THE CATALYTIC EFFECT OF ACTIVE CRYSTALLINE PAPAIN ON THE DENATURATION OF THYROGLOBULIN*

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It has been assumed that denaturation occurs preliminary to enzymatic hydrolytic fission in some proteins. The basis of this supposition is the generally accepted observation that these proteins are less readily attacked by proteolytic enzymes in their native state than when they are denatured (1–3). In the case of such readily digestible proteins as gelatin, prolamin, or casein, Linderström-Lang states that there is hardly any doubt that fission of peptide bonds is the initial and sole reaction, but in the case of the slowly digestible “crystalline or globular proteins” he believes that a secondary structure in the native protein blocks the peptide bonds to direct enzyme action. Such a blocking is thought to have its origin in steric hindrances or in the presence of chemical structures into which peptide bonds have themselves entered.

To account for the difference between the action of proteolytic enzymes on the native and denatured forms of the slowly digestible proteins and in the absence of the proof of a direct action of enzymes on the native proteins Linderström-Lang proposes that the process involves reversible denaturation. He assumes that native proteins as such are not attacked by the enzyme, but the change occurs as a result of an equilibrium between the native, \( N \), and denatured, \( D \), forms. Then, if the proteolytic enzyme promotes the removal of the denatured form by hydrolysis, the native form shifts to \( D \) to maintain the equilibrium as follows:

\[
N \rightleftharpoons D \xrightarrow{\text{enzyme}} \text{hydrolysis}
\]

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In this investigation the rôle which a proteolytic enzyme plays in denaturation prior to enzymatic hydrolysis has been determined through use of newer physicochemical methods. In this paper there are reported the results of a study on the effect of crystalline active papain on a system in which the changes occurring in denaturation are already known and can be followed under rigid experimental control. Highly purified hog thyroglobulin offered the ideal substrate.

In previous studies on the stability characteristics of thyroglobulin (4, 5) it was found by ultracentrifugal analysis that given appropriate conditions it is possible to detect the formation from the native form of the protein of an unstable more slowly sedimenting protein, called for convenience α-protein. An equilibrium is finally established. This unstable form was found to behave as an intermediate between the native protein on the one hand and the denatured form on the other. It appears to involve an unfolding of the regular structure in the native protein molecule preliminary to a change into the new structure of the denatured form (5). A similar mechanism has been found to hold in the case of several other proteins so far investigated in this laboratory. The effect of the addition of salt is to reverse the process with formation of native protein. This accounts for the fact that the α form is not observed in electrolyte concentrations ordinarily used in the sedimentation analysis of proteins. Conditions favoring denaturation such as heat treatment or addition of increasing amounts of sodium salicylate to the solution cause the disappearance of α-protein with the simultaneous formation of denatured protein. The denatured form sediments at the same rate as the original native protein and in the presence of salt the denatured protein appears to be heterogeneous. Aside from this, the denatured form can be distinguished from the native protein by differences in solubility and electrophoretic mobility.

After denaturation caused by the heating of the salt-free native thyroglobulin solution, the protein possesses different solubility, because it precipitates in borax-succinic acid buffers at pH 5.2, ionic strength = 0.1. The native protein is soluble under these conditions. On standing in this solution, especially at elevated temperatures, the native form changes to the denatured form and precipitates. On the other hand if the cooled heat-denatured
protein is allowed to stand before addition of the buffer, the precipitability is decreased as before. On reheating, followed by cooling, the precipitability again decreases on standing. These experiments point to the reversibility of denaturation in this protein under these conditions.

Effect of Active Papain on Denaturation in the System, $N \rightarrow \alpha \rightarrow D$, under Conditions Favoring Retention of Denatured Form in Solution: Ultracentrifugal Analysis

Salt-free solutions of thyroglobulin, with a total protein concentration of 2.0 per cent and containing the equilibrium mixture,

![Ultracentrifugal diagrams](image)

Fig. 1. Ultracentrifugal diagrams obtained by using the Philpot schlieren optical system, showing the effect of active papain on $\alpha$-thyroglobulin. The left-hand diagram represents the results of an experiment performed before incubation but immediately after mixture with papain at 40°. Native protein is shown below and $\alpha$-protein above. (Thyroglobulin sediments to the bottom of the centrifuge cell before papain comes into the optical field; therefore the papain peak is not to be observed in these pictures. Thyroglobulin $s_{20} = 19.2 \times 10^{-12}$; papain $s_{20} = 2.7 \times 10^{-12}$.) The center diagram represents the results of an experiment after incubation with papain for 3 hours at 40°. No $\alpha$-protein is present. The right-hand diagram represents the results of an experiment after incubation with papain for 12 hours at 40°. Hydrolytic fission is shown. Thyroglobulin concentration = $2.3 \times 10^{-4}$ mole per liter; papain concentration = $8.8 \times 10^{-4}$ mole per liter; NaCN concentration = $8.0 \times 10^{-4}$ mole per liter; pH = 5.6.

$N \rightarrow \alpha$, established at pH 5.8, were incubated at temperatures which previously had been found to cause the slow disappearance of $\alpha$-protein into the denatured form. At intervals samples were removed and subjected to ultracentrifugal analysis in a Svedberg oil turbine ultracentrifuge. With the Philpot modification of the schlieren optical system it was found that the presence of active papain catalyzed the disappearance of $\alpha$-protein (Fig. 1).
Control experiments proved that this effect was due to the active enzyme present and not to the presence of electrolytes, including HCN. Furthermore, papain preparations which had been denatured by heat treatments in a water bath at 100° were not effective. With native protein at the start and with no α form present, incubation at temperatures between 30-50°, with or without active papain, caused no appearance of α-protein as shown by centrifugal analysis. This is explained on the basis of the relative reaction rates, by assuming that the α form changes to

Table I

<table>
<thead>
<tr>
<th>Thyroglobulin</th>
<th>Papain</th>
<th>NaCN</th>
<th>pH</th>
<th>Time of standing</th>
<th>Relative amount of α-protein present</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8 moles per l. X 10⁶</td>
<td>5.6</td>
<td></td>
<td>25</td>
<td></td>
<td>About 50% normal sedimenting component</td>
</tr>
<tr>
<td>5.8 moles per l. X 10⁶</td>
<td>5.6</td>
<td></td>
<td>40</td>
<td>28</td>
<td>About same relative amount</td>
</tr>
<tr>
<td>3.7 moles per l. X 10⁶</td>
<td>5.6</td>
<td></td>
<td>25</td>
<td></td>
<td>About 50% normal component</td>
</tr>
<tr>
<td>3.7 moles per l. X 10⁶</td>
<td>5.6</td>
<td></td>
<td>40</td>
<td>47</td>
<td>Slightly less than native sedimenting component</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 (Active)</td>
<td>5.8</td>
<td>25</td>
<td>About 25% normal sedimenting component</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>5.8</td>
<td>26.6</td>
<td>Very little, less than 5% normal sedimenting component</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>5.8</td>
<td>26.6</td>
<td>All gone</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>5.00</td>
<td>5.65</td>
<td>25</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>5.00</td>
<td>5.65</td>
<td>30</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>4.0</td>
<td>5.6</td>
<td>25</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>4.0</td>
<td>5.6</td>
<td>30</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>4.0</td>
<td>5.6</td>
<td>30</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 (Denatured, 100°, 10 min.)</td>
<td>4.0</td>
<td>5.6</td>
<td>25</td>
</tr>
<tr>
<td>2.3 moles per l. X 10⁶</td>
<td>8.0</td>
<td>8.8 &quot;&quot; &quot;&quot;</td>
<td>4.0</td>
<td>5.6</td>
<td>30</td>
</tr>
</tbody>
</table>
the denatured form in papain catalysis. Under these conditions the rate of change of $\alpha$-protein to the denatured, $D$, form is greater than the change of native protein to the $\alpha$ condition.

When the temperature is below 30°, the rate of the reaction $\alpha \rightarrow D$ becomes slower than that of the reaction $N \rightarrow \alpha$, since $\alpha$-protein accumulates in the solution. Thus the change $\alpha \rightarrow D$ appears to be the rate-determining step in denaturation.

The time intervals involved in these experiments were in no case long enough to cause significant hydrolytic fission, since no lighter material was detected in the sedimentation field. The results of a typical sequence of experiments are given in Table I.

Although the catalytic disappearance of $\alpha$-protein in the presence of papain is assumed to involve the simultaneous formation of the soluble denatured form of the protein (as had previously been shown to occur in the absence of enzyme) confirmation was necessary, since the denatured protein cannot be distinguished from the normal native protein by centrifugal analysis alone. The system was studied further by using the methods of differential solubility and electrophoresis.

Effect of Active Papain on Denaturation of Thyroglobulin under Conditions Favoring Precipitation of Denatured Protein As Rapidly As It Is Formed: Kinetics of Denaturation

To render the differential solubility method more adaptable for the kinetic investigation of the system, it was decided to follow the precipitation in borax-succinic acid buffers by means of changes in light absorption as detected by using a photoelectric colorimeter. Preliminary experiments showed that for all practical purposes the light absorption of the suspension of the precipitated denatured, $D$, form (especially when the initial concentration of $D$ was not too high) varied with concentration according to Beer's law.

An apparatus was designed for studying the light absorption in thyroglobulin systems at various temperatures. This consisted of a General Electric photoelectric cell (No. 88 $\times$ 565), a Rawson milliammeter, a constant light source, and a constant temperature bath with arrangements for passing monochromatic light through the solutions and onto the photoelectric cell.

A standardized experimental procedure was developed. The
water bath was regulated to the desired temperature ±0.05°. To insure complete temperature equilibrium the buffer was attempered in the experimental tube before each experiment. With 10 cc. of buffer attemperated at 46.3°, the addition of 0.5 cc. of 3.5 per cent thyroglobulin at room temperature caused no change in temperature of any practical significance. In most experiments not more than 0.2 cc. of 3.5 per cent thyroglobulin and not more than 0.04 cc. of 0.68 per cent papain solution were added for any experiment. Readings were taken at appropriate time intervals with intermittent stirring. The light intensity, regulated at scale reading 100 on the milliammeter with 10 cc. of buffer alone, was checked at the end of the reaction or, if the reaction was very slow, from time to time with a matched tube containing 10 cc. of buffer solution. In all experiments reported here, there was used a borax-succinic acid buffer at pH 5.2, with ionic strength = 0.1.

In a typical experiment, a solution of 3.5 per cent thyroglobulin was denatured by heating in a boiling water bath for 10 minutes. The solution was cooled to room temperature and 0.2 cc. aliquots were added immediately to 10 cc. of buffer attemperated at 46.3°. The precipitation curves were reproducible to within 2 per cent. There is shown in Fig. 2 the curve for precipitation of denatured protein under these conditions. Here the ordinate represents the concentration of denatured protein precipitated, and is equal to \( \log \left( \frac{I_0}{I} \right) / K \). In this equation \( I_0 \) is the original light intensity as registered by the milliammeter, \( I \) is the intensity of light passing through the suspension at time \( t \), and \( K \) is the extinction coefficient.

When the solutions of denatured protein were allowed to stand, an appreciable and regular decrease in the over-all rate of precipitation was observed. When the material was reheated, cooled rapidly, and analyzed, the precipitation rate was as fast as before. This again indicates that on standing reversible denaturation occurs in the cooled solution.

In the presence of active papain the precipitation of denatured protein was not affected. However, solutions which had stood for a period of time and which precipitated somewhat more slowly than the freshly prepared denatured form now precipitated as fast as the freshly denatured protein, showing that papain catalyzes the denaturation of the reversed protein.
With native protein as the starting material, with or without active papain, the rate of precipitation follows the general form of the curves shown in Fig. 3. From kinetic considerations, it is possible to show the effect of papain on the step involving the denaturation of the protein. It has been well established that the denaturation reaction follows the first order reaction law. Hence the rate of formation of denatured protein is expressed as follows:

\[
(1) \quad (dD/dt)_f = N_0 e^{-k_1 t}
\]

**Fig. 2.** Precipitation of denatured thyroglobulin, with the denatured form in succinic acid-borax buffer as starting material; pH 5.2; \( \mu = 0.1; t = 46.3^\circ \).  

In this equation \( N_0 \) is the original concentration of native protein and \( k_1 \) is the first order reaction velocity constant. This equation expresses the rate of the slowest reaction governing the change, \( N \rightarrow \alpha \rightarrow D \).

When precipitation occurs, the rate of change of concentration of denatured soluble protein is equal to the rate of formation of denatured protein minus its rate of precipitation, or,

\[
(2) \quad \left( \frac{dD}{dt} \right)_f = \left( \frac{dD}{dt} \right)_r - \left( \frac{dD}{dt} \right)_p,
\]
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But when the rate of formation of denatured protein is equal to the rate of precipitation, the system is in the steady state and

\[
\left( \frac{dD}{dt} \right)_i = 0
\]

so that

\[
\left( \frac{dD}{dt} \right)_p = \left( \frac{dD}{dt} \right)_f = N_0 e^{-k_1 t}
\]

![Graph showing precipitation of denatured thyroglobulin.](image)

**Fig. 3.** Precipitation of denatured thyroglobulin, with the native form in succinic acid-borax buffer as starting material; pH 5.2; \( \mu = 0.1; t = 46.3^\circ \). Curve I represents the control (\( N \rightarrow D \rightarrow \) precipitate); Curve II, the control + 4.5 \( \times 10^{-4} \) mole of active papain; Curve III, the control + 9.0 \( \times 10^{-4} \) mole of active papain. Papain activity units by the milk clotting method per mg. of protein nitrogen = 21.3.

Therefore if the steady state can be recognized, it is possible to determine the reaction velocity constant, \( k_1 \), for the first order denaturation reaction in this system. By plotting the logarithm of the concentration of the precipitating denatured protein against time, the steady state should exist in the region where the curves are linear. This region was found to occur early in the reaction. The \( k_1 \) values corresponding to the slopes of the straight lines are tabulated in the last column of Table II. With the \( k_1 \) values for reactions at two temperatures and with the van't Hoff-Arrhenius
equation, a value of about 100,000 calories was calculated for the critical thermal increment. Since this value is characteristic of the denaturation reaction in protein systems, the validity of the foregoing interpretation is confirmed. Not only is the reaction constant, $k_1$, for the denaturation reaction increased by the enzyme as shown by Table II but as the rate is increased by the enzyme

\[ \text{TABLE II} \]

**Kinetics of Denaturation of Thyroglobulin in Succinic Acid-Borax Buffers, pH 5.2, $\mu = 0.1$**

<table>
<thead>
<tr>
<th>Thyroglobulin</th>
<th>Papain</th>
<th>NaCN</th>
<th>$t$</th>
<th>$\frac{\Delta \log C}{\Delta t} = k_1$</th>
</tr>
</thead>
<tbody>
<tr>
<td>moles per l. $\times 10^6$</td>
<td>moles per l. $\times 10^6$</td>
<td>moles per l. $\times 10^6$</td>
<td>$^\circ C.$</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>4.5 (Active)</td>
<td>8.0</td>
<td>46.3</td>
<td>0.28</td>
</tr>
<tr>
<td>2.0</td>
<td>9.0</td>
<td>8.0</td>
<td>46.3</td>
<td>0.67</td>
</tr>
<tr>
<td>2.0</td>
<td>9.0 (Heat-denatured, 100°, 10 min.)</td>
<td>8.0</td>
<td>46.3</td>
<td>2.50</td>
</tr>
<tr>
<td>2.0</td>
<td>8.0</td>
<td>46.3</td>
<td>0.384</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>9.0 (Inactive, oxidized)</td>
<td>46.3</td>
<td>0.29</td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>46.3</td>
<td>0.29</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.0</td>
<td>50.4</td>
<td>2.5</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\[ \text{TABLE III} \]

**Electrophoretic Mobility of Denatured Thyroglobulin Catalyzed by Papain**

3.02 per cent thyroglobulin (pH 5.8, electrolyte-free), 0.07 per cent active papain, and 0.02 M NaCN were incubated at 40° for 3 hours and diluted 1:15 with succinic acid-borax buffer at 0°; pH, 5.2; $\mu = 0.1$.

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Average mobility of boundary</th>
<th>Average mobility of thyroglobulin, $\mu = 0.02$, pH 5.2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dialyzed 10 hrs. at 5° ............</td>
<td>5.14</td>
<td>4.95</td>
</tr>
<tr>
<td>No dialysis run immediately ......</td>
<td>5.10</td>
<td>4.91</td>
</tr>
</tbody>
</table>

the S-shaped curves of Fig. 3 approach the characteristic shape of the curve for the precipitation of denatured thyroglobulin which is shown in Fig. 2. The $k_1$ values for the effect of inactive and denatured papain and for the presence of cyanide alone (Table II) prove that only the active papain catalyzes the denaturation of thyroglobulin.
**Electrophoretic Mobility of Papain-Catalyzed Denatured Thyroglobulin**

Evidence that α-protein changes to the denatured form on heat treatment in the absence of enzyme was presented in another paper (5). By the addition of buffer to the solution and analysis in a Tiselius electrophoresis apparatus, mixtures of the heat-treated protein and native protein in equal concentration showed two boundaries of approximately equal size. These boundaries were found to have mobilities which correspond to values found earlier for native and denatured thyroglobulin by Heidelberger and Pedersen (6).

Solutions containing the equilibrium system $N \rightleftharpoons \alpha$ were incubated with active papain until they showed no α-protein on centrifugal analysis. They were then treated with buffer solution and analyzed in the Tiselius equipment. A single boundary with mobility corresponding to that of the denatured form prepared without enzyme was obtained.

Judging from the electrophoretic field, the extent of hydrolytic fission during the denaturation under these conditions appears negligible.

The results of these experiments are given in Table III.

**DISCUSSION**

The studies of the kinetics of precipitation and of the electrophoretic behavior in thyroglobulin systems confirm the observations made by ultracentrifugal analysis which show that active papain catalyzes the denaturation of thyroglobulin under conditions in which hydrolysis is negligible. The catalysis involves the reaction between the unstable or α form of the protein and the denatured form. Since the denaturation reaction follows the first order law, the reaction, native protein to α form, must occur rapidly under the conditions in these experiments. Accordingly, under conditions in which the native protein is stable, the enzyme would have no effect. Since the production of α-protein appears to involve an unfolding of the protein in salt-free solutions, the assumption of Linderström-Lang that structural blocking opposes enzyme action in the native protein is confirmed. In salt solution, unfolding itself is not observed on sedimentation
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analysis. It has not yet been determined whether, under ordinary conditions, this formation of \( \alpha \)-protein occurs too fast to be followed or perhaps involves a less profound change (cf. Annetts (7)) of the molecule.

The results confirm the assumption that denaturation is an initial reaction in proteolysis. It appears non-essential in the system under investigation whether or not the change from native protein to the denatured form is reversible.

SUMMARY

Ultracentrifugal, kinetic, and electrophoretic experiments show that active papain catalyzes the denaturation of thyroglobulin prior to hydrolytic fission of the protein. Inactive native or denatured papain does not possess this activity. An initial non-enzymatic conditioning reaction occurs before the enzymatic reaction. This involves a structural change in the native protein which presumably liberates groups to serve as points of attack for the enzyme. The results are in agreement with the Linderström-Lang theory for the mechanism of enzyme action in protein systems.

The author wishes to express sincere thanks to J. W. Williams for his interest in this problem. Grateful acknowledgment is made to the Wisconsin Alumni Research Foundation and to the University Research Committee for financial assistance which has made this work possible. The author wishes to thank Dr. A. K. Balls for his kindness in placing at his disposal a supply of crystalline papain from fresh papaya latex.

BIBLIOGRAPHY

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