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Mechanism of Sirtuin Inhibition by Nicotinamide: Altering the NAD+ Cosubstrate Specificity of a Sir2 Enzyme

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Summary

Sir2 enzymes form a unique class of NAD+-dependent deacetylases required for diverse biological processes, including transcriptional silencing, regulation of apoptosis, fat mobilization, and lifespan regulation. Sir2 activity is regulated by nicotinamide, a noncompetitive inhibitor that promotes a base-exchange reaction at the expense of deacetylation. To elucidate the mechanism of nicotinamide inhibition, we determined ternary complex structures of Sir2 enzymes containing nicotinamide. The structures show that free nicotinamide binds in a conserved pocket that participates in NAD+ binding and catalysis. Based on our structures, we engineered a mutant that deacetylates peptides by using nicotinic acid adenine dinucleotide (NAAD) as a cosubstrate and is inhibited by nicotinic acid. The characteristics of the altered specificity enzyme establish that Sir2 enzymes contain a single site that participates in catalysis and nicotinamide regulation and provides additional insights into the Sir2 catalytic mechanism.

Introduction

Sir2 enzymes, also known as sirtuins, comprise an ancient family of NAD+-dependent deacetylases (Imai et al., 2000; Landry et al., 2000; Smith et al., 2000) that are conserved from bacteria to humans and play a role in a wide variety of important biological processes, including transcriptional silencing (Brachmann et al., 1995), DNA recombination (Gottlieb and Esposito, 1989; McMurray and Gottschling, 2003) and repair (Bennett et al., 2001), apoptosis (Brunet et al., 2004; Luo et al., 2001; Vaziri et al., 2001), axonal protection (Araki et al., 2004), fat mobilization (Picard et al., 2004), and aging (Kaeberlein et al., 1999; Lin et al., 2000). Overexpression or hyperactivation of Sir2 enzymes increases lifespan in yeast (Kaeberlein et al., 1999), worms (Tissenbaum and Guarente, 2001), and flies (Wood et al., 2004), whereas deletion or inhibition of sirtuins shortens lifespan (Kaeberlein et al., 1999). Consistent with their diverse roles in biology, a variety of proteins are deacetylated by sirtuins, including histones (Imai et al., 2000), acetyl-coA synthetase (Starai et al., 2002), α-tubulin (North et al., 2003), myoD (Fulco et al., 2003), p53 (Luo et al., 2001; Vaziri et al., 2001), Foxo3 (Brunet et al., 2004; Motta et al., 2004), Ku70 (Cohen et al., 2004), and NF-κB (Yeung et al., 2004). Sirtuins deacetylate lysine residues in an unusual chemical reaction that allows them to be tightly regulated in the cell. The deacetylation reaction catalyzed by these enzymes is coupled to the cleavage of NAD+, yielding nicotinamide and O-acetyl ADP-ribose (OAADPr) along with the deacetylated lysine (Denu, 2003; Sauve et al., 2001; Sauve and Schramm, 2004). The nicotinamide product is a noncompetitive inhibitor of sirtuins (Bitterman et al., 2002), thereby allowing theses enzymes to be modulated by nicotinamide levels in the cell as well as by NAD+.

Sirtuin inhibition by nicotinamide has emerged as an important regulatory mechanism of sirtuin activity in vitro and in vivo. Budding yeast grown in the presence of added nicotinamide have defects in Sir2-mediated transcriptional silencing, increased rDNA recombination, and a significantly shorter lifespan (Bitterman et al., 2002). Depletion of nicotinamide by PNC1, a yeast enzyme that converts nicotinamide into nicotinic acid, is sufficient to activate Sir2 to extend longevity and prevent nicotinamide-induced inhibition of telomeric and rDNA silencing (Anderson et al., 2003; Gallo et al., 2004). Nicotinamide can inhibit p53 deacetylation by Sir2α upon DNA damage in mouse embryonic fibroblast cells (Luo et al., 2001). In human embryonic kidney cells, nicotinamide inhibits the deacetylation of histones H3 and H4 by SirT1, which leads to the loss of transcriptional repression mediated by COUP transcription factor-interacting proteins (Senawong et al., 2003). Interestingly, the sensitivity of yeast Sir2 to nicotinamide differs when it binds to Sir4 or when it is part of the RENT complex (Tanny et al., 2004), suggesting that different cellular partners can further modulate the inhibition of sirtuins by nicotinamide.

Nicotinamide inhibits the deacetylation activity of sirtuins by reacting with a reaction intermediate. The NAD+-dependent deacetylation carried out by sirtuins is thought to begin with a nucleophilic attack of the carbonyl oxygen of acetyl-lysine on the C1′ of the nicotinamide ribose (N-ribose) of NAD+, which results in release of nicotinamide and formation of a positively charged O-alkyl-amidate intermediate (Denu, 2003; Sauve et al., 2001; Sauve and Schramm, 2004) (Figure 1A). Subsequent steps in the reaction lead to the production of OAADPr and deacetylated lysine. If, however, nicotinamide binds to the enzyme when it contains the O-alkyl-amidate intermediate, nicotinamide can react with the intermediate in a process known as nicotinamide exchange, in which NAD+ and acetyl-lysine are reformed (Jackson et al., 2003; Sauve et al., 2001; Sauve and Schramm, 2003) (Figure 1A). High concentrations of nicotinamide increase the rate of the nicotinamide exchange reaction, which occurs at the expense of the deacetylation activity. The structural basis for the mechanism of this inhibitory side reaction and the way in which it is regulated by sirtuins are not

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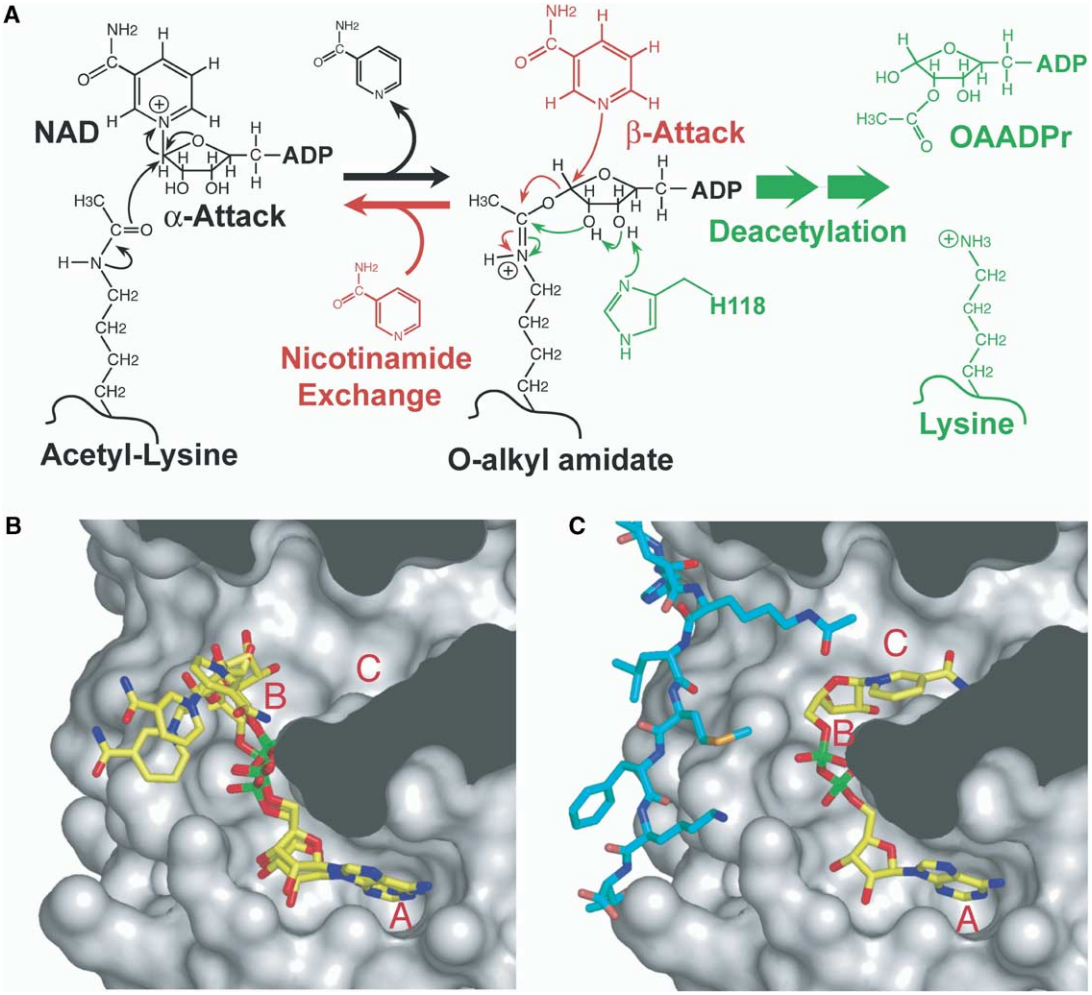

Figure 1. Overview of the Mechanism of the Sirtuin-Catalyzed NAD$^+$-Dependent Deacetylation and Nicotinamide Regulation

(A) The initial step of catalysis involves a nucleophilic attack of the carbonyl oxygen of acetyl-lysine on the C1' of the N-ribose of NAD$^+$ (black arrows). This step forms an O-alkylamidate intermediate that is consumed by the internal attack of its 2′ OH, activated by a conserved histidine, leading to deacetylation (green arrows), or by the attack of a nicotinamide molecule on the β face of its C1', which leads to nicotinamide exchange and inhibition of deacetylation (red arrows).

(B) In the absence of substrate peptide, NAD$^+$ can bind in the A and B pockets of sirtuins in alternative, nonproductive conformations.

(C) In the presence of a substrate peptide, NAD$^+$ binds in a precise productive conformation that buries its nicotinamide moiety in the highly conserved C pocket of sirtuins.

known. Crystal structures of sirtuins have shown that NAD$^+$ can bind in various "nonproductive" conformations that are not suitable for catalysis (Avalos et al., 2004; Min et al., 2001) (Figure 1B). However, simultaneous binding of NAD$^+$ and substrate peptide to the enzyme promotes binding of NAD$^+$ in a distinct "productive" conformation that places the nicotinamide ring in a highly conserved pocket, called the C pocket, where it is activated for catalysis (Avalos et al., 2004; Zhao et al., 2004) (Figure 1C). It has been a matter of debate as to whether the C pocket is also used in the exchange reaction that results in nicotinamide inhibition (Avalos et al., 2004; Bitterman et al., 2002), or if an alternative pocket serves as a nicotinamide binding site for this purpose (Zhao et al., 2004). The two models have different implications for the mechanisms of NAD$^+$ cleavage, nicotinamide exchange, and the regulation of sirtuins by nicotinamide.

In order to address the structural basis for the enzymatic mechanism by which nicotinamide regulates the deacetylation activity of sirtuins, we have determined crystal structures of sirtuins bound to nicotinamide and used the findings to engineer an altered specificity enzyme that can catalyze NAAD-dependent deacetylation and that is inhibited by nicotinic acid. The structures of archaeal Sir2Af2 and bacterial Sir2Tm reported here show that free nicotinamide binds in the same conserved C pocket in which NAD$^+$ is activated for catalysis, supporting a dual role for the C pocket in both nicotinamide exchange and deacetylation. In order to test whether the C pocket is indeed responsible for NAD$^+$ cleavage and nicotinamide exchange, we engineered a

Table 1. Crystallographic Statistics

Crystal	Sir2Af2	Sir2Tm
Diffraction Data		
Space group	$P2_12_12$	$P2_12_12_1$
Unit cell (Å)	105.1, 181.6, 79.0	46.1, 59.7, 106.1
Resolution (Å)	2.4	1.4
Measured reflections	422,928	394,258
Unique reflections	59,851 [4907]	58,321 [5,707]
Completeness (%)	98.1 [82.1]	99.4 [99.0]
Average I/σ (merged data)	19.7 [2.6]	26.1 [3.7]
Multiplicity	7.1	6.8 [4.6]
Mosaicity	0.40	0.46
R_{sym}[a] (%)	10.8 [48.1]	5.3 [41.4]
Refinement Statistics		
Resolution range (Å)	50.0–2.4	50.0–1.4
Reflections (I/σ > 0)	59,360	58,259
Working set	56,349	55,273
Test set (5.0%)	3011	2903
Total atoms	10,414	2219
Protein monomers	5	1
Nicotinamide molecules	5	1
Residues of p53 peptide	0	13
NAD^+ molecules	4	0
ADP-ribose molecules	1	0
Zn^{2+} atoms	9	1
PEG molecules	9	0
Sulfate ions	12	0
Water molecules	249	266
R_{factor}[b] (%)	20.9	18.5
R_{free}[c] (%)	25.5	20.2
B-factor (Å^2)	44.7	16.8
rmsd		
Bond lengths (Å)	0.010	0.010
Bond angles (°)	1.35	1.33

Values in brackets correspond to the highest resolution shell, 2.49–2.40 Å for Sir2Af2 and 1.45—1.40 Å for Sir2Tm.
[a] $R_{sym} = \Sigma|I - \langle I\rangle|/\Sigma I$, where I is the observed intensity and <I> the average intensity of multiple observations of symmetry-related reflections.
[b] $R_{factor} = \Sigma||Fo| - |Fc||/\Sigma|Fo|$, where Fo is the amplitude of the observed structure factor and Fc is the structure factor calculated from the model.
[c] R_{free} is the R_{factor} calculated with 5% of the reflection data randomly omitted from the refinement.

single point mutation in the C pocket that was designed to enable the enzyme to use NAAD in place of NAD^+ to deacetylate lysine residues. The mutant acquired the predicted NAAD-dependent deacetylation activity while retaining some NAD^+-dependent activity. Importantly, the mutant lost sensitivity to nicotinamide inhibition while acquiring sensitivity to nicotinic acid inhibition and the ability to catalyze nicotinic acid exchange. The results of these biochemical and structural studies allow us to propose a structure-based mechanism for the noncompetitive inhibition and regulation of sirtuins by nicotinamide and shed light on the mechanism of NAD^+ cleavage by sirtuins and their cosubstrate specificity.

Results

Structures of Sir2Af2 and Sir2Tm Bound to Nicotinamide

We have determined structures of archaeal and bacterial sirtuins bound to nicotinamide (Table 1). Two independent structures of *Archaeoglobus fulgidus* Sir2Af2 bound to nicotinamide were determined from a single crystal diffracting to 2.4 Å resolution. The crystal was grown in the presence of NAD^+, PEG400, and nicotinamide and was isomorphous to crystals grown in the absence of nicotinamide that were reported in a previous study (Avalos et al., 2004). The crystals contain five crystallographically independent monomers in the asymmetric unit that are in differently liganded states. Two of the five monomers in the asymmetric unit are ternary complexes containing nicotinamide bound in the C pocket of the active site of Sir2Af2. One of these structures (which we shall call "structure I") is also bound to NAD^+ in a nonproductive conformation (Figures 2A and 3A). Another ternary complex (which we call "structure II") also contains α-ADP-ribose bound in the active site (Figures 2B and 3B). The nicotinamide in structure II is well ordered, whereas that in structure I appears to be present at somewhat lower occupancy (Figures 2E and 2F and Table S1 available online with this article). The density corresponding to the carboxamide and part of the pyridine ring, along with the assumption that the molecule is planar, was used to position the structure I nicotinamide in the map. A third complex in the crystal, which contains NAD^+ bound in a nonproductive conformation, has density suggestive of nicotinamide bound in the C pocket at low occupancy and is therefore not used in our analysis. The

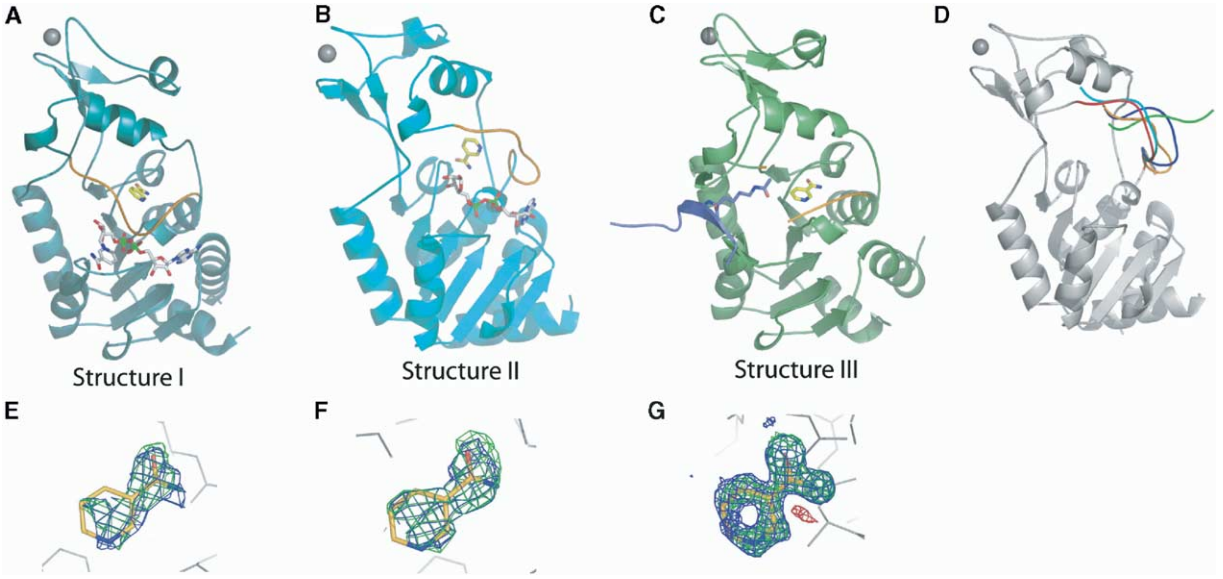

Figure 2. Crystal Structures of Sir2Af2 and Sir2Tm Bound to Nicotinamide

(A) Structure I: Sir2Af2 bound to nicotinamide (yellow) and nonproductive NAD^+ (white) with a highlighted flexible loop (orange).
(B) Structure II: Sir2Af2 bound to nicotinamide (yellow) and ADP ribose (white), flexible loop highlighted (orange).
(C) Structure III: Sir2Tm bound to nicotinamide (yellow) and acetylated p53 peptide (blue), flexible loop highlighted (orange).
(D) Superposition of the different conformations of the flexible loop observed in the known structures of Sir2Af2.
(E–G) Simulated annealing omit maps showing nicotinamide density. 2Fo – Fc map is contoured at 1σ (blue) and the Fo – Fc map at 3σ (green). Structure I (E), Structure II (F), and Structure III (G).

remaining two monomers in the asymmetric unit are bound to NAD^+ in a productive conformation that occupies the C pocket and to a PEG molecule that lies in the acetyl-lysine binding tunnel. These two complexes are virtually identical to one another as well as to the previously reported structure of these complexes determined from crystals grown in the absence of nicotinamide (Avalos et al., 2004). There are two additional nicotinamide molecules far from the active site that mediate apparently nonspecific interactions between monomers in the asymmetric unit and are not shown.

The 1.4 Å structure of Sir2Tm from the thermophilic bacterium *Thermotoga maritima* was determined in complex with nicotinamide and an acetylated p53 peptide, which is an in vitro substrate for Sir2Tm (structure III, shown in Figure 2C). A single, well-ordered nicotinamide molecule is bound to the C pocket of Sir2Tm (Figures 2C, 2G, and 3C). The peptide and acetylated lysine bind to the enzyme in a manner similar to that observed in the structure of Sir2Af2 bound to the same peptide (Avalos et al., 2002). We shall refer to the numbering of sirtuins residues according to the Sir2Af2 protein except where otherwise noted (Figure 3D).

Previous studies (Avalos et al., 2002; Finnin et al., 2001; Min et al., 2001) have shown that sirtuins contain a highly flexible region, called the flexible loop, which adopts a variety of conformations in different crystal structures and is in some cases partially disordered (Figure 2). This 15–30 amino acid flexible loop includes some of the most highly conserved residues in sirtuins, which form the front wall of the C pocket (Figure 3D). Structures I and II of Sir2Af2 bound to nicotinamide have ordered flexible loops that adopt different conformations (Figures 2A and 2B). In contrast, the structure of Sir2Tm bound to nicotinamide (structure III) has a partially disordered flexible loop from Arg34 to Ser44 (Figure 2C) but still shows strong electron density for the conserved Phe33 that forms part of the C pocket. These structures suggest that the flexible loop is influenced by NAD^+ binding, which can trigger the assembly and disassembly of the C pocket.

Nicotinamide Interactions in the C Pocket of Sirtuins

The structures presented here show that nicotinamide can bind to sirtuins simultaneously with peptide, ADP ribose, or NAD^+ that is in a nonproductive conformation. These ternary structures show that nicotinamide can bind in a collection of alternative positions that are anchored by the carboxamide group but leave the pyridine ring free to pivot inside the C pocket. The C pocket is a largely hydrophobic cavity that contains the most highly conserved residues in the catalytic core of sirtuins (Figure 3D). In all structures reported in this study, the carboxamide group of nicotinamide forms a conserved set of interactions that anchor the nicotinamide in the C pocket. The carboxamide amino of nicotinamide interacts with the side chain of a conserved aspartic acid in the C pocket (Asp103), whereas the carboxamide oxygen interacts with the backbone amino group of a conserved isoleucine (Ile102) (Figure 4). Additional conserved interactions with the carboxamide include van der Waals contacts with the side chains of Ile102 and Asn101, which are highly conserved. By contrast, the pyridine ring of nicotinamide can adopt a variety of conformations (Figure 4), as reflected in the different

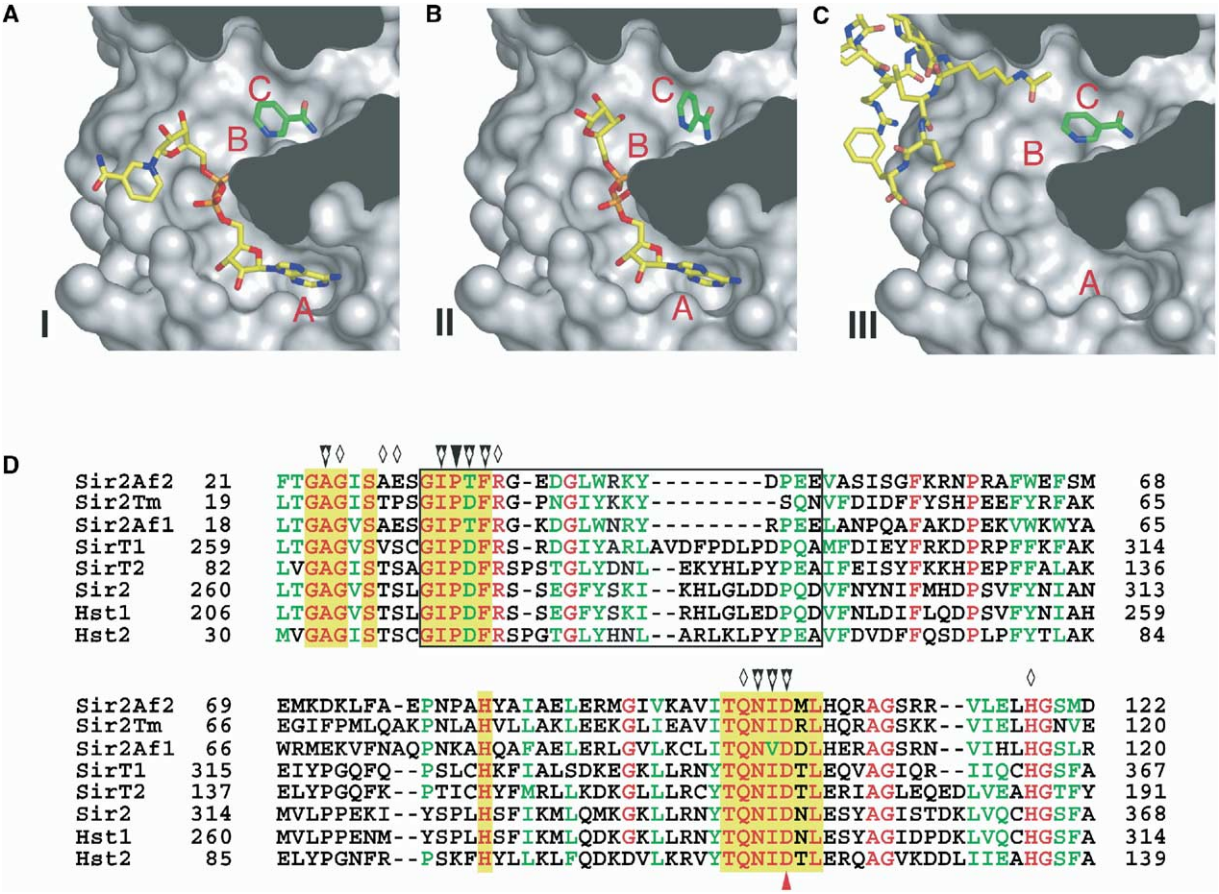

Figure 3. Surface Representation of the Sirtuin Active Site Pockets A, B, and C with Bound Ligands and Conservation of the C Pocket

(A) Structure I: Sir2Af2 bound to nicotinamide (green) and nonproductive NAD^+ (yellow).

(B) Structure II: Sir2Af2 bound to nicotinamide (green) and ADP ribose (yellow).

(C) Structure III: Sir2Tm bound to nicotinamide (green) and acetylated p53 peptide (yellow).

(D) Multiple sequence alignment of eight sirtuins showing highly conserved residues (red) and conserved residues (green). Residues that are involved in making the C pocket are highlighted in yellow, flexible loop is indicated by box. Residues that contact nicotinamide (black triangles) or contact NAD^+ bound in the productive conformation (white diamonds) are indicated above the alignment. The Asp that confers NAAD-dependent deacetylation activity and nicotinic acid sensitivity when mutated to Asn is marked below (red triangle).

positions of the ring in structures II and III and as suggested by the weaker electron density for the ring in structure I (Figure 2E). This variability is due not only to differences in the positioning of the pyridine ring inside the pocket but also to variations in the conformation of the flexible wall of the C pocket itself (Figure 2D). The differences in the C pocket flexible wall and, hence, the variability of its interactions with nicotinamide are due at least in part to variations in the conformation of the flexible loop. Consequently, the nicotinamide can make alternative interactions with the highly conserved residues Ala24, Ile32, Phe35, and Ile102 (Figures 4A–4C). Phe35 is of particular interest, because it displays the largest conformational differences within the C pocket and the greatest diversity of interactions with the nicotinamide (Figure 4). As discussed below, this side chain appears to play an important role in the catalytic mechanism.

The interactions of free nicotinamide with the C pocket have some similarities with those of the nicotinamide moiety of NAD^+ but also differ in important ways. In the structure of Sir2Af2 bound to productive NAD^+ (Figures 1C and 4D) (see also Avalos et al., 2004), the interactions of the carboxamide group of nicotinamide with Asp103, Ile102, and Asn101 are very similar to those formed by free nicotinamide (Figures 4A–4C). This similar interaction occurs even though the nicotinamide moiety, when part of NAD^+, is constrained by its glycosidic bond with the C1′ of the N-ribose (Figure 4D), which leads to a rotation of the NAD^+ carboxamide by approximately 150° from its most favorable conformation (Bell et al., 1997; Olsen et al., 2003). In contrast, the ring in free nicotinamide can adopt the most favored conformation, with the carboxamide nearly coplanar with the nicotinamide ring (Olsen et al., 2003). The N1 and C5 of nicotinamide cannot be distinguished in the electron density map, and the two possible low-energy rotamers are probably in equilibrium. However, when the N1 of nicotinamide is in the *cis* position with the carboxamide amino (N1-NH_2-*cis* rotamer), the N1

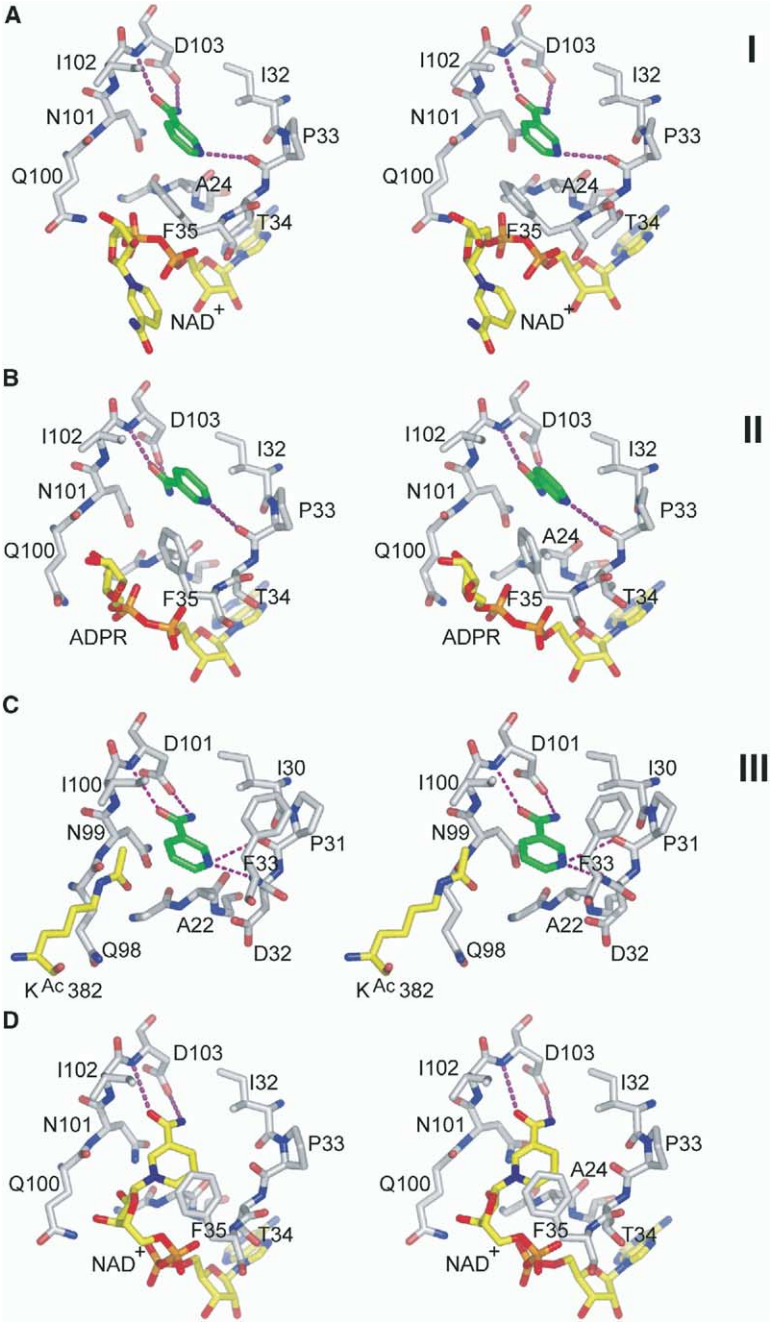

Figure 4. Stereo Figures of the Interactions of Nicotinamide and NAD+ Bound in the C Pocket of Sirtuins

The nicotinamide rotamer shown in (A)–(C) was chosen to maximize favorable interactions, as described in the text.

(A) Structure I: C pocket of Sir2Af2 (white) bound to nicotinamide (green) and nonproductive NAD+ (yellow).

(B) Structure II: C pocket of Sir2Af2 (white) bound to nicotinamide (green) and ADP ribose (yellow).

(C) Structure III: C pocket of Sir2Tm (white) bound to nicotinamide (green) and acetyllysine (yellow) from the p53 bound peptide.

(D) C pocket of Sir2Af2 (white) bound to productive NAD+ (yellow).

can form favorable contacts with conserved residues in the C pocket that could shift the equilibrium toward this rotamer. The N1-NH_2-*cis* rotamer, which is opposite to the rotamer in the productive NAD+ complex (Figure 4D), places the nicotinamide N1 3.3 Å from the backbone oxygen of the conserved Pro33 in structure II (Figure 4A), with a similar distance predicted for the less well-determined structure I (3.5 Å, Figure 4B). In structure III, the N1 of nicotinamide in this rotamer is 3.1 Å from the amino backbone of Phe33 (Sir2Tm numbering) and 3.5 Å from the backbone oxygen of Pro31 (Figure 4C). The alternative nicotinamide rotamer (N1-NH2-trans) does not permit the N1 to form significant interactions with the enzyme in any of the complexes. This suggests that, upon cleavage of NAD+, the pyridine ring of the nicotinamide may flip about its carboxamide group to relieve the stress induced on the NAD+ and form new interactions between the N1 of nicotinamide and residues deep inside the C pocket.

Engineering an Altered Cosubstrate Specificity in a Sirtuin

Our structural studies suggest that the C pocket is the sole binding site for free nicotinamide and thus the regulatory site for nicotinamide inhibition. To test the relevance of the observed interactions with nicotinamide

to the NAD^+-dependent deacetylation and nicotinamide exchange activities of sirtuins, we engineered a point mutant in the C pocket designed to alter the enzyme's cosubstrate specificity. Based on our structures, we reasoned that a mutation of the conserved aspartic acid in the C pocket (Asp101 in Sir2Tm) to asparagine would confer on the mutant an NAAD-dependent deacetylation activity, because the amino group of asparagine could hydrogen bond with the carboxylate of NAAD just as the wild-type (wt) aspartic acid hydrogen bonds with the amino group of NAD^+ (Figure 5A). If the C pocket is important not only for NAD^+ cleavage but also for nicotinamide inhibition, this mutation should also lead to a loss of sensitivity to nicotinamide inhibition and a gain of sensitivity to nicotinic acid inhibition due to an acquired ability to catalyze nicotinic acid exchange.

As expected, the Sir2Tm enzyme containing an Asp101 to Asn substitution (D101N) exhibits a significant loss in NAD^+-dependent deacetylation activity. The Sir2Tm-D101N mutant has significantly reduced catalytic power, with an apparent k_{cat} of (1.8 ± 0.1) × 10^{-3} s^{-1}, two orders of magnitude lower than the wt k_{cat} of 0.170 ± 0.006 s^{-1} (Figure 5B). In addition, the apparent K_M for NAD^+ of the mutant enzyme, 1.17 ± 0.18 mM, represents a 22-fold increase from the 53 ± 11 μM K_M value of the wt Sir2Tm (Figure 5B). A significant loss in NAD^+-dependent deacetylation activity was also found when the analogous substitution was introduced into Sir2Af2 (data not shown).

To test the effect of the C pocket aspartic acid mutation on cosubstrate specificity, we assayed the ability of the Sir2Tm-D101N mutant to deacetylate a p53-derived peptide by using NAAD as a cosubstrate instead of NAD^+. As predicted, mutation of the conserved aspartic acid in the C pocket to asparagine enables the mutant enzyme to carry out NAAD-dependent deacetylation, whereas the wt enzyme exhibits no detectable deacetylation activity with NAAD as a cosubstrate (Figure 5B). The acquired NAAD-dependent deacetylation activity of the Sir2Tm-D101N mutant, with an apparent k_{cat} of (1.1 ± 0.1) × 10^{-3} s^{-1} is comparable to the mutant enzyme's NAD^+-dependent activity. The mutant has an apparent K_M for NAAD of 617 ± 43 μM, which is approximately half its K_M for NAD^+ (Figure 5B). Furthermore, the apparent second order rate constant of the Sir2TmD101N mutant (k_{cat}/K_M) for NAD^+ of (1.5 ± 0.4) × 10^{-3} s^{-1} mM^{-1} is comparable to its apparent k_{cat}/K_M for NAAD of (1.8 ± 0.3) × 10^{-3} s^{-1} mM^{-1}, and they are three orders of magnitude lower than the wt apparent k_{cat}/K_M for NAD^+ of 3.2 ± 0.8 s^{-1} mM^{-1}. The D101N point mutation in Sir2Tm therefore results not only in a significant drop in catalytic power and loss of specificity for NAD^+ but also in the loss of cosubstrate selectivity, as the mutant appears to be unable to discriminate between NAD^+ and NAAD.

Mutation of the aspartic acid in the C pocket also alters the sensitivity of the enzyme to inhibition by nicotinamide and nicotinic acid. As predicted, the Sir2Tm-D101N mutant has significantly reduced sensitivity to nicotinamide inhibition. In fluorescence-based assays monitoring deacetylation activity, the mutation causes the IC_{50} of nicotinamide to increase an order of magnitude from 1.0 ± 0.2 mM in the wt to 9.0 ± 2.0 mM in the Sir2Tm-D101N mutant (Figure 5C). In addition, the NAD^+-dependent deacetylation activity of the Sir2Tm-D101N mutant can be inhibited by nicotinic acid, with an IC_{50} of 11.3 ± 3.3 mM, whereas nicotinic acid added to concentrations of up to 100 mM fail to inhibit the wt enzyme (Figure 5C). Importantly, the acquired NAAD-dependent deacetylation activity of the Sir2Tm-D101N mutant is also inhibited by nicotinic acid, as well as nicotinamide, with IC_{50} values of 6.2 ± 2.0 mM and 14.6 ± 3.4 mM, respectively (Figure 5C). The nicotinamide and nicotinic acid IC_{50} values for both the NAD^+- and NAAD-dependent activities of the Sir2Tm-D101N mutant are similar, suggesting that the mutant has not only lost sensitivity to nicotinamide but also its ability to discriminate between nicotinamide and nicotinic acid (Figure 5C). Similar results were obtained when deacetylation activity was assayed by monitoring NAD^+ consumption (see Supplemental Data).

To assay directly the nicotinamide and nicotinic acid exchange activities of the wt and mutant Sir2Tm, we incubated the enzymes with unlabeled NAD^+ and acetylated peptide in the presence of ^{14}C-labeled nicotinamide or nicotinic acid and separated the products by thin layer chromatography (TLC) (Figure 5D). As expected from the inhibition experiments, both enzymes could catalyze formation of labeled NAD^+ through the nicotinamide exchange activity. Most remarkably, the Sir2Tm-D101N mutant is able to synthesize ^{14}C-labeled NAAD by using the base exchange reaction to catalyze replacement of the unlabeled nicotinamide ring of NAD^+ with ^{14}C-labeled nicotinic acid, which the wt enzyme cannot do. This striking new enzymatic activity must be the consequence of a nicotinic acid exchange activity conferred by the D101N mutation, consistent with its acquired NAAD-dependent deacetylation activity and sensitivity to nicotinic acid inhibition.

Discussion

Regulation of Sirtuins by Nicotinamide

We have shown that sirtuins contain a multifunctional site that is directly involved in NAD^+ cleavage, base exchange activity, and nicotinamide regulation. This conclusion rests on our crystallographic studies showing free nicotinamide bound in this site, known as the C pocket (Min et al., 2001), and on our ability to introduce a point mutation in the C pocket that alters the cosubstrate specificity and inhibitor sensitivity of the enzyme. Our striking finding that a single Asp → Asn change in the C pocket enables the enzyme to catalyze NAAD-dependent deacetylation, be inhibited by nicotinic acid, and synthesize NAAD from NAD^+ and nicotinic acid strongly supports the role of the C pocket as the sole nicotinamide binding site in sirtuins. We had previously shown that the same C pocket binds the nicotinamide moiety of NAD^+ when this cosubstrate binds to the enzyme in a productive conformation that is poised for catalysis (Avalos et al., 2004). Our findings therefore argue against the presence of a second regulatory binding site for nicotinamide, as has been proposed (Zhao et al., 2004). The details of the interactions of sirtuins with both NAD^+ and free nicotinamide provide a mechanism for regulating the enzyme's activity in response to these molecules.

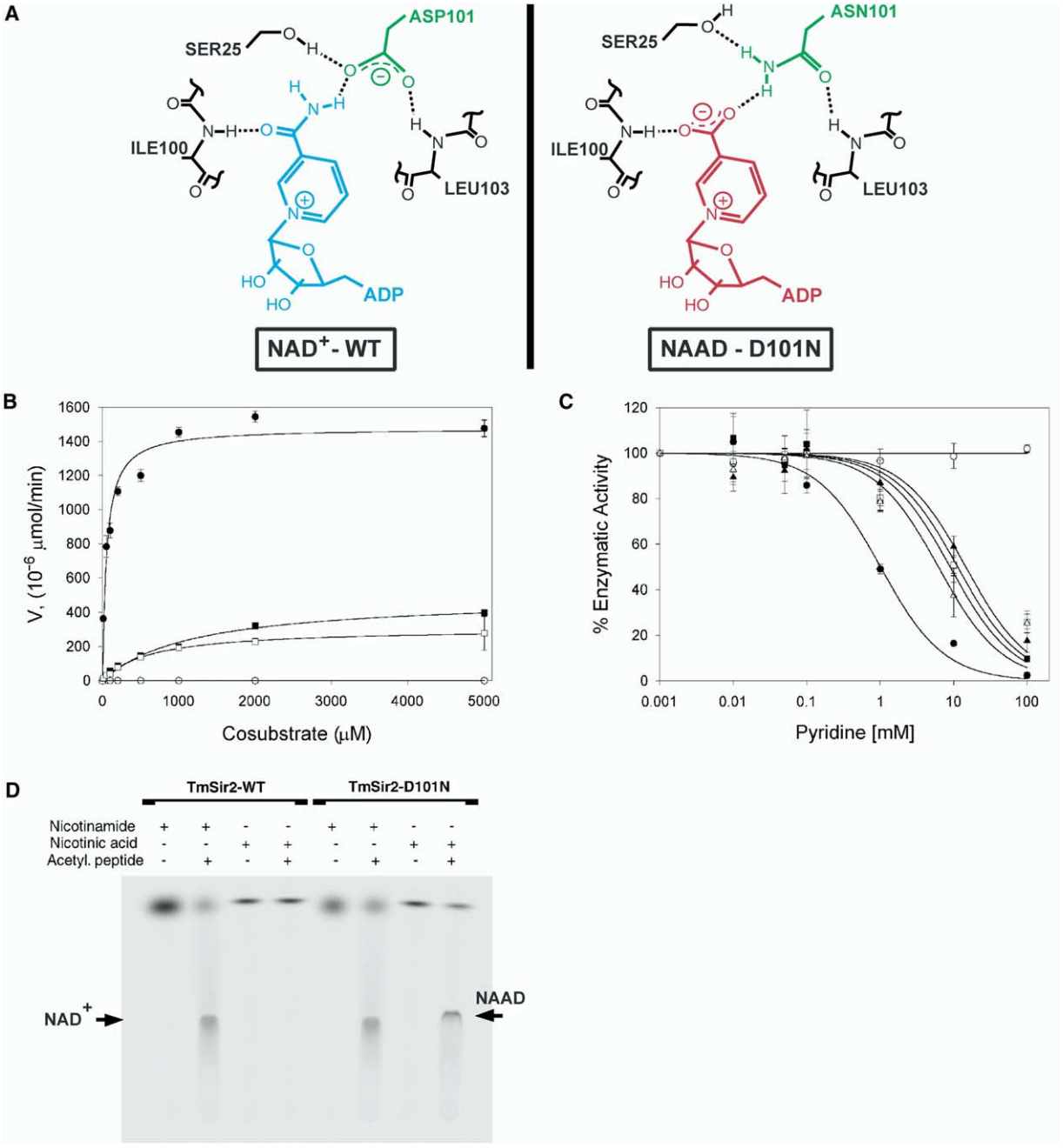

Figure 5. Effects of the D101N Mutation on the Enzymatic Activity and Regulation of Sir2Tm

(A) Schematic representation of the H bond network observed between productive NAD^+ (blue) and Asp101 (green, left) and the hypothetical H bond network between productive NAAD (red) and the substituted Asn101 (green, right).

(B) Initial rates of NAD^+-dependent (black) and NAAD-dependent (white) deacetylation activities of the wild-type (wt) (circles) and D101N mutant (squares) Sir2Tm. Error bars represent one SD of experiments done at least in triplicate.

(C) Inhibition of Sir2Tm wt's NAD^+-dependent deacetylation activity (circles) and the Sir2Tm-D101N mutant's NAD^+-dependent (squares) and NAAD-dependent (triangles) deacetylation activities by nicotinamide (black) and nicotinic acid (white). Error bars represent one SD of experiments done at least in triplicate.

(D) TLC plate showing the products of the base exchange reactions of Sir2Tm wt and D101N mutant with unlabeled NAD^+, acetylated p53 peptide, and either ^{14}C-nicotinamide or ^{14}C-nicotinic acid. The migration of NAD^+ and NAAD are marked with arrows.

Our results highlight the role of the C pocket, and the conserved aspartic acid it contains, in conferring the specificity for both NAD^+ and nicotinamide that are critical for sirtuin function in vivo. Sirtuins have very high specificity for NAD^+, as relatively small changes in the nicotinamide ring of NAD^+ result in the most dramatic

losses of binding affinity and reactivity, as is the case for NADH and NAAD (Schmidt et al., 2004). Similarly, the fine specificity of sirtuins for nicotinamide in the base exchange reaction makes them insensitive to inhibition by other metabolites, especially nicotinic acid (Schmidt et al., 2004). The latter property is particularly relevant to the role of the yeast PNC1 enzyme in transcriptional silencing and replicative lifespan, where it is believed to relieve inhibition of Sir2 by converting nicotinamide into nicotinic acid (Anderson et al., 2002; Anderson et al., 2003; Sandmeier et al., 2002). The ability of sirtuins to discriminate between NAD^+ and NAAD as cosubstrate, as well as between nicotinamide and nicotinic acid as inhibitors, is therefore crucial for the activity and regulation of sirtuins in the cell.

If sirtuins contain a single nicotinamide binding site that functions in both deacetylation and base exchange, why is nicotinamide inhibition noncompetitive (Bitterman et al., 2002; Jackson et al., 2003; Sauve and Schramm, 2003)? One possible explanation is that the affinity of nicotinamide for the C pocket is very low in the absence of an O-alkyl amidate intermediate and that we were able to see it in our electron density maps only because the crystals were grown at high nicotinamide concentrations (see Experimental Procedures). Indeed, there is some evidence that high, nonphysiological concentrations of nicotinamide (approaching 0.1 M) may inhibit the deacetylation reaction in a manner that is competitive with NAD^+ (our unpublished data).

We had previously suggested (Avalos et al., 2004) that binding of an acetylated peptide to sirtuins promotes binding of NAD^+ in a strained conformation required for catalysis, which is supported by the nicotinamide interactions in the structure of Hst2 bound to carba-NAD^+ and acetylated peptide (Zhao et al., 2004) and is consistent with recent kinetic studies showing that NAD^+ binds after acetylated peptide (Borra et al., 2004). Our structures showing that the nicotinamide product binds in the C pocket in conformations that are energetically lower than that of the productive NAD^+ supports this model of ground state destabilization. However, the increase in K_M for cosubstrate observed in the TmSir2-D101N mutant suggests that other factors involving Asp101, probably transition-state stabilization, are also important for NAD^+ cleavage. The 100-fold loss in catalytic power of the mutant may be due to its inability to distort the carboxamide of NAD^+ or the carboxylate of NAAD. The out-of-plane rotation of the carboxamide could play a role in catalysis by disrupting the electronic resonance between the carboxamide and the pyridine ring, which could alter the electronic distribution on the pyridine ring in a way that weakens the glycosidic bond and promotes NAD^+ cleavage. It is possible that the NAAD-dependent deacetylation activity of the mutant is not as robust as the wt enzyme's NAD^+-dependent activity because of inherent differences in the charge and electronic distribution between the positively charged NAD^+ and the electronically neutral NAAD.

The structures of Sir2Af2 and Sir2Tm with bound nicotinamide reveal how sirtuins sequester the nicotinamide that is cleaved from NAD^+ in the initial step of catalysis, reducing base exchange and thus promoting deacetylation. The enzymatic reaction begins with cleavage of nicotinamide from NAD^+ and formation of a reactive O-alkyl amidate intermediate (Sauve et al., 2001) (Figure 1A). Because the fast rate of nicotinamide condensation with the O-alkyl amidate intermediate is similar to the rate of NAD^+ cleavage (Jackson et al., 2003), the enzyme needs to release the cleaved nicotinamide quickly or sequester it in order to attenuate the immediate reversal of NAD^+ cleavage, as has been proposed for ADP ribosyltransferases (Han et al., 1999). A comparison of complexes containing cleaved nicotinamide with those containing productive NAD^+ suggests how the enzyme could entrap the cleaved nicotinamide. Although a common set of hydrogen bonding interactions with the carboxamide always anchors nicotinamide in the C pocket, free nicotinamide binds in a low-energy conformation in which the carboxamide and pyridine ring are almost coplanar, whereas a less favorable out-of-plane rotamer is found in the productive complex with NAD^+ (Avalos et al., 2004; Zhao et al., 2004). This suggests that the positively charged nicotinamide moiety of NAD^+ shifts from a distorted, high-energy conformation to a collection of low-energy states upon NAD^+ cleavage. The enzyme may simply use the energy gained from the release of strain upon NAD^+ cleavage to allow the pyridine ring of nicotinamide to rotate—or "flip"—along the carboxamide group dihedral until it adopts its most stable rotamer, allowing the N1 of nicotinamide to make favorable interactions with conserved residues in the C pocket.

The kinetic significance of the alternative modes of nicotinamide binding remains to be determined. It is formally possible that the energy released by relieving NAD^+ strain is enough to expel nicotinamide out of the active site completely and that the alternative nicotinamide binding conformations are the consequence of the high nicotinamide concentrations used in the crystallizations. Nevertheless, the observed alternate modes of nicotinamide binding could be used by the enzyme to entrap nicotinamide and attenuate the reformation of NAD^+.

Our findings allow us to propose a structure-based mechanism for the regulation of sirtuins by nicotinamide (Figure 6). The initial steps in the NAD^+-dependent deacetylation reaction lead to formation of the O-alkyl amidate intermediate and free nicotinamide, which has been cleaved from NAD^+ (Figure 6, step i and ii). The pyridine ring of the cleaved nicotinamide can flip to more favorable conformations in which the pyridine ring is free to adopt a variety of positions that allow its N1 to interact with residues inside the C pocket, whereas the carboxamide remains anchored through hydrogen bond interactions (Figures 4 and 6, step iii). The bound nicotinamide can exist in either an entrapped or a reactive state that are interchangeable through a flipping mechanism (Figure 6, steps ii and iii). In the entrapped state, the nicotinamide places the N1 on the distal side of the C pocket, preventing reaction with the O-alkyl-amidate intermediate and allowing the deacetylation reaction to proceed (Figure 6, step iii). If the pyridine ring flips about its carboxamide group, the N1 of nicotinamide can move into a position to react with the C1′ of the alkyl-amidate intermediate, leading to base exchange (Figure 6, step ii). Based on crystal structures of sirtuins bound to acetylated peptide

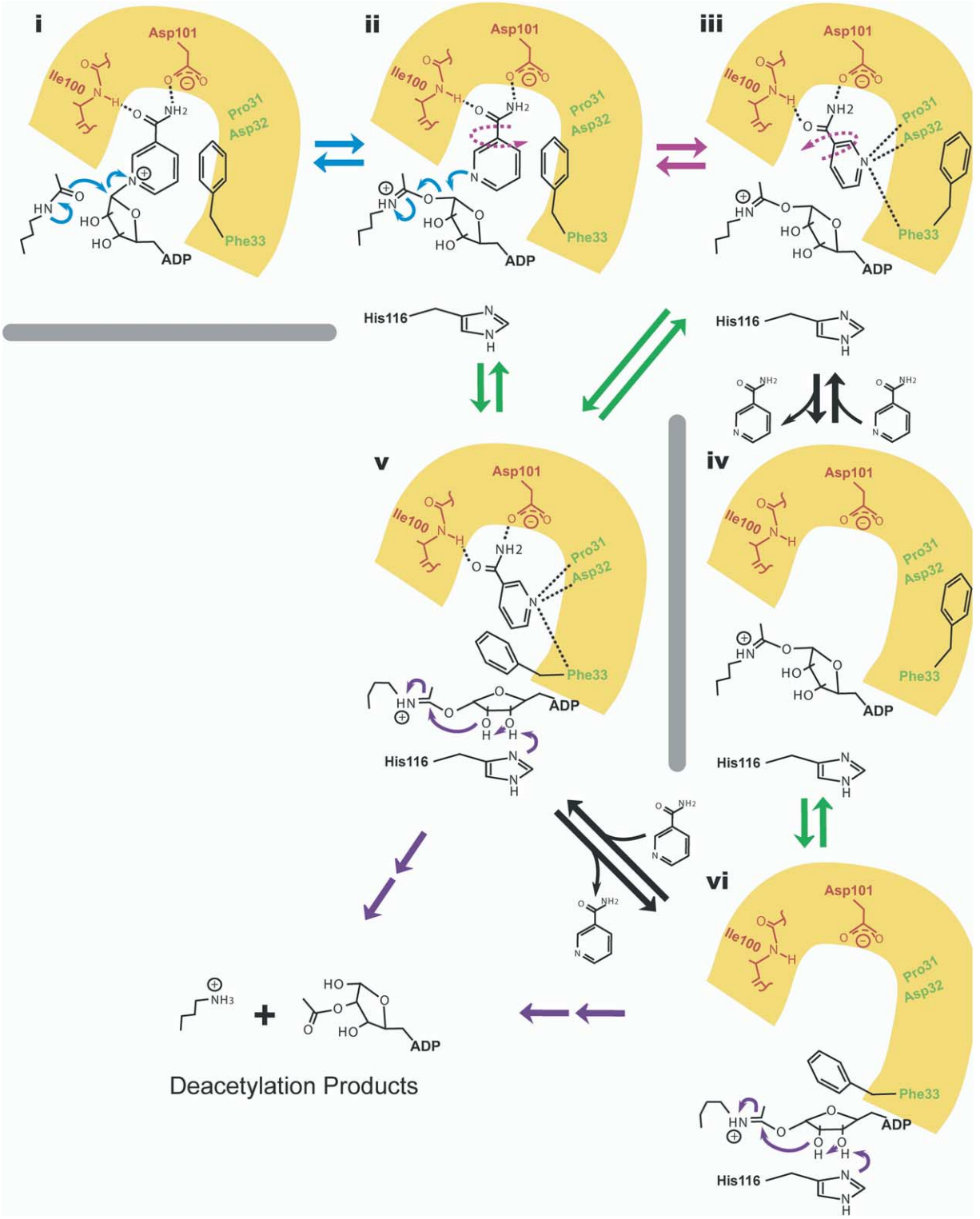

Figure 6. Structure-Based Mechanism of the Enzymatic Activity and Regulation of Sirtuins

Step i: NAD^+ binds in a productive conformation in the C pocket (yellow), making hydrogen bonds with the rigid wall of the pocket (red residues), which promotes NAD^+ cleavage (blue arrows). Step ii: The produced O-alkyl amidate intermediate in the extended conformation can reform NAD^+ with a reactive nicotinamide in the C pocket unless the nicotinamide is entrapped by flipping (pink arrows) or the intermediate shifts to a contracted conformation (green arrows). Step iii: The entrapped nicotinamide buries its N1 against residues in the flexible wall of the C pocket (green residues), thereby preventing it from reacting with the O-alky amidate, even if this intermediate is in the extended conformation. From this position, nicotinamide can either flip out of entrapment (pink arrows) or be released by the enzyme (black arrows).

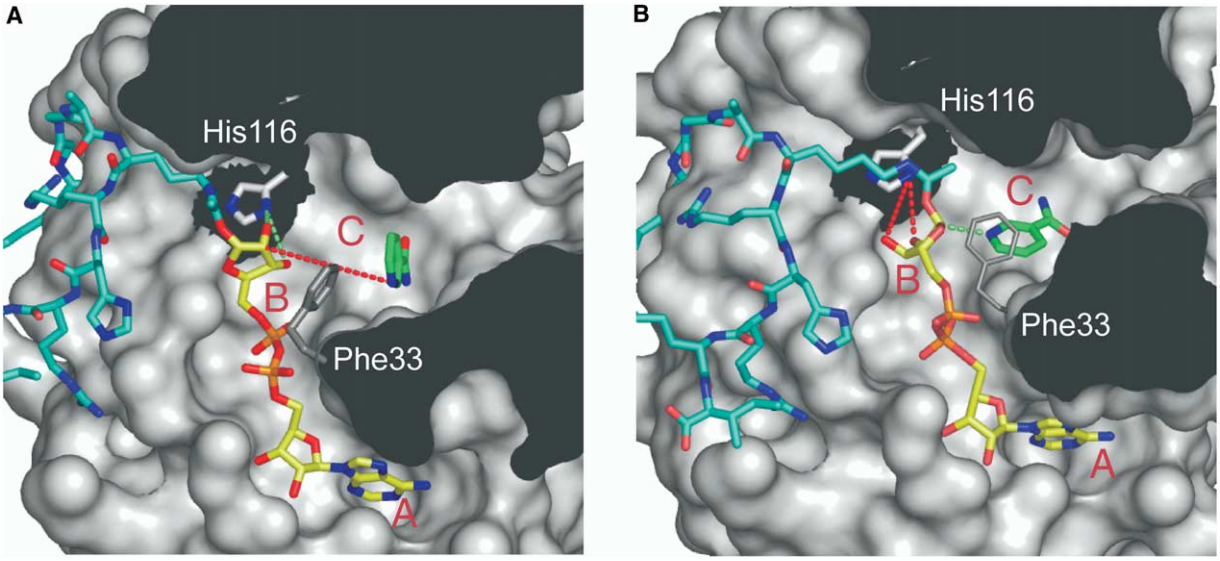

Figure 7. Proposed Alternative Conformations of the O-alkyl Amidate Intermediate

(A) The contracted conformation of the O-alkyl amidate intermediate is too far from the nicotinamide in the C pocket (red dotted line) and is shielded by Phe33. However, in this conformation, the 2′ and 3′ OH groups of the intermediate are at a suitable distance and orientation from His116 to promote deacetylation (green dotted lines). This conformation was modeled from the structure of Hst2 bound to acetylated histone peptide and 2′O-acetyl-ADP ribose.

(B) The extended conformation of the O-alkyl amidate intermediate is further from His116 (red dotted lines), closer to the reactive nicotinamide in the C pocket (green dotted line), and exposed by Phe33, thereby promoting nicotinamide exchange. This intermediate and the reactive nicotinamide were modeled from the position of NAD^+ in the productive conformation and the acetyl-lysine position in the structures of Sir2Af2 and Sir2Tm bound to acetylated peptide.

(Avalos et al., 2002), 2′O-acetyl ADP ribose (Zhao et al., 2003) and NAD^+ (Avalos et al., 2004), we propose that the alkyl-amidate intermediate exists in two alternative conformations, either contracted or extended (Figure 7), that could influence whether bound nicotinamide reacts with the intermediate to reform NAD^+. The contracted conformation would favor deacetylation, because its C1′ is far from nicotinamide bound in the C pocket, and the N-ribose 2′ and 3′ OH groups are near His116 (Sir2Tm numbering), which catalyzes subsequent steps in the deacetylation reaction (Denu, 2003; Sauve et al., 2001; Sauve and Schramm, 2004) (Figures 6, step v and 7A). Furthermore, the O-alkyl amidate intermediate in the contracted conformation is protected from the solvent and nicotinamide bound in the C pocket by Phe33 (Figures 6, step v and 7A). When the intermediate is in the extended conformation, it is further from His116, closer to the C pocket, and exposed by Phe33 (Figures 6, step ii and 7B), making it more likely to react with the bound nicotinamide and reform NAD^+ and acetyl-lysine. The conformational variability of the flexible loop (Figure 2D) may allow nicotinamide release by allowing the partial disassembly of the C pocket. Rebinding of nicotinamide can lead to the inhibitory base exchange reaction if the rebinding occurs when the enzyme is bound to the O-alkyl amidate intermediate.

Control of Sirtuin Activity by Small Molecules and Protein Partners

Our findings regarding the central regulatory role of the C pocket suggest a structural basis for the action of small molecules that either inhibit or stimulate sirtuin activity. Molecules that can bind in the C pocket and prevent NAD^+ from adopting its productive conformation could act as competitive inhibitors, whereas those that are able to participate in the flipping mechanism and react with the O-alkyl amidate intermediate would probably act as noncompetitive inhibitors of the sirtuin deacetylation reaction. The latter instance is likely to be the case in the inhibition of Hst2 by thionicotinamide and 3-hydroxypyridine, two nicotinamide analogs that can participate in pyridine base exchange reactions in Hst2 (Jackson et al., 2003). Conversely, molecules that bind in the C pocket while the enzyme is bound to the O-alkyl amidate intermediate but are either inert or unable to flip for activation could stimulate the deacetylation reaction by reducing the inhibitory effect of intracellular nicotinamide. The latter mechanism is consistent with the observed stimulatory effect of isonicotinamide (Sauve et al., 2005), which also binds in the C pocket (J.L.A., M. Tang, and C.W., unpublished data).

The proposed role of the flexible loop in nicotinamide binding and release suggests a mechanism by which

Step iv: The empty C pocket will have certain affinity for nicotinamide in the cell. Step v: By shifting to a contracted conformation, the O-alkyl amidate intermediate is shielded by Phe33 and brings its 2′ and 3′ OH groups closer to His116, both of which promote deacetylation (purple arrows). Step vi: The empty C pocket will have certain affinity for nicotinamide, but not necessarily the same as in step iv.

protein partners may modulate sirtuin activity. Although the carboxamide group of nicotinamide is anchored to the rigid inner wall of the C pocket, the pyridine ring makes a variety of contacts with residues in the flexible loop, whose conformation is highly variable in the different sirtuin structures. Binding of proteins or small molecules that affect the conformation or mobility of the flexible loop could therefore affect deacetylation activity and nicotinamide exchange. This feature of the protein could therefore be exploited to design or select for proteins or small molecules that increase or decrease sirtuin activity by interacting with the flexible loop. Interestingly, approximately one-third of the flexible loop comprising the flexible wall of the C pocket contains some of the most conserved residues in sirtuins, whereas the remainder of the flexible loop is one of the most variable regions of the catalytic core of sirtuins (Figure 3D). This peculiarity hints at the possibility that the variable region of the flexible loop participates in interactions that various sirtuins make with different cellular partners, each of which may have distinct consequences for enzyme activity. This hypothesis could explain the differing nicotinamide sensitivity of yeast Sir2 when it forms different protein complexes as well as the stimulation of Sir2 activity when it is bound to Sir4 (Tanny et al., 2004). It is also possible that the binding of sirtuins to certain substrates could similarly affect the flexible loop and, hence, enzyme activity. Interestingly, the human SirT1 protein interacts with a variety of cellular proteins (Brunet et al., 2004; Motta et al., 2004; Picard et al., 2004; Takata and Ishikawa, 2003; Vaquero et al., 2004; Vaziri et al., 2001) and is sensitive to nicotinamide in vitro. The various interactions of human sirtuins with their cellular partners have the potential to play an important role in regulating this important class of deacetylase enzymes by modulating their catalytic activity and nicotinamide sensitivity.

Experimental Procedures

Protein Expression and Purification

The Sir2Af2 from *Archaeoglobus fulgidus* and Sir2Tm from *Thermotoga maritima*, wt, and mutant enzymes were expressed in *E. coli* and purified as described previously (Smith et al., 2002). The mutagenesis of Sir2Af2 and Sir2Tm was carried out by using QuikChange (Stratagene).

Crystallization of Sirtuin Complexes with Nicotinamide

Purified Sir2Af2 enzyme was dialyzed into 10 mM HEPES (pH 7.4) with 1mM Tris (2-carboxyethyl)-phosphine TCEP and concentrated to 20 mg/ml. Prior to crystallization trials, 5.5 μl of a neutralized solution of 100 mM NAD^+ was added to 50 μl of the Sir2Af2 solution to a final concentration of 10 mM NAD^+. Crystals were grown by the hanging drop method in 0.1 M HEPES (pH 7.4) with 1.8 M ammonium sulfate, 1% PEG400, and 70 mM nicotinamide and formed in space group $P2_12_12$ with unit cell dimensions a = 105.1 Å, b = 181.6 Å, and c = 79.0 Å. Crystals were flash frozen in nujol oil (Plough Inc.) and stored in liquid nitrogen until use.

Purified Sir2Tm enzyme was dialyzed into 10 mM HEPES (pH 7.4) and concentrated to 16 mg/ml, and 5 μl of a 40 mM solution of acetylated p53 peptide (372-KKGQSTSRHK-K[Ac]-LMFKTEG-389) was added to a final concentration of 10 mg/mL Sir2Tm, and 4 mM peptide. Crystals were grown by the hanging drop method in 100 mM CHES (pH 9.6) with 16% PEG 3350 and 100 mM nicotinamide and formed in space group $P2_12_12_1$ with unit cell dimensions a = 46.1 Å, b = 59.8 Å, and c = 106.2 Å. Crystals were flash frozen in mother liquor containing 20% ethylene glycol and stored in liquid nitrogen until use.

Structure Determination

Diffraction data on Sir2Af2 crystals were recorded at beamline X25 of the National Synchrotron Light Source (NSLS) with a Quantum CCD detector and reduced with HKL2000 (Otwinowski and Minor, 1997) and CCP4. The crystals are isomorphous to the structure of Sir2Af2 bound to NAD^+ and ADP ribose (Avalos et al., 2004) (accession number 1S7G), which was used to calculate phases, compute a difference Fourier map with CNS (Brunger et al., 1998), and locate the electron density corresponding to the nicotinamide and additional NAD^+ molecules. The density corresponding to nicotinamide could not otherwise be accounted for by bound water or ions. The structure was built by using Xfit (McRee, 1999) and refined with simulating annealing and energy minimization in CNS (Brunger et al., 1998). The positions of the nicotinamide molecules, as well as flexible regions in the proteins, were verified with simulated annealing omit maps (Brunger et al., 1998) (Figures 2E and 2G). The final model contains five Sir2Af2 monomers, four molecules of NAD^+, one of ADP ribose, five of nicotinamide, nine of PEG, nine zinc atoms, 12 sulfates, and 249 waters. The crystallographic statistics are summarized in Table 1. Values for protein and ligand B factors are shown in the Supplemental Data (Table S1).

Diffraction data on Sir2Tm crystals were collected at NSLS beamline X4A and processed as described above. The structure was solved by molecular replacement with MOLREP (Vagin and Teplyakov, 1997) using as a search model the structure of the Sir2Tm apoenzyme (J.L.A. and C.W., unpublished data) broken into two segments: the Rossmann domain and the small domain. The structures were built by using Xfit (McRee, 1999) and refined with simulating annealing and energy minimization in CNS (Brunger et al., 1998). The position of the nicotinamide was verified with simulated annealing omit maps (Figure 2G). Residues Arg34–Ser44 of the flexible loop showed no corresponding electron density, indicating that this region is disordered. The final model contains one monomer of Sir2Tm, 13 residues from the acetylated p53 peptide, one nicotinamide, one zinc atom, and 266 waters. Crystallographic statistics are summarized in Table 1.

Measurement of Deacetylation Activity Using a Fluorolabeled Peptide

The deacetylation activity was measured by using the Fluor de Lys-SirT1 assay (Biomol), using a peptide containing amino acids 379-RHK-K(Ac)-382 of p53 as substrate. The initial rates of the NAD^+- and NAAD-dependent deacetylation activities of Sir2Tm wt and D101N mutant enzymes were measured at different concentrations of dinucleotide. The reactions were carried out at 37°C in a 50 μl reaction volume containing 50 mM Tris, (pH 8), 50 mM NaCl, and 400 μM flourolabeled peptide (~10 times K_M). The enzyme concentration of the wt enzyme was 80 μg/mL and that of the D101N mutant was 2.56 mg/ml. The NAAD-dependent activity of the wt enzyme was undetectable even at enzyme concentrations of 2.56 mg/ml and incubation periods of 4 hr. Reactions were done at least in triplicate. The data were fitted to the Michaelis-Menten equation by using SigmaPlot to obtain the kinetic constants. This assay was also used to measure the inhibition by nicotinamide and nicotinic acid, with 500 μM NAD^+ for the wt enzyme and 2 mM of NAD^+ or NAAD for the mutant. The initial rates were measured at different concentrations of nicotinamide and nicotinic acid, and the reaction conditions were the same as above, except the D101N mutant enzyme was used at 1.6 mg/ml. The data were fitted to Equation 1 by using SigmaPlot (SYSTAT) to calculate the IC_{50} values:

$$v_I = v_0(1 - [I / (IC_{50} + I)]), \quad (1)$$

where v_0 is the initial rate of the uninhibited reaction and v_I is the initial rate of the reaction at concentration I of inhibitor.

TLC Detection of Base Exchange Activities

The nicotinamide and nicotinic acid exchange reactions were carried out in 20 μl containing 50 mM sodium phosphate, (pH 8.0), 0.5 mM DTT, 2 mM NAD^+, and 500 μM of the acetylated p53 peptide used in the crystallographic studies and NAD^+ consumption assay.

The reactions contained 0.1 mM of ^{14}C-nicotinamide (Moravek Biochemicals MC1427, 53 mCi/mmol) or ^{14}C-nicotinic acid (Moravek Biochemicals MC1324, 54 mCi/mmol), with 80 μg/mL of the wt enzyme or 2.5 mg/mL of the D101N mutant. After incubation at 37°C for 3 hr, 8 μl of each reaction was spotted on a Polygram SIL-G TLC plate (Machevey-Nagel, 20 × 20). The plate was developed in a preequilibrated chamber with 80:20 ethanol:2.5 M ammonium acetate. After chromatography, the plate was air dried and exposed to a phosphorimaging screen overnight. The reaction using 2.5 mg/ml of wt enzyme and ^{14}C-nicotinic acid showed no detectable formation of NAAD.

Supplemental Data

Supplemental Data include Supplemental Experimental Procedures and two figures and are available with this article online at http://www.molecule.org/cgi/content/full/17/6/855/DC1/.

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Accession Numbers

Coordinates for the structures of Sir2Af2 bound to NAD^+, ADP ribose, and nicotinamide (accession number 1YC2) and the structure of Sir2Tm bound to acetylated p53 peptide and nicotinamide (accession number 1YC5) have been deposited in the Protein Data Bank.