Unstructured Conformations Are a Substrate Requirement for the Sir2 Family of NAD-dependent Protein Deacetylases*\textsuperscript{S}

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The regulation of protein function is often achieved through post-translational modifications including phosphorylation, methylation, ubiquitination, and acetylation. The role of acetylation has been most extensively studied in the context of histones, but it is becoming increasingly evident that this modification now includes other proteins. The Sir2 family of NAD-dependent deacetylases was initially recognized as mediating gene silencing through histone deacetylation, but several family members display non-nuclear subcellular localization and deacetylate non-histone protein substrates. Although many structural and enzymatic studies of Sir2 proteins have been reported, how substrate recognition is achieved by this family of enzymes is unknown. Here we use \textit{in vitro} deacetylase assays and a variety of potential substrates to examine the substrate specificity of yeast homologue Hst2. We show that Hst2 is specific for acetyl-lysine within proteins; it does not deacetylate small polycations such as acetyl-spermine or acetylated amino termini of proteins. Furthermore we have found that Hst2 displays conformational rather than sequence specificity, preferentially deacetylat ing acetyl-lysine within unstructured regions of proteins. Our results suggest that this conformational requirement may be a general feature for substrate recognition in the Sir2 family.

Protein phosphorylation has long been accepted as a key mechanism in the regulation of diverse cellular processes. Lately, other post-translational modifications including methylation, ubiquitination, and particularly, acetylation, are being recognized as playing key roles in protein function (1). Histone acetylation, for example, mediated by the interplay between histone acetyltransferases and histone deacetylases (HDACs),\textsuperscript{2} has proven to be vital for control of gene silencing, transcription, replication, and repair (2). The Sir2 family of NAD-dependent HDACs has homologues in organisms ranging from bacteria to human (3). Since histones are absent in bacteria, it seems likely that the activity of this family is not restricted to histones, for example some family members display cellular localization outside of the nucleus and deacetylate non-histone protein substrates (3). It is as yet unclear how this family of enzymes achieves its broad substrate specificity. Here we demonstrate that the yeast homologue Hst2 displays conformational rather than sequence specificity, deacetylating acetyl-lysine within unstructured regions of proteins. This suggests that conformational specificity may be a substrate determinant for all members of the Sir2 family.

\textbf{MATERIALS AND METHODS}

\textit{Expression and Purification of Recombinant Proteins}—Yeast HST2 and HOS3 open reading frames were amplified by PCR and cloned into PET30a at the XhoI/KpnI and BamHI/HindIII restriction sites, respectively, to produce NH\textsubscript{2}-terminal His\textsubscript{6}-tag fusion proteins. Plasmids were transformed into BL21(DE3) cells, and the cells were grown at 37 °C to log phase. HST2 expression was induced by the addition of 0.1 mM isopropyl β-D-thiogalactopyranoside and overnight incubation at 18 °C. HOS3 expression was induced by addition of 1 mM isopropyl β-D-thiogalactopyranoside and overnight incubation at 30 °C. Cells were harvested and lysed by sonication and protein purified by nickel-chelate chromatography. Hst2 lysate was bound in 50 mM HEPES-KOH, pH 8.0, 300 mM NaCl, 10 μM ZnCl\textsubscript{2}, 10 mM imidazole, 1 mM 2-mercaptoethanol, and protease inhibitor-EDTA mixture (Roche Applied Science); eluted in 50 mM HEPES-KOH, pH 8.0, 300 mM NaCl, 10 μM ZnCl\textsubscript{2}, 1 mM 2-mercaptoethanol, 300 mM imidazole; and dialyzed into 10 mM HEPES-KOH pH 8.0, 10 μM ZnCl\textsubscript{2}, 1 mM diethiothreitol, 10% glycerol. Hst3 was bound in 500 mM NaCl, 20 mM Tris, pH 8.0, 1 mM phenylmethylsulfonyl fluoride; eluted in 500 mM NaCl, 20 mM Tris pH 8.0, 10 μM ZnCl\textsubscript{2}, 300 mM imidazole, and 1 mM phenylmethylsulfonyl fluoride; and dialyzed into 500 mM NaCl, 20 mM Tris, pH 8.0, 10 μM ZnCl\textsubscript{2}, 10 mM 2-mercaptoethanol. The purity of both proteins was estimated to be >90% by SDS-PAGE and Coomassie Blue staining.

\textit{Expression and Purification of TAP-tagged Proteins}—TAP containing yeast strains were a gift from J. Greenblatt. All strains were grown in YPD (1% yeast, 2% peptone, 2% glucose) to log phase, harvested, and then lysed by vortexing with glass beads and the TAP-tagged protein purified as described (4). Beads were resuspended in deacetylase assay buffer described below. Assays were carried out directly on TAP protein bound to IgG-Sepharose for 3 h at 30 °C.

\textit{Preparation of Acetylated Substrates}—Peptides corresponding to the NH\textsubscript{2}-terminal sequences of the yeast core histones (SCH2B1, SAKAEKPKASKAPEKPPAAC; SCH2A12, SGGKGKKAGSAKASQSR-SAC; SCH3, ARTKQTARKSTGGKAPRKQKLASKAC; SCH4, SGRKGK-GGKLGKGGAKRHC) were synthesized by a PerSeptive Biosystems Pioneer peptide synthesizer. Crude peptide was purified by reverse phase high performance liquid chromatography on a C18 column using a linear gradient of water-acetonitrile containing 0.06% trifluoroacetic acid. Melittin (M7129), protamine (P3880), cytochrome c (C7752), poly-l-lysine (P0879), poly-d-lysine (P0296), and spermine were purchased from Sigma. Urotensin II was purchased from American Peptide Co. Substrates were lightly acetylated by means of sulfo-N-hydroxysuccinimidyl \textsuperscript{14}C]acetate or \textsuperscript{15}C]acetate, which specifically reacts with lysine, and purified as described previously (5). \textsuperscript{14}C-Acetylated substrate concentration was determined by scintillation counting. Peptide concentration was determined by microburet protein assay (6).

\textit{Preparation of Denatured Proteins}—RNase A was reduced and denatured by incubation for 30 min at 37 °C in 8 M urea, 37.5 mM Tris, pH 8.8, 10 mM DTT and then alkylated by addition of 5 mM iodoacetamide and...
incubation for 30 min at 37 °C. Protein was precipitated by addition of trichloroacetic acid to 20% and subsequent incubation at −20 °C for 15 min. The precipitate was recovered by centrifugation, washed with 20% trichloroacetic acid, acetone-0.1% HCl, and finally with dry acetone. Samples were air-dried and redissolved in 10 mM Tris, pH 8.0. Control samples were only trichloroacetic acid-precipitated. Acetyl-cytosine c was denatured by heating at 100 °C for 10 min and then immediately transferring to ice. Control samples were kept on ice.

**Deacetylase Assays**—Deacetylase assays were carried out in 10 μL total volume in buffer containing 10 mM sodium phosphate, pH 7.4, 5 mM 2-mercaptoethanol. Hst2 reactions contained 0.2 μM purified recombinant enzyme (unless otherwise indicated), with 0.5 mM NAD and 0.2–10 μM [14C]acetyl-substrate or 0.02 nmol [14C]NAD (Amer sham Biosciences, CFA497) and 80 μM acetyl-substrate and were incubated 5–15 min at room temperature (within the linear range of enzyme activity). Hos3 reactions were incubated for 30 min at 30 °C. Reactions were stopped by addition of LDS sample buffer and electrophoresed on a 15% polyacrylamide gel for 12 min at 200 V in 0.25 M Tris, 100 mM Tricine, 0.1% SDS buffer. [14C]Acetate of known specific activity was included for quantitation. Gels were dried onto filter paper, exposed to a phosphorscreen overnight, and then imaged using the STORM phosphorimager (Amer sham Biosciences). Bands were quantified using IPLabGel software (Scanalytics). Competition assays were carried out in the presence of varying amounts of competitors with NAD and [14C]acetyl-pol-y-lysine substrates. Pol-y-glutamate (P1818), pol-y-asparagine (P8137), pol-y-arginine (P46633), Ne-acetyl-l-lysine (A4021), l-lysine (L5626), Nε-acetyl-derminate (P1714), and RNase A were purchased from Sigma. Acetylurotensin II substrate was preincubated with or without 5 mM DTI for 30 min at 37 °C, then the reaction was initiated by addition of 1[14C]NAD and Hst2 and incubated for 20 min at room temperature. All assays were performed at least twice in duplicate.

**Analysis of Kinetic Data**—Data were fitted using Enzyme Kinetics Pro software (ChemSW SyneChem), which uses a least square method to determine kinetic parameters and associated error.

**Structure Diagrams**—Cytosine c (Protein Data Bank ID: 1AKK) and RNase A (Protein Data Bank ID: 1AFU) structural data were obtained from the Research Collaboratory for Structural Bioinformatics Protein Data bank (www.pdb.org), and illustrations were created with RasWin Molecular Graphics program (R. Sayle).

**Circular Dichroism Analyses**—Spectra were measured on a JASCO circular dichroism spectrophotometer. Samples were 0.15–0.2 mg/ml. Ellipticity was measured from 190 to 260 nm at room temperature, except for heat-denatured acetyl-cytosine c, which was measured at 90 °C.

**Protein Disorder Prediction**—Regions of intrinsic disorder were predicted using (i) Regional Order Neural Network (7) (www.strubi.ox. ac.uk/RONN), (ii) GlobPlot (8) (globplot.embde.de), and (iii) disEMBL Intrinsic Protein Disorder Prediction 1.4 web-based software (9) (dis.embl.de).

**RESULTS**

The Sir2 family of NAD-dependent deacetylases is involved in a variety of cellular processes, including gene silencing, transcription, replication, and control of life span (2, 3). Although new substrates for Sir2 proteins are being discovered (3), no proven consensus sequences or other substrate determinants have been identified. Our study aims to uncover the mode by which Sir2 proteins recognize their substrates by analyzing whether various compounds are deacetylated by or inhibit Hst2 activity in vitro. Since the NH2-terminal tails of histones are known to be a physiological target for various HDACs, we first compared the ability of recombinant Hst2 to deacetylate histone peptides and a completely unrelated peptide, melittin, found in bee venom. As melittin functions in cell lysis (10), it is not expected to interact with cellular enzymes in vivo. However, deacetylase assays clearly show that Hst2 is able to deacetylate acetylated histone peptides as well as melittin (Fig. 1A). In contrast, a non-NAD-dependent HDAC, Hos3 (2), specifically deacetylates only histone peptide substrates (Fig. 1A). The activity with melittin suggested to us that Hst2 may be able to deacetylate any acetyl-lysine-containing substrate. To test this, we assayed for deacetylase activity with acetyl-pol-y-lysine. Again, Hst2 efficiently deacetylated this unnatural substrate while Hos3 did not (Fig. 1A). Furthermore, kinetic analyses with these substrates revealed similar specificity constants, kcat/Km, of ~6–8 × 105 M−1 min−1 (Fig. 1B), indicating that Hst2 deacetylates these substrates with comparable catalytic efficiency. To be certain this activity was not a result peculiar to the recombinant protein, we also assayed TAP-tagged enzymes purified from yeast (Fig. 1C). Hst2-TAP deacetylated acetyl-H3 peptide and acetyl-pol-y-lysine. Similarly, Sir2-TAP deacetylated both histone and pol-y-lysine substrates, whereas Hos3-TAP was only able to deacetylate the histone substrate. The ability of both Hst2-TAP and Sir2-TAP to deacetylate acetyl-pol-y-lysine suggests an extreme lack of sequence specificity and that this may be a general feature of the Sir2 family. This is further supported by a recent peptide library study by Guarente and co-workers (11) that demonstrates human homologue SIRT1 shows no sequence specificity surrounding the acetyl-lysine residue.

Polycations such as spermine are also acetylated in vivo (12). Given their structural similarity to the lysine side chain and the lack of sequence specificity they could also be substrates for Sir2 enzymes. Deacetylase assays with acetyl-spermine showed that Hst2 did not deacetylate this substrate even at high enzyme concentration (Fig. 2), suggesting that polycations are unlikely targets in vivo. Acetyl-spermine and acetyl-spermidine were also unable to compete for activity with acetyl-pol-y-lysine (data not shown), implying that simple similarity to the lysine sidechain is not enough to bind to the enzyme. Remarkably, even a vast excess of lysine or acetyl-lysine amino acids was unable to outcompete the acetyl-pol-y-lysine peptide (data not shown), implying that the peptide backbone is necessary for insertion into the active site. In agreement with this notion, crystal structure studies of Hst2 in complex with an acetyl-histone H4 peptide reveal few residue-specific contacts outside of the acetyl-lysine but numerous contacts with the peptide backbone (13).

To further examine the specificity for lysine residues, we assayed for deacetylase activity with NH2-terminally acetylated protamine. Protamine is a highly arginine-rich, lysine-deficient, histone replacement in sperm cells. As expected, Hst2 did not deacetylate acetyl-protamine even at high enzyme concentrations (Fig. 2), demonstrating that acetylated NH2 termini are not targets for this enzyme. However, protamine effectively outcompeted acetyl-pol-y-lysine for Hst2 activity (Fig. 3), suggesting the possibility that Hst2 can associate with any peptide even in the absence of lysine residues. Thus, we tested the ability of poly-l-arginine, poly-l-glutamate, and poly-l-asparagine to outcompete the acetyl-pol-y-lysine substrate and found that only poly-l-arginine efficiently inhibited Hst2 activity (Fig. 3). These results indicate that Hst2 preferentially binds positively charged proteins.

Interestingly, all of the compounds (poly-l-lysine, protamine, poly-l-arginine, histone peptides, and melittin), which we have shown to be substrates and/or inhibit Hst2 activity, are known to be disordered under our assay conditions (14). Circular dichroism analysis showed acetyl-pol-y-lysine also retains random coil structure (Fig. 4C). This led...
us to hypothesize that a disordered or flexible conformation may be key to substrate binding. To test this, we compared the ability of Hst2 to deacetylate native or denatured acetyl-cytochrome c. Cytochrome c is a globular protein containing many lysine residues that are within α-helices or immobile loops on the surface of the protein (15), which should be freely accessible for an enzyme to bind (Fig. 4C). Our results clearly showed that Hst2 did not deacetylate native acetyl-cytochrome c but did deacetylate the heat-denatured substrate (Fig. 4A). Likewise, RNase A, which was denatured by reduction and alkylation, but not native RNase A, inhibited Hst2 activity (Fig. 4B). Since RNase A is a globular protein with significant α and β content (16) (Fig. 4C), this suggests that Hst2 does not bind lysines within α or β structures. Circular dichroism confirmed that the acetylated native proteins were folded and had indeed lost their secondary structures upon denaturation (Fig. 4C). These results further support a complete lack of sequence specificity for this enzyme and clearly demonstrate the inability of this enzyme to deacetylate or bind structured proteins.

Lack of activity with acetyl-poly-D-lysine (Fig. 2) and its inability to inhibit Hst2 activity (Fig. 3) show that stereospecificity is also important. Thus, although the adjacent sequence is unimportant, the substrate side chains must be in a precise orientation when inserted into the active site.

Crystal structure data shows that the acetyl-H4 peptide binds to the

FIGURE 1. Hst2 shows no preference for acetylated histone peptides over other lysine-containing peptides. A, deacetylase assays of Hst2 (panel i) and Hos3 recombinant enzymes (panel ii) with 14C-acetylated substrates. Lane 1, [14C]acetate standard; lane 2, H2A peptide; lane 3, H2B peptide; lane 4, H3 peptide; lane 5, H4 peptide; lane 6, Melittin; lane 7, poly-L-lysine. The positions of the Hst2 product O-acetyl-ADP-ribose (OAADPR) and Hos3 product (acetate) are indicated. B, Lineweaver-Burk analyses of Hst2 reaction with 14C-acetylated substrates. Panel i, H2A peptide; panel ii, H2B peptide; panel iii, H3 peptide; panel iv, H4 peptide; panel v, Melittin; panel vi, poly-L-lysine. Specificity constant, kcat/Km (M⁻¹ min⁻¹) for each substrate is also indicated. C, deacetylase assays of purified TAP-tagged enzymes with [14C]Ac-H3 peptide (panel i) and [14C]Ac-poly-L-lysine (panel ii). Lane 1, Sir2-TAP; lane 2, Hst2-TAP; lane 3, Hos3-TAP; lane 4, no enzyme; lane 5, recombinant Hst2.
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Hst2 active site in an elongated manner (13). Likewise, acetyl-p53 peptide binds to Sir2 homologue AF2 in an extended conformation (17). We propose that an unstructured conformation is necessary to allow binding of the acetyl-lysine substrate in this fashion. Results with acetylcytocchrome c demonstrate that simple accessibility is insufficient for activity and suggest that the substrate must be free to conform to the enzyme active site. To examine this further, we tested the ability of Hst2 to deacetylate acetyl-urotensin II peptide either in its conformationally restrained cyclic (oxidized) form or free acyclic (reduced) form. We found that acetyl-lysine was much more efficiently deacetylated when the peptide conformation was unrestricted (in the free acyclic form) than when the acetyl-lysine was in a restrained loop (cyclic form) (Fig. 4D). Thus our results strongly suggest that the key to substrate binding by Hst2 is flexibility, rather than simple accessibility, provided by the disordered conformation of the substrate.

Similar conformational specificity has been demonstrated by cyclic-AMP-dependent protein kinase A, which also binds substrates in an extended conformation (18). Systematic analysis of protein kinase A phosphorylation sites within various substrates shows these sites to be preferentially contained within regions of predicted or known disorder (19). We have conducted a similar analysis of acetylated non-histone proteins, using available structural data and several web-based programs (Regional Order Neural Network (7), GlobPlot (8), and disEMBL (9)) (supplemental Table S1). It is well known that histone NH2-terminal tails protrude from the nucleosome core in a flexible conformation (20). Structural studies of p53 reveal that the COOH terminus, which contains the deacetylation sites, is disordered (21). Analysis of known Sir2 substrates, including Ku70, HIV Tat, E2F1, tubulin, and PGC-1α, suggests intrinsic disorder within the region of deacetylation and is verified by structural studies where available. In fact, intrinsic disorder surrounding the acetyl-lysine is predicted and structurally confirmed for many acetylated proteins for which the deacetylase is unknown. Our results suggest that these proteins, which include Importin α1, high mobility group proteins, and transcription factors MyoD, GATA1, and dTTF, are potential targets for Sir2 deacetylation and support a broad role for Sir2 proteins in a variety of cellular processes. Knowledge of this conformational specificity may therefore be useful in structure-based design of Sir2 inhibitors with potential for therapeutic application.

DISCUSSION

The lack of sequence specificity displayed by Hst2, Sir2, and SIRT1, taken together with the fact that Hst2 is the yeast member most closely related to the mammalian Sir2 proteins (22), suggests that conformational rather than sequence specificity is a general property of the Sir2 family. This is in clear contrast to the non-NAD-dependent HDACs such as Hos3, which display definite substrate specificity (2). The question of how Sir2 proteins achieve substrate specificity in vivo remains.

As with other modifying enzymes, one possibility is through recruitment of cofactor proteins that mediate the subcellular localization of the enzyme and interaction with its substrate. Notably, the eukaryotic Sir2 proteins commonly feature sequence-divergent NH2- and COOH-terminal regions that lie outside of the conserved catalytic core, which could mediate interactions with other proteins (22). This is exemplified by yeast Sir2, which achieves silencing at telomeric and mating-type loci through interaction with Sir3 and Sir4, and at rDNA loci through nucleolar localization facilitated by binding to Net1 (23). Although no in vivo substrates or binding partners for Hst2 have yet been identified, it too has unique NH2- and COOH-terminal extensions (22) that could mediate interactions with other proteins. It is likely that the cytosolic localization of Hst2 prevents this relatively abundant enzyme from interfering with the activity of the other less abundant Sir2 family members who function in the nucleus (22). A lack of sequence specificity would also explain the dominant negative effect that Hst2 overexpression has on telomeric silencing (22), allowing it to outcompete Sir2 for a common substrate (22).

Many proteins contain regions of intrinsic disorder or flexible structure, which are important to protein function. One example where acetylation within a disordered region mediates protein function is the interaction of p53 with cyclic AMP response element-binding protein (CBP). Upon DNA damage, p53 is acetylated at Lys382 within its disordered COOH terminus. The bromodomain of CBP binds the acetylated lysine inducing a conformational change in the p53 COOH terminus.
which allows residues surrounding Lys<sup>382</sup> to make specific contacts with the CBP bromodomain (24). Association with CBP promotes p53-mediated transcriptional activation (25). Acetyl-Lys<sup>382</sup> is also a substrate for SIRT1 (26), so CBP and SIRT1 may compete for binding to the same disordered region. This example shows how the presence of intrinsically disordered regions within proteins allows for the ability to structurally adapt and bind various partners (27). Thus, another factor in regulating Sir2 enzyme activity is the disordered state of the substrate itself, which could be affected by the presence of other post-translational modifications, and association with binding partners, which compete for the same site or induce changes in substrate conformation.

Equally important is the role of NAD. Since there are many other non-NAD-dependent deacetylases, and the expensive consumption of NAD seems biologically wasteful, it is a mystery as to why a family of NAD-dependent deacetylases exists. A tight link to NAD availability might regulate the activity of these enzymes. In the example given above, the competing activity of SIRT1, which would prevent the interaction between acetyl-p53 and CBP, inhibiting p53 function, is likely restricted by the dramatic reduction in NAD levels which occurs upon DNA damage (28). Thus the activity of Sir2 proteins is exquisitely controlled by the metabolic state of the cell, which would restrict the availability of NAD, its association with protein binding partners, and the conformational state of the substrate itself.

Our findings suggest that the traditional view of protein substrate
recognition by consensus sequence is inadequate. Recognition of disordered regions by protein kinase A and Sir2 enzymes suggests this may be a mechanism employed by many different enzymes and emphasizes the importance of natively unfolded regions in protein function (27).

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