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Co-enzyme Specificity of Sir2 Protein Deacetylases: Implications for Physiological Regulation

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The abbreviations used are: SIR2, silent information regulator 2; NAD+, Nicotinamide Adenine Dinucleotide; NADH, reduced Nicotinamide Adenine Dinucleotide; OAADPr, O-Acetyl-ADP-ribose; NMN+, Nicotinamide Mononucleotide; NAMN+, Nicotinic Acid Mononucleotide; NAAD+, Nicotinic Acid Adenine Dinucleotide; NADP+, Nicotinamide Adenine Dinucleotide Phosphate; Thio-NAD+, Thionicotinamide Adenine Dinucleotide; 3-AcPAD, 3-Acetyl Pyridine Adenine Dinucleotide; 3-hPAD+, 3-Hydroxypyridine Adenine Dinucleotide; NGD+, Nicotinamide Guanine Dinucleotide; NHD+, Nicotinamide Hypoxanthine Dinucleotide.

Keywords: SIR2, Deacetylation, Inhibition, NAD, Histone

Running title: Co-enzyme Specificity of Sir2 Protein Deacetylases
Summary

Sir2 (silent information regulator 2) enzymes catalyze a unique protein deacetylation reaction that requires the coenzyme NAD$^+$ and produces nicotinamide and a newly discovered metabolite, O-acetyl-ADP-ribose (OAADPr). Conserved from bacteria to humans, these proteins are implicated in the control of gene silencing, metabolism, apoptosis and aging. Here we examine the role of NAD$^+$ metabolites/derivatives and salvage pathway intermediates as activators, inhibitors, or co-enzyme substrates of Sir2 enzymes \textit{in vitro}. Also, we probe the co-enzyme binding site using inhibitor binding studies and alternative co-enzyme derivatives as substrates. Sir2 enzymes show an exquisite selectivity for the nicotinamide base co-enzyme, with the most dramatic losses in binding affinity/reactivity result from relatively minor changes in the nicotinamide ring, either by reduction, as in NADH, or by converting the amide to its acid analogue. Both ends of the dinucleotide NAD$^+$ are shown to be critical for high selectivity and high affinity. Among the NAD$^+$ metabolites tested none were able to allosterically activate, although all led to various extents of inhibition, consistent with competition at the coenzyme binding site. Nicotinamide was the most potent inhibitor examined, suggesting that cellular nicotinamide levels would provide an effective small-molecule regulator of protein deacetylation and generation of OAADPr. The presented findings also suggest that changes in the physiological NAD$^+$:NADH ratio, without a change in NAD$^+$, would yield little alteration in Sir2 activity. That is, NADH is an extremely ineffective inhibitor of Sir2 enzymes (Ave. IC$_{50}$ of 17 mM). We propose that changes in both free nicotinamide and free NAD$^+$ afford the greatest contribution to cellular activity of Sir2 enzymes, but with nicotinamide having a more dramatic effect during smaller fluctuations in concentration.
INTRODUCTION

The Sir2 (silent information regulator 2) family (or Sirtuins) of protein deacetylases have emerged as important regulators of seemingly diverse cellular processes, such as gene silencing, apoptosis, metabolism and aging (1-6). These enzymes catalyze a unique reaction that requires the coenzyme NAD$^+$ and produces deacetylated product, nicotinamide and O-acetylated ADP-ribose (OAADPr) (7-11). Yeast Sir2 is required for gene silencing at the silent genetic loci and is thought to maintain a hypoacetylated chromatin state by localized histone deacetylation (1). The catalytic core domain is conserved from bacteria to humans, with 5 homologous genes in yeast and 7 in humans (12). Mammalian SIRT1 is localized in the nucleus and has been reported to regulate p53 (reviewed in (13)) and FOXO transcription factors (3,4), promoting survival under cell stress. Human SIRT2 is cytoplasmic and is reported to deacetylate $\alpha$-tubulin (14), whereas SIRT3 is localized to the matrix of mitochondria (15,16), though cellular targets have not been reported. Substrates and potential functions of the remaining mammalian homologs have not been demonstrated.

Sir2 has been linked to increased longevity in yeast (17) and C. elegans (18), and early reports suggest that SIRT1 and SIRT3 may play a similar role in mammals. The molecular basis for aging has not been established, however, it does appear that increased Sir2 activity is required. This has led to the investigation of regulatory mechanisms that might control Sir2 activity. Currently, there are two schools of thought: One that proposes the cellular NAD/NADH ratio is critical (19,20), while another suggests that free nicotinamide levels act to suppress Sir2 activity (21).

Recently, a natural product activator of Sir2 activity, resveratrol, was found to activate Sir2 both in vitro and in vivo (22). Resveratrol is found in red wine and has been
linked to the health benefits of consuming such drinks. Several other related compounds also appear to activate Sir2 in vitro, suggesting that not only might Sir2 be the functional target of resveratrol and similar compounds, but that the cell may regulate Sir2 activity through the binding of other naturally occurring allosteric regulators.

Several reports have implicated a nuclear NAD⁺ salvage pathway in yeast as a regulatory control point for ySir2 (23-26). This pathway (Figure 1) conserves the pyridine ring of nicotinic-acid/nicotinamide to regenerate β-NAD⁺, and is distinct from the β-NAD⁺ biosynthetic pathway. Anderson et al. have demonstrated that increased dosage of several salvage pathway genes in yeast results in increased gene silencing and life-span extension (24). Moreover, altering the levels of cellular nicotinamide, either genetically or by exogenous addition to yeast cells, decreased gene silencing and accelerated aging in a Sir2 dependent manner (25).

Here we examine the role of NAD⁺ metabolites/derivatives and salvage pathway intermediates as activators, inhibitors, or co-enzyme substrates of Sir2 enzymes using three distinct Sir2 homologs and a variety of in vitro assays. Also, we probe the co-enzyme binding site using inhibitor binding studies and alternative co-enzyme derivatives as substrates. The presented findings have important implications for the role of cellular NAD/NADH ratios and free nicotinamide concentrations in controlling Sir2 activity, as well as provide the mechanistic basis for co-enzyme specificity.
MATERIALS AND METHODS

Reagents. Synthetic 11-mer H3 peptide and acetylated H3 peptide (AcH3), corresponding to the 10 amino acid residues surrounding lysine-14 of histone H3; H2N-KSTGGK(Ac)APRKQ-CONH2, were purchased from SynPep Corporation (Dublin, CA), 3H-Labeled Acetyl H3-peptide (3H-AcH3) was prepared as previously reported (27). 3-hPAD+ was made enzymatically by utilizing the nicotinamide-exchange reaction of yeast HST2 and 3-hydroxypyridine as described previously (28). All other chemicals were of the highest purity commercially available, were purchased from Sigma (St. Louis, MO), Aldrich (Milwaukee, WI), or Fisher Scientific (Pittsburgh, PA), and were used without further purification.

Expression and Purification of His-Tagged HST2, hSIRT2, and ySIR2. The procedure used in the expression and purification of histidine-tagged full-length HST2 has been reported previously (27) with only minor changes and was applied to the purification of the SIR2 enzymes. Fractions containing protein greater than 90% purity, based upon densitometry, were pooled, concentrated, and dialyzed in 50 mM Tris (pH 7.5 @ 37 °C), 10% glycerol and 1 mM DTT. For yeast SIR2 (ySIR2), 800 mM NaCl was also included in the final dialysis. Enzyme concentrations were determined using the method of Bradford et al. (29).

Charcoal Binding Assays. Charcoal binding assays were performed as reported previously (28). Briefly, 70 μL reaction volumes containing 3H-AcH3 were quenched by adding to charcoal slurry (1 vol. activated charcoal to 2 vol. 2.0 M Glycine buffer, pH
Mixture was heated to 95 °C for one hour, and then centrifuged. The $^3$H-acetate is liberated from the product OAADPR under these conditions, and remains in the supernatent. The supernatent was collected and counted by liquid scintillation counting. Rates of deacetylation are obtained from the ratio of free $^3$H-acetate in the supernatent to total radioactivity in the reaction mixture.

Inhibition Reactions. Inhibition reactions were performed in 50 mM Tris-CI or 50 mM sodium phosphate buffer (pH 7.5 @ 37 °C) with 50 μM NAD$^+$, 225 μM $[^3]$H-acetylated H3 peptide, 1 mM DTT, 0.2–0.5 μM HST2, inhibitor concentrations from 5 μM–100 mM, in a total volume of 80 μL. Reactions were initiated by addition of enzyme, incubated at 37 °C from 0–6 minutes (within the linear range of product formation under steady-state conditions), and quenched with activated charcoal. Inhibition was determined by comparing the rate of product formation between inhibited and non-inhibited reactions. Equation 1 was used to fit initial velocities at various inhibitor concentrations (28). The resulting $K_{i,app}$ is reported as an IC$_{50}$ value; the $v_{app}$ and $v_{max}$ are represented by % deacetylase activity, where $v_{max}$ is 100% and $v_{app}$ is % activity at the given [I]. Reported values are the average of at least 3 independent experiments. Fits were obtained using Kaleidagraph (Synergy software, Reading PA).

$$v_{app} = v_{max}[1- ([I]/(K_{i,app} + [I])))$$

(Eq. 1)

Analysis of NAD$^+$ Analogues as Substrates for the Formation of OAADPr, or its Respective Analogue. NAD$^+$ analogues were used as an alternate substrate for NAD$^+$ in HST2-catalyzed deacetylase reactions. For saturation curves of NAD$^+$, NAAD$^+$, 3-
AcPAD\(^+\), NGD\(^+\), and NHD\(^+\), reactions were performed in 50 mM Tris-Cl or 50 mM sodium phosphate buffer (pH 7.5 @ 37 °C), with 129 - 300 µM \(^3\)H-acetylated H3 peptide, 1 mM DTT, 1–100 µM HST2, in a total volume of 80 µL. NAD\(^+\) analogue concentrations ranged from 150 µM to 14 mM. Reactions were initiated by addition of enzyme, incubated at 37 °C from 0–6 minutes (within the linear range of product formation under steady-state conditions), and quenched with activated charcoal. To obtain the kinetic parameters of Table 2, initial velocity data were fit to the Michaelis-Menten equation using non-linear least squares analysis (Kaleidagraph software), Equation 2. In the case of 3-hPAD\(^+\), the rate increased linearly with substrate concentration up to 6.2 mM. The data was fit to a line using linear least-squares regression, and the slope reported as the \(k\text{cat}/K_m\). Two NAD\(^+\) analogues, NADP\(^+\) and Thio-NAD\(^+\), were subjected to a bisubstrate kinetic analysis, where both [AcH3] and [NAD\(^+\) analogue] were varied. Peptide concentrations ranged from 2.2–50 µM for Thio-NAD\(^+\) assays, and 225 µM to 1 mM for NADP\(^+\) assays. [Thio-NAD\(^+\)] ranged from 50 µM to 1.5 mM, and [NADP\(^+\)] from 300 µM to 3 mM. Since it has been shown that SIR2 enzymes follow a sequential mechanism (30), the initial velocity data were fitted to the equation for a sequential mechanism, Equation 3, using KinetAssyst software (IntelliKinetics, State College, PA) and algorithms of Cleland (31). Reported values are the average of at least 3 independent experiments.

\[
v_o = \frac{(k\text{cat} [E_0] [S])}{(K_m + [S])} \quad (\text{Eq. 2})
\]

\[
v_o = \frac{(v_{\text{max}} \cdot [A] \cdot [B])}{((K_a \cdot K_b) + (K_{ma} \cdot [B]) + (K_{mb} \cdot [A]) + ([A] \cdot [B]))} \quad (\text{Eq. 3})
\]
RESULTS

Evaluation of Potential of NAD\(^+\)-derived regulators of Sir2 Enzymatic Activity.

Given the requirement for the co-enzyme NAD\(^+\) and the genetic evidence for NAD\(^+\) metabolites controlling Sir2 activity, we first investigated whether a variety of NAD\(^+\) metabolites could directly modulate these enzymes, either by direct competition with NAD\(^+\), allosteric binding, or as a substrate surrogate of NAD\(^+\). Although NADH and nicotinamide have been suggested to directly regulate Sir2 enzymes (19-21), a comprehensive and quantitative analysis including these and other NAD\(^+\) derivatives was lacking. We tested yeast NAD\(^+\) salvage pathway intermediates (nicotinamide, nicotinic acid, \([\beta]\)-NAMN, \([\beta]\)-NAAD (Fig 1)) and other NAD\(^+\) metabolites (\([\alpha]\)-NADH, \([\alpha]\)-NMN and \([\alpha]\)-NAD) as potential regulators of the in vitro Sir2 reaction (Table 1). The yeast Sir2 homolog HST2 was used as the primary test case, however ySir2 and human SIRT2 were also examined to confirm general trends observed with HST2. The rate of OAADPr product formation was monitored under steady-state conditions at varying concentrations of these potential regulators. Substrate concentrations were both fixed: 50 \(\mu\)M NAD\(^+\) (4-fold above \(K_m\)), and 225 \(\mu\)M AcH3 (saturating). Of the compounds examined, none stimulated the forward reaction (Table 1). Instead, all compounds inhibited the reaction to varying extents. Interestingly, nicotinamide was the most potent inhibitor (IC\(_{50}\) = 0.126 mM), while nicotinic acid (IC\(_{50}\) = 250 mM) was the worst inhibitor, displaying almost a 2000-fold difference in efficiency (Table 1 and Figure 2). It is notable that three of the worst inhibitors (IC\(_{50}\) ~ 26-250 mM) tested were the three acid analogues (\([\beta]\)-NAMN, \([\beta]\)-NAAD and nicotinic acid) of the nicotinamide amide. Although an IC\(_{50}\) of 250 mM for nicotinic acid was determined, the uncertainty for this value is likely greater as a
consequence of the lack of data points above the IC$_{50}$, which was experimentally impractical. Of the mononucleotides, NAMN$^+$ inhibited ~60-fold less effectively than its amide analogue, NMN$^+$. Of the three dinucleotides tested [γ-NAD$^+$ ranks the best (IC$_{50}$ = 1.08 mM), inhibiting about 10-fold less effectively than nicotinamide, but >20-fold better than either NADH or γ-NAAD$^+$. Given the poor inhibition of HST2 by NADH, and its proposed role in directly regulating ySir2, we examined NADH inhibition with ySir2 as well as human SIRT2 under identical assay conditions. NADH was a poor inhibitor of both human SIRT2 and ySir2, yielding IC$_{50}$ values of approximately 11 mM and 15 mM, respectively. These findings were quite similar to the IC$_{50}$ of 28 mM obtained with HST2, indicating that Sir2 enzymes bind NADH with extremely low affinity.

**Probing the Co-Enzyme Binding Site.**

To probe the co-enzyme binding site of Sir2 enzymes, we determined whether structurally diverse mono- and dinucleotides, including some NAD$^+$ salvage pathway intermediates, could support the deacetylase reaction when substituted for NAD$^+$. Ten different NAD$^+$ analogues were analyzed as co-enzyme substrates for HST2. Formation of OAADPr (or the respective analogue) was monitored, and when possible, the steady-state kinetic values of $k_{cat}$, $K_m$, and $k_{cat}/K_m$ were determined for each compound tested (Table 2). The values of $k_{cat}$ represent the maximal rate of the reaction, and the $K_m$ value represents the concentration of substrate at 1/2 the maximal velocity. The values of $k_{cat}/K_m$ are the apparent second-order rate constant for the reaction between free enzyme and free substrate and include all processes up to and including the first irreversible step in the overall reaction. Therefore, the $k_{cat}/K_m$ is a direct measure of an enzyme’s
specificity toward a given substrate, as it reflects both substrate binding and the first chemical step in the reaction, the cleavage of the nicotinamide–ribosyl bond. We and others have shown that nicotinamide release, under steady-state conditions, is the first irreversible step in the overall reaction \((28,30,32-34)\). The kinetic constants obtained with the various co-enzymes were determined from substrate saturation curves (Figure 3) and compared to those derived from an NAD\(^+\) saturation curve at high [Ac-H3 peptide].

As substrates, the two best NAD\(^+\) analogues tested were NADP\(^+\) and Thio-NAD\(^+\). The kinetic parameters for both analogues were determined using bi-substrate kinetic analysis where both [Ac-H3 peptide] and [co-enzyme] were varied. NADP\(^+\) exhibited a \(k_{cat}/K_m\) value 130-fold lower than that of NAD\(^+\), while yielding the same \(k_{cat}\) and an increased \(K_m\), suggesting that the lower \(k_{cat}/K_m\) is due to poor binding affinity for NADP\(^+\) (Table 2). Compared with NAD\(^+\), Thio-NAD\(^+\) exhibited a \(k_{cat}/K_m\) value 235-fold lower, with a 4-fold decrease in \(k_{cat}\), and a 60-fold increase in \(K_m\) (Table 2, Figure 4). Although small, the 4-fold change in \(k_{cat}\) may reflect a significantly decreased rate of the thio-nicotinamide-ribosyl bond cleavage, or slowed thio-nicotinamide release. Thus, the \(k_{cat}/K_m\) for Thio-NAD\(^+\) represents both lower binding affinity and a slower step at or before thio-nicotinamide release.

As a result of the extremely low activity, kinetic parameters for additional analogues were determined by constructing co-enzyme saturation curves at high, but constant [Ac-H3 peptide]. Of the two NAD\(^+\) salvage pathway intermediates, NAAD\(^+\) and NAMN\(^+\), only NAAD\(^+\) yielded measurable product with a \(k_{cat}/K_m\) nearly \(1.4 \times 10^6\)-fold lower than with NAD\(^+\). Both \(k_{cat}\) and \(K_m\) were affected, making NAAD\(^+\) the worst product-forming dinucleotide substrate tested (Table 2). NAMN\(^+\), along with its amide
analogue NMN⁺, yielded no measurable product (3 mM substrate, 100 μM HST2, 20 min.). As might be predicted with stereo-selective enzymes, △-NAD⁺ yielded no product (20 mM △-NAD⁺, 0.5 μM HST2, 6 min.) as the nicotinamide group is in the opposite orientation compared with △-NAD⁺. However, as an inhibitor, △-NAD⁺ bound with reasonable affinity (IC₅₀ = 1 mM). Four additional dinucleotide substrates were tested; two were NAD⁺ analogues with respect to the nicotinamide base, and two were NAD⁺ analogues with respect to the adenine base. Of the two nicotinamide base analogues, 3-AcPAD⁺ and 3-hPAD⁺, the former exhibited a $k_{cat}/K_m$ value nearly 1300-fold lower compared with NAD⁺, effecting both $k_{cat}$ and $K_m$ values (Table 2). The 3-hPAD⁺ substrate displayed a 6 x 10⁵-fold decrease in $k_{cat}/K_m$. Because the reactions with 3-AcPAD⁺ and 3-hPAD⁺ proceed in identical fashion to that of NAD⁺ after the base is released, the observed change in the kinetic constants represents a change in any or all of the following: initial co-enzyme binding, cleavage of the glycosidic bond, and/or nicotinamide analogue release. NGD⁺ and NHD⁺ displayed $k_{cat}/K_m$ values 1400- and 2400-fold lower than with NAD⁺ respectively; and both displayed $k_{cat}$ and $K_m$ changes. The significant decrease in $k_{cat}$ (10-60-fold) here implies that a chemical step and/or release of product is affected since the final product is an analogue of OAADPr. The dramatic decrease in $k_{cat}/K_m$ suggests that co-enzyme binding is weak and/or nicotinamide-ribosyl bond breakage is slowed.

**DISCUSSION**

*Implications for Regulation of Sir2 Enzymatic Activity by NAD⁺ metabolites.*

Over the last couple of years, there has been a growing debate on how Sir2 enzymes are
regulated within the cell. In yeast, several reports have implicated the NAD$^+$ salvage pathway (Figure 1) in regulating activity (23-25). De-repression of Sir2 inhibition by nicotinamide (21,25,28,32) and increased NAD$^+$ flux through the pathway have been proposed to activate Sir2. Others have argued that changes in the NAD$^+$ to NADH ratio either inhibit (decreased ratio) or increase (increased ratio) Sir2 activity (19,20). However, in several cases where Sir2 activity is required for the observed phenotypes, the level of NAD$^+$ and NADH show little change, and increased NAD$^+$ correlated with rapid aging (24). Here, we explored the potential regulation of Sir2 enzymes by NAD$^+$ salvage pathway intermediates (nicotinamide, nicotinic acid, NAMN$^+$, and NAAD$^+$) and well as by NADH and a variety of other NAD$^+$ metabolites. With the use of multiple mono- and di-nucleotide co-enzyme substrates, the specificity of the NAD$^+$ binding site was elucidated. Our results are discussed in the context of proposed Sir2 regulatory mechanisms and the structural evidence for co-enzyme binding.

Of all compounds tested, none were found to activate the enzymes. Instead, varying levels of inhibition were observed. Nicotinamide was by far the most potent inhibitory molecule tested. This inhibition results from the facile condensation with a high energy ADPr-like intermediate formed from the reaction with acetylated substrate and NAD$^+$; the reaction cannot proceed through deacetylation and the intermediate is forced back toward re-formation of substrates (28,34). Although other pyridine/nicotinamide analogues can act in this transglycosidation reaction, the observed inhibition of the forward reaction is orders of magnitude lower than compared with nicotinamide (28), suggesting an exquisitely specific nicotinamide binding site. Among the inhibitory compounds examined in this study, those possessing an altered
nicotinamide ring, such as NADH, NAAD, NAMN, and nicotinic acid, exhibited the poorest ability to inhibit. A second trend is also noteworthy. The potency of inhibition was dependent on the number of effective nucleoside binding sites, as seen between similar mono- and dinucleotides. Though the acid form of nicotinamide was the most detrimental factor for binding, removing the adenosine moiety in $\beta$-NAAD$^+$ to form $\beta$-NAMN$^+$ further reduced the binding affinity. In addition, $\beta$-NMN$^+$ and $\alpha$-NAD$^+$ have similar IC$ _{50} $ values, indicating that both ends of a dinucleotide are important for binding affinity. Given the disparity between $\alpha$-NAD$^+$ (IC$ _{50} $ = 1.08 mM) and $\beta$-NAAD$^+$ (IC$ _{50} $ = 26 mM), a negatively charged nicotinate in the beta configuration is more detrimental than nicotinamide in the alpha configuration. This observation can be explained by examining the available crystal structures, discussed below.

Perhaps the most notable finding was the poor binding seen with NADH. With an IC$ _{50} $ ranging from 11-28 mM for three different Sir2 enzymes (ySir2, yeast HST2 and human SIRT2), NADH was one of the least effective nucleotide inhibitors. These results have important implications for the proposed role of NAD$^+$ and NADH levels in controlling Sir2 activity. The K$ _m $ values for Sir2 enzymes generally fall between 10-100 µM NAD$^+$ ((30) and Table 2). The exception appears to be human SIRT1, where Howitz et al (22) have measured a significantly higher K$ _m $ of 560 µM. Our results indicate that the relative binding affinity between NAD$^+$ and NADH is approximately 1000-fold, favoring NAD$^+$. Recently, Lin et al. (19) report that caloric restriction in yeast leads to a 2-fold decrease in NADH (from 0.85 to 0.39 mM), without any significant change in NAD$^+$ (1.2 mM), compared to normal growth conditions. Anderson et al. demonstrate a similar lack of NAD$^+$ fluctuation during yeast caloric restriction; however, they report no
detectable change in NAD$^+$ levels (~4 mM), and estimate [NADH] to be below 0.2 mM (24). The disparity in results may reflect the difference in the method of measurement. From other reports, the ratio of NAD$^+$:NADH in yeast has been estimated to be >20 (refs in (35)). The NAD$^+$:NADH of a typical eukaryotic cell is estimated to be between 60-700 for the cytoplasm and 7 for the mitochondria (36). In muscle cells, intracellular concentrations of 0.54 and 0.05 mM for NAD$^+$ and NADH, respectively, have been reported (37). A recent report by Fulco et al. has suggested that Sir2 regulates skeletal muscle cell differentiation and that Sir2 activity is regulated by an ~4-fold reduction (from 125 to 29) in NAD$^+$:NADH as muscle cells differentiate (20).

Using data from our studies and those of others, we set out to calculate the magnitude change on the enzymatic activity of Sir2 when NAD$^+$/NADH is significantly altered. These estimates suggest that several fold changes in the NAD$^+$ to NADH ratio have only minor effects on the Sir2 deacetylation rate. For instance, if we assume an average $K_m$ of 50 $\mu$M for NAD$^+$ (which is reasonable given the estimates from various labs), an average $IC_{50}$ of 17 mM for NADH (reported here), and an initial NAD$^+$:NADH ratio of 10, then even a dramatic 10-fold reduction in the NAD$^+$:NADH ratio would change the rate of deacetylation by only ~0.2 % whether the intracellular [NAD$^+$] was 1 mM or as low as 100 $\mu$M. This effect becomes even smaller as the total concentration of co-enzymes drops. It is important to note that Zhang et al. (38) estimated cellular concentrations of free NADH at ~110 nM. Using only the data presented by Lin et al. (19), which proposes to link changes in [NADH] from caloric restriction to Sir2 function, we calculated that under caloric restriction ySir2 activity would increase by just 5 %.
The huge disparity between the NAD$^+$ $K_m$ values for Sir2 enzymes and the IC$_{50}$ values with NADH blunts the inhibitory effects when NADH levels rise. Accordingly, the changes in NAD$^+$:NADH ratios during muscle cell differentiation that are reported to inhibit Sir2 activity (20) are likely due to depletion in levels of free NAD$^+$ and not an increase in free NADH levels. However, given that there is some variability in NAD$^+$ $K_m$ values among Sir2 homologs (Table 2 and (22,30)), drastic changes in cellular [NAD$^+$] may elicit a greater effect on some homologs than others.

In summary, we propose that changes in both free nicotinamide and free NAD$^+$ will contribute to the observed activity of cellular Sir2 enzymes. However, the relative inhibitory contribution of nicotinamide is likely to be more substantial than the small increases in activity predicted with non-catastrophic alterations in NAD$^+$ or NAD$^+$:NADH ratios. The nature of nicotinamide inhibition indicates that it cannot be alleviated by increased levels of NAD$^+$ (28). The concentration of nicotinamide required for significant Sir2 inhibition is within physiological concentrations, which have been reported to range from 11–400 $\mu$M (see Refs in (25)).

**Kinetic and Structural Basis for the Observed Co-Enzyme Specificity.** Until very recently, crystal structures of bacterial (39), archael (40-42) and eukaryotic (43-45) Sir2 homologs with various ligands had not revealed an intact nicotinamide base within NAD$^+$, and thus the exact location and mode of the nicotinamide binding site was simply suggested. However, new crystal structure data from Avalos et al. (46) and Zhao et al. (47) have shown Sir2Af2 with NAD$^+$, and HST2 with carba-NAD$^+$ and acetylated histone H4 peptide where the nicotinamide ring was positioned in the highly conserved C pocket,
previously suggested to contain a latent nicotinamide-group binding site (42). In the following discussion, we utilize this new structural data to provide the rationale for the observed substrate and inhibitor results presented in this study. Using HST2 as the reference for discussion, we compared the structure of HST2 with bound 2’-O-acetyl ADP ribose (44) and the structure of Sir2Af2 with bound NAD⁺ (46). Fitting the backbone chains overlies 156 of 251 residues of Sir2Af2 onto HST2 with an r.m.s. deviation of 1.40 Å.

Perhaps the most striking result is the relative inability of analogs containing nicotinic acid to serve as substrates or to act as inhibitors of the Sir2 enzymes. We postulated this was due to a negatively charged residue in the NAD⁺ binding pocket that would form a hydrogen-bonding interaction with the exocyclic amide of NAD⁺, but when replaced with a carboxylate group (as is the case for NAAD⁺ and other nicotinic acid analogs), charge repulsion would greatly reduce binding. Indeed, the crystal structure of Sir2Af2 reveals that the nicotinamide exocyclic amide of NAD⁺ forms a hydrogen-bond to the side chain carboxylate of Asp-103 (equivalent to Asp-118 in HST2) (46). In addition to Asp-118, the nicotinamide ring forms important hydrogen-bonding interactions with the invariant residues Ser-36, Ile-117, and Leu-120 and van der Waal’s interactions with the invariant residues Ile-41, Pro-42, Phe-44, Asn-116, and Ile-117 in HST2 (Figure 5).

The analog α-NAD⁺ showed no detectable deacetylase activity as a co-enzyme but displayed the second lowest IC₅₀ value (1.08 mM) of the compounds tested. The difference between α-NAD⁺ and β-NAD⁺ lies in the orientation of nicotinamide about the ribose ring. Accordingly, when bound to enzyme, the nicotinamide ring of α-NAD⁺
would not be placed in site C, but rather would protrude away from this pocket, and occupy a position where the acetylated-lysine substrate would attack the ribose ring (28). The acetylated-lysine peptide binds in a hydrophobic tunnel that ends at the C1′ carbon of the nicotinamide ribose ring (41,44) (Figure 6). It is likely that [-NAD⁺ inhibits the reaction by prohibiting the acetylated-lysine from accessing the appropriate face of the co-enzyme. The fact that ADP-ribose yielded similar IC₅₀ values (~1 mM) (30) to that of [-NAD⁺ is consistent with this proposed mode of [-NAD⁺ binding and inhibition.

The two mononucleotides, [-NMN⁺ and [-NAMN⁺, yielded no detectable deacetylase activity as substrates, and poor inhibition when NAD⁺ was the co-enzyme (Table 1), consistent with low binding affinity. In HST2, the AMP portion of NAD⁺ is hydrogen bonded to Ala-33, Thr-37, Arg-45, Ser-225, Asn-248, and Ser-270, and forms van der Waals interactions with Leu-249 and Tyr-269 (Figure 5). These interactions would be absent in the nicotinamide mononucleotide compounds, resulting in the substantial loss of binding affinity observed.

As discussed above, [-NADH was shown to be a poor inhibitor of the reaction. Although the reduction of NAD⁺ to NADH removes the positive charge from the nicotinamide ring, and therefore might be expected to bind tighter to a hydrophobic pocket, the reduced ring also loses its planar geometry and aromatic character, preventing the specific interactions with Phe-44 and Asp-118 present with NAD⁺. Phe-44 is one of the most conformationally mobile residues in the nicotinamide binding pocket and is thought to be involved in shielding from solvent reactive intermediates (46). In addition, we propose that Phe-44 could serve as a gate to this pocket and could form stabilizing π-stacking interactions with the nicotinamide ring upon binding (Figure 6), an interaction...
lost with NADH. In addition, the nicotinamide ring of NADH has been shown both by \textit{ab initio} calculations (48,49) and X-ray crystal structure data (48) to adopt a boat or twist-boat conformation upon reduction. The reduction of nicotinamide also changes the hybridization of the endocyclic nitrogen from $sp^2$ to $sp^3$, which greatly alters the position of the nicotinamide ring. As a result, the hydrogen-bonding interactions between Asp-118 and the nicotinamide amide (Figure 5) will be weakened or completely absent in NADH, limiting the ability of NADH to act as an inhibitor.

The substrate $b$-NADP$^+$ differs from $b$-NAD$^+$ only in the presence of a phosphate group at the 2'-hydroxyl on adenosine. Because the chemical steps in the reaction occur at the nicotinamide ribose, the adenosine nucleoside might be expected to function in increasing the binding affinity of the substrate. This idea was confirmed by our results, as we found a 130-fold increase in the $K_m$, and the $k_{cat}$ was unchanged. The high $K_m$ with $b$-NADP$^+$ is likely due to two factors: 1) switching from a hydrogen-bond donating hydroxyl to a hydrogen-bond accepting phosphate and 2) increasing the steric bulk. In HST2 the 2'-hydroxyl of adenosine is hydrogen-bonded to the carbonyl oxygen of the side chain of Asn-248 (Figure 5). Phosphorylation results in the loss of this interaction accounting, along with the increased steric clash, for the observed increase in $K_m$.

Substrates with substitutions at the nicotinamide ring, $b$-thio-NAD$^+$, $b$-3-AcPAD$^+$, and $b$-3-hPAD$^+$, exhibited lower $k_{cat}$ values and higher $K_m$ values compared to those of $b$-NAD$^+$. This can be explained from the loss of important binding interactions and the reduced reactivity of the glycosidic bond. Sir2Af2 crystal data has shown that hydrogen-bonds between the nicotinamide amide and Ile-117 and Asp-118 pull the amide 30° out of the plane of the nicotinamide ring, 150° from the most stable position (46,50).
Therefore, the ϖ-electron stabilization of the nicotinamide ring and the amide group is broken upon binding to the enzyme, which causes the NAD⁺ to adopt a higher energy conformation. In θ-thio-NAD⁺, the hydrogen-bond predicted to form between the amide nitrogen of Ile-117 and the sulfur in the thioamide will be weaker because sulfur serves as a poor hydrogen-bond acceptor compared to oxygen. Therefore, the ability of the enzyme to pull the amide group out of the plane of the nicotinamide ring will be lessened, causing a decrease in ground-state destabilization of θ-NAD⁺ making the glycosidic bond between the nicotinamide and the ribose rings less reactive.

A similar rationale can be applied to θ-3-AcPAD⁺ as a substrate. Substitution of the amide nitrogen in θ-NAD⁺ with a methyl group results in the loss of a hydrogen-bond donor at that position (Figure 5). This contrasts with θ-thio-NAD⁺ in which the present hydrogen-bonding interactions are only weakened and consequently the changes in $k_{cat}$ and $K_m$ seen between θ-3-AcPAD⁺ and θ-NAD⁺ are magnified compared to those between θ-thio-NAD⁺ and θ-NAD⁺.

In θ-3-hPAD⁺, the exocyclic amide is replaced with a hydroxyl group, which would be too far away from Ile-117 and Asp-118 to form hydrogen-bonding interactions like those seen in θ-NAD⁺, explaining the increase in $K_m$ observed with θ-3-hPAD⁺. Also, the electron-donating character of the hydroxyl will help to stabilize the positive charge on the nicotinamide ring, making 3-hydroxypyridine less reactive as a leaving group compared to nicotinamide. The leaving group ability is reflected by the pKₐ values of the endocyclic nitrogens of nicotinamide (3.54) and 3-hydroxypyridine (4.86). This along with the loss of the hydrogen-bonding interactions causes a decrease in the reactivity of the glycosidic bond, explaining the decrease in $k_{cat}$. Conversely, we have
shown that among a variety of pyridine derivatives, 3-hydroxypyridine can serve as a potent nucleophile in the Sir2 catalyzed transglycosidation reaction (28).

CONCLUSION

In conclusion, several trends are apparent from these investigations. The first is the exquisite selectivity for the nicotinamide base of the dinucleotide co-enzyme. The most dramatic losses in binding affinity/reactivity are from relatively minor changes in the nicotinamide ring, either by reduction, as in NADH, or by converting the amide to its acid analogue, as in NAAD\(^+\), NAMN\(^+\), and nicotinic acid. The second trend is the specificity of the adenine binding site, which is reflected in the poor ability of $\beta$-NHD\(^+\) and $\beta$-NGD\(^+\) to serve as substrates for the deacetylation reaction. Thus, both ends of the dinucleotide NAD\(^+\) are critical for high selectivity and high affinity. In addition, this study has important implications for the cellular regulation of Sir2 enzymes by NAD\(^+\) metabolites. Among the NAD\(^+\) metabolites tested none were able to allosterically activate the enzyme, although all led to various extents of inhibition, consistent with competition at the coenzyme binding site. Our findings suggest that cellular nicotinamide levels would provide an effective small-molecule regulator of protein deacetylation and generation of OAADPr. On the other hand, these findings also suggest that changes in the physiological NAD\(^+\):NADH ratio, without a change in NAD\(^+\), would yield little difference in Sir2 activity. That is, NADH is an extremely ineffective inhibitor of Sir2. Therefore, we propose that changes in both free nicotinamide and free NAD\(^+\) afford the greatest contribution to the observed activity of Sir2 enzymes, but with nicotinamide having a more dramatic effect during smaller fluctuations in concentration.
References

Table 1. Inhibitory Effects of NAD⁺ Analogues and Salvage Pathway Intermediates on the formation of O-Acetyl-ADP-Ribose. All reactions were carried out in 50 mM phosphate or 50 mM Tris buffer, pH 7.5 at 37 °C, 225 μM AcH3 peptide, 50 μM NAD⁺, 1 mM DTT, 0.2 – 0.5 μM HST2, and inhibitor concentrations ranging from 5 μM – 100 mM. IC₅₀ values are derived from inhibition curves fitted in Figure 2 with SEM errors (see Fig 2 legend).

<table>
<thead>
<tr>
<th>Structure</th>
<th>Name</th>
<th>IC₅₀ (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td><img src="image" alt="Nicotinamide" /></td>
<td>Nicotinamide</td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td><img src="image" alt="a-NAD⁺" /></td>
<td>a-NAD⁺</td>
<td>1.08 ± 0.10</td>
</tr>
<tr>
<td><img src="image" alt="b-NMN⁺" /></td>
<td>b-NMN⁺</td>
<td>2.76 ± 0.20</td>
</tr>
<tr>
<td><img src="image" alt="b-NADH" /></td>
<td>b-NADH</td>
<td>27.9 ± 1.4</td>
</tr>
<tr>
<td><img src="image" alt="b-NAAD⁺" /></td>
<td>b-NAAD⁺</td>
<td>26.1 ± 4.5</td>
</tr>
<tr>
<td><img src="image" alt="b-NAMN⁺" /></td>
<td>b-NAMN⁺</td>
<td>158 ± 40</td>
</tr>
</tbody>
</table>

Nicotinic Acid 250 ± 24
Table 2. Steady-State Kinetic Analysis Using \( \beta \)-NAD\(^+\) Analogues as Substrates for HST2. All reactions were carried out in either 50 mM phosphate or 50 mM Tris buffer, pH 7.5 at 37 °C, 1 mM DTT, up to 20 mM NAD\(^+\) analogue, up to 1 mM AcH3, and HST2 concentrations ranging from 1-106 \( \mu \text{M} \). Values are derived from fits to the Michaelis-Menten equation, or bisubstrate analysis fit to a sequential mechanism (Eq. 3). Data are an average of 3 or more independent experiments (\( n \geq 3 \) with SD reported).

<table>
<thead>
<tr>
<th>Name</th>
<th>( k_{\text{cat}}/K_M ) (M(^{-1})s(^{-1}))</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_M ) (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \beta )-NAD(^+)</td>
<td>( 1.75 \pm 0.17 \times 10^4 )</td>
<td>( 0.21 \pm 0.01 )</td>
<td>( 1.2 \pm 0.1 \times 10^{-2} )</td>
</tr>
<tr>
<td>( \beta )-NADP(^+)</td>
<td>( 135 \pm 33 )</td>
<td>( 0.21 \pm 0.04 )</td>
<td>( 1.6 \pm 0.7 )</td>
</tr>
<tr>
<td>( \beta )-NAAD(^+)(^{(b)})</td>
<td>( 1.3 \pm 0.3 \times 10^{-2} )</td>
<td>( 4.8 \pm 0.4 \times 10^{-5} )</td>
<td>( 3.8 \pm 0.9 )</td>
</tr>
<tr>
<td>( \beta )-NAMN(^+)</td>
<td>N/D(^{(a)})</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \beta )-NMN(^+)</td>
<td>N/D(^{(a)})</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \beta )-NAD(^+)(^{(a)})</td>
<td>N/D(^{(a)})</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \beta )-Thio-NAD(^+)</td>
<td>( 74.6 \pm 24.5 )</td>
<td>( 5.2 \pm 0.7 \times 10^{-2} )</td>
<td>( 0.70 \pm 0.21 )</td>
</tr>
<tr>
<td>( \beta )-3-AcPAD(^+)(^{(b)})</td>
<td>( 13.6 \pm 1.9 )</td>
<td>( 3.0 \pm 0.1 \times 10^{-2} )</td>
<td>( 2.2 \pm 0.3 )</td>
</tr>
<tr>
<td>( \beta )-3-hPAD(^+)(^{(b,c)})</td>
<td>( 2.9 \pm 0.2 \times 10^{-2} )</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>( \beta )-NGD(^+)(^{(b)})</td>
<td>( 12.2 \pm 0.6 )</td>
<td>( 2.8 \pm 0.1 \times 10^{-2} )</td>
<td>( 2.3 \pm 0.1 )</td>
</tr>
<tr>
<td>( \beta )-NHD(^+)(^{(b)})</td>
<td>( 7.20 \pm 1.45 )</td>
<td>( 3.6 \pm 0.2 \times 10^{-3} )</td>
<td>( 0.50 \pm 0.10 )</td>
</tr>
</tbody>
</table>

\(^{(a)}\) Not detectable.
\(^{(b)}\) Assays performed at 130 – 300 \( \mu \text{M} \) peptide.
\(^{(c)}\) Unable to saturate with NAD\(^+\) analogue.
FIGURE LEGENDS

Figure 1. Schematic diagram showing the proposed $\phi$-NAD$^+$ salvage pathway in yeast.

Figure 2. Inhibition of HST2 deacetylation activity. From left to right are nicotinamide (solid squares), $\phi$-NAD$^+$ (solid diamonds), NMN$^+$ (open circles), NADH (solid circles), NAAD$^+$ (open diamonds), NAMN$^+$ (open squares), and nicotinic acid (solid triangles). Reactions were carried out in either 50 mM phosphate or 50 mM Tris buffer, pH 7.5 at 37°C, 1 mM DTT, 225 $\mu$M AcH3 peptide, 0.2–0.5 $\mu$M HST2, and inhibitor concentrations ranged from 10 $\mu$M–100 mM. Inhibition data were fitted to Eq. 1. Error bars are SD and are determined from at least three independent experiments (n $\geq$ 3).

Figure 3. Co-enzyme saturation curves with NAD$^+$ and NAD$^+$ analogue, 3-AcPAD$^+$. HST2 deacetylase reactions at: 0.1 $\mu$M HST2, 225 $\mu$M AcH3, 1 mM DTT, with varying [NAD$^+$] in plot (A), and 3.0 $\mu$M HST2, 300 $\mu$M AcH3, 1 mM DTT, with varying [3-AcPAD$^+$] in plot (B). Experiments were carried out as described in Materials and Methods. Initial velocities were plotted and fitted to Eq. 2. Error bars are SD from three experiments (n =3).

Figure 4. Bisubstrate analysis of HST2 with Thio-NAD$^+$ and AcH3 Peptide. Double reciprocal plots are shown with varied [Thio-NAD$^+$] at constant fixed [AcH3] (A), and varied [AcH3] at constant [Thio-NAD$^+$] (B). Experiments were carried out as described in Materials and Methods. In plot (A), [Thio-NAD$^+$] varies from 50 $\mu$M–1.5 mM at
various fixed [AcH3]: 2.2 mM (open diamonds), 4.3 mM (solid circles), 7.0 mM (open squares), 10.8 mM (open circles), 32.3 mM (solid diamonds), and 53.8 mM (open triangles). In (B) [AcH3] is varied between 2.2 mM and 50.8 mM at various fixed [Thio-NAD\(^+\)]: 50 mM (open diamonds), 125 mM (solid circles), 250 mM (open squares), 500 mM (open circles), 750 mM (solid diamonds), and 1.5 mM (open triangles). Lines represent a complete fit to a sequential kinetic mechanism (Eq. 3).

**Figure 5. Schematic Drawing of the NAD\(^+\)-Binding Pocket of HST2.** These interactions were modeled by superimposing the Sir2Af2 NAD\(^+\) conformation (46) onto the HST2 crystal structure with 2'-O-Acetyl ADP ribose bound (44). Hydrogen bonds are shown as dashed lines. An arc next to the residue name indicates van der Waal’s interactions. A single box indicates a sidechain interaction, a double box indicates a mainchain interaction. Invariant residues, conserved, and nonconserved residues are colored red, blue, and black, respectively.

**Figure 6. Model of NAD\(^+\) and Acetyl-lysine Binding.** The conformation of NAD\(^+\) bound to Sir2Af2 (46) is modeled with the acetyl-lysine substrate from the crystal structure of p53 bound to Sir2Af2 (41). Negatively charged, positively charged, hydrophobic, and hydrogen-bonding residues are shown in red, blue, grey, and yellow, respectively. (A) The structure is shown with Gly-37 (corresponding to Ser-46 in HST2) removed and the sidechains of Phe-35, Arg-36, Glu-48, and Phe-165 in Sir2Af2 (corresponding to Phe-44, Arg-45, Lys-58, and Phe-184 in HST2) removed in order to better visualize the substrates. (B) The model from (A) is shown without residues
removed. This figure was generated using Swiss Pdb Viewer v3.7 (51) and POV-Ray v3.6.
Figure 1

Nicotinic Acid

Nicotinamide

B-Nicotinic Acid MonoNucleotide (B-NAMN)

Nicotinate phosphoribosyltransferase

nicotinate mononucleotide adenylytransferase

Nicotinate glycohydrolase

Exogenous Nicotinamide

O-Acetyl-ADP-Ribose

SIR2-like Enzymes

Acetylated Substrate

B-Nicotinamide Adenine Dinucleotide (B-NAD)

B-NAD Synthetase

Nicotinamidase

B-Nicotinic Acid Adenine Dinucleotide (B-NAAD)

Nicotinic Acid Adenine Dinucleotide

Nicotinic Acid

Nicotinate phosphoribosyltransferase

nicotinate mononucleotide adenylytransferase

Exogenous Nicotinamide

O-Acetyl-ADP-Ribose

SIR2-like Enzymes

Acetylated Substrate

B-Nicotinamide Adenine Dinucleotide (B-NAD)

B-NAD Synthetase

Nicotinamidase

B-Nicotinic Acid Adenine Dinucleotide (B-NAAD)

Nicotinic Acid

Nicotinate phosphoribosyltransferase

nicotinate mononucleotide adenylytransferase

Exogenous Nicotinamide

O-Acetyl-ADP-Ribose

SIR2-like Enzymes

Acetylated Substrate

B-Nicotinamide Adenine Dinucleotide (B-NAD)

B-NAD Synthetase

Nicotinamidase

B-Nicotinic Acid Adenine Dinucleotide (B-NAAD)

Nicotinic Acid

Nicotinate phosphoribosyltransferase

nicotinate mononucleotide adenylytransferase

Exogenous Nicotinamide

O-Acetyl-ADP-Ribose

SIR2-like Enzymes

Acetylated Substrate

B-Nicotinamide Adenine Dinucleotide (B-NAD)

B-NAD Synthetase

Nicotinamidase

B-Nicotinic Acid Adenine Dinucleotide (B-NAAD)
Figure 2

[Graph showing inhibition of % deacetylase activity by various inhibitors across different concentrations (mM).]

- Nicotinamide
- alpha-NAD
- NMN
- NADH
- NAAD
- NAMN
- Nicotinic Acid
Figure 3

A

\[ v (s^{-1}) \]

\( [\text{NAD}^+] \) (mM)

B

\[ v (s^{-1}) \]

\( [3-\text{AcPAD}^+] \) (mM)
Figure 4

(A) Graph showing the relationship between $1/v$ (s) and $1/\text{[Thio-NAD}^+]$ (M$^{-1}$). The plot includes multiple data points and lines indicating different conditions or concentrations.

(B) Graph showing the relationship between $1/v$ (s) and $1/\text{[AcH3]}$ (M$^{-1}$). The plot includes multiple data points and lines indicating different conditions or concentrations.
Figure 5
Figure 6

A)  

B)