Purification and Characterization of a Dynorphin-processing Endopeptidase*

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Dynorphin B (Dyn B-13, also known as rimorphin) is generated from Dyn B-29 (leumorphin) by the cleavage at a single Arg residue. An enzymatic activity capable of processing at this monobasic site has been previously reported in neurosecretory vesicles of the bovine pituitary and pituitary-derived cell lines. This enzyme termed “the dynorphin-converting enzyme” (DCE) has been purified to apparent homogeneity from the neurointermediate lobe of the bovine pituitary using hydrophobic chromatography, preparative isoelectrofocusing in a granulated gel between pH 4 to 6.5, and non-denaturing electrophoresis on 5% polyacrylamide gel. DCE exhibits a pI of about 5.1 and a molecular mass of about 54 kDa under reducing conditions. DCE is a metallopeptidase and exhibits a neutral pH optimum. Specific Inhibitors of soluble metallopeptidases such as enkephalinase (EC 3.4.24.11) or enkephalin generating neutral endopeptidase (EC 3.4.24.15) do not inhibit DCE activity indicating that DCE is distinct from these two enzymes. Cleavage site determination with matrix-assisted laser desorption ionization mass spectrometry shows that DCE cleaves the Dyn B-29 N terminus to the Arg14 generating Dyn B-13 and Dyn B-(14–29). Among other peptides derived from Dyn B-29, DCE cleaves only those peptides that fit the predicted “consensus motif” for monobasic processing. These data are consistent with a broader role for the dynorphin converting enzyme in the biosynthesis of many peptide hormones and neuropeptides by processing at monobasic sites.

The majority of the neuropeptides are synthesized as larger precursors that undergo endoproteolysis at specific sites (1). These sites are usually multiple basic amino acids (2–4) although some cleavage sites are single basic (“monobasic”) residues that usually fit a consensus sequence (5).

The dynorphin precursor, Prodyn, contains both dibasic and monobasic cleavage sites; cleavage at these sites generates a variety of potent opioid peptides. The cleavage at dibasic sites gives rise to α- and β-neo-endorphin, dynorphin (Dyn) A-17, Dyn B-29, and leucine6-enkephalin (Leu-Enk). The formation of Dyn B-13 from Dyn B-29 and Dyn A-8 from Dyn A-17 requires cleavage at monobasic sites. Several neuropeptide-processing enzymes have been identified in mammalian cells (2–6). Endopeptidases such as furin, PC1, and PC2 are thought to preferentially cleave peptide precursors at multibasic residues due cleavage sites (4, 7). An endopeptidase designated “dynorphin converting enzyme” (DCE)1 cleaves neuropeptides at monobasic sites (5, 8). Following endopeptidase activity, carboxypeptidase E (also known as carboxypeptidase H) removes the basic amino acids from the C terminus of the peptides (9).

DCE is a peptide processing enzyme that converts Dyn B-29 into Dyn B-13 by cleavage at single basic residue Arg14 (5, 6). In the brain and pituitary, the distribution of the DCE activity generally matches that of Dyn B-13 (10, 11). These data suggest that DCE is physiologically involved in the processing of dynorphin peptides at single arginine cleavage sites.

In the bovine pituitary, DCE activity is enriched in the highly purified secretory vesicles and co-sediments with Leu-Enk and carboxypeptidase E (12). The DCE activity is also found in the regulated pathway of secretion in a number of cell lines (13, 14). The presence of DCE activity in structurally similar (12) and functionally similar (13–16) neuropeptide-containing secretory granules is also consistent with a function in opioid peptide processing since it has been shown that secretory granules and trans Golgi network are the sites of peptide processing (17).

In this study we used bovine neurointermediate lobe (NIL) of the pituitary to purify DCE to apparent homogeneity. Using hydrophobic chromatography, preparative isoelectrofocusing, and non-denaturing electrophoresis, we have purified DCE approximately 20,000-fold. We find that the purified DCE is a neutral metallopeptidase with a molecular mass of 54 kDa under reducing conditions. DCE cleaves Dyn B-29 N-terminal to the Arg14 directly generating Dyn B-13. These data support the premise that DCE is a monobasic processing endopeptidase involved in the generation of Dyn B-13.

MATERIALS AND METHODS

Assay for DCE Activity—In a typical assay, the reaction mixture consisted of 50 mM sodium phosphate, pH 7.5, 0.5–1 μM Dyn B-29 (Peninsula), 100 μM Dyn B-29–(9–22), and enzyme (1–100 μg of extracted membrane proteins or 1–10 ng of purified enzyme protein) in a final volume of 100 μl. The reaction mixture was incubated for 20 min at 37°C and terminated by boiling for 5 min. Reactions using enzyme preparation boiled before the addition of peptide were performed (“control”). Bailing leads to irreversible inactivation of the enzyme activity without affecting Dyn B-13. Typically, 5–10 μl of the reaction mixture were analyzed by radioimmunoassay as described (11). Antiserum “IS” was used for the detection of Dyn B-13. It is a highly specific antiserum for Dyn B-13 from Dyn B-29 and Dyn A-8 from Dyn A-17.

1 The abbreviations used are: DCE, dynorphin-converting enzyme; NIL, neurointermediate lobe; IEF, isoelectrofocusing; MALDITOF, matrix-assisted laser desorption ionization mass spectrometry.

2 YGGFLRRQFKVVT+5SQEDPNAYEEFLDV, Dyn B-29; YGGFLRRQFKVVT+5Dyn B-13; FKVVT+5SQEDPNAY, Dyn B-29–(9–22).

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selective and sensitive antiserum (8). Radioimmunoassay with this antiserum allows the detection of picomolar concentrations of Dyn B-13 in the presence of nanomolar concentrations of Dyn B-29.

Purification of DCE Activity—Approximately 10–20 g of bovine neuron intermediate lobe of the pituitary glands (Pel-Freez) were homogenized (Polytron, Brinkmann) in 5 volumes of 50 mM sodium phosphate, pH 7.5, containing 0.1 mM dithiothreitol. The homogenate was centrifuged at 50,000 x g for 1 h, and the supernatant was adjusted to 15% saturation of ammonium sulfate. This results in the precipitation of some of the proteins; most of the DCE activity is retained in the supernatant. The pH of the supernatant was adjusted to 7.5, and the supernatant was subjected to hydrophobic chromatography on phenyl-Sepharose CL-4B (Pharmacia Biotech Inc.). For a typical purification, 10–15 ml of phenyl-Sepharose beads were washed with 100–150 ml of 50 mM sodium phosphate buffer, pH 7.5, containing 15% saturation ammonium sulfate (loading buffer), and the supernatant containing DCE activity in the 15% ammonium sulfate supernatant was applied. Following washing with 60–100 ml of loading buffer, DCE was eluted with a linear gradient of 15%-0% ammonium sulfate at a rate of 1 ml/min; 2-ml fractions were collected. DCE activity in each fraction was measured as described above. Protein estimation was using BCA reagent (Pierce). DCE elutes in the second elution with 0% ammonium sulfate and is about 59-fold pure. The peak of activity from the phenyl-Sepharose chromatography was then concentrated by ultrafiltration on Amicon UM 100 membranes and subjected to preparative IEF at pH 4-6.5.

For preparative IEF, a gel slurry was prepared by mixing 4 g of Sephadex G-75 IEF-grade (Sigma) with 95 ml of 10% glycerol (v/v), 2.5 ml of ampholyte (Pharmacia) at pH 4.0–6.5 and 1 ml dithiothreitol. The slurry was poured onto a glass plate (24 x 11 cm) and dried with a light stream of cool air until the weight decreased by 35%. The plate was placed on the cooling unit of an LKB 2117 Multiphor platform. Electrode strips were soaked in either 40 mM glutamic acid (anode) or 200 mM histidine (cathode) and placed at the end of the IEF plate. The plate was prefocused for 2 h at 8 watts/plate at 4 °C. The concentrate containing peak of DCE activity from the phenyl-Sepharose column was applied in the pH 6.0 region of the gel and focused for 8 h at 37°C before the addition of substrate. DCE activity was then measured as described above. Protein estimation was using BCA reagent (Pierce). In addition, we estimated protein concentration on the silver-stained gel following electrophoresis of the fractions containing the peak of activity. The pH gradient was determined by extracting a small segment of each fraction with deionized water and then directly measuring the pH.

The preparative IEF chromatography procedure alone gives another 80–100-fold purification and DCE runs with a pI of about 5.1. The DCE is extracted from the gel, concentrated on a Centriprep 100 (Amicon) concentrator, and the protein subjected to non-denaturing electrophoresis (18). For this, 5% polyacrylamide gels were used and the samples were not denatured by boiling and SDS or β-mercaptoethanol were excluded during sample preparation. 0.5 ml thioglycollate was prerun to remove ammonium persulfate and other free radical reaction products generated in the gel system (19). The electrophoresis was carried out at 4°C. Following electrophoresis, the activity in 0.5-mm sections was extracted with 50 ml sodium phosphate buffer, pH 7.5, containing 0.1 mM dithiothreitol and 0.1% Triton X-100. The DCE activity in each fraction was determined as described above. The peak of DCE activity corresponded to a protein band around 180 kDa as visualized by silver staining (data not shown). This is interesting because DCE activity by gel-exclusion chromatography also exhibited a molecular mass of about 180 kDa (12).

The peak of activity was pooled, concentrated on Centricon-100 (Amicon), and subjected to electrophoresis under denaturing conditions and visualized by silver-staining.

Characterization of DCE Activity—DCE in the fractions representing the peak of activity following the non-denaturing electrophoresis was used for characterization. Protease inhibitors (Sigma) at the concentration indicated in Table I were preincubated with the enzyme for 20 min at 37 °C before the addition of substrate. DCE activity was then measured as described above. For the determination of the optimum pH for DCE activity, the following buffers were used (at 50 mM final concentration): sodium citrate, pH 4.4–6.0; sodium phosphate, pH 6–8.5; Tris-Cl, pH 7.4–8.8.

| Table I | Purification of DCE activity from bovine pituitary soluble fraction |
|---------|---------------------------------------------------------------|
| **Step**| **Activity** | **Specific activity** | **Fold purification** | **Yield** |
|---------|--------------|----------------------|----------------------|---------|
|         | nmol/min     | nmol/min/mg          | %                    |         |
| Soluble fraction | 550          | 0.16                 | 1.0                  | 100     |
| Ammonium sulfate supernatant | 520          | 0.2                  | 1.3                  | 77      |
| Phenyl-Sepharose | 408          | 9.5                  | 59                   | 74      |
| Isoelectrofocusing | 90           | 600                  | 3,800                | 15      |
| Non-denaturing polyacrylamide gel electrophoresis | 10           | 4,000                | 20,000               | 2       |

**Fig. 1. Purification of DCE activity from bovine pituitary soluble fraction.** A, hydrophobic chromatography on phenyl-Sepharose. DCE activity (hatched bars) is expressed as Rel. Units are in nanomoles of immunoreactive-Dyn B-13 formed/min/200 µl. Protein (solid line) is in mg/200 µl; protein estimation was using Bradford reagent (Bio-Rad). The ammonium sulfate gradient is in dashed lines. B, preparative isoelectrofocusing. DCE activity (hatched bars) expressed as Rel. Activity is in nanomoles of irradiated-Dyn B-13 formed/min/fraction. Protein (solid line) relative units (mg/ml); protein estimation was using BCA reagent, pH gradient (dotted line) was determined by extracting a small segment of each fraction with deionized water and then directly measuring the pH.
To determine the cleavage site specificity of DCE in Dyn B-29 and in Dyn B-29-derived peptides (shown in Table VI), 1 nmol of peptide was incubated with 100 ng of purified enzyme in 50 mM sodium phosphate buffer, pH 7.5, containing 0.1% Triton X-100 for 20 min, 1 or 18 h at 37°C. The reaction was terminated by incubation at 100°C for 10 min. The reaction mixture was subjected to MALDITOF-mass spectrometry (Dr. Ronald Beavis, Skirball Institute, NYU Medical Center, New York).

The intramolecularly quenched fluorescent peptides were synthesized as described previously (20); these peptides have ortho-aminobenzonic acid (Abz) and N-(2,4-dinitrophenyl)ethylamine as a donor-acceptor pair at the N and C termini of the peptides, respectively. N-(2,4-Dinitrophenyl)ethylamine was attached to glutamine in all peptides, a necessary result of the solid-phase peptide synthesis strategy employed. An automated bench-top simultaneous multiple solid-phase peptide synthesizer (PSSM 8 system from Shimadzu) was used for the solid-phase synthesis of all the peptides by the Fmoc procedure.

RESULTS

Purification of the DCE from Bovine Pituitary—We used a selective assay that allows specific detection of Dyn B-13 to isolate the DCE; this assay makes use of Dyn B-29-(9–22), a Dyn B-29-derived peptide that is not recognized by DCE and thus protects the Dyn B-29 and Dyn B-13 from hydrolysis by other peptidases (21). We chose bovine NIL as the source of DCE since previously we had shown that in the NIL the DCE is contained within the neuroepitope containing secretory vesicles and that in the NIL DCE is at a higher specific activity as compared with the anterior pituitary. Ammonium sulfate at 60% saturation results in complete precipitation of DCE (data not shown). Attempts to solubilize the 60% ammonium sulfate precipitate result in substantial loss of DCE activity. Therefore, we did not use precipitation with 60% ammonium sulfate as a step in the purification of DCE. 15% saturation of ammonium sulfate results in complete retention of bovine NIL DCE in the supernatant and results in a 1.3-fold purification. More importantly, this step results in the elimination of lipophilic proteins that tend to cause inefficient binding of DCE to the phenyl-Sepharose beads. The majority of DCE activity binds to the phenyl-Sepharose in 15% ammonium sulfate, and the proteins that do not bind are removed during extensive washes. DCE elutes in 0% ammonium sulfate (Fig. 1A); this step alone gives about a 59-fold purification with ∼74% yield (Table I). The preparative IEF on a granulated gel between pH 4–6.5 gives another 70-fold purification and about a 10–20% yield (Table I). DCE runs with a pI of about 5.1 (Fig. 1B). At this point only three major protein bands can be visualized in the fraction containing peak of DCE activity (Fig. 2). The non-denaturing electrophoresis in 5% gel results in approximately 4–6-fold purification and results in the homogeneous purification of DCE. DCE runs as a 180-kDa protein under these non-denaturing electrophoresis conditions (data not shown), and when the peak of DCE activity is subjected to denaturing electrophoresis followed by silver-staining, a single band of 54 kDa is detected (Fig. 2). Increasing the reducing conditions by boiling in 2% SDS, 5% β-MeC, treatment with iodoacetamide, or treatment with guanidine hydrochloride (22) do not result in further changes in the size of this protein (data not shown).

Characterization of the DCE Activity Purified from the Bovine Pituitary—The purified bovine pituitary enzyme is active over a broad pH range, with the optimum between 7–7.5 (Fig. 3). The enzyme retains about 40–50% activity at pH 6 in sodium phosphate buffer. It is interesting to note that the enzyme prefers Tris-CI buffer over sodium phosphate buffer and shows substantially lower activity with citrate buffer. This is consistent with the metalloprotease nature of the enzyme as evidenced by the protease inhibitor profile (Table II). The metal-chelating agents substantially inhibit the activity (Tables II and III). The enzyme is completely inhibited by 1 mM 1,10-phenanthroline and not by 4,7-phenanthroline. There is only a 30–40% inhibition of activity by 1 mM EDTA or EGTA, respectively (Tables II and III). Inhibitors of aspartyl proteases, aminopeptidases, and other lysosomal hydrolases do not inhibit the activity (Table II). In addition, the inhibitors of trypsin, chymotrypsin, and other serine proteases do not inhibit the enzyme activity. Although cysteine protease inhibitors do not inhibit the activity, sulfhydryl reagents substantially inhibit the enzyme activity. The enzyme is especially sensitive to p-chloromercuribenzenesulfonic acid exhibiting about 50% inhibition at 0.3 μM.

Among the chelating agents the enzyme is more sensitive to 1,10-phenanthroline (Table III). 1 mM 1,10-phenanthroline completely inhibits the activity whereas 5 mM EDTA or EGTA cause only a 73–78% inhibition of activity. 5 mM CA 50% inhibition of activity. In order to see if the DCE activity is modulatable by cations, we examined the effect of divalent cations on DCE activity (Table IV). CaCl2, MgCl2, or MnCl2 do not show appreciable activation of DCE; ZnCl2 and CoCl2, in contrast, cause substantial inhibition of activity at 1 or 5 mM, respectively. Inhibition by these heavy metal ions could be due to the ability of these metal ions to bind free SH groups in the cysteine residue. These data suggest that DCE is...
a thiol-sensitive metalloprotease.

Other well characterized thiol-sensitive metalloproteases include soluble neutral endopeptidases EC 3.4.24.15 (23), EC 3.4.24.16 (24), EC 3.4.24.11 (25), N-arginine dibasic convertase (26), and amidorphin Gly-generating enzyme (27). We used specific peptide inhibitors of EC 3.4.24.15, EC 3.4.24.16, or EC 3.4.24.11 and potent inhibitors of N-arginine dibasic convertase or amidorphin Gly-generating enzyme in order to examine their effects on DCE activity. DCE activity is not inhibited by these inhibitors at 100 μM (Table V). In addition the size, pH profile, and the protease inhibitor profiles for these enzymes are distinct from that of DCE (Table V). These data suggest that DCE is distinct from these other neutral metallopeptidases.

DCE is fairly stable at 23 and 37 °C and retains essentially 100% activity during 30 min of incubation at these temperatures; however, a 30-min incubation at 45 °C causes 60% loss of activity. DCE is also differentially sensitive to organic solvents. 1% of ethanol or isopropanol causes up to 30% of inactivation, whereas, 5% Me2SO causes no inactivation. Approximately a 20% loss of activity is detected only when Me2SO is increased to 10% (data not shown).

The cleavage site specificity of DCE was determined using MALDITOF mass spectrometry. Dyn B-29 alone was detected as a single peak with a mass ion of about 3528 (Fig. 4). Upon incubation with the purified DCE for 1 h at 37 °C, two additional peaks of mass ions 1570 and 1975 are detected; this mass is identical to the predicted mass of Dyn B-13 and Dyn B-29-(14–29), respectively (Fig. 4). Longer incubation (18 h) of enzyme with the substrate results in substantial conversion of the substrate to only two products with masses identical to Dyn B-13 and Dyn B-29-(14–29) (data not shown). These data suggest that DCE cleaves Dyn B-29 N-terminal to Arg14. DCE displayed typical Michaelis-Menten kinetics with an apparent K_{m} of about 0.1 mM and an apparent V_{max} of about 0.1 μmol/min/μg (Fig. 5).

In order to test the hypothesis that DCE recognizes peptides that fit the consensus for monobasic processing, we synthesized a number of peptides with substitutions at sites predicted to be involved in the recognition by the enzyme. The peptide representing Dyn B-29-(6–16) was efficiently cleaved by DCE. By MALDITOF mass spectrometry the site of cleavage was determined to be N-terminal to the Arg6 (Table VI). The substitutions of the Arg-7 to a Gly or Nle resulted in the absence of cleavage of the peptide as determined by MALDITOF spectrometry (Table VI). Similarly, substitution of the Ser-1 to Val or Phe and Thr-1 to Tyr resulted in absence of processing. These results suggest that the residues around the cleavage site play an important role in the recognition of Dyn B-29 by DCE.

**DISCUSSION**

In the present study we have purified the monobasic processing endopeptidase designated DCE from NIL to apparent homogeneity. Using only a few steps including preparative isoelectrofocusing, the enzyme was purified 20,000-fold. The fairly high yield and the quick purification protocol has made possible studies characterizing the enzyme. The limited amount of the enzyme in the NIL is consistent with a selective role for the enzyme in neuropeptide biosynthesis. The evidence for such a role derives from our earlier finding that within NIL the enzyme appears to be associated with neurosecretory vesicles where it is associated with Dyn B-13 (12). Also, in the brain and in peripheral tissue, the distribution of DCE shows about 10-fold variation, which has similarities to the distribution of neuropeptides (11). DCE is distinct from any of the previously described neutral metallopeptidases (23–28). Unlike the other metallopeptidases, DCE is extremely sensitive to PCMBS being inhibited completely 5 μM. Additionally, selective inhibitors that inhibit other neutral metallopeptidases do not have any effect on the DCE activity (Table V).

The molecular mass of the purified enzyme under denaturing conditions is determined to be 54 kDa. Under non-denaturing conditions the enzyme was purified 20,000-fold. The purified enzyme was shown to have a molecular mass of 54 kDa under non-denaturing conditions, which is similar to the molecular mass of other metalloproteases. The enzyme was shown to be sensitive to organic solvents, with 1% of ethanol or isopropanol causing up to 30% of inactivation, whereas, 5% Me2SO causes no inactivation. Approximately a 20% loss of activity is detected only when Me2SO is increased to 10% (data not shown).

| Cations   | Conc. | % activity |
|-----------|-------|------------|
| CaCl2     | 1.0   | 111 ± 13   |
|           | 5.0   | 84 ± 6     |
| MgCl2     | 1.0   | 106 ± 9    |
|           | 5.0   | 114 ± 6    |
| MnCl2     | 1.0   | 111 ± 10   |
|           | 5.0   | 93 ± 6     |
| CoCl2     | 1.0   | 66 ± 6     |
|           | 5.0   | 40 ± 1     |
| ZnCl2     | 0.1   | 55 ± 3     |
|           | 1.0   | 0          |
| CuSO4     | 0.1   | 50 ± 2     |
|           | 1.0   | 16 ± 2     |

**TABLE II**

Effect of protease inhibitors on the DCE activity

The abbreviations used are: TPCK, L-1-tosyl-amido-2-phenylethyl chloromethyl ketone; TLCK, 1-chloro-3-tosylamido-7-amo7-hexanone; SBTI, soybean trypsin inhibitor; CETI, chicken egg white trypsin inhibitor; E-64, trans-epoxysuccinyl-L-leucinamido-(4-guanidino)butane; IAA, iodo-acetic acid; PCMBS, p-chloromercurybenzenesulfonfonic acid; DTNB, 5,5'-dithio-bis(2-nitrobenzoic acid).

| Inhibitor                  | Conc. | % inhibition |
|---------------------------|-------|--------------|
| 1,10-phenanthroline       | 1.0   | 100 ± 1      |
| 4,7-phenanthroline        | 1.0   | 0            |
| EDTA                      | 1.0   | 31 ± 1       |
| EGTA                      | 1.0   | 41 ± 4       |
| Pepstatin A               | 1.0   | 0            |
| Bestatin                  | 1.0   | 0            |
| Leupeptin                 | 0.1   | 0            |
| TPCK                      | 0.1   | 0            |
| Phenylmethylsulfonyl fluoride | 1.0   | 0            |
| TLCK                      | 0.1   | 11 ± 1       |
| SBTI                      | 0.1   | 18 ± 1       |
| CETI                      | 0.1   | 10 ± 1       |
| E-64                      | 1.0   | 0            |
| IAA                       | 1.0   | 0            |
| Cystatin                  | 0.1   | 0            |
| PCMBS                     | 0.01  | 100 ± 17     |
|                           | 0.001 | 78 ± 7       |
|                           | 0.0001| 42 ± 7       |
| DTNB                      | 1.0   | 40 ± 3       |

**TABLE III**

Effect of chelating agents on DCE activity

| Inhibitor   | Conc. | % inhibition |
|-------------|-------|--------------|
| 1,10-phenanthroline | 0.1  | 7.0 ± 0.5   |
|             | 1.0   | 100 ± 1     |
|             | 5.0   | 100 ± 1     |
| EDTA        | 0.1   | 15 ± 2      |
|             | 1.0   | 31 ± 1      |
|             | 5.0   | 73 ± 9      |
| EGTA        | 0.1   | 4 ± 4       |
|             | 1.0   | 41 ± 4      |
|             | 5.0   | 78 ± 17     |
| CDTA        | 0.1   | 40 ± 0.3    |
|             | 1.0   | 60 ± 0.3    |
|             | 5.0   | 34 ± 4      |

**TABLE IV**

Effect of divalent cations on DCE activity

| Cations   | Conc. | % activity |
|-----------|-------|------------|
| CaCl2     | 1.0   | 111 ± 13   |
|           | 5.0   | 84 ± 6     |
| MgCl2     | 1.0   | 106 ± 9    |
|           | 5.0   | 114 ± 6    |
| MnCl2     | 1.0   | 111 ± 10   |
|           | 5.0   | 93 ± 6     |
| CoCl2     | 1.0   | 66 ± 6     |
|           | 5.0   | 40 ± 1     |
| ZnCl2     | 0.1   | 55 ± 3     |
|           | 1.0   | 0          |
| CuSO4     | 0.1   | 50 ± 2     |
|           | 1.0   | 16 ± 2     |
conditions and upon gel-filtration chromatography, the activity exhibits a molecular mass of 180 kDa (this report and 12). It is possible that the enzyme activity exists in vivo as an oligomer resulting in a larger apparent size; this larger size also could be due to anomalous migration of the enzyme during gel-filtration and in non-denaturing gels.

DCE is a monobasic processing enzyme that processes Dyn B-29 at a single Arg residue. Other dynorphin processing enzymes have been shown to predominantly process dynorphins at dibasic processing sites (29, 30). DCE is unique in that the enzyme processes Dyn B-29 N-terminal to the Arg. We had previously found that the 2800-fold purified DCE from bovine anterior pituitary cleaved Dyn B-29 N-terminal and to the C-terminal of the Arg14 (12); we have not observed such a cleavage in the present study. This could be either due to the absence of another enzyme that was copurified with DCE from the anterior pituitary or due to regional variations in the properties of DCE. The latter possibility is supported by the finding that some of the properties of anterior pituitary DCE are similar but not identical to the NIL DCE; for example, NIL DCE is inhibited by EDTA whereas the anterior pituitary or DCE was not (12).

It is interesting to note that metalloproteases have been predominantly found to cleave peptide hormone precursors N-terminal to basic sites (26–28). We find that DCE cleaves Dyn B-29 at a monobasic cleavage site, Thr-Arg (this report).

FIG. 4. Cleavage site determination using MALDITOF mass spectrometry. Purified DCE (1 ng) was incubated with 1 nmol of Dyn B-29 in 50 mM Tris-Cl buffer, pH 7.5, containing 0.1% Triton X-100, for 1 h at 37 °C. The reaction was terminated by boiling for 10 min. Reaction mixture with boiled enzyme was used as control. The samples were subjected to MALDITOF mass spectrometry. Top panel, Dyn B-29 with buffer; bottom panel, Dyn B-29 with DCE. MALDITOF, m/z (M + H)+ for Dyn B-29 is 3528 (formula weight is 3527), for Dyn B-13 is 1571 (formula weight is 1569), and for Dyn B-29-(14–29) is 1975 (formula weight is 1974).

TABLE V

| Enzyme          | Predominant location | Molecular weight | pH optimum | Potent inhibitor* |
|-----------------|----------------------|------------------|------------|------------------|
| EC 3.4.24.15    | Testis/brain         | 67               | 7.0        | N-Cpp-Ala-Ala-Phe-pAB |
| EC 3.4.24.16    | Liver                | 73               | 7.5        | N-Cpp-Ala-Ala-Phe-pAB |
| EC 3.4.24.11    | Kidney               | 90               | 7.0        | N-Cpp-Phe-pAB     |
| NRD convertase  | Testis               | 110              | 8.5        | Bestatin, amastatin |
| AGE             | Adrenal medulla      | 45               | 8.0        | EDTA             |
| DCE             | Brain/NIL            | 54               | 7.5        | PCMBS            |

* Potent inhibitor is defined as the inhibitor that causes >95% inhibition of the enzyme activity at a concentration of 0.1 mM or lower.

DCE is a monobasic processing enzyme that processes Dyn B-29 at a single Arg residue. Other dynorphin processing enzymes have been shown to predominantly process dynorphins at dibasic processing sites (29, 30). DCE is unique in that the enzyme processes Dyn B-29 N-terminal to the Arg. We had previously found that the 2800-fold purified DCE from bovine anterior pituitary cleaved Dyn B-29 N-terminal and to the C-terminal of the Arg14 (12); we have not observed such a cleavage in the present study. This could be either due to the absence of another enzyme that was copurified with DCE from the anterior pituitary or due to regional variations in the properties of DCE. The latter possibility is supported by the finding that some of the properties of anterior pituitary DCE are similar but not identical to the NIL DCE; for example, NIL DCE is inhibited by EDTA whereas the anterior pituitary or DCE was not (12).

It is interesting to note that metalloproteases have been predominantly found to cleave peptide hormone precursors N-terminal to basic sites (26–28). We find that DCE cleaves Dyn B-29 at a monobasic cleavage site, Thr-Arg (this report). The adrenorphin Gly-generating enzyme cleaves bovine adrenal peptide 12P at a monobasic cleavage site, Gly-Arg (27). The NRD convertase cleaves prosomatostatin at a dibasic cleavage site, Glu-Arg-Lys (26). The cleavage site selectivity (N-terminal to an Arg) of these neutral metalloproteases suggest that these enzymes are related and belong to a distinct class of peptide-processing enzymes.

Monobasic enzymes are widely varied including, cholecystokinin-8-processing serine protease (31), prosomatostatin-processing serine protease (32), somatostatin-28-converting aspar-
TABLE VI
Analysis of Cleavage site using MALDITOF Mass Spectrometry

The numbers on the top line correspond to position of the amino acid in relation to the cleavage site Arg designated as 0. The arrow points to the site of cleavage determined by mass spectrometry. Abz, o-aminobenzoyl; EDDnp, ethylenediamine dinitrophenyl. Purified DCE (1 ng) was incubated with 1 nmol peptide in 50 mM Tris-Cl buffer, pH 7.5, containing 0.1% Triton X-100, for 1 h at 37 °C. The reaction was terminated by boiling for 10 min. Reaction mixture with boiled enzyme was used as control. The samples were subjected to MALDITOF mass spectrometry.

| Site of cleavage | Thr-Arg |
|------------------|---------|
| | Not cleaved |
| | Not cleaved |
| | Not cleaved |
| | Not cleaved |
| | Not cleaved |

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Furin mRNA is expressed at similar levels in brain, intestine, and kidney (42), whereas DCE activity is 10–20-fold lower in lung and kidney as compared with brain and intestine (11). The subcellular distribution of these two enzymes is also different. Whereas furin has been localized to the Golgi apparatus (43, 44), DCE activity is enriched in the secretory vesicle fraction of bovine pituitary glands (12) and in neuroendocrine cell lines (13, 14).

The unusual specificity of DCE had previously led us to propose that this enzyme recognizes secondary structure around the cleavage site rather than the primary structure at the cleavage site (8). By comparing sequences that are processed at analogous cleavage sites, we proposed that a compositional motif (or consensus) could govern the secondary structure around the cleavage site, and such a motif is recognized by DCE and DCE-like enzymes (45). The consensus for recognition by the monobasic processing enzyme is hypothesized to require basic residues at −3, −5, or −7 position, not tolerate an aliphatic residue at +1 position, or an aromatic residue at −1 position. It is exciting to see that DCE recognizes peptides that fit only the consensus motif and does not recognize peptides with amino acid substitutions at sites that are predicted to be necessary for recognition. This makes DCE an ideal candidate for the enzyme involved not only in the generation of Dyn B-13 but also in the processing of other precursors that require cleavage at monobasic sites such as growth factors, neuroptides, and other peptide hormones.

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