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Cloning and Characterization of NAD-Dependent Protein Deacetylase (Rv1151c) from *Mycobacterium tuberculosis*

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Abstract—Sir2 family proteins are highly conserved and catalyze a well-characterized NAD-dependent protein deacetylation reaction that regulates multiple cellular processes including aging, gene silencing, cellular differentiation, and metabolic pathways. Little is known about Sir2 family proteins in bacteria. The Sir2 homolog Rv1151c of *Mycobacterium tuberculosis* was cloned and over-expressed in *Escherichia coli*, and the protein then purified by Ni^{2+}-affinity chromatography to homogeneity. The purified recombinant protein showed a typical NAD-dependent protein deacetylase activity that could be inhibited by nicotinamide and other known Sir2 inhibitors. The optimal temperature and pH for activity of Rv1151c are 25°C and pH 9 ± 1, respectively. Rv1151c is capable of deacetylating the acetyl-CoA synthetase from *M. tuberculosis*. However, unlike Sir2 family proteins identified from other bacteria, Rv1151c shows a substrate-independent NAD glycohydrolase activity in accordance with its auto-ADP ribosylation activity.

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Key words: ADP-ribosyltransferase, deacetylase, *Mycobacterium tuberculosis*, NAD glycohydrolase, Sir2

Tuberculosis (TB) is a major cause of illness and death worldwide. Recently more challenges emerged including HIV-associated TB, multidrug-resistant TB (MDR-TB), and extensively drug-resistant TB (XDR-TB). Deeper insight into the metabolism regulation system of the pathogen *Mycobacterium tuberculosis* could provide a potential theoretical strategy for TB therapy, especially those key regulators could be drug target candidates.

Sir2 (silent information regulator 2) enzymes catalyze a unique protein deacetylation reaction that requires the coenzyme NAD and produces nicotinamide (NAM) and O-acetyl-ADP-ribose (OAADPr). Conserved from bacteria to humans [1], these proteins are implicated in the control of life span extension [2, 3], DNA repair and recombination [4], gene silencing [5], and apoptosis [6, 7] in eukaryotic cells.

In bacteria, the lack of histones and silent chromatin suggested that Sir2 proteins might have more general functions related to metabolism. For example, the Sir2 homolog from the archaean *Sulfolobus solfataricus* deacetylates the nonspecific DNA binding protein Alba to mediate transcription repression [8], and the Sir2 homolog CobB of *Salmonella enterica* was shown to specifically release the acetyl group from an active site acetyl-lysine of acetyl-CoA synthetase (ACS) to increase its activity [9].

In addition to the well-defined NAD^+-dependent deacetylase activity of Sir2 proteins, several reports have shown that some Sir2 proteins can catalyze protein ADP-ribosylation, i.e. *Salmonella typhimurium* Sir2 homolog CobB could substitute for the loss of CobT, a known

Abbreviations: ACS, acetyl-CoA synthetase; GDH, glutamate dehydrogenase; HNA, 2-hydroxy-1-naphthaldehyde; LB medium, Luria–Bertani medium; NA, nicotinic acid; NAM, nicotinamide; OAADPr, O-acetyl-ADP-ribose; POA, pyrazinoic acid; PZA, pyrazinamide; Sir2, silent information regulator 2; TB, tuberculosis; ZMAL, (S)-[5-acetylamino-1-(4-methyl-2-oxo-2H-chromen-7-ylcarbamoyl)-pentyl]-carbamic acid benzyl ester.

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phosphoribosyltransferase in cobalamin biosynthesis [10]. This led to the assumption that some Sir2 proteins might act as ADP-ribosylating enzymes. Subsequently, it was shown that *E. coli* CobB and human SIR2 both could transfer ^{32}P from NAD to BSA [11], and that yeast Sir2 could also transfer labeled phosphate from NAD to BSA, histones, and itself *in vitro* [12]. However, some eukaryotic Sir2 proteins had no measurable deacetylase activity, but only ADP-ribosyltransferase activity, i.e. human SIRT4 was shown to ADP-ribosylate and down-regulate glutamate dehydrogenase (GDH) activity [13], while some other eukaryotic Sir2 proteins including yeast Sir2 [14], Hst2 [15], *Trypanosoma brucei* Sir2 ortholog Tb-SIR2rp1 [16-18], and *Plasmodium falciparum* Pf-Sir2 [19, 20], possess both protein deacetylase and ADP-ribosyltransferase activities.

So far, most studies on Sir2 family proteins have been done in eukaryotic organisms. Despite the demonstration of a potentially important role of CobB in general cellular metabolism in *Salmonella* [10] and the crystallization of *E. coli* CobB structure [21], the function of bacterial Sir2-like proteins remains largely unknown, both biochemically and physiologically. To get more insight into the Sir2 proteins in pathogenic bacteria, the Sir2 homolog from *M. tuberculosis* (Rv1151c, accession number BX842575) was characterized. The gene was cloned and over-expressed, purified, and proved to have NAD-dependent protein deacetylase activity, substrate independent NAD glycohydrolase activity, and also self-ADP-ribosyltransferase activity, which is not found in Sir2-like proteins from other prokaryotic organisms.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. *Escherichia coli* strain AD494(DE3) and plasmid pET32a(+) (Novagen, USA) were used for the expression of the Rv1151c. For the selection and maintenance of plasmids, *E. coli* was grown at 37°C in Luria–Bertani (LB) broth or on an agar plate supplemented with the appropriate amount of antibiotics when necessary (15 μg/ml kanamycin and 50 μg/ml ampicillin).

DNA manipulations. DNA manipulations and analyses were performed using standard techniques. DNA sequencing was performed commercially. The *rv1151c* gene was amplified from *M. tuberculosis* H37Rv genomic DNA (from Wuhan Tuberculosis Hospital). The primers for *sir2* amplification were as below: forward, 5′-TATAGGATCC(*Bam*HI)**ATG**CGAGTGGCGGTGCTCAG-3′; reverse, 5′-TATTCTCGAG(*Xho*I)CTATTTCAGCAGGGCGGGCA-3′. The PCR product (734 bp) was cloned into the *Bam*HI and *Xho*I sites of pET32a(+) to generate pRv1151c. The recombinant expression vector was confirmed by restriction enzyme digestion and sequencing, and then introduced into *E. coli* AD494(DE3) by $CaCl_2$ transformation, and the antibiotic resistant transformants were selected for expression experiments.

Expression and purification of *M. tuberculosis* Sir2. *Escherichia coli* AD494(DE3)/pRv1151c was incubated in 5 ml LB medium. The overnight culture was transferred into 500 ml fresh LB medium containing ampicillin and kanamycin and incubated at 37°C till A_{600} reached 0.6, then IPTG was added to a final concentration 0.2 mM and the incubation was continued for 4 h at 25°C. Cells were harvested by centrifugation, resuspended in binding buffer (20 mM Tris-HCl, pH 7.9, 500 mM NaCl, and 5 mM imidazole), and sonicated on ice with an Ultrasonic Cell Disruptor VCX750 (Sonics, USA). The lysate was centrifuged (10,000 rpm for 30 min at 4°C), and the supernatant was applied to an affinity Ni^{2+} column. After loading, the column was washed with the binding buffer, followed by washing buffer (20 mM Tris, 500 mM NaCl, and 100 mM imidazole, pH adjusted to pH 7.9 with HCl). The protein was eluted with elution buffer (same as binding buffer except for 250 mM imidazole concentration). Peak fractions, according to SDS-PAGE, were pooled and concentrated using a 30,000 MWCO concentrator (Millipore, USA) in storage buffer (10 mM Tris-HCl, pH 8.0). Protein concentration was determined by Bradford using Protein Assay Kit (Beyotime Institute of Biotechnology, China).

Deacetylase activity. Two different types of substrates were used to determine the NAD-dependent protein deacetylase activity of *M. tuberculosis* Rv1151c. One was (S)-[5-acetylamino-1-(4-methyl-2-oxo-2H-chromen-7-ylcarbamoyl)-pentyl]-carbamic acid benzyl ester [22], also termed ZMAL (kindly provided by Manfred Jung, Department of Pharmaceutical Sciences, University of Freiburg, Germany), the other was purified recombinant acetyl-CoA synthetase (ACS) of *M. tuberculosis* (provided by Ru Li of our laboratory). The reactions were performed in 96-well plates at 25°C for several hours in 50 μl of sirtuin buffer (50 mM Tris-HCl, pH 8.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$) with 400 μM NAD^+, 5-10 μg Sir2 enzymes, and 8 μM ZMAL or 10 μg Ac-ACS protein. The reaction with ZMAL was terminated by adding 60 μl of stop solution containing 500 μg/ml trypsin to each well, incubated for 30 min at 37°C, and the fluorescence was measured in a plate reader (excitation at 360 nm, emission at 460 nm).

Western blotting was carried out using standard protocols for detection of the deacetylation intensity of purified recombinant ACS. The samples were resolved on SDS-PAGE and transferred to polyvinylidene difluoride membrane, with blocking by 5% nonfat milk in TBS buffer (20 mM Tris-HCl, pH 8.0, 150 mM NaCl) with 0.1% Tween 20 (TBS-T). The membrane was incubated with acetyl-lysine-specific antibodies (diluted 1 : 1000 in TBS-T; Cell Signaling Technology, USA), followed by incubation with alkaline phosphatase (AP)-labeled goat anti-rabbit antibodies (diluted 1 : 3000 in TBS-T; Boster

Bio-Technology Co. Ltd, China). Color development was achieved by reacting with chromogenic substrates 5-bromo-4-chloro-3-indolyl-phosphate/nitroblue tetrazolium (BCIP/NBT).

Inhibition assays. Inhibition experiments of the Rv1151c deacetylation were performed with known Sir2 inhibitors and their analogs, including nicotinamide (NAM) and nicotinic acid (NA) (dissolved in deionized water), pyrazinamide (PZA), and pyrazinoic acid (POA) (dissolved in dimethylsulfoxide), and 2-hydroxy-1-naphthaldehyde (HNA) (dissolved in methanol).

NAD glycohydrolase activity. NAD, NAM, and potential products of the deacetylase reaction were separated by capillary electrophoresis (PrinCE 560 CE instrument; Prince Technologies B.V., The Netherlands) with detection at 260 nm using a programmable UV/VIS detector (Ce-cell for Bischoff detector Lambda 1010, The Netherlands). After reaction, samples with or without acetylated substrates were diluted with deionized water and injected into the capillary by a pressure pulse of 30 mbar × 30 sec. Electrophoresis was run in pH 6.5 phosphate buffer containing 25 mM SDS by an electric field of 20 kV with positive polarity at the injection end for 10 min prior to each run.

ADP-ribosyltransferase activity. Biotinylated NAD (6-biotin-17-NAD) (R&D Systems, Inc., USA) was used to detect the ADP-ribosyltransferase activity of Rv1151c. The reaction mixture including sirtuin buffer (pH 8.5), 25 μM 6-biotin-17-NAD^+, 400 μM NAD^+, and 10 μg Sir2 was incubated at 25°C for 4 h and then resolved by SDS-PAGE. The inhibition effect of NAM was also tested. The product biotin-ADP-ribose was analyzed by Western blotting with horseradish peroxidase (HRP)-labeled avidin (Boster Bio-Technology Co. Ltd.).

RESULTS AND DISCUSSION

Rv1151c overexpression and purification. The gene *rv1151c* from *M. tuberculosis* was successfully cloned to generate the expression vector pRv1151c and expressed in *E. coli*. SDS-PAGE indicated that the protein was overexpressed abundantly *in vivo*, but partially lost as inclusion bodies in the insoluble fraction of the cell lysate. Ni-NTA chromatography of the soluble cell lysate fraction yielded approximately 2 mg protein per liter of culture. The SDS-PAGE analysis (Fig. 1) revealed a 95% pure band consistent with the calculated mass of His-tagged Rv1151c (46 kDa).

NAD-dependent deacetylase activity of *M. tuberculosis* Rv1151c. Trypsin recognizes the ZMAL oligopeptide as a substrate only when its lysine residue is deacetylated. Upon cleavage by trypsin, the aminocoumarin moiety of ZMAL is released and fluorescence is produced. As shown in Fig. 2a, fluorescence intensities of the positive samples were gradually increased, but those

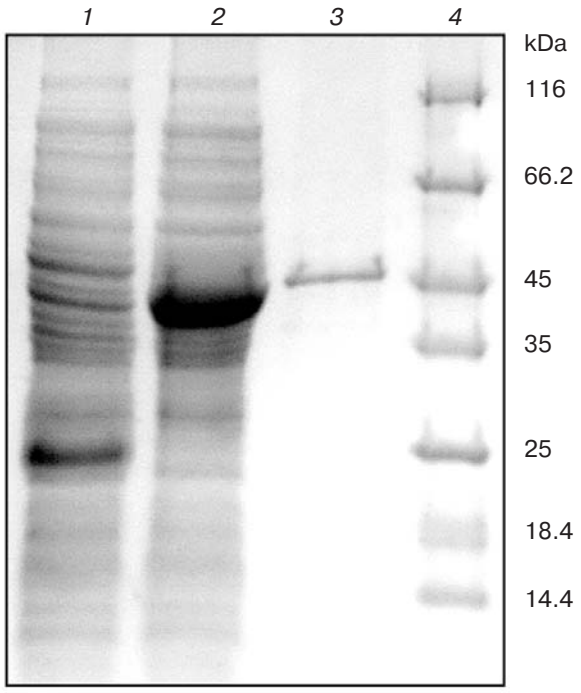

Fig. 1. SDS-PAGE of recombinant Rv1151c of *M. tuberculosis*. Lanes: *1*) total cell lysate of *E. coli* AD494 with pET32a(+) vector; *2*) total cell lysate of *E. coli* AD494 with pRv1151c; *3*) purified recombinant Rv1151c; *4*) protein molecular weight standards.

of the negative controls (no added NAD or Rv1151c) were steady, illustrating that the measured deacetylase activity of the Rv1151c was not caused by possible contaminating proteinase or amidase. However, the activity is lower compared with other reported Sir2-like proteins [9, 23], which could be explained by longer generation time of *M. tuberculosis* in comparison with other related bacteria.

So far, the optimal temperature and pH have not been reported for bacterial Sir2 family proteins. They were determined for Rv1151c to be 25°C and 9 ± 1 (Fig. 2, b and c).

Inhibition of Rv1151c by known Sir2 inhibitors. Many compounds have been identified to be inhibitors of Sir2-like proteins, including NAM, the natural non-competitive inhibitor of Sir2 [24]. Interestingly, a very important front-line TB drug pyrazinamide (PZA) was discovered as an analog of NAM. Thus the inhibitory effects of NAM and its analogs were tested. As shown in Fig. 3, NAM and HNA did exhibit significant inhibition against Rv1151c. However, PZA and its active form POA did not show any inhibition against Rv1151c, which suggests that NAM and PZA may act quite differently *in vivo* despite their common properties, such as requirement of the same nicotinamidase for hydrolysis, similar chemical structure, and similar acting concentrations for *M. tuberculosis* [25].

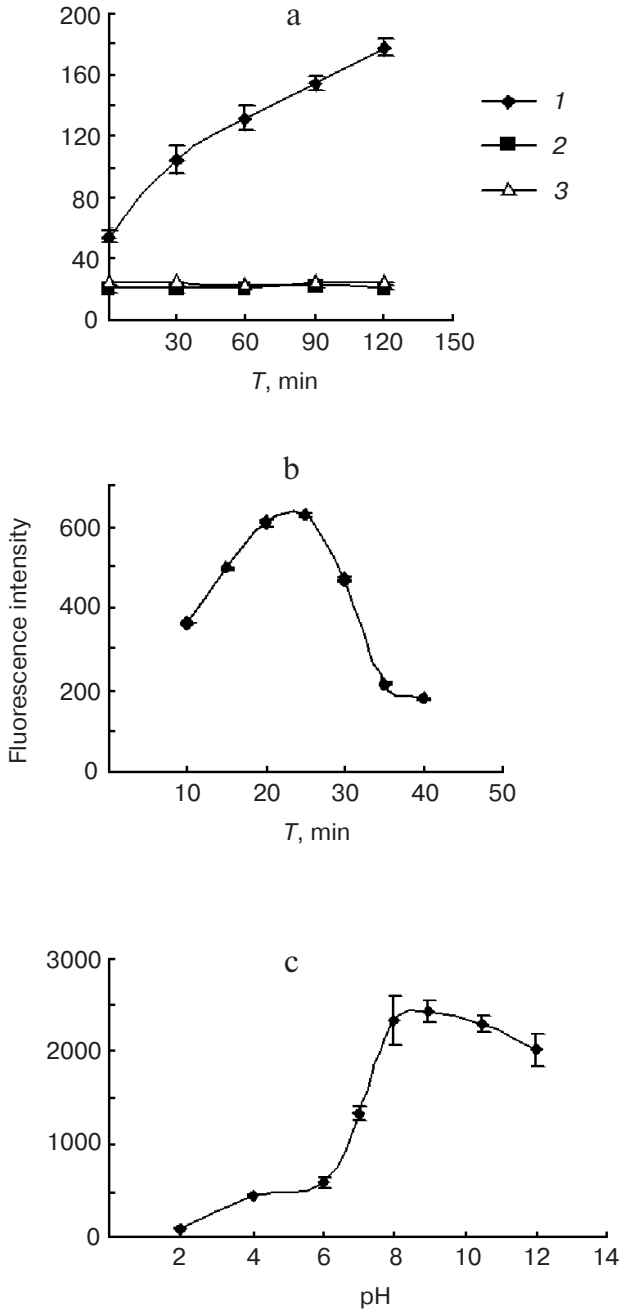

Fig. 2. NAD-dependent deacetylase activity of Rv1151c and optimal reaction conditions. a) Fluorescence signals produced from incubation of Rv1151c with the substrate ZMAL and NAD: *1*) typical reaction samples; *2*) reaction without Rv1151c; *3*) reaction without NAD. b) Temperature profile of Rv1151c. c) pH profile of Rv1151c.

Rv1151c deacetylates acetyl-CoA synthetase of *M. tuberculosis*. Rv1151c remarkably reduced acetylation levels of ACS compared with controls, and this effect could be gradually alleviated as the concentration of NAM increased (Fig. 4). This result indicated that Rv1151c is an NAD-dependent protein deacetylase, and suggested that ACS is also a substrate for the Sir2 protein in *M. tuberculosis*.

Auto-NAD hydrolysis activity and auto-ADP-ribosylation of Rv1151c. The deacetylation activity of Sir2 protein is usually accompanied by NAD glycohydrolase activity. When Rv1151c was mixed with acetylated substrates and NAD (peak *1*), the positive samples produced two additional peaks (peaks *2* and *3*), and peak *2* was due to production of nicotinamide, a byproduct of the deacetylation reaction (Fig. 5). Peak *3* was an unidentified product (Fig. 5, c and d), which is speculated to be OAADPr. Surprisingly, Rv1151c hydrolyzed NAD in the absence of acetylated substrates, which was not been reported for its homologs, indicating a substrate-independent NAD hydrolysis activity (Fig. 5e). This substrate-independent NAD glycohydrolase activity could be explained by Rv1151c having auto-ADP-ribosylation activity, as was observed with yeast Sir2 and human Sirt6, or NAD glycohydrolase activity, which might suggest some intriguing function of this protein *in vivo*. To test these hypotheses, auto-ADP ribosylation activity of Rv1151c was determined using 6-biotin-17-NAD and subsequent immunoblotting.

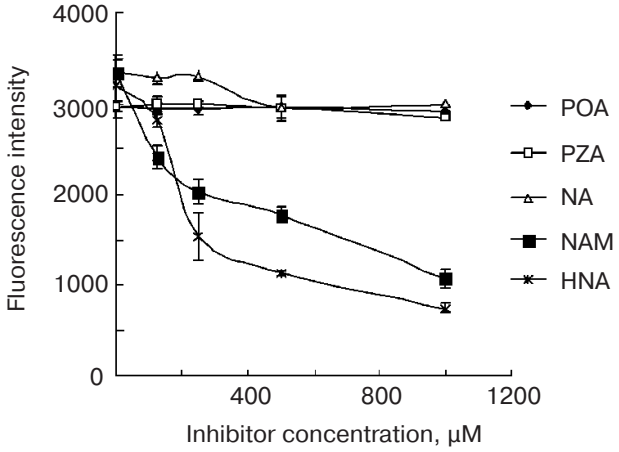

Fig. 3. Inhibition of the deacetylase activity of Rv1151c by known Sir2 inhibitors.

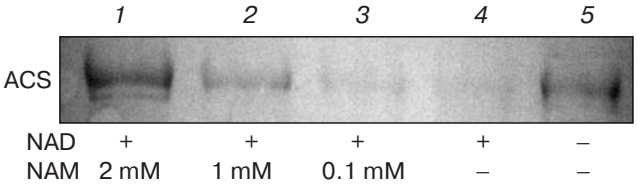

Fig. 4. Western blotting demonstration of ACS deacetylation by recombinant Rv1151c. Standard reaction mixture is composed of 50 mM Tris-HCl, pH 8.5, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, 400 μM NAD, 10 μg Rv1151c enzymes, and 10 μg ACS supplied with NAM. Lanes: *1-4*) inhibition decreased as concentration of NAM was decreased; *5*) negative control (no NAD^+).

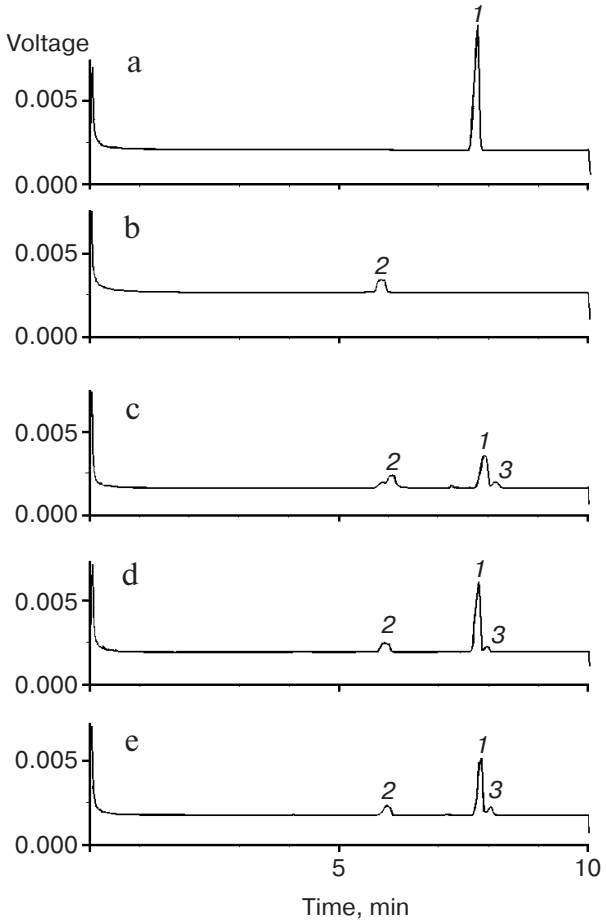

Fig. 5. Detection of NAD hydrolysis activity of Rv1151c by capillary electrophoresis: a) NAD standard (peak *1*); b) NAM standard (peak *2*); c) reaction mixture with Rv1151c, ACS, and NAD; d) reaction mixture with Rv1151c, ZMAL, and NAD; e) ADP-ribosyltransferase reaction mixture with Rv1151c and NAD, without acetylated substrates.

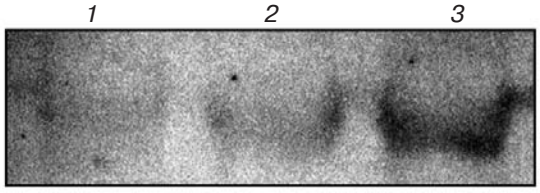

Fig. 6. Auto-ADP ribosylation of Rv1151c. The Western blotting results. Lanes: *1*) control (auto-ADP ribosylation using heat inactivated Rv1151c); *2*) auto-ADP ribosylation of Rv1151c in the presence of 1 mM NAM; *3*) auto-ADP ribosylation of Rv1151c in the absence of NAM.

The results in Fig. 6 show that *M. tuberculosis* Sir2 could transfer biotinylated ADP-ribose to itself, and the effect could be strongly inhibited by NAM. This suggests that the auto-NAD hydrolysis activity of Rv1151c is mostly due to its auto-ADP ribosylation activity.

To our knowledge, this is the first characterization of a Sir2 homolog from *Mycobacterium*. Rv1151c of *M. tuberculosis* is an NAD-dependent protein deacetylase that can deacetylate ACS, and this activity can be inhibited by known Sir2 inhibitors like NAM. In addition, Rv1151c may be the first NAD-dependent protein deacetylase that also has substrate-independent NAD glycohydrolase activity and auto-ADP ribosylation activity in bacteria. Further studies are needed to determine the physiological roles of Rv1151c in *M. tuberculosis*.

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REFERENCES

1. Frye, R. A. (2000) *Biochem. Biophys. Res. Commun.*, **273**, 793-798.
2. Lin, S. J., Defossez, P. A., and Guarente, L. (2000) *Science*, **289**, 2126-2128.
3. Tissenbaum, H. A., and Guarente, L. (2001) *Nature*, **410**, 227-230.
4. Tsukamoto, Y., Kato, J., and Ikeda, H. (1997) *Nature*, **388**, 900-903.
5. Brachmann, C. B., Sherman, J. M., Devine, S. E., Cameron, E. E., Pillus, L., and Boeke, J. D. (1995) *Genes Dev.*, **9**, 2888-2902.
6. Motta, M. C., Divecha, N., Lemieux, M., Kamel, C., Chen, D., Gu, W., Bultsma, Y., McBurney, M., and Guarente, L. (2004) *Cell*, **116**, 551-563.
7. Vaziri, H., Dessain, S. K., Ng Eaton, E., Imai, S. I., Frye, R. A., Pandita, T. K., Guarente, L., and Weinberg, R. A. (2001) *Cell*, **107**, 149-159.
8. Bell, S. D., Botting, C. H., Wardleworth, B. N., Jackson, S. P., and White, M. F. (2002) *Science*, **296**, 148-151.
9. Starai, V. J., Celic, I., Cole, R. N., Boeke, J. D., and Escalante-Semerena, J. C. (2002) *Science*, **298**, 2390-2392.
10. Tsang, A. W., and Escalante-Semerena, J. C. (1998) *J. Biol. Chem.*, **273**, 31788-31794.
11. Frye, R. A. (1999) *Biochem. Biophys. Res. Commun.*, **260**, 273-279.
12. Tanny, J. C., Dowd, G. J., Huang, J., Hilz, H., and Moazed, D. (1999) *Cell*, **99**, 735-745.
13. Haigis, M. C., Mostoslavsky, R., Haigis, K. M., Fahie, K., Christodoulou, D. C., Murphy, A. J., Valenzuela, D. M., Yancopoulos, G. D., Karow, M., Blander, G., Wolberger, C., Prolla, T. A., Weindruch, R., Alt, F. W., and Guarente, L. (2006) *Cell*, **126**, 941-954.

14. Imai, S., Armstrong, C. M., Kaeberlein, M., and Guarente, L. (2000) *Nature*, **403**, 795-800.
15. Tanner, K. G., Landry, J., Sternglanz, R., and Denu, J. M. (2000) *Proc. Natl. Acad. Sci. USA*, **97**, 14178-14182.
16. Garcia-Salcedo, J. A., Gijon, P., Nolan, D. P., Tebabi, P., and Pays, E. (2003) *EMBO J.*, **22**, 5851-5862.
17. Alsford, S., Kawahara, T., Isamah, C., and Horn, D. (2007) *Mol. Microbiol.*, **63**, 724-736.
18. Kowieski, T. M., Lee, S., and Denu, J. M. (2008) *J. Biol. Chem.*, **283**, 5317-5326.
19. Merrick, C. J., and Duraisingh, M. T. (2007) *Eukaryot. Cell*, **6**, 2081-2091.
20. French, J. B., Cen, Y., and Sauve, A. A. (2008) *Biochemistry*, **47**, 10227-10239.
21. Zhao, K., Chai, X., and Marmorstein, R. (2004) *J. Mol. Biol.*, **337**, 731-741.
22. Heltweg, B., Dequiedt, F., Verdin, E., and Jung, M. (2003) *Anal. Biochem.*, **319**, 42-48.
23. North, B. J., Marshall, B. L., Borra, M. T., Denu, J. M., and Verdin, E. (2003) *Mol. Cell.*, **11**, 437-444.
24. Bitterman, K. J., Anderson, R. M., Cohen, H. Y., Latorre-Esteves, M., and Sinclair, D. A. (2002) *J. Biol. Chem.*, **277**, 45099-45107.
25. Zhang, Y., Permar, S., and Sun, Z. (2002) *J. Med. Microbiol.*, **51**, 42-49.