



## Review

## Sirtuin chemical mechanisms

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## ABSTRACT

Sirtuins are ancient proteins widely distributed in all lifeforms of earth. These proteins are universally able to bind  $\text{NAD}^+$ , and activate it to effect ADP-ribosylation of cellular nucleophiles. The most commonly observed sirtuin reaction is the ADP-ribosylation of acetyllysine, which leads to  $\text{NAD}^+$ -dependent deacetylation. Other types of ADP-ribosylation have also been observed, including protein ADP-ribosylation,  $\text{NAD}^+$  solvolysis and ADP-ribosyltransfer to 5,6-dimethylbenzimidazole, a reaction involved in eubacterial cobalamin biosynthesis. This review broadly surveys the chemistries and chemical mechanisms of these enzymes.

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Sirtuins are a broadly conserved family of enzymes found in all phyla of life including archaea, eubacteria, yeast, plasmodia, metazoans, mammals and even viruses [1–3]. These are ancient proteins that have a common catalytic architecture, which allows these proteins to recognize universally a metabolically central and abundant compound,  $\text{NAD}^+$ . The most common reaction catalyzed by sirtuin enzymes is that of  $\text{NAD}^+$  dependent protein deacetylation, which consumes a mole equivalent of  $\text{NAD}^+$  per acetyl group removed [1]. The reaction effects an acetyl group transfer to ADPR to form a novel compound called 2'-O-acetyl-ADPR (2'-AADPR, Scheme 1 [1]). Select sirtuins also catalyze other reactions, such as protein ADP-ribosyltransfer,  $\text{NAD}^+$  hydrolysis and many if not all seem to catalyze acetyllysine-dependent nicotinamide base-exchange into  $\text{NAD}^+$ . Sirtuins have been implicated in organism adaptations to nutrient intake, and are regulators of aging in a variety of organisms, ranging from yeast, flies, worms and probably in mammals as well [4,5].

The detailed active site recognition of  $\text{NAD}^+$  has been delineated by X-ray crystal structures of sirtuins co-complexed with this substrate [6,7].  $\text{NAD}^+$  complexed to a sirtuin active site is a chemically reactive form of  $\text{NAD}^+$  [6], capable of undergoing chemistry with nucleophiles such that ADP-ribosyltransfer occurs with inversion of stereochemistry [8–12] (Scheme 2). Characterized general nucleophiles for  $\text{NAD}^+$  include acetyllysine substrates or thioacetyllysine inhibitors [11–13]. Close proximities of  $\text{NAD}^+$  and the acetyllysine group (or thioacetyl group) enforced by the active site structure leads to ADP-ribosylation of acetyllysine to form an imidate (or thioimidate, Scheme 2, top). The imidate is proposed to account for deacetylation as well as base-exchange catalyzed by these enzymes [9,12,14,15]. On several

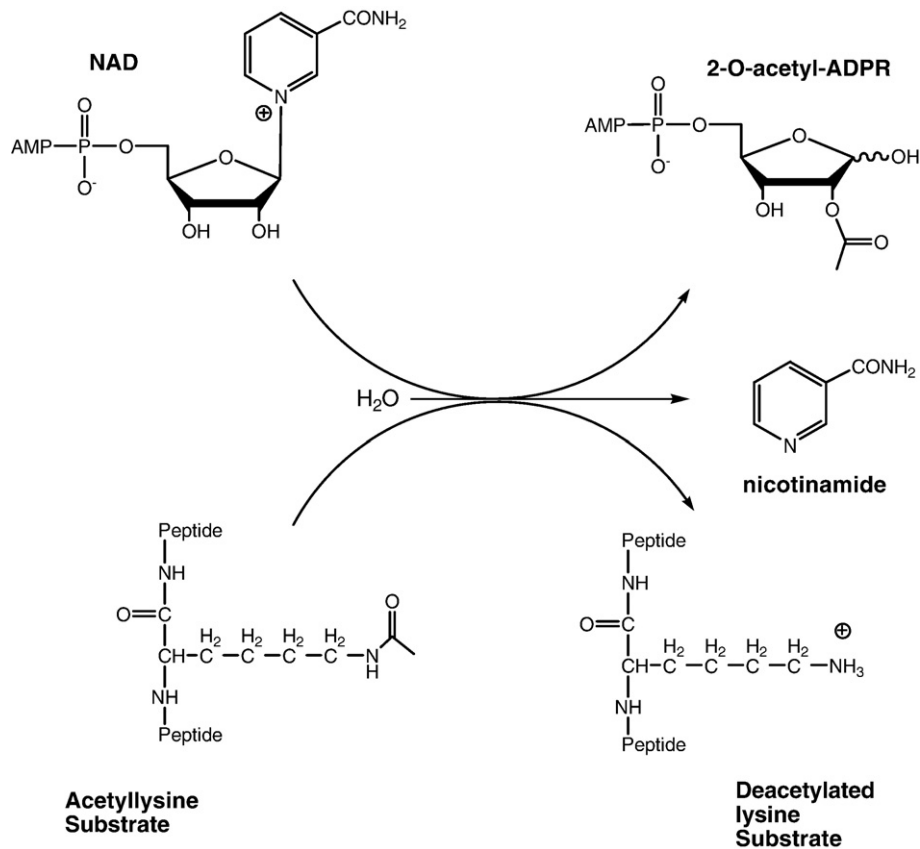
sirtuin enzymes other nucleophiles have also been found to react directly with  $\text{NAD}^+$  such as methanol [10] (Scheme 2, middle), and water [10], characteristic of an  $\text{NAD}^+$  glycohydrolase activity.  $\text{NAD}^+$  (and possibly NaMN) can also react with inversion with the nucleophile 5, 6-benzimidazole catalyzed by CobB, a Sir2 found in eubacteria (Scheme 2, bottom) [8,16]. It has been suggested that some sirtuins catalyze a reaction where  $\text{NAD}^+$  reacts directly with protein nucleophilic amino acid side chains [16,17]. Indeed, there are multiple examples in which sirtuins have been reported to catalyze ADP-ribosyltransfer to proteins [18–22].

The common mechanistic thread in all of these chemistries is that sirtuins activate  $\text{NAD}^+$  as a chemical partner to react with nucleophiles to effect different reaction outcomes. We continue to learn more about how sirtuin chemistry varies from species to species and from isoform to isoform. Nature has honed the chemistry platform provided by sirtuin enzymes to accomplish specificity, regulatory control, and linkage to metabolism, which in turn disposes these enzymes to regulate a variety of fundamental cellular processes. In this review, we examine the mechanisms of sirtuin chemistries with an emphasis on the relationships to known chemistries, the differences of chemistries discovered for different sirtuins, and we discuss the functional and chemical components of sirtuin reactions.

## 1. Sirtuin deacetylation reaction

The most biologically relevant of the reactions that sirtuin enzymes catalyze is protein deacetylation [1,3]. The reaction has been demonstrated for sirtuins isolated from a variety of phylogenetically distributed species, from archaea to human enzymes [2,7,9,23]. The reaction stoichiometry in Scheme 1 is catalyzed by sirtuins derived from yeast, archaeal, eubacterial and mammalian

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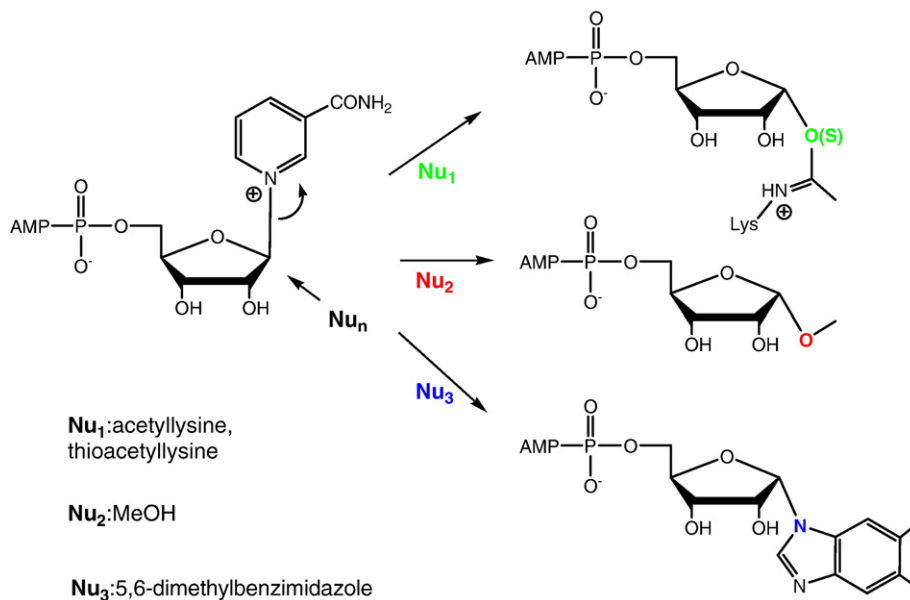


**Scheme 1.** Overall stoichiometry determined for the  $\text{NAD}^+$ -dependent deacetylation reaction.

sirtuins [9,24,25]. Among other things, the reaction is unusual in that it generates 2'-AADPR [1,9,24]. The full characterization of 2'-AADPR established that sirtuins catalyze the synthesis of an ester from an amide, a thermodynamically challenging reaction paid for by  $\text{NAD}^+$  degradation [1]. This reaction outcome, the biological distribution of the reaction and the structural and sequence similarities of sirtuins provide evidence that sirtuins utilize a common mechanistic strategy

to achieve deacetylation. The function of 2'-AADPR in cells, which spontaneously and non-enzymatically equilibrates with the 3'-AADPR isomer [9,24], is currently poorly understood, but is the topic of an extended review in this journal issue.

The sirtuin deacetylation reaction, in addition to restoring the free amino group of lysine and producing AADPR, also generates nicotinamide as a product in all cases [9,26,27] indicating that the deacetylation



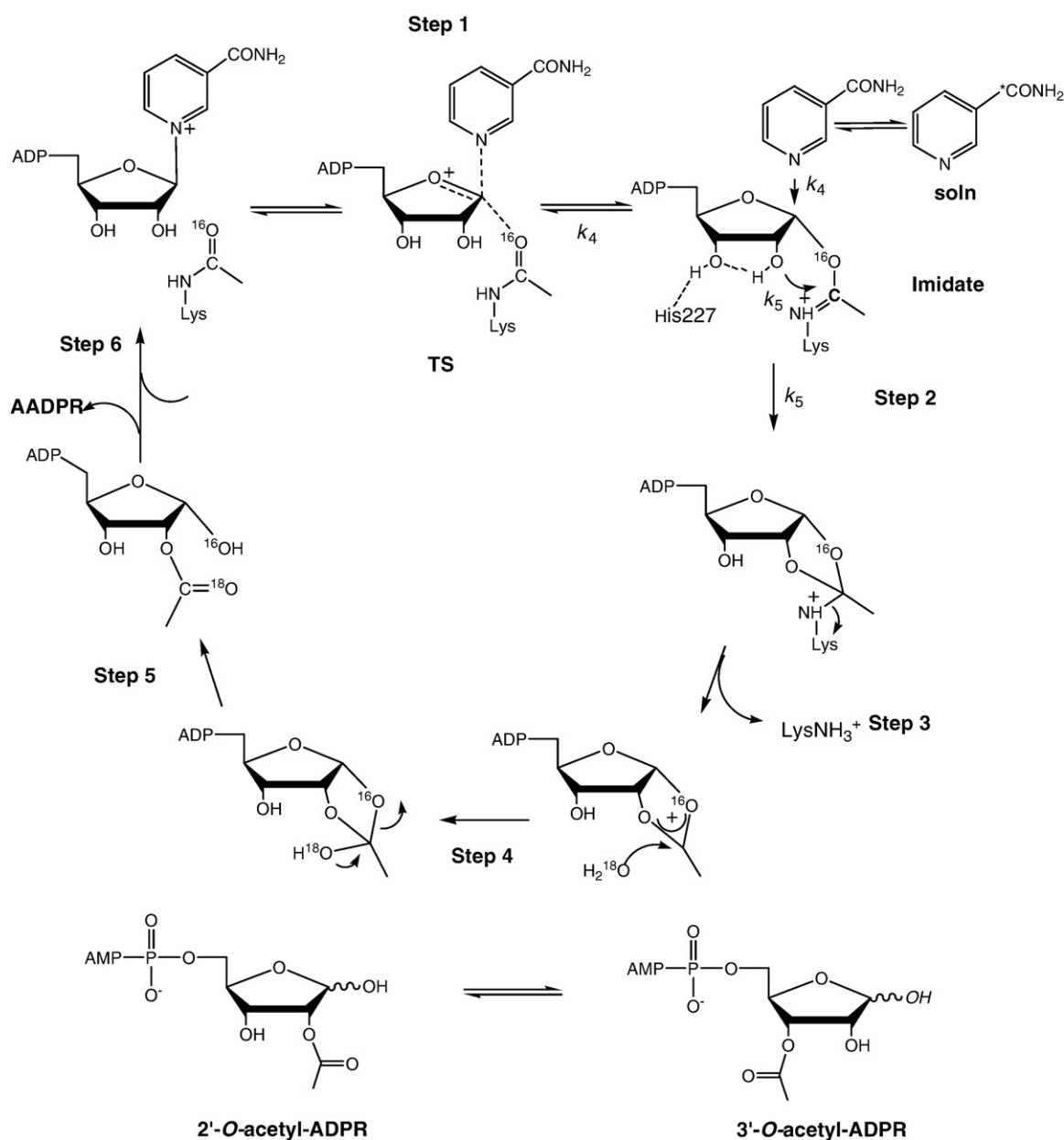
**Scheme 2.** Direct reaction of  $\text{NAD}^+$  with nucleophiles determined for distinct sirtuin enzymes. The top reaction depicts imidate and thioimidate formation. The second reaction depicts a direct solvolysis reaction characterized for a *Plasmodium falciparum* sirtuin enzyme. The bottom reaction represents an ADP-ribosyltransfer reaction determined for the CobB enzyme, a sirtuin from eubacteria.

reaction necessarily cleaves the nicotinamide bond to the anomeric carbon (C1') of ADPR (Scheme 1). It is also true that sirtuin enzymes are generally capable of reversible breakage and reformation of the N1-ribose bond [14,26,28]. The reversible cleavage of nicotinamide is observable by incubation of sirtuin reactions with [carbonyl- $^{14}\text{C}$ ] nicotinamide and by detection of newly formed  $^{14}\text{C}$ -NAD $^{+}$  from initially unlabeled substrate NAD $^{+}$ . Interestingly, this base-exchange reaction requires the acetylated substrate to be present [26]. A detailed explanation of this mechanism will be presented in the following section. Finally, the overall sirtuin reaction requires stoichiometric consumption of water from solvent, as shown by  $^{18}\text{O}$  incorporation into the acetyl group of AADPR when reactions are performed in  $^{18}\text{O}$  water (Scheme 3, [9]).

A detailed mechanism for the deacetylation reaction was proposed that we have since referred to as the ADPR-peptidyl imidate mechanism [1,9] (Scheme 3). Several mechanistic clues led to this proposal, including the proper identification of 2'-AADPR. The regiochemistry of the acetyl group in this molecule implied that the

acetyllysine was likely to react at the  $\alpha$ -face of the NAD $^{+}$  molecule. Isotope labeling experiments supported a reaction mechanism wherein ADP-ribosyltransfer occurs between acetyllysine oxygen and the C1' atom of the ADP-ribose moiety of NAD $^{+}$  [1,9]. An abundance of X-ray crystal structures demonstrates that the proposed direct reaction between acetylated lysine and NAD $^{+}$  is readily accommodated at the active site, including a termolecular complex of acetyllysine and NAD $^{+}$  [6]. The proposed bond formation between the acetyl carbonyl-oxygen and the ADPR moiety was definitively established with synthesis of an  $^{18}\text{O}$ -isotopically labeled acetyllysine peptide substrate. This substrate was reacted with NAD $^{+}$  catalyzed by a yeast HST2 and the  $^{18}\text{O}$ -isotope in the amide carbonyl oxygen was found to be transferred to the hydroxyl substituent at the C1'-position of the product AADPR, as predicted by the peptidylimidate mechanism [29].

As Scheme 3 shows, the first chemical step in the sirtuin deacetylation mechanism generates nicotinamide and a novel intermediate called a peptidylimidate intermediate (an intermediate



**Scheme 3.** Proposed reaction mechanism of sirtuin-catalyzed NAD $^{+}$ -dependent deacetylation. The asterisk depicts radioactively labeled nicotinamide that can be used to monitor the base-exchange reaction (reversal of imidate to NAD $^{+}$ ). Bottom scheme shows spontaneous non-enzymatic equilibration of AADPR isomers that occurs in solution.

we originally called an alkylamidate) [9]. Sauve et al. suggested that this intermediate has special properties, including the property that it could be formed reversibly, and thereby, could explain both the ability of sirtuins to catalyze base-exchange, as well as the requirement for acetyllysine peptide to perform exchange chemistry [9]. In addition, the imidate was envisioned to react as a reactive intermediate that is subject to intramolecular attack by the 2'-OH to eventually yield 2'-AADPR (Scheme 3). This downstream mechanism accounted for deacetylation, production of 2'-AADPR and explained solvent labeling of  $^{18}\text{O}$  into the acetyl of AADPR (Scheme 3; [1,9,29]).

The proposed enzymatic mechanism in Scheme 3 is now supported by a variety of structural, kinetic, isotope labeling, computational and other studies. It has been exceptionally versatile for explaining a variety of features of sirtuin enzymes and is supported by a considerable body of experimental data. Some of these studies are reviewed here more specifically, and structural studies are extensively reviewed in this issue. Discussion on aspects of the mechanism that are less well understood will be included.

## 2. NAD<sup>+</sup> binding geometry and affinities

The reaction of NAD<sup>+</sup> and a nucleophile is mediated by recognition of the NAD<sup>+</sup> in an elongated *trans*-conformation, within a domain that has a Rossmann-like fold [6,7]. There are a variety of reports of NAD<sup>+</sup>  $K_m$  values for sirtuins. These measures establish that the enzyme affinity for NAD<sup>+</sup> is not of high affinity and in the range of 100–550  $\mu\text{M}$  for human enzymes. For example, several independent determinations of the  $K_m$  of human SIRT1 have been published, ranging from 132  $\mu\text{M}$  to 550  $\mu\text{M}$  [30,31]. The Sauve laboratory has determined a  $K_m$  for NAD<sup>+</sup> of 165  $\mu\text{M}$  using a p53 derived peptide substrate (Sauve, unpublished data). The  $K_m$  value of SIRT2 was reported to be near 100  $\mu\text{M}$  [32]. Corresponding values for yeast Sir2 can be estimated from reported data or taken from published values to be in the range of 29–70  $\mu\text{M}$  [26,32,33]. The likelihood that NAD<sup>+</sup> levels in cells can regulate sirtuins is dependent on several factors, one of which is the  $K_m$  for NAD<sup>+</sup>. Importantly, the value of  $K_m$  (NAD<sup>+</sup>) for yeast Sir2 is quite low relative to the reported NAD<sup>+</sup> levels in the yeast which are reportedly near or above mM concentration [33,34], even when yeast are depleted of nicotinic acid [35]. These values suggest that NAD<sup>+</sup> fluctuations alone in yeast are not sufficient to regulate sirtuin function, although Belenky et al. found that at sub-mM (near 700  $\mu\text{M}$ ) NAD<sup>+</sup> concentrations, the biological functions of yeast Sir2 appeared to be responsive to NAD<sup>+</sup> increases [35]. These findings could imply that other factors are important in the yeast that raise the apparent  $K_m$  for NAD<sup>+</sup>. Among these factors could be allosteric effects, mediated by other proteins, which could be quite relevant since Sir2 is found complexed to other proteins *in vivo*. In addition, non-competitive inhibition effects on  $K_m$  by nicotinamide can raise the apparent  $K_m$  for NAD<sup>+</sup> [26]. Guarente has suggested that NADH levels in yeast might also increase apparent  $K_m$  for NAD<sup>+</sup> *in vivo*, raising it sufficiently so that alterations in NADH ratio or increases in NAD<sup>+</sup> could provide Sir2 stimulatory effects [33]. In contradiction, the Denu laboratory, found  $K_i$  values for NADH to be in the 15 mM range, well above what would be relevant *in vivo* for this model of sirtuin regulation to be true [36]. A review on yeast regulation of Sir2 is presented in this issue.

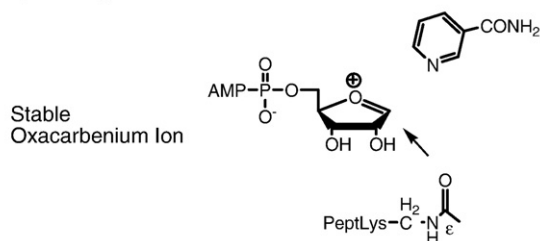
Mammalian cell NAD<sup>+</sup> concentrations are typically well below those found in yeast and are typically in the range from 200 to 600  $\mu\text{M}$  (for recent measurements in mammalian cells and tissues see [37–39]). However, the mammalian  $K_m$  values for NAD<sup>+</sup>, and for SIRT1 in particular, are high enough to suppose that direct NAD<sup>+</sup> regulation of sirtuin biochemical activity in mammalian cells is likely and that the biochemical properties of at least some sirtuins in mammalian cells are consistent with roles as NAD<sup>+</sup> sensors, suggesting that they link directly to NAD<sup>+</sup> fluctuations *in vivo*. Increasingly biological evidence supports this point of view [37,38,40,41], although it is clear that limitations on

accurately determining compartmentalized (organelle-specific) NAD<sup>+</sup> concentrations still limits our ability to precisely link NAD<sup>+</sup> concentration dynamics to regulation of sirtuins. The effect of NAD<sup>+</sup> concentrations on sirtuin function in physiologic settings is beyond the scope of this review, but the idea of modulating NAD<sup>+</sup> levels to influence sirtuin function is being pursued pharmacologically [42]. Nevertheless, the concept that NAD<sup>+</sup> levels can regulate sirtuin activity provides one rationale for consuming metabolically expensive NAD<sup>+</sup> as a price for deacetylation. Acetylation of proteins is metabolically expensive in its own right, and perhaps it is biologically logical to regulate removal of these groups by sensing centralized metabolic information in the cell. In time it appears that metabolic inputs transduced through sirtuin enzymatic activity provided a basis for profound adaptive responses to nutritional intake, via alteration of chromatin function and nuclear transcriptional programs which are highly sensitive to acetylation status.

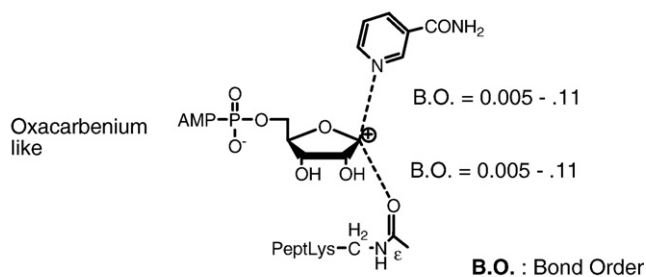
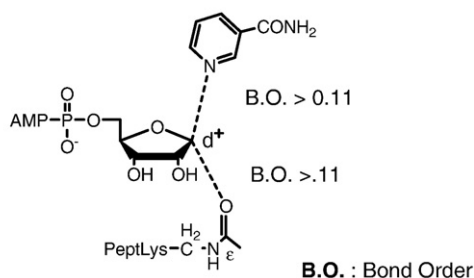
The binding of NAD<sup>+</sup> to sirtuins is mediated by a set of highly conserved catalytic residues. Some of the roles that these residues play in mediating NAD<sup>+</sup> recognition have been elucidated. For example, a universally conserved catalytic histidine has been identified that recognizes the 3'-OH group of NAD<sup>+</sup>. This residue is important for substrate binding, and for activation of the 2'-OH group for nucleophilic attack on the imidate to initiate downstream chemistry [7,9,29]. Furthermore, a conserved phenylalanine appears to be important for organizing and recognizing the nicotinamide riboside moiety of NAD<sup>+</sup> into the active site [6,12]. Both of these residues have also been argued to play important roles in catalysis. It has been proposed that the active site residues enforce a destabilization of the NAD<sup>+</sup> which facilitates nicotinamide bond cleavage and downstream reaction of NAD<sup>+</sup> with acetyllysine substrate or other nucleophiles [6,12]. Currently the evidence that such a destabilization is important for catalytic activity of sirtuin enzymes is mostly from X-ray crystallographic data, and computational and other experimental evidence to support this model of NAD<sup>+</sup> reactivity is currently not available. Ground state destabilization is one way to facilitate enzymatic catalysis, which leads indirectly to reducing the transition state barrier. The manner in which NAD<sup>+</sup> is reacted in the first chemical step of deacetylation catalysis is now considered, with respect to views that have been presented on the nature of the transition state.

## 3. Transition state and mechanistic proposals for ADP-ribosylation reaction with acetyllysine

The proposed first chemical step catalyzed by sirtuins leading to acetyllysine deacetylation is ADP-ribosylation of acetyllysine, leading to formation of an ADP-ribosyl-peptidylimidate intermediate (Scheme 3). The formation of this species can be imagined to proceed via several distinct mechanistic processes, all of which can achieve the formation of the imidate. For simplification, we will present three mechanistic choices (Scheme 4) which have been proposed in the literature, although additional variations of these are possible. The first mechanism is a stepwise dissociative mechanism (formally  $\text{S}_{\text{N}}1$ ), in which full dissociation of the nicotinamide forms an enzyme-stabilized oxocarbenium ion, followed by collapse of the oxocarbenium ion by reaction with acetyllysine (Scheme 4, top). A second mechanism can be considered also, in which an oxocarbenium ion is formed at the transition state, but with extensive bond cleavage to the leaving group, and weak bond formation to the nucleophile (Scheme 4, middle). This dissociated, reaction mechanism is not stepwise, as it lacks a discrete intermediate between the reactants and the imidate. The reaction is asynchronous, since the leaving group is largely cleaved prior to bond formation with the nucleophile. The third mechanism is characterized by associative effects of the nucleophile at the transition state, consistent with an  $\text{S}_{\text{N}}2$  mechanism (Scheme 4, bottom). This mechanism typically is characterized by a

Stepwise  $S_N1$  mechanism

## Dissociative asynchronous mechanism

Concerted  $S_N2$  mechanism

**Scheme 4.** Different transition states or intermediates proposed for ADP-ribosylation of acetyllysine discussed in the text. Bond angles as drawn between nicotinamide and C1' and carbonyl-oxygen and C1' are not strictly defined in these representations.

pentacoordinate transition state with significant bond orders to both nucleophile and leaving group. Data in support of each of these mechanisms has been produced, although, there is a lack of firm consensus on which of the mechanisms best explains sirtuin chemistry.

Sauve et al. first proposed the direct displacement reaction between  $NAD^+$  and acetyllysine and considered the reaction to proceed via a mechanism featuring a highly dissociated transition state (Scheme 4, middle) [9]. Acetyllysine is not a good nucleophile, and the authors proposed that electrophilicity of an oxacarbenium ion-like transition state accounted for the ability of sirtuins to ADP-ribosylate this unlikely sidechain nucleophile [9,43]. An old example by Borch and co-workers demonstrated that *O*-alkylimidates could be generated from amides by use of very electrophilic alkylating agents, such as triethyloxonium tetrafluoroborate [44]. Interestingly Borch and co-workers determined that these imidates have a tendency to decompose to esters and free amines when exposed to water, directly analogous to the chemistry proposed for sirtuins. The authors realized that  $NAD^+$  was not itself particularly electrophilic, but that enzyme catalyzed reactions of  $NAD^+$  involving ADP-ribosyltransfer commonly proceed through transition state oxacarbenium ions [45–48]. It is important to point out that in every case where there is a solved ADP-ribosyl transition state, the structures are highly dissociated, but still have very small bond orders between 0.001 and 0.11 to both nicotinamide leaving group and to nucleophile (consistent with Scheme 4, middle) [45–48]. These highly oxacarbenium-like transi-

tion states are characterized by extremely electrophilic sugars that would be envisioned to capture the acetyloxygen to form the envisioned imidate species [1,9,43].

The concept that the reaction might proceed differently than a conventional dissociated transition state was recently suggested by data obtained by Smith and Denu, in which it was suggested that an  $S_N2$ -type reaction (Scheme 4, bottom) might better explain sirtuin chemistry in the initial ADP-ribosylation step [49]. To assess the nature of the transition state, Smith and Denu found that the rate of the ADP-ribosyltransfer reaction was strongly dependent on the nature of substitution at the  $\alpha$ -position of the acetyllysine group, with a powerful decelerating effect associated with electron withdrawing groups such as fluorine [49]. Mono-fluoro, difluoro and trifluoro modifications of acetyl of acetyllysine reacted at relative rates  $5.5 \cdot 10^{-4}$ ,  $6.9 \cdot 10^{-6}$  and  $1.6 \cdot 10^{-6}$  versus that of the underivatized acetyllysine. This was interpreted to be an indication of “considerable nucleophilic participation” at the transition state. The authors argued that Sir2 deacetylase ribosyl-nicotinamide cleavage is consistent with an  $S_N2$  like mechanism where considerable bonding to the nucleophile occurs at the transition state (Scheme 4, bottom). The authors concluded that it was unlikely that a fully dissociated “stable” oxacarbenium ion (Scheme 4, top) as proposed by Marmorstein [50] and initially favored by Denu [14] would form at the transition state or prior to the ADP-ribosylation of the acetyllysine [49]. The authors argued that a stepwise mechanism involving a fully formed oxacarbenium ion (Scheme 4, top) would not be expected to provide rate differences for nicotinamide formation from  $NAD^+$  in the presence of different amide nucleophiles, as was observed. Interestingly, a fully dissociated yet enzyme-stabilized oxacarbenium ion has been favored by some authors in other instances of ADP-ribosylation, for instance, to explain the promiscuous active site chemistry of CD38 [51]. The existence of an enzyme-stabilized oxacarbenium ion on sirtuin enzymes is still an open question. Interestingly, the catalytic properties of *Plasmodium falciparum* Sir2, which has promiscuous ADP-ribosyltransferase activity [10], could be explained by such an intermediate. Such an intermediate seems unlikely to be formed on yeast Hst2, the enzyme used in the Smith and Denu study [49]. The authors did not clearly suggest if the transition state was oxacarbenium-like or non-oxacarbenium-like [49]. Thus the meaning of “considerable nucleophilic participation” was left quantitatively undefined in terms of transition state structure.

Hu et al. used computational methods to address this problem and obtain a more quantitatively defined geometry of the transition state [52]. These authors used QM/MM methods to determine that the *Thermotoga maritima* Sir2 (TmSir2) transition state for imidate formation is highly dissociated, and highly oxacarbenium-like, analogous to other solved ADP-ribosyltransfer transition states [45–48] with bond lengths of 2.60 Å to nicotinamide and 2.35 Å to the acetyllysine oxygen [52]. Their structure invokes weak nucleophilic participation at the transition state, to the extent of 0.14 bond order [52]. The Hu et al. result is largely consistent with our original suggestion that a highly dissociated oxacarbenium ion is likely formed at the transition state [1,9,43] and is in agreement with the result of Smith and Denu that there is no fully dissociated oxacarbenium at the transition state [49]. In fact this computational result provides evidence that the sirtuin ADP-ribosyltransfer reaction is not stepwise ( $S_N1$ , Scheme 4, top), but is a dissociative asynchronous mechanism (Scheme 4, middle) since the nicotinamide bond is largely cleaved before the acetyllysine bond is formed.

It is of interest to ask if the Smith and Denu rate data is more consistent with a highly-associated  $S_N2$  like transition state, or is consistent with the  $0.14 \pm 0.04$  bond order to the nucleophile at the transition state as suggested by the Zhang study. A clear explanation of the Smith and Denu rate data remains to be provided, although the data at face value suggests a high degree of nucleophile associativeness, consistent with a concerted  $S_N2$  type mechanism [49]. Selectivity for the nucleophile can be understood to arise from the extent of



nucleophilic involvement at the transition state in the bond forming reaction. Since the transition state marks the point of no return for the barrier crossing, it is intuitive that the more a bond is required to be formed to reach the transition state, the more selectivity there can be for making that bond. An interesting example at the minimal extreme is the selection of methanol versus water of 1:1 (taking into account mole fractions) for  $\text{NAD}^+$  solvolysis, suggesting a very weak bond to the nucleophile at the transition state [53]. Indeed Berti and Schramm found the nucleophile bond order at the transition state for this reaction was 0.005, consistent with extremely poor nucleophile selection in this reaction [54]. The Smith and Denu data set shows that the nicotinamide formation rate from  $\text{NAD}^+$  on the sirtuin HST2 displays a strong dependence on the nucleophile, seemingly inconsistent with a very weak bond to the nucleophile at the transition state.

However, there are some very important considerations and caveats for the modifications that Smith and Denu [49] tested. These modifications may have caused the transition state position to shift to later and later character, requiring increased nucleophile participation to form an increasingly destabilized imidate. Weakening of the nucleophile basicity and application of Hammonds postulate would be the chemical explanation for this phenomenon. If so, the least basic of the amide groups (for example, the trifluoro-substituted) would require the most significant bonding at the transition state. Improvement of the imidate leaving group in the  $\text{NAD}^+$  reformation reaction (as predicted for the electron deficient amides) also increases the possibility that the rate of internal return to the Michaelis complex is enhanced for the least stable imidates, perhaps reducing the observed rates of nicotinamide formation. Finally, the geometric and electronic changes in substrates introduces the very real possibility that the modifications cause substrates to bind in altered geometries from those of the normal substrate, which could substantially reduce observed rates of reaction.

The Wolberger and Marmorstein laboratories have also produced X-ray crystallographic evidence for a highly dissociated transition state occurring on sirtuin enzymes [12,50]. Since these are reviewed extensively elsewhere in this issue, we will not survey these data here, except in short. The Wolberger laboratory developed evidence for a dissociated transition state on the basis of structural analysis of distances between nucleophile and leaving group in several X-ray crystal structures complexes, including a sirtuin-complexed with a “dissociated  $\text{NAD}^+$ ” mimic [12]. They argued that the sirtuin ADP-ribosylation step is an example of “nucleophilic displacement by electrophilic migration” [12]. Electrophilic migration contends that the breakage of the leaving group bond largely precedes the formation of the nucleophile bond, and is caused by the migration of the sugar electrophile between leaving group and nucleophile positions that are largely fixed through the reaction coordinate [55]. This mechanism is found for a number of N-ribosyltransferase enzymes and explains asynchronous mechanisms for substitution reactions in this family of enzymes [55,56].

The debate on transition state structure of the ADP-ribosylation of acetyllysine catalyzed by sirtuins is still unresolved, partly because there is no clear intellectual reconciliation of the variety of different data sets that have emerged on this question. In addition, the possibility that distinct sirtuin isoforms might stabilize variants of the theoretically available transition states, or even proceed through different mechanisms, remains experimentally untested. Although it is clearly of interest to explain the biochemical and structural basis for the ability of sirtuins to catalyze ADP-ribosyltransfer, investigations to address this issue arguably require a more thorough elucidation of sirtuin transition states. An expected solution is in the experimental determination of the transition states by kinetic isotope effects with interpretations supported by computational analyses. Such methods have the potential to probe the transition state structure directly, without perturbation of substrate steric or electronic structure. The

Schramm laboratory in particular has shown that this approach, combined with computational methods, can provide remarkably well determined structural determinations of transition states, including several ADP-ribosyltransfer transition states [45–48]. In several cases, using the transition state structures as blueprints, inhibitors of N-ribosyltransferase enzymes have been designed and synthesized and bind their targets with nM–fM affinity [57,58]. It is expected that similar studies will provide a more time resolved and mechanistically defined view of the ADP-ribosyltransfer step mediated by specific sirtuin enzymes, and can lead logically to studies which probe the enzymatic features that direct and accelerate sirtuin ADP-ribosyltransfer chemistry.

#### 4. Imidate and chemistries from imidate

Whatever the transition state of reaction between acetyllysine and  $\text{NAD}^+$  it is now widely agreed that the ADPR-peptidyl imidate (originally called an 1'-O-alkylamidate) [1,9] is the species that is formed by the reaction of the two main reaction participants after the first chemical step (Scheme 3). Nevertheless direct evidence for the existence of this catalytic species is still unavailable. Supportive but indirect evidence that this intermediate is formed on the enzyme has come from a number of observations. For example, the amide is required for base-exchange [26], and 1-O-glycosyl imidates are now commonly used in coupling of sugars to nucleophiles in organic synthesis [59,60], demonstrating the intrinsic reactivity of this type of functionality at the anomeric center. The imidate formation is relatively insensitive to removal of an active site histidine from the enzyme, which coordinates to the 3' and 2' OH in different crystal structures and does not significantly decelerate sirtuin base-exchange [7,29]. This suggests that the enzyme is not typically reliant on acid-base catalysis to form or reverse the proposed imidate. Consistently, the Denu laboratory has shown that  $\beta$ -2'-deoxy-2'-fluororibo- $\text{NAD}^+$  can be accepted as a base-exchange substrate with the HST2 enzyme [14].

The most direct evidence in support of the imidate is a crystal structure of TmSir2 with the adduct formed between  $\text{NAD}^+$  and a thioacetylated peptide on the enzyme [12]. The crystal structure reveals a 1'-thioimidate formed on the enzyme (Scheme 2, top, where S is bonded to C1'). This analogue of the reactive imidate is surprisingly unreactive, and causes potent inhibition of sirtuin enzymes [13,61]. Moreover, in several sirtuins sensitive to inhibition by thioacetylpeptides, base-exchange cannot be supported by a thioacetylpeptide (Sauve, unpublished results), arguing that the thioimidate is formed irreversibly from  $\text{NAD}^+$ . The competency of the thioimidate to eventually complete deacetylation chemistry has been demonstrated by Denu and coworkers, who showed that the thioacetyl groups on sirtuin enzymes eventually yield a compound consistent with the structure 1'-thio-2'-AADPR [61]. Collectively these results provide compelling evidence that an imidate species formed on sirtuin enzymes is responsible for the sirtuin deacetylation mechanism.

The imidate is proposed to have two fundamental reaction pathways. The first is collapse of the 2'-OH group onto the imidate by nucleophilic attack (Scheme 3, Step 2), and the other is reversal to  $\text{NAD}^+$  via  $\beta$ -face attack of nicotinamide on the anomeric carbon (Scheme 3, Step 1 reversed). Evidence in favor of the 2'-OH being involved in the decomposition of the imidate as a means to obtain deacetylation has come from mutation of a universally conserved catalytic histidine that was first observed crystallographically at the 3'-OH position of complexed  $\text{NAD}^+$  and AF1Sir2 [7]. The mutation His to Ala does not appear to significantly alter base-exchange, but significantly slows deacetylation catalysis [7,29]. This histidine is posited to play an important role as an activating base that deprotonates the 2'-OH group during Step 2, Scheme 3 downstream of imidate formation, leading to imidate decomposition [9,12,29]. In

addition,  $\beta$ -2'-deoxy-2'-fluororibo-NAD<sup>+</sup> which lacks a 2'-OH group, reacts to form an apparent imidate on HST2, but only releases acetate when treated with strong alkaline quench, consistent with a requirement of the 2'-OH to complete deacetylation chemistry under multiple turnover conditions of the enzyme [14].

The other important chemistry of the imidate is nucleophilic attack at the  $\beta$ -face. In the presence of nicotinamide, the imidate reverses back to NAD<sup>+</sup> (Scheme 3, reverse Step 1). This reversal from the imidate has been demonstrated to be the mechanism whereby nicotinamide can inhibit deacetylation chemistry [14,15]. Consistent with the idea that the imidate has reactivity to nucleophilic substitution at the anomeric carbon is the observation that histidine mutants which cannot achieve deacetylation and have low nicotinamide in media, instead react by hydrolysis at the  $\beta$ -face [29]. This was shown for the Hst2 enzyme, wherein addition of methanol caused the imidate to form  $\beta$ -1'-O-methyl-ADPR with selection of methanol over water of 26:1 [29]. The Sauve laboratory has also identified a wildtype enzyme from *P. falciparum* which is a slow deacetylase, but can perform base-exchange very efficiently [10]. In the absence of nicotinamide, but in the presence of NAD<sup>+</sup> and acetylated peptides the enzyme makes ADPR more rapidly than it deacetylates the peptides [10]. As such, it is the first wildtype sirtuin to have a demonstrated NAD<sup>+</sup> glycohydrolase function [10]. When methanol is present the enzyme forms  $\beta$ -1'-O-methyl-ADPR consistently with the reaction of the imidate with methanol at C1 [10]. Interestingly, when nicotinamide is added to reaction mixtures  $\beta$ -1'-O-methyl-ADPR formation is inhibited, consistent with nicotinamide and methanol competing for the same anomeric carbon in the same active site pocket (Scheme 5) [10].

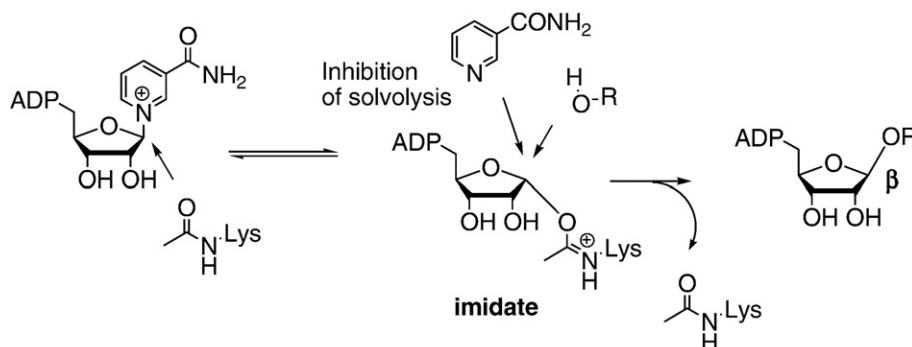
### 5. Partitioning of the intermediate (nature of nicotinamide inhibition)

Of interest to the understanding of the mechanism of sirtuins, and for understanding their biological function is the appreciation that a variety of sirtuins in different organisms are sensitive to physiologic nicotinamide concentrations [62,63]. Nicotinamide is the first product formed in sirtuin reaction chemistry, as has been demonstrated by rapid-mix rapid-quench studies [32] and from observations that nicotinamide product formation is reversible [26]. In depth studies of the nicotinamide base-exchange process and the nicotinamide inhibition of deacetylation have determined them to be intimately connected [14,15]. For example, independent laboratories have shown that nicotinamide  $K_i$  and base-exchange  $K_m$  values are similar [14,15]. Inhibition depletes the imidate intermediate (Scheme 3) and leads to return of the imidate to the Michaelis complex, which explains the correspondence of the two kinetic parameters, since they both measure this effect using inhibition and base-exchange respectively as readouts. This type of inhibition mechanism is expected to produce non-competitive inhibition [64]. Consistently, sirtuins are non-competitively

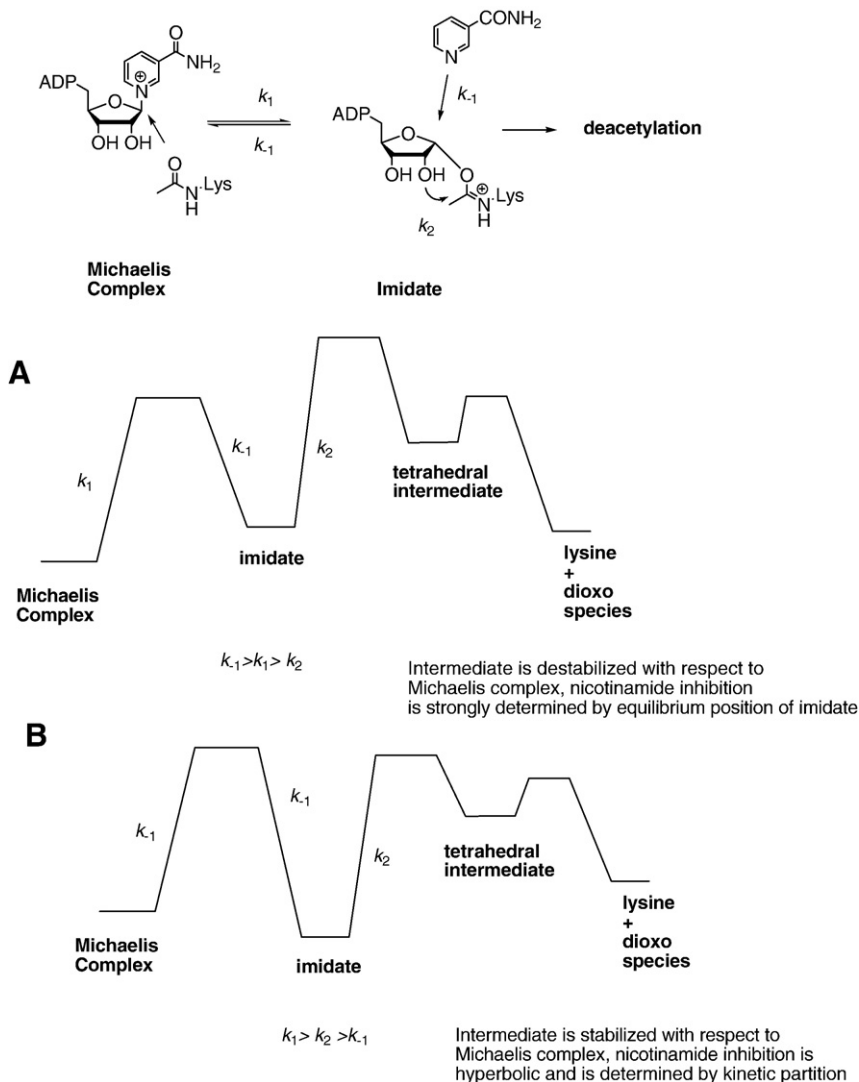
inhibited [26,63], and  $K_i$  values are in the 200  $\mu$ M range or below for a number of these enzymes. Influential work by the Sinclair laboratory has shown that nicotinamide is a potent disruptor of yeast silencing and can prevent lifespan extension as measured by replication of mother yeast cells [62,63]. There is sufficient biological and biochemical data to suggest that nicotinamide is an endogenous negative regulator of sirtuin activity in yeast, flies and even in mammalian cells.

Several structural studies have been performed looking at how nicotinamide reacts on sirtuin enzymes, and they are reviewed elsewhere in this issue [65]. We look now at the biochemical and kinetic studies that have been performed to look more in depth at how inhibition of sirtuins results from the reactivity of nicotinamide with the imidate complex (Scheme 3). A condition of the ability of sirtuins to sense nicotinamide concentrations within a cell is that nicotinamide has to have the opportunity to dissociate and reassociate prior to the intermediate on the enzyme progressing forward (Scheme 3). This condition is required for base-exchange to be observed and base-exchange has been observed for a variety of sirtuin enzymes [7,14,15,26]. This intermediate stability speaks to the existence of an alpha-ADPR covalent complex, capable of reversal, and is consistent with the proposed imidate [9,14,15]. Interestingly, other stabilized ADPR intermediates have been identified on enzymes like CD38, where nicotinamide also causes base-exchange and can inhibit enzyme activity, such as NAD<sup>+</sup> glycohydrolase activity [64]. The CD38 intermediate is stabilized by an active site Glu residue [66] which has been claimed by different groups to stabilize covalent [66] or non-covalent [51] forms of ADPR on the enzyme. Interestingly covalent intermediates have been formed on CD38, using ara-F-NMN [66] and 2'-deoxy-nicotinamide riboside [67]. Interestingly, these two compounds, which covalently inhibit CD38 by ribosylating the catalytic residue, are less reactive and more reactive respectively, compared with their 2'-OH substituted counterparts [68]. The enzyme-bound ribosyl intermediates formed from these compounds are able to support base-exchange chemistries occurring on CD38 [66,67]. The covalent complex formed from ara-F-NMN has been identified by MS [66] and has been solved by X-ray crystallography [51]. In spite of the demonstration of covalent intermediates that can support chemical mechanisms observed with NAD<sup>+</sup> on CD38, the identity of the ADP-ribosyl intermediate on these enzymes remains undetermined and controversial. Although a report of a crystallized non-covalent ribosyl cation on CD38 has appeared [51], the thorough demonstration that this species is a cation is not available. Sauve et al. reported KIE data for reactions catalyzed by CD38 suggesting the equilibrium isotope effect (versus NAD<sup>+</sup>) for an intermediate with a 2'-OH group is not consistent with a cation [66].

Studies indicate that in general the nicotinamide bond cleavage step is usually faster than the downstream chemistry of the imidate intermediate (Fig. 1A) [14,15,32]. Indeed not only is the nicotinamide bond cleavage step not rate limiting, but in a variety of cases it has been shown that the nicotinamide base-exchange rate proceeds faster



**Scheme 5.** Methanolysis of imidate observed for a mutant and wildtype sirtuin. Sensitivity to nicotinamide inhibition is depicted by competitive attack of nicotinamide in base-exchange.



**Fig. 1.** Depictions of fate of imidate complex partitioned between base-exchange and deacetylation pathway. The formation of imidate is governed by the rate constant  $k_1$ , its reversal to the Michaelis complex is  $k_{-1}$  and  $k_2$  is the rate of attack of the 2'-hydroxyl of the imidate. The two reaction coordinates are for enzymes that are sensitive to nicotinamide inhibition of deacetylation, and the bottom is proposed for the enzyme Af2Sir2, which is relatively insensitive to nicotinamide inhibition of deacetylation. The significance of these reaction coordinates is discussed in the text.

than the deacetylation reaction rate [14,15]. Both the Schramm and Denu laboratories have agreed that the efficiency of nicotinamide inhibition typically depends on the ability of nicotinamide to react faster ( $k_{-1}$ ; Fig. 1) than the attack of the 2 hydroxyl on the imidate ( $k_2$  Fig. 1) [14,15] and it has been argued that  $k_2$  can often be rate limiting for the enzyme [15], an idea that appears not to be universal since HST2 is limited by product release [29] but this situation has been demonstrated for the *P. falciparum* Sir2 enzyme [10]. If  $k_2$  is usually limiting or close to limiting for deacetylation rate, then by rule of thumb, nicotinamide inhibition is typically efficient when nicotinamide base-exchange rate greatly exceeds the deacetylation rate. Exceptions to this pattern have proven interesting for testing the model. For example, on the enzyme Af2Sir2 the base-exchange process proceeded slower than the deacetylation rate [15]. Inhibition of deacetylation on this enzyme caused by nicotinamide does not exceed 50%, even when nicotinamide saturates base-exchange [15]. This type of inhibition is called hyperbolic or non-linear inhibition. With reference to reaction coordinate Fig. 1B, this is best explained by having a slower rate of return for the imidate than for forward chemistry ( $k_{-1} < k_2$ ), given that both base-exchange and deacetylation share the imidate forming step  $k_1$ . This causes nicotinamide to be slower in reacting with the intermediate, relative to forward

chemistry and results in hyperbolic inhibition. In this case, the inhibition can be viewed as a purely kinetic phenomenon determined by the partitioning of the imidate between the two reaction pathways (Fig. 1B).

We have suggested that for most sirtuins, in which nicotinamide functions as a potent inhibitor, that both barriers of the nicotinamide formation and reversal are lower than the hill that controls the rate of the deacetylation reaction downstream of the intermediate (Fig. 1A [15]). We have contended that under these conditions, the intermediate must also be destabilized with respect to the Michaelis complex (Fig. 1A) [10,15]. Since generally sirtuins have very fast rate constants  $k_1$  and  $k_{-1}$  barriers relative to  $k_2$ , pseudoequilibration of the imidate and Michaelis complex would be predicted to occur when base-exchange is at maximum rate. If the energies of imidate and Michaelis complex were similar, the imidate would only be depleted 50% at maximal exchange and less so if the imidate was more stable, causing hyperbolic inhibition. What phenomenon can account for the imidate being relatively unstable to  $\text{NAD}^+$ ? This is not completely understood, but one likelihood is that the imidate remains protonated during catalysis, and since an amide leaving group has negligible basicity even when compared to nicotinamide, the amide remains the superior leaving group and correspondingly the imidate is



thermodynamically less stable. Interestingly, base-exchange rates for sirtuins do not vary much across extended pH ranges (Sauve, unpublished data) suggesting that the imidate  $pK_a$  on the sirtuin enzyme is well above 8, since deprotonation of it would likely stabilize the intermediate to base-exchange. Interestingly, protonation of the imidate also activates this imidate for attack by the 2'-OH on the imidate. Imidates are normally easily deprotonated by amines, such as triethylamine, suggesting that the active site kinetically and/or thermodynamically stabilizes this proton to deprotonation even at pHs as high as 9. In fact the Wolberger laboratory has identified a residue which is likely important for interacting with the imidate proton, a Val 160 in the TmSir2 structure in which a backbone carbonyl is within hydrogen-bonding distance to the N of the imidate in the structurally determined thioimide [12].

In the interesting case of Af2Sir2, where nicotinamide is a poor inhibitor of this enzyme and fails to inhibit deacetylation fully we have hypothesized that the intermediate is stabilized relative to the Michaelis complex in this case (see discussion above and Fig. 1B). We do not have evidence that this stabilization is due to deprotonation of the imidate, since deacetylation remains active. We suppose instead that the intermediate is likely stabilized with respect to the Michaelis complex by some other means, which causes nicotinamide attack to be poorly competitive with 2'-OH attack on the imidate (Fig. 1B). What accounts for this stabilization is also not well understood. One possibility has been suggested by structural work done by the Wolberger laboratory, who showed that a Phe residue interacts with the  $\beta$ -face of the ribose. This residue is highly conserved in sirtuins and can regulate the imidate reaction with nicotinamide [12]. Mutation of this residue was found to profoundly accelerate the base-exchange rate of the TmSir2 enzyme, suggesting that this residue can shield the intermediate kinetically, and consequently produce stabilization [12]. It is also possible that the Phe forms a pi-cation interaction with the imidate which also contributes to stabilization, given that the sugar is calculated to retain an approximately 0.5 positive charge in the imidate complex [52]. Evidence that this Phe residue is mobile and adopts different geometries in different types of sirtuin complexes solved crystallographically fully supports the idea that this residue can modulate stability of imidate complexes [12]. It has also been suggested that this Phe residue can prevent indiscriminate reactivity of the imidate with solvent, although increased hydrolysis relative to deacetylation in a Phe mutant has not been demonstrated [12].

## 6. Nicotinamide derepression

The realization that nicotinamide regulates sirtuins *in vivo*, and that it negatively impacts sirtuin deacetylation activity through the base-exchange reaction led to the idea that competitive inhibition with nicotinamide in the base-exchange reaction could lead to relief or derepression of sirtuin inhibition [1,15,28,42]. A requirement for this would be a small molecule that would only bind to the imidate complex and only compete for the nicotinamide binding pocket and would not itself react with the imidate, or otherwise inhibit deacetylation. The author identified a small molecule that had the required property, in the form of a nicotinamide isostere, called isonicotinamide [28]. Isonicotinamide does not inhibit deacetylation, it does not compete with  $NAD^+$  or acetyllysine binding to yeast Sir2, and it does not react with the imidate, but it can competitively inhibit nicotinamide binding to this sirtuin (Scheme 6). As such it inhibits the base-exchange reaction selectively and thereby activates sirtuin activity that is inhibited by nicotinamide (Scheme 6) [28]. The authors showed that isonicotinamide increases the apparent  $K_m$  value for nicotinamide base-exchange, and also increases apparent  $K_i$  values of nicotinamide inhibition of yeast Sir2 [28]. This causes nicotinamide to be weakened as an inhibitor, a phenomenon we have called nicotinamide derepression [42]. The compound isonicotinamide is a

weak binder to yeast Sir2 and only derepresses nicotinamide inhibition at millimolar concentrations. However, this compound is inexpensive, is relatively non-toxic to yeast and to mammalian cells and can be dissolved in nearly molar concentrations in water. It also readily penetrates cells and is very stable.

In a biological test designed to test if nicotinamide is a relevant negative regulator in yeast cells, yeast were treated with isonicotinamide to determine if this compound could cause Sir2 activation. These yeast experienced significant enhancements in gene silencing and could even correct for deletion of *PNC1*, which confers a silencing defect because of highly increased endogenous nicotinamide, measured to be 10 fold over wildtype [42]. There is no published data on the effects of isonicotinamide on human sirtuins, although isonicotinamide acts on mammalian cells in a manner consistent with activation of SIRT1, and can duplicate some of the biological effects of resveratrol, albeit at much higher concentrations [69–72].

## 7. Possible mechanisms of ADP-ribosyl transfer

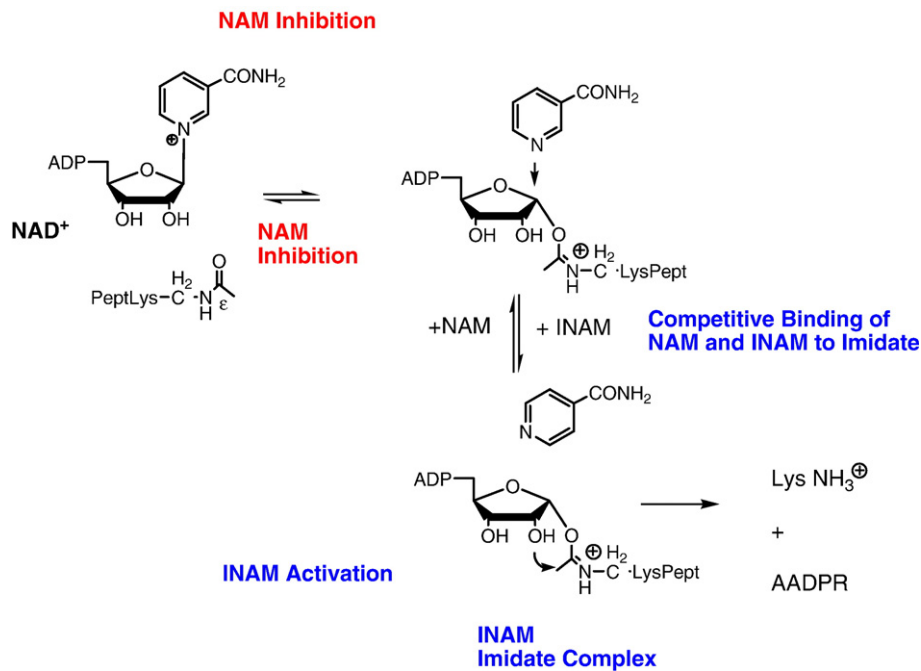
In the final section we consider a set of observations that have been made for sirtuin enzymes, specifically that they are able to catalyze stable ADP-ribosylation of proteins. Interestingly an ADP-ribosyltransferase activity of yeast Sir2 was determined prior to the discovery that  $NAD^+$  could also stimulate histone deacetylation [16,17]. Numerous reports have appeared since that time reporting sirtuin dependent ADP-ribosylation, although there is little agreement on its possible mechanism [10,18–22,73,74] nor are there definitive characterizations of the reaction products themselves. For example, the identities of residues that have been subject to this type of ADP-ribosylation have not been determined, thus complicating the assessments of the nature of this activity. Rather, most of the data obtained has been by observation of radiolabeled proteins when sirtuins and proteins are co-incubated with  $^{32}P$  NAD.

We will not provide an extensive review of the reported claims of ADP-ribosylation of sirtuins, but merely focus on several examples: SIRT6, *Trypanosoma brucei* Sir2, SIRT4 and *P. falciparum* Sir2. We will review each of these examples, taking a critical view of what has been learned and what remains undetermined about protein ADP-ribosylation by sirtuins.

### 7.1. SIRT6

The Guarente laboratory authored a very interesting report on Sirt6, which was reported to auto ADP-ribosylate itself [21], and interestingly enough in this same report was presented evidence that SIRT1 could catalyze ADP-ribosylation of histones [21]. Later workers have shown that SIRT6 also has an  $NAD^+$  dependent histone deacetylase activity [75–78]. ADP-ribosylation activities characterized by Liszt et al. were deduced by protein labeling with  $^{32}P$   $NAD^+$ , wherein it could be shown that label was transferred covalently to the respective proteins [21]. Skepticism about the ADP-ribosylation activity attributed to sirtuins has focused on the likelihood of ADP-ribosylation being a consequence of non-enzymatic reaction of ADP-ribose, which had been shown to react with lysine histones at pH 7.5 and increasingly at higher pH values [79,80]. This was in fact recently, re-demonstrated for histones by the Denu laboratory [18].

The observed SIRT6 auto-ADP-ribosylation was very weak, and did not exceed 20% of the available SIRT6 in solution after single hour incubations of protein and  $[^{32}P]NAD^+$  [21]. Interestingly the activity is stimulated by the presence of core histones [21]. The auto-ADP-ribosylation required enzymatic activity, as demonstrated using catalytically inactive mutants, and of some note the reaction appeared to be intramolecular [21]. This was determined by co-incubation of catalytically active and inactive SIRT6 together, in which it was demonstrated that the label could not be transferred to the catalytically inactive SIRT6. This argues that the active enzyme does



**Scheme 6.** Mechanism of activation of sirtuin deacetylation reaction by isonicotinamide (INAM). INAM is able to compete with nicotinamide (NAM) to occupy position above  $\beta$ -face of imidate which pushes deacetylation reaction forward, and avoids NAM induced chemical reversal of the imidate, which is inhibitory to the deacetylation reaction.

not transfer the label to another protein, but rather ADP-ribosylates itself with NAD<sup>+</sup> [21]. The amino acid residue that is so modified is unknown. The effect of this ADP-ribosylation on catalytic function of SIRT6 has not been determined, although the ubiquitous presence of NAD<sup>+</sup> in cells and the ability of SIRT6 to bind NAD<sup>+</sup> in the absence of other substrates suggests the possibility that this modification could occur *in vivo*, and could be of regulatory significance to SIRT6 itself. This would be especially true if the ADP-ribosylation occurred at or near the active site of the enzyme, which would be likely for an intramolecular mechanism.

### 7.2. *T. brucei* Sir2

This enzyme was cloned from a trypanosome that causes sleeping sickness in humans. This Sir2 is a telomere associated protein that was initially reported to have both deacetylase and ADP-ribosylation activities [22]. Reactions containing <sup>32</sup>P NAD<sup>+</sup> were used to show sirtuin-catalyzed ADP-ribosylation of BSA and histones at high pH = 8.8 [22]. Both deacetylase and ADP-ribosylation activities were confirmed independently by another laboratory [18]. The Denu laboratory investigation of this enzyme focused on the relative strengths of the two activities, and determined that the deacetylase activity was a much more robust activity than the ADP-ribosylation activity [18]. Addition of acetylated peptide was able to stimulate the observed ADP-ribosylation [18]. However, a priori if the mechanism of ADP-ribosylation is catalytic generation of AADPR or ADPR, followed by a non-enzymatic pathway of protein ADP-ribosylation from these compounds, then the stimulation by acetylpeptide is predicted. The question then is what is the likelihood that the enzyme itself directly mediates some or all of the observed ADP-ribosylation. It was concluded that part of the observed ADP-ribosylation was likely to be non-enzymatic, and that a major part was due to reaction of lysine residues that can be ADP-ribosylated by an imidate-dependent ADP-ribosylation mechanism, wherein the imidate transfers ADPR with  $\beta$ -stereochemistry in the final product [18]. Most importantly, the overall ADP-ribosylation activity was found to proceed 5 orders of magnitude slower than the deacetylase reaction [18]. With such a meager activity in evidence Kowieski et al. advocated skepticism

[18] about the potential biological relevance of reported protein ADP-ribosyltransferases of sirtuins, a view also advocated by another laboratory [73]. Nevertheless, the caveat still applies that the actual ADP-ribosylation target was not identified, and that a still unidentified substrate could provide a higher catalytic rate. Also possible is that the modification is supposed to be slow, and performs a currently unidentified regulatory function.

### 7.3. SIRT4

The most potentially interesting of the ADP-ribosylations identified to date is attributed to the enzyme SIRT4. SIRT4 ADP-ribosylation is implicated in an important biological process, that of regulating the activity of glutamate dehydrogenase (GDH) [20]. Glutamate dehydrogenase was known to be ADP-ribosylated *in vivo*, although the actual responsible ADP-ribosyltransferase was unidentified [81]. ADP-ribosylation was found to inhibit this enzyme which interconverts glutamate and  $\alpha$ -keto-glutarate. The latter metabolite is a part of the TCA cycle. Increased activity of GDH can increase mitochondrial activity by increasing the dominant upstream feeder of oxidative phosphorylation, the TCA cycle. Thus, ADP-ribosylation of GDH is predicted to down-regulate ATP synthesis in mitochondria, and to weaken ATP production from fuel sources such as glutamine and glutamate.

SIRT4 was found to interact directly with GDH in cells using immunoprecipitation, and it was also demonstrated that SIRT4 and GDH colocalize in tissues in mice and within cells, to mitochondria [20]. SIRT4 ADP-ribosylation of proteins was demonstrated by <sup>32</sup>P blots and this ADP-ribosylation was found to inhibit the activity of the enzyme [20] consistent with the effects of ADP-ribosylation identified previously [81]. The specific residue on GDH that is ADP-ribosylated has not been determined, and the catalytic mechanism for SIRT4 ADP-ribosylation also remains undetermined. However, SIRT4 knockout animals have increases in amino acid-induced insulin release, consistent with the concept that GDH activity is upregulated, which is predicted to increase ATP synthesis in the pancreatic  $\beta$ -cell [20]. ATP synthesis is an important metabolic process in nutrient sensing by these cells, and is directly coupled to the insulin releasing pathway.

SIRT4 has proven difficult to study in vitro, and a deacetylation activity for this human sirtuin isoform is thus far unreported. This suggests that this sirtuin could indeed be an ADP-ribosyltransferase. It is of interest to consider that in the PARP family, many of the PARP isoforms are in fact not polymerase enzymes but are protein mono-ADP-ribosyltransferases, providing an example where common scaffolds that catalyze ADP-ribosyltransfer can achieve divergent chemical functions. One laboratory concluded that it is unlikely that SIRT4 has an ADP-ribosyltransferase activity after finding no activity. However, with use of modified NAD<sup>+</sup>s, and no activity for SIRT4 determined, it is difficult to determine if the SIRT4 used in these studies was catalytically functional or compatible with the modified substrates [73].

#### 7.4. *P. falciparum* Sir2

The parasite *P. falciparum* is the pathogen most responsible for human malarial infections. It encodes two Sir2s implicated in regulating silencing in a large set of functionally redundant genes called *var* that encode isoforms of a virulence factor [82–84]. This virulence factor is expressed on the surface of infected erythrocytes. Persistence of malaria in the host can occur by a mechanism wherein *var* gene expression is switched so that the epitopic identity of the *var* product is changed, allowing the malarial parasite to avoid the host immunity. Thus, while one gene is typically expressed, all other *var* genes are kept silent. Deletion of Sir2 leads to dysregulated silencing for a large set of *var* genes [82–84]. It has been suggested that the activity most responsible for doing this is deacetylation of core histones within the parasite nucleus at sites of chromatin that encode these genes [82–84]. In fact independent laboratories have confirmed histone deacetylase activity for this enzyme [10,19].

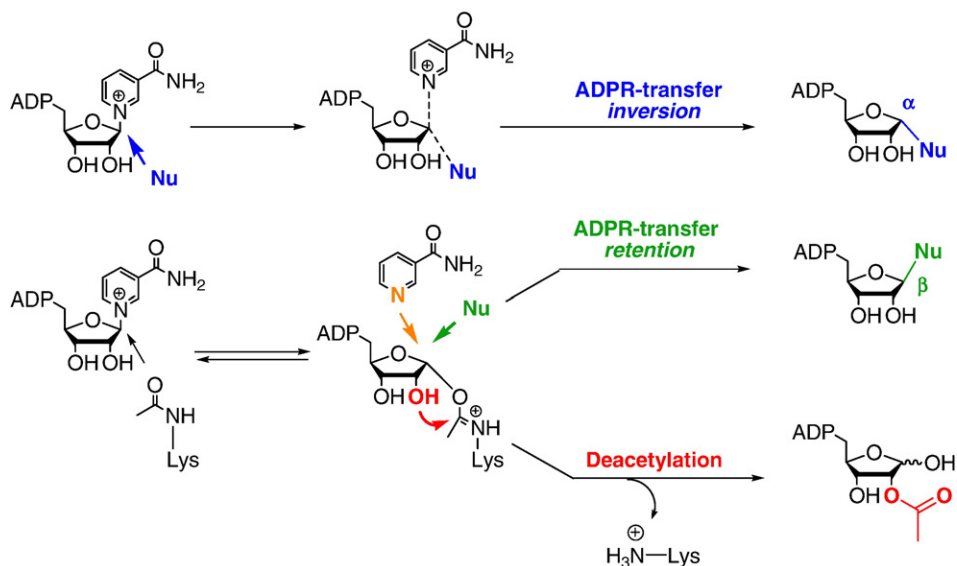
In addition to deacetylase activity, the enzyme appears to be able to ADP-ribosylate histones as shown by labeling of histones when incubated with <sup>32</sup>P NAD and recombinant PfSir2 at pH 8 [19]. This activity was found to be mostly inhibited by 10 mM nicotinamide but not completely [19], implying that the bulk of the ADP-ribosylation mechanism requires formation of the imidate, but another imidate independent mechanism could also be contributing. Given the known ability of histones to be ADP-ribosylated by ADPR, or AADPR [18,85] it is quite possible that the ADP-ribosylation process observed is non-

enzyme mediated ADP-ribosyltransfer of ADPR and AADPR, as we have previously discussed.

Interestingly, PfSir2 has an acetyllysine peptide dependent and an acetyllysine peptide independent NAD<sup>+</sup> glycohydrolase activity, both of which exceed the deacetylase activity of the enzyme [10]. These activities could be important in explaining the ADP-ribosylation of histones observed for this enzyme [19]. Our work found that the acetyllysine-dependent hydrolysis activity was much greater than the acetyllysine independent hydrolysis activity [10]. The acetyllysine-dependent activity proceeds through hydrolysis of the imidate, which only slowly proceeds forward in the deacetylation reaction [10]. The imidate hydrolysis could be inhibited by nicotinamide, which leaves a residual hydrolytic activity uninhibited which proceeds through the peptide independent route [10]. A scheme that accounts for all of these activities is shown in Scheme 7. The high production of ADPR from the imidate by this enzyme could explain the observed histone ADP-ribosylation and the observed incomplete sensitivity to inhibition by nicotinamide [19]. However, the alternative is still possible that some of the observed ADP-ribosylation is directly enzyme catalyzed [10,19]. This is a topic of current investigation in our laboratory.

## 8. Summary

We have reviewed here the varieties of chemistries that sirtuins catalyze and possible mechanisms by which these reactions proceed. Investigations into this class of enzymes continue to expand as new enzymatic activities are discovered and new biology develops. Important questions remain unresolved. This includes the mechanisms of sirtuin activation, especially by small molecules such as resveratrol. The mechanisms of ADP-ribosyltransfer are still in debate. In addition, mechanistic studies to further clarify the inner workings of these complex enzymes are ongoing in several laboratories to resolve questions about transition states and what regulates the chemical reactivities of imidate complexes. Importantly, elucidating the biochemical mechanisms that link these enzymes to NAD<sup>+</sup> metabolism is highly relevant to understanding how these enzymes function as transducers of metabolic information in cells. Undoubtedly, this research will impact ongoing medicinal chemistry efforts and drug development that target this very interesting enzyme class.



**Scheme 7.** General mechanistic scheme that accounts for ADP-ribosyltransfer with inversion, retention and deacetylation as observed for *Plasmodium falciparum* Sir2 enzyme. In addition, nicotinamide reaction from imidate causes inhibitions of imidate-solvolysis and deacetylation. Nicotinamide does not inhibit the direct ADP-ribosyltransfer reaction shown by top arrow.

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