Use of Substrate Analogs and Mutagenesis to Study Substrate Binding and Catalysis in the Sir2 Family of NAD-dependent Protein Deacetylases

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The Sir2 family of enzymes is highly conserved throughout evolution and functions in silencing, control of life span, apoptosis, and many other cellular processes. Since the discovery of the NAD-dependent deacetylase activity of Sir2 proteins, there has been a flurry of activity aiming to uncover the mode of substrate binding and catalysis. Structural and biochemical studies have led to several proposed reaction mechanisms, yet the exact catalytic steps remain unclear. Here we present in vitro studies of yeast homolog Hst2 that shed light on the mechanism of Sir2 proteins. Using acetyl-lysine substrate analogs, we demonstrate that the Hst2 reaction proceeds via an initial SN2-type mechanism with the direct formation of an ADP-ribose-acetyl-lysine intermediate. Kinetic studies further suggest that ADP-ribose inhibits the Hst2 reaction in a biologically relevant manner. Through biochemical and kinetic analyses of point mutants, we also clarify the role of several conserved core domain residues in substrate binding, stabilization of the ADP-ribose-acetyl-lysine intermediate, and catalysis. These findings bring us a few steps closer to understanding Sir2 activity and may provide a useful platform for the design of Sir2-specific inhibitors for analysis of Sir2 function and possibly therapeutic applications.

The Sir2 (also known as Sirtuin) family of protein deacetylases catalyzes the removal of an acetyl moiety from the ε-amino group of lysine residues in an NAD-dependent manner. The reaction proceeds via an ordered Bi-Ter reaction mechanism, requiring the formation of a ternary enzyme-NAD-acetyl-lysine substrate complex prior to any catalysis (1). The reaction occurs in two major steps: 1) cleavage of the high energy glycosidic bond in β-NAD⁺ that joins the ADP-ribose (ADPR) moiety to nicotinamide and 2) transfer of the acetyl group to the 2′-hydroxyl of ADPR to form a unique product, 2′-O-acetyl-ADPR (OAADPR) (2, 3).

The Sir2 family is broadly conserved from archaea to eukaryotes (4), and members exhibit great diversity in their subcellular localization and protein targets (5). Nuclear Sir2 proteins deacetylate histones (6) and transcription factors, such as p53 (7). Other Sirtuins are cytosolic and even mitochondrialy localized (5), acting on tubulin (8) and as yet unknown substrates. Sir2 enzymes function in a variety of cellular processes, including gene silencing, control of life span, maintenance of genomic stability, and apoptosis (5). Several homologs have been crystallized, and their structures have been solved, including Archaeoglobus fulgidus Sir2 Af1 (9, 10) and Sir2 Af2 (11, 12), yeast Hst2 (13, 14), and human SIRT2 (15). These studies reveal two conserved core domains, a large domain consisting of a Rossmann fold-like structure and a small domain that contains a structural zinc ion. The active site is located at the interface between the large and small domains, with the acetyl-lysine substrate inserting into a tunnel on one side while NAD binds a large groove on the opposite side. These studies suggest that the two substrates meet with the acetyl group positioned below the α face of nicotinamide ribose. Sequence analyses show a high degree of conservation of residues that make contact with the two substrates and those that are contained within the active site (Fig. 1).

Several mechanisms by which the Sir2 proteins catalyze the NAD-dependent deacetylase reaction have been proposed (2, 3, 9–12, 14). However, it remains unclear whether the initial step, cleavage of nicotinamide from NAD, occurs via an S_n1- or S_n2-type mechanism (Fig. 2). In an S_n1-type mechanism, the nicotinamide group leaves prior to attack by the acetyl carbonyl oxygen. This kind of release of nicotinamide from NAD would result in formation of an oxacarbenium ADPR intermediate. C1′ of nicotinamide ribose is then more susceptible to nucleophilic attack by the acetyl carbonyl oxygen. Alternatively, in an S_n2-type mechanism, the acetyl carbonyl oxygen directly attacks C1′ of nicotinamide ribose, displacing the nicotinamide group, directly forming a 1′-O-peptidylamide-ADPR (acetyl-lysine-ADPR) intermediate. This is followed by the deacetylation event, where the general consensus is that the 2′-oxygen of nicotinamide ribose is activated and attacks the acetyl carbonyl carbon, forming a cyclic intermediate. This intermediate is finally resolved by nucleophilic attack by a “structurally conserved” catalytic water molecule (2, 9–11, 14), to produce OAADPR and the deacetylated lysine products (Fig. 2).

Despite numerous structural and mutagenesis studies of different Sir2 family members, it is not absolutely known which residues are involved in substrate binding and/or specific catalytic steps in the Sir2 deacetylase reaction. It is also uncertain what role the acetyl-lysine substrate plays in the initial nicotinamide cleavage step. Yeast homolog Hst2 has been the subject of structural, mutagenesis, and kinetic studies (1, 9, 13–16) given its high degree of conservation and in vitro catalytic activity. In vivo, Hst2 appears to play similar roles as Sir2; it affects silencing (4) and has recently been shown to mediate life span extension (17). Thus, to better understand the Sir2 enzymatic mechanism, we analyzed reactions with acetyl-lysine substrate analogs and determined the effect of mutating several highly conserved residues in Hst2. Our results strongly suggest an initial S_n2-type reaction mechanism for Hst2, in which an acetyl-lysine-ADPR intermediate is directly formed, and further clarify the role of several highly conserved core domain residues in Hst2 function.
MATERIALS AND METHODS

Sequence Alignment—Sir2 protein sequences were obtained from ExPASy (available on the World Wide Web at ca.expasy.org/uniprot). Primary accession numbers are as follows: Sir2 Af1, O28597; Sir2 Af2, O30124; Hst1, P53685; Hst2, P53686; Sir2, P06700; SIRT1, Q96EB6; SIRT2, Q8IXJ6. Alignment was performed using ClustalW (18) at the Pole Bioinformatique Lyonnais Network Protein Sequence Analysis (19) Web site (npsa-pbil.ibcp.fr/cgi-bin/npsa_automat.pl?page=npsa_clustalw.html).

Structure Diagrams—Hst2 ternary product inhibition complex (14) (Protein Data Bank code 1Q1A) and NAD-Sir2 Af2 complex (12) (Protein Data Bank code 1S7G) structural data were obtained from the RCSB Protein Data Bank (available on the World Wide Web at www.pdb.org/), and illustrations were created with RasWin Molecular Graphics program (R. Sayle).

Expression and Purification of Proteins—Yeast HST2 open reading frame was amplified by PCR using oligonucleotides (5'/3') CATGGG-TACCATGTCTGTTTCTACCGC and TGGACTCGAGTTATTCTT-GAAGTGGATATCCCGGCAGCCACCATAAAGATTAC, S36A (GTGGGTGCCGGGATAGCCACTTCTTGGGATAC and GTATCCACAAGAAGTGGCTATCCCGGCACCAC), R45A (GGGATAC-GAGACTTCGCATCTCCGGGAACTGGC and GCCAGTTCCCGGAGATGGAAGTGAACGTATC), N116A (GTTTTGAAGAGATTATATACCCAGGCTATAGACACTTTAGAAAGACAGGCC and GGCTGTCTTTCTAAAGTGTCTATAGCCTGGGTATATACTCTTCAAAAC), H135A (CTGATCATCGAGCCGCTGGCAGTTTTTGCTCAC and GTGAGCAAAACTGCCAGCGGCCTCGATGAC), K178A (GTGCGGTGAACTGGTAGCGCCGGCAATAGTTTTTTTG and CAAAAAAAACTATTGCCGGCGCTACCAGTCACCGGAC), V228A (GGCACCTCACTAGCCGCTTATCCCTTTGCGTCTC and GAGACGCAAAGGGATAAGCGGCTAGTGAGTGCC), P230A (CTCACTAGCCGTTTATGCCTTTGCGTCTCTGCCC and GGGCAGAGACGCAAAGGCATAAACGGCTAGTGAGTGCC, and GCCACCGTTTCCCAAGGGCACAGAAGCTCTTTTAC), D263A (GATTTTAAAGCTAACAAGAGGCCTACAGCTTTAATAGTTCATCAGTACTCGG and CCGAGTACTGATGAACTATTAAAGCTGTAGGCCTCTTGTTAGCTTTAAAATC), and H338A (CAGCGTCTCAATGGCGCTGACAGCGATGAAGATG and CATCTTCATCGCTGTCAGCGCCATTGAGACGTG). All mutations were confirmed by sequence analysis. Plasmids were transformed into BL21(DE3) cells, and the cells were grown at 37°C to log phase. HST2 expression was induced by the addition of 0.1 mM isopropyl 1-thio-D-galactopyranoside from 4 h to overnight at 18°C. Cells were harvested and lysed by sonication and protein purified by nickel-chelate chromatography.

Lysates were bound in 50 mM HEPES-KOH, pH 8.0, 300 mM NaCl, 10 μM ZnCl2, 1 mM 2-mercaptoethanol, and protease inhibitor mixture (Roche

FIGURE 1. Sequence conservation in the catalytic core domain of Sir2 family members. The ClustalW multiple alignment program was used to align the yeast Sir2, Hst2, and Hst1, human SIRT1 and SIRT2, and A. fulgidus Sir2 Af1 and Sir2 Af2 protein sequences. Positions at which five or more sequences are identical, strongly similar, or weakly similar are indicated by dark, medium, and light shading, respectively. The numbering above corresponds to the Hst2 protein sequence. Residues 35–63 comprise the β1-α2 loop in Hst2 that forms the roof of the NAD binding cleft (14). Residues that have been mutated in Hst2 are indicated by an asterisk.
FIGURE 2. Proposed reaction mechanisms for Sir2 enzymes. Schematic diagram showing proposed steps involved in the Sir2 reaction mechanism. (Residue numbers refer to the Hst2 sequence.) The initial nicotinamide cleavage step can proceed via an $S_N1$- or $S_N2$-type mechanism. With an $S_N1$-type mechanism, nicotinamide dissociates, leaving behind an oxacarbenium ADP-ribose intermediate, which is then attacked by the acetyl carbonyl oxygen to form a 1'-O-$\alpha$-peptidylamidate-ADP-ribose intermediate. In an $S_N2$-type...
RESULTS

For Sir2 enzymes, there is an absolute requirement for the acetyl-lysine substrate to be bound before any catalysis of NAD occurs (23). However, since there are currently no structures with a Sir2 enzyme in a true Michaelis biubstrate ternary complex or with a trapped intermediate, it is unclear whether the acetyl-lysine substrate directly participates in the initial nicotinamide cleavage step or whether particular catalytic residues within the enzyme act at this stage. To address this question, we assayed the activity of Hst2 with acetyl-lysine substrate analogs. We have previously shown that acetyl-poly-L-lysine is a substrate for Hst2 (22); thus, we prepared analogs of this compound, acetyl-poly-L-ornithine, and carbamoyl-poly-L-lysine, depicted in Fig. 3A.

Precise Positioning of the Substrate Acetyl Group Relative to NAD Is Essential for Catalysis—Ornithine, an amino acid with a side chain one methylene shorter than lysine, can be acetylated in a similar manner to lysine, and this allows one to study the importance of the length of the lysine side chain in substrate binding and catalysis. As seen in Fig. 3B, poly-L-ornithine and acetyl-poly-L-ornithine efficiently out-competed the acetyl-poly-L-lysine substrate for Hst2 activity, suggesting that these compounds bind to the Hst2 active site. We next asked whether Hst2 can deacetylate this analog, by assaying activity in the presence of [14C]NAD (radiolabeled on adenine) and analyzing the reaction by TLC. Fig. 3D shows the formation of OAADPR upon reaction with acetyl-poly-L-lysine substrate; however, we did not detect OAADPR or any reaction intermediates with the acetyl-poly-L-ornithine analog. These results demonstrate that simple binding of an acetyl-substrate is not sufficient for the initial nicotinamide cleavage step to occur. It further suggests that the acetyl group must be precisely positioned within the active site for catalysis to take place and that close proximity of the acetyl group to NAD is required for release of nicotinamide from NAD. Consistent with these results, crystal structure studies of Hst2 in a ternary complex with an acetyl-histone H4 peptide and OAADPR (product) showed a distance of 3.1 Å between the acetyl carbonyl oxygen and C1’ of nicotinamide ribose (14). Similarly, modeling of an acetylated peptide onto the Sir2 A2-NAD structure indicated a distance of 2.8 Å between C1’ of nicotinamide ribose and the acetyl carbonyl oxygen (12). Thus, these results suggest that the acetyllysine substrate plays a critical role in nicotinamide cleavage and point toward the direct participation of the acetyl group in cleavage of nicotinamide from NAD.

To examine the proximity issue further, we created another analog of acetyl-poly-L-lysine by reacting the ε-amino group of lysine with ammonium cyanate to produce carbamoyl-poly-L-lysine. This compound has an amide group in place of the acetyl group and should be sufficiently similar to acetyl-poly-L-lysine to be positioned in correct proximity to NAD. As with the ornithine peptides, carbamoyl-poly-L-lysine out-competed acetyl-poly-L-lysine for Hst2 activity, indicating that it binds to the enzyme (Fig. 3C). We then assayed for activity with carbamoyl-poly-L-lysine and found that, in contrast to the reaction with acetyl-poly-L-ornithine, which did not result in cleavage of NAD, and acetyl-poly-L-lysine, which produced OAADPR, reaction with this substrate analog produced a slower migrating product, consistent with ADPR (23) (Fig. 3D).

To confirm the identity of the observed products, reaction components were separated by HPLC and analyzed by electrospray ionization mass spectrometry (Fig. 3E). Compounds of molecular weight 123 (not
FIGURE 3. Activity of Hst2 with acetyl-lysine substrate analogs. 
A, chemical structures of acetyl-lysine (i), acetyl-ornithine (ii), and carbamoyl-lysine (iii). 
B and C, deacetylase assays using Hst2 with [14C]acetyl-poly-L-lysine in the presence of various amounts of competitors: poly-L-lysine (○), poly-L-ornithine (●), acetyl-poly-L-ornithine (△), acetyl-poly-L-lysine (▲), carbamoyl-poly-L-lysine (□). Percentage activity is relative to the amount of activity with no competitor. 
D, activity with substrate analogs. Hst2 reactions were incubated for 1 h at

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Substrate Analogs and Mutagenesis of Hst2
shown) and 664, which correspond to nicotinamide and unreacted NAD, respectively, were detected in both the acetyl-polyl-lysine and carboxamidopoly-lysine reactions, indicating that nicotinamide was indeed released from NAD. A product of molecular weight 603 was also identified in the acetyl-polyl-lysine reaction, which is the expected molecular weight of OAADPR. In the carboxamidopoly-lysine reaction, a compound of molecular weight 559 was detected; this is the expected molecular weight of ADPR. Thus confirming that ADPR is a product of the reaction with carboxamidopoly-lysine. Only unreacted NAD was observed in the reaction with acetyl-polyl-ornithine (data not shown).

Since it was unclear whether the observed ADPR was a direct result of nicotinamide cleavage (as would occur in an S_N1-like mechanism) or was due to hydrolysis of a reaction intermediate or product, we also examined whether any cleavage or modification of the carboxamidopoly-lysine analog occurred. Reaction of Hst2 with carboxamidopoly-lysine radiolabeled on the carboxamidogroup revealed no removal of the carboxamidogroup in a gel-based assay (Fig. 4) or by TLC (data not shown). This suggests that the observed ADPR is not a result of decarboxamidoylation of the substrate and hydrolysis of a resultant carboxamidoadPR product. Since the additional nitrogen of the carboxamidamide probably increases the nucleophilicity of its carbonyl oxygen, it is probable that the formation of a peptidylamidate-ADPR intermediate does occur, suggesting that the observed ADPR is a result of hydrolysis of an unstable carboxamidopoly-lysine-ADPR intermediate. The presence of the amide substituent in the carboxamidopoly-lysine analog may prevent attack by the 2'-OH of nicotinamide ribose by decreasing the electrophilicity of the carbonyl carbon. Since no other reaction products were detected, clearly the reaction with carboxamidopoly-lysine did not proceed beyond the release of nicotinamide.

**ADPR-ribose Is a Dead End Inhibitor of Hst2 Deacetylase Activity**—We further tested whether exogenous ADPR was reactive in the presence of an acetyl-lysine substrate. Hst2 was reacted with ADPR and [14C]acetyl-polyl-lysine, and the reaction was analyzed by a gel-based assay (Fig. 4) and TLC (data not shown). No change in the radiolabeled substrate was observed, indicating that the substrate was not deacetylated in the presence of ADPR. ADPR is thus nonproductive in the deacetylation reaction, suggesting that the presence of the nicotinamide group is essential for attack by the acetyl carbonyl oxygen. These results suggest that an oxacarbenium ADPR intermediate is not formed and thus support an S_N2-type cleavage of nicotinamide from NAD. Moreover, the ADPR generated by reaction with carboxamidopoly-lysine would thus abort the deacetylation reaction.

Although ADPR is nonproductive in the deacetylation reaction, kinetic studies demonstrate that ADPR inhibits Hst2 activity, suggesting that it binds the active site (Table 1, supplemental Fig. S1). Kinetic analyses showed that ADPR inhibits acetyl-polyl-lysine in a pure noncompetitive manner, whereas it displayed mixed inhibition with NAD. Pure noncompetitive inhibition ($K_i = 1.54 \pm 0.12$ mM) with the acetyl-lysine substrate is expected, since ADPR could be bound at a separate site. The mixed inhibition pattern seen with NAD highlights the predominantly competitive component ($K_i = 173 \pm 33$ mM) that results from ADPR directly competing with NAD for its binding site as well as the effect this compound has on the catalytic rate ($K_i = 4.85 \pm 1.02$ mM).

**The Role of Conserved Core Domain Residues in Substrate Binding and Catalysis**—All Sir2 family members contain several blocks of highly conserved residues within the catalytic core domain (Fig. 1). Structural studies reveal that these blocks contain residues that make up the substrate binding pockets, making direct or indirect contact with NAD, the acetyl-lysine substrate, or both (12, 14). However, due to the variation observed in NAD conformation in different Sir2 homolog binary complexes (9, 10, 12, 14) and the frequently inferred position of the nicotinamide moiety that results from a lack of electron density (9, 10, 14), the function of many invariant core residues is not yet clearly understood. One report comparing the structures of binary enzyme-NAD and the ternary product inhibition complexes suggested that no structural rearrangement of NAD occurs upon binding and catalysis of the acetyl-lysine substrate (14). This is in contrast to other structural studies that proposed that in the absence of the acetyl-lysine substrate, NAD binds to the enzyme in a nonproductive conformation and that only binding of the acetyl-lysine substrate allows NAD to adopt a productive (high energy state) conformation (9, 11, 12). Since the carboxamidopoly-lysine substrate analog allows for the separation of the preliminary nucleophiles cleavage step from the remaining of the reaction, we used this effect to study the role of specific residues in catalysis. Thus, we mutated several highly conserved residues within the catalytic core domain of Hst2 (Fig. 1) and compared the activity of these mutants with the acetyl-polyl-lysine substrate and carboxamidopoly-lysine analog (Fig. 5A). The positions of mutated residues relative to OAADPR and the acetyl-lysine substrate in the Hst2 product inhibition complex structure (14) and relative to NAD in the NAD-Sir2 A2 productive complex structure (12) are illustrated in supplemental Fig. S2. In addition, we performed enzyme kinetic analyses with these mutants to determine the effect on the turnover number ($k_{cat}$) and the Michaelis constant ($K_m$) for NAD and the acetyl-polyl-lysine substrate (supplemental Fig. S3). No significant changes in the secondary structure of these mutants were detected using far UV circular dichroism analyses (data not shown). Combining our analyses with available structural and mutational data, we are able to clarify the role of these residues in substrate binding and catalysis. These results are summarized in Table 2 and discussed below.

**Residues Involved in NAD Binding but Not Catalysis**—The block of residues containing Gly 1152–Ser 1158 is extremely well conserved and is

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**Table 1**

| Varied substrate | Type of inhibition | $K_i$ (mM) |
|-----------------|-------------------|------------|
| Acetyl-polyl-lysine | Noncompetitive | 1.54 ± 0.12 |
| NAD | Mixed | 0.173 ± 0.033 | 4.85 ± 1.02 |

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*room temperature in the presence of [14C]NAD and the indicated substrate: acetyl-polyl-ornithine (7), polyl-ornithine (2), carboxamidopoly-lysine (3), polyl-lysine (4), acetyl-polyl-lysine (5), or none (6). Reaction products were resolved by TLC and analyzed by phosphorimaging. E, analysis of the products of acetyl-polyl-lysine (Ac) and carboxamidopoly-lysine (Cb) reactions with NAD by electro spray ionization mass spectrometry. Products were first separated by HPLC on a C18 column. amu, atomic mass units.*
located in an interesting region of the enzyme, the β1-α2 loop, which is highly mobile and conformationally flexible, adopting different conformations in the apoenzyme or when bound to NAD (12–14, 24). In the ternary Hst2 product inhibition complex, the 1–2 loop forms the roof of the NAD binding cleft, with residues Ala33 and Thr37 making hydrogen bond interactions with the phosphate and adenine groups, respectively, and Gly34 making Van der Waals contact with the adenine rings (14). In the productive NAD-Sir2 Af2 complex structure (12), the strictly conserved Gly23-Ala24-Gly25 motif (corresponding to Gly32-Ala33-Gly34 in Hst2) makes hydrophobic contacts with the nicotinamide ring. Mutation of Gly32 to alanine completely abolished Hst2 deacetylase activity. Furthermore, no ADPR was detected by reaction with the carbamoyl analog. This suggests that mutation of Gly32 to alanine renders the enzyme incapable of binding NAD, presumably due to the introduced methyl group causing steric hindrance of the nicotinamide ring.

Another invariant residue in this block, Ser36, has been proposed to play a role in catalysis, given the fact that its mutation results in severely decreased levels of activity (9). Early structural studies (9, 15) suggested that Ser36 acts as a nucleophile that attacks the glycosidic bond to displace nicotinamide, resulting in the formation of an enzyme-ADPR intermediate. More recent structural studies, however, do not support a direct catalytic function but rather a role for Ser36 in supporting other residues involved in positioning of the nicotinamide group for cleavage (12) or in maintaining a hydrogen bonding network that positions Asn116, a residue proposed to be involved in the resolution of the cyclic intermediate in the final steps of catalysis (14). Mutation of Ser36 to alanine resulted in a low yield of ADPR produced by reaction with car-
bamoyl-poly-\text{-}l\text{-}lysine in comparison with the WT enzyme. This is consistent with a decrease in affinity for NAD and a role in NAD binding for Ser\textsuperscript{36} and is further supported by our kinetic analyses that reveal a \textsim 5\text{-}fold increase in \( K_m \) for NAD compared with the WT enzyme. Reaction with acetyl-poly-\text{-}l\text{-}lysine showed that nearly all of the NAD was converted to OAADPR, indicating that the Ser\textsuperscript{36} mutant was not defective in steps beyond the initial nicotinamide cleavage. Thus, the observed decrease in \( k_{cat} \) is not due to a defect in catalysis but only in unproductive binding of NAD, and our results confirm that Ser\textsuperscript{36} does not play a direct catalytic role.

Mutation of another highly conserved residue, Asn\textsuperscript{244}, had similar effects on Hst2 activity as did mutation of Ser\textsuperscript{36}. Again, there was little ADPR observed by reaction with the carbamoyl-poly-\text{-}l\text{-}lysine analog, but WT-like production of OAADPR by reaction with the acetyl-poly-l-lysine substrate. Mutation of Asn\textsuperscript{248} also caused an increase in \( K_m \) for NAD and a decrease in \( k_{cat} \). This indicates that mutation of Asn\textsuperscript{248} results in inefficient cleavage of nicotinamide, correlating with a marked decrease in overall catalytic activity, and is a result of decreased binding affinity for NAD rather than a defect in catalysis. Structural studies show that Asn\textsuperscript{248} hydrogen-bonds with the adenine ribose in the Hst2 ternary complex (14). The corresponding residue (Asn\textsuperscript{217}) in the NAD-Sir2 A\text{2} complex makes electrostatic contact with N3 of adenine (12). Thus, Asn\textsuperscript{248} is not in a position to perform any catalytic function, in agreement with our results.

**Mutation of Arg\textsuperscript{45} Prevents Completion of the Deacetylase Reaction**—The \( \beta\text{-}\alpha\text{-}2 \) loop contains another highly conserved block of residues, Gly\textsuperscript{40}–Ser\textsuperscript{46}, that also makes contact with NAD. In the ternary Hst2 product inhibition complex, Phe\textsuperscript{44} and Arg\textsuperscript{45} hydrogen-bond directly with an NAD phosphate (14). In the productive NAD-Sir2 A\text{2} structure, the equivalent residue (Phe\textsuperscript{43}) makes Van der Waals and hydrogen bond contacts with nicotinamide and phosphate groups, respectively. The corresponding arginine residue (Arg\textsuperscript{45}) electrostatically interacts with the adenine ribose and hydrogen-bonds with surrounding residues, its side chain wrapping around the diphosphate moiety (12). Reaction of the Arg\textsuperscript{45} mutant with carbamoyl-poly-\text{-}l\text{-}lysine showed that the majority of NAD was converted to ADPR similarly to WT, suggesting that this residue is dispensable for the initial nicotinamide cleavage step. In stark contrast to WT, however, reaction of the Arg\textsuperscript{45} mutant with the acetyl-poly-l-lysine substrate also produced the ADPR intermediate rather than OAADPR product. Consistent with this result, we also observed a severe decrease in \( k_{cat} \), but remarkably, \( K_m \) values were unaffected.

These results indicate that the overall loss in enzymatic activity is not due to a defect in binding of substrates or an inability to cleave nicotinamide. They further suggest that Arg\textsuperscript{45} plays a role in catalytic steps beyond the initial nicotinamide cleavage and/or in stabilizing the acetyl-lysine-ADPR intermediate. The position of Arg\textsuperscript{45} in the crystal structures supports the latter conclusion and suggests that this residue undergoes a shift in position upon cleavage of nicotinamide to protect the resultant acetyl-lysine-ADPR intermediate and orient it for the next steps in catalysis (12).

Consistent with the tight binding of ADPR to the enzyme active site, the product of the carbamoyl-poly-\text{-}l\text{-}lysine reaction, ADPR, can be detected in association with the wild type enzyme in a dot blot assay (Fig. 5B). Bound ADPR was only detected by spotting the reaction directly onto the membrane and not by SDS-PAGE (data not shown), indicating that this interaction is not covalent. This further validates the hypothesis that there is no enzyme-ADPR intermediate formed by nucleophilic attack by Ser\textsuperscript{26} or any other residue. Although nearly all radiolabeled NAD was cleaved, little of the ADPR produced by the Arg\textsuperscript{45} mutant remained associated with the protein; this is in stark contrast to the wild type enzyme or other mutants not defective in the initial nicotinamide cleavage step (V228A and H338A). This suggests that Arg\textsuperscript{45} is essential for the association of ADPR with the enzyme. This further suggests a role for Arg\textsuperscript{45} in stabilizing the acetyl-lysine-ADPR intermediate and is consistent with the inability of this mutant to proceed with the deacetylase reaction in the presence of an acetylated substrate. Notably, our results are in disagreement with previous mutagenesis studies that show mutation of the corresponding residue in SIRT2 (Arg\textsuperscript{275}) (15) and in Sir2 (Arg\textsuperscript{270}) (25) did not affect deacetylase activity.

**Asn\textsuperscript{116} Is Required for Binding of NAD**—As mentioned above, Asn\textsuperscript{116} is thought to play a key role in the final catalytic steps of the Sir2 reaction. In the ternary Hst2 structure, Asn\textsuperscript{116} hydrogen-bonds with a structurally conserved catalytic water molecule (14) that is proposed to carry out nucleophilic attack to resolve the cyclic intermediate as illustrated in Fig. 2. In the productive NAD-Sir2 A\text{2} structure, the equivalent residue (Asn\textsuperscript{101}) makes Van der Waals contacts with nicotinamide and hydrogen bonds to the 2'-OH of nicotinamide ribose (12). Thus, this residue is appropriately positioned to play a role in catalysis. Mutation of the corresponding residues in SIRT2 (Asn\textsuperscript{105}) (15) and in Sir2 (Asn\textsuperscript{105}) (25, 26) resulted in a severe decrease in catalytic activity. Likewise, our mutation of Asn\textsuperscript{116} completely abrogated Hst2 deacetylase activity. Moreover, we did not detect any ADPR product formed upon reaction with carbamoyl-poly-\text{-}l\text{-}lysine. The lack of any reactivity suggests that the observed loss in activity is due to a failure in NAD binding; thus, we cannot distinguish a role for Asn\textsuperscript{116} in catalysis based on our results.

**Efficient NAD Binding and Deacetylation Requires His\textsuperscript{135}**—Structural studies also support a role for His\textsuperscript{135} in catalysis. In the ternary Hst2 product inhibition complex, His\textsuperscript{135} hydrogen-bonds to the 3'-OH of nicotinamide ribose and is also closely situated to the acetyl-lysine substrate (14). This would allow His\textsuperscript{135} to act as a base, deprotonating the 3'-OH of ribose and activating the 2'-oxygen for nucleophilic attack of the acetyl carbonyl carbon (Fig. 2). It would also put His\textsuperscript{135} in a position to reprotoenate the deacetylated lysine product at the end of the reaction (14). Mutagenesis of His\textsuperscript{135} and the equivalent residues in SIRT2 (His\textsuperscript{187}) (15), Sir2 (His\textsuperscript{264}) (25), and Sir2 A\text{2} (His\textsuperscript{116}) (10) have been shown to result in moderate to background levels of deacetylation activity. Our mutation of His\textsuperscript{135} similarly resulted in a dramatic decrease in \( k_{cat} \). We also observed a low amount of ADPR formed by reaction with the carbamoyl-poly-\text{-}l\text{-}lysine substrate analog and a modest increase in \( K_m \) for NAD, which suggests that His\textsuperscript{135} may be important for NAD binding. We also observed an incomplete resolution of ADPR to OAADPR upon reaction with acetyl-poly-\text{-}l\text{-}lysine, which supports the proposed role for His\textsuperscript{135} in base catalysis following the nicotinamide cleavage step.

**Positioning of the Small Domain Affects Overall Activity but Not Binding of Substrates**—Another interesting highly conserved residue is Lys\textsuperscript{278}. This residue is located in the small zinc-binding domain, away from the enzyme’s active site. It participates in a salt bridge with Glu\textsuperscript{263} and is probably involved in positioning of the small domain (9), which appears to be conformationally flexible (12) relative to the large domain. Mutagenesis of Glu\textsuperscript{263} significantly reduced deacetylase activity (9). Similarly, our mutation of Lys\textsuperscript{278} to alanine resulted in a dramatic decrease in deacetylase activity. However, we did not observe a significant change in \( K_m \) for either NAD or acetyl-poly-\text{-}l\text{-}lysine substrates, indicating that this loss in activity was not due to a defect in substrate binding. Reaction of this mutant with carbamoyl-poly-\text{-}l\text{-}lysine resulted in little production of ADPR, consistent with a reduced ability to release nicotinamide due to an imperfect arrangement of substrates. This mutant retained the ability to complete the deacetylase reaction, however, producing OAADPR by reaction with acetyl-poly-l-lysine. These results confirm that Lys\textsuperscript{278} does not play a direct role in
catalysis and suggest that the correct positioning of the small domain is essential for proper orientation of the two substrates in the active site.

**Residues That Are Important for Binding of the Acetyl-lysine Substrate**—Val\(^{228}\) and Pro\(^{230}\) are two absolutely conserved residues that are believed to be involved in acetyl-lysine binding based on their positions in various crystal structures. In the ternary Hst2 structure, Val\(^{228}\) and Pro\(^{230}\) make Van der Waals contacts with the aliphatic portion of the acetyl-lysine side chain, and Val\(^{228}\) also contacts nicotinamide ribose (14). Mutation of Val\(^{228}\) to alanine resulted in a slight decrease in \(k_{\text{cat}}\) and an \(\sim 2\)-fold increase in \(K_m\) for the acetyl-poly-L-lysine substrate. However, we could detect no significant differences from WT with our TLC assays. This is probably due to the relatively conservative nature of the mutation made but suggests a role for Val\(^{228}\) in acetyl-lysine binding and not in catalysis. In comparison, mutation of Pro\(^{230}\) to alanine resulted in a substantial decrease in deacetylase activity and a higher \(K_m\) value for the acetyl-poly-L-lysine substrate. Remarkably, we did not detect any ADPR intermediate upon reaction with the carbamoyl-poly-L-lysine analog or the acetyl-poly-L-lysine substrate but did observe OAADPR produced by reaction with the acetyl-poly-L-lysine substrate, ruling out a role for this residue in catalysis. These results imply that the Pro\(^{230}\) mutant is unable to bind the carbamoyl analog but can inefficiently bind the acetyl-lysine substrate, consistent with Pro\(^{230}\) mutation causing a defect in acetyl-lysine binding. Mutation of Pro\(^{230}\) may affect the positioning of residues that contact the acetyl group, or the enzyme may undergo a conformational change to position Pro\(^{230}\) itself near the acetyl group during catalysis.

**Analysis of Residues Located Away from the Active Site**—We also studied two other residues in Hst2, Asp\(^{263}\) and His\(^{338}\). Asp\(^{263}\) is a strictly conserved residue that is buried within the large domain away from the Hst2 active site (13, 14). Mutation of this residue to alanine completely abrogated Hst2 deacetylase activity. We were also unable to detect any catalysis of NAD by our TLC assay. Since its position in the crystalline structures precludes involvement in catalysis, we therefore propose that Asp\(^{263}\) is important for the structural integrity of the enzyme. As a negative control for our assays, we also mutated His\(^{338}\), a nonconserved residue located outside of the conserved deacetylase domain. Since structural studies have only been successful with C-terminally truncated versions of Hst2, the position of this residue is not known; however, these truncated enzymes retain full activity (13). Not surprisingly, mutation of His\(^{338}\) to alanine resulted in WT-like behavior in our assays.

**DISCUSSION**

Here we provide strong evidence that the Sir2 deacetylase reaction proceeds via an initial S\(_n\)-2-type catalytic mechanism. In contrast to a dissociative S\(_{1/2}\)-1-type reaction, which would require release of nicotinamide prior to nucleophilic attack by the acetyl carbonyl oxygen, an associative S\(_{1/2}\)-2-type mechanism with direct attack by the acetyl carbonyl oxygen would cause displacement of the nicotinamide moiety directly forming a peptidylamidate-ADPR intermediate (Fig. 2). Structural studies indicate that only upon binding of the acetyl-lysine substrate is NAD able to adopt a catalytically competent configuration (12). In this productive conformation, the positively charged nicotinamide moiety is buried within a hydrophobic pocket, making numerous contacts with highly conserved residues, and in an orientation that puts considerable strain on the glycosidic linkage. This high energy strained conformation would facilitate release of the nicotinamide group and thus promote attack by the acetyl carbonyl oxygen, a relatively weak nucleophile, on C1’ of nicotinamide ribose. Invariant residues Asn\(^{116}\) and Asp\(^{118}\) may further assist in the nicotinamide cleavage step by inductive polarization of the nicotinamide ribose group through their interaction with the 2’-OH (15).

The lack of activity seen with the acetyl-poly-L-ornithine substrate analog demonstrates that binding of the acetylated substrate is not sufficient to cause dissociation of nicotinamide. The correct positioning of the acetyl carbonyl group seems to be required for this step to occur; its close distance from C1’ of nicotinamide ribose suggests that the carbonyl group acts to stabilize the strained conformation and the developing positive charge, thus promoting associative cleavage of the glycosidic bond. Structural studies of Hst2 in complex with ADPR and an acetyl-H4 peptide indicate that the acetyl carbonyl oxygen is within hydrogen bonding distance of C1’ in ADPR (23), indicating the appropriate geometry for nucleophilic attack. Despite this observation, the inability of ADPR to act as a productive substrate further suggests a direct link between nicotinamide cleavage and attack by the acetyl carbonyl oxygen. The absence of any negatively charged residues in close proximity to C1’ of nicotinamide ribose, which would be favorable for stabilization of a positively charged oxacarbenium intermediate formed by an S\(_{1/2}\)-1-type mechanism, further suggests that nicotinamide cleavage proceeds via an S\(_{n}\)-2-type mechanism.

S\(_{2}\)-2 attack by the acetyl carbonyl oxygen from the α face would result in inversion of stereochemistry at C1’, producing a 1’-O-α-peptidyl-adiamidate-ADPR intermediate. Recently, biochemical evidence for this intermediate has been demonstrated (16). This would then permit the 2’-oxygen of nicotinamide ribose to attack the peptidylamidate intermediate from the α face to produce the cyclic intermediate. Isotope labeling experiments have shown that a single water molecule is involved in catalysis, with the acetyl carbonyl oxygen being replaced by a solvent oxygen in the final product (2). An S\(_{2}\)-2 mechanism, followed by final resolution of the cyclic intermediate by the structurally conserved catalytic water molecule as illustrated in Fig. 2, would be consistent with these data.

Our solution studies are compatible with the available structural data and provide further insight into the function of key residues. Analyses of strictly conserved residues Gly\(^{32}\) and Ser\(^{26}\) underscore the importance of this block of residues in NAD binding and, specifically, positioning of the nicotinamide group in a productive conformation but suggest no direct catalytic role. Mutation of Asn\(^{248}\), which contacts the adenine ribose, reveals that interactions with the entire NAD molecule must be maintained for efficient catalysis to occur. This is reinforced by the results from the Arg\(^{45}\) mutation, which clearly demonstrate an important role following the nicotinamide cleavage step and suggest that this role involves stabilization of the peptidylamidate-ADPR intermediate. Our analyses of Val\(^{228}\) and especially Pro\(^{230}\) emphasize the importance of precise positioning of the acetyl-lysine substrate for catalysis. Mutagenesis of Lys\(^{378}\) suggests that the relative position of the zinc binding module affects the orientation of the two substrates within the substrate binding pocket. Given the high degree of conservation of these residues, our findings are probably applicable to other Sir2 family members. Clearly, the Sir2 enzyme active site is highly discriminating with respect to the conformation of both NAD and acetyl-lysine substrates, allowing catalysis to occur only when both substrates have the appropriate relative orientation.

Mutational analyses of the two proposed catalytic residues, His\(^{338}\) and Asn\(^{116}\), uncovered additional functions for these residues. Our results agree with a role in base catalysis for His\(^{338}\) but also suggest that this residue is important for NAD binding. Recent pH studies further support a role for His\(^{338}\) in NAD binding and activation of the 2’-hydroxyl for nucleophilic attack of the peptidylamidate-ADPR intermediate (16). The position of Asn\(^{116}\) in the crystal structures strongly sug-
gests that it plays a key role in catalysis. Whereas our results support a role for Asn116 in NAD binding, they certainly do not rule out an additional catalytic function for this residue.

Studies have shown nicotinamide to be a potent inhibitor of Sir2 activity and suggest that this family of enzymes is metabolically regulated (1). Nicotinamide exerts its inhibitory effect by causing a reverse reaction termed nicotinamide exchange to occur (12, 27). In this reaction, nicotinamide attacks the 1'-O-α-peptidylamidate intermediate from above, reforming β-NAD. Our results would also explain the low level of nicotinamide exchange activity seen with mutation of Ser36 and His135 (9), which would result from inefficient NAD binding and instability of the peptidylamidate-ADPR intermediate. We would predict a similar defect in exchange activity for the Arg45 mutant and any mutation that directly or indirectly affects the ability to productively bind NAD or the peptidylamidate-ADPR intermediate.

We have also shown that ADPR inhibits the Hst2 deacetylase reaction. With a $K_I$ value in the micromolar range with respect to NAD, this inhibition is likely to be of physiological significance. Notably, similar studies with SIRT2 revealed a 10-fold higher inhibition constant for ADPR (1). However, another Hst2 study determined dissociation constants for ADPR in the low micromolar range (24), supporting our results. Inhibition of Sir2 enzymes by ADPR in the nucleus may be regulated by the action of poly-ADPR-polymerase and its antagonist poly-ADPR-glycohydrolase, which control the levels of free NAD and ADPR (28). Similarly, enzymes such as ADP-ribosyl cyclases (29) in the cytoplasm, would probably affect Sirtuin activity by affecting the concentration of free ADPR, as has been shown for NAD salvage pathway enzymes like Nampt (nicotinamide phosphoribosyltransferase) (30) and PNC1 (pyrazinamidase/nicotinamidase 1) (31), which regulate nicotinamide levels.

Given the array of cellular processes with which Sirtuins are involved, it is not surprising that this family of enzymes is being targeted for therapeutic treatments. Splitomicin and sirtinol are two drugs that were identified through phenotypic screening to inhibit Sir2 activity. Splitomicin is believed to act by blocking access of the acetyl-lysine substrate to its binding pocket (32). Alternatively, mechanism-based inhibitors could be used to specifically target Sir2 enzymes. Compounds that mimic the peptidylamidate-ADPR intermediate or the trigonal bipyramidal geometry of an $S_N$2 transition state, for example, may be extremely effective in inhibiting Sir2 activity. Moreover, knowledge of the precise function of active site residues may allow the design of compounds that specifically and potently inactivate Sir2 enzymes. For instance, inhibitors could be targeted to a residue that is critical for the interaction between enzyme and substrate/intermediate, such as Arg45. Alternatively, given the lack of sequence specificity demonstrated by Sir2 proteins (22, 33), molecules that resemble the carbamoyl-lysine or ornithine analogs may be useful as inhibitors and may also provide a means of studying intermediate reaction states through structural studies of enzyme-NAD-acetyl-lysine-analog complexes.

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