Expression of the Acidic Stretch of Nardilysin as a Functional Binding Domain†

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ABSTRACT: Kinetic evidence suggests an acidic region in nardilysin binds polyamines and acts as a regulatory domain. The binding of ∼5 mol of spermine/mol of nardilysin was demonstrated. The binding curve was sigmoidal exhibiting an IC_{50} of ∼118 μM and a Hill coefficient of 1.8. Spermine diminished the tryptophan fluorescence of the enzyme and increased its sensitivity to protease V8. The acidic stretch from mouse and human nardilysin were expressed as glutathione transferase fusion proteins. All fusion proteins bound spermine with an IC_{50} of 40 to 110 μM. The mouse fusion protein bound ∼7 mol of spermine exhibiting a sigmoidal binding curve and a Hill coefficient of 1.4. The human acidic stretch, containing fewer acidic residues, bound ∼5 mol of spermine/mol with a hyperbolic binding curve. Chimeric fusion proteins containing the N-terminus of the mouse acidic region fused to the C-terminus of the human acidic region bound ∼10 mol of spermine, while the opposite chimera bound ∼4 mol of spermine/mol. The N-terminal region of the mouse acidic domain binds 3−4 mol spermine/mol exhibiting a Hill coefficient of 1.4, while the same region from human nardilysin binds 1 mol of spermine/mol. Spermine enhanced the sensitivity of the mouse acidic domain, but not the human acidic domain, to protease V8. Together the data support a model where the acidic stretch of nardilysin functions as an autonomous domain.

Nardilysin (*N*-arginine dibasic convertase, NRDc)[1] is a 140-kDa zinc metalloendopeptidase first identified based on its ability to cleave peptides at arginine residues in the paired sequences Arg−Arg or Arg−Lys (*1*, *2*). Although the enzyme exhibits a preference for cleavage at arginine residues, recent studies indicate a more complex specificity including cleavage between an aromatic residue and a single basic residue (*3*). To date, the physiological substrates and exact functions of nardilysin have not been identified. However, the accumulation of the enzyme in spermatides during the late stages of spermatogenesis has led to the suggestion that nardilysin is involved in the processing of peptides associated with the morphological transformation seen in spermatogenesis (*4*). In vitro substrates of the enzyme include dynorphins A and B, α-neoendorphin, somatostatin 28, and pre-proneurotensin (*1*, *5*).

The cDNA and deduced amino acid sequences of mouse, rat, and human nardilysin show the presence of an inverted Zn-binding motif HXXEH, placing the enzyme in the inverzincin family of zinc metallopeptidases (*6*). A unique feature of nardilysin is the presence of an extended "acidic domain" consisting of approximately 76 amino acids in which 57 (mouse nardilysin), 59 (rat nardilysin), or 45 (human nardilysin) of these residues are glutamate or aspartate (*6*, *7*). This domain, which is located just N-terminal to the zinc binding catalytic domain, may play a role in substrate recognition and binding. Alternatively, it has been suggested that this acidic domain might play a role in the appropriate routing and targeting of the enzyme to a subcellular compartment (*8*). We previously reported (*6*) that simple amines such as ammonium chloride, Tris, and Bis-Tris propane as well as the physiological polyamines putrescine, spermidine, and spermine could activate or inhibit nardilysin activity depending on the substrate utilized. We have suggested that the acidic domain was the site of amine binding and proposed that the acidic domain might serve as a regulatory domain for polyamines and perhaps for protein−protein interactions (*9*).

In the present study, the acidic domain of mouse and human nardilysin have been expressed as fusion proteins with glutathione *S*-transferase (GST) and compared to the native enzyme with respect to spermine binding. Our data suggest that the acidic domain of nardilysin can indeed function as an autonomous domain.

MATERIAL AND METHODS

^{3}H-spermine (40 Ci/mmol) was purchased from American Radiolabeled Chemicals Inc. Spermine and glutathione-agarose were purchased from Sigma Chem. Co. Bio-gel P_6-DG was obtained from Bio-Rad. An anti-His_6 antisera was obtained from Clontech. Pfu DNA polymerase was purchased from Gibco, BRL. *Staphylococcus aureus* protease V8 was obtained from Sigma. Chem. Co. Antisera to recombinant nardilysin, to the acidic domain peptide SGAEIQDD (residues 166 to 173), and to the peptide DRLAHEIEALKSF, (residues 1061 to 1073), were produced in rabbits by Bethyl Labs Inc., Montgomery, TX.

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[1] Abbreviations: NRDc, nardilysin, *N*-arginine dibasic convertase; GST, glutathione transferase; PCR, polymerase chain reaction.

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Expression of Recombinant Mouse Nardilysin. A mouse nardilysin cDNA (*6*) was subcloned into the pFASTBAC-HTa vector (Gibco-BRL) using *Nco I* and *Sal I* restriction sites such that a His_6-affinity tag and linker region became fused to the N-terminus of the protein. The nardilysin portion of the fusion protein started at methionine 1. A second construct was made in which expression began at glycine 51. To accomplish this, Pfu DNA polymerase was used to amplify nucleotide 151 to nucleotide 1161 by PCR employing the following primers: forward primer, GTACCCATGG-GAAGAAACAAGGCGAAG and reverse primer, GTTA-AATGCTGGTGTGTCAAAA. The PCR product was cut with *Nco* I and *BamH I* and used to replace an *Nco I*−*BamH I* fragment from the original construct. Generation of recombinant bacculovirus and expression of recombinant nardilysin in Sf-9 cells was done according to the manufacturer's instructions. For the purification of recombinant nardilysin containing a His_6-affinity tag, a 1/10 (wt/vol) suspension of Sf-9 cells expressing the enzyme was prepared in 50 mM Tris-HCl buffer, pH 8.5, containing 150 mM KCl, 10 μg/mL aprotinin, 2 μg/mL leupeptin, 2 μg/mL pepstatin, 0.2 mM phenylmethylsulfonylfluoride, and 40 μg/mL trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane (E−64). The cells were sonicated using a Branson sonifier (setting 3 at 30%) 10 times, each time for one second. The sonicate was centrifuged at 30000*g* for 30 min at 4 °C, and the supernatant was loaded onto a nickel-nitrilotriacetic acid column (Qiagen) that had been equilibrated with 50 mM Tris-HCl buffer, pH 8.5, containing 150 mM KCl. After extensive washing of the column with starting buffer, and then with 50 mM Tris-HCl buffer, pH 8.5, containing 2 M NaCl, the enzyme was eluted with 250 mM imidazole in 50 mM Tris-HCl, pH 8.5. The His_6 affinity tag and most of the polylinker was removed by treating the purified enzyme with 15 units of His_6-affinity tagged TEV protease (Gibco-BRL) per mg of protein overnight at 4 °C. The TEV protease and any residual His_6-affinity tagged nardilysin were removed by rechromatography on a nickel-nitrilotriacetic acid column.

Recombinant nardilysin in which the His_6-affinity tag was removed during expression (see Results) was purified as follows. The enzyme was first chromatographed on a Pharmacia Mono Q column in 50 mM Tris-HCl buffer, pH 7, using a 0 to 1 M NaCl gradient to elute the enzyme. Active fractions were pooled, concentrated, and applied to a Pharmacia S200 molecular sieve column (20 × 60 cm) in 50 mM Tris-HCl buffer, pH 7. The pool from the S200 column was adjusted to 30% ammonium sulfate and applied to a Pharmacia FPLC phenyl column equilibrated with 30% ammonium sulfate in 50 mM Tris-HCl buffer, pH 7. The enzyme was eluted with a decreasing gradient of 30% to 0 ammonium sulfate, dialyzed against 10 mM sodium phosphate buffer, pH 7, and concentrated with a Centricon Plus-20 spin concentrator.

Construction of GST-Nardilysin Acidic Domain Expression Vectors. The acidic domain of mouse nardilysin encompassing amino acid residues Asp^{139} to Glu^{209} or human nardilysin encompassing amino acid residues Asp^{141} to Glu^{197} were fused to the C-terminus of GST. For the mouse acidic domain, this was accomplished by PCR amplification of the nucleotide sequence encoding the acidic domain of mouse nardilysin using a mouse nardilysin cDNA clone (*6*) as a template. The sequence of the forward primer was TCG GAA TTC AGA TCT AAG TAA TGT GG while the reverse primer was ATC GTC GAC TAT TTC TCA GTA GTT TTC TT. The nucleotides encoding the human acidic domain were amplified from a partial human cDNA clone (*6*) using the forward primer CGT GAA TTC AGA CCT AAG TAA TAT GG and the reverse primer TCA GTC GAC TTA TTC AGT AGT TTT TTT. The PCR primers included an *EcoR I* site located at the 5′ end and an in-frame stop codon followed by a *Sal I* site at the 3′ end. These sites are underlined. The PCR fragments were digested with *EcoR I* and *Sal I* and ligated into the PGEX-5X-3 vector (Pharmacia) at the same restriction sites to produce an expression vector for the GST-nardilysin acidic domain constructs. Chimeras of the mouse and human acidic domains were generated by ligation of cDNA fragments at a common *Hinf I* site. Deletion mutants were also generated using the *Hinf I* site. The resulting constructs were designated as m-pGEX-AD, which encodes the mouse nardilysin acidic domain fused to GST; h-pGEX-AD, which encodes the human nardilysin acidic domain fused to GST; pGEX-m-h, which is a chimera encoding the N-terminal region of the mouse nardilysin acidic domain and the C-terminal region of the human nardilysin acidic domain fused to GST; pGEX-h-m, which is a chimera encoding the N-terminal region of the human nardilysin acidic domain and the C-terminal region of the mouse nardilysin acidic domain fused to GST; pGEX-m-N-terminus, which contains the N-terminal region of the mouse nardilysin acidic domain fused to GST; and pGEX-h-N-terminus, which contains the N-terminal region of the human nardilysin acidic domain fused to GST. Each construct was sequenced with the Thermo-Sequenase Kit (Amersham) with oligos: TCA GTC AGT CAC GAT GCG and ATC GAA GGT CTG GGG ATC CCC.

Expression of GST Fusion Proteins. E. coli strain BL-21 was transformed with the desired cDNA construct, plated on LB agar plates, and cultured overnight at 37 °C. An isolated colony was used to inoculate 50 mL of LB media containing ampicillin at 100 μg/mL. Following overnight growth at 37 °C, 1.5 mL of this culture was used for plasmid isolation and digested with *EcoR I* and *Sal I* to confirm the presence of the insert. Twenty milliliters of this culture was used to inoculate 1 L of LB media. When the cell density reached an OD_{600} of 0.5−1.0, isopropy-β-D-thiogalactoside was added to 0.5 mM and the GST fusion protein was induced at 37 °C for 3−4 h. The induced cells were harvested and washed with PBS, pH 7.4. Cells were stored at −80 °C until use.

Purification of GST-Nardilysin Acidic Domain Fusion Proteins. Frozen cells were suspended in 10 vol of PBS, pH 7.4, containing 1 mM EDTA, 1 mM phenylmethylsulfonylfluoride, and 1 mM dithiothreitol (PBS-EPD). Cells were broken with a French pressure cell and then centrifuged at 30000*g* at 4 °C for 30 min. The supernatant was applied to a glutathione-agarose column and washed with PBS-EPD. The fusion protein was eluted with 25 mM glutathione in PBS-EPD. Fractions containing fusion protein were pooled and concentrated/dialyzed with 10 mM sodium phosphate buffer, pH 6.5, using a Centricon Plus-20 spin column (Millipore). The concentrated fusion protein was stored at −20 °C until use. GST was purified in the same manner.

Measurement of Spermine Binding. Recombinant nardilysin (final concentration ∼20 μM) or GST-nardilysin

acidic domain fusion proteins (final concentration 10 to 20 μM) were incubated at 30 °C for 10 min with [^{3}H]spermine ($0.6-1.1 \times 10^5$ cpm) in the presence of varying concentrations of nonradioactive spermine in a final volume of 80 μL of 10 mM sodium phosphate buffer, pH 6.5. The mixture was applied to a 0.8 mL Bio-gel P_6-DG (Bio-Rad) spin column prepared in a 1 mL syringe and previously equilibrated in the 10 mM sodium phosphate buffer. The column was centrifuged for 2 min at 2000 rpm in a Sorvall GLC-1 centrifuge at room temperature, according to the method of Penefsky (*10*). Bound spermine was determined by the radioactive content of the excluded volume based on the specific radioactivity of the added spermine. Eluted protein was determined using the Coomassie Plus protein assay reagent (Pierce Chem. Co.). Blank samples in which either GST, BSA, or no protein was added were used as controls. Blank values represented less than 0.2% of input radioactivity. Binding data were fit to either a hyperbolic or a sigmoidal curve using Sigma plot. When appropriate, data were analyzed according to the nonlogarithmic form of the Hill equation (*11*): $Y = BS^h/(K_{0.5}^h + S^h)$; where Y is the degree of saturation of the fusion protein by spermine, h is the hill coefficient, and $K_{0.5}$ is the concentration of spermine at which 50% saturation is achieved, B = maximum bound, and S is the substrate concentration.

Fluorescence Binding Measurements. The fluorescent spectra of nardilysin was determined using a Hitachi F-2000 spectrofluorometer.

N-Terminal Sequencing. N-terminal sequencing was performed by the automated Edman degradation method at the University of Kentucky Molecular Structure Analysis Facility.

RESULTS

We have previously provided kinetic evidence that an acidic stretch found in nardilysin can serve as a regulatory domain binding polyamines (*9*) and proteins (K. M. Chow, Z. Ma, and L. B. Hersh, manuscript in preparation). To directly demonstrate the binding of polyamines to nardilysin, we expressed the mouse enzyme in Sf-9 cells through the baculovirus system. Our initial construct was one in which methionine 1 of nardilysin was fused to a His_6 affinity tag through a polylinker region containing a TEV protease site. Although this construct produced active nardilysin, Western blot analysis with an anti-His_6 antisera showed that the His_6 affinity tag was absent. The use of protease inhibitors during cell disruption failed to preserve the affinity tag. Purification by conventional techniques (see Methods) yielded pure enzyme which when subjected to N-terminal sequence analysis yielded the sequence Pro−Gly−Arg−Asn−Lys−Ala−Lys−Ser−Thr, which indicates the enzyme was cleaved between Met 49 and Pro 50. Since this form of the enzyme appeared kinetically identical to the wild type enzyme purified from rat testes, we prepared a second construct in which glycine 51 was fused in frame to the polylinker. This form of the enzyme yielded good expression in Sf-9 cells and was easily purified by chromatography on a nickel-nitrilotriacetic acid column. The His_6 affinity tag and most of the polylinker was subsequently removed by treating the purified nardilysin with TEV protease as described in Materials and Methods. It is worth noting that Hospital et al. (*12*) recently reported the presence of a truncated form of rat nardilysin in testes, which had valine 77 as the N-terminal amino acid. They also observed that recombinant nardilysin expressed in the BSC 40 cell line was cleaved between leucine 80 and glycine 81. Thus, the N-terminus of nardilysin is likely to contain unstructured regions which are susceptible to proteolysis.

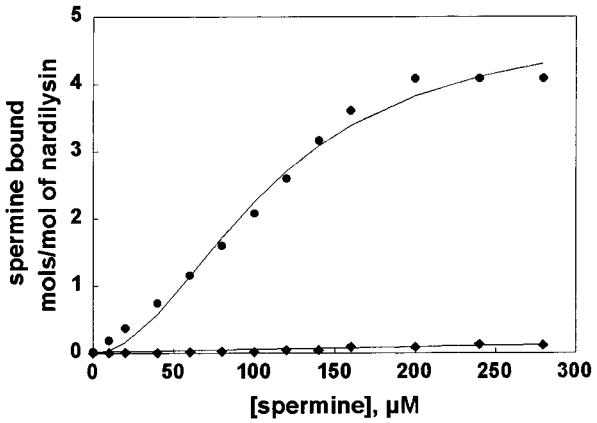

FIGURE 1: Binding of spermine to mouse nardilysin. Mouse nardilysin (17.3 μM) (●) or BSA (20 μM) (◆) was incubated with the indicated concentration of spermine in the presence of a constant amount of ^{3}H-spermine (6×10^4 cpm) in 10 mM sodium phosphate buffer, pH 6.5, for 30 min at room temperature. Bound spermine was separated from free spermine by the method of Penefsky (*10*) as described in the methods. The data were plotted according to the Hill equation $Y = BS^h/(K_{0.5}^h + S^h)$ as described in Materials and Methods.

Table 1: Kinetic Parameters for Spermine Binding to Nardilysin and the Nardilysin Acidic Domain GST Fusion Proteins[a]

sample	maximal number of binding sites B	Hill coefficient h	$K_{0.5}$ (μM)
nardilysin	5.1 ± 0.4	1.8 ± 0.2	118.2 ± 13.6
GST-mAD	7.4 ± 0.4	1.4 ± 0.1	103.0 ± 10.2
GST-hAD	5.4 ± 0.4	1.1 ± 0.1	110.3 ± 18.2
GST-m−h	9.8 ± 0.4	1.5 ± 0.1	44.7 ± 4.3
GST-h−m	3.6 ± 0.2	1.3 ± 0.1	64.4 ± 6.2
GST-m−N-terminus	3.6 ± 0.1	1.4 ± 0.1	60.9 ± 3.5
GST-h−N-terminus	1.0 ± 0.1	1.1 ± 0.11	37.0 ± 5.5

[a] Binding data was fit to the Hill equation $Y = BS^h/(K_{0.5}^h + S^h)$ as described in Materials and Methods. Values given are ± their standard error.

The binding of spermine to recombinant nardilysin was determined by incubating varying concentrations of ^{3}H-spermine with the enzyme, and separating the protein−spermine complex from free spermine on a Bio-gel P_6-DG spin column according to the method of Penefsky (*10*). As can be seen in Figure 1, nardilysin maximally bound 5 mol of spermine/mol of enzyme. The data best fit a sigmoidal binding curve indicating cooperativity and were fit to the Hill equation. As shown in Table 1 a Hill coefficient of 1.8 was obtained with a $K_{0.5}$ value of 118 μM. This cooperative binding of spermine is reminiscent of the cooperativity previously observed when measuring the effect of spermine on the enzyme activity and thermal stability of rat nardilysin (*9*).

Previous kinetic data (*6*) indicated that polyamines bind to nardilysin with the relative affinity being spermine > spermidine > putrescine. We confirmed this directly by testing the ability of these polyamines to displace ^{3}H-spermine from recombinant mouse nardilysin. As shown in

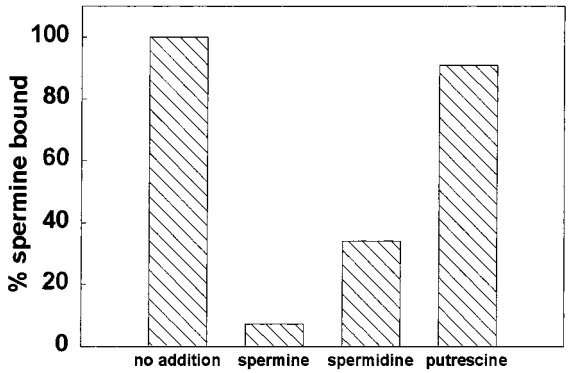

FIGURE 2: Comparison of the relative affinity of mouse nardilysin for polyamines. The ability of a 5 mM solution of the indicated polyamine to compete for 100 μM ^{3}H-spermine (8.7×10^4 cpm). Binding to mouse nardilysin was measured as described in Figure 1. The values obtained in the absence of added polyamine (2 mol of spermine bound/mol of nardilysin) was taken as 100%.

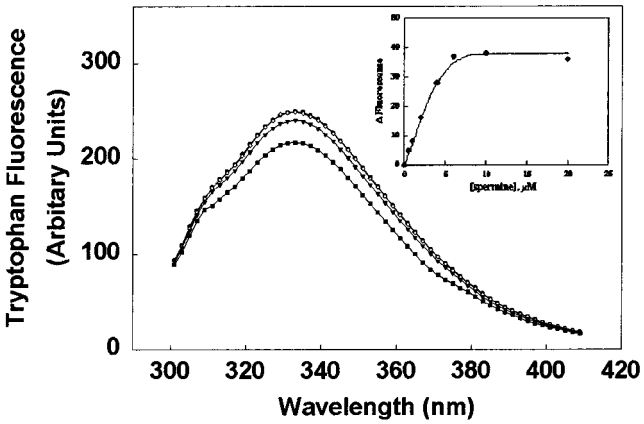

FIGURE 3: Tryptophan fluorescence emission spectra of nardilysin in the absence and presence of 100 μM polyamine. Emission spectra of 76 nM mouse nardilysin in 10 mM potassium phosphate buffer, pH 7.0, were obtained following excitation at 275 nm and emission at 333 nm. Nardilysin alone (○), nardilysin + 100 μM putrescine (◆), nardilysin + 100 μM spermidine (▼), and nardilysin + 100 μM spermine (■). The insert shows the change in nardilysin fluorescence as a function of spermine concentration.

Figure 2, at a concentration of 5 mM, spermine is more effective than spermidine, which is more effective than putrescine, in displacing 100 μM spermine.

The cooperative nature of spermine binding suggests the possibility that the interaction of spermine with nardilysin induces a conformational change, which facilitates further spermine binding. To provide evidence for such a conformational change, we compared the tryptophan fluorescence of the enzyme alone to that in the presence of polyamines. As shown in Figure 3, the addition of 100 μM spermine to nardilysin produced a decrease in tryptophan fluorescence indicating the environment around one or more tryptophans has changed. A smaller, but reproducible, decrease in tryptophan fluorescence was seen in the presence of 100 μM spermidine; however, at this concentration putrescine had no effect on tryptophan fluorescence, Figure 3. The insert to Figure 3 shows a titration of the fluorescence change as a function of spermine concentration. The data fit a hyperbolic binding curve, yielding a K_d of ~5 μM spermine. This suggests that the observed fluorescent change represents the binding of one of the first molecules of spermine.

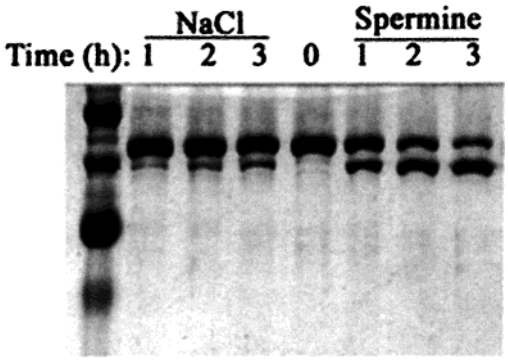

FIGURE 4: Spermine enhances cleavage of nardilysin by protease V8. Nardilysin (5 μg) was digested with protease V8 at a molar ration of 1/100 for the indicated time in 10 mM sodium phosphate buffer, pH 6.5, containing either 100 μM spermine or 400 μM NaCl.

To obtain additional evidence for a spermine-induced conformational change, we measured the protease sensitivity of nardilysin in the presence or absence of spermine. As shown in Figure 4, V8 protease cleaved nardilysin (molecular mass ~ 130 kDa) producing one major product (molecular mass ~ 105 kDa), and this cleavage was enhanced by the presence of 100 μM spermine relative to 400 μM NaCl. We estimated the concentration dependence on spermine for the enhancement in V8 protease sensitivity by measuring the fraction of nardilysin cleaved in 3 h as a function of spermine concentration. A saturating effect was seen, with 50% of the maximal enhancement occurring at about 60 μM spermine. To determine where protease V8 cleaved nardilysin we used Western blot analysis with three different antisera: a polyclonal antisera to mouse nardilysin, an antipeptide antisera directed toward an epitope within the acidic domain of mouse nardilysin, and an antipeptide antisera directed toward an epitope within the C-terminal region of mouse nardilysin. This analysis showed that the N-terminal region of nardilysin, including the epitope in the acidic domain, was removed by protease V8. This finding is consistent with our unpublished studies which show a protease sensitive site in nardilysin located near the C-terminal end of the acidic domain.

To determine whether it is the acidic stretch that serves as a functional amine binding domain, we expressed this region from mouse and from human nardilysin as fusion proteins with glutathione transferase, Figure 5. These fusion proteins were used to compare their polyamine binding properties to that of the intact protein. The fusion proteins were expressed in *Escherichia coli* and purified by affinity chromatography on a glutathione column. We initially measured the binding of spermine to the GST-mouse nardilysin acidic domain fusion protein (GST-mAD). Spermine binding to GST-mAD appeared similar to that seen with the native protein, with a sigmoidal binding curve. As shown in Table 1, a maximum of ~7 mol of spermine bound/mol of fusion protein, with a Hill coefficient of 1.4 and a $K_{0.5}$ of ~100 μM. These values are similar to those obtained with the intact enzyme. To determine whether the GST-mAD retains the same selectivity for polyamines as the native enzyme, spermidine and putrescine were compared to spermine for their ability to displace ^{3}H-spermine from GST-mAD. Although not shown, the same order of selectivity of spermine > spermidine > putrecine seen with the native enzyme is retained in the GST-mAD.

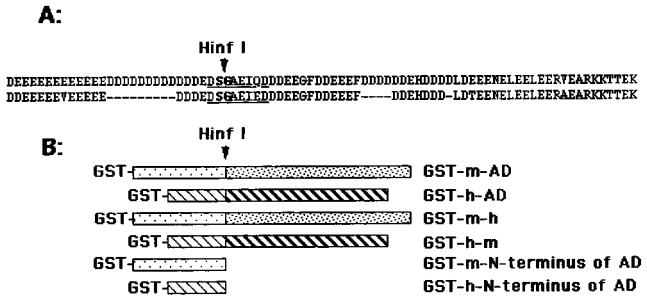

FIGURE 5: Structure of the human and mouse nardilysin acidic domain and nardilysin GST fusion proteins. (A) Comparison of the amino acid sequence of mouse (upper sequence) and human (lower sequence) nardilysin. The cleavage of the respective cDNAs by *Hinf I* was used to produce the indicated fragment. (B) Schematic of the GST-fusion proteins generated in this study. The abbreviations used: GST-mAD is the mouse acidic domain fused to the C-terminus of GST. GST-hAD is the human acidic domain fused to the C-terminus of GST. GST-m-h represents a chimera containing the N-terminal 28 amino acids of the mouse acidic domain joined to the C-terminal part of the human acidic domain and fused to the C-terminus of GST. This was accomplished by ligating the cDNAs at the *Hinf I* restriction site. GST-h-m represents a chimera containing the N-terminal 19 amino acids of the human acidic domain joined to the C-terminal part of the mouse acidic domain and fused to the C-terminus of GST. This was accomplished by ligating the cDNAs at the *Hinf I* restriction site. GST-m-N-terminus is the N-terminal 28 amino acids of the mouse acidic domain fused to the C-terminus of GST. GST-h-N-terminus is the N-terminal 19 amino acids of the human acidic domain fused to the C-terminus of GST.

Since human nardilysin has fewer acidic residues within its acidic domain (see Figure 5) we measured the binding of spermine to the GST-human nardilysin acidic domain fusion protein (GST-hAD). GST-hAD bound ~5 mol of spermine/mol in contrast to the 7 mol/mol bound by GST-mAD. The $K_{0.5}$ for spermine binding was ~100 μM. Spermine binding to the human acidic domain appeared hyperbolic rather than sigmoidal, with a Hill coefficient of ~1, Table 1. Although not shown, the human acidic domain showed an order of selectivity toward polyamines of spermine > spermidine > putrecine, the same as seen with both the intact mouse enzyme and the mouse acidic domain.

The mouse and human acidic domains show a high degree of sequence conservation with the major difference being the deletion of 10 acidic residues in the N-terminal portion of the human acidic domain, Figure 5. We thus explored the possibility that this difference could account for the both the difference in the number of moles of spermine bound and the cooperativity seen with the mouse acidic domain. Chimeras were generated in which the N-terminal region of the mouse nardilysin acidic domain was fused to the C-terminal region of the human nardilysin acidic domain and vice versa, see Figure 5. The GST chimeric fusion protein containing the N-terminal region of the mouse nardilysin acidic domain fused to the C-terminal region of the human nardilysin acidic domain (GST-m-h) bound ~10 mol of spermine/mol of fusion protein with an $K_{0.5}$ of ~45 μM. The data fit a sigmoidal binding curve with a Hill coefficient of 1.5, Table 1. The reverse chimera, in which the N-terminal part of the human acidic domain was fused to the C-terminal part of the mouse acidic domain (GST-h-m), bound ~3−4 mol of spermine/mol with a $K_{0.5}$ of ~60 μM, and a Hill coefficient of 1.4, Table 1.

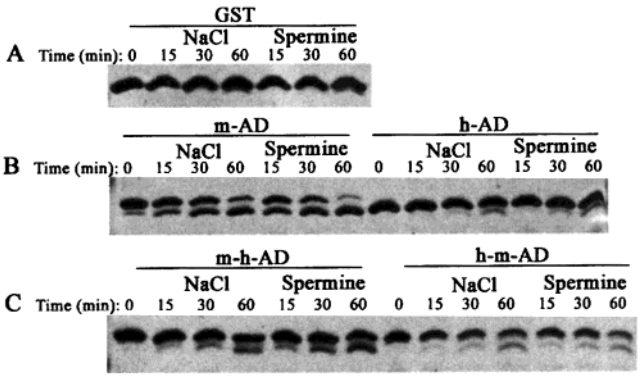

FIGURE 6: Digestion of nardilysin acidic domain-GST fusion proteins in the presence and absence of spermine. Nardilysin acidic domain GST fusion proteins were digested with protease V8 in the presence or absence of spermine as described in Figure 4 for the indicated times. (A) GST alone; (B) GST-mAD (left) and GST-hAD (right); (C) GST-m-h (left) and GST-h-m (right).

We also expressed the N-terminal region of mouse and human nardilysin as GST fusion proteins. As shown in Table 1, the N-terminal region of the mouse acidic domain binds 3−4 mol of spermine/mol. The binding curve appears cooperative with a Hill coefficient of 1.4, and a $K_{0.5}$ of ~60 μM. In contrast the N-terminal region of the human nardilysin acidic domain binds 1 mol of spermine/mol in a noncooperative manner, a $K_{0.5}$ of ~40 μM.

To determine whether the binding of spermine to these fusion proteins could produce a conformational change, we compared their sensitivity to V8 protease in the presence or absence of spermine. As shown in Figure 6, panel B, the sensitivity of the mouse acidic domain fused to GST to protease V8 digestion was enhanced by spermine. In contrast, GST alone was not cleaved by protease V8 (Figure 6, panel A). The human acidic domain fused to GST was relatively resistant to protease V8, and spermine had no effect (Figure 6, panel B). The chimeric fusion protein containing the N-terminal region of the mouse nardilysin acidic domain fused to the C-terminal region of the human nardilysin acidic domain mimicked the mouse acidic domain in both sensitivity to protease V8 and enhancement of this sensitivity by spermine (Figure 6, panel C). Similarly, the chimeric fusion protein containing the N-terminal region of the human nardilysin acidic domain fused to the C-terminal region of the mouse nardilysin acidic domain mimicked the human acidic domain, (Figure 6, panel C). To provide insight as to where protease V8 cleaved the fusion proteins, we conducted Western blot analysis with the anti-acidic domain anti-peptide antisera described above. In each case where protease V8 cleavage was observed, the epitope remained attached to GST, indicating cleavage in the C-terminal region of the acidic domain of the fusion protein.

DISCUSSION

A sigmoidal saturation curve was observed for the binding of spermine to nardilysin indicating cooperative binding. This suggests a model in which binding of the first molecule of spermine induces a conformational change, which facilitates the binding of additional spermine molecules. Since nardilysin is a monomeric protein, this conformational change differs from that seen in most allosteric proteins where such a conformational change is generally mediated through

subunit interaction (*13*). In nardilysin, a conformational change occurs within the same molecule and is likely a result of changes in electrostatic interactions within the acidic domain. Evidence favoring a conformational change is the observed decrease in tryptophan fluorescence and the enhanced protease sensitivity produced by spermine. As described below, the acidic stretch of nardilysin functions as an autonomous domain displaying many of the characteristics of native nardilysin in terms of binding spermine. Although it is likely that spermine binding causes a conformational change within the acidic domain, this region is devoid of any tryptophans. Thus, the observed change in tryptophan fluorescence likely reflects a conformational change elsewhere in the molecule. This is consistent with our previous observation that polyamines, particularly spermine, can act as noncompetitive inhibitors of substrate hydrolysis (*9*). The binding of spermine thus causes a conformational change that not only affects the binding of additional spermine molecules, but also alters the conformation of the active site of the enzyme. Since we did not observe a difference in the circular dichroism spectrum of nardilysin in the presence or absence of spermine (E. Csuhai, unpublished results) any spermine-induced conformational changes are localized and do not affect the structure globally. Using antisera generated to a peptide within the acidic domain, we have found that the protease V8 site lies between the acidic domain and the active site as defined by the HFLEH motif. Since protease sensitive sites are associated with "open" or unstructured regions of a protein, the effect of spermine binding to the acidic domain appears to decrease the secondary structure in this region. We suggest that this localized structural change results in a subtle change in the conformation of the active site making the enzyme less active.

A GST-fusion protein containing the acidic stretch of mouse nardilysin binds ~7 mol of spermine, retains cooperative spermine binding, and its sensitivity to protease V8 is also enhanced by spermine. These findings provide evidence that the acidic stretch of nardilysin acts as a functional domain. These data further suggest that the mouse acidic domain is structured and that its structure is independent of, but linked to, the conformation of the active site. Within the fusion protein, the structure of the acidic domain may be less structured than within the native enzyme permitting more molecules of spermine to bind. However, the findings that this domain is relatively resistant to protease V8 digestion, and that spermine binding increases protease V8 sensitivity, are consistent with the proposal that the acidic domain becomes less structured upon spermine binding facilitating additional spermine binding. This facilitated spermine binding is observed kinetically as a Hill coefficient of greater than one.

The acidic domain from human nardilysin, which contains fewer acidic residues than does the mouse acidic domain, binds fewer molecules of spermine (5 versus 7), does not exhibit cooperativity for spermine binding, and is more resistant to protease V8 even in the presence of spermine. These observations suggest that the human acidic domain is more structured and that spermine binding to it does not produce a detectable structural change.

The chimera between the N-terminal part of the mouse acidic domain and the C-terminal part of the human acidic domain binds ~10 mol of spermine/mol and retains cooperativity. Spermine binding increases protease sensitivity of this chimera as seen with the intact mouse acidic domain. The opposite chimera consisting of the N-terminal part of the human acidic domain and the C-terminal part of the mouse acidic domain also shows cooperativity but is resistant to protease digestion even in the presence of spermine. These findings suggest that it is spermine binding to the N-terminal region of the mouse acidic domain that produces the structural change that alters protease sensitivity. It is likely that spermine binding to the C-terminal part of the mouse acidic domain produces the conformational change that produces cooperativity in spermine binding. The difference between the mouse and human acidic domains in terms of a spermine induced conformational change are consistent with the decreased sensitivity of the human enzyme to polyamine inhibition (*6*).

Taken together, these studies demonstrate that the acidic domain of nardilysin serves as an functional polyamine binding domain. Binding to this domain produces a conformational change in the protein that alters catalytic activity. Preliminary studies (K. M. Chow, unpublished results) indicate that there are cellular proteins that specifically bind to the acidic domain of nardilysin. The role of these proteins in regulating nardilysin activity is currently under investigation.

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