INVESTIGATION OF THE FIRST EUKARYOTIC NICOTINIC ACID UTILIZATION PATHWAY ON THE MODEL ORGANISM *ASPERGILLUS NIDULANS*; STUDY OF REGULATION AND ENZYME ACTIVITIES

PH.D. THESIS

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Introduction

In the 1990s, the investigation of catabolism of heterocyclic rings by microbes increased significantly in order to make the production of bioactive drugs (herbicides and human therapeutic drugs) more cost-effective.

Although many prokaryotic organisms have been identified in the last few years, which can utilize nicotinic acid (NA) as nitrogen and/or carbon sources, the eukaryotic NA catabolic pathway(s) have not been studied yet. During the work on the purine utilization pathway, Scazzocchio *et al.* recognized that in the model organism *A. nidulans*, PHII plays an essential role in NA utilization and at the same time it shares functional similarities with the purine catabolic pathway enzyme, purine hydroxylase I (PHI). In addition to the similarities, PHII differs from PHI in many aspects. PHII is able to carry out the hydroxylation of hypoxanthine to xanthine (as PHI), but unable to catalyze the further hydroxylation of xanthine. Uniquely, PHII has a new substrate, the NA, which is hydroxylated to 6-NA acid. Notably, during their experiments they could detect PHII enzyme activity only when the non-repressing nitrogen source media was supplemented with inducer concentration of NA or 6-NA. Therefore, PHII seemed to be devoted to the NA catabolic route instead of the purine catabolic pathway.

For the first time, the investigation of the eukaryotic NA catabolic process has been started by our research group.

Aims

The aims of the present study were as follows:

- to prove that PHII is coded by the *hxnS* gene and HxnR is identical to the product of AN11197 gene
- structure–and function analysis of HxnS and HxnR
- (i) exploring the role of HxnR; (ii) expression analysis of the neighbouring genes of *hxnR* and *hxnS*; (iii) and studying the role of AreA as a co-regulator of the cluster genes by transcript analysis
- the exploration of the possible role of the clustering genes
- study of the intracellular localization of HxnR

Methods

Cultivation of cells used in experiments

Maintenance of *E. coli* and *A. nidulans* strains, cultivation on solid and in liquid medium.

Molecular techniques:

DNA, RNA and protein isolation from filamentous fungus, cDNA synthesis, protoplasts isolation and transformation of *A. nidulans*, competent cell preparation and chemical transformation of *E. coli*, plasmid isolation from bacteria, PCR, qRT-PCR, Double-Joint PCR, DNA gel electrophoresis, molecular cloning

Other methods

Southern-hybridization, heterothallic crossing of *A. nidulans*, UV mutagenesis, enzyme assay, nitrogen source utilization test and inducer formation assays, fluorescence microscopy, laser scanning microscopy, *in silico* analysis

Results

Identification of HxnS and HxnR encoding genes (Amon *et al.*; 2017)

Since the PHI is the paralog of PHII, we have been able to identify the open reading frame of the supposed *hxnS* gene and deleted. We also deleted the neighboring Zin-finger transcription gene of *hxnS*, *hxnR*, which is assumed to encode the specific transcription factor of the NA degradation pathway. We carried out growth tests and enzyme activity assays with the available PHII and HxnR mutants (*hxnS29, hxnS35, hxnS41, hxnR2*) as well as with the *hxnS, hxnR* deleted strain (*hxnSΔ, hxnRΔ*) and we demonstrated that *hxnS* is the coding gene of PHII and AN11197 is the coding gene of HxnR.

We sequenced the available loss of function mutants and identified those nucleotide changes, which are responsible for the loss of enzyme activity. Chain termination mutations in *hxnS35* and *hxnS41* caused the complete inhibition of the PHII enzyme, however the mis-match mutation within the conserved region of *hxnS29* resulted in amino acid (AA) substitution that reduced the activity of the PHII enzyme most probably by weakening the stability of the enzyme.

We identified the cDNA sequence of HxnS, and we made a detailed comparison between PHI (HxA), PHII (HxnS) and the biochemically well-characterized xanthine dehydrogenase (XDH) of *Bos taurus.* We defined AA residues, which are conserved in not only HxnS, but all the orthologs of HxnS found in the fungal genom database. According to the comparative analysis of more than one hundred HxnS orthologs, the identified conserved AA residues, which differ in HxA or XDH of *B. taurus* are thought to be important for the HxnS substrate specificity.

We investigated the structure of the NA catabolic route specific transcription factor, HxnR. HxnR is a Cys2His2 Zinc-finger protein, with two Cys2His2 boxes at the amino-terminus followed by NLS and NES signals and a fungal specific transcription factor domain PF04082.

We started the functional analysis of HxnR by the isolation and analysis of HxnR constitutive mutants. Since we reached saturation of the constitutive mutations, we think that we identified all those nucleotide changes, which resulted in constitutivity. We proposed that an aromatic AA at positions 226 and 228 and a basic AA in the position 605 is pivotal to maintain the protein in its physiological inactive state in the absence of an actual inducer.

We made CONSURF alignment of HxnR with 123 putative Pezizomycotina orthologues and mapped all the known loss-of-function and constitutive mutations onto the protein and to the 3-dimensional model we made according to the consurf data. Those AA residues, which are responsible for the loss-of-function or gain-of–function mapped to the two opposite ends of the outer surface of the protein.

Discovery of a highly conserved gene cluster on the chromosome VI. (Amon *et al.*; 2017)

On the basis of the close genomic linkage of *hxnR* and *hxnS*, we reasoned that similarly to prokaryotic NA degradation pathway genes, the *A. nidulans* NA catabolic pathway genes also might be organized in a cluster. Through the systematic expression profile analysis of *hxnS*, *hxnR* and the neighbouring genes, further 4 genes (*hxnP*, *hxnT*, *hxnZ* and *hxnY*) showing co-regulation were identified. The cluster of six genes on chromosome VI was named the *hxn* cluster. All the cluster genes were expressed only in response to NA and 6-NA induction in wild type control and their expression depended on HxnR. The gene expression studies revealed that *hxnR* is derepressed under non-repressed conditions and is able to enhance its own gene expression in the presence of an inducer (autoregulation). We also demonstrated that urea (previously considered as neutral nitrogen source) acts as an inhibitory nitrogen source in our experimental system.

We also found out that besides HxnR, the AreA (often acting as a co-regulator in the nitrogen source utilization pathways (e.g. nitrate), is also essential for the expression of the *hxn* cluster genes and acts as a positive regulator.

In silico analysis of *hxn* genes

We confirmed that the sequence of HxnP protein is incorrect in the database, and we designated the real position of the fourth intron.

Using computional analysis of HxnP we determined that this protein is a member of the Major facilitator superfamily, which has typically 12 transmembrane segments.

HxnZ is also a predicted 12-TMS transporter of the MFS1 superfamily, most probably it transports the aromatic metabolites of the route.

hxnT gene encodes a flavin oxidoreductase with NADH dehydrogenase activity. NADH dehydrogenase activity of HxnT was experimentally confirmed by enzyme assays.

HxnY is an α-ketoglutarate dependent dioxygenase. It is probable that the substrate of HxnY is a nicotinate derivative, with a closed pyridine ring.

Construction of deletion mutant strains and examination of their NA utilization properties in the presence of different NA derivatives

We created a deletion mutant for each *hxn* cluster gene and investigated their NA utilization ability. In growth tests of deletion mutants, NAA and 2.5-DP (known from the prokaryotic pathways) act as the inducer of the pathways. It was found that 2,5-DP is the intermediate product of the pathway, and the NAA is feeded in and degraded by the NA catabolic pathway after a deamination. Since none of the deletions caused the complete loss of 6-NA utilization capacity, we supposed that the metabolism of 6-NA occurs through multiple pathways involving genes outside this cluster, which may also be under the control of *hxnR*.

Examination of the intracellular localization of HxnR by expressing and analyzing *hxnR-gfp* fusion constructs

Intracellular localization of the NA degradation pathway regulator HxnR was studied through C-terminal and N-terminal Gfp fusions under non-induced, induced and induced-repressed conditions.

For the C-terminal Gfp fusion we developed three types of constructions, in which the operating promoter was different. In addition to the native promoter, we also made constructions with the constitutive *gpdA* and the proline inducible *prnD* promoters. None of the constructions provided transformants, which were able to complement the deletion phenotype in a single copy number. Those transformants, which contained the construct in high copy number showed a partial complementation only on 6-NA and the HxnS diagnostical hypoxanthine medium while showed no complementation on NA.

In fact, we introduced our constructions in the first available *hxnRΔ* mutant and found out that the mutant carries other *hxn* genes flanking the *hxnR* locus, in multycopy. We surmised that the low and partial complementation of deletion phenotype by the *hxnR-gfp* fusion on 6-NA might be due to the increased number of *hxn* promoter sites in the *hxnRΔ* strain, which practically dilute the available HxnR molecules from its promoter regions. We explained the lack of complementation on NA medium by supposing that HxnR-Gfp does not work appropriately on HxnS promoter, but is able to interact with other *hxn* promoters at a better level (e.g. *hxnY, hxnP*). This hypothesis correlates to our result, in which we reveal that beside HxnR, AreA also acts as a positive coregulator on the *hxn* promoters. It means that depending on where the AreA binding sites are positioned on an *hxn* promoter, the HxnR-Gfp fusion protein might have less or no access to its natural binding site. In the worst scenario, AreA binding site is so close to the HxnR binding site that the fusion protein does not gain access to the HxnR site.

Upon the failure with the C-terminal fusion we developed amino-terminal fusion of HxnR (Gfp-HxnR). Unfortunately, we got similar result as with the carboxy-terminal fusion. The single copy transformants showed no complementation on the tested NA or, 6-NA media and a little complementation on the diagnostical hipoxanthine medium. However, the multycopy transformants showed a partial complementation on hypoxanthine medium, while no complementation was seen on NA and 6-NA media. We explain the complementation results by supposing that Gfp-HxnR operates very well with the HxnS promoter, hence the particularly good growth on hypoxanthine medium is observed. Since NA and 6-NA utilization need the proper expression of other *hxn* genes, failure of Gfp-HxnR on even one of the downstream pathway related *hxn* promoters can result in the failure on NA or 6-NA utilization. These results further support our hypothesis of different arrangements of Are and HxnR binding sites on the different *hxn* promoters.

All in all, both C-terminal and N-terminal GFP fusion of HxnR interferes with the HxnR function, thus they are inadequate to examine the physiological intracellular localization of the protein. The constitutive nuclear localisation seen in the *prnD* promoter driven *hxnR-gfp* carrying transformant was probably due to the overproduction of HxnR-GFP and the localization did not necessarily reflect the natural physiological events. Knowing that beside NLS a NES is also present on HxnR, shifting of the protein between nucleus and cytoplasm would be reasonable to presume.

Summary

During this project we have

- proved that the PHII enzyme is encoded by the *hxnS* gene and HxnR is encoded by the AN11197 gene
- identified those AA regions which responsible for the activity and substrate specificity of HxnS and HxnR enzymes
- identified two Cys2His2 zinc finger domains, NLS and NES signals and a fungal specific transcription factor domain PF04082 in the sequence of HxnR
- identified the correct cDNA sequence of *hxnS* and *hxnP*
- identified a gene cluster consisting of 6 genes on the chromosome VI
- confirmed in expression analysis:
  - HxnR is the transcription factor of the NA catabolic pathway
  - *hxnR* is derepressed under non-repressed conditions and is able to enhance its own gene expression in the presence of an inducer
  - urea acts as an inhibitory nitrogen source in our experimental system
  - AreA is a positive regulator of the *hxn* cluster genes
- determined the possible functions of the clustering genes
- confirmed that the metabolism of NA occurs through several routes involving genes outside this cluster, which may also be under the control of *hxnR*
- the HxnR C-terminal and N-terminal GFP fused protein also interfere with the HxnR function and hence failed to investigate the physiological intracellular localization of HxnR. In some cases, the constitutive nuclear presence in fluorescence microscopy was probably due to the protein overproduction and does not necessarily reflect the natural physiological location.

Publications

Publications in referred journals summarizing the results of this Ph.D. Thesis

Ámon J, Fernandez-Martin R, Bokor E, Cultrone A, Kelly JM, Flipphi M, Scazzocchio C, Hamari Z; A eukaryotic nicotinate-inducible gene cluster: convergnt evolution in fungi and bacteria, *Open Biology* 7:(12) Paper 170199. 17 p. (2017); **IF: 3,286**

Additional publication

Ámon J, Keisham K, Bokor E, Kelemen E, Vágvölgyi C, Hamari Z; Sterigmatocystin production is restricted to hyphae located in the proximity of hülle cells, Journal Of Basic Microbiology 58:(7) pp. 590-596. (2018); **IF: 1,580**

Cumulative impact factor: 4,866

Conference abstracts summarizing the results of this Ph.D. Thesis:

Ámon J, Bokor E, Hamari Zs; Structure and function analysis of the nicotinic acid catabolic pathway specific transcription factor, HxnR in *Aspergillus nidulans*; 19th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Program and abstracts 65.p. (ISBN:978-963-306-535-8)

Judit Ámon, Eszter Bokor, Csaba Vágvölgyi, Zsuzsanna Hamari; Comprehensive analysis of HxnT, an enzyme of the nicotinate catabolic route; Acta Microbiologica Et Immunologica Hungarica 64:(Supplement 1) p. 107. 1 p. (2017); 5th Central European Forum for Microbiology

Ámon J, Bokor E, Keisham K, Vágvölgyi C, Hamari Z; Study of the intracellular localization of the nicotinate catabolic pathway specific transcription factor, HxnR of *Aspergillus nidulans*; 18th Danube-Kris-Mures-Tisa (DKMT) Euroregional Conference on Environment and Health: Book of abstracts, p.49. (ISBN:978-86-6253-059-2)

Judit Ámon, Eszter Bokor, Kabichandra Keisham, Csaba Vágvölgyi, Zsuzsanna Hamari; Expression of the HxnR-Gfp protein driven by three different promoter; 5th CESC 2016 Central European Summer Course on Mycology and 2nd Rising Stars in Mycology Workshop: Biology of pathogenic fungi. 74 p. (ISBN:978-963-306-493-1)

Judit Ámon; Regulation of the nicotinic acid degradation pathway and study of the evolution of the pathway enzymes; Acta Biologica Szegediensis 60**:**(1) pp. 77-78. (2016); Conference For Doctoral Students In Biology.

Judit Ámon, Eszter Bokor, Kabichandra Keisham, Csaba Vágvölgyi, Zsuzsanna Hamari; The intracellular localization of the transcription factor HxnR in *Aspergillus nidulans*; A Magyar Mikrobiológiai Társaság 2016. évi nagygyűlése és a XII. Fermentációs Kollokvium: absztraktkötet, p.4.

Judit Ámon, Eszter Bokor, Kabichandra Keisham, Csaba Vágvölgyi, Zsuzsanna Hamari; Otaining and characterization of constitutive HxnR mutants - structure-and-function analysis; A Magyar Mikrobiológiai Társaság 2016. évi nagygyűlése és a XII. Fermentációs Kollokvium: absztraktkötet, p.4.

Ámon Judit, Bokor Eszter, Vágvölgyi Csaba, Hamari Zsuzsanna; Study of HxnR, the transcription factor of the nicotinic acid degradation pathway in *Aspergillus nidulans*; Acta Microbiologica Et Immunologica Hungarica 62:(Suppl 1) p. 1. (2015); Annual Meeting of the Hungarian Society for Microbiology

Ámon Judit, Bokor Eszter, Michel Flipphi, Rafael Fernández-Martín, Claudio Scazzocchio, Hamari Zsuzsanna; The nicotinate utilization pathway of *Aspergillus nidulans;* 12th European Conference on Fungal Genetics (ECFG12). 358 p.