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QUANTIFICATION OF ENDOGENOUS SIRTUIN METABOLITE *O*-ACETYL-ADP-RIBOSE (*O*AADPR)

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Abstract

Sirtuins are NAD^+-dependent deacetylases that mediate cellular processes such as lifespan extension and metabolic regulation. Sirtuins form a unique metabolite, 2′-*O*-acetyl-ADP-ribose (*O*AADPr), shown to block oocyte maturation, bind to chromatin-related proteins, and activate ion channels. Given the various sirtuin phenotypes, the potential of *O*AADPr as a signaling molecule is extensive. However, exploring of the biological roles of *O*AADPr has been hindered by the lack of *in vivo* evidence and a reliable method for quantification. Here, we provide the first direct evidence and quantification of cellular *O*AADPr. Compared to endogenous *O*AADPr levels (0.56 ± 0.13 μM) in wildtype *S. cerevisiae*, deletion of all five yeast sirtuins (Sir2, Hst1-4) yielded essentially no detectable *O*AADPr. The single deletion of Hst2 yielded 0.37 ± 0.12 μM *O*AADPr. Deletion of an enzyme, Ysa1, previously shown *in vitro* to hydrolyze *O*AADPr resulted in a significant increase (0.85 ± 0.24 μM) in *O*AADPr. Together, these data provide evidence that cellular levels of *O*AADPr are controlled by the action of sirtuins and can be modulated by the Nudix hydrolase Ysa1. Our methodology consisting of internal standard ^{13}C-*O*AADPr and LC-MS/MS analysis, displays excellent sensitivity and a linear dynamic range from 0.2 to 500 pmol. Moreover, extraction efficiencies were >75 %. This methodology is an essential tool in probing the biological roles of *O*AADPr, especially under conditions in which sirtuin phenotypes are well established.

Keywords

2′-*O*-acetyl-ADP-ribose; *O*AADPr; sirtuin; Sir2; Hst2; Ysa1

Introduction

Sirtuins, class III histone deacetylases (HDACs), are found in all kingdoms of life and have been implicated in a growing number of diverse cellular processes such as gene silencing [1; 2;3], lifespan extension [4;5;6;7], and direct metabolic regulation [8;9;10]. In contrast to class I and II HDACs, which generate free acetate [11], sirtuins require NAD^+ and subsequently generate a unique metabolite, 2′-*O*-acetyl-ADP-ribose (*O*AADPr) [12;13;14;15;16;17]. Though Sir2 homologs from yeast, *Drosophilia*, and humans have been shown to produce *O*AADPr *in vitro* [18], there is no experimental evidence of its existence or roles *in vivo*. To

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elucidate the biological roles of *O*AADPr and sirtuins, a method to identify and quantify endogenous *O*AADPr is crucial.

In yeast there are five sirtuins which may contribute to endogenous *O*AADPr, the founding member yeast silent information regulator 2 (ySir2) and Hst1 - 4 [2;3;13;19]. HST2 is of particular interest because it has been reported to be the most active yeast sirtuin [13] and has been extensively characterized [13;20;21;22;23;24;25]. Additionally, endogenous *O*AADPr may be degraded by a variety of enzymes such as Nudix hydrolases or esterases [18;26;27]. Nudix hydrolase YSA1 not only hydrolyzes *O*AADPr but also degrades its related metabolite ADPr [26].

Though many sirtuins have been well characterized with respect to their acetylated protein targets (e.g. histones, transcription factors, α-tubulin and metabolic enzymes), little is known about the sirtuin metabolite *O*AADPr. Initial studies by Borra et al. showed that microinjection of *O*AADPr can block oocyte maturation in starfish [18]. Additional *in vitro* studies demonstrated that *O*AADPr binds targets such as histone macroH2A1.1 [28], the SIR complex [29], and cation channel TRPM2 [30]. These *in vitro* studies suggest *O*AADPr may function as a signaling molecule or second messenger; however, there is a paucity of information that directly links cellular *O*AADPr with sirtuin functions. To explore the roles of sirtuins at the molecular level, it will be essential to establish a robust method to identify and quantify endogenous *O*AADPr.

Here we have developed the first method to detect and quantify endogenous *O*AADPr in biological extracts. First, the isotopically labeled internal standard, ^{13}C-*O*AADPr, was synthesized enzymatically using yeast sirtuin HST2 and a histone H3 peptide substrate. Second, a method for detection and quantification of *O*AADPr was developed using LC-MS/MS with HILIC chromatography coupled to Multiple Reaction Monitoring (MRM) MS/MS. This methodology has a lower limit of detection of 0.2 pmol and displays a linear response over three orders of magnitude from 0.2 to 500 pmol. The utility of this method is evidenced by the first direct verification of *O*AADPr in yeast. Furthermore, *O*AADPr was quantified in wild-type *S. cerevisiae* and compared to a strain lacking all sirtuins and to a strain lacking an *O*AADPr-consuming enzyme. .

Materials and Methods

Materials

Materials were purchased from Sigma-Aldrich (St. Louis, MO) or Fisher Scientific (Pittsburgh, PA) and were of the highest quality available, unless otherwise indicated. ^{13}C- and ^{12}C-acetyl-H3 peptides (TGGK(ac)APR), corresponding to histone H3 tail residues 11-17, were synthesized at the UW Madison Biotechnology Peptide Synthesis Facility using acetic acid-1-^{13}C (99 atom %) or acetic acid. NADase was obtained from Sigma-Aldrich. Yeast strains, *S. cerevisiae* BY4742, were obtained from Open Biosystems (Huntsville, AL).

Synthesis of ^{13}C-OAADPr and Native OAADPr

^{13}C-*O*AADPr and *O*AADPr (Figure 1) were synthesized enzymatically using yeast sirtuin HST2, following procedures previously described for *O*AADPr [18]. Briefly, the reaction consisted of 40 μM HST2, 0.8 mM NAD^+, 1.6 mM ^{13}C- or ^{12}C- acetyl-H3 peptide (TGGK (ac)APR) in 50 mM Tris, pH 8 and was purified over a small-pore Vydac C18 column using an acetonitrile gradient (A: 0.05 % (v/v) trifluoroacetic acid (TFA) in water, B: 0.02 % (v/v) TFA in acetonitrile). Identity and purity were confirmed by mass spectrometry at the UW Madison Human Proteomics Program, supported by the Wisconsin Partnership Fund for a Healthy Future.

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Yeast samples

Wild-type, *Δhst2*, and *Δysa1* BY4742 *S. cerevisiae* yeast were obtained from Open Biosystems (Huntsville, AL). The complete sirtuin deletion strain (*Δsir2Δhst1Δhst2Δhst3Δhst4*) was a generous gift from Brian Kennedy at University of Washington. This strain also harbored *Δfob1*. Yeast were grown in YPD media overnight to OD_{600} of 0.6. Cells were harvested by centrifugation (1500 g, 10 min, 4 °C), weighed, and 2 nmol of ^{13}C-*O*AADPr standard was added to each extract. Cell pellets were lysed by vortexing with glass-beads in 10 % (v/v) TFA, at maximum speed for 10 min at 4 °C [26]. Using this method, over 90 % of the yeast were lysed, as confirmed by microscopy. Next, cell debris was pelleted by centrifugation (17000 g, 60 min, 4 °C) and the supernatant immediately analyzed by LC-MS/MS, corresponding to approximately 4×10^7 cells per injection.

LC-MS/MS Analysis

Metabolites were analyzed at the UW Madison Biotechnology Center using an Agilent 1100 HPLC coupled to an ABI 3200 Q-trap mass spectrometer. Chromatography consisted of a HILIC column (Nest Group, 300Å 5μm polyhydroxyethyl A, 4.6 × 200 mm) eluted with a gradient of A: acetonitrile versus B: 10 mM ammonium acetate at 0.2 mL/min. The HPLC gradient was held at 20 % B for 15 min before ramping to 80 % B over 20 min. The gradient then ramped to 100 % B over 20 min where it was held for 5 min before ramping back to 20 % B over 5 min. The system was then equilibrated in 20 % B for 40 min before the next run.

The Q-trap mass spectrometer parameters were optimized using a direct infusion of 0.1 μM *O*AADPr in 50 % acetonitrile using a flow of 80 μL/min. The parameters were set as follows: curtain gas 20, probe temperature 350 °C, nebulizer gas 20, auxillary gas 5, interface heater on, collision gas medium, ion spray -4500V, declustering potential -55V, entrance potential -10V, collision energy -35V, and collision exit potential -4V.

The mass spectrometer was operated in negative MRM (multiple reaction monitoring) mode and monitored the following MRM transitions, 600.3/346.0 and 601.3/346.0, for *O*AADPr and ^{13}C-*O*AADPr, respectively.

Data Analysis

Data were manually analyzed using the ABI Analyst Software 1.4.1. All data were quantified and normalized by comparison to the ^{13}C-*O*AADPr internal standard.

Results

Synthesis of Internal Standard ^{13}C-OAADPr and Native OAADPr

To begin investigating the biological roles of *O*AADPr, we developed a specific, sensitive method to identify and quantify *O*AADPr from biological samples. The first step was to generate an internal standard, ^{13}C-*O*AADPr (Fig. 1). Using methodologies previously developed for *O*AADPr [18], ^{13}C-*O*AADPr and native *O*AADPr (Fig. 1) were synthesized enzymatically and purified by reverse-phase chromatography. Briefly, yeast sirtuin HST2 was used to deacetylate ^{13}C- or ^{12}C-acetyl-H3 peptide (TGGK(ac)APR) in the presence of NAD^+, generating the corresponding *O*AADPr standards. ^{13}C-*O*AADPr and *O*AADPr were then individually purified over a small-pore Vydac C18 column [18]. Purity and identity were confirmed by analytical HPLC (data not shown) and MS. Mass spectra demonstrating the isotopic purities of *O*AADPr and ^{13}C-*O*AADPr are shown in Figure 2.

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Development of LC-MS/MS Method

Next, the internal standard ^{13}C-*O*AADPr and native *O*AADPr were used to develop the LC-MS/MS methodology. Mass spectrometer parameters were optimized by directly infusing *O*AADPr as described in the Methods section. Under these conditions, *O*AADPr, ^{13}C-*O*AADPr, or related metabolite ADPr, all sharing the same major fragment ion of 346.0, had similar MRM intensities (data not shown). Using the internal standard ^{13}C-*O*AADPr, our methodology was shown to have excellent sensitivity, with a lower limit of detection of 0.2 pmol (Fig. 3). Additionally, the linear dynamic range extends over three orders of magnitude from 0.2 to 500 pmol (Fig. 3).

Direct Evidence of Endogenous OAADPr

Here we provide the first direct evidence of endogenous *O*AADPr (Fig. 4). As described in the Methods section, ^{13}C-*O*AADPr was spiked into yeast cell pellets prior to cell lysis and LC-MS/MS analysis. Figure 4 shows example MRM chromatograms with intensity versus time for *O*AADPr (solid line) within wild-type yeast (Fig. 4). There is a distinct signal from *O*AADPr (solid line, Fig. 4) that co-elutes exactly with the internal standard ^{13}C-*O*AADPr (dashed line, Fig. 4) in all samples types tested (Fig. 4 and data not shown). Control extracts without the internal standard ^{13}C-*O*AADPr display significant intensities from native *O*AADPr only (data not shown). The split peaks seen in Figure 4 are the 2′ and 3′ isomers of both *O*AADPr and ^{13}C-*O*AADPr. Though sirtuins enzymatically generate the 2′ isomer, this is rapidly transesterified to form an ~1:1 mixture of 2′: 3′ isomers [31;32].

Calculation of Extraction Efficiency

To calculate exact *O*AADPr concentrations, ^{13}C-*O*AADPr intensities were used to calculate extraction efficiencies and to normalize between biological replicates (Fig. 5). At least three separate yeast cultures (biological replicates) were analyzed for each experimental group. Figure 5 shows example MRM chromatograms for two biological replicates from wild-type yeast (Fig. 5). Not only do the chromatograms in Figure 5 display the same retention times and split peaks as shown in Figure 4, it is apparent that the extraction efficiency was similar for all of the biological replicates. The average extraction efficiencies of the ^{13}C-*O*AADPr standard were 77 ± 8 % for wild-type, 75 ± 16 % for *Δhst2*, and 130 ± 19 % for *Δsir2Δhst1Δhst2Δhst3Δhst4*, and 77 ± 4 % for *Δysa1*samples, with n = 3 - 6 and errors given as one standard deviation. Additionally, we have shown that the ratio of ^{12}C/^{13}C MRM intensities is reproducible across technical replicates, with an average variability of less than 3 % (data not shown).

Rigorous Quantification of OAADPr

Here we report the first cellular concentrations for the sirtuin metabolite *O*AADPr. *O*AADPr concentrations were determined by comparison to the ^{13}C-*O*AADPr standard curve (Fig. 3) and the samples were normalized for extraction efficiency using the internal standard ^{13}C-*O*AADPr.

It is important to address two minor points regarding the isotopic distributions between *O*AADPr and ^{13}C-*O*AADPr. The natural abundance ^{13}C-isotope within endogenous *O*AADPr contributes to the intensity of the ^{13}C-*O*AADPr standard. This contribution was theoretically and experimentally determined to be 19 % of the *O*AADPr intensity (Fig. 2A). Additionally, the small amount of *O*AADPr present in the ^{13}C-*O*AADPr standard was determined to be 2 % of the ^{13}C-*O*AADPr intensity (Fig. 2B). Under our conditions of approximately 5-fold more internal standard than endogenous *O*AADPr, the small amount of *O*AADPr arising from the ^{13}C-*O*AADPr standard was subtracted from the total *O*AADPr present in the sample.

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To calculate the *O*AADPr concentration in yeast cells, the number of cells were estimated by OD_{600} [33]. All results are from 3 - 6 biological replicates, with errors given as one standard deviation. The *O*AADPr concentration was 0.56 ± 0.13 μM in wild-type yeast (Fig. 6). In the sirtuin deletion strain (*Δhst2*), the *O*AADPr concentration was lower at 0.37 ± 0.12 μM (Fig. 6). Comparing wildtype to *Δhst2* strains, an unpaired t test yielded a p value of 0.064. Deletion of all five yeast sirtuins (*Δsir2Δhst1Δhst2Δhst3Δhst4*) resulted in no detectable *O*AADPr levels (-0.14 ± 0.33 μM, Fig. 6). Essentially, the levels were within the detection limit of the method. The p value of this data set as compared to the wild-type data is 0.0006. Deletion of YSA1, one of the enzymes implicated in *O*AADPr hydrolysis, resulted in a significant increase in *O*AADPr concentration (0.85 ± 0.24 μM). Comparing these samples to the wild-type control yielded a p value of 0.0456.

Discussion

Though NAD^+ and its related metabolite ADPr have been studied by a variety of methods, such as enzyme cycling assays [34;35;36], HPLC [37;38;39;40], MS [41], and LC-MS/MS [42], there has been a lack of information about the cellular levels of novel sirtuin metabolite *O*AADPr. Accordingly, we have developed an LC-MS/MS method to identify and quantify endogenous *O*AADPr.

In yeast, we found cellular levels of *O*AADPr to be 0.4 - 0.8 μM. For comparison, NAD^+ levels in yeast are estimated to be 1 - 3 mM (summarized in [43], also [44]). In mammalian cells, NAD^+ has been estimated to be ~370 μM, both in red blood cells and tissue culture cells [41; 42]. Moreover, ADP-ribose, one of the major products that may be formed from degradation of *O*AADPr or directly from NAD^+ hydrolysis, has been calculated to be 44 - 73 μM in tissue culture cells [40]. If indeed *O*AADPr functions as a second messenger or signaling molecule, levels in the low micromolar range are not unexpected. Steady-state levels of *O*AADPr *in vivo* will depend on the rate of synthesis by sirtuins, its slow degradation by spontaneous hydrolysis [18], and on the rates of utilization by a variety of endogenous *O*AADPr-metabolizing enzymes [18;26;27].

Significantly, we show sirtuin deletion (partial in *Δhst2*, and complete in *Δsir2Δhst1Δhst2Δhst3Δhst4*) or Nudix hydrolase deletion (*Δysa1*) affect *O*AADPr levels (Fig. 6). Hst2, a well-characterized yeast sirtuin [13;20;21;22;23;24;25] has been implicated in transcriptional regulation [20;25]. Yeast *Δhst2* samples show 30 % less *O*AADPr than the control samples (0.37 ± 0.12 μM versus 0.56 ± 0.13 μM, p = 0.064), under the conditions tested. Dramatically, deletion of all five yeast sirtuins results in *O*AADPr levels within the limits of detection (-0.14 ± 0.33 μM, Fig. 6). Comparison of the complete sirtuin deletion strain to wild-type yields a p-value of 0.0006, indicating a highly significant difference between the data sets.

Previously, we have described three different enzymatic activities in yeast extracts that are capable of utilizing *O*AADPr as a substrate. These enzyme activities include (1) an esterase that removes the acetyl-group to form ADPr, (2) a Nudix hydrolase that cleaves the pyrophosphate bond to yield AMP and acetylated phospho-ribose, and (3) an acetyltransferase that transfers the acetate to an unknown acceptor [18;26;27]. Interestingly, these same *O*AADPr-utilizing activities have been observed in mammalian tissue culture cells ([18;26; 27]). Here we have shown that deletion of one of these *O*AADPr-utilizing enzymes, Nudix hydrolase Ysa1, results in a significant increase (p value of 0.0456) in *O*AADPr concentration (0.85 ± 0.24 μM versus 0.56 ± 0.13 μM). These data provide direct evidence that Ysa1 can modulate the cellular levels of *O*AADPr.

As pointed out in the Results section, there is minor isotopic spillover between the endogenous *O*AADPr and the ^{13}C-*O*AADPr standard. This is easily taken into account to correct the

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observed levels from biological samples, however, future use of an isotopic standard labeled at multiple positions, such as $^{13}C_x$, $^{15}N_x$-*O*AADPr would alleviate this necessary correction.

In summary, we have developed the first methodology to detect and quantify the unique sirtuin metabolite *O*AADPr, using LC-MS/MS and internal standard ^{13}C-*O*AADPr. This system results in rigorous quantification by correlating chromatographic retention times, parent masses, and fragment masses for both *O*AADPr and the internal standard ^{13}C-*O*AADPr. Additionally, ^{13}C-*O*AADPr was used to normalize for extraction efficiency and to quantify *O*AADPr levels. The MRM methodology provides higher confidence in metabolite identification versus traditional enzyme cycling or UV-based HPLC methods, which are affected by contaminants or co-eluting species [42]. We have demonstrated that ^{13}C-*O*AADPr exactly co-elutes with native *O*AADPr with a lower limit of detection of 0.2 pmol and a linear range over 3 orders of magnitude from 0.2 to 500 pmol. Using our methodology, average extraction efficiencies were ≥ 80 %.

Most importantly, we have provided the first direct evidence of endogenous *O*AADPr in biological extracts. We also demonstrate that genetic manipulation of *O*AADPr producing and degrading enzymes regulate the cellular levels of *O*AADPr. Decreased *O*AADPr levels were observed in yeast sirtuin deletions strains, *Δhst2* and *Δsir2Δhst1Δhst2Δhst3Δhst4*, while increased *O*AADPr was observed with the *Δysa1* strain. We anticipate this methodology will be essential in determining the biological roles of *O*AADPr, studying its roles as a possible secondary messenger, and establishing the regulatory pathways that affect the production and degradation of *O*AADPr.

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Abbreviations

*O*AADPr, 2′-*O*-acetyl-ADP-ribose; LC-MS/MS, liquid chromatography coupled to tandem mass spectrometry; NAD^+, nicotinamide adenine dinucleotide; TFA, trifluoroacetic acid; MRM, multiple reaction monitoring; YPD, yeast extract peptone dextrose.

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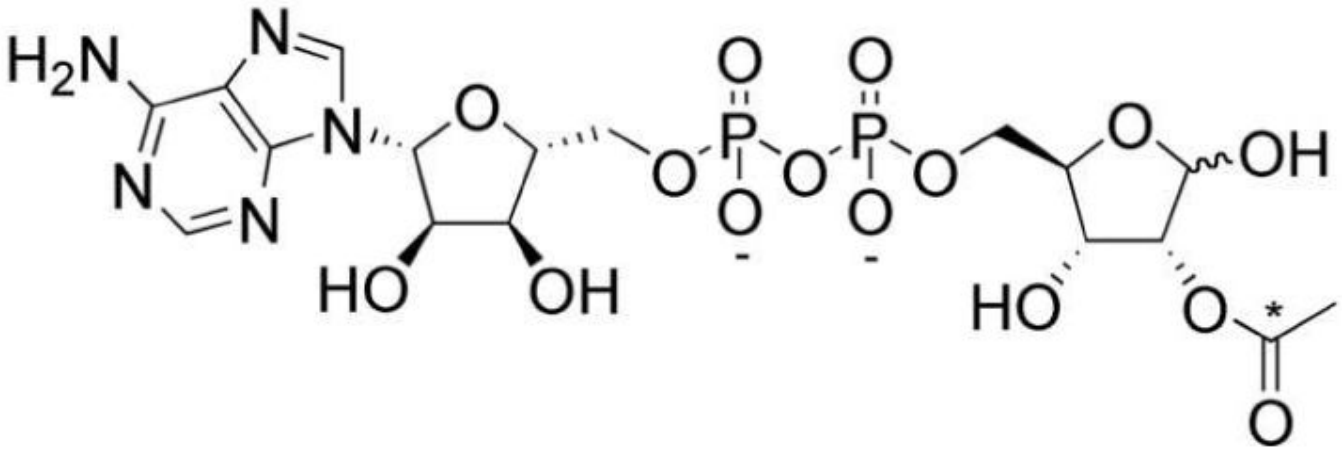

Figure 1.
Structure of Novel Sirtuin Metabolite *O*AADPr, 2′-*O*-acetyl-ADP-ribose. The ^{13}C carbon in internal standard ^{13}C-*O*AADPr is indicated by an asterisk.

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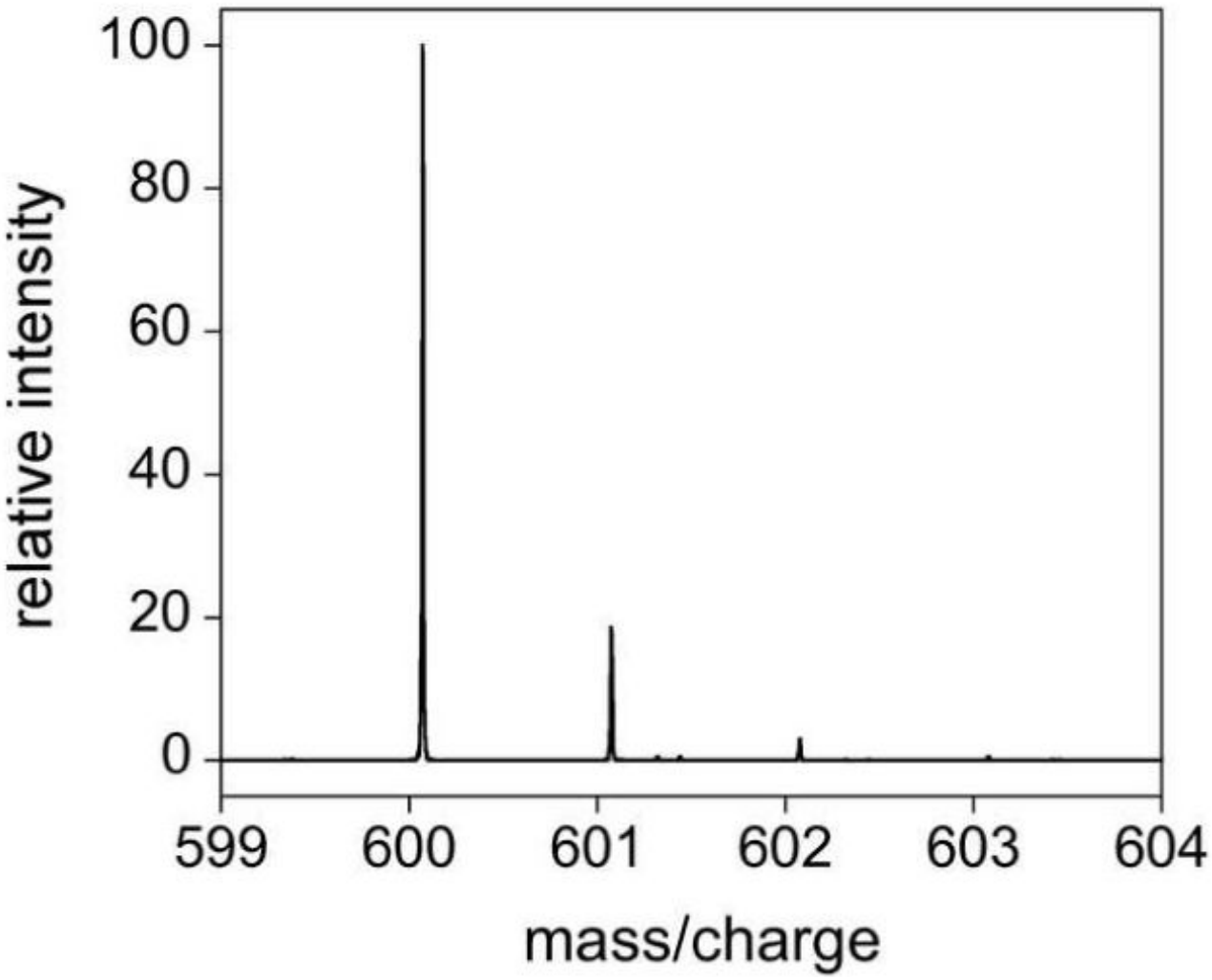

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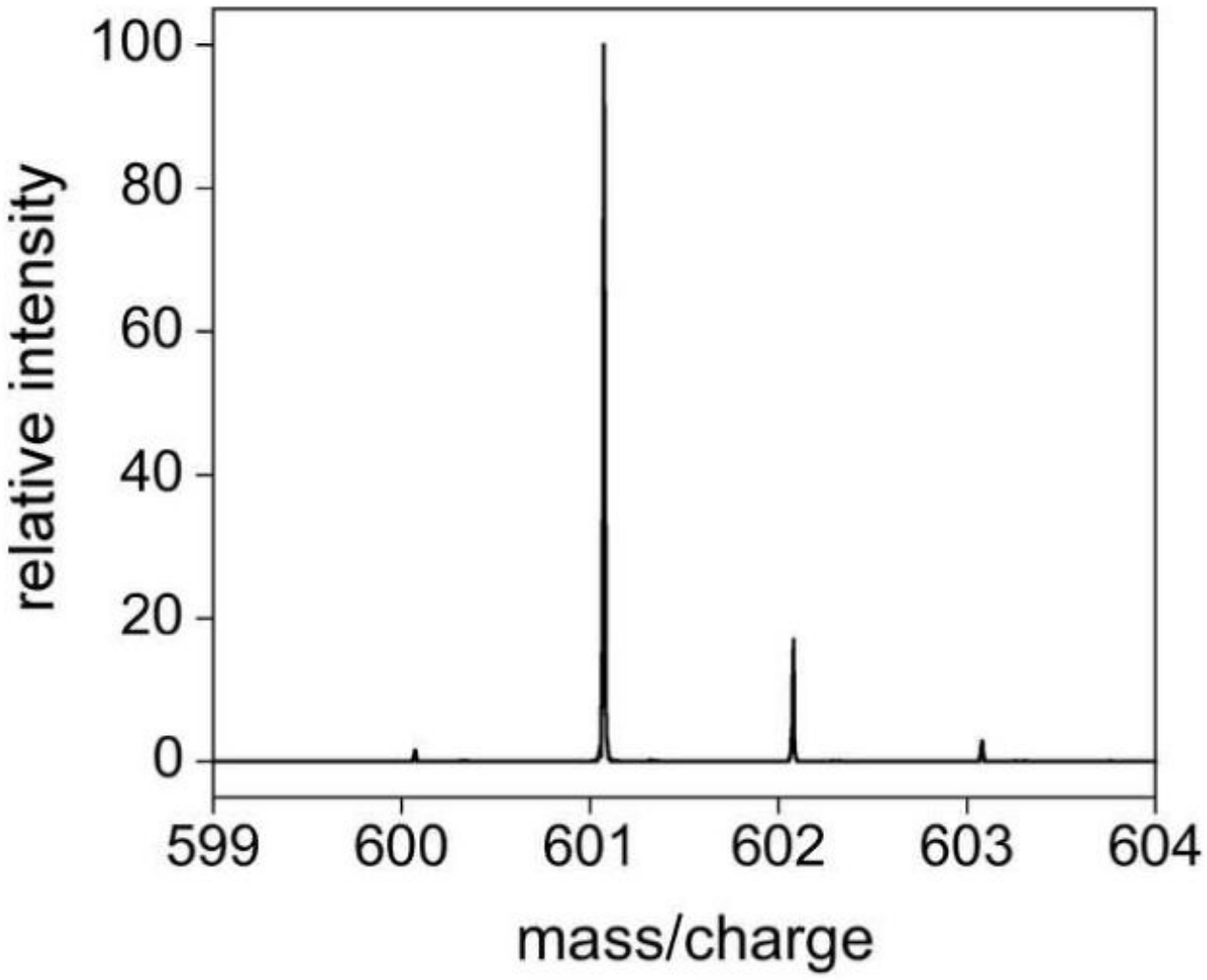

Figure 2.

Mass spectra of sirtuin metabolite *O*AADPr and internal standard ^{13}C-*O*AADPr. Mass spectra were acquired using 10 μM of A) *O*AADPr (m/z 600.072) and B) ^{13}C-*O*AADPr (m/z 601.075) in 50 % acetonitrile, using a TriVersa NanoMate coupled to a Thermo Scientific LTQ-FT operated in negative mode.

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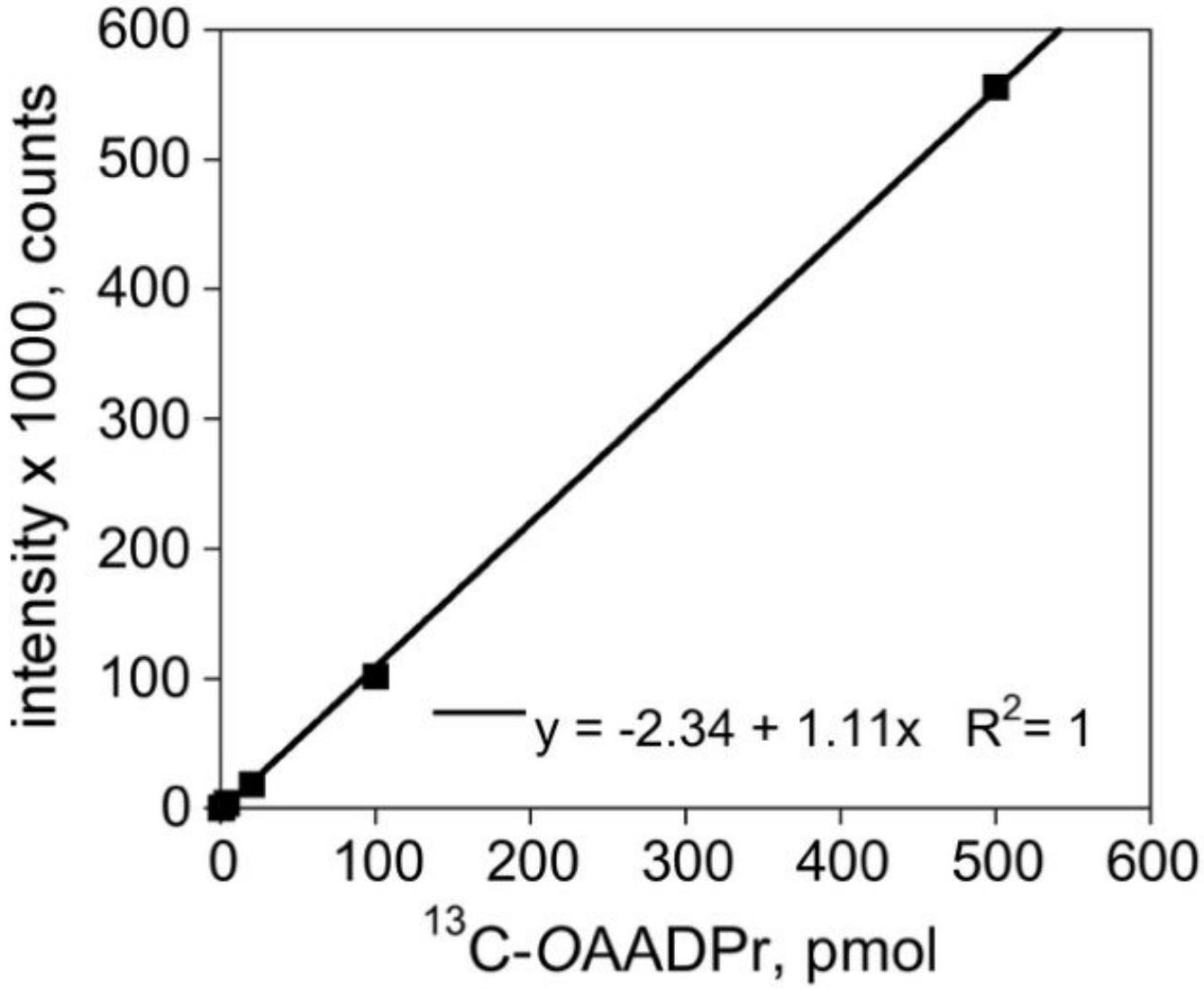

Figure 3.
Detection Limits of *O*AADPr LC-MS/MS Methodology. MRM intensity of internal standard ^{13}C-*O*AADPr is linear over 3 orders of magnitude from 0.2 to 500 pmol, using the LC-MS/MS methodology. The equation of the standard curve is shown inset.

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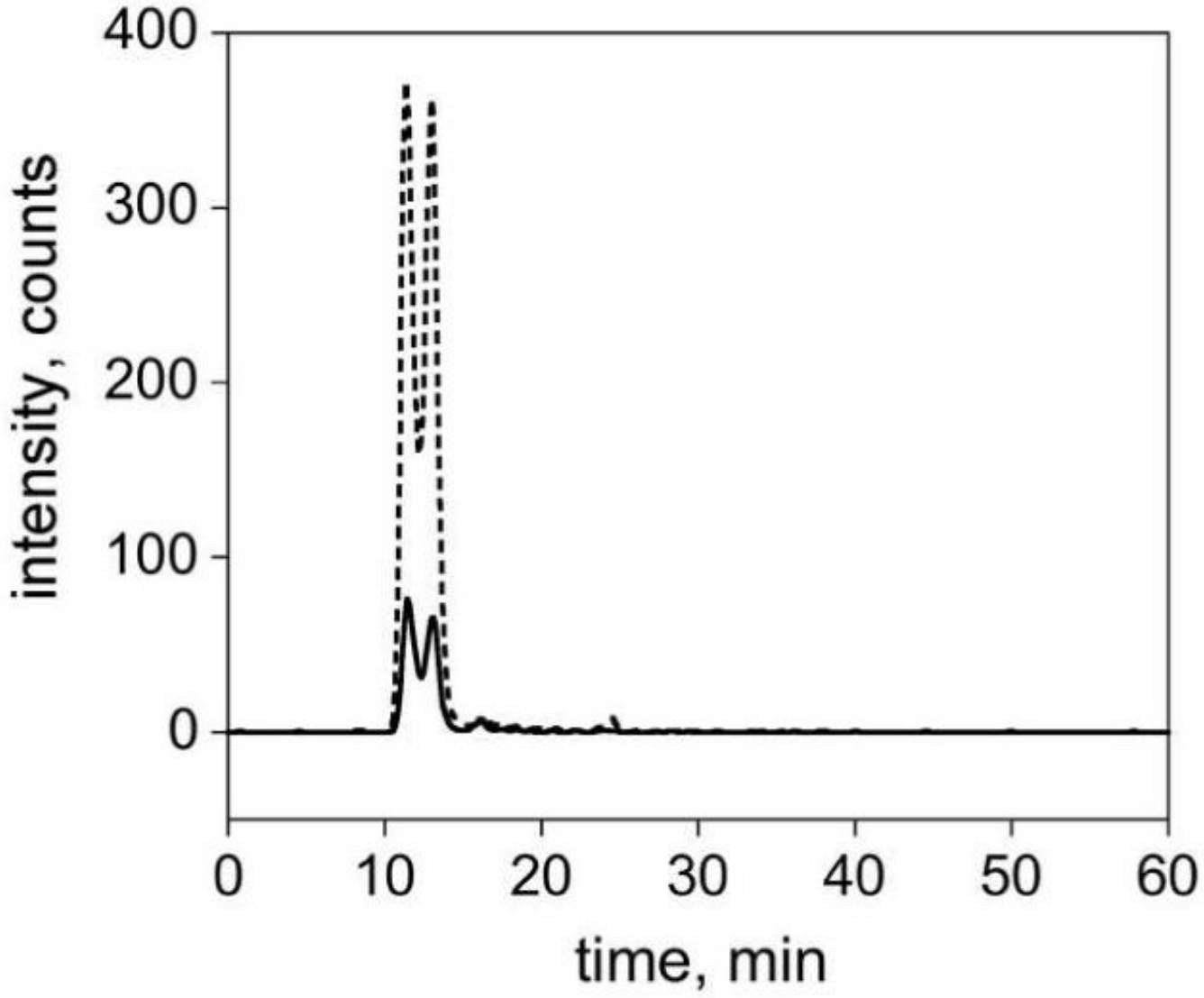

Figure 4.
Direct Evidence of Endogenous *O*AADPr. Example MRM chromatograms show intensity versus time for native *O*AADPr (solid line) and ^{13}C-*O*AADPr standard (dashed line) and in wild-type *S. cerevisiae* extracts.

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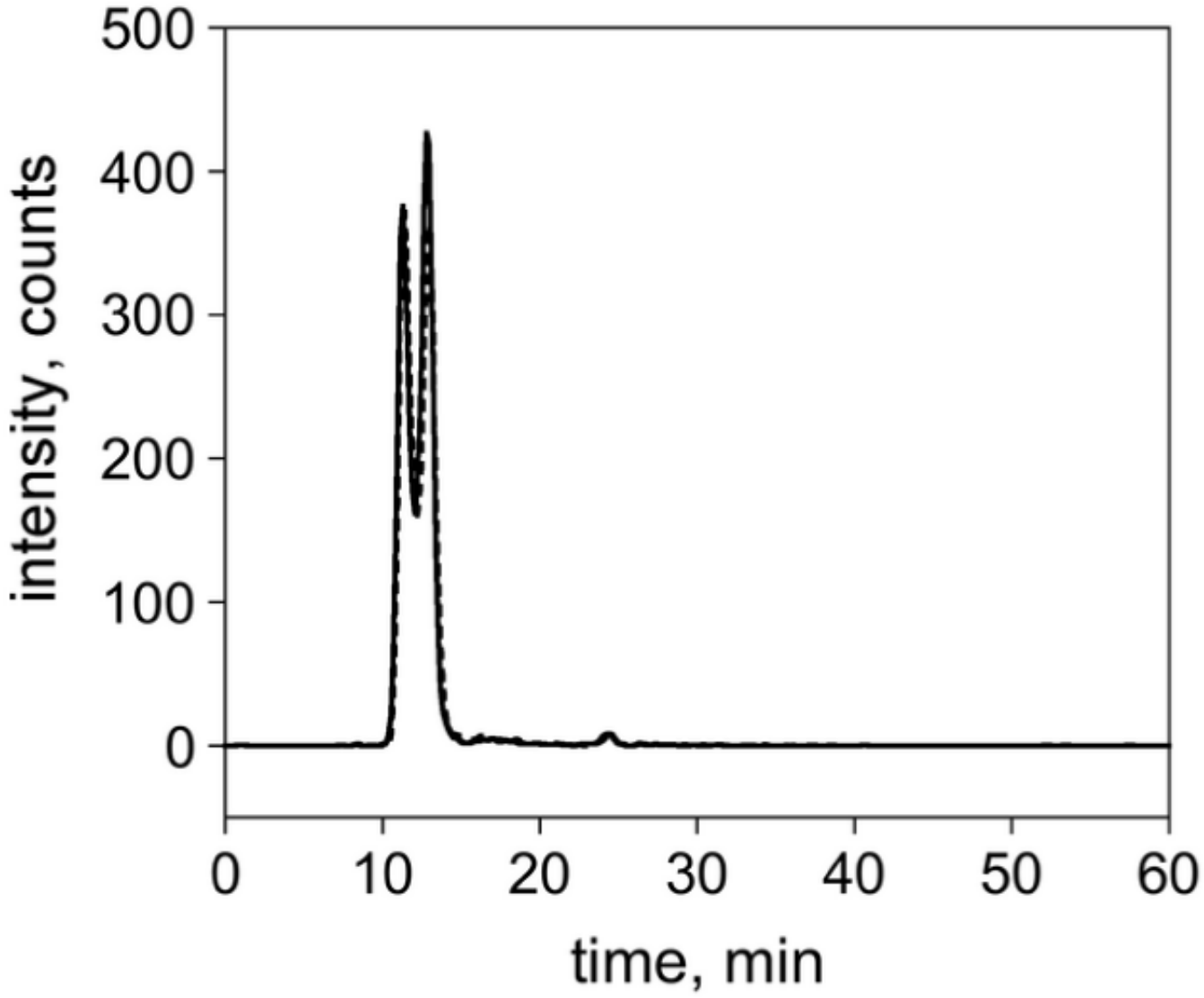

Figure 5.
Direct Comparison of Biological Extracts using Internal Standard ^{13}C-*O*AADPr. Example MRM chromatograms show intensity versus time for ^{13}C-*O*AADPr internal standard within two different biological replicates in wild-type *S. cerevisiae* extracts.

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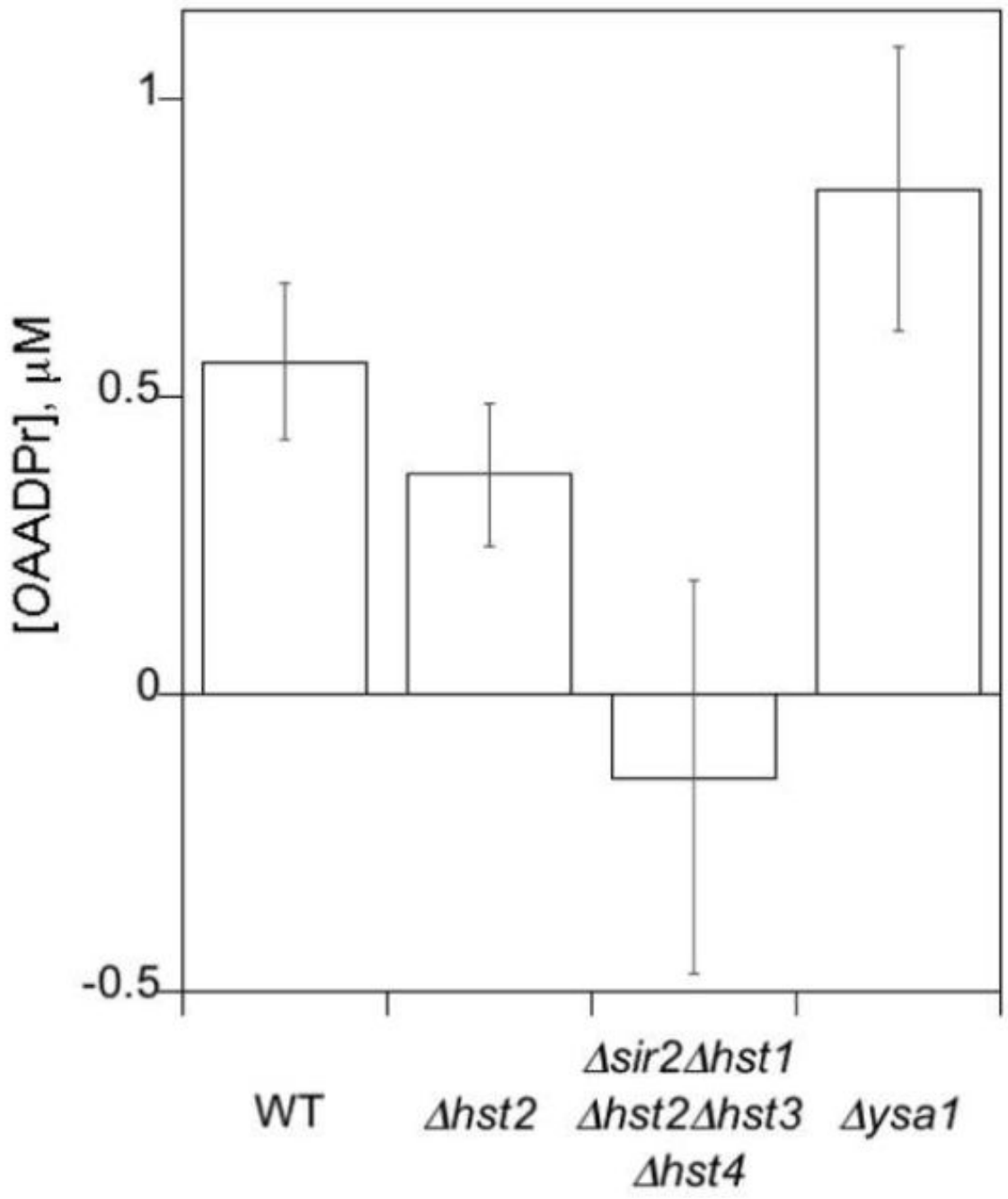

Figure 6.

Endogenous *O*AADPr Concentrations in BY4742 *S. cerevisiae*. *O*AADPr concentrations in wild-type (0.56 ± 0.13 μM), *Δhst2* (0.37 ± 0.12 μM), complete sirtuin deletion strain *Δsir2Δhst1Δhst2Δhst3Δhst4* (-0.14 ± 0.33 μM), and Nudix hydrolase deletion strain *Δysa1* (0.85 ± 0.24 μM) were determined as described in the text. All values are averages from 3 - 6 biological replicates, with errors given as one standard deviation.