Induction and maintenance of mucosal enterokinase activity in proximal small intestine by a genetically determined response to mediated sodium transport

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SUMMARY Mucosal enterokinase activity was established at intervals throughout the small intestine in guinea-pigs; maximum activity was present in the duodenum and proximal jejunum in new born as well as adult animals. Transposition of 5 cm lengths of small gut from the high enterokinase containing proximal region to the distal intestine and vice versa showed that mucosal enterokinase activity in the transposed segments was little changed after several weeks of healthy life. Isolation of proximal jejunal loops from luminal continuity resulted in the fall of mucosal enterokinase activity to minimal levels within 16 hours. Low levels of mucosal enterokinase activity were identified in loops of both proximal and distal jejunum 12 weeks after isolation. Luminal perfusion studies in vivo in proximal jejunal loops 24 hours after isolation showed that mucosal enterokinase activity could be restored to near normal levels within four to six hours by luminal sodium in the presence of active pancreatic endopeptidases, oligopeptides, L-amino acids, or D-glucose but not D-amino acids or D-fructose. Near normal mucosal enterokinase activity persisted in the loops for as long as luminal perfusion with 144 mM sodium and L-lysine or trypsin was maintained (24 hours). The time course of the restoration of mucosal enterokinase activity was compatible with an initial precursor activation as well as biosynthesis. The requirement for luminal sodium appeared to be absolute regardless of the co-substrate and supports the conclusion that mucosal enterokinase activity is dependent on mediated sodium transport. The ability of proximal intestinal enterocytes to respond to sodium flux with an increase in enterokinase activity is a property determined in intrauterine life: distal intestinal enterocytes may have functioning structural genes for enterokinase but appear to be unable to respond.

Enterokinase (enteropeptidase E.C. 3.4.21.9) is a regulatory enzyme which triggers the activation of pancreatic exocrine secretion. It is produced by the enterocytes of the small intestine and is expressed on the luminal brush border membrane from which it may be released by bile acids and proteolysis during digestion.

Immunofluorescent studies in man and biochemical assays in man and animals have shown that the greatest enterokinase activity is present in the duodenum and upper jejunum with low or undetectable mucosal activity thereafter in the distal small intestine.

The present study in guinea-pigs was designed to determine what luminal factors may control mucosal enterokinase activity; a preliminary report of some aspects of this work has appeared elsewhere.

Methods

Bovine trypsin, acetyltrypsin, bovine chymotrypsin, porcine pancreatic elastase, amino acids, and other reagents were all from Sigma Ltd., Poole, Dorset.
TLCK (Tosyl-lysyl-chloromethyl ketone) trypsin was prepared as described by Shaw et al; the absence of residual TLCK or trypsin activity was established before use. N-acetyl-Tyr-Lys, N-acetyl-Tyr-Tyr-Lys, and Tyr-Tyr-Lys were kindly synthesised by Dr Brian Austen.

**Enzyme Assays**

Reproducible assays were developed for mucosal enterokinase and aminopeptidase (E.C. 3.4.11.2), the latter to act as an internal control. Two to 6 mg samples of fresh intestinal mucosa were washed immediately with 0·15 M NaCl, gently blotted dry and weighed. For enterokinase, the mucosal sample was placed in 0·2 ml 70 mM-Na succinate buffer pH 5·6 containing 6·8 mM glycodeoxycholate (GDOC), 0·25 ml of a solution of bovine trypsinogen 1 mg/ml in 1 mM HCl containing 2 mM CaCl₂ and incubated for 30 minutes at 30°C. The reaction was stopped by the addition of one drop of 2 M HCl, and 0·2 ml of the incubate assayed for trypsin using Nα-benzoyl-L-arginine ethyl ester (Bz-Arg-OEt) as substrate. Specific enterokinase activity was expressed as units where 1 unit is that amount of mucosal enterokinase activity resulting in 1 pmol trypsin/min/mg wet weight mucosa in the incubate. Control tubes containing washed mucosal biopsies and Bz-Arg-OEt showed no trypsic activity. For aminopeptidase assay the mucosal sample was placed in 1 ml 50 mM-Na phosphate buffer pH 7·2 to which 0·1 ml 16·6 mM leucine-p-nitroanilide (Leu-NHPhNO₂) was added. After incubation for 15 minutes at 30°C the reaction was stopped by the addition of one drop of 2 M-HCl and the absorbance of the liberated p-nitroaniline read at 405 nm. Specific aminopeptidase activity was expressed as units where 1 unit is that amount of mucosal aminopeptidase activity resulting in 1 pmol nitroanilide/min/mg wet weight mucosa in the incubate.

Mucosal enterokinase and aminopeptidase activities at 5 cm intervals throughout the small intestine distal to the duodenojejunal flexure were established by multiple biopsies in three adult and three newborn guinea-pigs.

**Biliary Diversion**

In six guinea-pigs at operation mucosal enzyme activities were established by biopsy immediately distal to the duodenojejunal flexure and at a point about half way along the small gut. The common bile duct was ligated and the biliary stream diverted to the site of distal biopsy by cholecystenterostomy. Mucosal enzyme activities close to the original jejunal biopsy site and immediately distal to the cholecystenterostomy were determined three months later when the patency of the anastomosis was also checked.

**Intestinal Transposition**

Dunkin Hartley specific pathogen free guinea-pigs 300–500 g were used. In each of six adult guinea-pigs at operation, a 5 cm segment of distal small intestine with a low mucosal enterokinase activity was transposed on its intact mesentery into the divided proximal jejunum where mucosal enterokinase activity was high. The transposed segment was inserted in luminal continuity with the jejunum immediately distal to the duodenojejunal flexure. Anaesthesia and operative details of such procedures in guinea-pigs are reported elsewhere.

At the time of operation mucosal enzyme activities were established in duplicate biopsies at the proximal and distal ends of the transposed segment and in the jejunum at the site of its insertion. The animals were allowed to recover and given their normal diet (RGP, Labsure Animal Diet, Poole, Dorset) on which they continued to thrive. Three months later mucosal enterokinase and aminopeptidase activities were determined in the transposed segment and throughout the small intestine in each animal. In six guinea-pigs, a similar operation was carried out transposing a 5 cm length of jejunum from immediately distal to the duodenojejunal flexure into a low enterokinase secreting region about half way along the small intestine. Three months later mucosal enzyme activities in the transposed segment and small intestine were determined as before. The values for mucosal enzyme activities before and after transposition were compared using Student's paired t test and the results expressed as 95% confidence intervals. Intervals not including zero were significant at the 5% level.

In 21 guinea-pigs at operation, a 5 cm length of high enterokinase secreting proximal jejunum on its intact mesentery was isolated from luminal continuity as a Thiry-Vella loop, each end being brought out as a separate stoma on the anterior abdominal wall. Mucosal enzyme activities at each end of the isolated segment were established at the time of operation. Continuity of the remaining small intestine was restored by end to end anastomosis. Mucosal enzyme activities in the isolated jejunal loops were measured in duplicate in each of three animals at four, eight, 12, 16, 20, and 24 hours after operation. Tissue from the loop was obtained at the time of operation and 24 hours after isolation for histology. Mucosal en-
zyme activities in the isolated loops of three additional animals were determined three months later.

**LUMINAL PERFUSION OF ISOLATED JEJUNAL LOOPS**

*In vivo* luminal perfusion of isolated small intestinal loops was carried out in a series of animals to identify factors which might restore mucosal enterokinase activity after it had fallen to minimal levels as a result of being isolated from luminal continuity. The loop was fashioned at operation as before; a fine Teflon tube was inserted along its length and the distal stoma closed. After operation the loop was flushed out with distilled water, the animal fitted with a collar to prevent it biting the tube and returned to its cage. Perfusion was carried out 24 hours later at a flow rate of 3 ml/h for 24 hours, the perfusate being allowed to run to waste from the proximal stoma. Restoration of enterokinase activity in the mucosa of jejunal loops three months after isolation was also tested in three guinea-pigs. Mean mucosal enterokinase activities were determined in duplicate biopsies near each end of the loop at operation and after 24 hours' perfusion; the mean mucosal enterokinase activity in the loop after perfusion was expressed as a percentage of the initial value. The principal substances tested and details of their concentrations and the buffers used are given in Table 4.

The time-course of the restoration of mucosal enterokinase activity in the isolated jejunal loops in response to luminal perfusion using sodium and active trypsin or sodium and L-lysine was studied in an additional series of experiments using three animals for each point tested (Figure).

### Results

**MUCOSAL ENZYME ASSAYS**

Catalytic assay of enterokinase with minimal delay in fresh mucosal biopsies gave consistently reliable results; this was not the case for snap-frozen samples. It is probable that intracellular as well as cell surface enterokinase activity was detected. Although luminal enterokinase itself is resistant to tryptic cleavage,^1^*^1^* considerable lysis of the mucosal sample took place during the assay on incubation with trypsinogen. Enterokinase was released into the supernatant and transfer of the partly digested biopsy to a second incubate showed minimal residual enterokinase activity in the tissue. The aminopeptidase assay in fresh tissue was less consistent, considerable variation being found in adjacent biopsies.

Mean mucosal enterokinase activities (±1 SD) at each level of the intestine in the three normal adult guinea-pigs the small intestinal enzyme profiles of which were determined were 33.5±2.1, 30.1±2.1, and 29.1±3.6 units for about 15 cm distal to the duodenojejunal flexure. Mucosal enterokinase activity then fell sharply over about 10 cm to 0.1-1.6 units, a level of activity which was then maintained throughout the rest of the small intestine. Mucosal aminopeptidase activity was more evenly distributed throughout the small gut, the range in each animal being 0.9-1.5, 0.4-0.8, and 0.4-1.1 units. In the three newborn animals mean mucosal enterokinase activities were 31.8±3.1, 34.2±4.4, and 28.8±3.7 units in the proximal jejunum, falling sharply to 0.4-1.4 units in the rest of the small gut. Mucosal aminopeptidase levels in each animal were 0.6-0.9, 0.7-1.0, and 0.8-1.0 units.

### Table 1  Mucosal enterokinase and aminopeptidase activities in duplicate biopsies from proximal jejunum and distal small gut before and three months after biliary diversion

<table>
<thead>
<tr>
<th>Activity in proximal jejunum</th>
<th>Activity at site of cholecystenterostomy</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Enterokinase</strong></td>
<td><strong>Aminopeptidase</strong></td>
</tr>
<tr>
<td><strong>Before</strong></td>
<td><strong>After</strong></td>
</tr>
<tr>
<td>1</td>
<td>21.8, 20.7</td>
</tr>
<tr>
<td>2</td>
<td>32.7, 30.1</td>
</tr>
<tr>
<td>3</td>
<td>18.5, 16.8</td>
</tr>
<tr>
<td>4</td>
<td>13.5, 13.0</td>
</tr>
<tr>
<td>5</td>
<td>14.6, 13.7</td>
</tr>
<tr>
<td>6</td>
<td>25.4, 22.7</td>
</tr>
</tbody>
</table>

95% confidence interval

Mucosal enzyme activities given as units as defined in the text under enzyme assays, and in Tables 2 and 3.
**Effect of Biliary Diversion**

In the biliary diversion studies mucosal enterokinase and aminopeptidase activities in duplicate biopsies from the proximal jejunum and immediately distal to the site of cholecystenterostomy were not significantly changed three months after biliary diversion (Table 1).

**Effect of Segmental Transposition**

Mucosal enterokinase and aminopeptidase activities in duplicate biopsies from the recipient region of intestine, and the small intestinal segments before and three months after their transposition in luminal continuity, are shown in Tables 2 and 3. Mucosal enzyme activities in the transposed segments and recipient regions of intestine were not significantly changed except in the case of the distally transposed proximal jejunum in which mucosal enterokinase activity fell by about 10% of the control value.

Mucosal enterokinase activity in proximal jejunal loops isolated from luminal continuity fell sharply to <2.3 units within 16 hours. Mean aminopeptidase levels in the loops fell from 0.85±0.25 units before isolation to 0.65±0.20 units in the animals four to 12 hours after operation, and from 0.85±0.17 to 0.56±0.12 units in the animals 16 to 24 hours after operation. The fall in aminopeptidase, though small by comparison with enterokinase, was statistically significant in both periods (p<0.001). Histology of the loops 24 hours after isolation showed no evidence of tissue damage. Mean enterokinase levels in the proximal jejunal loops three months after isolation from luminal continuity were 0.9, 0.8, and 1.1 units; mean aminopeptidase levels were 0.5, 0.5, and 0.3 units.

**Luminal Perfusion of Isolated Jejunal Loops**

The percentage restoration of mucosal enterokinase activity in isolated proximal jejunal loops after 24 hours in vivo luminal perfusion with pancreatic endopeptidases, amino acids, oligopeptides, and sugars is shown in Table 4. With pancre-
Mucosal enterokinase activity in isolated loops of proximal jejunum by in vivo luminal perfusion in guinea-pigs

<table>
<thead>
<tr>
<th>Perfusate</th>
<th>%</th>
<th>Perfusate</th>
<th>%</th>
<th>Perfusate</th>
<th>%</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>2*</td>
<td>Na : L-Ala</td>
<td>83</td>
<td>Na : Tyr-Lys</td>
<td>88</td>
</tr>
<tr>
<td>Na : trypsin</td>
<td>3*</td>
<td>Na : L-Arg</td>
<td>41</td>
<td>Na : acetyl-Tyr-Lys</td>
<td>58</td>
</tr>
<tr>
<td>Na : trypsin</td>
<td>79*</td>
<td>Na : L-Gln</td>
<td>61</td>
<td>Na : acetyl-Tyr-Tyr-Lys</td>
<td>34</td>
</tr>
<tr>
<td>Li : trypsin</td>
<td>3*</td>
<td>Na : L-Glu</td>
<td>87</td>
<td>Na : pyroGlu-His-ProNH2</td>
<td>10*</td>
</tr>
<tr>
<td>Na : TLCK-trypsin</td>
<td>6*</td>
<td>Na : L-Gly</td>
<td>64</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na : acetyl-trypsin</td>
<td>59*</td>
<td>Na : L-His</td>
<td>78</td>
<td>D-glucose</td>
<td>1*</td>
</tr>
<tr>
<td>Na : chymotrypsin</td>
<td>38*</td>
<td>Na : L-Ile</td>
<td>74</td>
<td>Na : D-glucose</td>
<td>47*</td>
</tr>
<tr>
<td>Na : elastase</td>
<td>62*</td>
<td>Na : L-Leu</td>
<td>69</td>
<td>Li : D-glucose</td>
<td>1*</td>
</tr>
<tr>
<td>Na : trypsin</td>
<td>12*</td>
<td>Na : L-Met</td>
<td>61</td>
<td>Na : D-fructose</td>
<td>17*</td>
</tr>
<tr>
<td>Na : L-Lys</td>
<td>13*</td>
<td>Na : L-Phe</td>
<td>83</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na : L-Lys</td>
<td>90*</td>
<td>Na : D-Phe</td>
<td>13*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Li : L-Lys</td>
<td>2*</td>
<td>Na : L-Pro</td>
<td>91</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na : L-D-Lys</td>
<td>13*</td>
<td>Na : L-Trp</td>
<td>74</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na : L-D-Lys</td>
<td>13*</td>
<td>Na : L-Tyr</td>
<td>77*</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Na : L-D-Lys</td>
<td>13*</td>
<td>Na : L-Val</td>
<td>61</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Enzymes perfused at 1 mg/ml in 144 mM NaCl, 10 mM Na acetate, 2 mM CaCl2, pH 6.5. Amino acids (200 mM), D-glucose (10 mM) and oligopeptides perfused in the same buffer but without CaCl2. Oligopeptide concentrations Tyr-Lys 200 μM, α-N-acetyl Tyr-Tyr-Lys 300 μM, Tyr-Tyr-Lys 67 μM, α-N-acetyl Tyr-Tyr-Lys 300 μM, and pyroGlu-His-ProNH2 (TRH) 10 mg/ml. All perfusions carried out 24 hours after isolation of proximal jejunal loops except + three months after isolation in vivo. *Mean value from studies in three guinea-pigs.

Mucosal enterokinase activity was greatest using a combination of trypsin and sodium, appeared less with chymotrypsin and sodium or elastase and sodium, and did not occur when lithium was substituted for sodium or when the trypsin had been inactivated with TLCK. Restoration of enterokinase activity in the loops by luminal perfusion with trypsin and sodium was independent of pH in the range 5 to 8. Mucosal enterokinase activity was not restored by luminal perfusion with either trypsin or sodium alone. The ability of the mucosa in the proximal jejunal loops to respond to luminal perfusion with trypsin and sodium three months after isolation was lost. Recently isolated distal small intestinal loops showed no increase in mucosal enterokinase activity in response to perfusion with trypsin and sodium, the mean value for each animal before and after perfusion being 1.2 and 1.1 units, 0.7 and 0.7 units, and 0.9 and 0.8 units respectively.

All the L-amino acids that were tested restored mucosal enterokinase activity in the presence of sodium, but not on their own or when lithium was substituted for sodium; perfusion with D-amino acids and sodium was without effect. Both di- and tri-peptides tested were active, though in each case restoration by the N-acetylated derivative (which would protect against degradation by mucosal aminopeptidases in the loop) appeared to be lower. Thyrotropin releasing hormone (TRH), the tripeptide-amide pyroGlu-His-ProNH2, and sodium was inactive. Mean restoration of mucosal enterokinase activity by D-glucose and sodium in the three perfusions was 47% and by D-fructose and Na was 17%. Once again, substitution of lithium for sodium or the use of D-glucose alone abolished the effect.

The time course of the restoration of mucosal enterokinase activity in proximal jejunal loops in response to luminal perfusion with trypsin and sodium or L-lysine and sodium is shown in the figure. In each case, between 25% and 40%...
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restoration occurred after 20 minutes luminal perfusion, and maximal levels in the range 70% to 95% were reached in four to six hours.

Discussion

As biosynthetically complete enterokinase is resistant to tryptic digestion and the mucosal samples became extensively degraded during incubation with trypsinogen, it is probable that the assay reported total trypsin-resistant enterokinase activity in the mucosa both as mature brush border enzyme and biosynthetic precursor. Mucosal enterokinase activity in guinea-pigs showed remarkably little change after small intestinal transposition in continuity as well as after biliary diversion, despite what must have been considerable changes in the local environment of the lumen. The rapid fall in activity after isolation of high enterokinase secreting segments in the absence of a similar reduction in aminopeptidase activity or histological evidence of tissue damage, suggested that the maintenance of enterokinase in proximal intestine depended on the presence of a luminal stimulus. Trypsin releases enterokinase from the brush border on the luminal surface of the enterocyte. Luminal perfusion with active trypsin (in 2 mM Ca²⁺) did not restore mucosal enterokinase activity, so that the maintenance of high levels of enterokinase in jejunal mucosa was not simply a response to the loss of cell surface enzyme.

The luminal perfusion studies show that the high levels of enterokinase activity in proximal small intestinal mucosa are dependent on mediated sodium transport, a conclusion compatible with the information available in the field of electrolyte and non-electrolyte absorption. In the present study the requirement for luminal sodium was absolute; the cosubstrate was an amino acid of correct stereospecificity, a susceptible oligopeptide of appropriate configuration, a mixture of protein degradation products produced in the loops by a pancreatic endopeptidase with brush border aminopeptidases and oligopeptidases, or D-glucose. TRH may have been neither hydrolysed nor transported as it contains a C-terminal proline-amide and lacks a free α-amino group shown to be necessary for the transport of some dipeptides. Furthermore, unlike D-glucose, the uptake of D-fructose, which had little enterokinase restoring activity, is not sodium driven.

Intestinal aminopeptidases have been shown to exist in the cytosol of rat jejunal enterocytes in precursor form and the sucrase-isomaltase complex is the subject of limited proteolytic processing at the luminal surface by pancreatic elastase. There is some preliminary evidence in man to suggest that enterokinase may exist intracellularly as an incompletely active precursor of high molecular weight. There is also preliminary evidence that this may be the case in the pig, although these authors used low concentrations (0·2%) of Triton X100 to extract freeze dried mucosa and similar low concentrations of this detergent produced an artefactual form of brush border dipeptidyl peptidase IV in the rat. The time course of the restoration of mucosal enterokinase activity within the first 20 minutes of luminal perfusion in guinea-pigs is, however, difficult to explain on the basis even of translational control, as it was faster, for example, than the induction of ornithine decarboxylase (a high turn-over protein) in fetal rat liver cells or rat hepatoma cells in response to dibutyril cyclic AMP. The rapid response to luminal perfusion is more compatible with the activation of an enterokinase precursor as suggested to explain glucagon-induced stimulation of mucosal enzyme activity in man. Near normal levels of mucosal enterokinase in the loops after 24 hours luminal perfusion with trypsin and sodium, however, signify transcriptional activity, although additional studies using inhibitors of transcription and translation are clearly indicated.

Records of electrical potential across the brush border membrane of intestinal enterocytes in the mudpuppy Necturus maculosus have demonstrated the membrane depolarisation associated with the sodium dependent transport of L-leucine and its dimer. If similar events occurred in guinea-pig enterocytes in the present study it would be one of few examples so far as enzyme induction linked to membrane depolarisation. Induction of tyrosine hydroxylase activity has been reported in mouse sympathetic ganglia in organ culture after depolarisation due to high extracellular potassium. It remains to be shown whether the regulation of other brush border enzymes, such as sucrase induction by sucrose, is associated with a similar mechanism.

Enterokinase first appears in fetal mucosa in the 26th week of human gestation and after the 20th day in the rat. The presence of an adult type enterokinase profile in 1 day old guinea-pigs suggests that the ability of enterocytes to respond to sodium transport with enterokinase synthesis is determined in intrauterine life for any level of the developing intestine and is a property which is thereafter refractory to induction. The persist-
ence for months of a low level of enterokinase activity in unstimulated isolated loops of proximal jejunum in the guinea-pig suggests that this activity reflects a basal rate of expression of enterokinase structural genes. Enterokinase activity in isolated loops of distal jejunum suggests basal activity of a similar nature and shows that enterocytes in this region of the intestine in guinea-pigs also possess functioning enterokinase genes; the enzyme is not present merely as a consequence of adsorption to the glycocalyx from luminal content. Mucosal enterokinase activity in the proximal intestine appears to be determined by the ability of the enterocytes to respond, and not by the presence or absence of a functioning structural gene. Like bacteria, therefore, gene control in eukaryotic enterocytes may be achieved by regulation of a regulator; congenital enterokinase deficiency may be a consequence of abnormalities in genes associated with such regulatory components.

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