Mechanism of Human SIRT1 Activation by Resveratrol*

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Received for publication, February 2, 2005

Published, JBC Papers in Press, March 4, 2005, DOI 10.1074/jbc.M501250200

The NAD⁺-dependent protein deacetylase family, Sir2 (or sirtuins), is important for many cellular processes including gene silencing, regulation of p53, fatty acid metabolism, cell cycle regulation, and life span extension. Resveratrol, a polyphenol found in wines and thought to harbor major health benefits, was reported to be an activator of Sir2 enzymes in vivo and in vitro. In addition, resveratrol was shown to increase life span in three model organisms through a Sir2-dependent pathway. Here, we investigated the molecular basis for Sir2 activation by resveratrol. Among the three enzymes tested (yeast Sir2, human SIRT1, and human SIRT2), only SIRT1 exhibited significant enzyme activation (∼8-fold) using the commercially available Fluor de Lys kit (BioMol). To examine the requirements for resveratrol activation of SIRT1, we synthesized three p53 acetylpoly- peptide substrates either lacking a fluorophore or containing a 7-amino-4-methylcoumarin (p53-AMC) or rhodamine 110 (p53-R110). Although SIRT1 activation was independent of the acetylpeptide sequence, resveratrol activation was completely dependent on the presence of a covalently attached fluorophore. Substrate competition studies indicated that the fluorophore decreased the binding affinity of the peptide, and, in the presence of resveratrol, fluorophore-containing substrates bound more tightly to SIRT1. Using available crystal structures, a model of SIRT1 bound to p53-AMC peptide was constructed. Without resveratrol, the coumarin of p53-AMC peptide is solvent-exposed and makes no significant contacts with SIRT1. We propose that binding of resveratrol to SIRT1 promotes a conformational change that better accommodates the attached coumarin group.

The silent information regulator 2 (Sir2) family of proteins (sirtuins) are NAD⁺-dependent histone/protein deacetylases that tightly couple the cleavage of NAD⁺ and deacetylation of protein substrates to form nicotinamide, the deacetylated product, and a novel metabolite, 2'-O-acetyl-ADP-ribose (OAADPr)³ (1–7). This family of proteins is evolutionarily conserved, with five homologs in yeast (ySir2 and HST1–4) and seven in humans (SIRT1–7) (8, 9). The founding member of this family, ySir2, is essential for gene silencing at the three silent loci in yeast (10–19). Besides gene silencing, Sir2 proteins are important for many processes, such as cell cycle regulation (20), fatty acid metabolism (21), and life span extension (22–24). SIRT1, the most extensively studied human homolog, mediates p53-dependent processes (25–27), transcription regulation (28–31), muscle differentiation (32), adipogenesis (33), protection from axonal degeneration (34), and life span extension (35, 36).

The importance of Sir2 enzymes in many cellular processes presents the need to understand their regulatory mechanisms. Substrate and product analogs as well as small molecules have been screened for Sir2 regulatory activity. Of all the NAD⁺-like metabolites and salvage pathway intermediates analyzed for regulatory activities on Sir2 enzymes, only nicotinamide displayed a level of inhibition which was consistent with a physiological role (37). Nicotinamide is the most potent inhibitor of Sir2 enzymes to date (38–40) and was shown to inhibit Sir2-dependent life span extension (41). NADH was shown previously to be a competitive inhibitor of NAD⁺ in vitro (42); however, the high mKᵢ binding constant for NADH indicates that cellular NADH levels are unlikely to regulate Sir2 activity under most physiological conditions (37). Through phenotypic screening, small molecules such as sirtinol (43), splitomicin (44), and splitomicin analogs (45, 46) were identified as inhibitors of Sir2. In addition, 15 plant phenols, including quercetin, piceatannol, and resveratrol, were shown to have SIRT1 activating properties (35). Of all the small molecules identified, resveratrol (3,4',5-trihydroxy-trans-stilbene) (Fig. 1) was found to be the most potent activator of SIRT1 and to a lesser extent, of ySir2 (35).

Resveratrol is a polyphenol found in grapes and grape products. It possesses the ability to scavenge oxidatively generated free radicals (47–54), and it exhibits cancer preventative properties (for recent reviews, see Refs. 55–59). Resveratrol has been shown to increase SIRT1 activity by as much as 8-fold, lowering the Kᵢ value for acetylated substrate and to a much lesser extent than that of NAD⁺, with no reported effect on the overall turnover rate of the enzyme (35). Resveratrol was shown to enhance SIRT1-dependent cellular processes such as axonal protection (34), fat mobilization (33), and inhibition of NF-κB-dependent transcription (31). In yeast, resveratrol was shown to mimic Sir2-dependent life span extension during calorie restriction, although the effects of calorie restriction and resveratrol were not additive (35). One study on yeast aging suggested that Sir2 acts independently of pathways mediated by calorie restriction (60). Resveratrol extended life span in Caenorhabditis elegans and Drosophila melanogaster through a Sir2-mediated process (36). The mechanism by which resveratrol activates Sir2 enzymes was unknown.

In the current study, we sought to elucidate how resveratrol

* This work was supported by American Cancer Society Grant RSG-01-029-01 and National Institutes of Health Grant R01 GM65386 (to J. M. D.), National Institutes of Health Biotechnology Training Grant NIH 5 T32 GM08349 (to B. C. S.), and National Institutes of Health Predoctoral Fellowship F31 GM066366 (to M. T. B.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1754 solely to indicate this fact.

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‡ The abbreviations used are: OAADPr, 2'-O-acetyl-ADP-ribose; AcH3, acetylated histone H3; AMC, 7-amino-4-methylcoumarin; Bec, butoxyxycarbonyl; HPLC, high performance liquid chromatography; MALDI MS, matrix-assisted laser desorption ionization mass spectrometry; R110, rhodamine 110.
activates SIRT1. Our results indicate that resveratrol is not a
general activator of Sir2 enzymes and that activation requires
the presence of a fluorophore covalently attached to the peptide
substrate. To validate our findings, four different deacetylation
assays were used, and three p53 acetylpeptide substrates ei-
ther lacking a fluorophore or containing either coumarin (p53-
AMC) or rhodamine (p53-R110) fluorophores were synthesized.
The commonly used BioMol (Plymouth, PA) assay kit, Fluor de
Lys, which employs a similar fluorescent detection method,
was included in the analysis. We show that the peptide se-
quence is inconsequential for resveratrol activation, but the
covalent attachment of the fluorophore on the peptide is nec-
essary to produce the resveratrol-mediated activation. A model
of p53-AMC peptide bound to SIRT1 was developed to visualize
the interaction of the fluorophore with the enzyme. We propose
that resveratrol binding to SIRT1 promotes conformational
changes in the enzyme which allow tighter binding of the
fluorophore.

EXPERIMENTAL PROCEDURES

Materials—Fluor de Lys-SIRT1 peptide, Fluor de Lys-H4 AcK16
peptide, and Fluor de Lys Developer II 5× concentrate, were purchased
from BioMol. Resveratrol was purchased from Sigma. The p53 peptide
was purchased from the University of Wisconsin-Madison Peptide Syn-
thesis Center. All other reagents were purchased from Sigma and
Fisher, or as otherwise noted, and were of the highest quality available.

Expression and Purification of SIRT1—Histidine-tagged SIRT1 was
cloned into a pQE80 vector (Qiagen, Valencia, CA). The plasmid
was transformed into BL21DE3 cells, which were grown to an
A600 of 0.6–0.8 prior to induction with isopropyl-
D-thiogalactopyranoside for 3–8 h. Cells were harvested and stored at
20 °C until use.

Cells were lysed using a French pressure cell in 50 mM Tris, pH 8,
300 mM NaCl with 1 mM β-mercaptoethanol, 0.1 mM phenylmethysul-
fonyl fluoride, 10 μg/ml leupeptin, and 5 μg/ml aprotinin. Cell debris
was removed by centrifugation. The supernatant was rocked with
nickel-nitritotriacetic acid resin for 1 h at 4 °C. The resin was then
loaded onto a column and washed with 50 mM Tris, pH 8.0, 300 mM
NaCl, and 1 mM β-mercaptoethanol. SIRT1 was eluted with a gradient
of 0–200 mM imidazole in 50 mM Tris, pH 8.0, 300 mM NaCl, and 1 mM
β-mercaptoethanol. SIRT1 fractions were pooled and lyophilized in 25
mM Tris, pH 7.5, 100 mM NaCl, 10% glycerc, and 5 mM diithothreitol
and stored at −20 °C prior to use.

SIRT1 Deacetylase Assays—Four different deacetylase assays were
used to assess resveratrol activation of SIRT1 in vitro: the Fluor de Lys
fluorescence assay, coumarin and rhodamine-based fluorescence as-
says, charcoal binding assay, and high performance liquid chromatog-
raphy (HPLC)-based assays. All the reactions were performed with 10%
final dimethyl sulfoxide concentration at 37 °C.

The Fluor de Lys fluorescence assay was performed as indicated in
the BioMol product sheets. Briefly, assays were performed using Fluor
de Lys-SIRT1 or Fluor de Lys-H4 AcK16 peptides, NAD⁺, and SIRT1, in
the absence and presence of resveratrol in SIRT1 assay buffer (25 mM
Tris-Cl, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, as indicated in the BioMol product sheets). The buffer,
dimethyl sulfoxide, resveratrol, and SIRT1 were preincubated for 10
min. Reactions were initiated by the addition of 2 × concentrations of
the Fluor de Lys peptide and NAD⁺. Prior to quenching the reaction, 2
mM nicotinamide was added to 1× Developer II in the histone deacety-
lase assay buffer (25 mM Tris, pH 8.0, 137 mM NaCl, 2.7 mM KCl, 1 mM
MgCl₂, as indicated in the BioMol product sheets). At each time point,
50 μl of the reaction was removed and mixed with 50 μl of the developer
solution. The quenched samples were kept at 37 °C for 45 min prior to
fluorescence reading. Fluorescence readings were obtained using the
CytoFluor series 4000 fluorometer (Perceptive Biosystems Inc., Fram-
ingham, MA), with the excitation wavelength set to 360 nm and the
emission set to 460 nm (according to BioMol specification).

For the charcoal binding assay and the HPLC assay, conditions
similar to those in the fluorescence assay were used. The enzyme was
preincubated in a mixture of dimethyl sulfoxide, resveratrol, and the

Fig. 1. Chemical structures of compounds used in this work.
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FIG. 2. Synthesis of p53-AMC and p53-R110. p53-AMC was synthesized by first removing the Boc group of Boc-Lys(Ac)-AMC with trifluoroacetic acid (TFA). The resulting H-Lys(Ac)-AMC was then coupled to the protected Ac-RHK peptide with O-benzotriazol-1-yl-tetramethyluronium hexafluorophosphate (HBTU) and N-methyl morpholine (NMM) in N,N-dimethylformamide (DMF). The resulting full-length peptide was deprotected with trifluoroacetic acid yielding p53-AMC.

SIRT1 assay buffer prior to addition of the substrates to initiate the reaction. For the charcoal binding assay, [3H]acetylated histone H3 peptide (5H]AcH3) was used as a substrate instead of the Fluor de Lys peptide. Generation of [3H]AcH3 was described previously (61). Briefly, for the charcoal binding assay, at each time point, the reaction was quenched by the addition of charcoal slurry containing activated charcoal in 2 M glycine, pH 9.5. Samples were incubated at 95 °C for 1 h. The samples were centrifuged and the supernatant transferred into a new microcentrifuge tubes containing charcoal slurry. After centrifugation, the radioactivity of the supernatant was determined by scintillation counting.

For the HPLC assay, Fluor de Lys-SIRT1 and [14C]NAD+, in which the 14C label is located on the nicotinamide ring, are used in the reaction (synthesis of [14C]NAD+ and the HPLC assay were described previously (61)). The reactions were quenched with trifluoroacetic acid to a final concentration of 1%. Time points were injected into a reversed phase, C18, small pore column (Vydac, Hesperia, CA). The substrates and products were resolved using increasing concentrations of acetonitrile. Radioactivity of the collected fractions was determined by scintillation counting.

Competition Assay between the Fluor de Lys-SIRT1 and p53 Peptide with [3H]AcH3—Competition assays were performed using the charcoal binding assay to determine whether resveratrol enhances the binding of the Fluor de Lys-SIRT1, p53-AMC, or p53 4-mer peptides. The charcoal binding assay was carried out with 20 μM [3H]AcH3, 100 μM NAD+, varying concentrations of the Fluor de Lys-SIRT1, p53-AMC, or p53 4-mer peptides, with and without 200 μM resveratrol. The reactions were quenched, and the amount of O-[3H]ADPr product was quantified as described previously (61).

Synthesis of p53-AMC and p53-R110 Peptides (Figs. 1 and 2)—Boc-Lys(Ac)-AMC (28 mg, 63 μmol; Bachem, Switzerland) was deprotected with 1:1 trifluoroacetic acid:CH2Cl2. The resulting clear oil was further dried using hexane to form an azeotrope. The product was purified by reversed phase HPLC using a C18, small pore preparative column (Vydac), eluting with increasing concentrations of acetonitrile in water. The product, Ac-Arg-His-Lys(Ac)-AMC, was lyophilized to yield a white flocculent powder (30 mg, 63 μmol) in 59% overall yield. MALDI MS calculated for C38H56N12O8 (M+H+), 809.8; found, 809.8.

Boc-Lys(Ac)2-R110 (a generous gift from L. Lavis and R. Raines, University of Wisconsin-Madison) was converted to p53-R110 in a manner similar to the p53-AMC peptide. MALDI MS calculated for C38H56N12O8 (M+H+), 1597.8; found, 1597.8.

Molecular Modeling of SIRT1 and p53-AMC—The SIRT1 crystal structure was built by homology modeling using the SWISS-MODEL function in Swiss PDB viewer version 3.7 (62). The p53-AMC peptide was modeled from the crystal structure of p53 bound to Sir2-Af2 (63). Using Sybyl (version 6.8, Tripos, Inc., St. Louis, MO), an amide bond was built between the amino group of AMC and the main chain carboxyl group of the acetylysine residue of p53 bound to Sir2-Af2. The built SIRT1 structure and the Sir2-Af2 structure containing the modeled p53-AMC peptide were aligned in Swiss PDB viewer version 3.7 (62) using sequence homology thereby dock the p53-AMC peptide into the SIRT1 structure. The resulting crystal structure of p53-AMC peptide bound to SIRT1 was used for all subsequent analysis.

RESULTS AND DISCUSSION

Resveratrol Activates SIRT1 but Not Other Sir2 Homologs—To determine whether resveratrol activated Sir2 homologs, ySir2, human SIRT2, and human SIRT1 were analyzed using the commercially available Fluor de Lys-SIRT1 as a peptide substrate, with and without 200 μM resveratrol. Samples were quenched with the Developer II solution with added 2 mM nicotinamide, and the fluorescence was measured according to the manufacturer’s protocol. With SIRT1, SIRT2, and ySir2, fluorescence increased over time, indicating deacetylation of the Fluor de Lys-SIRT1 peptide (Fig. 3). Comparison of the rate of fluorescence increase in the presence and absence of resveratrol showed that resveratrol activated SIRT1 by 8-fold (Fig. 3A), consistent with previous studies using this assay...
However, no significant activation was observed when SIRT2 (Fig. 3B) and ySir2 (Fig. 3C) were assayed with the Fluor de Lys-SIRT1 peptide. Moreover, a higher concentration of ySir2 was required to observe significant deacetylation of the substrate, indicating that Fluor de Lys-SIRT1 peptide is a poor substrate for ySir2. Because resveratrol activation was specific for SIRT1, subsequent mechanistic studies were performed using this enzyme.

**Resveratrol Activation Requires Fluor de Lys-SIRT1 as a Substrate**—To verify resveratrol activation of SIRT1, an alternative assay was employed. The charcoal binding assay (61) was carried out under the same conditions as those for the Fluor de Lys fluorescence assay, except that the Fluor de Lys-SIRT1 peptide was replaced by [3H]AcH3 peptide, which corresponds to the sequence of the histone H3 N-terminal tail including and surrounding the acetylated lysine 14 (sequence: KSTGG([3H]AcK)APRKQ). In the charcoal binding assay, the [3H]acetyl group from the [3H]AcH3 is transferred to the ADP-ribose portion of NAD\(^+\) to form O-[3H]AADPr, which is quantified as described previously (61). The assays were carried out with and without 200 μM resveratrol, and the concentrations of product formed were quantified and plotted versus time. As shown in Fig. 4A, the product formed increased over time; however, the rate of product formation was identical (within error) in the presence and absence of resveratrol, indicating that resveratrol had no effect on the enzymatic activity when [3H]AcH3 was employed as a substrate and when the charcoal binding assay was used.

Because of the conflicting results from the Fluor de Lys fluorescence assay and the charcoal binding assay, other possible explanations for resveratrol activation were examined. To determine whether resveratrol induces an increase in product fluorescence, the deacetylated Fluor de Lys-SIRT1 peptide was incubated in the SIRT1 assay buffer with and without resveratrol. The fluorescence was measured, and no difference with and without resveratrol was observed (data not shown), indicating that resveratrol does not increase the fluorescence of the product fluorophore. To test whether resveratrol activated the Developer II solution, SIRT1 reactions were performed without resveratrol. Instead, resveratrol was added with the Developer II solution. The rate of fluorescence increase was similar in the presence and absence of the resveratrol (data not shown), indicating that the observed resveratrol activation did not stem from activation of the Developer II solution.

To examine the possibility that resveratrol activation required the use of the Fluor de Lys-SIRT1 peptide, an alternative HPLC assay was performed with the Fluor de Lys-SIRT1 substrate. In this assay, [14C]NAD\(^+\) was used as the coenzyme substrate. Samples at various time points from the reaction were analyzed by reversed phase HPLC, and radioactivity of the nicotinamide and NAD\(^+\) fractions was determined by scintillation counting. Results showed increased nicotinamide formation over time (Fig. 4B), and a comparison of the rates showed that resveratrol activated SIRT1 by an average of 7-fold, consistent with the activation observed in the Fluor de Lys fluorescence assay.

**p53-AMC and p53-R110 Peptide Substrates Allow for Resveratrol Activation of SIRT1**—Because the Fluor de Lys substrate and assay kit are commercial products from BioMol, details of the Fluor de Lys fluorescence assay are proprietary information. However, it is most likely the Fluor de Lys assay is similar to other methods published previously (64, 65), which take advantage of a newly created trypsin cleavage site after lysine deacetylation, liberating the attached fluorophore and generating the increased fluorescence. The Fluor de Lys-SIRT1 substrate is a 4-mer peptide based on the p53 sequence, with a

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**Fig. 3.** Fluor de Lys fluorescence assay to determine resveratrol activation of SIRT1 and other Sir2 homologs. All assays were carried out in the presence of 64 nM SIRT1 (A), 0.5 μM SIRT2 (B), or 1 μM ySir2 (C) with 20 μM Fluor de Lys-SIRT1, 100 μM NAD, in the absence (filled circles) and presence (filled squares) of 200 μM resveratrol. The corrected arbitrary fluorescence units (corr AFU) were plotted versus time.
fluorophore at the C terminus adjacent to the acetylated lysine residue, (sequence: Arg^{379}-His-Lys-Lys^{382}(Ac)-fluorophore). With this in mind, we synthesized two fluorophore-containing p53 peptides, utilizing the same p53 sequence, with either 7-amino-4-methylcoumarin (p53-AMC) or rhodamine 110 (p53-R110) (for details, see "Experimental Procedures" and Figs. 1 and 2) adjacent to the acetylated lysine.

Initially, the synthesized p53-AMC peptide was tested as a substrate for SIRT1, and its ability to exhibit resveratrol activation was examined. Reactions were carried out using 20 μM p53-AMC peptide and 100 μM NAD\(^{+}\) in the presence and absence of 200 μM resveratrol. As shown in Fig. 5A, the fluorescence increased over time, indicating deacetylation of the p53-AMC peptide. The addition of resveratrol increased the rate of product formation by 9-fold. This result indicates that not only can the p53-AMC peptide be used in the fluorescence assay, but that it also behaves similarly to the Fluor de Lys-SIRT1 peptide, allowing resveratrol activation. Subsequently, our synthesized p53-AMC peptide was used to probe for the mechanism of resveratrol activation.

Similarly, the synthesized p53-R110 peptide was used as a substrate in the SIRT1 reaction. The p53-R110 contains two p53 4-mer peptides, covalently attached to rhodamine 110, and contains the same sequence as the p53-AMC peptide (Fig. 1). The reactions were carried out with 20 μM p53-R110 peptide and 100 μM NAD\(^{+}\), in the presence and absence of 200 μM resveratrol. As shown in Fig. 5B, the rhodamine fluorescence increased over time in the presence of SIRT1, indicating deacetylation of the p53-R110 peptide. The addition of resveratrol in the assay increased the rate of product formation by a significant 2.3-fold (Fig. 5B). The p53-AMC, p53-R110, and Fluor de Lys-SIRT1 peptides all demonstrate the ability to be deacetylated more efficiently by SIRT1 in the presence of resveratrol.
The Fluorophore of Fluor de Lys-SIRT1, p53-AMC, and p53-R110 Peptides Are Required for the Observed Resveratrol Activation—The fact that p53-AMC, p53-R110, and Fluor de Lys-SIRT1 peptides, but not AcH3, allowed resveratrol activation indicates that the either the peptide sequence or the fluorophores were necessary for resveratrol activation. To determine whether the sequence of the p53 peptide was necessary for the observed activation, Fluor de Lys-H4 AcK16 was purchased from BioMol. The Fluor de Lys-H4 AcK16 is a 5-mer peptide based on the sequence of histone H4 tail, with the acetylated lysine 16 adjacent to the same fluorophore as Fluor de Lys-SIRT1 (sequence: Lys\(^{12}\)-Gly-Gly-Ala-Lys\(^{16}\)(Ac)-fluorophore). The Fluor de Lys fluorescence assay was performed using 20 \(\mu\)M Fluor de Lys-H4 AcK16, 100 \(\mu\)M NAD\(^{+}\), in the presence and absence of 200 \(\mu\)M resveratrol. Using SIRT1, the fluorescence increased over time, indicating deacetylation of the substrate (Fig. 6A). Comparison of the rate of fluorescence increase demonstrated that resveratrol activated SIRT1 by 7-fold (Fig. 6A), which was similar in magnitude to the activation observed using Fluor de Lys-SIRT1 and p53-AMC peptides. The sequence of the peptide, therefore, was not critical for resveratrol activation.

To determine whether resveratrol activation required the fluorophore, a non-fluorophore-containing p53 4-mer peptide (Fig. 1), with the same sequence as Fluor de Lys-SIRT1, p53-AMC, and p53-R110 peptides, was used as a SIRT1 substrate in the presence and absence of 200 \(\mu\)M resveratrol. HPLC assays were performed using 20 \(\mu\)M p53 4-mer peptide and 100 \(\mu\)M \(^{14}\)C NAD\(^{+}\) and analyzed as discussed above. The amounts of nicotinamide formed increased over time, but the presence of resveratrol had no significant effect on the rate of product formation (Fig. 6B). These data indicate that the fluorophore was required to observe resveratrol activation.

To probe whether covalent attachment of the fluorophore is required for resveratrol activation, HPLC assays were performed using the p53 peptide \(^{14}\)C NAD\(^{+}\), in the presence and absence of AMc, resveratrol, or AMc and resveratrol combined. At each time point, samples were quenched and analyzed by reversed phase HPLC. As shown in Fig. 6C, the amount of \(^{14}\)C nicotinamide formed over time increased, indicating deacetylation of the p53 4-mer peptide, but the addition of resveratrol and AMc, individually or combined, does not show activation on the rate of product formation. These data indicate that resveratrol activation depends on the presence of AMc that is covalently attached, as in the p53-AMC peptide.

Resveratrol Promotes Tighter Binding of the Fluor de Lys-SIRT1 and p53-AMC Peptides to SIRT1—It was shown previously that resveratrol lowered the \(K_m\) values for the Fluor de Lys-SIRT1 peptide without affecting the overall turnover rate (\(V_{max}\)) of the enzyme (35). Therefore, it is possible that resveratrol allows tighter binding of the fluorophore-containing peptide into the active site of the enzyme. To test this hypothesis, a competition study was performed, in which Fluor de Lys-

*Fig. 6. Resveratrol activation is independent of the peptide sequence but dependent on the presence of the fluorophore. A. Fluor de Lys-H4 AcK16 was used as a substrate for SIRT1. The reactions were carried out in the presence of 20 \(\mu\)M Fluor de Lys-H4 AcK16, 100 \(\mu\)M NAD\(^{+}\), with and without 200 \(\mu\)M resveratrol, and 0.3 \(\mu\)M SIRT1. The corrected arbitrary fluorescence unit (corr AFU) of the samples was determined and plotted versus time in the presence (filled squares) and absence (filled circles) of resveratrol. B, SIRT1 assay was performed with 20 \(\mu\)M p53 peptide, 100 \(\mu\)M \(^{14}\)C NAD\(^{+}\), and 0.3 \(\mu\)M SIRT1 in the presence (filled squares) and absence (filled circles) of 200 \(\mu\)M resveratrol. Samples were analyzed by reversed phase HPLC. C, SIRT1 assays were performed with 20 \(\mu\)M p53 4-mer peptide, 100 \(\mu\)M \(^{14}\)C NAD\(^{+}\), 0.3 \(\mu\)M SIRT1, and the concentrations of \(^{14}\)C nicotinamide formed were determined over time. The reactions were carried out in the presence of 200 \(\mu\)M resveratrol (dark gray columns), 200 \(\mu\)M coumarin (light gray columns), 200 \(\mu\)M resveratrol and coumarin combined (white columns), or just the p53 4-mer peptide alone (black columns).*
weakly competed with \[^{3}H\]AcH3 as a substrate. In the presence of resveratrol, a greater decrease of O-\[^{3}H\]AADPr was observed, indicating that the Fluor de Lys-SIRT1 peptide displays higher affinity for the enzyme in the presence of resveratrol.

Substrate competition assays were also carried out using the p53-AMC and p53 4-mer peptides as binding competitors of \[^{3}H\]AcH3, in the presence and absence of resveratrol. As shown in Fig. 7B, both the p53-AMC and p53 4-mer peptides decreased the amount of O-\[^{3}H\]AADPr formed. Interestingly, the p53-AMC peptide in the absence of resveratrol was the least effective competitor, suggesting that covalent attachment of AMC lowered the affinity compared with binding of the free p53 4-mer peptide. Resveratrol had no effect in the competition assay when the non-AMC-containing p53 4-mer peptide was used, indicating that resveratrol does not increase the binding efficiency of free p53 4-mer peptide substrate. However, in the presence of resveratrol, the ability of p53-AMC to compete directly with \[^{3}H\]AcH3 was enhanced to a level similar to that seen with the free p53 4-mer peptide substrate.

From the competition study, it is evident that resveratrol increases the binding affinity for the fluorescently labeled peptides, but not for peptides lacking a fluorophore. To quantify the resveratrol binding enhancement of SIRT1 for Fluor de Lys-SIRT1 peptide, the dissociation constants for Fluor de Lys-SIRT1 and free p53 4-mer peptides, with and without resveratrol, were determined from substrate inhibition analyses. Inhibition analysis was carried out in the presence of increasing concentrations of p53 4-mer or Fluor de Lys-SIRT1 peptides as substrate competitors, at two fixed concentrations of \[^{3}H\]AcH3 in the presence and absence of resveratrol. Using the charcoal binding assay, initial velocities were measured, and for each \[^{3}H\]AcH3 concentration, the 1/Kd values were plotted versus the competing substrate concentration. Each data set is fitted into a linear equation, and the –Kd value corresponds to the value on the –x axis where the lines intersect, as described previously (66). The dissociation constants for the Fluor de Lys-SIRT1 peptide were 504 and 75 μM, in the absence and presence of resveratrol, respectively. For the p53 peptide, the corresponding dissociation constants were 204 and 210 μM, in the absence and presence of resveratrol, respectively. The 7-fold tighter binding of Fluor de Lys-SIRT1 peptide in the presence of resveratrol suggests that the observed resveratrol activation is largely the result of the increased affinity of the p53-AMC and Fluor de Lys-SIRT1 peptides. The lack of a resveratrol effect with free p53 4-mer peptide substrate indicates that the covalently attached fluorophore (coumarin in the case of p53-AMC) is required to manifest the increased binding affinity induced by resveratrol. Collectively, these data suggest that resveratrol induces a conformational change in SIRT1 which better accommodates the binding of coumarin or other fluorophores within related peptide substrates, as those displayed in Fig. 1.

Structural Model for the Observed Resveratrol Activation—Having determined the attachment of the coumarin in the p53-AMC peptide necessary for resveratrol activation, we developed a model of activation based on the available structural data. Because no crystal structure of SIRT1 was available, a homology model was built using the SWISS-MODEL function in Swiss PDB viewer version 3.7 (62). The crystal structure of a p53 peptide bound to Sir2-A2 (63) allowed us to build a model of the p53-AMC peptide into a SIRT1 structure generated by aligning the two structures using sequence homology. Our biochemical data showed that the presence of coumarin at the C terminus of the p53 peptide is slightly deleterious for binding, and the addition of resveratrol appears to compensate
for this effect by increasing the binding efficiency of the p53-AMC peptide. From the modeled crystal structure, the coumarin ring is significantly solvent-exposed prior to resveratrol binding (Fig. 8). In this energetically unfavorable configuration, the hydrophobic aromatic rings of coumarin are held in the hydrophilic environment on the surface of the enzyme. In addition, the coumarin lacks significant contacts to the enzyme that would facilitate binding. This may account for lower binding affinity observed for p53-AMC compared with the p53-4-mer peptide alone.

Resveratrol activation of SIRT1 appears to be a binding phenomenon as indicated by the decreased dissociation constant (this work) and decreased $K_d$ value (35). We proposed that resveratrol binds to SIRT1 and induces a protein structural change near the coumarin group of bound p53-AMC peptide. This alternate conformation creates a binding pocket to accommodate the attached coumarin better, resulting in the enhanced binding of p53-AMC peptide. Residues that would likely interact with coumarin are Gln$^{102}$, Met$^{104}$, Phe$^{221}$, Phe$^{222}$, and Arg$^{254}$, which are depicted in Fig. 8. These residues were chosen from the built model because of their close proximity to the coumarin ring (<10 Å). This figure was generated using Sybyl version 6.8, Swiss PDB viewer version 3.7 (62), and POV-ray version 3.6.

![Fig. 8. Putative binding site of the p53-AMC peptide.](image)

for the general use of resveratrol as an in vivo activator of Sir2 homologs from a wide variety of organisms. In particular, because resveratrol activation appears to be specific for SIRT1, it would seem prudent to readdress the previously published studies linking yeast Sir2 and resveratrol to common cellular processes, such as life span increase via calorie restriction and gene silencing. Also, at this stage, it is unclear whether the fluorophore-specific activation of SIRT1 by resveratrol has revealed an intrinsic property of SIRT1. Resveratrol may serve as a mimic to endogenous regulators, which could alter SIRT1 structure and function, perhaps yielding an apparent "up-regulated" SIRT1. "Activated" SIRT1 may display altered specificity for a distinct acetylated substrate or may demonstrate a higher affinity for this target substrate. Because SIRT1 is reported to harbor no substrate selectivity on its own (67), SIRT1 may need the help of an activator to take advantage of this inherent property and discriminate among possible substrate targets.

Acknowledgments—We acknowledge Dr. Mihail Iordanov from Oregon Health and Science University for providing resveratrol for our initial studies, Luke D. Lavis for the synthesis of Boc-Lys(4Ac) rhodamine and for valuable discussions, and Grant D. Geske for computer time and help with Sybyl. We are also grateful to Terri Kowieski for cloning and purifying SIRT1.

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