**Endopeptidase-24.11: a cell-surface enzyme for metabolizing regulatory peptides**

A. JOHN KENNY, MICHAEL A. BOWES, NICOLAS S. GEE and REBECCA MATSAS

M. R.C. Membrane Peptidase Research Group, Department of Biochemistry, University of Leeds, Leeds LS2 9JT, U.K.

Regulatory peptides interact with specific receptors on the surface of target cells. Any molecules that are subsequently internalized are degraded by unknown intracellular mechanisms. However, a larger proportion of each peptide is degraded extracellularly, and this process is an important control mechanism since it is essential that the signal generated by a regulatory peptide should be of limited duration. The time scale may well vary for different peptides and at different target sites. While little is known of the rates of hydrolysis in the micro-environments of cell surfaces, it is known that the half-lives of injected peptide hormones in vivo are often remarkably short. Cell-surface peptidases are responsible for this rapid hydrolysis and these appear to have a wide distribution. So far, the apical brush-border membrane of the renal proximal tubule contains the highest concentration of membrane peptidases, which encompass a wide range of specificities that, in theory, should permit virtually complete hydrolysis of most simple peptides. Several of these peptidases are major components of the membrane and they have been isolated and characterized in considerable detail. A comparable battery of enzymes is known to be present on the brush-border of the small intestine, where they may be assumed to be involved in the final steps of protein digestion. The peptidases in brush-border membranes are ecartoenzymes, with the part of the peptide that is removed being located at the external surface. Peptidases in the brush-border membrane of the renal proximal tubule contain both lysosomal and brush-border membrane peptidases. They are glycoproteins of large subunit size (in comparison with most other cytosolic or secreted proteinases and peptidases) and are usually dimeric (for reviews of their structure, topology and biosynthesis, see Kenny & Maroux, 1982; Danielsen et al., 1984). One of this group of enzymes is endopeptidase-24.11 (EC 3.4.24.11), which in most species is the only endopeptidase in kidney microvilli, though a second has been observed in rat (Kenny et al., 1981) and some strains of mice (Benyon et al., 1981). Endopeptidase-24.11 was first purified from rabbit kidneys (Kerr & Kenny, 1974a,b) and subsequently from rat kidneys (Varandani & Shroyer, 1977), bovine pituitary (Orlowski & Wilk, 1981), pig kidneys (Mumford et al., 1981; Fulcher & Kenny, 1983), intestine (Fulcher et al., 1983) and brain (Relton et al., 1983). In summary, the enzyme has a subunit $M_r$ of about 90000 and contains approx. 15% carbohydrate and one zinc atom at the active site. It hydrolyses peptide bonds involving the amino groups of hydrophobic residues provided that they are not $N$- or $C$-terminal. In this property, it resembles thermolysin and other microbial zinc-metalloendopeptidases and, like them, is very susceptible to inhibition by phosphoramidon (Kenny, 1977). While much is known about the structure of purified endopeptidase-24.11, only recently has it been possible to begin to understand its probable role.

**Distribution of endopeptidase-24.11 in peripheral tissues and organs**

There is no specific substrate for the endopeptidase, hence in crude tissue extracts it can be recognized only as a phosphoramidon-sensitive peptidase in an assay with a 'general' substrate such as $\text{D}-\text{Ala}^2\text{Leu}^3\text{enkephalin}$, the tripeptide product, $\text{D}-\text{Ala}^2\text{Leu}^3\text{Gly}$, being identified by high-performance liquid chromatography (Matsas et al., 1983). However, in tissues that are very abundant in peptidase activity, a more specific IRMA is preferable. A monoclonal antibody, GK7C2, has been characterized (Gee et al., 1983) and has enabled N. S. Gee, M. A. Bowes & A. J. Kenny, unpublished work) to set up an IRMA, the first stage of which is the absorption of GK7C2 by serially diluted homogenates, which are then subjected to a solid-phase IRMA (Gee et al., 1983). The results of a survey of pig tissue are shown in Fig. 1. Kidney is clearly the richest source, and the small intestine also contains considerable amounts, though most of this is concentrated in the jejunum and none was present in the stomach. However, lymph nodes contain more endopeptidase-24.11 than any region of the gut. The value in the lymph nodes is of the order of 25% of the activity present in the jejunum, and none was present in the renal proximal tubule. In several tissues, no activity was detectable by the IRMA, the first stage of which is antibody absorption, which are then subjected to a solid-phase IRMA (Gee et al., 1983). The results of a survey of pig tissues are shown in Fig. 1. Kidney is clearly the richest source, and the small intestine also contains considerable amounts, though most of this is concentrated in the jejunum and none was present in the stomach. However, lymph nodes contain more endopeptidase-24.11 than any region of the gut. The value in the lymph nodes is of the order of 25% of the activity present in the jejunum, and none was present in the renal proximal tubule. Assays of lymph nodes from adult pigs revealed higher activity. Moreover, we have observed considerable variability from node to node (compared with more consistent results with other tissues and organs) which suggests that the activity may relate to the functional state of the lymph node. In comparison with these three organs, the activity present in other sites is small, but glandular tissue, such as salivary glands, pancreas, anterior pituitary and adrenals, contains significant quantities. Tongue (which contains both lymphoid and salivary glandular tissue), spleen and thymus contain only very low levels, as does lung. In several tissues, no activity was detectable by the IRMA. Chondrocytes harvested from articular cartilage contained high activity, which diminished rapidly during subsequent culture of the cells.

**Distribution in the central nervous system**

Fig. 2 shows the distribution by IRMA in membrane fractions prepared from brain pituitary and spinal cord. The same fractions were also assayed with $\text{D}-\text{Ala}^2\text{Leu}^3\text{enkephalin}$, and the phosphoramidon-sensitive component correlated well with these IRMA results ($r = 0.98$). The first point to note is that, even in the richest regions, the activity for the membrane fractions is about 15% of that of the kidney.

Abbreviations used: IRMA, immunoradiometric assay; CCK, cholecystokinin.
homogenate and hence only about 0.1% of that in kidney microvillar membranes. The second point is that the enzyme has distinctly non-uniform distribution, being most abundant in the spinal cord and striatum (caudate and globus pallidus). Cerebral cortex was a poor source.

We have recently shown that striatal synaptic membranes hydrolyse [D-Ala²,Leu⁵]enkephalin and substance P in a manner similar to that by purified kidney endopeptidase-24.11. Thus, the same bonds are split, the hydrolyses are equally sensitive to inhibition by phosphoramidon and thiorphan, indicating that endopeptidase-24.11 may play a significant role in the hydrolysis of peptides generated by gastric and pancreatic enzymes. In this respect it has no relevance to any control mechanisms. In the kidney, the likely substrates are peptide hormones filtered at the glomerulus, but it is not yet understood why their total hydrolysis in the proximal tubule should be a mandatory function. The reabsorption of the constituent amino acids, while desirable, would seem to be nutritionally insignificant in relation to overall excretion of free and conjugated amino acids in the urine. It is more likely that the microvillar peptidases subserve a protective function in ensuring the inactivation of one or more peptides that might otherwise exert a non-physiological effect when presented to the luminal membranes of distal nephron or bladder epithelium. However, this proposal awaits experimental confirmation.

Although the amounts of the enzyme in the central nervous system are small in comparison with several other tissues, it is easier to argue the case for it playing an important role in neuropeptide metabolism (Matsas et al., 1983). The hydrolysis of enkephalin, substance P and CCK-8 by synaptic membrane preparations are potently inhibited by phosphoramidon and thiorphan, indicating that endopeptidase-24.11 may play a significant role in the hydrolysis of each of these peptides (Deschodt-Lanckmann & Strosberg, 1983; Matsas et al., 1983, 1984b). In vitro, thiorphan has been shown to potentiate enkephalin-induced analgesia (Roques et al., 1980) though the effectiveness of a series of other inhibitors in such tests did not correlate with their potency as inhibitors of endopeptidase-24.11 (Murphy et al., 1984).

Our more recent observations showing that lymph nodes and chondrocytes are very rich in the enzyme pose many interesting questions concerning the nature and function of the possible peptide substrates for it. Chondrocytes not only lay down the matrix of cartilage, but, if appropriately

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**Fig. 1. Distribution of endopeptidase-24.11 in pig peripheral tissues and organs**

Homogenates of tissues and cells were subjected to an IRMA based on the depletion by the antigen of a monoclonal antibody (GK7C2) to endopeptidase-24.11. All assays are shown relative to kidney cortex homogenate (= 100).

**Fig. 2. Distribution of endopeptidase-24.11 in the central nervous system of the pig**

The central nervous system regions were assayed and the results expressed as in Fig. 1.

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The efficiency of hydrolysis of a group of naturally occurring peptides and some synthetic analogues (Matsas et al., 1984a,b). The results are summarized in Table 1, which shows the bonds hydrolysed by purified kidney endopeptidase-24.11 and the kinetic constants $K_m$ and $k_{cat}/K_m$, the latter being shown relative to that for [Leu⁵]enkephalin. All the bonds hydrolysed are those involving hydrophobic residues: phenylalanine, leucine, tyrosine and tryptophan. In three substrates where a bond adjacent to methionine could have been attacked (the extended [Met]enkephalins and CCK-8) no hydrolysis was observed. In each case the mode of attack by the enzyme is known to be capable of inactivating the peptide. Both the $K_m$ and the $k_{cat}/K_m$ values vary over two orders of magnitude. These large differences emphasize the important role of amino acid residues remote from those in the $P_1$ and $P_3$ positions, a feature of the enzyme which has also been noted by Orlowski & Wilk (1981) and Almenoff & Orlowski (1983) in regard to a series of synthetic substrates. Thermolysin seems to be less exacting in its specificity requirements (Pozsgay et al., 1985) and we may conclude that endopeptidase-24.11 has a more extended active site than the microbial enzyme. This feature could predispose it to hydrolyse oligopeptides in preference to large polypeptides and proteins.

**Role of endopeptidase-24.11**

For the most part, this is a topic for educated speculation. In the jejunum it is strategically located, in association with various microvillar exopeptidases, to complete the hydrolysis of peptides generated by gastric and pancreatic enzymes. In this respect it has no relevance to any control mechanisms. In the kidney, the likely substrates are peptide hormones filtered at the glomerulus, but it is not yet understood why their total hydrolysis in the proximal tubule should be a mandatory function. The reabsorption of the constituent amino acids, while desirable, would seem to be nutritionally insignificant in relation to overall excretion of free and conjugated amino acids in the urine. It is more likely that the microvillar peptidases subserve a protective function in ensuring the inactivation of one or more peptides that might otherwise exert a non-physiological effect when presented to the luminal membranes of distal nephron or bladder epithelium. However, this proposal awaits experimental confirmation.
Solid and dotted arrows represent sites of cleavage under initial-rate conditions and after extensive substrate hydrolysis, respectively. The one-letter code for amino acids is used. NH₂ at the C-terminus indicates an amidated residue. The abbreviations dA and NorL refer to D-alanine and norleucine respectively. DiMeC7 is an analogue of substance P (5-11), methylated at peptide bonds 7-8 and 8-9. Data from Matsas et al. (1984a,b).

Table 1. Hydrolysis of neuropeptides by endopeptidase-24.11

<table>
<thead>
<tr>
<th>Enkephalins and analogues</th>
<th>Bonds hydrolysed</th>
<th>KM (µM)</th>
<th>kcat/Km (relative to Leu⁵-enkephalin)</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Leu⁵]enkephalin</td>
<td>Y G G¹F L</td>
<td>86</td>
<td>1.00</td>
</tr>
<tr>
<td>[Leu⁵]enkephalinamide</td>
<td>Y G G¹F LNH₂</td>
<td>680</td>
<td>0.04</td>
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<tr>
<td>d-Ala²,Leu⁵-enkephalin</td>
<td>Y dA G¹F L</td>
<td>88</td>
<td>1.46</td>
</tr>
<tr>
<td>d-Ala²,Leu⁵-enkephalinamide</td>
<td>Y dA G¹F LNH₂</td>
<td>773</td>
<td>0.05</td>
</tr>
<tr>
<td>[Leu⁵]enkephalin-Arg⁶</td>
<td>Y G G¹F L R</td>
<td>111</td>
<td>0.95</td>
</tr>
<tr>
<td>Dynorphin (1-9)</td>
<td>Y G G¹F L R R</td>
<td>119</td>
<td>0.56</td>
</tr>
<tr>
<td>[Met⁷]enkephalin</td>
<td>Y G G¹F M</td>
<td>62</td>
<td>0.95</td>
</tr>
<tr>
<td>d-Ala²,[Met⁷]enkephalinamide</td>
<td>Y dA G¹F MNH₂</td>
<td>432</td>
<td>0.32</td>
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<tr>
<td>[Met⁷]enkephalin-Arg⁶</td>
<td>Y G G¹F M R</td>
<td>39</td>
<td>1.22</td>
</tr>
<tr>
<td>[Met⁷]enkephalin-Arg⁶-Phe⁸</td>
<td>Y G G¹F M R F</td>
<td>50</td>
<td>2.67</td>
</tr>
</tbody>
</table>

| Tachykinins and analogues | | | |
|---------------------------| | | |
| Substance P                | R P K P Q Q¹F F G/MNH₂ | 32 | 3.62 |
| Substance P (deamidated)   | R P K P Q Q¹F F G¹L M | 38 | 4.62 |
| [NorLeu¹¹] substance P     | R P K P Q Q¹F F G¹L NorL NH₂ | 84 | 1.93 |
| Physalaemin                | E A D P N K F²F²Y G¹L MNH₂ | 106 | 1.59 |
| DiMeC7                     | E Q F (MeF) (MeG)¹L MNH₂ | 2567 | 0.10 |

| Others | | | |
|--------| | | |
| Bradykinin     | R P P G¹F S P¹F R | 92 | 1.57 |
| CCK-8 (sulphated)| D Y M G¹W M D¹F¹NH₂ | 296 | 0.68 |

stimulated, can resorb it. One such stimulus is a product of the synovium (Fell & Jubb, 1977) which subsequent work has shown to be a 21kDa protein (isolated from the monocye culture medium, thought to be homologous to interleukin-1; Saklatvala et al., 1983a,b). It remains to be seen whether catalin is a susceptible substrate for endopeptidase-24.11. Lymph nodes contain many different cell types, which exchange information by a variety of humoral factors, many of which are known to be peptides. Among these are the lymphanes, including interleukins and interferons. Others include chemotactic factors of undefined structure, although it is relevant to note that a synthetic analogue, f-Met-Leu-Phe, possessing chemotactic activity, has been reported to be a substrate for a phosphoramidon-sensitive peptidase (Yuli & Snyderman, 1984). Blalock (1984) has recently reviewed the complex inter-relationships of the neuroendocrinological and immunological systems. For example, interferons may mimic the effects of certain peptide hormones (e.g. corticotropin and endorphins) and conversely peptide hormones have been shown to affect the production of interferon-γ; lymphocytes have been shown to produce corticotropin- and endorphin-like peptides in response to external stimuli. Although this area needs much clarification, there is clearly scope for enzymes such as endopeptidase-24.11 in regulating the action of these peptide factors.

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