Acetylation-dependent ADP-ribosylation by *Trypanosoma brucei* Sir2*

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Sir2 (Silent information regulator 2) NAD\(^{+}\)-dependent enzymes (sirtuins) are found in all kingdoms of life and have been implicated in a variety of cellular processes such as gene silencing (1–3), lifespan extension (4–7), and direct metabolic regulation (8–10). Founding member ySir2p is essential for maintaining transcriptionally silenced chromatin in yeast (1–3), whereas overexpression of Sir2 leads to life span extension in diverse organisms (4, 5). Mammalian studies have focused on the seven human sirtuins, SIRT1–SIRT7 (11–13). The most studied human sirtuin, SIRT1, is reported to act on a number of enzymes and transcription factors that are dynamically regulated by reversible acetylation (14). For example, SIRT1 has been shown to affect the functions of p53 (15–17), NFκB (18), FoxO (19–21), Mef2 (22), PGC-1\(\alpha\) (23, 24), human immunodeficiency virus transcription by Tat (25), acetyl-CoA synthetase 1 (9); play a role in lifespan extension (6, 7); and increase glucose response to insulin (26).

Although there is general consensus that many sirtuins affect biological pathways by catalyzing the NAD\(^{+}\)-dependent deacetylation of target proteins, a number of reports have suggested that some sirtuins catalyze protein ADP-ribosylation, either exclusively or in conjunction with their inherent deacetylation activity. The initial idea that sirtuins are indeed enzymes and could mediate phosphoribosyl transfer came from work on *Salmonella typhimurium* CobB, a Sir2 homologue that could compensate for the loss of CobT, a phosphoribosyltransferase involved in cobalamin biosynthesis (27). Subsequent studies reported the abilities of CobB and human SIRT2 to transfer radiolabel from \[^{32}P\]NAD\(^{+}\) to bovine serum albumin (11). Another study reported the ability of ySir2 to transfer ADP-ribose to both bovine serum albumin and histones (1). These early reports suggested sirtuins possessed intrinsic ADP-ribose transferase activity.

However, additional reports demonstrated that a majority of sirtuins were robust NAD\(^{+}\)-dependent histone/protein deacetylases, coupling deacetylation to the formation of a novel metabolite, O-acetyl-ADP-ribose (OAADPr)\(^{3}\) (28–33). However, not all human sirtuins had measurable *in vitro* deacetylase activity on histone substrates (34, 35). Among mammalian sirtuins with little or no detectable histone deacetylase activity, SIRT4 was shown to ADP-ribosylate and down-regulate glutamate dehydrogenase and has been implicated in insulin regulation (35, 36). Also, SIRT6 functions in DNA base-excision repair and can mediate auto-ADP-ribosylation (37).

Other studies report that sirtuins, including ySir2, Hst2, and Sir2 orthologues from the parasites *Trypanosoma brucei* and *Plasmodium falciparum*, possess both protein deacetylase and mono-ADP-ribosyltransferase activity (28, 31, 38). *T. brucei* Sir2 orthologue TbSIR2rp1 localizes to the nucleus and plays a role in DNA repair and silencing in the insect and bloodstream stages of the parasite (38, 39). However, the mechanistic relationship between protein deacetylation and ADP-ribosylation has not been elucidated.

Here we perform biochemical and kinetic studies to probe the mechanisms of ADP-ribosylation using sirtuin *T. brucei* Sir2.
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TbSIR2rp1 as a model for sirtuins that display both deacetylase and ADP-ribosyltransferase activities. Steady-state kinetic analysis revealed a highly active histone deacetylase, but protein ADP-ribosyltransferase activity was ~5 orders of magnitude lower. Moreover, protein/histone ADP-ribosylation by TbSIR2rp1 required an acetylated substrate. We propose that the dependence on acetylated substrate occurs through two distinct pathways. The first pathway involves a direct, non-catalytic reaction between the unique deacetylation product OAADPr (or its hydrolysis product ADPr) and histones. The second pathway is responsible for the majority of TbSIR2rp1-dependent ADPr transfer and involves a mechanism in which a side-chain nucleophile from bound histone attacks an intermediate from the catalytic pathway that normally leads to deacetylated protein and OAADPr.

EXPERIMENTAL PROCEDURES

Plasmid Construction—T. brucei genomic DNA was generously provided by Dr. J. Bangs from University of Wisconsin, Madison, and used as a template for the subsequent PCR. TbSir2rp1 was amplified using primers 5′-GGGATCCATGACAGAACGGAATGTTAGCAACC-3′ and 5′-CCGTCGAGACCCCTCAACGACTTTTTC-3′ that introduced BamHI and Xhol, upstream and downstream recognition sites, respectively. PCR products were gel-purified, digested with BamHI and Xhol, and cloned into pGEX-KG (40) upstream and in-frame of a sequence encoding a glutathione S-transferase (GST) fusion protein. Using BamHI and HindIII, the TbSir2rp1 fragment was released and subcloned into pQE80 (Qiagen) downstream and in-frame of a sequence encoding an His6 tag.

Eukaryotic expression plasmid pcDNA3.1 SirT1 was kindly provided by Dr. Eric Verdin from University of California, San Francisco, and used as a template for the following PCR. SirT1 was amplified using primers 5′-GGTTCATGAGTTCATGACAGAACGGAATGTTAGCAACC-3′ and 5′-CCTCAGTCGAGACCCCTCAACGACTTTTTC-3′ that introduced BamHI and Xhol, and cloned into pGEX-KG (40) upstream and in-frame of a sequence encoding a glutathione S-transferase (GST) fusion protein. Using BamHI and HindIII, the TbSir2rp1 fragment was released and subcloned into pQE80 (Qiagen) downstream and in-frame of a sequence encoding an His6 tag. All plasmids were sequence-verified. The SirT1 template contained a deletion of amino acid residues 6–84 resulting in a truncated form of bacterially expressed SIRT1.

Protein Expression—Plasmids encoding TbSIR2rp1 and SIRT1 were transformed into Escherichia coli BL21DE3 and grown in 2× YT media containing 100 mg/liter of ampicillin at 37 °C until an A600 nm ~ 0.7 was reached. Cultures were induced with 0.1 g/liter isopropyl β-d-thiogalactopyranoside for 4–6 h at room temperature. Harvested cells were lysed by sonication in 50 mM Tris (pH 8.0), 300 mM NaCl, 1.0 mM β-mercaptoethanol, and protease inhibitors (0.1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 5 μg/ml apropin). Histidine-tagged proteins were purified over nickel-nitritolactric acid resin (Qiagen), as described by the manufacturer, and eluted with a 0–250 mM imidazole gradient. TbSIR2rp1-GST cells were induced as described above, processed in 1× phosphate-buffered saline, and purified using glutathione-agarose resin (GE Healthcare) as described by the manufacturer. Purified proteins were dialyzed in the following storage buffer: 50 mM Tris-HCl (pH 7.5), 1.0 mM dithiothreitol, and 10% glycerol.

Histone Acetylation—Recombinant Xenopus laevis histones were expressed and purified from E. coli as described previously (41) and acetylated using the histone acetyltransferase complex, piccolo-NuA4 (picNuA4) (42). Reactions containing 0.2 μM picNuA4, 5 mM DTT, 75 μM acetyl-CoA, and 300 μg recombinant histones in 50 mM Tris, 150 mM NaCl were incubated at 24 °C for 1 h. Histone acetyltransferases were then heat-inactivated by boiling at 95 °C for 20 min. picNuA4-acetylated histones (i.e. AcH2A) were used for all the experiments, unless otherwise indicated. Histones H2A and H4 were also chemically acetylated using acetic anhydride in 50 mM HEPES (pH 7.4) using 100× molar excess anhydride. Enzymatic and chemical acetylation were confirmed by electrospray ionization-mass spectrometry. Histone concentrations were determined by the BCA protein assay (Pierce) using bovine serum albumin as a standard or by molar extinction coefficients (ε276 nm, H4 = 4050 M⁻¹ cm⁻¹, ε276 nm, H2A = 5400 M⁻¹ cm⁻¹).

Peptide Synthesis—Acetylated histone H3 11mer peptide corresponding to the residues around lysine 14 (AcH3, KSTGGK(ac)APRKQ) was synthesized at the University of Wisconsin Peptide Synthesis Facility.

Mass Spectrometry—Mass spectrometry was performed at the University of Wisconsin Madison Biotechnology Center on an ABI 3200 Q-trap.

Deacetylation Assays—Charcoal-binding deacetylase assay was used as described previously (43) to determine the activity of the TbSIR2rp1 recombinant proteins.

Saturation kinetics were performed with 5–750 μM [NAD⁺] with a fixed concentration of [3H]AcH3 (700 μM). A second set of saturation kinetic curves was performed with a fixed concentration of NAD⁺ (1 mM), with varying concentrations of [3H]AcH3 peptide from 10 μM to 1 mM. Reactions were incubated at 37 °C for 10 min and contained 0.7 μM TbSIR2rp1 in 1 mM DTT and 50 mM Tris-HCl (pH 7.5).

Acetylated recombinant histone H2A (AcH2A) saturation curves were generated using a fixed concentration of NAD⁺ (500 μM) and varying concentrations of [3H]AcH2A (5–100 μM). All reactions were composed of 0.7 μM TbSIR2rp1, 10 mM DTT, 50 mM Tris-HCl (pH 8.8), and 150 mM NaCl. Reactions were incubated at 37 °C for 10 min and were performed under initial velocity conditions where product formed was linear with time.

The deacetylation reaction was also monitored by an HPLC method, essentially as described (43). In these assays, time points from a deacetylase reaction were quenched at 0, 2, 4, 8, and 10 min before being analyzed by HPLC. The initial deacetylation rates were calculated by comparing the nicotinamide peak area from each time point to a nicotinamide standard curve. Rates were calculated over the linear portion of the reactions.

ADP-ribosylation Assays—Reactions, unless otherwise indicated, contained 3 μM recombinant Sir2 homologues in 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 10 mM DTT, 25 μM unlabeled NAD⁺, and 1–10 μCi of [α-32P]NAD⁺ (1000 Ci mmol⁻¹; GE Healthcare), for a total reaction volume of 100 μl. As specified, reactions contained 20 μg total (5 μg of each histone subunit)
unacetylated or acetylated recombinant histones. Samples were incubated at 37 °C for 4 h or times indicated. Reactions that contained Ach3 peptide were incubated with TbSIR2rp1 at 37 °C for 10 min prior to addition of unacetylated recombinant histones.

Unincorporated [\(^{32}\)P]NAD\(^+\) was removed by trichloroacetic acid protein precipitation followed by three acetone washes. Precipitated proteins were resuspended in SDS loading buffer, boiled at 95 °C for 15 min, and resolved on an 18% SDS-polyacrylamide gel. Gels were stained with Coomassie Blue, dried, and exposed to film or PhosphorImaging screen. Quantification was performed by densitometry using lane-specific background subtraction and a standard curve of [\(^{32}\)P]NAD\(^+\) with the same specific activity.

Reactions containing [\(^{32}\)P]OAADPr or biotin-NAD\(^+\), used in place of [\(^{32}\)P]NAD\(^+\), were the same as specified above. Biotin,NAD\(^+\)-6-bio-17-NAD\(^+\), was synthesized as described previously (44) and confirmed by mass spectrometry.

Synthesis of [\(^{32}\)P]OAADPr—[\(^{32}\)P]OAADPr was synthesized and purified as reported previously (45). The [\(^{32}\)P]-labeled product was derived from a stock of [\(^{\alpha-\mathrm{32}}\)P]NAD\(^+\) with known concentration and specific activity to ensure identical specific activities among the reaction substrates.

Snake Venom Phosphodiesterase Cleavage—The cleavage reaction was conducted as described (46).

RESULTS

TbSIR2 Deacetylase Activity—T. brucei Sir2-related protein (TbSIR2rp1) was cloned from genomic DNA. Bacterial constructs expressing TbSIR2rp1 either as an His\(_6\) or a GST fusion protein were generated. A previous study of the T. brucei SIR2-related protein utilized a GST fusion protein (38). Although NAD\(^+\)-dependent deacetylase activity of TbSIR2rp1-GST had been observed previously (38), a quantitative steady-state analysis has not been reported.

To verify that both bacterial constructs generated enzymes with similar deacetylase activity, the steady-state kinetic parameters of both purified proteins, termed TbSIR2-His (predicted molecular mass of 38.5 kDa) and TbSIR2-GST (predicted molecular mass of 64.5 kDa), were determined (see Fig. 1, A and B). In these reactions, 0.7 \(\mu\)M enzyme was reacted with 700 \(\mu\)M acetylated histone H3 peptide (KSTGGK(ac)APRKQ) and varied [NAD\(^+\)] (Fig. 1A) or 1 mM [NAD\(^+\)] and varied [AcH3] (Fig. 1B). TbSIR2-His and TbSIR2-GST displayed almost indistinguishable deacytlation activity under varied [NAD\(^+\)], with \(K_m\) and \(k_{cat}/K_m\) values of 42 ± 3.2 \(\mu\)M, 0.075 ± 0.001 s\(^{-1}\), and 1800 ± 139 M\(^{-1}\)s\(^{-1}\), and 45 ± 3.5 \(\mu\)M, 0.090 ± 0.002 s\(^{-1}\), and 2000 ± 200 M\(^{-1}\)s\(^{-1}\), respectively (Fig. 1A).

Similarly, the steady-state kinetic parameters with varied [AcH3], yielded \(K_m\) and \(k_{cat}/K_m\) values of 82 ± 5.9 \(\mu\)M, 0.060 ± 0.001 s\(^{-1}\), and 730 ± 54 M\(^{-1}\)s\(^{-1}\) for TbSIR2-His and 46 ± 5.8 \(\mu\)M, 0.050 ± 0.001 s\(^{-1}\), and 1100 ± 141 M\(^{-1}\)s\(^{-1}\) for TbSIR2-GST (Fig. 1B). Most importantly, the kinetic values are comparable with those reported for other highly active sirtein deacetylases (34, 47), establishing TbSIR2rp1 as a bona fide protein deacetylase. Because no significant differences in activity were observed between the two constructs, subsequent experiments were performed with TbSIR2rp1-His, abbreviated TbSIR2, unless otherwise indicated.

TbSIR2 Displays ADP-ribosyltransferase Activity—After characterizing TbSIR2 NAD\(^+\)-dependent deacetylase activity, we explored the ability of the enzyme to catalyze protein ADP-ribosylation. In several reports, sirtuins incubated with [\(^{\alpha-\mathrm{32}}\)P]NAD\(^+\) resulted in [\(^{32}\)P]-labeling of proteins (histones, bovine serum albumin, and GDH) or of sirtuins themselves (1, 11, 35, 37, 38), suggesting that these sirtuins possess ADP-ribosyltransferase activity. To begin examining the requirements and mechanism(s) of ADP-ribosylation, we performed initial TbSIR2 assays under conditions similar to those that previously yielded [\(^{32}\)P]-labeling of histones (38).

Consistent with published work (38), TbSIR2 was capable of transferring the radiolabel from [\(^{\alpha-\mathrm{32}}\)P]NAD\(^+\) to calf thymus histones (see supplemental Fig. S1A, lane 4). However, because

![](https://example.com/image1.png)

**FIGURE 1.** Saturation kinetics of deacetylation by recombinant T. brucei SIR2. A, TbSIR2-His (○) and TbSIR2-GST (□) deacetylase reactions containing 700 \(\mu\)M [\(^{\mathrm{3H}}\)AcH3 peptide with varying [NAD\(^+\)] (5–750 \(\mu\)M). Plots of initial velocities versus [NAD\(^+\)] were fitted to the Michaelis-Menten equation, yielding \(K_m\), \(k_{cat}\), and \(k_{cat}/K_m\) values of 42 \(\mu\)M, 0.075 s\(^{-1}\), and 1800 M\(^{-1}\)s\(^{-1}\) for TbSIR2-His and 45 \(\mu\)M, 0.090 s\(^{-1}\), and 2000 M\(^{-1}\)s\(^{-1}\) for TbSIR2-GST, respectively. B, deacetylase reactions containing 1 mM NAD\(^+\) with varying concentrations of [\(^{\mathrm{3H}}\)AcH3 (10–1000 \(\mu\)M). Plots of initial velocities versus [AcH3] were fitted as in A, yielding \(K_m\) and \(k_{cat}/K_m\) values of 82 \(\mu\)M, 0.060 s\(^{-1}\), and 730 M\(^{-1}\)s\(^{-1}\) for TbSIR2-His and 46 \(\mu\)M, 0.050 s\(^{-1}\), and 1100 M\(^{-1}\)s\(^{-1}\) for TbSIR2-GST, respectively. Reactions contained 0.7 \(\mu\)M enzyme and 1 mM DTT in 50 mM Tris-HCl (pH 7.5). Error bars are S.D. from three experiments (n = 3).
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commercially available calf thymus histones are relatively heterogeneous preparations, we repeated the above experiment with purified, recombinant X. laevis histones (H3, H2A, H2B, and H4), which lack detectable post-translational modifications. In dramatic contrast, TbSIR2 had a much lower capacity to transfer the label from \(^{32}\)P\(\text{NAD}^+\) to recombinant purified histones (see Fig. S1A, lane 5). Note that both preparations of histones showed a low level of nonspecific, background labeling with the \(^{32}\)P\(\text{NAD}^+\) (Fig. S1A, lanes 1 and 2), also previously observed by Liszt et al. (37).

Native histone preparations, such as commercially available calf thymus histones, include various amounts of post-translational modifications, including acetylation. We hypothesized that the observed increase in \(^{32}\)P labeling (putative ADP-ribosylation) upon incubation with calf thymus histones versus purified recombinant histones may be due to endogenous acetylation of calf thymus preparations, creating a possible link between the \(\text{NAD}^+\)-dependent deacetylation activity of sirtuins and the putative ADP-ribosyltransferase activity.

Acetylated Substrate Required for TbSIR2 ADP-ribosylation—To test this hypothesis, we acetylated pure recombinant X. laevis histones with the acetylation complex picNuA4 (42), and we incubated these acetylated histones with TbSIR2 and \(^{32}\)P\(\text{NAD}^+\) as described above (Fig. 2A). Both picNuA4-acetylated and unmodified recombinant histones were incubated with \(^{32}\)P\(\text{NAD}^+\) in the absence (Fig. 2A, lanes 1 and 2 and lanes 3 and 4, respectively) or presence of TbSIR2 (Fig. 2A, lanes 6 and 7, respectively).

Acetylation of the recombinant histones greatly increased the TbSIR2-mediated ADP-ribosylation (Fig. 2A, lane 6) over controls without TbSIR2 (Fig. 2A, lanes 1 and 2). Unmodified recombinant histones showed no TbSIR2-dependent increase in labeling compared with control reactions without TbSIR2 (Fig. 2A, lane 7 versus lanes 3 and 4). These findings suggested that histone acetylation is required to observe the putative ADP-ribosylation activity of TbSIR2.

Sir2 homologues from different organisms, human SIRT1 (see supplemental Fig. S1B) (37) and yeast HST2 (data not shown) (31), also displayed putative ADP-ribosyltransferase ability using \(^{32}\)P\(\text{NAD}^+\) and acetylated histones. Interestingly, the observed auto-ADP-ribosylation of sirtuin SIRT6 also increased in the presence of core histones (37).

Mechanisms of TbSIR2 Enzymatic ADP-ribosylation—Next we explored the mechanisms behind the dependence of TbSIR2 on acetylated histones for ADP-ribosylation. Previously, we proposed three possible mechanisms for sirtuin-dependent ADP-ribosylation (Scheme 1) (48). Because TbSIR2 is an active histone deacetylase, we determined whether a portion of the observed ADP-ribosylation might result from reaction of the deacetylation product OAADPr with the protein (Scheme 1c). OAADPr was generated in situ by incubating 3 \(\mu\)M TbSIR2 with 300 \(\mu\)M AcH3 peptide and 25 \(\mu\)M [\(\alpha\)-\(^{32}\)P]\(\text{NAD}^+\) for 10 min prior to the addition of 10 \(\mu\)g of unmodified recombinant histones. Under these conditions, all of the \(^{32}\)P\(\text{NAD}^+\) was converted to \(^{32}\)P\(\text{OAADPr}\). Reactions were quenched after 2 and 4 h (Fig. 2B, lanes 1 and 2, respectively). The degree of labeling was compared with that of background reactions containing 25 \(\mu\)M [\(\alpha\)-\(^{32}\)P]\(\text{NAD}^+\) incubated with 10 \(\mu\)g of recombinant histones in the presence of (Fig. 2B, lanes 3 and 4) or absence of (lanes 5 and 6) 3 \(\mu\)M TbSIR2.

As before, TbSIR2 was incapable of catalyzing ADP-ribosylation of unmodified histones (Fig. 2B, compare lanes 3 and 5 or lanes 4 and 6), although there was a slight increase of
nonenzymatic incorporation of $^{32}$P with time (Fig. 2B, lane 4 versus lane 3 or lane 6 versus lane 5). However, the amount of $^{32}$P incorporation increased substantially when TbSIR2 was incubated with an acetylated substrate prior to the addition of recombinant unmodified histones (Fig. 2B, compare lanes 1 and 2 to lanes 3 and 4), suggesting that the formation and subsequent reaction of OAADPr was responsible for a portion of the TbSIR2-dependent labeling of histones. After 2 h, the TbSIR2-mediated $^{32}$P labeling on histones was 3-fold greater than the control without enzyme (Fig. 2B, lane 1 versus lane 3), although at 4 h the labeling had increased 5-fold over the control (Fig. 2B, lane 2 versus lane 4).

It is important to point out that we cannot distinguish between labeling by OAADPr or its hydrolysis product ADPr (Scheme 1c), so both may contribute to the observed signal. However, previous work indicated that less than 20% of OAADPr hydrolyzed to ADPr over 3 h at 37°C (pH 7.5) (45).

To investigate whether OAADPr directly reacted with the histone substrate or whether TbSIR2 facilitated the OAADPr transfer, purified $[^{32}]$P]OAADPr and unmodified histones were incubated either in the absence or presence of TbSIR2, and the labeling was quantified (Fig. 2C). The amount of $^{32}$P labeling did not increase when $[^{32}]$P]OAADPr was incubated in the presence of TbSIR2 (Fig. 2C, compare lanes 7 and 10), indicating that the observed labeling resulted from a direct, nonenzymatic reaction between $[^{32}]$P]OAADPr and unmodified histones. When acetylated recombinant histones were used in place of unmodified histones, inclusion of TbSIR2 also had no effect on the extent of $[^{32}]$P]OAADPr-dependent labeling of acetylated histones (Fig. 2C, compare lanes 6 and 9). Because $[^{32}]$P]OAADPr showed less reactivity (2-fold) with acetylated histones than with unmodified histones (Fig. 2C, compare lanes 6 and 7), these data suggest that $[^{32}]$P]OAADPr and/or $[^{32}]$P]ADPr reacted nonenzymatically with histone lysine side chains. Note that this mechanism of modification requires TbSIR2 to first deacetylate substrate to generate OAADPr (Scheme 1c).

Because $[^{32}]$P]NAD$^+$ can react or associate with histones in the absence of enzyme (Fig. S1A and Fig. 2A; see also Scheme 1a), we measured the nonenzymatic association of NAD$^+$ with both acetylated and unmodified histones (Fig. 2C, compare lanes 1 and 2), and we compared its reactivity with that of OAADPr (Fig. 2C, lanes 6 and 7), using preparations of $[^{32}]$P]NAD$^+$ and $[^{32}]$P]OAADPr with identical specific activity. Overall, in the absence of enzyme, $[^{32}]$P]NAD$^+$ was more reactive with histones than was $[^{32}]$P]OAADPr (Fig. 2C, lanes 6 and 7 compared

SCHEME 1. Proposed pathways for sirtuin-dependent ADP-ribosylation. Pathway a does not require acetylated substrate and involves the direct transfer of the ADP-ribose moiety from NAD$^+$. Pathway b uses acetylated substrate and NAD$^+$ to generate an O-alkylamidate intermediate, which subsequently reacts with a nearby side-chain nucleophile. Pathway c uses acetylated substrate and NAD$^+$ to generate deacetylation product OAADPr, which subsequently reacts with the protein substrate.
with lanes 1 and 2). Interestingly, $^{32}$P-OAADPr was 7-fold more reactive than $^{32}$P-NAD$^+$ for the nonenzymatic labeling of unmodified histones (Fig. 2C, lane 7 versus lane 2). This is consistent with the 5-fold increase observed with in situ-generated $^{32}$P-OAADPr versus nonenzymatic $^{32}$P-NAD$^+$ (Fig. 2B, lanes 2 and 4). With acetylated histones, the $^{32}$P-OAADPr reaction (Fig. 2C, lane 6) showed 11-fold more labeling than the NAD$^+$ reaction (lane 1).

Next, we examined whether the enzymatic ADP-ribosylation of acetylated histones required an ADPr donor from NAD$^+$ and/or OAADPr (Fig. 2C, lanes 4 and 9). In the presence of TbSIR2, there was 7-fold more labeling of acetylated histones with $^{32}$P-NAD$^+$ than with $^{32}$P-OAADPr (Fig. 2C, lanes 4 and 9). Most strikingly, addition of TbSIR2 resulted in a 60-fold increase in ADP-ribosylation over acetylated histones and $^{32}$P-NAD$^+$ alone (Fig. 2C, compare lane 4 with lane 1). This dramatic increase in ADP-ribosylation reveals the difference between nonenzymatic NAD$^+$ association (Fig. 2C, lane 1) and the TbSIR2, NAD$^+$-dependent ADP-ribosylation on acetylated histone substrate (Fig. 2C, lane 4).

**Histone Substrate Specificity for Deacetylation and ADP-ribosylation**—To assess the efficiency of TbSIR2-catalyzed deacetylation on histone substrates, the rates of deacetylation among histones H2A and H4 were determined. We chose these histones because H2A was implicated as a target for ADP-ribosylation (38), and our data indicated both H2A and H4 were ADP-ribosylation targets (i.e. Fig. 2A). The deacetylation reactions were conducted as described above using an HPLC assay (43). Both enzymatically acetylated (42) and chemically acetylated histones were tested. Mass spectrometry showed the enzymatic acetylation yielded 1–3 acetyl groups per protein, whereas the chemical acetylation yielded an average of 10–13 acetyl groups per protein (data not shown). In these reactions, 0.7 μM TbSIR2 was incubated with 25 μM acetylated histone and 1 mM NAD$^+$ at 37 °C for 10 min. Independent of substrate or acetylation method, the rates of deacetylation under saturating substrate were similar at ~0.1 s$^{-1}$ (data not shown).

Next we tested core histones individually as substrates for ADP-ribosylation. We sought to determine which of the four core histones were essential for ADP-ribosylation and, additionally, if acetylation on the same histone was required (cis) or if an acetyl group from a different histone could support ADP-ribosylation (trans). Acetylated recombinant histones (5 μg each) were incubated with 3 μM TbSIR2 and 25 μM $^{32}$P-NAD$^+$ either alone or with various combinations of acetylated or unmodified histones (Fig. 3). Upon incubation of acetylated core histones in the absence (Fig. 3, lanes 1–4) or presence (lanes 5–8) of additional unmodified histones, only acetylated H2A (AcH2A) was able to undergo substantial labeling (lanes 1 and 5), indicating that AcH2A is the preferred target of ADP-ribosylation by TbSIR2, and that the majority of ADP-ribosylation might be occurring in cis. However, it is notable that the inclusion of AcH3 and AcH4 yielded some weak ADP-ribosylation of unmodified H2A or H2B (trans) (Fig. 3, lane 12). Interestingly, the presence of recombinant H3 and H4 inhibited TbSIR2 activity (data not shown) and likely contributed to the lower amount of labeling on AcH2A when incubated with unmodified H3 and H4 (Fig. 3, lanes 5 and 11).

**Confirmation of ADP-ribosylation**—Because previous characterization of nearly all sirtuin-dependent ADP-ribosylation activity has relied on detection of $^{32}$P transferred from $[^{32}$P]-NAD$^+$ (1, 11, 28, 35, 37, 38), we performed two additional assays to support the conclusion that ADPr is indeed being covalently transferred to acceptor histones. In these experiments, we used the preferred ADP-ribosylation target histone H2A.

First, to confirm the enzymatic transfer of the adenine ring into the ADP-ribosylated protein, the adenine ring of NAD$^+$ was labeled with a biotin moiety to generate 6-bio-17-NAD$^+$ (50). When the biotin-NAD$^+$ analogue was used in place of...
[\text{32P}]NAD^+ in the H2A ADP-ribosylation reactions, the biotin label was successfully incorporated into the histones, confirming the transfer of the adenine ring onto the histones (Fig. S2). The upper panel of Fig. S2 shows equal protein loading by Coomassie staining, and the lower panel of Fig. S2 shows enzyme-dependent incorporation of the biotin-NAD^+ by streptavidin-horseradish peroxidase Western blot.

Second, we confirmed the presence of the phosphate diester of ADPPr in TbSIR2-dependent H2A ADP-ribosylated protein using snake venom phosphodiesterase (PDE) to cleave the pyrophosphate bond (46). The PDE reaction was analyzed by both HPLC and SDS-PAGE. As expected, the HPLC chromatogram and scintillation counting of HPLC fractions are consistent with AMP release after PDE treatment (data not shown). Furthermore, the SDS-polyacrylamide gel shows >50% of the \text{32P} radiolabel from the TbSIR2 ADP-ribosylated H2A is released after PDE treatment (Fig. S3). The upper panel of Fig. S3 displays protein loading via Coomassie staining, whereas the lower panel displays the autoradiogram of the same gel.

\textit{TbSIR2 Shows Greater Deacetylase than ADP-ribosyltransferase Activity on AcH2A—}A direct comparison of the relative rates of TbSIR2 protein deacetylation and ADP-ribosylation with the preferred ADP-ribosylation target AcH2A (Fig. 3) was performed. First, the initial velocities with varied [AcH2A] (5–100 \text{ M}) were determined in the presence of a saturating [\text{NAD}^+] (500 \text{ M}), 500 \text{ M}\text{NAD}^+ \text{, } 10 \text{ mM DTT, } 150 \text{ mM NaCl in } 50 \text{ mM Tris-HCl (pH 8.8). Plots of initial deacetylation velocities versus [AcH2A] were fitted to the Michaelis-Menten equation, yielding } K_{cat}, k_{cat}/K_{m} \text{ values of } 65 \pm 6.0 \text{ M, } 0.138 \pm 0.002 \text{ M, and } 2100 \pm 210 \text{ M}^{-1}\text{s}^{-1}, \text{respectively} \text{ (Fig. } 4\text{). These numbers are in excellent agreement with the AcH3 peptide kinetic data} \text{ (Fig. } 1\text{, A and B)} \text{ and consistent with HPLC assay data} \text{ (data not shown). For the histones tested, TbSIR2 displays a relatively broad deacetylation specificity.}

To quantify the relative efficiencies of deacetylation versus ADP-ribosylation, rates of each reaction were measured under identical conditions using 500 \text{ M} \text{NAD}^+ \text{ or } [\text{32P}]\text{NAD}^+,\text{ 3 \text{ M}} \text{TbSIR2, and varied concentrations of chemically acetylated H2A for 10 min at 37 °C (Fig. 5, A and B). The deacetylation reactions were analyzed by HPLC as described above, and the rate of nicotinamide formation is plotted in Fig. 5A. The ADP-ribosylation reactions were analyzed using densitometry of the } [\text{32P}]\text{labeled SDS-PAGE bands and plotted in Fig. 5B. The } [\text{32P}]\text{labeled bands were compared with a } [\text{32P}]\text{NAD}^+ \text{ standard curve, using stocks of identical specific activity.}

Both enzyme-dependent activities yielded increased product formation with increasing concentrations of substrate (Fig. 5, A and B). Auto-ADP-ribosylation of TbSIR2 (Fig. 5B, shaded bars) also followed the same trend. However, for identical substrate, the calculated rate of deacetylation (Fig. 5A, \text{ } 50 \text{ M/min}) \text{is at least 5 orders of magnitude faster than the highest rate of ADP-ribosylation (Fig. 5B, } \sim 0.0003 \text{ M/min, dark bars}) \text{ under identical conditions. This vast difference in rates, in addition to the small amounts of ADP-ribosylated protein detected by } [\text{32P}]\text{ labeling, contributed to}
the technical challenges of precisely quantitating ADP-ribosylation (Fig. 5B). Consistent with the above observations, qualitative mass spectrometry revealed complete deacetylation of histone H2A with no detectable levels of ADP-ribosylation during these TbSIR2-mediated reactions (data not shown).

**DISCUSSION**

In summary, we have biochemically characterized and compared the protein deacetylase and ADP-ribosyltransferase activities of TbSIR2, a sirtuin reported previously to harbor both enzymatic properties (38). Most notably, TbSIR2 displays histone deacetylation efficiencies (i.e. $k_{\text{cat}}/K_{m}$ and $k_{\text{cat}}$ values) that are comparable with other sirtuins that exhibit robust deacetylation activity (34, 47); however, TbSIR2 ADP-ribosylation of histones was $\sim$5 orders of magnitude slower than deacetylation. We find that TbSIR2-dependent ADP-ribosylation of histones requires acetylated histones and proceeds through two plausible mechanisms.

Previously, we proposed three possible mechanisms for the observed sirtuin-dependent ADP-ribosylation (Scheme 1) (48). The first possibility is that some sirtuins possess ADP-ribosyltransferase activity, directly transferring the ADP-ribose moiety of NAD$^+$ to an acceptor protein without the acetylated substrate normally required for sirtuin deacetylase activity (Scheme 1a). Enzymes that exhibit this type of ADP-ribosylation may include SIRT6 and SIRT4, which have no known deacetylase activity (34, 35). In a second model, both an acetylated substrate and NAD$^+$ are required to initiate the formation of an ADP-ribose-acetylpeptide (O-alkylamidate) intermediate, which is formed during the initial catalytic step of NAD$^+$-dependent deacetylation (33, 51, 52). This intermediate is susceptible to nucleophilic attack by a distinct acceptor protein (trans) or by the acetylated protein itself (cis) (Scheme 1b). It has been shown that this intermediate can be intercepted by exogenous nucleophiles, yielding ADP-ribose transfer to various small molecule acceptors (52). Thus, it is conceivable that amino acid side chains, like the e- amino of lysine, may intercept this intermediate and result in the observed ADP-ribosylation. A third model also requires both NAD$^+$ and an acetylated substrate, but the enzymatic activity of the sirtuin is required only to produce the end product of the NAD$^+$-dependent deacetylation, OAADPr. OAADPr, or its hydrolyzed product, ADP-ribose, can then react nonenzymatically with an acceptor protein resulting in ADP-ribosylation (Scheme 1c).

The results presented in this study support the two mechanisms of histone ADP-ribosylation (Scheme 1b or Scheme 1c) that require an acetylated substrate (Fig. S1A and Fig. 2A). Reactions with commercially available calf thymus histones yielded ADP-ribosylation, whereas purified, recombinant, unmodified histones showed negligible activity (Fig. S1A). Only after acetylating purified recombinant histones with either piccolo-NuA4 acetyltransferase or acetic anhydride (chemical acetylation) was TbSIR2 ADP-ribosylation of histones restored (Fig. 2A). We conclude that endogenous acetylation of native protein preparations (1, 11) can serve as the source for acetylation needed for these pathways of ADP-ribosylation.

In the second acetylation-dependent mechanism, TbSIR2 deacetylation product OAADPr (or ADPr) reacts nonenzymatically with histones. This process is 7-fold more efficient with unmodified histones than the reaction between NAD$^+$ and histones, and inclusion of TbSIR2 did not enhance the OAADPr-dependent labeling (Fig. 2C). Decreased OAADPr labeling with acetylated histones versus unmodified histones suggests that lysine side chains are a major site of modification. Collectively, these results support the mechanism shown in Scheme 1c, where OAADPr (or ADPr) reacts directly with histones to yield apparent ADP-ribosylation. The physiological relevance of this OAADPr-mediated ADP-ribosylation has not been established, although it is interesting to note that in vivo levels of OAADPr/ADPr may be regulated by a number of hydrolases (53, 54).

Furthermore, we tested whether the nonenzymatic reaction of OAADPr/ADPr with histones was consistent with previously described glycation/glycooxidation reactions as reported by Jacobson and co-workers (55, 56). Preparations of H2A ADP-ribosylated with $[^{32}\text{P}]\text{OAADPr}$ showed a 50% decrease in $^{32}\text{P}$ label after incubation at pH 9, whereas the H2A labeled using TbSIR2 and $[^{32}\text{P}]\text{NAD}^+$ showed no significant release of $^{32}\text{P}$ compared with controls (data not shown). We conclude that a significant portion of the OAADPr-dependent ADP-ribosylation is due to glycation or glycooxidation.

Although TbSIR2 can mediate low levels of protein ADP-ribosylation through indirect reactions with OAADPr, a second, direct enzymatic mechanism for ADP-ribosylation (Scheme 1b) appears to be the most efficient pathway, as it is responsible for the majority of the ADP-ribosylation (Fig. 2C, lane 4). Whereas OAADPr-dependent ADP-ribosylation shows no enzymatic involvement, the TbSIR2-mediated ADP-ribosylation with NAD$^+$ displays a 60-fold increase with acetylated substrate compared with the no enzyme control (Fig. 2C).

This second pathway of TbSIR2 ADP-ribosylation also requires acetylated substrate and NAD$^+$ (Scheme 1b). For this mechanism, the ADP-ribose-acetylpeptide (O-alkylamidate) intermediate, formed during the deacetylation reaction, is attacked by a nucleophilic group on the protein acceptor (Scheme 1b) (48). In the case of TbSIR2, the preferred protein acceptor is H2A, which must be acetylated for efficient transfer, likely through a cis reaction (Fig. 3).

Direct comparison of the two activities of TbSIR2 reveals that the deacetylase activity is $\sim$5 orders of magnitude greater than ADP-ribosylation on its preferred ADP-ribosylation target, histone H2A (Fig. 5). Consistent with this dramatic difference, the yeast Sir2 homologue HST2 exhibited at least 1000× more deacetylase than ADP-ribosyltransferase activity (31). This large difference in catalytic efficiency as well as the observed noncatalytic reaction between OAADPr and histone proteins raise important questions about the physiological significance of ADP-ribosylation by sirtuins that display protein deacetylation (1, 11, 28, 30, 31, 37, 38). It is possible that the relative efficiencies of ADP-ribosylation and deacetylation could be regulated in vivo through accessory proteins, such as those associated with histones on chromatin, other post-translational modifications, or in response to cellular events.
Additional work is needed to uncover the ADP-ribosylation mechanisms for sirtuins that have not displayed significant protein deacetylase activity but are capable of NAD\(^+\)-dependent, protein ADP-ribosylation. Reports indicate that human SIRT6 and SIRT4 display negligible deacetylase activity in vitro (34, 35) but are capable of ADP-ribosyltransferase activity (35, 37). SIRT6 has been shown to ADP-ribosylate itself, although surprisingly, core histones were able to increase this auto-ADP-ribosylation (37). SIRT4 has been shown to regulate glutamate dehydrogenase by ADP-ribosylation (34, 35) but are capable of ADP-ribosyltransferase reactions. These sirtuins, with little or no in vitro deacetylase activity, may ADP-ribosylate their substrates through a direct mechanism (Scheme 1a), distinct from those observed with TbSIR2 (Scheme 1b and Scheme 1c).

The studies presented here should help provide the framework for future mechanistic investigations on the relationship between deacetylation and ADP-ribosylation, mediated by various members of the sirtuin family.

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