The Origin of the Alkaline Inactivation of Pepsinogen†

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ABSTRACT: Above pH 8.5, pepsinogen is converted into a form which cannot be activated to pepsin on exposure to low pH. Intermediate exposure to neutral pH, however, returns the protein to a form which can be activated. Evidence is presented for a reversible, small conformational change in the molecule, distinct from the unfolding of the protein. At the same time, the molecule is converted to a form of limited solubility, which is precipitated at low pH, where activation is normally seen. The results are interpreted in terms of the peculiar structure of the pepsinogen molecule. Titration of the basic NH_2-terminal region produces an open form, which can return to the native form at neutral pH, but which is maintained at low pH by neutralization of carboxylate groups in the pepsin portion.

In his initial studies on the properties of swine pepsinogen, Herriott (1938) showed that between pH 8.5 and 10.5 the molecule was converted by a reversible equilibrium to a form which could not be activated at low pH, to form pepsin, and which was insoluble in concentrated salt solution. At that time and by these criteria, he seemed justified in assuming that he was studying the reversible denaturation of the protein. More recent studies have shown that large changes in physical properties (viscosity, optical rotatory dispersion, fluorescence) usually associated with the unfolding of proteins do not become detectable until the pH rises above 9 (Perlmann, 1963; Frattali et al. 1965). The latter authors did find an increase in the fluorescence of a dansyl-labeled pepsinogen, which occurred over the same pH range as the transition reported by Herriott. At higher pH a decrease in fluorescence was seen. They took this to indicate the presence of two consecutive transitions occurring in pepsinogen at high pH, but did not attempt any interpretation of their results in molecular terms.

There is a great deal of evidence that the activation of pepsinogen to pepsin at low pH is preceded and accompanied by conformational changes (Funatsu et al. 1971; Wang and Edelman, 1971; McPhie, 1972) but this has not yet yielded much information on the actual mechanism of activation. The present study was undertaken in the hope that a better understanding of the inactivation reported by Herriott would give more information on interactions within the pepsinogen molecule.

Experimental Procedure

Pepsinogen (lot PG1JA) and hemoglobin substrate powder (lot HB3CA) were obtained from the Worthington Biochemical Corp. Concentrations of pepsinogen were measured, at neutral pH, using a molar absorbance of 5.1×10^4 at 278 nm.

Pepsinogen was estimated with a 25-mg/ml solution of hemoglobin in 0.1 *M* hydrochloric acid, in a one-stage assay as described previously (McPhie, 1974). Activities were converted into Anson units by multiplication by a predetermined conversion factor of 2.43. Spectrophotometric measurements were performed in a Cary 15 spectrophotometer. Values of pH were read with a Radiometer pH meter 26, using a G.K. 2321.C combined electrode. All experiments were performed at 25°.

Effect of High pH on Potential Activity against Hemoglobin. Pepsinogen was dissolved in distilled water to a concentration of 0.7 mg/ml, pH 6. Aliquots of 250 μl of this stock solution were then added to 1-ml samples of 0.05 *M* Tris-HCl buffers varying in pH from 8 to 10. After 5 min, 100 μl of each mixture was assayed with acid-denatured hemoglobin for 30 min. In a second experiment the stock solution of pepsinogen was adjusted to pH 10.5 with 0.1 *M* sodium hydroxide, before incubation with Tris buffer.

Effect of High pH on Conformational Changes Accompanying Activation. Under certain conditions the activation of pepsinogen to pepsin can be followed by measuring the absorbance at 287 nm as a function of time (McPhie, 1972). An attempt was made to detect activation of pepsinogen using this method.

A 4-mg/ml solution of pepsinogen (500 μl) was mixed with an equal volume of 10 m*M* Tris buffer (pH 9.8) in a spectrophotometer cuvette. After 5 min, 1 ml of activating solution was added. The activating solution consisted of 0.333 *M* potassium chloride–0.667 *M* potassium hydroxide, adjusted to pH 4.2 with glacial acetic acid. The absorbance of the mixture was followed. However, instead of the slow sigmoidal decrease in absorbance expected, a rapid and large increase was seen. Inspection of the cuvette showed that this was due to precipitation of protein. The origin of this precipitation was investigated by repeating the experiment under a number of different conditions. In a first series of experiments, the pH of the Tris solution was lowered by the addition of hydrochloric acid, while the activating solution was kept at pH 4.2. In a second series, the Tris buffer was kept at pH 9.8, while the pH of the activating solution was varied from 8 to 4, the concentration of potassium ion remaining constant. The pH of the solution in the cuvette was always measured before and after the addition of the activating solution.

A 0.7-mg/ml solution of pepsinogen (6 ml), in distilled water, was adjusted to pH 10.5 by the addition of 10 μl of 1 *N* potassium hydroxide. After 5 min, 5 ml of this alkaline solution was mixed with an equal volume of activating solution (pH 4.2) described above. The precipitate was removed by centrifugation and suspended in 5 ml of distilled water and the pH raised to 7 by dropwise addition of potassium hydroxide. After stirring for 5 min, the solution was clarified by centrifugation.

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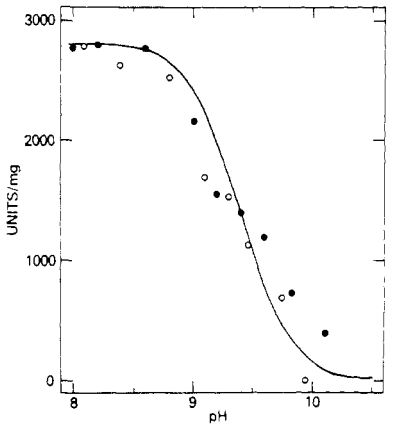

FIGURE 1: The specific activity of pepsinogen against acid-denatured hemoglobin (pH 1.8) following incubation at the indicated pH: (●) increasing pH from 6; (○) decreasing pH from 10.5. The curve shows the pH dependence found by Herriott (1938).

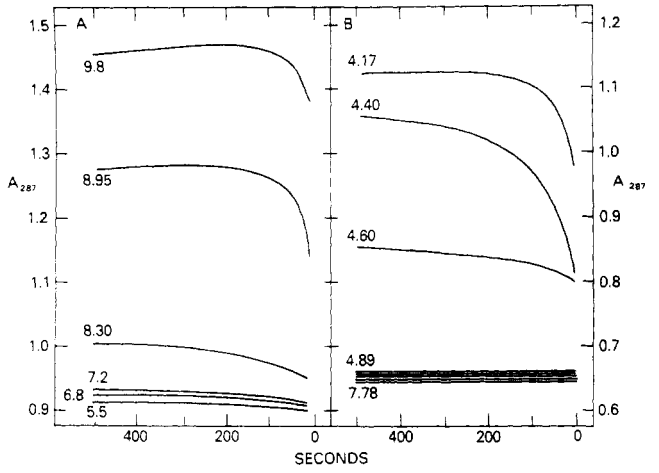

FIGURE 2: (A) The precipitation of pepsinogen, measured by absorbance, at pH 4.2, following incubation at the indicated pH's. Pepsinogen concentration, 21.4 μM. (B) The precipitation of pepsinogen, measured by absorbance, at the indicated pH's, following incubation at pH 9.8. Pepsinogen concentration, 15.5 μM.

The stock solution, the alkaline solution, the supernatant, and the final solution were all assayed against acid-denatured hemoglobin. Protein content was estimated from their absorbance at 280 nm.

Spectrophotometric Titrations. These were performed on salt-free solutions of pepsinogen, in a thermostated cuvette holder. Dilute potassium hydroxide was added to the solution from a Micro Metric syringe microburet. The solution was stirred with a Teflon rod and the difference spectrum was recorded against a suitable diluted, neutral, reference solution. Two series of experiments were performed: (1) at a protein concentration of approximately 1 μM, difference spectra were recorded between 320 and 220 nm, and (2) at a protein concentration of 10 μM, difference spectra were recorded between 320 and 240 nm.

Results

Within experimental error, the results shown in Figure 1 are in complete agreement with Herriott's demonstration (between pH 8.5 and 10.5) of a reversible equilibrium of pepsinogen between two forms, one of which fails to develop activity when assayed against denatured hemoglobin at pH 1.8. The reaction is completely reversible on reincubation at pH 8, after exposure to pH 10.5.

Table I: Recovery of Activity from Acid Precipitated Inactive Pepsinogen.

	Protein (mg/ml)	Pepsin (units/ml)	Specific activity
Stock solution, pH 7	0.65	1750	2700
Stock solution, pH 11	0.65	0	0
Acid supernatant	0	0	0
Redissolved ppt.	0.56	820	1450

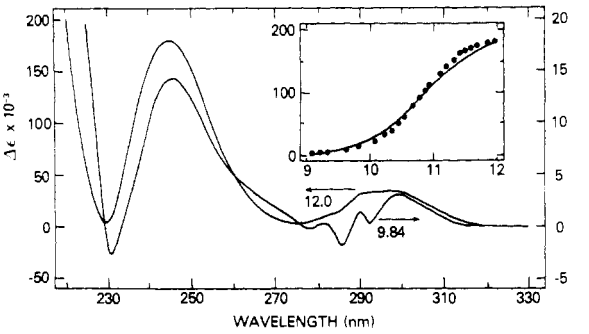

FIGURE 3: Characteristic difference spectra during alkaline titration of pepsinogen: samples at pH 9.84 and pH 12.0, reference at pH 7.0. Note the different scales for the two spectra. Inset: The dependence of the difference in molar absorbancy, at 245 nm, on pH. The curve follows the titration of one group, $pK = 10.80$, $\Delta\epsilon = +190000$. Pepsinogen concentration, 1.6 μM.

An attempt was made to detect activation of pepsinogen by the spectrophotometric assay, described previously (McPhie, 1972). However, transfer of pepsinogen from pH 9.8 to pH 4.2 produced a rapid rise in absorbance of the solution, which proved to be due to irreversible precipitation. Figure 2A shows that as the pH of the initial incubation was reduced the extent of precipitation at pH 4.2 also decreased. Solutions incubated below pH 8 showed no precipitation and when their absorbances were followed on the 0–0.1 A scale, the expected fall in absorbance was observed (not shown). The half-time of activation was 2000 sec, in agreement with previous results (McPhie, 1972). Figure 2B shows the effect of varying the final pH of a solution of pepsinogen previously incubated at pH 9.8. Precipitation did not occur until the pH fell below 4.7, where activation of the protein would be expected.

The potential pepsin activity of these solutions was determined by assaying them with denatured hemoglobin at pH 1.8. It fell from 2700 Anson units, for a solution which was incubated at pH 6 for 10 min, to 200 units after 10 min at pH 4.2.

In a larger scale experiment, the nature of the precipitate was investigated (Table I). Pepsinogen at pH 7, the species that shows full activity against denatured hemoglobin, is transformed at pH 10.5 to a form which develops no pepsin activity at low pH. When transferred to pH 4.2, this form of the protein is completely precipitated, leaving no detectable protein or activity in the supernatant. Almost 90% of the precipitate can be redissolved at pH 7 and when assayed against hemoglobin at low pH shows almost 60% of the specific activity of the original pepsinogen.

Figures 3–5 show the results of the spectrophotometric titration experiments. In agreement with the results of Perlmann (1964), the difference spectra developed are very sim-

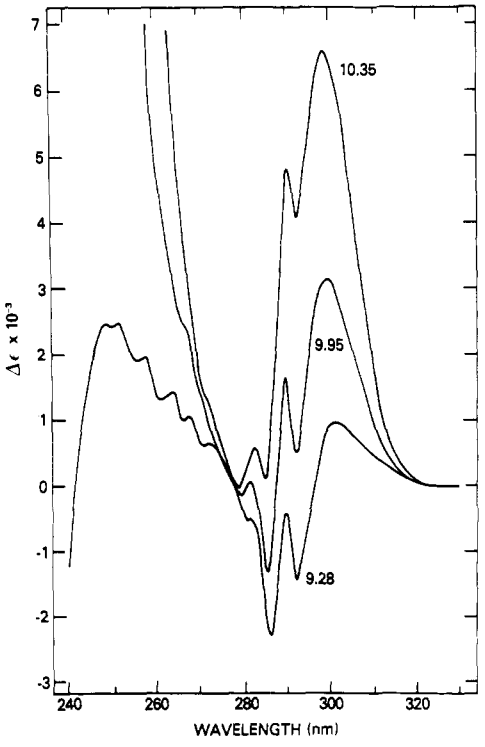

FIGURE 4: Characteristic difference spectra during alkaline titration of pepsinogen: samples at pH 9.28, 9.95, and 10.35, reference at pH 7.0. Pepsinogen concentration, 10 μM.

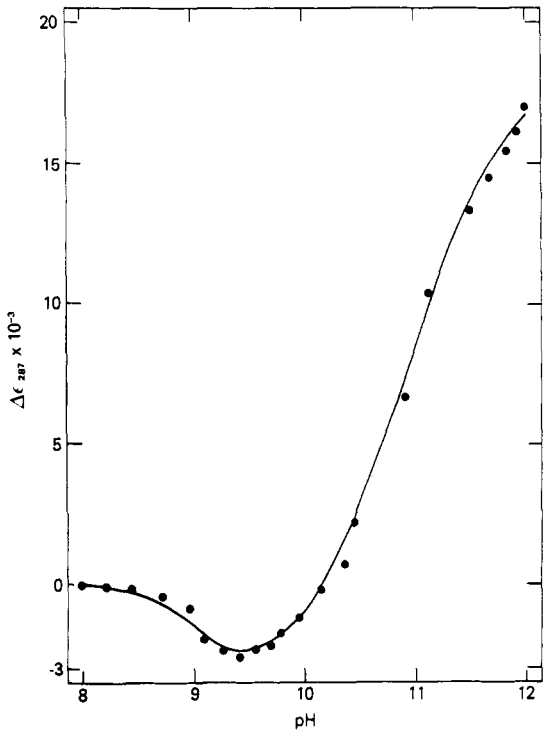

FIGURE 5: The pH dependence of the difference in molar absorbancy, at 287 nm. The curve follows the absorbance of two groups, pK = 8.95, $\Delta\epsilon$ = −3200 and one group pK = 10.88, $\Delta\epsilon$ = +21000.

ilar to those produced by the ionization of tyrosine residues (Figure 3). However, it is especially clear in spectra between pH 9 and 10 that there are a number of deviations from a normal tyrosine ionization spectrum. These are most marked in the wavelength range of 270–300 nm, where changes usually indicate perturbation of aromatic side chains. To investigate these deviations more closely, experiments were repeated at a higher protein concentration, in this region of the spectrum. The variation of absorbance at 287 nm, a wavelength indicative of the perturbation of unionized tyrosine residues, is shown in Figure 5. In all cases, it was found that absorbance changes were independent of time, at least in this time scale, and readily reversible, if exposure to high pH was kept within a few minutes.

Discussion

The essential conclusion to be drawn from these studies is that when pepsinogen is exposed to pH values above 8, it is changed into a form which is precipitated when brought to a pH at which pepsin activity would be expected to develop. Intermediate exposure of this alkaline form to neutral pH allows reversal of the transformation and pepsin activity then develops as expected, at low pH. It is perhaps more interesting that the precipitated, inactive pepsinogen can be recovered from acid conditions and be redissolved at neutral pH, leading to extensive reappearance of potential pepsin activity. The spectrophotometric titration experiments indicate a conformational change in the molecule, distinct from the unfolding reaction at higher pH, which occurs over the same pH region as the transformation to an inactive form. Circular dichroism experiments show that, in agreement with Grizzuti and Perlmann (1969), extensive changes in secondary structure do not appear below pH 9.

Transient precipitation of pepsinogen, during activation, was reported in earlier work (McPhie, 1972) and other workers have also reported difficulties due to aggregation of pepsinogen (Al-Janabi et al., 1972). At that time, this precipitation was regarded as an artifact, arising from the fact that the experiments were performed close to the isoelectric point of the protein.

Transient precipitation was not a major problem until activation experiments were attempted below pH 4, yet persisted all the way down to pH 1. The effect was thus not symmetrical about pepsinogen's isoelectric point (pH 3.7). However, pH 4 was also the onset of a rapid, reversible conformational change in the molecule, indicated by the appearance of a difference spectrum due to perturbation of aromatic side chains. Thus there is evidence for conformational changes occurring in the pepsinogen molecule above pH 8 and below pH 4; both are in the same ranges as a reduction in solubility. The high pH change is reversible between pH 5 and 8, but is not reversed when the pH is taken below pH 5. The low pH conformational change occurs much more rapidly than pepsin activity appears and seems to be readily reversible at pH 7. However, extensive studies on the pH dependence of this reversibility are complicated by the denaturation of pepsin which is formed during any exposure to a low pH (Sanny et al., 1975).

Nevertheless, it is possible to postulate a simple model which explains these results and is consistent with current ideas of the structure of pepsinogen. The main difference in structure between pepsin and its zymogen is the presence of a highly basic NH_2-terminal 44 residue sequence in the latter. A number of authors have suggested that activation of pepsinogen is initiated by titration of carboxyl groups in the pepsin region of the molecule, thereby reducing electrostatic interactions within the molecule. The positively charged region can then leave the surface of tlhe molecule, uncovering the active site (Kassell and Kay, 1973). On the other hand, Perlmann (1963) has postulated that unfolding of

pepsinogen at high pH might be initiated by titration of basic residues in the NH_2-terminal region, again allowing it to leave the surface, by loss of electrostatic interactions. Thus essentially similar conformational changes might occur at both high and low pH. For the alkaline transition, lowering the pH to neutrality would recharge the basic residues and allow a return to the native conformation. However, a larger pH change to below pH 5 will also allow protonation of carboxylate groups, which titrate in this region and the molecule will thus be propped in an open configuration. If this form of the molecule is of low solubility, precipitation will occur either in high salt or close to its isoelectric point, as is observed. The ultimate fate of the pepsinogen will depend on the relative rates of precipitation and activation. Starting from the neutral form, the ionized carboxylate groups will be stabilized by the neighboring basic residues and the conformational change and precipitation will not become apparent until the pH falls below 4, at which point the rate of activation to pepsin is quite rapid. However, in the alkaline form of the zymogen the carboxylate groups are less perturbed and titration and precipitation will begin at higher pH. Consequently, the apparent pH dependence of the alkaline inactivation will depend on the pH at which the pepsinogen is activated. This may be why the inactivation and the conformational change do not show exactly the same pH dependence (see above and also Frattali et al., 1965).

These results indicate the presence of at least two forms of the pepsinogen molecule: the soluble native form, between pH 4 and pH 8, and insoluble open forms which occur outside these limits, by dissociation of the NH_2-terminal region from the pepsin portion of the molecule. The open forms found at high and low pH may be similar, but are not identical. Pepsinogen molecules taken from neutrality to pH 1.8 can still activate themselves by a unimolecular mechanism, whereas those taken from above pH 8 to pH 1.8 cannot. The differences between those molecules originating from neutral pH and high pH must also be maintained by this propping mechanism. Another form of pepsinogen, the unfolded form, exists at high temperature, pH's above 10, and in the presence of denaturants (Neumann and Shinitzky, 1971).

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