REVIEW ARTICLE

Mechanism of activation of the gastric aspartic proteinases: pepsinogen, progastricsin and prochymosin

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The gastric aspartic proteinases (pepsin A, pepsin B, gastricsin and chymosin) are synthesized in the gastric mucosa as inactive precursors, known as zymogens. The gastric zymogens each contain a prosegment (i.e. additional residues at the N-terminus of the active enzyme) that serves to stabilize the inactive form and prevent entry of the substrate to the active site. Upon ingestion of food, each of the zymogens is released into the gastric lumen and undergoes conversion into active enzyme in the acidic gastric juice. This activation reaction is initiated by the disruption of electrostatic interactions between the prosegment and the active enzyme moiety at acidic pH values. The conversion of the zymogen into its active form is a complex process, involving a series of conformational changes and bond cleavage steps that lead to the unveiling of the active site and ultimately the removal and dissociation of the prosegment from the active centre of the enzyme. During this activation reaction, both the prosegment and the active enzyme undergo changes in conformation, and the proteolytic cleavage of the prosegment can occur in one or more steps by either an intra- or inter-molecular reaction. This variability in the mechanism of proteolysis appears to be attributable in part to the structure of the prosegment. Because of the differences in the activation mechanisms among the four types of gastric zymogens and between species of the same zymogen type, no single model of activation can be proposed. The mechanism of activation of the gastric aspartic proteinases and the contribution of the prosegment to this mechanism are discussed, along with future directions for research.

INTRODUCTION

This review describes the activation of the gastric aspartic proteinases, with an emphasis on the role of the prosegment in the activation pathway. More specifically, the structural features of the gastric zymogens and the steps involved in the activation pathway are presented, along with a discussion of how the structure of the prosegment contributes to the activation reaction of the gastric zymogens. Directions for future research are also considered. Although the activation mechanism has been studied in other aspartic proteinases, such as the intracellular proteinases cathepsins D and E and proteinases from viral (HIV proteinase) and microbial (yeast proteinase A, aspergillopepsinogen 1, rhizopuspepsinogen) sources, this review is limited to the activation mechanism of the gastric proteinases, since this subclass has been examined in great detail in a wide range of species. For example, the amino acid sequences of a diverse range of gastric zymogens are known, the crystal structures of pig pepsinogen A, human pepsinogen A, human progastricsin and an intermediate in the activation pathway have been determined, and the conformational changes and proteolytic processing of the prosegment have been studied in depth [1–5]. For a recent review of the activation mechanism of other classes of proteinases (the metalloproteinases and the serine and cysteine proteinases), the reader is referred to Khan and James [6].

The gastric juice of vertebrates contains proteinases that are responsible for the digestion of dietary protein; these are classified into the following four groups, based on enzymic and immunochemical properties: pepsin A (EC 3.4.23.1), pepsin B (EC 3.4.23.2), pepsin C or gastricsin (EC 3.4.23.3), and chymosin (EC 3.4.23.4). Pepsin A, pepsin B and gastricsin are found in the gastric juice of adult vertebrates, while chymosin is found exclusively in neonates. As members of the aspartic proteinase class of enzymes, these gastric proteinases exhibit optimal activity at acidic pH, are inhibited by pepstatin, and possess two active-site aspartate residues that are required for catalysis. In order to protect host tissue from damage and to prevent self-autolysis, the gastric proteinases are synthesized as inactive precursors known as zymogens. The zymogen forms of pepsin A, pepsin B, gastricsin and chymosin are referred to as pepsinogen A, pepsinogen B, progastricsin and prochymosin respectively. Relative to the active enzymes, these zymogens have additional residues at the N-terminus. This type of N-terminal extension is known by a variety names, the most common being prosegment (which will be used here), propart, propeptide and activation peptide [1–5].

The gastric zymogens are synthesized and stored in the chief cells of the gastric mucosa. Upon ingestion of food, the zymogens are secreted into the lumen of the stomach and are converted into their respective active forms in the acidic gastric juice of the lumen [6]. Conversion of the gastric zymogens into active enzymes requires proteolytic removal of the prosegment. This conversion process is initiated by the acidic conditions in the lumen, is carried out by the enzyme itself and is accompanied by conformational changes in both the zymogen and the active enzyme moieties [1–3,5,7].

STRUCTURAL FEATURES OF THE GASTRIC ZYMOGENS

Primary structure

The gastric zymogens have been isolated and sequenced, at either the protein or the DNA level, from a wide range of vertebrates, including mammals, avians, reptiles, amphibians and fish. The complete amino acid sequences have been determined for the

Abbreviation used: TNS, 6-(*p*-toluidinyl)naphthalene-2-sulphonate.

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Table 1 Amino acid composition of the gastric zymogens

ND, not determined.

Enzyme	No. of residues			Refs.
	Total	Prosegment	Basic residues in prosegment	
Human pepsinogen A-3	373	47	13	[8]
Monkey pepsinogen A	373	47	12	[10]
Bovine pepsinogen A	ND	45	12	[35]
Pig pepsinogen A	370	44	13	[17]
Rabbit pepsinogen A-2	372	44	12	[20]
Bear pepsinogen A	ND	43	13	[36]
Chicken pepsinogen A	367	42	14	[18]
Turtle pepsinogen A	361	46	10	[21]
Musk shrew pepsinogen A	ND	41	9	[39]
Pig pepsinogen B	ND	43	11	[45]
Human progastricsin	372	43	12	[24]
Monkey progastricsin	374	43	12	[25]
Pig progastricsin	ND	43	12	[38]
Guinea pig progastricsin	378	49	16	[22]
Rat progastricsin	376	46	12	[26]
Bullfrog progastricsin	368	36	9	[23]
Musk shrew progastricsin	ND	43	10	[39]
Calf prochymosin	365	42	9	[28]
Pig prochymosin	365	42	10	[32,33]
Tuna pepsinogen 1	ND	41	5	[40]
Tuna pepsinogen 2	360	37	10	[34]
Tuna pepsinogen 3	ND	35	8	[40]

	1p *	10p *	20p *	30p *	40p *	Refs.
Pig pepsinogen A	--LV-KVPLVRK-KSLRQNLI-KIGKLKD-FL-KT--HKH--NP-AS-KY-F-PEAAA------L---					[17]
Bovine pepsinogen A	S-VV-KIPLVKK-KSLRQNLIEN-GKLKE-FM-RT--HKY--NL-GS-KY-I-REAA------TL---					[35]
Monkey pepsinogen A	--IIYKVPLVRK-KSLRRNLSEH-GLLKD-FL-KK--HNR--NP-AS-KY-F-PQTEA----PTL---					[9, 10]
Rabbit pepsinogen A	--IVHKVPLVRK-KSLRKNLIEK-GLLQD-YL-KT--HTP--NL-AT-KY-F-PKETF----------					[20]
Human pepsinogen A-3	--IMYKVPLIRK-KSLRRTLSER-GLLKD-FL-KK--HNL--NP-AR-KY-F-PQWEA----PTL---					[8]
Bear pepsinogen A	F-II-KVPLVKK-KSLRKNLKEH-GLLKD-FL-KK--HSP--NP-AS-KY-F-PQEAA------V---					[36]
Chicken pepsinogen A	S--IHRVPL-KKGKSLRKQLKDH-GLLED-FL-KK--HPY--NP-AS-KYH--PV---------L---					[18]
Turtle pepsinogen A	--LVTKVPLQ-KGKSLRQNLKEAF-LLED-FK-KK--HPY--NP-AS-RY-F-PSL---GDE--F---					[21]
Monkey progastricsin	A-VV-KVPL-KKFKSIRETMKEK-GLLGE-FL-RT--HKY--DP-AW-KYHF-------GD---L---					[25]
Human progastricsin	A-VV-KVPL-KKFKSIRETMKEK-GLLGE-FL-RT--HKY--DP-AW-KYRF-------GD---L---					[24]
Rat progastricsin	S-LL-RVPL-RKMKSIRETMKEQ-GVLKD-FL-KT--HKY--DP-GQ-KYHFGNF----GD---Y---					[26]
Pig progastricsin	S-VI-KVPL-KKLKSIRQAMKEK-GLLEE-FL-KT--HKY--DP-AQ-RYRI-------GD---I---					[38]
Guinea pig progastricsin	TQI--KVPL-KKIKSIREVLREK-GLLGD-FL-KN--HKP-QH--AR-KF-FRNRLAKTGD---F---					[22]
Bullfrog progastricsin	--II-KVPL-KKFKSMREVMRDH-GI-------K----APVVDP-AT-KY-Y-NN---------F---					[23]
Musk shrew progastricsin	--KVTKVTL-KKFKSIRENLREQ-GLLED-FL-KTN-HY---DP-AQ-KYHF-------GD---F---					[39]
Monkey pepsinogen III-3	--IIYKVPLVRK-KSLRRNLSEH-GLLKD-FL-KK--HNL--NP-AS-KY-F-PQAEA----PTL---					[11]
Monkey pepsinogen III-1/2	--IIHKVPLVRK-KSLRRNLSEH-GLLKD-FL-KK--HNF--NP-AS-KY-F-PQAEA----PTL---					[11]
Monkey pepsinogen I/II	--IIYKVPLVRK-KSLRRNLSEH-GLLKD-FL-KK--HNL--NP-AS-KY-F-PQAEA----PTL---					[11]
Rabbit pepsinogen II-2/3	--IVHKVPLVRK-KSLRKNLIEK-GLLQD-YL-KT--HTP--NP-AT-KY-F-PKET-------F---					[20]
Rabbit pepsinogen III	--IIHKVPLVRK-KSLRKNLIEK-GLLKD-YL-KT--HTP--NL-AT-KY-L-PKAA-------F---					[20]
Rabbit pepsinogen II-4	--IVHKVPLVRK-KSLRKNLIEK-GLLQD-YL-KT--HTP--NP-AT-KY-F-PKET-------F---					[20]
Rabbit pepsinogen F	--LVT-IPLM-KVKSMRENLREND-ILLD-YLEK---HPYR--P--TYKL-L----S--GQQD-----					[20]
Tuna pepsinogen 1	--LLQ-VPLE-KGQSAREYLQEQ-G-LWEQY--RL-KYPY--NPMA--K--FDP--S-------F---					[40]
Tuna pepsinogen 2	FH---KLPLI-KGKTAREELQER-G-LWEDY--RKQY-PY--HPMA--K--F----------------					[34]
Tuna pepsinogen 3	--IN--VPLTRH-KSMRESLREK-GIELP-YQDPAI----------KYR--P-E---------F---					[40]
Embryonic chicken pepsinogen	--IT-RLPLE-RGKKLREILREK-GLLHH-FLQH---HRY-D-IGT--K--F-PHAFP--DV--LTVV					[19]
Bovine prochymosin B	AEIT-RIPLY-KGKSLRKALKEH-GLLED-FLQKQQYGIS-----S--KY------S--G-F------					[29,30]
Pig prochymosin	SGIT-RVPL-RKGKSLRKELKER-GLLED-FLQKQPYALS-----S--KY------S--S-F------					[32,38]
Bovine prochymosin A/B	TEIT-RIPLY-KGKSLRKALKEH-GLLED-FLQKQ--QYG---ISS--KY------S--G-F------					[31]

Figure 1 Alignment of the amino acid sequences of prosegments of the gastric zymogens

The numbering is based on the sequences of pig pepsinogens, and begins at the N-terminus of the prosegment. The suffix 'p' denotes residues in the prosegment. The highly conserved residues at positions 5p (Pro), 6p (Leu), 10p (Lys), 13p (Arg), 20p (Gly) and 22p (Leu) are highlighted.

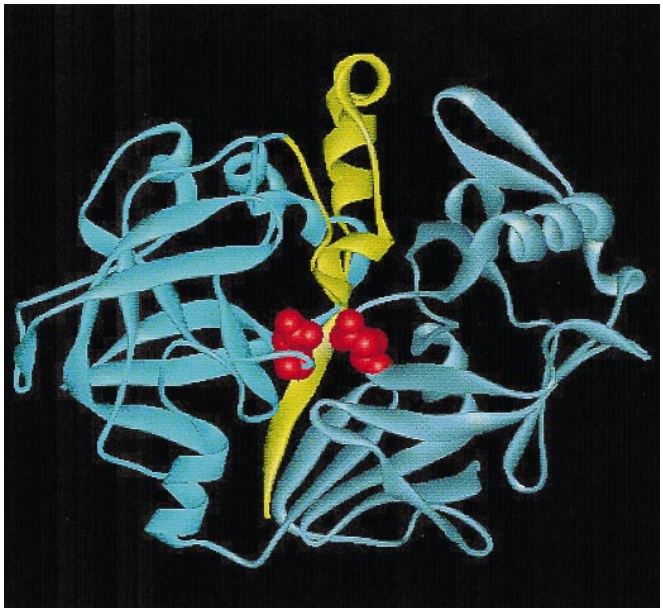

Figure 2 Tertiary structure of pig pepsinogen A

The active enzyme moiety is shown in blue, the prosegment in yellow and the two active-site aspartates (Asp-32 and Asp-215) in red. The structure of human progastricsin is similar (r.m.s. difference = 1.31 Å).

following: (1) pepsinogens A from human [8], rhesus monkey [9], Japanese monkey [10,11], pig [12–17], chicken [18], embryonic chicken [19], rabbit [20] and turtle [21]; (2) progastricsins from guinea pig [22], bullfrog [23], human [24], Japanese monkey [11,25] and rat [26,27]; (3) prochymosins from calf [28–31] and pig [32,33]; and (4) tuna pepsinogen 2 [34], which could not be classified as any of pepsinogen A, pepsinogen B, progastricsin or prochymosin. In addition, partial sequences, including the entire prosegment, are known for bovine pepsinogen A [35], bear pepsinogen A [36], pig pepsinogen B [37], pig progastricsin [38], house musk shrew pepsinogen A and progastricsin [39], and tuna pepsinogens 1 and 3 [40], neither of which could be classified as any of pepsinogen A, pepsinogen B, progastricsin or prochymosin.

The gastric zymogens consist of single polypeptide chains of approx. 370 amino acids. As indicated in Table 1, the prosegment moieties at the N-terminus of the zymogens range in length from 35 to 49 amino acid residues and are highly basic. In contrast, the active enzyme moieties contain a large number of Asp and Glu residues and are highly negatively charged. As shown in Figure 1, the prosegments of the gastric zymogens show considerable identity. More significantly, residues Pro-5p, Leu-6p, Lys-10p, Arg-13p, Gly-20p and Leu-22p are conserved in nearly all gastric proteinases whose sequences are known. (The numbering of the amino acid residues is based on the sequence of pig pepsinogen A, and the suffix 'p' is used to denote residues in the prosegment.) Furthermore, non-polar residues are present at positions 1p, 4p, 12p, 16p, 22p, 25p and 37p, basic residues at positions 3p, 8p and 36p, and hydroxylated residues at position 11p. Differences in the sequences of the prosegments are most notable at the C-terminus, in the region connecting the prosegment to the active enzyme moiety. The crystal structures of pig pepsinogen A [41–43], human progastricsin [44] and human pepsinogen A [5], to be described below, indicate that many of these conserved residues are critical to the structure and function of the prosegment. For example, the conserved non-polar amino acids at positions 12p, 16p, 22p and 25p form the hydrophobic core of the prosegment, the conserved basic residues at positions 3p, 8p, 10p, 13p and 36p are engaged in electrostatic interactions, and the conserved hydroxylated residue at position 11p and the conserved Gly at 20p cap the N- and C-terminus respectively of the first helix of the prosegment.

Three-dimensional structures

Despite the fact that the primary structures of a number of diverse gastric zymogens have been determined, the three-dimensional structures are known only for pig pepsinogen A [41–43], human progastricsin [44] and human pepsinogen A [5]. The overall structures of the three zymogens are similar; however, the similarity between the pig and human pepsinogens [root mean square (r.m.s.) = 0.91 Å for 362 α-carbons) is greater than between human pepsinogen and human gastricisin (r.m.s. = 2.52 Å for 336 α-carbons).

The active enzyme portion of the zymogens consists of two domains (shown in blue in Figure 2), which are related by a two-fold axis of symmetry and consist predominantly of β-sheet structure. At the junction of the two domains is the extended substrate binding cleft, which contains the two catalytic aspartate residues, Asp-32 and Asp-215 (shown in red in Figure 2). This binding cleft is covered and occupied by the prosegment (shown in yellow). Since the active site is fully formed in pig pepsinogen A and presumably also in the other gastric zymogens, the prosegment serves to block entry of substrate to the active site and is critical to maintaining the zymogen in its inactive form at neutral pH. The prosegments of the gastric zymogens have also been implicated in protein folding [2,47].

The prosegment in both pig pepsinogen A [41–43] and human progastricsin [44] consists of a single β-strand at the N-terminus followed by three short helical regions. In pig pepsinogen, the β-strand spans residues Leu-1p to Leu-6p, while the three α-helices comprise residues Ser-11p–Asp-19p, Lys-21p–Thr-28p and Pro-33p–Tyr-37p respectively [41–43]. Given the identity in the amino acid sequences in these regions of the prosegment, this folding is probably similar in the prosegments of the other gastric zymogens. The C-terminus of the prosegment has little secondary structure and exhibits high conformational flexibility. The N-terminus of the prosegment forms the first strand of a six-stranded anti-parallel β-sheet at the back of the molecule, while the remainder of the prosegment and the first 12 residues of the active enzyme moiety cover and occupy the substrate binding cleft (Figure 2). The prosegment is bound to the active enzyme mainly through electrostatic interactions, although hydrogen bonds and hydrophobic interactions also play a role. In pig pepsinogen A and human progastricsin, there are four and seven electrostatic interactions respectively between basic residues in the prosegment and acidic residues in the active enzyme moiety. Electrostatic interactions between Arg-13p and Asp-11 and between Lys-36p and Asp-32/Asp-215 are conserved in the crystal structures of pig pepsinogen and human progastricsin [44].

pH-DEPENDENCE OF ACTIVATION

The gastric zymogens are stable at neutral pH, but are converted into the active enzymes at pH values below 5.0. It has been proposed that this pH-dependence of activation is due to electrostatic interactions between basic residues in the prosegment and acidic residues in the active enzyme portion of the molecule [41–44]. At neutral pH, the electrostatic interactions, particularly the ion pairs between the highly conserved pro-

segment residue Lys-36p and the two catalytic aspartates, Asp-215 and Asp-32, maintain the zymogen in its inactive form, by stabilizing the position of the prosegment in the substrate binding cleft. This interaction, along with the hydrogen bonds between Tyr-37p and Asp-215 and between Tyr-9 and Asp-32, render the catalytic aspartates unavailable for catalysis at neutral pH (Figure 3). At pH values below 5.0, the acidic residues in the active enzyme portion become protonated, which disrupts the electrostatic interactions between the prosegment and the active enzyme, thus allowing the prosegment to undergo the conformational changes that initiate the activation reaction. Therefore negligible activation occurs above pH 5.0, and the rate of activation increases as the pH is lowered [48–54].

The importance of prosegment residues to the pH-dependence of the activation reaction has been demonstrated in our lab using recombinant pig pepsinogen A (C. Richter, T. Tanaka and R. Y. Yada, unpublished work). Replacement of Lys-36p with either Arg or Met resulted in a mutant that was capable of activation at more alkaline pH values than the wild-type. Molecular modelling of the pepsinogen active site indicated that the Arg and Met mutations disrupted or altered the electrostatic interactions between Lys-36p and the catalytic aspartates Asp-32 and Asp-215. Therefore, in the mutants, the prosegment may not be bound as tightly to the active enzyme moiety, and because of this increased flexibility the zymogen would not require as acidic a pH in order to undergo activation.

PROTEOLYTIC PROCESSING OF THE PROSEGMENT

The conversion of the gastric zymogens into active enzymes requires the proteolytic cleavage of the prosegment from the N-terminus of the zymogen and the dissociation of the cleaved prosegment from the active enzyme. The cleavage of the prosegment is carried out by the enzyme itself, occurs by either a direct or a sequential pathway, and involves both intramolecular and intermolecular reactions.

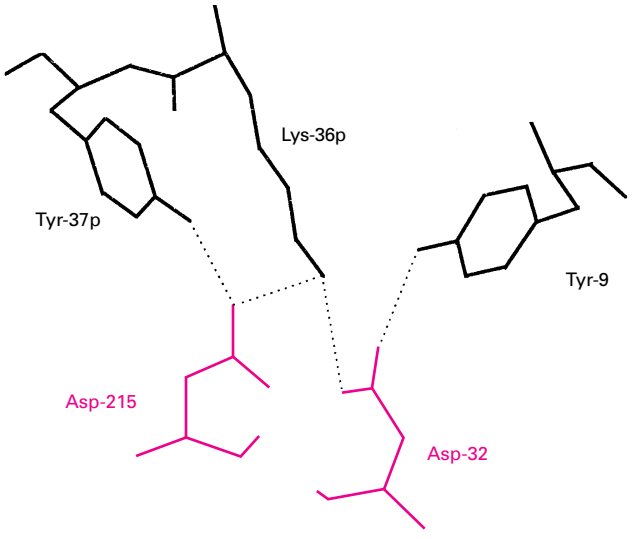

Figure 3 Interactions that block access to the catalytic aspartates (Asp-32 and Asp-215) at neutral pH

Electrostatic interactions occur between the two catalytic aspartates (shown in red) and Lys-36p. In addition, hydrogen bonds are formed between Tyr-37p and Asp-215 and between Tyr-9 and Asp-32. See the text for details.

Direct pathway

In the direct pathway, the entire prosegment is released in one step by proteolytic cleavage of the peptide bond between the C-terminus of the prosegment and the N-terminus of the active enzyme. As shown by the isolation of the intact prosegment and/or the absence of intermediate forms, the direct pathway of activation has been demonstrated for monkey pepsinogen [68,69], chicken pepsinogen A [55], bear pepsinogen A [35], tuna pepsinogen 3 [16], rabbit pepsinogen A-3 [46], bullfrog pepsinogen A [70] and bullfrog progastricsin [23,70].

Sequential pathway

In the sequential pathway, the prosegment is released in a stepwise manner by proteolytic cleavage of one or more peptide bonds within the prosegment, followed by cleavage of the peptide bond connecting the prosegment to the active enzyme. With the exception of tuna pepsinogen 1 [40] and turtle pepsinogen A [21], where the release of the prosegment proceeds through four and two intermediates respectively, the sequential pathway occurs in two steps via one intermediate; this has been demonstrated for human pepsinogen A [71–73], bovine pepsinogen A [35], house musk shrew pepsinogen A [39], pig progastricsin [38], monkey progastricsin [74], human progastricsin [72,73,75], house musk shrew progastricsin [39] and tuna pepsinogen 2 [40]. The intermediate form in the conversion of monkey progastricsin into gastricsin has been shown to have proteolytic activity [76].

Concurrent sequential and direct pathways

At pH 2.0, the removal of the prosegment from pig pepsinogen A [77] and rabbit pepsinogen A-2 [46] occurs simultaneously by both a direct and a two-step sequential pathway, as demonstrated by the isolation of both the intermediate form and the intact prosegment. In the case of pig pepsinogen A, both pathways occurred to an equal extent when the initial pepsinogen concentration was 0.16 mg/ml. However, as the initial pepsinogen concentration was increased, the proportion of prosegment released via the direct pathway increased [46]. The intermediate in the two-step sequential pathway of pig pepsinogen A is known as pseudopepsin A. Similar to pepsin A, pseudopepsin A has proteolytic activity and is alkali-labile [78,79].

Incomplete sequential pathway

In the case of pig pepsinogen B [37], bovine prochymosin [52] and rabbit pepsinogen A-1 [46], proteolytic cleavage of the prosegment at pH 2.0 results in a highly stable intermediate that is unable to undergo further processing to the mature enzyme by cleavage of the peptide bond between the prosegment and the active enzyme. Conversion of the intermediate into the mature enzyme for pig pepsinogen B [37] and bovine prochymosin [52] occurred only when the pH was raised to 5.5. This may reflect a pH-dependent change in the substrate specificity of the active enzyme [37], or possibly that the peptide bond at the junction between the prosegment and the active enzyme is blocked at pH 2.0, but becomes exposed at a more alkaline pH [52]. The stable intermediates in the activation pathway of pig pepsinogen B and bovine prochymosin are referred to as pseudopepsin B [37] and pseudochymosin [52] respectively. Pseudochymosin has been shown to have proteolytic activity [52].

Sites of cleavage in the direct and sequential pathways

The cleavage sites in the direct and sequential pathways have been identified by amino acid and N-terminal sequencing of the

protein and peptide products of activation. The major sites of cleavage at pH 2.0 in a diverse range of gastric zymogens are summarized in Figure 4. It is evident that there is considerable variability in the sites of cleavage, both among the four different types of gastric zymogens and between species within the same zymogen type; this is believed to be a function of the primary structure of the prosegment and the substrate specificity of the active enzyme [80]. For example, in the sequential pathways of bovine prochymosin and the progastricsins from human, monkey and pig, the initial site of cleavage is the peptide bond between Phe-25p and Leu-26p. However, cleavage of this bond in the activation pathway of pepsinogens A from pig, cow, human, monkey and bear is precluded by the presence of a Lys residue at the P3 position [55]. A Lys at this position is incompatible with the substrate specificity of the active site of pepsin A.

Despite the marked variability in the cleavage sites of the gastric zymogens, several similarities are evident (Figure 4). First, the majority of the cleavage sites in the sequential and direct pathways occur at peptide bonds containing non-polar residues at the P1 and P1′ positions. This is consistent with the hydrophobic character of the S1 and S1′ binding sites of the active enzyme. Although the acidic residues at the P1 or P1′ position in pig pepsinogen B and the rabbit pepsinogens appear to be exceptions, these residues will be protonated and, therefore, uncharged at pH 2.0. Secondly, in both the direct and the sequential pathways, cleavage of the peptide bond between the prosegment and the active enzyme occurs in nearly all cases on the N-terminal side of a residue three to seven amino acids upstream from a conserved Pro at position 5 in the active enzyme [34]. Finally, the initial cleavage site in the sequential pathway occurs at a peptide bond located at the C-terminal end of either the first or the second helix in the prosegment. In pig pepsinogen, these two helices span residues 11p–19p and 21p–28p [42,43].

A few studies have examined the effects of mutations in the prosegment on proteolytic processing. For example, in bovine prochymosin, simultaneous replacement of Leu-26p with Pro, Gln-27p with Arg and deletion of Lys-28p resulted in a mutant incapable of proteolytic cleavage at Phe-25p–Leu-26p at pH 2.0 [82]. However, when seven residues that span the cleavage site between the prosegment and the active enzyme were deleted, cleavage at the C-terminus of the prosegment still occurred, although at a new site and at a substantially reduced rate [86]. These findings suggest that the amino acid sequence of the internal proteolytic processing site is more critical to the activation reaction than is the sequence at the cleavage site between the prosegment and the active enzyme. This possibility needs to be examined further with regard to the cleavage sites of the pepsinogens A and the progastricsins.

Molecularity of cleavage sites in the direct and sequential pathways

Some attempts have been made to identify the molecularity of the cleavage sites in the sequential [73,79] and the direct [61,69] pathways. These studies indicate that both intramolecular and intermolecular reactions are involved in the proteolytic cleavage of the prosegment. In the intramolecular reaction, the zymogen cleaves its own prosegment, whereas in the intermolecular reaction the prosegment is cleaved by a molecule of mature enzyme or an active intermediate. Intramolecular cleavage of the prosegment is assumed to be carried out by the same active site that is responsible for the hydrolysis of substrates. For example, in pig pepsinogen A, mutating Asp-32 (one of the catalytic aspartates) to Ala produced a mutant that was incapable of being converted into pepsin at acidic pH [17].

Molecularity of cleavage sites in the sequential pathway

The molecularity of the cleavage sites in the two-step sequential pathway at pH 2.0 has been examined for pig pepsinogen A [69,79] and human pepsinogen A [73]. Hydrolysis of the initial peptide bond, Leu-16p–Ile-17p in pig pepsinogen A and Leu-22p–Lys-23p in human pepsinogen A, appears to occur via an intramolecular reaction, whereas subsequent hydrolysis of the intermediate at the peptide bond between the prosegment and the active enzyme seems to occur via an intermolecular reaction. The intramolecular reaction at the initial cleavage site has been demonstrated directly by the appearance of a product with an N-terminal Ile-17p upon activation of Sepharose-bound pig pepsinogen A at pH 2.0 [79], and indirectly for both zymogens by the finding that the addition of exogenous pepsin resulted in cleavage exclusively at the peptide bond between the prosegment and the active enzyme [69,73]. Intermolecular interactions between the zymogen and active enzyme are minimized in Sepharose-bound pepsinogen, and the addition of exogenous pepsin results in predominance of the intermolecular reaction. Cleavage of the intermediate at the peptide bond between the prosegment and the active enzyme proceeds by an intermolecular reaction, which was demonstrated by the concentration-dependence of the rate of conversion of the intermediate into the mature enzyme [79]. The molecularity of the cleavage sites in the two-step sequential pathways of the other gastric zymogens is not known.

It is interesting to note that the intermolecular cleavage at the peptide bond between the prosegment and the active enzyme was eliminated when a thioredoxin molecule was fused to pig pepsinogen A in order to improve the soluble expression of this recombinant protein in *Escherichia coli*. The presence of thioredoxin at the N-terminus of the prosegment resulted in a prosegment of increased length and bulkiness that may have inhibited the intermolecular cleavage by blocking access to the scissile bond separating the prosegment from the active enzyme, or by stabilizing an intermediate in the intramolecular reaction, thus making this reaction more energetically favourable [83].

Molecularity of cleavage sites in the incomplete sequential pathway

With respect to the incomplete sequential pathway of prochymosin, there is kinetic evidence that cleavage of the Phe-25p–Leu-26p bond at pH 2.0 occurs predominantly by an intermolecular reaction between two zymogen molecules [52]. The intermolecular mechanism is supported by the finding that prochymosin has proteolytic activity at pH 2.0. This was determined using a mutant prochymosin that was unable to undergo cleavage at the Phe-25p–Leu-26p bond [82].

Molecularity of the cleavage site in the direct pathway

The molecularity of the cleavage site in the direct pathway at pH 2.0 has been evaluated for pig pepsinogen A [61,69] and monkey pepsinogen A [69]. In the case of pig pepsinogen A, the rate of cleavage of the peptide bond between the prosegment and the active enzyme fits a first-order rate equation, indicating that cleavage of the intact prosegment is predominantly intramolecular [61]. However, the observation that this reaction rate is partially concentration-dependent suggests that intermolecular cleavage at this peptide bond can also occur [69]. In contrast, the rate of cleavage of the peptide bond between the prosegment and the active enzyme in monkey pepsinogen A was highly concentration-dependent, and was accelerated by the addition of exogenous pepsin. This suggests that, in monkey pepsinogen, the

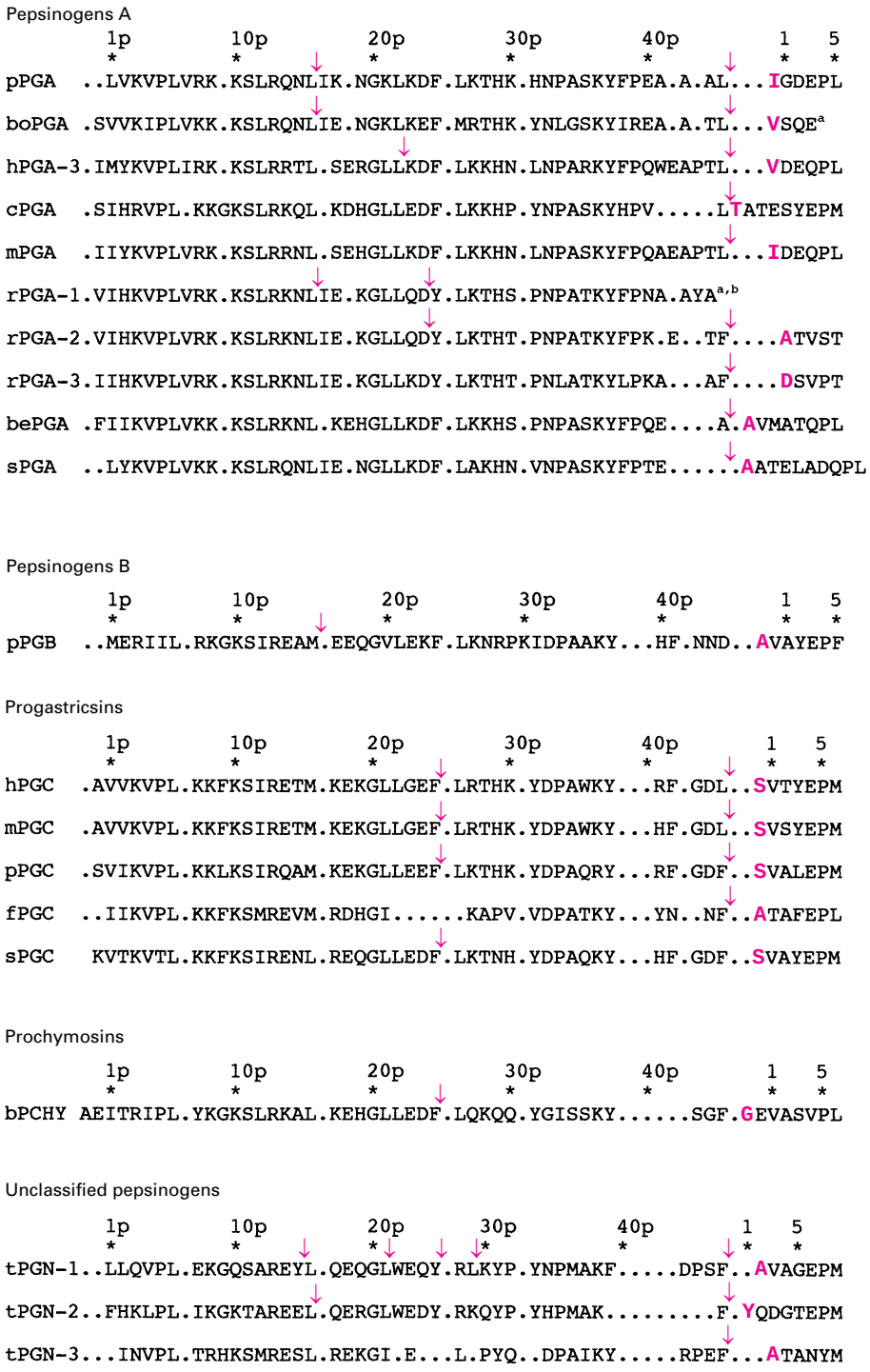

Figure 4 Major cleavage sites in prosegments of the gastric aspartic proteinases during activation at pH 2.0

Numbering is based on the sequence of pig pepsinogen A. Residues of the prosegment are numbered from the N-terminus of the zymogen and are denoted by the suffix 'p'. Numbering begins again at residue 1 at the N-terminus of the active enzyme. The N-terminus of the active enzyme is shown in red. Arrows above the sequence indicate major sites of cleavage on activation at pH 2.0. Deletions of amino acids are indicated with dots. PGA, pepsinogen A; PGB, pepsinogen B; PGC, progastricsin; PCHY, prochymosin; p, pig; bo, bovine; h, human; c, chicken; m, monkey; r, rabbit; be, bear; s, house musk shrew; f, bullfrog; t, tuna. References for the cleavage sites are: pig pepsinogen A [77–79], bovine pepsinogen A [35], human pepsinogen A [71,73], chicken pepsinogen A [55], monkey pepsinogen A [68], rabbit pepsinogen A-1, A-2 and A-3 [46], bear pepsinogen A [36], house musk shrew pepsinogen A [39], pig pepsinogen B [37], human progastricsin [75], monkey progastricsin [74], pig progastricsin [38], bullfrog progastricsin [23], bovine prochymosin [52], tuna pepsinogens 1, 2 and 3 [40], house musk shrew progastricsin [39]. [a] Remainder of sequence not determined; [b], N-terminus of active enzyme unknown.

cleavage site is predominantly intermolecular. However, intramolecular cleavage at this site was also possible, as shown by the ability of Sepharose-bound monkey pepsinogen to cleave the intact prosegment [69]. It was, therefore, hypothesized that, in monkey pepsinogen A, intramolecular cleavage of the peptide bond between the prosegment and the active enzyme is required in the early stages of the activation reaction to produce the first molecules of active enzyme, while subsequent intermolecular attack of the remaining pepsinogen by active enzyme is required to accelerate and complete the activation reaction [69]. In conclusion, both intramolecular and intermolecular reactions are involved in the direct pathway at pH 2.0. In pig pepsinogen A, the peptide bond between the prosegment and the active enzyme is more susceptible to intramolecular attack, whereas in monkey pepsinogen A this bond is more susceptible to intermolecular attack.

The finding that hydrolysis of the peptide bond between the prosegment and the active site occurs by both intra- and intermolecular reactions, whereas hydrolysis at the initial cleavage site in the sequential pathway occurs exclusively by an intramolecular reaction (with the exception of prochymosin), is consistent with the position of these peptide bonds in the crystal structures of pig pepsinogen A [42,43] and human progastricsin [44]. The peptide bond at the initial cleavage site (16p–17p and 22p–23p) and the peptide bond between the prosegment and the active enzyme are both located in close proximity to the active site, and a conformational change in the prosegment would allow the placement of the scissile bonds into the active site. The peptide bonds of the initial cleavage sites in the sequential pathway are, however, located within α-helices in the substrate binding cleft, whereas the peptide bond between the prosegment and the active enzyme is located on the surface of the molecule, in a region of the polypeptide chain that is exposed to solvent and exhibits high conformational flexibility. Consequently, only the peptide bond between the prosegment and the active enzyme is available for intermolecular attack. The accessibility of the Phe-25p–Leu-26p bond to intermolecular attack in prochymosin suggests that the prosegment of prochymosin adopts a different conformation in this region as compared with pepsinogen A and progastricsin. Therefore the position of the scissile bond relative to the active site and relative to solvent is one of the factors determining the pathway of proteolytic processing of the prosegment.

Proteolytic processing of the prosegment at pH 3

Few studies have followed the cleavage of the prosegment at pH values above pH 2.0. With pig pepsinogen A [76], monkey progastricsin [11] and human progastricsin [73], increasing the pH to 3.0 increased the proportion of the prosegment released via the direct pathway. In the case of prochymosin, increasing the pH to 4.5 resulted in direct conversion of prochymosin into chymosin without the formation of an intermediate [52]. Therefore it appears that, at pH values of 3.0 and above, the direct pathway becomes more predominant. Given that activation has been shown to occur mostly via an intermolecular reaction at pH $\geqslant$ 3 in pig pepsinogen A [48,49] and chicken pepsinogen A [51], cleavage of the prosegment by the direct pathway at pH 3 probably occurs via an intermolecular reaction.

CONFORMATIONAL CHANGES THAT ACCOMPANY THE CONVERSION PROCESS

Conversion of the gastric zymogens into their active forms at acidic pH requires changes in the conformation of the zymogen. These conformational changes have been detected in chicken pepsinogen A [51,55] and pig pepsinogen A [50,56–62] by monitoring changes in the UV absorbance or fluorescence of the zymogen upon exposure to acidic conditions.

Conformational changes that precede cleavage of the prosegment

The initial steps in the conversion of the gastric zymogens into active enzymes are conformational changes. Glick and coworkers [63] have identified three distinct conformational changes that precede cleavage of the prosegment in pig pepsinogen A at pH values between 2.0 and 3.0. The first two conformational changes occurred very rapidly and could only be detected using stopped-flow fluorescence techniques [59,60]. The first conformational change was observed as a change in the intrinsic fluorescence of pepsinogen, and was detected 5–100 ms after acidification [59]. The second conformational change was the unmasking of the active site; this was detected 2–3 s after acidification using a fluorescent analogue of pepstatin, a potent inhibitor of pepsin [60]. This second conformational change results in the intermediate δ described by Marciniszyn et al. [64]. Both the intrinsic change in fluorescence and the unmasking of the active site were first-order reactions, which were observed as two concurrent conformational changes occurring at different rates in two pepsinogen species differing in the protonation of an acidic residue with a pK_a of approx. 2.0. It has been proposed that one of the concurrent conformational changes leads to intramolecular cleavage of the prosegment at Leu-16p–Ile-17p, while the other culminates in intramolecular cleavage of the prosegment at Leu-44p–Ile-1 [63]. The cleavage sites involved in the conversion of the gastric zymogens into active enzymes are described in detail below.

The third and slowest conformational change proceeds by a first-order reaction and occurs at the same rate as cleavage of the peptide bond separating the prosegment from the active enzyme [61]. Since the bond cleavage step was inhibited by pepstatin, a potent inhibitor of pepsin, while the conformational change was not, it was concluded that this conformational change precedes bond cleavage and limits the rate at which cleavage can occur. This conformational change is believed to be the insertion of the scissile bond of the prosegment into the active site [61,62], which results in the intermediate θ, the intramolecular enzyme–substrate complex postulated by Marciniszyn et al. [64]. Therefore, prior to cleavage of the prosegment, conformational changes occur that are assumed to reflect the uncovering of the active site and the placement of the scissile bond into the active site.

It is evident that the conversion of the gastric zymogens into their active forms is a complicated process that is carried out by the enyzme itself and requires a series of conformational changes and bond cleavage steps. In general, when the pH is lowered to 2.0, the protonation of acidic residues in the active enzyme moiety initiates a number of conformational changes, including the unmasking of the active site and the placement of the scissile bond of the prosegment into the active site. These conformational changes are followed by the proteolytic cleavage of the prosegment and, finally, the dissociation of the prosegment from the active enzyme and the rearrangement of residues at the N-terminus of the active enzyme. The pathway of proteolytic cleavage shows considerable variability. Depending on the gastric zymogen, it can proceed by either a direct or a sequential pathway, and involves intermolecular and/or intramolecular reactions. Consequently, no single model of activation can be proposed to account for the mechanism of activation of the gastric zymogens. The mechanism of activation at more alkaline pH values is not well understood.

Conformational changes that follow cleavage of the prosegment

There are two conformational changes that follow proteolytic cleavage of the prosegment. The first is the dissociation of the prosegment from the active enzyme, and the second is the dramatic change in the conformation of the first 13 residues at the N-terminus of the active enzyme. The dissociation of the prosegment has been examined in pig pepsinogen A [84] and chicken pepsinogen A [85] by spin-labelling of Lys residues in the prosegment and monitoring changes in the ESR spectrum upon acidification to pH 2.0. These studies indicate that dissociation of the prosegment in both species of pepsinogen A proceeds by a first-order reaction at a rate which is one order of magnitude slower than the rate of prosegment cleavage. This suggests that the prosegment, once cleaved, dissociates slowly from the active enzyme. It is not known whether this slow dissociation of the prosegment affects the activity of the active enzyme [86].

In pepsinogen, the N-terminus of the active enzyme (residues 1–13) is located in the active site in a random-coil conformation, while the N-terminus of the prosegment (residues 1p–6p) forms the first strand of a six-stranded anti-parallel β-sheet at the opposite side of the molecule. A comparison of the crystal structures of pig pepsinogen A [41–43] and pig pepsin A [65–67] indicates that, during activation, the N-terminus of the active enzyme moves approx. 40 Å to replace the N-terminus of the prosegment in the six-stranded β-sheet. There is strong evidence that this conformational change follows cleavage of the prosegment. First, Nielsen and Foltmann [45] have observed non-covalent complexes between pig pepsin A and prosegment peptides (1p–16p and 1p–44p) during activation, and were unable to reconstitute these complexes by mixing pepsin with either of the prosegment peptides, suggesting that the exchange of residues 1p–6p of the prosegment with residues 1–6 of the active enzyme may not occur until after cleavage of the prosegment [45]. Secondly, and more important, is the recent determination of the crystal structure of an activation intermediate in the activation pathway of human progastricsin [87]. This structure shows that, after cleavage of Phe-25p–Leu-26p and the peptide bond between the prosegment and the active enzyme, residues 1p–20p of the prosegment and residues 1–6 of the active enzyme are in the same position as in the zymogen. This indicates that the final steps in the activation reaction are the dissociation of the N-terminus of the prosegment from the active enzyme and the replacement of the prosegment residues in the β-sheet with the residues at the N-terminus of the active enzyme [86].

In summary, at least four conformational changes occur during the conversion of the gastric zymogens into active enzymes at pH 2.0. These are the unmasking of the active site, the placement of the scissile bond into the active site, the dissociation of the prosegment, and the movement of the N-terminus of the active enzyme from the active site to the six-stranded anti-parallel β-sheet. The first two conformational changes precede cleavage of the prosegment, while the latter two occur after cleavage.

RATES OF ACTIVATION OF GASTRIC ZYMOGENS

Methods of determining activation rates of gastric zymogens

The overall rate of activation of the gastric zymogens has been determined by the following methods: (1) measuring the rate of conversion of the alkali-stable zymogen into an alkali-labile product by denaturing the active enzyme produced and determining the activity of the remaining zymogen [48,49,53,54,58,61,64,84]; (2) measuring the rate of cleavage of the prosegment by densitometry of activation products separated by SDS/PAGE [11,61,69,73,76]; (3) measuring the rate of change in spectroscopic properties of the zymogen (either UV absorbance or fluorescence) [50,55–57,61,62]; and (4) measuring the rate of appearance of proteolytic activity using haemoglobin [51], skim milk [13,79,85,88] or a synthetic peptide [55,89]. It is noteworthy that the rate of conversion to alkali lability, the rate of cleavage of the peptide bond between the prosegment and the active enzyme and the rate of change in TNS [6-(*p*-toluidinyl)naphthalene-2-sulphonate] fluorescence are essentially the same for pig pepsinogen A between pH 2.0 and 3.0 [63]. Therefore the first three methods appear to be measuring the same step in the activation process. According to Glick [63], this step is a conformational change that immediately precedes cleavage of the prosegment and limits the rate at which it proceeds.

The traditional method of quantifying the rate of activation of the gastric zymogens is to measure the rate of conversion to alkali lability. This method takes advantage of the difference in the stability of the gastric zymogen and its active form at pH 8.5. More specifically, with the exception of chicken pepsin(ogen) and (pro)chymosin, the zymogen is stable at pH 8.5, while the active enzyme is denatured. In order to determine the rate of conversion to alkali lability, the activation reaction is terminated at timed intervals by raising the pH to 8.5. This increase in pH not only quenches the activation reaction, but also rapidly and irreversibly denatures the active enzyme produced. After incubation at pH 8.5, the samples are then brought to pH 2.0, to convert the remaining molecules of zymogen into active enzyme. The loss of capacity for activation at pH 2.0 is used as a measure of the conversion of zymogen into active enzyme. Although the conversion to alkali lability is one of the most common methods of determining rates of activation, it has several limitations. First, the method cannot be used to determine the activation rate of chicken pepsinogen or calf prochymosin, since chicken pepsin is stable at pH 8.5 and the difference in the alkaline stabilities of prochymosin and chymosin is too small. Secondly, the activation reaction cannot be monitored in real time. Thirdly, since both the active intermediate and the mature enzyme are denatured at pH 8.5 [79], the rates of appearance of these protein products cannot be differentiated. And fourthly, when the pH is raised to 8.5, the formation of non-covalent complexes between the active enzyme and the released peptides increases the stability of the active enzyme at pH 8.5 [45]. Consequently the active enzyme may not be completely inactivated at pH 8.5, and the rate of activation may be underestimated. Methods have been developed to circumvent these problems. For example, changes in the spectroscopic properties of the zymogen, such as TNS fluorescence [61], allow activation to be followed in real time, and densitometry of activation products separated by SDS/PAGE [61,69] permits determination of the rate of appearance of the individual products of activation and eliminates non-covalent complexes between active enzyme and prosegment peptides.

Comparison of the activation rates of gastric zymogens

Since the rates of activation of the gastric zymogens are sensitive to pH, temperature, ionic strength and zymogen concentration, it is not possible to compare rates of activation determined by different researchers under different activation conditions. However, in a few cases, the rates of activation of different gastric zymogens have been compared under the same conditions. The following conclusions can be drawn from these comparisons. (1) At pH 2.0, progastricsins are activated at a higher rate than pepsinogens A. This has been shown to be the case for zymogens from human [72,73], monkey [11,76] and bullfrog [23,70]. (2) Human pepsinogen A-3 is converted into its active form at a higher rate than human pepsinogen A-5 at pH 2.0 [71–73]. (3)

With the exception of chicken pepsinogen A, non-mammalian gastric zymogens appear to be activated at higher rates than mammalian pepsinogens. For example, at pH 2.0, the activation rates of pepsinogens A from trout [54], cod [88] and turtle [21] were more rapid than that of pig pepsinogen A. In addition, bullfrog progastricsin was shown to be activated faster than human pepsinogen A-5 [23]. However, for trout pepsinogen the activation rate was similar to that of pig pepsinogen when compared at physiological temperatures of activation, i.e. 15 °C for trout and 3 °C for pig pepsinogen [54]. (4) Pig pepsinogen B is activated far more slowly than the other pig gastric zymogens [36].

It has been shown that replacement of certain basic residues in the prosegment with negatively charged, neutral or more basic groups, by chemical modification or site-directed mutagenesis, results in substantial increases in the rate of activation (C. Richter, T. Tanaka and R. Y. Yada, unpublished work) [62,81]. In these experiments, some of the electrostatic interactions between the prosegment and the active enzyme were disrupted or weakened, resulting in the prosegment being less tightly bound to the enzyme and thus more free to undergo the conformational changes required for activation. Therefore differences in the activation rates of the gastric zymogens, both between zymogen types and between species for the same zymogen type, may reflect differences in the number of electrostatic interactions between the prosegment and the active enzyme. With respect to the higher rates of activation of progastricsins relative to pepsinogens A, the crystal structures indicate that the prosegment of human progastricsin [44] is engaged in three fewer electrostatic interactions with the active enzyme than is the prosegment of pig pepsinogen A [42,43]. In addition, molecular modelling shows that there is a smaller number of possible electrostatic interactions between the prosegment and the active enzyme in human progastricsin than in human pepsinogen A [90]. For the human pepsinogen isozymogens A-3 and A-5, the prosegment sequences are identical except for a Glu at position 43 in the former and a Lys at this position in the latter [71,91]. Therefore the lower activation rate of pepsinogen A-5 may be due to the fact that the prosegment of A-5 is theoretically capable of one extra electrostatic interaction with the active enzyme moiety. With regard to the higher activation rates of the non-mammalian pepsinogens as compared with their mammalian counterparts, Table 1 clearly shows that, with the exception of chicken pepsinogen A, fewer basic residues are present in the non-mammalian prosegments, and consequently fewer interactions with the active enzyme moiety are likely. Therefore it is possible that the fewer the number of electrostatic interactions between the prosegment and the active enzyme, the less tightly the prosegment is bound to the active enzyme and the more rapidly the prosegment can undergo the conformational changes and proteolytic processing steps required for activation [90]. In contrast, the low activation rate of pig pepsinogen B is probably attributable to the low general proteolytic activity of the active enzyme [36].

What is the rate-limiting step in the activation reaction?

As discussed above, the conversion of the gastric zymogens into active enzymes involves a series of conformational changes and bond cleavage steps, one of which is the rate-limiting step in the activation reaction. The activation kinetics indicate that the rate-limiting step in the activation pathway at pH $\leqslant 3$ occurs predominantly by a first-order, and thus intramolecular, reaction in pig pepsinogen A [49,58,61,62,64,69,79,92,93], chicken pepsinogen A [51,55,85], dog pepsinogen A [53] and trout pepsinogen A [53]. In these gastric zymogens, first-order activation kinetics were shown by linear semi-logarithmic plots of pepsinogen remaining against time and/or the independence of the first-order rate constant of the initial concentration of zymogen. Early research assumed that this rate-limiting step was the intramolecular cleavage of the prosegment. However, research by Glick et al. [61,62] indicates that this rate-limiting step is a conformational change in the prosegment that immediately precedes cleavage of the prosegment and limits the rate at which cleavage occurs. This conformational change is thought to be the insertion of the scissile bond of the prosegment into the active site. In contrast, the rate-limiting step for monkey pepsinogen A [65] and bovine prochymosin [52] occurs predominantly by an intermolecular reaction at pH $\leqslant 3$. For monkey pepsinogen A, this step is the intermolecular cleavage of the peptide bond between the prosegment and the active enzyme by a molecule of pepsin, while in bovine prochymosin it is the intermolecular cleavage of Phe-25p–Leu-26p by a molecule of zymogen that has adopted an active conformation.

At pH $\geqslant 4$, the rate-limiting step in the activation pathway proceeds predominantly by an intermolecular reaction. This has been shown to be the case for pig pepsinogen A [48–50], chicken pepsinogen A [51] and bovine prochymosin [52] by the concentration-dependence of the activation rate and/or the presence of a lag-phase in the appearance of the active enzyme. This rate-limiting intermolecular step at pH $\geqslant 4$ is most probably the cleavage of the prosegment by a molecule of pepsin or active intermediate at the peptide bond separating the prosegment from the active enzyme.

FUTURE RESEARCH

This review clearly shows that our understanding of the mechanism of activation of the gastric zymogens is not complete. The conformational changes that occur during activation need to be examined in other types and species of gastric zymogens besides pig pepsinogen A, and the dramatic change in conformation at the N-terminus of the active enzyme needs to be further investigated. The cleavage sites in the prosegment have been identified in a wide range of gastric zymogens; however, the molecularity of these cleavage sites is known in only a few of these zymogens and thus needs to be studied further, particularly in the progastricsins. Mutagenesis studies of the prosegment need to be carried out in order to identify which residues are critical to the conformational changes and which are essential to proteolytic processing. Also of importance is the need to examine the activation reaction under conditions that more closely resemble those *in vivo*, i.e. activation in the presence of protein substrate and in the presence of a mixture of zymogens. Lastly, the determination of the crystal structures of gastric zymogens from different species, as well as the structures of the intermediates in the activation pathway, will further our understanding of the activation process and provide structural evidence for the similarities and differences in the activation processes of the gastric zymogens.

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