Calcium-binding constants of trypsin and trypsinogen

A Reassessment

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The Ca^{2+} -binding constants for trypsin and trypsinogen have been reassessed by using enzyme that has been purified by affinity chromatography and measuring the distribution of $^{45}\text{Ca}^{2+}$ between the protein and a cation exchanger. The $\text{p}K_{\text{Ca}^{2+}}$ value of 4.5 for the high-affinity site on trypsin was 1 logarithmic unit greater than that previously reported.

The dependence on Ca^{2+} for trypsinogen auto-activation and subsequent stabilization of the active enzyme is well established (Abita *et al.*, 1969; Colomb & Figarella, 1979). It has been proposed that there is a high-affinity site common to both trypsin and trypsinogen that maintains the enzyme in its active form and simultaneously protects it from autodegradation (Delaage & Lazdunski, 1967). Trypsinogen has an additional low-affinity site, which has been localized to the tetra-aspartic acid sequence of the *N*-terminal activation peptide (Delaage *et al.*, 1967). Estimations of the binding constants for these two sites have been attempted by several groups (Delaage & Lazdunski, 1967; Epstein *et al.*, 1974; Abbott *et al.*, 1975; Colomb & Figarella, 1979). However, a variation in the $\text{p}K_{\text{Ca}^{2+}}$ value for the high-affinity site of between 2.8 and 3.8 reflects the different methods of measurement used, some of which relied on indirect assay of the Ca^{2+} bound. In addition, it is uncertain whether the enzyme preparations were free of inactive material.

The distributions of trace amounts of radio-labelled Ca^{2+} between Ca^{2+} -binding proteins and cation-exchange resins has been well described for the estimation of binding constants (Wasserman *et al.*, 1968; Harmeyer & DeLuca, 1969). The present paper describes the application of this technique to the measurement of Ca^{2+} binding by trypsin and trypsinogen that have been purified by affinity chromatography with ligands binding only enzymically active material.

Abbreviations used: Gly-(Asp)₄-Lys-Nap, glycyl-tetra-L-aspartyl-L-lysine 2-naphthylamide; Tos-Lys-CH₂Cl, 7-amino-1-chloro-3-L-tosylamidoheptan-2-one [(tosyl-lysyl)chloromethane; 'TLCK'].

Materials and methods

Bovine trypsin (twice-crystallized) and trypsinogen (once-crystallized) were from Sigma Chemical Co., Poole, Dorset, U.K.; Gly-(Asp)₄-Lys-Nap was obtained from Bachem Feinchemikalien, CH-4416 Bubendorf, Switzerland; Trasylol was from Bayer (U.K.) Ltd., Haywards Heath, Surrey, U.K.; $^{45}\text{Ca}^{2+}$ (specific radioactivity 10–40 mCi/mg of Ca) was from The Radiochemical Centre, Amersham, Bucks., U.K. and Dowex 50W (X8; 100–200 mesh) was from BDH Ltd., Poole, Dorset, U.K.

Further purification of trypsin, trypsinogen and activation peptide

The commercial preparation of bovine trypsin was separated into enzymically active and inactive fractions by affinity chromatography, with *p*-aminobenzamidine as the immobilized ligand as described by Hixson & Nishikawa (1973). The concentration of eluted enzyme was estimated by active-site titration with 4-methylumbelliferyl *p*-guanidinobenzoate hydrochloride as the fluorimetric titrant (Jameson *et al.*, 1973). Commercial bovine trypsin was estimated to contain 42% active enzyme by weight.

Some of this material was used to prepare either inactive Tos-Lys-CH₂Cl-treated trypsin (Shaw *et al.*, 1965) or autolysed trypsin, which was made by incubating the enzyme in 10 mM-imidazole/HCl, pH 7.3, containing 0.15 M-NaCl for 100 h at 37°C. Less than 1% residual activity remained in each case.

Commercial bovine trypsinogen was further purified by affinity chromatography with immobilized bovine lung trypsin inhibitor (Trasylol) as described

by Colomb *et al.* (1979); they showed that the inactive zymogen will bind to this inhibitor even in the presence of 1 mM-benzamidine, which was present in the applied material, but not in the chromatography buffer. The concentration of purified trypsinogen was calculated by taking $A_{1\text{cm}, 280}^{1\%}$ as 15.4 (Keil, 1970) and it was calculated that the commercial preparation contained 60% (w/w) enterokinase-activatable zymogen.

An activation-peptide analogue, Gly-(Asp)₄-Lys, was prepared by tryptic digestion of 10 mg of the artificial enterokinase substrate, Gly-(Asp)₄-Lys-Nap (Grant & Hermon-Taylor, 1979). The free peptide was separated from β -naphthylamine by reverse-phase high-pressure liquid chromatography, as previously described by Grant & Cliffe (1980), and was characterized by high-voltage paper electrophoresis at pH 6.5 (Webster & Offord, 1977).

Estimation of calcium-binding constants

Dowex 50W (X8; Na⁺ form) was prepared by successive washes with 100 bed volumes of water, 1 M-NaOH, 1 M-NaCl, and finally water. All buffers were run through some of this resin before use, and portions of the exchanger were added to dialysis buffers in order to minimize endogenous [Ca²⁺]. Polypropylene beakers and centrifuge tubes were used throughout the present study.

Binding constants were determined by the method of Schubert *et al.* (1950). The equilibrium distribution of a trace amount of ⁴⁵Ca²⁺ between the exchanger and a Ca²⁺-binding protein is defined as follows:

$$K_d = \frac{\text{Amount (\%) in exchanger}}{\text{Amount (\%) in solution}} \times \frac{\text{volume of solution}}{\text{mass of exchanger}}$$

If K_d^0 and K_d represent the values of the distribution coefficient obtained in the absence and presence respectively of the protein complex, then they may be written as:

$$K_d^0 = \frac{[\text{⁴⁵Ca}^{2+} \text{ bound}]}{[\text{⁴⁵Ca}^{2+} \text{ free}]}$$

$$K_d = \frac{[\text{⁴⁵Ca}^{2+} \text{ bound}]}{[\text{⁴⁵Ca}^{2+} \text{ free}] + [\text{⁴⁵Ca}^{2+} \text{ protein-bound}]}$$

Hence:

$$\frac{K_d^0}{K_d} = 1 + \frac{[\text{⁴⁵Ca}^{2+} \text{ protein-bound}]}{[\text{⁴⁵Ca}^{2+} \text{ free}]}$$

The values of K_d^0 and K_d are independent of changes in the mass of exchanger or volume of solution due to normal pipetting error and also of the

concentration of radiotracer, which is of the order of 100 mM (Schubert *et al.*, 1950).

For any one class of binding sites in trypsin:

$$K_{\text{diss.}} = \frac{[\text{⁴⁵Ca}^{2+} \text{ free}][\text{free trypsin}]}{[\text{⁴⁵Ca}^{2+} \text{ trypsin-bound}]^n}$$

which may be rewritten:

$$K_{\text{diss.}} = \frac{[\text{free trypsin}]^n}{(K_d^0/K_d - 1)}$$

where $K_{\text{diss.}}$ is the dissociation constant for the enzyme, and n is the molar ratio of Ca²⁺ to trypsin in the Ca²⁺-trypsin binding site. In the presence of trace amounts of ⁴⁵Ca²⁺, the molar concentration of free trypsin approximates to the total trypsin concentration.

For the Ca²⁺-binding studies, a portion of the Dowex resin was washed with 0.2 M-imidazole/HCl, pH 7.3, containing 0.15 M-NaCl, and finally with 0.01 M-imidazole/HCl, pH 7.3, containing 0.15 M-NaCl and 1 mM-*p*-aminobenzamidine. About 1 g of the resin was suspended in 100 ml of buffer by vigorous magnetic stirring, and approx. 1 μ Ci (3 nmol) of ⁴⁵Ca²⁺ was added. At this concentration of ⁴⁵Ca²⁺ the distribution of the cation was approx. 4:1 between the resin and the supernatant respectively. A 500 μ l portion of this mixture was added to capped 1-ml-capacity polypropylene centrifuge tubes, which contained 500 μ l of buffer alone or buffer containing a known concentration of the appropriate trypsin derivative. The tubes were rotated end over end for 30 min at room temperature, which was sufficient for equilibrium to be re-established. After centrifugation at 10000 *g* for 1 min, two 0.2 ml samples of the supernatant were withdrawn for liquid-scintillation counting, and 0.4 ml of buffer containing 20 mM-Na₂EDTA was added to the remaining solution. The tubes were rotated end over end for a further 15 min, re-centrifuged and a further two 0.2 ml samples taken for counting. All samples were counted for radioactivity in 5 ml of PCS scintillation fluid (Hopkins and Williams, Romford, Essex, U.K.) on an Inter-technique SL 3000 instrument. Values for K_d and K_d^0 could be calculated from these results.

Results and discussion

The data was analysed by linear regression analysis. Values of $K_{\text{ass.}}$ (association constant) and $K_{\text{diss.}}$ for commercial trypsin, purified trypsin, Tos-Lys-CH₂Cl-treated trypsin, autolysed trypsin and trypsinogen were obtained from plots of $(K_d^0/K_d) - 1$ versus different molar concentrations of each (Fig. 1). Owing to lack of material, an estimate of $K_{\text{diss.}}$ was made at a single activation-peptide concentration (0.5 mM) under the

assumption that $n = 1$. Values of $pK_{Ca^{2+}}$ for each of the trypsin derivatives studied are listed in Table 1. Double-logarithmic plots of the same parameters for commercial trypsin, trypsin, Tos-Lys-CH₂Cl-treated trypsin and trypsinogen showed that the regression coefficients, which were equivalent to the slope, n , were 0.94, 0.86, 0.98, and 0.84 respectively, indicating one high-affinity calcium-binding site on each derivative. The $pK_{Ca^{2+}}$ value for trypsin was 1 logarithmic unit greater than those previously reported and this was probably for two reasons; 100% active enzyme could be prepared by affinity chromatography, and the ion-exchange technique for measuring the distribution of $^{45}Ca^{2+}$ between the enzyme and the resin was less prone to error in comparison with other methods. This site was maintained in the catalytically inactive but structurally intact Tos-Lys-CH₂Cl-treated trypsin, whereas it was lost in the autolysed inactive trypsin.

Fig. 1. Plot of $(K_d^0/K_d) - 1$ against different molarities of trypsin (▲) or Tos-Lys-CH₂Cl-treated trypsin (□). Each datum point represents the mean of at least two determinations and the line was obtained by linear-regression analysis. In the same way, data for three different concentrations of commercial trypsin (120, 160 and 200 μM) and five different concentrations of purified trypsinogen (10, 20, 30, 40 and 50 μM) were analysed.

The difference in $pK_{Ca^{2+}}$ values for the high-affinity site on trypsinogen (3.8) and trypsin (4.5) was most probably due to the difficulty in accurately measuring the molar concentration of the purified zymogen, since the $A_{1cm,280}^{1\%}$ value would not necessarily correlate with the concentration of enterokinase-activatable material.

A second Ca^{2+} -binding site could not be detected on trypsinogen by the ion-exchange method, since the difference in $pK_{Ca^{2+}}$ of the two sites was at least 2 logarithmic units. However, a $pK_{Ca^{2+}}$ value of 2.2 was determined by using the activation-peptide analogue Gly-(Asp)₄-Lys. Delaage & Lazdunski (1969) reported a $pK_{Ca^{2+}}$ value of 1.8 for this site by monitoring the effect of Ca^{2+} on the initial rate of autoactivation and the formation of inert protein. Colomb & Figarella (1979) used the same technique to estimate $pK_{Ca^{2+}}$ values of 3.3 and 2.7 for human trypsinogens 1 and 2 respectively.

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Table 1. Values of Ca^{2+} -binding constants for various trypsin derivatives

Trypsin derivative	$K_{ass.} (M^{-1})$	$K_{diss.} (M)$	$pK_{Ca^{2+}}$	n
Commercial trypsin*	3.05×10^3	3.3×10^{-4}	3.5	1.05
Commercial trypsin†	7×10^3	1.4×10^{-4}	3.9	0.94
Purified trypsin	3.4×10^4	2.94×10^{-5}	4.5	0.86
Tos-Lys-CH ₂ Cl-treated trypsin	3.8×10^4	2.6×10^{-5}	4.6	0.98
Autolysed trypsin	<0.1			
Trypsinogen	6×10^3	1.7×10^{-4}	3.8	0.84
Activation peptide‡	1.4×10^2	7×10^{-3}	2.2	

* Molarity based on weight, not active-site titration.

† Molarity based on active-site titration.

‡ Based on mean of four determinations; $K_{diss.} = 7 \times 10^{-3} \pm 1.8$ (1 s.d.).

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