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The Human Histone Acetyltransferase P/CAF is a Promiscuous Histone Propionyltransferase

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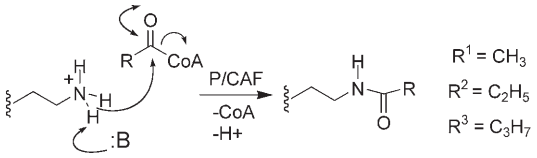
The histone-code hypothesis[1] suggests that specific histone protein modifications act as "marks" in chromatin and determine gene expression. These marks are brought about by covalent modification (for example, acetylation, methylation, and phosphorylation) of the histone N-terminal tails. The resulting epigenetic pattern defines transcriptional activation and silencing by the recruitment of specific effector proteins which have structural or enzymatic consequences for the surrounding region of chromatin. The acetylation of lysine residues on all four core histones, one of the most abundant and highly characterised modifications, is associated with transcriptionally active regions of chromatin, though this acetylation also plays roles in chromatin assembly and repair. In common with all histone modifications, the location and abundance of histone acetylation is dynamically regulated by two opposing classes of enzymes, histone acetyltransferases (HAT) and histone deacetylases (HDAC). These activities are typically found in multisubunit complexes, which are recruited to their target loci by interactions with transcriptional activators or repressors, respectively.[2] This is consistent with the distribution of many activating histone modifications, which are typically restricted to the promoter regions of actively transcribed genes.

Histone acetylation exerts its functional effect by two mechanisms. The charge neutralisation associated with lysine acetylation (see Scheme 1) reduces the interaction of histone tails



Scheme 1. The acyltransferase reactions catalysed by P/CAF by using native (R^1) and promiscuous (R^2, R^3) acyl donors.

with DNA, thereby facilitating the accessibility of chromatin structure, and enabling transcription factor access to the underlying DNA template. In addition, specific acetyl-lysine residues (R^1) are recognised by "bromodomains", a specific protein fold found in many chromatin-associated proteins including transcriptional regulators and chromatin remodellers.[3]

Revisiting[4] previous mass spectrometric fragmentation patterns of nuclear proteins from human HeLa S3 cells[5] revealed the existence of two new histone marks, namely propionylated and butyrylated lysines on H4 histones. This observation raises the question how these modifications were brought about. In vitro analysis indicates that several HATs can both acetylate and propionylate H4 peptides, although the lysine specificity of these modifications was not explored.[4,6] We now report that the human histone acetyltransferase P/CAF has the ability to transfer propionyl and acetyl groups with similar efficiency and specificity. When a histone H3 peptide (corresponding to the N-terminal H3 tail) and P/CAF enzyme were incubated with propionyl-CoA (ProCoA, R^2) a mass increase of 56 Da was observed in MALDI-TOF MS spectra (Figure 1), corresponding to

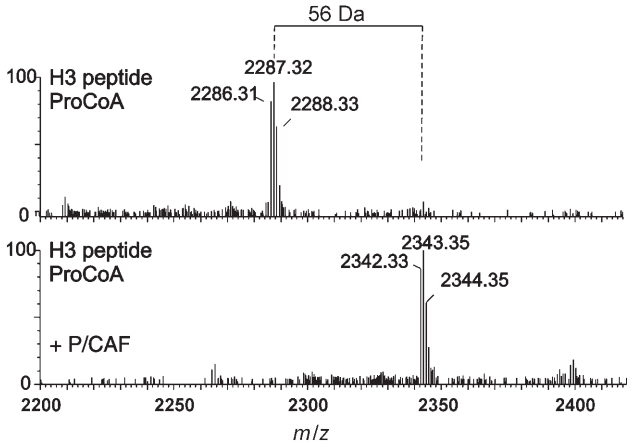


Figure 1. Propionylation of histone H3 peptide by the histone acetyl transferase P/CAF. MALDI-TOF MS spectrum of the H3 peptide (ARTKQTARKSTGGKAPRKQLC) incubated with ProCoA in the presence and absence of P/CAF, showing full turnover. Conditions: 1 h incubation, pH 7.5, 25 °C, 3.8 μM P/CAF, 300 μM H3 peptide, 400 μM ProCoA. See Figures S1 and S2 for the full spectra and analogoues spectra for incubations with BuCoA.

addition of one propionyl group. Analogously, P/CAF also catalysed butyrylation (BuCoA, R^3; Supporting Information). In the absence of P/CAF (Figure 1), no significant propionylation or butyrylation was detected.

A kinetic assay in which acyl transfer was detected spectrophotometrically by CoA formation[7] was used to quantify the rates of transfer of various acyl donors to the histone H3 tail peptide, the preferential substrate of P/CAF.[8] Activity was observed not only for AcCoA, but also for ProCoA and, to a lesser extent, with BuCoA.[9] No activity was detected with malonyl-

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CoA, methylmalonyl-CoA, isovaleryl-CoA, or hexanoyl-CoA. Figure 2 shows the saturation profiles obtained for Ac- and ProCoA.

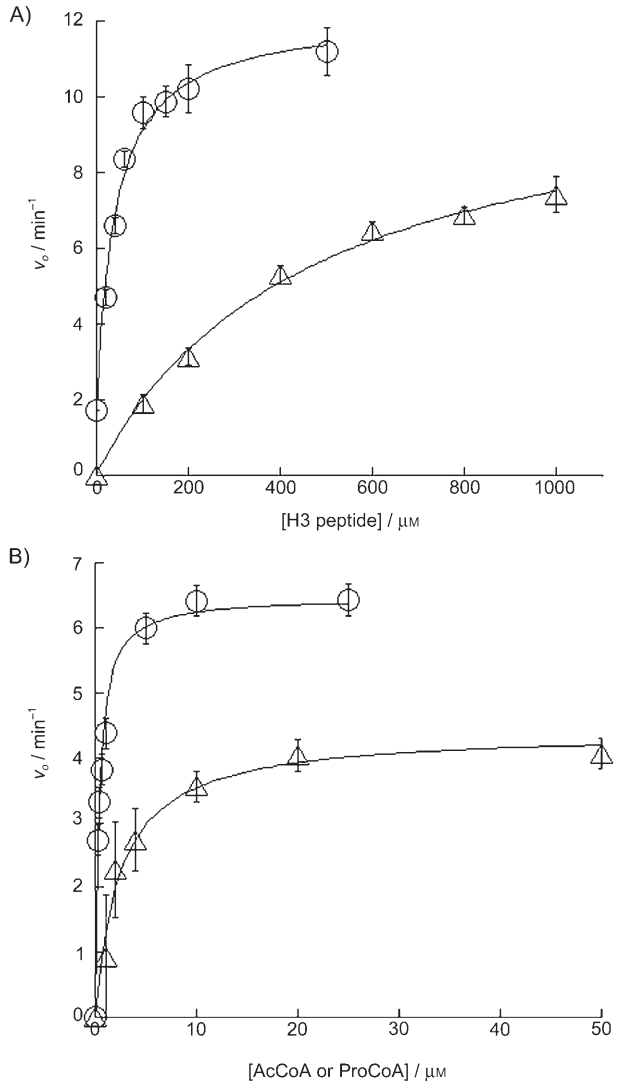

Figure 2. Michaelis–Menten plots for acyl transfer with a histone H3 peptide substrate (ARTKQTARKSTGGKAPRKQLC). A) Rates at varying histone H3 peptide concentrations and saturating AcCoA (○, 30 μM) and ProCoA (△, 100 μM). B) Rates at varying concentrations of AcCoA (○) versus ProCoA (△) in the presence of H3 peptide (50 or 250 μM, approximately at the respective K_M). Conditions: pH 7.5, 25 °C, [P/CAF] = 0.83 μM.

The k_{cat} for propionylation is 8.8 ± 1.5 min^{-1}, only slightly lower than the rate of acetylation (12.2 ± 0.4 min^{-1}) determined under identical conditions (extrapolated to saturating H3 peptide concentration, Figure 2A). The K_M^{acyl} increased from 0.38 ± 0.04 μM for AcCoA to 1.6 ± 0.9 μM for ProCoA (Figure 2B),[10] rendering the k_{cat}/K_M^{acyl} sixfold smaller. There is a marked decrease in the affinity for the peptide acceptor substrate: the K_M^{H3} increases tenfold (from 41 ± 5 to 453 ± 49 μM), leading to an approximately 15-fold lower second-order rate constant.

An active site mutation, Glu570Gln,[11] reduces both the acetylation and propionylation rates to the background rate of AcCoA and ProCoA hydrolysis, providing evidence that the same catalytic machinery is involved in catalysis of both substrates.

The histone H3 tail has four potential acetylation sites, but only one, Lys14, is acetylated by P/CAF.[8a] We determined the specificity of propionyl transfer by mass spectrometry (MS). MS/MS fragmentation of the intact propionylated peptide was unproductive as the internal Arg residues inhibited fragmentation. The peptide was therefore treated with acetic anhydride, to acetylate free lysines, cysteine, and the N terminus, and cleaved with trypsin (after Arg only). The resulting tryptic peptide masses indicated that the propionylated lysine was located in a single peptide containing Lys9 and Lys14 (Figure 3). MS/MS fragmentation of this peptide clearly showed the propionyl group to be on Lys14 (Figure 3). This indicates that acceptor specificity remains unchanged for the alternative acyl donor.

Enzymes are normally thought to be both highly efficient and specific for one single cognate substrate. However, there is a growing body of evidence that enzymes can acquire the ability to perform a new function, while still maintaining high activity for the original reaction.[12] Promiscuous activities have been invoked to explain the evolutionary mechanism of gene duplication, in which additional activities play a role for functional diversification by providing a "head start" activity for catalysis of new reactions.[12a,13] However, evolution of a promiscuous enzyme may also lead to a catalyst with multiple functions in one active site.[14] The observation of an alternative acyl donor for P/CAF provides further evidence that promiscuous activities can occur with efficiencies similar to their native activity. Such multispecificity may have a well-defined biological function: For example, substrate promiscuity by the enzymes of the Entner-Doudoroff metabolic pathway enables the archaeon *Sulfolobus solfataricus* to grow on glucose and galactose, forming pyruvate with only a single set of enzymes.[15] Other acetyltransferases related to the HAT GCN5 have been shown to transfer acetyl and propionyl groups to modify the activity of propionyl-CoA-synthetase by introducing propionyllysine modifications that can in turn be removed by sirtuins.[16]

The high efficiency of the reaction makes the enzymatic propionylation of histone tails feasible under in vivo conditions. ProCoA is readily available in cells, for example, as a result of the breakdown of odd-chain fatty acids and the amino acids isoleucine, methionine, threonine, and valine. In vivo HAT activities are actively recruited to target loci by protein–protein interactions, such that the effective local concentration of histone tails will be high, and should easily overcome the relatively high K_M^{H3} measured for the H3 peptide in the propionylation reaction. To date the biological effects of histone propionylation are not known. Lysine propionylation, like lysine acetylation, leads to charge neutralisation and is likely to induce similar structural changes in chromatin. However, the two groups differ in the steric bulk of the additional methylene group, offering the possibility of differential recognition of propionylated and acetylated histones by bromodomains or other proteins.

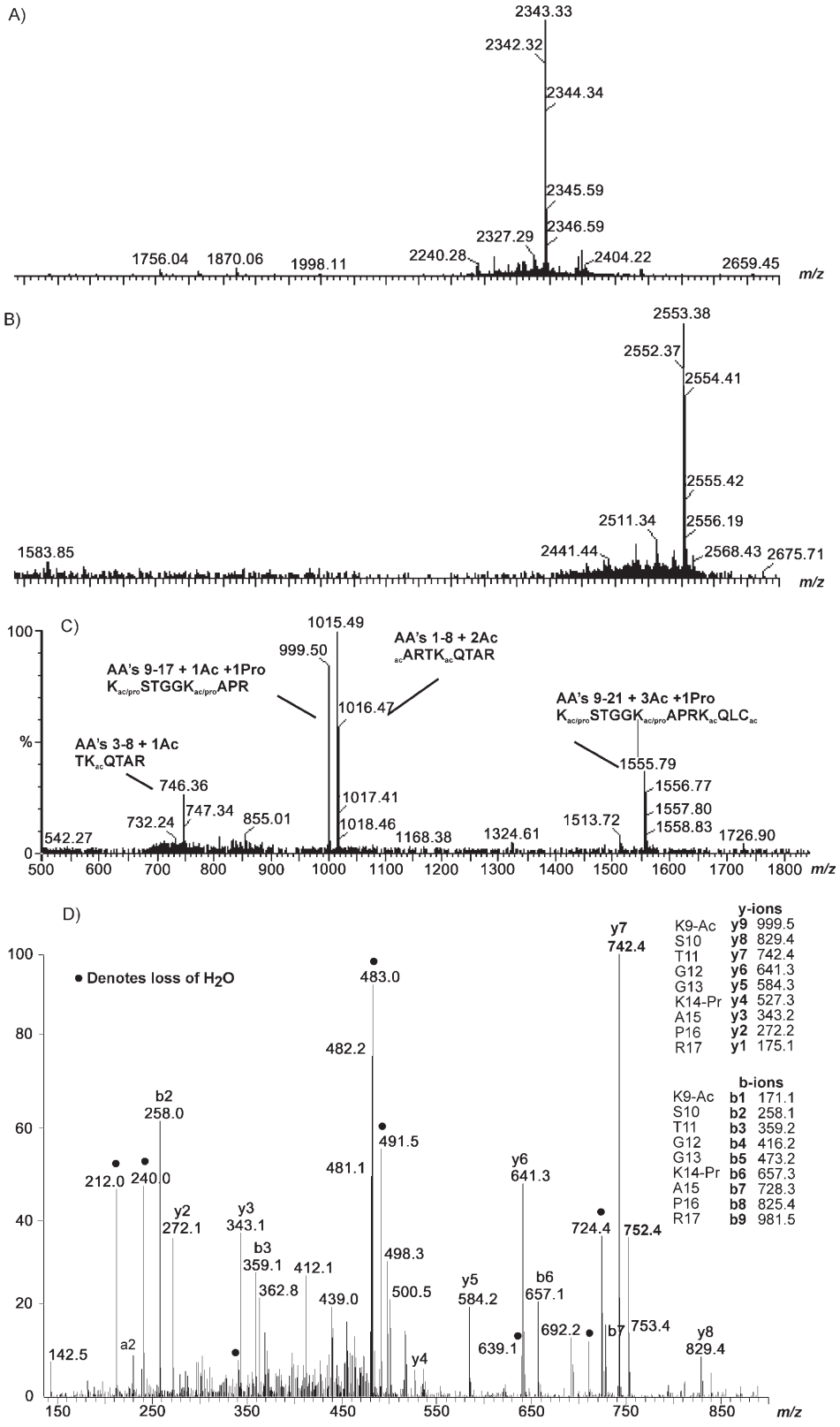

Figure 3. Specificity of propionylation. A) MALDI-TOF MS of the H3 peptide propionylated by P/CAF; B) the propionylated H3 shown in panel A after treatment with acetic anhydride, and C) following subsequent trypsin digestion of the sample shown in panel B. D) ESI-MS/MS spectrum of the acylated 998.5 Da fragment (KSTGGKAPR; amino acids 9–17) obtained after trypsin digestion. The MS spectrum in B shows that the mass of the peptide increases by 210 Da upon acetic anhydride treatment revealing acetylation at five sites, four amines, and one thiol. Spectrum C (all singly charged species) shows that the N-terminal peptide ARTKQTAR (amino acids 1–8; mass 1014.49 Da) is acetylated at its N terminus and at Lys4. The C-terminal peptide KSTGGKAPRKQLC (amino acids 9-21; 1554.79 Da) carries one propionyl group and two acetyl groups. The mass of the smaller peptide KSTGGKAPR (amino acids 9-17; 998.5 Da) shows that the propionyl moiety is located here, on either Lys9 or Lys14. The *b*-ion and *y*-ion series data in spectrum D are consistent with propionylation of Lys14 only.

Histone modifications are dynamic marks, regulated by enzymes with opposing functions (that is, acetylases and deacetylases). This suggests that if histone propionylation is biologically relevant it will also require depropionylating enzymes. This is consistent with findings that both an HDAC activity obtained from rat liver,[17] and two members of the human sirtuin family of HDACs (SIRT2 and SIRT3) can catalyse the depropionylation of lysine residues,[16] suggesting that these may act as the other half of a regulatory system for this modification. It is possible that additional unidentified enzymes are required for the depropionylation of histones.

The observation that a metabolite, ProCoA, can act as a substrate for histone modification is consistent with suggestions that cellular metabolism may impact on gene expression via epigenetic mechanisms.[18] In addition to cofactor concentrations as a regulatory mechanism, the chemical nature of alternative cofactors may generate distinct epigenetic marks, with specific effector proteins and different regulatory outcomes. This notion links cell nutrition and transcriptional outcome directly.[18] Taken together, the observation of propionyl marks in biological samples,[4] the substantial and residue-specific promiscuous histone propionyl-transferase activity of a human enzyme identified here, and the existence of depropionylating activities make it conceivable that histone propionylation activity has a regulatory biological role. We speculate that histone propionylation may prove to be a distinct epigenetic mark, recognised by either a subset of bromodomains, or by a novel protein fold.

Experimental Section

Peptides and chemicals: Histone H3 tail peptide, ARTKQTARKSTGGKAPRKQLC and H4 tail peptide, SGRGKGGKGLGKGGAKRHRKVGGK-biotin were synthesised by the PNAC facility (Cambridge University, UK). Acetyl-CoA, propionyl-CoA, butyryl-CoA, malonyl-CoA, methylmalonyl-CoA, succinyl-CoA, and hexanoyl-CoA were from Sigma–Aldrich.

Expression and purification: The catalytic domain of P/CAF (amino acids 492–658) was expressed in *Escherichia coli* BL21(DE3) from the pRSET-P/CAF[19] plasmid in liquid medium (NaCl (10 g L^{-1}), yeast extract (10 g L^{-1}), and bacto tryptone (20 g L^{-1})) at 25 °C for 12–14 h. Expression was induced by the addition of IPTG (0.25 mM). Cells were harvested by centrifugation, resuspended in sodium phosphate buffer (50 mM, pH 6.5) supplemented with DTT (1 mM), EDTA (1 mM), and one tablet of protease inhibitor cocktail (Roche) per 30 mL. After cell lysis and centrifugation (20000 *g*, 45 min), the supernatant was filtered (0.45 μM) and applied on a 5 mL HiTrap SP-FF cation exchange column (Pharmacia). Protein was eluted with a gradient of 0–1 M NaCl in sodium phosphate buffer (50 mM, pH 6.5) supplemented with DTT (1 mM) and EDTA (1 mM). Fractions containing P/CAF protein were pooled and further purified using a 120 mL GL16/60 Sephadex 75 size exclusion column (Pharmacia) run with sodium phosphate buffer (50 mM, pH 7.5) supplemented with NaCl (150 mM), DTT (1 mM), and EDTA (1 mM). Fractions containing pure P/CAF, as judged by SDS-PAGE, were pooled and stored at 4 °C or at −80 °C in the presence of glycerol (10%).

Site-directed mutagenesis: An E570Q active site mutant of P/CAF was created using the QuickChange Kit (Stratagene) with the oligonucleotides E570Q-for 5′-CAA GGA TTC ACA CAG ATT GTC TTC TGT G-3′ and E570Q-rev 5′-CAC AGA AGA CAA TCT GTG TGA ATC CTT G-3′ and the pRSET-P/CAF plasmid as PCR template.

Continuous HAT assay: HAT activity was followed in time using a coupled enzyme assay.[7] Briefly, the transfer of the acyl moiety from acyl-CoA to an acceptor molecule (ε-amino group of a lysine) generates CoA as a by-product. The CoA is then converted to succinyl-CoA by α-ketoglutarate dehydrogenase (or acetyl-CoA by pyruvate dehydrogenase), which is accompanied by NAD^+ reduction to NADH. Pyruvate dehydrogenase was only used to measure the K_M for acetyl-CoA; this enzyme could not be used for assay using acyl-CoA compounds other than acetyl-CoA as it generates acetyl-CoA from CoA. The absorbance of NADH at 340 nm was followed in time as a measure of the amount of CoA produced. Reactions were carried out in 300 μL volumes in 96-well plates at 25 °C. Reaction mixtures contained MES buffer (100 mM, pH 7.5), 0–1000 μM H3 peptide, NAD^+ (0.2 mM), thiamine pyrophosphate (0.2 mM), $MgCl_2$ (5 mM), DTT (1 mM), α-ketoglutarate (2.4 mM; or pyruvate), and α-ketoglutarate (0.1 units; or pyruvate) dehydrogenase (Sigma). P/CAF enzyme was added to a final concentration of 0.83 to 10 μM and reactions were started by the addition of the acyl-CoA compounds. Initial rates were measured at 340 nm and kinetic data were fitted to the Michaelis–Menten equation ($rate = k_{cat}[S]/(K_M + [S])$) and the rates of spontaneous acyl-CoA hydrolysis (no enzyme) were subtracted from the initial rates. The extinction of NADH coefficient was 4984 Abs M^{-1} under the conditions used. Rates were calculated by dividing the measured slope by the protein concentration and the ε_{NADH} ([slope (Abs min^{-1})]/[protein (M) * ε_{NADH} (Abs M^{-1})]).

Mass-spectrometric data and experimental details: Propionylation of the H3 peptide by P/CAF was carried out in MES buffer (25 mM, pH 7.5) containing H3 peptide (300 μM), propionyl-CoA (400 μM), and P/CAF enzyme (3.8 μM) at 25 °C for 1 h. Butyrylation of the H3 peptide by P/CAF was carried out in the same buffer containing H3 peptide (300 μM), butyryl-CoA (600 μM), and P/CAF enzyme (40 μM) at 25 °C for 4 h. Negative controls did not contain the P/CAF enzyme. Reactions were stopped by heating the samples to 65 °C for 5 min to inactivate the enzyme. Samples were then analysed by MALDI-TOF mass spectrometry on a Waters Micromass TofSpec2E instrument using α-cyano-4-hydroxycinnamic acid matrix; a propionylation/butyrylation event will increase the mass of the H3 peptide by 56/70 Da respectively. Lysine residue specificity was determined by treating the propionylated H3 peptide (50 μM in 0.5 M Tris-Cl, pH 7.5) with 0.2 μL acetic anhydride (5 min, 21 °C) to acetylate all free amine and thiol groups. Mass analysis confirmed complete reaction; 210 Da added = 5 acetyls (4 amine, 1 thiol). The reaction mixture was titrated back to pH 7.5 with Tris-Cl (1 M, pH 7.5) and modified trypsin (5 μg, Promega) was added. Digestion, now at the C-terminal side of Arg only as the Lys residues are acylated, was at 37 °C for 15 min and it produced four peptide products. Following desalting by μC18 ZipTip (Millipore), MALDI-TOF MS showed propionylation in one peptide only, *m/z* 999.5 $[M+H]^+$, at either Lys9 or Lys14 in the H3 peptide. ESI-MS/MS of this peptide, on a ThermoFinnigan LCQ Classic mass spectrometer, showed *b*- and *y*-ion series corresponding to propionylation of Lys14.

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[9] A butyryl group is transferred at a ~100-fold lower rate than acetyl and propionyl groups, at a high H3 peptide concentration of 1 mM.
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