ON THE ACTIVATION OF TRYPSINOGEN. A STUDY OF PEPTIDE MODELS RELATED TO THE N-TERMINAL SEQUENCE OF THE ZYMOGEN.

M.Delaage, P.Desnuelle, M.Lazdunski
Institut de Chimie Biologique, Faculté des Sciences,Marseille,France
E.Bricas, J.Savrda
Institut de Biochimie, Faculté des Sciences, Orsay, France.

Received September 21, 1967

The N-terminal peptides liberated in the course of the activation of bovine (Davie and Neurath (1955), Desnuelle and Fabre (1955)) porcine (Charles and al. (1963)) and ovine (Bricteux-Grégoire and al. (1966)) Tg all have in common the sequence Asp-Asp-Asp-Asp just preceding the Lys-Ile bond which is hydrolyzed as the first step in the activation. Several roles might be attributed to this sequence (Lazdunski and Delaage 1967, Delaage and Lazdunski 1967). In this paper the influence of the aspartyl residues on the tryptic specificity for the Lys-Ile bond is described. The kinetic behavior of several peptides related to the N-terminal sequence of bovine Tg, Val-Asp_4-Lys-Ile-Val-Gly, is studied.

Methods : The peptides have been obtained as sumarized in Figure 1. Their purity has been checked by electrophoresis, thin layer chromatography on silica gel and by enzymatic degradation with leucine aminopeptidase. Details of their synthesis will be described elsewhere (Savrda and Bricas in preparation).

The kinetic parameters for the hydrolysis by trypsin of the

The following abbreviations are used : Tg, Trypsinogen ; Ti, Trypsin ; ChTg A, Chymotrypsinogen A.

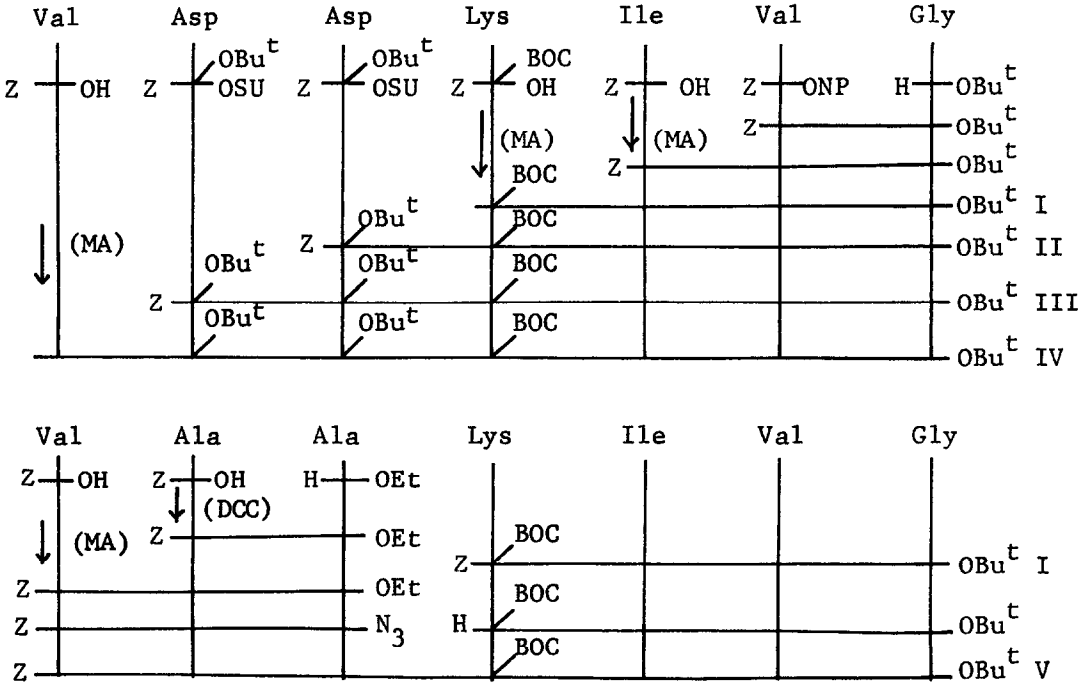

Fig. 1 During peptide bond synthesis, the carboxyl groups were protected by conversion to the t-butyl ester (-OBu^t) or the ethyl ester (-OEt). The α- and ε-amino groups were protected with benzyloxycarbonyl (Z-) and t-butyloxycarbonyl (BOC-) respectively. For the formation of peptide bonds the carboxyl groups (-OH) of the Z-aminoacids were activated either by conversion into the p-nitrophenyl ester (-ONP), the N-hydroxysuccinimide ester (-OSU) or the acid azide (N_3-) or were activated by the coupling agents dicyclohexylcarbodiimide (DCC) or carbonic mixed anhydride (MA). At each step of the synthesis the Z-protecting group was removed by catalytic hydrogenation and the free "amino component" (H-) was coupled to the following carboxyl activated Z- aminoacid or peptide. -OBu^t and BOC- were removed by treatment with trifluoroacetic acid.

Lys-Ile bond in the peptides were obtained by two methods. (1) A direct evaluation by the pH-stat technique of the number of liberated protons at pH 9 (fig. 2 A). (2) A study of the competition for bovine Ti between the peptides and ChTg A. The initial rate of activation of ChTg A in the presence of peptides is :

$$v_2 = \frac{V_2 K_1 S_2/K_2}{S_1 + K_1'} \qquad \text{with } K_1' = K_1 \left(1 + \frac{S_2}{K_2}\right)$$

where V_2, K_2 (1.2×10^{-3}M at pH 8.0, 1° C) and S_2 ; V_1, K_1 and S_1 are respectively the maximal rates, Michaelis constants and concentrations of ChTg A and the peptides. A plot of $1/v_2$ against S_1 (fig. 2 B) gives K_1' and then K_1.

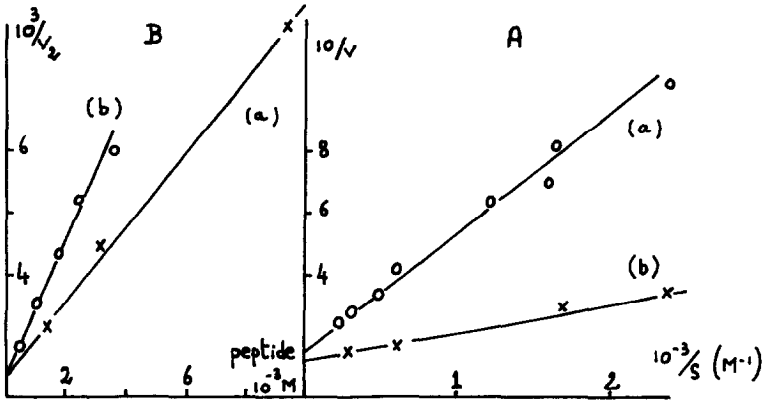

Fig. 2 A Lineweaver-Burk determination of V_1 and K_1 for peptide (IV) without (a) or with Ca^{2+} 65 mM (b), pH 9.0, 25°, ionic strength 0.3. v is expressed in µmole of peptide hydrolysed/min/mg Ti. Fig. 2 B Determination of V_1 and K_1 by competition for Ti between ChTg A and peptide (I) (a) or ChTg A and peptide (IV) (b), ionic strength 0.1. v_2 is expressed in chymotrypsin units (with N-acetyl-L-tyrosine ethyl ester as a substrate)/min/mg Ti.

Results and Discussion : In table I, a comparison of peptides I and II and of peptides III and IV shows that slight variations of K_1 are accompanied by large changes in k_{cat}. Conversely, large variations of K_1 are accompanied in the case of peptides IV and V by small changes in k_{cat}. These independent changes of K_1 and k_{cat}

TABLE I

Peptides	Ca^{2+} (mM)	K_1 (mM)	k_{cat} (sec^{-1})
Lys-Ile-Val-Gly (I)	0	4.25 (a)	0 (c)
Asp-Lys-Ile-Val-Gly (II)	0	8.53 (a)	0.39 (c)
	50	no effect	
$(Asp)_2$-Lys-Ile-Val-Gly (III)	0	2.51 (a)	0.46 (c)
	50	positive effect	
Val-$(Asp)_2$-Lys-Ile-Val-Gly (IV)	0	1.92 (a)	
	0	3.35 (b)	2.8 (b)
	65	1.11 (b)	3.6 (b)
Val-$(Ala)_2$-Lys-Ile-Val-Gly (V)	0	.0255 (a)	1.45 (a)
	0	.0905 (b)	7.0 (b)
	50	.0905 (b)	7.0 (b)

(a) 1°, pH 8.0 ; (b) 25°, pH 9.0 ; (c) 1°, pH 9.0.

suggest that the Michaelis constants are true equilibrium constants, and that k_{cat} is the rate constant k_2 for the acylation of the active serine in trypsin*.

The results shown in Table I indicate that the accumulation of aspartyl residues near the Lys-Ile bond does not favor the formation of the Michaelis complex. (1) A comparison of the K_1 for

* The mechanism of action of Ti involves the following steps $E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES_1 \xrightarrow[+ P_1]{k_2} ES_2 \xrightarrow{k_3} E + P_2$, ES_1 is the Michaelis complex, ES_2 the acylated complex on Ser 183. $V = k_{cat}[E_o]$; $k_{cat} = \frac{k_2 k_3}{k_2 + k_3}$; $K = K_S \frac{k_3}{k_2 + k_3}$ and $K_S = \frac{k_{-1} + k_2}{k_1}$. When $k_2 \ll k_3$ and k_{-1}, $k_{cat} = k_2$, $K = \frac{k_{-1}}{k_1}$ and these parameters vary independently.

peptides I, II, III and IV shows that this constant is largely independent of the number of aspartyl residues. (2) The replacement of these residues by alanyl residues in peptide V increases the binding constant, 75 times at 1° pH 8 and 37 times at 25° pH 9 ; k_{cat} is increased by a factor 2.5. Peptide V, is an excellent substrate for trypsin, its K_1 and k_{cat} are much more favourable than for the usual amide substrates as N-benzoyl-L-argininamide.

The presence of Ca^{2+} in the activation medium has an important effect on the formation of inert proteins and on the rate of hydrolysis of the strategic bond Lys(6)-Ile (7) of bovine Tg. This double effect is due to the binding of the cation at two different sites (Delaage and Lazdunski (1967)). The effect on the hydrolysis of the Lys (6)- Ile (7) bond is apparently due to the binding of Ca^{2+} on the 4 aspartyl residues. The rate of hydrolysis of the Lys-Ile bond is increased by a factor of about 7 for native Tg in 5×10^{-2} M Ca^{2+} (Mc Donald and Kunitz (1941)) and an analysis of the results of Pechère and al. (1958), shows that the initial rate of the hydrolysis of the same bond in denatured S-sulfo Tg is also increased 6 to 7 times under these same conditions. This Ca^{2+} effect appears then to be independent of any influence on the spatial structure of the zymogen. In accordance with that conclusion we find a very similar effect of Ca^{2+} on the hydrolysis of peptide IV where 6.5×10^{-2} M Ca^{2+} (at this concentration the effect is maximum) lowers K_1 by a factor of 3 and increases k_{cat} by a factor of 1.3 thus increasing the rate at low substrate concentration ($S_1 \ll K_1$) by a factor of 3.9.

The Ca^{2+} effect only appears with peptides III and IV which have two aspartyl residues. Our results are in agreement with the recent observation that calcium is not necessary for the activation of a Tg derivative in which the carboxylate groups are blocked (Radhakrishnan and al. (1967)).

Financial help by Délégation Générale à la Recherche Scientifique et Technique (Convention 66.00.056), Centre National de la Recherche Scientifique (RCP 23) and National Institute of Health (Grant AM 04642) is gratefully acknowledged.

References

Bricteux-Grégoire, S., Schynd, R. and Florkin, M., Biochim.Biophys. Acta 127, 277 (1966).
Charles, M., Rovery, M., Guidoni, A. and Desnuelle, P., Biochim. Biophys.Acta 69, 115 (1963).
Davie, E.W. and Neurath, H., J.Biol.Chem., 212, 818 (1955).
Delaage, M. and Lazdunski M., Biochem.Biophys.Research Comm.,28, 390 (1967).
Desnuelle, P. and Fabre, C., Biochim.Biophys.Acta, 18, 29 (1955).
Mc Donald, M.R. and Kunitz, M., J.Gen.Physiol., 25, 53 (1941).
Gabeloteau, C. and Desnuelle, P., Arch.Biochem.Biophys., 69, 475 (1957).
Lazdunski,M. and Delaage,M., Biochim.Biophys.Acta, 140, 417 (1967).
Pechère, J.F., Dixon, G.M., Maybury, R.M. and Neurath, H., J.Biol. Chem., 233, 1364 (1958).
Radhakrishnan, T.M., Walsh,K.A. and Neurath, H., J.Am.Chem.Soc., 89, 3059 (1967).