Biophysical Chemistry 9 (1979) 281–287

THE ALKALINE TRANSITION OF SWINE PEPSINOGEN

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Received 10 October 1978

At alkaline pH, swine pepsinogen is reversibly inactivated in a transition which involves the cooperative release of two protons from the molecule and is governed by a pK = 9. Stopped flow kinetic studies on the absorbance changes accompanying this reaction show that it can be resolved into two steps, with increasing pH; a slow conformational change, whose amplitude follows the ionisation curve of one group of pK = 9.9, followed by a rapid pH dependent conformational change, linked to a group of pK = 8.2. The pH dependence of the rate of the slow step is interpreted to show the presence of a protonated group which cannot ionise in the neutral form of the zymogen, but is in slow equilibrium with a form where it titrates with a pK = 6.8. At the same time, a histidine in the amino terminal region of the protein becomes reactive to diethyl pyrocarbonate, suggesting this to be the group which triggers the reaction.

1. Introduction

Above pH 8, swine pepsinogen is changed to a form which cannot be activated to pepsin at low pH [1]. This reversible alkaline transition is accompanied by changes in the near ultra-violet spectrum and fluorescence of the protein, but not by changes in viscosity or circular dichroism [2,3]. Consequently, it may be regarded as a subtle conformational change involving only a limited rearrangement of secondary structure. Its pH dependence indicates that it involves the cooperative release of two protons by the zymogen. In contrast to the wealth of information on protein unfolding and refolding [4], little is known of the kinetics and mechanisms of limited changes of this kind, although these are certainly more relevant to physiological conditions. This paper reports the results of pH jump kinetic and chemical modification studies on this transition and their interpretation in terms of the structure of pepsinogen. The region of the molecule involved in the conformational change and the origin of its cooperative pH dependence are identified.

2. Materials and methods

Pepsinogen (lot #PG34D903) was from Worthington. Diethylpyrocarbonate (DEP) was from Eastman. All other reagents were analytical grade or equivalent.

Kinetic experiments were performed in an Aminco-Morrow stopped flow apparatus or in a Cary 15 spectrophotometer. The stopped flow apparatus was mounted on a Beckman DU monochromator. Transmission changes were measured with a R-136 photomultiplier and were stored and digitized in an Aminco Dasar Memory System. The data were analyzed using the MLAB system for curve fitting, on a PDP-10 computer system [5].

Pepsinogen was dissolved in distilled water to a concentration of 4–20 μM. The pH values of the solutions were between 6 and 6.5. In stopped flow experiments, the protein solution was mixed with an equal volume of sodium pyrophosphate or sodium bicarbonate buffer to give a final pH between 9 and 12. Below pH 10 the reaction was sufficiently slow that absorbance changes could be followed in a spectrophotometer. In these cases, 100 μl of buffer were added to 2 ml of protein solution using an "adder-mixer" from Precision Cells, Inc. For pH jumps in the opposite direction, the protein solution was first ad-

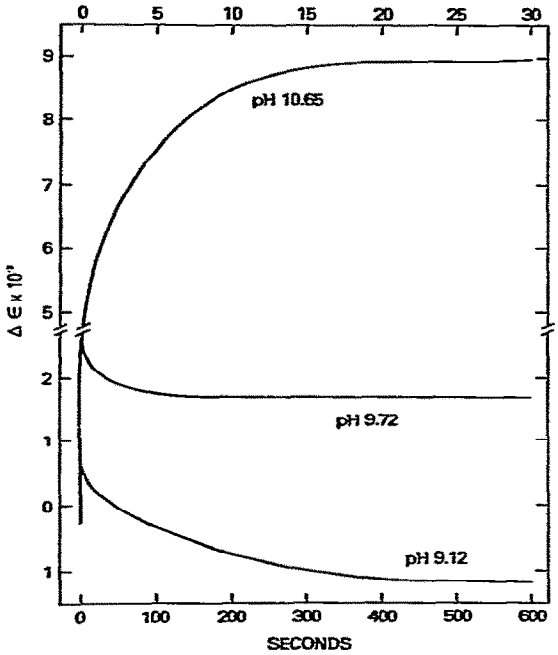

Fig. 1. Representative curves showing the time course of the changes in molar extinction at 287 nm, after jumping from neutrality, to the indicated final pH. (note the different time scale for the upper curve.) A 20 μM solution of pepsinogen in distilled water was mixed with an equal volume of 0.1 M buffer of the required pH, in the stopped flow apparatus.

justed for pH 12 by the addition of 25 μl of 1 N sodium hydroxide. Kinetic measurements were begun after 30 s by the addition of 100 μl of sodium pyrophosphate or sodium phosphate to give final pH values below 9. The composition of the buffer was such that the final concentration of sodium ion was 0.05 M. The pH of the reaction mixture was measured after each experiment, using a Radiometer pH meter 26 with a GK 2321C combined electrode. Spectrophotometric titrations were performed as before [3]. Sodium hydroxide was used as the titrant and the ionic strength of the solution varied by the addition of sodium perchlorate. All experiments were performed at 25°C.

The reaction between pepsinogen and DEP was studied in two ways. In initial experiments, 2 ml of a 20 μM solution of protein, in the appropriate solvent, were placed in a spectrophotometer cuvette. At zero time, 50 μl of a 1% solution of DEP in ethanol were added. The absorbance of the mixture at 242 nm, which specifically measured modification of histidine [6], was followed as a function of time. To demonstrate exposure of a histidine residue at high pH a different strategy was applied. In a thermostated cuvette, 10 μl of 1 N sodium hydroxide were added to 1 ml of a 20 μM solution of pepsinogen in distilled water. Working as quickly as possible, (within 1 min) up to 250 μl of a 5% solution of DEP in ethanol were mixed with 4.75 ml of 0.1 M sodium phosphate at pH 7, and 1 ml of this mixture was added promptly to the contents of the cuvette. Again, reaction was followed by absorbance at 242 nm. The extent of histidine modification was calculated using $\epsilon(m) = 3200$ [6]. Pepsin was prepared from modified pepsinogen using the method of Rajagopalan et al. [7]. Protein was hydrolyzed for 24 hr at 100°C, in constant boiling hydrochloric acid (Pierce) and its amino acid composition determined in duplicate runs in a Beckman model 120 C analyzer.

3. Results

3.1. Amplitudes of absorbance changes on increasing pH

Absorbance changes were followed at three wavelengths, 300 nm, 287 nm and 245 nm. Their behavior was most complex at 287 nm. However, this complexity aids in the interpretation of results, since it can be related to previously published spectrophotometric titration curves and difference spectra [3].

The results obtained at this wavelength on rapidly increasing the pH of a pepsinogen solution from neutrality depended on the final pH. They can be summarized as follows (see fig. 1).

i) Above pH 8, there is a slow decrease in absorbance, whose amplitude increases with pH.

ii) Above pH 9, the slow decrease is preceded by an "instantaneous" increase, whose amplitude increases with pH. (Instantaneous implies completion of the change within the mixing time of the kinetic experiments, i.e., 10 ms in the stopped flow apparatus and 3 s in the Cary).

iii) The amplitude of the slow decrease in absorbance

reaches a maximum around pH 9.2. At higher pH, its amplitude decreases, becoming zero at pH 10.

(iv) Above pH 10, the instantaneous increase in absorbance is followed by a slow increase. The amplitude of both rises markedly with pH until, at pH 12, they are equal in size.

At 300 nm and 245 nm both the instantaneous and slow changes in absorbance, which specifically measure the ionization of tyrosine residues, were always positive.

3.2. Amplitudes of absorbance changes on decreasing pH

Attempts to measure the kinetics of pH jumps in the opposite direction in the stopped flow apparatus were not successful. Absorbance changes on neutralization were complex and not reproducible. Control experiments showed that inactivation became increasingly irreversible both as judged by absorbance and by potential pepsin activity, if the protein was kept at pH 12 for more than 1 minute. It was found that renaturation could be conveniently studied in the Cary spectrophotometer within 30 s after raising the pH. Since this was much longer than the time required for complete reaction at this pH (see fig. 3), it was chosen as the standard time of exposure to high pH. Rapidly changing the pH of the protein solution from 12 to below 9 produced a large, instantaneous decrease in absorbance followed by a small, slow increase at 287 nm. The size of this increase found to decrease with the final pH (fig. 2). Observations at 300 nm and 245 nm showed only instantaneous decreases in absorbance. Kinetic measurements could not be extended below pH 6, since inactivation becomes irreversible under these conditions [3].

A rigorous description of any conformational change in kinetic terms requires good agreement between the total absorbance changes measured in kinetic and equilibrium experiments. The final absorbance in the kinetic experiments always agreed with values obtained in spectrophotometric titration curves measured at the same ionic strength.

3.3. Rates of absorbance changes in kinetic experiments

Within experimental error, the time course of the slow absorbance change always followed a single exponential curve. The relaxation time of this curve, τ, was the same at all wavelengths and where comparable, the same value was found by both procedures. The relaxation time was a marked function of the final pH, being a maximum at pH 8.7 and decreasing markedly to either side. No specific buffer effects were detected. The pH dependence of the reciprocal relaxation time, $1/\tau$ is shown in fig. 3. The curve through the points is derived from the model presented in the discussion.

Both the rates and sizes of the resolved absorbance changes were found to be independent of initial conditions in the pH range 6 to 8 for jumps to high pH and the range 10 to 12 for jumps to neutral pH. The reaction rates were independent of protein concentration over the 5-fold range used.

3.4. Reaction of DEP with pepsinogen

As explained in the discussion, the pH dependence of the relaxation time suggests that the conformational change involves the uncovering of a histidine residue which is buried and charged in the neutral form of pepsinogen. Consequently, the reactivities of the histidine residues in the protein towards DEP were investigated. With pepsinogen solutions initially at neutral pH, a very slow reaction was detected spectrophotometrically. Unfolding of the protein in 1% sodium dodecyl sulphate (SDS) at pH 7 made all three residues react rapidly (not shown). However, when the protein is initially at high pH, neutralization and addition of DEP at the same time produced a competition between the modification reaction and burying of any exposed histidines. Under the appropriate conditions, rapid modification of up to one histidine residue could be demonstrated (fig. 4). The increase in absorbance at 242 nm could be reversed by overnight dialysis against 1 M hydroxylamine hydrochloride, pH 7, confirming the presence of carbethoxy-histidine [7].

Pepsinogen which had been modified in this way was activated to pepsin by the method of Rajagopalan et al. [7]. No carbethoxyhistidine could be detected spectrophotometrically in the product of this procedure. However, amino acid analyses indicated the presence of one residue of histidine per mole of pepsin formed.

4. Discussion

Equilibrium spectrophotometric studies on the behavior of pepsinogen at high pH indicated the presence of two consecutive transitions [3]. Above pH 8 a small negative difference spectrum showed the presence of a conformational change to the inactive "open" form of the zymogen. Its pH dependence showed that this transition accompanied the cooperative ionization of two groups in the protein of apparent pK = 9. Above pH 9, a large increase in absorbance accompanied ionization of the tyrosine residues and unfolding of the protein. Preliminary kinetic studies with increasing pH were made by Frattali et al. [2] who interpreted their results in terms of two overlapping transitions, the first slow and the second fast. The present results obtained on jumping to high pH seemed consistent with this model and could be interpreted accordingly. Let the slow transition be between the neutral (N) and open (O) forms of the protein and the fast be between the open and unfolded (U) forms. Then, neglecting protons released for the moment, we may write a minimum scheme;

$$N \rightleftharpoons O \rightleftharpoons U. \qquad (1)$$

A pH jump from neutrality to above pH 8 will enter the region of the first conformational change, indicated by a slow decrease in absorbance at 287 nm, due to the perturbation of tyrosine and tryptophan residues. A pH jump to above pH 9 will also cause ionization of the tyrosine residues in the protein: those on the surface will ionize instantaneously, producing an initial increase in absorbance; the ionization of buried tyrosines will depend on the rate and extent of unfolding of the molecule [8]. If unfolding is much faster than the first change then eq. (1) predicts that the system will show only one relaxation time, their rate of ionization being limited by the first transition. The increase in absorbance due to tyrosine ionization will oppose the decrease due to the first transition and eventually overwhelm it, producing a large, slow increase in absorbance at high pH. The total change in absorbance at pH 12 indicates that all the tyrosine residues in pepsinogen are ionized. Kinetic resolution of this absorbance change into equal fast and slow components indicates that half of these residues are buried and only titrate as the protein unfolds. Solvent perturbation and chemical modification

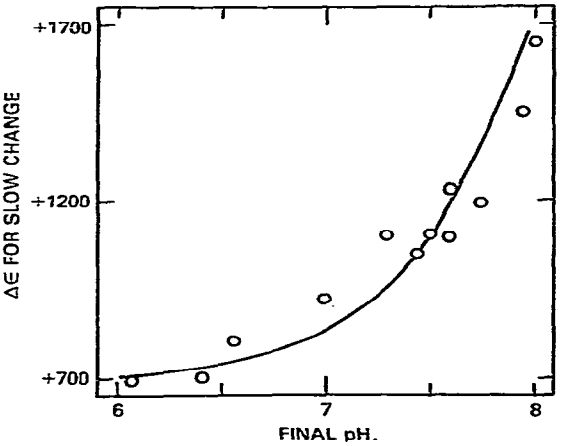

Fig. 2. The pH dependence of the size of the slow absorbance increase, at 287 nm, resolved after rapidly changing the pH of pepsinogen solutions from 12 to the indicated value. A 20 μM solution of pepsinogen was adjusted to pH 12. After 30 s, low pH buffer was added and absorbance followed as a function of time. The total change was evaluated by extrapolation of first order plots back to the mixing time.

experiments on pepsin and pepsinogen gave similar conclusions [9,10].

However, the results obtained following jumps from high to neutral pH show that eq. (1) is too simple to describe the behavior of pepsinogen. Consider a jump from pH 12 to below pH 8, where the protein is completely in the neutral form at equilibrium. The tyrosine residues in the unfolded protein all protonate instantaneously, with a large decrease in absorbance. Refolding of the protein chain to the native structure will involve burying half of the tyrosine and tryptophan residues, with a sizeable absorbance increase. Refolding of pepsinogen from the urea denatured form at neutral pH gave $\Delta\epsilon \approx 7000$ at 287 nm [11], much larger than the measured values here (fig. 2). If the $U \rightleftharpoons O$ reaction is complete within the mixing time of these experiments (3s), the resolved absorbance change should be that of the $O \rightleftharpoons N$ transition, $\Delta\epsilon = 3200$ [3]. In fact, the change detected is only half this value at pH 8 and decreases to 700 at pH 6.

The only explanation for these observations is that refolding is too fast to detect in these experiments and that pepsinogens alkaline transition is itself com-

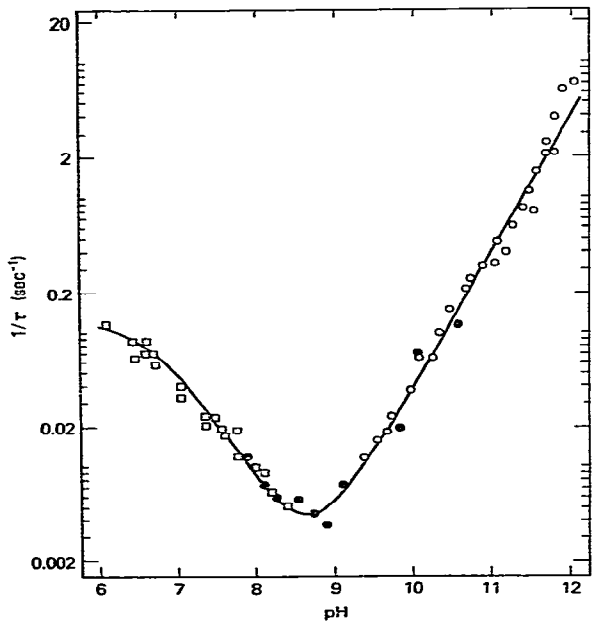

Fig. 3. The pH dependence of the reciprocal relaxation time of the slow process observed after pH jumps in pepsinogen solutions. Experiments were performed in (□) sodium phosphate, (●) sodium pyrophosphate and (○) sodium bicarbonate buffers.

posed of two pH dependent steps, with an unstable intermediate, I, represented by eq. (2) (again ignoring ionizations);

$$N \rightleftharpoons I \rightleftharpoons O. \qquad (2)$$

If the first step is much slower than the second, it again follows that jumps to high pH from the neutral form of the zymogen will show only a slow phase. In contrast, jumps across the transition from the open form to the neutral form will show both fast and slow phases. The relative sizes of these phases will reflect the pH dependence of the fast step [cf. 12]. The line drawn through the points in fig. 2 assumes that the equilibrium absorbance change of the fast $I \rightleftharpoons O$ step follows a pK of 8.2 and has a maximum value $\Delta\epsilon = 2500$. Curve fitting calculations showed that the observed equilibrium pH dependence of the total alkaline transition could be reproduced by assigning to the equilibrium absorbance change of the slow $N \rightleftharpoons I$ step at an apparent pK of 9.9 and $\Delta\epsilon = 700$.

These considerations indicate that the rate being measured under all conditions is that of this slow conformational change in the native protein. It is not possible to identify the ionizing group involved in this change from its apparent pK of 9.9, since this must also include the unknown equilibrium constant of the structural change [13]. However, under favorable circumstances, it is possible to estimate this by kinetic measurements [14–16]. The bimodal pH dependence of the reciprocal relaxation time shown in fig. 3 can be most readily interpreted in terms of the "trigger group" model [17], in which structural interactions cause one (or more) ionizing group to have different pK's in the two states of the protein. Let N, N^-, I and I^- be the protonated and unprotonated forms of the two states, K_N and K_I the ionization constants of the trigger group in the two states and k_1, k_2, k_{-1}, and k_{-2} the rate constants of isomerization for the protonated and unprotonated forms of the molecule. The reaction scheme can be written:

$$\begin{array}{ccc} N & \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} & I \\ \uparrow K_N & & \uparrow K_I \\ H^+ + N^- & \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} & I^- + H^+ \end{array} \qquad (3)$$

If the ionizations are fast, the system will show one relaxation time given by

$$1/\tau = \frac{k_1(H^+) + k_2K_N}{(H^+) + K_N} + \frac{k_{-1}(H^+) + k_{-2}K_I}{(H^+) + K_I}. \qquad (4)$$

Models of this type are subject to various limitations. Most specifically, the rate constants and ionization constants are all related by

$$(k_1/k_{-1})K_I = (k_2/k_{-2})K_N = 10^{-9.9} \qquad (5)$$

and cannot be assigned arbitrarily in fitting the data to a model [17]. The line drawn through the points in fig. 3 assumes values of $pK_I = 6.75$, $k_{-1} = 0.13\ s^{-1}$, $k_1 = 0.0001\ s^{-1}$. The upper branch of the curve shows no sign of reaching a plateau, indicating that $k_2 > 40\ s^{-1}$, $k_{-2} < 0.01\ s^{-1}$ and $pK_N > 13$. Such a pK_N implies that this group never titrates in the native protein. Assuming that the group ionizes normally after the transition, a pK_I of 6.75 indicates that the trigger group might be a histidine residue or the α-amino group.

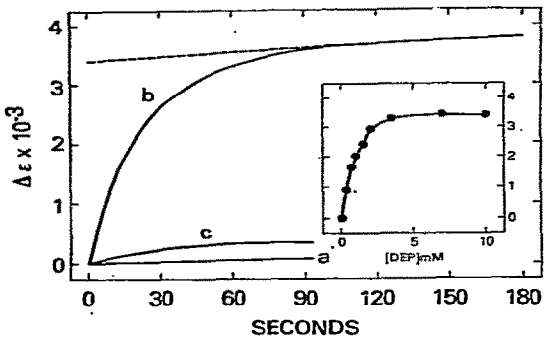

Fig. 4. The reaction of pepsinogen with DEP. (a) 1 ml of 20 μM pepsinogen, pH 11.5 plus 1 ml of 0.1 M phosphate buffer pH 7 (b) 1 ml of 20 μM pepsinogen, pH 11.5 plus 1 ml of 7 mM DEP, 0.1 M phosphate buffer, pH 7. (c) 1 ml of 20 μM pepsinogen pH 6 plus 1 ml of 7 mM DEP, 0.1 M phosphate buffer, pH 7. The final pH in the first two experiments was 7.25. All buffers contained 1% ethanol. Absorbance changes were followed at 242 nm. In five repeats of experiment (b) the burst was equivalent to 1.13 ± 0.03 histidine residues. Inset: Showing the effect of increasing concentration of DEP on the absorbance change following mixing as in experiment (b).

Finlay [18] showed that the amino terminal residue of pepsinogen reacted readily with pyridoxal phosphate at pH 7. While most other amino groups only reacted above pH 8 where the protein was "mildly denatured". Perlmann et al. [19] suggested that the histidines of native pepsinogen were stabilized in the protonated state; in agreement with their hypothesis, none of the histidines in native pepsinogen reacted readily with DEP at neutral pH. However, when the protein was unfolded in 1% SDS, all three reacted rapidly, producing a quantitative increase in absorbance at 242 nm. Attempts to identify the residue involved in the slow conformational change, by reaction at a higher pH were unsuccessful, because of the instability of DEP and the reaction product, carbethoxyhistidine [6]. However, exposure of a histidine residue in the open form of the protein is demonstrated by the experiment shown in fig. 4. At high pH, pepsinogen will be distributed between the open and unfolded forms. A jump to pH 7 will rapidly refold the protein to the I form which will then slowly change to the neutral form. If a sufficiently high concentration of DEP is present, the rate of substitution at any exposed histidine residues can be faster than the rate of isomerization.

Holbrook and Ingram [20] studied the rate of reaction of imidazole with DEP as a function of pH. From their results, one can calculate that, at pH 7, the rates of the conformational change and of the substitution reaction at a normal histidine will be equal when the concentration of DEP is 1.3 mM. Consequently, initial experiments were made in this concentration range. Fig. 4 shows that when an alkaline solution of pepsinogen is neutralized in the presence of 3.5 mM DEP, an increase in absorbance equivalent to one histidine residue is seen. On the other hand, an equal concentration of reagent had no effect on the protein already at equilibrium at neutral pH. The absence of carbethoxyhistidine from the pepsin derived from the modified zymogen indicates that the reaction occurred at one of the two almost adjacent histidine residues in the amino terminal region, which are lost during activation [18].

Modification of a histidine residue in pepsinogen is clearly demonstrated by the large absorbance change at 242 nm compared to unmodified protein. It is of interest that these solutions showed no difference spectrum in the region of 260–300 nm. This indicates not only that no modification of tyrosine residues by DEP had occurred [6] but also that the alkaline transition could still be reversed after histidine modification. The extreme lability of carbethoxyhistidine at high pH [6] has prevented further studies on the effect of this reaction on the alkaline transition.

It has often been suggested that pepsinogen is stabilized by ionic or salt bridges between its highly basic amino terminus sequence and the very acidic pepsin region of the molecule [e.g. 3, 19]. Such a structure will become increasingly unstable as the pH of the solution rises above the intrinsic pK's of these basic residues. Since histidines have much lower pK's than lysines or arginines, they would be expected to be the sites where breakdown of this complex will begin. From the rate constants derived above, one can calculate the equilibrium constant for the $N \rightleftharpoons I$ reaction to be 8×10^{-4}, with a free energy change of 4.3 kcal $mole^{-1}$. It was previously shown that the free energy change for unfolding of pepsinogen, at neutral pH and 25°C, is in the range 6–12 kcal mol^{-1} [11]. Clearly, the structure destroyed by this reaction makes a major contribution to the stability of pepsinogen and its removal will have marked effects on the properties of the protein. By the loss of these inter-

actions, the first step of the alkaline transition can cause a reduction of the free energy change of the second step, from its hypothetical value in the neutral form of the zymogen, making it appear to be linked to a group of lower pK. As pointed out by Weber [21], this will give rise to positive interactions between the two ionizing groups and to the cooperative pH dependence of pepsinogens alkaline transition.

It remains to be determined how this structure is involved in the activation of pepsinogen. Wang and Edelman [22] isolated a dodecapeptide containing these two histidines from activation mixtures. They showed that it could reduce pepsin's milk clotting activity, through a conformational change in the enzyme induced by binding of the peptide away from the active site. Christensen et al. [23] showed that "pseudopepsin", an activation intermediate, which contains this peptide, had proteolytic activity. The inability of the I form of pepsinogen to activate, after jumping from high to low pH, indicates that it is not a direct intermediate in activation. However the presence of ionic bridges in the zymogen suggested above, implies the existence of a similar isomerization in the native zymogen at low pH, coupled to the titration of some anionic side chain [16]. Just as, at high pH, the $N \rightleftharpoons I$ conformational change must occur before the $I \rightleftharpoons O$ transition, this low pH change may prevent the premature breakdown of some other structure necessary for activation. Studies on this problem are hampered by the marked insolubility of inactive pepsinogen at low pH [3].

Acknowledgement

I would like to thank George Poy for performing the amino acid analyses.

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