Characterization of a metalloprotease from ovine chromaffin granules which cleaves a proenkephalin fragment (BAM12P) at a single arginine residue

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A metalloprotease has been identified in ovine chromaffin granules which cleaves the proenkephalin fragment BAM12P to produce adrenorphin-Gly. This cleavage occurs at a single arginine residue and is an intermediate step in the production of the opiate adrenorphin *in vivo*. The identity of the product was confirmed by reverse-phase and ion-exchange chromatography. The adrenorphin-Gly-generating enzyme (AGE) was determined by chromatofocusing to have a pI value of 5.2 and bound strongly to a metal-chelate affinity column. After purification by gel-filtration and ion-exchange chromatography AGE was free of contaminating activities, as cleavage of radiolabelled BAM12P generated a single product as judged by reverse-phase and ion-exchange chromatography. The enzyme has a molecular mass of

approx. 45 kDa and a pH optimum of 8.6 in Mops, Taps and Hepes buffers, but was inhibited by phosphate buffers. It was inhibited by micromolar concentrations of copper and zinc ions, but not by millimolar concentrations of calcium or manganese ions. The addition of BAM22P, dynorphin 1–13 or dynorphin 1–8 to the incubation mixture inhibited the cleavage of radiolabelled BAM12P. The cleavage was also inhibited by the presence of catecholamines at concentrations similar to those found within the chromaffin granule. This may explain the known effect of reserpine on chromaffin cells of reducing catecholamine levels and simultaneously increasing adrenorphin levels. It may also indicate a function for AGE and adrenorphin as reporters of intragranular conditions.

INTRODUCTION

Peptide hormones and neurotransmitters are synthesized as larger precursors that are proteolytically processed to form mature bioactive peptides, an event which largely takes place in the secretory granule (Gainer et al., 1985). This processing occurs at specific sites in the precursor sequence, which are often pairs of basic residues such as lysine-arginine (Lindberg and Hutton, 1991). However, processing also occurs at single basic residues (principally arginine) and approx. 110 such cleavage sites have been identified (Devi, 1991a), including multiple sites within a single precursor. Not all single arginine sites in prohormones are subjected to proteolytic attack and therefore the target sequences must include the surrounding amino acids. Several suggestions have been made as to which sequences might indicate proteolytic sites (Schwartz, 1986; Benoit et al., 1987; Devi, 1991a).

Two proteases from the subtilisin family, PC2 and PC3 (also known as PC1), have been identified (Smeekens and Steiner, 1990; Seidah et al., 1990, 1991; Smeekens et al., 1991) which are likely to be responsible for the specific cleavages of precursors at pairs of basic residues in endocrine cells. These enzymes are expressed in the same tissues as the prohormone precursors and regulated in parallel with them (Birch et al., 1991; Day et al., 1992; Alarcon et al., 1993), they are capable of cleaving the appropriate precursors (Thomas et al., 1991; Benjannet et al., 1991; Bailyes et al., 1992; Zhou et al., 1993) and, most tellingly, the expression of the antisense message of PC1 inhibits processing in a pituitary cell line (Bloomquist et al., 1991). However, several other proteolytic activities, which are also capable of processing prohormones at pairs of basic residues, have been described in

secretory granules, including aspartyl protease (Loh et al., 1985; Parish et al., 1986) and thiol protease (Krieger and Hook, 1991; Toomim and Hook, 1992) activities. It may be that these activities act in concert with, or in the place of, PC2 and PC3 in some situations. The aspartyl is probably the mammalian homologue of the yeast protease YAP3, which processes yeast α -mating factor in cells that are deficient in the subtilisin-related protease KEX2 (Azaryan et al., 1993).

It seems probable that a separate enzyme would be required for cleavage at single arginine residues, both because PC1 and PC2 have a high specificity for pairs of basic residues (Thomas et al., 1991; Bailyes et al., 1992) and because the peptide products of cleavage at pairs of basic residues and single basic residues are found in different proportions in different endocrine tissues and brain areas, for example in the cases of prodynorphin and proenkephalin (Devi, 1991b). Furthermore cultured canine pancreatic cells retain the ability to process pancreatic polypeptide at pairs of basic residues while losing the ability to process at single arginine residues (Schwartz, 1987), suggesting that different enzymes are responsible for these activities. Several enzymes have been identified that are capable of cleaving precursors or precursor fragments at single arginine residues, for example a dynorphin-cleaving enzyme (DCE) in rat brain and pituitary (Devi et al., 1991) and in an anterior pituitary cell line (Greco et al., 1992) and also a metalloprotease that has been found in the atrial gland of *Aplysia* (Wallace et al., 1984) which cleaves dynorphin at a single arginine residue.

The production of the peptide adrenorphin (also known as metorphamide) from proenkephalin involves cleavage at a single arginine residue at the C-terminus of the sequence followed by

Abbreviations used: AGE, adrenorphin-Gly-generating enzyme; BAM12P, bovine adrenal medullary dodecapeptide; BAM22P, bovine adrenal medullary docosapeptide; DCE, dynorphin-cleaving enzyme; TFA, trifluoroacetic acid.

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amidation through the removal of a C-terminal glycine. The proportion of adrenorphin with respect to other proenkephalin products varies widely among different areas of the brain (Sonders et al., 1984; Miyata et al., 1984; Sonders and Weber, 1987), strongly suggesting that differential expression of the processing enzymes is occurring. Since adrenorphin is a strong mu-agonist, while other proenkephalin fragments are delta-agonists (Weber et al., 1983), it is possible that the opiate peptide signal transmitted by an enkephalinergic cell depends on the processing enzymes it expresses.

The adrenal medulla has been used as a model system from which to isolate proenkephalin-processing enzymes and several have been identified in the medulla which are capable of cleaving proenkephalin, e.g. PC2 and PC3 (Christie et al., 1991; Kirchmair et al., 1992), thiol (Krieger et al., 1992) and aspartyl (Krieger and Hook, 1992) proteases. The ovine adrenal medulla is a suitable model system to study processing at single arginine residues as it contains adrenorphin (Tezapsidis and Parish, 1989a) and we have previously described a metalloprotease from ovine chromaffin granules which cleaves at a single arginine residue. This protease, which we termed adrenorphin-Gly-generating enzyme (AGE) (Tezapsidis and Parish, 1989b), generates the intermediate in adrenorphin production from the proenkephalin fragment bovine adrenal medullary dodecapeptide (BAM12P). Since most processing occurs within the secretory granule it is to be expected that processing enzymes should be located in the subcellular compartment and be active under conditions found within that compartment. The main characteristics of the interior of the chromaffin granule under resting conditions are high concentrations of catecholamines, proteins and peptides and relatively low pH. Thus in this paper we characterize AGE further and examine the effect of such conditions on AGE activity.

MATERIALS AND METHODS

Subcellular fractionation

This was performed as described previously (Tezapsidis and Parish, 1989b). In brief, homogenates of sheep adrenal medullas were prepared in 0.25 M sucrose/10 mM Hepes, pH 7.4. A vesicle pellet was isolated by differential centrifugation (homogenate centrifuged at 4000 *g* for 15 min at 4 °C and the resulting supernatant at 26000 *g* for 15 min at 4 °C). The pellet was resuspended, applied to an iso-osmolar metrizamide gradient (Russell, 1981) with steps of 0.15, 0.28 and 0.45 M metrizamide and centrifuged at 105000 *g* for 1 h at 4 °C in an SW40 rotor Beckman L2 65B centrifuge. The vesicle-enriched fractions from this gradient were then applied to a 1.8 M sucrose cushion and centrifuged at 200000 *g* for 2 h in the same rotor. This yielded a highly purified granule pellet almost free from lysosomes and other subcellular organelles (Tezapsidis and Parish, 1989b).

Enzyme assay

Proteolytic activity was measured by the incubation of fractions with the model substrate ¹²⁵I-BAM12P (20000–30000 c.p.m.) in a final volume of 200 µl of 150 mM Mops, pH 8.0, at 37 °C for 2 h and was stopped by the addition of HCl to a final concentration of 0.1 M. For the determination of the pH optimum, 100 mM phosphate (pH 6.2–8.0), Mes (pH 5.4–6.8), Mops (pH 6.4–8.0), and Taps (pH 7.8–9.2) buffers were used. Peptides, bivalent cations and catecholamines were included in some incubations as indicated in the Results section. All iodinated

peptides used in this study were produced by the Chloramine T method (Hunter and Greenwood, 1962) and purified by reverse-phase h.p.l.c. An aliquot of the incubation mixture was applied to an LKB Spherisorb ODS C18 reverse-phase column and was eluted with 23 % acetonitrile/0.1 % trifluoroacetic acid (TFA) for 20 min and then with a linear gradient up to 32 % acetonitrile/0.1 % TFA over 5 min at a flow rate of 1 ml/min. Fractions (0.5 ml) were collected and their radioactivity measured. Enzyme activity was expressed as the percentage of iodinated substrate converted into product according to the equation: AGE activity = (c.p.m. of ¹²⁵I-adrenorphin-Gly product × 100)/(c.p.m. of unreacted substrate + c.p.m. of products). The identity of the product was confirmed by cation-exchange chromatography. The fractions corresponding to ¹²⁵I-adrenorphin-Gly from the reverse-phase h.p.l.c. separation were lyophilized and resuspended in 0.1 M ammonium formate/acetic acid, pH 5.5 (buffer A) and then applied to an LKB Ultrapac TSK SP-5PW cation-exchange h.p.l.c. column (7.5 mm × 75 mm). The column was pre-equilibrated with 20 % (v/v) buffer B (1 M ammonium formate/acetic acid, pH 5.5) and the sample was eluted with a linear salt gradient from 20 to 100 % buffer B in 60 min. The flow rate was 1 ml/min and 1 ml fractions were collected and radioactivity was measured. The elution positions of iodinated marker peptides was determined for both chromatographic procedures in parallel, but separate, runs.

Purification of AGE

The pellet obtained after the sucrose density centrifugation was resuspended in 10 mM ammonium formate, pH 7.0, and subjected to two freezing and thawing cycles. The resuspension was centrifuged at 10000 *g* for 30 min and the supernatant was taken as the soluble granule fraction and the pellet as the granule-membrane fraction. Only the soluble fraction contained AGE activity and this was used for the isolation and characterization of the enzyme. This supernatant was then subjected to ultrafiltration using Amicon-10 filters, this step reduced the volume of the sample to 300–400 µl and removed low-molecular-mass contaminants.

Gel-filtration and ion-exchange chromatography were performed essentially as previously described (Tezapsidis and Parish, 1989b). In short, the supernatant was applied to an LKB TSK G3000SW gel-filtration column and was eluted with 0.1 M ammonium formate, the active fraction from gel filtration was then applied to an LKB TSK-DEAE 5PW ion-exchange column and was eluted with a gradient from 0 to 0.4 M NaCl in 10 mM Mes, pH 5.5.

The active fraction after gel filtration was also subjected to chromatofocusing using a Pharmacia f.p.l.c. Mono P HR5/20 column and applying a pH gradient of 6–4. After an initial injection of 5 M NaOH the column was equilibrated with 25 mM Tris, pH 6.3 (starting buffer). The pH gradient was formed by eluting with 10 % (v/v) Polybuffer 74, pH 4 (Pharmacia). The flow rate was 0.5 ml/min, 1 ml fractions were collected and the pH value and AGE activity were determined.

A Cu²⁺-chelate affinity column (1 cm × 3.5 cm) was prepared by passing a 1 mg/ml solution of CuSO₄ through imino-diacetic acid-epoxy-activated Sepharose 6B (Sigma) and this was then pre-equilibrated with buffer that contained 0.15 M NaCl/50 mM Tris, pH 8. The active fraction obtained after gel filtration was applied and bound proteins were eluted with a linear gradient up to 0.8 M NaCl/0.1 M sodium acetate, pH 4.5, over 60 min with a flow rate of 0.2 ml/min. The column was finally washed with

50 mM EDTA/0.5 M NaCl, pH 7. Fractions (1 ml) were collected, dialysed and analysed for AGE activity.

Chromatographic fractions were prepared for gel electrophoresis by concentration of 500 μ l aliquots by ultrafiltration and lyophilization. These were then each resuspended in 20 μ l of 2.5% SDS/1 mM EDTA/5% mercaptoethanol/10 mM Tris, pH 8.0. Samples were boiled for 5 min and applied to 12.5% homogeneous SDS gels in the Pharmacia Phast system and separated according to the manufacturer's instructions. The gels were then silver stained according to a method derived from Heukeshoven and Dernick (1985).

RESULTS

Enzyme assay

AGE purified by gel-filtration and ion-exchange chromatography generated a single product peak after incubation with 125 I-BAM12P and separation by reverse-phase h.p.l.c. (Figure 1a). This product co-eluted with synthetic 125 I-adrenorphin and 125 I-adrenorphin-Gly, which were not separated by this method. When this product was collected and applied to an ion-exchange column it was eluted in the position of 125 I-adrenorphin-Gly and not that of 125 I-adrenorphin (Figure 1b); the two markers were well resolved on this system as they carry different charges.

Purification of AGE

AGE activity was eluted, after gel filtration, as a single peak corresponding to a molecular mass of approx. 45 kDa (Tezapsidis and Parish, 1989b). When this was applied to a MonoQ chromatofocusing column the activity was eluted as a single peak at a pH of 5.2 (Figure 2); however, on the basis of absorbance at 280 nm this activity was not separated from the bulk of the protein. AGE activity purified by gel filtration bound to a Cu^{2+} -chelate affinity-chromatography column and was eluted with buffers of relatively low pH and high salt concentrations. After dialysis the activity was detected as a single peak, which corresponded to a prominent absorbance peak. However, when this material was examined by SDS/PAGE it was found to contain multiple protein bands (results not shown). Furthermore, the recovery of AGE activity was relatively low. On ion-exchange chromatography the activity separated into two principal peaks (Figure 3) and when these fractions were examined by SDS/PAGE one of the fractions (fraction 33) containing activity was found to contain a single major protein band of apparent molecular mass 45 kDa (Figure 4). The other active fractions contained multiple protein bands; however, it could be seen that the intensity of the 45 kDa band in adjacent fractions paralleled the distribution of activity (i.e. more intense in fractions which had high activity and of low intensity in fractions which had little activity).

Characterization of AGE activity

AGE purified by gel-filtration and ion-exchange chromatography was used in all characterization experiments. Except where otherwise specified, incubations were conducted in Mops buffer, pH 8, with 20000 c.p.m. of 125 I-BAM12P. Incubation mixtures were separated by reverse-phase h.p.l.c. and the only major product detected in each case co-eluted with synthetic 125 I-adrenorphin-Gly. Where incubation of activity is shown (e.g. by catecholamines or peptides), a control incubation was conducted

Figure 1 Chromatographic identification of the product of incubation of 125 I-BAM12P with AGE

(a) AGE activity purified by gel-filtration and ion-exchange chromatography was incubated with 125 I-BAM12P and the incubate separated on a C18 reverse-phase column (see Materials and methods section). The elution position of radiolabelled peptides is indicated for 125 I-adrenorphin-Gly (1), 125 I-adrenorphin (2) and 125 I-BAM12P (3). (b) Fractions 25–27 from (a) were lyophilized and applied to an ion-exchange column (see Materials and methods section) and 1 ml fractions were collected and radioactivity measured. The elution position for radiolabelled peptides is indicated as in (a).

Figure 2 Chromatofocusing of AGE activity

AGE activity purified by gel filtration was applied to a Mono P column and eluted with a pH gradient. Fractions (1 ml) were collected and assayed. AGE activity is expressed relative to the highest activity found in these fractions, which is taken as 1.0.

Figure 3 Ion-exchange chromatography of AGE activity

AGE activity purified by gel filtration was applied to a DEAE column in four injections of 500 μ l using a 1 ml loading loop and eluted with a gradient of NaCl (see Materials and methods section). Fractions (1 ml) were collected and assayed. AGE activity is expressed relative to the highest activity found in these fractions, which is taken as 1.0.

Figure 4 Gel electrophoresis of AGE purified by gel-filtration and ion-exchange chromatography

AGE purified by gel filtration was applied to a DEAE column (Figure 3) and fraction 33 from this column was concentrated, separated on an SDS/12.5% polyacrylamide gel and silver stained (see the Materials and methods section). The position of molecular-mass markers is shown on the left.

at the same time with the same preparation and inhibition is expressed relative to the activity in the control sample. The conversion of substrate into product in these control samples was normally approx. 20%.

AGE was incubated in several buffer systems covering a wide pH range (Figure 5). Levels of activity were similar in Mes, Mops and Taps buffers at similar pH values and the enzyme had a pH optimum around pH 8.6. However, it was active over a broad pH range and had 30% of the maximal activity at pH 6. Activity in phosphate buffers was markedly lower than in Mes or Mops buffers at the same pH, suggesting that phosphate is inhibitory for this enzyme.

AGE was incubated with different concentrations (8 μ M–500 mM) of bivalent cations; the chloride salts of all

Figure 5 pH dependency of AGE activity

AGE activity purified by gel-filtration and ion-exchange chromatography was incubated with 125 I-BAM12P and conversion into 125 I-adrenorphin-Gly was measured over a range of pH values in the following buffers: Mes ($\circ$), Mops ($\bullet$), Taps ($\square$) and phosphate ($\blacksquare$).

Figure 6 Effect of bivalent cations on AGE activity

AGE activity purified by gel-filtration and ion-exchange chromatography was incubated with 125 I-BAM12P in the presence of the following bivalent cations (in order of effectiveness): Cu^{2+} ($\circ$), Zn^{2+} ($\bullet$), Ni^{2+} ($\blacklozenge$), Co^{2+} ($\diamond$), Mn^{2+} ($\square$), Ca^{2+} ($\blacktriangle$) and Mg^{2+} ($\blacksquare$). 125 I-adrenorphin-Gly-producing activity is expressed as a percentage of a control incubation with no added ions.

cations were used (Figure 6). Mg^{2+} , Ca^{2+} or Mn^{2+} ions did not interfere with the level of activity at the concentrations tested, while Co^{2+} , Ni^{2+} and Zn^{2+} inhibited the activity in a concentration-dependent manner and Cu^{2+} ions inhibited AGE activity even at the lowest concentration tested (8 μ M).

AGE was also incubated with a series of opiate-peptide fragments derived from proenkephalin and prodynorphin. A sample (10 μ g) of peptide was added to each incubation mixture. The most potent peptides both on a weight and a molar basis were bovine adrenal medullary docosapeptide (BAM22P), dynorphin A 1–9 and dynorphin A 1–13 (Table 1).

The enzyme was also incubated in the presence of dopamine and noradrenaline and AGE activity was inhibited by these

Table 1 Effect of peptides on AGE activity

AGE activity purified by gel-filtration and ion-exchange chromatography (0.2 μ g protein/tube) was incubated with 125 I-BAM12P in the presence of various peptides. Percentage inhibition of 125 I-adrenorphin-Gly-producing activity is expressed as a percentage of a control incubation with no added peptide. Peptide sequences are given in the one-letter code.

Peptide	% Inhibition of peptide	% Inhibition/nmol
[Met]Enkephalin (YGGFM) (87 μ M)	47	2.7
[Met]Enkephalin-Arg-Phe (YGGFMRF) (57 μ M)	52	4.5
Adrenorphin (YGGFMRRV-amide) (51 μ M)	51	5.0
Adrenorphin-Gly (YGGFMRRVG) (49 μ M)	47	4.9
BAM12P (YGGFMRRVGRPE) (35 μ M)	48	6.8
BAM22P (YGGFMRRVGRPEWMDYQKRYG) (18 μ M)	77	22.0
[Leu]Enkephalin (YGGFL) (90 μ M)	4	0.2
Dynorphin 1-6 (YGGFLR) (70 μ M)	35	3.2
Dynorphin 1-7 (YGGFLRR) (58 μ M)	26	2.3
Dynorphin 1-8 (YGGFLRRI) (51 μ M)	45	4.4
Dynorphin 1-9 (YGGFLRRIR) (44 μ M)	84	9.6
Dynorphin 1-13 (YGGFLRRIRPKLK) (31 μ M)	58	9.3

catecholamines. The inhibition was concentration-dependent and dopamine was the more potent (Figure 7). Adrenaline was also inhibitory, with a 30% inhibition at a concentration of 50 mM ($n = 2$). BSA when added to incubations at a concentration of up to 5 mg/ml had no effect on activity and neither did reserpine at concentrations up to 1 mM (the solubility limit of reserpine in our hands).

DISCUSSION

It has been predicted that cleavage will occur on the amino side of a single arginine processing site when there is a proline residue on the carboxyl side (Schwartz, 1986). Thus the sequence Gly-Arg-Pro in BAM12P would be cleaved between glycine and arginine and the product would be adrenorphin-Gly. The reverse-phase chromatography system employed separates 125 I-adrenorphin-Gly from the substrate 125 I-BAM12P and from other possible products such as [Met⁵]enkephalin but not from 125 I-adrenorphin (Tezapsidis and Parish, 1989b). Purified AGE generates a single major product from 125 I-BAM12P (Figure 1a) which elutes in the position of adrenorphin-Gly and adrenorphin.

Figure 7 Effect of catecholamines on AGE activity

AGE activity purified by gel-filtration and ion-exchange chromatography was incubated with 125 I-BAM12P in the presence of dopamine (■) or noradrenaline (●). 125 I-adrenorphin-Gly-producing activity is expressed as a percentage of a control incubation with no added catecholamine and the data is averaged from two independent experiments.

It seems unlikely that this is adrenorphin as for this to be produced either the enzyme would have to cleave within the glycine residue rather than at the peptide bond or an amidating enzyme would have to co-purify with the enzyme and be active in the incubation conditions. Since adrenorphin is amidated on the C-terminus there is a difference in the charge carried by these two molecules and they can be separated by ion-exchange chromatography. Therefore the product peak was collected from reverse-phase chromatography and applied to a cation-exchange column. The radiolabel eluted in the position of 125 I-adrenorphin-Gly rather than 125 I-adrenorphin (Figure 1b) and the two markers were widely separated on this system. The combination of these two chromatography systems, which separate by different properties of the compounds, confirmed that the identity of the product is adrenorphin-Gly. Although preparations of enzyme purified only by gel filtration generate several products from 125 I-BAM12P (Tezapsidis and Parish, 1991), after further purification by ion-exchange chromatography only a single major product is seen, even when virtually all the substrate has been converted into product. The radiolabelled substrate is present at a low concentration which means that a high proportion of the peptide could be converted into other products by quite low levels of contaminating proteolytic activities if they were present in the preparation. In studies of another single-arginine-cleaving enzyme, DCE, competing proteolytic activities were saturated with a peptide composed of part of the dynorphin sequence which was not cleaved by DCE (Devi and Goldstein, 1985; Devi et al., 1991). Since only a single product is detected after h.p.l.c. separation of the AGE incubation mixture it seems that such measures are not necessary with this preparation. Furthermore the pair of basic residues in the BAM12P sequence (arginine-arginine) are not cleaved by AGE; by contrast another study on the bovine adrenal medulla has identified a thiol protease activity (termed PTP) in this tissue which cleaves both at pairs of basic residues and at single basic residues (Krieger et al., 1992). While multiple proteolytic activity may well be involved in prohormone

processing it seems likely that there should be at least one which will be specific for single basic residues only, as such processing does not occur in all tissues where paired basic processing occurs (Devi, 1991b) and the two are separable in tissue culture (Schwartz, 1987).

Chromatofocusing of the activity revealed a single peak eluting at a pH of 5.2 (Figure 2). In this form of chromatography this pH must be equivalent to the pI of the protein. It may be that the AGE activity could be separated into multiple peaks having closely related pI values by a shallower pH gradient, but this result demonstrates that there are no major heterogeneities in the pI value of this enzyme. On the basis of the absorbance profile there was a large amount of protein eluting at this point in the pH gradient, suggesting that chromatofocusing would not be a useful purification step. Another approach which was investigated was metal-chelate affinity chromatography, which has been successfully applied to the purification of a number of proteins. Copper was used as a metal ligand as this was most effective in purifying other proteins (Nakagawa et al., 1988). The enzyme activity was eluted in a single peak under high-salt and low-pH conditions. This strong binding to the column probably indicates the presence of histidine residues on the surface of the enzyme (Nakagawa et al., 1988). The fractions containing the enzyme activity contained multiple bands when assessed by SDS/PAGE (results not shown); however, the absorbance profile suggests that large amounts of protein were excluded from these fractions and it may be that this step, in combination with another technique such as ion-exchange chromatography, could produce material of sufficient purity to obtain sequence data. However, the recovery of activity was very poor (approx. 10%), which may be due to leaching of copper from the column into the fractions [which is known to occur (Nakagawa et al., 1988)] as we have shown that copper can be an inhibitor of this activity even at micromolar concentrations. Therefore this step is not suitable for preparing enzyme for the characterization of its activity. The ion-exchange separation we had previously reported (Tezapsidis and Parish, 1989b) was modified by reducing the buffer pH to 5.5 and eluting with a shallower salt gradient with the result that the activity was spread out over a larger portion of the gradient and partially separated into two peaks (Figure 3). It is interesting that ion-exchange chromatography was more successful in separating the activity from other proteins, and into two peaks, than was chromatofocusing, which separates on a similar principle. This may be because the shallow gradient in the ion-exchange chromatography was able to separate molecules of closely related pI or it may be because of non-ionic interactions occurring on the ion-exchange column. Most of the fractions from ion-exchange chromatography which contained activity also contained multiple bands, as judged by SDS/PAGE; however, one fraction (fraction 33) only contained a single band, which corresponded to a molecular mass of 45 kDa. This is consistent with the enzyme activity eluting on gel filtration with a position corresponding to 45 kDa and it was observed that the intensity of staining of the 45 kDa band in adjacent fractions corresponded to the intensity of activity in those fractions. This suggests, but does not prove, that this band is AGE; however, a complete proof would depend on isolating sufficient pure material to obtain a partial protein sequence and then producing specific antibodies. The use of non-denaturing gels for purifying AGE has been investigated but so far it has not been possible to recover AGE activity from the gels after electrophoresis. The enzyme activity used for characterization was taken from the fractions containing the highest activity, which would normally contain more than one protein as judged by SDS/PAGE; however, they only contained a single proteolytic activity which

cleaved BAM12P as judged by the h.p.l.c. separations of the products.

AGE activity had a pH optimum of 8.6, which is not consistent with the normally expected intragranular pH of around 5 or 6 (Gainer et al., 1985). However, the enzyme did have 30% of its maximal activity at pH 6 and therefore could be active within the granule. Furthermore this incubation does not mimic all the factors within the granule and it may be that other granule components could alter the pH optimum of the enzyme. It should also be noted that several of the activities which have been described as cleaving prehormones at single arginine residues have pH optima of 7 or above, including DCE (Devi and Goldstein, 1984), somatostatin-converting enzyme (Beinfeld et al., 1989) and pro-atrial natriuretic factor-processing enzyme (Wypij and Harris, 1988). This might mean that single arginine processing occurs in a more basic environment than is normally assumed, either as an early event before granules become fully acidified or in a subpopulation of granules with a less acidic interior.

AGE activity was strongly inhibited by phosphate buffers (Figure 5); however, there is thought to be little free phosphate inside the secretory granule, all the phosphorus being in the form of ATP (Ornberg et al., 1988). The inhibition of AGE by phosphate buffers may explain why this activity was not observed in other studies of single arginine cleavages, as both the somatostatin-converting enzyme and DCE were detected in assay systems using phosphate buffers in which AGE would be inactive. It is also worth noting that another single arginine cleaving enzyme, PTP, was detected with an assay system using an EDTA-containing buffer, which would also inhibit AGE activity.

Although AGE is a metalloprotease it is inhibited by incubation with some bivalent cations (Figure 6). This phenomenon also occurs with other metalloproteases, including the prohormone-processing enzyme carboxypeptidase E (Fricker, 1991) and may be due to interaction with residues outside the active site and, like the binding to the metal chelation column, may indicate the presence of histidine residues at the surface (Nakagawa et al. 1988). It is not clear what the true concentration of bivalent cations is within the secretory granule as most will be complexed with chromogranins (Winkler et al., 1986); however, by far the most abundant is calcium, which had no inhibitory effect on AGE at concentrations up to 500 mM.

The specificity of a protease can be determined by incubation with substrate in the presence of other peptides which may be cleaved and hence act as competitive inhibitors. Since it is possible that a peptide could contain more than one peptide bond which is sensitive to AGE, a constant amount of peptide (10 µg) was added to each incubation, giving a similar 'concentration' of peptide bonds for peptides of different length. For comparison the results are also expressed on a molar basis in Table 1 (as % of inhibition/nmol of protein) which would be more relevant if there were a single peptide bond in each peptide that acted as a competitive inhibitor. The most potent inhibitors on both criteria were BAM22P, dynorphin A 1-9 and dynorphin A 1-13. Both dynorphin A 1-13 and BAM22P contain the sequence Arg-Pro, which has been suggested as a specific sequence for single arginine cleavages (Schwartz, 1986), although dynorphin A 1-9 does not. However, all three peptides fit another of the suggested criteria (Devi, 1991a) by having a basic residue at the -3 position from a potential cleavage site. Since dynorphin A peptides were inhibitory it is possible that AGE processes both proenkephalin and prodynorphin fragments *in vivo*. However, an enzyme has already been described which cleaves both dynorphin A and dynorphin B at single arginine residues (Devi and Goldstein, 1984, 1985; Devi et al., 1991).

When the incubation mixtures were separated by h.p.l.c. their absorbance profile at 223 nm was monitored to detect peptide fragments generated from the unlabelled peptides by AGE. An absorbance peak in the elution position of adrenorphin-Gly was generated from unlabelled BAM12P with a peak area of 10% of that of BAM12P; however, no fragments were detected from the other unlabelled peptides. This may indicate that the peptides were not cleaved by AGE, although it may also arise from technical limitations. First, an assay using a radioactive substrate is more sensitive than one using unlabelled peptides, and it may be that fragments were generated in low amounts below the detection threshold of the h.p.l.c. system used. This point could only be addressed with a large study in which each peptide was labelled. Secondly the h.p.l.c. separation and the gradient were optimized to separate the fragments of ^{125}I -BAM12P and may not have resolved the fragments arising from other peptides.

At first sight it seems paradoxical that catecholamines should inhibit the activity of AGE as this enzyme should be active in the granule and resting conditions in the granule include high levels of catecholamines [up to 0.6 M (Winkler and Carmichael, 1982)]. However, there are well-established links between adrenorphin production and catecholamine levels. Treatment of chromaffin cells in culture with reserpine depletes the cells of catecholamines by blocking uptake into the granule and simultaneously causes a large increase in the cellular adrenorphin content (Eiden and Zamir, 1986; Lindberg, 1986). There is no increase in the level of proenkephalin mRNA after reserpine treatment (Eiden et al., 1984) and the increase in adrenorphin is due to increased post-translational processing of higher-molecular-mass proenkephalin fragments (Eiden and Zamir, 1986; Lindberg, 1986). The cellular content of other small proenkephalin fragments such as [Met⁵]enkephalin and [Leu⁵]enkephalin (Eiden and Zamir, 1986; Lindberg, 1986), [Met⁵]enkephalin-Arg-Phe (Adams and Boarder, 1987) and amidorphin (Lindberg, 1986) were increased to a much lesser extent, suggesting a specificity for adrenorphin, the only one of these peptides produced by a single arginine cleavage. One possible explanation of these observations is that catecholamines inhibit proteolysis at single arginines, so that when reserpine decreases intragranular catecholamine concentrations then processing at these residues increases with a consequent increase in adrenorphin concentration. The inhibition of AGE by catecholamines is consistent with this model. Further support for this hypothesis is provided by the observation that processing of proenkephalin to adrenorphin is much higher in neurons in the striatum, where the processing is not occurring in the presence of catecholamines, than it is in the adrenal medulla (Eiden and Zamir, 1986).

A biological role can be proposed for the inhibition of AGE by catecholamines. It is known that adrenorphin inhibits the release of catecholamines from chromaffin cells, is 100 times more potent than [Met⁵]enkephalin in this role and may be acting through a non-classical opiate receptor (Marley et al., 1986). Thus adrenorphin could be an autocrine factor inhibiting release which would respond to intragranular catecholamine levels. Under resting conditions relatively little adrenorphin would be produced; however, when the adrenal medulla was depleted of catecholamines by intense stimulation then AGE activity would no longer be inhibited, adrenorphin would be produced and would inhibit further release, allowing catecholamine levels to be restored. In effect AGE would be a 'reporter' of intragranular conditions. Predictions can be made from this model, one is that if the adrenal medulla is depleted by intense stimulation then enkephalin levels should be restored before catecholamine levels. This is known to happen in the cat adrenal after intense stimulation by subarachnoid haemorrhage (Klein et al., 1986).

Another prediction is that *in vivo* depletion of the adrenal should result in elevated adrenorphin levels; however, at present such depletion experiments have been confined to the use of reserpine in cell culture. This is the first time it has been possible to suggest mechanisms co-ordinating classical transmitter and neuropeptide signals for the same cell from an examination of the properties of a processing enzyme.

AGE is capable of making the cleavage in BAM12P which would be made by a single-arginine-processing enzyme and makes no other in BAM12P. It is found in the subcellular compartment where processing occurs and could be active under some intragranular conditions. Hence it is a suitable candidate to be considered a single-arginine-processing enzyme. Furthermore its inhibition by catecholamines provides an explanation for the observed effects of reserpine on adrenorphin levels and allows a biological role to be suggested for AGE and adrenorphin.

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