Heat and Photochemical Inactivations of Taka-Amylase A and Mechanisms of Protection by Substrate

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The heat, ultraviolet and riboflavin-sensitized visible inactivations of taka-amylose A are strongly inhibited by the presence of its substrate. The stabilization of the secondary structure of enzyme protein by the conformation change due to the formation of enzyme-substrate and -product complexes is responsible for the protection of enzyme from the heat inactivation. The photoinactivations are brought about by the combined effects of heat and photochemical processes. The observed protections from photoinactivations are due to the inhibition effects of substrate and its decomposition products on both processes. The thermodynamic quantities determined for the inactivation reactions throw some light on the relationship between the heat and photochemical processes, and on the mechanisms of the protective action of substrate.

Under the aerobic conditions, the irradiation of taka-amylose A (TAA) solution by the ultraviolet light\(^1\) absorbed by the enzyme protein itself gives rise to the irreversible inactivation. The inactivation is mainly derived from the cleavage of \(-S-S-\) linkage to \(-S\cdot S-\) in cystine and the oxidation of the other amino acids by the photochemical reaction. The visible light\(^2\) is also effective for the inactivation of TAA, if a suitable sensitizer is present. Riboflavin (RF) is a favorable photosensitizer for the inactivation of TAA, and its photosensitizing action brings about the oxidation of amino acids in TAA, leading to the inactivation. When TAA solution is heated, TAA undergoes certain characteristic changes in its structure. Such structural changes bring about the loss of activity irreversibly\(^3\). Therefore, the photoinactivation in the high temperature range is caused by the collaboration of heat and photochemical processes. The exact relationship between these two processes is considered to be indeed very complicated one. The presence of substrate, soluble starch strongly protects TAA from the heat\(^3\) and photo\(^4\)-inactivations. It is considered that the substrate plays an important role for the protection of TAA both in the heat and photochemical processes. The phenomena of protection by substrate are of very interest, but the detailed mechanisms remain still open.

In the present article, the heat, ultraviolet and RF-sensitized visible inactivations are dealt with, by determining the thermodynamic quantities for TAA inactivations, to find the relationship between the heat and photochemical processes and to make clear the mechanisms of protection from inactivations by substrate.

Materials and Experimental

TAA was prepared from “taka-diastase Sankyo” and recrystallized three times by the Akabori’s method\(^5\). The about 0.2% stock solution of TAA was prepared by dissolving the crystals with 0.02 M acetate buffer at \(pH\) 5.6 (the \(pH\) of the optimum activity) and was kept at 4 °C in the dark. The solution was used for the experiments after suitable dilution. The concentration was determined spectroscopically assuming the extinction coefficient\(^6\) of TAA in acetate buffer (\(pH\) 5.6; \(\mu = 0.1\)) to be \(E_{\text{1\,cm}}^{1} = 22.1\) at 278.5 nm.

The treatment for the heat inactivation was made by the following procedure. The TAA solutions, in the absence and presence of starch, were incubated for various periods at various temperatures and rapidly cooled at 0 °C after incubation.

For the photoinactivations, the TAA solutions, in the absence and presence of starch, were irradiated for various periods at various temperatures by the ultraviolet light or, in the presence of RF (3.3 \(\cdot\) \(10^{-5}\) M), by the visible light.

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The ultraviolet light for the ultraviolet inactivation was obtained from the 100 watt high pressure mercury lamp. The visible light for the excitation of 445 nm-band of RF was isolated from a 500 watt projection lamp by means of an appropriate glass filter system. The activity of TAA was measured by the blue value method at 0 °C to avoid the thermal effect as much as possible. Chemicals used were of special grade for analytical use.

Results and Discussion

The initial velocities of heat- and photo-inactivations of TAA were measured at various temperatures in the absence and presence of starch. The treatments for the inactivations in the presence of starch were started immediately after mixing the substrate with TAA solution. The presence of substrate strongly inhibits the inactivation reactions. The degree of protection defined by the ratio of the difference between the initial velocities of inactivations in the absence \( R_0 \) and presence \( R \) of starch to the initial velocity in the absence of starch, for the heat, ultraviolet and RF-sensitized visible inactivations, is shown in Fig. 1 as a function of the substrate concentration. The degree of protection increases linearly with the increase of the concentration of substrate in the region of low substrate concentration. However, in the region of high substrate concentration, the degree of protection gradually saturates to the limiting value, unity (complete protection) under the present experimental conditions.

Since the heat inactivation of TAA occurs measurably only above about 50 °C, curves 2 and 3 in which the irradiation treatments were made at 20 °C do not involve the heat inactivation, and hence show the protection from the photochemical inactivations.

The overall rate constants for heat- and photo-inactivations, in the absence and presence of starch, were determined from the initial velocities of inactivations. The logarithm of these values was plotted against the reciprocal of absolute temperature \( T \) in Fig. 2. The plot for the heat inactivation (curves 1 and 2) is linear and obeys the Arrhenius law. However, the curve for the photoinactivation has a knick, consisting of the low and high temperature portions. The low temperature portion is mainly due to the photochemical inactivation and does not appreciably involve the heat inactivation. However, the high temperature portion is considered to be brought about by the combined effects of heat and photochemical processes. We can roughly eliminate the photochemical contribution from the high temperature portion by subtracting the extrapolated values of the sufficient low-temperature portion from the overall rate constants, although the exact relationship between the heat and photochemical inactivation processes in the high temperature range.

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my be indeed very complicated one. Namely, the differences (curves 3', 4', 5' and 6') between the overall rate constants (curves 3, 4, 5 and 6) and the extrapolations of the sufficient low-temperature portions (a, b, c and d) are considered to stand for the inactivations by the thermal processes. Curves 3', 4', 5' and 6' are all linear as seen in Fig. 2. Therefore, the overall rate constant \( k \) can be expressed by the addition of two exponential forms, the thermal and photochemical parts,
\[
k = A_1 e^{-E_1/RT} + A_2 e^{-E_2/RT}
\]
where \( A_1 \) and \( A_2 \) are constants and \( R \) gas constant and \( E_1 \) and \( E_2 \) respectively the activation energies for the heat and photochemical processes in the inactivation reactions.

However, since the heat and photochemical processes are not completely independent with each other, the first term is considered to include an effect of the interference between the heat and photochemical processes. The activation energies calculated from curves 1, 2 and 3', 4', 5', 6' and a, b, c, d, are given in Table 1. The protein structure of TAA composed of a single polypeptide chain is held by \( \text{—}S—S— \) links and hydrogen bridges between peptide chains. These links may be broken by the thermal vibration at higher temperatures or by the photochemical reactions to bring about the destruction of enzyme structure, leading to the inactivation and denaturation. The spontaneous breakage of \( —S—S— \) links in cystine to \( —S’—S’— \) radicals and the self-oxidation of amino acids in TAA are the main events for the ultraviolet inactivation. For this reason, it is considered that the ultraviolet inactivation in the low temperature region is independent of temperature. The obtained activation energy \( E_2 = 0 \) explains the temperature independency. The presence of substrate decreases the value of \( k \), but does not affect the value of \( E_2 \).

The RF-sensitized visible inactivation, however, is brought about by the oxidation of amino acids by the reactive oxygen which may be produced by the coupling of oxygen with the excited sensitizer, probably the metastable triplet species. Such photochemical processes are temperature-dependent and hence \( E_2 \) is not zero. \( E_2 \) in the presence of substrate is higher than that in the absence of substrate. The substrate inhibits the photo-oxidation process of amino acids by decreasing the collision frequency between reactive oxygen and enzyme, increasing the viscosity of medium. Furthermore, the reactive oxygen may be destroyed by the reaction with substrate or products around the enzyme, creating a favorable condition for enzyme protection.

In the ultraviolet inactivation, curve 6' almost coincides with curve 5' and \( E_1 \) as well as \( E_2 \) do not change appreciably by the presence of substrate. These facts imply that the screening of the exciting light by substrate and products may bring about the apparent protection observed. \( E_1 \) for the ultraviolet inactivation is much lower than that for the heat inactivation. This means that the interference between heat and photochemical processes is very strong. Such a strong interference may prevent the substrate from protecting the heat inactivation.

In the visible inactivation, however, the interference between heat and photochemical processes must be weaker than in the ultraviolet inactivation as supposed from the inactivation mechanisms. This weaker interference gives the higher value of \( E_1 \) (57 Kcal/mol) than \( E_1 \) for the ultraviolet inactivation (but lower than that for the heat inactivation). The presence of substrate inhibits the thermal inactivation and suppresses the interference between heat and photochemical processes. Therefore, the presence of substrate makes \( E_1 \) higher.

By applying the theory of absolute reaction rate to curves 1, 2, 3', 4', 5' and 6', the heat of activation \( (\Delta H^*) \), the free energy of activation \( (\Delta F^*) \) and the entropy of activation \( (\Delta S^*) \) were calculated and are given in Table 2. These values decrease in

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**Table 1. Activation energies for heat, ultraviolet and visible inactivations, in the absence of starch.**

<table>
<thead>
<tr>
<th>Inactivation</th>
<th>Thermal process</th>
<th>Photochemical process</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>85.2</td>
<td>—</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>41.6</td>
<td>0</td>
</tr>
<tr>
<td>Visible</td>
<td>57.0</td>
<td>8.5</td>
</tr>
<tr>
<td></td>
<td>77.6 (s)</td>
<td>12.8 (s)</td>
</tr>
</tbody>
</table>

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The order of heat, visible, ultraviolet inactivations, depending on the degree of the magnitude of interference between heat and photochemical processes. The important feature of the results in Table 2 is the abnormally high entropies of activation. This explains the abnormally high activation energy for the heat inactivation. The high entropy of activation suggests that a profound structural change is involved. The values of thermodynamic quantities for ultraviolet inactivation do not change appreciably by the presence of substrate. This means the lack of substrate effect on the ultraviolet inactivation, as stated above.

The protective effect of substrate from the heat inactivation is thought to be attributable to the stabilization of the secondary structure of enzyme protein by the conformation change from disorder (labile) to order (rigid) due to the formation of enzyme-substrate complex. The formation of such a complex gives rise to the decrease of entropy. This decrease of entropy may explain the observed increase of $\Delta S^*$ by the presence of substrate. The difference of 14 cal/mol between the entropies for the heat inactivation in the absence and presence of substrate (Table 2) is considered to be the reasonable order of magnitude as the entropy change upon the formation of enzyme-substrate complex.

The visible inactivation (curve 3') is brought about by the combined effects of heat process and interference between heat and photochemical processes. For this reason, $\Delta S^*$ is much lower than that in the case of heat inactivation. The presence of substrate not only protects the heat process but suppresses the interference between heat and photochemical processes by inhibiting the photochemical process, so that $\Delta S^*$ increases remarkably by the presence of substrate.

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**Table 2.** Free energies of activation ($\Delta F^*$), heats of activation ($\Delta H^*$) and entropies of activation ($\Delta S^*$) for thermal processes of various inactivations. (s) in the presence of starch (0.6%).

<table>
<thead>
<tr>
<th>Inactivation</th>
<th>Temp. [°C]</th>
<th>$\Delta F^*$ [Kcal/mol]</th>
<th>$\Delta H^*$ [Kcal/mol]</th>
<th>$\Delta S^*$ [cal/mol]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>55</td>
<td>24.7</td>
<td>84.5</td>
<td>183</td>
</tr>
<tr>
<td></td>
<td>25.9 (s)</td>
<td>90.5 (s)</td>
<td>197 (s)</td>
<td></td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>50</td>
<td>23.7</td>
<td>40.9</td>
<td>53</td>
</tr>
<tr>
<td></td>
<td>23.7 (s)</td>
<td>41.2 (s)</td>
<td>54 (s)</td>
<td></td>
</tr>
<tr>
<td>Visible</td>
<td>50</td>
<td>24.9</td>
<td>56.4</td>
<td>99</td>
</tr>
<tr>
<td></td>
<td>25.5 (s)</td>
<td>77.0 (s)</td>
<td>160 (s)</td>
<td></td>
</tr>
</tbody>
</table>

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TAA may form enzyme-product complex as well as enzyme-substrate complex, and the observed protection are considered to be derived from the superimposed effects of substrate and its decomposition products. In order to find the difference between substrate and product effects, the substrate was digested before the heat and irradiation treatments. The degree of protection in the cases of zero and 20 min digestions is given in Table 3. In the heat inactivation, the protective action of products is smaller than that of substrate. (The substrate is completely hydrolysed by 20 min digestion.) Therefore, the stabilization of the secondary structure of enzyme protein by the formation of enzyme-product complex seems to be less effective for the protection from the heat inactivation than that by the formation of enzyme-substrate complex. However, for the visible inactivation (at 20 °C), we can not find the large difference between the value of degree of protection in the presence of substrate and that in the presence of product, showing that the effectiveness of substrate and product is nearly same for the protection from the photo-oxidation of enzyme. The degree of protection for ultraviolet inactivation (at 20 °C) shows that the screening of the exciting ultraviolet light by starch does not change measurably by the digestion treatment.

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**Table 3.** Effect of digestion of substrate on degree of protection. Concentration of starch, 0.6%. Digestion was made at 20 °C before inactivation treatments. Conditions are the same as for Fig 1 unless otherwise stated. * temperature of the inactivation treatment.

<table>
<thead>
<tr>
<th>Inactivation</th>
<th>Temperature * [°C]</th>
<th>Digestion time [min]</th>
<th>Degree of protection [1−R/R0]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Heat</td>
<td>60</td>
<td>0</td>
<td>0.68</td>
</tr>
<tr>
<td>Visible</td>
<td>20</td>
<td>0</td>
<td>0.89</td>
</tr>
<tr>
<td>Ultraviolet</td>
<td>20</td>
<td>0</td>
<td>0.56</td>
</tr>
</tbody>
</table>

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