The Bicyclic Intermediate Structure Provides Insights into the Desuccinylation Mechanism of Human Sirtuin 5 (SIRT5)*

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Background: Human SIRT5 was recently identified as an NAD-dependent demalonylase/desuccinylase.

Results: We have determined the crystal structures of SIRT5 with a succinyl peptide and with a bicyclic reaction intermediate.

Conclusion: A structure-based mechanism of SIRT5 desuccinylation is delineated.

Significance: The structures provide insights into the sirtuin-catalyzed deacylation reaction and benefit the design of specific inhibitors for SIRT5.

Sirtuins are pivotal regulators in various cellular processes, including transcription, DNA repair, genome stability, and energy metabolism. Their functions have been generally attributed to NAD-dependent deacetylase activity. However, human SIRT5 (sirtuin 5), which has been reported to exhibit little deacetylase activity, was recently identified as an NAD-dependent demalonylase and desuccinylase. Biochemical studies suggested that the mechanism of SIRT5-catalyzed demalonylation and desuccinylation is similar to that of deacetylation catalyzed by other sirtuins. Previously, we solved the crystal structure of a SIRT5-succinyl-lysine peptide-NAD complex. Here, we present two more structures: a binary complex of SIRT5 with an H3K9 succinyl peptide and a binary complex of SIRT5 with a bicyclic intermediate obtained by incubating SIRT5-H3K9 thiosuccinyl peptide co-crystals with NAD. To our knowledge, this represents the first bicyclic intermediate for a sirtuin-catalyzed deacylation reaction that has been captured in a crystal structure, thus providing unique insights into the reaction mechanism. The structural information should benefit the design of specific inhibitors for SIRT5 and help in exploring the therapeutic potential of targeting sirtuins for treating human diseases.

Protein lysine acetylation is an important and reversible post-translational modification that regulates protein function. Sirtuins, widely recognized as a family of NAD-dependent deacetylases that remove acetyl groups from protein lysine residues, have been shown to play crucial roles in the regulation of numerous cellular processes, including DNA repair, cell survival, apoptosis, and energy metabolism (reviewed in Refs. 2 and 3). Sirtuins have been associated with human health and diseases, including life span extension, cancers (6–9), neurodegenerative diseases (10–13), and metabolic diseases (14, 15).

Extensive biochemical and structural studies have provided insights into the deacetylation mechanism (2, 16, 17). Upon the binding of both acetylated substrate and NAD, the nicotinamide group of NAD is released when the carbonyl oxygen of the acetyl group attacks the C1′ position of the nicotinamide ribose (N-ribose), forming the alkylamidate intermediate (intermediate I). Upon deprotonation by the enzyme, the ribose 2′-OH then attacks the amide at the carbonyl carbon, generating the 1′,2′-cyclic intermediate (intermediate II). The bicyclic intermediate is hydrolyzed to produce 2′-O-acetyl-ADP-ribose, which can be non-enzymatically isomerized to 3′′-O-acetyl-ADP-ribose. The absolutely conserved histidine residue among sirtuins serves as a general base to deprotonate the 2′-OH directly or indirectly through the deprotonation of the 3′-OH to attack the 1′-O-alkylamide. Kinetic and mass spectrometry experiments suggested the existence of the alkylamide and the bicyclic intermediates (18, 19). Using a mechanism-based inhibitor (a thioacetyl-lysine peptide), the S-alkylamide intermediate was captured in Sir2Tm and SIRT3 crystals (20, 21). To date, no bicyclic intermediate has yet been directly observed.

Among the seven sirtuins in mammals, SIRT1–3 have been demonstrated to have robust deacetylase activity, whereas SIRT4–7 show little or undetectable deacetylation activity (22–29). However, in contrast to its weak deacetylation activity, SIRT5 was identified as an efficient desuccinylase and demalonylase (30). Furthermore, many mitochondrial proteins were found to contain lysine malonylation and succinylation. In mice, SIRT5 plays a role in ammonium disposal by catalyzing the desuccinylation and activation of carbamoyl-phosphate synthetase I (30, 31). Independently, Zhao and co-workers (32, 33) also identified lysine succinylation and malonylation as novel post-translational modifications.

Among the seven human sirtuins, SIRT5 is the only one that has been shown to possess efficient demalonylase and desuccinylase activity (30). The unique activity of SIRT5 enabled us...
to develop thiosuccinyl peptides as mechanism-based inhibitors specific for SIRT5. Using a thiosuccinyl peptide, we were able to obtain the crystal structure of SIRT5 in complex with the 1',2'-cyclic intermediate. To our knowledge, this is the first piece of direct evidence supporting the existence of the 1',2'-bicyclic intermediate in sirtuin-catalyzed reactions. We also obtained the crystal structure of SIRT5 in complex with a succinyl peptide. These structures, together with the SIRT5-H3K9 succinyl peptide (succH3K9)-NAD ternary complex structure (30), which mirrors the Michaelis-Menten complex, provide step-by-step snapshots of SIRT5-catalyzed reactions.

**EXPERIMENTAL PROCEDURES**

**Protein Cloning, Expression, and Purification—**Truncated SIRT5(34–302) was cloned using TOPO and Gateway cloning technology (Invitrogen) into pDEST-F1 for expression, expressed in *Escherichia coli*, and purified as described previously (34). Purified protein was dialyzed into crystallization buffer (20 mM Tris pH 8.0, 20 mM NaCl, and 5% glycerol), concentrated to 16 mg/ml, flash-frozen in liquid nitrogen, and stored at −80 °C for crystallization.

**Protein Crystallization—**Histone H3K9 succinyl or thiosuccinyl peptides (suchH3K9 or tsuH3K9), 4KQTAR(succinyl-K/thiosuccinyl-K)STGGKA<sup>15</sup>, were used for co-crystallization. SIRT5/peptide mixtures were prepared at a 1:20 protein/peptide molar ratio and incubated for 30–60 min on ice. The final protein concentration was 10 mg/ml. Crystals were grown by hanging drop vapor diffusion method. SIRT5-suchH3K9 co-crystals were obtained with 16% PEG 4000 and 6% glycerol at 18 °C, and SIRT5-tsuH3K9 co-crystals were obtained with 30% PEG 10,000 and 0.1 M Tris (pH 8.5) at room temperature.

**Data Collection and Structure Determination—**SIRT5-suchH3K9 co-crystals were soaked in cryoprotectant solution containing 30% PEG 10,000, 0.1 M Tris (pH 8.5), and 15% glycerol with 10 mM NAD for 0.5–16 h at 4 °C and flash-frozen in liquid nitrogen for data collection. All x-ray diffraction data were collected at the Cornell High Energy Synchrotron Source (CHESS) F1 station. The data were processed using the program package HKL2000 (35). The two structures of SIRT5 complexes were solved by molecular replacement using the program MOLREP from the CCP4 suite of programs (36). The SIRT5-suchH3K9-NAD structure (Protein Data Bank code 3RIY) served as the search model. Refinement and model building were performed with REFMAC5 and Coot from CCP4. The x-ray diffraction data collection and structure refinement statistics are provided in Table 1.

**RESULTS**

**Overall Structure of SIRT5-suchH3K9—**We first set out to obtain structural insights into the enzyme-substrate binding step of SIRT5-catalyzed desuccinylation by co-crystallizing SIRT5 with a 12-mer H3K9 peptide (4KQTAR-succinyl-K-stGGKA<sup>15</sup>) containing succinylated lysine 9, which was previously shown to be an efficient in vitro substrate (30). The SIRT5-suchH3K9 crystal is in the P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> space group with two SIRT5 molecules in the asymmetric unit. The NAD-stabilizing loop is partially disordered such that there is no electron density to trace either residues 71–74 in one SIRT5 molecule or residues 64–74 in the other molecule.

At least three residues on each side of the succinyl-lysine of the H3K9 peptide are visible in the structure. The pattern of binding of suchH3K9 to SIRT5 is the same as that of acetyl peptides to other sirtuins (37, 38). suchH3K9 forms an antiparallel β-sheet with one loop from the zinc-binding domain and the other loop from the Rossmann fold domain (Fig. 1A). This β-sheet is stabilized by the main chain hydrogen bonds between the enzyme and substrate peptide. The structural alignment of the three SIRT5 structures SIRT5-ADP-ribose (ADPR) (27), SIRT5-thioacetyl (tac)H3K9 (30), and SIRT5-suchH3K9 suggested that the interactions within the β-sheet drive the zinc-binding

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**TABLE 1**

| Crystallographic data collection and refinement statistics | SIRT5-suchH3K9 | SIRT5-bicyclic intermediate |
|-----------------------------------------------------------|----------------|---------------------------|
| **Space group**                                           | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> | P2<sub>1</sub>2<sub>1</sub>2<sub>1</sub> |
| **Cell dimensions**                                       | 52.69, 67.03, 157.63 | 52.40, 66.76, 156.86 |
| **Resolution (Å)**                                        | 30-2.00 | 50-1.70 |
| **R<sub>merge</sub> (%)**                                 | 21.80/26.09 | 16.87/23.27 |
| **Completeness (%)**                                      | 99.6 (97.4) | 98.5 (93.5) |
| **Redundancy**                                            | 6.8 (6.1) | 6.3 (3.3) |
| **No. of protein residues**                               | 526 | 539 |
| **No. of ligand/ion molecules**                           | 2 | 2 |
| **r.m.s.d.**                                              | 0.007 | 0.010 |
| **Bond lengths (Å)**                                     | 0.995° | 1.530° |
domain to rotate clockwise to the Rossmann fold domain, resulting in SIRT5 movement from an inactive open state to an active closed state upon substrate binding (Fig. 1B). This movement is independent of the interactions made by the acyl group of the acylated lysine because the zinc-binding domain in the SIRT5-thioacetylH3K9 structure moved to the same extent as it did in the SIRT5-sucH3K9 structure (Fig. 1B).

At the entrance of the lysine-binding pocket, we found that the lysine residue is surrounded by three hydrophobic residues from SIRT5, namely Phe-223, Leu-227, and Val-254 (Fig. 1A), with each of these residues being highly conserved within the sirtuin family. These three residues form a small triangle and define the entrance for the acyl-lysine group of the substrate.

**Comparison between SIRT5-sucH3K9 and Other Sirtuins**—Other studies have reported that the acetyl-lysine was surrounded by hydrophobic residues (37). In SIRT5, however, two non-hydrophobic residues, Tyr-102 and Arg-105, are positioned in the deep end of the succinyl-lysine-binding pocket, where they interact with the succinyl group (Fig. 2A) (30), suggesting that there is a specific recognition of negatively charged acyl groups by SIRT5. SIRT5 harbors a larger acyl-lysine-binding pocket than does SIRT3 due to the replacement of a smaller residue, specifically Ala-86 of SIRT5 compared with Phe-180 of SIRT3 (Fig. 2B). The structural alignment (Fig. 2C) also shows that the succinyl-lysine peptide binds in the same place as the acetyl-lysine peptides in other sirtuins, such as SIRT3 (21), Sir2Tm (38), and yeast Hst2 (39).

**Comparison between SIRT5-sucH3K9 and SIRT5-sucH3K9-NAD**—Biochemical studies have demonstrated that the acetyl substrate binds first to the sirtuins, followed by the binding of NAD (40). This ordered binding mechanism ensures that NAD adopts a productive conformation that proceeds to the completion of the deacetylation reaction (41). Previously, we were able to capture the Michaelis-Menten complex of SIRT5 with both the succinyl-lysine peptide and NAD bound to the enzyme (SIRT5-sucH3K9-NAD). In this structure, the NAD-binding loop is ordered, stabilizing NAD at the active site (Fig. 3A). As described above, the binding of the succinyl-lysine substrate caused the movement between the two domains of SIRT5. The alignment of Cα atoms between the succinyl-lysine substrate-bound structure and the Michaelis-Menten complex structure for SIRT5 yielded a root mean square deviation of 0.357 Å (Fig. 3A), suggesting that the NAD binding did not cause any further movement between the two domains of SIRT5. The nicotina-
mide group of NAD forms hydrogen bonds with the side chain of the invariant residue Asp-143 and the main chain nitrogen of Ile-142, as well as with two water molecules (Fig. 3B). These interactions cause the amide group of nicotinamide to rotate off the pyridine plane, promoting the cleavage of nicotinamide. Phe-70 from the NAD-binding loop is situated roughly parallel to the pyridine ring and almost perpendicular to the ribosyl ring of NAD, making room for the release of nicotinamide. The other two conserved residues, Gln-140 and Asn-141, form hydrogen bonds via their side chains with the 3'-OH and 2'-OH of N-ribose, respectively (Fig. 3B). The carboxyl oxygen of the succinyl group forms an additional hydrogen bond with the 3'-OH of the ribose. These interactions collectively position N-ribose in an orientation that favors the cleavage of nicotinamide. The catalytic residue His-158 does not contact NAD at this stage, consistent with the finding that the catalytically deficient mutant H135A of yeast Hst2 had little effect on the rate of nicotinamide release (42).

**Structure of SIRT5-Bicyclic Intermediate**—Extensive studies have established the mechanism of deacetylation of sirtuins, which includes the formation of two intermediates: the O-alkylamidate intermediate I and the bicyclic intermediate II (2, 16, 17). Thioacetyl-lysine peptides have been reported as inhibitors for sirtuins with deacetylase activities and have been used to capture the intermediate形式 do not interfere with the binding and stabilization of the ADPR moiety. However, Phe-70 on the NAD-binding loop adopts two different orientations. In the Michaelis-Menten complex structure, Phe-70 is perpendicular to the ribosyl ring of NAD (Figs. 3B and 5A), which is proposed to favor nicotinamide escape (20). In the intermediate II structure, Phe-70 is parallel to the ribose face, which prevents the base-catalyzed exchange reaction that generates NAD from nicotinamide (Fig. 4D and 5A). Compared with the ADPR in the SIRT5-ADPR structure (27), the bicyclic intermediate exhibits...
the same conformation as that obtained with the ADPR fragment, whereas NAD in the Michaelis-Menten complex is oriented in a different way, especially with regard to the phosphates and N-ribose moiety (Fig. 5B). These differences suggest that NAD first binds to SIRT5 in a conformation that favors the cleavage of nicotinamide, and then after releasing nicotinamide, the N-ribose flips to the succinyl-lysine, repositioning the two phosphates to facilitate the formation of intermediate II. C, the lysine side chain is rotated $-18^\circ$ to form intermediate II.

Comparison between SIRT5-Bicyclic Intermediate and SIRT3-Alkylamidate Intermediate—Kinetic studies and mass spectrometry have demonstrated the existence of the alkylamidate and 1',2'-cyclic intermediates (18, 19). In 2008, Hawse et al. (20) trapped an S-alkylamidate intermediate in Sir2Tm at 2.5 Å resolution using a thioacetyl peptide. That was the first direct observation of an intermediate I. Later, a similar alkylamidate intermediate was obtained in human SIRT3, which aligned well with the intermediate in Sir2Tm (21). When comparing the SIRT5-bicyclic intermediate structure with the SIRT3-alkylamidate intermediate structure (Fig. 6), we see that the ADPR moiety of the alkylamidate and
the bicyclic intermediates superimpose well except for the orientation of the N-ribose (Fig. 6A). The ribose plane rotates some degree toward the succinyl group, which facilitates the nucleophilic attack of the succinyl carbon by the 2′-OH of the N-ribose.

FIGURE 6. Comparison of SIRT5-bicyclic intermediate and SIRT3-alkylamidate intermediate. A, the structural alignment shows that the N-ribose changes its conformation to facilitate the nucleophilic attack by the 2′-OH on the succinyl carbon. B, schematic representation of the bicyclic intermediate in SIRT5. The lysine is colored gray. C, schematic representation of the alkylamidate intermediate in SIRT3.

DISCUSSION

SIRT5 was recently identified as a novel desuccinylase and demalonylase (30), which is different from the widely known deacetylase activity of sirtuins. Biochemical studies suggested that the mechanism for SIRT5-catalyzed desuccinylation and demalonylation is similar to the deacetylation reaction catalyzed by other sirtuins. Previously, the alkylamidate intermediate was trapped in the Sir2Tm and human SIRT3 crystals (20, 21), supporting the ADPR-peptidylamidate mechanism of deacetylation. To our knowledge, we have now shown for the first time that a bicyclic intermediate can be directly observed in crystals, providing an additional piece of evidence that sirtuins utilize the ADPR-peptidylamidate mechanism to remove acyl groups from substrate lysine residues. The desuccinylation mechanism of SIRT5 is summarized in Fig. 7. Upon formation of the Michaelis-Menten complex, the oxygen atom of the carboxyl from the succinyl group forms a hydrogen bond with the 3′-OH of the N-ribose of NAD (Fig. 7A). The two residues of SIRT5, Gln-140 and Asn-141, which are invariant among sirtuins, form additional hydrogen bonds with the 3′-OH and 2′-OH of the N-ribose, respectively. The carboxyl amide of nicotinamide interacts with the absolutely conserved Asp-143 and the highly conserved Ile-142. These interactions collectively force the nicotinamide into a high energy conformation, which subsequently causes the cleavage of nicotinamide and generates the transient ionic intermediate (Fig. 7B). It is likely that this oxocarbenium ion intermediate is stabilized via hydrogen bonding between the 3′-OH of the N-ribose and the catalytic residue His-158 and via π-hydrophobic interactions between the ribose ring and Phe-70 (Fig. 7B). The release of nicotinamide leads to the rotation of the N-ribose (Fig. 5B), which disrupts the interactions between the ribose and Gln-140 and Asn-141 (Fig. 7B). The new conformation of the N-ribose favors the attack of the carboxyl oxygen of the succinyl group, producing the ADPR-peptidylamidate intermediate (Fig. 7C), followed by the attack of the 2′-OH on the carboxyl carbon of the succinyl group to form...
the bicyclic intermediate (Fig. 7D). The bicyclic intermediate is further hydrolyzed to a free lysine and succinyl-O-ADPR (Fig. 7, E and F).

We were unable to obtain the alkylamidate intermediate and the thiosuccinyl-O-ADPR product even though we soaked SIRT5-tsuH3K9 co-crystals in 10 mM NAD at 4 °C for 0.5–16 h. Regardless of the NAD soaking time we tested, we could trap the bicyclic intermediate only at the active site. This is quite different from the reported soaking experiments with Sir2Tm or SIRT3, which generated the alkylamidate intermediate and thioacetyl-O-ADPR product with relatively short and long soaking times with NAD, respectively (20, 21). This suggests that, although sirtuins share a similar mechanism to remove acyl groups from modified lysines, different sirtuins may preferentially stabilize distinct intermediates at the active site. SIRT5 favors the bicyclic intermediate as the more stable species as opposed to the alkylamidate intermediate. Additionally, the type of acyl group may affect the stability of these two intermediates.

Similar to lysine acetylation/deacetylation reactions, lysine malonylation/demalonylation and succinylation/desuccinylation reactions are likely to play important roles in regulating protein function. To date, only carbamoyl-phosphate synthetase 1 has been identified as a desuccinylation substrate of SIRT5 (30). SIRT5-specific inhibitors will help to further investigate the biological functions of SIRT5 (43). The structural study presented here will facilitate the design of specific inhibitors to better characterize the functions of SIRT5 and to offer lead compounds for the ultimate development of therapeutic reagents.

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