Mutational Analysis of Conserved Residues in the GCN5 Family of Histone Acetyltransferases*

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GCN5 is a critical transcriptional co-activator and is the defining member of a large superfamily of N-acetyltransferases. GCN5 catalyzes the transfer of an acetyl group from acetyl-CoA to the ε-amino of lysine 14 within the core H3 histone protein. Previous biochemical analyses have indicated a fully ordered kinetic mechanism. Recent structural studies have implicated several conserved residues in catalysis and substrate binding. Here we discuss the roles of Glu-173, His-145, and Asp-214 in yeast GCN5 catalysis and substrate binding. The results with wild type and E173Q, H145A, and D214A mutants are consistent with chemical catalysis being rate-determining in turnover. All mutants exhibited $K_{d}$ values (3.5–8.5 μM) for acetyl-CoA that were similar to wild type enzyme, indicating no functional role for these residues in AcCoA binding. The E173Q mutant demonstrated a ~500–600-fold decrease in $k_{cat}$ and $k_{cat}/K_{M}$ with Glu-173 acting as the general base catalyst as proposed previously. No significant effect was observed on substrate binding steps. His-145 was identified as a residue in the peptide binding cleft that must be unprotonated ($pK_{a} = 5.8$) for peptide binding and likely hydrogen-bonds to the Ser-10 hydroxyl of histone H3. His-145 also contributes to lowering the $pK_{a}$ value (by 0.8 units) of general base Glu-173 through a water-mediated hydrogen bond to the carboxylate side chain. Analysis of D214A revealed an obligate protein isomerization step that occurs after AcCoA binding and permits efficient peptide binding. Asp-214 is part of a conformationally flexible loop that mediates the isomerization by stabilizing distinct conformers of the protein.

Histone acetyltransferases (HATs)$^3$ are classified as enzymes capable of transferring an acetyl group from acetyl-CoA to an acceptor histone protein substrate. The acceptor site is the ε-amino group of lysine side chains within the amino-terminal tails of the core histones, H2A, H2B, H3, and H4. At least four gene families of HATS have been identified in mammals (1, 2). The largest family includes the defining member GCN5 whose catalytic domain is well conserved from yeast to humans. The GCN5 family of HATS are part of a larger superfamily of enzymes capable of acetyl transfer to amine-containing substrates (referred to as GNATs, for N-acetyltransferases). PCAF (p300/CBP-associating factor) (3–6) is also a member of the GCN5 family of HATS, displaying similar substrate specificity within the catalytic domain. In vitro, GCN5 displays a strong preference for Lys-14 of histone H3, although other acetylation sites on H3 and H4 have also been observed (7–10).

Yeast GCN5 has been known for some time to be essential for full transcriptional activation in a subset of genes (11–13). After the discovery of HAT activity in a related Tetrahymena enzyme (14), the causal link between histone acetylation and gene activation was codifying and has led to an explosion of HAT discoveries (reviewed in Ref. 15). In yeast, GCN5 promotes both global histone acetylation (i.e. genome wide) and gene loci-specific histone acetylation (9, 16–18). In mammals where global acetylation is less pronounced than in yeast, gcen5 appears to be an essential gene (19).

Given the importance of these enzymes in controlling transcription by both global and loci-specific acetylation, understanding the molecular mechanisms of catalysis, regulation, and substrate specificity has become a requisite part of understanding the biological functions of these enzymes and the development of specific inhibitors. To date, GCN5 is the best characterized HAT in terms of its catalytic mechanism and structure. With both GCN5 and PCAF, the basic mechanism was shown to involve the initial formation of a ternary complex before any chemical events occur (20–23). This implies the lack of an acetyl-enzyme intermediate that may form from the initial reaction of acetyl-CoA with enzyme. Also, attempts to trap an enzyme intermediate have not been successful (21, 22). The binding of substrates (histone and acetyl-CoA) and the release of products (acetylated histone and CoA) appear to be strictly ordered (21–23). It was demonstrated that conserved Glu-173 (yeast GCN5 numbering) is functioning as the general base catalyst, abstracting a proton from the ε-amino group of the bound lysine-containing substrate (20). This effectively increases the nucleophilicity of the amine nitrogen for efficient attack at the carbonyl carbon of bound acetyl-CoA, thus transferring the acetyl moiety to histone.

Several recent NMR (24) and x-ray structures (25, 26) have

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$ The abbreviations used are: HATs, histone acetyltransferases; Ac-CoA, acetyl coenzyme A; CoA, coenzyme A; DTT, dithiothreitol; PCAF, p300/CBP-associating factor; PAGE, polyacrylamide gel electrophoresis; tGCN5, tetrahymena GCN5; yGCN5, yeast GCN5; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol.

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corroborated these initial mechanistic conclusions. The structures of PACF and GCN5 from several species revealed an α/β globular fold where two roughly orthogonal hydrophobic troughs were found along the surface of the protein. The structure of the dead-end complex of Tetrathymina GCN5 with CoA and peptide identified these troughs as the binding sites for H3 histone peptide and coenzyme A (25). The conserved glutamic acid residues within a deep hydrophobic pocket, near the junction where Lys-14 of substrate and the thiol of CoA meet. The crystal structures are consistent with the biochemical evidence that demonstrated that the reaction is ordered and that a conserved glutamate residue acts as the general base. However, many mechanistic questions remain. The structure determinations have provided some additional insight into the possible roles played by other conserved residues that line the active site and the substrate-binding grooves.

We have undertaken a detailed kinetic analysis to provide biochemical and chemical evidence for the functional role of several invariant residues. Here we examine the functions of His145, Asp-214, and Glu-173 in yeast GCN5 using site-directed mutagenesis, pH analysis, steady state and pre-steady state kinetics, equilibrium dialysis, and isotope partitioning methods. Our findings are discussed in terms of previous structural and biochemical data.

EXPERIMENTAL PROCEDURES

Materials—Histone H3 peptide, ARTKQTARKSTGGKAPRKL (representing the 20 amino-terminal residues of human histone H3 and an additional carboxyl-terminal cysteine), and the corresponding H3 peptide with phosphoserine at position 10 were synthesized by the Protein Chemistry Core Lab at the Baylor College of Medicine. [3H]Acetyl-CoA (1.88 Ci/mmol) was from PerkinElmer Life Sciences.

Expression and Purification—Mutations in the catalytic domain of yGCN5 were generated using the Bio-Rad Muta-gene method, and then subjected to size-exclusion chromatography on G-75 phosphocellulose disks were from Life Technologies, Inc. Dispo-Equilibrium Dialyzers were from Amika Corp. All other reagents were from Sigma, Pierce, or Fisher.

Enzymatic Assays for yGCN5—yGCN5 HAT activity was measured using [3H]Acetyl-Coa and histone H3 peptide or the phosphorylated (Ser-10) H3 peptide as substrates using a radioactive P81 filter binding assay as described previously (20).

Bi-substrate Kinetic Measurements—Bi-substrate kinetic analyses were performed on the E173Q, D214A, and H145A mutants at AcCoA concentrations spanning 0.1 to 40 μM and H3 peptide concentrations spanning 25 to 600 μM. The data were fit to the sequential (ternary complex) mechanism equation (Equation 1), using the algorithms of Cleland (29) and the computer program KinetAssay (IntelliKinetics, State College of Pennsylvania), using a nonlinear least squares approach.

\[ v = V_{m} \times \frac{[A] 	imes [B] \times (K_{mH3} + [B]) 	imes (K_{mAc} + [A] + [B])}{(K_{mH3} + [B]) + (K_{mAc} + [A] + [B])} \]  

Determination of the Dissociation Constant for AcCoA Binding to yGCN5 via Equilibrium Dialysis—Equilibrium conditions were 50 μM Tris, pH 7.5, and 15–20 μM yGCN5. Equilibration was performed using Dispo-Equilibrium Dialyzers (Amika Corp.), which contain two 75-μl chambers separated by a 5-kDa cut-off dialysis membrane. The \( K_d \) value for AcCoA in the presence of yGCN5 was determined by aliquoting 5–400 μM AcCoA (20–40 cpm [3H]CoA) into the buffer chamber, and yGCN5 into the sample chamber. After 40 h of equilibration on a level shaker, samples were recovered from each chamber and counted by scintillation counting to determine the amount of radioactivity in the buffer chamber ([AcCoA]_buffer) and the sample chamber ([AcCoA]_sample) in order to determine the amount of bound AcCoA. The data were fit to Equation 2 and are presented in hyperbolic form.

\[ \text{[AcCoA]yGCN5} = \frac{([\text{AcCoA}]_{\text{buffer}})}{(K_{d} + ([\text{AcCoA}]_{\text{buffer}}))} \]  

Isotope Trapping Experiments—Pulse-chase experiments were done in order to obtain information regarding both kinetic and equilibrium aspects of the yGCN5-AcCoA (30–32). Experiments were performed at 24 ± 1 °C, in 5 mM DTT, 50 mM Tris, pH 7.5 and 9.5, by preparing solutions containing 37.5 μM of 1.0 μM [3H]-acetyl-CoA and 20 μM wild type yGCN5 (pulse) and 20 μM of 10 μM unlabeled AcCoA and 0.0–4.0 mM histone H3 peptide (chase). Under these conditions, the unlabeled AcCoA and histone H3 peptide react slowly yet spontaneously and therefore must be mixed immediately before use. Additionally, the equilibrium conditions for the pulse solution were chosen such that no further reactions take place after the [3H]-acetyl-CoA is bound to yGCN5 by equilibrium dialysis. 5–10 s after preparing the chase solution, 12.5 μl of the chase was added to the pulse solution to obtain 50 μl of 15 μM yGCN5, 0.75 μM [3H]-acetyl-CoA, 2.5 mM AcCoA, and 0.0–1.0 mM histone H3 peptide. The reactions were rapidly mixed for ~5 s with a pipette; 40 μl of the reaction volume was spotted onto P81 phosphocellulose, and the amount of [3H]-acetyl-H3 peptide was determined as described above (20).

To determine the amount of radioactivity incorporated into [3H]-acetyl-H3 peptide after the initial partitioning event, control experiments were performed at 24 ± 1 °C, in 5 mM DTT, 50 mM Tris, pH 7.5 and 9.5, by preparing solutions containing 3.33 mM unlabeled AcCoA, 1.0 μM [3H]-acetyl-CoA, and 20 μM yGCN5 (pulse) and 20 μl of 0.0–4.0 mM histone H3 peptide (chase). Under these conditions, the unlabeled AcCoA and the unlabeled AcCoA are in rapid equilibrium with yGCN5, such that the labeled substrate is effectively diluted with unlabeled substrate. After pre-equilibrating the reaction solutions to 24 ± 1 °C, 12.5 μl of the chase was added to the pulse solution to obtain 50 μl of 15 μM yGCN5, 0.75 μM [3H]-acetyl-CoA, 2.5 mM AcCoA, and 0.0–1.0 mM histone H3 peptide, the identical reaction conditions as the actual pulse-chase experiment. As before, the reactions were rapidly mixed for ~5 s with a pipette; 40 μl of the reaction volume was spotted onto P81 phosphocellulose, and the amount of [3H]-acetyl-H3 peptide was determined. The amount of labeled product generated in these control experiments was used to correct for the amount of labeled product obtained in the partitioning samples after the initial partitioning event due to enzymatic conversion of the labeled label substrate.

Determination of the Rate-limiting Step, Pre-steady State Analysis—The rate-limiting step for wild type yGCN5 was examined using a Hi-Tech quench-flow device (Hi-Tech Ltd., Salisbury, UK). Experiments under single turnover conditions were performed using 20 and 30 μM yGCN5, 0.5 μM AcCoA, and 200 μM H3 peptide at 24 ± 1 °C, pH 7.5 (concentrations given are post-mixing). Two concentrations of wild type yGCN5 were used to ensure that maximal rate was achieved for the single turnover reaction. After various reaction times between 0.01 and 10 s, the reactions were quenched with 2 N HCl, and the amount of [3H]-acetyl-H3 peptide was determined using the filter binding assay by spotting 50 μl of the 200-μl reaction slug on P81 phosphocellulose paper and washing away the free acetyl-CoA in sodium bicarbonate (20). The amount of the reaction slug was used as the internal control of total [3H] radioactivity by spotting 50 μl of the reaction slug onto P81 phosphocellulose paper and allowing it to dry without washing prior to scintillation counting. Control experiments with yGCN5 and [3H]-acetyl-CoA quenched prior to the addition of H3 peptide yielded insignificant counts, consistent with the lack of an acetyl-enzyme intermediate being involved in the reaction. Data were fit to a first-order kinetic model.
first-order rate of product formation; \( k \)

values spanning 6.5–23. In addition, at pH values spanning 6.5–9.5, the \( k_{a} / K_{M,AcCoA} \) values were determined at near-saturating levels of H3 peptide (1.5 mM) while varying AcCoA (0.5–2 mM). A 50 mM Tris, 50 mM Bis-Tris, and 100 mM sodium acetate (TBA) buffer was employed to keep the ionic strength constant over the pH range examined. Typically full saturation curves were fit to the basic Michaelis-Menten equation. For the pH profiles however, initial velocities were measured at varied sub-saturating concentrations for the substrate of interest. Consequently, the data were fit to a modified Michaelis-Menten equation (Equation 4) in order to directly obtain statistically valid values for \( k_{a} / K_{M,AcCoA} \) and \( k_{a} / K_{M,AcCoA} \). 

\[
 v_0 = \left( \frac{k_{a}}{K_{M,AcCoA}} - [S] \right) / (1 + ([S]/K_{M})) 
\]

Equation 4

Once the \( k_{a} / K_{M,AcCoA} \) and \( k_{a} / K_{M,AcCoA} \) values were obtained at the indicated pH, each parameter was fit to Equation 5 or 6.

\[
 k_{a}/K_{M} = C/(1 + H/K_{ca}) 
\]

Equation 5

\[
 k_{a}/K_{M} = C/(1 + H/K_{ca}) 
\]

Equation 6

RESULTS AND DISCUSSION

Mutational Analysis of yGCN5—Initial kinetic analyses of wild type yGCN5 and the highly homologous human PCAF were previously published (21–23). These analyses indicated an ordered Bi-Bi kinetic mechanism as follows: AcCoA binds to the free HAT enzyme; H3 histone binds second to form a ternary complex; chemistry occurs by facilitating the transfer of the acetyl group from AcCoA to the ε-amino group acceptor of Lys-14 of histone H3, generating CoA and Ac-Lys-14 H3 histone products; Ac-Lys-14 histone H3 is released from the ternary complex; and CoA is released to regenerate the free HAT (21–23). In addition, biochemical analysis of an E173Q mutant of yGCN5 implicated the invariant Glu-173 as the general base, abstracting a proton from Lys-14 of histone H3 to facilitate acetyl transfer from AcCoA (20).

The crystal structures of Tetrahymena GCN5 (tGCN5) bound to coenzyme A, and tGCN5 bound to both coenzyme A and a histone H3 peptide (representing the amino-terminal tail of histone H3) have been solved (25). These structures have provided valuable structural information that are consistent with Glu-173 functioning as the general base in catalysis and that have suggested the involvement of other conserved residues in catalysis; or substrate binding. For example, the conserved Asp-214 of yGCN5 resides on a conformationally flexible loop. Its location near the presumed active site had suggested a potential role in catalysis, perhaps as a general base and/or general acid catalyst (Fig. 1) (24, 25).

In addition to Glu-173 and Asp-214, which may be involved in acetyl transfer to histone H3, His-145 appeared to be within hydrogen bonding distance of Ser-10 of histone H3 peptide in the structure of the ternary complex (Fig. 1) (25). Ser-10 of histone H3 has been demonstrated to be phosphorylated during mitosis (33, 34) and transiently during mitogen stimulation of quiescent cells (35, 36). Mutation of Ser-10 impairs condensation and segregation of chromosomes during mitosis (37).
phosphorylation of Ser-10 on histone H3 has been demonstrated to be synergistically coupled to acetylation of Lys-14 and is mediated by an increased affinity for the phosphorylated substrate by wild type yGCN5 (36). Due to the proximity of His-145 to Ser-10 of histone H3 peptide, we hypothesized that this His residue may play a role in peptide binding and possibly in recognition of the phosphorylated substrate.

To elucidate the functions of Glu-173, Asp-214, and His-145, we generated and kinetically characterized the E173Q, D214A(D214N), and H145A mutated forms of yGCN5. As one of the conclusions from this analysis, we provide data for an isomerization step in the catalytic mechanism subsequent to AcCoA binding and prior to H3 peptide binding (Scheme 1). The experimental evidence for this isomerization will be discussed below, along with a discussion of the functions of Glu-173, Asp-214, and His-145.

Bi-substrate Kinetics—In order to elucidate the function of conserved amino acids in the catalytic mechanism of GCN5, initial bi-substrate saturation kinetics were performed to obtain the steady state kinetic parameters for the mutated forms of yGCN5: E173Q, D214A, and H145A. Initial velocities were determined as a function of AcCoA concentrations at different fixed H3 peptide concentrations (Fig. 2). These data were plotted in double-reciprocal form and, for all three mutated forms, displayed an intersecting line pattern that intersects to the left of the 1/velocity axis. Entire data sets were fit directly to Equation 1, using a nonlinear least squares method. The steady state parameters obtained from duplicate experiments for the various forms of yGCN5 are summarized in Table I.

The results for the mutant forms of yGCN5 were compared with the kinetic parameters obtained with wild type yGCN5. At pH 7.5, the E173Q mutant exhibited a 680-fold decrease in $k_{cat}$ and a 540-fold decrease in $k_{cat}/K_{m}$ for wild type yGCN5: E173Q, D214A, and H145A. Initial velocities were determined as a function of AcCoA concentrations at different fixed H3 peptide concentrations (Fig. 2). These data were plotted in double-reciprocal form and, for all three mutated forms, displayed an intersecting line pattern that intersects to the left of the 1/velocity axis. Entire data sets were fit directly to Equation 1, using a nonlinear least squares method. The steady state parameters obtained from duplicate experiments for the various forms of yGCN5 are summarized in Table I.

The experimentally determined kinetic mechanism of yGCN5 involves the following steps:

1. $k_{cat}$ = $k_{A}$
2. $k_{cat}$ = $k_{B}$
3. $k_{cat}$ = $k_{C}$

Thus, the overall rate equation for yGCN5 can be written as:

$$k_{cat} = k_{A} K_{A} + k_{B} K_{B} + k_{C} K_{C}$$

where $k_{A}$, $k_{B}$, and $k_{C}$ are the rate constants for each step, and $K_{A}$, $K_{B}$, and $K_{C}$ are the corresponding dissociation constants. The experimental evidence for this isomerization will be discussed below, along with a discussion of the functions of Glu-173, Asp-214, and His-145.

For the D214A mutant, we observed an ~200-fold decrease in $k_{cat}$ and an ~150-fold decrease in $k_{cat}/K_{m}$ for wild type yGCN5. Asp-214 functions during chemical catalysis ($k_{cat}$) and that Glu-173 functions as a general base by deprotonating the ε-amino group of the histone lysine, thereby facilitating nucleophilic attack of AcCoA (20).
decrease in \( k_{\text{cat}}/K_{m,\text{AcCoA}} \) may therefore suggest a decrease in AcCoA binding affinity. In addition to changes in \( k_{\text{cat}}/K_{m,\text{AcCoA}} \), we observed a significant 2-fold increase in the \( K_{m,\text{H3}} \) suggesting that His-145 may contribute slightly to peptide binding.

Equilibrium Dialysis Binding Assays—The initial observations with the D214A and the H145A mutants had suggested that the AcCoA dissociation constant (\( K_{d} \)) for these mutants may have been significantly increased. In order to determine the AcCoA affinity of the yGCN5 mutants, binding assays were performed using equilibrium dialysis. The dissociation constant for AcCoA binding to wild type yGCN5 was previously determined in the absence of histone H3 peptide via equilibrium dialysis (22). In this study, equivalent experiments were performed on the E173Q, D214A, D214N, H145A, and R164K mutants. The dissociation constant (\( K_{d} \)) was determined by dispensing varying concentrations of \(^{3} \text{H}\)AcCoA into the buffer chamber and fixed concentrations of the various mutated forms of yGCN5 into the sample chamber. The data were collected and analyzed as described under “Experimental Procedures.” A representative binding experiment is shown in Fig. 3. The average \( K_{d} \) values from duplicate experiments are summarized in Table II. The mutations E173Q, D214A(D214N), H145A, and R164K appeared to have little or no effect on AcCoA binding (values ranged from 3.5 to 8.5 \( \mu M \)). In addition, the data were in excellent agreement with the calculated enzyme concentrations determined by the method of Bradford (28).

The equilibrium binding results for the E173Q mutant are consistent with Glu-173 playing no direct role in AcCoA binding. Since there is no observable change in \( k_{\text{cat}}/K_{m,\text{AcCoA}} \) for the E173Q mutant, the observed decrease in \( k_{\text{cat}} \) is consistent with an ~600-fold decrease in \( k_{T} \) (Scheme I), and in agreement with the conclusion that Glu-173 functions as the general base in catalysis. Also, the lack of an effect with the R164K mutant is consistent with Arg-164’s proposed role in phosphoserine 10 recognition on H3 substrate. The steady state parameters of R164K were indistinguishable from those of wild type enzyme when unmodified H3 peptide is the substrate (36). For the D214A and H145A mutated forms, these results strongly suggest that changes in the steady state kinetic parameters, namely \( k_{\text{cat}}/K_{m,\text{AcCoA}} \), may be caused by affecting steps in the kinetic mechanism (such as enzyme isomerization) prior to H3 peptide binding but distinct from initial AcCoA binding (\( k_{1} \) and \( k_{2} \)).

Isotope Trapping Experiments, Evidence for Isomerization—To provide evidence for an enzyme isomerization, isotope trapping experiments (pulse-chase experiments) (30–32) were performed on wild type yGCN5. This method provides a technique for evaluating (under various concentrations of second substrate) the relative amount of labeled \(^{3} \text{H}\)acetyl-CoA substrate from a \(^{3} \text{H}\)acetyl-CoA-yGCN5 complex that dissociates to a large pool of unlabeled AcCoA substrate (effectively irreversible) versus the amount of labeled substrate from the complex that is trapped as product (31). Since an equilibrium binding study does not preclude the possibility of a catalytically dysfunctional complex, and since improper binding is not easily revealed under steady state conditions, this method provides information that cannot be obtained from either a steady state or an equilibrium analysis. The ability to trap all of the \(^{3} \text{H}\)acetyl-CoA-yGCN5 complex at infinite concentrations of histone H3 peptide would suggest that all of the complex exists as a relevant Michaelis complex capable of catalysis, and from this, \( k_{\text{cat}},K_{m,\text{AcCoA}} \) may be determined (30). Alternatively, the failure to trap any or all of the \(^{3} \text{H}\)acetyl-CoA-yGCN5 at infinite concentrations of histone H3 peptide would lead to two possible interpretations as follows: not all species of complex are competent Michaelis complexes or \(^{3} \text{H}\)acetyl-CoA dissociates from

### Table I

| Enzyme | \( K_{M,\text{AcCoA}} \) (\( \mu M \)) | \( k_{\text{cat}}/K_{M,\text{AcCoA}} \) (M\(^{-1}\)s\(^{-1}\)) | \( K_{M,\text{H3}} \) (\( \mu M \)) | \( k_{\text{cat}}/K_{M,\text{H3}} \) (s\(^{-1}\)) | \( k_{\text{cat}} \) (M\(^{-1}\)s\(^{-1}\)) |
|--------|-----------------|-----------------|-----------------|-----------------|-----------------|
| Wild type | 2.5 ± 1.4 | 6.8 × 10\(^{5}\) | 490 ± 80 | 3.5 × 10\(^{3}\) | 1.7 ± 0.12 |
| E173Q | <0.025 | >1.0 × 10\(^{5}\) | 384 ± 119 | 6.5 | 2.5 ± 4.0 × 10\(^{-3}\) |
| D214A | 4.2 ± 0.9 | 2.0 × 10\(^{3}\) | 362 ± 59 | 2.3 × 10\(^{1}\) | 8.6 ± 0.6 × 10\(^{-3}\) |
| H145A | 3.1 ± 0.9 | 3.9 × 10\(^{4}\) | 833 ± 211 | 1.4 × 10\(^{2}\) | 1.2 ± 0.2 × 10\(^{-1}\) |

\(^{a}\) Previously published results for wild type yGCN5 (22).

### Table II

| Enzyme | \( K_{d,\text{AcCoA}} \) (\( \mu M \)) |
|--------|-----------------|
| WT | 8.5 ± 2.6\(^{a}\) |
| E173Q | 4.0 ± 1.7 |
| D214A | 8.3 ± 2.8 |
| D214N | 3.5 ± 1.1 |
| H145A | 4.9 ± 0.8 |
| R164K | 4.4 ± 1.3 |

\(^{a}\) Previously published results for wild type yGCN5, pH 7.5 (22).
The percent of 0.75 μM total [3H]AcCoA partitioned to product during the first turnover of 15 μM wild type yGCN5 relative to the amount that dissociates and is diluted into 2.5 mM unlabeled AcCoA was determined for 0–1.0 mM histone H3 peptide at 24 ± 1 °C, in 5 mM DTT, 50 mM Tris, pH 7.5 (closed diamonds) and 9.5 (data not displayed). The data were plotted and fit to Equation 6. The experiments were performed in duplicate with a representative plot displayed.

Asp-214 Provides Structural Stability to a Conformationally Sensitive Loop of yGCN5—The x-ray crystallographic structures of free (apo) tGCN5, of tGCN5 bound to AcCoA substrate, and of tGCN5 bound as a dead-end complex to CoA and a histone H3 peptide have provided insight into the role of many conserved residues in the GCN5 family of enzymes (25). Upon initial consideration of these structures, it has been observed that the β5-loop-α4 region of GCN5 undergoes distinct conformational changes upon formation of the binary and ternary complexes (Fig. 5). The results of the isotope partitioning experiment suggest that this isomerization is an important step in the catalytic mechanism of yGCN5, resulting in a fully ordered mechanism (Scheme 1). Evidence for this isomerization is provided by the D214A mutant of yGCN5. Asp-214 is located directly in the loop portion of the conformationally flexible β5-loop-α4 region of GCN5 and appears to make a number of stabilizing hydrogen bonding interactions with neighboring residues (Fig. 5). As the β5-loop-α4 portion of GCN5 shifts to accommodate substrate binding, Asp-214 actually swings out of the active site, and the side chain makes a new hydrogen bonding interaction with the backbone amide nitrogen of the neighboring Asn-215, providing additional conformational stability.

Based on structural data and our current biochemical investigation, mutation of Asp-214 to an alanine impedes the ability of the β5-loop-α4 region of yGCN5 to isomerize toward a stable conformation that can effectively bind peptide. This conclusion is further corroborated by the results of the kinetic analysis performed on the D214A mutant. We observed an ~200-fold decrease in $k_{\text{cat}}$ and an ~150-fold decrease in $k_{\text{cat}}/K_{\text{m},\text{H3}}$ both of which contain $k_3$ (isomerization) and $k_4$ (chemistry) rate constants (Scheme 1). We also observed an ~340-fold decrease in the $k_{\text{cat}}/K_{\text{m},\text{AcCoA}}$ which reflects AcCoA binding ($k_5$/$k_7$) and $k_9$. Since AcCoA affinity ($k_5$/$k_7$) has not been affected in the D214A mutant (Table II), and because changes in the steady state kinetic parameters were independent of pH in comparison to wild type yGCN5, this implies that $k_9$ must be the perturbed step. It should be duly noted, however, that changes in $k_9$ do not preclude the possibility that mutation of Asp-214 also influences the rate of other steps in the mechanism, such as peptide binding or chemistry. Pre-steady state reaction kinetics were required to probe this possibility.

Pre-steady State Reaction Kinetics at pH 7.5—In order to elucidate further the function of specific amino acids in the catalytic mechanism of GCN5, pre-steady state experiments were used to determine whether substrate binding, chemical catalysis, or product release is rate-limiting for yGCN5 and its various mutated forms. Two forms of pre-steady state experiments were employed as follows: single turnover experiments ([enzyme] > [substrate]) and multiple turnover experiments ([enzyme] < [substrate]). Under single turnover conditions, the first-order exponential rate of product formation yields the rate of chemistry when substrate binding is not rate-limiting. Therefore, if the observed rate of product formation is equivalent to $k_{\text{cat}}$, then chemistry is rate-limiting relative to substrate binding. If a pronounced lag exists in the initial stage of the reaction, causing multiphasic kinetics, then substrate binding or an enzyme isomerization step may be rate-limiting. Under multiple turnover conditions, if substrate binding or an enzyme isomerization is rate-limiting, a lag phase should again be evident in the kinetic trace, followed by a steady state linear phase. If chemistry is rate-limiting, then the rapid kinetic trace should be linear. If release of product is rate-limiting, an exponential burst phase should begin the kinetic trace followed by a linear steady state phase.

A quench flow apparatus was used to determine the rate-limiting step in the catalytic mechanism of wild type yGCN5 at pH 7.5. Under single turnover conditions, wild type yGCN5 (20 μM) was rapidly mixed with a fixed concentration of H3 peptide (200 μM) and substoichiometric amounts of [3H]AcCoA (0.5 μM). The reaction was quenched with 2 N HCl at various reaction times, and the concentration of [3H]acetyl-H3 was determined as described under “Experimental Procedures.” The concentration of product was plotted versus time and was fitted to a first-order exponential (Fig. 6) with an observed rate constant of 0.44 ± 0.08 s⁻¹. This value is in excellent agreement with the
steady state turnover rate of 0.47 ± 0.05 s⁻¹ obtained under equivalent conditions (Table III). Similar to human PCAF (21, 23), this suggests that chemistry (k₇) is the rate-limiting step in the reaction for wild type yGCN5.

In order to provide additional evidence for rate-limiting chemistry, multiple turnover experiments were also performed at pH 7.5 (Fig. 6). Wild type yGCN5 (5–10 μM) was rapidly reacted with a fixed concentration of H3 peptide (200 μM) at saturating concentrations of [³H]AcCoA, and the formation of acetyl-H3 was determined as before. Over the course of 30 ms to 2 s, the data produced a linear kinetic trace starting at the origin, with no apparent burst or lag phase. The rate constant determined from the slope of the line (0.41 ± 0.02 s⁻¹) is entirely consistent with the values obtained under single turnover and steady state conditions (Table III), suggesting that for wild type yGCN5, chemistry (k₇) is rate-limiting relative to product release steps.

Similar experiments were performed to elucidate the rate-limiting step for the E173Q, D214A, D214N, and H145A mutated forms of yGCN5 (Fig. 6). Under single turnover conditions, the mutated forms of yGCN5 were rapidly reacted with a fixed concentration of H3 peptide (500 μM) and substoichiometric amounts of [³H]AcCoA (0.5 μM) for varied amounts of time. Enzyme concentrations were varied (20–50 μM) to ensure that the experiments were performed under single turnover conditions (i.e. that all of the AcCoA is initially bound) such that increased enzyme concentrations did not cause an increase in the observed first-order rate constant. Multiple turnover experiments were also performed for the mutated forms of yGCN5 (5–10 μM) using 500 μM H3 peptide at saturating concentrations of [³H]AcCoA. As with wild type yGCN5, the concentration of product was plotted versus time for both types of experiment. For all of the mutated forms of yGCN5, single turnover experiments produced first-order exponential traces, and multiple turnover experiments generated linear traces starting at the origin, with no apparent burst or lag phase (Fig. 6). In addition, the rate constants obtained from these experiments correlated well with the rate constants obtained under steady state conditions (Table III). As with wild type yGCN5, the results suggest that chemistry (k₇) is rate-limiting for the E173Q, D214A, D214N, and H145A mutants. It should be noted, however, that we cannot rule out the possibility that another step may partially limit turnover. In this case, a small lag would not be discernible with this approach.

Although substitutions at Asp-214 clearly have deleterious effects on the enzyme isomerization step (k₃), the pre-steady

![Fig. 5. Comparison between apoGCN5, GCN5/AcCoA, and GCN5/CoA/histone H3.](http://www.jbc.org/)
Mutational Analysis of Conserved Residues in GCN5

The Role of Glu-173 as General Base—Previously, the conserved Glu-173 was implicated as an essential catalytic residue for yGCN5 HAT activity, functioning as the general base catalyst through deprotonation of Lys-14 from the histone (20). Here this conclusion has been further corroborated using bi-substrate steady state kinetics, equilibrium binding assays, and pre-steady state kinetic analysis. The E173Q mutant retains the ability to bind AcCoA as effectively as wild type yGCN5, while exhibiting an ~600-fold decrease in $k_{\text{cat}}$ and $k_{\text{cat}}/K_{m,\text{AcCoA}}$ as evinced by both bi-substrate and pre-steady state kinetic analysis. Although the HAT assay provided insufficient sensitivity to obtain an accurate assessment of $K_m$ for the E173Q mutated form is approaching the same magnitude as the $k_{\text{cat}}/K_{m,\text{AcCoA}}$ for wild type yGCN5. Although the $k_{\text{cat}}/K_{m,\text{AcCoA}}$ has decreased greater than 100-fold, the affinity of the E173Q mutant for AcCoA ($K_a$) has not been affected. These results are consistent with Glu-173 functioning as a general base in the transfer of the acetyl moiety from AcCoA to Lys-14 of histone H3 peptide and that mutation of Glu-173 to a glutamate causes an ~600-fold decrease in $k_{\text{cat}}$.

pH Dependence of HAT Activity for Wild Type yGCN5 and the H145A Mutant—In order to understand the molecular basis for changes in the steady state parameters conferred upon mutation of His-145 to an alanine, $k_{\text{cat}}/K_{m,\text{AcCoA}}$ pH profiles were obtained for wild type yGCN5 and the H145A mutant (Fig. 7). The second-order rate constant $k_{\text{cat}}/K_{m,\text{AcCoA}}$ describes the reaction between free enzyme and free substrate, thereby reflecting both substrate binding and catalysis. The $k_{\text{cat}}/K_{m,\text{AcCoA}}$ pH profile will yield the intrinsic $pK_a$ values of critical ionizations that are necessary for binding and/or catalysis. Previously, a $k_{\text{cat}}/K_{m,\text{AcCoA}}$ profile of the related human PCAF, using H3 peptide as a substrate, revealed the presence of two critical ionizations with $pK_a$ values of 6.9 and 7.5 (21). The group with a $pK_a$ value of 7.5 was assigned to be the general base for PCAF, Glu-570 (Glu-173 for yGCN5), and the second ionization at pH 6.9 was suggestive of the $pK_a$ of a critical histidine residue. The identity of this residue was not confirmed.

In the structure of the ternary complex of tGCN5 bound to CoA and H3 peptide (25), it appeared that His-145 (yGCN5 numbering) resides in the H3 peptide-binding cleft and is potentially within hydrogen bonding distance from Ser-10 of histone H3 peptide. His-145 is also oriented toward Glu-173 (Fig. 1). We hypothesized that the second critical ionization observed in the $k_{\text{cat}}/K_{m,\text{AcCoA}}$ profile of PCAF (with a $pK_a$ 6.9) is caused by the necessity for the corresponding histidine residue to be unprotonated in order to provide a stabilizing hydrogen bond with histone H3 peptide.

The pH rate profiles of the steady state kinetic parameter $k_{\text{cat}}/K_{m,\text{AcCoA}}$ were generated for wild type yGCN5 as well as for the H145A mutant by varying the concentration of H3 peptide at saturating concentrations of AcCoA over the pH range 5.6–10 (Fig. 7). Wild type yGCN5 demonstrated two critical ionizations with a $pK_a$ value of 8.3 ± 0.1, a $pK_a$ value of 5.8 ± 0.1, and a pH-independent $k_{\text{cat}}/K_{m,\text{AcCoA}}$ value of $2.4 \pm 0.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$. The pH profile of the H145A mutant, however, indicated only a single critical ionization with a $pK_a$ value of 9.1 ± 0.1, and a pH-independent $k_{\text{cat}}/K_{m,\text{AcCoA}}$ value of $1.0 \pm 0.1 \times 10^4 \text{M}^{-1}\text{s}^{-1}$. The data suggest that mutating His-145 to an alanine results in a loss of the critical ionization with a $pK_a$ value of 5.8 and a concurrent shift of the $pK_a$ value from 8.3 to 9.1 ± 0.1. The shift in the $pK_a$ value from 8.3 to 9.1 suggests that His-145 may be responsible for effectively lowering the $pK_a$ of Glu-173 in the active site of wild type yGCN5. In addition, the pH-independent value for H145A is approximately 2-fold lower than the pH-independent value for wild type yGCN5 which is.

![Fig. 6. Determination of the rate-limiting step under pre-steady state conditions at pH 7.5.](http://www.jbc.org/)

The state analysis indicated that chemical catalysis ($k_2$) is also less efficient by 2–3 orders of magnitude. This observation is likely the result of improper orientation of the two substrates within the binding pockets, precluding efficient attack of the e-amino on the carbonyl oxygen of AcCoA. It is interesting to point out that an asparagine at position 214 is 10-fold worse (in terms of $k_{\text{cat}}$) than having alanine at this position. We hypothesized that the second critical ionization observed in the $k_{\text{cat}}/K_{m,\text{AcCoA}}$ profile of PCAF (with a $pK_a$ 6.9) is caused by the necessity for the corresponding histidine residue to be unprotonated in order to provide a stabilizing hydrogen bond with histone H3 peptide.

The pH rate profiles of the steady state kinetic parameter $k_{\text{cat}}/K_{m,\text{AcCoA}}$ were generated for wild type yGCN5 as well as for the H145A mutant by varying the concentration of H3 peptide at saturating concentrations of AcCoA over the pH range 5.6–10 (Fig. 7). Wild type yGCN5 demonstrated two critical ionizations with a $pK_a$ value of 8.3 ± 0.1, a $pK_a$ value of 5.8 ± 0.1, and a pH-independent $k_{\text{cat}}/K_{m,\text{AcCoA}}$ value of $2.4 \pm 0.2 \times 10^4 \text{M}^{-1}\text{s}^{-1}$. The pH profile of the H145A mutant, however, indicated only a single critical ionization with a $pK_a$ value of 9.1 ± 0.1, and a pH-independent $k_{\text{cat}}/K_{m,\text{AcCoA}}$ value of $1.0 \pm 0.1 \times 10^4 \text{M}^{-1}\text{s}^{-1}$. The data suggest that mutating His-145 to an alanine results in a loss of the critical ionization with a $pK_a$ value of 5.8 and a concurrent shift of the $pK_a$ value from 8.3 to 9.1 ± 0.1. The shift in the $pK_a$ value from 8.3 to 9.1 suggests that His-145 may be responsible for effectively lowering the $pK_a$ of Glu-173 in the active site of wild type yGCN5. In addition, the pH-independent value for H145A is approximately 2-fold lower than the pH-independent value for wild type yGCN5 which is,
The experiments were performed in triplicate with representative data being fit to Equation 6, and the pH profile for H145A was fit to Equation 7.

The steady state pH profile for H3 peptide formation (0.97 ± 0.01 cat./s, AcCoA, 20 μM wild type yGCN5 and 0.5 μM AcCoA, 50 μM H3) or the H145A mutated form (2.4 ± 0.2 cat./s, AcCoA, 20 μM mutated yGCN5, 0.5 μM AcCoA, 500 μM H3) was due to a change in the rate-limiting step at high pH. The single turnover experiment was performed at pH 9.5 by mixing either wild type yGCN5 (20 μM) or the H145A mutated form (20 μM) with a fixed concentration of H3 peptide (50 μM) and substoichiometric amounts of [3H]AcCoA (0.5 μM). As in the previous single turnover reactions performed at pH 7.5, the kinetic trace did not exhibit a lag phase, and the observed first-order exponential rate constants (t1/2cat,AcCoA) for wild type yGCN5 and the H145A mutant, respectively) were 0.97 ± 0.01 and 0.2 ± 0.02 s−1 for wild type yGCN5 and the H145A mutant, respectively (Table III). These data indicate that the observed kcat/Km values obtained for steady state experiments correspond to kcat values obtained from AcCoA saturation curves at H3 peptide concentrations equivalent to those used for the single and multiple turnover experiments.

Within error, the difference between the kcat/Km values obtained for wild type yGCN5 and H145A at pH 7.5 (Table I).

Due to the increased approximate Km value of H145A, obtaining a full kcat pH profile was not plausible. Instead, a single turnover pre-steady state control experiment was performed at pH 9.5 for both wild type yGCN5 and the H145A mutant (Fig. 6), in order to verify that the plateau in the kcat/Km pH profile (Fig. 7) was not a result of changing the rate-limiting step at high pH. The single turnover experiment was performed at pH 9.5 by mixing either wild type yGCN5 (20 μM) or the H145A mutated form (20 μM) with a fixed concentration of H3 peptide (50 μM) and substoichiometric amounts of [3H]AcCoA (0.5 μM). As in the previous single turnover reactions performed at pH 7.5, the kinetic trace did not exhibit a lag phase, and the observed first-order exponential rate constants (t1/2cat,AcCoA) for wild type yGCN5 and the H145A mutant, respectively) were 0.97 ± 0.01 and 0.2 ± 0.02 s−1 for wild type yGCN5 and the H145A mutant, respectively (Table III). These data indicate that the observed kcat/Km′ shift in the pH profile reflects an intrinsic change in the pKa of Glu-173 and not an apparent shift due to a change in the rate-limiting step for H145A catalysis.

At pH 7.5, it appeared that the kcat/Km for the H145A mutant was ~17-fold less than that for wild type yGCN5. In order to assess whether this change was a result of the pKa shift, was caused by an inherent decrease in AcCoA binding, or was due to a change in the rate of isomerization (as with the D214A mutant), a pH profile was obtained for H145A and the H145A mutated form (Fig. 7) from single turnover experiments performed at pH 9.5 (Fig. 9). The pH rate profiles for both wild type yGCN5 and the H145A mutant exhibited pH dependence for kcat/Km value of 2.8 ± 0.2 cat./s−1 for wild type yGCN5 at 24 ± 1 °C, 5 mM DTT, 50 mM Tris, pH 9.5 (concentrations given are post-mixing). The reactions were quenched and analyzed as described under “Experimental Procedures.” Rate constants obtained from duplicate experiments are summarized in Table III.

Experimental conditions, 20 μM H145A, 0.5 μM AcCoA, 50 μM H3.

### Table III

| Enzyme | kcat (s−1) from single turnover experiment | kcat (s−1) from multiple turnover experiments | kcat (s−1) from steady state experiments |
|--------|------------------------------------------|---------------------------------------------|------------------------------------------|
| WT, pH 7.5 | 4.4 ± 0.7 × 10−12 | 4.1 ± 0.2 × 10−1 | 4.7 ± 0.5 × 10−1 |
| WT, pH 9.5 | 9.7 ± 0.5 × 10−13 | 2.3 ± 0.8 × 10−3 | 2.1 ± 0.5 × 10−3 |
| E173Q | 2.4 ± 0.2 × 10−8 | 4.9 ± 0.8 × 10−7 | 4.8 ± 0.2 × 10−3 |
| D214A | 4.3 ± 0.2 × 10−8 | 4.6 ± 0.2 × 10−6 | 4.9 ± 0.4 × 10−4 |
| D214N | 4.4 ± 0.4 × 10−8 | 4.2 ± 0.8 × 10−1 | 4.2 ± 0.8 × 10−1 |
| H145A, pH 7.5 | 2.8 ± 0.2 × 10−8 | 3.0 ± 0.5 × 10−2 | 3.0 ± 0.5 × 10−2 |
| H145A, pH 9.5 | 4.3 ± 0.2 × 10−8 | 4.2 ± 0.8 × 10−1 | 4.2 ± 0.8 × 10−1 |

* The kcat values obtained for steady state experiments correspond to kcat values obtained from AcCoA saturation curves at H3 peptide concentrations equivalent to those used for the single and multiple turnover experiments.

Experimental conditions, 10 μM wild type yGCN5, 50 μM AcCoA, 200 μM H3.

Experimental conditions, 20 μM wild type yGCN5, 0.5 μM AcCoA, 50 μM H3.

Experimental conditions, 20–50 μM mutated yGCN5, 0.5 μM AcCoA, 500 μM H3.

Experimental conditions, 2.5–10 μM mutated yGCN5, 100 μM AcCoA, 500 μM H3.

Experimental conditions, 20 μM H145A, 0.5 μM AcCoA, 50 μM H3.
the H145A mutant is as catalytically efficient as wild type in terms of AcCoA binding and isomerization.

To examine whether the ionization in the $k_{cat}/K_m,AcCoA$ profiles reflected the Glu-173 residue or was a distinct residue important for AcCoA binding, we performed equilibrium dialysis at pH 6.5 where the $K_v$ value would be expected to decrease if this ionization is critical for AcCoA binding (Fig. 10). The acetyl moiety of AcCoA is labile in alkaline conditions, so the affinity of yGCN5 for AcCoA could not be assessed at pH > 7.5 with this technique. The average $K_v$ values at pH 6.5 obtained from duplicate experiments with wild type yGCN5 and the H145A mutant were compared with values obtained at pH 7.5 (Table II). At pH 7.5, the AcCoA dissociation constants ($K_{d,AcCoA}$) for wild type yGCN5 and the H145A mutant were 8.5 $\pm$ 2.6 and 4.9 $\pm$ 0.8 $\mu M$, respectively. At pH 6.5, the $K_{d,AcCoA}$ values for wild type yGCN5 and the H145A mutant were 6.6 $\pm$ 1.1 and 6.7 $\pm$ 1.0 $\mu M$, respectively. Both forms of the enzyme have similar affinity for AcCoA, and these affinities appear to be pH-independent. In addition, these data suggest that the critical ionization observed in the $k_{cat}/K_m,AcCoA$ profiles reflects the Glu-173 ionization which must be unprotonated for activity.

The Role of His-145 in Recognition of a Phosphorylated Histone H3 Peptide Substrate—When a phosphoserine is modeled into position 10 of histone H3 in the co-crystal structure of the ternary complex of GNC5 bound to CoA and H3 peptide (Fig. 1) (25), it appeared that His-145 and Arg-164 are within hydrogen bonding distance of phosphoserine 10. Previously, it has been shown that wild type yGCN5 exhibits a 5–10-fold lower $K_m$ value for H3 peptide phosphorylated at Ser-10 compared with the unmodified H3 peptide (36). Mutation of Arg-164 of yGCN5 to a lysine residue (R164K) effectively eliminates this preference for the phosphorylated substrate (36).

Due to the proximity of His-145 to Ser-10 of histone H3, it was of interest to determine whether His-145 was also involved in recognition or binding of the phosphorylated H3 histone substrate. HAT assays were performed with the H145A mutant at fixed concentrations of AcCoA (15 $\mu M$) using unmodified H3 peptide (25–1000 $\mu M$) and phosphorylated (Ser-10) H3 peptide (25–1000 $\mu M$). The data were plotted in double reciprocal form with linear fits to illustrate a 3–6-fold difference in $K_m$ value for H3 peptide phosphorylated at Ser-10 compared with the unmodified H3 peptide (36). Mutation of Arg-164 of yGCN5 to a lysine residue (R164K) effectively eliminates this preference for the phosphorylated substrate (36).

Conclusions—Previous biochemical analyses have indicated a fully ordered kinetic mechanism for the GNC5 family of HATs (20–23). Recent structural studies with GNC5 HATs have implicated several conserved residues within the active site and the proposed substrate binding pockets (24–26, 38). By using a variety of kinetic and biochemical approaches, we have evaluated the functional roles of invariant residues Glu-173, His-145, and Asp-214 in yeast GNC5. The results with wild type GNC5 and E173Q, H145A, and D214A mutants are consistent with chemical catalysis being rate-determining in turnover. All mutants exhibited $K_d$ values within 3.5–8.3 $\mu M$ for AcCoA, which were similar to the value of 8.5 $\pm$ 2.6 $\mu M$ ob-
performed at 24°C in 5 mM DTT, 50 mM Tris, pH 7.5. The experiments were performed in duplicate with a representative plot displayed.

tained for wild type enzyme. Therefore, these residues play no significant functional role in AcCoA binding. These data also indicated that these substitutions had no gross effect on protein structure, allowing unencumbered interpretation of alterations on other steps on the reaction pathway. The E173Q mutant demonstrated ~500–600-fold decreases in $k_{\text{cat}}$ and $k_{\text{cat}}/K_m$ of H3 peptide, consistent with Glu-173 acting as the general base catalyst as proposed previously (20). No significant effect was observed on either substrate binding steps. His-145 was identified as a residue within the peptide-binding cleft that must be unprotonated ($pK_a = 5.8$) for peptide binding and that likely hydrogen-bonds to the Ser-10 hydroxyl of histone H3. His-145 also contributes to lowering the $pK_a$ value (by 0.8 units) of general base Glu-173 through a water-mediated hydrogen bond to the carboxylate side chain. Detailed analysis of the D214A mutant revealed an obligate protein isomerization step that occurs after AcCoA binding and permits efficient peptide binding, explaining the ordered addition of substrates (20–23) and the inability to bind peptide efficiently in the absence of coenzyme (21). Asp-214 is located within a conformationally flexible loop that mediates the isomerization of distinct enzyme conformers.

REFERENCES

1. Mizzen, C. A., and Allis, C. D. (1998) Cell 90, 6–20
2. Kouzarides, T. (1999) Curr. Opin. Genet. Dev. 9, 40–48
3. Neuwald, A. F., and Landsman, D. (1997) Trends Biochem. Sci. 22, 154–155
4. Yang, X. J., Ogryzko, V. V., Ni, H., Howard, B. H., and Nakatani, Y. (1996) Nature 382, 319–324
5. Ogryzko, V. V., Schiltz, R. L., Russanova, V., Howard, B. H., and Nakatani, Y. (1996) Cell 87, 953–959
6. Bannister, A. J., and Kouzarides, T. (1996) Nature 384, 641–643
7. Kuo, M. H., Brownell, J. E., Sobel, R. E., Ranalli, T. A., Cook, R. G., Edmondson, D. G., Roth, S. Y., and Allis, C. D. (1996) Nature 383, 269–272
8. Grant, P. A., Eberhartner, A., John, S., Cook, R. G., Turner, B. M., and Workman, J. L. (1999) J. Biol. Chem. 274, 5895–5900
9. Zhang, W., Bone, J. R., Edmondson, D. G., Turner, B. M., and Roth, S. Y. (1998) EMBO J. 17, 3155–3167
10. Schiltz, R. L., Mizen, C. A., Vassilev, A., Cook, R. G., Allis, C. D., and Nakatani, Y. (1999) J. Biol. Chem. 274, 1189–1192
11. Brownell, J. E., and Allis, C. D. (1998) Curr. Opin. Genet. Dev. 6, 176–184
12. Wade, P. A., Pruss, D., and Wolffe, A. P. (1997) Trends Biochem. Sci. 22, 128–132
13. Grunstein, M. (1997) Nature 389, 349–352
14. Brownell, J. E., and Allis, C. D. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 6364–6368
15. Roth, S. Y., Denu, J. M., and Allis, C. D. (2001) Annu. Rev. Biochem. 70, 81–120
16. Krebs, J. E., Fry, C. J., Samuels, M. L., and Peterson, C. L. (2000) Cell 102, 587–598
17. Vogelauer, M., Wu, J., Suka, N., and Grunstein, M. (2000) Nature 408, 485–498
18. Kuo, M. H., vom Hau, E., Struhl, K., and Allis, C. D. (2000) Mol. Cell 6, 1309–1320
19. Xu, W., Edmondson, D. G., Evrard, Y. A., Wakamiya, M., Hehringer, R. R., and Roth, S. Y. (2000) Nat. Genet. 26, 229–232
20. Tanner, K. G., Trievel, R. C., Kuo, M.-H., Howard, R., Berger, S. L., Allis, C. D., Marmorstein, R., and Denu, J. M. (1999) J. Biol. Chem. 274, 18157–18160
21. Tanner, K. G., Langer, M. R., and Denu, J. M. (2000) Biochemistry 39, 11961–11969
22. Tanner, K. G., Langer, M. R., Kim, Y., and Denu, J. M. (2000) J. Biol. Chem. 275, 22048–22055
23. Lau, O. D., Courtney, A. D., Vassilev, A., Marzilli, L. A., Cotter, R. J., Nakatani, Y., and Cole, P. A. (2000) J. Biol. Chem. 275, 21953–21959
24. Lin, Y., Fletcher, C. M., Zhou, J., Allis, C. D., and Wagner, G. (1999) Nature 400, 86–89
25. Rojas, J. R., Trievel, R. C., Zhou, J., Mo, Y., Li, X., Berger, S. L., Allis, C. D., and Marmorstein, R. (1999) Nature 401, 93–98
26. Clements, A., Rojas, J. R., Trievel, R. C., Wang, L., Berger, S. L., Allis, C. D., and Marmorstein, R. (1999) EMBO J. 18, 3521–3532
27. Kim, Y., Tanner, K. G., and Denu, J. M. (2000) Anal. Biochem. 280, 308–314
28. Bradford, M. M. (1976) Anal. Biochem. 72, 248–254
29. Cleland, W. W. (1977) Adv. Enzymol. Relat. Areas Mol. Biol. 45, 273–387
30. Rose, I. A. (1980) Methods Enzymol. 64, 47–59
31. Rose, I. A., O’Connell, E. L., and Litwin, S. (1974) J. Biol. Chem. 249, 5163–5168
32. Rose, I. A. (1985) Methods Enzymol. 149, 315–340
33. Wei, Y., Mizzen, C. A., Cook, R. G., Gorovsky, M. A., and Allis, C. D. (1998) Proc. Natl. Acad. Sci. U. S. A. 95, 7480–7484
34. Hendzel, M. J., Wei, Y., Mancini, M. A., Van Hosser, A., Ranalli, T., Brinkley, B. R., Bazzet-Jones, D. F., and Allis, C. D. (1997) Chromosoma 106, 348–360
35. Mahadevan, L. C., Willis, A. C., and Barratt, M. J. (1991) Cell 65, 775–783
36. Cheung, P., Tanner, K. G., Cheung, W. L., Sassone-Corsi, P., Denu, J. M., and Allis, C. D. (2000) Mol. Cell 5, 905–915
37. Wei, Y., Yu, L., Bowen, J., Gorovsky, M. A., and Allis, C. D. (1999) Cell 97, 99–109
38. Trievel, R. C., Rojas, J. R., Sterner, D. E., Venkataramani, R. N., Wang, L., Zhou, J., Allis, C. D., Berger, S. L., and Marmorstein, R. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 8931–8936
39. Cleland, W. W. (1975) Biochemistry 14, 3220–3224
Mutational Analysis of Conserved Residues in the GCN5 Family of Histone Acetyltransferases

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