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Protonation and Dimerization Equilibria of the Basic Tripsin Inhibitor (Kunitz Base)

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Introduction

The basic pancreatic trypsin inhibitor (kallicrein inactivator) polypeptide consists of 58 amino acids and contains in all 18 different amino acids. Its amino acid sequence has been known for some time [1–3] and by X-ray diffraction studies also its atomic structure has been determined [4–6]. These latter investigations also show the intramolecular hydrogen bridges in the crystalline peptide. A great part of these H-bonds are formed between the main chain peptide nitrogens and the corresponding peptide oxygens, rather few H-bridges connect main chain peptide nitrogens or oxygens with side chain donor groups. Only three of these latter type of H-bonds contain donor groups with protonation constants low enough to be studied by pH-metric equilibrium measurements in aqueous solutions. These intramolecular H-bridges are:

Tyr(10)-OH...Val(34)-CO,
Tyr(35)-OH...Cys(38)-NH and
Arg(1)-NH...Cys(55)-CO

bridges with bond distances 0.3, 0.3 and 0.28 nm respectively.

The X-ray investigations did not reflect the effect of intermolecular hydrogen bridges in this system. Molecular weight determinations have shown however [7] that BPTI dimerizes in solutions. The mechanism proposed for the dimerization process is, however, not convincing. Anderer and Hörnle [7] assumed the presence of low molecular weight components connecting the two macromolecular BPTI peptides in the dimer. The pH-dependence of the dimerization suggests, however, that this process is due to the formation of hydrogen bridges connecting the BPTI peptides.

Previous investigations have shown [8] that hydrogen-bond formation changes the dissociation constants of the protons involved in this bond, and of protons affected in other ways by the formation of the H-bridges.

If two protonated donor atoms (*e.g.* A and B) are so arranged on a macromolecule that after dissociation of the proton of the less basic one (A) the two donor atoms are connected by the proton of the originally more basic donor atom (B):

$$H\text{–}A + H\text{–}B \rightleftharpoons H^{+} + A\cdots H\text{–}B$$

then due to the formation of this hydrogen bond, dissociation of the A–H group is favoured, and that of B–H hindered, thus the protonation constant of A becomes smaller and that of B larger than they would be in the absence of the hydrogen bridge. In this way both, intra- and intermolecular H-bridge formation influences the protonation constants of the donor atoms participating in these bonds. It seemed probable therefore that the pH-metric equilibrium study of the protonation equilibria of BPTI would reflect the effect of both types of H-bonds. On the basis of this type of investigations not only could the exact pH range of the dimerization process be determined but the assignment of the functional groups taking part in the dimer formation also could be attempted.

For this purpose a protonation study of BPTI in aqueous solutions using the precision pH-metric method outlined in our previous paper [8] was performed. The results are presented in the following.

Experimental

The experimental procedure worked out for the equilibrium study of the protonation of corticotropin fragments [8] was used for the investigations.

The solutions to be examined have to be adjusted to a constant ionic strength high enough (0.3 *M* KNO_3) to allow for the neglect of the contribution of the polypeptide content. The peptide concentration was kept constant in each series of measurements. The peptide was protonated with a known quantity of acid. The emf change caused by the pH increase of the solution during titration with a standard NaOH solution was measured.

In the knowledge of the total peptide content (5×10^{-5}–10^{-3} *M* dm^{-3}) of the solutions, the quantities of acid added, the quantities of alkali consumed and the experimental mV values could be used to calculate the equilibrium constants describing the system. A computer evaluation procedure [9] served this purpose. The essence of this was that the experimental mV *vs.* ml sodium hydroxide standard solution curves are simulated on the basis of models assuming the stepwise formation of proton complexes of various compositions. The good fit of the experimental points to the calculated curve was accepted as a proof of the correctness of the equilibrium constants derived by this method (Fig. 1).

TABLE I. The Protonation Constants of BPTI.

Proton:Peptide Ratio	Successive Protonation Constants		Assigned Functional Groups
2:1	$lgK_{1,1}K_{2,1}$	22.28	Phenolic OH
4:1	$lgK_{3,1}K_{4,1}$	21.16	Phenolic OH
6:1	$lgK_{5,1}K_{6,1}$	19.88	ϵ-NH_2
7:1	$lgK_{7,1}$	9.47	ϵ-NH_2
8:1	$lgK_{8,1}$	8.97	ϵ-NH_2
9:1	$lgK_{9,1}$	7.90	terminal NH_2
19:2	lgK[a]	8.27	Dimer formation[a]
20:2	$lgK_{20,2}$	4.36	-COOH
22:2	$lgK_{21,2}K_{22,2}$	8.71	-COOH
24:2	$lgK_{23,2}K_{24,2}$	7.41	-COOH
27:2	$lgK_{25,2}K_{26,2}K_{27,2}$	9.94	-COOH

Standard deviation: 1.88 mV.

[a] $K = \dfrac{[H_{19}P_2]}{[H_9P]\,[H_{10}P]}$.

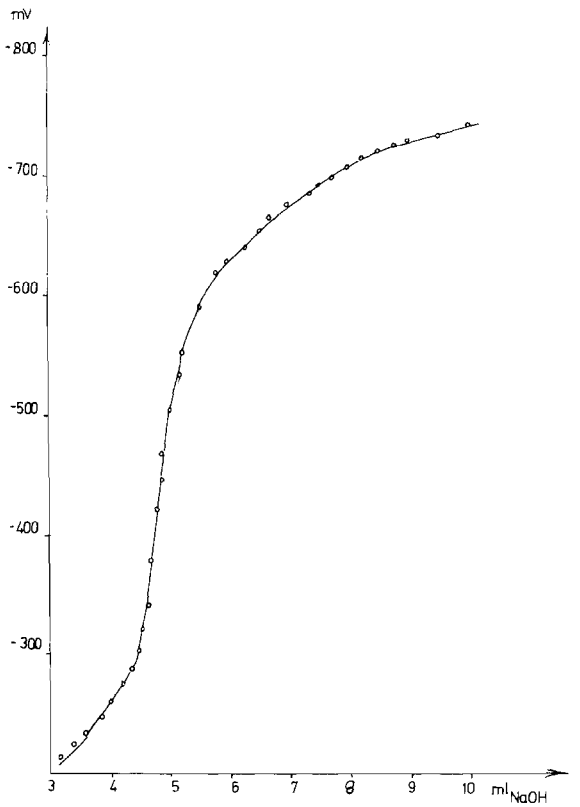

Fig. 1. Potentiometric titration curve of BPTI. Total peptide concentration: 1.003×10^{-3} *M* dm^{-3}, ionic strength 0.3 *M* made up with KNO_3. The circles represent the experimental points, the full line the calculated curve.

Discussion

The computer treatment of experimental data has shown that the system contains differently protonated monomeric and dimeric species. The composition of the protonated species (bound proton: peptide ratios), the protonation equilibrium constants and the dimer formation constant are shown in Table I. In the knowledge of the total peptide concentration and pH of the solutions the concentration of each species can be calculated with the help of these constants. The results of such a calculation are shown in Fig. 2. The curves show the concentration distribution of the different species in solution of different pH. It is also to be seen that starting from the completely protonated monomer the pH increase resulting in the dissociation of the first carboxylic acid proton is accompanied by formation of the dimer. A further pH increase results first in the successive deprotonation of the dimer, later at about pH 7 when the last protonated carboxyl group releases its proton the dimer dissociates and the successive deprotonation of the monomer peptide sets in. Thus, it became clear that the deprotonation of the terminal carboxyl group and the protonation of all other carboxyls and that of the amino groups are essential for the formation of the BPTI dimer in aqueous solution. It is however impossible to determine on the basis of only equilibrium measurements the exact location of the hydrogen bridges in the dimer.

The carboxylate oxygen can be bound by H-bonding to any protonated group which has a basicity high enough to prevent its deprotonation in the pH range investigated. Such functional groups could be the protonated primary amino groups, arginin guanidino groups and any peptide NH group. Similarly the protonated carboxyl and amino groups can form H-bridges not only with the carboxylate but with any donor atom having such a low basicity that

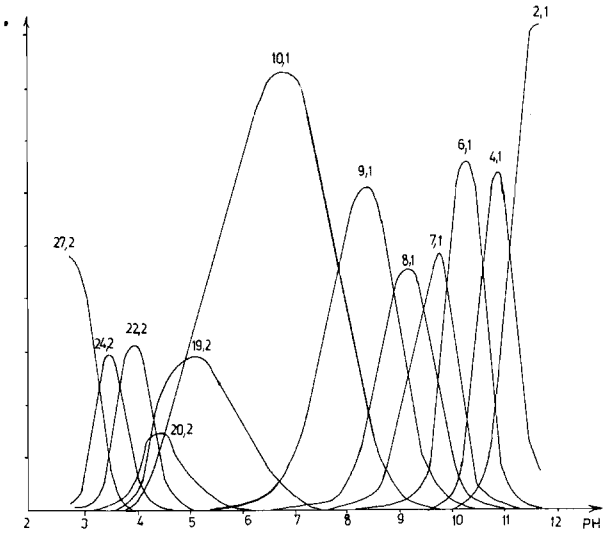

Fig. 2. The distribution of the BPTI species of different protonation as a function of the pH of the solution.

formation of intermolecular hydrogen bridges and the pH-dependence of this process is determined by the protonation–deprotonation equilibria of the peptide. it remains unprotonated in the pH range of the investigations. Such groups could be *e.g.* the peptide oxygens.

These investigations have proved however unambiguously that the dimerization of BPTI is due to the

The results of these investigations may serve also as the basis for a further study of the metal complex formation equilibria of BPTI.

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