Electrophoretic Demonstration of Specific Enzyme-Substrate Complex between Pepsin and Serum Albumin

II. INHIBITION OF COMPLEX FORMATION BY ACETYL-L-TRYPTOPHAN AND FATTY ACIDS†

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The first paper of this series (1) described an electrophoretic demonstration of a complex between pepsin and bovine serum albumin during the course of the enzymatic hydrolysis of the latter. Several independent lines of evidence were presented that this complex is the Michaelis-Menten complex capable of being activated to give rise to reaction products. This communication reports the results of electrophoretic experiments on inhibition of complex formation between pepsin and bovine serum albumin by acetyl-L-tryptophan and fatty acids. These experiments not only afford further evidence for the specificity of the electrophoretically demonstrable pepsin and bovine serum albumin complex but also have a bearing on the general concepts of proteolytic mechanisms.

EXPERIMENTAL PROCEDURE

The pepsin, Pentex 3 times crystallized Lot No. 2223, had an electrophoretic mobility of \(-9.04 \pm 0.13 \times 10^{-5}\) cm² volt⁻¹ sec⁻¹ in phosphate buffer, pH 5.32, ionic strength 0.1. It is curious that this value is somewhat smaller than the value of \(-9.42 \pm 0.05 \times 10^{-4}\) obtained in our previous experiments (1) with the same lot of pepsin. A second batch of Lot No. 2223 supplied by the Pentex Corporation during the course of the present investigation also had the lower mobility. In contrast, the two preparations of crystalline BSA, Pentex Lot No. 9F07 and Armour Lot No. V68802, gave the same values of mobility, within experimental error, in these experiments as in our previous ones. The human serum albumin was Merck Sharp and Dohme 25% solution of HSA¹ stabilized with 0.02 M sodium caprylate and 0.02 M sodium acetyltryptophanate.

In certain experiments, the BSA was treated with HCl before studying its interaction with pepsin. The prior treatment with HCl was carried out as follows: a 2% solution of the Armour BSA in 1 M HCl was incubated for 6 hours at 25°. The slurry of precipitated protein in its acid supernatant was then dialyzed in a rocking dialyzer for 4 hours at room temperature against hourly changes of phosphate buffer, pH 7.5, ionic strength 0.1, followed by 3 hours against hourly changes of phosphate buffer, pH 5.3, ionic strength 0.1, and finally by 11 hours of static dialysis at 3° against the latter buffer. The precipitate redisolved during dialysis against the pH 7.5 buffer, and the final solution was clear. Occasionally, the protein was dialyzed against water and then dried by lyophilization. In the case of prior treatment with urea, a 2% solution of Armour BSA in 8 M urea was incubated at 26° or 30° for 1 hour followed by removal of the urea by dialysis in much the same manner as in the case of the HCl-treated material. Since the electrophoretic pattern of urea-treated BSA changes slowly on storage of its solution at 3°, the standard procedure was adopted of treating the BSA with urea the day previous to studying its interaction with pepsin. The prior treatment of HSA with urea was as follows: an HSA solution previously dialyzed overnight against water was mixed in suitable proportion with an 8 M urea solution to give a 2% protein solution of 0.6 M urea which was incubated for 3 hours at 37° and then dialyzed in much the same manner as in the case of HCl-treated BSA.

Electrophoretic analyses of pepsin-albumin mixtures were carried out for 90 minutes at a field strength of 5 to 6 volts cm⁻¹ with the Perkin-Elmer Tiselius apparatus or the Spinco model-H diffusion-electrophoresis apparatus. The buffer solvent was sodium acetyltryptophanate. Protein concentrations were determined spectrophotometrically or by a combination of refractometric and spectrophotometric measurements. The procedure used for preparing and handling of the pepsin-albumin mixtures, assembling and filling the Tiselius cell, etc., were the same as described previously (1). The electric field was applied about 25 minutes after mixing the pepsin and albumin solutions. As shown previously, the rate of proteolysis under the experimental conditions is sufficiently slow so as to have a negligible effect on the electrophoretic patterns. Mobility values, cm² volt⁻¹ sec⁻¹ × (10⁵), and apparent values are shown above or beside the corresponding peaks in the patterns presented in the various figures.

As described in detail previously (1), the electrophoretic patterns of mixtures of pepsin and albumin are typical of interacting systems. The reaction boundaries in the patterns are designated by brackets. The mean mobility of the descending reaction boundary gives the constituent mobility of pepsin in the equilibrium mixture; the greater the extent of complex formation between pepsin and albumin, the lower the constituent mobility of pepsin as compared to the mobility of uncombined pepsin. Experience has shown that the relative area of the faster migrating peaks in the rising reaction boundary is a qualitative

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1 The abbreviations used are: HSA, human serum albumin; BSA, bovine serum albumin.
index of the extent of binding of albumin by pepsin. Quantitative information as to the extent of complex formation between pepsin and albumin was obtained from electrophoretic experiments carried out at a pepsin-albumin ratio sufficiently low to assure the predominance of a complex containing a single molecule each of pepsin and albumin (1). By application of the concepts of weak electrolyte moving boundary theory (2) the following expression can be derived for the dissociation constant, $K$, of the enzyme-substrate complex:

$$K = \frac{m^0 S m^0 E}{m_{ES}} = \frac{m^0 S - m^0 + m^0 E}{m_{ES} - m^0}$$

where $m^0$ and $m^0$ are the molar concentrations of uncombined enzyme, $E$, and uncombined substrate, $S$, in the equilibrium mixture, i.e. the $\alpha$ phase beneath the descending reaction boundary; $m_{ES}$, the concentration of the complex $ES$; $m^0$, the constituent concentration of enzyme; $m^0$, the constituent concentration of substrate; $u^0$ and $u^0$, the mobility values of uncombined $E$ and $S$. In the case of HCl-treated BSA which is electrophoretically heterogeneous, $u^0$ is taken as the mean mobility of the protein. Consequently, the computed value of $K$ is an average one. The concentration of $S$ in the $\beta$ phase separating the two descending boundaries, $m^0$, is obtained from the area of the slower moving boundary.

**RESULTS**

An important result of electrophoretic experiments on the formation of a complex between pepsin and serum albumin is that different preparations of BSA interact to different extents with pepsin. Thus, the two BSA preparations, Pentex BSA Lot No. 9F07 and Armour V68802, used for the experiments reported in this communication formed a complex to a much lesser extent with pepsin than the Pentex BSA Lot No. 1207 previously subjected to extensive investigation (1). The three preparations gave $K$ values of $6.5 \times 10^{-6}$ mole liter$^{-1}$, $18 \times 10^{-6}$, and $20 \times 10^{-6}$ respectively. Not only do these preparations differ in their $K$ values but also in their rates of peptic digestion: the rate of peptic formation of trichloroacetic acid-soluble material from Pentex BSA Lot No. 1207 was about 23 times greater than that with Armour V68802 under the conditions of the electrophoretic experiments. While exploring possible explanations of this difference in behavior it was found that acetyl-l-tryptophan inhibits complex formation. A 2% solution of Pentex BSA Lot No. 9F07 in $0.035 M$ acetyl-l-tryptophan, pH 4.5, was incubated for 3 hour at room temperature before dialysis for 20 hours against phosphate buffer, pH 5.3, ionic strength 0.1. A combination of refractometric and spectrophotometric analysis showed that the dialyzed solution contained 1.6 moles of acetyl-l-tryptophan per mole of BSA. The electrophoretic pattern of a mixture of this material with pepsin is compared with the control pattern obtained with untreated BSA in Fig. 1. Computations of $K$ values for the treated and untreated BSA are presented in Table I. Clearly, acetyl-l-tryptophan is an effective inhibitor of complex formation. Furthermore the inhibition is reversible. Within the error of the analytical method, dialysis of a solution

**Table I**

<table>
<thead>
<tr>
<th>Substrate†</th>
<th>$m^0 E$</th>
<th>$m^0 S$</th>
<th>$m^0 S + m^0 E$</th>
<th>$m^0 E$</th>
<th>$K$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pentex BSA Lot No. 9F07</td>
<td>1.36</td>
<td>0.21</td>
<td>1.80</td>
<td>0.768</td>
<td>7.27</td>
</tr>
<tr>
<td>Pentex BSA prevously treated with acetyl-l-tryptophan</td>
<td>1.37</td>
<td>0.21</td>
<td>1.80</td>
<td>0.837</td>
<td>7.76</td>
</tr>
<tr>
<td>Armour BSA Lot No. V68802</td>
<td>1.33</td>
<td>0.20</td>
<td>1.81</td>
<td>0.761</td>
<td>7.16</td>
</tr>
<tr>
<td>&quot;Fatty-acid free&quot; Armour BSA</td>
<td>1.33</td>
<td>0.20</td>
<td>1.51</td>
<td>0.440</td>
<td>6.43</td>
</tr>
<tr>
<td>Armour BSA previously treated with $1 M$ HCl</td>
<td>1.37</td>
<td>0.21</td>
<td>1.57</td>
<td>0.439</td>
<td>6.38</td>
</tr>
</tbody>
</table>

* Phosphate buffer, pH 5.32 ionic strength 0.1. Mobility of pepsin, $-9.04 \pm 0.13 \times 10^{-6}$ cm$^2$ volt$^{-1}$ sec$^{-1}$.† Mobility values of BSA: Pentex BSA Lot No. 9F07, $-2.94 \times 10^{-6}$; Pentex BSA previously treated with acetyl-l-tryptophan, $-2.95 \pm 0.02 \times 10^{-6}$; Armour BSA V68802, $-2.93 \pm 0.01 \times 10^{-6}$; "fatty-acid-free" Armour BSA, $-3.06 \pm 0.06 \times 10^{-6}$; Armour BSA previously treated with $1 M$ HCl, average value of $-2.63 \times 10^{-6}$. 

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Fig. 1. Inhibition of complex formation between pepsin and BSA by acetyl-l-tryptophan. Electrophoretic patterns of mixtures containing about 1% pepsin and about 1% of: A, Pentex BSA Lot No. 9F07; B, Pentex BSA previously treated by exposure to 0.035 M acetyl-l-tryptophan followed by 20 hours of dialysis against buffer. Apparent mobility values of two unlabeled peaks in rising reaction boundary of A: $-7.5 \times 10^{-6}$ cm$^2$ sec$^{-1}$ volt$^{-1}$ and $-6.2 \times 10^{-6}$. Mobility of pepsin, $-9.04 \times 10^{-6}$. Buffer solvent used in these experiments and those shown in the other figures was phosphate buffer, pH 5.32, ionic strength 0.1.
of BSA in 0.035 M acetyl-L-tryptophan against buffer for 7 days (on a rocking dialyzer at room temperature during the day and statically at 3° during the night) removed all the acetyl-L-tryptophan from the protein. A mixture of this material with pepsin gave the same electrophoretic pattern and the same value of \( \alpha_2 \) as the control experiment.

Complex formation is also inhibited by sodium caprylate. A 2% solution of Pentex BSA 9F07 in 0.02 M sodium caprylate containing a small amount of caprylic acid (about \( 10^{-3} \) M), pH 5.8, was incubated for \( \frac{1}{2} \) hour at room temperature before 24 hours of dialysis (6 hours at room temperature on rocking dialyzer with four changes of buffer and 18 hours of static dialysis at 3° with one change of dialysate). Electrophoretic analysis of a mixture of this material with pepsin revealed considerable inhibition of complex formation. The patterns were very similar to those shown in Fig. 2C; the value of \( \alpha_2 \) was \(-8.48 \times 10^{-4}\) cm\(^2\) volt\(^{-1}\) sec\(^{-1}\) as compared to the control value of \(-7.68 \times 10^{-5}\). In contrast to acetyl-L-tryptophan, inhibition by sodium caprylate is difficult to reverse. Thus, even after 7 days of dialysis, BSA, treated previously by exposure to sodium caprylate, gave a \( \alpha_2 \) value of \(-8.15 \times 10^{-4}\), which is still 0.5 of a mobility unit smaller than that of the control.

These results suggest that the differences in the extent of complex formation of the several preparations of native BSA with pepsin are related to possible differences in their fatty acid content (3, 4). Experiments with "fatty-acid-free" BSA prepared by the extraction method of Goodman (5) show that this is indeed the case. Comparison of the constituent mobility and the area sustained by the faster migrating peaks in the rising reaction boundary of a mixture of the "fatty-acid-free" BSA with pepsin, Fig. 2B, with those of the control experiment, Fig. 2A, reveals a striking enhancement of complex formation of BSA with pepsin upon removal of bound fatty acid from the albumin. Furthermore, replacement of the fatty acid by exposure of the "fatty-acid-free" BSA to 0.02 M sodium caprylate containing a small amount of caprylic acid reversed the effect (Fig. 2C). As shown in Table I, the value for the dissociation constant of the pepsin-"fatty-acid-free" BSA complex is only about \( \frac{1}{4} \) that of the pepsin-untreated BSA complex. Upon replacement of bound fatty acid to the "fatty-acid-free" BSA, \( \alpha_2 \) increased to a value even greater than that of the control (compare Figs. 2C and A).

Complex formation of BSA with pepsin is enhanced not only by solvent extraction of fatty acid from BSA but also by transitory exposure of the albumin to either \( 1 \) M HCl or \( 8 \) M urea. Furthermore, the effect of these agents is also reversed by sodium caprylate-caprylic acid. The electrophoretic patterns of mixtures of pepsin with untreated BSA or HCl-treated BSA are presented in Figs. 2A and 3A, respectively. As in the case of the "fatty-acid-free" BSA, the value of \( K \) for the complex formed between pepsin and HCl-treated BSA is only about \( \frac{1}{4} \) that of the pepsin-untreated BSA complex. As shown by Fig. 3D, exposure of the HCl-treated BSA to 0.02 M sodium caprylate containing a small amount of caprylic acid completely reversed the effect of the HCl treatment. In fact, the value of \( \alpha_2 \) increased from 7.8 to 8.8, the latter value being 0.5 of a mobility unit greater than in the control experiment. This observation indicates that the effect of HCl treatment is simply one of removal of bound fatty acids from the protein molecule and not irreversible denaturation. Indeed, Williams and Foster (6) have devised a procedure for removal of fatty acid from BSA by exposure to low pH.

The situation with urea-treated BSA is somewhat more complex. The electrophoretic pattern of this material showed two major boundaries in about 50-50 proportions. One of the boundaries migrated with about the same mobility as untreated BSA, the other with a slower mobility. As might be expected, complex formation of pepsin with the two components actually gave rise to a bimodal descending reaction boundary, Figs. 3B and C. Although it is apparent from inspection that pepsin forms a complex much more strongly with urea-treated BSA than with untreated BSA, the computation of the dissociation constant of the former complex is precluded by the fact that sedimentation measurements indicate that the effect of HCl treatment is simply one of removal of bound fatty acids from the protein molecule and not irreversible denaturation. Indeed, Williams and Foster (6) have devised a procedure for removal of fatty acid from BSA by exposure to low pH.

Similar results have been obtained with HSA. In agreement with the results of the sedimentation experiments of Foster, Baronowsky, and Rachinsky (7) on mixtures of pepsin and human mercaptalbumin, pepsin forms a complex very weakly with HSA. The value of \( \alpha_2 \) shown by a mixture containing 1% pepsin and 1% HSA was \(-8.47 \times 10^{-4}\) cm\(^2\) volt\(^{-1}\) sec\(^{-1}\). In contrast, urea-treated HSA gave \( \alpha_2 \) of \(-7.40 \times 10^{-5}\).
The effect of transient exposure of BSA to 1 M HCl or 8 M urea at 26° on its extent of complex formation with pepsin and reversal of the effect of HCl by sodium caprylate-caprylic acid. Electrophoretic patterns of mixtures of pepsin with: A, HCl-treated Armour BSA Lot No. V68802; B, urea-treated Armour BSA; C, urea-treated Armour BSA; D, HCl-treated Armour BSA exposed to 0.02 M sodium caprylate-caprylic acid followed by 24 hours of dialysis against buffer before mixing with the enzyme. Composition of mixtures: A, B, and D, about 1% pepsin-1% substrate; C, 0.473% pepsin-1.16% urea-treated BSA. See Fig. 2A for control patterns.

Neurath, Cooper, and Erickson (8) have studied the denaturation of serum albumin by 8 M urea at pH 5. These workers found that upon removal of the denaturing agent, the protein could be separated into two fractions differing from one another in solubility. The more soluble fraction, comprising about 85% of total protein, was characterized physically as "reversibly" denatured protein, whereas the more insoluble fraction was irreversibly denatured material.

**DISCUSSION**

The results of the experiments described above not only afford further evidence for the specificity of the pepsin-BSA complex demonstrable by electrophoresis but are also informative concerning the more detailed mechanism of peptic hydrolysis of BSA.

Linderström-Lang, Hotchkiss, and Johansen (9) originally proposed the hypothesis that proteolytic enzymes exert their action upon a denatured form of the substrate (D) rather than directly upon the native protein molecule (N) according to the scheme, $N \rightarrow D \rightarrow$ enzyme $\rightarrow$ products. In this context, denaturation refers to conformational changes which expose susceptible peptide bonds to the hydrolytic action of the given enzyme. It has been suggested (10, 11) that, under conditions in which native protein molecules do not transform spontaneously to reversibly denatured ones, the initial action of a protease might be nonhydrolytic induction of a conformational change in the substrate. Green and Neurath (12) have suggested that the enzyme may attack the N form as well as the D form of the substrate, the product of limited proteolysis of N being in equilibrium with a corresponding denatured form more susceptible to enzymatic action than is N.

Fig. 4. Reversal by sodium caprylate-caprylic acid of the effect of transient exposure of BSA to 8 M urea at 30° on its extent of complex formation with pepsin. Electrophoretic patterns of mixtures containing about 1% pepsin and 1% of: A, urea-treated BSA; B, urea-treated BSA exposed to 0.02 M sodium caprylate-caprylic acid followed by 24 hours of dialysis against buffer before mixing with enzyme. See Fig. 2A for control patterns.
Our observations are consistent with a Linderström-Lang mechanism for the initial phase of the peptic digestion of BSA. Thus, acetyltryptophan or sodium caprylate, both of which inhibit complex formation between pepsin and BSA, also stabilize serum albumin against denaturation by heat, urea, and guanidine salts (13-18) and against isomerization at pH 4 (19). This strongly suggests that pepsin forms complexes predominately, if not solely, with a D conformation of BSA which may or may not be in spontaneous equilibrium with the native conformation at pH 5.3. Binding of acetyltryptophan or fatty acids to BSA presumably inhibits complex formation with pepsin via stabilization of the albumin molecule in its native conformation.4

Recently, Schlamowitz and Peterson (20) have found that, whereas peptic digestion of untreated BSA is optimal at pH 1.7, digestion of BSA, after treatment with HCl, urea, or combinations of these agents, is optimal at pH 3.0 to 3.5. In the pH range 2.5 to 5.5, the proteolytic activity of pepsin on treated BSA is greater than on the untreated protein. In the experiments with urea, this agent was not removed from the substrate solution before addition of pepsin. However, experiments made in our laboratory have shown that the rate of peptic production of trichloroacetic acid-soluble material from BSA, exposed to 8 M urea followed by removal of the urea by dialysis, is about 60% greater than with untreated BSA at 0°C in phosphate buffer, pH 5.3, ionic strength 0.1. The observed enhancement of complex formation between pepsin and HCl- or urea-treated BSA as compared with the untreated protein is quite consistent with these results and affords still another line of evidence for the specificity of the electrophoretically demonstrable pepsin-BSA complex.

Schlamowitz and Peterson interpreted their above mentioned observations in terms of denaturation of BSA by HCl or urea. However, negation by sodium caprylate-caprylic acid of the enhancing effect of HCl or urea treatment of BSA on complex formation with pepsin indicates an alternative explanation. The very low optimal pH for peptic digestion of untreated BSA may be an apparent one resulting from enhanced enzyme-substrate complex formation under those conditions due to spontaneous release of bound fatty acids from the BSA molecule. When the fatty acids are removed from BSA by a transitory exposure of the protein to HCl or urea before the addition of pepsin, the activity-pH curve is shifted to a higher optimal pH determined solely by factors such as dissociation of groups on the enzyme, on the substrate, or on both, which require a particular state of ionization for substrate activation. The results of the electrophoretic studies reported herein should serve as a guide in designing experiments to test these ideas.

**SUMMARY**

Electrophoretic analyses of pepsin-albumin mixtures have revealed inhibition of complexing between pepsin and bovine serum albumin (BSA) by acetyl-L-tryptophan and fatty acids. Enhanced complex formation between pepsin and “fatty-acid-free” BSA and HCl- or urea-treated BSA is negated by exposure of the substrates to sodium caprylate-caprylic acid. These experiments, which afford further evidence for the specificity of the electrophoretically demonstrable pepsin-BSA complex, are interpreted within the framework of the Linderström-Lang mechanism of proteolysis.

**REFERENCES**

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