# INDUCTION OF THE BOVINE TRYPSINOGEN–TRYPSIN TRANSITION BY PEPTIDES SEQUENTIALLY SIMILAR TO THE N-TERMINUS OF TRYPSIN

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## 1. Introduction

It is well known that the activation of bovine trypsinogen is brought about by the enzymatic removal of its N-terminal hexapeptide [1,2]. As has been demonstrated in the crystal structures of β-trypsin [3,4] and before in the homologous α-chymotrypsin [5], the newly formed α-ammonium group of Ile 16 intrudes into the molecule and binds to the carboxylate side chain of Asp 194. The importance of this internal salt bridge for the stability and function of these serine proteases has already been proved by a series of titration, activity and modification experiments [6–10], indicating that any discharge, removal or blocking of the Ile 16 amino-group leads to a transition from the active enzyme conformation to an inactive conformation [11,12].

Recently it has been demonstrated by Neurath and associates that the zymogens of serine proteases like, for example, trypsinogen and chymotrypsinogen, also show a very weak intrinsic activity toward certain active site titrants [13–16]. Besides its low autocatalytic activity [17], a weak binding capacity for competitive trypsin inhibitors [15,18] and a specific interaction with the basic pancreatic trypsin inhibitor (PTI) have been observed for trypsinogen [19,20]. We have been engaged in crystallographic structural studies of the trypsin–trypsinogen–PTI system [4,21,22,22a]. We found indeed that the complex between PTI and trypsinogen crystallizes isomorphously to the trypsin–PTI complex and is structurally extremely similar. The Ile 16 binding pocket is formed but filled with some water molecules instead of the Ile 16 N-terminus as in trypsin and the trypsin–PTI complex [21]. In contrast the crystal structure of free bovine trypsinogen, which has been determined and refined at 1.8 Å resolution [22], shows that the chain segments forming the specificity pocket of the active trypsin and the Ile 16 binding pocket are disordered. The N-terminal segment including residues 10 to 19 (i.e. more than the split off hexapeptide) is not fixed.

This apparent absence of any specific interaction of the N-terminus with the trypsinogen moiety leads us to the conclusion that the trypsin(ogen) molecule exists in two interconvertible states, namely in a partially disordered trypsinogen-like conformation or in an ordered trypsin-like conformation, characterized by the presence of rigid specificity- and Ile 16 binding pockets. Thus the question arises whether this interconversion could also be induced in the zymogen simply by peptides sequentially analogue to the N-terminus of trypsin. For two reasons we tested this with the *p*-guanidinobenzoate (pGB)-modification instead with trypsinogen itself. As Kerr et al. [16] have shown the pGB-chromophor displays a considerable Cotton-effect when it is inserted in the specificity pocket. Moreover the pGB-group due to its high affinity to the trypsin specificity site should be able to support the transition to the trypsin like conformation as observed for the trypsinogen–PTI complex.

## 2. Materials and methods

Crystalline salt-free homogenous bovine trypsinogen was from Merck, Darmstadt. About 90% pure β-trypsin

*Footnote*: Amino acids of trypsin are identified by the residue number of the homologous amino acid in chymotrypsin [1].

was prepared from bovine trypsin (Boehringer, Mannheim) according to Schroeder and Shaw [23]. The chromatographically pure Ile–Val-dipeptide was a generous gift from Dr Eugen Schaich, Tutzing (Boehringer Mannheim) that is greatly appreciated. All other peptides of purest grade available were purchased from Serva (Heidelberg) and were used without further purification. Quantitative amino acid analysis and thin-layer chromatography confirmed the homogeneity of these peptides.

The circular dichroism (CD) spectra were recorded with a Cary 60 spectropolarimeter equipped with a Cary 6002 CD attachment (Varian, Monrovia, USA), using fused thermostated cells of 10 and 1 mm path-length (Hellma, Müllheim). The data were expressed as mean molar ellipticity per residue $[\theta]$ in (deg × $cm^2$)/dmol. pGB-Trypsinogen and pGB-trypsin were prepared as described by Kerr et al. [16]. The percentage of active sites in β-trypsin, pGB-trypsin, trypsinogen and pGB-trypsinogen (after activation with trypsin in 0.1 M Tris buffer, pH 8.0, containing 0.02 M $CaCl_2$ and 0.002 M $NdCl_3$) and the degree of acylation were determined with *p*-nitrophenole-*p'*-guanidinobenzoate (NPGB) at pH 8.3 [24]. The degree of acylation was usually about 60% for trypsinogen and higher than 90% for β-trypsin. The concentrations of the non-modified enzymes were determined from absorbance at 278 nm ($\epsilon_{278}$: 3.6 × $10^4$ $M^{-1}$ $cm^{-1}$) and of the pGB-derivatives with a calibrated micro-Biuret-method.

## 3. Results

Figure 1 shows the CD-spectra in the aromatic region for β-trypsin, trypsinogen, pGB-trypsin and pGB-trypsinogen, those of the pGB-derivatives being corrected for incomplete acylation. These spectra are qualitatively similar to those published by Kerr et al. [16] but differ in their absolute values being more negative according to our measurements. Whereas the spectra of the first three species are quite similar to one another, the molar ellipticity of trypsin changes considerably upon acylation with NPGB. As was also pointed out by Kerr et al. [16], the CD-spectrum of pGB-trypsin and its difference-CD-spectrum with trypsin show large minima at 269 nm, identical with the maximum of their difference absorption spectra. Therefore these ellipticity changes can be considered

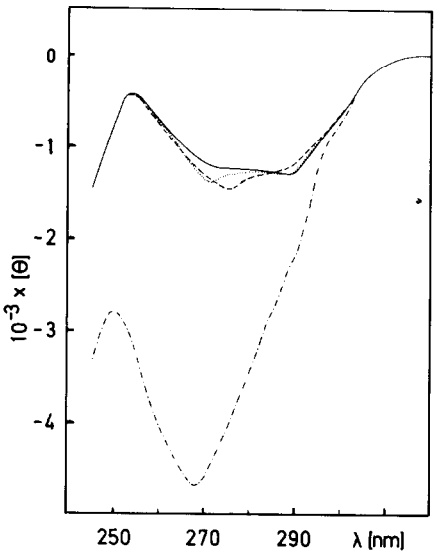


Fig.1. Circular dichroism spectra of trypsin (- - -), trypsinogen (——), pGB-trypsin (–·–·–) and pGB-trypsinogen (· · · · ·) in the aromatic region in 0.02 M $CaCl_2$, 0.5 M Tris–HCl buffer, pH 7.5, at 20°C.

to be largely due to environmental changes of pGB-chromophor. Furthermore, we could confirm their finding that after activation of pGB-trypsinogen with small trypsin amounts its CD spectrum changes completely to that expected for pGB-trypsin.

Exactly the same increase of the negative ellipticity around 270 nm could, however, be observed for pGB-trypsinogen in the presence of $10^{-1}$ M Ile–Val, a peptide with the starting sequence of active trypsin. At lower dipeptide concentrations only a partial increase in ellipticity is obtained. We take the change in ellipticity from $\theta_{min}$ at 269 nm (in the absence of Ile–Val) to $\theta_{max}$ (at high Ile–Val concentrations) as a measure of the conversion from the trypsinogen conformation to the trypsin-like conformation. The degree of conversion α upon induction with Ile–Val can be calculated for varying total peptide concentrations $P_o$ with $\alpha = (\theta - \theta_{min})/(\theta_{max} - \theta_{min})$. Figure 2 shows a plot of α versus the total Ile–Val concentration at pH 7.5. Evidently these values follow a curve expected for a single binding site with an association constant $K_a$ = 2.3 × $10^4$ $M^{-1}$. This value can be also directly derived from the fitting curve since it should be $K_a = (P_o - 1/2\ T_o)^{-1}$ at the point of half satura-

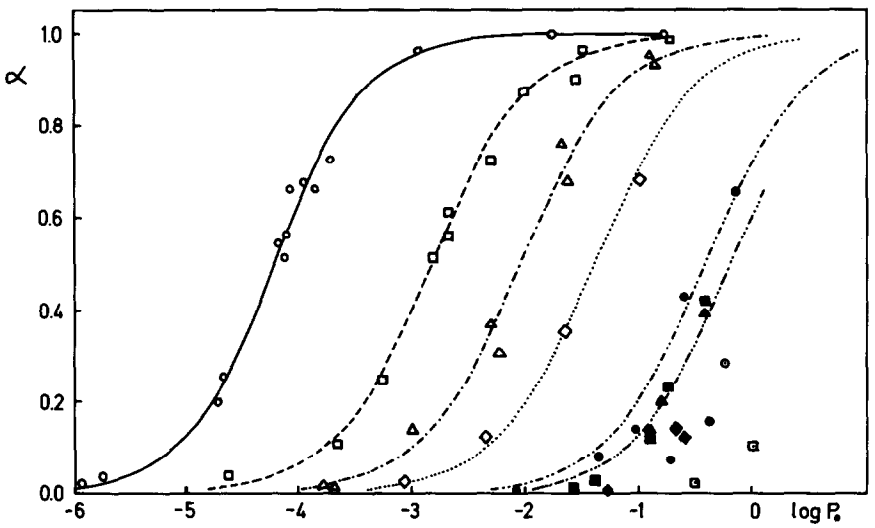


Fig.2. Halflogarithmic plot of the conversion degree $\alpha$ versus total peptide concentration $P_0$ in 0.02 M $CaCl_2$, 0.5 M Tris–HCl buffer, pH 7.5 at 20°C and optimal fit by single-site binding curves. pGB-trypsinogen concentration 1.9 × $10^{-5}$ M. Ile–Val (——○——); Val–Val (- - -□- - -); Leu–Val (- · · ·△- · · ·); Val–Leu (· · · · ·◇· · · · ·); Val–Ala (- ·· - ··●- ·· - ··); Val–Gly (- ··· -■··· -); Val–Gly–Gly (- ···▲- ···); Ile (◆); Ile-*O*-Methylester (⊛); Ala–Val (⊡).

tion, $T_0$ being the total pGB-trypsinogen concentration.

Similar experiments at pH 7.5 with related peptides in which the first, the second or both residues have been replaced by other amino acids showed the very high specificity of this interaction between Ile–Val and trypsinogen. The transition curves induced by these peptides are also included in fig.2. Most of these compounds induce comparable effects on pGB-trypsinogen, but at much higher characteristic concentrations. The corresponding association constants $K_a$ are presented in table 1. The estimated errors mostly due to systematic errors are ± 10 to 20%. As can be seen from fig.2 and table 1, replacing the N-terminal Ile by the isomeric Leu, reduces $K_a$ by a factor of 190. No binding at all could be recorded for a replacement by Phe up to 0.5 M concentrations. However, with Val at the first position $K_a$ is only 30-fold reduced. Evidently a slightly smaller amino acid but with the branch at $C_\beta$ is more easily accepted than the equally large but differently branched amino acid. However, for the slightly smaller Ala $K_a$ is drastically reduced for more than 5 orders of magnitude.

Table 1 shows that the association is equally affected by a variation in the second residue. Its absence or replacement by the methyl ester group reduces $K_a$ for about 30 000. From the series of peptides in table 1 with Val in the first position, the binding contribution of the second residue can be estimated. Replacing the second Val by the larger amino acid Leu leads to a 30-fold reduction of $K_a$,

Table 1
Association constants $K_a$ for binding of different peptides to pGB-trypsinogen

| Peptide | $K_a$ $M^{-1}$ |
|---|---|
| Ile–Val | 23 000 |
| Leu–Val | 120 |
| Val–Val | 770 |
| Ala–Val | ~ 0.1 |
| Phe–Val | << 0.1 |
| Ile-*O*-methylester | ~ 0.7 |
| Ile | ~ 0.7 |
| Val–Leu | 25 |
| Val–Val | 770 |
| Val–Ala | 2.6 |
| Val–Gly | 1.7 |
| Val–Gly–Gly | 1.7 |

but with the less bulky Ala in this position $K_a$ is even 300-fold less. Gly or Gly–Gly at this position has almost no additional effect on $K_a$ compared to Ala. From these data it may be derived that these small side chains do not adequately fit to the binding site and that the binding is not seriously influenced by the third residue. In addition, Ala–Pro and Phe–Pro being the N-termini in the activation peptides of some trypsinogens (e.g. of porcine trypsinogen, [25]) showed no binding effect up to 0.7 and 0.4 M concentrations.

Furthermore, the pH-dependence for the transition induced by Ile–Val was examined. At extreme pH-values comparable transition curves were obtained but shifted to higher peptide concentrations compared to pH 7.5. In fig.3 the logarithms of the derived apparent association constants are plotted versus the pH, yielding a bell shaped curve. At pH-values below 6.5 this curve seems to be governed by the protonizable group with an apparent p$K$ of 5.6. The slope of $d\log K_a/dpH > 1$ seems to indicate the involvement of more than one single acid-base pair. At the alkaline side the transition and binding seem to be affected by one ionizable group with an apparent p$K$ of 8.3. Most probably this group is the free α-ammonium group of

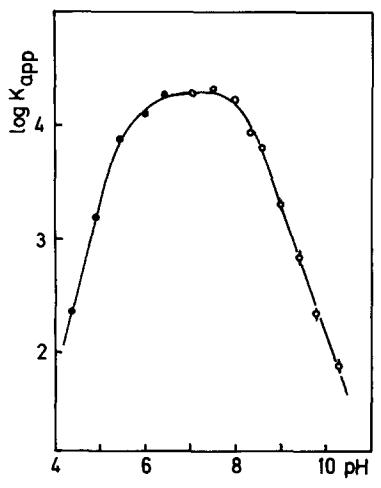


Fig.3. Dependence of the apparent association constant from pH for Ile–Val at 20°C in 0.2 M piperazine-*N,N'*-(2-ethanesulfonic acid) –NaOH (○), 0.2 M *N*-morpholine-3-propanesulfonic acid–NaOH (●), 0.2 M Tris(hydroxymethyl)aminomethan–HCl (○) and 0.2 M borate–HCl–NaOH (ϕ), all buffers excepting the alkaline borate buffer containing 0.02 M $CaCl_2$.

Ile–Val and the other assayed peptides to which typically such p$K$-values can be assigned (s. [26]). On the other hand, anomalous p$K$-values of about 10 have been assigned to the ammonium group of chymotrypsin and trypsin in the salt bridge conformation and about 7.9 for the free N-terminus [7,27].

## 4. Discussion

As shown in fig.2 the transition induced in pGB-trypsinogen upon interaction with Ile–Val and related compounds can satisfactorily be interpreted by simple binding at one site. Upon this binding and ion pair formation, a conformational change must be induced in the specificity pocket, bringing the pGB-group into a stereospecific environment equal or similar to that in trypsin. The opposite change accompanied by disappearance of the Cotton-effect has been observed after the specific carbamylation of ε-guanidinated pGB-trypsin [9].

No increased catalytic activity toward specific substrates could yet be registered for unmodified trypsinogen after addition of 0.2 M Ile Val, applying the usual activity assay with *p*-toluene sulfonyl-L-argininemethylester. More sensitive experiments have to be performed in order to detect minor increases in activity. A first attempt to demonstrate the bound Ile–Val and the conformational changes induced in trypsinogen crystals failed even in the presence of 2.0 M Ile–Val and 2.0 M *p*-aminobenzamidine (in glutaraldehyde cross-linked trypsinogen crystals). Even the concerted interaction of strongly binding, but not covalently linked, inhibitors and Ile–Val seems to be too small to bring the equilibrium between trypsinogen and trypsin-like conformations close to unity. In active trypsin the binding of the Ile 16 α-ammonium group to Asp 194 is so strong that the trypsin conformation is favoured even in the absence of supporting groups in the specificity site. The tremendously increased binding affinity of the covalently bound Ile–Val N-terminus to the trypsin molecule can be explained by its increased effective concentration, by a reduction in its rotational and translational freedom and by some orientation that it may have compared to the free peptides. Similar effects are discussed for the rate and binding enhancement of intramolecular and enzymatic reactions respectively [28–30].

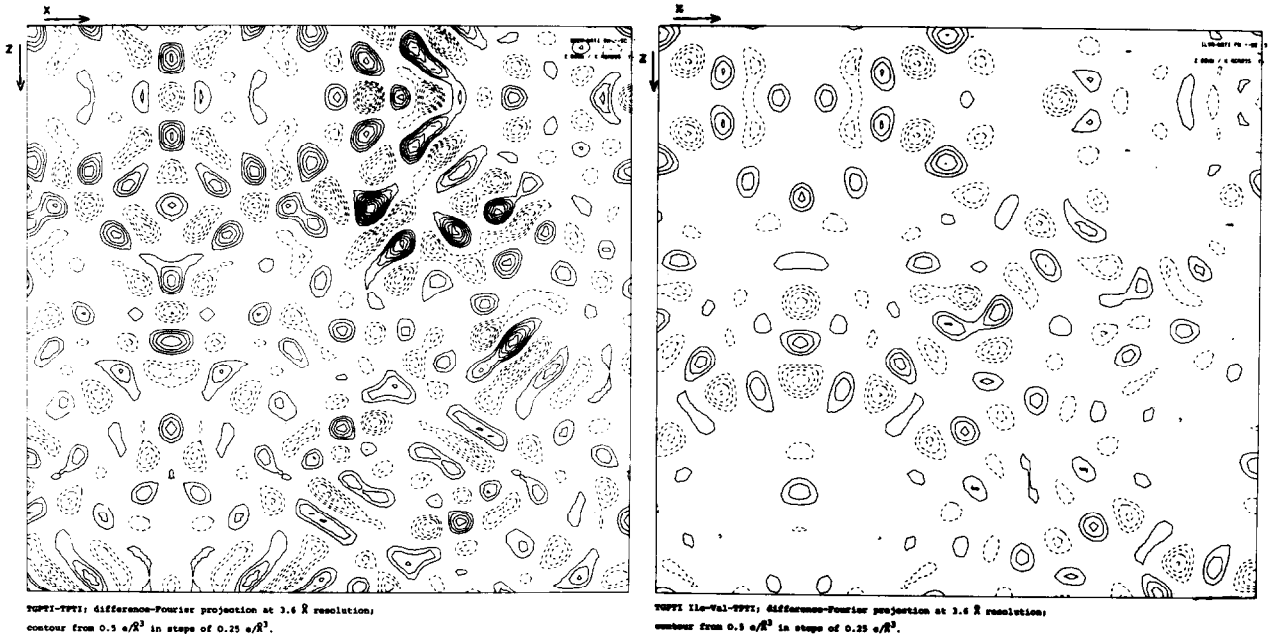

Fig.4. Difference Fourier projection on the $x$, $z$-plane at 3.6 Å resolution. Contours starting at 0.5 e/Å$^3$ in 0.25 e/Å$^3$ steps. Phases of the trypsin–PTI complex. The size of the part plotted is: x from −11.8 to 37.8 Å, z from −5.8 to 40.4 Å. Left: Trypsinogen–PTI minus trypsin–PTI. Right: Trypsinogen–PTI+Ile–Val minus trypsin–PTI.

However, the free energy of binding of the covalently linked and stereochemically fitting pGB-group is evidently large enough to shift the equilibrium far enough to the trypsin side so that upon additional interactions with Ile–Val and other related peptides the trypsin-like conformation exists in detectable amounts.

These data strongly suggest that the protein segments forming the Ile 16 binding pocket and the specificity pocket are conformationally linked and the Ile–Val peptide (or the N-terminus of trypsin) and the pGB-group act together cooperatively. This is also underlined by the PTI–trypsinogen complex structure where due to the high binding affinity of PTI both the Ile 16 and the specificity pocket are formed in spite of the absent N-terminus. By adding about 0.1 M Ile–Val to the mother liquor, the diffraction pattern of the trypsinogen–PTI crystals changes almost completely to that found for PTI–trypsin crystals [21]. A difference Fourier synthesis calculated in projection for the PTI–trypsin complex versus PTI–trypsinogen+Ile–Val complex (fig.4, right) is almost featureless in contrast to the difference map of PTI–trypsin complex versus PTI–trypsinogen complex (fig.4, left). These data again indicate that that the Ile–Val peptide plays the structural role of the N-terminus in the active species.

From a comparison of the phylogenetically related serine proteases sequenced so far it can be expected that the overall structures of all these enzymes are quite similar (e.g. [31]). Most remarkable is the strong coincidence in their N-terminal sequences being mostly Ile–Val and only in some cases Val–Val. So it may be predicted that in all these related serine proteases the activation and ion pair formation will be similar to trypsinogen. The conversion of the zymogens to the active enzyme conformations should be inducable by Ile–Val and Val–Val as in pGB-trypsinogen. Our finding leads to a quite interesting hypothesis about the activation of the related plasminogen by streptokinase. Streptokinase itself does not seem to display any hydrolytic activity but forms with plasminogen a stable complex, the active site of which seems to develop before the plasminogen

moiety is converted to plasmin [32]. Streptokinase after binding to plasminogen near the Ile 16 binding pocket (i.e., far away from the contact site) could intrude its N-terminus, Ile–Ala–Gly [33] into the pocket (molecular sexuality). Like in trypsinogen in this way the specificity pocket of the complexed plasminogen could become structured being accompanied by activity. This hypothesis may be easily tested by a specific blocking of the N-terminal α-amino group of streptokinase.

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