-CoA at 1.05 Å resolution. Compared with other lysine acetyltransferases of known structure, α-tubulin acetyltransferase displays a relatively well-conserved cosubstrate binding pocket but is unique in its active site and putative α-tubulin binding site. Using acetylation assays with structure-guided mutants, we map residues important for acetyl-CoA binding, substrate binding, and catalysis. This analysis reveals a basic patch implicated in substrate binding and a conserved glutamine residue required for catalysis, demonstrating that the family of α-tubulin acetyltransferases uses a reaction mechanism different from other lysine acetyltransferases characterized to date.

Results and Discussion

Overall Structure Characterization. Human αTAT1 (residues 1–196) was overexpressed in Escherichia coli, purified, and crystallized. The crystallized construct encompasses the catalytic domain and catalytic mechanism. Overall Structure Characterization. Human αTAT1 (residues 1–196) was overexpressed in Escherichia coli, purified, and crystallized. The crystallized construct encompasses the catalytic domain and catalytic mechanism.
Cosubstrate Binding by αTAT1. Although the cosubstrate was not added before crystallization, the electron density unambiguously revealed the presence of an AcCoA coenzyme molecule in the active site of the protein that likely copurified with the recombinantly expressed protein in *E. coli* (Fig. 2B). The cosubstrate is buried deeply within a groove located at the center of the core domain and makes extensive contacts with αTAT1 mainly via helices α2, α4, and α6 and β-strands β6–7 as well as with the connecting loops (Figs. 1 and 2). Because the structure presented here was determined to atomic resolution, the AcCoA binding geometry can be determined accurately (Fig. 2C). αTAT1 makes more than 20 interactions of both hydrophobic and hydrophilic nature with AcCoA (Fig. 2C). Comparison with members of different HAT families reveals that the overall binding site for AcCoA is conserved among HATs and TATs, although the specific residues involved in cosubstrate recognition have diverged substantially (Fig. 2A and Fig. S5). For example, αTAT1 does not contain the unusually long L1 loop observed to contribute to cosubstrate binding in the p300/CBP and Rtt109 families of KATs (24, 26). The binding mode of AcCoA in different KATs is such that the activated acetyl groups are in similar positions, but the phosphoribose adenine (3′,5′-ADP) moieties occupy different positions (Fig. 2C). In the case of αTAT1, the adenine base of AcCoA has a binding mode not previously observed in members of other KAT families. In αTAT1, the adenine base of AcCoA is sandwiched between the side chains of K162 and R132 (Fig. 2B). R132 serves a dual role as it also forms multiple interactions with the 3′ phosphate group of the ribose ring of the AcCoA molecule (Fig. 2C). As K162 and R132 are well conserved among αTAT1 proteins (Fig. S5), the interactions that hold the adenine moiety in place are likely to be a structural hallmark of the αTAT1 family. To test for the functional importance of K162 and R132 in binding and positioning of AcCoA, acetylation assays with polymerized MT were carried out by using αTAT1 R132A and K162A single-point mutants and the R132A/K162A double-point mutant (Fig. 2D). All αTAT1 mutants used in this study are soluble and properly folded as judged by size exclusion chromatography (Fig. S6). Quantifications within the linear range of the enzyme activity show that whereas the single-point mutants have approximately 50% of wild-type (wt) activity, the R132A/K162A double mutant has activity reduced to near background levels (Fig. 2E). As expected for AcCoA-binding mutants, the acetylation activity could be partly restored by adding 20x more cosubstrate (Fig. 2D and E). The structural and biochemical data are thus consistent with a role for R132 and K162 in AcCoA binding and positioning in the αTAT1 family of KATs.

**Substrate Recognition.** Conserved basic patch of αTAT1 is required for the acetylation of α-tubulin K40. TATs and HATs share a common evolutionary origin reflected by a conserved core fold and AcCoA coenzyme binding site. However, HATs and TATs act on different substrates and are thus expected to have evolved different substrate-binding sites specific for histones and α-tubulin, respectively. This assumption is supported by the fact that αTAT1 is specific for MT and does not acetylate core histones in vitro (16). Crystal structures of HATs in complex with histone substrate peptides reveal that the substrate adopts a random coil conformation and binds to an extended groove that runs parallel to the AcCoA cosubstrate binding site (22, 28). αTAT1 acetylates K40 of α-tubulin, a residue that is located in a highly conserved loop region of α-tubulin (α-LoopK40) found at the luminal side of MT (29). Comparison of the surface properties of the αTAT1 structure reveals a groove in a similar position to that of the histone peptide-binding groove in HATs (Fig. 3). The residues lining this groove are highly conserved among αTAT1 proteins (Fig. 3B), suggesting that it could be the binding site for the α-LoopK40. In agreement with this notion, the position of histone peptide seen in HATs fits the groove of αTAT1 without major clashes (Fig. 3 C and D). However, when LoopK40 in the conformation observed in the α-tubulin structure (30) is superposed onto the HAT histone substrate, only the residues located C-terminally to K40 of α-tubulin share a similar binding mode with the histone peptide (the residues located C-terminally to K40 diverge substantially in structure; Fig. 3C). However, LoopK40 may adopt a different conformation or be more flexible in the context of polymerized MT compared with Zn2+-induced tubulin sheets (which was the basis for the structural analysis in ref. 30). The residues flanking K40 of α-tubulin are mainly acidic, which is in contrast to histone peptides that are mainly basic (Fig. 3 C and D). Consistently, the predicted α-tubulin binding groove of αTAT1 has a prominent positively charged patch formed by residues R69, H75, and K102 that is well suited to bind a negatively charged substrate. To test the functional importance of these residues, acetylation assays were performed with point-mutated proteins. Mutation of R69, H75, or K102 to glutamates results in a reduction of MT acetylation to background levels, an effect that cannot be significantly rescued by adding 5x more substrate, suggesting that substrate binding is severely impaired in these mutants (Fig. 3 E and F). Because R69, H75, and K102 are all located too far away from the acetyl group of the cosubstrate (>10 Å) to participate directly in catalysis or cosubstrate binding, the lack of activity upon mutation is most consistent with a role for these residues in α-tubulin substrate binding.

**Lysine-binding cleft.** The active site of αTAT1 harboring the acetyl group of the cosubstrate is located in a cleft into which the substrate target lysine must be inserted to become acetylated. The structure presented here does not have an α-tubulin substrate bound, but examination of crystal packing reveals that a
neighboring αTAT1 molecule inserts the side chain of an arginine residue (R86 from the β2–β3 loop) into the active site (Fig. 4A) resembling the recognition of substrate lysines by HATs (22). This potential lysine-binding cleft is lined by the side chains of the well-conserved residues I64 and R158 (Fig. 4A and Fig. S5). The side chain of I64 and the aliphatic part of the R158 side chain form a hydrophobic cleft that could serve as a binding pocket for the hydrophobic part of the α-tubulin K40 side chain. In addition, the guanidinium group of R158 forms a hydrogen bond with the main-chain carbonyl of the loop from which the arginine pseudosubstrate protrudes, further stabilizing its position (Fig. 4A). To investigate the importance of I64 and R158 in αTAT1 function, single alanine point mutations were tested in MT acetylation assays. These experiments show that the activity of the I64A mutant is at background levels, and the activity of the R158A mutant is approximately 20% of wt activity (Fig. 4C and D). Consistent with a role in substrate binding, the activities of the I64A and R158A are partly rescued by the addition of 5× more substrate (Fig. 4C and D). The structural and mutational data are consistent with a function of I64 and R158 in forming a pocket that binds and positions α-tubulin-K40 for acetylation.

Catalytic Mechanism of αTAT1. Several structures of HATs in complex with cosubstrates, histone-peptide substrates, and products of the acetylation reaction have elucidated that different HAT families operate via different reaction mechanisms. The classical catalytic mechanism described for GCN5/PCAF is relatively well understood and involves a glutamate residue (general base) that
activates a water molecule to remove a proton from the ε-amino group of the target lysine residue (22). The neutral lysine side chain can then perform a nucleophilic attack on the acetyl group of the cosubstrate, thus releasing the acetylated protein product and the CoA coenzyme. In contrast, the MYST family of HATs uses a ping-pong mechanism that requires a catalytic cysteine and autoacetylation of an active site lysine (23, 31). The Rtt109 family also uses autoacetylation of a buried lysine residue but has a divergent active site configuration compared with other KAT families. Further details of the catalytic mechanism of Rtt109 are not well understood (24). Another difference of the Rtt109 family is the reliance on either of the two histone chaperones Asf1 or Vps75 for activity. However, another different mechanism was suggested for the P300/CBP family that lacks the catalytic glutamate and appears to use a so-called hit-and-run reaction that does not rely on a general base (26).

Inspection of the active site of the αTAT1 structure presented here reveals a different configuration from that of other KAT families. Both the catalytic glutamate and cysteine residues found in HATs are not present in αTAT1. A cysteine is found at β6 of the αTAT1 structure colored according to sequence conservation within the αTAT1 family. A highly conserved cleft located close to the acetyl group of the cosubstrate is a prime candidate for α-tubulin binding. The positions of conserved amino acids lining the cleft are indicated. (C) Electrostatic surface potential of αTAT1 with the cosubstrate shown as sticks and a modeled H3 substrate peptide shown in pink (from the GCN5 structure; PDB ID code 1PU9) and a modeled α-Loop peptide (superimposed on the H3 peptide of GCN5) shown in green. (D) Electrostatic surface potential of the GCN5 HAT bound to H3 histone peptide (PDB ID code 1PU9). A region similar to the positively charged patch in Fig. 3C is encircled and shows a distinctively negatively charged area, providing a possible explanation for substrate specificity between histone and tubulin acetyltransferases. The sequence of the bound H3 histone peptide is indicated below the image. Results of in vitro acetylation assays of MT by basic patch αTAT1 mutants (E) and quantification of the results (F). Acetylation assays were done with either 5 or 25 μM substrate concentration. For further details, see Fig. 2 D and E.
αTAT1 but is located too far away (11 Å) from the acetyl group of AcCoA to be involved in the catalytic mechanism (Fig. S7). However, a conserved aspartate residue (D157 in Hs-αTAT1) is found at a similar structural position as the catalytic glutamate in GCN5, suggesting that it could serve as the general base. In support of this notion, mutation of D157 to asparagine renders αTAT1 completely inactive (16). In the structure of αTAT1 presented here, D157 is positioned close to where the K40 target lysine is predicted to enter the active site (Fig. 4A). Additionally, D157 forms a salt bridge with and positions the side chain of R158, one of the residues suggested to line the α-tubulin K40-binding pocket. The structural data are thus both compatible with a role for D157 in catalysis as the general base and with a role in structuring the active site for substrate binding. To evaluate these possibilities, D157A mutant αTAT1 was tested in MT acetylation assays, demonstrating that the mutant is not catalytically dead but retains approximately 10% of wt protein acetylation activity (Fig. 4E and F). This result is incompatible with D157 as an essential catalytic residue in the reaction mechanism. The complete lack of activity observed for the D157N mutation (16) is likely a result of R158 occupying a conformation that occludes the substrate in the D157N mutant protein. To test whether the main function of D157 is in substrate binding, assays with 5× the substrate concentration were carried out but were unable to rescue the reduction in activity (Fig. 4E and F). Another interesting property of the D157A mutant is that this protein behaves as a mixture of monomers and dimers in size exclusion chromatography (Fig. S6). The data are not consistent with the D157 as the general base in the reaction mechanism and suggest that mutation of this residue leads to significant structural changes resulting in dimerization of the enzyme.

This analysis raises the question of the nature of the potential general base in αTAT1. Examination of the αTAT1 active site reveals that a conserved glutamine (Q58 in hs-αTAT1) occupies a prominent position close to the acetyl group of the cosubstrate and could serve a role in keeping the cosubstrate in a productive conformation for catalysis or in stabilization of the reaction intermediate (Fig. 4A and Fig. S5). Additionally, Q58 coordinates

Fig. 4. Substrate lysine binding cleft and catalysis of αTAT1. (A) αTAT1 structure is shown as cartoon with AcCoA (white) and active site residues (gray) displayed as sticks. A loop region from a neighboring αTAT1 molecule in the crystal inserts the side chain of an arginine residue (blue) into the active site mimicking the substrate target lysine. A well-ordered water molecule that could participate in catalysis is shown as a red ball. A zoom-in with interaction distances indicated is shown within the dashed box. (B) Active site configuration of the Q58A point mutation. Two well-ordered water molecules are observed in the active site of the Q58A mutant structure (each approximately 2 Å away from the water molecule observed in the wt structure). The αTAT1 Q58A mutant crystallized in a different spacegroup where the insertion of a pseudosubstrate into the active site is not observed. (C and E) Acetylation assays of MT with potential lysine channel and catalytic residue mutants using either 5 or 25 μM substrate concentration. (D and F) Quantification of the results in C and E. For further details, see Fig. 2 D and E.
a well-ordered water molecule located at the end of the proposed target lysine-binding cleft (Fig. 4A), indicating that Q58 could serve the role as the general base in the reaction mechanism. To test whether Q58 is important for activity, αTAT1-196 Q58A mutant protein was purified and assayed for its ability to acetylate MT. The result of this experiment shows that αTAT1-196 Q58A is catalytically dead (Fig. 4 E and F). One possible explanation for the lack of αTAT1-196 Q58A activity is that this mutant is unable to bind AcCoA or that AcCoA binds but with the acetyl group positioned in a catalytically unproductive way. To assess this issue, the αTAT1-196 Q58A was crystalized and the structure determined at 1.6 Å resolution, demonstrating that AcCoA is still bound in the mutant protein. Wild-type and Q58A αTAT1-196 superimpose with an rmsd of 0.4 Å over all Co atoms and the acetyl groups of the cosubstrate are positioned less than 0.3 Å apart, demonstrating that Q58 is not required for the correct positioning of AcCoA for catalysis (Fig. 4B). These results are consistent with a direct role for Q58 in catalysis, either as the general base required for activation of a water molecule or in stabilization of the reaction intermediate. In agreement with this notion, the activity of the Q58A mutant is not restored when increasing the substrate concentration (Fig. 4 E and F). Additional structural studies of αTAT1 in complex with substrates, reaction intermediates, and products of the reaction will be required for a more complete understanding of the catalytic mechanism of this family of acetyltransferases.

Acetylation of Ciliary MT by αTAT1. The fact that αTAT1 acts on α-tubulin K40 found at the luminal side of polymerized MT presents a logical problem of how the enzyme gets access to the substrate in vivo. In the case of the cilium, αTAT1 likely enters this organelle via intraflagellar transport and is released inside the cilium where it has to diffuse into the lumen of MT. Given the dimensions of the αTAT1 catalytic domain of 3–6 nm, diffusion through the 1.7-nm pores between MT protofilaments does not seem possible. The only entry point for αTAT1 into the MT lumen thus appears either to be at the MT plus end openings or through lateral openings created by MT defects (i.e., missing protofilaments). Once inside the MT lumen (inner diameter of 14 nm), αTAT1 can diffuse freely and would have a very high effective substrate concentration, which could contribute toward the higher efficiency toward MT substrates compared with free αβ-tubulin (16). Studies of how αTAT1 is transported into the cilium and into the lumen of MT should be the focus of future studies.

Materials and Methods

Recombinant protein expression in bacteria and subsequent crystallization and X-ray diffraction data collection were carried out as described in SI Materials and Methods. Acetyltransferase activity assays on polymerized microtubules were used to assess the effect of various mutations on enzymatic activity. Details about microtubule polymerization, the acetyltransferase assay, and quantification of enzyme activities can also be found in SI Materials and Methods.

ACKNOWLEDGMENTS. We thank Vincent Olieric and Jerome Basquin for help with X-ray diffraction data collection; the crystallization facility of the Max Planck Institute of Biochemistry (Munich) for access to crystallization screening and Atlanta Cook; and Ingrid Schaefer for carefully reading and correcting the manuscript. We acknowledge Michaela Morawetz for technical assistance with molecular biology and Sagar Bhogaraju for assistance with Fig. 3. This work was funded by Emmy Noether Grant Deutsche Forschungsgemeinschaft LO1627/1-1, European Research Council Grant 310343, and by the European Molecular Biology Organization Young Investigator program. M.T. is the recipient of an Erwin Schroedinger stipend granted by the Austrian Science Fund J3148-B12.

Supporting Information

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SI Materials and Methods

Purification and Crystallization of αTAT1-196. Isoform 5 of human αTAT1 (323 residues) was cloned from the MegaMan Human transcriptome cDNA library (Agilent) into different pET-based vectors and subsequently used to clone a truncated version of αTAT1 containing only the catalytic domain (residues 1–196). αTAT1-196 (wild-type and point mutants) was overexpressed with an N-terminal hexa-histidine tag in Escherichia coli and purified by Ni-NTA affinity, Q-Sepharose ion-exchange, and Superdex75 size exclusion chromatography (final buffer: 10 mM Tris-HCl at pH 7.5, 150 mM NaCl, and 1 mM DTT). The selenomethionine substituted protein was purified as the native protein but with 5 mM DTT added to buffers used in the Q-Sepharose and size exclusion chromatography steps. For crystallization, wild-type αTAT1-196 at 45 mg/mL concentration was mixed with an equal volume of precipitant solution containing 50 mM Tris-HCl at pH 8.0, 200 mM NaCl, and 15% (wt/vol) PEG 8000. Crystals grew in 4–6 d at 18 °C to a maximum size of 0.5 mm and were flash cooled (liquid nitrogen) in mother liquor supplemented with 15% glycerol. αTAT1-196 Q58A mutant protein was crystallized at 74 mg/mL concentration by mixing the protein with an equal volume of 2.8 M Na-acetate at pH 7.0. The mother liquor was supplemented with 20% glycerol before flash-cooling in liquid nitrogen.

X-Ray Diffraction Data Collection and Structure Refinement. Seleniummethionine substituted αTAT1 crystals diffracted X-rays to approximately 1.5 Å resolution by using an attenuated beam. A SAD dataset of 720° rotation was collected at the Se peak wavelength at PXIII at the Swiss Light Source (Villigen, Switzerland) and processed by using the SHELX package (2). Native crystals diffracted X-rays extremely well, and data were collected and processed to 1.05 Å resolution. Because the mosaicity was estimated as low as 0.1° for the best native crystal, finely sliced data of 4,500 frames (0.05° rotation per frame) were collected and processed by using XDS. The wavelength of the X-rays was reduced to 0.8 Å to capture all high-resolution reflections that would otherwise fall outside the detector space. Refinement was carried out in PHENIX (3) by using the model obtained from the selenomethionine SAD data as a starting point. The final model resulted from iterative cycles of model building in COOT (4) and refinement in PHENIX. The model contains all residues from 1 to 195 except a small disordered loop between residues 88–91 that could not be modeled. Residues 105–115 were clearly observed to be flexible and were modeled in the two most prominent conformations. After completing the building of the polypeptide, clear electron density remained at the core of the protein and could be unambiguously identified as a bound acetyl-CoA molecule. Because of the atomic resolution of the data, all heavier atoms (C, N, O, S, and P) were refined with anisotropic atomic displacement parameters (ADPs) in PHENIX. Well-ordered parts of the protein and the acetyl-CoA molecule clearly displayed positive electron density peaks at the theoretical positions for hydrogens, which were consequently added to the model (but not to solvent molecules). Hydrogens were refined in riding positions by using isotropic ADPs. αTAT1-196 Q58A mutant protein crystals diffracted to 1.6 Å resolution and belonged to a different space group than crystals of αTAT1-196 wild-type protein (Table 1). The structure of αTAT1-196 Q58A was determined by molecular replacement using the structure of the wild-type protein and refined in PHENIX with isotropic ADPs and hydrogens in a riding position.

MT Acetylation Assay. For assembly of microtubules, 50 μM β-tubulin dimers (bovine brain, Cytoskeleton) were incubated at 37 °C for 40 min in the presence of 2 mM GTP and 5% glycerol and used in acetylation reactions with αTAT1. The reaction mix contained either 5 μM or 25 μM polymerized microtubules, 2.5 μM αTAT1 (either wild type or mutant), 80 mM Pipes at pH 6.9, 0.5 mM EGTA, 2 mM MgCl2, 10% glycerol, 1 mM DTT, and either 20 μM or 400 μM acetyl-CoA. Total reaction volumes of 50 μL were set up and incubated at 37 °C. Seven-microliter samples were removed at the indicated time points, mixed with an equal volume of 2× SDS loading dye, and incubated at 95 °C for 5 min to stop the reaction. Three microliters of the obtained sample were spotted on nitrocellulose membranes, and K40-acetylated α-tubulin was subsequently detected by using the anti-acetylation antibodies (Sigma), followed by incubation with Alexa Fluor 647 rabbit anti-mouse antibody. The fluorescent signals were detected by using a Typhoon FLA 7000 scanner (GE Healthcare) and quantified by using the ImageQuant software. The acetylation activity of each mutant was tested in three independent experiments.

Fig. S1. Purification, crystallization, and activity of αTAT1<sup>1-196</sup>. (A) Schematic representation of the hs-αTAT1 protein. The 323-aa protein contains the catalytic domain at the N terminus (1–196) and a C-terminal tail predicted to be unstructured. (B) Purification of hs-αTAT1<sup>1-196</sup>. The final elution profile from size exclusion chromatography (HiLoad Superdex75) is shown, together with a Comassie stained gel. The protein fragment eluted as a sharp single peak corresponding to monomeric protein and was highly pure. (C) Native protein crystals formed by the protein preparation shown in B in the initial crystallization screen. (D) The purified hs-αTAT1<sup>1-196</sup> is catalytically active toward α-tubulin K40 in microtubules. Five micromolar αTAT1<sup>1-196</sup> was incubated either without microtubules or with 2 μM polymerized α/β-tubulin dimers for the indicated times. K40-acetylated α-tubulin was detected by Western blot using an acetylation-specific antibody, and the presence of equal amounts of protein (both αTAT1<sup>1-196</sup> and tubulin) was confirmed by Coomassie staining.

Fig. S2. Experimental electron density map at 1σ displayed around the cofactor binding site. The AcCoA is shown as sticks (carbons in turquoise color), and amino acids of the αTAT1 protein are displayed as black sticks.
Fig. S3. Cartoon representation of $\alpha$TAT1$^{1-196}$ displaying the N-terminal part of the protein. Three conserved phenylalanines (F3, F5, F11) pack into a hydrophobic pocket created by residues from the N-terminal half of $\alpha_2$ (Q42 and I46), thereby stabilizing this helix. The AcCoA molecule is shown as sticks; the N terminus of $\alpha$TAT1$^{1-196}$ is labeled.

Fig. S4. Superposition of three different families of KATs: $\alpha$TAT1 is colored salmon, yeast Esa1 HAT (PDB ID code: 3TO7) is shown in blue, and yeast Hat1 (1BOB) in yellow. The cofactors are shown as stick models. The structural conservation between HATs and $\alpha$TAT1 is restricted to the very core of the enzymes. The boxed region is shown in Fig. 2A.
Fig. S5. Sequence alignment of αTAT1 from various species. The positions of secondary structure elements from human αTAT1 are shown above the alignment. Important residues (as determined in this study) are highlighted. AcCoA binding residues are marked in blue, potential substrate-binding residues are marked in yellow, and the glutamine proposed here to function in catalysis marked in red. Sequences from two HATs (ScHat1 and ScEsa1) are also shown at the appropriate positions (as determined by a structure-based alignment using the DALI server).
Fig. S6. Size-exclusion chromatograms (Superdex75) of the wild type and all of the mutant versions of α\text{TAT}1\textsuperscript{1-196} used in this study. With the exception of the D157A mutant, all proteins elute at the same volume as the wild type, indicative of proper folding of the mutants. In the case of D157A, we observe a second peak at a lower elution volume in addition to the peak observed for the wild type, which is most likely caused by dimerization of the mutant protein. We note that these two forms of the D157A mutant protein are in equilibrium and are interconverted after storage for several hours at 4 °C.

Fig. S7. Position of Cys120 relative to the catalytic site. (A) Structure of the α\text{TAT}1 active site reveals that Cys120 is located approximately 11 Å from the acetyl group of the cosubstrate. Cys120 is part of a hydrophobic pocket together with Leu104 and Phe183 and, thus, unlikely to be a catalytic cysteine in a ping-pong mechanism. (B) Acetylation assays of MT with C120A and C120S mutants, both at 2.5 μM and 12.5 μM enzyme concentration. Note that both cysteine mutants displayed a pronounced tendency to aggregate, likely due to a destabilization of the hydrophobic core. (C) Quantification of the results shown in (B). The C120A α\text{TAT}1 mutant protein has approximately 8% of wild-type activity under steady-state conditions and approximately 36% under single turnover conditions. These results further speak against Cys120 as a catalytic residue.
Table S1. Data collection and refinement statistics for Hs αTAT1

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