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Activity of α-Aminoadipate Reductase Depends on the N-Terminally Extending Domain

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L-α-Aminoadipic acid reductases catalyze the ATP- and NADPH-dependent reduction of L-α-aminoadipic acid to the corresponding 6-semialdehyde during fungal L-lysine biosynthesis. These reductases resemble peptide synthetases with regard to their multidomain composition but feature a unique domain of elusive function—now referred to as an adenylation activating (ADA) domain—that extends the reductase N-terminally. Truncated enzymes based on NPS3, the L-α-aminoadipic acid reductase of the basidiomycete *Ceriporiopsis subvermispora*, lacking the ADA domain either partially or entirely were tested for activity in vitro, together with an ADA-adenylation didomain and the ADA domainless adenylation domain. We provide evidence that the ADA domain is required for substrate adenylation: that is, the initial step of the catalytic turnover. Our biochemical data are supported by in silico modeling that identified the ADA domain as a partial peptide synthetase condensation domain.

In the context of the biosynthesis of the proteinogenic α-amino acid L-lysine, it is a remarkable feature that fungi evolved a distinct metabolic route that is entirely unrelated to the bacterial and plant pathway.[1] Fungi use α-ketoglutaric acid and acetyl-CoA to synthesize L-lysine de novo in eight steps (Scheme 1).[2] One remarkable event in this biosynthetic route is the ATP- and NADPH-dependent reduction of the intermediate L-α-aminoadipic acid into L-α-aminoadipate 6-semialdehyde by a multifunctional aminoacyl-adenylate-forming reductase, prototypically represented by Lys2 of *Penicillium chrysogenum* and *Saccharomyces cerevisiae* (Scheme 1).[3]

Reminiscent of nonribosomal peptide synthetases (NRPSs), Lys2 reductases are organized as multidomain enzymes, each including an adenylation (A) domain, a thiolation (T) domain, which serves for covalent binding of the substrate, and a C-terminal short-chain reductase (R) domain (Figure 1). Notably, the A domain of a Lys2 enzyme is N-terminally extended by an additional sequence of about 250 aa, the function of which has remained obscure.[4] Hereafter, these domains are referred to as adenylation activating (ADA) domains. They show some sequence similarity to portions of NRPS condensation domains; however, they do not include their active site motif $HH(X)_3DG$.[5]

Other adenylating reductases involved in fungal metabolism have recently been described and include *Aspergillus flavus* L-tyrosine reductases LnaA and LnbA, *Ceriporiopsis subvermispora* L-serine reductase NPS1, and an orsellinic acid reductase of *Aspergillus terreus*, encoded by the ATEG_03630 gene.[6] They show—like Lys2—the A-T-R domain layout, but curiously lack the ADA domain. Still, they follow mechanistically identical biochemistry: that is, aminoacyl adenylate formation, covalent substrate tethering through a thioester, and subsequent reductive product release. Given that the ADA domain is dispensable for the functionality of these enzymes, we investigated its role by using *C. subvermispora* NPS3, a model basidiomycete Lys2 α-aminoadipate reductase that had been biochemically characterized previously.[6b] It was hypothesized that the function of ADA domains has relevance for the catalytic functionality of the reductase.[4] Alternatively, they might not fulfil any function and, thus, might be dispensable. This hypothesis is supported by the existence of the recently discovered ADA-domainless reductases mentioned above.

To test these hypotheses and to shed further light on the relevance of ADA domains, we created a set of four truncated *nps3* genes that were expressed heterologously (Figure 1). The enzymes were produced in *Escherichia coli* KRX, transformed with the appropriate expression plasmids. The pET21a-based expression plasmid pMR1 was used to produce an N-terminally tagged ADA-less version of NPS3, lacking the first 247 aa, thus resulting in a 132.6 kDa protein. The gene expressed from plasmid pDK22 encodes an N-terminally hexahistidine-tagged NPS3 in which about the first half of the ADA domain—that is, 152 aa—is absent. *E. coli* was transformed with plasmids pDK24 and pDK25, both based on expression vector pET28a, to produce the 102.8 kDa ADA-A didomain (lacking the C-terminal 515 aa) and the 75.7 kDa standalone NPS3A domain, respectively, in which the first 247 and terminal 515 aa are absent. These two truncated NPS3 versions were produced as N-terminally tagged fusions. To produce the 159.6 kDa C-terminally tagged hexahistidine-fusion full-length protein, plasmid pDK21 was used.[6b]

The four truncated NPS3 variants were purified by immobilized metal affinity chromatography and assayed in vitro for adenylating activity by quantification of the amino-acid-dependent ATP-[^{32}P]pyrophosphate radioisotope exchange, with L-α-aminoadipic acid as substrate. Full-length NPS3 served as positive control, and a reaction mixture with water instead of L-α-aminoadipic acid as negative control. Both the full-length NPS3 and the ADA-A didomain were active and showed clear L-α-aminoadipic acid turnover (301 700 and 239 200 cpm, re-

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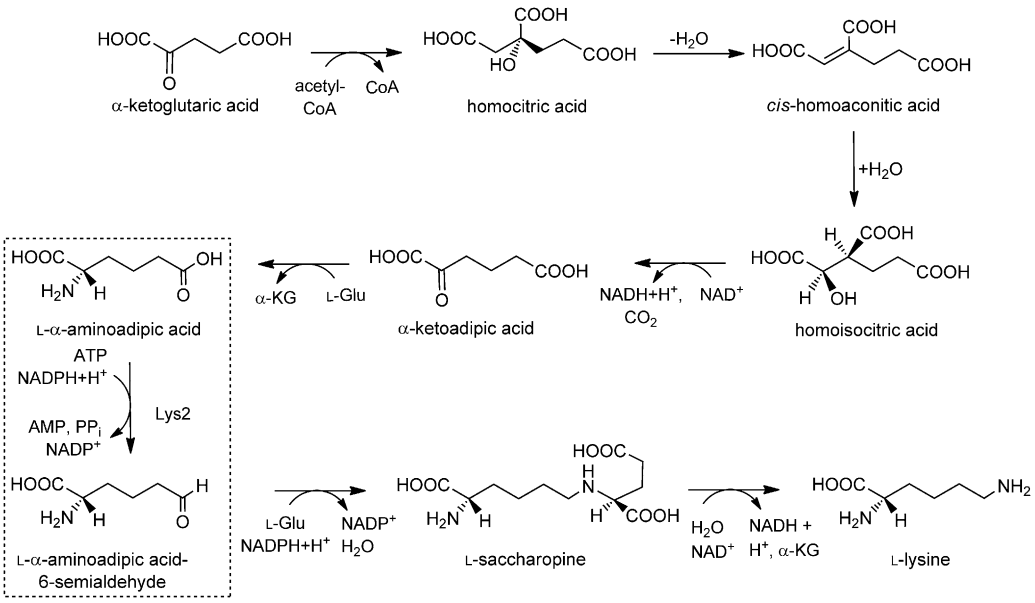

Scheme 1. Fungal de novo L-lysine biosynthesis. The Lys2-catalyzed reduction of L-α-aminoadipic acid to L-α-aminoadipic acid 6-semialdehyde is shown in the box. α-KG: α-ketoglutaric acid.

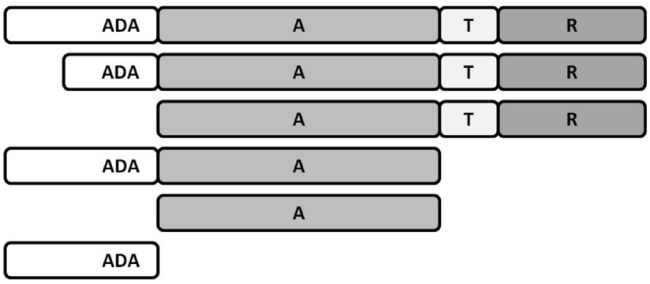

Figure 1. Domain compositions of full-length and truncated NPS3 reductases. Top to bottom: full-length NPS3, partially and completely removed ADA domain, ADA-A didomain, standalone adenylation domain and ADA domain. ADA: adenylation activating domain. A: adenylation domain. T: thiolation domain. R: reductase domain.

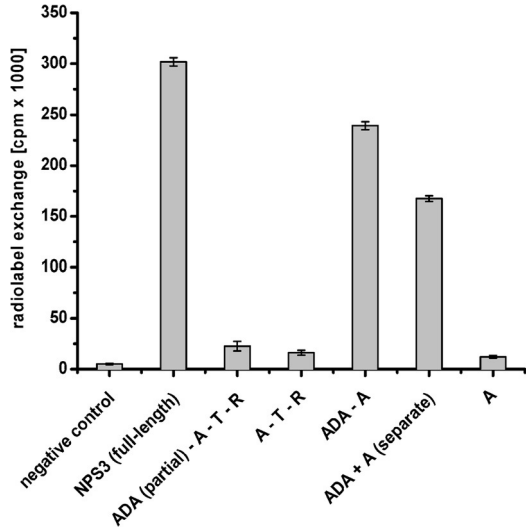

Figure 2. Adenylation of L-α-aminoadipic acid by the *C. subvermispora* NPS3 full-length enzyme and truncated versions, as determined in vitro by the substrate-dependent ATP-[^{32}P]pyrophosphate radioisotope-exchange assay. The bar diagram represents exchange based on the arithmetic means of triplicate reactions. Error bars indicate the standard deviations.

spectively, Figure 2). In stark contrast, partial or full deletion of the ADA domain severely impacted substrate adenylation, as is evident from a pyrophosphate exchange of $<10\%$ (22700 and 16100 cpm, respectively), relative to full-length NPS3. An even lower value (12100 cpm), about twice the value of the negative control (5100 cpm), was found for the isolated A domain. Taken together, these results suggest that NPS3 depends on the ADA domain to adenylate the amino acid substrate.

To corroborate these findings, the NPS3 ADA domain was produced as a separate N-terminally histidine-tagged 247 aa protein, by use of the expression plasmid pDK28. Strikingly, the adenylating activity of the standalone A domain was qualitatively restored (167600 cpm, Figure 2) in assay mixtures containing both the A domain and an equimolar concentration of physically separate ADA domain. To test for possible protein–protein interactions, we also performed size-exclusion chromatography with either the NPS3A or the ADA domain, or with an equimolar mixture of both, to determine the apparent molecular masses of the eluting proteins. The recombinantly produced A domain and ADA have calculated masses of 75.7 and 30.6 kDa, respectively, and these were verified experimentally. An additional peak at about 106 kDa appeared when the mixture was chromatographed, and is in plausible agreement with the expected mass of the ADA·A complex (Figure 3A). This peak was analyzed by SDS-PAGE and showed both the ADA and the A domain as components (Figure 3B). These results clearly point towards a structural interaction of the two domains.

For more insight into the nature of the ADA domain, its primary protein sequence was subjected to Phyre2 calculation for

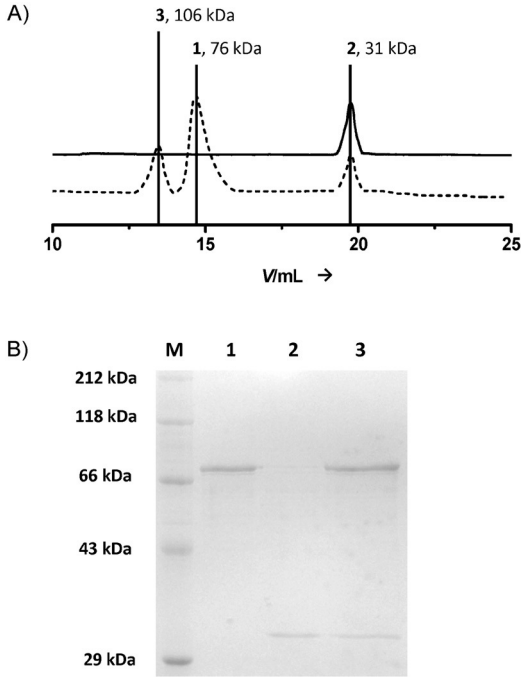

Figure 3. A) Determination of the molecular mass of the ADA·A complex by size-exclusion chromatography. Solid line: pure ADA domain (eluting at about 19.5 mL). Dotted line: ADA and A domain. The complex elutes at about 13.5 mL, the A domain at about 14.5 mL. B) Polyacrylamide gel of the peak eluting at about 106 kDa (lane 3). M: molecular weight standard, lane 1: NPS3A domain, lane 2: ADA domain.

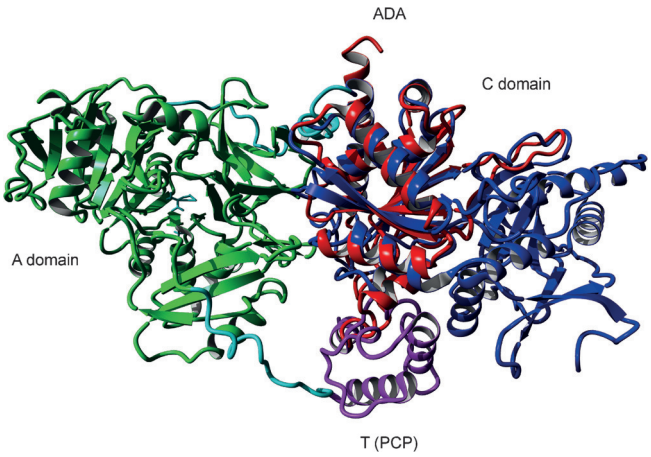

Figure 4. Structural superposition of a homology model of the NPS3 ADA domain with the crystal structure of SrfA–C, the termination module of surfactin synthetase (PDB ID: 2VSQ). The ADA domain aligns well with subdomain 2 of the C domain of SrfA–C. Subdomain 2 is in close contact with the A domain through a large set of protein–protein interactions. The thioesterase domain of SrfA–C and the reductase domain of NPS3 are not shown. Red: ADA domain. Blue: condensation domain. Green: adenylation domain. Pink: thiolation domain.

a prediction of the three-dimensional structure.[7] Strikingly, this software identified ADA as an NRPS condensation domain that adopts a CoA-dependent acyltransferase fold. Notably, condensation domains are pseudo-dimers, each consisting of two structurally related subdomains with an acyltransferase fold.[8] Structural alignment of the ADA domain homology model with the surfactin synthetase (SrfA–C) condensation domain revealed that the ADA domain aligns well with the complete second subdomain of the condensation domain (Figure 4), which is located at the N-terminal interface of the SrfA–C A domain. The second subdomain has been shown to participate in intimate protein–protein interactions with the A domain, whereas subdomain 1 does not.[8b] These results strongly support the view of the ADA domain interacting closely with the NPS3A domain, perhaps to stabilize an active conformation. These findings confirm our conclusion that the NPS3A domain requires the ADA domain for activity and point to a close structural interaction of both domains. In an alternative conceptualization, our results also support the view of the ADA-A didomain representing one single domain and functional unit. Lys2 enzymes, such as *C. subvermispora* NPS3, do not occur outside the fungal kingdom. However, the existence of an additional or extended domain required for functionality distantly resembles the situation in some bacterial A domains with activities that depend on the presence of MbtH-like proteins, which are about 70 aa in length and facilitate some (but not all) adenylating reactions in actinobacterial secondary metabolism by forming presumably stoichiometric complexes with A domains.[9]

Further work is warranted to elucidate the reason why L-α-aminoadipic acid reductases require ADA domains whereas other fungal adenylating reductases and NRPSs do not possess these N-terminal extensions. Given the overall similarity of L-α-aminoadipic acid reductases and NRPSs, the ADA domain could represent a rudimentary ancient condensation domain that has lost its catalytic function but maintained its impact for the structural integrity of the adjacent A domain. Support for this hypothesis is provided by structural data that show close and extended interaction between C and A domains in the surfactin A–C NRPS.[9b] This scenario would imply that these reductases originate from a more complex NRPS-like synthetase, and that the ADA domain was lost as other adenylating reductases evolved. In this context it should be noted that known synthetases from fungi also contain C-A-T-R tetradomain units. These include TENS (the tenellin synthetase in which the C-terminal reductase domain acts as a Dieckmann-type cyclase), FUSS (fusarin synthetase, which releases its product reductively), the NG-391 synthetase NGS1, and others.[10] Although resembling NPS3, they are dissimilar in that they are integral to larger hybrid polyketide synthase/peptide synthetase enzymes and feature a complete C domain that serves to fuse the T domain-tethered polyketide with the amino acid, thus elaborating complex natural products. Alternatively, the ADA domain might have been acquired by preexisting ADA-less reductases. The evolutionary history of L-α-aminoadipic acid reductases is intricate[11] and, ultimately, our current study does not favor one or the other scenario.

In conclusion, our results showed that A domain activity depends on the presence of the ADA domain. They further suggest that, in general, the production of di- or multidomain pro-

teins might be advantageous if the production of single domains yields enzymatically inactive protein.

Experimental Section

General: *C. subvermispora* sequence data were taken from the published genomic sequence.[12] Molecular biology procedures were carried out according to the manufacturers' instructions (Fermentas, NEB). Chemicals were purchased from Sigma–Aldrich and Roth; [^{32}P]pyrophosphate was from PerkinElmer.

Construction of expression plasmids: Plasmid pDK21[6b] is a pET21b-based expression construct harboring the full-length *nps3* gene, which served as PCR template to produce truncated *nps3* versions. The gene lacking the ADA-domain-encoding portion entirely was amplified by use of forward primer oDK15 (5′-TATAT AGAAT TCGTT GGTGC AGTTC CG-3′) and reverse primer oDK17 (5′-ACGAT ACATA CGATC GCGCA CACC-3′). The reaction mixture contained $MgCl_2$ (2 mM), dNTP (0.2 mM each), primer (40 pmol each), and Phusion DNA-polymerase (1 U) with the buffer supplied with the enzyme, in a total volume of 50 μL. Thermal cycling conditions: initial denaturation: 2 min at 94 °C; amplification: ten cycles (94 °C for 30 s, 55 °C for 30 s, 72 °C for 120 s), followed by 25 cycles (94 °C for 30 s, 57 °C for 30 s, 72 °C for 120 s); terminal hold: 5 min at 72 °C. The PCR product was cloned into pDK21 from which the EcoRI/AgeI portion had been removed, to create plasmid pMR1. The EcoRI site was introduced by use of primer oDK15; the AgeI site occurs naturally in *nps3*. The construct to express *nps3* with a partial ADA domain (pDK22) was created identically, except for using oDK16 (5′-TATAT AGAAT TCAAA GCAGA ACCGA CCG-3′) as forward primer and a 140 s PCR extension time. The gene encoding the ADA-A didomain was amplified with primers oDK30 (5′-CGCGG ATCCG AATTC GCCGA AGAAC GTCTG CAG-3′) and oDK31 (5′-GATCA AAAAG CTTTA ATCCA GCGGC AGCGG AC-3′) under the above PCR conditions, but with the annealing temperature set to 60 °C during the first 10 and 62 °C during the subsequent 25 cycles, and 90 s extension. The PCR product was cloned between the EcoRI and AgeI sites of pET28a to create plasmid pDK24. The construct to express the A domain gene (pDK25) was created as described for the ADA-A didomain gene, but with use of primers oDK15 and oDK31 and with the annealing temperature set to 54 °C during the first 10 and 57 °C during the subsequent 25 cycles, and with 65 s extension time. The gene to express the ADA domain was amplified by using primers oDK30 (above) and oDK40 (5′-TATAT AAAGC TTTTA CTGTG CCAGC GGATT G-3′), and application of the PCR parameters used for the single A domain gene. The PCR product was cut with EcoRI and HindIII and cloned into pET28a, restricted equally, to create plasmid pDK28.

Enzyme production and purification: Seed cultures of *E. coli* KRX, transformed variously with pDK21, pDK22, pDK24, pDK25, pDK28, and pMR1, were grown overnight in liquid lysogeny broth (LB), amended with the appropriate antibiotics (kanamycin or ampicillin). The production culture (400 mL LB with the appropriate antibiotic) was inoculated and grown at 37 °C and 180 rpm to an $OD_{600} = 0.35$. Subsequently, the incubation temperature was lowered to 16 °C for a further 30 min, followed by induction with L-rhamnose (0.1 %, *w/v*) for 16 h. The cells were pelleted, resuspended in phosphate buffer (10 mL) containing imidazole (10 mM), and disrupted as described.[13] The supernatant containing soluble proteins was loaded onto an Äkta Pure FPLC instrument (GE Healthcare) equipped with a HisTrap FF crude 1 mL column volume. The flow rate was 1 mL min^{-1}, buffer A was NaH_2PO_4 (50 mM), NaCl (300 mM), pH 7.5, and buffer B was buffer A+imidazole (500 mM). After application of the sample in 5 % buffer B (i.e., 25 mM imidazole), the column was washed with buffer A/buffer B (93:7, 35 mM imidazole, 4 mL) and with buffer A/buffer B (92:8, 40 mM imidazole, 4 mL) before elution with B (100 %, 6 mL). The fraction containing soluble NPS3 protein was loaded onto a PD-10 buffer exchange column (GE Healthcare) and eluted with sodium phosphate buffer (200 mM, 3 mL). For size-exclusion chromatography, a GE Healthcare Superdex 200 Increase 10/300 GL column (24 mL bed volume) and phosphate buffered saline (Na_2HPO_4 (10 mM), KH_2PO_4 (10 mM), NaCl (150 mM), pH 7.6) at a flow of 0.35 mL min^{-1} were used. To calculate the apparent molecular mass of the ADA·A complex, a standard curve referenced to myosin, β-galactosidase, albumin, ovalbumin, carboanhydrase, and lysozyme was used.

Radioisotope-exchange assay: The adenylating activities of full-length and truncated NPS3 enzyme domains were determined by substrate-dependent ATP-[^{32}P]pyrophosphate exchange assay with use of a previously described procedure and parameters[14] but with Tris·HCl buffer (200 mM) and L-α-aminoadipic acid (water in the negative control) as sole substrate. The pyrophosphate exchange reaction was quantified with a PerkinElmer TriCarb 2910TR scintillation counter. All reactions were carried out in triplicate.

In silico modeling: The search for structural homologues and construction of a homology model was performed by use of the Phyre2 server.[7] The homology model based on VibH (PDB ID: 1L5A) as a template was visualized with YASARA 14.[15] A structural alignment with the crystal structure of the SrfA–C module (domains A-C-T-TE, PDB ID: 2VSQ) was performed by use of MUSTANG[16] implemented in YASARA 14.

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