Studies on the Irreversible Step of Pepsinogen Activation†

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ABSTRACT: The bond cleavage step of pepsinogen activation has been investigated in a kinetic study in which the denatured products of short-term acidifications were separated on SDS–polyacrylamide gels and the peptide products were quantitated by densitometry. Although several peptide products were observed, under the conditions of the experiments (pH values between 2.0 and 2.8, 22 °C), the only one that was a product of an initial bond cleavage was the 44-residue peptide, which upon removal from pepsinogen yields pepsin. The rate constant for this bond cleavage is 0.015 s^{-1} at pH 2.4, which is the same as that at which the alkali-stable potential activity of pepsinogen had been found to convert to the alkali-labile activity of pepsin. When the conversion of zymogen to enzyme was followed by the change in fluorescence of adsorbed 6-(*p*-toluidinyl)naphthalene-2-sulfonate (TNS), the rate of change in TNS fluorescence was the same as the conversion to alkali lability. However, pepstatin blocked the bond cleavage of pepsinogen to pepsin, but it permitted the fluorescence change to proceed. In fact, it accelerated the apparent rate of change of TNS fluorescence by shifting the pK_a of an essential conjugate acid from 1.7 to 2.6. The conversion to alkali lability, therefore, may be considered to be a composite of a relatively slow conformational change (at the measured rate), followed immediately by a relatively fast bond cleavage.

The conversion[1] of pepsinogen has been experimentally dissected into a series of stages, characterized by progressively decreasing rate constants, that follow protonation: (a) a rapid change in intrinsic fluorescence, the basis of which is presumed to be a conformational change (Auer & Glick, 1982); (b) the unmasking of the binding site (Glick et al., 1986); (c) the conversion of the potential enzymatic activity of the zymogen, which is stable at alkaline pH values, to alkali lability (Al-Janabi et al., 1971); and (d) the dissociation of the amino-terminal propart (Twining et al., 1981). It seems logical to assume that the pepsin-like binding site is involved in conversion of pepsinogen; its unmasking, therefore, should precede bond cleavage. Although the rate of bond cleavage had not yet been measured, we suspected it is identical with the conversion to alkali lability, but this, of course, was a matter for experimentation, not conjecture.

Complicating the issue has been the controversy over the identity of the scissile bond. Dykes and Kay (1976) reported that, in the presence of pepstatin to prevent any proteolysis of the dissociated propart, they had detected a peptide that represents the amino-terminal 16 residues of pepsinogen. Christiansen et al. (1977) confirmed this by detection of the protein product of that cleavage, named pseudopepsin, in conversions performed at 0 °C in dilute solutions to minimize proteolysis of the initial products of activation. It was thought, therefore, that conversion begins by unimolecular cleavage of the Leu_{p16}–Ile_{p17} bond[2] of pepsinogen to generate pseudopepsin. Subsequent bimolecular processing would lead to cleavage of the Leu_{p44}–Ile_1 bond, removing the remaining 44 residues of the propart and forming pepsin. Kageyama and Takahashi (1983) then reported that acidification at 14 °C yielded, besides the 16-residue peptide, the complete 44-residue peptide, presumably by a single-step conversion to pepsin. They concluded, therefore, that there are concurrent pathways, resulting in initial cleavages at, alternatively, the p16–p17 or the p44–1[3] bonds. X-ray crystallography data indicate that during conversion there must be a significant movement of the amino-terminal portion of the pepsin moiety, if it is to occupy the position in pepsin that homologous residues occupy in microbial pepsins (James & Sielecki, 1986), but this is not considered to be an obstacle to either proposed activation pathway. It was possible that the 44-residue peptide had been a product of the action on pepsinogen of pepsin or pseudopepsin that had been generated in the acidified reaction mixture. Nevertheless, the proposal of Kageyama and Takahashi was indirectly supported by the finding that two early stages of the conversion, the fast change in intrinsic fluorescence (Auer & Glick, 1984) and the unmasking of the binding site (Glick et al., 1986), occur as pairs of concurrent first-order events. The zymogen is apparently locked into two conformers that are separated by protonation of a single group of pK_a near 2 and that are interconverted only slowly relative to the rates of these events.

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[1] Although the title of this paper uses "pepsinogen activation" because that phrase is immediately understood by biochemists, the word "activation" is ambiguous. We shall refer hereafter to the transformation of the zymogen to the enzyme as its conversion. Similarly, the "activation peptide", whether still attached to the protein or already cleaved, will be the propart so that we may reserve the term "activation" for the thermodynamic parameters E_a, $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, the energy, enthalpy, and entropy of activation, respectively. This also avoids the awkwardness of referring to an "activation" in the presence of pepstatin, when all resulting enzyme activity would be inhibited.

[2] Following the usage of Foltmann (1981), we designate the residues of the amino-terminal propart of pepsinogen p1, p2, ..., p44 and the residues of the pepsin moiety 1, 2, ..., 371.

[3] Abbreviations: DMF, dimethylformamide; P, pepsin; ΨP, pseudopepsin; Pg, pepsinogen; Ps, pepstatin; p1–p16 and p1–p44, 16- and 44-residue peptides, respectively, that represent the amino-terminal sequence of pepsinogen; PTH, phenylthiohydantoin; SDS, sodium dodecyl sulfate; TNS, 6-(*p*-toluidinyl)naphthalene-2-sulfonate.

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Since a central issue of pepsinogen conversion has remained the site of initial bond cleavage and its rate, we have undertaken to identify these together in a study of the kinetics of bond cleavage, in which the appearance of individual peptides is monitored following acidification. We have also adapted the observation that the fluorophore TNS emits differently in the presence of pepsinogen than in the presence of pepsin (Wang & Edelman, 1973), to devise an alternative assay for the conversion of pepsinogen. This method allows observation of the system in real time and is useful in the presence of the pepsin inhibitor pepstatin. We conclude that the irreversible step in the overall process, the conversion of the alkali-insensitive potential activity of pepsinogen to the alkali-labile activity of pepsin, may be dissected into two different stages: the first is a relatively slow conformational change which is compatible with pepstatin; the second follows immediately and is a cleavage of the Leu_{p44}–Ile_1 bond, which is inhibited by pepstatin.

Materials and Methods

Materials. Pepsinogen was a product of Sigma Chemical Co. (St. Louis, MO). Solutions of the protein were quantitated by its extinction coefficient, $\epsilon_{280} = 1.25 \times 10^4\ M^{-1}\ cm^{-1}$ (Wang & Edelman, 1973). Pepstatin was also purchased from Sigma. Its solutions were standardized by titration against the activity of acid-treated pepsinogen. TNS was also a Sigma product; its concentration in solution was determined by its absorbance, $\epsilon_{317} = 1.89 \times 10^4\ M^{-1}\ cm^{-1}$ (Wang & Edelman, 1973).

Pepsin Activity. The turbidometric milk-clotting assay of McPhie (1976) was performed using a Gilford 2400 recording spectrophotometer.

Bond Cleavage. Solutions of pepsinogen in 100 mM NaCl–25 mM glycine, pH 7.3, were passed over a Bio-Gel P-30 (Bio-Rad Laboratories, Inc., Richmond, CA) gel filtration column (2.5 × 24.7 cm) immediately before use. To initiate activation, zymogen solutions, 40–125 μM in the glycine–NaCl buffer, were mixed with equal volumes of acid solutions prepared by adjusting the pH of 100 mM monobasic sodium phosphate solutions with concentrated HCl to values, after mixing with the zymogen solution, between 1.8 and 3.2. The mixing was effected with an Update Instrument Inc. (Madison, WI) System 777 rapid mixing apparatus that mixed precisely 31.5 μL of each solution and at precisely timed intervals delivered the mixture to quenching tubes containing 63 μL of 2% SDS–20% (v/v) glycerol–600 mM NH_4OH, to give solutions with pH values, after mixing, of 9–10. The pH values of identically acidified zymogen solutions were determined with a Radiometer (Copenhagen) Model 26 pH meter.

Electrophoresis. Samples quenched in this manner, containing 20 μg of protein, were separated on 1.5 mm thick, 10 cm wide polyacrylamide gels, following Laemmli (1970), that had been cast with an exponential 5–20% gradient cross-linking. Electrophoresis was at 70–80 mA (for two electrophoresis plates). After electrophoresis, the gel slabs were soaked in 5% formalin for 20 min, then stained in 0.1% Coomassie brilliant blue–45% methanol–10% acetic acid, and destained in 15% ethanol–10% acetic acid.

Quantitative Analysis of Electrophoretograms. The gels were scanned in an apparatus built around a Gilford spectrophotometer that was designed by Dr. Vaughn Jackson of The Medical College of Wisconsin, and the data were analyzed by using the 3.6C version of the integration program of Nelson Analytical, Inc. (Cupertino, CA), to yield band intensities as a percent of the total density for each lane of the gel. As the peptide intensities were greater than could be accounted for by the number of residues in the peptides, the intensities were weighted according to the sum of intensities of all peptide bands in relatively long-term activations, compared to the fraction of amino acid residues represented by the peptides, i.e., 44 of 371 residues. The intensities of the peptide bands were then recalculated as percentages of total density. It was these data that were used to evaluate rate constants for their appearance.

Peptide Sequence Analysis. Sequencing was performed on an Applied Biosystems Model 477A pulsed liquid-phase automated sequencer. Samples were loaded onto precycled Biobrene-impregnated glass fiber disks and subjected to repetitive Edman degradation (Hewick et al., 1981), using the "normal" program for reaction and conversion cycles, which involves double cycling. PTH derivatives of amino acids were identified by subjecting 50 μL of the 132 μL recovered from each conversion cycle to reversed-phase HPLC using an on-line Model 120A PTH-amino acid analyzer.

Rates of Conversion. Rates of conversion to alkali lability were performed substantially as described by Al-Janabi et al. (1972). Typical solutions were composed of 0.50 mL of 2 mg/mL pepsinogen in 0.05 M sodium acetate (pH 5.3)–0.2 M NaCl, 0.05 mL of 2 mM TNS in 50% DMF–50% 0.05 M sodium acetate (pH 5.3)–0.2 M NaCl, or the same solution without TNS and 0.05 mL of 2-propanol. Conversion was initiated by addition of 0.50 mL of 0.05 M citric acid–0.2 M NaCl and HCl (0–0.06 M) sufficient to achieve the desired final pH value. Aliquots of 0.10 mL were removed at timed intervals, quenched in 0.30 mL of 0.125 M Tris-HCl, pH 8.5, and then acidified and assayed for pepsin activity. Least-squares regression analysis of the logarithm of activity vs time yielded first-order rate constants.

Fluorometric Assay. In the fluorescence assay, the solution contained 1.00 mL of 1.2–2.4 μM pepsinogen in 0.05 M sodium acetate (pH 5.3)–0.2 M NaCl, 0.10 mL of 2 mM TNS in 50% DMF–50% 0.05 M sodium acetate (pH 5.3)–0.2 M NaCl, and 0.10 mL of 490 μM pepstatin in 2-propanol or an equal volume of the alcohol without pepstatin. Conversion was initiated by addition of 1.00 mL of 0.05 M citric acid–0.2 M NaCl and HCl (0–0.06 M) sufficient to achieve the desired final pH value, which was measured on a pH meter at the end of the experiment. Fluorescence was monitored on a Perkin-Elmer MPF-445B spectrofluorometer. Excitation was at 366 nm and emission at 445 nm. Relative fluorescence decreased with time in a manner well described by a single exponential (but see below). The decrease, as a percent of the total fluorescence (the value extrapolated to zero time minus the value with a closed shutter), was 22 ± 2% in the absence of pepstatin and 42 ± 5% in its presence. Experiments were also run at one-fifth the TNS concentration, with identical results. The data were collected on a strip chart recorder and then transcribed onto semilogarithmic plots; a least-squares regression analysis of the data yielded a first-order rate constant.

Temperature Dependence of Conversion. To calculate the thermodynamic activation parameters for the reaction that is monitored by the fluorometric assay, a minimum of two rate determinations at each of four temperatures between 10 and 22 °C were performed.

Results

The electrophoretic method was able to separate the peptides formed during activation (Figure 1). An appropriate choice of times between acidification and quenching allowed observation of the greatest part of the activation process. The 16-residue peptide derived from the amino-terminal sequence of pepsinogen was not detected, either because it was lost from

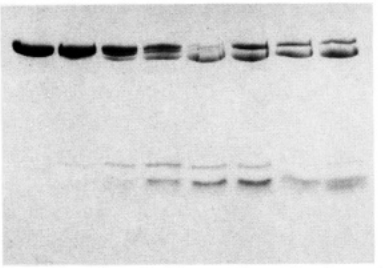


FIGURE 1: Polyacrylamide gel electrophoresis of pepsinogen and products of short-term acidification. Pepsinogen was brought to pH 2.39, 22 °C, for varying periods of time before being quenched in NH_4OH–SDS. Times in acid were (left to right) 0.00, 4.05, 8.05, 12.05, 20.05, 30.05, 60.05, and 90.05 s.

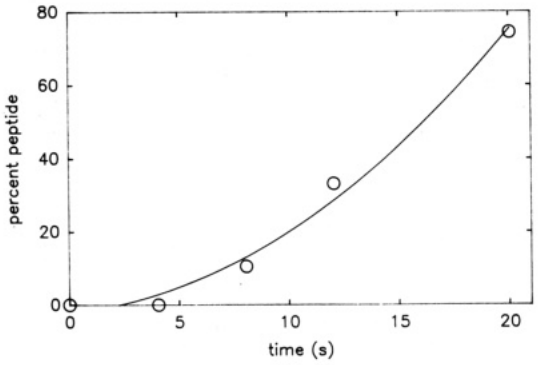


FIGURE 2: Appearance of the more mobile peptide formed during pepsinogen conversion. Data are from densitometry of the gel in Figure 1. A line is arbitrarily chosen to guide the eye.

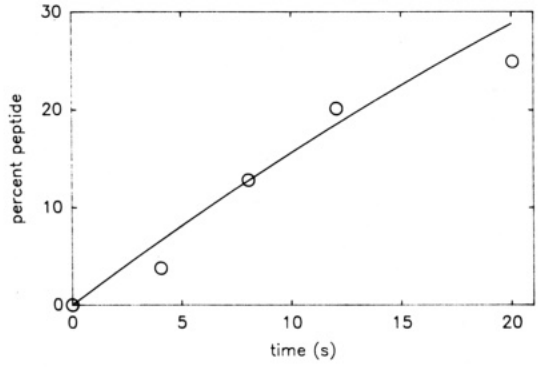


FIGURE 3: Appearance of the less mobile peptide formed during pepsinogen conversion. Data are from densitometry of the gel in Figure 1. The line is calculated for a first-order rate constant of 0.0170 s^{-1}.

the gel during electrophoresis or after electrophoresis or because it did not stain with Coomassie blue. The fastest detected band was determined by sequence analysis to be a mixture of peptides representing residues Ile_{p17}–Leu_{p44} and Leu_{p1}–Lys_{p30}. The former is clearly the result of two peptide bond cleavages, and their appearance after a distinct lag period is consistent with their being secondary products of conversion (Figure 2). Our failure to detect the 16-residue peptide makes it impossible for us to evaluate at this time the rate of initial cleavage of the Leu_{p16}–Ile_{p17} bond.

The slower of the two peptides represents the 44 amino-terminal residues of pepsinogen (Figure 1). This peptide, p1–p44, appeared without any lag time, in a manner well described by first-order kinetics (Figure 3). At several pH values, the data from quantitative analysis of the electrophoretograms were analyzed to extract rate constants for appearance of the peptide by linear regression of the logarithms of the percent of peptide not yet appearing vs the time of exposure to acid (Figure 4).

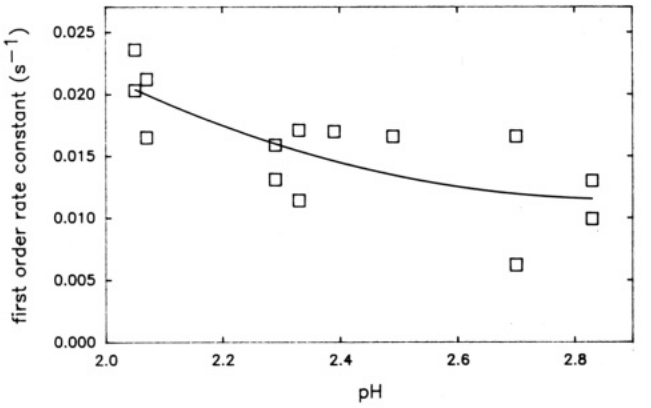


FIGURE 4: First-order rate constant vs pH value for the cleavage of the Leu_{p44}–Ile_1 bond. Rate constants were calculated from densitometry data, as in Figure 3.

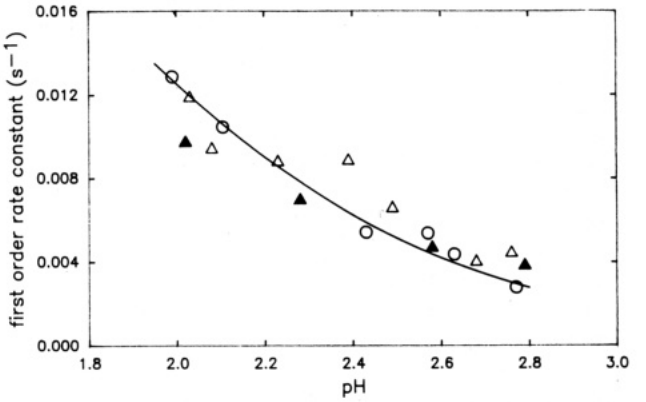


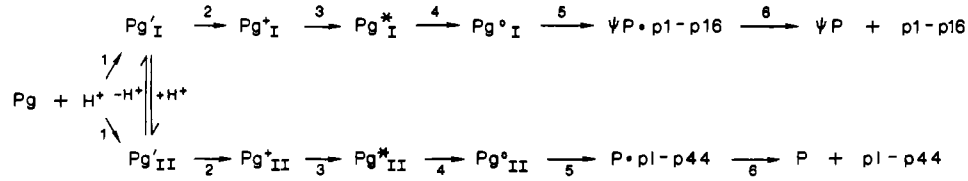
FIGURE 5: Comparison of assays for conversion of pepsinogen. The first-order rate constants are shown as a function of pH. All assays were performed at 22 °C and contained 0.047 M acetate, 0.186–0.213 M chloride, 0.047 M citrate, 2.3% DMF, and 4.5% 2-propanol. (▲) Turbidometric assay (22 μM pepsinogen); (△) turbidometric assay (22 μM pepsinogen, 91 μM TNS); (○) fluorescence assay (1–2 μM pepsinogen; 91 μM TNS). The line is calculated for a limiting rate constant for the fully protonated zymogen of 0.0375 s^{-1}, dependent upon an essential conjugate acid of pK_a 1.7.

When these experiments were performed in the presence of pepstatin at three pH values between pH 1.8 and 3.0, we found that as the molar ratio of pepstatin to pepsinogen increased, the rate of appearance of peptides decreased, so that when the ratio reached unity (25 μM pepstatin), there remained no detectable cleavage.

In order to compare the rate of change of TNS fluorescence with the rate of conversion to alkali lability, we performed both assays under comparable conditions. It was first shown that TNS itself (at two different concentrations) has no effect on the rate of conversion to alkali lability. Then, by the fluorometric method which measures the change in TNS fluorescence, the rates were found to be indistinguishable from those for the conversion to alkali lability (Figure 5), even though the protein concentration in the latter assays was 20–40 times that in the former assays. In an extensive study, it was shown that the conversion to alkali lability is a first-order event at these protein concentration, and in this range of pH values (Al-Janabi et al., 1972).

As the TNS fluorescence assay does not depend upon measurement of enzymatic activity, it was possible to follow conversion in the presence of the pepsin inhibitor, pepstatin. We were surprised to note that the rates were considerably enhanced by inclusion of this inhibitor (Figure 6). The observed first-order rate constants were obviously dependent upon pH, but in Figures 5 and 6, the constants have been presented

Scheme I: Pathways of Pepsinogen Activation[a]



[a] Values for the rate constants characterizing steps 1–6 are shown in Table II. The subscripts I and II refer, respectively, to the relatively less and more protonated events previously detected for steps 2 and 3.

in a pH-dependent form because that avoids presupposing a particular mechanism of pK_a shifting and particular pK_a values that cannot always be determined with confidence. The solid lines in these figures are calculated curves for rates dependent on a conjugate acid of pK_a 1.7 (without pepstatin) or 2.6 (with pepstatin) with the same limiting rate constant for the fully protonated form. Use of nonsaturating concentrations of pepstatin yielded an estimate of the K_d for the zymogen–inhibitor complex of about 1 μM, which assures that the higher concentration of pepstatin routinely used (22 μM) achieved essentially complete saturation.

In some cases, at 22 °C when pepstatin was included and even in its absence at 12 °C, a faster transient was seen. This appeared as an increase in fluorescence at the higher end of the pH range and as a decrease in fluorescence below pH 2.1. As the rate of this event was of the same order of magnitude as was reported for unmasking of the binding site (Glick et al., 1986), we attribute this to an effect on TNS fluorescence associated with this change and especially to the binding of pepstatin, when present. Data are presented for only the slower transients.

An explanation for the acceleration of the rate-limiting step in the presence of pepstatin is considered to be a shift in the pK_a of a conjugate acid that is essential for the reaction. On the basis of that interpretation of the data, it is possible to calculate thermodynamic activation parameters for the reaction of the fully protonated zymogen. Rate constants were measured at three pH values (2.1, 2.6, and 3.1) from 11 to 18 °C in the absence and the presence of pepstatin, to be considered along with the data at 22 °C (Figure 6). Because the temperatures at the different pH values were not chosen to be identical, for each pH value in the presence of pepstatin an Arrhenius plot was constructed to allow calculation of rate constants at specific intermediate temperatures; 12 and 17 °C were chosen. At each of these two temperatures, rate constants for the three pH values were extrapolated to yield a limiting rate constant for the fully protonated zymogen, as was done for 22 °C (Figure 6). The pK_a of the essential conjugate acid appeared to remain constant at 2.6. Arrhenius plots of these limiting rate constants (at 12, 17, and 22 °C) yielded E_a, the energy of activation. $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$, the enthalpy and entropy of activation, respectively, were calculated according to the theory of absolute reaction rates (Jencks, 1969b). These data are presented in Table II. Assuming that the limiting rate constant in the absence of pepstatin is the same as in its presence, the estimated pK_a of the essential conjugate acid in the absence of pepstatin varied from 1.7 at 22 °C, to 1.9 at 17 °C, to 2.1 at 12 °C.

Discussion

The finding that the 44-residue peptide appears as an initial product of conversion confirms the proposal of Kageyama and Takahashi (1983) of a single-step pathway from pepsinogen to pepsin (bypassing pseudopepsin).[4] It also lends support to the scheme of parallel activation pathways that was proposed to account for the concurrent changes of intrinsic fluorescence (Auer & Glick, 1984) and of the unmasking of the binding site (Glick et al., 1986) (Scheme I). A tabulation of rate constants for the several stages of the transformation of pepsinogen to pepsin is presented in Table II. Inspection of Table II reveals that, as was long suspected, the first irreversible step

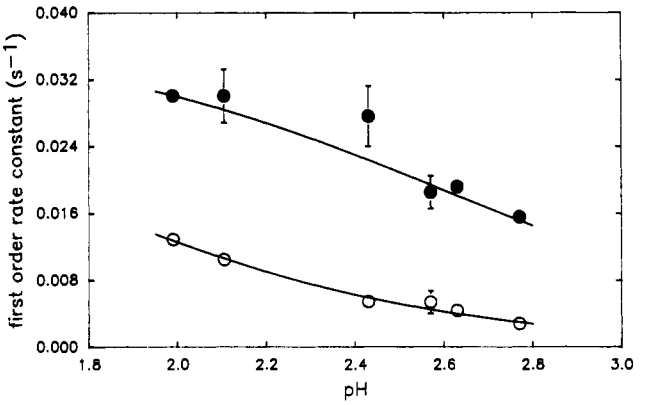


FIGURE 6: Effect of pepstatin on the rate of conversion of pepsinogen. The first-order rate constants, ±standard deviations for duplicate or triplicate determinations, are shown as functions of pH. Assays were performed at 22 °C by the fluorometric method as in Figure 5. (○) In the absence of pepstatin; (●) in the presence of 22 μM pepstatin. Both lines are calculated for a limiting rate constant for the fully protonated zymogen of 0.0375 s^{-1}, dependent upon an essential conjugate acid of pK_a 2.6 (upper line) or of pK_a 1.7 (lower line).

Table I: Thermodynamic Activation Parameters for the Change in TNS Fluorescence Associated with the Conversion of Pepsinogen to Pepsin (Step 4 in Scheme I)[a]

parameter	value
k (s^{-1})	0.0375
E_a (kcal/mol)	21.1
$\Delta H^{\ddagger}$ (kcal/mol)	20.5
$\Delta S^{\ddagger}$ (cal K^{-1} mol^{-1})	−2.5

[a] k is the limiting first-order rate constant for the fully protonated form of the zymogen, estimated by extrapolation of the data of Figure 6; $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ are the enthalpy and entropy of activation, respectively, calculated according to the theory of absolute reaction rates (Jencks, 1969b). All values are given for 22 °C.

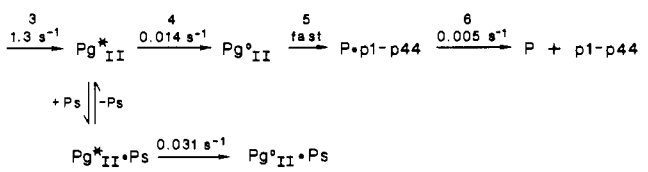
[4] Further support for an initial cleavage at the Leu_{p44}–Ile_1 bond lies in the observation that transpeptidation accompanies conversion of pepsinogen. In a single experiment, a sample of pepsinogen, that had accidentally not been first passed over a gel filtration column, was acidified and separated by polyacrylamide gel electrophoresis. The slower of the peptide bands was eluted, and its amino acid sequence was determined. It proved to be a mixture of 2 peptides, each having the 44 amino-terminal residues of pepsinogen, but one continuing with the Lys_{p27}-Ser_{p35} sequence and the other with the Tyr_{p37}-Ala_{p41} sequence. (They were recovered in a combined yield of at least 25%.) A repeat of the experiment on material that was purified by gel filtration yielded only the 44-residue peptide, as reported above. We conclude that the first sample was contaminated with peptides p27–p35 and p37–p41 and that an initial cleavage at the Leu_{p44}–Ile_1 bond makes the 44-residue peptide available for transfer to water (hydrolysis) or to the peptides (transpeptidation).

Table II: Events of Pepsinogen Activation

step[a]	process	k (s^{-1})[b]	ref
1	diffusion-limited protonation	4×10^7	Jencks, 1969a
2	change in intrinsic fluorescence	200 70	Auer & Glick, 1984
3	unmasking of binding site	1.3 0.3	Glick et al., 1986
4[c]	change in TNS fluorescence	0.015	this paper, Figure 5
	conversion to alkali lability	0.014[d]	Al-Janabi et al., 1972
5	p44–1 bond cleavage	$\gg$0.015	this paper
6	dissociation of propart	0.005[e]	Twining et al., 1981

[a] Steps refer to events depicted in Scheme I. [b] The rate constants are listed for pH 2.4, 22 °C. [c] Two values are given for step 4, measured by two different methods. [d] This rate constant was estimated from the published rate constant at pH 2.4, 28 °C, assuming an E_a of 15.5 kcal/mol (Glick et al., 1986). [e] Measured on spin-labeled pepsinogen, for which the rate constant for conversion to alkali lability is 0.05 s^{-1} at pH 2.4, 22 °C.

Scheme II: Dissection of the Irreversible Step of Pepsinogen Conversion to Pepsin[a]



[a] See legend for Scheme I.

of activation, the conversion to alkali lability, is, in fact, bond cleavage, at least of the p44–1 peptide bond. We have yet to determine the rate of cleavage of the Leu_{p16}–Ile_{p17} bond.

The measurement of the conversion of pepsinogen to pseudopepsin, based on TNS fluorescence, has two major advantages over the conventional assay: (a) it permits direct, continuous monitoring of conversion, i.e., observation in real time; (b) it can be performed in the presence of a pepsin inhibitor. We have exploited this assay to observe the effect of pepstatin on the conversion. An explanation for the apparent acceleration of the rate-limiting step lies in the observation that the data of Figure 6 are adequately described as a change of the conversion from dependence on a conjugate acid of pK_a 1.7 in the absence of pepstatin to dependence on one of pK_a 2.6 in its presence. In this case, there is not necessarily a true acceleration of the limiting rate for the fully protonated form of the zymogen. The value of E_a for the pH-independent rate (Table I) is identical with the E_a determined for the cis–trans isomerization of an acylproline peptide bond (Brandts et al., 1975). This suggests that the irreversible step of the transformation of zymogen to enzyme may involve such an isomerization, possibly that of the Val_{p4}–Pro_{p5} bond, close to Lys_{p3}, which is known to be involved in one of the salt bridges that hold the propart to the pepsin moiety at neutral pH values (James & Sielecki, 1986). This suggestion, however, is highly speculative.

Consideration of the effect of pepstatin on the change in TNS fluorescence (Figure 6) leads us to conclude that this change is due to an event that is distinct from bond cleavage. Inclusion of pepstatin leads to an increase in the apparent first-order rate constant for the change in TNS fluorescence, yet when we investigated the effect of pepstatin on the rate of bond cleavage we found it to inhibit quite strongly. We conclude that the fluorescence change is due to a conformational change that is distinct from bond cleavage and that precedes it and that there is no bond cleavage of pepsinogen when bound to pepstatin (Scheme II). The finding of no bond cleavage in the presence of pepstatin removes the apparent paradox of having a pepstatin-insensitive conversion of pepsinogen to pepsin due to the action of a pepsin-like active site.

This proposal, obviously, puts us in conflict with Dykes and Kay (1976), who found production, in low yield, of the 16-residue peptide in an acidified pepsinogen–pepstatin mixture. We suspect that their observation may have been due to a combination of the low affinity of the inhibitor for the zymogen and the slow development of the tight-binding complex of pepstatin with pepsin (Rich & Sun, 1980). The finding of an obligatory stepwise conversion of chicken pepsinogen to pepsin by this same approach, acidification in the presence of pepstatin (Keilova et al., 1977), has now been shown to have been misleading, conversion to chicken pepsin now being recognized as possible via a one-step process (Pichova et al., 1985). We do not suggest, however, that cleavage at the Leu_{p16}–Ile_{p17} bond is entirely artifactual because the appearance of pseudopepsin at 0 °C was observed in the absence of pepstatin in an unambiguous experiment (Christiansen et al., 1977).

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