THE BINDING OF Ca^{2} TO TRYPSINOGEN AND ITS RELATION TO THE MECHANISM OF ACTIVATION

M.Delaage et M.Lazdunski
Institut de Chimie Biologique,Faculté des Sciences,Marseille,France

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The presence of Ca^{2+} during the activation of Tg increases the rate of hydrolysis of the strategic Lys (6) - Ile (7) bond and decreases the formation of large quantities of inert proteins which are produced in the absence of the cation at the expense of active Ti (Mc Donald and Kunitz (1941), Desnuelle and Gabeloteau (1957)).

Conformational properties of Tg and Ti have been described and different isomeric forms of trypsinogen have been identified and placed in state diagrams (Lazdunski and Delaage(1965) (1967), Delaage and Lazdunski (1965 b)). Figure 1 A shows the effect of Ca^{2+} on the reversible transitions $I' \rightleftharpoons II' \rightleftharpoons III'$ as followed by differential spectrophotometry at 293 mμ (tryptophan pertubation). The transitions $I' \rightleftharpoons II'$ is displaced by Ca^{2+} whereas the $II' \rightleftharpoons III'$ transition is only slightly affected. This suggests that only the I' isomer binds Ca^{2+} firmly, the II' isomer considerably less and the III' isomer does not bind Ca^{2+} at all. These interpretations are in agreement with the observation that below pH 4, Ca^{2+} is without effect on urea or thermal denaturations.

The following abbreviations are used : Tg, trypsinogen ; Ti, trypsin ; DPTi, diisopropylphosphoryl trypsin. All of these proteins are of bovine origin. U ; urea.

The displacement of $II' \rightleftharpoons I'$ by Ca^{2+} at constant pH is shown in figure 1 B and may be expressed as follows :

$$II' \rightleftharpoons I' \underset{n\,Ca^{2+}}{\rightleftharpoons} I'\,Ca_n^{2+}$$

where $$K = \frac{(II')}{(I')} \quad \text{and} \quad K_{Ca^{2+}} = \frac{(I')(Ca^{2+})^n}{(I'\,Ca_n^{2+})} \qquad (1)$$

The slope experimentally determined at the inflection point is 0.57 in agreement with the theoretical value obtained when n = 1. At the mid-point of the curve the abcissa is equal to $pCa^{2+} = pK_{Ca^{2+}} - \log (K + 1)$. In the absence of Ca^{2+} and at pH 6.1 and 30° C, K is 1.9 ; pCa^{2+} is 2.82 (figure 1 B) and therefore $pK_{Ca^{2+}}$ is 3.25 ± 0.1.

The binding constant was also established by studying the protecting effect of Ca^{2+} on urea denaturation of Tg (figure 1 C). The rate of denaturation of a mixture of $I' + I'\,Ca^{2+}$ is always a first order reaction. The rate constants λ have been determined by following the unmasking of the 4 buried tryptophans (Abita and Lazdunski, unpublished results) at 293 mμ. One can demonstrate that

$$\lambda = \lambda_o\, h_{I'} + \lambda_1\, h_{I'Ca^{2+}} \qquad (2)$$

$h_{I'}$ and $h_{I'Ca^{2+}}$, are the molar fractions and λ_o and λ_1 are the rate constants for the denaturation of I' and $I'\,Ca^{2+}$. From (1) when n = 1 and (2)

$$\lambda = \frac{\lambda_o K_{Ca^{2+}} + \lambda_1 Ca^{2+}}{K_{Ca^{2+}} + Ca^{2+}} \qquad (3)$$

When $\lambda = \frac{\lambda_o + \lambda_1}{2}$, $pK_{Ca^{2+}} = pCa^{2+} = 3.20$; this value is again in agreement with that previously determined. The slope is 0.57 and indicates that only one Ca^{2+} is involved in this stabilization (one cannot distinguish the case where two Ca^{2+} would bind

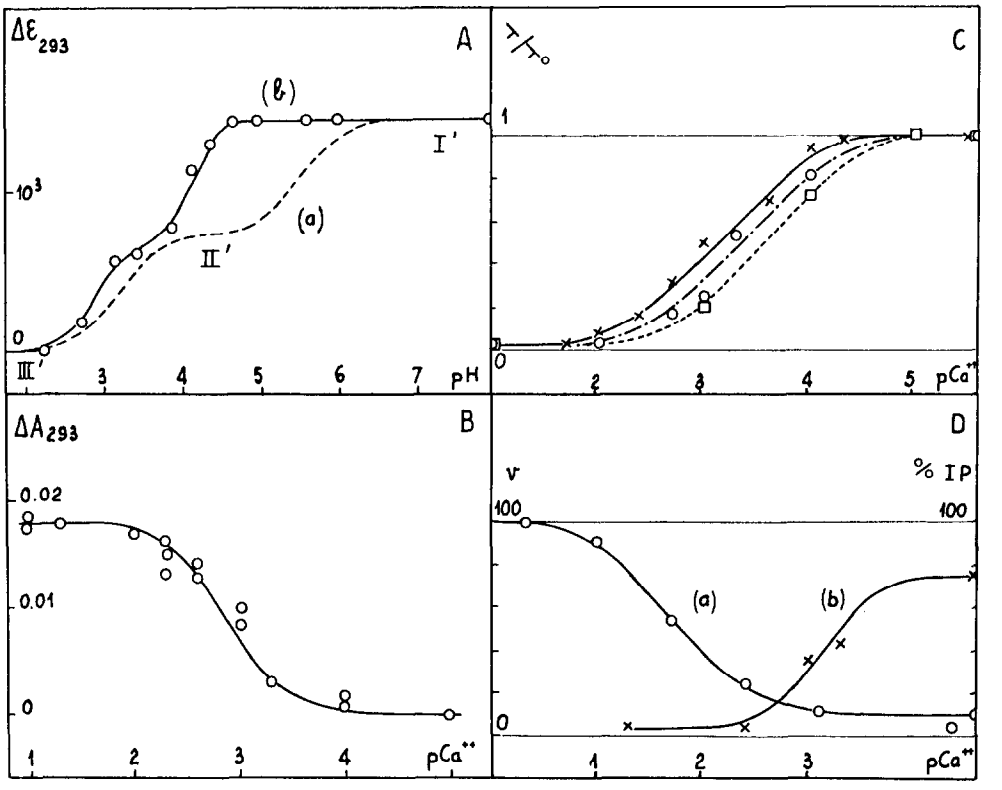

Fig. 1 A : Influence of Ca^{2+} on the isomerisations $I' \rightleftharpoons II' \rightleftharpoons III'$ (ε_{293} : molar absorbancy at 293 mμ). Curve (a) Tg in 0.1 M NaCl, curve (b) Tg in 0.1 M NaCl + 0.1 M $CaCl_2$. Temperature 10° C. No difference spectra was obtained between I' and I' Ca^{2+}.

Fig. 1 B : Influence of pCa^{2+} (-log Ca^{2+}) on ΔA_{293}. Tg 1.26 mg/ml, 30° C, pH 6.1, ionic strength was adjusted to 0.3 with NaCl.

Fig. 1 C : Influence of pCa^{2+} on λ / λ_o. Tg (X) pH 8.4, U 5.9 M. Ti (□) pH 7.7, U 7.4 M. DPTi (●) pH 8.4, U 7.4 M. λ_o is 0.16, 0.36 and 1.45 mm^{-1} respectively. NaCl 0.4 M, 20° C.

Fig. 1 D : Influence of pCa^{2+} on the rate of hydrolysis v of $Lys_{(6)}$- $Ile_{(7)}$ (a), and on the percentage of inert proteins (IP) formed (b). Curve (a) is drawn from the results of Mc Donald and Kunitz 1941. The conditions for curve (b) are Tg 10 mg/ml, pH 7.8, 0° C. The method for evaluating IP has been described by Gabeloteau and Desnuelle 1957. The preparation of Tg and the determination of difference spectra have been described previously (Lazdunski and Delaage 1965).

to 2 distinct sites with the same affinity constants and with additive effects on λ ; this situation seems quite improbable however). Similar experiments have also been done with Ti and DPTi (fig. 1 C). The pK_{Ca}^{2+} was found to be 3.5 and 3.3 respectively. The ratios λ_o/λ_1 are 60, 56 and greater than 32 respectively for Ti (in 7.4 M urea) DPTi (in 7.4 M urea) and Tg (in 5.9 M urea). In view of the similarity of the pK_{Ca}^{2+} found for Tg, Ti, and DPTi it seems reasonable to conclude that the binding site is the same in all three molecules. It must therefore be in a part of the zymogen structure which is preserved in the activation and it cannot be constituted by any of the 4 adjacent aspartyl residues of the N-terminal hexapeptide even though the affinity of Ca^{2+} suggests a binding site composed of 2 to 3 carboxylates. Since the formation of the active center in the course of the activation is nearly without effect on Ca^{2+} binding it is not surprising that blocking the active center by diisopropylfluorophosphate does not impair the binding of the cation.

Figure 1 D shows the Ca^{2+} effect on the formation of the inert proteins and on the rate of hydrolysis of the Lys-Ile bond. At low Ca^{2+} concentrations, only the rate of formation of inert proteins is affected. At the mid-point of curve (a) a value for pCa^{2+} of 3.2 is obtained. This value is close to the pK_{Ca}^{2+} previously determined. This suggests that the specific binding of one Ca^{2+} on I' not only increases its stability in alkali (Delaage and Lazdunski (1965 a)), in acid (fig. 1 A) and urea solutions but also masks the bonds the hydrolysis of which produce the inert proteins. The rate of hydrolysis of the Lys (6)-Ile (7)

bond only is affected at high concentrations of Ca^{2+}. The analysis of the slope of the curve indicates a less specific binding of the second Ca^{2+} with a $pK_{Ca}2+$ of about 1.8. This binding probably occurs on the carboxylates of the aspartyl residues adjacent to the Lys-Ile bond since a similar effect of Ca^{2+} has been found with model peptides related to the N-terminal sequence of Tg (Savrda, Bricas, Delaage, Lazdunski in preparation). The enhancing effect of Ca^{2+} on the rate of hydrolysis of Lys-Ile is probably due to the partial neutralization of the negative charges on these neighbouring carboxylates. This binding induces no detectable structural change of the molecule in agreement with the finding that the N-terminal hexapeptide of Tg is apparently free in the solvent and does not participate in the thermodynamic stabilization of the zymogen (Lazdunski and Delaage (1967)).

Summary : Trypsinogen possesses 2 different binding sites for Ca^{2+}. The first site has a high affinity for the cation and the binding induces a conformational change which protects the molecule against the formation of inert proteins, this site also exists in trypsin. The second site has a lower affinity for Ca^{2+} and is probably located in the 4 adjacent N-terminal aspartyl residues. This binding induces no conformational change but is responsable for the acceleration of the hydrolysis of the Lys (6) -Ile (7) bond.

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