Native and Unfolded States of Pepsinogen

II. THE KINETICS OF THE STRUCTURAL TRANSITION INDUCED BY UREA*

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In the preceding communication, the molecular properties of pepsinogen in water and water-urea solutions were evaluated (1). In this report, the rates of unfolding of pepsinogen as a function of pH and temperature in urea solutions are presented. A comparison can then be made between the influence of these variables on pepsinogen denaturation and the results of a similar study reported several years ago for pepsin, the enzymatically active form of this protein (2-4). Since only about 20% of the molecule is cleaved off in the conversion of zymogen to enzyme, it should be possible to assess its influence on the kinetics of transformation of pepsinogen.

It is instructive to preface an account of the kinetic properties of pepsinogen in solution with a brief summary of the relatively well-studied behavior of its active derivative, pepsin. The first order rate constant of pepsin inactivation increases very rapidly in the neutral pH range, varying inversely with the 3.4th power of the hydrogen ion activity (3). The transformation from the active to the inactive molecule results in the acquisition of hydrodynamic properties, including a high and ionic strength-dependent viscosity, which are suggestive of a relatively loose and flexible configuration (2).

A number of experimental observations, including the high pH dependence of inactivation rates in the neutral pH zone, the dependence of the temperature coefficient on pH in this zone, and the effects of various metallic cations such as Cu++, Pb++, and Cd++ in accelerating the rate, suggest that the "trigger groups" of denaturation are the carboxyl groups (3, 4). The rupture of the noncovalent bonds involving these groups reduces the kinetic energy barrier stabilizing the molecule, thereby resulting in the inactivation process. It has been proposed that up to five or six abnormal carboxyl groups may serve to stabilize the native molecule in this way (3, 4). The transformation is accompanied by an ionization, which results in a time-dependent release of protons from these groups that parallels the loss of enzymatic activity and changes in optical rotation and viscosity (5).

Of particular interest to the present study is the question of whether the abnormal carboxyl groups which have been identified as "trigger groups" in pepsin inactivation also exist in pepsinogen and the role they play in its inactivation.

EXPERIMENTAL PROCEDURE

Methods

The methods used to measure fluorescence and absorption are given in Paper I of this series (1). The optical rotation measurements were performed with a Rudolph model 80 spectropolarimeter according to standard procedures.

pH-stat Measurement—The release of hydrogen ions accompanying denaturation was determined by means of a pH-stat measurement, with the use of a Radiometer TTT1 pH meter in conjunction with an Ole Doh automatic recorder. The surface of the titration vessel was flushed continuously with N2. Continuous plots of the volume of standard base added as a function of time were obtained. From the limiting values obtained, the number of moles of [H+] released per mole of pepsinogen was computed.

Analysis of Kinetic Data—The first order rate constants reported in this paper were computed by standard first order plots of log (x - xo)/(x - xo) versus time, where xo, xo, and xo are the value of the observed parameter at the given time, at zero time, and the final limiting value, respectively. The parameters utilized were tryptophan fluorescence at 350 mp, change in ab-
At a constant urea level (8 M), the rate increases rapidly with urea concentration (Fig. 2). A logarithmic relationship is followed, the slope of log $k$ versus log [urea] being 6. The cations Pb$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$ were found to be effective. In all three cases, the rate constant showed a region of linear increase with the logarithm of the molarity of cation. The relative effectiveness paralleled their affinity for the carboxylate anion (8). The similarity to the case of pepsin is quite suggestive (4).

The addition of metallic cations known to be bound strongly by changes in ultraviolet fluorescence and absorption for the carboxylate anion enhances the rate to an important extent (Fig. 4). The cations Pb$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$ were found to be effective. In all three cases, the rate constant showed a region of linear increase with the logarithm of the molarity of cation. The relative effectiveness paralleled their affinity for the carboxylate anion (8). The similarity to the case of pepsin is quite suggestive (4).

### Materials

The materials were the same as reported in the preceding paper (1). Salts were reagent grade. Glass-distilled water was used throughout.

### RESULTS

**pH-stat Measurements**—One of the most interesting characteristics of pepsin inactivation is the release of protons associated with the reaction (3). Since pepsin inactivation is essentially an irreversible process whereas pepsinogen inactivation has been shown to be reversible, conditions were determined under which the pepsinogen reaction would go to completion. In this way the number of protons released for the complete conversion could be obtained at a single pH as was done with pepsin. This was found to be the case for urea levels of 4 M or greater and pH values above 6.0.

The kinetic results reported here and in the following sections are confined to conditions for which the transition went to completion, as manifested by the adherence to simple first order kinetics up to the highest degrees of conversion observed. The rate of release of hydrogen ions was measured in the pH-stat and is reported in Table I. The number of protons liberated per mole was found to decrease significantly with increasing ionic strength and appeared to level off at a value near 3 (Table 1).

**Kinetics of Inactivation**—Three different physical criteria were employed to follow the reaction. These were the quenching of tryptophan fluorescence, the development of a difference spectrum, and the increase in levorotation, all of which are discussed in the preceding paper (1). For the range of conditions cited above, the change with time of all three parameters was in accord with simple first order kinetics. First order plots of the change in tryptophan fluorescence as a function of temperature are presented in Fig. 1. Since the fluorescence intensity of tryptophan and tryptophan peptides decreases with increasing temperatures, the fluorescence intensity of the fully denatured molecule had to be evaluated at each temperature (6, 7). The fact that the concentrations used for fluorescence measurements (0.1 g per liter) were an order of magnitude smaller than those employed for difference spectra or optical rotation (1 to 2 g per liter) provided an additional test for obedience to strictly first order kinetics.

The adherence to first order behavior by three quite different criteria does not of course exclude the possibility that more than one step occurs in the transition. It does, however, indicate that only a single rate-determining step is present. As shown in the preceding communication (1), the molecular change which occurs rapidly below pH 6 in urea represents at least one additional step.

At pH 7 and 25°, the first order rate constant ($k$) increases rapidly with urea concentration (Fig. 2). A logarithmic relationship is followed, the slope of log $k$ versus log [urea] being 6. At a constant urea level (8 M), the rate increases rapidly with pH, changing from very slow at pH 6 to immeasurably fast at pH 8. Log $k$ varies linearly with pH with a slope of 1.6 (Fig. 3).

The addition of metallic cations known to be bound strongly by the carboxylate anion enhances the rate to an important extent (Fig. 4). The cations Pb$^{2+}$, Cd$^{2+}$, and Cu$^{2+}$ were found to be effective. In all three cases, the rate constant showed a region of linear increase with the logarithm of the molarity of cation. The relative effectiveness paralleled their affinity for the carboxylate anion (8). The similarity to the case of pepsin is quite suggestive (4).

### Table I

**Release of protons from pepsinogen during denaturation**

<table>
<thead>
<tr>
<th>KCl</th>
<th>$[H^+]$/pepsinogen</th>
<th>$k$ min$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1</td>
<td>4.59</td>
<td>0.087</td>
</tr>
<tr>
<td>0.25</td>
<td>4.07</td>
<td>0.083</td>
</tr>
<tr>
<td>0.49</td>
<td>3.78</td>
<td>0.068</td>
</tr>
<tr>
<td>1.075</td>
<td>3.29</td>
<td>0.001</td>
</tr>
<tr>
<td>1.40</td>
<td>3.31</td>
<td>0.041</td>
</tr>
<tr>
<td>1.675</td>
<td>3.03</td>
<td>0.036</td>
</tr>
</tbody>
</table>

**Fig. 1.** Representative first order plots for the urea denaturation of pepsinogen at a series of temperatures, as monitored by tryptophan fluorescence. The pepsinogen concentration was 0.10 g per liter; the solvent was 8.0 M urea-0.1 M KCl-0.02 M phosphate, pH 6.97. The temperature is indicated by the number placed by each curve. The ordinate is equal to the fraction of native material (see "Methods").
FIG. 2. Dependence upon urea molarity of first order rate constant for the denaturation of pepsinogen at 25°. The solvent was 0.1 M KCl-0.01 M phosphate, pH 7.0. ○, ultraviolet difference spectra; ●, tryptophan fluorescence; Δ, optical rotation.

FIG. 3. pH dependence of the first order rate constant for the urea denaturation of pepsinogen in 8 M urea-0.01 M phosphate, 25°. ○, ultraviolet difference spectra; ●, tryptophan fluorescence; Δ, optical rotation.

However, both methods agree in indicating an important accelerating effect of the metal ions (Fig. 4).

**Thermodynamic Activation Parameters of Urea Denaturation**

The classical Arrhenius energy of activation, \( E^* \), was computed as a fraction of pH and urea level by using the relations

\[
\frac{d \ln k}{dT} = \frac{E^*}{RT^2}
\]

\[
\ln k = \left( -\frac{E^*}{RT} \right) + \text{constant}
\]

where \( k \) = first order rate constant, \( R \) = gas constant, and \( T \) = absolute temperature. In Fig. 5 are shown the first order rate constants as a function of pH and temperature in 6 M urea.

The enthalpy of activation is given by \( \Delta H^* = E^* - RT \). Values of \( \Delta H^* \), as computed from the thermal variation of rate constants evaluated from fluorescence measurements, are shown in Fig. 6. The enthalpy of activation decreases in a roughly linear manner from pH 6.2 to 6.8. This region of linear decrease is followed by a plateau region. Determinations could not be extended beyond pH 7.2 since the rates became too fast for measurement. At constant pH, the enthalpy of activation decreased with molarity of urea, indicating a reduction in the energy barrier to denaturation with increasing urea level. Increasing levels of guanidine have been shown to produce similar reductions in the enthalpy of activation in the case of pepsin (9). Pepsin also shows a linear decrease in enthalpy of activation between pH 6.0 and 6.9 with constant regions on either side (9). The pepsinogen determinations could not be extended to sufficiently acid pH values to test for the presence of a second constant region similar to that found for pepsin.

The free energy of activation (\( \Delta F^* \)) was computed from the equation

\[
k = \frac{k^b T}{h} e^{-\Delta F^*/RT}
\]

where \( k^b \) = Boltzmann's constant and \( h \) = Planck's constant.

The entropy of activation (\( \Delta S^* \)) is given by

\[
\Delta F^* = \Delta H^* - T \Delta S^*
\]

The thermodynamic parameters characterizing the urea denaturation of pepsinogen are summarized in Table II. The entropies of activation are large and positive, as is generally the case for protein denaturation. While this is formally in accord with the entirely plausible picture of an activation step which involves a transition to a less highly ordered configuration, the interpretation is by no means straightforward, in view of the uncertain contribution of such factors as a change in the state...
FIG. 4. Left, logarithmic dependence of first order rate constant for urea denaturation of pepsinogen upon molarity of Pb\(^{++}\) and Cd\(^{++}\), as monitored fluorometrically. \(\square\), 0.01% pepsinogen in 0.05 M glycine ethyl ester-5 M urea, pH 7.0, plus lead diacetate, at 14.2\(^{\circ}\); \(\bigcirc\), same conditions, plus CdCl\(_2\). Right, logarithmic dependence of first order rate constant upon molarity of metal ion, as monitored by difference spectra. \(\square\), Pb(NO\(_3\))\(_2\), 0.00% protein, 0.02 M imidazole-5.5 M urea, pH 6.75, 27\(^{\circ}\); \(\bigcirc\), CdCl\(_2\), same as for Cu(NO\(_3\))\(_2\); \(\triangle\), Cu(NO\(_3\))\(_2\), 0.047% protein, 0.02 M imidazole-6 M urea, pH 6.7, 25\(^{\circ}\). \(k_0\) is the rate constant under identical experimental conditions in the absence of heavy metal ions.

FIG. 5. The effect of temperature and pH on the first order rate constants for pepsin denaturation in 6 M urea, 0.10 M KCl, and 0.02 M phosphate.

of ionization. The pH profile of entropy of activation parallels closely that of enthalpy of activation.

Reversibility—In harmony with the results of Perlmann (10), the urea denaturation of pepsinogen was found to be reversible by the criterion of peptic activity. When the product of the prolonged action of 8 M urea at pH 7 was diluted 20-fold with buffer, a time-dependent recovery of activity occurred over a 24-hour period at 0\(^{\circ}\) to a level of 80% of that for native pepsinogen. After a 45-minute exposure to 31\(^{\circ}\), the activity increased to 98%. Peptic activity was determined as described in the accompanying paper (11).

Discussion

It has been necessary to make measurements in concentrated urea solutions, in which the conversion from Form IV to V (1) goes to completion, since the two transitions that occur in aqueous alkaline solution are pH-dependent equilibrium reactions. Moreover, as shown in the preceding paper (1), the product of the transition in urea (Form V) is largely devoid of intramolecular constraints and therefore more nearly resembles the inactive form of pepsin than do the forms produced in aqueous solution at alkaline pH values. It should be possible to compare the
two reactions even though the pepsin inactivation occurs in water and that of pepsinogen occurs in urea-water solution. Studies of amino acids and other model substances have shown that the influence of urea appears to be largely in producing small changes in the pK values of the dissociable groups (12) and in their solubility (13, 14).

Examination of the detailed kinetic picture reveals many parallels with the case of pepsin. The steep dependence of $k_1$ upon pH, the nature of the variation of enthalpy with pH, and the accelerating effect of certain metallic cations all resemble the behavior of pepsin and are suggestive of a basically similar underlying mechanism. As in the latter case, it is likely that the time-dependent transition is accompanied by an ionization of several abnormal carboxyls. This is consistent with the observed evolution of hydrogen ions and the accelerating influence of Pb$^+$ and Cd$^{++}$, both of which are known to be bound by carboxylate ions.

The only ionizable group other than carboxyl which has an considerable amount of recent evidence indicates that the ionization constant that occurs at a pH near neutrality, and which can therefore be responsible for the liberation of protons in pepsin or pepsinogen, is imidazole. Since there is only one imidazole group in pepsin, it cannot account for the number of protons released. Moreover, the relative influence of the heavy metal cations studied clearly relates to their affinity for carboxylate anions. The protons released in the alkaline inactivation of pepsinogen in water might come from carboxyl, imidazole, α-amino, or even ε-amino groups. However, when the reaction occurs in concentrated urea solution at pH 7, the only groups which can contribute protons are carboxyl and imidazole. Since the intrinsic pK of the imidazole group of proteins in aqueous solution is generally about 6 (15) and should be approximately 0.4 unit higher in 5 M urea (12), the 3 imidazole residues in pepsinogen can hardly suffice to account for the 3 to 4 protons evolved during the transition at pH 7. Consequently, most, if not all, of the protons must come from the carboxyl groups.

It was suggested in an earlier communication that the carboxyl groups responsible for the liberation of protons during pepsin inactivation are stabilized by hydrogen bonding in the active form of the enzyme (3). The principal molecular and kinetic features which characterize pepsin inactivation have also been shown to apply to pepsinogen. It seems plausible, therefore, to identify the critical carboxyl groups in pepsinogen with those in pepsin. These groups commence to lose their stability in aqueous solution at 25° at about pH 6 in pepsin and pH 9 in pepsinogen. If this difference in pH represents the difference in their free energies of ionization, then they are stabilized by 3 logarithmic units of hydrogen ion activity or about 1800 cal ($\Delta (\Delta F) = RT \Delta (pK)$) in the zymogen relative to the enzyme. Whether this extra stabilization is provided by the large number of basic groups lost or is somehow linked to the hydrolysis of peptide bonds in the conversion of precursor to enzyme is not evident from the data. A similar stabilization of two of the carboxyl groups in β-lactoglobulin has been reported (16).

A considerable amount of recent evidence indicates that the traditional picture of urea as a potent breaker of hydrogen bonds
is incorrect and that its activity as a protein denaturant may depend instead upon its capacity to rupture hydrophobic bonds (13, 14, 17). Thus, it is unlikely that the labilization by urea of the hydrogen bonding of the critical carboxyl groups results from the direct action of urea. It is more probable that the stability of these hydrogen bonds depends upon the integrity of other structural elements, which are in turn stabilized by hydrophobic bonds. Rupture of these by urea eliminates these structural features and thus indirectly labilizes the hydrogen-bonded carboxyls. In other words, the hydrogen and hydrophobic bonds may constitute an interdependent system which cannot survive the loss of either source of stability. The significance of cooperative interactions of this kind in providing an additional source of free energy has been discussed recently (18). It has been proposed previously that the stability of the carboxyl hydrogen-bonded groups in pepsinogen, then the greater stability of the carboxyl hydrogen bonds in the latter molecule can be rationalized. In addition, the acid shift of the pH region of pepsinogen stability with urea concentration may be reconciled with a weakening of the strength of the critical carboxyl groups as a result of increasing losses in hydrophobic interactions. The reduction in the enthalpy of activation with increasing urea concentration could be a reflection of the disappearance of the more labile hydrophobically bonded structures. An intermediate of this kind has been identified for pepsinogen in 8 M urea at pH 5.0 (1).

**SUMMARY**

1. The kinetics of pepsinogen denaturation has been determined by pH-stat, fluorescence, difference spectra, and optical rotation measurements. Rates of reaction as found by the latter three methods were found to be equivalent.

2. Kinetic activation energy constants have been determined for the molecular transition occurring in concentrated urea solutions.

3. The behavior of pepsinogen has been shown to resemble that of pepsin in its alkaline inactivation, and a single mechanism, involving the rupture of several stabilized carboxyl groups, has been proposed to account for both reactions.

**REFERENCES**


Native and Unfolded States of Pepsinogen: II. THE KINETICS OF THE
STRUCTURAL TRANSITION INDUCED BY UREA
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