Acetylation of proteins by p300 histone acetyltransferase plays a critical role in the regulation of gene expression. The prior discovery of an autoacetylated regulatory loop in the p300 histone acetyltransferase (HAT) domain prompted us to further explore the mechanisms of p300 autoacetylation. Here we have described a kinetic and mass spectrometric analysis of p300 HAT autoacetylation. The rate of p300 HAT autoacetylation was approximately fourth order with respect to p300 HAT domain concentration and thus appeared to be a highly cooperative process. By showing that a catalytically defective p300 HAT domain could be efficiently acetylated by active p300 HAT, we deduced that autoacetylation occurs primarily by an intermolecular mechanism. This was further confirmed using a semisynthetic biotinylated p300 HAT domain that could be physically separated from the catalytically defective p300 HAT by avidin affinity chromatography. Autoacetylation catalyzed by p300 HAT was ~1000-fold more efficient than PCAF (p300/CREB-binding protein-associated factor)-mediated acetylation of catalytically defective p300 HAT. Using a novel tandem mass spectrometric approach, it was found to be possible to observe up to 17 autoacetylation events within the intact p300 regulatory loop. Kinetic analysis of the site specificity of p300 autoacetylation reveals a class of rapid events followed by a slower set of modifications. Several of these rapid autoacetylation sites correlate with an acetyltransferase-activating function based on prior mutagenesis analysis.

 Protein acetylation is now understood to play key roles in the regulation of transcription, cell growth, and differentiation. Histone acetyltransferases (HATs)\(^8\) catalyze the transfer of acetyl groups from acetyl coenzyme A to lysine residues in histones and other proteins (1). Among the HAT families, p300 and its paralog, CREB-binding protein (CBP), have been identified as major enzymes in the acetylation of a myriad of protein targets with a range of biological functions (2, 3). For example, p300/CBP acetyltransferase activity has been implicated in the transcriptional coactivation mechanism of p53 (4), p73 (5), steroid hormone receptors (6), NFκB (7, 8), the STATs (9), and GATA1 (10). Moreover, dysregulation of p300/CBP has been implicated in several types of cancer, cardiac disease, and inflammatory processes (11–23). The detailed contributions of these enzymes to biological function remain poorly understood.

One of the challenges in characterizing the enzymatic behavior of p300 HAT domain has been the difficulty in generating p300 HAT protein in bacteria due to host cell toxicity from promiscuous acetyltransferase hyperactivity. Moreover, recombinant protein prepared using standard expression methods has been complicated to study because of its tendency to undergo autoacetylation in vivo (24, 25). These two obstacles have recently been largely overcome by using the technique of expressed protein ligation (26, 27). In this method, a recombinant N-terminal thioster fragment is produced by intein fusion, and it is ligated via a chemoselective reaction to a C-terminal peptide fragment to produce the semisynthetic p300 HAT protein (see Fig. 1). In this way, it was shown to be possible to generate milligram quantities of semisynthetic p300 HAT in a hypoacylated form (less than three acetyl modifications) as opposed to 10–15 acetylations observed in protein generated in standard recombinant fashion (25). Careful comparison of the hypoacylated and hyperacylated forms reveals catalytic activation, primarily reflected in reduced \(K_m\) of the substrates, upon autoacetylation. Using mass spectrometric methods involving post-source decay, 13 sites of autoacetylation have been mapped, and many of these were localized to a proteolytically sensitive loop (Fig. 1) (25). Contributions of individual lysines to activation were analyzed by site-directed mutagenesis and several residues appeared to be important in catalytic activation (25). It also proved possible to generate a loop-deleted form, and this protein appeared to be constitutively activated, suggesting the loop behaves to limit acetyltransferase action in its hypoacylated state (25). Evidence that this regulatory loop is relevant to an in vivo setting with full-length p300 protein was obtained by examining cellular acetylation and transcriptional regulation with wild-type and loop-deleted forms (25). These studies suggest a potentially significant biological role for p300 loop acetylation. Nevertheless, several important unanswered questions remain regarding the nature of p300 HAT domain...
loop acetylation. Is the autoacetylation intermolecular, intramolecular, or both? If intermolecular, can the p300-associated HAT PCAF catalyze p300 acetylation? Are there preferences for the order of sites of autoacetylation, or is it purely stochastic? Here we have investigated the biochemistry of p300 HAT domain autoacetylation to attempt to address these issues.

**EXPERIMENTAL PROCEDURES**

**Chemicals**—N-α-Fmoc-amino acids were purchased from Novabiochem (San Diego, CA), and 14C-labeled acetyl-CoA was purchased from Amersham Biosciences. Acetylated bovine serum albumin, dithiothreitol, and biotin were from Sigma. Avidin monomer was obtained from Pierce. Phenol and trifluoroacetic acid were obtained from Fisher Scientific. 1,2 Ethanedithiol was purchased from Fluka Chemie, and thioanisole was obtained from Acros Organics.

**Peptide Synthesis**—Peptides CMLVELHTQSQDRF and CMLVELHTQSQDRFK(e-biotin)G were synthesized by solid-phase peptide synthesis using the Fmoc strategy with Wang resin on a Rainin PS3 machine. Biotinylation of the NH2-amino group of the Lys was carried out using orthogonal protection with dimethyldioxocyclohexylidene removal with 2% hydrazine, and coupling with N-hydroxysuccinimidyl-biotin. The peptides were cleaved and deblocked from the resin with Reagent K (trifluoroacetic acid/thioanisole/water/phenol/ethanedithiol 82.5:5:5:5:2.5 v/v) and subsequently precipitated with ice-cold diethyl ether. Precipitates were collected by centrifugation (3000 × g, 10 min), the supernatants discarded, and the pellets washed twice with cold diethyl ether (30 ml). Precipitated peptides were dissolved in 5 ml of double-distilled water, flash-frozen, and lyophilized. Peptides were purified (>95% homogeneity) by reversed phase (C-18) high performance liquid chromatography, as described previously, using a gradient of water-acetonitrile (0.05% trifluoroacetic acid). Electrospray mass spectrometry of each peptide confirmed the correct structure.

**Wild-type (wt) p300 HAT and wt p300 HAT-Btn**—These semisynthetic proteins were prepared and purified following previously described procedures (25). Briefly, pTYB2 expression plasmid encoding p300 HAT protein (aa 1287–1652) containing an M1652G mutation fused to VMA intein-chitin binding domain was grown in Escherichia coli BL21(DE3)-RIL cells.
to and A_{600} of 0.45, at which point, the incubator temperature was reduced to 16 °C and the medium allowed to cool. After 15 min, protein expression was induced by the addition of isopropyl 1-thio-β-D-galactopyranoside to a final concentration of 0.5 mM. Cells were then grown for 16 h at room temperature with 50 mM cysteine and 200 mM MESNA, at which point p300 HAT-ΔC protein was eluted from the column. Fractions containing p300 HAT-ΔC domain were pooled and concentrated before being applied to a Mono-S HR5/5 (Amersham Biosciences) strong cation exchange column for further purification. Fractions containing purified protein (>90%), as determined by SDS-PAGE analysis, were pooled and concentrated to ~5 mg/ml, as measured by Bradford assay. Following concentration, 10% glycerol was added before flash freezing in liquid N\textsubscript{2}, and samples were stored at −80 °C. Semisynthetic proteins showed the correct molecular weights as determined by matrix-assisted laser desorption ionization.

**Preparation of p300 HAT-ΔC—C-terminally deleted p300 HAT domain (aa 1287–1652) containing the M1652G mutation fused to VMA intein-chitin binding domain** was immobilized on chitin as described above. The reaction was incubated for 16 h at room temperature with 50 mM cysteine and 200 mM MESNA, at which point p300 HAT-ΔC protein was eluted from the column. Fractions containing p300 HAT-ΔC domain were pooled and concentrated before being applied to a Mono-S HR5/5 (Amersham Biosciences) strong cation exchange column for further purification. Fractions containing purified protein (>90%), as determined by SDS-PAGE analysis, were pooled and concentrated to ~5 mg/ml, as measured by Bradford assay. Following concentration, 10% glycerol was added before flash freezing in liquid N\textsubscript{2}, and samples were stored at −80 °C. p300 HAT-ΔC protein showed the correct molecular weights as determined by matrix-assisted laser desorption ionization.

**Autoacetylation Kinetic Assay**—Autoacetylation assays of p300 HAT were carried out using [14C]-acetyl-CoA, and the production of [14C]-acetylated protein was quantified after separating the components of the reaction on SDS-PAGE. The p300 HAT domains were preincubated in assay buffer (50 mM HEPES, pH 7.9, 500 mM NaCl, 10% glycerol, 1 mM MgSO\textsubscript{4}, and 2 mM phenylmethylsulfonyl fluoride), and lysed by two passages through a French press cell. The lysate was cleared by centrifugation and applied to a 10-ml chitin column after extensive washing. Excess buffer was drained, and this immobilized fusion protein was treated with 200 mM mercaptoethane sulfonate to generate the thioester and ligated to synthetic peptide aa 1653–1666 (CMLVELHTQSQRFRF(e-biotin)G) over 16 h at room temperature. Fractions containing semisynthetic p300 HAT were pooled and concentrated before being applied to a Mono-S HR5/5 (Amersham Biosciences) strong cation exchange column for further purification. Fractions containing purified protein (>90%), as determined by SDS-PAGE analysis, were pooled and concentrated to ~5 mg/ml, as measured by Bradford assay. Following concentration, 10% glycerol was added before flash freezing in liquid N\textsubscript{2}, and samples were stored at −80 °C. Semisynthetic proteins showed the correct molecular weights as determined by matrix-assisted laser desorption ionization.

**Autoacetylation Kinetic Assay**—Autoacetylation assays of p300 HAT were carried out using [14C]-acetyl-CoA, and the production of [14C]-acetylated protein was quantified after separating the components of the reaction on SDS-PAGE. The p300 HAT domains were preincubated in assay buffer (50 mM HEPES, pH 7.9, 0.1 mM EDTA, 1 mM dithiothreitol, and 50 μg/ml bovine serum albumin, 30-μl volumes) for 10 min at 30 °C prior to initiation of reaction by the addition of [14C]-acetyl-CoA (20 μM, unless otherwise specified) for 0.5–60 min,
whereupon the reaction was quenched with 6 μl of 5× SDS gel loading buffer. After running out on 10% SDS-PAGE, the gels were dried and the radioactivity quantified by phosphorimage analysis (Molecular Dynamics) relative to a 14C-bovine serum albumin standard. Assays were performed in duplicates, and these generally agreed within 20%.

For acetyl-CoA $K_m$ experiments, autoacetylation assays were performed at a fixed concentration of p300 HAT domain (40 nM) and varying acetyl-CoA concentrations (0–20 μM) for 30 s. The rates obtained from these assays were fit by nonlinear least squares to equation 1, using the Kaleidagraph version 3.5 software package.

$$V_m = v^*[\text{acetyl-CoA}]/(K_m + [\text{acetyl-CoA}])$$  
(Eq. 1)

For autoacetylation inhibition experiments, by Lys-CoA (28), varying concentrations of Lys-CoA (0–2 μM) were used with fixed p300 HAT domain (40 nM) and acetyl-CoA (20 μM) for 30 s. In all cases, the concentration exceeded p300 HAT concentration by at least 4-fold. The inhibition constant $K_i$ for Lys-CoA was estimated using equation 2.

$$K_i = (IC_{50})/[1 + [\text{acetyl-CoA}]/K_m \text{ acetyl-CoA}]$$  
(Eq. 2)

For the electrospray ionization quantitative-Fourier transform mass spectrometry (FTMS) mapping experiments, wt p300 HAT (50–58 μM) along with acetyl-CoA (1.6 or 6 mM) were incubated in reaction buffer (20 mM sodium HEPES, pH 7.9, 200 mM NaCl, 10 mM dithiothreitol) for the time indicated and quenched with 1% aqueous trifluoroacetic acid.

**Pulldown Assays with Biotinylated p300 HAT Domain—** Autoacetylation reactions were performed as above, except instead of quenching with denaturant, p300 inhibitor Lys-CoA (40 μM final) was used (28). In control experiments, it was demonstrated that, in this way, auto-acetyltransferase activity could be blocked (>95%) within 30 s. Following Lys-CoA addition, 10 μl of monomeric avidin beads were added to the reaction mixtures at room temperature for 20 min (29). The supernatants were collected by centrifugation at 2100 revolutions/min for 40 s at 4 °C. The beads were washed twice with 0.5 ml of wash buffer (50 mM HEPES, pH 7.9, 1 mM dithiothreitol, 1 mM EDTA, 0.1% Triton-X, 1 M NaCl). Following washing, the supernatants and beads were treated separately with 5× SDS gel loading buffer and the fractions analyzed using 10% SDS-PAGE. The dried gels were analyzed by phosphorimage analysis (Molecular Dynamics).

**PCAF Acetylation of p300 HAT-ΔC—** PCAF HAT was expressed and purified as described previously (30, 31). PCAF HAT-catalyzed acetylation of p300 HAT-ΔC was performed in the autoacetylation reaction buffer described above including 20 μM 13C-acetyl-CoA using 400 nM p300 HAT-ΔC and variable concentrations of PCAF HAT (0–2 μl) over 5 min at 30 °C. Quantitation of acetylation using autoradiography was carried out as described for autoacetylation reactions.

**Circular Dichroism—** Circular dichroism spectra were recorded on wt p300 HAT and p300 HAT-ΔC using a JASCO J-810 circular dichroism spectropolarimeter instrument. The wt p300 HAT and p300 HAT-ΔC (~1.37 μM and 1.66 μM individually) were dissolved in buffer containing 50 mM NaF, 50 mM sodium phosphate, and spectra were recorded over a range of 185–260 nm at 42 °C. For thermal stability studies, circular dichroism spectra were monitored at fixed wavelengths (210 or 220 nm) in the range of 20–80 °C at 0.2 °C intervals. In addition, full spectra (185–260 nm) were recorded every 10 °C in the range of 20–80 °C.

**CNBr Digestion—** Protein samples from different time points were digested by incubating with 1 M CNBr in 15% trifluoroacetic acid at 4 °C for 12–18 h in the dark. After digestion, samples were flash frozen in liquid N2 and lyophilized. The lyophilized pellets were resuspended in 100 mM NH4OAc, pH 4, 6 M urea, 5 mM tris(2-carboxyethyl)phosphine and 10% CH3CN for 1 h at room temperature prior to being injected onto a Jupiter C4 reverse-phase column (4.6 × 150 mm). The digests were fractionated in a linear gradient of 30–70% CH3CN in 0.1% trifluoroacetic acid over 40 min at 1 ml/min. 1-min fractions were collected, lyophilized, and stored at −20 °C before mass spectrometry analysis.
Electrospray Ionization Quantitative-FTMS—The lyophilized fractions were resuspended in 20–40 μl of electrospray solvent (49% CH₃CN, 50% water, 1% formic acid). The peptides were directly infused into a custom-built quadrupole-FTMS hybrid instrument through nanoelectrospray nozzles using an Advion nanospray robot. The resulting ions were passed through a resistively heated metal capillary and skimmer before being externally accumulated for a total of 2 s in an accumulation octupole. After accumulation, the ions were shuttled to the ion cyclotron resonance cell through a quadrupole that functions either as a simple ion guide or as a selective filter (32).

Individual acetylation forms were enhanced by selected ion accumulation and SWIFT isolation (33). Each acetylation form was dissociated using electron capture dissociation (ECD) (34). Electrons were introduced to the cell for 100–200 ms using a dispenser cathode 35 inches from the center of the magnet. The kinetic energy of the electrons was controlled by placing a 1–2-V bias potential on the filament of the dispenser cathode.

Mass Spectrometric Data Analysis—Data were collected using the modular ion cyclotron resonance data acquisition system (MIDAS) (35). The isotopic distributions of each ion were fit using a least squares algorithm provided by the FTMS data analysis program THRASH (36). Upon comparing the least squares fit to a theoretical fit of the distribution, the m/z values of both the monoisotopic and the most abundant isotopic peaks of the ion were determined. The loop region peptide mass spectrometry and tandem mass spectrometry data were analyzed by THRASH, resulting in a protein list and fragment ion list that were uploaded onto the ProSight PTM web server for single protein mode (37). The mass difference between observed ion and theoretical ion indicates modifications on the observed ion.

RESULTS

Effect of Varying Acetyl-CoA and Lys-CoA Concentration on p300 Autoacetylation—Previous studies revealed that p300-mediated acetylation of peptide substrates proceeds with apparent K_m values for acetyl-CoA in the range of 1–30 μM (24, 25). We thus investigated the K_m of acetyl-CoA for the autoacetylation process. The K_m for acetyl-CoA was measured to be 3.3 ± 0.8 μM, and the IC₅₀ for Lys-CoA < 100 nM (Fig. 2). These studies indicate that coenzyme A binding is likely to be similar for p300-mediated autoacetylation reactions compared with peptide and histone acetylation catalyzed by p300.

p300 HAT Concentration Dependence of Autoacetylation—A classical method for analyzing the kinetic mechanism of self-catalyzed chemical reactions is to examine their concentration dependence. If the self-catalyzed reaction rate is first order with respect to substrate concentration, it is consistent with an intramolecular process. A self-catalyzed reaction rate that is second order or higher with respect to substrate concentration would support an intermolecular mechanism. We thus measured the rate of autoacetylation as a function of time and p300
HAT concentration using a fixed and saturating concentration of acetyl-CoA (20 μM) (Fig. 3). As can be seen, autoacetylation shows a rapid burst phase followed by a slower accumulation of product formation (Fig. 3B). Because it was not possible to find a true initial rate, we used the shortest time point (30 s) that was within a near linear range for most of the concentrations investigated. Under these conditions, a plot of product (acetylated p300 HAT) versus p300 HAT concentration showed highly cooperative behavior (Fig. 3C). A logarithmic replot of this data shows a slope of ~4 in the range of 10–80 nM (Fig. 3D). This suggests that the reaction mechanism is approximately fourth order with respect to p300 HAT concentration. There are at least two explanations for these results. The first is that oligomerization of p300 HAT is needed for autoacetylation. In this model, four or more molecules of p300 HAT come together to facilitate protein acetylation. A second potential explanation is that initial autoacetylation of p300 HAT molecules leads to the enhanced catalytic power of the modified enzyme and/or enhanced p300 HAT intermolecular interaction. This behavior would be consistent with observations previously obtained for activation of p300 HAT activity by loop autoacetylation (25). However, these results do not resolve the issue of whether p300 autoacetylation is intra- or intermolecular.

Acetylation of p300 C-terminally Truncated HAT Domain—To further investigate the possibility of intermolecular versus intramolecular acetylation, we examined the C-terminally deleted p300 HAT domain (p300 HAT-ΔC). As described above, p300 HAT-ΔC is highly defective as a catalyst but generates soluble protein. Using circular dichroism, we showed that p300 HAT-ΔC appears to be folded and has approximately the same temperature of denaturation (Fig. 4). These data suggest that the gross structure of p300 HAT is unaffected by C-terminal deletion and that the loop of p300 HAT-ΔC is three-dimensionally similar to wild type. We thus investigated the properties of p300 HAT-ΔC in autoacetylation. As expected, it was highly defective compared with wt p300-HAT (Fig. 5, A and B). As shown, the rate of autoacetylation was 150-fold lower when measured with 2.5 μM p300 HAT-ΔC compared with 40 nM wt p300 HAT (Fig. 5B). This shows that the catalytic defect of p300 HAT-ΔC extends beyond peptide substrates and also should allow for cleaner interpretation of intermolecular acetylation experiments. Using excess p300 HAT-ΔC (40–300 nM) in the presence of a low concentration (10 nM) of wt p300 HAT, efficient protein acetylation was observed, which rose with increasing concentrations of p300 HAT-ΔC, plateauing in the region of 300 nM (Fig. 5C).

The simplest explanation of this behavior was that the relatively small concentration of wt p300 HAT was responsible for the observed acetylation catalyzed by p300 HAT-ΔC intermolecularly. However, it was formally possible that p300 HAT-ΔC was allosterically activating wt p300 HAT to undergo intramolecular autoacetylation. To distinguish among these possibilities, we needed a method to physically separate p300 HAT-ΔC from wt p300 HAT, which run very close together using SDS-PAGE. To accomplish this goal, we used EPL (26, 27) to ligate a synthetic peptide containing a biotin group to generate wt p300 HAT-Btn. It proved possible then to achieve substantial separation of wt p300 HAT-Btn from p300 HAT-ΔC using avidin affinity chromatography (Fig. 6). As shown in the control experiments, although p300 HAT-Btn was largely retained (>50%) on the avidin resin during pulldowns, the nonbiotinylated p300 HAT domain almost completely (>95%) remained in the supernatant under these conditions (Fig. 6A). In this way, it is established that the vast majority (>90%) of the observed autoacetylation could be accounted for by invoking an intermolecular process (Fig. 6). The trace-acetylated p300 HAT pulled down with the resin (Fig. 6B) was consistent with the slight nonbiotinylated p300 HAT “crossover” observed in Fig. 6A.

PCAF-catalyzed Acetylation of p300 HAT-ΔC—Given that p300 HAT autoacetylation appears to be intermolecular, we investigated whether PCAF HAT domain (30, 31) could efficiently catalyze the p300 acetylation of p300 HAT-ΔC. As shown in Fig. 5D, with 2 μM PCAF, slight acetylation of 400 nM p300 HAT-ΔC was observed. The overall rate was 18 nmol/min, which represents a 5-fold lower rate than when 10 nM p300 HAT is used as the catalyst. By dividing the acetylation rate by the concentration of active HAT enzyme (2 μM PCAF versus 10 nM p300 HAT), it can be estimated that acetylation of p300 HAT-ΔC is 1000-fold less efficient by PCAF compared with p300. These results are compatible with the known high specificity of purified PCAF for histone H3 and the especially high efficiency of p300 autoacetylation.

Site-specific Kinetic Analysis of Loop Autoacetylation—Prior mass spectrometric analysis of the wt p300 HAT domain led to the identification of 13 autoacetylation sites (10 within the proteolytically sensitive loop) (Fig. 1). However, stoichiometries and kinetics at individual sites cannot be readily obtained using these earlier methods. We turned to Fourier transform tandem mass spectrometry, which allows for large protein fragments (>100 aa) to be analyzed with high resolution. It proved possible to chemically degrade wt p300 HAT using cyanogen bromide fragmentation, which led to the isolation of a 103-aa fragment containing the entire loop (Fig. 1). This allowed
identification of numbers and distribution of acetylation sites as a function of time (see below).

Autoacetylation was carried out on a scale suitable for mass spectrometric analysis with high concentrations of p300 HAT (50–58 μM) and acetyl-CoA (1.6 mM and 6 mM) employed. It should be noted that, even at time zero, although the unacetylated loop form is the major species, there are modest amounts of monoo-, di-, and triacetylated (1Ac, 2Ac, 3Ac) species and even small amounts of tetraacetylated (4Ac) p300 HAT (Figs. 7 and 8). Significant differences were observed in the rate and extent of acetylation in these two experiments (Figs. 7 and 8). In the 1.6 mM acetyl-CoA time course, it is noteworthy that 5 min were required before the zero acetylation state (0Ac) was no longer the largest component. In contrast, in the 6 mM acetyl-CoA run, the zero-acetylated form (0Ac) was already smaller than the tetraacetylated (4Ac) form after 30 s. There were up to 12 loop acetylation modifications in the 1.6 mM acetyl-CoA reaction after 2 h, whereas there were 17 (almost all available lysines) in the 6 mM acetyl-CoA reaction at 2 h (Figs. 7 and 8). Thus, the efficiency of autoacylation was at least partially rate-limiting at 1.6 mM acetyl-CoA with such a large p300 HAT concentration. Because the $K_m$ value of autoacetylation is 3.3 μM (see Fig. 2), this suggests that acetyl-CoA depletion and/or an unfavorable acetyl-CoA/CoASH ratio contribute to rate decrease in the 1.6 mM acetyl-CoA time course.

Common among the 1.6 and 6 mM acetyl-CoA reactions, there were a set of more rapid acetylation events followed by a slower phase of autoacetylation, giving a bimodal appearance of autoacetylation in the 1.6 mM acetyl-CoA reaction (Fig. 7). Although the bimodal appearance of the 1.6 mM experiment can be at least partially rationalized as related to acetyl-CoA substrate depletion, results with the 6 mM acetyl-CoA run argue against this being the case. In the 6 mM acetyl-CoA reaction, complexity increased further after 10 min, suggesting several clusters of favorable multicetylated forms (Fig. 8). Focusing on
the 1.6 mM acetyl-CoA reaction, there appeared to be a rather rapid accumulation of a subpopulation of hexa- and hepta-acetylated (6Ac and 7Ac) forms, even by 5 min (Fig. 7). However, at 5 min, the majority of p300 HAT protein still had two or less acetylation modifications and the 3–5 acetylated (3–5Ac) forms were relatively smaller than the 6–7 acetylated (6–7Ac) states.

Using high resolution tandem mass spectrometry, distinct acetylated forms at each time point can, in principle, be further analyzed to decipher the populations of acetylation events at the individual sites. Because the broadband mass spectrometry data showed that 1Ac–5Ac forms changed significantly in the first 30 s, even for the 1.6 mM acetyl-CoA reaction, their ECD data will be discussed in more detail. ECD produces c and z' ions, which denote N- and C-terminal fragment ions, respectively. By comparing the observed mass of c or z' ions to their theoretical mass, the acetylated lysine residue could be localized. ECD results showed that some fragments have more than one acetylation state, which indicates that there are different isomers present in each acetylation form. For example, Fig. 9 shows that the 1Ac peak is actually a mixture of five isomers. For peaks with two acetylations or more, the isomeric composition can be very complex.

Although combinations of acetylations can be challenging to measure (38), it is possible to show the change of acetylation level at individual lysine sites (Fig. 10). For example, by comparing the acetylation on z'15 and z'17, it was found that, in successive acetylation increases from the 1Ac to the 5Ac forms, Lys-1558 was acetylated 30, 45, 60, 100, and 100%, respectively (Fig. 10). It should be stated that the accuracy of these percentages can be estimated as ±5% based on comparison to standards (38). About 90% of Lys-1555 was acetylated in the 3Ac form, and the remaining 10% was added to it in the 4Ac form (Fig. 10). Nearly 60% of Lys-1554 was acetylated in the 4Ac form, and an additional 25% was observed in the 5Ac form (Fig. 10). About 15% of Lys-1546 was acetylated at 2Ac and kept the same from 2Ac to 5Ac (Fig. 10). For Lys-1542, the change of acetylation level was <5% at 5Ac (Fig. 10). For Lys-1549, -1550, and -1551, it was difficult to determine the acetylation level on each of them because of technical limitations, but the overall acetylation on them can be deduced. The results showed that, at 3Ac, the overall acetylation level reached 100% (of 300% total), and an additional 40% was added to them at 5Ac (Fig. 10). In summary, in the pentaacetylated protein, p300 HAT acetylations were as follows: 30% of Lys-1560, 100% of Lys-1558 and Lys-1555, 85% of Lys-1554, 140% of Lys-1549, -1550, and -1551, 15% of Lys-1546, and 35% of Lys-1542 (Fig. 10).

ECD was also performed on the 6Ac–12Ac forms, and acetylations were localized to individual lysine residues. In the C-terminal portion of the loop region, Lys-1570 acetylation was not observed; however, the next 10 lysine residues (Lys-1569–1564) were found to be acetylated. In the N-terminal portion, only Lys-1499 could be acetylated, which was acetylated after ~2 h and only in the 12Ac form. A previous study showed that Lys-1473 could also be acetylated (25), but our results showed that it is not acetylated until the 12Ac form. Compared with prior mass spectrometry analysis, two additional acetylation sites were observed, Lys-1568 and -1569. Consistent with previous site-directed mutagenesis studies where individual Lys→Arg mutations were analyzed, except Lys-1499, all of the lysine residues that were shown to affect catalytic activity were observed to be acetylated in 30 s. Acetylations on these lysine residues probably are essential and could facilitate the further acetylation on other lysine residues and thus affect enzyme activity.

**DISCUSSION**

Post-translational modifications provide key regulatory switches for cell signaling pathways and in gene regulation. Most well established in this regard are kinase regulatory cascades in which phosphate addition and removal can be the basis for signal flow. Many protein kinases have phosphorylatable flexible regions known as activation loops, which are modulated by autophosphorylation or upstream kinases (39–44).
In general, there are 1–3 phosphorylation events that occur on these kinase loops, and phosphate modifications induce conformational switches. Evidence of an autoregulatory loop in p300 HAT extended this paradigm to acetyltransferases (25, 40), and here we have further explored the nature of the autoacetylation process.

Analysis of the concentration dependence of p300 autoacetylation led to the unusual finding of an apparently highly cooperative process. Given the prior studies that autoacetylation can stimulate catalytic activity, it was thus impossible to distinguish whether the cooperativity resulted from autoactivation or the requirement for intermolecular autoacetylation. However, by using an acetyltransferase-deficient p300 HAT mutant, unequivocal evidence was obtained for intermolecular autoacetylation as the predominant mechanism. The very low apparent $K_m$ (110 nM) for "trans" autoacetylation is unlikely to represent a high affinity among p300 HAT domains, because p300 HAT appears to run as a monomer by size exclusion chromatography in the micromolar range (data not shown). Rather, the low apparent $K_m$ more likely reflects additional catalytic rate constants, such as conformational changes or slow product release, which can cause $K_m$ to be nonequivalent to $K_d$. It should also be mentioned that the catalytic efficiency measured here far exceeds prior studies of p300-mediated acetylation of synthetic peptide, which was based on the p300 loop (25). This suggests that distal protein–protein contacts or a particular p300 loop conformation present in the intact protein may influence autoacetylation.

Interestingly, with potentially a couple of exceptions, autophosphorylation is also an intermolecular process (39–44). It is perhaps generally difficult for an intramolecular enzymatic modification to occur because of the conformational rearrangement necessary to accommodate substrate entry into the active site (43, 44). The biological significance of trans modifications in proteins is that regulation will depend on the local concentration of the partners involved. Because p300/CBP are not abundant cellular proteins, the basal state of acetylation is unlikely to be high given the presence of deacetylase enzymes. However, it is possible at transcriptional promoters that multiple p300/CBP molecules are recruited that would enhance autoacetylation.

Prior to the tandem mass spectrometry measurements described here, the preferences for autoacetylation within the HAT regulatory loop were unknown. Given that these autoacetylation sites, even within the loop, span >50 residues, a standard mass spectrometric analysis using trypsin digestion would not permit full insight into the interdependency of autoacetylation, because typically small peptide fragments are examined. By examining the entire p300 loop en bloc facilitated by ECD and FTMS, it proved straightforward to measure the abundance of acetylation at each of the sites in the loop. In this way, it could be determined that rapid autoacetylation of approximately five sites, several of functional importance (25), precedes more global autoacetylation. Interestingly, four of these sites have recently been observed by mass spectrometry on endogenous p300 and CBP (45). However, our current studies do not show strict order of acetylation, as demonstrated by partial stoichiometries at multiple sites from mass spectrometry. Thus, it appears that even for the rapid events, several initial acetylation sites are possible.

Unraveling the detailed kinetic complexity of 17 different autoacetylation reactions on the p300 HAT domain, even with...
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Kinetic and Mass Spectrometric Analysis of p300 Histone Acetyltransferase Domain Autoacetylation
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