FEBS Letters 588 (2014) 1016–1023

journal homepage: www.FEBSLetters.org

Characterization of bacterial NMN deamidase as a Ser/Lys hydrolase expands diversity of serine amidohydrolases

Leonardo Sorci [a], Lucia Brunetti [b], Lucia Cialabrini [b], Francesca Mazzola [a], Marat D. Kazanov [c], Sabato D'Auria [d], Silverio Ruggieri [b], Nadia Raffaelli [b,*]

[a] Department of Clinical Sciences, Polytechnic University of Marche, Ancona, Italy
[b] Department of Agricultural, Food and Environmental Sciences, Polytechnic University of Marche, Ancona, Italy
[c] A.A. Kharkevich Institute for Information Transmission Problems, Russian Academy of Sciences, Moscow, Russia
[d] Laboratory for Molecular Sensing, IBP-CNR, Napoli, Italy

ARTICLE INFO

Article history:
Received 6 December 2013
Revised 23 January 2014
Accepted 26 January 2014
Available online 11 February 2014

Edited by Miguel De la Rosa

Keywords:
NMN deamidase
Pyridine nucleotide
Catalytic dyad
Amidohydrolase
Site-directed mutagenesis

ABSTRACT

NMN deamidase (PncC) is a bacterial enzyme involved in NAD biosynthesis. We have previously demonstrated that PncC is structurally distinct from other known amidohydrolases. Here, we extended PncC characterization by mutating all potential catalytic residues and assessing their individual roles in catalysis through kinetic analyses. Inspection of these residues' spatial arrangement in the active site, allowed us to conclude that PncC is a serine-amidohydrolase, employing a Ser/Lys dyad for catalysis. Analysis of the PncC structure in complex with a modeled NMN substrate supported our conclusion, and enabled us to propose the catalytic mechanism.

1. Introduction

Amidohydrolases represent a numerous group of hydrolases acting on amide carbon–nitrogen bonds. They are categorized under EC numbers 3.4 and 3.5, according to whether they act on peptide bonds (peptidases and proteases) or other carbon–nitrogen bonds. Amidohydrolases comprise thousands of members with diverse chemistry and functional roles. The salient mechanistic hallmark of these enzymes is the structure-aided activation of a nucleophile, either a water molecule or aminoacid side chain, that can attack the amide group for cleavage. The water molecule activation can be achieved through complexation with a mononuclear or binuclear metal center as in metalloproteases [1], or by interaction with an acid residue in conjugate base form as in aspartate/glutamate proteases [2]. In other cases, a serine, threonine, or cysteine residue acts as the major nucleophile after its activation triggered by one or more conserved residues in the active site. In this latter group, a covalent acyl-enzyme intermediate is generated. The first identified and most studied active site architecture is the "classic" Ser/His/Asp catalytic triad of serine proteases [3]. Subsequently, variations of this characteristic triad have been reported, either in the residues' identity, like the Ser/Ser/Lys triad characterizing the "amidase signature superfamily" [4,5], or in the number of participating residues, as seen in the dyads or in the Ser-only configurations [6].

Recently, we have identified and functionally characterized a novel member of the amidohydrolase group, the enzyme nicotinamide mononucleotide (NMN) deamidase (PncC, EC 3.5.1.42), formerly known as Competence/damage-inducible protein CinA [7]. PncC catalyzes the hydrolysis of the carbamide bond in the nicotinamide moiety of NMN yielding nicotinic acid mononucleotide (NaMN), a key intermediate in NAD biosynthetic pathway. Our previous study showed that PncC is both phylogenetically and structurally separate from other known amidohydrolases [7]. Indeed,

Abbreviations: NMN, nicotinamide mononucleotide; NaMN, nicotinic acid mononucleotide; NaAD, nicotinic acid adenine dinucleotide; HPLC, high pressure liquid chromatography; PMSF, phenylmethylsulfonylfluoride; CD, circular dichroism

* Corresponding author. Address: Via Brecce Bianche, 60131 Ancona, Italy. Fax: +39 0712204677.
E-mail address: n.raffaelli@univpm.it (N. Raffaelli).

http://dx.doi.org/10.1016/j.febslet.2014.01.063

PncC does not align with any characterized amidase sequence, nor contains a signature typical of known amidohydrolase families. Its three-dimensional structure, which is available for the apo-protein from *Agrobacterium tumefaciens*, as determined by the Midwest Center for Structural Genomics, represents a unique and distinctive fold. In the SCOP database it is classified as the only member of the CinA-like superfamily of amidohydrolases, here renamed as PncC superfamily.

In the present study, we combined mutational and structural analysis to gain insight into the PncC active site architecture. Our results point to a Ser/Lys dyad as a catalytic center for the PncC superfamily, as seen in several proteases [8,9] and some amidases [10,11]. Overall, these features characterize PncC as a novel example of convergent evolution.

2. Materials and methods

2.1. In silico analysis

PncC protein sequences (see Supplementary information) were retrieved from The SEED comparative genomics database (http://pubseed.theseed.org) [12]. Due the large number of sequences (760 at the time of the analysis), a final set of 447 sequences was obtained after removing fragments and redundancy among strains coming from the same species. Prior to the alignment using Muscle [13], the COG1058 domain of the bifunctional COG1058/PncC proteins [14] was manually removed. The most divergent sequences (30) were obtained by decreasing redundancy (85% max similarity threshold). Multiple sequence alignment and secondary structure elements were displayed using ESPript/ENDscript [15].

2.2. Site-directed mutagenesis and mutants' expression and purification

Site-directed mutagenesis of *Escherichia coli* PncC was carried out using the QuikChange XL Site-Directed Mutagenesis Kit (Agilent Technologies), according to the manufacturer's instructions. Sequences of mutagenic primers are listed in Table S1. The plasmid pCA24N-pncC, used as the PCR template, was purified from the *E. coli* ASKA clone [16]. The mutagenized plasmids were sequenced to verify incorporation of the desired modification and to ensure the absence of random mutations. For mutants and wild-type protein expression, the plasmids were transformed into electrocompetent *E. coli* BL21(DE3) cells. Cells were grown at 37 °C in Luria Bertani medium supplemented with 0.030 mg/ml chloramphenicol. After reaching an OD_{600} of 0.3, cultures were shifted at 25 °C and expression was induced with 1 mM isopropyl β-D-thiogalactopyranoside (IPTG), at an OD_{600} of 0.6. After 3 h induction, cells from 20-ml cultures were harvested by centrifugation at 5000×*g* for 10 min, resuspended in 1 ml buffer A (50 mM TRIS/HCl buffer, pH 7.5, 0.15 M NaCl, 1 mM DTT) containing 1 mM phenylmethylsulfonylfluoride (PMSF) and 0.002 mg/ml leupeptin, antipain and chymostatin. The suspensions were sonicated for 3 min at 50 watt, with 30 s intervals, and centrifuged at 15000×*g* for 30 min. The supernatants representing the soluble fractions and the corresponding pellets were analyzed by SDS–PAGE [17]. The soluble fractions containing the recombinant proteins were assayed for the enzymatic activity and applied to a 5-ml HisTrap HP column (GE Healthcare), equilibrated with buffer A, containing 10 mM imidazole. The column was washed with the equilibration buffer, and elution was performed with an imidazole gradient from 10 mM to 350 mM in buffer A. Fractions containing the recombinant proteins (eluted at about 100 mM imidazole) were pooled and purity of the preparations was assessed by SDS–PAGE. Pools were dialyzed against 50 mM TRIS/HCl buffer, pH 7.4, 0.15 mM NaCl, and used for the kinetic characterization.

2.3. PncC activity determination and kinetic experiments

NMN deamidase activity was measured with a continuous spectrophotometric assay, as described in [7]. Briefly, NaMN formation by the enzyme is coupled to the conversion of NaMN to NADH, in the presence of recombinant *E. coli* NadD (converting NaMN to NaAD), NadE (amidating NaAD to NAD) and yeast alcohol dehydrogenase. For kinetic analyses, the HPLC-based assay relying on direct quantitation of NaMN was used, as described [7]. Briefly, reaction mixtures contained 50 mM HEPES buffer, pH 7.5, NMN concentrations ranging from 1 μM to 3 mM, and appropriate amounts of purified proteins. After incubation at 37 °C, reactions were stopped with 0.6 M $HClO_4$, and after 10 min on ice, the samples were centrifuged for 1 min at 12000×*g*. The supernatants were neutralized with 0.8 M K_2CO_3, kept on ice for 10 min, and centrifuged as described above, before injection into an ion-paired analytical Supelcosil LC18-S column (5 μM, 4.6 × 250 mm). Elution conditions were 4 min at 100% buffer A (100 mM potassium phosphate, pH 6.0, 8 mM tetrabutylammonium hydrogen sulfate), 6 min up to 7% buffer B (buffer A containing 30% methanol), returning to 100% buffer A in 1 min, and holding at 100% buffer A for 5 min. The amount of enzyme in the reaction mixture was maintained at a level between 0.1 and 500 μg/ml, leading to 1–10% substrate consumption within the incubation time. The linearity of response was assessed by the analysis of aliquots taken at 2 time points (10 and 20 min) over the course of reaction. Apparent values of K_m and k_{cat} were calculated by fitting initial rates to a standard Michaelis–Menten model using the software Prism 4 (GraphPad).

2.4. PMSF and fluorophosphonate inhibition studies

The influence of PMSF and a biotynilated fluorophosphonate (Desthiobiotin-FP, Thermo Scientific, Rockford, IL USA) on PncC activity was tested by incubating the pure enzyme (final concentration 0.1 μg/ml) in the presence of 1.0 mM PMSF or 0.1 mM desthiobiotin-FP, and 1 mM NMN. At different incubation times at 37 °C, NaMN product was quantified using the HPLC assay described above.

2.5. Thermal stability assay

Purified T31A, E28A, and wild type PncC (final concentration 0.5 mg/ml) were incubated at 70 °C in 50 mM TRIS/HCl buffer, pH 7.4, 0.15 mM NaCl. Aliquots were taken at different times, and enzymatic activity was assayed as described above.

2.6. Circular dichroism analysis

CD measurements were performed on purified R142A, Y56A, and wild type PncC at protein concentration of 0.1 mg/ml for far-UV measurements and 0.3 mg/ml for near-UV measurements, in 2.0 mM Tris–HCl buffer, pH 7.4, at 25 °C. A spectropolarimeter model J-810 (Jasco, Tokyo, Japan) equipped with the temperature-controlled liquid systems Neslab RTE-110 (Neslab Instruments, Portsmouth, NH) calibrated with a standard solution of 10-camphor sulfonic acid was used. Circular quartz cuvettes (Helma, Jamaica, NY) with 0.1 cm and 0.5 cm path length were used for the measurements in the far-UV (200–250 nm) and near-UV