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The *p*H Dependence of the Equilibrium Constant K_{Hyd} for the Hydrolysis of the Lys[15]–Ala[16] Reactive-Site Peptide Bond in Bovine Pancreatic Trypsin Inhibitor (Aprotinin)

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The pH dependence of the equilibrium constant K_{Hyd} for the hydrolysis of the Lys[15]-Ala[16] reactive-site peptide bond of the bovine pancreatic trypsin inhibitor (aprotinin) was investigated over the pH range 2.3-6.5. Solutions of aprotinin, modified aprotinin with the Lys[15]-Ala[16] peptide bond cleaved and mixtures of both species were incubated with 10 mol% porcine β-trypsin. The state of equilibrium was determined by analytical cation-exchange HPLC. The K_{Hyd} values obtained did not exactly obey the simple equation of Dobry et al. (1952), which had to be used in an extended form with two additional parameters for a satisfactory fit. The pH-independent equilibrium constant is 0.90 and the pK values of the Lys[15] carboxyl group and of the Ala[16] amino group are 3.10 and 8.22, respectively. The pK of an additional group is apparently perturbed by the peptide-bond hydrolysis. It is 4.60 in the native and 4.40 in the modified aprotinin.

KEY WORDS: proteinase inhibitor; aprotinin; reactive-site peptide bond hydrolysis; equilibrium constant.

1. INTRODUCTION

The incubation of proteinase inhibitors with catalytic amounts of proteinases, which have a mutual affinity, leads to hydrolytic cleavage of the reactive-site peptide bond in the inhibitor. This conversion of the native inhibitor I to the modified inhibitor I^*, with the reactive-site peptide bond hydrolyzed, can be characterized by the equilibrium constant K_{Hyd}:

$$K_{Hyd} = (I^*)/(I)$$

The existence of an equilibrium between I and I^* has been demonstrated for several inhibitors, such as the trypsin inhibitor from soybeans (Niekamp *et al.*, 1969;

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Mattis and Laskowski, 1973), the secretory Kazal inhibitor from bovine pancreas (Sealock and Laskowski, 1973), the third domains of various ovomucoids (Ardelt and Laskowski, 1983; Kato *et al.*, 1987), and the bovine pancreatic trypsin inhibitor or aprotinin (Tschesche and Kupfer, 1976; Estell *et al.*, 1980). A minimal overall mechanism for the interaction of a proteinase with a proteinase inhibitor has been established (Luthy *et al.*, 1973):

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} L \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} C \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} L^* \underset{k_{-4}}{\overset{k_4}{\rightleftharpoons}} E + I^*$$

where E is the proteinase, I and I^* are the native and modified inhibitor respectively, C is the stable enzyme inhibitor complex, and L and L^* are loose noncovalent complexes of I and I^* with E.

The equilibrium constant K_{Hyd} does not depend on the enzyme used to catalyze the conversion; it only depends on the inhibitor and the pH of the solution (Finkenstadt *et al.*, 1974). Our particular interest was to investigate the pH dependence of K_{Hyd} of aprotinin. Measurements have already been carried out for this inhibitor by Estell *et al.* (1980) over a pH range of 6.2–8.9, using trypsin 1 from the starfish *Dermasterias imbricata* (Estell and Laskowski, 1980). This enzyme can rapidly hydrolyze the reactive-site Lys^{15}-Ala^{16} peptide bond. Thus equilibrium was reached at pH 8.2 within about 4 days. Measurements at lower pH values could not, however, be performed due to the instability of the starfish enzyme.

In a previous paper (Tschesche and Kupfer, 1976), K_{Hyd} was estimated at pH 5.0 with plasmin using enzymatic methods for the calculation of the ratio $(I^*)/(I)$. A considerable difficulty was the long incubation time; here 300 days were needed to attain equilibrium. This is a possible source of some systematic errors. Thus we used an improved technique, described in this paper, to determine K_{Hyd} in dependence on the pH over the range of 2.3–6.5.

2. MATERIALS AND METHODS

2.1. Materials

Native aprotinin was kindly provided by Bayer AG, D-5600 Wuppertal. Porcine β-trypsin was purchased from Merck, D-6100 Darmstadt, and plasmin from porcine blood was obtained from Sigma, D-8640 Deisenhofen.

2.2. Methods

2.2.1. Preparation of Modified Aprotinin

Modified aprotinin with the reactive-site Lys^{15}-Ala^{16} peptide bond hydrolyzed was prepared by the method of Jering and Tschesche (1976). The disulfide bridge Cys^{14}-Cys^{38} of the native inhibitor was selectively reduced with sodium borohydride, and the Lys^{15}-Ala^{16} peptide bond split with porcine trypsin. After reoxidation of the reduced disulfide bridge with oxygen, the modified inhibitor could be isolated by subsequent ion-exchange chromatography on CM-Sephadex C-25.

2.2.2. Preparation of the Incubation Mixtures

All incubations were carried out at room temperature, using 10 mol% porcine β-trypsin, and in some cases plasmin from porcine blood in 0.05 M buffers containing 0.02 M $CaCl_2$ and 0.02% NaN_3. Over the *p*H range 2.3–3.9, sodium citrate–HCl was used as the buffer system, over the range 4.0–6.1 succinic acid–NaOH, and at *p*H 6.5 triethanolamine–HCl. All buffers were filtered using a sterilizing Millipore Millex-GS filter of mesh size 0.22 μm. At the end of incubation, all *p*H values were controlled using a microelectrode.

In preparing the incubation mixtures, solutions of native and modified aprotinin (1.54×10^{-4} M = 1 mg/ml for the reaction with trypsin and 3.07×10^{-5} M = 0.2 mg/ml for the reaction with plasmin) in the appropriate buffers were pipetted directly to a weight quantity of the lyophilized enzyme. Mixtures ($\alpha = 0.5$ and 0.75) of the native and modified inhibitor could be also prepared with the solutions so obtained. All the reactions were carried out, using a total volume of 4 ml, in closed-glass 5-ml vessels.

2.2.3. HPLC Analyses

A Hewlett-Packard liquid chromatograph 1084 A equipped with an LC terminal 79850 A, variable wavelength UV detector 1030 B, and an automatic sample transport system 79842 A were used for all HPLC separations. Volumes of 20 μl for all incubations with trypsin and 50 μl for incubations with plasmin were analyzed directly by high-performance liquid chromatography (HPLC) using a cation-exchange system (Wenzel *et al.*, 1985).

Chromatographic conditions: column LKB Ultropac TSK SP-5 PW, 7.5× 75 mm. Solvent A: 0.01 M phosphate buffer, *p*H 7.0; solvent B: 1 M NaCl in 0.01 M phosphate buffer, *p*H 7.0. Elution: 10% B for 5 min, then linear gradient from 10% B to 100% B over 45 min. Flow rate 1 ml/min, oven temperature 30°C, detection at 220 nm. All samples for HPLC analysis were taken from the incubation mixtures under sterile conditions (laminar flow).

3. RESULTS AND DISCUSSION

3.1. Determination of the Equilibrium Constant K_{Hyd}

The equilibrium constant K_{Hyd} was investigated over the *p*H range 2.3–6.5. For this purpose, 1.54×10^{-4} M solutions (1 mg/ml buffer) were incubated with 10 mol% porcine trypsin. Because long incubation times were to be expected, not only native and modified aprotinin were used but also mixtures of both species. Thus, the time required to reach equilibrium could be shortened.

The conversion reactions $I \rightarrow I^*$ and $I^* \rightarrow I$ were followed by the use of analytical cation-exchange HPLC. Volumes of 20 μl were analyzed directly by HPLC. Free aprotinin and aprotinin* of the solutions could be clearly separated (Fig. 1). The absorbance was monitored at 220 nm and the peaks automatically integrated. Previous measurements at 220 nm had demonstrated that solutions of aprotinin and aprotinin*, both of equal concentration, gave the same HPLC integral for the

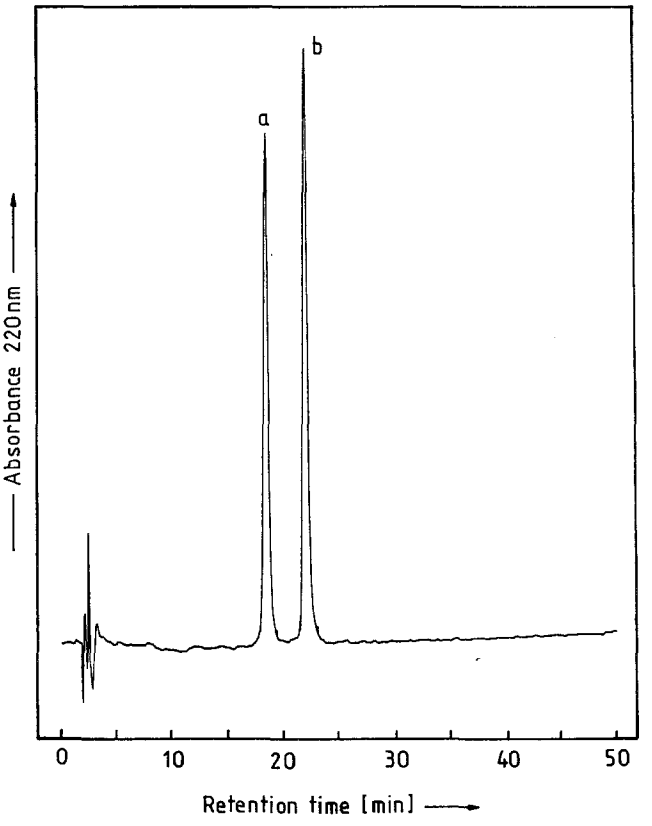

Fig. 1. Separation of modified aprotinin (a) and aprotinin (b) by analytical cation-exchange HPLC using a LKB TSK SP-5 PW column. Chromatographic system: see Materials and Methods.

corresponding peaks. Thus, $K_{Hyd} = (I^*)/(I)$ could be calculated from the integrals of the HPLC signals.

In addition, it was possible to estimate the concentration of active enzyme in the mixtures by HPLC. The HPLC measurements indicated that trypsin-aprotinin complexes are stable at *p*H 7 and under the given chromatographic conditions. Thus, we were able to calculate the amount of active enzyme by comparing the integrals of aprotinin solutions with or without trypsin.

The principle underlying the investigation of the state of equilibrium is shown in Fig. 2. This shows the time course of α, the fraction of modified inhibitor, $\alpha = (I^*)/[(I)+(I^*)]$, at *p*H 4.1. It is seen that the state of equilibrium could not be attained by using pure samples of aprotinin and aprotinin* ($\alpha = 0$ and 1). Even after an incubation time of 200 days, equilibrium was not reached. The resynthesis reaction (starting condition: $\alpha = 1$) has been completed, however, giving the same equilibrium concentration indicated by the line starting at $\alpha = 0.5$ (interrupted line). A line corresponding to a mixture, under the starting condition $\alpha = 0.75$, tended to the same value.

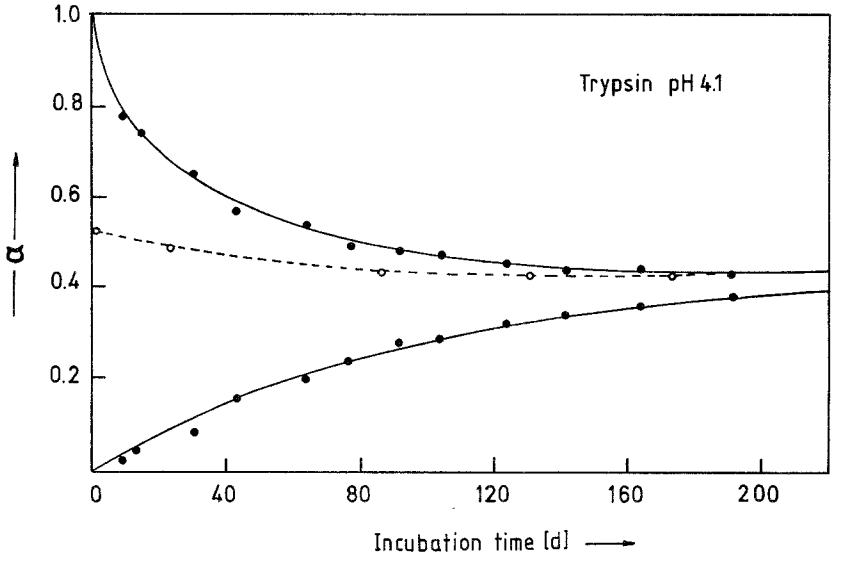

Fig. 2. Time course of the fraction α of modified aprotinin at *p*H 4.1 using porcine β-trypsin. Starting conditions: $\alpha = 0$, 0.5, and 1.

Using these solutions (starting conditions: 0, 0.5, 0.75, and 1), K_{Hyd} could be calculated within an incubation time of 200 days at all *p*H values over the range 2.3–6.5. When this time was extended, by-products were formed, as indicated by HPLC. If porcine β-trypsin was substituted by plasmin from porcine blood, shorter incubation times were needed. Equilibrium was reached at *p*H 5.0 after 90 days

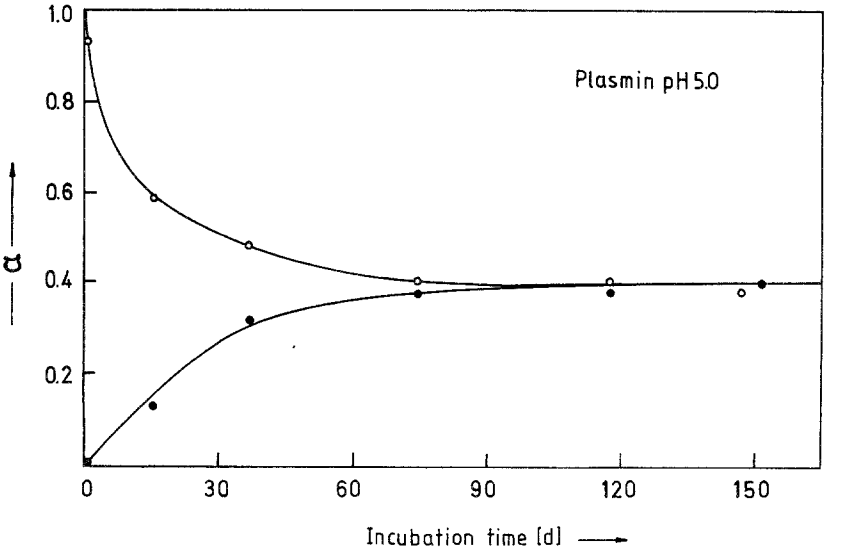

Fig. 3. Time course of the fraction α of modified aprotinin at *p*H 5.0 using plasmin from porcine blood. Starting conditions: $\alpha = 0$ and 1.

(Fig. 3). Only solutions of pure aprotinin and aprotinin* (starting conditions: $\alpha = 0$ and 1) were used.

3.2. *p*H Dependence of K_{Hyd}

The K_{Hyd} values obtained should obey the equation of Dobry *et al.* (1952), in which the *p*H dependence of the equilibrium constant is expressed:

$$K_{Hyd} = K_{Hyd}^{\circ}\{1+[(H^+)/K_1]+[K_2/(H^+)]\}$$

K_{Hyd}° denotes the *p*H-independent equilibrium constant for hydrolysis of the Lys^{15}-Ala^{16} reactive-site peptide bond of the inhibitor, with the Lys^{15} carboxyl group in the COO^- and with the Ala^{16} amino group in the NH_3^+ form. K_1 and K_2 are the corresponding ionization constants of these groups. This equation was drawn up for the enzymatic hydrolysis of a blocked dipeptide and can be used, if the hydrolysis of the peptide bond does not perturb the *p*K values of any pre-existent ionizable group (Mattis and Laskowski, 1973).

The K_{Hyd} values obtained were fitted using this equation in combination with data at higher *p*H values (*p*H range: 6.2–8.9) reported by Estell *et al.* (1980). Fitting was carried out by computer analysis based on the Gauss-Newton method for nonlinear least-squares problems. As shown in Fig. 4, however, the fit (fitting parameters: $K_{Hyd}^{\circ} = 0.86$; $pK_1 = 2.86$; $pK_2 = 8.27$) is not satisfactory because the measured K_{Hyd} values run through a minimum over the *p*H range of 3.5–5.0. It was therefore presumed that an ionizable group was perturbed; the expression of Dobry *et al.* (1952) was used in an extended form:

$$K_{Hyd} = K_{Hyd}^{\circ}\{1+[(H^+)/K_1]+[K_2/(H^+)]\}\{[1+(H^+)/K_3^*]/[1+(H^+)/K_3]\}$$

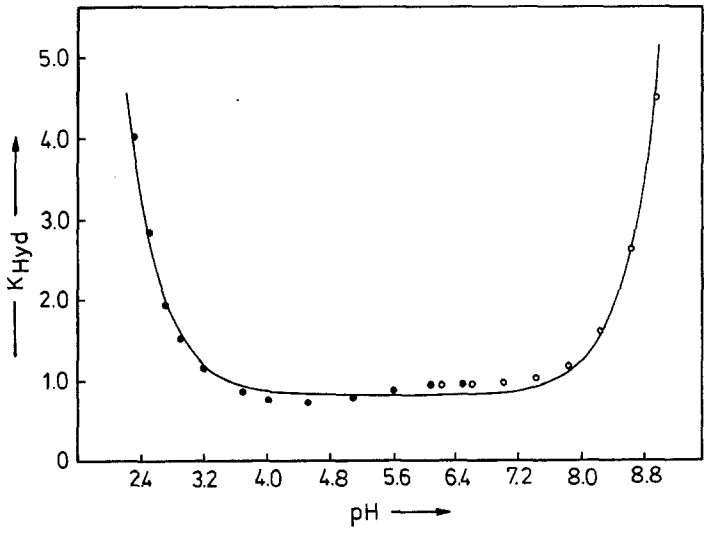

Fig. 4. The *p*H dependence of the equilibrium constant K_{Hyd} for the reaction aprotinin $\rightleftharpoons$ aprotinin*. (——) Calculated using the equation of Dobry *et al.* (1952). (○) Experimental data published by Estell *et al.* (1980).

The additional parameters K_3 and K_3^* represent apparent ionization constants of an acidic group of the native and modified inhibitor, respectively. The additional factor

$$\left\{\frac{[1+(H^+)/K_3^*]}{[1+(H^+)/K_3]}\right\}$$

was inserted in analogy to the corrections reported for the soybean trypsin inhibitor (Mattis and Laskowski, 1973).

The improved fit is shown in Fig. 5. The best-fitting parameters are $K_{Hyd}^\circ = 0.90$; $pK_1 = 3.10$; $pK_2 = 8.22$; $pK_3 = 4.60$; $pK_3^* = 4.40$. The ionization constants K_1 and K_2 have the expected values. These are similar to those described for the soybean trypsin inhibitor, the secretory Kazal inhibitor from bovine pancreas and several ovomucoid inhibitors (for references, see Introduction). The K_{Hyd}° value of 0.90 is essentially the same as the value of 0.95 calculated by Estell *et al.* (1980) and indicates that native and modified aprotinin show similar free energies. The values pK_3 and pK_3^* are in the order of those expected for a carboxyl side chain.

A perturbation of a system, as discussed in this paper, was described by Mattis *et al.* (1973) for the soybean trypsin inhibitor. Here the pK value of a histidine residue was changed by the conversion reaction from native to modified inhibitor. This could be clearly demonstrated by [^{1}H]-NMR data (Markley *et al.*, 1973).

An explanation for a perturbation in the case of aprotinin could be the conformational differences between native aprotinin and aprotinin*, described by Quast *et al.* (1975). It was reported that several amino acid residues gain rotational freedom during the conversion from aprotinin to aprotinin*, as demonstrated by solvent perturbation spectra, circular dichroism, and fluorescence. In addition, this could be confirmed, in some instances, by the use of an [^{1}H]-NMR technique (Wagner *et al.*, 1979). Furthermore, it was proved by [^{1}H]-NMR experiments (Brown *et al.*,

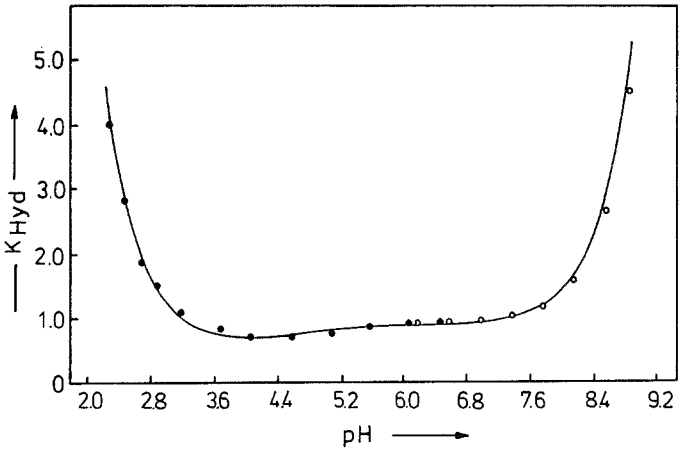

Fig. 5. The pH dependence of the equilibrium constant K_{Hyd} for the reaction aprotinin $\rightleftharpoons$ aprotinin*. (——) Calculated using the equation of Dobry *et al.* in the extended form with the additional parameters pK_3 and pK_3^*. (○) Experimental data published by Estell *et al.* (1980).

1978) that a salt bridge exists between the C- and N-terminals of aprotinin and that this salt bridge perturbs the pK values of the participating groups.

It must be emphasized that the effect of perturbation is very small. A K_{Hyd} value of 0.66, determined with plasmin at pH 5.0 (see Fig. 3), corroborates the minimum in the pH range of 3.5–5.0. Furthermore, the K_{Hyd} values in the pH range of 6–7 are in agreement with those of Estell *et al.* (1980), thus giving weight to the argument for combining both sets of data.

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