SIRT6 is a member of the evolutionarily conserved sirtuin family of NAD$^+$-dependent protein deacetylases and functions in genomic stability and transcriptional control of glucose metabolism. Early reports suggested that SIRT6 performs ADP-ribosylation, while more recent studies have suggested SIRT6 functions mainly as a histone deacetylase. Thus, the molecular functions of SIRT6 remain uncertain. Here, we perform biochemical, kinetic and structural studies to provide new mechanistic insight into the functions of SIRT6. Utilizing three different assays, we provide biochemical and kinetic evidence that SIRT6-dependent histone deacetylation produces O-acetyl-ADP-ribose (OAADPr) but at a rate ~1,000 times slower than other highly active sirtuins. To understand the molecular basis for such low deacetylase activity, we solved the first crystal structures of this Class IV sirtuin in complex with ADP-ribose (ADPr) and the non-hydrolyzable analog of OAADPr, 2'-N-acetyl-ADP-ribose (NAADPr). The structures revealed unique features of human SIRT6, including a splayed zinc-binding domain and the absence of a helix bundle that in other sirtuin structures connects the zinc-binding motif and Rossmann fold domain. SIRT6 also lacks the conserved, highly flexible, NAD$^+$-binding loop, and instead contains a stable single helix. These differences led us to hypothesize that SIRT6, unlike all other studied sirtuins, would be able to bind NAD$^+$ in the absence of an acetylated substrate. Indeed, we found that SIRT6 binds NAD$^+$ with relatively high affinity ($K_d = 27 \pm 1 \mu$M) in the absence of an acetylated substrate. Isothermal titration calorimetry and tryptophan fluorescence binding assays suggested that ADPr and NAD$^+$ induce different structural perturbations, and that NADH does not bind to SIRT6. Collectively, these new insights imply a unique activating mechanism and/or the possibility that SIRT6 could act as an NAD$^+$ metabolite sensor.

Sirtuins comprise an ancient and diverse family of nicotinamide adenine dinucleotide (NAD$^+$)-dependent protein deacetylases that are evolutionarily conserved from bacteria to eukaryotes (1). Unlike other classes of histone deacetylases (HDACs), which utilize an active-site Zn$^{2+}$ and involve direct attack of a water molecule on acetylated lysines, sirtuins transfer the acetyl group from the lysine side chain of a protein or peptide substrate to the co-factor NAD$^+$, generating nicotinamide, 2'-O-acetyl-ADP ribose (OAADPr) (2,3) and a deacetylated substrate. This unique requirement of NAD$^+$ suggests sirtuins might act as sensors of the cellular metabolic state (4), relaying changes in cellular metabolism to reverse acetylation-mediated pathways, which include transcription, cell cycle progression, genome maintenance, apoptosis and organism longevity. The founding member of the sirtuin family – yeast Sir2 (silent information regulator 2) has emerged as an important regulator in extending the lifespan of S. cerevisiae (5). In other organisms such as C. elegans and D. melanogaster, sirtuin-activated pathways promote longevity (6,7). It was recently demonstrated that mammalian SIRT3 deacetylates isocitrate dehydrogenase 2, reduces oxidative damage and prevents age-related hearing loss in response to caloric restriction (8). This result provides a direct mechanistic link between mammalian sirtuins and aging.

There are seven members in the human sirtuin family, SIRT1-7; these exhibit diversity and complexity in their cellular localization patterns and targets (9). SIRT1, being the putative ortholog to yeast Sir2, has been the most studied. A recent
study reported that SIRT6-deficient mice have a striking degenerative phenotype leading to shortened lifespan (10). At the cellular level, SIRT6 deficiency leads to marked metabolic and genomic instability caused by hypoglycemia and potential defects in base excision repair (BER) or DNA double-strand break (DSB) repair (10). However, the molecular mechanism of SIRT6's protective role in preventing these processes is not fully understood.

The early in vitro analysis suggested that SIRT6 was not an active deacetylase and instead could undergo intramolecular mono-ADP-ribosylation utilizing NAD$^+$ as a substrate (11). More recently however, it was reported that SIRT6 does harbor NAD$^+$-dependent deacetylase activity towards the H3K9Ac (12) and H3K56Ac (13,14) histone marks as well as towards the DSB resection protein, CtIP [C-terminal binding protein (CtBP) interacting protein] (15). Hundreds of genes are differentially expressed in SIRT6−/− mouse cells when compared with wild-type controls (16) and the modulation of NF-κB (16) and HIF1α-dependent pathways (17) were reported to be achieved through H3K9Ac deacetylation by SIRT6.

Despite these recent advances in elucidating the biological functions of SIRT6, the lack of detailed biochemical and structural studies have hindered our mechanistic understanding of this class IV sirtuin. Three human sirtuins - SIRT2 (18), SIRT3 (19) and SIRT5 (20), have crystal structures available. Along with structures from other sirtuin homologs (21-32), these results have provided important insights into the mechanism for activity, inhibition, and substrate specificity of individual sirtuins. Crystal structures of the class IV sirtuins, which include the mammalian SIRT6 and SIRT7, have not been solved.

Here we explore the biochemical function and structural details of human SIRT6 protein. We provide the first quantitative assessment of SIRT6 deacetylation and give direct evidence for OAADPr as one of the end products of a very inefficient SIRT6-catalyzed reaction. We also report the first sets of crystal structures of human SIRT6 – a complex structure of SIRT6 with ADP-ribose (ADPr) and another complex with a synthetic OAADPr analog – 2'-N-acetyl-ADP-ribose (NAADPr) (33). Comparison of SIRT6 with other sirtuin structures reveals several unique features that provide rationale for the low deacetylase activity and the ability to bind NAD$^+$ with high affinity in the absence of acetylated substrate. Furthermore, isothermal titration binding and tryptophan fluorescence assays suggest that NAD$^+$ and ADPr may induce different structural changes upon binding. These novel features provide insight into the class IV sirtuins, which have poorly defined molecular functions.

**EXPERIMENTAL PROCEDURES**

**Chemicals and reagents** - NAD$^+$, NADH, ADP-ribose, Dithiothreitol (DTT), TCEP, Glutamate dehydrogenase from bovine liver and α-ketoglutarate were purchased from Sigma. Tris-HCl, Tris base, sodium phosphate monobasic, sodium phosphate dibasic and sodium chloride were purchased through Fischer. [3H]-acetic anhydride (50-100 mCi/mmols) was purchased from American Radiolabeled Chemicals, Inc. Site-directed mutagenesis kits from Stratagene were utilized to introduce a point mutation at His131 of SIRT6. 2'-'-NADPr was synthesized as previously described (33).

**Expression and purification of recombinant WT SIRT6, H131Y SIRT6 and Hst2 for deacetylation assays and ITC** - pQE-80L (Qiagen, Valencia, CA) His-tagged WT or H131Y SIRT6 were transformed into the competent E. coli strain, BL21 DE3. Overexpression was initiated by growing cells to an OD$_{600}$ of 0.6-0.8 at 37 °C. To induce expression, 0.5 mM isopropyl-1-thio-D-galactopyranoside (IPTG) was added and cells were grown at room temperature for 18 hours. Cells were harvested by centrifugation at 5000 rpm for 10 minutes and stored at -20°C. For purification, cells were resuspended in 50 mM sodium phosphate pH 7.2, 250 mM NaCl, 5 mM imidazole and 1 mM β-mercaptoethanol, lysed by sonication and purified by nickel resin affinity chromatography. WT and H131Y SIRT6 were further purified via a HiTrap SP Sepharose Fast Flow column (GE) using a linear gradient from 50 mM to 750 mM NaCl in 50 mM sodium phosphate pH 7.2 and 1 mM β ME. Fractions containing purified WT or H131Y SIRT6 were pooled, concentrated, and dialyzed into 50 mM Tris pH 8.0 (4°C), 150 mM NaCl, 100 μM TCEP and 5% (w/v) glycerol. Hst2 was expressed and purified as
described previously (34). Protein concentrations were determined by the Bradford reagent assay.

Synthesis and analysis of the H3K9 peptide - A peptide corresponding to residues 5-13 of histone H3 (QTARKSTGG) was synthesized by the University of Wisconsin-Madison Biotechnology Center (UWBC) and purified over a preparative C18 HPLC column. The chromatographic purity of the peptide was determined to be \( \geq 95\% \) and mass spectrometric analysis on a Bruker REFLEX II: MALDI-TOF (matrix-assisted laser desorption ionization time-of-flight) instrument confirmed the identification of the peptide.

Generation of a \([3H]\)-labeled H3K9Ac peptide - To produce a \([3H]\)-labeled H3K9Ac peptide, 4 mM H3K9 peptide was reacted with 20 mM tritium-labeled acetic anhydride in 50 mM HEPES pH 8.0 and incubated at 4 °C for 4 hours. The reaction was quenched by the addition of 100 mM Tris-HCl pH 7.4 (final concentration). During synthesis the N-terminus was left unblocked and therefore, acetic anhydride reacted with the \( \varepsilon \)-amine of the lysine residue as well as the N-terminal amine to produce a peptide that was acetylated at both the lysine residue and the N-terminus. The presence of the doubly acetylated peptide was confirmed by mass spectrometric analysis on a Bruker REFLEX II: MALDI-TOF of a peptide acetylated with unlabeled acetic anhydride. The molar concentration of \([3H]\)-H3K9Ac (3.7 mM) was determined by the amount of tritium in 2-3 µL of the resuspended peptide and calculated as described previously (35).

Charcoal-binding assay determination of SIRT6 activity - The charcoal-binding assay was performed as described previously (35). Reactions were performed in 400 µL containing 600 µM NAD\(^+\), 300 µM \([3H]\)-H3K9Ac, 1 mM DTT, and 4 µM WT SIRT6 or H131Y SIRT6 in 50 mM Tris pH 7.5 at room temperature. Time points (0.5 h, 1 h, 1.5 h, 2 h, 2.5 h, 3 h) were taken by adding 60 µL of the reaction mixture to 60 µL activated charcoal pH 9.5 (1/3 w/v of charcoal (Sigma, C3345) and 2/3 (v/v) of 2 M glycine, pH 9.5).

HPLC assay to monitor OAADPr formation - Reactions were performed in 40 µL containing 2 mM NAD\(^+\), 300 µM \([3H]\)-H3K9Ac, 1 mM DTT, and 4 µM WT SIRT6, Hst2 or no enzyme in 50 mM Tris pH 7.5 at room temperature. The reaction was quenched after 3 hours by the addition of 1% (v/v) TFA to 20 µL of the reaction mixture. The reaction mixture was separated on an analytical C18 HPLC column by a gradient separation at 0.5 ml/min; 0-8% solvent B (0.02% TFA in acetonitrile) over 25 minutes, 8-100% solvent B over 3 minutes, followed by 100% solvent B for 10 minutes. Fractions (1 min) were collected and radioactivity was quantified by counting 150 µL of each fraction by scintillation counting and plotted versus the fractions. The peak corresponding to \([3H]\)-OAADPr was confirmed by alignment with purified OAADPr.

Sirtuin enzyme coupled assay - SIRT6 activity was measured continuously using the method previously reported (36) with minor variations. The assays solution contained 2 µM MBP-PncA (nicotinamidase), 0.2 mM NADH, 0.6 mM NAD\(^+\), 3.3 mM \( \alpha \)-ketoglutarate, 1 mM DTT, 2 units Glutamate dehydrogenase from bovine liver, 300 µM H3K9Ac peptide and 0 or 8 µM SIRT6 enzyme in 20 mM phosphate buffer (pH 7.5). Continuous readings were taken every 20 seconds for a total duration of one hour and 30 minutes. The rate of nicotinamide formation was determined by the use of the coupled enzymes nicotinamidase and Glutamate dehydrogenase. Nicotinamidase converts nicotinamide, formed by the cleavage of NAD\(^+\), into nicotinic acid and ammonia. The ammonia is then utilized by Glutamate dehydrogenase to convert \( \alpha \)-ketoglutarate to glutamate, consuming NADH in the process. The oxidation/consumption of NADH was monitored continuously at 340 nm and subsequently the rate of nicotinamide formation could be determined.

Protein cloning, expression and purification for crystallization - Human SIRT6(3-318) was amplified by PCR and sub-cloned into the pET28a-LIC vector downstream of the poly-histidine coding region. The recombinant protein was overexpressed in E.coli BL21(DE3)-R3-pRARE2 cells in Terrific Broth (TB) in the presence of 50 µg/mL kanamycin at 37°C to an OD600 of 0.8. Protein expression was induced by the addition of IPTG to a final concentration 0.5 mM, and the cells were then incubated overnight at 15°C. Cells were harvested by centrifugation. The cell pellets were frozen in liquid nitrogen and stored at -80°C. During purification, the cell paste was thawed and resuspended in lysis buffer (1X...
PBS, pH 7.5, 0.5 M NaCl, 5% glycerol) with protease inhibitor (0.1mM phenylmethyl sulfonyl fluoride, PMSF). Cell lysis was accomplished by sonication (Virtis408912, Virsonic) on ice: the sonication protocol was 10 s pulse at 80% maximal frequency (8.0), 10 s rest, for 5 min total sonication time. The cell lysate was clarified by centrifugation using a Beckman JLA-16.250 rotor at 15,500 rpms for 45 min at 4°C. The clarified lysate was loaded onto 5 mL HiTrap chelating column (Amersham Biosciences), charged with Ni²⁺. The column was washed with 10 column volumes (5) of 20 mM Hepes, pH 7.0, containing 250 mM NaCl and 5% glycerol, and the protein was eluted with elution buffer (20 mM Hepes buffer, pH 7.0, 250 mM NaCl, 5% glycerol and 250 mM imidazole). The protein was loaded onto Superdex200 column (26x60) (Amersham Biosciences), equilibrated with 20 mM Pipes buffer, pH 6.5, and 150 mM NaCl, at flow rate 4 mL/min. Thrombin was added to combined fractions containing SIRT6 at 4°C overnight to cleave off the N-terminal poly His-tag. The protein was further purified to apparent homogeneity by ion-exchange chromatography on Source 30S column (10x10) (Amersham Biosciences), equilibrated with buffer 20 mM MES pH 6.5 and 150 mM NaCl, and then adjusted to pH 7.0 with a final concentration of 100mM. Subtilisin A (Sigma) was dissolved in 10 mM NaOAC and 5 mM Ca (37)² to a final concentration of 1mg/mL. The protein solution contained 10 mg/mL purified SIRT6 mixed with 10mM NAD⁺/ADPr (Sigma)/NAADPr and 3mM H3K9Ac and 0.01mg/mL subtilisin A (37) in 20mM Mes buffer, pH 6.5. SIRT6+ADPr and SIRT6+NAADPr crystals were obtained using hanging drop vapor diffusion at 20°C by mixing 2 µl of the protein mix with 2 µl of the reservoir solution containing 1.8-1.9 M (NH₄)₂SO₄, 2% PEG 400, Bis-Tris pH 5.6-6.2. All crystals were soaked in the corresponding mother liquor supplemented with 15% ethylene glycol as cryoprotectant before freezing in liquid nitrogen.  

Data collection and structure determination - X-ray diffraction data were collected at 100 K at beamline 19ID of Advanced Photon Source (APS) at Argonne National Laboratory and a Rigaku FR-E home source. Data were processed using the HKL-2000 software suite (38). The SIRT6+ADP ribose structure (PDB:3K35) was solved by molecular replacement using the program Phaser (39). A mixed model of Sir2Tm (PDB:1Y5C) generated by SCWRL and Jackal modeling method on the FFAS03 search server (http://ffas.ljcrf.edu) was used as template. Refinement was carried out with Refmac (40). SIRT6+ADPr (PDB:3PKJ) and SIRT6+NAADPr (PDB: 3PKJ) were solved by molecular replacement using the initial SIRT6 structure (PDB:3K35, ADPr and all other small molecules were removed) as a search model. Subsequent refinement was performed using Refmac and Buster-TNT(41), respectively. Graphics program COOT (42) was used for model building and visualization. Data statistics are reported in Table 1. Isothermal titration calorimetry studies of WT and H131Y SIRT6 with NAD⁺ metabolites - Isothermal titration calorimetry (ITC) measurements were taken using a VP-ITC microcalorimeter from MicroCal, LLC (Northampton, MA). Binding experiments were all performed in 50 mM Tris pH 7.5 (25 °C), 150 mM NaCl, 100 µM TCEP and 5% (w/v) glycerol. Purified WT and H131Y SIRT6 were dialyzed against the previously described buffer and ligands were suspended in the dialysis buffer. Experimental data were fitted to a one-site binding model using Origin scientific plotting software. Enthalpy (ΔH), binding constant (Kₐ) and number of binding sites (n) were all flexible parameters, while the free energy (ΔG) and entropy (ΔS) were calculated according to the following equation: 

$$\Delta G = \Delta H - T \Delta S = -RT \ln K_a \quad (1)$$

For each experiment, 37 automatic injections (1-8 µL) were titrated into the cell (initial cell volume 1.42 mL) while being stirred at 300 rpm. NAD⁺ (1.40 mM) was titrated into 27 µM WT SIRT6. NAD⁺ (1.63 mM) was titrated into 50 mM His2. NADH (450 µM) was titrated into 25 µM WT SIRT6. ADPr (300 µM) was titrated into 28 µM WT SIRT6. 2'-'NAADPr (2.96 µM) was titrated into 38 µM WT SIRT6. NAD⁺ (767 µM) was titrated into 33 µM H131Y SIRT6 and 1.45
µM was titrated into 37 µM H131Y SIRT6. All titrations were also performed in the absence of enzyme to account for the heat caused by ligand dilution. Intrinsically tryptophan fluorescence emission studies of WT and H131Y SIRT6 with NAD⁺ metabolites – Fluorescence emission was monitored on a FluoroMax®-4 spectrofluorometer from HORIBA Jobin Yvon. Samples were excited at 295 nm (slit width 2 mm) and emission was monitored from 320 nm to 390 nm (slit width 3 mm). Experiments under native conditions were performed in 50 mM Tris pH 7.5 at room temperature. Increasing concentrations of NAD⁺ from 0 µM to 600 µM or ADPr from 0 µM to 400 µM were added to 1 µM WT SIRT6 and the emission spectrum was monitored. Likewise, increasing concentrations of NAD⁺ (0 – 200 µM) or ADPr (0 – 1.2 mM) were added to 1 µM H131Y SIRT6. The peak maximum was determined for each concentration. To account for decreases in fluorescence from non-specific quenching due to increasing concentration of ligand, the same experiments were carried out in 7 M urea. The peak maxima were obtained and the correction factor was determined by \( \frac{F_{\text{obs}}}{F_{\text{fr}}} \) where \( F_{\text{obs}} \) is the maximum intensity at each concentration and \( F_{\text{fr}} \) is the maximum intensity at 0 µM ligand. The intensities from experiments under native conditions were divided by their correction factors and the fraction of substrate bound (FSB) was determined by:

\[
FSB = \frac{F_{\text{obs}} - F_{\text{fr}}}{F_{s} - F_{\text{fr}}}
\]  

(2)

where \( F_{s} \) is the maximum intensity under saturating ligand and \( F_{\text{obs}} \) and \( F_{\text{fr}} \) are the same as stated previously. The FSB values were plotted versus ligand concentration and the data were fitted to a one-site binding equation to determine the \( K_d \):

\[
F_{\text{fsb}} = \frac{K_d + P_c + L_c}{2P_c} \times \left( \frac{(K_d + P_c + L_c)^2 - 4L_cP_c}{2P_c} \right) + C
\]  

(3)

where \( K_d \) is the binding constant, \( P_c \) is the protein concentration (1 µM), \( L_c \) is the varying ligand concentration, and \( C \) is the y-axis maximum.

RESULTS

SIRT6 deacetylase activity - It was initially reported that SIRT6 was not an active deacetylase, but rather was capable of performing intramolecular auto-ADP-ribosylation using NAD⁺ as a co-substrate (11). More recently, Michishita et al. used mass spectrometry and western blot analysis to support SIRT6 as a histone H3K9 deacetylase (12). However, SIRT6 did not deacetylate H3K9 with high efficiency; non-catalytic amounts of SIRT6 (~100 pmol) were needed to observe deacetylation of ~65 pmol acetylated histone by western blot analysis, which detects loss of immunoreactivity from an anti-acetyl-lysine antibody. In mass spectrometry assays, ~100 pmol SIRT6 was needed to convert ~10% of 360 pmol of H3K9Ac peptide to the deacetylated form (12). It is still unknown whether SIRT6 can form 2’-O-acetyl-ADP-ribose as the product of the deacetylase reaction. Thus, the molecular function(s) of SIRT6 remains unclear. A detailed quantitative assessment of the SIRT6 reaction is essential toward our understanding of its cellular functions.

To monitor the deacetylase activity of purified recombinant SIRT6, we used an in vitro charcoal-binding assay (35) that measures acetate released from deacetylation of a tritium-labeled H3K9Ac peptide. [³H]-Acetate, formed through the hydrolysis of [³H]-OADPr, was measured in the presence of 300 µM [³H]-H3K9Ac, 600 µM and 4 µM SIRT6. After subtracting the control reaction without SIRT6, reactions containing SIRT6 exhibited a linear increase in acetate formation over time (Fig. 1A). The rate of deacetylation was 3.2 ± 0.2 µM/h, with a specific activity of 0.80 ± 0.05 h⁻¹ (0.00022 ± 0.00001 s⁻¹). This low level of activity is in general agreement with the previous qualitative assays (12), and indicates that SIRT6 is approximately 1000 times less active than the well-characterized yeast Hst2 enzyme, which deacetylates the same H3K9Ac peptide with a kcat of 0.32 ± 0.08 s⁻¹ (34).

Such low activity raised the concern that the deacetylation activity could be due to a contaminating E. coli sirtuin co-purifying with SIRT6. To ensure the activity measured was dependent on SIRT6, the invariant catalytic base histidine residue (3,43) (His131) was mutated to a tyrosine (H131Y). If the low level of deacetylase activity emanated from SIRT6, the H131Y mutant purified in exactly the same manner (supplemental Fig. 1) should yield no [³H]-H3K9 deacetylation. H131Y SIRT6 deacetylase activity was monitored by the charcoal-binding assay. No detectable
activity was observed above a control reaction containing no SIRT6 (Fig. 1A), thereby providing further evidence that the low level of deacetylase activity observed in the WT reactions stemmed from catalytically active SIRT6.

To provide direct evidence that the acetate formed through the charcoal-binding assay was due to deacetylation by SIRT6 and subsequent hydrolysis of OAADPr, an HPLC assay was utilized to monitor the formation of [3H]-OAADPr. Reactions containing 300 μM [3H]-H3K9Ac, 2 mM NAD\(^+\) and either 4 or 8 μM SIRT6, Hst2, or no enzyme were incubated for three hours and quenched by the addition of 1% (v/v) TFA. As a positive control for OAADPr formation, deacetylation of the H3K9 peptide by Hst2 (34) was monitored. Hst2 deacetylated the H3K9Ac peptide after three hours as demonstrated by the loss of a peak corresponding to [3H]-H3K9Ac peptide and generation of peaks corresponding to [3H]-OAADPr and [3H]-acetate, which was formed through the hydrolysis of [3H]-OAADPr (Fig. 1B).

Compared to Hst2, SIRT6 (4 μM) was able to weakly deacetylate the [3H]-H3K9Ac peptide (Fig. 1B), producing 13 μM OAADPr during the three-hour incubation. Thus, SIRT6 deacetylated H3K9Ac at a rate of 4.3 μM/h and had a specific activity of 1.1 h\(^{-1}\) (0.0003 s\(^{-1}\)), which is consistent with the results from the charcoal-binding assay (Fig. 1A). Along with the charcoal-binding assay, the results provide the first evidence that SIRT6 can perform multiple turnovers and can generate OAADPr through NAD\(^+\)-dependent protein deacetylation.

To provide an independent method for determining SIRT6 activity towards acetylated peptides, a continuous assay was utilized (36). The continuous assay measures nicotinamide formation, which is the first product released from either the NAD\(^+\)-dependent deacetylation or an ADP-ribosylation reaction. Therefore, the assay provides a good indication of the coupling between nicotinamide cleavage and OAADPr formation. H3K9Ac peptide (300 μM) was incubated with 0.6 mM NAD\(^+\) and either 0 or 8 μM SIRT6 and a linear increase of nicotinamide was observed over 1.5 hours. The rate of nicotinamide formation was greater in the presence of SIRT6 compared to spontaneous NAD\(^+\) hydrolysis (10.5 ± 0.4 μM/h vs. 5.9 ± 0.8 μM/h), indicating that the SIRT6-dependent rate of NAD\(^+\) cleavage is 4.6 ± 0.9 μM/h with a specific activity of 0.6 ± 0.1 h\(^{-1}\) (Fig. 1C). The three different assays (Figures 1A, 1B & 1C) are in excellent agreement and confirm the extremely low level of deacetylase activity displayed by SIRT6. Importantly, the fact that the rate of nicotinamide cleavage is similar to that for OAADPr formation indicates that NAD\(^+\) cleavage is tightly coupled to deacetylation, and argues against a significant contribution from protein ADP-ribosylation that does not require deacetylation.

**SIRT6 protein construct for crystallization -** It is unclear why SIRT6 displays such low deacetylase activity (~1000-fold lower) compared to other sirtuins. To investigate the molecular basis for this low activity, the crystal structures of human SIRT6 bound to ADP and 2'-NAADPr were solved. Full length human SIRT6 has 355 amino acids and consists of a putative catalytic sirtuin core with N- and C-terminal flanking extensions. In our attempts to obtain SIRT6 protein crystals, we generated several minimal truncations of SIRT6 as well as full-length protein. Among the various constructs examined, the SIRT6 (3-318) construct was expressed and purified from E. coli in milligram quantities, compared to lower yields obtained with the full-length construct. Therefore, SIRT6 (3-318) was selected for crystallization trials. This construct has both the N-terminal extension (NTE) and the core catalytic domain, but lacks the C-terminal extension (CTE). It was previously reported that a SIRT6 construct lacking the CTE is enzymatically active, but is impaired in its ability to associate with chromatin (44).

**In situ proteolysis and the two ADPr bound SIRT6 structures -** We initially attempted to co-crystallize SIRT6 with H3K9Ac and the cofactor NAD\(^+\), but no crystals were observed. We then attempted to promote crystallization using *in-situ* proteolysis (37). After adding trace amounts of the protease - subtilisin A, into the protein mix, protein crystals appeared within 24 h after setting up the crystallization experiment. The crystals were refined to achieve X-ray diffraction quality. In the solved structure (PDB: 3K35), we did not observe the nicotinamide moiety of NAD\(^+\). The nicotinamide ribose ring adopted a C3'-endo
conformation and remained in the β-form. We repeated the crystallization experiment substituting ADPr for NAD$^+$ in the protein mix, and obtained crystals within 24 h. Although the space group changed from P2$_1$ to P1, the number of molecules in the asymmetric unit remained the same and monomer structural features were nearly identical. The only difference was that the ADPr now assumed an α-form. Since both the α and β anomers of the ribose ring interconvert in aqueous solution, we do not believe there is any significant biological differences between the two SIRT6+ADPr structures. However, at similar resolution (~2 Å), the model (PDB: 3PKI) was built on a more complete dataset with higher resolution (Table 1). In describing the structure of SIRT6+ADPr, we will be referring to the model/dataset in which ADPr was used for co-crystallization.

**Overall structure of SIRT6 in complex with ADP-ribose -** To gain insight into the structural basis for SIRT6 enzymatic function, we initially solved a 2.0 Å crystal structure of the human SIRT6 protein in complex with ADPr. The asymmetric unit consists of 6 molecules and each monomer contains one zinc atom, one molecule of ADPr and several sulfate molecules, which were present in the crystallization buffer. Residues 11 to 295 of SIRT6 (3-318) were visible in the crystal structure. It is possible that the N- (3-10) and C-terminal (295-318) residues were cleaved by trace amounts of the subtilisin that was added to promote crystallization(37), or alternatively these regions are disordered. Both the N- and C-terminal residues visible in the crystal structure form long unstructured coils, providing support that the unseen residues of the SIRT6 constructs are highly flexible and disordered.

Consistent with other solved sirtuin structures, SIRT6 contains two globular domains composed of 8 α-helices and 9 β-strands – a large Rossmann fold for NAD$^+$-binding (residues 25-128, and 191-266) and a smaller domain, which contains a zinc-binding motif (residues 129-190) (Fig. 2). The large Rossmann fold domain is formed by a six-stranded (β1, β2, β3, β7, β8, and β9) parallel β-sheet sandwiched between two helices - α6 and α7 on one side, and four helices - α1, α4, α5, α8 on the other side. The small domain is formed by two extending loops (connecting β3 and α6) from the large domain and consists of a three-stranded antiparallel β-sheet (β4, β5, β6). Interestingly, while most sirtuins contain a Cys-X-X-Cys-(X)$_{15}$, 20-Cys-X-X-Cys sequence motif for Zn$^{2+}$-binding, SIRT6 contains a 10-residue insertion between the second set of cysteines, resulting in an extended long loop (Supplemental Fig. 2). The six molecules in the asymmetric unit appear to be almost identical (Fig. 2B), with variations in the extended loop of the zinc-binding motif as well as in the N- and C-termini. The extended loop is highly flexible and only one out of the six molecules in the asymmetric unit has an ordered extended loop.

The NAD$^+$ binding pocket of sirtuins has been divided into three regions – site A, B and C (24). Similar to all other solved sirtuin structures, the adenosine moiety of ADPr is bound in the A site of SIRT6 and the nicotinamide ribose moiety is bound in the B site. Many of the residues involved in ADPr-binding are conserved between SIRT6 and the other sirtuins (Supplemental Fig. 2). The ADPr interactions in the SIRT6 active site are shown in Fig. 2C. For all six molecules in the asymmetric unit, the ribose moiety of ADPr assumes the C2’ exo conformation in its α-form (Fig. 2D).

Although acetylated H3K9Ac peptide was included during co-crystallization, we did not observe the peptide in the solved SIRT6 structure. However, almost perpendicular to the ADPr electron density, we observed elongated density (Fig. 2D). Given that this location is where peptide binding is predicted (19), we attempted to model a lysine side chain or a PEG molecule into this density. However, we were unable to unambiguously fit the density and therefore we left these atoms unassigned.

**Structural comparison of SIRT6+ADP-ribose with SIRT6+2’-N-acetyl-ADP-ribose -** As we have demonstrated in Fig. 1, SIRT6 forms 2’-OAADPr as one product of the reaction, and therefore to gain insight into the interactions between 2’-OAADPr and SIRT6, we co-crystallized SIRT6 with 2’-NAADPr (Fig. 3A) – a non-hydrolyzable analog of OAADPr in which the O-acetyl moiety is substituted with an N-acetyl group (33). The overall structural features are identical between the SIRT6+ADPr and SIRT6+NAADPr crystal structures (Fig. 3B). This is not surprising as NAADPr only contains an extra N-acetyl group compared to ADPr. There are no significant
changes in the orientation of residues surrounding the NAD$^+$ binding site (Fig. 3C). It is interesting to note that the unknown density observed in the SIRT6+ADPr structure was not present in the SIRT6+NAADPr structure (Fig. 3D). The N-acetyl group protrudes into the space that is otherwise occupied by the unassigned chemical entity in the SIRT6+ADPr structure.

Structural comparison with other solved human sirtuins (SIRT2, SIRT3, and SIRT5)-Sirtuins have been phylogenetically divided into five subclasses: I through IV, and U (45-47). Among the seven human sirtuins: SIRT1-3 belong to Class I; SIRT4, and 5 belong to Class II, and III respectively; SIRT6 and 7 are both Class IV sirtuins. The three previously characterized human sirtuins provide structural insight into Classes I and III. The SIRT6 structure constitutes the first solved sirtuin structure in Class IV. Structural features revealed by SIRT6 provide additional information on how the different evolutionary lines developed in the sirtuin family.

Although SIRT6 shares the overall domain architecture with SIRT2 (18), SIRT3 (19), and SIRT5 (20), there are several differences on the surface of the protein. In all previously solved sirtuin structures, a conserved "cofactor binding loop" (Supplemental Fig. 2) (48) is involved in NAD$^+$ binding; this loop adopts several different conformations depending on the ligand(s) bound in the active site. A small region of the cofactor-binding loop is disordered in the ADPr-bound SIRT5 structure, but in the suramin-SIRT5 complex structure, this loop becomes ordered, and even forms β strands and a small helix which directly interact with suramin - an anti-parasitic drug that also binds sirtuins with micromolar affinity (20). The changes in the cofactor-binding loop are also observed in the set of SIRT3 structures (19). SIRT6 lacks the cofactor-binding loop, which is replaced by a single helix (α3) containing several NAD$^+$ binding residues (Fig. 4A). Unlike the cofactor-binding loop in the ADPr-bound SIRT5 structure and the SIRT3 apo structure, helix α3 appears to be ordered in the SIRT6+ADPr and SIRT6+NAADPr structures. This is an indication that the SIRT6 substrate-binding pocket may be less flexible and does not significantly vary its conformation when different ligands are bound. In addition, helix α1 in SIRT6 is much longer and the N-terminal unstructured coil folds back towards, and may structurally stabilize, the NAD$^+$ binding site (Fig. 4B), although there is no direct interaction between the random coil and ADPr.

In contrast to Classes I, II and III human sirtuins, Class IV sirtuins contain a deletion in the sequence immediately following the α3 helix (Supplemental Fig. 2). The result is that SIRT6 does not have a helix bundle in its small domain (Fig. 4A). The helix bundle is replaced by a short loop, which interacts with the loop between α2 and α3 and contacts a small region on the zinc-binding module. The lack of a helix bundle to form extensive interactions with the β-sheets in the zinc-binding module provides one explanation for why SIRT6 has a splayed small binding domain. This unique structural module provides one explanation for why SIRT6 has a splayed small binding domain. This unique structural feature is likely to be adopted by SIRT7, another class IV sirtuin that also contains a deletion in this region (Supplemental Fig. 2), but that has not yet been structurally and functionally characterized. This unique feature may be responsible for the observed lower activity (1000 times) of SIRT6. To date, there are no reports of detectable deacetylase activity for SIRT7.

In addition to the missing helical bundle, another reason for the splayed small domain in SIRT6 is the loss of a conserved salt bridge, which has been reported to contribute to the positioning of the zinc-binding motif with respect to the Rossmann fold domain and substrate-binding site (20,24). Based on precedent from other sirtuins, a salt bridge should be formed between Arg180 and Asp188 (Fig. 4C) in SIRT6. However, this interaction is not observed. The side chain of Arg180 is directed towards the surface of the protein rather than towards the Asp188 residue. Instead, Arg124 on the loop connecting α5 and β3 and Gln145 on β5 form a hydrogen bond to tether the zinc-binding motif and the large domain together (Fig. 4C). The loss of the previously described salt bridge removes the constraint on the two loops, which would otherwise result in a more closed conformation. The new Arg124-Gln145 hydrogen bond in SIRT6 pulls the two domains together on the opposite side and therefore, the small domain is not directly above the Rossmann fold, but rather in an open conformation tilted in a 45° angle.

It is generally observed that peptide substrates interact with sirtuins via non-specific backbone
interactions (19). There is a conserved FGExL loop that forms hydrogen bonds with the substrate peptide (27). However, in the case of SIRT6, the sequence is WEDsL (Supplemental Fig. 2). The amide nitrogen of Trp186 appears to be flipped while its analogue in other sirtuins is involved in a hydrogen bond with a carbonyl group from the peptide substrate backbone. Upon superimposition of the SIRT6 structure with the SIRT3-acetylated peptide structure (PDB:3GLT)(19), we observed that the backbone amide between the tryptophan and glutamate side chains is further away and cannot form a hydrogen bond with the carbonyl in the amide backbone of the lysine (Fig. 4D). This poses an issue for the peptide-protein anti-parallel β-sheet formation. It is possible that a β-sheet configuration might still form, but a kink near the active site would adversely affect the interactions between the peptide substrate and the protein. Therefore, a less stable substrate-protein interaction might be the cause of the poor deacetylase activity for SIRT6 and why the H3K9Ac peptide does not exhibit binding saturation by isothermal titration calorimetry (Supplemental Fig. 3).

Sirtuins catalyze deacetylation through a sequential mechanism in which the substrate peptide binds first to form an ordered NAD⁺- binding pocket for the subsequent co-substrate binding. SIRT1, SIRT2 and SIRT3 were all found to require a substrate/ligand binding prior to NAD⁺ binding (19,34,49). The unique SIRT6 structural features suggest that SIRT6 utilizes a different binding mechanism, which does not require peptide substrate binding prior to NAD⁺ binding. These features include: an ordered helix regardless of substrate binding; large open channel in the substrate and NAD⁺ binding site due to missing helix bundle; NAD⁺ site potentially reinforced by the N-terminal loop. To test this hypothesis, we performed a series of equilibrium binding experiments with SIRT6.

NAD⁺ metabolite binding - Isothermal titration calorimetry was used to monitor the binding of NAD⁺ in the absence of an acetyl lysine substrate. Consistent with previous kinetic results, Hst2, SIRT1-3 and 5 did not bind NAD⁺ in the absence of the acetyl lysine substrate (Fig. 5A, supplemental Fig. 4). Strikingly, NAD⁺ bound to SIRT6 with a Kd of 27 ± 1 µM (Fig. 5B), providing the first physical evidence that SIRT6, unlike other characterized sirtuins, can bind NAD⁺ efficiently in the absence of an acetylated substrate. We detected no binding when NADH was titrated into SIRT6 (supplemental Fig. 5A), suggesting that SIRT6 cannot accommodate the puckered conformation of the nicotinamide ring of NADH. Interestingly, ADPr bound to SIRT6 with a Kd of 4.7 ± 0.5 µM (supplemental Fig. 5B, Fig. 5E), suggesting that the binding pocket of SIRT6 distinguishes structural differences between NAD⁺ and ADPr. The construct (3-318) used for SIRT6 crystallization was able to bind NAD⁺ and ADPr with similar affinities compared to the full-length SIRT6 construct (Supplemental Fig. 6), suggesting that there is negligible variability between the full-length construct used for kinetic assays and the crystallized constructs.

Consistent with the crystal structure, the non-hydrolyzable product analog 2'-NAADPr bound to SIRT6 with a Kd of 22 ± 2 µM (supplemental Fig. 5C, Fig 5B). Interestingly, unlike NAD⁺ and ADPr, 2'-NAADPr bound with a negative –TΔS (Fig. 5A & 5B) suggesting there is more disorder when 2'-NAADPr binds. This result is consistent with the crystal structure, which showed a high degree of variability and poor electron density for the 2'-N-acetyl-ribose moiety of 2'-NAADPr.

The invariant catalytic base histidine residue of Hst2 is important for both NAD⁺ binding and activation of the 2'-hydroxyl of the α1'-O-alkylamidate intermediate during the deacetylation reaction (43). To investigate the role of SIRT6 His131 in NAD⁺ and ADPr binding, ITC was used to monitor the effects of the H131Y substitution on the ability of SIRT6 to bind NAD⁺ metabolites. The substitution to tyrosine led to a dramatic effect in SIRT6’s ability to bind ADPr. The Kd of 69 ± 4 µM was ~15 times higher than the Kd for WT SIRT6 (supplemental Fig. 5D, Fig. 5B). The enthalpy increased 6 ± 1 kcal/mol, consistent with the loss of hydrogen bonds to the 2' and 3'-hydroxyl groups of the ribose, observed in the crystal structure of SIRT6 bound to ADPr (Fig. 2C). Interestingly, the H131Y mutant bound NAD⁺ with a Kd of 8.4 ± 0.5 µM, or ~3 times lower than the Kd for WT SIRT6 (supplemental Fig. 5E, Fig. 5B). This is a 47-fold change in specificity for NAD⁺ over ADPr from WT SIRT6 to H131Y SIRT6. The enthalpy of binding NAD⁺ decreased 15 ± 1 kcal/mol, while -TΔS became
less favorable as it increased 14 ± 1 kcal/mole from WT to H131Y (Fig. 5B). These changes likely reflect increased hydrogen bonding and van der Waal’s contacts between the nicotinamide moiety of NAD⁺ and H131Y of SIRT6 causing the ΔH to become more negative and subsequent ordering of both NAD⁺ and SIRT6, leading in turn to the increase in the entropy noted in the ΔS value.

As an independent method for analyzing NAD⁺ metabolite binding, we monitored changes in tryptophan fluorescence as a function of ligand binding. SIRT6 contains two tryptophan residues near the active site (Trp69 and Trp186). We observed a decrease in the tryptophan fluorescence signal of SIRT6 with increasing concentrations of NAD⁺ (supplemental Fig. 7A), suggesting a change in the local environment surrounding the tryptophan residues upon NAD⁺-binding. The fluorescence intensities were adjusted for non-specific quenching of tryptophan residues located outside of the active site and the data were fitted to a one-site binding curve (Equation 3). The K_d of 24 ± 7 μM is in excellent agreement with the K_d determined by ITC (K_d of 27 μM). However, at increasing concentrations of ADPr no change in fluorescence was observed (supplemental Fig. 7B), despite the fact that by ITC, ADPr binds to SIRT6 with a K_d of 4.7 μM. The results suggest that there are different structural rearrangements in the active site depending on the NAD⁺ metabolite.

In agreement with the ITC results, a decrease in the fluorescence of H131Y SIRT6 was observed at increasing concentrations of NAD⁺, yielding a K_d of 3.5 ± 0.6 μM for NAD⁺ (supplemental Fig. 7C). Interestingly, an increase in fluorescence was observed with H131Y SIRT6 upon binding ADPr (supplemental Fig. 7D), suggesting that the environment surrounding the tryptophan residues are more non-polar when ADPr binds. The K_d of 51 ± 8 μM is in agreement with the previous ITC results (K_d of 69 μM, Fig. 5B). These results further highlight the conclusion that NAD⁺ and ADPr induce different structural perturbations on SIRT6 upon binding.

**DISCUSSION**

Several physiological and cellular investigations have suggested that SIRT6 plays a role in genome maintenance and metabolic regulation (10,12,15-17,50-53). While these studies have provided insight into the biological role of SIRT6, the molecular functions of SIRT6 remain unclear. Here we have reported the first quantitative assessment of SIRT6 activity, providing direct evidence that OAAADPr is formed as a product of an extremely inefficient deacetylase reaction. Unlike other sirtuins, SIRT6 displays tight binding of NAD⁺ in the absence of an acetylated substrate. Furthermore, tryptophan fluorescence results suggest that NAD⁺ and ADPr induce different structural arrangements upon binding. We have also solved the first crystal structures of SIRT6, a member of the Class IV sirtuins. The crystal structures provide insight into the biochemical functions presented in this study and reveal several significant differences between SIRT6 and the other solved sirtuin structures.

SIRT6 was reported to act as an H3K9 deacetylase (12). Here, we utilized three quantitative assays to establish that SIRT6 forms OAAADPr, but at a specific activity of ~ 0.0002 s⁻¹ towards H3K9Ac, which is ~1000 times slower than other highly active sirtuins (34). Furthermore, the rate of nicotinamide cleavage is similar to the rate of OAAADPr formation, suggesting that SIRT6 functions as a NAD⁺-dependent deacetylase rather than as an ADP-ribosyltransferase. It is unlikely that the low deacetylase activity is caused by inhibition of the undefined molecule observed in the acetyl lysine-binding site of the SIRT6-ADPr structure. The SIRT6+NAADPr structure shows no evidence of this molecule, suggesting that it was either readily displaced by NAADPr or was not present during crystallization. It is important to note that the acetyl group of NAADPr occupied the same space as that of the undefined molecule in the SIRT6+ADPr structure. If this molecule were responsible for the low activity of SIRT6, we would expect to observe major differences in the binding affinities among NAADPr, NAD⁺ or ADPr. The fact that NAADPr and NAD⁺ displayed nearly identical binding affinities suggests that this molecule, if present, did not impede NAADPr binding and therefore would not significantly contribute to inhibiting SIRT6 activity. In addition, the results are consistent with the previous analyses, which demonstrated that SIRT6 is not an efficient H3K9 deacetylase (12). The fact that SIRT6 has been implicated in important processes
including several metabolic pathways (17,50,51,53), double-strand break repair (15,52), base-excision repair (10) and telomere maintenance (12,13) raises the question of why a seemingly important sirtuin would display such low deacetylase activity.

The newly solved crystal structures of SIRT6 in complex with ADPr and NAADPr provide some insight. SIRT6 shares an overall similar architecture with the previously solved sirtuin structures and contains the conserved catalytic residues. However, the SIRT6 structure revealed many unique features. SIRT6 exists in a more open conformation, containing a zinc-binding motif in the small domain, which is splayed from the larger Rossmann fold domain. Several structural features contribute to the displacement of the small domain relative to its position on other sirtuins including the lack of a conserved salt bridge between the zinc-binding motif and the substrate-binding loop. This conserved interaction was previously demonstrated to be important for maintaining the proper orientation of the small domain with respect to the large domain and is important for forming the acetylated substrate-binding pocket (20,24). In SIRT6 however, a new hydrogen bond connects the zinc-binding motif to the lower portion of the Rossmann fold, stabilizing SIRT6 in an open conformation. Furthermore, the Class IV sirtuins, which include SIRT6, lack a conserved helix bundle region that forms contacts between the small domain and the Rossmann fold domain. The consequence of this feature contributes to the open conformation observed in the SIRT6 crystal structure. The open conformation might disrupt the structural integrity of the substrate-binding pocket, providing one explanation for why H3K9Ac does not exhibit binding-site saturation, which in turn leads to poor deacetylase efficiency.

Instead of containing a highly conserved cofactor binding loop (48) that aids in NAD\textsuperscript{+} binding, SIRT6 contains a single helix that forms interactions with ADPr and NAADPr. In several sirtuin structures, the orientation of the loop and the ordering of the residues depend on the substrate(s) bound in the active site (26,29). The single helix in SIRT6 however, appears to be ordered in both structures, indicating that the substrate-binding pocket is less flexible and does not widely vary its conformation. This lead us to hypothesize that, unlike all other studied sirtuins, SIRT6 would be able to bind NAD\textsuperscript{+} in the absence of an acetylated substrate.

We found that SIRT6 can bind NAD\textsuperscript{+} with a relatively high affinity ($K_d = 27$ µM) in the absence of an acetylated substrate. Furthermore, we determined that SIRT6 can bind both ADPr (4.7 µM) and NAADPr (22 µM) with relatively similar affinities compared to NAD\textsuperscript{+}. However, the fact that a change in tryptophan fluorescence was observed when the concentration of NAD\textsuperscript{+} was increased and not when ADPr was increased suggests that there may be differing structural changes near the tryptophan residues when the ligands bind; this provides evidence that SIRT6 is able to distinguish between the ligands that are bound in the active site. To investigate NAD\textsuperscript{+}-binding more closely, we mutated the catalytic histidine residue (His131), which was previously reported to be important for not only catalysis, but also for NAD\textsuperscript{+}-binding (43). We found that H131Y SIRT6 can still bind NAD\textsuperscript{+} (8.4 µM), but has a decreased ability to bind ADPr (69 µM). In addition, while a decrease in the tryptophan fluorescence signal was seen with increasing NAD\textsuperscript{+}, an increase in fluorescence was seen with increasing ADPr. Together these results further support the conclusion drawn for WT SIRT6, that there is an inherent structural difference near the tryptophan residues in SIRT6 between NAD\textsuperscript{+} and ADPr binding. Therefore, this structural difference may play an important role in controlling SIRT6 activity.

The fact the NAD\textsuperscript{+} binds to SIRT6 in the absence of an acetyl lysine substrate is a unique feature of this sirtuin. Also, the low basal activity and the unique structural features suggest that regulatory mechanisms may be needed to activate SIRT6 in cells. The open structure of SIRT6 might provide a surface for regulatory protein binding and stimulation of activity. Similar mechanisms have been demonstrated for other histone modifying proteins including the yeast acetyltransferase RTT109, which requires the histone chaperones Asf1 (54) and Vps75 (55) for efficient activity. SIRT6 interacts with several transcription factors including NF-κB(16) and Hif1α(17); these interactions might stabilize an active conformation of SIRT6 and subsequently stimulate histone deacetylation at the site of
action. It is also possible that SIRT6 is indeed a robust enzyme, but we and others have not supplied the appropriate protein substrate. Alternatively, the deacetylase activity of SIRT6 might not be regulated by activation, but instead this intrinsically low deacetylase activity might suggest that SIRT6 has a different molecular function.

Interestingly, SIRT6 has been implicated in many of the same pathways as SIRT1, including the targeting of H3K9 for deacetylation (56). SIRT1 is a highly active deacetylase, begging the question of why purified SIRT6 would also deacetylate H3K9, but very inefficiently. The ability of SIRT6 to bind NAD$^+$ in the absence of an acetylated substrate is unique and the possibility that SIRT6 may adopt different structural arrangements depending on which ligand is bound suggests that an alternative hypothesis for SIRT6’s molecular function is plausible: SIRT6 has evolved away from a functioning NAD$^+$-dependent deacetylase and toward a function as a NAD$^+$ metabolite sensor. There are many examples of enzymes that have evolved away from their catalytic role and toward a regulatory function that takes advantage of the ability to bind ligand. These include signaling proteins that belong to the large family of protein kinases (57) and phosphatases (58).

SIRT6 controls flux into glycolysis by inhibiting Hif1α-dependent transcription of several glycolytic genes to promote mitochondrial respiration (17). In addition, SIRT6 has also been reported to negatively regulate PPARγ-mediated transcription to prevent triglyceride formation (59). These are two pathways controlled by cellular NAD$^+$ levels and furthermore are also pathways in which SIRT1 has been reported to play an important role (60,61). SIRT6 is found at the promoters of several metabolic genes involved in these pathways and its interaction with transcription factors suggests that NAD$^+$ levels might control SIRT6 function. Presumably, under high levels of NAD$^+$, SIRT6 could bind to transcription factors at promoters of metabolic genes preventing triglyceride formation and flux into glycolysis. Alternatively, below $K_d$ levels of NAD$^+$, the interaction would be destabilized, allowing transcription factors to access the targeted genes. Therefore, SIRT6 might ensure metabolic homeostasis and promote more efficient energy usage through its ability to sense the levels of NAD$^+$ (and related metabolites) and regulate protein:protein interactions with transcription factors. Future in vivo studies will be needed to explore these possibilities.

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REFERENCES


**FOOTNOTES**

The abbreviations used are: OAADPr, O-acetyl-ADP-ribose; NAADPr, N-acetyl-ADP-ribose; ADPr, ADP-ribose; CtIP, [C-terminal binding protein interacting protein]; BER, base excision repair; DSB, DNA double-strand break repair; ITC, isothermal titration calorimetry.

**FIGURE LEGENDS**
Fig. 1. SIRT6 deacetylation assays. A, charcoal-binding assay measuring the production of OAADPr. All assays carried out in the presence of 4 µM SIRT6 WT (●) or H131Y (■), 300 µM [³H]-H3K9Ac peptide and 600 µM NAD⁺. Shown is the average of three experiments for WT and H131Y. Rate of OAADPr production was 3.2 ± 0.2 µM/h based on linear regression analysis with an R² of 0.98612. B, HPLC separation of [³H]-labeled products monitoring deacetylation of a [³H]-H3K9Ac peptide. Reactions were carried out in the presence of 300 µM [³H]-H3K9Ac peptide, 2 mM NAD⁺ and either 4 µM SIRT6, Hst2, or no enzyme. Showing all counts for Hst2 deacetylation reaction (green), SIRT6 (red) and a no enzyme control (blue) as well as a zoomed in view displaying counts for [³H]-acetate, [³H]-OAADPr and H3K9 ([³H]-N-termAc)-. C, continuous assay monitoring the release of nicotinamide over time. Average of three trials with assays carried out in the presence of 8 µM (gray) or 0 µM (white) SIRT6, 300 µM H3K9Ac, and 0.6 mM NAD⁺. Rate of nicotinamide release in the presence of SIRT6 was 10.5 ± 0.4 µM/h and in the absence was 5.9 ± 0.8 µM/h.

Fig. 2. Structure of human SIRT6 in complex with ADP-ribose. A, overall structural features of SIRT6 monomer. B, superimposition of the six molecules in the asymmetric unit. Chain A: red; Chain B: green; Chain C: dark blue; Chain D: orange; Chain E: cyan; Chain F: yellow. C, schematic illustration of the hydrogen bonding network surrounding ADPr; hydrogen bonds are indicated as dashed lines, water molecules are spheres. D, left panel: SIRT6+ADPr 2Fo-Fc electron density map (blue mesh, 1.5σ) of the residues within 4Å of ADPr; right panel: Fo-Fc omit electron density map(green mesh, 2σ) of ADPr molecule; the putative peptide binding site contains an unidentified electron density.

Fig. 3. Comparison of SIRT6+ADP-ribose with SIRT6+2’-N-acetyl-ADP-ribose structure. A, left panel: SIRT6+NAADPr 2Fo-Fc electron density map (purple mesh, 1.5σ) of the residues within 4Å of NAADPr; right panel: Fo-Fc omit electron density map(green mesh, 1σ) of NAADPr molecule. B, superimposition of SIRT6+ADPr (blue) onto SIRT6+NAADPr(purple), the unknown density (Fo-Fc omit electron density map contoured at 2σ, green mesh) observed in SIRT6+ADPr structure is not present in the SIRT6+NAADPr structure, the N-acetyl group occupies part of the unidentified density space. C, residues immediately surrounding the N-acetyl group of NAADPr (purple) and their corresponding residues (blue) in the SIRT6+ADPr structure.

Fig. 4. SIRT6+ADPr structure compared to other solved human SIRTs. A, SIRT6 structure (3PKI, orange) superimposed onto SIRT3 (3GLR, green); left panel: highlighting differences in the zinc-binding domain; right panel: showing a missing helix bundle. B, comparison of N-terminal loop of SIRT6 (3PKI, orange), SIRT2 (1J8F, pink), SIRT3 (3GLR, green) and SIRT5 (2B4Y, blue); SIRT6 contains a long loop covering NAD⁺ binding site. C, SIRT6 lacks the conserved salt-bridge; left: SIRT3 (3GLR) structure with conventional salt bridge in the sirtuin family; right: the green pair (Arg180 and Asp188) is where the bridge would be predicted to form on the SIRT6 structure according to other sirtuin crystal structures; pink pair (Arg124 and Gln145) : the actual hydrogen bonding pair found in SIRT6 structure. D, Stereo diagram of flipped FGExL(WEDsL) loop of SIRT6(3PKI, orange)+ADPr(orange) compared with SIRT3(3GLT, green) with an ADPr-thioAceCS2 (green) complex.

Fig. 5. Isothermal titration calorimetry studies. A, representative ITC trace. Top graph shows data obtained for 37 automatic injections (1-8 µL) of 1.4 mM NAD⁺ titrated into 27 µM SIRT6. Bottom graph represents integrated curves of the experimentally generated heats. The data were fitted to a one-site binding curve (—). NAD⁺ (1.40 mM or 1.63 mM) was titrated into 27 µM WT SIRT6 (●) or 50 µM Hst2 (■) respectively. B, dissociation constants and column
A graph displaying thermodynamic parameters for WT and H131Y SIRT6 binding to NAD⁺, ADPr, and 2’-NADPr. NAD⁺ (1.40 mM or 767 µM) was titrated into WT or H131Y SIRT6 respectively. ADPr (390 µM or 1.45 mM) was titrated into 28 or 37 µM WT or H131Y SIRT6 respectively. 2’-NADPr (2.96 mM) was titrated into 38 µM WT SIRT6. Change in Gibbs free energy (ΔG) is shown in blue. Change in enthalpy (ΔH) is shown in green and –TΔS is displayed in brown.

Table 1. Data collection and refinement statistics

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† R_work is defined as \(\Sigma |F_{o}\text{-}F_{c}| / \Sigma |F_{o}|\), where \(F_{o}\) and \(F_{c}\) are observed and calculated structure-factor amplitudes, respectively.
‡ R_free is the R factor for the test set (1-10 % of the data).
Figure 1

A

B

C

[Graphs and diagrams showing data analysis]
Figure 5