Regulation of *N*-Arginine Dibasic Convertase Activity by Amines: Putative Role of a Novel Acidic Domain as an Amine Binding Site†

Eva Csuhai, Guojin Chen, and Louis B. Hersh*

Department of Biochemistry, College of Medicine, Chandler Medical Center, University of Kentucky, 800 Rose Street, Lexington, Kentucky 40536-0084

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ABSTRACT: Peptide sequence analysis and cDNA cloning indicate that a previously described mouse arginine-specific dibasic cleaving enzyme (dynorphin converting enzyme) [Csuhai et al. (1995) *Biochemistry 34*, 12411] is the homologue of *N*-arginine dibasic convertase (NRDc) isolated from rat testis [Chesneau et al. (1994) *J. Biol. Chem. 269*, 2056]. A mouse NRDc cDNA exhibited 98% amino acid identity with the rat cDNA. However, within a 74 residue acidic stretch, this identity drops to 82%. Likewise, the corresponding acidic stretch of human NRDc is only 73% identical with that of rat NRDc. To reconcile previously observed kinetic differences between rat and mouse NRDc, the hydrolysis of peptide substrates by the rat, human, and mouse enzymes was compared using phosphate and Tris as buffers. Although the three NRDc's behaved similarly, Tris had a pronounced effect on the kinetics of peptide hydrolysis. With BAM-8, α-neoendorphin, and dynorphin B as substrates, Tris increased K_M up to 40-fold with little change in V_{max}, while with dynorphin A or somatostatin 28 as substrate, Tris caused a decrease in K_M of up to 100 fold, again with only a modest change in V_{max}. Other amines, including the polyamines putrescine, spermidine, and spermine, all affected NRD convertase activity. It is proposed that amines bind to the acidic stretch found in NRDc, and that quantitative differences in the sensitivity to amines between the rat, mouse, and human enzymes can be at least partially accounted for by differences in their acidic stretch. The role of polyamines as physiological modulators of *N*-arginine dibasic convertase is considered.

Endogenous opioid peptides are presumed to play a role in the regulation of not only nociceptive responses but also cell proliferation and the immune response (*1*, *2*). These peptides regulate biological functions through interactions with opioid as well as nonopioid receptors (*3*, *4*). The regulation of the activities of these peptides takes place mainly through cleavage by ectopeptidases found on the cell surface or by peptidases secreted into the extracellular environment. Intracellular cleavage of some neuropeptides by cytosolic or lysosomal peptidases is also likely, after receptor-mediated internalization (*5*, *6*). Endopeptidase cleavage of dynorphins A and B and α-neoendorphin to yield enkephalin derivatives yields peptides with differing receptor specificity and altered physiological activity. Thus, the processing of dynorphins and α-neoendorphin by endopeptidases is of potential significance in the regulation of the immune response and cell proliferation as well as nociceptive responses.

A group of enzymes believed to be involved in the processing of dynorphin A and/or dynorphin B to generate Leu-enkephalin-Arg[6] were classified as dynorphin converting enzymes (*7*, *8*). We previously reported the purification and characterization of a secreted arginine-specific dibasic cleaving enzyme with dynorphin converting enzyme activity from the EL-4 mouse lymphoma cell line (*9*). The secretion of this peptidase represents a possible new mechanism for the regulation of extracellular dynorphins. The arginine-specific dibasic cleaving enzyme we isolated appeared similar, but kinetically distinct, from a previously described rat enzyme, *N*-arginine dibasic convertase (NRDc)[1] (*10*). NRD convertase was described as a 140 kDa metalloendopeptidase belonging to the inverzincin family of Zn metalloenzymes, in which the putative Zn-binding motif differs from that of other zinc metallopeptidases in being inverted to HXXEH. The enzyme cleaves peptides at the N-terminus of arginine residues in dibasic sequences. Its in vitro substrates include dynorphins A and B, α-neoendorphin, somatostatin 28, and preproneurotensin and their smaller fragments. Based on its abundance and localization in testes, it was suggested that NRD convertase is involved in spermiogenesis (*11*), although its physiological substrates have not been identified.

In the present study, we show that the EL-4 arginine-specific dibasic cleaving enzyme we previously described is the mouse homologue of rat NRD convertase. A kinetic analysis of the rat, mouse, and human NRDc suggests that the previously observed kinetic differences between mouse and rat NRD convertase can be ascribed in part to differences

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* Address correspondence to this author. Phone: (606) 323-5549. Fax: (606) 323-1727. E-mail: lhersh@pop.uky.edu.

[1] Abbreviations: BAM, bovine adrenal medulla peptide; Bis-Tris propane, 1,3-bis[tris(hydroxymethyl)methylamino]propane; HPLC, high-pressure liquid chromatography; NRDc, *N*-arginine dibasic convertase; PBS, phosphate-buffered saline; RT PCR, reverse transcription polymerase chain reaction; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; Tris, tris(hydroxymethyl)aminomethane.

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in assay conditions, but also in part to differences in their primary sequence.

MATERIALS AND METHODS

Materials. Chemicals were purchased from Sigma Chemical Co., unless otherwise indicated, and were used without further purification. Tissue culture media and reagents were from Gibco BRL. Peptides were obtained from Bachem California and Peninsula Laboratories. [α-^{35}S]dATP for sequencing was purchased from ICN.

Cell Culture. EL-4, Jurkat, and MOLT-4 cells were grown in RPMI 1640 with 5% fetal bovine serum in the presence of 100 units/mL penicillin and 100 μg/mL streptomycin at 37 °C with 10% CO_2. Cultures were maintained between 0.5 and 5 × 10^6 cells/mL. Cells were harvested by centrifugation at 450*g* for 10 min and then washed 2 times with PBS and stored at −80 °C until further use.

Assay of NRDc Activity. NRD convertase activity was routinely assayed with bovine adrenal medulla peptide (1−8) (BAM-8) as substrate. Reaction mixtures contained 20 mM potassium phosphate buffer, pH 7.0, 60 μM BAM-8, and enzyme, in a final reaction volume of 100 μL. Following a 15 min preincubation period, the reaction was initiated with substrate and allowed to proceed to 10−25% conversion, generally 5−30 min, at 37 °C. The reaction was stopped by boiling for 5 min, and then 10 μL of 5% trifluoroacetic acid was added. The samples were centrifuged for 2 min in an Eppendorf 5415C centrifuge and injected onto a Vydac C_4 reverse phase HPLC column for analysis with detection at 214 nm. Activity was quantitated by measuring the peak area of the product, Met-enkephalin-Arg^6.

Alternative substrates and inhibitors were studied in similar reaction mixtures. Kinetic constants were obtained by measuring the dependence of the reaction rate on substrate or inhibitor concentration. The kinetic constants K_M, V_{max}, and K_I were calculated using the kinetic programs of Cleland (*12*). Cleavage products were identified by co-injection with commercially available authentic peptides. K_M values for dynorphin A and somatostatin 28 were determined using these peptides as alternative substrate inhibitors of bovine adrenal medulla peptide (1−8) hydrolysis (*13*).

Protein Purification. EL-4 cells were grown and the dibasic specific endopeptidase (mouse *N*-arginine dibasic convertase, NRDc) was purified as previously described (*9*). *N*-Arginine dibasic convertase was purified from rat testes as described by Chesneau et al. (*10*).

Human NRD convertase was purified from 15 g of Jurkat T cells analogously to the procedure described for purification of EL-4 cell derived NRD convertase (*9*). Briefly, Jurkat T cells were homogenized at 4 °C in 100 mL of 20 mM Tris-HCl, pH 7.5, first with 10 strokes of a tight-fitting Potter−Elvehjem homogenizer, followed by sonicating the homogenate 3 times for 10 s with an Ultrasonics W-220F cell disrupter set at 30% maximal power. The homogenate was cooled on ice for 2 min between each round of sonication. This homogenate was centrifuged at 30000*g* for 70 min at 4 °C, and then loaded at a flow rate of 1.5 mL/min onto a 15 mL QMA Accell Plus column connected to a Waters 650 Protein Purification system. Following this step, the purification scheme was identical to the one described in Csuhai et al. (*9*).

Immunoblotting was carried out with a rabbit anti-NRDc antiserum, kindly provided by Dr. Paul Cohen, as described (*10*).

Cloning a Mouse NRDc cDNA. An oligo(dT)-primed Uni-Zap XR cDNA library from adult mouse testis (*14*) was used for isolating a mouse NRD convertase cDNA. All steps of the screening procedure were carried out according to protocols developed by Stratagene. Screening was carried out using radiolabeled PCR-generated DNA probes prepared from EL-4 mRNA and primers based on the rat NRD convertase sequence (*15*). Probe 1, corresponding to nucleotides 312−811 of the rat NRDc sequence, was prepared with the PCR primers 5′ TGAATCTGAGGAGGAGGGACG and 3′ CATGTGCTCCAAAAAGTG. A second probe (probe 2), which includes nucleotides 2664−3162 of the rat NRDc cDNA, was prepared in the same manner using PCR primers 5′ TTGTTGAGGGCCTGGTGCA and 3′ ATCAGAGCTGTGACCTGAGT. Fifteen filters containing approximately 30 000 plaques each were hybridized with probe 1 at 42 °C in 6 × SSC, 1 × Denhardt's solution, 100 μg/mL yeast tRNA, and 0.05% sodium pyrophosphate based on published protocols (*16*). Sixty-four putative positive clones were isolated and rescreened with probe 2. From this second screen two clones were isolated, both ∼3.7 kb in size. One of these was rescued using phage-mediated in vivo excision as a pBSK II plasmid, and sequenced from both ends. Oligonucleotides were synthesized on an Oligo 1000 DNA synthesizer (Beckman).

RT-PCR Conditions for the Amplification of a Human NRDc cDNA Fragment. The oligonucleotide primers 5′ TTCAGATCTAAGTAATATGGAAGGTAAAA and 3′ TGGTATCTGAGAGAAGATCTCCGTCA were used to amplify a fragment of the human NRDc gene by RT-PCR (*17*). Using mRNA isolated from the MOLT-4 cell line, this procedure resulted in the amplification of a 0.81 kb partial clone, spanning bases 438−1309 of the rat NRD convertase sequence. This PCR fragment was subcloned into the *Eco*RV site of the pBSK II vector and sequenced.

DNA Sequencing. Oligonucleotide primers of 22−26 nucleotides each, based on the rat, mouse, and human NRDc cDNA sequences, were synthesized on an Oligo 1000 DNA synthesizer (Beckman). Plasmid DNA in the pBSK II vector was isolated from 2 mL cultures of *E. coli* XL-1 Blue using the Wizard Plus Minipreps (Promega) DNA Purification System, based on the manufacturer's instructions. Sequenase Version 2.0 DNA Sequencing Kit (United States Biochemical) was used for sequencing reactions, which were developed on 6% polyacrylamide gels.

Other Methods. LysC digestion and peptide sequencing was done at the Macromolecular Structure Analysis Facility at the University of Kentucky. SDS−PAGE and silver staining of the gels were carried out according to published procedures (*18*, *19*).

RESULTS

This laboratory reported the isolation and characterization of an arginine-specific dibasic cleaving enzyme that exhibited dynorphin converting enzyme activity from the mouse EL-4 cell line. This enzyme cleaves peptide substrates between dibasic amino acids when arginine occupies the P1′ position (*9*). Mouse arginine-specific dibasic cleaving enzyme re-

sembled a previously described activity purified from rat testes and referred to as NRD convertase (*10*). The published data indicated that the two enzymes exhibit the same molecular weight, the same sensitivity to metallopeptidase inhibitors, and the ability to cleave the same peptide substrates. However, the published kinetic constants for peptide hydrolysis by the mouse arginine-specific dibasic cleaving enzyme and the rat NRD convertase differed significantly. For example, dynorphin B was cleaved ~2 times faster than dynorphin A by rat NRD convertase, but >100-fold faster than dynorphin A by the mouse dibasic cleaving enzyme. Similarly, rat NRD convertase cleaved α-neoendorphin at approximately the same rate as dynorphin B, while the mouse dibasic cleaving enzyme cleaved α-neoendorphin 3 times faster than dynorphin B.

To establish whether the mouse arginine-specific dibasic cleaving enzyme we described was indeed an NRD convertase, N-terminal sequencing was attempted on the enzyme isolated from EL-4 cells. However, a blocked N-terminus was encountered. The EL-4 enzyme was then subjected to lysC digestion, and N-terminal sequences were obtained for the two resulting peptide fragments: peptide 1, ANLVLLSGANEGRCDL; and peptide 2, SDLVSWFK. Peptide 1 corresponds exactly to residues 619−634 and peptide 2 to residues 1077−1084 of rat NRD convertase (*15*). The identity of these peptide sequences made it very likely that the enzyme we isolated is the mouse homologue of rat NRD convertase.

To substantiate this conclusion, a mouse testis cDNA library (*14*) was screened using probes constructed by RT-PCR based on the sequence of rat NRD convertase. Two clones of approximately the size of the rat cDNA (3.7 kb) were isolated, one of which was sequenced and found to represent a full-length clone. The complete sequence of the mouse and rat enzymes was found to be 94% identical at the nucleotide level and 98% identical at the amino acid level. The differences in the predicted amino acid sequence are shown in Figure 1. Although the identity between the mouse and rat sequences is extremely high, a notable exception is in a 74 residue acidic stretch of the protein encompassing residues 139−212. This region contains 57 (77%) glutamate plus aspartate residues in rat NRD convertase. Within this region, the identity of the mouse and rat enzymes decreases to 82%. The changes in this acidic region include a deletion of three glutamates, an insertion of three aspartates, and a change of a stretch of five glutamates to five aspartates. This was the only region where there were any considerable differences between the rat and mouse NRD convertases.

A search of computer databases revealed a partial clone (372 nucleotides) of what appeared to be a human NRD convertase (Genbank #T25336), which encompassed a portion of this acidic stretch. Based on this finding, we utilized oligonucleotide primers for RT-PCR and mRNA isolated from the human MOLT-4 cell line, which exhibits a high NRDc specific activity, to obtain a 474 bp DNA fragment containing the entire acidic stretch and the active site inverted zinc binding domain. We also sequenced a DNA fragment, obtained by RT-PCR, from human mRNA which spans nucleotides 438−1309 in the rat sequence. This part of the translated human protein sequence, which is also shown in Figure 1, indicates that within the acidic stretch the human NRD convertase sequence diverges even more from the rat sequence than does the mouse sequence. This region of the human enzyme has a total of 13 fewer negatively charged amino acids, which are derived primarily from deletions of stretches of 5, 3, and 4 consecutive acidic residues. Various other human cDNA fragments of a few hundred base pairs each that were highly homologous to the mouse and rat NRD convertase sequences have been deposited in databases, mostly as part of the Merck−Washington University sequencing project. The overlapping database fragments cover almost the complete cDNA clone for the human enzyme, with the exception of the N-terminus of the protein and residues 470−570. The predicted amino acid sequence is included in Figure 1.

It is worth noting that Prat et al. (*20*) recently reported the isolation of a variant NRDc cDNA which contains a 68 amino acid insert located between the acidic stretch and the active site zinc binding motif, HXXEH. We have used RT-PCR with primers that encompass the acidic stretch and the zinc binding motif to test for this variant in the cell lines used in this study. We were unable to detect this variant, indicating that a single NRDc form is produced predominantly if not exclusively in the cell lines used in this study.

The above results establish that the NRDc sequence is highly conserved between rat, mouse, and human with the exception of an acidic stretch which shows considerably greater variability in amino acid conservation. Thus, either differences in this acid region or differences in assay conditions could account for the observed kinetic differences noted between rat and mouse NRDc. Since the human NRDc was the most divergent, we decided to include this enzyme in our studies. We thus isolated human NRDc from Jurkat T cells using the purification scheme given in Table 1. We established that the rat, mouse, and human endopeptidases used in this study cross-reacted with an antiserum developed against rat NRD convertase, and that all three enzymes exhibited the same subunit molecular weight. The relative molecular weights for the secreted and intracellular forms of the mouse and human enzymes were identical based on SDS−PAGE and immunoblotting.

Since the published kinetic experiments conducted with rat NRD convertase utilized Tris buffer (*10*), while those conducted with the mouse enzyme (*9*) utilized phosphate buffer, we examined the possibility that kinetic differences could result from a buffer effect. We thus studied in more detail the effect of Tris and other amines on the kinetics of the NRD convertase reaction. At the same time, we compared the rat, mouse, and human enzymes to see if they differed from each other. Any differences between species could be attributed to sequence differences.

Using dynorphins A and B, α-neoendorphin, BAM-8, and somatostatin 28 as substrates, the kinetic parameters K_M and V_{max} were determined in potassium phosphate buffer. This is the buffer previously used to characterize the mouse enzyme. The results are shown in Table 2. All three enzymes appear similar, with BAM-8, α-neoendorphin, and dynorphin B being cleaved at similar rates, and with dynorphin A and somatostatin being cleaved at a slower rate. We next determined these same kinetic parameters in 100 mM Tris buffer, the assay condition used by Chesneau et al. (*10*) for rat NRD convertase. As can be seen in Table 3, Tris had a rather dramatic effect on the kinetics of all three NRD convertases. This effect was manifested primarily as

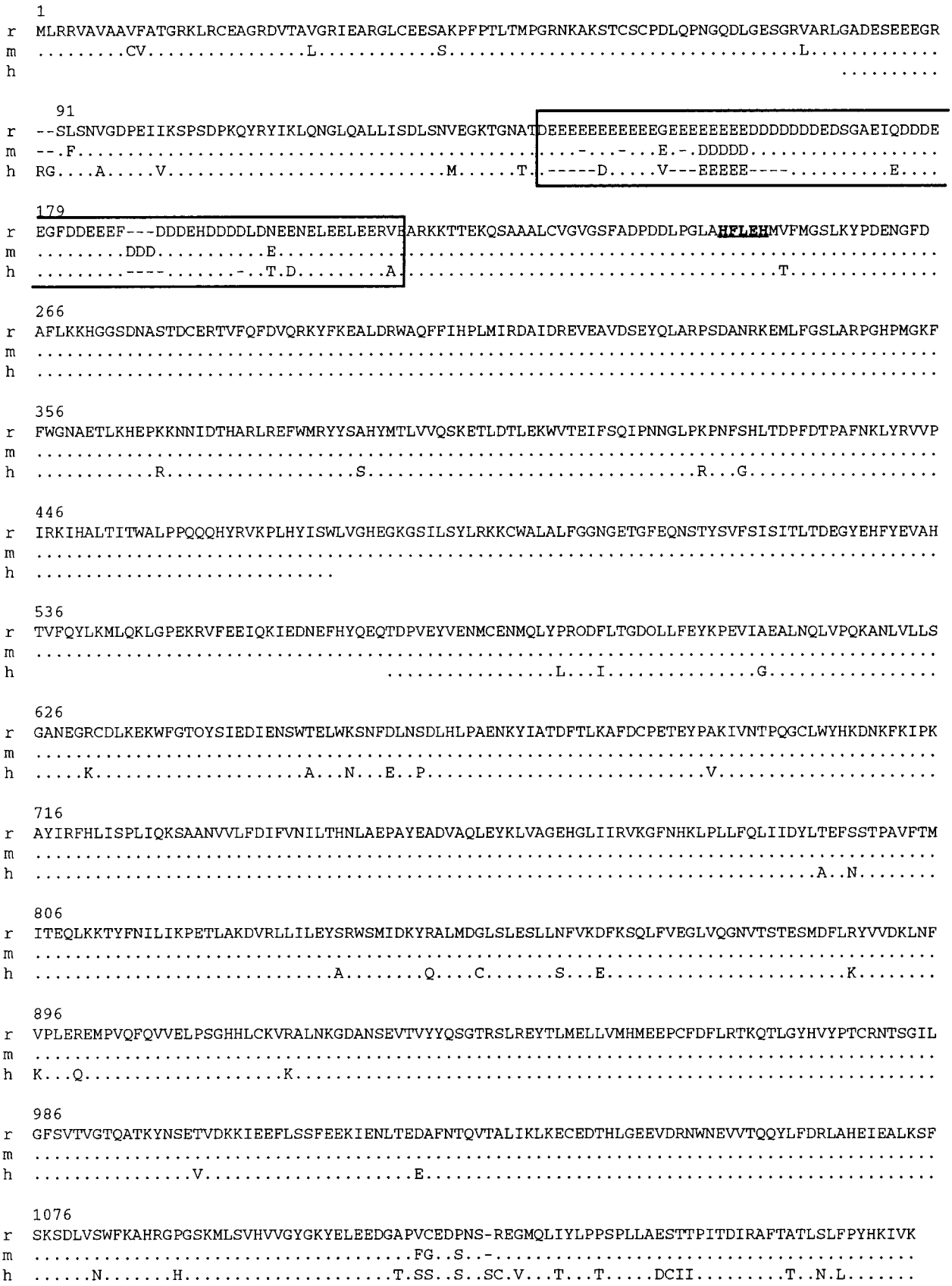


FIGURE 1: Comparison of partial amino acid sequences of rat, mouse, and human *N*-arginine specific dibasic convertases. The numbering shown corresponds to that of the rat testis enzyme (*7*). The human sequence is identified only in the region shown on this figure, whereas the rat and mouse cDNA's are fully sequenced. Dots in the figure indicate identical amino acids; horizontal lines indicate deletion of residues. The putative zinc binding site is underlined. The acidic stretch is boxed.

an increase in K_M for the smaller, more reactive peptides BAM-8, α-neoendorphin, and dynorphin B. In contrast, with the larger, less reactive peptides dynorphin A and somatostatin, Tris actually decreased K_M.

Table 1: Purification of NRD Convertase from Jurkat T Cells

purification step	total protein[a] (mg)	total activity[b] (μmol/min)	sp act. [μmol/(min·mg)]	recovery (%)	purification (*x*-fold)
homogenate	310	2139	6.9	100	1
QMA chromatography	18.9	1057	55.9	49.4	8.1
Phenyl 650S chromatography	0.97	1285	1325	60.1	192
Superdex 200 chromatography	0.26	264	1015	12.3	147
Mono Q chromatography	0.021	62.5	2900	2.9	420

[a] Protein concentrations were measured by using Coomassie Plus Protein Assay Reagent (Pierce) with BSA as a standard. [b] Enzyme activity was assayed with bovine adrenal medulla peptide (1−8) as a substrate at 60 μM final concentration in 20 mM potassium phosphate buffer, pH 7.0, by incubating 5−10 μL of the homogenate or appropriate column fractions for 10−15 min at 37 °C. The reaction rate was calculated based on the appearance of the product, Met-enkephalin-Arg^6, as determined by HPLC.

Table 2: Kinetic Parameters for the Hydrolysis of Peptide Substrates by Rat, Mouse, and Human NRD Convertases in Phosphate Buffer[a]

	mouse NRD convertase		rat NRD convertase		human NRD convertase	
peptide	K_M (μM)	V_{max} [μmol/(min·mg)]	K_M (μM)	V_{max} [μmol/(min·mg)]	K_M (μM)	V_{max} [μmol/(min·mg)]
BAM−8 (YGGFM**R**^**R**V)	6.0 ± 0.7	3.6 ± 0.10	28 ± 0.8	3.9 ± 0.07	19.1 ± 1.2	2.9 ± 0.087
α-neoendorphin (YGGFL**R**^**K**YPK)	3.6 ± 0.05	2.0 ± 0.01	6.5 ± 0.75	1.3 ± 0.03	17.4 ± 2.9	1.2 ± 0.126
dynorphin B (YGGFL**R**^**R**QFKVVT)	0.6 ± 0.07	3.5 ± 0.42	0.9 ± 0.007	2.1 ± 0.3	0.72 ± 0.11	2.3 ± 0.34

	mouse NRD convertase		rat NRD convertase		human NRD convertase	
peptide	K_M (nM)	V_{max} [μmol/(min·mg)]	K_M (nM)	V_{max} [μmol/(min·mg)]	K_M (nM)	V_{max} [μmol/(min·mg)]
dynorphin A[b] (YGGFL**R**^**R**IRPKLKWDNQ)	8.4 ± 1.5	0.03 ± 0.005	1.7 ± 0.29	0.1 ± 0.015	13 ± 0.9	0.16 ± 0.014
somatostatin 28[b] (SANSNPAMAPRE^**RK**AGCKNFFWKTFTSC)	21 ± 2.5	0.003 ± 0.0005	55 ± 6	0.002 ± 0.0003	5 ± 0.6	0.003 ± 0.0005

[a] Enzyme activity was assayed as described under Materials and Methods using 20 mM potassium phosphate buffer, pH 7.0, with ∼0.1 μg of enzyme per sample. [b] The K_M values for dynorphin A and somatostatin 28 were determined based on their inhibition of the cleavage of bovine adrenal medulla peptide (1−8) as previously described (*13*).

Table 3: Kinetic Parameters for the Hydrolysis of Peptide Substrates by Rat, Mouse, and Human NRD Convertases in Tris Buffer[a]

	mouse NRD convertase		rat NRD convertase		human NRD convertase	
peptide	K_M (μM)	V_{max} [μmol/(min·mg)]	K_M (μM)	V_{max} [μmol/(min·mg)]	K_M (μM)	V_{max} [μmol/(min·mg)]
BAM−8 (YGGFM**R**^**R**V)	261 ± 36	3.5 ± 0.38	158 ± 10	1.9 ± 0.12	219 ± 29	1.7 ± 0.19
α-neoendorphin (YGGFL**R**^**K**YPK)	49 ± 8	1.1 ± 0.09	86 ± 9.4	1.0 ± 0.05	90 ± 11	1.1 ± 0.13
dynorphin B (YGGFL**R**^**R**QFKVVT)	15.7 ± 1.2	2.4 ± 0.1	30.6 ± 1.8	2.0 ± 0.08	8.2 ± 1.1	1.4 ± 0.22

	mouse NRD convertase		rat NRD convertase		human NRD convertase	
peptide	K_M (nM)	V_{max} [μmol/(min·mg)]	K_M (nM)	V_{max} [μmol/(min·mg)]	K_M (nM)	V_{max} [μmol/(min·mg)]
dynorphin A (YGGFL**R**^**R**IRPKLKWDNQ)	1.4 ± 0.22	0.05 ± 0.009	<5	0.14 ± 0.02	0.31 ± 0.04	0.26 ± 0.04
somatostatin 28 (SANSNPAMAPRE^**RK**AGCKNFFWKTFTSC)	10.2 ± 0.7	0.005 ± 0.0008	0.95 ± 0.16	0.004 ± 0.0007	9.5 ± 1.7	0.011 ± 0.002

[a] Assay conditions were as described in Table 2 except 100 mM Tris buffer, pH 7, was used in place of phosphate buffer.

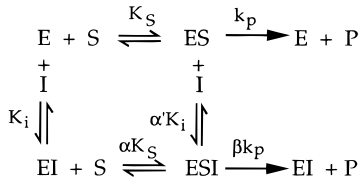
The generality of the effect of Tris was investigated by testing several amines for their effect on the rate of cleavage of BAM-8. We included in this study the physiological polyamines spermine, spermidine, and putrescine, as well as ammonium chloride and Bis-Tris propane. BAM-8 was used as substrate at 60 μM, which is a saturating level in phosphate buffer, but below the K_M in the presence of Tris. As shown in Table 4 such diverse amines as ammonium ion, Bis-Tris propane, and the polyamines all were inhibitory toward this substrate. In the presence of polyamines, the kinetic properties of the three NRD convertases were clearly distinguishable. With the mouse enzyme, the IC_{50} decreased with increasing number of amino groups per molecule (IC_{50} putrescine > spermidine > spermine) such that a ∼1000-fold difference in IC_{50} was observed between putrescine and spermine. With the rat and human enzymes, spermine was less effective as an inhibitor. Spermine was a more potent effector, by a factor of 6, with mouse NRDc compared to the rat enzyme, and a more potent effector by a factor of 600 with mouse NRDc compared to human NRDc. With the rat enzyme, both spermine and spermidine were equally effective and ∼400 times more effective than putrescine. With the human enzyme, putrescine, spermidine, and spermine exhibited similar IC_{50} values.

Table 4: Inhibition of the Cleavage of BAM-8 by Amines [a]

amine	IC_{50} (mM) mouse NRDc	rat NRDc	human NRDc
NH_4Cl	8.5	18.4	12
Tris	2.5	15	2.5
Bis-Tris propane	1.0	6.4	4.0
putrescine	1.5	5.0	4.0
spermidine	0.020	0.013	3.5
spermine	0.002	0.012	1.2

[a] Assay conditions were as described in Table 2. Amines were prepared in 20 mM potassium phosphate, pH 7.0, and adjusted to pH 7.0 at 37 °C with HCl. The ionic strength of the reaction mixture was made equal to that of 100 mM spermine by adding NaCl at the appropriate concentrations. Bovine adrenal medulla peptide (BAM-8) was used as substrate at its K_M of 6, 28, and 8 μM for mouse, rat, and human NRD convertases, respectively.

Scheme 1



The effect of polyamines differed from that of the simple amines in that polyamines increased the K_M not just for BAM-8 and dynorphin B but also for dynorphin A. At a saturating concentration of spermine, the K_M for BAM-8 increased 15-, 1.6-, and 13-fold for rat, mouse, and human NRDc, respectively. With dynorphin B, this increase was more dramatic, being 118-, 11-, and 260-fold, while for dynorphin A K_M increased 8-, 65-, and 4-fold for rat, mouse, and human NRDc, respectively. Thus, although the *N*-arginine dibasic convertases from the three species behaved similarly, they were quantitatively different. The mouse enzyme was affected the least with BAM-8 and dynorphin-B as substrate, but showed the greatest change in K_m with dynorphin A as substrate.

Further analysis of the concentration dependence for inhibition by amines showed them to act as partial inhibitors. That is, a plot of enzyme activity as a function of amine concentration shows that initially activity decreases as the amine concentration increases, but the activity then plateaus and the residual activity is not further reduced by increasing amine concentration. Classical Dixon plots of 1/rate vs amine concentration are thus nonlinear. This incomplete inhibition is the result of the inhibitor affecting both K_M and V_{max}, but with both K_M and V_{max} reaching new limiting values. This is illustrated in Scheme 1 (*21*). The distinguishing features of this system are that $\beta > 0$ and $\alpha < 1$ or $\alpha > 1$; that is the K_M for binding of substrate to the EI complex can increase ($\alpha > 1$) or decrease ($\alpha < 1$) relative to free enzyme, and the ESI complex can catalyze the reaction, but at a slower rate than from the ES complex ($\beta > 0$). The limiting K_M and V_{max} values, and thus α and β, can be obtained at high inhibitor concentrations (*21*). In the case of NRDc, the values for α are greater than 1 for the peptides dynorphin B, α-neoendorphin, and BAM-8, while they are less than 1 for dynorphin A and somatostatin 28. Similarly, the β values are less than 1 for dynorphin B, α-neoendorphin, and BAM-8 and greater than 1 for dynorphin A and somatostatin 28. For example, from the data in Tables 2 and 3, it can be calculated that with Tris as the amine and dynorphin B as the substrate α values range from 11 for the human enzyme to 33 for the rat enzyme, while β values ranged from 0.6 to 0.9. With dynorphin A as substrate, α values ranged from 0.01 for the human enzyme to <0.3 for the rat enzyme, while β values ranged from 1.3 to 1.6. Since the NRDc reaction involves only a single substrate, this mechanism requires that amines bind at a site other than the substrate binding site. Thus, amines appear to act as effector molecules of the NRDc reaction.

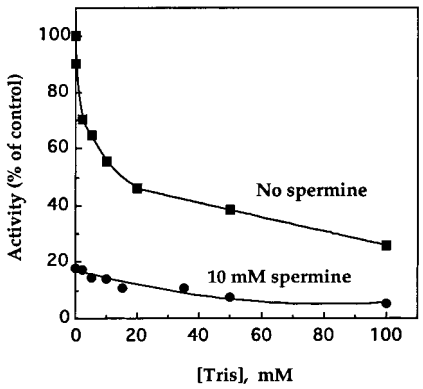


FIGURE 2: Inhibition of the cleavage of BAM-8 by Tris in the presence of 10 mM spermine. The rate of cleavage in the absence of amines was set as 100. Ionic strength was held constant by the addition of NaCl. All solutions were adjusted to pH 7.0 at 37 °C.

An experiment was conducted with BAM-8 as substrate to examine the effect of adding increasing concentrations of Tris to a fixed spermine concentration of 10 mM. As shown in Figure 2, 10 mM spermine alone produced ~70% inhibition of activity. Adding increasing concentrations of Tris further reduced this activity to ~4% residual activity. Thus, Tris can bind to the enzyme in the presence of spermine.

DISCUSSION

The previously described mouse arginine-specific dibasic cleaving enzyme (dynorphin converting enzyme) has been identified as an NRD convertase. Comparison of the amino acid sequences of the rat, mouse, and human enzymes shows they are highly conserved with the exception of an acidic region within which the three NRDc-s are considerably more divergent. The differences in the published kinetic properties between the mouse and rat enzymes can be largely, but not completely, attributed to differences in assay conditions utilized, the rat enzyme being studied in Tris buffer and the mouse enzyme being studied in phosphate buffer. We have shown that peptide hydrolysis catalyzed by rat, mouse, and human NRD convertases is affected by Tris and other amines. Simple amines such as Tris can act as inhibitors with a substrate such as dynorphin B, by increasing the K_M 10−30-fold dependent on the origin of the NRDc, and decreasing the V_{max} less than 1.5-fold. Alternatively, Tris can act as an activator with a substrate such as dynorphin A, decreasing K_M 3−100-fold and increasing V_{max} 2−4-fold. In contrast, polyamines act as inhibitors with all substrates. The effects of Tris and spermine were found to be additive, suggesting these two amines can interact differently with the enzyme.

The observed effect of amines requires a mechanism involving binding to a site other than the active site; that is,

amines act as effectors or regulatory molecules. Although not yet proven, the simplest model which accounts for the kinetic behavior of amines is one in which the positively charged amine binds to the anionic acidic stretch found in NRDc, and causes a conformational change in the active site. With an amine like Tris, this conformational change can either diminish or enhance substrate binding, depending on the substrate. Polyamines, which bind in a different mode than simple amines, inhibit the binding of all substrates. The observation that the kinetics of reaction of peptide substrates with rat, mouse, and human NRDc show relatively small quantitative differences indicates the active sites of all three enzymes are similar. In contrast, there are relatively large variations in the ability of amines, particularly polyamines, to inhibit rat, mouse, and human NRDc. Polyamines have the highest affinity for mouse NRDc and the lowest affinity for human NRDc. The sequence differences between the rat and human NRD convertases identified so far cluster in the acidic stretch region of these endopeptidases. The mouse enzyme contains 59 negative charges in a 74 residue stretch, while the human enzyme has only 43 total negative charges in the same region. Thus, differences in sensitivity to amine inhibition correlate with the divergence of the acid stretch within the three enzymes, and support the hypothesis that amines bind to the acidic stretch. We suspect that differences in sensitivity to amines may result not only from a difference in net charge but also from a difference in tertiary structure which could affect amine binding. Based on the known binding of spermine to a relatively few (1−4) negatively charged residues in other proteins (see below), it would appear that the number of negatively charged residues in the acid stretch of NRD convertase is sufficient to create more than one binding site for amines in the enzyme.

The role of acidic residues in spermine binding is illustrated by a prostatic spermine binding protein that contains a very highly negatively charged stretch of 125 residues (*22*, *23*). The published crystal structure of spermidine bound to PotD, the primary receptor of the polyamine transport system (*24*), shows spermidine bound by two glutamates and two aspartates with several aromatic residues contributing to the interaction between the protein and its ligand. However, spermine binding through a single aspartate residue has also been demonstrated with the spermine-regulated potassium channel IRK1 (*25*) and the NMDA-receptor (*26*, *27*). Thus, the tight binding of multiply positively charged amines to the extended negatively charged acidic stretch of NRD convertase seems highly likely.

Although Tris cannot be considered a physiological regulator of NRDc, the effects of polyamines occur at or below their physiologically relevant concentrations. The potential importance of putrescine, spermidine, and spermine in determining the in vivo characteristics of NRD convertase is highlighted by the fact that these amines are known to reach their highest physiological concentrations in rapidly reproducing tissue (*28*) and the highest level of both NRDc mRNA and enzymatic activity was found in testis among the tissues tested (*10*, *15*). Although the highest intracellular concentrations of polyamines are reported in the cell nucleus (*29*), the overwhelming majority of the polyamines can be found in the rest of the cell body and also in plasma (*29*). Polyamines have also been shown to be secreted from various cell lines and tissues (*30*, *31*), and have been shown to be localized to the secretory granules in peptide and protein secreting cells (*32*, *33*). This makes their interaction with endopeptidases possible in the extracellular space as well as in the cytosol, where most (70−80%) of the intracellular NRDc activity is localized (Csuhai and Hersh, unpublished results).

We have reported that NRD convertase is secreted during the normal growth of the mouse lymphoma EL-4 cells (*9*) and found the same phenomenon for human Jurkat T cells. Rat NRD convertase was also reported to be associated with the secretory machinery of rat pituitary endocrine cells and found in conditioned media of spermatocytes and spermatids (*11*). It is generally accepted that lack of polyamine synthesis or depletion of polyamine reserves causes growth retardation and arrest of cell cycle progression in the S- and G_2-phases of the cell cycle (*34*−*36*). Thus, it is conceivable that the physiological activity of NRD convertases might be regulated with the fluctuating polyamine concentration during cell cycle progression. Putrescine and spermidine, but not spermine, were found to be secreted from mammalian cells (*30*, *37*). Thus, secreted NRDc might be influenced by these secreted polyamines.

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