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Nardilysin facilitates complex formation between mitochondrial malate dehydrogenase and citrate synthase

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Abstract

Gel filtration chromatography showed that nardilysin activity in a rat testis or rat brain extract exhibited an apparent molecular weight of ~300 kDa compared to ~187 kDa for the purified enzyme. The addition of purified nardilysin to a rat brain extract, but not to an *E. coli* extract, produced the higher molecular species. The addition of a GST fusion protein containing the acidic domain of nardilysin eliminated the higher molecular weight nardilysin forms, suggesting that oligomerization involves the acidic domain of nardilysin. Using an immobilized nardilysin column, mitochondrial malate dehydrogenase (mMDH) and citrate synthase (CS) were isolated from a fractionated rat brain extract. Porcine mMDH, but not porcine cytosolic MDH, was shown to form a heterodimer with nardilysin. Mitochondrial MDH increased nardilysin activity about 50%, while nardilysin stabilized mMDH towards heat inactivation. CS was co-immunoprecipitated with mMDH only in the presence of nardilysin showing that nardilysin facilitates complex formation.

Keywords: Acidic domain; Protein–protein interaction; Metabolon; *N*-arginine dibasic convertase; Centaurin-alpha; Tyrosyl-tRNA ligase

1. Introduction

Nardilysin (*N*-arginine dibasic convertase, EC 3.4.24.61) is a zinc metalloendopeptidase of the M16 pitrilysin family that cleaves peptides at dibasic residues. The scissile bond can be either between the dibasic peptide bond or at the amino side of the dibasic pair, and is influenced by the nature of the residue at the amino side of the dibasic pair [1]. As its original name implies, nardilysin was believed to be a convertase [2] because the activation of many peptide hormones requires cleavage at dibasic residues. Although its endogenous substrates are unknown, nardilysin in vitro releases Leu-enkephalin-Arg[6] from dynorphins (A or B) and α-neoendorphin, and Met-enkephalin-Arg[6] from BAM12 [2,3]. Additionally, nardilysin can hydrolyze several neuropeptides including β-endorphin at monobasic sites [4], suggesting that the enzyme may be involved in a wider range of biological processes.

Highly expressed in mammalian testes, nardilysin mRNA has also been detected in the heart, skeletal muscle, and brain [5,6]. The expression level is especially high in the germ cells of the testis suggesting a role in spermatogenesis [7]. The enzyme has also been found in neural tissues of mouse embryos indicating a role in neural development [5]. Although nardilysin is primarily found in the cytosol, it has been reported that nardilysin is located on the cell surface [8] acting as a receptor for heparin-binding EGF-like growth factor [9]. In addition, nuclear nardilysin has also been reported [10], and its function may

Abbreviations: Abz, 2-aminobenzoyl; CM, carboxymethyl; CS, citrate synthase; DTNB, 5,5′-dithio-bis (2-nitrobenzoic acid); EDDnp, ethylenediamine-2,4-dinitrophenol; GO, glucose oxidase; GST, glutathione *S*-transferase; LDH, lactate dehydrogenase; MALDI, Matrix Assisted Laser Desorption Ionization; mMDH, mitochondrial malate dehydryogenase; Ni-NTA, nickle-nitrilotriacetic acid

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be important for the maturation of oocytes [11]. Therefore, nardilysin may have multiple cellular functions at different cellular locations or in different tissues.

Nardilysin is unique in that it contains an acidic stretch highly enriched in glutamate and aspartate residues that precedes the active site motif HXXEH [5]. In rat nardilysin this domain consists of 57 Glu or Asp residues within a stretch of 74 residues, while in mouse nardilysin 59 Glu or Asp residues are found within this 74 residue stretch. It was originally observed that amines, particularly the polyamine spermine, can activate or inhibit nardilysin activity dependent on the particular substrate [12]. Evidence was obtained that spermine binds to the acidic stretch and induces a conformational change [13]. Most recently the acidic stretch has been expressed as a GST-fusion protein and shown to function as an autonomous binding domain of spermine [14]. The binding of spermine to the acidic domain fused to GST induced a conformational change mimicking the binding of spermine to the intact enzyme. In this communication, we provide evidence that the acidic domain of nardilysin can act as a protein binding domain that might serve to regulate the activity of both nardilysin as well as the interacting protein.

2. Materials and methods

2.1. Materials

Glucose oxidase (GO) was purchased from Boehringer Mannheim and before use was further purified on a Pharmacia Superdex 200 gel filtration column equilibrated with 10 mM potassium phosphate buffer, pH 7.0. Porcine mitochondrial citrate synthase (CS), porcine mitochondrial malate dehydrogenase (mMDH), porcine cytosolic malate dehydrogenase (cMDH), lactate dehydrogenase (LDH) and horseradish peroxidase were purchased from Sigma Chem. Co. CS and MDHs were dialyzed against 10 mM potassium phosphate buffer, pH 7.0, before use. Frozen rat brains and testes were purchased from Pel Freeze (Rogers, Ark.). Polyclonal rabbit anti-mitochondrial malate dehydrogenase was a generous gift from Dr. Arnold W. Strauss (Vanderbilt University).

2.2. Preparation of tissue extracts

Rat brain or rat testes extracts were prepared by homogenization in 10 volumes of 10 mM potassium phosphate buffer, pH 7.0, using a Tissumizer (Tekmar) at a setting of 7 for 15 to 20 s. The extracts were centrifuged at $20{,}000 \times g$ for 20 min, and the supernatants dialyzed against the same buffer overnight. The rat brain extract was fractionated on a 15 ml column of Accell CM resin (Waters) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. The flow-through containing neutral and anionic proteins was collected and then bound proteins were eluted batchwise with 0.25 M, 0.5 M, and 1 M NaCl. A negligible amount of protein was eluted with 1 M NaCl and this fraction was not utilized. The flow-through (unbound), low salt (0.25 M) and the high salt (0.5 M) fractions were dialyzed against 10 mM phosphate buffer, pH 7.0, and then concentrated 10–20 fold.

2.3. Purification of nardilysin and a GST-nardilysin acidic domain fusion protein

Wild type nardilysin from rat testis was purified as described by Csuhai et al. [3]. Recombinant mouse nardilysin containing an N-terminal hexa-histidine affinity tag, a linker region, and the nardilysin coding sequence starting at amino acid 51 [4] was expressed in Sf9 cells using the bacculovirus expression system as described by Gibco-BRL. The recombinant enzyme was purified on a Ni-nitrilotriacetic acid column (Ni-NTA, Qiagen) and most of the linker region and the hexa-histidine affinity tag were removed by cleavage with TEV protease (Gibco-BRL). A fusion protein consisting of the acidic domain of mouse nardilysin fused to the carboxy terminus of glutathione *S*-transferase (GST) was expressed in *E. coli* and purified on a glutathione-agarose column as previously described [14]. Glutathione *S*-transferase was expressed and purified similarly.

2.4. Isolation of nardilysin binding proteins

Purified hexa-histidine containing mouse nardilysin was bound to Ni-NTA resin at a ratio of 3.3 mg of enzyme per milliliter of resin. Protein (0.2 mg) from a fractionated rat brain extract (see above) was mixed with 0.15 ml of hexa-histidine nardilysin (0.3 mg) bound to Ni-NTA resin on a rotating platform for 60 min at 4 °C. The loading buffer was composed of 10 mM potassium phosphate buffer, pH 7.0, containing 10 mM imidazole. The mixture was then centrifuged at $300 \times g$ for 2 min and washed with loading buffer until the absorbance at 280 nm reached the baseline value. Bound proteins were eluted with 0.15 ml of 0.4 M NaCl.

2.5. Molecular sieve chromatography

Molecular sieve chromatography was conducted using a 1.6 cm × 60 cm Superdex 200 column (Pharmacia) equilibrated with 10 mM potassium phosphate buffer, pH 7.0. One milliliter fractions were collected at a flow rate of 0.4 ml/min. Rabbit muscle lactate dehydrogenase (LDH) and/or *Aspergillus niger* glucose oxidase (GO) were used as internal molecular weight standards in each run. To minimize diffusion, protein samples contained 20% glycerol and no more than 0.25 ml of sample was loaded onto the column. In separate chromatography runs the column was calibrated with ferritin (400 kDa), sweet potato β-amylase (200 kDa), glucose oxidase (160 kDa), recombinant