The First and Second Dissociation Constants of Subtilisin BPN'–Plasminostreptin Complex Determined by a Polarographic Method, and the Effects of pH on Them

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A polarographic method based on the Brdicka current (the polarographic catalytic hydrogen evolution current produced by proteins in the presence of cobalt salts) was applied to direct titration of subtilisin BPN' (S.BPN') with plasminostreptin (PS) at a concentration level of 10^{-8} M. The first and second dissociation constants of the S.BPN'–PS complex were determined by fitting theoretical curves to the titration data, in which the multiple equilibria involving microscopically distinct forms of S.BPN'–PS complex were taken into account. The intrinsic free energy change in the first binding of S.BPN' to dimeric PS was larger than that in the second binding. The dependence of the microscopic dissociation constants of S.BPN'–PS complex on pH indicates the participation of ionizable groups of pK_a 8.0 and 9.4 in the complex formation. The repulsive effect between negatively charged molecules of S.BPN' and PS in the complex formation at elevated pH is suggested.

Plasminostreptin (PS)¹⁾ is a protein proteinase inhibitor of microbial origin, which inhibits plasmin [EC 3.4.21.7], trypsin [EC 3.4.21.4], subtilisin BPN' (S.BPN') [EC 3.4.21.14], and other microbial alkaline proteinases. The amino acid sequence of PS is identical with that of *Streptomyces* subtilisin inhibitor (SSI) at about 70% of the positions.²⁾ PS as well as SSI consists of two identical subunits and is able to bind one molecule of proteinase per subunit of the inhibitor to form enzyme–inhibitor complexes, for which an accurate determination of the dissociation constants has been the subject of intensive studies.^{3,4)} In previous papers,⁵⁾ we have shown that the polarographic method based on the Brdicka current can be successfully applied to direct titration of S.BPN' with SSI

or PS at the concentration level of 10^{-7} to 10^{-10} M, and determined the first and second dissociation constants of dimeric S.BPN'–SSI complex. In this study, we have determined the microscopic dissociation constants of S.BPN'–PS complex using a fast polarographic method, and investigated the effects of pH on the microscopic dissociation constants.

MATERIALS AND METHODS

Materials. Plasminostreptin (PS) was donated by Dr. A. Kakinuma of Takeda Chemical Industries, Ltd. and subtilisin BPN' (S.BPN') was commercially obtained (Sigma Chemical Co., lot No. 89C-0528), and both were used as received. The purity of the enzyme preparation was determined to be 75.0% according to the method of Inouye *et al.*⁶⁾ All other chemicals were of reagent grade quality.

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Apparatus. Polarograms were recorded in a fast d.c. mode with a Yanagimoto P-1000 voltammetric analyzer, equipped with a Watanabe WX-4401 X-Y recorder. Two dropping mercury electrodes (DME) were used. Their characteristics were $m = 0.852 \text{ mg s}^{-1}$ and $t = 11.08 \text{ s}$ at $h = 50.0 \text{ cmHg}$ for DME I and $m = 1.471 \text{ mg s}^{-1}$ and $t = 4.25 \text{ s}$ at $h = 48.3 \text{ cmHg}$ for DME II, both at -1.5 V in ammoniacal buffer.

Electrochemical measurements. As the base solutions, 0.2 M Tris-HCl buffer (pH 7.6~8.5), and 0.2 M $\text{NH}_3\text{-NH}_4\text{Cl}$ buffer (pH 8.8~10.2), both containing $2 \times 10^{-4} \text{ M}$ $\text{Co}(\text{NH}_3)_6\text{Cl}_3$, were used. The ionic strength of the base solution was adjusted to 0.2 M with KCl. The Brdička current was measured from the limiting current of cobalt reduction. Amperometric titration was performed at a fixed potential, usually at -1.35 V . All potentials were measured against a saturated calomel electrode (SCE). Other details of electrochemical measurements have been described in a previous paper.^{5b)}

RESULTS AND DISCUSSION

Brdička current of PS and S.BPN'-PS complex

In d.c. polarography, PS gave a well defined Brdička wave (Fig. 1, curve 2). The Brdička current, usually measured at -1.35 V , increased linearly with the concentration of PS up to $8.0 \times 10^{-7} \text{ M}$ at pH 8.0. S.BPN' also gave a small wave, but its wave height was very much smaller than that of PS (Fig. 1, curve 1). When S.BPN' was added to a PS solution, the Brdička wave decreased in height as shown, for example, by curve 3 in Fig. 1. It has been shown⁷⁾ that generally the adsorption of protein on a mercury electrode surface is strong and the adsorption process is controlled by diffusion at the potential more positive than -1.5 V . Under the conditions of diffusion-controlled adsorption, the Brdička current increases linearly with the bulk concentration of protein and the Brdička current, i_B , produced by a mixture of proteins is given by the sum of the Brdička currents, $(i_B)_j$, produced by each protein, j .

$$i_B = \sum (i_B)_j = \sum \kappa_j [j] \quad (1)$$

where κ_j is the proportionality constant converting the concentration of the protein j , $[j]$, to its Brdička current $(i_B)_j$. Accordingly, the

FIG. 1. Polarographic Tast Waves (with DME I) of (1) $2.0 \times 10^{-7} \text{ M}$ S. BPN', (2) $1.0 \times 10^{-7} \text{ M}$ Dimeric PS and (3) $2.0 \times 10^{-7} \text{ M}$ S. BPN' + $1.0 \times 10^{-7} \text{ M}$ Dimeric PS in the Base Solution of pH 8.0.

The current was measured from the cobalt limiting current of the base solution. The broken line (curve 4) represents the $\Delta i_B([E]_0, [I_2]_0)$ vs. potential curve for the mixture of $2.0 \times 10^{-7} \text{ M}$ S. BPN' and $1.0 \times 10^{-7} \text{ M}$ dimeric PS.

decrease of the Brdička wave height of PS with the addition of S.BPN' should be attributed to the formation of a complex of PS with S.BPN', with the κ value of the complex being much smaller than κ_{PS} .^{5a)}

Curve 4 in Fig. 1 represents a difference Brdička current, $\Delta i_B([E]_0, [I_2]_0)$, defined by Eq. (2);

$$\Delta i_B([E]_0, [I_2]_0) = i_B([E]_0) + i_B([I_2]_0) - i_B([E]_0 + [I_2]_0) \quad (2)$$

where $i_B([E]_0)$, $i_B([I_2]_0)$, and $i_B([E]_0 + [I_2]_0)$ are the Brdička currents produced by the enzyme E (S.BPN' in our case) at the concentration of $[E]_0$, the dimeric inhibitor I_2 (dimeric PS in our case) at the concentration of $[I_2]_0$, and their mixture at the concentrations of $[E]_0$ and $[I_2]_0$, respectively. We further define the relative difference Brdička current, $\Delta I_B([E]_0, [I_2]_0)$, by

$$\Delta I_B([E]_0, [I_2]_0) = \Delta i_B([E]_0, [I_2]_0) / i_B([I_2]_{\text{equiv}}) \quad (3)$$

where $i_B([I_2]_{\text{equiv}})$ is the Brdička current produced by the inhibitor at the concentration of $[I_2]_{\text{equiv}}$ which is equal to $[E]_0/2$. We titrated $2.86 \times 10^{-7} \text{ M}$ S.BPN' with PS in a Tris buffer of pH 8.5 amperometrically by measuring the Brdička current heights at -1.35 V . The titration curve is shown in Fig. 2, where $\Delta I_B([E]_0, [I_2]_0)$ is plotted against $2[I_2]_0/[E]_0$, $[I_2]_0$ being

FIG. 2. Polarographic Titration Curve ($\Delta I_B([E]_0, [I_2]_0)$ vs. $2[I_2]_0/[E]_0$ plot) of S.BPN' with PS at $[E]_0 = 2.86 \times 10^{-7}$ M and at pH 8.5, $I = 0.2$ M and 25°C .

The currents were measured at -1.35 V with DME I.

the amount in M of dimeric PS added and $[E]_0 = 2.86 \times 10^{-7}$ M. The $\Delta I_B([E]_0, [I_2]_0)$ value initially increased linear with $2[I_2]_0/[E]_0$ and approached a certain maximum value, $\Delta I_B([E]_0, [I_2]_0)^{\text{max}}$, with an inflection point at $2[I_2]_0/[E] = 1.0$, indicating that the complex formation between S.BPN' and PS proceeds according to

Here it is assumed that the complex is present as a dimeric S.BPN'-PS complex in analogy with the formation of S.BPN'-SSI complex,^{5b)} though Kakinuma *et al.*¹⁾ have reported that a monomeric trypsin-PS complex is also formed under certain conditions in Tris buffer of pH 7.5.

Determination of the dissociation constants of S.BPN'-PS complex

Titration of S.BPN' with PS was also carried out at $[E]_0 = 4.56 \times 10^{-8}$ M in Tris buffer of pH 8.5. A representative $\Delta I_B([E]_0, [I_2]_0)$ vs. $2[I_2]_0/[E]_0$ plot is shown in Fig. 3. The curve changes gradually near the equivalent point ($2[I_2]_0/[E]_0 = 1$), indicating that the dissociation of the S.BPN'-PS complex is not negligible at such a low concentration of proteins.

For the dimeric S.BPN'-PS complex, we consider that a bifunctional dimeric PS, I_2 successively binds two molecules of S.BPN', E,

FIG. 3. Polarographic Titration Curve ($\Delta I_B([E]_0, [I_2]_0)$ vs. $2[I_2]_0/[E]_0$ plot) of S.BPN' with PS at $[E]_0 = 4.56 \times 10^{-8}$ M and at pH 8.5, $I = 0.2$ M and 25°C .

The currents were measured at -1.35 V with DME I. The solid line is the theoretical curve calculated on values of $K_1' = 6.7 \times 10^{-9}$ M, $K_2' = 1.2 \times 10^{-8}$ M and $\Delta\kappa = 0.55$ according to Eqs. (6) and (8).

to form enzyme-inhibitor complexes, according to the following scheme⁵⁾:

where K_1' and K_2' are the microscopic dissociation constants.⁸⁾ Then, as described in a previous paper,^{5b)} $\Delta I_B([E]_0, [I_2]_0)$ for given analytical concentrations of E and I_2 , $[E]_0$ and $[I_2]_0$, respectively, is expressed by

$$\Delta I_B([E]_0, [I_2]_0) = \Delta\kappa([E]_0 - [E])/[E]_0 \quad (6)$$

with

$$\Delta\kappa = (2\kappa_E + \kappa_{I_2} - \kappa_{EI_2E})/\kappa_{I_2} \quad (7)$$

Here κ_E , κ_{I_2} and κ_{EI_2E} are the proportionality constants converting the protein concentrations to the Brdicka current intensities for E, I_2 and EI_2E , respectively (see Eq. (1)). The proportionality constants for EI_2 and I_2E , κ_{EI_2} and κ_{I_2E} , are assumed to be $\kappa_{EI_2} = \kappa_{I_2E} = (\kappa_{I_2} + \kappa_{EI_2E})/2$. $[E]$ is the concentration of free enzyme and is given by the solution of Eq. (8);

$$\begin{aligned} & [E]^3 + (2[I_2]_0 - [E]_0 + 2K_2')[E]^2 \\ & + K_2'(2[I_2]_0 - 2[E]_0 + K_1')[E] \\ & - K_1'K_2'[E]_0 = 0 \end{aligned} \quad (8)$$

We determined $\Delta I_B([E]_0, [I_2]_0)$ for different $[I_2]_0$'s ($0.37 \sim 5.22 \times 10^{-8}$ M) for a constant $[E]_0$ (4.56×10^{-8} M) at pH 8.5 and fitted the $\Delta I_B([E]_0, [I_2]_0)$ vs. $2[I_2]_0/[E]_0$ plot (Fig. 3) to the theoretical curve represented by Eqs. (6) and (8) by adjusting the three parameters K_1' , K_2' and $\Delta\kappa$ using Facom M-200 computers in the Data Processing Centers of Nagoya University and Kyoto University. The results of non-linear least-squares analysis using the SALS program⁹⁾ were $K_1' = (6.7 \pm 4.8) \times 10^{-9}$ M, $K_2' = (1.2 \pm 0.7) \times 10^{-8}$ M and $\Delta\kappa = 0.55 \pm 0.11$. The solid line in Fig. 3 is a regression curve calculated on values of $K_1' = 6.7 \times 10^{-9}$ M, $K_2' = 1.2 \times 10^{-8}$ M and $\Delta\kappa = 0.55$.

Equation (6) shows that $\Delta I_B([E]_0, [I_2]_0)$ approaches $\Delta\kappa$ at $[E]/[E]_0 \rightarrow 0$. On the other hand, $\Delta I_B([E]_0, [I_2]_0)$ approaches a maximum value, $\Delta I_B([E]_0, [I_2]_0)^{\max}$, as $2[I_2]_0/[E]_0$ exceeds the equivalent point greatly (see Figs. 2 and 3). Since $[E]/[E]_0 \ll 0$ when $2[I_2]_0/[E]_0 \gg 1$, $\Delta\kappa$ must be equal to $\Delta I_B([E]_0, [I_2]_0)^{\max}$. The agreement between the adjusted $\Delta\kappa$ value of 0.55 and the $\Delta I_B([E]_0, [I_2]_0)^{\max}$ value of 0.55 estimated from the titration curve in Fig. 2 is excellent.

Effects of pH on the dissociation constants of S.BPN'-PS complex

The microscopic dissociation constants of S.BPN'-PS complex were determined in the way described above in buffer solutions of pH 7.6 to 10.2. The results are shown in Fig. 4. Let us consider that the enzyme and the complexes exist in two states of ionization as follows:

for the first step of complex formation and

for the second. In this case, $K_{a,1}^\alpha$ and $K_{a,1}^\beta$

FIG. 4. Effects of pH on pK_1' ($= -\log K_1'$) (●) and pK_2' ($= -\log K_2'$) (○) of S.BPN'-PS Complex.

The vertical bars indicate the standard deviation. The solid lines are the theoretical curves calculated on values of $pK_1' = 8.96$, $pK_{a,1}^\alpha = 7.81$ and $pK_{a,1}^\beta = 9.34$ according to Eq. (13) for the upper curve, and $pK_2' = 8.34$, $pK_{a,2}^\alpha = 8.08$ and $pK_{a,2}^\beta = 9.42$ according to Eq. (14) for the lower curve. The broken lines represent guide lines of Dixon plots.

should be equal. Alternatively, we may consider that the inhibitor and the complexes exist in two states of ionization as follows,

for the first step of complex formation and

for the second. Here the subscripts n , n' , m and m' represent the number of ionizable protons in the protein species and $K_{a,1}^\alpha$, $K_{a,1}^\beta$, $K_{a,2}^\alpha$ and $K_{a,2}^\beta$ are the acid dissociation constants of the respective ionizable groups. These two schemes (9, 10) and (11, 12) result in the same pH-dependence of the first and second apparent dissociation constants, pK_1' and pK_2' , respectively, as given by

$$pK_1' = p\bar{K}_1' + \log(1 + 10^{pH - pK_{a,1}^\alpha}) - \log(1 + 10^{pH - pK_{a,1}^\beta}) \quad (13)$$

$$pK_2' = p\bar{K}_2' + \log(1 + 10^{pH - pK_{a,2}^\alpha}) - \log(1 + 10^{pH - pK_{a,2}^\beta}) \quad (14)$$

We fitted the curve represented by Eqs. (13) and (14) to the experimental plots of pK_1' and pK_2' vs. pH by adjusting two sets of three parameters $p\bar{K}_1'$, $pK_{a,1}^\alpha$, and $pK_{a,1}^\beta$, and $p\bar{K}_2'$, $pK_{a,2}^\alpha$, and $pK_{a,2}^\beta$, respectively. The results of the non-linear least-squares analysis using the SALS program⁹⁾ were $p\bar{K}_1' = 8.96 \pm 0.16$, $pK_{a,1}^\alpha = 7.81 \pm 0.22$, and $pK_{a,1}^\beta = 9.34 \pm 0.18$ for the first binding step (the upper curve), and $p\bar{K}_2' = 8.43 \pm 0.15$, $pK_{a,2}^\alpha = 8.08 \pm 0.24$, and $pK_{a,2}^\beta = 9.42 \pm 0.23$ for the second binding step (the lower curve). The broken lines are so-called guide lines of Dixon plots,¹⁰⁾ with two inflection points at $pK_{a,1}^\alpha = 7.81$ and $pK_{a,1}^\beta = 9.34$, and $pK_{a,2}^\alpha = 8.08$ and $pK_{a,2}^\beta = 9.42$, respectively, for the upper and lower curves.

As seen in Fig. 4, pK_1' and pK_2' change with pH, but the difference between them is nearly constant, independently of pH; the mean difference $p\bar{K}_1' - p\bar{K}_2' = 0.53$ implies that the free energy change of the first binding of the enzyme to the inhibitor is 3 kJ mol^{-1} larger than that of the second binding. A similar result has been obtained with S.BPN'-SSI complex with the difference of 7.1 kJ mol^{-1} .^{5b)}

$pK_{a,1}^\alpha$ and $pK_{a,1}^\beta$ coincide with $pK_{a,2}^\alpha$ and $pK_{a,2}^\beta$, respectively, within experimental error. This result can reasonably be explained by the above schemes, when we consider that in both the first and second bindings of S.BPN' to PS dimer the same ionizable group α with pK_a^α of about 8.0 and the same ionizable group β with pK_a^β of 9.4 are relevant to the ionization of the free enzyme (or inhibitor) and bound enzyme (or inhibitor), respectively. If we further assume that the group α and the group β are an identical ionizable group, the above result indicates that pK_a^α of this ionizable group of S.BPN' (or PS) in the free ($pK_a^\alpha \approx 8.0$) is increased by 1.4 unit ($pK_a^\beta \approx 9.4$) by the complex formation between S.BPN' and PS. Note

that according to Inouye *et al.*¹¹⁾ pK_a of a tyrosine in S.BPN' is shifted from 9.7 to 11.5 by the complex formation between S.BPN' and SSI. However, assignment of this ionizable group to a specific group is difficult. Among the candidates for this ionizable group, tyrosine ($pK_a = 9.5 \sim 10.5$)¹²⁾ and lysine ($pK_a = 9.5 \sim 10.5$)¹²⁾ have much higher pK_a values, while histidine ($pK_a = 5.0 \sim 7.4$)^{12,13)} has a much lower pK_a value than the observed value of pK_a^α . Uehara *et al.*³⁾ have reported the involvement of an ionizable group of pK_a 8.5 in the association of S.BPN' and dimeric SSI, though these authors did not observe the second inflection point.

Another possible interpretation of the pH-dependence of these dissociation constants is as follows. The isoelectric point (pI) of S.BPN' is 7.8,¹⁴⁾ which is close to the value of pK_a^α . PS is always negatively charged in the pH range studied, since the pI of PS is 6.3.²⁾ Therefore the repulsion between the net negative charges of S.BPN' and PS may be a possible cause of the decreased affinity between them at elevated pH.

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