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Sir2 Deacetylases Exhibit Nucleophilic Participation of Acetyl-Lysine in NAD^+ Cleavage

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Sir2 protein deacetylases (or sirtuins) play a critical role in a variety of biological processes including glucose homeostasis, lifespan extension, apoptosis, and neurodegeneration, suggesting that sirtuins may be targets for treatment of diabetes, aging, cancer, and neurodegenerative diseases.[1] Sirtuins couple the deacetylation of lysine residues with conversion of NAD^+ to *O*-acetyl-ADP-ribose (*O*AADPr) and nicotinamide.

A major unresolved portion of the chemical mechanism is the initial catalytic step resulting in formation of the *O*-alkylamidate intermediate (Scheme 1). One possible mechanism is the concerted attack of acetyl-lysine at the 1′-carbon of the nicotinamide ribose, displacing nicotinamide in a direct-displacement S_N2 (A_ND_N) reaction. Another possibility is that the acetyl group is not chemically involved in nicotinamide cleavage, but instead serves to position NAD^+ in a destabilizing conformation that allows nicotinamide cleavage to occur in an S_N1 ($D_N + A_N$) mechanism, forming a distinct oxocarbenium intermediate (Scheme 1). Subsequent attack of acetyl-lysine on the oxocarbenium would yield the *O*-alkylamidate intermediate.[2] The actual mechanism of NAD^+ cleavage lies on a continuum between these two possibilities. Subsequently, the 2′-hydroxyl is activated by an active-site histidine to attack the *O*-alkylamidate, forming a 1′,2′-cyclic intermediate. Addition of water yields deacetylated peptide and *O*AADPr (Scheme 1).[1] Here, we present evidence that the nucleophilicity of the acetyl-oxygen is directly tied to the rate of nicotinamide−ribosyl bond cleavage, consistent with an S_N2-like mechanism.

Other NAD^+-consuming enzymes such as ADP-ribosyltransferases and NAD^+ hydrolases are postulated to proceed through oxocarbenium intermediates in which the incoming nucleophile is minimally involved in nicotinamide formation (bond order of the nucleophile is between 0 and 0.11 where kinetic isotope effects are known).[3] With the Sir2 reaction, an acetylated lysine substrate is required to break the nicotinamide−ribosyl bond,[4] suggesting that the acetyl-lysine residue might be chemically involved in nicotinamide−ribosyl bond cleavage. To determine how acetyl-lysine is involved, we measured the rate of nicotinamide formation under single-turnover conditions (Figure 1A) for six acetyl-lysine analogues that differ greatly in the electron-withdrawing nature of the substituents but are similar in steric size (Scheme 1). A rapid quenching approach was used so that the rate of chemical cleavage, independent of the physical dissociation of nicotinamide from the active site, could be determined. All acetyl-lysine analogues were incorporated into 11-mer peptides based on the N-terminal tail of histone H3 acetylated at lysine-14 (H_2N-KSTGGK(acetyl-analogue)APRKQ-OH). If an S_N2-like mechanism were operative, the rate of nicotinamide formation would be directly tied to the nucleophilicity of the acetyl group. With an S_N1 mechanism, attack of acetyl-lysine occurs after nicotinamide formation and therefore the nucleophilicity of the amide oxygen would not greatly affect the rate of nicotinamide cleavage from NAD^+.

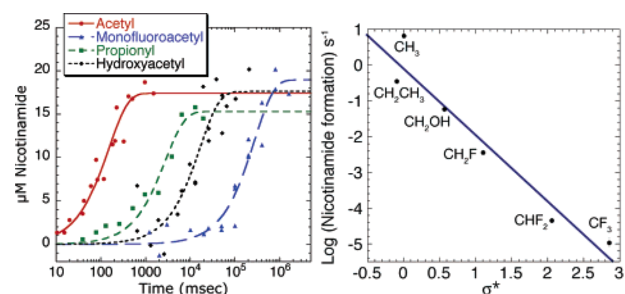

Figure 1. (Left) Single-turnover kinetics of acetyl-lysine analogues monitoring nicotinamide formation. (Right) Plot of the log rate of nicotinamide formation versus the Taft σ^* constant.

Among the acetyl-lysine analogues, there was a dramatic dependence of rate (>5 orders of magnitude) on the electron-withdrawing potential of the substitution. The log of the rate of nicotinamide formation was plotted versus the inductive Taft constant, σ^*,[5] revealing a linear free energy relationship with a steep negative slope of $\rho^* = -1.9$ (Figure 1B). Notably, all analogues in this study were converted to the corresponding *O*-acetyl-ADP-ribose analogues, without significant formation of ADP-ribose[6] (Supporting Information Table 1). Where the steady-state turnover rate k_{cat} could be measured, the first-order rate of nicotinamide formation was similar to that of the k_{cat} values, indicating that the rate-limiting step was altered from product release[2] to nucleophilic attack of the carbonyl oxygen on NAD^+ (Table 1).

To ensure that the rate of nicotinamide formation was not reflective of diminished binding of the acetyl-lysine analogues in the ground state, we measured the dissociation constant (K_d) of each analogue to free enzyme, Hst2, by isothermal titration calorimetry (Table 1, Supporting Information Figure 1). All acetyl-lysine analogues exhibited K_d values that were as low or lower than that of acetyl-lysine, with the exception of the α-hydroxyacetyl analogue. Thus, the binding differences (<30-fold change among analogues) cannot account for the ~600 000-fold range in nicotinamide formation rates observed. Instead, the negative slope likely reflects decreased nucleophilicity of the acetyl-oxygen as the electron-withdrawing nature of the substituents was increased. This is best exemplified with the trifluoroacetyl analogue that yielded the slowest rate of nicotinamide formation but displayed the tightest binding to the enzyme.

Few precedented examples exist where an amide oxygen acts as a nucleophile in an enzyme-catalyzed reaction. In only one distantly related case has Taft free-energy analysis been performed. With *β-N*-acetylglucosamidases, which utilize anchimeric assistance of the 2′-acetamide for glycoside hydrolysis, ρ^* values ranging from −0.4 to −1.6 were determined for several homologues.[7] These ρ^* values provided evidence for direct nucleophilic participation of the amide carbonyl oxygen in attack at the anomeric position. The large ρ^* value of −1.9 reported here suggests that Sir2 deacetylases

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Scheme 1. Possible Chemical Mechanisms for Initial Acetyl-Lysine Analogue Attack and Complete Deacetylation Mechanism

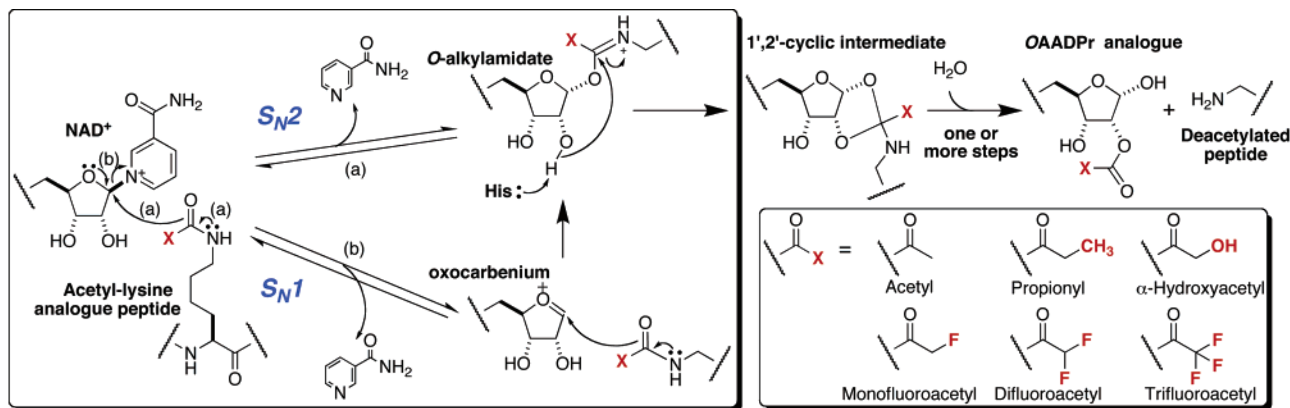

Table 1. Physical and Kinetic Parameters of Acetyl-Lysine Analogues

analogue	K_d (μM)	nicotinamide formation (s^{-1})	k_{cat} (s^{-1})
acetyl	21 ± 4	$(6.7 \pm 0.9) \times 10^{0}$	$(2.0 \pm 0.3) \times 10^{-1}$
propionyl	8.6 ± 0.2	$(3.6 \pm 0.7) \times 10^{-1}$	$(1.7 \pm 0.3) \times 10^{-1}$
α-hydroxyacetyl	90 ± 50	$(6.0 \pm 1.5) \times 10^{-2}$	$(6.6 \pm 0.6) \times 10^{-2}$
monofluoroacetyl	22 ± 5	$(3.7 \pm 0.6) \times 10^{-3}$	$(3.3 \pm 0.5) \times 10^{-3}$
difluoroacetyl	20 ± 1	$(4.6 \pm 0.8) \times 10^{-5}$	not determined
trifluoroacetyl	3.3 ± 0.7	$(1.1 \pm 0.4) \times 10^{-5}$	not determined

utilize similar nucleophilic participation of the amide carbonyl in ribosyl−nicotinamide cleavage, consistent with an S_N2-like mechanism.

We also examined the ability of the acetyl-lysine analogues to support Sir2-catalyzed transglycosidation (nicotinamide exchange) in which exogenously added nicotinamide reacts with an intermediate to re-form NAD^+ in the presence of an acetyl-lysine substrate.[4,8] With acetyl-lysine analogues that bind tightly but are weak nucleophiles (e.g., fluorinated analogues), efficient transglycosidation might be predicted in an S_N1 mechanism as discrete oxocarbenium formation would be independent of the attacking nucleophile. Measurement of the transglycosidation rate with the acetyl, monofluoroacetyl, and trifluoroacetyl analogues revealed facile exchange with the acetyl substrate ($V_{max} = 2.9 \pm 0.2$ s^{-1}; $K_m = 406 \pm 70$ μM) but >400-fold slower exchange rates with the fluorinated analogues (Supporting Information Figure 2). These extremely low exchange rates argue against formation of a stable oxocarbenium but instead support an S_N2-like mechanism where nicotinamide captures the *O*-alkylamidate to efficiently reverse the reaction. We cannot rule out the possibility that the steady-state oxocarbenium level in an S_N1 mechanism is vanishingly low with the fluorinated analogues because of a fast subsequent catalytic step. However, this is unlikely as the next catalytic step would involve the attack of acetyl analogues on the oxocarbenium, a competing reaction with respect to nicotinamide exchange. Thus, nicotinamide exchange would be expected to be faster with the less nucleophilic fluorinated analogues, opposite to what we observe.

An S_N2-like mechanism for Sir2 deacetylases is unusual, as many studies of enzymatic nicotinamide−ribosyl bond cleavage postulate formation of an oxocarbenium intermediate.[3,9] Our results suggest that Sir2 deacetylases use a NAD^+-consuming reaction whereby nicotinamide−ribosyl bond cleavage involves considerable participation of the incoming nucleophile (the amide oxygen) to form an *O*-alkylamidate intermediate. These results have important implications on the selective inhibition of Sir2 over other NAD^+-metabolizing enzymes. Design and development of compounds that mimic the attack of acetyl-lysine may be potent and selective inhibitors of Sir2 deacetylases.

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Supporting Information Available: Experimental procedures, nicotinamide exchange data, representative isothermal titration calorimetry data, and mass spectral data. This material is available free of charge via the Internet at http://pubs.acs.org.

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