Acetyl-lysine Analog Peptides as Mechanistic Probes of Protein Deacetylases

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Class III histone deacetylases (Sir2 or sirtuins) catalyze the NAD⁺-dependent conversion of acetyl-lysine residues to nicotinamide, 2'-O-acetyl-ADP-ribose (OADDPr), and deacetylated lysine. Class I and II HDACs utilize a different deacetylation mechanism, utilizing an active site zinc to direct hydrolysis of acetyl-lysine residues to lysine and acetate. Here, using ten acetyl-lysine analog peptides, we have probed the substrate binding pockets of sirtuins and investigated the catalytic differences among sirtuins and class I and II deacetylases. For the sirtuin Hst2, acetyl-lysine analog peptide binding correlated with the hydrophobic substituent parameter \( \pi \) with a slope of -0.35 from a plot of log \( K_d \) versus \( \pi \). Interestingly, propionyl- and butyryl-lysine peptides were found to bind tighter to Hst2 compared with acetyl-lysine peptide and showed measurable rates of catalysis with Hst2, Sirt1, Sirt2, and Sirt3, suggesting propionyl- and butyryl-lysine proteins may be sirtuin substrates in vivo. Unique among the acetyl-lysine analog peptides examined, homocitrulline peptide produced ADP-ribose instead of the corresponding OADDPr analog. The electron-withdrawing nature of each acetyl analog had a profound impact on the deacylation rate between deacetylase classes. The rate of catalysis with the acetyl-lysine analog peptides varied over five orders of magnitude with the class III deacetylase Hst2, revealing a linear free energy relationship with a slope of -1.57 when plotted versus the Taft constant, \( \sigma^* \). HDAC8, a class I deacetylase, displayed the opposite trend with a slope of +0.79. These results are applicable toward the development of selective substrates and other mechanistic probes of protein deacetylases.

Histone deacetylases (HDACs)² remove the acetyl moiety from the ε-amino group of protein lysine residues. HDACs are separated into four main classes: class I (e.g. human HDAC 1–3, 8); class II (e.g. human HDAC 4–7, 9–10); class III (e.g. yeast Sir2, Hst1–4; human Sirt1–7); and class IV (HDAC11) which is homologous to both class I and II (1). The Sir2 or sirtuin family of deacetylases constitute class III HDACs, catalyzing the NAD⁺-dependent conversion of acetyl-lysine residues to nicotinamide, deacetylated-lysine, and 2'-O-acetyl-ADP-ribose (OADDPr) (2, 3). Class I, II, and IV HDACs possess a considerably different deacetylation mechanism, utilizing an active site zinc to direct hydrolysis of acetyl-lysine residues to deacetylated-lysine and free acetate (4).

Although these enzymes are referred to as histone deacetylases, they also deacetylate non-histone substrates. For example, the human homolog Sirt1 is reported to catalyze deacetylation of PGC-1α (5, 6), FOXO proteins (7–9), PPARγ (10), AceCS1 (11), NF-κB (12), p53 (13–15), and many other substrates implicating sirtuins in a variety of cellular processes including glucose homeostasis and stress resistance. Sirt2 is localized primarily to the cytoplasm where it deacetylates α-tubulin (16). Sirt3, Sirt4, and Sirt5 are located in the mitochondrial matrix (17–19), where Sirt3 deacetylates AceCS2 (11, 20) and Sirt4 ADP-ribosylates and inhibits glutamate dehydrogenase (21). Sirt6 and Sirt7 are found in the nucleus (17), where Sirt6 is reported to possess ADP-ribosyltransferase activity (22) and Sirt7 may regulate cellular growth and metabolism (23).

HDACs are targets for therapeutic intervention in a variety of human diseases. Aberrant expression of class I and II deacetylases is linked to malignancies in leukemias, lymphomas, and solid tumors (24). Class III deacetylases have been associated with pathways that oppose diseases associated with aging including obesity, type-II diabetes, and neurodegenerative disorders such as Alzheimer’s and Parkinson’s disease (25). Class I and II inhibitors, such as trichostatin A (TSA) and suberoylanilide hydroxamic acid (SAHA) that target the active site zinc of class I and II HDACs, are promising antitumor compounds. Indeed, SAHA recently received FDA approval to treat cutaneous T-cell lymphoma (26). However, TSA and SAHA are inactive against sirtuins. Rational design of selective HDAC inhibitors would be greatly aided by further understanding of inhibitor mechanisms and the differences in chemical mechanism between deacetylase classes. Sirtuins catalyze a sequential mechanism in which the acetyl-lysine substrate binds first followed by NAD⁺ to form the productive Michaelis complex (27). Chemical catalysis then occurs in two main steps: 1) initial attack of acetyl-lysine cleaving the nicotinamide ribosyl bond of NAD⁺ to form nicotinamide and an ADP-ribose-peptidyl intermediate (\( \alpha^-1'-O\)-alkylamidate) and 2) attack of the 2'-hydroxyl at the O-alkylamidate and subsequent addition of water to form OADDPr and deacetylated peptide (28). More recently, we have shown that the nucleophilicity of the acetyl-oxygen is...
directly tied to the rate of nicotinamide-ribosyl bond cleavage in the first chemical step (29).

Here, we utilize a series of ten acetyl-lysine analog peptides to probe the determinants for efficient binding and catalysis within the acetyl-lysine binding site of sirtuin deacetylases. Taking advantage of the unique chemical mechanisms and acetyl-lysine substrate binding sites between different deacetylase classes, we demonstrate the potential utility of these mechanistic probes in the development of selective substrates for class I/II/IV versus class III deacetylases.

**EXPERIMENTAL PROCEDURES**

**General Materials and Methods**—\(^{18}\)O-Labeled water was purchased from Cambridge Isotope Laboratories, Inc. (Andover, MA). Fmoc-\(\epsilon\)-homocitrulline was purchased from Advanced Synthetics (Millstadt, IL). All other chemicals used were of the highest purity commercially available and were purchased from ChemImpex (Wood Dale, IL), Sigma, Aldrich (Milwaukee, WI), or Fisher Scientific. The concentrations of enzyme were determined using the method of Bradford using bovine serum albumin as the standard (30). Enzyme aliquots were stored at \(-20^\circ\)C.

**Expression and Purification of Sirtuins**—Expression and purification of Hst2 (2, 31), Sirt1 (11, 32), Sirt2 (33), and Sirt3 (11) were performed as described previously.

**Expression and Purification of GST-tagged HDAC8**—The plasmid pGEX4T-3-HDAC8 (34), a generous gift from Edward Seto (H. Lee Moffitt Cancer Center and Research Institute; Tampa, FL), was used to express a glutathione transferase classes, we demonstrate the potential utility of these mechanistic probes in the development of selective substrates for class I/II/IV versus class III deacetylases.

**Acetyl-lysine Analogs as Mechanistic Probes of HDACs**

**Synthesis of Acetyl-lysine Analog Peptides**—The acetyl, propionyl, \(\alpha\)-hydroxycetyl, monofluoroacetyl, difluoroacetyl, thioacetyl, and trifluoroacetyl peptides with the sequence NH\(_2\)-KSTGGK(acetyl-analog)APRKQ-OH were synthesized according to previously published procedures (29). The homogarginine and homocitrulline analog peptides were synthesized similarly (29) using Fmoc-hArg(Pmc)-OH or Fmoc-hCitr-OH. The acetimidoyl-lysine and butyryl-lysine peptides were synthesized in a similar manner as previously published (29) utilizing Fmoc-Lys(ivDde)-OH. For these analogs, the full-length peptide was synthesized in a 0.1-mmol scale and then ivDde was orthogonally deprotected with 2% hydrazine in DMF. For acetimidoyl-lysine peptide, the liberated \(\epsilon\)-amino group was then reacted with ethylacetamide hydrochloride (38 mg, 0.3 mmol, 3 equiv) and triethylamine (61 \(\mu\)l, 0.44 mmol, 4.4 equiv) in 2.5 ml DMF for 1 h. For butyryl-lysine peptide, the liberated amine was reacted with butyric acid under standard peptide coupling conditions. The resin was then rinsed with dichloromethane and dried.

After completion of each synthesis, each peptide was cleaved, purified, and characterized as previously described (29). Homogarginine: MS (ESI): calculated for \(C_{49}H_{90}N_{19}O_{16}\) + [M+H]\(^{+}\) : 1199.7 found: 1199.6. Homocitrulline: MS (ESI): calculated for \(C_{49}H_{90}N_{19}O_{16}\) + [M+H]\(^{+}\) : 1200.7 found: 1200.9. Imidoyl-lysine: MS (ESI): Calcul’d for \(C_{49}H_{90}N_{19}O_{16}\) + [M+H]\(^{+}\) : 1198.7 found: 1198.7. Butyryl-lysine: MS (MALDI): calculated for \(C_{49}H_{90}N_{19}O_{16}\) + [M+H]\(^{+}\) : 1227.7 found: 1227.8.

**Determination of Catalytic Turnover Rate of Acetyl-lysine Analog Peptides with Sirtuin Homologs**—An HPLC-based assay that measures either the rate of \([14C]\)nicotinamide formation from \([14C]\)NAD\(^{+}\) or the rate of OAADPr analog formation at 260 nm was employed as previously described (36). The quenched timepoints were then separated on a C18 column (Grace Vydac, Deerfield, IL; 90 Å, 10 \(\mu\)m) using a gradient of 0 to 100% acetonitrile (with 0.02% v/v trifluoroacetic acid) to a final concentration of 1% v/v.

**Determination of Products Formed by Homocitrulline and Butyryl-lysine Peptides by Mass Spectrometry**—Reactions done in 20 \(\mu\)l containing 1 mM DTT, 400 \(\mu\)M homocitrulline, or acetimidoyl-lysine peptides in Hst2 were determined as described previously (29) using a MicroCal VP-ITC instrument. Reaction products were identified using a Micromass Quattro Ultima. Reactions done in 20 \(\mu\)l containing 1 mM DTT, 400 \(\mu\)M homocitrulline, or butyryl-lysine peptides in Hst2 were determined as described previously (29).
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dine buffer adjusted to pH 7 with formic acid were reacted for 30 min at room temperature. Reactions were flash-frozen and stored at −20 °C until ready for mass spectral analysis as previously described (37).

Nicotinamide Exchange Reactions with Homocitrulline Peptide—Exchange reactions for were performed in 80 µl containing 500 µM NAD⁺, 300 µM homocitrulline peptide, 1 mM DTT, 1 µM Hst2, and [14C]nicotinamide concentrations ranging from 25 µM to 1.6 mM in 50 mM Tris-Cl, pH 7.5 at 25 °C. Reactions were initiated by enzyme addition, incubated at 25 °C for 5 min, quenched with trifluoroacetic acid to a final concentration of 1% v/v, and analyzed as previously described (29, 31, 36). The plot of exchange rates versus nicotinamide concentration was fitted to the Michaelis-Menten equation (Equation 1) using KaleidaGraph (Synergy Software, Reading, PA).

Solvent Isotope Effects with Homocitrulline Peptide—Solvent isotope effects were determined as previously described.3 Reactions contained 1 mM DTT, 325 µM [14C]NAD⁺, 650 µM homocitrulline peptide, 50 mM Tris, pH 7.5 at 25 °C, and 2–6 µM Hst2. Four µl of Hst2 (in H₂O) to a final volume of 160 µl were added to initiate reactions.

18O-labeling of Homocitrulline Peptide Reaction—18O-labeling experiments were done as previously described for the Hst2 H135A mutant enzyme (37). 60-µl reactions containing 1 mM DTT, 200 µM homocitrulline peptide, 200 µM NAD⁺, 10 µM Hst2, in natural abundance water or 85% 18OH₂, and 20 mM pyridine buffer adjusted to pH 7 with formic acid were used.

Characterization of Methyl-ADPr From Hst2 Catalyzed Methanalysis of NAD⁺ using Homocitrulline Peptide—A 1-ml reaction containing 1 mM homocitrulline peptide, 2 mM NAD⁺, 1 mM DTT, 100 µM Hst2, 5 mM methanol, and 50 mM Tris-HCl, pH 7.5, was reacted for 2 h at 25 °C and quenched with trifluoroacetic acid to a final concentration of 1% v/v. The 1’-O-methyl-ADPr formed was purified and analyzed by 1H NMR as previously described for the Hst2 H135A mutant enzyme (37).

Determination of Overall Turnover Rate with HDAC8—The reactions were performed with 300–600 µM acetyl-lysine or propionyl-lysine peptide, 300–9600 µM homocitrulline, monofluoroacetyl-lysine, difluoroacetyl-lysine, trifluoroacetyl-lysine, or ω-hydroxyacetyl-lysine peptide. Reactions containing the desired analog peptide in buffer (25 mM Tris, pH 7.5 at 37 °C, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 1 mg/ml bovine serum albumin) were initiated by addition of GST- HDAC8 (0.5 μM for difluoroacetyl and trifluoroacetyl, 1 μM for monofluoroacetyl, propionyl, and acetyl, 2 μM for homocitrulline and ω-hydroxyacetyl). Reactions were quenched by addition of aqueous HCl and acetic acid to a final concentration of 250 mM and 40 mM, respectively. Time points of between 4 and 60 min were chosen such that less than 20% conversion was maintained in all reactions. The quenched time points were then separated by RP-HPLC (Grace Vy dac, Deerfield, IL; C18 column, 90 Å, 10 µm, 4.6 × 200 mm) by running H₂O (with 0.05% v/v trifluoroacetic acid) for 5 min followed by a gradient of 0–15% acetonitrile (with 0.02% v/v trifluoroacetic acid) over 30 min at a flow rate of 0.5 ml/min. Under these conditions, deacetylated peptide eluted at ~21 min and the acetyl analog peptides eluted from ~24 to ~30 min. Product conversion was determined through comparison of deacetylated peptide and acetyl analog peak areas detected at 214 nm. Saturating peptide conditions were not obtained for the α-hydroxy, monofluoro, difluoro, and trifluoroacetyl analogs, therefore Vmax values reported for these analogs are from fits of the initial rates at varying peptide concentrations to the Michaelis-Menten equation (Equation 1) using KaleidaGraph (Synergy Software, Reading, PA).

Fitting of Log Kd to Physiochemical Parameters—Log Kd values of the acetyl-lysine analog peptides were fit versus the inductive Taft constant (σ*), the volume of the acetyl group in Å³, and the hydrophobicity parameter π. The σ* values used were from the literature (38). The volumes were calculated for the acetaldehyde analogs using the JME molecular editor (Molinspiration Property Calculation Service). The π values for the acetyl group (-C(=O)CH₃) or corresponding analog (-C(X)Y) were either reported in the literature (38) or calculated from the corresponding acetyl analog substituted benzenes using Equation 2 where log P_H is the log P for benzene. MarvinSketch (version 4.1.6, 2007, ChemAxon) was used for prediction and calculation of πX values in Equation 2.

RESULTS

Binding Affinity of Acetyl-lysine Analog Peptides Correlates with Hydrophobicity—A series of ten acetyl-lysine analogs were incorporated into a 11-mer peptide based on the human histone H3 sequence modified at Lys-14 (NH₂-KSTGGK(acetyl-analog)APRKQ-OH) (Fig. 1). These analogs vary in the nature...
of the nucleophile, the electron-withdrawing potential, size, charge at neutral pH, and hydrophobicity of the substituents. To probe the determinants for acetyl-lysine binding to Sir2 deacetylases, the binding constant ($K_d$) for each peptide was measured with the archetypal Sir2 homolog from yeast, Hst2, using isothermal titration calorimetry (Fig. 2). The $K_d$ values for these peptides varied more than 75-fold, ranging from $3.3 \pm 0.7 \text{ M}$ to $260 \pm 4 \text{ M}$. Utilizing linear free energy relationships between binding affinity and either the electron-withdrawing nature ($\sigma^*$) (38), hydrophobicity ($\pi$) (38), or size of the acetyl analog (volume Å$^3$), we analyzed the correlation of the log $K_d$ values to each of these parameters. The hydrophobicity parameter $\pi$, which is a measure of the relative hydrophobicity of each substituent distinct from electronic and steric effects, yielded a strong linear correlation to log $K_d$ with a slope of $-0.33 \pm 0.06$ and an $R^2$-value of 0.77 (Fig. 3A). In contrast, the log $K_d$ values did not correlate well with the electron-withdrawing nature or the size of the substituents (Fig. 3, B and C), as revealed by the slopes of $-0.16 \pm 0.15$ and $-0.026 \pm 0.015$ and $R^2$-values of 0.18 and 0.24 for these fits, respectively. In the fit of log $K_d$ values versus volume, removal of butyryl-lysine peptide, a possible outlier, provided a marginally better fit with a slope of $-0.055 \pm 0.022$ and an $R^2$-value of 0.44.

**Modeling Acetyl Analogs into the Hst2 Active Site**—The correlation of binding affinity with hydrophobicity led us to model trifluoroacetyl-lysine and thioacetyl-lysine into the active site of Hst2 (Fig. 4). For modeling, the 1.5-Å resolution structure of acetyl-lysine peptide and ADP-ribose bound to Hst2 (39) was used replacing the bound acetyl-lysine with the appropriate acetyl-lysine analog using Sybyl (ver. 7.3). This modeling gave a clear rationalization for the observed binding results. In particular, the hydrophobic trifluoromethyl group of trifluoroacetyl-lysine peptide filled empty space not occupied by acetyl-lysine in the hydrophobic pocket defined by Phe-67, Ile-117, and Ile-181 resulting in an increase in binding affinity compared with acetyl-lysine peptide ($3.3 \pm 0.7$ versus $21 \pm 4 \text{ M}$) (29). The modeling also placed the hydrophobic thioacetyl group within the hydrophobic pocket defined by Phe-184 and Val-228, consistent with the lower $K_d$ value of $4.7 \pm 1.0 \text{ M}$ displayed by thioacetyl-lysine peptide. Importantly, all of these residues are conserved among sirtuin homologs, suggesting the trends presented here are applicable to other sirtuins.

The surprising ability of Hst2 to bind both the propionyl-lysine and butyryl-lysine peptides ($K_d$ values of $8.6 \pm 0.2 \text{ M}$ (29) and $16 \pm 3 \text{ M}$) with affinity greater than that of acetyl-lysine peptide ($21 \pm 4 \text{ M}$) (29) led us to model their binding within the Hst2 active site. The Hst2 structure can readily accommodate the extra volume of the propionyl group with only minimal rearrangement of the neighboring Phe-67, Ile-117, and Ile-181 after energy minimization (r.m.s.d. of 0.14 Å). This result was similar to a previous modeling study of a *Thermatoga maritime* sirtuin, suggesting that propionyl-lysine binding is conserved among sirtuins (40). However, modeling indicated significant...
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FIGURE 4. Models of acetyl-lysine analogs bound in the Hst2 active site. Space-filling models of (A) acetyl-lysine, (B) trifluoroacetyl-lysine, (C) thioacetyl-lysine, (D) propionyl-lysine, and (E) butyryl-lysine docked into the Hst2 active site were based on the structure of acetyl-lysine and ADPr bound to Hst2 (PDB code 1SZD) (39). The enzyme surface shown is prior to energy minimization with the orange surface representing hydrophobic residues. F, sticks representation of the energy-minimized Hst2 active site following docking of acetyl-lysine (orange), trifluoroacetyl-lysine (cyan), thioacetyl-lysine (yellow), propionyl-lysine (purple), and butyryl-lysine (blue). Modeling was performed by replacing the acetyl group in the crystal structure with the appropriate acetyl analog using Sybyl (ver. 7.3), hydrogens were added, and the structure was energy-minimized using the Powell gradient with the Tripos force field and Gasteiger-Huckel charges.

FIGURE 5. A, relative reactivity of acetyl-lysine (black), propionyl-lysine (dark gray), and butyryl-lysine (light gray) peptides with Sirt1, Sirt2, Sirt3, and Hst2. Activity was measured using an HPLC-based assay that determines the rate of [14C]nicotinamide formation from [14C]NAD+. The reactions were performed with 50–1600 μM [14C]NAD+, 325–1200 μM acetyl-lysine, propionyl-lysine, or butyryl-lysine peptide, 1 mM DTT, and Hst2, Sirt1, Sirt2, or Sirt3 in 50 mM Tris-Cl pH 7.5 at 25 °C. Time points were chosen such that steady-state initial velocities were maintained in all reactions. Error bars represent standard deviations. B, NAD+ saturation curves with the human Sir2 homolog, Sirt2. At saturating acetyl-lysine (circles), propionyl-lysine (triangles), or butyryl-lysine peptide (squares), [14C]NAD+ was varied from 50 to 1600 μM in 50 mM Tris-Cl pH 7.5 at 25 °C with 1 mM DTT. Data were fit to the Michaelis-Menten equation using Kaleidagraph to determine kinetic constants. Time points were chosen such that steady-state initial velocities were maintained in all reactions.

Acetyl-lysine analogs were chosen such that steady-state initial velocities were maintained in all reactions. Time points were fit to the Michaelis-Menten equation using Kaleidagraph to determine kinetic constants. Actinomycin D and propargylglycine (ActD and Parg, respectively) were added at 0.4 μM and 0.2 mM, respectively, to test Hst2 and the human homologs Sir2, Sirt2, and Sirt3 for depropionylase and debutyrylase activity. We have previously shown that Hst2 catalyzes the formation of 2′-O-propionyl-ADPr (OPADPr) with a rate of 0.17 s⁻¹ compared with 0.2 s⁻¹ for acetyl-lysine peptide (29). Recently, Garrity et al. (40) revealed that a S. enterica propionyl-CoA synthetase enzyme can be propionylated in vivo, and can be depropionylated with the Sir2 homolog, CobB. Here, all the sirtuin homologs tested were able to catalyze the formation of OPADPr or 2′-O-butyryl-ADPr (OBADPr) with varying efficiency (Fig. 5A). The formation of OBADPr was verified for Hst2, Sirt1, Sirt2, and Sirt3 catalyzed debutyrylation by ESI-MS as revealed by a major peak at 628.2 to 628.4 m/z for these Sir2 homologs (calculated for C₁₅H₂₆N₅O₁₅P₂ - [M-H]: 628.1 m/z; Supplemental Fig. S9). We and others (29, 40) have previously confirmed the formation of OPADPr by HPLC and mass spectral methods utilizing propionyl-lysine peptides or proteins as sirtuin substrates. In all cases, sirtuin deacetylase activity decreased with increasing chain length with depropionylase and debutyrylase activity ≥ 28% and ≥ 2% that of deacetylation. Rates measured by liberation of [14C]nicotinamide from [14C]NAD⁺ were nearly identical to the rates measured from integration of the peak areas from HPLC separation of NAD⁺ and the OPADPr or OBADPr, indicating that all the cleaved NAD⁺ was converted to OPADPr or OBADPr.

To further characterize the reactions catalyzed using propionyl- and butyryl-lysine peptides, the Kₗₜₜ for NAD⁺ was determined utilizing Sirt2 as the representative sirtuin and compared with acetyl-lysine peptide. Monitoring the steady-state...
rate of $[14C]nicotinamide$ formation, we measured NAD$^+$ $K_m$ values of $239 \pm 15$, $358 \pm 39$, and $133 \pm 16 \mu M$ under saturating acetyl-, propionyl-, and butyryl-lysine peptide, respectively (Fig. 5B).

Homocitrulline Forms ADPr—Thus far we have shown that propionyl-, butyryl-, $\alpha$-hydroxycetyl-, monofluoroacetyl-, or thioacetyl-lysine peptides are all converted to their corresponding 2'-O-acetyl-analog-ADPr products (29). However, when utilizing homocitrulline peptide, NAD$^+$ was converted exclusively into ADPr as revealed by the major MS peak at 558.1 $m/z$ (Supplemental Fig. S9) consistent with a previous report by Khan and Lewis (41) (Fig. 6). The unusual ADPr formation with the homocitrulline peptide warranted further mechanistic analysis.

ADPr Is Formed from Hydrolysis of the O-Alkylisourea Intermediate with Homocitrulline Peptide—We hypothesized that the formation of ADPr with the homocitrulline peptide was due to one of two possibilities: hydrolysis of the corresponding 2'-O-carbamoyl-ADPr product or hydrolysis of the O-alkylisourea intermediate during catalysis. To distinguish between these two possibilities, we performed reactions in $^{18}$OH$_2$ with Hst2, NAD$^+$, and homocitrulline peptide. If ADPr resulted from hydrolysis of the 2'-O-carbamoyl-ADPr product, then the ADPr formed should contain no $^{18}$O-label, whereas if ADPr resulted from hydrolysis of the O-alkylisourea intermediate, then the $^{18}$O-label from water might be transferred to ADPr (37). Indeed, the Hst2-labeling reaction revealed the incorporation of one $^{18}$O label into ADPr as seen by the major ESI-MS peak at 560.0 $m/z$ (Supplemental Fig. S10). Furthermore, the transferred $^{18}$O label could be exchanged when a lyophilized aliquot of the original reaction was redissolved in 10% formic acid in natural abundance water, as shown by the major peak at 558.1 $m/z$ (Supplemental Fig. S10). This exchange indicated that the $^{18}$O-label was located at the 1'-position because only the 1'-hydroxyl can exchange with bulk solvent (37).

If homocitrulline peptide were stalled at the corresponding $\alpha$-1'-O-alkylisourea intermediate, then reaction in the presence of methanol should produce $\beta$-1'-O-methyl-ADPr through a double-displacement mechanism. By $^1$H-NMR characterization of the purified $\beta$-1'-O-methyl-ADPr from Hst2 catalyzed methanlysis (data not shown) the stereochemistry was assigned as $\beta$ by comparison with previously published spectra of $\beta$-1'-O-methyl-ADPr (42). The formation of exclusively $\beta$-1'-O-methyl-ADPr is identical to what we have previously shown for the mutant enzyme Hst2 H135A (37). Therefore, the ADPr formed was due to an altered enzymatic pathway in which the O-alkylisourea was hydrolyzed to ADPr, regenerating the homocitrulline peptide. This reaction essentially converts Hst2 into a NAD$^+$ glycohydrolase utilizing the homocitrulline peptide as an essential cofactor (Fig. 6).

Homocitrulline Peptide Displays Rapid Nicotinamide Formation but Slow Overall Turnover Rates—to determine the individual rate constants in the mechanism of ADPr formation with homocitrulline peptide, the rate of nicotinamide formation was measured using a rapid-quench flow apparatus. These single turnover reactions yielded a first-order nicotinamide formation rate of 1.9 $s^{-1}$ for homocitrulline peptide (Fig. 7A) compared with 6.7 $s^{-1}$ for acetyl-lysine peptide (29). At saturating homocitrulline peptide and NAD$^+$ concentrations, the turnover rate ($k_{cat}$) was $(1.2 \pm 0.4) \times 10^{-2} s^{-1}$ for homoc-
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Homocitrulline Peptide Solvent Isotope Effect—The rapid nicotinamide exchange reaction at varying concentrations of acetyl-lysine (squares) or homocitrulline (circles) peptide. Exchange reactions contained 500 μM NAD⁺, 300 μM acetyl-lysine analog peptide, 1 mM DTT, 1 μM Hst2, and 25–1600 μM [¹⁴C]nicotinamide in 50 mM Tris, pH 7.5 at 25 °C. Exchange rates were determined as outlined under “Experimental Procedures.” Error bars represent standard deviations. Points for acetyl-lysine have been published previously (29).

DISCUSSION

Acetyl-lysine Analog Peptide Binding to Sirtuins Correlates with Hydrophobicity—To effectively design probes that bind the acetyl-lysine binding site within sirtuins, we determined the parameter(s) that effect acetyl-lysine analog binding. The strongest binding (lowest Kd) was observed for the most hydrophobic analogs, thioacetyl and trifluoroacetyl-lysine, whereas the weakest binding was observed for the most hydrophilic analogs, homoarginine and imidoyl-lysine that are positively charged at neutral pH. Therefore, the hydrophobicity of the analogs gave the best correlation to binding compared with electronic and steric effects. However, steric effects become important with butyryl-lysine peptide as this peptide has increased hydrophobicity compared with propionyl-lysine peptide, but exhibited weaker binding to free Hst2 (Kd value of 16 versus 6.7 μM). This suggests that the propionyl group is the greatest volume that will fit without requiring significant rearrangement of the acetyl-lysine binding pocket. Molecular modeling supports this hypothesis as significant steric clashes were observed for the butyryl-lysine peptide, but no significant structural rearrangements of the enzyme were necessary to accommodate the propionyl-lysine peptide. These observations have important implications for the design of inhibitors,
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$$K_m = \frac{k_{cat}(k_4 + k_5)}{k_5 k_s} \quad \text{(Eq. 3)}$$

where $k_s$ and $k_5$ are the association and dissociation rate of NAD$^+$ to the sirtuin:peptide complex. This suggests two competing effects are expressed in the $K_m$ value: 1) poorer binding of NAD$^+$ (increase in $k_s$ or decrease in $k_5$) to the sirtuin:peptide complex increases the $K_m$ value and 2) slower reaction of the ternary complex to form nicotinamide and the O-alkylamidate (decrease in $k_5$) which decreases the $K_m$ value. This suggests that the higher $K_m$ with propionyl-lysine compared with acetyl-lysine peptide is due to initial NAD$^+$ binding being adversely affected without significantly affecting the reaction to form nicotinamide. However, the lower $K_m$ with butryryl-lysine peptide suggests that reaction to form nicotinamide is more adversely affected compared with NAD$^+$ binding.

Mechanism of ADPr Formation by Homocitrulline Peptide and Implications for ADP-ribosylation—Distinct among the acetyl-lysine analog peptides, homocitrulline peptide did not convert NAD$^+$ to the corresponding 2'-O-carbamoyl-ADPr analog product. Instead, homocitrulline peptide formed exclusively ADPr through water attack at the β-face of the corresponding α-1'-O-alkylisourea thereby converting Hst2 into a NAD$^+$ glycohydrolase (Fig. 6). The formation of the O-alkylisourea occurs rapidly at 1.9 s$^{-1}$ for homocitrulline peptide (compared with 6.7 s$^{-1}$ for acetyl-lysine peptide), but the overall turnover with homocitrulline peptide was much slower than acetyl-lysine peptide (0.012 versus 0.2 s$^{-1}$). Therefore, the attack of the 2'-hydroxyl is prohibitively slow with the homocitrulline peptide, which is likely due to decreased electrophilicity of the carbonyl carbon through extra π-electron donation from the additional NH$_2$ group in the O-alkylisourea intermediate (Fig. 6) (41). This results in stalling at the O-alkylisourea, allowing sufficient time for hydrolysis to yield ADPr as the dominant reaction. In the nicotinamide exchange reaction, the lower apparent $K_m$ for nicotinamide with homocitrulline versus acetyl-lysine peptide (139 versus 406 μM) is consistent with the homocitrulline peptide being stalled at the O-alkylisourea.$^4$

Several studies have suggested that some Sir2 homologs possess protein ADP-riboyltransferase activity (22, 49–52). However, substrates, or other biophysical probes that bind within the acetyl-lysine binding site.

Propionyl- and Butyryl-lysine Peptides Are Sirtuin Substrates—A recent proteomics study by Chen et al. (46) revealed that propionyl- and butyryl-lysine are bona fide histone modifications. In addition, the authors showed that two acetyltransferases, p300 and CREB-binding protein, catalyze lysine propionylation and lysine butyrylation in vitro. Berndsen et al. (47) have also shown that the histone acetyltransferase Esa1 is able to efficiently catalyze lysine propionylation. More recently, Garrity et al. (40) have observed propionylation of propionyl-CoA synthetase by several acetyltransferases. Here, among the sirtuins tested, Hst2 exhibited the greatest depropionylase activity compared with deacetylase activity. Interestingly, Hst2 also exhibited the slowest debutrylase activity compared with deacetylase activity suggesting a more sterically-limited active site within Hst2 compared with the human homologs Sirt1–3. In addition, the activity of Hst2 most closely mimicked that of its closest human homolog, Sirt2.

To examine if propionyl- or butyryl-lysine peptide negatively affected NAD$^+$ binding or alternatively cleavage of the nicotinamide-ribosyl bond, the $K_m$ values were measured by varying NAD$^+$ at saturating peptide. For acetyl-, propionyl-, and butyryl-lysine peptide with Sirt2, we determined $K_m$ values of 239, 358, and 133 μM, respectively. Using the net rate constant method of Cleland (48), the $K_m$ value of NAD$^+$ can be expressed in terms of the individual rate constants in Equation 3.

$\text{FIGURE 8. Taft plot of the log acetyl-lysine analog deacylation rate (k_{cat} s^{-1}) for HDAC8 (squares, solid line), and Hst2 (circles, dashed line) versus the inductive Taft constant } \sigma^*(38). \text{ HDAC8 reactions were performed with 325 μM [14C]NAD$, 325–650 μM acetyl-lysine analog peptide, 1 mM DTT, and Hst2 in 50 mM Tris-Cl, pH 7.5 at 25 °C. Reactions were initiated by addition of 0.5–6 μM GST-HDAC8. Time points were chosen such that initial rate conditions were maintained in all reactions. Error bars represent standard deviations.}$

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$\text{K_m = \frac{k_{cat}(k_4 + k_5)}{k_5 k_s} \quad \text{(Eq. 3)}}$

$\text{When nicotinamide formation (k_5) is the rate-limiting step (i.e. k_{cat} = k_5) this formula reduces to Equation 4,}$

$\text{K_m = \frac{k_4 + k_5}{k_5}} \quad \text{(Eq. 4)}$

$\text{where k_s and k_5 are the association and dissociation rate of NAD^+ to the sirtuin:peptide complex.}$

$\text{The stalling of the O-alkylisourea with homocitrulline peptide can be explained kinetically by expression of the apparent K_m for nicotinamide exchange in terms of individual rate constants using the net rate constant method of Cleland (48) in Equation 5.}$

$\text{K_m = \frac{k_4}{k_5 k_s} E_i \quad \text{(Eq. 5)}}$

$\text{In this case, the nicotinamide formation rate (k_5) is slower with homocitrulline compared to acetyl-lysine peptide and the rates of nicotinamide dissociation (k_s) and association (k_5) are predicted to be similar for both acetyl-lysine and homocitrulline peptide.}$

$\text{Therefore, in order for homocitrulline peptide to display a lower K_m for nicotinamide exchange the steady-state level of the O-alkylisourea (E_i) must be greater than that of the O-alkylamidate.}$
Acetyl-lysine Analogs as Mechanistic Probes of HDACs

ever, sirtuin catalyzed ADP-ribosylation activity has been difficult to characterize as it has not yet been demonstrated to be catalytic. The results presented in this study provide some additional insight toward potential mechanisms of ADP-ribosylation. In particular, the formation of ADPr or β-1′-O-methyl-ADPr with the homocitrulline peptide suggests that other nucleophiles could attack the stalled O-alkylisourea intermediate, as was recently hypothesized (53). If this nucleophile were a protein side-chain, then mono-ADP-ribosylation would result. Recently, it was shown that protein ADP-ribosylation mediated by Sir2 homologs that possess deacetylase activity requires the presence of an acetyl-lysine peptide/protein substrate.5 How-ever, the human homologs Sirt4 and Sirt6 do not display deacylation activity, but are suggested to possess ADP-ribosyl-transferase activity in the absence of acetyl-lysine substrates (21, 22). In these cases, it is possible that a protein sidechain such as Asn or Gln could fulfill the role of acetyl-lysine in the formation of the high energy O-alkylamidate-like intermediate. This intermediate could then accept another nucleophile from a protein sidechain (e.g. Lys, Arg, Cys, Ser, or Thr) to catalyze ADP-ribosylation. Homocitrulline peptide could be a valuable probe to distinguish between potential ADP-ribosylation mechanisms due to its ability to stall at the O-alkylisourea intermediate, not proceed to deacylation, and accept alternative nucleophiles. In particular, substitution of homocitrulline for acetyl-lysine in a peptide or protein might be expected to increase ADP-ribosylation in a mechanism that reacts with the O-alkylisourea (or O-alkylamidate) intermediate, but decrease ADP-ribosylation in a mechanism that reacts with the OADPr (or ADPr) product.

Selective Substrates for Different Deacetylase Classes—The differing ability of each acetyl-lysine analog peptide to participate in sirtuin catalyzed deacylation led us to examine their activity with other deacetylase classes. We hypothesized that differences in mechanism between deacetylase classes would result in large differences in deacylation efficiency. In particular, greater electron-withdrawing substituents (larger σ* values, e.g. trifluoroacetyl) should increase the electrophilicity of the carbonyl carbon, the site of water attack in class I, II, and IV deacetylases, thereby increasing their deacylation rate (k_cat). The greater electron-withdrawing substituents would also decrease the nucleophilicity of the acetyl carbonyl oxygen, the nucleophile for attack of NAD^+ in Sir2 deacetylases (28, 29), thereby decreasing sirtuin deacetylase rate as the electron-withdrawing nature is increased. Indeed, we observed a striking difference between deacetylase classes as seen in the Taft plots with complete opposite slopes of +0.79 for HDAC8 versus –1.57 for Hst2. Most remarkably, the trifluoroacetyl analog yielded a 300,000-fold rate difference between the two enzyme classes, whereas acetyl-lysine displayed only a 7-fold change in rate (Fig. 8). For comparison with Sir2 deacetylases, Taft free-energy analyses have been performed with the distantly related β-N-acetylglucosamidases, which utilize anchimeric assistance of the 2′-acetamide in glycoside hydrolysis. For this family of enzymes, Taft-plot slopes of –0.4 to –1.6 were measured for several homologs (35, 55–58). For comparison with class I and II deacetylases, free energy analysis has been carried out in the related chitin deacetylase, which hydrolyzes N-acetyl-glucosa-mine monomers to glucosamine and acetate within chitin polymers in a metal-dependent manner. In that case, log k_cat was plotted versus an alternative Hammett constant σ_1 with a large positive slope of +1.7 (54).

In summary, this work provides a starting point to develop chemical tools for molecular investigations of protein deacylases. Future work will utilize the mechanistic differences between protein deacetylase classes as well as peptide sequence selectivity to formulate class selective deacetylase substrates and mechanistic probes. For example, substitution of an analog for acetyl-lysine within existing fluorescent deacetylase substrates would provide a fluorescent readout for the activity of a particular subset of HDACs. The correlation of binding with hydrophobicity lays the groundwork for the development of fluorescence polarization (FP) probes. These FP probes will be useful for high-throughput screening of compounds that bind to the acetyl-lysine binding site of sirtuins as well as elucidating the activity of sirtuin homologs that have diminished deacylation activity but may retain the ability to bind acetyl-lysine peptides or proteins. Furthermore, attachment of a fluorophore and photolabeling agent to a high affinity acetyl-lysine analog peptide would allow photolabeling and identification of sirtuin-associated proteins. Therefore, the results presented here are critical steps toward elucidating the roles of protein deacylases in a variety of human disease states such as cancer, diabetes, and neurodegeneration.

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