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DECHEMA e.V.
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Göttingen, 30.09.2014

Betreff: Max-Buchner Forschungsbericht #3338

Sehr geehrte Frau Frömel,

Herzlichen Dank für die finanzielle Unterstützung unseres Forschungsprojekts. Anbei finden Sie meinen Forschungsbericht. Die Thematik des Projektes hat sich kurzfristig etwas geändert. Dies ist darauf zurückzuführen, dass es sich hier um Vorarbeiten für einen geplanten SFB handelt. Das ursprünglich geplante Thema "Orthogonales Heterochromatin" bot zu wenige Vernetzungsmöglichkeiten. Der Vorantrag für den SFB wurde im vergangenen Jahr eingereicht, scheiterte jedoch knapp in der Auswahl für die Aufforderung zur Einreichung eines Vollantrags (Koordinator: Prof. Dr. G. Braus, Göttingen). Derzeit berät das Kollegium die Strategie für eine erneute Antragstellung.
Die im Bericht dargestellten Forschungsergebnisse sind vollständig unveröffentlicht und wurden bislang nur in Form eines Posters bei der DECHEMA Tagung "Trends in Metabolomics" (03.-05.06.2014, Frankfurt am Main) vorgestellt. Wir beabsichtigen, die Daten im Verlauf des kommenden Jahres zu veröffentlichen. Auch eine Patentierung des Enzyms, das in diesem Projekt entwickelt wurde, erscheint mir möglich. Ich möchte Sie deshalb bitten, von einer Veröffentlichung der Langfassung meines Berichts vor Ende nächsten Jahres abzusehen.

Mit freundlichen Grüßen,

Heinz Neumann

Anlagen:
Abstract für das Internet,
Kurz- und Langfassung des Forschungsberichts

Abstract für das Internet:

Sirtuins are conserved lysine deacetylases that produce a unique by-product, O-acetyl-ADP-ribose (OAADPR). We have engineered an enzyme producing OAADPR from cell endogenous metabolites, facilitating experiments addressing its physiological role. We demonstrate the efficient *in vitro* production of this metabolite by OAADPR synthase and analyse its impact on the physiology of *E. coli* and baker's yeast. We expect that this tool will help elucidating the physiological role of OAADPR.

# Engineering of an O-acetyl-ADP-ribose synthase


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***Max-Buchner Research Report (#3338)***



## Abstract

Sirtuins are lysine deacetylases conserved throughout all three domains of life. They catalyze a reaction that produces a unique by-product, O-acetyl-ADP-ribose (OAADPR). The physiological role of this metabolite is strongly debated. Biochemical studies have ascribed functions in gene expression and the formation of silent heterochromatin in yeast. However, limitations in the ability to manipulate *in vivo* levels of OAADPR have hampered any experiments addressing the *in vivo* validation of such models. To circumvent this problem we have begun to engineer an enzyme that is able to produce OAADPR from cell endogenous metabolites. We demonstrate the efficient *in vitro* production this metabolite by OAADPR synthase and characterize its enzymatic properties. The maximal rate of this enzyme is similar to turnover rates reported for other Sirtuins under substrate saturating conditions. In *E. coli*, OAADPR synthase is highly active, depleting $NAD^+$ levels when overexpressed. At low levels of expression, microarray analyses did not reveal an impact on gene expression in *E. coli* besides the upregulation of $NAD^+$ biosynthesis pathways. Currently, we are exploring the influence of OAADPR synthase on the formation and spreading of silent heterochromatin in baker's yeast. We have developed methods to target the enzyme to defined sites on the genome and are evaluating its influence on the expression of nearby genes. We expect that the tools created in this study will help elucidating the physiological role of OAADPR and may provide a means to manipulate the formation of silent chromatin in yeast and other fungi.


## Introduction

Sirtuins are a phylogenetically ancient class of lysine deacetylases (KDACs) found in all three kingdoms of life with connections to cancer development, aging and metabolic disorders. Sirtuins utilize the ubiquitous redox cofactor $NAD^+$ as a stoichiometric co-substrate (1), cleaving it in nicotinamide (NAM) and O-acetyl-ADP ribose (OAADPR) (2-4). Why these enzymes couple the deacetylation of their substrate to the cleavage of a high-energy bond is mysterious since KDACs of class I, II and IV achieve them same using water instead. This led to the postulation that OAADPR acts as a signalling molecule in the regulation of metabolic flux, gene expression, cellular redox processes and ageing, cell cycle control and apoptosis (5). To date, however, technical limitations have prevented a detailed analysis of the physiological functions of this metabolite. The first indication of

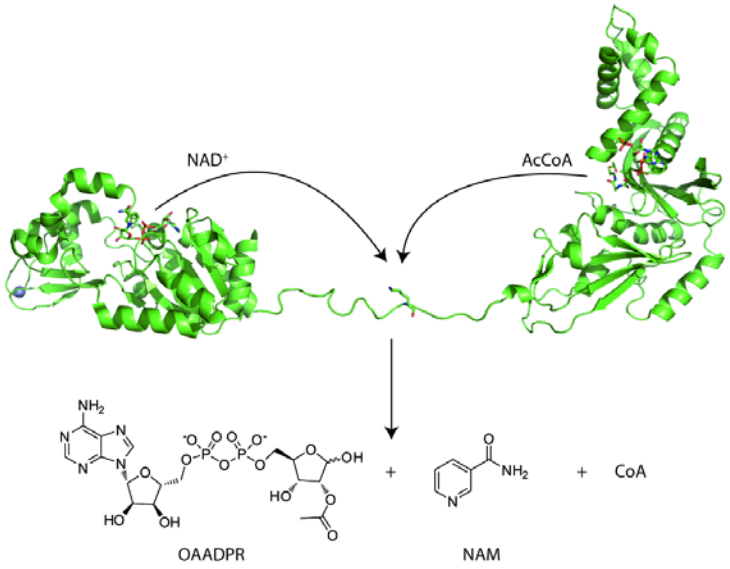


**Figure 1: Design of an O-Acetyl-ADP-ribose synthase (OAADPRS). Three functional parts are fused in a single open reading frame encoding a Sirtuin deacetylase, a lysine acetyltransferase and a peptide linker that serves as a mutual substrate.**

a pivotal role of OAADPR in cell cycle regulation and developmental processes was obtained by injecting starfish oocytes and blastomeres with purified OAADPR, which blocks maturation and cell cycle progression, respectively (6). While this suggests a role in cell cycle regulation, the mechanistic underpinning of this effect has so far remained elusive. In budding yeast, OAADPR may contribute to the formation of heterochromatin-like structures that repress transcription by enhancing the loading of the Sir2/3/4 silencing complex onto chromatin (7-9) *via* binding to a catalytically inactive AAA+ domain of Sir3. OAADPR also binds to a splice variant of the Macro domain containing histone 2A variant MacroH2A, which is highly enriched in heterochromatin regions (10). Hence, lysine deacetylation might indirectly regulate gene expression *via* binding of the metabolite to specific sensor domains, like macro or AAA+ domains, on DNA binding proteins. Furthermore, the NUDIX homology domain of the mammalian cation channel Trpm2 binds OAADPR or ADP-ribose to control channel gating and thereby apoptosis (11). Finally, elevated levels of OAADPR have been shown to provide increased resistance to oxidative stress and to control cellular respiration (12). Unsurprisingly, OAADPR levels are tightly controlled, and several classes of degrading enzymes have been identified. NUDIX hydrolases, such as Ysa1 and NudT5, cleave the pyrophosphate bond (13), while ADP-ribosyl hydrolases, e.g. ARH3, remove the acetyl group of OAADPR (14). Furthermore, a cytosolic esterase activity has been detected that hydrolyses the acetyl group of OAADPR and a nuclear acetyltransferase that uses it as an activated form of acetate (13).

All insights into the biological role of OAADPR so far have been obtained from *in vitro* experiments or from organisms with deletions in genes involved in the turnover of the metabolite. The cellular concentration of the metabolite cannot be controlled from outside because of its inability to permeate the plasma membrane. It is therefore impossible to evaluate these *in vitro* findings by genetic or cell biological experiments. A system that generates the metabolite *in situ*, ideally localised to specific sites within the cell, would circumvent these technical problems and facilitate the design of experiments to address the *in vivo* function of OAADPR. Unfortunately, an enzyme that generates the metabolite constitutively has not been identified yet and probably does not exist in nature. However, it might be possible to assemble such an enzyme from individual components in an approach that employs the principles of tethered catalysis and protein engineering.

Here we create and characterize an OAADPR synthase from individual enzymatic activities by directed design and show that this enzyme produces the metabolite *in vivo*. This approach allowed us for the first time to investigate its impact on the physiology of yeast cells.

## Results

The individual parts required to build an OAADPR synthase can be found in nature. Under physiological conditions OAADPR is produced by the sequential action of lysine acetyltransferases (KATs) and class III lysine deacetylases, sirtuins, on an acetylation substrate. If these parts were engineered to reside within the same molecule or complex, oriented correctly, the KAT could mediate the transfer of an acetyl group from acetyl-CoA to the target lysine. Subsequently, the sirtuin would remove this modification by cleaving the amide bond with $NAD^+$, generating the metabolite (Figure 1). To create an enzyme that catalyzes the formation of OAADPR from $NAD^+$ and acetyl-CoA we first explored the individual parts provided by nature. Sirtuins are conserved in all organisms examined from bacteria (1 homologue), archea (1 homologue) and yeast (5 homologues) to flies (5 homologues) and man (7 homologues). The structures of several Sir2 homologues have been solved, revealing a conserved two-domain architecture that combines a larger $NAD^+$-binding open α/β, Rossmann-fold domain and a smaller, roofing $Zn^{2+}$-binding domain. The core-enzymes are monomeric and bind the substrate peptide in a cleft formed between the two domains. Substrate selectivity is rather limited, especially for isolated core-enzymes (15-16). This is reflected by the fact that most enzymes bind their substrate peptides via backbone interactions and show a limited extend of side chain burial. Selectivity for certain lysine residues *in vivo* is probably mediated by surrounding domains, additional subunits or targeting proteins. We decided to concentrate on the bacterial sirtuin CobB. The protein has been crystallized and is stably expressed in its native environment. As we intend to investigate the functions of OAADPR in eukaryotic cells, it might be an advantage to employ this bacterial homologue since it is unlikely to find specific interaction partners or substrates in the foreign host.

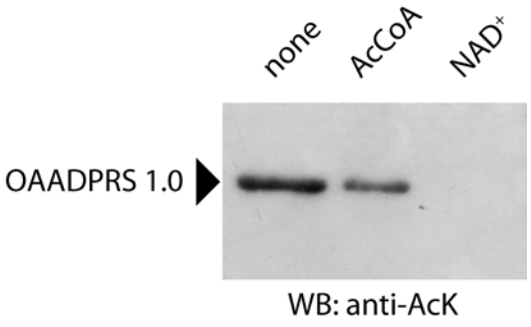


**Figure 2: OAADPRS 1.0 from *E. coli* is acetylated in vivo and self-deacetylates in the presence of $NAD^+$. Purified OAADRS 1.0 was incubated in the presence of buffer, AcCoA or $NAD^+$ and analysed by SDS-PAGE and Western blot.**

There is a wide choice of different KAT enzymes. We initially focussed on yeast variants Esa1, Gcn5, Hat1 and Rtt109. These proteins have been crystallized in monomeric form, indicating that they express functionally in *E. coli*. The best-known substrates for these KATs are histone tails. Histone tails are unstructured, positively-charged peptides and therefore ideally suited to form a linker between the two types of enzymes. We generated a set of constructs that were composed of an N-terminal $His_6$-tag followed by the catalytic core of CobB (aa 40-279). Downstream of CobB we fused the coding

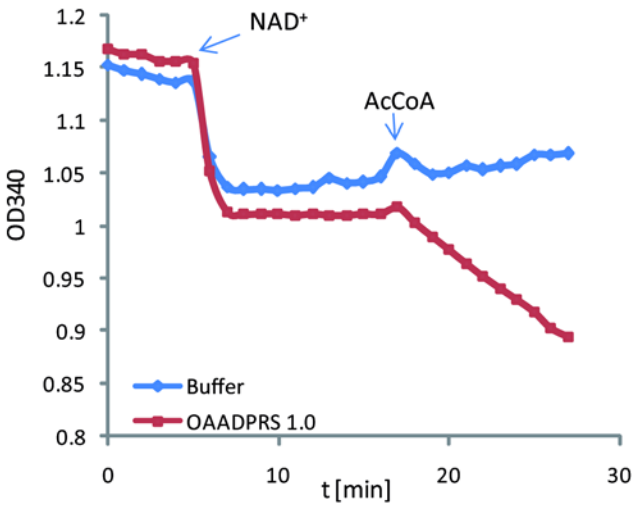


**Figure 3: Robust turnover of $NAD^+$ by OAADPRS 1.0. A coupled enzymatic assay (converting $NAD^+$ to nicotinamide and further to nicotinic acid and ammonia, which is finally coupled to the consumption of NADPH by glutamate dehydrogenase) shows sirtuin activity only upon addition of both substrates. Absorbance of NADPH at 340 nm is followed over time at 25°C.**

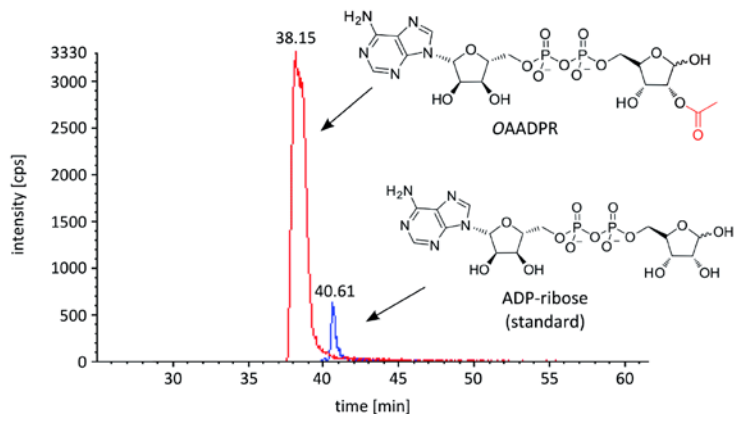


**Figure 4: Identification of OAADPR produced by OAADPRS in vitro by UPLC-MS. Reaction products of the coupled enzymatic reaction were analysed by UPLC-MS.**

sequence of either the *X. laevis* H3 (aa 1-56) or the *D. melanogaster* H4 N-terminal tail (aa 1-20). The C-terminus of the construct formed the catalytic core of either Esa1 (aa 160-435), Gcn5 (aa 95-262), Hat1 (aa 1-320) or Rtt109 (aa 1-436). These eight construct were initially screened for stable expression in *E. coli*. Here the constructs containing Hat1 had far superior properties compared to the others (data not shown). We isolated CobB-H3-Hat1 and CobB-H4-Hat1 from *E. coli* cells and investigated their acetylation status after incubation with acetyl-CoA or $NAD^+$. We observed that CobB-H4-Hat1 was acetylated under all conditions, implying that the substrate peptide was accessible for Hat1 but not for CobB. Vice versa, the CobB-H3-Hat1 construct showed only very weak acetylation after incubation with acetyl-CoA that was sensitive to subsequent incubation with $NAD^+$. This indicated that the H3 peptide was not a good substrate for Hat1 (according to published observations Hat1 specifically acetylates K5 and K12 on free histone H4). We therefore created a chimeric protein in which residues 36-56 of H3 in CobB-H3-Hat1 are replaced by the consensus sequence of Hat1 in H4 (aa 8-16). This protein was almost quantitatively acetylated after purification since the level of acetylation was not significantly increased by treatment with acetyl-CoA (Figure 2). Incubation with $NAD^+$, however, removed the acetylation beyond detection by anti-acetyllysine antibodies. This indicates that this construct is able to cycle between an acetylated and an unacetylated state and should therefore be able to constitutively produce OAADPR. We purified the protein to near homogeneity in two chromatography steps and tested the performance of this construct in a continuous sirtuin assay (17). In this assay the nicotinamide produced during the deacetylation reaction is further converted by a nicotinamidase into nicotinic acid and ammonia. The latter is a co-substrate of glutamate dehydrogenase, which produces glutamate from α-ketoglutarate and ammonia under consumption of NADPH. The decline in absorbance from NADPH at 340 nm is a direct measure of sirtuin activity. Upon addition of both substrates, acetyl-CoA and $NAD^+$, we observed a robust turnover in the presence of the engineered enzyme (Figure 3). Omitting either substrate or mutating the substrate lysine (a K12G mutation in the H4 recognition peptide) abolished the activity, demonstrating that the reaction is on pathway. Analysis of the reaction products by UPLC-MS confirmed the formation of OAADPR (Figure 4). We calculated the turnover number under saturating substrate concentrations. The enzyme consumes 0.046 ± 0.0014 µmol substrates / s / µmol enzyme, which is close to the value of 0.06 $s^{-1}$ published for the human sirtuin homologue SirT1 (17). In order to optimize the enzyme's activity we created variants with altered linker length between the substrate lysine and CobB. The activity of a variant with a linker shortened by 5 amino acids (OAADPRS 1.1) was increased 30% relative to progenitor enzyme (Figure 5). Removing further 5 amino acids (OAADPRS 1.2) significantly reduced the activity indicating that the linker length was too short for the substrate lysine to efficiently reach the active site of CobB. We therefore decided to use the former variant, which we refer to as OAADPR synthase or OAADPRS from here on, for further experiments.

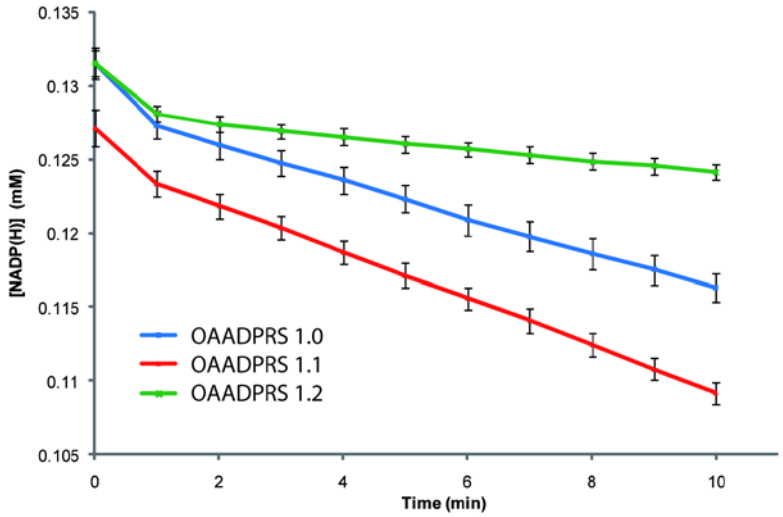


**Figure 5: Optimization of the enzymatic activity of OAADPRS. Variants of OAADPRS with different linker lengths containing the substrate lysine were assayed in the coupled enzymatic Sirtuin assay. Measured turnover rates: OAADPRS 1.0: 0.046 $s^{-1}$; OAADPRS 1.1: 0.059 $s^{-1}$; OAADPRS 1.2: 0.016 $s^{-1}$.**

Next, we analysed the kinetic parameters of OAADPRS by the sirtuin assay. We measured $K_M$ values for $NAD^+$ and acetyl-CoA of 21.0 ± 1.8 µM and 3.4 ± 0.3 µM, respectively, which is in the expected range for sirtuins and KATs. Additionally, the turnover number ($k_{cat}$) of the enzyme was comparable to published values for sirtuins, indicating that the enzymatic properties of the individual parts are not disturbed and that the sirtuin is working almost under substrate saturating conditions in the fusion construct. The kinetic parameters of OAADPRS also indicate that the enzyme will work at maximal rate *in vivo* since typical cellular concentrations of substrate molecules are at least 100 fold higher than $K_M$ (18).

Next, we analysed the effect of OAADPRS expression on the growth rate of *E. coli* (Figure 6). While an OAADPRS enzyme with a mutation of the substrate lysine did not reduce the growth rate of a control strain containing an empty plasmid, cells expressing the active enzyme stalled growth immediately after induction of protein expression. To identify the source of this effect, we analysed the levels of NAD+ present in these cells. Already at low levels of expression (which do not affect growth rates), $NAD^+$ levels were significantly reduced, indicating that the OAADPRS enzyme is highly active. The consumption of an important co-enzyme likely explains the growth defects of cells that overexpress OAADPRS. We have begun to explore possible impacts of OAADPRS on gene expression in *E. coli*. Microarray analysis of mRNA levels of all known open reading frames did not reveal any reproducible changes upon induction of OAADPRS expression besides genes with known roles in $NAD^+$ biosynthesis.

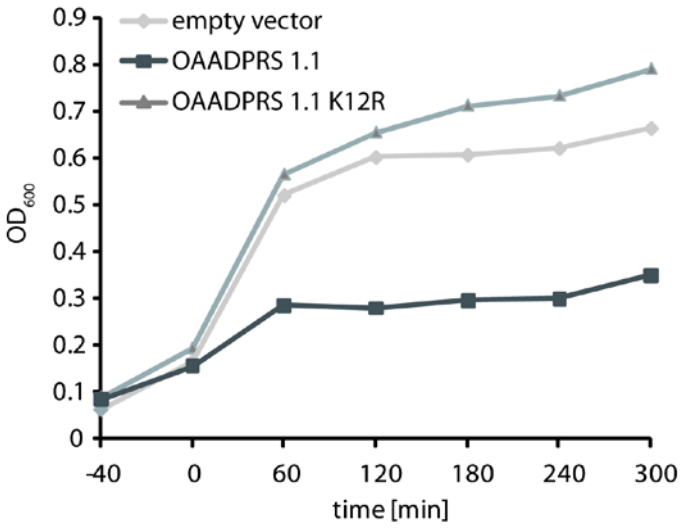


**Figure 6: OAADPRS 1.1 expression results in severe growth delay in E. coli. Growth rates of E. coli BL21 DE3 cells expressing the indicated constructs from a T7 promoter were measured in LB medium at 37°C.**

## Outlook

We have begun to explore the role of OAADPR in eukaryotic cells, starting with baker's yeast. Here, we are addressing the question whether OAADPR promotes the spreading of silent chromatin by stabilizing the association of the Sir complex with chromatin. Therefore, we have established the expression and targeting of OAADPRS to defined loci on the *S. cerevisiae* genome. We plan to use chromatin immunoprecipitation to investigate the impact of the presence of the enzyme on Sir protein levels close to the binding site of OAADPRS. Furthermore, we will analyse gene expression levels in the presence or absence of the enzyme. These experiments should help us to clarify whether OAADPR has any of the ascribed *in vivo* functions and may allow us to create tools for the manipulation of silent chromatin in yeasts and other fungi.

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