PCAF Histone Acetyltransferase Processing of a Peptide Substrate: Kinetic Analysis of the Catalytic Mechanism*

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Running Title: PCAF Catalytic Mechanism
**Summary**

PCAF is a histone acetyltransferase which plays an important role in the remodelling of chromatin and the regulation of gene expression. It has been shown to catalyze preferentially acetylation of the ε-amino group of lysine-14 in histone H3. In this study, the kinetic mechanism of PCAF was evaluated with a 20 amino acid peptide substrate derived from the amino terminus of histone H3 (H3-20) and recombinant bacterially expressed PCAF catalytic domain (PCAFcat). The enzymologic behavior of full length PCAF and PCAFcat were shown to be similar. PCAF catalyzed acetylation of the substrate H3-20 was shown to be specific for Lys-14, analogous to its behavior with the full length histone H3 protein. Two substrate kinetic analysis displayed an intersecting line pattern, consistent with a ternary complex mechanism for PCAF. The dead-end inhibitor analog desulfo-CoA was competitive versus acetyl-CoA and noncompetitive versus H3-20. The dead-end analog inhibitor H3-20 K14A was competitive versus H3-20 and uncompetitive versus acetyl-CoA. The potent bisubstrate analog inhibitor H3-CoA-20 was competitive versus acetyl-CoA and noncompetitive versus H3-20. Taken together, these inhibition patterns support an ordered BiBi kinetic mechanism for PCAF in which acetyl-CoA binding precedes H3-20 binding. Viscosity experiments suggest that diffusional release of product is not rate-determining for PCAF catalysis. These results provide a mechanistic framework for understanding the detailed catalytic behavior of an important subset of the histone acetyltransferases and have significant implications for molecular regulation of, and inhibitor design for these enzymes.
Histone acetyltransferases (HATs) appear to modulate gene expression by catalyzing targeted acetylation of the ε-amino group of lysine residues on histones and other proteins (1-3). Several families of HATs have recently been identified including GCN-5, SRC-1, p300, and TAF250 (1, 2). Among the more exciting aspects of this emerging field is the recognition that protein substrates for HATs extend outside of the histone family and include such key transcription factors as p53 (4-6), HIV TAT (7, 8), TFIIFβ (9), GATA-1 (10), and MyoD (11). Despite their importance, the molecular bases for substrate recognition and the catalytic mechanisms of the group of HAT enzymes have not been established. In this study we focus our attention on the histone acetyltransferase PCAF (p300/CBP-associated factor) (3, 12).

PCAF is a member of the GNAT (GCN-5 related N-acetyltransferases) superfamily which includes a variety of acetyltransferase enzymes such as serotonin N-acetyltransferase, gentamicin N-acetyltransferase, GCN-5, spermidine N-acetyltransferase, HAT 1 and approximately 150 other "motif A/B" acetyltransferases in the DNA sequence database (13-16). Of these superfamily members, the most well characterized mechanistically is serotonin N-acetyltransferase. It was shown to follow an ordered BiBi ternary complex mechanism where acetyl-CoA binds first, most likely with direct attack of the serotonin amine nitrogen on acetyl-CoA (17, 18). Using viscosity experiments, the rate-determining step appears to be product release (19). Moreover, AANAT appears to bind the substrate as the protonated (ammonium species) and nucleophilicity of the amine seems to be important (19). The sequence homology among the superfamily members is relatively weak and significant differences exist among their three-dimensional structures (9-12). Consequently, how related the AANAT catalytic mechanism will be to that of other superfamily members is unclear.

PCAF was discovered in 1996 as a histone acetyltransferase that interacts with the transcriptional coactivator p300, which is also a HAT (12, 20, 21). PCAF is a 90 kDa
protein which contains several domains as outlined in Figure 1. A high resolution X-ray structure of the PCAF HAT domain in a complex with acetyl-CoA shows the conserved structural features of the GNAT superfamily (22). Functions for the other PCAF domains including the bromodomain (23) are not yet understood but they may mediate protein-protein interactions or alternatively allosterically regulate PCAF HAT activity.

PCAF has been shown to catalyze highly selective acetylation of lysine-14 of histone H3 both in free histones and nucleosomes (24). To date, there has been little detailed evaluation of PCAF's catalytic mechanism. In this report, we reveal several new aspects of the catalytic mechanism of, and substrate recognition by PCAF using purified recombinant enzyme and a synthetic peptide substrate. We also describe a detailed analysis of the inhibition of PCAF by a potent and selective bisubstrate analog inhibitor (25).

Materials and Methods

General

Unlabelled acetyl-CoA was purchased from Pharmacia; desulfo-CoA, acetylated BSA, and trypsin were obtained from Sigma. Other reagents were obtained from commercial sources unless otherwise described. Full length PCAF was overproduced and purified as previously described (24). H3-CoA-20 was prepared as described previously (25). Mass spectrometry experiments were performed on either a PE Biosystems API150EX (Foster City, CA) electrospray single quadrupole instrument or a Kratos Kompact MALDI4 (Manchester England) time-of-flight mass spectrometer. HPLC was done on Varian instruments with Varian reverse phase HPLC columns.

PCAFcat Production and Purification

The production of PCAFcat was carried out analogously to that described by Marmorstein and colleagues (18). Briefly, the human PCAF catalytic domain (amino acid
residues 493-676) DNA was subcloned into a modified pET28a vector (confirmed by DNA sequencing), engineered so that the His6 tag could be cleaved by TEV protease, and the protein was overproduced in the *E. coli* strain BL21DE3 (14). A frozen permanent cell line was used to inoculate 2 x 30 mL of Luria Broth in Erlenmeyer flasks containing kanamycin (25 µg/ml) and allowed to grow overnight at 37°C in a floor shaker. This culture (10 mL) was used to inoculate each liter of Luria Broth (total 6 L) in flasks with kanamycin (25 µg/ml) and grown at 37°C in a floor shaker until the A595 = 0.5-0.6. The flasks were cooled to room temperature by standing at 4°C for 15 min and then induced with isopropyl-1-thio-β-D-galactopyranoside (0.2 mM final concentration). The culture was grown overnight in a floor shaker at 16°C for approximately 20 h. The cells were pelleted by centrifugation (4°C, 5000 X g, 5 min) and then resuspended in 20 mL lysis buffer (50 mM NaHepes pH 7.0, 500 mM NaCl, 5 mM dithiothreitol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride). The suspension was lysed by passage through a French pressure cell at 900 p.s.i. The insoluble protein and debris were removed by centrifugation (4°C, 27,000 X g, 30 min followed by 4°C, 100,000 X g, 120 min), and the supernatant was quick frozen in liquid nitrogen and stored at –80°C. The thawed solution was passed through 20 mL of Chelating Sepharose resin (30 mL of suspension, washed with 60 mL of water, followed by 100 mL of 50 mM ZnSO4 solution, and finally equilibrated by washing with 60 mL of IMAC 30 [20 mM NaHepes pH 7.0, 1 M NaCl, 30 mM imidazole, 1 mM phenylmethylsulfonyl fluoride]) in a 60 mL column packed with the aid of a peristaltic pump at a rate of 0.5-1 mL/min. The loaded resin was washed first with 80 mL of washing buffer A (20 mM NaHepes pH 7.0, 1 M NaCl, 72.5 mM imidazole, 1 mM phenylmethylsulfonyl fluoride) then with 80 mL of washing buffer B (20 mM NaHepes pH 7.0, 1 M NaCl, 98 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). Fractions of 10 mL were collected as the protein was eluted off with ~60 mL of IMAC-EDTA buffer (20 mM NaHepes pH 7.0, 1 M NaCl, 10% glycerol, 200 mM imidazole, 100 mM EDTA). The PCAFcat-containing fractions (as determined by 12%
SDSPAGE) were combined and dialyzed overnight at 4°C (10,000 MW cut-off) against 1 L solution of 50 mM NaHepes, 250 mM NaCl, and 5 mM dithiothreitol. The dialyzed protein solution (volume = 65mL) was treated with TEV protease (0.1 mg / mL final concentration) at 16°C overnight. The excised His6 tag was removed by passing the protein solution through 20 mL Chelating Sepharose resin (loaded with 100 mL of 50 mM ZnSO4) and washing the resin with 30 mL of IMAC-30 (20 mM NaHepes pH 7.0, 1 M NaCl, 30 mM imidazole, 1 mM phenylmethylsulfonyl fluoride), then 50 mL of IMAC-200 (20 mM NaHepes pH 7.0, 1 M NaCl, 200 mM imidazole, 1 mM phenylmethylsulfonyl fluoride). The fractions containing the purified PCAFcat (>90% pure by Coomassie stained SDSPAGE) were combined and dialyzed against 1 L solution of 25 mM NaHepes pH 7.0, 100 mM NaCl, 1 mM EDTA and 10 mM dithiothreitol overnight at 4°C. The solution was concentrated to a final volume of 20 mL (2.1 mg/mL as determined by Bradford assay) by Centricon ultrafiltration and then stored at –80°C.

Peptide Synthesis

Peptides were synthesized on an automated solid phase peptide synthesizer (Rainin, PS3) using the Fmoc strategy and purified by preparative C-18 reversed phase HPLC (using a gradient of H2O:CH3CN:0.05% trifluoroacetic acid). H3-CoA-12 was prepared analogously to H3-CoA-20 according to previously described procedures (25). All synthetic peptides appeared >95% pure by HPLC and their structural identities were confirmed by electrospray mass spectrometry.

Acetyltransferase Activity Assays

HAT kinetic assay procedures were adapted from previously described methods (12, 20, 24, 25). Briefly, substrate concentrations were 0.5-20 µM acetyl-CoA (NEN, 14C, 0.02 µCi/µL), 5-120 µM peptide and buffer conditions included 50 mM Tris-HCl (pH 8), 1 mM dithiothreitol, 0.1 mM EDTA, and 50 µg/mL acetylated bovine serum
albumin. Reactions employed purified recombinant full length PCAF or PCAFcat at concentrations of 0.1-100 nM as needed. Assays were carried out in 0.5 mL plastic tubes at 30°C and the reaction volumes were 30 μL. Reactions were initiated with enzyme or acetyl-CoA after allowing the other components to equilibrate at 30°C, and reactions quenched after 1-10 min with 6 μL 6x SDS-PAGE loading buffer. Mixtures were run out on 16% SDS Tris-Tricine polyacrylamide gels, dried, and radioactivity quantified by phosphorimage analysis (Molecular Dynamics) by comparing to known quantities of 14C-labelled bovine serum albumin standard (NEN). In all cases, background acetylation (in the absence of enzyme) was subtracted from the total signal. All assays were performed at least twice and duplicates generally agreed within 20%. Enzyme activities were demonstrated to be linear versus enzyme concentration and time in the ranges used. Rate measurements were based on initial conditions (less than 10% consumption of the limiting substrate).

Comparison of apparent k_{cat} and K_{m} for full length PCAF and PCAFcat

For K_{m} (apparent) measurements, a range of at least 5 substrate concentrations were employed at fixed concentration of the second substrate (10 μM ATP or 100 μM H3-20). The data were fitted to the Michaelis-Menten equation

\[ v = \frac{V_{m} \cdot S}{K_{m} + S} \]

using a non-linear least squares approach (Macintosh computer program-Kaleidograph™) and the kinetic constants ± standard errors are reported in Table 1.

Two-substrate kinetic measurements with PCAFcat

Two-substrate kinetic analysis was performed with substrate concentrations given in Figure 3 and the data were fitted to the sequential (ternary complex) mechanism equation below using the computer program KinetAsyst II™ (IntelliKinetics) based on the algorithms of Cleland (26):

\[ v = \frac{V_{m1} \cdot [S1] + V_{m2} \cdot [S2] \cdot [S1]}{K_{m1} + [S1] + K_{m2} \cdot [S2] + [S1] \cdot [S2]} \]
\[ v = V_m \times A \times B \times (K_{ia} \times K_{mb} + K_{ma} \times B + K_{mb} \times A + A \times B) \]

using a non-linear least squares approach. Kinetic constants ± standard error are shown in Table 2. \( K_{ma} = K_m \) of acetyl-CoA in this work, \( K_{ia} = \) dissociation constant for acetyl-CoA (dissociation constant to free enzyme where acetyl-CoA binds prior to H3-20), \( K_{mb} = K_m \) of H3-20. Fitting to a ping-pong mechanism gave a significantly larger sum of squares of the residuals.

**Steady-State Kinetic measurements with inhibitors**

Competitive inhibition kinetic analysis was done by fitting all of the data points to the linear competitive inhibition equation of KinetAsyst II™ based on the algorithms of Cleland (26):

\[ v = V_m \times S / [K_m \times (1 + I/K_{is}) + S] \]

using a non-linear least squares approach. The fixed substrate was assumed to be saturating. Kinetic constants ± standard error are shown in Table 3.

Noncompetitive inhibition kinetic analysis was done by fitting all of the data points to the linear noncompetitive inhibition equation of KinetAsyst II™ based on the algorithms of Cleland (26):

\[ v = V_m \times S / [K_m \times (1 + I/K_{is}) + S \times (1 + I/K_{ii})] \]

using a non-linear least squares approach. Kinetic constants ± standard error are shown in Table 3.

Uncompetitive inhibition kinetic analysis was done by fitting all of the data points to the linear uncompetitive inhibition equation of KinetAsyst II™ based on the algorithms of Cleland (26):

\[ V = V_m \times S / [K_m + S \times (1 + I/K_{ii})] \]

using a non-linear least squares approach. Kinetic constants ± standard error are shown in Table 3.
The abbreviations are $K_{ji} = K_i$ intercept and $K_{is} = K_i$ slope based on double reciprocal plot analysis according to the nomenclature of Cleland (27). The data for individual experiments with each inhibitor versus a varied substrate was fit to all three inhibitor models. Choice of kinetic fit was based on a combination of visual inspection and comparison of standard errors and residuals for all 3 inhibition types applied to the data sets (26). The lines drawn through the data points in the figures are derived from the fitted equations above.

**Calculation of $k_{diss}$**

Data for the dissociation rate constant ($k_{diss}$) for the bisubstrate analog H3-CoA-20 were fitted to the equation:

$$P = v_s(t - \{1 - e^{-k_{diss}t}\} / k_{diss})$$

where $P$ is the concentration of acetylated product, $v_s$ is the steady-state velocity, $k_{diss}$ is the off rate for the inhibitor from the enzyme, and $t$ is time (28, 29).

**Viscosity Experiments**

Microviscosity was adjusted by varying the concentration of the microviscogen sucrose as described previously (19, 30). There were no significant changes in $K_m$ of acetyl-CoA or H3-20 at the extremes of viscosity evaluated.

**Mapping the acetylation site on H3-20**

PCAF$_{cat}$ catalyzed acetylation of H3-20 was carried out on a preparative scale as follows: 300 μM H3-20, 300 μM acetyl-CoA, 1 μM PCAF$_{cat}$, 5 mM phosphate (pH ~7.5) mixed in 1 mL and incubated for 10 min at 30°C. The reaction mixture was injected immediately on to a preparative HPLC (C-18 reversed phase column) and separated using a H$_2$O: CH$_3$CN:0.05% CF$_3$CO$_2$H gradient which showed predominantly one peak (>90%). The resultant product was lyophilized to dryness. The reaction product was
analyzed by mass spectrometry. A peak consistent with the monoacetylated H3-20 peptide (+42 Da) was observed.

Tryptic digestion of both the unmodified H3-20 and acetylated H3-20 peptide were examined by MALDI-TOF mass spectrometry. Tryptic digestions were performed by combining 2 µL of trypsin (1.0 µg/µL), 2 µL of peptide solution (control peptide: 100 pmol/µL, acetylated peptide: 25 µL of distilled water was added to dry sample) and 2 µL of buffer solution (25 mM ammonium bicarbonate pH 8). The above reaction mixture (0.3 µL) was applied to the sample plate followed by 0.3 µL of the saturated α-cyano-4-hydroxycinnamic acid matrix solution (1:1 (v/v) ethanol:water). The matrix was added in timed increments (1, 5, 10 and 60 minutes) and served to quench the enzymatic digestion. The mixture was allowed to air dry (10 min) prior to introduction into the mass spectrometer. This product and an equivalent amount of unreacted H3-20 peptide were subjected to trypsin digestion followed by analysis using MALDI mass spec (31). Comparison of the tryptic fragments led to the unequivocal identification of Lys-14 as the site of acetylation.

Results

H3-20 as a PCAF Substrate

Previous work has shown that a bisubstrate analog based on the N-terminal sequence of histone H3 is a potent inhibitor of full length PCAF (25). In that study, it was observed that H3-CoA-20 (Figure 2), a peptide CoA conjugate with the 20 N-terminal residues of histone H3, was a potent PCAF inhibitor (IC50 ~ 0.3 µM) whereas H3-CoA-7 was a comparatively weak inhibitor (IC50 > 50 µM) (25). Recently, an X-ray structure of the PCAF homolog was reported in which an 11 amino acid peptide co-crystallized with GCN-5 (32). Consequently, the 12 residue peptide CoA conjugate (H3-CoA-12) was prepared, but this too was found to be only a weak PCAF inhibitor (IC50~50 µM). From
this finding, it was deduced that greater than 12 residues but less than or equal to 20 residues are likely necessary for high affinity binding.

We thus investigated the corresponding unmodified H3 related peptide (H3-20) as a PCAF substrate. As with the previously described histone acetylation assay, $^{14}$C-acetyl-CoA was used as substrate and radioactive $^{14}$C incorporation into the peptide product was determined. Separation and quantification was achieved using a 16% Tris-Tricine SDS-PAGE followed by phosphorimage analysis. In this way, it was found that H3-20 afforded a reasonably sharp band (data not shown) that was readily quantified. Furthermore it was found that, unlike with the case of histones, PCAF enzyme activity was linear with respect to both enzyme concentration (0.5-5 nM range) and time (up to 10 min). It was therefore possible to measure $k_{cat}$ (0.7 s$^{-1}$) and $K_m$ values for acetyl-CoA (2.1 µM) and H3-20 (10 µM) (see Table 1).

Given the difficulty in obtaining large quantities of full length PCAF from eukaryotic expression systems, we turned to the study of recombinant PCAF catalytic domain (PCAF$\text{cat}$) which was overproduced in *E. coli* (22). PCAF$\text{cat}$ was inhibited by H3-CoA-20 with a similar IC$_{50}$ to full length PCAF (data not shown). Steady-state kinetic studies showed that it had similar but non-identical parameters using H3-20 peptide as substrate. In particular for PCAF$\text{cat}$, the apparent $k_{cat}$ was approximately 4-fold greater, the apparent $K_m$ for H3-20 substrate was about 3-fold higher, and the apparent $K_m$ for acetyl-CoA was about 3-fold lower compared to full length PCAF (see Table 1). Because of the relatively small magnitude of the differences we felt confident that mechanistic and substrate selectivity parameters determined for PCAF$\text{cat}$ HAT activity would likely be relevant for full length PCAF. The experiments described below were therefore carried out with PCAF$\text{cat}$.

*PCAF$\text{cat}$ HAT Assays with H4-20*
In order to quantitatively assess PCAF's selectivity for H3-20, H4-20 as evaluated as a PCAF\textsubscript{cat} substrate. H4-20 is a peptide derived from the first 20 N-terminal residues of histone H4 (25) and it has been reported for the full length proteins that histone H4 is less efficiently acetylated compared to histone H3 (24). As expected, H4-20 was a much weaker PCAF\textsubscript{cat} substrate than H3-20. Acetylation showed somewhat complex kinetics (data not shown) and was not amenable to a simple Michaelis-Menten fit. However, direct comparison of acetyltransferase rate at 80 µM of both peptide concentration shows that H3-20 is processed ~500-fold faster than H4-20.

Mapping the H3-20 Acetylation Site

In order to map H3-20's acetylation site, a mass spectrometric approach was applied. A mass increase of 42 Daltons from the unmodified peptide indicated the addition of a single acetyl group. The acetylated peptide was treated with trypsin and produced a number of tryptic fragments. This digestion mixture was then analyzed directly by MALDI-TOF mass spectrometry and served to map the location of the acetyl group. The peptide map showed that the acetylated residue of H3-20 was Lys-14.

\textit{PCAF\textsubscript{cat} HAT Assays with H3-20 K14A}

To confirm and quantitate the site-selectivity of H3-20 for Lys-14 and to develop a dead-end analog inhibitor for the steady-state kinetic analysis below, H3-20 K14A was synthesized and evaluated as a substrate for PCAF\textsubscript{cat} HAT activity. As expected, H3-20 K14A was a very weak substrate for PCAF\textsubscript{cat}. Although precise k\textsubscript{cat} and K\textsubscript{M} values were difficult to measure with this peptide substrate because of its low activity, a direct comparison of rates for acetylation of 100 µM of both substrates showed that H3-20 K14A was processed ~200-fold slower than H3-20 itself.

\textit{Two Substrate Kinetic Studies with PCAF\textsubscript{cat}}
In order to establish that PCAF follows a ternary complex mechanism as opposed to a ping-pong kinetic mechanism, we examined the effects of varying the concentrations of both acetyl-CoA and H3-20 on the acetyltransferase rate (Figure 3). As can be seen from the double reciprocal plot, a clear intersecting line pattern is observed consistent with a ternary complex mechanism. The $K_m$, $K_i$, and $k_{cat}$ values (see Table 2) obtained by a global fit of the data are in reasonable agreement (within 2-fold) with those apparent values obtained for PCAF$_{cat}$ at fixed second substrate concentrations as described above.

**Inhibition of PCAF$_{cat}$ by Desulfo-CoA**

In order to determine the order of binding of the two substrates for catalysis, inhibition experiments were initiated with desulfo-CoA, a dead-end analog of acetyl-CoA. Desulfo-CoA was clearly shown to be a linear competitive inhibitor of the PCAF$_{cat}$ HAT reaction versus varying concentration of acetyl-CoA with $K_{iS} = 0.57$ µM (Figure 4, Table 3). The low $K_i$ suggests that the acetyl carbonyl group of acetyl-CoA likely contributes little to binding. In contrast, desulfo-CoA exhibited a linear noncompetitive pattern versus varying H3-20 concentration (Figure 4). While in this case the fit was significantly better for noncompetitive versus uncompetitive inhibition, the $K_{iS}$ was about ~5-fold higher than the $K_{ii}$ (Table 3). This suggests significant binding synergy between desulfo-CoA and H3-20 and that enzyme inhibition by desulfo-CoA is enhanced by the peptide substrate. The noncompetitive inhibition pattern suggests that binding of acetyl-CoA either precedes H3-20 or that substrate binding is random. It effectively rules out obligatory ordered binding of peptide substrate prior to acetyl-CoA for catalytic turnover.

**Inhibition of PCAF$_{cat}$ by H3-20 K14A**

The peptide H3-20 K14A was employed as a dead-end analog of the peptide substrate to determine its apparent affinity as well as to further dissect the kinetic mechanism. H3-20 K14A was found to be a linear competitive inhibitor versus varying
H3-20 (Figure 5, Table 3). The $K_{i_S}$ value of 225 µM for H-20 K14A was somewhat higher than the $K_m$ of H3-20 (50 µM) deduced from the two-substrate kinetic studies. H3-20 K14A versus varying acetyl-CoA concentration revealed a linear uncompetitive pattern of inhibition. Inclusion of a $K_{i_S}$ term for this inhibitor showed no enhancement of fit. Taken together with the desulfo-CoA results, this inhibitory pattern argues in favor of an ordered BiBi kinetic mechanism in which acetyl-CoA binding precedes H3-20 binding and effectively rules out a random substrate binding mechanism.

*Inhibition of PCAF_{cat} by H3-CoA-20*

In previous work, it was shown that H3-CoA-20 exhibited potent inhibition versus acetyl-CoA but the mode of inhibition was not determined. In principle, H3-CoA-20 could exhibit 'slow-binding' behavior of PCAF_{cat} as is common for tight-binding inhibitors. We thus investigated carefully the time course of inhibition. In the presence of H3-CoA-20, the rate of the PCAF_{cat} catalyzed reaction was linear within 20 s after initiation and the slope did not change with increasing time (data not shown). This suggests that binding of the inhibitor to the enzyme and/or conformational changes necessary to reach maximal inhibition are achieved relatively rapidly compared to the time scale of the assay.

To assess the dissociation rate of H3-CoA-20 from PCAF_{cat}, an experiment was performed in which the fully inhibited enzyme (0.5 µM PCAF_{cat}, 10 µM H3-CoA-20) was rapidly diluted 150-fold into a solution containing near-saturating concentrations of acetyl-CoA and H3-20 to minimize the inhibitory effect (<20%) of H3-CoA-20 on PCAF_{cat} after initial dissociation from the enzyme. In this way, it was shown that there was a slight lag phase in product formation before the steady-state rate was reached (Figure 6). In control experiments in the absence of inhibitor, no lag phase is apparent (data not shown) ruling out an artifact of mixing as the source of this lag. This lag phase
was interpreted therefore as the time course for inhibitor dissociation from PCAF\textsubscript{cat}. The extrapolated dissociation rate constant ($k_{\text{diss}}$) was shown to be 0.028 s\(^{-1}\).

Given the relatively fast dissociation rate and the fast "on rate" for H3-CoA-20 with PCAF\textsubscript{cat}, a steady-state inhibitor analysis was feasible. In these experiments, H3-CoA-20 was shown to be a linear competitive inhibitor versus varying acetyl-CoA concentration ($K_{iS} = 28$ nM) and a linear noncompetitive inhibitor versus varying H3-20 concentration (Figure 7, Table 3). This pattern of inhibition is most consistent with an ordered BiBi sequential kinetic mechanism (18).

**Viscosity Effect on the PCAF\textsubscript{cat} Acetyltransferase Reaction**

In order to investigate the nature of the rate-limiting step, the effects of sucrose microviscosity on the steady-state kinetic parameters were measured. At the highest relative viscosity ratio tested, the $K_m$ values for acetyl-CoA or H3-20 were not appreciably affected (data not shown). A plot of the $k_{\text{cat}}$-control/$k_{\text{cat}}$-viscogen versus relative viscosity ratio showed only a minimal positive slope of 0.03 (Figure 8). The viscosity effect on the GNAT superfamily member serotonin $N$-acetyltransferase (slope = +0.75) is shown for comparison. This lack of a microviscosity effect argues against diffusional release of products being rate-determining for the PCAF\textsubscript{cat} reaction.

**Discussion**

A detailed understanding of the catalytic mechanism and substrate selectivity of HAT enzymes is an important component of defining the molecular bases of their biological functions. Furthermore, such understanding is likely to enhance the design of potent and selective HAT inhibitors. Prior to this investigation, a preliminary mechanistic analysis on the Tetrahymena HAT enzyme GCN-5 was reported. In this study, mixed histone substrates were used as the acetyl-CoA acceptor (33). While this study revealed an intersecting line pattern for GCN-5 suggestive of a ternary complex mechanism, more
detailed studies investigating order of substrate binding were not described. The complexity of the mixed histone substrate may have made detailed mechanistic studies difficult. In our hands, PCAF does not show linearity in plots of enzyme activity versus time or enzyme concentration using mixed histone substrates. Furthermore, high background acetyltransferase activities accompany these mixed histone preparations which also complicate kinetic studies.

In the current analysis, a short 20 amino acid peptide sequence taken from the N-terminus of histone H3 was employed as a PCAF substrate. The length of this sequence was based on previous and current data showing that H3-CoA-20 was a potent bisubstrate analog whereas H3-CoA-7 and H3-CoA-12 were weakly inhibitory. Unlike mixed histones, this peptide displayed classical steady-state kinetic behavior as a substrate, which made it most useful for kinetic studies. Among the first experiments carried out with this peptide substrate was the measurement of the apparent $k_{\text{cat}}$ and $K_m$ values for acetyl-CoA and peptide. It should be mentioned that these values (Table 1) differed significantly from those reported for Tetrahymena GCN-5 with mixed histones as substrates (ref. 32, $k_{\text{cat}} = 0.1$ s$^{-1}$, $K_m$ (acetyl-CoA) = 20 µM, and $K_m$ (histones) = 28 µM) and it remains to be seen if this is related to the different substrates employed or the modest dissimilarities in the protein amino acid sequences.

The catalytic domain of PCAF (PCAF$_{\text{cat}}$) showed comparable histone acetyltransferase kinetic parameters to the full length enzyme, validating the utility of PCAF$_{\text{cat}}$ for further kinetic experiments. It also suggests that all of the key recognition elements for peptide substrate selectivity are present in the PCAF catalytic domain and the non-catalytic PCAF domains do not contribute much to this recognition. This is not meant to imply that protein substrate recognition by PCAF would not be influenced by the non-catalytic domains of PCAF. For example, a 25 amino acid peptide derived from the known acetylation site of p53 is a very weak PCAF (full length) and PCAF$_{\text{cat}}$ substrate ($k_{\text{cat}}/K_m$ is reduced ~10,000-fold compared to H3-20) (O. Lau, unpublished
data) and it appears likely that the molecular recognition of p53 by PCAF will involve more complex interactions if it is physiologically relevant. As p53 is acetylated in a DNA damage-dependent manner in cultured cells (6), the binding mode of PCAF to p53 might be regulated via a signalling network.

The known selectivity of PCAF for Lys-14 of histone H3 was demonstrated for the peptide substrate H3-20 by showing that the corresponding K14A substituted peptide was a very poor PCAF substrate, and that a similar length peptide derived from histone H4 (H4-20) was also a poor substrate. Furthermore, the specificity was confirmed by mass spectrometric analysis of PCAFcat mediated acetylation of H3-20 which indicated a single acetylation event on Lys-14 had occurred.

In order to confirm the expected ternary complex mechanism for this GNAT family member, a steady-state kinetic analysis was undertaken where both substrate concentrations were varied within the same experiment. These results showed a clear intersecting line pattern, consistent with a sequential (ternary complex) mechanism and disfavoring a ping-pong mechanism. Inhibition studies with the dead-end analog inhibitors desulfo-CoA and H3-20 K14A established an ordered BiBi kinetic mechanism in which acetyl-CoA binding precedes peptide binding. The kinetics of inhibition of PCAFcat by the bisubstrate analog H3-CoA-20 were also confirmatory of the ordered BiBi kinetic mechanism.

The finding that PCAF obeys a sequential ordered BiBi kinetic mechanism is similar to the previous results with the GNAT superfamily member serotonin N-acetyltransferase (17, 18), despite their wide amino acid sequence divergence. Although not rigorously evaluated with other GNAT superfamily members, it seems plausible that this will be the rule for the superfamily. A key loop movement in serotonin N-acetyltransferase has been suggested to be the structural basis for ordered binding (34) in that enzyme and analogous conformational changes likely explain the ordered binding in PCAF. Establishing this mechanism may have significant implications for HAT
regulation and inhibitor design. For example, the presumed conformational change associated with acetyl-CoA binding may impact upon interactions with other proteins in transcriptional complexes. Likewise, the stability of the protein may be modulated by alterations in acetyl-CoA concentration (35). Moreover, it is likely that peptide substrate inhibitor analogs would require the initial binding of acetyl-CoA (or a CoA analog) in order to potently inhibit PCAF. The fluctuations in concentrations of acetyl-CoA in the cell could therefore cause variations in inhibitor potency in vivo. This makes the bisubstrate analog approach to inhibitor design particularly appealing for PCAF.

The bisubstrate analog H3-CoA-20 is a potent inhibitor of PCAFcat ($K_{iS} = 28$ nM). However, since the $K_i$ of desulfo-CoA is ~600 nM, this suggests that the attachment of the H3-20 peptide only provides approximately a factor of 20-fold in enhanced affinity (additional binding energy of 1.7 kcal/mol) compared to desulfo-CoA. Since bisubstrate analogs can in optimal cases provide additive binding energy (or greater) compared to the binding energy of the individual substrates, a theoretical $K_i$ less than 1 nM might have been anticipated for H3-CoA-20. Indeed, the bisubstrate analog for the related enzyme serotonin N-acetyltransferase displayed a $K_i$ (91 nM) which was equivalent to the sum of the expected binding energy for both substrates ($K_m$ (app) tryptamine = 0.26 mM, $K_m$ (app) for acetyl-CoA = 0.36 mM) (14). Why this is not the case for H3-CoA-20 and PCAF is unclear but one possibility is that the acetyl bridge in H3-CoA-20 between the two substrate moieties is not ideal for PCAF binding. Future objectives will involve optimizing this tethering functionality as well as exploring approaches to enhance the cell permeability of potent analogs (28).

The results of the viscosity effect studies also differ between serotonin N-acetyltransferase and PCAF. While product release appears to be rate-limiting in the former case, with PCAF it seems that the rate-determining step is likely to be the chemical step. The overall PCAF $k_{cat}$ is approximately 10-fold lower compared to that of serotonin N-acetyltransferase (17). Furthermore, for the true PCAF substrate,
nucleosomes, it is likely that the catalytic rate constant is slower still (28). Perhaps the plodding catalytic rate of PCAF histone acetylation is biologically advantageous because the state of histone acetylation has to be finely tuned to prevent the critical consequences of dysregulated gene expression.

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References


Footnotes

EDTA, N,N,N',N'-ethylenediamine tetraacetic acid; PCAF, p300/CBP-associated factor; PCAF_{cat}, catalytic domain of PCAF; SDSPAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis; K_{ji}, K_i intercept, K_{is}, K_i slope; H3-20, 20 amino acid peptide derived from histone H3 N-terminus; H4-20, 20 amino acid peptide derived from histone H4 N-terminus; H3-20 K14A, 20 amino acid peptide derived from histone H3 with an alanine for lysine replacement at residue-14; MALDI-TOF, matrix assisted laser desorption time-of-flight.
Table 1. Steady-State Kinetic Comparison of PCAF and PCAF_cat HAT Activity with H3-20 Substrate. Assays were carried out with fixed and near-saturating concentration of the second substrate. See Materials and Methods for Details. Values are shown ± standard error.

<table>
<thead>
<tr>
<th>Enzyme Form</th>
<th>Acetyl-CoA $K_m(app), \mu M$</th>
<th>Tryptamine $K_m(app), \mu M$</th>
<th>$k_{cat(app)}$, s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>full length PCAF</td>
<td>2.1±0.6</td>
<td>10±2</td>
<td>0.7±0.1</td>
</tr>
<tr>
<td>PCAF_cat</td>
<td>0.64 ±0.18</td>
<td>30±3</td>
<td>2.8 ±0.2</td>
</tr>
</tbody>
</table>
Table 2. Two Substrate Steady-State Kinetic Analysis for PCAFcat. Data is shown in Figure 3. See Materials and Methods for Details.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$K_m, \mu M$</th>
<th>$k_{cat}, s^{-1}$</th>
<th>$K_{ia}, \mu M$</th>
</tr>
</thead>
<tbody>
<tr>
<td>H3-20</td>
<td>50±10</td>
<td>4.0±0.3</td>
<td></td>
</tr>
<tr>
<td>Acetyl-CoA</td>
<td>0.46±0.23</td>
<td></td>
<td>1.1±0.48</td>
</tr>
</tbody>
</table>
Table 3. Steady-State Kinetic Analysis of Inhibitors for PCAF<sub>cat</sub>. Data is shown in Figures 4, 5, and 7.

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>Varied Substrate</th>
<th>Constant Substrate</th>
<th>Inhibitor Pattern</th>
<th>$K_{iS}$ (μM)</th>
<th>$K_{ii}$ (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Desulfo-CoA</td>
<td>Acetyl-CoA</td>
<td>H3-20 (100 μM)</td>
<td>Competitive</td>
<td>0.57 ± 0.09</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H3-20</td>
<td>Acetyl-CoA (1 μM)</td>
<td>Noncompetitive</td>
<td>6.2 ± 2.4</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>H3-20 K14A</td>
<td>Acetyl-CoA</td>
<td>H3-20 (30 μM)</td>
<td>Uncompetitive</td>
<td></td>
<td>389 ± 43</td>
</tr>
<tr>
<td></td>
<td>H3-20</td>
<td>Acetyl-CoA (10 μM)</td>
<td>Competitive</td>
<td>225 ± 52</td>
<td></td>
</tr>
<tr>
<td>H3-CoA-20</td>
<td>Acetyl-CoA</td>
<td>H3-20 (100 μM)</td>
<td>Competitive</td>
<td>0.028 ± 0.011</td>
<td></td>
</tr>
<tr>
<td></td>
<td>H3-20</td>
<td>Acetyl-CoA (2 μM)</td>
<td>Noncompetitive</td>
<td>0.14 ± 0.07</td>
<td>0.5 ± 0.3</td>
</tr>
</tbody>
</table>
Figures

1. Architectural layout of Full Length PCAF Protein. The abbreviations above the PCAF domains represent genes that have been shown to interact with PCAF mapped to these regions. "Br" - bromodomain (see text).

2. Structures of peptides/compounds used in this work.

3. Two Substrate Kinetic Analysis of PCAFcat HAT Activity. E/V vs. 1/[acetyl-CoA] at three fixed [H3-20] concentrations. Acetyltransferase assays and data fitting were performed as described in Materials and Methods. The catalytic parameters are displayed in Table 2.

4. Desulfo-CoA Inhibition of PCAFcat. A, E/V vs. 1/[acetyl-CoA] at fixed H3-20 concentration (100 µM) and varying concentrations of desulfo-CoA. B, E/V vs. 1/[H3-20] at fixed acetyl-CoA (1 µM) and varying concentrations of desulfo-CoA. Acetyltransferase assays and data fitting were performed as described in Materials and Methods. The inhibition parameters are displayed in Table 3.

5. H3-20 K14A Inhibition of PCAFcat. A, E/V vs. 1/[H3-20] at fixed acetyl-CoA concentration (10 µM) and varying concentrations of H3-20 K14A. B, E/V vs. 1/[acetyl-CoA] at fixed H3-20 (30 µM) and varying concentrations of H3-20 K14A (I). Acetyltransferase assays and data fitting were performed as described in Materials and Methods. The inhibition parameters are displayed in Table 3.

6. Recovery from H3-CoA-20 Inhibition of PCAFcat. Acetylated H3-20 product vs. time after dilution of the inhibited enzyme into substrate mix. PCAFcat (0.5 µM) was incubated with 10 µM H3-CoA-20 for 10 min at 30°C, then diluted 150-fold into a 300
µL solution containing 120 µM H3-20 and 10 µM 14C-acetyl-CoA. Product formation was monitored every 20-30 s as shown. Recovery constant to reach steady-state (k_diss) was 0.028±0.008 s⁻¹ calculated as described in Materials and Methods.

7. Steady-State Kinetic Analysis of H3-CoA-20 Inhibition of PCAFcat. A, E/V vs. 1/[acetyl-CoA] at fixed H3-20 peptide substrate concentration (100 µM) and varying H3-CoA-20. B, E/V vs. 1/[H3-20] at fixed acetyl-CoA concentration (2 µM) and varying H3-CoA-20. Acetyltransferase assays and data fitting were performed as described in Materials and Methods. The inhibition parameters are displayed in Table 3. Rates were analyzed after 4 min reaction times to ensure the measurement of steady-state kinetic constants.

8. k_cat-control vs. k_cat-viscogen for PCAFcat Processing of H3-20. Near-saturating concentrations of acetyl-CoA (10 µM) and H3-20 (100 µM) were employed. The solid line represents a linear least-squares fit of the experimental data (slope +0.03). See Materials and Methods for further details. The dashed line (slope +0.75) represents the fit for the wild-type serotonin N-acetyltransferase reaction with tryptamine substrate taken from reference 15.
Figure 1

Domain Structure of PCAF
Figure 2

H3-CoA-7

H3-CoA-12

H3-CoA-20

H3-20: AcHN-ARTKQTARKSTGGKAPRKQL-CO$_2$H
H4-20: AcHN-SGRGKGGKGLGKGGAKRNRA-CO$_2$H
H3-20 K14A: AcHN-ARTKQTARKSTGGAAPRKQL-CO$_2$H

desulfo-CoA
Figure 3

$E/V (s)$

$1/[\text{acetyl-CoA}] (1/\mu\text{M})$

$[H3-20] = 10 \ \mu\text{M}$

$[H3-20] = 20 \ \mu\text{M}$

$[H3-20] = 120 \ \mu\text{M}$
PCAF histone acetyltransferase processing of a peptide substrate: Kinetic analysis of the catalytic mechanism

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