The MYST family of histone acetyltransferases (HATs) plays critical roles in diverse cellular processes, such as the epigenetic regulation of gene expression. Lysine autoacetylation of the MYST HATs has recently received considerable attention. Nonetheless, the mechanism and function of the autoacetylation process are not well defined. To better understand the biochemical mechanism of MYST autoacetylation and the impact of autoacetylation on the cognate histone acetylation, we carried out detailed analyses of males-absent-on-the-first (MOF), a key member of the MYST family. A number of mutant MOF proteins were produced with point mutations at several key residues near the active site of the enzyme. Autoradiography and immunoblotting data showed that mutation of these residues affects the autoacetylation activity and HAT activity of MOF by various degrees demonstrating that MOF activity is highly sensitive to the chemical changes in those residues. We produced MOF protein in the deacetylated form by using a nonspecific lysine deacetylase. Interestingly, both the autoacetylation activity and the histone acetylation activity of the deacetylated MOF were found to be very close to that of wild-type MOF, suggesting that autoacetylation of MOF only marginally modulates the enzymatic activity. Also, we found that the autoacetylation rates of MOF and deacetylated MOF were much slower than the cognate substrate acetylation. Thus, autoacetylation does not seem to contribute to the intrinsic enzymatic activity in a significant manner. These data provide new insights into the mechanism and function of MYST HAT autoacetylation.

Histone acetyltransferases (HATs), also often referred to as protein lysine acetyltransferases (KATs), catalyze the addition of acetyl groups in histone and non-histone proteins (1–6). The acetylation catalyzed by HATs occurs on the ε-amino group of specific lysine residues with the cosubstrate acetyl-coenzyme A (acetyl-CoA (AcCoA)) as the acetyl donor. First discovered in nucleosomal histones, protein acetylation is now widely recognized extending far beyond the chromatin realm and orchestrates other diverse biological functions and processes, including cell cycle, cytoskeleton remodeling, chaperones, ribosome, and metabolic pathways (7–12). In the past decade, significant progress has been made in various aspects of HAT biology, from enzyme kinetics, protein structures, gene regulation, signal transduction, and cell development to disease mechanism and inhibitor development (13–19). Based on the sequence and structural similarities, HATs are grouped into several major families, including GCN5/PCAF family, MYST family, p300/CBP, and RTT109 (15, 20, 21). The MYST family contains the largest number of HAT proteins in humans, including MOF (males-absent-on-the-first, KAT8, MYST1), which has specific HAT activity for Lys-16 on histone H4, Tip60, MORF, MOZ, and HBO1 (22). Recently, increasing biochemical, proteomic, and crystal structural evidence shows that overexpressed MYST proteins exist in the acetylated form. For instance, our data showed that when recombinant Tip60 or MOF was mixed with radiolabeled AcCoA, the proteins exhibited strong bands on the radioactive gel. Confirmative evidence supporting MYST autoacetylation comes from the structural reports showing that in several MYST protein crystal structures a conserved lysine residue residing near the active site (i.e. Lys-274 in MOF, Lys-327 in Tip60, Lys-815 in MORF, Lys-604 in MOZ, and Lys-262 in yEsa1) was found predominantly in the acetylated form (23–26). Such a unique spatial feature prompted several studies toward sorting out the functionality of this lysine autoacetylation in the regulation of the enzymatic activities of the MYST HATs. Although some studies showed that MOF Lys-274 autoacetylation increases substrate binding and acetylation (23, 27), others found that it first; MOZ, monocytic leukemia zinc finger protein; MORF, MOZ-related factors; HBO1, histone acetyltransferase binding to origin recognition complex 1; yEsa1, yeast essential Sas2-related acetyltransferase 1; Sirt1, silent mating type information regulation 2 homolog 1; AcCoA, acetyl-CoA.
blocks enzyme recruitment to chromatin (26). It is noteworthy to mention that almost all the previous studies relied on site mutagenesis to decipher the function of autoacetylation that might be prone to producing false-positive results. In addition to the active site lysine acetylation, there are other acetylated lysine residues, albeit to lesser degree. These alternative autoacetylation events have yet to be well studied. Because elucidation of MYST autoacetylation is of critical significance for understanding the regulatory mechanism of the activities of this class of epigenetic enzymes, in this work we carried out a detailed study of the autoacetylation of MOF; in particular, we investigated the effect of autoacetylation on its intrinsic enzymatic activities. This work provides new insights into the mechanism and function of MYST autoacetylation.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Reagents**—pET19b-hMOF(125–458) DNA plasmid was a gift from Dr. John Lucchesi, Emory University. *Escherichia coli* BL21(DE3) cell was purchased from Stratagene. [14C]Acetyl-CoA was purchased from PerkinElmer Life Sciences. Anti-acetyl-lysine antibody (ST1027) was obtained from Calbiochem. Goat anti-rabbit IgG-HRP antibody (sc-2004) was purchased from Santa Cruz Biotechnology.

**Site-directed Mutagenesis and Protein Expression**—Site-directed mutagenesis was achieved using the QuikChange protocol (Stratagene). All mutations were confirmed by DNA sequencing. Each His6-tagged hMOF(125–458) DNA plasmid was introduced into BL21(DE3) through the heat shock transformation method. Protein expression was induced with 0.3 mM isopropyl-1-thio-β-D-galactopyranoside at 16 °C for 20 h. After protein expression, cells were harvested by centrifugation, suspended in lysis buffer (25 mM Na-HEPES, pH 8.0, 150 mM NaCl, 1 mM PMSF, 1 mM MgSO4, 5% ethylene glycol, 5% glycerol), and lysed by a French press. The protein supernatant was purified on nickel-nitriilotriacetic acid resin (Novagen). Before protein loading, the beads were equilibrated with column buffer (25 mM Na-HEPES, pH 8.0, 500 mM NaCl, 1 mM PMSF, 10% glycerol, and 30 mM imidazole). After protein loading, the beads were washed thoroughly with washing buffer (25 mM Na-HEPES, pH 8.0, 500 mM NaCl, 1 mM PMSF, 10% glycerol, and 70 mM imidazole), and then the protein was eluted with elution buffer (25 mM Na-HEPES, pH 7.0, 500 mM NaCl, 1 mM PMSF, 100 mM EDTA, 10% glycerol, and 200 mM imidazole). Different elution fractions were individually checked on SDS-PAGE, followed by concentration using Millipore centrifugal filters. Concentrated protein solution was applied to size-exclusion chromatography on Superdex 75 (GE Healthcare) in the buffer containing 25 mM Na-HEPES, pH 7.0, 200 mM NaCl, 1 mM EDTA, 1 mM DTT, and 5% glycerol at 4 °C. The protein peak was collected and concentrated by Millipore centrifugal filters.

His6-tagged human Sirt1(1–555) protein was expressed and purified using similar procedures as described above. Protein was dialyzed against dialysis buffer (25 mM Na-HEPES, pH 7.0, 500 mM NaCl, 1 mM EDTA, 10% glycerol, and 1 mM DTT) and concentrated using Millipore centrifugal filters. The Bradford assay was utilized to determine the concentration of protein.

Final proteins were aliquoted and stored at −80 °C for future use.

**Protein Digestion and MS Analysis**—One-dimensional gel slices of MOF were submitted to the Harvard University Mass Spectrometry and Proteomics Resource Laboratory (Cambridge, MA) for protein identification and site of modification (acetylation) analysis. Each slice was reduced and alkylated (tris(2-carboxyethyl)phosphine and iodoacetamide) before enzymatic digestion. Three enzymes were chosen to obtain complementary peptides of expected sites of acetylation, endoproteinase Glu-C, chymotrypsin, and elastase. After digestion, peptides were extracted and pooled for MS analysis.

All samples were analyzed on a Thermo Fisher LTQ-Orbitrap XL equipped with a Waters nano-Acquity UPLC and nanocapillary chromatographic components. The Integrafrit trapping column was packed with 5 cm of Michrom Bioresources Magic C18 AQ (5 μm beads with 200-Å pore size) and the Picofrit analytical column was packed with 20 cm of Michrom Bioresources Magic C18 AQ (3 μm beads with 100-Å pore size). HPLC buffers were 0.1% formic acid in water (A buffer) and acetonitrile (B buffer). Sample trapping was accomplished by loading 12 μl of the digested peptides and trapping for 20 min at 2 μl/min at 1% B with eluent passing through the vent valve via a tee place between the trapping and analytical column.

Full MS data were collected in the Orbitrap at a resolution of 60,000, with the top 20 ions targeted for MSMS analysis in the linear ion trap. After the run, DTAs were produced using Extract Msr and were then searched using Sequest against both a large database (UniProt Human) for full protein identification, as well as the target molecule for site of modification analysis. For the modification analysis, the DTAs were searched against the target protein sequence with no enzyme specificity, and the additional variable added a mass of 42.01056 Da on lysine for the acetylation analysis.

Manual validation of peptides identified as acetylated was performed using functions included in the Proteomics Browser Suites (PBS). Fuzzylons was used to display and annotate the spectra as well as confirm the optimal position for modifications on a peptide regardless of amino acid specificity (GraphMod), which confirmed the acetylation was preferred at the indicated lysine residues. Muquest was used to search for any unknown peptide modification (including no modification) on spectra obtained during these experiments that were not specifically designated during the initial Sequest search. This process reliably shows unexpected modification (including amino acid truncations or missed cleavages) of spectra related to the model spectra used for the Muquest analysis.

**Radioactive Acetylation Assays**—In the acetylation assay, [14C]Acetyl-CoA was used as the acetyl donor. In the MOF autoacetylation, the reaction was quenched by 5× protein loading dye. Samples were resolved on 12% SDS-PAGE or 16% peptide gel. The gel was dried in vacuum and then exposed to a phosphorscreen (GE Healthcare). Autoradiograph was scanned on a Typhoon scanner and analyzed by QuantityOne software. Quantitation was accomplished according to 14C-labeled BSA as standard. For the substrate histone H4 acetylation, a synthetic peptide corresponding to the first 20 amino acid
sequence of the histone H4 N-terminal tail, i.e. H4-20 (ac-SGRGKGGKGLGKGGAKRHRK), was used as the substrate. In the filter binding assay for measuring H4-20 acetylation, the reaction was quenched by spotting the reaction mixture on a P81 filter paper disc (Whatman). After the paper discs were washed with 50 mM NaHCO3, pH 9.0, and air-dried, liquid scintillation counting was performed to measure the amount of acetylated products. Both MOF autoacetylation and histone acetylation assays were carried out at 30 °C in the reaction buffer containing 50 mM HEPES, pH 8.0, 0.1 mM EDTA, and 1 mM DTT.

Western Blot Analysis of MOF Autoacetylation—Autoacetylation of wild-type MOF and the mutants was analyzed by Western blot using a pan anti-acetyl-lysine antibody as the primary antibody. Each MOF protein was denatured and then resolved on 12% SDS-PAGE gel in equal amounts and transferred to nitrocellulose membrane (0.45 μm, Bio-Rad). The membrane was blocked with 5% (w/v) milk/TBST (50 mM Tris base, pH 7.4, 200 mM NaCl, 0.1% (v/v) Tween 20) for 1 h, followed by incubation with 5% (w/v) milk/TBST containing 1:1,000 dilution of anti-acetyl-lysine antibody for 1 h. The membrane was washed with TBST three times for 5 min. Development was performed using SuperSignal West Pico substrate (Pierce) and exposed to autoradiography film (Denville Scientific, Inc.). All the procedures were carried out at 25 °C. The acetylation level was quantified using QuantityOne software and normalized according to the gel input.

Preparation of Deacetylated MOF—MOF deacetylation was carried out in the reaction mixture containing 50 mM HEPES, pH 8.0, 1 mM DTT, 15 μM Sirt1, 7.5 mM NAD+, and 10 μM MOF at 25 °C for 1 h. Separation of deacetylated MOF was achieved by using size-exclusion chromatography on Superdex 75 in the buffer containing 25 mM HEPES, pH 7.0, 200 mM NaCl, 5% glycerol, and 1 mM DTT. The target peak was collected and concentrated by Millipore centrifugal filters. Concentration of protein was measured by Bradford assay. The final protein samples were aliquoted, flash-frozen, and stored at −80 °C. For quantitative comparison, the parallel control of MOF was prepared following the same procedure in the absence of Sirt1. Deacetylation efficiency was tested by Western blot using anti-acetyl-lysine antibody.

RESULTS

Mutational Effect of Key Residues on the Autoacetylation Activity of MOF—Autoacetylation in the MYST HATs has received increased attention recently, but the mechanism and function of the autoacetylation are not well understood. To better define the biochemical mechanism of MYST autoacetylation and the impact of autoacetylation on the cognate substrate acetylation, we produced MOF mutants with point mutations at several key residues in the active site of the enzyme. In the crystal structure, Lys-274 was found to be nearly stoichiometrically acetylated; thus we mutated this residue to other residues, e.g. Arg, Gln, Ala, Met, and Cys. All the proteins were purified with the nickel-affinity chromatography. With the availability of the purified wild-type and mutant MOF proteins, we first measured the autoacetylation activity by reacting each protein with [14C]AcCoA. As shown in Fig. 1A, WT-MOF and mutant MOF exhibited varied levels of autoacetylation activity. K274R mutant almost completely lost autoacetylation activity, which is consistent with previous reports (26, 27). However, several other MOF mutants still maintained significant amounts of autoacetylation activity. Intriguingly, MOF-K274M
Effect of Autoacetylation in MOF Catalysis

TABLE 1
MS analysis of lysine acetylation in WT-MOF protein
Quantitation of lysine acetylation was achieved by spectral counting, in which the number of times a peptide is isolated and fragmented was counted during the analysis.

| Site no. | Confidence level | Approximate no. of spectra observed | No. of acetylated spectra | Approximate % acetylated |
|---------|-----------------|-------------------------------------|--------------------------|--------------------------|
| 274     | High confidence | 25                                  | 25                       | 100                      |
| 175     | High confidence | 50                                  | 8                        | 16                       |
| 177     | Possible        | 22                                  | 3                        | 14                       |
| 143     | Possible        | 45                                  | 3                        | 8                        |
| 154     | Possible        | 100                                 | 1                        | 1                        |
| 449     | Possible        | 100                                 | 1                        | <1                       |
| 141     | Possible        | 300                                 | 1                        | <1                       |
| 454     | Possible        | 300                                 | 1                        | <1                       |
| 457     | Possible        | 300                                 | 1                        | <1                       |

and MOF-K274C showed significantly high autoacetylation activity. These data suggest that MOF autoacetylation is very sensitive to chemical changes of the side chain of Lys-274. The fact that strong autoacetylation activity was seen in certain MOF-K274 mutants is a clear indication that there are additional acetylated lysine residues in MOF other than Lys-274. Indeed, from tandem MS analyses, in addition to Lys-274 acetylation, we found that several other lysine residues in MOF were acetylated as well, including Lys-125, Lys-143, Lys-145, Lys-154, Lys-168, Lys-175, Lys-177, Lys-410 Lys-449, Lys-454, and Lys-457 (Table 1 and supplemental Tables S1 and S2). In addition to Lys-274, in the active site of MOF, Cys-316 and Glu-350 are two prominent residues likely essential for the enzyme activity, because of their proximity to the acetyl group of the cofactor. Thus, we produced MOF with mutation at these two residues as well (e.g. C316A, C316S, and E350Q). Radioactive assays were conducted on the autoacetylation of MOF-C316 and MOF-E350 mutants. The results showed that MOF enzymes with mutation at Cys-316 or Glu-350 exhibited decreased autoacetylation activity (Fig. 1B), highlighting that these two residues play roles in MOF autoacetylation.

In Cellulo Autoacetylation of MOF Examined by Western Blot—Interpretation of the MOF autoacetylation data from the radiometric assays is complicated because significant amounts of autoacetylation have already been introduced in the protein overexpression step in E. coli. Direct measurement of the autoacetylation level of the purified proteins may more precisely reflect the autoacetylation capability of each MOF mutant. In this regard, we examined lysine acetylation in MOF and MOF mutants by using Western blot with a pan anti-acetyl-lysine antibody (ST1027, Calbiochem). As expected, strong acetylation was observed in WT-MOF (Fig. 2). MOF-K274R mutant had no autoacetylation observed, consistent with the radioactive assay result. However, significant and varied levels of autoacetylation were seen in the other Lys-274 mutants, e.g. K274M, K274A, and K274C, which further support that MOF contains additional lysine autoacetylation sites. Again, autoacetylation of MOF-C316A/C316S and MOF-E350Q mutants decreased in comparison with WT-MOF but was not abolished. These results suggest that Cys-316 and Glu-350 regulate the autoacetylation activity of MOF, but their function is dispensable.

HAT Activity of MOF Mutants—To provide insights into how autoacetylation correlates with or regulates the cognate substrate acetylation activity of MOF, we carried out HAT reactions for each MOF protein in the presence of [14C]AcCoA and H4-20 peptide. As shown in Fig. 3, all the tested MOF mutants showed impaired HAT activity compared with WT-MOF. In particular, the enzymatic activity of K274R mutant was completely abolished, highlighting that replacement of Lys-274 with arginine is lethal to the enzyme. With the exception of MOF-K274R, other Lys-274 mutants still retained certain HAT activity. Interestingly, mutation of Cys-316 and Glu-350 decreased but did not abolish the HAT activity of MOF.

MOF Can Be Deacetylated by Sirt1—Although the mutational studies offered valuable information about Lys-274 autoacetylation, caution should be taken for interpreting the functional correlation of Lys-274 acetylation to the intrinsic enzymatic activity of MOF. In particular, the introduced Lys-
to-Arg mutation might not strictly mimic the unacetylated lysine residue, and the Lys-to-Gln mutation might not well mimic the acetylated lysine. If this happens, the observed impact of Lys-274 mutation on autoacetylation activity and cognate histone acetylation activity of MOF could be an artificial effect. To more rigorously calibrate the function of MOF autoacetylation, we set out to produce deacetylated MOF by using Sirt1, a NAD\(^+\)/H\(_{1001}\)-dependent nonspecific lysine deacetylase (28, 29). In this experiment, MOF was treated with recombinant Sirt1 in the presence of 7.5 mM NAD\(^+\). The reaction mixture was then subjected to size-exclusion chromatography on Superdex 75. From Fig. 4A, it is seen that MOF was well separated from Sirt1. The efficiency of MOF deacetylation was checked by Western blot analysis and was found to be complete (Fig. 4B).

**Autoacetylation of MOF Slightly Modulates HAT Activity**—The deacetylated MOF provides a mechanistic tool to evaluate the biochemical effect of lysine autoacetylation in MOF. We measured the cognate substrate acetylation activity of the deacetylated MOF (abbreviated as MOF\(^{\text{deac}}\)) and compared it with nondeacetylated MOF (i.e. without treatment with Sirt1). The acetylation reaction was carried out with 10 \(\mu\text{M}\) \(^{14}\text{C}\)AcCoA, 200 \(\mu\text{M}\) H4-20 peptide, and 20 nM enzyme. As depicted in Fig. 4C, MOF\(^{\text{deac}}\) exhibited a slightly decreased substrate acetylation activity in comparison with MOF. These data gave strong evidence that acetylation of Lys-274 does not significantly alter MOF activity, and deacetylation at this lysine residue does not abrogate MOF activity. To corroborate this result in more detail, we measured MOF activity at different concentrations of one substrate while fixing the other. As shown in Fig. 5, both MOF and MOF\(^{\text{deac}}\) showed Michaelis-Menten-type hyperbolic curves. In comparison with MOF, MOF\(^{\text{deac}}\) showed decreased but still substantial amounts of activity at any selected concentrations of AcCoA and H4-20. By fitting the data to the Michaelis-Menten equation, we found that both apparent \(k_{\text{cat}}\) and apparent \(K_m\) values of MOF\(^{\text{deac}}\) modestly differ from that of MOF (Table 2), which suggests that acetylation of Lys-274 slightly affects the substrate binding affinity as well as the turnover rate of MOF catalysis. Similar results were found in Tip60, another MYST protein (30).

Because there is a possibility that the deacetylated MOF might be re-acetylated during the process of substrate acetylation, we determined the autoacetylation rates of the deacetylated MOF at different time points under the same experimental condition. As shown in Fig. 6, MOF\(^{\text{deac}}\) was re-acetylated over time in the presence of \(^{14}\text{C}\)AcCoA, and the autoacetylation rate of MOF\(^{\text{deac}}\) was determined from the initial linear time region to be 0.017 min\(^{-1}\). Such an autoacetylation rate is much slower than the substrate acetylation rates (8.55 min\(^{-1}\), Table 2). To further validate these results, we directly compared the autoacetylation and substrate acetylation activity on the

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**FIGURE 3. HAT activity of MOF proteins.** The HAT activity of MOF proteins was tested using the radioactive assay. 10 \(\mu\text{M}\) \(^{14}\text{C}\)AcCoA was incubated with 200 \(\mu\text{M}\) H4-20 at 30 °C for 5 min, and the reaction was initiated by addition of 0.1 \(\mu\text{M}\) HATs.

**FIGURE 4. Deacetylation of MOF and comparison of HAT activity.** A, FPLC profile for separation of deacetylated MOF from reaction mixture (peak 1 represents Sirt1; peak 2 represents MOF protein; peak 3 represents NAD\(^+\)). B, Western blot detection of deacetylation efficiency using anti-acetyl-lysine antibody. Protein loading amount is 2.7 \(\mu\text{g}\). C, comparison of HAT activity between deacetylated MOF (MOF\(^{\text{deac}}\)) and nondeacetylated MOF.
same autoradiographic gel. It is clear that although strong substrate acetylation was observed, less than 2% MOF-deac was autoacetylated (Fig. 7 and Table 3). Thus, the majority of MOF-deac was still in the deacetylated form during the catalysis, and autoacetylation of the enzyme was not kinetically competent in comparison with substrate acetylation.

MOF Autoacetylation Occurs through an Intramolecular Process—Autoacetylation of MOF may undergo either an intramolecular or intermolecular mechanism. This can be determined by examining the kinetic relationship between autoacetylation rate and enzyme concentration. An intramolecular autoacetylation should follow a first-order rate law, and an intermolecular autoacetylation will comply with second-order kinetics. Thus, we measured the initial autoacetylation rates of different concentrations of MOF-deac at a fixed concentration of AcCoA (20 μM). Because K274M mutant exhibited strong autoacetylation, we also studied the autoacetylation of deacetylated MOF-K274M versus enzyme concentration. The deacetylated MOF-K274M mutant was obtained by following the same Sirt1/NAD⁺ treatment procedure described above (Fig. 8, A and B). The initial autoacetylation velocity of MOF-deac versus enzyme concentration was plotted in the log-log format, which gave rise to a linear line with a slope of 0.86 ± 0.13 (Fig. 8C), in agreement with a first-order reaction mechanism. The apparent autoacetylation rate was calculated to be 0.005 ± 0.0002 min⁻¹. For the deacetylated MOF-K274M mutant, the logarithmic plot of initial autoacetylation rates with respect to enzyme concentration also showed a linear relation with a slope of 0.74 ± 0.01 (Fig. 8D), consistent with a first-order reaction mechanism. The apparent autoacetylation rate of MOF-K274M mutant was calculated to be 0.045 ± 0.01 min⁻¹. These results support that autoacetylation likely is a unimolecular process. This conclusion is supported by several other experimental observations. For instance, in the size-exclusion chromatography of MOF, no dimeric or oligomeric state of MOF was observed (see an example in Fig. 4A). Along with, when MOF was mixed with the catalytically inactive MOF-K274R protein in the presence of [¹⁴C]AcCoA, no intermolecular acetylation was observed (Fig. 8E).

| TABLE 2  | Steady-state kinetic parameters of MOF and deacetylated MOF |
|----------|------------------------------------------------------------|
| Enzyme   | Substrate        | appKₐ   | appKᵦ  | appkₐKᵦ       |
|          |                  | (µM)    | (µM)   | (µM⁻¹min⁻¹)   |
| MOF      | H4–20           | 37.75 ± 0.63 | 788.8 ± 27.4 | 0.048 ± 0.0002 |
|          | AcCoA           | 14.28 ± 0.45  | 0.55 ± 0.07  | 25.96 ± 3.45    |
| MOFdeac  | H4–20           | 22.20 ± 0.79  | 897.7 ± 63.9 | 0.025 ± 0.0002  |
|          | AcCoA           | 8.55 ± 0.40   | 0.47 ± 0.09  | 18.19 ± 3.73    |

DISCUSSION

Autoacetylation of the active site lysine in the MYST HATs (i.e., Lys-274 in MOF) has elicited great interest of study recently. One of the most appealing facts is that, in the crystal structures of several MYST proteins (23–27), this conserved lysine residue was found to be almost stoichiometrically in the acetylated form. However, because recombinant MOF protein is predominantly in the acetylated form after overexpression and purification from E. coli, further quantitative analysis of MOF autoacetylation activity is a technical challenge. Because of its unique position in the enzyme structure, it has been speculated that acetylation of this active site lysine residue is essential for MYST activity and might regulate enzyme-substrate interaction (23, 26). This proposition is most supported by the mutagenesis studies showing that mutation of Lys-274 to Arg or Ala almost completely abolishes MYST’s cognate H4 acetylation activity (Fig. 3) (25, 27). Although these data are highly valuable, a potential pitfall for the mutagenesis approach is that arginine and glutamine are only moderately mimicked substitutes for the unacetylated lysine and the acetylated lysine, respectively (31, 32). Because the Lys-274 residue resides near the head group of AcCoA, a chemical change to this residue may alter the active site microenvironment, thus compromising the catalytic activity of MYST proteins. Indeed, our data showed that all the tested Lys-274 mutants have impaired activity compared with WT-MOF (Figs. 2 and 3). Therefore, a method independent from the mutagenesis method would be warranted to more authentically address the function of MYST autoacetylation. In this study, we were able to produce MOF in the completely deacetylated form by treatment with Sirt1/NAD⁺. The high efficiency of Sirt1 in deacetylating MYST proteins such as MOF and Tip60 was also confirmed by several
other recent studies (30, 33, 34). Enzymatic analysis with the deacetylated MOF showed that, in the deacetylated state, the cognate substrate acetylation activity of MOF decreased only slightly, less than 1-fold (e.g. Figs. 4C and 5). Therefore, acetylation of the active site lysine has a marginal effect on the enzymatic activity of the MYST HATs. Changes within a 1-fold range were observed in the apparent $k_{cat}$, $K_m$, and $V/K$ values, suggesting that Lys-274 acetylation may subtly modulate substrate binding and the chemical turnover rates, thereby affecting substrate specificity. Nevertheless, the observed strong activity of deacetylated MOF approximate to that of MOF suggests that Lys-274 acetylation regulates MOF activity but is unlikely a major determining factor obligatory for the cognate enzymatic activity of MOF. Structurally, acetylated Lys-274 (K274Ac) is located on a flexible/H9251/H9252/H9253 loop, and the side chain points inward to the active site (Fig. 9). The side chain of unacetylated Lys-274 (form b) switches outward about 90° from the active site. Notably, the side chain of the adjacent residue His-273 in these two enzyme forms almost superimposes each other (Fig. 9) (23). However, mutation of Lys-274 to arginine changes the loop conformation into a helix structure. Now the side chain of Arg-274 points toward the bulky solvent, which is quite divergent from the side chain of the acetylated Lys-274. His-273

| Enzyme            | MOF | MOF$^{\text{deac}}$ |
|-------------------|-----|--------------------|
| $[^{14}\text{C}]\text{AcCoA}$ | +   | +                  |
| H4-20             | -   | +                  |

**TABLE 3**

Rate comparison of MOF autoacetylation with substrate acetylation

| Rate $(\mu\text{M}\text{min}^{-1})$ | MOF $^{\text{deac}}$ | MOF$^{\text{deac}}$ |
|-------------------------------------|-----------------------|-----------------------|
| 1                                  | 0.07 ± 0.01           | 0.01 ± 0.003          |
| 2                                  | 0.032 ± 0.002         | 0.080 ± 0.004         |
| 3                                  | 0.002 ± 0.000         | 0.030 ± 0.008         |
| 4                                  | 0.004 ± 0.000         | 0.080 ± 0.008         |

**FIGURE 6.** Autoacetylation activity of MOF and MOF$^{\text{deac}}$. Time-dependent autoacetylation was performed at different time points at 30 °C. 2 μM MOF or MOF$^{\text{deac}}$ was added to the $[^{14}\text{C}]\text{AcCoA}$ (20 μM) mixture to initiate the reaction. Samples were resolved on the 12% SDS-PAGE. A and B, time-dependent curves for autoacetylation of MOF and MOF$^{\text{deac}}$, respectively. C, radioactive gel for the acetylated protein band. D, initial rates of autoacetylation of MOF and MOF$^{\text{deac}}$.

**FIGURE 7.** Rate comparison of MOF autoacetylation with substrate acetylation. Acetylation assay was initiated by adding 2 μM of MOF enzyme to the mixture of 20 μM $[^{14}\text{C}]\text{AcCoA}$ in the absence or presence of 400 μM H4-20. Samples were resolved on the 16% peptide gel.
in the MOF-K274R mutant moves inward; this orientation may block the active site (27). Likely, the dramatic conformational change of the loop region and the orientation of His-273 in MOF-K274R is the major reason for the enzyme activity loss of this mutant. Deacetylation of Lys-274 only induces negligible differences to the loop and the spatial orientation of His-273,

FIGURE 8. Deacetylation of MOF-K274M and intramolecular reaction of autoacylation. A, FPLC profile for separation of deacetylated MOF-K274M from reaction mixture (peak 1 represents Sirt1; peak 2 represents MOF-K274M protein; peak 3 represents NAD+). B, Western blot detection of deacetylation efficiency using anti-acetyl-lysine antibody. C and D, logarithmic plot of concentration-dependent re-autoacylation of deacetylated MOF and deacetylated MOF-K274M, respectively. The autoacylation rate was measured as a function of protein concentration ranging from 1.25 to 10 μM. [14C]AcCoA concentration was fixed at 20 μM. E, acetylation of MOF-K274R by WT-MOF. Acetylation reactions were carried out in the presence of 20 μM [14C]AcCoA. Final protein concentration was 5 μM each.

FIGURE 9. Comparison of the active site of WT-MOF (Protein Data Bank code 3TOA) with that of MOF-K274R mutant (Protein Data Bank code 2PQ8). (The letter in front of residue represents a different form of MOF. a, Lys-274 in the acetylated form; b, Lys-274 in the unacetylated form; c, K274R mutant.)
leaving the active site still in the open state. Taken together, the structural analysis explains why K274R cannot be regarded as a mimetic substitute for the deacetylated Lys-274.

From our concentration-dependent kinetic assays, auto-acetylation of MOF seems to be a unimolecular process. This conclusion, however, needs to be cautioned because the MOF construct we used does not contain the chromodomain. It will be reasonable to investigate whether and how the chromodomain affects the kinetics of MOF autoacetylation. Compared with the cognate histone acetylation, the rate of MOF autoacetylation was relatively slow. Nevertheless, the MS studies and crystal structural data show that Lys-274 is acetylated in high abundance, almost stoichiometrically (Table 1 and supplemental Table S2). We cannot exclude the possibility that, in the cell, other endogenous lysine acetyltransferases may acetylate this lysine as well. While our biochemical assays showed that deacetylated MOF still remained as strong enzymatic activities as the acetylated MOF, it might be possible that, in the cell, the autoacetylation event may regulate other functionalities of the enzyme, such as protein stability and protein–protein interaction.

During the manuscript submission process, we noticed that Seto and co-workers (34) also obtained deacetylated MOF by treatment with Sirt1/NAD⁺. Interestingly, their studies showed that deacetylation of MOF reduces the cellular protein stability of MOF. It is known that the MYST HATs frequently exist as multiprotein complexes (25, 35–37). The presence of interactive proteins could provide biochemical cues regulating and altering the degree of MYST autoacetylation. Reciprocally, autoacetylation may affect the interaction of a MYST protein with its interacting partners. It remains intriguing to investigate how MYST autoacetylation is utilized by the cell to regulate the diverse biological pathways in which MYST proteins are involved, such as transcriptional regulation, DNA damage response, apoptosis, and cell cycles.

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