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Analytical Biochemistry 332 (2004) 90–99
ANALYTICAL BIOCHEMISTRY
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Fluorescence assay of SIRT protein deacetylases using an acetylated peptide substrate and a secondary trypsin reaction

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Received 10 March 2004
Available online 3 July 2004

Abstract

A novel fluorescent substrate was devised for the sirtuin (SIRT) class of human protein deacetylases comprised of a peptide sequence containing a single acetyl-lysine residue, with a fluorescent group (tetramethylrhodamine-6-carboxylic acid, 6-TAMRA) near the carboxyl terminus and a nonfluorescent quenching group (QSY-7) near the amino terminus. The peptide sequence is modeled after the p53 acetylation site but is unreactive toward trypsin because all other lysine and arginine residues have been replaced by serine. However, the SIRT-deacetylated peptide is readily cleaved by trypsin, resulting in a maximal 30-fold enhancement of the 6-TAMRA fluorescence. Nicotinamide at millimolar concentrations stops the deacetylation but does not inhibit trypsin, and a microtiter plate assay of the SIRTs has been devised using the fluorescent substrate and these reagents. Using this method, the kinetics of the reaction of the cosubstrate nicotinamide adenine dinucleotide and the competitive inhibitor nicotinamide with SIRT1 and SIRT2 has been analyzed. Several nicotinamide analogs have also been tested as inhibitors and found to have much lower affinity for these enzymes than does the parent compound.

Keywords: Sirtuin deacetylase; Histone deacetylase; HDAC; Fluorescence assays; FRET assays

The SIRTs are members of a family of nicotinamide adenine dinucleotide (NAD)[1]-dependent enzymes that catalyze the deacetylation of acetyl-lysine residues of histones and other proteins [1,2]. They were first characterized as the "silent information regulator" class 2 (SIR2) gene products in yeast [3], and at least seven members of this protein family, termed "sirtuins" or SIRTs, have been identified in humans [4]. The SIRTs are often referred to as class III histone deacetylases [1] because this was the first enzymatic activity identified for these proteins [2,5], although they are structurally and mechanistically distinct from the class I and class II histone deacetylases (HDACs) [6]. In contrast to these zinc-dependent HDACs, the deacetylation reaction catalyzed by SIRTs is metal independent and requires stoichiometric quantities of NAD, with the nicotinamide portion of the cosubstrate being displaced from the ribose as the protein is deacetylated [7].

The sirtuins are closely related in structure to the NAD-dependent deacetylases of other species [8], and acetylated proteins in addition to histones, most notably p53 [9,10], are believed to be natural substrates for SIRTs. It has been shown that p53 binding to DNA is activated by its acetylation within the C-terminal domain [11]; thus, deacetylation of p53 by SIRT could be important in the regulation of cellular responses to DNA-damaging agents [12]. Because SIRT functions to silence apoptotic responses mediated by p53 [13,14],

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[1] *Abbreviations used:* NAD, nicotinamide adenine dinucleotide; SIR2, silent information regulator class 2; HDAC, histone deacetylase; RP-HPLC, reversed phase high-performance liquid chromatography; 6-TAMRA, tetramethylrhodamine-6-carboxylic acid; Fmoc, 9-fluorenylmethoxycarbonyl; TFA, trifluoroacetic acid; MALDI MS, matrix-assisted laser desorption/ionization mass spectrometry; L-Nle, L-norleucine; PCR, polymerase chain reaction; OD, optical density; DTT, dithiothreitol; GST, glutathione-*S*-transferase.

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doi:10.1016/j.ab.2004.05.039

inhibitors of these proteins are of interest as potential anticancer drugs.

SIRTs have previously been assayed by monitoring radioactivity released from a protein labeled with radioactive acetate [2,15–17] or by analyzing with reversed phase high-performance liquid chromatography (RP-HPLC) an acetylated peptide substrate and the deacetylated product [7,18]. The assay of human SIRT1 and yeast Sir2, using a commercially available fluorescence assay designed for histone deacetylases, has been reported [19]. We synthesized a SIRT peptide substrate based on a segment of the p53 sequence containing the acetylated lysine [9] and then modified this peptide to devise a fluorogenic substrate suitable for screening. The polypeptide was rendered insensitive to trypsin proteolysis by replacing all arginine and underivatized lysine residues with serine; thus, the substrate contains one acetylated lysine residue that is readily deacetylated by SIRT2. A fluorogenic substrate for SIRTs was devised by attaching fluorescent tetramethylrhodamine-6-carboxylic acid (6-TAMRA) and quenching (QSY-7) groups on opposite sides of this trypsin-resistant sequence. No increase in fluorescence is observed on incubation of the substrate with SIRT1 or SIRT2. The deacetylated product, but not the substrate, is readily cleaved by trypsin, and the fluorescence increase after the addition of trypsin is a measure of the deacetylase activity. We report this method as a sensitive new assay of human SIRT1 and SIRT2. We have also determined the dissociation constants of NAD and nicotinamide (product inhibitor) and performed a preliminary survey of the inhibition of these enzymes by nicotinamide analogs.

Materials and methods

Synthesis and characterization of peptides

All peptides were synthesized by solid phase peptide synthesis using standard 9-fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems model 433 peptide synthesizer (Foster City, CA). QSY-7-succiniminyl ester (QSY-7-NHS) and tetramethylrhodamine-6-carboxysuccinimidyl ester (6-TAMRA-NHS) were obtained from Molecular Probes (Eugene, OR). For the labeled substrate, the QSY-7 group was introduced after selectively removing the 4-methyltrityl-protecting group (1% trifluoroacetic acid (TFA), 4% triisopropylsilane, 95% methylene chloride) from the epsilon-amino of the lysine residue at position 3 of the resin-bound protected peptide. After cleavage from the resin and RP-HPLC purification of the QSY-7-labeled peptide, the 6-TAMRA group was added to lysine 20. The peptides were purified by RP-HPLC, the purities were assessed by analytical RP-HPLC, and the identities were verified by matrix-assisted laser desorption/ionization mass spectrometry (MALDI MS). The labeled peptide (substrate A) was found to have the correct mass ($m/z = 3572.8$) for the following sequence: Ac-EE-K(QSY-7)-GQSTS SHS-K(Ac)-L-Nle-STEG-K(6-TAMRA)-EE-NH_2.

Expression and purification of recombinant SIRT2

SIRT2 was expressed in *Escherichia coli* as a fusion protein with glutathione *S*-transferase, using a methodology similar to that reported previously [15]. In short, SIRT2 cDNA (OriGene Technologies, Rockville, MD) was amplified by polymerase chain reaction (PCR), and the resulting polynucleotide was cloned into a modified pGEX4T-1 vector (Amersham Biosciences, Piscataway, NJ). The sequence was confirmed, and the contruct was transformed into competent DH5α cells grown in NZYM media with carbenicillin (100 μg/ml). The cell culture was grown at 30 °C until the optical density (OD) at 600 nm reached approximately 0.9, and expression was then induced by the addition of 1 mM IPTG at 20 °C for 8 h. Cells were harvested and lysed using a microfluidizer (Microfluidics Corporation, Newton, MA) in lysis buffer (20 mM Na_2HPO_4, pH 7.4; 130 mM NaCl; 1% Triton X-100; 5 mM dithiothreitol (DTT)). The glutathione-*S*-transferase (GST) fusion protein of SIRT2 was purified using glutathione–Sepharose followed by mono-Q ion exchange chromatography (Amersham Biosciences). After the second chromatography, the preparation was found to contain a single protein by SDS–PAGE.

Other proteins and reagents

Human HDAC from K562 cells was prepared as described previously [20]. Recombinant human SIRT1 and the Fluor-de-Lys HDAC substrate and developer were purchased from Biomol (Plymouth Meeting, PA). Endoproteinase Lys-C was purchased from Roche Molecular Diagnostics (Indianapolis, IN). The buffer used for assays was 25 mM Tris–HCl, 137 mM NaCl, 2.7 mM KCl, 1 mM $MgCl_2$, pH 7.5, with 2% glycerol added. A stock solution of 1 mg/ml trypsin (Sigma, St. Louis, MO) in 10 mM acetic acid was stored for up to 1 month at 4 °C and diluted in buffer immediately before use. Stock solutions of 100 mM nicotinamide and 2 mM NAD (Sigma) were prepared in the assay buffer and stored at −20 °C.

Analyses of deacetylation reactions by RP-HPLC

The unlabeled peptides KKGQSTSRHKK(Ac)LM FKTEG and SSGQSTSSHSK(Ac)LMFSTEGK-NH_2 at 1 mg/ml (~0.3 mM) were incubated with purified GST–SIRT2 (60 μg/ml) and NAD (0.5 mM) for up to 3 h. Aliquots of the incubations were quenched at intervals by the addition of 0.5% TFA, and the reaction was analyzed by injection onto a YMC C-18 RP-HPLC column. The acetylated and deacetylated peptides are

resolved by approximately 1 min in a gradient elution from 100% water to 30% acetonitrile/water, with 0.1% TFA added to the mobile phases. Detection was by uv absorbance at 220 nm.

A solution of 8 μM substrate A was incubated with 6 μg/ml GST–SIRT2 and 0.1 mM NAD for up to 6 h. A lower concentration of this peptide was used due to its limited aqueous solubility and intense absorbance. The analysis of conversion of substrate to product was performed similarly with the gradient elution from 30% acetonitrile/water to 60% acetonitrile/water, with 0.1% TFA added to the mobile phases. Detection was by visible absorbance at 586 nm.

Determination of K_m of NAD and K_i of nicotinamide

Stock solutions of NAD and nicotinamide were diluted in half-log steps (two dilutions for each 10-fold decrease in concentration) as described previously for inhibitors of matrix metalloproteinases [21]. The range of NAD concentrations in the final assay was 1 μM–1 mM. The nicotinamide concentrations used were 0, 3.16, 10, 31.6, 100, and 316 μM. The complete reaction mixture (120 μl) in a white Microfluor 96-well plate contained buffer (0.2 mg/ml bovine serum albumin added), SIRT1 or SIRT2, nicotinamide, and NAD and was initiated by the addition of substrate A (0.54 μM final). After 1 h reaction, a solution (30 μl) of 2.5 μg/ml trypsin in 100 mM nicotinamide was added both to stop further deacetylation and to cleave the deacetylated product. The fluorescence of each well was recorded 1 h after the trypsin/nicotinamide quench using a Molecular Devices (Sunnyvale, CA) f_{max} fluorescence plate reader (excitation 540 nm, emission 585 nm). The background fluorescence (from wells without NAD) was subtracted from all wells, and the data were analyzed using Lineweaver–Burk plots. The series without nicotinamide was used to calculate the K_m of NAD.

Assay conditions and inhibitor experiments

The concentration range of inhibitors was 1 μM–1 mM, and each determination was performed in duplicate as described for the K_m determinations except that the concentration of NAD was held constant at 20 μM and the substrate solution was added after a 30-min preincubation of SIRT1 or SIRT2, inhibitor, and NAD. The fluorescence readings were used to calculate the percentage inhibition at each inhibitor concentration relative to control wells without inhibitor.

Comparative experiments with HDAC and SIRT with two fluorescent substrates

Nuclear extract HDAC or SIRT2 was incubated with the fluorescent substrates A (Ac-EE-K(QSY-7)-GQSTSSHS-K(Ac)-L-Nle-STEG-K(6-TAMRA)-EE-NH_2) and B ((QSY-7)-RGGRGLG-K(Ac)-GGARRHR-K(6-TAMRA)NH_2) at 0.54 μM in the buffer described above for 1 h. For SIRT assays, NAD was also added at 20 μM. The volume in the deacetylase assay was 0.12 ml. To quench the enzymes, trichostatin A (1 μM) was added to HDAC assays and nicotinamide (20 mM) was added to SIRT assays. To detect deacetylation of substrate A, trypsin (75 ng/well) was added to each well and the plates were read after 1 h. For substrate B, endoproteinase Lys-C (10 ng/well) was added and the plates were read after 4 h. The final volume in each well after the addition of the quench reagents and secondary enzymes was 0.15 ml.

Results

Design and synthesis of a fluorogenic substrate for SIRT deacetylases

The carboxyterminal sequence (amino acids 371–393) of human p53 is SKKGQSTSRHKKLMFKTEGPDSD [22]. It has been shown [9] that the lysine at position 382 is acetylated in the activated form of p53 and that SIRT1 will remove this acetyl group. The first step in the substrate design was to determine whether a peptide containing this sequence can be deacetylated by recombinant enzyme and also whether the enzyme has a high degree of sequence specificity in its reaction.

The deacetylation of the following two peptides by SIRT2 was evaluated by RP-HPLC:

Peptide 1: KKGQSTSRHKK(Ac)LMFKTEG

Peptide 2: SSGQSTSSHSK(Ac)LMFSTEGK-NH_2

The first peptide follows exactly the sequence of p53 (amino acids 372–387), whereas the second peptide has all of the arginines and unacetylated lysines of the first peptide substituted with serine. The carboxyterminal lysine was added to the second peptide to determine whether the presence of this group would affect deacetylation because the TAMRA group was to be introduced at this position. A comparison of the hydrolysis of peptides 1 and 2 (Figs. 1 and 2, respectively) with SIRT2 shows that peptide 2 is cleaved at approximately half the rate of peptide 1. The RP-HPLC traces show that NAD, added at 1.5-fold molar excess to peptide, is consumed as the peptide is deacetylated. These experiments demonstrate that this modification of the p53 sequence can be used to produce an acetylated peptide substrate for SIRT2 that would not be sensitive to cleavage by trypsin.

To devise a fluorogenic substrate, a peptide based on sequence 2 was synthesized with 6-TAMRA (fluorescent) and QSY-7 (quenching) groups linked to lysine residues on opposite ends of the peptide chain. Because both dyes are hydrophobic, and the substitution of

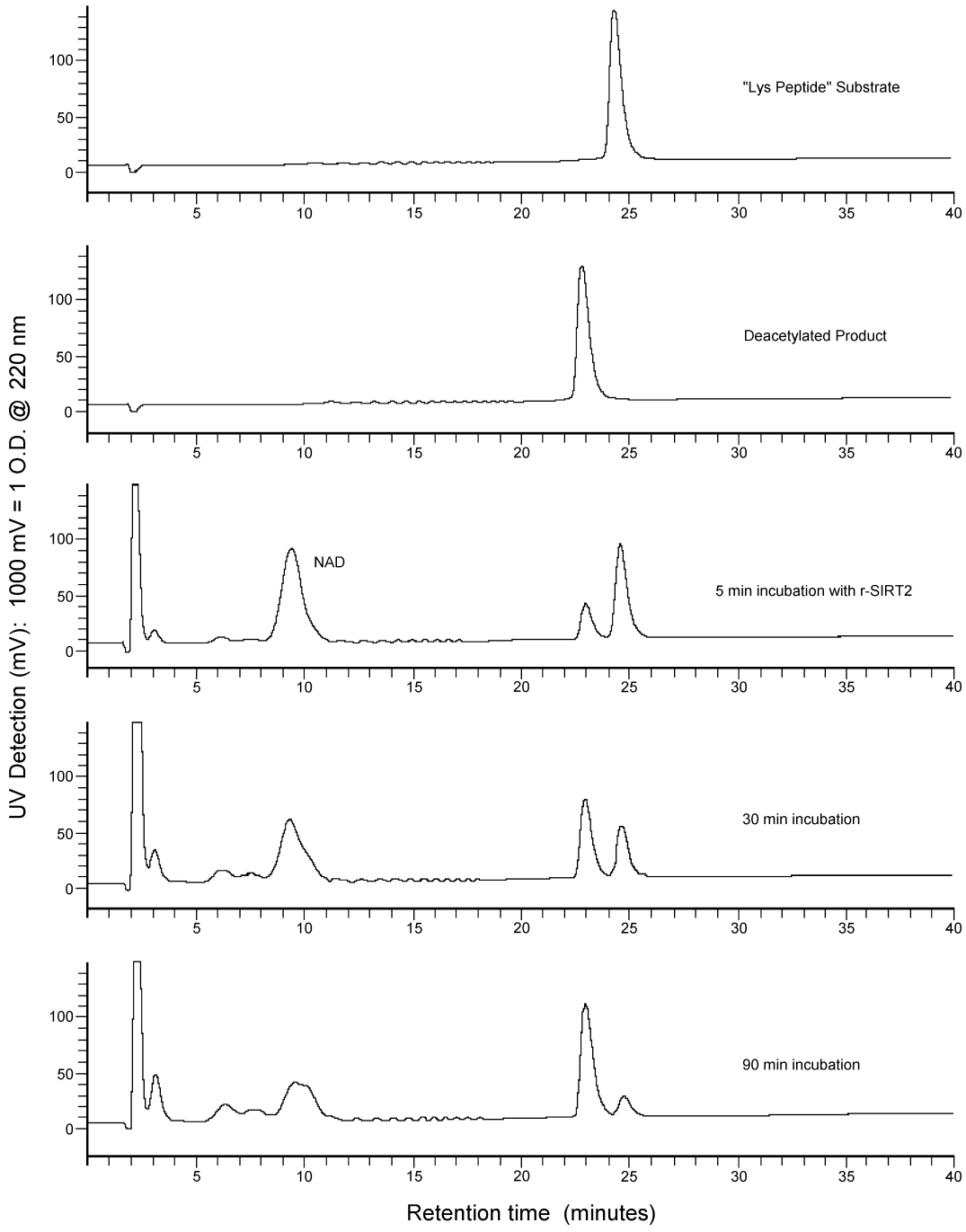

Fig. 1. RP-HPLC determination of the time course of reaction of GST–SIRT2 with peptide 1. Gradient elution: 5–35 min. Detection by UV absorbance @ 220 nm.

serine for lysine and arginine in the peptide removes charged residues, two additional glutamate residues were added to each end of the substrate to increase its aqueous solubility. In the final substrate design, the sole methionine residue was replaced by the isosteric norleucine because we have found that TAMRA-labeled substrates containing methionine are subject to oxidation during synthesis and purification, resulting in a more difficult purification by RP-HPLC and a lower yield of labeled peptide. The complete sequence of the SIRT substrate (substrate A) is Ac-EE-K(QSY-7)-GQSTSSHS-K(Ac)-L-Nle-STEG-K(6-TAMRA)-EE- NH_2.

The deacetylation of the labeled substrate was monitored by HPLC (Fig. 3), demonstrating that substrate A is progressively converted to a single product by recombinant SIRT2. The gradient elution and detection parameters were modified from those used previously due to the properties of the labeled peptide.

The reaction of substrate A with SIRT1 or SIRT2 results in no increase in fluorescence because the

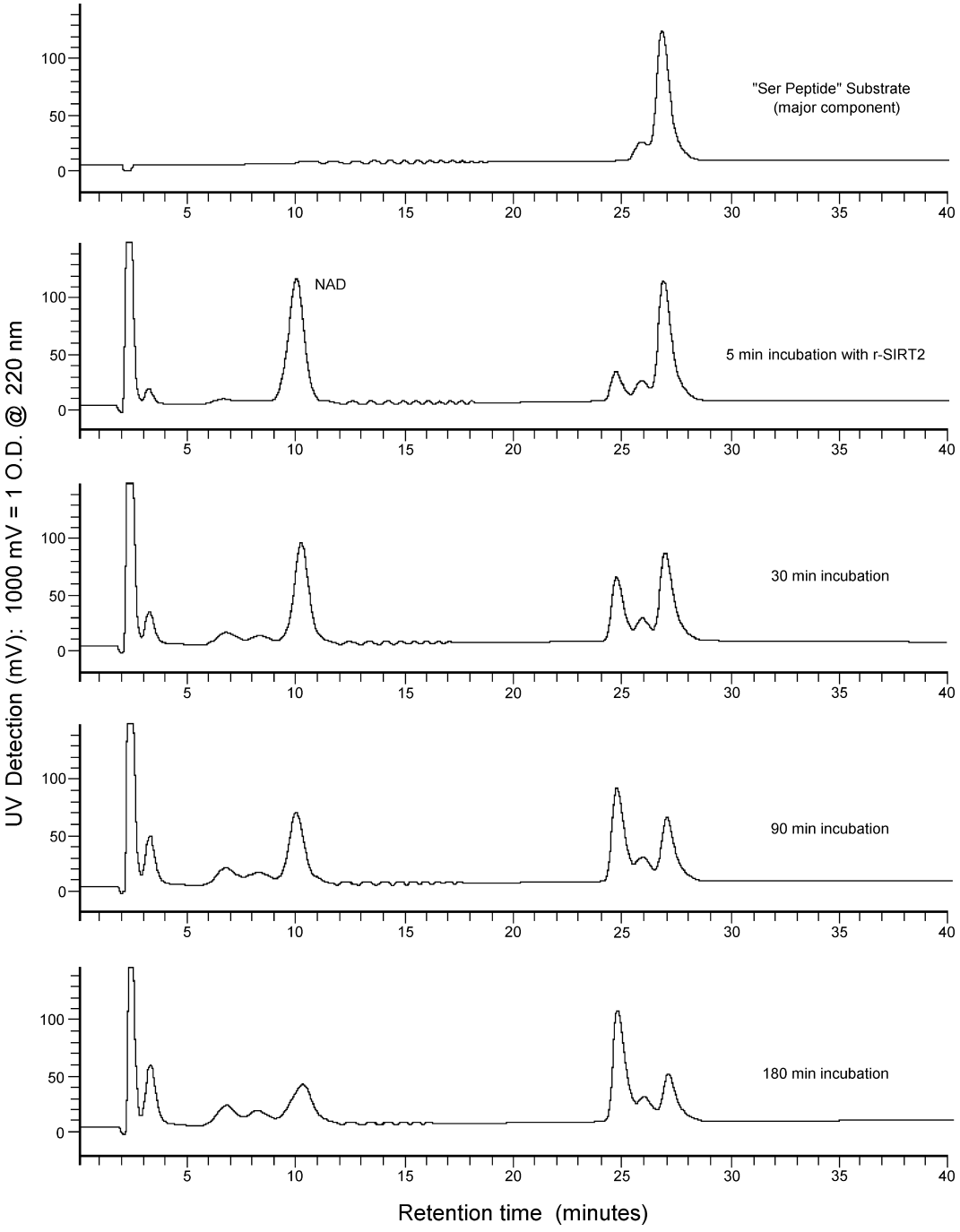

Fig. 2. RP-HPLC determination of the time course of reaction of GST–SIRT2 with peptide 2. Gradient elution: 5–35 min. Detection by UV absorbance: @ 220 nm.

removal of the acetyl group from the lysine in the peptide has no effect on the quenching of the fluorescence of the 6-TAMRA by the QSY-7 group. The purified SIRT preparations also contain insignificant levels of proteinases that nonspecifically cleave this substrate. At the end of the incubation, the addition of trypsin at 0.5 μg/ml to each well completely cleaves the deacetylated peptide in 30–60 min, whereas intact substrate A is unreactive. Nicotinamide is a micromolar inhibitor of SIRT [19] and is a useful quenching agent because it is very soluble and does not inhibit trypsin even at high (20 mM) concentration. As shown in Fig. 4, the deacetylation reaction can be driven to completion with sufficient quantities of GST–SIRT2.

K_m of NAD and K_i of nicotinamide with SIRT1 and SIRT2

The Lineweaver–Burk analyses of the reaction of these enzymes with various concentrations of NAD and nicotinamide are plotted in Figs. 5 and 6, and the data

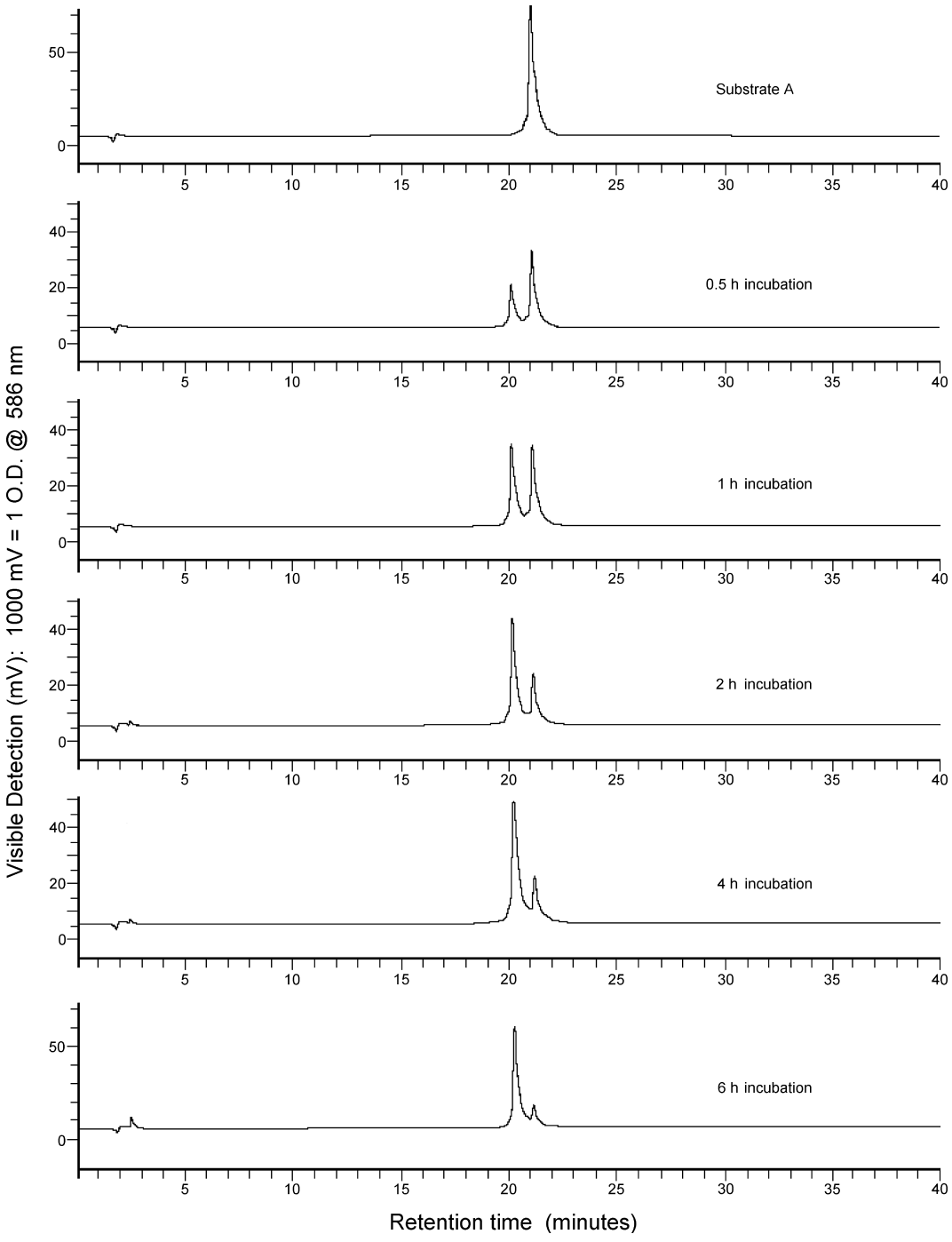

Fig. 3. RP-HPLC determination of the time course of reaction of GST–SIRT2 with substrate A. Gradient elution: 5–35 min. Detection by visible absorbance @ 586 nm.

are summarized in Table 1. In contrast to a recent publication in which mixed or noncompetitive inhibition was reported [19], we have found that the inhibition of both enzymes by nicotinamide is competitive with NAD. The previous researchers used a higher concentration of substrate (250 μM) due to the insensitivity of the Biomol assay used to measure SIRT activity. Because stoichiometric amounts of the NAD cosubstrate are required for reaction, lower concentrations of NAD than of the acetylated substrate cannot be used. The sensitivity of our assay, and the low concentration (<1 μM) of fluorescent substrate employed, allows determination of activity over a wider concentration range of NAD and nicotinamide than in the previous research [19]. These factors may explain the difference in kinetic profile and also why we calculate a lower K_m for NAD (90 μM) than that

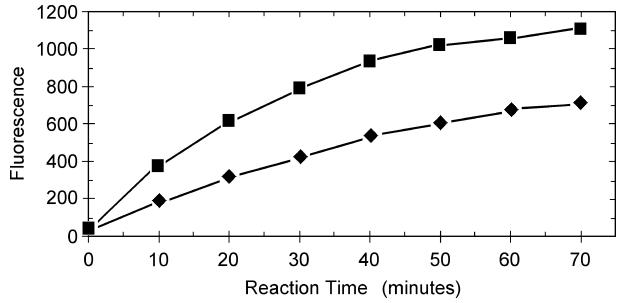

Fig. 4. Time course of reaction of GST–SIRT2 with substrate A. The reaction was quenched at the indicated time, with nicotinamide and trypsin were added to all wells at the end of the time course. ■, 0.7 μg protein/well; ◆, 0.35 μg/protein/well.

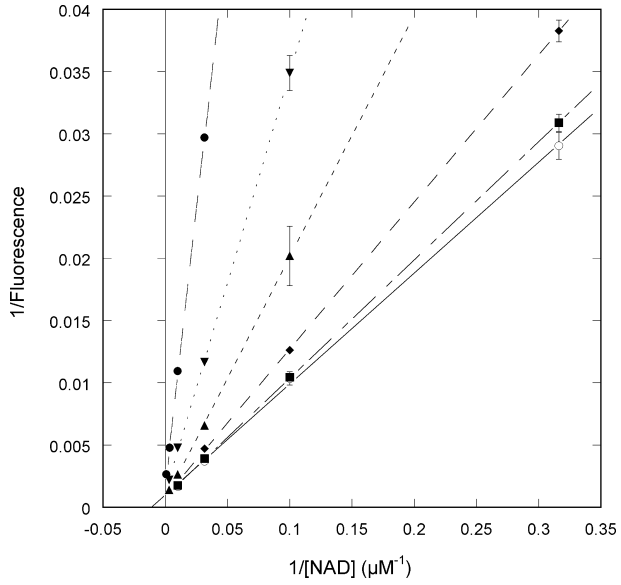

Fig. 5. Lineweaver–Burk plot of assay of SIRT1 with varying concentrations of NAD and nicotinamide. ○, NAD K_m determination; ■, 3.162 μM nicotinamide; ◆, 10 μM nicotinamide; ▲, 31.62 μM nicotinamide; ▼, 100 μM nicotinamide; ●, 316.2 μM nicotinamide. Shown are means and standard deviations of duplicate determinations. The standard deviations not shown are within the dimensions of the plot symbol.

observed for SIRT1 (~250 μM from Fig. 6B of [19]). The K_m determined for NAD using our fluorescent assay is similar to that found previously (70 μM) by another laboratory in which a chromatographic assay for SIRT1 was employed [7].

Both NAD and nicotinamide have higher affinity (lower dissociation constants) for our preparation of GST–SIRT2 than for the commercial SIRT1. In both cases, the enzyme has a slightly higher affinity for nicotinamide than for NAD, indicating that the binding at the nicotinamide site results in most of the productive interactions of NAD with the protein. Although the construct employed to produce these recombinant proteins might affect the kinetic parameters, our results show that this methodology can be used to assay SIRTs from various sources and that both proteins are similar NAD-dependent deacetylases.

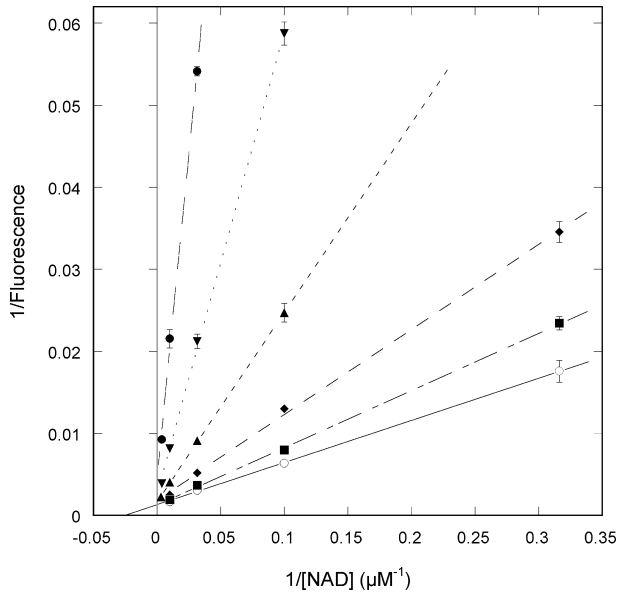

Fig. 6. Lineweaver–Burk plot of assay of SIRT2 with varying concentrations of NAD and nicotinamide. ○, NAD K_m determination; ■, 3.162 μM nicotinamide; ◆, 10 μM nicotinamide; ▲, 31.62 μM nicotinamide; ▼, 100 μM nicotinamide; ●, 316.2 μM nicotinamide. Shown are means and standard deviations of duplicate determinations. The standard deviations not shown are within the dimensions of the plot symbol.

Table 1
Kinetic constants derived from Figs. 5 and 6

	SIRT1 (μM)	SIRT2 (μM)
K_m (NAD)	90	42
K_i (nicotinamide)	38	10

Assay of nicotinamide analogs as SIRT inhibitors

The inhibition by nicotinamide and some derivatives is shown in Table 2. These determinations were performed at a single concentration of NAD (20 μM); therefore, only IC_{50} values are reported. Because the concentration of NAD is below its K_m for both enzymes, the calculated IC_{50} values should approximate the K_i values, and this was confirmed by the nicotinamide IC_{50} values determined in an independent experiment from the K_i values calculated in Table 1. Simple derivatization of nicotinamide, such as replacement of the carboxamide with the corresponding carboxylic acid or methylamide as well as methylation of the pyridine nitrogen, results in inactive molecules. These results imply that these structural features of the nicotinamide molecule are required for binding to the enzyme. Two other derivatives, 6-iodonicotinamide and 5,6-benzofused nicotinamide, are very weak inhibitors of SIRT1 and SIRT2, with IC_{50} values $\geqslant 200$ μM.

Table 2
Inhibition of SIRT1 and SIRT2 by nicotinamide analogs

Compound	IC_{50} of SIRT1 @ 20 μM NAD	IC_{50} of SIRT2 @ 20 μM NAD
Nicotinamide	40 μM	10 μM
Nicotinic acid	No inhibition @ 1 mM	No inhibition @ 1 mM
Nicotinic acid methyl amide	No inhibition @ 1 mM	No inhibition @ 1 mM
N1-Methylnicotinamide hydrochloride	No inhibition @ 1 mM	No inhibition @ 1 mM
6-Iodonicotinamide	880 μM	200 μM
5,6-Benzonicotinamide	30% inhibition @ 1 mM	310 μM

Comparative assay of SIRT and HDAC with two fluorescent substrates

We have reported the use of a fluorescent peptide substrate containing one acetyl-lysine and QSY-7 and 6-TAMRA groups for analysis of inhibition of HDAC by synthetic compounds [23–26]. This substrate is modeled on amino acids 5–20 of the histone H4 sequence [27] and is a modification of an HDAC substrate described previously [28,29]. All unblocked lysine residues have been replaced by arginine, but our substrate employs QSY-7 instead of Rhodamine green [28] attached to the amino terminus.

The amino-terminal sequence of human histone H4 (20 residues) [27] is as follows:

(1) SGRGKGGKGLGKGGAKRHRK (20)

The sequence of HDAC substrate (substrate B) is as follows:

(QSY-7)-RGGRGLG-K(Ac)-GGARRHR-K(6-TAMRA)NH_2

As in the previous work [28], endoproteinase Lys-C was used to selectively cleave the deacetylated product. Because of the expense of this enzyme, a maximum of 10 ng/well was added, and the secondary reaction does not proceed to completion in an overnight incubation. However, we have found that the rate of the secondary reaction is proportional to the amount of deacetylated product, and a fluorescent measurement after partial reaction (2–4 h) can be used to determine the relative amount of deacetylated product in each well. We have found that this histone sequence substrate can be used to assay SIRTs as well as HDACs, with the requirement that NAD is needed for the assay of SIRT. However, the arginine-free substrate (substrate A) described in this article is much more convenient for assay of SIRTs because the secondary trypsin reaction (added at 75 ng/well) proceeds to completion in less than 1 h.

In contrast, nuclear extract HDAC (containing HDACs 1 and 2) does not react with acetylated substrate A (peptide based on the p53 sequence), whereas it readily deacetylates substrate B (peptide based on the histone H4 sequence). We have found that our HDAC assay using substrate B is of comparable sensitivity to the commercially available fluorescent assay from Biomol. However, our assay of SIRT1 or SIRT2 using substrate A is 20-fold more sensitive than the commercial assay, in large part due to the much higher concentration of Biomol substrate used in each well.

Discussion

We have devised a novel peptide substrate for SIRT1 and SIRT2, the deacetylation of which can be used in a sensitive fluorescence-based assay. Although the peptide sequence near the lysine acetylation site of p53 was the starting point in the design of the substrate, the final peptide does not closely resemble the p53 sequence. It has been shown by crystallographic analysis [8] that an acetylated p53 peptide and an archaeal Sir2 enzyme interact through flanking β-sheet interactions of the amide peptide bonds of the protein and the peptide, with the acetylated lysine side chain held in a hydrophobic pocket. The other side chains of the peptide extend away from the active site. Therefore, it is likely that other peptide sequences containing a single acetylated lysine would also serve as substrates for SIRT.

In the absence of the NAD cosubstrate, the peptide is not deacetylated and no increase in fluorescence is observed with the addition of trypsin, showing that this assay measures the activity of the SIRT deacetylase and not a contaminating proteinase or amidase. All of our fluorescence assays have been carried out in a 96-well microtiter plate format and can be used for evaluation of the activity of preparations of recombinant SIRT proteins, as well as for evaluation of SIRT inhibitors, because few compounds interfere with the intense, long-wavelength fluorescence of 6-TAMRA. The fluorescence of 6-TAMRA is efficiently quenched by QSY-7, and this fluorescent/quencher pair may have broader use in fluorescence-based assays of hydrolytic enzymes. Except for the substrate, all reagents used in this assay are readily available and inexpensive. Because trypsin is used as the secondary enzyme, the deacetylated product can be completely cleaved and the assay plate can be read in less than 1 h.

Although we have not fully explored the peptide sequence specificity of these deacetylases, the results of our experiments using our two fluorescent peptide substrates and the Biomol substrate indicate that it should be possible to prepare acetyl-lysine-containing peptides that react preferentially with HDACs or SIRTs.

The activities of HDACs and SIRTs are also easily differentiated by the requirement of NAD for deacetylation by SIRTs and the selective inhibition of HDACs by trichostatin A and of SIRTs by nicotinamide.

Inhibition of SIRTs is an active area of research in the pharmaceutical industry because such compounds could modulate cellular processes by inducing hyperacetylation of histones, p53, and possibly other proteins. Sirtinol and M15 [15], as well as splitomicin [17], have been identified through chemical library screening as inhibitors of the human or yeast enzymes. However, these compounds have limited aqueous solubility and are less potent than nicotinamide. Because our preliminary survey of nicotinamide analogs indicates that minor structural variations of this molecule greatly decrease inhibitory activity, it will likely be necessary to find compounds of other classes to devise more potent inhibitors of the SIRTs.

Acknowledgments

We are grateful to Rohinton Edalji and Richard Smith for the preparation of the nuclear HDAC and to Kent Stewart for helpful suggestions.

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