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Catalytic Mechanism and Function of Invariant Glutamic Acid 173 from the Histone Acetyltransferase GCN5 Transcriptional Coactivator*

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Within chromatin, reversible acetylation of core histones is critical for transcriptional activation of eukaryotic target genes. The recent identification of intrinsic histone acetyltransferase (HAT) catalytic activity from a number of transcriptional co-activators (including yeast GCN5, p300/CBP, P/CAF, and TAFII250), has underscored the importance of protein acetylation in transcriptional control. The GCN5 family is the prototype for a diverse group of at least four distinct human HATs families. Although there is now a clear link between *in vivo* HAT catalytic activity and gene activation, little is known about the molecular mechanisms of histone acetylation. Herein, we report the first detailed biochemical study that probes the catalytic mechanism and the function of invariant glutamic acid 173 within the GCN5 family of HATs. Our results suggest that the HAT reaction involves the formation of a ternary complex (histones, acetyl-CoA, and enzyme) where the ϵ-amino group of histone lysine residues directly attacks the bound acetyl-CoA. The acetylation reaction requires deprotonation of the ϵ-amino group prior to nucleophilic attack. Employing site-directed mutagenesis, chemical modification, steady-state, and pH-dependent rate analysis, it is demonstrated that glutamic acid 173 is an essential catalytic residue, acting as a general base catalyst by deprotonating the histone substrate.

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Histone acetyltransferases (HATs)[1] catalyze acetyl group transfer from acetyl-CoA to the ϵ-amino group of specific lysine residues within histone amino termini. Recent identification of intrinsic HAT catalytic activity from a number of transcriptional co-activators, including yeast GCN5 (1), p300/CBP (2), P/CAF (3), and TAFII250 (4), has prompted the evaluation of histone acetylation in transcriptional control. Reversible acetylation of core histones is critical for transcriptional activation of eukaryotic target genes (5–7). By alleviating the transcriptionally repressive interaction between positively charged histone amino termini and negatively charged DNA and thus providing direct access of the transcription machinery, hyperacetylation of distinct loci within chromatin is thought to give rise to gene activation (reviewed in Refs. 5–7). However, several aspects of this model have proven to be more complex. Some evidence supports the concept that the amino termini participate primarily in protein-protein interactions rather than protein-DNA interactions (reviewed in Ref. 8). It has been suggested that the physical consequence of histone tail acetylation is the disruption of structured domains in the amino termini, rather than the strict neutralization of positive charge (reviewed in Ref. 8). Enrichment of acetylation on specific lysine residues suggests that differential acetylation within distinct chromatin loci may play a key role in regulation (5). Also, the notion that nonhistone proteins may be the actual targets of many HATs has been proposed (9, 10), because acetylation of the general transcription factors by various HATs (9) and the acetylation of the tumor suppressor p53 by p300 (10) have been demonstrated *in vitro*. Despite the explosion of recent reports linking transcription and reversible protein/histone acetylation, the molecular mechanisms remain elusive.

At least four distinct human HAT families have been identified (11). The prototypical GCN5 family of HATs constitutes a large group of enzymes found in such diverse organisms as yeast, *Tetrahymena*, and humans (5). The GCN5 family exhibits specificity toward lysine 14 of H3 and lysines 8 and 16 of H4 (12). GCN5 enzymes are members of a superfamily of *N*-acetyltransferases that appear to share a common structural core (13). Although the sequence identity is limited among these diverse enzymes, this region is predicted to form a similar acetyl-CoA binding structure (13–16). Several x-ray structures of other CoA-dependent transferases in this superfamily have recently been reported (14–16); however, the catalytic mechanism has not been established.

Here, we report the first detailed biochemical study that probes the catalytic mechanism and the function of invariant glutamic acid 173 within the GCN5 family of HATs. Our results suggest that the HAT reaction does not involve the formation of a covalent acetyl-enzyme intermediate but rather involves the formation of a ternary complex (histones, acetyl-CoA, and enzyme) where the ϵ-amino group of histone lysine residues directly attacks the bound acetyl-CoA. We demonstrate that glutamic acid 173 is an essential catalytic residue, acting as a general base catalyst by deprotonating the histone substrate.

[1] The abbreviations used are: HAT, histone acetyltransferase; DEPC, diethyl pyrocarbonate; EDAC, 1-ethyl-3(3-dimethylaminopropyl) carbodiimide.

EXPERIMENTAL PROCEDURES

Reagents—All chemicals were of the highest grade commercially available. Calf thymus histones where purchased from Calbiochem. Amino acids 99–262 of yeast GCN5 corresponding to the HAT catalytic domain were overexpressed in BL21-DE3 bacteria. T7 based protein expression of GCN5 was induced for 3.5 h. Harvested cells were lysed by French pressure in 50 mM sodium phosphate (pH 8), 300 mM NaCl, 1 mM β-mercaptoethanol, 10% glycerol with protease inhibitors (1 mM phenylmethylsulfonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin). GCN5 from clarified extract was adsorbed over a 4-ml column of Ni^{2+} affinity resin (Qiagen) and eluted with a 0–50 mM imidazole gradient in the above buffer. GCN5 eluted at 12 mM imidazole. Fractions with HAT activity where pooled and concentrated to a volume of 1–2 ml. Concentrated sample was then applied to a G-75 size exclusion column and eluted in a buffer of 30 mM HEPES (pH 8), 150 mM NaCl, 1 mM dithiothreitol, and 10% glycerol. E173Q was generated by site-directed mutagenesis and was purified using SP-Sepharose (Amersham Pharmacia Biotech), CoA-agarose (Sigma) and Superdex 75 (Amersham Pharmacia Biotech) gel filtration chromatography. Pure fractions (assessed by SDS-polyacrylamide gel electrophoresis) were pooled and stored at either 4 °C or −20 °C until use. Protein concentration was determined by the method of Bradford (28).

HAT Assays—Extensive development of the HAT assays were required for the detailed kinetic analysis. Assays were performed in 100 mM acetate, 50 mM Bis-Tris, and 50 mM Tris, a three-component buffer that maintains constant ionic strength at a function of pH. Briefly, the assay involves the determination of [^{3}H]acetate transfer from [^{3}H]acetyl-CoA to the histone substrate, catalyzed by GCN5. Labeled [^{3}H]acetyl-CoA (NEN Life Science Products) was mixed with cold acetyl-CoA to generate a stock solution with 40–200 cpm/pmol specific activity. Final acetyl-CoA concentrations of 2–30 μM were employed depending on the assay. Histones were resuspended separately at ~10 mg/ml in the assay buffer. The reactions (55 μl) were initiated by either the addition of [^{3}H]acetyl-CoA or enzyme (final concentration, 20–100 nM). The reaction was allowed to proceed for 5–25 min at 22 °C before quenching the reaction by spotting the solution onto Whatman P81 (phosphocellulose) filter paper discs. The absorbed histones were then washed three times in 600 ml of 50 mM $NaHCO_3$ buffer (pH 9). Discs were dried briefly in acetone, and the amount of tritium retained was determined by liquid scintillation counting. Initial velocities were determined by varying both substrate (histones and acetyl-CoA) concentrations. Usually, histone concentrations were varied (0.5–50 μM) at different fixed levels of acetyl-CoA (1–30 μM). Because the nonenzymatic histone acetylation reaction resulted in significant background counts, we developed a series of controls to take this into account. For every GNC5 enzymatic reaction, a corresponding nonenzymatic control reaction was performed. The nonenzymatic rate was then subtracted from each sample, prior to fitting the data. When one substrate was varied at saturating levels of the second, the data were fitted to the following equation using KinetAsyst (IntelliKinetics, State College, PA.).

$$v = (k_{cat} \cdot S)/(K_m + S) \quad \text{(Eq. 1)}$$

When both substrates were varied, the data were fitted to the following equation.

$$v = [k_{cat} \cdot A \cdot B/(K_{ia} \cdot K_b + K_a \cdot B + K_b \cdot A + A \cdot B)] \quad \text{(Eq. 2)}$$

Alternatively, the data were plotted in double reciprocal form (as in Fig. 1), and the kinetic parameters were determined from intercept and slope replots (17) using the computer program Kaleidagraph (Abelbeck Software).

Chemical Modification—The chemical modifying agents iodoacetate, diethyl pyrocarbonate (DEPC), and 1-ethyl-3(3-dimethylaminopropyl)-carbodiimide (EDAC) were used to inactivate GCN5. GCN5 was incubated in the presence of the reagent for an extended period (10–60 min) at pH 7.5 and 22 °C. The remaining GCN5 activity was then determined at saturating levels of histones and acetyl-CoA, as described above.

pH Analysis—At different pH values between 5.9 and 10.5, the k_{cat} and $k_{cat}/K_{m(histones)}$ values were determined by varying both histone and acetyl-CoA concentrations and fitting the resulting data to Equation 2. Alternatively, saturating levels of acetyl-CoA (30 μM) were used, and histone concentration was varied. The k_{cat} and $k_{cat}/K_{m(histones)}$ values were then determined from a fit to Equation 1. Once the k_{cat} and $k_{cat}/K_{m(histones)}$ values were obtained at the indicated pH, each parameter was fitted to the following equation.

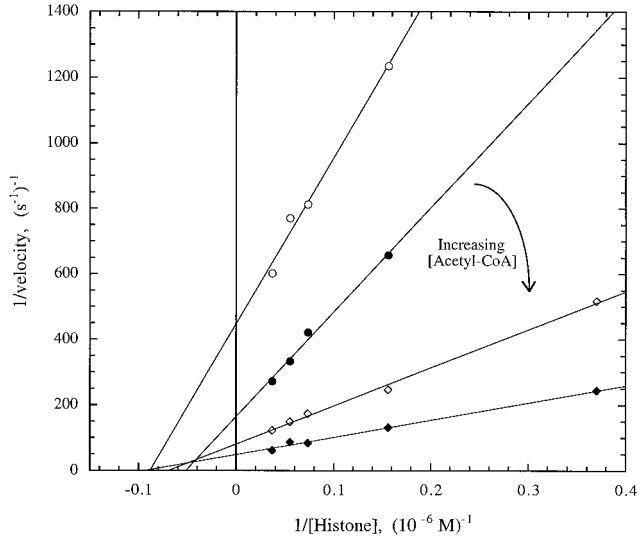

FIG. 1. **Ternary complex between yeast GCN5 histone acetyltransferase, acetyl-CoA, and histones is required for catalysis.** The data are presented in double reciprocal form, where 1/velocity is plotted against 1/[histone] at various fixed concentrations of acetyl-CoA. ○, 2.2 μM; ●, 4.4 μM; ◇, 13 μM; ◆, 24 μM. The conditions were 25 °C, pH 7.5, and 30 nM GCN5.

$$v = C/(1 + H/K_a) \quad \text{(Eq. 3)}$$

where C is the pH-independent value, and K_a is the ionization constant, and H is the proton concentration.

RESULTS AND DISCUSSION

CoA-dependent transferases are known to utilize one of two distinct mechanisms to catalyze acetyl transfer. One mechanism involves acetyl transfer from CoA to an enzyme nucleophile prior to transfer to the second substrate (18). In a subsequent step, the enzyme then transfers this group to the acceptor substrate. The alternative mechanism involves direct acetyl transfer from CoA to the substrate acceptor, without the formation of a covalent enzyme intermediate (19). The latter mechanism requires that both substrates and enzyme must form a ternary complex before catalysis can occur. For yeast GCN5 HAT, these two mechanisms were distinguished from a steady-state kinetic analysis. Both substrate (histones and acetyl-CoA) concentrations were varied, and the resulting initial velocities were determined. Fig. 1 displays the data in double reciprocal form where 1/velocity is plotted against 1/[histone] at various fixed concentrations of acetyl-CoA. The observed intersecting line pattern is diagnostic for the mechanism in which both substrates must bind to the enzyme before catalysis (Fig. 1). These data argue against a mechanism involving initial acetylation of an enzyme residue, followed by acetyl transfer to lysine. Instead, the data suggest that the ϵ-amino group of lysine directly attacks the acetyl-CoA within the active site of the enzyme. Supporting evidence comes from the recent x-ray structure of the related HAT1/acetyl-CoA complex, which revealed that the acetyl group of acetyl-CoA was not transferred to the enzyme in the complex (14). To date, there is no evidence for a covalent acetyl-enzyme intermediate. Consistent with our data (Fig. 1), similar intersecting line patterns were reported for the native rat liver nuclear HAT (20). Thus the GCN5 HAT family will likely utilize a catalytic mechanism that will optimize this direct attack on acetyl-CoA by lysine. The HATs must overcome the fact that at physiological pH values, the ϵ-amino of the lysine substrate is protonated and therefore unreactive. The ϵ-amino group of lysine must first be deprotonated by an active site amino acid residue acting as a general base catalyst.

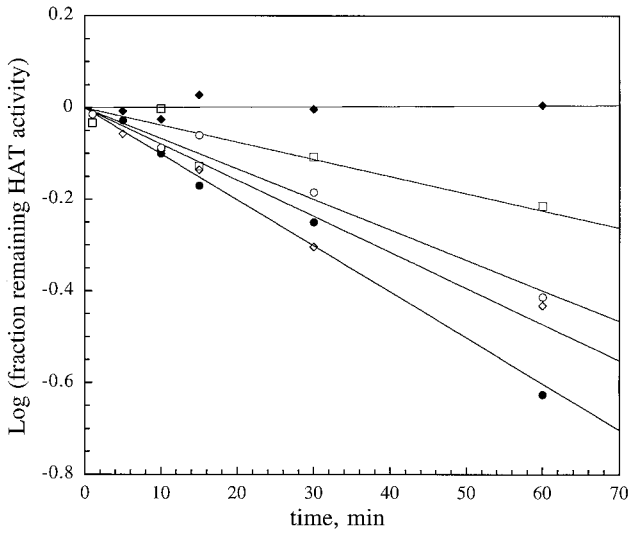

FIG. 2. **Carboxylate-directed inactivation of GCN5 HAT activity.** The carboxylate-specific reagent EDAC inactivated GCN5 in a concentration-dependent fashion. ♦, 0 mM EDAC; □, 10 mM; ○, 30 mM; ◇, 50 mM; ●, 80 mM.

To identify critical active site residues, we utilized a variety of side chain-specific amino acid modifying reagents to inactivate GCN5. Chemical modifying agents DEPC and iodoacetate, which are specific for histidine and cysteine residues, respectively, failed to inactivate GCN5 HAT activity. Although thiol group sensitivity has been previously reported with native (full-length) HATs from *Tetrahymena* (21) and rat liver (22), we observe no adverse effect of iodoacetate treatment when only the HAT catalytic domain is employed. It is likely that these differences in thiol sensitivity result from reactivity of structurally important cysteine residues outside the putative catalytic domain. In contrast, the carboxylate-directed reagent EDAC was effective at inactivating GCN5 catalytic activity in a concentration-dependent manner (Fig. 2), suggesting the importance of one or more carboxylic acid-containing residues.

If a side chain carboxylate is responsible for deprotonating the ϵ-amino group of a histone lysine, then the enzyme would be active only when this acidic residue is unprotonated in the active ternary complex. The pH dependence of the reaction rate should indicate the presence of an ionization that must be unprotonated for binding/catalysis. To explore the possibility of general base catalysis by an acidic residue, we generated pH rate profiles of the steady-state kinetic parameters, k_{cat} and k_{cat}/K_m. The second order rate constant k_{cat}/K_m describes the reaction between free enzyme and free substrate. Reflecting both substrate binding and catalysis, the k_{cat}/K_m pH profile will yield the intrinsic pK_a values of critical ionizations. The k_{cat} parameter describes the unimolecular catalytic steps subsequent to enzyme/substrate complex formation and will therefore yield pK_a values of the complex. Because of high nonenzymatic histone acetylation at alkaline pH, the background reaction rate at each substrate concentration was determined and subtracted from each data point. The pH profile of both k_{cat}/K_m and k_{cat} revealed a critical ionization that must be unprotonated for catalysis (Fig. 3). As the pH increases there is a corresponding increase in catalytic activity that plateaus above the critical ionization with pK_a value of 8.1 ± 0.2 for k_{cat} and 8.3 ± 0.2 for k_{cat}/K_m. Below the pK_a, the rate of catalysis drops 10-fold for every pH unit decrease, toward a limit of zero. These data indicate the catalytic requirement of an unprotonated active site residue and are consistent with a mechanism in which this residue may abstract a proton from the ϵ-amino group of substrate.

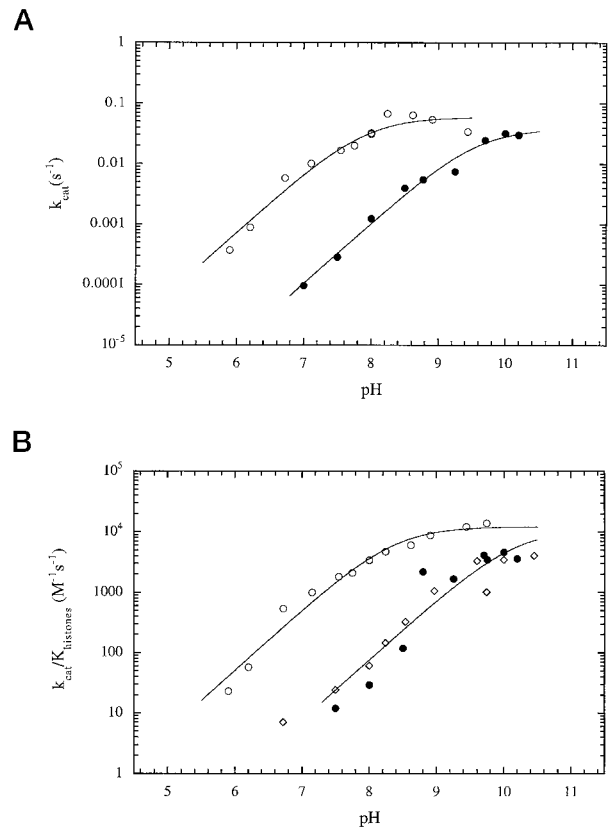

FIG. 3. **The pH-dependent HAT activity of GCN5 and E173Q mutant.** At the indicated pH values, the k_{cat} (*A*) and k_{cat}/K_m (*B*) values were determined for both wild type GCN5 (○) and E173Q (●) enzymes. The wild type GCN5 profile indicates the presence of an enzyme residue with a pK_a value of 8.1 ± 0.2 for k_{cat} and 8.3 ± 0.2 for k_{cat}/K_m, which must be unprotonated for activity (Equation 3). The E173Q mutant has lost this ionization but instead displays an ionization with a pK_a value of 9.5 ± 0.1 for k_{cat} and 10.1 ± 0.6 for k_{cat}/K_m, consistent with the expected ionization of lysine from the histone substrate. The pH dependence of the nonenzymatic histone acetylation was determined to verify the ionization of histone lysine residues (◇, *panel B*). The nonenzymatic rate yielded an apparent pK_a of 9.6 ± 0.2.

The minimal catalytic domain of GCN5 has been mapped to region between amino acids 170 and 253 (1, 23). Within this stretch of amino acids, there are only three invariant acidic residues, Glu-173, Glu-183, and Asp-214. Previous single-site mutational analysis had indicated the detrimental effect of substitution at Asp-214 but a lack of an effect upon Glu-183 substitution (24). Although substitution at Asp-214 resulted in decreased activity, this mutant was not among the most debilitating in the study (24, 25). A triple mutant involving residues 171–173 was one of the most debilitating mutations *in vivo* and *in vitro*, suggesting an important role for Glu-173 (25). To determine the role of Glu-173, a GCN5 mutant protein harboring a Glu-173 to Gln substitution was generated and probed as the potential catalytic base in the reaction. The steady-state kinetic parameters were determined and compared with the wild type enzyme (Table I). Strikingly, the k_{cat} value for the E173Q mutant was 320-fold lower than that determined for the wild type enzyme at pH 7.5. But the K_m values for histones and acetyl-CoA were not significantly different between wild type ($K_{m(histones)}$ = 28 μM; $K_{m(AcCoA)}$ = 20 μM) and mutant protein ($K_{m(histones)}$ = 17 μM; $K_{m(AcCoA)}$ = 21 μM). These data suggest that the E173Q mutant does not alter the ability of protein to bind substrates but rather has a profound effect on catalysis. No difference in binding affinity also suggests that the overall structural integrity of the protein is maintained.

TABLE I
Kinetic analysis for GCN5 and E173Q mutant histone acetyltransferases

Errors are the standard errors of the fitted parameters determined from least squares fitting from one representative data set. The conditions were 100 mM acetate, 50 mM Bis-Tris, 50 mM Tris, and 22 °C.

Enzyme	k_{cat}	K_m histones	K_m AcCoA
	s^{-1}	μM	
GCN5, pH 7.5	$8.0 \times 10^{-2} \pm 3.7 \times 10^{-2}$	28 ± 2.1	20 ± 6.6
E173Q, pH 7.5	$2.5 \times 10^{-4} \pm 2.3 \times 10^{-4}$	17 ± 6.8	21 ± 8.6
GCN5, pH 9.75	0.18 ± 0.03	1.9 ± 1.1	57 ± 13
E173Q, pH 9.75	$2.8 \times 10^{-2} \pm 0.3 \times 10^{-2}$	8.0 ± 4.7	15 ± 3.2

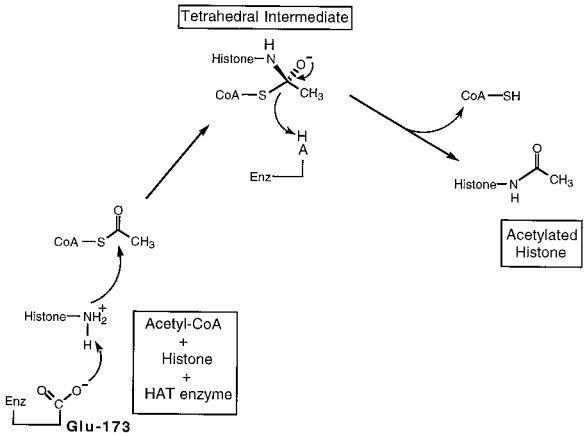

FIG. 4. **Proposed mechanism of catalysis involving glutamic acid-173 as the general base.**

If Glu-173 is the general base responsible for deprotonating the lysine (Fig. 4), one would predict that at elevated pH values where the concentration of unprotonated lysine is significant, the E173Q mutant activity will converge toward wild type activity. Because the reaction requires an unprotonated lysine to act as the nucleophile within the active site, this can either be accomplished by active deprotonation by Glu-173 at neutral pH (general base catalysis) or by artificially raising the pH to deprotonate the free histone lysine residues. Indeed, at pH 9.75 the E173Q mutant k_{cat} value is only 6-fold lower than wild type, as compared with 320-fold at pH 7.5 (Table I). To explore this further, a complete pH rate analysis of the E173Q mutant was performed (Fig. 3). The E173Q mutant does not display the ionization with pK_a value of 8 found with the wild type enzyme but rather exhibited an ionization with a pK_a value of 9.7 (and 10.1 in k_{cat}/K_m) for a group that must be unprotonated for activity. This pK_a 9.7–10.1 group is consistent with the ionization of the ϵ-amino group of histone lysines (26). To verify that the ionization displayed in the E173Q pH profile is the ϵ-amino group from histone lysine residues, the pH-dependent rate for the nonenzymatic reaction was determined (Fig. 3). The nonenzymatic reaction will be fully dependent upon the ionization of histone lysines and should yield an average pK_a value of all reactive lysine residues. The apparent second order rate constant for the reaction of acetyl-CoA and histones in the absence of GCN5 was measured as a function of pH and is displayed in Fig. 3. Identical to the E173Q catalyzed reaction, the reaction rate decreases 10-fold for every pH unit decrease below a pK_a value of 9.8. As predicted, the rate of nonenzymatic histone acetylation depends solely upon the solution ionization state of lysine residues. Similarly, the E173Q mutant enzyme depends upon the solution ionization state of histone lysine residues. At high pH where the majority of free histone lysine residues are unprotonated, the rate of catalysis by E173Q reaches values observed with wild type enzyme. That is, the requirement for lysine deprotonation by Glu-173 is lost at alkaline pH, and the E173Q can function as efficiently as wild type GCN5 (Fig. 4). However, at physiological pH the E173Q mutant is 320-fold less efficient as a catalyst, compared with wild type GCN5. In the cell, we would predict this mutant to be functionally inactive as a transcriptional activator.

The recent x-ray structure of *N*-myristoyl transferase revealed that a conserved glutamic acid (Glu-173) residue is positioned between two hydrophobic pockets (27). Based upon its location within the protein, the authors have implicated this residue in catalysis. Chloramphenicol acetyltransferase utilizes a histidine residue as a general base to abstract a proton from the primary hydroxyl group of chloramphenicol (19). Amazingly, glumatic acid replacement at this position can substitute as the general base, albeit with lower catalytic activity (19). The effect of burying the glutamic acid within the hydrophobic active site resulted in a dramatic shift in the pK_a of the substituted general base, from a pK_a of 6.6 for histidine to a pK_a of 8.3 for glutamic acid. Similar to our measured value of 8.3 for Glu-173 of GCN5, Glu-173 would be expected to reside within a hydrophobic active site pocket. Knowledge of the molecular details of HAT-catalyzed reactions will lead to a better understanding of their specific physiological functions and will augment the design of specific HAT inhibitors. This work provides the foundation for elucidating the role of protein/histone acetylation in the control of gene activation.

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