Characterization of Dynorphin A-converting Enzyme in Human Spinal Cord

A highly specific proteinase, converting dynorphin A (1–17) to enkephalins, was isolated from the human spinal cord and subjected to further characterization. The enzyme was found to be a thiol-dependent enzyme with a relative molecular mass of 50 kDa and a pH optimum between 5.0 and 5.5. This proteinase appears to exclusively convert dynorphin A (1–17) to Leu-enkephalin and its COOH-terminal extensions Leu-enkephalin-Arg6 (which was a major conversion product) and Leu-enkephalin-Arg6-Arg7 but not the other prodynorphin- or proenkephalin-derived peptides. This high specificity toward a single structure is suggested to be involved in a distinct processing pathway associated with the generation of the opioid peptides with selectivity for δ-opioid receptors.

The general processing pattern for neuropeptides includes trypsin-like cleavages of the inactive precursor (preprohormone) at paired basic amino acids, followed by the action of a variety of enzyme to generate bioactive products (1). The prodynorphin precursor contains three Leu-enkephalin-containing structures: dynorphin A, dynorphin B, and α-neoendorphin. These peptides can be converted to the pentapeptide by the action of a variety of proteinases (2). The product, however, is not unique in this particular preprohormone, as another opioid peptide precursor, proenkephalin A, also contains the Leu-enkephalin sequence in its structure. The prodynorphin-derived peptides also contain the COOH-terminal extension of the pentapeptide Leu-enkephalin-Arg6 which has previously been detected at relatively high concentrations, e.g., in the cerebrospinal fluid (9). Therefore, it is likely that the formation of Leu-enkephalin also occurs subsequent to the release of this hexapeptide, i.e., via the sequential action of an endopeptidase and a carboxypeptidase B-like enzyme.

The dynorphin-converting enzymes (DCE) constitute recently identified group of proteinases that are capable of transforming prodynorphin-related opioid peptides to enkephalins (4). Although the conserved sequence of the NH2-terminal pentapeptide (Leu-enkephalin), flanked by the dibasic stretch, is present in all of these structures (see Table 1), they are processed differently by the aforementioned proteinases. Recently, the recovery and characterization of highly selective dynorphin B convertases from the bovine and human spinal cord were described (5, 6), suggesting a distinct processing pathway for at least one of these peptides. Such unusual specificity has also been found for the proteolysis of dynorphin B-29 in the rat brain (7).

Several authors (8, 9) have described the conversion of dynorphin A to Leu-enkephalin in the central nervous system, but the reported proteinases were also capable of converting other nonrelated structures. In the present work we demonstrate the presence of an endoproteinase, here designated DCE-A, in human spinal cord with a distinct specificity against dynorphin A but not the other prodynorphin-related peptides, dynorphin B and α-neoendorphin.

EXPERIMENTAL PROCEDURES

Materials—All peptides used in this work were obtained from Bachem (Bubendorf, Switzerland). Their purity was tested by reversed-phase HPLC and fast atom bombardment-mass spectrometry, as described below. Iodo[1-14C]acetamide (50–60 mCi/mmol, code CFA326) was purchased from Amersham (Buckinghamshire, United Kingdom). Other reagents were of the highest available purity and were purchased from various commercial sources.

Human spinal cord was kindly supplied by the Department of Pathology, University Hospital, Uppsala and was collected at autopsy from normal males, after accidental death. Each enzyme preparation was obtained from a spinal cord tissue collected from one donor.

Recovery of Dynorphin A Converting—The enzyme activity was purified to apparent homogeneity by a rapid, two-step procedure involving ion-exchange chromatography and HPLC molecular sieving. All steps were performed at 4 °C, unless otherwise stated. Briefly, the tissue (16 g) was homogenized in a glass Teflon homogenizer in 160 ml of 20 mM Tris-HCl buffer, pH 7.4, for 60 s, followed by centrifugation (30,000 × g, 20 min). The extract was applied directly on a DEAE-Sepharose CL-6B column (22 × 130-mm) and eluted with a linear gradient of NaCl (0–0.3 M) in 20 mM Tris-HCl buffer, pH 7.4. Fractions of 6 ml were collected at a flow-rate of 1 ml/min. Aliquots of the active fractions were further concentrated and subjected to molecular sieving on a TSK G-3000 SW column (7.5 × 600-mm) in 20 mM Tris-HCl buffer, pH 7.4, at a flow rate 0.5 ml/min. 0.5-ml fractions were collected and stored for further analysis. The column was calibrated using protein standards (Pharmacia/LKB, Uppsala, Sweden).

Protein content was measured either by the method of Bradford (10) or by UV detection at 280 nm, respectively.

Enzyme Assay—The proteolytic activity was measured at pH 5.0 (0.1 mM citrate-phosphate buffer) using dynorphin A (120 pmol/assay) as substrate in a total volume of 40 μl according to the procedure described previously (5). The conversion product Leu-enkephalin-Arg6 was measured using a specific radioimmunoassay as described.

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1 The abbreviations used are: DCE, dynorphin-converting enzyme; DYN A, dynorphin A; HPLC, high pressure liquid chromatography; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
the major conversion product of spinal cord dynorphin-converting enzyme. To assure homogeneity of preparation, this product was separated on SP-Sephadex C-25 minicolumns and quantitated by counting in a gamma-counter, as described previously (5). The recovery of radiolabeled product was approximately 85%.

Reversed-phase HPLC Separation—This step was performed in order to identify all products that may be released from the substrate peptide. The HPLC instrument used a reversed-phase column (Hichrom, Reading, United Kingdom, 4 × 200 mm, 5 μm) in conjunction with photodiode-array UV detection, as described previously (12). Fragments were separated using a 40-min linear gradient of acetonitrile (15-45%), supplemented with 0.04% trifluoroacetic acid. The flow-rate was maintained at 0.5 ml/min, and 0.5-ml fractions were collected and assayed for DCE activity as described above. Arylsulfatase A activity was measured with a microplate assay described above, prior to autoradiography (3 weeks, Kodak X-AR5).

Fast Atom Bombardment-Mass Spectrometry (FAB-MS)—Experiments were performed with a Finnigan MAT 90 double-focusing instrument with B,E geometry, equipped with a continuous-flow FAB interface. Magnetic scans in positive ion mode were acquired at a full accelerating voltage of 5 kV. Details of the method were described in a previous paper (19).

RESULTS

Separation of the crude spinal cord extract by ion-exchange chromatography resulted in the recovery of proteolytic activity (here designated DCE-A) capable of releasing Leu-enkephalin-Arg6 from dynorphin A as shown in Fig. 1a. The pattern represents one of the typical separation profiles (n = 3), and the major activity was eluted between fractions 40–52. The presence of the minor peak (fractions 36–40) may suggest the existence of an isoform or an aggregate of this enzyme. To avoid any confusion, all further steps were performed using fractions 45–50 (Fig. 1a). During this procedure, over 98% of the protein contaminants present in the crude extract were removed (see Table I). Further purification was achieved on an HPLC gel filtration column (Fig. 1b) where additional amounts of inactive proteins were removed (Table II). Aliquots obtained during the last step were rerun on the same gel column to assure homogeneity of preparation. This latter procedure did not affect either the enzyme specificity or the other properties of the proteinase. Recovery and yield in this micropurification procedure are given in Table II.

| Peptide       | Sequence                  | Step | Total protein | Total activity | Specific activity | Factor |
|---------------|---------------------------|------|---------------|----------------|------------------|--------|
| Dynorphin A   | Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Ile-Arg-Pro-Lys-Leu-Lys-Asp-Asn-Gln | Extract | 1,600 | 30.0 | 0.02 | 1 |
|Dynorphin B   | Tyr-Gly-Gly-Phe-Leu-Arg-Arg-Gln-Phe-Lys-Val-Val-Thr                | DEAE | 30   | 11.0 | 0.4  | 20 |
| α-Neodynorphin| Tyr-Gly-Gly-Phe-Leu-Arg-Lys-Tyr-Pro-Lys                             | G 3000SW | 0.04 | 4.0  | 100  | 5,000 |

Obviously, by this two-step procedure the enzyme activity displayed an approximate 5,000-fold purification.

With regard to sensitivity to group-specific activators or inhibitors, purified dynorphin A convertase resembles a cysteine proteinase. Thus, it is strongly inhibited by low concentrations of thiol-blocking agents, such as p-hydroxymercuribenzoic acid, iodoacetate, and N-ethylmaleimide (Table III). Enzyme activation was achieved by diethiothreitol, 2-mercaptoethanol, and chelating agents. The protein was also sensitive to heavy metal ions.

The optimal activity of the enzyme was found to be between pH 5.0-5.5, however, subcellular fractionation, on a stepwise sucrose gradient did not follow the arylsulfatase A activity, which is a lysosomal marker (Fig. 2).

A relative molecular mass of 50 kDa of the purified enzyme was estimated from HPLC molecular sieving experiments as well as from SDS-PAGE (Fig. 3c). The purity of the preparation and MI value were additionally confirmed by autoradiography (Fig. 3b) where a single radioactive band was recorded.

Michaelis-Menten kinetics (Km) of dynorphin A hydrolysis revealed Km = 9 μM and Vmax = 0.8 s⁻¹. The most striking feature of the enzyme is its selective cleavage of only one peptide belonging to the prodynorphin family. Fig. 4 presents...
formation of Leu-enkephalin-Arg⁶ as a major product when equimolar amounts of various substrates were applied. The only effective cleavage was achieved with dynorphin A as substrate, and this specificity was further explored using different dynorphin and enkephalin fragments as shown in Table IV. The most potent inhibitors of ¹²⁵I-dynorphin A conversion to Leu-enkephalin-Arg⁶ were the dynorphin A fragments containing a pair of arginines. Moreover, the dynorphin A fragment (9-17) was found to be somewhat effective, probably due to the presence of an NH₂-terminal arginine residue. Dynorphin B also inhibits proteolysis of the substrate but is poorly converted by the enzyme, as confirmed by radioimmunoassay for Leu-enkephalin-Arg⁶ and by reversed-phase HPLC (see below). This latter technique also allowed us to verify that no other cleavage sites within dynorphin B or α-neoendorphin are affected by the isolated enzyme. Several dynorphin B fragments were also tested to determine the shortest structure still retaining the inhibitory activity (see Table IV). All these fragments contain a dibasic stretch, and they differ in a successively elongated NH₂ terminus, while the COOH-terminal portion remains constant. It is worth noting that the conserved Leu-enkephalin structure (YGGFL) also seems to play at least a minor role in substrate recognition of the enzyme although it is present in all opioid-related peptides. The latter fragments were not or negligibly (10–15%) cleaved by the enzyme, as revealed by reversed-phase HPLC studies.

Conversion studies with larger amounts were guided by reversed-phase HPLC. Fig. 5 presents a typical separation pattern recorded from an experiment with dynorphin A. Several products were identified as peptides with β-opioid activity: Leu-enkephalin-Arg⁶, Leu-enkephalin, and Leu-enkephalin-Arg⁶-Arg². This conversion was possible only when dynorphin A was applied as a substrate. Other peptides tested were not or negligibly degraded as shown in Table V.

**DISCUSSION**

Enzymatic conversion and inactivation are well-established mechanisms for maturation and degradation of biologically active peptides. Research on the biosynthesis of the opioid peptides has revealed that this requires several proteolytic steps, involving trypsin-like actions, followed by carboxypeptidase B-like cleavage at COOH-terminal basic amino acids to yield the mature peptide (20). In a previous report (5) we described a highly specific converting activity recovered from spinal cord, directed selectively toward dynorphin B. The present work provides evidence for the existence of a distinct dynorphin A processing enzyme in the same tissue. This proteinase exhibits unique properties, and its activity seems to be limited to this particular structure among other prodynorphin-related peptides. It is still not known whether there is a unique endogenous substrate for the enzyme. Its physiological significance is not understood, but the properties and possible localization within secretory granules do not exclude that dynorphin A may be a natural substrate for DCE-A. Although the turnover number (kcat) was found to be relatively low, the total proteolytic activity toward dynorphin A present in the spinal cord can convert at least 10 nmol of substrate/minute (see Table II), enough to process, within less than 1 min, the entire peptide pool present in the tissue (approximately 130 pmol of DYN A/g of tissue) (21). On the other hand, DCE-A may also play a dual role in the spinal cord by releasing endogenous β-ligands (enkephalins) on the one hand and by regulating the dynorphin A level in the tissue on the other; all this in order to control or mediate the spinal analgesic/anti-analgesic system.

The possible existence of a specific proteolytic pathway for dynorphin A at the spinal level is of particular interest since this peptide has been shown to act as an endogenous mediator in a descending antianalgesic system (22). Thus, the heptapeptide was found to produce an anti-analgesic effect following intrathecal administration in mice (23), an effect not shared by dynorphin B and α-neoendorphin (24). It is

**TABLE III**

| Agent          | Conc. (mM) | % control |
|----------------|-----------|-----------|
| EDTA           | 1.0       | 140       |
| 1,10-Phenanthroline | 1.0     | 180       |
| PMSF           | 1.0       | 94        |
| PHMB           | 0.25      | 3         |
| Iodoacetate    | 1.0       | 27        |
| N-Ethylmaleimide | 1.0    | 20        |
| DTT            | 1.0       | 160       |
| 2-Mercaptoethanol | 1.0   | 147       |
| Captopril      | 0.01      | 92        |
| Amastatin      | 0.02      | 100       |
| Phosphoramidon | 0.1       | 90        |
| Bestatin       | 0.05      | 80        |
| Leupeptin      | 0.1       | 5         |
| SDS            | 0.005     | 3         |
| ATP            | 1.0       | 100       |
| Na⁺           | 1.0       | 100       |
| K⁺            | 1.0       | 100       |
| Zn²⁺          | 1.0       | 21        |
| Ca²⁺          | 1.0       | 35        |
| Mg²⁺          | 1.0       | 80        |
| Fe³⁺          | 1.0       | 5         |
| Cu²⁺          | 1.0       | 6         |
| Co²⁺          | 1.0       | 12        |

*Fig. 2. Subcellular fractionation of human spinal cord homogenate on sucrose gradient. The assay for DCE-A activity (expressed in terms of Leu-enkephalin-Arg⁶ formed/min) was performed at two different time intervals. Arylsulfatase activity (bore) was measured with p-nitrocatechol sulfate as substrate (20). Liberated p-nitrocatechol was measured at 510 nm in an enzyme-linked immunosorbent assay plate reader (18).*
Conversion of Dynorphins

FIG. 3. Panel a, SDS-PAGE of the purified enzyme. Sample load: 1 μg (lane A) and 0.5 μg (lane B) of the enzyme. Panel b, autoradiography of the enzyme-inhibitor complex after incubation with [1,14C]acetamide. Separation conditions were the same as in panel a. For details see “Experimental Procedures.” The band below 30 kDa, migrating at the front of a gel, is always present during separations and during blank runs (no protein). The band present between stacking and running gels appears due to the radioactive contamination caused during sample application.

FIG. 4. Conversion of various prodynorphin-derived substrates to Leu-enkephalin-Arg

The results obtained by the rerun on HPLC gel-filtration and by SDS-PAGE analyses suggest that DCE-A is purified to homogeneity. However, the presence in the purified preparation of similar-sized contaminating proteins can still not be excluded. On the other hand, according to data shown in Table II, the present two-step procedure seems to be effective in the recovery of this particular enzyme, and an about 5,000-fold purification was achieved. To finally settle the question of purity for this enzyme, additional work requiring larger amounts of purified enzyme are necessary. Due to limited amounts of human spinal cord, we have here applied a micro-purification procedure, which in fact was successful in providing purified material for a proper characterization of DCE-A.

We have previously identified a dynorphin-converting enzyme in human choroid plexus (25), cerebrospinal fluid (26) and, more recently, in the human pituitary (27). Although all these enzymes have many properties in common with DCE-

![Figure 5](image_url)

FIG. 5. Reversed-phase HPLC of the converted fragments, after dynorphin A incubation with the enzyme (150 ng of protein). Products, eluted at 14, 17, and 18 min belong to dynorphin A(7–17), dynorphin A(8–17), and dynorphin A(6–17), respectively. A small peak, belonging to dynorphin A(1–7) is eluted at 19 min. The inset represents FAB-MS identification of the major converted product Leu-enkephalin-Arg

Therefore inviting to speculate whether or not the present enzyme may have a function uniquely directed toward dynorphin A.

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We have previously identified a dynorphin-converting enzyme in human choroid plexus (25), cerebrospinal fluid (26) and, more recently, in the human pituitary (27). Although all these enzymes have many properties in common with DCE-A, they do not seem to be identical to this proteinase. The specificity of the isolated enzyme, directed toward only one peptide belonging to a prodynorphin family, is quite unusual. Recently, we reported unique processing of dynorphin B in bovine and rat spinal cords (5, 28). These proteases, including DCE-A, have essential thiols in their active sites. The dynorphin B convertase seems to be involved in pain modulation, as it was recently shown that its activity displays an ~2-fold...
Conversion of Dynorphins

Table V

HPLC recorded conversion of dynorphin A and other substrates

Peptides (10 nmol) were incubated with the enzyme (0.6 µg) for 3 h at 37 °C. Reaction mixtures were analyzed on reversed-phase HPLC in conjunction with photodiode-array technique. The recovery of the peptides from the column was greater than 95%. For details, see "Experimental Procedures."

| Peptide                  | % conversion |
|-------------------------|--------------|
| Dynorphin A             | 70           |
| Dynorphin B             | 10           |
| Dynorphin B(5-9)        | <10          |
| Dynorphin B(5-10)       | <10          |
| Dynorphin B(1-9)        | 10           |
| α-Neendorphin           | <10          |
| α-Neendorphin (1-8)     | <10          |
| Substance F             | <10          |
| Bradykinin              | <10          |

Decrease in the spinal cord of rats with adjuvant-induced arthritis (28).

Recently, Devi (29) presented an interesting hypothesis concerning prediction of the monobasic cleavage site(s) in peptide precursor molecules. According to this theory, the cleavages follow certain sequence motifs, and the tendencies and rules can be applied for prediction of mono- or multibasic sites within peptides. Similar analyses have been described for the processing in dibasic stretches (30). Our previous (6, 9) and present observations suggest that the cleavage of prodynorphin-related structures in the spinal cord occurs in the vicinity of dibasic stretches, and we have not observed cleavage around other basic amino acids. Dynorphin B was found to be a potent inhibitor of DCE-A, although it was not cleaved by the enzyme. It seems to us that the presence of paired arginine residues in a sequence similar to that of dynorphin A is essential but not enough for a given peptide to be processed by the present enzyme. The COOH-terminal sequence beyond the actual cleavage site also seems to be of importance. This observation might be useful in further research on synthetic inhibitors of DCE-A. Inhibitory studies with N-peptidyl-O-acyl hydroxylamines reveal the importance of phenylalanine at the P2 position. On the other hand, the dynorphin convertases seem to recognize the entire NH2-terminal sequence of enkephalins, left of the basic pair of amino acids, although the shorter structures are not hydrolyzed by the spinal cord enzymes (5).

In addition to their action in the release of bioactive peptides from their precursors, the convertases may also serve as a specific switch for altering the receptor binding profile. This process is suggested to occur within the secretory granules during peptide maturation (31). Dynorphin A-converting enzyme from the spinal cord may be a good candidate for such function, given its pH optimum and localization. The enzyme is capable of converting the δ-receptor-specific dynorphin A to δ-specific Leu-enkephalin-Arg6 and two other minor products with selectivity for δ-receptors, Leu-enkephalin and Leu-enkephalin-Arg6-Arg7. If DCE-A is involved in a pathway for the generation of Leu-enkephalin, it seems likely that its action requires the participation of a carboxypeptidase B-like enzyme to achieve the final conversion to the pentapeptide. Moreover, the isolated proteinase is, to our knowledge, the first reported protein cleaving dynorphin A with such high specificity at acidic pH. Accordingly, it appears from our findings that conversion of prodynorphin-related peptides may occur via distinct proteolytic pathways restricted to the particular peptide, the physiological importance of which is yet unclear.

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