

The protein acetyltransferase PatZ from *Escherichia coli* is regulated by autoacetylation-induced oligomerization

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Background: PatZ is the main *Escherichia coli* acetyltransferase and control acetyl-CoA synthetase (Acs) activity.

Results: The kinetic and structural PatZ oligomer characteristics were determined.

Conclusion: PatZ is a stable tetramer and forms an active octamer by autoacetylation to increase its stability.

Significance: PTMs by acetylation have structural and functional roles in the cell.

Lysine acetylation is an important post-translational modification in the metabolic regulation of both prokaryotes and eukaryotes. In *Escherichia coli* (*E. coli*), PatZ (formerly YfiQ), is the only known acetyltransferase protein and is responsible for Acs (acetyl-CoA synthetase) acetylation. In this study, we demonstrated PatZ positive cooperativity in response to acetyl-CoA and the regulation of Acs activity by the acetylation level. Furthermore, functional analysis of an E809A mutant showed that the conserved glutamate residue is not relevant for the PatZ catalytic mechanism. Biophysical studies demonstrated that PatZ is a stable tetramer in solution and is transformed to its octameric form by autoacetylation. Moreover, this modification is reversed by the sirtuin CobB. Finally, an *in silico* PatZ tetramerization model based on hydrophobic and electrostatic interactions is proposed and validated by 3D hydrodynamic analysis. These

data reveal, for the first time, the structural regulation of an acetyltransferase by autoacetylation in a prokaryotic organism.

Post-translational modification (PTM) of proteins by lysine acetylation has been traditionally associated with eukaryotic organisms. However, in recent years, interest in PTM in prokaryotic organisms has increased greatly (1). Many metabolic enzymes are known to be acetylated in *E. coli* (2), although the physiological importance of acetylation has not been studied in detail. Acetylation has traditionally been described as a reversible PTM catalysed by an acetyltransferase (KAT) with acetyl-CoA as acetyl donor. However, recently the importance of non-enzymatic acetylation has been discovered, using acetyl-CoA or acetyl-phosphate as acetyl donors in prokaryotic and eukaryotic organisms (2–4).

In *E. coli*, the N ϵ -acetyltransferase PatZ is the only enzyme known to be involved in the post-

translational acetylation of proteins. PatZ is an acetyltransferase belonging to the GCN5-related N-acetyltransferases (GNAT) superfamily, which catalyses the transfer of an acetyl group from acetyl-CoA to a primary amine (5). PatZ is a large multidomain protein with 886 residues (~98 kDa) that has a C-terminal acetyl-CoA binding fold whose predicted structure belongs to the large GNAT superfamily of acetyltransferases (residues 725 to 884). The N-terminal domain is a predicted domain with high similarity to the acyl-CoA synthetase (NDP-forming) superfamily of enzymes (residues 3 to 625) (6). Many members of this family are oligomeric proteins (7). In fact, *SePat*, *Salmonella enterica* (*S. enterica*) PatZ ortholog, oligomerizes to a tetramer due to its binding to acetyl-CoA (8).

It has recently been described that in *E. coli*, PatZ is acetylated *in vivo* (2, 9), although the consequences of PatZ acetylation are not known. Regarding the acetyltransferase catalytic mechanism, it has not been studied in bacteria, although studies carried out in other GNAT, suggest the existence of two possible mechanisms, a Bi-Bi sequential and a ping-pong catalytic mechanism (10–14).

One of the best known PatZ substrates is acetyl-CoA synthetase (Acs), an enzyme regulated by acetylation in bacteria such as *S. enterica*, *E. coli*, *Bacillus subtilis*, *Rhodopseudomonas palustris*, and *Mycobacterium tuberculosis* (15–19). In *E. coli*, Acs reversible lysine acetylation is mainly regulated by two enzymes, the GCN5-like acetyltransferase Pat/YfiQ (15–19) and the NAD⁺-dependent sirtuin-like deacetylase CobB (16–20). Acs is a key enzyme in prokaryotic and eukaryotic metabolism since it synthesizes acetyl-CoA from acetate, ATP and CoA via an acetyl-adenosine monophosphate (acetyl-AMP) intermediate and is involved in metabolism homeostasis due to its direct link with acetyl-CoA. Moreover, acetyl-CoA is involved in the energy metabolism, acting as substrate and playing a critical role in overall metabolism modulation, which makes it a key operator in the regulation of metabolic fluxes. It is important to note that in exponentially growing aerobic cultures of *E. coli*, cytosolic acetyl-CoA concentrations vary from 0.5 to 0.6 mM with glucose and from 0.2 to 1.7 mM with acetate as the sole carbon sources (21). In this sense, the Acs enzyme is essential in limited

carbon source conditions accompanied by a low extracellular concentration of acetate (22, 23).

Hence, the main objective of this study was to ascertain the kinetic and biochemical characteristics of PatZ and its effect on the regulation of Acs by lysine acetylation. This characterization will provide a deeper understanding of protein acetylation in *E. coli*.

EXPERIMENTAL PROCEDURES

Construction of PatZ overexpression plasmids—The 2658-bp *patZ* gene of *E. coli* BW25113 was PCR-amplified and cloned into the pRSETA plasmid. The resulting plasmid was named pRSET*patZ*. Single amino acid mutant E809A was obtained by site-directed mutagenesis from pRSET*patZ* using PCR. The resulting plasmid was named pRSET*patZ*^{E809A}. The quintuple-mutant, (substitution of lysines at 146, 149, 391, 447, 635 for arginines) was obtained by sequential site-directed mutagenesis from pRSET*patZ*. The resulting plasmid was named pRSET*patZ*^(K→R). The 2100 bp fragment corresponding to a truncated acetyltransferase defective *patZ* gene (1-2100 bp) was PCR-amplified and cloned into the pRSETA plasmid. The resulting plasmid was named pRSET*patZ*^{Acs-like}. The 558 bp fragment encoding the GNAT domain of PatZ (2100-2658 bp) was cloned in the same way, resulting in plasmid pRSET*patZ*^{GNAT}. The Acs and CobB proteins were overexpressed using the ASKA collection plasmids (24). All molecular biology enzymes used were from Thermo Fisher Scientific. The strains, plasmids and primers used are listed in Table 1.

Overproduction and purification of proteins—Chemically competent *E. coli* BL21(DE3) wild type or Δ *patZ* strains were transformed by heat shock at 42 °C. Cultures were grown overnight at 30 °C with orbital shaking (200 rpm). The culture medium used was Terrific broth (TB) (12 g/L tryptone, 24 g/L yeast extract, 4 % v/v glycerol, 0.17 M KH₂PO₄ and 0.72 M K₂HPO₄) containing ampicillin (100 µg/mL) (for pRSETA) or chloramphenicol (30 µg/mL) (for ASKA plasmids). The expression was induced with isopropyl-β-D-thiogalactopyranoside (IPTG), 1 mM (for pRSETA) or 0.1 mM (for ASKA plasmids). Cells were harvested by centrifugation, thoroughly washed with 0.9 % NaCl and resuspended in 10 mL of binding buffer (50 mM potassium phosphate pH 7.5 containing 500 mM

NaCl and 20 mM imidazole), supplemented with EDTA-free protease inhibitor (SigmaFast Protease Inhibitor Cocktail Tablet, from Sigma Aldrich). Cells were lysed by sonication for 2 min (20 s each pulse) on ice using a Vibra Cell sonicator (Sonicator Sonics & Materials,). The lysates were clarified by centrifugation at 10000 x g for 15 min at 4 °C.

Recombinant proteins were purified by immobilized metal affinity chromatography (IMAC). The cell free extract was loaded onto a 5 mL HisGraviTrap column (GE Healthcare), and washed with washing buffer (50 mM potassium phosphate buffer pH 7.5 containing 500 mM NaCl and 50 mM imidazole). The His6-tagged proteins were eluted with an elution buffer (50 mM potassium phosphate buffer pH 7.5 containing 500 mM NaCl and 500 mM imidazole). The salts and imidazole from protein-containing fractions were removed with a PD-10 Sephadex G-25 column (GE Healthcare). Purified proteins were kept in storage buffer (50 mM potassium phosphate buffer pH 7.5 containing 100 mM NaCl and 10 % v/v Glycerol) at -80 °C until used. Finally, Amicon Ultra centrifugal-15 filters (Millipore) were used to concentrate the proteins.

SDS-PAGE and native electrophoresis— Proteins were analysed by SDS/PAGE electrophoresis on 10 % acrylamide gels using a Mini-Protean cell (Biorad). For native electrophoresis, NativePAGE 4-16 % Bis-Tris gels and Native unstained Protein Standard (Life Technologies) were used. The proteins were detected by Coomassie Blue staining (Thermo Fisher Scientific).

Detection of lysine acetylated proteins by western blot analysis— Lysine acetylated proteins were separated by SDS-PAGE or native-PAGE. The proteins were transferred to PVDF membranes using a semidry transfer unit (Trans-Blot® SD Semi-Dry Transfer Cell, Bio-Rad). The membranes were incubated with a primary rabbit monoclonal anti-acetyl-lysine antibody (ImmuneChem) and a goat anti-rabbit IgG secondary antibody (Santa Cruz Biotechnology). Finally, the membrane was incubated for 10 min with Amersham ECL Western blotting detection reagent (Thermo Scientific).

Acetylation and deacetylation assays—All acetyltransferase/deacetylase enzymatic experiments were performed at 37 °C in 50 mM potassium phosphate buffer at pH 7.5 with a reaction volume of 200 µL. A blank assay without enzyme under the same conditions was carried out

to subtract the chemical hydrolysis of acetyl-CoA. The concentration of the TNB²⁻ anion was determined using a molar extinction coefficient of 15.53 mM⁻¹ cm⁻¹, which was experimentally determined from the slopes of three independent experiments using 62.5 to 500 µM cysteine and 0.3 mM DTNB (5,5'-dithiobis-2-nitrobenzoic acid) as standard. The amount of purified PatZ enzyme was optimized. Experiments were carried out at a PatZ concentration of 60 nM. The initial rates of colour development, obtained as milliunits of absorbance per minute at 412 nm (Synergy HT spectrophotometer, Bio-Tek), were converted to units of absorbance per minute by means of the PathCheck Sensor feature. Pseudo-first-order kinetic parameters were determined using Prism v6 (GraphPad) analytical software. A non-enzyme control was used to correct the background. Data for Acs and PatZ autoacetylation were fitted to the equation $V_0 = (V_{\max} \times [S]) / (K_M + [S])$, where V_0 is the initial velocity, V_{\max} is the maximum velocity, $[S]$ is the substrate concentration, and K_M is the substrate concentration for half-maximal velocity.

Data for acetyl-CoA were fitted to the equation $V_0 = V_{\max} \times [S]^h / (K_{0.5}^h + [S]^h)$, where h represents the Hill coefficient and $K_{0.5}^h$ denotes the substrate concentration for half-maximal velocity. The parameters were determined from curves with an r^2 value of 0.98.

Deacetylation assays were carried out with a 3:1 CobB:PatZ molar ratio and a 1 mM NAD⁺ concentration.

Acetyl-CoA synthetase activity— The acetyl-CoA synthetase assay used was based on the coupled assay reported by Williamson and Corkey (25). AMP production was detected via a coupled-enzyme assay in which myokinase (MK), pyruvate kinase (PK) and lactate dehydrogenase (LDH) couple AMP production to NADH oxidation. Acs was purified from *E.coli* BL21(DE3) $\Delta patZ$ and preincubated with different PatZ concentrations at 0.1 mM acetyl-CoA for 1h prior to measuring its synthetic activity. Then, standard acetyl-CoA synthetase assays (0.2 mL) were performed at 37 °C in 50 mM potassium phosphate buffer at pH 7.5 containing 3 mM PEP (phosphoenolpyruvate), 5 units MK, 1 unit PK, 1.5 units LDH, 5 mM MgCl₂, 2.5 mM ATP, 1.5 mM CoA, 0.1 mM NADH, 5 mM acetate and 1 mM dithiothreitol. This reaction was

started with the addition of 40 μL of 0.1 μM Acs. All reactions were performed in triplicate. Specific activity was calculated from the extinction coefficient of 6.22 $\text{mM}^{-1} \text{cm}^{-1}$ for the oxidation of two molecules of NADH for each AMP released. One unit of Acs activity is defined as 1 μmole acetyl-CoA formed per minute at pH 7.5 and 37 $^{\circ}\text{C}$. The specific activity of Acs is expressed as a percentage.

Differential Scanning Calorimetry (DSC)— A differential scanning calorimeter DSC 2920 (TA Instruments) was used for the calorimetric analysis of PatZ in different media. Samples were analysed using a programmed heating scan rate of 1 $^{\circ}\text{C} \text{min}^{-1}$ from 30 $^{\circ}\text{C}$ to 120 $^{\circ}\text{C}$. All experiments were carried out at 0.1 mg/mL in 50 mM phosphate buffer pH 7.5, and 0 to 1.5 mM acetyl-CoA. Phosphate buffer without protein was used as reference. No peaks were visible on the rescan in any case, which means that all transitions were irreversible. For data analysis and conversion Universal Analysis Software (TA Instrument v 0.4) was used and collected DSC data were normalized for protein concentration. Heat capacity (C_p) was expressed in $\text{kcal mol}^{-1} \text{K}^{-1}$ (1 cal = 4.184 J).

Gel permeation chromatography (GPC)— Gel permeation analysis was performed with an HPLC (Agilent technologies) using a Supelco Discovery Bio GFC 300 column ($4.6 \times 50 \text{ mm}$ and 5 μm particle, Sigma Aldrich, USA). The eluent was 50 mM phosphate buffer pH 7.5, containing 100 mM NaCl, with a flow rate of 0.05 ml/min . The injection volume was 50 μL . The elution of proteins was monitored by absorbance at 280 nm . A set of protein standards (Sigma Aldrich, USA) was used to generate standard curves from elution times of molecules with known molecular masses: thyroglobulin (670 kDa), catalase (250 kDa), γ -globulin (150 kDa), bovine serum albumin (67 kDa), ovalbumin (44.3 kDa) and ribonuclease type A I (13.7 kDa). Blue dextran and p-aminobenzoic acid were used to determine column exclusion limits. The void volume (V_0) and the total volume (V_T) of the column were 0.35 mL and 0.8 mL , respectively. A standard curve using linear regression analysis was obtained: $K_{av} = -0.02 (M_w) + 1.44$ with an $r^2 = 0.97$. Data represent mean values with standard deviations for three separate experiments.

Liquid Chromatography–Tandem Mass Spectrometry Assay (LC-MS)— Samples were

alkylated with 100 mM iodoacetamide (IAA) for 30 min at room temperature in the dark. Proteins were digested with 0.5–1 μg of Trypsin Gold Proteomics Grade (Promega) for 3 h at 37 $^{\circ}\text{C}$. The reaction was stopped with 0.1 % formic acid and samples were dried using a vacuum evaporator. Tryptic peptides generated from the samples were separated and analyzed by LC/MS. An Agilent 1100 (Agilent Technologies) was equipped with a Zorbax SB-C18 HPLC column (Agilent Technologies) and connected to an Agilent Ion Trap XCT Plus mass spectrometer (Agilent Technologies) using an electrospray (ESI) interface. Two mobile phases were used, phase A, composed of water/acetonitrile/formic acid (94.9:5:0.1, v/v) and phase B, consisting of water/acetonitrile/formic acid (10:89.9:0.1, v/v).

The digested peptides were resuspended in 20 μL of phase A and eluted using a linear gradient from 0–80% phase B for 180 min and at a flow rate of 10 $\mu\text{L/min}$. The mass spectrometer was operated in the positive mode with a capillary spray voltage of 3500 V, and a scan speed of 8100 (m/z)/sec from 50 to 2200 m/z , with a target mass of 1000 m/z , and 3 spectra averaging. The nebulizer gas pressure was set at 15 psi, and the drying gas a flow rate at 5 l/min at a temperature of 350 $^{\circ}\text{C}$. MS/MS data were collected in an automated data-dependent mode (AutoMS mode). Data processing was performed with Data Analysis program for LC/MSD Trap Version 3.3 (Bruker Daltonik) and Spectrum Mill MS Proteomics Workbench (Agilent Technologies) (26, 27). After automatic validation of the results, the identified proteins with the sequence of the digested peptides were compiled. Peptides were considered valid with a score threshold of 8, and a percentage-scored peak intensity higher than 70%.

Dynamic light scattering (DLS)— DLS measurements were carried out in a NanoSizer ZS (Malvern Instruments), operating a laser of 632.8 nm at an angle of 173 $^{\circ}$. The autocorrelation function was processed by using the ZetaSizer v7.03 software associated with the instrument in the CONTIN mode. This procedure determines a diffusion coefficient that is presented as a distribution of the hydrodynamic radius, R_h . The hydrodynamic measurements were carried out at 20 $^{\circ}\text{C}$ ($T = 293 \text{ K}$) in 100 mM NaCl, 50 mM potassium phosphate buffer at pH 7.5 with a PatZ protein concentration of 10 μM . The experiment consisted of 12 measurements of 11 runs each, with 30

seconds per run, which amounted to 11 minutes of data acquisition time. The CONTIN analysis allows for two modalities in the distribution, expressing the amount of solute of a given R_h in terms of either contribution to scattering intensity or as mass fraction. The former is particularly sensitive to the presence of large particles even if their population is very small, while the latter detects the sample's major species. A limitation of DLS-derived distributions is that R_h is handled in a logarithmic scale, and peaks have, even for monodisperse samples, a merely instrumental width, so that components of a paucidisperse sample cannot be differentiated if their R_h do not differ widely. Nonetheless, the Protein Workshop mode, specific for protein samples, of the ZetaSizer software was useful for our purposes.

Analytical ultracentrifugation (AUC)— Analytical ultracentrifugation experiments were performed in a Beckman Coulter Optima XL-I analytical ultracentrifuge (Beckman-Coulter) using an An50Ti eight-hole rotor, 12-mm path-length charcoal-filled Epon double-sector centrepieces, employing the UV-visible detection system. The experiments were carried out in the same buffer and temperature mentioned above with a PatZ protein concentration of 10 μM . A wavelength of 280 nm was used in the absorbance optics. Sedimentation velocity (SV) runs were carried out for 8 hours at a rotor speed of 40000 rpm using 400 μl samples in the above mentioned solvent. A series of 400 scans, without time intervals between them, were acquired for each sample. Least squares boundary modelling of the sedimentation velocity data was used to calculate sedimentation coefficient distributions with the size-distribution $c(s)$ method (28) implemented in the SEDFIT v13.0b software (29). As the measurements are made at 20 $^\circ\text{C}$, the sedimentation coefficients determined are practically identical to the standard coefficient, $s_{20,w}$. Unlike DLS, AUC-SV has excellent resolution, enabling the differentiation of components (peaks in the $c(s)$ distribution) with close $s_{20,w}$ values. In addition to rather reliable sedimentation coefficients, AUC-SV also provides approximate estimations of the molecular weights.

RESULTS

PatZ shows positive cooperativity in response to acetyl-CoA substrate— Acs N ϵ -lysine acetylation

by PatZ was kinetically characterized. PatZ kinetic analysis showed a typical hyperbolic response *versus* Acs substrate (0 to 40 μM) at a fixed saturating concentration of acetyl-CoA (1 mM) (Figs. 1A, B).

In contrast, when Acs was held at a fixed saturating concentration (40 μM) and the acetyl-CoA concentration was varied (50 to 800 μM) (Fig. 1C), PatZ activity showed a sigmoidal dependence. The kinetic cooperativity observed in PatZ was revealed by the double-reciprocal plot of the rate data, which was upwardly concave (Fig. 1D) and could be described by the Hill equation (30). The calculated Hill coefficient (h) was 7.91 ± 0.22 ($r^2 = 0.99$), which describes the PatZ positive cooperativity on acetyl-CoA. The kinetic parameters determined are displayed in Table 2. These data showed that enzymatic rate was slightly higher for the substrate Acs than for acetyl-CoA, while the $K_{0.5}$ was much higher for acetyl-CoA.

Acs activity is regulated by the acetylation level.— Western blot was performed to confirm PatZ N ϵ -acetyl-transferase activity on Acs using anti-acetyl-Lys antibody. Acs was incubated with PatZ and 0-1 mM acetyl-CoA concentration for 30 minutes (Fig. 1E). As can be seen, there was an increase in Acs acetylation in response to increasing acetyl-CoA concentrations.

In order to identify the acetylated lysines of Acs by PatZ, a mass spectrometry assay was conducted after Acs incubation at different acetyl-CoA concentrations with PatZ. The Acs purified from a $\Delta patZ$ strain was acetylated at K199 and K226. These acetylations were independent of PatZ, probably due to non-enzymatic acetylation. Moreover, these acetylated lysines explain the low signal shown by Acs in the first line of the western blot (Fig. 1E). Acs showed sixteen acetylated lysines at 1 mM acetyl-CoA in the presence of PatZ. The identified lysine acetylation sites were 50, 54, 68, 111, 130, 131, 199, 200, 207, 221, 226, 400, 401, 604, 609 and 617. To determine whether the Acs acetylation was mediated chemically or enzymatically, an acetylation assay of Acs incubated at 1 mM acetyl-CoA concentration in the absence of PatZ was carried out. The lysine acetylation sites identified by chemical acetylation were 130, 131, 199, 200, 207, 226 and 221. Therefore, nine site-specific of lysine acetylation in Acs were due to enzymatic acetylation by PatZ. The results are given in Table 3. An example of the

assignment of acetylation to Lys 54 and 68 of Acs by LC-MS/MS analysis is shown in Fig. 2A.

Purified Acs from *E. coli* BL21(DE3) $\Delta patZ$ was incubated with different PatZ concentrations and 0.1 mM acetyl-CoA at 37 °C for 30 min before measuring Acs activity. The acetyl-CoA synthetase activity decreased in response to the acetylation level by PatZ (Fig. 1F). The Acs synthetic activity was also measured at 0 and 0.1 mM acetyl-CoA in the absence of PatZ and was found to be the same in both cases, 620.86 ± 12.6 U/mg.

Potential role for the E809 residue of PatZ in the catalytic mechanism— Although the GNAT family acyltransferases mechanism is not completely understood, most evidence suggests a sequential mechanism operates to transfer the acetyl group from acetyl-CoA to the targeted protein via a ternary complex. The reaction mechanism is initiated by the nucleophilic attack of the deprotonated amine on the carbonyl functional group of acetyl-CoA (10–12, 31). This proposed mechanism of catalysis involves a conserved glutamic acid (E809 in PatZ) acting as a general base, deprotonating the amine. To determine the role of E809, a PatZ mutant protein harbouring an E809 to alanine mutation was generated. The pH dependence of the acetyltransferase activity with Acs and acetyl-CoA as substrates was determined and compared with the wild type enzyme (Fig. 3A). As can be seen, PatZ^{E809A} showed lower activity than PatZ at low pH (5–7). However, this activity increased with the pH, reaching similar activity levels to those of the wild type enzyme. At high pH values (9–10) lysine residues were spontaneously deprotonated ($pK_a = 9.6$).

Phyre2 (32) and 3D ligand site (33) web servers were used to predict acetyl-CoA ligand-binding sites of the PatZ catalytic domain (sequence from 725 to 884) in multi-template/*ab initio* mode (Fig. 3B). Five templates were selected to model PatZ GNAT domain (sequence from 725 to 884), which were from *Kribbella flavida* (4MY3, Pdb id code), *Salmonella typhimurium* (3DR8, Pdb id code), *Mycobacterium smegmatis* (4ORF, Pdb id code), *Mycobacterium tuberculosis* (4AVC, Pdb id code), *Salmonella enterica* (4U5Y, Pdb id code) and *Saccharomyces cerevisiae*, based on heuristics to maximize confidence (99 % in all cases), percentage identity (21 %, 26 %, 29 %, 19 % and 19%, respectively) and alignment coverage by each template (Fig. 4). The Coenzyme A binding pocket

included V812, L813, V814, G824 and R825 residues, as detected by the fpocket2 program (Fig. 3B) (34, 35). WebLab Viewer Lite software was used to display the electrostatic surface of the PatZ catalytic domain (Fig. 3C).

Autoacetylation/Deacetylation of PatZ— It has recently been reported that PatZ is acetylated *in vivo* in *E. coli* (2, 9). However this acetylation has not been studied in detail and the consequences of this regulation are unknown. The kinetic of PatZ autoacetylation was determined at a fixed PatZ concentration (60 nM) and different acetyl-CoA concentrations (0–1 mM) using the DTNB assay. The activity plot was fitted to a nonlinear of Michaelis-Menten enzyme kinetics ($r^2 = 0.98$) with a K_M of 0.11 ± 0.01 mM (Fig. 5A). To test the kinetic mechanism of self-catalysed PatZ, its concentration dependence was examined, and a linear concentration dependence of log PatZ autoacetylation velocity on the log PatZ concentration was observed; $y = 1.16x - 2.39$, with $r^2 = 0.97$ (Fig. 5C). Western blotting confirmed that PatZ showed deacetylation by CobB (Fig. 5D) with a 1:3 (PatZ:CobB) molar concentration ratio and a reaction time of 5 hours. Therefore, PatZ acetylation is reversible.

To characterize the self-catalysed reaction of PatZ, the acetyltransferase activity of PatZ^{GNAT} (GNAT C-terminal catalytic domain of PatZ) on Acs and PatZ^{Acs-like} (truncated N-terminal PatZ fragment lacking the GNAT domain) was analyzed by western blot (Figs. 6A, B), using wild type PatZ (PatZ^{wt}) and PatZ^{GNAT} at 60 nM. PatZ^{GNAT} was seen to be catalytically inactive on both substrates, Acs and PatZ^{Acs-like}, which suggest that the N-terminal domain of PatZ is essential for acetyltransferase activity. Moreover, Acs was acetylated by PatZ^{wt}, while PatZ^{wt} could not acetylate PatZ^{Acs-like}.

A mass spectrometry assay of PatZ incubated with a saturating acetyl-CoA concentration (1 mM) was conducted. The results are shown in Table 3. Six acetylated lysine residues were identified, five on the surface of the protein core (146, 149, 391, 446 and 635) and one in the catalytic (GNAT) domain (819). An example of the assignment of acetylation to 146 and 149 of PatZ by LC-MS/MS analysis is shown in Fig. 2B.

Tetrameric PatZ oligomerizes to an octameric form by acetylation— In order to understand the changes induced in PatZ by acetylation, biophysical studies were conducted, determining the molecular weight

of the native protein by gel filtration chromatography (Fig. 7A). In the absence of acetyl-CoA, purified PatZ eluted mainly at 8.2 min, with a left shoulder peak appearing at 7.3 min, and with an apparent molecular weight of 410 ± 21 kDa and 798 ± 40 kDa, respectively, which correspond to tetrameric and octameric forms. In contrast, at a saturating acetyl-CoA concentration (1.5 mM) the main peak eluted at 7.3 min, with a right shoulder at 8.2 min, corresponding to a protein population in which the octamer species is predominant.

Purified PatZ was also analysed by dynamic light scattering (DLS) and analytical ultracentrifugation (AUC). Structural information can be extracted from both the diffusion coefficient, D , and the sedimentation coefficient, s . These are related to the friction coefficient, f , by the expressions $D = RT/(N_A f)$ and $s = M(1-\nu\rho)/(NA f)$, which are combined in the well-known Svedberg equation $s/D = M(1-\nu\rho)/RT$. R is the ideal gas constant, T the absolute temperature, N_A the Avogadro number, ν the specific volume of the protein solute and ρ the density of the buffer solvent, while f is proportional to the solvent viscosity, η_0 , and it depends on the conformation (size and shape) of the solute molecules. The friction coefficient is customarily expressed as an equivalent hydrodynamic radius, R_h , defined as $R_h = f/(6\pi\eta_0)$, which can be obtained as either, $R_h = RT/(N_A 6\pi\eta_0 D)$ or $R_h = M(1-\nu\rho)/(N_A 6\pi\eta_0 s)$. The hydrodynamic measurements were carried out at 20 °C ($T = 293\text{K}$), in the same buffer as used for gel permeation. The SEDNTERP software (29) could be used to estimate the values $\eta_0 = 1.00$ cP and $\rho = 1.01$ g/cm³ from the solvent composition. The same program was used to estimate the required protein solute properties from the amino acid sequence. Using the sequence of the monomer, the following properties were obtained for PatZ: $M = 98.0$ kDa, and $\nu = 0.742$ cm³/g.

Figure 7B (a,b) shows the results of the DLS measurements. Disregarding the appearance of a small amount of aggregated material, the single peak in the mass distribution corresponded to the protein in its oligomeric forms. The analysis of this peak by the ZetaSizer software showed that it corresponds to a polydisperse material (the Protein Workshop mode of the instrument software indicating the coexistence of different oligomers) with $M = 420 \pm 150$ kDa and $R_h = 7.7 \pm 2.4$ nm.

Figure 7C (a, b and c) shows the AUC results. The main feature was the peak in $c(s)$ with $s_{20,w} = 12.4$ S and $M \sim 446$ kDa. The results also showed two other minor but significant peaks, one with $s_{20,w} = 18.0$ S and $M \sim 800$ kDa, and another with $s_{20,w} = 4.6$ S and $M \sim 97$ kDa (Fig. 6C, c). As indicated in the Methods section, M values in AUC-SV are approximate.

The mass and intensity distribution of the hydrodynamic radius obtained by DLS revealed the existence of some aggregated protein but, as reported by the ZetaSizer software, the weight fraction of aggregates is smaller than 2 % (Fig. 6B). Indeed, that material does not showed up in the mass distribution. Results of the AUC measurements (Fig. 7C) showed that they are quite small and non-biased, indicating the good quality of the fit.

With the same purpose of understanding the PatZ oligomeric states, interconversion between the two oligomeric forms of PatZ was monitored by native PAGE at different acetyl-CoA concentrations (Fig. 8A). In the absence of acetyl-CoA the tetramer was the main form observed but, when the acetyl-CoA concentration was increased, an octameric structure was formed and the tetramer band was less abundant. Western blot with an anti-acetyl-Lys antibody revealed that only the octameric form was acetylated (Fig. 8B). To confirm that PatZ autoacetylation induces a change in its oligomerization status, lysine acetylated residues of PatZ were mutated to arginine (non-acetylatable mutation). These lysine residues were 146, 149, 391, 447 and 635 (PatZ^{5(K→R)}). The oligomerization of PatZ^{5(K→R)} and PatZ^{E809A} was studied by native PAGE. In both cases, the tetrameric form was observed in the absence of acetyl-CoA (Fig. 8C). However, an octameric structure was formed at 1mM acetyl-CoA for PatZ^{E809A} mutant but it was not observed for PatZ^{5(K→R)}.

To study intersubunits electrostatic interactions in the PatZ tetramer, purified and mainly tetrameric PatZ was incubated at 25 °C for 2 days in solutions containing different KCl concentrations (0 to 2.0 M) and analyzed by native PAGE (Fig. 8D) (36). The native PAGE analysis showed that the homotetramer molecule dissociated to homodimers at a KCl concentration of 0.5 M or higher. The maximal dissociation occurred at 2.0 M KCl, but homotetramers were observed at all KCl

concentrations, while monomers were not observed in any of the conditions assayed. The addition of KCl probably disrupted interdimer electrostatic interactions in the PatZ tetramer, while intermonomers interactions were not affected.

To further clarify PatZ oligomerization by acetylation and its physiological function, differential scanning calorimetry (DSC) was used. DSC measures the heat capacity of states and the excess heat associated with transitions induced by temperature changes. Purified PatZ DSC assays were performed at 0, 0.5 and 1.5 mM acetyl-CoA (Fig. 9A). Table 4 shows the PatZ thermodynamic parameters estimated from the DSC data. Tetrameric PatZ showed a single peak upon thermal denaturation in the absence of acetyl-CoA, reflecting a first-order protein denaturation pattern, $N \rightarrow D$ (N is the native state and D the denatured state) characterized by a T_m^0 of 80.19 ± 2 °C. In contrast, the protein showed a double-peaked denaturation curve in the presence of 0.5 mM acetyl-CoA (non-saturating concentration considering the concentration of PatZ in the assay), characterized by a T_m^0 of 81.15 ± 2 °C and a T_m^1 of 104.81 ± 4 °C (Table 4). Finally, the protein showed a single peak at a saturating acetyl-CoA concentration (1.5 mM), with a T_m^1 of 102.23 ± 3 °C, similar to the T_m^1 value for 0.5 mM acetyl-CoA. Subsequently, denaturation of PatZ was studied at different concentrations of acetyl-CoA, from 0.25 to 1.5 mM (thermograms not shown) and in all non-saturating acetyl-CoA concentrations, two peaks were observed in the thermograms. The data were analysed according to the van't Hoff equation (37) and the model (Fig. 9B), based on a relationship between the total protein concentration and the concentration of ligand or substrate, and the melting temperature, as expressed by the following equation (Equation 1) (38);

$$\ln \frac{[L]}{[enzyme]} = \frac{A}{n} - \frac{(\Delta H^\circ - \Delta C_p^\circ T_m^\circ)}{nR} \frac{1}{T_m} + \frac{\Delta C_p^\circ \ln(T_m/T_m^\circ)}{nR}$$

where R is the gas constant (8.31×10^{-3} kJ / mol K), n is the number of binding sites for substrate and A is a constant (-1284.1 ± 0.2). Using the above equation for PatZ thermodynamic analysis with several acetyl-CoA concentrations, a value of 7.2 ± 0.02 was obtained for n . This indicates that between seven and eight binding sites for this ligand exist on the active oligomer, which agrees with the formation of an active PatZ octamer.

In silico protein-protein docking— In order to propose a PatZ model for the tetrameric state based on the above results, *in silico* protein-protein docking was performed by ClusPro 2.0 server (39–42). Phyre 2 server (32) was used to predict the PatZ monomer structure (Supplementary pdbS1). This model was generated with 95 % of the residues modelled at > 90 % confidence level and only 44 residues were modelled by *ab initio*. The PDB file generated by Phyre2 was uploaded on ClusPro2 for monomer-monomer interaction analysis. The output in the form of the structure of a predicted complex was obtained. Five structures based on the best scores were retrieved. Taking into account the scores obtained and favouring hydrophobic interaction between monomers, the best model was chosen (Supplementary pdbS2). In the proposed dimer, the link between the C-terminal PatZ monomers occurs via a large hydrophobic surface which matches with the CoA binding domain (Fig. 10A). Finally, ClusPro2 was also used to generate a dimeric structure of this dimer based on electrostatic interactions corresponding to the tetramer form. The output of the predicted complex was obtained. Five structures based on best scores were downloaded. The best candidate structure was selected taking into account the scores and the electrostatic surface interaction (Supplementary pdbS3). In this model, the electrostatic interaction, demonstrated experimentally by the dissociation of the homotetrameric PatZ in the presence of KCl (Fig. 8D), occurs at most of the interface between dimers (Fig. 10B).

In order to deepen our knowledge on PatZ tetramer acetylation, Phyre2 server (32) was also used to model the PatZ sequence with the acetylated lysine residues in the core protein (146, 149, 391, 447, 635) changed to glutamine residues to simulate the effect of acetylation on the PatZ structure. Glutamine was selected because of its similar side-chain conformational entropy to acetyl-lysine (43, 44). This model was generated with 94 % of the residues modelled at $> 90\%$ confidence level and only 45 residues were modelled by *ab initio*. Then, this model was compared with the native PatZ model (Fig. 11). The mutant PatZ model presented a more compact structure (model dimensions (Å); X= 77.21; Y= 82.39; Z=94.65) than the native model (model dimensions (Å); X= 74.85; Y= 116.23; Z=93.76). This structural change resulting from the acetylation of PatZ could promote the

formation of the octamer. It seems likely that the predominant effect of the acetylation is to alter the shape and charge characteristics of a surface patch allowing contact between monomers that was not permitted in the tetramer wild type protein.

Validation of in silico PatZ tetramerization model using three-dimensional hydrodynamic analysis—

The essential value of hydrodynamic studies is the information that they provide on the structure of proteins. For many years, hydrodynamic coefficients have been used to probe the overall conformation (e.g. globular, fibrous, denatured, etc) of proteins in solution. In recent years, methodologies and computational tools have been developed to calculate the solution properties from detailed, residue- and even atomic-level structures (45–47). Apart from data for the solute and solvent properties, the input necessary for the calculation of solution properties is simply a PDB formatted file of atomic coordinates. For this reason such tools are now widely used to validate structures obtained from crystallography, NMR or *in silico* methods, by comparing the results experimentally determined with those obtained computationally. For the simple hydrodynamic coefficient, as in the present case, the most widely used tool is HYDROPRO (45, 47). Calculation of the sedimentation coefficients requires the values for ρ , T , η_0 , v , and M to be known. These are available in our case, but, note that the result for R_h is independent of these quantities and is directly calculated from the set of atomic coordinates.

Based on our PatZ modelled structures for the tetramer and the monomer by ClusPro2 and Phyre2 servers, respectively, using HYDROPRO version 10 (47) the results listed in Table 5 were obtained. The agreement between the experimental and calculated sedimentation coefficients and hydrodynamic radius was excellent: differences of less than 5%. Considering the diversity of aspects involved in (i) the *in silico* structure prediction of PatZ oligomeric forms, (ii) the DLS and, especially, the AUC experiments and (iii) data processing, it can be affirmed that the hydrodynamic experiments and calculations fully validate the *in silico* molecular modelling and docking studies of the PatZ monomeric subunit for the tetrameric assembly.

With an even simpler approximate methodology, the hydrodynamic radius of the tetramer model was calculated using the Protein

workshop viewer (48) and taking into account the macromolecular surface (49). The calculated average radius was 7.03 ± 0.5 nm, which is in accordance with the DLS and AUC results and the more precise HYDROPRO calculation.

DISCUSSION

PatZ activity is controlled by positive cooperativity and Acs activity is regulated by the degree of acetylation.— PatZ kinetic data for acetyl-CoA (Fig. 1C; Table 2) pointed to atypical sigmoidal activity, reflecting positive cooperativity (Hill coefficient = 7.91 ± 0.22). Positive cooperativity is unusual in enzymes belonging to the GNAT family, although this behaviour has been described in PatZ homologous enzyme, *SePat*, with a Hill coefficient of 2.2 ± 0.2 (8) suggesting that the structural basis for this behaviour is protein tetramerization in the presence of acetyl-CoA.

The kinetic parameters of PatZ were within the range of those reported for other KAT proteins (50). The acetylation of Acs by PatZ was confirmed by western blot and mass spectrometry analysis (Fig. 1E and Table 3). Moreover, the acetyl-CoA synthetase activity assay (Fig. 1F) confirmed that *E. coli* Acs activity was dependent on the acetylation degree caused by PatZ. Besides, the Acs synthetic activity measured at 0 and 0.1 mM acetylCoA in the absence of PatZ, was the same in both cases.

It is well known that in *S. enterica*, Pat-catalysed acetylation of Acs results in the modification of its K609 residue, which inhibits AMP-acetyl forming activity (15). In contrast, we observed that the number of site-specific lysine residues of Acs acetylated by PatZ increased with acetyl-CoA concentration (Table 3) and a correlation between Acs activity and its acetylation degree was established. In fact, our group has recently reported quantitative changes in the acetylation ratios of some of these residues after deacetylation with CobB *in vitro* (9).

PatZ kinetics for Acs acetylation (Table 2) indicated that the limiting factor was the acetyl-CoA concentration, since the $K_{0.5}$ for this substrate was 12.5 times higher than for the Acs protein. These results suggest a mechanism for linking the physiological state of the cell with the acetylation state of Acs, so the concentration of acetyl-CoA in the cell will determine the acetylation of Acs by PatZ. One could speculate that the post-translational

regulation of Acs prevents the accumulation of acetyl-CoA while avoiding the depletion of free coenzyme A and metabolic energy, as previously proposed (23, 51). In addition, it has recently been demonstrated that depletion of *patZ* gene leads to high *E. coli* proteome acetylation in acetate cultures. This increase in chemical acetylation could be the consequence of non-regulated Acs activity by PatZ (9).

Conserved glutamic acid 809 from PatZ is not important for catalysis— To date, studies on the acetyltransferases kinetic mechanism suggest, for most MYST and GNAT families, a direct-attack mechanism within a ternary complex. In this mechanism, a conserved glutamic acid residue functions as a general base by assisting in the deprotonation of the ϵ -amino group of the acetyltable lysine residue (10, 31, 52, 53). This was demonstrated in the MYST family member Esa1, where the conserved active-site E338 deprotonates the N- ϵ -lysine of histone, facilitating the nucleophilic attack on the bound acetyl-CoA (14). In the GNAT family member GCN5, the E173 deprotonates the ϵ -amino group of the targeted lysine of the protein (12). Based on this premise, local sequence alignment of histone acetyltransferase GCN5 (1YGH, pdb id code) and GNAT domain of PatZ showed that this residue is conserved in position 809 (Fig. 4) (54).

To determine the role of the E809 in PatZ catalytic activity, we generated a PatZ mutant protein harbouring an E809 to A and checked that it acted as a potential catalytic base in the reaction (Fig. 3A). The activity of the mutant enzyme was 20 % lower at pH 7 than for the native enzyme, although activity gradually recovered at pH values above 9 as a result of the spontaneous deprotonation of the ϵ -amino group of the targeted lysine residue on Acs. This result suggested that the E809 residue was not relevant for PatZ activity.

It is important to note that there are examples where an invariant glutamic is not necessary for catalysis (55). For example, the yeast Hpa2 GNAT has no obvious catalytic residue that might function as a general base, and it has been proposed that a net positive electrostatic potential of the active site would favour an uncharged state of lysine residue (56). This positive potential was not found in the GNAT catalytic PatZ domain (Fig. 3C). Other acetyltransferases, such as Hat1, AAT and AAC6, also have a net negative charge of the active site

(57–59). Thus an alternative mechanism must be necessary for proton transfer. This has been proposed for the GNAT family member Serotonin N-Acetyltransferase, in which two histidines residues are involved in the deprotonation (60).

In addition, the mutant structure without acetyl-CoA determined by native PAGE presented an apparent molecular mass of ~ 410 kDa (Fig. 8C), suggesting that the E809 does not alter the ability of protein to correctly fold and oligomerize. In the same way, CD spectra of the yGCN5-E173Q protein were identical to those of the native protein, suggesting that the protein did not undergo significant structural changes that could be attributed to the glutamate-to glutamine substitution native (10).

The GNAT catalytic domain model of PatZ was generated with 99% of the residues modelled and no residue was modelled by *ab initio* (Fig. 3B). The coenzyme A binding pocket included V812, L813, V814, G824 and R825 residues, which are also conserved in the GNAT family (Fig. 4) (55). These residues create a hydrophobic core for acetyl-CoA attachment, and it is also possible that acidic side-chains in the vicinity of the PatZ active site could play a role in deprotonation of lysine residue (Figs. 3B, C).

PatZ is a homotetramer that forms a thermostable octamer by autoacetylation— In contrast to the eukaryotic enzymes, prokaryotic acetyltransferases adopt various quaternary structures. A number of structurally characterized GNAT superfamily members have been found to be multimeric (55). A similar oligomeric behaviour has been described for *S. enterica*. *SePat* oligomerizes from a monomer to a tetramer form in the presence of acetyl-CoA (8). However, the fact that PatZ was mainly in stable tetrameric form in the absence of acetyl-CoA demonstrated that the tetramerization process was independent of acetyl-CoA ligand binding. In contrast, the presence of acetyl-CoA was required for the formation of the octamer state (Fig 7). Hydrodynamic experiments using the two techniques, DLS and AUC, confirm the oligomeric nature and composition of the protein, which is predominately tetrameric, with some octamers, and an even smaller but still detectable amount of monomers observed by AUC. As seen in Table 5, the approximate molecular weights observed from the AUC are in a 1:4:8 proportion. The DLS value, which must correspond

to an average, is slightly above than that of the tetramer because, compared with the other two species, there is more octamer than monomer. Regarding the hydrodynamic radius, the values for each species were determined from the sedimentation coefficient, using the exact values of M and those of v , ρ , T , and η_0 (Table 5). Again, the DLS hydrodynamic radius was slightly higher than that of the tetramer. Therefore, the results of the two hydrodynamic techniques are consistent. In addition, it is important to highlight that hydrodynamic results agree with those of gel filtration chromatography analysis. This technique showed that, in the absence of acetyl-CoA, purified PatZ eluted mainly as a tetramer with a certain presence of octamers and an apparent molecular weight of 410 ± 21 kDa and 798 ± 40 kDa, respectively.

Native PAGE showed that the formation of the octamer occurred above a 0.05 mM acetyl-CoA concentration (Fig. 8A), which agrees with the Michaelis-Menten kinetic constant for PatZ autoacetylation of 0.11 ± 0.01 mM (Fig. 5A). Moreover, western blot of native PAGE showed that only the PatZ octamer form has acetylated lysine residues. These results suggest that the formation of the octamer occurred via tetramer acetylation and not from the monomer, since the tetramer band disappeared gradually with a concomitant increase of the octamer band (Fig. 8B). In addition, Native PAGE of PatZ^{5(K→R)} showed that an octamer form was not formed at 1 mM acetyl-CoA (Fig. 8C). This result confirms that the autoacetylation of these lysine residues (146, 149, 391, 447 and 635) is responsible for inducing PatZ octamerization.

The acetylation kinetics of Acs by PatZ (Fig. 1A) showed that the octamer form was a functionally active form since the acetyltransferase activity was measured at a fixed saturating acetyl-CoA concentration. A linear concentration dependence of log PatZ autoacetylation rate vs log Pat concentration at saturating concentration of acetyl-CoA (1 mM) was detected (Figs. 5B, C). This suggests that the reaction mechanism is first order, indicating an intramolecular process (61). The autoacetylation of histone acetyltransferases, such as p300, Tip-60 or the MYST acetyltransferases Esa1 and hMOF (46–49), has been described. Furthermore, the acetylation of these

acetyltransferases is reversed by deacetylases as described for PatZ in this study (Fig. 5D).

In order to obtain a deeper understanding of the oligomeric nature of PatZ, the Predict protein server (65) was used to predict PatZ monomer solvent accessibility. The result showed that 31.38 % of the residues are exposed, 9.37 % are intermediate and 59.26 % are buried within the protein. This largely hydrophobic interface could be involved in monomer contact to form the stable dimer in solution. This would explain the limited presence of PatZ monomers in equilibrium sedimentation studies, DSC analysis, and their absence in native PAGE and the elution profile of PatZ from a gel filtration column. Moreover, the results of the dissociation studies of homotetrameric PatZ in the presence of KCl (Fig. 8D) suggest that the PatZ homotetramer is stabilized, mainly by favourable electrostatic contacts between the two homodimers (36). In contrast, most of the interactions between monomers are hydrophobic. A similar behaviour has been demonstrated for histone acetyltransferase Hpa2, which forms dimers via an extensive, interdigitated, and largely hydrophobic interface and which forms a tetramer in the presence of acetyl-CoA (56).

With respect to PatZ domains, PatZ^{Acs-like} showed a tetrameric form, suggesting that dimers and tetramers are formed from the N-t domain (Fig. 8C). However, the Nt-domain was also necessary for catalytic PatZ activity (Fig. 6B). In fact, it has been demonstrated that full-length *SePat* is necessary for efficient binding to acetyl-CoA (8) and that the specificity of the GNAT-protein interaction is dictated by an extensive interaction surface (66). Moreover, the tetramer PatZ^{Acs-like} could not be acetylated by PatZ^{wt} (Fig. 6B). From this result, we hypothesize that a PatZ^{wt} tetramer cannot recognize another tetramer, suggesting that the autoacetylation mechanism consists of an intramolecular self-catalysis based on an acetylation between monomers, although more studies are needed to confirm this.

DSC experiments were carried out to understand the physiological consequences of this octamerization induced by tetramer autoacetylation. The results showed that PatZ has 8 acetyl-CoA binding sites in the octameric form at a saturating concentration of acetyl-CoA, and is characterized by a T_m of 104.81 ± 4 °C, while the PatZ tetramer has a T_m of 80.19 ± 2 °C in the absence of acetyl-

CoA. These data (Fig. 9) and PatZ acetyltransferase activity analyses (Fig. 1A) revealed that the octamer is functionally active and more thermostable than the tetramer. In fact, in other protein acetyltransferases, it has been described that multiple acetylated lysines play critical role in protein stability (67). In the same way, K709 of component HIF-1 transcriptional complex is acetylated by p300, which increases protein stability (68). This specific environmental function of protein acetylation might explains the association of tetramers to form an active PatZ octamer by acetylation and to increase its stability in response to cell stress situations (69).

The kinetic, structural and functional characteristics of the PatZ activity have physiologically relevant consequences. Kinetic data revealed that acetyl-CoA concentration limits the acetylation of Acs. This is possible due to the positive cooperativity of PatZ towards its substrate acetyl-CoA, which, in turn, is related to changes in protein structure and its oligomeric state. When cellular concentrations of acetyl-CoA increase (e.g. as a consequence of high Acs activity during growth on acetate), PatZ tetramers self-acetylate, which promotes the formation of PatZ octamers, which are more stable and active. This fact would prevent the accumulation of acetyl-CoA while avoiding the depletion of free coenzyme A (23, 38). This work supports a major role for PatZ as a molecular sensor of acetyl-CoA concentration.

In conclusion, this study demonstrates for the first time the structural regulation of an acetyltransferase by autoacetylation in a prokaryotic organism, a common post-translational event in eukaryotic organisms. Surprisingly PatZ autoacetylation is correlated with its oligomerization and structural stability, as well as with its activity. This study represents a breakthrough in prokaryotic protein modification studies.

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CONFLICT OF INTEREST

The authors declare that they have no conflicts of interest with the contents of this article.

AUTHOR CONTRIBUTIONS

TDP and JGJ conceived and coordinated the study and wrote the paper. TDP and JGJ designed, performed and analyzed all experiments in paper. JGT provided technical assistance and contributed to the preparation of Fig. 7. All authors reviewed the results and approved the final version of the manuscript.

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Footnote

¹Abbreviations: PTMs, post-translational modifications; GNAT, GCN5-related N-acetyltransferases; Pat, protein acetyltransferase; PatZ, *Escherichia coli* Pat; SePat, *Salmonella enterica* Pat; Acs, acetyl-CoA synthetase; CobB, NAD⁺-dependent sirtuin class deacetylase, NAD⁺, Nicotinamide adenine dinucleotide; TB,

Terrific broth; DTNB, 5,5'-dithiobis-2-nitrobenzoic acid or Ellman's reagent; IAA, Iodoacetamide; PatZ^{GNAT}, GNAT C-terminal catalytic domain; PatZ^{Acs-like}, truncated N-terminal domain of PatZ without GNAT domain; PatZ^{wt} wild type *E. coli* Pat; Differential scanning calorimeter, DSC; Dynamic light scattering, DLS; Analytical ultracentrifugation, AUC; Cyclic-adenosine 3',5'-monophosphate, cAMP.

FIGURE LEGENDS

FIGURE 1. PatZ activity on Acs. **A.** Substrate saturation curve of the PatZ-dependent acetylation reaction rate at different concentrations of Acs. **B.** Double-reciprocal plot of the kinetic data. PatZ was used at 60 nM, and acetyl-CoA at 1.0 mM. **C.** Substrate saturation curve of the PatZ-dependent acetylation reaction velocity at different concentrations of acetyl-CoA. **D.** Double-reciprocal plot of the kinetic data indicating a concave curve. PatZ enzyme was present at 60 nM, and Acs was present at a saturating concentration of 40 μ M. **E.** Western blot of the Acs protein. Acs untreated (lane 1) and lysine acetylated by PatZ at 0.1mM (lane 2), 0.2 mM (lane 3), 0.5 mM (lane 4) and 1.0 mM (lane 3) of acetyl-CoA. **F.** Acs activity *versus* acetylation degree. Percentage of acetyl-CoA synthetase activity (Acs) incubated at different concentrations of PatZ for 30 min and 0.1 mM of acetyl-CoA at 37°C. The insert shows the loss of Acs activity *versus* acetylation time as a result of the incubation with 68 nM PatZ and 0.1 mM acetyl-CoA.

FIGURE 2. Identification of Lysine acetylation by LC-MS/MS analysis. **A.** Assignment of acetylation to Lys 54 and 68 of Acs. MS/MS spectrum of precursor ion m/z 944.67³⁺ of modified peptide (ILDWIKPYQ**k**VKNTSFAPGNVSI**k**) from Acs. **B.** Assignment of acetylation to Lys 146 and 149 of PatZ. MS/MS spectrum of precursor ion m/z 1806.73³⁺ of modified peptide (LLGPNLGLLAPWQGLNASFSPVPI**k**RG**k**LAFISQSAAVSNTILDWAQQ**R**) from PatZ. The labelled peaks correspond to masses of b and y ions series shown in red and blue, respectively. Manual inspection of b and y ions series of these spectra indicates mass increments of 42 Da for ion⁺ and 21Da for ion²⁺ in some cases compared with the corresponding ion series of the unmodified peptides, suggesting that lysine is acetylated. Acetylation at the location is indicated in bold lowercase letter, “**k**”.

FIGURE 3. PatZ activity versus pH. **A.** pH dependent activity of PatZ (●) and PatZ^{E809A} mutant (■) on Acs. The PatZ wt and mutant concentration was 60 nM. The Acs concentration was 40 μ M at 1mM of acetyl-CoA. **B.** PatZ catalytic domain model generated by Phyre2 server. Coenzyme A binding pocket and conserved glutamic acid are shown in wireframe mode coloured green and purple, respectively. Acetyl-CoA substrate is shown in red. The figure was made with Swiss Model PDB viewer (70). **C.** Electrostatic surface PatZ catalytic domain (red represents negative electrostatic potential; blue represents positive electrostatic potential, and white is neutral).

FIGURE 4. Multiple alignment of GNAT domain from *E. coli* (PatZ), *Kribbella flavida* (4MY3, PDB ID code), *Salmonella typhimurium* (3DR8, PDB ID code), *Mycobacterium smegmatis* (4ORF, PDB ID code), *Mycobacterium tuberculosis* (4AVC, PDB ID code), *Salmonella enterica* (4U5Y, PDB ID code) and *Saccharomyces cerevisiae* (1YGH, PDB ID code). *, fully conserved residue; :, residues with high similarity; .., residues with low similarity. Sequence alignment generated in T-Coffee web server (v11).

FIGURE 5. PatZ acetylation/deacetylation. **A.** Substrate saturation curve of the PatZ-dependent acetylation reaction rate at different concentrations of acetyl-CoA. The PatZ concentration was 60 nM. **B.** Nonlinear concentration dependence of PatZ autoacetylation rate at 1mM acetyl-CoA. **C.** log-log of data in B was fitted to a linear regression, $y = 1.16 x - 2.38$, $r^2 = 0.98$. **D.** PatZ deacetylation by CobB western blot. The concentration ratio was 1:3 (PatZ:CobB).

FIGURE 6. Western blot of Acs and PatZ^{Acs-like} incubated with PatZ^{GNAT} and PatZ^{wt}. **A.** Multidomain PatZ protein that belongs to the GNAT superfamily, indicating the PatZ^{GNAT} (truncated GNAT C-terminal catalytic domain) and PatZ^{Acs-like} (truncated N-terminal domain of PatZ within GNAT domain). **B.** Acs and PatZ^{Acs-like} western blot incubated in presence of 1 mM acetyl-CoA with 60 nM PatZ^{wt} or PatZ^{GNAT}.

FIGURE 7. PatZ biophysical studies. **A.** Oligomeric state of PatZ in the absence of acetyl-CoA (—) and 1 mM acetyl-CoA (---) by gel filtration chromatography, at a flow rate of 0.05 mL/min. The insert shows a typical linear regression of a standard curve with a set of protein standards of known molecular mass. **B.**

Size distributions from DLS: (a) by scattering intensity (b) by mass **C.** AUC Results (a) scans in absorbance optics (b) Residuals of fitted scans (c) fitted distribution coefficients with s and M values as provided by SEDFIT.

FIGURE 8. Deacetylated PatZ native western blot. **A.** Deacetylated PatZ native PAGE at different acetyl-CoA concentration: lane 1 (0 mM), lane 2 (0.01 mM), lane 3 (0.02 mM), lane 4 (0.05 mM), lane 5 (0.1 mM), lane 6 (0.2 mM), lane 7 (0.5 mM) and lane 8 (1 mM) **B.** Western-blot analysis using anti-acetyl-Lys antibody. **C.** Native PAGE analysis of PatZ^{E809A} and PatZ^{5(K→R)} in the absence and in the presence of 1 mM acetyl-CoA. **D.** PatZ native PAGE incubated with KCl. Homotetrameric PatZ dissociation in the presence of KCl. The incubations were carried out at 25 °C for 2 days in solutions containing final concentrations of KCl from 0 to 2.0 M.

FIGURE 9. PatZ differential scanning calorimetry. **A.** DSC thermograms of PatZ. Experiments were run at different acetyl-CoA concentrations: 0 (—), 0.5 (---) and 1.5 mM (···). **B.** van't Hoff plot according to Equation 1 for PatZ DSC experiments in the presence of different concentrations of acetyl-CoA. The logarithm of the ligand concentration is plotted as a function of T_m^{-1} , the reciprocal of the temperature of maximal excess specific heat. The standard deviation of the points from the least squares curve corresponds to $\pm 0.20^\circ\text{C}$ in T_m ($r^2 = 0.99$).

FIGURE 10. In silico PatZ oligomerization by monomer and dimer docking simulations performed by the ClusPro 2.0 server. **A.** PatZ monomers dimerization by favoured hydrophobic interactions. The triangulated macromolecular surface (49) is shown in hydrophobic colour scale (32 colours) from yellow-brown (most hydrophobic) to blue (most hydrophilic). **B.** PatZ dimers tetramerization by favoured electrostatic interactions. Dimers of the tetramer are coloured in red and blue. All figures were created using Protein Workshop (48).

FIGURE 11. Deacetylated and acetylated PatZ models generated by Phyre2 server. PatZ models of native (A) and mutant (B). Catalytic domain is indicated in red and lysine residues changed to glutamine are indicated in purple. Coordinates indicate the model dimension in Å. The figure was made using Protein Workshop (48)

Table 1. Strains, plasmids and primers used in this study. Restriction nuclease sites are in grey. Bold typeface indicates the modified codon during site-directed-mutagenesis

<i>E. coli</i> Strain	Genotype	Source
BL21(DE3)	F ⁻ ompT gal dcm lon hsdSB(rB ⁻ mB ⁻) λ(DE3)	Agilent Technologies
BL21(DE3) Δ <i>patZ</i>	F ⁻ ompT gal dcm lon hsdSB(rB ⁻ mB ⁻) λ(DE3) <i>patZ</i> :kan	This study (74)
DH10B	F ⁻ mcrA Δ(mrr-hsdRMS-mcrBC) Φ80dlacZΔM15 ΔlacX74 endA1 recA1 deoR Δ(ara,leu)7697 araD139 galU galK nupG rpsL λ-	Invitrogen
Plasmid	Genotype	Source
pRSETA	N-terminal, rTEV-cleavable His6-tag overexpression vector, Amp ^R	Invitrogen
ASKA <i>acs</i>	N-terminal, His6-tag overexpression vector, Cam ^R . Encodes Acs wt	ASKA collection (26)
ASKA <i>cobB</i>	N-terminal, His6-tag overexpression vector, Cam ^R . Encodes CobB wt	ASKA collection (26)
pRSETA <i>patZ</i>	Encodes PatZ wt	This study
pRSETA <i>patZ</i> ^{E809A}	Encodes PatZ ^{E809A} mutant	This study
pRSETA <i>patZ</i> ^{S(K→R)}	Encodes PatZ ^{K146, 149, 391, 447, 635R} mutant	This study
pRSETA <i>patZ</i> ^{GNAT}	Encodes PatZ ^{GNAT} variant	This study
pRSETA <i>patZ</i> ^{Acs-like}	Encodes PatZ ^{GNAT} variant	This study
Primer	Sequence	
pRSETA <i>patZ</i> R	GGTGGTAAGCTTTATGATTCCTCGCGCTGGGC	
pRSETA <i>patZ</i> F	GGTGGTCTCGAGACCATGAGTCAGCGAGGACTGGA	
pRSETA <i>patZ</i> ^{E809A} R	GAACCAGTAC AG C ^A AAATGCGGCATCGATGTTATC	
pRSETA <i>patZ</i> ^{E809A} F	GATAACATCGATGCC GC ATTTGCTGTACTGGTTC	
pRSETA <i>patZ</i> ^{K146, 149R} R	CAGCCTGCCCGCTCTAATCGGCACAG	
pRSETA <i>patZ</i> ^{K146, 149R} F	CTGTGCCGATTAGACGCGGC AGG CTG	
pRSETA <i>patZ</i> ^{K391R} R	CATATTTGCTGCGGGGATGATGT CTT ACCGCTTCAATTAATAC TTG	
pRSETA <i>patZ</i> ^{K391R} F	CAAGTATTAATTGAAGCGGTA AG ACATCATCCCCGCAGCAAA TATG	
pRSETA <i>patZ</i> ^{K447R} R	GTTTCGCGTAGTTGCCTCTGATTACGCCGG	
pRSETA <i>patZ</i> ^{K447R} F	CCGGCGTAATCAG AGG CAACTACGCGAAAC	
pRSETA <i>patZ</i> ^{K635R} R	CACGAATCTTTTACT TCT GATCCCCTGAATAACC	
pRSETA <i>patZ</i> ^{K635R} F	GGTTATTCAGGGGATCAG A AGTAAAAAGATTTCGTG	
pRSETA <i>patZ</i> ^{GNAT} R	GGTGGTAAGCTTTATGATTCCTCGCGCTGGGC	
pRSETA <i>patZ</i> ^{GNAT} F	GGTGGTCTCGAGAACGAGAGTCGGCTGGCAG	
pRSETA <i>patZ</i> ^{Acs-like} R	GGTGGTAAGCTTGCAGCGTTCACCGTTTTTCAAT	
pRSETA <i>patZ</i> ^{Acs-like} F	GGTGGTCTCGAGACCATGAGTCAGCGAGGACTGGA	

Table 2. Kinetic parameters of PatZ with Acetyl-CoA and Acs as substrates

Substrate	V_{max} (U/mg)	K_M (μ M)	$K_{0.5}$ (μ M)	k_{cat} (s^{-1})	$k_{cat}/K_M(s^{-1}M^{-1})$
Acetyl-CoA	0.63 ± 0.006	-	185.2 ± 2.5	1.02 ± 0.05	5.49×10^3
Acs	0.73 ± 0.024	14.78 ± 0.8	-	1.19 ± 0.04	8.1×10^2

Table 3. Mass Spectrometry Assay of Acs and PatZ proteins

Protein	Acetyl-CoA (mM)	Acetylated Lysine residues
		0
Acs	0.1	50, 68 , 130, 131, 199, 226, 609
	0.5	50, 54, 68, 111 , 130, 131, 199, 207, 221, 226, 400, 401, 609
	1	50, 54, 68, 111 , 130, 131, 199, 200, 207, 221, 226, 400, 401, 604, 609, 617
PatZ	1	146, 149, 391, 447, 635, 819

Site-specific of enzymatic lysine acetylation of Acs by PatZ are shown in bold.

Table 4. Thermodynamic parameters of PatZ obtained from DSC thermograms

PatZ	Acetyl-CoA (mM)	T_m^0 ($^{\circ}$ C)	ΔH^0 (J/mol)	ΔCp^0 (W/g)	T_m^1 ($^{\circ}$ C)	ΔH^1 (J/mol)	ΔCp^1 (W/g)
Tetramer	0	80.19 ± 2	1560 ± 8	-0,1	-	-	-
	0.5	81.15 ± 2	1614 ± 10	-0,15	104.81 ± 4	1453 ± 7	-0.002
Octamer	1.5	-	-	-	102.23 ± 3	1668 ± 11	-0.005

Table 5. Experimental and calculated properties from hydrodynamic studies

Property	Technique	Tetramer	Monomer	Octamer
M (kDa)	DLS	$< 420^{(a)}$	-	-
	AUC	$\sim 446^{(b)}$	$\sim 97^{(b)}$	$\sim 800^{(b)}$
	Sequence	392	98	784
s (S)	AUC	12.4	4.6	18.0
	HYDROPRO	12.9	5.2	-
R_h (nm)	DLS	$< 7.7^{(a)}$	-	-
	AUC	7.3	4.1	9.9
	HYDROPRO	6.9	4.3	-

(a) Average over oligomers. The actual value should be slightly smaller

(b) Approximate values from AUC-SV

Figure 1

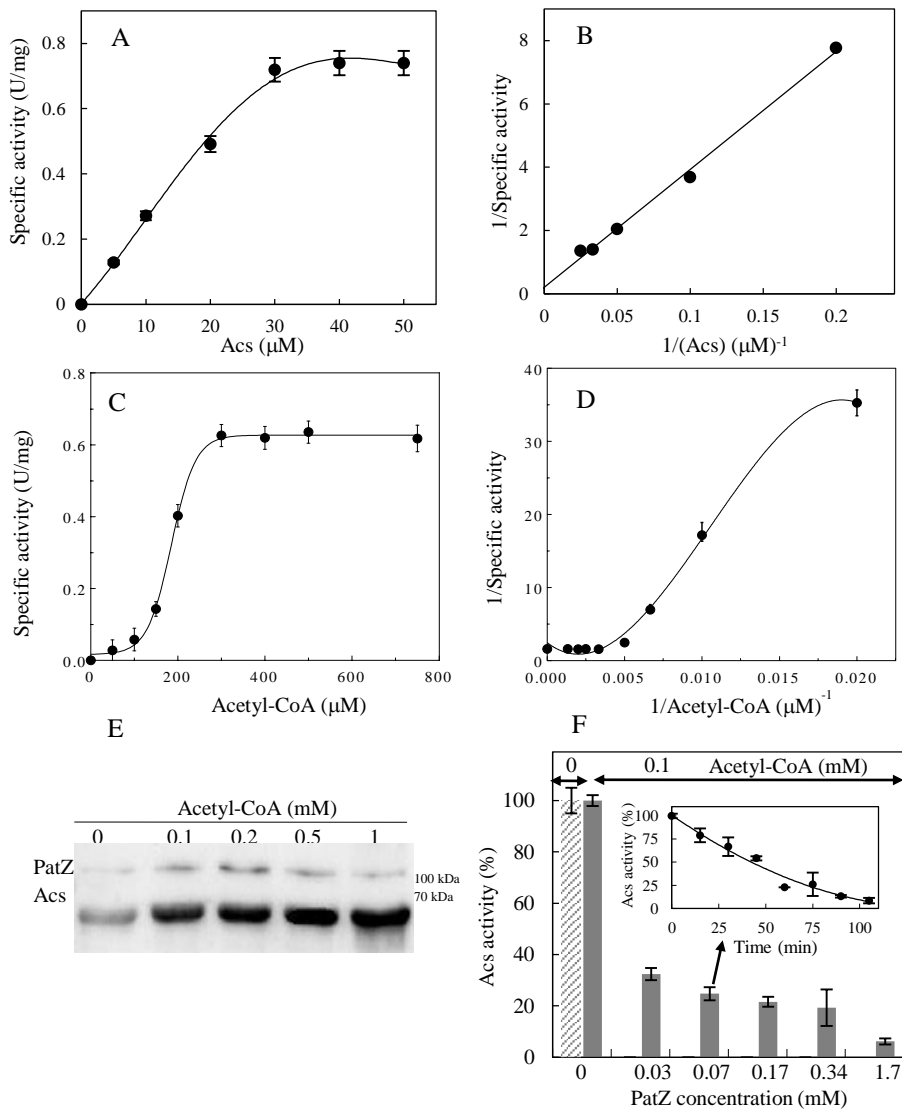


Figure 2

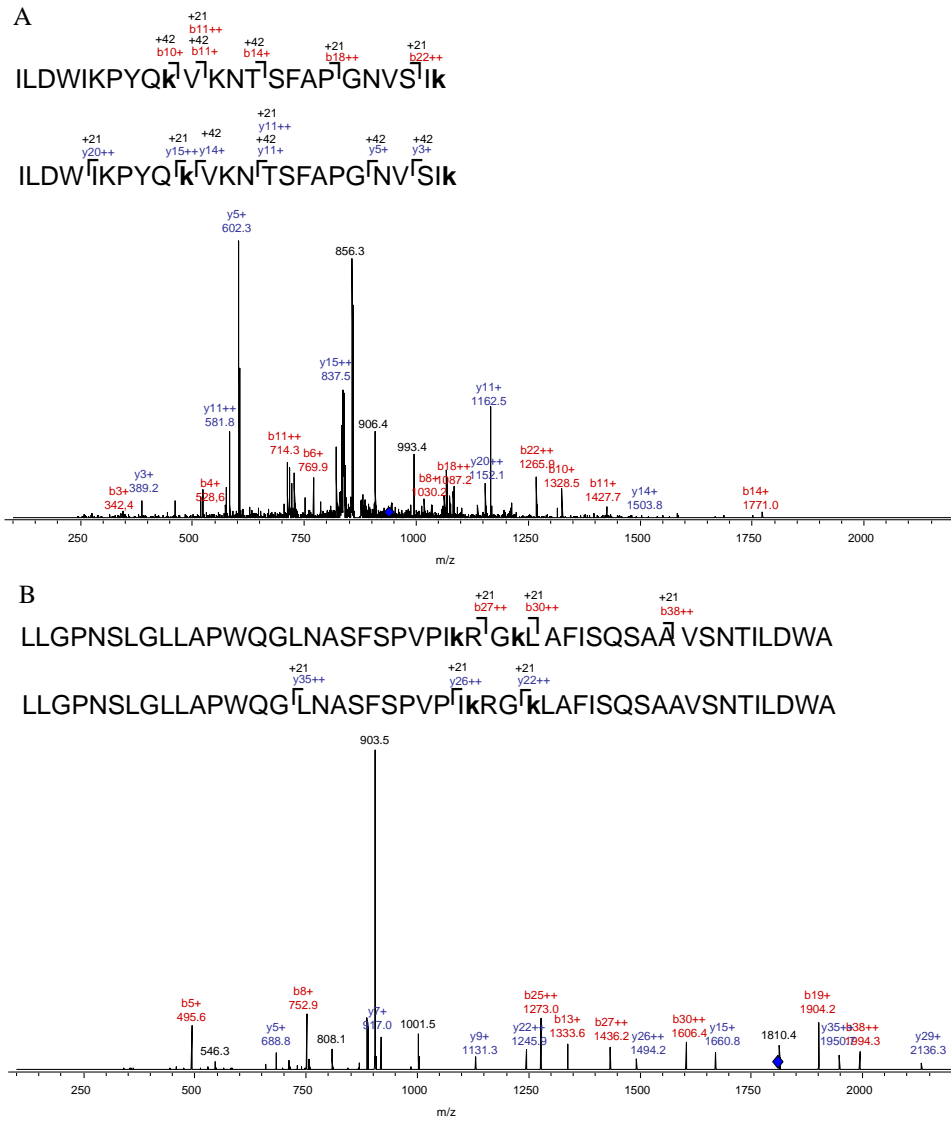


Figure 3

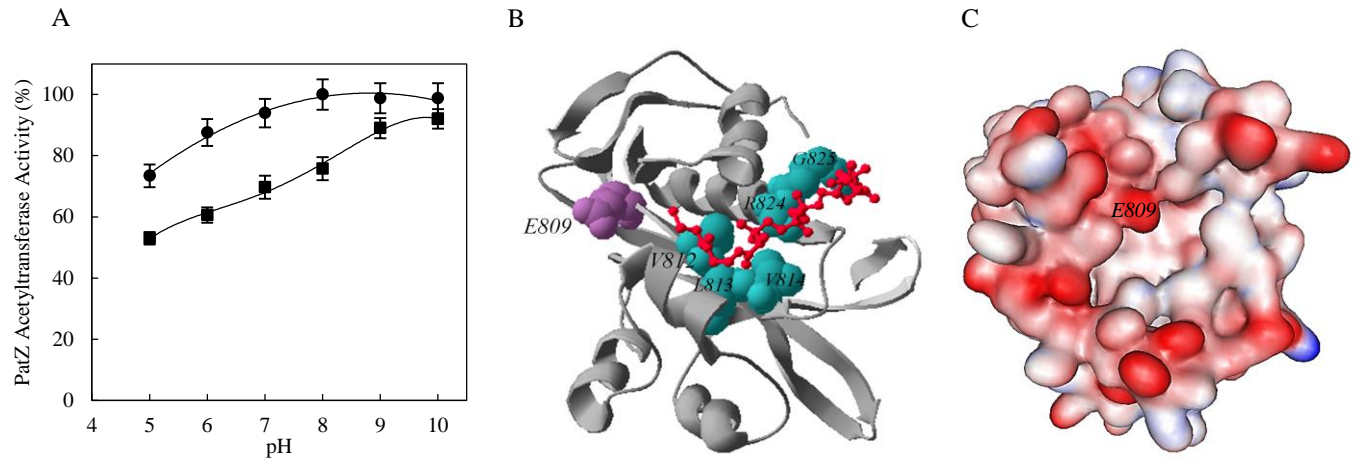


Figure 4

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PatZ -----RCLFRPIL-----PEDEPQLQQFISRVTKEDL 751
4MY3 -----EALQVRDAE-----DADWPAILPPFREIVSAGE 30
3DR8 -----AXTIRFAD-----KADCAAITEIYNHAVLHTA 28
4ORF LVRIARQRLAAFITPIPVQVRTGEWFYLRPVL-----PGDVER-----TSETL 169
4AVC LLRTARQRLAAFVSPVPVRLADGTQLXLRPVL-----PGDRERTVHGHIQFSGETL 182
4U5Y -----EADVLRDGGTARVRPIT-----VDDAERLVSFYEQVSDSK 62
1YGH -----I-EFRVVNNDNTKENMMVLTG-----LKNI 123
      *

PatZ YYRYFSEINEFTHEDLANMTQIDYDR-EMAF-----VAVRRIDQTEEILGVTRAI- 800
4MY3 TYAYDPELTDEQARSLW-XTPSGPQS-RTTV-----AV--DA--DGTVLGSANXYP 75
3DR8 -----AIWNDRTVDTDN-RLAWYEARQLLGYPVLVS--EE--NGVVTGYASFGD 72
4ORF YRRFQSV-RKPTRALLEYLFVVDYAD-HFVW-----VM--TEGALGPVIADARFV- 215
4AVC YRRFXSA-RVPSPALXHYLSEVDYVD-HFVW-----VV--TD--GSDPVADARFV- 226
4U5Y YRRFFAPYPRLCAKDVHRFTHHDFVD-RVGL-----AA--TI--GGEFIATVRYD- 107
1YGH ---FQKQLPKMPKEY---IARLVYDRSHLSM-----AVI--RKPLTVVGGITYR- 161
      *

PatZ -S--DPDNIDAEFAV-LVRSDLKGLGLGRRLMEKLITYTRDHGLQRLNG-ITMPNNRGMVALA 858
4MY3 NRP-GPGAHVASASF-XVAAAARGRGVGRALCQDXIDWAGREGFRAIQFNAVVE TNTVAVKLW 136
3DR8 WRSFDGFRYTVEHSV-YVHPAHQKGGRKLLSRLIDEARRCGKHVXVA-GIESQNAASIRLH 133
4ORF -RE-GHNATMAEVAF-TVGDDYQGRGIGSFLMGALIVSANYVGVQRFNA-RVLTDPNMMARKIM 274
4AVC -RD-ETDPTVAEIAF-TVADAYQGRGIGSFLIGALSVAARVDGVERFAA-RXLSDNVPXRTIX 285
4U5Y -R----IADEAEVAF-LVQDAHQGRGVSALLEHIAAVAREGIRRFAA-EVLPANNKMIKVF 163
1YGH -P--FDKREFAEIVFCAISSTEQVRGYGAHLMNHLKDYVRNTSNI-KYF-LTYADNYA-IGYF 221
      .. : : * : * . . *

PatZ RKLGFNVDIQLEEGI-----VGLTLNLAQR---E----- 884
4MY3 QSLGFRVIGTVPEAF-----HHPTHG-----YVGLHVX-----HRPL----- 168
3DR8 HSLGFTVTAQXPQVG-----VKFGR-----WLDLTFXQ-LQL--DEHAAPDAC-- 173
4ORF DRLGAVVW-----V-----VMTEVDVPPVDTVPFPELIDQIRDATRKVIRAVSQ 319
4AVC DRYGAVWQRE-DVGV-----ITTXIDVPGPELSLGREXVDQINRVARQVIEAVG- 333
4U5Y MDAGYTQKRSFEDGV-----VRLEFD-----L 185
1YGH KKQGFTKEITLDKSIWMGYIKDYEGGTLMQCSMLPRIRYLD----- 261
      *
    
```

Figure 5

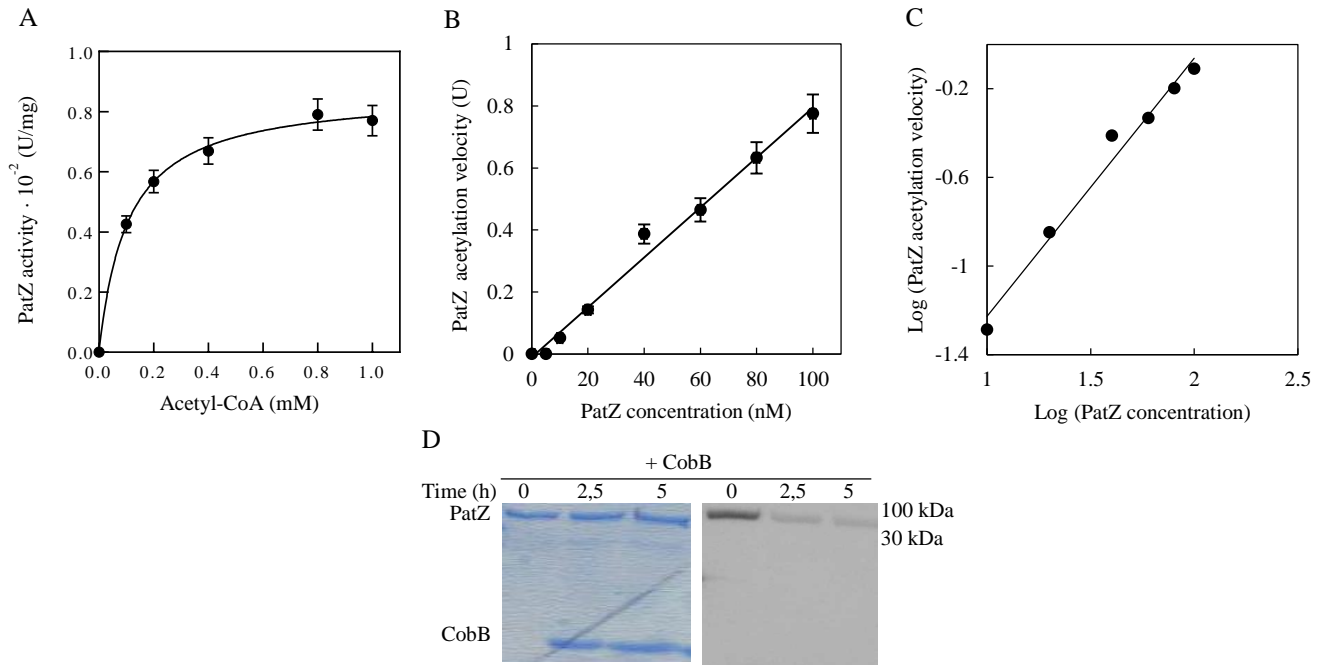


Figure 6

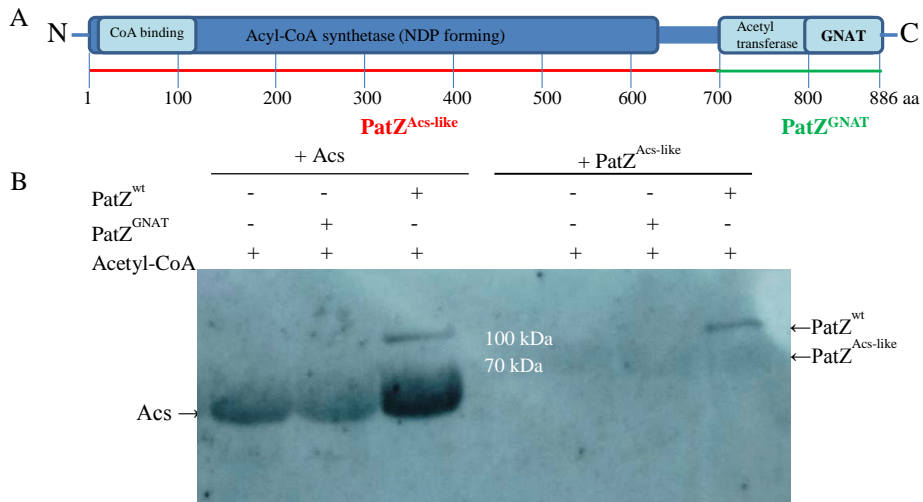


Figure 7

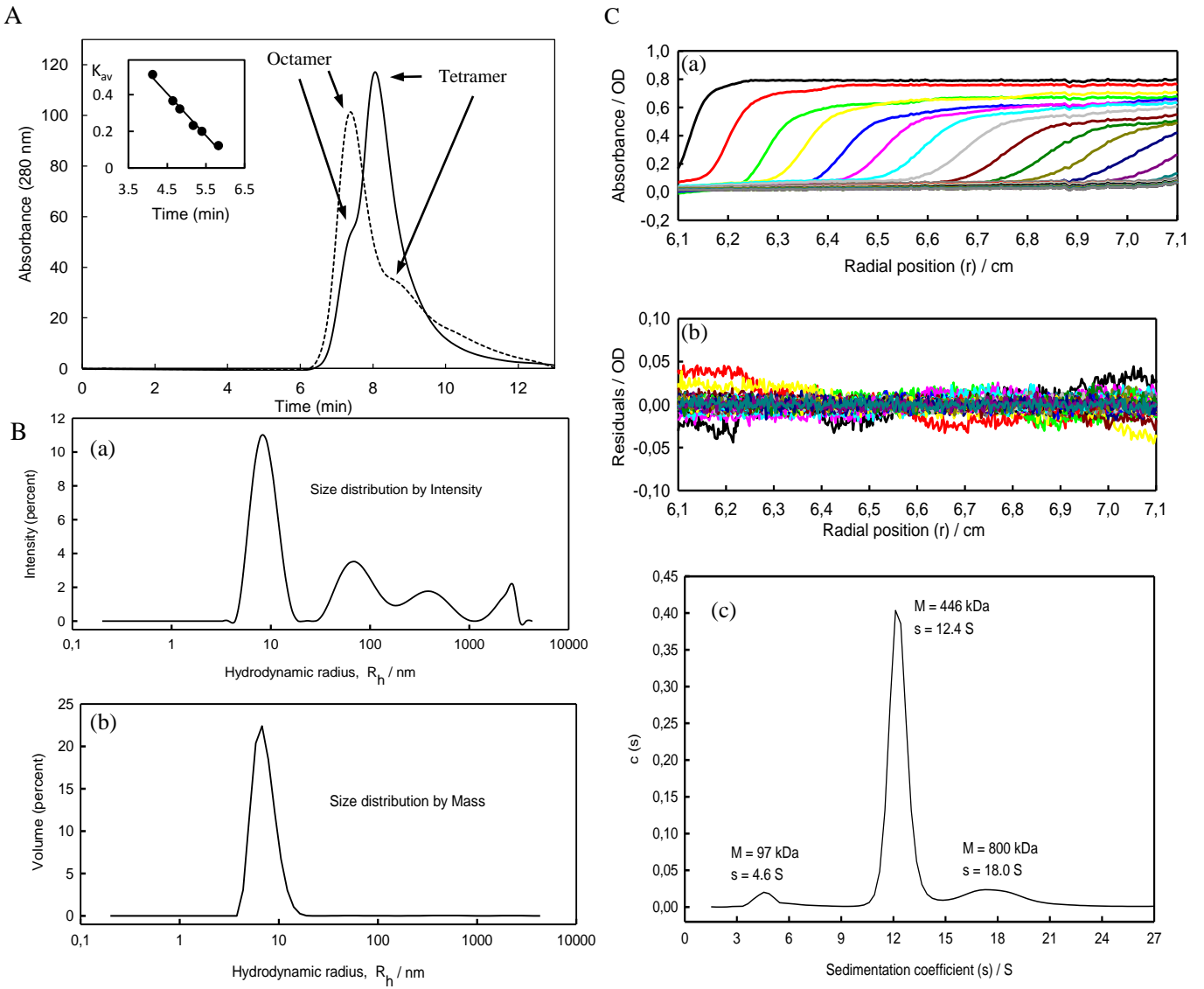


Figure 8

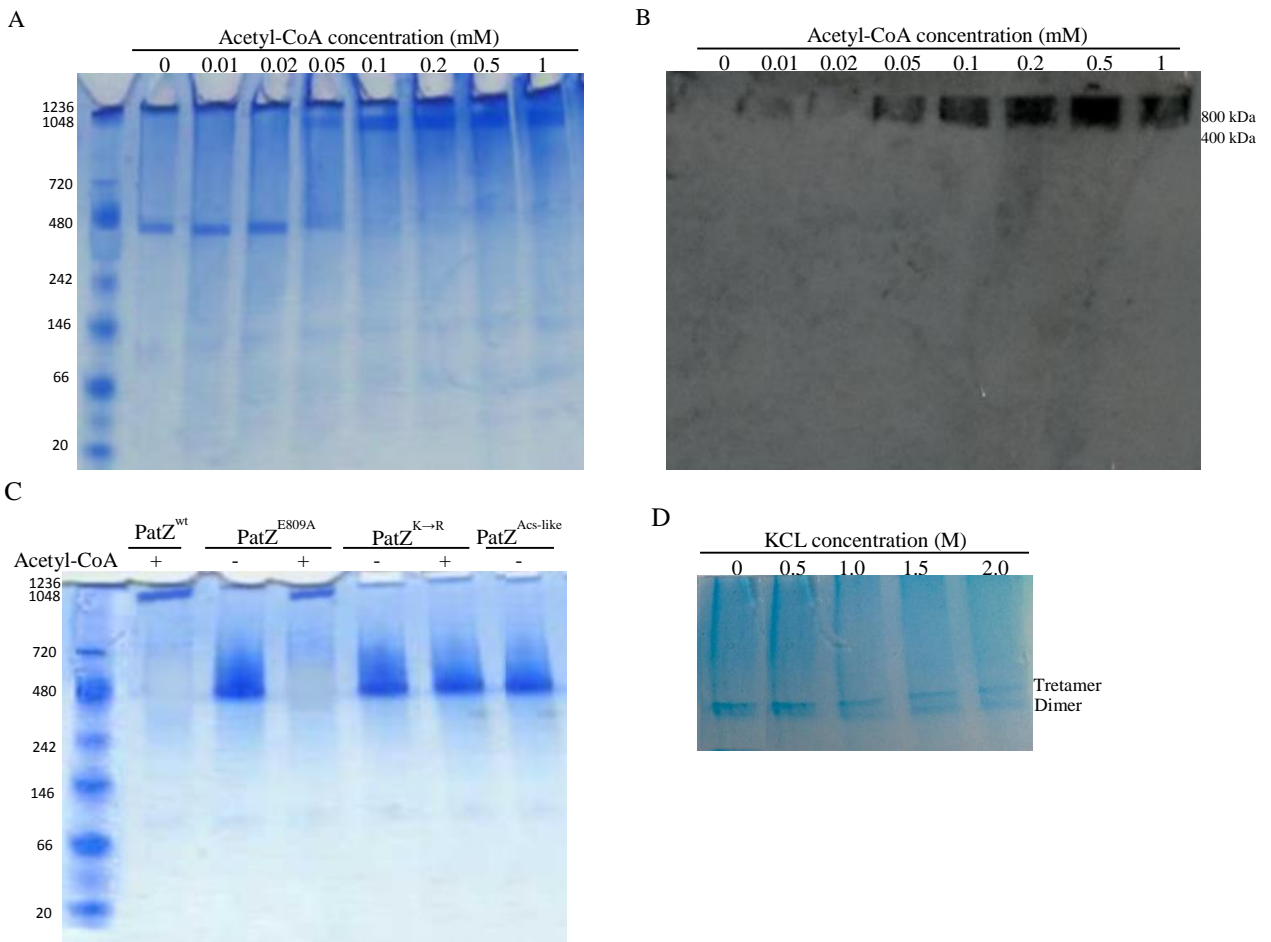


Figure 9

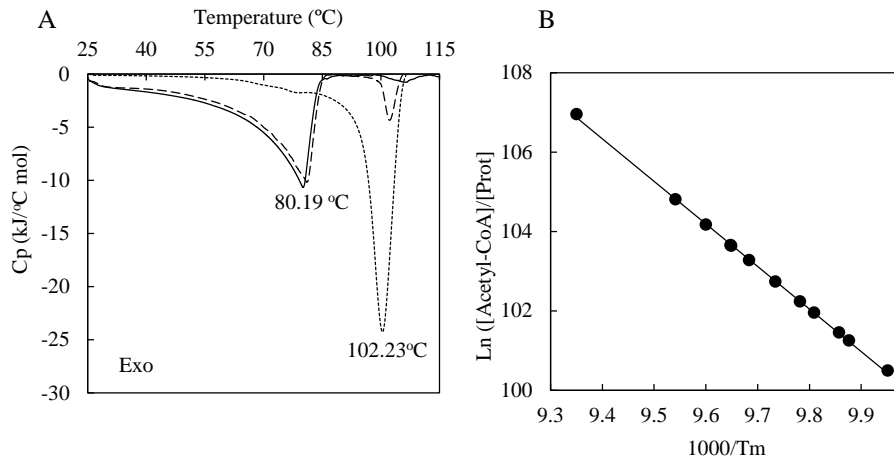


Figure 10

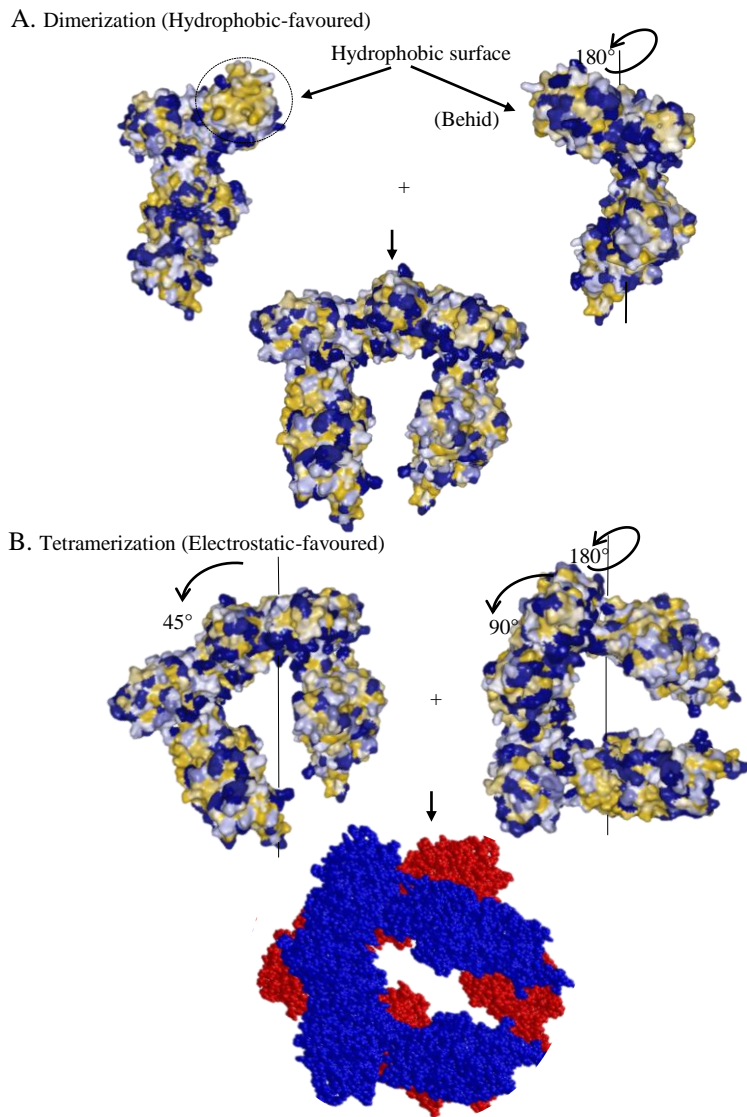
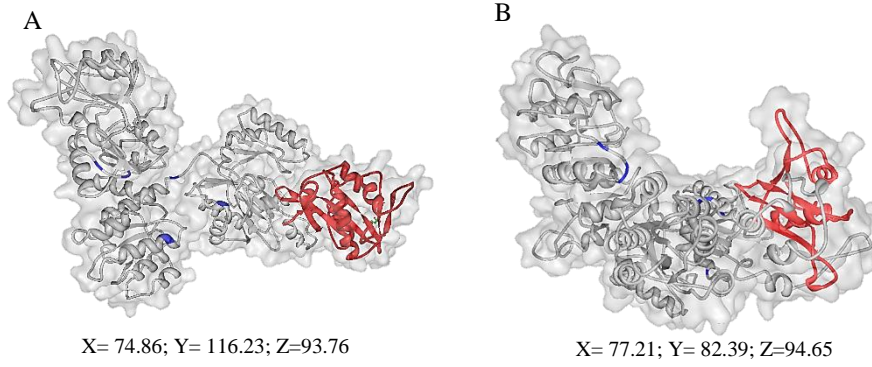


Figure 11



The protein acetyltransferase PatZ from *Escherichia coli* is regulated by autoacetylation-induced oligomerization

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